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Curriculum

CIVIL AND ENVIRONMENTAL PROTECTION

TOXICITY OF MECHANICALLY AND CHEMICALLY DISPERSED OILS IN MARINE ORGANISMS

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1. INTRODUCTION

1.1 General introduction

The limited production of petroleum in particular areas of the world, determines intense transport activities of oil, particularly from Middle East, Russia and United States (Martínez-Gómez et al., 2010).

Transport of crude oil occurs mainly by sea, posing some critical issues related to possible oil spills at sea due to accidents during navigation, equipment failure, fire and explosion, loading/discharging, bunkering, ballasting/de-ballasting, tank cleaning and some other unknowing causes.

Recent reports (Chapman et al., 2007) reveal that in the last decades, the number of accidents and the amount of spilled oil showed a decreasing trend, undoubtedly due to a better design of oil tankers, i.e. equipped by double hull, new stringent international maritime safety regulations, and the effects of controls provided by port security personnel.

The attention to oil spill issues is currently focused on technologies used in offshore platforms to minimize the risk of accidental discharges, and on the best and innovative techniques to counteract potential environmental damage. The accident of *Deepwater Horizon (DWH)*, occurred in the Gulf of Mexico in 2010, have questioned the validity of security systems and the adequacy of emergency plans for offshore oil extractions. The *DWH* disaster caused 11 victims, the release at sea of about 500 million liters of crude oil, and the use of more than 7 million liters of dispersants: fate and impact of these compounds and of dispersed oil still remain largely unknown despite oil spread has been reported up to 2000 m depth, contaminating and affecting deepwater habitats, in addition to 2100 km of shoreline and coastal areas (Beyer et al., 2016).

The impact of an oil spill can be reduced by appropriate and timely interventions, which typically include physical and/or chemical removal of oil slick. The goal of oil-spill response techniques is to remove the floating oil, to reduce the potential damage to the environment, marine organisms, socio-economic activities such as fishing, mariculture and tourism.

Mechanical and manual removal of oil from sea surface are based on the use of floating barriers, skimmers, pumps and absorbent products, which allow to contain, remove and recover the oil slick. In-situ burning can be integrated when large amounts of oil are released far from the shore (IPIECA and IOGP, 2015). Physical removal of oil from the sea surface is intended

to reduce the contamination risk for marine environment and biodiversity; it is recommended when the thickness of the oil slick is high (at least more than a dozen microns) and for oils with high viscosity. On the other hand, particular environmental conditions such as high wave velocity and wind speed, do not allow the effectiveness and applicability of mechanical and physical removal techniques.

Compared mechanical removal, a different approach to reduce the propagation of petroleum hydrocarbons, is based on the use of chemical substances such as dispersant products, which in contact with oil slick promote its dispersion in the water column. The use of dispersant products is still highly controversial. Potential advantages include the rapid use, ability to handle large areas, prevent formation of emulsions, and much lower costs compared to the mechanical techniques. On the other hand, the effectiveness of dispersants has clear limits, such as in the presence of heavy or weathered oil, and they have been often reported to enhance toxicity of dispersed oil (Lessard and DeMarco, 2000; Luna-Acosta et al., 2011; Milinkovitch et al., 2011c; Ramachandran et al., 2004).

Crude oil is composed of a large number of heterogeneous chemical compounds, including both aromatic and aliphatic hydrocarbons.

Aromatic hydrocarbons consist of one or more benzene rings while aliphatic hydrocarbons can be cyclic, straight or branched, saturated or unsaturated depending on the presence of simple, double and/or triple carbon-carbon bonds.

When oil spills occurs, the behaviour of the slick can be simulated according to the prevailing winds, currents, and wave conditions. Mathematical and oceanographic modelling are nowadays essential tools to simulate atmospheric agents and oil transport, to better prepare the emergency plans before of a potential event, and to mitigate the impact in case of oil spillage.

Crude oil undergoes rapid transformations of the chemical and physical structure, generally termed as ‘weathering’, and including processes such as evaporation, oxidation, dissolution, dispersion, sedimentation and biodegradation.

The original features of hydrocarbons combined with the above-mentioned modifications strongly influence the choice of intervention methodologies that can be applied. For example, light products, such as gasoline, tend to evaporate, thus requiring no recovery action. On the contrary, heavy oils have low evaporation, low solubility and spread on the surface of water, requiring direct containment and removal.

The relative proportions of original chemical compounds, and 'weathering' processes are also key factors for the efficiency of dispersant during the oil spill responses.

The dispersants can be surfactants or solvents. The surfactants are chemical compounds characterized by a hydrophilic and a lipophilic part (Figure 1), with chemical affinity for both oil and water respectively, which allow them to diffuse to the oil/water interface and, reducing the surface tension of the two fluids, facilitate their miscibility (Lessard and DeMarco, 2000).

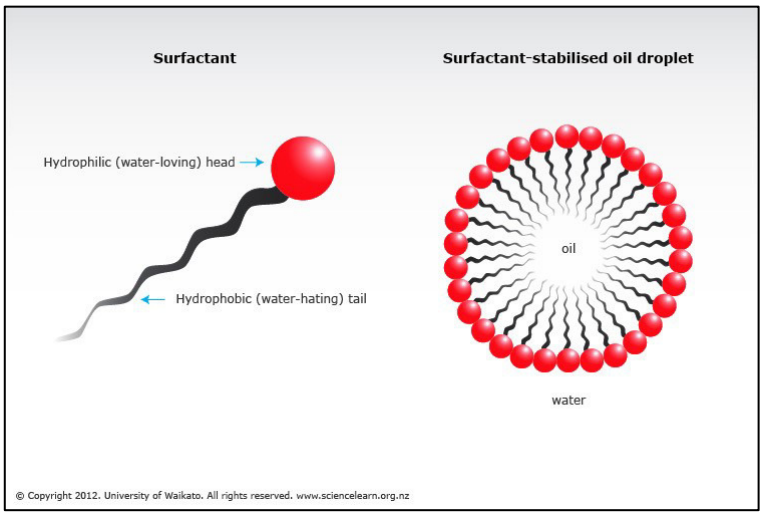


Figure 1: Surfactant structure and surfactant-stabilized oil droplet.

In general, the composition and the nature of surfactants is unknown and protected by licence. The US Environmental Protection Agency (EPA), publishes on its web site the 'Alphabetical list of National Contingency Plan product schedule (Products available for use during an oil spill)', specifying that listing a product does not mean that EPA approves, recommends, licenses, certifies or authorizes the use of that product on an oil discharge (https://www.epa.gov/emergency-response/alphabetical-list-ncp-product-schedule-products-available-use-during-oil-spill). The schedule of each dispersant show a lot of characteristics including the 'Surface Active Agents' but, for all the products, the chemical formulation is declared as 'confidential'.

Solvents can be composed by water, water mixed with hydroxyl compounds or hydrocarbons, and their role is to enhance the solubility of the surfactant into the petroleum (REMPEC, 2011). As an example, EPA has posted a full list of solvents of two dispersants, Corexit 9527 and Corexit 9500, used during the Deepwater Horizon accident: 1,2-Propanediol, 2-butoxyethanol (not included in the composition of Corexit 9500), Butanedioic acid, 2-sulfo-1,4-bis(2-ethylhexyl) ester, sorbitan, mono-(9Z)-9-octadecenoate, mono-(9Z)-9-octadecenoate,

poly(oxy-1,2-ethanediyl) derivatives, tri-(9Z)-9-octadecenoate, poly(oxy-1,2-ethanediyl) derivatives, 2-Propanol, 1-(2-butoxy-1-methylethoxy), distillates (petroleum) (Wise and Wise, 2011).

The dispersants favour the reduction of oil into micellar aggregates ranging between 1 and 70 μm (Lessard and DeMarco, 2000), and preventing the re-aggregation of oil particles, keep them suspended in water column and allow dispersion.

The *Torrey Canyon* tanker sank along the coast of United Kingdom in 1967, and it was the first major disaster to draw universal attention on the risks of chemical dispersants. A distinguishing feature of the *Torrey Canyon* response operation was the huge and indiscriminate use of 'first generation' dispersants, characterized by high toxicity, that caused a devastating impact on marine life (ITOPF, 2014; REMPEC, 2011).

After that, new formulations of dispersants were made, and the 'second' or 'third generation' dispersants appeared on the market. These new products have high content of surfactants and less solvent, increasing efficiency and, at least in theory, decreasing the toxicity.

Assessing toxicity of dispersants has been the main concern for scientific community and several laboratory experiments were focusses on this aim (Manfra et al., 2017; Wise and Wise, 2011), both in terms of acute toxicity and sub-lethal effects. However studies on the toxicity of dispersants alone do not give any information on the ecological and environmental impact of these products when used on oil, and their utility is thus greatly hampered.

In this contest DISCOBIOL, one of the main project promoted by CEDRE organization, aims to improve practical recommendations on dispersant used in coastal and estuarine areas by providing information on the effects of chemically and naturally dispersed oil on organisms living in different areas. To achieve these goals, several experiments are performed in order to compare the toxicity and impacts on different marine organisms, coastal and estuarine ecosystems, when expose to either dispersed oil naturally (by mechanical agitation by the sea) or chemically (by spraying dispersant).

These knowledges will then be used not just to improve operational guidelines on dispersion but also to develop a solid base of technical information on the toxicity of dispersed oil.

The toxicity of dispersed oil is a complex topic. Dispersants transform a surface slick of oil to a plume of very small droplets in the water column, which might be more bioavailable and easily ingested by filter feeding organisms. On the other hand, successful application of dispersants should reduce oil-spill impacts to wildlife and shoreline habitats, thus making a

switch of potential impact to water column organisms. Because of that, the use of dispersants is highly controversial and debated within scientific community. Potential synergistic effects of chemical dispersants with oil are still under-explored (Kujawinski et al., 2011) although many studies, (Lessard and DeMarco, 2000; Ramachandran et al., 2004; Milinkovitch et al., 2011) have reported that chemical dispersion of hydrocarbons, can frequently lead to a, at least temporary increase and local toxicity compared to not-treated and not-dispersed oil. Observed increased toxicity of dispersed oil (chemically or mechanically) has been attributed variously to particle size in the dispersion (Bobra et al. 1989), and to aromatic hydrocarbon content (Anderson et al., 1987). In truth, however, acute oil toxicity is brought about by a number of interacting chemical, physical and physiological factors, and is highly dependent on the specific conditions of exposure.

Estimates of toxicity is dependent upon the test conditions and populations being exposed. Comparisons among species, life stages or toxicants are difficult, when there are significant differences in the methodologies used to produce data.

One of the most fundamental parameters affecting any toxicity test is the exposure medium itself. Test media must be reproducible over time and between laboratories. In literature, there are two main methods used to assess the toxicity of mechanically and chemically dispersed oil: one simulates oil dispersion, considering the presence of oil droplets in the water column during all the exposure time, the mixture was homogenized using a funnel (at the surface of a seawater tank), which was linked to a water pump (at the bottom of the tank) in order to homogenize the mixture despite the hydrophobic nature of the oil (Frantzen et al., 2016; Luna-Acosta et al., 2011; Milinkovitch et al., 2011c); the other use the Water-Accommodated Fraction (WAF) and the Chemically Enhanced Water-Accommodated Fraction (CEWAF) (Singer et al., 2000). A research group, known as the Chemical Response to Oil Spills Ecological Effects Research Forum (CROSERF) group has specified the technical definition of 'Water-Accommodated Fraction' (WAF) as a laboratory-prepared medium derived from low energy mixing of a poorly soluble test material (e.g., an oil or petroleum product) which is essentially free of particles of bulk material (Aurand and Coelho, 1996; Coelho and Aurand, 1997).

As with WAF, Singer and co-authors (2000), have specified also a technical definition of oil/dispersant mixtures (i.e., dispersed oil); a 'Chemically Enhanced Water-Accommodated Fraction' (CEWAF), is a laboratory-prepared medium derived from vortex mixing of test material and chemical dispersant in which a relatively stable population of bulk material droplets is present (Aurand and Coelho, 1996; Coelho and Aurand, 1997). Mixing energy

sufficient to create a vortex is essential for dispersant/oil interactions consistent with the intended use of the dispersant which had better simulate a real oil spill scenario.

1.2 Ecotoxicological approach

Effects of contaminants are often consequence of concentrations in target organs and tissues; the degree to which a contaminant accumulates in biota depends on the typology of the contaminant, the organism, and the environmental conditions under which the organisms and contaminants are interacting.

Analyses carried out at different biological levels provide some important and different information on biological impact. The ecological relevance of an environmental disturb increase at higher biological levels but they typically have a lower sensitivity (Figure 2). On the other hand, the effect on an environmental stressor begins to act at the lowest levels of biological organization (i.e. molecular, biological, cellular and physiological levels) also known as *early-warning signals* or *biomarkers* (van der Oost et al., 2003). The ideal biomarker should be easily measurable in a broad range of sentinel species, either endemic or caged, used to assess the impact of contaminants. Such biomarkers can also useful to monitor the recovery to a normal environmental state, i.e. after a clean-up or remediation of a contaminated site. The filter feeder *Mytilus galloprovincialis* is a well-known and widely used bioindicator in the Mediterranean Sea (Bocchetti et al., 2008; Gorbi et al., 2008; Regoli et al., 2014). Invertebrates are characterized by a less efficient metabolization pathway of PAHs compared to vertebrates that does not involve the Cytochrome P450 biotransformation system. As consequence of the low biotransformation and excretion of PAHs, mussels can accumulate high levels of these contaminants.

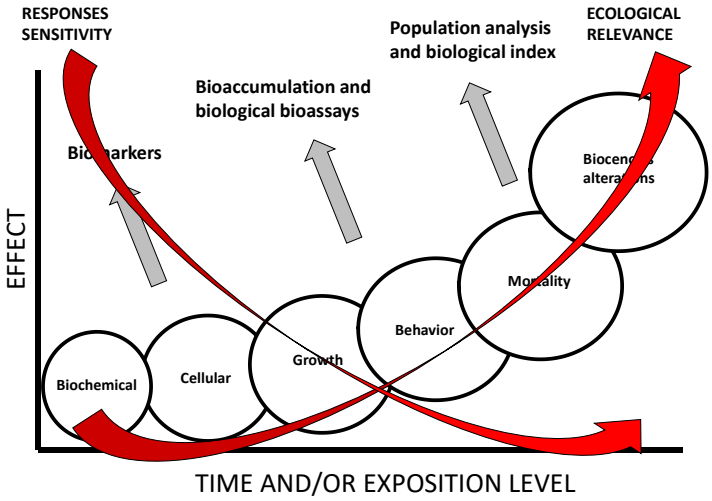


Figure 2: Different levels of biological organization and their different biological impact.

The immune system is extremely vulnerable to insult from exogenous chemicals, such as PAHs, organometals and heavy trace elements, especially after chronic exposures or repeated short-term exposures (Weeks et al., 1992).

A decrease of cell membrane stability has been reported in many studies after exposure of invertebrates to dispersed oil (Baussant et al., 2009) and produced water (Hannam et al., 2009).

More particularly, lysosomes are extremely sensitive to toxic effects of these substances and lysosomal alterations, such as the reduction of membranes stability, are widely reported as an important early-warning signal. Responses of the lysosomal system have been used as biomarkers of effect, especially in mussels where the lysosomal compartment is extremely developed both at the level of digestive tissues and in haemocytes freely circulating in the haemolymph.

In mussels, haemocytes are also responsible for cell-mediated immune defences, i.e. through high phagocytosis capacity. Phagocytosis is the main immune event in sea bivalve molluscs and haemocytes represent cells that are responsible for recognition, adhesion, incorporating and degradation processes of the exogenous particles. Such cells were subdivided microscopically into granulocytes and hyalinocytes.

Phagocytic processes are strictly dependent on the membrane properties of haemocytes; PAHs can interfere with these function and, oil exposure has been shown to modulate the phagocytosis activity even after low-dose exposure (Hannam et al., 2010b).

The acetylcholinesterase (AChE) is an enzyme that plays a key role in the nervous transmission; although its activity was considered to be specifically inhibited by organophosphates and carbamates, its modulation in haemolymph and gills of bivalve molluscs has been demonstrated for a wide spectrum of environmental pollutants (Moreira et al, 2004).

Certain classes of environmental contaminants (including many polycyclic aromatic hydrocarbons, organohalogenated and metals) are potentially genotoxic, able to interact with the genetic material either directly or following metabolic activation on onset of oxidative stress.

The reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are produced by aerobic metabolism and other processes. Free radical such as superoxide radical ($^{\bullet}O^{2-}$) and hydroxyl radical ($^{\bullet}OH$) can damage lipids, proteins, DNA and other biomolecules.

These molecules are naturally produced during aerobic metabolism and their toxicity is, in normal conditions, counteracted by the presence of a complex antioxidant defences system based on enzymes and low molecular weight molecules (both cytosolic and localized on the membranes) capable of neutralizing the free radicals. The presence of both organic and metallic contaminants can alter the physiological balance between pro-oxidants and antioxidant defences, causing a pathological condition.

One of the main approaches to reveal the onset of oxidative stress conditions, is based on the analysis of antioxidants including low molecular weight compounds (such as total

glutathione) and the main antioxidant enzymes, such as catalase, glutathione S-transferases, glutathione reductase, Se-dependent and Se-independent forms glutathione peroxidases (Gorbi et al., 2008). In addition, the measurement of the total oxyradical scavenging capacity (TOSC) toward peroxyl radicals (ROO$^{\bullet}$) and hydroxyl radicals ($^{\bullet}$OH) provides a quantitative parameter on the overall efficiency of the entire antioxidant defense system to neutralizing various forms of free radicals (Regoli and Winston, 1999).

A consequence of oxidative stress is the increase of lipid peroxidation processes. When ROS interact with a membrane, they sequester a hydrogen atom causing a molecular rearrangement and giving rise to a radical lipid. Damage is able to propagate through a chain reaction, as radical lipids tend to reintegrate electron by subtracting them from contiguous molecules. Lipid oxidation gives rise to a number of secondary products including malondialdehyde (MDA), which is a typical product of damaged membranes.

Peroxisomes are cellular organelles involved in many cellular functions including lipid metabolism and the maintenance of the redox homeostasis. Organic contaminants can lead to a drastic increase in the volume of these organelles and to induction of enzymes involved in fatty acid oxidation, such as acyl-CoA oxidase (AOX) (Cajaraville et al., 2003, 2000). The size, abundance, and enzyme composition of peroxisomes vary with the type of cell and the considered species (Cancio and Cajaraville, 2000). Peroxisomal proliferation is considered an important biomarker to evaluate the effects of organic compounds such as PAHs, PCBs, phthalates, polybrominated, some pesticides etc.

To better understand the effects of mechanically and chemically dispersed oils on different levels of biological organization, bioaccumulation and multi biomarker approach can be integrated with ecotoxicological bioassays that taking in account the sublethal and lethal effects on organisms level using different species and different end-point. Typically used species are the bacterium *Vibrio fischeri*, for which toxicity is detected by the decrease of bioluminescence, the unicellular algae *Phaeodactylum tricornutum* where the end-point is the growth inhibition, the crustacean *Tigriopus fulvus* where is used the mortality as end point, the echinoderm *Paracentrotus lividus* and the bivalve *Crassostrea gigas* that give information on embryotoxicity.

The incorporation of bioaccumulation, exposure and effect biomarkers, and bioassays can provide some suggestions about the causality of the higher-level adverse effects (Martínez-Gómez et al., 2010).

Recently, a Weight of Evidence (WOE) model has been developed through specific mathematical algorithms to integrate and "weight" adequately different Lines Of Evidence (LOEs), including chemical analysis, biomarkers and bioassays, considering several chemical and biological parameters, normative guidelines, and/or scientific evidence. This allow to summarizing of the data into specific synthetic indices, which are finally integrated into an overall evaluation of hazard (Benedetti et al., 2012; Piva et al., 2011). A similar approach can be useful to provide an environmental risk assessment associated with the oil spills, and to obtain an index of environmental risk for the different treatments and times.

1.3 Regulations and normative guidelines on dispersants

Actual regulations on the use of dispersants reveal frequent gaps and contradictions.

A review by ITOPF (International Tanker Owners Pollution Federation) on past oil spill events, revealed that of 258 marine incidents occurred between 1995 and 2005, the 18% of case were treated with chemical dispersants (Chapman et al., 2007). In addition, considering the regional incidence of dispersants, Countries from Southeast Asian and Middle East are more incline to use these products in respect to Europe and Americas (Chapman et al., 2007). However also in Europe the actual policies on the use of dispersants greatly differ across the states as recently highlighted in a study on national practices and policies on the use of dispersants as a response to oil spills, carried out by the European Maritime Safety Agency (EMSA, 2010). In order to provide an accurate and up-to-date information on the pollution preparedness and response mechanisms and capabilities of the European Union coastal States, EMSA contacts the competent authorities in each State and prepares specific inventories. In the majority of States, the use of chemical dispersants is secondary to mechanical containment and recovery actions; in other counties, the use of dispersants is either not allowed or very limited, especially in the Baltic States. Only in UK the use of chemical dispersants is considered as the primary intervention option, partly due to the meteorological conditions of the area, which frequently preclude the use of containment and mechanical recovery techniques for most part of the year (Chapman et al., 2007).

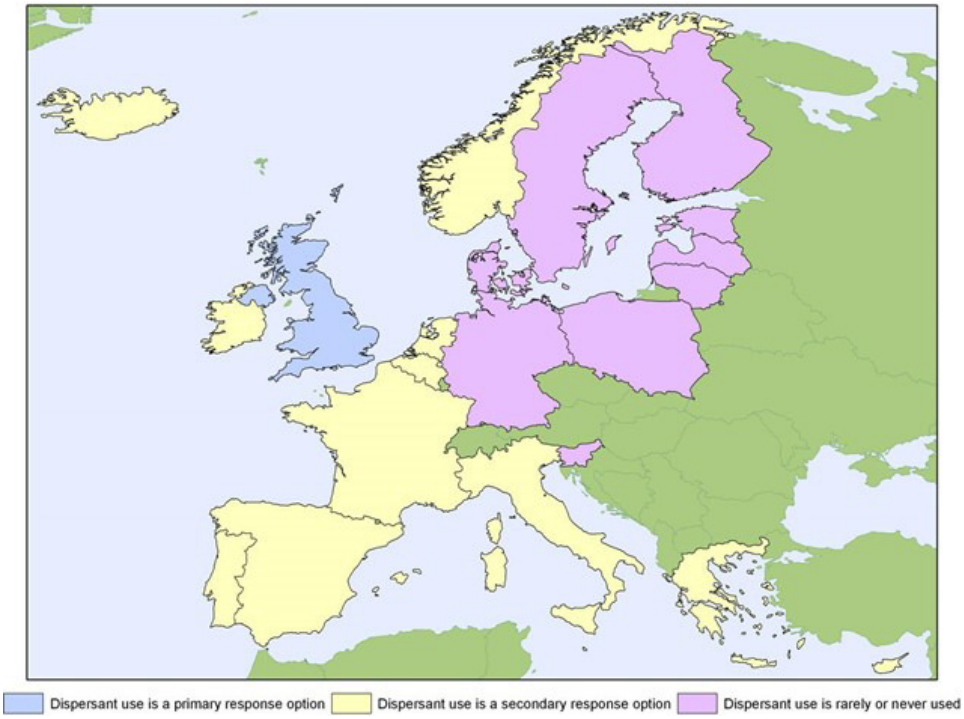


Figure 3: Summary of European policy in terms of use of chemical dispersants (Chapman et al., 2007).

In addition, the UK approval scheme for dispersants differs from those of other national protocols: the main difference regards the toxicity tests that must be carried out to evaluate also the effect of dispersed oil in addition to those of the dispersant alone.

The test compare the toxicity of treated oil (i.e. treated with dispersant) against that of oil alone (no product added, oil that is mechanically dispersed). It is generally accepted that the (dispersed) oil is more toxic than the dispersant alone and the UK does not consider the inherent toxicity of the dispersant alone because, if used correctly, organisms should only be exposed to it in the environment in combination with oil; so it is important to assess how the application of a chemical product changes the toxicity of oil (EMSA, 2016).

The Italian regulations on the use of dispersants is based on the precautionary principle of Barcelona Convention (1976), and these chemical products are considered only as *extrema ratio*, when all other reclamation procedures are inapplicable and/or have failed.

According to the Decree of 25th February 2011, all products possibly used for oil decontamination in seawater should be previously assessed, tested and authorized by the Italian Ministry of Environment (*Ministero dell'Ambiente e della Tutela del Territorio e del Mare,* MATTM), before being included in a list of potentially suitable products.

Detailed specifications (see Annex 1 – *Allegato 5*) are given on how the tests must carried out to assess:

- the effectiveness and stability of dispersant products are determined in terms of suspended and emulsified oil (*Arabian light*) after the addition of the dispersant under either standardized calm or agitation conditions following the method ISO 9377-2 (2002).
- the biodegradability of dispersants are determined on organic compound following the "close bottle" method as indicated by guideline OECD n. 306 (17 July 1992).
- the bioaccumulation is determined providing a bioconcentration value (BCF) for every substance present in the dispersant products. If the BCF value is not available must be provided the *log* K_{OW} value empirically determined.
- the toxicity tests requires a battery of at least three marine organisms belonging to different trophic levels (algae, crustacean and fish), performed according to specified accredited protocols.

The toxicity test with algae must follow the method UNI EN ISO 10253: 2006, while for crustaceans the decree allows to choose one of the following five species and relative protocol:

SPECIES	METHOD	EXPOSURE TIME
Acartia tonsa	UNICHIM pr MU 2365 (2010)	48 h
Artemia franciscana	APAT-IRSA-CNR 8060 (2003)	96 h
Amphibalanus Amphitrite	UNICHIM pr MU 2245 (2010)	48 h
Corophium orientale	UNICHIM pr MU 2246 (2010)	96 h
Tigriopus fulvus	UNICHIM pr MU 2396 (2010)	96 h

Table 1: List of species, method and exposure time to be used to test dispersants products according to the Italian Decree of 25th February 2011.

For fish, it is possible to use *Dicentrarchus labrax* or *Sparus aurata* according to the method indicated in the guideline OECD n. 203 (17th July 1992).

Although there is a particular focus on the quality of dispersant products that can be used after an oil spill events, the limit of this approach requires that the analyses of effectiveness and stability, are tested on an 'Arabian light' oil. However oil spill events are characterized by molecules with different chemical-physical properties that can not be represented just by one kind of oil. Furthermore, biodegradability, bioaccumulation and toxicity tests are made on the dispersant alone rather than chemically dispersed oil. Thus, the normative does not consider

that the biological effects and bioaccumulation of chemically dispersed oil can potentially increase compared to exposure to mechanically dispersed or not dispersed oil.

Furthermore the bioaccumulation approach, required from the Italian legislation, based on the determination of bioconcentration factor (BCF) or $log\ K_{OW}$ value, are now obsolete and they have been replaced with the more reliable measurement of levels of chemical agents in organisms' cells, tissues and organs.

2. AIMS OF THE THESIS

The use of dispersants is a strongly controversial and discussed approach by the scientific community, since it may cause a temporary increase of toxicity respect to untreated oil. However, the use of dispersants also has apparent advantages, including quick application, ability of treat large areas and to increase the biodegradation.

Despite this, in Italy, dispersants are used as *extrema ratio*, only when all other reclamation systems are inapplicable or failed.

The Italian law requires that all products possibly used for oil decontamination in seawater should be previously assessed, tested and authorized by Ministry of the environment, before they can appear on the market.

One of the main shortcoming of the Italian law is to consider only the ecotoxicological potential of the dispersant alone rather than the oil and dispersant mixture.

For this reason this PhD thesis aims to increase the knowledge on the possible synergistic effects and toxicity of dispersants and oil mixtures on marine organisms that can be useful in the decision-making and operational processes during oil spill events and to set up a new protocol in order to evaluate the impact of dispersants on the environment as realistically as possible.

In this respect, compared to current Italian legislation, the proposed experimental protocol provides that laboratory tests are performed on oils extracted or refined in the Mediterranean area that have different physical and chemical characteristics than the *Arabian light* which is currently provided by the Decree of 25th February 2011. The new protocol also includes an integrated approach, considering interactive effects of chemical dispersants and different types of oil on bioaccumulation, early biochemical and cellular effects in mussels *Mytilus galloprovincialis*, integrated with a wide battery of ecotoxicological bioassays in different marine organisms belonging to different trophic levels. This approach also evaluate the reversibility of the effects measured in organisms considering recovery periods in clean water. One of the main outcoming of this research, aims to further elucidate the possible aspects that could be investigated during and after an accidental oil spills event. The innovative procedure suggest to compare the results obtained with combination of bioassays and biomarker methods coupled with chemical analysis to better assess the toxicity of chemical dispersants before commercializing.

3. METHODS

3.1. Experimental designs with *Mytilus galloprovincialis*

The Mediterranean mussel, *Mytilus galloprovincialis*, represents one of the most widely used bioindicators in the Mediterranean Sea and this filter feeding bivalve is extensively used in biomonitoring programmes (Bocchetti et al., 2008; Gorbi et al., 2008; Regoli et al., 2014). Due to its limited ability to metabolize PAHs and aliphatic compounds, and its capability to bioaccumulate relatively higher levels of organic molecules (Frantzen et al., 2015; Lee and Anderson, 2005), it has been chosen as one of the target organism in this study.

Two similar experimental plans have been carried out exposing the invertebrate *Mytilus galloprovincialis*, to the Water-Accommodated Fractions (WAF) of oil, dispersant, oil+dispersant. In the first experimental design (see paper in submission) organisms were exposed to crude oil with high viscosity (723.6 mm^2/s at 50°C) (hereafter CRUDE OIL); in the second one experiment, a "blend" was used, that is an extremely sticky crude oil with the addiction of gasoline to reduce the viscosity (746.5 mm^2/s at 40°C) (hereafter BLEND); this product is produced directly on offshore platforms to reduce the oil viscosity and to facilitate its scrolling through pipelines. The anionic dispersant (hereafter DISPERSANT) CHIMSPERSE 4000 was kindly obtained from manufacturer; it represents one of the Italian approved products according to the Decree of 25th February 2011, having passed all the tests for evaluation of dispersant efficacy, stability, biodegradability, bioaccumulation and toxicity. The chemical composition of this dispersant is protected by patent.

Both oils test solutions were prepared based on the methods described by Singer et al., (2000) with some modifications. Each test solution was prepared by stirring with magnetic stir bar 5 L of artificial salt water (ASW) brought to a 35-40% vortex in three beakers glass on a magnetic stir plate.

In the first beaker, the mechanical dispersion condition was set up by pouring 9 g of oil; in the second beaker, the dispersant alone condition was set up by pouring 0.9 g of chemical dispersant and in the third beaker, the chemical dispersion condition was set up by pouring 9 g of oil and the amount of dispersant recommended by the manufacturer (dispersant/oil ratio 1:10), i.e. 0.9 g of dispersant.

In order to simulate a real condition during and after an oil spill event, all the solutions were mixed for 24 h, in darkness and at 18°C followed by 1 h of settlement to favor the separations

of water and oil. After this time, test solutions are removed from the bottom of the beakers and transferred into respective 45 L glass-tanks a diluted with synthetic sea water in order to obtained a nominal oil concentration of 200 ppm.

Organisms were exposed at 18°C for 2 days at each experimental condition ("oil", "dispersant" and "oil+dispersant") and then transferred to clean ASW for other 21 days (first experiment) or 35 days (second experiment). Water changes were carried out every two days and mussels were fed 12 h prior the water change with commercial mixture of zooplankton for filter-feeding organisms. No mortality was observed during the experiments.

