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PhD thesis:

"Investigating the potential of ephyrae Jellyfish (Cnidaria) as model organism in ecotoxicology for sea water quality assessment"

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SUMMARY

1. GENERAL INTRODUCTION

1.1 Jellyfish in marine ecosystems

Jellyfish are an important and conspicuous biotic component of marine ecosystems and play a central role in the trophic organization of many marine food webs (Fowler et al. 2004; Richardson et al. 2009; Epstein et al. 2016; Boero, 2013; Gibbonsn et al. 2016). In addition, they have strong top-down control on plankton communities and are considered to have a "large footprint" on lower trophic levels (Brodeur et al., 2011). Indeed, recognized historically as trophic dead ends, nowadays jellyfish are considered the fourth pathway of the ecosystem functioning of marine ecosystems (represented by carnivorous gelatinous plankton) as dislplayed in Figure 1 (Boero, 2013; Boero et al. 2016) due to jellyfish feeding on eggs and larvae of dominant nekton species providing food to many invertebrate species (Purcell et al. 2007; Richardson et al. 2009; Boero, 2013; Faimali et al. 2014).

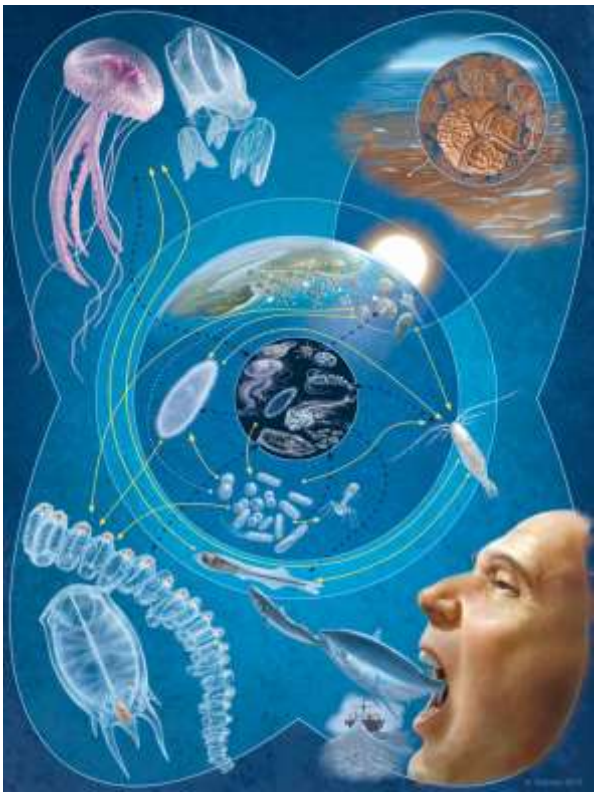

Figure 1. The four main pathways of ecosystem functioning in marine ecosystems (Concepts: F. Boero; Art: A. Gennari; Boero et al. 2016)

However, the jellyfish blooms, increasingly frequent during the last decades, have drawn attention on the tourism, fishing and power industries for many deleterious consequences. Indeed, jellyfish at high densities can clog fishing nets and contaminate fish catches, they can block the pump filters associatet with coastal power and desalination plants (Purcell et al. 2007; Gibbons et al. 2016). They can sting people, sometimes fatally, and impact on coastal tourism. They can also have indirect impacts on commercial resources by virtue of their zooplanktivorous diet, and can be predators on, and competitors with, valuable finfish and their larvae (Gibbons et al. 2016). Consequently the impact of gelatinous blooms on fish populations can be positive, when fish and jellyfish coevolved in the same environmental context and if the jellyfish are abundant just for short periods, consequently playing a keystone role that prevents the monopolization of overly successful fish species at the expenses of others, so maintaining fish biodiversity high. In the same way, if the jellyfish are not coevolved with the resident fish or if the fish populations are not "healthy", due to overfishing, and the jellyfish blooms are abnormally large and long-lasting, due to predation on and competition with fish larvae and juveniles, the impact of jellyfish blooms can be negative on fish populations (Boero, 2013). The drivers of these increased blooms seemed to be linked to various anthropogenic factors such as overfishing, eutrophication, climate change, translocation and habitat modification, including their tolerance to marine pollution (Bakun and Weeks, 2006; Richardson et al 2009; Condon et al. 2012; Purcell et al. 2012; Gibbson et al. 2016). Regarding overfishing, the decline of large, long-lived predators and the transition to short-lived invertebrates and planktivorous fishes may have potentially serious effects on ecosystems that could lead to a top-down control of marine food webs by this gelatinous predators (Pauly et al. 1998; Mills, 2001).

These indirect and direct effects may be the mainly cause of a suppression of high-energy (fish and whales) food chains (Figure 2) with a possible subsequent de-evolution of the pelagic marine ecosystem back to a gelatinous dominance (Mills, 2001; Purcell et al. 2007, 2012; Richardson et al. 2009; Boero, 2013).

In the same way, however, a decrease of jellyfish populations could have unexpected negative impacts on marine ecosystem (Brotz, 2016 Gibson et al. 2016). Indeed, the blooms result to play such a keystone role, reducing the success of dominant nekton species, since they eaten their eggs and larvae, thereby releasing resources for previously outcompeted species, thus enhancing local diversity. Moreover, jellyfish can act as nurseries for juvenile fish and can be agents for carbon sequestration (Gibbons et al. 2016). Thus, it should be taken into occount that the recent increase to use jellyfish as source of food and consequently an increase of their fishing, does not have negative results. In this regard, Gibbons and co-authors, (2016) suggested to mitigate and minimize the anthropogenic impacts, main cause of increasing blooms rather than to eat more jellyfish or even fishing them, considering also that jellyfish seemed to be more tolerant to these impacts than many other marine species. Indeed, jellyfish are the oldest animals among the ones that are currently present on the planet, they were present since the Pre-Cambrian and they not changed their body organization since then (Boero, 2013). These findings suggest that jellyfish probably have a suite of successful attributes such as broad diet, fast growth rates, the ability to shrink when starved, the capacity to fragment and regenerate, and the ability to tolerate hypoxia, that enable them to survive in disturbed marine ecosystems and to rebound rapidly as conditions improve, including their tolerance to marine pollution (Arai, 1997; Eiane et al. 1999; Bakun and Weeks, 2006; Palomares and Pauly, 2009; Richardson et al 2009; Condon et al. 2012; Boero et al. 2016; Gibson et al. 2016).

The most obvious effects of pollution on jellyfish is that it typically causes acute death of chronic toxicity in other species, effectively leaving jellyfish the last men standing.

However, it seemed that this organisms are not entirely immune to direct effects from contaminants.

Figure 2. The decrease of large fish releases jellyfish from competition with their larvae. Increased jellyfish availability favors medusivorous species, whose populations increase at the expenses of gelatinous plankton (Concepts: F. Boero; Art: A. Gennari)

Indeed, several recent studies have demonstrated that chemical compounds can exert toxic effects on jellyfish (Almeda et al. 2013; Faimali et al. 2014; Costa et al. 2015; Gambardella et al. 2015; Templeman and Kingsford, 2015; Echols et al. 2016; Gadreaud et al. 2016; Klein et al. 2016). Recently, some authors highlighted the strong potential of jellyfish as bioindicators to monitor and assess the health of marine ecosystem (Echols et al. 2014; Templeman and Kingsford, 2015) and the use of their different life stages (adult, ephyrae and polyp) as model organisms to predict the ecological effects of contaminants (Almeda et al. 2013; Faimali et al. 2014, 2016 Lucas and Horton, 2014; Costa et al. 2015; Gambardella et al. 2015; Gadreaud et al. 2016; Giussani et al. 2016).

Netherless, our knowledge of the direct biological effect of contaminants such as pesticides, plastics and radiation on jellyfish is lacking. In this scenario, due to the key role of jellyfish in the marine

food chain, a better investigation in this way could be provided a basis not only for understanding the population jellyfish dynamics but in the some way for exploring the wider impact of pollution in marine ecosystem.

1.2 Jellyfish, taxonomy, ecology and life cycles

The word "jellyfish" is a popular term defining what marine biologists call gelatinous macrozooplankton (Boero, 2013). The word "gelatinous" refers to the general consistency of these animals: their body is mostly made of extracellular matrix (often called mesoglea), i.e. the matrix that holds cells together and that is present in all animals, including us, but that, in these organisms, is the greatest portion of the whole body. They are referred to the phylum Cnidaria and Ctenophora, or comb jellyfish (Cartwright et al. 2007; Boero, 2013).

Although many Cnidaria actively swim through muscular contraction of their bells and Ctenophora propel themselves through the sequential beating of cilia, neither can progress against currents and are, thus, defined as zooplankton. Jellyfish range in size from a few millimeters (*Aglaura* and *Obelia* spp.) to 2m (*Nemopilema nomurai*) in diameter. The Cnidaria comprise four classes: anthozoa (corals and anemones), hydrozoa (hydroids and hydromedusae), Scyphozoa ("true" jellyfish) and Cubozoa ("box" jellyfish) all of which can pose problems to human health.

Most species of the Class Scyphozoa, which includes the large, bell-shaped jellyfish commonly washed up on beaches, have a polyp stage that strobilate small medusae. They are found in all pelagic environments, but attain greatest abundances near the coast. Scyphozoans, the jellyfishes, are cnidarians in which the medusoid generation is large and noticeable and the polypoid is small and inconspicuous.

The medusae, known as scyphomedusae, tend to be large, mobile, pelagic, drifting, solitary carnivores and all are marine. The mesoglea of the medusa is thick, gelatinous, and accounts for most of the mass of the organism, although it is mostly water. Cnidocytes are present, concentrated on tentacles, and are used for prey capture, and defense. Locomotion is by muscular contraction

antagonized by elastic recoil of the mesoglea. The sense organs are arrayed around the periphery of the organism. The sexes are separate. Polyps are small benthic scyphistomae. Scyphozoa includes about 200 species in five taxa: Semaeostomae, Rhizostomeae, Cubomedusae, Coronatae, and Stauromedusae (Arai, 1997).

The Semaeostomeae with simple of branched radial canals and ring canal, with or without subgenital pits, include the genera *Aurelia* and *Sanderia* whose species are used as biological model in this thesis (see Matherial and Methods). Scyphozoan jellyfish display a complex life cycle (Figure 3) comprising an alteration of generation with a planktonic sexsually- reproducing medusa and a benthic asexually reproducing polyp (Arai, 1997). Medusae tend to be gonochoristic, broadcast spawners with the resulting fertile planule being motile for a few days to weeks before settling to a hard substrate. After settlement of the planulae, young polyps developed within 2 days (Arai, 1997).

Polyps of all species reproduced asexually by buds that rise laterally from the calyx and produced chitinous layered cysts (podocysts) under their stalks. However, the asexual reproduction of Scyphozoan is widely investigated and the results may be found in the diversity of asexsual reproductive strategies existing among scyphozon (e.g. Han and Uye 2010; Schiariti et al. 2014).

While some species exhibit the potential to display several asexual modes, even simultaneously, others only exhibit a few or just one (Adler and Jarms 2009).

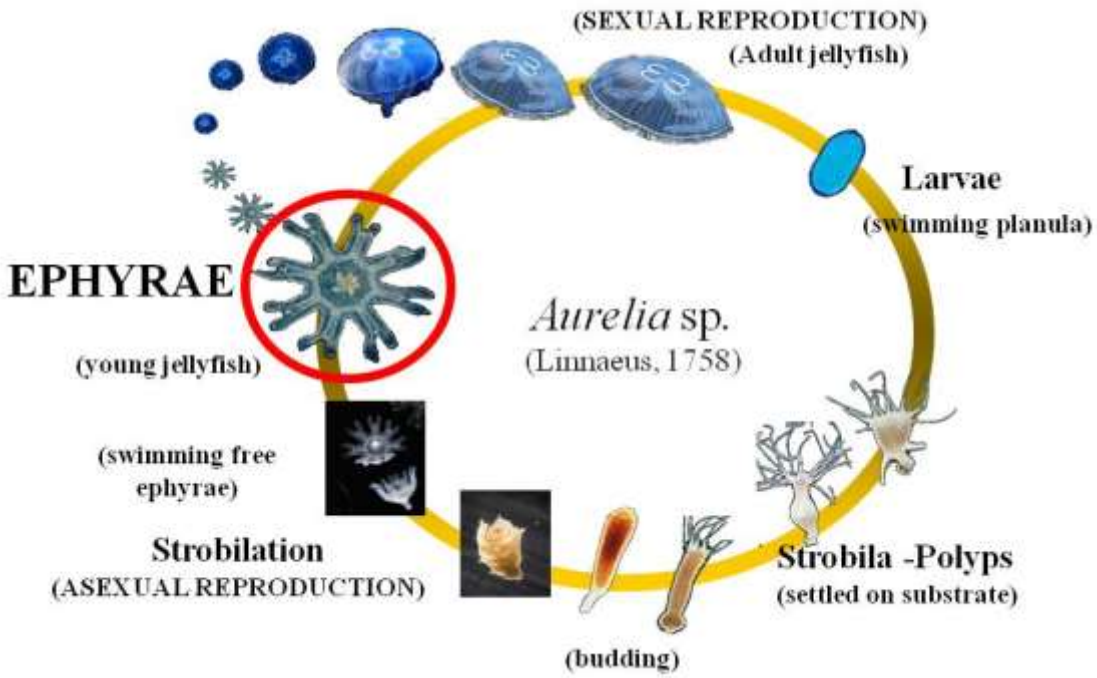

Figure 3. Life cycle of *Aurelia sp.* as example of the Scyphozoans jellyfish. Ephyrae (red circle is the life stage used in this thesis as biological model).

Therefore, considering the reproductive strategy to be the number and type of asexual reproduction modes displayed and the relative importance of each mode to polyp density, we hypothesize that those species capable of switching their reproductive strategy in response to environmental fluctuations will have a greater blooming potential (Purcell et al. 2012; Schiariti et al. 2014). Polyps are able to develop the early stage of jellyfish called ephyrae via mono-or-poly strobilation and consequentially the ephyrae develop into the much larger medusa.

1.3 Jellyfish in marine ecotoxicology.

1.3.1 Marine ecotoxicology

Aquatic ecosystems are often impacted by chemical pollution, originating from municipal and industrial wastewater effluents (point sources), airborne deposition as well as runoff from urban and agricultural areas (diffuse sources).

One of the core missions of ecotoxicology is to understand the mechanisms by which contaminants perturb normal biological performances (their mode of action), in order to develop appropriate

measures to prevent adverse outcomes resulting from environmental contaminants (Cairn and Pratt, 1993; Chapman et al. 2002; Moisenko, 2008; Connon et al. 2012). A definition or characterisation of the field of ecotoxicology is to study ecological and ecotoxicological effects of pollutants on populations, communities and ecosystems in conjunction with the fate of pollutants in the environment (Forbes and Forbes, 1994; Connon et al. 2012). In aquatic ecosystems there is a wide range of possible contaminant effects that can compromise the ecological fitness of individual organisms or populations. Toxic substances can cause effects at different levels of biological organization, from molecular to ecosystem levels (Figure 4). Of primary concern is the protection of aquatic organisms at the population or ecosystem level; it is therefore important to bridge the gap between the relatively short-term (acute) effects that can realistically be quantified in laboratory or field experiments, and longer-term (chronic) ecological effects. Ultimately, the impact of a toxic contaminant or contaminant mixture depends on the relative sensitivity of a species, community or ecosystem, and the intensity and timing of exposure.

Currently available approaches (Figure 5) for assessing the toxicity of chemical mixtures include theoretical models, each with different levels of data requirements (Backhaus and Faust, 2012) and biological tools (Escher and Leusch, 2012). Biological tools based on indicator species were used to detect environmental hazards, and establish the relationship between contaminant concentration and toxicity (establishment of dose-response relationships).

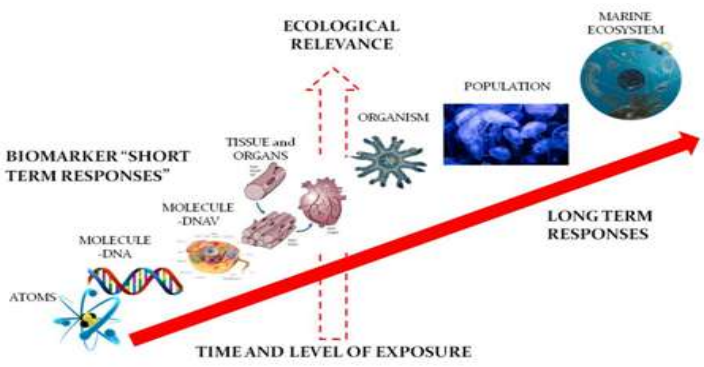

Figure 4. The field of ecotoxicology studies the effects of anthropogenic chemicals on ecosystems at different levels of biological organization, from the molecular and cellular level to entire ecosystems.

Standardized biological test methods to measure water quality developed quickly after the US Environmental Protection Agency (EPA) initiated a national policy in 1984 to control toxic substances based on a water quality approach aimed at the protection of environmental health using biotests. Biotests can measure integrative toxic effects and provide the data needed for the derivation of EQS (Environmental Quality Standards) and risk assessment. To date, however, most environmental monitoring programmes rely primarily on the analysis of chemical substances in water column, sediment and biota. The resulting assessment was performed on a single-substance basis. Biotests can target different levels of biological organization, from the level of molecules and cells, to tissues and organs, individuals, populations and communities.

Biotests can be performed under standardized conditions in laboratories using single cell systems (*in vitro*), organisms (*in vivo*) or simple communities (micro- mesocosms), or where the focus is on ecological relevance in the field, by means of *in situ* exposures, health assessment of resident organisms using biomarkers (molecular, biochemical, cellular and/or physiological alterations) and (histo-) pathological evaluation, and/or community indices.

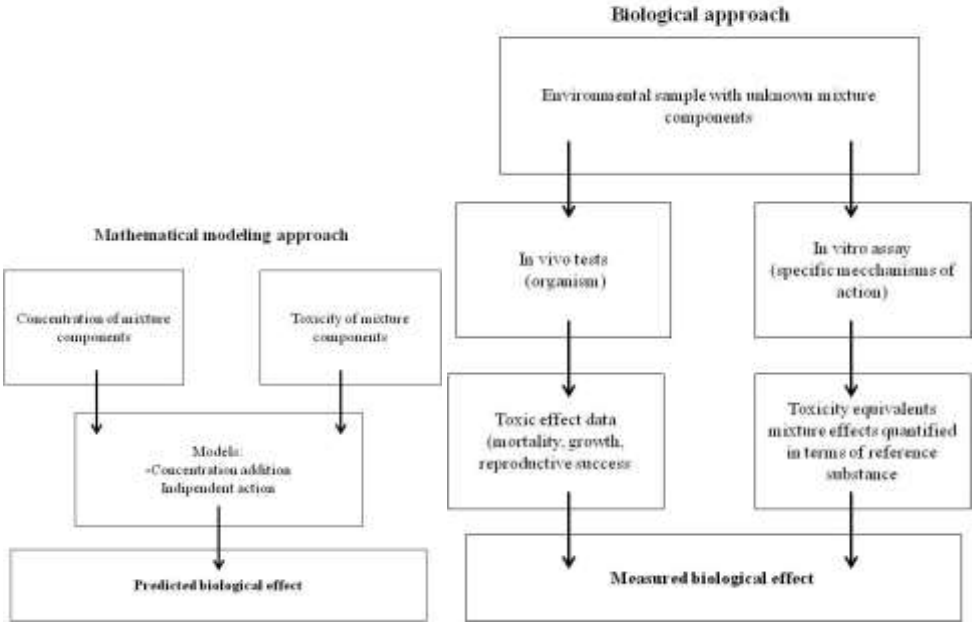

Figure 5. Two approaches to mixture toxicity assessment: effect prediction using mathematical modelling and single-substance toxicity data, and effect measurement using effect-based biological tests (Connon et al. 2012).

1.3.2 Model organisms used in marine ecotoxicology

The selection of suitable model organisms is one of the most important steps in aquatic toxicology, able to provide information on the acute and chronic toxicity of marine pollutants. The main objective for the environmental monitoring is to use batteries of species from different trophic levels, typically a primary producer (e.g., green algae), a detritivore or filter feeder (e.g., water flea), and a consumer (e.g., larval fish). In comparison to single tests, advantages of using a battery of assays include higher sensitivity and selectivity, higher ecological relevance, and the integrative interpretation of effects and mechanisms of action.

In addition, test batteries can prove useful to select the most sensitive and selective biotest (Repetto et al. 2000; Holdway et al. 2001) or sentinel species (Madoni 2000) for a particular application and/or for the contaminants present in a given environmental situation.

On the other hand, the conduct of test batteries may require considerable expenditures, resources, and operator training (Harby et al. 2017).

The current Italian legislation (D. Lgs. 152/99) requires the use of bioassays for the assessment of water quality, in addition to the monitoring of physicochemical and microbiological parameters. Likewise, the EU Water Framework Directive (2000/60/ EC) requires data from ecotoxicological tests to derive environmental quality standards and subsequently assess the chemical status of waters, by using a base-set of taxa belonging to three trophic levels (algae and macrophytes, *Daphnia* or representative organisms for saline waters, fish). Previous studies have commonly used test batteries composed of a number of taxa that were not fully representative of the whole marine trophic web (Jiangning et al. 2004) or used different species belonging to the same trophic level (Davoren and Fogarty, 2004). As a result, the characterization of substances or the environmental monitoring was often offering an ecotoxicologically incomplete picture.

A representative, cost-effective, and quantitative ecotoxicological in vitro test battery should instead include organisms of all trophic levels, that is, decomposers, primary producers, consumers, and predators.

1.3.2.1 Invertebrates as model organisms in marine ecotoxicology

In this context, invertebrate species are being used extensively in laboratory tests for their usefulness for seeking mechanistic links between effects occurring at the individual level and consequences for higher levels of biological organization. In addition, compared to vertebrates, they are also easy to maintain under laboratory conditions, widely distributed and ecologically relevant (Aschuer et al. 2007; Faimali et al. 2017; Gouveia et al. 2017).

The development of bioassays using invertebrates has been stimulated by both biological and toxicological characteristics of these organisms.

Many invertebrate species can be cultured easily under laboratory conditions because they are of small size and display high fecundity and short life span. These characteristics make the simultaneous breeding of various species easier than for vertebrates. Handling of animals is also easy and the number of replicates for each tested concentration or dose may increase, thus improving the statistical significance of test results without a significant increase in testing costs. The short life span of numerous invertebrate species provides an opportunity to save time and money. Indeed, a short time in invertebrates refers to a few days (from 2 to 4 days), whereas short-term tests in rodents require 7 to 14 days to be completed (Ren et al. 2017) Moreover, invertebrates occupy key positions in the food webs of aquatic and terrestrial ecosystems, and some species or groups of species (e.g., daphnids) are present throughout a wide range of habitats. The hazards of environmental chemicals, therefore, can be evaluated on a large panel of species with specific ecologic characteristics.

1.3.3 Behavioral in marine ecotoxicology

Trough the setup of bioassays the toxicity of contaminants can be measured in many different ways as the measure of different end-points (biological responses). Among the biological responses, the death, is more frequently expressed as lethal concentration or dose at which 50% of individuals are killed in a given period of time (usually 48 to 96h) (Median Lethal Concentration - LC_{50}).

This is of limited value in the marine environment, lethal concentrations being very rarely encountered in the field. The main use of these lethal tests is to allow an easy comparison of the relative toxicity of very different compounds. Sub-lethal effects are also used (e.g. embryotoxicity tests), the results being expressed as the concentration of compound where 50% of its maximal effect is observed (Median Effective Concentration – EC_{50}).

Among the different sub-lethal responses, the behavioural features have been used for decades as method for environmental monitoring (Cairns and Gruber, 1980; Kramer et al. 1989; Diamond et al. 1990; Gerhardt et al. 1998; Van der Schalie et al. 2001). In addition, aquatic vertebrate and invertebrate behavior such as predator-prey interactions, avoidance, and spatial orientation have been shown to be impacted by chemicals at sub-lethal concentrations and, for that, have a great potential as ecologically relevant end-points for contributing in ecological risk assessment especially in the weight of evidence approach (Dodson and Hanazato, 1995; Boyd et al. 2002; Gerhardt, 2007; Stanley et al. 2007; Berninger et al. 2011; Valenti et al. 2009; Regoli et al. 2014). Behavior is the result of the cumulative interactions of different biotic and abiotic factors and represents the major mechanism by which organisms, by its modulation, adapt to changes in their environment, including exposure to contaminants.

1.3.3.1 Swimming behavior in marine ecotoxicology.

Among the different behavioral features, the ability to move is an essential component of an organism's fitness. An organism's speed determines the rate at which it encounters objects within its environment, including food and predators (Visser and Kiorboe, 2006). Thus, any factor influencing an organism's ability to swim through its environment may have strong ecological implications.

In particular, there are four major areas in which swimming behavior is an important mechanism (Dodson et al. 1997): (i) swimming behavior is the underlying mechanism for population level behavior such as horizontal and vertical migration; (ii) individual behavior affects the outcome of predator-prey interactions, especially in the pelagic environment, where prey movement is important both as a cue to predators (Brewer and Coughlin, 1995) and a determinant of encounter rate (Gerritsen and Strickler, 1977), (iii) individual feeding rate may be linked to swimming behavior, (iv) toxic chemicals (natural or anthropogenic) affecting the swimming behavior can have indirect effects on the whole pelagic community (Dodson et al. 1995).

Swimming activity is a fundamental feature in many organisms and for that changes in the movement behavior can be used as a suitable indicator in ecotoxicological risk assessment (Tahedl and Header, 2001). Swimming alteration is one of the most frequently used responses in aquatic ecotoxicology and its evaluation has proved to be a valuable end-point in ecotoxicological studies with aquatic vertebrates (Little and Finger, 1990; Vogl et al. 1999; Beauvais et al. 2000; Gerhardt et al. 2005; Kane et al. 2004; Barbieri, 2007; Georgalas et al. 2007), invertebrates (Janssen et al. 1994; Charoy et al. 1995; Baillieul and Blust, 1999; Charoy and Janssen, 1999; Shimizu et al. 2002; Goto and Hiromi, 2003; Untersteiner et al. 2003; Gerhardt et al. 2005; Amsler et al. 2006; Faimali et al. 2002, 2006; Venkateswara Rao et al. 2007), and protists (Tahedl and Header, 2001).

1.3.3.2 Swimming behavior in marine invertebrates as ecotoxicological end-point.

In the literature subject to this section of the introduction that focuses on marine invertebrates (Table 1), over 50% of the studies use crustaceans to investigate swimming behavior and its alteration as toxicological response immediately followed by cnidarians (11%), echinoderms (7%) and mollusks (6%). Only a few papers have investigated swimming of annelids (4%) and rotifers (3%) (Faimali et al. 2017). All the literature analyzed showed that swimming alteration could be used as an ecologically relevant tool and a sensitive endpoint to assess and complement the study on the effects of contaminants on marine organisms. It is evident that this field of study has a strong technological component and, for that, before a more deep analysis of swimming alterations as a behavioral end-point, it is useful to move the attention on the existing system to analyze the swimming performance and how some of these have been applied in ecotoxicological investigations.

Also considering jellyfish, the swimming activity represents a fundamental feature to capture food, avoid predators an maintain orientation in the water column, hence the changes in the movement behaviour could be used as suitable indicator of a stress condition for this model organism. However, as reported in Table 2 only recently great attention has been devoted to cnidarians, and in particular to ephyra stage swimming behavior, and used in bioassays as ecotoxicological end-points (Costa et al. 2015; Faimali et al. 2014; Gambardella et al. 2015; Giussani et al. 2016). The last concern will be elucidated in the next section of this introduction.

1.3.4 Jellyfish as model organisms in ecotoxicological bioassays

Although Cnidarian jellyfish (Scyphozoans) are known to play an important role in marine food webs and are often conspicuous components in marine ecosystems, as widely discussed in the previous section of introduction, they are not yet employed in routine ecotoxicology (Table 2).

