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"Amedeo Avogadro"

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ECOTOXICOLOGICAL EFFECTS OF ATMOSPHERIC PARTICULATE ON AQUATIC AND EDAPHIC ORGANISMS

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Anna Volta

Supervised by: Prof. Marco Cucco and Prof. Aldo Viarengo

Ph.D. Program Co-Ordinator: Prof. Luigi Panza

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GENERAL INTRODUCTION

The atmospheric particulate

Environment, by almost universal definition, is the dynamic set of everything around us. It is an active complex of biotic and abiotic components moving in a common context in which they affect each other. Every natural phenomenon, as well as any human intervention on the environment has repercussions firstly on the quality of the atmosphere, the main reservoir of innumerable substances' mixtures.

The presence and continuous increase of anthropic activities, industrial and technologicalinnovations, have generated new sources of pollution, releasing chemicals and wastes in larger amounts. These substances are usually not present in the normal composition of the air, or there are at a lower concentration level (Raes, et al., 2000). The increase in specific air pollutants involves a serious risk factor influencing the ecosystems global balance.

It is therefore clear the urgency to keep up with the emergence of new environmental concerns that may have a multi-level impact.

Figure 1. Anthropogenic atmospheric pollution

Particulate Matter (PM), together with carbon monoxide (CO), sulphur dioxide (SO_2), nitrogen oxides (NO_x), and hydrocarbons, represent more than 95% of the total pollutant load emitted in the atmosphere (Yoo et al., 2015). An anthropized environment is an almost inexhaustible source of PM, defined by USEPA (2004) as "a complex mixture of chemically and physically different substances existing in the form of discrete particles (liquid or solid drops)" that travels into the atmosphere until it settles on the soil or in contact with water bodies.

Although natural phenomena contribute globally to more than 90% of air emissions (https://earthobservatory.nasa.gov/Features/Aerosols), the multiple anthropogenic sources have the salient characteristic of conveying large quantities of particles containing substances able to harm human and environmental health, with toxic effects on microorganisms, plant and animal species (Fuzzi, 2009) which vary according to the mixture.In particularly polluted areas, the frequency of formation of new atmospheric PM can reaches considerable percentages over a few years (Hamed et al. 2007).

Despite the growing awareness of institutions and public opinion on prevention and environmental protection and the remarkable advances of the scientific community

in the research field, one of the major obstacles to the development of attitudes decisively aimed at reducing the risk factors (where elimination is not possible) is the reasonable motivation that the main sources of emission are the product of technologies indispensable by now. The reduction of the atmospheric concentration of some pollutants ubiquitous even just thirty years ago (see the case of SO_2 as reported by Henschel et al., 2013), was only possible thanks to the spread of new, less impacting compounds that replaced themwhere possible, and/or the introduction of more stringent standards for the regulation of emissions. Following the adoption of a unitary regulatory framework (Directive 2008/50/ EC) which governs and manages the objectives to be pursued for the reduction of risk factors, the emissions of some priority pollutants (such as sulfur dioxide, benzene, oxide of carbon, lead, cadmium and nickel) have significantly decreasedto comply with regulatory standards for the protection of human health and environment. However, despite annual trends shows encouraging decreases the concentration of atmospheric PM is one of those forms of emission that it is hard to lower below regulatory standards (EEA, 2018).

The chemical composition of the atmospheric particulate matter is highly variable and linked to the characterization of the particles. Their size distribution varies from few nanometers (lower limit of instrumental detection) up to several tens of micrometers; after this threshold, the coarse particles cannot overcome the gravitational force and they rapidly deposit on the ground. It is assumed that only ideal particles have a perfectly spherical shape, in reality both their morphology and density are very variable so, to dimensionally describe them, we refer to an "equivalent" (or aerodynamic) diameter (Jennings and Parslow, 1988), directly proportional to the rate of sedimentation and therefore to that of particles persistence in the environmental compartments.

The PM concentration in the atmosphere follows a multimodal distribution (Ulrich et al., 2012) described for the first time by Whitby (1978), and identifies three large dimensional classes:

- **Ultrafine particles** (UFPs), with an aerodynamic diameter <0.1 µm and formed by nucleation from the gas phase to the solid one (Kreyling 2003);
- **Fine particles** (FP), which have an aerodynamic diameter of between 0.1 and 2.5 µm and growing by condensation and/or coagulation (Gelbard et al., 1980);
- **Coarse particles** (CP) that have a diameter >2.5 µm and formed mostly due to mechanical processes.

Instead, the classification adopted by the Environmental Regulation Agencies subdivides the particles by dimensional criteriacalled "cut points" based on their detectability from specific sampling systems, in a certain range of values that identify:

-**TSP** (total suspended particles) with aerodynamic diameter less than 100 µm;

-**PM_{10}** that identifies the particulate fraction in which the particles of aerodynamic diameter equal to 10 µm are sampled with an efficiency of 50%, according to the standard UNI EN123419/2014.

-**$PM_{2.5}$** that identifies the fraction of particulate matter (equal to about 60% of PM_{10}) in which the particles with an aerodynamic diameter equal to 2.5 µm are sampled with an efficiency of 50%, according to the standard UNI EN 123419/2014.

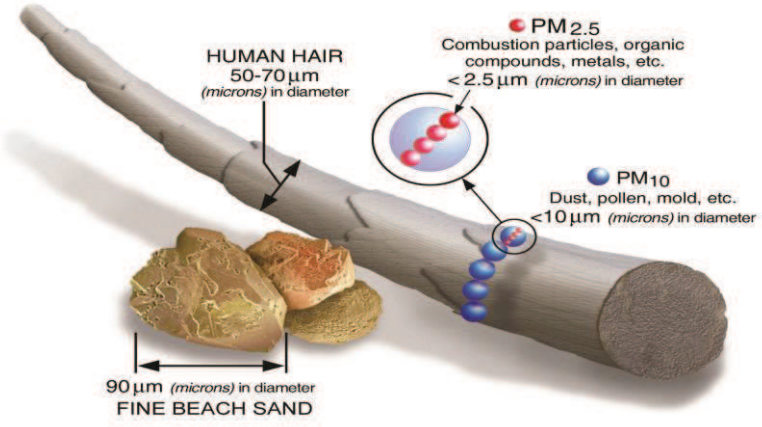

Figure 2. Size comparisons for PM particles (©epa.gov, 2016)

The directive 2008/50/CE of the European Parliament and the Council, implemented in Italy with the legislative decree n.155/2010, define the threshold values established for the concentration of PM_{10} and $PM_{2.5}$ (together with other atmospheric pollutants).

		Air Quality Directive		WHO guidelines	
Pollutant	**Averaging period**	**Objective**	**Comments**	**Objective**	**Comments**
$PM_{2.5}$	One day			25 µg/m³ (*)	99th percentile (3 days/year)
$PM_{2.5}$	Calendar year	Limit value, 25 µg/m³		10 µg/m³	
PM_{10}	One day	Limit value, 50 µg/m³	Not to be exceeded on more than 35 days per year.	50 µg/m³ (*)	99th percentile (3 days/year)
PM_{10}	Calendar year	Limit value, 40 µg/m³ (*)		20 µg/m³	
O_3	Maximum daily 8-hour mean	Target value, 120 µg/m³	Not to be exceeded on more than 25 days per year, averaged over three years	100 µg/m³	
NO_2	One hour	Limit value, 200 µg/m³ (*)	Not to be exceeded more than 18 times a calendar year	200 µg/m³ (*)	
NO_2	Calendar year	Limit value, 40 µg/m³		40 µg/m³	

Table1. Air quality standards under the Air Quality Directive, and WHO air quality guidelines for the most critical atmospheric contaminant (© European Environment Agency, 2017)

The study of the dimensional distribution and chemical composition of the air-dispersed particulate is of particular interest because it provides useful information on atmospherical residence times and the consequent environmental impact of the various fractions (iMonitraf, 2012).

It should be kept in mind that the concentrations of atmospheric PM vary, based on demographic concentration, from a few tens to hundreds of µg/m^3 in big cities (Marcazzan et al., 2001) and in industrial districts. In these areas, which are geographically very limited on a planetary scale, extremely high concentrations of polluting particles are often reached and due to atmospheric transport and deposition phenomena (Glišović et al., 2018) they can be carried over a great distance (from tens to hundreds of Km according to their size) in peri-urban or

extra-urban environments, often after having undergone physical-chemical transformations which increase their toxicity.

From a quali/quantitative point of view, the composition of atmospheric particulate responds to certain proportions among the soluble inorganic components, metals, carbonaceous fraction and organic compounds. Specifically, the most relevant components of the inorganic fraction are nitrates, sulfates, ammonium, sodium, calcium, magnesium and chlorides; their percentage availability varies seasonally within PM_{10} (Chen et al., 2013).

The atmospheric concentrations of airborne metals, their distribution and chemical composition depend on the type of emission sources (natural or anthropic) and on their balance at a local level.

Metals are naturally released into the atmosphere because of processes such as erosion, combustion or volcanic emissions, while the most common anthropic sources are mainly due to industrial activities and gas condensation at high temperatures (Fuzzi, 2009).

The carbonaceous fraction of PM is more difficult to define and characterize. It is possible to identify substances consisting of only elementary carbon, stable and mostly inert, in its various allotropic forms. Elemental carbon also exists as black carbon, a constitutively similar variant but with very different thermal and optical properties (Lepore et al., 2003); it is a primary pollutant emitted as it is in the atmosphere following incomplete combustion events, in the form of black powder (soot). This is able to absorb other toxic combustion products, such as volatile organic compounds (VOCs) and dioxins, increasing the harmful health potential.

One of the most representative class of molecular only carbon-based compounds present in the atmosphere as components of soot is certainly that of Fullerenes, emerging pollutants whose environmental increase is a consequence of the rapid expansion of nanotechnology. Fullerenes are spheroidal cages with unique characteristics in terms of physical/chemical properties and practically ubiquitous in the environment due to the innumerable sources of release. However, they are

difficult to analyze especially in a complex matrix such as the atmosphere, in which the carbonaceous particles bind and transport various chemical species.

Among these, metals and organic compounds with which carbon atoms bind together, with hydrogen and various other elements (including nitrogen, sulfur, phosphorus and chlorine), have great relevance, giving rise to tens of thousands of species (Goldstein and Galbally, 2007) often of great impact for human and environmental health, as in the case of polycyclic aromatic hydrocarbons (PAHs).

PAHs are emitted as sub-micron primary pollutants following incomplete combustion processes, both adhering to particulate matter and as constituents in the gaseous phase (Gregoris et al., 2014). Altough their presence represents a negligible fraction of the total mass of atmospheric PM (<0.1%) (Di Monte, 2009) they has an important (eco)toxicological interestas one of the main causes of particle hazardness. Their strong photochemical reactivity leads to the formation of secondary pollutants following seasonal trends (Zhang et al., 2016).

It is understandable how air pollution is an extremely complex phenomenon, made even more unpredictable by meteorological and climatic factors intervening in the dynamics of transformation and distribution of pollutants in the various environmental compartments.

Beyond industrial activities, one of the main anthropic contributions to the emission of PM and its associated pollutants (like metals and benzo[a]pyrene) is the road traffic (Stroe et al., 2014).

Vehicles can originate particulate material mainly through:

- Emission of exhaust gas;
- Tyre abrasion;
- Brake wear.

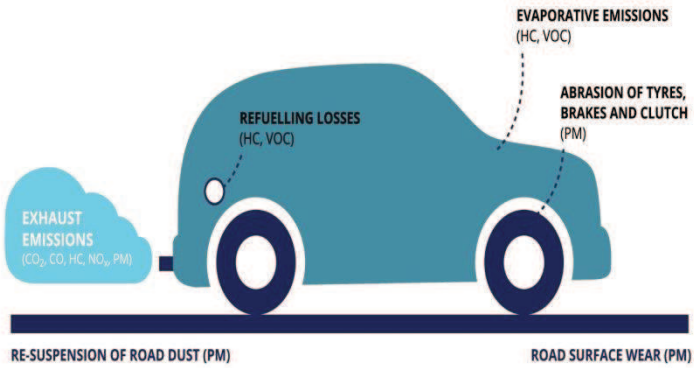

Figure 3. Different types of emissions from vehicles (©eea.europa.eu, 2016)

National laws and regulations (Italian Legislative Decree 155/2010) have been particularly targeted in reducing exhaust emissions of road vehicles, traditionally considered to be the main causes of air pollution in urban environments (Ionescu et al., 2013).

Conversely, atmospheric levels are still above the regulatory targets for particulate matter emitted from non-exhaust sources (particles with an aerodynamic diameter of 2.5-10 μm), and attention is always paid to the benzo[a]pyrene emission trends, due to its ascertained carcinogenicity (IARC, 2005), the innumerable sources of emission (Fuller et al., 2014) and the impossibility of defining minimum risk-free thresholds.

Several years ago, the data collected by Air Quality in Europe (EEA, 2013) had pointed out the wear of brakes and tyres as an important source of trace metals in the urban environment. Because of their movement and the action of the wind, all the vehicles contribute to wear down the surface and resuspend the heterogeneous road dust, with high levels of penetration indoor, as evidenced by the consistent presence of particulate deriving from traffic found in domestic environments (Urso et al., 2015; ZauliSajani et al., 2016).

Air pollution also leads to several important environmental impacts, which affect vegetation and fauna directly, as well as the quality of water and soil, and the ecosystem services they support (EEA, 2018).
Despite the innumerable researches, it remains a great obstacle: the difficulty of understanding the mechanisms of interaction of the atmospheric particulate within the biotic and abiotic compartments.

Environmental impact of the atmospheric particulate and ecotoxicological approach

Atmospheric transport causes the particulate matter, in the form of dry or wet deposition (i.e. mediated by aqueous condensations), to come into contact with aquatic and terrestrial ecosystems, damaging their biodiversity (EEA, 2013). There is the problem of the persistence of such contaminants, difficult to remove when introduced in the environment and which, when accumulating, increase the toxic load at the expense of the organisms.
A single contaminant can exert its toxicity in multiple manifestations of damage but, since in the natural environment organisms are exposed mostly to mixtures of contaminants, they can act in many ways (synergistically, antagonistically or in additive form). A whole series of transformations can therefore occur resulting in increase or decrease in the toxic potential of the starting substance, or its activation/deactivation (Newman and Unger, 2003). This means that investigations into the toxic effects of heterogeneous mixtures such as atmospheric particulate matter are much more demanding than the analysis of the single compounds (Benedetti et al., 2007).
The coexistence of the different grain-size fractions within the PM makes them interact at different levels of biological complexity, based on their aerodynamic

dimensions. The coarse PM can act like adsorbent exerting toxic effects both as such, and as a vector for the finer fractions which can interact at the sub-cellular level as genotoxicants, citotoxicants or immunotoxicants (Jha, 2008).

Therefore, it is difficult to choose the biological parameters to analyze in order to embrace the entire interval between the first symptoms that appear in a healthy system until the emerging of irreversible damage (Köhler et al., 2002).

For this reason ecotoxicology (Butler, 1978) stands as a field of wide-ranging investigation, studying the toxic effects of environmental potentially dangerous substances that come into contact with living organisms, starting from the single individuals up to define the dynamics of populations and communities within their proper biotope.

The study of the spread modalities of these agents and their interactions is based on considerations regarding their bioavailability and possible accumulation in biological systems (i.e. bioconcentration, biomagnification). For example, the main form of storage of heavy metals in nature is that of oxides or sulphides with solubility coefficients so low as to render them almost inert; one of the most negative environmental impacts because of the increase in human activities is precisely the release of ionic metals, whose solubility makes them highly interactive with living systems. Instead, the organic substances show affinity for the apolar components within the environmental matrices (mostly they are adsorbed by the particulate fractions and/or by the sediments) and of the organisms (strongly welded to the lipid fractions, they are biomagnified along the trophic network). Therefore, knowledge of the specific characteristics and dynamics of the environmental compartment of release is essential to predict the contaminant behavior and fate.

In many cases, the destination of substances emitted into the atmosphere is the aquatic (marine or freshwater) environment (Hering et al., 2015).

The aquatic ecosystem is a very complex dynamic system. Overlooking the natural contaminations, water pollution can be defined as any alteration of the chemical,

physical, biological or microbiological quality of the water that causes an unacceptable depreciation for the purposes of normal use or conservation of the environment (Amendola and Cerioli, 2006).

Pollution is generated by substances released into the water, which can fulfill their action directly (e.g. oxygen depletion due to an excess of reducing substances) or through biological processes (e.g. bioactivation with formation of derivatives or toxic metabolic intermediates).

The particulate suspended along the water column, on the other hand, can hinder the absorption of light by inhibiting photosynthesis, and interfere with the life cicle of aquatic organisms, without taking into account the accumulation into the sediments of potentially dangerous residues that can be reintroduced within the water column following seabed movimentation.

Historically, the aquatic ecosystem has always been at the center of the scientific community's interest (Penny and Adams, 1863; Weigelt et al., 1885; Carpenter, 1924) and most of the ecotoxicological tests have been developed primarily for waters and aquatic organisms.

The development of techniques useful for understanding the mechanisms affecting or disturbing the edaphic compartment, especially with reference to biological tests, is a relatively more recent research activity. The current anthropization rhythms substantially and progressively affect thecondition of the soil, contaminated mainly by the use of fertilizers and/or pesticides indispensable for agriculture, industrial emissions and urban vehicular traffic without counting the improper use of soils as storage depots for waste materials or toxics. The ascertained relevance of the studies concerning the soil system ecology has given a strong propulsive boost to the characterization of biodiversity through bioassays on autochthonous organisms and eco-toxicological investigations.

As already mentioned, determining the actual extent of environmental pollution phenomena is really complicated and the challenge that scientific community and institutions have prefixed over time is to be able to identify effective remediation

and restoration interventions. This cannot be feasible without an early assessment of alterations at the cellular, physiological, morphological and behavioral level on target species (or bioindicators) that through easily verifiable variations of their status (biomarkers) provide information on the quality of an environment based on the answers to stressful situations. In fact, every living species is linked to a particular set of conditions in the environment in which it lives, therefore reflecting its characteristics.

A good bioindicator must react in an observable way, be sensitive to the environmental disturbance factor of interest, it must preferably be native and available in the reference biotope, with a life cycle that makes it possible to integrate the short-term environmental variability (Desrosiers et al., 2013) and a significant ecological role. If the biological indicator stores pollutants in concentrations higher than those of the original environmental compartment it also acts as a "bioaccumulator"; in this case the organism must have a high resistance to contaminants to guarantee a quantitative analysis that can be an indicative integrator in space-time terms of the toxicological input of a specific area.

The effects of contaminants can be progressively identified at various levels of complexity within a system, as a result of a concatenation of events usually induced by subcellular biomolecular dysfunctions (Sforzini et al., 2018). In this context, the use of multivariate analyzes in identifying biomarkers that provide better risk assessment, especially in the early stages, is increasingly relevant (Moore et al., 2006; Jenkins et al., 2011; Ortiz et al., 2011).

In environmental monitoring, since 1970s (Bayne et al., 1976, Payne, 1977) biomarkers represent the main system in evaluate the biological impact of pollutants, quickly detecting the effects of single compounds or complex mixtures able to arouse stressful events, identified at early stages before the damage becomes detectable at higher ecological levels (Banni et al., 2005; Viarengo et al., 2007; Moore et al., 2012).

Biomarkers find application in many branches of ecology providing information and provisions on a population health and its change in wellness (McCarthy and Shugart, 1990; Depledge and Fossi, 1994; Au, 2004; Fränzle, 2006). When a biomarker is properly applied, it is possible to identify (and often quantify) the exposure to a contaminant by giving information on spatial-temporal concentration changes, in order to predict the occurrence and magnitude of adverse effects (Au, 2004).

The use of predictive biomarkers of stressful events at an early stage presupposes a scientific approach that is often not possible to implement directly in the field experiments. This gap is overcome with the optimized use of experimental biological assays in the laboratory, with a multi-species (on microbial, plant and animal species) and a multi-matrices (effluents, soils, sediments andwaste) approach, representing natural conditions as faithfully as possible.

If it is true that ecotoxicological studies in the field allow assessments of environmental health based on surveys at populations or communities level through direct quali-quantitative analyses, they have the limitation of not being able to provide information on the relationship between concentration of contaminant and related effects, nor indications on the bioavailability of the substances of interest.

Toxicity laboratory-tests are performed by selecting model organisms belonging to different taxonomic classes, and exposing them to individual substances, mixtures or environmental samples under standardized conditions. Different types of end-points are considered: lethal (mortality) or sublethal (growth, reproduction, physiological or metabolic anomalies), not only to test the environmental quality in a specific time range but to predict the course and evolution of the processes tested, projecting them in a natural context. The results are strictly defined by cause-effect relationships valid only in each narrow window of experimental evaluation; this means that it is not possible to extend the predictions based on the results obtained on a single species to other elements of the biome, due to the complex interactions and dynamic equilibrium within each ecosystem. For this reason it is important to

deepen and diversify each ecotoxicological approach, by including widened batteries of model organisms, able to broaden the spectrum of investigation on the possible environmental repercussions of each contaminant under examination, to obtain the most complete and realistic result as possible. An accurate assessment of the toxic effects and their interconnections is at the base of an aware prediction of the risks, in a contest in which humans are both cause and potential victims of the homeostasis disturbance into natural biota.

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AIMS OF THE THESIS

Air pollution is well known to be harmful to human and environmental health. Its characteristics are constantly evolving and the same should be for the horizons of the scientific community's research. Atmospheric events ensure that the contaminants in the air, mostly concentrated in urban areas, are dispersed and distributed in the various environmental compartments, in areas that are also very far from those of emission.

There is a vast literature regarding the effects of air pollution on aquatic and edaphic ecosystems, but the focus is increasingly on identifying biological effects by developing biomarkers at different levels of functional complexity, suitable in monitoring the stress syndrome evolution from the early alarm signals at the subcellular level up to the manifestation of dysfunctions detectable at the organism/population level.

For this reason, the scientific investigation during the PhD followed two different lines of research:

A first approach was useful to detect the mode of action atsubcellular level of specific contaminants whose presence in the atmospheric matrix is ascertained. Subsequent investigations of a more holistic nature were carried out considering a source of increasingly widespread air pollution and of general interest that is the one related to PM produced by road traffic (specifically the wear of the brake systems), which possibly cause a detectable impact on biodiversity. In this way, the present study can provide diversified information, embracing a wide spectrum of effects related to exposure to atmospheric contaminants starting from the sub-cellular level up to detecting the possible impact on the health of the population/communities in an ecosystem.

The initial step of the PhD project was therefore to identify the cytotoxic effects of the well-known organic xenobiotic benzo[a]pyrene (b[a]p), examining a methodological approach based on the responses of a battery of stress biomarkers with particular attention to the the study of cytochemical mechanisms related to the induction of autophagic pathways. The lysosomal membrane stability (LMS) and the increase in the lysosome/cytoplasm ratio, as well as the production of lipofuscins (oxidative stress index) and neutral lipids (which indicate metabolic alterations) have been verified on the widespread used aquatic model *Mytilus galloprovincialis*.

Experiments were performed on samples of *M. galloprovincialis* digestive gland for the evaluation of toxic effects and metabolic disorders caused by the b[a]p. The results obtained were used as a substrate for immunohistochemical investigations, aimed at detecting changes or alterations induced by b[a]p in the mTOR kinase complex pathway, whose inactivation is directly related to the onset of autophagy events.

The study of alterations in DNA and lysosomal vacuolar system will have the task of clarifying the meaning of changes affecting the cell from early stress signals to loss of physiological functions that prevents a proper "scope for growth".

It was also verified the possibility of the occurrence of a "Trojan Horse effect" in co-exposure with nanoparticles of fullereneC60, progenitor of the extended family of carbon nanostructures easily available in atmosferical emissions from both natural and anthropogenic sources.

The main purpose of the subsequent research phases was to identify the possible toxic effects of fullereneC60 also in order to verify the hypothesis of its interaction with b[a]p that could cause changes in the properties of the single compounds and their bioavailability.

Regarding the role of mTOR, although many studies on mammals have largely demonstrated its involvement in the regulatory mechanisms of various pathologies, variations in its functions and the resulting implications on cellular signalling in

aquatic and terrestrial invertebrates exposed to contaminants are a significant frontier of environmental research. The study was part of a larger project funded by the Natural Environment Research Council (NERC), "Trojan Horses-Elucidating the Potential Interaction of Nanoparticles Produced with Polycyclic Aromatic Hydrocarbons: an Integrated Toxicogenomic Approach", conducted at the University of Plymouth in collaboration with King's College London, the University of Nottingham and the University of Piemonte Orientale, Italy.

The research line was subsequently focused on investigating a specific case that included data obtained within a more detailed survey, aimed at researching new technologies to improve the quality of atmospheric emissions, vector of toxic substances such as aggregates of metallic and carbon-based nanocompound and ubiquitous organic contaminants. The collaboration with the Mario Negri Institute of Pharmacological Research in Milan, has offered the possibility to be part of an investigation that combines pure scientific research with applications in external partnerships of industrial research and risk assessment. Therefore, after opening a window on the cytological and immunohistochemical implications of the induced contact of specific contaminants with biological systems, the attention has been paidtothe ecotoxicological evaluation of matrices in which the same contaminants are certainly found, with a view to developing technologies aimed at their progressive abatement.

In particular, the Mario Negri Institute contribution to the LOWBRASYS Horizon2020 project, offered the opportunity to study the environmental impact produced by the nano-micro particulate, generated as a result of the abrasion/wear of vehicle brake devices. Once released into the atmosphere it can directly affect the quality of the air but, following transport and deposition phenomena, it can come into contact with the edaphic and aquatic ecosystems, determining risks for the living organisms.

In this context, the industrial development of new solutions to obtain more eco-friendly braking systems with better emission performance in terms of mass,

composition and particle size, can draw attention to a problem which until recently was decidedly neglected, making a significant contribution to improving air quality, and consequently to human and environmental health.

To assess whether and to what extent the experimental technology under examination can improve the quali/quantitative emissions a battery of ecotoxicological assays has been prepared with model organisms of terrestrial and aquatic ecosystems at different trophic levels, exposed directly to the particles or their soluble fraction.

CHAPTER 1. A contribution from atmospherical contamination: Biological investigation on the cytological impact of benzo[a]pyrene and fullereneC60 using an aquatic model organism (*Mytilus galloprovincialis*)

1.1 Benzo[a]pyrene: The PAHs landmark

Among the many substances that are continuously introduced into the environment, polycyclic aromatic hydrocarbons (PAHs) are a family of organic micro-pollutants now ubiquitous mainly because of anthropic activity. They form during incomplete combustion processes especially in oxygen depletion (pyrolysis) and at very high temperatures (Haritash and Kaushik, 2009). A common feature is the poor solubility expressed in a high lipophilicity (U.S. EPA, 2008). In indoor environments, the emissions are mainly represented rom cigarette smoke, flames-cooked or smoked foods and from all the household tools or fuel-burning appliances, including increasingly popular wood-burning fireplaces. Outdoor, they naturally originate by forest and rangeland fires, oil spills and seeps, volcanic eruptions and trees exudates, whereas among the anthropogenic sources of release are the burning of fossil fuels, industrial processes, waste incineration and, not least, vehicular traffic (Kaushik and Haritash, 2006; Chłopek et al., 2016). It seems that with the increase of the vehicle wear, total aromatic content and paraffinic components in the fuel even the emission factors of PAHs increase (Tricarico, 2005).

The predominant presence of PAHs released during fuel combustion, in vehicles' exhausted discharges has been widely documented for a long time (Marr et al.,

1999; Nelson et al., 2008; Kam et al, 2012; Simpson, 2018 among many others). Only more recently the attention has focused on the organic components associated with the wear particulate of the vehicles' mechanical components (Plachà et al., 2016) and their environmental repercussions. Although PAHs originate from many different sources related to road traffic, and it is very difficult discriminating the exact participation of each ones, it has been provided evidence that the processes related to the wear of the tribological elements in the braking systems represent a substantial contribution (Chłopek et al., 2016).

The interest from both analytical and environmental point of view derives from the fact that, although most of them have no practical use and any commercial importance, they are widely spread in the environment.