After 2, 7, 14, 21 and 35 days 39 mussels were sacrificed and dissected rapidly. Whole tissues, were used to evaluate the bioaccumulation of PAHs and aliphatic hydrocarbons. Gills, digestive glands and haemolymph were collected for biological analyses: tissues were pulled in 9 replicates, each constituted by 3 individuals and rapidly frozen in liquid nitrogen and stored at -80°C. An aliquot of haemolymph was immediately processed for the analyses of lysosomal membrane stability, phagocytosis capability, granulocytes-hyalinocytes ratio and genotoxic damage.

3.2. Bioaccumulation of hydrocarbons

Bioaccumulation of PAHs and aliphatic hydrocarbons was analysed according to previous methods (Bocchetti et al., 2008; Piva et al., 2011; Regoli et al., 2014).

For the analysis of polycyclic aromatic hydrocarbons (PAHs), about 3 g (wet weight) of tissues were extracted in 10 mL 0.5 M potassium hydroxide in methanol with microwave at 55°C for 20 min (800 Watt) (CEM, Mars System). After centrifugation at 3.000 × g for 10 min, the methanolic solutions were concentrated using a SpeedVac and purified with solid-phase extraction (Octadecyl C18, 500 mg × 6 mL, Bakerbond). A final volume of 1 mL was recovered with pure, analytical HPLC gradient grade acetonitrile, and HPLC analyses were carried out in a water and acetonitrile gradient by fluorimetric and diode array detection. The PAHs were identified according to the retention times of an appropriate pure standards solution (EPA 610 Polynuclear Aromatic Hydrocarbons Mix), and classified as low molecular weight (LMW: naphthalene, acenaphthylene, 1-methyl naphthalene, 2-methyl naphthalene, acenaphthene, fluorene, phenanthrene, anthracene) or high molecular weight (HMW: fluoranthene, pyrene, benzo(a)antrhacene, chrysene, 7,12-dimethyl benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3,c,d)pyrene).

Aliphatic hydrocarbons were extracted treating tissues (about 3 g, wet weight) with hexane:acetone (2:1) in a microwave (110°C for 25 min, 800 Watt) (Mars CEM, CEM Corporation, Matthews NC). After centrifugation at 3.000 × g for 10 min, the supernatants were purified with solid-phase extraction (Phenomenex Strata-X, 500 mg × 6 mL plus Phenomenex Strata-FL, 1000 mg × 6 mL) and then concentrated using a SpeedVac (RC1009; Jouan, Nantes, France) to dryness. Samples were finally recovered with 1mL of pure, analytical GC grade n-hexane and analysed with a gas chromatograph (Perkin Elmer) equipped with an Elite-5 capillary column (30 m × 0.32 mm ID × 0.25 μm-df) and a flame ionization detector. For quantitative determination, the system was calibrated with an unsaturated pair n-alkane standard mixture according to EN ISO 9377-3 (Fluka 68281).

3.3. Biomarkers analysis

For the first experimental design, where the effect of mechanically and chemically dispersed CRUDE OIL were tested, a wide and complete battery of biological responses were carried out in different tissues such as haemolymph, gills and digestive glands, to assess all the possible variations in term of immunological responses, the neurotoxic damage, genotoxic damage, lipid peroxidation and oxidative stress biomarkers.

Lysosomal membrane stability, granulocytes-hyalinocytes ratio and phagocytosis capacity was evaluated as immunological responses, and DNA fragmentation was evaluated at molecular level as biomarkers of genotoxic damage in haemocytes (Gorbi et al., 2013, 2008).

The *lysosomal membrane stability* was measured as Neutral Red Retention Time (NRRT); the cationic probe Neutral Red (NR) was used to evaluate the capability of the lysosomal membranes to retain the dye (Lowe et al., 1995). At least 8 mussels were individually analysed for each sampling time and treatment. Haemocytes were incubated on a glass slide with a freshly prepared NR working solution (2 μl/ml filtered seawater) from a stock solution of 20 mg neutral red dye dissolved in 1 ml of dimethyl sulfoxide, and microscopically examined at 20 min intervals (for up to 120) to determine the time at which 50% of cells had lost the dye to the cytosol previously taken up by lysosomes

The *granulocytes-hyalinocytes ratio* was evaluate using 50 μl of haemolymph dispersed on glass slides, dried and fixed in Baker's fixative (+2.5% NaCl). The slides were washed with water, stained with H&E and mounted in Eukitt. Observations were carried out with a light

microscope (20X) and the ratio was evaluated after counting almost 200 cells for each sample (Gorbi et al., 2013).

Phagocytosis capacity assay was microscopically evaluated in haemolymph incubated for 2 h with Fluorescein-labelled Zymosan A bioparticles (Invitrogen), added at 10:1 target:haemocyte ratio; phagocytosis was expressed as percentage of cells that internalized at least 3 fluorescent particles (Gorbi et al., 2013).

The DNA integrity was evaluated at molecular level as single strand breaks (SB) by the *Comet assay*. The comet assay was immediately carried out on haemocytes collected from the adductor muscle of organisms according (Gorbi et al., 2008). Cells were included in 1% normal-melting-point agarose on glass slides, followed by treatment in lysing solution, DNA denaturation, electrophoresis and staining with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI); 100 randomly selected "nucleoids" per slide, and two replicates per sample, were examined under fluorescence microscopy (200 × magnification; Olympus BX-51) and the captured images (Image-Pro-Plus package) were analyzed by the Comet Score software. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.

Acetylcholinesterase activity (AChE), in haemolymph and gills samples, was assessed with Ellman's reaction at 412 nm, with acetylthiocholine and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to investigate neurotoxic effects (Gorbi et al., 2008).

As reported in various studies, the antioxidants mechanisms play, a primary role in marine organisms in the modulation of toxic effects induced from environmental contaminants such as PAHs (Giuliani et al., 2013; Gorbi et al., 2008; Hannam et al., 2010a).

Antioxidants defenses were measured in mussels gills and digestive glands following standardized assay conditions, at a constant temperature of 18 ± 1°C (Bocchetti et al., 2008). *Catalase* (CAT) was determined by the decrease in absorbance due to H_2O_2 consumption; *glutathione S-transferases* (GST) were determined following the reaction between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, *glutathione reductase* (GR) activity was measured by the oxidation of NADPH during the reduction of GSSG; *glutathione peroxidases* (GPx) activities were assayed in a coupled enzyme system where β-nicotinamide adenine dinucleotide (NADPH) is consumed by glutathione reductase to convert the oxidized glutathione (GSSG) to its reduced form using hydrogen peroxide or cumene hydroperoxide as substrates, respectively, for the selenium-dependent and for the sum of Se-dependent and Se-independent forms.

Levels of *total glutathione* were enzymatically assayed after acidic deproteinization with sulphosalicilic acid (Akerboom and Sies, 1981).

The *Total Oxyradical Scavenging Capacity* (TOSC) was measured in mussels gills and digestive glands by the capability of cellular antioxidants to inhibit the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas in the presence of different forms of oxyradicals, like peroxyl radicals ($ROO^{\bullet}$) and hydroxyl radicals ($^{\bullet}OH$) which are artificially generated at constant rate (Regoli and Winston, 1998; 1999). Ethylene formation was determined by gas-chromatographic analyses and TOSC values were quantified from the equation: TOSC = 100-(∫SA/ ∫CA x 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curve produced during the reaction course for respective sample (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay (Regoli and Winston, 1999).

The content of malondialdehyde as a product of lipid peroxidation and the acyl-CoA oxidase activity biomarkers of peroxisomal proliferation were detected in gills and digestive glands as well (Bocchetti et al., 2008; Gorbi et al., 2013).

The content of malondialdehyde (MDA) was measured in samples homogenized (1:3 and 1:5 w/v respectively for gills and digestive gland) in 20 mM Tris–HCl pH 7.4, centrifuged at 3000 × g for 20 min and then derivatized in 1 ml reaction mixture containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1), HCl 32%, 100 µl water and an equal volume of sample or standard (standard range 0–6 µM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl, pH 7.4). The tubes were vortexed and incubated at 45 °C for 40 min. Samples were cooled on ice, centrifuged at 15000 ×g for 10 min and read spectrophotometrically at 586 nm; levels of MDA were calibrated against a malondialdehyde standard curve and expressed as nmol/g wet weight (Shaw et al., 2004).

The activity of the peroxisomal enzyme Acyl-CoA oxidase (AOX) was measured in samples homogenized in 1 mM sodium bicarbonate buffer (pH 7.6) containing 1 mM EDTA, 0.1% ethanol, 0.01% Triton X-100 and centrifuged at 500 ×*g* for 15 min. The H_2O_2 production was measured in a coupled assay (Bocchetti and Regoli, 2006) by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 µM sodium azide, 0.01% Triton X-100, 1.2 U/ml HRP in a final volume of 1 ml. After a pre-incubation at 25 °C for 5 min in the dark with an appropriate volume of sample, reactions

were started adding the substrate Palmitoyl-CoA, at final concentrations of 30 μM; readings were carried out against a blank without the substrate at 502 nm.

Protein concentrations were determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

Based the results, of the first experimental design, we decided to investigate the toxicity of the different treatments of BLEND dosed alone and in combination with dispersant (BLEND+DSPERSANT), only in the most sensitive tissues and biological responses. In this respect, the attention was focused on hemolymph and gills, where the more evident immunological responses, genotoxic damage and the neurotoxic biomarker had been detected.

3.4. Ecotoxicological bioassays

A large battery of toxicity tests, among those indicated by the Italian legislation with the Decree of February 25th 2011 (Annex 1), was applied not only to the dispersant dosed alone (as required by the legislation), but also to oils (mechanically dispersed) and to combinations of oils (crude oil and blend) and dispersant.

The species tested belong to different trophic levels and take into consideration different end points, such as *Vibrio fischeri* (bacterial bioluminescence), *Phaeodactylum tricornutum* (algal growth), *Tigriopus fulvus* (mortality) and *Paracentrotus lividus* and *Crassostrea gigas* (embryo development). The concentrations of oil and dispersant tested in each bioassay are provided in Table 2.

Tested-species	**Oils nominal concentrations (mg/L)**	**Dispersant nominal concentration (mg/L)**
Vibrio fischeri	1620 – 1080 – 720 – 480 – 320 – 142 - 0	162 – 108 – 72 – 48 – 32 – 14 - 0
Phaeodactylum tricornutum	350 – 250 – 200 – 150 – 100 – 50 – 20 – 10 - 0	35 – 25 – 20 – 15 – 10 – 5 – 2 – 1 - 0
Tigriopus fulvus	1800 – 900 – 450 – 200 – 100 - 0	180 – 90 – 45 – 20 – 10 - 0
Paracentrotus lividus	450 – 200 - 100 – 50 – 30 – 20 - 0	45 – 20 – 10 – 5 – 3 – 2 - 0
Crassostrea gigas	500 – 400 – 300 – 200 - 100 – 50 - 0	50 – 40 – 30 – 20 – 10 – 5 - 0

Table 2: Concentration of oils (CRUDE OIL and BLEND) and dispersant used in each toxicity test. The concentrations used for oils+dispersant treatments are not show in this table and the ratio of dispersants:oils are 1:10.

3.4.1. Bacterial test

The toxicity tests with the marine luminescent bacteria *Vibrio fischeri* were performed according to ISO 11348-3 (ISO, 2007) and the methodology developed for Microtox 500 equipment. The toxic effect of a sample is determined as the concentration causing a 50% reduction on the light emitted by the bacteria, after a predetermined exposure time.

Dilutions of WAF obtained by oils, dispersant and oils+dispersant were tested applying the Basic 90% protocol (Azur Environmental, 1994) considering 7 samples dilutions starting from 90%, and measurements were made at 5, 15 and 30 minutes.

The dose-responses relationship was elaborated with a specific software Microtox OmniTM v. 1.16, which provide the EC_{50} values.

3.4.2. Algal test

The algal growth test was carried out according to the ISO 10253 method (2006) using monospecific algal cultures of *Phaeodactylum tricornutum*.

A mass culture of *P. tricornutum* was kept in a thermal-static chamber at 20 ± 2 °C with a 12hL/12hD light cycle, at 10000 lx light (cool white) intensity.

The individuals were reared in synthetic seawater (Instant Ocean®) at 33 ‰, filtered with 0.45 µm cellulose membranes. The organisms were kept in 250 mL glass flask and the culture were renew every week.

A test was conducted with potassium dichromate ($K_2Cr_7O_3$) as reference toxicant at different concentrations (from 4 to 60 mg/L) in order to verify the sensitivity of the algal batch. The test was performed in sterile capped polystyrene twelve-well microplates at 20°C ± 2, a continuum light 10000 lux.

A concentration of 10^4 cells/mL was inoculated in 5 ml of solution in each well of a 12-well plate (three replicate per each concentrations).

Cell density was measured at the end of 72h by using Burker's chamber.

3.4.3. Crustacean test

Toxicity tests with *Tigriopus fulvus* were performed according to UNICHIM (2396: 2014). A mass culture of *T. fulvus* was kept in a thermal-static chamber at 20±2 °C with a 16hL/8hD light cycle, at 500–1200 lx light intensity. The individuals were reared in synthetic seawater (Instant Ocean®) at 36 ‰, filtered with 0.45 µm cellulose membranes. The organisms were

kept in 0.5 L polystyrene tissue culture flasks with 0.22 µm filter screw caps and were fed weekly with Tetramarin® (fish food) ad *libitum*. Nauplii (24–48 h after hatching) originating from synchronized cultures were used for toxicity tests. The nauplii were released by ovigerous females and selected 3–7 days prior the test, transferred on a 80 µm mesh plankton net fixed to a plexiglas tube and fed with a mix of *Tetraselmis suecica* and *Isochrysis galbana* algae cultures at 1.5×10^8 and 3.0×10^8 cells/L density, respectively (UNICHIM, 2014). Population sensitivity was evaluated by testing a concentration of 0.05 mg Cu^{2+}/L, corresponding to 96h EC_{50} value for *T. fulvus* (Faraponova et al., 2016), prepared starting from 1000 mg Cu^{2+}/L in HNO_3 standard solution for atomic absorption spectroscopy.

Thirty test organisms divided into three groups (replicates) were exposed to each concentration, to the positive and negative controls in a static test. Ten individuals of one replicate were immersed in 3 ml of solution in each well of a 12-well plate.

The transfer of the test organisms in the wells was the crucial phase of the experiment. The excess of water around the nauplii was carefully recovered by aspiration, using a rubber tube fitted with a 200 µl micropipette tips and closed at the opposite end. The operations were carried out by means of a stereo dissection microscope with a bottom cool light.

The acute exposure tests were carried out in tissue culture plates (12-well, flat bottom with low evaporation) without feeding and lasted 96h, with intermediate inspections of the mortality of *T. fulvus* nauplii every 24h. Organisms were considered dead when they were unable to move any external appendage o any internal member in a period of up to 20 s of observation and light stimulation of well solution (Faraponova et al., 2016).

3.4.4. Embryo toxicity tests

The embryo toxicity test with sea urchin *Paracentrotus lividus* was performed in according with standard procedure US EPA (1994, 1995, 2000); ASTM (2004).

Adults of sea urchins were collected in a subtidal zone located along the coastal area of Ancona, Adriatic Sea, Italy. Immediately after collection the animals were transported in laboratory and placed in 200 L tanks in flowing seawater at a temperature of 14 ± 1° C, a salinity of 38 ‰ and natural photoperiod.

Three male and three females were induced to spawn by injecting 1 ml of 0.5 M KCl through the perioral membrane.

After fertilization, 1 ml of fertilized egg suspension (1000 embryos per ml) was added to 9 ml aliquots of test solutions and incubated in a dark room at 20°C for 48 h using sterile capped

polystyrene six-well microplates. At the end of the experiment, the samples were preserved in 10 µl of solution of Lugol and ethanol 1:3 and 100 embryos were counted.

The bioassay was conducted exposing embryo suspension at 5 increasing concentrations of each test solution (3 replicates per concentration) as reported in Table 2 prepared by dissolving hitch concentrated test solutions in 38‰ and 0.45 µm filtered seawater (FSW) collected at the same site and date as the adult sea urchins and was also used for negative control.

After 48 h of incubation at 20°C, the percentage of normally developed plutei were observed.

The acceptability of the test results was fixed as a percentage of normal plutei ≥ 70% in control tests (Volpi Ghirardini et al., 2005) and only if the EC_{50} using the reference toxicant (Cu^{2+}) fell within previously defined acceptability ranges for both tests (34.598 – 68.344 µg/l).

Another embryo toxicity test was carried out with the conditioned oyster according to (Environmental Protection Agency, 2007).

Mature oysters *Crassostrea gigas* were purchased from a commercial hatchery specialized in year-round production of mature oysters (Guernsey Sea Farms, UK). Oysters were kept at around 10°C for transportation and then acclimatized in filtered seawater (FSW) before the experiments. Oyster gametes were obtained by stripping after oyster were opened with a standard oyster knife. The eggs and/or sperm were collected with a clean Pasteur pipette inserted into the gonad tissues to a depth of 1 - 2 mm. The gametes were be transferred to separate volumes of artificial seawater and held at 22 ± 2°C. A small sample of sperm was transferred to a small vessel, together with a few volume of seawater.

After 15 - 30 minutes the activity of the sperm was be assessed using a microscope.

The female eggs were suspended in a single beaker; the egg suspension was then be adjusted with an appropriate volume of seawater to achieve an egg density of approximately 4000 - 5000 eggs/ ml. Within 30 minutes from obtaining the egg and sperm suspensions, the egg suspension was fertilized with the sperm suspension. The volume ratio was 2 - 3 ml of sperm suspension to 300 ml of egg suspension. After mixing, the eggs were checked within 15 minutes to ensure fertilization was occurring. By this time, sperm should surround each egg and polar bodies be evident.

After that, 1 ml of fertilized egg suspension was added to 9 ml aliquots of test solutions and incubated in a dark room at 20°C for 24 h using sterile capped polystyrene 6-well microplates. At the end of the experiment, the samples were preserved in 10 µl of solution of Lugol and ethanol 1:3 and 100 embryos were counted.

The bioassay was conducted as described for sea urchins embryo toxicity test. The acceptability of the test results was fixed as a percentage of normal D-larvae ≥ 70% in control tests and only if the EC_{50} using the reference toxicant (Cu^{2+}) fell within previously defined acceptability ranges (12.32 – 15.95 µg/l).

3.5. Data analysis

Statistical analyses were performed with GraphPad Prism 5® (Ver. 5.01). Analysis of variance (1-way ANOVA) was applied to test the differences among treatments followed by a Dunnett's post hoc test to determine significant differences between the exposed groups and the control group, at each sampling time. Level of significance was set at $p<0.05$, homogeneity of variance was checked by Cochran C and mathematical transformation applied if necessary.

The commercially available statistical software package Mathlab was used to calculate EC_{50} values and 95% confidence intervals included Trimmed Spearman-Karber methods for *P. lividus* test, *C. gigas* test and *P. tricornutum* test.

For *V. fischeri* test, the dose-response relationship, that is, the interaction of the sample-inhibition of bioluminescence, has been elaborated using the dedicated software Microtox OmniTM v. 1.16.

The LC_{50} for *T. fulvus* were calculated with 95% confidence limits by using the Probit Analysis Program (Version 1.5). The EC_{50} and LC_{50} values are reported as mg/L of oils, dispersant and oils+dispersant.

Data on bioaccumulation, biological responses and ecotoxicological bioassays were further elaborated within a weight of evidence (WOE) model Sediqualsoft (Piva et al., 2011) which summarizes large heterogeneous data from different line of evidence (LOE), providing a synthetic hazard index (Benedetti et al., 2012; Piva et al., 2011).

The quantitative and software-assisted WOE model (Sediqualsoft model; Benedetti et al., 2014, 2012; Piva et al., 2011; Regoli et al., 2014) has been developed to integrate and differently weight data from various Lines of Evidence (LOEs), which include sediment chemistry (LOE 1), bioavailability of chemicals in bioindicator species (LOE 2), ecotoxicological effects measured at subcellular level (biomarkers; LOE 3), toxicity at organism level (laboratory bioassays; LOE 4) and ecological effects on benthic communities (LOE 5). Logical flow charts, based on expert judgment and legislative constraints, have been converted into mathematical algorithms to elaborate synthetic hazard indices and classify different indicators of quality. The elaboration and integration of data occur at two consecutive levels: within each module, a specific hazard quotient is provided for the type of dataset corresponding to individual LOEs (Module output); the hazard quotients elaborated for individual LOEs are then integrated in a Risk Index, which assigns an increasing weight according to the ecological relevance of various LOEs.

In this study data on bioaccumulation (LOE 2) were calculated, for each parameter, by the variation of the concentration compared with proper controls corrected for the typology of pollutant and the statistical significance of the difference.

Briefly, the elaboration of bioavailability hazard within LOE 2 is based on the initial calculation, for each pollutant, of the Ratio to Reference (*RTR*), i.e. the increase of concentration compared to control organisms; from this value, a *RTRw* is obtained by the application of a correction factor (*w*) which depends on the statistical significance of the difference and the typology of chemical (if "non priority", "priority" or "priority and hazardous". Based on expert judgement, the model assigns the *RTRw* of each parameter to 1 of 5 classes of effect (Absent, Slight, Moderate, Major, Severe); the calculation of the cumulative Hazard Quotient for bioavailability (HQ_{BA}) does not consider parameters with *RTRw* <1.3 (effect Absent, i.e. concentrations≤control value for a priority and hazardous pollutants), calculates the average for those with *RTRw* ranging between 1.3 and 2.6 (Slight, i.e. up to 2 fold increase compared to controls), and adds the summation (∑) of all those with *RTRw*≥2.6 (i.e. effects Moderate, Major and Severe; Figure 4).

$$HQ_{BA} = \frac{\sum_{n=1}^{j} RTR_W(n)_{1.3 \le RTR_W(j) < 2.6}}{j} + \sum_{n=1}^{K} RTR_W(n)_{RTR(k) \ge 2.6}$$

The level of cumulative HQ_{BA} is summarized in one class of hazard for bioavailability, from Absent to Severe, depending on the distribution of analyzed chemicals within the different classes of effect (Benedetti et al., 2012; Piva et al., 2011).

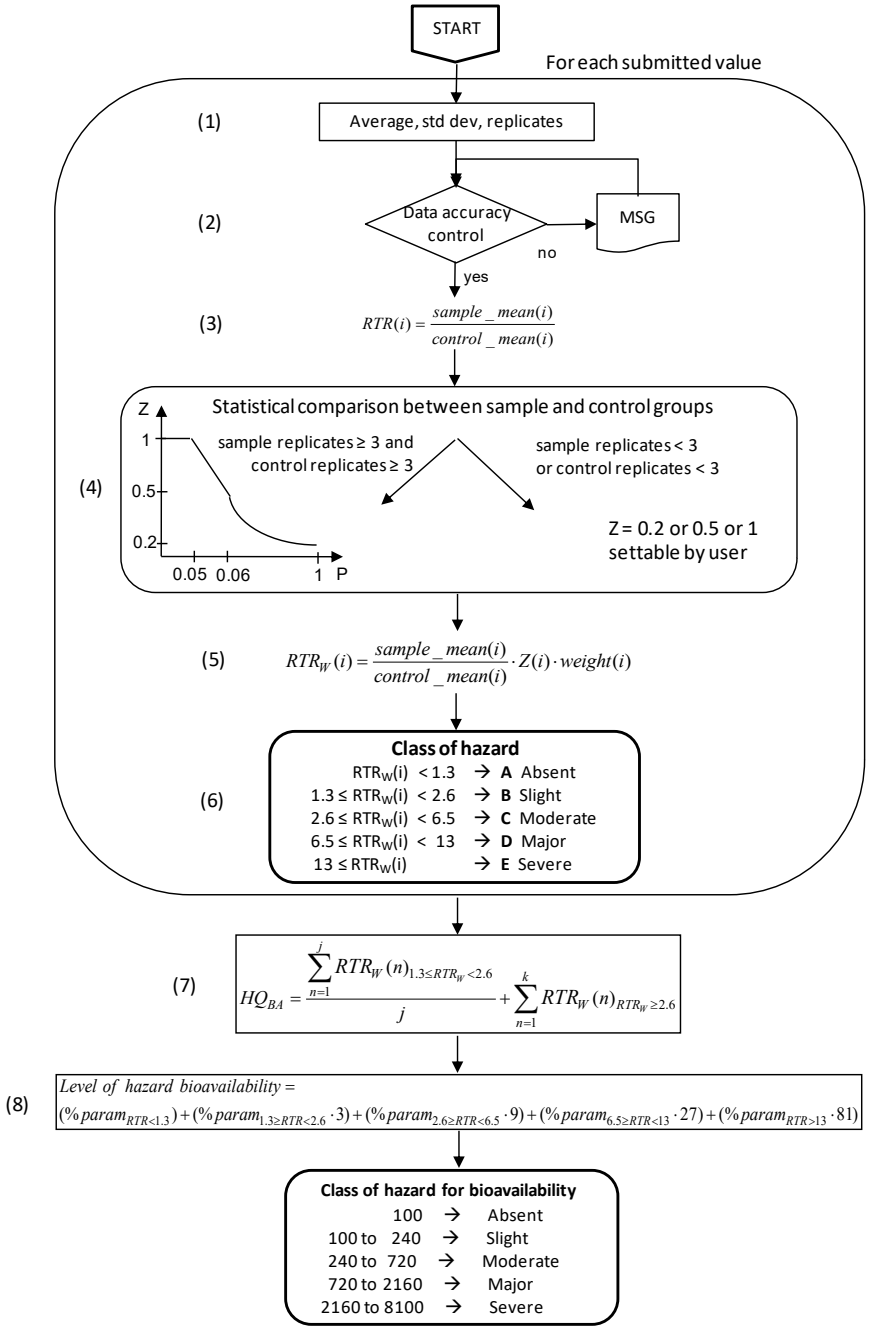


Figure 4. Flow-chart of LOE 2 (Bioavailability).

The module for the elaboration of biomarker (LOE 3) results contains a wide battery of responses, each assigned with a weight (based on the relevance of biological endpoint) and a threshold for changes of biological relevance which considers species or tissue differences, and the possibility of both induction and/or inhibition for biomarkers potentially showing biphasic responses (Piva et al., 2011). For every analysed biomarker, the measured variation is compared to the threshold (*effect*), then corrected for the weight of the response and the statistical significance of the difference compared to controls. The calculation of the Hazard Quotient for

biomarkers (HQ_{BM}) does not consider the contribution of responses with an effect <1 (lower than threshold), calculates the average for those with an effect up to two-fold compared to the threshold and adds the summation (Σ) for the responses more than 2 fold greater than the respective threshold (Piva et al., 2011):

$$HQ_{BM} = \frac{\sum_{j=1}^{N} Effect_W\ (j)_{1<Effect(j)\leq 2}}{num\ biomark_{1<Effect(j)\leq 2}} + \sum_{k=1}^{M} Effect_W(k)_{Effect(j)>2}$$

According to variations measured for various biomarkers, the model summarizes the level of cumulative HQ_{BM} in one of the five classes of hazard for biomarkers, from Absent to Severe (Piva et al., 2011; Figure 5).

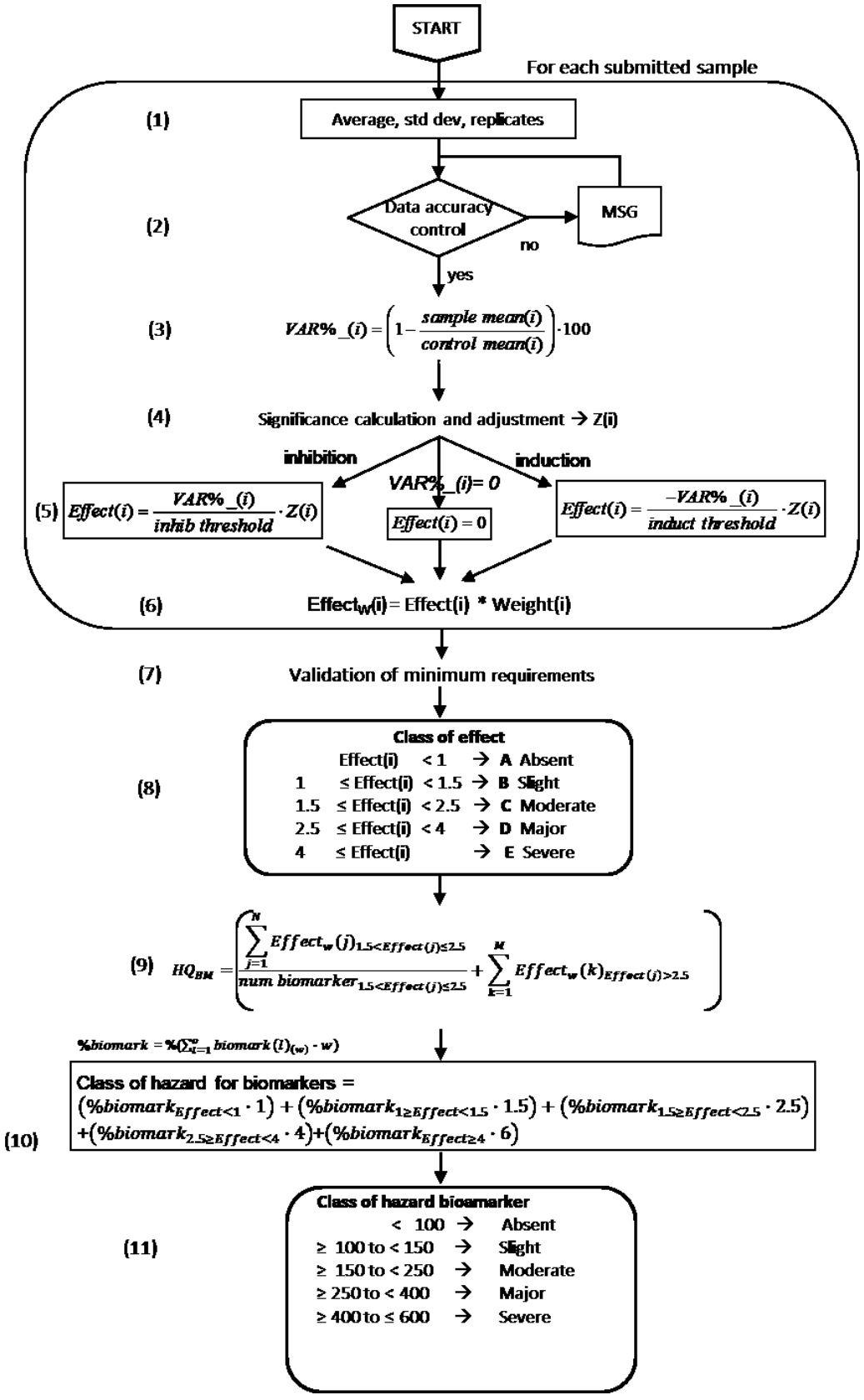


Figure 5. Flow-chart of LOE 3 (Biomarkers).

Several standardized laboratory tests are included in the module on ecotoxicological bioassays (LOE 4). Weighted criteria elaborate also the results from batteries of ecotoxicological assays at 200 mg/L of the exposure concentration of oils and oils+dispersants and at 20 mg/L of dispersant. Specific thresholds and weights are assigned to each bioassay depending on the biological endpoint, tested matrix, time of exposure, and the possibility of hormetic responses.

In the module for ecotoxicological bioassays, the cumulative hazard quotient ($HQ_{Battery}$) is obtained by the summation (Σ) of the weighted effects (*Ew*), i.e., the variations measured for each test compared to specific thresholds, corrected for the statistical significance of the difference (*w*), biological importance of the endpoint and exposure conditions (w_2):

$$HQ_{BATTERY} = \sum_{k=1}^{N} Effect_w(k) \cdot w_2$$

The $HQ_{Battery}$ is normalized to a scale ranging from 0 to 10, where 1 is the battery threshold (when all the measured bioassays exhibit an effect equal to the threshold, 10 when all the assays exhibit 100% of effect); the $HQ_{Battery}$ is then assigned to one of five classes of hazard, from Absent (if <1) to Severe (if >6); Figure 6).

