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Environmental toxicology by assessing biological responses after wastewater and sediment recovery actions in mesocosm scale experiments, and *in vitro* techniques

Tesi di Dottorato

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Abstract

In the last decades, great attention has focused on the impact of oil refinery wastewaters and discharged effluents, which are released to the environment as gases, particles, sludge, and liquid effluents, and therefore represent potential sources of pollution. In environmental biomonitoring programmes, marine mussels have been often used as bioindicator species because of their ability to bioaccumulate toxic compounds and their tolerance towards a huge variety of environmental conditions. To recover polluted sites, several remediation actions were made. In this work, two remediation techniques (BF-MBR and Soil Washing) versus wastewater and natural polluted sediments, collected from a contaminated area in Augusta (eastern Sicily, Italy), were evaluated. To this aim, in the first part of thesis, mesocosm scale-up experiments were settled in order to measure the recovery effects on mussels *Mytilus galloprovincialis*. Both for wastewater and sediments, three different environmental conditions were simulated: a control area (W, white group), a petrochemical polluted area (B, black group), and a polluted area subjected to the remediation actions (G, grey group). Mussels were exposed to each condition for 15 days, and then the biological effects of technological actions for the potential recovery of petrochemical contamination were evaluated by a multi-biomarker approach, including histology, metabolomics, immunohistochemistry, molecular and enzymatic investigations. Mussel gills, mainly involved in nutrient uptake and gas exchange, were chosen as target

organs. In respect to the W group, the gills of mussels from the B mesocosms, both for wastewater and sediments, showed marked morphological alterations with loss of cilia. Changes in serotoninergic (i.e. serotonin, 5-HT, and its receptor, 5-HT_3R) and cholinergic (i.e. acetylcholinesterase, AChE, and acetylcholintransferase, ChAT) systems were observed in mussel gills from the B group by immunohistochemistry, as well as supported by the enzymatic analysis of AChE activity, metabolomics and molecular assay, which revealed changes in neurotransmitters. Contrarily, in the G mesocosms, the same battery of biomarkers indicated a general recovery trend in mussel gills. Overall, the application of a multi-biomarker panel results effective in assessing the environmental influences of petrochemical pollutants on the health of aquatic organisms. Furthermore, findings from this study confirmed that the remediation actions herein applied on wastewaters and natural polluted sediments might be good tools to recovering a petrochemical polluted ecosystem. Some of the results presented in this PhD thesis are already submitted for publication in international peer-reviewed journals.

Since in the first part of thesis it was demonstrated the high sensitivity and the extensive alterations induced to the respiratory organs (gills), in parallel a brief internship at the Swedish Toxicology Sciences Research Center (SWETOX) in Södertälje, Sweden, was done under the supervision of Dr. Ernesto Alfaro-Moreno, in order to acquire competences on the use of cell culture in toxicology. So, in the second part of Thesis, two different techniques were assessed for the

evaluation of tight junction disruption induced by pollutants (in our case NaClO) on human airway epithelial cells. Overall, the use of the *in vitro* technique is a promising tool for future investigations on the effects of environmental toxic compounds on human airway epithelial cells, which confirmed also the high sensitivity of this kind of cells versus pollutants.

CHAPTER I

Introduction

1.1 General Introduction

In the last years, various biomonitoring programmes have been carried out with the aim to evaluate the environmental effects of various pollutants, in particular those from oil refinery activity. The activity of petrochemical industry and oil refinery is responsible for the emission of a huge amount of pollutants in the water in form of gases, particles, sludge, and liquid effluent. In these wastes, it is possible to observe a substantial amount of polycyclic aromatic hydrocarbons (PAHs), phenols, heavy metals and their derivatives, sulphides, naphthenic acids, and other chemicals (Dorris et al., 1972). It is well known that most of these pollutants are even toxic at low concentration (Long et al., 1995). Hence, petrochemical pollutant is able to provoke serious damages to the marine environment and human health (Fasulo et al., 2012a; Sureda et al., 2011). Particularly negative effects of these pollutants are observed in harbour areas, where the quality of water, the water exchange and the biodiversity are reduced (Guerra-García and García-Gómez, 2005). Therefore, harbour areas (for instance the Augusta-Melilli-Priolo petrochemical area in Sicily, Italy) became a target for various biomonitoring programmes (Cappello et al., 2017; Fasulo et al., 2015; Maisano et al., 2017). The data collected within

several of these research programmes have offered interesting results which are useful not only in the evaluation of the real damage caused by these kind of wastes, but also in the development of new remediation approaches and improvement of the existing techniques. The main aim of remediation is to reduce the amount and the effects of wastes in the environment. It is possible to list various techniques of remediation, which can be based on various approaches (i.e. chemical, engineering and microbiological). The main aim of this thesis, which is part of the project ”SYSTEMS BIOLOGY” (PRIN2010-2011) (Fasulo et al., 2015), is principally evaluate efficiency of two recovery techniques, the first one versus petrochemical polluted wastewater (BF-MBR) and the second one versus petrochemical polluted sediments (Soil Washing), by assessing different biological responses elicited by marine mussels *Mytilus galloprovincialis* treated in mesocosm scale experiments

Fig 1.1 Area of Augusta-Melilli-Priolo (Sicily, Italy)

1.2. Remediation

The recovery of polluted areas is becoming a very urgent and relevant problem. In some cases, as for instance in petrochemical areas such as the Augusta-Melilli-Priolo (Sicily, Italy), the concentration of pollutants such as Polycyclic Aromatic Hydrocarbons (PAHs) and heavy metals, like mercury, reach elevated concentrations. In fact, in the Augusta-Melilli-Priolo petrochemical area, the level of mercury in sediment exceeds the standard limit reported by national and international sediment quality guidelines (SQGs) (Ministerial Decree No. 260/2010 De Domenico et al., 2013). This contamination degree is related to the anthropogenic activities in the area, responsible to provoke a severe alteration of the environment. In light of this, it has become fundamental to develop techniques able to remove pollutants or mitigate their effects. During last years, various methods were produced based on engineering, physic-chemical and biological procedures. The various remediation actions could reduce the effect and the concentration of various organic and inorganic pollutant in a different matrix (Water and Soil).

1.2.1. Soil Washing

An example of remediation activity is “Soil Washing”. The term “Soil Washing” is used to describe an *ex situ* technique whose aim is to extract contaminant from soil. It therefore differs from the “Soil Flushing”, which is an *in-situ* technique. It is a very useful technique able to reduce the amount of contaminant in the soil, in particular the level of heavy metals. Various techniques of soil washing could be listed. It is possible to classify them in physical techniques, which try to use different physical properties of contaminants, and chemical techniques, where the different chemical properties of the contaminants are used for the extraction. It is also possible to observe even combined techniques. In the soil washing, the knowledge of the contaminated soil properties is very relevant in order to apply the better technique (Dermont et al., 2008). The aim of this kind of technique is to concentrate contaminants into a smaller volume of soil by employing the common physical differences with regards to characteristics (size, density, magnetism, and hydrophobic surface properties) between the pollutant particles and soil particles. The foremost physical separation techniques are:

- Mechanical screening
- Hydrodynamic classification
- Gravity concentration
- Froth Flotation

The choice of the physical separation technology is heavily related to the soil and site types to be treated (Williford et al., 2000). These kind

of techniques are principally appropriate to “anthropogenic” soils placed in urban or industrial areas, and soil with a good amount of sand of 50-70% (USEPA, 1997). On the other hand, physical separation techniques are not appropriate for treating the “natural” soils or agricultural soils affected by a diffuse contamination. Hence, these soils typically have a high content of silt/clay and organic matter the metals present in soils are mostly in sorbed forms as opposed to discrete particles. For these reasons, physical separation is often associated with chemical procedures to improve pollutant removal (Dermont et al., 2008). Chemical extraction (CE) tries to remove pollutants by chemical reagents in order to transfer metals or other kind of pollutants from soils to an aqueous solution. Since it uses an aqueous solution that “wash” the soil, the chemical extraction is considered from some authors as the proper "Soil Washing. In this technique, in order to increase solubility leaching solutions in which the contaminants are dissolved can be used. Another way to increase solubility could be the conversion to soluble salts by valence change (Dermont et al., 2008). It is possible to consider different solutions able to dissolve pollutants:

- Acids (FRTR 2007)
- Salts and high concentration chloride solutions (Tampouris et al., 2001; Kuo et al., 2006)
- Chelating Agents (Peters, 1999)
- Surfactant (Ehsan et al., 2006)
- Redox Agents (Van Benschoten et al., 1997).

Also, in this case, it is very important to have good information about the chemical aspect of the soil because this knowledge could address versus the best technique. In various situation, chemical extraction looks better in respect to physical extraction (as for instance for sorbed metals) but, at the same time, the application of chemical reagents can increase processing cost and generate further environmental problems (Dermont et al., 2008).

1.2.2. Membrane Bioreactor (MBR)

Membrane Bioreactor (MBR) is a technology that affords biological approach with a membrane separation. The two main steps of MBR are:

- Biological degradation of organic pollutants by adapted microorganisms in a bioreactor;
- Physical separation by a membrane able to segregate microorganisms, which permit either recycling the activated sludge and creating a clean matter.

The position of membranes in important in MBR organisation. Hence, the membrane could be located outside or inside in respect to the bioreactor. In the first case, there is a recirculated configuration with an external membrane unit. Mixed liquor is circulated outside of the reactor to the membrane module, where is filtered under pressure. The concentrated sludge recycling occurs back into the reactor. In the second one, the membrane module is submerged in the activated sludge (Submerged configuration). This last system looks more economical concerning energy expenditure (Huang et al., 2001). The MBR, in

respect to other technique, looks more efficient respect to the common clarifier (Ng et al., 2007), because it is possible to use a membrane module more compact. Furthermore these systems permit to work with a higher biomass concentration than other systems (Yamamoto et al., 1989; Jefferson et al., 2000) The use of a bioreactor is related to the acquired ability of different bacteria stain to biodegrade several pollutants, as for example hydrocarbons (Cappello et al., 2016). An excellent example of treatment technology is the Moving Bed Biofilm Reactor (MBBR). In this method biomass is able to develop either as suspended flocs or as biofilm, implementing a higher total biomass concentration in the reactor. Newly, MBR and MBBR have been employed together, known as Biofilm Membrane Bioreactor (BF-MBR), substituting the secondary settler by means of MBR (Di Trapani et al., 2014).In these conditions the removal concentration of hydrocarbons refractory is considerably higher. The bioreactors for bioremediation procedures are tank where living organisms are able to perform their biological reactions. A reactor should be easy to maintain and operate (Evangelho et al., 2001) and should be able to work both in aerobic and anaerobic conditions. Their effectiveness is guaranteed by the capacity of bacteria to attach on the inert material, such as granular activated carbon, to generate high biomass at interfaces (Bouwer and McCarty, 1982; Teitzel and Parsek, 2003). By the rupture of solid aggregates and the dispersion of insoluble supports, desorption of hydrocarbons and contact with the aqueous phase is raised, with consequent improvement of biodegradation. Several models of bioreactors are broadly applied in a large kind of aerobic bioprocesses

such as aerobic fermentation, biological wastewater and hydrocarbon impacted soil/sediments treatments (Van Hamme et al., 2003). The filtration of oil-contaminated water with a porous membrane bioreactor (MBR) is a novel improvement in wastewater discharges bioremediation. In particular marine wastewater discharges are related to operations deriving from slops, dirty ballast, sewage and bilge water management are held responsible of high oil load and consequent risk of marine pollution (Ciacci et al., 2012; Fasulo et al., 2015; Mancini et al., 2017). A membrane bioreactor couples the activated sludge process among a membrane separation method. The work of the reactor is comparable to a traditional activated sludge process but, in this case, secondary clarification and tertiary steps like sand filtration are not necessary. Especially, in MBR treatment, native, water-borne micro-organisms in a controlled habitat performed the bioremediation, so the choice and identification of microbial consortia with high capability to degrade hydrocarbons is a primary step for the optimization of the process. Nowadays, various aspects of these techniques are considered in different scientific works aimed at improving the wastewater discharges treatment (Cappello et al., 2016; Mancini et al., 2017; Pirrone et al., 2018; Yakimov et al., 2007).

1.3 Mesocosm: definition and application

A simple definition of mesocosm is an experimental set-up able to combine the complexity of an environmental experiment and the controllability provided by lab context. Mesocosm should be considered as a useful tool that give explanations when it is difficult to obtain results in extremely controlled oversimplified conditions or complex and largely uncontrolled natural conditions (Sagarin et al., 2016). This experimental set-up offers various opportunities in several research topics. For instance, in the ecological field, the mesocosm permits to have an easier observation and setting of several parameters in respect to nature. In addiction, the possibility to create perturbation is more pleased. For these reasons, mesocosm in the ecological field it is hugely applied. Indeed, in this field various scientific works were developed (Davis et al., 1996; Scheinin et al., 2015; Kelly et al., 2014). Another interesting context in which mesocosm is applied is the assessment of the effects of various pollutants on living organisms in an enclosure environment (Oviat et al., 1984, Vethaak et al., 1996, Sanchis et al., 2018. Oviat et al. (1984) tested the recovery capacity of an ecosystem with polluted sediments. Vethaak et al. (1996) directed their investigations on possible pathologies found on *Platichthys flesus* and their direct correlation to pollution. In the work of Sanchis et al. (2018), the metabolic responses of *M. galloprovincialis* to fullerenes were evaluated. On other interesting use of mesocosm is in scale-up experiments to better evaluate the efficiency of remediation techniques. For instance, Pirrone et al. (2018), used a microcosm approach to measure BF-MBR efficiency in mitigating the impact of oily-

wastewater discharge into marine environments using *M. galloprovincialis.* In all these cases, the experimental use of mesocosms allowed to getting notable results.

1.4. Enviromental Biomonitoring

As mentioned earlier, the anthropogenic activity is one of the main causes, for various reasons, of adverse repercussions on the state of the wellness of the environment, and in particular, of the marine environment. Aquatic ecosystems resulted deeply disrupted by anthropogenic interferences by pollution. In particular, estuaries and coastal waters are commonly exposed to contamination because, during history, they have often been fundamental areas for human settlement and resource use (Lotze et al., 2006; Marean et al., 2007) among the biological effects of pollutants it is possible to add physiological alterations processes related to the accumulation of substances at toxic levels and movement of these substances through different trophic levels. For monitoring pollution levels It is possible applying several techniques, in relation to the specific target of the study. Direct chemical analysis of water and sediment in order to Measure the concentration of inorganic (e.g., heavy metals, radionuclides, rare earth elements) and organic pollutants (dichloro-diphenyltrichloroethane, DDT; hexachlorocyclohexanes, HCHs; polychlorinated biphenyls, PCBs; polycyclic aromatic hydrocarbons, PAHs etc.) in the environment is very common approach (Superville et al., 2014) For several years, “Mussel Watch”-like programmes (Farrington et al., 1983; Goldberg et al., 1978; 1983; Goldberg and Bertine, 2000; Mee et al., 1995) have used chemical analyses of pollutant concentrations in

bioindicator organisms as the tool to evaluate water quality.. Nevertheless, this kind of results contributed little data with regards to the concentration of bioavailable toxins. Bioavailable toxins are the available pollutants for uptake and accumulation by living organism and their evaluation is one the most relevant aspects in environmental toxicology (Rainbow, 1995; Soto et al., 1995). Hence they are linked to a potential health risk to humans and the food reservoirs that they depend upon (Schöne and Krause, 2016). It is a common knowledge that various contaminants cause negative effects on the aquatic organisms. On the other hand, the living organisms react to the pollutant by different biological responses (i.e. biochemical, molecular, cellular, and physiological). These biological responses are usually named as "biomarkers". Biomarker is defined as a change in a biological response starting at the sub-cellular level (e.g. interference with molecular pathways) and ultimately leading to adverse effects at higher levels of biological organization (De Coen and Janssen, 2003), which can be related to exposure to or toxic effects of environmental chemicals (Peakall, 1994). The living organisms able to produce these kinds of responses are called “biondicators”, which can be considered as “sentinel organisms” (Walker et al., 2006). The organisms belonging to the genus Mytilus are very common bioindicators. Hence, it is possible to find several research works about their use as bioindicators (Bebianno et al., 2007; Ciacci et al., 2012; Cappello et al 2013a; Cappello et al, 2017; Fasulo et al., 2008, 2012, 2015; Fernández et al., 2012; Hellou and Law, 2003; Maisano et al., 2017; Manduzio et al., 2004; Shaw et al., 2011; Sureda et al., 2011; Viarengo et al., 2007). The

reason of their wide use is associated to several aspects of these organisms. Mussels are filter-feeding organisms able to withstand also baseline levels of pollutant. Accordingly, they may be exposed to a large range of concentration of chemical pollutants even if these chemical compounds are found in moderately diluted concentrations. Another interesting aspect of the mussels is their ability to bioconcentrate xenobiotics up to numerous thousand times in respect to the biotope background. They are sessile species, a property particularly helpful for bioindicators since they are likely to reflect changes in the pollution status of a point sampling area (Manduzio et al., 2004). Mussels are known to accumulate high levels of metals and organic contaminants including PAHs and PCBs in their tissues with observable cellular and physiological responses (Livingstone et al., 2000; McDowell et al., 1999). Alterations in cellular metabolism are used as biomarkers for the detection of pollutant-induced cellular effects and help as early warning signals of exposure to contaminants in environmental monitoring.

Other interesting aspects of these bioindicators are:

- Their wide geographic distribution
- They are easy to be collected
- They are copious in estuarine waters
- They are able to endure a range of environmental conditions and to accumulate toxic chemicals in their tissues
- They are suitable for caging experiments at field sites and mesocosm experiment (Andral et al., 2004; Fasulo et al., 2012a; 2015; Gornati et

al., 2018; Maisano et al., 2017; Pirrone et al., 2018; Romeo et al., 2003; Tsangaris et al., 2010; Viarengo et al., 2007; Wu and Shin, 1998; Sanchis et al., 2018).

1.5. Mussels: Mytilidae

The mussel *Mytilus galloprovincialis*, chosen as bioindicator in the first part of thesis, is endemic to the Mediterranean coast and the Black and Adriatic Seas. Mussels belong to one of the major classes of molluscs, Bivalvia. Mussels are essentially marine bivalves. The valves are equal in size and shape with an elongated oval-triangular form. It has been able to set itself up at broadly spread points in several seas around the world. Most of these introductions have happened in temperate areas and at sites where there are extended shipping ports (Branch and Stephanni, 2004). A relevant reason of this huge spread is also related to shipping hull fouling and transport of ballast water as indicated by a number of studies and observations (Carlton, 1992; Robinson and Griffiths, 2002; Geller, 1999). The mussel tissue investigated in the present study was the gills, which is a first organ exposed to various pollutants. In Mytilus, the two shell valves are similar in size with a roughly triangular shape, connected together at the anterior of the shell (Fig. 1.2). The shell, as a skeleton, permits the attachment of muscles and defends against predators. The opening and the closing of the shell valves are set by the two muscles, the anterior and the posterior adductors. The mantle is composed of connective tissue with haemolymph vessels, nerves and muscles. This organ encloses the animal within the shell contained most of the gonads. Moreover, the

mantle represents the main site for storage of nutrient. Cilia on the inner surface of the mantle move particles onto the gills and pull away heavier material towards the inhalant opening, where it can be discharged (Gosling, 2003; Bayne et al., 1976a). The pair of gills (ctenidia) is made up o of ciliated filaments through which branchial blood vessels move. The opposite face is called ventral or frontal. The gills perform different functions, i.e. respiratory gas exchange, blood haematosis, the capture of blood particles, uptake of nutrients and dissolved organic particles. All these functions are mainly performed by ciliary and mucous cells of the gill epithelium (Gosling, 1992; Auffret et al., 2003). In particular, these mucociliary mechanisms are used in the mussel gills and labial palps in order to filter and ingest particles suspended in the ambient water, such as bacteria, phytoplankton, detritus, microzooplankton and dissolved organic matter (Widdows and Donkin, 1992; Gosling, 2003). This way of nutrient acquisition is known as filter feeding. Water flows into the mantle cavity through the inhalant syphon, is carried through the gill filaments where particles are captured, and exits through the exhalant syphon. The quantity of particles captured depends on the volume of water transported across the gills (pumping rate) and the efficiency with which the particles are retained on the gills (Bayne et al., 1976a). After capture, the particles are moved towards the ventral ciliated particle channels on the gill filaments, incorporated into mucus strings and further transported along ciliated grooves to the labial palps for particle sorting (Beninger and StJean, 1997). The particles are both directed towards the mouth for ingestion or discarded as pseudofaeces (Beninger et al., 1992; Foster-Smith, 1978). The ingested material

undergoes extracellular digestion in the gut, and selected particles are moved from the stomach to the tubules of the digestive gland for a more complete intracellular digestion (Bayne et al., 1976a). Afterwards, the material is addressed to the intestine where absorption can occur throughout its length before elimination as faeces (Reid, 1968). in the terminal intestine part is located anus, and faecal pellets are swept away through the exhalant opening of the mantle.

1.6. Neuronal gill activity regulation

The activity of gills results regulated by the sympathetic and parasympathetic innervations of the autonomic nervous system located in the connective tissue (Catapane et al., 1974). The ciliary beating is regulated by various neurotrasmitters (Stefano 1990). Serotonin, or 5-hydroxytryptamine (5-HT), involved in the serotonergic system, plays a cilio-excitatory activity in the gills of lamellibranchiates (Gosselin, 1961; Carroll and Catapane, 2007). The molluskan gills are able to produce endogenous 5-HT, which stimulate in prompt, sustained, and reversible way. A wide range of 5-HT level is able to grade the magnitude of the response (Gosselin, 1961). The exposure to toxicant compounds is responsible of impairment in the serotoninergic system as observed in gills of the mussel *M. galloprovincialis* exposed to chromium and copper, resulting in increase of the 5-HT-stimulated adenylate cyclase activity in vivo and over-expression of 5-HT receptors (Fabbri and Capuzzo, 2006). In efferent nervous system acetylcholine is used as neural transmitter, it is synthesized in the cytoplasm of cholinergic neurons by the enzyme choline

acetyltransferase (ChAT) and split into choline and acetate in cholinergic synapses and neuromuscular junctions by acetylcholinesterase (AChE) (Matozzo et al., 2005). AChE plays a fundamental role for the regular function of the central and peripheral nervous system (Lionetto et al., 2013). The measure of AChE activity is a well known biomarker of neurotoxic compounds in aquatic organisms (Cajaraville et al., 2000; Matozzo et al., 2005; Ciacci et al., 2012; D'Agata et al., 2014). In particular, Lionetto et al. (2013) showed that AChE activity is directly inhibited by pollutants like organophosphate and carbamate pesticides, but other chemicals, such as heavy metals and hydrocarbons, may be responsible of its responsiveness (Rank et al., 2007; Ciacci et al., 2012, Cappello et al., 2015).