The main chemical feature lies in the presence of two and up to seven fused benzene rings. In the air, the compounds with lower molecular weight generally remain in gaseous form and after a brief stay in the atmosphere are rapidly degraded (especially in the presence of light and oxidizing chemical species such as ozone) through a sequence of cascade reactions, beginning with the addition of an OH radical to a double bond. This process is highly dependend from seasonality and temperature (OMS, 2000). The strong photochemical reactivity of these molecules leads to the formation of secondary pollutants spread in the aerosol, increasing its toxicity level. These organic byproducts are mostly made up of species such as nitro-PAHs, a class of highly mutagenic compounds (Chuesaard et al., 2014), generated by the photo-catalyzed reactions with nitric acid (ISPRA, 2010) and oxi-PAHs deriving from the reaction with ozone (Perrone, 2010).

PAHs characterized by five or more rings (the species with the highest toxicological impact) tend to exist mostly in solid phase, adsorbed to fine particulate (Yamasaki et al., 1982). Many of them are particularly persistent in the environment, inside natural waters, soil, sediments and even in foods (Lee et al., 1981; Bamforth and Singleton, 2005). This widespread diffusion determines that

every individuals are exposed to the certainly unhealthy action of this class of substances.

The International Agency for Research on Cancer (IARC) has associated the presence of PAHs to an increase of cancer cases, both in human and experimental animals (Dipple et al., 1984; Harvey, 1991; Warshawsky, 1992). The danger arising from exposure is justified by the fact that some of these compoundsexert their harmful effects at very low concentrations.

Sixteen hydrocarbons have been identified as “priority pollutants” by the United States Environment Protection Agency (USEPA) starting from the smallest naphthalene, and some of them belongs to different classes of possible or probable human carcinogenicity, as defined by the IARC; one of the most damaging is benzo[a]pyrene (b[a]p) (Mastrangelo et al., 1996).

The estimate of the b[a]p atmospheric concentration is based on the annual exceedances number of the limit value of 1 ng/m^3, based on analytical determinations on PM_{10} carried out at the air quality monitoring stations.

Nowadays these are located not only near the road and industrial sources, but also in urban, sub-urban and extra-urban background areas, allowing a more complete assessment of the quality of the air throughout the territory as planned, in Italy, by Legislative Decree 155/2010.

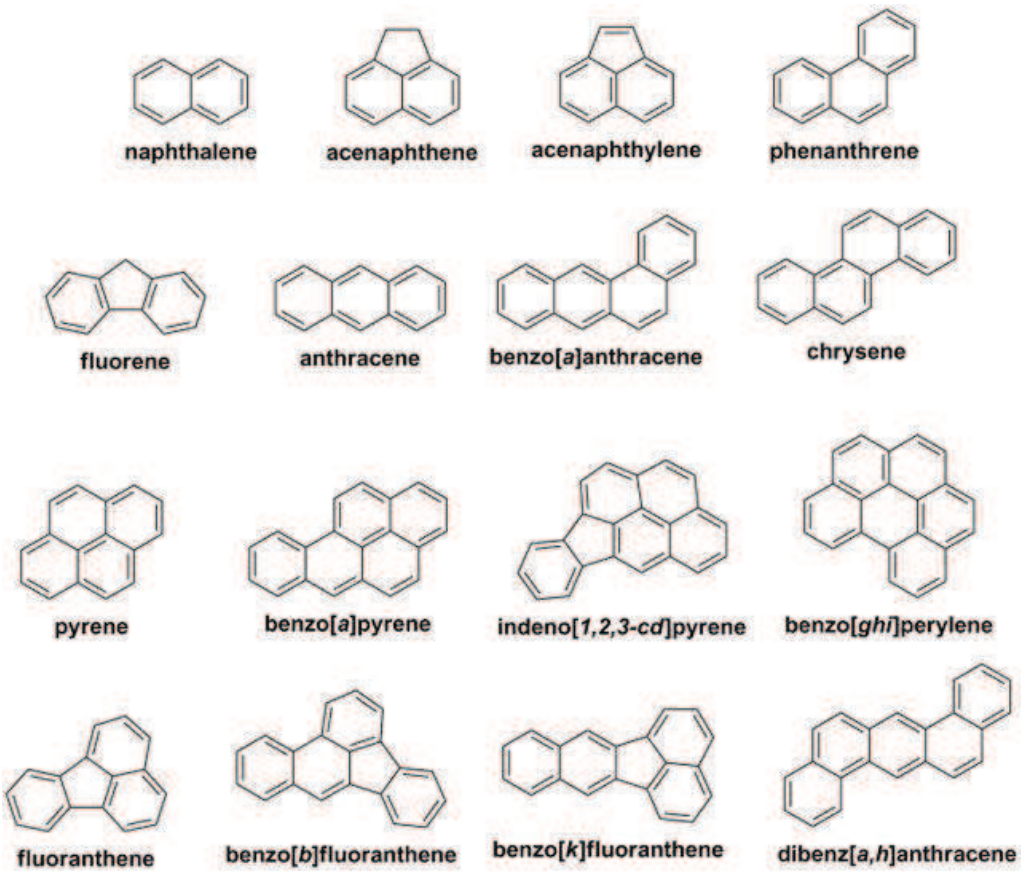

Figure 1.1 Molecular structure of the 16 PAHs considered priority pollutants by the US EPA (© Shukla et al., 2014).

Precisely because of its environmental ubiquity, that unfortunately accompanies a well known carcinogenicity (indeed, the first to be ascertained), it has been proposed as a unitary reference for the toxicity of the entire class of PAHs (Nisbet et al. 1992), recognized by identifying the relative carcinogenic action of individual hydrocarbons expressed as a toxic equivalence factor (TEF) compared to b[a]p.

The story of its discovery sees its roots at the dawn of the Industrial Revolution. Although it was not yet possible to understand the real cause, the scientists of that time began to correlate the increase in mortality between the ranks of chimney sweeps and, later, of workers assigned to the preparation of carbon-coke with their

greater exposure to coal tar (Nebbia, 2011). It will be necessary to wait until the 1930s to obtain appreciable results. Finally, after years of hard work, in 1933, from two tons of tar, it was possible to isolate seven grams of a crystalline substance which, later purified, could be synthesized (Phillips, 1983). From that moment on it was possible to investigate its entire structural and chemical characteristic.

The b[a]p is five-ring molecule with raw formula $C_{20}H_{12}$, formed by a benzene ring fused to a pyrene structure. It is surely the most studied and tested member of this class, particularly because of its cytotoxic, mutagenic, and carcinogenic properties for both terrestrial and aquatic organisms (Juhasz and Naidu, 2000; Binelli et al., 2008; Tung et al., 2014; Williams and Hubberstey, 2014). Different species have different responses to b[a]p contamination because of the variations in exposure pathways or in the rate and route of its biotransformation (Duan et al., 2015). The distribution of b[a]p (as well as that of most PAHs interacting with biological systems) is related to the route of intake and the tissues'lipidic content. Lipophilic compounds are rapidly absorbed due to their capability of easily crossing biological membranes through simple diffusion or receptor-mediated endocytosis in association with low-density lipoprotein (Moore et al., 2012), without the need for active transport; then they can be subjected to bioaccumulation processes that is much higher when lipid content increases.

After the b[a]p has been distributed inside the tissues, various enzymes trigger metabolic reactions. Metabolic enzymes are almost ubiquitous, but the highest activities are generally found in tissues involved in food processing (Livingstone, 1991). B[a]pis a chemically inert molecule and the mutagenic action starts right from its metabolism: generally the first step in this processing, to increase its hydrophilicity and facilitate the excretion, is the oxidation of a double bond catalysed by cytochrome P450 enzymes (CYPs) to obtain an unstable arene oxides. Then, the hydrolysis of this reaction intermediate by microsomal epoxide hydrolase to trans dihydro-diols occurs and finally, a second CYP catalyses the oxidation of

the double bond adjacent to the diol function to generate the benzo[*a*]pyren-7,8-dihydrodiol-9,10-epoxide(Poirier and Beland, 1992).

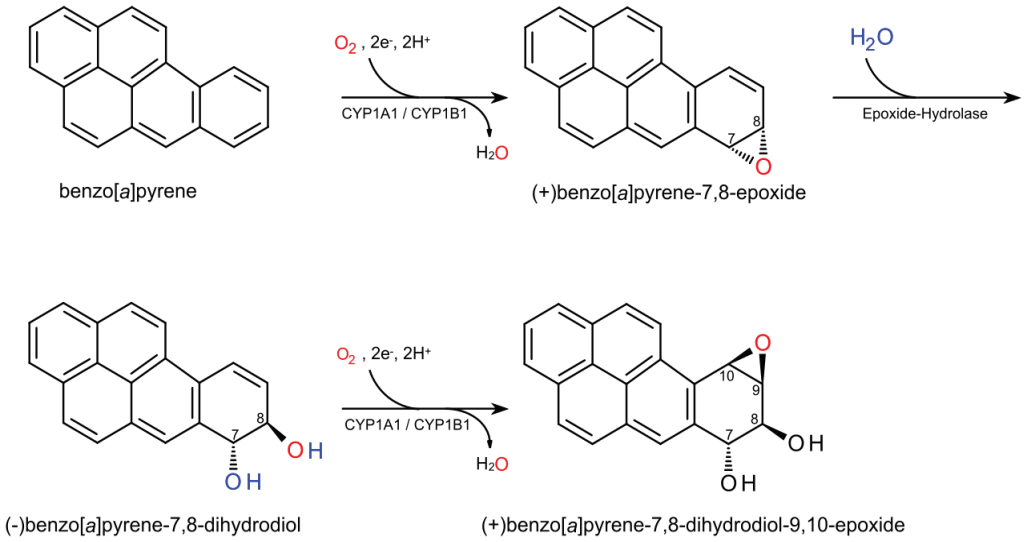

Figure 1.2 Metabolism of benzo[a]pyrene yielding cancerogenic benzo[a]pyrene-7,8-diol-9,10-epoxide.

Therefore, although the benzo[a]pyrene primary toxicity is actually very low (it is a pre-cancerogenic agent actually, which requires bioactivation), during the biotransformation reactions that should eliminate it, can be formed electrophilic intermediates, able to interact with various biological macromolecules, including DNA with multi-organ carcinogenic and genotoxic effects. This is through the formation of bulky adducts that binds with guanine nucleotide, that are considered exposure biomarkers. Some adducts can be removed by endogenous repair systems others not, causing strand breaks, mutation (Kucab et al., 2015; Long et al., 2016), changes in gene expression (Banni et al., 2014), chromosomal aberrations and many other cellular disfunctions with mechanisms that may lead to neoplastic formations (Denissenko et al., 1996).

The search for effective solutions to reduce emissions of impacting pollutants such as b[a]p must be considered among the priority actions to be undertaken considering also the possible interaction with nanoparticles and other emerging pollutants that would alter the properties and bioavailability of each ones, differentially modifying their potential toxicity (Maria et al., 2103; Farkas et al., 2015; Di et al., 2016; Barranger et al., 2019).

1.2 FullereneC60: The forerunner of carbon nanostructures

Increasing knowledge on the consequences of exposure to atmospheric PM has confirmed the need to monitor the aerosol components made up of particles of the smallest aerodynamic dimensions: PM_1, $PM_{0.1}$ up to nanometric size, according to the accredited thesis that the possible toxic interactions of the particles with living organisms are greater the smaller they are (Chow, 1996).

Nanomaterials (NMs) can be summarily classified as a peculiar subgroup of chemicals, substantially defined by their size. Although the European Commission (EC, 2011) has proposed a recommendation for a regulatory specification of the term, a universally accepted definition has not yet been actually agreed; then, the only common feature among all nanomaterials is considered the presence of at least one external dimension that falls into the nanoscale, *id est* less of 100 nm (Lövestam et al., 2010).

Concerning their physicochemical properties, the increasing of the specific surface area to volume ratio can make nanocompounds chemically more reactive in respect with the same macroscale material and with unique catalytic properties, since a different surface structure can lead to changes in the surface reaction kinetics (Fadeel et al., 2017).

Material can be nanoscale in each of the three known dimensions respectively as thin platelets, nanowires/nanotubes and nanoparticles (NPs).

NPs fall into three major groups: natural, incidental, and engineered. Naturally-occurring nanomaterials such as volcanic ash, ocean spray, mineral composites or even viral particles and protein molecules (Oberdörster, 2004) are already present on earth for millions of years. Engineered nanoparticles (ENPs) are the most discussed and controversial component in nanomaterials because of their great heterogeneity in shapes, sizes, surface coating and dispersion properties that can determine structure and function (Fiorito et al., 2006). Incidental or, better-defined, waste nanoparticles are instead produced as undesirable result of industrial processes and urban traffic. From the environmental point of view, it is necessary to consider the contribution due to the release, intentionally and not, of nanomaterials in the atmosphere.

Among the wide range of nano-sized compounds, carbon-based particles have a peculiar attraction for researchers, because of their almost ubiquitous presence, in different forms, in practically all environmental and biological compartments. Nanoparticles produced in large amounts include carbon black, fullerenes and a range of carbon nanotubes.

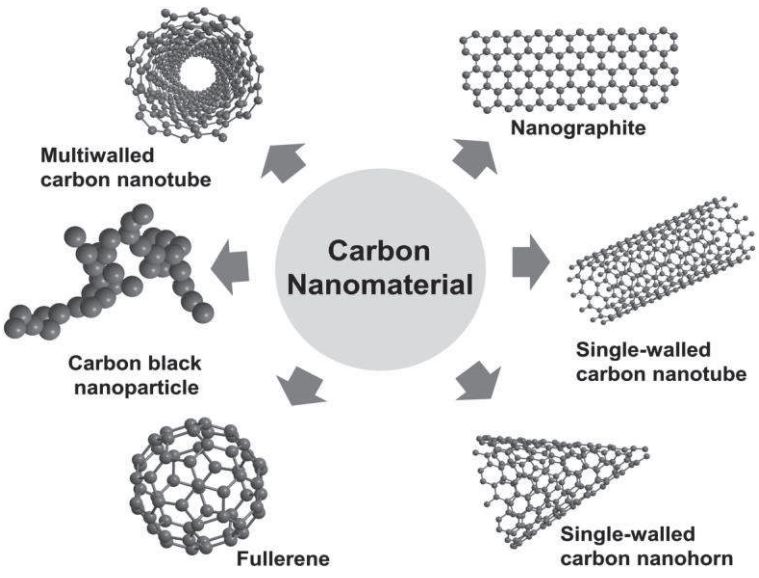

Figure 1.3 Various carbon-based nanomaterials (©Yuan et al., 2019)

Even if carbon nanoparticles (CNPs) exert toxicity at the cellular level through several mechanisms, determining this toxicity turns out to be rather complicated.

The difficulty arises *in primis* from the variability generated by the superficial coating of particles that, although not engineered, may exhibit changes at the external surface level by interacting with the environment or biological systems (Canesi and Corsi, 2016). By changing the surface coatings the nanomaterial toxicity can almost be completely altered, for example by transforming a hydrophobic particle, as usually the carbon nanomaterials are known to be (Riding et al., 2012), into a hydrophilic one. By the other hand, even the possible presence of metal catalysts, exposure to UV radiation and the tendency to deposit as aggregates due to high Van der Waals forces, are other factors that can greatly condition the behaviour of these compounds, often increasing their toxicity (Fiorito et al., 2006).

Over a third of the atoms in a nanoparticle are located at the surface, and this makes them very reactive systems that, in some cases, can promote the formation of radical species (Yang et al., 2009). There are numerous cytotoxicity studies, both *in vitro* and *in vivo*, that prove direct oxidation of cells and the occurrence of inflammation conditions, cytokine production, cytoskeletal changes, altered vesicular trafficking, as well as changes in gene expression and cell signalling in response to different types of NPs (Ding et al., 2005; Hussain et al., 2005; Ju-Nam and Lead, 2008; Magrez et al., 2006; Owen and Depledge, 2005; Xia et al., 2006).

All these effects are likely to occur through the generation of reactive oxygen species (ROS) which induce damage of membrane lipids and DNA (Clutton, 1997) with possible implications for cell viability and metabolic function.

ROS production is regarded as one of the primary mechanisms of nanotoxicity (Wani et al., 2011), which damages cells through oxidising the double bonds on fatty acids of phospholipids within the cell membrane (Kang et al., 2007; Klaine et al., 2008). This can lead to oxidative stress occurring in living cells if the quantity of ROS exceeds the ability of cellular protective mechanisms to defend against

such damage (Vileno et al., 2010). In addition, the peroxidized fatty acids themselves can generate further free radicals, subsequently oxidising subcellular components that can result in cell necrosis or apoptosis (Klaine et al., 2008; Vileno et al., 2010).

Among all the carbon nanoparticles, fullereneC60 (named also Buckminsterfullerene) is probably the best known because of the history of its discovery and the characteristics that make it a compound on the threshold between natural and engineered world.

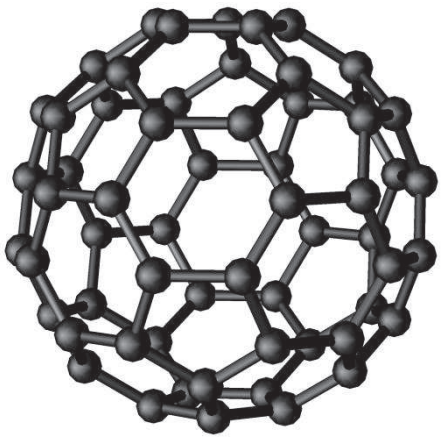

Figure 1.4 Molecular structure offullerene C60

The experiments that led to the finding of C60 were aimed at simulating in the laboratory the rich carbon atmosphere of a giant red star. So in 1985 Sir Harold Kroto, Richard Smalley and Robert Curl, firing a high frequency laser pulse on a graphite disc, came casually across in discovering a molecule that have literally opened the doors of nanotechnology. Before then, the only two known allotropic carbon forms were the diamond (in tetrahedric conformation) and the graphite (in planar disposition).

Carbon fullerenes represent the third carbon allotrope and in general terms are approximately spherical cages (Yang et al., 2010), derived from a bending process of graphite sheets, formed from a different number of hexagonally and pentagonally bonded carbon atoms. Those containing fewer hexagons possess more reactive surface sites (Mauter and Elimelech, 2008).

FullereneC60 represents the first, smallest, most commonly utilized and studied fullerene (Bagrii and Karaulova, 2001; Aw et al., 2006), with a diameter of approximately one nm and the shape of an elegant truncated icosahedron (Johnston et al., 1990). Due to its electron affinity and polyalkene-like nature, C60 is a worldwide produced nanoparticle (Ferreira et al., 2014) and a vast number of functional, fullerene-based materials have been synthetized starting from its structure.

There are several sources of release, and it is understandable for a molecule that have natural emission pathways in addition to those resulting by anthropogenic intervention (Franco et al., 2007; Handy et al. 2008; Palmbery et al. 2009). With increasing production and usage, accidental or uncontrollable release of these physically and chemically unique macromolecule becomes an ever-greater inevitability (Ferguson et al., 2008).

Incidental formation of C60 from combustion sources has recently been confirmed in the exhaust of common fuels (Sanchis et al., 2018). More, as engineered nanomaterial, C60 are expected to be released into aquatic environment through industrial and urban wastewaters. The behaviour of fullerenes in aqueous solutions have been thoroughly described in past (Fortner et al., 2005; Lee et al., 2009); at present is known that C60 can form water-stable nano-suspensions negatively charged with diameters from 5 to 500 nm (Andrievsky et al., 2002; Sayes et al., 2004;Brant et al., 2005; Khokhryakov et al., 2006) despite being overly hydrophobic and virtually non-wettable (Ruoff et al., 1993; Heymann, 1996). It has been reported (Colvin, 2003; Sayes et al., 2004) that stabilized C60-aggregates in the aqueous phase exhibited higher cytotoxicity in respect of other water-soluble

fullerenes. The mechanism through which this toxicity is explicit lies in chemical reactivity toward various radicals if in the presence of UV irradiation (Krusic et al., 1991; Arbogast et al., 1991). Under such conditions, C60 facilitates transfer of absorbed energy to oxygen leading to the scission of the molecule, and interferes with the common electronic transfer (Yamakoshi et al., 2003). In fact, at the mitochondrial level, carbon NPs can greatly stimulate ROS production by compromising the electronic transport chain, causing structural damage, activation of NADPH-like enzyme system, and depolarization of the mitochondrial membrane (Li et al., 2003; Xia et al., 2006). However, it is inaccurate to assume that ROS generation is a prerequisite to NP-induced toxicity since fullerene toxicity can be elicited by ROS-dependent mechanism only when photo-excited (Trpkovic et al., 2012) and precedent studies have attested the direct toxicity of NPs even without causing ROS (Wang et al., 2010).

Fullerenes toxicity is a controversial issue, actually (Ferreira et al., 2014). Although relatively little is known regarding quantification of C60 in environmental matrices, bioaccumulation factors and biochemical responses to living organisms, concerns have been raised about its potential impacts (Handy et al. 2008; Petersen et al. 2011). When fullerenes cross the external phospholipidic bilayer, once inside the cell compartments it preferentially locate to the mitochondria, cytoplasm, lysosome and cell nuclei (Porter et al., 2007) potentially affecting the functioning of enzymes and proteins. Biological responses of several organisms to C60 exposure include induced oxidative stress and lipid peroxidation (Oberdorster et al., 2006; Zhu et al., 2008) resulting in activation of signalling pathways, decreasing of protein synthesis and cytokine cascade contributing to a diverse range of cellular responses (Viarengo, 1989; Li et al., 2010).

Canesi et al. (2010) demonstrated that C60 can induced an increase of GSTs activity, due to a significant rise in lysosomal lipofuscin, lysosomal damage, oxidative stress and toxic cell injury in *Mytilus sp*., and Al-Subiai et al. (2012) showed in the same organism histological anomalies and DNA damage.

Oberdörster (2004) reported that juvenile largemouth bass exposed to 0.5 ppm aqueous uncoated C60 for 48h had a significant increase in lipid peroxidation of the brain, and glutathione (GSH) depletion in the gill. FullereneC60 have been shown to produce adverse effects even on *Daphnia magna* (Roberts et al., 2007), zebra-fish embryos (Usenko et al., 2008) and rainbow trout (Smith et al., 2007). Using similar concentration, however, other researchers have found no effects of this nanomaterial of freshwaters amphipods or copepods (Templeton et al., 2006).
Somewhat controversial results regarding the real presence of toxic effects on model organisms have opened a still active debate on the actual level of C60 toxicity. Nevertheless, there is a consensus that nanomaterials, especially in the aquatic environment, can potentially affect biological systems not only by themselves but also through interactions with other compounds (Christian et al., 2008; Henry et al., 2011).

1.3 *Mytilus galloprovincialis*: A "top model" of aquatic environments

Bivalve molluscs have long been used to estimate contamination levels in temperate water ecosystems and mussels, in particular, are long since widely used as aquatic pollution bioindicators (Bayne and Moore, 1979; Banni et al., 2007; Viarengo et al., 2007) due to their sessile nature, filter-feeding habits, suitability for caging experiments along coast lines and wide tolerance to a large range of environmental conditions and pollutants (Viarengo et al., 2007; Campos et al., 2012; Liu et al., 2014;Ji et al., 2016). They have a broad geographical distribution in both salt and freshwaters and their very low biotransformation metabolism allows the bioaccumulation of many different classes of contaminants that usually end up in the benthic environment (Widdows et al., 2002; Attig et al., 2010). This, in addition with their relevance as a food organism to humans, means they are a particularly effective model in ecological studies and bio-monitoring programmes

(Viarengo and Canesi 1991, Cajaraville et al. 2000; Jones et al., 2008). A well-known clear example was the “Mussel Watch Program” proposed by the US that has been employing these organisms for over 30 years to indicate the health of estuarine and coastal waters using a variety of indicators, including anatomical and physiological parameters, diseases and other pathologies (Cajaraville et al., 2000). Mussels readily bioaccumulate both organic (Moore, 1985) and metal (Viarengo, 1985) pollutants and extensive background information is now available on their biological responses to a wide range of chemicals (Dagnino et al., 2007).

Figure 1.5 *Mytilus spp.*

Mytilus spp. is one of the most employed sentinel organism since 1976, when Goldberg tested it in a bioassay for the first time. To understand why mussels are considered a reliable model it is necessary to know their anatomy. Bivalves belong to one of the six classes of the phylum Mollusca, that of the Lamellibranchs. They generally live in the infralitoral area, from the upper limit of the intertidal zone to a

depth of a few meters, attached to hard substrates or suspended materials. Mussels in this way tend to cluster together forming very large colonies.

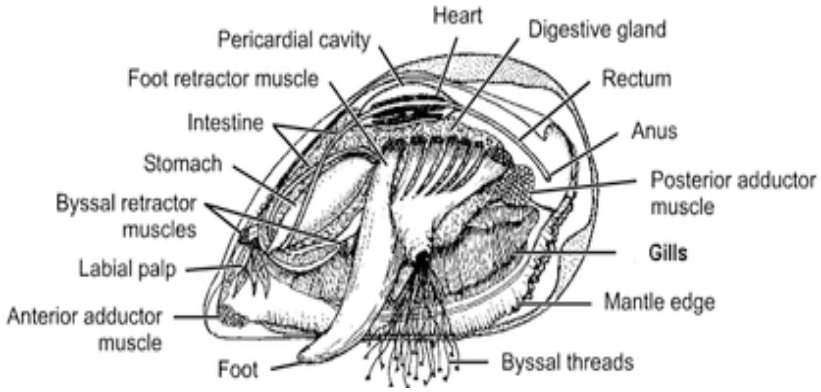

Figure 1.6 Internal anatomy of *Mytilus spp. (*©Saba, 2011)

These organisms are laterally compressed and their soft body is completely enclosed in a shell composed of two symmetrical valves (Riedl, 1991; Mengoli, 1998) consisting of an organic matrix made of proteins, mucopolysaccharides and calcium carbonate crystals

The adductor muscles, perpendicular to the valves, close the shell playing an important role in molluscs survival, because from the closure of their valves depends the possibility to defend themselves from predators, and to retain the hemolymph to keep the gills moist for as long as possible to favor the gas exchanges. The gills able to absorb oxygen for breathing and trap macromolecules, ions and food particles (predominantly phytoplankton and suspended organic matter), as they act as a filter in which particles attached to the mucus on the surface are retained and moved towards the labial palps and the mouth thus entering the gut, then reaching the digestive gland where digestion occurs.

The digestive tract of bivalve molluscs consists in a short esophageal tract, which opens into a large pleated stomach, and dotted with the orifices of the digestive

diverticula. These greatly branched structures end in small swollen bodies constituting the glandular acini (Mengoli, 1998). The digestive tubules are composed of ciliate cells and vacuolar cells rich in pigments such as carotenoids, glucoproteids and lipids.