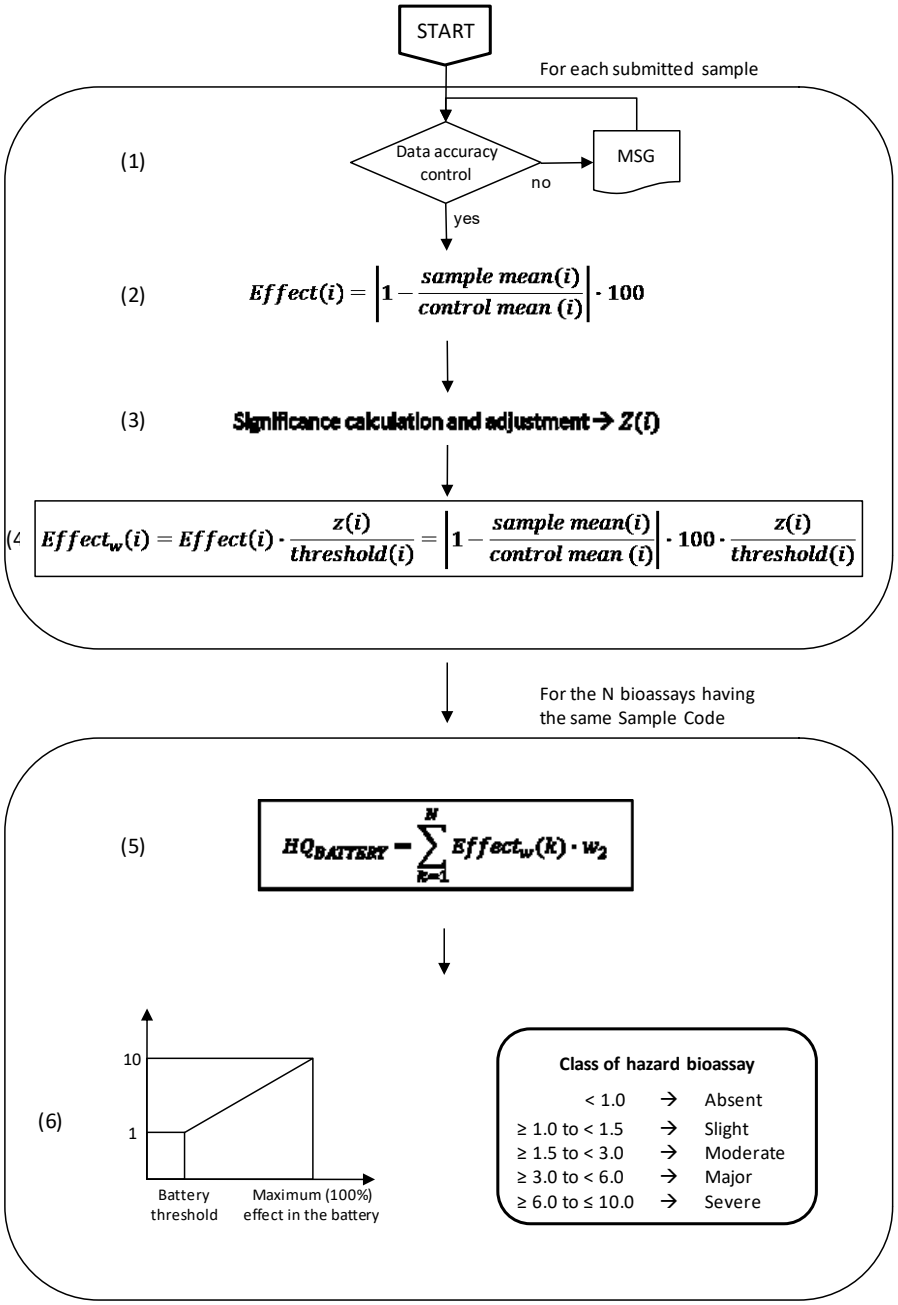


Figure 6. Flow-chart of LOE 4 (Bioassays).

The HQs obtained in single modules are finally elaborated through a classical WOE integration. After normalization of indices to a common scale, the assignment of a different weighting to LOEs is based on their different ecological relevance, and the capability of various datasets to directly unveil negative effects. An overall WOE level of risk is thus calculated and assigned to 1 of 5 classes from absent to severe (Figure 7).

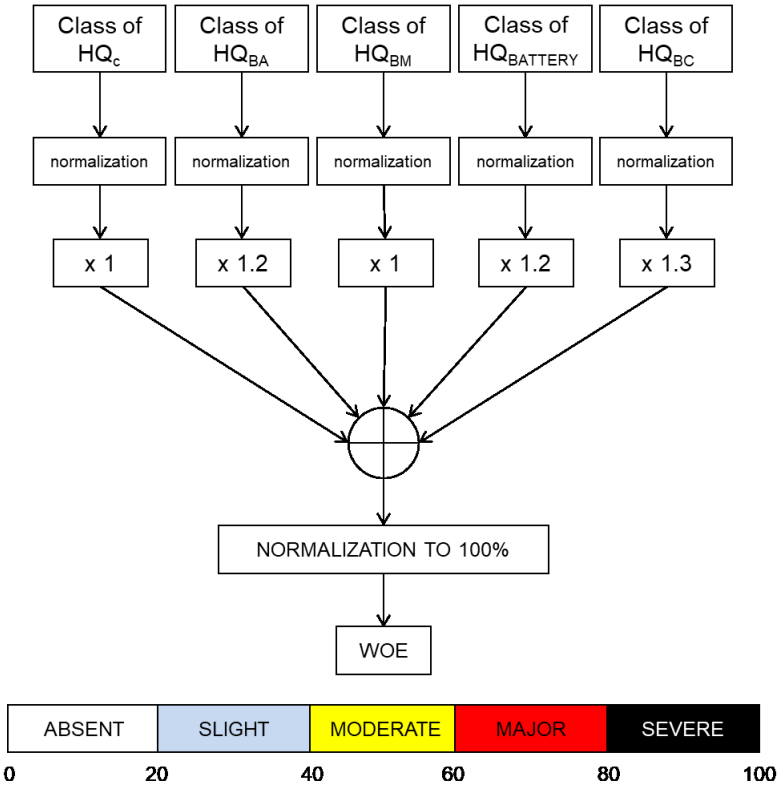


Figure 7. Flow-chart of WOE.

4. RESULTS

4.1. Bioaccumulation and biomarkers analyses in *M. galloprovincialis* exposed to Crude oil

The results on bioaccumulation, biochemical and cellular responses caused by the exposure of *M. galloprovincialis* to Water-Accommodated Fraction of CRUDE OIL, DISPERSANT and CRUDE OIL+DISPERSANT are reported in Figures 8 to 13. Organisms were exposed for 2 days and allowed to recover in clean seawater for the fallowing 21 days to simulate an acute oil spill scenario.

Obtained results highlighted that dispersant increased the accumulation of low and high molecular weight PAHs (Figure 8A-B). In particular, low molecular weight compounds (Figure 8A), which represent the dominant and more soluble component of the oil, markedly increased during the exposure period (2 days), with a value of 1518.9±98.3 ng/g d.w., approximately 5.7-fold higher than in controls, thus highlighting the capability of dispersants to make these hydrocarbons more bioavailable. Also low molecular weight PAHs were accumulated during exposure period in mussel treated with CRUDE OIL, with values of 720.0±73.0 ng/g d.w, 2.7-fold higher than in controls.

Concentrations of low molecular weight PAHs in mussels exposed to chemically dispersed oil tended to decrease during recovery period, although differences against control were still significant after 21 days, with a value of 386.8±47.4 ng/g d.w.

On the other hand, high molecular weight PAHs in mussels exposed to CRUDE OIL+DISPERSANT (Figure 8B), showed a great variability during the first phases of exposure/recovery, while significantly increased after 14 and 21 days (12.6±0.9 ng/g d.w.) of recovery probably due to their low excretion.

No significant variations were observed for tissue concentration of aliphatic hydrocarbons (Figure 8D) in different treatments and in all sampling times, with values ranging from 247.82±149 to 448.36±58 ng/g d.w.

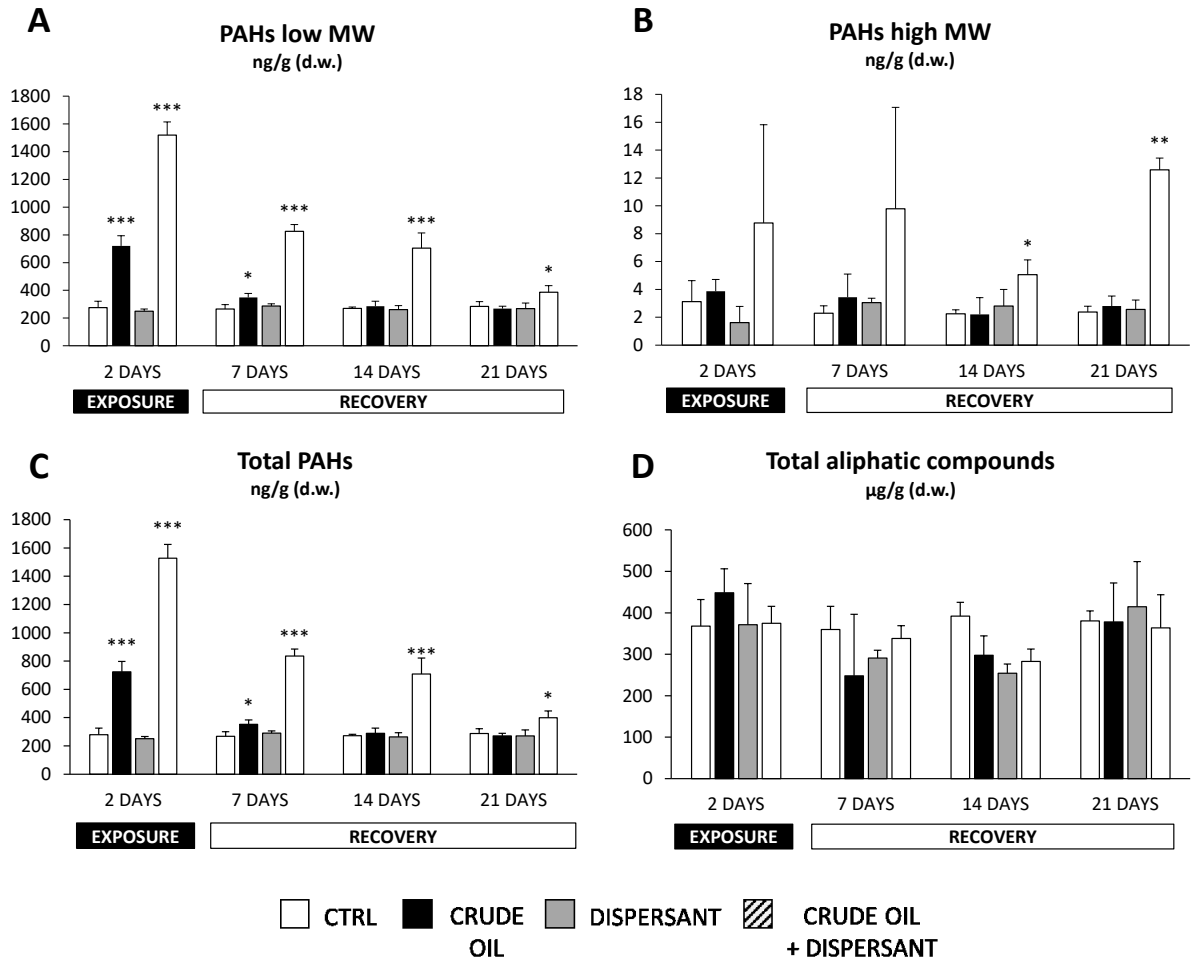


Figure 8: Bioaccumulation of low (A), high (B) molecular weight PAHs, total PAHs (C) and total aliphatic compounds (D) in whole soft tissues of *M. galloprovincialis* exposed to crude oil mechanically dispersed (CRUDE OIL), dispersant alone (DISPERSANT), oil chemically dispersed (CRUDE OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 days of recovery period in clean water. Values are shown as mean ± standard deviation (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Concerning the immunological parameters measured in haemolymph of mussels, a clear reduction of lysosomal membranes stability (Figure 9A) was observed in all treatments during the 2 days exposure time; this effect was strongly evident in mussels exposed to dispersant, either dosed alone and in combination with the oil, with a decrease of the neutral red retention time from 130 minutes in CONTROL to 82 and 84 minutes; mussels exposed to CRUDE OIL alone show a more limited but still significant decrease 108 minutes. This damage persisted during the first 2 weeks of recovery period, than returning to control levels after 21 days (Figure 9A).

Despite some fluctuations, no statistically significant effects were found in granulocyte/hyalinocytes ratio and in phagocytosis rate of mussels exposed to oil, dispersant and chemically dispersed oil (Figure 9B-C).

The acetylcholinesterase activity was not affected in gills (Figure 9D) while a significant enhancement was observed in haemolymph (Figure 9E) during the recovery period, thus revealing a delayed sensitivity of this neurotoxic marker in all the treatments.

A delayed effect was observed also in terms of DNA fragmentation (Figure 9F), which significantly increased during the recovery period, especially in organisms exposed to DISPERSANT and CRUDE OIL+DISPERSANT.

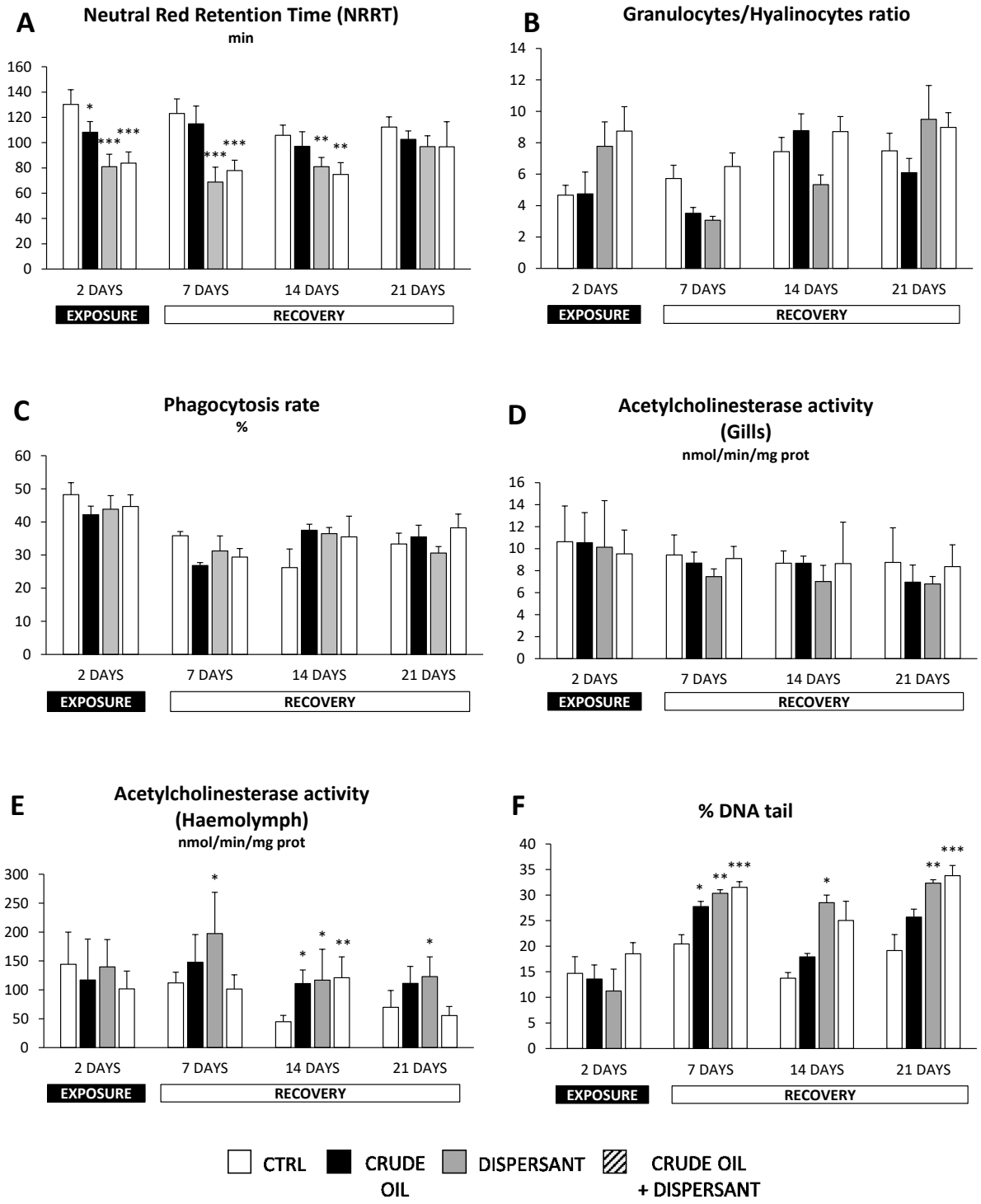


Figure 9: Lysosomal membrane stability, granulocytes-hyalinocytes ratio, phagocytosis rate, acetylcholinesterase activity and DNA damage in haemolymph (A, B, C, E and F) and acetylcholinesterase activity in gills (D) of *M. galloprovincialis* exposed to crude oil mechanically dispersed (CRUDE OIL), dispersant alone (DISPERSANT), chemically dispersed oil (CRUDE OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 days after depuration. Values are shown as mean ± standard error (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Slight modulations were observed for oxidative stress parameters in gills with a certain modulation of single antioxidant enzymes activity, such as catalase, glutathione reductase, Se-dependent glutathione peroxidases and of total glutathione level during exposure and recovery time but without a clear trend for any of various experimental treatments (Figure 10A-C-D-F).

The limited variations of antioxidant defences did not affect the total oxyradical scavenging capacity toward hydroxyl and peroxyl radicals (Figure 11A-B), with the only exception of gills in mussels exposed to CRUDE OIL+DISPERSANT during exposure time showing a temporary decrease in the capability to neutralize hydroxyl radicals.

While no variations were observed for Acyl-CoA oxidase activity in gills (Figure 11C), slight increases were measured for malondialdehyde content (Figure 11D), despite these effects were statistically significant only in gills of organisms exposed to CRUDE OIL+DISPERSANT after 14 days of recovery period.

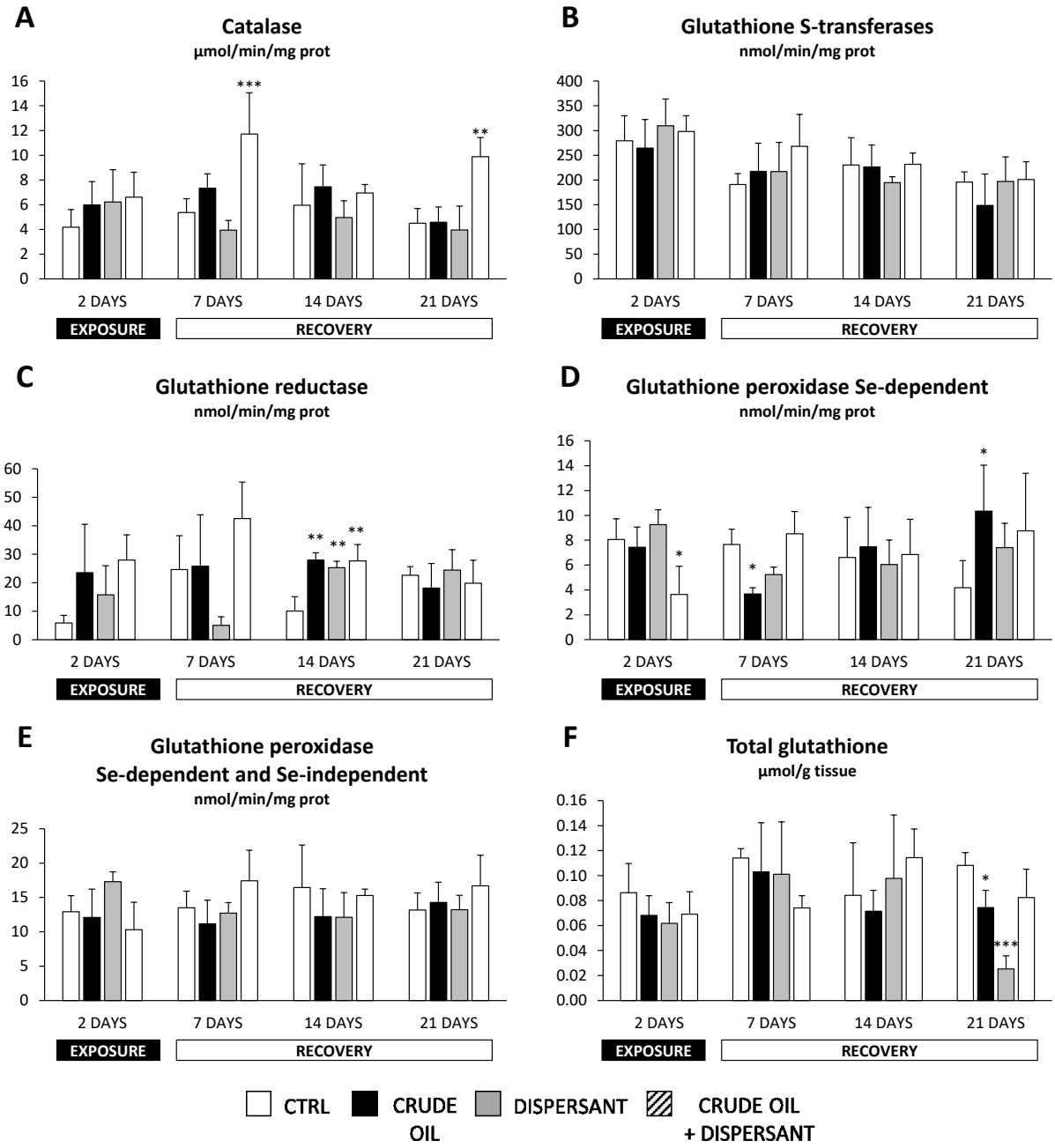


Figure 10: Activity of individual antioxidant enzyme activity (A-E) and total glutathione (F) measured in gills of *M. galloprovincialis* exposed to mechanically dispersed crude oil (CRUDE OIL), dispersant alone, chemically dispersed (CRUDE OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 days after depuration. Values are shown as mean ± standard deviation (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

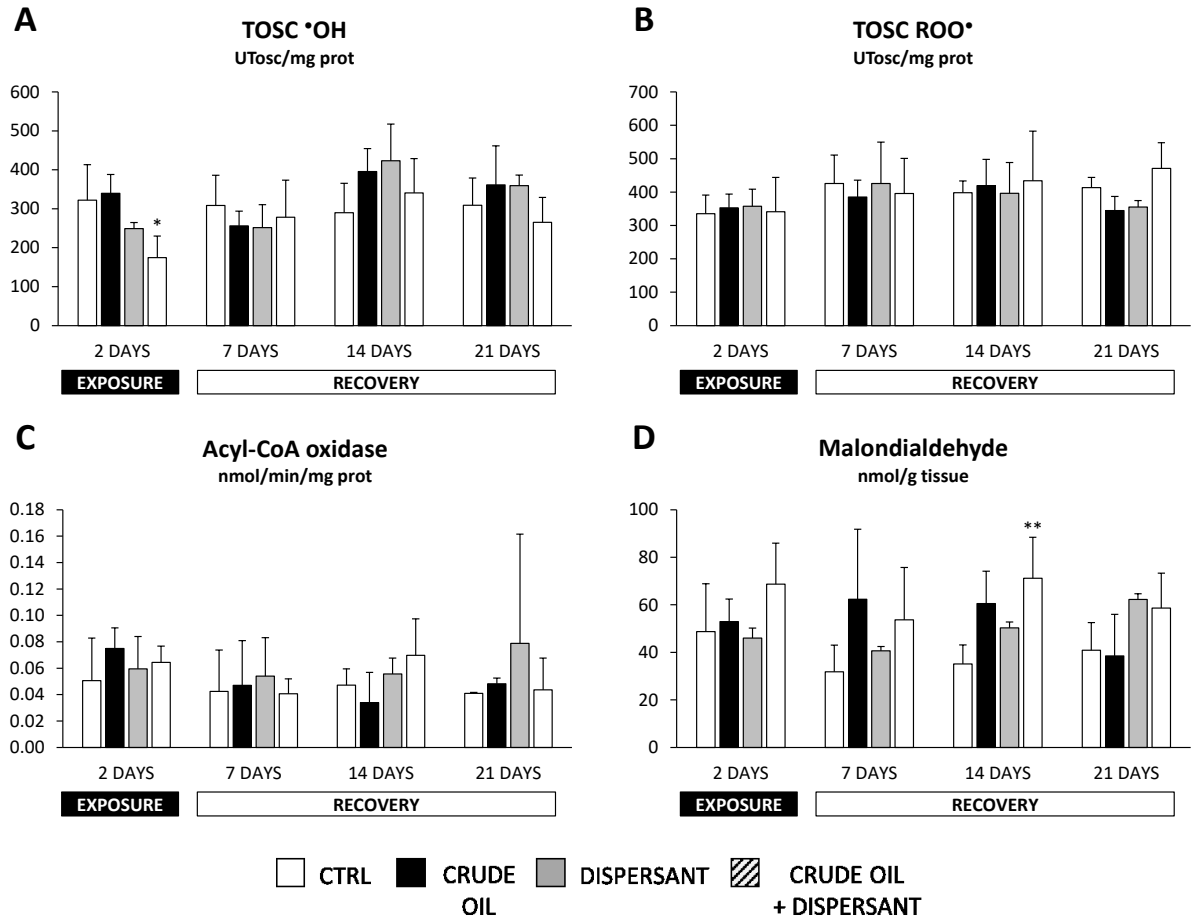


Figure 11: Total Oxyradical Scavenging Capacity toward hydroxyl and peroxyl radicals (A-B), activity of acyl-CoA oxidase (C) and level of malondialdehyde (D) measured in gills of *M. galloprovincialis* exposed to mechanically dispersed crude oil (CRUDE OIL), dispersant alone, chemically dispersed (CRUDE OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 days after depuration. Values are shown as mean ± standard deviation (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$).

Antioxidant responses measured in digestive glands, exhibit some significant inductions during exposure and early stage recovery period, especially in mussels exposed to dispersant dosed both alone and in combination with oil, in particular for catalase and glutathione peroxidase Se-dependent (Figure 12A-D). Total glutathione (Figure 12F) and the total oxyradical scavenging capacity against hydroxyl radical showed a similar increasing trend during the 2 days of exposure, which was statistically significant only for TOSC (Figure 13A). Slight and delayed variations in glutathione S-transferases (Figure 12B) were observed in organisms exposed to oil and dispersant dosed alone at the end of recovery period.

A delayed significant induction was observed in digestive gland also for acyl-CoA oxidase (Figure 13C) especially after 14 and 21 days of recovery in mussels exposed to chemically dispersed oil. Content of malondialdehyde (Figure 13D), increased after 7 and 14 days of

recovery period respectively in mussels exposed to CRUDE OIL and CRUDE OIL+DISPERSANT.

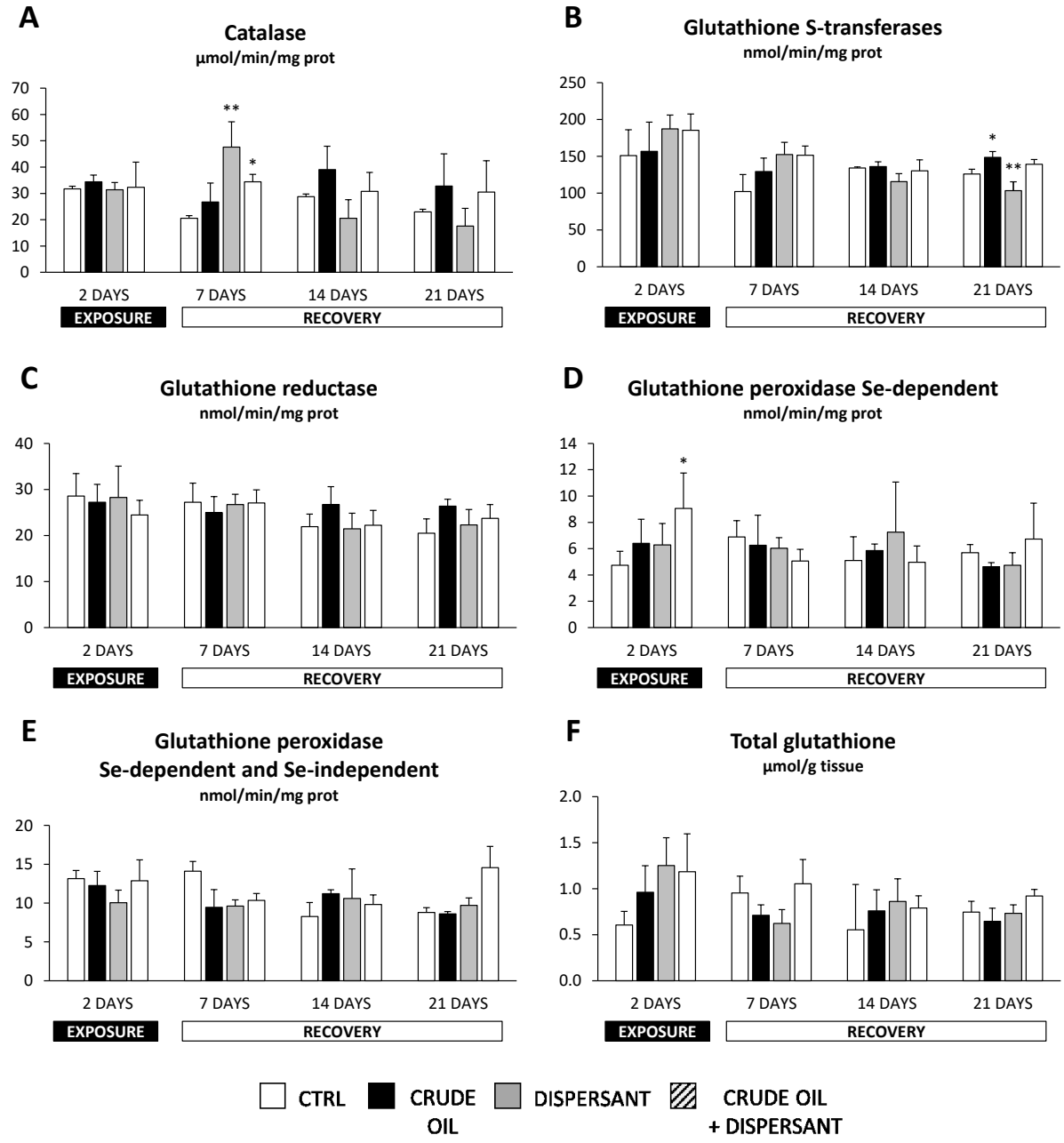


Figure 12: Activity of individual antioxidant enzyme activity (A-E) and total glutathione (F) measured in digestive glands of *M. galloprovincialis* exposed to crude oil mechanically dispersed (CRUDE OIL), dispersant alone (DISPERSANT), chemically dispersed (CRUDE OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 days after depuration. Values are shown as mean ± standard deviation (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$).