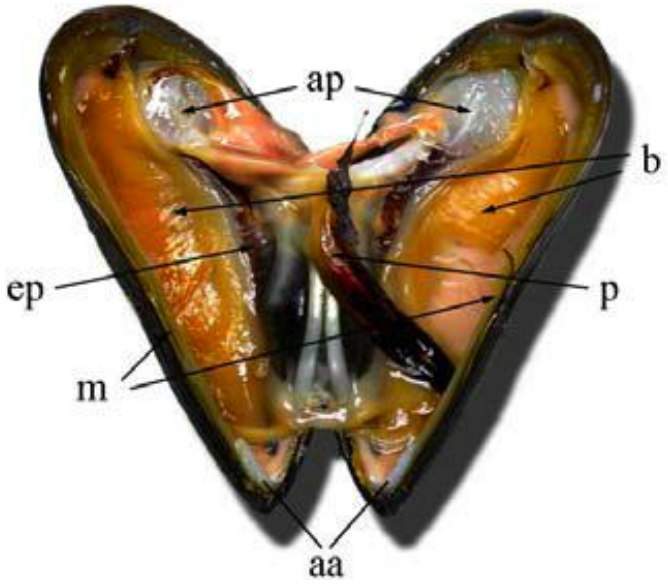


Fig. 1.2. General anatomy of Mytilus sp. Arrows indicate (aa) anterior adductor muscle; (ap) posterior adductor muscle; (m) mantle; (ep) hepatopancreas; (b) branchial epithelium.

CHAPTER II

Aim of the thesis

In this thesis, it is evaluated the performance of two remediation actions, the first one versus oil wastewater and the second one versus metal-polluted sediments, particularly by mercury. To this aim, mussels *Mytilus galloprovincialis*, well-known bioindicators, were used as the sentinel organisms, exposed in mesocosms and their responses assessed by a multi-biomarker approach.

The main aims of this thesis are:

1) The evaluation of a recovery technique (BF-MBR) versus wastewater discharges by performing mesocosm scale experiments and assessing the biological responses of mussels using a multi-biomarker approach on gills;

2) The evaluation of a remediation technique (Soil Washing) versus natural mercury-polluted sediments collected from the petrochemical site “Augusta-Melilli-Priolo” (Sicily, Italy) by performing mesocosm scale experiments and assessing the biological responses of mussels using a multi-biomarker approach on gills;

3) The evaluation of two techniques for the analysis of tight junction disruption in human airway epithelial cells induced by NaClO, a well known respiratory irritant compound.

CHAPTER III

BIOLOGICAL APPROACH TO EVALUATE THE EFFICIENCY OF-BF-MBR TREATMENT ON WASTEWATER

3.1 Material and methods

Set-up of mesocosms

All experiments were carried out in the "Mesocosm Facility" of the IAMC-CNR of Messina (Italy). Animals were housed in fiberglass tanks (150 x 150 x 150 cm, volume 3375 L) filled in continuous (125 L/h) with seawater (salinity 37-38‰) directly collected, by a pipeline, from the station “Mare Sicilia” (38°12.23’N, 15°33.10’E; Messina, Italy), in order to ensure daily water turnover (Della Torre et al., 2012; Cappello et al., 2015). Natural seawater was filtered through a 300 μm nylon mesh to remove large metazoans and detritus. To ensure a constant level of water, each mesocosm was equipped with a relief valve connected by a vertical conduct (PVC-u pn10, 200 mm Ø) placed laterally of the tank to continuously discharge the excess of seawater. Water within each mesocosm was gently mixed in a continuous mode with a pump (35 L/h) placed close to the entrance of each tank to provide more homogenous conditions within each mesocosm. The measurement of pH and temperature, performed through a multi-parametric probe, Waterproof CyberScan PCD 650 (Eutech Instruments, The Netherlands), revealed values of 19.5-20.5 °C (daily

temperature fluctuations not exceeded 1 °C) and approximatively constant pH values (around 7). The experimental set-up was conceived as follow:

1) White (W): mesocosm supplied only with seawater (uncontaminated system) at flow rate of 2 L/h.

2) Black (B): mesocosm conceived to simulate the effect of an untreated oily wastewater discharged to the marine environment. In order to achieve this goal, the mesocosm B was constantly supplied with a mixture of seawater, commercial Diesel (300 mL) and Bioversal HC (30 mL/g). The mixture was prepared by vigorously mixing in a 50 L-volume tank, 40 mL of the commercial Diesel and 4 mL of dispersant. A peristaltic pump (flow rate of 2 L/h) was used to supply all the volume of the continuously stirred tank every 24 h. The ratio between the simulated discharge and the incoming seawater flow-rate was 1:4.

3) Grey (G): this mesocosm was conceived to measure the biological effects of a BF-MBR treatment as a strategy to mitigate the marine impact of oily wastewater discharge to the sea. In this context, the same mixture used for the mesocosm B (seawater, commercial Diesel and Bioversal HC) was prepared in the continuously-stirred 50 L tank and then pre-treated through the BF-MBR as previously described (Pirrone et al., 2018). Permeate was withdrawn by using a vacuum pump; the flow rate, adjusted by opening or closing a valve before the vacuum pump, was set at 2 L/h transferring the permeate in the mesocosm G. The oxygen required for microorganism growth and membrane aeration was supplied by two sets of air diffusers, which were placed in the biological reactor and below the membrane module respectively.

Permeate flow was monitored by a rotameter placed upstream the vacuum pump. (Gornati et al., 2018)

Mixture of potential toxic compounds

Commercial Diesel (ENI Technology S.p.A.), as a mixture of potential toxic compounds, was used as described in Cappello et al. (2007).

Dispersant

Bioversal HC (BIOVERSAL INTERNATIONAL GmbH), density at 20 °C 1.02 g/ml, non toxic, pH 7.5, was here used in addition to the commercial Diesel to increase the dispersion of hydrocarbons in the entire water depth of the mesocosm, reducing its accumulation at the surface (Pirrone et al., 2018). This method was used to simulate the effect of the natural sea-water turbulence, which promotes the mixing of the oil over the water column (Gornati et al., 2018).

Mussel exposure

Adult *Mytilus galloprovincialis* Lamarck, 1819 (5.2 ± 0.4 cm shell length) were collected on March 2016 from an aquaculture plant located in the Faro lake (38°16'6.51"N; 15°38'18.57"E), one of the two brackish coastal lakes nearby Capo Peloro (Messina, Sicily). Mussels were then placed in large flow-through holding tanks (500 L) filled with natural seawater and maintained at 19-20 °C. After two weeks, almost 300 animals were transferred in each mesocosm and maintained for 15 days (experimental time) (Fig. 3.1.). At the end of the experimental time, 30

individuals were randomly sampled. from, each mesocosm (Gornati et al., 2018). Gills were frozen in liquid nitrogen and then stored at −80 ˚C for molecular, metabolomics and enzymatic analysis. For histological and immunohistochemical evaluation tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered solution (Maisano et al., 2017).

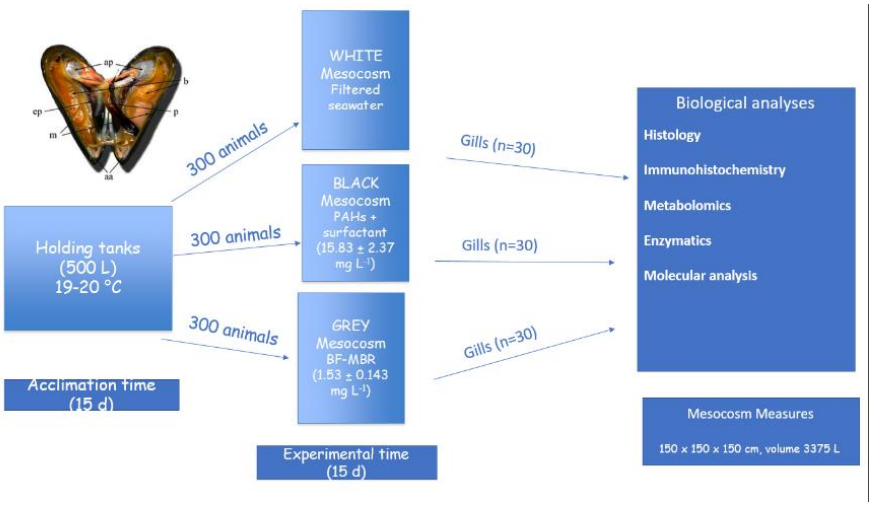


Fig. 3.1. Experimental design of Mesocosm experiment.

Measurement of the main physico-chemical parameters

The main physic-chemical parameters (pH, temperature) were measured daily in each of the three tanks through the use of a Waterproof CyberScan PCD 650 multi-parametric probe (Eutech Instruments, The Netherlands). Measurements of dissolved oxygen were likewise made by HI97196 multi-parameter probe (Hanna Instruments, Italy).

Chemical Oxygen Demand (COD) measures

The Chemical Oxygen Demand (COD) measurement was performed daily in triplicate on the three tanks by spectrophotometric measurement. This procedure provided the use of specific LCK 1014 cuvettes, that after appropriate preparation were incubated in a suitable digester (Digester HT 200 S, HACH Lange) for 15 minutes at 170 °C. The COD reading, expressed as mg/L, is obtained by the spectrophotometer (DR 3900 spectrophotometer, HACH Lange).

Quantitative analysis of hydrocarbons

Quantitative measurements of the hydrocarbons present in the experimental systems were carried out on the three mesocosms in triplicate, on days 7, 10 and 15 from the beginning of the experimentation in order to verify the BF-MBR efficiency during the experimental time. Hydrocarbon extraction and analysis was conducted

in accordance to the 3510 EPA (Environmental Protection Agency) method.

Histological analysis

Gills for histological assessment were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered solution (pH 7.4) at 4 °C, dehydrated in a graded series of ethanol and embedded in Paraplast (Bio-Optica, Italy). Histological sections, 5 µm thick, were cut with a rotary automatic microtome (Leica Microsystems, Wetzlar, Germany), glass-slide mounted and stained with Hematoxylin/Eosin (Bio-Optica, Italy) to evaluate morphological features. Observations were made on five fields of one section for sample using a 40X oil-immersion objective with a motorized Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Gottingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany)

Immunohistochemical analysis

Histological sections of mussel gills were also used for immunodetection of neurotransmission biomarkers applying an indirect immunofluorescence method (Cappello et al., 2015; Maisano et al., 2017) for localization of 5-HT and its receptor (5-HT_3R), AChE and ChAT. Briefly, sections were incubated for 1 h with normal goat serum (NGS) in PBS (1:5) to blocking non-specific binding sites for immunoglobulins. The sections were then incubated o.n. at 4° C in a

humid chamber with the primary antisera, namely mouse anti-5-HT antibody (Product No. M0758; Dako Cytomation, Milan, IT) diluted 1:50, rabbit anti-5-HT_3R antibody (Product No. S1561; Sigma-Aldrich, St. Louis, MO, USA) diluted 1:100, mouse anti-AChE antibody (Product No. MAB304; Chemicon International, Temecula, CA, USA) diluted 1:50, rabbit anti-ChAT antibody (Product No. AB6168; Abcam, Cambrige, UK) diluted 1:250. After a rinse in PBS for 10 min, sections were incubated for 2 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) or tetramethylrodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Sigma), diluted 1:50. Positive controls for labelling specificity of each peptide were performed by incubating sections with antiserum pre-absorbed with the respective antigen (10-100 g/mL). The pre-absorption procedures were carried out o.n. at 4°C. In addition, negative controls were also performed by substitution of non-immune sera (without antibodies) for the primary antisera. All observations were made on five fields of one section per sample using a 40X oil-immersion objective with a motorized Zeiss Axio Imager Z1 epifluorescence microscope (Carl Zeiss AG, Werk Gottingen, Germany), equipped with an AxioCam digital camera (Zeiss, Jena, Germany) for the acquisition of images. Sections were imaged using the appropriate filters for the excitation of FITC (480/ 525 nm) and TRITC (515/590 nm), and then processed by using AxioVision Release 4.5 software (Zeiss).

Metabolomics analysis

Gill metabolite extraction

Polar metabolites were extracted from gill tissues of mussels using a "two-step" methanol/chloroform/water procedure (Cappello et al., 2013b; Wu et al., 2008; Maisano et al 2017). In brief, a 100 mg sub-sample of each gills was homogenized in 4 mL/g of cold methanol and 0.85 mL/g of cold water by a TissueLyser LT bead mill (Qiagen) with 3.2 mm stainless steel beads, for 10 min at 50 vibrations/s. Homogenates were transferred into glass vials, and 4 mL/g chloroform and 2 mL/g water were added. After vortexed, samples were left on ice for 10 min for phase separation, and centrifuged for 5 min at 2000 g at 4° C. A volume of the upper methanol layer (600 µL) containing the polar metabolites were transferred into glass vials, dried in a centrifugal vacuum concentrator (Eppendorf 5301), and stored at -80 °C. Prior to Nuclear Magnetic Resonance (NMR)-based metabolomics analysis, the dried polar extracts were resuspended in 600 µL of a 0.1 M sodium phosphate buffer (pH 7.0, 10% D_2O; Armar AG, Dottingen, Switzerland) containing 1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (Sigma-Aldrich Co) as internal reference. The mixture was vortexed and transferred to a 5 mm diameter NMR tube. The DSS acted as an internal standard and provided a chemical shift reference (δ = 0.0 ppm) for the NMR spectra, whereas the D_2O provided a deuterium lock for the NMR spectrometer.

^{1}H NMR-based metabolomics analysis

Extracts of gill tissue were analyzed on a Varian-500 NMR spectrometer operating at a spectral frequency of 499.74 MHz at 298 K. One-dimensional (1-D) ^{1}H NMR spectra were obtained using a PRESAT pulse sequence to suppress the residual water resonance and 6983 Hz spectral width with a 2.0 s relaxation delay. A total of 256 transients were collected into 16,384 data points requiring a 19 min acquisition time. All data sets were zero filled to 32,768 data points and exponential line-broadenings of 0.5 Hz were applied before Fourier transformation. All ^{1}H NMR spectra were manually phased, baseline-corrected, and calibrated (DSS at 0.0 ppm) using Chenomx Processor, a module of Chenomx NMR Suite (version 5.1; Chenomx Inc., Edmonton, Canada) software. The peaks of interest, namely the metabolites related to serotonergic (i.e. serotonin) and cholinergic (i.e. acetylcholine) systems, were identified within the ^{1}H NMR spectra (Cappello et al., 2013b; Fasulo et al., 2012b) and quantified using Chenomx Profiler, another module of Chenomx NMR Suite software, which uses the concentration of a known DSS signal to determine the levels of individual metabolites (Brandao et al., 2015; Cappello et al., 2015).

Enzymatic analysis

Acetylcholinesterase (AChE) activity was estimated in the gills of mussels by using the colourimetric method of Ellman et al. (1961), with small changes, by UV–Vis–UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). In short, thiocholine derivatives are hydrolysed by acetylcholinesterase to yield thiocholine. The subsequent combination with 5,5-dithiobis-2-dinitrobenzoic acid (DTNB) forms the yellow anion 5-thio-2-nitrobenzoic acid, which absorbs strongly at 412 nm. AChE activity was expressed as μmol/min/mg (Maisano et al., 2017).

Molecular analysis

RNA extraction and cDNA synthesis

Tissue homogenization and RNA extraction from gill tissues of *Mytilus galloprovincialis* collected at each sampling site were performed using Qiazol reagent (Qiagen). RNA quantity and quality were evaluated as detailed by Giannetto et al. (2015). cDNA synthesis from 1 mg total RNA was performed by QuantiTect reverse transcription kit (Qiagen) after gDNA wipeout buffer treatment in order to remove any potential genomic DNA contamination, following manufacturer's instructions.

5-HT$_3$R quantitative gene expression (qPCR)

Degenerate primers (Table 3.1.) were designed on the conserved regions of 5-HT receptor genes isolated from other mussels.

PCR product was separated on 1% agarose gels. The region containing the expected size fragment was sliced, purified using QIAquick Gel Extraction kit (Qiagen) and sequenced using ABI PRISM BigDye Terminator 3.1 Cycle Sequencing kit (PE Applied Bio-system). The sequence (340 bp) was submitted to NCBI database. Quantification of 5-HT$_3$R gene expression in *M. galloprovincialis* gills was performed by real-time PCR using the Rotor-Gene Q 2plex Hrm thermocycler (Qiagen) with SYBR Green chemistry (Qiagen) as mentioned by Giannetto et al. (2014). qPCR primers, listed in Table 3.1., were designed using the Beacon Designer TM online tool (http://www.premierbiosoft.com/qOligo/ Oligo.jsp?PID=1) for the target and reference genes analysed.

The actin (act), 18S ribosomal RNA (18S rRNA) and elongation factor (ef1-α), were chosen as reference genes. The primers for the reference genes are reported in Giannetto et al. (2015). Twenty-fold diluted gill cDNA samples were run in duplicate and no template and minus reverse transcriptase controls were included in each reaction The PCR efficiency determination was made by a five-point standard curve of a 5-fold dilution series (1:1 to 1:32) from pooled RNA (Fernandes et al., 2006). To correct the raw data genes a Normalization Factor was used. This Normalization Factor was calculated from the two most stable genes (*18S rRNA* and *act*) by by geNorm software

(http://medgen.ugent.be/~jvdesomp/genorm/). The single-peak melting curves confirmed the specificity of the reaction.

Table. 3.1. Nucleotide sequences of primers, amplicons size (bp), methods, qPCR efficiencies (E%),

Primer	Primer sequence	Size (bp)	Methods	E (%)
5HT_ R _FWD	ATTNCGTTGGNTCGGTNCTG	340	Cloning	
5HT _R_REV	TANCGCCAGANCAATTNCAT			
q5HT _R _FWD	TAACGCCAGACCAATTCCAT	95	qPCR	98
q5HT _R_ REV	TGAAGCCATCTTGACTGACG			102

Statistical analysis

Statistical analyses were made by GraphPad software (Prism 5.0, San Diego CA, USA) for immunohistochemical, metabolomics and enzymatic data, while molecular data were analyzed using SigmaPlot (Systat software). Results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyse statistically the data, applying the Dunnett's multiple comparison test, in order to determine significant differences between control and treatment groups and the Student-Newman-Keuls post-hoc tests to assessing differences in 5-HT_3R expression levels between mussels from the three mesocosms. Data were considered statistically significant at $p < 0.05$.

3.2. Results

Physico-chemical parameters

No significant variations were noted throughout the experimental period of the measured values of the main physico-chemical parameters (pH, mean value 7.9 ± 0.1; temperature, mean value 15.2 ± 0.5 ° C; dissolved Oxygen, 5 ± 0.5 mg/L)**.**

COD measures

The contribution of the polluting load measured by the COD in comparison with the control showed COD average values of about 50-55 mg/L in the black mesocosm and about 30-35 mg/L in the grey mesocosm receiving the treated water. The control recorded average COD values around 22-24 mg/L. In all the tanks, the COD parameter initially recorded significant increases at the time of placing the mussels (values around 110-115 mg/L are evidence of suspended material - unfiltered sample), and then lowered to the aforementioned values after a couple of water changes within the systems.

Levels of hydrocarbons

The values of the hydrocarbons for the three days of water sampling (average on the triplicate of the samples taken) are shown in the following table (Table 3.2). In the control, all the values are below the limits of detection. The level of PAHs in the grey mesocosm (1.53 ± 0.143 mg/L) after the wastewater treatment, was reduced of almost 10 times (90% efficiency) than black mesocosm (15.83 ± 2.37 mg/L). This efficiency is maintained during the various measurements carried out in the selected days as indicated above. These data point out the efficiency of BF-MBR to reduce the PAH concentration in oil wastewater discharge.

Table 3.2. Quantitative analysis of hydrocarbons mean (µg/L) and Standard Deviation (SD); RE (Removal Efficiency) in BLACK and GREY mesocosms

Day		BLACK (Mean) (µg/L)	BLACK (SD)	GREY (Mean) (µg/L)	GREY (SD)	RE (%)
7th	**THC**	**13,527**	**855**	**1,820**	**168**	**87**
	C6-C10	7	3	6	4	
	C10-C20	10,930	838	1,453	154	
	C20-C30	2,600	88	350	40	
	C30-C40	40	22	31	18	
11th	**THC**	**14,954**	**664**	**1,689**	**280**	**89**
	C6-C10	4	3	6	2	
	C10-C20	12,120	365	1,353	142	
	C20-C30	2,840	334	309	116	
	C30-C40	54	33	28	22	
15th	**THC**	**15,836**	**2,376**	**1,531**	**143**	**90**
	C6-C10	9	3	6	4	
	C10-C20	12,803	1,791	1,233	103	
	C20-C30	3,040	568	273	68	
	C30-C40	51	25	26	14	

Histology

In the gills of *M. galloprovincialis* from the W mesocosm is possible to observe the regular morphology of gill tissue (Fig 3.2A.), consisting of parallel filaments whose whole surface is coated by various cilia. In the B mesocosm is showed a critical aberration of the morphology, with a relevant detachment of respiratory epithelium from the underlying connective tissue and loss of the cilia (3.2B.). In the G mesocosm instead, it is possible to observe a relevant reduction of cilia in respect to the control (3.2C.), but the tissue organization is maintained.

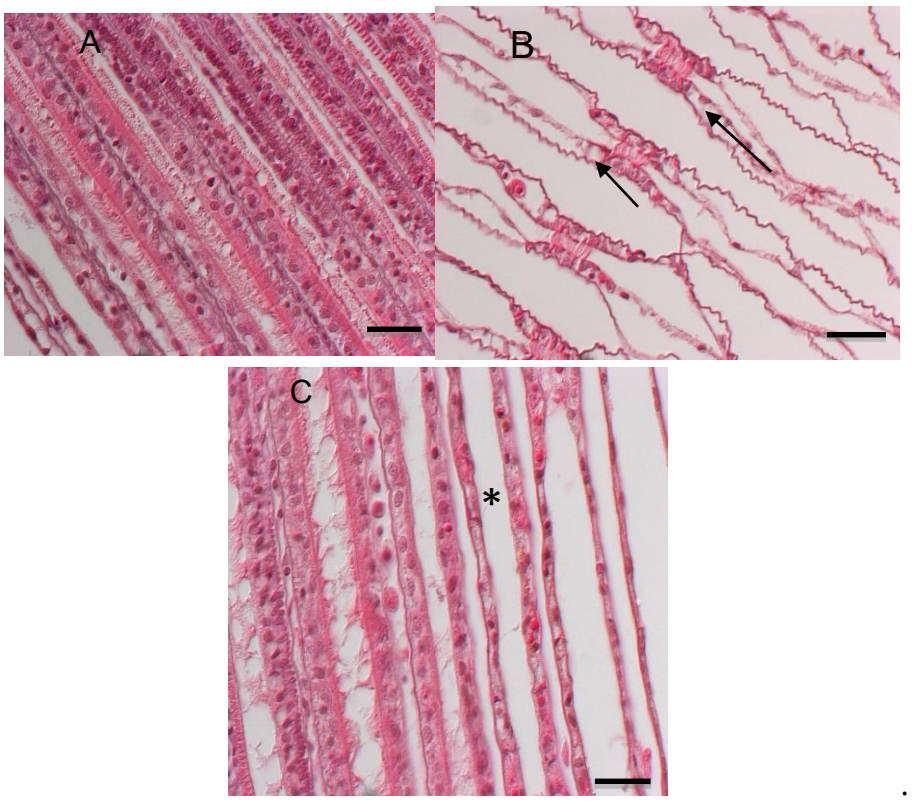


*Fig.3.2. Haematoxylin and Eosin (H&E) staining in the gills of Mytilus galloprovincialis in Mesocosm W(A), Mesocosm B (B) and Mesocosm G (C). The arrow shows detachement of cells and * indicates loss of cilia. Scale bars, 20 µm.*

Immunohistochemistry

With regards the serotonergic system, in the mesocosm of control, it is possible to observe an intense immunopositivity for serotonin (5-HT) (3.3A.) within the cells, whilst there is a moderate immunopositivity of serotonin receptor (5-HT_3R) (3.4A.). In the case of the B mesocosm, it is possible to recognize a drastic reduction of 5-HT immunopositivity (3.3B) and an intense positivity for 5-HT_3R in fibers and a very low signal in the cells (3.4B.). In the G mesocosm (3.3C.; 3.4C.) the signal of 5-HT is high in fibers while the immunopositivity of 5-HT_3R is high in the fiber and cells. Statistical analyses of immunohistochemical results for 5-HT and 5-HT_3R are shown in 3.3D and 3.4D. About the cholinergic system, the immunopositivity of acetylcholinesterase (AChE) and acetyltransferase (ChAT) is low and present only in the cells (Fig. 3.5A.;3.6A.). In the B mesocosm (3.5B; 3.6B.), it is possible to evaluate an increase of the signal for the two enzymes in the cells. In the G mesocosm (3.5C.; 3.6C.), the intensity of the signal is also high, and in addition, it is possible to observe an immunopositivity also in fibers. Statistical analyses of immunohistochemical results for AChE and ChAT are shown in 3.5D. and 3.6D.