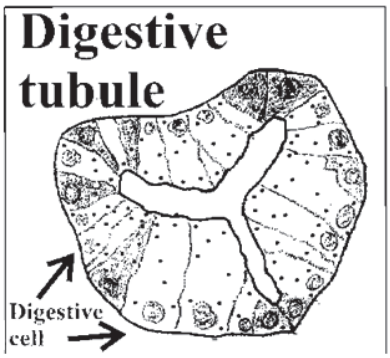

Figure 1.7 Representation of a digestive tubule with the cells that compose it, in the mussel hepatopancreas

The stomach is differentiated into a dorsal portion into which the esophagus and the ducts of the digestive gland open; and in a ventral, swollen region, in which the crystalline stylus is located, and which secretes a series of lytic enzymes such as amylases, cellulases and lipases. Digestive cells have an extremely developed lysosomal vacuolar system not only for intracellular digestion but also in the accumulation of lipofuscin granules, which are known to trap different types of compounds, including heavy metal cations (George and Viarengo, 1985; Viarengo, 1989; Gosling, 1992). In fact, in this organ they also happen significant detoxification functioning as well as innate cellular immunity (Canesi et al., 2010; Du et al., 2013). The portion that surrounds the mollusc bowels, constituted by two lobes of soft tissue closely in contact with the shell and responsible for its secretion is the mantle, which is also the site designed to contain gametes (ovules or spermatozoa). Mantle edges are prolonged by forming siphons for the inlet and outlet of the water: a superior inhaling siphon (which in female subjects allows the

entry of male seminal material) and a lower exhaling siphon with excretory function can be distinguished. The edge has extensions which, when the valves are opened, intertwine to form a sort of filter to prevent large particles from entering.
Mytilus spp. has an open circulatory system with a dorsal heart, constituted by a ventricle and two lateral atriums, enclosed in a pericardium bathed in haemolymph.
The impact of aquatic pollutants is thought to be significantly different in various organ/tissues along with their own biological function variability. It should also be taken into account that these organisms are subject to seasonal metabolic variations related both to the fluctuation of environmental parameters (temperature, salinity, oxygen levels) and to the physiological status of the animals, depending on food availability and on the gametogenic cycle (Gabbott, 1975; Widdows, 1978; Livingstone, 1981; Viarengo et al., 1991).
Chronic exposure of mussels to pollutants in water and sediments may ultimately impair their nutrient absorption ability and compromise their growth and reproduction (Smital et al., 2004). On cellular scale, several researches have proven oxidative damage, scavenger responses, genotoxicity and endocrine disruption in organism subjected to environmental contaminants, such as benzo[a]pyrene (Viarengo et aI., 1989; Livingstone et al., 1990; Viarengo et al., 1990; Akcha et al., 2000; Di et al., 2011; Banni et al., 2010; Tian et al., 2013). On these bases, as well highlighted by Moore (2006), it is easy to understand how mussels might represent a unique target group for evaluation of micro and nano-particles toxicology in aquatic matrices (Wessel et al., 2007; Banni et al., 2017)

1.4 Biomarkers: The early sentinels of environmental monitoring

The most accredited definition identifies biomarkers as that biochemical, cellular, physiological or behavioural variations measured in a tissue, biological fluid or whole organism (individual or population) that provide the evidence of an exposure

and/or effect to one or more polluting compounds (and/or radiation) (Depledge, 1994).

To obtain a reliable and predictive interpretation of the environmental risk related to the presence of harmful substances (as single compounds or complex mixtures), it is important to be able to recognize and determine the events that occur following an exposure on a molecular/cellular scale, before the biological effects are macroscopically recognizable to the highest ecological levels (Banni et al., 2005; Viarengo et al., 2007). In this context, the use of biomarkers is an effective tool for environmental quality assessments and monitoring, because in most cases different organisms share a common cellular, biochemical and functional substrate that makes this type of investigation adaptable to multispecies approaches within a large number of ecosystems (Shugart, 1995).

It is however true that in a natural environment, even within a single population, individuals can respond differently to an identical exposure scenario, activating compensatory mechanisms in order to maintain homeostasis and avoid the damages appearance; when these occur, the physiological repair mechanisms are activated, which make the organism more susceptible to other possible stressful events. In this perspective, it is clear that in nature the exposure to which the organisms are subjected is cumulative and that the different interacting stresses may influence the dynamics of expression of the observed response.

For this reason, it is really important to understand the dose/response relationship to correctly interpret the occurrence of biomarkers and use them for the study of environmental quality (Depledge, 1994).

In the laboratory, to obtain the dose/response curves the model organism is exposed to a single substance (or mixtures suitably prepared of several substances) and the structural variations or physiological functions are identified according to the exposure to an increasing series of doses. The information obtained therefore needs an interpretation so that it can be extrapolated and adapted to the variability and complexity of the natural environment.

The traditional eco-toxicological approaches focus on the measure of ordinary biomarkers suite, that must include rapid and sensitive responses to stress at molecular and cellular level, assessment of tissue damage and eventually also at the organism level, to obtain indication of the individual survival capacity, which may have impacts on extended ecological level (Viarengo et al., 2007).

The use of single biomarkers in an eco-toxicological assay may be limiting because of the possible lack of sensitivity and specificity (Campos et al., 2012), but if a biomarker battery is used, the effect can be amplified and become easier to be detected, and the result obtained is an integrated response to the overall exposure (Ji et al., 2013). Therefore, a broad employment of toxicological multi-level biomarkers, such as gene, protein and metabolite, can give a global view on the toxicological effects induced by the environmental contaminants in organisms (Akcha et al., 2000).

Since the increase in specificity and sensitivity of a biomarker goes hand in hand with the difficulty of obtaining low-cost analysis, two-tier approaches have been proposed in the past, consisting of early screening with very sensitive but low cost biomarkers; if significant effects are found in this first phase, you can proceed using the full battery of biomarkers to reduce costs and unnecessary efforts (Viarengo et al., 2007).

Among others, the most employed screeneng biomarkers, which indicate both exposure to a toxic compound and its toxicological effect (Losso and VolpiGhirardini, 2010) are lysosomal membrane stability, lipofuscin and neutral lipid accumulation in lysosomes, while damage to genetic material is routinely investigated using the the micronuclei frequency.

1.4.1. Biomarkers of stress: Lysosomal membrane stability

In polluted ecosystems, toxic substances alter the organisms health by causing a "stress syndrome", a measurable alteration of the physiological state induced by an environmental change. Subcellular pathological reactions to environmental stressors frequently involve destabilizing changes in the lysosomal membrane and the induction of autophagy (Moore et al., 2009), which is the degradation of cellular components implicated in many disease processes, cell death and adaptive responses (Cuervo 2004).

Lysosomes are subcellular organelles very active in stress conditions, involved in a number of essential processes such as digestion, membrane turnover, defence and reproduction (Moore, 1976; Ferreira and Dolder 2003). They are easy to visualize in all the nucleated cells and, in addition, being the target of a wide range of contaminants they can be aspecifically used as a preliminary investigation tools on cellular health.

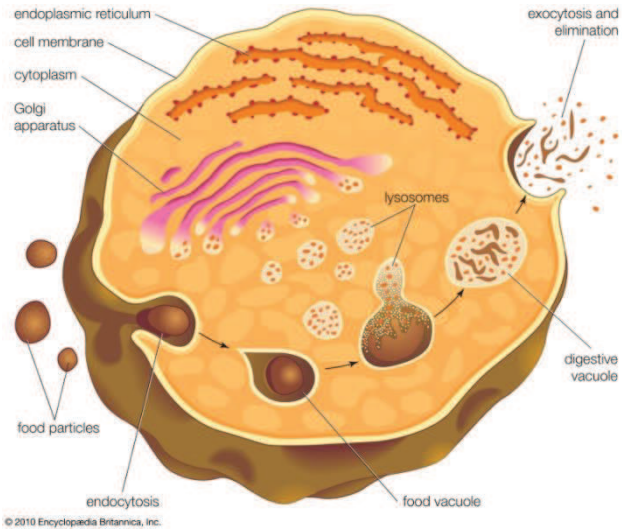

Figure 1.8 Representation of lysosomal formation and evolution within the cell (©2010 Encyclopaedia Britannica, Inc.)

A primary lysosome derives from a hydrolasic vesicle (i.e. containing a large quantity of inactive digestive enzymes) formed by gemmation from the Golgi apparatus, which is activated by merging with an endosome equipped with membrane proton pumps that lower the pH (up to 4.8) so as to activate the acid hydrolases contained in the primary organelle. Such enzymes are capable of degrading the cellular macromolecules up to the elemental constituents (proteins, lipids and carbohydrates), be they foreign material (heterophagy) or endogenous damaged components (autophagocytosis) to be expelled or recycle in the context of cell turnover. When the material to be phagocytized combines with a mature secondary endolysosome, the actual catabolic processes are activated, in order to preserve cellular homeostasis. Since lysosomes are the site of preferential storage within the cell, non-essential metals and toxic organic contaminants are accumulated in them (Moore, 1990). The catabolic activity of a cell enhances when subjected to stressors, both because it increases the energy requirements for both the need to degrade and dispose of the excess of harmful waste products. In the search for early signals of cellular disturbance the responses of the lysosomal compartment can provide an optimal observation point to realize the degree of vulnerability to toxic substances which, compromising the integrity of these organelles, cause alterations of the autophagic pathway causing loss of cellular function and, in extreme cases, death (Koehler et al., 2002). The latter case occurs when the hydrolytic enzymes (approximately sixty types as reported by Nolde et al., 2006) accumulated within the lumen-covering lipidic bilayer are poured into the cytoplasm with consequent irreversible impairment of the lysosomal membrane. Before this occurs it is possible to identify other biomarkers of stress accumulation: changes in lysosomal content (Moore, 1988) as well as their size increase following multiple vacuolar fusion (particularly evident in cells responsible for intracellular digestion as reported by Moore, 1990; Marigómez et al., 1991; Regoli, 1992), and membrane destabilization (Moore 1988, Wiston et al., 1991, Cajaraville et al., 1995, Nicholson 1999, Lowe and Fossato, 2000).

The integrity of the lysosomal membrane is among the first parameters investigated to assess the condition of cellular wellness.This procedure has long since become a routine on model organisms such as *Mytilus spp*. (Moore, 1976) and uses histochemical methods on frozen tissue sections or cellular extracts in vivo, preferably from digestive or detoxification organs where lysosomal activity is certainly higher. The *in vitro* histochemical assay, also called Lysosomal Latency assessment (Schneider et al., 1984), is performed to destabilize the membrane and make it permeable to a substrate that reacts with a specific lysosomal enzyme (N-acetyl-hexosaminidase), resulting in a detectable reaction with the coupling of an azo-dye. The principle of evaluation is that the already destabilized membranes will sequester the dye complex much faster than the intact ones.

Instead, the evaluation of cell damage *in vivo* is measured with the Neutral Red Retention time based on a reverse process to the former with the determination of the neutral red (NR) vital dye effusion from the inside to the external side of the lysosomes (Moore 1988, Wiston et al., 1991, Cajaraville et al., 1995, Nicholson 1999, Lowe and Fossato, 2000). Once in contact with the cells, NR in its deprotonated form is seized inside lysosomal membrane where, due to the sudden variation of pH (Nolde et al., 2006), undergoes a protonation process which, under normal conditions, should prevent its escapement in a short times. Indeed, since NR is itself a cytotoxic compound (Viarengo et al., 2007), even healthy lysosomes in contact with it suffer damage, but only after a longer period of time (150-180 min as reported by Lowe and Pipe, 1994) compared to conditions where stress was already present (about 15 min). Damage to the membrane, caused by the impact of cytotoxic agents, decreases the NR retention times. The release of the dye possibly together with the lysosomal content into the cytosol is probably due to the dysfunctional interaction of the NR with the ATP-dependent hydro-protonic pump of the lysosomal membrane, responsible for maintaining the low internal pH (Ohkuma et al., 1982). Its failure to function increases the pH in the lysosomal lumen, and the loss of the chemical gradient results in a trans-membrane

equilibrium which promotes the diffusion of the lysosomal content into the cytosol, with shorter timings as much as the previous damage was been extended (Lowe et al., 1995).

The lysosomal membrane stability (LMS) is a widely used indicator to measure responses to environmental perturbation caused by various contaminants including metal ions, PAHs, heterocycles and nanoparticles (Moore et al., 2012), which are sequestered from lysosomes once inside the cell (De Duve et al., 1974; Moore 1990; Rashid et al., 1991; Moore et al., 2004).

The advantage of the cytochemical approach on frozen sections in the lysosomal biomarker research is to be able to evaluate other interrelated parameters, such as accumulation of lipofuscines and unsaturated lipids. As already mentioned, a reduction of LMS is often associated with an increase in the lysosome/cytoplasm volume ratioin the target tissues, a parameter indicating an unphysiological level of cell catabolism (Moore and Viarengo, 1987; Moore et al., 2008). In the assessment of LMS, the same image analysis can be employed to quantify this ratio to find out whether or not stress syndrome development affects autophagy and, consequently, the physiology of the whole organ since an increased lysosome/cytoplasm ratio indicates that the cells are losing their proper functionality, along a catabolic path (Lowe et al., 1981). LMS and lysosome/cytoplasm volume ratio are of particular relevance because only when significant alteration of both parameters are observed the results indicate an unphysiological increase in autophagic-related sequestration of cellular components, a process that may lead to severe cell damage (e.g. cytoplasm shrinkage) and loss of tissue functions (e.g. reduction of the epithelial thickness) (Lowe,1988; Lowe et al., 1981; Moore, 1988; Moore and Clarke, 1982; Moore et al., 2007).

1.4.2. Oxidative stress and lipofuscin lysosomal content

Although ROS are produced through normal cellular metabolism, specific molecules such as PAHs and various metal species (e.g. Fe, Cu, Zn, Ni), as subject to redox cycles they are able to induce chain reactions that cause intracellular increases of superoxide radical (.O2-), hydrogen peroxide (H_2O_2) and, in particular, hydroxyl radical (.OH), damaging cellular macromolecules (Storey, 1996; Kelly, 2003; Ayres et al., 2008).

High amounts of ROS act on detoxifying enzymes (such as superoxide dismutase and catalase) and on scavenger molecules such as glutathione; if the oxidative damage they cause exceeds the antioxidant shield, the cell loses its homeostasis incurring even in lethal damages. One of the most well-known and observable manifestations of oxidative stress is the peroxidation of membrane lipids (Viarengo, 1989) and even one of the main causes of the cellular disfunctions.

Membrane fatty acids are among the most vulnerable macromolecules to attack by free radicals. The origin of the lipid peroxidation involves the removal of a hydrogen atom from a methylene group (-CH2-) usually promoted by a hydroxyl radical, which triggers a series of cascade reactions starting from the generation of an unstable lipid radical that, reacting with oxygen, originates a peroxylipidic radical; each of these can, in turn, extract a hydrogen atom from an adjacent fatty acid chain by converting into hydroperoxide lipids.

The accumulation of these latters is particularly dysfunctional, since they may trigger the activation of phospholipases and proteases with even drastic effects on the integrity of biological membranes (Halliwell, 1992). The final peroxidation product is an accumulation of insoluble, intralisosomic polymeric material usually referred to as lipofuscin, consisting mainly of protein and lipid residues, which cannot be degraded by lysosomal hydrolases (Kikugawa et al., 1989) neither excreted through exocytosis. The nature of these granules is highly heterogeneous, essentially consisting of residues of oxidatively modified proteins and lipid

degradation products (triglycerides, free fatty acids, cholesterol and phospholipids, among others) along with carbohydrates and metals especially iron (Brun and Brunk 1970; George and Viarengo, 1985; Viarengo and Nott, 1993; Jolly et al., 1995; Terman and Brunk, 2004). The presence of lipofuscins is an indicator of aging, especially in post mitotic cells or those with a low rate of cell division that accumulate them as chemically polymorphic biological waste, since they fail to effectively disperse their presence as the mitotically active cells do (Terman 2001). Oxidative stress and lipofuscins accumulation are cellular events in continuous cross-talk: ROS, which easily diffuse through the lysosomal membrane, are responsible for the appearance of lipofuscins, with a rate of formation as fast as intralisosomal degradation is slower (Terman and Brunk 1998). It follows that the lipofuscins deposit can compromise the integrity of the lysosomes since their poor degradability favors the incremental accumulation that ends up burdening on the lysosomal functionality and causing decreases in the degradation rate that feed this positive feedback (Viarengo et al., 2007; Moore et al., 2007). As already mentioned, the intracellular content of lysosomes is more pronounced in digestive or detoxifying tissues, so an ecotoxicological study to evaluate a potential increase in lipid peroxidation and the consequent increase in lipofuscines in aquatic organisms is carried out on fish hepatocytes or in mollusc's digestive gland (Viarengo and Nott, 1993). There is a great variety of histochemical methods to highlight the presence of lipofuscines (Terman and Brunk., 2004) but one of the simplest and inexpensive, often used for analysis on mussels, is the coloring reaction of Schmorl (Pearse 1972) which shows the presence of reducing substances in tissues.

1.4.3. Metabolic stress and neutral lipid accumulation

Another effect of the action of the pollutants at the cellular level is the alteration of the fatty acids metabolism that results in manifestation of lipidosis events often associated with the lysosomal vacuolar system (Lüllman-Rauch, 1979).

While the accumulation of lipofuscins seems to constitute a general response to environmental stress (Viarengo et al., 1990), the neutral lipids increase appears more closely related to organic contaminants exposure, which can alter cellular functionality with an increase in proteins catabolism and the formation of peptides and amino acids within the lysosomal compartment (Moore 1988, Domouhtsidou and Dimitriadis 2001).

The alteration of the neutral lipids content in the digestive organs of bioindicator organisms (and specifically molluscs) is a direct consequence of metabolic imbalances (Lüllman-Rauch, 1979) that cause a lipidosis initially localized in the cytosol. The cellular adaptation response is to sequester the lipid droplets within the lysosomal compartment, following contaminants-induced autophagic processes (Moore, 1988). These accumulations may be related respectively to increased concentrations of cytosolic lipids or dysfunctions in fatty acids processing. In both cases, the presence of disproportionate lipid content is a useful indicator of physiological cellular changes (Koehler, 2004).

Neutral lipid contents is often determined according to the method of Bancroft (1967), in a simple and low-cost way (Moore, 1988; Viarengo et al., 2007), using a specifical lysochrome (Oil Red-O, ORO) which stains neutral lipids and cholesteryl esters but not biological membranes (Ramirez-Zacarias et al., 1992).

The principle for staining is that ORO is almost insoluble in the solvent, and the solubility is further decreased by dilution in water before use. The hydrophobic dye will therefore move from the solvent to associate with the lipids allows their detection and localization in tissues (Mehlem et al., 2013).

1.4.4. Effects of b[a]p and fullereneC60 on cell signalling: The mTOR kinase complex

Cellular signalling occurs through rapid activation and/or inactivation of signal proteins, which result in more or less temporary gene expression modification. External inputs or those coming from inside the cell induce stimuli that can reach the nucleus and determine transcriptional changes to specific genes or act downstream, in the cytoplasm, blocking the translation of specific mRNAs.

Generalized stress conditions, lack of nutrient or growth factors involved cellular metabolism rearrangements that trigger autophagy to compensate and optimize energy resources (Jung et al., 2010). This essential process for cell survival in response to metabolic and toxic stress involves lysosomes through the seizure of cytoplasmic components, the removal of damaged and aggregated proteins and their subsequent degradation (Sforzini et al., 2017). Cellular waste products are used to generate new components and energetical substrates in response to nutritional needs.

Lysosomal biogenesis and functions are subject to transcriptional control, resulting in their ability to adapt to different environmental stimuli. The role of lysosomes as nutrient sensors broadens the definition of these organelles, from cellular waste disposal executives to regulators in the signaling pathways that are involved in metabolism, cell growth and autophagy (Zoncu et al., 2011).

Among the many components involved in cellular regulation and growth, mTOR (mechanistic Target of Rapamicyn), an evolutionarily-conserved serine/threonine protein kinase belonging to the phosphatitylinositol kinase-related kinase (PIKK) family (Jung et al., 2010).

It represents a key component that coordinates the balance between growth and catabolism in response to cellular physiological conditions and environmental stress, inhibiting the autophagic event when there is plenty of nutrients (Levine et al., 2005; Jung et al., 2010).

When, as a result of physiological or environmental stimuli, mTOR is inhibited (by dephosphorylation), the catabolic cell metabolism is triggered by a mechanism common to all eukaryotic cells from yeasts to mammals (Klionsky and Emr, 2000; Moore et al., 2012).

mTOR is a catalytic subunit of two molecular complexes called mTORC1 and mTORC2. Although these two complexes share the same three highly conserved subunits (i.e. mTOR, mLST8 and DEPTOR), they also present a specific protein-complexes composition (Luo et al., 2018). While mTORC1 contains Raptor (mTOR-associated regulatory protein) and PRAS40 (proline Akt substrate of 40 kDa) (Sancak et al., 2007, Vander Haar et al., 2007, Wang et al., 2007), mTORC2 is composed by Rictor (probably with a Raptor-like function, as reported by Sarbassov et al., 2004) as well as mSin1 and Protor 1/2 regulatory subunits (Frias et al., 2006, Jacinto et al., 2006, Luo et al., 2018). In addition to this compositional difference, the two complexes also differ in their response to rapamycin, which directly inhibits mTORC1 while mTORC2, insensitive to acute treatment, is inhibited only if exposed for prolonged periods (Sarbassov et al., 2004).

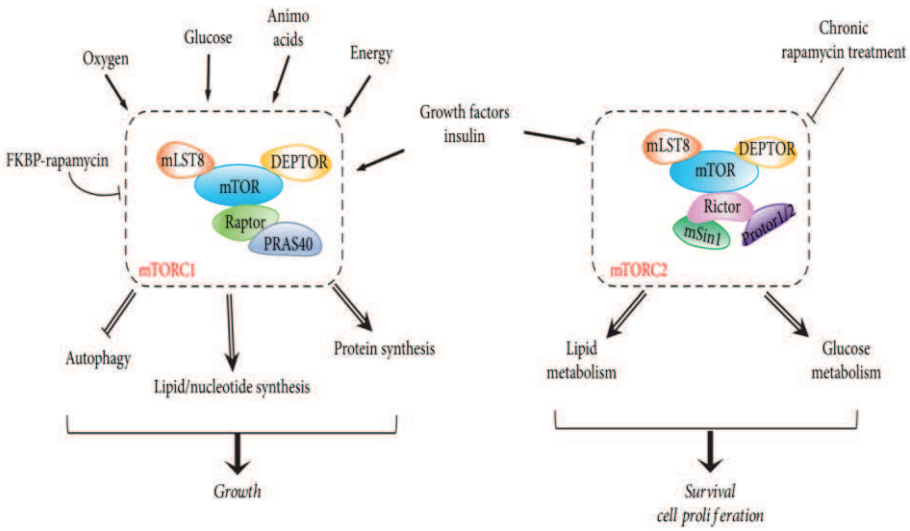

Figure 1.9. The composition of mTORC1 and mTORC2 protein complexes and their main molecular components, signals and processes to control cell growth and survival (© 2018 Yongting Luo et al.).

In particular, mTORC1 works mainly as a sensor of nutrients, oxygen, energy and growth factors to regulate cell growth by inhibiting autophagy coordinating the regulation of the main anabolic cellular processes starting from transcriptional events, ribosomal biogenesis and protein, lipids and proteins synthesis (Soulard et al., 2009; Dowling et al., 2010). When activated, it is mainly located near the nucleus, modulating the activity of transcription factors (Barquilla et al., 2008; Li et al., 2006).

Even mTORC2 plays a fundamental role in cell survival, proliferation and growth and its distribution is mainly cytoplasmatic; it has an important role in regulating the function of the actin cytoskeleton, thereby affecting the shape and mobility of cells (Gaubitz et al., 2016; Jacinto et al., 2004) and plays an important role in glucose metabolism (Kumar et al., 2010; Tang et al., 2016; Albert et al., 2016).

It is therefore clear that all the events that induce cellular stress with autophagy activation have the first impact on the regulation of the mTOR complex, within a highly conserved signaling network. An articulated "signalling" machinery composed of mTORC1 and other protein complexes, named LYNUS (Lysosome Nutrient Sensing), is located on the lisosomal surface (Sancak et al., 2010) suggesting a co-regulation between cell growth and catabolism (Settembre et al., 2013). It responds to the amino acid content in the lysosomes and reports information both to the cytoplasm and to the nucleus.

The regulation pathways of mTOR, as already mentioned, are multiple and often endogenous but many environmental contaminants such as b[a]p can influence their activity, e.g. by stimulating the production of ROS which act by dephosphorylating the kinase complex (Moore, 2008; Chen et al., 2010) and activating a cascade of events that can lead to sharpening the physiological conditions linked to oxidative stress, such as a decrease in the stability of lysosomal membranes with a consequent increase in the L/C ratio (Sforzini et al., 2018).

Less is known about the effect that nanoparticles such as the fullereneC60 can exert on the regulation of the mTOR pathway. Studies of bivalve exposure at different C60 concentrations show that fullerene passes through hepatopancreas cell membranes and lysosome appears to be at least one of the main concentration sites and an intracellular target for toxicity (Ringwood et al., 2009). It has already been pointed out that exposure to C60 can induce oxidative stress, lysosomal damage and a decrease in protein synthesis, in line with the activation of autophagic mechanisms. It is therefore conceivable that everything that influences the metabolism and lysosomal architecture, as well as everything that makes the organisms "catabolic", may be related to the changes induced on the mTOR pathway.

The role of mTOR as a nodal point of coordination of metabolic pathways and the study of its multiple regulatory mechanisms have been extensively investigated in studies on mammals, and on humans in particular since the dysregulation of mTOR signaling is related to the onset of human diseases including cancer (Dowling et al., 2010; Laplante and Sabatini, 2012).

On the other hand, similar studies related to invertebrates exposed to environmental stress conditions, to ascertain the role of mTOR in cell signaling and the regulation of cellular responses to contaminants, are currently a frontier zone in scientific research.

1.5 MATERIALS AND METHODS

1.5.1. Experimental design and sampling

The sampled mussels were transferred to glass beakers containing seawater and allowed to acclimatize for 48 h. The experiment consisted of a 3-day static exposure with no water changes, during which the mussels were not fed. Groups of mussels were exposed to several treatments, the first using solvent control (0.02% dimethyl sulfoxide -DMSO) and 5-50-100 µg/L b[a]p alone and in mixture withfullereneC60 (1 mg/L). After 3 days exposure period, digestive glands were rapidly removed, placed on aluminium cryostat chucks, chilled in super-cooled n-hexane and stored at -80°C.

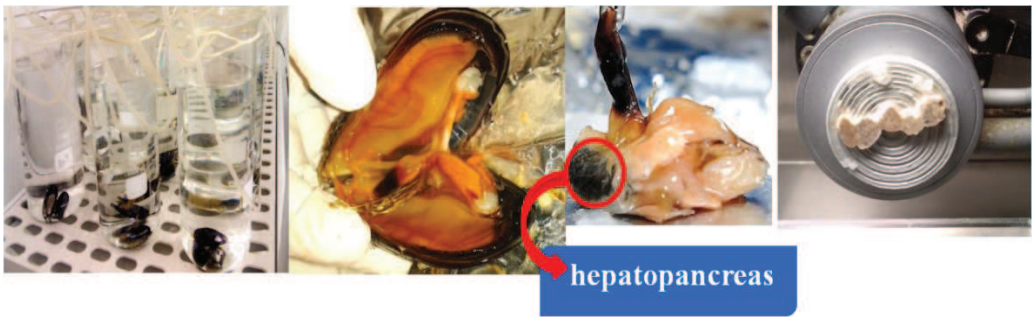

Figure 1.10. Experimental settings on *M. galloprovincialis* samples

1.5.2. Lysosomal alterations

Frozen digestive gland sections (10 µm) of mussels from each exposure condition were cut by cryostat and flash-dried by transferring them onto slides at room temperature. The determination of LMS was performed following essentially the

method described by Moore (1988). This cytochemical assay is based on acid labilization characteristics of latent hydrolase β-N-acetylhexosaminidase (NAH) a lysosomal acid hydrolase involved in the degradation and digestion process (Franzellitti et al., 2014). The digestive gland sections were incubated in a solution containing naphthol (AS-BI N-acetyl-β-Dglucosaminide) 0.04%and Polypep 7% in 0.1 M citrate buffer (pH 4.5) as a substrate for NAH. The sections were rinsed with NaCl 3% at room temperature and left to soak in a saturated solution of Fast Violet B dye (specific for the enzyme-lysosomal substrate complex) in 0.1M sodium phosphate buffer (pH 7.4) for 10 minutes in the dark. Finally, the slides were washed for 5 minutes under running water. The L/C volume ratio of the digestive gland tissue was evaluated following the method described by Moore (1976) and Moore and Clarke (1982).

Lysosomal lipofuscin content in the cells of the digestive glands was estimated on cryostat sections (10 μm) using the Schmorl reaction (Pearse 1972). The slides were first fixed in calcium formol for 15 minutes at 4°C and after rinsing in distilled water, they were immersed for 5 minutes in a dye aqueous solution consisting of 1% ferric chloride and 1% potassium ferrocyanide. Two washes were then carried out first in 1% acetic acid and then in distilled water.

The neutral lipids quantitative analysis was carried out through a cytochemical method, which provides for their selective coloring within the digestive tubules. The gland sections were fixed in Ca-formol for 15 minutes at 4°C, the slides rinsed with distilled water and transferred to 60% triethyl phosphate for 3 minutes they were then placed in the dark in a solution of Oil Red-O at 1% for 15 minutes at 20°C and then rinsed first in 60% triethyl phosphate at 4°C for 30 seconds and then in distilled water.