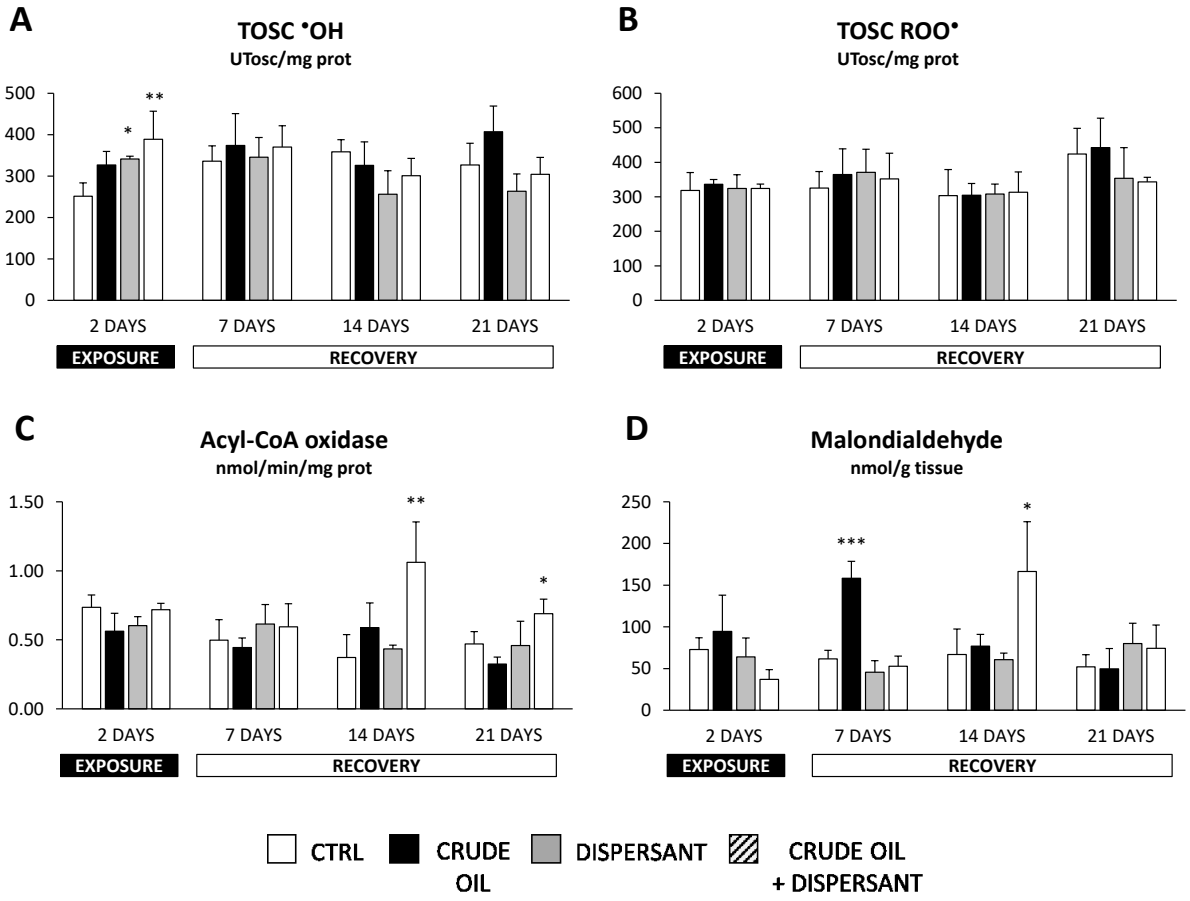


Figure 13: Total Oxyradical Scavenging Capacity toward hydroxyl and peroxyl radicals (A-B), activity of acyl-CoA oxidase (C) and level of malondialdehyde (D) measured in digestive glands of *M. galloprovincialis* exposed to crude oil mechanically dispersed (CRUDE OIL), dispersant alone (DISPERSANT), chemically dispersed (CRUDE OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 days after depuration. Values are shown as mean ± standard deviation (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

4.2. Bioaccumulation and biomarkers analyses in *M. galloprovincialis* exposed to Blend

Based on the results of the first experiment, the effects of the same dispersant were evaluated with a similar design using a different oil. In this respect organisms were exposed to blend dosed alone, to dispersant and to chemically dispersed blend. After 2 days exposure, mussels were recovered for 35 days in clean water. Analyses were focused on the most sensitive tissues and biological responses, including bioaccumulation, immunological responses, genotoxic damage and the neurotoxic biomarkers in haemolymph and gills.

Results confirmed the ability of the dispersant to increase the accumulation of PAHs with both low and high molecular weight. For low molecular weight PAHs (Figure 14A), the trend and the concentrations measured were similar to those obtained during the previous exposure

with crude oil: after two days of exposure, these values were 364.9±65.7, 104.3±92.3 and 1435.6±107.1 ng/g d.w. for BLEND, DISPERSANT and BLEND+DISPERSANT treatment. Such values corresponded to a bioaccumulation rate in organisms exposed to blend with and without dispersant of respectively 14.6 and 3.7-fold compared to controls, highlight again the general ability of dispersant to make these hydrocarbons more bioavailable. During the recovery period, levels of low molecular weight PAHs decreased over time returning to values comparable to controls after 14 days in clean water.

Even for high molecular weight PAHs (Figure 14B), the BLEND+DISPERSANT treatment determined a higher bioaccumulation both during the exposure and, evenmore, during the recovery period (34.4±2.6 and about 60.0 ng/g d.w. respectively).

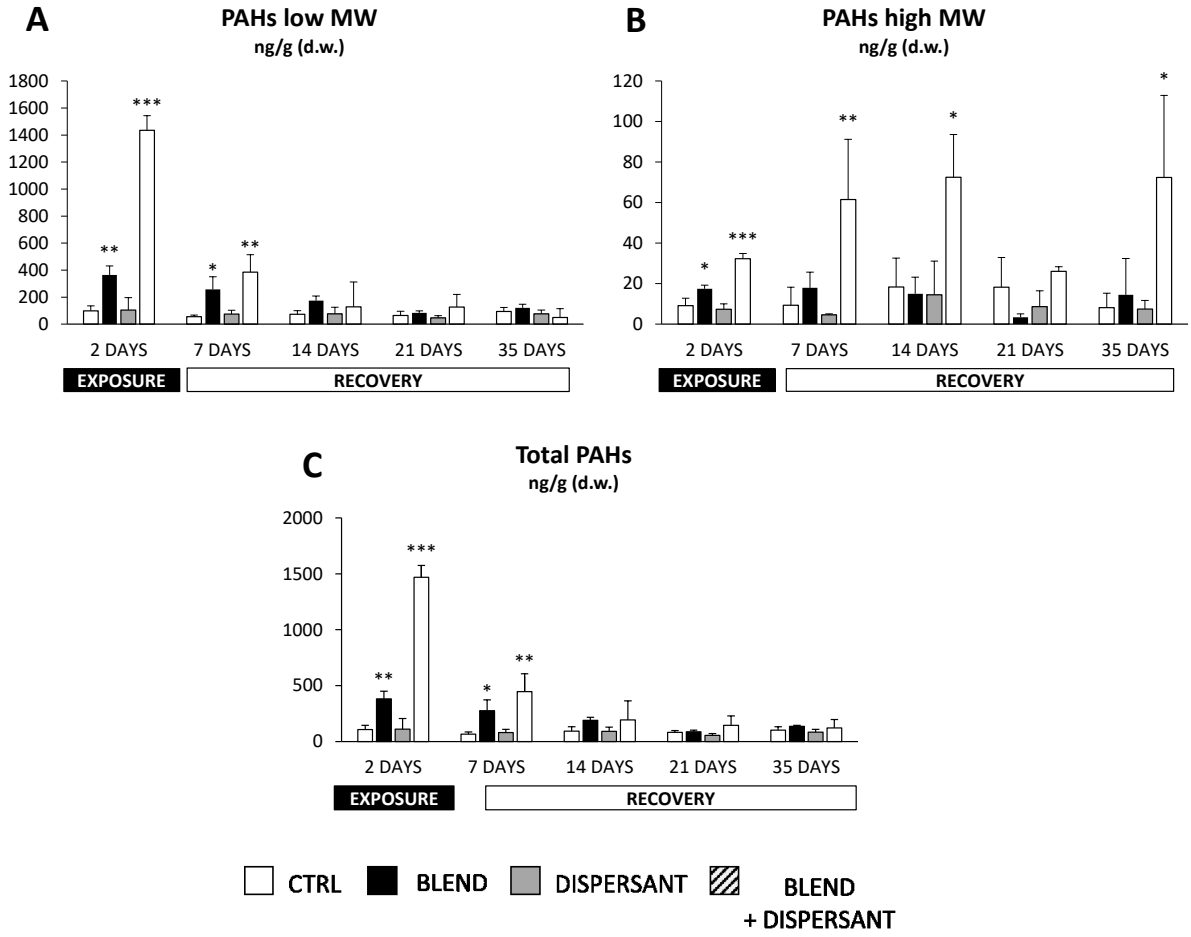


Figure 14: Bioaccumulation of low (A), high (B) molecular weight PAHs, total PAHs (C) in whole soft tissues of *M. galloprovincialis* exposed to blend mechanically dispersed (BLEND), dispersant alone (DISPERSANT), blend chemically dispersed (BLEND+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 and 35 days of recovery period in clean water. Values are shown as mean ± standard deviation (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

In light of the results obtained in the first experiment, which highlighted the high sensitivity of the haemolymph and gills, biomarkers were analyzed only in these tissues. Also during treatments with blend, a reduction of the lysosomal membranes stability (Figure 15A) was clearly observed after 2 days of exposure to the DISPERSANT and BLEND+DISPERSANT with values respectively of 73.6±5.8, 63.3±13.9 min; as comparison, CONTROL and BLEND dosed alone showed values of 100.0±4.2 and 102.4±4.0 min. After 14 days of recovery, organisms exposed to DISPERSANT returned to control levels, while those treated with BLEND+DISPERSANT maintained a reduction of the lysosomal membrane stability, still significantly lower than the other treatments (77.7±5.2 min).

As already observed in the first experimental design, no variations were detected in granulocytes-hyalinocytes ratio and in acetylcholinesterase activity in gills (Figure 15B-C). In addition, more limited effects were caused by blend (mechanically and chemically dispersed) for acetylcholinesterase activity in haemolymph and DNA damage in haemocytes, except for a significant increase in respectively DISPERSANT and BLEND during 21 days of recovery.

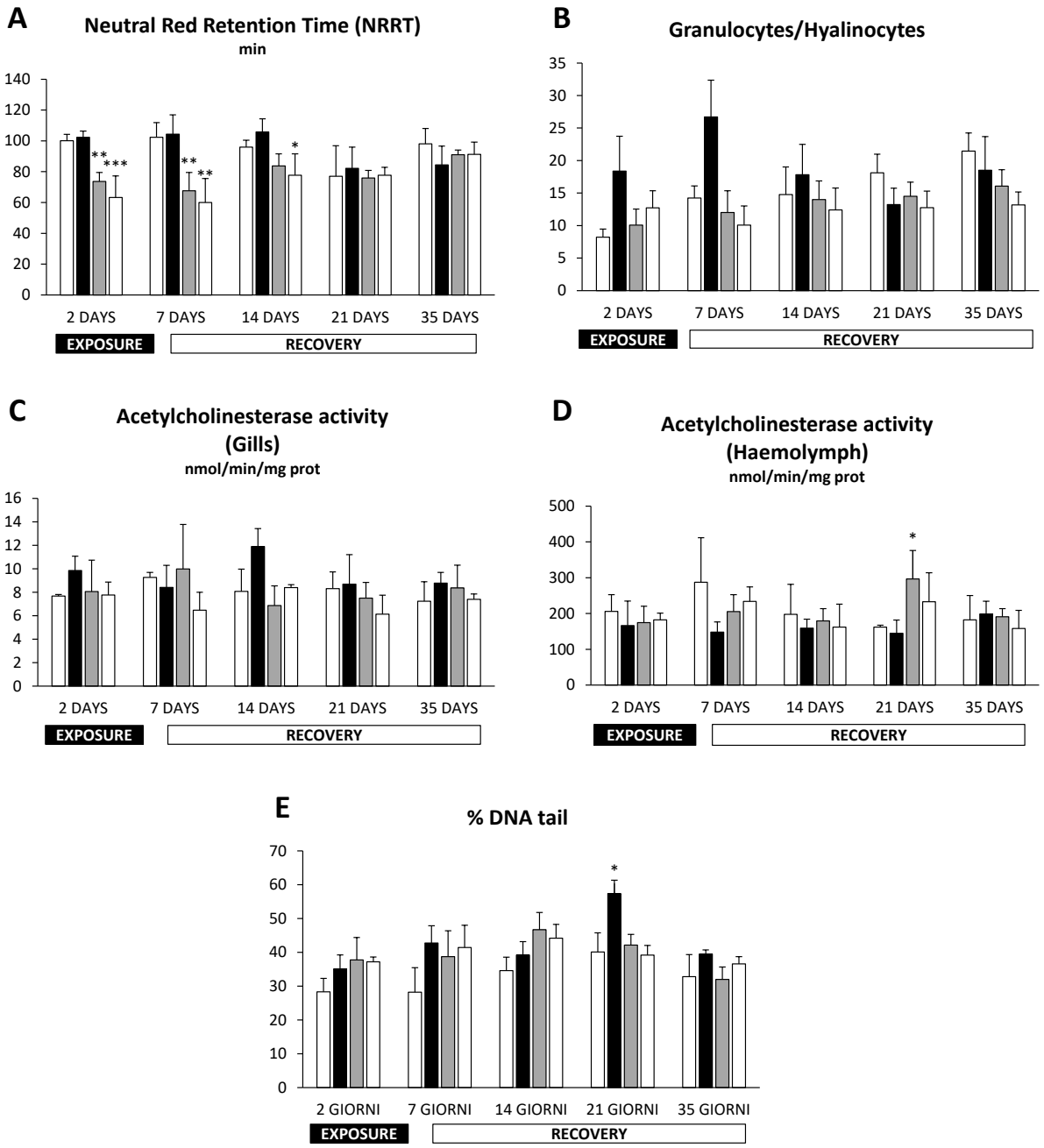


Figure 15: Lysosomal membrane stability, granulocytes-hyalinocytes ratio, acetylcholinesterase activity and DNA damage in haemolymph (A, B, D and E) and acetylcholinesterase activity in gills (C) of *M. galloprovincialis* exposed to blend mechanically dispersed (BLEND), dispersant alone (DISPERSANT), chemically dispersed blend (BLEND+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 and 35 days after depuration. Values are shown as mean ± standard error (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

4.3. Bioassays

4.3.1. Bacterial test

The 30 minutes EC_{50} for *Vibrio fischeri* exposed to CRUDE OIL is major of the maximum concentration tested (1800 mg/L), indicating the lack of acute toxicity of this oil for this species: the 30 minutes EC_{20} value was 938.9 (827.5 - 1065.4) mg/L. On the contrary, the 30 minutes EC_{50} values for DISPERSANT and CRUDE OIL+DISPERANT treatment were respectively 13.04 (8.52 - 19.96) and 504,4 (491,4 - 517,5) mg/L (Table 3). Thus indicating a higher acute toxicity compared to oil alone.

4.3.2. Algal test

The algal sensitivity, evaluated by testing the toxicity of potassium dichromate ($K_2Cr_7O_3$), resulted in 72h EC_{50} of 8.78 (7.02 - 10.98) mg/L, a slightly lower value than that reported in literature.

The 72h EC_{50} values for CRUDE OIL and BLEND were both higher than the higher tested dose (>350 mg/L); for CRUDE OIL+DISPERSANT, BLEND+DISPERSANT and DISPERSANT alone, the 72h EC_{50} values were 32.91 (31.97 - 33.87) mg/L, 36.31 (33.56 - 39.27) mg/L and 2.96 (2.79 - 3.14) mg/L respectively (Table 3), thus confirming higher toxicity compared to oils dosed alone.

4.3.3. Crustacean test

The results of the crustacean test with *Tigriopus fulvus* obtained after the exposure of different concentration of CRUDE OIL, DISPERSANT and CRUDE OIL+DISPERSANT, revealed a 96h LC_{50} values >1800 mg/L, 21.40 (n.d.) mg/L and 239.74 (n.d.) mg/L respectively (Table 3).

The sensitivity of this organism was evaluated by testing a concentration of 0.05 mg Cu^{2+}/L, corresponding to 96h EC_{50} value for *T. fulvus*. While the validity of all copepod assays was ensured by a 100% of control survival.

4.3.4. Embryotoxicity test

Results of the embryotoxicity test on *Paracentrotus lividus* were considered acceptable considering that the percentage of normal plutei was higher than 70% in the controls.

The bioassay has been performed on both the oils in different treatments. The sensitivity of the embryo was verified by testing the reference toxicant (Cu^{2+}) with a 48h EC_{50} of 33.57 (29.46 - 38.25) µg/L. The 48h EC_{50} values was >450 mg/L for CRUDE OIL, 279.57 (267.75 - 291.92) mg/L for CRUDE OIL+DISPERSANT, 289.17 (269.69 - 310.05) mg/L for BLEND, 156.45 (141.57 - 172.89) for BLEND+DISPERSANT and 23.51 (18.59 - 23.51) mg/L for DISPERSANT (Table 3).

Concerning the embryotoxicity test of *Crassostrea gigas*, the bioassays has been performed on the CRUDE OIL, CRUDE OIL+DISPERSANT and DISPERSANT in which the relative 24h EC_{50} were not calculated, being out of the concentrations range considered. Despite this, the bioassays gave some indication of the toxicity of various treatments: the 24h EC_{50} in the CRUDE OIL treatment was >500 mg/L conversely for CRUDE OIL+DISPERSANT and DISPERSANT the 24h EC_{50} was <50 mg/L and <5 mg/L respectively (Table 3). The sensitivity of the embryos was verified by testing the reference toxicant (Cu^{2+}) with a 24h EC_{50} of 15.95 (14.28 – 17.82).

	Vibrio fischeri	*Phaeodactylum tricornutum*	*Tigriopus fulvus*	*Paracentrotus lividus*	*Crassostrea gigas*
	Decreasing bioluminescence 30 min EC50 (mg/L)	Growth inibition 72h EC50 (mg/L)	Mortality 96h LC50 (mg/L)	Larval development 48h EC50 (mg/L)	Larval development 24h EC50 (mg/L)
Crude oil	>1800	>350	>1800	>450	>500
Dispersant	13.04 (8.52-19.96)	2.96 (2.79-3.14)	21.40 (n.d.)	23.51 (18.59-23.51)	<5
Crude oil + Dispersant	504.4 (491.4-517.5)	32.91 (31.97-33.87)	239.74 (n.d.)	279.57 (267.75-291.92)	<50

Table 3: E(L)C50 (mg/L) calculated for CRUDE OIL, DISPERSANT, CRUDE OIL+DISPERSANT. n.d. = not determinated.

	Phaeodactylum tricornutum	***Paracentrotus lividus***
	Growth inibition 72h EC50 (mg/L)	Larval development 48h EC50 (mg/L)
Blend	>350	289.17 (269.69-310.05)
Dispersant	2.96 (2.79-3.14)	23.51 (18.59-23.51)
Blend + Dispersant	36.31 (33.56-39.27)	156.45 (141.57-172.89)

Table 4: E(L)C_{50} (mg/L) calculated for BLEND, DISPERSANT, BLEND+DISPERSANT.

4.4. Weight of evidence elaboration

The bioaccumulation, biomarkers and bioassays results have been processed within the Sediqualsoft model which elaborated specific lines of evidence, providing quantitative and qualitative indices of hazard (from Absent to Severe).

The Figure 16 and 17 show the hazard indices elaborated for various LOEs, the final integration (WOE) for each treatment with the two different oils, exposures or recovery time.

Concerning the bioavailability, in organisms exposed to CRUDE OIL dosed alone, the level of hazard appeared to be “Moderate” during the two days of exposure, “Slight” and “Absent” respectively after 7, 21 and 14 days of recovery (Figure 16).

For organisms exposed to the DISPERSANT, the “Absent” hazard for bioaccumulation was observed at all experimental times with the exception of the last time of recovery; on the contrary, a “Major” hazard level was calculated in CRUDE OIL+DISPERSANT treatment respectively after two days of exposure and 14 days of recovery, and “Moderate” for the other experimental times.

The elaboration of biomarkers results in haemolymph, gills and digestive gland allowed to summarize the synthetic biological hazard index as “Slight” in CRUDE OIL dosed alone treatment after 2 days exposure and 7 and 21 days of recovery and “Moderate” after 14 days of recovery (Figure 16).

For mussels exposed to DISPERSANT, the biomarker hazard level was “Slight” after 2 days of exposure and “Moderate” during all the recovery period; a “Moderate” biomarker hazard level was measured in mussels exposed to the CRUDE OIL+DISPERSANT after 2 days of exposure and 14 days of recovery, “Slight” after 7 and 21 days of recovery.

The battery of bioassays performed on different species belong to different trophic levels showed an hazard level as “Absent” for CRUDE OIL dosed alone and “Major” for both DISPERSANT and CRUDE OIL+DISPERSANT treatments.

The final Weight Of Evidence integration of chemical, biological and ecotoxicological bioassays analysed in the different treatments and experimental times, showed a level of risk classified as “Slight” for organisms exposed to CRUDE OIL dosed alone, “Moderate” for DISPERSANT treatment during the exposure time and the purification, while organisms treated with CRUDE OIL+DISPERSANT showed a level of risk “Moderate” after 7 and 21 days of recovery and “Major” for the other experimental times.

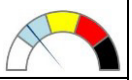
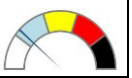
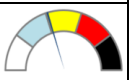
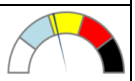
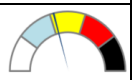
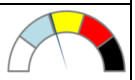
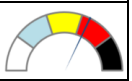
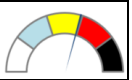
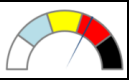
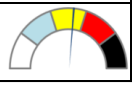
	Time	BIOAVAILABILITY	BIOMARKERS	BIOASSAYS	WOE	
Crude Oil	2 days	MODERATE	SLIGHT	ABSENT	SLIGHT	
	7 days	SLIGHT	SLIGHT		SLIGHT	
	14 days	ABSENT	MODERATE		SLIGHT	
	21 days	SLIGHT	SLIGHT		SLIGHT	
Dispersant	2 days	ABSENT	SLIGHT	MAJOR	MODERATE	
	7 days	ABSENT	MODERATE		MODERATE	
	14 days	ABSENT	MODERATE		MODERATE	
	21 days	SLIGHT	MODERATE		MODERATE	
Crude Oil + Dispersant	2 days	MAJOR	MODERATE	MAJOR	MAJOR	
	7 days	MODERATE	SLIGHT		MODERATE	
	14 days	MAJOR	MODERATE		MAJOR	
	21 days	MODERATE	SLIGHT		MODERATE	

Figure 16: Qualitative Weight of Evidence (WOE) classification of single Lines on Evidence witch integrated bioavailability, biomarkers and bioassays in organisms exposed to CRUDE OIL, DISPERSANT and CRUDE OIL+DISPERSANT.

Data of the second exposures with BLEND and DISPERSANT (Figure 17), showed in BLEND treatment a “Moderate” hazard level for bioaccumulation after 2 days of exposure and after 7 days of recovery, followed by a “Absent” and “Slight” hazard level after 21 and 14-35 days respectively.

The bioaccumulation hazard level, in DISPERSANT treatment was always “Absent” for all the times of exposure.

The bioaccumulation hazard level for BLEND+DISPERSANT treatment was “Major” after 2 and 7 days of exposure and recovery, “Slight” after 14, 21 days, “Moderate” after 35 days of recovery.

Concerning the biomarkers results measured in haemolymph and gills of organisms exposed to BLEND, a “Moderate” level of hazard appeared during 2 days of exposure and after 7 days

of recovery, otherwise the level of hazard appear to be “Slight” after 14, 21 and 32 days of recovery.

For mussels exposed to DISPERSANT, the biomarker hazard level was “Slight” after 2 days of exposure and in all the time of recovery with the exception of 21 days of recovery in which the level of hazard appeared “Moderate”.

Similarly, in BLEND+DISPERSANT treatment, the “Slight” hazard level was observed at all experimental times.

The battery of bioassays performed on different species, showed an hazard level as “Absent” for BLEND dosed alone and “Major” for both DISPERSANT and BLEND+DISPERSANT treatments.

The final Weight of Evidence integration of bioaccumulation, biomarkers and ecotoxicological bioassays, analysed in the different experimental times and treatments, showed a level of risk classified as “Slight” for exposed organisms to BLEND dosed alone after 2 days of exposure and 7 and 14 days of recovery, “Absent” for the same organisms after 21 and 35 days of recovery. Differently the level of risk was classified as “Moderate” for organisms treated with DISPERSANT and BLEND+DISPERSANT although, in the latter case, the level of risk was classified as “Major” after 2 days of exposure.

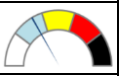
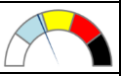
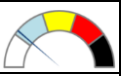
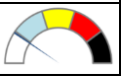
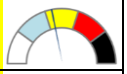
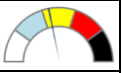
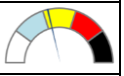
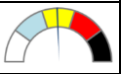
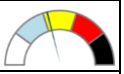
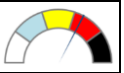
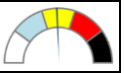
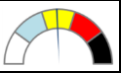
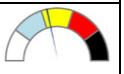
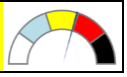
	Time	BIOAVAILABILITY	BIOMARKERS	BIOASSAYS	WOE
Blend	2 days	MODERATE	MODERATE	ABSENT	SLIGHT 
	7 days	MODERATE	MODERATE		SLIGHT 
	14 days	SLIGHT	SLIGHT		SLIGHT 
	21 days	ABSENT	SLIGHT		ABSENT 
	35 days	SLIGHT	SLIGHT		ABSENT 
Dispersant	2 days	ABSENT	SLIGHT	MAJOR	MODERATE 
	7 days	ABSENT	SLIGHT		MODERATE 
	14 days	ABSENT	SLIGHT		MODERATE 
	21 days	ABSENT	MODERATE		MODERATE 
	35 days	ABSENT	SLIGHT		MODERATE 
Blend + Dispersant	2 days	MAJOR	SLIGHT	MAJOR	MAJOR 
	7 days	MAJOR	SLIGHT		MODERATE 
	14 days	SLIGHT	SLIGHT		MODERATE 
	21 days	SLIGHT	SLIGHT		MODERATE 
	35 days	MODERATE	SLIGHT		MODERATE 

Figure 17: Qualitative Weight of Evidence (WOE) classification of single Lines on Evidence witch integrated bioavailability, biomarkers and bioassays in organisms exposed to BLEND, DISPERSANT and BLEND+DISPERSANT.

5. DISCUSSION AND CONCLUSIONS

Considering the growing international demand of crude oil, the increase of oil extraction worldwide, and the consequent greater risk of oil spills, this PhD thesis focussed on the biological effects deriving from the use of dispersant products. In particular, the ambition of this work was to suggest a revision of the actual Italian legislation to evaluate of the potential toxicity of chemical dispersants.

Dispersant usage has been a topic of renewed interest for the European national administrations in recent years, in particular following the Deepwater Horizon incident in 2010 in the Gulf of Mexico (EMSA, 2016).

Every European countries adopt national protocols to test oil dispersants before their possible use/application at sea but, in the majority of cases, only the intrinsic toxicity of dispersant is evaluated.

The aim of this study was to increase the knowledge on the possible synergistic effects and toxicity of dispersants and oil mixtures on marine organisms and to propose a new experimental protocol to assess both the effects of chemically and mechanically dispersed oils.

The experimental exposures of *M. galloprovincialis* to the Water-Accommodated Fraction of oils, dispersant, and oils+dispersant had been performed to better understand the sublethal effects of these different treatments and how the different characteristics of crude oils differ in term of toxicity if treated or not with dispersant. Another aspect taken into account was the sublethal and lethal effects of the same treatments at higher biological organization, and possible changes on growth, reproduction, development etc. in different species.

An hypothetical oil spill scenario in natural marine environment consists of a first stage in which we would expect a period of maximum in PAHs concentrations in water column, followed by other phases of exposure to lower environmental levels due to natural processes, human clean-up activities, and recovery steps (Boehm and Page, 2007; Lee et al., 2013; Lessard and DeMarco, 2000).

In this thesis, data will be discussed on the different parameters considered, such as bioaccumulation of oil, biomarkers and bioassays measured in different treatments and using two types of oils.

5.1. Bioaccumulation of mechanically and chemically dispersed oils

The results on the bioavailability of PAHs, that is the fraction of petroleum associated with the long-term toxicity (Boehm and Page, 2007; Perrichon et al., 2016), after two days of

exposure of CRUDE OIL and BLEND, showed that the dispersant is able to increase bioaccumulation of both low and high molecular weight PAHs. In mussels exposed to CRUDE OIL+DISPERSANT, hydrocarbons with low molecular weight which represents the more soluble and dominant component of oil, increase of about 5.5 times compared to the control organisms, especially during the exposure period. These results highlight the ability of the chemical dispersant to make these molecules more bioavailable than in oil dosed alone, which determined a 2.6 fold increase compared to controls.

The same trend were also found in mussels exposed to BLEND+DISPERSANT and BLEND in which, once again, after the two days of exposure we noted the effect of dispersant to significantly increase the bioaccumulation of PAHs: concentration were 14.6 and 3.7-fold higher compared to controls in mussels exposed to chemically dispersed or mechanically dispersed oil.

Similar results, but with different tissue concentrations, had been highlighted also in the Icelandic scallop *Chlamys islandica* after two days of exposure at 333.3 mg/L nominal concentration of mechanically and chemically dispersed crude oil, where the measured levels were 35.4 and 42.3 times greater than in the controls (Frantzen et al., 2016); in Pacific oyster (*Crassostrea gigas*) exposed to 67 mg/L of nominal concentration of mechanically and chemically dispersed crude oil, PAHs increased of 28.3 and 75.7 folds compared to controls (Luna-Acosta et al., 2011). The apparent discrepancy between concentrations could be due to differences of investigated species, but also to the different kind of oil and dispersant used, and how the exposure media is generated, that make difficult the direct comparison of the experimental studies.

Differences in PAHs bioavailability between mechanically and chemically dispersed oils were evident also during the recovery period; after 21 days of recovery the low PAHs concentrations in whole tissues were higher in organisms co-exposed to oils and dispersant compared to those treated with oils dosed alone. Nevertheless, is interesting to note how the concentration of low molecular weight PAHs in mussels exposed to BLEND+DISPERSANT decrease faster than in organism exposed to CRUDE OIL+DISPERSANT, reaching concentrations comparable to control after only 14 days of recovery against the 21 days. This behaviour is probably due to the different composition of oils with a major predominance of low molecular weight PAH in blend compared to crude oil.

The capability of the PAHs to be easily bioaccumulated and slowly eliminated was also observed in the Pacific oyster *C. gigas*: despite a decrease of these molecules of the 97% and

93% in chemically dispersed and mechanically dispersed oil treatment respectively after 15 days of depuration, concentration still remained significantly higher than in controls (Luna-Acosta et al., 2011).

A partly different behaviour was observed for high molecular weight PAHs: despite they were accumulated at lower concentrations compared to low molecular weight PAHs in organisms exposed both to mechanically and chemically dispersed crude oil and blend, these compounds remained stable and were not excreted during recovery period, especially in organisms exposed to oils and dispersant. In general these compounds are more persistent, hydrophobic and less water soluble (Couillard and Lee, 2005; Perrichon et al., 2016), characteristics that promote their permanence in tissues. In addition, the absence of an efficient biotransformation pathway in invertebrates, like Cytochrome P450 for vertebrates, does not allow to excrete though Phase II enzymes these compounds in a short period (Stegeman and Lech, 1991).

Compared to PAHs, the bioavailability and the bioaccumulation of aliphatic compounds (C10-C40) did not show any variations between exposure to mechanically and chemically dispersed crude oil, with values, ranging between 247.82±148.5 and 448.4±57.8 µg/g d.w., comparable to those measured in control organisms (Benedetti et al., 2014).

