Department of Life and Environmental Sciences

Doctorate course in

Life and Environmental Sciences

Curriculum Biology and Marine Ecology

XXXII Cycle
2016 - 2019

Ecotoxicity studies on marine sediments

Tutor:

Prof. Oliana Carnevali (UNIVPM)

Student:

Dott. Daniele Ancillai

Co-tutors:

Dott.ssa Francesca Maradonna (UNIVPM)

Dott.ssa Alessandra Valenti (ARPAM)

Dott. Tristano Leoni (ARPAM)

Department of Life and Environmental Sciences

Doctorate course in
Life and Environmental Sciences
Curriculum Biology and Marine Ecology

XXXII Cycle
2016 - 2019

Ecotoxicity studies on marine sediments

Tutor:
Prof. Oliana Carnevali (UNIVPM)

Student:
Dott. Daniele Ancillai

Co-tutors:
Dott.ssa Francesca Maradonna (UNIVPM)
Dott.ssa Alessandra Valenti (ARPAM)
Dott. Tristano Leoni (ARPAM)

Index

General Introduction

1. The coast: a delicate environment

In Italy, as in Europe, many of the marine-coastal areas coexist together with large industrial settlements, including port facilities, which have deteriorated the neighbouring seabed (Gabellini et al., 2011). The Italian coastal strip has an extension of approximately 8000 km and represents an important strategic resource for the nation itself and for the other European Union countries. More than 50% of population lives within 50 kilometres from the sea and the resources of these areas produce most of the economic wealth of the EU. Activities such as fishing, marine transport, industry and tourism are competing for every meter of a territory that runs along the 89,000 kilometres of the European coasts, hosting some of the most fragile and precious habitats of the entire planet. A richness given not only by the environment biodiversity.

Due to the increasingly intense anthropic exploitation, coastal resources are fast degrading: the aquifers are lowered and are invaded by salt water, the rivers are intercepted along their path, thus failing to add sediment that the sea can process, factors such as erosion accelerate, pollution is pressing, fish resources are diminishing and the environment begins to change irretrievably, losing resources being vulnerable. Every year, the Earth Overshoot Day (EOD, the day in which humanity consumes entirely the resources produced by the planet over the whole year) falls more and more in advance: it was August 1st in 2018, it is July 29th in 2019; at this rate, in 2050 humanity will consume twice as much as Earth can produce. We are not realizing it, but we are losing our life insurance, we are destroying an environment that is no longer able to adapt to the sudden changes we have imposed with our activities.

2. The specific problems of the coastal environment: between society and environment

Understanding the deleterious effects that the environment is undergoing does not mean dwelling only on the purely ecological aspect of this problem, but also social and economic ones. Mainly the coastal areas, if compared to any other area of the European Union, face more environmental, social and economic problems. The European Commission listed some of the best known:

Poor planning of tourism development: if managed in a suitable manner, tourism can be decisive for economic recovery in coastal areas. However, in many coastal zone tourisms has developed in a chaotic manner, causing: strong pressure on local drinking water resources and causing serious difficulties, as occurs in some areas of southern Europe. There are several Mediterranean areas where, due to excessive exploitation of groundwater, sea water has managed to infiltrate aquifers, thus making them unsuitable for drinking. Then considering the unsuitable systems for the disposal of solid waste and the consequent spread of illegal landfills, how this contributes to cause serious pollution phenomena on environmental matrices. Tourism is certainly a strong economic boost for each country, but to carry out positive action it must be controlled;

Decreased catch: fishing, which for centuries has been the basis of the local economy of most nowadays faces serious difficulties throughout the European area. Overfishing has caused a sudden and drastic impoverishment of what is the fish heritage and consequently a cascade of economic consequences. In a desperate attempt to curb this phenomenon, the EU's common fisheries policy has imposed severe restrictions on the volume caught in EU waters, trying to reduce the number of boats. While this seemed positive, the downsizing of the fleet's capacity had the direct consequence of causing an increase in unemployment in coastal areas, with fishermen moving in search of other occupations and with the coastal cities that have so lost their tourist appeal. Furthermore, in those cities where fishing activity continues, it still has to contend with other sectors such as the now uncontrolled urbanization of the coastal strip. The increasingly intense use by man of the coastal areas has caused the disappearance of fishing sites and the loss of entire

marine habitats, as well as deterioration of water quality and damage to the environment. Recent activities such as aquaculture facilities, try to find a compromise between the different aspects of urbanization, tourism and agriculture. Aquaculture can have positive effects on coastal areas because it can only be practiced where there is excellent water quality. However, its presence can also have negative effects causing pollution and waste disposal problems;

Inadequate transport: the lack of adequate connections tends to slow down the development of local economies, preventing coastal regions from exploiting the economic advantages that could derive from them. Usually transports are insufficient for what is the large European catchment area, and networks designed only on the basis of tourist flows can also cause mobility problems to the population that instead resides there throughout the year. It is also true, however, that too many or poorly designed communication routes certainly cause greater pollution and overcrowding problems, causing the destruction of natural habitats. Creating sustainable and efficient transport systems that respect the local environment is currently a utopia, but one that must be achieved by ensuring that those who build infrastructures interface with local realities;

Urbanization: this phenomenon has particularly affected our country, also manifesting itself in the form of illegals actions. If planned with due environmental precautions, building development can also contribute to the rescue of those coastal regions that are at risk of economic decline. However, the incessant overbuilding in Europe is unsustainable, a practice that in addition to destroying fragile natural habitats and preventing people from accessing the beaches, also increases the load deriving from the waste disposal systems and the septic tanks. A load that is often greater than the natural absorption capacity of the environment;

Coastal erosion: this is a natural process that occurs in all coastal areas and prevents the sea from penetrating too far into the land. Today many watercourses have been intercepted along their path (from quarries, artificial banks, urban constructions, dams, ...) and sedimentary transport has been compromised. Nothing comes to the sea to be processed, and the equation is thus completely shifted towards erosion. This phenomenon is not very worrying for the environment, but it becomes a big problem in areas where human constructions are located. Trying to prevent erosion is a very arduous task and it is not always easy to consider all the variables involved, nor to try to predict what the long-term effects of human interventions might be. Traditional works such as breakwaters or panels involve high maintenance costs and are not always able to prevent coastal erosion, and in some cases cause far more serious secondary problems. For example, they favour the presence of steadier waters along the coast which, combined with a high intake of nutrients and late summer temperatures, facilitate the flowering of toxic algae (e.g. *Ostreopsis ovata*). Even the construction of large-scale works of any kind in areas prone to erosion can aggravate the problem: in fact, in many places, instead of fighting natural erosion, a sort of "controlled withdrawal" has been opted for, or a gradual reduction in the presence of human activities in coastal areas. This cannot be practiced anywhere, so we begin to opt for decidedly more ecological solutions such as the reintroduction of native plant species, which with their root system are able to slow down the erosion process considerably;

Pollution: large-scale maritime disasters, such as oil spills and release of chemical substances, and disposal of inland waste at sea represent the main threats for coastal areas. The pollution that is caused by maritime accidents is a problem that concerns to a greater extent the coastal areas located near the major international shipping routes: in the Mediterranean alone oil transport accounts for 20-30% of world maritime traffic and amounted to in 2000 , to over 360 million tons per year (data from REMPEC - Regional Marine Emergency Center for the Mediterranean Sea) with a movement of 8 million barrels per day, which require over 3,000 naval trips per year and as many return trips. With such numbers it is obvious to think also of the high statistical incidence of accidents: on April 10th 1991, in the port of Livorno the ferry Moby Prince rammed the AGIP Abruzzo tanker; more than 25,000 tons of oil spilled from the ship, in addition to environmental damage, oil caused the fire of the cruise ship Moby Prince. 140 people died. What is more aggravating is that the responsibility for these accidents is difficult to determine: the

companies to which the oil tankers belong for the most part are based outside the jurisdiction of the European Union and in this way the causes become complex and very long. Another serious cause for concern is also nitrate pollution caused by agricultural fertilizers. They are an essential component of fertilizers, due to the leaching of the soil they are poured in large quantities into rivers and streams, supplying nutrition to organisms such as microalgae, which cause extensive bloom and tend to suffocate other forms of aquatic life. The problem also has repercussions on the sea, where the proliferation of mucilage's makes bathing unpleasant;

Habitat destruction: in the European coastal areas there are some of the richest and at the same time most fragile habitats. These environments are seriously endangered given both by the climatic changes perpetuated by man, by the demographic increase, by the change in economic activities, the alteration of the seabed, beaches and coastlines in response to the needs of the community. In particular, wetlands are the most exposed to the risk of urban expansion. Urbanization has led to the total extinction of some animal species in different coastal regions: a continuous and unstoppable loss of biodiversity. Although the environment maintains requirements of resistance and resilience, it is extremely impossible to reconstruct a coastal habitat and give it the same functionality that it had before a disturbance event occurred. Making an environment functional again, even if different from the starting characteristics, requires extremely complex and expensive interventions. Those who are responsible for coastal planning do not seem to realize at all that the loss of habitats can also have negative effects on the availability of water resources and coastal erosion, and so continue to destroy coastal habitats. Protecting a habitat is still frowned upon both by production activities and by regional administrations, which see their industrial growth, construction, urban planning and tourism as the real opportunity for economic growth. We do not realize that habitat loss has serious consequences on the economy of coastal areas.

It is quite clear that there must be directives, national but above all European, for what concerns the quality of matrices such as water, sediment and air. For example, the EU directive on water quality, known as the water framework directive, tackles the problems of coastal pollution in a decisive but common sense, using innovative ideas and basing water protection on a subdivision into river basins, whose management is possible thanks to the coordination of national, regional and local subjects.

3. Characteristics of marine sediment

Sediment is represented by all those solid materials that are deposited on the bottom of a generic water body. A sediment particle is generally composed of:

Inorganic fraction	Characterized by clay and non-silicates, carbonates, iron and manganese oxides, phosphates and sulphides.
Organic fraction	Consisting of living organisms, xenobiotic compounds and natural organic substance, which is very variable and can consist of proteins, peptides, humic and fulvic material.
Interstitial Water	Which can be up to about 50% by volume.
Pollutant	Inorganic or organic.

Based on their genesis and composition, sediments can be divided into clastic and chemical. While the former derives from processes of disintegration and erosion of rocks carried out by atmospheric agents, the latter are due to the precipitation of salts or solutes due to physical variations in pressure, concentration, pH, temperature etc. The clastic sediments can be subdivided in turn into volcaniclastics (tuffs and ignimbrites) and clastic-terrigens (argillite's, siltstones, sandstones and conglomerates). Chemical sediments are comprised of siliceous, evaporitic,

carbonatic, iron-manganese, aluminiferous, carbonaceous and bituminous ones. Finally, it is also possible to mention biogenic sediments, which are formed by the remains of plant, animal or organisms produced by different human activities.

The physical-chemical composition of sediments is very heterogeneous and depends on both natural (erosion and decomposition) and anthropogenic (civil or industrial pollution) processes, as well as environmental conditions (temperature, salinity, tides, currents and depths).

Sediments play a role of primary importance in all aquatic ecosystems as they offer a variety of different habitats for many species in support of the entire trophic network; however, the microbiological processes that occur in them, mainly responsible for the degradation of organic matter and the nutrient cycle, are extremely influenced by toxicity due to the possible presence of pollutants. Sediments constitute the preferential site of accumulation for many contaminants, as they are very easily adsorbed on the suspended particulate and are deposited with it on the bottom. Furthermore the marine sediments are distinguished by peculiar characteristics, some of which are problematic for their re-use in an environment different from the aquatic one: the high salinity; the inhomogeneous granulometry (due to sedimentation dynamics and subsequent resuspension by sea currents); inhomogeneous nutrient content and high organic substance content (due to the presence of animal and vegetable organisms); low biological activity; heterogeneous presence of pollutants. Fortunately, sediments have properties that allow them to mitigate the toxic effects of pollutants, a capacity similar to that of the soil system: organic matter can bind and immobilize contaminants, reducing their harmful effects. Moreover, the granulometry of the sediment represents a relevant factor for the fate of the contaminants, given that the fine particles (clay) offer many binding sites for metals, facilitating the phenomena of adsorption (Campbell et al., 1988).

4. Source of pollution of marine sediment

In this thesis a focus on marine sediments deriving from dredging activities is placed, in the light of presenting this topic not only in terms of environmental impact (contaminated sediment), but also in the possible re-use that these sediments could offer, in conjunction with the social and economic aspects mentioned above.

Marine sediment does not only comprise an environment, but also the set of chemical, physical and biological conditions of the environment in which the sediments accumulate (from the definition of Krumbein/Sloos, 1963). These conditions represent “matter and energy introduced into the environment to cause a persistent and sometimes irreversible alteration” (Della Croce et al., 1997).

A more recent definition of pollution defines it as a group of substances derived from anthropic activities and present at a concentration level higher than normal (Clark, 2001). This alteration may be of a chemical type, such as anomalous concentrations of an element, but also of a physical type, such as anomalies of the electromagnetic field or of light radiation. In fact, these two meanings have been somewhat tied together, and the Barcelona Convention for the Protection of the Mediterranean Sea identifies marine pollution as the introduction or subtraction by man, directly or indirectly, of substances or energy, cause deleterious effects on living resources, a danger to human health and an obstacle to its activity, compromising the environmental quality in relation to its use, including recreational and aesthetic use. What it creates is a disturbance, and to distinguish whether it is natural or anthropic, it needs a monitoring activity that includes, idealistically, a before and an after. It can substantially give two types of effects: acute and chronic. Acute pollution is a massive phenomenon that occurs in tight spaces and times and is by its very nature unpredictable and potentially catastrophic (for example, the sinking of an oil tanker or the loss of radioactive material). Chronic pollution, on the other hand, is linked to the presence of constant agents over time, which may not cause immediate mortality, but are nevertheless capable of altering the functioning of an ecosystem (for example the continuous exploitation of the hydrosphere as a vehicle to dispose of substances of refusal, trusting in its self-depurative capacity). Pollutant

contaminants can enter in the environment following different paths: a distinction can be made between rural areas, urban areas and direct entrances. The entrance that takes place from the rural areas is through the erosion of the soils or the banks of the canals themselves, and indirectly by the atmospheric deposition on the land. Urban areas contribute through leaching from the building material and through sewer systems. Direct entries are instead mainly due to the industry, then follow the transport and other additional sources. For this purpose, it also makes sense to divide the sources into **punctiform** and **diffuse** (Figure 1), with the first ones including urban wastewater, sewage discharges, ballast water or even mining activities; the latter, are sources defined precisely by river supplies and atmospheric emission. In this regard it is worth specifying that rivers are among the main vectors of marine pollution, always used as a place of discharge of many harmful substances or waste determine an alteration in water quality and fluvial biological communities, and radical changes in the environmental characteristics of the coastal strip.

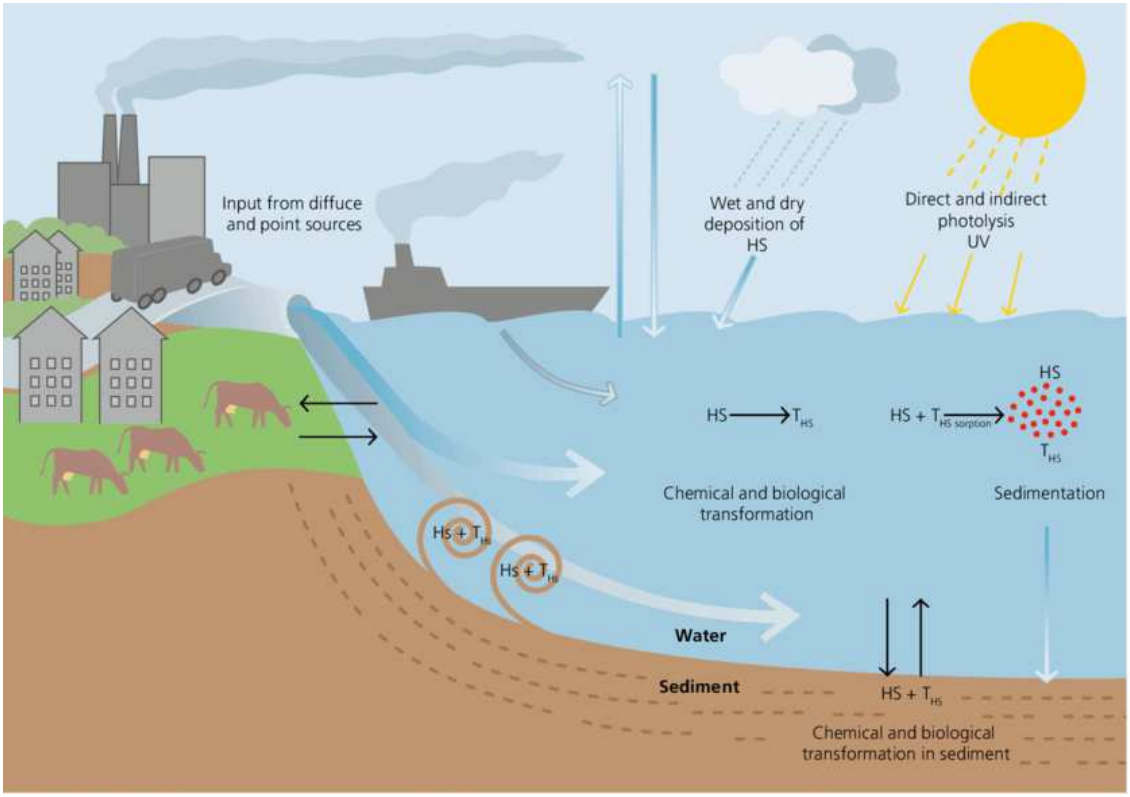


Figure 1. Conceptual model of the sources of pollution inputs to the marine environment and the fate of hazardous substances (HSs) and their transformation products (T_{HS}), (Helcom, 2010).

Dangerous substances can enter the in the environment through different sources, and these are not stored only within the sediments, but can be activated by interacting with other molecules, or by photo-activation, generating even more dangerous molecules. Among various environmental matrices, the sediment represents an extremely complex entity in which there are many interactions between the abiotic and biotic components and in which complicated processes of degradation and recirculation of the organic substance and nutrients takes place. Moreover, soils are among the largest reserves of biodiversity on Earth, important reservoirs of prokaryotes and eukaryotes, and a great variety of invertebrates. In addition to their biodiversity role, sediments are also fundamental for providing many ecosystem functions, which become much more complicated at sea than on land. From a geochemical point of view, marine sediments are the final deposit of everything that runs through and passes through the emerged lands (Frignani and Turci, 1981; Pellegrini et al., 1999; Del Valls et al., 2004; Casado Martinez et al., 2006): from the material that is washed away naturally from the rains and transported by the rivers, up to the contaminants used in the anthropic

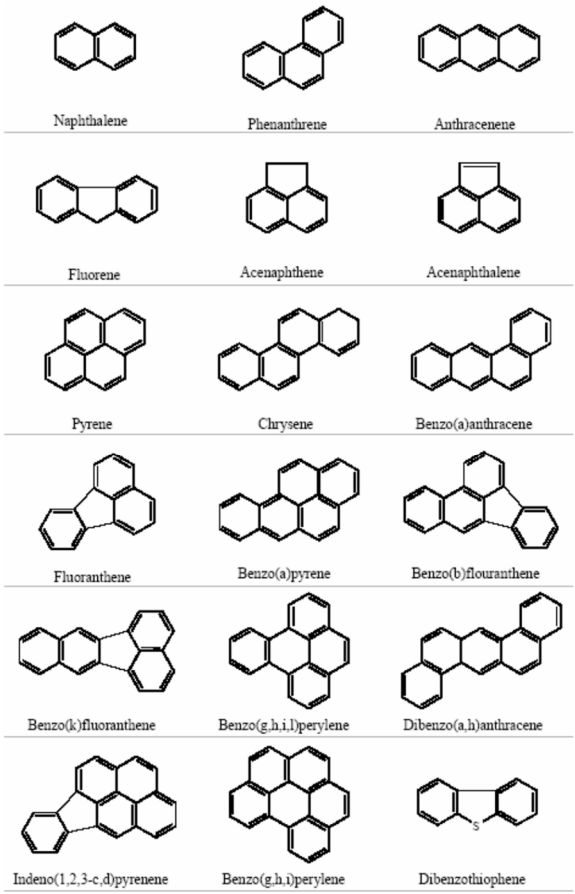


Figure 2. More common PAHs.

productive activities and dragged to the sea. Marine sediments are conservative matrices and, especially along the coasts and in the vicinity of industrial, port and urban settlements, they represent the chemical memory of the processes that took place there. Despite the appearance of sediment is a stable sector, it is not really so, and its degree of contamination can vary not only on the basis of sedimentation rates and hydro-dynamism, but also to more particular phenomena such as bio-disturbance, or Dredging activities are even more important. Moreover, these substances can cause a serious dysfunction in the ecosystem, due to bioaccumulation and biomagnification, with their transfer to the various levels of the trophic chain of the biotic communities present and the consequent risk to human health, following the consumption of products coming from these areas. Recent studies conducted on Western European countries indicate that the sediments of coastal areas present in 5% of the cases risks for human health and 10% for the environment. Therefore, their management represents a problem of global dimensions both from the ecological and political point of view (Chapman and Wang, 2001) and for this reason they need to be analysed and monitored. To assess the quality of sediments in a water body, tests are usually performed on both the chemistry of sediments, to understand the overall degree of contamination, but also toxicological tests, to quantify the effect these have on organisms, especially benthic ones, and their bioavailability.

The most widespread contaminants in dredged, marine and river sediments are oils and hydrocarbons, heavy metals and synthetic products (such as agricultural drugs, chemical compounds used in agriculture), derived from the most varied productive activities. In particular, regarding port sediments, we can divide the contaminants present in two groups, according to their nature**:** organic and inorganic compounds.

Organic compounds: hydrocarbons of petroleum origin, polycyclic aromatic hydrocarbons (PAHs) (Fig. 2), polychlorinated biphenyls (PCBs) and other chlorinated, dioxins, furans, pesticides, pesticides, halogenated organ compounds and stannic organ. Even nutrients, if present in high quantities, can be considered potential contaminants, as they can lead to eutrophication problems.

Inorganic compounds: heavy metals and metalloids (arsenic, aluminium, cadmium, cobalt, chromium, iron, manganese, mercury, molybdenum, nickel, lead, copper, selenium, tin and zinc).

Among the before mentioned contaminants, those most present in port areas are heavy metals and hydrocarbons. The substances that can be found there, for example, are:

HEAVY METALS	They are many and present everywhere, and it is right that it is so as fundamental constituents of the earth's crust. In some areas of the globe they are even responsible for "geological anomalies", places that apparently seem very polluted, but which in reality is their natural starting conditions (e.g. the Tyrrhenian presents natural high levels of mercury sulphide). However, their presence in the environment is also largely due to man:

	metals have a limited atmospheric transport (with the exception of lead and mercury) and arrive at sea mainly through rivers, discharges and urban, agricultural and industrial run-off (Homady et al., 2002). They have common features such as: density above 4.5-5 g/cm^3 and atomic number greater than 30; they have different oxidation states, depending on the pH; they form hydroxides with low solubility; have an aptitude for forming complexes; they have a high affinity for sulphides; and as ionic behaviour they are generally cations (Sequi, 1989). Many metalloids (As, Cd, Cr, Co, Cu, Hg, Pb, Sn, Zn) are, to a small extent, essential elements for living organisms, but become toxic at high concentrations, since they tend to bio-magnify in adipose tissues, exerting also a carcinogenic effect. In fact, they can cause damage to cell membranes and DNA and can modify the enzymatic specificity and cellular functions, interacting, through ionic and / or covalent bonds, with essential cellular components (Bruins et al., 2000). Heavy metals present in an environmental matrix cannot be metabolized, unlike organic contaminants (Sequi, 1989). Another thing to keep in mind is that in nature there are hyper-accumulating species, that is, organisms that concentrate in their tissues so high concentrations of these elements that they have even tried to use these species to reclaim contaminated environments.
HYDROCARBONS	Hydrocarbons are represented by a heterogeneous set of organic compounds presenting hydrogen and carbon atoms, even though they may have oxygen, nitrogen, sulfur, metal or halogen atoms (Farnè, 2003). Many hydrocarbons derive from the refining of oil, which consists of a set of physical-chemical processes with the aim of obtaining products such as combustible mixtures, asphalt, lubricants starting from crude oil. They are therefore of natural origin, but the effects of anthropic contamination are linked to the various processing phases (extraction, transport, refining and combustion). Hydrocarbons includes also complex mixtures. In crude oil are aliphatic hydrocarbons (no complex structures) and aromatic hydrocarbons (with 10 benzene rings): the former are linear, branched or cyclic molecules, saturated or unsaturated, composed of carbon, hydrogen and, in some cases halogens, have a medium- low molecular weight and are in the liquid or gaseous state. Some examples are the alkanes, alkenes, alkynes and dienes; the latter can be natural or synthetic and have a characteristic structure with condensed aromatic rings, the benzene rings, which give it stability. They can be found in the solid or liquid state and have high vapor pressure and reduced solubility. BTEXs (benzene, toluene, ethylbenzene, xylenes) and PAHs (polycyclic aromatic hydrocarbons) belong to this group. Hydrocarbons are non-mixing substances with water and given their poor solubility in the marine environment, they can be immobilized in sediments, thanks to their ability to be easily absorbed by the solid matrix, thus becoming available to be degraded by the microbial community present. They are substances that are not biomagnified, but only bioaccumulated. And fortunately, they are pollutants with a medium/limited transport (10-100 km depending on weather conditions). They become global pollutants only because of their global anthropic use. Once reached the sea, PAHs form emulsions, which tend to dry out and become of the tar balls that can then reach the bottom or reach the shore. So, the water is only a transitory matrix, and the real phenomena take place in the sedimentary sector. Water acts as a lift, transporting the particles to the sediments, thus entering the

	trophic network.
HALOGENATED ORGANIC COMPOUNDS	They are hydrocarbons to which halogens are added (e.g. Chlorine). They are not natural compounds, but synthetic. Used in industrial applications, such as polychlorinated biphenyls (banned for years but still found in the environment), in plastics and in the products used in agriculture (pesticides and herbicides) and finally also halogenated organic compounds that are not voluntarily produced, but which are reaction by-products, such as dioxins that form if they have organo-halogenated substrates. They do not degrade and are easily transported. They are POPs (Persistant Organic Pollutants), or molecules that are extremely resistant to degradation, with degradation rates of up to 100,000 years. This persistence also has a biological effect as they accumulate in the tissues of organisms (e.g. the adipose tissue) and this has important repercussions in the organisms that live at the poles, where there are animals with the greatest number of fats. These molecules can be transported from the atmosphere and this makes them very dangerous. Dioxins were widely used in the 60s and 70s as a defoliant in the Vietnam War (Orange agent). They had their boom in the 80s and 90s as they were released during the disposal of substances such as DDT and herbicides, anti-mold, ... These substances travel across each compartment, but it is through the atmospheric substances that they travel more and potentially enter into food webs, making themselves more dangerous for multilevel predators (like humans).
PCBs (polychlorinated biphenyls)	Those defined as non-ortho substituted are very dangerous (unlike mono and bi-ortho substituted) and biologically aggressive. Also called PCB dioxin like not from the structural point of view but because of their biological mode of action. They greatly increase when an oxygen bridge is formed in the biphenyl (furans, they are 135 and differ in the position of chlorine on the molecule). Dioxin is formed when a second oxygen bridge is created. Basically, dioxins interact with AHR receptors that are normally inactive. Once active, they trigger cellular responses such as apoptosis.
PLASTICS	In the last few years (2016) total demand for plastics in Europe has grown strongly with values of around 50 million tons: 39.9% required for packaging polymers, 19.7% for building constructions, 8, 9% for vehicles, 5.8% for the electricity industry and 3.3% used in agriculture. It is a very efficient material, easy to work with and characterized by a low production cost. Such an increase in production (even higher before the 2006-2007 crisis) has meant that plastic has become the predominant fraction of waste found at sea (from 60 to 80% of the total, in some areas up to 90%). This material does not biodegrade in a short time, and through the mechanical action of wave motion and photodegradation it is transformed into micro-fragments. The Expedition MED program (2010-2013) was the first scientific program at European level that set out to study pollution from plastic micro-fragments and with a monitoring campaign that included 40 stations it was discovered that around 50 billion plastic micro-fragments contaminate currently the surface waters of the Mediterranean. Not to think about what could be stored inside the sediments. In addition to the physical damage that these micro-fragments can cause (by ingestion or simply due to obstruction of movement) it should be remembered that plastics are added with plasticizing additives. These compounds are organic esters which allow them to increase their flexibility and viscosity. Among the most used ones is the phthalates class, and given their worldwide use, the entire

	population is constantly exposed (Mikula et al., 2005). This is especially true for phthalates with lower molecular weight, characterized by higher volatility and migration than high molecular weight compounds. High molecular weight phthalates, such as diisononyl phthalate (DINP), are characterized by a dorsal with 7 carbon atoms or a ring structure. The longer their alkyl side chain, (high molecular weight), the greater their hydrophobicity. Having a low volatility and low migration, they are used as pasta makers and mainly added as softeners in toys and baby products, precisely because of their "safety". These characteristics make them extremely important also in the manufacture of footwear, raincoats, electrical insulation, vinyl flooring, and other industrial products too. If ingested, phthalates are rapidly absorbed and eliminated without being bioaccumulated by organisms. However, like lipophilic molecules, they are reworked in the body before being expelled. The processes to which they are subjected include hydrolysis from diesters to monoesters by pancreatic or hepatic enzymes in the intestine (Kim and Park, 2014). This reaction originates bioactive metabolites, able to exert their toxic activity: these are absorbed and reach the bloodstream, through which they can reach the liver. Because of their hydrophobicity, they are subjected to other reactions: generally, they oxidize, in order to obtain more soluble compounds ready to be excreted; the next step may involve the conjugation of these monoesters (or their oxidized metabolites) with glucuronic acid. The lower molecular weight phthalates and their metabolites are then expelled through the urine, while the medium and high ones can also be found in the faeces. The fact that they are not bioaccumulated has created the false belief that they were harmless also due to their very low acute toxicity, but risk assessments within the EU have shown that some of them can cause important sub-chronic and chronic toxic effects. as a result, they are now recognized as toxic molecules. A typical target organ of phthalates is the liver, but an exposure to them also decreases sperm quality in men and seems to induce endometriosis in women. Several studies, in Italy, have clearly detected the presence of phthalates both in inland waters and in sediments, at concentrations not exactly irrelevant (Vitali et al., 1997).

The sediment is therefore a real chemical reactor in which chemical-environmental processes and interactions are mainly carried out with the water compartment above it, which acts as a necessary passage for the passage of toxic substances and not to biota. Dynamic equilibria are created at the water-sediment interface, which play a decisive role in the exchanges between the various sectors. All this makes us understand that the sediments are a very complex matrix which in strategic areas for human activities preserves contaminants of various kinds. It is for this reason that this matrix cannot be analysed only from the chemical point of view.

5. Dredging activity

Coastal construction, land reclamation, beach nourishment and port/harbour construction, all of which involve dredging, are increasingly required to meet the growing economic and societal demands in the coastal zone worldwide (Erftemeijer et al., 2012). Nowadays, for example, more than 90% of the economic exchanges are performed by sea channels. In order to improve harbour infrastructures, ports managers organize dredging operations which generate a large quantity of sediments. Only in France, a volume of 1.6 million m^3 of sediments is dredged each year by the French Channel ports to maintain the water depth within their infrastructure (Maherzi et al., 2017).

In a few simple words, dredging consists of physical excavation that allows the removal of sediments (for the most part sand, gravel, debris and everything that can end up in a port) from the bottom of water basins, of distinct nature, through the use of specific machines called dredgers (mechanical or hydraulic), and their subsequent placement in prepared structures, such as reclaimed tanks. This operation can be divided into at least 4 main categories, depending on the aim pursued (Galassi, 2016):

Infrastructural dredging	Aimed at the construction of new ports and canals or the extension of those that are already existing.
Maintenance dredging	It consists in solving the problem of sanding up the seabed, so as to guarantee the maintenance of their navigational depth.
Rehabilitation dredging	Which is carried out in order to restore the sediment removed by distinct sources of contamination.
Dredging for the beach nourishment	The latter takes place to meet the needs of the Regions to try to solve, or at least reduce, the problem of coastal erosion.

Dredging activities were also present in the past (Marriner and Morhange, 2006), activities whose sole purpose was to guarantee the navigability of ports, rivers and canals, preserving their natural flow. Today, in addition to having maintained this traversability task, a more typically ecological purpose is also associated: the rehabilitation of contaminated matrices to guarantee environmental health and human health. Dredging activity change according to his type and by the project (Dotto, 2013) but substantially there is a first phase of excavation, a lifting of the sediment material towards the liquid surface, a transport of this to the treatment area and, finally, placement of the sediment in the designated area based on the quality class of the material. The equipment used to carry out the dredging is chosen according to the nature, quantity and level of contamination of the sediment, as well as to the type of physical environment of the excavation site and to the distance from the final positioning area.

One of the most common problems occurring, during the dredging phases, is the mixing of the bottom from which the sediment is taken, leading to the suspension of large quantities of material, and generating a strong cloudiness of the water column. Nutrient dispersion can also occur with consequent reduction of dissolved oxygen, not to mention the handling of pollutants accumulated on the seabed. Therefore, before proceeding with this activity, it would be advisable to check the conditions of the area and the surrounding areas, through bibliographic investigations or specific field research, which aim to exclude significant interferences on marine communities, as well as on the environment in general and on human health.

Several studies have been carried out on the negative effect caused by dredging on organisms of ecological interest, with works concerning in particular the fertility and reproductive cycle of corals (Jones et al., 2015), on reef vitality (Gailani et al. , 2016) and on the Posidonia meadows (Capello et al., 2014). Other studies instead highlighted the effects that dredging activities have on the redistribution of contaminants, particularly heavy metals and bio-accumulation process on marine biota and molluscs (Rocha et al., 2016; Shefer et al., 2015). Given these various problems, methods are currently being devised to implement operations, based mainly on the study of current and hydrodynamics, which reduce environmental impact and limit the contamination of the water column during dredging (Wasserman et al., 2016). Alternative methods to conventional dredging are currently under development, aiming at a “remediation” of the sediments: some of them are based on resuspension techniques that eliminate small percentages of sediments instead of large quantities: through this operation only the finest particles are removed (known to adsorb the contamination more effectively) by blowing air (Mulligan et al., 2001).

On the one hand dredging has also a positive effect representing a useful way to restore water bodies with eutrophic problems by reducing the concentration of some nutrients such as nitrogen and phosphorus (Yu et al., 2016; Zhong et al., 2010) thus avoiding the formation of potentially toxic algal blooms. On the other hand, however, problems remain and certainly one of this is represented by the creation of huge quantities of sediments (some millions of m^3 a year only in Europe) that must be carefully managed. This phenomenon, which is crucial and has a significant economic impact, presents a pressing problem of global interest, given the tendency to increase the storage of dredged materials over time. This issue has generated the idea of exploiting these sediments, apparently unusable, as a resource not to be wasted.

6. Chemistry versus Ecotoxicology

The chemical analysis of a matrix (e.g. the chemical characterization of a sediment) lead to break it down into smaller elements in order to examine them, identify them and quantify them, if possible. This allows to have quantitative data on the presence of certain pollutants in the environment, but observing their presence is not enough: we must also know their bioavailability and possible effects on organisms. This assumes a high relevance considering the huge amount of substances that can pollute the environment (heavy metals, organometals, polycyclic aromatic hydrocarbons, halogenated organic compounds, nitroaromatics, organophosphorus, carbamates, pharmaceuticals, ...) and which are poured into our Mediterranean Sea every year: 120,000 tons of oils, 60,000 tons of detergents, 85,000 tons of metals (20,000 tons of Cu only, 3,860 of Pb, 100 of Hg and 80 of Cd), 1,960,000 tons of hydrocarbons, and unfortunately more uncertain estimates for pesticides and organochlorines. All this because the anthropic pressure on the Mediterranean is very high, especially for industrial purposes. The maritime traffic is also impressive (for example in the Sicily Strait alone), and at global level the situation becomes even more complex. This is also cause by all those new substances produced every year (at least 1500 each year), and at least 11,000 are produced in such quantities as to cause damage to the environment. But what does ecotoxic substance mean? In each state, different indexes are used but conventionally toxicity including EC_{50}, or the concentration of a substance that determines 50% of the effect (for example mortality) on the population, is the most commonly adopted. The lower it is, the more it means that a low concentration of the test substance can have an effect on the exposed organisms. But how is the danger of a substance estimated? Different types of tests are used in which concentrations of various unknown substances are taken and administered to organisms (biomarkers) to evaluate their toxicity. A distinction has to made between acute and chronic toxicity, the former causes short-term damage even at low concentrations, chronic toxicity occurs on longer time scales, making measurement more difficult by tests, which instead prove to be much more adept at discriminating an acute toxicity. Toxicity evaluation is further confounded by the fact that substances used in the various sectors are regulated by regulations (including European law n.1907/2006, also called REACH: Registration, Evaluation, Authorization and Restriction of Chemical). These regulations require provision of a detailed card for every new synthetic compound; however, these cards do not take into account compound toxicity by compound and the fact that many compounds can interact with each other, with possible additive, synergistic or antagonistic effects. This represent a very important problem because it is very difficult to assess the effect of complex mixtures in normal environmental conditions.

Finally, finding the presence of a contaminant in a given matrix is important and it is absolutely urgent to understand the possible effects on biological systems. However, the two type of data must be integrated, since chemical contamination may not always lead to an ecotoxic response, as the presence of ecotoxicity in the environment can also occur with a low chemical risk. For this reason an approach called “Weight Of Evidence” has been adopted, that is an integrated chemical-ecotoxicological-biological approach reflecting the need to overcome the limitations of the “pass to fail” approach of a pure tabular model to evaluate the state of danger of a complex system such as

the sedimentary compartment, characterized by phenomena of mobility of contaminants between the matrices in relation to the change in the chemical-physical parameters of the environment, phenomena of chemical speciation, as well as effects due to synergistic and/or antagonist interaction of complex mixtures of pollutants, whose effects on biological communities cannot be described or predicted through simple linear "dose-response" relationships.

7. The regulatory framework (laws)

Polluted marine sediments (especially those present in harbours) have been traditionally documented in terms of contaminating chemical concentrations (His et al., 1997). From a legislative point of view, the problem of dredging sediment management was treated in a complex and confused manner, as there were no specific international conventions or Community directives on this issue. There were only legal texts that focused mainly on the prevention of impacts on the aquatic environment, caused by distinct discharge activities at sea. Among the international conventions we must remember:

The London Convention (1972)	For the prevention of marine pollution, following the spill of waste or other materials.
The Oslo Convention (1972)	For the prevention of marine pollution from shipments and from aircraft.
The Paris Convention (1974)	For the prevention of marine pollution from land.
The Barcelona Convention (1976)	For the protection of the marine environment and the coastal region of the Mediterranean.
The OSPAR Convention (1992)	For the protection of the marine environment of the North-East Atlantic.
The Bucharest Convention (1992)	For the protection of the Black Sea.
The Helsinki Convention (1992)	For the protection of the Baltic Sea.
The Barcelona Convention (1995)	Dumping protocol on discharges in the marine environment.

Instead, with regard to the European Directives, we have:

Directive 1975/442/CE	on waste
Directive 1976/464/CE	on marine pollution
Directive 1999/31/CE	on the landfill of waste
Directive 2000/60/CE	water framework directive
Decision 2455/2001/CE	on priority substances in the water policy sector
Directive 2008/56/CE	framework directive on the strategy for the marine environment
Directive 2008/98/CE	waste framework directive

In some countries, such as France and Spain, these Directives are adopted without modification and in a rigorous manner: they usually provide for treatment that makes the sediments inert and their reuse, in port maintenance or as building materials. Similar virtuous examples can also be found in countries with an important tourist industry such as Croatia, Greece, Malta and Portugal. In our country, waste management is still controversial, due to a lack of coordination between the various regulatory sources of reference. One of the problems that should be solved is to find a synergy in the actions of the various subjects involved in this management, such as the port

authorities, the dredging companies, the consulting companies and the Research Institutions. Legislative gaps have also prevented an adequate implementation of the treatment of sediments aimed at recovery. This caused, for example, very expensive sediment disposal, little or not at all contaminated, at a great distance from the dredging site or even in other countries.

Until the end of the last century, most of the sediments were taken and poured indiscriminately into the open sea. In 1996, a legislation (D.M. 24/01/96) was emanated to regulate the spillage of sediments coming from the dredging of sea beds, brackish or coastal land emerged, at sea or in adjacent places (beaches, lagoons, brackish ponds and coastal embankments). This legislation was followed by Legislative Decree 152/1999, presenting an article (n. 35) established that the authorization for discharge of excavation material of sea or brackish waters into the sea could only be obtained when these materials were proved to not be reusable for coastal nourishment.

They must also be mentioned (in the Italian legislation):

Law 179/2002	In the Art. 21 states that the regions are the competent authorities for issuing authorization for coastal protection interventions (beach nourishment), as well as for the immersion of excavation material of seabed or brackish waters inside reclaimed tanks, tanks of collection or containment facilities, located in the coastal area.
The D.M. 02/05/2006	That defines the sampling criteria for excavated earth and rocks.
Law 296/2006	In Art. 1, par. 996 and in its implementation decree (Ministerial Decree of 7/11/2008) defines some criteria for the execution of the dredging activity and for the management of sediments within the SIN (Sites of national interest). In 2012, this law was further amended.
The Legislative Decree 152/2006	Known as the "Consolidated Environmental Law" (and modified, in subsequent updates, such as Legislative Decree 4/2008): in the fourth part the reclamation of polluted sites is regulated.

In the latter, two relevant parameters were also established, such as: the Contamination Threshold Concentration, or the level of contamination of the environmental matrices above which the site characterization and a risk analysis are required; and the Risk Threshold Concentration: that is the level of contamination of the environmental matrices to be determined, case by case, with the site-specific risk analysis procedure and on the basis of the characterization plan. In this decree it was also reported that the dredging project had to be presented by the port authority to the Ministry of Infrastructure, which, after approval within 30 days, sends it to the Ministry of the Environment, which has the task of resolving definitively on this. This project had to contain the following: the project of the reclaimed tank, a detailed physical, chemical, microbiological and eco-toxicological characterization, according to the Ministerial Decree of 7/11/2008 and the appropriate techniques of work for dredging (which must minimize the sediment dispersion), sediment transport and implementation of a correct management option. Within this legislation, the possible management options for dredged sediments inside the SIN (sites of national interest), such as re-use in situ (beach nourishment, formation of coastal land, improvement of the seabed through activities of capping, re-use on the ground) were also proposed. The first solution is implemented "if they have similar physical-chemical and microbiological characteristics to the natural background with reference to the sampling site and suitable with reference to the destination site and do not present positivity to eco-toxicological tests". The delivery in the reclaimed case occurs if they are not

dangerous at the origin or following treatments aimed at the sole removal of pollutants. For re-use on the ground the material must not have, at the origin or following decontamination treatments, levels of contaminants higher than those established in columns A and B of table 1 of annex 5 to part IV of Legislative Decree 152/2006 , depending on the intended use and must conform to the assignment test, based on Ministerial Decree 5/02/1998.