Indeed, as reported in Table 2, most toxicity tests were previously carried out on benthic cnidarians (e.g., hydras, colonial hydroids, hydrozoans, sea anemones, scleractinian corals), considering

budding, regeneration, gametogenesis, mucus production, and larval metamorphosis as end-points. However, in recent years, several investigations have been performed on different life stages of jellyfish, suggesting their potential use as model organisms to predict ecological effects of chemical and other stressors in the marine environment (Table 2).

In this regard, the high sensitivity of jellyfish to a wide range of stressors (i.e. chemical and organic compounds, nanomaterials, pesticides, crude oil compared to other marine invertebrates, i.e. algae, crustaceans, sea urchins, has been well documented.

Among the species of jellyfish, the scyphozoan genus *Aurelia* is used by several authors as model organism in ecotoxicology (Table 3). and has been used in different phases of its life cycle, displayed in Figure 1.3 of this thesis (polyps, ephyare stage and adults)

This species was proposed by Spangenberg (1984) as an ecotoxicological model for the first time in 1984 after a series of preliminary studies in the previous years on its metamorphosis and strobilation (Spangenberg , 1964, 1967, 1974; Spangemberg et al. 1980).

Spangenberg and co-authors developed a specific experimental protocol (*Aurelia* Metamorphosis Test System, AMTS) to highlight sublethal effects of hydrocarbons on morphological and behavioral end-points (rate of strobilation initiation, teratological malformations, statolith synthesis alteration, ephyra pulsing and swimming alteration) on both benthic and planktonic life stages. Several developmental, behavioural, and genetic effects caused by marine hydrocarbons could be detected with this system, which proved to be a good early warning system to detect subtle effects also for other marine pollutants.

Lucas and Horton, (2010) investigated different end-points related to polyp conditions: budding (including stolon development), strobilation (including constrictions), deformities (such as an unusually wide mouth and extruding, pale and inactive gastric cavity), and mortality, indicated by a complete loss of tentacles, mouth closing (or inability to close), decomposition. Results show that 200 μg Cu L^{-1} was the most harmful concentration to *Aurelia* sp. polyp, with 100% mortality before

the end of the 21-day experiment. The study also highlighted that silver had a greater impact on polyp condition than copper in low concentrations, suggesting an antagonistic relationship.

Exposure time seems to play an important role, highlighting lower reactions to metals for long lasting contact times between polyps and metals, with mortality and deformity values decreasing over time.

Most of the literature focused on the pelagic (ephyra) rather than on the adult stage of this species (Table 3). Recently, Almeda and co-authors, (2013), have investigated the effects of Louisiana light sweet crude oil spills on survival and PAHs bioaccumulation in *Aurelia* sp. and *Pelagia noctiluca*, highlighting the sensitivity of the larval stage compared to adult.

Table 1 Marine model invertebrates investigated for their behavioral responses (by Faimali et al. 2016).

Group	Organisms	Behavioral response	References
Porifera	Sponges	larval swimming	Abdul Wahab et al. 2011
Cnidarians	Jellyfish	larval frequency of pulsation, larval swimming	Conley and Uye, 2015; Costa et al. 2015a; Faimali et al. 2014; Gambardella et al. 2015; Giussani et al. 2016
	Corals	larval motility,	Kwok and Ang Jr., 2013; Martinez-Quintana et al. 2015; Reichelt-Bruschett and Harrison, 2004; Vermeij et al. 2006
Crustaceans	brine shrimps	larval swimming	Alyuruk et al. 2013; Anufriieva and Shadrin, 2014; Costa et al. 2015b; Gambardella et al. 2014; Garaventa et al. 2010; Huang et al. 2016; Kokkali et al. 2011; Manfra et al. 2016; Mesarič et al. 2015; Venkateswara Rao et al. 2007
	Barnacles	larval swimming, pre-settlement and settlement	Amsler et al. 2006; Costa et al. 2015b; Faimali et al. 2006; Gambardella et al. 2015; Mesarič et al. 2013; Piazza et al. 2014, 2016; Wu et al. 1997
	Copepods	Swimming	Bianco et al. 2013; Bradley et al. 2013; Cailleaud et al. 2011; Chen et al. 2012; Dur et al. 2011; Goetze and Kiørboe, 2008; Lenz et al. 2015; Mc Allen and Taylor, 2001; Michalec et al. 2013
	Decapods	larval and adult swimming	Cohen et al. 2015; Garcia de la Parra et al. 2006; Oliveira et al. 2012, 2013; Smith and Jensen, 2015; Unterstainer et al. 2005
	Amphipods	Swimming	Wallace and Estephan, 2004
	Mysids	Swimming	Roast et al. 2000a,b, 2001
Mollusks	Bivalves	larval swimming	Beiras and Widdows, 1995; Cragg, 1980; Hubbard and Reidenbach, 2015
Anellids	Polychaetes	Swimming	Behaulieu et al. 2015; Hansen et al. 2010; Ward et al. 2000
Rotifers	Rotifers	Swimming	Costa et al. 2015b; Garaventa et al. 2010
Echinoderms	sea urchins	sperm motility, larval swimming	Chan et al. 2011, 2015; Fabbrocini et al. 2010; Fabbrocini and D'Adamo, 2011; Gambardella et al. 2015; Morgana et al. 2016

In addition, also some of the most toxic crude oil PAHs had accumulated in these species, being potentially transferred up the food web with subsequent contamination of apex predators. Indeed, among cnidarians, jellyfish play a very important role in the trophic chain, since while they prey on vertebrates (i.e. fish larvae) and planktonic invertebrates, they are the main prey for some species of sea turtles, fish, and sea birds.Jellyfish accumulated PAHs (Almeda et al. 2013) and also other toxic compounds in their tissues, such as metals and heavy metals (Caurant et al. 1999; Mitchelmore et al. 2003) through uptake of dissolved compounds, diffusion, ingestion of food, or contact with suspended solids and sediments. The ephyrae stage of *Aurelia* sp. were used by Echols et al. (2015, 2016) in toxicity tests for evaluated lethal response to Macondo crude oils from the Deepwater Horizon (DWH) incident in the Gulf of Mexico (GOM), Corexit 9500, and oil-dispersant mixtures highlighting behavioral abnormalities on ephyrae exposed to chemical disperdant. Recently, Gadreaud et al. 2016; showed some alterations of the morphogenesis of ephyrae of *Aurelia sp.* in response to low concentrations of silver nanoparticle exposures.

1.3.4.1 Ephyrae stage of *Aurelia aurita* as model organism in ecotoxicological bioassay

The first to propose the ephyra stage of *Aurelia aurita* jellyfish (xxx) as a new biological model in ecotoxicological bioassays have been Faimali and coauthors in 2014 at laboratory of ISMAR-CNR, using an automated recording systems for evaluate the behavioral swimming responses in ephyrae exposed to environmental condition and contaminants.

This system called Swimming Behavour Recorded-SBR, is a a video camera-based system (Figure 6), coupled with an image analysis software, specially designed to track and analyse linear swimming speed of aquatic invertebrates. The experimental set up consists of a video camera with a macro-objective, recording the paths of a sample of larvae swimming in a small recording black box chamber monitored under infrared light.

Table 2. Ecotoxicological studies on cnidarians exposed to different chemicals and stressors. The only reference related to gelatinous zooplankton (jellyfish) is reported in grey (From Faimali et al. 2016 and other literatureinformation).

Cnidarians	Species	Ecotoxicological response	Chemicals and stressors	Ref.
Hydras	*Hydra viridissima*	Mortality, population growth	4-chlorophenol, hydrocarbons, metal	Pollino and Holdway, 1999
	Hydra vulgaris	Mortality, population growth, feeding, budding regeneration	4-chlorophenol, metals, drugs	Pollino and Holdway, 1999
	Hydra attenuata	Mortality, change in morphology, developmental toxicity	Pesticides, industrial effluents, heavy metals, antidepressants	Demetrio et al 2014, Blaise and Kusui, 1997 Minguez et al. 2014 Ronco et al. 2000
Colonial hydroids	*Hydractinia echinata*	Metamorphosis	Organic compounds	Chicu et al. 2000
	Campanularia flexuosa	Colony growth	Chemicals	Stebbing, 1981 Stebbing and Santiago Fandillo, 1983
	Eirene viridula	Morphological effects, colony growth	Heavy metals	Karbe, 1972
Hydrozoans	*Gonothyrea loveni*	retraction	Metals	Theede et al. 1972
Sea anemones	*Aiptasia pallida*	Biomarkers, histological changes on zooxanthellae	Biocides, metals	Brack and Bielmyer, 2013
	Nematostella vectensis	Mortality, change in weight, egg production, metamorphosis	Chemicals, nanocrystals	Mercier et al. 1997 Ambrosone et al. 2014
	Aiptasia pulchella	Mortality, immobilization, development, reproduction	Metals, effluents	Harter and Matthews, 2005 Howe et al. 2012 Howe et al. 2014a,2014b Howe et al. 205
Scleractinian corals	*Pocillopora damicornis*	Biomarkers	Chemicals	Downs et al. 2010
	Acropora millepora	Fertilisation, larval metamorphosis	Biocides, metals	Negri and Heyward, 2001
	Montipora capitata, Porites compressa	Growth, reproduction	Steroidal estrogens	Tarrant et al. 2004
	Stylophora pistillata	Mortality, settlement rate, morphological and behavioural deformation	Oil dispersants	Epstein et al. 2000
Jellyfish	*Aurelia sp.*	Mortality, immobility, frequency of pulsation	$Cd(NO_3)_2$, chemicals, organic compounds, surfactants, pesticides, nanomaterials, harmful algae	Almela et al. 2013 Faimali et al. 2014 Lucas and Horton, 2014 Gambardella et al. 2015 Costa et al. 2015 Giussani et al. 2016 Echols et al. 2016
		Metal accumulation	Metals	Templeman and Kingsford, 2015
	Cassiopea sp.	Metal accumulation	Metals	Templeman, 2012 Templeman and Kingsford, 2015
		Growth and Photosynthetic efficiency of zooxantelle	Herbicides	Rowen et al. 2017
	Pelagia noctiluca	Mortality	Organic compounds, crude oil	Almela et al. 2013

Swimming behavior is digitally recorded by a frame grabber plugged into a PC. Images are analysed through an advanced image processing software (SBR System developed by e-magine IT, Genoa, Italy) reconstructing individual path-tracks and measuring the average swimming speed (mm/s) for each sample (Faimali et al. 2006; Garaventa et al. 2010; Morgana et al. 2016). A specific software (Figure 6). for recording and analysing swimming behaviour of ephyrae has been duly adjusted to measure their Frequency of pulsations (Fp). In this work, experiments with ephyrae were performed using conventional multi-well plates with round-shaped wells (Figure 1.8) in order to remove refractive effects of infrared light and ensure that organisms are not mechanically damaged. Recording time was 1 min; the SBR may record both under light and dark conditions. Faimali and co-authors (Faimali et al. 2014) have conducted a series of sequential experiments in order to investigate the impact of different culturing and methodological parameters (temperature, photoperiod, ephyrae density and age) on behavioural end-point (frequency of pulsations) and standardized a protocol for organism maintenance and testing (Table 4).

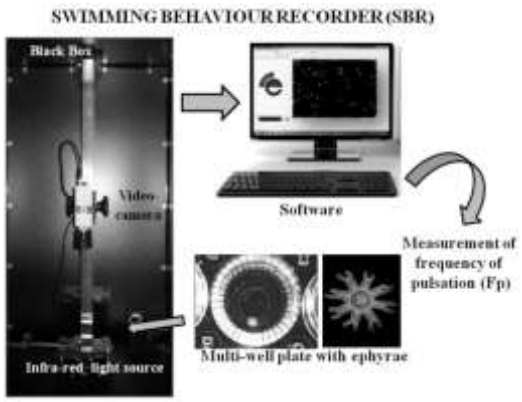

Figure 6. Swimming Behaviour Recorder (SBR) system for measuring *Aurelia aurita* ephyra Frequency of pulsations (Fp)

Table 3. Ecotoxicological studies on the different phases of *Aurelia* sp. life cycle exposed to pollutants and stressors (from Faimali et al. 2016 and other literature information).

Aurelia sp. life cycle		End-point	Pollutants and Stressors	Ref.
Benthic phase	Polyp	Metamorphosis	Hydrocarbons, Petroleum Oil	Spangengemberg , 1984
		Strobilation	Gentamycin	McAfee et al., 2015
		Budding, strobilation, deformities, mortality	Heavy metal, Silver, Copper	Negri and Heyward, 2001 Lucas and Horton, 2014
		Feeding response, survival	Interaction with toxic dinoflagellates	Huang et al. 2015
		Mortality, Polyps contraction	Harmful algae	Giussani et al. 2016
Pelagic phase	Ephyra	Motility	Glutamate	Spangenberg et al. 2004
		Motility	Gentamycin	McAfee et al., 2015
		Motility, growth	Feeding on toxic dinoflagellates *Alexandrium catenella*	Huang et al. 2013
		Mortality	Crude oil, PAH bioaccumulation	Almeda et al. 2013
		Immobility, Frequency of pulsation	Cadmium nitrate, SDS	Faimali et al. 2014
		Immobility, Frequency of pulsation	Eserine, CPF	Costa et al. 2015
		Immobility, Frequency of pulsation	Silver nanoparticles	Gambardella et al. 2015
		Immobility, Frequency of pulsation	Harmful algae	Giussani et al. 2016
		Morfological changes Mortality	PAH	Echols et al. 2016
	Adult	Molecular biomarkers, adaptation	Environmental parameters (temperature, oxygen)	Schroth et al. 2005
		Mortality	Cadmium chloride, potassium chloride, copper sulphate	Echols et al. 2015

In order to validate the new bioassay develop, they also exposed the organisms to two well known reference toxicants (Cadmium Nitrate and Sodium Dodecyl Sulfate, SDS) in order to compare the results to those found in the literature and obtained with other marine invertebrates exposed to the same toxicant.

Figure 7. Multy -well plate, where ephyrae were exposed by ecotoxicological test

Table 4. Test parameters to be used in the Toxicity test wit ephyrae of *Aurelia* sp. (Faimali et al. 2014)

Test parameters	
Density	2 organism for well
Recording light conditions	Dark
Photoperiod	24 h Dark
Ephyrae age	Not significant at 20°C
Temperature	20°C

The experiments allowed to identify two end-points (sub-lethal, frequency of pulsation and acute, immobilization) with different levels of sensitivity and to optimize the use of SBR system. In addition, the comparison of the EC_{50} values obtained exposing ephyrae jellyfish to reference toxic compounds highlighted that ephyrae could be an interesting and promising invertebrate model with a very high ecological relevance to be used in ecotoxicological research suggesting further investigation.

2. AIMS OF THE STUDY

The jellyfish occupy a key evolutionary position as basal metazoans and are ecologically important both as predators and preys in the aquatic ecosystems. However, they are not represented in routine ecotoxicology even though over the past few decades the importance of its role in marine ecosystems balance has become widely recognized. Beside, recently several study have demonstrated that chemical compounds can exert toxic effects on jellyfish, suggesting their potential use as model organisms to predict the ecological effects of contaminants and their strong potential as bioindicators to monitor and assess the health of marine ecosystem. Moreover, Faimali and co-author in 2014, at ISMAR-CNR have developed a new toxicity bioassay using ephyrae of *Aurelia aurita* highlighting their potential use in the ecotoxicological studies.

However, the knowledge of the direct biological effect of contaminants on these gelatinous organisms and the relation between jellyfish and pollution in marine ecosystem is lacking. In this scenario, a better investigation could be provide a basis not only for understanding the population jellyfish dynamics but in the some way for exploring the wider impact of pollution in marine ecosystem.

The overall aim of this PhD thesis was to assess the potential of ephyrae jellyfish as biological models in ecotoxicology exposing this organisms to a wide toxic compounds such as metals, neurotoxic pesticides, natural organic molecules and emerging contaminants, and investigating on their swimming behavior as suitable indicators of a stress condition in marine ecosystem.

The experimental activities performed to reach the goals of the PhD thesis were:

- **Experimental activities on *Aurelia* sp.**

to the effects of neurotoxic compounds (Eserine and Chlorpyriphos) and the harmful dinoflagellate (*Ostreopsis cf. avata*) on model organism proposed by Faimali et al. (2014), ephyrae of *Aurelia* sp.

- **Experimental activity on *S. malayensis***

to investigate on ephyrae of *Sanderia malayensis* to use as new model organism in ecotoxicology

- **Experimental activity on *Aurelia* sp. *and S. malayensis:* ecotoxicological comparison with referenc toxic compounds.**

to validate the bioassay with ephyrae jellyfish of *S.malayensis* and compare the results with *Aurelia* sp. by two different methods of exposition to contaminants.

- **Experimental activity on *Aurelia* sp. *and S. malayensis:* ecotoxicological comparison with emerging contaminants**

to 24valuate the effect of emerging contaminants, like microplastics on *Aurelia* sp. and *S.malayensis* ephyrae by different methods of exposition.

- **Experimental activity on *Aurelia* sp. *and S. malayensis:* trophic transfer of contaminants**

to assess the effect of trophic transfer of contaminants on ephyrae jellyfish (*Aurelia* sp. and *S.malayensis*) through a development of simplified marine food webs.

In addition, the results of the first part of this PhD thesis, were used to investigate the possibility to suggest the ephyrae jellyfish as model organism to quality sea water assessment in private and public aquarium. The experimental activity performed to aim this goal were carried out in collaboration of Acquario di Genova.

- ***Aurelia* sp. and *S.malayensis* model organism for rapid assessment of seawater: A case study at Acquario di Genova (Italy)**

3. MATERIAL AND METHODS

3.1 Experimental activities on *Aurelia* sp.

In the first part of this PhD thesis the ecotoxicological bioassay proposed by Faimali et al. (2014) were used to evaluate the effects of neurotoxic compounds (Eserine and Chlorpyriphos) and natural organic molecules (*Ostreopsis* cf. *Ovate)* on the biological model *Aurelia* sp.

3.1.1 Model organism : *Aurelia* sp.

The moon jellyfish *Aurelia* was represented for decades by two different species (Kramp, 1961; Russell, 1970): the cosmopolitan *Aurelia aurita* (Linnaeus, 1758) with broad temperature and salinity tolerance (Kramp, 1961; Russell, 1970) and the cold-water boreal *Aurelia limbata* (Brandt, 1835). However, recently Scorrano et al. 2016, by morphological and molecular analyses indentified three species in the Mediterranean Sea. Two clades of *Aurelia* that can be referred to exting valid species: *Aurelia coerula* (von Lendenfeld, 1884) and *Aurelia solida* (Browne, 1905) and a third clade described as a new species *Aurelia reclicta* sp. nov. (Scorrano et al. 2016).

The ephyrae of jellyfish *Aurelia* (Figure 8) selected as model organism in this first experimental activity and in the previous studies reported by Faimali et al. 2014 (see section 1.3.4.1) arrived from Acquario di Genova. Here, there are collected in separate tanks 15 differents types species of *Aurelia aurita* polyps arrived from other Italian and European Aquariums. Among them, in this thesis was selected the polyps of *Aurelia aurita* that allowed to provide in planned way a large number of ephyrae by strobilation needed for all ecotoxicological tests performed.

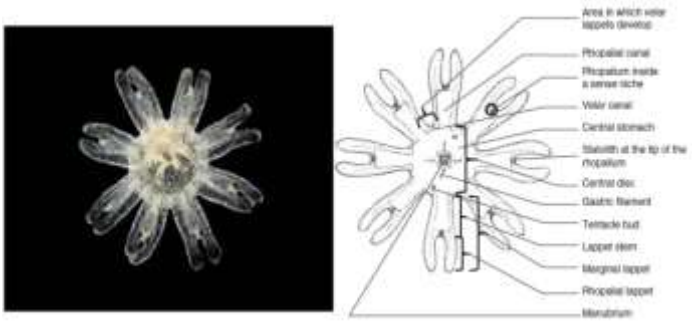

Figure 8. Ephyrae of *Aurelia* sp.

However, considering the recently (Scorrano et al. 2016) a revision of the species used in this thesis is requested. Among the diagnostic characthers observed on some organism used for the experiments , the meausures of bell diameter, and lappets proportion are comparable with those *Aurelia solida* (Browne, 1905) in according to Scorrano et al. (2016). However, an integrative molecular investigation to confirm this pathway is needed. Thus the term of *Aurelia* sp. was used in this thesis given this recent revision of the *Aurelia* genus.

3.1.2 Obtainement of ephyrae of *Aurelia* sp.

For this study some colonies of polyps attached on PVC tubes were received from the laboratories of the "Acquario di Genova, Costa Edutainment S.p.A.", and transported to CNR-ISMAR and placed in the thermostatic room at 20 °C in 1.5 L dark plastic tanks, covered with a lid in order to keep polyps in dark conditions. Tanks were filled with FNSW (37‰ salinity) and gently aerated.

Polyps were fed daily with nauplii of *Artemia salina* (about 40 nauplii mL^{-1}) and seawater was changed every two days.

Strobilation was induced by thermic shock: PVC tubes with polyps were moved into 1.5 L dark plastic tanks filled with FNSW at 10°C. During this period, in order to reduce stress, polyps were not fed and seawater was not changed. Once released by strobilation, ephyrae were poured into a

beaker and immediately used for the assay or bred by keeping them in a 6 L plastic tank filled with gently aerated FNSW (salinity 32‰) at 20 °C (photoperiod 12:12 light:dark).

3.1.3 End-point evaluated

In this experimental activity an acute and sub-lethal end-points were evaluated,(Faimali et al. 2014) The acute end-point consisted in the evaluation of the organism ability to perform any kind of movements (immobilization), defined as % of Immobilization (% I), while the sub-lethal end-point was measured as the number of pulsations (Frequency of pulsation, Fp) made by the ephyra.

The number of pulsations made by each ephyra were used to obtained the percentage of Alteration of Frequency pulsation according to the following formulae:

$$\%AFp= [(Fptreated-Fpcontrol)/Fpcontrol]/100;$$

where, Fp treated and Fp control are the number of pulsation counted in ephyrae fed with prey contaminated and no contaminated respectively.

Both end-points were measured within a defined time-unit (one minute) and evaluated using an automatic recording system coupled with a specifically designed video graphics analyser (Swimming Behavioural Recorded – SBR; Faimali et al.2014). The SBR developed at ISMAR-CNR to track and analyse linear swimming speed of aquatic invertebrates (Faimali et al.2006; Garaventa et al.2010) and optimized to recorded the Frequency pulsation in ephyrae as described in the introduction of this thesis (see paragraph 1.3.4.1).

3.1.4 Neurotoxic compounds

In this experiment were tested a carbamate pesticide as Eserine (physostigmine, ES) and a broad-spectrum organophosphorothioate insecticide as Chlorpyrifos (CPF). The solution of CPF was prepared in Dimethyl sulfoxide_DMSO 0.01% (v/v); this concentration of DMSO did not show toxic effects for ephyrae of *Aurelia sp.*, in agreement with that reported by Baharaona et al.(1994) for other marine organisms exposed to this organic solvent. Eserine stock solution (1 mg mL^{-1}) was prepared in 0.22 µm of filtered natural sea water (FNSW, 37‰ salinity). All test solutions were prepared in 0.22 µm FNSW at 37‰ at increasing concentrations (0-0.1-0.5-1-5-10-50 mg L^{-1}) of both toxic compounds and for CPF was included a DMSO negative control.

3.1.4.1 Bioassays

In this experiment in agreement with that reported by Faimali et al. (2014) were evaluated one acute and one sub-lethal end-point, in order to verify the neurotoxicity of ES and CPF on *Aurelia sp.*ephyrae.

Toxicity tests were prepared using ephyrae collected immediately after strobilation (0 days old ephyrae) and organisms were placed individually into a multi-well plate containing 2 mL of test solution (one individual for each well). For each concentration, three replicates were prepared, each replicate consisting of 8 wells containing one ephyra. Plates were then sealed and kept in the thermostatic room at 20 °C in dark conditions. After 24 and 48h the acute and sub-lethal end-points were evaluated as described in section 3.1.3 by SBR (see section 1.3.4.1 of introduction).

3.1.4.2 Morphological observations

Morphological changes were observed in order to establish a general ranking of stress caused by neurotoxic compounds in ephyrae of *Aurelia* sp. After 48 h of exposure, the organism in the control and treatment (concentration of ES and CPF) were removed from multi-well plate and washed with fresh FNSW three time to remove the toxic compound and were fixed in 4% paraformaldhehyde

solution and observed under a LEICA optical microscope. Image were acquired using a DFC420C Leica CCD camera and Leica software. The stress parameters considered in this study to evaluate the morphological changes caused by neurotoxic compounds follow the categories proposed by Echols et al. (2016).

3.1.5 Ostreopsis cf.avata strains

In this experiment were tested on ephyrae of *Aurelia* sp. the effect of harmful dinoflagelalte *Ostreopsis cf. ovate*. Dinoflagellate strains (CBA29 strain), were isolated from natural samples collected in Quarto dei Mille (Ligurian Sea) from the NW Mediterranean Sea. Microalgae were maintained into polystyrene flasks (50 mL for stock cultures, 200 mL for experiments) with transpiring caps, containing f/2 enriched seawater medium (Guillard, 1962) made on GF/F filtered and sterilized seawater. The stock and experimental cultures were kept under identical environmental conditions, although the settings were slightly different in the two laboratories. Experiments were conducted at 20± 0.5 °C, 16:8 light:dark (L:D hours) cycle and 85–135 μmol light intensity.

Periodic examination of the stock and experimental cultures was performed by visual inspection of the flasks and estimation of the cell concentrations using Sedgewick-Rafter chambers and inverted microscope. Aliquots of *O*. cf. *ovata* experimental cultures, collected during stationary phase, were used for ecotoxicology bioassays since toxin intracellular concentrations reach the highest values in that physiological state (Guerrini et al. 2010; Giussani et al. 2015). Chemical analyses were performed in according to Ciminiello et al. 2010 and 2011.

3.1.5.1 Bioassay

Ecotoxicological tests were performed according to Faimali et. al (2014) and as described in the introduction of this thesis (see sections 1.3.4.1 of the introduction).

In detail, ephyrae of *Aurelia* sp. were exposed to the different concentrations of *O.* cf. *ovata* strain culture (namely, 5, 10, 50, 100 and 500 cells mL-1) covering a wide range of densities, corresponding to both usual abundances and large blooms which can occur during the summer season in Mediterranean coastal areas. For each level of concentration of O. cf. *ovata*, 24 ephyrae were placed individually (one ephyra per well) into a polystyrene multi-well plate (24 well per plate each with a capacity of 2 ml volume) to avoid interactions among organisms (Faimali et al. 2014), and 2 mL of algal culture were then added to each well.

A treatment control was set up with 24 wells, each containing a single ephyra and 2 mL of filtered seawater. A total of 144 ephyrae were used, over a total of six multi-well plates. Plates were then sealed (to avoid evaporation) and kept in a thermostatic room at 20 ± 0.5 °C with a 16:8 (L:D) photoperiod. After 24 and 48 hours of exposure, acute and sub-lethal end-points, Immobility and Frequency pulsation respectively were evaluated by SBR (Faimali et al. 2014) as described in section 3.1.3.

3.1.6 Data processing and statistical analysis

The median Effective Concentrations (EC_{50}_ the concentration of compound resulting in 50% Immobility or Alteration of Frequency pulsation effect in the exposed ephyrae after 24 and 48 h) on % I and %AFp and related 95% Confidence Limits (CL) were calculated using Trimmed Spearman–Karber analysis (Finney, 1978) after 24 and 48 h.

Significant differences between controls and treated samples were determined using one-way analysis of variance (ANOVA) followed by Tukey test. When data failed to meet the assumption of normality, non parametric Kruskal Wallis test and Mann Whitney test were used to compare individual treatments. For AFp statistical analysis were performed using Frequency pulsation data.