5.2. Biomarkers responses in mussels

The immune system is extremely vulnerable to the xenobiotic stressors in a wide range of organisms (Galloway and Depledge, 2001). Alterations, such as lysosomal membrane stability, phagocytosis activity and granulocytes/hyalinocytes ratio, represent important early warning indicators of the environmental disturbance and they are an important tool for assessing the sub-lethal effects of contaminant exposure (Hannam et al., 2010b). Lysosomal membrane stability bivalve haemocytes can be impaired by PAHs or, alternatively, by the metabolic phase of PAH detoxification that can enhance the production of reactive oxygen species (ROS), with direct damaging to cell membrane through lipid peroxidation. In this respect, the lysosomal responses, including the destabilization of these membranes, are widely used because extremely sensitive to a large range of environmental contaminants, including PAHs (Grundy et al., 1996; Hannam et al., 2010a; Lowe et al., 1995; Regoli, 1992). In our work, the stability of lysosomal membranes decreased significantly during the period of exposure in all organisms exposed to

different experimental conditions with both the oils, with the exception of BLEND dosed alone. The same effects were also found in *Chlamys islandica* where the reduction of this biomarker was significant in organisms exposed for 24h to both mechanically (44.25±13.8 min) and chemically dispersed oil (21.0±35.9 min) compared to controls (109.5±35.9 min) (Frantzen et al., 2016). In our study, the decrease of the membrane stability persisted even after the 14 days of recovery exclusively in organisms exposed to crude oil dosed with dispersant and dispersant alone only. Despite this, after 21 days of recovery, all exposed organisms reached levels similar to those of controls, supporting similar results reported in *Chlamys islandica* exposed to dispersed oil in which the reduction of lysosomal membrane stability was reversed after the post-exposure recovery period (Hannam et al., 2009).

Although dispersants are considered harmless, non-toxic and biodegradable (US EPA 2011; Luna-Acosta et al., 2011), with low acute toxicity on marine organisms (Hansen et al., 2012; Wise and Wise, 2011), few studies have been carried out to evaluate the action of dispersants on cell membranes. Our results suggest a possible interference of surfactant within the membrane lipid bilayer, leading to the impairment of its compactness and integrity. This evidence is also supported by Dasgupta and McElroy (2017) who observed an alteration of cellular physiology in rainbow trout liver cell lines exposed to anionic surfactants such as Corexit, follow by an inhibition of a key enzyme involved in hydrocarbon detoxification, CYP1A.

Reduction in the stability of lysosomal membranes is often associated with a transient decrease of phagocytic capacity, potentially recovered during the purification period, as observed for haemocytes of *Mya arenaria* (Pichaud et al., 2008). In our study, no significant variation was observed for the phagocytosis ability measured in mussels exposed to crude oil and for the granulocyte-hyalinocytes ratio measured in mussels exposed to both crude oil and blend. This evidence could be explained by the higher labilization of membrane exerted by the dispersant used in this study compared to hydrocarbons.

Among biomarkers of neurotoxicity, acetylcholinesterase (AChE) is an enzyme that plays a key role in the transmission of nervous impulses, and a decrease of its activity is generally considered a sensitive biomarker toward pollutants, partially for organophosphate and carbamate exposure. Modulation of AchE in haemolymph and gills of bivalve molluscs has been demonstrated for a wide spectrum of environmental pollutants (Payne et al., 1996) including PAHs (Moreira et al, 2004). However, the sensitivity of AchE activity in *M. galloprovincialis* exposed to oils and dispersant is currently poorly documented and still

contradictory. Geraudie et al., (2016) have demonstrated that muscular AchE activity was strongly inhibited in *C. islandica* exposed to marine diesel compared to the control animals; also juvenile wolfish *Anarhichas denticulatus* exposed to mechanically and chemically dispersed oil showed a significant inhibition of this enzyme in brain (Sandrini-Neto et al., 2016).

In this study, organisms exposed to crude oil treatments, did not exhibit any variation of AChE activity in gills, while significant increase were measured in haemolymph during the recovery period, showing a general delayed effect of the different treatments and an higher sensitivity of this tissue. Similar AChE responses were measured in mussels exposed to blend treatments with no variations in gills, confirming a lower sensitivity of this tissue to all treatments considered. Slight changes were observed in haemolymph only in dispersant treatment after 21 days of recovery, supporting the more limited effects of BLEND and BLEND+DISPERSANT treatments on this enzyme, and the absence of a delayed effect over the time.

Similarly to haemolymph AChE, delayed effects were observed in this study also for DNA fragmentation, measured in haemocytes of mussels exposed to different crude oil experimental conditions. Even in this case, the results showed a significant increase in DNA fragmentation especially in organisms exposed to the dispersant alone and in combination with the crude oil throughout the recovery period. Comparable results were observed by Martinović and Co-Authors. (2015) indicating an higher genotoxic damage in *M. galloprovincialis* exposed to dispersant (Superdispersants-25) dosed alone and in combination with diesel oil; also Le Dû-Lacoste and Co-Authors. (2013) observed that DNA fragmentation in blood of juvenile turbots *Scophthalmus maximus* exposed to elutriate from Erika heavy fuel oil was significantly enhanced only after 4 days of exposure in fish. This delay in response may be attributed to the time necessary for the production of a threshold amount of genotoxic metabolites required to cause a positive response in the comet assay. In our study, the results on delayed genotoxic effects could be due by the relative higher value of high and low molecular weight PAHs which persist in organisms exposed to chemically dispersed oil until 21 days of recovery in clean water.

Surprisingly, the results on DNA damage in blend treatments did not show any relevant variation against treatments, with the exception of blend dosed alone after 21 days of recovery. This result could be explained by the reduction of concentration of low molecular weight PAHs.

The antioxidant mechanisms, play a primary role in marine organisms, in the modulation of toxic effects induced from environmental contaminants and their analysis is, therefore, useful to verify the onset of a disorder and the possible health status impairment.

In this study, the oxidative status was measured in gills and digestive gland of mussels exposed to different experimental treatments of crude oil, integrating the analysis of individual antioxidants with the measurement of the total antioxidant capacity against hydroxyl radicals (•OH) and peroxyl radicals (ROO•) (Regoli and Winston, 1998).

The results obtained on individual antioxidants, revealed a slight oxidative stress condition that continued over the time, even after 2 days of exposure in both gills and digestive glands. As highlighted for other parameters, gills appeared more sensible also in terms of antioxidant enzyme activities and total glutathione, suggesting that this tissue is more affected by presence on hydrocarbons because directly exposed to the external environment and consequently in contact with these pollutants. Few enzymes were modulated in digestive glands (i.e. catalase and glutathione transferases) during recovery period, while catalase and glutathione peroxidase Se-dependent were modulated in gills probably to counteract the slight production of H_2O_2 in mussels exposed to oil mechanically and chemically dispersed. A biphasic response was observed in organisms exposed to oil only for Se-dependent glutathione peroxidase highlighting an initial inhibition and subsequent induction of this enzyme. This result is partially confirmed by the responses obtained in *Chlamys islandica* exposed to low concentrations of oil mechanically dispersed and dispersant alone (Frantzen et al., 2016) with a lack of response in GST, Superoxide dismutase (SOD) and GR in digestive gland. No variations were also found for the activity of glutathione S-transferases and glutathione peroxidase Se-dependent and Se-independent in the liver of the golden grey mullet *Liza aurata* exposed to mechanically and chemically dispersed oil (Milinkovitch et al., 2011b).

These limited variations of the antioxidant enzyme activities were confirmed by the lack of responses of the total antioxidant capacity against peroxyl radical (ROO•), in both tissues, and hydroxyl radical (•OH): a few exceptions were measured in gills for mussels exposed to CRUDE OIL+DISPERSANT, and in digestive gland for mussels exposed to dispersant dosed alone and in combination with oil during the exposure time.

Considering the high bioaccumulation of PAHs during the exposure period, in *M. galloprovincialis* exposed to crude oil mechanically and chemically dispersed, the slow responses of oxidative cellular pathway in both gills and digestive gland, could be explained by the short period of exposure itself that did not allow the production of ROS and therefore the

induction of antioxidant enzymes; however delayed effects on these parameters cannot be excluded.

The increased levels of malondialdehyde, biomarker of lipid peroxidation, during the exposure period in gills and digestive gland, corroborate the slight and delayed effects of the oxidative disturbance. This aspects is further confirmed by previous studies, in which slight or no variations were observed for malondialdehyde, suggesting a slight oxidative disorder, both in bivalves and fishes (Frantzen et al., 2016; Milinkovitch et al., 2011a).

The peroxisomal proliferation has been observed in many invertebrates and vertebrates exposed to various organic contaminants from the soluble fraction of oils, lubricants, PAHs, PCBs and phthalates (Bocchetti et al., 2008; Cajaraville et al., 2003). The peroxisome are organelles involved in lipid metabolism through oxidative reactions and for this reason they are localized in metabolic active organs such as liver or digestive gland in vertebrate and invertebrate organisms respectively. In this study, the acyl-CoA oxidase (AOX) activity, an index of peroxisomal proliferation, did not show any significant variation between different treatments, both during exposure and the recovery period in gills; however, it is interesting to note how the basal levels of the activity of this peroxisomal enzyme is much lower in gills in respect to digestive gland. On the contrary, AOX in digestive gland showed significant variations in mussels exposed to CRUDE OIL+DISPERSED treatment during the second half of recovery period. Complementary results were obtained by Cajaraville et al., (1997): mussels exposed to Water-Accommodated Fraction of two kind of crude oils showed an induction of volume, surface and numerical densities of peroxisomes in the digestive epithelium after 21 days of exposure.

5.3. Lethal and sublethal effects: ecotoxicological bioassays

A very wide spectrum of organisms belonging to different classes, such as bacteria, algae/plant and animals can be used to better understand the acute toxicity of chemical dispersants. The toxicity of these compounds can depend on their chemical composition, but also on the variability of testing methods, such as sensitivity of different species, exposure time and physical-chemical parameters (Manfra et al., 2017; Wise and Wise, 2011).

Italy, together with France, Greece, Norway, Spain and the United Kingdom are currently the countries in the EU with established procedures for testing dispersants for governmental approval (EMSA, 2016): only United Kingdom include in the risk assessment of dispersants,

also the evaluation of potential of these products to increase the toxicity of chemically dispersed oil.

In this study, the intent was to follow the test procedures recommended by Italian legislation (Decree 25th February 2011 – Annex 1 – *Allegato 5*), but implementing the approach to assess not only the intrinsic toxicity of dispersant done, but also of the different types of oils mechanically and chemically dispersed.

Concerning the toxicity of CRUDE OIL dosed alone, obtained results clearly indicated a very low acute toxicity for all the organisms considered, with EC_{50} or LC_{50} values higher than the highest concentration tested.

Regarding BLEND, while in *Phaeodactylum tricornutum* the 72h EC_{50} was above the highest concentration tested (>350 mg/L), in *Paracentrotus lividus* 48h the EC_{50} has been calculated to be 289.17 mg/L. This results suggest that the both the oils dosed alone show a low level of toxicity, or a level of toxicity that is not environmental relevant for all the considered organisms.

On the other hand, different results were observed for the all the organisms exposed to CRUDE OIL+DISPERSANT, BLEND+DISPERSANT and DISPERSANT alone that, although some variations in species sensitivity revealed a typically higher toxicity compared to that of oils dosed alone. A slightly higher toxicity was observed in *Paracentrotus lividus* exposed to BLEND+DISPERSANT compared to CRUDE OIL+DISPERSANT, while no differences have been obtained between the same treatments in *Phaeodactylum tricornutum* which was thus not affect by the different composition of oils.

The results of ecotoxicological bioassays support once again, a marked increases in bioavailability and biological reactivity of the PAHs in chemically dispersed oils compared to mechanically dispersed oils, corroborating the hypothesis that the toxicity of chemically dispersed oils is from one to ten times greater than of mechanically dispersed oils, as also reported in many studies (Fuller et al., 2004).

The Italian criteria for dispersant approval are among the most restrictive compared to other EU countries (EMSA, 2016; Manfra et al., 2017), because they require that EC_{50} or LC50 values must be above 10 mg/L for all the organisms tested. In this study, the only species which did not meet this criteria were *P. tricornutum* and *C. gigas* which represent the most sensitive species to this kind of treatment.

Comparison of toxicity data between studies is challenging because not all the treatments and bioassays were designed under similar experimental conditions, despite results are

commonly pooled when regulatory or oil spill response decisions are to be made. Comparisons are further complicated by differences in the chemical composition of oils used in toxicity testing, the temperature at which these test are conducted and temperate tolerance of the tested species, the degree of mixing in preparing the solutions, and other procedural details that influence the partitioning of oil constituents into the aqueous exposure media (Bejarano et al., 2014).

Fern and co-authors (2015) that exposed the blue crab *Callinected sapidus* to three dispersants alone and in combination with crude oil. Their study revealed a similar toxicity for the chemically dispersed oil, the dispersant dosed alone and the crude oil; the 48 h LC50 values for Corexit 9500, Corexit 9500-dispersed oil and crude oil were 59.1 mg/L, 51.8 mg/L and 55.9 mg/L respectively.

Conversely, Fuller and co-autors (2004) tested the toxicity of the dispersant Corexit 9500, the Arabian medium crude oil dosed alone and in combination with dispersant on different species of organisms such as *Americamysis bahia*, *Menidia beryllina*, *Cyprinodon variegatus* and *Vibrio fischeri*. They concluded that the dispersant toxicity would be negligible compared to oil, while an even greater to toxicity was evident for the oil+dispersant, highlighting a greater tolerability of fish than crustaceans.

The toxicity of dispersants has been widely studied (Wise and Wise, 2011), revealing variability depending on the dispersant and species. Some studies suggest that dispersants are highly toxic, whereas others indicate only a mild toxicity (Wise and Wise, 2011). As an example, Manfra and co-authors (2017) tested two typologies of dispersant with different species, reporting elevated differences from both: they found a 72h EC_{50} for *Phaeodactylum tricornutum* of 6.60 (4.40-8.80) mg/L for Dispersant A, while 82.70 (63.40-102.00) mg/L for Dispersant B; in the same way, 96h LC50 was 5.89 (4.86-7.04) and 49.66 (40.36-62.42) for *Tigriopus fulvus* exposed to Dispersant A and B respectively.

5.4. Multidisciplinary Weight Of Evidence (WOE) model

The quantitative and software-assisted WOE model (Sediqualsoft model; Benedetti et al., 2014; Piva et al., 2011; Regoli et al., 2014) has been recently developed to integrate and differently weight, data from various Lines of Evidence (LOEs), which include sediment chemistry, bioavailability of chemicals in bioindicator species, ecotoxicological effects measured at subcellular level (biomarkers), toxicity at organism level (laboratory bioassays) and ecological effects on benthic communities. In this study, we combined results related to bioaccumulation

of PAHs, biomarkers and bioassays which represent complex and heterogeneous data. These data were elaborated through weighted criteria of the quantitative and software-assisted WOE model Sediqualsoft recently introduced in the Italian legislation (Benedetti et al., 2014, 2012; Piva et al., 2011; Regoli et al., 2014). The overall quantitative effects ranged between 'Absent' to 'Major' in exposed mussels, with differences according to time and typology of treatment.
Concerning the bioavailability, the model confirmed a higher hazard for accumulation of hydrocarbons during the exposure period in organisms exposed to CRUDE OIL dosed alone, followed by a slow recovery during the permanence of mussels in clean water. A more pronounced degree of hazard for bioaccumulation has been observed for organisms exposed to CRUDE OIL+DISPERSANT without on evident improvement during depuration of the organisms. Mussels exposed to BLEND showed a "Moderate" hazard index after two days of exposure and 7 days of recovery, followed by a general improvement during the remaining days of recovery. Mussels exposed to BLEND+DISPERSANT showed a "Major" hazard index after 2 days of exposure and 7 days of recovery, followed again by a fast recovery after 14 days.
These innovative results suggest that oils with low and high viscosity tend to have the same level of hazard in term of bioaccumulation, but those with low viscosity are more rapidly recovered as reported in previews studies (Luna-Acosta et al., 2011).
The mathematical elaboration of biomarkers data confirmed the delayed effect of all the treatment with CRUDE OIL alone and in combination with dispersant and the DISPERSANT alone over time; on the contrary this effect is less prominent in organisms treated with BLEND and BLEND+DISPERSANT.
The integration of data had allowed to define a 'Slight' hazard index for both oil and dispersant treatment and a 'Moderate' hazard level for organisms exposed to the OIL+DISPERSANT mixture.

The integration of overall results from bioassays showed the same result for organisms exposed to both oils and relative treatments, highlighting an "Absent" hazard index in organisms exposed to CRUDE OIL and BLEND dosed alone, and a "Major" hazard for organisms exposed to DISPERSANT and CRUDE OIL/BLEND+DISPERSANT.

Finally, the Weight Of Evidence confirm the small differences on the effects that the two type of oils has against the treated organisms; however, the use of this model allows to confirm, once again, the less persistence and a lower risk of the oil with low viscosity compared to that of high viscosity.

In conclusion, the present work emphasized the ability of mussels exposed to chemically dispersed oils to bioaccumulate more PAHs than those treated with oil alone; moreover, excretion times were very different between low and high molecular weight PAHs, with higher recovery times for chemically dispersed oils exposed organisms compared to those exposed to mechanically dispersed oils.

The biological responses measured in the gills and in the haemolymph turned out very sensitive compared to digestive gland but sometimes with transient responses, especially in the presence of the dispersant, both dosed alone and in combination with the oils. Our result confirm that the gills and haemolymph are the most sensitive organs in *M. galloprovincialis* and we suggest to consider these tissues as the target compartments to take in account during monitoring program after an oil spill event. These effects are more evident in mussels exposed to oil with high viscosity, less emphasized in organisms exposed to oil with low viscosity.

Ecotoxicological bioassays confirmed the higher chemical toxicity of the chemically dispersed oils than the mechanically dispersed, and further highlight the importance of evaluating not only the intrinsic toxicity of the dispersant, as indicated by the Italian legislation, but also of oils and dispersants mixture.

Finally, the Weight Of Evidence model, which represent an useful tool for integrating and interpreting a wide range of heterogeneous data sets, confirmed a higher risk associated with chemically dispersed oils. WOE model represent a fundamental tool for stakeholder, which allow to summarizing and interpreting large datasets from a multidisciplinary studies.

This research gave a more realistic point of view, suggesting a more articulated and integrated evaluation of eligibility of dispersant products, differently from that currently considered by Italian legislation.

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7. SCIENTIFIC PRODUCT

- **Lanzoni I.**, Benedetti M., d'Errico G., Fattorini D., Di Carlo M., Regoli F. Biochemical and cellular responses to assess toxicity of mechanically and chemically dispersed crude oil in *Mytilus galloprovincialis*. In preparation.

Biochemical and cellular responses to assess toxicity of mechanically and chemically dispersed crude oil in *Mytilus galloprovincialis*.

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1. Introduction

Oil spills can severely affect the marine environment causing toxicity in marine organisms through both physical smothering and adverse effects that their constituents such as aliphatic and aromatic hydrocarbons can lead (Beyer et al., 2016; Teal and Howarth, 1984). The severity of impact and the social-economical damages (i.e. mariculture, fishing and tourism), typically depends on the concentration and typology of spilled oil, environmental characteristics of the area and associated biota, in terms of species and life stages.

The consequences of the impact of oil spills can be reduced significantly by implementing appropriate and timely intervention responses. Up to now, the physical removal of oil, such as skimmers, buoyant booms and adsorbed products, is the technique mostly used. However in the right circumstances and when used judiciously resorting to chemical dispersants can be an effective means (Chapman et al., 2007).

Oil dispersants are chemical compounds, which in contact with oil slick promote its dispersion in the water column. They are composed of amphipathic molecules, that reduce the surface tension increasing oil miscibility in water and favouring the reduction of oil into micellar aggregates between 1 and 70 μm. Due to their conformation, these particles prevent the re-aggregation of oils particles, keeping them suspended in water and allowing dispersion (Lessard and DeMarco, 2000).

All response techniques have limitations; physical removal of oil is useful for small spills or under calm wave and weather conditions, but other kind of response techniques must be taken in account when the goal is to reduce the overall human and environmental impact (Coelho et al., 2013).

Dispersants is a rapid tool, to minimize the oil slick on the sea surface, thus reducing the impact on birds and mammals. Further, their application can prevent the oil slick to reach the coastal area and promote the biodegradation of oil in the water column (Fingas, 2011); in off shore area and in certain condition of hydrodynamism, dispersed oil dilution is very fast, ensuring environmental harmless oil concentrations to be reached in the water column within a short time period (Frantzen et al., 2015; Lessard and DeMarco, 2000). A sad example is represented by the accidents of the platform *Deepwater Horizon*, occur in the Gulf of Mexico in 2010, that have questioned the validity of security

systems and the adequacy of emergency plans for offshore oil extractions. This represent the largest ecological disaster never happened, where about 500 million liters of crude oil release at sea and about 7 million liters of dispersant used have caused the worst marine environmental disaster (Beyer et al., 2016).

Despite this, the use of dispersants is highly controversial and debated within scientific community, because of their potential synergistic effects with oils (Kujawinski et al., 2011). As reported in many studies, (Lessard and DeMarco, 2000; Ramachandran et al., 2004; Milinkovitch et al., 2011) the transfer of hydrocarbons, from the sea surface to the water column, can lead to a temporary increase of local toxicity when compared to not treated and dispersed oils. On the contrary, from a legislative point of view (EMSA, 2010), these compounds are not adequately tested in terms of synergistic toxicity toward marine organisms because many national rules and regulations test only the toxicity of dispersants alone instead combined effects of oil and dispersants on marine organisms. The synergistic toxicity complexity allow us to understand which different mechanisms occur at various levels, from biochemical to cellular level. Variations of biomarkers are influenced by both the intensity and the duration of the insults, and adaptive or compensatory mechanisms, can occur, especially in chronic conditions, possibly influencing survival of populations (Benedetti et al., 2015).

After Deep Water Horizon accident occurred in 2010 in the Gulf of Mexico, many projects were funded to better understand the ecotoxicological impact of oil and dispersants. Most of the studies were performed in laboratory conditions, in order to assess biological and biochemical responses of several marine organism (at different life stages and trophic level) treated with many types and concentrations of oils and dispersants. Therefore, most of the results of in vivo studies cannot be easily referred to real contamination situations because of the complexity of oil composition and of the natural weathering processes which affect oil behaviour after a spill (Della Torre et al., 2012)

The most relevant marine organisms impact and toxicity are due to polycyclic aromatic hydrocarbons (PAHs) which involved effect in medium and long term, such as immunosuppression, diseases and mutagenesis, carcinogenesis and death of organisms respectively (Benedetti et al., 2015; Stegeman and Lech, 1991).

Considering the growing international attention about oil spills phenomena, the aim of this study is to increase our knowledge on the possible synergistic effects and toxicity of dispersants and oil mixtures on marine organisms, setting up a more realistic evaluation of environmental impact of dispersants that can be useful in the decision-making and operational processes during oil spill events but also to suggest revising the currently legislations in term of evaluation of the potential toxicity of the dispersants.

For these reasons, the Mediterranean mussel *Mytilus galloprovincialis*, extensively used as bioindicator species in biomonitoring program (Marigómez et al., 2013; Nigro et al., 2006; Zorita et al., 2007) due to its limited ability to metabolize PAHs and aliphatic compounds, its capability to bioaccumulate relatively higher levels of organic molecules (Frantzen et al., 2016; Lee and Anderson, 2005) and its characterization of biological responses, was exposed to the soluble fractions of Mediterranean oil with or without the use of dispersant, regularly chosen from products officially authorized by the Italian Ministry of the Environment. Despite chemical analyses on bioaccumulation of PAHs and aliphatic compounds, a wide and complete battery of biological responses were carried out in different tissues such as haemolymph, gills and digestive glands, to assess all the possible variations in term of immunological responses, the neurotoxic damage, genotoxic damage, lipid peroxidation and oxidative stress biomarkers.

This allow to assess not only the effects of the dispersant alone but also to take in account for the synergistic effects that oil and dispersant have on marine organisms. Furthermore it includes the reversibility of the effects measured in organisms considering recovery periods in clean seawater.

To provide an environmental risk assessment associated with the oil spills, the data obtained were integrated and elaborated within a multidisciplinary Weight Of Evidence (WOE) model to obtain an index of environmental risk for the different treatments and times. All the results were elaborated using a recently developed model (Sediqualsoft) based on a multidisciplinary weight of evidence (WOE) approach integrating adequately wide range of data, including chemical and biological analysis, providing a quantitative hazard index (Benedetti et al., 2012; Piva et al., 2011; Regoli et al., 2014).

2. Materials and Methods

2.1. Experimental design

Mussel, *Mytilus galloprovincialis* (6 ± 1 cm shell length) were collected from a shellfish farm, localized in central Adriatic Sea, in January 2016. A total of 720 mussels were randomly and equally distributed into four 45 L glass-tanks and they were acclimatized for 7 days to laboratory conditions (at 18 ± 1 °C, salinity 37‰, pH 8.0 ± 0.5) with aerated artificial seawater (ASW, Instant Ocean®).

Organisms were exposed to ASW as control (CTRL) and three water-accommodated fraction of crude oil mechanically dispersed (OIL), dispersant alone (DISPERSANT) and chemically dispersed (OIL+DISPERSANT). Crude oil extracted in Adriatic Sea, was obtained by Edison SpA, was maintained at +4°C in the darkness until use and was not weathered. The anionic dispersant CHIMSPERSE 4000 was kindly obtained from Chimec SpA, was maintained at room temperature in the darkness until use.

The test solutions were prepared based on the methods describe by Singer et al., (2000) with some modifications. Each test solution was prepared by stirring with magnetic stir bar 5 L of ASW brought to a 35-40% vortex in three beakers glass on a magnetic stir plate.

In the first beaker, the mechanical dispersion condition was set up by pouring 9 g of oil; in the second beaker, the dispersant alone condition was set up by pouring 0.9 g of chemical dispersant and in the third beaker, the chemical dispersion condition was set up by pouring 9 g of oil and the amount of dispersant recommended by the manufacturer (dispersant/oil ratio 1:10), i.e. 0.9 g of dispersant.

In order to simulate a real condition during and after an oil spill event, all the solutions were mixing for 24 h, in darkness and at 18°C followed by 1 h of settlement to favor the separations of water and oil. After this time, test solutions are removed from the bottom of the beakers and transferred into respective 45 L glass-tanks a diluted with synthetic sea water in order to obtained a nominal oil concentration of 200 ppm.

Organisms were exposed at 18°C for 2 days at each experimental condition and then transferred to clean ASW for other 21 days. Water changes were carried out every two days and mussels were fed 12 h prior the water change with commercial mixture of zooplankton for filter-feeding organisms. No mortality was observed during the experiments.

After 2, 7, 14 and 21 days mussels were sacrificed and dissected rapidly. Whole tissues, used to evaluate the bioaccumulation of PAHs and aliphatic hydrocarbons, were pooled in 3 sample constituted by 6 organisms each and stored at -20°C. Gills, digestive glands and haemolymph were collected for biological analyses and to determine the sensitivity of various biological responses; these tissues and organs were pulled in 9 samples, each constituted by 3 individuals and rapidly frozen in liquid nitrogen and stored at -80°C. A portion of haemolymph was immediately processed for the analyses of lysosomal membrane stability, phagocytosis capability, granulocytes-hyalinocytes ratio and processes for genotoxic damage.

2.2. Chemical analyses

Bioaccumulation of PAHs and aliphatic hydrocarbons was analysed according to previous methods (Bocchetti et al., 2008; Piva et al., 2011; Regoli et al., 2014).

For analysis of polycyclic aromatic hydrocarbons (PAHs), about 3 g (wet weight) of tissues were extracted in 10 mL 0.5 M potassium hydroxide in methanol with microwave at 55°C for 20 min (800 Watt) (CEM, Mars System). After centrifugation at 3.000 × g for 10 min, the methanolic solutions were concentrated using a SpeedVac and purified with solid-phase extraction (Octadecyl C18, 500 mg × 6 mL, Bakerbond). A final volume of 1 mL was recovered with pure, analytical HPLC gradient grade acetonitrile, and HPLC analyses were carried out in a water and acetonitrile gradient by fluorimetric and diode array detection. The PAHs were identified according to the retention times of

137 an appropriate pure standards solution (EPA 610 Polynuclear Aromatic Hydrocarbons Mix), and
138 classified as low molecular weight (LMW: naphthalene, acenaphthylene, 1-methyl naphthalene, 2-
139 methyl naphthalene, acenaphthene, fluorene, phenanthrene, anthracene) or high molecular weight
140 (HMW: fluoranthene, pyrene, benzo(a)antrhacene, chrysene, 7,12-dimethyl benzo(a)anthracene,
141 benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene,
142 benzo(g,h,i)perylene, indeno(1,2,3,c,d)pyrene).
143 Aliphatic hydrocarbons were extracted treating tissues (about 3 g, wet weight) with hexane:acetone
144 (2:1) in a microwave (110°C for 25 min, 800 Watt) (Mars CEM, CEM Corporation, Matthews NC).
145 After centrifugation at 3.000 × g for 10 min, the supernatants were purified with solid-phase
146 extraction (Phenomenex Strata-X, 500 mg × 6 mL plus Phenomenex Strata-FL, 1000 mg × 6 mL)
147 and then concentrated using a SpeedVac (RC1009; Jouan, Nantes, France) to dryness. Samples were
148 finally recovered with 1mL of pure, analytical GC grade n-hexane and analysed with a gas
149 chromatograph (Perkin Elmer) equipped with an Elite-5 capillary column (30 m × 0.32 mm ID × 0.25
150 µm-df) and a flame ionization detector. For quantitative determination, the system was calibrated
151 with an unsaturated pair n-alkane standard mixture according to EN ISO 9377-3 (Fluka 68281).
152 2.3. Biochemical analyses
153 Lysosomal membrane stability, granulocytes-hyalinocytes ratio and phagocytosis capacity in
154 haemocytes was evaluated as immunological responses. The *lysosomal membrane stability* was
155 measured as Neutral Red Retention Time (NRRT); the cationic probe Neutral Red (NR) was used to
156 evaluate the capability of the lysosomal membranes to retain the dye (Lowe et al., 1995). At least 8
157 mussels were individually analysed for each sampling time and treatment. Haemocytes were
158 incubated on a glass slide with a freshly prepared NR working solution (2 µl/ml filtered seawater)
159 from a stock solution of 20 mg neutral red dye dissolved in 1 ml of dimethyl sulfoxide, and
160 microscopically examined at 20 min intervals (for up to 120) to determine the time at which 50% of
161 cells had lost the dye to the cytosol previously taken up by lysosomes
162 To evaluate the *granulocytes-hyalinocytes ratio* was used 50 µl of haemolymph dispersed on glass
163 slides, dryed and fixed in Baker's fixative (+2.5% NaCl). The slides were washed with water, stained
164 with H&E and mounted in Eukitt. Observations were carried out with a light microscope (20X) and
165 the ratio was evaluated after counting almost 200 cells for each sample (Gorbi et al., 2013).
166 *Phagocytosis capacity assay* was microscopically evaluated in haemolymph incubated for 2 h with
167 Fluorescein-labelled Zymosan A bioparticles (Invitrogen), added at 10:1 target:haemocyte ratio;
168 phagocytosis was expressed as percentage of cells that internalized at least 3 fluorescent particles
169 (Gorbi et al., 2013).

Acetylcholinesterase activity (AChE), in haemolymph and gills samples, were assayed with Ellman's reaction at 412 nm, with acetylthiocholine and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Gorbi et al., 2008).

The DNA integrity was evaluated at molecular level as single strand breaks (SB) by the *Comet assay*. The comet assay was immediately carried out on haemocytes collected from the adductor muscle of organisms according (Gorbi et al., 2008). Cells was included in 1% normal-melting-point agarose on glass slides, followed by treatment in lysing solution, DNA denaturation, electrophoresis and staining with with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI); 100 randomly selected "nucleoids" per slide, and two replicates per sample, were examined under fluorescence microscopy (200 × magnification; Olympus BX-51) and the captured images (Image-Pro-Plus package) were analyzed by the Comet Score software. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.