At the end of each of the aforementioned cytochemical methods, the slides were mounted with glycerine gel. The sections thus obtained were observed using an inverted optical microscope (Zeiss Axiovert 100M) with a magnification of 400×,

connected to a digital camera (Zeiss AxioCam), and quantitatively evaluated for each biomarker using image analysis software Scion Image 4.0.2 (Scion Corporation Frederick, MD, USA). The results were expressed as a percentage change with respect to the control.

1.5.3. Immunofluorescence analysis

Cryostat frozen digestive gland sections (10 μm) obtained as described above were flash-dried by transferring them onto poly-L-lysine-coated microscope slides at room temperature and fixed in paraformaldehyde (PFA) solution.

Immunofluorescent anti-PAHs staining was carried out as described by Sforzini et al., (2014) both to identify the only b[a]p at different exposure concentrations and its mixture with 1 mg/L of fullereneC60. Immunofluorescence co-localization of b[a]p and the lysosomal enzyme cathepsin-D was performed following immunolabelling with the first primary and secondary antibodies (as described above for single labelling).

For the immunofluorescent anti-mTOR staining, sections were incubated in a permeabilisation and blocking solution and then with the primary antibody (anti m-TOR (phospho S2448) antibody) overnight at 4°C in a moist chamber. Sections were then washed in PBS and the secondary antibody was applied for 1 h at 20 ± 1°C in the dark. Finally, sections were rinsed in PBS, counterstained with propidium iodide and mounted.

1.6 RESULTS

1.6.1. Cytochemical and immunohistochemical analysis

After the 3 days of exposure, the concentrations of b[a]p and fullerene-C60 to which the *M. galloprovincialis* specimens were exposed did not have any impact on their viability (data not shown).

B[a]p was stored in digestive tubule of epithelial cells as revealed by the immunofluorescence reactions that showed positivity to the anti-PAH antibody (Fig. 1.11b-d) on the tissue samples taken from the digestive gland of exposed mussels, according to the method already used by Sforzini et al. (2014); in the sections coming from the control organisms no immunopositivity was found (Fig. 1.11a).

To confirm the intra-lysosomal b[a]p accumulation a double immunolabel of the sections was carried out with anti-PAH and anti-cathepsin D antibodies, which have had positive response (Fig. 1.12). The quantification of the fluorescence signal by digital imaging for both b[a]p alone and in mixture with fullereneC60 (Fig. 1.14) showed a significant increase in fluorescence intensity in animals exposed to all experimental conditions, compared to controls; however, the most intense emission was found at the lower concentration (5 μg/L) of b[a]p, alone or in co-exposure (Fig. 1.13b-d). Examination of unstained serial sections of mussels exposed to b[a]p and fullereneC60 under UV light showed the presence of numerous white-blue fluorescent droplets; fluorescence was minimal in the digestive glands of mussels exposed to 5 μg/L and increased from 50 μg/L to 100 μg/L b[a]p (Fig.1.15).

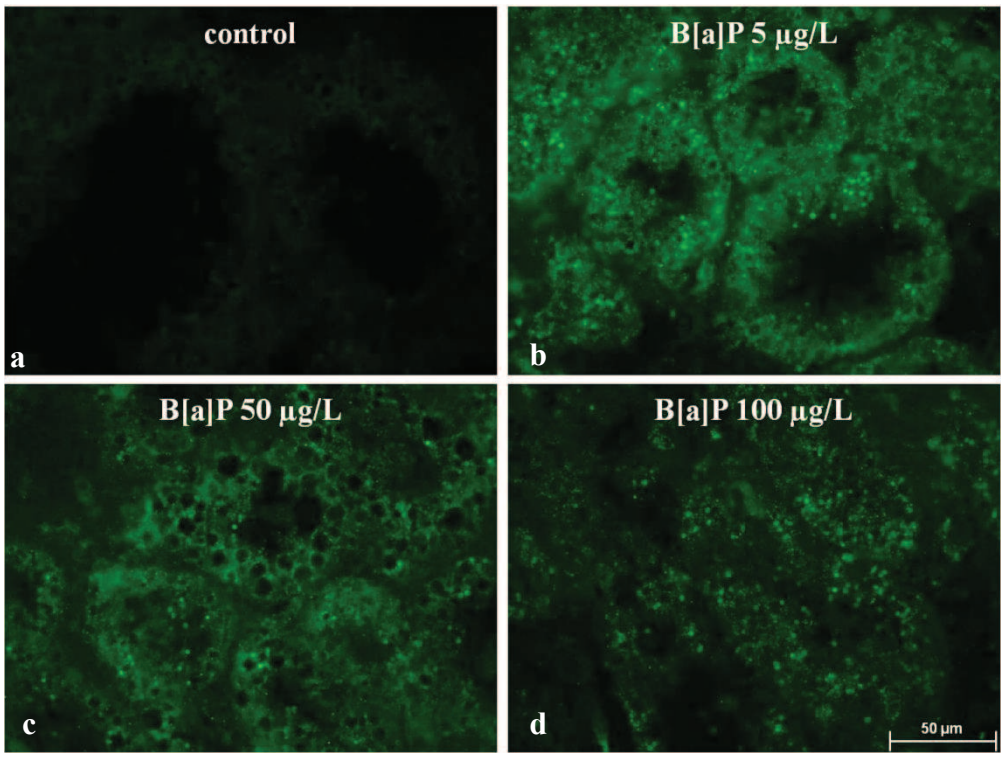

Figure 1.11 Anti-PAHs immunohistochemical staining (green: FITC conjugated secondary antibody) of digestive gland tissue sections from mussels exposed to different experimental conditions (a. Control; b. 5 µg/L b[*a*]b; c. 50 µg/L b[*a*]p; d. 100 µg/L b[*a*]p).

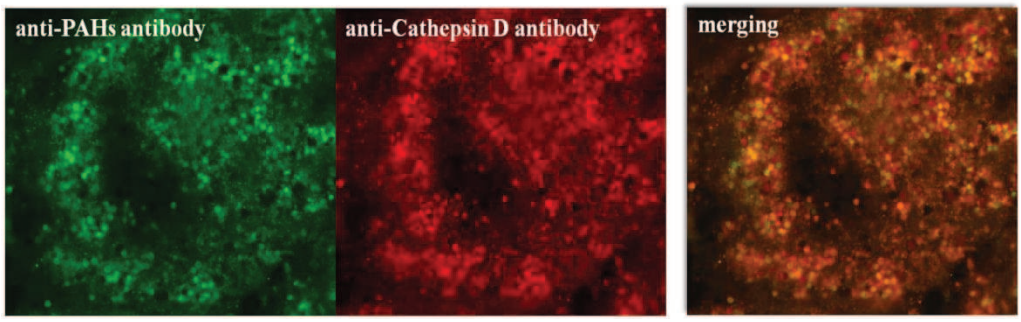

Figure1.12. Double immunohistochemical staining of digestive glands from mussels exposed to 5 µg/L b[a]p with anti-PAHs (FITC, green) and –Cathepsin D antibodies (DyLight594, red). The two images obtained were melted in a composite image, making the two labels coincide in a yellow one that allowed to detect the colocalization of both antigens in the mussel lysosomes exposed to b[a]p (5 µg/L).

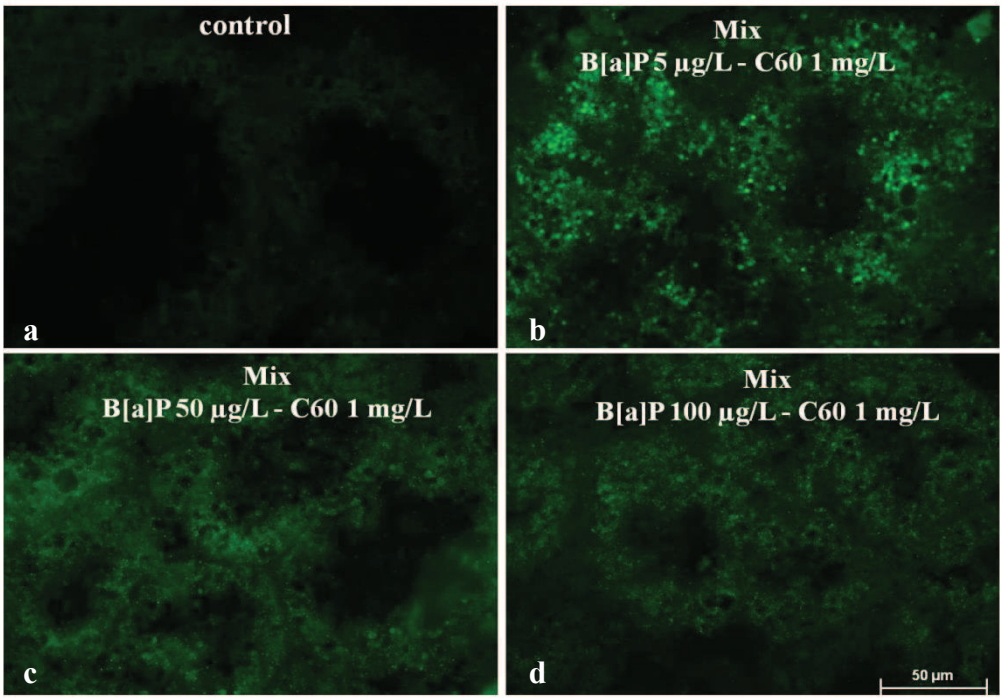

Figure1.13. Anti-PAHs immunohistochemical staining (green: FITC conjugated secondary antibody) of digestive gland tissue sections from mussels exposed to different experimental conditions (a. Control; b. 5 µg/L b[*a*]p- C60 1 mg/L; c. 50 µg/L b[*a*]p- C60 1 mg/L; d. 100 µg/L b[*a*]p- C60 1mg/L).

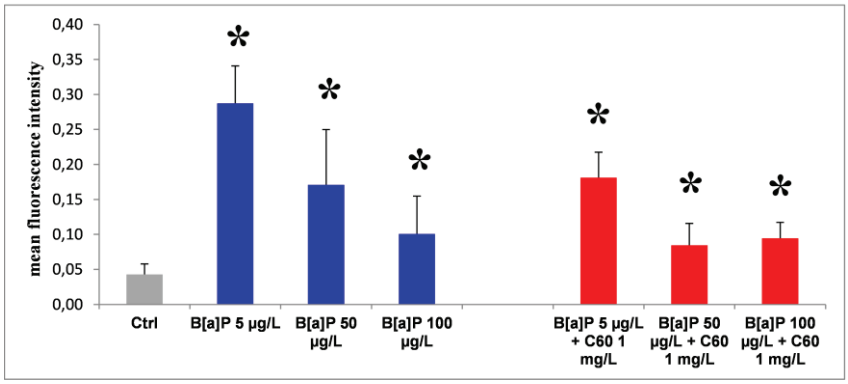

Figure 1.14. Quantitative fluorescence analysis of the anti-PAH immunoreaction for the experimental conditions of exposure to b[a]p alone (5 µg/L; 50 µg/L; 100 µg/L) and in mixture with fullereneC60 (5 µg/L b[*a*]p- C60 1 mg/L; 50 µg/L b[*a*]p- C60 1 mg/L; 100 µg/L b[*a*]p- C60 1mg/L). The data are mean ± SD of at least five replicates; * = $p < 0.05$ (Mann-Whitney U test.

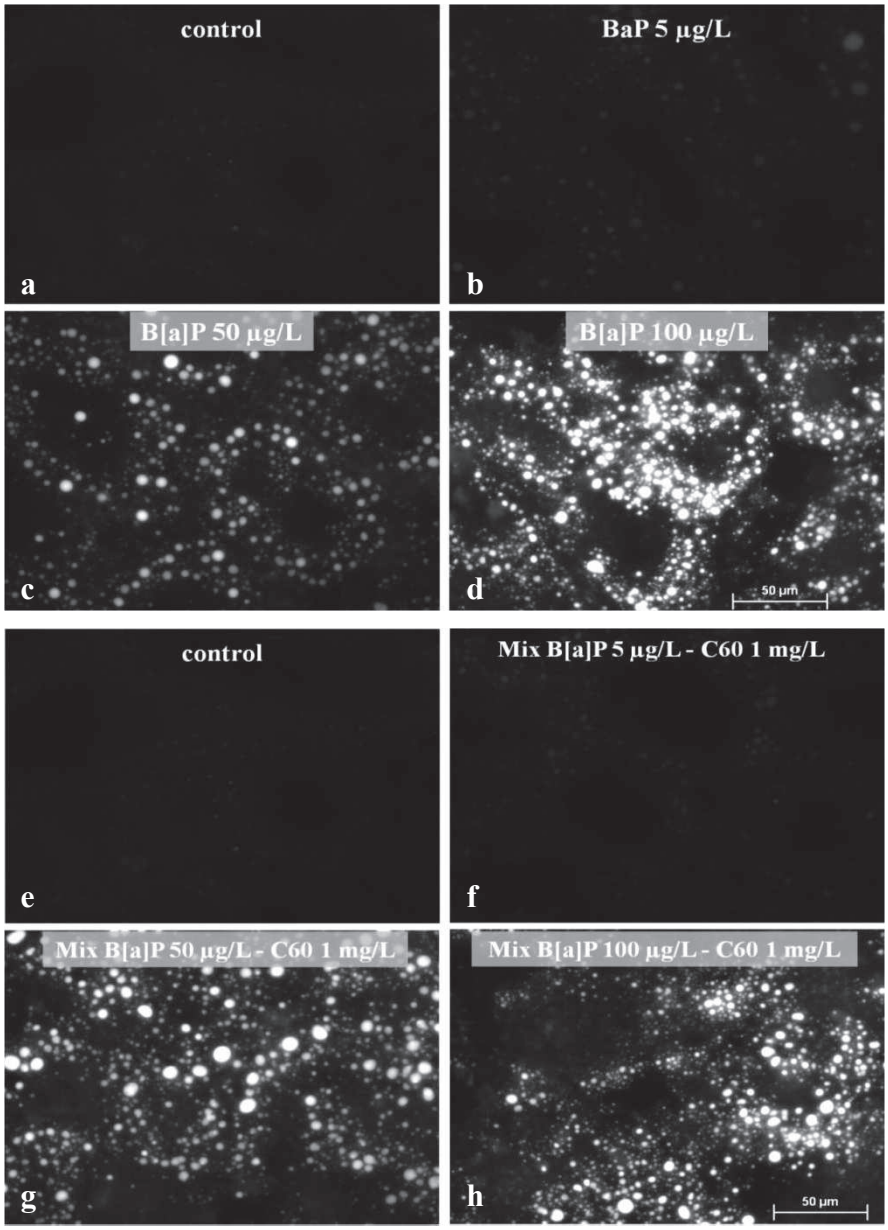

Figure 1.15. Cryostat unstained sections of digestive glands from mussels exposed to different experimental conditions (a,e: controls; b: 5 µg/L b[a]p; c: 50 µg/L b[a]p; d: 100 µg/L b[a]p; f: 5 µg/L b[*a*]p- C60 1 mg/L; g: 50 µg/L b[*a*]p- C60 1 mg/L; h: 100 µg/L b[*a*]p- C60 1mg/L) examined with UV excitation: white-blue fluorescent deposits, in form of droplets, were evident particularly at the higher b[a]p concentrations (c, d) confirming the observation of no noticeable trojan horse effect in b[a]p accumulation when in co-exposure with fullereneC60 (grayscale images).

B[a]p and the fullereneC60 accumulated in the tubules of the digestive glands of exposed mussels have caused remarkable alterations of the lysosomal vacuolar system (Fig. 1.16), as demonstrated by the decrease of LMS in all the exposure conditions, and particularly significant at 100 µg/L of b[a]p and even more at the same concentration in mixture with fullereneC60. The evaluation of the lysosomal/cytoplasmic volume ratio, biomarker of tissue damage, resulted in a significant increase in the volume of lysosomal vacuoles compared to controls (Fig. 1.16B).

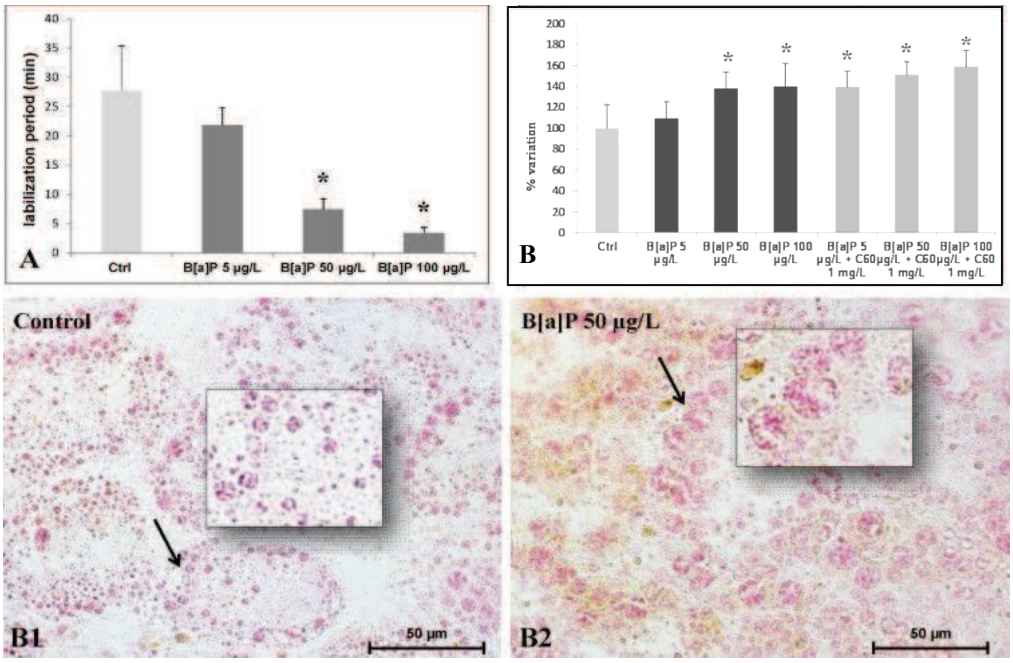

Figure 1.16. Lysosomal biomarker responses in digestive gland of mussels exposed to b[a]p (5, 50, 100 µg/L). A: Lysosomal membrane stability (cytochemical assay based on acid labilization characteristics of latent hydrolase β-N-acetylhexosaminidase); B: lysosomal/cytoplasmic volume ratio (lysosomes reacted for the lysosomal enzyme β-N-acetylhexosaminidase: when compared to controls (B1), in mussels exposed to b[a]pand Fullere C60 an enlargement of autolysosomes was observed (B2), see arrows and insets) Images obtained by Sforzini et al., 2018. Data represent the mean ± SD of at least five replicates. * indicates statistically significant differences ($p < 0.05$ Mann-Whitney U-test).

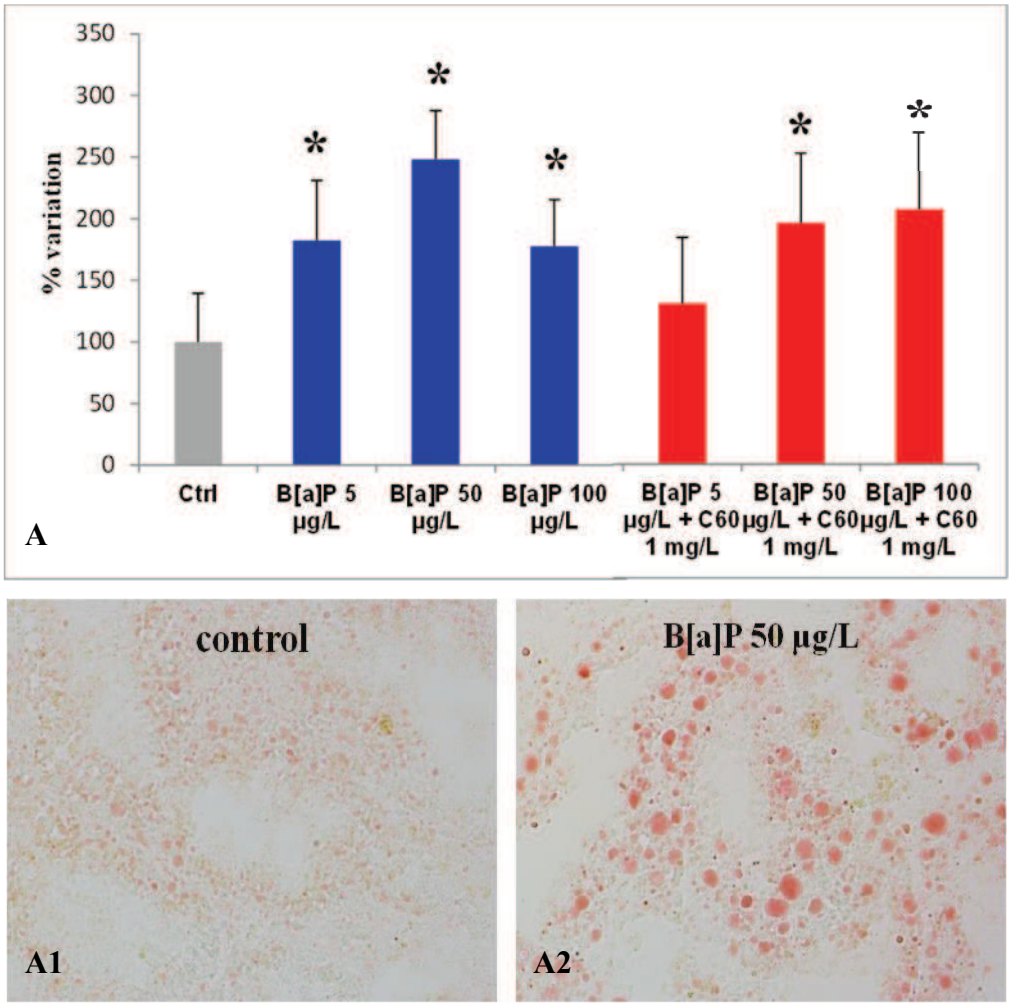

Figure 1.17. A: Effects of b[a]p (5, 50, 100 µg/L) alone and in co-exposure with fullerene C60 (1 mg/L) on neutral lipid lysosomal content in the epithelial cells of M. galloprovincialis digestive gland, evaluated in cryostat tissue sections by the Oil red-O staining. When compared to controls (A1), an increase in lipid droplet formation has been observed (A2: an illustrative image of the histological variation in the neutral lipids content relative to exposure to B [a] P 50ul / L). Data represent the mean ± SD of at least five replicates. * indicates statistically significant differences ($p < 0.05$ Mann-Whitney U-test).

These alterations are related to significant changes in cellular metabolism, identified in the general increase of the lysosomal neutral lipids content in the hepatopancreas digestive tubules of the mussels under investigation.

As shown in Fig. 1.17, exposure to b[a]p and co-exposure with fullereneC60, especially at higher concentrations (50-100 ug/L b[a]p), caused a clear deviation in respect to the control values.

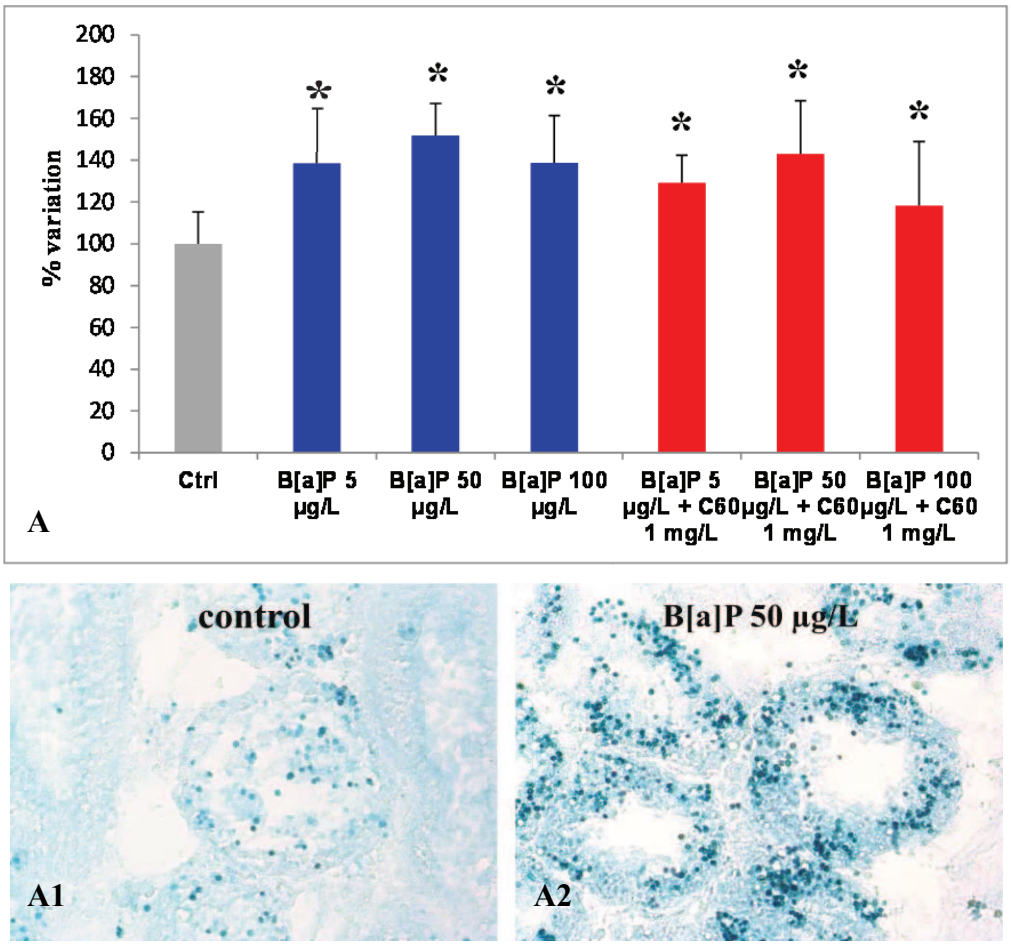

Figure 1.18. A: Effects of b[a]p (5, 50, 100 µg/L) alone and in co-exposure with fullereneC60 (1 mg/L) on lipofuscin lysosomal content in the epithelial cells of *M. galloprovincialis* digestive gland, evaluated in cryostat tissue sections by the Schmorl reaction. When compared to controls (A1), an increase in lipofuscin intravacuolar formation has been observed (A2: an illustrative image of the histological variation in the lipofuscins content relative to exposure to b[a]p 50ul / L). Data represent the mean ± SD of at least five replicates. * indicates statistically significant differences ($p < 0.05$ Mann-Whitney U-test).

Significant changes were observed also when considering a general stress-related biomarker such as the formation of intra-vacuolar lipofuscins. In this case, as shown in Figure 1.18, even the lowest concentrations of b[a]p have triggered the accumulation of material not further degradable by the lysosomal compartment; the co-exposure with fullerene C60 did not seem to have influenced the evolution of stress syndrome. As hypothesized, the phosphorylated anti-mTOR antibody on S2448 inside the analyzed tissue sections triggered immunopositivity, particularly localized in the perinuclear region of the digestive tubules epithelial cells (Fig. 1.19).