The Lowest Observed Effect Concentration for the percentage of Immobility ($LOEC_I$) and for the percentage of Alteration of Frequency of pulsation ($LOEC_{AFp}$ $p < 0.05$) were deducted by ANOVA

results. Data were considered significantly different when $p < 0.05$. SPSS statistical software (Statistical Package for the Social Sciences, Version 20) was used for data analysis.

3.2 Experimental activities on *Sanderia malayensis*

In this section in order to expand the knowledge about the using of jellyfish as model organism in ecotoxicology, was investigated on ephyrae of S*anderia malayensis.* Experimental activities were performed to investigate the effects of environemental and methodological parameters on Frequency pulsation in order to define the protocol testing with this species of jellyfish.

3.2.1 Model organism *: S. malayensis*

The scyphozoan *Sanderia malayensis* (Goette, 1886) belongs to the family Pelagidae displayed a different and narrower geographical area compared to the widespread species of *Aurelia* sp., indeed records of this tropical species were reported from Indo-Pacific, Japan, Suez-Canal, South China Sea and Malay Archipelago (Straehler-Pohl and Jarms, 2010). Recently new records were reported also from Pakistan (Gul and Morandini, 2013; Gul and Morandini, 2015; Morandini and Gul, 2016). However, *S. malayensis* shows a reproduction strategy similar to *Aurelia* sp. (Schiariti et al. 2014). exhibiting an high reproduction rate under laboratory condition and showing a wide spectrum of asexual reproduction (polyp stage) as reported by Adler and Jarms (2009). All these findings have been take into account in this study to select this species in order to investigate its potential as model organism in ecotoxicology.

3.2.2 Obtainement of ephyrae of *S. malayensis*

Ephyrae of *S. malayensis* (Figure 9) were released from the stock culture population of the Acquario di Genova laboratory. They were reared on small pieces of PVC tubes into a darkened tank of 2,5L of natural seawater with an airstone. The chemical – physical parameters were:

temperature 24±1°C, salinity 31±1 ‰, ammonium less than 0,05 mg*L-1^{-1}, nitrite less than 0,05 mg*L-1^{-1} , nitrates less than 10 mg*L-1^{-1} and pH 8-8,2. Polyps were fed one time per day with enriched *Artemia salina* nauplii. One time per week the tank was cleaned and there was a 90 % of water change.

Figure 9: Ephyrae stage of *S.malayensis.* 13-16 lappet stems, 26-32 bread knife-life shopalial lappets, 1-2 gastric filaments per socket, no marginal tentacles or tentacle bulbs, long manubrium (Straehler-Pohl and jarms, 2010).

In order to trigger the strobilation phase the polyps were moved into another darkened incubator tank of 2,5L at 18 °C with air and the ame chemical-physical conditions. During this phase polyps were fed 3 times for week,there wasn't change of water and no control of salinity increase due to evaporation. Usually strobilation happened after 2-3-weeks. Once released by strobilation ephyrae were poured into a beaker and immediately used for the assay (ephyrae 0 days old).

3.2.3 Methodological parameters investigated to define the protocol testing with ephyrae of *S. malayensis*

This experimental activity, aimed to assess the influence of different methodological parameters like temperature (*Experiment 1*), volume (*Experiment 2*) and photoperiod of exposition (*Experiment 3*) on ephyrae swimming performance (Frequency of pulsation, Fp), after 24 hours of exposition in natural filtered (0,22 µm) seawater FNSW in order to standardize the protocol testing. To identify

the optimal parameters, these bioassays were perfomed in sequence, for that the informations obtained from results of the first one were apllied to the second third.

All test were carried out using ephyrae collected immediately after strobilation (0 days) according to Faimali et al. 2014 *Experiment 1- Volume of exposition.* The organisms, placed individually (one individual for each well) into a mult-well plates since, previous study on ephyrae jellyfish highlighted how the Frequency of pulsation decreases proportionally to the increase of number of ephyrae in the well (Faimali et al. 2014). In this study, considering that ephyrae of *S.malayensis* resulted to be bigger than *Aurelia* sp., was evaluated the influence of volume on Frequency pulsation exposing the organism in 2 ml (Faimali et al. 2014) 5ml and 10ml of FNSW (0,22 µm and 37‰) for 24 hours.

Experiment 2- Temperature. The ephyrae were placed individually in each well containing 2 ml of FNSW (0,22 µm and 37‰) and the plate covered with 1 layer of transparent film to prevent evaporation were kept in different incubators at four different temperatures (10°, 20°, 25° and 30°C) for 24 hours.

Experiment 3- Photoperiod of exposition. In the last experiment the ephyrae were placed individually in each well containing 2 ml of FNSW and the plates were kept in the thermostatic room at 20°C for 24 hours in different photoperiods: totally dark (24 h D), 12 h light:12 h dark (12 h L: 12 h D) and totally light (24 h L).

For all experiments 3 replicates with 8 ephyrae were performed and after 24 hours for each ephyra the Freuency pulsation was recorded in dark conditions by SBR according to Faimali et al. (2014) The results of this experiment, have led to the development of a protocol to use the ephyra stage of *S. malayensis* as model organism in ecotoxicological bioassays (Table 10).

3.3 Experimental activities on *Aurelia* sp. and *S.malayensis* : ecotoxicological comparison with refence toxic compounds

The experimental activity aimed to compare the sensitivity of a new model proposed, ephyrae of *S.malayensis* with *Aurelia* sp. The comparison was performed by exposition of ephyrae to reference toxic compounds following the protocol for toxicity test reported by Faimali et al. 2014 for *Aurelia* sp. and defined in this thesis for *S. malayensis* (see section 3.2 of Material and methods) and a new bioassay exposing the organisms in a semi-dynamic exposition. Conventionally the toxicity test in static condition and the new bioassay in semi-dynamic exposition were called Ephyra Test_ET and Ephyra Test in semi-dynamic_ETsd respectively.

3.3.1 Ephyra Test_ET

3.3.1.1 Reference toxic compounds

The selected reference toxic compounds were Cadmium Nitrate $(CdNO_3)_2$ and Sodium Dodecyl Sulphate (SDS).

SDS ($CH_3(CH_2)_{11}OSO_3Na$) is an anionic surfactant capable of emulsifying lipids and low-surface tension aqueous solutions, SDS was purchased from Sigma Aldrich (St. Louis MO).

Cadmium Nitrate Cd(NO3)2 was purchased from Sigma Aldrich (St.Louis, MO). It is commercially available in a 2% HNO_3 standard solution for AA at a concentration of 1000 ppm. Stock solutions of SDS and Cadmium Nitrate were prepared in ASW (Artificial Sea Water, Instant Ocean_, 37&). Tested concentrations were: 0 (Ctr) e 0.01- 0.05- 0.1- 0.5- 1- 5 $mg*L^{-1}$ for $Cd(NO_3)_2$ and 0 (Ctr) e 0.1- 0.5- 1--5-10- 50 $mg*L^{-1}$ for SDS.

3.3.1.2. Bioassays

After the first experimental phase (described in section 3.2), where test parameters of the toxicity assay were defined, a bioassay using the two different reference toxicants (Cadmium nitrate and SDS) has been performed in order to assess the possibility of using ephyrae of *S.malayensis* as a proxy in ecotoxicological testing. Experimental parameters adopted in this phase were established from results obtained in the first part of the study (described in 3.2) and other in according to Faimali et al. 2014 (see sections 1.3.4.1 of the introduction) .

In detail ephyrae of 0 days old, were placed individually into a multi-well plate containing 2 ml of test solution (one individual for each well). For each concentrations of toxic selected, 3 replicates with 8 ephyrae (one ephyrae in each well) were prepared (Figure 10)

Plates were covered with 1 layer of transparent film to prevent evaporation and were kept in the thermostatic room at 20 °C in dark conditions, after 24 and 48 h both the acute end-point and the sub-lethal one were evaluated by SBR (Faimali et al. 2014) as described in section 3.1.3 for *Aurelia* sp. At the same time was perfomed a bioassay with ephyrae of *Aurelia* sp. following the parameters reported by Faimali et al. 2014 (Table 10)

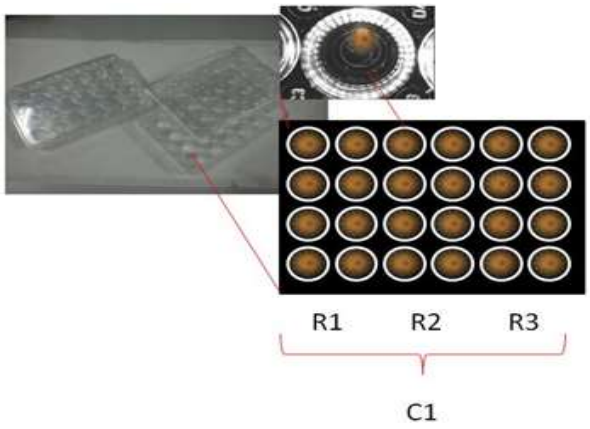

Figure 10 Multy-well plate used to expose the ephyrae (Ephyra Test_ET). One ephyra for each well, 8 ephyrae for each replica (R1-R2-R3), 24 ephyrae for each concentration (C1) stage of *S.malayensis.*

3.3.2 Ephyra Test in semi-dynamic exposition_ ETsd

At the same temperature and photoperiod conditions of Ephyra Test (20°C and total dark condition) for both species of jellyfish 10 ephyrae (in order to maintain the same number of ephyrae per replica used in the static exposition,8 ephyrae for replica; see section 3.3.1.2) were placed in beakers with 100 ml of volume, with an aeration system in order to have an excellent current balance for the organism (Figure 11 and Table 12).

Changes to current setting should be one of the first thing to check when some problem in the jellyfish system occurred. To balance the current, the ephyrae were not feed for twenty-four hours beforehand in according to Widmer, 2008, since unfed jellies extend their tentacles and oral arm (lappets in ephyrae), whereas the organism that are eating retract them, affecting their placement in the tank.

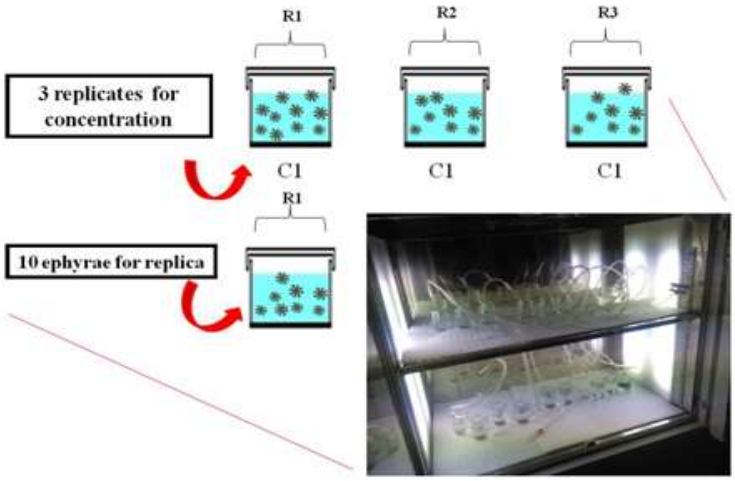

Figure 11 Experimental design to perfom the Ephyra Test in semi-dynamic condition with ephyrae of *Aurelia* sp. and *S.malayensis* The test consisted in 10 ephyrae for each replica R1 (beaker) and 30 ephyrae (3 replica) for concentration (C1).

For each concentrations (0 - 0.01- 0.05- 0.1- 0.5- 1- 5 $mg*L^{-1}$) of $Cd(NO_3)_2$ 3 replicates (one beaker with 10 ephyrae for replica) were prepared. A control was performed using NSW (salinity

37‰) filtered 0,22 µm. The experiments were performed in the thermostatic room at 20 °C in dark conditions (Table 12) . Bioassays were repeated three times.

3.3.2.1 Behavioural end-points investigated

After 24 and 48 hours of exposition, for each concentration of Cadmium nitrate tested the ephyrae were individually transferred from beaker to multi-well plates, placing one organism for each well in 2ml of the same concentration of exposition (see section 3.1.3). In detail, the aeration was stopped and each ephyrae was prelevated with 2ml of volume from beaker and placed in the well. The multi-well plates were used to evaluate the acute and the sub-lethal end-points (Immobility and Frequency of pulsation) using the SBR as described in session 3.1.3, since it was not possible to observed the pulsation directly in the beakers. Data of Immobility and Frequency pulsation obtained from the three repetition of the bioassay were used to evaluate the EC_{50} values.

3.3.2.2 Data processing and statistical analysis

The EC_{50} values from Immobility and Alteration of Frequency pulsation and related 95% Confidence Limits (CL) were calculated using Trimmed Spearman–Karber analysis (Finney, 1978) after 24 and 48 h. Significant differences between controls and treated samples were determined using one-way analysis of variance (ANOVA) followed by Tukey test. When data failed to meet the assumption of normality, non parametric Kruskal Wallis test and Mann Whitney test were used to compare individual treatments.

For AFp statistical analysis were performed using Frequency pulsation data.The LOEC values for Immobility and Alteration of Frequency of pulsation were deducted by ANOVA results. Data were

considered significantly different when $p<0.05$. SPSS statistical software (Statistical Package for the Social Sciences, Version 20) was used for data analysis.

3.4 Experimental activities on *Aurelia* sp. and *S.malayensis* : ecotoxicological comparison with emerging compounds

The purpouse of this experimental activity was to carried out the boassays in static (ET) and semi-dynamic condition (ETsd) proposed in the previous experiment (sections 3.2 and 3.3) in order to investigate the effect of emerging contaminants like microplastics on *S.malayensis* and *Aurelia* sp. ephyrae. An exhaustive investigation was performed to evaluate the sub-lethal effects (Immobility and Frequency of pulsation) and ingestion or accumulation of MPs of polyethylene on ephyrae of *S. malayensis* and *Aurelia* sp. In detail microparticles of different size ranges were used in this study: i) MPs of polyethylene 1-4 µm size (clear and fluorescent green microspheres); ii) LDPE (Low density polyethylene) microplastic of 4-6 µm size. In addition in order to investigate the role of microplastics as vector of contaminants in marine ecosystem, either isolated or in combination, further experiments were performed using LDPE 4-6 µm size spiked with Oxybenzone (BP-3) at high and low concentration.

3.4.1 Behavioral response investigated

Also in this experimental activity as described in previous, the Immobility and Frequency pulsation were the end-points evaluated after 24 and 48h of exposition by both ET and ETsd. For each concentration besides for the control, the average Fp was calculated, and the % alteration of Fp (% AFp) was derived for each concentration against the Control. Both end-points were evaluated by SBR for both method of exposition. (see sections 3.1.3)

3.4.2 Materials and chemicals

The toxic compounds and microplastic particles used in this study were described below and reported in Table 5.

3.4.2.1 Reference toxic compounds

Cadmium Nitrate $(CdNO_3)_2$: was selected as reference toxic compound for *Aurelia* sp. and *S.maalayensis* ephyrae (see 3.2 and 3.3). It was purchased from Sigma Aldrich (St.Louis, MO). It is commercially available in a 2% HNO_3 standard solution for AA at a concentration of 1000 ppm. Stock solutions of Cadmium Nitrate were prepared in ASW (Artificial Sea Water, Instant Ocean_, 37&). Tested concentrations were: 0 (Ctr) e 0.01- 0.05- 0.1- 0.5- 1- 5 $mg*L^{-1}$.

Oxybenzone Waterborne BP-3: was supplied by Aldrich (Milwaukee, WI, USA) and Merck (Darmstadt, Germany). Oxybenzone or benzophenone-3 (BP3); which formula was displayed in Figure 8.1 is an organic compound and belongs to the class of aromatic ketones known as benzophenones. It used in sunscreens and cosmetic and plastics additive (SCCP, 2008) because they help prevent potential damage to sunlight. As a sunscreen, it provides broad-spectrum ultraviolet coverage, including UVB and short-range UVA rays. As a photoprotective agent, it has an absorption profile ranging from 270 to 350 nm with absorption peaks at 288 and 350 nm. A stock solution of BP-3 was prepared by dilution in DMSO, and diluted in distilled water and in 0.22 µm natural ultra-filtered sea water (NFSW, 37‰ salinity, 7 pH). The final concentration of DMSO never exceeded the NOEC previously found for this chemical for ephyrae jellyfish. Tested concentration were : 0 (ctr)-0.5-1-2.5-5-10 $mg*L^{-1}$.

3.4.2.2 Microplastics

MPs of polyethylene 1-4 µm: were commercially available polyethylene spherical particles, provided by Cospheric industry. The Mps used in this study were supplied in solid form as particles of size 1-4 µm, transparent and green capable of emitting a green fluorescence that were used for documenting particle ingestion in ephyrae jellyfish.

LDPE 4-6 µm: Low density polyethylene (LDPE) microplastics of 4-6 µm were also tested. In this case the Mps were provided by one of the partners Ephemare project (University of Orebro, Sweden), as virgin particles (uncontaminated) and spiked with BP-3. In this case were selected two concentration of Waterborne BP-3 spiked MP (100ng/g and 10ng/g), according to literature data (Blüthgen et al. 2012; Fent et al. 2010; Paredes et al. 2014), concentration most likely showing effects (hereafter termed 'high' level), and a more environmental relevant concentration ('low' level) (Table 5).

Table 5: Toxic compounds and microplastics used in this study

Microplastics and toxic compounds	**Diameter**	**Density**	**Colour**	**Tested concentrations/dilutions**
Cadmium nitrate	-	-	Transparent (not solid)	0-0.01-0.05-0.1-0.5-1-5 $mg*L^{-1}$
Waterborne BP-3	-	-	White powder	0-0.5-1-2.5-5-10 $mg*L^{-1}$
MP	1-4 µm	0.96 $g*cm^{-3}$	White/Green	0-0.01-0.1-1-10 $mg*L^{-1}$
LDPE	4-6 µm	-1 $g*cm^{-3}$	White	0-0.01-0.1-1-10 $mg*L^{-1}$
LDPE + BP-3 Low concentration	4-6 µm	-1 $g*cm^{-3}$	White	0-0.01-0.1-1-10 $mg*L^{-1}$
LDPE + BP-3 High concentration	4-6 µm	-1 $g*cm^{-3}$	White	0-0.01-0.1-1-10 $mg*L^{-1}$

3.4.3 Bioassays

3.4.3.1 Static condition (ET)

Ecotoxicological tests were performed in static condition as described in 3.3 of Material and Methods. Ephyrae of *S.malayensis* and *Aurelia* sp. were individually (one individual for each well)

placed into a multi-well plate containing 2 ml of test solution, 3 replicates with 8 ephyrae (one ephyrae in each well) for each concentrations of reference toxic compounds and microplasic dilutions , were prepared. For all test the controls were performed using FNSW (salinity 37‰) filtered 0,22 µm. Plates were kept in the thermostatic room at 20 °C in dark conditions for 24 and 48 hours.

3.4.3.2 Semi-dynamic condition (ETsd)

Ecotoxicological tests in semi-dynamic exposition were performed following the protocol set-up defined in this thesis and described in section 3.3 of Material and Methods. The organisms were exposed into a beaker (10 individual for each beaker); containing 100ml of test solution gently aerated, 3 replicates were prepared for each concentration of reference toxic compounds and microplastic dilutions were prepared. For all test the controls were performed using FNSW (salinity 37‰) filtered 0,22 µm. All experiments were performed in the thermostatic room at 20 °C in dark conditions.

3.4.3.3 End-points evaluated

After 24 and 48 hours of exposition for both species and bioassays (ET and ETsd) the acute and the sub-lethal end-point like Immobility and Alteration of Frequency of pulsation were evaluated by SBR (Faimali et al. 2014) as described in the previous esperiments of this thesis. (see section 3.1.3)

3.4.3.4 MPs accumulation

Mps accumulation was evaluated using the fluorescently green micropheres 1-4 µm of polyethylene (Table 5). After 24 and 48 h of exposition, the organisms were removed from the test solution and washed with fresh FSW four times to remove MP bound to the gelatinous body.

This is according to Nasser and Lynch (2015), in order to assess only ingested particles by ephyrae. The organism were fixed in 4% paraformaldhehyde solution in phosphate-buffered saline (PBS, pH 7.4) and observed under a Olympus BX61 epi-fluorescence microscope.

3.4.4 Data processing and statistic analysis

The EC_{50} values from Immobility and Alteration of Frequency pulsation and related 95% Confidence Limits (CL) were calculated using Trimmed Spearman–Karber analysis (Finney, 1978) after 24 and 48 h. Significant differences between controls and treated samples were determined using one-way analysis of variance (ANOVA) followed by Tukey test. When data failed to meet the assumption of normality, non parametric Kruskal Wallis test and Mann Whitney test were used to compare individual treatments. For AFp statistical analysis were performed using Frequency pulsation data. The LOEC values for Immobility and Alteration of Frequency of pulsation were deducted by ANOVA results. Data were considered significantly different when $p<0.05$. SPSS statistical software (Statistical Package for the Social Sciences, Version 20) was used for data analysis.

3.5 Experimental activities on *Aurelia* sp. and *S.malayensis* : trophic transfer of contaminants

In the experimental activity the jellyfish species proposed in this thesis *Aurelia sp.* and *S. malayensis* and the bioassay in semi-dynamic exposition, were used to perform simplified food chains by experimental feeding of gelatinous zooplankton on crustaceans. The “ingestion rate” and “predatory performance methods” were used to define the optimal prey/predator (ephyrae/nauplii) ratio and to evaluate the effect of Cadmium nitrate by feeding treatment of ephyrae with *Artemia* nauplii contaminated.

In addition, beside the behavioural end-point proposed like Immobility and Frequency pulsation, biometrics and bioenergetics parameters (Disch diameter, Ash-Free Dry Weight_AFDW and Gross Growth Efficiency_GGE%) were used to assess the "Cadmium enriched-diet" effects on ephyrae jellyfish.

3.5.1 Predators organisms for food chain

Ephyrae of *S. malayensis* and *Aurelia* sp. were used in this study, as predators to perform an experimental simplified food chain in laboratory. This organisms arrived from polyp cultures reared at Acquario di Genova as described in previous section of the Material and Methods (see paragraph 3.1.2 for *Aurelia* sp. and section 3.2.2 for *S. malayensis*). Once released by strobilation, ephyrae no-fed were transferred at laboratory of ISMAR-CNR where poured into a tank with FSNW in a thermostatic room at 20°±0,5 and immediately (unfed) used to perform the experimental feeding (ephyrae 0 days old).

3.5.2 Prey organisms for food chain

The ephyrae jellyfish were fed with different concentration (number organisms) of brine shrimp nauplii, *Artemia* sp. 100-200 µm size (Figure 12). Prey organisms were obtained by incubating 500mg of cysts for 24h at 28°C under light source (3000-4000lx) and continuous aeration of the cyst suspension in seawater (37‰ salinity). The newly hatched larvae (Instar I stage), were separated from hatched cysts based on their phototaxis and then transferred with a Pasteur pipette into a beaker containing 0.22 µm FSNW.

Figure 12 *Artemia* sp. nauplii used in this thesis as prey food

3.5.3 Experimental set-up to perform a simplified food chain.

3.5.3.1 "Ingestion method" and "predatory performance" to set prey/predators ratio.

The optimal prey/predators ratio was chosen in order to eliminate any variability due to a non-saturated feeding condition.

For each species of jellyfish, the experimental set (different number of prey) consisted of three replicate jars with 100ml filtered seawater (0.22 µm FSNW, 37‰ salinity) and ten ephyrae for each replicate. In this experiment was carried out the test following the protocol develop for Ephyra Test in semi-dynamic conditions (ETsd) in the section 3.3.2 and Figure 11.

Have been used four different number of prey : 10, 20, 50 and 100 nauplii of brine shrimp given daily to ephyrae. All experimental jars were gently mixed through an aeration system. The experiments were conducted for one week and consisted of five feeding treatment (one feeding a day) and at the end were measured the Ingestion rate and the Predatory performance. Temperature was maintained constant for all experiment, keeping the jars in a thermostatic room at 20°C ± 0,5 where experimental water was able to adjust to the appropriate temperature condition. Also the salinity was measured at the beginning and at the end of experiment.

The Ingestion rate I , namely the number of prey ingested per medusa, as reported by Riisgard et al. (2011) for steady-state experiment (same number of prey given each day of experiment) was determinate in this study as:

$$I= G/E,$$

where G was the number of prey eat by ephyrae, per day of experiement (E).

The Predatory performance (Pp) was expressed as percentage of prey ingested relatively to the number of prey offered (OP) during the feeding experiment:

$$PP= (OP-CP)*100/OP,$$

where CP resulted the number of prey remained in the jars at the end of experiment; as reported by Fonte et al. (2016).

3.5.4 Evaluation of "Cadmium enriched –diet effect" on ephyrae jellyfish.

3.5.4.1 Contamination of *Artemia* sp. with Cadmium nitrate.

The Cadmium nitrate concentrations were selected in according to Faimali et al. 2014 and the results obtained in the section 4.3 about the reference toxicants tested on ephyrae of *Aurelia* sp. and *S.malayensis*. First, hatched larvae (Instar I stage) of *Artemia* sp. obtained as reported in paragraph 7.2.2, were transferred into each well of 24 multi-well plates containing 1 mL of different metal concentrations using a small 80 μm mesh filter. They were incubated in the dark, for 6, 24 and 48 hours at 25 °C according to Gambardella et al. (2015a) and APAT IRSA CNR (8070,2003). After the times selected mortality analysis was performed under a stereomicroscope: completely motionless larvae were counted as dead organisms, and the percentage of mortality was compared to the controls to define the LC_{50} value (ephyrae were fed only with prey alive).

In the second step, *Artemia* nauplii were contaminated with Cadmium nitrate at different concentrations below the LC_{50} value (Table 17) exposing the organisms in semi-dynamic condition, in jars with 100ml of volume, gently aerated, at dark and 25° C for 6 hours according to Batel et al. (2016).

In this case Swimming Speed Alteration (SSA) a sub-lethal behavioral end-point was also evaluated by SBR (used also for ephyrae jellyfish). Briefly, swimming behavior was monitored in dark conditions, under infrared light, for three seconds. The resulting digital images were analyzed using

an advanced image processing software to reconstruct individual swimming paths and measure the average swimming speed (mm/s) for each test population organism (10–20 organisms) (Garaventa et al. 2010).

Data were expressed as percentages of swimming speed alteration (SSA) normalized to controls' swimming speed (S), as follows:

$$SSA\ (\%) = [(S\ Treated - S\ Control\ /S\ Control) \times 100].$$

where S Treated is the swimming speed evaluated on nuplii contaminated and S Control is the swimming speed measured in the control (nauplii exposed to sea water) (Garaventa et al. 2010; Gambardella et al. 2016; Morgana et al. 2017). In addition, a bioassay in static (according to ARPAT IRSA CNR, 8070,2003) and semi-dyanamic condition exposing *Artemia* sp. to potassium dichromate (reference toxicant) were performed.

3.5.4.2 Feeding of ephyrae jellyfish of *Aurelia* sp. and *S. malayensis* with *Artemia* nauplii contaminated with Cadmium nitrate.

Two simplified food chains were performed following the results obtained during the set-up of prey/predators ratio described in section 3.5.3.1 and summarized in the Table 16.

After 6 h of contamination with Cadmium nitrate (see section 3.5.4.1), for each concentration *Artemia* nauplii were washed in FNSW 0, 22 µm and offered to ephyrae of *Aurelia* sp. and *S.malayensis* , 20 and 50 nauplii respectively. For each concentration of Cadmium nitrate to which the *Artemia* nauplii were exposed, three replicate jars with 100ml of volume and ten ephyrae for each replicate were performed as displayed in Figure 13

The control group of ephyrae was fed with nauplii of *Artemia* nauplii no contaminated. The experimental design were performed exposing the ephyrae jellyfish in semi-dynamic condition, following the ETsd reported in section 3.3.2 of this thesis (Figure 13).

The feeding treatment was performed always every 24h for a week (5 feeding treatment) and no additional food was given to ephyrae during the day.

