UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente/Department of Agronomy Animals Food Natural Resources and Environment

Dipartimento di Biologia/Department of Biology

Corso di Laurea magistrale / Second Cycle Degree (MSc) in Scienze e Tecnologie per l'Ambiente ed il Territorio – LM75

Evaluation of biomarkers in the European nightcrawler *Dendrobaena veneta* exposed to PFAS under controlled conditions

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ANNO ACCADEMICO/ACCADEMIC YEAR 2020/2021

INDEX

ABSTRACT (Versione Italiana)

Dai riscontri sulla rilevanza scientifica degli effetti dei PFASs sull'uomo, risulta necessario ampliare la conoscenza scientifica in merito agli effetti che questa classe di sostanze può avere sugli organismi dell'ambiente naturale in modo da non compromettere gli ecosistemi. A tal fine nel presente studio di ricerca sono stati valutati diversi biomarker di stress in modo da determinare quali possibili effetti abbiano tre miscele distinte di PFASs a concentrazione rilevata in natura sull'organismo modello *Dendobaena veneta*. I risultati hanno sottolineato come nel breve periodo (30 giorni), questi inducano un aumento di ROS a livello mitocondriale con conseguente aumento di danno genomico confermato dal Comet Assay. E' stato osservato una riduzione della capacità antiossidante negli organismi esposti a perfluoroalchilici, indicando come la risposta fisiologica sia impegnata a ridurre l'effetto di stress indotto da xenobiotici. Inoltre è stato riportato che l'esposizione a PFASs aumenti sia i livelli trascrizionali che i livelli traduzionali delle metallotioneine implicando un loro possibile coinvolgimento nella risposta antiossidante allo stress ossidativo nei mitocondri. Alla luce dei seguenti risultati, risulta opportuna la valutazione degli effetti di un esposizione cronica a sostanze perfluoroalchiliche. Inoltre è da investigare più nel dettaglio la funzione scavenger delle metallotioneine e il contributo del glutatione nella risposta antiossidante. Infine, sarebbe interessante determinare i meccanismi con cui questi xenobiotici permeano la membrana cellulare.

ABSTRACT (English Format)

From the scientific relevance findings concerning the PFASs effects on humans, it is necessary to extend the scientific knowledge about the effects that they may have on organisms in the natural environment. With this aim, in the present research study, different stress biomarkers have been evaluated to assess the possible effects that three different PFASs' mixtures at environmentally detected concentrations may have on the model organism *Dendobaena veneta*. The results underlined that, in the short term (30 days), these compounds induce an increase in ROS levels in the mitochondria with a consequent increase in genomic damage. It has been observed a reduction of the antioxidant capacity concerning perfluoroalkyls exposure, indicating the activation of the physiological stress response induced by xenobiotics. Furthermore, it has been reported that exposure to PFASs enhances both transcriptional and translational levels of metallothioneins implying their possible involvement in the response to oxidative stress in mitochondria. Looking ahead, it would be interesting to evaluate the effects of chronic exposure to perfluoroalkyl substances. Furthermore, it should be better investigated the scavenger function of MTs and the glutathione contribution in the antioxidant response. Moreover, it would be interesting to determine the mechanisms by which these xenobiotics permeate the cell membrane.

1 INTRODUCTION

In the last decades, several environmental problems linked to uncontrolled socio-economical development of multiple human activities around the world have emerged. In the last century, the average living standards of the so-called "first world", or rather developed, capitalist, industrialized countries aligned with the United States and shared more or less common political and economic interests, increased exponentially. Consequently, higher consumer demand led to increased production of pollutants due to a rise in industry productions, transport, agriculture, and urbanisation. To satisfy every human need, thousands, or better, millions of new substances have been produced and consequently released into the environment.

Unlike known contaminants, such as CO_2, NO_x, SO_2 and other greenhouse gases, oil spills, leachate and nutrients, another class of contaminants has undergone rapid development. For these compounds, in addition to the growing release into the environment, it is not even known how they act and how to remove them. These substances, also known as “contaminants of emerging concern” (CECs), include various chemicals such as pesticides, pharmaceuticals, cosmetics, personal and household care products and many others. The potential impact on the environment and human health was not specified before and is still unknown. It has been reported that between 1930 and 2000, global production of anthropogenic chemicals increased from 1 million to 400 million tons per year with an increasing production rate every year. In 2013 EUROSTAT, the statistical office of the European Union published statistics regarding the production of chemicals between 2002 and 2011. This report shows that environmentally harmful compounds represent more than 50% of the world production of chemicals and that over 70% of this 50% are chemicals with significant environmental impact (Gavrilescu et al., 2015; Geissen et al., 2015).

There are several anthropogenic sources from which CECs can be released into the environment that are primary point sources or diffuse sources. Once in the environment, pollutants distribute in different environmental matrices based on their physical-chemical properties. Their intrinsic pollutants properties are invasive because they can move and persist in different environmental compartments such as soil, sediments, air and water. Most of these substances have only been discovered in the last decades and further have not been discovered yet. Even the ones that have been discovered and classified as persistent pollutants are poorly regulated and adequate scientific knowledge is lacking. According to NORMAN (Network of reference

laboratories, research centres and related organisations for monitoring of emerging environmental substances), more than 700 different chemicals, appertaining to 20 different classes, have been identified in the European aquatic environment, for which poor or no disposal regulations have been settled (Geissen et al., 2015).

Moreover, the ecotoxicological assessment of most CECs has not been done yet and the effective environmental risk associated with some CECs release in the environment is still unknown. It is estimated that almost 2 million industrial and agricultural waste sewages are discharged into the environment every day worldwide. Furthermore, it is estimated that the annual global wastewater production is around 1500 km^3, or rather plus or minus six times more than the existing water in the rivers of the whole world (Geissen et al., 2015).

Although technical and scientific progress has led to the development and construction of good quality wastewater treatment plants (WWTPs) and wastes disposal in general, the environmental release of pollutants is still too high. Therefore, it is necessary to broaden the scientific knowledge about CECs and institute an early warning international system able to play the role of the "watchdog". Such a system might forecast the environmental risks associated with newly manufactured chemicals and prevent possible environmental contamination (Dulio et al., 2018).

1.1 The PFASs

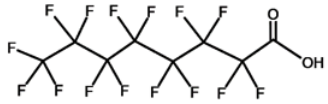
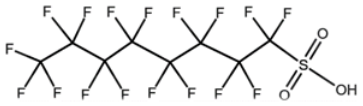
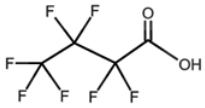
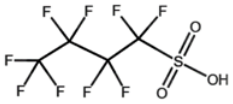
Within the class of Contaminants of Emerging Concern, Per and Poly-fluorinated compounds showed off. Perfluoroalkyl and Polyfluoroalkyl substances, also known by the acronym PFASs, are a broad family of human-produced compounds whose danger to the environment and human health has only been demonstrated in the last twenty years.

1.1.1 PFASs characteristic

PFASs class includes all compounds characterized by a carbon chain whose hydrogen atoms have been completely replaced by fluorine atoms, which render them highly hydrophobic, and by a hydrophilic functional group at the end of the chain. These fluorinated compounds of synthetic origin normally range between four and sixteen carbon atoms, and they can be divided into two different classes based on their functional groups. Indeed, it can be distinguished acid form compounds with a carboxyl group [R-C(=O)-OH] at the end of the chain and sulphonate form compounds with a

sulphonic group [R-S(=O)$_2$-OH] at the end of the chain. According to OECD (Organisation for Economic Co-operation and Development), PFASs can be divided, based on the length of the carbon chain, into "Short-chain PFASs" and "Long-chain PFASs". Long-chain PFAS include sulfonic perfluoroalkyl ($C_nF_{2n+1}SO_3H$, $n \geq 6$, PFSAs, where PFSAs stands for perfluoroalkane sulfonates) and carboxylic perfluoroalkyl acids ($C_nF_{2n+1}COOH$, $n \geq 7$, PFCAs, where PFCAs stands for perfluoroalkyl carboxylates) as well as their corresponding anions (USEPA, 2009; OECD, 2011). It has been proved that the chain length, as well as the functional group, influences the interaction properties of the molecules. "Short-chain" PFASs tend to be hydrophilic which influence their high rate of mobility. Oppositely, "long-chain" perfluoroalkyl molecules are inclined to give bioaccumulation phenomena due to their higher hydrophobicity.

Table 1.1 Example of PFASs classification.

PFASs classification based on functional groups and chain length		
Carbon chain length	**Perfluoroalkyl carboxylic acids R-C(=O)-OH**	**Perfluoroalkyl sulfonic acids R-S(=O)$_2$-OH**
Long-chain PFASs ***(8 carbon atoms)***	 *Perfluorooctanoic acid (**PFOA**)*	 *Perfluorooctanesulfonic acid (**PFOS**)*
Short-chain PFASs ***(4 carbon atoms)***	 *Pentafluorobenzoic acid (**PFBA**)*	 *Perfluorobutanesulfonic acid (**PFBS**)*

A more detailed classification of PFAS was first proposed by Buck et al., 2011 and subsequently revised in Ahrens et al., 2014. This classification was more comprehensive by dividing the PFAS into three broad classes: Perfluoroalkyl substances (PerFASs), Poly-fluoroalkyl substances (PolyFASs), and Fluorinated polymers.

PerFASs are characterised by a fully fluorinated alkyl chain, such as perfluoroalkane sulfonates (PFSAs; $C_nF_{2n+1}SO_3^-$), perfluoroalkyl carboxylates (PFCAs; $C_nF_{2n+1}COO^-$),

perfluoroalkyl phosphonates (PFPAs; $C_nF_{2n+1}[O]P[OH]O^-$), perfluoroalkyl sulphonamides (FASAs; $C_nF_{2n+1}SO_2NH_2$), perfluoroalkyl sulphonamidoethanols (FASEs; $C_nF_{2n+1}SO_2NHCH_2CH_2OH$), and perfluoroalkyl sulphonamidoacetic acids (FASAAs; $C_nF_{2n+1}SO_2NHCH_2COOH$). Oppositely, PolyFASs are characterized by a partially fluorinated alkyl chain that contains at least 1 fluorine atom. Such class includes polyfluoroalkyl phosphoric acid esters (PAPs; $[O]P[OH]_{3-x}[OCH_2CH_2CnF_{2n+1}]_x$), fluorotelomer alcohols (FTOHs; $C_nF_{2n+1}CH_2CH_2OH$), x:2 fluorotelomers sulfonates (FTSAs; $C_nF_{2n+1}CH_2CH_2SO_3^-$), x:2 fluorotelomer carboxylates (FTCA; $C_nF_{2n+1}CH_2COO^-$), x:2 fluorotelomer unsaturated carboxylates (FTUCA; $C_{n-1}F2_{n-1}CF¼CHCOO–$), n:2 fluorotelomer saturated aldehydes (FTALs; $C_nF_{2n+1}CH_2CHO$), and n:2 fluorotelomer unsaturated aldehydes (FTUALs; $C_{n-1}F2_{n-1}CF¼CHCHO$). The third class are Fluorinated polymers which include a broad variety of chemical substances, divided into three subclasses: Perfluoropolyether, Fluoropolymers and Side-chain fluorinated polymers (Ahrens and Bundschuh, 2014). These xenobiotic compounds have been widely applied in several usages thanks to their chemical-physical properties, which confer them:

- High molecular stability due to the carbon-fluorine bonds
- High heat resistance
- Low boiling point
- Weak surface tension
- Hydrolysis resistance at extreme pH

As a consequence of their intrinsic properties, they are highly resistant to biological, chemical and photochemical degradation. Furthermore, the amphipathic nature of these substances, due to the coexistence of a hydrophilic group and a hydrophobic group, attributes to PFASs treated materials water and oil repellent properties (Kissa, 2001; Buck et al., 2011).

1.1.2 PFASs history and environmental disasters

The PFASs are a family of synthetic organic chemicals that have been produced since the mid-20th century. According to their astonishing physical-chemical properties such as friction-reducing agents, oil and water repellent and temperature resistance, they have been widely implemented in the industries. PFASs have been mostly used as textiles coatings, of which the best-known example is the Gore-Tex. Other applications are in firefighting foams, cookware, food packaging, paper products and insulating

sheaths, they have so a wide range of possible applications in food, photographic automotive, electronics, construction, aviation and aerospace industries.(KEMI 2015; USEPA 2017b). It has been estimated that between PFASs invention till nowadays, a number ranging from 5 to 10 thousand PFAS species have been fabricated and that more than 4,700 PFAS have still been identified on the global market (USEPA 2018) (OECD 2018). Due to the wide range of PFASs classes and structures, several production processes have been implemented. The two most used processes for PFASs molecules production such as fluorosurfactants and side-chain fluorinated polymers are electrochemical fluorination (ECF) and telomerization (KEMI, 2015). Electrochemical fluorination (ECF) is the first reported process for PFASs production, and it has been patented in the 1940s by 3M (Minnesota Mining and Manufacturing Company), an American multinational leader in the production of chemicals (Banks and Tatlow, 1994). Although the ECF has been used for some time, the yield of the linear products was around 70% while the remaining 30% was made up of branched-chain polymers which had no use and were therefore disposed of as waste (Concawe, 2016). On the other hand, the fluorotelomerization process has been developed only in the 1970s, this PFASs production technique allowed to obtain an optimal yield of linear chain polymers (Kissa, 2001).

Only at the beginning of the 70s, the increase in tumours and other related pathologies in workers exposed to the production of perfluoroalkyl has led to a greater awareness of the potential health effects of human exposure PFASs. Several studies have found perfluoroalkyl substances in the blood of workers exposed during the production cycle. However, it was not possible to link the increase in the incidence rate of tumour pathologies with PFASs in the blood. Subsequently, this investigation has been extended to populations near the production plants but not primarily involved in PFASs production. In the 1990s PFASs were also found in their blood (Buck et al., 2011). Following these findings, global awareness of PFASs has increased exponentially and several nations have taken steps to monitor these substances. It should be emphasized that the unique surfactant properties of PFAS hindered the analytical detection, which in those years was also not yet sufficiently developed for the detection of these complex molecules (Giesy and Kannan, 2001).

In this regard, since then, various researches have primarily confirmed the presence of PFASs in underground water intended for human consumption. Secondly, their discovery has also been reported in various environmental matrices, including

sediments, surface waters in the oceans, at the poles and in the biota, including animals from different ecological niches and environmental distribution (Higgins et al., 2005; Yamashita et al., 2005; Rankin et al., 2016).

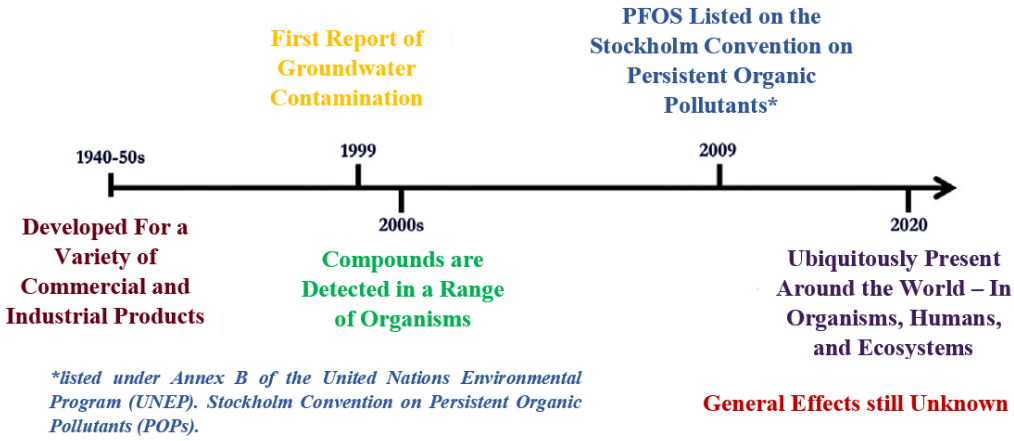


Figure 1.1 PFASs timeline and historically important events. From Sinclair et al., 2020

The turning point on the regulation of PFAS came following a class action implemented by the community of Little Hocking, Ohio U.S.A. towards one of the biggest multinationals company producing PFASs, or rather the DuPont. Since it was known that DuPont used to release PFOA (Perfluorooctanoic acid) into the river and that the population relied on local wells for water supply, it was easy to link people heath issues in the Ohio river basin to PFOA. DuPont has been therefore found guilty of PFASs environmental contamination in the Ohio river basin. For this purpose, a community advisory committee (CAC) was formed with residents served by the water supply, state and federal government representatives, local doctors and researchers. Peoples blood had been tested and the PFASs concentrations were 80 folds normal people's concentration in other counties. As a consequence, the Minnesota Department of Health (MDH) developed "Health-Based Values for PFOS and PFOA", following, health-based values have been settled also for PFBS (Perfluorobutanesulfonic acid), PFBA (Pentafluorobenzoic acid), and PFOS (Perfluorooctanesulfonic acid) surrogates such as PFHxS (Perfluorohexanesulfonic acid) (ITRC, 2020).

In the meantime, in 2000 the 3M company announced a voluntary phase-out of perfluorooctanoic chemistries, which included PFOS, PFOA, PFHxS and related precursors, as a consequence of personal internal verifications of PFOS and PFOA possible risks. In 2002, 3M reported completed most of the phase-out of long-chain production and declared the remaining phase-out completed by 2008. Furthermore, It

was proved that PFOS might bind blood proteins. It was associated with several health issues, and it showed bioaccumulation phenomena and long half-life in humans. Furthermore , it has been evaluated as dangerous for human health and in 2009 it was inserted in Annex B of the Stockholm Convention as a persistent organic pollutant. (Olsen et al., 2003; Kannan et al., 2004; Kannan et al., 2006).

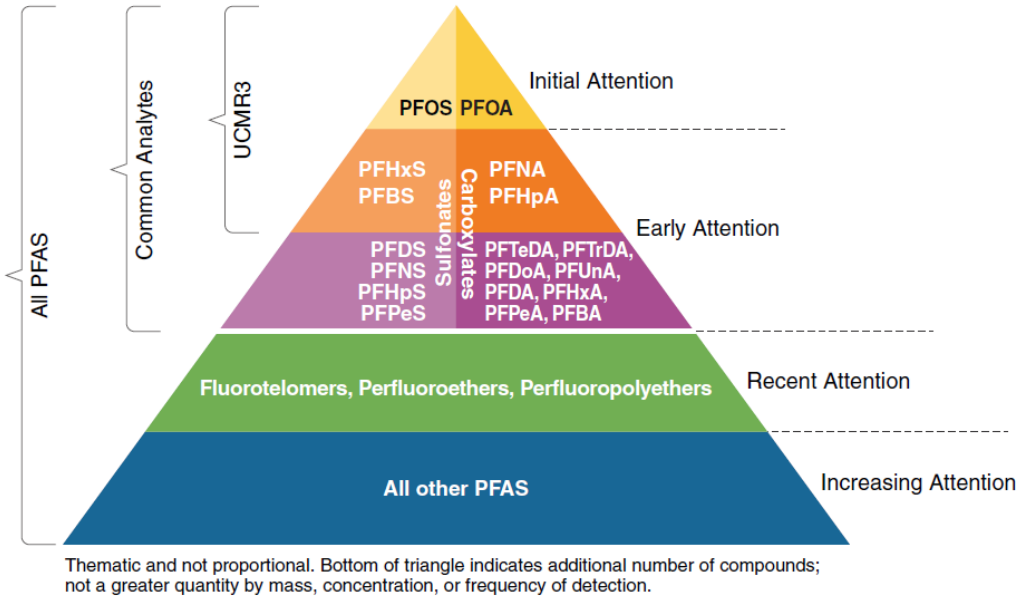


Figure 1.2 PFASs classification based on their knowledge and on increasing awareness of their presence in the environment. From Hale, 2016.

In the following years, awareness and attention on PFAS have increased exponentially. The detailed study initiated on PFOS and PFOA gradually presented itself to other compounds, including classes of attention compounds, monitoring classes and possible contaminants. In 2006, USEPA, the U.S. Environmental Protection Agency, started the PFOA Stewardship Program. The eight major manufacturing companies aimed to reduce PFOA, longer-chain PFASs and related precursors (USEPA 2006). In 2016, the USEPA finally issued a Lifetime Health Advisory (LHA) for the PFOS and PFOA. The limit set for these compounds was 70 nanograms per litre, equivalent to parts per trillion (ppt). This limit was then applied to each PFAS, as well as PFASs combination, in water intended for drinking (USEPA 2016). In 2017, USEPA reported that all eight companies successfully satisfied the program goals, met a 95% reduction by 2010, and eliminated 2015 (USEPA 2017). Despite so, in 2016, PFOS and derivatives continued to be undisturbed produced in Germany, Italy, and China and continued to be produced for several follow years.

On February 13, 2017, the 2001 class action stirred up by Little Hocking Community regarding DuPont, ended. The jury judged DuPont responsible for the environmental PFOA disaster, for the health effects of workers implemented in PFASs production and the personal injury caused to the entire Little Hocking community. For these reasons, DuPont agreed to pay $ 671 million compensation to approximately 3,550 people for personal injury.

Since PFASs play a unique role and no surrogates have yet been invented to replace them, they are still being produced. The manufacturers reformulated and substituted "Long-chain" PFASs with alternative technologies such as shorter or branched-chain perfluoroalkyl substances. Examples of these new technologies are PFBS, PFBA, GenX and ADONA (per- and polyfluoroalkyl ethers) (Wang et al., 2015). Even if it seems that such compounds achieve the same performance effectiveness as some of their predecessors, however, it has not been confirmed for all replacement PFASs yet. Furthermore, some replacement chemicals might or might not be less hazardous than their respective longer-chain predecessors. Indeed, publicly available information about hazardous properties on most replacement chemicals are still limited (Wang et al., 2015). In addition, there is no information about PFASs replacement' environmental contamination due to both the knowledge gap on these substances and difficulties in detecting them with the analytical methods currently available (Wang et al., 2013). Furthermore, unlike Long-chain PFASs, which WWTPs treatment processes have well trapped, these new replacement substances are less effectively trapped and so their environmental loads are much less controllable (Sun et al., 2016).

1.1.3 PFASs environmental trends

As mentioned in the previous chapters, PFASs release into the environment causes their partitioning in different compartments. The PFAS can be directly introduced into the environment through the production cycle or indirectly through the transformation of their precursors. PFASs diffusion into the environment can take place through point-like sources or non-point sources, or widespread. Point sources include, for example, production plants, sewage treatment plants and landfills. In contrast, non-point sources are mainly represented by leaching of contaminated sites, transport or dry and wet deposition of the volatile component of perfluoroalkyl substances present in the atmosphere (Ahrens and Bundschuh, 2014). PFASs transport and fate in the environment have been widely investigated at multiple scales and levels. Until

nowadays, researches about PFASs impacts have primarily focused on their environmental presence and transport in the atmosphere, surface water, and groundwater. Only in recent years, perfluoroalkyl substances contaminated soil has been considered a source of primary pollution (Brusseau et al., 2020). According to ongoing research on soil and vadose zone of the aquifers, it is possible to address soil as PFASs' reservoir (Anderson et al., 2016).

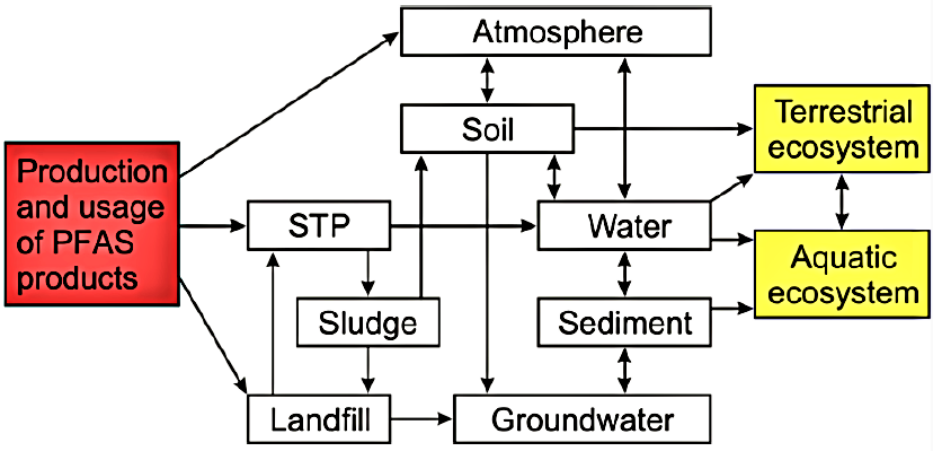


Figure 1.3 PFASs pathway from production to their environmental fates. STP = Sewage treatment plant. From Ahrens and Bundschuh, 2014.

The surfactant nature of PFASs and the wide number of properties that they might have, make it difficult to predict the transport and fate of PFASs. It was first shown that PFASs molecular chain-length and functional groups affect the interactions with media. Secondly, their repartition between aqueous and solid phases is strongly influenced by the aqueous phase's ionic composition and solid surfaces' characteristics. Indeed, the intermolecular interaction between PFASs and soil solids depends principally on electrostatic forces and by solids chelating properties (Brusseau et al., 2020).

Despite the potential accumulation of PFASs in soil, water and atmosphere remain the compartments and pathways through which these substances diffuse into the environment. Some research has shown that most of the emissions (more than 95%) are due to wastewater from industrial production factories that are directly released into the water environment. In comparison, emissions into the atmosphere are considered negligible (less than 5%). Other studies have shown that PFASs precursors are typically transported through the atmosphere due to their volatile or semi-volatile properties. In the atmosphere, these precursors can undergo various abiotic reactions, thus forming different transformation products that are highly reactive. These degradation products can precipitate in the aqueous phase or be transported into the atmosphere linked to

atmospheric particles or incorporated into the marine spray. Thanks to atmospheric transport, the presence of PFASs has also been detected in remote regions of the Arctic Ocean and in Antarctica where there are no PFASs production plants, and their presence is therefore only attributable to atmospheric transport. In addition to the degradation products of the precursors of PFASs, the presence of ionizable PFASs has also been found in these remote regions. Despite various researches on this topic, it is still debated whether atmospheric transport to remote regions is the dominant transport path for ionizable PFASs. At the same time, it is almost certain that the gas phase is the dominant path for neutral and volatile PFASs transport (Ahrens and Bundschuh, 2014).

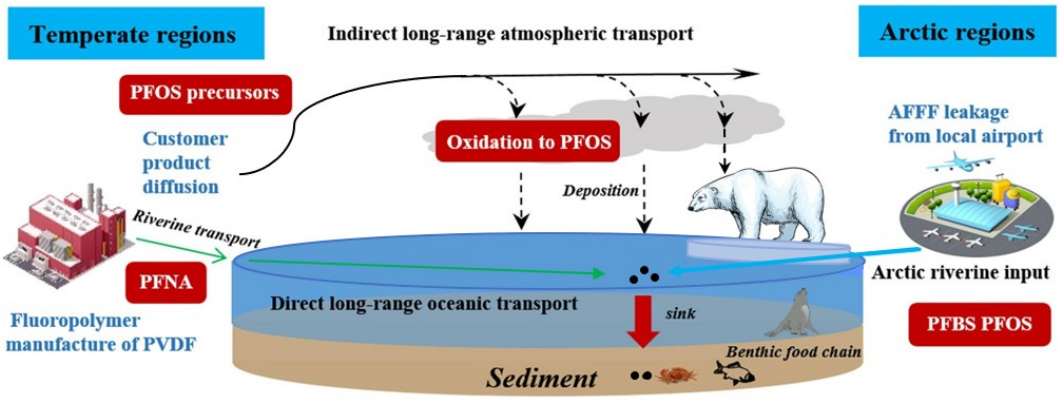


Figure 1.4 Example of possible PFASs pathway in order to explain PFASs presence in the Arctic ocean and in Antarctica. Modified from Lin et al., 2020. AFFF = Aqueous Film-Forming Foam.