In addition to the legislation just mentioned above, drawn up by APAT and ICRAM in 2007, there was the "Manual for the handling of marine sediments, a technical document that defines the guiding criteria for the dredging process, from characterization to their final destination, identifying different quality classes and possible management options. This document has been strongly revised. Subsequently, in the Ministerial Decree 161/2012 the qualitative criteria to be met are reported so that the excavated materials (which also include materials from marine dredging) are considered by-products and non-waste, pursuant to Art.183, paragraph 1 of the D. Legislative Decree 152/2006. When identified as a by-product, respecting the limits imposed by the Decree, the dredged sediment can be reused for the realization of fillings, nourishment, sea operations or other forms of restoration - environmental improvements.

But now all is changed: it has been demonstrated that the merely chemical characterization of the sediment is not enough to predict toxicological effects, as well as the transfer of chemical substances from sediment to organisms through bioaccumulation processes (Benedetti et al., 2012). Therefore multidisciplinary approaches have been followed to gain knowledge on how the toxicity of a sediment works and monitoring has been carried out on entire Italian areas using the Weight-of-Evidence approach (Chapman et al., 2002; Burton et al., 2002) based on the integration of so-called "Lines of Evidence", or rather on the integration of data from different fields of investigation, e.g. chemical, ecological and ecotoxicology, has been used. Marine organisms living in and on the marine surface may be exposed to certain substances through direct contact with those adsorbed to sediment particles and understanding the bioavailability of pollutants is the key issue for assessing sediment quality (Prato, 2015). Bioassay using biological systems to detect toxicity in different environmental samples (water, soil or sediment) are now an established practice used for ecotoxicological evaluation of marine matrices. Unfortunately, a sensitive species to all the environmental contaminants does not exist, and so the use of a battery of bioassays with different species of organisms is required for toxicity screening for ecological risk assessment (OSPAR, 1998; Nendza, 2002; D.M.173 and Technical Annex to D.M. 173/2016). These bioassays require less technological equipment than the sophisticated physical and chemicals analyses, and they don't explain the toxicological results (Burton, 1995). We must remember that harbour sediment contains a very complex mixture of anthropogenic compounds, but these don't cause, necessarily, a toxic effect upon biota (Pellegrini et al., 1999). This situation required a recent evolution of the regulatory framework concerning the assessment of the quality of marine sediments. The Decree of the Ministry of the Environment n. 173/2016 of 15 July 2016 (Official Italian Gazette No. 208 of 06/09/2016) responds to the need to have a new guideline, and approves the methods and technical criteria for authorizing the immersion in the sea of the excavation materials from the seabed (this decree is related to the D.M. 172, concerning the dredging in the SIN). *This Decree represent a really cut with the past: thanks to this 173, the old "Pass-To-Fail" model is passed in favor of a "Weight-Of-Evidence" approach. In fact the adoption of WOE demonstrate how this approach allows to overcome the limitation of a conventional tabular approach (present in the old law "Manuale Per la Movimentazione dei Sedimenti Marini") and it's better for classify the different harbor zone by integrating ecotoxicological and chemical parameters, using a ISPRAs software.* The D.M. 173 explains these fundamental points: **a)** the modalities for the release of the authorization for the deliberate immersion in the sea of the excavation materials of seabed, brackish or coastal; **b)** homogeneous criteria for the whole nation, for the use of materials for the purpose of nourishment or storage in *conterminated* environments (e.g. sediment tank), in accordance with the characterization, classification and acceptability of the materials in terms of environmental quality; **c)** the management of materials coming from the dredging of the port and coastal marine areas.

In this way, the D.M. 173 directs the process of assessment of the quality of the sediment in a univocal path, based on the integration of several lines of evidence such as chemical and biological data (Giaime, 2016). Through this analysis, with the definition of an Hazard Chemical Quotient (HQc) and an Hazard Ecotoxicological Quotitent (HQe), and thanks to a particular software called Sediqualsoft® (by ISPRA and UNIVPM of Ancona) the sediment falls into one of five possible quality classes, each of which provides different management options (Fig. 3): class A) is the best quality, which can be used both for beach nourishment and for submerged beach nourishment; class B) this typology can be immersed only in non-coastal marine areas (over 3 nautical miles); class C) the quality begins to be worse and this type of sediment must be placed in a sediment tank in the harbour area; the last two classes, D and E, are the worst and sediments of this type must be stored in confined waterproof environments or, in worst cases, even removed safely from the marine environment after the risk assessment. Therefore, the true novelty of D. M. 173 no longer results in treating the dredged sediment as a waste, but it defines it as a real resource to be recovered and reused.

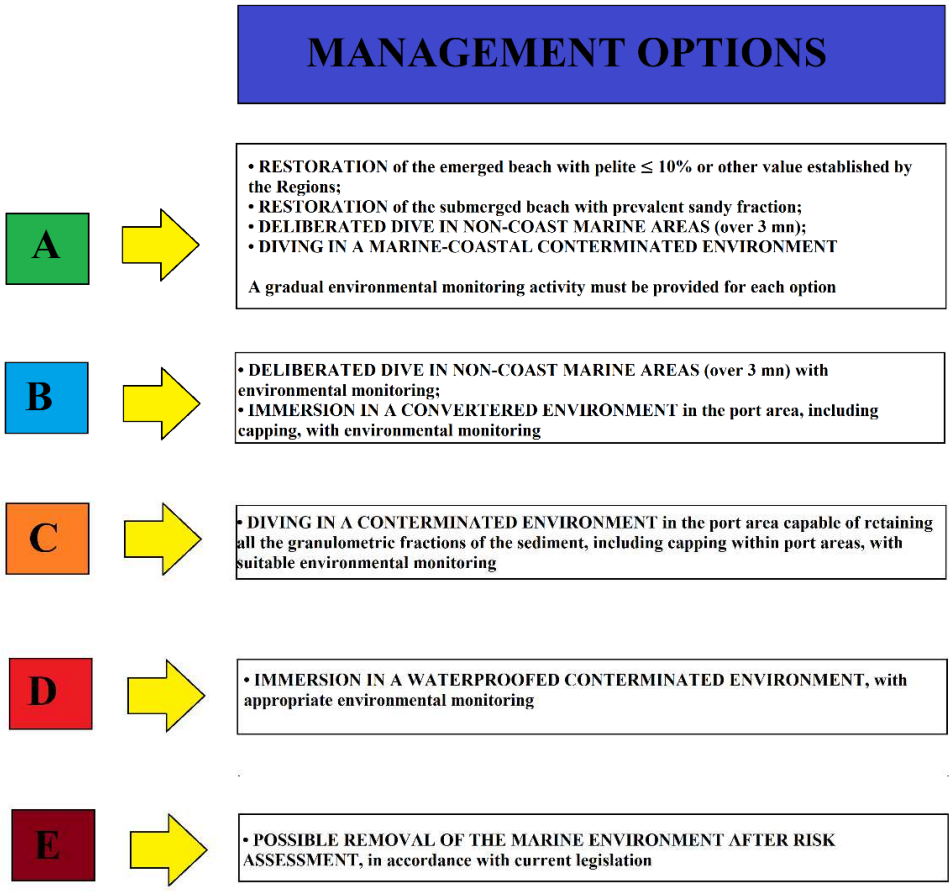


Figure 3. Compatible management options with the quality classification of the materials to be dredged (source: Technical Annex to D. M. 173/2016)

The Decree also contains a difficult task for the different regions: to go and try to economically manage a resource that, in some cases, may not be a resource. In fact, if in the A class fall the sediments in high environmental quality, in the following classes the quality decreases, as well as the management options, that are gradually more limited up to the worst class (E) which even involves removal. Surely the latter represents the most unmanageable economic situation for the Region systems. The question becomes very delicate, because we try to match environmental

issues with those of economic management. And if the cost becomes too important, there is the risk that the regions no longer consider dredged sediment as a possible resource.

As regards of the Marche Region, the management of sediments is a problem of considerable importance: in fact the anthropic dredging activity is very developed and the problems connected with these dredging activities and with the storage of sediments on the ground are becoming increasingly complex and the need to solve them, with sustainable interventions, both from an environmental and economic point of view, is leading to the tendency to consider them increasingly as a resource to be exploited. But how can these sediments be reused? If from a scientific point of view there is a multi-year experience on environmental characterization, monitoring and analysis in this field, on the other there is a lack of technological knowledge of treatment and the potential for effective re-use of sediments. In the bibliography it is possible to find recent and not yet consolidated experiments of reuse of sediments in different areas, obviously after careful decontamination processes, for instance: they have been reused in the field of civil engineering, as aggregates in cemented mortars (Couvidat et al., 2016) , for the production of bricks (Cappuyns et al., 2015), as a secondary construction material or for the creation of a road surface (Banoune et al., 2016; Maherzi and Abdelghani, 2014). Experiments on their potential use have also been carried out in more environmental and ecological fields: they have been tested for the maintenance of a wetland (Mchergui et al., 2014), as *tecnosuolo* (Macía et al., 2014; Masciandaro et al., 2014) as a growth substrate for marine macrophytes or ornamental plants and an in vitro test was also carried out for the possible commercialization of fulvic acids extracted from the sediments of the arid regions, in order to amortize the dredging costs in these areas in development path (Galassi, 2016).

8. Molecular investigation

Although Ministerial Decree 173 of 2016 represents a sure and remarkable leap forward in terms of management of sediments deriving from dredging activities, this decree does not evaluate some aspects that could be investigated more specifically through molecular analysis. In this work we focused the attention on those sediments that showed non-overlapping chemical and ecotoxicological HQs, thus going to look for an answer to this different evidence arising from the analyses proposed by the decree. It was therefore decided to examine the response given by contaminants in the sediment both in a very sensitive moment such as embryonic development and to visualize the effect of pollutants in the diet of higher organisms.

In the first case we investigated the embryonic response, at different time points after fertilization, analysing molecular markers related to development and the immune response, in order to observe possible alteration cause by pollutants present in the elutriates and eventually, understand which could be the most sensitive stage. The use of *Crassostrea gigas* embryos has represented a real challenge, mainly because of the scarce presence of studies of this type. The mRNAs analysed were *lysozyme*, *transglutaminase*, *galectin* and *defh1* - involved in toxic response - and *vasa* - to establish the developmental pattern of germline cells during oyster ontogenesis development and finally, *heat shock protein 70*, *superoxide dismutase* and *glutathione peroxidase* for antioxidant mechanism.

Even more hazardous, but certainly fascinating was trying to understand how these contaminants, once resuspended in the water column after being stored in the sediments, could enter the trophic web. Among all the possible pollutants, interest fell into phthalates, mainly because they are widely considered and environmental issue throughout the world. The dangerousness of these compounds is recognized, and many regulations and studies have led to generate new, less toxic compounds that could replace their use: for this reason it was decided to evaluate the effects of exposure to different concentrations of diisononyl phthalate (DiNP) administered through the diet, in *Sparus aurata*. In recent decades, DiNP has become a common substitute for 2-ethylhexyl phthalate (DEHP), due to their similar structure. Thus, it is extremely important to better understand

its potential negative effects on organisms, especially if exposed after anthropic activity. As liver is the first organ to be involved in the detoxification process, the effects of the contaminated diet were evaluated at hepatic level focusing of three different cellular processes: the antioxidant defence, the immune response, and the presence of apoptotic signals.

Aims and organization of the thesis

This study aimed at developing a novel approach to classify sediments when non correspondence between the chemical and ecotoxicological risk, identified by D.M. 173/2016 was found.

The identification of molecular biomarkers in oyster larvae exposed to sediment elutriates, could help in the classification of sediment quality and the results could be to integrate with the common chemical and ecotoxicological approach, significantly improving the evaluation system. In addition, despite not requested by the decree, an additional focus on phthalate detection was laid, and the effects of their environmental presence was explored in seabream analysing the effects of their dietary administration on the fish metabolism.

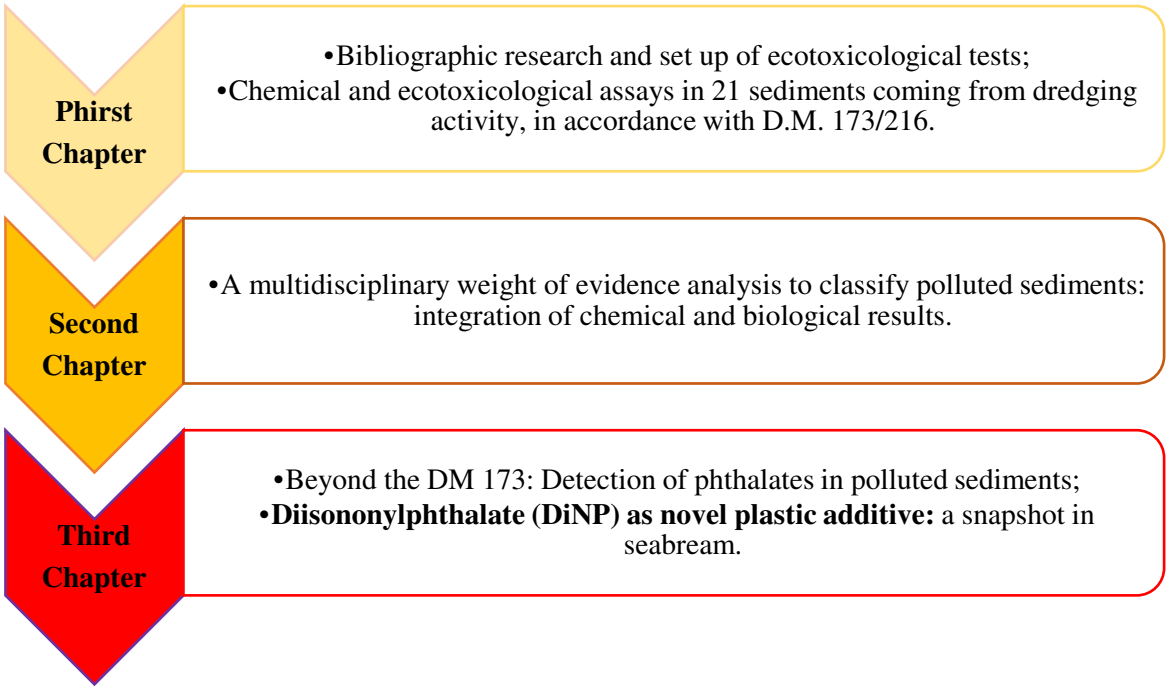
The **first chapter** of the project consists of a bibliographic research concerning the application of the new Decree n. 173 and its implementation. Twenty-one (21) sediment samples sampled at the entrance of a port in central Italy were used to carry out chemical analysis and three ecotoxicological test on *Vibrio fischeri* (solid phase, acute toxicity) with modified method UNI EN ISO 11348-3, *Phaeodactylum tricornutum* (UNI EN ISO 10253) and *Crassostrea gigas* (chronic toxicity) as well as from Decree n. 173, following the respective ISO standards for the microalgae and the luminescent bacterium. The toxicity test on *C. gigas* embryonic development was developed according to EPA/600/R-95-136 (U.S. EPA, 1995) method. In addition to ecotoxicological tests, physical and chemical analyses were also conducted, in accordance with current legislation (D.M. 173/2016). The greatest difficulty found with the *C. gigas* test, was the optimization of the protocol, since the EPA considers the use of generic seawater. Preliminary studies revealed that the best results were obtained using 30‰ natural seawater filtered with 0.45 µm membrane pores. The use of the ISPRA's software for the integration of ecotoxicological and chemical data allowed the classification of the quality of the sampled sediments within three different quality categories (A, B and C). It is really important to notice the integration between chemical and ecotoxicological data as no one can replaces others. Very significant are the areas 5 and 7 of the port, both in C quality: in Area 7 the chemical data (L2 values for benzo(a)pyrene, and the sum of DDT that is between L1 and L2) is more relevant compared to the low ecotoxicological values. In contrast to the Area 5, where a high critical level of the values obtained from ecotoxicological tests are not supported by high levels of chemicals contaminant. Moreover, in the deep sample from Area 2, a significant amount of PAHs was measured, (L2 overflow for Fluorene and Phenanthrene), associated to a lack of ecotoxicological effect. In conclusion, not always high levels of contaminants correspond to observable ecotoxicological effects, and viceversa.

In the **second chapter**, interest was focused in finding a solution to the lack of match between chemical risk and ecotoxicological risk in dredged sediments. Given the high sensitivity of oyster embryos in ecotoxicological assays, this model was chosen to perform addition molecular analysis. Three sediments, although classified within the same quality class, had different chemical and ecotoxicological risk hazard quotient: sediment 18/SM has a medium ecotoxicological risk and a low chemical risk. The 19/SM presents a high chemical risk associated to an average ecotoxicological one. The sediment 20/SM has a high chemical risk but a low ecotoxicological risk index. Oyster samples were exposed to these three sediments elutriates and sampled at 5 (blastula) and 18 (trochophore) hours post fertilization. A set of genes specifically involved in the development, in immune response and oxidative stress were analysed to gain info on the effects of these sediments on oyster embryonic stages. These mRNA were lysozyme, transglutaminase, galectin and defh1 - involved in toxic response - and vasa - to establish the developmental pattern of germline cells during oyster ontogenesis development -, *Hsp70*, *GPx* and *Sod* for oxidative stress.

Third Chapter: As novelty, despite the decree does not foresees the evaluation of phthalates within the sediments, but considering that these molecules have a great environmental importance being recognized as one of the leading causes of tumours as well as metabolic diseases, the possible effect of Di-iso-nonylphtalate (DiNP) in the diet of seabream, an excellent experimental model for marine toxicological studies, was investigated. In this work, the adult sea bream (*Sparus aurata*) was exposed for 21 days to DiNP at 15 and 1500 µg kg^{-1} per day^{-1} through the diet. The analysis of a series of iomarkers involved in oxidative stress and immune response provided evidence of liver toxicity by DiNP.

These molecular evidences were complemented with features obtained by Fourier Transform Infrared Imaging (FTIRI) analysis regarding the hepatic distribution of the main biological macromolecules. Finally, the results also suggested the onset of hepatic oxidative stress and the activation of immune response, adding new knowledge to the already described hepatic DiNP toxicity.

The different chapters of this study are reported in the block diagram:



Chapter 1
Chemical and ecotoxicological analysis of 21 sediments from harbour dredging activity

1. Introduction

The normative aspect that guides a country, or a group of nations such as the EU, is constantly being updated: both for the new materials that invade the market every day, and for the new knowledge that is acquired in the field of human health. In this "race", a third participant is added, which is the world of norms, with the goal to combine health and technology with the aim of generating a healthy environment. In this continuous administrative evolution, the new Ministerial Decree n. 173/2016 recognized the role of ecotoxicology. It's contribution, in synergy with chemical data, defines the quality of marine sediments deriving from dredging activities, to be considered an asset to be reused. In this way, with an eco-sustainable ideology, there are several risks: the relevance given to ecotoxicological tests could strongly condition the quality of the sediment, even in the absence of a chemical risk. Too "sensitive" ecotoxicological tests allow to define a very low-quality sediment, with a difficult economic impact to manage.

This work was carried out on 21 sediments coming from 7 areas at the entrance of a commercial port in Central Italy. The decision to study marine sediments from dredging activities was taken because harbours are a known reservoir of contaminants: once in the water column, metals and other organic contaminants are quickly adsorbed onto particles, deposited and accumulated in sediments, which therefore act as a sink. However numerous studies have demonstrated that sediments are not only a sink but also a possible source of contamination to the water column due to desorption and remobilization processes (Tessier, 2011).

The ecotoxicological, chemical and physical parameters useful for the definition of the sediment quality class are indicated respectively in tables 2.3, 2.4 and 2.6 of the Technical Annex to the Ministerial Decree 173/2016, while the national reference chemical levels (L1 and L2) are shown in Table 2.5.

1.1 Sediqualsoft® Software

For statistical analyses, the Ministerial Decree 173/2016 referring to all the analyses of this work involves the use of a special software, Sediqualsoft®, developed by ISPRA and Università Politecnica delle Marche (Ancona). The software allows the qualitative classification of marine and brackish sediments, as provided for in the D.M. 173/2016. Sediqualsoft® is organized in 3 modules: the first and second are related to the ecotoxicological characterization and chemical characterization, while the third deals with integrating the first two to provide the final quality class of the sediment. The sediments can thus be divided into 5 classes of sediment quality from A to E, each class with the respective management options (from A d E quality decreases). The chemical and effect data are thus integrated in order to obtain a single qualitative class; in more complex cases, a weighted integration of the results of the analyses carried out is foreseen that allows to weigh adequately the dangerousness of the different substances analysed as regards chemical concentrations and the relevance of the biological endpoint, matrix and exposure time, for regarding ecotoxicological tests. The 2D Appendix to the decree contains the local environmental reference levels ($L1_{loc}$). Appendix 2E also introduces a synthetic bioavailability index which allows to establish which and how many bio-accumulative contaminants are associated with sediments, as well as the risk associated with their possible transfer to the biotic sector. Please refer to the tables for data processing of ecotoxicological and chemical data. In more detail, the ecotoxicological classification is based on the application of a battery of 3 biological assays whose results are integrated and lead to the formulation of the toxicity judgment. The level of danger (from absent to very high) is thus assigned based on the overall danger index of the battery of ecotoxicological tests (Hazard Quotient, $HQ_{battery}$). Instead the chemical classification is based on the comparison of the

concentrations measured in the sediment with the reference concentrations L1 and L2 referred to in the Table below (values reported in D.M. 173), with the values of L1 and L2 that can be modified to take into account the characteristics site-specific of the area in question. The values of L1 correspond to those of the environmental quality standards (EQS) foreseen for the sediment by the Water Framework Directive and by the national transposition regulations (D.M. 260/2010 - Table 1). The values of L2 correspond to a quality level lower than L1 but still corresponding to an acceptable ecological risk. The procedure for estimating the chemical hazard level (Hazard Quotient, HQC), from absent to very high, is based on the calculation of the variation with respect to the limit, i.e. the Ratio-To-Reference (RTR), with the RTR subsequently corrected according to the weight of the contaminant that takes into account the importance of the observed variations for the most dangerous contaminants (i.e. Cd and Hg, persistent pollutants). Finally, the two results, ecotoxicological and chemical, are integrated to attribute the quality class of the excavation sediments, according to tabular criteria.

1.1.1 Weighted integration criteria for the evaluation of ecotoxicological results

The weighted integration criteria consider important aspects and specific characteristics of the biological assays inserted in the battery used, including the statistical significance of the difference effect between the sample and the control (taking into consideration the variability between the replicates, both in the control and in the sample); the severity effect (understood as the severity of the biological damage measured by the specific end-point); the type of exposure (acute or short-term, chronic or long-term); the environmental representativeness of the tested matrix. For each of the assays proposed in the different types of usable batteries, an effect "threshold" is indicated which represents the minimum variation considered biologically significant for each experimental condition (Tab. 1); the "weights" attributed to each assay are also reported as a function of the biological relevance of the measured end-point, of the duration of the exposure, of the tested matrix (Tab. 2).

Threshold values attributed to biological assays foreseen in the batteries				
Species	**Endpoint (E)**	**Threshold (%)**	**Type of exposition (T)**	**Type of sample (M)**
Acartia tonsa	Larval development	20	Chronic/sub lethal	A, D
	Death rate	15	Acute	B, C
Amphibalanus amphitrite	Death rate	10	Acute	B, C
Corophium insidiosum	Death rate	15	Acute	A, D
Corophium orientale	Death rate	15	Acute	A, D
Crassostrea gigas	Development	15	Chronic/sub lethal	C
Dunaliella tertiolecta	Algal growth	10	Chronic/sub lethal	B, C
Mytilus galloprovincialis	Development	15	Chronic/sub lethal	B, C
Paracentrotus lividus	Fertilization	15	Chronic	B, C
	Development	15		
Phaeodactylum tricorutum	Algal growth	10	Chronic	B, C
Skeletonema costatum	Algal growth	10	Chronic	B, C
Tigriopus fulvus	Death rate	10	Acute	B, C

Vibrio fischeri	Bioluminescece	15	Acute	B, C
		25		A, D
A = whole sediment; B = interstitial water; C = elutriate; D = wet sediment (without interstitial water)				

Table 1. Threshold values attributed to biological assays foreseen in the batteries (source: Technical Annex to D. M. 173/2016)

BIOLOGICAL ENDPOINT (En)		**TYPE OF SAMPLE (M)**	
Fertilization	1,5	Sediment (100%)	1
Development	1,9	Interstitial water	0,8
Algal growth	2,1	Elutriate	0,7
Bioluminescence	2,4	Wet sediment (e.g. centrifuged)	0,6
Death rate	3		
EXPOSURE (T)		**ALAGAL BIOSTIMULATION (Ei)**	
Acute	1	$E \leq 40\%$	0
		$40 < E \leq 100\%$	1,25
Chronic	0,7	$E > 100\%$	1,5

Table 2. Weights assigned according to the relevance of the biological Endpoint, the matrix, the exposure time, and used for the calculation of the W2 coefficient. Values for algal biostimulation (hormesis) are also reported (source: Technical Annex to D. M. 173/2016).

The steps and the calculation procedures for the integration of the results and the formulation of the toxicity judgment of which an overall diagram is shown in Fig. 1 and are described below.:

2. after the data verification, the effect (E_i) is calculated for each biological assay, understood as the percentage change of the endpoint measured and compensated through the Abbott correction with respect to the variations observed in the control (eq. 2 of the flow chart of Figure 1);
3. the E_i effect is corrected based on the statistical significance of the variation with respect to the controls, applying the Z coefficient which is calculated as a function of the value obtained from the T test for data with inhomogeneous variance (point 3 of the flow-chart of Figure 1). The Z coefficient has a value of 1 (no reduction in the effect) when the sample is significantly different from the control (p <0.05); it decreases with decreasing significance, passing linearly from 1 to 0.5 when p grows from 0.05 to 0.06. For p values higher than 0.06, the Z coefficient rapidly decreases non-linearly up to 0.2, when p tends to 1. This correction progressively reduces the overall weight of a non-statistically significant assay, but does not completely eliminate the contribution to the battery;
4. each effect (E_i) multiplied by its coefficient Z, is compared with the specific "threshold" for that assay (eq. 4 of the flow-chart of Fig. 1); the correct effect (E_{iw}) thus obtained indicates how many times the variation measured in an assay exceeds that considered biologically relevant;
5. only for algal bioassays, in the case of a biostimulation effect, an E_{iw} value of 0 is assigned if the effect is < 40%, 1.25 if the effect is > 40% but < 100%, equal to 1.5 if the effect is > 100%;
6. the overall danger index of the ecotoxicological bioassay battery (Hazard Quotient, $HQ_{Battery}$) is calculated as the sum of the weighted effects (E_{iw}) of the individual assays (eq. 5 of the flow-chart of Fig. 1), further corrected according to the W2 factor which corresponds to the product of the weights assigned as a function of the biological relevance of the considered endpoint, of the ecological relevance of the tested sample, of the acute or chronic exposure of the organisms (Table 2).

7. for the attribution of the danger level deriving from the ecotoxicological assay battery, the value obtained for the $HQ_{Battery}$ index is normalized to a scale between 0 and 10 (eq. 6 of the flow-chart of Fig. 1), where 1 corresponds to the threshold value of the battery (i.e. the value of HQ that would be obtained if all the tests of the battery showed an effect equal to the respective threshold) and 10 corresponds to the maximum value of the battery (when all the tests show 100% of effect). Depending on the normalized $HQ_{Battery}$ value, the ecotoxicological hazard level is attributed to a gravity class (from absent to very high), identified by a different color: Absent/white if $HQ_{Battery} < 1$; Low/blue if $HQ_{Battery} \geq 1$ and < 1.5; Medium/yellow if $HQ_{Battery} \geq 1.5$ and < 3; High/red if $HQ_{Battery} \geq 3$ and < 6; Very High/black if $HQ_{Battery} \geq 6$ (Tab. 3).

HQ battery bioassay	Hazard class
< 1	Absent
≥ 1 – 1,5	Low
≥ 1,5 – 3,0	Medium
≥ 3,0 – 6,0	High
≥ 6,0 – 10,0	Very High

Table 3. Ecotoxicological hazard classes compared to Hazard Quotient (HQ) values of the battery assay (source: Technical Annex to D. M. 173/2016).

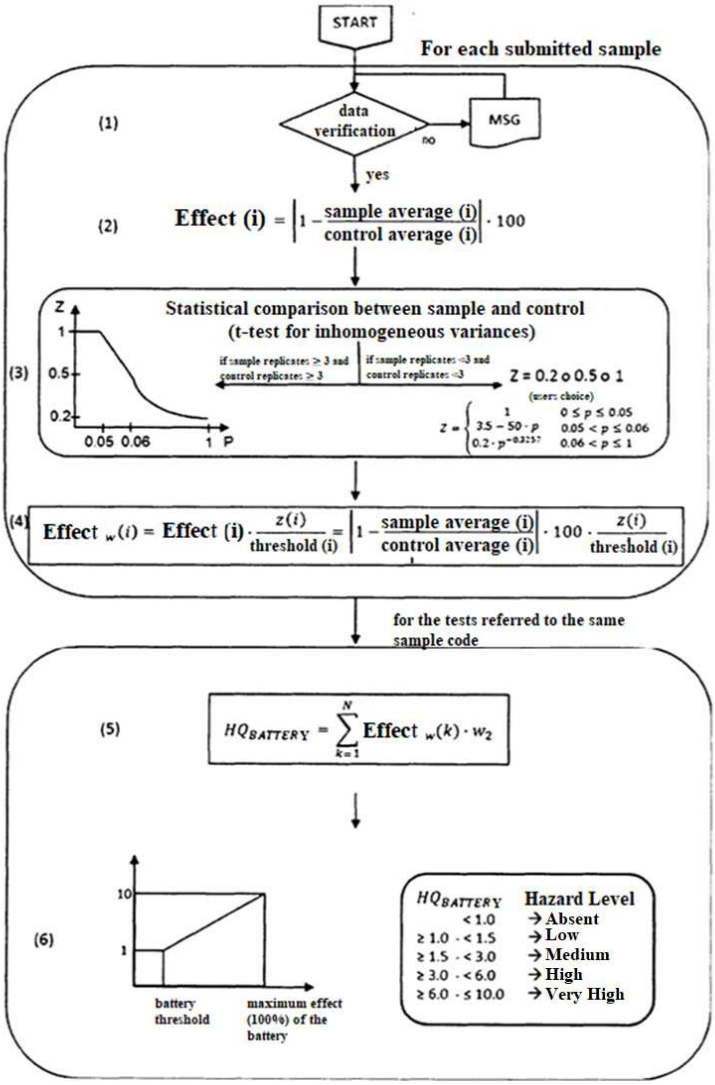


Figure 1. Procedure for processing ecotoxicological tests data (source: Technical Annex to D. M. 173/2016).

1.1.2 Weighted integration criteria for the processing of chemical results

The weighted integration criteria consider the type of parameters, the number of contaminants that exceed the specific reference, as well as the extent of these overruns compared to the limits set. The logic of the simply exceeding of the tabular values, even minimal and by a single parameter, is therefore leaved as a fundamental principle for chemical classification. All the chemical parameters for which analysis is planned have a "weight" (from 1 to 1.3) depending on whether they are covered by Directive 2013/39/EU (weight 1), or that, on the contrary, they are included in the list of "priority" substances (weight 1.1) or in that of "dangerous and priority" substances (weight 1.3), or are included in the Stockholm POPs Convention (weight 1.3). The different weight assigned to the various compounds has the purpose of giving greater relevance in the chemical classification of sediments to the variation of those pollutants that are characterized by a higher toxicity, tendency to bioaccumulate and persistence in the environment or that must be subjected to a progressive reduction in the environment according to the objectives set by the Water Framework Directive (Tab. 4).

List of parameters and relative weights, recommended for processing chemical data

Chemical substances	Weight	CAS Number	Chemical substances	Weight	CAS Number
As	1	7784-42-1	**PCB-81**	1.3	70362-50-4
Cd	1.3	7440-43-9	**PCB-101**	1	37680-73-2
Total Cr	1	7440-47-3	**PCB-118**	1.3	31508-00-6
Cu	1	7440-50-8	**PCB-126**	1.3	57465-28-8
Hg	1.3	7439-97-6	**PCB-128**	1	38380-07-3
Ni	1.1	7440-02-0	**PCB-138**	1	35065-28-2
Pb	1.1	7439-92-1	**PCB-153**	1	35065-27-1
Zn	1	9029-97-4	**PCB-156**	1.3	38380-08-4
Acenaphtene	1	83-32-9	**PCB-169**	1.3	32774-16-6
Anthracene	1.3	120-12-7	**PCB-180**	1	35065-29-3
Benzo(a)anthracene	1	56-55-3	**ΣPCB**	1.3	n.a.
Benzo(a)pyrene	1.3	50-32-8	**Aldrin**	1.3	309-00-2
Benzo(b)fluoranthene	1.3	205-99-2	**A-Hexachlorocyclohexane**	1.3	319-84-6
Benzo(k)fluoranthene	1.3	207-08-9	**β-Hexachlorocyclohexane**	1.3	319-85-7
Benzo(g,h,i)perylene	1.3	191-24-2	**γ-Hexachlorocyclohexane**	1.3	581-89-9
Crysene	1	218-01-9	**Total Hexachlorocyclohexane**	1.3	n.a.
Debenz(a,h)anthracene	1	53-70-3	**Clordan**	1.3	57-74-9
Phenanthrene	1	85-01-8	**Σ DDD**	1.3	72-58-8+53-19-0
Fluorene	1	86-73-7	**Σ DDE**	1.3	82413-20-5+72-55-9
Fluorantene	1.1	206-44-0	**Σ DDT**	1.3	50-29-3+789-02-06
Indeno(1,2,3,c,d)pyrene	1.3	193-39-5	**Σ DDD DDE DDT**	1.3	n.a.
Nafthalene	1.1	91-20-3	**Dieldrin**	1.3	69-57-1
Pyrene	1	129-00-0	**Endrin**	1.3	72-20-8
ΣIPA	1.3	n.a.	**Heptachlor epoxide**	1.3	1024-57-3
PCB-28	1	7012-37-5	**Σ organostannic**	1.3	n.a.

			compounds (Sn)		
PCB-52	1	35693-99-3	**Hexachlorobenzene (HCB)**	1.3	118-74-1
PCB-77	1.3	32598-13-3	**Σ PCDD, PCDF (TE-I)**	1.3	n.a
			Σ PCDD, PCDF, dioxin-like PCB (TE-I)	1.3	n.a.

Table 4. List of parameters and relative weights recommended for processing chemical data (source: Technical Annex to D. M. 173/2016).

The steps and calculation procedures for the integration of results and chemical classification are described below; the overall scheme is summarized in Figure 2.

The processing of chemical data begins with the comparison of the concentrations measured in the sediments with L1 and L2 referred to in Table 2.5 (and its subsequent updates, DM 173); the comparison can be made with site-specific "references" (for example $L1_{loc}$ and $L2_{loc}$), if these levels have been defined at the local level according to the criteria set out in the 2D Appendix. According to the reference, for each chemical parameter analysed, the variation with respect to the limit is calculated, i.e. the Ratio To Reference (RTR) (eq. 3 of the flow-chart of Figure 2); the RTR value is corrected as a function of the "weight" of the contaminant to obtain a value of RTR_w (eq. 4 of the flow-chart of Figure 2), in order to emphasize the importance of the observed variations for the most dangerous contaminants. The calculation of the quantitative danger index (Hazard Quotient), specific for the chemical characterization of the sediments (HQC), is obtained from the average of all the RTRw of the parameters with RTR ≤ 1 (i.e. values lower than the reference limit), added with the summation Σ of the RTRs of all the contaminants with RTR> 1 (eq. 5 of the flow-chart of Figure 2):

$$HQ_C = \frac{\sum_{j=1}^{N} RTR_W(j)_{RTR(j)\leq 1}}{N} + \sum_{k=1}^{M} RTR_W(k)_{RTR(k)>1}$$

Where N and M are the number of parameters with RTR respectively ≤ or> 1, while j and k are indices that allow to repeat the calculation for N or M times. With this calculation procedure, the chemical danger index (HQ_C) can change in according to the number of parameters that exceed the references (whose RTRs are added together in the summation ϵ), the extent of the exceedance and the type of contaminants. The HQ_C chemical index is assigned to a hazard class (from absent to very high), identified by a different color: Absent/white if $HQ_C < 0.7$; Negligible/green if $0.7 \geq HQ_C < 1.3$; Low/blue if $1.3 \geq HQC < 2.6$; Medium/yellow if $2.6 \geq HQ_C < 6.5$; High/red if $6.5 \geq HQ_C < 13$; Very High/black if $HQ_C \geq 13$ (eq. 6 of the flow-chart of Figure 2 and Tab. 5).

Since the calculation procedure does not change according to the type of reference chosen for the comparison, the chemical data are processed simultaneously to obtain an HQ_C value and a chemical hazard class with respect to all the references adopted.

HQc	Hazard class
0 – < 0,7	Absent
0,7 – < 1,3	Negligible
1,3 – < 2,6	Low
2,6 – < 6,5	Medium
6,5 – < 13,0	High
≥ 13,0	Very High

Table 5 Chemical hazard classes compared to HQ_C values (source: Technical Annex to D. M. 173/2016)

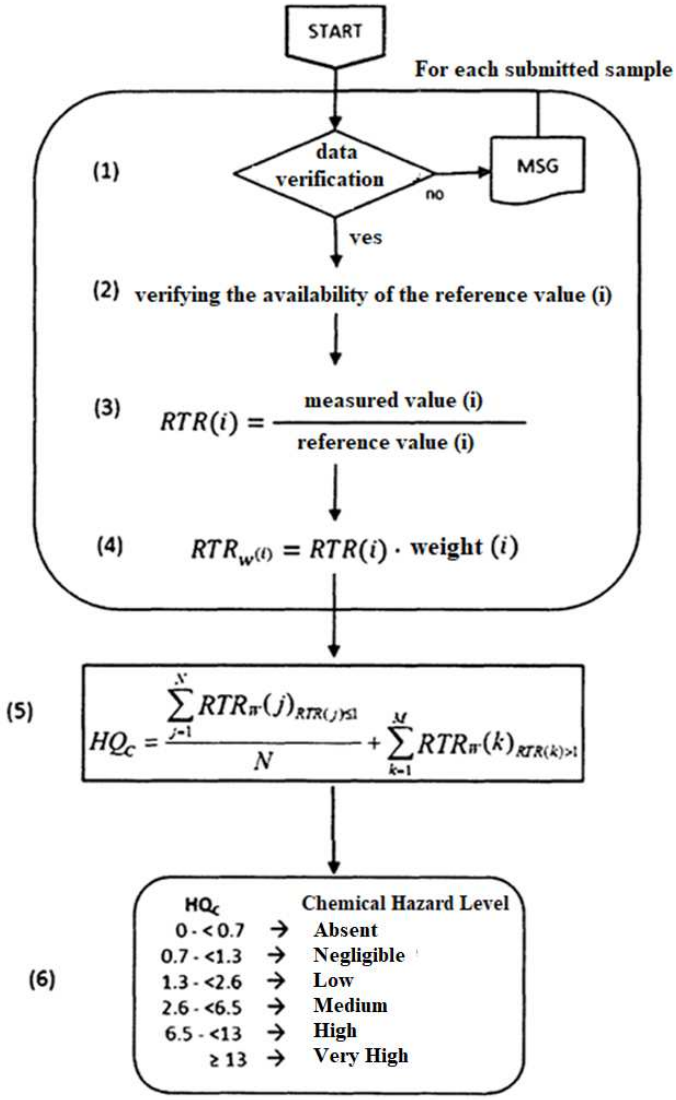


Figure 2. Procedure for processing the chemical characterization data of the sediment (source: Technical Annex to D. M. 173/2016).

1.1.3 Identification of local chemical reference levels from the environmental view ($L1_{LOC}$)

Chemical Level L1 is the concentration of a certain substance present in the sediment sample, mixed with other possible contaminants, at which generic toxic and bioaccumulation effects are expected with low probability. For each substance $L1_{loc}$ is given by the 90th percentile of the distribution of "non-toxic" data. It is essential to use a sample size of at least 30 samples without ecotoxicological danger (HQ < 1) according to the weighted integration criteria (Appendix 2B, D.M. 173/2016), of which at least 15 with bioaccumulation falling into the "Absent" or "Slight" class. Only the data of samples for which both chemical and ecotoxicological analyses are available can be used. These analyses can also refer to different times, provided they are not older than 10 years and based on "pairs" of associated data (chemical and ecotoxicological referred to the same sample), regardless of the period in which they were collected. The use of recent data will allow us to describe a more "faithful" situation in the current state of places. Each reference value thus identified has an optimal field of application with respect to local sediments with concentrations falling within the same range identified by the data set used for processing. Therefore, the extension of the use of the reference values to sediments with different characteristics must take into consideration the magnitude of these differences, evaluating the opportunity of a re-elaboration of

the data that includes all the measurements made, possibly obtained also by integrative investigations.

1.1.4 Final material quality class

Finally, the ecotoxicological and chemical classifications are integrated to attribute the quality class of the excavation sediments. This classification is based on the weighted integration criteria based on the ecotoxicological and chemical HQs reported in Table 6. There are 5 sediment quality classes from A to E, with their respective management options: environmental quality sediments are included in class A higher while in subsequent classes the quality decreases, as well as the management options that are gradually more limited. In the worst class, E, a possible safety removal from the marine environment after planned a risk assessment.

Ecotoxicological hazard class developed for the entire HQ Battery	**Chemical classification**	**Material quality class**
Absent	HQc (L2) ≤ Negligible	A
	Slight ≤ HQc (L2) ≤ Medium	B
	HQc (L2) = High	C
	HQc (L2) > High	D
Low	HQc (L1) ≤ Low	A
	HQc (L1) ≤ Medium and HQc (L2) ≤ Low	B
	Moderate ≤ HQc (L2) ≤ High	C
	HQc (L2) > High	D
Medium	HQc (L2) ≤ Low	C
	HQc (L2) ≥ Medium	D
≥ High	HQc (L2) ≤ Low	D
	HQc (L2) ≥ Medium	E
$HQ_{Battery}$: Ecotoxicological Hazard Quotient HQ_c : Chemical Hazard Quotient		

Table 6. Sediment quality classification according to weighted integration criteria

2. Material and methods

2.1 Sediment collection

Sediments were sampled at the entrance of a harbour in Central Adriatic Sea (Fig. 3), by ARPAM – Department of Ancona, according to the technical annex of the Decree 173/2016. Sampling was carried out in January 2017 in 7 different and contiguous areas, 4 replicates per area (R1 – R4) were collected and analysed. Each excavation area has a side length of 50 meters, 2 meters of excavation depth, for a total volume of 35.000 m^3 Sediment samples were taken, homogenized in order to include all granulometric fractions and aliquots were stored according to the analysis. Samples for chemical analysis were transported at 4° C, frozen to – 20°C in glass decontaminated vessels. Sample for bioassays and for granulometric analysis were stocked at 4° C.

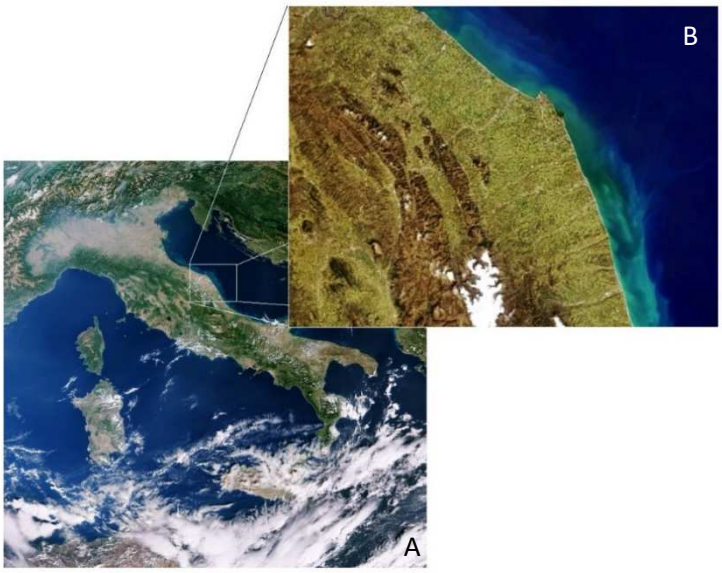


Figure 3. Photo of Italy from space (visibleearth.nasa.gov - March 2003). B) Photos of the Marche region from satellite (maps.google.it - 2014).