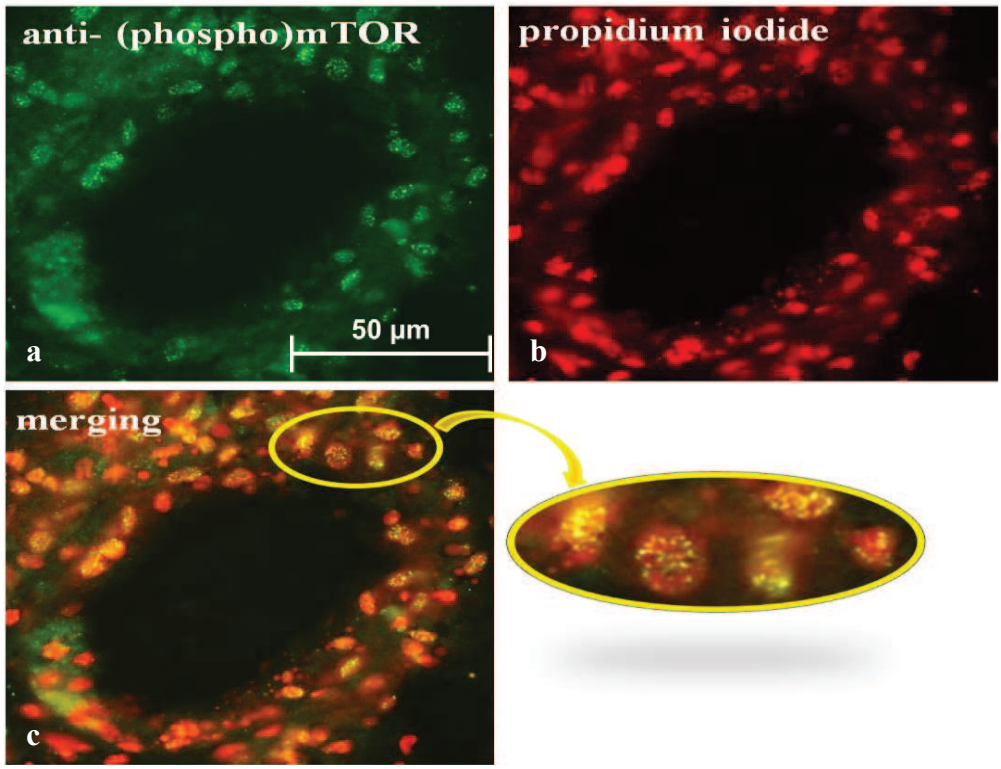

Figure 1.19. Anti-mTOR (phospho S2448) immunohistochemical staining a: green (Chromeo conjugated secondary antibody)) and the nuclear counterstain propidium iodide (b: red) were merged into a composite image (c), whereby the yellow colour highlights the localization of mTOR in perinuclear region of the tubule epithelial cells.

Conversely, exposure to different concentrations of b[a]p alone or in mixture with 1 mg/L fullereneC60 significantly reduced the fluorescence signal emitted by phosphorylated mTOR, with dose-dependent effects (Fig. 1.20).

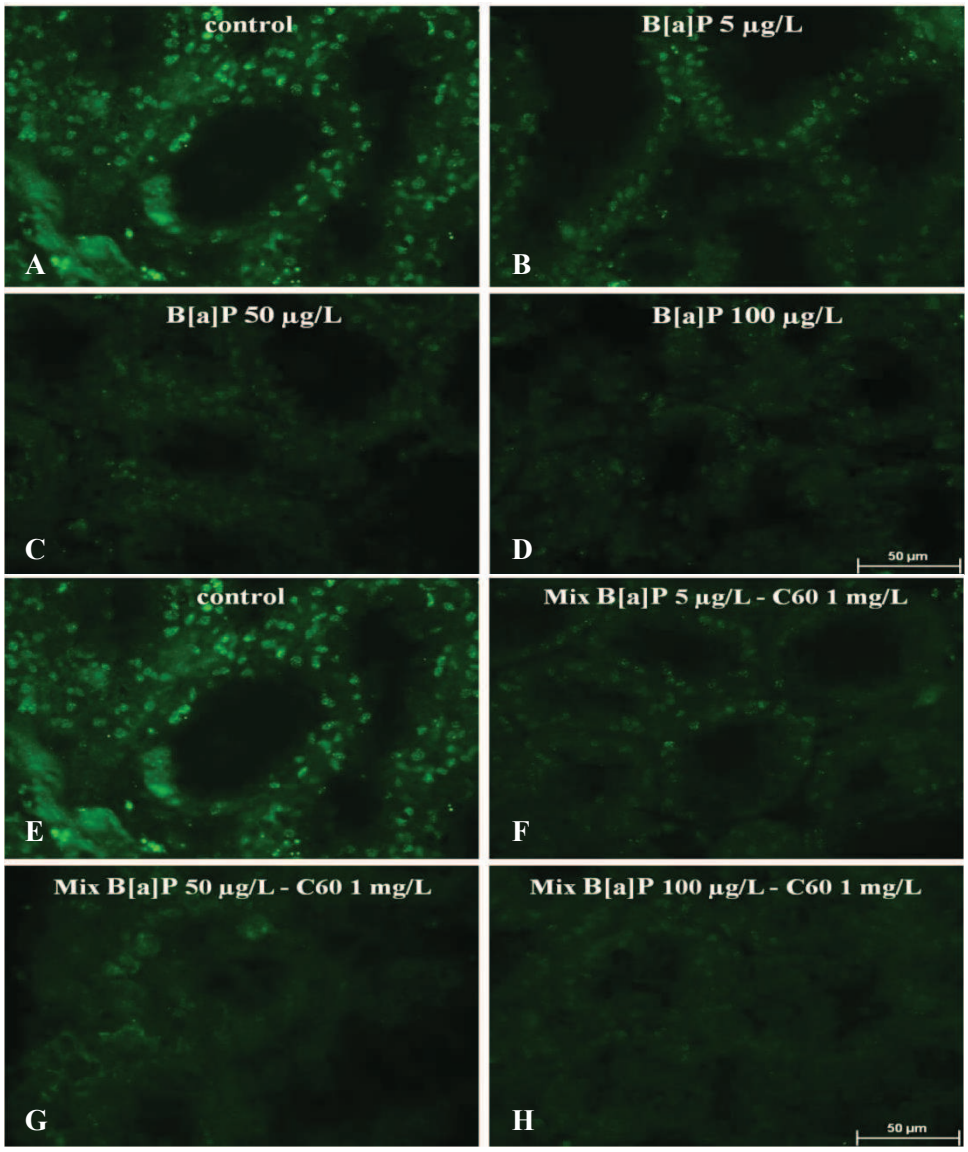

Figure 1.20. Anti-mTOR (phospho S2448) immunohistochemical staining (green: Chromeo conjugated secondary antibody) of digestive gland tissue sections from control mussels (A,E) exposed to different experimental conditions (B-D: 5 -50 -100 µg/L b[a]p; F-H: 5 -50 -100 µg/L b[a]p in mixture with fullerene C60 1mg/L).

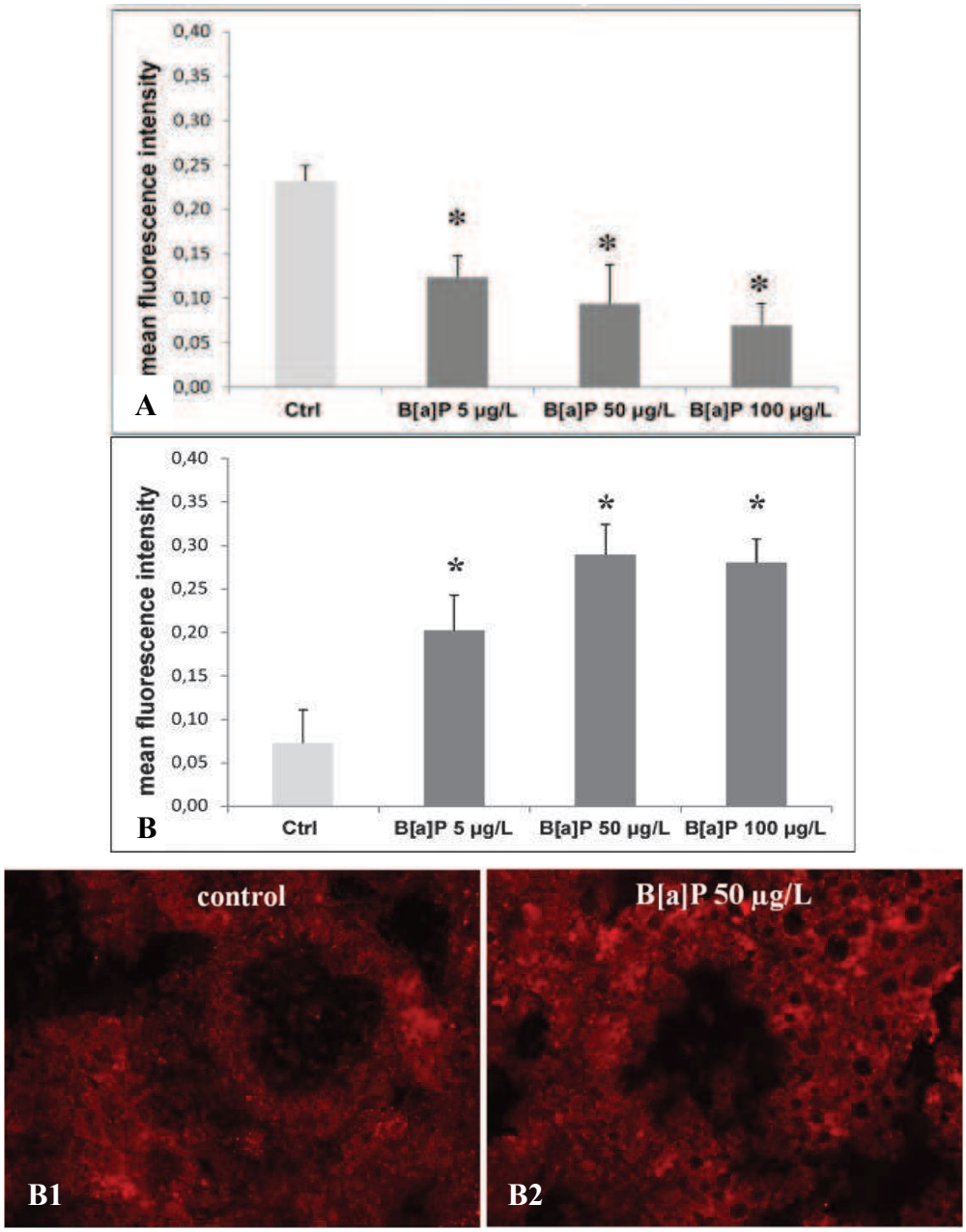

Figure 1.21. Quantitative fluorescence analysis of anti-mTOR (phospho S2448) and total anti-mTOR immunoreaction of digestive gland tissue sections from mussels exposed to b[*a*]p. Data represent the mean ± SD of at least five replicates. * indicates statistically significant differences ($p < 0.05$ Mann-Whitney *U*-test). Representative images of tissue sections of controls (B1) and 50 µg/L b[*a*]p exposed mussels (B2) (red: DyLight594 conjugated secondary antibody).

The quantitative analysis of the fluorescence generated respectively by the use of the anti-mTOR (phospho2448) and total anti-mTOR antibodies clearly demonstrated the inactivation (Fig. 1.21A) of the phosphorylatedkinase complex after exposure to b[a]p alone (in a dose-dependent decremental trend). If the presence of activated mTOR decreases, an increase in the total cytoplasmic presence of the kinase complex proteins was detected also in this case with higher values at higher concentrations (Fig. 1.21B)

1.7 DISCUSSION

The presence and continuous increase of human activities, industrial and technological innovation with the consequent new sources of pollution, have produced an almost exponential release of chemicals and wastes. There are several groups of anthropogenic contaminants present in the environment and they can interact with each other in different ways to induce a biological response. In the wide range of environmental contaminants, some are of great interest to the scientific community or due to their obvious ability to produce significant toxic effects, others because of the lack of knowledge of their ability to produce them. The first case concerns the most discussed and studied member of PAH class, i.e. b[a]p particularly because of its universally recognized cytotoxic, mutagenic, and carcinogenic properties for both terrestrial and aquatic organisms (Tung et al., 2014). Considering its persistence, a question arises about what can happen when this ubiquitous pollutant is in co-exposure with other emerging contaminants such as nano compounds. Thanks to their very small size (1-100 nm) and high reactivity, they easily penetrate the cellular compartments of organisms with paths that are not yet completely understood. Among all the carbon nanoparticles, fullerene-C60 is probably the best known (Aw et al., 2006). There are several sources of release, both natural and anthropogenic-made (Palmbery et al. 2009).

One of the objectives of this study was to verify the interactions that b[a]p has in co-exposure with fullerene C60, to investigate possible variations in its toxicity triggered by a possible Trojan Horse effect, with fullerene C60 as a vector, and b[a]p adsorbed on nano-aggregates that form spontaneously in aqueous solutions.

The novelty element introduced within this research was precisely that of analyzing for the first time the absorption of b[a]p (at three different exposure concentrations) in the digestive gland of *Mytilus galloprovincialis* in the presence of fullereneC60

nanoparticles. B[a]p was stored in digestive tubule epithelial cells of exposed mussels, as revealed by immunohistochemical analysis using an anti-PAHs antibody to visualise the cellular distribution of this chemical ondigestive gland tissue sections, according to the method already developed by Sforzini et al. (2014).

The double method for co-localization of b[a]p and cathepsin D (a major cellular highly conserved protease that has numerous functions within the lysosomal compartment) has provided a further confirmation of the lysosomal accumulation of these molecules.

Quantification of the b[a]p fluorescence signal by digital imaging showed a significant increase in fluorescence intensity in animals exposed to all the experimental conditions, with respect to controls. The preferential absorption of b[a]p within the the digestive gland tissues was visibly perceptible but at the same concentrations in co-exposure with 1 mg/L of fullereneC60 the accumulation data showed no detectable cumulative effect, nullifying the eventuality of a Trojan-Horse effect. Conversely, in some cases the co-exposure gave diminutive results with respect to the effect exercised by b[a]p alone.

It is interesting to observe, both in single exposure and in mixture, that the intensity of the anti-PAHs antibody fluorescent signal was more intense in the sections of digestive gland excised from animals exposed to the lower b[a]p concentration (5 µg/L). Considering that previous studies have shown the tendency of several PAHs (including b[a]p) to compartimentalize within high lipid content vesicles when present in tissues at high concentrations, and that these vesicles have dose dependent fluorescent properties, histological sections were excited using the UV fluorescence. The examination of the unstained serial sections inverted the previous trend highlighting the presence of numerous droplets that emitted fluorescence of increasing intensity when increasing the b[a]p dose, (with or without C60) with the maximum peak at 100 µg/L. The droplets corresponded to the clusters of neutral

lipids incorporated into the lysosomal vesicles within the cells of the digestive tubules.

In the analyzed sections a significant increase in the neutral lysosomal lipid content was observed, in response to exposure to all the different concentrations of contaminant; this was verified by comparison with other sections (observed both in bright-field and by UV-fluorescence modes) exposed to b[a]p and stained with Oil-Red O, the commonly used liposoluble dye for the histochemical identification of neutral lipids in cryostat tissue sections, as reported by Bayliss High (1984) and Moore (1988). The explanation of this phenomenon lies in the ability of b[a]p, like many other organic contaminants, to induce lipidosis (alterations in the metabolism of fatty acids) (De Coster and van Larebeke, 2012; Moore et al., 2007) to following which the excess of lipid material is autophagocytized in lysosomes (Moore, 1988; Podechard et al., 2009); the highly lipophilic b[a]p, follows the cellular path and storage inside the vesicles.

The chemical results support this hypothesis, showing that the amount of b[a]p accumulated in the digestive gland of exposed mussels increases with increasing dose; furthermore, the lipid concentration also increased with the increase in exposure concentrations (Banni et al., 2017). The reduction of immunofluorescence detection of the PAH molecules has already been clarified in a previous work by Sforzini et al. (2014), basing on the difficulty of the antibodies to access inside the lipid droplets, which results in a signal so low that it is not possible to label the target molecules.

Even in the presence of fullereneC60 the same results occurred, but to a lesser extent. This may be due to the fact that the aggregates of fullereneC60 on which the b[a]p presumably adheres will lower its bioavailability, due to a series of phenomena attributable to variations in physico-chemical factors triggered by co-exposure (Linard et al., 2017; Barranger et al., 2019). In one case or another, the final storage site remains the lysosomal compartment. Such abundant intra-lysosomal accumulations can disrupt the natural physiology of these organelles,

heavily affecting the stability of their membranes (Moore et al., 2006b; Sforzini et al., 2014; Viarengo et al., 1981) which preserve the cellular environment from the mixture of lytic enzymes contained within them.

Since lysosomes are one of the first targets in the toxic action of various pollutants, the results obtained showed significant perturbations, with a reduction in LMS starting from the lowest concentrations (5 µg/L b[a]p), and incremental up to 100 µg/L. The explanation of this destabilization is to be found in the dynamic interactions that b[a]p triggers within the lysosomal compartment.

In addition to the aforementioned lipid hyperproduction, a significant increase in lipofuscin production has been demonstrated (Banni et al., 2017), whose intra-lysosomal concentration had already reached the plateau at the lowest exposure conditions. The diminutive effect in co-exposure, with respect to the presence of b[a]p alone, can be explained by a peculiar characteristic of fullerenes, capable both of acting as generators of reactive oxygen species (ROS) but also as scavengers (Rondags et al., 2017). Thus, when C60 and b[a]p are closely associated or bound together within the lysosomal compartment of the mussel digestive cells their interactions can alter both scavenging activities and ROS generation (Della Torre et al., 2018; Di et al., 2016). Reactive free radicals are one of the most ascertained biogenesis sources for lipofuscins (Viarengo, 1989; Moore, 2008; Winston et al., 1996).

In the digestive tissues of mussels (Livingstone et al., 1988; Stegeman, 1985), as well as in those of higher vertebrates (Kim and Lee, 1997) ROSs can derive from redox cycles of secondary metabolites generated by the action of monooxygenases during phase-I of cellular metabolism of PAHs from the cytochrome P450 microsomal biotransformation complex.

In vertebrates, however, this enzymatic system converts b[a]p into an epoxy intermediate that easily forms adducts to DNA while in mussels the molecule is mainly metabolized in much less reactive quinonic microsomal derivatives, but which nevertheless enter the redox cycles proliferating in peroxisomes, where they

strengthen the generation of oxiradicalic species (Livingstone et al., 1990; Orbea et al., 2002).

The increase in the L/C volume ratio is another clear symptom of the catabolic conversion to which stress has subjected the test organisms. The activation of the autophagic pathway acts as a shield against oxidative stress through the digestion and subsequent rehash of oxidized proteins and damaged organelles (Cuervo, 2004). Nevertheless, it is also true that the prolongation of autophagic processes can prove to be deleterious (Levine and Kroemer, 2008), and the pathological reactions starting from lysosomes can lead to dysfunctions at the tissue level or in the whole organism.

Upstream of the observed effects it was possible to identify the signal transduction pathway of mTOR (mechanistic target of rapamycin), a highly conserved kinase complex which, if the mTORC1 functional complex is active, acts as a nodal biosensor promoting temporal regulation of cellular biosynthetic processes in response to exogenous and endogenous stimuli (Jung et al., 2010), inhibiting the autophagic pathway (Dowling et al., 2010; Soulard et al., 2009). Structural differences in the activation of the complex have been demonstrated which when associated with TORC1, instead that with mTORC2, is phosphorylated mainly in the catalytic site S2448 (Cheng et al., 2004; Copp et al., 2009).

Immunofluorescence labeling of the digestive glands sections used as a control in the investigation by phosphorylated anti-mTOR antibody on S2448 revealed immunopositivity particularly in the perinuclear region of the epithelial cells of the tubules, where the fluorescence signal was stronger, giving a clear demonstration of the b[a]p effect on the phosphorylated mTOR levels within the tissues under analysis.

When the cells exposed to increasing concentrations of b[a]p reacted with the anti-mTOR antibody (phosphos S2448), the fluorescence signal is reduced in a dose-dependent manner and at higher concentrations which caused a prolonged increase of the cellular catabolic rate, substantial changes were observed. Although the

mechanisms that regulate the dephosphorylation of mTOR have not yet been completely understood, the oxidative stress caused by the presence of b[a]p, alone and in co-exposure with fullereneC60, can have a positive feedback that inhibits mTORC1, promoting the triggering of autophagic processes (Chen et al., 2010). The consequences are borne by the lysosomal system (Boya, 2012), both as a target and accumulation site of toxic chemicals. However, whether it is present in active or dephosphorylated form it is important to note that the total production of mTOR present in the tissues has been intensified by exposure to b[a]p, confirming the key role of this enzymatic complex in regulating cellular metabolism.

Evenin this case the presence of fullereneC60 did not involve substantial variations. It is true that in this experimental design the co-exposure was arranged using a single concentration of fullerene which obviously represents only a narrow window of its toxicological action. It is possible that interactions with b[a]p will change by selecting different other dose ranges (Groten et al., 2001) or by changing the duration of exposure (Gomes et al., 2011).

In conclusion, the target role of the cellular lysomal compartment within the mussels' digestive gland, as site of accumulation of toxic species such as b[a]p (preferably stored within lipid droplets) was confirmed. In addition to lipidosis, high doses of b[a]p stimulated the triggering of the entire autophagic pathway, starting from signal transduction of the mTOR kinase complex. It was found that the dephosphorylation of the mTORC1 fraction over-stimulates the induction of catabolic pathways that can determine a set of pathological effects in tissues, making organisms no longer able to sustain a correct scope for growth.

In this context, the use of fullereneC60 in co-exposure with the three different concentrations of b[a]p did not confirm the original hypothesis on the generation of potential synergistic effects, but future studies will be able to verify if and to what extent this chemical species can interact with b[a]p by altering the mutual toxic potential.

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CHAPTER 2. Ecotoxicological effects of particulate materials produced by differents types of brake systems on aquatic and edaphic organisms

2.1 Atmospheric particulate from vehicular traffic

Emissions from vehicles are an important source of environmental contamination. The urban environment is an extremely heterogeneous tank of particulate matter (PM), suspended in the air or deposited on the ground, which, despite the research developments, is still difficult to characterize, given the highly variable chemical composition bound to the dimensional characterization of the particles. Although globally the amount of particle mass is mainly attributable to natural production, anthropogenic sources have the characteristic of conveying a greater number of particles containing substances capable of undermining human health (causing respiratory problems and tumours) and the environment (acid rain and toxic effects on living organisms such as plants and animals).

Figure 2.1 Atmospheric release of particulete emitted by urban traffic

The PM emitted by vehicles can be directly derived from exhaust emissions (fuel combustion), contributing to the fine fraction of the atmospheric particulate (Kam et al., 2012), or due to the non-exhauste emission of mechanical wear of tyres and brakes (with dimensions included in the nano and micro scale) (Wahid 2018; Perricone 2019). The latter type has only recently attracted the attention of the scientific community following the publication of numerous studies (Ketzel et al., 2007; Harrison et al., 2011, 2012b; Denier van der Gon et al., 2013) which highlighted its impact on air quality and on human/environmental health.

Particulates from vehicle wear and resuspended road dust can act as carriers for toxic and carcinogenic compounds such as PAHs, nanomaterials and heavy metals (Amato et al., 2011). Although the problem is significant, there are still no standardized guidelines for the assessment of the risk related to emissions and the accumulation of road particulate and its subsequent containment, and without precise regulation the accumulation rate increases undisturbed.

Already in 2009 (Rexeis and Hausberg) it had been hypothesized that in just over a decade 90% of road traffic emissions would have been attributable to sources other than direct fuel combustion. This impasse condition is a direct consequence of two factors: on the one hand, the disinterest a long manifested towards the problem, expressed in a lack of scientific knowledge available on this matrix, on the other the complexity of its chemical composition that does not allows to identify and classify distinctly the constituent species. Brake wear (considered by EEA and EPA as a substantial source of pollutants emissions in the air), leads to the production of PM generally smaller than 10μ and often composed of nanoparticles aggregates (Grigoratos& Martini, 2014) that facilitate the concentration of pollutants, heavy metals and carbon nanostructures (Wahlin et al., 2006; Varrica et al., 2012), with effects on health and environment still largely unknown.

2.2 LOWBRASIS H2020 Project

The challenge is therefore to develop innovative and eco-friendly transport technologies, able to reduce the incidence of vehicular traffic on total particulate matter and, in perspective, to comply with future and more stringent legislations on vehicle emissions and on air quality. In this scenario it is inserted the LOWBRASYS (LOW environmental impact BRAkeSYStem) project, an articulated research that has obtained funding from the European Commission' Horizon2020 program. This project combinethe most authoritative realities of the automotive sector (Brembo, Ford, Continental Teves, Federal Mogul, Flame Spray) with prestigious Research Institutes and Universities (Mario Negri Institute, Technical University of Ostrava, KTH Royal Institute of Technology, University of Trento-Department of Industrial Engineering and Joint Research Center of the European Commission). The goal of LOWBRASYS is to study tools, materials and driving procedures capable of producing a considerable reduction (at least 50%) of particulate emissions from the braking system, to make it more efficient from an environmental point of view. The main technologies developed focused on testing newly formulated pad+disc (FM4-BD2 and FMB-BD7) in bench test comparing to the standard already on the market (FM1-BD1), to highlight the differences in the composition of the resulting powders and assess their toxicity, given that the newformulations were designed to reduce total emissions and limit the negative effects due to the dispersion of nano and microparticles (with a collection system placed near the braking system itself).

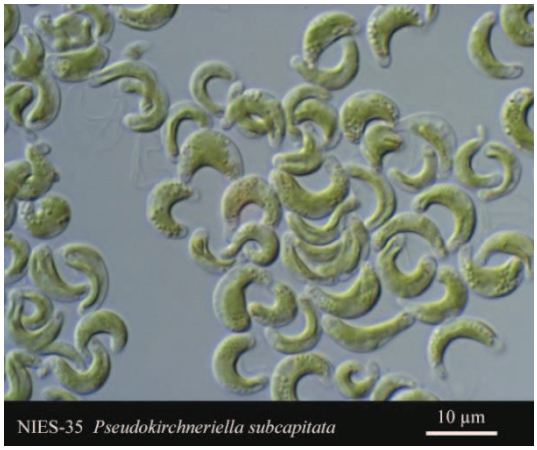
Figure 2.2 Representative pads (FMx) and disc (BDx) formulations from automotive brake system, within LOWBRASYS project

2.3 Evaluation of ecotoxicological risk on aquatic and soil organisms

To study the effect of the emission of toxic substances into the environment, some species are chosen as indicators able to summarize the general characteristics of the environmental phenomenon. In ecotoxicological essays, living organisms in optimal conditions are placed in contact with the matrix to be tested. The battery of ecotoxicological tests must be selected based on ecological representativeness and in relation to the trophic chain, i.e. it must at least include models belonging to the grazing and detritus chain, at different levels. In this context, the unicellular algae *Pseudokirchneriella subcapitata,* the crustacean *Daphnia magna,*and the bacteria *V. fisheri* were selected as bioindicators of the aquatic environment, while for the edaphic ecosystem we used the seeds of *Lepidium sativum* and *Sorghum saccharatum*, the nematode *Caenorhabditis elegans*, the earthworm *Eisenia andrei* and the social ameba *Dictyostelium discoideum.*

2.4 Organisms employed

2.4.1. *Pseudokirchneriella subcapitata*



Algae, at the base of food chains in aquatic ecosystems, have been widely used as model organisms in toxicity tests because experimental activities are relatively simple, rapid and cheap (Wong and Coulture, 1986). Present both as unicellular and multicellular organisms are primary producers involved in the self-cleaning activity of all aquatic ecosystems. Through photosynthesis, they produce the indispensable oxygen for the life of animal species and represent the main source of energy for consumer organisms. As always happens when the level of environmental equilibrium is exceeded, their excessive presence (favored by the release of high concentrations of nutrients based on nitrogen and phosphorusin the aquatic compartment) can cause oversize blooms (eutrophication) that interfere with the quantity of oxygen dissolved, with the consequent generation of toxic species that make aquatic environments inhospitable to the biota.

In the field of biomonitoring, the ecotoxicological tests involving the use of unicellular green algae are valid tools for detecting alterations of the phytoplankton communities due to toxic substances potentially capable of destabilizing the structure and functioning of the entire ecosystem (Passarelli and Sbalchiero, 2005).

The most commonly used freshwater microalgae for performing the inibition biotests is the *Pseudokirchneriella subcapitata* (Chlorophycea), with its characteristic sickle shape, ubiquitous in fresh water and easily grown even out of the natural environment. In laboratory coltures, its life cycle can be monitored keeping under control temperature, lighting and nutrient availability; under these conditions the population growth, from the time of inoculation to that of decline, is described by an exponential curve as a function of time from which it is possible to derive the growth rate (UNI EN ISO 8692: 2005) and the main phases of the cell cycle that identify:

- Latency, that is, acclimatization of the cells to the growth medium and the breeding conditions, after the inoculation and for the following 48 hours;
- Exponential growth, with a cellular increase greater than mortality (48-96 hours);
- Plateau, during which cell growth and mortality are equivalent (96-168 hours);
- Decline, with an increase in mortality no longer offset by cell reproduction (over 168 hours).