In addition to wastewater of the production plants, a considerable amount of PFAS-containing consumer products contributes to PFASs upload in the environment. For these reasons, a brief description will be made below of landfills, which are known to be one of the primary sources of PFASs contamination. Once these compounds, such as cooking ware, food packaging, paint, textiles, arrive at the end of life, they are directly disposed of in landfills. Due to water solubility property, several PFASs can dissolve in landfill leachates and then be released due to water solubility property. It has been proved that PFASs-containing products in landfills are likely to release PFASs continuously for years. Cui et al in 2020 referred that it is estimated that between 563 and 638 kg of total PFASs mass were released in landfill leachate in 2013 in the US. This is possible due to no regulations on the disposal of PFAS-containing products. The real problem is that landfill leachate is usually managed by WWTPs or by leachate treatment facilities but in some cases. It is directly released into surface or groundwater sources. It is precisely for this last case that landfill leachate is considered one of the main sources of PFASs release into the environment (Cui and Quinete, 2020). Since

these substances have been found in almost every environmental compartment, in animals and humans, they have been considered ubiquitous contaminants. It is estimated that, among the environmental media, the largest reserves of these compounds are sediments and oceans.

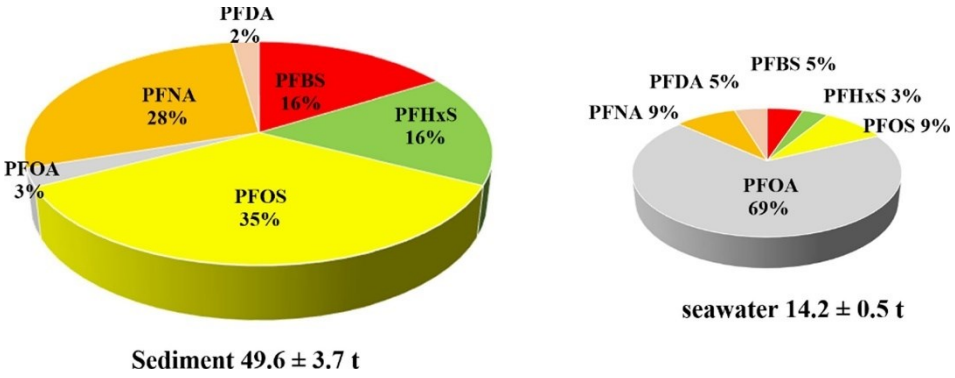


Figure 1.5 Example of possible PFASs inventories and compositions in seawater and sediments of the Bering Shelf and the Chukchi Sea. From Lin et al., 2020.

As long as the effects that these substances might cause have not yet been entirely determined. The ubiquitous presence of PFAS in soil, air, water and biota poses a potential danger to nature and public health. It is, therefore, of primary importance to reduce the load of PFASs in the environment until the risk assessments for these substances emerge (Mussabek et al., 2020).

1.1.4 PFASs health effects

The studies of the effects of PFASs, a high-interest actual topic, in truth, were firstly conducted by Taves in the late 1960s and early 1970s. Although they did not have much resonance at the time, these pioneering studies detected perfluoroalkyl substances in the blood of humans and non-humans bodies. (Taves, 1968; Taves, 1971). Subsequently, Gilliland studied the effects of Long-chain PFASs on employees of a perfluoroalkyl acids production plant. Its results reported no associations between the cause of death and length of employment in employees, but a mortality ratio of 2.03 for prostate cancer has been observed in exposed males to PFOA in comparison to 0.58 in non-exposed males (Gilliland and Mandel, 1993; Gilliland and Mandel, 1996). Only in the early 2000s, many epidemiological studies have been conducted following the ubiquitous worldwide detection of PFAS. It emerged that perfluoroalkyl substances have been found in all human tissues and that they tend to bioaccumulate in particular in the bones, the blood and the liver tissue (Buck et al., 2011; Pérez et al, 2013). It has been demonstrated that prolonged exposure to PFAS can cause alterations to the

respiratory, nervous and reproductive systems or give rise to immunotoxic and hepatotoxic effects. in addition, alterations of hormones at the endocrine level have been relieved. Moreover, it was observed alterations to enzymes of hepatic tissues such as acetyl-CoA, oxidase and dehydrogenase, which result in liver oxidative damage to the DNA due to hydrogen peroxide production (Guruge et al., 2006; EFSA 2008; Lau et al., 2007; Hu and Hu, 2009; Bjork et al., 2011; Pedersen et al., 2016). It has also been proved the ability of these substances to cross the placenta from the mother's blood to the child (Kristensen et al., 2013). Other researches showed that PFASs presence in the human body might cause various types of dysfunctions such as reaching puberty at a younger age, thyroid diseases, hypercholesterolemia, ulcerative colitis, prostate cancer, testicular and kidney tumours (Olsen et al., 2001; Melzer et al., 2010; Wen et al, 2013; Watkins et al, 2013; Goudarzi et al., 2017).

1.1.5 Cleaning technologies and knowledge gaps

Once the effect of PFASs on human health has been demonstrated, awareness of possible environmental and ecosystem damage has accordingly increased. To ensure a sufficient level of environmental quality of rivers and oceans water, the role of wastewater treatment plants (WWTPs) is fundamental. To reduce the contribution of perfluoroalkyl substances to the environment, various techniques have been tested. To extract and therefore reduce perfluoroalkyl concentrations from the wastewater, it was initially necessary to place filters. These filters were mainly made up of activated carbon, a substance capable of binding large quantities of PFASs. Several relevant research articles have been published concerning PFOS and PFOA removal, while technics regarding their substitution compounds removal have been almost ignored. This might be the cause of both knowledge gaps about specific features of these materials and how to reduce their concentration in the water. (Fujii et al., 2007; Vecitis et al., 2009). The problem concerning PFASs in WWTPs is about the high load that they receive. Indeed manufacturing and industrial wastewater, as well as household wastewater all stream into WWTPs (Cui and Quinete, 2020).

In traditional wastewater treatment plants, the load of pollutants is reduced through sand filtration, through chemical reactions and mainly through biological reactions that allow the precipitation of different substances. Due to the stability of the PFASs, they do not tend to react and do not degrade through biological reactions. This is why a

normal WWTP is not enough to reduce the environmental load of perfluoroalkyl. Several techniques have been adopted over the years.

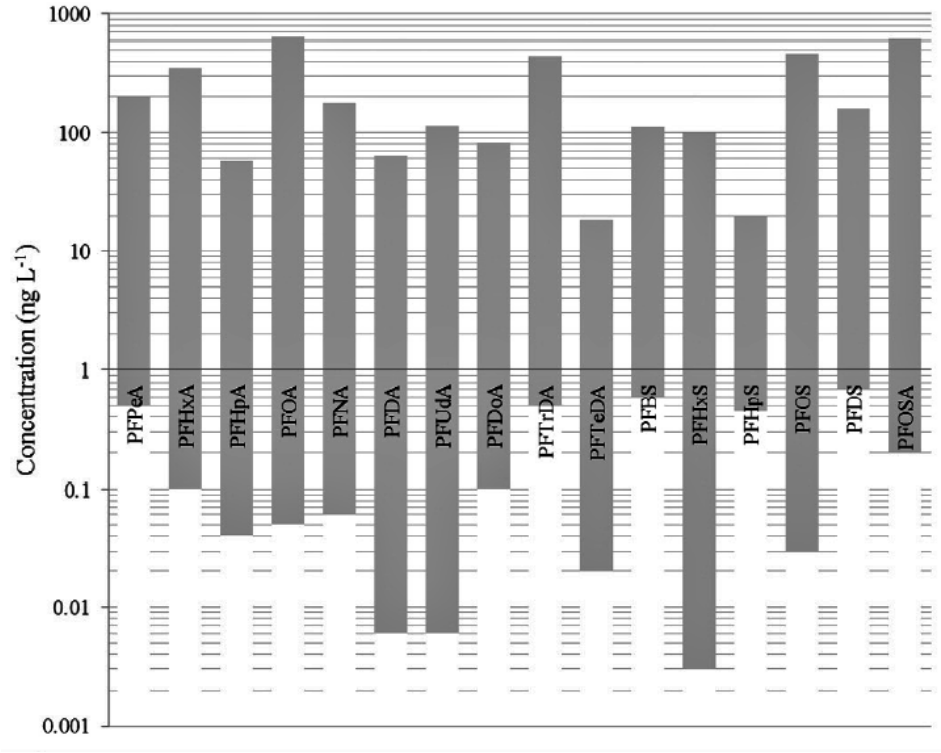


Figure 1.6 PFASs concentration ranges in influent wastewaters worldwide. From Arvaniti et al., 2015.

Among the best known is the use of activated carbon in filters of different porosity. The PAC (Powdered Activated Carbon) and the GAC (Granular Activated Carbon) stand out. In 2014 Appleman reported the high removal efficiency on longer chain PFASs such as PFOA, PFOS, PFHxS by using coal-based GAC. On the other hand, partial removal rates, 33%, 74%, and 91%, were obtained for the shorter chain PFASs PFBA, PFPeA, and PFHxA. These results agreed with the assumption based on previous studies about the higher affinity of long-chain PFASs for organic carbon. This study also reported the application of RO (Reverse Osmosis) filters, or rather polyamide Hydranautics ESPA2 membranes-based filters. It results to be the best PFASs removal by removing the totality of both long and short-chain PFASs. The complications of RO filters are mainly their high costs (Appleman et al., 2014).

In 2015, Arvaniti et al. reported several techniques for PFASs removal such as adsorption, filtration, chemical oxidation and reduction. The most known adsorbent materials, as mentioned before, are activated carbons. This study compared the efficiency rate due to adsorbent competition between activated carbon and organic materials present in the wastewater. The results indicate that organic matters reduce

filter efficiency. Other adsorbents have also been tested such as anion exchange resins, zeolite mineral materials (alumina, silica, goethite), carbon nanotubes, molecularly imprinted polymer and cross-linked chitosan beads. The results suggest that all the techniques were valid options for PFASs removal but still, the best options were GAC and anion exchange resins which have the higher removal rate of long-chain PFASs such as PFOS and PFOA (Arvaniti et al., 2015).

As for Appleman, Arvaniti also tested the potential of RO filtration for PFASs removal in comparison to nanofiltration techniques (NF). The results indicated that NF removal rates ranged between 90% and 99% while RO filtration, due to the smaller pores and thicker rejection layers, had even higher effectiveness in PFASs removal.

In another study, the NF membranes were tested for both long and short-chain PFASs removal. The authors reported that effectiveness higher than 95% has been registered for perfluoroalkyl compounds with a molar weight higher than 300 g/mol, in optimal experimental conditions, using deionized water (Steinle-Darling and Reinhard, 2008).

PFASs removal through oxidation methods includes Advanced oxidation processes (AOPs) by using hydrogen peroxides as oxidant. Since PFASs don't have any hydrogen molecules bonded on their main body, it results in no possible attachment sites for oxidation processes. The oxidation methods resulted so ineffectively for PFASs removal. A decent removal yield was only obtained under extreme laboratory conditions, which are hardly applicable to a full-scale plant.

Very little research has been published on reduction processes for PFASs removal, the available ones reported the use of Zero-Valent Iron (ZVI). This methodology resulted in ineffective PFOS removal since partial degradation has been obtained only under subcritical experimental conditions, temperature higher than 250°C and pressure higher than 20MPa. PFOA removal yield ranged around 70%, the overall results suggested the inefficiency of this methodology for PFASs removal from wastewater (Arvaniti et al., 2015).

Another recent study reported the use of innovative technology for PFASs removal such as cross-linked poly DMAPAA-Q hydrogel, composed by the cation monomer of N-[3 (dimethylamino)propyl] acrylamide, methyl chloride quaternary (DMAPAA-Q). This ingenious technology has been tested as removal option for 5 long-chain PFASs, ranging from 8 to 10 carbon atoms, and for 11 short-chain PFAS, ranging from 4 to 7 carbon atoms including PFBS, PFBA, NaDONA and GenX. To verify the possible application of this technology to large-scale water treatment plant (WTP), different

types of water containing the PFASs mixtures mentioned above have been tested. It was tested simultaneously deionized water, as laboratory reference, two surface water bodies water such as lake water and water influent to a (WTP) and treated water from a WWTP.

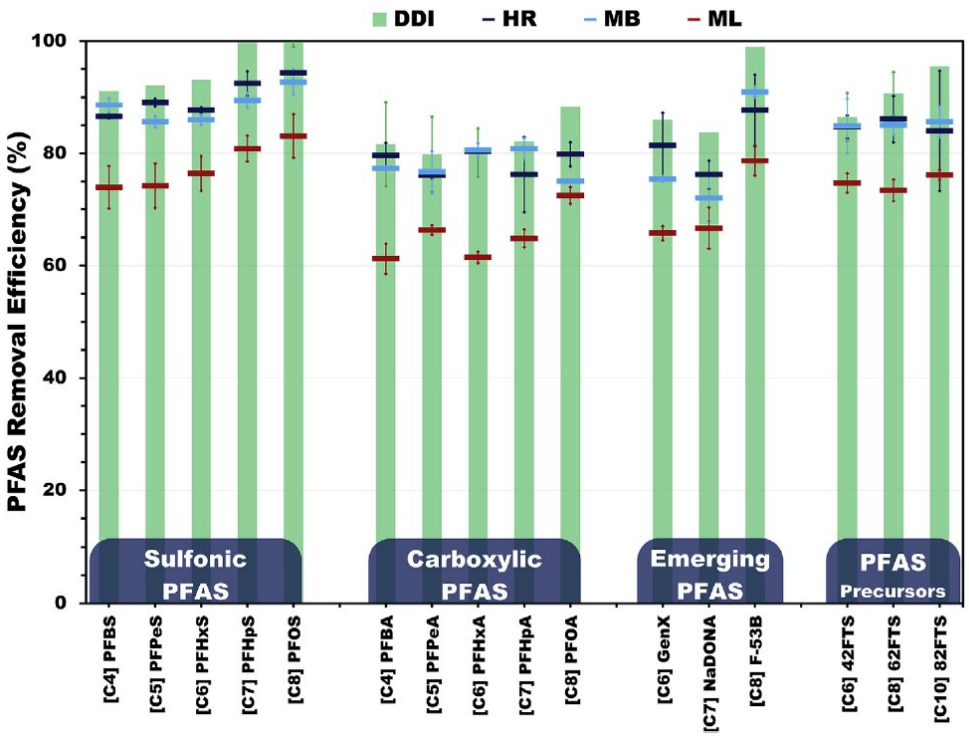


Figure 1.7 Graphical explanation about the 16 tested PFASs molecules (1000ng/L) yield of removal by poly DMAPAA-Q hydrogel (70 mg/L) in distilled deionized water (DDI), lake water (HR), water influent to WTP (MB), and treated wastewater (ML). C4 to C10 represent the number of carbon atoms in each compound. Vertical error bars show the standard deviation from the triplicated experiments. From Ateia et al., 2019.

The results showed the high efficiency of the hydrogel as a PFASs binder for both Long and short-chain PFASs. Furthermore, it has been tested the possibilities of hydrogel regeneration and its efficiency after several cycles of regeneration by obtaining good results. It is therefore possible to conclude that hydrogel is one of the most valid technologies currently on the market for the removal of PFASs from water (Ateia et al, 2019). However, it must be emphasized that long-chain PFASs have a greater affinity to absorbents, of whatever media they are made of. For these reasons, any filter material, which is used to filter a PFAS long and short chains mixture, initially tends to bind both, but over time will tend to release previously adsorbed short-chain PFASs replacing them with longer chain PFASs with higher affinity.

In addition to PFASs problematics in water, another huge problem that renders WWTPs one of the main sources of PFASs'environmental contamination is the production of

contaminated sewage treatment sludge. These sludges are treated as hazardous in the USA and most of the European countries such as Germany, Spain, Greece, Switzerland, the Netherlands, Denmark, and Sweden, for which the environmental PFASs' loads are regulated. Data about sewage treatment sludge from the rest of European countries are missing, for which is known that up to 80% of the produced sludge is reused in agriculture as fertilizer. It has been observed that these sludges released the PFASs adsorbed in with the time, and they contribute so as diffuse source of environmental pollution (Semerád et al., 2020). In Italy for example, they are still used as agricultural fertilizer and only recently a debate has been opened on their possible hazardous properties for human and environmental health.

1.2 THE SITUATION IN THE VENETO REGION (ITALY)

From the results obtained by the studies carried out in America on perfluoroalkyl substances to clarify the dynamics of environmental dispersion and the consequences that can lead to humans, the attention on PFASs pollution is increased. It was therefore activated several research projects to monitor the presence of these pollutants in the European environment since several factories producing PFASs are distributed onto this territory.

In 2006 it was implemented the project PERFORCE, which included the examination of waters and sediments of the major European river. As a consequence, it was found that the Po River is heavily polluted by perfluoroalkyl substances, and it results to be the most polluted river by PFAS of Europe. IRSA-CNR (Water Research Institute, National Research Council) implemented, on behalf of the Ministry of Health, three successive monitoring campaigns in the Veneto region, or rather in 2011, 2012 and 2013. The high ecological and health risk have been then confirmed. Furthermore, it has been revealed that the pollution plume extended not only to surface waters but also to deep fault waters from which water is extracted for human consumption, to irrigate crops and for livestock consumption, thus implying potential risks for humans (ENEA, 5/5/2016).

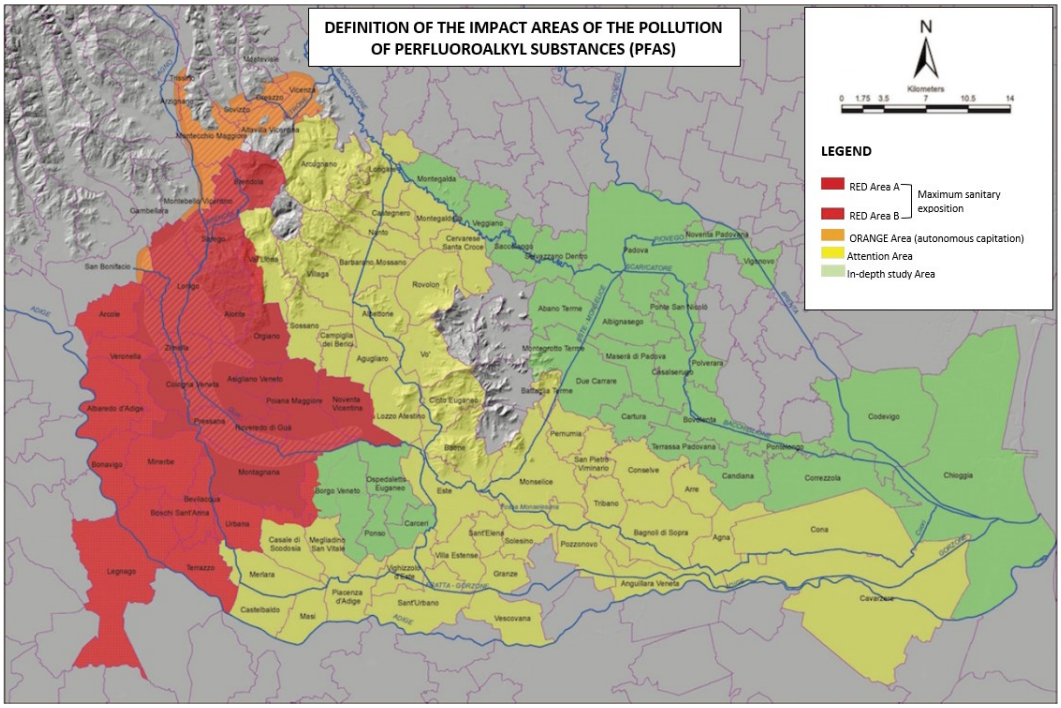


Figure 1.8 PFASs contaminated area in Veneto region (venetoeconomia, May 2018)

Subsequently, ARPAV (Regional Agency for Environmental Protection in the Veneto) embarked on an in-depth analysis campaign extended to the entire water network in the Veneto. The analysis campaign conferred priority to wells intended to supply the aqueducts of multiple municipalities. The investigation area included the lower Agno valley (Vicenza), Vicenza, Verona and Padua provinces. Subsequently, it has been stated that the polluted area extends to 150 Km^2, affecting 30 municipalities and a population of about 300 thousand citizens. It was also found that 24 municipalities have perfluoroalkyl concentrations higher than those proposed by the Ministry of Health.

Following a cross-analysis on the peaks concentration of PFASs, it was possible to attribute the cause of this environmental disaster to the company MITENI S.p. A, a company with headquarters in Trissino (VI). It also appears to be the only company producing perfluoroalkyl substances for industrial use in northern Italy. MITENI S.p.A was a company founded in 1965, specialized in the production of perfluoroalkyl substances for industrial use, it has produced long-chain compounds such as PFOS and PFOA until 2011, the year in which new regulations have banned the production of long-chain perfluoroalkyl in Italy. Afterwards, it produced short-chain compounds only such as PFBA, PFPeA, PFBS, PFHxA, PFHpA, PFBS and GenX till September 2018. In October 2018, the MITENI S.p.A was considered permanently bankrupt.

This environmental contamination is one the largest reported cases of PFASs high residential exposure. Due to similarity in characteristics, extent, and origin, it resembles the PFASs contamination disaster that occurred in the Mid-Ohio Valley, in the United States, approximately between 1984 and 2004 (Frisbee et al., 2009). To protect the population' health, activated carbon filters have been installed on the aqueducts intended for human consumption. Activated carbon filters have the purpose of retaining and therefore reducing the concentration of PFAS in the water. When this environmental disaster happened, in Italy there was no specific legislation concerning the release of PFAS in the environment. Therefore, it was allowed to discharge into the water network PFASs concentrations equal to surfactants (amphipathic nature molecules, used as wetting agents or suspensants in the production of paints, emulsifiers, detergents and inks), which law limit is 500µg/litre.

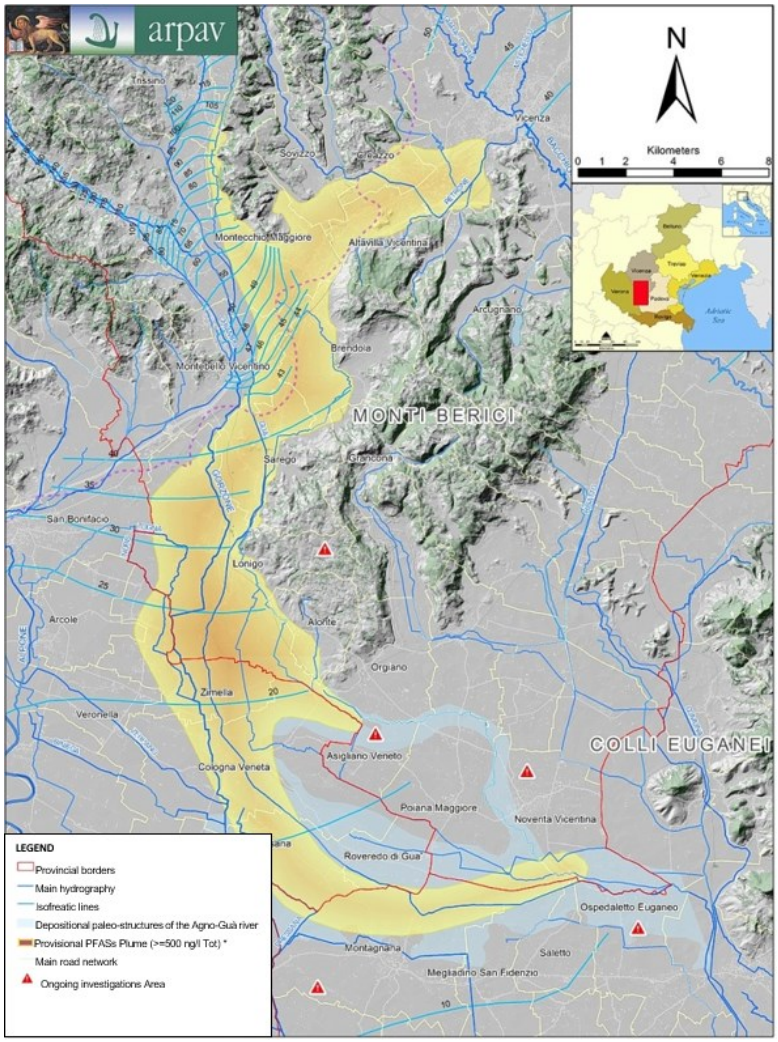


Figure 1.9 Plume of underground aquifer pollution (ARPAV)

On 29 January 2014, the National Ministry of Health, through the opinion of the Superior Institute of Health (ISS), established target performance values for perfluoroalkyl substances in the water for human and zootechnical consumption:

- PFOA ≤ **500 ng/ L**
- PFOS ≤ **30 ng/ L**
- Other PFAS (sum of the remaining 10 perfluoroalkyl compounds) ≤ **500 ng/L**

Afterwards, it was made known that the activated carbon filters have poor effectiveness to short-chain compounds. The Superior Institute of Health have then defined specific limit values for two other short chains (4 carbon atoms) perfluoroalkyl substances by considering the potential danger to health:

- PFBS ≤ **500 ng / L**
- PFBA ≤ **500 ng / L**

The PFASs values measured in the aqueducts affected by contamination after 2013 are around 100 ng / L for PFBA, around 50 ng / L for PFBS, around 80 ng / L for PFOA, around 100 ng / L for Other PFASs. Furthermore, it has been detected that PFOS average concentration of about 4 ng / L against a performance value of 30 ng / L (Regione del Veneto).

1.3 THE LIFE PHOENIX PROJECT

Perfluorinated compounds HOlistic ENvironmental Interinstitutional eXperience

WITH THE CONTRIBUTION OF THE LIFE FINANCIAL INSTRUMENT OF THE EUROPEAN UNION
LIFE16/ENV/IT/000488 - LIFE PHOENIX

For the scope of implementing a monitoring campaign aimed at protecting the health of the population in the Veneto region, it has been implemented the Life Phoenix Project. On August 08, 2017, with the Veneto Regional Council Deliberation n°1245, the Life16 / ENV / IT / 000488 Phoenix "Perfluorinated compounds Holistic ENvironmental Interistitutional eXperience" project was approved.

The goal of the project is to implement and develop a new governance model for the Veneto region, which allows controlling the risk of contamination by Persistent Mobile Organic Emerging Contaminants (PMOC) with particular attention to short-chain PFASs. The project is the normal consequence of avoiding or reducing public health

costs due to damage to people caused by pollutants of anthropogenic nature, with particular reference to drinking water and irrigation water. The project has been coordinated by the Veneto Region, lead partner, and it had the collaboration of Azienda Zero, Veneto Regional Agency for Environmental Prevention and Protection (ARPAV), CNR Water Research Institute (IRSA-CNR) and the University of Padua.

The project has been co-funded by the European Union (EU) under the LIFE-Environment program. The overall project duration was firstly supposed to runs from 1st September 2017 to 30 September 2020, the deadline has been then postponed due to Sars-Covid 19 issue to 31st March 2021. The following guiding principles inspire the Life Phoenix actions:

- Promptly and effectively prevent the risks liked to the spread of emerging contaminants in the environment.
- Guaranteeing the safety of drinking water, the protection of consumer health by monitoring the water quality.
- Promote conscious and sustainable use of water, following the general European guidelines of safeguarding water resources.

The Goals of the project are:

- Draw up specific guidelines on how to face up environmental and public health risks caused by PMOC contamination.
- Promptly provide innovative tools to estimate the presence, distribution and risk of PMOCs, to support the risk assessment analysis.
- Establish a permanent regional commission on PFASs, supported by a multidisciplinary group of experts and stakeholders. This council aim to implement policy measures and actions to limit and prevent problems caused by PFAS.
- Suggest contamination mitigation strategies based on innovative technologies and on natural solutions.

- Raise awareness among the population and stakeholders on the importance of an effective water resource system by prevention and protection.
- Replicate the working methodology, or rather, transfer the design approach and solutions applied in this project to other geographical contexts characterized by similarity of characteristics, extent, and origin of the environmental contaminants.