2.2 Textural and chemical analysis

Drain size analysis were performed according to the ICRAM Methodologies (ICRAM, 2001). Samples were pre-treated with a hydrogen peroxide and distilled water solution (2:8) for 48 hours at room temperature to facilitate granule separation, and then separated using a 63 μm mesh, stewed with distilled water to obtain two fractions which were dried in a stove at 60 ° C and weighed. The > 63 μm fraction was checked on a set of sieves. The analytical results are expressed as a percentage (ratio between the weight of the granulometric fraction and the weight of the total sample) and are represented in tabular form by dividing the sample, according to granulometry into gravel, sand, silt and clay. Total organic matter was determined by loss of ignition at 450 °C of dried sediment for 4 hours in a muffle furnace UNI EN 15936 (2012) method.

Trace metal concentrations were measured following the Environmental Protection Agency (EPA) method 3051A-2007 (U.S. EPA 3051A, 2007) using the Ethos Easy Advanced Microwave Digestion System and applying the UNI EN ISO 11885 protocol, using an Inductively Coupled Plasma– Optical Emission Spectrometry (ICP-OES), by Agilent technologies. Mercury (Hg) was determined following the EPA Method 7471B (U.S. EPA 7471B), based on Cold Vapor-Atomic Absorption Spectrophotometry (CVAAS).

Phytopharmaceutical determination **(pesticide organochlorine)**, **polycyclic aromatic hydrocarbons (PAHs)** and **polychlorinated biphenyls (PCB)** were determined following the EPA methods (U.S. EPA Method 3545A-3630C-3660B-8270D, 2007, 1996, 1996, 2014) using Varian GC 3800 gas chromatograph and Saturn 2000 mass spectrometer by Agilent. Briefly, samples were lyophilized and extracted with an Accelerate Solvent Extractor (ASE) working at high pressure. After concentration, sample was purified by columns with silica gel and finally reconstituted and analyzed by gas-chromatography.

C > 12 hydrocarbons were analyzed using the protocol UNI EN ISO 16703 (UNI EN ISO 16703, 2004). The extraction was carried out starting from 20 g of sediments in a solution of heptane/acetone with ASE. Purification in Florisil® and final reading was performed at the gas chromatograph with Flame Ionization detector (FID), model Perkin Elmer Autosistem XL.

Organostannic compounds (MBT, DBT and **TBT)** determination was performed according to (Devos et al., 2005).

Organic contaminants (DDT, DDE and **DDD)** were determined following the EPA methods 3545/3630C/3660B/8270D. Lyophilized sediments were extracted using the ASE, purified on a silica gel column. The sample was loaded and subsequently eluted with hexane. A subsequent dilution was then carried out in a hexane dichloromethane mixture, till gas-chromatographic analysis.

Dioxin like molecules (polychlorodibenzofuran (PCDF), **polychlorinated dibenzopdioxins (PCDD)** and **dioxin-like PCB)** were determined following the EPA method 1613 (U.S. EPA, 1994). Lyophilized sample were extracted with toluene at ASE, dried, concentrated and pre-purified with a series of acidic and basic washing, followed by a purification with FMES Dioxine Power Prep equipment and analysed by high-resolution Triple Quadrupole GC-MS (TSQ spectometer 8000 Evo from Thermo Scientific, and GC Trace 1310).

2.3 Bioassays

The preparation of **sediment elutriates** was performed in accordance with the ASTM E-1391-03 Guidelines (ASTM, 2014). Briefly, elutriates were prepared by combining different ratio of water and sediment (4:1 ratio) and mixing for 1 h by magnetic agitation. Filtration of the supernatant through cellulose acetate 0.45 µm filters (for microalgae bioassay) and in mixed esters of cellulose (for oyster test) was performed.

2.3.1 Phaeodactylum tricornutum bioassay (acute toxicity)

Phaeodactylum tricornutum, considered of low ecological relevance, has become one of the key organisms for the study of diatom biology due to its ability to grow easily in laboratory conditions. Differently from other diatoms, *P. tricornutum* can exist in different morphotypes (fusiform, triradiate and oval), stimulated by different environmental conditions. A further peculiarity is that during asexual reproduction, frustules do not undergo a dimensional reduction: this allows a continuous culture without the need for sexual reproduction (currently never documented in the laboratory).

Test

The toxicity test of elutriate with the microalgae ***Phaeodactylum tricornutum*** was performed as described in the ISO method (UNI EN ISO 10253) and according to (Libralato et al., 2011; Surricchio et al., 2019). *P. tricornutum*, strain number CCAP 1052/1A was provided by the Scottish Marine Institute, Oban, Argyll, UK.

For the bioassay, *P.tricornutum* initial cell density shall not exceed 10^4 cells/ml, to allow exponential growth in the control culture throughout the experiment duration. Algal inocula, in triplicate, were inoculated into each well of a multiwell plate (Fig. 4). Control is represented by the algae reared in sea water, without the addition of elutriate, while the effects of sediment was assayed incubating the algae with the selected elutriates. Elutriates were used undiluted.

The experiment was carried out in a ventilated thermostatic chamber for 72 hours at 20 ° C ± 2, with continuous lighting regime (10.000 lux). At the end of the established period, algal growth was determined by using the Bechman coulter Z2 electronic particle counter, particle count and size analyzer. Optimal growth rate was reached after 72 hours incubation period when algal growth in each control replicate is at least 16 times higher than the initial concentration. The coefficient of variation among control replicates must not exceed the 10%.

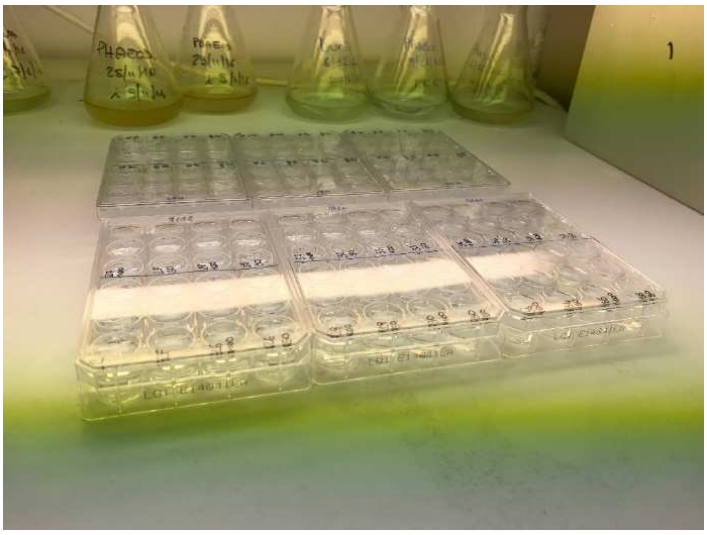


Figure 4. Multiwell plates for algal bioassay.

2.3.2 Vibrio fischeri bioassay (solid phase, acute toxicity)

This assay allows to evaluate the acute toxicity of a sample using as a response the inhibition of the naturally bioluminescence emitted by Gram-negative marine bacteria called *Vibrio fischeri*. This bacterium can emit a blue-green light with a maximum wavelength of 490 nm, in the presence of oxygen. The sequence of reactions that causes the emission of light is associated with the respiratory chain of transport of electrons and is catalysed by the enzyme luciferase. The enzyme simultaneously oxidizes luciferin (a cyclic aldehyde that contains more than eight carbon atoms) and riboflavin 5-P, with a reaction that can be synthesized as follows:

$$FMNH_2 + O_2 + R\text{-}CHO \rightarrow FMN + H_2O + R\text{-}COOH + hv$$

Where FMNH2 = reduced flavin mononucleotide, R-CHO = long chain of fatty aldehyde, RCOOH = fatty acid, hv = light. From the reaction between luciferase and the flavin coenzyme, together with oxygen and luciferin, an active complex form which degrades and emits the protons responsible for bioluminescence (Perin, 2004). When bacteria encounter toxic substances, there is a tendency to have a bioluminescence decrease which can be quantified, as each xenobiotic carries out its toxicity on specific action sites. Therefore, the ability to emit light is inversely proportional to the degree of toxicity of the analysed sample. In some cases, certain compounds may also cause the opposite effect, or stimulate light production (a hormesis phenomenon is similar to the stimulation of algal growth in the homonymous inhibition test).

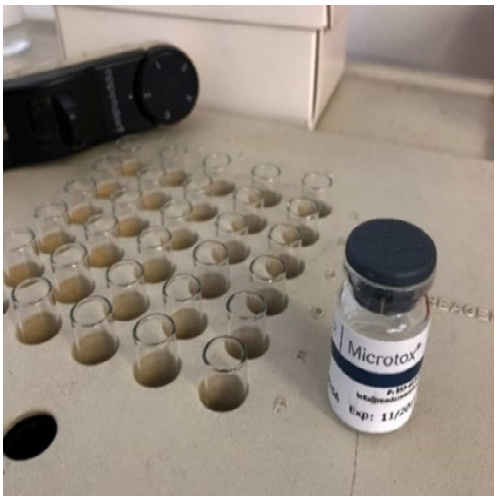


Figure 5. Reconstitution of *V. fischeri.*

Test

Microtox® microbial luminescence bioassay (Fig. 5) was performed on solid-phase (< 1 mm sediment fraction) according to the ISO Method (UNI EN ISO 11348-3, 2009) and following (Adams et al., 2015; Froehner et al., 2000) using the marine luminescent bacteria ***Vibrio fischeri.*** Selected sediments were diluted in 0.2 µm filtered synthetic seawater (dilution medium). Dilutions series were made with factor = 2 in special SPT tubes and let settle at 15 ± 2 ° C for 20 minutes. The

supernatant was decanted and 20 µL of Microtox® reagent containing the bacteria (Acute Reagent, Modern Water for Microtox® with Reconstitution Solution at final salinity of 20‰) was added. After 20 minutes the bacterial suspension is separated from the sediment particles. Bioluminescence is measured 30 minutes after separation. Controls are represented by luminescent bacteria suspended in the dilution medium. Bacterial light intensity (I_{30}) was measured by a Microtox Modern Water M500 Analyzer luminometer and the dedicated Microtox® Omni Software. The toxicity derived from concentration-effect curves using linear regression is expressed as EC_{50} (concentration that produces 50% of the effect in the population). Similarly, to other toxicity parameters, the lower the value, the more toxic it will be. To convert this into a positive indicator, where a higher value indicates greater toxicity, the EC50 values were converted to toxicity units (TU). The conversion was obtained with the following: $TU = (1 / EC_{50}) \times 100$. In addition, TU value must undergo a pelitic correction since *V. fischeri* tends to be adsorbed by the silty sediment particles and the decrease of the bioluminescence can be caused by the bacterium entrapment into the silt. On the basis of the natural toxicity of the sediment, considering the percentage of pelite, a convention rate should be proposed by applying the formula $Y = 0.28 + 3.49 X$ (where Y is the estimate of natural toxicity and X is the percentage of pelite). The conversion between observed and estimated TU, generate the Sediment Toxicity Index - STI (Icram, 2000). ($0 \leq STI \leq 1$ Absent; $1 < STI \leq 3$ Slight; $3 < STI \leq 6$ Moderate; $6 < STI \leq 12$ Major; > 12 Severe).

2.3.3 Crassostrea gigas bioassay

Thanks to its rapid growth and its great ease of adaptation, the Pacific concave oyster (*Crassostrea gigas*) is - today - the most widely bred oyster in the world. It is a hermaphrodite species: the individuals that are born are male and subsequently become females, with the possibility of changing sex every year (based on environmental conditions) (Nehring, 2011). During the reproductive season, the sexual organs constitute 50% of the volume of their body; they are extremely fertile and produce between 50 and 100 million eggs which, like sperms, are released into the water where they will then be fertilized (Nehring, 2011). From the fertilized egg develops a larva, the veliger, characterized by a protein matrix plate and by a large ciliate lobe called velum. At one day of life, the plate bends along the median line and calcifies on both sides, giving rise to the two valves of the larval shell. The velum allows the D-larva (is the stage at 48 hours post fertilization) to float and, through cilia, to filter the nutritive particles in the water column (Cataudella and Bronzi, 2001). The D-larvae lead planktonic life for about 3/4 weeks, during which they are dispersed by the sea currents, only then will they descend to the bottom to perform the metamorphosis. Sea water is the natural environment of this filtering organism and must present certain chemical and physical conditions to allow them a normal development: a salinity between 20 and 30 ‰ and an optimal temperature that varies from a vital minimum of 8-9 ° C up to a maximum of 30 ° C (Fratini, 2012).

Figure 6. *C. gigas* separate in different bekers.

Test

The toxicity test with the oyster ***Crassostrea gigas*** (Fig. 6) was based on the EPA protocol EPA/600/R-95-136 (U.S. EPA, 1995) with the appropriate modifications adopted under laboratory conditions. The seawater used for the test was collected 2 miles from the coast, filtered with mixed esters of cellulose 0.45 µm membrane and diluted to a final salinity of 30 ‰. The same water, as well as to conduct the test, was also used for preparing elutriates.

For the toxicity test, sexual mature oysters were purchased at Guernsey Sea Farms (Guernsey Sea Farms Ltd. Parc Lane, Vale. Guernsey) and were induced to spawning by alternating 30' of 18° and 32° C baths. Sperm was sieved through a 32 µm mesh to remove possible particulate matter, while oocytes where was sieved through a 100 µm mesh. Before fertilization, small aliquote of male and female gametes were checked for quality.

Fertilization was set up diluting approximately 5.000-8.000 eggs/mL in a final volume of 500 ml. Once fertilization occurred, as stated by the appearance of the membrane fertilization, 200 embryos (20 to 50 µl of embryo suspension) were reared in 10 ml of elutriates sediments, four replicates for each sample were set up, in a multiwell plate. The multiwell plates with the sample were incubated at 20 ± 2 °C for 48 hours, under 100 candle - 1076 lux - light conditions, with a photoperiod of 18L/6D. Morphological abnormalities was checked in larvae at 48 hours post fertilization after fixation in 37% PFA under inverted microscope. Before the beginning of the bioassays, O_2, pH and salinity were measured.

2.4 Statistical Analysis

Data obtained were analysed using Sediqualsoft® 109.0, developed by ISPRA and Università Politecnica delle Marche (Ancona).

2.4.1 Ecotoxicological bioassays

Regarding the expression of the results: in the bioassay from the reduction of the bioluminescence of *Vibrio fischeri* to 30 minutes, the results were expressed as a % of effect respect to the control, and the statistical treatment of the data was carried out directly by the software that manages the instrument (Microtox Omni Windows Software v. 1.19 by Azur Environmental). For the algal test and the embryotoxicity test, the% inhibition compared to the control is considered. According to the following formula:

$$I_{\mu i} = \frac{\overline{\mu_c} - \mu_i}{\overline{\mu_c}} \times 100$$

Where:

$I_{\mu i}$ is the percentage inhibition for each test

μi is the value (e.g. the growth rate) for each test replica

$\overline{\mu_c}$ is the mean value for the control

For marine microalgae, based on the reference methods, values that deviate from the control by more than 20% were considered different from this one. Therefore, samples with an inhibition rate ≥ 20% were considered toxic and ≥ 25% for the oyster bioassay. For samples considered toxic respect to the control, analysis of variance (one way ANOVA) was used to test the significance of the effects caused by exposure to various elutriates sediment (level of significance $P < 0.05$).

3. Results

3.1 Sediment granulometry and environmental variables

Sediment samples are mostly composed of sand, resulting the 0.25-10.125 fraction the most abundant. The gravelly fraction is generally poorly represented, some samples (1, 2, 6, 8 and 12) have 20% content of pelite, which reaches over 35% in samples 3, 13, 14 and 19 (Fig. 7). Results clearly shows that the pelitic fraction is higher in the sediments. The values show how the pelitic fraction is greater in the sediments taken inside the port, and close to the docks, associated to the higher sedimentation of material. In Table 7 physical data of the different samples taken in the harbor are reported and the graph shows the textural composition.

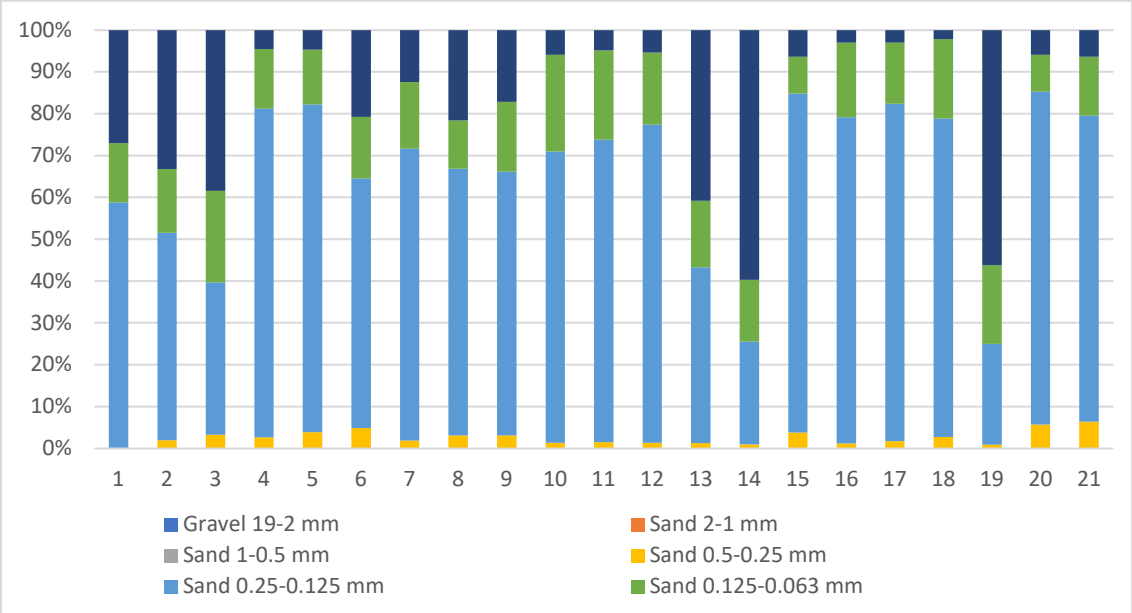


Figure 7. Sediment granulometry measured in the 21 sample of the 7 areas in the harbour in the Centre of Italy.

Geological characterization of the samples (%, w/w)									
Sample	**Humidity**	**Gravel 19-2 mm**	**Sand 2-1 mm**	**Sand 1-0.5 mm**	**Sand 0.5-0.25 mm**	**Sand 0.25-0.125 mm**	**Sand 0.125-0.063 mm**	**Total Sand**	**Silt and clay, or total pelite < 0.063 mm**
1/SM	36.0	0.00	0.00	0.37	4.02	56.22	13.55	74.16	25.84
2/SM	27.80	0.00	0.00	0.11	1.95	49.53	15.22	66.81	33.19
3/SM	24.00	0.83	0.81	1.35	3.17	35.34	21.24	61.91	37.26
4/SM	22.10	0.00	0.00	0.16	2.61	78.52	14.21	95.49	4.51
5/SM	20.80	0.00	0.00	0.25	3.87	78.14	13.05	95.31	4.69
6/SM	32.30	0.00	0.00	0.00	4.87	59.66	14.71	79.24	20.76
7/SM	25.00	0.92	0.00	0.00	1.82	69.16	15.78	86.77	12.31
8/SM	29.70	0.00	0.00	0.00	3.08	63.78	11.53	78.39	21.61
9/SM	23.20	0.85	0.00	0.00	3.05	62.51	16.60	82.17	16.99
10/SM	17.40	0.00	0.00	0.00	1.34	69.60	23.18	94.12	5.88
11/SM	20.40	0.00	0.00	0.00	1.47	72.30	21.38	95.16	4.84
12/SM	16.50	0.00	0.00	0.00	1.33	76.07	17.26	94.65	5.35
13/SM	26.40	0.00	0.00	0.00	1.23	42.00	15.99	59.23	40.77
14/SM	28.10	0.00	0.00	0.00	0.99	24.51	14.82	40.32	59.68

15/SM	17.70	0.00	0.00	0.00	3.75	81.10	8.77	93.62	6.38
16/SM	20.20	0.00	0.00	0.00	1.17	78.01	17.78	96.96	3.04
17/SM	20.30	0.28	0.00	0.00	1.75	80.37	14.58	96.70	3.02
18/SM	20.30	0.00	0.00	0.00	2.69	76.14	19.00	97.83	2.17
19/SM	30.80	0.00	0.00	0.00	0.85	24.12	18.86	43.83	56.17
20/SM	23.30	0.00	0.00	0.00	5.72	79.56	8.80	94.08	5.92
21/SM	21.70	0.37	0.34	0.78	6.27	72.11	13.81	93.32	6.31

Table 7. Geological characterization of the samples

3.2 Chemical analysis

As reported in tables 2, 3, 4 and 5 chemical characterization of sediments revealed the presence of all major contaminant classes. With regard to metals, all comply with the chemical reference level L1 (pursuant to the Decree of 15 July 2016, No. 173), (Tab. 8).

Chemical characterization of sediment samples										
	Metals in the sediment (mg/kg d. w.)									
Sediment Sample	**Arsenic**	**Cadmium**	**Chrome**	**Copper**	**Nickel**	**Lead**	**Zinc**	**Aluminium**	**Vanadium**	**Mercury**
1/SM	5.37	0.16	13.59	13.45	11.76	5.14	33.24	3810	12.27	0.05
2/SM	4.93	0.18	16.90	11.32	11.24	6.67	35.53	3957	11.85	0.06
3/SM	5.85	0.17	16.47	10.62	12.62	6.72	32.03	4474	12.21	0.07
4/SM	4.84	0.15	7.06	3.19	5.20	2.49	12.64	1550	6.23	0.03
5/SM	4.93	0.13	7.06	3.21	5.73	3.81	11.50	1783	6.46	0.03
6/SM	5.14	0.14	9.53	6.88	7.74	3.62	16.78	2315	8.54	0.04
7/SM	4.86	0.13	7.74	4.98	6.20	2.84	14.44	1780	7.23	0.04
8/SM	4.72	0.15	10.06	8.21	8.64	4.69	19.62	3022	9.61	0.04
9/SM	4.67	0.18	11.82	7.54	9.31	4.88	20.18	2722	9.67	0.06
10/SM	4.43	0.11	6.01	2.49	4.89	2.31	11.86	1512	5.93	0.04
11/SM	4.23	0.14	6.51	2.82	5.49	2.28	11.59	1830	6.67	0.04
12/SM	4.72	0.13	6.20	2.73	5.31	2.35	11.50	1708	6.45	0.03
13/SM	5.95	0.17	18.21	14.49	14.93	6.82	42.10	6207	16.07	0.05
14/SM	5.43	0.28	21.44	15.65	15.15	11.76	45.11	6259	16.26	0.07
15/SM	3.61	0.12	8.49	3.02	5.67	2.99	12.20	1876	5.79	0.03
16/SM	4.09	0.15	5.22	2.09	4.45	2.05	8.88	1381	5.29	0.03
17/SM	4.05	0.14	5.58	1.98	4.42	2.12	9.17	1361	5.35	0.03
18/SM	4.50	0.15	7.18	2.70	5.24	2.45	11.19	1681	6.50	0.04
19/SM	6.60	0.17	21.15	18.37	17.32	8.46	53.52	7878	19.49	0.06
20/SM	5.18	0.14	7.66	5.35	6.78	3.26	13.57	1893	7.47	0.04
21/SM	4.01	0.16	7.65	4.40	6.01	3.51	12.82	2273	6.48	0.04
L1 Law DM 173/2016	12	0.3	50	40	30	30	100	/	/	0.3
L2 Law DM 173/2016	20	0.80	150	52	75	70	150	/	/	0.80

Table 8. Chemical characterization of sediment

Similarly, to what observed for organic contaminants, most of the value are below of the reference limit L1. In samples # 13/SM to 21/SM DDT concentrations exceed the reference limit L1, remaining lower than the reference limit L2. See Tab. 9.

Chemical characterization of sediment samples					
Sediment Sample	**Organic Contaminants in the sediment (µg/kg d. w.)**				
	Σ PCB[3]	**Σ DDD[4]**	**Σ DDT[4]**	**Σ DDE[4]**	**Organostannic compounds**
1/SM	4.4223	0.7	0.8	0.7	2.54
2/SM	6.8384	2.4	< LOD	1.7	0.701
3/SM	3.8923	2.4	1.7	1.0	2.46
4/SM	0.1595	< LOD	< LOD	< LOD	0.25
5/SM	0.3881	< LOD	< LOD	< LOD	< LOD
6/SM	2.385	0.6	4.2	0.8	1.86
7/SM	2.5286	< LOD	0.2	0.3	1.11
8/SM	2.8156	0.5	0.7	0.6	1.15
9/SM	3.1217	1	0.7	0.5	0.97
10/SM	2.8118	0.2	< LOD	0.4	0.44
11/SM	0.2384	< LOD	0.3	< LOD	< LOD
12/SM	0.07	< LOD	0.4	0.2	< LOD
13/SM	10.7738	0.6	1.8	0.5	7.57
14/SM	7.4388	0.7	2.4	0.7	7.73
15/SM	0.4149	0.6	2.2	0.6	0.3
16/SM	0.07	0.6	1.9	0.5	0.71
17/SM	0.07	0.7	1.9	0.7	1.09
18/SM	0.2144	0.6	2.2	0.6	2.3
19/SM	14.2655	0.6	2.1	0.7	10.26
20/SM	0.3468	0.6	2.1	0.7	4.06
21/SM	0.3649	0.5	2	0.7	1.85
L1 Law DM 173/2016	8	0.8	1.8	1.0	5[1]
L2 Law DM 173/2016	60	7.8	3.7	4.8	72[2]

[1] referring only to TBT;
[2] referred to the summation of MBT, DBT, TBT;
[3] as the sum of the follower's congeners: 28, 52, 77, 81, 101, 118, 126, 128, 138, 153, 156, 169, 180;
[4] as a summation of isomers: 2,4 and 4,4.
In yellow values between L1 and L2 limits.

Table 9. Chemical characterization of sediment samples

Concerning PAHs: 13 sediment samples (3/SM, 4/SM, 5/SM, 7/SM, 10/SM, 11/SM, 12/SM, 13/SM, 15/SM, 16/SM, 17/SM, 18/SM and 21/SM) show concentrations below the reference limits L1, but with significant detectable values. In the remaining sediments values fall between the reference limits L1 and L2. Some contaminants exceeded the reference L2 limit: this occurs for sample 6 regarding levels of Fluorene and Phenanthrene; for sediment 14 regarding Indenopyrene and Benzo(g,h,i)perylene; values in sample 19 excess of Benzo(a)pyrene was observed and in sample 20, excess L2 limits was detected for Indenopyrene, Benzo(g,h,i)perylene and Benzo(a)pyrene. See Tab. 10.

Chemical characterization of sediment samples																			
Sediment Sample	**PAHs in the sediment (µg/kg d. w.)**																		
	Naphthalene	**Acenaphthylene**	**Acenaphthene**	**Fluorene**	**Phenanthrene**	**Anthracene**	**Fluoranthene**	**Pyrene**	**Benz(a)anthracene**	**Crysene**	**Benzo(b)fluoranthene**	**Benzo(k)fluoranthene**	**Benzo(e)pyrene**	**Benzo(a)pyrene**	**Perylene**	**Indenopyrene**	**Dibenz(a,h)anthracene**	**Benzo(g,h,i)perylene**	**Σ PAH[1]**
1/SM	0,25	0,25	8,95	17,69	75,58	9,31	82,38	69,52	38,71	35,58	41,12	22,77	39,64	55,43	49,87	14,38	2,31	15,64	489,87
2/SM	21,56	0,25	5,74	14,55	51,07	13,34	62,29	101,00	33,79	35,12	36,90	17,42	36,44	42,91	38,14	24,28	5,35	33,59	499,16
3/SM	0,25	0,25	0,25	19,77	76,86	22,11	59,56	54,50	22,33	17,38	23,93	12,21	17,08	14,35	51,39	18,60	3,59	21,67	367,61
4/SM	0,25	0,25	1,84	2,56	9,26	0,97	6,60	6,77	3,96	3,79	5,62	3,16	4,78	5,88	2,93	3,94	0,25	4,04	59,14
5/SM	1,87	0,25	1,22	1,08	10,52	1,10	7,37	9,16	3,32	3,16	4,72	2,20	4,06	4,96	4,56	3,15	0,25	6,24	60,57
6/SM	39,17	0,25	88,19	146,30	584,70	28,80	107,40	100,90	38,89	36,90	42,00	21,94	36,19	57,76	30,90	30,68	35,81	36,99	1396,68
7/SM	1,83	0,25	1,81	2,14	12,60	1,28	9,86	9,81	4,03	4,31	8,11	3,65	6,22	6,26	4,71	3,95	0,80	4,35	75,04
8/SM	3,46	3,72	5,06	7,11	47,71	7,52	55,50	101,10	5,75	26,25	42,36	21,70	37,80	51,02	38,22	32,48	8,08	44,03	462,85
9/SM	6,22	15,80	18,12	30,79	201,10	27,72	123,80	121,10	8,62	48,40	65,76	32,50	47,85	70,30	43,86	40,88	9,80	41,73	862,64
10/SM	0,25	0,25	5,67	10,66	35,05	2,44	18,39	35,10	1,72	10,16	28,43	13,26	25,14	22,24	8,16	14,02	0,25	28,84	226,73
11/SM	0,25	0,55	2,10	2,52	13,18	2,08	16,75	15,05	12,88	12,03	27,56	10,65	14,04	20,98	6,64	11,06	0,25	9,08	156,97
12/SM	0,89	0,25	2,56	5,55	13,20	1,20	11,39	42,49	4,57	3,56	7,04	3,44	7,12	8,42	3,12	4,94	0,25	13,13	122,88
13/SM	1,69	0,25	6,06	5,15	28,25	4,06	29,56	33,35	12,83	12,04	20,03	9,49	17,09	21,56	72,99	13,12	0,25	23,05	220,74
14/SM	7,01	0,25	13,43	16,88	110,20	18,57	121,00	116,10	54,47	49,43	74,18	36,45	56,05	83,35	156,40	346,40	57,29	379,90	1484,91
15/SM	0,25	0,25	1,31	1,23	16,54	2,53	15,92	17,07	7,19	8,46	12,63	5,91	9,49	9,42	12,28	6,22	1,53	6,61	113,07
16/SM	0,25	0,25	1,82	0,25	22,74	1,65	4,52	4,17	2,11	2,85	4,14	2,12	2,84	3,34	1,64	1,84	0,25	1,68	53,98
17/SM	0,25	0,25	3,03	5,92	16,17	2,06	18,09	19,06	11,68	12,65	21,30	10,61	16,58	19,48	6,23	11,70	2,73	15,05	170,03
18/SM	1,94	0,25	6,12	6,49	39,41	2,49	19,05	52,25	8,65	8,28	2,28	1,11	2,04	2,24	0,82	8,51	0,25	17,73	177,05
19/SM	0,25	22,09	12,48	33,52	94,35	47,07	220,50	215,30	100,10	85,63	107,30	55,83	83,49	119,60	101,10	86,13	22,30	72,16	1294,61
20/SM	0,25	110,60	14,16	48,29	378,70	83,06	428,00	402,70	175,90	154,20	193,30	105,50	145,80	229,80	68,35	111,40	24,03	108,10	2567,99
21/SM	0,25	2,65	1,43	1,59	10,59	1,07	8,80	9,81	4,48	4,73	9,09	4,17	6,33	6,25	13,94	4,32	1,01	4,41	74,65
L1 DM 173/2016	35	/	/	21	87	24	110	153	75	108	40	20	/	30	/	70	/	55	900
L2 DM 173/2016	391	/	/	144	544	245	1494	1398	500	846	500[2]	500[2]	/	100	/	100[(2)]	/	100[(2)]	4000

[(1)] as the sum of the 16 major environmental PAHs indicated by the USEPA (Acenaphtylene, Benz(a)anthracene, Fluoranthene, Naphthalene, Anthracene, Benzo(a)pyrene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(g,h,i)perylene, Acenaphthene, Fluorene, Phenanthrene, Pyrene, Dibenz(a,h)anthracene, Crysene, Indeno(1,2,3,c-d)pyrene;
[(2)] valid concentration only for emerged nourishment activities;
In green values under L1 limits, in yellow values between L1 and L2 limits, in red values higher than L2 limits.

Table 10. Chemical characterization of sediment sample.

Regarding the related toxicity equivalent factors T.E. of PCDDs, PCDFs (Dioxins and Furans) and similar dioxin PCBs concentrations are well below the limit values (Tab. 11). The chemical parameters were searched not in all samples: as they are very expensive and time-consuming analyzes, in a first step we search for the presence of dioxins on a vertical profile, considering all the depths of the sampled area. If one of these proves to have a high presence, then the sampling will be performed completely for each point of the work area.

Chemical characterization of sediment		
Sediment Sample	**Σ T.E. PCDD, PCDF[(1)] (Dioxins and Furans) and PCB dioxin-like (µg/kg d. w.)**	
	Sum of PCDD, PCDF and PCB	**Sum of PCDD and PCDF**
1/SM	0.00039	0.00036
2/SM	/	/
3/SM	/	/
4/SM	/	/
5/SM	0.00012	0.00012
6/SM	/	/
7/SM	/	/
8/SM	/	/
9/SM	0.0001	0.00006
10/SM	/	/
11/SM	/	/
12/SM	< LOD	< LOD
13/SM	/	/
14/SM	0.00062	0.00058
15/SM	/	/
16/SM	/	/
17/SM	< LOD	< LOD
18/SM	/	/
19/SM	0.00031	0.00016
20/SM	/	/
21/SM	/	/
L1 DM 173/2016	0.002	-
L2 DM 173/2016	-	0.01[(2)]
[(1)] the list of congeners and related toxicity equivalent factors T.E. (EPA, 1989) and the list of similar dioxin PCB congeners (WHO, 2005) and the one reported in the notes of table 3/A pursuant to Legislative Decree 172/2015; [(2)] relative to the summation of PCDD and PCDF		

Table 11. Chemical characterization of sediment

3.3 Toxicity with V. fischeri

The solid phase test (performed on the sediment fraction <1 mm) with the bioluminescent marine bacterium *V. fischeri* gives us the toxicity derived from the concentration-effect curves using linear regression and is expressed as EC_{50}. The EC_{50} values are converted into toxic units (TU) to obtain an expression of toxicity and this value must undergo a pelitic correction to finally get a Sediment Toxicity Index - STI (ICRAM, 2001). In all sediments STI ranged between 0 and 1,

excluding sediments 20 and 21 (but their values correspond to a very slight toxicity), showing lack of toxicity. The EC_{50} of the test was calculated on 7 dilutions (see method) and the dose/response curves of the samples tested are shown below (Fig. 8 and Fig. 9), numerical data are shown in Table 12.

Ecotoxicological values of the *V. fischeri* bioassay					
Sediment Sample	**EC_{50} Concentration (%)**	**R^2 Coeff. of determination**	**TU**	**Y**	**STI**
1/SM	6.157	0.9531	16.2	90.46	0.18
2/SM	2.648	0.9970	37.8	116.11	0.32
3/SM	1.944	0.9979	51.4	130.32	0.39
4/SM	28.26	0.9947	3.5	16.02	0.22
5/SM	26.20	0.9734	3.81	16.65	0.23
6/SM	9.368	0.9843	10.7	72.73	0.15
7/SM	6.453	0.9508	15.5	43.24	0.36
8/SM	31.95	0.8590	3.13	75.70	0.04
9/SM	5.43	0.9213	18.4	59.57	0.31
10/SM	110.6	0.9149	0.90	20.80	0.04
11/SM	58.77	0.9873	1.70	17.17	0.10
12/SM	49.67	0.9867	2.01	18.95	0.11
13/SM	1.537	0.9962	65.1	142.57	0.46
14/SM	1.571	0.9885	63.6	208.56	0.31
15/SM	11.15	0.9606	8.96	2.55	0.40
16/SM	10.74	0.5763	9.3	10.89	0.85
17/SM	37.66	0.9785	2.65	10.82	0.24
18/SM	14.86	0.9286	6.73	7.85	0.86
19/SM	1.162	0.9922	86.05	196.31	0.44
20/SM	2.59	0,9835	38.61	20.94	1.84
21/SM	2.347	0.9788	42.6	22.30	1.91

Legend:
TU: toxic units (observed toxicity)
Y: estimated natural toxicity [y = 0.28 + 3.49(x)] where x is pelitic %
STI: Sediment Toxicity Index

Table 12. Ecotoxicological values of the *V. fischeri* bioassay

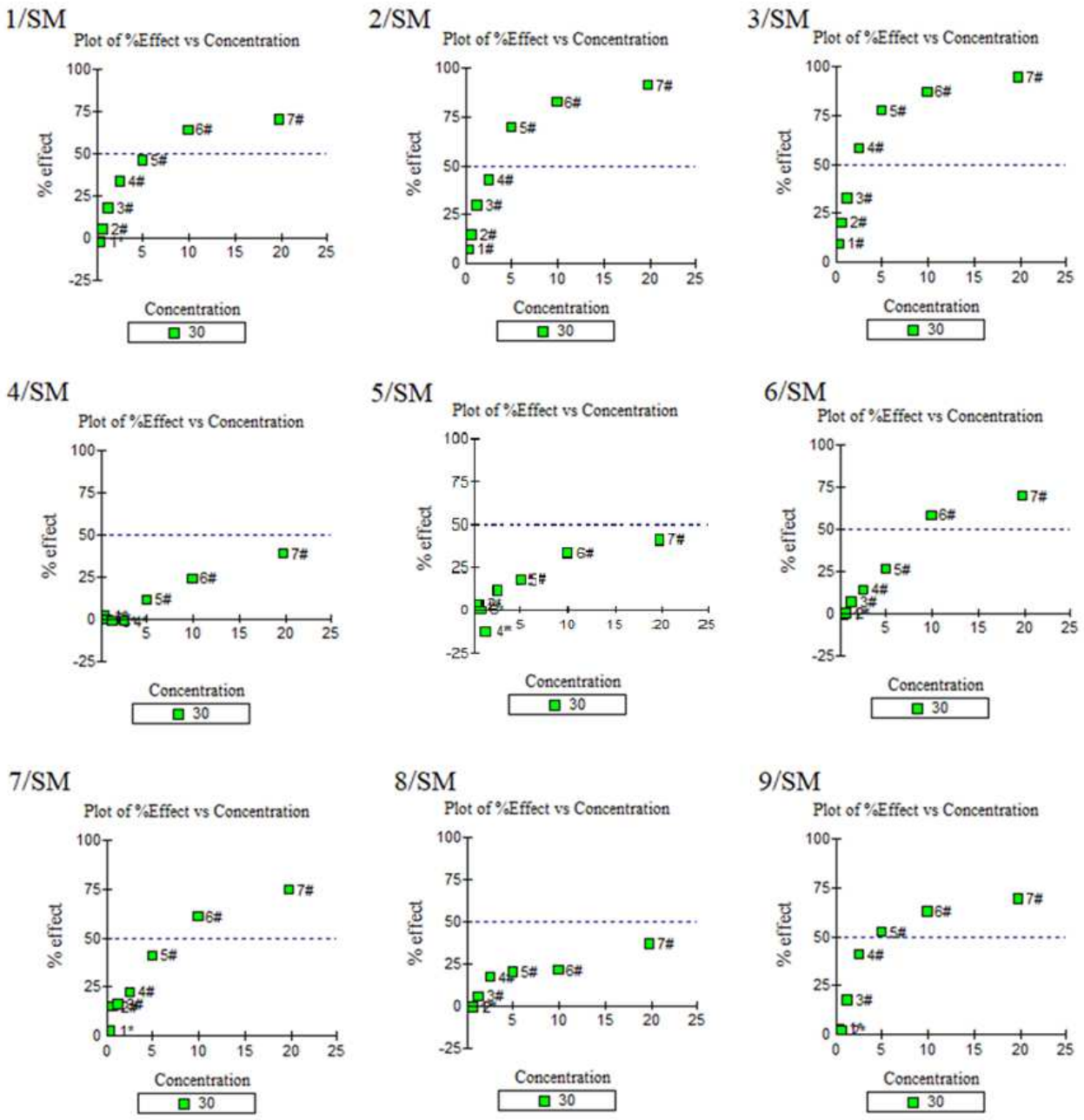


Figure 8. Dose/response curves of the *V. fischeri* test on the 21 sediment samples analysed (Microtox® Omni Software, developed by Azur Environmental, version 1.18).

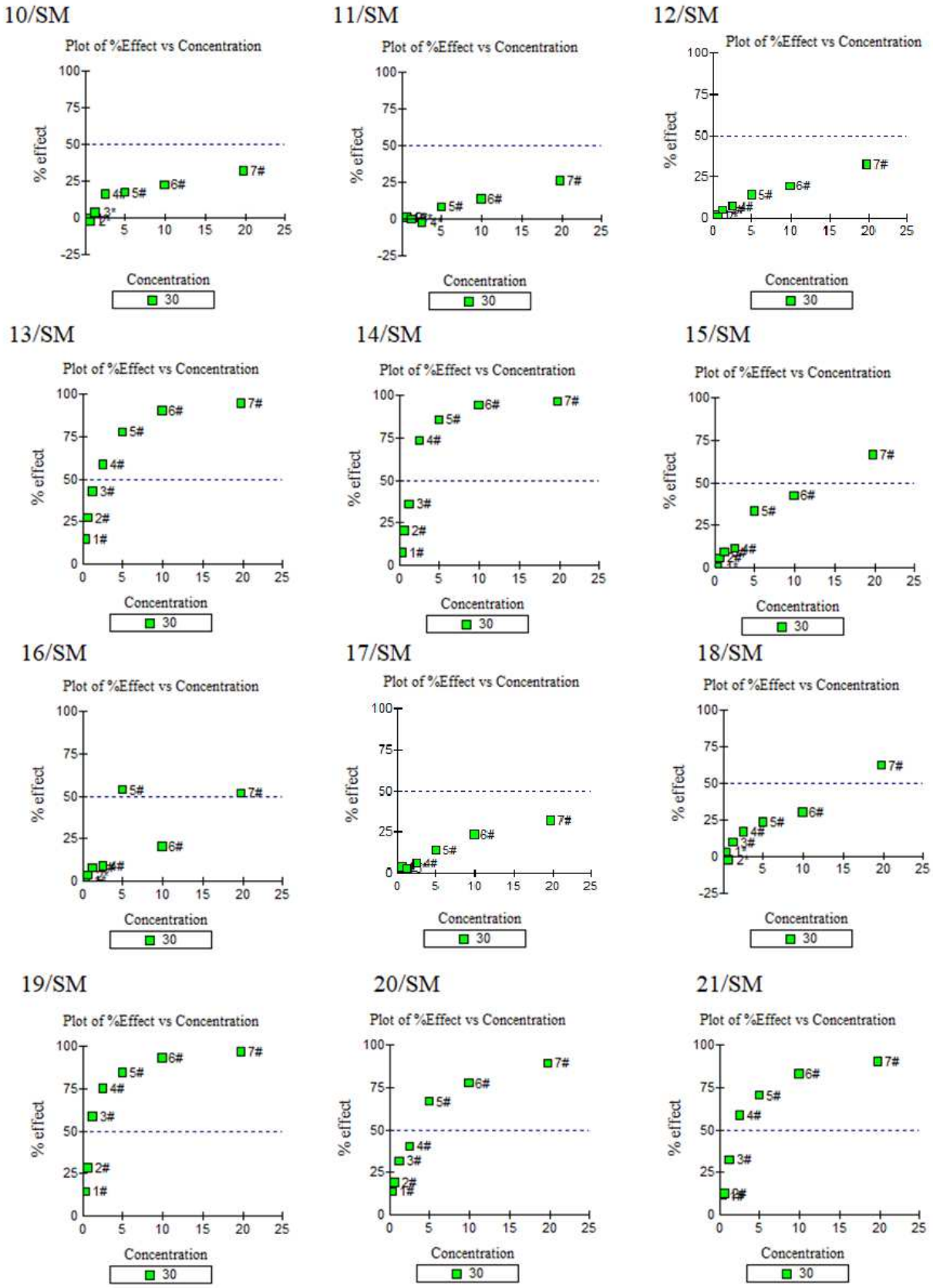


Figure 9. Dose/response curves of the *V. fischeri* test on the 21 sediment samples analysed (Microtox® Omni Software, developed by Azur Environmental, version 1.18).