Temperature was maintained constant for all experiments, keeping the jars in a thermostatic room at 20°C ± 0,5 where experimental water was able to adjust to the appropriate temperature condition. Also the salinity was measured at the beginning and at the end of experiment.

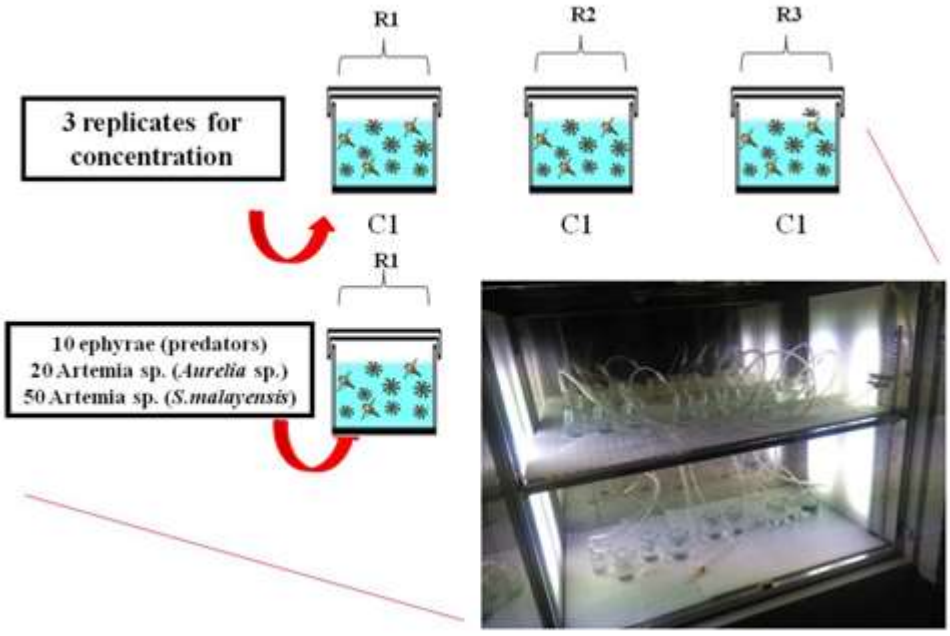

Figure 13 Experimental design to perfom the simplified food web with ephyrae of *Aurelia* sp. and *S.malayensis* fed with Artemia sp. nauplii.

3.5.4.3 "Ingestion method" and "predatory performance"

The ingestion rate I, (prey d^{-1}) and the Predatory performance (%Pp) were determinate as reported in the paragraph 3.5.3.1 at the end of the feeding treatment (5 days) for each concentration of Cadmium nitrate to which *Artemia* nauplii were exposed.

3.5.4.4. Biometric and bioenergetic parameters

All parameters were evaluated following the biometric and bioenergetic measurements proposed by Bamstedt et al. 1999. At the end of feeding experiment (5 days) ephyrae were individually sucked up with a pipette and measured alive in a petri disch under a optical microscope with a measuring ocular. For the diameter was used the distance between opposite rhopaliae (Disc diameter, Figure 14).

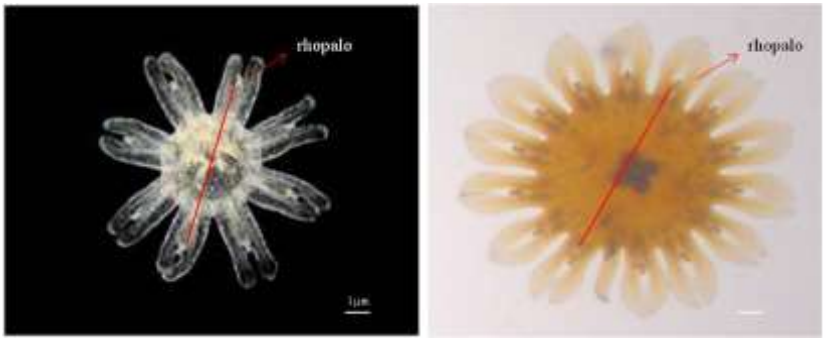

Figure 14 Distance between opposite rhopalie to define Disc diameter in ephyra of *Aurelia* sp. and *S.malayensis*

Subsequently the ephyrae were placed in individual pre-weighed alluminium pans and bried for 24h at 60°C. After weighing on a microbalance the samples were burned in a laboratory stove at 250°C for 48h and weighed again. The weight loss from dry to burnt sample was defined as the ash-free dry weight (AFDW).

In addition was measured the gross growth efficiency (GGE%), according to the following formulae:

$$GGE\% = [\ \Delta AFDW_{ephyrae} / (N_{prey} * AFDW_{prey})] * 100$$

where AFDWephyrae is the change in average body mass of the predator at the beginning (unfed) and end of experiment. Disc diameter, AFDW and GGE% were measured in ephyrae of *Aurelia* sp.

and *S.malayensis* fed with *Artemia* nauplii for each concentration of Cadmium nitrate (to which prey were contaminated) and for the control (ephyrae fed with prey no contaminated). The average individual AFDW for *Artemia* nauplii, 3.2 µg, was taken from Bamstedt et al. (1999a).

3.5.4.5 Ephyrae swimming performance

At 24, 48, 72 and 96 hours from beginning of feeding experiment the ephyrae of *Aurelia* sp. and *S. malayensis* placed in beaker, were individually transferred in a multi-well plates (one ephyra for each well). It was performed for each concentration of Cadmium nitrate to which *Artemia* nauplii were exposed and for the controls (ephyrae fed with prey no contaminated). At each time considered were evaluated two end-points: the Immobility and Frequency of pulsation by the SBR according to Faimali et al. 2014 and described in section 3.3.2.1.

3.5.5 Data processing and statistic analysis

Analysis of Variance (ANOVA) by IBM SPSS, was employed to test the difference between feeding treatments performed and the effect of Cadmium nitrate transfer on "ingestion rate" , "predatory performance" and bioenergetic parameters.

Also was applied on analysis data about the end-points evaluated (Frequency of pulsation and Immobility). Prior to analysis the assumption of the normality and homogeneity of variances was tested by Shapiro–Wilk and Levene tests. Kruskal–Wallis non parametric test and post hoc Mann-Withney test were used when data did not meet the normality and homogeneity of variances assumptions for ANOVA.

In this study was also evaluated: the EC_{50} and LC_{50} values (the concentration of Cadmium nitrate that caused the 50% deaths of exposed Artemia nauplii after 24 h and 48 h), and related 95%

Confidence Limits (CL) using trimmed Spearmane Karber analysis (Finney, 1978) after 24 and 48 h of exposure.

For AFp statistical analysis were performed using Frequency pulsation data. The LOEC values for Immobility and Alteration of Frequency of pulsation were deducted by ANOVA results. Data were considered significantly different when $p<0.05$. SPSS statistical software (Statistical Package for the Social Sciences, Version 20) was used for data analysis.

4 RESULTS

4.1 Experimental activities on *Aurelia* sp.

4.1.1 Neurotoxic compounds

4.1.1.1 Bioassays

The results obtained exposing 0 days old ephyrae to different concentrations of ES and CPF are reported in Figure 15 and 16, respectively, while the 24, 48 h-EC_{50} and LOEC values for % I and % AFp are summarized in Table 6 for both tested neurotoxic compounds.

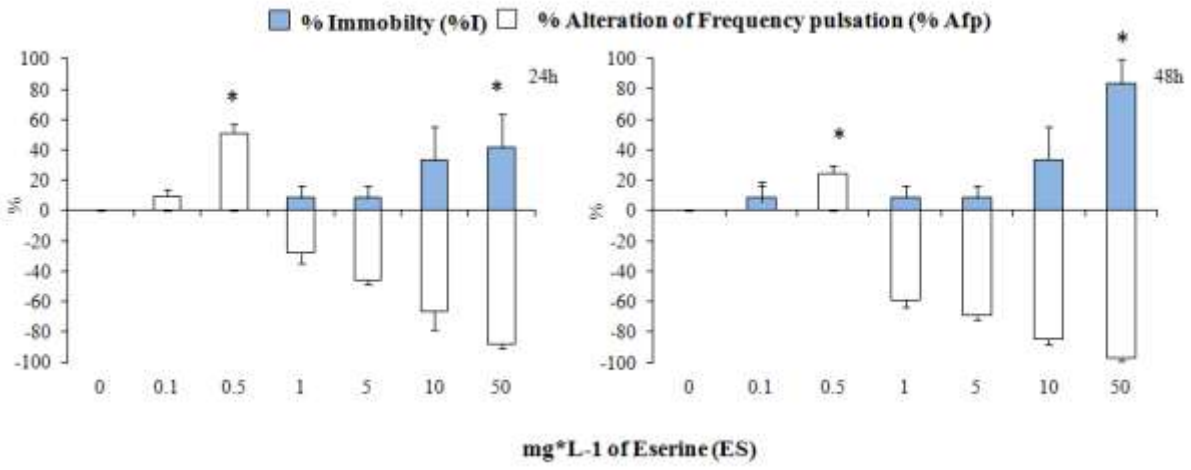

Figure 15 Alteration of frequency of pulsation (% AFp) and immobility (% I) of 0 days old ephyrae of *Aurelia* sp. after 24 and 48 h of exposure to increasing concentrations of ES (M ±se, n = 3). *= $p< 0.05$, (one-way ANOVA)

As highlighted in Figure 15, ES on ephyrae of *Aurelia* sp. caused a significant effect on both end-points; moreover, after 24 h and 48 h of exposure, a difference in sensitivity between the two end-point was observed. The $LOEC_I$ was 50 mg L^{-1} while the $LOEC_{AFp}$ was 0.5 mg L^{-1}. Moreover, after 24 h, the median effect of the neurotoxic carbamate pesticides was not calculable considering the Immobility, while the bioassay pointed out a clear sub-lethal effect on Frequency of pulsation (Table 6). In addition, after 24 and 48 h, a slight but statistically significant hormetic effect on the sub-lethal end-point in ephyrae exposed to 0.5 mg L^{-1} of ES was observed.

Results obtained exposing ephyrae to CPF are reported in Figure 16.

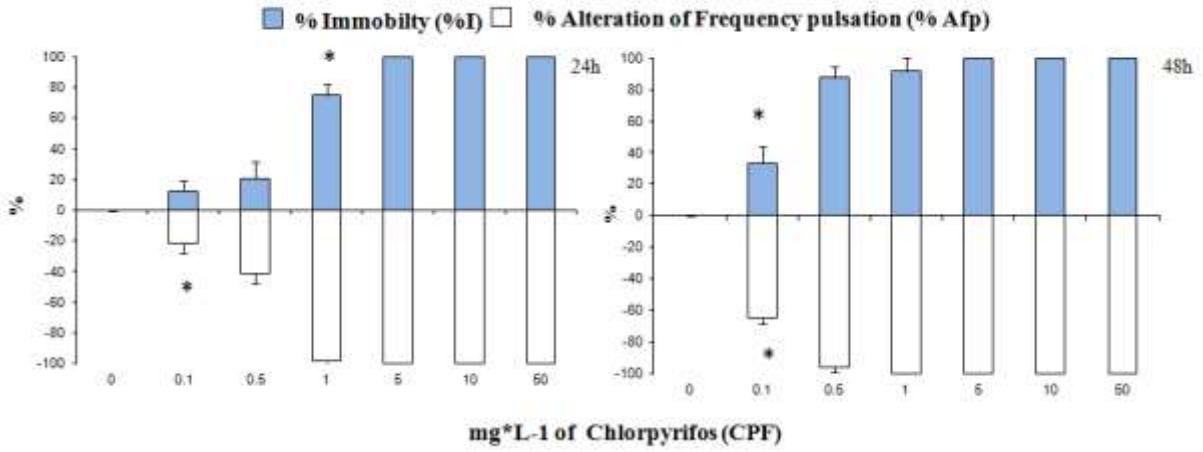

Figure 16 Alterations of frequency of pulsation (% AFp) and immobility (% I) of 0 days old ephyrae of *Aurelia* sp. after 24 and 48 h of exposure to increasing concentrations of CPF (mean ± SE, n = 3). *= $p<0.05$ (one-way ANOVA).

Also this compound caused a significant effect on both the end-points; moreover, after 24 h of exposure, the sub-lethal end-point (% AFp) was more sensitive compared to acute one (% I).The $LOEC_I$ was 1 mg L^{-1} while the $LOEC_{AFp}$ was 0.1 mg L^{-1}. This difference was no more evident after 48 h of exposure, when the LOEC value was 0.1 mg L^{-1} for both end-points. It is also evident how for both end-points starting from the concentration of 5 mg L^{-1} a 100% response was obtained and in the range of concentrations and between 0.1 and 1 mg L^{-1} the AFp was the most sensitive (in

terms of magnitude) end-point if compared to Immobility after 24 h and 48 h of exposure. Moreover, all concentrations of CPF caused an increase in % I and a decrease in % AFp in a dose dependent manner at 24 h and 48 h of exposure time.

Table 6 24 and 48 h-EC_{50}values with 95% confidence limits derived from % I and % AFp of ephyrae of *Aurelia sp.*exposed to ES and CPF.

***Aurelia* sp.**	**Eserine (ES) mg L^{-1}**	**Chlorpyrifos (CPF) mg L^{-1}**
24H		
Immobility (%I)	> 50	0.70 (0.59-0.84)
EC_{50} (CL 95%)	50	1
$LOEC_I$		
% Alteration of Frequency of pulsation (%AFp)	4.12 (2.98-5.69)	0.44 (0.35-0.55)
EC_{50} (CL 95%)	0.5	0.1
$LOEC_{AFp}$		
48H		
Immobility (%I)	17.43 (14.36-21.16)	0.17 (0.14-0.20)
EC_{50} (CL 95%)	50	0.1
$LOEC_I$		
% Alteration of Frequency of pulsation (%AFp)	1.02 (0.78-1.34)	<0.1
EC_{50} (CL 95%)	0.5	0.1
$LOEC_{AFp}$		

4.1.1.2 Morphological observations

Microscopy observation showed that both neurotoxic compounds caused different morphological changes in ephyrae of *Aurelia* sp. In Figure 17, were reported the images of ephyrae control (FNSW) and treatment , in particular were selected the organisms exposed to 50 mg*L^{-1} of ES and CPF. All control organisms were considered “Normal” with clear to transparent coloration,eight distinct pairs of lappets, evident rhopalia and manubrium, overall round shape and regular, steady muscle contractions. Ephyrae treated with different concentration of ES and CPF showed morphological changes.

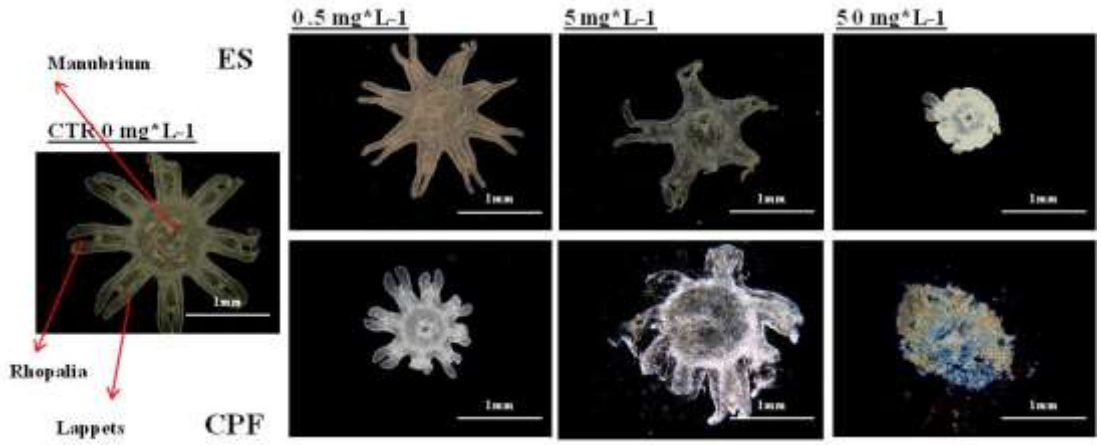

Figure 17 Representative microscopy images of *Aurelia* sp. ephyrae exposed to FNSW (CTR 0 mg*L-1) and to different concentrations of ES and CPF (0.5- 5- 50 mg*L-1). Note morphological changes in ephyrae treated compared to the control.

The signs of the stress increase with the increasing of concentration of ES and CPF to which *Aurelia* sp. ephyrae were exposed. Minimal morphological changes were evident at 0.5mg*L^{-1}, while at highest concentration (50 mg*L^{-1}) the organisms showed the tissue degradation, shriveled tissue in the central disc, lappet stem, and manubrium areas, inward curling of the body. In particular CPF seems to be more toxic, since that ephyrae resulted to have a total degradation of tissue. The LC_{50} and EC_{50} obtained for other marine invertebrate exposed to ES and CPF were reported in the tables below (Table 7 and 8)

Table 7 24 and 48 h-EC_{50}values with 95% confidence limits derived from % I and % AFp and 24h, 48h LC_{50} (median Lethal Concentration) values reported in literature for some marine model organisms exposed to ES.

Marine organisms	**Species**	**Stage**	**End-point**	**Exposure time**	**EC_{50}-LC_{50} (mg*L-1^{-1})**	**References**
Cnidarians	*Aurelia* sp.	Ephyra	%Immobility	24h 48h	>50 17.43 (14.36-21.16)	This study
			% Alteration of Frequency of pulsation	24h 48h	4.12 (2.98 -5.69) 1.02 (0.78-1.34)	
Crustaceans	*Amphibalanus amphitrite*	Nauplii II	% Mortality	24h	>10.24	Faimali et al.2006
	Artemia salina	Nauplii I	% Mortality	24h 48h	>100 >100	Garaventa et al. 2010
Rotifers	*Brachionus plicatilis*	-	% Mortality	24h 48h	>100 >100	Garaventa et al. 2010

Table 8 24 and 48 h-EC_{50} values with 95% confidence limits derived from % I and % AFp and 24h,48h- EC_{50}, LC_{50} (median Lethal Concentration) values reported in literature for some marine model organisms exposed to CPF.

Marine organisms	Species	Stage	End-point	Exposure time	EC_{50}-LC_{50} (mg L^{-1})	References
Cnidarians	*Aurelia* sp.	ephyra	% Immobility	24h	0.7 (0.59-0.84)	This study
				48h	0.17 (0.14-0.20)	
			% Alteration of Frequency of pulsation	24h	0.44 (0.35-0.55)	
				48h	> 0.1	
	Hydra attenuata	Polyp	% Mortality	96h	1 (0.80-1.2)	Demetrio et al.2012
Bacteria	*Vibrio fischeri*	Cell	% Inhibition luminescence	72h	2.84 (n.c)	Palma et al.2008
Crustaceans	*Artemia salina*	Nauplii I	% Mortality	24h	3.19 (1.35-6.34)	Varó et al.2002
Molluscs	*Mytilus galloprovincialis*	Adult	% Mortality	96h	22.5 (22.9-22.1)	Serrano et al.1995
Rotifers	*Brachionus plicatilis*	-	% Mortality	24h	1.7 (1.75-1.6)	Guzzella 1997
Echinoderms	*Paracentrotus lividus*	Larval	% AChE Inhibition	48h	0.3 (0.35-0.4)	Bellas et al.2005

4.1.3 Ostreopsis cf.avata strains

3.1.5.1 Bioassay

Data regarding the effects of direct contact with the whole culture of *O.* cf. *ovata* (CBA29 strain), on ephyrae of *Aurelia* sp. performed at several cell concentrations are shown in Figure 18

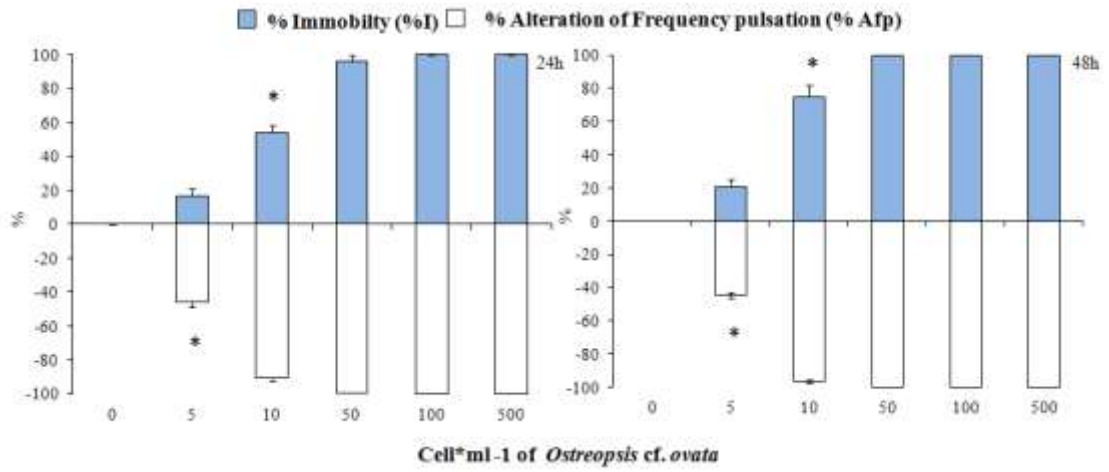

Figure 18 Alteration of frequency of pulsation (% AFp) and immobility (% I) of 0 days old ephyrae of *Aurelia* sp. after 24 and 48 h of exposure to increasing concentrations of *Ostreopsis* cf. *ovata* culture (M ±se, n = 3). **=p<0.05 (one-way ANOVA).

In general, ephyrae were severely affected by the presence of this dinoflagellate, thus EC_{50} regarding the two end points taken into account show very low values even after only 24 h of exposure. In particular, EC_{50}-24h referred to the percentage of pulsation frequency (% AFp) was equal to 5.32 cells/mL while EC_{50}-24h of immobility resulted in 10.50 cells/mL. EC_{50} values are reported in Table 9, together with previous data referred to other end-points or different model organisms. Moreover, some algal cells through filaments were observed attached onto the body surface of ephyrae and around the lappet (Figure 19).

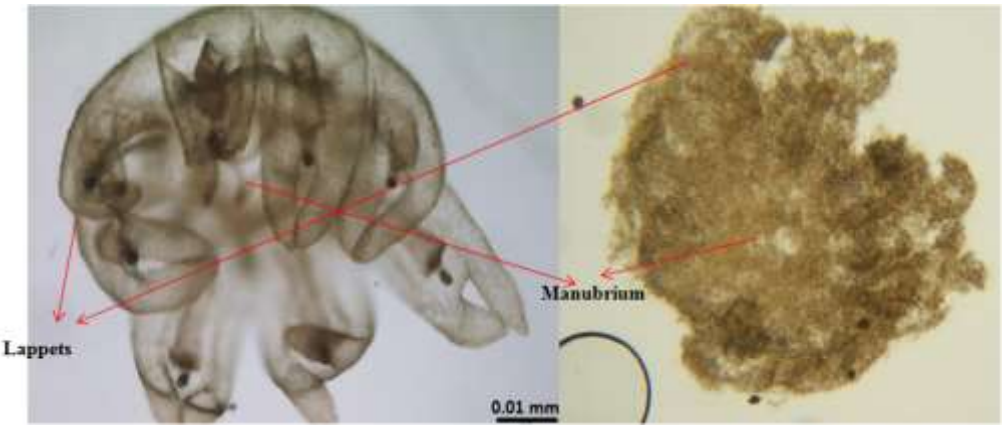

Figure 19. From the right to left, *Aurelia* sp. ephyra in control condition (0.22 µm filtered natural sea water) after 48 h. and ephyra exposed to 100 cells/mL of *O.* cf. *ovata* whole culture after 48 h (same size bar).

Statistical analyses on immobility data highlighted a significant effect ($p < 0.05$) from 10 cell*ml-^{1} after both time of exposure, while the Frequency pulsation results significantly affect ($p < 0.05$) already at the lowest concentration of *Ostreopsis* cf. *ovate* tested.

Table 9 24 and 48 h-EC_{50}values with 95% confidence limits derived from % I and % AFp measured in ephyrae of *Aurelia* sp. and 24h, 48h LC_{50} (median Lethal Concentration) values reported in literature for some invertebrates marine model organisms exposed to *Ostreopsis* cf. *Ovate.*

Marine organisms	**Species**	**Stage**	**End-point**	**Exposure time**	**EC_{50}-LC_{50} (cell*ml-1)**	**References**
Cnidarians	*Aurelia* sp.	Ephyra	% Immobility	24h	10.50(8.9-12.4)	This study
				48h	7.31(6.6-8.1)	
			% Alteration of Frequency of pulsation	24h	5.35 (4.7-6.1)	
				48h	5.32(4.6-6.1)	
Crustaceans	*A. salina*	Nauplii I	% Mortality	24h	>4000	Faimali et al. 2012
				48h	12.43	
			% Mortality	24h	>400	Giussani et al. 2015
				48h	<4	
			% Immobility	24h	>400	
				48h	<4	
			% Mortality	24h	15.11	Faimali et al. 2012
				48h	>6	
	A.amphitrite	-	% Mortality	24h	>400	Faimali et al. 2012
				48h	>1416.95	
	Tigriopus Fulvus	Larval	% Mortality	24h	>4000	Faimali et al. 2012
				48h	>1486.74	

4.2 Experimental activities on *Sanderia malayensis*

4.2.1 Methodological parameters investigated to develop a protocol testing with ephyrae of *S.malayensis*

Experiment 1- Volume of exposition. Results of the first experiment, aimed to investigate the influence of the sub-lethal end-point (Frequency pulsation, Fp) of volume of exposition were reported in Figure 20. The one-way ANOVA, does not highlighted a significant effect ($p < 0.05$) of 2 and 5 ml volume of exposition on sub-lethal end-point, indeed the ephyrae reported the same number of pulsations in both volumes of exposition considered (Figure 20). Only significantly ($p < 0.05$) different was reported in ephyrae exposed to 10ml of volume.

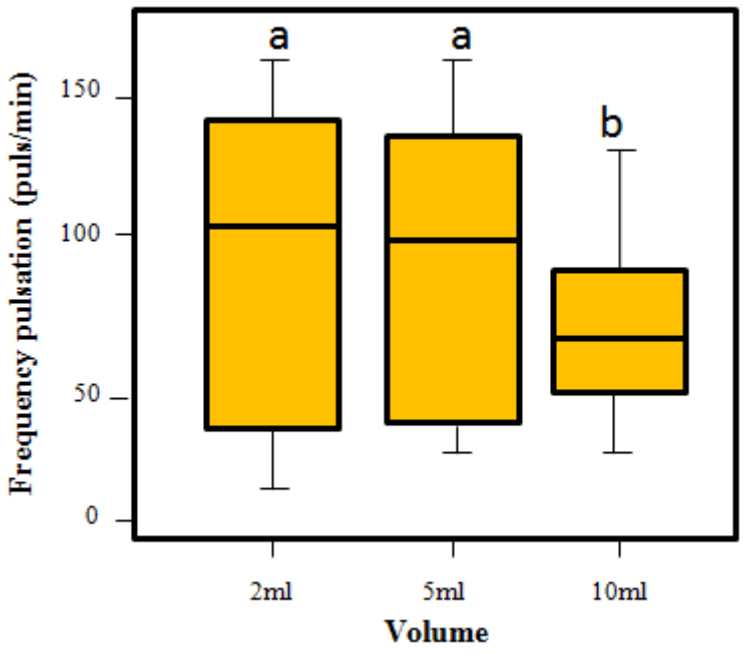

Figure 20 Frequency of pulsation (Fp) made by ephyrae of *S. malayensis* exposed to different volumes (2ml, 5ml and 10ml) of filteres natural sea water (FNSW, 0,22 µm at 37 ‰). The horizontal line in the box shows the median and the whiskers show the range. The letter a, b indicate significantly different (P <0.05) groups with Kruskal-Wallis post-hoc test. Any groups sharing the same letter are not significantly different.

Experiment 2-Temperature. Results of the experiment performed to assess the influence of the temperature on the Frequency of pulsation (Fp) are reported in Figure 21.