In order to achieve the objectives listed above, the project structure has been divided into different subprogrammes, or rather, operational/implementation actions (**action B**), monitoring actions (**action C**) and public awareness and dissemination of the results (**action D**). Each subclass has been further divided in specific goals in order to achieve each one independently.

Action B1: Organizing the control of danger and risk analysis system
The implementation of an inter-institutional system that will monitor and manage the issues concerning PFASs issue. This Permanent Regional Commission is supported by working groups of experts who will interpret the perceived information and elaborate specific results based on the information and statistical system. Action B.1 will draw up control and management guidelines for problems relating to PMOC contamination. Furthermore, it will define a prevention system and an action plan to manage PMOC post-contamination. The results of the project will be possibly proposed as potential options to other European contexts affected by similar PMOC contamination.

Action B2: Implementing information and statistical system
The informational system will be upgraded by numerous databases from various regional and national institutions. This action aims to make information easy and immediately accessible to everybody, through a dedicated portal. The databases will be organized in different thematic areas to facilitate the finding of data.

Action B3: Innovation and technological development
The purpose of this action is to compare and validate some innovative short-chain PFAS mitigation and containment systems. This action was carried out through the construction of pilot plants for the purification of drinking water based on resin filters, and pilot plants for the purification of water for irrigation purposes by implementing phytodepuration techniques.

Action B4: Innovative and integrated forecasting tools to support the decision-making

The use of innovative and integrated forecasting tools will help to estimate the diffusion of PFASs in the various environmental matrices. It will set biological and eco-toxicological systems of early warning to risk management support.

Action C1: Environmental monitoring

The environmental monitoring action will be aimed at measuring the performance and progress of the project. The monitoring will be carried out by validation activities of the target analytical methods with the study of the effectiveness of the technological tools adopted. Moreover, it will be performed fieldwork to test the performance of the action in all environmental matrices by sampling water, soil, plants, and animals.

Action C2: Socio-economic impact

Environmental impact assessment is a complex multi-dimensional process, involving multiple actors. To identify the needs of the population involved in the target area, in this project, a multi-criteria technique has been adopted. To perform it, it has been necessary to define the objectives capable of satisfying the needs of the stakeholders, the resources necessary to achieve the objectives, the results it has been decided to achieve.

Action D1: Communication and dissemination to the public

Communication and dissemination activities will be focussed on non-technical audiences such as schools and citizens. This action aims to disseminate the results of the project, attract attention and raise awareness on water issues, environmental sustainability and encourage more ecological choices of lifestyles.

Action D2: Communication and dissemination towards stakeholders

Specific communication and dissemination activities addressed to the technical-scientific area such as researchers, technical officials of the public administration and stakeholders. This action will develop targeted activities to increase stakeholder awareness of the problems caused by emerging pollutants (Life Phoenix).

1.4 BIOINDICATOR

To evaluate the state of a natural environment, the combination of chemical measurement and biological tests has been demonstrated to be optimal. Chemical measurements are fundamental to understand the movement of the contaminants in the area of interest and the toxicokinetic relationships. On the other hand, intending to

assess the real danger for the ecosystem, it turns out to be essential the role of bioindicators. In ecotoxicology, a bioindicator is defined as an organism that can be detected in the natural state in the environment, furthermore, the organism must belong to a specific animal or plant community and that it is possible to detect morphological or physiological changes if exposed to a pollutant or mixtures of pollutants. These variations could cause modification of the community structure, physiological changes into the organism, or in the worst of the scenarios, to death. An organism can be considered a good bioindicator if there is a correlation between the physiological state of the organism and the entity of exposure to a certain pollutant. Moreover, a good bioindicator must have the following characteristics:

- Widely distributed in the area of interest
- Easy to sample
- Poor mobility
- Easy availability and handling
- Easy to breed in the laboratory
- Pollutant sensibility
- Good representativeness, or rather, the possible correlation with a phenomenon or a characteristic that it has been decided to detect or control.

Most of the terrestrial ecosystems are developed onto soil fauna as the first component of the food chain; soil fauna plays an essential role in numerous aspects of the decomposition of organic matter, contributes to microbial activity regulation, to the soil structure and the nutrient cycle. As Cortet et al. wrote in 1999, the soil fauna is involved in pollutants monitoring into the environment includes Nematodes, Enchitreidae, Gastropods, Collembola, Isopods, Arachnids and Oligochaetes. Within the class of oligochaetes, organisms of the Lumbricidae family are the most important biotic components since they make up the majority of soil biomass and they are essential for maintaining its structure and fertility (Cortet et al., 1999). Nightcrawlers, belonging to the oligochaetes class, perfectly satisfying the qualities of a good bioindicator mentioned above since they have a wide geographical distribution, a relatively long-life cycle and a high reproductive rate. Nightcrawlers are also resistant to a wide range of temperatures and humidity rates, they are easy to breed and they are sensitive to a large number of contaminants present in the substrate in which they live. Thanks to their excellent characteristics, earthworms have been recognized among the 5 key

bioindicators for the ecotoxicological assessment of persistent chemicals in the environment (OECD, 1984).

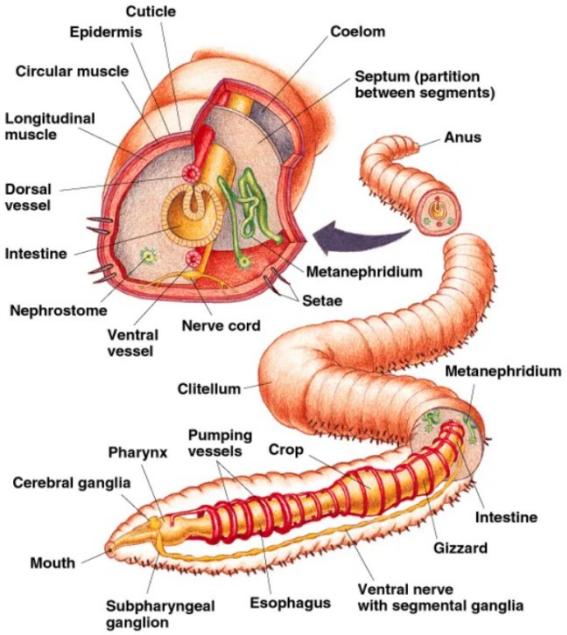


Figure 1.10 COPYRIGHT 1999 ADDISON WESLEY LONGMAN INC.

Earthworms are invertebrates' animals with an elongated cylindrical body divided into segments, with bilateral symmetry. they display a small number of setae on each segment, a mouth and an anus at the end of the body. To differentiate the mouth and anus, the swollen ring-shaped external gland, known as clitellum, is located in the top part of the body and has reproductivity functions. The presence of clitellum is fundamental for sexually mature individuals' identification.

Earthworms are insufficient hermaphroditic animals that practice cross-fertilization within eggs protected by cocoons. Earthworms usually become sexually mature over a period of time between two months and one year of life, after which they deposit the cocoons near the surface of the soil, except in periods of drought they can be deposited in deeper layers. The short reproductivity cycle of earthworms is possible in favourable environmental conditions, in which earthworms mate continuously and form cocoons every three to four days. In controlled environmental conditions, as *in vivo* experiments, the hatching time of eggs varies only according to the species of earthworm and by possible induced external factors (Dominguez and Edwards, 2011). Over the years, numerous researches have been conducted using earthworms of different species as a bioindicator, following which it turns out that the main variable to grow them under controlled conditions in the laboratory is the type of food. One of the most studied earthworm species is *Eisenia fetida*. This thick earthworm loves nitrogen-rich substrates such as animal manure, municipal sludge and household waste while it does not adapt well in poorly fertilized substrates. Although these substrates are ideal for growing *E. fetida*, they are not perfect for maintaining controlled conditions as their composition is variable. Another well-studied earthworm is *Lumbricus terrestris*. It is considered a good bioindicator of the soil fauna but despite being cited in various

scientific researches, its maintenance in the laboratory is not the simplest. It requires a frequent supply of fresh, readily available organic matter to observe biomarkers such as growth or reproduction (Fayolle et al., 1997). Aiming to have a good bioindicator whose biomarkers cannot be influenced by poor management of its physiological needs, the *Dendrobaena veneta* earthworm proved to be ideal.

Figure 1.11 D. veneta

Classification:

Kingdom: Metazoa
Phylum: Annelida
Class: Clitellata
Subclass: Oligochaeta
Order: Crassiclitellata
Suborder: Lumbricines
Family: Lumbricidae
Subfamily: Lumbricinae
Genus: Dendrobaena
Species: *Dendrobaena veneta*
(NCBI: txid332521)

Dendrobaena veneta has been firstly called *Eisenia hortensis* and only in a second period it was changed to *D. veneta*. It is a fast-growing sturdy earthworm classified as a medium-small earthworm, which could reach 1,5g at full grown. It is recognised as an optimal bioindicator since it tolerates wide moisture ranges, unlike many other species. It normally gets to sexual maturity in almost two months and its life cycle might be completed in four or five months. Given his tolerance, *D. veneta* is easily bred in the laboratory without special equipment and substrates. Normal organic origin soil and oatmeal as food is enough for its breeding.

1.4.1 Coelomocytes

In the natural environment, the earthworms are constantly exposed to various pathogenic pollutants through dermal contact and ingestion. If not protected, the body cavity might be continuously in danger of infection and consequent damages. The whole defence system of earthworms is based on celomatic fluids and organelles. If an infection occurs, it is commonly inactivated through non-specific defensive reactions such as phagocytosis, encapsulation and nodulation. Among the organelles involved in

the response to infection, there are coelomocytes. Coelomocytes are immunocompetent cells suspended and circulating in the coelomic fluid characterized by a pronounced polymorphism. The coelomocytes and coelomic fluid are shared by all oligochaete annelids, including earthworms, and together they play the hydrostatic skeleton function. One of the defence mechanisms involving coelomocytes and coelomic fluid in earthworm is the rapid extrusion of coelomic fluids. When earthworms are under stressful conditions by external factors, they can increase the coelomic pressure and therefore expel the coelomocytes through inter-segmental dorsal pores in the body wall (Adamowicz and Wojtaszek, 2001; Plytycz et al., 2006; Sforzini et al., 2012).

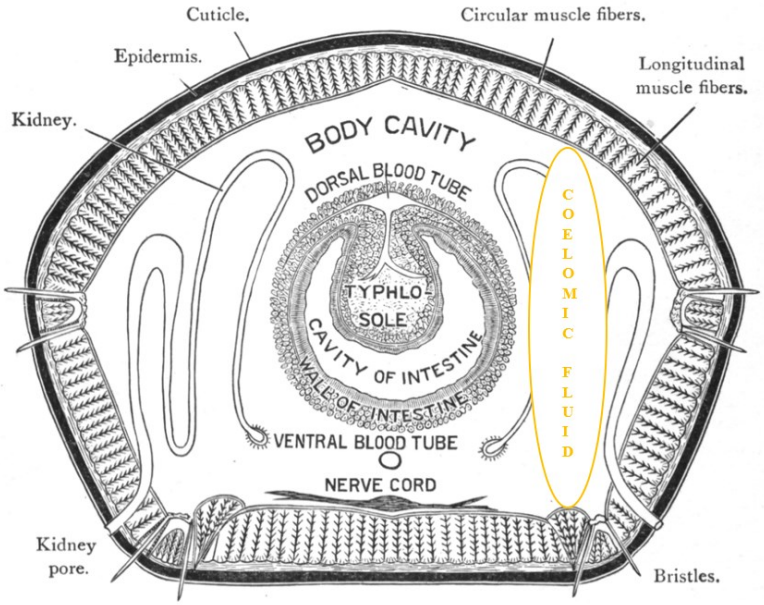


Figure 1.12 Cross Section of Earthworm. Modified from Colton, 1903.

Coelomocytes are also involved in waste substances accumulation and transport. These cellular elements, therefore, fulfil a primary importance role in the immune responses to xenobiotic substances. Earthworms' coelomocytes' quantitative/qualitative composition might change depending on external environmental factors, stage of development and physiological condition (Avel, 1959).

Coelomocytes can be broadly differentiated into two lineages, namely amoebocytes and eleocytes, and the proportions between these can vary according to earthworm species. Their main difference is that they originate from different tissues of their body. Indeed, the amoebocytes derive from the mesenchymal lining of the coelom. At the same time, eleocytes, also known as chloragocytes, differentiate from the chloragogen cells that cover the coelomic surfaces of the digestive tract and major blood vessels. In

some species, as in *D. veneta*, it is possible to distinguish the third line of coelomocytes, or rather the granulocytes (Plytycz et al., 2006).

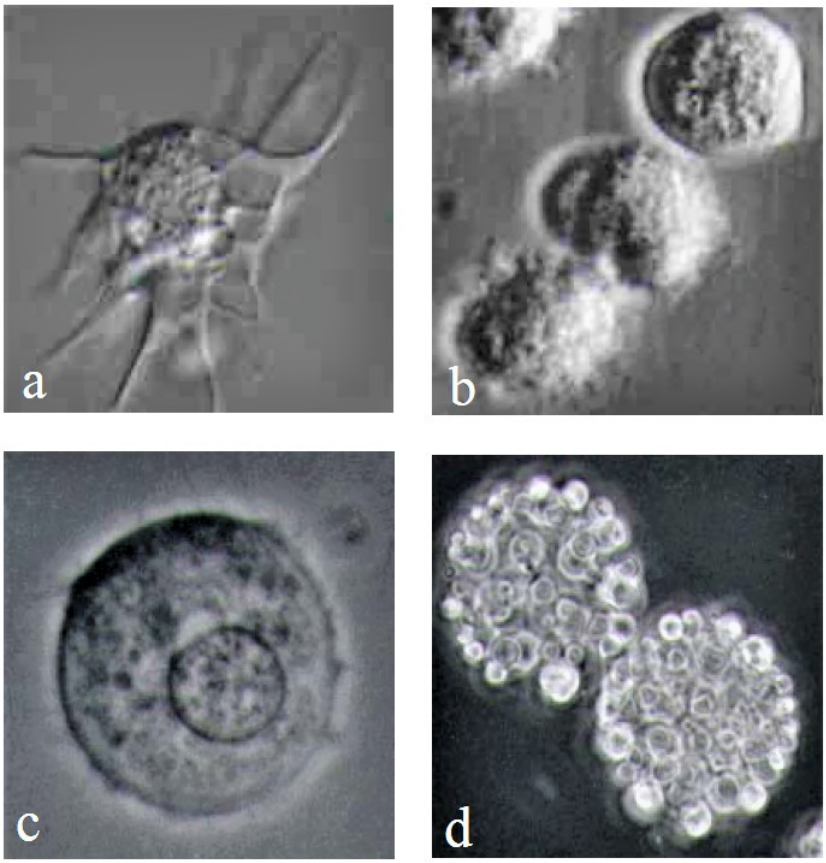


Figure 1.13 Coelomocytes of D. veneta. A) is amoebocyte of A I type, 870X. b) is amoebocyte of A II type, 1335X; c) is granulocyte, 945X and d) is eleocytes, 600X. From Adamowicz Adamowicz and Wojtaszek, 2001.

The granulocytes are spherical cells of a mean surface area of 180 μm^2. They are around 28% of total coelomocytes, and they are characterized by centrally located nuclei and by the presence of fine, strongly eosinophilic granules in the cytoplasm. Eleocytes are roundly or oval shape cells of a mean surface area of 1770 μm^2. Chloragogenic cells are around 30% of all coelomocyte's population. They are characterized by small, located eccentrically spherical nuclei and by the presence of chloragosomes, or rather polymorphic granules. In *in vivo* cell observations, eleocytes displayed a tendency to form cells aggregations of a few to about a dozen cells. Eleocytes are involved in pH and ionic balance maintenance for both coelomic fluid and haemolymph. It has been observed that they also involved in immune processes such as bactericidal substances production. Furthermore, they are implicated in extracellular respiratory pigments synthesis, and they are capable of storing exogenous substances, such as pigments or metals and endogenous substances, such as glycogen and lipids.

The third and last lines of coelomocytes are the amoebocytes. Amoebocytes are polymorphic cells of surface area ranging from 112 to 270 μm^2. They are around 42% of total coelomocytes, and they are characterized by centrally or peripherally located nuclei, whose shape varies from oval to kidney-like and by cytoplasm containing granules. It is possible to distinguish two types of amoebocytes, or rather A I type and A II type, which differs in number and shape of cytoplasmic processes. Type A I are characterized by a short lobopodian shape, they form numerous pseudopodia, and they tend to distribute regularly on the cell periphery.

Differently, Type A II is characterized by a long filopodia shape, they tend to form irregularly distributed pseudopodia by concentrating on one pole of cells. Despite all types of coelomocytes might phagocytize xenobiotic substances. As a result of various studies on this topic, it turned out that amoebocytes are more efficient than other types of coelomocytes (Adamowicz and Wojtaszek, 2001). Phagocytosis is the process through which cells ingest and neutralize by removing xenobiotic materials or substances. In annelids, this multi-phase reaction mechanism is activated only after immunological recognition, which is performed by specialized cells dedicated to distinguishing between organisms' components and exogenous substances. Following the recognition of intrusion into the body by alien substances, the phagocytosis process continues through chemotaxis and adhesion. These stages might modulate in the intensity depending on the kind and quantity of phagocyted material, in addition, the presence or absence of "plasma" factors might also interfere. Subsequently, adhesion processes, the final enzymatic destruction through biotic factors take place by the formation of a phagolysosome (Adamowicz and Wojtaszek, 2001).

1.5 BIOMARKER

The main problem of environmental contamination is that most sites are polluted by mixtures of toxicants whose effects are poorly known. Environmental pollution monitoring relied only on chemical measurements for several decades before introducing biological monitoring. To understand with certainty the relationship between chemical measurements data and environmental health status, it has become indispensable to adopt the biomonitoring approach. Toxicant bioavailability and toxicokinetic interactions are fundamental parameters to assess the impact of pollutants on the environment. However, these parameters do not prove the final effect of the pollutants on the organism of interest. In this regard, organism’s responses to toxicants

are assessed by the biological approach through the evaluation of biomarkers (Galay-burgos et al., 2003).

Biomarkers are specific measurements of variations in body fluids, cells or tissues that indicate physiological changes induced by the presence and magnitude of various compounds (NRC, 1989). The World Health Organization (WHO) upgraded this definition of biomarker by including almost any measurement reflecting an interaction between a biological system and a potential hazard, which might be physical, chemical or biological. Subsequently, in 1989, the US National Academy of Sciences defines a biomarker as 'a xenobiotically induced variation in cellular or biochemical components or processes, structures or functions measurable in a biological system or sample (NRC, 1989).

Biomarkers have been further investigated by several authors, in 1994 Van Gastel and Van Brummelen subdivided biomarkers in terms of their use at each level of the ecological hierarchy. They proposed to divide biomonitoring levels into four classes, biomarkers, bioassays, bioindicators and ecological indicators defined as follow:

- Biomarkers measure biochemical and physiological processes and deviations from the normal situation ('health') at the sub-organismal level.
- Bioassays measure the survival, growth and reproduction of individuals in the face of contaminants using classic laboratory ecotoxicity tests at the population level.
- Bioindicators measure changes in genetic structure, age structure or abundance of a population.
- Ecological indicators measure changes in species composition, abundance and diversity that may indicate the effects of pollution on communities'. (Van Gastel and Van Brummelen, 1994).

Moreover, in 1994 Peakall defined biomarkers as 'a biological response to a chemical or chemicals that give a measure of exposure and sometimes, also, of toxic effect'. In this definition, it has to be underlined the grade of "biological responses", indeed it can range from the molecular level to organism level, and even to ecosystems' structure and functions. As reported by Van Gastel and Van Brummelen, Peakall also distinguished the level at which the 'biological responses' are evaluated by introducing the term 'ecological indicator' to evaluate ecosystems' status while he kept the term biomarker at the molecular level. (Peakall, 1994).

Pollutants' effects determination through biomarkers is the most accurate way to assess organisms 'stress. The aim is to identify the primary toxicity attachment of a contaminant. The individuation of the primary target of action of any toxic compound is fundamental for stress assessment. In general, toxicants first act at the molecular level and might cause enzymatic activity modifications or DNA alterations if not controlled. Afterwards, with a subsequent cascade mechanism, the damaging effects can be detected at all the levels of the hierarchical internal organization of organisms, or rather organelles, cell, tissue, organ and organism up to the population level. Concurrently, to the external stressors' attachment, the organisms start-up adaptive responses through the activation of antioxidant multi-enzymatic systems to re-establish the internal parameters' homeostasis. Based on organisms' responses, it is possible to differentiate the biomarkers in specific or non-specific. Specific biomarkers features might be that due to a specific contaminant attachment, the organism defence systems activate specific enzymes such as acetylcholinesterase deactivation due to pesticides exposure. On the other hand, non-specific biomarkers include all kind of parameters' modification and antioxidants activation that is possible to observe after xenobiotic interactions, an example might be the evaluation of lysosomal membrane stability.

1.5.1 Genomic damage evaluation

As reported above, organisms' antioxidant system failure against ROS oxidative actions may lead to cellular components damage such as lipid peroxidation, protein oxidation, and DNA damages. Several environmental stressors have shown genotoxic properties by causing single- and double-strand breaks in DNA through the formation of ROS. Different biomarkers have been routinely used as single tests for specific environmental pollutants, nevertheless, in the environment, toxicant compounds occur as complex mixtures. In the last decades, several reports have been published, in which multiple biological endpoint approaches have been used. The study of several biomarkers including biomarkers of genotoxicity resulted effective in environmental stressor evaluation. Since DNA is a key target of environmental stressors in both aquatic and terrestrial organisms, it results fundamental to evaluate eventual genomic damages. Indeed, possible DNA compromise might stimulate the induction of genetic mutations and further irremediable consequences such as invertebrates' "genotoxic disease syndrome". Since DNA strand breaks might occur by a wide range of chemicals and as a result of several internal mechanisms, it has been reported as an efficient

biomarker for genetic damage. DNA strand breakage levels have been adopted as a sensitive and effective biomarker for environmental biomonitoring. To evaluate these sensitive genotoxic effects biomarker, it has been broadly used the Comet assay.

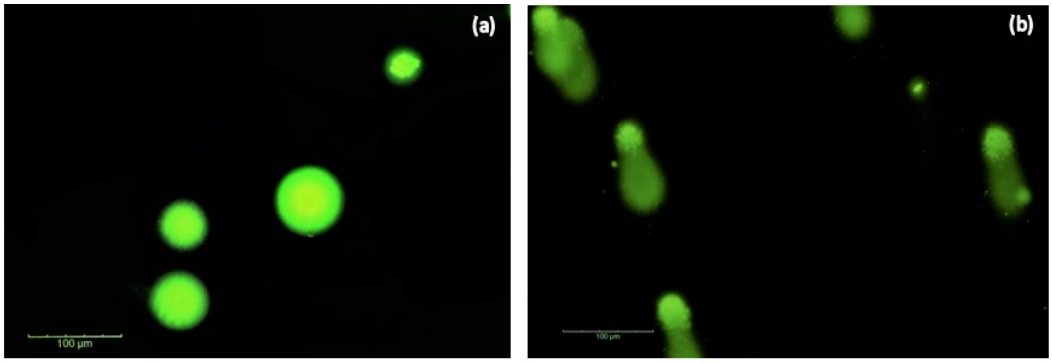


Figure 1.14 Example of Comet assay in which it is possible to observe healthy cells (a) whose nucleus are intact. In b it is possible to observe cells exposed to oxidative reactive species whose effect is visible as a break in the DNA strand and consequent comet shape due to DNA fragments that run in the gel following the electric field.

The comet assay, sometimes cited in the publication as single-cell gel electrophoresis (SCGE), is a sensitive and efficient technique for cellular DNA damage analysis. The comet assay is a simple and rapid method to detect DNA strand breaks in all kinds of cells of all living organisms. The theory on which this test is settled is based on cleaved DNA migration in an electric field. As the intact DNA remains inside the nucleoid, the damaged DNA migrates into the gel following the electric field. The resulting DNA cloud, also called "comet", thus formed depends on the DNA degree of damage (Reinecke and Reinecke, 2003; Song et al, 2009; Wang et al., 2015; Braafladt et al., 2016).

1.5.2 ROS (Reactive Oxygen Species)

The main molecule of aerobic living on earth is oxygen (O_2). Oxygen takes part in all the oxidation reactions that occur during aerobic metabolism. It participates as the final acceptor of the electrons in the chain reaction through which the ATP (Adenosine Triphosphate) is formed at the mitochondrial level. By oxidizing the organic substance, oxygen is progressively reduced to water. During this process, based on the reaction that occurs, reaction intermediates, also called Reactive Oxygen Species may form (ROS). ROS family include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). These oxygen species have specific chemical properties that confer reactivity to determining biological targets. The main characteristic concerning the danger of ROS is their reactivity, some molecules are extremely reactive such as

hydroxyl radicals while others are less reactive such as superoxide anion and hydrogen peroxide (Schieber and Chandel, 2014).

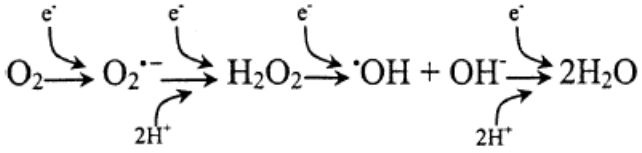


Figure 1.15 Reactive Oxygen Species (ROS) pathway (Nordberg and Arnér Elias, 2001).

In a homeostatic state, the production rate of ROS should be comparable to the degradation rate by the antioxidant system, which keeps ROS concentration at a physiological level. When the internal concentration of ROS exceeds the homeostatic concentration, the so-called oxidative stress state occurs. The overproduction of oxygen intermediates threatens the integrity of various biomolecules, including proteins, lipids, lipoproteins and DNA. In addition to reactive oxygen species, intracellular free radicals, or low molecular weight molecules with an unpaired electron, also have high reactivity and behave as oxidizing species. For these reasons, these two terms are therefore commonly used as equivalents. To interrupt the chain reaction between ROS and free radicals, two radicals must react together, eliminating the unpaired electrons. This way of eliminating dangerous radicals is random and does not allow control of these intracellular concentrations. A more effective way to eliminate free radicals is their reaction with a free radical scavenger or primary antioxidant.

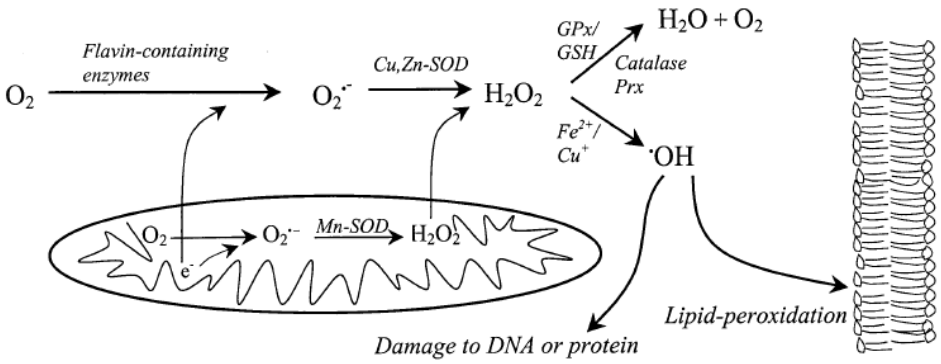


Figure 1.16 Simplified scheme of ROS production and antioxidative systems in cells (Nordberg and Arnér Elias., 2001).

ROS steady-state level maintenance is ensured not only through their controlled production but also through the elimination system. ROS elimination system is specialized in eliminating free radicals and minimizing their negative effects. The

antioxidant system is multilevel and highly differentiated. There are several approaches to classify antioxidant molecules, one option is to base the subdivision on molecular masses. Based on this assumption, antioxidant molecules can be divided into two classes. Low molecular mass antioxidants are characterized by molecular masses below one kilodalton (kDa). On the other hand, high molecular mass antioxidants are characterized by molecular masses higher than ten KDa (Lushchak, 2014).