3.4 Toxicity with P. tricornutum

Toxicity test on elutriate was performed. The toxic effect of all sediments was demonstrated by comparing the growth rate of the algae cultivated with the elutriate to that of alga cultured in control medium. However, in some of them the phenomenon defined as hormesis was observed with the tendency of the microalga exposed to the sediments to grow more than the control. In particular, this effect can be evidenced in the samples 4/SM, 5/SM, 6/SM, 7/SM, 10/SM and 11/SM. The hormetic effect is a problem in ecotoxicological tests and in their accreditation procedure, in fact they can mask the toxic effect since the microalga could increase its metabolism in order to contrast toxicity. N, P or C could justify an increase in growth, however the presence of TOC in these sediment samples is very low, resulting not higher than 1.1% and frequently below the limit of determination set at 0.5 % (data not shown in the table 13). The toxic nature of the hormetic effect is still strongly debated.

Ecotoxicological values of the *P. tricornutum* bioassay

Sediment Sample	**R1 (cell/ml) x 1000**	**R2**	**R3**	**R average**	**% inhibition***	**DS**
C	**501**	**483**	**452**	**479**	/	25
1/SM	332	342	353	342	28	11
2/SM	291	266	253	270	44	19
3/SM	231	220	223	225	53	6
4/SM	712	730	806	749	-57	50
5/SM	539	545	571	552	-15	17
6/SM	441	527	529	499	-4	50
7/SM	685	668	625	659	-38	31
C	**701**	**747**	**754**	**734**	/	29
8/SM	543	625	715	628	14	86
9/SM	643	597	523	588	20	61
10/SM	835	800	935	857	-17	70
11/SM	745	704	781	743	-1	39
12/SM	653	603	706	654	11	52
13/SM	446	387	342	392	47	52
14/SM	369	401	422	397	46	27
C	**1017**	**1055**	**1069**	**1047**	/	27
15/SM	524	601	522	549	48	45
16/SM	769	853	821	814	22	42
17/SM	675	690	717	694	34	21

18/SM	418	558	550	509	51	79
19/SM	390	490	493	458	56	59
20/SM	667	732	715	705	33	34
21/SM	428	467	482	459	56	28
* compared to the respective control test						

Table 13 Ecotoxicological values of the *P. tricornutum* bioassay

3.5 Toxicity with C. gigas

The embryotoxicity test with the *C. gigas* oysters was evaluated only on the elutriate at concentration 100%, as this is required by the Sediqualsoft® software. The assay is very sensitive, and these results fit with the chemical results (Fig. 11). The number of malformed D larvae was counted in four replicates per sample. Of the 21 sediment samples, there was always a % effect compared to the control (Tab. 14): it is important to note that the essay does not intend to divide the malformations by type, but simply the result is given in a number of malformed larvae (any aberration respect to the normal D-larvae shape). The test turns out to be too conservative and goes to condition the battery of the essays in many cases, thus becoming central in the evaluation and in the downgrading in the quality of the sediment. The typical malformations found in the tests were photographed and shown in Fig. 10.

Ecotoxicological values of the *C. gigas* essay							
Sediment Sample	**R1 (% normoformed embryo)**	**R2**	**R3**	**R4**	**R average**	**% Effect***	**DS**
C	96	90	86	93	91	/	4
1/SM	75	69	78	74	74	19	4
2/SM	63	68	65	73	67	26	4
3/SM	78	64	71	80	73	20	7
4/SM	89	90	81	84	86	6	4
5/SM	88	91	87	90	87	5	4
6/SM	75	66	69	79	72	21	6
7/SM	89	91	76	89	86	5	7
8/SM	81	79	73	90	81	12	7
9/SM	76	65	73	82	74	19	7
10/SM	89	90	80	94	88	3	6
11/SM	87	92	85	90	89	3	3
12/SM	95	81	86	88	88	4	6
13/SM	64	71	66	60	65	28	5

14/SM	45	61	50	58	54	41	7
15/SM	87	72	79	84	81	12	7
16/SM	87	90	94	80	88	4	6
17/SM	92	88	78	80	85	7	7
18/SM	85	93	87	81	87	5	5
19/SM	56	71	60	58	61	33	7
20/SM	61	58	74	78	68	26	10
21/SM	92	75	78	79	81	11	8
* compared to the control test							

Table 14. Ecotoxicological values of the *C. gigas* essay

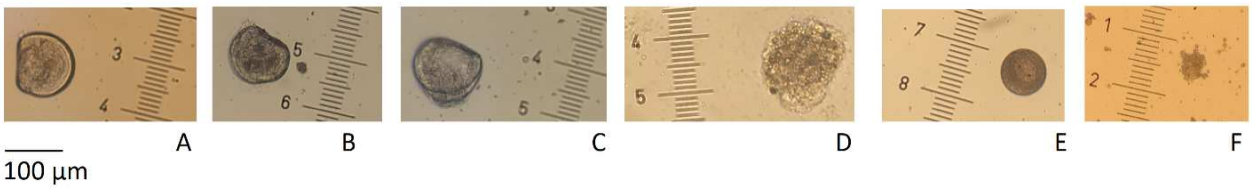
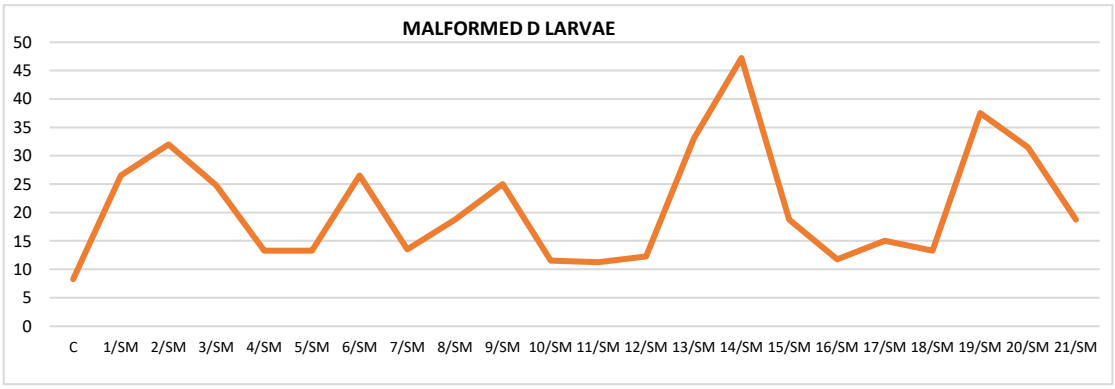
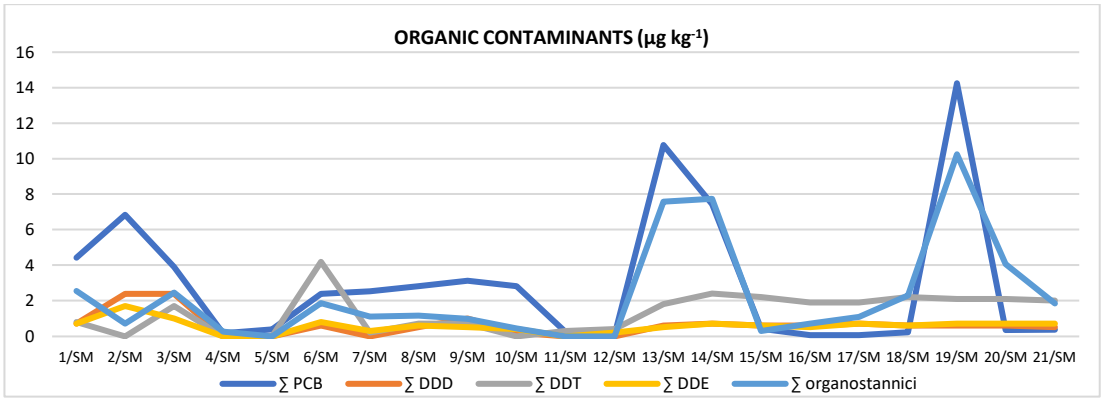


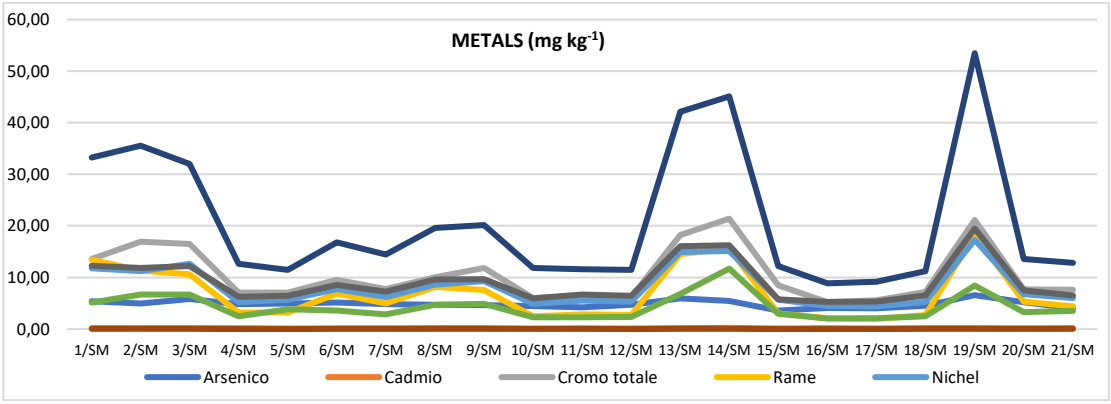
Figure 10 Light microscopy images showing various morphological developmental defects at 2 days post fertilization (48 hours post fertilization): A) control D-larvae; B-F) D-larvae exposed to 20/SM; B) D-larvae showing abnormal shell; C) D-larvae presenting hypertrophied mantle; D) D-larvae with both shell and mantle malformations; E) embryo showing arrested development at early stage; F) "degenerated" D-larvae.



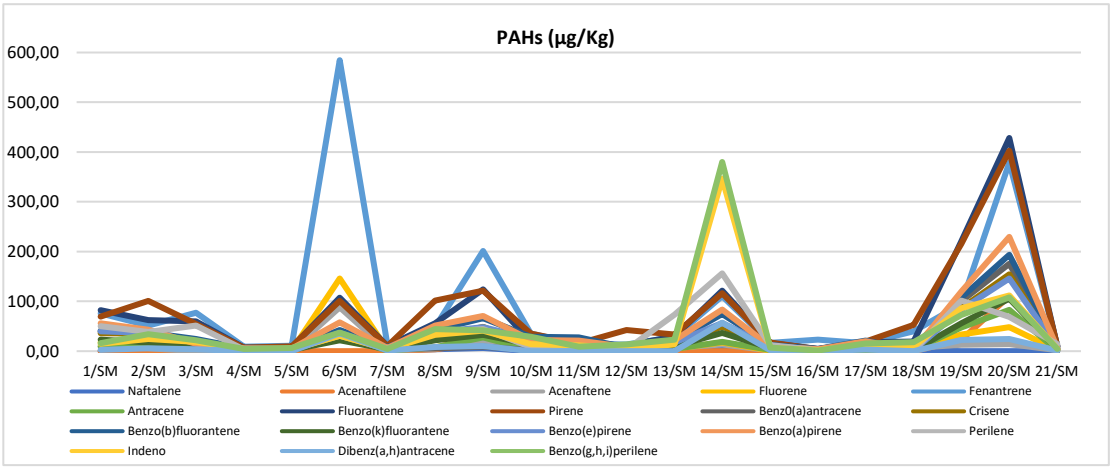
a



b



c



d

Figure 11 The graphs show the trend of the number of malformed larvae (a), compared to the presence of organic contaminants (b), metals (c) and PAHs (d) in the 21 sediments.

3.6 Sediqualsoft® analysis

The sediments of the harbour here studied, fall into 3 (of 5) different categories of quality: A, B and C. The results obtained through the use of the ISPRA software, Sediqualsoft®. Data are summarized in the table below.

Sediqualsoft® results									
Sediment Sample	**Ecotoxicology**				**Chemistry**				
	Test	**HQe Specific**	**HQe Battery test**	**HQe**	**HQc L1**	**HQc L2**	**Chemical danger L1**	**Chemical danger L2**	**Sediment quality class**
1/SM	*C. gigas*	1.16	1.31	Low	5.594	0.125	Medium	Absent	B
	P. tricornutum	2.94							
	V. fischeri	0							
2/SM	*C. gigas*	1.64	2.21	Medium	6.197	0.139	Medium	Absent	C
	P. tricornutum	4.49							
	V. fischeri	0							
3/SM	*C. gigas*	1.23	2.45	Medium	6.489	0.132	Medium	Absent	C
	P. tricornutum	5.45							
	V. fischeri	0							
4/SM	*C. gigas*	0.07	0.4	Absent	0.087	0.035	Absent	Absent	A
	P. tricornutum	1.29							
	V. fischeri	0							
5/SM	*C. gigas*	0.06	0.02	Absent	0.085	0.034	Absent	Absent	A
	P. tricornutum	0							
	V. fischeri	0							
6/SM	*C. gigas*	1.29	0.38	Absent	29.563	2.259	Very High	Low	B
	P. tricornutum	0							
	V. fischeri	0							
7/SM	*C. gigas*	0.07	0.02	Absent	0.121	0.046	Absent	Absent	A*
	P. tricornutum	0							
	V. fischeri	0							
8/SM	*C. gigas*	0.11	0.12	Absent	5.316	0.101	Medium	Absent	A*
	P. tricornutum	0.29							
	V. fischeri	0							
9/SM	*C. gigas*	1.16	0.43	Absent	15.758	0.138	Very High	Absent	A*
	P. tricornutum	0.31							
	V. fischeri	0							
10/SM	*C. gigas*	0.05	0.01	Absent	0.238	0.057	Absent	Absent	A
	P. tricornutum	0							
	V. fischeri	0							
11/SM	*C. gigas*	0.03	0.01	Absent	0.172	0.046	Absent	Absent	A
	P. tricornutum	0							
	V. fischeri	0							
12/SM	*C. gigas*	0.05	0.08	Absent	0.133	0.044	Absent	Absent	A
	P. tricornutum	0.21							
	V. fischeri	0							
13/SM	*C. gigas*	1.77	2.41	Medium	4.382	0.119	Medium	Absent	C
	P. tricornutum	4.8							
	V. fischeri	0							
14/SM	*C. gigas*	2.52	2.7	Medium	31.983	0.197	Very High	Absent	C
	P. tricornutum	4.72							
	V. fischeri	0							
15/SM	*C. gigas*	0.11	1.71	Medium	3.018	0.068	Medium	Absent	C
	P. tricornutum	4.9							
	V. fischeri	0							

16/SM	*C. gigas*	0.05	0.69	Absent	2.59	0.059	Low	Absent	A
	P. tricornutum	2.29							
	V. fischeri	0							
17/SM	*C. gigas*	0.08	1.07	Low	2.687	0.073	Medium	Absent	B
	P. tricornutum	3.47							
	V. fischeri	0							
18/SM	*C. gigas*	0.06	1.86	Medium	3.034	0.074	Medium	Absent	C
	P. tricornutum	5.29							
	V. fischeri	0							
19/SM	*C. gigas*	2.05	2.97	Medium	33.005	1.736	Very High	Low	C
	P. tricornutum	5.79							
	V. fischeri	0							
20/SM	*C. gigas*	0.29	1.19	Low	56.175	3.187	Very High	Medium	C
	P. tricornutum	3.36							
	V. fischeri	0.17							
21/SM	*C. gigas*	0.13	2.21	Medium	2.744	0.07	Medium	Absent	C
	P. tricornutum	5.78							
	V. fischeri	0.23							
* Percentage of pelite higher than indicated for emerged nourishment (Technical Annex to the Ministerial Decree 173 Figure 7)									

Table 15 Sediqualsoft® software results

4. Discussion

The granulometric analysis of the 21 sediments analysed in this study shows that they were mostly formed by fine components: sand (in particular the components 0.25 - 0.125 mm and 0.125 - 0.063 mm) and pelitic fraction (< 0.063 mm). So composed sediment was more able to adsorption contaminants present in the water column, rather than a sandy sediment. The pelitic fraction was usually more enriched in heavy metals and the fine-grained sand was a very important repository of contaminants (Giusti, 2001). Curiously, although sediment quality problems within harbours were becoming increasingly significant in the design of new ports and in the planning of modifications to existing basins, there were a very few number of studies in literature about the pollution levels in harbour sediments, and significantly lower than the number of work focused on sediment qualities of other environments such as estuaries, bays, etc (Garcia-Guerra and Garcia Gomez, 2005).

Chemical analyses indicate that coastal sediments in some areas of the World were contaminated (Sprovieri et al., 2007; Long et al., 1995) and the chemical profile of an harbour was particularly complex, as a result of the classification category of the port (D.M. 1213 of 03/24/1960) and therefore from the activities carried out by man.

In this study, focusing on chemical data, different situations were found for the different contaminants: the heavy metals detected in the samples were all below the reference limit L1 recommended by Decree 173 (for each substance L1 is given by the 90th percentile of the distribution of data deemed non-toxic, Appendix 2D to D.M. 173). Very high values were found for Aluminium and Vanadium, which - however - were considered as additional parameters and with no L1 and L2 reference values, although their toxic effect on aquatic organisms was surely proven (Jaishankar et al., 2014), such as seaweeds and crawfish, is also affected by Aluminium toxicity (Bezak-Mazur et al., 2001). Continuing with the other parameters, it was evident that the harbour was particularly affected by a polycyclic aromatic hydrocarbons contamination: in fact most PAH inputs in the environment were linked to the anthropogenic activity (Soclo et al., 2000) that is generally considered to be the major source of these compounds (e.g., wastes from industrialized and urbanized areas, off-shore petroleum hydrocarbons production or petroleum transportation), with the harbours that fully follow this description. Nothing to be noted regarding dioxins, furans and dioxin-like PCBs.

Different situation regarding the organic contaminants (PCB, DDT, DDD, DDE and Organostannic compounds) and PAHs, that constitute the persistent organic pollutants (POPs). Many POPs are believed to be possible carcinogens or mutagens and probably they are dangerous to human and environmental health. Although most of these compounds are no longer in use, the persistence of many organic compounds in the environment has requested continuous studies to evaluating environmental quality for wildlife and humans (Barakat et al., 2002). In this investigation, several sediments showed levels of organic contaminants above the reference L1 limit set by the Decree, a situation that subsequently required a greater level of detail.

Finally, observing in its entirety, the chemical quality class of sediment samples (although there were overruns as regards the L1 reference limit) there were shown a no significant pollution conditions regarding the L2 reference levels. Only three sediments were dangerous for exceeding the L2 limit: 6/SM, 19/SM and 20/SM. Two of these have also been investigated at the molecular level (see Chapter 2).

The interpretation of ecotoxicological data was more complicated respect to chemical data. Interpretative problems were also present in the ecotoxicological tests, and they require a good knowledge of both the test and the historical situation of the sampling point, so that contradictory data are not provided and far from reality.

The *V. fischeri* test was performed on the granulometric fraction <1 mm. In this fine fraction there was present the silt (< 0.063 mm), which gives problems because the bacterium tends to adsorb on it, with a consequent drastic lack of bioluminescence. This effect was called non-specific toxicity, since the decrease of bioluminescence cannot be due to the toxicity of the sediment, but to a sediment physical factor itself. Since the solid phase test was subjected to various interferences related to the variability of the matrix, its results were normalized to the pelitic fraction of the samples and expressed as STI index (Index of Sediment Toxicity) (ICRAM, 2001; Onorati et al., 1999; Cibic et al., 2008). Because of this correction, the test with *V. fischeri* never proves to be decisive as regards the ecotoxicological result, in all sediment sample tested in this work. In a sense, the pelitic correction doesn't allow the essay to condition the battery.

The other two battery tests better conditioned the ecotoxicological result, despite some incongruences: in the assay using the marine microalgae *P. tricornutum*, exposure to the sediment elutriate does not always generate a toxic effect so that there was a decrease in growth compared to control. In the majority of the sediments, an effect of inhibition of the algal growth is noticed, but in 6 cases a biostimulation occurs, due to the presence of nutrients (Glass and Silvestrini, 1998; Harkey et al., 1994; Stimson and Larned, 2000) or other factors (e.g. physical) capable of exerting a stimulating effect on algal growth. It is called hormesis (Calabrese and Baldwin, 2001) which can lead to a masking of toxicity (Sbrilli et al., 2003). This behaviour, was known as the "matrix effect", which produces significant algal developments in the elutriates and is due to the fact that the preparation of the elutriate allows the hydrophilic nutrients to enter into solution, and to add their nutritional contribution to that of the soil of growth. In the case of these sediments (4/SM, 5/SM, 6/SM, 7/SM, 10/SM and 11/SM), exchanges at the water-sediment interface between soluble nutrients and water column would not have been able to overcome the toxic effects induced by the other substances adsorbed to sediment granules. The ISPRA Sediqualsoft® software is programmed to assign a certain score to the algal assays with biostimulation effect: a value equal to 0 if the effect is < 40%, 1.25 if the effect is > 40% but <100%, equal to 1.5 if the effect is > 100%. In our case, the weight is 0 for 5 sediments, only the 4/SM, due to a more significant biostimulation, receives a slightly higher HQe. So, the hormesis phenomenon was assigned a low weight within the test: the problem was that the toxicity could be much higher and the biostimulation would mask it, so the weight assigned to the hormesis could be upwards revised. It must be remembered that hormesis was a dose-response relationship that is characterized by low dose stimulation and high dose inhibition (Xiao-Ying and Jiang, 2013; Calabrese, 2008).

In the *Crassostrea gigas* test, the results obtained shown how the number of D-larvae malformations rate at 48 hours per sediment has a direct correspondence to the level of

contamination in each sample. The embryotoxicity test proves to be very sensitive possible affected by the amount of ammonia and sulphides possibly present in the sediment (Losso et al., 2007). Ammonia presence in the sediment is a known "confusion factor", as has been denoted in various studies carried out in the Venice area due to anthropogenic ammonia discharges (Volpi Ghirardini et al., 2005; Losso et al., 2009; Picone et al., 2009). The ISPRA software should also include a correction for *C. gigas* based on the levels of ammonia present in the sediment sample tested for the test in its calculation algorithm, as it proceeds with the pelitic correction of *V. fischeri*. As it is thought, the essay tends to give an extreme toxicity response, which can lead to a wrong negative classification, of the quality class of the sediment sample.

The set of physical, chemical and ecotoxicological data was analysed by the ISPRA software, Sediqualsoft. The integration of all these data made it possible to divide the treated sediments in this study into three of the five quality classes available: A, B and C (Fig. 12).

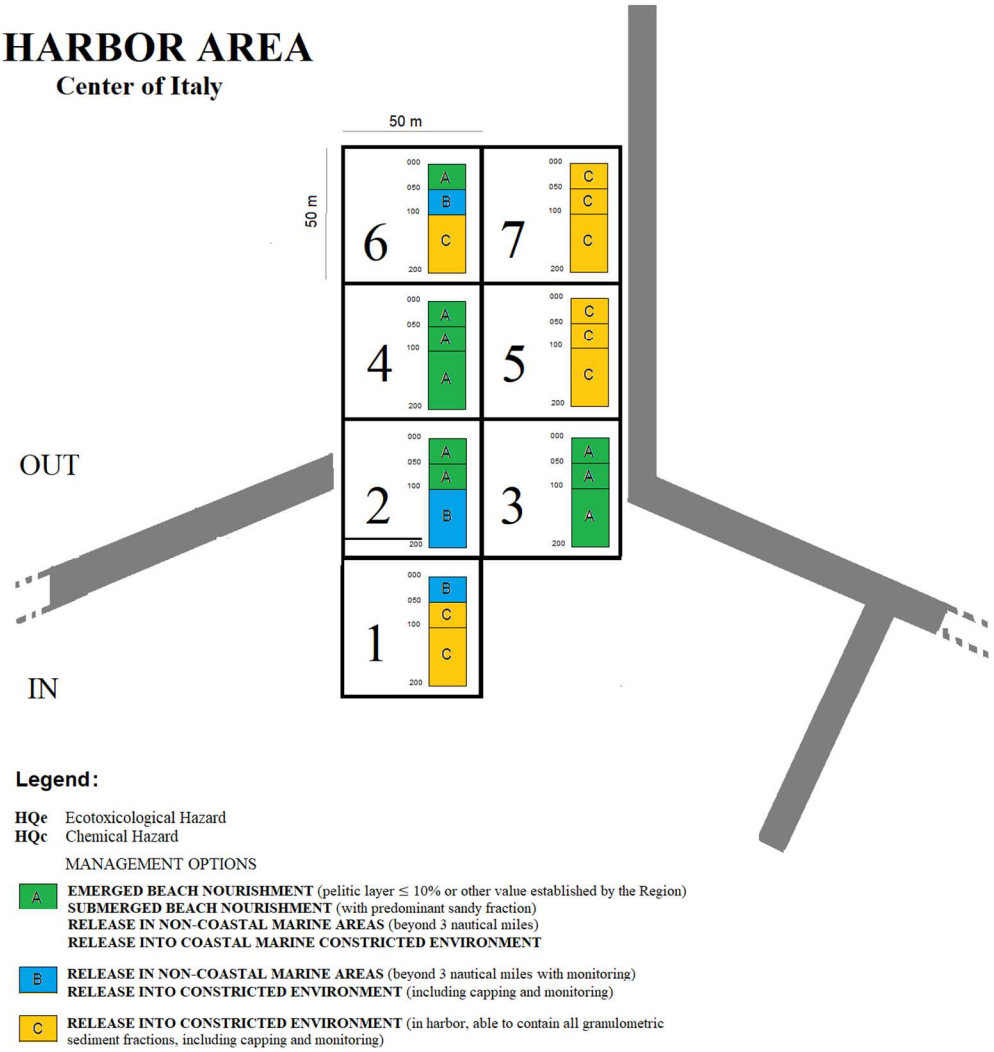


Figure 12. Quality of excavation materials with different management options.

From the simple observation of the quality classes, the results suggest that the sampling area cannot be considered an extreme polluted area, and all the sediments can be reused and on merely classified as waste. What is most carefully observed was not the final quality class assigned to each sediment, but how it was obtained: if in areas 1, 2, 3, 4 and 6 a low chemical risk is equally associated with a low ecotoxicological risk, this is not true for areas 5 and 7. In these two cases it can be observed that both areas have sediments considered as C quality level, the worst quality class

obtained in this work, but although the result is the same, not it is the way it was obtained: in fact in the area 5 the ecotoxicological data have a more impact in the final evaluation, while in the area 7 the chemical parameters deeply influenced the quality class.

At this point, if we compare the data of ecotoxicological tests with those of chemical tests and observe the two types of Hazard Quotient, it is possible to see that to high contaminant levels not necessarily corresponds a clear ecotoxicological effects, and viceversa. This strongly suggests that the approaches so far recommended by the decree should be integrated with *in vivo* biological evidences obtained analysing the direct effects of the exposure at molecular levels. Changes of nucleic acid/protein levels clearly suggest the damage occurring within the organism and can be use an early warning signal of environmental contamination.

Chapter 2
An integrated approach to evaluate sediment quality: from to chemical characterization to multispecies bioassays.

1. Introduction

Protection of ecological resources, including wildlife and natural habitat is a responsibility of National authorities. Local authorities are involved in projects to evaluate contaminant concentrations in sediment and to assess their potential associated risk. Contaminants, in fact, when concentrations exceed the natural background level for the area and for the organism, can be pollutants, and pose detrimental impact on the environment (Masindi and Muedi, 2018). They belong to a multifaceted and multispecies family of compounds of both natural or anthropogenic origin. Those of natural origin include metals from weathered rock or organic compounds such as polycyclic aromatic hydrocarbons (PAHs), which are by-products of fuel burning processes and are also naturally produced during forest fires (Abdel-Shafy and Mansour, 2016). Concerning those of anthropogenic origin, they derive from different sectors, such as industrial, agricultural, and pharmaceutical, and can be found in water bodies with considerable endocrine disruptors potency thus potentially damaging the biotic components of the environment (López-Pacheco et al., 2019).

At date, the level of pollution of marine sediments (in particular of harbour sediments) has been mainly documented in terms of contaminant chemical concentrations (His et al., 1997; Wang et al., 2019) and the effect of their toxicity on live organisms has been rarely investigated (Benedetti et al., 2012). These last authors demonstrated that the merely chemical characterization of the sediment is not sufficient to predict their toxic effects, and a more biological approach, integrating a set of biological tests, should be considered (Benedetti et al., 2012). Therefore, multidisciplinary approaches have been followed to gain knowledge on how a sediment exerts its toxic effect at biological level. In the last years, monitoring programs have been carried out in the entire Italian area following the Weight-of-Evidence approach (Burton et al., 2002; Chapman et al., 2002), based on the multidisciplinary integration of chemical, and in field - or laboratory - based ecotoxicological evidences. In this scenario, a recent progress of the regulatory framework is the Decree of the Italian Ministry of Environment, n. 173 (2016), concerning terms and technical criteria for the authorization to dump the dredged sediments in the marine environment. The decree establishes rules and methods to characterize and classify dredged sediments and provides technical requirements for their management and reuse.

On the bases of the definition of Hazard Chemical Quotient (HQc) and Hazard Ecotoxicological Quotient (HQe), the decree classifies sediments within 5 classes, each ruled by different management options. Class A and B sediments are non-polluted sediments and can be dredged and used for coastal beach nourishment (class A), or for nourishment of non-coastal marine areas (class B); class C sediments, of worst quality, can be used in specific, well confined areas, to avoid the release of contaminants in the ecosystem/harbour area; class D and E sediments, must be stored in confined water-proofed environments or should be removed from the marine environment following a risk assessment approach. Therefore, as novelty, the decree considers dredged sediments no longer as a waste, but as a real resource to be recovered and reused.

Worldwide international organizations with competence in environmental management (OSPAR Commission, International Council for the Exploration of the Sea (ICES)….) handled the results of chemical monitoring of pollution, integrated them with the sediment geological features, and as results the so called “geochemical background” (Matschullat et al., 2000) contributed in building up the marine sediment recovery strategy. It promoted the application of biological techniques to evaluate the effects of pollutants on living resources. Specifically, because of their sensitivity, ecological relevance, cost-effectiveness and rapid response, marine invertebrate embryo-larval bioassays developed as biological tools to provide important information regarding coastal monitoring approaches (Bellas et al., 2011).

The Ministery decree 173 establishes that the assessment of the sediment HQe should result from the integration of data obtained with a test battery on at least three different taxonomic organisms. The toxicity of complex matrices must therefore be evaluated using a set of bioindicators, in order to analyse the broad spectrum of effects on organisms. The strength of these analyses therefore relies in the careful selection of the assays to be performed, the key organisms to be used and the endpoints to be evaluated. In this light, to better characterize the ecotoxicological risk associated with the exposure to contaminated sediments, the marine bioluminescent bacterium *Vibrio fischeri,* for the acute toxicity test on solid phase, the marine microalga *Phaeodactylum tricornutum* for the chronic toxicity test on liquid phase and the bivalve *Crassostrea gigas,* to assess the chronic/long-term effects of the exposure, were selected as sentinel species from the decree technical annex. The rationale of the organism selection relies in the fact that toxicity is usually measured on liquid matrices (e.g. surface waters, drainage, solid matrices), which differently from solid ones, are not affected by the non-negligible interactions between the soil and the soil bioavailable toxic component (Van Leeuwen et al., 1995; Calow, 2009). On the contrary, the bioassay on the solid matrix has the advantage of using the whole matrix and not just the aqueous extract, being closer to the real situation.

Considering their trophic role, ecological relevance and sensitivity to physical and chemical environmental parameters, ecotoxicological assays using phytoplankton organisms represent one of the most valuable tool in ecotoxicological studies (Sbrilli et al., 2003; Walsh, 1988). Among the assays so far used, in this study, the chronic toxicity test on sediment elutriate using the diatom *Phaeodactylum tricornutum* was chosen. This diatom presents an excellent distribution all over the world, has a good sensitivity toward hydrophilic and hydrophobic contaminants, and above all, has an excellent adaptability and stability under laboratory controlled conditions (Chan et al., 2019; Niehus et al., 2018).

Regarding *Vibrio fischery* bioassay, the assay using this marine bacterium is a simple and inexpensive technique, often applied for the screening and ecotoxicological evaluation of a wide range of solid, liquid, gaseous substances but also complex sample (Abbas et al., 2018). Respect to those tests using algae, fish or crustaceans, it guarantees a higher sensitivity level, which combined with ease of application, reliability and reproducibility, is commonly used for the quality assessment for most of matrix.

Concerning the chronic/long-term effects of the exposure to polluted sediments, oysters, as sessile and filter-feeder organisms, were chosen since are constantly in contact with various chemical products and have been largely used so far as a marine sentinel species (Chan and Wang, 2019; Edge et al., 2012). To cope with this type of challenges, bivalve molluscs possess the two types of innate responses: cellular and humoral (Girón-Pérez, 2010). Exposure to chemical or organic pollution determines an increase of oxidative stress, in term of an increase of reactive oxygen species (ROS). ROS content in the organism can be increased by the presence of contaminants and is regulated by antioxidant enzymes, such as superoxide dismutase (*Sod*) and glutathione peroxidase (*GPx*) (Cheung et al., 2004; Verlecar et al., 2007). Other immune parameters such as lysozyme activity, may also be modulated by contaminants (Hart et al., 2016)

The main goal of the present study was to investigate the toxicity of three different "C" class sediments following a Weight of evidence approach, integrating data regarding the chemical characterization, with biological results obtained exposing sentinel species to contaminated sediments, according to the recent Italian legislation for a better management of dredged marine sediments.

2. Material and Methods

2.1 Sediment collection

Sediments were sampled at the entrance of a harbour in Central Adriatic Sea (Fig. 1), by ARPAM – Department of Ancona, according to the technical annex of the Decree 173/2016. Sampling was carried out in January 2017 in 7 different and contiguous areas, 4 replicates per area (R1 – R4) were collected and analysed. Each excavation area has a side length of 50 meters, 2 meters of excavation depth, for a total volume of 35.000 m^3 Sediment samples were taken, homogenized in order to include all granulometric fractions and aliquots were stored according to the analysis. Samples for chemical analysis were transported at 4° C, frozen to – 20°C in glass decontaminated vessels. Sample for bioassays and for granulometric analysis were stocked at 4° C.

2.2 Textural and chemical analysis

Drain size analysis were performed according to the ICRAM Methodologies (ICRAM, 2001). Samples were pre-treated with a hydrogen peroxide and distilled water solution (2:8) for 48 hours at room temperature to facilitate granule separation, and then separated using a 63 µm mesh, stewed with distilled water to obtain two fractions which were dried in a stove at 60° C and weighed. The > 63 µm fraction was checked on a set of sieves. The analytical results are expressed as a percentage (ratio between the weight of the granulometric fraction and the weight of the total sample) and are represented in tabular form by dividing the sample, according to granulometry into gravel, sand, silt and clay. Total organic matter was determined by loss of ignition at 450 °C of dried sediment for 4 hours in a muffle furnace UNI EN 15936 (2012) method.

Trace metal concentrations were measured following the Environmental Protection Agency (EPA) method 3051A-2007 (U.S. EPA 3051A, 2007) using the Ethos Easy Advanced Microwave Digestion System and applying the UNI EN ISO 11885 protocol, using an Inductively Coupled Plasma– Optical Emission Spectrometry (ICP-OES), by Agilent technologies. Mercury (Hg) was determined following the EPA Method 7471B (U.S. EPA 7471B), based on Cold Vapor-Atomic Absorption Spectrophotometry (CVAAS).

Phytopharmaceutical determination **(pesticide organochlorine)**, **polycyclic aromatic hydrocarbons (PAH)** and **polychlorinated biphenylis (PCB)** were determined following the EPA methods (U.S. EPA Method 3545A-3630C-3660B-8270D, 2007, 1996, 1996, 2014) using Varian GC 3800 gas chromatograph and Saturn 2000 mass spectrometer by Agilent. Briefly, samples were lyophilized and extracted with an Accelerate Solvent Extractor (ASE) working at high pressure. After concentration, sample was purified by columns with silica gel and finally reconstituted and analyzed by gas-chromatography.

C > 12 hydrocarbons were analyzed using the protocol UNI EN ISO 16703 (UNI EN ISO 16703, 2004). The extraction was carried out starting from 20 g of sediments in a solution of heptane/acetone with ASE. Purification in Florisil® and final reading was performed at the gas chromatograph with Flame Ionization detector (FID), model Perkin Elmer Autosistem XL.

Organostannic compounds (MBT, DBT and **TBT)** determination was performed according to (Devos et al., 2005)

Organic contaminants (DDT, DDE and **DDD)** were determined following the EPA methods 3545/3630C/3660B/8270D. Lyophilized sediments were extracted using the ASE, purified on a silica gel column. The sample was loaded and subsequently eluted with hexane. A subsequent dilution was then carried out in a hexane dichloromethane mixture, till gas-chromatographic analysis.

Dioxin like molecules (polychlorodibenzofuran (PCDF), **polychlorinated dibenzodioxins (PCDD)** and **dioxin-like PCB)** were determined following the EPA method 1613 (U.S. EPA, 1994). Lyophilized sample were extracted with toluene at ASE, dried, concentrated and pre-purified with a series of acidic and basic washing, followed by a purification with FMES Dioxine Power Prep equipment and analysed by high-resolution Triple Quadrupole GC-MS (TSQ spectrometer 8000 Evo from Thermo Scientific, and GC Trace 1310).

Phthalates were determined following the ISO method (UNI EN ISO 18856, 2006) using Gas Chromatograph Trace 1310 and TSQ 8000 EVO for the mass spectrometry (Thermo Scientific). For di(2-ethylhexyl) phthalate (DEHP) determination, water samples were filtered using silica gel columns (SPE C18). 100 µl of standard DEHP-d4 to the concentration of 1 ppm (ex. 1 mg/L) was added. Samples were analysed by GC-MS.

2.3 Bioassays

The preparation of **sediment elutriates** was performed in accordance with the ASTM E-1391-03 Guidelines (ASTM, 2014). Briefly, elutriates were prepared by combining different ratio of water and sediment (4:1 ratio) and mixing for 1 h by magnetic agitation. Filtration of the supernatant through cellulose acetate 0.45 µm filters (for microalgae bioassay) and in mixed esters of cellulose (for oyster test) was performed.

2.3.1 Phaedocatylum tricornutum bioassay (acute toxicity)

The toxicity test of elutriate with the microalgae ***Phaeodactylum tricornutum*** was performed as described in the ISO method (UNI EN ISO 10253) and according to (Libralato et al., 2011; Surricchio et al., 2019). *P. tricornutum*, strain number CCAP 1052/1A was provided by the Scottish Marine Institute, Oban, Argyll, UK.

For the bioassay, *P.tricornutum* initial cell density shall not exceed 10^4 cells/ml, to allow exponential growth in the control culture throughout the experiment duration. Algal inocula, in triplicate, were inoculated into each well of a multiwell plate. Control is represented by the algae reared in sea water, without the addition of elutriate, while the effects of sediment was assayed incubating the algae with the selected elutriates.

The experiment was carried out in a ventilated thermostatic chamber for 72 hours at 20 ° C ± 2, with continuous lighting regime (10.000 lux). At the end of the established period, algal growth was determined by using the Bechman coulter Z2 electronic particle counter, particle count and size analyzer. Optimal growth rate was reached after 72 hours incubation period when algal growth in each control replicate is at least 16 times higher than the initial concentration. The coefficient of variation among control replicates must not exceed the 10%.

2.3.2 Vibrio fischeri bioassay (solid phase, acute toxicity)

Microtox® microbial luminescence bioassay was performed on solid-phase (< 1 mm sediment fraction) according to the ISO Method (UNI EN ISO 11348-3, 2009) and following (Adams et al., 2015; Froehner et al., 2000) using the marine luminescent bacteria ***Vibrio fischeri.*** Selected sediments were diluted in 0.2 µm filtered synthetic seawater (dilution medium). Dilutions series were made with factor = 2 in special SPT tubes and let settle at 15 ± 2 ° C for 20 minutes. The supernatant was decanted and 20 µL of Microtox® reagent containing the bacteria (Acute Reagent, Modern Water for Microtox® with Reconstitution Solution at final salinity of 20‰) was added. After 20 minutes the bacterial suspension is separated from the sediment particles. Bioluminescence is measured 30 minutes after separation. Controls are represented by luminescent bacteria suspended in the dilution medium. Bacterial light intensity (I_{30}) was measured by a Microtox Modern Water M500 Analyzer luminometer and the dedicated Microtox® Omni software. The toxicity derived from concentration-effect curves using linear regression is expressed as EC_{50} (concentration that produces 50% of the effect in the population). Similarly, to other toxicity parameters, the lower the value, the more toxic it will be. To convert this into a positive indicator, where a higher value indicates greater toxicity, the EC50 values were converted to toxicity units (TU). The conversion was obtained with the following: $TU = (1/EC_{50}) \times 100$. In addition, TU value

must undergo a pelitic correction since *V. fischeri* tends to be adsorbed by the silty sediment particles and the decrease of the bioluminescence can be caused by the bacterium entrapment into the silt. On the basis of the natural toxicity of the sediment, considering the percentage of pelite, a convention rate should be proposed by applying the formula Y = 0.28 + 3.49 X (where Y is the estimate of natural toxicity and X is the percentage of pelite). The conversion between observed and estimated TU, generate the Sediment Toxicity Index - STI (Icram, 2000). ($0 \leq STI \leq 1$ Absent; $1 < STI \leq 3$ Slight; $3 < STI \leq 6$ Moderate; $6 < STI \leq 12$ Major; > 12 Severe).

2.3.3 Crassostrea gigas bioassay

The toxicity test with the oyster ***Crassostrea gigas*** was based on the EPA protocol EPA/600/R-95-136 (U.S. EPA, 1995) and modified according to laboratory conditions. Sexual mature oysters were purchased at Guernsey Sea Farms (Guernsey Sea Farms Ltd. Parc Lane, Vale. Guernsey) and were induced to spawning by alternating 30' of 18° and 32° C baths. Sperm was sieved through a 32 µm mesh to remove possible particulate matter, while oocytes where was sieved through a 100 µm mesh. Before fertilization, small aliquote of male and female gametes were checked for quality.