The *peroxisomal Acyl-CoA oxidase activity* (AOX) was measured with a coupled assay following the production of H_2O_2 by the oxidation of dichlorofluorescein-diacetate in the presence of an exogenous horseradish peroxidase (Bocchetti et al., 2008).

Antioxidants defenses were measured in mussels gills and digestive glands following standardized assay conditions, at a constant temperature of 18 ± 1°C (Bocchetti et al., 2008). *Catalase* (CAT) was determined by the decrease in absorbance due to H_2O_2 consumption; *glutathione S-transferases* (GST) were determined following the reaction between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, *glutathione reductase* (GR) activity was measured by the oxidation of NADPH during the reduction of GSSG; *glutathione peroxidases* (GPx) activities were assayed in a coupled enzyme system where β-nicotinamide adenine dinucleotide (NADPH) is consumed by glutathione reductase to convert the oxidized glutathione (GSSG) to its reduced form using hydrogen peroxide or cumene hydroperoxide as substrates, respectively, for the selenium-dependent and for the sum of Se-dependent and Se-independent forms.

Levels of *total glutathione* were enzymatically assayed after acidic deproteinization with sulphosalicilic acid (Akerboom and Sies, 1981).

The content of *malondialdehyde* (MDA) was measured after derivatization with 1-metyl-2-phenylindole and spectrophotometrically determined after calibration against a malondialdehyde standard curve (Gorbi et al., 2013).

The *Total Oxyradical Scavenging Capacity* (TOSC) was measured in mussels gills and digestive glands by the capability of cellular antioxidants to inhibit the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas in the presence of different forms of oxyradicals, like peroxyl radicals ($ROO^{\bullet}$) and hydroxyl radicals ($^{\bullet}OH$) which are artificially generated at constant rate (F Regoli and

Winston, 1998; Regoli and Winston, 1999). Ethylene formation was determined by gas-chromatographic analyses and TOSC values were quantified from the equation: TOSC = 100-(∫SA/ ∫CA x 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curve produced during the reaction course for respective sample (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay (Regoli and Winston, 1999). Protein concentrations were determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

2.4. Statistical analyses

Statistical analyses were performed with GraphPad Prism 5® (Ver. 5.01). Analysis of variance (1-way ANOVA) was applied to test the differences among treatments followed by a Dunnett's post hoc test to determine significant differences between the exposed groups and the control group, at each sampling time. Level of significance was set at $p < 0.05$, homogeneity of variance was checked by Cochran C and mathematical transformation applied if necessary.

Data of bioaccumulation and biological responses were further elaborated with a weight of evidence (WOE) model Sediqualsoft (Piva et al., 2011) which summarizes large heterogeneous data sets for different line of evidence (LOE), providing a synthetic hazard index. Whole calculations, detailed flow-charts, rationale for weights, thresholds and expert judgements, as well as validated in field and laboratory conditions (Bebianno et al., 2015; Benedetti et al., 2016, 2014, 2012; Mestre et al., 2017; Mezzelani et al., 2016; Nardi et al., 2017; Piva et al., 2011; Regoli et al., 2014) and recently introduced in the Italian legislation.

Data on bioaccumulation (LOE 2) are calculated, for each parameter, by the variation of the concentration compared with proper controls corrected for the typology of pollutant and the statistical significance of the difference. Similarly, the elaboration of biomarkers data (LOE3) is based on the possibility to select among a wide battery of responses, each assigned with a weight (based on the relevance of biological endpoint) and a threshold for changes of biological relevance (Piva et al., 2011).

Results obtained from individual LOEs (LOE 2 and LOE3) are integrated assigning different weights to the various lines of evidence according to the weight of evidence concept. The overall processing WOE leads to a risk characterization that provides both a quantitative value and qualitative risk index to absent to severe (Piva et al., 2011).

3. Results

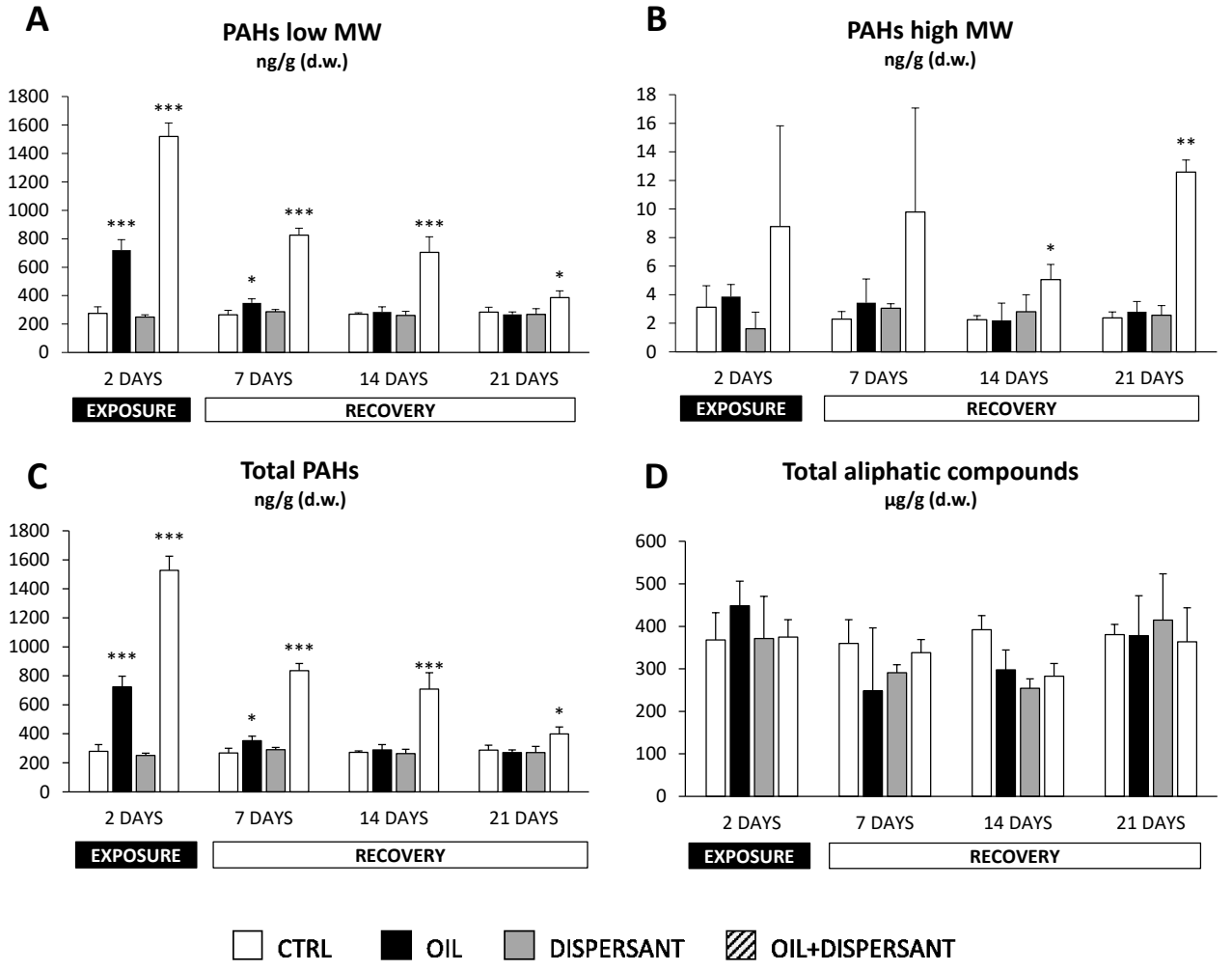
Dispersant increased the accumulation of low and high molecular weight PAHs (Fig. 1A, B). In particular, low molecular weight compounds, which represent the dominant and more soluble

component of the oil, increased especially during the exposure period (2 days) with value of 1519±98 ng/g d.w. or 5.7-fold higher than control, highlighting the capability of dispersants to make these molecules more bioavailable. In addition, the concentration of low molecular weight PAHs during exposure period are accumulated in mussel exposed to OIL 2.7-fold higher than the control (720.0±73.0 ng/g d.w).

The effects of dispersant showed a temporal decrease trend during recovery period, with differences against control still significant after 21 days with value of 387±47 ng/g d.w, while in mussels exposed to OIL alone reach the control levels already after 14 days.

In the OIL+DISPERSANT treatment, high molecular weight PAHs, showed a wide variability with marked increase after 21 days (12.58±0.85 ng/g d.w.) probably due to their low excretion, on the contrary no significant bioaccumulation was observed for the mussels exposed to OIL alone both during exposure and recovery period.

No significant bioaccumulation variations was observed for aliphatic hydrocarbons (Fig. 1D) in different treatments in all sampling times with a range values from 247.82±149 to 448.36±58 ng/g d.w.



254

255 **Fig. 1.** Bioaccumulation of low (A), high (B) molecular weight PAHs, total PAHs (C) and total aliphatic compounds (D)
256 in whole soft tissues of *M. galloprovincialis* exposed to crude oil mechanically dispersed (OIL), dispersant alone
257 (DISPERSANT), oil chemically dispersed (OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days
258 of exposure and 7, 14, 21 days of recovery period in clean water. Values are shown as mean ± standard deviation
259 (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$;
260 *** $p<0.001$).

261

262 Among immunological parameters measured in haemolymph of mussels exposed to laboratory
263 conditions shows a reduction of lysosomal membranes stability (Fig. 2A) in all treatments during the
264 exposure time; this effect was strongly evident in mussels exposed to dispersant, either dosed alone
265 or in combination with the oil, and this damage persisted during the recovery period, until returning
266 to control levels. Despite some fluctuations, no statistically significant effect was found in
267 granulocyte/hyalinocytes ratio and phagocytosis rate (Fig. 2B-C).
268 Acetylcholinesterase activity, was not affected in gills (Fig. 2D) while a significant enhancement was
269 observed in haemolymph (Fig. 2E) during the recovery period, thus revealing a delayed sensitivity of
270 this neurotoxic matter.
271 A delayed effect was observed also for haemocyte DNA fragmentation (Fig. 2F), which increased
272 during the recovery period, especially for organisms exposed to dispersant and oil+dispersant.

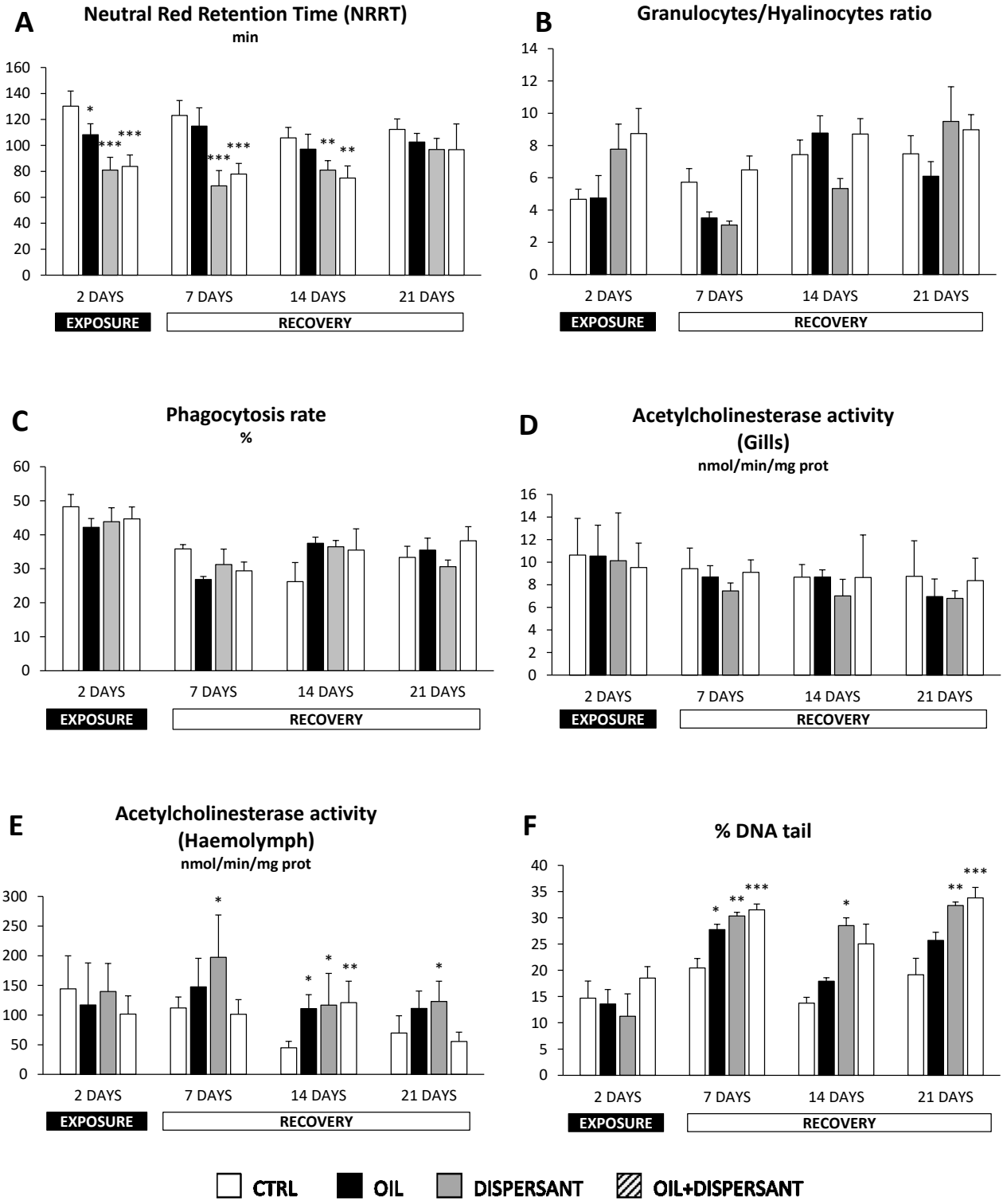


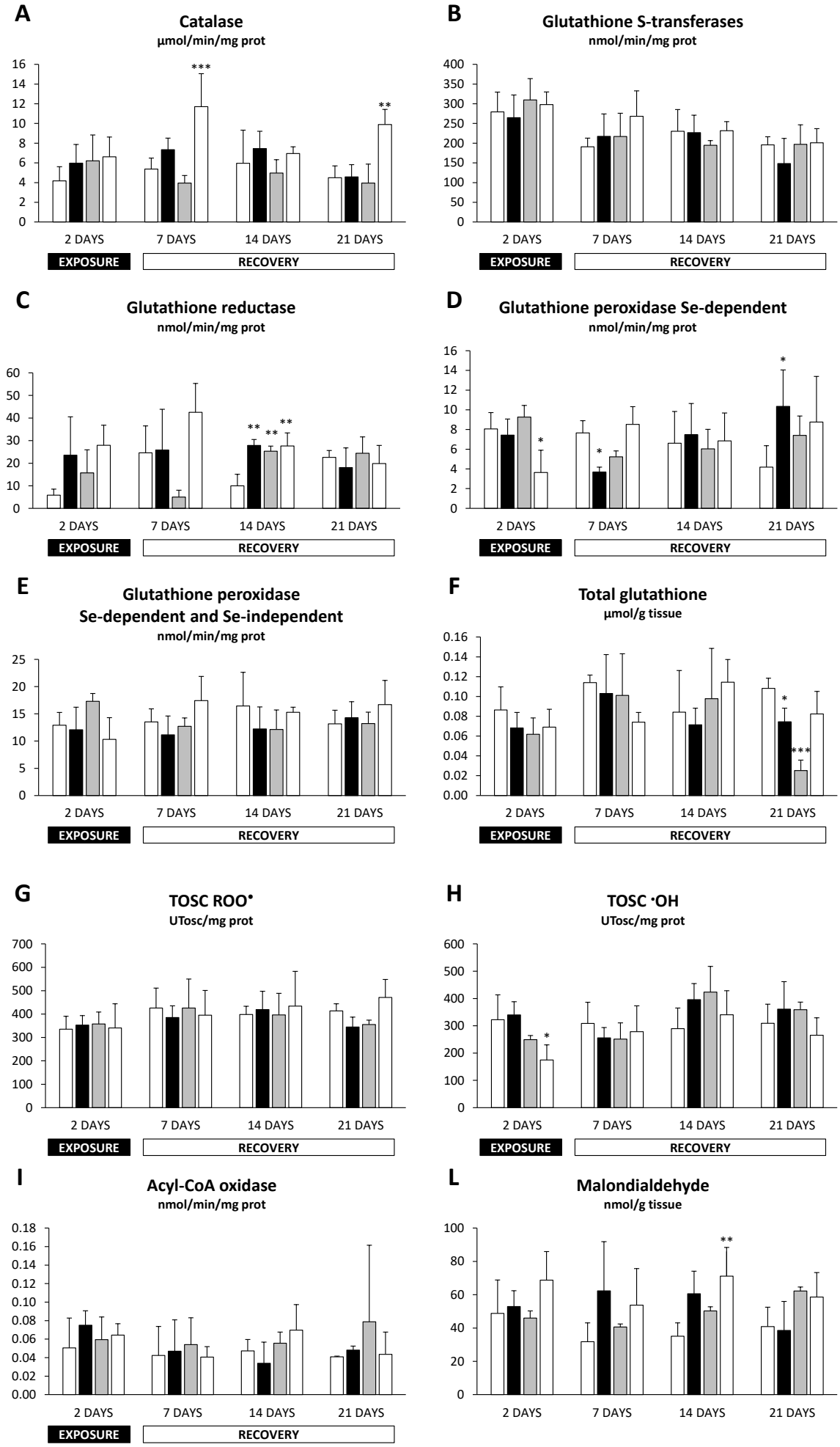
Fig. 2. Lysosomal stability, granulocytes-hyalinocytes ratio, phagocytosis rate, acetylcholinesterase activity and DNA damage in haemolymph (A, B, C, E and F) and acetylcholinesterase activity in gills (D) of *M. galloprovincialis* exposed to crude oil mechanically dispersed (OIL), dispersant alone (DISPERSANT), chemically dispersed oil (OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 days after depuration. Values are shown as mean ± standard error (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Antioxidant defences showed a certain variability in their responsiveness depending on the analysed parameter, tissue and stress factor.

Oxidative stress parameters showed a certain modulation of single antioxidant enzymes activity, such as catalase, glutathione reductase, Se-dependent glutathione peroxidases and of total glutathione level during exposure and recovery time (Fig. 3A, C, D, F). In particular catalase activity showed a biphasic responses in the treatment OIL+DISPERSANT which increase after 7 and 21 days. Otherwise, glutathione reductase showed a significant increase compared to its relative control only after 14 days of recovery. Se-dependent glutathione peroxidases showed an initial significant inhibition during the two days of exposure in OIL+DISPERSANT treatment while in organisms exposed to OIL alone showed a significant inhibition of its activity follow by a slow increase during all the depuration time. The total glutathione showed a not clear trend with significant variation after 21 days of recovery in OIL and DISPERSANT treatment.

However these limited variations did not affect the total oxyradical scavenging capacity toward peroxyl and hydroxyl radicals (Fig. 3G, H), with the only exception of mussels exposed to OIL+DISPERSANT during exposure time (Fig. 3G).

Slight increases in malondialdehyde content were observed only in organisms exposed to OIL and OIL+DISPERSANT during exposure and recovery period (Fig. 3L).

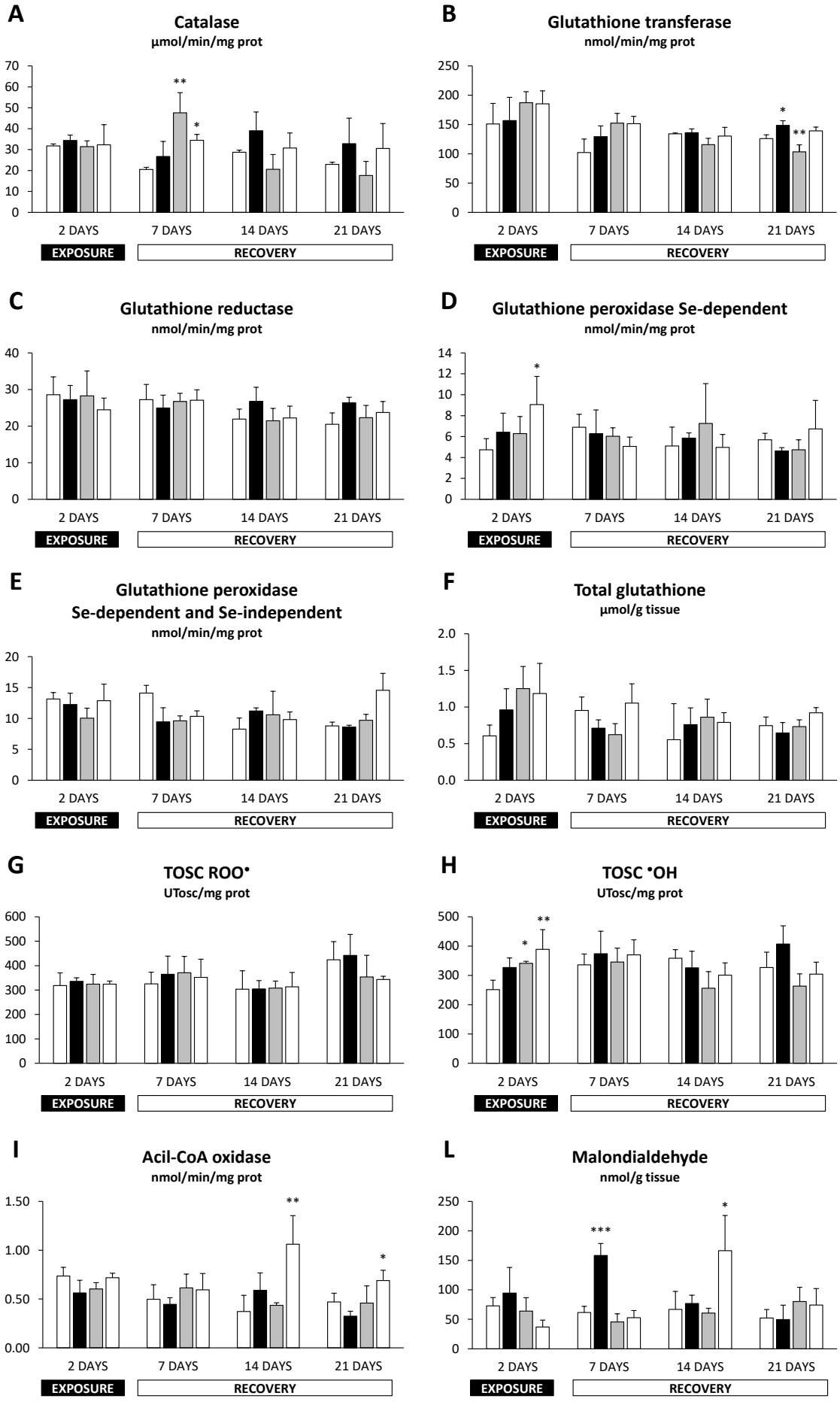

A
Catalase
µmol/min/mg prot
B
Glutathione S-transferases
nmol/min/mg prot
C
Glutathione reductase
nmol/min/mg prot
D
Glutathione peroxidase Se-dependent
nmol/min/mg prot
E
Glutathione peroxidase
Se-dependent and Se-independent
nmol/min/mg prot
F
Total glutathione
µmol/g tissue
G
TOSC ROO•
UTosc/mg prot
H
TOSC •OH
UTosc/mg prot
I
Acyl-CoA oxidase
nmol/min/mg prot
L
Malondialdehyde
nmol/g tissue
2 DAYS
7 DAYS
14 DAYS
21 DAYS
EXPOSURE
RECOVERY
CTRL
OIL
DISPERSANT
OIL+DISPERSANT

300

Fig. 3. Activity of individual antioxidant enzyme activity (A-E), total glutathione (F), Total Oxyradical Scavenging Capacity toward peroxyl and hydroxyl radicals (G-H), activity of acyl-CoA oxidase (I) and level of malondialdehyde (L) measured in gills of *M. galloprovincialis* exposed to mechanically dispersed crude oil (OIL), dispersant alone, chemically dispersed (OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 days after depuration. Values are shown as mean ± standard deviation (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

A few minor changes are related to biological responses about digestive gland parameters. Antioxidant responses in this tissue, exhibited some significant induction during exposure and early stage recovery period, especially in mussels exposed to dispersant both dosed alone and in combination with oil, in particular for catalase and glutathione peroxidase Se-dependent (Fig. 3 A and D). The total glutathione, although shows an increase but not significant values during the 2 days of exposure (Fig. 3 F), may affect the total oxyradical scavenging capacity against hydroxyl radical during exposure period (Fig. 3 H). Slight delayed variations in glutathione transferase activity (Fig. 3 B) were observed in organisms exposed to oil and dispersant dosed alone in the end of recovery period.

A delayed significant induction is observed also for acyl-CoA oxidase especially during 14 and 21 days of recovery (Fig. 3 I), and two different time responses are observed in malondialdehyde content, which increase after 7 and 14 days of recovery period respectively for mussels exposed to OIL alone and OIL+DISPERSANT treatment before to return to control level (Fig. 3 L).


A
Catalase
µmol/min/mg prot
B
Glutathione transferase
nmol/min/mg prot
C
Glutathione reductase
nmol/min/mg prot
D
Glutathione peroxidase Se-dependent
nmol/min/mg prot
E
Glutathione peroxidase
Se-dependent and Se-independent
nmol/min/mg prot
F
Total glutathione
µmol/g tissue
G
TOSC ROO•
UTosc/mg prot
H
TOSC •OH
UTosc/mg prot
I
Acil-CoA oxidase
nmol/min/mg prot
L
Malondialdehyde
nmol/g tissue
2 DAYS
7 DAYS
14 DAYS
21 DAYS
EXPOSURE
RECOVERY
CTRL
OIL
DISPERSANT
OIL+DISPERSANT

321

Fig. 3. Activity of individual antioxidant enzyme activity (A-E), total glutathione (F), Total Oxyradical Scavenging Capacity toward peroxyl and hydroxyl radicals (G-H), activity of acyl-CoA oxidase (I) and level of malondialdehyde (L) measured in digestive glands of *M. galloprovincialis* exposed to crude oil mechanically dispersed (OIL), dispersant alone (DISPERSANT), chemically dispersed (OIL+DISPERSANT) and control (CTRL). Mussels were sampled after 2 days of exposure and 7, 14, 21 days after depuration. Values are shown as mean ± standard deviation (n=4/treatment). Asterisks indicate differences between control and treatment for each given time (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

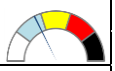
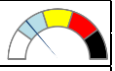
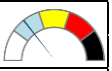
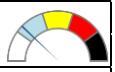
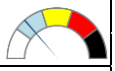
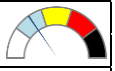
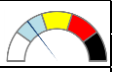
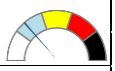
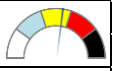
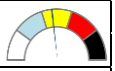
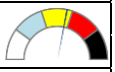
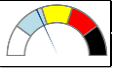
	Time	BIOAVAILABILITY	BIOMARKERS	CLASS OF HAZARD	
Oil	2 days	MODERATE	SLIGHT	SLIGHT	
	7 days	SLIGHT	SLIGHT	SLIGHT	
	14 days	ABSENT	MODERATE	SLIGHT	
	21 days	SLIGHT	SLIGHT	SLIGHT	
Dispersant	2 days	ABSENT	SLIGHT	SLIGHT	
	7 days	ABSENT	MODERATE	SLIGHT	
	14 days	ABSENT	MODERATE	SLIGHT	
	21 days	SLIGHT	MODERATE	SLIGHT	
Oil + Dispersant	2 days	MAJOR	MODERATE	MODERATE	
	7 days	MODERATE	SLIGHT	MODERATE	
	14 days	MAJOR	MODERATE	MODERATE	
	21 days	MODERATE	SLIGHT	SLIGHT	

Fig. 4. Qualitative Weight of Evidence (WOE) classification of single Lines on Evidence witch integrated bioavailability and biomarkers from gills, hemolymph and digestive glands, in mussels exposed to different treatments and experimental times.

The bioaccumulation and biomarkers results have been appropriately processed with the Sediqualsoft risk analysis model through specific lines of evidence, respectively LOE 2 and LOE 3 providing quantitative and qualitative (from Absent to Severe) environmental risk index that allow to simplify the interpretation of the results.

The Figure 4 showed the various LOEs, the final integration (WOEs) for each treatments and exposure or recovery time and the relative risk level.

Concerning the bioavailability (LOE 2), in organisms exposed to oil dosed alone, the hazard level appears to be "Moderate" during the two days of exposure and 'Slight' and "Absent" respectively after 7, 21 and 14 days of recovery (Figure 4).

As regards the organisms exposed to the dispersant, the "Absent" risk level was observed for all experimental times with the exception of the last time of recovery; on the contrary, a "Major" hazard

344 level was calculated in OIL+DISPERSANT treatment respectively after two days of exposure and 14
345 days of recovery, and "Moderate" for the other experimental times considered.
346 Also, the elaboration of results of biomarkers (LOE 3) obtained in haemolymph, gills and digestive
347 gland allowed to summarize in a synthetic biological hazard index that was found to be "Slight" in
348 oil dosed alone treatment after 2 days exposure and 7 and 21 days of recovery and 'Moderate' after
349 14 days of recovery (Figure 4).
350 For mussels exposed to dispersant, the hazard level has been "Slight" after 2 days of exposure and
351 "Moderate" during all the recovery period; otherwise, a "Moderate" hazard level was measured for
352 mussels exposed to the oil dosed in combination with dispersant after 2 days of exposure and 14 days
353 of recovery and "Slight" after 7 and 21 days of recovery.
354 The final integration Weight Of Evidence of chemical and biological parameters analysed in the
355 different treatments and experimental times, shows a level of risk classified as "Slight" for exposed
356 organisms both in oil dosed alone and dispersant during the exposure time and the purification.
357 Instead, organisms treated with OIL+DISPERSANT show a level of risk "Moderate" for the other
358 experimental times considered except at the last time of recovery period when it was lowered to
359 'Slight'.
360 **4. Discussion**
361 Although the use of dispersants is considered a valid alternative to the use of physical removal
362 procedure, since it is a rapid technique application and can be used when all other procedures are
363 inapplicable, it must take into account the possible effects that these may have on the environment
364 and marine ecosystems. However is interesting to note, that there is a divergence of opinion and a
365 different inclination between individual states of the European Union (Chapman et al., 2007) to use
366 this remediation technique for petroleum hydrocarbons but also a multitude of protocols to test the
367 toxic effects of these substances on marine organisms.
368 For this reason the aim of this study was to assess the possible synergistic effects and the toxicity of
369 mixtures of oil and dispersant in the marine organism filter feeder *Mytilus galloprovincialis*. It was
370 applied an integrated ecotoxicological approach, that includes bioaccumulation analysis and specific
371 biological responses at biochemical and cellular level, able to anticipate changes in the higher levels
372 of biological organization and therefore early identification of biological risks (Regoli et al., 2014).
373 The chemical and biochemical results were also integrated and processed with an ecological risk
374 analysis model, in order to have an early identification of impairment phenomena of the health status
375 of organisms.
376 An hypothetical oil spill scenario in natural marine environment consists on a first stage in which we
377 would expect a period of maximum PAHs water column concentration, follow by other steps, in

which involved natural processes and human clean-up activities that speed up the recovery steps (Boehm and Page, 2007; Lee et al., 2013; Lessard and DeMarco, 2000).