The class of low mass molecular antioxidants comprehend broadly chemically different compounds such as vitamin C or ascorbic acid, vitamin E or tocopherol, anthocyanins, carotenoids, polyphenols, thiols and uric acid. Most of these compounds are integrated with diet by organisms. Nevertheless, other compounds are internally synthesized by most living organisms. One of the most essential internally synthesized antioxidants is glutathione (GSH) which is essential to control ROS level either as a direct interceptor or as a ROS-detoxifying enzymes substrate (Lushchak, 2014). One of the most common known ROS elimination pathways by high molecular mass antioxidants is begun by superoxide dismutase (SOD) by catalysing the reaction ($O_2^{\cdot -}$ + $O_2^{\cdot -}$ + $2H^+ \rightarrow O_2 + H_2O_2$). The formed hydrogen peroxide is further enzymatically dismutated by catalase into water and oxygen ($H_2O_2 \rightarrow 2H_2O + O_2$). On the other hand, H_2O_2 might be reduced into water by peroxidases such as glutathione peroxidase (GPx) (H_2O_2 + 2GSH $\rightarrow$ H_2O + GSSG). Furthermore, GPx might reduce lipid peroxides reducing so the oxidative damage (LOOH + 2GSH $\rightarrow$ LOH + GSSG). Once the GSH is oxidated to GSSG, it is re-reduced by the enzyme glutathione reductase, which participate in the reaction, in conjunction with NADPH, as a catalyser (GSSG + NADPH $\rightarrow$ 2GSH + $NADP^+$).

Oxidative damage prevention is essential for living organisms to minimize deleterious effects. Since hydroxyl radical cannot be eliminated by any enzymatic systems, it turns fundamental to prevent its formation by eliminating its precursors. However, it happens that a small number of free radicals overtake high molecular mass defence systems and might be converted to $HO^{\cdot}$. The eventual failure of this system implies the intervention of the low molecular mass antioxidants such as ascorbate, tocopherol and GSH, which minimize hydroxyl radical damage by reacting with them.

To understand the detoxification system, it has to be taken into consideration where ROS could form. Mitochondria, cytoplasm and extracellular space are the sites where superoxide anion ($O_2^{\cdot -}$) could firstly form.

($O_2^{\cdot -}$) is normally scavenged by the first line of defence, or rather, by the enzyme SOD and converted to hydrogen peroxide (H_2O_2). Subsequently, other enzymes such as catalase or peroxidase could further convert H_2O_2 to water. An eventual failure of the first-line defence system implies the intervention of the second line of antioxidants. These molecules are small molecules that tend to be oxide rather than essential cell components. The most well-known molecules included in the second line defender are GSH, vitamin C, and vitamin E. Vitamin C is a hydrophilic antioxidant mostly implied in preserving against protein oxidation and lipid peroxidation. Meantime, vitamin E is a lipophilic antioxidant that, thanks to its tendency to dissolve in lipids, is mostly implied in protecting membranes. In addition to these antioxidant molecules, there are several other molecules that despite not being antioxidant, act as antioxidants. This molecule category includes vitamin A cytochrome c and ubiquinol (Van Raamsdonk and Hekimi, 2010).

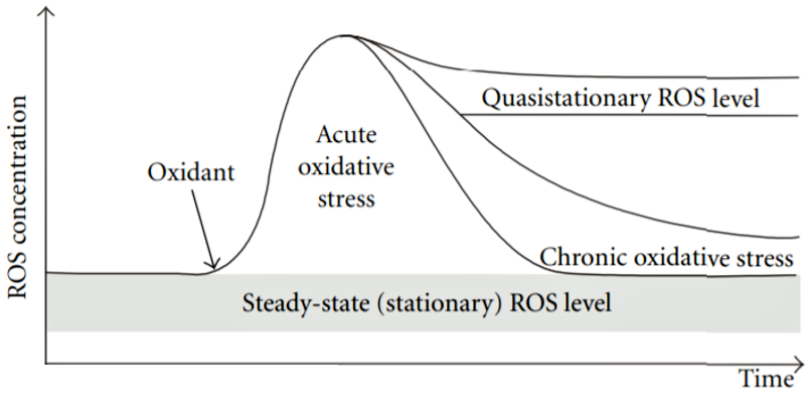


Figure 1.17 ROS dynamic level in biological systems. Modified from Lushchak, 2012.

Despite living organisms have developed high-efficiency antioxidant systems that keep shallow internal ROS level, it has been calculated that approximately 3% of the O_2 used for cell respiration is mistakenly converted into ROS. Nevertheless, this rate of conversion can easily increase in numerous physiological and pathological (Bonato et al., 2020). By observing Figure 5 it is possible to deduce how easily ROS internal level could increase beyond the normal range resulting in acute or chronic oxidative. The normal stationary level of ROS could be influenced by mechanical and thermal stress, high partial pressure of oxygen, hypoxia, xenobiotic and ionizing radiation exposure. These factors could cause inactivation of antioxidant enzymes, depletion of reserves of low molecular mass antioxidants, decrease in the production of antioxidant enzymes and low molecular mass antioxidants and in addition, combinations of two or more factors listed above (Lushchak, 2014; Bonato et al, 2020).

It has to be marked that increased ROS levels resulting from an imbalance between generation and elimination processes may affect many, if not all, living processes. Nevertheless, it is not enough to have an increased ROS level for a certain time to develop oxidative stress. ROS increased level consequences differ and depend on the level and place of ROS generation. In normal circumstances, the antioxidant system can return the ROS level to a steady state. However, in some cases, the cell cannot neutralize increased ROS amounts and oxidative stress occur. Oxidative stress could disturb cell homeostasis. Possible consequences of oxidative stress could be the expansion of the initial steady-state level, leading to the so-called ‘‘chronic oxidative stress’’. Under special conditions, the cell homeostasis could be highly disturbed to the point that ROS levels may not return close to the initial range and stabilize at a new quasistationary level (Lushchak, 2014).

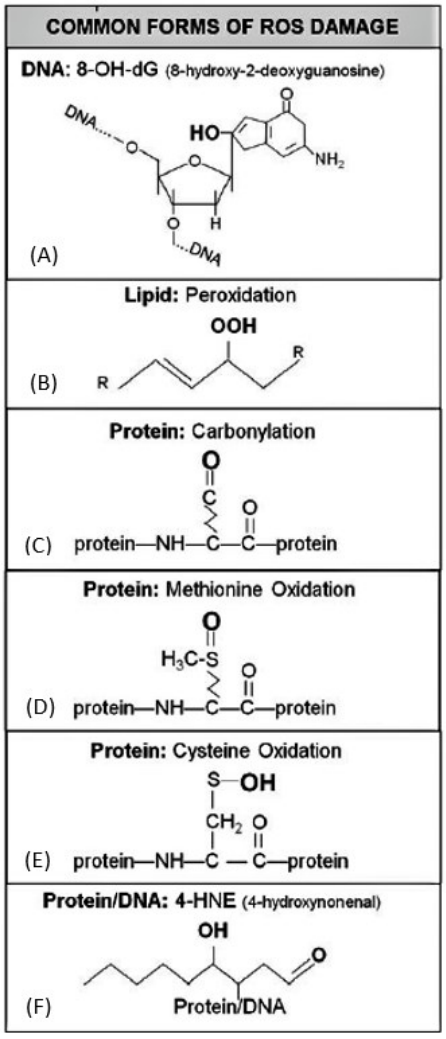


Figure 1.18 Examples of ROS damage to building blocks of the cells. Modified from Van Raamsdonk and Hekimi, 2010.

Figure 1.17 report the most common types of ROS damage to cell building blocks. Lipid peroxidation (Figure 6 B) could take place either in free lipids as in the membranes’ contained ones. Membrane's lipids compromise could cause membrane permease of substances and homeostasis deficiency. Lipids are firstly attached by hydroxyl radical, which removes hydrogen from a lipid to generate a lipid radical (R·). The lipid radical can then react with molecular oxygen to form a lipid peroxyl radical (ROO·) and increase its stability. Like a chain reaction, the peroxyl radical (ROO·) can then remove the hydrogen from another lipid to form a lipid peroxide (ROOH) and a consequent new lipid radical (R·). In special cases, lipid peroxidation product such as 4-hydroxynonenal (4-HNE) can further react with DNA or proteins causing genomic damage and eventually cell death (Figure 17 F). Protein oxidative damage (Figure 17) can manifest as protein carbonylation (C), or rather the addition of a double-bonded

oxygen atom. Protein carbonylation is site-dependent, indeed it results to affect specifically amino acid side chains, including threonine, arginine, proline and lysine. Specific amino acids such as methionine can be oxidized to form methionine sulfoxide, which can be occasionally further oxidized to methionine sulfone (D). Another specific case is the one regarding cysteine. Cysteine could be attached several times by free radical undergoes multiple oxidations and sequentially forming cysteine sulfenic acid, cysteine sulfinic acid, and cysteine sulfonic acid (E). Genomic damage is caused due to hydroxyl radical attachment to DNA. By the addition of a hydroxyl group to the guanine base of the guanosine nucleotide, it forms 8-hydroxy-2-deoxyguanosine (Figure 17 A) (Van Raamsdonk and Hekimi, 2010).

1.5.3 Total Antioxidant Capacity (TAC)

Since the origin of aerobic life, living organisms developed different strategies to cope with it. By the strategy adopted it is possible to divide organisms into two categories. Anaerobic organisms remained confined to places where the O_2 cannot penetrate. On the other hand, aerobic organisms differentiated and evolved different protections against oxygen in order to face up its toxicity. Furthermore, some of the aerobic organisms as well as protecting themselves from oxygen evolved aerobic respiration. In aerobic respiration, oxygen participates as the terminal electron acceptor in the electron transport chain, which is implied in the mitochondrial metabolism of food sources in cells. Based on these assumptions, it is easy to deduce how the evolution of antioxidant systems is highly connected with the evolution of aerobic respiration. (Halliwell, 1999).

As mentioned in the previous chapter, during cell metabolism, that leads to ATP (adenosine triphosphate) formation and in which O_2 is reduced to water, may happen that free electron reacts with O_2 forming intermediates, also called ROS (Bonato et al, 2020). Endogenous processes are not the only source of ROS, indeed several exogenous factors such as xenobiotics and pathogen exposition, heat shock and UV radiation could increase internal ROS levels (Ferro et al., 2017). ROS are physiologically produced in several cellular biochemical reactions, indeed it has been demonstrated that certain concentrations of ROS are vital beneficial for intracellular signalling and redox regulation. As early as 1987 nitric oxide (NO) was identified as a signalling molecule, subsequently, it was demonstrated to be determinants for gene expression and a regulator of transcription factor activities. Afterwards, it was

discovered that H_2O_2 and O_2^- have similar intracellular effects. Nowadays, it is known that ROS are involved as secondary messengers in the intracellular signal transduction for several hormones, neurotransmitters, cytokines and growth factors (Nordberg and Arnér Elias, 2001; Ferro et al., 2018; Tolomeo et al., 2019). As an adequate content of ROS is vital for the functioning of an organism, as a high content of ROS may lead to irreversible cell damages or to uncontrolled cell death which eventually could cause tissues damages and organs failure (Ferro et al., 2018; Tolomeo et al., 2019).

To prevent damages caused by ROS, living organisms evolved complex multilevel antioxidant systems based on the reducing activity of enzymes and the scavenging activity of different smaller molecules (Ferro et al., 2018; Jakubczyk et al., 2020). Different species evolved differently over time but as it is reported in Tolomeo et al., 2019, completely different eukaryotic organisms such as animals and yeast share evolutionarily conserved enzymes and non-enzymatic compounds to keep ROS homeostasis (Tolomeo et al., 2019; Muhtadi et al., 2020). With the purpose of ensuring normal vital functions, it is fundamental to keep oxidation-reduction reactions balanced. This balancing process also called ROS homeostasis equivalents to internal adequate amounts of antioxidants quantities corresponding to the amounts of free radicals formed. The antioxidant system includes all the mechanisms involved in the deal with free radicals (Jakubczyk et al., 2020). Depending on the characteristics that are given more importance, antioxidant components could be differently divided. According to Lushchak, it is possible to subdivide the components of the antioxidant system based on the molecular mass. In this regard, high molecular mass antioxidants are characterized by molecular masses higher than ten kilodaltons (KDa) while low molecular mass antioxidants are characterized by molecular masses below one KDa (Lushchak, 2014).. The high mass molecular antioxidants group is formed by antioxidant enzymes such as superoxide dismutase SOD, Catalase (CAT), GPx, Peroxiredoxins (Prxs) and Methionine sulfoxide reductase (Msr). Low mass molecular antioxidants include vitamin C, vitamin E, anthocyanins, carotenoids, polyphenols, thiols, uric acid and others more (Lushchak, 2014). As claimed by Jakubczyk et al., 2020 Antioxidant pathways could be divided based on the mechanisms involved and categorised as preventive, reparative and eliminative. Preventive antioxidant pathways consist in the organism' ROS first line of defence. This category of molecules includes enzymes such as SOD, CAT and GPx. These enzymes are involved in ROS homeostasis to avoid oxidative stress. When ROS level increase, other enzymes and

antioxidant elements come into action to cope up with oxidative damage. These elements belong to the antioxidant reparative system, and they are targeted at interrupting free-radical reactions. Enzymes belonging to the eliminative system are involved in damaged cells repair and liquidation to avoid necrosis and further tissues damage (Jakubczyk et al., 2020). Moreover, in 2014 Lushchak proposed an antioxidant molecules classification based on oxidative stress intensity. This classification is the result of the study of different oxidative stress biomarkers and their validity opposed to possible erroneous interpretation of the results. Until then, previous classifications of the antioxidant system and its constituent elements were based on purely physical and observable aspects such as molecular weight. In many previous studies, different markers have been used to evaluate oxidative stress. While the evaluation of oxidatively modified lipids, proteins and nucleic acids has been largely confirmed, the evaluation of antioxidant activity and enzymes may loom in problems. The idea of developing the classification on the intensity of oxidative stress derives from the fact that there is no univocal response of the enzymatic activity when, due to internal or external conditions, the organism 'internal concentration of ROS increases. Indeed, the enzymatic activity might behave differently depending on the situation by increasing, decreasing or not change from the basal level. Several processes are involved in enzymatic activity. The modification of only one process may result in enzymatic up-regulation, resulting in increased enzyme biosynthesis levels. On the other hand, enzymatic down-regulation can lead to ROS-induced enzyme inactivation or consequent cease enzyme biosynthesis.

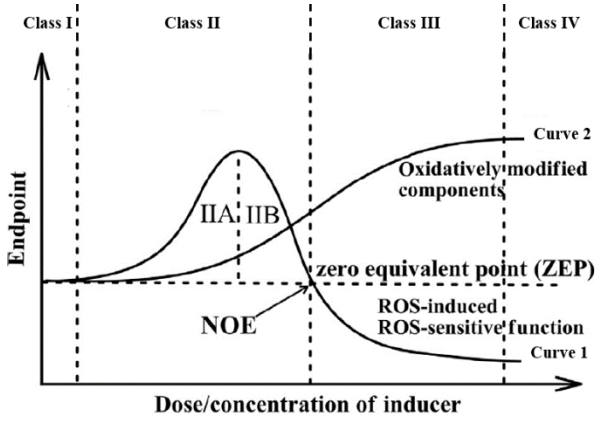


Figure 1.19 Intensity-based oxidative stress schematic classification (Lushchak, 2014).

As described in Figure 1.18, oxidative stress may be divided into four classes, or instead Class I basal (BOS), Class II low intensity (LOS), Class III intermediate intensity (IOS) and Class IV high intensity (HOS). Basal oxidative stress (BOS) is coped up by the antioxidant system since ROS is always generated in living organisms. Even more, a surplus of antioxidant is normally exploited to cope with small additional amounts of external ROS or other oxidative stress inducers. External factors

could cause higher internal ROS doses/concentrations that might induce LOS. Higher ROS level could cause two possible scenarios explained by curve 1, which show the dynamics of oxidatively modified components increased level. On the other hand, curve 2 explain the increased endpoint level of the measured ROS-induced/sensitive parameter. These two pathways of action could be distinguished and monitored by conventional analytical techniques. Curve 2 trend is easy to accept from a logical point of view. It explains a standard system response, or rather, due to an increase in ROS internal concentration, the oxidative damage increase to a plateau. Contrariwise, curve 1 needs an especial explanation to be understood. The LOS zone can be divided into two separate areas or rather, the increasing zone (IIA) and after it passes the maximum the decreasing zone (IIB). Furthermore, the curve continues to decrease passing through NOE point (no observable effect), in which, despite ROS-dependent parameter level seems to be the same as in basal zone, the level of oxidatively modified component is substantially higher. A further increase of inducer dose/concentration led to the passage of curve 1 to the IOS zone. Even though the induction of oxidative modifications is greater, ROS-induced ROS-sensitive components clearly show a decrease. The decrease can be explained by the fact that at this level, the oxidative stress is as high as the up-regulation of the monitored parameter can be counterbalanced by its inactivation. Furthermore, it can also be correlated with secondary effects (not evaluated) linked to a certain extent to the evaluated parameter. An example of this situation could be ROS-induced upregulated inactivation of antioxidant enzymes and associated components, which reflect enzymes degradation and inhibition of de novo enzymes' biosynthesis. High Oxidative Stress induce both functions to converge to a different plateau. HOS induces near maximum response, which consequence is the oxidation of all available potential substrates. The border between the IOS zone and the HOS zone can be calculated as 90% of the maximum of the curve (plateau). Despite this exhaustive explanation of oxidative stress subdivisions, it is difficult to evaluate the general oxidative stress in an organism. The best way to deal with it is to identify the target or critical organ or tissue responsible for the organism' survival (Lushchak, 2014).

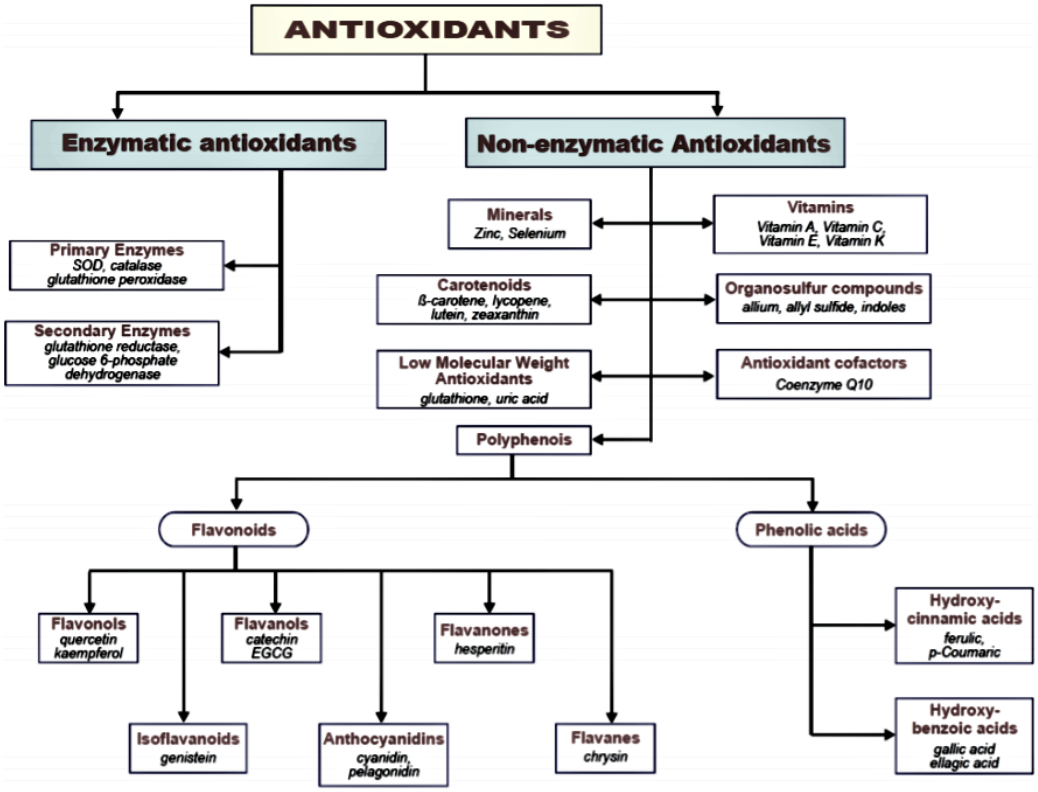


Figure 1.20 Classification of antioxidant elements. (Bunaciu et al., 2015).

As reported in Bunaciu et al., 2015, antioxidant elements can be divided into two broad families or rather enzymatic and nonenzymatic antioxidants. There are some endogenously produced antioxidants including enzymes, enzyme cofactors, and low molecular-weight molecules. Other antioxidants need to be supplemented by the diet such as vitamins, minerals, polyphenols, carotenoids and organosulfur compounds (Bunaciu et al., 2015). Enzymatic antioxidants could be further divided based on the time of intervention in primary and secondary enzymes. Primary Enzymes includes SOD, CAT, GPx, peroxiredoxins (Prdx) and methionine sulfoxide reductases (Msr). The ubiquitous family of SODs can be classified based on the metal present in the catalytic core. SODs are considered ancient enzymes indeed they have been identified either in bacterial cells either in all eukaryotic cells. These metalloenzymes are involved in the catalysation of the superoxide anion ($O_2^{-\cdot}$) dismutation to hydrogen peroxide (H_2O_2). Three different types of SODs were found in animals, or rather manganese SOD (Mn-SOD) or SOD2, which is localized in the mitochondrial matrix and two Copper/Zinc SODs, named intracellular SOD (IC-SOD) and extracellular SOD (EC-SOD) or respectively SOD1 and SOD3, concerning their cellular localisation (Ferro et al., 2017; Chatzidimitriou et al., 2020).

Since oxygen appeared on earth about 3.5 billion years ago, aerobic organisms developed antioxidant defences. The catalase enzymes family is evolutionary designed proceeded with the development of the aerobic biosphere. CAT are proteins designed for inducing hydrogen peroxide resistance, indeed they are considered the main canonical peroxide scavenger (Mutoh et al., 1999; Sharma and Ahmad, 2014; Muhtadi et al., 2020).

Glutathione peroxidases (GPxs) are a broad enzymatic family that structurally share the alfa/beta fold of thioredoxin. GPxs have been discovered in organisms of all living kingdoms although relatively different from each other. GPxs play an essential role in catalysing hydroperoxides (H_2O_2) reduction to alcohols and the concomitant oxidation of thiols to disulphides. By removing H_2O_2, GPxs are implied in preventing reactive radicals' formation by homolytic or heterolytic decomposition of hydroperoxides. Despite structural similarities, GPx homologues might be reduced by GSH either thioredoxin (Trx) or related proteins. It has been observed that the majority of nonvertebrate share structural features indicating a preferential GPxs reactivity with thioredoxin (Tosatto et al., 2008; Ferro et al., 2018).

Another family of enzymes that take place in ROS detoxification is peroxiredoxins (Prxs). Prxs are involved in H_2O_2, organic secondary peroxides and peroxynitrite ($ONOO^-$) reduction to less reactive products. All Prxs stand out for a redox-active Cys residue known as the peroxidatic Cys which is the catalytic core of these enzymes. During Prxs catalysis, the reduced form of cysteine (thiolate) reacts with the peroxide substrate and becomes oxidised to Cys sulphenic acid. Afterwards, as for invertebrate GPxs, the peroxidatic cysteine reacts by forming a disulphide bridge with a second cysteine subunit giving rise to a disulphide-linked dimer. Subsequently, the disulphide bond might be disaggregated by thioredoxin (Trx), or a similar redox protein, whose consequence is restoring cysteine to its original thiolate state which is ready to begin another cycle of catalysis. Nevertheless, excessive concentration of ROS might induce further oxidation of sulfenic acid to sulphinic acid which leads to Prxs functionality loss and possible oxidative damages (Tosatto et al., 2008; Al-Asadi et al., 2019; Tolomeo et al., 2019).

Another family of enzymes of essential importance but which play their role in the second line of defence against oxidative stress are the methionine sulfoxide reductases (Msr). Methionine (Met) is an essential amino acid which sulphide group (R-S-R) render it one of the most oxidation-sensitive amino acids. Met oxidation produces two,

R and S, stereoisomers at the sulphur atom, which are useless for physiological functions. To restore the methionine oxidative state, two structurally distinct classes of methionine sulfoxide reductases (MsrA and MsrB) catalyses respectively the reduction of the methionine-S-sulfoxide (Met-S-SO), and methionine-R-sulfoxide (Met-R-SO) (Ricci et al., 2017). These enzymes are fundamental against ROS but do not guarantee complete oxidative stress risk coverage. There are necessary joint and coordinated actions of other various antioxidant components to prevent successive intracellular damage caused by primary enzymes degradation products such as hydrogen peroxide (H_2O_2) produced by SOD.

Through diet, living organisms refuel themselves with an adequate supply of exogenous antioxidants and enzymatic cofactors. The best-known exogenous antioxidants and cofactors include antioxidant vitamins such as vitamin A (retinol), B-carotene (provitamin A), vitamin C (ascorbic acid), vitamin E (tocopherol), GSH and polyphenolic compounds (Jakubczyk et al., 2020).

GSH is regarded as the most important exogenous antioxidant thanks to its powerful redox properties. GSH plays an essential role in maintaining intracellular homeostasis through xenobiotics detoxification. Apart from free radicals 'detoxification, it takes part in vitamin C and vitamin E regeneration. This peptide is made up of three main amino acid residues, or rather cysteine (Cys), glycine (Gly) and glutamic acid (Glu). Its detoxification role is carried out by non-enzymatic reactions and by GSH-dependent enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST). It is also considered responsible, in case of deficiency, for ageing and various degenerative diseases (Lushchak, 2012; Jakubczyk et al., 2020).

A further category of low molecular weight antioxidants is Metallothionein (MTs) family. MTs are cysteine-rich metal-binding proteins widely distributed in nature. Since their conservation and multiple gene presence in the different phylum, it has been supposed that they play important cellular functions. MTs, such as other ligands, have been reported to be metals induced. Subsequently, it has been demonstrated to be essential for heavy metal excess detoxification, metals storage and homeostasis in organisms. Moreover, it has been proved their free radical scavenging role (Formigari et al., 2010; Santovito et al., 2015; Zhao et al., 2018).

An additional class of essential antioxidant elements is vitamins. In this class of compounds, it has to be underlined the role of vitamin A, vitamin C and vitamin E. Vitamin A is best known as carotenoids, it includes mainly b-carotene and retinol

esters. These compounds are capable of quenching singlet oxygen and interrupting the lipid peroxidation process by binding peroxide radicals. Through several studies over the years, carotenoid levels have been shown to decrease with age, which can intensify free radical processes and would explain the acceleration of ageing (Jakubczyk et al., 2020).

Vitamin C or ascorbic acid is a water-soluble compound, which thanks to its hydrophilic properties does its antioxidant tasks in aqueous compartments such as cytosol and plasma. Its power is well studied indeed it has been proved to terminate oxidation processes by deactivation of several free radicals such as superoxide and hydroperoxyl radicals, singlet oxygen, ozone, peroxynitrite, nitrogen dioxide, nitroxyl radicals, hypochlorous acid and lipid peroxides. In addition, it is known that vitamin C has protective and regenerative effects on other antioxidant elements with the common purpose of lipid and DNA protection against oxidation damages. However, apart from fundamental antioxidant actions, it has been proved that vitamin C might act as an oxidant enhancer in the presence of ions of specific metals (Jakubczyk et al., 2020).

Unlike vitamin C, vitamin E is liposoluble and so it does its antioxidant tasks in lipidic compartments such as phospholipids present in cells membranes. Vitamin E or α-tocopherol deaden singlet oxygen and suppresses lipid peroxidation. Its main purpose is to protect lipids, lipoproteins and in particular cell membranes from lipid peroxidation, which increase the permeability of cellular membranes and their consecutive loss of functionality. Despite α-tocopherol antioxidant functionality has been widely studied, it has been observed that in in vivo studies it does not exhibit antioxidant properties concerning hydroxyl and thyil radicals, to alkoxyl radicals, ozone and nitrogen dioxide. In several studies, an interaction between vitamin C and vitamin E has been observed, which depending on experimental conditions led to different outputs. Of course, their interaction can be confirmed but the modality and the consequences of their interaction should be studied in greater details (Jakubczyk et al., 2020).