Fertilization was set up diluting approximately 5,000-8,000 eggs/mL in a final volume of 500 ml. Once fertilization occurred, as stated by the appearance of the membrane fertilization, 200 embryos (20 to 50 µl of embryo suspension) were reared in 10 ml of elutriates sediments, four replicates for each sample were set up, in a multiwell plate. The multiwell plates with the sample were incubated at 20 ± 2 °C for 48 hours, under 100 candle - 1076 lux - light conditions, with a photoperiod of 18L/6D. Morphological abnormalities was checked in larvae at 48 HPF after fixation in 37% PFA under inverted microscope. Before the beginning of the bioassays, O_2, pH and salinity were measured.

2.3.4 Oyster exposure based on C. gigas EPA protocol for bioassay test

Approximately one million of embryos were transferred in 100 ml solution, made of 50 ml filtered natural sea water and 50 ml of the sediment 18/SM, 19/SM and 20/SM elutriates (1:1 ratio). Control is represented by embryos reared in 100 ml of filtered natural sea water. Developing embryos were sampled at 5 hpf (blastula) and 18 hpf (trocophora), centrifuged at 3000 RPM for 5 minutes, the supernatant was gently collected and thrown, and pelleted embryos were stored in RNA Later solution.

2.4 RNA extraction and cDNA synthesis.

Total RNA was extracted from oyster embryos using RNAzol solution (Sigma Aldrich, Milan, Italy). RNA concentrations were determined with a NanoDrop™ 1000 Spectrophotometer (NanoPhotometerTM P-Class, IMPLEN, Germany). RNA quality was assessed with 1% agarose gel electrophoresis. one microgram of total RNA was used for cDNA synthesis using iScript cDNA Synthesis Kit (Bio-Rad, Milano, Italy) and stored at 20°C.

2.5 Real-time PCR.

qRT-PCRs were performed with SYBR green in a CFX thermal cycler (Bio-Rad) as described in (Carnevali et al., 2019). Ubiquitin-60S ribosomal protein L40 (rpl40) and elongation factor1a (ef1α) were used as housekeeping genes in order to standardize the results and eliminate variation in mRNA and cDNA quantity and quality. Primer sequences, GenBank accession numbers and annealing temperature for the genes analysed are reported in Table 1. No amplification products were observed in negative controls and no primer-dimer formations were observed in the control

templates Primers used at a final concentration of 10 pmoL/mL. Data were analyzed using iQ5 Optical System version 2.1 (Bio-Rad) including Genex Macro iQ5 Conversion and Genex Macro iQ5 files. Modification of gene expression among the experimental groups are reported as relative mRNA abundance (Arbitrary Units). Primers used at a final concentration of 10 pmoL/mL (Tab. 1).

Gene	For Sequence 5'-3'	Rew Sequence 5'-3'	T M	Accession number	Source
vasa	CATACCACCCTTCACCCATC	ACGTCAGTGCAAGCACCA	60	AY423380.1	(Fabiou x et al., 2004)
defensin *(defh 1)*	TTACATTGGTCGTTCTCCTGATGG	ATGGATTGGCAGTGACTGTT	60	DQ 400101	(Hart et al., 2016)
Lysozyme	CAACTGTGTCAGAGCCTACAT	CCATTGTGGATTCGTGCATAAC	60	AB179775	(Hart et al., 2016)
galectin	GGGTTACTCTCCAGGCAATTC	TGGAAGGCAATATCTCCACTTT	60	AB308370	(Hart et al., 2016)
Transglutaminase *(Tg)*	GTTCAGTGGGTGGTGAAGAA	CATCCTCTTGGTGCTGATGTAG	60	BQ426592	(Hart et al., 2016)
Heat shock protein 70 *(hsp70)*	AGCAAGCCAGCACAGCA	GCGATGATTTCCACCTTC	57	AJ305315	(Farcy et al., 2007)
Glutathione peroxidase *(gpx)*	GAC CGT GGA ACC AATGGA CAT C	GTT GGA TTCGGA CAC AGA TAG GG	57	EF692639	(Jo et al., 2008)
Superoxide dismutase *(sod)*	AACCCCTTCAACAAAGAGCA	TTTGGCGACACCGTCTTC	55	AJ496219	(Farcy et al., 2007)
Elongation factor 1 alfa *(ef1a)*	GAGCGTGAACGTGGTATCAC	ACAGCACAGTCAGCCTGTGA	60	EKC330663	(Hart et al., 2016)
Ubiquitin-60S ribosomal protein L40 *(rpl40)*	AATCTTGCACCGTCATGCAG	AATCAATCTCTGCTGATCTGG	60	AJ563473	(Hart et al., 2016)

Table 1 Primers list

2.6 Statistical Analysis

2.6.1 Ecotoxicological bioassays

Regarding the expression of the results: in the bioassay from the reduction of the bioluminescence of *Vibrio fischeri* to 30 minutes, the results were expressed as a % of effect respect to the control, and the statistical treatment of the data was carried out directly by the software that manages the instrument (Microtox Omni Windows Software v. 1.19 by Azur Environmental). For the algal test and the embryotoxicity test, the% inhibition compared to the control is considered. According to the following formula:

$$I_{\mu i} = \frac{\overline{\mu_c} - \mu_i}{\overline{\mu_c}} \times 100$$

Where:

$I_{\mu i}$ is the percentage inhibition for each test

μi is the value (e.g. the growth rate) for each test replica

$\overline{\mu_c}$ is the mean value for the control

For marine microalgae, based on the reference methods, values that deviate from the control by more than 20% were considered different from this one. Therefore, samples with an inhibition rate ≥ 20% were considered toxic and ≥ 25% for the oyster bioassay. For samples considered toxic respect to the control, analysis of variance (one way ANOVA) was used to test the significance of the effects caused by exposure to various elutriates sediment (level of significance $P < 0.05$).

2.6.2 Real Time PCR

Real Time PCR data were analyzed by Two-Way ANOVA followed by the Tukey test as a multiple comparisons test to compare experimental groups (male and female during reproductive and non-reproductive season). All statistical analyses were performed using the statistical software package Prism5 (GraphPad Software, Inc. USA) with significance accepted at $P < 0.05$.

2.6.3 *Sediqualsoft® Software*

For the quality class of the sediment samples, *Sediqualsoft® Software* was used. The main novelty of the D.M. 173/2016 consists of the integration of chemical exposure data with the effect data, therefore the assessment of the sediment quality derives from integrating the results of the ecotoxicological and chemical analyzes.

A special software, SediqualSoft 109.0®, developed by the Institute for Environmental Protection and Research (ISPRA) and by the Polytechnic University of Marche (Ancona), implements the methodology for the classification of the quality of marine and brackish sediments as foreseen by the DM 173/2016. This software is organized in 3 modules: the first two are related to ecotoxicological characterization and chemical characterization, the third to their integration and to the sediment quality classification. The ecotoxicological classification is based on the performance of a battery of biological assays whose results are integrated and lead to the formulation of the toxicity judgment in accordance with the scheme of Figure 1.

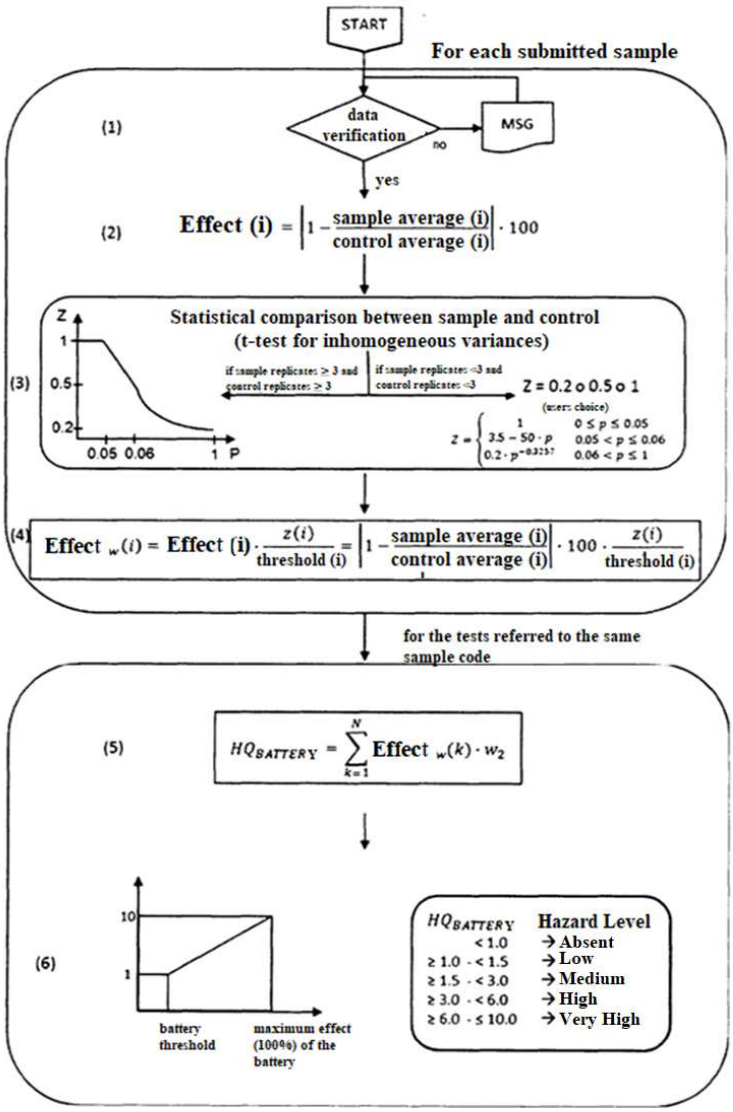


Figure 1 Procedure for processing data on ecotoxicological tests. The figure reproduces the scheme shown in D.M. 173/2016.

The attribution of the danger level is based on the overall danger index of the ecotoxicological assay battery (Hazard Quotient, $HQ_{battery}$): Absent if $HQ_{Battery} < 1$; Low if $HQ_{Battery} \geq 1$ and < 1.5; Medium if $HQ_{Battery} \geq 1.5$ and < 3.0; High if $HQ_{Battery} \geq 3.0$ and < 6.0; Very High if $HQ_{Battery} \geq 6.0$.

The chemical classification is based on the comparison of the concentrations measured in the sediment with the reference concentrations L1 and L2 (present in the D.M. 173). The values of L1 correspond to those of the environmental quality standards (EQS) envisaged for sediment by the water framework directive and by national transposing regulations (Ministerial Decree 260/2010 - Table 1). The L2 values correspond to a quality level lower than L1 but still corresponding to an acceptable ecological risk. The procedure for estimating the level of chemical danger (Hazard Quotient, HQC), from absent to very high, shown in Figure 2, is based on the calculation of the variation with respect to the limit, or the Ratio-To-Reference (RTR), with the RTR subsequently corrected according to the weight of the contaminant which takes into account the importance of the observed variations for the most dangerous contaminants. The HQ_C chemical index is assigned to a hazard class: Absent if $HQ_C < 0.7$; Negligible if $0.7 \geq HQ_C < 1.3$; Low if $1.3 \geq HQC < 2.6$; Medium if $2.6 \geq HQ_C < 6.5$; High if $6.5 \geq HQ_C < 13.0$; Very High if $HQ_C \geq 13.0$.

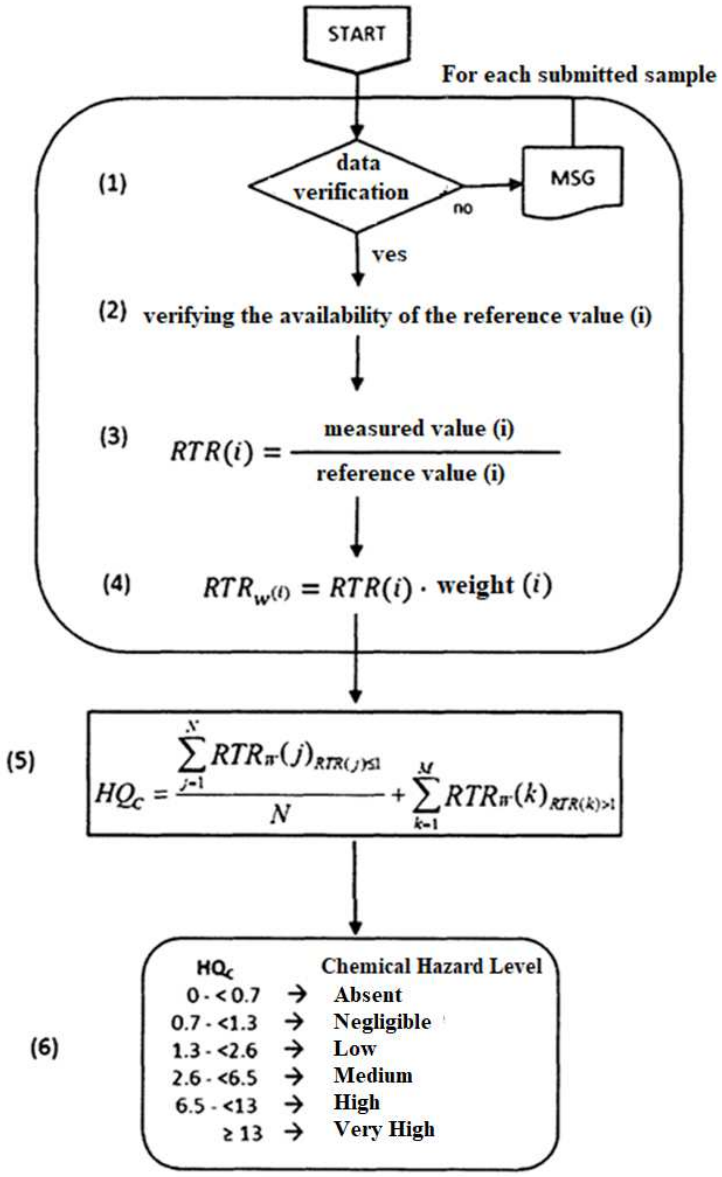


Figure 2 Procedure for processing data on chemical tests. The figure reproduces the scheme shown in D.M. 173/2016.

Finally, the ecotoxicological and chemical classifications are integrated to attribute the quality class of the excavation sediments. This classification is based on the weighted integration criteria based on the ecotoxicological and chemical HQs reported in Table 2. There are 5 sediment quality classes from A to E, with their respective management options: environmental quality sediments are included in class A higher while in subsequent classes the quality decreases, as well as the management options that are gradually more limited. In the worst class, E, a possible safety removal from the marine environment after planned a risk assessment.

Ecotoxicological hazard class developed for the entire HQ Battery	**Chemical classification**	**Material quality class**
Absent	HQc (L2) ≤ Negligible	A
	Slight ≤ HQc (L2) ≤ Medium	B
	HQc (L2) = High	C
	HQc (L2) > High	D

Low	HQc (L1) ≤ Low	A
	HQc (L1) ≤ Medium and HQc (L2) ≤ Low	B
	Moderate ≤ HQc (L2) ≤ High	C
	HQc (L2) > High	D
Medium	HQc (L2) ≤ Low	C
	HQc (L2) ≥ Medium	D
≥ High	HQc (L2) ≤ Low	D
	HQc (L2) ≥ Medium	E
$HQ_{Battery}$: Ecotoxicological Hazard Quotient HQ_c : Chemical Hazard Quotient		

Table 2 Sediment quality classification according to weighted integration criteria.

3. Results

3.1 Sediment granulometry and environmental variables

As shown in Table 3, in all sediments sand fraction ranges from 0.5 to 0.063 mm with the silt and clay fraction also present. Sediment 19/SM is characterized by the highest pelite content. In addition, in this sample, a higher humidity rate and a lower sand content is present, resulting the sediment surface an excellent absorption area for most of contaminants. Sediment 18/SM and 20 SM share a similar humidity rate, as well as the granulometric sand content.

Geological characterization of the samples (%, w/w)									
Sediment Sample	**Humidity**	**Gravel 19-2 mm**	**Sand 2-1 mm**	**Sand 1-0.5 mm**	**Sand 0.5-0.25 mm**	**Sand 0.25-0.125 mm**	**Sand 0.125-0.063 mm**	**Total Sand**	**Silt and clay, or total pelite < 0.063 mm**
18/SM	20.30	0.00	0.00	0.00	2.69	76.14	19.00	97.83	2.17
19/SM	30.80	0.00	0.00	0.00	0.85	24.12	18.86	43.83	56.17
20/SM	23.30	0.00	0.00	0.00	5.72	79.56	8.80	94.08	5.92

Table 3 Physical characterization of sediment samples (%, w/w)

3.2 Chemical analysis

As shown in Table 4, metal content in all sediments results significantly lower than the limits set by the Decree. As commonly observed in all harbor sediments, Aluminum and Vanadium content is extremely abundant, even though the decree does not set a toxicity detection limit.

Chemical characterization of sediment samples - Trace Element										
	Metals in the sediment (mg/kg d. w.)									
Sediment Sample	**Arsenic**	**Cadmium**	**Chrome**	**Copper**	**Nickel**	**Lead**	**Zinc**	**Mercury**	**Aluminium**	**Vanadium**
18/SM	4.50	0.15	7.18	2.70	5.24	2.45	11.19	0.04	1681	6.50
19/SM	6.60	0.17	21.15	18.37	17.32	8.46	53.52	0.06	7878	19.49
20/SM	5.18	0.14	7.66	5.35	6.78	3.26	13.57	0.04	1893	7.47
L1 Law DM 173/2016	12	0.3	50	40	30	30	100	0.3	/	/
L2 Law DM 173/2016	20	0.80	150	52	75	70	150	0.80	/	/

Table 4. Chemical characterization of sediment samples

Concerning organic contaminants, PCB, DDD, DDT, DDE and organostannic compounds have been measured in the selected sediments. Details regarding congeners and isomers have been provided in footnotes of Table 5. In sediment 19/SM, PCB, DDT and organostannic compound levels exceed the detection limit L1 but are still significantly below the detection limit L2. In sediment 18/SM and 20/SM, DDT level exceeds the L1 limit.

Chemical Characterization of sediment samples - Organic Contaminants					
Sediment Sample	Organic Contaminants in the sediment (µg/kg d. w.)				
	Σ PCB[(3)]	Σ DDD[(4)]	Σ DDT[(4)]	Σ DDE[(4)]	Organostannic compounds
18/SM	0.2144	0.6	2.2	0.6	2.3
19/SM	14.2655	0.6	2.1	0.7	10.26
20/SM	0.3468	0.6	2.1	0.7	4.06
L1 Law DM 173/2016	8	0.8	1.8	1.0	5[(1)]
L2 Law DM 173/2016	60	7.8	3.7	4.8	72[(2)]
[(1)] referring only to TBT; [(2)] referred to the summation of MBT, DBT, TBT; [(3)] as the sum of the follower's congeners: 28, 52, 77, 81, 101, 118, 126, 128, 138, 153, 156, 169, 180; [(4)] as a summation of isomers: 2,4 and 4,4. In grey values between L1 and L2 limits.					

Table 5. Chemical characterization of sediment – Organic Contaminants

Concerning PAH content (Table 6), in sediment 18/SM, all forms detected have a concentration below the detection limit L1. In sediment 19/SM, all PAHs measured, except for naphthalene and chrysene exceeded the L1 detection limit and only benzo(a)pyrene exceeded L2 limit. Sediment 20/SM presented Benzo(a)pyrene, Indenopyrene and Benzo(g,h,i)perylene levels exceeding the L2. Except for Naphthalene, all other PAH, exceed the limit L1. Acenaphthylene, Acenaphthene, Benzo(e)pyrene, Dibenz(a,h)anthracene have been detected and quantified in all sediments, but since are not prioritized by the Decree, detection limits are not provided.

Chemical characterization of sediment																			
Sediment Sample	PAHs in the sediment (µg/kg d. w.)																		
	Naphthalene	**Acenaphthylene**	**Acenaphthene**	**Fluorene**	**Phenanthrene**	**Anthracene**	**Fluoranthene**	**Pyrene**	**Benz(a)anthracene**	**Crysene**	**Benzo(b)fluoranthene**	**Benzo(k)fluoranthene**	**Benzo(e)pyrene**	**Benzo(a)pyrene**	**Perylene**	**Indenopyrene**	**Dibenz(a,h)anthracene**	**Benzo(g,h,i)perylene**	**Σ PAH[1]**
18/SM	1.94	0.25	6.12	6.49	39.41	2.49	19.05	52.25	8.65	8.28	2.28	1.11	2.04	2.24	0.82	8.51	0.25	17.73	177.05
19/SM	0.25	22.09	12.48	33.52	94.35	47.07	220.50	215.30	100.10	85.63	107.30	55.83	83.49	119.60	101.10	86.13	22.30	72.16	1294.61
20/SM	0.25	110.60	14.16	48.29	378.70	83.06	428.00	402.70	175.90	154.20	193.30	105.50	145.80	229.80	68.35	111.40	24.03	108.10	2567.99
L1 DM 173/2016	35	/	/	21	87	24	110	153	75	108	40	20	/	30	/	70	/	55	900
L2 DM 173/2016	391	/	/	144	544	245	1494	1398	500	846	500[2]	500[2]	/	100	/	100[(2)]	/	100[(2)]	4000

[(1)] as the sum of the 16 major environmental PAHs indicated by the USEPA (Acenaphtylene, Benz(a)anthracene, Fluoranthene, Naphthalene, Anthracene, Benzo(a)pyrene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(g,h,i)perylene, Acenaphthene, Fluorene, Phenanthrene, Pyrene, Dibenz(a,h)anthracene, Crysene, Indeno(1,2,3,c-d)pyrene;

[(2)] valid concentration only for emerged nourishment activities.

In green values under L1 limits, in yellow values between L1 and L2 limits, in red values higher than L2 limits.

Table 6. Chemical characterization of sediment samples.

Regarding the related toxicity equivalent factors T.E. of PCDDs, PCDFs (Dioxins and Furans) and PCB dioxin like, concentrations are well below the limit values; in sediment 19/SM, PCDD, PCDF (Dioxins and Furans) and PCB dioxin-like (µg/kg d. w.) were measured and while the former (PCDF+PCB), did not exceed the L1, the latter did not exceed the L2 (Table 7).

Chemical characterization of sediment		
Sediment Sample	**Σ T.E. PCDD, PCDF(1) (Dioxins and Furans) and PCB dioxin-like (µg/kg d. w.)**	
	Sum of PCDD, PCDF and PCB	**Sum of PCDD and PCDF**
18/SM	/	/
19/SM	0.00031	0.00016
20/SM	/	/
L1 DM 173/2016	0.002	-
L2 DM 173/2016	-	0.01(2)
(1) the list of congeners and related toxicity equivalent factors T.E. (EPA, 1989) and the list of similar dioxin PCB congeners (WHO, 2005) and the one reported in the notes of table 3/A pursuant to Legislative Decree 172/2015; (2) relative to the summation of PCDD and PCDF		

Table 7. Chemical characterization of sediment- PCDD, PCDF and PCB dioxin-like

Concerning phthalates, as shown in Table 8, DEHP value detected are in the same range of those measured in the internal waters of the Marche Region (ARPAM data for the year 2017 range from 0.110 µg/L to 1.018 in rivers, and from <0,01 to 0.08 µg/L in lakes; for the year 2018 range from 0.016 µg/L to 0.720 in rivers, and from 0.016 to 0.703 µg/L in lakes).

Chemical characterization of sediment	
Sediment Sample	Calculated Concentration Units in the elutriate (µg/L)
	Di(2-ethylhexyl) phthalate (DEHP)
18/SM	0.091
19/SM	0.236
20/SM	0.109
SQA-MA(1) in internal surface waters and in other surface waters	1.3(*)
SQA-CMA(2)	Not applicable (3)
(*) According to the D. Lgs. 172 del 13/10/2015; (1) SQA-MA: environmental quality standards expressed as an average annual value; (2) SQA-CMA: environmental quality standards expressed as maximum admissible concentration; (3) it is believed that the SQA-MA values protect from short-term pollution peaks, in continuous discharges, because they are significantly lower than the values derived on the basis of acute toxicity.	

Table 8. Chemical characterization of sediment Di(2-ethylhexyl) phthalate (DEHP).

3.3 Ecotoxicological tests

3.3.1 Vibrio fischeri bioassay

Based on this assay, both sediment 18/SM and 19/SM lack of toxicity, being the STI ranged between 0 and 1. In sample 20/SM, STI value suggests a slight toxicity. The EC_{50} of the test was calculated on 7 dilutions and the dose/response curves of the samples tested are shown below (Fig. 3) numerical data are shown in Table 9.

Ecotoxicological values of the *V. fischeri* bioassay					
Sediment Sample(*)	**EC_{50} Concentration (%)**	**R^2 coeff of determination**	**TU**	**Y**	**S.T.I.**
18/SM	14.86	0.9286	6.73	7.85	0.86
19/SM	1.162	0.9922	86.05	196.31	0.44
20/SM	2.59	0.9835	38.61	20.94	1.84
Legend: **TU**: toxic units (observed toxicity) **Y**: estimated natural toxicity [y = 0.28 + 3.49(x)] where x is pelitic % **STI**: Sediment Toxicity Index (*) Since a control sediment cannot be used, in addition to dilutions, two controls with the diluent solution are used for each sediment.					

Table 9. Ecotoxicological values of the *V. fischeri* bioassay

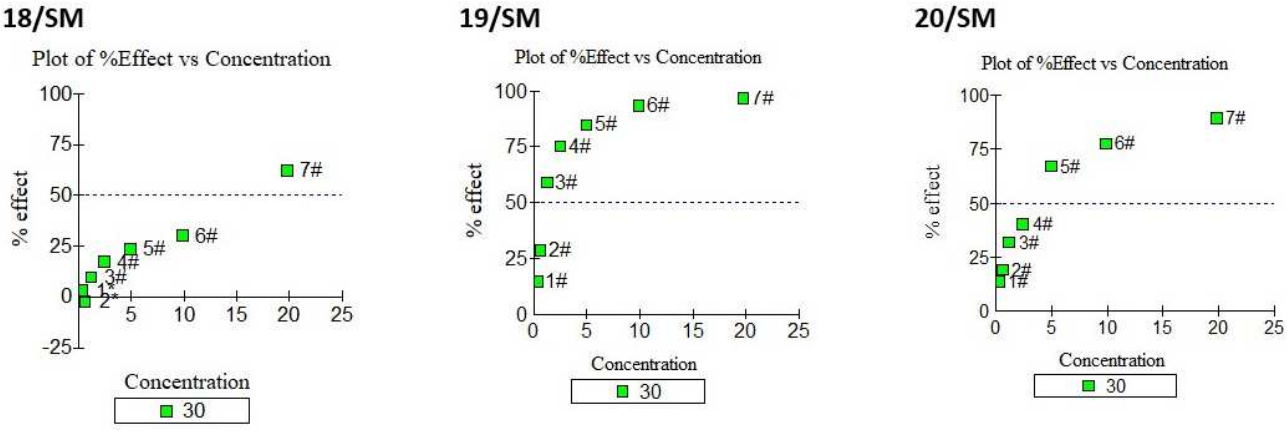


Figure 3. *Vibrio fischeri* bioassay results for the three samples.

3.3.2 Phaeodactylum tricornutum bioassay

As result of the *Phaeodactylum tricornutum* assay, the three samples inhibited the growth of the microalgae respect to the control (see Table 10). In particular, the sediment 19/SM has an inhibition capacity of 56% respect to the control. A similar score (51%) was measured for the sample 18/SM. Sediment 20/SM caused a 33% of growth inhibition.

Ecotoxicological values of the *P. tricornutum* essay						
Sediment Sample	**R1 (cell/ml) x 1000**	**R2**	**R3**	**R average**	**% inhibition***	**DS**
C	1017	1055	1069	1047	/	27
18/SM	418	558	550	509	51	79
19/SM	390	490	493	458	56	59
20/SM	667	732	715	705	33	34
* compared to the control test value						

Table 10. Ecotoxicological values of the *P. tricornutum* essay

3.3.2 Crassostrea gigas bioassay

In the test with *Crassostrea gigas*, the three elutriate exposure significantly interfered with the normal embryonic development. The 19/SM sediment exposure induced the greatest percentage of malformed embryos respect to the control, followed by the sample 20/SM and finally by the 18/SM, which caused only a 5% effect. These results fit with the chemical results, being the sediment 18/SM the less contaminated, and the 19 SM the worst quality one. During these tests, the physical and chemical parameters (O_2, pH and salinity) remained constant (data not shown).

Ecotoxicological values of the *C. gigas* essay							
Sediment Sample	**R1 (% normoformed embryos)**	**R2 (% normoformed embryos)**	**R3 (% normoformed embryos)**	**R4 (% normoformed embryos)**	**R average**	**% Effect***	**DS**
C	96	90	86	93	91	/	4
18/SM	85	93	87	81	87	5	5
19/SM	56	71	60	58	61	33	7
20/SM	61	58	74	78	68	26	10
* compared to the control test							

Table 11. Ecotoxicological values of the *C. gigas* essay

3.4 Statistical Analyses with Sediqualsoft® Software

The all three sediments fall into the C quality class (for which release is expected in sediment tank within the ports). Although classified within the same quality class, they have different chemical and ecotoxicological risk hazard quotient: sediment 18/SM has a medium ecotoxicological risk and a low chemical risk. On the contrary, the sediment 20/SM has a high chemical risk but a low ecotoxicological risk index. The 19/SM presents a high chemical risk associated to an average ecotoxicological one (Table 12).

Sediqualsoft® results									
Sediment Sample	**Ecotoxicology**				**Chemistry**				
	Test	HQe Specific	HQe Battery test	HQe	HQc L1	HQc L2	Chemical danger L1	Chemical danger L2	Sediment quality class
18/SM	*C. gigas*	0.06	1.86	Medium	3.034	0.074	Medium	Absent	C
	P. tricornutum	5.29							
	V. fischeri	0							
19/SM	*C. gigas*	2.05	2.97	Medium	33.005	1.736	Very High	Low	C
	P. tricornutum	5.79							
	V. fischeri	0							
20/SM	*C. gigas*	0.29	1.19	Low	56.175	3.187	Very High	Medium	C
	P. tricornutum	3.36							
	V. fischeri	0.17							

Table 12. Sediqualsoft® results

3.5 Molecular Analyses

For the molecular analyses were analysed the expression of a set of biomarkers of germ cell differentiation and in immune response.

Defensin (Dehf-1) mRNA levels significantly increase only in oysters exposed to 19/SM sediment at both time points, respect to controls. The exposure to 18/SM and 20/SM sediments seems to induce a slight, but not statistically significant, decrease of mRNA levels respect to control at both time points (Fig. 4A).

Regarding *galectin*, at 5 hrs, exposure to sediments did not induce significant variation of mRNA levels respect to control. At 18 HPF only the exposure to samples 19/SM and 20/SM significantly upregulated mRNA levels respect to control oysters as development proceeds mRNA levels significantly increase in oysters exposed to 19/SM or 20/SM sediments (Fig. 4B).

Concerning *Lysozyme* mRNA levels (Fig. 4C), lack of significant differences was detected among experimental groups at 5 hrs. On the contrary, differences were observed among experimental groups at 18 hrs. The exposure to all sediments induced a similar decrease of mRNA levels respect to control oysters. As development proceeds, from 5 to 18 hrs, an increase of mRNA expression was observed in oysters exposed to 19/SM and 20/SM sediments.

Transglutaminase (Tg) mRNA levels were significantly decrease in oysters exposed to all sediments at 5 hrs. A similar decrease, respect to control, was also observed in specimens at 18 hrs (Fig. 4D). In control oysters, Tg mRNA levels increase with development. An increase of mRNA levels was also induced in oysters exposed to sediment 19 SM and 20 SM. On the contrary, the exposure to 18 SM sediment did not induce changes of mRNA levels at the two time points analysed (Fig. 4D).

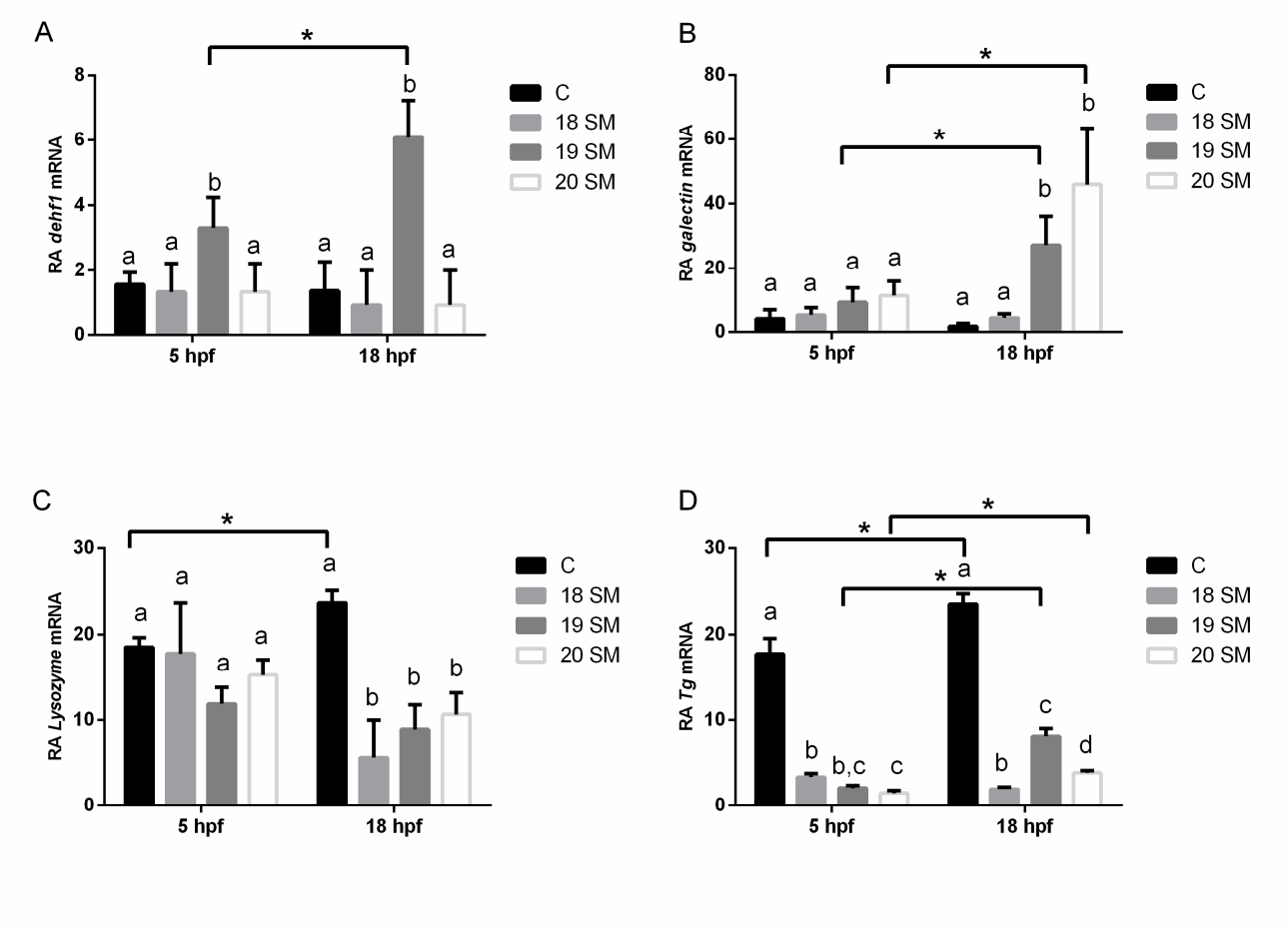


Figure 4. Dehf1, galectin, Lysozyme and Tg mRNA relative abundance (RA). Different letters above each column denote significant differences among the experimental at 5 or 18 hrs. Asterisks (*) denote statistically significant differences between oysters at 5 and 18 hrs, exposed to the same sediment

Hsp70 mRNA was significantly increased only in embryos exposed to 19 SM sediment respect to control. At 18 hpf the expression increases in oysters exposed to 18 SM and 19 SM sediments respect to control oysters and this increase was also significantly higher than that measured at 5 hpf. Sediment 20 SM did not induce any significant changes neither at 5 nor 18 hpf (Fig. 5A).

Concerning *GPx* mRNA abundance, at 5 hrs all the sediments are able to induce a significant increase of the mRNA abundance, but the true upregulation occurs at 18 hours above all for the sample 19/SM which shows itself as the greatest increase (Fig. 5B). A similar trend was observed for *Sod* mRNA relative abundance, with a visible effect for all three sediments already at 5 hours of exposure (Fig. 5C), and a markedly higher increase in oysters at 18 hpf exposed to the 19/SM elutriate (Fig. 5C).

In control oysters, *vasa*, as biomarker expressed in early migratory germ cells, peaks at 5 hrs with the expression decreasing, even though not significantly at 18 hrs. A decrease of the expression was observed after the exposure to sediments 18/SM and 19/SM, despite statistically significant with the 19/SM one. A slight not significant decrease was also observed with the sediment 20/SM. At 18 hrs, no significant differences were detected among experimental groups. Only the exposure to sediment 19/SM induced a significant mRNA increase between 5 hrs and 18 hrs developing oysters (Fig. 5D).

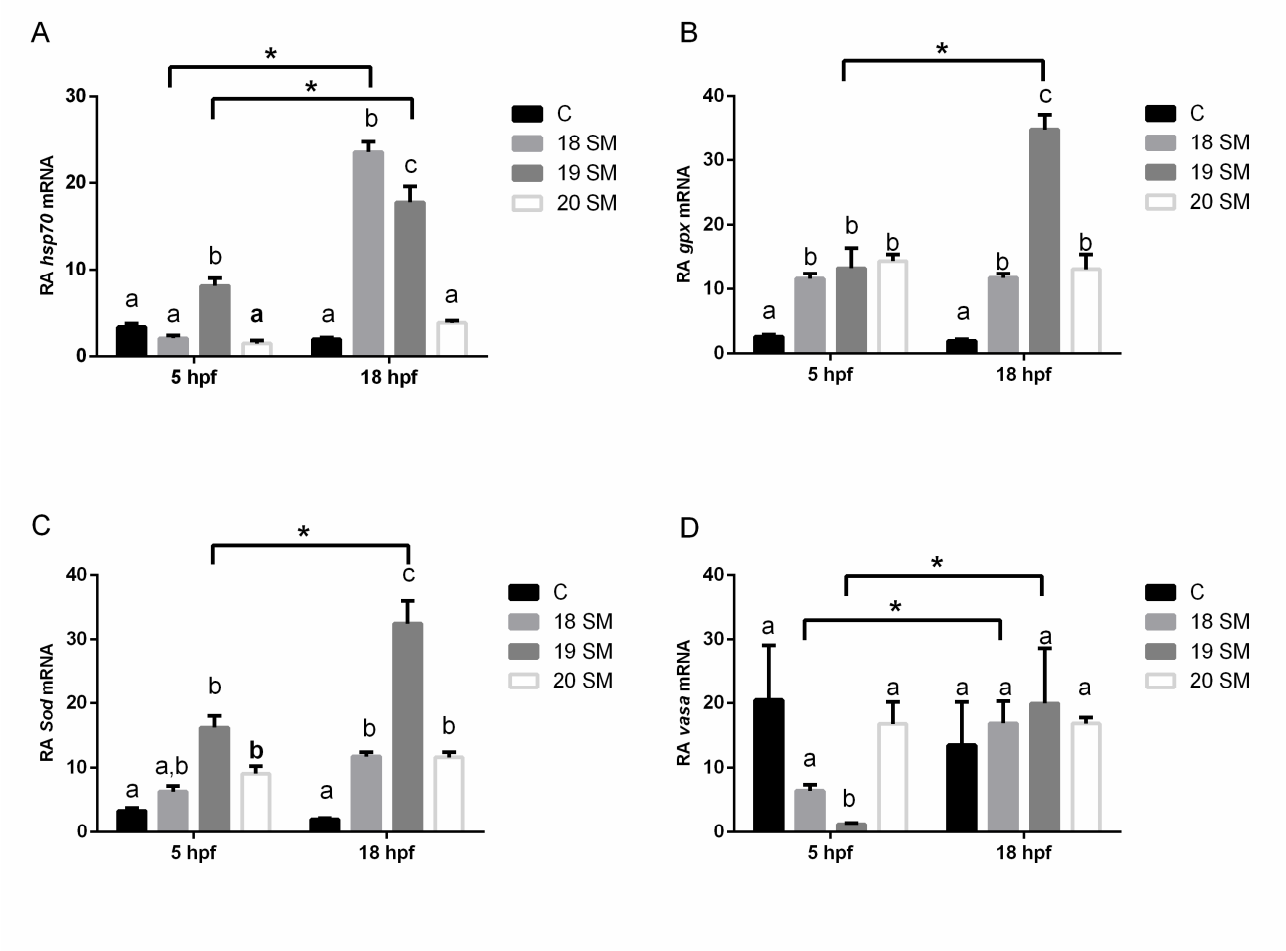


Figure 5. hsp70, gpx, Sod and vasa mRNA relative abundance (RA). Different letters above each column denote significant differences among the experimental at 5 or 18 hrs. Asterisks (*) denote statistically significant differences between oysters at 5 and 18 hrs, exposed to the same sediment.

4. Discussion

The application of DM 173 follows a weight of evidence approach (WOE) and has been adopted after several years of results based on different LOEs. WOE methods combine different approaches (chemicals, ecotoxicology's and physicals parameters) to define an ecological risk assessment (Benedetti et al., 2012). DM 173 application brought to the standardization of procedures, and results are managed using a software, Sediqualsoft®, developed by ISPRA, in collaboration with the Polytechnic University of Marche.

Based on this software, the integration of chemical and biological results classifies the three sediments within the C quality class, for which release is expected in sediment tank within the ports. Although classified within the same quality class, this result is extrapolated from ISPRAs software through very different chemical and ecotoxicological results: in fact, the samples have different chemical and ecotoxicological risk hazard quotient. Sediment 18/SM has a medium ecotoxicological risk and a low chemical risk. On the contrary, the sediment 20/SM has the highest chemical risk associated to a low ecotoxicological risk index. The 19/SM presents a high chemical risk associated to an average ecotoxicological one.

Regarding chemical analysis, values reflect the granulometric nature of the samples representing the sediment surface as an excellent absorption area for most of contaminants especially for heavy metals (Buccolieri A. et al., 2006). Despite the differences in the granulometric composition, heavy metals concentration in all samples resulted below the L1 detection limit set by

the Decree, similar to that found in unpolluted sites (or with moderate impact), especially if compared to those of industrialized areas such Barcelona, Rotterdam, Bilbao and Piombino ports (Bocchetti et al., 2008; Guevara-Riba et al., 2004; Neff, 2002).

On the bases of the evaluation organic and organostannic compound content: chemical risk resulted low in 18/SM sediment, and significantly higher in the 20/SM and 19/SM samples.
Regarding phthalates, values detected in elutriates are in the same range of those measured by ARPAM (in 2017 and 2018) in internal waters. Considering that phthalates are hydrophobic molecules with a longer half-life in sediments rather than in the water column (Net et al., 2015), we can imagine that in port sediments their concentration should be higher. This last aspect should be considered in a wider contest mainly because phthalates are ubiquitous compounds and can be bioaccumulated in organism by both direct exposure or by dietary accumulation. For this reason, their environmental presence should be monitored and evaluated in a wider ecotoxicological contest and local/national organization should increase the list of chemicals to be measure establish the HQe of sediments.

Concerning ecotoxicological data, the essay with microalgae, rather than the *V. fischeri* test, gives the highest contribution to HQe in the test battery.