Its growth is quiterapid (the chronic toxicity test involves reading after 72 hours of incubation) and, since it has an appreciable sensitivity to many types of contaminants, it is a particularly useful species in eco-toxicological field.

2.4.2. *Daphnia magna*

Daphnia magna is a small freshwater crustacean (adults do not exceed 5 mm in length), belonging to the Brachiopoda class and to Cladocera order, with a good level of sensitivity to contaminants, used for biomonitoring since 1934 (Naumann) to assess toxicity of matrices with minimum or absent salinity.

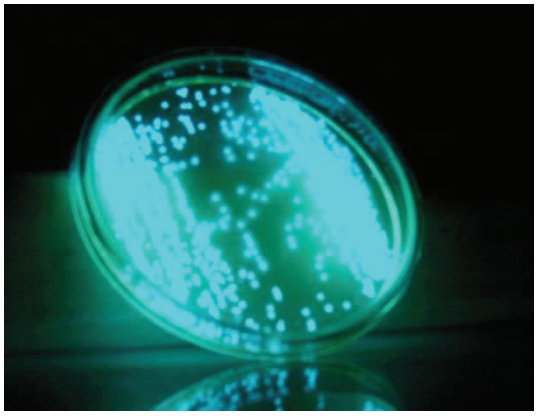
The organism present a latero-compressed oval morphology and the transparent dorsally welded carapace encloses the entire organism excluding the cephalic region, in which a single compound eye, a small ocellus and two pairs of antennas are distinguished, the latter of which are much more developed with a natatorial function. The heart, middle intestine and ovaries are located in the dorsal post-cephalic region. The thorax presents a series of five bristly filtering appendages for the capture of food particles, subsequently conveyed to the buccal apparatus, processed and disposed of from the intestine. The abdominal region does not have appendages and ends in a forked structure. The daphnia's exoskeleton is replaced, starting from the first 7-10 days, after each moult (coinciding, in mature females, with the emission of small daphnids) allowing the stadial growth of the organism.

Organisms live, on average, from two to four months at 20°C, and the reproduction strategies depend on the environmental conditions.

One of the salient features, which justify the widespread use of these organisms for *in vivo* ecotoxicological tests, is that in the absence of stressful conditions a population is made up of individuals exclusively belonging to the female gender who reproduce by parthenogenesis giving rise to genetically identical copies, which they follow the same cycle. If the conditions in the surrounding environment become unfavorable (e.g. in the event of overpopulation or lack of food), male individuals develop spontaneously (smaller and with appreciable sexual dysmorphism). Since the eggs from which males and females develop have an identical series of chromosomes, two different genetic pathways are activated by mechanisms of sexual differentiation based on changes in chromatin structure induced by specific environmental signals (Ruvinsky et al., 1986). Males are capable of sexually fertilizing pairs of non-parthenogenetic eggs that are encapsulated, inside the incubation chamber, inside a thick resistance structure called ephippio, emitted following the moult. The ephippia are able to be preserved until optimal conditions are restored, and parthenogenetic female organisms will be generated from their hatching (EN ISO 6341:2013).

The small size, combined with the short life cycle and ease of reproduction in the laboratory, offer considerable operational advantages. The acute toxicity test with *D. magna*, foreseen by the legislative decree 152/99 and subsequent modification of the legislative decree 258/2000, is particularly sensitive to heavy metal pollution (Khangarot and Ray, 1989) and allows to calculate the concentration of substances (or matrices) that in a time-window between 24 and 48 hours cause effects on the immobilization of the organisms employed. The results can be expressed as a percentage of dead/immobilized individuals or as a value of EC50 (the toxic substance concentration that determines 50% mortality/immobilization).

2.4.3. *Vibrio fischeri* (Microtox® test)



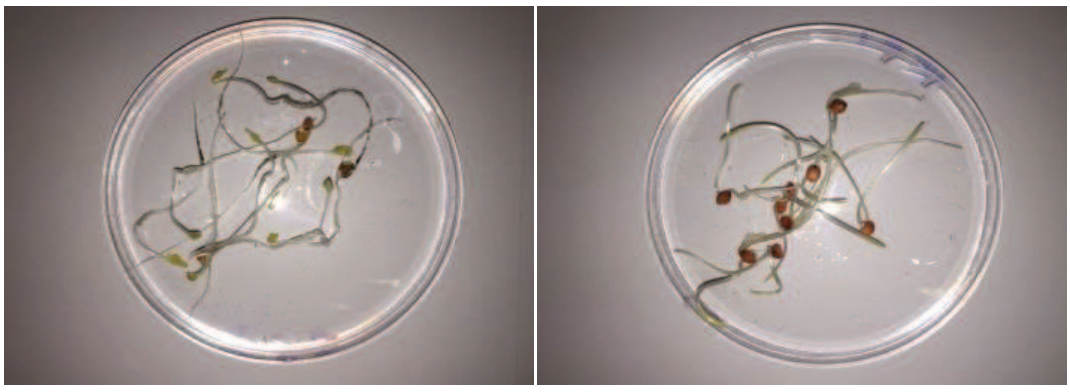
Vibrio fischeri (Beijerinck, 1889) is a rod-like luminescent gamma-proteobacterium of the Vibrionaceae family, common in marine environments throughout the world, but with greater diffusion in temperate and subtropical areas. When it is not associated in symbiosis by staying inside photophores on the surface of the host organism (i.e. *Euprymna scopoles*), it survives in free form by decomposing organic matter (Nealson and Hastings, 1979).

Its bioluminescence involves the oxidation of an organic substrate (luciferin) mediated by enzymatic catalysis (enzyme luciferase) to generate light emission in the visible spectrum. The genes involved in the induction of the whole process are grouped in the Operon Lux and regulated by a quorum sensing system. In response to environmental stimuli, the luxR gene encodes the transcription factor LuxR that, in turn, regulates the operon expression. The homoserine lactone (HSL) signal molecule, after reaching a threshold value diffuses into the cytoplasm communicating to the cells the induction of quorum sensing and binding to the transcription factor LuxR. Its activation induces the expression of the luxA and luxB genes that encode respectively for the synthesis of the alpha and beta chains of the luciferase enzyme; this couples to the luciferin substrate (consisting of a long

chain aldehyde and a reduced riboflavin phosphate), oxidizing it and converting the chemical energy into light emission (Ruby et al., 2005; Myashiro and Ruby, 2012). The acute assay (ISO 11348) with which the inhibition of bioluminescence is evaluated, since the 1970s is considered among the most effective and reliable bio-tests (Mariani et al., 2006) and a valid alternative to tests with fish and invertebrates, as a screening tool for the quality control of liquid (fresh water, sea water or brackish water) or solids samples (extracts and eluates of sediments and muds). With the Microtox® method the light emission is measured in defined times using a luminometer.

The presence of pollutants produces a reduction in bioluminescence proportional to the toxicity of the sample under examination. This makes it possible to assess the degree of toxicity of the substance or matrix tested in terms of EC50, which represents the concentration for which there is a 50% decrease in the luminescence initially emitted by the bacteria (APAT, IRSA-CNR, 2003).

2.4.4. *Lepidium sativum* and *Sorghum saccharatum*



The germination and root lengthening tests of the terrestrial plants seeds have been used since the 1950s (Hunter, 1952) in the classic batteries of eco-toxicological assays.

Phytotests allow monitoring of water and soil health, with established experimental evidence and easily reproducible methodologies (Ratsch, 1983).

It is known that the initial stages of development are often the most sensitive to environmental alterations (Wang, 1990), precisely because they are subject to more rapid changes that make them more susceptible to environmental stresses.

The end-points evaluated in the ecotoxicological tests on plants are germination, seed growth rate and subsequent growth modes.

Since the roots represent the first organ of the plant to come into contact with soil pollutants, the development of the root apparatus has often been used to assess the plants tolerance to the cumulative and/or synergistic effects of various pollutants, mainly metals (Salt et al., 1995), by measuring the growth inhibition of germinated seeds under controlled conditions.

The seeds, protected by integuments that enclose the endosperm and the embryo (radicle, hypocotyl and cotyledons), contain the cellular reserve products (lipids, proteins and carbohydrates). During development, the seed generally dehydrates and the embryo enters a state of quiescence until it absorbs water from the environment, starting the cascade of metabolic reactions that culminate in germination.

Two of the most used terrestrial plant species for evaluate the phytotoxicity are *Lepidium sativum* (watercress) and *Sorghum saccharatum* (sorghum), as they are commonly available in commerce and offer a remarkable sensitivity to pollutants and a high reproducibility in response (Wong and Bradshaw, 1982; An, 2004; Montvydienė et al., 2004).

Lepidium sativum is a dicotyledonous herbaceous plant, belonging to the Brassicaceae family, with a wide range of distribution (Nappi, 1986). It is easy to cultivate and also grows spontaneously, but suffers the direct radiation.

Sorghum saccharatum is a monocotyledonous species belonging to the Poaceae family, mainly cultivated for the production of grain and forage (Newman et al.,

2010). It adapts to different types of soil, with wide pH and salinity ranges but is affected by water stagnation (Buccafusca et al., 2013).

2.4.5. Eisenia andrei

Soil fauna is an important component of edaphic ecosystems, as it is involved in the decomposition processes of organic matter, contributing to the regulation of microbial activity and the nutrient cycle. The polluting substances present in the soil significantly affect earthworms, the most abundant invertebrates in the soils of temperate regions (Edwards, 2004). Earthworms are bilateral organisms with elongated cylindrical morphology, divided into annular bristled segments, homonomes with the exception of the cephalic prostomium with a sensory lobe, and the terminal pygidium at which the anus opens.

One of the segments, distinctive of sexually mature organisms, differenziates in a swollen glandular structure (clitellum) positioned behind the genital pores with the function of secreting cocoons (Dominguez and Edwards, 2011) during mating. Since earthworms are insufficient hermaphrodites, two sexually synchronized organisms practice cross-fertilization, intertwining together so that the cocoons slip first on the female gonopores, where the eggs are cocooned and then inseminated after passing over the seminal receptacles.

According to Reynolds and Wetzel (2004) there are more than 8300 species of Oligochaetes, and among these *Eisenia andrei* (Bouché, 1972) is one the most widespread and used for many years to monitor the toxicity levels of contaminated soils (Sforzini et al., 2015).

It is an epigeal species that grows optimally in presence of abundant organic matter at temperatures between 18 and 25 °C, and with humidity values close to 85%. The life cycle and biology of *E. andrei* species have been the subject of extensive study for many years (Watanabe and Tsukamoto 1976; Hartenstein et al., 1979; Edwards 1988, Reinecke and Viljoen 1990; Domínguez and Edwards 1997; Monroy et al., 2006; Dominguez and Edwards, 2011). As key and cosmopolitan species of the pedofauna (Edwards and Bohlen, 1996), they participate in the formation of the soil profile from a physical, chemical and microbiological point of view.

Earthworms, constantly in contact with the particles that make up the soil, are significantly influenced by the pollutants found there and are therefore models indicated for biomonitoring. Because of this sensitivity, they have been described as the bioaccumulation site of various organic pollutants (Jager et al., 2005), heavy metals (Lourenco et al., 2011) and new-generation compounds such as nanoparticles (Li et al., 2010; Laycock et al., 2015).

2.4.6. *Caenorhabditis elegans*

Caenorhabditis elegans is a non-parasitic soil nematode, affirmed as a model organism mainly in the field of genetic investigations since in 1998 the sequencing of its genome was completed for the first time for a multicellular organism.

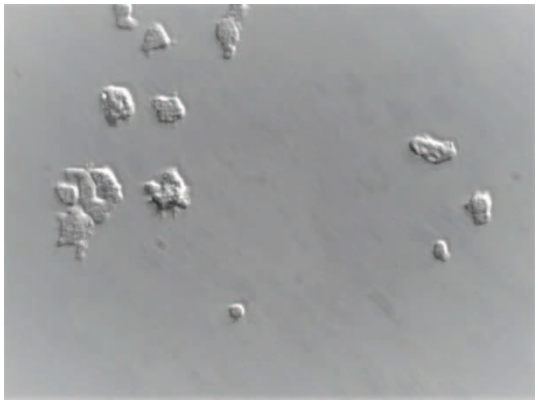
Nematodes are the most abundant and species-rich metazoan organisms, reaching high densities in soils and shallow aquatic sediments (Traunspurger, 1996; Sochová et al., 2006) that decrease with increasing depth.

Its fully mapped genome and nervous system make this organism particularly suitable for cell biology and neurobiology investigations (Hodgkin, 2001; Lin and Rankin, 2010) but it is often used as a bioindicator of the pedofauna' health status during ecotoxicological investigations.

This species has the advantage of being practical to cultivate in the laboratory and, at temperatures between 15 and 25°C, it grows in even small spaces and rapidly (the organisms double in about a day and a half) in liquid cultures or agarized plates supplemented with *E. coli.* It reproduces through self-fertilization or even sexually. If necessary, organisms can be frozen and stored for quite a long time without incurring genetic drift (Ledoux, 2015).

For their relevance in the edaphic environment, the ecological and ecotoxicological role of nematodes can supplement the classic soil toxicity tests, because their sensitivity is comparable to that of other model organisms such as earthworms, plants and microorganisms (Kammenga et al., 1994; Kammenga et al., 1996; Peredney and Williams 2000a; Peredney and Williams, 2000b; Boyd et al., 2001).

2.4.7. *Dictyostelium discoideum*



The increase in the variety of bioindicators is a fundamental requirement for the evolution and augmentedsensitivity of eco-toxicological investigations. One of the organisms that has contributed to the development of ecotoxicological research in recent years is *Dictyostelium discoideum*, a soil ameba easily found in nature, isolated and described by Raper in 1935. Since then, it has become a consolidated model in cellular biology, and only in a more recent past has begun to be used in the laboratory for the evaluation of the toxicity of potentially contaminated environmental matrices (Sforzini et al., 2008). It is a eukaryotic haploid organism that normally lives in unicellular state but chemical stimuli may induce it to form a cooperative multicellular macroscopic structure. In presence of nutrients, it divides and moves similarly to leukocytes. This similarity is not limited to morphological and biochemical characteristics, as it has been shown that, among organisms whose genomes have been completely sequenced (Eichinger et al., 2005), *Dictyostelium* is more similar to vertebrates, with which it shares many metabolic pathways, which at the yeasts (King, 2012). This, together with a unique combination of biochemical, genetic and molecular tools (which have been lost by other eukaryotic organisms, such as fungi) make it a versatile model for the study of chemotaxis, cell differentiation, signal transduction, phagocytosis and autophagy (Dominguez-Martin et al., 2017).

The simple cellular structure, the ease in cultivation and life cycle reproduction (it is possible to obtain 2-3 generations in 24 hours) make *D. discoideum* suitable for laboratory studies as a relatively simple experimental system in detecting sensitive and early sub-lethal responses, using the same stress biomarkers found in eukaryotic cells (Moore, 2002; Moore et al. al., 2004) to detect the effects of environmental pollutants on cell physiology (Sforzini et al., 2008). In fact, as organisms they can interact directly with the surrounding environment but as isolated cells that expose receptors directly outside, are more sensitive to environmental variations than cells of a higher organism, differentiated and organized into complex structures that give responses mediated by their different function (Amaroli et al., 2005). For this reason, it was decided to subject *D. discoideum* to essays for evaluation of very sensitive sublethal biomarkers such as the lysosomal membrane stability as well as the micronuclei detection. Under these experimental conditions, the amebic cells showed a remarkable sensitivity to the treatments, suggesting a greater susceptibility to the effects of stress factors such as the presence of environmental chemical contaminants.

2.5 Genotoxicity and Mutagenicity biomarkers

2.5.1. Chromosomal damage: Micronuclei Test

The micronuclei (MNi) test is by now a routine cytogenetic technique for the evaluation of genotoxic effects caused by environmental stress factors that involve the chromosomal DNA. Micronuclei were first observed more than a century ago in cytoplasm of mammalian blood cells as round structures recognizable as fragments of nuclear material that do not succeed to be incorporated into the daughter nuclei during cell division. Evans (1959) was the first researcher to

discover that chromatic and chromosomic breaking, as well as symmetrical and asymmetric exchanges, could give rise to acentric fragments then expelled from the nuclei of the cells in formation, appearing in the cytoplasm during the subsequent interphase where they remain throughout the life of the cell. The presence of micronuclei is an indicator of chromatin breakage, which may be provoked by clastogens or spindle dysfunctions, ultimately caused by toxic compounds (Heddle et al., 1983; Carrano and Natarajan, 1988).

By correlating the average life expectancy of each type of cell with their mitotic partition rate in a given tissue, it is possible to obtain the micronucleic occurrence frequency, considered a good indicator of genotoxic damage accumulated throughout cell life.

The micronuclei test has been extensively applied to a variety of cell types and species belonging to the aquatic environment: fish, amphibians, bivalve molluscs and crustaceans (Majone et al., 1990; Scarpato et al., 1990; Bolognesi et al. 1999, 2004; Siu et al., 2004). Treatments with genotoxic agents (such as b[a]p) have also highlighted in these species chromosomal damage mechanisms common to those of higher organisms. Because of its versatility, and the ease and speed of execution, the micronuclei test is the most widely used, and been proposed as the main low-cost biomarker of contamination by genotoxic agents (Bolognesi et al., 1996).

2.5.2. Mutagenicity: Ames test

Evaluation of the ecosystems quality using mutagenesis assays is a biomonitoring technique widely employed in the predictive determination of sub-cellular effects induced by environmental contaminants, since it allows an early identification of stress on the genetic heritage. The accumulation of mutations is closely related to the induction of carcinogenic events, therefore the increase of mutagens in the environment is indicative of a significant genotoxic risk (APAT, 2002). The strains of the Gram-negative bacterium *Salmonella typhimurium*, suitably mutated in the

gene coding for the biosynthesis of histidine and then auxotroph for this amino acid, they are used in the Ames test (Ames et al., 1973, 1975) the most widely used in the world for genotoxicological screening. The positivity of the test is based on the evaluation of the capability of a contaminant, if mutagenic, to cause reversion in the compromised gene, making the strain capable of resynthesizing histidine again, according to the principle of retromutation (Maron and Ames, 1983).

The Ames test has been advocated by international organizations as a reliable test for discriminate genotoxic events in environmental studies (Canada, 1986; McGeorge et al., 1983).

2.6 MATERIALS AND METHODS

2.6.1. BW-PM collection and storage

Brake-wear particulate matter (BW-PM) for commercial and innovative brake disc and pads samples were collected during specific inertia brake dynamometric tests. A full-scale dynamometer adapted to measure the particle emission was used to generate and collect wear dust (Perricone et al., 2016). The test procedure is a sub-section of the industry-used Los Angeles City Traffic (LACT) test cycle, called 3h-LACT, comprising 217 stops, simulating actual driving conditions (Mathissen et al., 2019). Three different pairs of brake pads were used during the experiments with a cast iron brake disc: one reference disc and pads already on the market (FM1-BD1) and two innovative ones (FM4-BD2 and FMB-BD7). A pooled sample was generated with all three mixing PM_{10} particles and coarser fractions collected during the bench tests. X-ray fluorescence spectroscopy (XRFS) was used to analyse the chemical composition of the brake linings of the pads.

2.6.2. Water accommodated fraction (WAF)

The water accommodated fraction (WAF) is an aqueous medium containing only the fraction of the substance that is dissolved or present as a stable dispersion or emulsion (OECD, 2000). The powder suspensions were sonicated for 1h then left under shaking for 24h at room temperature (about 20°C). The solutions were centrifuged at 500 rpm for 5 minutes then filtered on a 0.2 µm filter (OECD, 2002).

2.6.3. BW-PMphysical/chemical characteristics and size distribution

A scanning electron microscopy-(SEM) (FEI ESEM Quanta 200, with attached EDAX electronic microwave) was used to analyse the morphological characteristics of wear particulate with Genesis interface software. The observation was done in low vacuum mode, with a variable pressure in a chamber equal to 90 Pa, without any further manipulation. The analyses were carried out at 20kV acceleration voltage, 15 nA beam current and a working distance of about 10 mm. The energy dispersive X-ray spectrometry (EDS) to determine the chemical composition were carried out considering as fixed parameters 80 L sec and a Death Time between 15 and 35%.

The particle size and the presence of agglomerates/aggregates were analyzed by Dynamic Light Scattering (DLS). Measurements were done with a ZetasizerNanoZS (Malvern Instruments) operating in a particle size range from 0.6 nm to 6 μm, equipped with a He-Ne laser with λ633 nm. Before analysis, samples were dispersed in ultrapure water and the dispersions were stirred at room temperature for 10 min, and then sonicated for another 10 minutes.

2.6.4. Ecotoxicological analyses

2.6.4.1. *Growth inhibition of P. subcapitata*

The bioassay followed the standard ISO, 8692:2012 (Water quality-freshwater algal growth inhibition test with unicellular green algae) using the unicellular Chlorophycea *Pseudokirchneriella subcapitata*, ubiquitous in fresh water and easily grown in the laboratory. Algal cultures in exponential growth phase (1×10^6 cells/mL) were used. Growth inhibition was measured after 72h exposure to samples.

In a parallel set of experiments, a test was done to verify the algal growth inhibition by exposing the organisms directly to the brake particles, using as control two types of quartz sand of different granulometry in a size range comparable to that of the PM resulting from the wear of braking systems (BCR-066: quartz 0.35-5 µm; silica glass spheres 9-13 µm). WAF, BW-PM and quartz sand suspensions were prepared, using the specific nutrient stock solution (in MilliQ water) as laboratory control.

Algal solution (20 µL) was inoculated in sterile 24-well plates with 2 mL of each treatment (from 0.1 to 1 mg/L) for 3 days at 25±1°C in a thermostatic chamber. Algal density was measured at the beginning and end of treatment with a hemocytometer chamber (Bürker).

2.6.4.2. Acute test of immobilization on D. magna

The immobilization assay on *Daphnia magna* was used to evaluate the acute aquatic toxicity of the BW-PM. The crustacean bioassay was done following the standard ISO, 6341:2012 (Water quality – determination of the inhibition of the mobility of D. magna Straus (Cladocera, Crustacea)–acute toxicity test). For each replicate, ten daphnids were exposed to 50 mL of treated solutions (WAF, BW-PM and Quartz Sand) at the concentrations previously described for 24 and 48h at 20±2°C, with a 16/8h light-dark cycle. Their immobilisation/death was recorded and compared with control values.

2.6.4.3. V. fischeri bioluminescence inhibition (Microtox®) test

The bioassay with the luminescent bacterium *V. fischeri* followed the standard ISO 11348-3:1998 (Water quality – determination of the inhibitory effect of water

samples on the light emission of *V. fischeri* (luminescent bacteria test) - part 3: method using freeze-dried bacteria). The reduction of light emitted after 15 min exposure to samples was recorded with a Microtox® luminometer.

2.6.4.4. Germination rate and root elongation of L. sativum and S. Saccharatum

Acute toxicity to terrestrial plants was investigated with the phytotoxicity test following the method UNICHIM No. 1651 (2003), which provides for the use of *Lepidium sativum* (a dicotyledonous species) and *Sorghum saccharatum* (a monocotyledonous species). Their seeds were grown in artificial soils (quartz sand and standard OECD soil -5% peat, 10% kaolin and 85% sand), used as lab controls, to which increasing concentrations of BW-PM obtained from FM1-BD1, FM4-BD2 and FMB-BD7 formulations were added.

For each replicate, an amount of 10 g (dry weight) of soil per plate was placed in Petri dishes and moistened with 5 mL of sample dilutions in MilliQ water; Whatman #1 filter paper was then put on the wet soil and ten seeds were randomly placed on the filter. The plates were incubated for 72h in the dark at 25±2°C. After incubation, the germinated seeds with root length ≥1 mm were measured, and data from each plate were used to derive the germination index (GI). The test is considered valid if the seed germination in the lab control is >90%. For the WAF samples, a Whatman #1 filter paper was placed in a Petri dish and 5 mL of the sample was added. Then, ten seeds for each species were put on the filter.

2.6.4.5. Toxicity test on the soil earthworm -E. andrei

Earthworms were cultured as described in the OECD guidelines (OECD, 1984; OECD, 2004), in a breeding medium (at 20±1°C) made up of a mixture of horse

manure and peat. Organisms were selected from a synchronized culture with a homogeneous age structure. Adult worms with clitellum of similar size and weight (400-500 mg) were used. The Filter Paper test was done as described in the OECD guideline for testing chemicals (OECD, 1984). Worms were kept on clean moist filter paper for 3h before being placed in test dishes to allow them to void their gut contents. They were then washed with deionised water and dried before use (Sforzini et al., 2017). One mL of the BW-PM suspension was spread on a filter paper (Whatman #1) which was placed on the bottom of a Petri dish. Control filter papers were treated with 1 mL of deionized water. The dishes were put in a climatic chamber at 20±1°C. The test was done in the dark and for 48h and 72h. At least ten replicates per treatment, consisting of one worm per dish, were used. At the end of the incubation period, live earthworms were counted.

2.5.4.6. Toxicity test on the soil nematode -C. elegans

The acute toxicity test (Ura et al., 2002) involves exposing the synchronous organisms (to prevent age from affecting their response to exposure) to the WAF samples for 24h at 20°C. Ten one-day-old worms were dispensed into 24 well culture plates containing 0.5 mL of K-medium (32 mM KCl, 51 mM NaCl) and exposed to different BW-PM concentrations, without feeding the organisms. At the end of the incubation period, live nematodes were counted.

2.6.4.7. Acute and chronic tests on D. discoideum

The amebic cells were cultured as described by Sforzini et al. (2016) until their concentration reached 2-4 × 10^6 cells/mL. After centrifugation, the cells (0.75 × 10^6 cells/mL) were incubated for 3h and 24h in the samples (25% AX-2 medium with

50 µM $CaCl_2$ and 10 µg/mL tetracycline) to assess their vitality and reproduction rate, respectively. Cell viability was estimated incubating the cells with propidium iodide (Invitrogen Molecular Probes, Eugene, OR, USA); cells were observed at 200× magnification (Zeiss Axio Observer) using a rhodamine emission filter (setting and transmission light simultaneously) to distinguish dead cells (with a fluorescent nucleus) from living ones. Reproduction rate was assessed using a hemocytometer chamber (Bürker).

Lysosomal membrane stability (LMS) was evaluated after 3h of exposure as described by Sforzini et al. (2016). Amebae were put in adhesions on a glass coverslip for 10 minutes inside a humidity chamber at 21±1°C. The cells were then added in a working solution of Neutral Red (NR), obtained by diluting a stock solution of NR (20 mg of NR in 1 mL of dimethyl sulfoxide) 1/800 in Page's Ameba Saline (PAS) solution. After 3 minutes incubation, the dye in excess was removed, and the cells were washed and kept moist with PAS. The retention time of NR dye in the lysosomes was checked after 1h. Slides were observed under 400× magnification with an inverted photomicroscope (Zeiss Axio Observer) equipped with a rhodamine emission filter for fluorescence investigations. Images were analysed with the Scion Image analysis system, for the quantification of lysosomal NR leakage, expressed as a percentage change in fluorescence intensity with respect to the controls.

2.6.4.8. Genotoxicity: Micronuclei Test on D. discoideum

For the evaluation of micronuclei frequency on *D. discoideum* cells, amebae were placed on a glass coverslip where they were allowed to adhere for 10 minutes in a humidity chamber at 21 ± 1 °C. The cells were washed with PAS and then fixed in paraformaldehyde solution (4% in PAS) for 10 minutes. The cells were washed with PAS again, air dried, and stained for 1 minute with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen Molecular Probes, Eugene, OR, USA), a DNA-specific

fluorescent probe. Two thousand cells with preserved cytoplasm were scored for each sample with an inverted photomicroscope (Zeiss Axio Observer) equipped for fluorescence microscopy at 630× magnification (Sforzini et al., 2012, 2016).