Another class of supporting antioxidant compounds, which has a positive influence on living organisms, are polyphenols. In particular, flavonoids, which are a subclass of polyphenolic compounds, are capable of capturing singlet oxygen, peroxide anions and lipid and hydroxyl radicals. Flavonoids' antioxidant potential is related to the presence of glycosidic bonds and methyl and hydroxyl groups arrangement. Thanks to flavonoids conformation, it has been proved that they exhibit chelating properties.

Flavonoids include flavonols, flavanols, flavones and anthocyanidins. They show strong oxidation-reduction potential, moreover, it has also been demonstrated that they inhibit the activity of phosphodiesterase and cyclooxygenase. Several studies confirm that a diet polyphenols rich is associated with a reduced risk of free radical-mediated degenerative and chronic diseases. Furthermore, they have a positive impact on the deceleration of the ageing process and decrease neurodegenerative age-related risk (Jakubczyk et al., 2020).

1.5.4 Metallothionein

MTs are low molecular weight non-enzymatic proteins, which molecular weight can range from 6000 to 8000 Da. MT are particular proteins consisting of a single polypeptide chain that does not have both aromatic amino acids and histidine (His) (Santovito et al., 2012). MTs are also characterized by a high content of cysteine (Cys) (around 30%), which implies its high thermal stability (Brulle et al., 2007; Bell and Valee, 2009). MTs were first identified in the middle of the last century by studying the bioaccumulation of cadmium (Cd^{2+}) in the horse's adrenal cortex (Margoshes and Vallee, 1957). After that, several research groups have studied and reported MT presence in various vertebrates and invertebrates. Thanks to science 'progress, it is possible to state that this family of proteins is ubiquitous in the animal kingdom. MTs' nomenclature system has been firstly adopted in 1978 and widened in 1985 by the institution of three classes of MTs subdivision (Kägi and Kojima, 1979; Fowler et al., 1987).

Following the latter classification class I includes proteinaceous MTs having the Cys in positions closely related to those of the mammalian forms, it belongs to this class both vertebrates and some invertebrates MTs such as crustaceans and molluscs. Class II comprehend proteinaceous MTs having the Cys in positions poorly correlated to mammalian MTs forms, it appertains to this class MTs of several invertebrates, yeasts, protozoa, cyanobacteria and some plants. Class III MTs include some plants and fungi MTs, distinguished by metallopolyisopeptides containing gamma-glutamyl-cysteinyl units comparable to proteinaceous MTs mentioned before (Binz and Kägi, 1999).

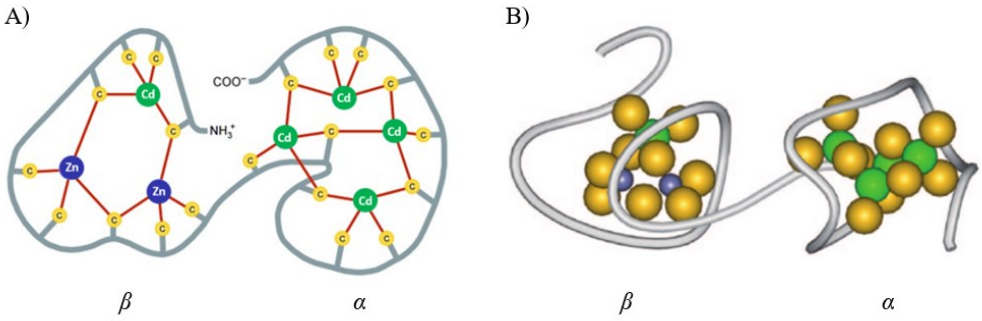


Figure 1.21 A) α and β-blinding domains of Metallothioneins; B) Rat MT structure of Cd5Zn2MT-2 (PDB ID: 4MT2). The α domain binds four cadmium (Cd) atoms (Green), while β domain binds one cadmium and two zinc (Zn) atoms (Blue) by means of sulphur (S) atoms (Gold). Modified from Bell and Valee, 2009.

In accordance with Figure 1.21, subsequent in-depth studies have shown that MTs are formed by an α (-C terminal) domain consisting of 11 Cys and a β (-N terminal) domain consisting of 9 Cys. MTs two-domain structure is essential for its overall conformation which implies its interaction with ligands and its consequent reactivity (Bell and Valee, 2009). Hand in hand with science's progress, many other MTs gene sequences have progressively discovered, as a consequence the latter MTs subdivision become outdated. Indeed, it has been observed that the aminoacidic composition and in particular cysteine number and distribution are extremely variable. In 1999 Binz and Kägi established a new classification system based on MTs sequence similarities and their phylogenetic connections. It has therefore been defined MTs' Superfamilies, which are further divided into Families, Sub-families, Sub-groups, Isoforms and Clans. As for the previous classification, the superfamilies division could be compared to the Class subdivision proposed by Fowler et al. in 1987. Or rather, it includes all polypeptides which resemble several features of the renal equines MTs. The classification into families presupposes the possible alignment of the aminoacidic sequences for all members. The division into families is supposed to be due to evolution and confers to the members the sharing of some specific characters. In addition to the characteristics that distinguish families, furthermore stringent phylogenetic characters subdivide Sub-families. MTs that share the same Sub-family have correspondence between the 5'UTR or 3'UTR regions of the gene or between the nucleotide sequences, even between exons and introns. Subgroups specify a branch of MTs sequences of a subfamily that is distinguishable from the others by its monophyletic character as the result of statistically validated phylogenetic analyses.

MTs allelic forms, also known as isoforms, are defined as all members of subgroups, subfamilies, families which occur naturally in a single species. Isometallothioneins share each other's genetically determined differences in the primary structure. The most specific level of classification is the clan level. Clan level includes sets of partial or total aminoacidic or polynucleotides sequences that share specific characters that are not defined by the above classification criteria. A clan is defined by a specific property in common to its members which could be common metal-binding properties, spatial structure, thermodynamic properties, functional characters or other specific features (Binz and Kägi, 1999).

MTs characteristic cysteine (Cys) residues' distribution in the primary structure is Cys-Cys, Cys-X-Cys, Cys-X-Y-Cys where X and Y are non-specific amino acids. A specific feature of MTs is that they might be induced both in vertebrate and invertebrate organisms, by essential metals such as copper (Cu) and zinc (Zn) but also by heavy or non-essential metals. MTs affinity for metals follows the following order: $Zn^{2+} < Pb^{2+} < Cd^{2+} < Cu^{+} < Ag^{+} = Hg^{+} = Bi^{3+}$ (Amiard et al., 2006). As a consequence of MTs affinity for metals, it turned out that they play an essential role in cell homeostasis processes. It has been shown that they are essential metals reserve components, in particular for copper and zinc. On the other hand, it has been proved MTs act in the detoxification mechanisms of essential metals in excess and heavy or non-essential metals presence (Mason and Jenkins, 1995; Roesijadi, 1996). To understand the effective roles of MTs subsequent studies have been focused on MTs. It unfolded that MTs are excellent scavenger's proteins that play an important role as antioxidant agents against ROS. In several in vivo experiments, the MTs antioxidant capacity has been confirmed against superoxide anion, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite. As consequences of MTs antioxidant action, they release the binding metal and consequent internal reorganization with the formation of disulphide bonds between cysteine components. This internal reorganization is the result of its antioxidant action, which implies differences between binding forces between the oxidized form and the reduced form. Furthermore, it resulted in that oxidative stress induced by ROS could increase the expression levels of MTs as the response of element-binding transcription factor MTF-1, which is the prevailing regulatory protein for MTs' induction (Santovito et al., 2008; Bell and Valee, 2009).

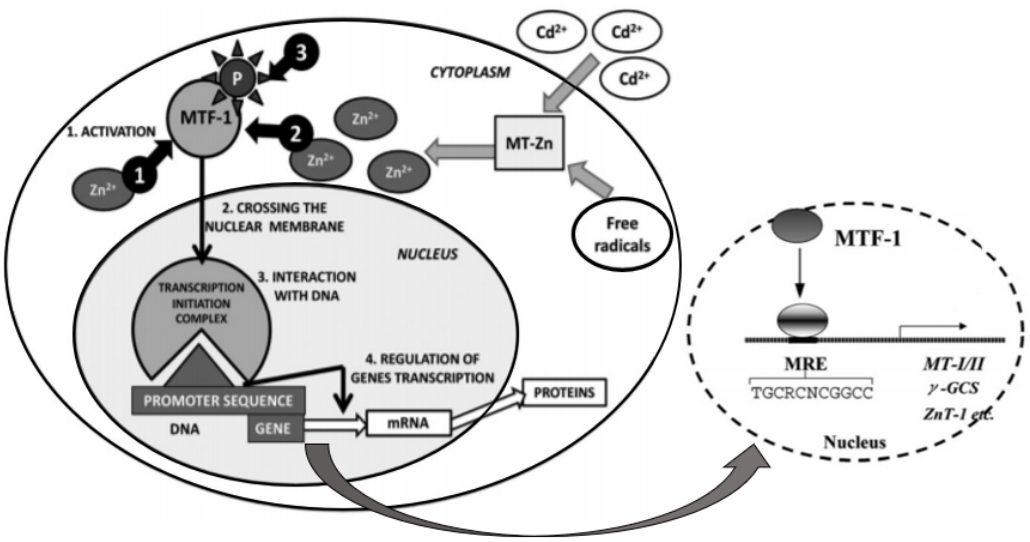


Figure 1.22 Regulation of MT-1 and MT-2 genes transcriptional activation mechanisms MTF-1 mediated. ROS and heavy metals induce MTs zinc release. Free zinc atoms in the cytoplasm act as signalling for MTF-1 factor, which ones phosphorylated might cross the nuclear membrane and perform its function of transcription activation mediator. Modified from Otsuka et al., 2007 and Grzywacz et al., 2015.

As early as 1988, Westin and Schaffner discovered and defined the MREs (Metal Response Elements). MREs are short DNA sequences characterized by a strictly conservative zone (TGCRCNC) also called the core region on the sides of which are present two flanking portions more variables. MREs promote genes encoding MTs' transcription due to metals induction. Mammals are the most studied class of animals, indeed, it is known that owing to stress conditions induced by metals, the activation of the coding genes occurs thanks to MTF-1 (Metal Transcription Factor -1), which is a six-finger zinc component of the $Cys_{(2)}$-$His_{(2)}$ family. This MTs family is characterized by three functional domains which bind specifically to MREs are essentials for coding activation (Saydam et al., 2001). In addition to MRE, the genes encoding MTs' transcription might be promoted by other regulatory elements such as the ARE (Antioxidant Responsive Elements). The ARE, also called electrophile response element, plays the role of induction mediators of glutathione S-transferase Ya subunit and the quinone reductase genes as a consequence of oxidative stress. As explained in Figure 13, oxidative stress ROS induced activates the transcription of MTs both directly, stimulating the ARE sequences and indirectly by increasing the free intracellular zinc concentration. The oxidation of MTs, as an antioxidant response to ROS aggression, causes MTs structural modifications due to zinc release and consequent disulphide bridges formed between the cysteine components. MTs, therefore, lose their function of binding zinc, whose increase of intracellular

concentration acts as a second messenger for the activation of the MTF-1-DNA complex. In greater details, the ARE might interact with the USF (Upstream Stimulator Factor), also called E box, which is a ubiquitously expressed protein and it can affect the expression of a myriad of genes. Once ARE are superimposed by the USF form the combined sequence USF/ARE, which is an essential condition for MT-1 baseline expression (Andrews, 2000).

1.5.5 Housekeeping Genes

In ecotoxicology, the degree of expression of some stress target genes is often evaluated as biomarkers of stress induced by pollutants. To evaluate the expression of a gene and whether this is conditioned by external factors, the role of housekeeping genes is strictly fundamental. Housekeeping genes also called reference genes (RGs), are specific genes that organisms produce whose expression rate is not modifiable by external factors.

In a utopia, RG should be constantly expressed at the same rate in different organisms undergone to a various range of experimental conditions. The valuation of the appropriate RG is one of the trickiest parts of gene expression evaluation since the same gene can act slightly different in different species, and so it might be a good RG for one species and a weak reference for another species. The choice of the appropriate RG is crucial for assessing the accuracy of target gene expression by quantitative real-time PCR analysis. The evaluation of gene expression is gaining increasing interest in recent years, and various scientific researches have evaluated which of the possible reference genes is the best one for a specific target species. Recent researches have demonstrated that RG in addition to species variabilities, might have variabilities in RG expression also due to different biotic and abiotic conditions such as different temperatures, pollutant concentrations or different developmental stages (Li et al., 2018). The housekeeping genes are normally present in all cell types since they are indispensable for basic cell survival. Reference genes that are commonly used include beta-actin (B-actin), ribosomal genes such as 18S rRNA and 28S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and eukaryotic elongation factors such as beta-tubulin (β-tubulin) (Kosakyan et al., 2019).

Since in this study, the expression of MTs was evaluated as a biomarker of stress response due to exposure to perfluoroalkyl substances by using GAPDH as a reference gene, it was decided to dedicate a brief description to it. Like any good reference gene, GAPDH has been used in several types of research due to its basal activity in every

organism. GAPDH, a polypeptide chain of 335 amino acids, is a ubiquitous enzyme involved in glycolysis. In particular, it plays the catalytic role simultaneously in both the phosphorylation and the oxidation of glyceraldehyde-3-phosphate to 1,3 biphosphoglycerate. This reaction is possible thanks to its tetrameric structure formed by four identical 37 kDa subunits, of which the amino acids Cys152 and His179 play an essential role in glycolysis (Colell et al., 2009).

Over the years, the function of GAPDH as RG has been questioned and carefully evaluated, as a change in its expression has been observed implied by various internal and external factors. In 2018 Li H-B. et al. evaluated what was the best housekeeping gene for the separate Mythimna moth, since conflicting versions of its functionality as an RG have been raised by Thellin et al., 1999; Silver et al., 2006 and Pan et al., 2015 (Thellin et al., 1999; Silver et al., 2006; Pan et al., 2015). Li et al. in 2018 concluded that of all the reference genes they evaluated, GAPDH was found to be the least stable gene in the different developmental states of the sample organism (Li et al. in 2018). On the other hand, Kosakyan et al., 2019 reported its suitability as a good reference gene for gene expression normalization in various life stages of red algae, plants and cnidaria (Kosakyan et al., 2019).

Furthermore, despite the widespread use of GAPDH as a reference gene in pesticide exposure studies, it has been observed that this enzyme might play a role in DNA repair following DNA oxidative damage. This role of GAPDH is insinuated by the uracyl-DNA glycosylase activity of the monomers of GAPDH, which is cell cycle-regulated (Meyer-Siegler et al., 1991). This latter role of the GAPDH enzyme has to be taken into consideration in evaluating its efficacy as a reference gene, even if its role as DNA repairer has not been fully demonstrated in all species yet.

2 SCOPE

In the last decades, the attention on the effects and fates of perfluoroalkyl substances on the environment and human health increased by several orders of magnitude. The awareness about long-chain PFASs' effects on human health induced special attention studying the effects that other perfluoro-compounds might have. In particular, it became a thematic of first interest in the Veneto region since perfluoroalkyl-contaminations have been detected in all environmental compartments. Only on March 21, 2018, during the recurrence of the world water day anniversary, the Italian Council of Ministers declared the state of emergency for the Veneto region, with the consequent nomination of a special commissioner whose purpose was to supervise the health situation in the Veneto region. Due to the high environmental persistence of PFASs, it turned out the essentiality to in-deep study the possible long-term effects that perfluoroalkyl might have on chronically exposed organisms. In addition, unlike long-chain PFASs for which ecotoxicological evaluations have already been done, no in-deep studies have been performed for several "new generation" PFASs such as NaDONA, GenX, PFBS and PFBA.

For the just mentioned motivations, it turned out to be fundamental to understand the ways of actions of these substances by focussing on the cellular level. Indeed, it is still unknown how they use to permeate the cell membranes, which receptors they tend to bind, and how they bind receptors. Furthermore, it is still unknown what induces PFASs hazardous properties. Indeed, they are thought to interact directly with cell components by inducing their consequent modifications or they might increase the internal concentration of ROS and consequent induction of oxidative damages as normal xenobiotics. By studying several biomarkers, it might be possible to increase the knowledge about PFASs effects on organisms.

The present research study evaluated the effects that environmental concentrations of PFASs have on earthworms. Earthworms have been used for this ecotoxicological evaluation since they are globally recognised by OECD as one of the 5 key model organisms for environmental ecotoxicological evaluation. To have the most adequate assessment of the health status of organisms in the Veneto region, in this in vivo laboratory experiment nightcrawlers of the species *Dendrobaena veneta* have been exposed for 30 days to three different mixtures of PFASs. To

perform the present exposure experiment, all the ethics measures have been respected by following the OECD guidelines for testing chemicals on earthworms (OECD, 1984). The concentration of the PFASs mixtures has been derived as the average concentration of each PFAS compound in the three sanitary exposure zones defined by the ministry of health and reported in Figure 1.8.

In this study, coelomocytes have been used for biomarkers evaluation since they are easily extrudable without causing excessive stress to the earthworms. Moreover, coelomocytes have in turn been recognized by the OECD as excellent tools for the evaluation of various biomarkers, thanks also to their immune responses functions to xenobiotic elements.

Based on the just mentioned assumptions, it has been decided to focus this research study on the following research questions:

- Is it possible to detect different stress responses between PFASs treated organisms and the references organisms?
- Are there any stress response differences between the three PFASs treatment groups of nightcrawlers exposed to different concentrations?

To answer the research questions of the present ecotoxicological assessment, it has been evaluated:

- The internal reactive oxygen species production;
- The organisms antioxidant capacity;
- The induced genomic damages;
- The total MTs content in the tissues and the ratio between oxidated and reduced MTs;
- The expression levels of MTs have also been evaluated.

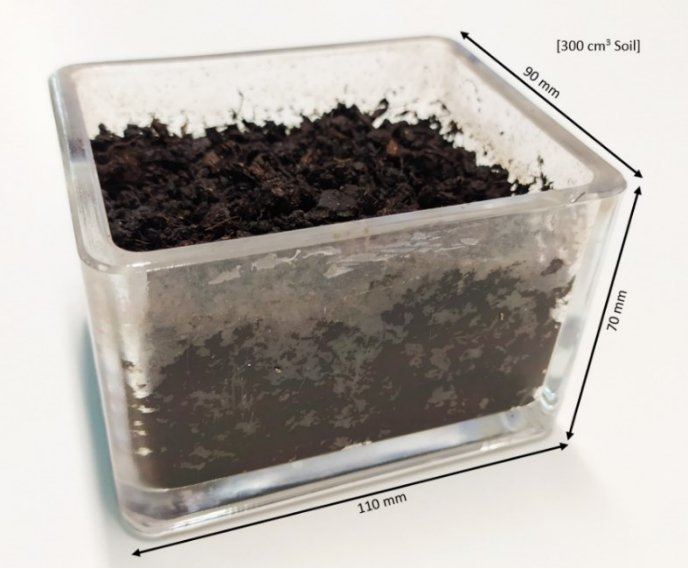
In addition, intending to answer the research questions of the present ecotoxicological assessment, it has been necessary to design and develop a new, easily applicable methodology for the genomic damage evaluation. In the present study, it has been designed a new methodology for genomic damages evaluation through the Comet assay. The comet assay is a sensitive method to evaluate single and double-strand breaks in DNA. The comet assay has been firstly adopted in 1988 by Singh et al. The modifications to the original methodology have been done to develop an easy and feasible methodology that might be replicated in different

laboratories without special equipment and tools. This new methodology to perform the Comet assay has been based on the principle to have a good screening method to detect genomic damage to all types of cells. All the procedure's tools and passages have been adapted in order to undergo Operetta®, High Content Imaging System, PerkinElmer microscope detection.

3 MATERIAL AND METHODS

3.1 EXPERIMENTAL CONDITIONS

To perform the ecotoxicological experiment proposed in this research study, eighty nightcrawlers of the species *Dendrobaena veneta* have been exposed for 30 days to PFASs mixtures composed of the same PFASs that were detected in the Veneto environment during the environmental sampling performed during the European LIFE PHOENIX project Life16/ENV/IT/000488-Life Phoenix. The nightcrawlers were bought in a fishing and hunting shop, then they have been acclimated for 14 days before the experiment in uncontaminated soil composed of organic material with a humidity rate of around 30%. The nightcrawlers have been divided into four groups (twenty each), or rather the Control group, the Zone 1 group, Zone 2 group and Zone 3 group where the concentration of each PFAS differs between groups and is reported in Table 3.2 as the average concentration of each PFAS founded in nature for each area.



For each group, the nightcrawlers have been divided into four boxes with 300 cm^3 of soil, using the same soil that was used for the acclimation to avoid ulterior stress and in each box 5 earthworms have been placed. The earthworms have been fed with apple peel every four days and the humidity rates have been controlled every two days for the entire duration of the experiment.

Table 3.1 List and specifications of Perfluoroalkyl compounds that have been manipulated in the present research study.

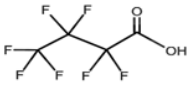
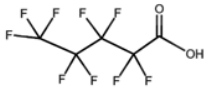
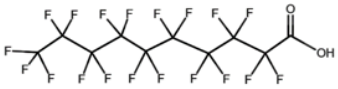
PFAS acronym	PFAS	Carbon Atoms	Structure	Molecular Weight (g/mol)
PFBA	Perfluorobutanoic acid	4		214.04
PFPeA	Perfluoropentanoic acid	5		264.05
PFHxA	Undecafluorohexanoic acid	6		314.05
PFHpA	Perfluoroheptanoic acid	7		364.06
PFNA	Perfluorononanoic acid	9		464.08
PFDA	Perfluorodecanoic acid	10		514.08
PFBS	Perfluorobutane sulfonic acid	4		300.1

Table 3.2 PFASs concentration for each exposition group where the concentration is the average of the environmental PFASs concentrations for each zone.

PFAS acronym	*Carbon Atoms*	*PFAS Concentration (ng/L)*			
		Control	*Zone 1*	*Zone 2*	*Zone 3*
PFBA	4	0	18,538	39,428	46,967
PFPeA	5	0	8,000	21,473	22,047
PFHxA	6	0	5,333	20,368	38,535
PFHpA	7	0	0	9,000	13,470
PFNA	9	0	0	0	5,000
PFDA	10	0	0	0	5,000
PFBS	4	0	13,727	51,520	114,437

3.2 CELOMOCYTES EXTRACTION

After thirty days of exposure, the coelomocyte's extraction from nightcrawlers was made to perform subsequent analysis. Sample organisms have been picked up from the exposition media and, to purge the earthworms, they have been stored for one day in sterile Petri dishes with wet laboratory paper. The day after, to remove the possible remaining intestinal content, they have been washed with a salty solution (0,85mg/ml NaCl) and massaged from the clitellum to the rear end. The organisms have then been placed in 15 ml polypropylene test tubes where it has been added 2 ml of extrusion solution formed by: 5% ethanol, 95% salty solution, 3,18mg/ml EDTA, 1,0 g Guaiacol glyceryl ether. After 3 minutes of extrusion, the earthworms have been removed from the test tubes and have been frozen into liquid nitrogen. To perform subsequent analysis the earthworms have been stored in a -80°C freezer. To keep cells alive, to the extruded solution it has been added 1,0 ml of HBSS (Hank's Balanced Salt Solution), the solutions have been then centrifuged for 2 minutes at 4°C and 360 rpm to separate the mucus from the cells suspension. Then the cell suspensions present in the supernatants have been transferred into a new test tube. The cell suspensions have then undergone another centrifuge with the same experimental condition for 10 minutes after which a cell pellet should be visible at the bottom of the tube. Part of the supernatants have been discarded and the cell pellets have been resuspended. Cell concentrations have been assessed by counting on a Bürker chamber with an optical microscope. The concentrations have been then adjusted to $1x10^5$ cells/ml. The cell solutions have been washed with cold PBS before freezing the samples into liquid nitrogen and stored in a -80°C freezer for subsequent analysis. For the Reactive Oxygen Species (ROS) determination analysis the cells suspensions have been directly analyzed after the cell count by optical microscopy (Eyambe et al., 1991).

3.3 COMET ASSAY

To evaluate organisms' stress responses, scientists evaluated several biomarkers. Since stress evaluation is a complex endpoint, a multi-biomarker approach including genotoxicity biomarkers has been broadly used. A simple and effective genotoxicity biomarker is the evaluation of DNA strand breaks by the Comet Assay. The comet assay is also known as single-cell gel electrophoresis (SCGE), and it has been firstly used by Singh et al. in 1988. This assay is DNA damage sensitive, even at a low concentration of damage.

Following the methodology described by Singh et al. in 1988, cells are freshly extracted from the organism under analysis. The cells are then incorporated in a 0.5% low melting temperature agarose (LMA) at 37°C and plated on microscope slides, which have been already disposed of with a thin layer of normal melting agarose (NMA) to promote the attachment of the second layer. The microscope slides have been then covered with cover glass and incubated at 4°C for 5 minutes to allow the LMA to solidify. Subsequently, the cover glass has been removed and the cell suspensions have been covered with a third layer of LMA and re-incubated at 4°C for 5 minutes. When this layer has solidified too, the plates are immersed for 1 hour in the lysis solution (100 mM Na_2-EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate, 2.5 M NaCl, and 1% Triton X-100, added fresh) to permit DNA unfolding by lysing the cells. Following, the slides are transferred to a horizontal gel electrophoresis chamber. The slides are then allowed to set in the alkaline electrophoresis buffer (1 mM Nay EDTA and 300 mM NaOH) for 20 minutes. The high pH of the buffer enables the unwinding of DNA before electrophoresis. Nuclear DNA strand breaks presences increase the DNA fragments mobility during electrophoresis. Through alkaline electrophoresis, the single-stranded DNA fragments and alkali-labile sites migrate away from the damaged nuclei within the gel following the electric flow, and based on the degree of damage, forms a comet-like pattern. The electrophoresis has been then performed for the next 20 min at 25 V. Following electrophoresis, the microscope slides are smoothly washed to remove detergent and alkali residues which might interfere with staining passage. The "comets" are then stained by pouring 25 µl of 20 µg/ml ethidium bromide on each slide and then covered with a cover glass. The evaluation of the results has been made by Axiomat microscope (Zeiss, RG), fitted out with a 515-560 nm excitation filter and a barrier filter of 590 nm. Single cells images have been obtained thanks to TRI X 135 black and white film, ASA 400. For each exposure group 20 randomly selected cells have been evaluated by measuring the nuclear DNA and the migrating DNA (Singh et al. in 1988).

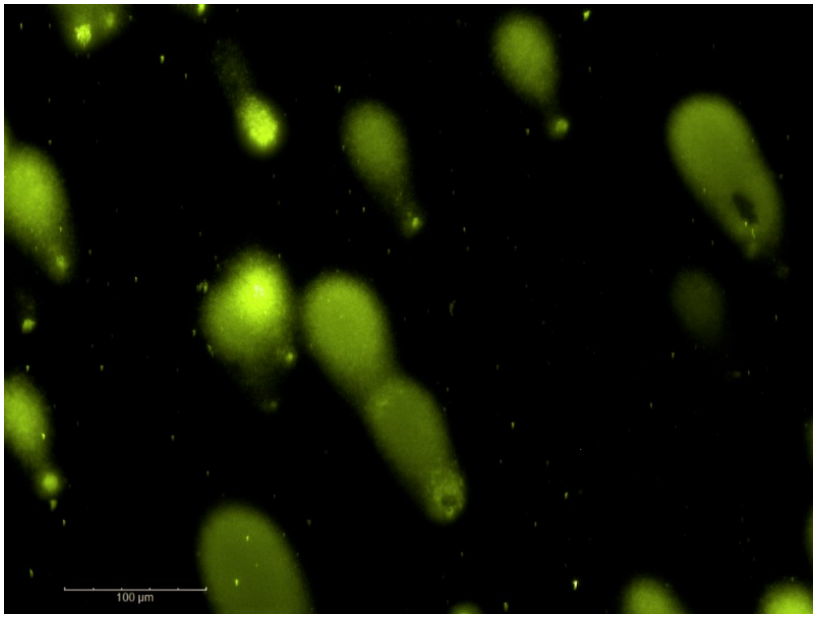


Figure 3.1 Comet assay example. The DNA have been stained with 1X Vista Green dye in Buffer TE (10 mM Tris, pH 7.5, 1 mM EDTA).