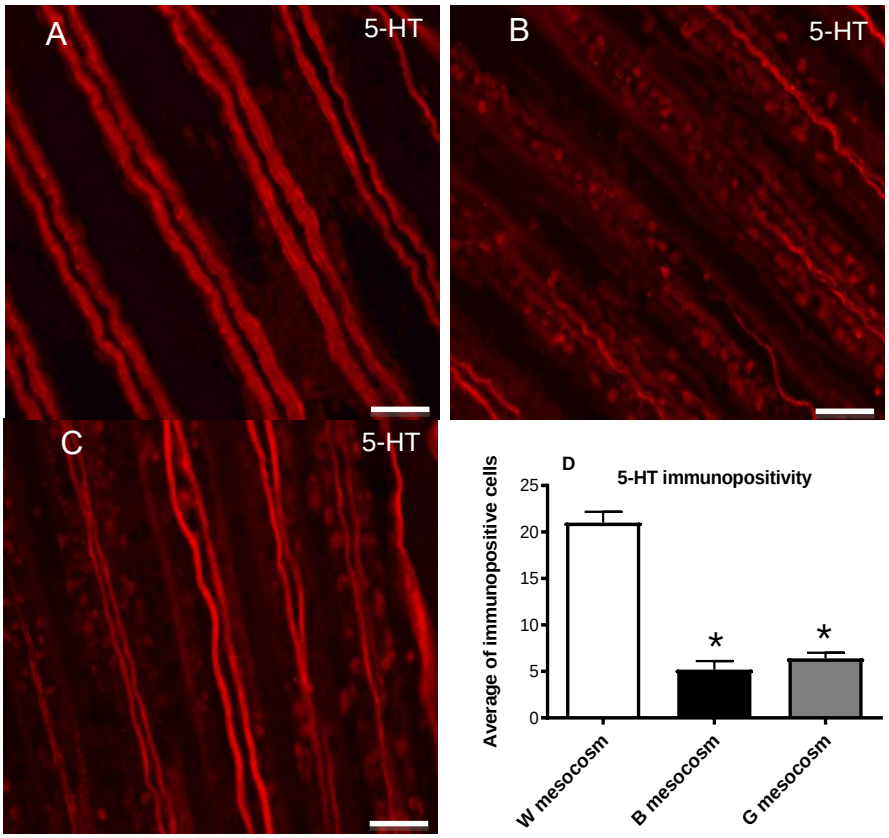


Fig 3.3. Immunohistochemical labeling for 5-HT in mussel gills in Mesocosm White (A), Mesocosm Black (B) and Mesocosm Grey (C). Mean and standard deviation (S.D.) of immunopositive cells (D). Significant difference $p < 0.05$.between Control (White Mesocosm) and the Black and Grey Mesocosm is indicated by an asterisk. Scale bars 20 μm.

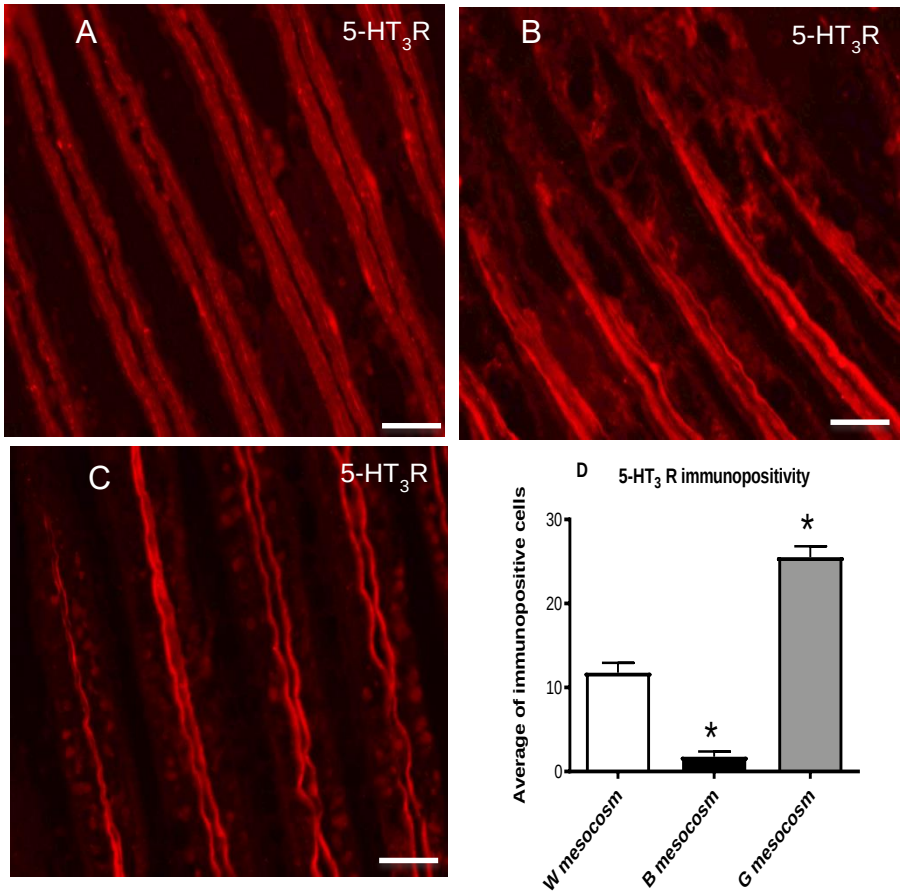


Fig 3.4. Immunohistochemical labeling for 5-HT$_3$ R in mussel gills in Mesocosm White (A), Mesocosm Black (B) and Mesocosm Grey (C). Mean and standard deviation (S.D.) of immunopositive cells (D). Significant difference $p < 0.05$.between Control (White Mesocosm) and the Black and Grey Mesocosm is indicated by an asterisk. Scale bars 20 μm.

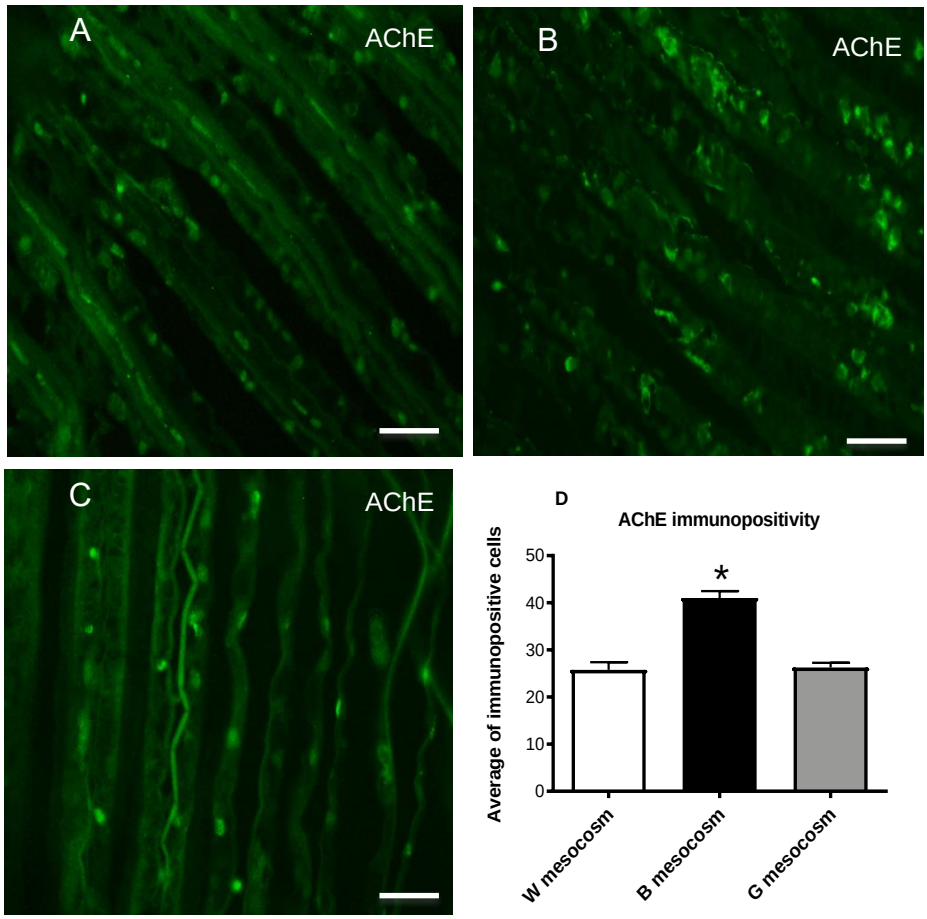


Fig3.5 .Immunohistochemical labeling for AChE in mussel gills.in Mesocosm White (A),Mesocosm Black (B) and Mesocosm Grey (C).). Mean and standard deviation (S.D.) of immunopositive cells (D). Significant difference $p < 0.05$.between Control (White Mesocosm) and the Black and Grey Mesocosm is indicated by an asterisk. Scale bars 20 μm.

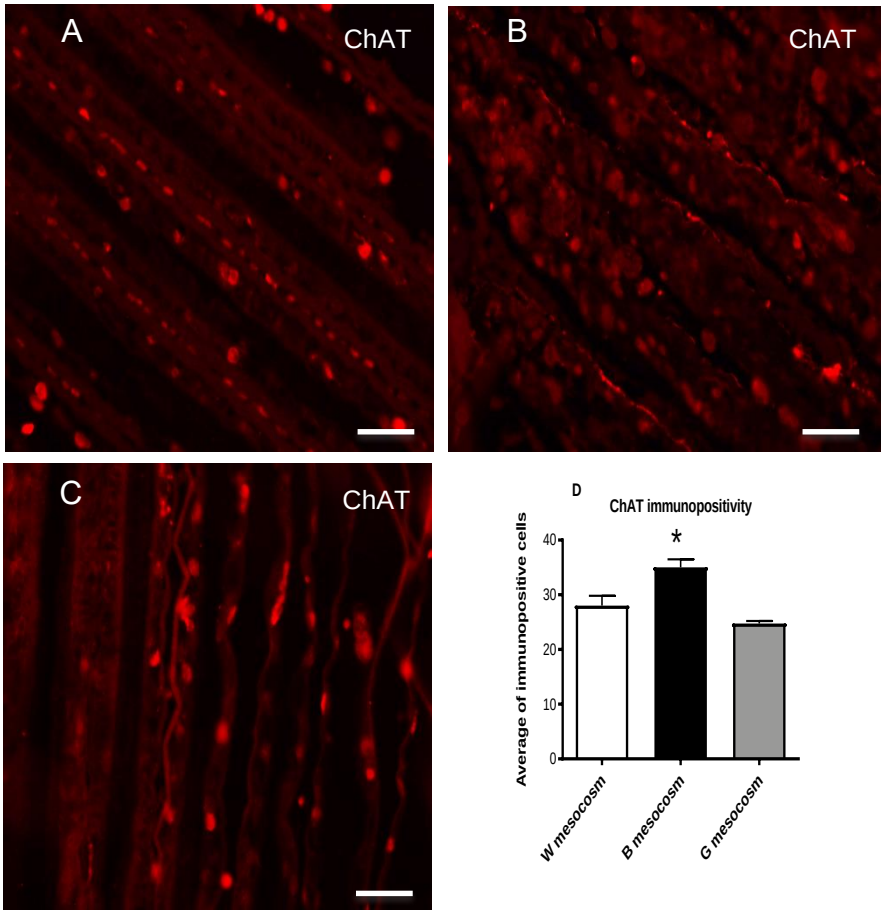


Fig. 3.6. Immunohistochemical labeling for ChAT in mussel gills in mesocosm White (a), mesocosm Black (B) and mesocosm Grey (C). Mean and standard deviation (S.D.) of immunopositive cells (D). Significant difference $p < 0.05$.between Control (White Mesocosm) and the Black and Grey Mesocosm is indicated by an asterisk. Scale bars 20 μm.

Metabolomics

With regards to the amount of the two neurotransmitters, in the case of serotonin, in the B mesocosm it is possible to observe an almost half reduction of serotonin compared to the W mesocosm, while in the G mesocosm, the concentration is slight higher than the control. The level of acetylcholine is slight higher than the control in the B mesocosm, while there is a three time increase of acetylcholine in respect to the control in the G mesocosm (Fig 3.7.). All reported data are statistically significant data.

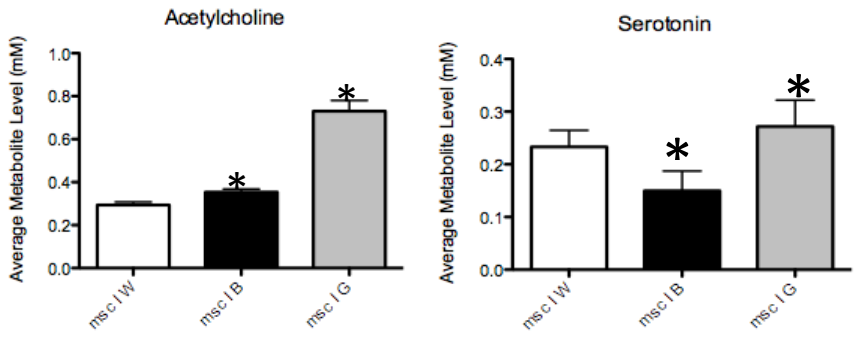


Fig 3.7. Average metabolite level of Serotonin and Acetylcholine in the three mesocosms.:p value < 0.05*

Enzymatics

From the comparison of the enzymatic activity in the three different mesocosms it is possible to observe a significant reduction of about 20% of AChE activity in the gills of mussels exposed to high concentrations of PAHs in the B mesocosm. In the G mesocosm, where wastewater was treated by BF-MBR, the enzymatic activity of AChE does not look inhibited hence the level of AChE activity appears equivalent to that recoreded in the W mesocosm (Fig. 3.8.).

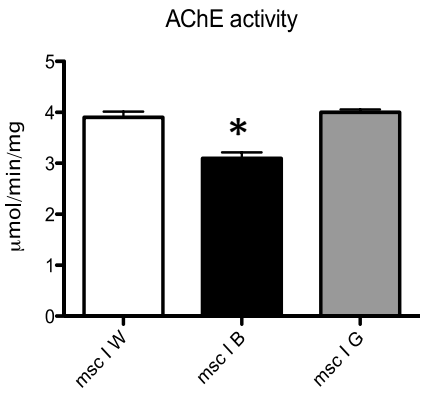


*Fig. 3.8. Evaluation of AChE activity in the three mesocosms. *:p value < 0.05*

Molecular results

In regard to the gene expression of the serotonin receptor 5-HT_3R, it is possible to observe variations in the different experimental conditions. In the black mesocosm, the gene expression of 5-HT_3R is three times higher than that observed in the white mesocosm. In the grey mesocosm, where wastewater was treated by BF MBR, a three time increase in gene expression is observed compared to the W mesocosm, while the increase compared to the B mesocosm is slight (statistically significant data) (Fig 3.9.).

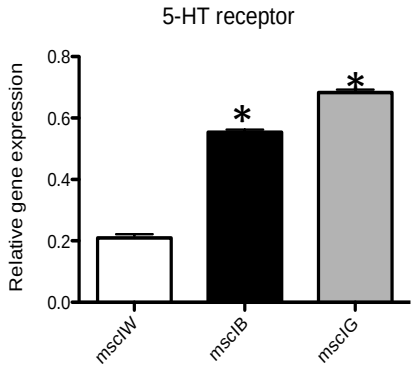


Fig 3.9. Evaluation of 5-HT receptor in the three mesocosms. * p. value< 0.05

3.3. Discussion

The pollution of water is the cause of severe alteration in the environment with serious damage to the ecosystem and living organisms. In particular, a great attention was focused over the pollution concerning the impact of the oil wastewater. Hence, despite events like oil spills look very impressing with regards to marine pollution, the higher oil load and consequent risk for marine pollution is mainly associated with routine operations deriving from slops, dirty ballast, sewage and bilge water management (Ciacci et al., 2012; Fasulo et al., 2015, Mancini et al., 2017). A common method used for oil wastewater treatment is a chemical de-emulsification followed by secondary clarifications. This kind of technique needs the use of a variety of chemical compounds such as sulphuric acid, iron and alumina sulphates, etc. (Al-Shamrani et al., 2002; Verma et al., 2010). The BioFilm Membrane Bio Reactor (BF-MBR), a growing biofilm system, became a very interesting method in the wastewater treatment sector (Barwal and Chaudhary, 2014; Gornati et al., 2018). The evaluation of BF-MBR efficiency is object of various studies. For instance, a pilot BF-MBR, inoculated with halophilic activated sludge and *Alcanivorax borkumensis*, was able to remove 72 and 90% of COD from low strength bilge water (1 g COD/L) in 30 days (Mancini et al., 2012). In Pirrone et al. (2018), the efficiency of a BF-MBR in reducing oily-wastewaters discharge impact to marine environment was investigated by small-scale (100 L) artificial systems (microcosms). In this work, the efficiency of BF-MBR is confirmed in a scale-up system mesocosom (3375 L). A 10-time reduction of PAH was observed.

Therefore, with regards to the chemical aspect, the BF-MBR looks useful to decrease the concentration of the petrochemical pollutants in the wastewater discharges. In this experiment, the efficiency of BF-MBR treatment was evaluated by a multi-biomarker approach in mussel's gills in a mesocosm scale experiment. The use of mesocosm represents a very good tool to evaluate the effects of pollutants (Sanchis et al., 2018), and the microcosm and Mesocosm has just offered also good brilliant results in the evaluation of BF MBR (Pirrone et al., 2018; Gornati et al.,2018).

The mussel, in particular *M. galloprovincialis*, is a very common "sentinel organism" used in several biomonitoring programmes (Cappello et al., 2015; Fasulo et al. 2015; Maisano et al., 2017). The gills are important for various functions, i.e. respiratory gas exchange, blood haematosis, the capture of blood particles, uptake of nutrients and dissolved organic particles. All these functions are mainly performed by the ciliary and mucous cells of the gill epithelium, so the evaluation of their function is fundamental. The histomorphological analysis showed a severe aberration of the gill morphology in samples from the black mesocosm in respect to control, with a severe detachment of epithelium from the connective tissue. A better morphology, even if not regular such as in the control and with loss of cilia, was observed in the gills of samples from the G mesocosm. The observation of histomorphological alteration represents a signal of impairment of the functional integrity of gills (Cappello et al., 2013b; Maisano et al., 2017).

To better evaluate the alteration of cilia activity, which plays a key role in most of the gill functions (Sunila, 1998), the neurotransmission system, i.e. serotoninergic and cholinergic, was analysed by a multi-biomarker approach since the cilia activity in mussels is under control of the neurotransmission system (Gosselin, 1961; Carroll and Catapane, 2007; Matozzo et al., 2005). With regards to the serotoninergic system, the level of serotonin, evaluated by metabolomics and immunohistochemical approach, was lower in the B mesocosm, and higher in the G mesocosm in respect to the control. This data suggests that the BF-MBR treatment is able to create good condition for the synthesis of serotonin and partial improvement for functionality of the serotoninergic system in the G mesocosm than in B mesocosm. Instead, the evaluation of 5-HT receptor, performed by molecular and immunohistochemical analysis, showed an up-regulation in samples from both B and G mesocosms. The rise of 5-HT_3R could be related to an adaptive response mediated by the paracrine signaling activities in order to recover a regular physiological activity in gills. Indeed, it has been reported that gill epithelial cells containing the lateral cilia present 5-HT receptors to increasing the ciliary beating rate (Cappello et al., 2015; Maisano et al., 2017).

The movement of cilia is also under control of the cholinergic system, responsible for the physiological functioning of the efferent nervous systems (Matozzo et al., 2005). The acetylcholinesterase (AChE) is a well known enzyme in ecotoxicology, since it possible to observe its inhibition by the presence of contaminants in complex mixtures (Matozzo et al., 2005; Cajaraville et al., 2000; Tsangaris et al., 2010;

Cravo et al., 2012; D'Agata et al., 2014), by pollutants such as organophosphate and carbamate pesticides (Lionetto et al., 2013), or heavy metals and hydrocarbons (Rank et al., 2007; Ciacci et al., 2012). With regards to AChE activity, it was evaluated by enzymatic assay combined with the metabolomic evaluation of acetylcholine. In addition, AChE level and distribution were observed by an immunohistochemistry assay. AChE enzymatic activity was reduced while the level of acetylcholine and AChE immunopositivity increased in the B mesocosm compared to the control. The increase of acetylcholine level is probably related to the inhibition of AChE. The enhancement of AChE level is a possible adaptive compensatory mechanism in order to heal a regular physiological function of gills. Except for the enzymatic activity, the observed results related to AChE appeared different from those reported by Cappello et al. (2015) and Maisano et al. (2017) in mussels caged in a petrochemical polluted site for 30 and 60 days. Choline acetyltransferase (ChAT), the key enzyme responsible for the synthesis of acetylcholine, was also evaluated. The ChAT immunopositivity increased in the B mesocosm. This enhancement, observed in Cappello et al. (2015), suggests the induction of adaptive compensatory responses mediated by the paracrine signalling activities in order to recover a regular physiological function of gills. In the G mesocosm, the AChE activity was not inhibited and the immunopostivity for AChE and ChAT was lower, while the acetylcholine level relevantly increased than in W and B mesocosms. Overall, this response pattern suggests that the BF MBR for wastewater

treatment is likely able to partly restore the condition observed in the W mesocosm.