The results on the bioavailability of PAHs, that is the fraction of petroleum associated with the long-term toxicity (Boehm and Page, 2007; Perrichon et al., 2016), after two days of exposure, showed that the dispersant is able to increase bioaccumulation of both low and high molecular weight PAHs. Hydrocarbons with low molecular weight which represents the more soluble and dominant component of crude oil, increase of about 5.5 times compared to the control organisms, especially during the exposure period. These results highlight the ability of the chemical dispersant to make these molecules more than in oil dosed alone, which determined a 2.6 fold increase compared to controls. Similar results, but with different tissue concentrations, had been highlighted also in the Icelandic scallop *Chlamys islandica* after two days of exposure at 333.3 mg/L nominal concentration of mechanically and chemically dispersed crude oil, where the measured levels, were 35.4 and 42.3 times greater than in the controls (Frantzen et al., 2016); in Pacific oyster (*Crassostrea gigas*) exposed to 67 mg/L of nominal concentration of mechanically and chemically dispersed crude oil, PAHs increased of 28.3 and 75.7 fold compared to controls (Luna-Acosta et al., 2011). The apparent discrepancy between concentrations could be due to differences of investigated species, but also to the different kind of oil and dispersant used, and how the exposure media is generated, that make difficult the direct comparison of the experimental studies.

Differences in PAHs bioavailability between mechanically and chemically dispersed oils were evident also during the recovery period; after 21 days of recovery the low PAHs concentrations in whole tissues were higher in organisms co-exposed to oil and dispersant compared to those treated with oil dosed alone. The capability of the PAHs to be easily bioaccumulated and slowly eliminated was also observed in the Pacific oyster *C. gigas*: despite a decrease of these molecules of the 97% and 93% in chemically dispersed and mechanically dispersed oil treatment respectively after 15 days of depuration, concentrations still remained significantly higher than in controls (Luna-Acosta et al., 2011).

A partly different behaviour was observed for high molecular weight PAHs: despite they were accumulated at lower concentrations compared to low molecular weight PAHs in organisms exposed both to mechanically and chemically dispersed oil, these compounds remained stable and were not excreted during recovery period, especially in organisms exposed to oil and dispersant. In general these compounds are more persistent, hydrophobic and less water soluble (Couillard and Lee, 2005; Perrichon et al., 2016), characteristics that promote their permanence in tissues. In addition, the absence of an efficient biotransformation pathway in invertebrates, like Cytochrome P450 for

411 vertebrates, does not allow to excrete though Phase II enzymes these compound in a short period
412 (Stegeman and Lech, 1991).
413 Compared to PAHs, the bioavailability and the bioaccumulation of aliphatic compounds (C10-C40)
414 did not show any variations between exposure to mechanically and chemically dispersed oils, with
415 values, ranging between 247.82±148.5 and 448.4±57.8 µg/g d.w., comparable to those measured in
416 control organisms (Benedetti et al., 2014).
417 The immune system is extremely vulnerable to the xenobiotic stressors In a wide range of organisms
418 (Galloway and Depledge, 2001). Alterations, such as lysosomal membrane stability, phagocytosis
419 activity and granulocytes/hyalinocytes ratio, represent important early warning indicators of the
420 environmental disturbance and they are an important tool for assessing the sub-lethal effects of
421 contaminant exposure (Hannam et al., 2010b). Lysosomal membrane stability in bivalve haemocytes
422 can be impaired by PAHs or, alternatively, by the metabolic phase of PAH detoxification that can
423 enhance the production of reactive oxygen species (ROS), with direct damaging to cell membrane
424 through lipid peroxidation. In this respect the lysosomal responses, including the destabilization of
425 these membranes, are widely used because extremely sensitive to a large range of environmental
426 contaminants, including PAHs (Grundy et al., 1996; Hannam et al., 2010a; Lowe et al., 1995; Regoli,
427 1992). In our work, the stability of lysosomal membranes decreased significantly during the period
428 of exposure in all organisms exposed to different experimental conditions also included the dispersant
429 dosed alone. The same effects were also found in *Clamys islandica* where the reduction of this
430 biomarker was significant in organisms exposed for 24h to both mechanically (44.25±13.8 min) and
431 chemically dispersed oil (21.0±35.9 min) compared to controls (109.5±35.9 min) (Frantzen et al.,
432 2016). In our study, the decrease of the membrane stability persisted even after the 14 days of
433 recovery exclusively in organisms exposed to the dispersant dosed alone and in combination with the
434 oil. Despite this, after 21 days of recovery, all exposed organisms reached levels similar to those of
435 controls, supporting similar results reported in *Clamys islandica* exposed to dispersed oil in which
436 the reduction of lysosomal membrane stability was reversed after the post-exposure recovery period
437 (Hannam et al., 2009).
438 Although dispersants are considered harmless, non-toxic and biodegradable (US EPA 2011; Luna-
439 Acosta et al., 2011), with low acute toxicity on marine organisms (Hansen et al., 2012; Wise and
440 Wise, 2011), few studies have been carried out to evaluate the action of dispersants on cell
441 membranes. Our results suggest a possible interference of surfactant within the membrane lipid
442 bilayer, leading to the impairment of its compactness and integrity. This evidence is also supported
443 by Dasgupta and McElroy (2017) who observed an alteration of cellular physiology in rainbow trout

liver cell lines exposed to anionic surfactants such as Corexit, follow by an inhibition of a key enzyme involved in hydrocarbon detoxification, CYP1A.

Reduction in the stability of lysosomal membranes is often associated with a transient decrease of phagocytic capacity, potentially recovered during the purification period, as observed for haemocytes of *Mya arenaria* (Pichaud et al., 2008). In our study, no significant variation was observed for the phagocytosis ability and for the granulocyte-hyalinocytes ratio. This evidence could be explained by the higher labilization of membrane exerted by the dispersant used in this study compared to hydrocarbons.

Among biomarkers of neurotoxicity, acetylcholinesterase (AChE) is an enzyme that plays a key role in the transmission of nervous impulses and a decrease of its activity is generally considered a sensitive biomarker toward pollutants, particularly for organophosphate and carbamate exposure. Modulation of AChE in haemolymph and gills of bivalve molluscs has been demonstrated for a wide spectrum of environmental pollutants (Payne et al., 1996) including PAHs (Moreira et al, 2004). However, the sensitivity of AchE activity in *M. galloprovincialis* exposed to oils and dispersant is currently poorly documented and still contradictory. Geraudie et al., (2016) have demonstrated that muscular AchE activity was strongly inhibited in *C. islandica* exposed to marine diesel compared to the control animals; also juvenile wolfish *Anarhichas denticulatus* exposed to mechanically and chemically dispersed oil showed a significant inhibition of this enzyme in brain (Sandrini-Neto et al., 2016).

In this study, despite no variations were observed for AChE activity in gills, significant increase were measured in haemolymph during the recovery period, showing a general delayed effect of the different treatments and an higher sensitivity of this tissue . as been already observed in *M galloprovincialis* exposed to pharmaceuticals (Mezzelani et al., 2016).

Similar delayed effects were observed for the DNA fragmentation, measured in haemocytes of mussels exposed to different experimental conditions. Even in this case, the results showed a significant increase in DNA fragmentation especially in organisms exposed to the dispersant alone and in combination with the oil throughout the recovery period. Similar results are observed by Martinović and Co-Authors., (2015) with an higher genotoxic damage in *M. galloprovincialis* exposed to dispersant (Superdispersants-25) dosed alone and in combination with diesel oil.

In our study, the results on delayed genotoxic effects could be due by the relative higher value of high molecular weight PAHs which persist in organisms exposed to chemically dispersed oil until 21 days of recovery in clean water. However, the toxicity of dispersant is still debate. The toxicity of dispersants can be high or low, it depend on type and chemical composition of dispersant, organism

involved and test of methods tested which prevents us to know how dispersants is most or less toxic (Wise and Wise, 2011).

The antioxidant mechanisms play a primary role in marine organisms in the modulation of toxic effects induced from environmental contaminants and their analysis are, therefore, useful to verify the onset of a disorder and the possible health status impairment.

In this study, the oxidative status was measured in gills and digestive gland of mussels exposed to different experimental treatments of crude oil, integrating the analysis of individual antioxidants with the measurement of the total antioxidant capacity against hydroxyl radicals ($^{\bullet}OH$) and peroxyl radicals ($ROO^{\bullet}$) (Regoli and Winston, 1998).

The results obtained on individual antioxidants, revealed a slight oxidative stress condition that continued over the time, even after 2 days of exposure in both gills and digestive glands. As highlighted for other parameters, gills appeared more sensible also in terms of antioxidant enzyme activities and total glutathione, suggesting that this tissue is more affected by presence on hydrocarbons because directly exposed to the external environment and consequently in contact with these pollutants. Few enzymes were modulated in digestive glands (i.e. catalase and glutathione transferase) during recovery period, while catalase and glutathione peroxidase Se-dependent were modulated in gills probably to counteract the slight production of H_2O_2 in mussels exposed to oil mechanically and chemically dispersed. A biphasic response was observed in organisms exposed to oil only for Se-dependent glutathione peroxidase highlighting an initial inhibition and subsequent induction of this enzyme. This result is partially confirmed by the responses obtained in *Chlamys islandica* exposed to low concentrations of mechanically dispersed oil and dispersant alone (Frantzen et al., 2016) with a lack of response in GST, Superoxide dismutase (SOD) and GR in digestive gland. No variations were also found for the activity of glutathione S-transferases and glutathione peroxidase Se-dependent and Se-independent in the liver of the golden grey mullet *Liza aurata* exposed to mechanically and chemically dispersed oil (Milinkovitch et al., 2011b).

These limited variations of the antioxidant enzyme activities were, confirmed by the lack of responses of the total antioxidant capacity against peroxyl radical ($ROO^{\bullet}$), in both tissues, and hydroxyl radical ($^{\bullet}OH$): a few exceptions were measured in gills for mussels exposed to oil+dispersant, and in digestive gland for mussels exposed to dispersant dosed alone and in combination with oil during the exposure time.

Considering the high bioaccumulation of PAHs during the exposure period, in *M. gallorpovincialis* exposed to mechanically and chemically dispersed oil, the slow responses of oxidative cellular pathway in both gills and digestive gland, could be explained by the short period of exposure itself

510 that did not allow the production of ROS and therefore the induction of antioxidant enzymes,
511 however, delayed effects on these parameters cannot be excluded.
512 The increased levels of malondialdehyde, biomarker of lipid peroxidation, during the exposure period
513 in gills and digestive glands, corroborate the slight and delayed effects of the oxidative disturbance.
514 This aspects is further confirmed by previous studies, in which slight or no variations were observed
515 for malondialdehyde, suggesting a slight oxidative disorder, both in bivalves and fishes (Frantzen et
516 al., 2016; Milinkovitch et al., 2011a).
517 The peroxisomal proliferation has been observed in many invertebrates and vertebrates exposed to
518 various organic contaminants from the soluble fraction of oils, lubricants, PAHs, PCBs and phthalates
519 (Bocchetti et al., 2008; Cajaraville et al., 2003). The peroxisome are organelles involved in lipid
520 metabolism through oxidative reactions and for this reason they are localized in metabolic active
521 organs such as liver or digestive gland in vertebrate and invertebrate organisms respectively. In this
522 study, the acyl-CoA oxidase (AOX) activity, an index of peroxisomal proliferation, did not show any
523 significant variation between different treatments, both during exposure and the recovery period in
524 gills; however, it is interesting to note how the basal levels of the activity of this peroxisomal enzyme
525 is much lower in gills in respect to digestive gland. On the contrary AOX in digestive gland showed
526 significant variations in mussels exposed to OIL+DISPERSED treatment during the second half of
527 recovery period. Complementary results were obtained by Cajaraville et al., (1997): mussels exposed
528 to Water-Accommodation Fraction of two kind of crude oils showed an induction of volume, surface
529 and numerical densities of peroxisomes in the digestive epithelium after 21 days of exposure.
530 The quantitative and software-assisted WOE model (Sediqualsoft model; Benedetti et al., 2014; Piva
531 et al., 2011; Regoli et al., 2014) has been recently developed to integrate differently weight, data from
532 various Lines of Evidence (LOEs), which include sediment chemistry, bioavailability of chemicals
533 in bioindicator species, ecotoxicological effects measured at subcellular level (biomarkers), toxicity
534 at organism level (laboratory bioassays) and ecological effects on benthic communities. In this study,
535 we combined results related to bioaccumulation of PAHs and biomarkers represent complex and
536 heterogeneous data. These data were elaborated through weighted criteria of the quantitative and
537 software-assisted WOE model Sediqualsoft recently introduced in the Italian legislation (Benedetti
538 et al., 2014, 2012; Piva et al., 2011; Regoli et al., 2014). The overall quantitative effects ranged
539 between 'Absent' to 'Major' in exposed mussels, with differences according to time and typology of
540 treatment.
541 Concerning the bioavailability, the model confirmed an higher hazard accumulation of hydrocarbons
542 during the exposure period in organisms exposed to oil dosed alone and in combination with the
543 dispersant, fallowed by a slow recovery during the permanence of mussels in clean water. On the

544 contrary, the mathematical biomarkers data elaboration confirm the delayed effect of all the treatment
545 over time. Finally, the overall integration of data had allow to define a 'Slight' risk index for both oil
546 and dispersant treatment and a 'Moderate' risk level for organisms exposed to the
547 OIL+DISPERSANT mixture.
548
549 **5. Conclusions**
550 The present work emphasized the ability of mussels exposed to chemically dispersed oil to
551 bioaccumulate more PAHs than those treated with oil alone; moreover, excretion times were very
552 different between low and high molecular weight PAHs, with higher recovery times for chemically
553 dispersed oil exposed organisms compared to those exposed to mechanically dispersed oil.
554 The biological responses measured in the gills and in the haemolymph turned out very sensitive
555 compared to digestive gland but sometimes with transient responses, especially in the presence of the
556 dispersant, both individually and in combination with the oil.
557 Our result confirm that the gills and haemolymph are the most sensitive organs in *M. galloprovincialis*
558 and we suggest to consider these tissues as the target compartments to take in account during
559 monitoring program after an oil spill event.
560 Finally, the Weight Of Evidence model, which represent a useful tool for integrating and interpreting
561 a wide range of heterogeneous data sets, confirmed a higher risk associated with chemically dispersed
562 oil. WOE model represent a fundamental tool for stakeholder, which allow to summarizing and
563 interpreting large datasets from a multidisciplinary studies.
564 These findings indicate a chronic rather than acute toxicological responsiveness to chemically
565 dispersed oil, highlighting that the measurement of biological effects immediately after the exposure
566 period may under-estimate the ecotoxicological relevance of these compounds.
567

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ANNEX 1

DECRETO 25 febbraio 2011.

Definizioni delle procedure per il riconoscimento di idoneità dei prodotti assorbenti e disperdenti da impiegare in mare per la bonifica dalla contaminazione da idrocarburi petroliferi.

IL DIRETTORE GENERALE
PER LA PROTEZIONE DELLA NATURA E DEL MARE

Vista la legge 31 dicembre 1982, n. 979, recante Disposizioni per la difesa del mare;

Vista la legge 8 luglio 1986, n. 349, istitutiva del Ministero dell'ambiente;

Visto il decreto legislativo del 30 marzo 2001, n. 165, recante norme generali sull'ordinamento del lavoro alle dipendenze delle amministrazioni pubbliche;

Visto il decreto direttoriale 11 dicembre 1997 di approvazione delle procedure per l'autorizzazione all'uso dei prodotti disinquinanti in mare;

Visto il decreto del Direttore generale per la difesa del mare del 23 dicembre 2002 di definizione delle procedure per il riconoscimento di idoneità dei prodotti disperdenti ed assorbenti da impiegare in mare per la bonifica dalla contaminazione da idrocarburi petroliferi;

Visto il decreto del Direttore generale per la protezione della natura del 24 febbraio 2004 di modifica del citato decreto direttoriale 23 dicembre 2002;

Visto il decreto direttoriale 31 marzo 2009 con il quale si riconosce l'impiegabilità in mare di prodotti composti da materiali inerti di origine naturale o sintetica, ad azione assorbente, per la bonifica dalla contaminazione da idrocarburi petroliferi;

Visto il decreto direttoriale DEC/DPN/1928 del 29 ottobre 2007 di istituzione, presso la Direzione per la protezione della natura, di un tavolo tecnico finalizzato alla formalizzazione di uno schema di revisione del citato decreto direttoriale del 23 dicembre 2002;

Considerati gli esiti dei lavori del suddetto tavolo tecnico relativamente alle specifiche tecniche dei prodotti assorbenti e disperdenti da impiegare in mare per la bonifica dalla contaminazione da idrocarburi petroliferi e alla definizione dei nuovi metodi analitici finalizzati a valutare l'efficacia e l'ammissibilità all'impiego in mare dei suddetti prodotti;

Ritenuto, pertanto, necessario procedere alla definizione di nuove procedure per il riconoscimento dell'idoneità tecnica e della ecocompatibilità dei prodotti assorbenti e disperdenti con l'ambiente marino;

Decreta:

Art. 1.

Campo di applicazione

1. Il presente decreto, comprensivo degli allegati 1, 2, 3, 4, 5 e 6 che ne fanno parte integrante, definisce le procedure amministrative e tecniche necessarie per conseguire il riconoscimento di idoneità esclusivamente dei prodotti da impiegare per la bonifica dell'ambiente marino dalla contaminazione da idrocarburi petroliferi, appartenenti alle seguenti tipologie:

prodotti assorbenti di origine sintetica o naturale non inerti;

prodotti disperdenti di origine sintetica o naturale;

2. Sono esclusi dal campo di applicazione del presente Decreto i prodotti ad azione biologica e i prodotti di origine sintetica ad azione disinquinante non compresi nel precedente comma.

Art. 2.

Definizioni

Ai fini del presente decreto si applicano le seguenti definizioni:

prodotti assorbenti di origine sintetica o naturale non inerti: materiali insolubili di natura chimica utilizzati per rimuovere gli idrocarburi petroliferi sfruttando le loro capacità adsorbenti;

prodotti disperdenti di origine sintetica o naturale: agenti chimici che, attraverso un meccanismo di micellazione, favoriscono la dispersione degli idrocarburi petroliferi nella colonna d'acqua;

prodotti ad azione biologica: colture microbiologiche (batteri, funghi o lieviti), additivi enzimatici o additivi nutritivi ad azione biodegradante sugli idrocarburi petroliferi;

prodotti di origine sintetica ad azione disinquinante: prodotti con meccanismo di azione diverso da quello assorbente o disperdente.

Art. 3.

Istanza di riconoscimento di idoneità per i prodotti assorbenti o disperdenti

1. Le società produttrici ovvero le società che intendano immettere sul mercato nuovi prodotti appartenenti alle tipologie di cui all'art. 1 del presente decreto devono presentare istanza di riconoscimento di idoneità degli stessi al Ministero dell'ambiente e della tutela del territorio e del mare - Direzione generale per la protezione della natura e del mare, via Cristoforo Colombo, 44 - 00147 Roma.

2. Tali istanze devono essere corredate dalla documentazione di cui all'allegato 1, comprensiva della scheda tecnica del prodotto redatta in conformità alle indicazioni di cui all'allegato 2 o 3, alla documentazione relativa ai test e alle prove di laboratorio di cui all'allegato 4 o 5 e la documentazione relativa alle informazioni da apporre sull'involucro esterno del prodotto redatta in conformità alle indicazioni di cui all'allegato 6.

3. La documentazione relativa ai test e alle prove di laboratorio di cui al precedente comma 2 dovrà pervenire da Enti e/o Istituti pubblici conformi ai requisiti della norma UNI CEI EN ISO/IEC 17025 e che dimostrino di partecipare a circuiti di intercalibrazione nazionali e/o internazionali, ovvero da laboratori privati accreditati ai sensi della norma UNI CEI EN ISO/IEC 17025.

Art. 4.

Istruttoria per il riconoscimento di idoneità

1. Il Ministero dell'ambiente e della tutela del territorio e del mare verificata la conformità delle istanze di cui al precedente art. 3, trasmette all'Istituto superiore per la

protezione e la ricerca ambientale e all'Istituto superiore di sanità la documentazione tecnica necessaria ai fini dell'acquisizione di un parere congiunto sull'idoneità dei prodotti assorbenti o disperdenti.

2. Il Ministero dell'ambiente e della tutela del territorio e del mare, sulla base della conformità della documentazione di cui all'art. 3 e dei pareri tecnici di cui al precedente comma 1, provvede, entro 90 (novanta) giorni dal ricevimento dell'istanza, ad emanare un provvedimento che riconosce l'idoneità dei prodotti assorbenti o disperdenti all'impiego in mare per la bonifica dalla contaminazione da idrocarburi petroliferi.

3. Qualora nel corso della istruttoria si renda necessaria l'acquisizione di documentazione integrativa, il termine di novanta giorni di cui al comma 2, è sospeso fino alla data di ricevimento della suddetta documentazione.

4. Il Ministero dell'ambiente e della tutela del territorio e del mare renderà pubblico sul sito internet istituzionale l'elenco dei prodotti assorbenti o disperdenti riconosciuti idonei ai sensi del presente decreto.

Art. 5.

Impiego dei prodotti riconosciuti idonei

1. Il provvedimento di cui al precedente articolo 4, comma 2 non costituisce formale autorizzazione all'effettivo impiego in mare dei prodotti assorbenti o disperdenti riconosciuti idonei. Tale autorizzazione deve essere appositamente rilasciata, di volta in volta, dal Centro operativo antinquinamento operante presso il Ministero dell'ambiente e della tutela del territorio e del mare - Direzione generale per la protezione della natura e del mare - Divisione VII[a].

2. Il Ministero dell'ambiente e della tutela del territorio e del mare, sulla base di eventuali indicazioni fornite dall'Istituto superiore per la protezione e la ricerca ambientale e/o dall'Istituto superiore di sanità, e attraverso il provvedimento di riconoscimento di idoneità, può imporre prescrizioni all'impiego in mare dei prodotti assorbenti o disperdenti riconosciuti idonei.

3. I prodotti assorbenti o disperdenti riconosciuti idonei dovranno riportare sull'involucro esterno le informazioni di cui all'allegato 6 al presente decreto.

Art. 6.

Durata e rinnovo del provvedimento di riconoscimento di idoneità

1. Il provvedimento del Ministero dell'ambiente e della tutela del territorio e del mare, di cui al precedente art. 4, comma 2, ha durata quinquennale ed è rinnovabile, all'atto della scadenza, con le modalità di cui ai successivi commi 2 e 3.

2. Qualora il prodotto già riconosciuto idoneo ai sensi del presente decreto non abbia mutato la sua composizione chimica e le sue caratteristiche fisiche alla data di scadenza del provvedimento di riconoscimento di idoneità, la società produttrice o che immette sul mercato il prodotto assorbente o disperdente, deve presentare una dichiarazione in cui si attesta che il prodotto ha conservato immutate la composizione chimica e le caratteristiche fisiche dalla data del suddetto provvedimento di riconoscimento di idoneità. È fatta salva la facoltà del Ministero dell'ambiente e della tutela del territorio e del mare, sentito il parere dell'Istituto superiore per la protezione e la ricerca ambientale e dell'Istituto superiore di sanità, di richiedere alla società produttrice o che immette sul mercato il prodotto assorbente o disperdente, documentazione tecnica integrativa che sia di supporto alla procedura del rinnovo del provvedimento di riconoscimento di idoneità.

3. Qualora il prodotto già riconosciuto idoneo ai sensi del presente decreto abbia mutato la sua composizione chimica e le sue caratteristiche fisiche alla data di scadenza del provvedimento di riconoscimento di idoneità, la società produttrice o che immette sul mercato il prodotto assorbente o disperdente, deve presentare una nuova istanza secondo le procedure di cui all'art. 3 del presente decreto al fine di ottenere il rinnovo del suddetto provvedimento.

Art. 7.

Aggiornamento degli allegati

1. L'aggiornamento delle procedure tecniche contenute negli allegati al presente decreto è effettuato con decreto del direttore generale per la protezione della natura e del mare.

Art. 8.

Disposizioni transitorie e finali

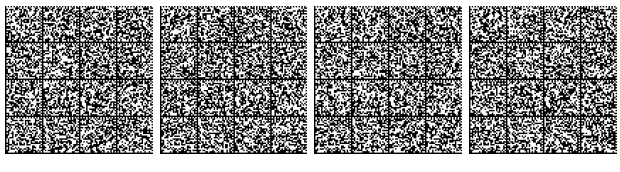
1. Sono fatte salve le istanze per il riconoscimento di idoneità dei prodotti assorbenti o disperdenti presentate ai sensi del decreto direttoriale 23 dicembre 2002 e s.m.i. nei novanta giorni successivi alla pubblicazione del presente decreto.

2. I prodotti assorbenti e disperdenti riconosciuti idonei ai sensi del decreto direttoriale 23 dicembre 2002 e s.m.i. mantengono la loro idoneità sino alla naturale scadenza del provvedimento con il quale tale idoneità è stata riconosciuta. Il relativo rinnovo seguirà le procedure amministrative e tecniche di cui al presente decreto.

3. Il decreto del direttore generale dell'ispettorato centrale per la difesa del mare dell'11 dicembre 1997 «Approvazione delle procedure per l'autorizzazione all'uso dei prodotti disinquinanti in mare», il decreto del direttore generale per la difesa del mare del 23 dicembre 2002 «Definizione delle procedure per il riconoscimento di idoneità dei prodotti disperdenti ed assorbenti da impiegare in mare per la bonifica dalla contaminazione da idrocarburi petroliferi» e il decreto del direttore generale per la protezione della natura del 24 febbraio 2004 «Provvedimento di modifica del decreto 23 dicembre 2002 concernente ''Definizione delle procedure per il riconoscimento di idoneità dei prodotti disperdenti ed assorbenti da impiegare in mare per la bonifica dalla contaminazione da idrocarburi petroliferi''» sono abrogati.

Roma, 25 febbraio 2011

Il direttore generale: GRIMALDI



ALLEGATO 1

Documentazione da allegare all'istanza di idoneità

La documentazione che deve essere allegata alla istanza con la quale si richiede il riconoscimento di idoneità ai sensi del presente Decreto di un prodotto assorbente o disperdente è quella di seguito elencata:

a) scheda tecnica del prodotto assorbente o disperdente di cui agli Allegati 2 o 3;

b) documentazione attestante che il laboratorio che ha eseguito le prove, se Ente e/o Istituto Pubblico sia conforme ai requisiti della norma UNI CEI EN ISO/IEC 17025 ovvero che partecipi a circuiti di intercalibrazione nazionali e/o internazionali, ove fosse privato sia accreditato ai sensi della norma UNI CEI EN ISO/IEC 17025;

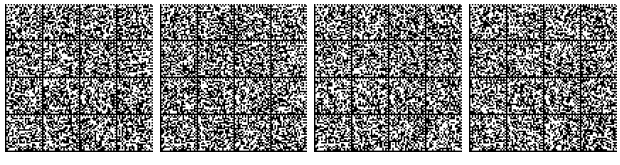
c) documentazione relativa alle prove di laboratorio effettuate ai sensi degli Allegati 4 o 5, comprendente i rapporti di prova corredati dai risultati dei test, elaborazione statistica, dati grezzi relativi a tutte le misure effettuate, schede tecniche A, B, C e D di seguito riportate;

d) documentazione relativa alle informazioni da riportare sull'involucro esterno del prodotto di cui all'Allegato 6.

SCHEDA A – Scheda tecnica riassuntiva delle informazioni

Nome del prodotto				
Forma con cui il prodotto è commercializzato (se assorbente)				
Produttore				
Responsabile dell'immissione nel mercato				
Elenco dei componenti chimici:				
Nome	numero CAS	percentuale in peso		
		minima	media	massima
1) 2) 3) …				

Sostanze in tracce	
Classificazione di pericolosità	
Stato fisico	
Peso Specifico	
Densità a 20°C	
Viscosità cinematica a 20°C	
Intervallo di viscosità entro cui il prodotto risulta efficace	
Punto di intorbidimento	
Infiammabilità	
Data di scadenza	
Precauzioni per lo stoccaggio	
Precauzioni per la conservazione	
Temperature di conservazione (min, max intervallo ottimale)	
Temperature che potrebbero causare la separazione dei componenti in soluzione, il cambiamento di stato chimico o fisico o un'alterazione dell'efficacia del prodotto	
Modalità per la manipolazione in sicurezza da parte dell'operatore	
Modalità di preparazione (tal quale o diluito)	
Modalità di impiego, recupero ed eventuale riutilizzo	
Condizioni meteo-marine ottimali per l'impiego	
Indicazioni di possibili restrizioni all'impiego	



SCHEDA B – Scheda restituzione dati del test di efficacia

Nome del prodotto				
	Replica 1	Replica 2	Replica 3	Replica 4
P = quantità di petrolio espressa in grammi (g)				
V = volume del campione (0,5 L)				
C = concentrazione iniziale del petrolio (g/L)				
p_1 = petrolio disperso espresso in grammi (g) nella prima aliquota (punto 2.1.2 lettera e)				
v_1 = volume della prima aliquota da saggio (50 mL)				
c_1 = concentrazione (g/L) di petrolio disperso misurato nella prima aliquota (50 mL)				

SCHEDA C – Scheda per la restituzione dati del test di stabilità

Stabilità in condizioni di agitazione:				
	Replica 1	Replica 2	Replica 3	Replica 4
p_2 = petrolio disperso espresso in grammi (g) nella seconda aliquota, di cui al par. 2.2.1				
v_2 = volume della seconda aliquota da saggio (100 mL)				
c_2 = concentrazione (g/L) di olio minerale disperso ed emulsionato misurato nella seconda aliquota da saggio (100 mL)				
S% = stabilità in condizioni di agitazione ovvero percentuale di petrolio disperso dopo un' ora.				
(E-S)% = scostamento della stabilità dall'efficacia				

Stabilità in condizioni di calma:				
	Replica 1	Replica 2	Replica 3	Replica 4
p_3 = petrolio disperso espresso in grammi (g) in ciascuna delle tre aliquote, di cui al par. 2.2.2				
v_3 = volume della aliquota da saggio (50 mL)				
c_3 = concentrazione (g/L) di petrolio disperso misurato nell'aliquota da saggio a un'ora (50 mL)				

S% = stabilità in condizioni di calma ovvero percentuale di petrolio disperso dopo un' ora.				
(E-S)% = scostamento della stabilità dall'efficacia				

SCHEDA D – Scheda per la restituzione dati dei saggi di tossicità

GENERALITA'

Denominazione prodotto	
Organismo test	
Metodo di prova	
Origine organismi[1]	

SENSIBILITA' SPECIFICA

Sostanza tossica di riferimento	
Range di riferimento e/o carta di controllo	
EC50 e limiti fiduciali (controllo positivo)	

SINTESI RISULTATI SAGGI

Data inizio prova						
Durata (ore)						
Concentrazioni testate (mg/L)	controllo	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5
Parametro di controllo 1						
Parametro di controllo 2						
Luce e fotoperiodo						
Temperatura (°C) di esposizione						
Acqua usata per il test	naturale/artificiale					
Metodo di calcolo EC50	pH					
	Salinità					
	Provenienza (*se naturale)*					
Sintesi dei Risultati						
EC 50 (mg/L)						
Parametri validazione[2]		L.F. INF	L.F. SUP			
Osservazioni[3]						

Dati grezzi

controllo	replica 1	replica 2	replica 3	Media ± s
	replica 4	replica 5	replica 6	
Concentrazione 1 (mg/L)	replica 1	replica 2	replica 3	Media ± s
	replica 4	replica 5	replica 6	
Concentrazione 2 (mg/L)	replica 1	replica 2	replica 3	Media ± s
	replica 4	replica 5	replica 6	
Concentrazione 3 (mg/L)	replica 1	replica 2	replica 3	Media ± s
	replica 4	replica 5	replica 6	
Concentrazione 4 (mg/L)	replica 1	replica 2	replica 3	Media ± s
	replica 4	replica 5	replica 6	
Concentrazione 5 (mg/L)	replica 1	replica 2	replica 3	Media ± s
	replica 4	replica 5	replica 6	
Concentrazione 6 (mg/L)	replica 1	replica 2	replica 3	Media ± s
	replica 4	replica 5	replica 6	

Considerazioni conclusive/Note

[1]Specificare se trattasi di popolazioni selvatiche, commerciali o allevate e la loro provenienza;

[2]Indicare i parametri di validazione quali ad esempio l'effetto sul controllo (mortalità, tasso di crescita, etc.);

[3]Riportare eventuali osservazioni utili sul comportamento degli organismi e/o sull'aspetto delle soluzioni durante l'esecuzione delle prove.