2.6.4.9. Mutagenicity: Ames test on S. typhimurium

The Ames test, with and without in vitro metabolic activation using the rat liver S9 microsomal fraction and cofactor mixture (Ames et al., 1975; Pant et al, 2016) was performed to detect the mutagenicity, following the OECD Guidelines for the testing of chemicals No. 471. The ability to induce gene mutation in *S. typhimurium* and/or *E. coli* was measured by the reversion of auxotrophic strains to prototrophy. The auxotrophic *Salmonella typhimurium* His-strains (TA98-TA100) and *E. coli* (WP2 uvrA strain) were employed to detect frame-shift or base-replacement mutations. Extraction of the test item was performed in sterile water for injection at 37°C for 24 hours. A single experiment was carried out using the preincubation method. The experiment included negative and positive controls, and at least eight doses of the test item, tested in the absence and presence of an S9 metabolising system.The test was repeated using three replicated wells for each sample.

2.6.4.10. Statistical analisys

For all the different tests used in this research, we analysed at least four replicates from controls and BW-PM exposed organisms. Due to the number of data available for the statistical significance evaluation of the biological effects produced by the different BW-PM concentrations, we employed the non-parametric Mann-Whitney U test to compare the data from treated organisms with those of the controls ones ($p < 0.05$).

2.7 RESULTS

The biological effects of the water accommodate fraction (WAF) and brake wear particulate (BW-PM) from FM1-BD1, FM4-BD2 and FMB-BD7 brake disc and pad formulations it has been studied, using bioassays with model organisms of aquatic and terrestrial ecosystems. The effects at organism/population level were investigated using high-level endpoints such as survival and/or reproduction rate. For the physical characterization of the particulate debris, the DSL technique was employed to identify the dimensional range of the three BW-PM samples through the variations in intensity of the diffused light. The results obtained showed a tendency to the aggregation of the particulate material as a function of time (the three successive, continuous scans were performed every two minutes), as easily seen from Figure2.3.

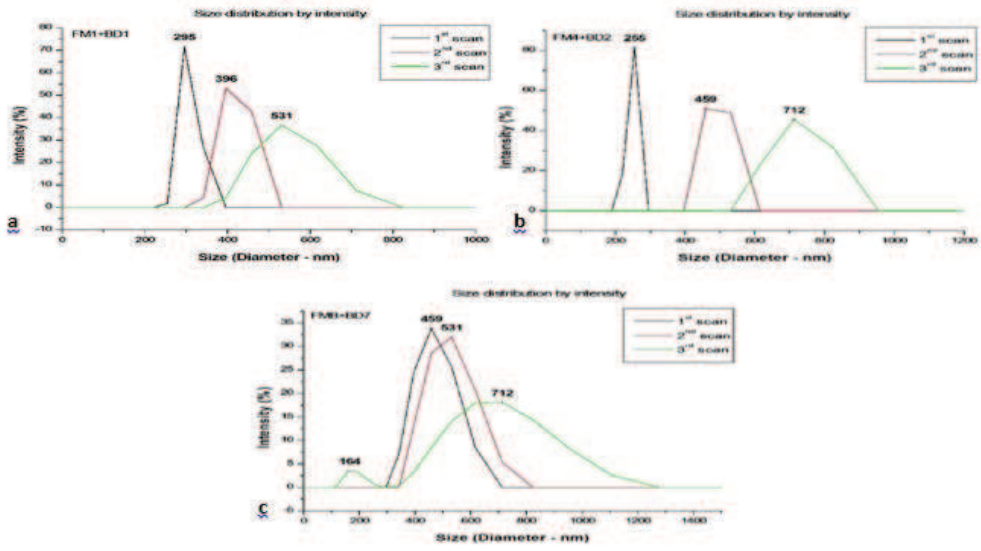


Figure 2.3.Particles size distribution by intensity obtained from the three brake-system particulate, FM1-BD1 (a), FM4-BD2 (b) and FMB-BD7 (c) as measured by DLS technique.

As can be seen in Figure 2.4 (acquired in the FM1-BD1 sample) there are several particles of sub-micrometric size with a tendency to aggregation, as evidenced by the arrows. Furthermore, in Figure 2.4A a morphology of fibrous nature is evident, indicated by the green arrow. The tendency to particle aggregation is even more evident at higher magnifications, as shown in Figure 2.4B: in this example, it is clear that the particles aggregate in clusters of micrometric dimensions

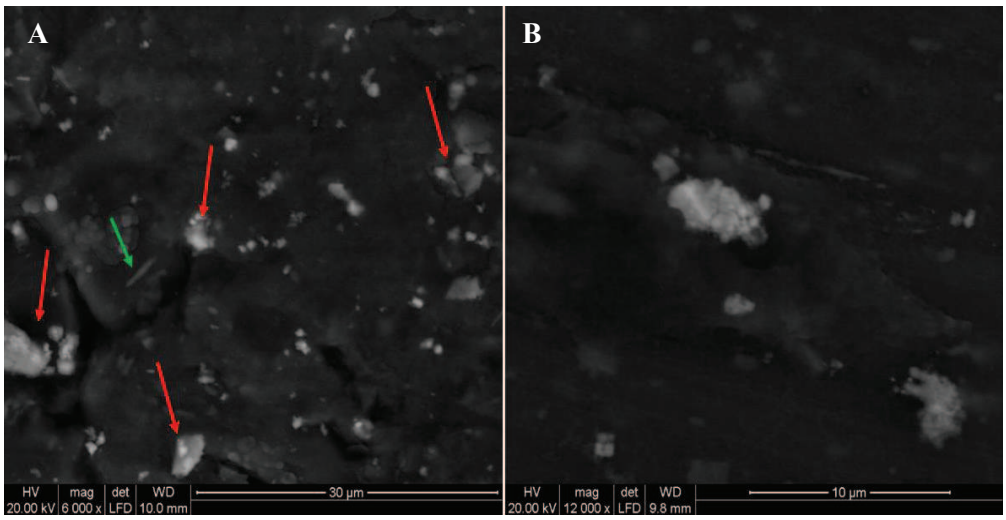


Figure 2.4 SEM images in secondary electrons acquired on an area of the sample FM1-BD1 (2.4A), and at higher magnification (2.4B)

The percentage values obtained by chemical analysis highlighted iron detection in great majority together with oxygen, suggesting a significant presence of iron oxides in the collected wear particles. In addition, Cu, Zn, Sn, Cr, Al, Si, and S were found in relevant amount.

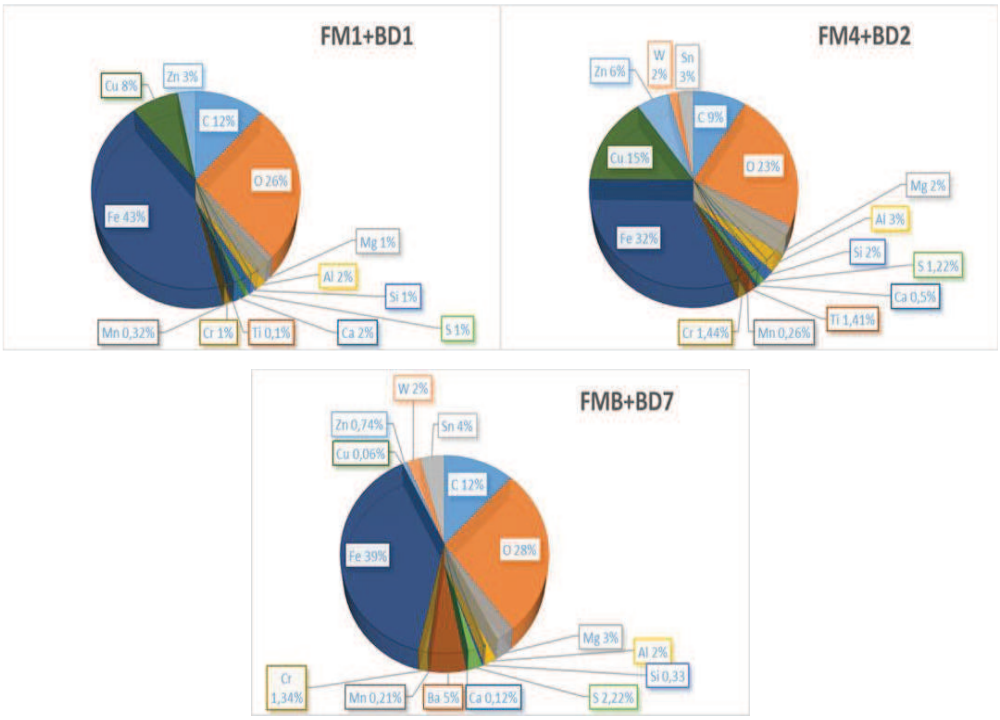


Figure 2.5. Chemical percentage composition obtained from the three brake-system particulate.

For what concerns the ecotoxicological effects on the freshwater compartment, it was found that the microalga *Pseudokirchneriella subcapitata* was sensitive to the WAF obtained from the particulate at concentrations higher than 100 mg/L, for two of the three formulations tested. One of the innovative pads'WAF solution (FM4-BD2) was weakly toxic at 200 mg/L (-40%) while the new formulation FMB-BD7 showed the lowest effect on algae reproduction, since even at 500 mg/L there was a reduction of about 41% in the replication rate.

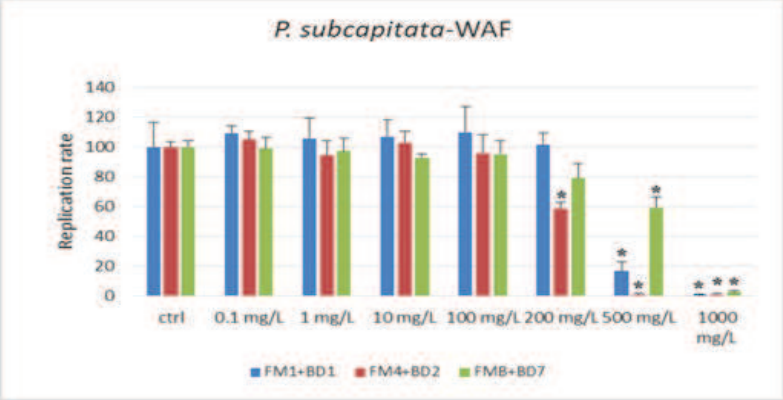


Figure 2.6. Growth of the alga *P. subcapitata* after exposure to the WAF from the three brake-system particulate. Data represent the mean ±SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney U test).

These results change significantly after exposure of the algal inoculum to the BW-PM, in order to simulate the potential interaction with the organisms into the water column during the transient of the particles deposition towards the bottom. The graph in Figure2.6 shows that the BW-PM of the FM1-BD1 and FM4-BD2 formulations had a clearly toxic effect already at the concentration of 1 mg/L while FMB-BD7 in the same conditions was only weakly toxic with evident effects at 100 mg/L.

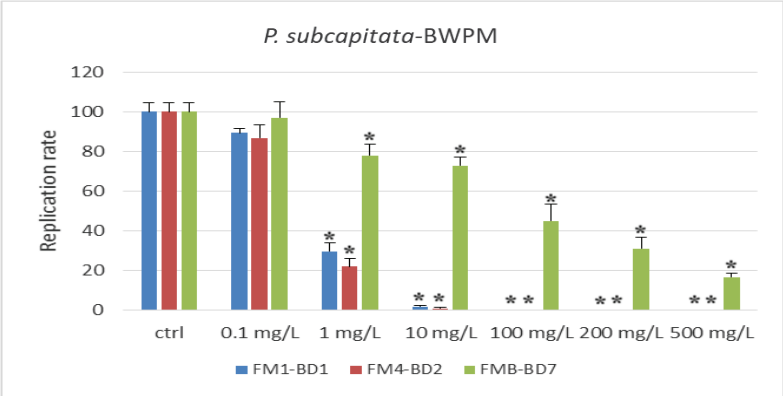


Figure 2.7. Growth of the alga *P. subcapitata* after exposure to the BW-PM solution from the three brake-system particulate. Data represent the mean ±SD of at least four replicates: *statistically significant differences compared to control values ($p < 0.05$ Mann-Whitney U test).

Quartz sand used as laboratory control at size of 0.3-5 µm (BCR-066) started to show a mild toxic effect at 200 mg/L. Glass spheres (9-13 µm) showed a tendency more similar to that of the FMB-BD7 suspension up to 10 mg/L, above which (100 mg/L) algal vitality was almost completely zeroed (Fig. 2.8).

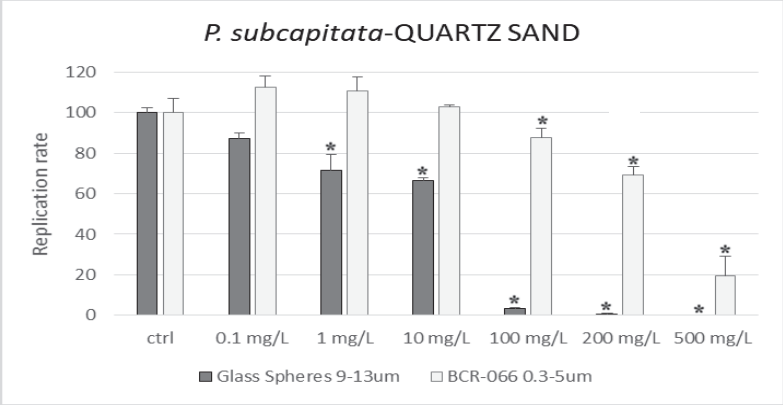


Figure 2.8. Growth of the alga *P. subcapitata* after exposure to the different size quartz particles (9-13 µm and 0.3-5µm) solutions. Data represent the mean ±SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney U test).

The same experimental set-up was employed for the acute toxicity tests on the water flea *D. magna.* The WAFs obtained from FM1-BD1 and FM4-BD2, had clear toxicity only at concentrations higher than 100 mg/L, while the exposure of the crustacean to FMB-BD7 did not inhibit its motility (Fig 4a). FM1-BD1 and FM4-BD2' BW-PM proved more toxic (as low as 10 mg/L) but even in this case the PM of the FMB-BD7 formulation had no effects on the crustacean's survival (Fig. 4b), neither did the solutions from the two different types of quartz sand (data not shown).

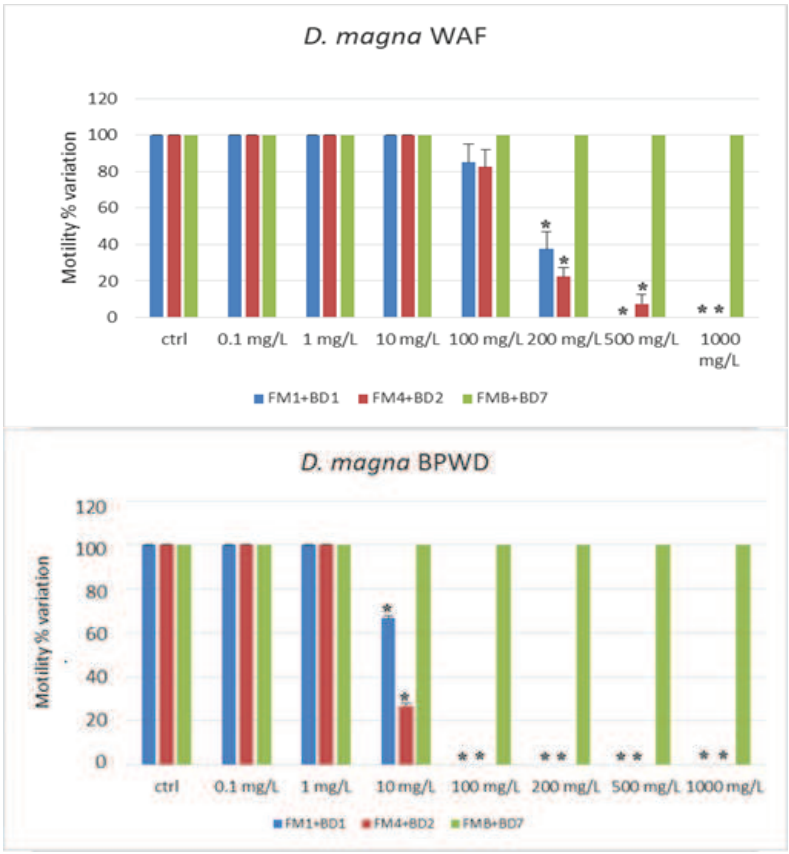


Figure 2.9-2.10. Motility of the crustacean *D. magna* after exposure to the WAF and BW-PM from the three brake-system particulate. Data represent the mean ±SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney U test).

In addition, the WAF solutions from all the brake pads formulations were not toxic for *Vibrio fischeri* until to 1000 mg/L, and FMB-BD7 showed again the most eco-friendly values.

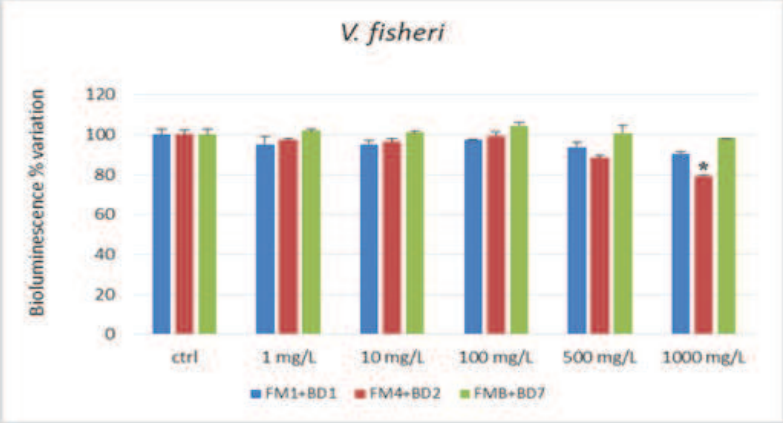


Figure 2.11. Bioluminescence variation of the bacterium *V. fischeri* after exposure to the WAF from the three brake-system particulate. Data represent the mean ±SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney U test).

The results about the BW-PM' effects on the edaphic organisms clearly demonstrate that the WAF solutions have not affected the germination of mono-dicotyledonous seeds (*S. saccharatum* and *L. sativum*) at the highest concentration tested of 1 g/L. Instead, the effects of these particulates in only quartz sand were evident from 500 to 1000 mg/kg for FM1-BD1 and FM4-BD2. Only FMB-BD7 did not affect the germination of the seeds; however, the BW-PM had no negative effects when the two seeds were exposed in more natural conditions, i.e. in standard OECD soil (Fig. 2.12).

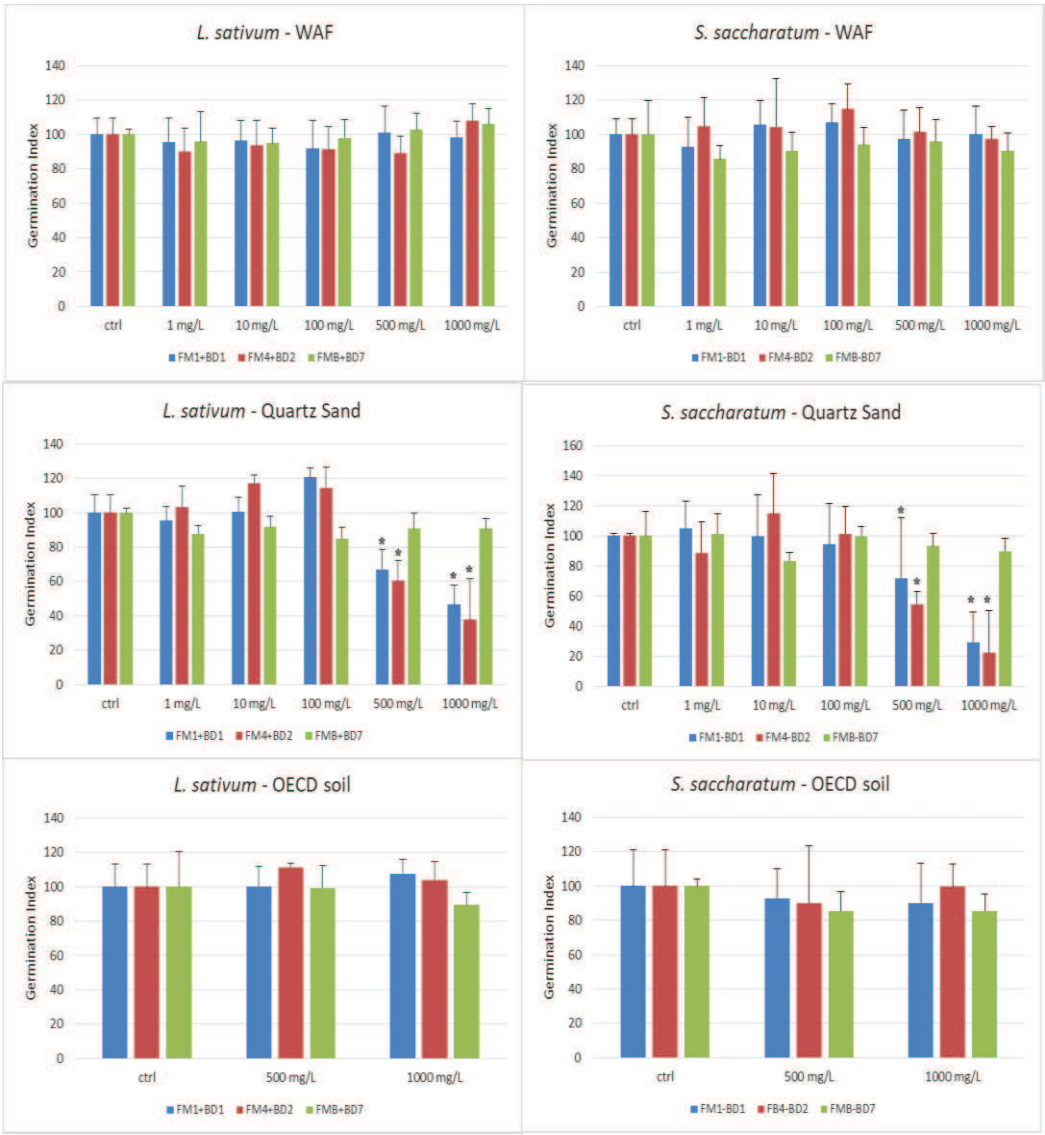


Figure 2.12. Germination index of *L. sativum* and *S. saccharatum* seeds at different concentrations of the three brake-system particulate, respectively in WAF, quartz sand and OECD soil. Data represent the mean ±SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney U test).

The same concentrations caused no toxic effects both on the nematode *C. elegans* (Fig. 2.13) and on the earthworm *Eisenia andrei* during a standard OECD 3 days' filter paper test (Fig.2.14).

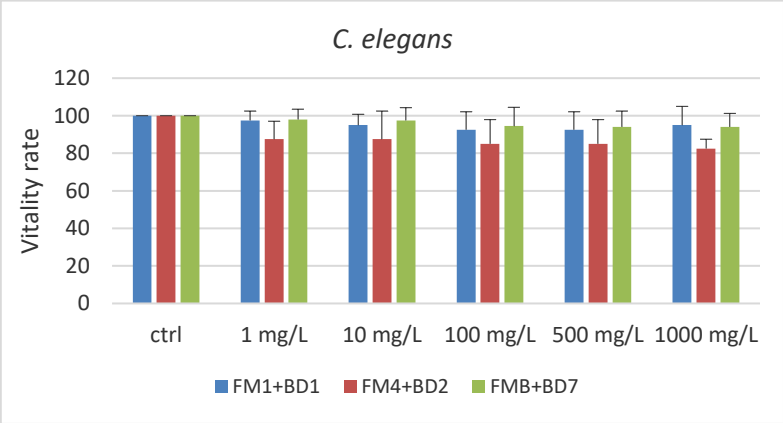


Figure 2.13. Survival rate of the nematode *C. elegans* after exposure to the WAF from the three brake-system particulate. Data represent the mean ±SD of at least four replicates.

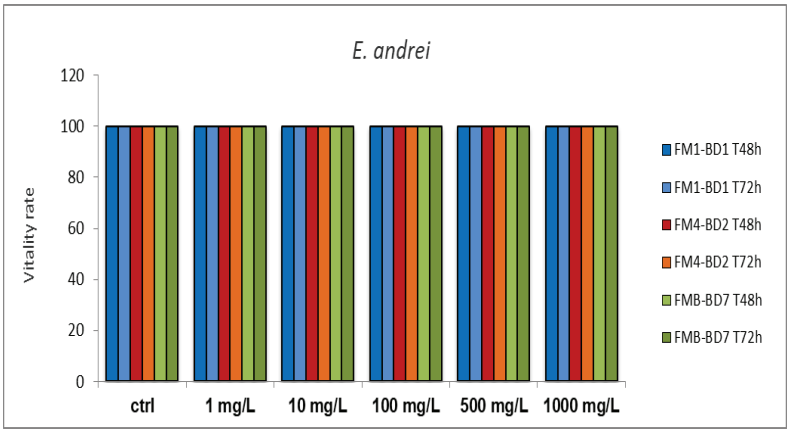


Figure 2.14. Survival of the earthworm *E. andrei* after exposure to the three brake-system particulate. Data represent the mean ±SD of ten replicates.

For the high-level endpoints, the results concerning effects of the BW-PM formulations on the pore-water ameba *Dictyostelium discoideum* have shown that the particulate did not affect the cell viability even at the highest concentration used (1000 mg/L).

However, the chronic toxicity test data had underlined a significant decrease in the reproduction rate of the protozoan, caused by FM1-BD1 and FM4-BD2 brake particles at the concentration of 100 mg/L; in the same conditions, FMB-BD7 showed slight lower inhibitory effects on cell division.

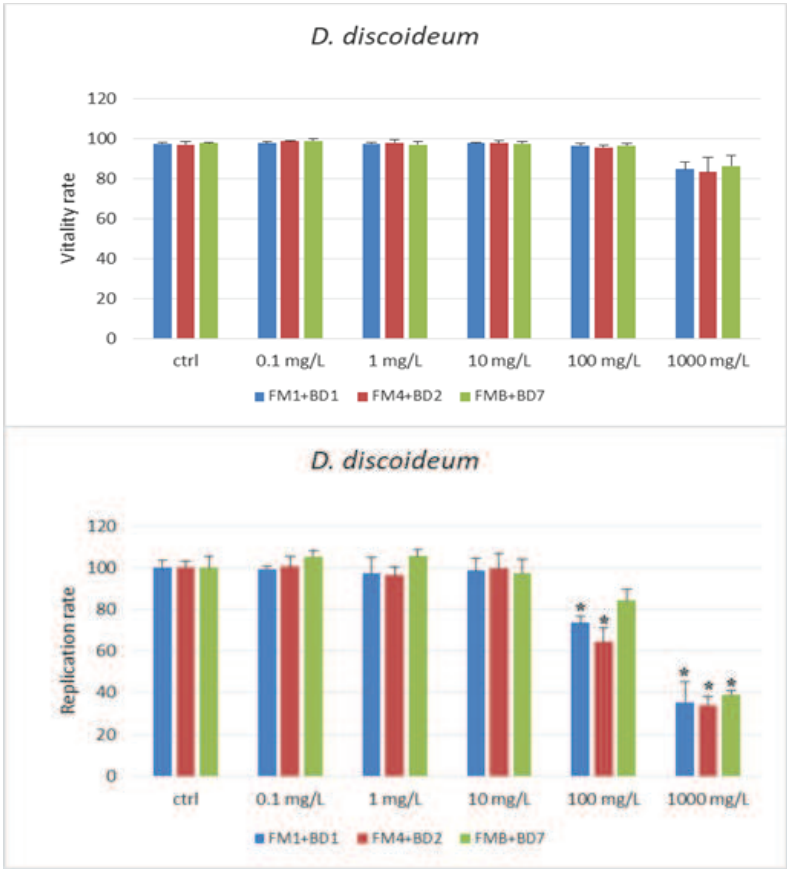


Figure 2.15-2.16. Survival and Reproduction rate of *D. discoideum* amebae after exposure to the three brake-system particulate. Data represent the mean ±SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney U test).

The evaluation of sublethal physiological parameters with the use of a very sensitive early stress biomarker such as the stability of the lysosomal membrane (LMS) has shown that, starting from the lowest concentration i.e. 0.1 mg/L, the particulate of the three brakes formulations caused in the amebae a significant decrease in the fluorescence intensity derived from the NR staining of the

lysosomes in the cells (respectively equal to 35% for FM1-BD1, 47% for FM4-BD2 and 31% for FMB-BD7).