The one-way ANOVA pointed out a significant effect ($p < 0.05$) of temperature and among the treatments as reported in Figure 21. Among the investigated temperatures, 20°C seems to be the best condition to produce a significant ($p< 0.05$) measurable values of Frequency of pulsation (Fp), while it was evident that ephyrae not pulsed after 24 hours of exposition at 10°C .

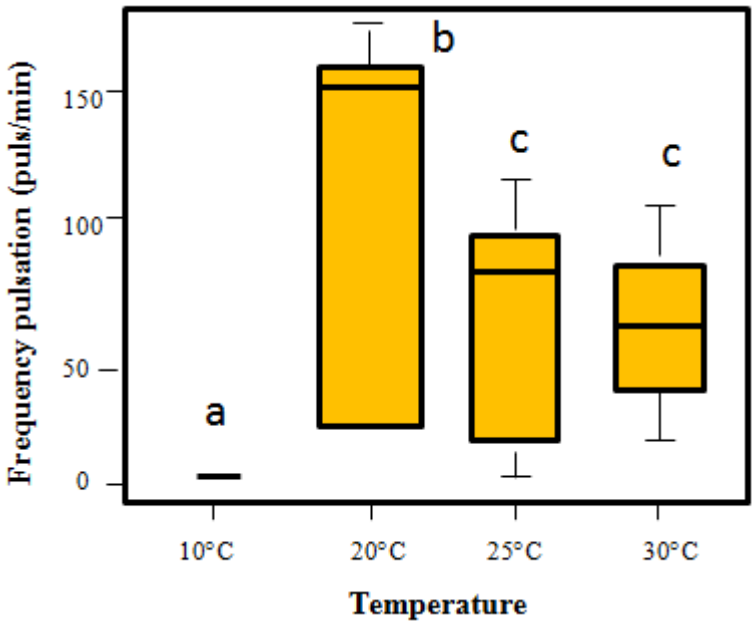

Figure 21 Frequency of pulsation (Fp) made by ephyrae of *S. malayensis* exposed to 2ml of FNSW (0,22 µm at 37 ‰) at different temperatures (10°C-20°C-25°C-30°C). The horizontal line in the box shows the median and the whiskers show the range. The letter a, b and c indicate significantly different ($P <0.05$) groups with Kruskal-Wallis post-hoc test Any groups sharing the same letter are not significantly different.

Experiment 3- Photoperiod. Results of the experiment performed to assess the influence of the photoperiod on the Frequency of pulsation (Fp) are reported in Figure 22.

The one-way ANOVA does not highlighted a significant effect of the Photoperiod on the sub-lethal end-point ($p < 0.05$), however the Frequency of Pulsation measured with exposition of ephyrae in total dark higher than other photoperiod conditions. Thus, to have the highest Frequency of

pulsation in ephyrae of *S.malayensis* the organism should be exposed in a volume of 2ml of sea water, at 20C° and total dark of temperature and photoperiod respectively.

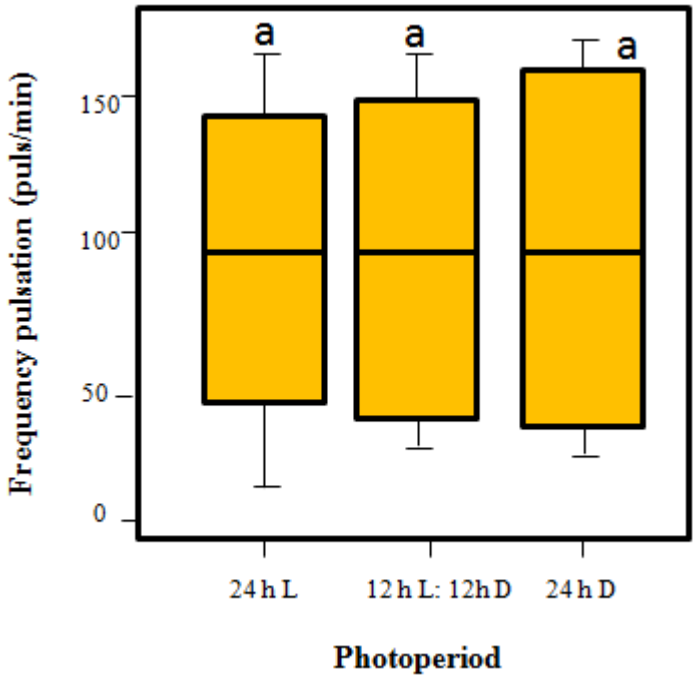

Figure 22 Frequency of pulsation (Fp) made by ephyrae of *S. malayensis* exposed to 2ml, of FNSW (0,22 µm at 37 ‰) at 20°C in different conditions of photoperiod (24h L, 12h L:12Hd, 24Hd). The horizontal line in the box shows the median and the whiskers show the range. The letter A, B and C indicate significantly different (P <0.05) groups with Kruskal-Wallis post-hoc test Any groups sharing the same letter are not significantly different.

On the basis of these results it was possible to define the following parameters to be used to perform the ecotoxicological test with ephyrae of *S. malayensis* (Table 10).

Table 10. Methodological parameters used to perform the toxicity test with ephyrae of *S. malayenis* defined in this study and those use to perfom the bioassay with *Aurelia* sp. described by Faimali et al. 2014

Model organism	Test parameters Ephyra Test_ET		References
S. malayensis	Organism density	1 ephyra/well	Faimali et al. 2014
	Volume	2ml for well	This study
	Recording light conditions	Dark	Faimali et al. 2014
	Photoperiod	24h Dark	This study
	Condition	Static	Faimali et al. 2014
	Ephyrae age	0 days	Faimali et al. 2014
	Temperature	20°C	This study
	Salinity	37‰	Faimali et al. 2014
Aurelia sp.	Organism density	1 ephyra/well	Faimali et al. 2014
	Volume	2ml for weel	
	Recording light conditions	Dark	
	Photoperiod	24h Dark	
	Condition	Static	
	Ephyrae age	0 days	
	Temperature	20°C	
	Salinity	37‰ (no defined)	

4.3 Experimental activities on *Aurelia* sp. and *S.malayensis* : ecotoxicological comparison with refence toxic compounds

4.3.1 Ephyrae Test_ET

The trend of the acute and sub-lethal end points evaluated on ephyrae of *S. malayenis*, were reported in Figure 23 as the percentage of Alteration of Frequency of pulsation (% AFp) compared to the control and the percentage of Immobility (% I) respectively. For *Aurelia* sp. were reported only the value of EC_{50} and summarized with those of *S.malayensis* in Table 11 for both toxic compounds.

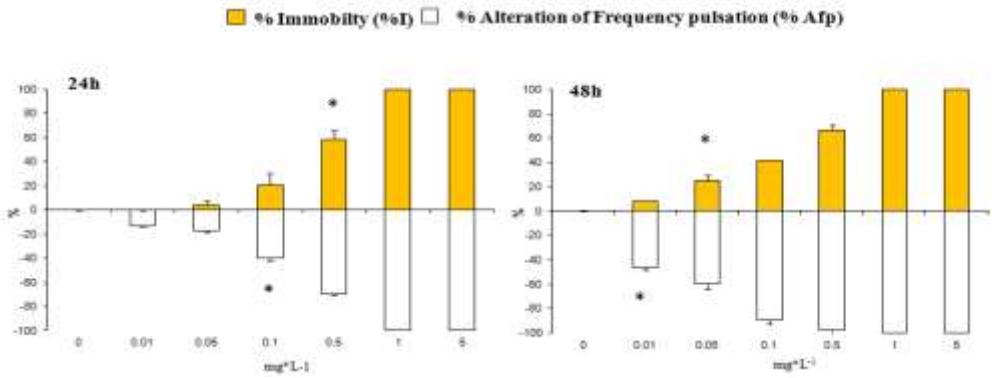

Figure 23. Alteration of Frequency of pulsation (% AFp) and immobility (% I) of ephyrae of *S. malayensis* after 24 h and 48 h of exposition at increasing concentration of Cadmium Nitrate (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

As showed in Figure 23 Cadmium nitrate on ephyrae of *S. malayensis* caused a significant effect ($p < 0.05$) on both end-points evaluated. This compound causes a significant effect ($p < 0.05$) on both end-points. LOEC (Lowest Observed Effect Concentration), calculated by the a posteriori pair-wise comparison for the % of immobility was 0,5 mg*L-1^{-1}, while for % Fp was 0,1 mg*L-1^{-1}.

This difference of sensitivity was less evident at 48 hours of exposure where the LOECI was 0,05 mg*L-1^{-1} while the $LOEC_I$ was 0,01 mg*L-1^{-1} (Table 5.2). Moreover, the results after 24 and 48 hours of exposition show how for both end-points from concentration of 1mg*L-1^{-1} a 100% of response and in the range of concentrations between 0.01 and 0.5 mg*L-1^{-1} the Alteration of Frequency of pulsation was the most sensitive end-point (in term of magnitude of the response) compared to Immobility. Overall all concentrations of Cadmium nitrate caused an increase in % I and a decrease in % AFp in a dose-dependent manner at 24 and 48 hours of exposure time.

Results obtained exposing ephyrae of *S. malayensis* to different concentrations of SDS was reported in Figure 24.

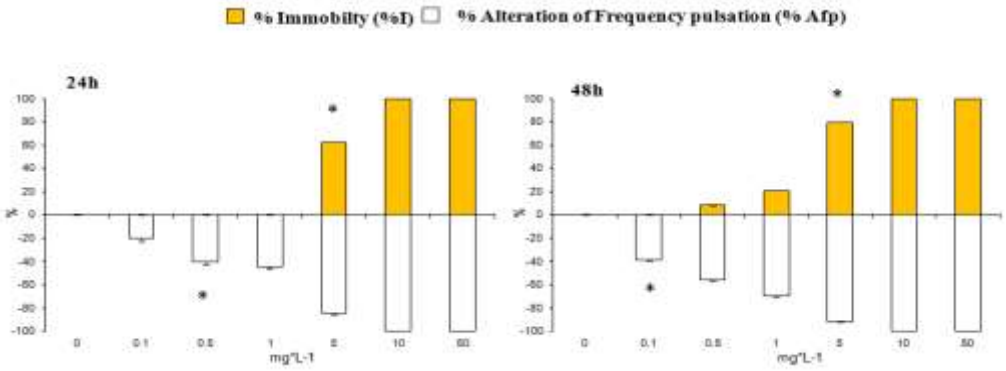

Figure 24 Alteration of Frequency of pulsation (% AFp) and immobility (% I) of ephyrae of *S. malayensis* after 24 h and 48 h of exposition at increasing concentration of SDS (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

Also this toxic compound caused a significant effect on both end-points in different manner at the 24 and 48 hours of exposition. In fact after both time of exposition the sub-lethal end-point was more sensitive compared with acute one at 24 hours the LOECI and LOECFp was 5 mg*L-1^{-1} and 0.5 mg*L-1^{-1} respectively while at 48 hours the difference of sensitivity was more evident because the LOECI values was 5 mg*L-1^{-1} and the LOECAFp 0.1 mg*L-1^{-1}. Moreover after 24 and 48 hours of exposition for both end-points, was evident how for both end-points starting from the concentration of 10 mg*L-1^{-1} a 100% of response was obtained and in the range of concentrations and between 0.1 and 1 mg*L-1^{-1} the AFp was the most sensitive (in term of magnitude) end-point if compared to immobility after 24 and 48 hours of exposition. On the contrary about the results of Cadmium nitrate, for SDS it was evident how all concentrations of this compound caused an increase in % I and a decrease in % AFp in a dose-dependent manner only after 48 hours of exposition.

Table 11 24and 48-h EC_{50} values with 95% confidence limits and $LOEC_{I,}$ $LOEC_{AFp}$ derived from % I and % AFp evaluaeted in this study on ephyrae of *S. malayensis* and *Aurelia* sp. exposed to Cadmium nitrate and SDS.

		Reference toxicants	
		Cadmium nitrate mg*L^{-1}	**SDS mg*L^{-1}**
	End-points		
Specie of jellyfish			
S.malayensis	24h-%I EC_{50}(CL 95%)	0,27 (0,23-0,32)	3.46(3.10-3.87)
	$LOEC_I$	0,5	5
	24h-%AFp EC_{50}(CL 95%)	0.17(0.14-0.22)	0.98(0.68-1.23)
	$LOEC_{Afp}$	0.1	0.5
	48h-%I EC_{50}(CL 95%)	0,15(0,12-0,19)	2.04(1.76-2.36)
	$LOEC_I$	0,05	5
	48h- %AFp EC_{50}(CL 95%)	0,02(0,01-0,04)	0.28(0.17-0.49)
	$LOEC_{Afp}$	0,01	0.1
Aurelia sp.	24h- %I EC_{50}(CL 95%)	0.40 (0.35-0.46)	4.89(4.34-5.39)
	$LOEC_I$	0.5	5
	24h - %AFp EC_{50}(CL 95%)		
	$LOEC_{Afp}$	0.13(0.10-0.15)	4.52(3.58-5.79)
		0.1	5
	48h- %I EC_{50}(CL 95%)	0.23(0.20-0.28)	5
	$LOEC_I$	0.05	5
	48h- %AFp EC_{50}(CL 95%)	0.07(0.05-0.07)	1.55(1.36-1.79)
	$LOEC_{Af}$	0.05	1

4.3.2 Ephyra Test in semi-dynamic exposition_ ETsd

Below were reported in Table 12 the parameter defined to perform the toxicity test in semi-dynamic exposition with ephyrae of *Aurelia* sp. and *S. malayensis*

Table 12 Methodological parameters used to perform the toxicity test in semi-dynamic condition ETsd with ephyrae of *Aurelia* sp. and *S. malayenis* defined in this study .

Test parameters Ephyra Test semi-dynamic_ETsd		**References**
Organism density	10 ephyra/beaker	This study
Volume	100ml	This study
Recording light conditions	Dark	Faimali et al. 2014
Photoperiod	24h Dark	This study
Condition	With aeration	This study
Ephyrae age	0 days	Faimali et al. 2014
Temperature	20°C	This study
Salinity	37‰	This study

Immobility results

The average of the *S. malayensis* and *Aurelia* sp. and ephyrae immobility percentage related to the three repetition (A,B,C) of 24 and 48 h toxicity test in semi-dynamic exposition were displayed in Figure 25.

The percentage of Immobility showed an increase proportional to the increase in Cadmium nitrate concentration from 1 and 0.1 mg*L-1 after 24 and 48 hours of exposition respectively for *S.malayensis* and from 0.1 mg*L-1 for both exposure times for *Aurelia* sp. Statistical analysis fixed the LOEC at 1 mg*L-1 and 0,5 mg*L-1 for *S.malayensis* and *Aurelia* sp. respectively after 48 hours of exposition highlighting a difference of sensitivity between the model organisms used. It was not possible to calculate EC_{50} values for *S.malayensis* and *Aurelia* sp. exposed to Cadmium nitrate (Table 13) , since ephyrae never show any > 50% effect on immobility for both exposure time. Two-way ANOVA releaved that there were no differences between repetitions ($F = 1.21$; $p < 0.544$), consistely with the concentrations (concentration x repetition: $F = 0.80$; $p < 0.660$).

Frequency pulsation results

The average of the *S. malayensis* and *Aurelia* sp. ephyrae Frequency pulsation percentage related to the three repetition (A,B,C) of 24 and 48 h toxicity test in semi-dynamic exposition were displayed in Figure 25.

Increasing concentrations of Cadmium nitrate resulted in a significant reduction ($p < 0.05$) in Frequency pulsation in ephyrae of *Aurelia* sp. and *S. malayensis* after 24 and 48 hours of exposition. The Frequency pulsation displayed a trend similar to that of the acute end-point with an increase proportional to the increase in Cadmium nitrate concentration.

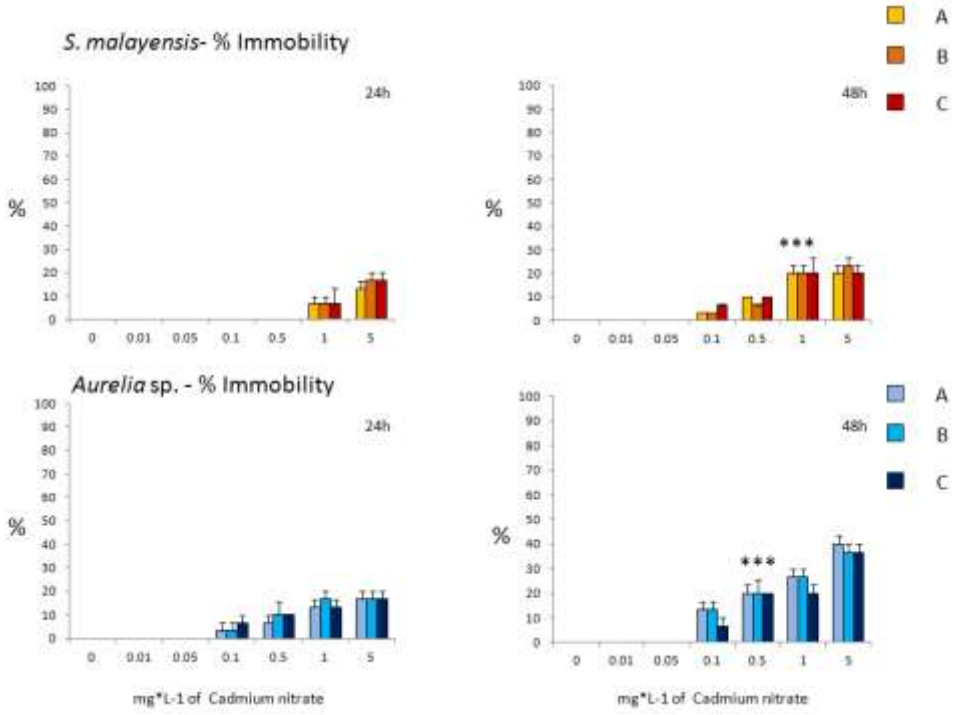

Figure 25 Percentage of Immobility (% I) of ephyrae of *S. malayensis* (orange bars) and *Aurelia* sp. (blue bars) after 24 h and 48 hours of semi-dynamic exposition at increasing concentration of Cadmium nitrate (M ±SE, n = 3).
*= $p < 0.05$ (one-way ANOVA). A,B and C were the three repletion of bioassay.

A clear significant effect of Cadmium nitrate was observed with a LOEC of 0.01 and 0.05 mg*L-1 after 24 and 48 hours of exposition respectively for *S. malayensis* and a LOEC of 0.01 mg*L-1 after both exposure times for *Aurelia* sp. for each bioassays (A,B and C). The EC_{50} values (± 95%CL) of

Frequency pulsation bioassay in semi-dynamic exposition to Cadmium nitrate after both exposure times were summarized in Table 13. The EC_{50} average of three repetitions was 0.21 (0.14-0.33) mg*L-1 and 0.07 (0.04-0.10) mg*L-1, for *S.malayensis*, while for *Aurelia* sp. 0.13(0.10-0.18) mg*L-1 and 0.03(0.02-0.09) mg*L-1 at 24 and 48 hours of exposition respectively. Two-way ANOVA releaved that there were no differences between repetitions ($F = 1.21$; $p < 0.544$), consistely with the concentrations (concentration x repetition: $F = 0.80$; $p< 0.660$).

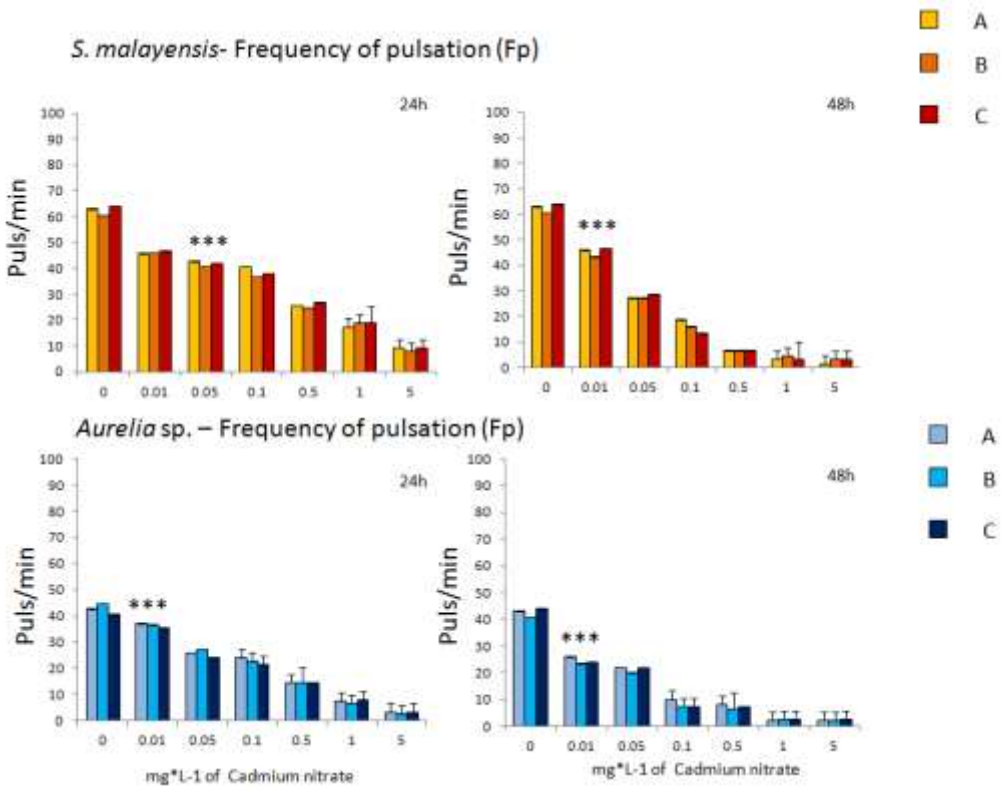

Figure 26 Trend of Frequency of pulsation (Fp) of ephyrae of *S. malayensis* (orange bars) and *Aurelia* sp. (blue bars) after 24 h and 48 hours of semi-dynamic exposition at increasing concentration of Cadmium nitrate (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA). A,B and C were the three repletion of bioassay.

Table 13 24- and 48-h EC50 values with 95% confidence limits and $LOEC_{I,}$ $LOEC_{AFp}$ derived from Immobility and Fp measured in ephyrae of *S. malayensis* and *Aurelia* sp. exposed by semi-dynamic condition to Cadmium nitrate. A,B and C were the three repetitions of the bioassay.

	Cadmium nitrate (mg/L)			
	S. malayensis		*Aurelia* sp.	
End-poin	Immobility	Frequency pulsation	Immobility	Frequency pulsation
A	24h-EC50= nc 24h-LOEC= nc 48h-EC50=nc 48h-LOEC= 1	24h-EC50= 0.23(0.15-0.36) 24h-LOEC= 0.05 48h-EC50= 0.07(0.05-0.11) 48h-LOEC=0.01	24h-EC50=nc 24h-LOEC=nc 48h-EC50=nc 48h-LOEC=0.5	24h-EC50=0.13(0.10-0.18) 24h-LOEC=0.01 48h-EC50=0.04(0.02-0.07) 48h-LOEC=0.01
B	24h-EC50= nc 24h-LOEC= nc 48h-EC50=nc 48h-LOEC= 1	24h-EC50= 0.21(0.14-0.32) 24h-LOEC= 0.05 48h-EC50= 0.06(0.04-0.09) 48h-LOEC=0.01	24h-EC50=nc 24h-LOEC=nc 48h-EC50=nc 48h-LOEC=0.5	24h-EC50=0.13(0.10-0.18) 24h-LOEC=0.01 48h-EC50=0.03(0.02-0.08) 48h-LOEC=0.01
C	24h-EC50= nc 24h-LOEC= nc 48h-EC50=nc 48h-LOEC= 1	24h-EC50= 0.19(0.12-0.30) 24h-LOEC= 0.05 48h-EC50= 0.07(0.04-0.10) 48h-LOEC=0.01	24h-EC50=nc 24h-LOEC=nc 48h-EC50=nc 48h-LOEC=0.5	24h-EC50=0.14(0.10-0.19) 24h-LOEC=0.01 48h-EC50=0.03(0.01-0.11) 48h-LOEC=0.01
Average	24h-EC50=nc 48h-EC50=nc	24h-EC50=0.21 (0.14-0.33) 48h-EC50=0.07 (0.04-0.10)	24h-EC50=nc 48h-EC50=nc	24h-EC50=0.13 (0.10-0.18) 48h-EC50=0.03 (0.02-0.09)

4.4 Experimental activities on *Aurelia* sp. and *S.malayensis* : ecotoxicological comparison with emerging compounds.

4.4.1 Reference toxic compounds

The results of Cadmium nitrate were reported only in Table 14 and 15 , while for BP-3 and Microplastics in the figures below were reported the % of Immobility and Alteration of Frequency pulsation measured in ephyrae of *S. malayensis* and *Aurelia* sp and in Table 14 and 15 respectively the EC_{50} and LOEC values.

4.4.1.1 Waterborne BP-3

Static condition (ET)

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by static condition to different concentrations of Waterborne BP-3 were reported in Figure 27.

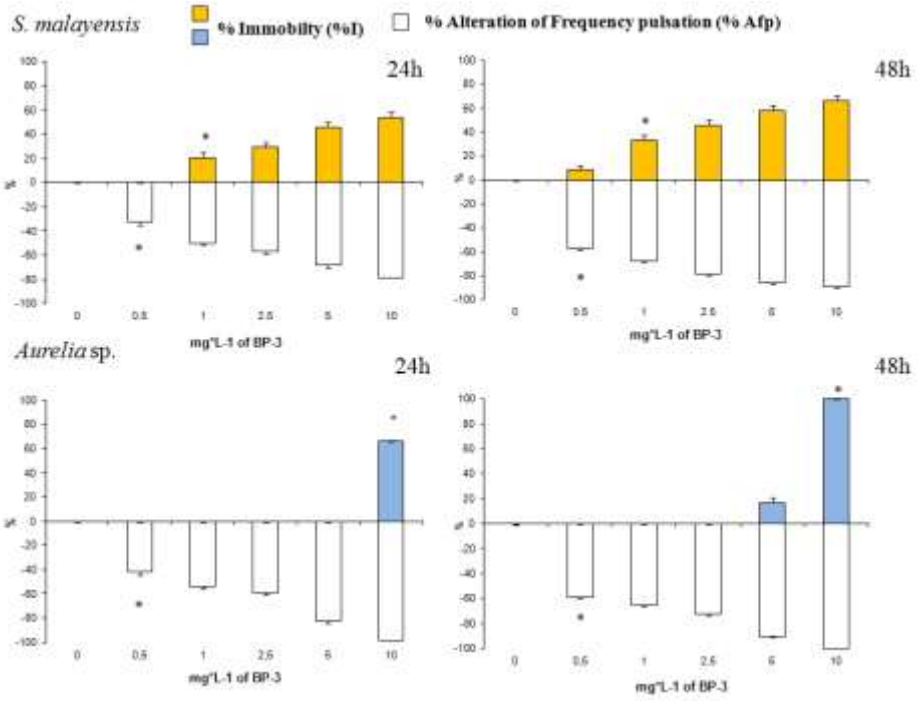

Figure 27 Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of BP-3 by static condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

Significant effect ($p< 0.05$) were observed in both species of jellyfish, end-points evaluated and exposure times considered. As highlighted in Figure 25, BP-3 on ephyrae of *S. malayensis* caused a significant effect ($p < 0.05$) on both end-points evaluated. After both exposure times, a difference in sensitivity between Immobility and Alteration of Frequency pulsation was observed: in fact the LOEC for the % I was 1 mg*L^{-1} while for % AFp was 0,5 mg*L^{-1} (Table 14). It was also evident how the effect of BP-3 on both end-points resulted to be in a dose-dependent manner already from the lowest concentration tested (0.5 mg*L^{-1}).