ALLEGATO 2

Indicazioni per la elaborazione della SCHEDA TECNICA di prodotti ASSORBENTI di origine sintetica o naturale non inerti

La Scheda Tecnica di un prodotto assorbente di origine sintetica o naturale non inerte per il quale si richiede il riconoscimento di idoneità ai sensi del presente Decreto deve contenere le seguenti informazioni:

a) nome e forma con i quali il prodotto sarà commercializzato;

b) identificazione del produttore e del responsabile dell'immissione sul mercato;

c) elenco dei componenti chimici e loro percentuale in peso (le percentuali devono indicare i pesi minimi, massimi e medi allo scopo di rappresentare il controllo della qualità e della variabilità del formulato);

d) numeri di registrazione attribuiti, ai sensi dell'art. 20, paragrafo 3 del regolamento (CE) n.1907/2006 ("regolamento REACH"), alle sostanze chimiche presenti nel prodotto e, qualora mancanti, indicazioni sulle motivazioni di tale mancanza;

e) assenza o presenza in tracce delle sostanze di cui alla tabella 1/A dell'Allegato 1 del Decreto 14 aprile 2009, n. 56 "Regolamento recante «Criteri tecnici per il monitoraggio dei corpi idrici e l'identificazione delle condizioni di riferimento per la modifica delle norme tecniche del Decreto legislativo 3 aprile 2006, n. 152, recante Norme in materia ambientale, predisposto ai sensi dell'articolo 75, comma 3, del Decreto legislativo medesimo»";

f) classificazione di pericolosità ai sensi del Regolamento (CE) n. 1272/2008 del Parlamento Europeo del Consiglio del 16 dicembre 2008 relativo alla classificazione, all'etichettatura e all'imballaggio delle sostanze e delle miscele che modifica e abroga le Direttive 67/548/CEE e 1999/45/CE e che reca modifica al Regolamento (CE) n. 1907/2006 ("regolamento REACH");

g) stato fisico;

h) peso specifico;

i) infiammabilità (secondo il metodo UNI EN ISO 2719: 2005 o secondo il metodo ASTM D93);

j) data di scadenza;

k) precauzioni per lo stoccaggio e la conservazione;

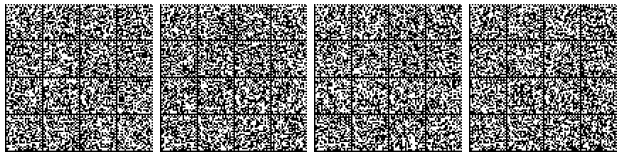
l) temperature minima e massima di conservazione (incluso il *range* ottimale);

m) modalità per la manipolazione in sicurezza da parte dell'operatore;

n) modalità di impiego, recupero ed eventuale riutilizzo;

o) condizioni meteo-marine ottimali per l'impiego;

p) indicazioni di possibili restrizioni all'impiego.



ALLEGATO 3

Indicazioni per la elaborazione della SCHEDA TECNICA di prodotti DISPERDENTI di origine sintetica o naturale

La Scheda Tecnica di un prodotto disperdente di origine sintetica o naturale per il quale si richiede il riconoscimento di idoneità ai sensi del presente Decreto deve contenere le seguenti informazioni:

a) nome con il quale il prodotto sarà commercializzato;

b) identificazione del produttore e del responsabile dell'immissione sul mercato;

c) elenco dei componenti chimici e loro percentuale in peso (le percentuali devono indicare i pesi minimi, massimi e medi allo scopo di rappresentare il controllo della qualità e della variabilità del formulato);

d) numeri di registrazione attribuiti, ai sensi dell'art.20, paragrafo 3 del regolamento (CE) n.1907/2006 ("regolamento REACH"), alle sostanze chimiche presenti nel prodotto e, qualora mancanti, indicazioni sulle motivazioni di tale mancanza;

e) elenco dei componenti principali suddiviso almeno nelle seguenti categorie: agenti ad azione disperdente, solventi e additivi;

f) assenza o presenza in tracce delle sostanze di cui alla tabella 1/A dell'Allegato 1 del Decreto 14 aprile 2009, n. 56 "Regolamento recante «Criteri tecnici per il monitoraggio dei corpi idrici e l'identificazione delle condizioni di riferimento per la modifica delle norme tecniche del Decreto legislativo 3 aprile 2006, n. 152, recante Norme in materia ambientale, predisposto ai sensi dell'articolo 75, comma 3, del Decreto legislativo medesimo»";

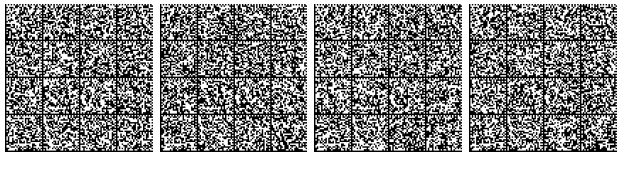
g) classificazione di pericolosità ai sensi del Regolamento (CE) n. 1272/2008 del Parlamento Europeo del Consiglio del 16 dicembre 2008 relativo alla classificazione, all'etichettatura e all'imballaggio delle sostanze e delle miscele che modifica e abroga le Direttive 67/548/CEE e 1999/45/CE e che reca modifica al Regolamento (CE) n. 1907/2006 ("regolamento REACH");

h) stato fisico;

i) peso specifico;

j) densità a 20°C;

k) viscosità cinematica a 20°C (secondo il metodo ASTM D 445);



l) punto di intorbidimento (secondo le norme IP 219/67 o secondo il metodo ASTM D2500);

m) intervallo di viscosità cinematica dell'idrocarburo a 20°C entro cui il prodotto risulta efficace;

n) infiammabilità (secondo il metodo UNI EN ISO 2719: 2005 o secondo il metodo ASTM D93);

o) data di scadenza;

p) precauzioni per lo stoccaggio e la conservazione;

q) temperature minima e massima di conservazione (incluso il *range* ottimale);

r) temperature che potrebbero causare la separazione dei componenti in soluzione, il cambiamento di stato chimico o fisico o un'alterazione dell'efficacia del prodotto;

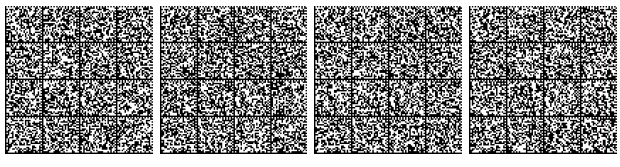
s) modalità per la manipolazione in sicurezza da parte dell'operatore;

t) modalità di preparazione (tal quale o diluito);

u) modalità di impiego, recupero ed eventuale riutilizzo;

v) condizioni meteo-marine ottimali per l'impiego;

w) indicazioni di possibili restrizioni all'impiego.



ALLEGATO 4

METODOLOGIE ANALITICHE e CRITERI DI ACCETTABILITÀ
delle risultanze dei test necessari per il riconoscimento di idoneità dei prodotti ASSORBENTI di origine sintetica o naturale non inerti

1. Al fine di riconoscere l'idoneità all'impiego in mare per la bonifica dalla contaminazione da idrocarburi petroliferi, un prodotto assorbente di origine sintetica o naturale non inerte deve essere sottoposto ai seguenti test:

a) test di efficacia di assorbimento da eseguirsi secondo quanto indicato al punto 2 del presente Allegato;

b) saggi di tossicità da eseguirsi secondo quanto indicato al punto 4 del presente Allegato.

2. **Test di efficacia di assorbimento**

Sono riportati i test per determinare l'idoneità del prodotto in termini di efficacia assorbente e di reversibilità del processo di assorbimento stesso. Quest'ultimo test può interpretarsi come una determinazione della stabilità dell'azione assorbente. I test proposti richiedono oltre alla normale strumentazione di laboratorio (inclusi termostato a circolazione sia interna, sia esterna di acqua ed estrattore Soxhlet), la seguente apparecchiatura specifica:

- dispositivo schiumatore-scolatore (Fig.1);
- dispositivo per test di cessione del petrolio assorbito (Fig. 2);
- agitatore oscillante termostatato, tipo Dubnoff.

2.1 Efficacia assorbente

2.1.1 Principio del metodo

Il metodo tende a determinare la capacità di assorbimento di solidi di tipo galleggiante nei confronti di petrolio stratificato su acqua di mare sintetica. Sono di seguito indicati due test da effettuare che consentono rispettivamente di:

A. determinare la quantità totale di petrolio eliminato come somma della quantità di petrolio assorbita dalle particelle assorbenti e di quella conglobata dalle particelle stesse;
B. determinare la quantità di petrolio eliminata per solo assorbimento dalle particelle assorbenti.

Test A

Preparare l'acqua di mare sintetica con la composizione in g/L di cui alla tabella 1. Pesare le quantità di sali utilizzando reattivi di grado analitico e solubilizzarle con acqua ultrapura (bidistillata e/o deionizzata).

Tabella 1. Composizione dell'acqua di mare sintetica

Sale	Concentrazione (g/L)
NaCl	22
$MgCl_2$ $6H_2O$	9,7
Na_2SO_4 (anidro)	3,7
$CaCl_2$ (anidro)	1,0
KCl	0,65
$NaHCO_3$	0,20
H_3BO_3	0,023

In una vaschetta di porcellana di forma rotonda (d.i. 21 cm, h 8,5 cm) introdurre 500 mL di acqua di mare sintetica; su di essa stratificare 150 mL di petrolio greggio *Arabian Light*, quindi spargere sulla superficie 10 g di prodotto assorbente. Porre la vaschetta su un termostato a 20°C ed agitare orizzontalmente per 5 minuti a 67 oscillazioni/minuto. Al termine raccogliere dalla superficie liquida l'assorbente imbevuto di petrolio, usando l'apposito schiumatore dotato di reticella metallica (400 maglie/cm^2; diametro del filo 0,1 mm) indicato in Fig. 3. Prelevare dalla quantità raccolta un'aliquota di 1- 2 g e sottoporla immediatamente a procedura di estrazione come di seguito riportata; trattare la parte rimanente come indicato nel test B.

Trattare l'aliquota di 1-2 g in un estrattore Soxhlet per 3 ore con 200 mL di una miscela estraente costituita da 80 volumi di *n*-esano e 20 volumi di metil-*t*-butiletere (MTBE). Effettuare la determinazione degli oli minerali sull'estratto seguendo il metodo ISO 9377-2 (2002). Seccare all'aria il campione di solido assorbente al termine dell'estrazione e pesarlo.

Analizzare l'assorbente non trattato con petrolio, con la stessa procedura analitica sopra riportata (bianco del metodo).

Calcolare la quantità di petrolio assorbito e conglobato come segue:

[Peso petrolio estratto(g) / Peso assorbente seccato (g)] – [Peso estratto del bianco (g) / Peso assorbente del bianco (g)].

Test B

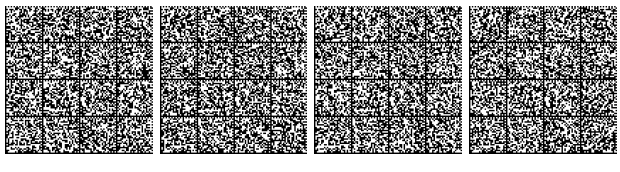
Lo schiumatore, con il prodotto assorbente ed il petrolio trattenuto utilizzato per il test A, viene posto in posizione verticale per 3 ore per permettere, attraverso la reticella di cui è dotato lo schiumatore stesso, l'eliminazione del petrolio conglobato fra le particelle assorbenti. Al termine di tale tempo si preleva un campione di assorbente più petrolio assorbito di 2-3 g e si procede alla determinazione degli oli minerali come indicato per il test A.

Calcolare la percentuale di petrolio assorbito attraverso il rapporto fra grammi di petrolio assorbito rispetto al peso del campione espresso in grammi secondo la formula riportata nel test A.

3. **Test di stabilità dell'azione assorbente**

Si esegue una prova con un campione ottenuto con le modalità indicate nei test A e B del punto 2.1.1 dell'Allegato 4. Al termine delle tre ore di sgocciolamento previste nel test B, si preleva un'aliquota di campione, per effettuare il test di cessione utilizzando l'apparecchiatura indicata in Fig. 2. Il tubo di vetro di Fig. 2, chiuso all'estremità inferiore, viene riempito per metà di acqua di mare sintetica e termostatato a 20°C. Sulla superficie dell'acqua viene versato un quantitativo di campione prelevato dallo schiumatore al termine delle 3 ore tale da produrre uno strato di 4 cm di altezza; si chiude l'estremità superiore del tubo con l'apposito tappo dotato di reticella metallica, dello stesso tipo di quella usata per lo schiumatore, al quale è collegato un tubo di efflusso. Si fanno scorrere nel tubo, dal basso verso l'alto, con l'ausilio di una pompa, 25 litri di acqua di mare sintetica termostata a 20°C attraverso il campione solido per 30 minuti. Al termine si prelevano 2-3 g di campione solido così trattato, si sottopongono ad estrazione in Soxhlet e sull'estratto si procede all'analisi degli oli minerali con il metodo ISO 9377-2 (2002) citato.

I risultati del presente test devono essere riportati sull'involucro esterno del prodotto, ai sensi dell'Allegato 6 (punto h) del presente Decreto.



4. **Saggi di tossicità**

Il saggio indicato verifica che non vengano rilasciati nell'acqua di mare, dal prodotto assorbente, composti in grado di esercitare un'azione tossica su organismi marini.

Il saggio di tossicità deve essere eseguito sull'eluato del prodotto, impiegando almeno una delle specie di crostacei di cui alla tabella seguente, con il relativo metodo (e suoi successivi aggiornamenti) e periodo di esposizione.

Specie	Metodo	Periodo di esposizione
Acartia tonsa	UNICHIM pr MU 2365 (2010)	48h
Artemia franciscana	APAT-IRSA-CNR 8060 (2003)	96h
Amphibalanus amphitrite	UNICHIM pr MU 2245 (2010)	48h
Corophium orientale	UNICHIM pr MU 2246 (2010)	96h
Tigriopus fulvus	UNICHIM pr MU 2396 (2010)	96h

4.1. Preparazione dell'eluato del prodotto

a) distribuire il prodotto assorbente in acqua marina sintetica, preparata secondo quanto indicato nella tabella al punto 2.1.1 del presente Allegato, alla concentrazione di 20 g/L;

b) tenere in agitazione la sospensione, termostatando a 25°C, per un periodo di 48 ore. Al termine di tale periodo filtrare su filtro in fibra di vetro di 1-2 µm. Il liquido così ottenuto dopo filtrazione costituisce l'eluato da utilizzare tal quale per l'esecuzione del saggio di tossicità prescelto.

5. **Criteri di accettabilità delle risultanze dei test ai fini del riconoscimento di idoneità di un prodotto assorbente di origine sintetica o naturale non inerte**

Sulla base del test di efficacia (test B) un prodotto è considerato accettabile, quando l'assorbente è in grado di trattenere almeno il 60% di petrolio rispetto al suo peso.

Sulla base del saggio di tossicità un prodotto è considerato accettabile quando non mostri effetti di tossicità statisticamente significativi (P >0,05 mediante test-t per campioni a varianza disomogenea) rispetto al controllo.

ALLEGATO 5

METODOLOGIE ANALITICHE e CRITERI DI ACCETTABILITÀ
delle risultanze dei test necessari per il riconoscimento di idoneità dei prodotti DISPERDENTI di origine sintetica o naturale

1. Al fine di riconoscere l'idoneità all'impiego in mare per la bonifica dalla contaminazione da idrocarburi petroliferi un prodotto disperdente di origine sintetica o naturale deve essere sottoposto ai seguenti test secondo le modalità di preparazione di cui all'Allegato 3, lettera s) del presente Decreto:

a) test di efficacia e stabilità da eseguirsi secondo quanto indicato al punto 2 del presente Allegato;

b) test di biodegradabilità da eseguirsi secondo quanto indicato al punto 3 del presente Allegato;

c) valutazione del potenziale di bioaccumulo da eseguirsi secondo quanto indicato al punto 4 del presente Allegato;

d) saggi di tossicità da eseguirsi secondo quanto indicato al punto 5 del presente Allegato.

2. Test di efficacia e stabilità

2.1. Test di efficacia

L'efficacia del disperdente viene determinata in termini di olio sospeso ed emulsionato dopo l'aggiunta del prodotto in esame in condizioni standardizzate di agitazione e in termini di stabilità dell'emulsione, determinata quest'ultima sia in condizioni standard di agitazione, sia in condizioni di calma. Entrambe queste caratteristiche (efficacia del disperdente e stabilità dell'emulsione) si basano sulla determinazione del parametro oli minerali, da effettuarsi seguendo il metodo ISO 9377-2 (2002).

2.1.1. Strumentazione

pH-metro, stufa, bilancia analitica, micropipetta, termometro (0-50 °C) e agitatore oscillante termostatato, (tipo Dubnoff, regolabile a 67 oscillazioni/minuto con una escursione orizzontale di 6 cm).
Per la parte relativa alla determinazione degli idrocarburi totali fare riferimento al metodo ISO 9377-2 (2002).

2.1.2. Modalità esecutive

a. Preparare una soluzione di disperdente e petrolio grezzo *Arabian Light*, miscelando una parte di disperdente con 10 parti di petrolio;
b. introdurre 500 mL di acqua marina preparata secondo lo schema riportato in tabella 1 di cui all'Allegato 4, in ciascuno dei 4 imbuti separatori da 500 mL (3 repliche e 1 controllo);
c. misurare e registrare la temperatura dell'acqua (20±1 °C);
d. aggiungere 500 µL della soluzione disperdente+petrolio nelle 3 repliche e 500 µL di petrolio senza disperdente nel controllo;

e. agitare energicamente ciascun imbuto separatore per 2 min. Al termine dell'agitazione prelevare un'aliquota di 50 mL su cui eseguire il dosaggio della quantità di petrolio grezzo disperso, effettuato in accordo con il metodo indicato al par 2.1 del presente Allegato;

f. prelevare un'ulteriore aliquota di 100 mL da ciascun imbuto su cui effettuare il test di stabilità in condizioni di agitazione;

g. porre gli imbuti separatori con l'aliquota rimanente (350 mL) in termostato a 20°C da utilizzare successivamente per il test di stabilità in condizioni di calma.

2.2. Test di stabilità

La stabilità del petrolio disperso deve essere misurata sia in condizioni di agitazione sia in condizioni di calma.

2.2.1. Test in condizioni di agitazione

Travasare l'aliquota di 100 mL in una beuta da 250 mL. Agitare per 6 ore a 67 oscillazioni/minuto usando l'agitatore Dubnoff termostatato a 20°C. Al termine delle 6 ore, prelevare un'aliquota 50 mL dal fondo della beuta e versarla in un imbuto separatore da 100 mL. Procedere al dosaggio degli oli minerali, secondo il metodo ISO 9377-2 (2002).

2.2.2. Test in condizioni di calma

Dagli imbuti separatori messi a riposo in termostato a 20°C prelevare in tempi successivi (1, 2 e 23 ore), per mezzo del rubinetto, tre aliquote di 50 mL ciascuna su cui effettuare la determinazione degli oli minerali come indicato al par. 2.1.

2.3. Calcoli

2.3.1. Efficacia

CALCOLO DELL'EFFICACIA

P = quantità di petrolio espressa in grammi (g)

V = volume del campione (0,5 L)

C = concentrazione iniziale del petrolio (g/L)

p_1 = petrolio disperso espresso in grammi (g) nella prima aliquota (punto 2.1.2 lettera e)

v_1 = volume della prima aliquota da saggio (50 mL)

c_1 = concentrazione (g/L) di petrolio disperso misurato nella prima aliquota (50 mL)

E% = efficacia dell'azione emulsionante

$C = \frac{P}{V}$ g/L

$c_1 = \frac{p_1}{v_1} \times 1000$ g/L

$E\% = \frac{c_1}{C} \times 100$

2.3.2. Stabilità

CALCOLO DELLA STABILITÀ

Stabilità in condizioni di agitazione:

p_2 = petrolio disperso espresso in grammi (g) nella seconda aliquota, di cui al par. 2.2.1

v_2 = volume della seconda aliquota da saggio (100 mL)

c_2 = concentrazione (g/L) di olio minerale disperso ed emulsionato misurato nella seconda aliquota da saggio (100 mL)

$c_2 = \frac{p_2}{v_2} \times 1000$ g/L

S%= stabilità in condizioni di agitazione ovvero percentuale di petrolio disperso al termine dell'agitazione

$S\% = \frac{c_2}{c_1} \times 100$

(E-S)% = scostamento della stabilità dall'efficacia

Lo scostamento della stabilità dall'efficacia viene determinato con la formula:

(E-S)% = 100 - *S%*

Ovvero, fatto 100 il quantitativo di petrolio disperso, misurato con il test di efficacia, si determina in termini percentuali la quantità di petrolio che è ancora rimasto disperso al termine dell'agitazione del test di stabilità di cui al par. 2.2.1

Stabilità in condizioni di calma:

p_3 = petrolio disperso espresso in grammi (g) in ciascuna delle tre aliquote, di cui al par. 2.2.2

v_3 = volume della aliquota da saggio (50 mL)

c_3 = concentrazione (g/L) di petrolio disperso misurato nell'aliquota da saggio a un'ora (50 mL)

S%= stabilità in condizioni di calma ovvero percentuale di petrolio disperso dopo un' ora.

(E-S)% = scostamento della stabilità dall'efficacia

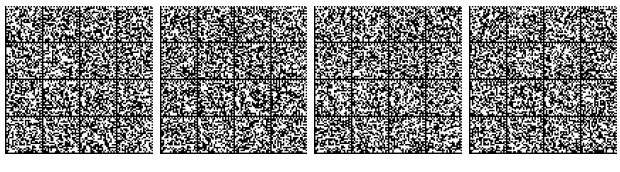
$c_3 = \frac{p_3}{v_3} \times 1000$ g/L

$S\% = \frac{c_3}{c_1} \times 100$

Lo scostamento della stabilità dall'efficacia viene determinato con la formula:

(E-S)% = 100 - *S%*

Ovvero, fatto 100 il quantitativo di petrolio disperso, misurato con il test di efficacia, si determina in termini percentuali la quantità di petrolio che è ancora rimasto disperso dopo un'ora di riposo.
Per il calcolo della stabilità a 2 ed a 23 ore utilizzare le formule sopra riportate sostituendo il valore del parametro c_3 con le rispettive concentrazioni di petrolio disperso nei due tempi di prelievo indicati.



3. **Test di biodegradabilità**

Effettuare il test di biodegradabilità sui componenti organici del prodotto seguendo la metodica "*closed bottle*" indicata nella linea guida OECD n. 306 (17 luglio 1992), utilizzando acqua di mare naturale caratterizzata come indicato nel metodo stesso.

4. **Valutazione del potenziale di bioaccumulo**

Per ciascuna sostanza presente nel prodotto deve essere fornito il valore di bioconcentrazione (BCF), ottenuto con metodi standardizzati e corredato da specifica documentazione.

In via subordinata, qualora il BCF non fosse disponibile, deve essere fornito il valore del *log* K_{ow} di ciascuna sostanza presente nel prodotto, determinato sperimentalmente, utilizzando metodi standardizzati.

Qualora non sia possibile determinare sia il valore di BCF sia quello del *log* K_{ow} dovrà essere giustificata, sulla base di adeguata documentazione, l'impossibilità tecnica di esecuzione dei test o la non rilevanza del potenziale di bioaccumulo.

5. **Saggi di tossicità**

La tossicità dei prodotti disperdenti deve essere valutata attraverso saggi di tossicità su organismi marini appartenenti a 3 livelli trofici (alghe, crostacei e pesci).

5.1 Preparazione del campione

La soluzione madre del prodotto disperdente deve essere preparata al momento dell'uso, sciogliendo o diluendo in matracci tarati in vetro borosilicato una quantità nota di prodotto (≤1000 mg/L) e portando a volume con acqua di diluizione.

Nella preparazione della soluzione madre, al fine di massimizzare la solubilizzazione e l'omogeneità della soluzione, dovranno essere seguite le indicazioni riportate in: "OECD *Series on Testing and Assessment* No. 23. *Guidance document on aquatic toxicity testing of difficult substances and mixtures*. Documento ENV/JM/MONO(2000)6".

Nei casi in cui nella soluzione siano presenti una o più sostanze con caratteristiche adsorbenti a concentrazioni <1 mg/L, si deve procedere al condizionamento dei contenitori utilizzando le soluzioni alle medesime concentrazioni che verranno impiegate nei test.

5.2 Saggio di tossicità su alghe

Effettuare il saggio di inibizione della crescita algale secondo il metodo UNI EN ISO 10253: 2006.

5.3 Saggio di tossicità su crostacei marini

Effettuare il saggio di tossicità su crostacei marini impiegando almeno una delle specie di cui alla tabella seguente, con il relativo metodo (e successivi aggiornamenti) ed utilizzando il periodo di esposizione specifico indicato.

Specie	Metodo	Periodo di esposizione
Acartia tonsa	UNICHIM pr MU 2365 (2010)	48h
Artemia franciscana	APAT-IRSA-CNR 8060 (2003)	96h
Amphibalanus amphitrite	UNICHIM pr MU 2245 (2010)	48h
Corophium orientale	UNICHIM pr MU 2246 (2010)	96h
Tigriopus fulvus	UNICHIM pr MU 2396 (2010)	96h

Nei saggi di tossicità a 96 ore su crostacei deve essere effettuato un rinnovo delle soluzioni dopo 48 ore avendo cura di sostituire il maggior volume operativamente possibile.

Il rinnovo delle soluzioni dovrà comunque essere effettuato ogni 24 ore nel caso di prodotti rapidamente degradabili o qualora si verifichino condizioni di disomogeneità delle soluzioni test.

Per verificare l'idoneità della popolazione di organismi all'impiego nei saggi con i prodotti disperdenti, la sensibilità specifica deve essere conforme a quanto indicato nei rispettivi metodi, in termini di controllo positivo rispetto ad una sostanza tossica di riferimento.

Il risultato deve essere espresso come EC50 (limiti fiduciali al 95%).

Qualora la EC50 risulti indeterminata perché maggiore di 1000mg/L, è sufficiente indicare la dicitura "EC50 >1000 mg/L".

5.4 Saggio di tossicità su pesci marini

Effettuare i saggi di tossicità su pesci marini impiegando *Dicentrarchus labrax* o *Sparus aurata,* riferendosi alla metodica indicata nella linea guida OECD n. 203 (17 luglio 1992), modificata dalle seguenti specifiche:

- utilizzare come controllo e diluente acqua di mare naturale o sintetica;
- l'acqua di mare naturale deve provenire da aree non soggette a scarichi o sversamenti originati da fonti diffuse di inquinamento e deve essere filtrata con filtri da 10 *µ*m prima dell'uso;
- l'acqua di mare sintetica può avere la composizione di cui alla tabella 1, Allegato 4, oppure essere ricostituita mediante utilizzo delle miscele di sali comunemente reperibili in commercio nel settore acquariologico;
- al momento dell'impiego la salinità sia dell'acqua di mare naturale, sia di quella sintetica deve essere compresa tra 20 e 22 PSU, previo condizionamento degli organismi;
- la taglia degli esemplari da sottoporre al saggio deve essere di 5,0±1,5 cm;
- ogni concentrazione deve essere allestita con almeno 3 repliche, ciascuna delle quali deve comprendere almeno 7 individui;
- il saggio deve svolgersi alla temperatura di 20±1 °C;
- il saggio deve avere un periodo di esposizione di 96h, con il rinnovo delle soluzioni (almeno per il 95%) dopo 48h;

- Il test è considerato valido se la mortalità nel controllo non eccede il 10% alla fine del test e se la concentrazione di ossigeno disciolto risulta ≥ 60% del valore di saturazione in aria per tutta la durata del test.

Il rinnovo delle soluzioni dovrà essere effettuato ogni 24 ore nel caso di prodotti rapidamente degradabili o qualora si verifichino condizioni di disomogeneità delle soluzioni test.

Per verificare l'idoneità della popolazione di pesci all'impiego nei saggi con i prodotti disperdenti, deve essere condotto un saggio di tossicità (organizzato con almeno 5 concentrazioni e almeno 3 repliche ciascuna) con la sostanza di riferimento Sodio Dodecil Solfato (SDS) (CAS NUMBER 4706-78-9), la cui LC50 a 48h deve risultare <10 mg/L.

La soluzione madre di SDS deve essere preparata al momento dell'uso.

Il risultato deve essere espresso come EC50 (limiti fiduciali al 95%). Qualora la EC50 risulti indeterminata perché maggiore di 1000 mg/L, è sufficiente indicare la dicitura "EC50 >1000 mg/L".

6. Criteri di accettabilità delle risultanze dei test ai fini del riconoscimento di idoneità di un prodotto disperdente di origine sintetica o naturale

1. Il punto di infiammabilità, determinato in accordo al metodo UNI EN ISO 2719: 2005, deve essere superiore a 55 °C;

2. La viscosità cinematica, misurata a 20 °C secondo il metodo ASTM D 445, deve essere inferiore o uguale a 400 cSt;

3. Il punto di intorbidimento, determinato secondo le norme IP 219/67, deve essere inferiore o uguale a -10°C;

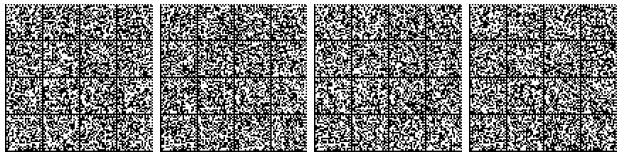
4. L'efficacia del prodotto, determinata secondo la metodica di cui al presente Allegato, deve essere tale da disperdere almeno il 60% del petrolio;

5. La stabilità della dispersione in condizioni di agitazione deve rimanere pressoché inalterata, mostrando scostamenti percentuali rispetto ai valori di efficacia contenuti entro il 10%; la stabilità della dispersione in condizioni di calma deve rimanere pressoché inalterata entro le 2 ore, con scostamenti accettabili entro il 10%, e può diminuire al massimo del 40% dopo le 23 ore. Qualora il prodotto non soddisfi integralmente i criteri di accettabilità sulla stabilità della dispersione in condizioni di calma, il Ministero dell'Ambiente e della Tutela del Territorio e del Mare valuterà l'eventuale riconoscimento di idoneità con prescrizioni all'impiego ai sensi dell'art. 5, comma 2, del presente Decreto;

6. La tossicità negli organismi marini, determinata secondo le indicazioni di cui al presente Allegato, deve prevedere per tutti gli organismi una EC50 maggiore di 10 mg/L;

7. Tutti i componenti del prodotto devono risultare biodegradabili con un consumo di ossigeno maggiore del 60% del ThOD.;

8. In riferimento al bioaccumulo, il *log* K_{ow} di tutti i componenti organici del prodotto deve essere inferiore o uguale a 3 oppure un BCF misurato minore di 500.



ALLEGATO 6

Informazioni da riportare sull'involucro esterno del prodotto assorbente o disperdente riconosciuto idoneo

1. Le informazioni che i prodotti assorbenti o disperdenti riconosciuti idonei ai sensi del presente Decreto dovranno riportare sull'involucro esterno sono le seguenti:

a) specifiche per la manipolazione del prodotto;

b) indicazioni di sicurezza per gli operatori (equipaggiamento o dispositivi di protezione individuali richiesti);

c) intervallo di temperatura di applicazione e temperatura ottimale;

d) condizioni di conservazione e scadenza;

e) mezzo idoneo per l'applicazione (aereo, imbarcazione, ecc);

f) percentuale della diluizione del prodotto con acqua o uso tal quale;

g) quantità da impiegare per unità di idrocarburi sversati;

h) stabilità.

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