3.4. Conclusion

The BF-MBR results a very good method to reduce the amount of the chemical concentration of pollutants in wastewater. The mesocosm scale experiment proved to be a very good tool to evaluate the effects of wastewater and BF MBR treated wastewater versus living organisms. Mussels, as seen in nature, were validated as very good biondicators also in this experimental approach. The multi-biomarker battery, applied in order to evaluate the condition of gills and its neurotransmission system, represents a good tool to understand better the health condition of samples in the three different experimental conditions. Hence, thanks to this approach, it is possible to report that the functionality of mussel gills in the G mesocosm was not so critical as that one observed in samples from the B mesocosm, but at the same time it was not possible to observe the same regular morphology and neurotransmission system activity found in the W mesocosm. Therefore, further studies are necessary for the improvement of this treatment.

CHAPTER IV

Biological evaluation of the Soil Washing remediation versus natural mercury-polluted sediments in a mesocosm scale experiment

4.1 Material and methods

Set-up of mesocosms

All tests were executed in the "Mesocosm Facility" of the IAMC-CNR of Messina (Italy). Animals were housed in fiberglass tanks (150 x 150 x 150 cm, volume 3375 L) filled in continuous (125 L/h) with seawater (salinity 37-38‰) directly collected, by a pipeline, from the station “Mare Sicilia” (38°12.23’N, 15°33.10’E; Messina, Italy), in order to guarantee periodic water turnover (Della Torre et al., 2012; Cappello et al., 2015). Natural seawater was filtered through a 300 μm nylon mesh to exclude large metazoans and detritus. To guarantee a constant level of water, each mesocosm was provided with a relief valve connected by a vertical conduct (PVC-u pn10, 200 mm Ø) located laterally of the tank to continuously remove the excess of seawater. Water within each mesocosm was lightly mixed in a continuous mode with a pump (35 L/h) placed close to the entrance of each tank to provide more homogenous conditions within each mesocosm. The measurement of pH and temperature, performed through a multi-parametric probe Waterproof CyberScan PCD 650 (Eutech Instruments, The Netherlands), revealed values of 19.5-20.5 °C (daily temperature

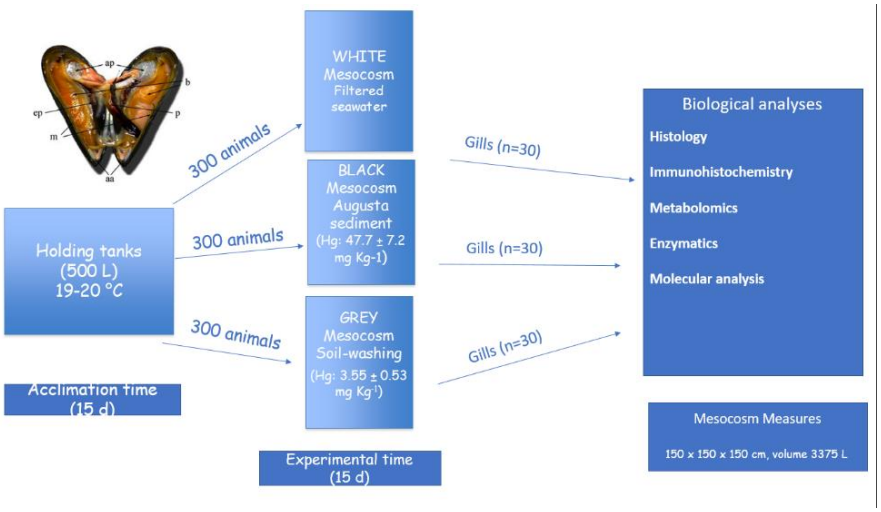
fluctuations not exceeded 1 °C) and approximatively constant pH values (around 7). The experimental set-up (Fig 4.1) was conceived as follow:

1. White mesocosm (WS): mesocosm supplied with seawater (uncontaminated system) at flow rate of 2 L/h. 125 kg unpolluted sediments were placed inside the mesocosm.
2. Black mesocosm (BS): approximately 125 kg of polluted sediments from the petrochemical site "Augusta-Melilli-Priolo" (Sicily, Italy) were placed at the bottom of the tank, showing a significant mercury contamination (about 50 mg/kg compared to an intervention limit value of 1 g/kg). Overall, the mercury in the sediment tank was about 860 mg by mass.
3. Grey mesocosm (GS): an equal quantity of sediments previously treated by numerous cycles of soil washing by EDTA, EDDS (Mancini et al., 2011) was arranged in order to reduce metal contamination above all of mercury.

Mussel exposure

Adult *Mytilus galloprovincialis* Lamarck, 1819 (5.2 ± 0.4 cm shell length) were collected in March 2016 from an aquaculture plant placed in the Faro Lake (38°16'6.51"N; 15°38'18.57"E), one of the two brackish coastal lakes nearby Capo Peloro (Messina, Sicily). Mussels were then stored in large flow-through holding tanks (500 L) filled with natural seawater and maintained at 19-20 °C. After two weeks of acclimation, about 300 individuals were transferred in each mesocosm

and kept for 15 days (Fig. 4.1). At the end of the experimental time, 30 animals from each mesocosm were randomly sampled (Gornati et al., 2018). Gills were frozen in liquid nitrogen and then stored at −80 ˚C for molecular, metabolomics and enzymatic analysis. For histological and immunohistochemical evaluation, tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered solution (pH 7.4) (Maisano et al., 2017).



F*ig 4.1. Experimental design of Mesocosm experiment.*

Measurement of the main physico-chemical parameters

The main physico-chemical parameters (pH, temperature) were measured daily in each of the three tanks through the use of a Waterproof CyberScan PCD 650 multiparametric probe (Eutech Instruments, The Netherlands). Measurements of dissolved oxygen (DO) were likewise made by a HI97196 multiparameter probe (Hanna Instruments, Italy).

Chemical Oxygen Demand (COD) measures

The Chemical Oxygen Demand (COD) measurement was performed daily in triplicate on the three tanks by spectrophotometric measurement. This procedure provided the use of specific LCK 1014 cuvettes, which after appropriate preparation were incubated in a suitable digester (Digester HT 200 S, HACH Lange) for 15 minutes at 170 °C. The COD reading, expressed as mg/L is obtained by a spectrophotometer (DR 3900 spectrophotometer, HACH Lange).

Quantitative analysis of sediment metals

For determination of metals, prior to analysis, sediment samples from Mesocosms were digested in concentrated nitric acid and concentrated hydrochloric acid using microwave heating (Ethos easy digestion system, Millestone) according to the US EPA 3051A sample preparation method (US EPA, 2007). The sample and acids were placed in a fluorocarbon polymer (PFA) microwave vessel, sealed and heated in the microwave unit. After cooling, the vessel contents were filtered and then diluted to volume and analysed by Inductively coupled plasma-mass spectrometry (PerkinElmer Nexion 350X Spectrometer) according to the EPA 6020A method. Reagent-grade chemicals were used in all the preparations (Maisano et al. 2017).

Histological analysis

Gills for histological evaluation were fixed in 4% paraformaldehyde in 0.1M phosphate buffered solution (pH 7.4) at 4 °C, dehydrated in a graded series of ethanol and embedded in Paraplast (Bio-Optica, Italy). Histological sections, 5 µm thick, were cut with a rotary automatic microtome (Leica Microsystems, Wetzlar, Germany), glass-slide mounted and stained with Hematoxylin/Eosin (Bio-Optica, Italy) to evaluate morphological components. Observations were made on five fields of one section per sample using a 40X oil-immersion objective with a motorized Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Gottingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany).

Immunohistochemical analysis

Histological sections of mussel gills were also utilized for immunodetection of neurotransmission biomarkers using an indirect immunofluorescence method (Cappello et al., 2015; Maisano et al., 2017) for localization of 5-HT and its receptor (5-HT_3R), AChE and ChAT. Summarily, sections were incubated for 1 h with normal goat serum (NGS) in PBS (1:5) to blocking non-specific binding sites for immunoglobulins. The sections were then incubated o.n. at 4° C in a humid chamber with the primary antisera, namely mouse anti-5-HT antibody (Product No. M0758; Dako Cytomation, Milan, IT) diluted 1:50, rabbit anti-5-HT_3R antibody (Product No. S1561; Sigma-Aldrich, St. Louis, MO, USA) diluted 1:100, mouse anti-AChE antibody

(Product No. MAB304; Chemicon International, Temecula, CA, USA) diluted 1:50, rabbit anti-ChAT antibody (Product No. AB6168; Abcam, Cambridge, UK) diluted 1:250. After a wash in PBS for 10 min, sections were incubated for 2 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Sigma), diluted 1:50. Positive controls for labelling specificity of each peptide were performed by incubating sections with antiserum pre-absorbed with the respective antigen (10 and 100 g/mL). The pre-absorption procedures were carried out o.n. at 4 °C. In addition, negative controls were also performed by substitution of non-immune sera (without antibodies) for the primary antisera. All observations were made on five fields of one section per sample using a 40X oil-immersion objective with a motorized Zeiss Axio Imager Z1 epifluorescence microscope (Carl Zeiss AG, Werk Gottingen, Germany), equipped with an AxioCam digital camera (Zeiss, Jena, Germany) for the acquisition of images. Sections were imaged using the appropriate filters for the excitation of FITC (480/ 525 nm) and TRITC (515/590 nm), and then processed by using AxioVision Release 4.5 software (Zeiss)

Metabolomics analysis

Gill metabolite extraction

Polar metabolites were extracted from gill tissues of mussels using a "two-step" methanol/chloroform/water procedure (Cappello et al., 2013b; Wu et al., 2008; Maisano et al., 2017). In summary, a 100 mg sub-sample of each gill was homogenized in 4 mL/g of cold methanol and 0.85 mL/g of cold water by a TissueLyser LT bead mill (Qiagen) with 3.2 mm stainless steel beads, for 10 min at 50 vibrations/s. Homogenates were carried into glass vials, and 4 mL/g chloroform and 2 mL/g water were added. After vortexed, samples were left on ice for 10 min for phase separation, and centrifuged for 5 min at 2000 g at 4° C. A volume of the upper methanol layer (600 µL) containing the polar metabolites were transferred into glass vials, dried in a centrifugal vacuum concentrator (Eppendorf 5301), and stored at -80 °C. Prior to Nuclear Magnetic Resonance (NMR)-based metabolomics analysis, the dried polar extracts were re-suspended in 600 µL of a 0.1 M sodium phosphate buffer (pH 7.0, 10% D_2O; Armar AG, Dottingen, Switzerland) containing 1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (Sigma-Aldrich Co) as the internal reference. The mixture was vortexed and transferred to a 5 mm diameter NMR tube. The DSS acted as an internal standard and provided a chemical shift reference ($\delta = 0.0$ ppm) for the NMR spectra, while the D_2O provided a deuterium lock for the NMR spectrometer.

^{1}H NMR-based metabolomics analysis

Extracts of gill tissue were analysed on a Varian-500 NMR spectrometer operating at a spectral frequency of 499.74 MHz at 298 K. One-dimensional (1-D) ^{1}H NMR spectra were obtained using a PRESAT pulse sequence to suppress the residual water resonance and 6983 Hz spectral width with a 2.0 s relaxation delay. A total of 256 transients were collected into 16,384 data points requiring a 19 min acquisition time. All data sets were zero filled to 32,768 data points and exponential line-broadening of 0.5 Hz was applied before Fourier transformation. All ^{1}H NMR spectra were manually phased, baseline-corrected, and calibrated (DSS at 0.0 ppm) using Chenomx Processor, a module of Chenomx NMR Suite (version 5.1; Chenomx Inc., Edmonton, Canada) software. The peaks of interest, namely the metabolites related to serotonergic (i.e. serotonin) and cholinergic (i.e. acetylcholine) systems, were identified within the ^{1}H NMR spectra (Cappello et al., 2013b; Fasulo et al., 2012b) and quantified using Chenomx Profiler, another module of Chenomx NMR Suite software, which uses the concentration of a known DSS signal to define the levels of individual metabolites (Brandao et al., 2015; Cappello et al., 2015).

Enzymatic analysis

Acetylcholinesterase (AChE) activity was estimated in the gills of mussels by using the colourimetric method of Ellman et al. (1961) with small changes by UV–Vis–UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). In short, thiocholine derivatives are

hydrolysed by acetylcholinesterase to yield thiocholine. The subsequent combination with 5,5-dithiobis-2-dinitrobenzoic acid (DTNB) forms the yellow anion 5-thio-2-nitrobenzoic acid, which absorbs strongly at 412 nm. AChE activity was expressed as µmol/min/mg (Maisano et al., 2017).

Molecular analysis

RNA extraction and cDNA synthesis

Tissue homogenization and RNA extraction from gill tissues of *Mytilus galloprovincialis* collected at each sampling mesocosm were performed using Qiazol reagent (Qiagen). RNA quantity and quality were assessed as detailed by Giannetto et al. (2015). cDNA synthesis from 1 mg total RNA was performed by QuantiTect reverse transcription kit (Qiagen) after gDNA wipeout buffer treatment in order to exclude any potential genomic DNA contamination, following manufacturer's instructions.

5-HT$_3$R quantitative gene expression (qPCR)

Degenerate primers (Table 4.1) were designed on the conserved regions of 5-HT receptor genes isolated from other mussels.

PCR product was separated on 1% agarose gels. The region containing the expected size fragment was sliced, purified using QIAquick Gel Extraction kit (Qiagen) and sequenced using ABI PRISM BigDye Terminator 3.1 Cycle Sequencing kit (PE Applied Bio-system). The sequence (340 bp) was submitted to NCBI database. Quantification of

5-HT_3R gene expression in *M. galloprovincialis* gills was performed by real-time PCR using the Rotor-Gene Q 2plex Hrm thermocycler (Qiagen) with SYBR Green chemistry (Qiagen) as mentioned by Giannetto et al. (2014). qPCR primers, listed in Table 4.1, were designed using the Beacon Designer TM online tool (http://www.premierbiosoft.com/qOligo/ Oligo.jsp?PID=1) for the target and reference genes analysed.

The actin (act), 18S ribosomal RNA (18S rRNA) and elongation factor (ef1-α), were chosen as reference genes. The primers for the reference genes are reported in Giannetto et al. (2015). Twenty-fold diluted gill cDNA samples were run in duplicate and no template and minus reverse transcriptase controls were included in each reaction The PCR efficiency determination was made by a five-point standard curve of a 5-fold dilution series (1:1 to 1:32) from pooled RNA (Fernandes et al., 2006). To correct the raw data genes a Normalization Factor was used. This Normalization Factor was calculated from the two most stable genes (*18S rRNA* and *act*) by geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/). The single-peak melting curves confirmed the specificity of the reaction.

Table 4.1 Nucleotide sequences of primers, amplicons size (bp), methods, qPCR efficiencies (E%).

Primer	Primer sequence	Size (bp)	Methods	E (%)
5-HTR _FWD	ATTNCGTTGGNTCGGTNCTG	340	Cloning	
5-HTR_REV	TANCGCCAGANCAATTNCAT			
q5-HTR _FWD	TAACGCCAGACCAATTCCAT	95	qPCR	98
q5-HTR_ REV	TGAAGCCATCTTGACTGACG			102

Statistical analysis

Statistical analyses were made by GraphPad software (Prism 5.0, San Diego CA, USA) for immunohistochemical, metabolomics and enzymatic data, while molecular data were analyzed using SigmaPlot (Systat software). Results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) applying the Dunnett's multiple comparison test, in order to determine significant differences between control and treatment groups and the Student-Newman-Keuls post-hoc tests to assessing differences in 5-HT_3R expression levels between mussels from the three mesocosms. Data were considered statistically significant at $p < 0.05$

4.2. Results

Physico-chemical parameters

No significant variations were noted throughout the experimental period of the measured values of the main physico-chemical parameters (pH, mean value 7.9 ± 0.1; temperature, mean value 15.8 ± 0.5 °C; dissolved oxygen, 5 ± 0.5 mg/L).

COD measures

Mean values of COD around 22 mg/L were recorded in the control mesocosm. The monitoring of the COD (unfiltered samples) showed average COD values of about 30-35 mg/L before agitation and very variable and different values in the two tanks of B and G sediment mesocosms following agitation (up to 270 mg/L). Values were elevated and comparable after agitation, and were recorded in the first two days of the experimentation in both tanks. The post-agitation values were in average lower after a couple of water changes within the systems. Further increases in COD were recorded in the last days of the experimentation even in the pre-stirring samples (values up to 190 mg/L in the B mesocosm and up to about 130 mg/L in the G mesocosm).

Metal concentration in sediment of B and G mesocosms

The monitoring of metal concentration in sediments of B and G mesocosms showed a reduction of metals in the G sediment after Soil Washing. In particular, the treatment did not allow to obtain mercury concentrations below the intervention limit value for the Bay of Augusta, but it permitted to record an average removal efficiency higher than 90% (mercury concentrations below 4 mg/kg) (Tab 4.2; Tab 4.3). Noteworthy, it is interesting to underline the very high treatment efficiency observed also versus other metals (Tab. 4.3).

Tab 4.2 Dutch Sediment Quality Guidelines (SQG):mg*k^{-1}

[Metal Concentration] < Target value, the material is class 0 (non-polluted); Target <[Metal Concentration] < Threshold, the material is class 1 (slightly polluted); Threshold < [Metal Concentration] <Test Value, the material is class 2 (moderately polluted);Test value <[Metal Concentration] <Action, material is class 3 (polluted) ;[MetalConcentration] >Action value, material is class 4 (heavily polluted).

Dutch SQG (mg/kg)	Target value	Threshold value	Test value	Action value
Chrome	100	380	380	380
Nickel	35	35	25	21.04
Copper	35	35	90	190
Zinc	140	480	720	720
Arsenic	29	55	55	55
Cadmium	0.8	2	7.5	12
Lead	85	530	530	530
Mercury	0.3	0.5	1.6	10

Table 4.3. Concentration of metals in sediments from B and G mesocosms

***Values exceeding the SQG.**

	16RP03402		16RP03403		
	BLACK SEDIMENT	**SD**	**GREY SEDIMENT**	**SD**	**Removal Efficiency (%)**
Nitrogen	0.44		< 0.1		78
Total Organic Carbon (TOC) (% p)	1.4		0.19		86
Chlorides (Cl^-) (mg/kg)	38900	5400	2,000	280	95
Sulphate ($SO4^-2$) (mg/kg)	3865.2		504.32		87
Sulphide (mg/kg)	< 0.1		< 0.1		
METAL					
Antimony. (Sb) (mg/kg)	0.48	0.072	0.255	0.034	48
Arsenic (As) (mg/kg)	12.4	1.9	1.98	0.3	85
Beryllium (Be) (mg/kg)	0.234	0.034	0.433	0.064	
Cadmium (Cd) (mg/kg)	0.197	0.03	0.05		75
Calcium(Ca) (mg/kg)	117000	35000	40000	12000	66
Cobalt (Co) (mg/kg)	10.8	3.2	3.6	1.1	66
Total Chrome (Cr) (mg/kg)	27.7	4.2	21.2	3.2	
Iron (Fe) (mg/kg)	32800	9800	34000	10000	
Total Phosphorus (P) (mg/kg)	1314.06		724.22		45
Magnesium (Mg) (mg/kg)	14000	2400	12600	2200	10
Mercury (Hg) (mg/kg)	**47.7***	7.2	**3.55***	0.53	90
Molybdenum (Mo) (mg/kg)	2.85	0.43	0.328	0.049	89
Nichel (Ni) (mg/kg)	12.6	1.9	9.4	1.4	27
Lead (Pb) (mg/kg)	23	3.5	18.9	2.8	18
Copper (Cu) (mg/kg)	60.3*	9.1	13.1	2	79
Vanadium (V) (mg/kg)	38.9	5.8	22.3	3.4	43
Zinc (Zn) (mg/kg)	86	13	25	3.8	71

Histology

In the gills of *M. galloprovincialis* from WS mesoscom it was observed the normal morphology of mussel gill tissue (Fig 4.2A), consisting of parallel filaments whose whole surface is coated by cilia. In the gills of the BS mesocosm, it was found a relevant loss of cilia (Fig 4.2B). In the GS mescosm, it was noticed a moderate alteration of cilia with a relevant presence of haemocyte (Fig. 4.2C).

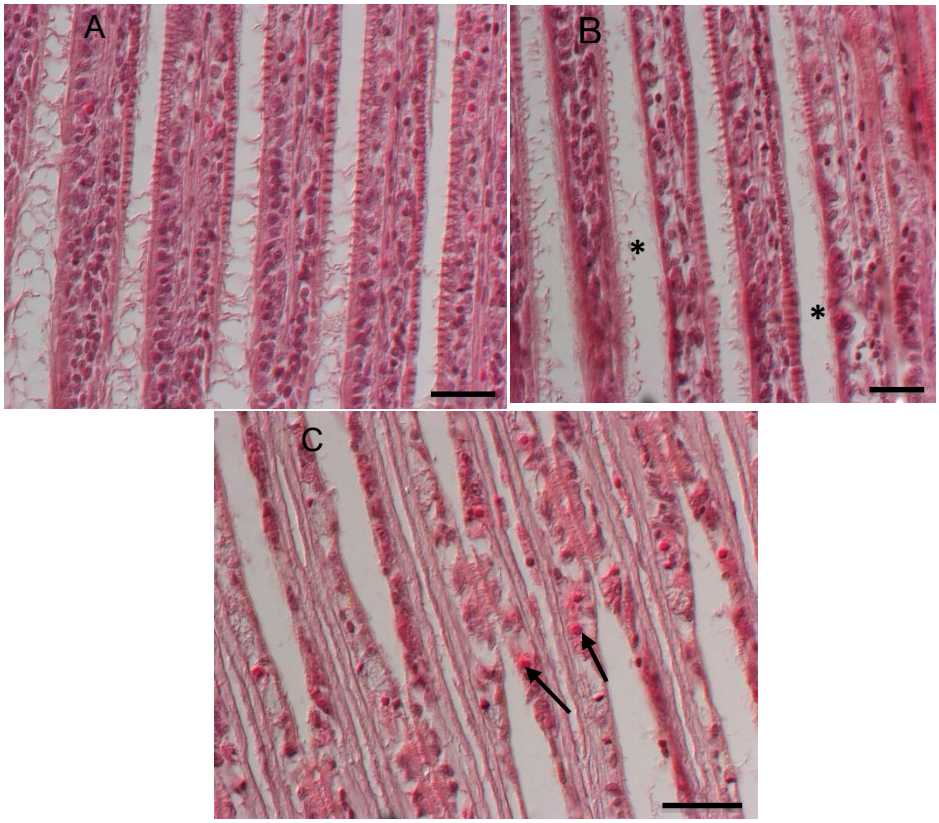


*Fig.4.2.The gills of Mytilus galloprovincialis stained with Haematoxylin and Eosin (H&E). Mesocosm W (msc II W) Mesocosm B (msc II B), Mesocosm G (msc II G). * indicate loss of clia , arrows show haemocyte. Scale bars, 20 μm*

Immunohistochemistry

With regards to the serotoninergic system in the WS mesocosm, it was observed a high cellular immunopositivity for 5-HT (4.3A.) and low expression of 5-HT_3R (4.4A.). In the BS mesocosm, it was recognized a reduction of immunopositivity for serotonin (4.3B.), and a concomitant increase of signal for the receptor in the cells and fiber (4.4B..). In the GS mesocosm, moderate increase of serotonin immunopositivity was found, as showed, in cells (4.3C.), in the GS mesocosm, its receptor is localized in cells (4.4C.). Statistical analyses of immunohistochemical results for 5-HT and 5-HT_3R are shown in Fig. 4.3D. and 4.4D. In the cholinergic system, the AChE and ChAT showed a high immunopositivity in the gill cells of control (4.5A.; 4.6A.). In the BS mesocosm, it is possible to observe a low cellular immunopositivity in AChE and ChAT and higher signal in fibers (4.5B.; 4.6B.). In the GS mesocosm, the immunopositivity for both enzymes is increased in respect to the BS mesocosm, and expressed mainly in cells (4.5C.; 4.6C.). Statistical analyses of immunohistochemical results for AChE and ChAT are shown in 4.5D. and 4.6D..