On the bases of the *Phaeodactylum tricornutum* bioassay, all samples significantly inhibited the growth of the microalgae resulting the 19/SM the most toxic. This evidence is only partially supported by chemical data, on which bases, in fact the 20/SM was the most contaminated. This incongruence between chemical and ecotoxicological data can be explained by the "matrix effect" that produces significant algal blooms in the elutriates. This relies in the fact that the preparation of the matrix itself allows hydrophilic contaminants to dissolve in the solution thus representing a source of nourishment for algae (Sbrilli et al., 2003). The higher pelite content of 19/SM sediment can be associated with a greater presence of hydrophilic molecules that can potentially enter in the aqueous solution of the elutriate representing a source of nutrients. A possible "mask effect" can be supposed in the 20 SM sample compared to the 18 SM: the first is richer in pelitic fraction than the second, and the possible hydrophilic "nutrients" contained in the 20/SM antagonizes the toxic effect of the contaminants resulting in an inhibition of growth lower than the sample 18/SM, which on the other side being poorer in pelite is therefore less rich of nutrients.

The strengthen the results obtained with algae bioassay, on the bases of the results on *V. fischeri*, both sediment 18/SM and 19/SM lack of toxicity. In sample 20/SM, STI value suggests a slight toxicity. The main reason is linked to the physical properties of the samples, the finest fraction is present, an increased adsorption can be observed. The sand, which has a low specific surface and a low surface charge density, is not very reactive and often has a lower contamination than the fine material (Mugnai et al., 2005). In sediments rich in pelite, a higher toxicity caused by contaminants adsorption on the surface of the small granulometric fraction is expected, as opposed to a sandy sediment. The problem of a pelitic sediment is that the low bioluminescence may not be caused only by the high quantity of contaminants associated with its low size particles, but also by the marine bacterium trapped in the fine component of the sediment: this preventing the instrument from detecting any bioluminescence. The low bioluminescence value is thus caused by a physical factor and not by the presence of contaminants. Differently from a sample with a lot of pelite (19/SM), in sandy samples (18/SM and 20/SM), *V. fischeri* is not adsorbed, and so its abundance in the water column will be higher and a higher bioluminescence will be detected. From a technical point of view, this result in a lower level of toxicity associated to sandy samples, as first stated by the toxic units (TU). But the application of pelitic correction, suggested by the method, reverses the situation and allows to obtain the STI Index in which the results, finally, shown that the two sandy samples (18/SM and 20/SM) give the greatest contribution of toxicity. The paradox concerning this assay relays in the fact that to a higher capacity of a sediment to absorb contaminants, with corresponds a lower bioluminescence, is less toxic than sandy sediment which - from its nature - should not be very contaminated.

Finally, also in this study, the test with the oyster *C. gigas* resulted the most sensitive test to detect the concentration of pollutants, as previously observed in (Pellegrini et al., 1999). The rate of malformations has a direct correspondence to the level of contamination in each sample, resulting sediments 19/SM and 20/SM the most detrimental in causing oyster toxicity, respect to 18/SM.

The suitability of oyster as model to reveal elutriate toxicity was further confirmed by molecular analyses, performed on *C. gigas* embryos sampled at 5 hours and 18 hours post fertilization. The expression of a set of genes specifically involved in the development, in immune response and oxidative stress were analysed to gain info on the effects of these sediments on oyster early development. Organisms Exposure to chemical or organic pollutants in the sediment or in the water determines an increase of oxidative stress, in term of an increase of reactive oxygen species (ROS). ROS content in the organism can be increased by the presence of contaminants and is regulated by antioxidant enzymes, such as superoxide dismutase (Sod) and glutathione peroxidase (*GPx*) (Cavaletto et al., 2002; Cheung et al., 2004; Verlecar et al., 2007). The molecular markers of antioxidant mechanisms are of primary importance in modulating the toxicological effects of chemical substances and their mixtures. Therefore, the measurement of biomarkers of oxidative stress in marine organisms could be extremely useful for the early diagnosis of a general disorder or even more serious health impairments (Regoli, 2000; Regoli et al., 2002, 2004).

Vasa gene establishes the developmental pattern of germline cells during oyster ontogenesis. In control oyster, the expression during the two time points analysed remains almost constant, while a down regulation was measured at 5 hrs following the exposure to all sediments, although significant only with the sediment 19 SM. At 18 hrs, levels are almost constant among all groups, suggesting that in treated oysters, the first cleavages could be delayed by the exposure to the 19/SM sediment. Previous studies in *C. gigas*, in fact revealed that vasa transcripts were inherited by a single blastomere as early as the 2-cell embryo stage and segregated into a unique blastomere along all cleavage stages, suggesting and confirming that vasa mRNAs might be maternal determinants of germline cells in oysters as observed for zebrafish and Ciona (Yoon et al., 1997; Fujimura et al., 2000).

Turning to immune-related genes, HSP70 mRNA levels were investigated. HSP70 mRNA expression suggests that oyster exposure to sediments 18/SM and 19/SM clearly induce stress. The increase of expression of this mRNA has been largely considered an early warning signal of the exposure to chemical compounds able to interfere with the organism physiology (Maradonna and Carnevali, 2007; Carnevali and Maradonna, 2003). As observed in different animal models, more specifically, Hsp70 family members are very strongly upregulated by heavy metals such as arsenic, cadmium, copper, mercury, etc., (Sun et al., 2019; Somasundaran et al., 2019), the abundance of which has been measured in both 18 and 19 SM sediment. Differently, the exposure to sediment 20/SM, did not induce a mRNA upregulation and let us speculate that the recovery system of the oyster could be compromised, mainly because of the highest levels of PAH measured in these sediments.

Galectin mRNA was upregulated only in oysters exposed to 19/SM and 20/SM sediments, suggesting that in these oysters, apoptotic process or an increase of the immune response occurs. These proteins are, in fact, mainly involved in the control of cell death, acting either extracellularly by cross linking glycans on the outside of cells and thus transducing signals across the membrane, or activating downstream signals that trigger the onset of apoptosis (Hsu et al., 2006). More specifically, their regulation of apoptosis consists in the selection of T cells in the thymus, avoiding the circulation of T cells that are self-reactive. Previous studies reported that transcripts encoding galectin in *C. gigas* have been documented independently of whether they received a bacterial challenge: resulting unresponsive to *V. tubiashii* infection but increasing after infection with a mixture of four pathogenic Vibrio bacterial strains (Yamaura et al., 2008).

The last analyzed immune-related genes was dehf1, which expression resulted upregulated at both time points, only oysters exposed to 19/SM sediment. Defensins are antimicrobial peptides that act mainly by disrupting the structure of bacterial cell membranes and are found in many

compartments of the body. Evidence is accumulating that defensins play a central role in defence against pathogens, and they are considered as a part of the innate immune response. Since in healthy oysters defensin, defh-1, contributes to the control of the endobiont microbiota, important to compete potential pathogens from the environment (Schmitt et al., 2012), this evidence might suggest, that the exposure to 19/SM sediment could alter the endogenous microbiome.

The organism antioxidant defence system protects against oxidative stress caused by pollutants of different chemical nature and is enhanced as an adaptation or a compensatory reaction to ROS formation or toxic effects (Livingstone et al., 1990). It consists of antioxidant enzymes, among them are superoxide dismutase (*Sod*) and glutathione peroxidase (*gpx*). They have a crucial role in maintaining cellular homeostasis and antioxidant defence by removing ROS (Rudneva, 1999), mainly the superoxide radical through the process of dismutation (Fridovich, 1975). The H2O2 produced from this process is reduced to H2O and O2 by CAT and GPX (Mruk et al., 2002). Specifically, GPX catalyzes the reduction of hydroperoxide and hydrogen peroxide to water and oxygen (Swiergosz-Kowalewska et al., 2006). The results obtained in this study, strongly suggest the ability of all sediments to cause oxidative stress in developing oysters and confirm the suitability of this system as a biomarker to detect oxidative stress in wild species.

Among other signals involved in detoxification, lysozyme mRNA, which expression, as observed in control oysters, in physiological condition increases with development, was significantly downregulated in 18 hrs oysters, exposed to all sediments. At the same stage, all the sediments significantly decrease its expression to similar levels. In a previous study using naïve oysters exposed to low levels of NP, despite the decrease of lysozyme-1 mRNA levels, an increase of its associated activity was measured (Hart et al., 2016). This was caused by the release of existing lysozyme stores and possibly suggests that similarly in our study, organisms fight using the stored protein thus preventing new mRNA requirements and subsequent enzyme synthesis. Further evidence of this altered immune response is given by the Transglutaminase mRNA: tg mRNA was downregulated by all sediment exposure at both time points selected. The role of tg in the immune response is very important, since the primary role of this enzyme is to form extensively cross-linked, generally insoluble protein polymers. These biological polymers are indispensable to create barriers and stable structures, including blood clots. Since, the formation of clots is another essential process of innate immunity as it prevents excess blood loss and microbial introduction during injury (Wang et al., 2001), we could speculate about a severe compromised development of the oyster immune system caused by the exposure to the different contaminated sediments

Overall, the molecular results observed, suggest that the 18 hrs stage seems to be more affected by exposure to contaminated sediments, possibly because at 5 hrs, the embryo is still developing inside the chorion and this could protect from direct contact with dissolved pollutants. In addition, we can speculate about the existence of a maternal immune system still working in the first stages of development, that could mitigate the toxicity of the sediments.

19/SM seems to be the most toxic sediment, probably due to its greater pelitic composition which makes it prone to the absorption of contaminants (in particular PCB, DEHP phthalate, organostannic compounds). Molecular data well support two of the three selected battery tests, the microalga and oyster bioassays, clearly indicating that 19/SM sediment has the highest ecotoxicological impact. On the contrary, the *V. fischeri* test, because of the scarce selectivity toward the type of contaminants and the great influence of the granulometric composition of the sediments to clearly define its toxicity level, should be carefully considered before being selected within a test battery aiming at defining the quality class of dredged sediments.

The integrative approach used in this study, which goes behind the evaluation of parameters proposed by DM 173, strongly support the need to integrate molecular data with ecotoxicological hazard quotient (HQe) and chemical hazard quotient (HQc) relative to sediment. This should be done in order to help individual environmental protection agencies in the management of sediments, especially when dealing with contrasting chemical and ecotoxicological results especially in absence of historical data or expert judgments.

The application of an integrative approach aimed at defining the quality class of the sediments, to facilitate the decisions for the best management of dredged sediments.

This integrative approach aims at considering dredged sediments as precious resources that, with the right classification and the following treatment, can be reused.

Chapter 3

Dietary Diisononylphthalate contamination induces hepatic stress: a multidisciplinary investigation in gilthead seabream (*Sparus aurata*) liver

1. Introduction

In recent decades, global plastic demand has increased dramatically, reaching 350 million tons in 2017 (Plastic Europe, 2018). Plastics are extremely easy to use and have a low-cost production, characteristics that have determined their incredible popularity in all sectors. Therefore, plastic represents the first enemy for the environment, and not for its possible toxicity (which is under the evaluation of many scientific studies because of the different plasticizing additives that compose it), but we are practically immersed 24 hours a day in these substances.

Plastic enters the sea in various ways (Fig. 1).

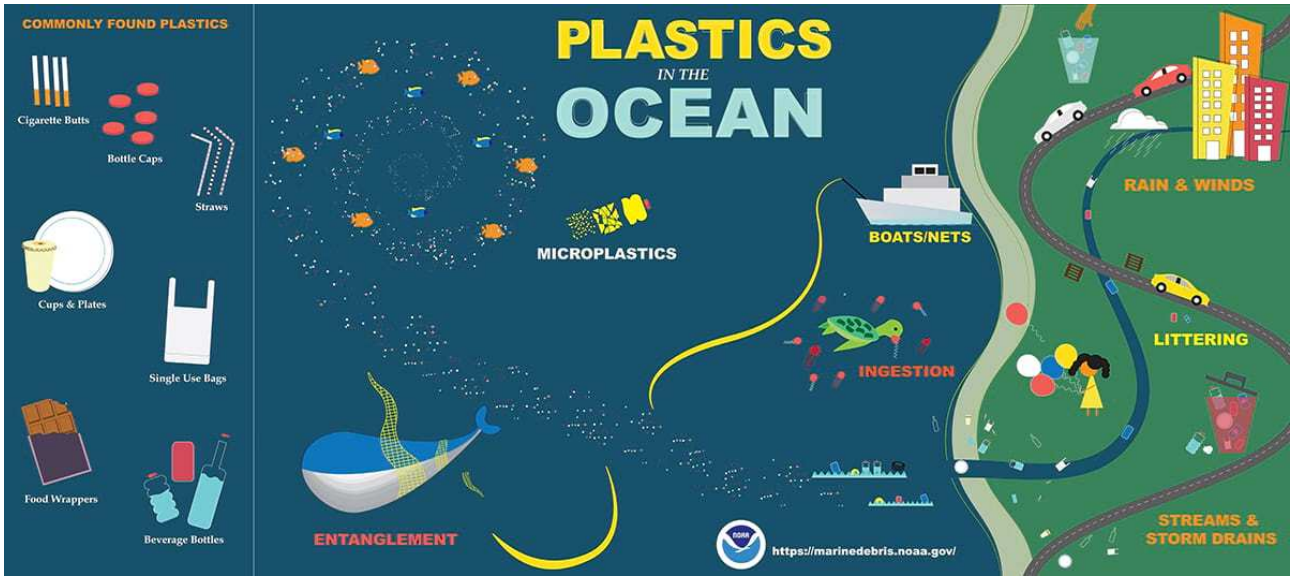


Figure 1. *Plastics are the most common form of marine debris and they can come from a variety of land and ocean-based sources. Once in the water (sea or lakes), plastic debris never fully biodegrades. Yellow text in the graphic shows sources of plastic that eventually end up in the ocean; the orange text shows ways that these plastics move into the ocean and red text provides examples of the harmful impacts of the plastic material debris (Source:* https://oceanservice.noaa.gov/hazards/marinedebris/plastics-in-the-ocean.html*).*

Global contamination due to plasticizing additives such as phthalates is a reality: the italian legislation on internal waters (Legislative Decree 172/2015) only includes Di(2-ethylhexyl)phtalate (**DEHP)** among phthalate and data from ARPAM detetct DEHP in internal waters with values in the same order to those found in elutriates from harbour sediments. Data from ARPAM for the year 2017 range from 0.110 µg/L to 1.018 in rivers, and from < 0.01 to 0.08 µg/L in lakes; for the year 2018 range from 0.016 µg/L to 0.720 in rivers, and from 0.016 to 0.703 µg/L in lakes, these values are in the same order as those found in sediment elutriates 18/SM, 19/SM e 20/SM. Therefore, the ubiquitous presence of phthalates in water, in food, in sediments, including harbours, should not be surprising. This omnipresence ensures that the list of these substances must be continually updated, and that the Ministerial Decrees concerning specific environmental problems go hand in hand with

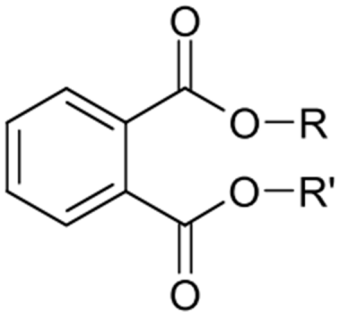


Figure 2. Phthalates general structure: a diester of 1,2-dicarboxy-benzene (CPSC, 2010)

this evolution. In fact, to better meet the needs of consumers, the new plastics implement additives that give the product additional features, such as flexibility and viscosity. This property is introduced by modifying the internal structure of the polymer: for example, in polyvinyl chloride (PVC) the use of plasticizing additives gives this product greater softness or resistance. In the world, 80% of the demand for plasticisers is destined only and exclusively to modify the properties of PVC, and among these the most used are phthalates, representing about 70% of global demand only in 2014. Their general structure is a diester 1,2-dicarboxy benzene (Fig. 2) and these are not covalently linked to plastic, therefore leaching and volatilization phenomena can easily occur and these substances can be released in the environment. This results in a practically ubiquitous exposure to these substances (Mikula et al. 2005; Serrano et al., 2014), especially for those compounds with lower molecular weight. Furthermore, a significant release from plastic containers into packaged foods and its further consumption was considered a significant route of human exposure to toxic substances (Cao, 2010; Rodgers et al., 2014). When ingested, phthalates are rapidly absorbed and eliminated, as they are not bioaccumulated. However, in the body they undergo various metabolic reactions: these metabolic processes (outlined in the figure 3, and which include a hydrolysis from diesters to monoesters by intestinal or pancreatic enzymes to then reach the liver and here conjugate with glucuronic acid) generate bioactive metabolites, usually monoesters, which are responsible for the toxic activity of phthalates, with the liver as a target organ (Mikula et al., 2005; Moon et al., 2012) and they are subsequently expelled in faeces or urine.

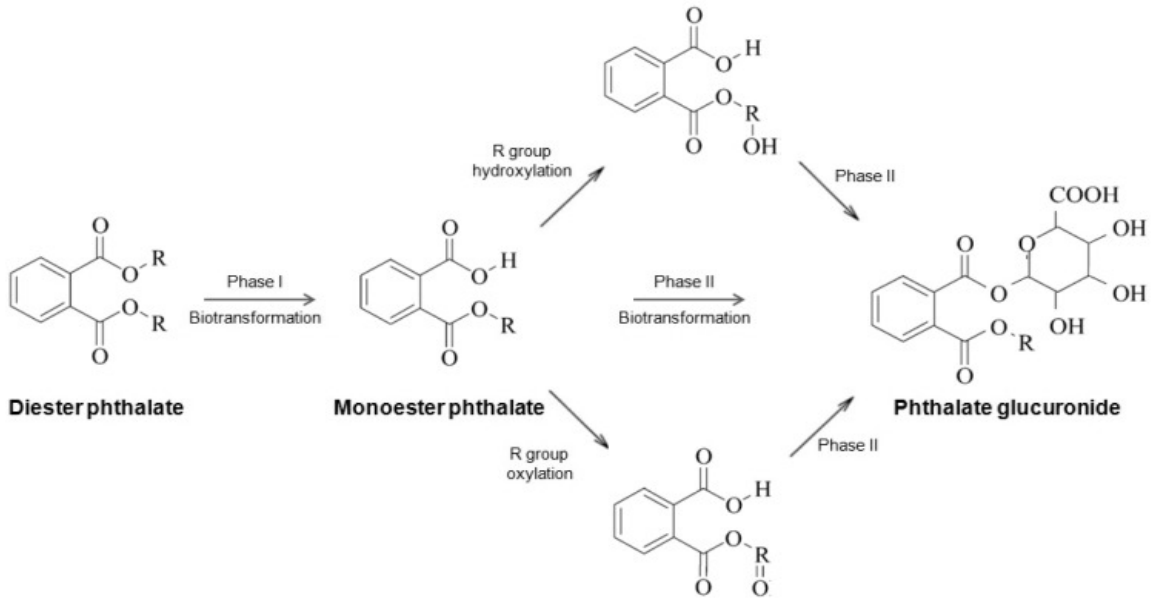


Figure 3. Metabolic pathways of phthalates (Kim and Park, 2014)

Initially it was thought that, given their low acute toxicity, they were harmless. But recent studies have shown their high chronic and sub-chronic toxicity and they have been recognized as toxic molecules. Several studies have shown that some phthalates induce an increase in the concentrations of reactive oxygen species (ROS) both in embryos of developing fish and in adult

specimens, with signs of liver stress due to greater lipid peroxidation and the occurrence of liver damage (Kang et al., 2010; Xu et al., 2013a). Therefore, in recent years, research organizations and the chemical industry have collaborated to produce new and safer additives. One of these new additives is Di-isononyl-phthalate (**DiNP**), produced to replace di-(2-ethylhexyl)phthalate (DEHP), whose carcinogenicity and toxicity have been demonstrated in various animal models where impaired reproduction and delayed development and growth were described (Gray et al., 2000; Parks et al., 2000; Carnevali et al., 2010; Zanotelli et al., 2010; Corradetti et al., 2013; Ye et al., 2014). However, of DiNP toxicity has been shown to cause hormonal imbalance and down-regulation of gene expression in cholesterol transport and androgen synthesis in male rat fetuses (Hannas et al., 2012).

Furthermore, the results obtained in the laboratory using fish as experimental models have demonstrated that exposure to DiNP can: 1) alter reproduction (Santangeli et al., 2017; Forner-Piquer et al., 2019); 2) influence lipid metabolism (Carnevali et al., 2019); 3) alter the functioning of the endocannabinoid system (Forner-Piquer et al., 2018a; 2018b). There are still many physiological aspects to study and investigate: in this study we wanted to evaluate the effects of exposure to different concentrations of diisononyl phthalate (DiNP) administered through diet, in a higher organism such as the adult sea bream (*Sparus aurata*). This aquatic organism was exposed to two doses of DiNP (at 15 and 1500 μg kg^{-1} bw day^{-1}) for 21 days, and subsequent analyses focused on its effects on the liver, since this represents the most sensitive organ to DiNP exposure (CPSC, 2010).

2. *Sparus aurata* as an experimental model

The gilthead seabream *Sparus aurata* is a demersal perciform fish that belongs to the family of the Sparids. Its body is oval, silver and laterally compressed. The head has a curved profile. It is easily identified and recognized due to a black spot present at the beginning of the lateral line and at the upper edge of the opercula. Moreover, it has a golden frontal band that is located between the eyes (FAO, 2018). It has a subtropical distribution between 62 ° N - 15 ° N, 17 ° W - 43 ° E and can be found in the Mediterranean Sea, or more rarely in the Black Sea and in the eastern Atlantic (Mosconi et al., 1998). Thanks to the fact that it is a euryhaline organism, it can survive in both salt and brackish water, such as coastal lagoons and estuary areas, where it usually spends the initial stages of its life cycle (Arabaci et al., 2010). *Sparus aurata* is a protandrous hermaphroditic species: it matures as a male until the age of 2 years old, when it reaches 20-30 cm in dimension and it becomes female when it turns 3 years old and it measures 33-40 cm in length. Females can lay up to 80,000 eggs every day for a period of 3 months. The young remain in the open sea from October to December, to then migrate to coastal waters to find warmer temperatures and greater food resources in spring. Once they become adults, they return to the open sea in autumn to start the reproductive cycle again.

They are mostly carnivorous fish as they eat mussels and oysters, but they can also act as herbivores. It is a species that inhabits rocky bottoms or *Posidonia oceanica* meadows, although this may be present in more sandy substrates (FAO, 2018). These organisms remain at a low depth, at most 30 meters, but some adults have been seen to go as deep as 150 meters (Arabaci et al., 2010).

Seabream aquaculture has been practiced, and is still a reality, in saltwater ponds and coastal lagoons. Since 1980 intensive farming systems (for example sea cages) have been developed, in order to satisfy the growing market demand. This led to a drastic drop in consumer prices of around 60% between 1990 and 2000. To date, the market price of *Sparus aurata* is around 5.50 euro/kg. Nowadays, the sea bream as well as the sea bass are the two most important species in Mediterranean aquaculture. In 2010, industries predicted an increase in European production of sea bass and sea bream by 20% for the following decade, with a further decrease in production costs of over 5% per year (Theodorou et al., 2010). Exploitation through aquaculture is still expanding and

Greece is the largest producer until 2002, followed by Turkey (15%), Spain (14%) and Italy (6%). In 2014 the global production of *Sparus aurata* aquaculture amounted to 158,389 tons, a slight increase compared to the 156,990 tons of 2013 (FAO data, 2018). Therefore, the commercial importance of sea bream is evident. Added to this is the important fact that the welfare of farm animals is becoming an important and significant social topic (Veissier et al., 2008) both from an ethical point of view for consumers, and for producers as stress and animal welfare is an important problem, as growth and reproductive performance, health status and susceptibility to disease are closely related to stress conditions (Conte, 2004). Therefore, improving aquaculture practices in order to minimize stress and preserve fish welfare is one of the main challenges in intensive farm research, considering that some aquaculture practices such as repetitive handling, confinement and crowding of fish represent potential chronic stressors that affect fish physiology and welfare status (Pagés et al., 1995; Mugnier et al., 1998; Arends et al., 1999; Barton et al., 2005). In line with this, the European Food Safety Authority recently identified certain hazards and risk factors that could potentially affect the welfare of different farmed species, including sea bream (EFSA, 2008). Sediments could also represent one of these hazards due to the enormous quantity of organic matter that is released into them from the overlying farms (uneaten food, faeces, ...), all the contaminants that could be associated with it (e.g. drugs, antibiotics, antifouling substances, ...) (Rico et al., 2012) and phthalates, given their presence in internal waters and their high life presence in sediments (Net et al., 2015), cannot be excluded.

3. Materials and methods

3.1 Experimental design

Two-year-old seabream males (weighing 459 ± 56.8 g; total length: 302 ± 10.7 mm), obtained from an Aquaculture Center (FORKYS SA, Crete, Greece), have been maintained in the AQUALABS facilities of the Institute of Marine Biology, biotechnology and aquaculture of the Hellenic Center for Marine Research (Heraklion, Crete, Greece), an approved facility for the maintenance of experimental animals (EL91-BIOexp-04) as previously described (Forner-Piquer et al., 2018a). The fish were fed 0.7% of their body weight (bw), divided into five meals a day, to ensure that all the food administered was consumed. The amount of DiNP added to the experimental feed was adjusted according to the size of the fish and the water temperature, in this way the right experimental dose containing the Endocrine Disrupting Chemicals (EDCs) was assigned (preparation according to the studies published by Maradonna et al., 2015; Forner-Piquer et al., 2018a). The experimental protocol was approved by the National Veterinary Services (AP 255361). All procedures involving animals were conducted according to the "Guidelines for the treatment of animals in behavioural research and teaching" (Anonymous, 1998; 2012), the "Ethical justification for the use and treatment of fish in research: an update" (Metcalfe and Craig 2011) and the "Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes" (European Council and European Parliament 2010).

After the acclimatization time, the fish were divided into 3 treatment groups in 2 m^3 duplicated tanks, consisting of 10 individuals per tank and fed on an EDC diet for 21 days. The treatments were as follows:

Control (C)	Fed with the commercial feed containing the phthalate vehicle (1.4 ml of EtOH kg^{-1} feed).

DiNP LOW (DiNP L)	Fed with commercial feed contaminated with DINP, to receive a daily ration of 15 µg DINP per kg of body weight per day.
DiNP HIGH (DiNP H)	Fed with commercial feed contaminated with DINP, to receive a daily ration of 1,500 µg DiNP per kg of body weight per day.

The dose quantity chosen for DiNP was based on: the lowest tolerable daily intake (TDI) for humans, suggested by the European Food Safety Authority, which is 150 µg/kg per day; while the higher dose, since few data are available on the physiological effects of DiNP, it was decided to compare a pharmacological dose to obtain further information on the compound MoA.

It has been assumed that each fish received the same amount of food. The experimentation was carried out under suitable conditions and recorded daily: water pH (7.52 ± 0.02) and dissolved oxygen levels (89.2 ± 1.8%), as well as NH3-N and NO2-N levels were always kept under security levels. After 21 days, all the fish in the tank were taken and anesthetized in a 30 mg/L bath of clove oil until they stopped breathing (Mylonas et al., 2005). So, the fish were quickly beheaded. Liver and plasma aliquots were stored at -80° C until further analysis, while other pieces of liver were fixed in a solution of formaldehyde: glutaraldehyde (4:1) for subsequent histological analysis. Instead for gene expression, livers were inserted into RNAlater (Ambion Inc., Texas, United States) and stored at 4° C until treatment.

3.2 RNA extraction and cDNA synthesis

Total RNA was extracted from 10 pieces of liver per group using the RNAzol solution (Sigma Aldrich, Milan, Italy). RNA concentrations were determined with a NanoDrop™ 1000 spectrophotometer (NanoPhotometerTM Class P, IMPLEN, Germany). The quality of RNA was evaluated by electrophoresis on 1% agarose gel. Two micrograms of total RNA were used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Milan, Italy) and stored at 20° C.

3.3 Real-Time PCR

qRT-PCR were performed with SYBR green in a CFX thermocycler (Bio-Rad) as described in Carnevali et al., (2017). Ss-actin (*act*) and elongation factor 1a (*ef1α*) have been used as housekeeping genes to standardize results and eliminate variations in the quantity and quality of mRNA and cDNA. The primer sequences, GenBank access numbers and annealing temperature for the analyzed genes are shown in Table 25. No amplification products were observed in the negative controls and no primer-dimer formations were observed in the control models. The primers were used at a final concentration of 10 pmoL/mL. Data were analyzed using iQ5 Optical System version 2.1 (Bio-Rad) including Genex Macro files iQ5 Conversion and Genex Macro iQ5. The modification of gene expression between the experimental groups is reported as a relative abundance of mRNA (Arbitrary Units). Primers used at a final concentration of 10 pmoL/mL.

Gene name	**Symbol**	**Primer For (5'-3')**	**Primer Rev (5'-3')**	**Annealing temperature (°C)**	**Accession Number**
Colony stimulating factor 1 receptor	*csf-1r*	ACG TCT GGT CCT ATG GCA TC	AGT CTGGTTGGGACATCTGG	60	AM050293
Glucose-6-Phosphate	*g6pdh*	TGA TGA TCC AAC AGT TCC TA	GCTCGTTCCTGACACACTGA	60	JX073711.1

Dehydrogenase					
Interleukin-1	*il1*	ATC CAG CTG TCT TTC CCT CA	TTGCATGTCATCTCGGATTC	60	AJ419178.1
Glucocorticoid receptor	*gr*	GTG CGA ACA GAT GCT GAA GA	ATGGCTTTACCCAGCTCCTT	54	DQ486890.1
Heat Shock Protein-70	*hsp70*	ACG GAG AGT CGA TTT CGA TG	GAAGGACATCAGCGACAACA	57	EU805481.1
Glutathione Reductase	*glut red*	TGT TCA GCC ACC CAC CCA TCG G	GCG TGATAC ATC GGA GTG AAT GAAGTC TTG	60	AJ937873.2
Glutathione Peroxidase-1	*gpx1*	GAA GGT GGA TGT GAA TGG AAA (A,G) GAT G	CTG ACG GGA CTC CAA ATG ATG (G,T)	54	KC201352.1
G Protein-coupled Receptor 75	*gpr75*	TC(C,T) GGT GTG GAT CTG ACC AAA GAC	TGT TTA GGC CCA GAA GCATCC ATG	56	DQ524993.1
Glucokinase	*gck*	CGT GTG ATG CTG GTG AAG	TGCTTGATATGATGTCTGTCC	54	AF053330.2
Cyclooxygenase-2	*cox2*	TGT CCC GTG GCA TCC TTT	ATCACGGAGCCCGTCTCTT	58	AM296029.1
Phospholipase-A2	*pla2*	CCA GAC CAT CTT CAC CAT CC	CACCCAATCCACAGGAGTTC	56	JX975710.1
5-Lipoxygenase	*5lox*	CCT GGC AGA TGT GAA CTT GA	CGTTCTCCTGATACTGGCTGA	60	FP334124

Table 1. Primers List

3.4 Fourier Transform Infrared Imaging (FTIRI) measurements and data analysis

Five liver samples were collected from each experimental group (DiNP L, DiNP H and C) and stored at 80° C. Shortly before the IR measurements, the samples were cut from a cryo-microtome. From each sample three sections were obtained (≈10 μm thickness) at 200 μm distance from each other, immediately deposited without any fixing process on CaF2 optical windows (thickness 1 mm, diameter 13 mm) and made to dry in the air for 30 minutes (Giorgini et al., 2015a; 2015b). The FTIRI measurements were performed within 48 hours on the IR beamline SISSI, ELETTRA-Sincrotrone Trieste, using a Bruker VERTEX 70 interferometer coupled with a Hyper-3000 Vis-IR microscope and equipped with a liquid nitrogen-cooled two-dimensional focal array (FPA) with a surface of the detector area of 164x164 μm^2, corresponding to 64x64 pixels (Bruker Optics GmbH, Germany). Similar samples prepared in the same laboratory showed good stability over time. 15 sections were analysed for each experimental group. The photomicrograph of each section was collected by means of a 15X condenser/lens and specific representative areas were chosen. On these areas, the IR maps were acquired in transmission mode in the MIR range from 4000 cm^{-1} to 800 cm^{-1}. Each map was 164x164 μm^2 in size and contained 4096 pixels/spectra (pixel resolution 2.56x2.56 μm^2); each spectrum was the result of 256 scans. Before each acquisition, the background spectrum was acquired on a clean portion of the CaF2 optical window. The RAW IR maps have been pre-processed with the OPUS atmospheric compensation routine (to correct the atmospheric contributions of carbon dioxide and water vapor), so the vectors have been normalized over the entire frequency range (to avoid artefacts due to variations in local thickness) (OPUS software package 7.1). The IR maps are false colour images that represent the topographical distribution of specific chemical groups were generated by integrating pre-processed IR maps in the following spectral regions: 3032-2995 cm^{-1} (absorption band at about 3018 cm^{-1}; representative of the alkyl lipids of unsaturated lipids; lengthening of the vibrations of CH in the alkyl lipid chains, called CH) (Christy and Egeberg, 2006; Coimbra et al., 2015); 2995-2952 cm^{-1} (absorption band at about 2959 cm^{-1}; asymmetric stretching vibration of CH3 groups in alkyl lipid chains, named CH3) (Vargas et al., 2018); 2952-2888 cm^{-1} (absorption band at about 2926 cm^{-1}; vibration of asymmetric stretching of CH2 groups in alkyl lipid chains, named CH2) (Vargas et al., 2018); 1720-1480 cm^{-1}

(absorption bands at about 1650 cm^{-1} and about 1545 cm^{-1}; vibrational modes of peptide bond, amide and II bands of proteins, called PRT) (Vargas et al., 2018); 1430-1370 cm^{-1} (absorption band at about 1400 cm^{-1}; symmetrical stretching vibration of the COO^- groups, called COO) (Rudbeck et al., 2009); 1270-1189 cm^{-1} (absorption band at about 1240 cm^{-1}; asymmetric stretching vibration of the $PO2^-$ groups, called PH) (Vileno et al., 2010); 1189-1129 cm^{-1} (absorption band at about 1155 cm^{-1}; elongation of the vibrations of the COH groups, called COH) (Carnevali et al., 2017); 1070-995 cm^{-1} (absorption band at about 1025 cm^{-1}; elongation of the vibrations of the CH2OH groups, mainly glycogen, called GLY) (Carnevali et al., 2017). To better highlight the distribution of each component, different scales have been adopted, light pink/white colours that indicate the highest absorbance values, while the blue colour represents the lowest values.

To obtain more information on the effects induced by DiNP on the biochemical composition of the gilthead liver, a semi-quantitative analysis was also performed. The integrated areas of the absorption bands defined above were obtained (OPUS 7.1 integration routine) and used to calculate the following band area ratios: CH2/TBM (degree of saturation of alkyl lipid chains); CH/TBM (degree of unsaturation of alkyl lipid chains); CH3/TBM (relative quantity of alkyl lipid chains); PRT/TBM (relative quantity of proteins); COO/TBM (relative amount of COO^- in amino acids); PH/TBM (relative quantity of phosphorylated proteins); COH/TBM (relative amount of carbohydrates) and GLY/TBM (relative amount of glycogen). The TBM, calculated as the sum of the integrated areas of the regions 3032-2995 cm^{-1}, 2995-2952 cm^{-1}, 2952-2888 cm^{-1} and 1780-950 cm^{-1}, was considered representative of the overall biomass of the tissues.

3.5 Histology, Caspase-3 Immunolabeling and Image analysis

The liver samples (5 mm) were fixed in formaldehyde: glutaraldehyde (in a 4: 1 ratio). After dehydration by ethanol serial graduations (70-96%), the samples were washed with "Histo-Clear" clarifying agent (Bio-Clear, Bio-Optica, Milan, Italy) and incorporated in paraffin (Bio-Optica, Milan, Italy). The paraffin blocks were cut with a microtome (Leica RM2125 RTS, GmbH, Wetzlar, Germany) and 5 µm sections were stained with Mayer's haematoxylin and Eosina Y (H&E, Sigma-Aldrich, Milan, Italy). For the immunolabeling of Caspase-3, the sections adjacent to that used for H&E staining, were treated according to a protocol of recovery of the antigen with citrate buffer in a microwave oven at 376 W (about 86° C) for 5 minutes. After antigen recovery, the slides were blocked with 40% calf serum to prevent aspecific antibody binding and incubated with the primary antibody (Anti-Caspase 3, # Ab13847; Abcam, 1: 200 dilution) during at night at 4° C. The following day, after rinsing the slides with PBS (phosphate buffered saline), the sections were then incubated with the secondary fluorescent antibody (Goat Anti-Rabbit IgG H&L, Alexa Fluor 488 n Ab150077; Abcam, dilution 1: 400) at room temperature for 1 hour and 30 min. After incubation, the slides were mounted with Fluoreshield Mounding Medium with DAPI (ab104139) for nucleus staining. The sections were observed with a Zeiss Axio Imager. M2 microscope and images captured with a high-resolution Zeiss Axiocam 105 colour camera. Negative controls were obtained by incubation without primary antibody. The captured images were processed using the Zen 2.6 version, Blue Edition.

Qualitative and histomorphometry analysis of MMCs were performed in images of liver sections (5 microns thick; 12 sections per animal, 60 sections per experimental group). The images were randomly captured at a magnification of 40X and the relationship between the MMC area and liver area was estimated with the free ImageJ software (https://imagej.nih.gov/ij/ NIH, USA). The area of the MMC was manually delimited and calculated with respect to the total area of the image. The results are reported as a percentage of the hepatic area covered by MMC.

3.6 Cortisol assay

Cortisol was extracted from 25 µl of plasma from each fish. The analyses were performed with a Cortisol EIS kit (Cayman Chemical Company, Arcore, Italy) using a standard curve in the range 7.8–1000 pg/mL, according to the kit instructions. The sensitivity of the test was 2 ng per tube; the inter and intra-assay coefficients of variation were 6.3% and 4.4% respectively. To validate the cortisol assay, parallelism was performed between the standard curve and the serial dilutions of the extracted solution.

3.7 Catalase activity

The Catalase activity (Cat) was measured according to Aebi (1984). The assay was performed with a final volume of 1 mL, containing 50 mM of NaH_2PO_4/Na_2HPO_4 pH 6.8 and 50 mM of 30% H2O2 as a substrate. After adding the sample, the reduction in absorbance to 240 nm due to the consumption of H2O2 (hydrogen peroxide) is followed for 3 minutes. The CAT activity was determined as a difference in absorbance per unit of time (e = -0.04 mM^{-1} cm^{-1}) and expressed as µmol H2O2 consumed min^{-1} mg^{-1} of the total protein concentration.

3.8 Statistical analysis

Normally distributed data deriving from the infrared band area ratios, from gene expression, from the enzyme activity and from the area occupied by the MMC were presented as mean ± Standard deviation (SD). Significant differences between the experimental groups were determined by a factorial analysis of variance (one-way ANOVA), followed by Tukey's multiple comparative test, using the statistical software package Prism6 (Graphpad Software, Inc. USA). Statistical significance was set at $p < 0.05$. Different letters at the apexes indicate significant differences between experimental treatments.

4. Results

4.1 Molecular markers of stress, antioxidant, immune and apoptotic response

With Real-Time PCR, the modulation of stress response in fish-fed DiNPs was evaluated and, as shown in Figure 4a and 4b, a significant increase of heat shock protein 70 (*hsp70*) and glucocorticoid receptor (*gr*) mRNA levels was detected in both treated groups with respect to control. A significant increase in plasma cortisol levels was measured only in DiNP L fish compared to the control (Fig 4c).

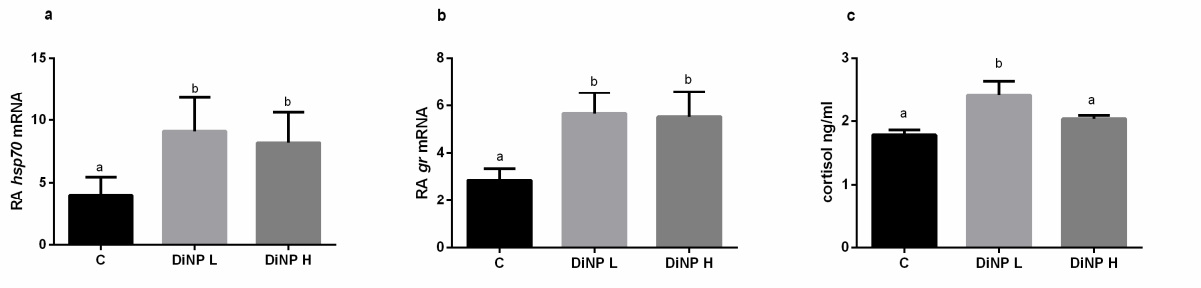


Figure 4. Levels of mRNA of *hsp70* (a) and *gr* (b) in the liver normalized against *act* and *ef1*. (c) Cortisol levels (ng/ml) in control livers and treated. Data are shown as mean ± S.D (N = 10). Different letters over each column indicate significant differences between the experimental groups ($p < 0.05$), which were

analyzed by ANOVA one way followed by the Tukey multiple comparison test. C: control; DiNP L: fish that receives 15 μg of DINP kg^{-1} bw day^{-1}; DiNP H: fish receiving 1500 μg of DINP kg^{-1} bw day^{-1}.

The variation in mRNA expression of genes encoding enzymes involved in antioxidant defence was also measured. The glucokinase mRNA level (*gck*) was reduced only with the administration of the lowest DiNP dose (Fig. 5a). Both DiNP doses significantly induced glucose 6 phosphate dehydrogenase (*g6pdh*), glutathione reductase (*glut-red*) and glutathione peroxidase 1 (*gpx1*) levels, compared to control (Fig. 5 b, c, d). Only for *gpx1*, the increase was dose dependent. Only the highest DiNP dose significantly increased the glutathione S transferase (*gst*) mRNA level (Fig. 5e). The catalase activity increased significantly in both exposed groups, with the highest activity measured in fish fed with the feed contaminated with the lowest dose of DiNP (Fig. 5f).