To develop a rapid and effective methodology to evaluate the DNA damage as a stress biomarker, the procedure described in Singh et al. 1988 has been modified to adapt to the microscope Operetta®, High Content Imaging System, PerkinElmer. The changes made to the aforementioned methodology were:

Two Comet assay kits have been tested, or rather TREVIGEN® Comet Assay silver kit 4251-050-K and CellBiolabs OxiSelectTM 96-well Comet Assay kit STA-355-5. To test the reproducibility of the experimental conditions in the laboratory, the kits protocols have been smoothly modified to fit laboratory experimental conditions and facilities. 96-well plates have been used instead of frosted normal microscopy slides.

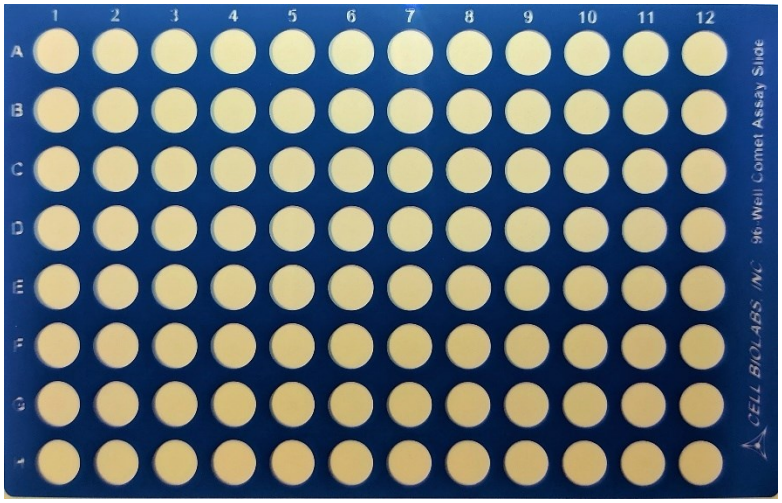


Figure 3.2 CELL BIOLABS INC 96 Well Comet Assay Slide

The plates have been covered with only one layer of 20 µL LMA 37°C previously mixed with sample cells (cells 1:10 agarose). Cell's lysis has been performed using the lysis solution (100 mM EDTA, 10 mM Tris, pH 10, 2.5 M NaCl, 1% Triton X-100 and 10% DMSO added fresh) for 1 hour at 4°C instead of overnight. Subsequently, the plates have been washed with pre-chilled Milli-Q water and then introduced in the electrophoresis chamber filled with the electrophoresis buffer (0.1M EDTA, 0.3M NaOH, pH> 13). The samples have been incubated for 1 hour in the unwinding buffer at 4°C before running the electrophoresis. The electrophoresis has been performed for 45 minutes at 25V (500mA). After the electrophoresis, the plates have been rinsed two times in water and one time in ethanol 70% for 10 minutes in total. The plates have then been dried for 10 minutes in the biological hood with laminar air flux and stained with SYBR GREEN in Buffer TE (10 mM Tris, pH 7.5, 1 mM EDTA). Observations have been done thanks to Operetta®, High Content Imaging System, PerkinElmer. The microscope has been equipped with a Rhodamine filter (excitation:490-510nm; emission:530-590nm) and the images have been taken at 20X enlargement, non-confocal for 250 ns. The images that have been evaluated were the maximum projection of a z stack 18.2 µm deep which was the result of the projection of 15 planes. In each well 12 fields have been considered for the scoring of the nucleus. The scoring of DNA damages has been performed by dividing the cells into four classes as reported in Figure 25. Healthy nuclei (a), Damaged nuclei (b), High damaged nuclei (c) and Completely damaged nuclei (d) which present DNA in a cloud (comet) form.

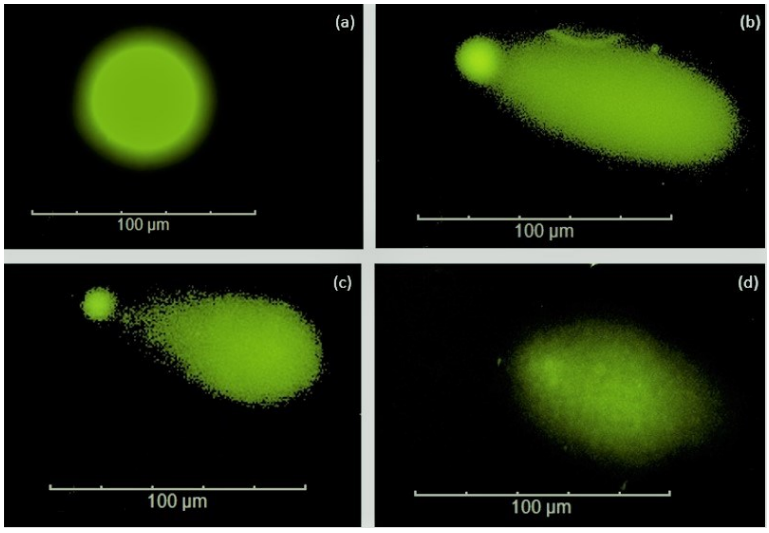


Figure 3.3 Scoring classes of damage: Healthy nuclei (a), Damaged nuclei (b), High damaged nuclei (c) and Completely damaged nuclei (d).

The image analysis was made possible thanks to the Harmony® high-content analysis software which is designed for PerkinElmer high-content screening systems.

3.4 REACTIVE OXYGEN SPECIES (ROS) DETERMINATION

Oxygen Reactive Species concentrations have been analyzed to understand if PFASs exposure may increase the ROS levels. Furthermore, two different fluorescent dyes probes have been used to determine the specificity of ROS levels into inner cells compartments. The fluorescent probes used in this assay were dihydroethidium (DHE) and dihydrorhodamine (DHR). It is known that DHE is oxidized into ethidium by the intracellular superoxide anion ($\bullet O_2^-$) into the nucleus, so its fluorescent detection indicates the nucleus specific ROS levels. On the other hand, DHR is oxidized in rhodamine 123 by the hydrogen peroxide (H_2O_2) and by the peroxynitrite ($ONOO^-$). It has been shown that these two types of ROS act site-specifically in the mitochondria. The ROS assay has been performed on freshly collected coelomocytes. Each coelomocytes sample has been divided into five test tubes, 0,5 mL each. The tubes included a positive control, negative control and three experimental replicas. The tubes were all treated in the same way until loading onto the reading plate. The coelomocytes solutions of each sample have been firstly centrifuged at 3000 rpm for five minutes at 4°C. Subsequently, the supernatants have been removed and the cells pellets have been resuspended with cold PBS by pipetting. The PBS has been used to clean the cell solutions, indeed, for each tube, 0.3 ml of PBS has been added and after resuspension, the samples have been centrifuged at 3000 rpm and 4°C for 5 minutes each time. This

passage has been repeated three consecutive times to ensure samples containing only the coelomocytes, improving the quality of subsequent images. Afterwards, the cell pellets have been newly resuspended with 0.3 ml of cold PBS and 6.0 µl of fluorescent probe paying attention that the negative controls have not to be treated with the fluorescent dyes. The test tubes have been then incubated for 30 minutes in the dark to favourite the staining process. Following, the test tubes have been newly centrifuged at 3000 rpm and 4°C for 5 minutes to remove the supernatant containing the fluorescent probe excess, which could interfere in the image analysis by increasing the background intensity. The cell pellets have been then resuspended in new cold PBS solutions. The content of each test tube has been then separately plated into a reading plate. The positive controls have been treated with 5 µl of FCCP (Trifluoromethoxy carbonylcyanide phenylhydrazone) 0,001 mM in Ethanol 1 µM to incentivize the ROS production. Observations have been done thanks to Operetta® CLS, High Content Imaging System, PerkinElmer. For DHE probe detection, the microscope has been equipped with a dihydroethidium filter (eccitation:490-510nm; emission:560-630nm and 650-760nm), the images have been taken at 40X enlargement, confocal for 250 ns. For DHR probe detection, the microscope has been equipped with a rhodamine filter (eccitation:490-510nm; emission:530-590nm and 650-760nm), the images have been taken at 40X enlargement, confocal for 250 ns. The ROS levels evaluation has been performed differently between the two fluorescent probes. The DHE analysis allowed observation and evaluation of ROS content in the nucleus and cytoplasm. The images have been evaluated thanks to Harmony® high-content analysis software.

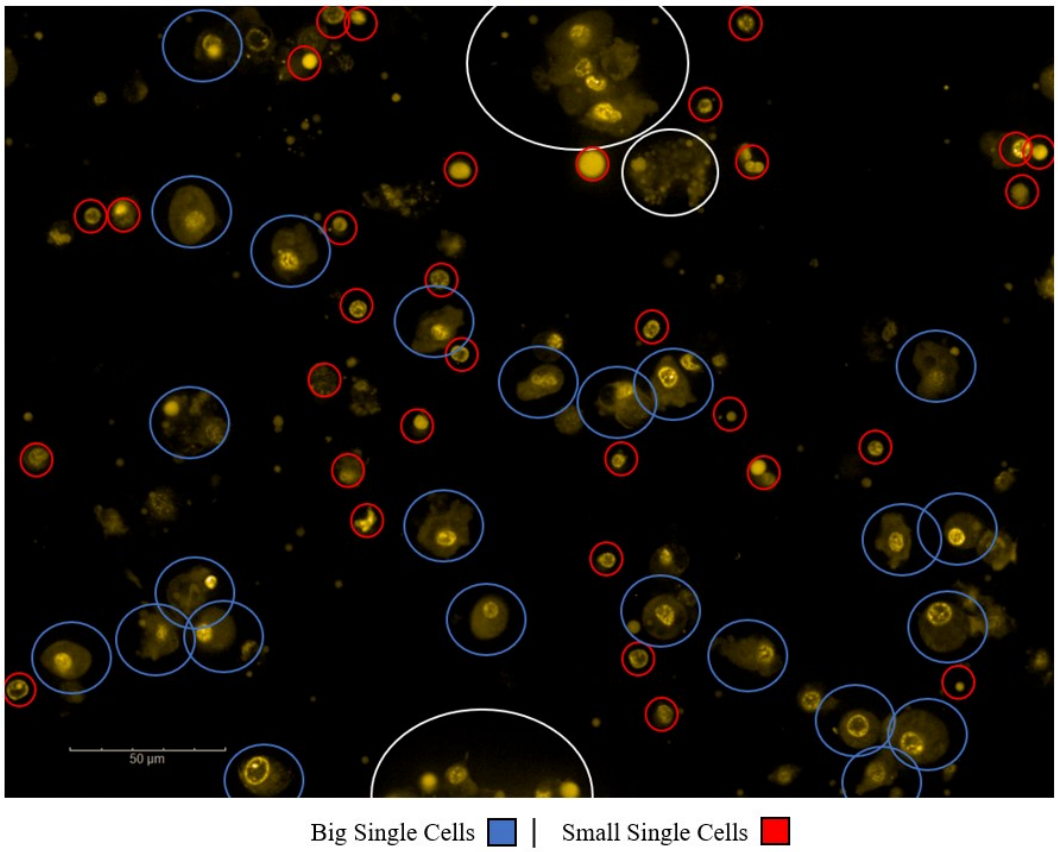


Figure 3.4 Example of Harmony® high-content analysis software DHE probe image training.

To carry out the analysis, it has been necessary to perform image training. In Figure 3.1 it is reported the specific evaluation of cells in this analysis. For this analysis, it has been differentiated the Big cells from Small cells, the two groups have been evaluated separately. The DHR analysis allowed observation and evaluation of ROS content in the mitochondria. The cells have been divided into four categories: Big single cells and Small single cells, Cluster cells and Cluster Dead cells, which have been separately evaluated. The image analysis has been performed thanks to the Harmony® high-content analysis software, which is designed for PerkinElmer high-content screening systems.

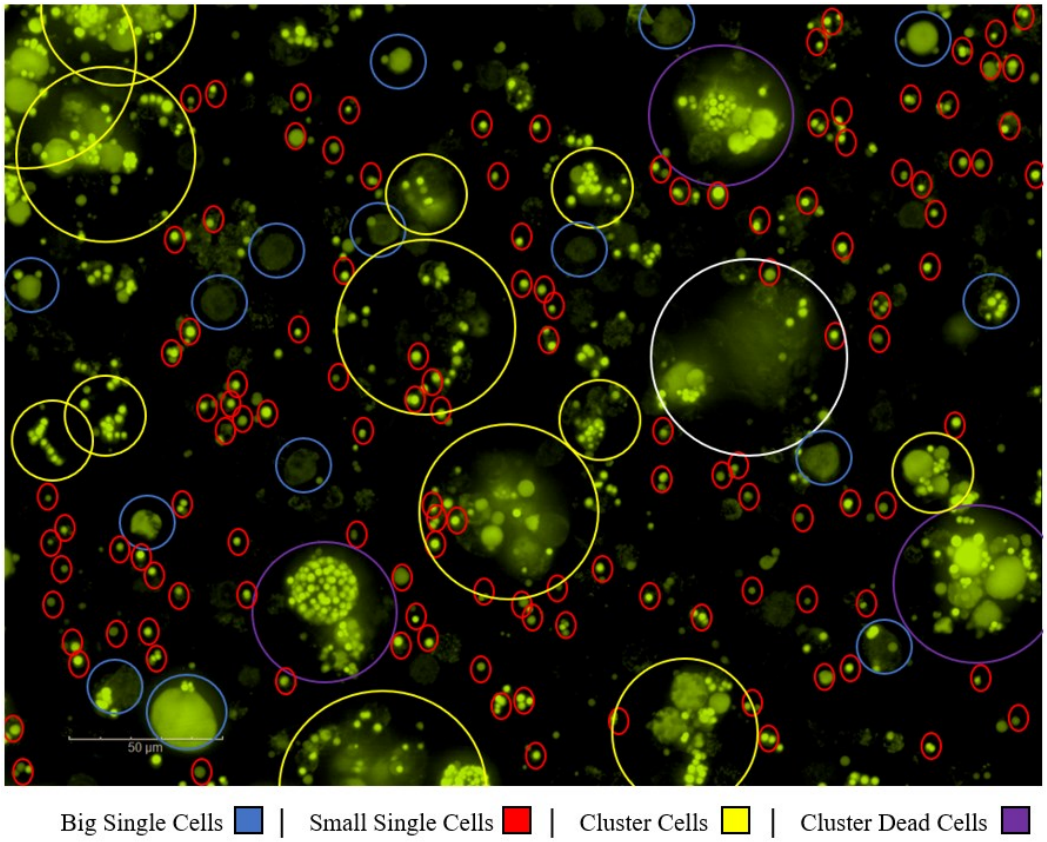


Figure 3.5 Example of Harmony® high-content analysis software DHR probe image training.

To carry out the analysis, it has been necessary to perform image training. In Figure 3.2 it is reported the specific evaluation of cells for DHR analysis. For this analysis, it has been differentiated Big single cells and Small single cells, Cluster cells and Cluster Dead cells, the four classes have been evaluated separately.

3.5 TOTAL ANTIOXIDANT CAPACITY DETERMINATION

The Total Antioxidant Capacity assay has been performed by the utilization of abcam® TAC 65329 kit assay, which allows the test of the total antioxidant capacity and also the antioxidant capacity of the small molecule only thanks to a protein mask. To perform the assay, the sample coelomocyte's solutions have been defrosted, counted and normalized to a concentration of $2x10^6$ cells/ml. A series of test tubes have been prepared by paying attention that each sample, blank included, have been present in double. The calibration standard curve has been constructed using 1µM Trolox Standard as reported in table X. In the case that it has been supposed to proceed with the total antioxidant capacity determination the coelomocytes solutions have been diluted with Milli-Q H_2O 1:1 considering that the final volume that has been introduced in each well is 100 µl. Subsequently, 100 µl of Copper (Cu^{2+}) working solution,

previously prepared by mixing one part of Cu^{2+} reagent with 49 parts of the Assay diluent, has also been added to each well of the analysis plate. The plate has been then closed and positioned on an orbital shaker at mid velocity, in the dark and at room temperature for 90 minutes. The sample's optical density (OD) has been then read by spectrophotometric analysis at 560 nm ± 10 nm thanks to the spectroscope TECAN Infinite® F200PRO.

In order to obtain sample antioxidant capacities, the sample's OD have been subtracted from the blank measurement and then has been applied the following formula:

$$Sample\ Total\ Antioxidant\ Capacity = \frac{Ts}{Sv} \times D$$

Where:

Ts: TAC amount in the sample well calculated from standard curve (nmol)

Sv: Sample volume added in the sample well (µl)

D: Sample dilution factor

Afterwards, the individual antioxidant capacities have been normalized onto the cell concentrations and reported as result. By the difference between the total antioxidant capacity and the antioxidant capacity due to small antioxidant molecules, it has been possible to determine the concentration of the antioxidant enzymes.

3.6 TOTAL PROTEIN DOSAGE

The total protein dosage determination has been performed following the Lowry methodology (Lowry O.H. et al., 1951) which involves the construction of a standard calibration line using bovine albumin. The reagents that have been used are:

- A solution: 2% Na_2CO_3 and NaOH 0.1N
- B solution: 0.5% $CuSO_4$ x $5H_2O$ in 1% sodium citrate ($Na_3C_6H_5O_7$)
- C solution: 25 ml of A solution + 0.5ml of B solution
- D solution: 2.72 ml of milliQ water + 2.0 ml of Folin-Ciocalteu
- Bovine albumin: 1 mg/ml

A set of test tubes has been set up so that each sample (blank included) is present in duplicate. The standard calibration line has been determined using 10, 20, 40, 60, 80 µl of bovine albumin solution and then the test tubes have been respectively filled up with

milliQ water to reach the final volume of 200 µl. For the samples, a specific volume of sample solution has been chosen so the reading from the spectrophotometer is included into the standard calibration line range, then fill up the test tubes with milliQ water to reach the final volume of 200 µl (for the control solution homogenization buffer have been used instead of sample solution). Subsequently 1ml of C solution has been added to each test tube and after vortexing the solutions have been incubated at room temperature for 10 minutes. Following, 100 µl of D solution have been added to each test tube and the solutions have been votexed again, then the solutions have been incubated for 30 minutes at room temperature. To conclude the absorbance of the samples and the controls have been determined by spectrophotometric reading using the UV-Visible spectrophotometer Cary 50 scan. Once the standard calibration line's equation have been determined it has been possible to calculate the total internal protein concentrations (µg/ml) of the samples.

$$Y = mX + q$$

Where: Y is the sample absorbance that have been red at 750 nm and X is the internal protein concentration of the sample.

3.7 METALLOTHIONEIN (MT) DOSAGE

The MTs concentrations into *Dendrobaena veneta* tissues have been determined thanks to Scheuhammer and Cherian (1991) methodology, or rather the silver saturation method which exploits the high affinity of silver and the high thermostability of MTs (Scheuhammer and Cherian, 1991). Since this methodology can be used to determine reduced MT only, it has been necessary to undergo the samples to a pre-treatment into reducing conditions (Santovito et al., 2008). Following the methodologies mentioned above it is possible to determine the total MT concentration and the reduced MT concentration, it is also possible to obtain the oxidized MT concentration by subtract the reduced MT concentration from the total MT concentration. The reagents that have been used are:

- Homogenization buffer Tris-HCl 20 mM pH 7.5; Leupeptin 0.006 mM; PMFS 0.5 mM; β-mercaptoethanol 0.01%
- Glycine buffer 0.5 M pH 8.5
- Silver solution (Ag) 20 ppm in glycine
- 2-mercaptoethanol 10 mmol/l
- Zinc chloride ($ZnCl_2$) 6 mmol/l

- Haemoglobin 2% in milliQ H_2O

The nightcrawlers, conserved into -80°C freezer, have been then divided into three segments; the central segment, which has been taken after the clitellum has been undergone MT content analysis while the other two segments of each worm have been replaced in the freezer for further analysis. The central segment has been weighted and placed in a test tube where it has been also added a volume of homogenization buffer equal to four times the weight of the sample (in grams), the samples have been then homogenised using a Polytron PT-MR 3000. Subsequently, the homogenized solutions have been divided into 1.5ml Eppendorf test tubes and they have been then centrifuged at 4°C and 16000 rpm for 60 minutes in a 5415D Eppendorf Centrifuge. The supernatant solutions have been divided into two parts, a volume of 500µl have been undergone to total MT content determination while the remaining volumes have been conserved in ice for the successive reduced MT content determination analysis.

The total MT content determination Analysis has been performed adding 0.35 µl of 2-mercaptoethanol to the 500 µl sample's solutions, the solutions have been then insufflated for 2 minutes with gaseous nitrogen (N_2) and subsequently, they have been incubated for 15 minutes into reducing atmosphere (nitrogen saturated). Furthermore, the sample's solutions have been added 1.70 µl of zinc chloride ($ZnCl_2$) and the solutions have been then insufflated with gaseous nitrogen for 2 minutes and incubated in reducing atmosphere as mentioned before for 2 hours. For the MT content determination, a series of test tubes have been prepared and it has been paid attention that each sample, blank included, has been present in double. In all the tubes, it has been added a certain amount of sample's solutions (homogenization buffer Tris-HCl for the blank) and glycine so that the final volume inside each test tube have been 800 µl. To each sample's solution, it has been added 500 µl of 20 ppm silver solution, the test tubes have been then vortexed and incubated for 10 minutes at room temperature. When the incubation time is terminated it has been possible to add 100 µl of 2% haemoglobin, whereupon the solutions have been vortexed and then immersed into a boiling water bath for 2 minutes, after which the test tubes have been centrifuged at room temperature and 2500 rpm for 10 minutes. The addition of haemoglobin, the immersion in the boiling water bath and the subsequent centrifuge in repeated two more times, paying attention to not vortex the test tubes anymore, these passages allow the silver bounded to the haemoglobin to precipitate making sure that the remaining silver in the solutions is the MTs' bounded. Afterwards, 1 mL of the supernatants have been

transferred into 15 mL polyethylene Falcon tubes and diluted to a final volume of 5 mL with Nitric Acid (HNO_3) 2.5%. At these conditions, the silver is stable for approximately 75 days. The samples have been then analysed thanks to optical emission spectrometry ICP-OES at a wavelength of 328,068nm. The results have been obtained in the order of magnitude of ppb (g/L).

Theoretically, each MT molecule (6500Da) has to be considered saturated by 17 mol of silver (MW: 107.87 g/mol); it is so possible to determine the sample's MT content thanks to the proportionality between the reading of the silver content and the content of MT in the sample considering that 1 µg/ml of silver correspond to 3.55 µg/ml of MT. Applying the following formula it is possible to determine the MT concentration in the samples:

$$\text{MT } \mu g/ml = ([A]-[B]) \times 3.55 \times 5 \times \left(\frac{Vtot}{Vs}\right)/1000$$

Where:

[A] = Silver concentration in the sample (ppb)

[B] = Silver concentration into the blank (ppb)

Vtot = Total volume (1.6 ml)

Vs = Sample volume used in the test

Once the reduced MT concentrations and the total MT concentrations have been obtained; it has been possible to extract the oxidized MT concentrations by difference. To conclude the test, all the MTs concentrations have been normalized to the total protein content for each sample.

3.8 RNA EXTRACTION

Nightcrawlers' RNAs have been extracted with the purpose of further analysis on MT expression. Earthworms' tissues have been taken from the -80°C freezer and tissue portions of about 100 mg have been placed into 1.5ml Eppendorf test tubes where it has been added 1 ml of TripleXtractor reagent for RNA isolation. The samples have been homogenized the best possible thank to the pestle and the solution have been then incubated at room temperature for 5 minutes after which has been introduced 200 µl of chloroform in each test tube. The tubes have been at first shook vigorously for 15 seconds and after 3 minutes of incubation at room temperature, they have been

centrifuged at 4°C and 13000 rpm for 15 minutes. The RNA precipitation has been possible by transferring the supernatant solutions obtained after the centrifuge in a new sterile 1.5 ml test tube where it has been added 500 µl of 2-propanol, then the tubes have been reversed upside down three times and after 10 minutes of incubation at room temperature they have been centrifuged at 4°C and 13000 rpm for 10 minutes at the end of which it has been possible to observe the RNA pellets on the bottom of the test tubes. The RNAs have been then washed two times with an aliquot of 1 ml of cold 75% ethanol to increase the purity by removing the supernatant, adding the ethanol, vortexing the solution for few seconds and by centrifuging at 4°C and 7500 rpm for 5 minutes. After the RNA purification, the pellets have been left to dry in a biological hood with laminar air flux for 10 minutes at the end of which the RNA have been resuspended with 100 µl RNAse free water and let to dissolve at 4°C for 2 hours and subsequently at 55°C for 10 minutes. The RNAs purity has been tested with the NANODROP spectrophotometer reader considering 280/260 rate and 260/230 rate. Samples that have not been considered pure enough have been undergone lithium chloride (LiCl) purification. This procedure includes the centrifugation of the samples at 4°C and 13000 rpm for 15 minutes, then the supernatants have been transferred to new sterile 1.5 ml Eppendorf test tubes. The solutions have been then mixed with 34 µl of LiCl, or rather ⅓ of the dissolution water used in the previous passage, the tubes have been then incubated in the fridge under ice at 4°C overnight. The day after the test tubes have been centrifuged at 4°C and 13000 rpm for 20 minutes at the end of which the supernatant has been discharged and the RNA pellets have been washed with 200 µl of 75% cold ethanol for two times. Terminated the purification, the samples have been left to dry as mentioned before and then the pellets have been resuspended into 45 µl of RNAse free water. The last passage includes the incubation of the samples into a 55°C water bath for 10 minutes after which it is possible to determine the RNA purity by NANODROP spectrophotometer.

3.9 DNA RETRO TRANSCRIPTION

The RNA samples that have been extracted from the nightcrawler, have been then retrotranscripted to DNA to undergo the sample's DNA to real-time PCR (Polymerase Chain Reaction) to identify possible MT expression differences between the treated organisms and the control ones. 1µl of RNA have been mixed with 2 µl of RNAse free water and 2 µl of Oligo DT primer(15) 1:10 diluted into a 0.2 ml Eppendorf test tube.

The test tubes have been incubated into a PCR thermocycler at 70°C for 10 minutes after which it has been added Mr Mix that for each sample contains: 2.5 µl of reaction buffer, 2.5 µl of magnesium chloride ($MgCl_2$), 1 µl of deoxyribonucleotide triphosphates (dNTPs), 0.5 µl RNAse inhibitor, 1 µl of reverse transcriptase and 7.5 µl of RNAse free water. The samples have been undergone the reverse transcription cycle formed by 5 minutes at 25°C, then 42°C for 60 minutes and then 70°C for 15 minutes, the samples have been then kept at 4°C until they have been stored in a -20°C freezer. The cDNA obtained as mentioned above can last for several weeks if stored correctly.