In ephyrae of *Aurelia* sp. was possible to observe a difference in sensitivity between the two end-points evaluated at both exposure times. However, ephyrae of *Aurelia* sp. showed a > 50% effect for Immobility only at the highest concentration of BP-3 tested (10 mg*L^{-1}). Frequency pulsation evaluated at both exposure times, resulted to be significantly inhibithed ($p < 0.05$) already at the lowest concentration of BP-3 tested (0.5 mg*L^{-1}). It was also evident how for the highest

concentration of BP-3 tested (10 mg*L^{-1}) a 100% response is obtained for both end-point considered (Table 15).

Semi-dynamic condition (ETsd)

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by semi-dynamic condition to different concentrations of Waterborne BP-3 were reported in Figure 28.

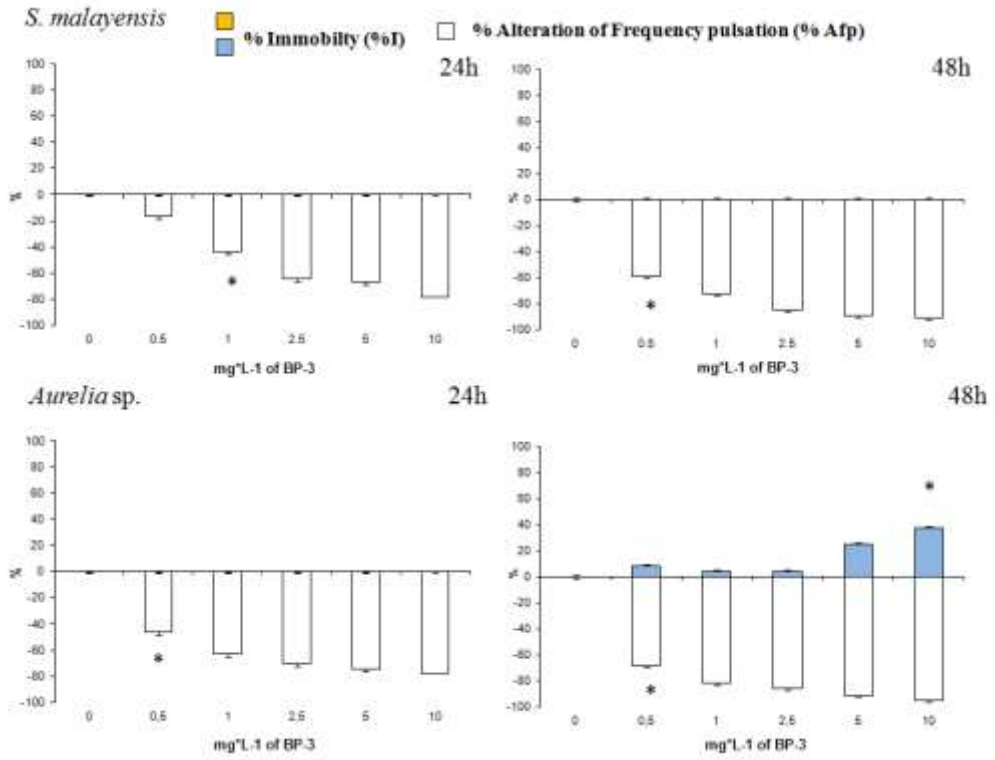

Figure 28 Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of BP-3 by semi-dynamic condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

Waterborne BP-3 did not affect *S. malayensis* ephyrae immobility at both exposure times considered. Only Frequency pulsation resulted to be significantly inhibited ($p < 0.05$) from 1 and 0.5 mg*L^{-1} after 24 and 48 h of exposition respectively. In addition, was also evident how the effect of

BP-3 on sub-lethal end-point resulted to be in a dose-dependent manner already from the lowest concentration tested (0.5 mg*L^{-1}).

It was not possible to calculate EC_{50} from Immobility for Aurelia *sp.*, (Table 15) since ephyrae never show any > 50% effect for acute end-point at both exposure times. However, at 10mg*L^{-1} the BP-3 affected significantly the Immobility after 48 hours of exposition, while no effects

were observed after short exposure time (24 h). Only Frequency pulsation, resulted to be significantly inhibited ($p < 0.05$) already at the lowest concentration of BP-3 tested (0.5 mg*L^{-1}) after both exposure times considered. In addition for all Waterborne- BP-3 concentrations tested, Frequency pulsation results to be the most sensitive end-point (in terms of magnitude of response) compared to Immobiltiy.

4.4.2 Microplastics

4.4.2.1 Mps 1-4 µm

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by static condition to different MP 1-4 µm concentrations were reported in Figure 29. Significant effect ($p < 0.05$) were observed in both species of jellyfish, end-point evaluated and exposure times.

Static condition (ET)

It was not possible to calculate EC_{50} from Immobility for *S. malayensis* , since ephyrae never show any > 50% effect for acute end-point at both exposure times. After 24 h, a difference in sensitivity between the two end-points evaluated was observed: in fact the LOEC for the % of I was 1 mg*L^{-1} while for % AFp was 0,1 mg*L^{-1}. This different was more evident after 48 h of exposure with a LOEC for the % of Immobility of 1 mg*L^{-1} and for % Alteration of Frequency pulsation of 0,01 mg*L^{-1}.

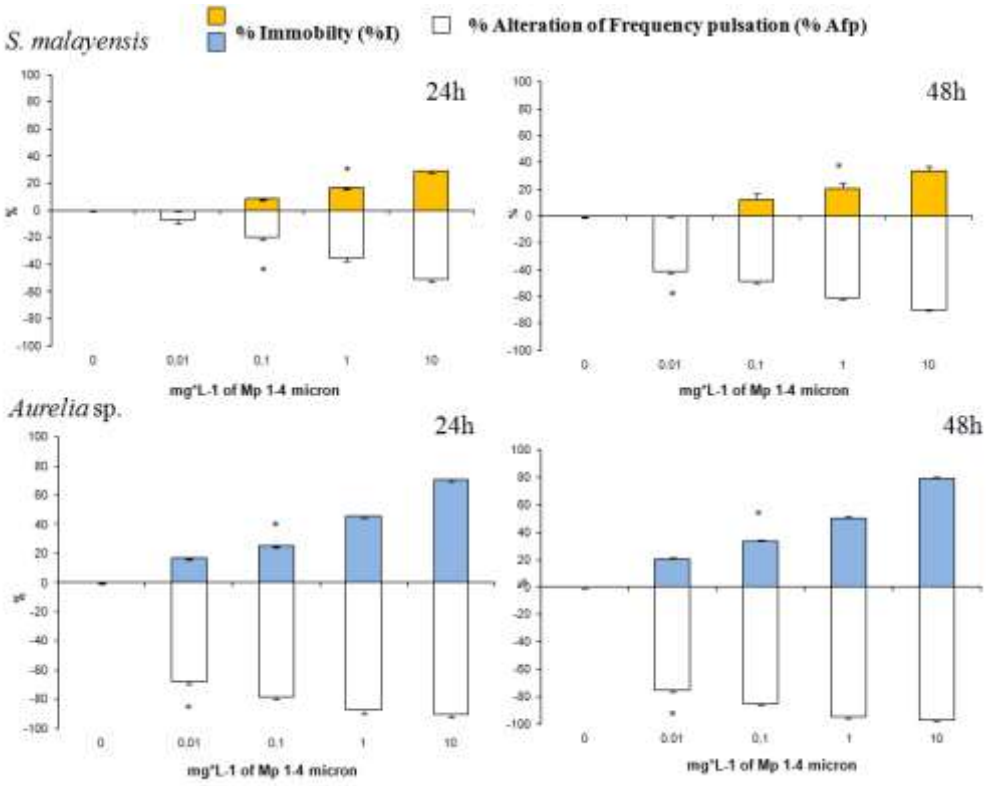

Figure 29 Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of Mp 1-4 µm by static condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

In ephyrae of *Aurelia* sp. (Table 15) was possible observe a > 50% effect for both end-point and exposure times. After both exposure times considered, a difference in sensitivity between the two end-points evaluated was observed: in fact the LOEC for the % of I was 0.1 mg*L^{-1} while for % AFp was 0,01 mg*L^{-1}.

Semi-dynamic condition (ETsd)

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by semi-dynamic condition to different MP 1-4 µm concentrations were reported in Figure 30.

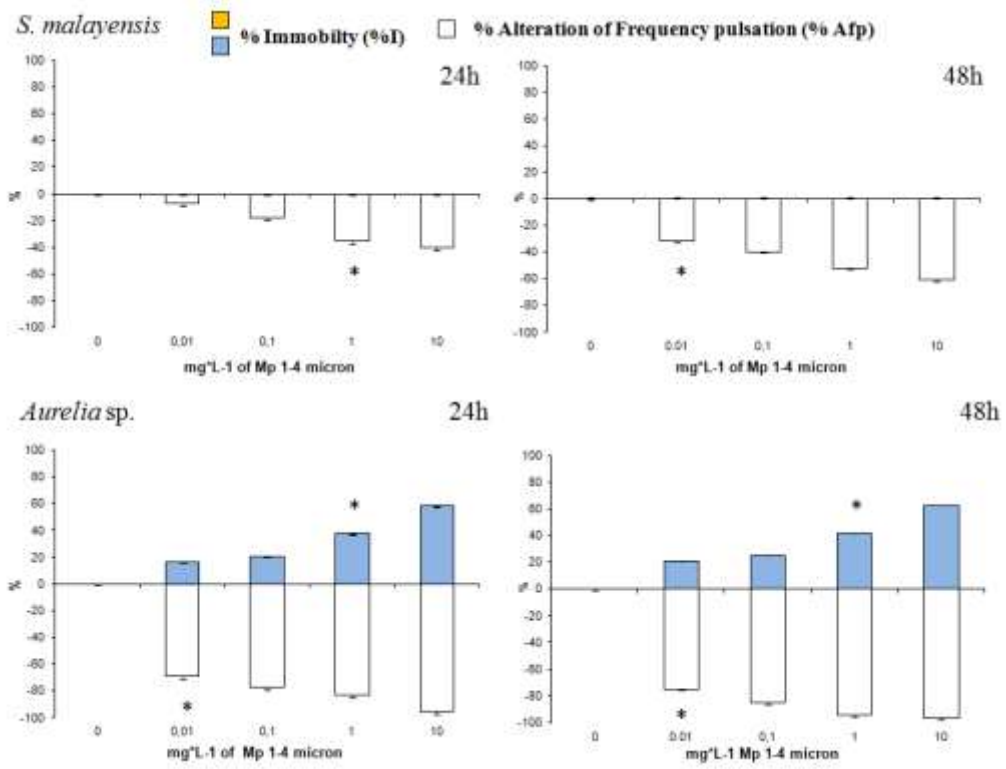

Figure 30 Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of Mp 1-4 µm by semi-dynamic condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

MP 1-4 micron did not affect *S. malayensis* ephyrae immobility at both exposure times considered. Only Frequency pulsation resulted to be significantly inhibited ($p < 0.05$) from 1 and 0.01 mg*L^{-1} after 24 and 48 h of exposition respectively.

In ephyrae of *Aurelia* sp. was possible observe a > 50% effect for both end-point and exposure times. A difference in sensitivity between the two end-points evaluated was observed: in fact the LOEC for the % of I was 1 mg*L^{-1} while for % AFp was 0,01 mg*L^{-1}. It also evident how th ephyrae of *Aurelia* sp. showed a > 50% effect for the sub-lethal end-point already at the lowest MP 1-4 µm concentration tested (0.01 mg*L^{-1}).

Mp accumulation

Microscopy observations showed that polyethylene fluorescent green microspheres were not found in ephyrae guts or gastric filaments, not even at highest concentration tested, after both exposures times and regardless of method of exposition performed. However, at 10 mg*L^{-1} by static exposition the MPs were attached onto the body surfaces of ephyrae. In *S.malayensis* (Figure 31) were also around the mouth (manubrio) and other MPs agglomerates were attached to the lappets and rhopals.

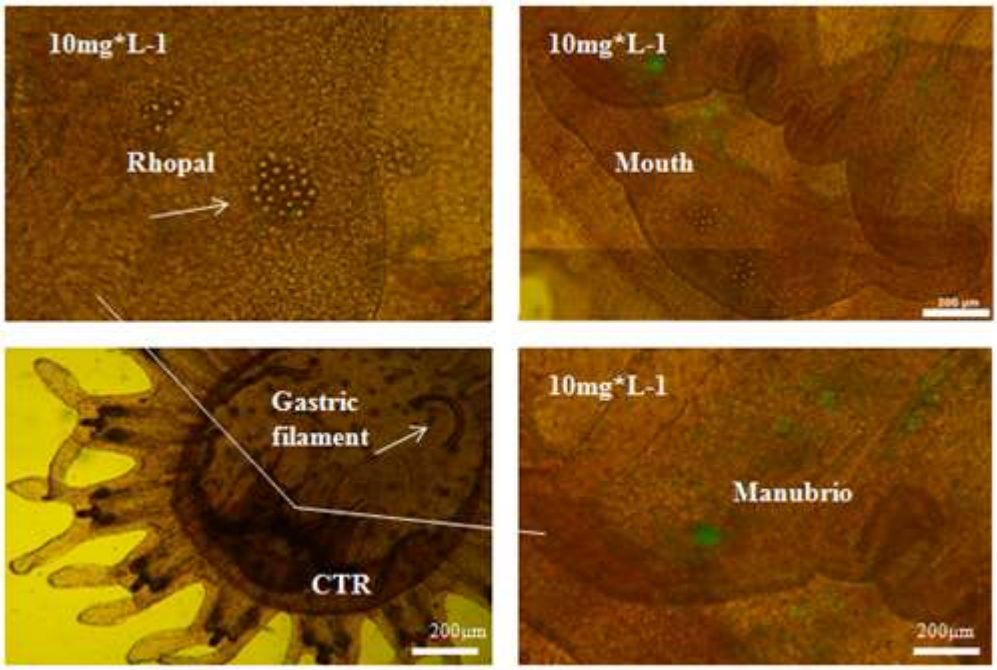

Figure 31 Ephyra of *S.malayenis* expose to 10mg*L-1 of MPs 1-4 µm for 48 hours. The MPs result to be around the mouth (manubrio) and rhopals.

While in ephyrae of *Aurelia* sp. MP were found attached on the body surface, around the mouth and lappets (Figure 32)

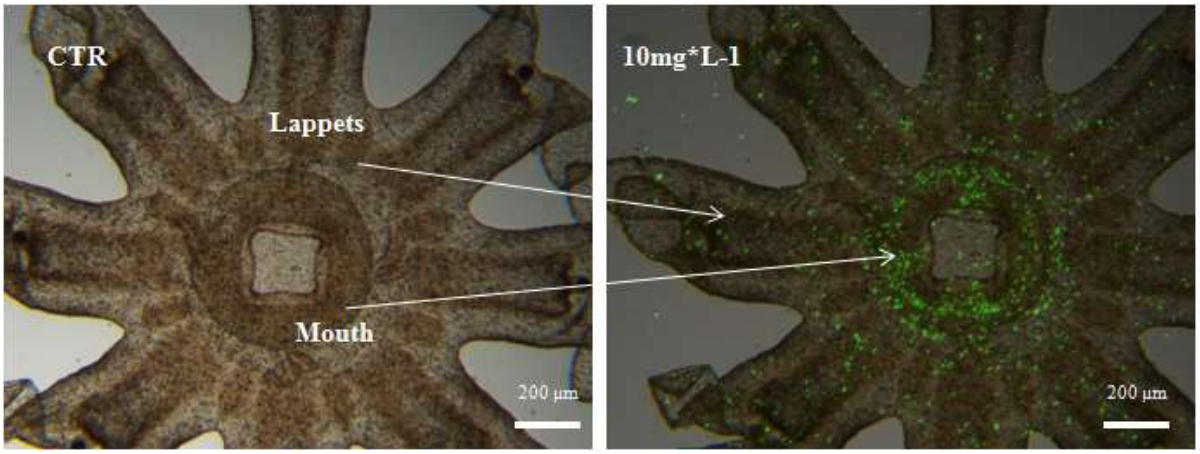

Figure 32 Ephyra of *Aurelia* sp. expose by static condition to 10mg*L-1 of MPs 1-4 µm for 48 hours. The MPs result to be attached on the body surface, around the mouth (manubrio) and on the lappets.

4.4.2.2 LDPE 4-6 µm

Static condition (ET)

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by static condition to different LDPE 4-6 µm concentrations were reported in Figure 33. It was not possible to calculate EC_{50} from Immobility for *S. malayensis* , since ephyrae never show any > 50% effect for acute end-point at both exposure times (Table 14). However, a significant effect ($p < 0.05$) on Immobility was observed in ephyrae of *S.malayensis* exposed to all concentrations at 48 h, while no significant effects were observed after short exposure time (24h). After 48 hours of exposition a difference in sensitivity between the two end-points evaluated was observed: in fact the LOEC for the % of I was 1 $mg*L^{-1}$ while for % AFp was 0,01 $mg*L^{-1}$.

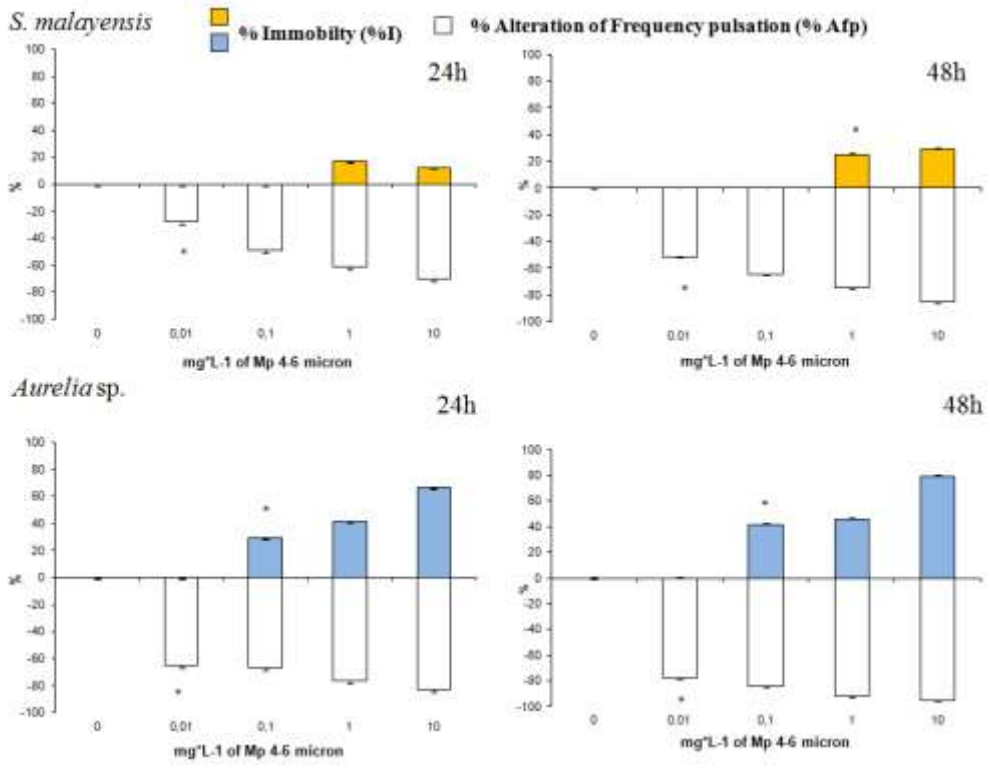

Figure 33 Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of LDPE 4-6 µm by static condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

In ephyrae of *Aurelia* sp. was possible observe a > 50% effect for both end-points evaluated and exposure times considered. A difference in sensitivity between the two end-points evaluated was observed for both exposure times, in fact the LOEC for the % of I was 0.1 mg*L^{-1} while for % AFp was 0,01 mg*L^{-1}.

Semi-dynamic condition (ETsd)

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by semi-dynamic condition to different LDPE 4-6 µm concentrations were reported in Figure 34. It was not possible to calculate EC_{50} from Immobility for *S. malayensis* , since ephyrae never show any > 50% effect for acute end-point at both exposure times.

However, a significant effect ($p < 0.05$) on both end-points at both exposure times was observed. After 24 h was evident a difference in sensitivity between Immobility and Frequency pulsation with a LOEC of 10 mg*L^{-1} and 1 mg*L^{-1} respectively.

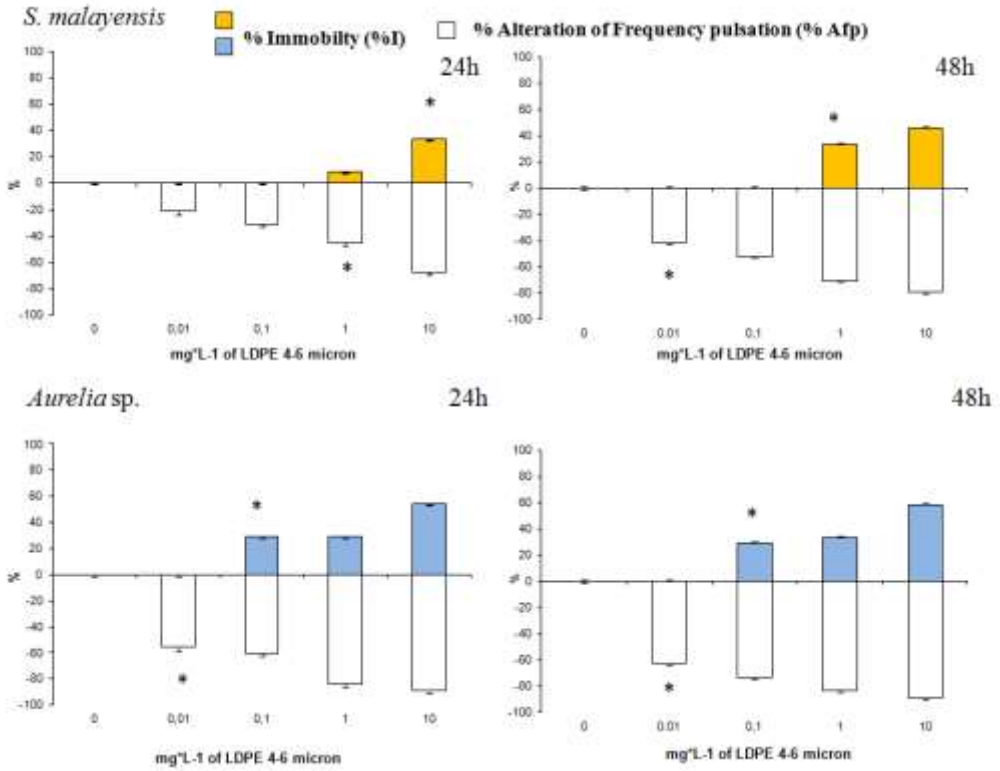

Figure 33 Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of LDPE 4-6 µm by semi-dynamic condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

This difference is more evident after 48 h of exposure with a LOEC of 0.01 mg*L-1 for Immobility and 0.01 mg*L-1 for Frequency pulsation. LDPE 4-6 µm caused a significant effect ($p < 0.05$) on both end-points evaluated on ephyrae of *Aurelia* sp. After both exposure times a difference in sensitivity between Immobility and Frequency pulsation with a LOEC of 0.01 mg*L^{-1} and 0.1 mg*L^{-1} respectively, was observed. The sub-lethal end-point results to be more sensitive (in terms of magnitude of response), compared to acute one, already from the lowest concentration of LDPE 4-6 µm tested at both exposure times.

4.4.2.3 LDPE 4-6 µm + BP-3 (Low concentration)

Static condition (ET)

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by static condition to different concentrations of LDPE 4-6 µm + BP-3 (Low concentration) were reported in Figure 35. Significant effect ($p < 0.05$) were observed in both species of jellyfish, end-point evaluated and exposure times. It was not possible to calculate EC_{50} from Immobility for *S. malayensis*, since ephyrae never show any > 50% effect for acute end-point at both exposure times. In addition, no significant effect ($p < 0.05$) in Immobility was observed in ephyrae exposed to all concentrations of LDPE + BP-3 low concentration at both exposure times. Only Frequency pulsation, resulted to significantly inhibited ($p < 0.05$) from 0.1 $mg*L^{-1}$ and 0.01 $mg*L^{-1}$ after 24 and 48 of exposure times respectively.

Oxibenzone (BP-3) LDPE low concentration caused a significant effect ($p < 0.05$) on both end-points evaluated at both exposure times. Considering results on *Aurelia* sp. After 24 h a difference in sensitivity between the two end-points was observed: in fact the LOEC for the % of Immobility was 1 $mg*L^{-1}$ while for % Alteration of Frequency pulsation was 0.01 $mg*L^{-1}$. This difference is more evident after 48 h of exposure with a LOEC for the % of I was 0.1 $mg*L^{-1}$ while for % AFp was 0.01 $mg*L^{-1}$. In addition the sub-lethal end-point results to be more sensitive (in terms of magnitude of response) then acute one at all the concentration of microplastics tested.

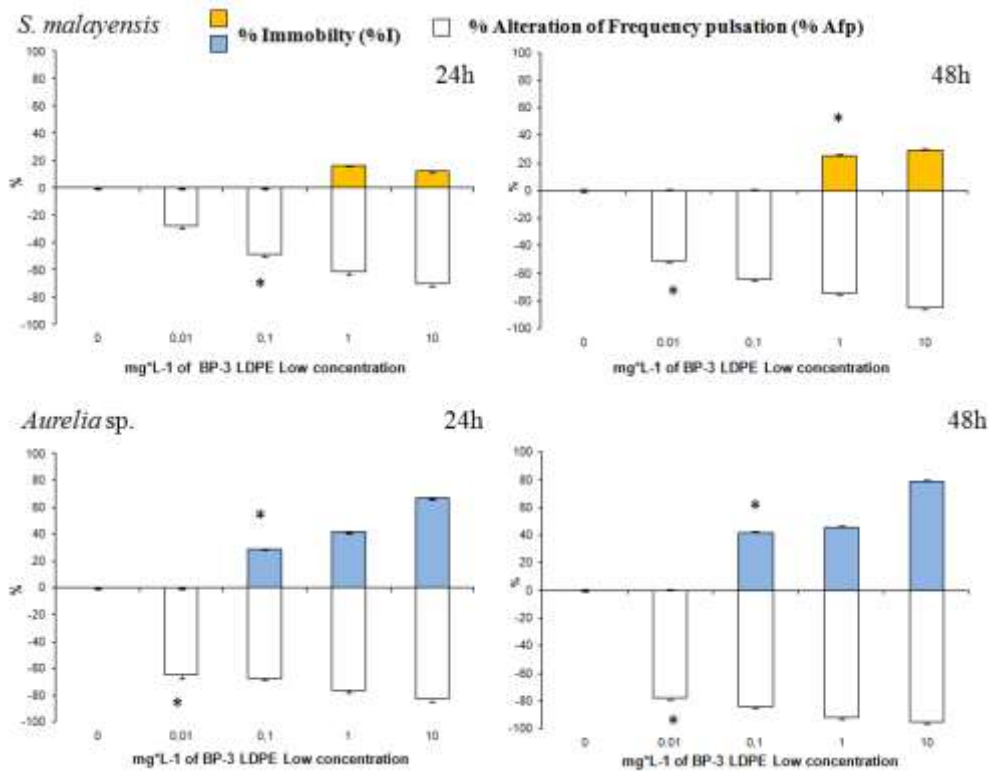

Figure 35 Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of LDPE + BP-3 low concentration by static condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

Semi-dynamic condition (ETsd)

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by semi-dynamic condition to different concentrations of Oxibenzone (BP-3) LDPE low concentration were reported in Figure 35. Significant effects ($p< 0.05$) were observed in both species of jellyfish, end-points evaluated at both exposure times considered. It was not possible to calculate EC_{50} from Immobility for *S. malayensis*, since ephyrae never show any > 50% effect for acute end-point at both exposure times (Table 14). However, significant effect ($p < 0.05$) in Immobility was observed in ephyrae exposed to LDPE + BP-3 low concentration at both exposure times.