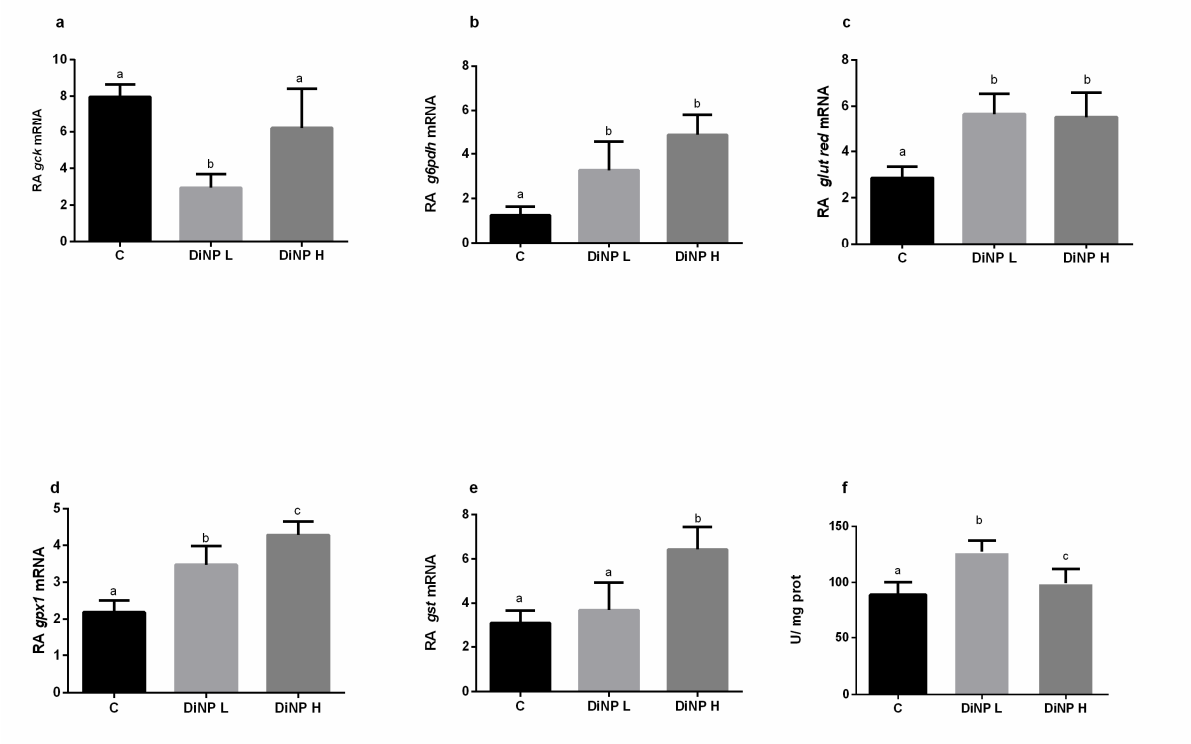


Figure 5. Levels of mRNA of *gck* (a), *g6pdh* (b), *glut-red* (c), *gpx1* (d), *gst* (e) in the liver, normalized against *act* and *ef1*. (f) Levels of catalase (U mg^{-1} prot). Data are shown as mean ± SD (N = 10). Different letters over each column indicate significant differences between the experimental groups (p <0.05), which were analszed with one-way ANOVA followed by the Tukey multiple comparison test. C: control; DiNP L: fish that receives 15 μg of DINP kg^{-1} bw day^{-1}; DiNP H: fish receiving 1500 μg of DINP kg^{-1} bw day^{-1}.

Regarding the markers involved in the immune response, the phospholipase 2 (*pla2*), 5-lipoxygenase (*5lox*) mRNA levels and the tumor necrosis factor (*tnfα*) were significantly upregulated only in fish fed with feed contaminated with high doses of DiNP (Fig 6a-c). The cyclooxygenase 2 mRNA levels (*cox2*) were not affected by the administration of both contaminated diets (Fig. 6d), while the interleukin1 gene (*il1*) was significantly upregulated by both DiNP doses (Fig. 6e). The *csf-1r* mRNA showed no changes in expression between the experimental groups (Fig. 6).

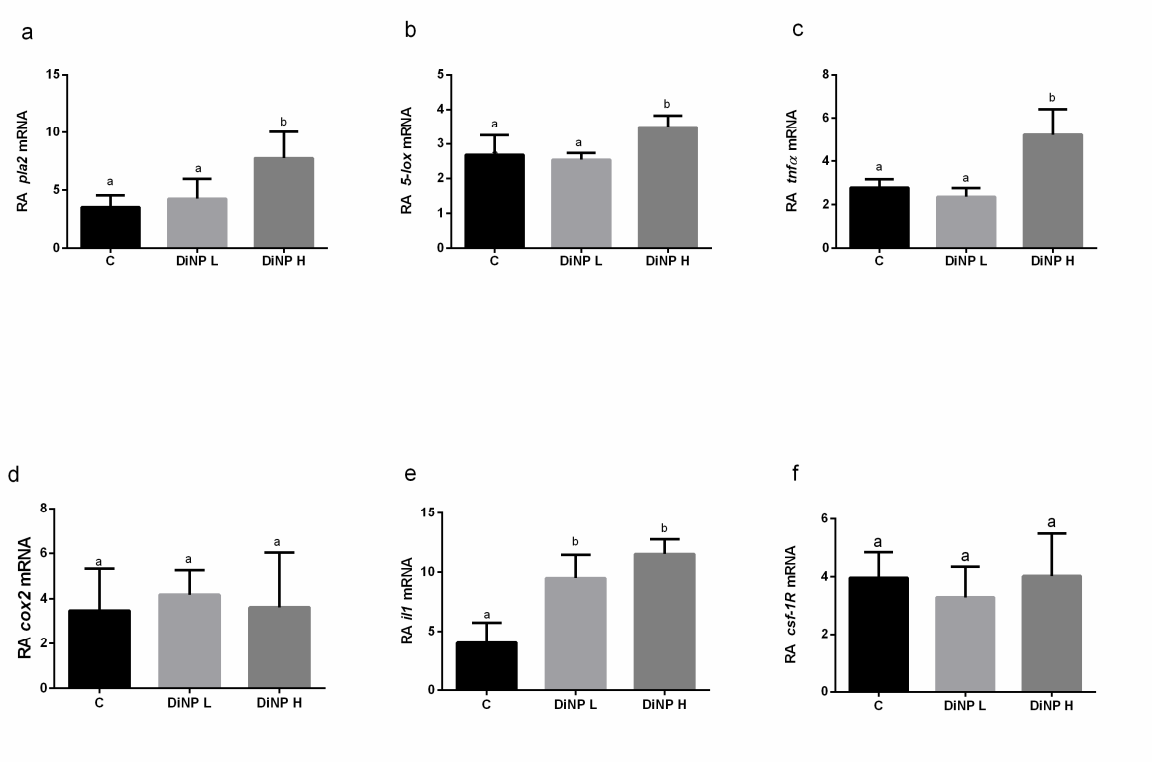


Figure 6. *pla2* (a), *5-lox* (b), *tnfa*, (c), *cox2* (d), *il1* (e) and csf-1R mRNA levels in the liver normalized against act and ef1. Data are shown as mean ± SD (N = 10). Different letters over each column indicate significant differences between the experimental groups (p <0.05), which were analyzed with ANOVA one way followed by the Tukey multiple comparison test. C: control; DiNP L: fish that receives 15 µg of DINP kg^{-1} bw day^{-1}; DiNP H: fish receiving 1500 µg of DINP kg^{-1} bw day^{-1}.

Expressions of genes related to the phenomenon of apoptosis have also been evaluated. The results showed that only the low DiNP doses induced a significant increase in mRNA levels of protein 75 (*grp75*) and tumour protein p53 (*p53*) regulated by glucose (Fig. 7a-b). The *bax* mRNA level was not influenced by treatments (Fig. 7c).

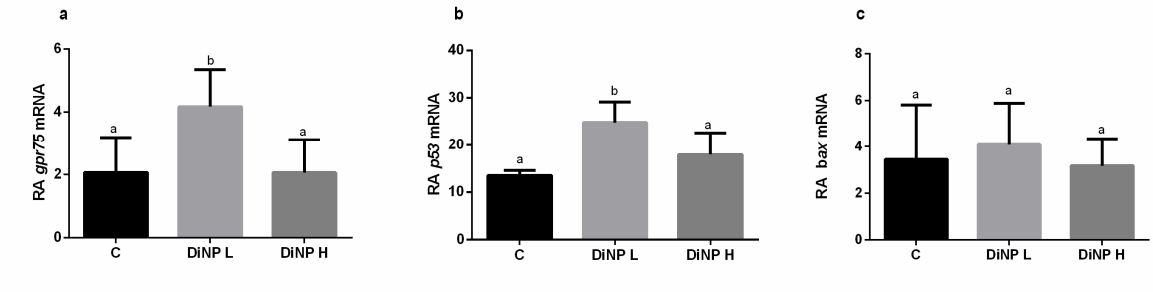


Figure 7. Levels of mRNA of *gpr75* (a), *p53* (b) and *bax* (c) in the liver normalized against act and ef1. Data are shown as mean ± SD (N = 10). Different letters over each column indicate significant differences between the experimental groups (p <0.05), which were analysed with one-way ANOVA followed by the Tukey multiple comparison test. C: control; DiNP L: fish that receives 15 µg of DINP kg^{-1} bw day^{-1}; DiNP H: fish receiving 1500 µg of DINP kg^{-1} bw day^{-1}.

4.2 Indirect immunofluorescence for Caspase-3

Histological staining of the hepatic sections showed the presence of small, slightly eosinophilic areas, identified by dark cells, due to the pigment content, of melano-macrophages in the hepatic parenchyma and considered centres of melano-macrophages (MMC). The CAS-3 immunolabeling technique was performed in adjacent sections of liver tissue and a marked immuno-staining was observed in the melano-macrophage centres (MMC) (Fig. 8 a-c). The CAS-3 reaction was calculated in all groups by summing the area covered by the positive for MMC (Fig. 8d) and no significant differences were observed between them.

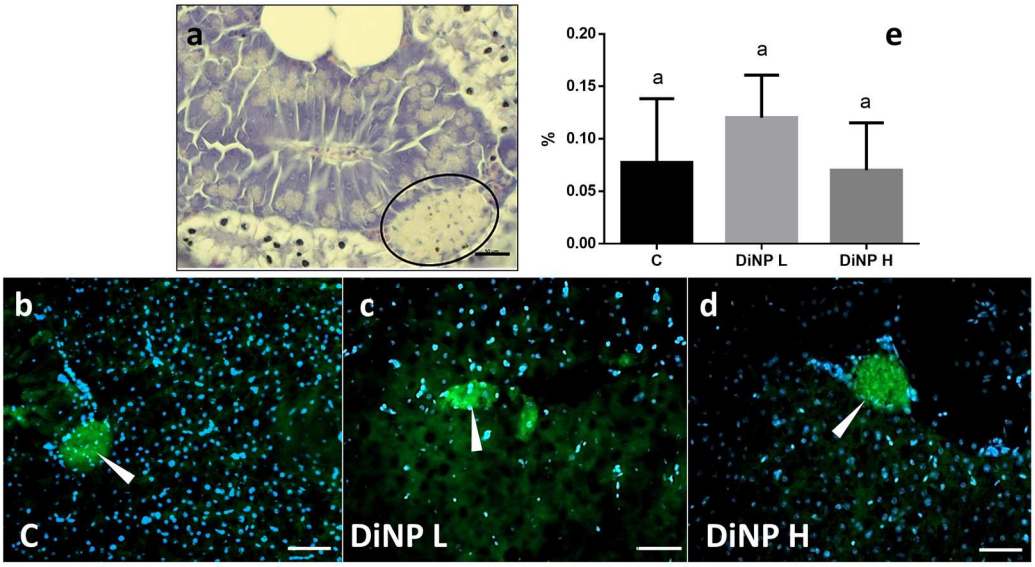


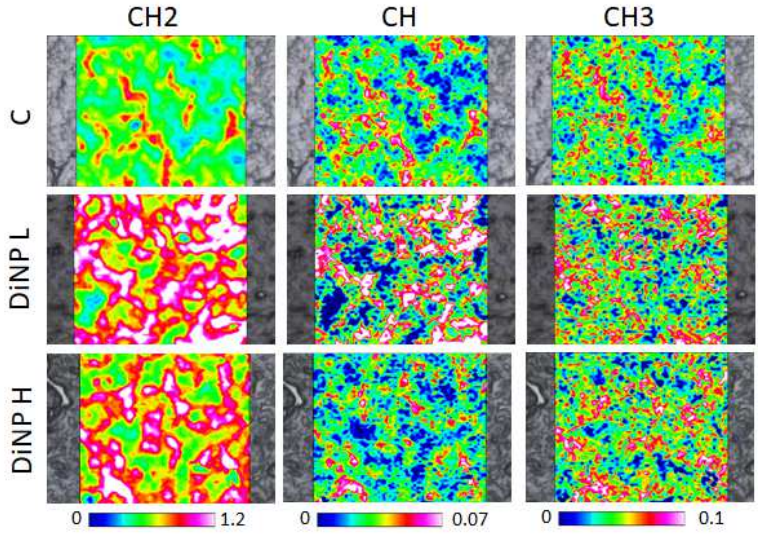
Figure 8. A) Representative histo-morphological aspect of the liver of the sea bream (H&E). MMC (circle) is often found near the exocrine pancreas, with a slight eosinophilic colouration with typical black spots due to the melanin content. Immunohistochemical detection of CAS-3 in the livers C (b), DiNP L (c) and DiNP H (d) The immunolabeling of Cas-3 was found exclusively at MMC (arrow). Scale = 50µm. E) Percentage of the hepatic area occupied by MMC in each experimental group. Data are reported as mean ± SD. Different letters indicate significant differences between the experimental groups (one-way ANOVA, post hoc test of Tukey, p <0.05).

4.3 Infrared imaging analysis

The biochemical composition of the liver sections of sea bream samples fed with the control diet and with the different DiNP doses was obtained by comparing the spectral outcomes of the different experimental groups. In fact, the FTIRI analysis allowed to map small and thin areas of liver sections and thus generate false colour images in which each pixel corresponds to an IR spectrum in the spectral range of 4000-800 cm^{-1} and represents the total absorbance of infrared radiation at that point on the map. The infrared imaging analysis has identified a different topographical location of lipids (maps CH2, CH and CH3) and proteins (maps PRT and PH) in all the mapped areas (figures 9a and 10a). As for lipids, a higher distribution was found in the samples treated with DiNP L both in terms of saturated lipidic alkyl chains (CH2) and unsaturated; no appreciable differences were observed in the CH3 groups (maps CH3) (Fig. 9a). In DiNP L, a greater distribution of phosphorylated proteins (PRT and PH maps) and amino acids containing COO^- groups (COO maps) was also detected (Fig. 10a). Carbohydrates and glycogen showed a lower distribution in both samples treated with DiNP L and DiNP H (maps COH and GLY) (Fig. 10a).

Furthermore, specific band area relationships with biological significance have been reported (Fig. 9b and 10b) and, from their statistical analysis, some considerations can be drawn. Compared to samples C and DiNP H, the livers DiNP L presented a statistically significant amount of lipids (CH3/TBM, Fig. 9b) (not appreciable in the maps CH3, Figure 9a), which had already been observed by Forner-Piquer et al., 2018a, also associated with an increase in both saturated (CH2/TBM) and unsaturated (CH/TBM) chains (Fig. 9b). In this experimental group, a statistically significant amount of proteins (PRT/TBM) and amino acids containing COO^- (COO/TBM) groups was also observed (Fig. 10b); furthermore, as phosphate groups (PH maps) and proteins (PRT maps) are located in the same areas of the IR map, the increase in the PH/TBM area-band ratio could be attributed to the presence of phosphorylated proteins (Fig. 10b). The minor presence of carbohydrates (COH/TBM) and, in particular, of glycogen (GLY/TBM), found in the DiNP L and DiNP H samples compared to the C control (Fig. 10b) is statistically significant.

A

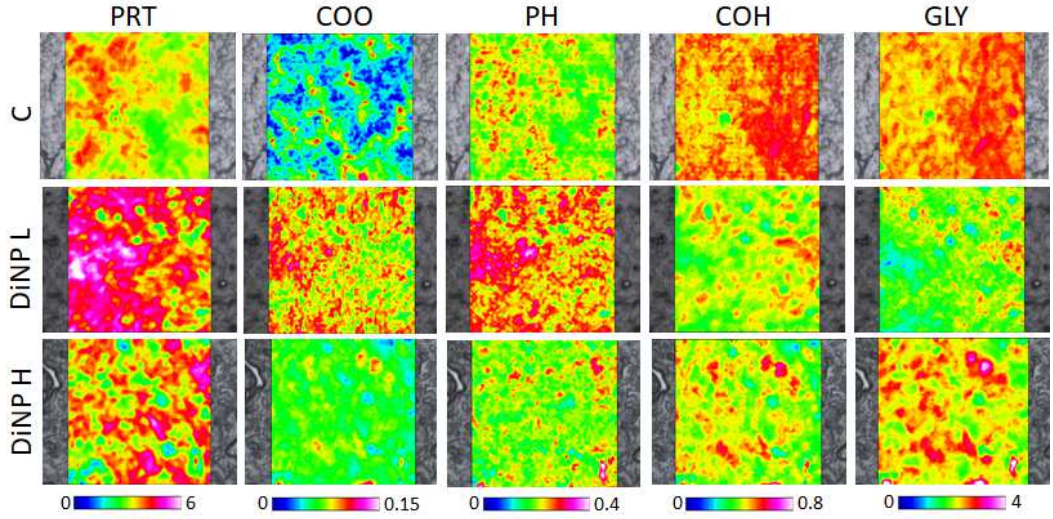


B

	C	DiNP L	DiNP H
CH2/TBM	0.0088 ± 0.0017 a	0.0109 ± 0.0019 b	0.0095 ± 0.0017 a
CH/TBM	0.0018 ± 0.0009 a,b	0.0023 ± 0.0012 a	0.0017 ± 0.0011 b
CH3/TBM	0.0023 ± 0.0008 a	0.0030 ± 0.0010 b	0.0023 ± 0.0009 a

Figure 9. A) Representative false colour images of gilthead liver (experimental groups C, DiNPL and DiNPH). Topographic distribution of: CH3 groups (alkyl lipid chains, CH3), CH2 groups (degree of saturation in lipid alkyl chains, CH2) and groups = CH (degree of unsaturation in lipid alkyl chains, CH). B) Effects of DiNP on hepatic lipid composition in terms of: alkyl lipid chains (CH3/TBM), saturation degree (CH2/TBM) and unsaturation (CH/TBM) in alkyl lipid chains. The ratios are expressed as mean ± SD. In arbitrary units (A.U.) (n = 5 for experimental group). Different letters indicate significant differences between the experimental groups (p <0.05), analysed with one-way ANOVA followed by Tukey's multiple comparison test.

A



B

	C	DiNP L	DiNP H
PRT/TBM	0.1506 ± 0.0231 a	0.1744 ± 0.0382 b	0.1455 ± 0.0246 a
COO/TBM	0.0049 ± 0.0008 a	0.0064 ± 0.0012 b	0.0044 ± 0.0009 a
PH/TBM	0.0088 ± 0.0013 a	0.0107 ± 0.0017 b	0.0089 ± 0.0014 a
COH/TBM	0.0215 ± 0.002442 a	0.0157 ± 0.0024 b	0.01675 ± 0.0024 b
GLY/TBM	0.1109 ± 0.01141 a	0.0755 ± 0.0099 b	0.0884 ± 0.0126 c

Figure 10. A) Representative false colour images of liver sections (experimental groups C, DiNPL and DiNPH). Topographic distribution of: proteins (PRT), groups COO^- (COO), phosphate groups (PH), carbohydrates (COH) and glycogen (GLY). B) Effects of DiNP on the hepatic lipid composition in terms of: proteins (PRT/TBM), groups COO^- (COO/TBM), phosphate groups (PH/TBM), carbohydrates (COH) and glycogen (GLY). The ratios are expressed as mean ± SD. In arbitrary units (A.U.) (n = 5 for experimental group). Different letters indicate significant differences between the experimental groups (p <0.05), analysed with one-way ANOVA followed by the Tukey multiple comparison test.

5. Discussion

The results obtained in this study are in agree with previous research showing activation of hepatic stress response after EDCs exposure (Jin et al., 2010a; 2010b; Zare et al., 2018). In particular, the modulation of antioxidant defence, the immune response and the possible onset of an apoptotic process were taken into consideration. We have therefore focused on different parameters that can be influenced by DiNP phthalate contamination.

Previous FTIRI results showed an increase in hepatic lipids, mainly triglycerides and fatty acids, in the gilthead fed with 5 µg of DINP kg^{-1} bw day^{-1} (Forner-Piquer et al., 2018a). The additional spectral characteristics obtained in this study enabled us to detect an increase of saturated fatty acids in fish contaminated with DiNP L, confirming th harmful action of this substance on the onset of liver disease. Evidence obtained from humans shows that saturated fat is more harmful than unsaturated fat and it triggers an increase in intrahepatic triglycerides, which predispose ti type 2 diabetes and cardiovascular diseases (Luukkonen et al., 2018). To prevent metabolic diseases, the hepatic lipid content should be reduced and a shift in the type of conservation, from saturation to poly-unsaturation, should also be promoted (Erickson et al.2018).

The liver plays a key role in the production of plasma proteins which then reach organs via blood circulation. Protein requirement increases during episodes of hepatocellular deterioration, for example when the liver drains nitrogen from other organs such as muscles. The increase observed in proteins levels in the livers of fish exposed to DiNP L correlates with a decrease in protein content measured in the muscle of the same samples (Carnevali et al., 2019) and it is hypothesized that the

amino acids released by the muscles can accumulate in the bloodstream of patients with liver disease, precisely because of reduced liver metabolism (Mezey, 1982). However, in DiNP L fish, the increase in protein levels is also associated with an increase in COO groups, which can be traced to an increase in the levels of aspartate, glutamate or pyruvate. An increase in aspartate, as a substrate of aspartate-aminotransferase (AST), has been documented in the case of liver lesions following exposure to EDC (Jiang et al., 2018a; Rangasamy et al., 2018; Abu-El-Zahab et al., 2019). Regarding pyruvate, in a previous study using the same fish, Forner-Piquer and collaborators (Forner-Piquer et al., 2018a) recorded a decrease of glycogen suggesting that an increase in pyruvate, the first designated substrate of the gluconeogenic pathway, (Rui, 2014) serves as a source of energy to generate ATP. Considering glutamate, which is also used for biosynthesis of the cellular antioxidant glutathione, it regulates cellular redox balance and the modulation of cell proliferation (Matés et al., 2002). However, all these hypotheses driven by the increase of COO groups mainly in DiNP L fish suggest a state of hepatic stress possibly associated with an increased need of ATP.

In fish exposed to DiNP L, the level of phosphorylated proteins suggests an increase in oxidative stress (Vileno et al., 2010). Oxidative stress can trigger the activation of multiple signalling pathways including phosphorylation cascades of mitogen-activated protein kinases, which could regulate cellular damage (Markou et al., 2009; Tomasi and Ramani, 2018).

As a primary response to stress, increases in levels of *gr* mRNA and cortisol are clear evidence of DiNP-induced stress. The increase in cortisol and its associated receptor has been widely observed in fish species exposed to different stress factors or changes in diet (Olivotto et al., 2002; Alves et al., 2010; Bermejo-Nogales et al., 2014; Piccinetti et al., 2015; Malandrakis et al., 2016; Carnevali et al., 2017; Piccinetti et al., 2017; Vargas et al., 2018; Zarantoniello et al., 2018).

A fast and reliable biomarker for the early detection of exposure to contaminants is HSP70 and the increase in mRNA in both groups fed with DiNP-contaminated diet, confirms its key role in stress response, as previously observed in *Gobius niger* (Carnevali and Maradonna, 2003; Migliarini et al., 2005; Maradonna and Carnevali, 2007). When the correct folding of proteins and cellular functions cannot be preserved, HSP70 and other chaperonins are able to determine the fate of cells interacting with the key molecules of the cell cycle as *p53* (Zylicz, 2001; Reyes-Becerril et al., 2013). In response to low levels of oxidative stress, *p53* exhibits antioxidant activity and ensures cell survival in cases of high oxidative stress, showing pro-oxidative activity that activates cell death (Jiang et al., 2015). Among the chaperonins, GRP75, a mitochondrial chaperone, acts directly on P53 and could mediate the beneficial and harmful effects depending on the type of cell and the pathological context (Guo et al. 2014). Furthermore, P53 can activate BAX, thus triggering the activation of the apoptotic pathway: the results obtained in this study show an increase in *grp75* and *p53*, suggesting that apoptosis could be activated however, the lack of variation of *bax* and *caspase 3* suggest instead that the organism is still able to counteract the damage induced by contaminants.

The in-situ hybridization of CAS-3 highlighted the attention on melano-macrophage centres (MMC) with the primary function of phagocytosis. They play a dual role, being involved both in immune defence and in normal non-immunological processes (Zuasti et al., 1998; Passantino et al., 2014; Steinel and Bolnick, 2017). Since no significant changes were observed, neither in the percentage of area covered by MMC nor in their abundance, the apoptotic process can be excluded. These results are also supported by the lack of variation of the *csf-1r* mRNA encoding a protein that acts as the receptor for *colony-stimulating factor 1*, a cytokine that controls growth, differentiation and function of macrophages (Roca et al., 2006) confirming that in the livers exposed to DiNP, the numbers of melanomacrophages are not affected. The lack of modulation of the signals described above in fish fed high-dose DiNP (DiNP H) supports the non-monotonic mode of action of this compound as previously described in Zebrafish (Santangeli et al., 2017).

Considering the immune response, the correct redox balance is guaranteed by the coordinated action of a series of signals including G6PDH, GPX1 and GLUT RED. In DiNP-fed fish, regulation of *g6dph* mRNA levels could be an explanation for the increase in glucose-6-

phospate (G6P) consumption to generate NADPH. G6P is necessary to trigger the pentose phosphate pathway, and it derives from the conversion of glucose into a reaction catalyzed by GCK; therefore, the decrease in glycogen measured in DiNP fish suggests that the glycogen content has been used to counteract the increase in cell energy demand. Under stressed conditions, fish mobilize energy reserves, thus activating hepatic glycogenolysis, which is the perfect source of glucose for the production of G6P. NADPH is used by GLUT-RED and mRNA levels increase significantly to maintain the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio. In DiNP-fed fish, the increase in glut-red mRNA is associated with an upregulation of *gpx1* which suggests that reduced glutathione was used as an electron donor to guide the conversion of H_2O_2 into water. In the same way, in this phase, in fish fed with DiNP, catalase could act in the conversion of hydrogen peroxide into water, suggesting a high-pressure pro-oxidant as previously observed in Zebrafish exposed to EDCs (Xu et al., 2013a, b; Jiang et al., 2018b). Furthermore, the increase in *gst* mRNA in DiNP H fed fish suggests the conjugation of GSH to a wide range of hydrophobic and electrophilic molecules including many xenobiotics and many products of oxidative metabolism, thus making them less toxic and easily removable from the cell (Allocati et al., 2018). A similar response had been previously observed in young bream exposed to food contamination by alkylphenols (Traversi et al., 2014), while no changes were detected when the fish were fed *bisphenol A* (BPA) (Maradonna et al., 2014) suggesting that this different response may depend on the type of xenobiotic and its degree of toxicity. Finally, in the groups exposed to both DiNP doses, the upregulation of mRNA expression of all these signals involved in the redox balance could explain the decrease in glycogen detected by the FTIRI method, which to a certain extent represents the fuel of the route described. The results are supported by previous studies on different species of fish exposed to different stress factors, in which a significant increase in plasma glucose levels was associated with a significant reduction in hepatic glycogen (Somaiah et al., 2014; Biswas, 2018). It is interesting to note that the gck mRNA was also significantly down-regulated similarly to the reduction in the conservation of hepatic glycogen, the key substrate for the catalysing reaction.

Pla2 has been shown to play a key role in the activation of TNF alpha-cytolytic activity in mammals (Adamson et al., 1994). In this study, *pla2* and *tnfα* show the same tendency and together with the increase in pro-inflammatory *il-1β* and *5 lox* mRNAs, it triggers the release of leukotrienes, the molecules implicated in immune and inflammatory responses (Serhan et al., 2015). Similar results showing alterations in the level of TNFα and *il-1β* have been shown in newly born Zebrafish exposed to BPA or NP (Xu et al., 2013b).

We can conclude that the results obtained in this study show that DiNP interrupts liver function in S. aurata. Both doses tested had negative outcomes on the antioxidant defence and on the body's immune system, providing various insights into the ability to prevent the onset of cellular apoptosis. DiNP-fed fish had higher cortisol levels and fatty liver, due to stress conditions induced by this pollutant, with the most damaging effects caused by the lowest DiNP concentration. These results confirm that DiNP cannot be considered a valid substitute for DEHP, since it is not a harmless compound. Future studies could improve basic knowledge about this emerging contaminant, hoping to raise awareness for its further applications.

General Conclusions

Environmental laws in force in a country, constantly updated, both for the new materials presented in the market - of which non-toxicity must be proved - and for the new knowledge acquired in the field of health. In Italy, an important milestone in environmental management has been represented by the Ministerial Decree n. 173, which finally recognized the importance of ecotoxicology. The necessity of integrating ecotoxicological data with chemical ones, in order to define the quality of marine dredged sediments deeply emerged. In this light the risk is related to the relevance given to ecotoxicological tests which could strongly condition the quality of the sediment, even in the absence of a chemical risk. Too "sensitive" ecotoxicological tests allow to define a very low-quality sediment, with a difficult economic impact to manage.

In this study it was demonstrated that comparing the results obtained with ecotoxicological assay with those of chemical analysis and observing the derived Hazard Quotient, it emerged that high levels of contaminants could not correspond evident ecotoxicological effects. From the chemical point of view, it could be done the fact that some polluting molecules have not been taken into consideration by D.M. 173, such as phthalates. The Italian legislation, and in particular the Environmental Consolidation Act (Legislative Decree 152/2006) and the subsequent Water Framework Directive (2000/60 /CE) did not include the search for phthalates in internal surface waters or in "other waters", at least until November 2015 when, on transposition of the European Directive 2013/39/EU, the Legislative Decree 172 was issued, including among the priority substances also Di(2-ethyl-hexyl)phthalate (DEHP) with Average Annual Quality Standard (SQA-MA) is 1.3 µg/L both in internal surface waters and in other waters. Considering that by now several scientific articles have highlighted the risk associated to these molecules, and also considering their environmental presence (data from ARPAM for the year 2018 range from 0.016 µg/L to 0.720 in rivers, and from 0.016 to 0.703 µg/L in lakes), its detection and those of its congeners in elutriates (Kicham et al., 2012) could be an important novelty for a better sediment management.

On the ecotoxicological side, it must be remembered that the use of biological assay was initially aimed at defining a perturbation that had already occurred; instead today, in the D.M. 173, they are used together with the chemical parameters to have a predictive approach, using the analyses as a forecasting tool for the evaluation of the environmental risk and the classification of the different matrices.

Ecotoxicological assays are used in the evaluation of the quality of the various matrices subject to contamination risks before, during and after the stress event (e.g. dredging activity) also to assess the effectiveness of the intervention during time (biomonitoring). Toxicity is usually saw on liquid matrices (e.g. surface waters, drainage, solid matrices), this is an advantage because the toxicity tests carried out directly on the solid matrix are affected by the non-negligible interactions between the soil and the bioavailable toxic component. It should also be considered that the bioassay on the solid matrix have the advantage of using the whole matrix and not only the aqueous extract, getting closer to the real situation. Although the use of living organisms in toxicity tests is well recognized and widely considered by methodologies and protocols developed by international environmental organization (EPA, ASTM, ISO, APAT), the occurrence of some limitation, also of ecological relevance, should be considered. An example could be that they do not perfectly simulate field conditions and don't consider the eventual gaseous contamination (e.g. from H_2S). The toxicity of complex matrices must therefore be evaluated using a battery of bioindicators, in order to analyse the broader spectrum of effects on organisms. The strength of these analyses therefore relies in the careful selection of the tests to be performed, the key organisms to be used and the endpoints to be evaluated. Considering the sensitivity of individual organisms to the particular classes of contaminants without the "matrix effect" is important: for many organisms and for some substances, a wide literature describing the dose-response curve in the laboratory, allowing to hypothesize the behaviour of the organism in the presence of these substances, exists. The

knowledge of these calibration curves with pure substances allows to study and characterize the effect of the matrix or of the sample manipulation (e.g. preparation of the elutriate), as well as the interaction with the other substances present. Unfortunately for some classes of contaminants (e.g. PAHs and phthalates) there is still not a large literature, above all for the difficulty in the control of the experimental conditions.

The evaluation of toxicity is one of the most current topics of discussion not only in the world of research, but also in the political-administrative one. The problem of defining the toxicity of a sample is crucial to establish qualitative criteria and apply remediation programs and administrative sanctions. There have been numerous efforts to define the selection criteria for an ecotoxicological test (Van Gestel et al., 1997), while very little exists on the interpretation of test battery results and on the development of reading scales. One of the many tests that can be cited has developed by Ahlf and Heise (2005) who used a 5-test battery for sediments (of freshwater), using the percentage of inhibition with respect to the control as the endpoint. For each test the percentage of inhibition was set (in an arbitrary way) for low/zero response, moderate and strong. However, this creates problems because the percentages so established can also be very different for different tests and organisms, as they reflect the different sensitivity and relevance of the different essays.

For all these reasons, chemical and ecotoxicological data should be supported by molecular data. In this study, the results obtained clearly demonstrated the suitability of *C. gigas* embryo as an excellent experimental model to obtain clear evidence about elutriated toxicity. In fact, despite the lack of correspondence between chemical and ecotoxicological data for the three selected sediment, which let hypothesize their different toxicity level, the molecular results obtained strongly suggest the ability of all sediments to cause oxidative stress in developing oyster.

In addition, to pinpoint the attention on the dangerous role of phthalates, considering their persistence in the environment and their ability to bioaccumulate in tissues as well as to biomagnificate along the food web, adult males of *S. aurata* were exposed, through the diet, to two doses of DiNP, showing the ability of this compound to affect both the fish metabolism and antioxidant system. The evidence obtained in this study, strongly suggest to environmental agencies that the list of compounds to be monitored and considered for a better environmental management should be implemented.

At the end, the results obtained in this study using an integrative approach allowed to gain a deeper knowledge on sediment toxicity, both from a chemical and a biological point of view.

Bibliography

- Abbas M., Adil M., Ehtisham-ul-Haque S., Munir B., Yameen M., Ghaffar A., Shar G.A., Asif Tahir M., Iqbal M. (2018). *Vibrio fischeri* bioluminescence inhibition assay for ecotoxicity assessment: A review. Science of the Total Environment, 626, pp. 1295–1309;
- Abdel-Shafy H.I., Mansour M.S.M. (2016). A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. Egyptian Journal of Petroleum, Volume 25, Issue 1, March 2016, pp. 107-123;
- Abu-El-Zahab H.S.H., Hamza R.Z., Montaser M.M., El-Mahdi M.M., Al-Harthi W.A. (2019). Antioxidant, antiapoptotic, antigenotoxic, and hepatic ameliorative effects of L-carnitine and selenium on cadmium-induced hepatotoxicity and alterations in liver cell structure in male mice. Ecotoxicology and Environmental Safety, 173, pp. 419–428;
- Adams R.H., Domínguez-Rodríguez V.I., Zavala-Cruz J. (2015) *Vibrio fischeri* Bioassay for Determination of Toxicity in Petroleum Contaminated Soils from Tropical Southeast Mexico. Sains Malaysiana 44, pp. 337–346;
- Adamson G.M., Carlson T.J., Billings R.E. (1994). Phospholipase A2 activation in cultured mouse hepatocytes exposed to tumor necrosis factor-α. Journal of Biochemistry and Toxicology, 9, pp. 181–190;
- Aebi H. (1984) Catalase in vitro. Methods in Enzymology, 105, pp. 121–126;
- Ahlf W. and Heise S. (2005). Sediment Toxicity Assessment. Rationale for effect classes. Journal of Soils and Sediments 5, pp. 16-20;
- Allocati N., Masulli M., Di Ilio C., Federici L. (2018). Glutathione transferases: substrates, inihibitors and pro-drugs in cancer and neurodegenerative diseases. Oncogenesis, 24;7(1), p. 8;
- Alves R.N., Cordeiro O., Silva T.S., Richard N., de Vareilles M., Marino G., Di Marco P., Rodrigues P.M., Conceição L.E.C. (2010). Metabolic molecular indicators of chronic stress in gilthead seabream (*Sparus aurata*) using comparative proteomics. Aquaculture, 299, pp. 57–66;
- Anonymous. (1998). Guidelines for the treatment of animals in behavioural research and teaching. Animal Behaviour, 55, pp. 251–257;
- Anonymous. (2012). Guidelines for the treatment of animals in behavioural research and teaching. Animal Behaviour, 83, pp. 301–309;
- Arabaci M., Yilmaz Y., Ceyhun S.B., Erdogan O., Dorlay H.G., Diler I., Akhan S., Kocabas M., Ozdemir K., Koyun H., Koncagul S. (2010). A review on population characteristics of Gilthead Seabream (*Sparus aurata*). Journal of Animal and Veterinary Advances;
- Arends R.J., Mancera J.M., Muñoz J.L., Wendelaar B.S.E., Flik G. (1999). The stress response of the gilthead sea bream (*Sparus aurata*) to air exposure and confinement. Journal of Endocrinology, 163, pp. 149–157;
- APAT. (2001). Elementi per la caratterizzazione fisicochimica biologica ed ecotossicologica dei parametri addizionali (D.Lgs. 152/99) nella matrice acquosa, nel sedimento e nel biota. RTI CTN-AIM 4/2001;
- ASTM E1391-03. (2014). Standard Guide for Collection, Storage, Characterization and manipulation of sediments for toxicological testing and for selection of samples used to collect benthic invertebrates;
- Banoune B., Melbouci B., Rosquoët F., Langlet T. Treatment of river sediments by hydraulic binders for valorization in road construction. Bulletin of Engineering Geology and the Environment. November 2016, Volume 75, Issue 4, pp. 1505–1517;
- Barakat O. A., Kim M., Qian Y., Wade T. L. (2002). Organochlorine pesticides and PCB residues in sediments of Alexandria Harbour, Egypt. Baseline/Marine Pollution Bulletin, 44, pp. 1421–1434;

- Barton B.A., Ribas L., Acerete L., Tort L. (2005). Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata*, to acute handling. Aquaculture Research, 36, pp. 172–179;
- Bellas J., Nieto Ó., Beiras R. (2011). Integrative assessment of coastal pollution: Development and evaluation of sediment quality criteria from chemical contamination and ecotoxicological data. Continental Shelf Research, 31, pp. 448–456;
- Benedetti M., Ciaprini F., Piva F., Onorati F., Fattorini D., Notti A., Ausili A., Regoli F. (2012). A multidisciplinary weight of evidence approach for classifying polluted sediments: integrating sediment chemistry, bioavailability, biomarkers responses and bioassays, Environment International, 38, pp. 17-28;
- Bermejo-Nogales A., Nederlof M., Benedito-Palos L., Ballester-Lozano G.F., Folkedal O., Olsen R.E., Sitjà-Bobadilla A., Pérez-Sánchez J. (2014). Metabolic and transcriptional responses of gilthead seabream (Sparus aurata L.) to environmental stress: New insights in fish mitochondrial phenotyping. General and Comparative Endocrinology, 205, pp. 305–315;
- Bezak-Mazur E., Widiak M., Ciupa T. (2001). A speciation analysis of aluminium in the River Silnica. Polish Journal of Environmental Studies, 10(4), pp. 263–268;
- Biswas S. (2018). Effect of Chlorpyrifos toxicity on non target fresh water fishes: A Review. 6, pp. 376–381;
- Bocchetti R., Fattorini D., Pisanelli B., Macchia S., Oliviero L., Pilato F., Pellegrini D., Regoli F. (2008). Contaminant accumulation and biomarkers responses in caged mussels, *Mytilus galloprovincialis*, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas. Aquatic Toxicology, 89, pp.257–266;
- Buccolieri A., Buccolieri G., Cardellicchio N., Dell'Atti A., Di Leo A., Maci A. Heavy metals in marine sediments of Taranto Gulf (Ionian Sea, Southern Italy). Marine Chemistry 99 (2006) pp.227-235;
- Burton G. A. (1995). Critical issues in sediment bioassays and toxicity testing. Journal of Aquatic Ecosystem Health, pp.151-156;
- Burton G.A., Chapman P.M., Smith E.P. (2002). Weight-of-Evidence approaches for assessing ecosystem impairment, Human and Ecological Risk Assessment, 8(7), pp. 1657-1673;
- Bruins M.R., Kapil, S., Oehme, F.W. (2000). Microbial resistance to metals in the environment. Ecotoxicol. Environmental Safety, 45, pp. 198-207;
- Calabrese E. J. and Baldwin L. A. (2001). Hormesis: a generalizable and unifying hypothesis. Critical Review in Toxicology, vol 31, pp. 353-424;
- Calabrese E. J. (2008). Hormesis: Principles and Applications for Pharmacology and Toxicology. American Journal of Pharmacology and Toxicology 3 (1), pp. 59-71;
- Calow P. (2009). Handbook of Ecotoxicology, Handbook of Ecotoxicology;
- Campbell P.G., Lewis, A. G., Chapman, P.M., Crowder, A., Fletcher W. K., Imber B., Luoma, S. N., Stokes, P. M. and Winfrey, M. (1988). Biologically Available Metals in Sediments. National Research Council of Canada, Ottawa, p. 298;
- Cao X.L. (2010). Phthalate Esters in Foods: Sources, Occurrence, and Analytical Methods. Comprehensive Reviews of Food Science and Food Safety, 9, pp. 21–43;
- Capello M., Cutroneo L., Ferranti M.P., Budillon G, Bertolotto R.M., Ciappa A., Cotroneo Y., Castellano M., Povero P., Tucci S. (2014). Simulations of dredged sediment spreading on a Posidonia oceanicameadow off the Ligurian coast, Northwestern Mediterranean. Marine Pollution Bulletin 79, pp. 196–204;
- Cappuyns V., Deweirt V., Rousseau S. (2015). Dredged sediments as a resource for brick production: Possibilities and barriers from a consumers' perspective. Waste Management Volume 38, April 2015, pp. 372-380;

- Carnevali O. and Maradonna F. (2003). Exposure to xenobiotic compounds: Looking for new biomarkers. General and Comparative Endocrinology, 131, pp. 203–209;
- Carnevali O., Tosti L., Speciale C., Peng C., Zhu Y., Maradonna F. (2010). DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis. PLoS One, 5, pp. 1–7;
- Carnevali O., Notarstefano V., Olivotto I., Graziano M., Gallo P., Di Marco Pisciottano I., Vaccari L., Mandich A., Giorgini E., Maradonna F. (2017). Dietary administration of EDC mixtures: A focus on fish lipid metabolism. Aquatic Toxicology, 185, pp. 95-104;
- Carnevali O., Giorgini E., Canuti D., Mylonas C., Forner-Piquer I., Maradonna F. (2019). Diets contaminated with Bisphenol A and Di-isononyl phtalate modify skeletal muscle composition: A new target for environmental pollutant action. Science of the Total Environment, 658, pp. 250–259;
- Casado Martinez M.C., Buceta J.L., Forja J.M., Del Valls T.A. (2006). Ejercicio interlaboratorio de bioensayos marinos para la evaluacion de la calidad ambiental de sedimentos costeros. Cien Mar 2006a; 32, pp. 121-128;
- Cataudella S. and Bronzi P. (2001). Acquacoltura Responsabile. Unimar-Uniprom, Roma, p. 683;
- Cavaletto M., Ghezzi A., Burlando B., Evangelisti V., Ceratto N., Viarengo A. (2002). Effect of hydrogen peroxide on antioxidant enzymes and metallothionein level in the digestive gland of *Mytilus galloprovincialis.* Comparative Biochemistry and Physiology Part C, 131, pp. 447–455;
- Chan C.Y., Wang W.X. (2019). Biomarker responses in oysters *Crassostrea hongkongensis* in relation to metal contamination patterns in the Pearl River Estuary, southern China. Environmental Pollution. 251, pp. 264–276;
- Chapman M.P. and Wang F. (2001) Assessing sediment contamination in estuaries. Environmental Toxicological Chemistry, 20, pp. 3-22;
- Chapman M.P., McDonald B.G., Lawrence G.S. (2002). Weight-of-Evidence issues and framework for sediment quality (and other) assessments, Human and Ecological Risk Assessment, 8(7), pp. 1489-1515;
- Cheung C.C.C., Siu W.H.L., Richardson B.J., De Luca-Abbott S.B., Lam P.K.S. (2004). Antioxidant responses to benzo[a]pyrene and Aroclor 1254 exposure in the green-lipped mussel, Perna viridis. Environmental Pollution;
- Christy A.A. and Egeberg P.K. (2006). Quantitative determination of saturated and unsaturated fatty acids in edible oils by infrared spectroscopy and chemometrics. Chemometrics and Intelligent Laboratory Systems, 82, pp. 130–136;
- Cibic T., Acquavita A., Aleffi F., Bettoso N., Blasutto O., De Vittor C., Falconi C., Falomo J., Faresi L., Predonzani S., Tamberlich F., Fonda S. (2008). Integrated approach to sediment pollution: A case study in the Gulf of Trieste. Marine Pollution Bulletin, 56, pp. 1650–1667;
- Clark James S., Steven R. Carpenter, Mary Barber, Scott Collins, Andy Dobson, Jonathan A. Foley, David M. Lodge, Mercedes Pascual, Roger Pielke Jr., William Pizer, Cathy Pringle, Walter V. Reid, Kenneth A. Rose, Osvaldo Sala, William H. Schlesinger, Diana H. Wall and David Wear (2001). Ecological Forecasts: An Emerging Imperative. Science, vol. 293, pp.657-660;
- Coimbra A.M., Peixoto M.J., Coelho I., Lacerda R., Carvalho A.P., Gesto M., Lyssimachou A., Lima D., Soares J., André A., Capitão A., Castro L.F., Santos M.M. (2015). Chronic effects of clofibric acid in zebrafish (*Danio rerio*): A multigenerational study. Aquatic Toxicology, 160, pp. 76–86;
- Conte F.S. (2004). Stress and welfare of cultured fish. Applied Animal Behaviour Science, 86, pp. 205–223;