3.10 PRIMER DESIGN

To analyse the MT expression trend in *Dendrobaena veneta* exposed to PFAS, the MT sequence has been studied since no primers for *Dendrobaena veneta*'s MT have been published yet. Since this species has not been studied enough, it has been necessary to build a primers pair with which is possible to amplify a segment of MT sequence in *Dendrobaena veneta*'s DNA. MT coding sequences of different annelids founded on the NCBI website have been compared by the BLAST NCBI program and then aligned thank to Muscle program with the aim of funding similarity between the sequences on which designing the primers. Similarities between *Eisenia foetida*, *Eisenia andrei* and *Lumbricus terrestris* MT sequences have been founded. The sequences have been then uploaded into the Primer3 program which, by setting specific parameters for the primers of interest, such as melting temperature (between 50 and 60°C), primer length(between 100 and 200 bp), probability of secondary structures and cytosine and guanine residual rate content (GC content around 55%), gives as the result of output different primers pair. The validity of the primer's pairs have been then tested thanks to the IDT OligoAnalizer program by performing self-dimer analysis, harping analysis and hetero-dimer analysis, only the primer's pair that passed this selection have been tested with *Dendrobaena veneta*'s DNA. The same procedure has been done to obtain a primer's pair for housekeeping genes in order to compare the expression of MT to the expression of a gene which expression shouldn't change due to PFAs exposure.

Table 3.3 MT primers sequences that have been tested.

Primer	Sequence 5'- 3'	Length of the amplicon (bp)	Annealing Temperature (°C)
RTPCR_Forw2_Lterrestris_MT	CACAATGTGGAAATGCAGGC	100	60
RTPCR_Rev2_Lterrestris_MT	CACTAGTCACCACAGCATCC		60

Table 3.4 Housekeeping genes primer sequences that have been tested.

Primer	Sequence 5'- 3'	Length of the amplicon (bp)	Annealing Temperature (°C)
GAPDH_E.fetida_F2	CAACTGCTTGGCTCCACTGG	129	60
GAPDH_E.fetida_R2	CGCCAGTCCTTGTTGCTTGG		60
28S_E.andrei_F2	CGTCGTCCAAAGAACAGACC	274	60
28S_E.andrei_R2	TAAGCAAACCGACTCGCCGT		60
18S_E.fetida_F	CATGCCACAAAGCTCCGACC	248	60
18S_E.fetida_R	AGCCGTTTCTCATGCTCCCT		60

3.11 cDNA AMPLIFICATION

To verify the primer's pairs functionality, the cDNAs have been undergone PCR amplification. Both primers for MTS and RGs have been tested by using the GAPDH, 28S and 18S as housekeeping genes. Since GAPDH, 28S and 18S are endogenous genes that are present in several phyla, their expression is not compromised by toxicants exposure. The nightcrawler's cDNAs have been mixed with the following PCR mixture reported in the table below to amplify the gene's sequence of interest.

Table 3.5 Polymerase Chain Reaction reagents specific mixture

Reagent	Volume for sample (µl)
PCR reaction buffer 10X (grisP®)	4.0
Calcium chloride ($mgCl_2$) 25mM (grisP®)	2.5
dNTPs 10mM (grisP®)	1.5
Forward primer 5pmol/µl	1.5
Reverse primer 5pmol/µl	1.5
Xpert Taq DNA polymerase 5U/µl (grisP®)	0.2
c-DNA	0.5
RNAse free water	Up to 25

The PCR thermal protocol has been as follows: 2 minutes at 95°C for the initial denaturation of the cDNA followed by 40 cycles of 30 seconds at 95°, 30 seconds at 60°C and 20 seconds at 72°C, followed by 10 minutes at 72°C.

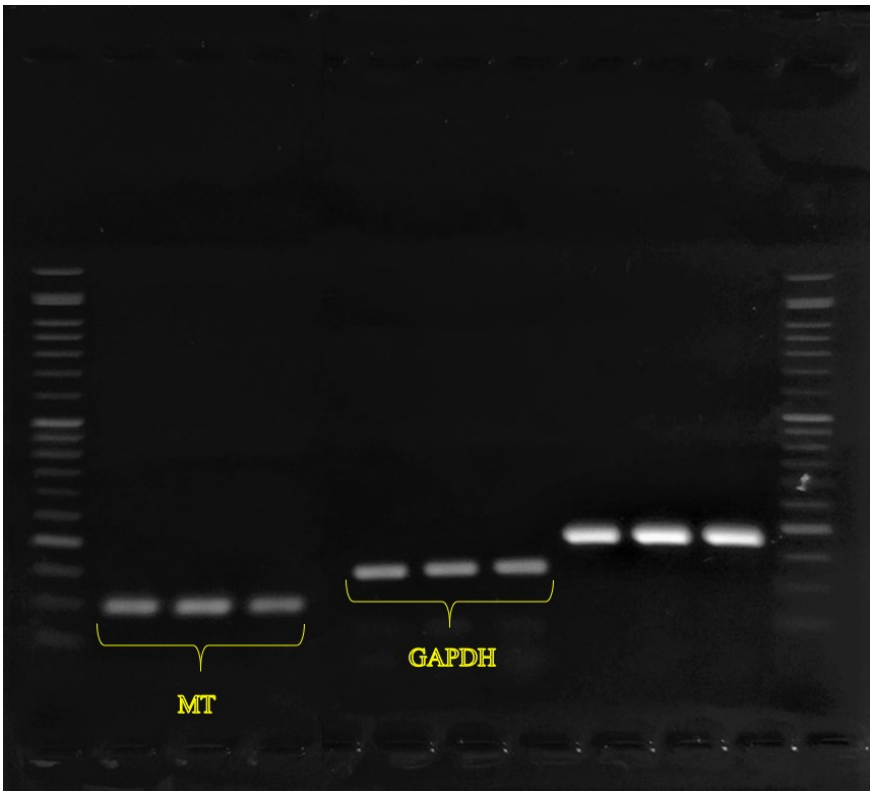


Figure 3.6 Electrophoretic stroke of the amplicons under analysis.

The amplicons obtained following the PCR have been run in a 1.5% agarose gel after being stained. Only the amplicons for which a positive result has been observed undergone Real-time PCR.

3.12 DETERMINATION OF THE EXPRESSION OF METALLOTHIONEIN (MT)

To evaluate the MTs expression rates, the cDNA of 8 organisms for every treatment group had undergone Real-time PCR analysis. To evaluate the possible under or up-regulation of MTs expression it has been necessary to compare and normalize the MTs expression rates to a housekeeping gene expression rate. To determine the specificity of the primers, the samples have been firstly tested at different concentrations. The primers that resulted to work specifically undergone RT-PCR analysis. In the present research study, the MTs expression rates have been normalized onto GAPDH expression rates by setting the threshold at 0.05. The RT-PCR cycle has been set as follows: initial denaturation at 95°C for 2 minutes followed by 38 cycles of 95°C for 5 seconds and 60°C for 35 seconds.

Table 3.6 RT-PCR specific mixture

Reagent	**Volume for sample (µl)**
2x qPCRBIO SyGreen Mix (PCRBIOSYSTEM®)	5.0
Forward primer 10µM	1.0
Reverse primer 10µM	1.0
c-DNA	1.5
RNAse free water	1.5

3.13 STATISTIC ANALYSIS

The results have been reported as the average ± SD (Standard Deviation). For the statistical analysis of the data, the Primer program has been used by applying the analysis of one-way variance followed by the Student-Newman-Keuls test to determine the significance.

4 RESULTS

4.1 DEVELOPMENT OF THE COMET ASSAY METHODOLOGY FOR THE EVALUATION OF THE GENOMIC DAMAGE

The secondary scope of this research study was to develop a new methodology, by modifying the procedure of Singh N. P. et al. (1988), of the comet assay for the genomic damage evaluation. The aim was to develop an easy and reproducible methodology in any research laboratory without special tools. The changes that have been made to Singh N. P. et al. (1988) methodology allowed to analyze a large number of samples in a relatively short time. Indeed, in total, the methodology development phase and the samples analysis phase required 20 hours of image acquisition and 22 hours of image analysis. Most of these hours have been implemented for the procedure set up and only 6 hours and 4 hours have been respectively used for the images acquisition of the tested samples and the image analysis. For the genomic damage evaluation in this research study, the coelomocytes of 6 organisms for each exposure group had undergone analysis. For each organism, a positive control, in which genomic damage was accentuated upon treatment with hydrogen peroxide, a negative control, and a technical triplicate have been analyzed. In total, approximately 60,000 cells have been analyzed, of which a false positive error of only 1.46% has been determined. The analysis methodology allowed to divide the nuclei into four classes of increasing damage. This classification allowed, instead of a simple qualitative presence-absence analysis of the comet, to perform a semi-quantitative analysis of the genomic damage. This methodology does not exclude a future development of a completely quantitative methodology, which would require more time for image analysis and methodology design. However, the results obtained by the genomic damage evaluation of this research study were satisfactory and allowed to discreetly evaluate the genomic damage due to xenobiotics exposure in organisms.

4.2 DNA DAMAGE EVALUATION

To evaluate the DNA damage, the coelomocytes had undergone the comet assay. The evaluation of the genomic damage has been done by dividing the cells into four classes of increasing damage. The classes that have been scored are Healthy nuclei, Damaged nuclei, High damaged nuclei and Completely damaged nuclei. Each class have been analyzed separately to detect even small differences in genomic damage. In addition,

the three classes of damaged cells have been summed with the scope of evaluating the general health status of cells.

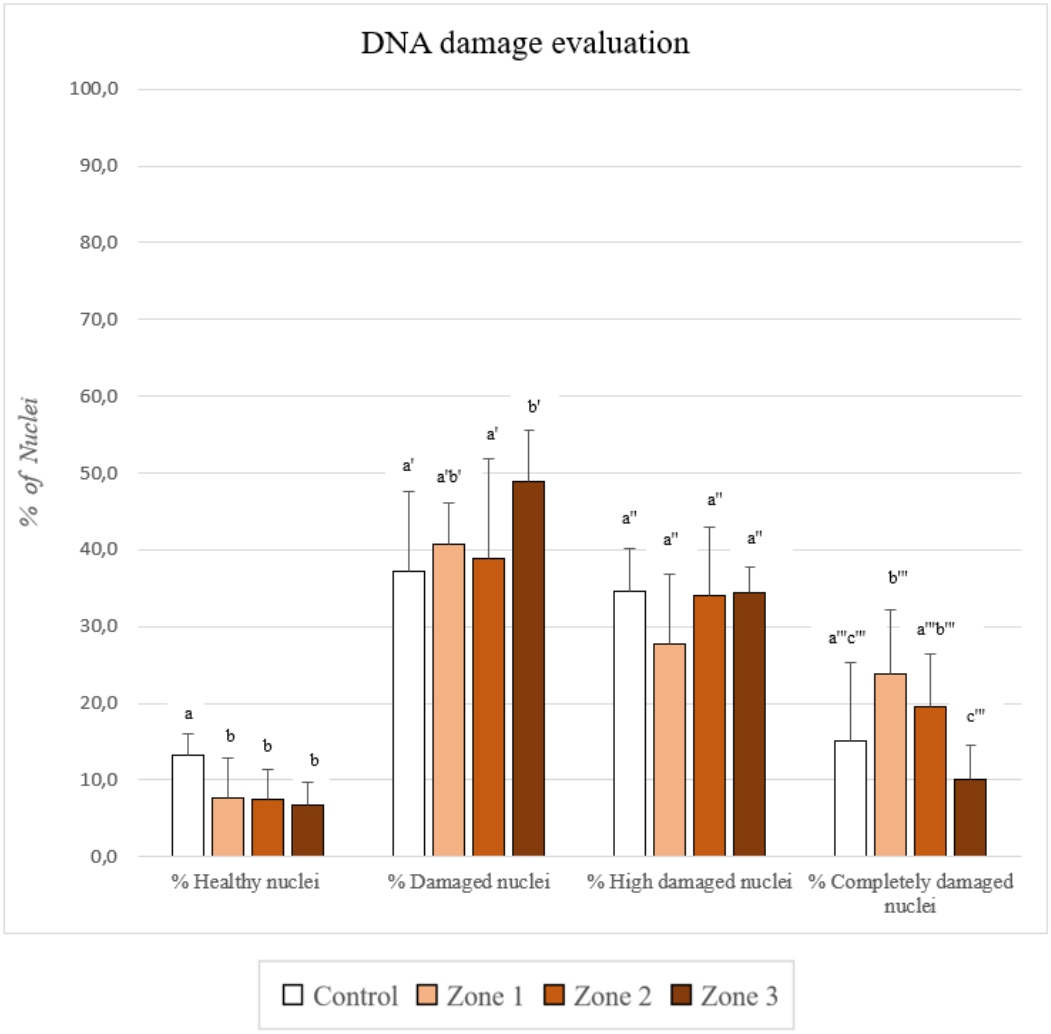


Figure 4.1 Evaluation of genetic damage to coelomocytes. The results are reported as percentages, and for each treatment, the cells have been divided into four classes: Healthy nuclei, Damaged nuclei, High damaged nuclei and Completely damaged nuclei. The four data sets have been statistically compared with each other separately. The presence of different letters in the graph underlines a statistically significant difference ($p < 0.05$). n =6.

In the graph above is reported that the percentage of healthy cells decrease significantly above 50% in all three treatment groups compared to the control. The comparison of Damaged nuclei shows that a statistically significant increase of about 30% in zone 3 treatment compared to the control, while zone 1 and zone 2 treatments do not differ from the control. The Highly damaged nuclei do not show any statistically significant differences between the control and the treatment zones. On the other hand, the comparison between Completely damaged nuclei shows a statistically significant increase of about 60% in the zone 1 treatment group compared to the control. Despite

it is not statistically confirmed, zone 3 shows a decrease of about 30% compared to the control.

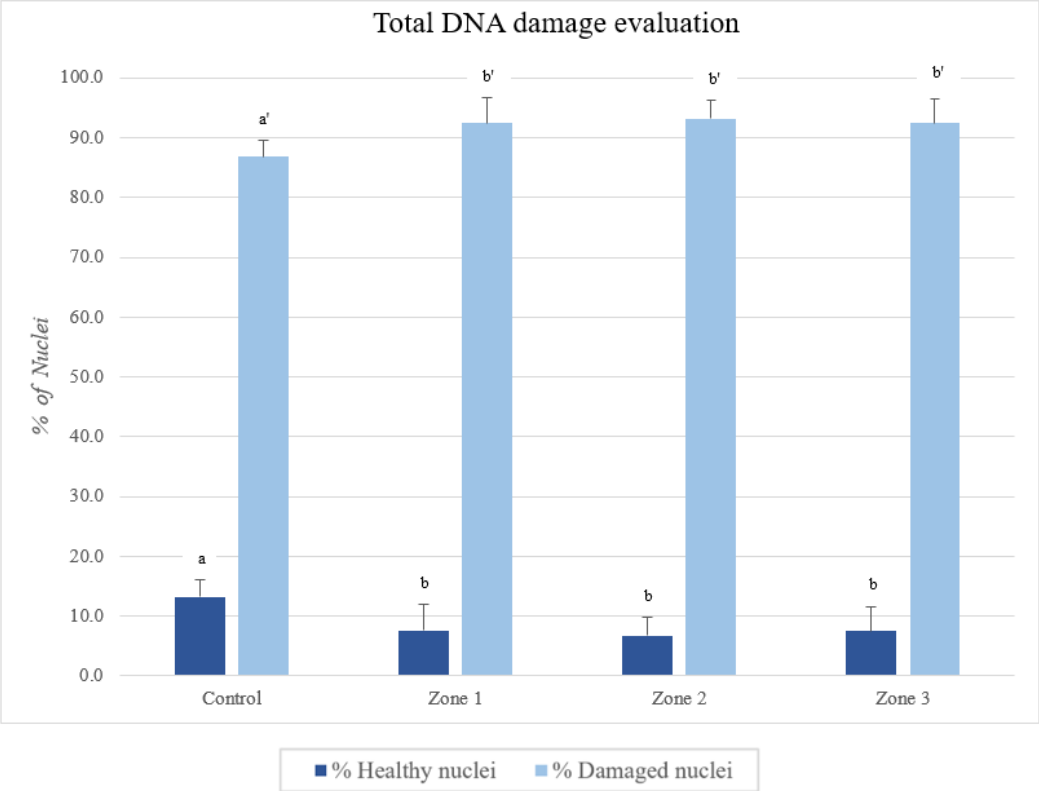


Figure 4.2 Evaluation of genetic damage to coelomocytes. The results are reported as percentages, and for each treatment, the cells have been divided into two classes: Healthy nuclei and Damaged nuclei, which are the sum of the three classes of cellular damage scored in the previous genetic damage evaluation. The two data sets have been statistically compared with each other separately. The presence of different letters in the graph underlines a statistically significant difference ($p < 0.05$). $n = 6$.

The graph above shows that a statistically significant decrease in the Healthy nuclei percentage between the control and the three treatment groups of about 50%. On the other hand, The damaged nuclei show a statistically significant increase of about 7% in all the treatment groups compared to the control.

4.3 REACTIVE OXYGEN SPECIES (ROS) DETERMINATION

To evaluate the target of oxidative stress by xenobiotics exposure, the evaluation of the presence of reactive oxygen species has been evaluated in both the nucleus and mitochondria. Indeed, for this assay, the coelomocytes have been treated separately with both dihydroethidium (DHE) and dihydrorhodamine (DHR). It is known that DHE is oxides by superoxide anion ($\bullet O_2^-$) into the nucleus, while DHR is oxidized by hydrogen peroxide (H_2O_2) and by peroxynitrite ($ONOO^-$). It has been observed that these last two reactive species seems to act site-specifically in the mitochondria.

4.3.1 *Superoxide anion ($\bullet O_2^-$) determination*

To determine the superoxide anion concentrations in the cell solutions the samples were treated with the DHE dye probe. The images have been obtained thanks to the Operetta® microscope, High Content Imaging System, PerkinElmer and evaluated thanks to Harmony® high-content analysis software. The images analysis has been performed by differential evaluation between the nucleus concentration of ROS and the cytoplasm content of ROS. The evaluation, as reported below, has been performed on both the classes of cells evaluated in this experiment.

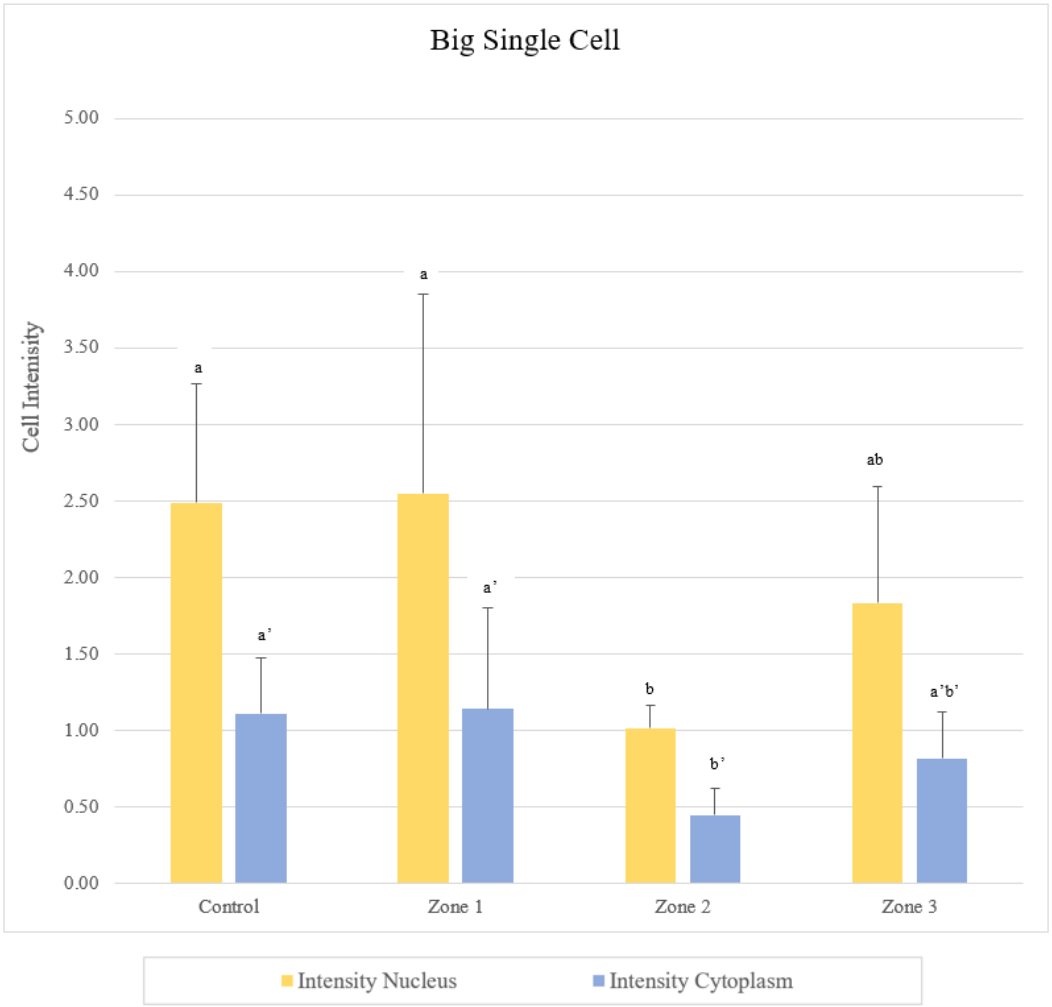


Figure 4.3 Coelomocytes ROS determination. The intensity of the probe normalized on the number of cells classified as "Big Single Nuclei". The intensity has been assessed both in the nucleus (yellow) and in the cytoplasm (blue). The two data sets have been statistically compared with each other separately. The presence of different letters in the graph underlines a statistically significant difference ($p < 0.05$). n = 3.

The graph above shows that the superoxide anion concentrations do not statistically differ between the control, zone 1 and zone 3. On the other hand, a significant decrease

in the concentration of (• O_2^-) in zone 2 of approximately 60% has been reported both in the nucleus and the cytoplasm.

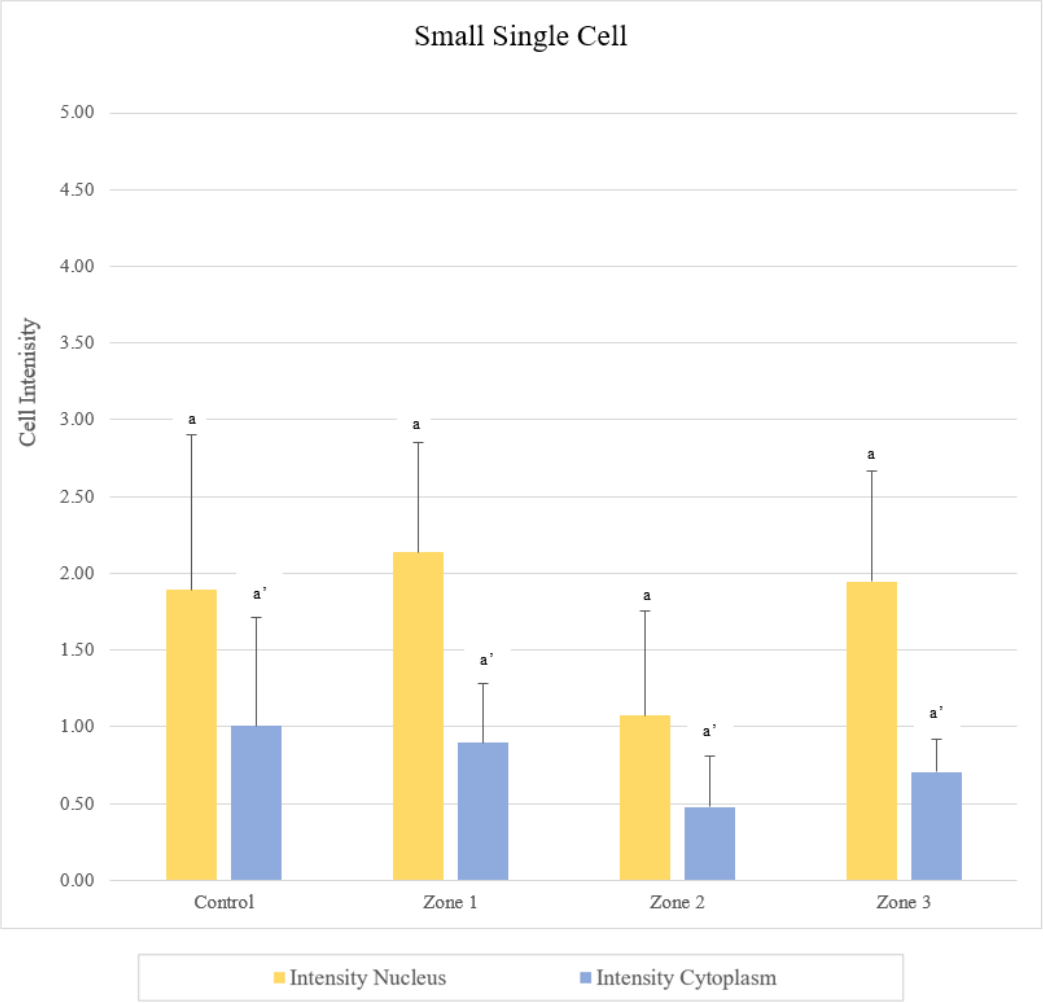


Figure 4.4 Coelomocytes ROS determination. Intensity of the probe normalized on the number of cells classified as "Small Single Nuclei". Intensity was assessed both in the nucleus (in yellow) and in the cytoplasm (in blue). The two data sets have been statistically compared with each other separately. The presence of different letters in the graph underlines a statistically significant difference ($p < 0.05$). $n = 3$.

The graph above shows that the superoxide anion concentrations in Small Single cells do not statistically differ between the control, zone 1, zone 2 and zone 3. It is possible to deduce that, in this experiment, the PFASs exposure does not influence the concentrations of (• O_2^-) in the nucleus and the cytoplasm

4.3.2 *Hydrogen peroxide (H_2O_2) and Peroxynitrite ($ONOO^-$) determination*

To determine the sum of hydrogen peroxide and peroxynitrite concentrations, the samples were treated with the DHR dye probe. The images analysis allowed the detection of the levels of ROS in the mitochondria of four different kinds of cells. The types of cells that have been counted for performing this analysis were Big cells, Small cells, Cluster cells and Cluster dead cells. The groups have been evaluated separately. The images have been evaluated with Harmony® high-content analysis software. For the analysis, it has been necessary to perform image training. In Figure 4.4 and in Figure 4.5 it is reported the specific evaluation of cells in this analysis. For this analysis, it has been differentiated Big cells, Small cells, Cluster cells and Cluster dead cells. The groups have been evaluated separately.

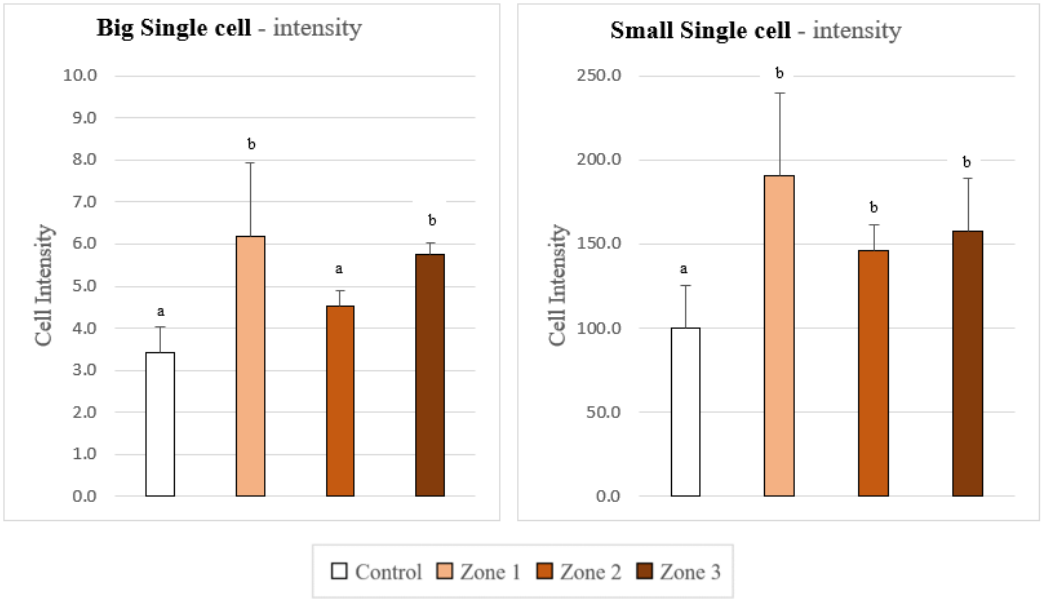


Figure 4.5 Coelomocytes ROS determination. The intensity of the probe normalized on the number of cells classified as "Big Single cell" and "Small Single cell". The two data sets have been statistically compared with each other separately. The presence of different letters in the graph underlines a statistically significant difference ($p < 0.05$). n = 3.