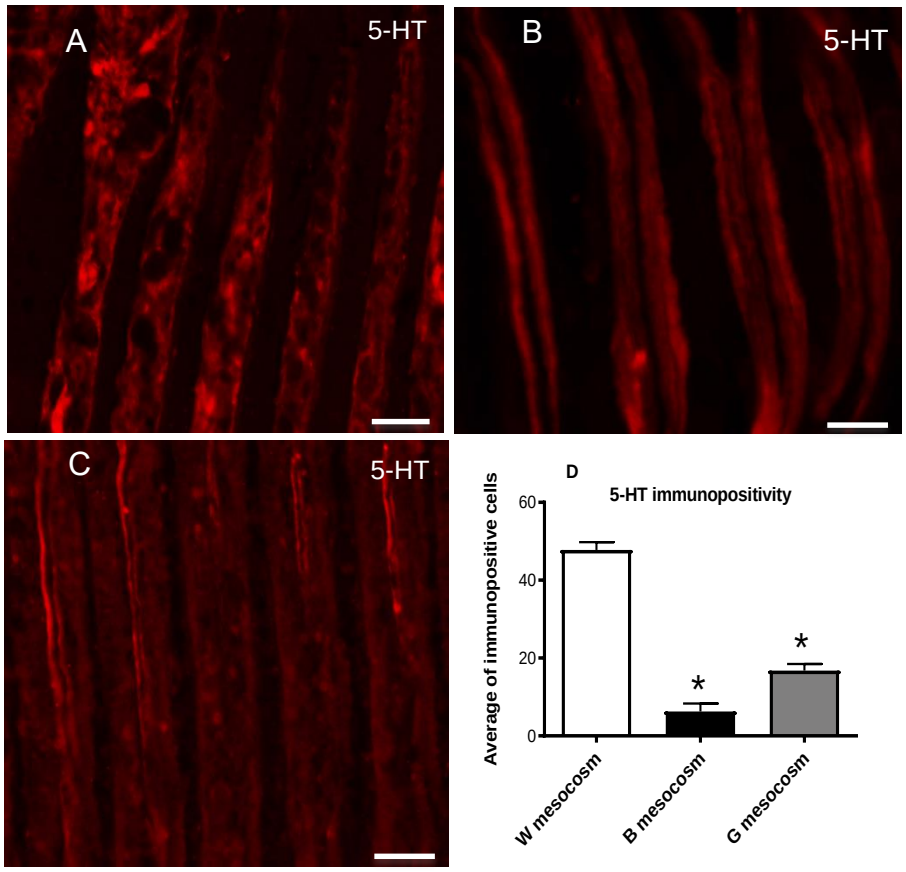


Fig.4.3. Immunohistochemical labeling for 5-HT in mussel gills in Mesocosm White (A), Mesocosm Black (B) and Mesocosm Grey (C). Mean and standard deviation (S.D.) of immunopositive cells (D). Significant difference $p < 0.05$.between Control (White Mesocosm) and the Black and Grey Mesocosm is indicated by an asterisk. Scale bars 20 µm.

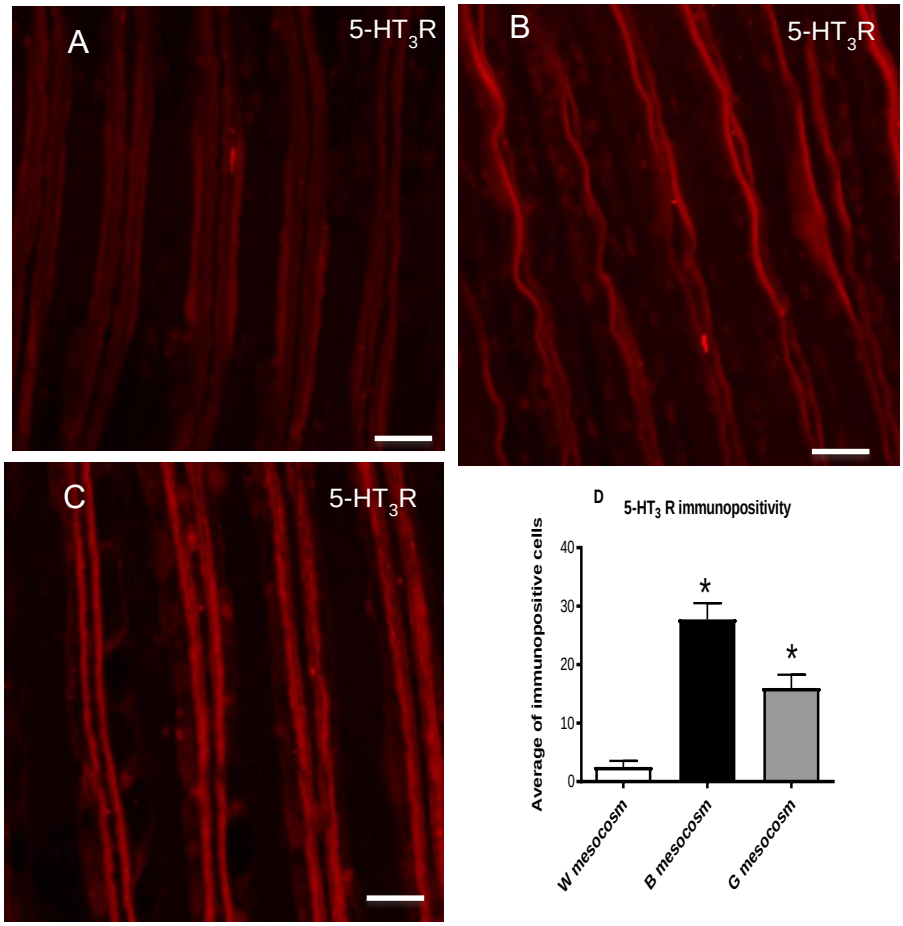


Fig4.4. Immunohistochemical labeling for 5-HT$_3$ R in mussel gills in Mesocosm White (A), Mesocosm Black (B) and Mesocosm Grey (C).Mean and standard deviation (S.D.) of immunopositive cells (D). Significant difference $p < 0.05$.between Control (White Mesocosm) and the Black and Grey Mesocosm I is indicated by an asterisk. Scale bars 20 μm.

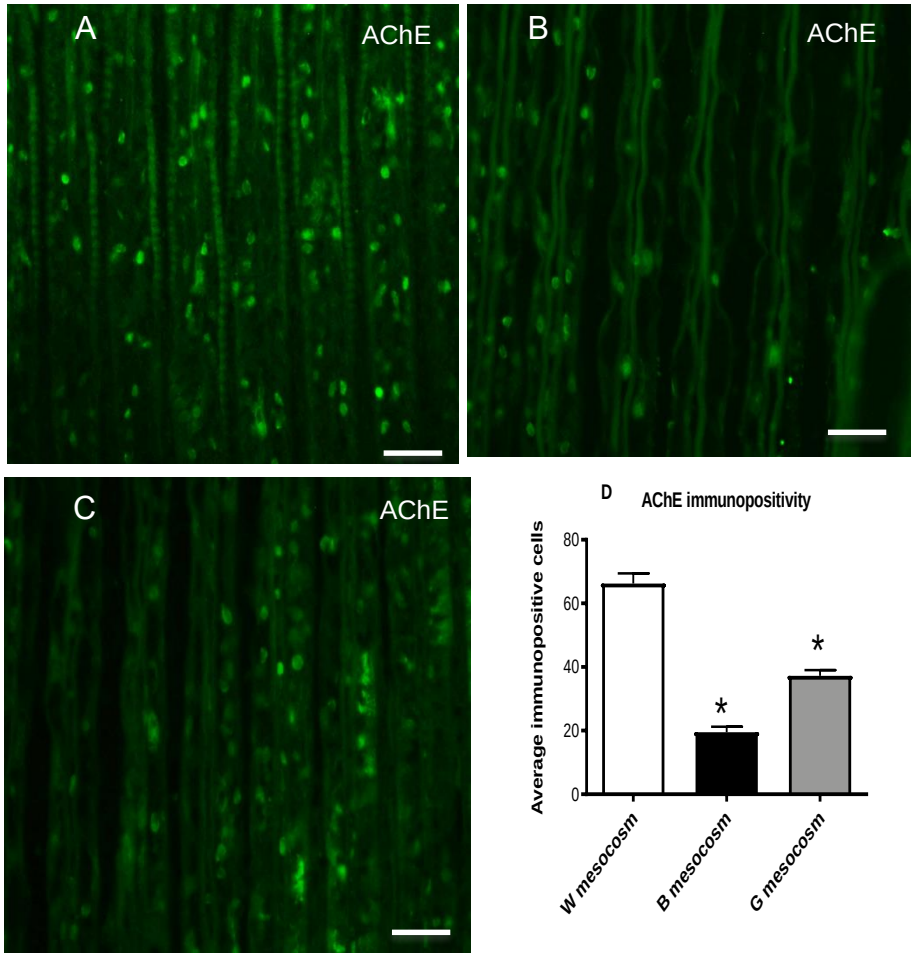


Fig.4.5. Immunohistochemical labeling for AChE in mussel gills.in Mesocosm White (A), Black Mesocosm (B) and Mesocosm Grey (C). Mean and standard deviation (S.D.) of immunopositive cells (D). Significant difference $p < 0.05$.between Control (White Mesocosm) and the Black and Grey Mesocosm is indicated by an asterisk. Scale bars 20 μm.

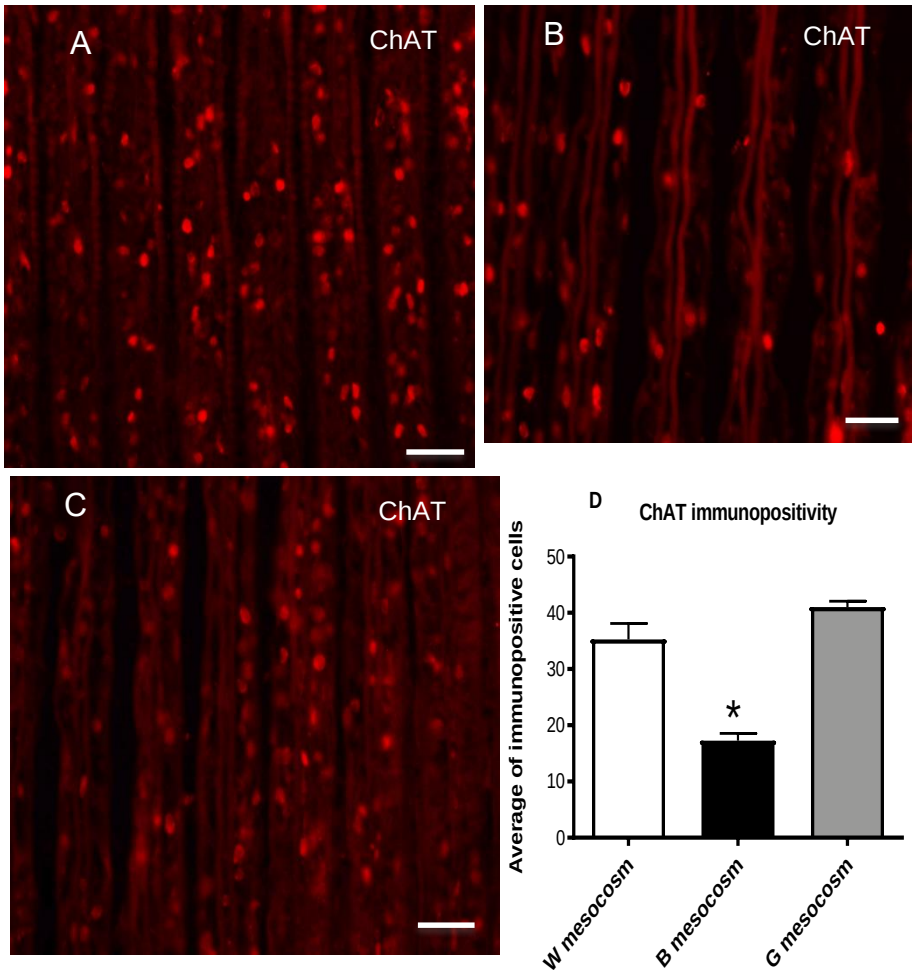


Fig 4.6. Immunohistochemical labeling for ChAT in mussel gills.in Mesocosm White (A),Mesocosm Black (B) and Mesocosm Grey (C). Mean and standard deviation (S.D.) of immunopositive cells (D). Significant difference $p < 0.05$.between Control (White Mesocosm) and the Black and Grey Mesocosm is indicated by an asterisk. Scale bars 20 μm.

Metabolomics

In the WS mesocosm, high levels of serotonin and acetylcholine were observed. In the BS mesocosm the level of acetylcholine was higher than that one in the WS mesocosm, while the level of serotonin was lower (statistically significant data). In the GS mesocosm, the level of serotonin was higher than in the BS mesocosm and nearer to the WS mesocosm, while the concentration of acetylcholine was lower in respect to the two WS and BS mesocosms. (Fig. 4.7.)

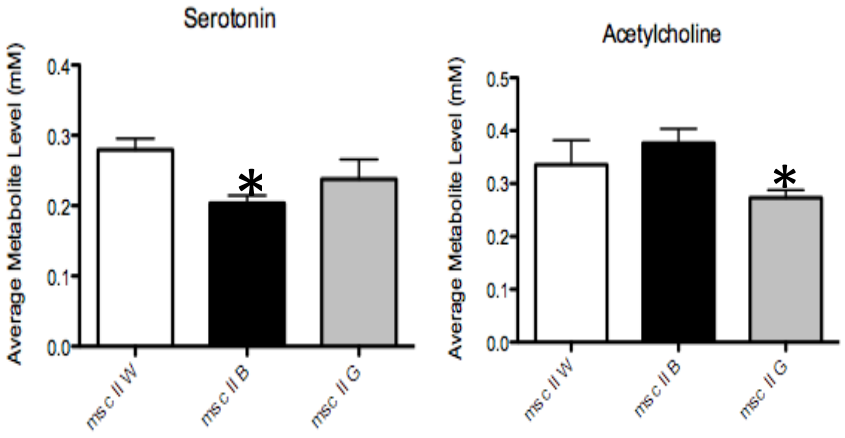


Fig. 4.7. Metabolomic evaluation of serotonin and acetylcholine in three mesocosms: * p value < 0.05

Enzymatics

From the comparison of the enzymatic activity in the three different mesocosms it was possible to observe a significant reduction of about 20% of AChE activity in the gills of mussels exposed to natural mercury-polluted sediments in the BS mesocosm (statistically significant data). In the GS mesocosm, where sediments were treated by Soil Washing, the enzymatic activity of AChE was not inhibited hence the level of AChE activity was equivalent to the W mesocosm (Fig. 4.8.).

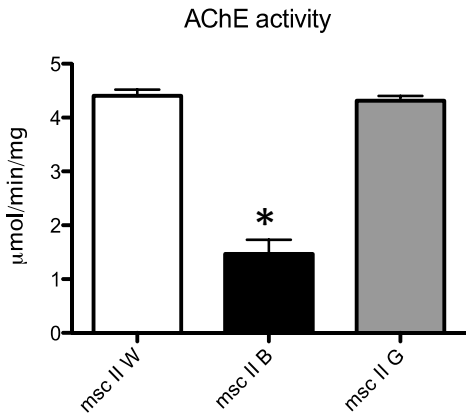


*Fig.4.8. AChE Activity in three mesocosms. * p value < 0.05*

Molecular results

In regards to the gene expression for the serotonin receptor 5-HT_3R, it was possible to notice variations in the different experimental conditions. In the BS mesocosm, the gene expression of 5-HT_3R was over three times higher than that observed in the WS mesocosm (statistically significant data). In the GS mesocosm, where natural mercury-polluted sediments were treated by Soil Washing, an almost three time increase in the gene expression was recorded compared to the WS mesocosm, but the gene expression compared to the BS mesocosm was slightly lower (statistically significant data). (Fig. 4.9.)

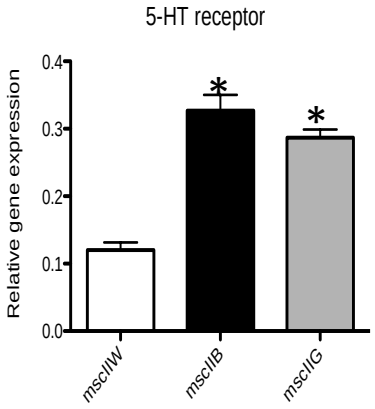


*Fig4.9. Gene expression of 5-HT receptor in three mesocosm. * P. Value <0.05*

4.3. Discussion

The petrochemical pollution of sediments in the harbour area represents a very relevant problem. For instance, the Augusta-Melilli-Priolo (Sicily, Italy) is characterized by high level of metals, in particular elevated concentrations of mercury overcoming the national and international guideline limits for sediment quality (Ministerial Decree No. 260/2010; Dutch SQGs ;De Domenico et al. 2013), though this area has been involved in the National Remediation Plan in 2002. The high level of these contaminants was confirmed by several works (De Domenico et al., 2011, 2013; Di Leonardo et al., 2014; Maisano et al., 2017; Sprovieri et al., 2011).

Soil washing, by physical and chemical approaches, is a technology particularly relevant for the remediation of metal-contaminated soils (Dermont et al., 2008). In this case, the evaluation of the efficiency of the Soil Washing Technique showed, with regards to the chemical analysis, a consistent 10 times reduction of mercury and other metals. The use of microscosm/mesocosm scale experiment is useful to evaluate the efficiency of remediation technique (Pirrone et al., 2018). The use a multi-biomarker approach in *M. galloprovincialis* gills has been widely reported (Cappello et al., 2015; Maisano et al., 2017; Pirrone et al., 2018). The histo-morphology of gills showed an important loss of cilia in the BS mesocosm. In the GS mesoscosm the condition of the tissue appeared good in respect to the gills in the BS mesocosm, even if it is possible to observe the presence of haemocyte. The toxic effects of mercury in aquatic organisms are still object of

research (Cappello et al., 2016a; 2016b). The view of histomorpholgical alteration represents a signal of damaging of the functional integrity of gills (Cappello et al., 2013b; Maisano et al., 2017). Hence the cilia activity play a key role in most of the gill functions (Sunila, 1998), while the hemocytes possess a complex cell signaling network with high homology with that of vertebrates, that allows them to modulate their own functions (Humphries and Yoshino, 2003; Franzellitti and Fabbri, 2013). The observation of haemocytes, a signal of inflammation, in the mesocosm with treated sediments is due to the presence of metals such as mercury. In fact, the soil washing was able to reduce the concentrations of heavy metals but in the case of mercury, the levels were still above of the allowed limits (Ministerial Decree No. 260/2010; Dutch SQGs). For this reason, such concentrations are likely to trigger a cytotoxic and inflammatory response in the tissue. Also in this case, the neurotransmission system, i.e. serotoninergic and cholinergic, which set the movement of cilia (Gosselin, 1961; Carroll and Catapane, 2007; Matozzo et al., 2005), was analysed by a multi-biomarker approach.

About the serotoninergic system, the amount of serotonin in the GS mesocosm was lower than in the WS mesocosm but higher than in the BS mesocosm. This trend was confirmed by the immunopositivity of 5H-T in the different mesosocosms. The reduction of serotonin, observed in the BS mesocosm, was previously reported in mussel gills exposed to petrochemical pollution in nature (Cappello et al., 2015; Maisano et al., 2017). The increase of the level serotonin is a signal that GS mesocosm condition is closer to that one observed in WS

mesocosm. The evaluation of 5-HT receptor, by immunohistochemistry assay and molecular analysis in the BS mesocosm shows an increase of the serotonin receptor than in the WS mesocosm. This result is an adaptive response mediated by the paracrine signalling activities in order to recover a regular physiological activity in mussel gills (Cappello et al., 2015; Maisano et al., 2017). Nevertheless, the reduction of the 5-HT_3R expression and immunopositivity, in the mesocosm with treated sediments, suggests a trend versus healthy condition. Hence, in the GS mesoscosm the expression and the immunopositivity are not stackable in respect to the WS mesocosm conditon but, at the same time, they are different in respect to the BS mesocosm.

The evaluation of cholinergic system was also made, since the cholinergic system is responsible for the physiological functioning of the efferent nervous systems (Matozzo et al., 2005). The acetylcholine level, evaluated by a metabolic approach, in the BS mesocosm was higher than control. As mentioned in the last chapter, this increase is not reported in mussels caged in petrochemical polluted areas for 30 days (Cappello et al., 2015) and 60 days (Maisano et al., 2017). In the GS mesocosm, the acetylcholine level was lower than in the BS mesocosm and control. The AChE activity was inhibited in the BS mesocosm, while in the GS mesocosm the same activity was comparable to control. The inhibition of AChE, which split acetylcholine into choline and acetate in cholinergic synapses and neuromuscular junction (Matozzo et al., 2005), in presence of pollutants is well documented (Rank et al., 2007; Ciacci et al., 2012). In addition

to these data, to evaluate deeper the efficiency and the functioning of cholinergic system, immunohistochemistry assay was used, as already seen in other works (Cappello et al., 2015; Maisano et al., 2017). By this assay, choline acetyltransferase (ChAT), which synthesizes in the cytoplasm the cholinergic neurons acetylcholine, and AChE, were evaluated. The immunopostivity of the two enzymes in the BS mesocosm was lower than in the control. This concomitance of signal was reported also in Maisano et al. (2017) and it is a signal of critical compromise of the cholinergic system. In the GS mesocosm the immunopositivity was quite similar to the WS mesocosm.

Overall, the Soil Washing of sediments was able to create healthier condition for mussels in the GS mesoscosm, with regards to gill histomorphology and neurotransmission system.