- Corradetti B., Stronati A., Tosti L., Manicardi G., Carnevali O., Bizzaro D. (2013). Bis-(2-ethylexhyl) phthalate impairs spermatogenesis in zebrafish (*Danio rerio*). Reproductive Biology, 13, pp. 195–202;
- Couvidat J., Benzaazoua M., Chatain V., Bouamrane A., Bouzahzah H. (2016). Feasibility of the reuse of total and processed contaminated marine sediments as fine aggregates in cemented mortars. Construction and Building Materials, 112, pp. 892–902;
- CPSC. (2010). Toxicity review of diisononyl phthalate (DiNP) Cosumer Product Safety Commission. Available online on: http://www.cpsc.gov;
- Decree n.173. (2016). Ministerial Decree of 15th July 2016: Regolamento recante modalità e criteri tecnici per l'autorizzazione all'immersione in mare dei materiali di escavo di fondali marini. 16G00184. Gazzetta Ufficiale, Serie Generale n. 208 del 06/09/2016 – Suppl. Ordinario n. 40;
- Del Valls T.A., Andres A., Belzunce M.J., Buceta J.L., Casado-Martinez M.C., Castro R. (2004). Chemical and ecotoxicological guidelines for managing disposal of dredged material. Trends Analytical Chemistry, 23, pp. 819-828;
- Della Croce N., Cattaneo Vietti R., Danovaro R. (1997). Ecologia e protezione dell'Ambiente marino costiero. UTET Università. p. 416;
- Devos C., Vliegen M., Willaert B., David F., Moens L., Sandra P. (2005). Automated headspace-solid-phase micro extraction-retention time locked-isotope dilution gas chromatography-mass spectrometry for the analysis of organotin compounds in water and sediment samples. Journal of Chromatography A 1079, pp. 408–414;
- Dotto M. (2013) Sulla tecnica del dragaggio ambientale. Alcuni casi di studio nella Regione Veneto. Tesi di Laurea Magistrale in Ingegneria Civile Idraulica. Università degli Studi di Padova;
- Edge K.J., Johnston E.L., Roach A.C., Ringwood A.H. (2012). Indicators of environmental stress: Cellular biomarkers and reproductive responses in the Sydney rock oyster (*Saccostrea glomerata*). Ecotoxicology 21, pp. 1415–1425;
- EFSA. (2008). Scientific report of EFSA on animal welfare aspects of husbandry systems for farmed European sea bass and Gilthead sea bream. (Question No EFSA-Q-2006- 149). Annex I to the EFSA Journal, 844, pp. 1–89;
- Erftemeijer Paul L. A., Bernhard Riegl, Bert W. Hoeksema, Peter A. Todd. (2012). Environmental impacts of dredging and other sediment disturbances on corals: A review. Marine Pollution Bulletin, 64, pp. 1737–1765;
- Erickson M.L., Haus J.M., Flask C., Kirwan J.P. (2018). Hepatic Lipid Profiling in Fatty Liver Disease—Degree of Saturation Matters. Diabetes, 67, p.1835;
- Fabioux C., A. Huveta C. Lelongc, R. Roberta S. Pouvreaua, J. Y. Daniela, C. Minguanta and M. Le Pennec. (2004). Oyster vasa-like gene as a marker of the germline cell development in *Crassostrea gigas*. Biochemical and Biphysical Research Communications 320, pp. 592-598;
- FAO. (2018). The State of World Fisheries and Aquaculture, 2018. Available on http://www.fao.org/3/i9540en/i9540en.pdf;
- Farcy E., Voiseux C., Lebel J.M., Fievet B. (2007). Seasonal changes in mRNA encoding for cell stress markers in the oyster *Crassostrea gigas* exposed to radioactive discharges in their natural environment. Science of the Total Environment, 374, pp. 328–341;
- Farnè L. (2003). Biorisanamento di suoli contaminati da idrocarburi policiclici aromatici: prove di biotrattabilità e caratterizzazione di un consorzio utile alla biodegradazione. Tesi di laurea in Biotecnologie Industriali, Facoltà di Scienze Matematiche Fisiche e Naturali, Università degli Studi di Bologna;
- Forner-Piquer I., Mylonas C., Calduch-Giner J., Maradonna F., Gioacchini G., Allarà M., Piscitelli F., Di Marzo V., Pérez-Sánchez J., Carnevali O. (2018a). Endocrine disruptors in the diet of male Sparus aurata: Modulation of the endocannabinoid system at the hepatic and

central level by Di-isononyl phthalate and Bisphenol A. Environment International, 119, pp. 54–65;
- Forner-Piquer I., Santangeli S., Maradonna F., Rabbito A., Piscitelli F., Habibi H.R., Di Marzo V., Carnevali O. (2018b). Disruption of the gonadal endocannabinoid system in zebra fish exposed to diisononyl phthalate. Environmental Pollution, 241, pp. 1–8;
- Forner-Piquer I., Mylonas C., Fakriadis I., Papadaki M., Piscitelli F., Di Marzo V., Calduch-Giner J., Pérez-Sánchez J., Carnevali O. (2019) Effects of diisononyl phthalate (DiNP) on the endocannabinoid and reproductive systems of male gilthead seabream (*Sparus aurata*) during the spawning season. Archives of Toxicology, 93(3), pp. 727-741;
- Fratini G. (2012). Effect of different farming sites and techniques on the growing performances and quality of Pacific oysters (*Crassostrea gigas*, Thunberg). Tesi di dottorato. Università degli Studi di Firenze. p. 162;
- Fridovich I. (2013). Superoxide Dismutase. Encyclopedy of Bioogical Chemistry. Second Edition, 65, pp. 963–974;
- Frignani M. and Turci C. (1981). Il ruolo dei sedimenti nei fenomeni di eutrofizzazione costiera. CNR AQ/2/12 pp.101;
- Froehner K., Backhaus T., Grimme L.H. (2000). Bioassays with Vibrio fischeri for the assessment of delayed toxicity. Chemosphere 40, pp. 821–828;
- Fujimura N. and Takamura K. (2000). Characterization of an ascidian DEAD-box gene, Ci-DEAD1: specific expression in the germ cells and its mRNA localization in the posterior-most blastomeres in early embryos, Development Genes and Evolution, 210, pp. 64–72;
- Gabellini M., Ausili A., Romano E., Geraldini S., Tornato A. and Dastoli S. (2011). Sedimenti Contaminati in Italia: Stato dell'Arte, Gestione e Bonifica. Conference Paper. Conference: REM TECH 2011, At Ferrara;
- Gailani J. Z., Lackey T. C., King Jr. D.B., Bryant D., Sung-Chan K., Shafer D. J. (2016). Predicting dredging-associated effects to coral reefs in Apra Harbor,Guam - Part 1: Sediment exposure modeling. Journal of Environmental Management 168, pp. 16-26;
- Galassi G. (2016). Il landfarming per la valorizzazione dei sedimenti di dragaggio al fine di un loro riutilizzo in orticoltura. Tesi di laurea magistrale in Conservazione ed Evoluzione. Università degli Studi di Pisa;
- Garcıa-Guerra J.M., Garcıa-Gomez J.C. (2005). Assessing pollution levels in sediments of a harbour with two opposing entrances. Environmental implications Journal of Environmental Management, 77, pp. 1–11;
- Giaime F., Onorati F., Sprovieri M., Quinci E.M., Ferrara F., Carere M. (2016). The management of marine sediments: towards a univocal path of quality assessment in the new regulatory instruments. Environmental Engineering Vol. 3 n. 4;
- Giorgini E., Sabbatini S., Conti C., Rubini C., Rocchetti R., Re M., Vaccari L., Mitri E., Librando V. (2015a). Vibrational mapping of sinonasal lesions by Fourier transform infrared imaging spectroscopy. Journal of Biomedical Optics, 20, 125003;
- Giorgini E., Tosi G., Conti C., Staibano S., Ilardi G., Sabbatini S. (2015b). FTIR micro spectroscopic characterization of *Spitz nevi*. Spectrochim Acta - Part A Molecular and Biomolecular Spectroscopy, 141, p.103;
- Girón-Pérez M.I. (2010). Relationships between innate immunity in bivalve molluscs and environmental pollution Abstract The immune system of invertebrates, such as molluscs consists of innate mechanisms very effective against antigens commonly present in the environment. Howeve. Invertebr. Surviv. J. 7, pp. 149–156;
- Giusti L. (2001). Heavy metal contamination of brown seaweed and sediments from the UK coastline between the Wear river and the Tees river. Environment International, 26, pp. 275-286;

- Glass C. and Silvestrini J. (1998). Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. Water Research, vol.32, n.3, pp. 831-839;
- Guevara-Riba A., Sahuquillo A., Rubio R., Rauret G. (2004). Assessment of metal mobility in dredged Harbour sediments from Barcelona, Spain. Science Total Environment, 321, pp. 241–255;
- Gray L.E. Jr, Ostby J., Furr J., Price M., Veeramachaneni D.N., Parks L. (2000). Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicological Science, 58, pp. 350–365;
- Guo W., Yan L., Yang L., Liu X., Qiukai E., Peiye Gao, Xiaofei Ye, Wen Liu, Ji Zuo. (2014). Targeting GRP75 improves HSP90 inhibitor efficacy by enhancing p53-mediated apoptosis in hepatocellular carcinoma. PLoS One 9, pp. 1–11;
- Hannas B.R., Lambright C.S., Furr J., Evans N., Foster P.M., Gray E.L., Wilson V.S. (2012). Genomic biomarkers of phthalate-induced male reproductive developmental toxicity: a targeted RT-PCR array approach for defining relative potency. Toxicological Sciences, 125, pp.544–57;
- Harkey G.A., Landrum P.F., Klaine S.J. (1994). Comparison of whole sediment, elutriate, porewater exposures for usein assessing sediment associated organic contaminants in bioassays. Environmental Toxicology and Chemistry, 13 (8), pp. 1315-1329;
- Hart C.E., Lauth M.J., Hunter C.S., Krasny B.R., Hardy K.M. (2016). Effect of 4-nonylphenol on the immune response of the Pacific oyster *Crassostrea gigas* following bacterial infection with Vibrio campbellii. Fish Shellfish Immunology 58, pp. 449–461;
- Helcom. (2010) Hazardous substances in the Baltic Sea - An integrated thematic assessment of hazardous substances in the Baltic Sea. Baltic Sea Environment, Proc. No. 120B;
- His E., Seaman M. N. L. and Beiras R. (1997). A simplification the bivalve embryogenesis and larval development bioassay method for water quality assessment. Wat. Res. Vol. 31, No. 2, pp. 351-355;
- Homady M., Hussein H., Jiries A., Mahasneh A., Farah A., Khleifat K. (2002) Survey of Some Heavy Metals in Sediments from Vehicular Service Stations in Jordan and Their Effects on Social Aggression in Prepubertal Male Mice. Environmental Research Volume 89, Issue 1, May 2002, pp. 43-49;
- Hsu D.K., Yang R.Y., Liu F.T. (2006). Galectins in Apoptosis. Methods of Enzymology. 417, pp. 256–273;
- ICRAM. (2001). Anna Maria Cicero (ICRAM, Roma) e Irene Di Girolamo (Ministero dell'Ambiente e della Tutela del Territorio). Metodologie Analitiche di Riferimento. 2001;
- Jiang T., Ding X., Wu Y., Ren B., Li W., Lv H., Chen J. (2018a), Hepatoprotective Effect of Loquat Leaf Flavonoids in PM2.5-Induced Non-Alcoholic Fatty Liver Disease via Regulation of IRs-1/Akt and CYP2E1/JNK Pathways. International Journal of Molecular Sciences, Vol 19, pp. 3005;
- Jiang J., Lv L., Wu S., An X., Wang F., Liu X., Zhao X. (2018b). Developmental toxicity of kresoxim-methyl during zebrafish (*Danio rerio*) larval development. Chemosphere, 219, pp. 517–525;
- Jiang L., Hickman J.H., Wang S.J., Gu W. (2015). Dynamic roles of p53-mediated metabolic activities in ROS-induced stress responses. Cell Cycle 14, pp. 2881–2885;
- Jin Y., Chen R., Liu W., Fu Z. (2010a). Effect of endocrine disrupting chemicals on the transcription of genes related to the innate immune system in the early developmental stage of zebrafish (*Danio rerio*). Fish Shellfish Immunology, 28, pp. 854–861.;
- Jin Y., Zhang X., Lu D., Sun L., Qian H., Liu W., Fu Z. (2010b). Histopathological and proteomic analysis of hepatic tissue from adult male zebrafish exposed to 17β-estradiol. Environmental Toxicology and Pharmacology, 29, pp. 91–95;

- Jo P.G., Choi Y.K., Choi C.Y. (2008). Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, Crassostrea gigas in response to cadmium exposure. Comp. Biochem. Physiol. - C Toxicology and Pharmacology, 147, pp. 460–469;
- Jones R., Ricardo G. F., Negri A. P. (2015). Effects of sediments on the reproductive cycle of corals. Marine Pollution Bulletin, vol. 100, Issue 1, 15 November 2015, pp. 13-33;
- Jaishankar M., Tseten T., Anbalagan N., Blessy B. M., Beeregowda K.N. (2014). Toxicity, mechanism and health effects of some heavy metals. Interdisciplinal Toxicologly, vol. 7(2), pp. 60–72;
- Kang J.C., Jee J.H., Koo J.G., Keum Y.H., Jo S.G., Park K.H. (2010). Anti-oxidative status and hepatic enzymes following acute administration of diethyl phthalate in olive flounder *Paralichthys olivaceus*, a marine culture fish. Ecotoxicology and Environmental Safety, 73, pp.1449–1455;
- Kicham P., Otton S. V., Moore M.M., Ikonomou G.M., Gobas F. A.P.C. (2012). Relationship between biodegradation and sorption of phtatalate esters and their metabolities in natural sediments. Environemntal oxicology and Chemistry;
- Kim S.H. and Park M.J. (2014). Phthalate exposure and childhood obesity. Annals of Pediatric Endocrinology & Metabolism, 19, p. 69;
- Krumbein W. and Sloss L. (1963) Stratigraphy and Sedimentation. W.H. Freeman and Co., San Francisco, 660 p.;
- Libralato G., Avezzù F., Volpi Ghirardini A. (2011). Lignin and tannin toxicity to Phaeodactylum tricornutum (Bohlin). Journal of Hazardous Materials, 194, pp. 435–439;
- Livingstone DR, Martinez PG, Michel X, et al (1990) Oxyradical Production as a Pollution-Mediated Mechanism of Toxicity in the Common Mussel, Mytilus edulis L., and Other Molluscs. Functional Ecology 4, pp. 415–424;
- Long E. R., MacDonald D., Sith S.L. and Calder F. (1995). Incidence of Adverse Biological Effects Within Ranges of Chemical Concentrations in Marine and Estuarine Sediments. Environmental Management Vol. 19, No. 1, pp. 81-97;
- López-Pacheco I.Y., Silva-Núñez A., Salinas-Salazar C., Arévalo-Gallegos A., Lizarazo-Holguin L.A., Barceló D., Iqbal H.M.N., Parra-Saldívar R. (2019). Anthropogenic contaminants of high concern: Existence in water resources and their adverse effects. Science Total Environment;
- Losso C., Arizzi Novelli A., Picone M., Marchetto D., Pantani C., Ghetti P.F., Volpi Ghirardini A. (2007) Potential role of sulfide and ammonia as confounding factors in elutriate toxicity bioassays with early life stages of sea urchins and bivalves. Ecotoxicology and Environmental Safety, 66, pp. 252–257;
- Losso C., Arizzi Novelli A., Picone M., Ghetti P. F., Volpi Ghirardini A. (2009). Porewater as a matrix in toxicity bioassays with sea urchins and bivalves: Evaluation of applicability to the Venice lagoon (Italy). Environment International, 35;
- Luukkonen P.K., Sädevirta S., Zhou Y., Kayser B., Ali A., Ahonen L., Lallukka S., Pelloux V., Gaggini M., Jian C., Hakkarainen A., Lundbom N., Gylling H, Salonen A., Orešič M., Hyötyläinen T., Orho-Melander M., Rissanen A., Gastaldelli A., Clément K., Hodson L.Y.H. (2018). Saturated Fat Is More Metabolically Harmful for the Human Liver Than Unsaturated Fat or Simple Sugars. Diabetes Care, 41, pp. 1732–1739;
- Macía P., Fernández-Costas C., Rodríguez E., Sieiro P., Pazos M., Sanromán M.A (2014). Technosols as a novel valorization strategy for an ecological management of dredged marine sediments. Ecological Engineering 67, pp. 182–189;
- Maherzi W. and Abdelghani F.B. (2014). Dredged Marine Sediments Geotechnical Characterisation for Their Reuse in Road Construction. VOL. 18, No. 4. Environment, Energy and Natural Resources;

- Maherzi W., Benzerzour M., Mamindy-Pajany Y., Van Veen E., Boutouil M. and Abriak N. E. (2017). Beneficial reuse of Brest Harbour (France) dredged sediment as alternative material in road building: laboratory investigations. Environmental Technology;
- Malandrakis E.E., Dadali O., Golomazou E., Kavouras M., Dailianis S., Chadio S., Exadactylos A., Panagiotaki P. (2016). DNA damage and differential gene expression associated with physical stress in gilthead seabream (*Sparus aurata*). General amd Comparative Endocrinology, 236, pp. 98–104;
- Maradonna F. and. Carnevali O. (2007). Vitellogenin, zona radiata protein, cathepsin D and heat shock protein 70 as biomarkers of exposure to xenobiotics. Biomarkers, 12, pp. 240–55;
- Maradonna F., Nozzi V., Dalla Valle L., Traversi I., Gioacchini G., Benato F., Colletti E., Gallo P., Di Marco Pisciottano I., Mita D.G., Hardiman G., Mandich A., Carnevali O. (2014). A developmental hepatotoxicity study of dietary bisphenol A in Sparus aurata juveniles. Comparative Biochemistry and Physiology. Toxicology and Pharmacology, 166, pp. 1–13;
- Maradonna F., Nozzi V., Santangeli S., Traversi I., Gallo P., Fattore E., Mita D.G., Mandich A., Carnevali O. (2015). Xenobiotic-contaminated diets affect hepatic lipid metabolism: Implications for liver steatosis in *Sparus aurata* juveniles. Aquatic Toxicology, 167, pp. 257–264;
- Marriner N. and Morhange C. (2006) Geoarchaeological evidence for dredging in Tyre's ancient harbour, Levant. Quaternary Research 65, pp. 164-171;
- Markou T., Dowling A.A., Kelly T., Lazou A. (2009). Regulation of Bcl-2 phosphorylation in response to oxidative stress in cardiac myocytes. Free Radical Research, 43, pp. 809-816;
- Masciandaro G., Di Biase A., Macci C., Peruzzi E., Iannelli R., Doni S. (2014) Phytoremediation of dredged marine sediment: Monitoring of chemical and biochemical processes contributing to sediment reclamation. Journal of Environmental Management 134, pp. 166-174;
- Masindi V. and Muedi K.L. (2018). Environmental Contamination by Heavy Metals, in: Hosam El-Din M. Saleh, R.F.A. (Ed.), Heavy Metals. Intech Open;
- Matés J.M., Pérez-Gómez C., Núñez de Castro I., Asenjo M., Márquez J. (2002). Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. The Internation Journal of Biochemistry & Cell Biology, 34(5), pp. 439-458;
- Matschullat J., Ottenstein R., Reimann C. (2000). Geochemical background - Can we calculate it? Environmental Geology, 39, pp. 990–1000;
- Mchergui C., Aubert M., Buatois B., Akpa-Vinceslas M., Langlois E., Bertolone C., Lafite R., Samson S., Bureau F. (2014). Use of dredged sediments for soil creation in the Seine estuary (France): Importance of a soil functioning survey to assess the success of wetland restoration in floodplains. Ecological Engineering 71, pp. 628–638;
- Metcalfe J.D. and Craig J.F. (2011). Ethical justification for the use and treatment of fishes in research: An update. Journal of Fish Biology 78, pp. 393–394;
- Mezey E. (1982). Liver Disease and Protein Needs. Annual Review of Nutrition, 2, pp. 21–50;
- Migliarini B., Campisi A.M., Maradonna F., Truzzi C., Annibaldi A., Scarponi G., Carnevali O. (2005) Effects of cadmium exposure on testis apoptosis in the marine teleost *Gobius niger*. General and Comparative Endocrinology, 142, pp. 241–247;
- Mikula, P., Svobodová, Z., Smutná, M. (2005). Phthalates: Toxicology and food safety - A review. Czech Journal of Food Science, 23, pp. 217–223;
- Moon M.K., Kim M.J., Jung I.K., Koo Y.D., Ann H.Y., Lee K.J., Kim S.H., Yoon Y.C., Cho B.J., Park K.S., Jang H.C., Park Y.J. (2012). Bisphenol A impairs mitochondrial

function in the liver at doses below the no observed adverse effect level. Journal of Korean Medical Science, 27, pp. 644–652;
- Mosconi G., Carnevali O., Carletta R., Nabissi M. (1998). Gilthead Seabream (*Sparus aurata*) Vitellogenin: Purification, Partial Characterization, and Validation of an Enzyme-Linked Immunosorbent Assay (ELISA). General and Comparative Endocrinology, vol. 110, Issue 3, pp. 252-261;
- Mugnai C, F. Pilato, D. Pellegrini, and G. Merico. (2005). Quality Assessment Of The Solid Matrix Obtained After A Process of Mechanical Separation Of Harbour Sediment. Aquatic Ecosystem Health & Management, 8(2), pp. 209-219;
- Mugnier C., Fostier A., Guezou S., Gaigon J.L., Quemener L. (1998). Effect of some repetitive factors on turbot stress response. Aquaculture International, 6, pp. 33–45;
- Mulligan C.N., Yong R.N., Gibbs B.F. (2001) An evaluation of technologies for the heavy metal remediation of dredged sediments. Journal of Hazardous Materials, 85, pp. 145–163;
- Mylonas C.C., Cardinaletti G., Sigelaki I., Polzonetti-Magni A. (2005). Comparative efficacy of clove oil and 2-phenoxyethanol as anesthetics in the aquaculture of European sea bass (Dicentrarchus labrax) and gilthead seabream (*Sparus aurata*) at different temperatures. Aquaculture, 246, pp. 467–481;
- Mruk D.D., Silvestrini B., Mo M.Y., Cheng C.Y. (2002). Antioxidant superoxide dismutase - A review: Its function, regulation in the testis, and role in male fertility. Contraception 65, pp. 305–311;
- Neff J.M. (2002). Bioaccumulation in marine organisms. Effect of contaminants from oil well produced water. Oxford, UK, Elsevier Science;
- Nendza M. (2002). Inventory of marine biotest methods for the evaluation of dredged material and sediments. Chemosphere, 48, pp. 865–883;
- Nehring S. (2011). Invasive Alien Species Fact Sheet *Crassostrea gigas*. Available on website: https://www.nobanis.org/globalassets/speciesinfo/c/crassostreagigas/crassostrea-gigas.pdf;
- Net S., Sempere R., Delmont A., Paluselli A., Ouddane B. (2015). Occurrence, fate, behaviour and ecotoxicological state of phthalates in different environmental matrices. Environmental Science and Technology, American Chemical Society, 49 (7), pp. 4019-4035;
- Niehus N.C., Floeter C., Hollert H., Witt G. (2018). Miniaturised Marine Algae Test with Polycyclic Aromatic Hydrocarbons – Comparing Equilibrium Passive Dosing and Nominal Spiking. Aquatic Toxicology 198, pp. 190-197;
- Olivotto I., Mosconi G., Maradonna F., Cardinali M., Carnevali O. (2002). *Diplodus sargus* interrenal-pituitary response: chemical communication in stressed fish. General and Comparative Endocrinology, 127, pp. 66–70;
- Onorati F., Pellegrini D., Bigongiari N., Ausili A. (1999). Valutazione della tossicità naturale nel saggio Microtox in fase solida. La normalizzazione pelitica. Acqua, Aria, vol 6, pp. 83–87;
- OSPAR. Oslo and Paris Commission. (1998). Guidelines for the management of dredged material. p. 32;
- Pagés, T., Gomez, E., Suner, O., Viscor, G., Tort, L. (1995). Effects of daily management stress on haematology and blood rheology of the gilthead seabream. Journal of Fish Biology, 46, pp. 775–786;
- Parks L.G., Ostby J.S., Lambright C.R., Abbott B.D., Klinefelter G.R., Barlow N.J., Gray L.E. Jr. (2000). The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicological Science, 58, pp. 339–349;

- Passantino L., Santamaria N., Zupa R., Pousis C., Garofalo R., Cianciotta A., Jirillo E., Acone F., Corriero A. (2014). Liver melanomacrophage centres as indicators of Atlantic bluefin tuna, Thunnus thynnus L. well-being. Journalof Fish Diseases, 37, pp. 241–250;
- Pellegrini D., Ausili A., Onorati F., Ciuffa G., Gabellini M., Bigongiari N., De Ranieri S. (1999). Characterisation of harbour and coastal sediments: specific destinations of dredged material. Aquatic Ecosystem Health and Management 2, pp. 455-464;
- Perin G. (2004). Metodi Biologici di Previsione degli Effetti Ambientali degli Stressori. Ecotossicologia, Ambiente e Salute, 5, pp. 1-41;
- Piccinetti C.C., Donati M., Radaelli G., Caporale G., Mosconi G., Palermo F., Cossignani L., Salvatori R., Lopez R.P., Olivotto I. (2015). The effects of starving and feeding on Dover sole (Solea solea, Soleidae, Linnaeus, 1758) stress response and early larval development. Aquaculture Research, 46, pp. 2512–2526;
- Piccinetti C.C., Grasso L., Maradonna F.Radaelli G., Ballarin C., Chemello G., Evjemo J.O., Carnevali O., Olivotto I. (2017). Growth and stress factors in ballan wrasse (Labrus bergylta) larval development. Aquaculture Research;
- Picone M., Bergamina M., Volpato E., Delaney E., Turetta C., Ranaldo M., Capodaglio G., Nascia C. (2009). Sequential toxicity identification evaluation (TIE) for characterizing toxicity of Venice Lagoon sediments: Comparison of two different approaches. Ecotoxicology and Environmental Safety, 72;
- Plastic Europe. (2018). Available online on the following link site https://www.plasticseurope.org/application/files/6315/4510/9658/Plastics_the_facts_2018_AF_web.pdf;
- Prato E., Isabella P. and Biandolino F. (2015). Ecotoxicological evaluation of sediments by battery bioassays: application and comparison of two integrated classification systems. Chemistry and Ecology, 31(7);
- Rangasamy B., Hemalatha D., Shobana C., Nataraj B., Ramesh M. (2018). Developmental toxicity and biological responses of zebrafish (*Danio rerio*) exposed to anti-inflammatory drug ketoprofen. Chemosphere, 213, pp. 423–433;
- Regoli F. Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. (2000). Aquatic Toxicology, 50, pp. 351–361;
- Regoli F., Gorbi S., Frenzilli G., Corsi I., Focardi S., Winston G.W. (2002). Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. Marine Environmental Research, 54, pp. 419–423;
- Regoli F., Frenzilli G., Bocchetti R., Annarumma F., Scancelli V., Fattorini D., Nigro M. (2004). Time-course variations of oxyradical metabolism, DNA integrity, and lysosomal stability in mussels, Mytilus galloprovincialis, during a field translocation experiment. Aquatic Toxicology, 68, pp. 167–178;
- Reyes-Becerril M., Guardiola F., Rojas M., Ascencio-Valle F., Esteban M.Á. (2013) Dietary administration of microalgae Navicula sp. affects immune status and gene expression of gilthead seabream (Sparus aurata). Fish Shellfish Immunology, 35, pp. 79–87;
- Rico A., Satapornvanit K., Haque M.M., Min J., Nguyen P. T., Telfer T. C: and Van Den Brink P.J. (2012). Use of chemicals and biological products in Asian aquaculture and their potential environmental risks: a critical review. Reviews in Aquaculture, 4, pp. 75–93;
- Rocha T. L., Sabóia-Morais S.M.T., Bebianno M. J. (2016). Histopathological assessment and inflammatory response in the digestive gland of marine mussel *Mytilus galloprovincialis* exposed to cadmium-based quantum dots. Aquatic Toxicology Volume 177, August 2016, pp. 306-315;
- Roca F.J., Sepulcre M.A., López-Castejón G., Meseguer J., Mulero V. (2006). The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. Molecular Immunology, 43, pp. 1418–1423;

- Rodgers K.M., Rudel R.A., Just A.C. (2014). Toxicants in Food Packaging and Household Plastics. Springer. Molecular and Integrative Toxicology, pp. 31–60;
- Rudbeck M.e., Kumar S., Mroginski M.A., Lill S.O.N., Blomberg M.R.A., Barth A. (2009). Infrared Spectrum of Phosphoenol Pyruvate: Computational and Experimental Studies. The Journal of Physical Chemistry, 113, pp. 2935–2942;
- Rudneva II (1999) Antioxidant system of Black Sea animals in early development Comparative Biochemistry & Physiology (CBP) Part C: Toxicology, Pharmacology and Endocrinology 122C, pp. 265–271;
- Rui L. (2014). Energy Metabolism in the Liver. Comparative Physiology, 4, pp. 177–197;
- Santangeli S., Maradonna F., Zanardini M. (2017). Effects of diisononyl phthalate on *Danio rerio* reproduction. Environ Pollution, 231, pp. 1051–1062;
- Sbrilli G., Calamati E., Boccalini S., Bimbi B. and Pistolesi F. (2003). Effects of Nutrients and Salinity on the Algal Assay Using P. subcapitata (Korshikov) Hindak. Bulletin of Environmental Contamination and Toxicology, 71, pp. 609-616;
- Schmitt P., Rosa R. D, Duperthuy M., de Lorgeril J., Bachère E. and Destoumieux-Garzón D. (2012). The antimicrobial defense of the Pacific oyster, Crassostrea gigas. How diversity may compensate for scarcity in the regulation of resident/pathogenic microflora. Frontal Microbiology, 21 May 2012;
- Serhan C.N., Chiang N., Dalli J. (2015). The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution. Seminars in Immunology, 27, pp. 200–215;
- Serrano S.E., Braun J., Trasande L., Dills R., Sathyanarayana S. (2014). Phthalates and diet: a review of the food monitoring and epidemiology data. Environmental Health, 13, pp.1–14;
- Sequi P. (1989). Chimica del suolo, Bologna: Patron Editore;
- Shefer E., Silverman J., Herut B. (2015). Trace metal bioaccumulation in Israeli Mediterranean coastal marine mollusks. Quaternary International 390, pp. 44–55;
- Soclo H.H., Garrigues P.H. and Ewald M. (2000). Origin of Polycyclic Aromatic Hydrocarbons (PAHs) in Coastal Marine Sediments: Case Studies in Cotonou (Benin) and Aquitaine (France) Areas. Marine Pollution Bulletin, vol. 40, No. 5, pp. 387-396;
- Somaiah K., Satish P.V.V., Sunita K., Nagaraju B., Oyebola O.O. (2014)- Toxic Impact of Phenthoate on Protein and Glycogen Levels in Certain Tissues of Indian Major Carp Labeorohita (Hamilton). IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT), 8, pp. 65-73;
- Somasundaran S, Abraham JS, Maurya S, et al (2019) Expression and molecular characterization of stress-responsive genes (hsp70 and Mn-sod) and evaluation of antioxidant enzymes (CAT and GPx) in heavy metal exposed freshwater ciliate, Tetmemena sp. Mol Biol Rep 46:4921–4931;
- Sprovieri M., Feo M.L., Prevedello L., Salvagio M. D., Sammartino S., Tamburrino S., Marsella E. (2007). Heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in surface sediments of the Naples harbour (southern Italy). Chemosphere 67, pp. 998–1009;
- Steinel N.C. and Bolnick D.I. (2017). Melanomacrophage Centers As a Histological Indicator of Immune Function in Fish and Other Poikilotherms. Frontiers in Immunology, 8, pp. 827;
- Stimson J. and Larned S.T. (2000). Nitrogen efflux from the sediment of a subtropical bay and the potential contribution to macroalgal nutrient requirements. Journal of Experimental Marine Biology and Ecology, 252, pp. 159-180;
- Sun M, Liu G, Lin H, Zhang T., Guo W. (2019) Effect of Feeding Strategies on Molecular Responses of Biotransformation Genes in Crassostrea gigas Exposed to Cadmium. J Ocean Univ China 18:883–888;
- Surricchio G., Pompilio L., Arizzi Novelli A., Scamosci E., Marinangeli L., Tonucci L., d'Alessandro N., Tangari A.C. (2019). Evaluation of heavy metals background in the

Adriatic Sea sediments of Abruzzo region, Italy. Science of the Total Environment. 684, pp. 445–457;
- Świergosz-Kowalewska R., Bednarska A., Kafel A. (2006). Glutathione levels and enzyme activity in the tissues of bank vole Clethrionomys glareolus chronically exposed to a mixture of metal contaminants. Chemosphere 65(6), pp. 963–974;
- Tessier E., Garnier C., Mullot J.U., Lenoble V., Arnaud M., Raynaud M., Mounier S. (2011). Study of the spatial and historical distribution of sediment inorganic contamination in the Toulon bay (France). Marine Pollution Bulletin, 62, pp. 2075–2086;
- Theodorou A. J., Kotsonis A., Sorgeloos P., Viaene J., Tzovenis I. (2010) Risk Assessment of the Mediterranena Seabass and seabream industry in Greece: a stochastic simulation approach based on insurance claims. IIFET 2010, Montpellier Proceedings;
- Tomasi M.L. and Ramani K. (2018). SUMOylation and phosphorylation cross-talk in hepatocellular carcinoma. Translational Gastroenterology and Hepatology, 3, p. 20;
- Traversi I., Gioacchini G., Scorolli A., Mita D.G., Carnevali O., Mandich A. (2014). Alkylphenolic contaminants in the diet: *Sparus aurata* juveniles hepatic response. General and Comparative Endocrinology, 205, pp. 185–196;
- UNI EN ISO 10253. 2017. Water Quality - Marine algal growth inibition test with *Skeletonema sp*. and *Phaeodactylum tricornutum*;
- UNI EN ISO 11348-3. 2009. Water Quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test). UNI EN ISO 11348-3;
- UNI EN ISO 11885. 2009 "Determination of selected elements by inductively coupled plasma optical emission spectrometry";
- UNI EN ISO 15936. 2012. Fanghi, rifiuti organici trattati e suolo - Determinazione del carbonio organico totale (TOC) mediante combustione secca;
- UNI EN ISO 16703. 2004. Soil Quality - Determination of content of hydrocarbon in the range C10 to C40 by gas chromatography.
- UNI EN ISO 18856, 2006. Water quality - Determination of selected phthalates using gas chromatography/mass spectrometry.
- U.S. EPA. 1994. Method 1613. Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS;
- U.S. EPA. 1995. Method 600/R-95-136. Gary A. Chapman, Debia C. Denton and James M. Lazorchak. 1995. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms;
- U.S. EPA. 1996. SW-846 3630C Method. Silica Gel Cleanup. Part of Test Methods for Evaluating Solid Waste, Physical/Chemical Methods;
- U.S. EPA. 1996. Method 3660B: Sulfur Cleanup, part of Test Methods for Evaluating Solid Waste, Physical/Chemical Methods;
- U.S. EPA. 2007. "Method 3051A (SW-846): Microwave Assisted Acid Digestion of Sediments, Sludges, and Oils," Revision 1. Washington, DC. "Microwave assisted acid digestion of sediments, sludges, soils, and oils;
- U.S. EPA. 2007. Method 7471B (SW-846): Mercury in Solid or Semisolid Wastes (Manual Cold-Vapor Technique);
- U.S. EPA. 2007. Method 3545A (SW-846): Pressurized Fluid Extraction (PFE)," Revision 1. Washington, DC;
- U.S. EPA. 2014. Method 8270D: Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry, part of Test Methods for Evaluating Solid Waste, Physical/Chemical Methods;
- Van Gestel C.A.M., Leon C.D. and Van Straalen N.M. (1997). Evaluation of soil fauna ecotoxicity tests regarding their use in risk assessment. In Soil ecotoxicology. Ed. Tarradellas J., Bitton G., Rossel D. CRC Press Chap. 11 pp. 291-314;

- Van Leeuwen C.J. (1995). Ecotoxicological Effects. In: van Leeuwen C.J., Hermens J.L.M. (eds) Risk Assessment of Chemicals. Springer, Dordrecht;
- Vargas A., Randazzo B., Riolo P., Truzzi C., Gioacchini G., Giorgini E., Loreto N., Ruschioni S., Zarantoniello M., Antonucci M., Polverini S., Cardinaletti G., Sabbatini S., Tulli F., Olivotto I. (2018). Rearing Zebrafish on Black Soldier Fly (Hermetia illucens): Biometric, Histological, Spectroscopic, Biochemical, and Molecular Implications. Zebrafish, 15(4), pp. 404-419;
- Veissier, I., Butterworth, A., Bock, B., Roe, E. (2008). European approaches to ensure good animal welfare. Applied Animal Behaviour Science, 113, pp. 279–297;
- Verlecar X.N., Jena K.B., Chainy G.B.N. (2007). Biochemical markers of oxidative stress in Perna viridis exposed to mercury and temperature. Chemico-Biologial Interaction 67(3), pp. 219-226;
- Vileno B., Jeney S., Sienkiewicz A., Marcoux P.R., Miller L.M., Forró L. (2010). Evidence of lipid peroxidation and protein phosphorylation in cells upon oxidative stress photo-generated by fullerols. Biophysical Chemistry, 152, pp. 164–169;
- Vitali M., Guidotti M., Macilenti G., Cremisini C. (1997). Phthalate esters in freshwaters as markers of contamination sources - a site study in Italy. Environment International, 1997, Elsevier;
- Volpi Ghirardini A., Arizzi Novelli A. and Tagliapietra D. (2005). Sediment toxicity assessment in the Lagoon of Venice (Italy) using *Paracentrotus lividus* (Echinodermata: Echinoidea) fertilization and embryo bioassays. Environment International, 31, pp. 1065–1077;
- Walsh G.E. (1988). "Principles of toxicity testing with marine unicellular algae". Environmental Toxicology and Chemistry, 7, pp. 979-987;
- Wang Q., Tsui M.M.P., Ruan Y., Lin H., Zhao Z., Ku J.P.H., Sun H., Lam P.K.S. (2019). Occurrence and distribution of per- and polyfluoroalkyl substances (PFASs) in the seawater and sediment of the South China sea coastal region. Chemosphere, 231, pp. 468–477;
- Wasserman J. C., Wasserman M.A., Barrocas P.R., Almeida A.M. (2016). Predicting pollutant concentrations in the water column during dredging operations: Implications for sediment quality criteria. Marine Pollution Bulletin 108, pp. 24–32;
- Xiao-Ying Q. and Jiang J.G. (203). Toxicity evaluation of two typical surfactants to *Dunaliella bardawil*, an environmentally tolerant alga. Environmental Toxicology and Chemistry, Vol. 32, No. 2, pp. 426–433;
- Xu H., Shao X., Zhang Z., Zou Y., Wu X., Yang L. (2013a). Oxidative stress and immune related gene expression following exposure to di-n-butyl phthalate and diethyl phthalate in zebrafish embryos. Ecotoxicology and Environmental Safety, 93, pp. 39–44;
- Xu H., Yang M., Qiu W., Pan C., Wu M. (2013b). The impact of endocrine-disrupting chemicals on oxidative stress and innate immune response in zebrafish embryos. Environmental Toxicology and Chemistry, 32(8), pp. 1793-1799;
- Yamaura K., Takahashi K.G., Suzuki T. (2008). Identification and tissue expression analysis of C-type lectin and galectin in the Pacific oyster, *Crassostrea gigas*. Comp. Biochem. Physiol. Part B Biochem. Molecular Biology, 149, pp. 168-175;
- Ye T., Kang M., Huang Q., Fang C., Chen Y., Shen H., Dong S. (2014) Exposure to DEHP and MEHP from hatching to adulthood causes reproductive dysfunction and endocrine disruption in marine medaka (Oryzias melastigma). Aquatic Toxicology, 146, pp. 115–126;
- Yoon C., K. Kawakami, N. Hopkins. (1997) Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells, Development, 1997 Aug; 124(16), pp. 3157-3165;
- Yu J., Fan C., Zhong J., Zhang Y., Wang C., Zhang L. (2016). Evaluation of in situ simulated dredging to reduce internal nitrogen flux across the sediment-water interface in Lake Taihu, China. Environmental Pollution 214, pp. 866-877;

- Zanotelli V.R.T., Neuhauss S.C.F., Ehrengrubera M.U. (2010). Long-term exposure to bis(2-ethylhexyl) phthalate (DEHP) inhibits growth of guppy fish (*Poecilia reticulata*). Journal of Applyied Toxicology, 30, pp. 29–33;
- Zarantoniello M., Bruni L., Randazzo B., Vargas A., Gioacchini G., Truzzi C., Annibaldi A., Riolo P., Parisi G., Cardinaletti G., Tulli F., Olivotto I. (2018) Partial Dietary Inclusion of Hermetia illucens (Black Soldier Fly) Full-Fat Prepupae in Zebrafish Feed: Biometric, Histological, Biochemical, and Molecular Implications. Zebrafish, 15, pp. 519–532;
- Zare A., Henry D., Chua G., Gordon P., Habibi H.R. (2018). Differential Hepatic Gene Expression Profile of Male Fathead Minnows Exposed to Daily Varying Dose of Environmental Contaminants Individually and in Mixture. Frontiers in Endocrinology (Lausanne)10,9, pp. 749;
- Zhong J., Fan C., Zhang L., Hall E., Ding S, Li B., Liu G. (2010). Significance of dredging on sediment denitrification in Meiliang Bay, China: A yearlong simulation study. Journal of Environmental Sciences, 22(1), pp. 68–75;
- Zuasti A., Jiménez-Cervantes C., García-Borrón J.C., Ferrer C. (1998). The melanogenic system of Xenopus laevis. Archives of Histology and Cytology, 61, pp.305–316;
- Zylicz M. (2001). New EMBO members' review: hsp70 interactions with the p53 tumour suppressor protein. The EMBO Journal, 20, pp. 4634–4638.