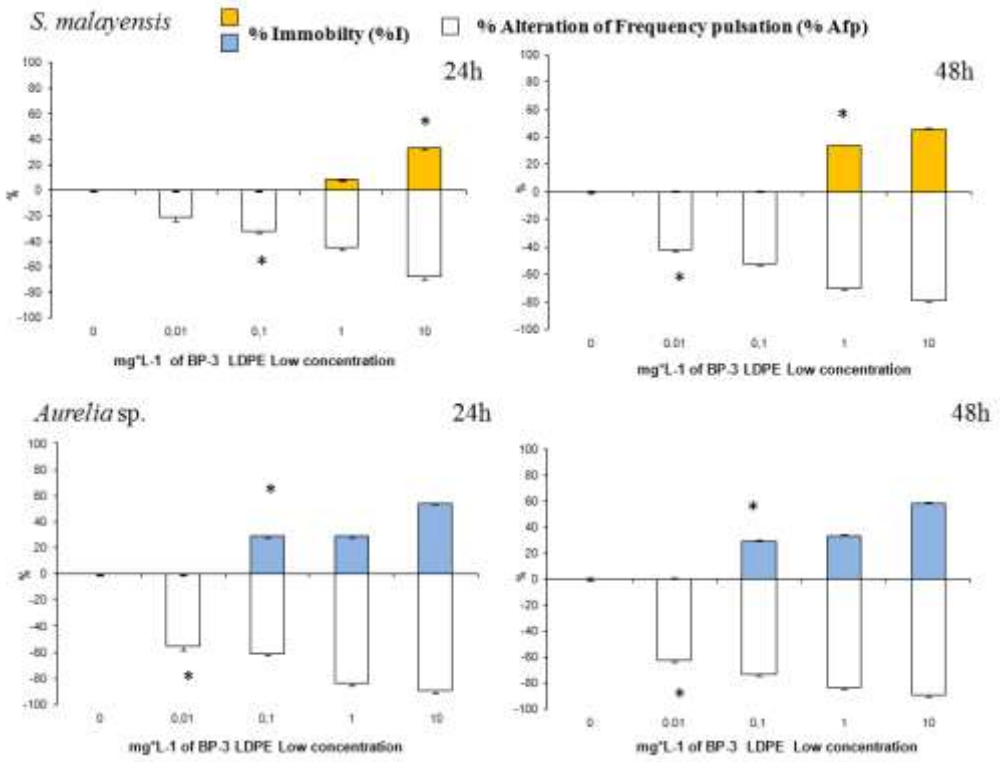

Figure 35 Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of LDPE + BP-3 low concentration by semi-dynamic condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

After 24 h a difference in sensitivity between the two end-points was observed: in fact the LOEC for the % of I was 10 mg*L^{-1} while for % AFp was 1 mg*L^{-1}. This difference is more evident after 48 h of exposure with a LOEC for the % of Immobility was 1 mg*L^{-1} while for % Alteration of Frequency pulsation was 0.01 mg*L^{-1}.

LDPE+ BP-3 low concentration caused a significant effect ($p < 0.05$) on both end-points evaluated at both exposure times on ephyrae of *Aurelia* sp. A difference in sensitivity between the two end-points was observed after both exposure times: in fact the Frequency pulsation resulted to be significantly inhibited at the lowest concentration tested (LOEC = 0.01 mg*L^{-1}), while Immobility only at the highest concentration of LDPE+ BP-3 low concentration. (LOEC = 10 mg*L^{-1}).

4.4.2.3 LDPE 4-6 µm + BP-3 (High concentration)

Static condition (ET)

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by static condition to different concentrations of LDPE+BP-3 high concentration were reported in Figure 37.

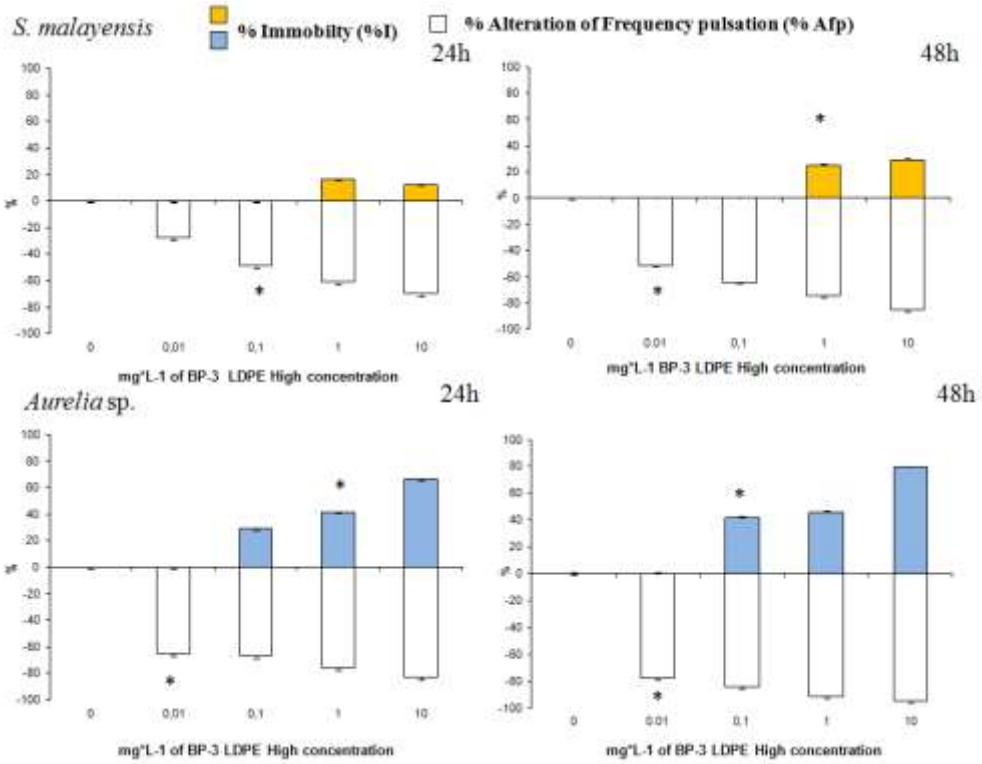

Figure 37. Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of BP-3 LDPE high concentration by static condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

It was not possible to calculate EC50 from Immobility for *S. malayensis*, since ephyrae never show any > 50% effect for acute end-point at both exposure times. Only Frequency pulsation, resulted to significantly inhibited ($p < 0.05$) from 0.1 mg*L^{-1} and 0.01 mg*L^{-1} after 24 and 48 of exposure times respectively. In ephyrae of *Aurelia* sp. a significant effect ($p < 0.05$) on both end-points evaluated at both exposure times. After 24 h a difference in sensitivity between the two end-points

was observed: in fact the LOEC for the % of I was 1 mg*L^{-1} while for % AFp was 0.01 mg*L^{-1}. This difference is less evident after 48 h of exposure with a LOEC for the % I was 0.1 mg*L^{-1} while for % AFpwas 0.01 mg*L^{-1}.

Semi-dynamic condition (ETsd)

Toxicity test results with ephyrae of *S. malayensis* and *Aurelia* sp. exposed by semi-dynamic condition to different concentrations of LDPE + BP-3 high concentration were reported in Figure 38. Significant effect ($p < 0.05$) were observed in both species of jellyfish, end-point evaluated and exposure times. It was not possible to calculate EC_{50} from Immobility for *S. malayensis*, since ephyrae never show any > 50% effect for acute end-point at both exposure times (Table 14). After 24 h a difference in sensitivity between the two end-points was observed: in fact the LOEC for the % of I was 10 mg*L^{-1} while for % AFp was 1 mg*L^{-1}. This difference is more evident after 48 h of exposure with a LOEC for the % of I was 1 mg*L^{-1} while for % AFp was 0.01 mg*L^{-1}.

On ephyrae of *Aurelia* sp. LDPE + BP-3 low concentration caused a significant effect ($p < 0.05$) on both end-points evaluated at both exposure times (Table 15). A difference in sensitivity between the two end-points was observed after both exposure times: in fact the Frequency pulsation resulted to be significantly inhibited at the lowest concentration tested (0.01 mg*L^{-1}), while Immobility only at the highest concentration of LDPE + BP-3 low concentration (10 mg*L^{-1}).

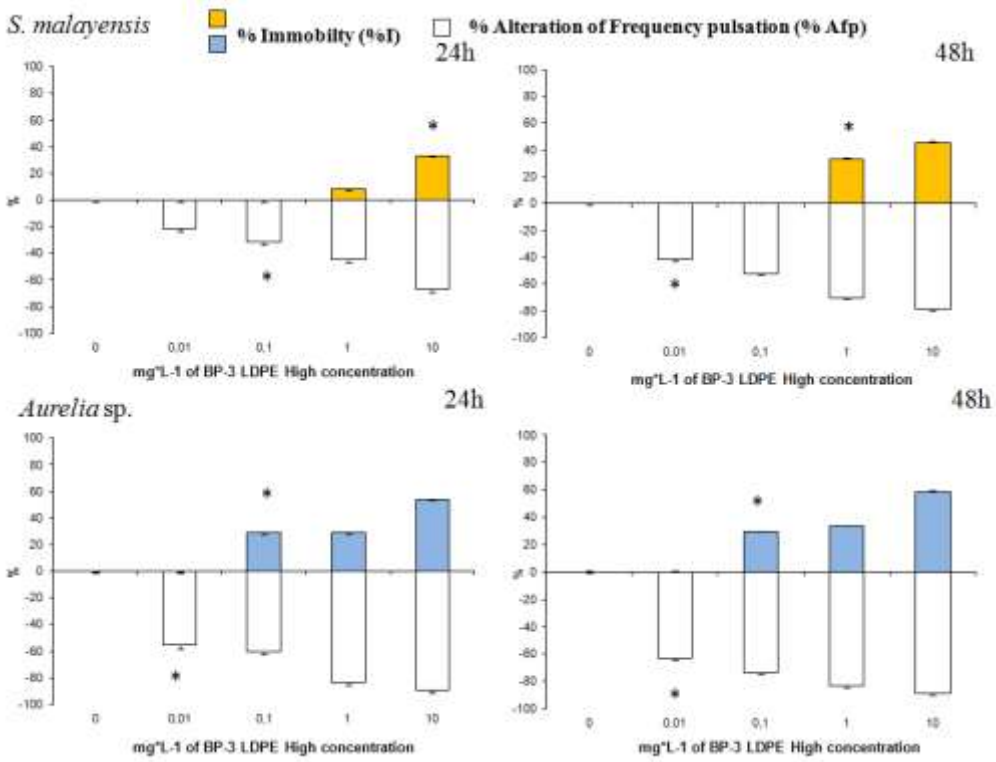

Figure 38. Alteration of Frequency of pulsation (% AFp) and immobility (% I) evaluated in ephyrae of *S. malayensis* and *Aurelia* sp. after 24 h and 48 h of exposition at increasing concentration of BP-3 LDPE high concentration by semi-dynamic condition (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

Table 14. Median effect concentration values with 95% Confidence Limits (CL) from the different tests with Mp vergin (1-4 and 4-6 µm), Oxibenezone (BP-3) and Mps spiked with Low and High BP-3 obtained from ephyrae of *S. malayensis* exposed by static and semi-dynamic conditions.

	Method of exposition			
	Static		**Semi-dynamic**	
	End-point			
Reference toxic compounds and MPs	**Immobility**	**Alteration of Frequency pulsation**	**Immobility**	**Alteration of Frequency pulsation**
Cadmium nitrate	24h-EC50=0,25 (0,23-0,30) 24h-LOEC=0.5 48h-EC50=0.16 (0.14-0.20) 48h-LOEC=0.1	24h-EC50= 0.14 (0.12-0.20) 24h-LOEC=0.05 48h-EC50= 0.02 (0.01-0.05) 48h-LOEC=0.01	24h-EC50= > 5 24h-LOEC= nc 48h-EC50= > 5 48h-LOEC= nc	24h-EC50= 0.16 (0.12-0.22) 24h-LOEC= 0.05 48h-EC50= 0.05 (0.03-0.06) 48h-LOEC= 0.01
Waterborne BP-3	24h-EC50= 6.40 (1.44-8.69) 24h-LOEC=1 48h-EC50= 3.19(1.59-4.52) 48h-LOEC=0.5	24h-EC50= 1.05(0.78-1.66) 24h-LOEC=1 48h-EC50= < 0.5 48h-LOEC=0.5	24h-EC50=> 10 24h-LOEC= nc 48h-EC50=> 10 48h-LOEC= nc	24h-EC50= 1.05(01.03-1.61) 24h-LOEC= 1 48h-EC50= < 0.5 48h-LOEC = 0.5
Mp 1-4 µm	24h-EC50= > 10 24h-LOEC=1 48h-EC50= 10 (9.88-12,36) 48h-LOEC=0.1	24h-EC50= > 10 24h-LOEC=1 48h-EC50= 0.30 (0.15-0.56) 48h-LOEC=0.01	24h-EC50=> 10 24h-LOEC=nc 48h-EC50=> 10 48h-LOEC=nc	24h-EC50= > 10 24h-LOEC=1 48h-EC50= 0.84 (0.21-3.30) 48h-LOEC=0.01
LDPE 4-6 µm	24h-EC50= > 10 24h-LOEC=nc 48h-EC50= >10 48h-LOEC=1	24h-EC50= 0.26 (0.39-4.48) 24h-LOEC=0.01 48h-EC50= < 0.01 48h-LOEC=0.01	24h-EC50=> 10 24h-LOEC=10 48h-EC50=> 10 48h-LOEC=1	24h-EC50= 1.38 (0.39-448) 24h-LOEC=1 48h-EC50= < 0.01 48h-LOEC=0.01
LDPE 4-6 µm + BP-3 Low concentration	24h-EC50= > 10 24h-LOEC=nc 48h-EC50= >10 48h-LOEC=nc	24h-EC50= 0.19 (0.08-0.45) 24h-LOEC=0.1 48h-EC50= < 0.01 48h-LOEC= 0,01	24h-EC50=> 10 24h-LOEC=10 48h-EC50= >10 48h-LOEC=1	24h-EC50=1.49(0.68-3.26) 24h-LOEC=1 48h-EC50= 0.95(0.93-1.11) 48h-LOEC=0.01
LDPE 4-6 µm + BP-3 High concentration	24h-EC50= > 10 24h-LOEC=nc 48h-EC50= >10 48h-LOEC=nc	24h-EC50= 0.14 (0.06-0.35) 24h-LOEC=0.1 48h-EC50= < 0.01 48h-LOEC=0.01	24h-EC50=> 10 24h-LOEC=10 48h-EC50= >10 48h-LOEC=1	24h-EC50= 0.95(1.03-1.30) 24h-LOEC=1 48h-EC50= 0.16(0.48-2.66) 48h-LOEC=0.01

Table 15. Median effect concentration values with 95% Confidence Limits (CL) from the different tests with Mp vergin (1-4 and 4-6 µm), Oxibenezone (BP-3) and Mps spiked with Low and High BP-3 obtained from ephyrae of *Aurelia* sp. exposed by static and semi-dynamic conditions.

	Method of exposition			
	Static		**Semi-dynamic**	
	End-point			
Reference toxic compounds and MPs	**Immobility**	**Alteration of Frequency pulsation**	**Immobility**	**Alteration of Frequency pulsation**
Cadmium nitrate	24h-EC50= 0,39 (0,36-0,46) 24h-LOEC=0.5 48h-EC50= 0.13 (0.10-0.25) 48h-LOEC=0.1	24h-EC50= 0.23 (0.20-0.27) 24h-LOEC=0.05 48h-EC50= 0.07 (0.06-0.08) 48h-LOEC=0.05	24h-EC50= > 5 24h-LOEC= nc 48h-EC50=2.99(2.96-4.78) 48h-LOEC= nc	24h-EC50=0.10(0.08-0.12) 24h-LOEC= 0.05 48h-EC50=0.05(0.03-0.06) 48h-LOEC= 0.01
Waterborne BP-3	24h-EC50= 8.39 (nc) 24h-LOEC=10 48h-EC50= 6.29 (5.79-6.62) 48h-LOEC=10	24h-EC50= 0.85(0.48-1.49) 24h-LOEC=0.5 48h-EC50= < 0.5 48h-LOEC=0.5	24h-EC50= > 10 24h-LOEC=nc 48h-EC50= > 10 48h-LOEC=nc	24h-EC50=0.59(0.43-0.81) 24h-LOEC=0.5 48h-EC50= < 0.5 48h-LOEC=0.5
Mp 1-4 µm	24h-EC50= 5.23(3.20-5.40) 24h-LOEC=0.1 48h-EC50=0.56 (0.32-0.99) 48h-LOEC=0.01	24h-EC50= < 0.01 24h-LOEC=0.01 48h-EC50= < 0.01 48h-LOEC=0.01	24h-EC50=6.33(6.05-11.10) 24h-LOEC=1 48h-EC50=3.16 (1.73-5.79) 48h-LOEC=1	24h-EC50= < 0.01 24h-LOEC=0.01 48h-EC50= < 0.01 48h-LOEC=0.01
LDPE 4-6 µm	24h-EC50= 2.10(1.20-3.10) 24h-LOEC=0.1 48h-EC50= 0.43(0.24-0.79) 48h-LOEC=0.1	24h-EC50= < 0.01 24h-LOEC=0.01 48h-EC50= < 0.01 48h-LOEC=0.01	24h-EC50=4.38 (4.05.10.04) 24h-LOEC=1 48h-EC50= 2.14 (0.72-6.41) 48h-LOEC=1	24h-EC50= < 0.01 24h-LOEC=0.01 48h-EC50= < 0.01 48h-LOEC=0.01
Oxibenzone BP-3 LDPE _Low concentration	24h-EC50= 2.38 (1.15-6.04) 24h-LOEC=1 48h-EC50= 0.82(0.20-2.30) 48h-LOEC=0.1	24h-EC50= < 0.01 24h-LOEC=0.01 48h-EC50= < 0.01 48h-LOEC=0.01	24h-EC50= 3.30(2.10-4.50) 24h-LOEC=10 48h-EC50=1.09(0.54-2.10) 48h-LOEC=10	24h-EC50= < 0.01 24h-LOEC=0.01 48h-EC50= < 0.01 48h-LOEC=0.01
Oxibenzone BP-3 LDPE _High concentration	24h-EC50= 3.10 (2.05-5.24) 24h-LOEC=1 48h-EC50= 1.02(0.90-1.25) 48h-LOEC=0.1	24h-EC50= < 0.01 24h-LOEC=0.01 48h-EC50= < 0.01 48h-LOEC=0.01	24h-EC50= 3.10(1.80-4.60) 24h-LOEC=10 48h-EC50=0.99(0.34-1.80) 48h-LOEC=1	24h-EC50= < 0.01 24h-LOEC=0.01 48h-EC50= < 0.01 48h-LOEC=0.01

4.5 Experimental activities on *Aurelia* sp. and *S.malayensis* : trophic transfer of contaminants

4.5.1 Experimental set-up to perform a simplified food chian.

4.5.1.1. "Ingestion method" and "predatory performance" to set prey/predators ratio.

Ingestion rate and Predatory performance trends of the feeding experiment with *Aurelia* sp. and *S. malayensis* ephyrae on *Artemia* sp. nauplii were reported in Figure 39 .

The Ingestion rate (blue and orange bars) was determinate as the average of the number of prey eat by *Aurelia* sp. and *S. malayensis* as function of time (5 day) at the end of feeding experiment.

In both species of jellyfish , significantly differences among the prey number chosen, was highlighted by Kruskal – Wallis analysis ($p < 0.05$) . For *Aurelia* sp., among the investigated regime of food (different number of prey), the highest Ingestion rate observed was for a concentration of 20 prey for day, with a decrease at the increasing availability of the food.

On the contrary in *S. malayensis*, the highest ingestion rate was measured with 50 prey for day, and was also observed a increase at the increasing availability of the food until this concentration. In this experiment, the mean (± se) number of prey (*Artemia* nauplii) eat by ephyrae was 13 ± 6,5 and 28 ± 13 for *Aurelia* sp. and *S.malayensis* respectively.

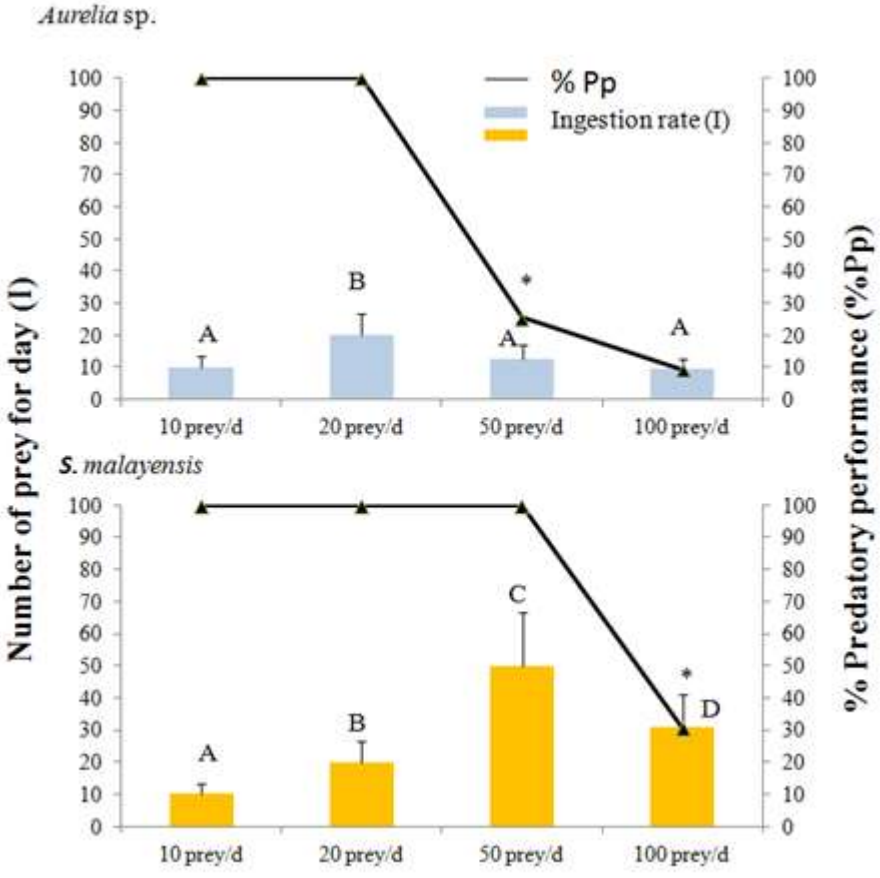

Figure 39 Ingestion rate I, (blu and orange bars with standard errors) and percentage of Predatory performance (black line) of *Aurelia* sp. and *S. malayensis* ephyrae respectively, on *Artemia* sp. nauplii (prey) measured for different number of prey for day (10-20-50 and 100 prey/d) evaluated at the end of the feeding experiment (one feeding a day). (M ±SE, n = 3). The letter A, B, C e D for Ingestion rate indicate significant differences among the groups with Kruskal-Wallis post-hoc test ($P < 0.05$). Any groups sharing the same letter are not significantly different. For Predatory performance *= $p< 0.05$ by one-way ANOVA.

The Predatory performance (black line) was significantly inhibited from the 50 prey for day in ephyra of *Aurelia* sp., while *S.malayensis* showed a significantly Predatory performance inhibition only at 100 prey of *Artemia* nauplii for day ($p < 0.05$).

The optimal number of prey 20 and 50 nauplii for *Aurelia* sp and *S.malayensis* respectively were used to perform a simplified food chain in order to evaluate the trophic transfer effect of Cadmium nitrate (Table 16).

Table 16. Test parameters and responses evaluated in the simplified food web with ephyrae of *Aurelia* sp. and *S.malayensis*

Predators	**Number of prey for each replica**	**Replicas for each concentration of reference toxicant**	**Feeding treatment**	**Parameters of exposition (volume, temperature, salinity)**	**Responses evaluated after 24h from feeding treatment (24-48-72-96 hours)**	**Responses evaluated at the end of feeding experiment (5 feeding treatment_96h)**
Aurelia sp.	20 nauplii of *Artemia* sp.	3	5 (1 feeding /day)	100 ml sea water 20°C ± 0,5 37‰±0,5	Immobiltiy Frequency of pulsation	"Ingestion rate" "Predatory performance" Disc diameter AFDW GGE%
S. malayensis	50 nauplii of *Artemia* sp.					

4.5.2 Evaluation of trophic transfer effect of Cadmium nitrate

4.5.2.1 Contamination of *Artemia* sp. with Cadmium nitrate.

In Table 17 were reported the EC_{50} and LC_{50} value from Swimming Speed Alteration and Mortality obtained exposing *Artemia* nauplii for 6 hours to Cadmium nitrate by static and semi-dynamic. In grey were highlighted the concentration of Cadmium nitrate selected to contaminate the prey to fed ephyrae jellyfish.

Table 17 EC50 and LC50 values with 95% Confidence Limits (CL) from Mortality and Swimming Speed Alteration measured on *Artemia* sp. nauplii exposed to Cadmium nitrate.

Referenc toxicant	**Tested concentration (mg*L-1-^{1})**	**Method of exposition**	**Time exposition**	**EC50-LC50(95% Confidence Limits) mg*L-1-1**
Cadmium nitrate	0-0.1-0.5-1-2-4-8-16	Static	6h	LC50= 5.56(5.10-6.40)
		Semi-dynamic	6h	LC50=5.05(4.89-5.40)
Potassium dichromate	0-0.5-1-5-10-50-100	Statico	24h	LC50= 36.98(32.26-45.84
		Semi-dynamic	24h	LC50= 34.86(32.04-42.64)
	Contamination with Cadmium nitrate mg*L^{-1}			
	0-0.1-0.5-1-2-4*	Semi-dynamic	6h	LC50= > 4 EC50=3.68(2.70-4.12)

*****the concentration (grey) were selected to contaminate the *Artemia* nauplii to fed ephyrae of *Aurelia* sp. and *S. malayensis*

4.5.2.2. "Ingestion method" and "predatory performance" .

Ingestion rate and Predatory performance trends of the feeding experiment with *Aurelia* sp. and *S. malayensis* ephyrae on *Artemia* sp. nauplii contaminated with Cadmium nitrate were reported in Figure 40. and for the control (ephyrae fed with prey not contaminated) were summarized the results in Table 18. For both species of jellyfish the effect of Cadiumin nitrate through the trophic transfer not caused significantly effect on Ingestion rate. The trends resulted to be steady independently of the metal concentrations, with an average of 18 and 45 nauplii/day for the treatment (concentration of Cadmium nitrate) compared to the controls with 20 and 50 nauplii/day, respectively for *Aurelia* sp. and *S. malayensis* (Table 18).

Considering Predatory performance (%Pp) no significant effect in ephyrae of *Aurelia* sp. were observed, while in *S. malayensis* resulted to be significantly inhibited ($p < 0.05$) from 0.5 mg*L-1-1 of Cadmium nitrate.

Table 18 .Ingestion rate (I), Predatory performance (%Pp), Disc diameter, Ash Free Dray Weigh (AFDW) and Growth Gross Efficiency (GGE%) measured in ephyrae of *Aurelia* sp. and S. *malayensis* fed with *Artemia* nauplii not contaminated .