4.4. Conclusion

The consistent amount of heavy metals in the sediments of a petrochemical site, such as "Augusta-Melilli-Priolo" (Sicily, Italy), represents a valid motivation to improve more efficient remediation techniques. The Soil Washing, used in this experiment, has proved to be able to reduce by 10 times the concentration of various metals. In the case of mercury, whose concentration overcame the national and international limits for sediments in Augusta-Melilli-Priolo, a 10 times reduction was observed. By the multi-biomarker approach and the mesocosm scale experiment, it is possible to state that the treatment for sediment it is not still able recreate the same condition of an unpolluted site, but at the same time the living organisms, in this case mussels, exposed to this treatment show better health condition than the polluted mesocosm. In addition, the morphology and the neurotransmission system of mussel gills showed condition and functionality closer to the White mesocosm. Overall, although the remediation approach needs further improvement, it was able to create good conditions for the life of aquatic organisms.

CHAPTER V Use of Cell Culture in Toxicology: Evaluation of alterations induced by pollutants (NaClO) on tight junction of human airway epithelial cells

5.1. General introduction

In view of the results obtained with the use of mussels in environmental recovery programs, which demonstrated the high sensitivity and the extensive alterations induced to the respiratory organs (gills), we decided to focus our interest to the direct assessment of the effects of environmental contaminants versus land organisms respiratory organs, including humans, by the use of airway epithelial cell lines. For this reason, I spent a training period and internship in the laboratory of Dr. Ernesto Alfaro-Moreno, Senior Researcher at SWETOX in Södertälje, Sweden. Here, I followed various activities, aimed at learning the use of cell culture in Toxicology. I learnt the basic methods of cell culture, from freezing to thawing cells, seeding and sub-culturing cells to the use of some common assays to evaluate cytotoxicity, cellular proliferation, epithelial barrier integrity and recovery of epithelial integrity after wound. For this, I did use two different cell lines, human bronchial epithelial cells, 16HBE, and human monocytic cell line,

THP-1.

In general, the impact of human activities have been responsible for the environmental release of significant amount of a wide range of organic and inorganic contaminants, directly and indirectly, to waters, soils and/or the air (Kim et al., 2016; Abdel Shufy and Mansour, 2016). Since the industrial revolution, the presence of chemical compounds in the environment has changed and in particular, air pollution has been widely recognized as the cause of negative effects on human health and other living organisms (Gerdol et al., 2014). Thus, air pollution became a real concern for the global community's due to the impact on humans and other living organisms. Due to these concerns, new approaches for the evaluation of the effects of pollutants on biological systems are required. For several years, animal models were among the most used tools in scientific research to evaluate the health effects of inhaled substances (Geiser et al., 1994; Phalen, 1976) because researchers thought that nonhuman animals as models would be able to predict toxicological response in Human Beings. Nevertheless, later the use of animal-based toxicity testing was not considered the best model for various reasons. First of all, the ethical problems to use animals, in 1959 British zoologist William Russell and microbiologist Rex Burch wrote the Book "The Principles of Humane Experimental Technique", and in this book, the authors underlined the importance of r*efinement*, *reduction*, and *replacement* (3Rs) of animal experimentation. In particular, the concept of *Replacement* suggests the use of non-animal model experiment respect animal model experiment for ethical reason. In addition, later various authors (Olson et al., 2000; Knight, 2008) showed that the ability of animal experiments to predict the real effect

of drugs is not so high (all animal experiments 71%, rodents 43% and no rodents 63%). Overall the increasing awareness that animal studies may not always be optimal predictors of human responses to inhaled substances, and the public concern about use of animals for research purposes in general, has prompted the search for alternatives (Hartung, 2009).

5.2. Use of Cell Culture in Toxicology

The first use of *Tissue Culture* was conceived in the first years of the twentieth century (Harrison, 1907; Carrel, 1912). The aim of these first experiments was to figure out the behaviour of animal cells free of systemic variations that might arise *in vivo* both during normal homeostasis and under the stress of an experiment. First researchers start to use lower vertebrate's cold-blooded animal tissue because these cells did not need incubation and the lower vertebrates have a better tissue regeneration then higher ones. Few years later, other researchers, driven by the medical science interest, started to focus their efforts to other species closer to the humans. Few years later, it was possible to establish the first continuous mammal cell line L-cell mouse Fibroblast (Earle et al., 1943) and HeLa, first human cell line (Gey et al., 1952). Nowadays, the cell culture technique is improved, and this improvement permitted the use of cell culture in different fields (Freshney, 2011). Currently, the research fields are represented by:

- Cell products
- Immunology
- Pharmacology
- Tissue Engineering
- Toxicology

The aim of Toxicology is to assess the biological effects of several chemicals on the environments. Chemicals play an important role in human daily life, and in the recent years, they furnish our society with tools able to improve manufacturing processes in different fields (i.e.

chemical industry, agriculture, cosmetic, food industry and pharmaceutical industry) (Limonciel, 2014). Nevertheless, the use of chemicals is also responsible for a higher exposure of the population, either directly or through contamination of the environment. As mentioned above the first test, in toxicology, used animal model but then, for various reasons, this model was not adequate for researchers who developed new in vitro toxicological models. In the second half of the 1980s, this kind of experimental model became widely used in toxicology. In the beginning, the aim of "in vitro toxicology" was to figure out what occurs inside the cells in contact with the toxic agents. Then, thanks to the strong improvement of tissue culture methodologies, researchers were able to focus on the toxic mechanisms of action. Lately, they were able to create models, which try to reproduce the in vivo environment (Zucco et al., 2004). Various *in vitro* systems were created for organ toxicity evaluation, hepatoxic model (Godoy et al., 2013), nephrotoxic (Soo et al., 2018), neurotoxic (Bal-Price et al., 2014) and human lung toxic (Hiemstra et al., 2018). The Lung airway epithelium is often exposed to inhaled particles, as for instance pollutants, toxins, and several airborne pathogens. Therefore, the integrity of this epithelium is fundamental to create a selective barrier, which permits the passage of solutes and ions through paracellular spaces, but avoid pathogen or pollutant migration from lumen to interstitium. Multiple mechanisms, including tight junctions, adherence junctions, and desmosome are crucial for the epithelial integrity (Matter and Bald 2003).

5.3. Aim of the Internship

The purpose of the abroad period was to learn *in vitro* techniques in order to use them, in toxicological tests. In order to better understand the mechanism of action of several pollutants against cells and tissues, we tried to understand if the *in vitro* techniques could be considered a useful tool for this type of investigation in the environmental toxicology. The first part of the internship was characterized by the learning basic knowledge and techniques in a cell culture lab (Aseptic method, Handling of cell, Cytotoxicity assay). After this period, we tried to test the efficacy of two techniques (TEER Measurement and Immunofluorescence staining) to evaluate the tight junction disruption induced by air pollutant particles. In particular, for these experiments the sodium hypochlorite (NaClO) was used. The NaClO is a common disinfectant agent, which is known to be responsible of acute lung injury in animals (Nemery et al., 2002; Van Den Broucke et al., 2018 Yadav et al., 2010).

5.4. Material and Methods

Cell Culture

The Human Bronchial Epithelium cells 16HBE (Cozens et al., 1994; Wan et al., 2000) were used in these experiments. The 16HBE were seeded in T75 flask with DmeM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) 10% Fbs (Fetal Bovine Serum).

Pollutant

A stock solution of commercial sodium hypochlorite (NaClO) (KLORIN original 27g/Kg) 2.7% was used. To create different concentration (0.000027%-2.7%) the stock solution was diluted in DMEM/F12 (no FBS).

Transepithelial electrical resistance (TEER) Measurement

The 16HBE (P1-P10) were seeded in 24 well plates with inserts (Transwell Permeable supports Costar, 6,5 mm insert-surface 0.33 cm^2, 0,4 μm Polyester membrane) with a densities of 100.000 cells/cm^2 in the upper chamber with 100 μl of medium (DMEM/F12 10% FBS), the down chamber of the well were filled with only 500 μl of the same medium. The cells were observed using an inverted microscope (Nikon TMS Inverted Microscope) to evaluate the confluence of the cells. Once the cultures look confluent, we measured the TEER every day by Evom^2 (Epithelial Volt Ohm Meter) with Stx2 chopstick. When the TEER reached a high value (550 Ω.cm^2), signal of tight junction development, it was decided to start the experiments. The experiments, conducted in duplicate, were repeated twice. The cells were exposed to

several concentration of sodium hypochlorite: 0.00027% - 0.27% in the first experiment and 0.000027% - 0.27% in the second experiment for 6 hours. During this time the TEER of the cells was measured by Evom^2 (Alfaro-Moreno et al., 2018).

Reagent Immunofluorescence staining of Zonula Occludens-1 (ZO-1)

For the immunofluorescence assay of Zo-1 the following reagents were used:

- Methanol Acetone 1:1 (V/V) solution for fixation
- PBS (pH 7.4) to wash
- BSA (Bovine Serum Albumin) 1% for blocking
- Primary Antibodies ZO-1 (2.5 μg/ml ZO-1 Polyclonal Antibody from Thermo Fisher Scientific, catalog # 61-7300, RRID AB_2533938) for the first incubation
- Secondary Antibodies Alexa Flour 488 coniugated (Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 from Thermo Fisher Scientific, catalog # A-21206, RRID AB_2535792)

Immunofluorescence staining of Zonula Occludens-1 (ZO-1)

In parallel, 16 HBE were seeded in 96 well plate (0.33 cm^2 surface) with the same densities of the inserts. When was registered an increase in the TEER values in the inserts, the 96 well plates were treated in the same condition. To evaluate alterations in the tight junctions we did immunofluorescence staining for ZO-1 (tight junction protein). After the NaClO exposure, the cells were fixed with a Methanol-Acetone 1:1 (V/V) solution. After the fixation the plates were incubated with the primary antibodies ZO-1. After washing, they were incubated 1 donkey

anti-rabbit secondary antibodies at 1:2000. ImageXpress Micro equipment was used for the imaging (Alfaro-Moreno et al., 2018).

Statistical analysis

Statistical analysis for TEER measurement was made by GraphPad software (Prism 7.0 San Diego USA). Results were expressed as mean ± standard deviation (SD). All data were statistically analyzed by two-way analysis of variance (ANOVA) by applying Dunnett Test. The datas were considered statistically significant at $p < 0.05$.

5.5. Results

TEER results

In the two experiments it is possible to observe a variation of the TEER measurement during the time exposure. But apart the highest concentration 0.27%(Fig. 5.2.; Fig. 5.4.) where the drop of the TEER after 1 hour, without recovery of it, is evident, in the other conditions, the value show results very variable how demonstrated by the SD, also in the negative control. The interaction was statistical significant only for the highest concentration (0.27%) in the two experiments (Fig. 5.2.; Fig. 5.4.). These results marked the high variability of the TEER Measurement (Fig. 5.1.-5.4.) (Tab. 5.1.; Tab. 5.2.)

Tab. 5.1; TEER Measurement I Experiment, SD (Standard deviation)

TEER Measurement I Experiment ($\Omega*cm^2$)										
Time	Neg		0.00027%		0.0027%		0.027 %		0.27%	
h	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	479.49	192.7	320.9	134	500.78	54.84	363.00	74.67	481.80	14.
1	402.6	60.6	325.	196	517.28	61.8	344.85	114.34	52.14	0.9
2	379.5	42	312.6	169	518.10	4.6	316.80	72.34	47.03	1.1
3	344.85	72.3	312.6	159.8	456.23	50.1	328.02	61.14	47.03	3.5
4	323.4	88.6	300.3	156.3	479.33	52.5	340.73	71.17	50.16	0.9
5	301.95	91	288.7	144.6	473.55	81.6	371.42	93.57	48.68	1.1
6	273.07	115.5	320.9	134.1	500.78	54.8	374.55	84.00	46.20	-

Tab. 5.2; TEER Measurement II Experiment, SD (Standard deviation)

TEER Measurement II Experiment($\Omega*cm^2$)												
T	neg		0.000027%		0.00027%		0.0027%		0.027 %		0.27%	
H	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	420.7	160.1	500.2	164.4	440.2	153.8	435.7	130.6	439.5	108.1	314.25	134
2	348.7	117.3	397.5	120.9	373.5	125.1	371.2	98.6	340.5	93.3	49.5	2.1
3	342	131.5	408	156.9	367.5	129.4	372	114.5	316.5	116.6	48	0
4	340.5	125.1	411	156.9	357.7	126.2	375	127.2	313.5	114.5	45	0
5	352.5	129.4	433.5	176.1	447	216.3	403.5	137.8	345	114.5	46.5	2.1
6	333	125.1	410.2	170.8	411.7	200.4	382.5	137.8	347.2	109.2	45.75	1

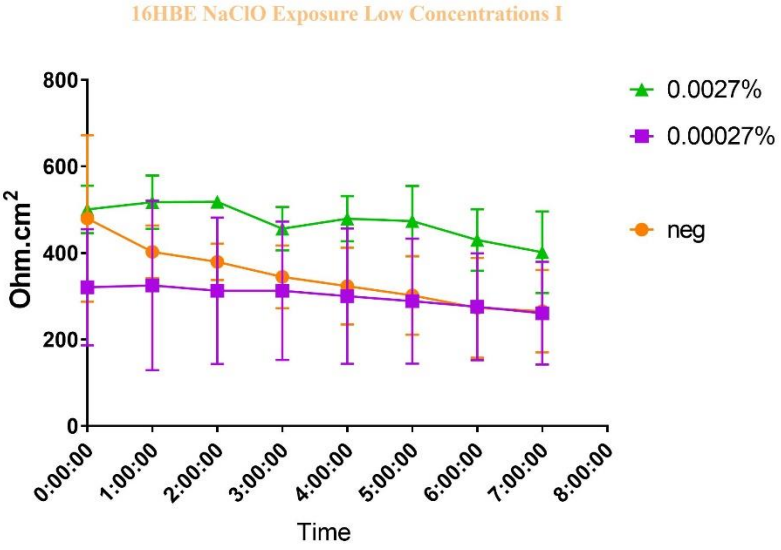


Fig. 5.1 TEER Measurement low concentrations (0.00027%; 0.0027%). neg (negative control).

The dot in the graph represents the mean while the bar the standard deviation (SD)

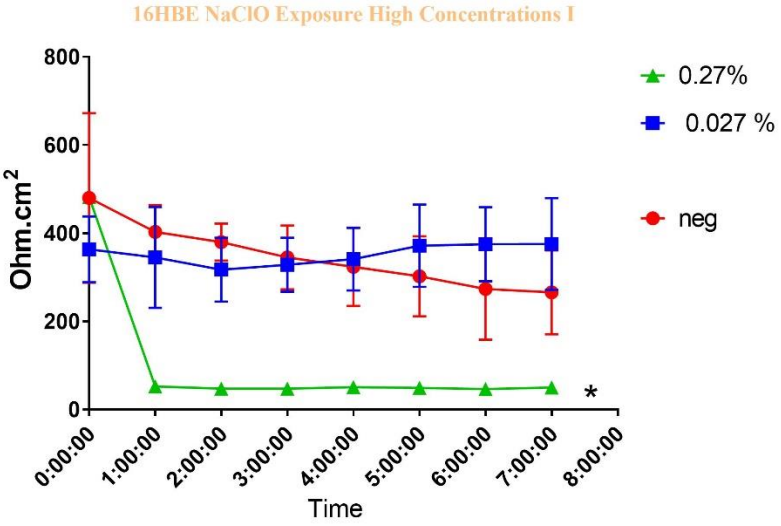


Fig .5.2. TEER Measurement for high concentrations (0.027%; 0,27%) neg (negative control).

The dot in the graph represents the mean while the bar represents the standard deviation (SD)

** statistical significant $p < 0.05$.*

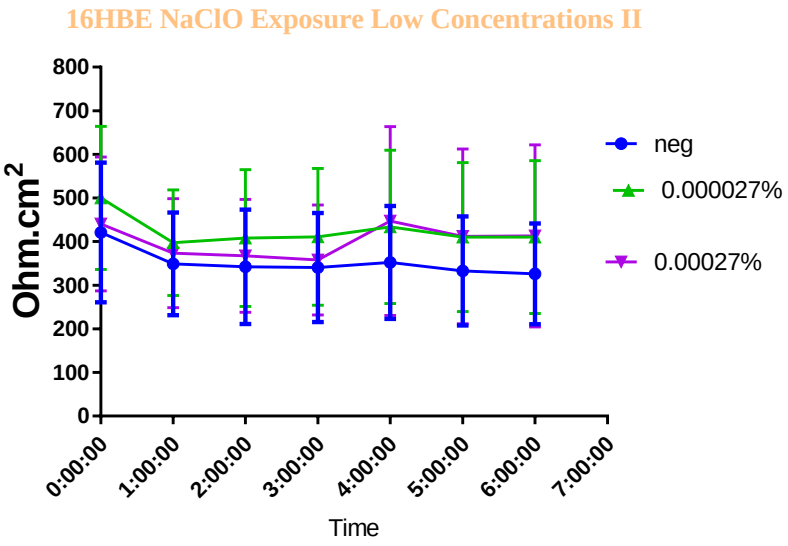


Fig 5.3. TEER Measurement for low concentrations (0.00027%; 0.000027%) neg (negative control). The dot in the graph represents the mean while the bar represents the standard deviation (SD)

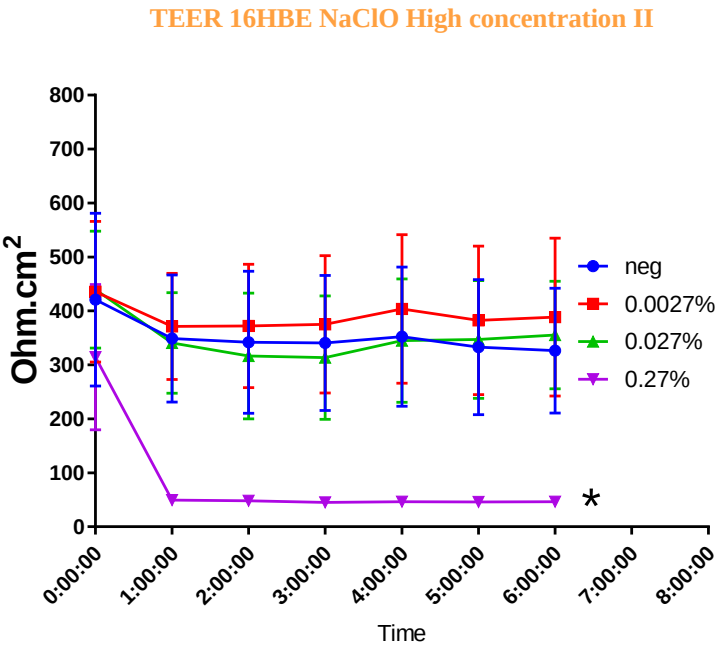


*Fig.5.4. TEER Measurement for high concentrations (0.0027%; 0.027 %; 0.27%) neg (negative control). The dot in the graph represents the mean while the bar represents the standard deviation (SD). * statistical significant $p < 0.05$.*

Zo-1 immunofluorescence

In the negative control (Fig. 5.5.) it was possible to observe the regular tight junction pattern of the cells. This pattern looks to maintain in the lowest concentration (0.00027%) (Fig. 5.6.), with few cells stretched. In the concentration 0.0027% (Fig. 5.7.) it was possible to notice an alteration of the shape, with fusiform cells, and in tight junction pattern. In the concentration 0.27% (Fig. 5.8.) it was possible to see an increase of the immunopositivity, with the presence of Gap and severe alteration of tight junction. In the highest concentration 2.7% (Fig.5.9.) the absence of signal is related to the death of the cells.

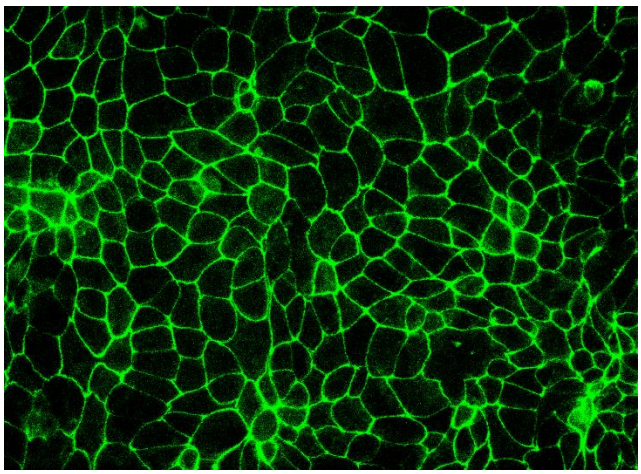


Fig 5.5. Immunohistochemical labeling for ZO-1 in 16HBE. Negative control with the regular Thight Junction pattern. Magnification 200X

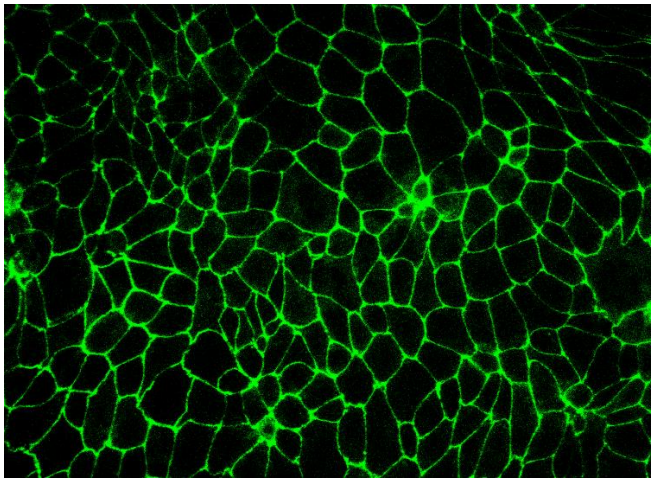


Fig 5.6. Immunofluorescence labeling for ZO-1 in 16HBE, 0.00027% NaClO exposure Regular pattern of the cells with some cells in the edge which look slightly stretching. Magnification 200X

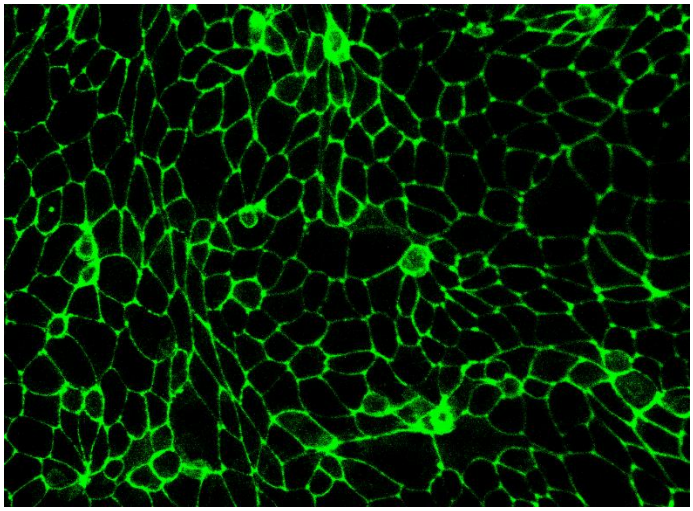


Fig. 5.7. Immunofluorescence labeling for ZO-1 in 16HBE, 0.0027% NaClO exposure. Cells with a interesting amount of Streched Cells. Magnification 200X