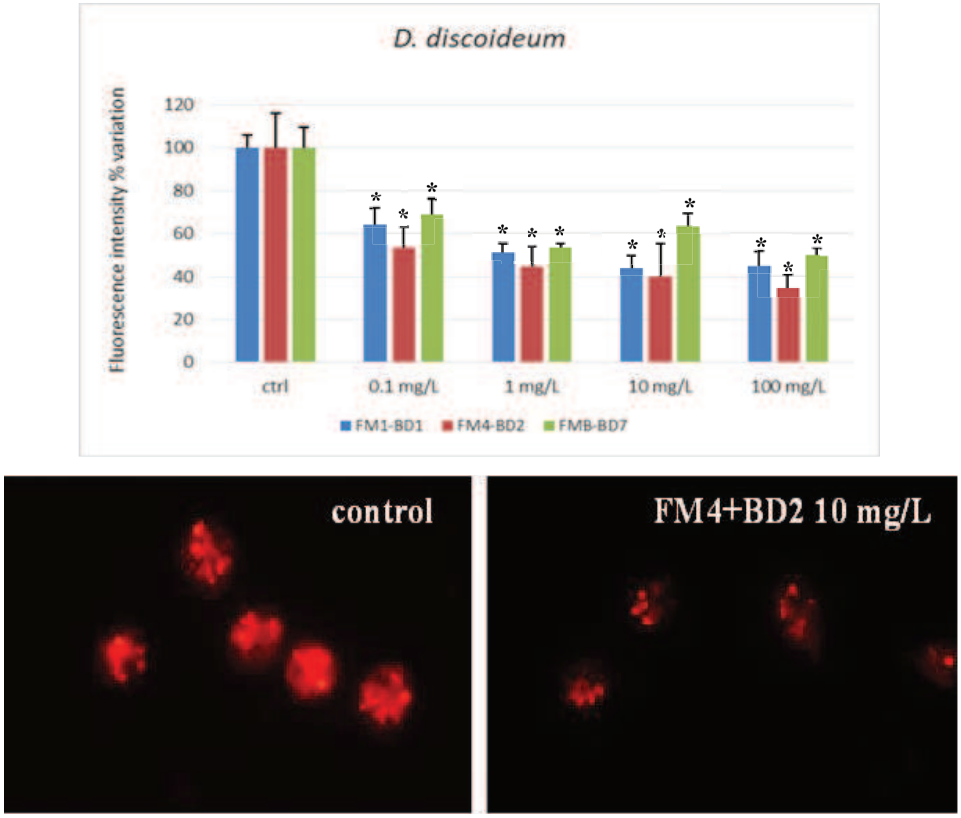
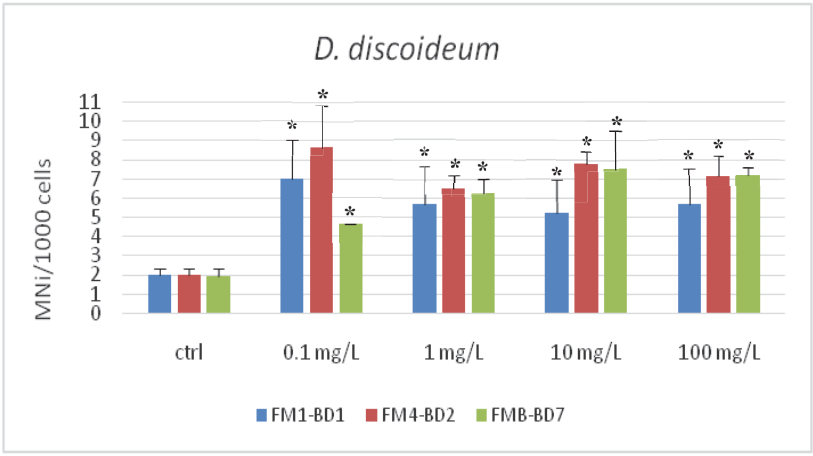


Figure 2.17. Lysosomal membrane stability of *D. discoideum* amebae after exposure to the three brake-system particulate. Data represent the mean ±SD of at least four replicates. Below, representative images of NR-derived fluorescent staining of the lysosomes in cells of control and brake debris-exposed cells (FM4-BD2 10mg/L).

The particulate matter from the three brake systems have also genotoxic effects on *D. discoideum*, as judged by the evaluation of the micronuclei (MNi) frequency. It should be noted that, for FM1-BD1, FM4-BD2 and at lower extent for FMB-BD7, the chromosomal alterations appear evident also in the organisms exposed to the lowest particulate concentration used in this study (0.1 mg/L).

The possibility that these genotoxic effects could be due to the physical action of the sub-micrometric component of the brake particulate material on the cells was demonstrated by using quartz particles with dimension ranging from 0.3 to 3.5 micrometres. The quartz sand particles (0.1 -1 mg/L) cause an increase in MNi frequency in the amebae (Figure 2.19).



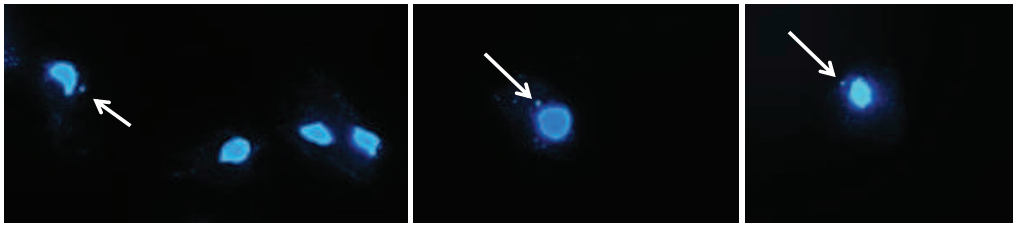


Figure 2.18. MNi induction in *D. discoideum* amebae after exposure to the three brake-system particulate. Data represent the mean ±SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney U test). In the figures, examples of micronuclei are marked with white arrows.

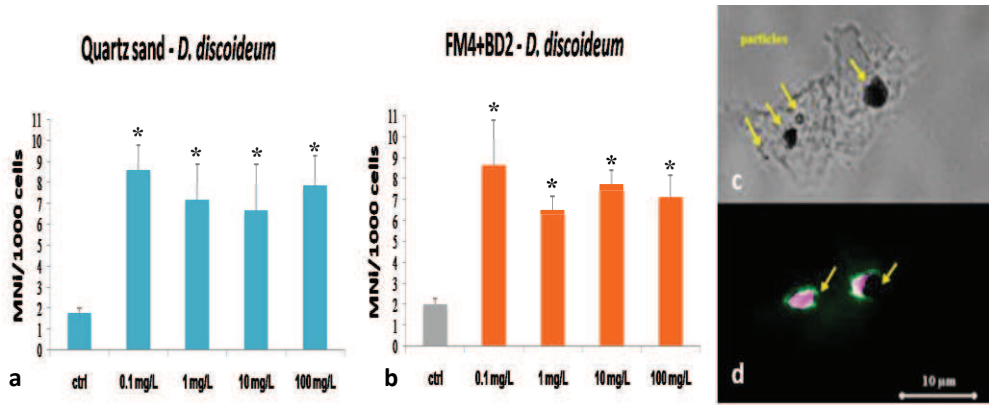


Figure 2.19. MNi induction in *D. discoideum* amebae after exposure to the quartz sand particles (a) in comparison to the FM4-BD2 brake-system particulate (b). Data represent the mean ±SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney U test). On the side, examples of wear particles inside the ameba cell (c) and within the nuclear membrane (d- in green the anti-nuclear membrane pore proteins, in violet the DAPI DNA stain)

The results from the Ames test demonstrate that the particulate debris from the three formulations are no mutagenic. It is concluded that the brakes particles does not induce reverse mutation in *S. typhimurium* (TA98, TA100) and *E. coli* (WP2 uvrA) in the absence or presence of S9 metabolism, following the OECD Guidelines for the testing of chemicals No. 471.

AVERAGE PM_{10} DRY DEPOSITION (mg m-2 yr-1)			
Brakesolution	**Roadside**	**200 m**	**500 m**
FM1-BD1	**18.65**	**2.95**	**0.80**
FM4-BD2	**17.60**	**2.64**	**0.73**
FMB-BD7	**27.95**	**4.05**	**1.13**

Tab. 2.1. Dry depositions (in mg m-2year-1) of $PM_{2.5}$ and PM_{10} for FM1-BD1, FM4-BD2 and FMB-BD7 in anextra-urban scenario, during cold and warm season and at three different distances from emission source.

Results of the dispersion model were clarified in a parallel study (manuscript in preparation). This study shows that, in the extra-urban environment, dry depositions related to the FMB-BD7' BW-PM_{10} fraction resulted to be 27.95 mg m-2year-1 at the roadside (i.e. approximately 20 m far from the side of motorway) and they decreased 7 and 30 times at respectively 200 m and 500 m from the side of the motorway. These values are (in average) 56% and 43% higher than depositions produced by FM4-BD2 and FM1-BD1 respectively (Tab. 2.1).

2.8 DISCUSSION

The particulate originated from the braking process (i.e. friction between brake disc and brake pads), due to atmospheric dispersion and deposition can interact with aquatic and edaphic ecosystems. It is therefore not possible to neglect the potential ecotoxicological impact; it is relevant both in terms of the quantitative representativeness of the PM contamination and for the presence of heavy metals and organic contaminants in every different formulations of braking devices (Thorpe and Harrison, 2008; Plachà et al., 2017). This explains the attention nowadays dedicated to the selection of more sustainable brake systems formulation in terms of materials composition and emissions abatement.

In this work, the ecotoxicity of wear particles from a commercial one and new supposed more eco-friendly formulations proposed within the LOWBRASYS project, were investigated using organisms typical of aquatic and soil ecosystems. Since potential risks associated with nano-micro particulate can be related to the shape and size of the particles together with their chemical composition (Nel et al., 2006), the first step was the physico-chemical characterization of the particulate.

The DLS analysis, in combination with the images obtained by SEM, allowed the determination of the particle size distributions in aqueous solutions. The tendency to aggregate over time increasing the dimensional range has been found, highlighted by the succession of DLS' series of scans. The SEM images, confirmed by optical microscopy, indicate that the PM is made of hundreds nanometres' particles, which agglomerate as a function of the interactive forces generated (Kukutschová et al., 2009). This can be an obstacle during the results' interpretation because some important physical parameters are influenced by the particles aggregation state (Van Hoecke et al., 2008).

By crossing the distribution data with the SEM images, it is possible to provide more information on both the particles' real dimensions and morphologies as well as the dynamics of their variations.

The chemical composition analysis of the BW-PMshowed the prevalence of metal oxides compounds and elemental carbon forms, as already reported in previous studies (Kukutschová et al., 2010; Kukutschová et al., 2011; Ciudin et al., 2014). For all the formulations, in slightly different percentages, Fe was the most representative element given that the discs are generally made of cast iron (Malachova et al., 2016). It is rare to find iron concentrations able to induce environmental toxic effects; it is a limiting factor for the development of plants and animals and it could be toxic only under certain conditions (i.e. excess of Fe^{2+} and Fe^{3+} free ions, bioavailability within the matrix) and at very high concentrations. Even iron oxides, present in large quantities in brake wear residues, are generally considered non-toxic. It is more relevant to note instead that brake pads are the main source of environmental pollution due to copper, generally present on average between 1-14% of the total composition (Haselden et al 2006; Straffelini et al., 2015). Many studies have focused attention on this topic, because of the potential toxic effects of this essential metal (Viarengo et al., 1988; Leili et al., 2008; Kothai et al., 2008; Lee and Hieu, 2011) on human and environmental health, especially with regard to the quality of water bodies (Armstrong 1994; Topping and Kuwabara, 2003).

Like Fe, Cu is an important component of the enzymatic systems of respiratory metabolism and photosynthesis, but it is also recognized as a priority pollutant according to the European criteria (Italian Legislative Decree n° 172/2015). Vehicle emissions and brake pad dust (Drapper et al., 2000) along with wide use in pesticides (USEPA 2005) and during industrial processes (Good 1993; Thomas and Greene 1993) have mobilized significant amounts of copper metal, Cu-oxides and Cu-sulphides into the environment, over time.

The copper concentration within living organisms is partly controlled by homeostatic mechanisms but if in overload and following long periods exposition it becomes toxic (De Boeck et al., 1995; Lundebye et al., 1999). However, the toxicity, rather than the total copper content, is due to the Cu^{2+} fraction readily bioavailable, that in turn depends on the physical, chemical and biological characteristics of the matrix in which the release takes place (Viarengo and Nott, 1993; Burlando et al., 2004; Negri et al., 2013). In fact, the high copper content' particulate reaching the aquatic compartment through washout is noxious when in the soluble Cu^{2+} form that may exert considerable toxic effects on algae and invertebrates (Kiaune andSinghasemanon, 2011), with possible alterations of the aquatic food chain.

In this work, the chronic aquatic toxicity assay has shown potential effects on *P. subcapitata* starting from concentrations of WAF solution of 200 mg/L (mainly for the FM4-BD2 prototype), and therefore the ecological risk should be considered limited. In fact, it is extremely difficult to reach these values in a lake or in the running water of a river. However, the exposure of the algal inocula directly to the BW-PM, in order to simulate the potential interactions occurring during the transient of the particles deposition towards the bottom, showed a general increase in the toxicity rate exercised by the three formulations, but in very different percentages between FM1-BD1 and FM4-BD2 compared to the most eco-friendly FMB-BD7.

The choice of quartz sand with various sizes as a laboratory control was aimed at ascertaining whether the particle dimensions could be a limiting factor for algal growth. Glass spheres with an average diameter of 9-13µm has indeed drastically affected algal growing already starting from the concentration of 1 mg/L, while BCR-066 (0.3-5 µm) was perceptibly toxic only exceeding the 200 mg/L threshold, with results more similar to those obtained for the brake wear particulate. According to Van Hoecke et al. (2008), although the quartz sand particles are not toxic by themselves, the SiO_2 solid colloids formation in a saturated solution may

exert toxicity through clustering around the algal cells. Particles and aggregates with a diameter bigger than that the microalgae cell-wall pores (ranging from 5 to 20 nm, as reported by Navarro et al., 2008) cannot enter cells but may manifest toxicity even through surface interactions, with direct effects on nutrient depletion, and/or shading with consequent photosynthesis inhibition (Van Hoecke et al., 2009; Ma et al., 2010).

However, the BW-PM of FM1-BD1 and FM4-BD2 were much more toxic than quartz sand, thus the answer must be sought more in-depth. Since the effects obtained from the dissolved fraction of the brakes particulate were less to those of the BW-PM the toxicity, this could be due to the physical interactions of the particles themselves more than to the release of free ions in solution. This, joined with more pronounced aggregation phenomena due to the lack of sonication and filtering (unlike the WAFs) and the simultaneous combination of the aforementioned factors, may have contributed to affecting algal growth. Even the classical acute toxicity test on *Daphnia magna,* a model species particularly sensitive to heavy metals (Khangarot and Ray, 1989), highlighted much more evident immobilization/death effects for the FM1-BD1 and FM4-BD2' BW-PM (10 mg/L), in respect to the WAF (200 mg/L). Experimental evidence on the environmental interactions of the vehicles' wear particulates (Wik and Dave, 2009) suggests that the most likely route of particle uptake for filter-feeding organisms is through ingestion. *Daphnia magna*has a range of active particle filtration approximately included between 200nm and 70µm (Burns, 1968; USEPA, 2002), depending on the animal size. Based on the clearance rate of the ingested materials, their accumulation could occur. The toxicity mechanism, which for *P. subcapitata* was hypothesized to be mostly related to a superficial physical-mechanical interference, in the case of *D. magna* has been linked to the processing of the actively ingested material. First, the size and the aggregation degree of the particles may have influenced or interfered with their capture by the crustaceans filtering apparatus. This may partly explain why the direct use of BW-PM has increased the

toxic effect: on the one hand, bulky aggregates may have compromised the respiratory activity, and moreover it has been shown (Peikertova and Filip, 2016) that nanoparticles produced by braking events easily stick to the wear debris and after ingestion could be released inside the cellular compartments.

There is an innumerable series of literature findings reporting the severe toxic effects on freshwater cladocerans, exerted by heavy metals and in particular by copper (Lewis, 1983; Vardia et al., 1988; Khangarot et al., 1987; Khangarot and Ray, 1987; Arambašić et al., 1995). In neutral aqueous solutions the dissolution processes of the copper compounds are not favoured (Wang et al., 2013) but, during the passage in the gastrointestinal tract, the enzymatic conditions would favour desorption processes even if the pH in the water fleas' gut is almost neutral (Pennak, 1978). Furthermore, passing through the cellular lumen, these compounds could be picked up inside the lysosomal vacuolar system; there, the acid pH (4-5) is able to break them down in free ions, whose greater solubility enhances their absorption, bioavailability and the consequent potential toxicity (Studer et al., 2010). If the lysosomal membranes become destabilized to the point of breaking, the intracellular release of free metal ions and their accumulation in the cytoplasm or in the nucleus can be harmful (Viarengo etal., 1981; Brunner et al., 2006; Midander et al., 2009; Semisch et al., 2014).

In addition to direct toxicity, another plausible hypothesis could be that the particles seized within the digestive tract could react or interact with the food compounds (microalgae and yeast), rendering them potentially useless for the organism (Rosenkranz et al., 2009).

The acute test with *Vibrio fischeri* did not allow the presence of particles in solution, which would have clearly interfered with the Microtox® detection system, but the results obtained by the WAF did not reveal any particular toxic effect. This data acquires a particular relevance taking into account the importance of the bacterial communities in the recirculation of the organic matter in the sediments, which are the ultimate deposit place of the particulate material in transit

along the water column. Taken together, these data indicate that the soluble substances dissolved within the WAF did not make water toxic except at very high concentrations. It is very important to note that the FMB-BD7 formulation was however very little toxic even when BW-PM was used. The results about quartz sand confirmed the increase of the particulate effect *per se* but, without taking into account the forecasts of environmental fallouts and the subsequent distribution of PM in large volumes of water, these results seem to be overestimated with respect to the potential exposure of natural environment in real conditions.

Data regarding the impact of the brakes particles on the edaphic organisms clearly demonstrate that the WAF solutions did not affect the germination of mono- and dicotyledonous seeds. Instead, the effects of the BW-PM added in a quartz sand substrate were more evident at the highest concentrations only for FM1-BD1 and FM4-BD2. In this case, it is not easy to give a univocal explanation of the results obtained. The absence of effects on the WAFs exposed seeds would seem to exclude that the phytotoxicity could depend exclusively on the particles chemistry; this would be confirmed by the germination rate perturbation in the presence of debris. The sensitivity of vegetal models to high quantities of heavy metals (Arambašić et al., 1995; APAT, 2004; Lin and Xing, 2007; Lee et al., 2008; Cuske et al., 2017) such as Cu and Zn, present in large amounts in the FM1-BD1 and FM4-BD2 formulations, is widely established. Therefore, even in this case it is possible that the physical interactions of the particles aggregates have allowed a greater accumulation of chemicals in adhesion to the seeds. This could have amplified the toxic effects at high concentrations or, alternatively it could have interfered with the uptake of water, limiting factor for seed germination (Ma et al., 2010). The dampening of these negative effects after the addition of BW-PM to the standard OECD soil, simulating natural conditions, could be related to the presence of organic matter (5% peat).

Both the nematode *C. elegans* and the earthworm *E. andrei* were not affected by the interaction with the particles of the braking formulations. They are key species

for the quality of terrestrial ecosystems (Sochovà et al., 2006; Sforzini et al., 2011) due to their quantitative relevance and ecological representativeness; therefore, their biological responses to a potential contaminants exposure are particularly significant in establishing expected risk levels. These species interact with the surrounding environment mainly through the ingested food but also by direct absorption through the permeable epidermal cuticle, so that their entire organism can quickly come into contact with the toxic substances dissolved in the interstitial water of the soil (and also of the sediments, in the case of *C. elegans*).

In this context, the link between the eco-toxicological investigations to assess the quality of both edaphic and freshwater environments was represented by the use of social ameba *Dictyostelium discoideum*, extremely sensitive to many contaminants starting from sub-cellular levels up to population dynamics (Sforzini et al., 2008). In nature, *D. discoideum* inhabits the pore waters of the soil where are concentrated amounts of chemicals and pollutants (Dueri et al., 2008; Gomiero et al., 2012; Magnusson et al., 2013) generally higher than in soils or in waters overlying the sediments (Simpson and Batley, 2015; Sforzini et al., 2016). Since *D. discoideum* is a professional phagocyte, able therefore to quickly and efficiently internalize a great variety of organisms as well as particles (Bozzaro and Eichinger, 2011), it was particularly significant to employ it as experimental tool to investigate the effects induced by the interaction with the brakes wear particulate. The use of classic ecotoxicological endpoints (cell viability and reproduction) did not show significant effects, maybe because the amebic cells are more sensitive to non-essential heavy metals and organic xenobiotic compounds (Sforzini et al., 2016), unrepresentative within the tested samples. All the results discussed up to this point indicate a very low eco-toxicity level of the studied brake particulates (especially regarding FMB-BD7) both for soil and freshwater organisms and consequently on the risk for biodiversity.

However, the evaluation of a very sensitive sublethal biomarker such as the lysosomal membrane stability (LMS) demonstrated that all the BW-PMs in study

cause a significant decrease of this parameterin the amebic cells, also in presence of the lowest debris concentration analysed (0.1 mg/L). The activation of the lysosomal vacuolar system represents an index of the autophagic activity overload (Sforzini et al., 2018) in response to the recognition of brake wear particles as extraneous bodies. In part, this may depend on the physical characteristics of the particulate fraction *per se*: in fact, quartz particles (0.35-3.50 μm) showed on *D. discoideum* similar sublethal effects as brake particulate obtained from all the formulations (data not shown).

For what concern the genotoxic effects, although the results from the Ames test demonstrate that brakes particulate did not induce mutations, it is important to mention that the micronuclei frequency test highlighted that the particulate from all the brake systems tested was genotoxic for the amebae also at the lowest concentration analysed (although FMB-BD7 has the fewer effects). This result has been put in relation to the effect of the mechanical action of the BW-PM' sub-micrometric components inside the cell nucleus; the exposure of *D. discoideum* to the quartz sand particles showed the formation of micronuclei by direct interaction with the nuclear chromatin (method not reported), with effects very similar to those obtained by exposure of the amebae to the particulate of the three braking systems. Further research will clarify the mechanism by which brake particulate can influence the cellular physiology of ameba (and human cells) and cause genotoxic effects.

In addition to low eco-toxicity evidence, FMB-BD7 was ranked as the least emitting brake solution in the urban context, according to the overall dynamometer results. The 3h-LACT cycle (Los Angeles City Traffic cycle) was chosen, within LOWBRASYS, as a representative reference of as wide use as possible for comparing solutions to reduce particulate emissions from brake wear.

In particular, compared to FM1-BD1, FMB-BD7 had 25% fewer BW-PM_{10} emissions (data not shown). However, predictive data on PM production and subsequent fallout in an extra-urban environment crossed by a motorway gave

greater deposition from the FMB-BD7 system (Table 1), reaching and exceeding the values obtained from the other two formulations. For eco-toxicological evaluations in a rural environment we used this more conservative model and found that the risk associated with the release and accumulation of BW-PM is negligible both for aquatic and edaphic ecosystems. Even assuming a 20 cm deep volume of natural soil (in which it is possible to find most of the biomass) near the motorway (i.e. roadside) it would take between 12.165 (for FMB-BD7) and 19.318 (for FM4-BD2) years to reach the maximum concentration tested for *E. andrei*[1]. These values are further overestimated since even in such a conservative scenario, the concentrations tested for the edaphic ecosystem are far higher than realistic environmental concentrations because of the source (brake systems). It is difficult to apply the same hypotheses for the aquatic ecosystem in order to contextualize the effect concentrations for *P. subcapitata*, *D. magna* and V. *fischeri*: water bodies are extremely dynamic environments and computing realistic concentrations starting from deposition values would call for specific models. Anyway, the order of magnitude of concentrations to which organisms are likely to be exposed is well below those tested. However, tests on biological models can detect the real ecotoxicity on different endpoints and in this case the in silico approach acts as a support.

In conclusion, the balance between the abatement efficiency in terms of PM_{10} and the lower environmental impacts identifies the innovative FMB-BD7 formulation as the most eco-friendly solution. This does show that the implementation of industrial research contribute to increase the compatibility between new braking system formulations and environmental safety. Evaluation of the eco-toxicological impacts related to the edaphic and aquatic environmental compartments showed that, compared to the commercial FM1-BD1 formulation (already in itself only

[1]0.055 (FM1-BD1), 0.052 (FM4-BD2) and 0.082 (FMB-BD7) mg kg-1 year-1 were computed starting from roadside dry deposition values (Tab. 1) assuming a 0.2 m3 soil volume (1 m x 1 m x 20 cm) with 1700 kg m-3 bulk density and no removal and BW-PM accumulation only.

weakly toxic), the new proposal always had the lowest harmful effects on biological models in acute and chronic bioassays.

In general, compared to the edaphics, aquatic organisms have proved to be more sensitive: while the toxic response to the soluble BW-PM fraction (WAF) was extremely low up to 200 mg/L, the direct mechanical interaction with the particulate gave more pronounced effects, confirmed in parallel by the results of exposure to quartz sand. The effects of an inert substrate on the viability and/or reproduction of aquatic models underlines a focal point: regardless of the origin and chemical composition, the particulate *per se*has an intrinsic background of toxicity.

The effects detected in edaphic model organisms were minimal, with the exception of the social ameba *D. discoideum*; this professional phagocyte, due to its greater capacity for particle internalization has undergone more accentuated effects on its reproduction rate but no effects on its viability.

It was interesting to use a model like ameba to ascertain the appearance of sub-lethal effects. One of the most sensitive early stress biomarkers, LMS, gave significant results with effects already visible at the lowest concentrations. Their early manifestation implies that, beyond any other consideration, it is necessary to take into account that even when present in very small amounts that do not have noxious effects at the organism/population level, the BW-PM may affect the organisms' physiological conditions. The appearance of early sub-lethal effects highlights the importance of considering the particulate a component of environmental contamination that may raise the level of stress, making the organisms more susceptible to other stressors (extreme temperatures and/or droughts and other forms of pollution), aggravating or triggering additional negative effects with substantial impact on biodiversity and environmental health.

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CHAPTER 3. General conclusions

This study aimed to investigate the ecotoxicological effects of the atmospheric toxi-chemicals and micro-nanoparticulate, with particular reference to the emissions coming from the wear of the braking systems of motor vehicles (a source of contamination of great current relevance).

The aquatic (marine and freshwater) and edaphic organisms were sensitive in varying degrees to exposure but the most significant effects were highlighted at the sub-cellular level, as evidenced by the investigations on *M. galloprovincialis* and *D. discoideum*.

This investigation highlighted that benzo[a]pyrene and fullereneC60, typical components of atmospheric particulate as well as brake wear residues, act in any case on model organisms at sublethal level, over-inducing the autophagic pathway. These effects, with reference to the tendency of the atmospheric particulate to form aggregates, may not necessarily be correlated to the particles chemical composition but refer to a mechanical stress dependent on the mass of the particulate per se, with cyto- and geno-toxic effects that add together contributing to increase the sensitivity of organisms to additional stress factors.

The effects of classical contaminants such as PAHs have been known for a long time, the novelty was to test the co-exposure with new generation contaminants and to relate it to the appearance of autophagy triggered by the inactivation of the mTOR kinase complex, a process widely studied on mammals but not yet fully clarified on invertebrate models exposed to environmental contaminants.

The evolution of the stress syndrome, starting from the first sub-cellular alert signals up to the alteration of the physiological parameters of the organisms, can have detrimental effects on their scope for growth, projecting non-negligible consequences on the population/community health. For this reason, in a current

scenario so permeated with awareness of the need to preserve the environment that surrounds us, it is desirable to incentivize and coordinate the cooperation of the scientific community and industrial development in seeking new and increasingly efficient strategies to reduce emissions and the environmental impact of a source of contamination as complex as atmospheric PM in association with common pollutants that may affects ecosystems and human health.

Scientific Publications of Associated Investigators

- Published:

Sforzini, S., Moore, M. N., Oliveri, C., **Volta, A**., Jha, A., Banni, M., & Viarengo, A. (2018). Role of mTOR in autophagic and lysosomal reactions to environmental stressors in molluscs. *Aquatic toxicology*, *195*, 114-128.

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