The graphs above show that hydrogen peroxide and peroxynitrite concentrations statistically differ between the control and the treatment underlining that PFASs may influence ROS production in the energy chain reactions in the mitochondria. In particular, it is possible to observe that the cell intensity of zone 1 is almost 2 fold more compared to the controls. Despite an increase of about 30% of cell intensity in zone 2 "Big cells", it is not statistically different from the controls. On the other hand, a statistically significant increase of 45% has been reported in the intensity of "Small

cells" zone 2. Following zone 1 and zone 2, zone 3 show a statistically significant increase of the cell intensity for both "Big cells" and "Small cells" by 70% and 60% respectively.

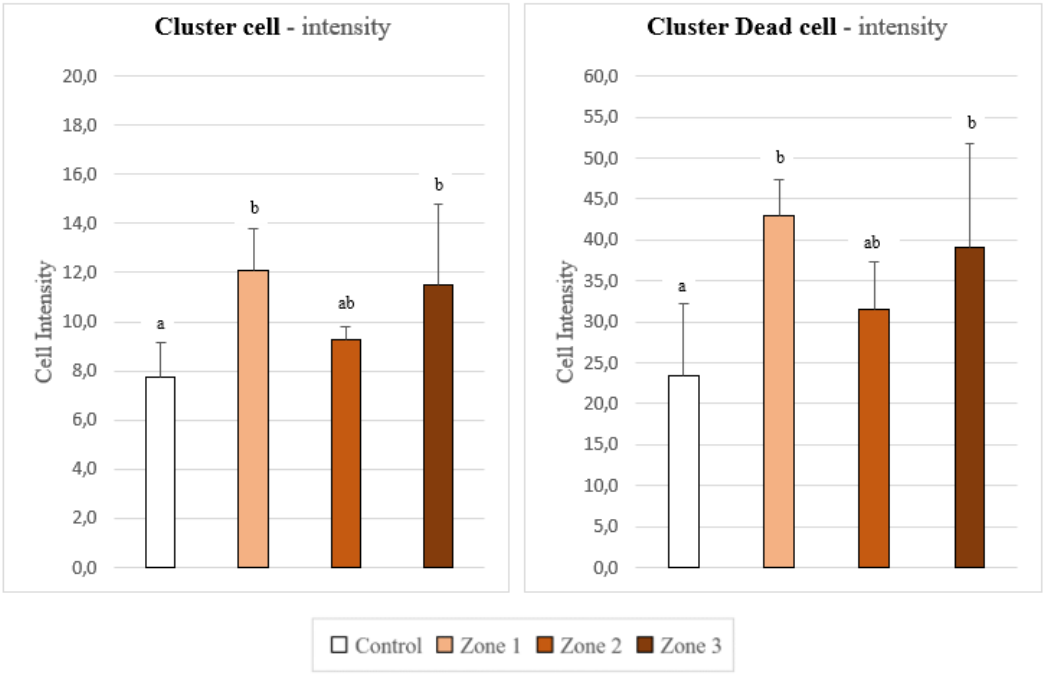


Figure 4.6 Coelomocytes ROS determination. The intensity of the probe normalized on the number of cells classified as "Cluster cell" and "Cluster Dead cell". The two data sets have been statistically compared with each other separately. The presence of different letters in the graph underlines a statistically significant difference ($p < 0.05$). n =3.

As mentioned above, the graphs above show hydrogen peroxide and peroxynitrite concentrations in "Cluster cells" and "Cluster Dead cells" in the mitochondria. By the observation of it, as for "Big cells" and "Small cells" evaluation, it is possible to observe a statistically significant increase of ROS in zone 1 treatment by 60% and 80% in the cluster cells and the cluster dead cells respectively. The zone 2 treatments, as for big cells, show an increase of about 20% and 35% in the cluster cells and the cluster dead cells respectively despite they are not statistically different from the control. Zone 3 treatments, in accordance with zone 1, show a statistically significant increase in mitochondria ROS production of about 50% and 65% in the cluster cells and the cluster dead cells respectively.

As general considerations, it is possible to deduce that PFASs exposure increases the ROS content at the mitochondrial level, however, the ROS content can't be linearly correlated to the exposure concentrations.

4.4 TOTAL ANTIOXIDANT CAPACITY (TAC)

To evaluate the Antioxidant capacity (AC) of the organisms, the abcam® TAC 65329 kit assay has been used. This kit resulted to be highly effective since it allows the test both the total antioxidant capacity and the antioxidant capacity of the small molecules only, by masking the antioxidant enzymes involved in detoxification processes. This test has been performed on coelomocytes extruded solution after been purified with PBS solution. The coelomocytes have been individually counted and the antioxidant capacity results have been normalized to the cell concentrations. The results are reported in the graph below as the average antioxidant capacity for the Control, Zone 1, Zone 2 and Zone 3 and the respective standard deviation.

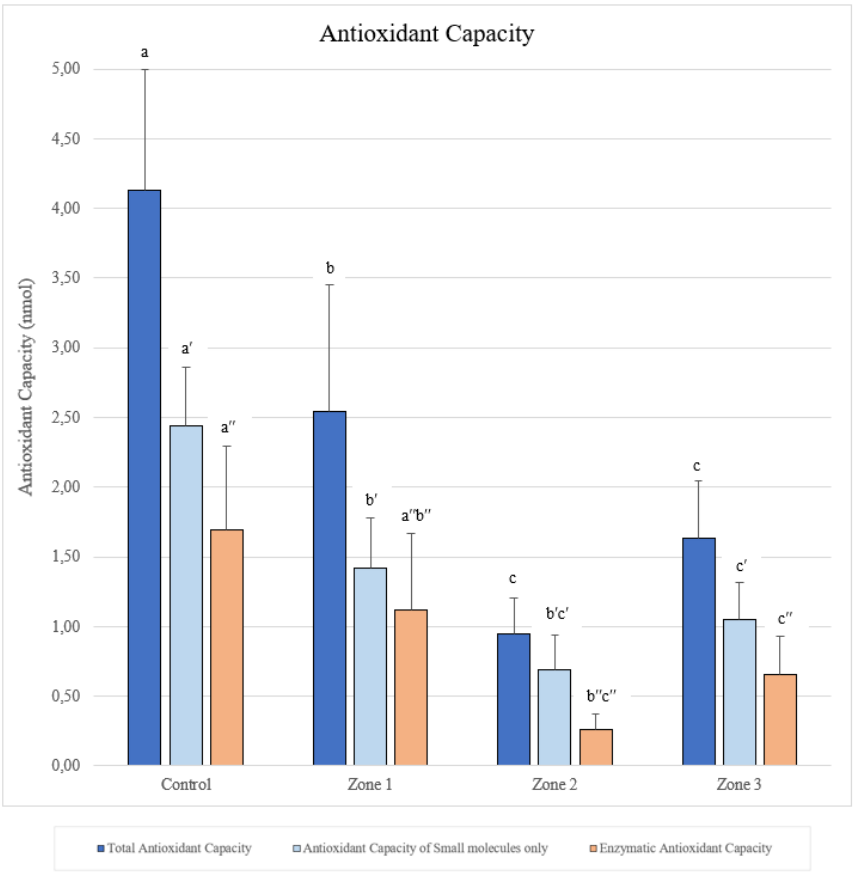


Figure 4.7 Coelomocytes total antioxidant capacity (in blue), antioxidant capacity of small antioxidant molecules only (in light blue) and derived enzymatic antioxidant capacity (in red). The three data sets have been statistically compared with each other separately. The presence of different letters in the graph underlines a statistically significant difference ($p< 0.05$). $n = 5$.

By observing the graph above it is possible to deduce that the antioxidant system is engaged by PFASs exposure. Indeed, a significant decrease in the total antioxidant capacity has been reported for all three treatments. In specific, a decrease of about 40%, 70% and 60% have been registered in zone 1, zone 2 and zone 3 respectively by control

group comparison. There is also a slightly linear correlation between the antioxidant capacity decrease and the PFASs concentration of exposure. The evaluation of low molecular mass antioxidant molecules concentration shows a trend comparable to the total antioxidant capacity. It is also possible to deduce therefore that they are the predominant part of the antioxidant system being about 60% of it, indeed their depletion leads to a consequent decrease in the total antioxidant capacity. Lastly, the enzymatic contribution to the antioxidant capacity show a similar trend comparable to low mass antioxidant molecules but, since they are derived data, the statistical differences between treatment groups can't be completely reliable.

4.5 METALLOTHIONEIN CONCENTRATION

The MTs concentrations into the tissues have been determined thanks to Scheuhammer and Cherian, 1991 methodology, or rather the silver saturation method. By this methodology, it has been possible to determine the Total MTs content and the Reduced MTs content. By the difference of these, it has been also possible to determine the Oxidized MTs concentrations (Scheuhammer and Cherian, 1991).

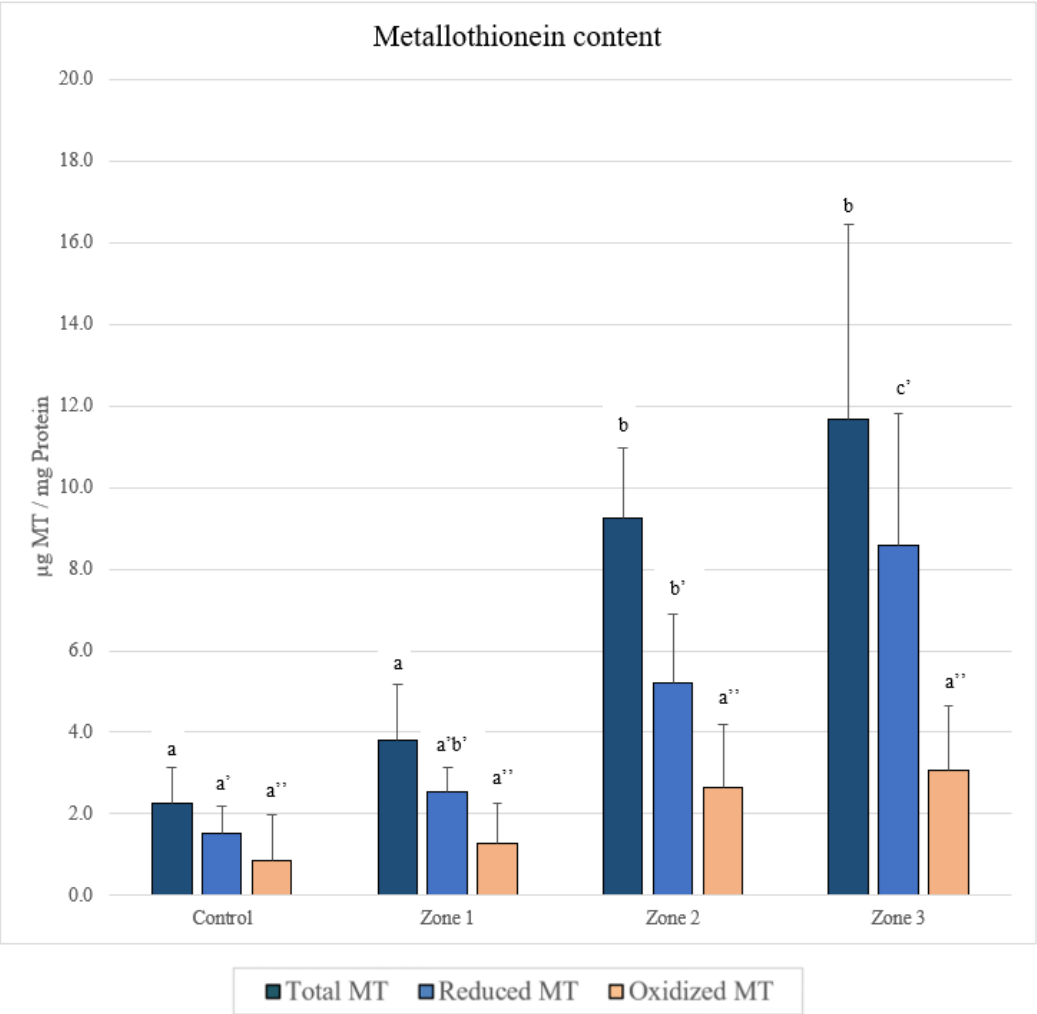


Figure 4.8 Evaluation of the tissues metallothionein's content. The results are reported as µg of MT normalized on mg of total protein content. The results are reported as Total MTs content, Reduced MTs content and by the difference of them, it has been possible to derivate the Oxidated MTs content. The three data sets have been statistically compared with each other separately. The presence of different letters in the graph underlines a statistically significant difference (p <0.05). n=6.

In the graph above it is possible to deduce that, by observing the Total MTs content, zone 1 Mts' levels do not statistically differ from the control even if an increase of about 60% have been registered. On the other hand, zone 2 and zone 3 show a statistically significant increase in the amount of MTs. Indeed it resulted in an increase of 4 and 5 folds respectively the levels of the control.

Regarding the Reduced MTs levels, it is possible to deduce by the graph that no statistical differences are reported for the zone 1 group compared to the control. Whereas, it is reported statistically increases in reduced MTs levels in both zone 2 and zone 3 groups, of about 3 and 5 folds respectively. Oxidated MTs levels instead do not statistically differ between all the treatment groups and the control.

4.6 METALLOTHIONEIN (MT) EXPRESSION LEVELS

MTs expression levels have been evaluated thanks to Real-time PCR analysis. To evaluate the expression of MTs it has been necessary to normalize the expression of MTs with a housekeeping gene. In the present research study, GAPDH has been used as a reference gene.

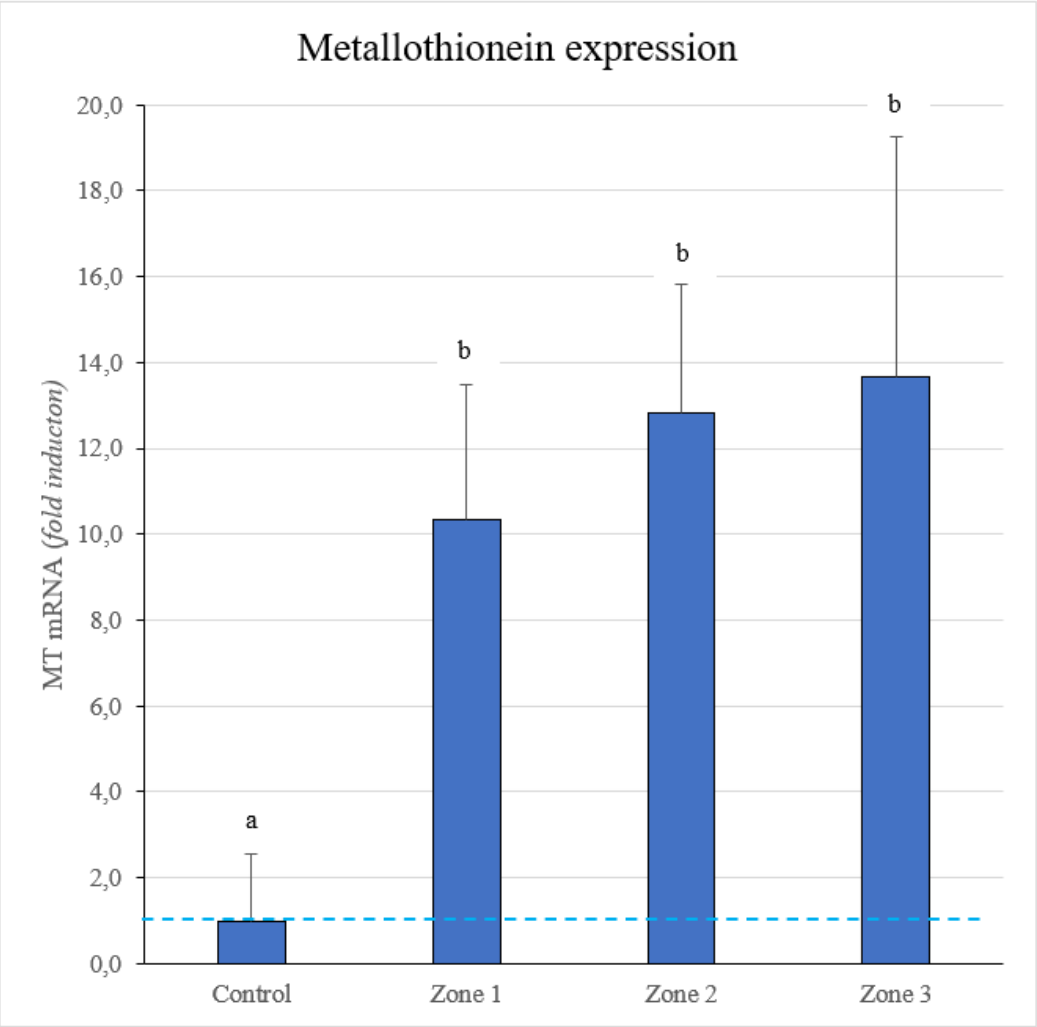


Figure 4.9 Evaluation of the tissues metallothionein expression levels. The results have been normalized on the expression levels of the reference gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The data set has been statistically evaluated. The presence of different letters in the graph underlines a statistically significant difference ($p < 0.05$). $n = 8$.

The graph above clearly shows the statistical difference that has been observed between the MTs' expression levels in the control group in comparison to all three treatment groups. As an assumption, the control group reports an expression level equal to 1 while the treatments groups of zone 1, zone 2 and zone 3 report expression levels 10, 13 and 14 times respectively higher than the control.

5 DISCUSSION AND CONCLUSION

In the last decades, the PFASs pollution issue has become a high scientific interest topic. Due to their wide usage in the production processes of various industrial products, these xenobiotic molecules are detected ubiquitously in the environment (Mussabek et al., 2020). It has been reported that PFASs exposure increases the onset of pathologies such as immunotoxicity, hepatotoxicity, neurotoxicity and alterations of the endocrine system (Guruge et al., 2006; EFSA 2008; Lau et al., 2007; Hu and Hu, 2009; Bjork et al., 2011; Pedersen et al., 2016). Furthermore, chronic exposure to PFASs may cause alterations to the respiratory, nervous and reproductive systems. Moreover, it has been shown that PFASs induce oxidative stress through the formation of ROS (Bonato et al., 2020).

In the present research study, the effects that environmental concentrations of PFASs may have on earthworms, which have been recognised as good soil bioindicators, has been investigated. For the DNA evaluation, it has been necessary to develop a new feasible methodology. The developed methodology was effective for DNA damage evaluation. It presented several favourable factors such as the short execution time, the high reproducibility without specific instrumentation and the analysis speed that allowed the evaluation of several samples.

The DNA damage evaluation reported that all three treatment groups show genomic damage. Indeed, the healthy nuclei percentage decreases significantly in all three treatments with the consequent statistical increase of the damaged nuclei percentage. By observing the DNA damage intensity, it shows a difference between the treatment groups, but the damage rates do not linearly follow the increasing concentration of PFASs. It indicates that other factors participate in the damaging reaction and that the DNA damage might be coped up by the antioxidant system or by the repair system. One of the main factors that induces genomic damage is ROS production. In the present study, it has been shown that PFASs exposure increases the formation of ROS. Furthermore, it has been possible to deduce that PFASs firstly tend to act on the mitochondria since the ROS level in these cells organelles increased significantly. This assumption has also been confirmed by literature, in which it is written that PFASs may cause mitochondrial membrane failure and consequent release of cytochrome c followed by apoptotic processes (Suh et al., 2017). On the other hand, no significant variations have been reported for ROS levels in the nucleus and the cytoplasm. The Life Phoenix project monitoring campaign reported, otherwise, increased ROS levels

in the cytoplasm and the nucleus of earthworms chronically exposed to PFASs (Life Phoenix).

The interpretation of these results might be that low exposure concentrations of PFASs affect ROS nucleus and cytoplasm concentration but, due to the short-term exposure of the present experiment, no significant ROS increases have been observed. The toxico-genetic action of PFASs due to the collapse of mitochondrial functions had been observed primarily by studying the effects of PFOA (Choi et al., 2016). The results of this study showed that a mixture of mainly carboxylic PFASs structurally similar to PFOA, at environmental concentrations, induces oxidative stress to the mitochondria and consequent increase in genetic damage. These results are supported by the assumption that PFASs actions in the mitochondria drive cascade reactions that terminate in the amplification of cell apoptosis (Choi et al., 2016).

The toxic effects of PFASs have also been confirmed by observing the levels of antioxidant capacity. Indeed, at the experimental conditions of this research study, the reduction of the antioxidant capacity has been attributed to PFASs exposure. However, only a slight linear correlation has been reported between the antioxidant capacity and the PFASs concentrations of exposure. Furthermore, the experiment showed that the antioxidant system of these organisms is mainly constituted by low molecular mass antioxidant molecules such as GSH, MT and vitamins. Indeed the depletion of the total antioxidant capacity is mainly driven by the oxidation of low molecular mass antioxidant molecules. Unlike enzymes' antioxidant activities, which catalyse oxidation-reduction reactions with consequent regeneration, non-enzymatic molecules are oxidized by their scavenging ROS reaction. Therefore, they do no longer contribute to the antioxidant capacity unless there are *ex-Novo* reintegrated or by the intervention of other physiological components. An example could be the case of GSH, which once being oxidized to GSSG may be reduced by the enzyme glutathione reductase, reintegrating so its antioxidant capacity (Zitka et al., 2012).

Considering the MT protein content, in this study, it is reported a significant increase, which is also supported by a statistically significant increase in MT expression levels. Despite the MTs scavenger role has been demonstrated by several studies (Santovito et al., 2008; Bell and Valee, 2009), in the present research it has not been confirmed. However, the scavenger role of MTs has to be further studied since no statistically different concentrations of oxidated MTs have been picked out between the treated and the controls organisms. Nonetheless, it has to be considered that even if the oxidate-

reduced MTs ratio has not shown any changes, the ratio of oxidized MTs normalized to the total protein content may presuppose their participation in the antioxidant response to PFASs exposure. Even if it has not statistically confirmed, it is possible to observe a linear increase of oxidated MTs concerning PFASs concentration. In future research, it will be necessary to deeply investigate the MTs scavenging role of mitochondrial ROS since the situation about their involvement is not clear (Kondoh et al., 2001). It is known that, as reported in this study, their expression is enhanced by increased mitochondrial ROS levels (Kondoh et al., 2001; Kadota et al., 2010; Ruttkay-Nedecky et al., 2013). However, it is also known that they are imported into the mitochondrial intermembrane space (IMS), but their specific role has to be further investigated (Ruttkay-Nedecky et al., 2013; Choi et al., 2016). Indeed, Koh et al., in 2020 reported that they are imported in the mitochondrial intermembrane as their oxidated form in order to bind excess free zinc atoms, anyhow, both free zinc and zinc coupled MTs inhibit the mitochondrial electron transport system activity (Koh et al., 2020). Furthermore, Ruttkay-Nedecky in 2013 cited that the ratio between oxidated and reduced MTs might be influenced by the oxidative ratio of GSH. Indeed it has been cited that the MTs' zinc release is mediated by the presence of GSSG. MTs are stabilized at relatively high cellular GSH concentrations and GSH presence inhibits MTs zinc release (Ruttkay-Nedecky et al., 2013). Concerning these findings, it results even more important to determine the internal GSH concentrations, the ratio of GSH/GSSG and the role that GSH has as a participant in the antioxidant system.

By the observation of the rates of MTs transcription and MTs translation, it is possible to underline that the increase in mRNA accumulation is much greater than the mRNA translates in MT protein. This further result confirms that, in our experimental conditions, the total cellular ROS levels do not need MTs' antioxidant intervention. However, they are preparing to implement its biosynthesis with conspicuous gene transcription.

Such a physiological response is typical of pollutants' exposed organisms. The risk of further cellular stress involves non-membranous cytoplasmic elements such as the stress granules (SGs). SGs are cytoplasmic granules defined as the sites of mRNA storage (Olszewska et al., 2012). The hypothesis is that part of the over transcribed mRNA could remain stored in intracellular structures such as SGs, in which these messengers are conserved and, in case of necessity, they might undergo future translation (Lavut and Raveh, 2012; Olszewska et al., 2012). It has been reported that

this physiological condition is characteristic of organisms living in unfavourable conditions' environment. Despite they are not influenced by acute stress, the antioxidant system is ready to extremely rapid respond to the sudden occurrence of acute stress. In this specific case, enhanced oxidative stress may lead to a greater biosynthesis of antistress proteins such as MTs, which are quickly traduced into protein and allowed to play their antioxidant role.

To conclude, it is necessary to underline the scientific relevance of this research study. The findings of the evaluation of several stress responses biomarkers indicate that organisms living in the natural environment may be affected by PFASs pollution. It is still unknown the real long-term effects that this class of compounds will have on the ecosystem, but it is possible to state that they tend to alter the internal physiological pathways of organisms. To fill up the knowledge gap about the long-term effects of PFASs, it turns out to be fundamental to deeply investigate their chronic exposure effects. With this aim, it will be useful to understand their effect as a function of time even in the short term and not only in the long one. It will lead to understanding which organism developmental stage is more in danger. For these purposes, it turns out to be essential to increase the biomarkers that should be investigated, such as the expression levels of several antioxidant enzymes such as SOD, CAT GPx. In addition, it should be investigated some physiological stage protein-specific such as vitellogenin and moulting hormones. Moreover, since the results of this study suggest an apoptotic pathway PFASs induced, it might be interesting to deeply studying cell apoptosis' involved protein and enzymes such as caspases enzymes, and BAX, BCL-2 and P53 proteins. On the other hand, it would be appropriate to investigate the mechanisms, currently discussed, by which these xenobiotic compounds manage to cross the cell membranes and consequently compromise the natural defence functions of cells.

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RINGRAZIAMENTI

Vorrei cogliere l'occasione per ringraziare innanzitutto il mio relatore, la Dott.ssa Paola Irato per avermi dato l'opportunita di poter esprimere la mia passione in quest'interessante progetto di tesi magistrale, per la fiducia che ha riposto in me e per la dedizione con cui mi ha supportato. Ringrazio vivamente il mio correlatore, il Dott. Gianfranco Santovito per avermi accolto nel suo gruppo di ricerca e per avermi trasmensso la passione per la ricerca sperimentale. Vorrei inoltre ringraziare le ricercatrici del CRIBI, ovvero la Dott.ssa Beniamina Pacchioni e la Dott.ssa Caterina Millino per la dedizione con cui mi hanno seguito e aiutato a raggiungere i miei obbiettivi. Ringrazio la Dott.ssa Giuseppina Covello e la Dott.ssa Marta Giacomello per le loro conoscenze e il supporto attraverso cui ho potuto ampliare il mio pensiero critico e di design sperimentale. Ringrazio i miei compagni di laboratorio per essermi stati accanto e per avermi supportato nei succesi e negli insuccessi della fase sperimentale di questo mio progetto. Un ringraziamento speciale va ai miei genitori, che hanno senpre creduto in me e mi hanno supportato in ogni fase della vita, a cui dedico quest'importante traguardo della mia carriera accademica. Un grande grazie e un abbraccio forte va a tutta la mia famigia per essere sempre stati al mio fianco e per avermi trasmesso i valori e i principi che mi hanno reso ciò che sono oggi. Infine volevo ringraziare i miei amici, con cui ho condiviso momenti e sentimenti durante questi anni, per il loro supporto e per avermi regalato tanti splendidi ricordi di quest'importante fase della mia vita.