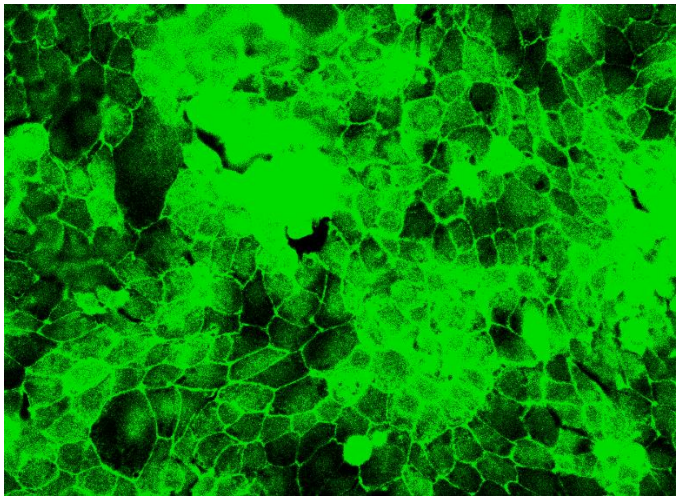


Fig. 5.8. Immunofluorescence labeling for ZO-1 in 16HBE, 0.27% NaClO exposure; It is possible to observe a severe alteration of the pattern and the immunopositivity. Magnification 200X

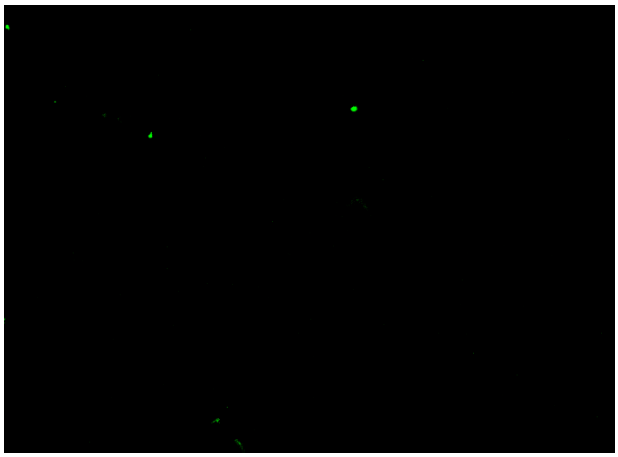


Fig. 5.9. Immunofluorescence labeling for ZO-1 in 16HBE, 2.7% NaClO exposure. The absence of signal is related to the death of the cells. Magnification 200X

5.6. Discussion

The tight junction or zona occludens is the intercellular junction that rules diffusion of several substances (Matter et al., 2003). Hence it permits both of these cell layers to form selectively permeable cellular barriers able to separate apical (luminal) and baso-lateral (abluminal) sides in the body. These barriers regulate the transport processes and support the maintenance of homeostasis. The epithelial barriers of several organs are continuously studied in order to develop new systems of therapeutic drug carrier across these barriers in order to reach the various target tissue (Srinivasan et al., 2015). Various researchers revealed the respiratory and systematic effect related to exposure to inhaled particles as Particular Matter (PM) and nanoparticles (Np), which are the main pollutants element in the air, (Lin et 2008; Nemmar et al., 2013; Nurkiewicz et al., 2011). This effect of PM and Np were evaluated by different approach (Alfaro-Moreno et al., 2002, 2008. Rueda-Romero et al., 2016) Geys et al. (2006) underlined the correlation between TEER and tight junction in relation to particles translocation. Some particles are able to modulate the tight junction and permeability of epithelial barrier (Lehaman et al., 2009; Nemmar et al., 2013). Therefore, the evaluation of the effect of toxic compounds on epithelial barrier permeability could be an interesting research field. The 16HBE (Cozens et al., 1994) are human immortalized bronchial epithelial cell lines, which are able to form tight junction (Luyts et al., 2015 Wan et al., 2000). One of the most common methods to evaluate the epithelial barriers integrity is the measure of the TEER. It is a kind of technique non-invasive able to permit the evaluation of the barrier

integrity during various steps of growth and differentiation. But it is a technique that shows a wide range of value for the same cell line. This high variability could be lead versus incorrect conclusions about the epithelial barriers alteration and disruption related to drugs or particles (Srinivasan et al., 2015). Our experiments seem to confirm this variability. Hence our measures show a wide range of value for the same cells in the same conditions, although NaClO was used, which represent a well-known enviromental respiratory irritant (McGovern et al. 2015, 2010; Tuck et al. 2008). For this reason, more efficient ways of estimating variations in tight junctions are required to assess and screen a large number of chemicals and particulate matter. Nevertheless, the evaluation of the Zo-1, a protein associated with the tight junction (zonula occludens) in a variety of epithelia (Stevenson et al., 1986; Fanning et al., 1998; Luyts et al., 2015 Wan et al., 2000) by immunofluorescence assay seems to show interesting results. The immunopositivity of ZO-1 seem to be sensitive to the exposure of NaClO, and results were comparable in the different plates. Overall, the 16HBE cells resulted sensitive to NaClO exposure, and these results confirmed their efficiency in the assessment of air pollutants effects (Luyts et al., 2015; Baulig et al., 2003).

5.7. Conclusion

In general, the cell culture technique represents a very useful and interesting tool to estimate the effect of particles and pollutant on cells. Hence, the use of cell culture in toxicology permits to investigate deeper the uptake and the action mechanism of several particles and chemical compound in easy setting microenvironment. About the experiments, the evaluation of the tight junction disruption induced by pollutants and particles represent a very interesting investigation field. With regards the approaches, the TEER measurement is a non-invasive technique able to give general information about the integrity of the epithelial barriers and the use of an immunofluorescence assay seems a very promising approach for the evaluation of tight junction disruption induced by pollutants on airway epithelial cells. Therefore, these preliminary results, obtained during a very brief internship, could represent the first step for future and specific investigations for the assessment of environmental toxic compound effects on human airway epithelial cells.

CHAPTER VI General Conclusion

The results obtained in this thesis highlight how the use of a multi-biomarker approach on aquatic organisms, such as mussels, is useful in evaluating the biological effects of environmental pollutants. The use of a scale-up experiment represented a good strategy to point out the degree of recovery treatments efficiency versus wastewater and contaminated sediments from a polluted area. Additionally, thanks to the approaches herein applied, it was possible to underline the capability of the aforementioned treatments in healing environmental conditions for the organisms. Overall, it was possible to foreground that sediment treatment produced results more significant compared to those obtained on wastewater, but in general, results provided useful suggestions for the optimization of both recovery action treatments. Moreover, during the traineeship at Swetox, evaluation on tight junction disruption on airway epithelial cells after exposure to NaClO, a well-known respiratory irritant, was performed. This work showed that the assessment of air pollutant effect on epithelial barrier permeability represents an interesting field of research. Furthermore, in general, cell culture techniques represent an important tool to investigate the action mechanisms of a variety of pollutants, that could surely represent a valid support in studies dealing with the biological effects of environmental toxic compounds in different ecosystems.

Reference

Alfaro-Moreno E, Martínez L, García-Cuellar C, Bonner JC, Murray JC, Rosas I, Ponce de León Rosales S, Osornio Vargas A.. (2002) Biologic effects induced in vitro by PM10 from three different zones of Mexico City. *Environ Health Perspect.;*110;715-20.

Alfaro-Moreno E, Nawrot TS, Vanaudenaerde BM, Hoylaerts MF, Vanoirbeek JA, Nemery B, Hoet HM P (2008) Co-cultures of multiple cell types mimic pulmonary cell communication in response to urban PM10. *Eur Respir J*.;32(5):1184.

Alfaro-Moreno E., Engel M., De Marco G., Kos V. M. (2018). Evaluation of tight junction disruption induced particles on airway epithelial cells using an automatized imaging method. *Tox. Letters*, 295, S208. doi:10.1016/j.toxlet.2018.06.910.

Al-Shamrani A.A., James A., Xiao H., (2002.) Destabilisation of oil-water emulsions and separation by dissolved air flotation. *Water Res*.. 36, 1503-1512.

Barwal A., Chaudhary R., (2014). To study the performance of biocarriers in moving bed biofilm reactor (MBBR) technology and kinetics of biofilm for retrofitting the existing aerobic treatment systems: a review. Rev. *Environ. Sci. Biotechnol*. 13(3), 285–299.

Abdel-Shafy H.I., Mansour M.S.M., (2016). A review on polycyclic aromatic hydro-carbons: source, environmental impact, effect on human health and remediation .*Egypt. J. Pet.* 25, 107–123.

Accomasso L, Cristallini C, Giachino C. (2018) Risk Assessment and Risk Minimization in Nanomedicine: A Need for Predictive, Alternative, and 3Rs Strategies. *Front in Pharma;* 9 228. https://doi.org/10.3389/fphar.2018.00228.

Andral B., Stanisiere J.Y., Sauzade D., Damier E., Thebault H., Galgani, F., Boissery,P., (2004). Monitoring chemical contamination levels in the Mediterranean based on the use of mussel caging. *Mar Pollut Bull* 49, 704-712.

Auffret M., Barille L., Besnard-Cochennec N., Blanc, F., Boucaud-Camou E., (2003). An atlas of histology and cytology of marine bivalve molluscs. Ed. Henri Grizel, Ifremer.

Bal-Price A., Hogberg H.T. (2014) In Vitro Developmental Neurotoxicity Testing: Relevant Models and Endpoints. In: Bal-Price A., Jennings P. (eds) In Vitro Toxicology Systems. Methods in Pharmacology and Toxicology. Humana Press, New York, NY.

Bayne B.L., (1976). Marine mussels: their ecology and physiology. Cambridge UniversityPress.

Bebianno M.J., Lopes B., Guerra L., Hoarau P., Ferreira, A.M., (2007). Glutathione S tranferases and cytochrome P450 activities in *Mytilus galloprovincialis* from the South coast of Portugal: effect of abiotic factors. *Environ Int* 33, 550-558.

Beninger P.G. and StJean S.D., (1997). The role of mucus in particle processing by suspension-feeding marine bivalves: unifying principles. *Mar Biology* 129, 389- 397.

Beninger P.G., Ward J.E., MacDonald B.A. and Thompson R.J., (1992). Gill function and particle-transport in *Placopecten-Magellanicus* (Mollusca, Bivalvia) as revealed using video endoscopy. *Mar Biology* 114, 281-288.

Bouwer E.J., McCarty P.L., (1982). Removal of trace chlorinated organic compounds by activated carbon and fixed-film bacteria. *Environ. Sci. Technol.* 16, 836-843. http://dx.doi.org/10.1021/es00106a003.

Branch G.M. and Steffani C.N. (2004). Can we predict the effects of alien species? A case history of the invasion of South Africa by *Mytilus galloprovincialis* (Lamarck). *J of E Mar Bio and Eco* 300, 189-215.

Brandao F., Cappello T., Raimundo J., Santos M.A., Maisano M., Mauceri A., Pacheco M., Pereira P. (2015). Unravelling mechanisms of mercury hepatotoxicity in wild fish (*Liza aurata*) through a triad approach: bioaccumulation, metabolomic profiles, and oxidative stress. *Metallomics* 7, 1352-1363.

Baulig A, Sourdeval M, Meyer M, Marano F, Baeza-Squiban A. (2003) Biological effects of atmospheric particles on human bronchial epithelial cells. Comparison with diesel exhaust particles. *Tox. in Vitro.*;17:567–73.

Cappello S, Denaro R, Genovese M, Giuliano L, Yakimov MM (2007) Predominant growth of Alcanivorax during experiments on "oil spill bioremediation" in mesocosms. *Microbiol Res* 162 (2):185–190.

Cappello S., Genovese M., Denaro R., Santisi S., Volta A., Bonsignore M., Mancini G., Giuliano L., Genovese L., Yakimov

M.M.(2015). Quick stimulation of *Alcanivorax* sp. by bioemulsificant EPS_{2003} on microcosm oil spill simulation. *Braz. J. Microbiol*. 45(4), 1317-23.

Cappello S., Volta A., Santisi S., Morici C., Mancini G., Quatrini P., Genovese M., Yakimov MM., Torregrossa M.(2016) Oil-degrading bacteria from a membrane bioreactor (BF-MBR) system for treatment of saline oily waste: Isolation, identification and characterization of the biotechnological potential. *I. Biodeterioration & Biodegradation.*110 ; 235-244.

Cappello T., Maisano M., D'Agata A., Natalotto A., Mauceri A., Fasulo S. (2013a). Effects of environmental pollution in caged mussels (*Mytilus galloprovincialis*). Mar. Env.l Research., 91, 52–60.

Cappello T., Mauceri A., Corsaro C., Maisano M., Parrino V., Lo Paro G., Messina G., Fasulo S. (2013b). Impact of environmental pollution on caged mussels *Mytilus galloprovincialis* using NMR-based metabolomics. *Mar. Pollut. Bull*. 77, 132-139.

Cappello T., Maisano M., Giannetto A., Parrino V., Mauceri A., Fasulo S. (2015). Neurotoxicological effects on marine mussel *Mytilus galloprovincialis* caged at petrochemical contaminated areas (eastern Sicily, Italy): ^{1}H NMR and immunohistochemical assays. *Comp. Biochem. Physiol. C* 169, 7-15.

Cappello T., Brandao F., Guilherme S., Santos M.A., Maisano M., Mauceri A., Canario J., Pacheco M., Pereira, P., (2016a). Insights into the mechanisms underlying mercury-induced oxidative stress in

gills of wild fish *Liza aurata* combining ^{1}H NMR metabolomics and conventional biochemical assays. *Sci.Total Environ.* 548-549, 13-24.

Cappello T., Pereira P., Maisano M., Mauceri A., Pacheco M., Fasulo S., (2016b). Advances in understanding the mechanisms of mercury toxicity in wild golden grey mullet (*Liza aurata*) by ^{1}H NMR-based metabolomics. *Environ. Pollut.* 219, 139–148.

Cappello T., Maisano, M., Mauceri, A., Fasulo, S., (2017). ^{1}H NMR-based metabolomics investigation on the effects of petrochemical contamination in posterior adductor muscles of caged mussel *Mytilus galloprovincialis*. *Ecotoxicol. Environ. Saf.* 142, 417-422. http://dx.doi:10.1016/j.ecoenv.2017.04.040.

Cajaraville M.P., Bebianno M.J., Blasco J., Porte C., Sarasquete C., Viarengo A., (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Sci. Total Environ*. 247, 295–311.

Carroll M.A., Catapane E.J. (2007). The nervous system control of lateral ciliary activity of the gill of the bivalve mollusc, *Crassostrea virginica*. *Comp. Biochem. Physiol. A* 148, 445–450.

Carlton J.T. (1992). The dispersal of living organisms into aquatic ecosystems: the mechanisms of dispersal as mediated by aquaculture and fisheries activities. In: Rosenfield A., Mann R. (Eds) Dispersal of living organisms into aquatic ecosystems. The University of Maryland, College Park, Maryland, pp 13-45.

Carrel A. (1912). On the permanent life of tissues outside the organism. *J. Exp. Med*. 15:516–528.

Catapane E., Aiello E., Stefano G.B., (1974). Ganglionic mediation mechanism of lateral cilia in *Mytilus edulis* gill. *Physiologist* 17–372.

Ciacci C., Barmo C., Gallo G., Maisano M., Cappello, T., D'Agata, A., Leonzio, C.,Mauceri, A., Fasulo, S., Canesi, L., (2012). Effects of sublethal, environmentally relevant concentrations of hexavalent chromium in the gills of *Mytilus galloprovincialis. Aquat. Toxicol*. 120, 109-118.

Cravo C., Pereira C., Gomes T., Cardoso C., Serafim A., Almeida C., Rocha T., Lopes B., Company R., Medeiros A., Norberto R., Pereira R., Araújo O., Bebianno M.J., (2012). A multibiomarker approach in the clam *Ruditapes decussatus* to assess the impact of pollution in the Ria Formosa lagoon, South Coast of Portugal. *Mar.Environ. Res*. 75, 23–34.

Cozens A. L., Yezzi M. J.,. Kunzelmann K,. Ohrui T, Chin L., Eng K., Finkbeiner W. E., Widdicombe J. H., and Gruenert D. C. (1994). CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol*. 10:38.

D'Agata A., Cappello T., Maisano M., Parrino V., Giannetto A., Brundo M.V., Ferrante M., Mauceri A., (2014). Cellular biomarkers in the mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae) from Lake Faro (Sicily, Italy). *Ital. J. Zool. 81 (1*), 43–54.

Davis M, Hodgkins GA, and Stoner AW. (1996). A mesocosm system for ecological research with marine invertebrate larvae. *Mar Ecol-Prog Ser* 130: 97–104.

De Coen W.M., and Janssen C.R., (2003). A multivariate biomarker-based model predicting population-level responses of *Daphnia magna*. *Env Tox and Chem* 22, 2195-2201.

De Domenico, E., Mauceri, A., Giordano, D., Maisano, M., Gioffre, G., Natalotto, A., D'Agata, A., Ferrante, M., Brundo, M.V., Fasulo, S., (2011). Effects of "in vivo" exposure to toxic sediments on juveniles of sea bass (*Dicentrarchus labrax*). *Aquat. Toxicol*. 105, 688-697.

De Domenico E., Mauceri A., Giordano D., Maisano M., Giannetto A., Parrino V., Natalotto A., D'Agata A., Cappello T., Fasulo S. (2013). Biological responses of juvenile European sea bass (*Dicentrarchus labrax*) exposed to contaminated sediments. *Ecotoxol. Environ. Safe* 97, 114-123**.**

Della Torre C., Tornambè A., Cappello S., Mariottini M. Perra G., Giuliani S., Amato E., Falugi C., Crisari A., Yakimov M.M., Magaletti E. (2012). Modulation of CYP1A and genotoxic effects in European seabass *Dicentrarchus labrax* exposed to weathered oil: A mesocosm study. *Mar env. res.* 76, 48-55.

Dermont G., Bergeron M., Mercier M., Richer-Laflèche M. (2008) Soil washing for metal removal: A review of physical/chemical technologies and field applications. *J. Hazard. Mater.* 152, 1–31.

Di Leonardo, R., Mazzola, A., Tramati, C.D., Vaccaro, A., Vizzini, S., (2014). Highly contaminated areas as sources of pollution for

adjoining ecosystems: the case of Augusta Bay (Central Mediterranean). *Mar. Pollut. Bull*. 89, 417-426.

Di Trapani D., Di Bella G., Torregrossa M., Viviani G., (2014). Comparison between moving bed-membrane bioreactor (MB-MBR) and membrane bioreactor (MBR) systems: Influence of wastewater salinity variation. *Bioresour. Technol*. 162,60-69.

Dorris T.C., Burks S.L., Curd M.R., Waller G.R., Broemeling L.D., (1972). Identification of Toxic Components in Oil Refinery Wastewaters and Determination of Their Effect upon the *Aquatic Biota. Technical Completion Report* (OWRR B- 017e0KLA), 113.

Earle W. R., Schilling E. L., Stark T. H., Straus N. P., Brown M. F., and Shelton E. (1943). Production of malignancy in vitro; IV: The mouse fibroblast cultures and changes seen in the living cells. *J. Natl. Inst*. 4:165–212.

Ehsan S., Prasher S.O, Marshall W.D., (2006) A washing procedure to mobilize mixed contaminants from soil. II. Heavy metals, *J. Environ. Qual*. 35 2084–2091.

Ellman G.L., Courtney K.O., Anders V., Featherstone R.M., (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol*. 7, 88-95.

Evangelho M.R., Gonçalves M.M.M., Sant'Anna Jr. G.L., Villas Boas R.C., (2001). A trickling filter application for the treatment of a gold milling effluent. Int. J. Miner. Process 62, 279-292. http://dx.doi.org/10.1016/S0301-7516(00)00059-4.

Fabbri E., Capuzzo A., (2006). Adenylyl cyclase activity and its modulation in the gills of *Mytilus galloprovincialis* exposed to Cr^{6+} and Cu^{2+}. *Aquat. Toxicol*. 76, 59–68.

Fanning AS, Jameson BJ, Jesaitis LA, Anderson JM. (1998) The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem*.;273: 29745–53.

Farrington J.W., Goldberg E.D., Risebrough R.W., Marlin J.H., Bowen V.T., (1983). US Mussel Watch 1976-1978: an overview of the trace-metal, DDE, PCB, hydrocarbon and artificial radionucleotide data. *Env Science & Techn* 17, 460-496.

Fasulo S., Mauceri A., Giannetto A., Maisano M., Bianchi N., Parrino V., (2008). Expression of metallothionein mRNAs by in situ hybridization in the gills of *Mytilus galloprovincialis*, from natural polluted environments. *Aquat. Toxicol.* 88, 62-68.

Fasulo S., Maisano M., Sperone E., Mauceri A., Bernabò I., Cappello T., D'Agata A. Tripepi S., Brunelli E., (2012a). Toxicity of Foroozan crude oil to ornate wrasse (*Thalassoma pavo*, Osteichthyes, Labridae): ultrastructure and cellular biomarkers. *Ital. J. Zool*. 79, 182-199.

Fasulo S., Iacono F., Cappello T., Corsaro C., Maisano M., D'Agata A., Giannetto A. De Domenico E., Parrino V., Lo Paro G., Mauceri A., (2012b) Metabolomic investigation of *Mytilus galloprovincialis* (Lamarck 1819) caged in aquatic environments. *Ecotoxicol. Environ. Saf*. 84, 139-146.

Fasulo S., Guerriero G., Cappello S., Colasanti M., Schettino T., Leonzio C., Mancini G., Gornati R., (2015). The "SYSTEMS BIOLOGY" in the study of xenobiotic effects on marine organisms for evaluation of the environmental health status: biotechnological applications for potential recovery strategies. *Rev. Environ. Sci. Biotechnol.* 14, 339-345.

Federal Remediation Technologies Roundtable (FRTR) (2007), Remediation Technologies Screening Matrix and Reference Guides, Version 4.0, http://www.frtr.gov/matrix2/top page.html,

Federal Remediation Technologies Roundtable (FRTR) (2007), RemediationTechnologies Screening Matrix and Reference Guides, Version 4.0, http://www.frtr.gov/matrix2/top page.html.

Fernandes J.M.O., MacKenzie M.G., Wright P.A., Steele S.L., Suzuki Y.,Kinghorn J.R., Johnston I.A., (2006). Myogenin in model pufferfish species: comparative genomic analysis and thermal plasticity of expression during early development. *Comp. Biochem. Physiol. Part. D.* 1, 35-45.

Fernandez B., Campillo J.A., Martinez-Gomez C., Benedicto J., (2012). Assessment of the mechanisms of detoxification of chemical compounds and antioxidant enzymes in the digestive gland of mussels, *Mytilus galloprovincialis*, from Mediterranean coastal sites. *Chemosphere* 87, 1235-1245.

Franzellitti S., Fabbri E., (2013). Cyclic-AMP mediated regulation of ABCB mRNA expression in mussel haemocytes. *PLoS ONE* 8 (4), e61634.

Freshney. **R. I. (2011)**. Introduction. In Culture of Animal Cells, R. I. Freshney (Ed.).

Geiser, M.; Baumann, M.; Cruz-Orive, L.M.; Im Hof, V.; Waber, U.; Gehr, P. (1994). The effect of particle inhalation on macrophage number and phagocytic activity in the intrapulmonary conducting airways of hamsters. *Am. J. Respir. Cell Mol. Biol., 10(6),* 594–603.

Geller J.B.(1999). Decline of a native species masked by dibbling species invasion. *Conserv Biol* 13, 661-664.

Gey G. O., Coffman W. D., and Kubicek M. T. (1952). Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res*. 12:364–365.

Geys J., Coenegrachts L., Vercammen J., Engelborghs Y., Nemmar, A. Nemery B., Hoet P.H.M. (2006) In vitro study of the pulmonary translocation of nanoparticles: A preliminary study. *Toxicol. Lett*., 160, 218–226.

Giannetto A., Fernandes J.M.O., Nagasawa K., Mauceri A., Maisano M., De Domenico E., Cappello T., Oliva S., Fasulo S., (2014). Influence of continuous light treatment on expression of stress biomarkers in Atlantic cod. *Dev. Comp. Immunol.* 44, 30-34.

Giannetto A., Maisano M., Cappello T., Oliva S., Parrino V., Natalotto A., De Marco G., Barberi C., Romeo O., Mauceri A., Fasulo S., (2015). Hypoxia-inducible factor α and Hif-prolyl hydroxylase characterization and gene expression in short-time air exposed *Mytilus galloprovincialis*. *Mar. Biotechnol*. 17, 768-781

Godoy P., Hewitt N.J., Albrecht U. et al. (2013) Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME *Arch Toxicol* 87: 13-15. https://doi.org/10.1007/s00204-013-1078-5.

Goldberg E.D., Bertine K., (2000). Beyond the mussel watch-new directions for monitoring marine pollution. *Sci. Total Environ*. 247, 165–174. http://dx.doi.org/10.1016/.

Goldberg E.D., Bowen V.T., Farrington J.W., (1978). The Mussel Watch. Environmental Conservation 5(2), 101-125.

Goldberg E.D., Koide M., Hodge V., Flegal A.R., Martin J. (1983). U.S. Mussel Watch:1977-1978 Results on Trace Metals and Radionuclides. *Estuarine, Coastal and Shelf Science* 16(1), 69-93.

Gornati R, Maisano M, Pirrone C, Cappello T., Rossi F., Borgese M., Giannetto A., Cappello S., Mancini G, Bernardini G, Fasulo S.(2018) Mesocosm system to evaluate Bf-MBR efficiency in mitigating oily-wastewater discharges: an integrated study on *Mytilus galloprovincialis*. *Aquatic Sciences* Submitted

Gosselin R.E., 1961. The cilioexcitatory activity of serotonin. *J. Cell. Comp. Physiol.* 58, 17–25.

Gosling, E., (1992) The mussel Mytilus: Ecology, physiology, genetics and culture. Ed.Gosling, E. Developments in Aquaculture and Fisheries Science, Elsevier, Amsterdam.

Gosling, E.M., (2003). Bivalve molluscs: biology, ecology and culture, Oxford, Fishing News Books.

Guerra-Garcia, J.M., Garcia-Gomez, J.C., (2005). Oxygen levels versus chemical pollutants: do they have similar influence on macrofaunal assemblages? A case study in a harbour with two opposing entrances. *Environ. Pollut*. 135, 281-291.

Harrison, R. G. (1907). Observations on the living developing nerve fiber. *Proc. Soc. Exp. Biol. Med*. 4:140–143.

Hartung T. (2009). Toxicology for the twenty-first century. *Nature* 460, 208–212.

Hellou J., Law R.J., (2003). Stress on stress response of wild mussels, *Mytilus edulis and Mytilus trossulus*, as an indicator of ecosystem health. *Environ. Pollut.* 126, 407-416.

Hiemstra P, Grootaers G, Van der Does A, Krul C, Kooter I (2018) Human lung epithelial cell cultures for analysis of inhaled toxicants: Lessons learned and future directions, *Tox. in Vitro*, 47,137-146.

Huang X., Gui P., and Qian Y. (2001). Effect of sludge retention time on microbial behaviour in a submerged membrane bioreactor, *Process Biochemistry*, 36, 1001–1006.

Humphries J.E., Yoshino T.P., (2003). Cellular receptors and signal transduction in molluscan hemocytes: connections with the innate immune system of vertebrates. *Integr. Comp. Biol*. 43, 305–312.

Jefferson B., Laine A. L., Judd S. J., and Stephenson T. (2000). Membrane bioreactors and their role in wastewater reuse, *Water Science and Technology*, 41(1), 197–204.

Kelly RP, Port JA, Yamahara KM, and Crowder LB. (2014). Using environmental DNA to census marine fishes in a large mesocosm. *PLoS ONE* 9: e86175.

Kim K.H., Kabir E., Jahan S.A., (2016). A review on the distribution of Hg in the environment and its human health impacts. *J. Hazard. Mater*. 306, 376–385.

Knight A (2008) Systematic reviews of animal experiments demonstrate poor contributions toward human healthcare. *Rev Recent Clin Trials* 3(2):89–96.

Kuo S, Lai M S, Lin C W, (2006) Influence of solution acidity and $CaCl_2$ concentration on the removal of heavy metals from metal-contaminated rice soils. *Env Poll*.144:918-925.

Lehmann A. D, Blank F.,Baum O, Gehr P., and Rothen-Rutishauser B. M. (2009) Diesel exhaust particles modulate the tight junction protein occludin in lung cells in vitro, *Part and Fibre Tox,* 6, 26.

Li, N., Xia, T., Nel, A.E., (2008). The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. *Free Radic. Biol. Med.* 44 (9), 1689–1699.

Lionetto M.G., Caricato R., Calisi A., Giordano M.E., Schettino T., (2013). Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. *Biomed Res. Int 2013*.1-8.

Limonciel A. (2014) The Past, Present, and Future of Chemical Risk Assessment. In: Bal-Price A., Jennings P. (eds) In Vitro Toxicology Systems. Methods in Pharmacology and Toxicology. Humana Press, New York, NY

Livingstone D.R., Chipman J.K., Lowe D.M., Minier C., Pipe R.K.(2000). Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (*Mytilus edulis L.*) and other mytilids. *Int J of Enviand Poll* 13(1-6), 56-91.

Long R.R., MacDonald D.D., Smith S.L., Calder F.D. (1995) Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. *Environ. Manag.* 19, 81-97.

Lotze, H.K., Lenihan, H.S., Bourque, B.J., Bradbury, R.H., Cooke, R.G., Kay,M.C., Kidwell, S.M., Kirby, M.X., Peterson, C.H., Jackson, J.B.C., (2006). Depletion, degradation, and recovery potential of estuaries and coastal seas. *Science* 312, 1806–1809.

Luyts K, Napierska D, Dinsdale D, Klein SG, Serchi T, Hoet P. (2015) A coculture model of the lung—blood barrier: The role of activated phagocytic cells. *Tox in Vitro* 29: 234–241

Maisano M., Cappello T., Natalotto A., Vitale V., Parrino V., Giannetto A., Oliva S., Mancini G., Cappello S., Mauceri A., Fasulo S. (2017). Effects of petrochemical contamination on caged marine mussels using a multi-biomarker approach: Histological changes,

neurotoxicity and hypoxic stress. *Mar. Environ. Res*. 128, 114-123. http://dx.doi:10.1016/j.marenvres.2016.03.008.

Mancini G., Bruno M., Polettini A., Pomi R. (2011). Chelant-assisted pulse flushing of a field Pb-contaminated soil. *Chem and Eco*, 27(3), 251–262. doi:10.1080/02757540.2010.547492.

Mancini G., Cappello S., Yakimov M.M., Polizzi A., Torregrossa M., (2012). Biological Approaches to the Treatment of Saline Oily Waste (waters) Originated from Marine Transportation. *Chem Eng*. 27, 37-42.

Mancini G., Panzica M., Fino D., Cappello S., Yakimov M.M., Luciano A., (2017). Feasibility of treating emulsified oily and salty wastewaters through coagulation and bio-regenerated GAC filtration. *J. of Envir Man. 203,* 817-824**.**

Manduzio H., Monsinjon T., Galap C., Leboulenger F., Rocher B., (2004). Seasonal variations in antioxidant defences in blue mussels *Mytilus edulis* collected from a polluted area: major contributions in gills of an inducible isoform of Cu/Zn-superoxide dismutase and of glutathione S-transferase. *Aquat Toxicol* 70, 83-93.

Marean C.W., Bar-Matthews M., Bernatchez J., Fisher E., Goldberg P., Herries A.I.R., Jacobs Z., Jerardino A., Karkanas P., Minichillo T., Nilssen P.J., Thompson E., Watts I., Williams H.M., (2007). Early human use of marine resources and pigment in South Africa during the Middle Pleistocene. *Nature* 449, 905–908.

Matter K, Balda MS. (2003) Functional Analysis of Tight Junctions. *Methods*. 30:228–234.

Matozzo V., Tomei A., Marin M., (2005). Acetylcholinesterase as a biomarker of exposure to neurotoxic compounds in the clam Tapes philippinarum from the Lagoon of Venice. *Mar. Pollut. Bull*. 50, 1686–1693.

McDowell J.E., Lancaster B.A., Leavitt D.F., Rantamaki P., Ripley B., (1999). The effects of lipophilic organic contaminants on reproductive physiology and disease processes in marine bivalve molluscs. *Lim. and Ocean.* 44(3), 903-909.

McGovern TK, Powell WS, Day BJ, White CW, Govindaraju K, Karmouty-Quintana H, Lavoie N, Tan J, Martin JG (2010) Dimethylthiourea protects against chlorine induced changes in airway function in a murine model of irritant induced asthma. *Respir Res 11*:138. https://doi.org/10.1186/1465-9921-11-138.

McGovern TK, Goldberger M, Allard B, Farahnak S, Hamamoto Y, O'Sullivan M, Hirota N, Martel G, Rousseau S, Martin JG (2015) Neutrophils mediate airway hyperresponsiveness after chlorine induced airway injury in the mouse. *Am J Respir Cell Mol Biol* 52(4):513–522. https://doi.org/10.1165/rcmb.2013-0430OC.

Mee L.D., Readmann J.W., Villeneuve J.P., Sericano J.L., Wade T.L., Jackson,T.J. Brooks J.M., Tripp B.W., Farrington J.W., Goldberg E.D., (1995). Trace organic contamination in the Americas: An overview of the US national status & trends and the international 'Mussel Watch' Programmes. *Mar. Poll. Bulletin* 31(4), 214-225.

Ministerial Decree of 8 november 2010, No. 260, 2010. Regolamento recante i criteri tecnici per la classificazione dei corpi idrici superficiali, per la modifica delle nrme tecniche del decreto legislativo 3 aprile 2006,

n. 152, recante norme in materia ambientale, predisposto ai sensi dell'articolo 75, comma 3, del medesimo decreto legislativo. Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Roma, 07 febbraio 2011.

Nemery B. Hoet P.H.M D. Nowak (2002) Indoor swimming pools, water chlorination and respiratory health. *Eur. Resp. Journal*. 19(5)790-793;doi:10.1183/09031936.02.00308602.

Nemmar A, Holme JA, Rosas I, Schwarze PE, Alfaro-Moreno E (2013).Recent advances in particulate matter and nanoparticle toxicology: a review of the in vivo and in vitro studies. *Biomed Res Int*.;2013:22 http://dx.doi.org/10.1155/2013/279371.

Ng, A. N. L., & Kim, A. S. (2007). A mini-review of modeling studies on membrane bioreactor (MBR) treatment for municipal wastewaters. *Desalination,* 212 261–281. doi:10.1016/j.desal.2006.10.013.

Nurkiewicz T.R., Porter D.W., Hubbs A.F., Stone S., Moseley A.M., Cumpston, J.L., Goodwill, A.G., Frisbee, S.J., Perrotta, P.L., Brock, R.W., Frisbee, J.C., Boegehold, M.A., Frazer, D.G., Chen, B.T., Castranova, V.(2011). Pulmonary particulate matter and systemic microvascular dysfunction. HEI Health Review Committee *Res. Rep. Health Eff. Inst*. 164, 3–48.

Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, Lilly P, Sanders J, Sipes G, Bracken W, Dorato M, Van Deun K, Smith P, Berger B, Heller A (2000) Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol* 32(1):56–67. doi: 10.1006/rtph.2000.1399.

Oviatt CA, Pilson MEQ, Nixon SW, Frithsen JB, Rudnick DT Kelly JR & Grassle JF Grassle J.(1984). Recovery of a polluted estuarine system – a mesocosm experiment. *Mar Ecol- Prog Ser* 16: 203–17.

Peakall DW.(1994). Biomarkers: the way forwardin environmental assessment. *Toxicol.Ecotoxicol. News*. 1, 55-60.

Peters R.W.(1999) Chelant extraction of heavy metals from contaminated soils, *J. Hazard. Mater*. 66 151–210

Phalen, R.F(1976). Inhalation exposure of animals. *Environ. Health Perspect*., *16*, 17–24.

Pirrone C., Rossi F., Cappello S., Borgese M., Mancini G., Bernardini G., Gornati R., 2018. Evaluation of biomarkers in *Mytilus galloprovincialis* as an integrated measure of BF-MBR efficiency in mitigating the impact of oily-wastewater discharge to marine environment: a microcosm approach. *Aquat. Toxicol.* 198,49-62, https://doi.org/10.1016/j.aquatox.2018.02.018.

Rainbow P.S., (1995). Biomonitoring of heavy metal availability in the marine environment. *Mar. Pollut. Bull.* 31, 183–192

Rank J., Lehtonen K.K., Strand J., Laursen M., (2007). DNA damage, acetylcholinesterase activity and lysosomal stability in native and transplanted mussels (*Mytilus edulis*) in areas close to coastal chemical dumping sites in Denmark. *Aquat. Toxicol*. 84, 50–61.

Reid, R.G.B., (1968). The distribution of digestive tract enzymes in lamellibranchiate bivalves. *Comparative Biochem and Physiol* 24**,** 727-744.

Robinson T.B., Griffiths C.L., (2002). Invasion of Langebaan Lagoon, South Africa, by *Mytilus galloprovincialis* – effects on natural communities. *Afr Zool* 37, 151-158.

Roméo M., Hoarau P., Garello G., Gnassia-Barelli M., Girard J.P., (2003). Mussel transplantation and biomarkers as useful tools for assessing water quality in the NW Mediterranean. *Environ Pollut* 122, 369-378.

Rueda-Romero C., Hernández-Pérez G., Ramos-Godínez P., Vázquez-López I., Quintana-Belmares R.O., Huerta-García E., Stepien E., López-Marure R., Montiel-Dávalos A., Alfaro-Moreno E., (2016). Titanium dioxide nanoparticles induce the expression of early and late receptors for adhesion molecules on monocytes. Part. *Fibre Toxicol. 13 (36).* https://doi.org/10.1186/s12989-016-0147-3.

Russell WMS, Burch RL (1959) The principles of humane experimental technique. Methuen,London.

Sagarin R.D., Adams J., Blanchette C.A., Brusca R.C., Chorover J., Cole J.E., Micheli F., Munguia-Vega A., Rochman C.M., Bonine K., van Haren J., Troch P.A., (2016). Between control and complexity: opportunities and challenges for marine mesocosms. *Front. Ecol. Environ*. 14, 389–396.

Sanchís J, Llorca M, Olmos M, Schirinzi GF, Bosch-Orea C, Abad E, Barceló D, Farré M. (2018) Metabolic Responses of Mytilus galloprovincialis to Fullerenes in Mesocosm Exposure Experiments. *Environ Sci Technol*. Feb;52(3) 1002-1013. doi:10.1021/acs.est.7b04089. PMID: 29244952.

Scheinin M, Riebesell U, Rynearson TA, et al. (2015). Experimental evolution gone wild. *J R Soc Interface* 12; doi:10.1098/ rsif.2015.0056.

Schöne B.R., Krause JR. R.A. 2016. Retrospective environmental biomonitoring - Mussel Watch expanded. *Global and Planetary Change*, 144: 228-251.

Shaw J.P., Dondero F., Moore M.N., Negri A., Dagnino A., Readman J.W., Lowe D.R., Frickers P.E., Beesley A., Thain J.E., Viarengo A., (2011). Integration of biochemical, histochemical and toxicogenomic indices for the assessment of health status of mussels from the Tamar Estuary, U.K. *Mar Environ Res* 72, 13-24.

Smith, L .A (1995).Contaminants and remedial options at selected metal-contaminated sites. Technical resource report. United States: N. p.Web.

Soo J, Jansen J, Masereeuw R Little M (2018) Advances in predictive in vitro models of drug-induced nephrotoxicity. *Nat Rev Nep*. *14,s378–393* doi:10.1038/s41581-018-0003-9.

Soto M., Kortabitarte M., Marigómez I., (1995). Bioavailable heavy metals in estuarine waters as assessed by metal/shell-weight indices in sentinel mussels *Mytilus galloprovincialis*. *Mar. Ecol. Prog. Ser*. 125, 127–136.

Sprovieri, M., Oliveri, E., Di Leonardo, R., Romano, E., Ausili, A., Gabellini, M.,Barra, M., Tranchida, G., Bellanca, A., Neri, R., Budillon, F., Saggiomo, R., Mazzola, S., Saggiomo, V., (2011). The key role played by the Augusta basin (southern Italy) in the mercury contamination of the Mediterranean Sea. *J. Environ. Monit*. 13, 1753-1760.

Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ, et al. (2015) TEER measurement techniques for in vitro barrier model systems. *J Lab Autom*;20: 107-26.

Stefano, G.B., (1990). Neurobiology of *Mytilus edulis*. Manchester University Press, ND (312 pp.).

Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. (1986); Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol*. 103: 755 –66.

Sunila, I., (1988). Acute histological responses of the gill of the mussel, *Mytilus Edulis*, to exposure by environmental-pollutants. J. Invertebr. Pathol. 52, 137-141.

Superville, P.-J., Prygiel, E., Magnier, A., Lesven, L., Gao, Y., Baeyens, W., Ouddane, B., Dumoulin, D., Billon, G., (2014). Daily variations of Zn and Pb concentrations in the Deûle River in relation to the resuspension of heavily polluted sediments. *Sci. Total Environ*. 470-471, 600–607.

Sureda, A., Box, A., Tejada, S., Blanco, A., Caixach, J., Deudero, S., (2011). Biochemical responses of *Mytilus galloprovincialis* as biomarkers of acute environmental pollution caused by the Don Pedro oil spill (Eivissa Island, Spain). *Aquat. Toxicol*.101, 540-549.

Tampouris S. Papassiopi N.,. Paspaliaris I, (2001) Removal of contaminant metals from fine grained soils, using agglomeration, chloride solutions and pile leaching techniques, *J. Hazard. Mater*. 84 297–319.

Teitzel G.M., Parsek M.R., (2003). Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 69, 2313e2320. http://dx.doi.org/10.1128/AEM.69.4.2313-2320.2003.

Tsangaris C., Kormas, K., Strogyloudi E., Hatzianestis I., Neofitou C., Andral B.,Galgani F., (2010). Multiple biomarkers of pollution effects in caged mussels on the Greek coastline. *Comp Biochem Phys C* 151, 369-378.

Tuck SA, Ramos-Barbón D, Campbell H, McGovern T, Karmouty-Quintana H, Martin JG (2008) Time course of airway remodelling after an acute chlorine gas exposure in mice**.** *Respir Res 9*:61. https://doi.org/10.1186/1465-9921-9-6.

USEPA, COGNIS TERRAMET (1997) Lead Extraction Process Twin Cities Army Ammunition Plant COGNIS Inc., Demonstration Bulletin, EPA/540/MR-95/535, Superfund Innovative Technology Evaluation (SITE) Program, Washington, DC.

US Environmental Protection Agency, 2007. Test Methods for Evaluating Solid Waste, Physical/chemical Methods, Office of Solid Waste and Emergency Responses.SW-846. Environmental Protection Agency, Washington, D.C., United States.

Van Benschoten J.E,. Matsumoto M.R, Young W.H., (1997) Evaluation and analysis of soil washing for seven lead-contaminated soils, *J. Environ. Eng*.123 (3) 217–224.

Van Den Broucke S., Pollaris L., Vande Velde G., Nemery B. Verbeken E.. Vanoirbeek J., Hoet P. (2018). Irritant-induced asthma

to hypochlorite in mice due to impairment of the airway barrier *Arch Toxicol* 92: 1551. https://doi.org/10.1007/s00204-018-2161-8.

Van Hamme J.D., Singh A., Ward O.P., (2003). Recent advances in petroleum microbiology. *Microbiol. Mol. Biol. Rev.* 67, 503-549. http://dx.doi.org/10.1128/.

Vethaak AD, Jol JG, Meijboom A, et al. (1996). Skin and liver diseases induced in flounder (Platichthys flesus) after long-term exposure to contaminated sediments in large-scale mesocosms.*Environ Health Perspect* 104: 1218–29.

Viarengo A., Lowe D., Bolognesi C., Fabbri E., Koehler A., (2007). The use of biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comp Biochem Phys C* 146, 281-300.

Walker C.H., Hopkin S.P., Sibly R.M., Peakall D.B., (2006). Principles of Ecotoxicology. Taylor & Francis Group, Boca Raton.

Wan H, et al. (2000) Tight junction properties of the immortalized human bronchial epithelial cells Calu-3 and 16HBE14o-. *Eur Respir J*. 15(6): 1058-1068.

Widdows, J., and Donkin, P., (1992). Mussels and environmental contaminants: Bioaccumulation and physiological aspects In: Gosling, E.M. (ed.) The Mussel *Mytilus*: ecology, physiology, genetics, and culture. Amsterdam: Elsevier.

Williford C.W, Bricka R.M. (2000) Physical separation of metal-contaminated soils, in: I.K. Iskandar (Ed.), Environmental Restoration

of Metals- Contaminated Soils, 1st ed., CRC Press LLC, Boca Raton, FL, pp. 121–165.

Wu H., Southam A.D., Hines A., Viant M.R., (2008). High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. *Anal. Biochem*. 372,204-212.

Yadav AK, Bracher A, Doran SF, Leustik M, Squadrito GL, Postlethwait EM, Matalon S. (2010) Mechanisms and modification of chlorine-induced lung injury in animals. *Proc Am Thorac Soc*;7: 278–283.

Yakimov, M.M., Timmis, K.N., Golyshin, P.N., (2007). Obligate oil-degrading marine bacteria. *Curr. Opin. Biotechnol*. 18, 257-266. http://dx.doi.org/10.1016/j.copbio.2007.04.006.

Yamamoto K., Hiasa M., Mahmood T., Matsuo, T. (1989). Direct solid-liquid separation using hollow fiber membrane in an activated sludge aeration tank". *Water Science and Technology*, 21(4 –5), 43–54.

Zucco, F., De Angelis I,. Testai E, Stammati A (2004) Toxicology investigations with cell culture systems: 20 years after, *Tox. in Vitro*, 18, 2, 153-163,