	Model organisms	
	Aurelia sp.	*S.malayensis*
Parameters evaluated		
Ingestion rate (I prey/d-1)	20 nauplii	40 nauplii
Predatory performance (%Pp)	100%	100%
Disc diameter (mm)	4,11± 0,5	8,3± 0,5
Ash Free Dray Weigh (AFDW µg)	275,3 ± 9,8	598,4 ± 4,6
Growth Gross Efficiency (GGE %)	14%	75%

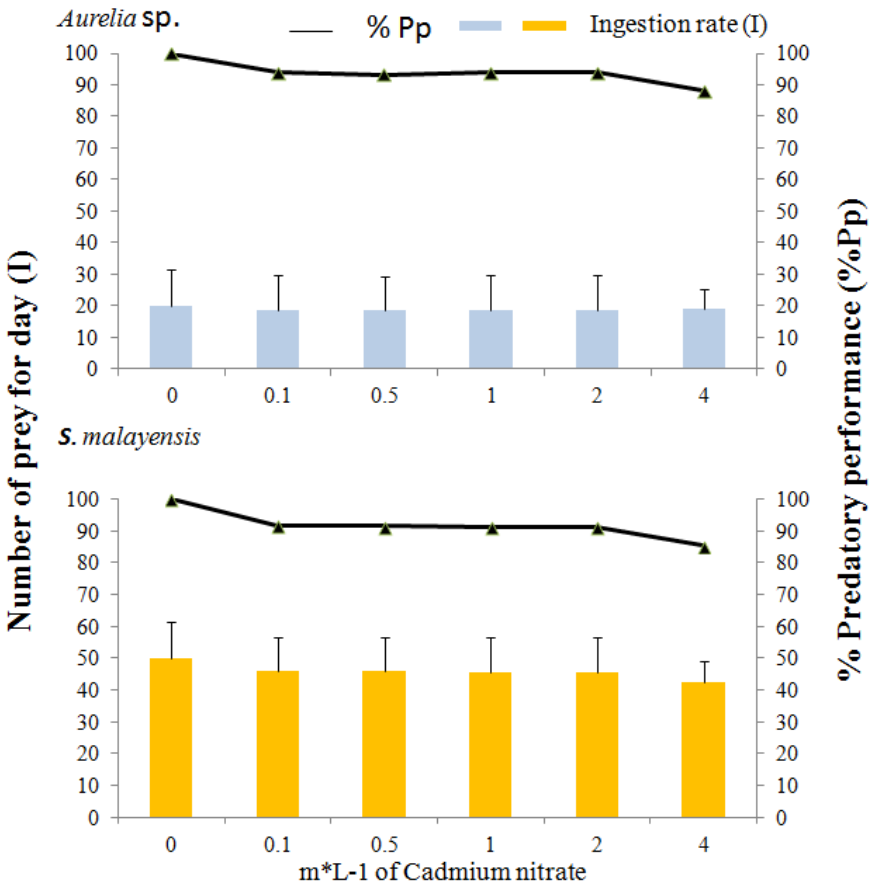

Figure 40 Ingestion rate I, (blu and orange bars with standard errors) and percentage of Predatory performance (black line) of *Aurelia* sp. and *S. malayensis* ephyrae respectively, on *Artemia* sp. nauplii (prey) measured for different concentration of Cadmium nitrate to which the prey were contaminated, evaluated at the end of the feeding experiment (one feeding a day). (M ±SE, n = 3). *= p< 0.05 by one-way ANOVA.

4.5.2.3 Biometric and bioenergetic parameters

The ash-free dry weight (AFDW) was related to the diameter (rhopalo-rhopalo) of ephyra of *Aurelia* sp. and *S.malayensis* as showed in the Figure 41 while the value measured at the end of experiment in the control (ephyrae with prey no contaminated) were summarized in Table 18.

In blue and orange were reported the results obtained in the ephyra control, measured at the end of the feeding experiment. With different colors of grey, were reported the relation between the AFDW and diameter measured for each group of ephyrae fed with *Artemia* sp. contaminated at different concentration of Cadmium nitrate.

Regarding *Aurelia* sp., the EC_{50} value was obtained only for ADFW (Table 7.4). However, significant effects ($p < 0.05$) were observed (Figure 42) in the disc diameter results from 1 $mg*L^{-1}$ and in ADFW results from 0.1 $mg*L^{-1}$ of Cadmium nitrate.

On the contrary, for Disc diameter and AFDW measured in ephyrae of *S.malayensis*, was not possible to calculate EC_{50} values, while a significant effect ($p < 0.05$) already at the lowest concentration of Cadmium nitrate to which *Artemia* nauplii were contaminated for both parameters measured (disc diameter and AFDW) as showed in Figure 43.

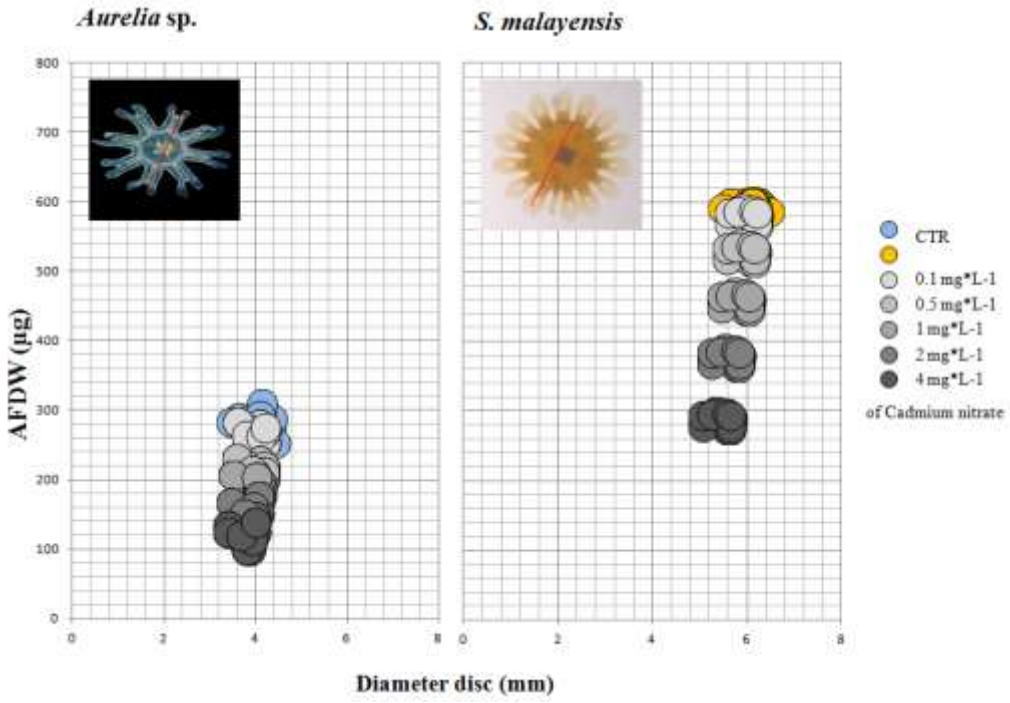

Figure 41 Relationship between respectively ash-free dry weight (AFDW) and diameter (rhopalo-rhopalo) measured in the ephyrae control (blue balls for *Aurelia* sp. and orange balls for *S.malayensis*) and in the different concentration (0.1-0.5-1-2-4 $mg*L-1^{-1}$) of Cadmium nitrate to which *Artemia* nauplii were contaminated (grey balls).

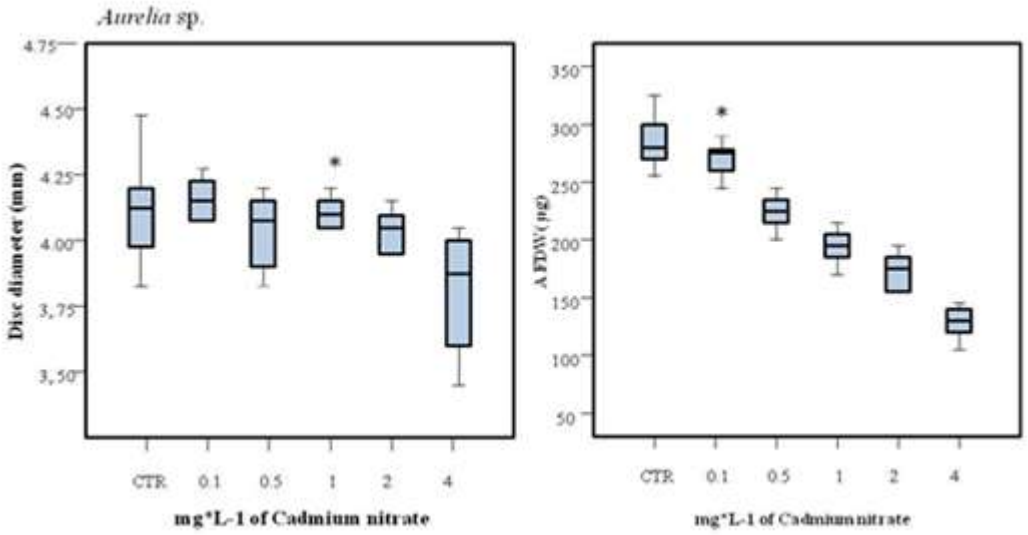

Figure. 42 Disc diameter (left graph) and ash-free dry weight (AFDW; right graph) measured in ephyrae of *Aurelia* sp. fed with Artemia nauplii no contaminated (ctr) and contaminated with different concentration of Cadmium nitrate (0.1-0.2-1-2-4 mg*L-1^{-1}) The horizontal line in the box shows the median and the whiskers show the range. *= p< 0.05 with Kruskal-Wallis post-hoc test.

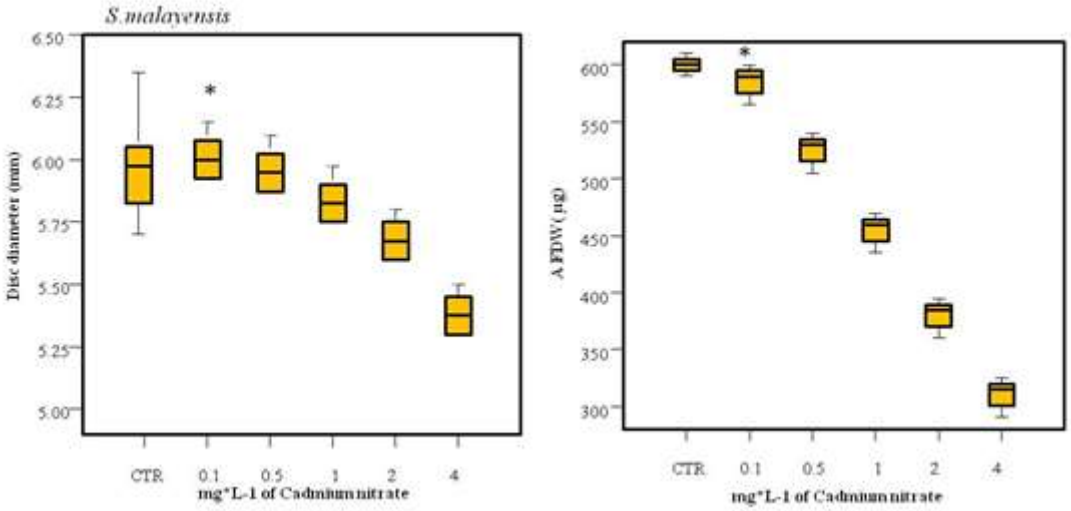

Figure 43 Disc diameter (left graph) and ash-free dry weight (AFDW; right graph) measured in ephyrae of *S.malayensis* fed with *Artemia* nauplii no contaminated (ctr) and contaminated with different concentration of Cadmium nitrate (0.1-0.2-1-2-4 mg*L-1^{-1}) The horizontal line in the box shows the median and the whiskers show the range. *= p< 0.05 with Kruskal-Wallis post-hoc test.

The Gross Growth Efficiency (GGE%) results evaluated on *Aurelia* sp. and *S. malayensis* ephyrae were reported in Figure 44. For both species of jellyfish the GGE% decreased in a dose-dependent manner to increase of Cadmium nitrate concentrations to which *Artemia* nauplii were contaminated. Cadmium nitrate through the throphic transfer, affected significantly ($p < 0.05$) the GGE% from 0.5 mg*L-1 [1]as highlighted in Figure 44.

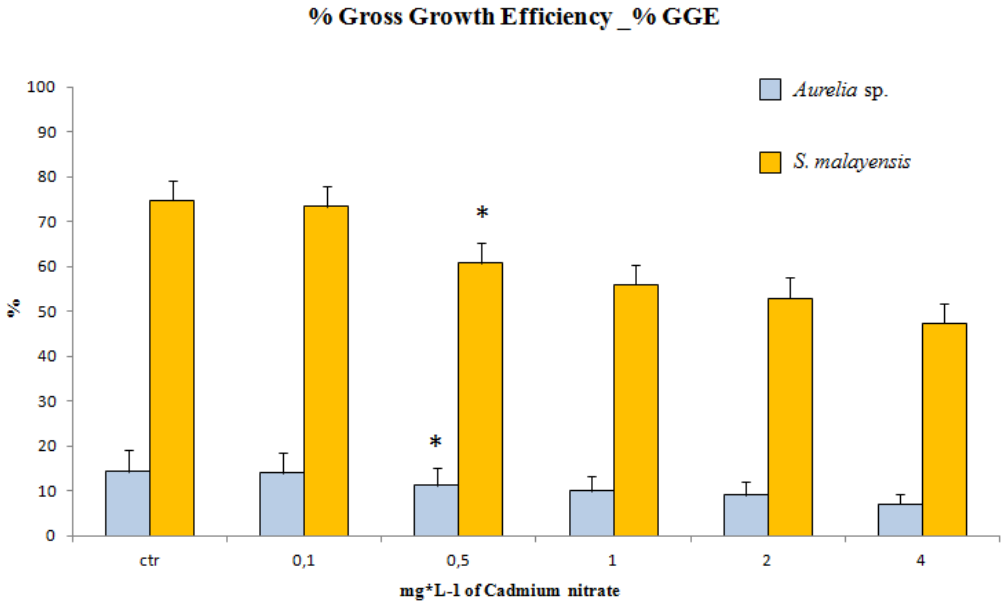

Figure 44 Percentage of Gross Growth Efficiency (GGE%) measured in ephyrae of *Aurelia* sp. (blue bars) and *S.malayensis* (orange bars) at the end of feeding experiment where the prey, *Artemia* nauplii were contaminated to different concentration of Cadmium nitrate.

Comparing the %GGE measured in ephyrae fed with prey contaminated to the control (ephyrae fed with Artemia nauplii no contaminated) was possible observed > 50% effect on GGE for *Aurelia* sp. ephyrae obtained a EC_{50} value (Table 19)

4.5.2.3 Ephyrae swimming performance

The trend of the percentage of Immobility and Alteration of Frequency of pulsation evaluated on ephyrae of *Aurelia* sp. and *S. malayensis,* after 24-48-72 and 96 hours of the feeding treatment were reported in Figure 45 and 46 respectively.

Regarding *Aurelia* sp. (Figure 45) at the first 48 hours of the feeding treatment, only the sub-lethal end-point (Alteration of Frequency pulsation) resulted to be significantly inhibited ($p < 0.05$) from 1 mg*L-1-1 and from 0.1 mg*L-1-1 respectively, while no significant effects were observed for Immobility. In the next 48 hours of experiment (72 and 96h), Cadmium nitrate through the trophic transfer seemed to cause significant effects ($p < 0.05$) on both end-points.

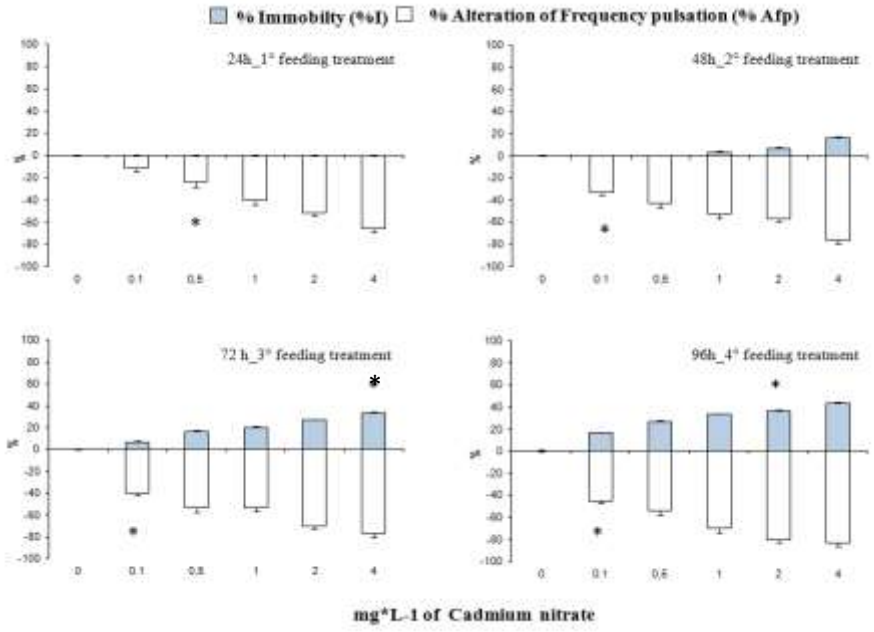

Figure 45 Alteration of Frequency of pulsation (% AFp) and immobility (% I) of ephyrae of Aurelia *sp.* after the feeding treatment (24-48-72-96 hours) with *Artemia* nauplii contaminated at increasing concentration of Cadmium nitrate (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

However, during the feeding experiment, the sub-lethal end-point result to be always more sensitive (in term of magnitude of the response) than immobility.

It was in agreement with the LOEC (Table 19a) that was for Immobility 4 mg*L-1^{-1} and 1 mg*L-1^{-1} at the 72 and 96 hours, while for Frequency pulsation was 0.5 mg*L-1^{-1} at 24 hours and 0.1 mg*L-1^{-1} from 48 hours from first treatment feeding.

In addition, it was possible to calculate EC_{50} only for the sub-lethal end-point, since ephyrae never show any > 50% effect for Immobility and for any time of the feeding treatment (Table 19a).

S.malayensis in Figure 45 showed as only at 96 hours of the feeding experiment , the Immobility resulted to be significantly affected ($p < 0.05$) from 1 mg*L-1-1 of Cadmium nitrate, while no significant effects were observed after short time considered (24- 48-72h).

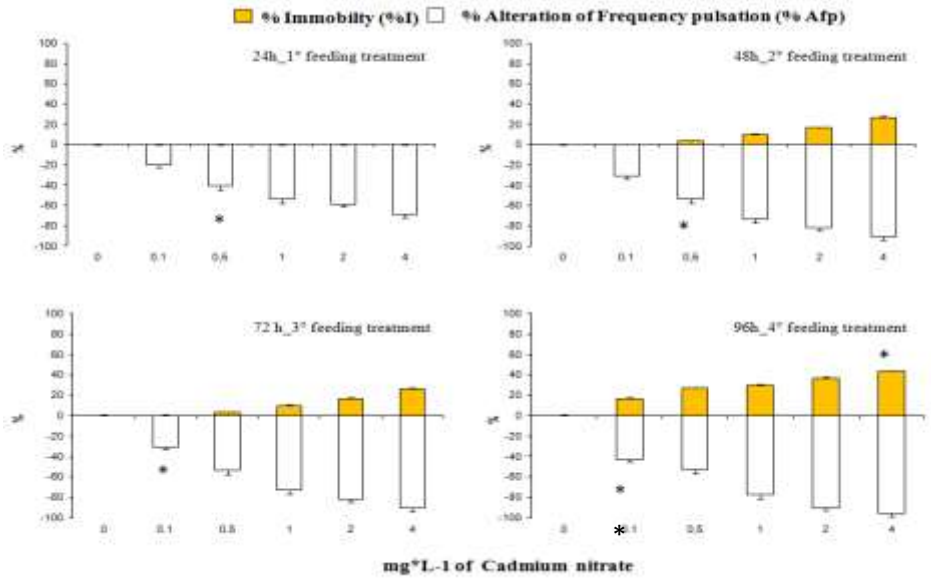

Figure 46. Alteration of Frequency of pulsation (% AFp) and immobility (% I) of ephyrae of *S.malayensis* after the feeding treatment (24-48-72-96 hours) with *Artemia* nauplii contaminated at increasing concentration of Cadmium nitrate (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

It was not possible to calculate EC_{50} for immobility, since ephyrae never show any > 50% effect for any feeding time considered, but it was possible to calculate a LOEC at 96h (1 mg*L-1^{-1}) (Table 15a).

Only ephyrae swimming performance already at 24 hours from the first feeding treatment resulted to be significantly inhibited ($p < 0.05$) at 0.5 mg*L^{-1}. It was possible to calculate EC_{50} and the LOEC for the sub-lethal at all time of feeding treatment considered (Table 19a). Also for *S. malayensis* as well as for *Aurelia* sp. the Frequency pulsation resulted to be more sensitive than immobility in all time of feeding treatment considered.

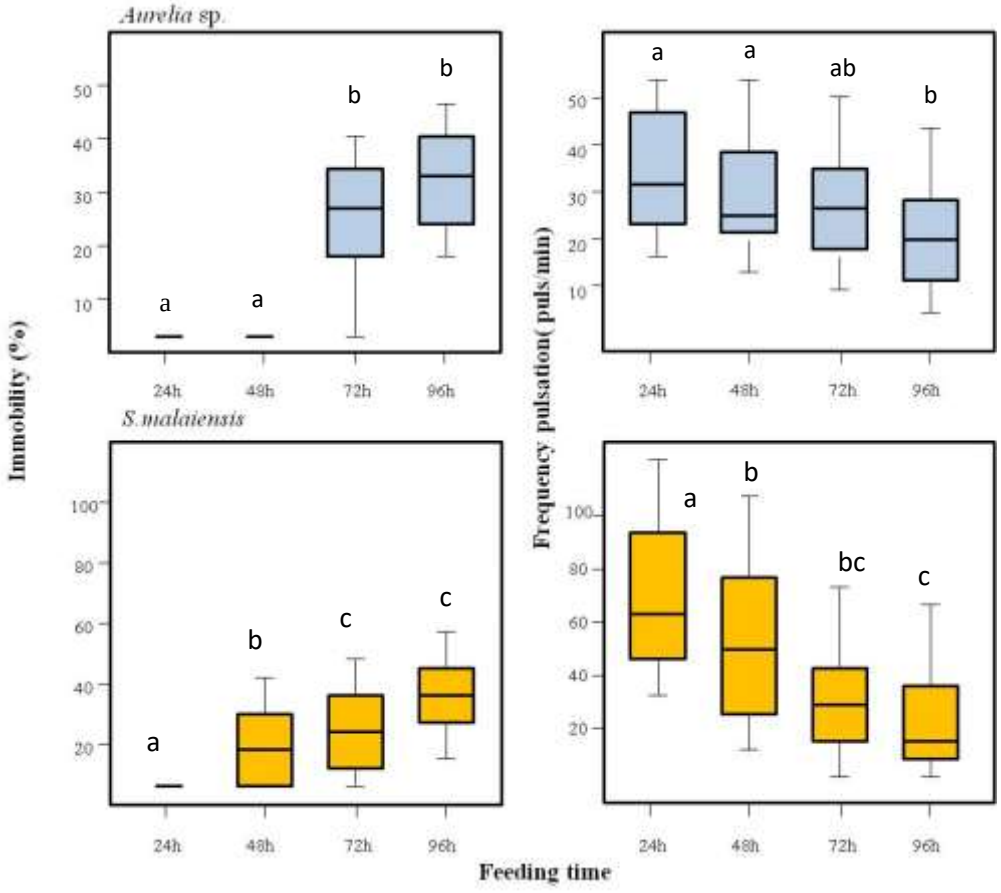

Figure 47 Percentage of Immobility and Frequency of pulsation measured in ephyrae of *Aurelia* sp. and *S.malayensis* (fed with *Artemia* nauplii contaminated with Cadmium nitrate) after different sampling time considered in the feeding experiment (24-48-72-96 hours from the first feeding treatment). The horizontal line in the box shows the median and the whiskers show the range. The letter a, b and c indicate significantly different groups with Kruskal-Wallis post-hoc test (P <0.05). Any groups sharing the same letter are not significantly different.

The results, showed as the effect of Cadmium nitrate through the trophic transfer increased to increase the hours of feeding experiment. Statistical analysis on Immobility and Frequency pulsation data highlighted significantly differences ($p < 0.05$) among the feeding time considered for both end-point evaluated (Figure. 47).

Table 19. EC_{50} and LOEC values with 95% Confidence Limits (CL) evaluated for different end-point measured in ephyrae of *S. malayensis* and *Aurelia* sp. fed with *Artemia* nauplii contaminated by Cadmium nitrate (a) and by direct exposition in semi-dynamic bioassay (b) *Boxes red, results at the same time of exposition.

a)

		Model organisms			
	Time (hours)	*Aurelia* sp.		*S.malayensis*	
Toxic compound		**Cadmium nitrate**			
End-point		LOEC (mg*L-1-1)	EC50 mg*L-1-1 (95% CL)	LOEC (mg*L-1-1)	EC50mg*L-1-1 (95% CL)
Ingestion rate (%Ir)	96	-	-	-	-
Predatory performance (%Pp)	96	-	-	0.1	-
Disc diameter	96	1	Nc	0.1	Nc
AFDW	96	0.1	3.06(2.09-4.50)	0.1	Nc
%AGGE	96	0.5	3.82(2.48-4.88)	0.5	Nc
%Immobility	24	nc	Nc	nc	Nc
	48	nc	nc	nc	nc
	72	4	nc	nc	nc
	96	1	nc	1	2.83(1.25-6.39)
%Altration of Frequency pulsation	24	1	1.75(1.30-2.55)	0.5	0.94(0.66-1.35)
	48	0.1	0.81(0.50-1.32)	0.1	0.35(0.25-0.51)
	72	0.1	0.40(0.19-0.84)	0.1	0.29(0.12-0.68)
	96	0.1	0.26(0.08-0.87)	0.1	nc < 0.1

b)

		Aurelia sp.		*S.malayensis*	
→	24	Nc	>5	Nc	>5
	48	1	2.99(1.86-4.80	Nc	>5
→	24	0.05	0.10 (0.07-0.13)	0.05	0.16(0.13-0.20)
	48	0.01	0.05 (0.04-0.07)	0.01	0.05(0.04-0.07)

5. DISCUSSION

5.1 Experimental activities on *Aurelia* sp.

5.1.1 Neurotoxic compounds

Most of carbamate (CB) pesticides and organophosphate (OP) insecticides, like Eserine (ES) and Chlorpyrifos (CPF), are neurotoxic compounds and their toxicity to aquatic organism is well known (Pesando et al. 2003; Pope, 2010; Eamkamon et al. 2012).

Recently, the inhibition of Acetylcholinesterase (AchE) enzyme in the nervous system of vertebrate and invertebrate aquatic organisms has been established as a useful indicator of OP and CB pesticides exposure (Key et al.2013). It has been shown that these neurotoxic compounds exert their effect at nervous system level, affecting the AchE, an enzyme that ensures the neurotransmission at synapses of neuromuscular and interneuronal junctions (Pope et al. 2010). Moreover, it has been demonstrated that CPF binds irreversibly the enzyme AchE, while the ES competes with its natural substrate (Pesando et al.2003). In this study the results on ephyra of *Aurelia sp.* (Figure 15 and 16) exposed to ES and CPF highlighted that the sub-lethal end-point (% AFp) seemed to be more sensitive than immobility (% I) for both neurotoxic compounds, in agreement with the observation by Faimali et al. (2014), who exposed this model organism to Cadmium nitrate and SDS. In particular the ES (Figure 15) showed a marked differences between acute and sub-lethal responses, more evident after 24 h than 48 h of exposure. Indeed, the %AFp seemed to be at least of two orders of magnitude lower than the sensitivity threshold showed by % I. This difference was evident comparing the $LOEC_{AFp}$ (0.5 mg L^{-1}) to the $LOEC_I$ one (50 mg L^{-1}, Table 6). On the contrary, after 48h of exposure the % AFp seemed to be at least of one order of magnitude lower than the sensitivity threshold showed by % I. The $LOEC_{AFp}$ values were found to be 0.1 mg L^{-1} while the $LOEC_I$ value was 1 mg L^{-1} (Table 6). Furthermore, ephyrae after both the time of exposure to low concentration of ES (0.05 mg L^{-1}) showed a significant stimulation (ANOVA, $p<0.05$) of the %

AFp; this phenomenon could be ascribed to the so-called hormesis, as suggested by Calabrese and Baldwin (2001). Such results could be interpreted considering that many biological systems, at low levels of toxicity, displaying an overcompensation response to homeostasis disruption, with a response curve, showing an apparent low-dose stimulation (Calabrese and Blain 2005; Costantini et al. 2010; Mesarič et al. 2013). This phenomenon is now widely recognized in the field of ecotoxicology (Calabrese and Baldwin, 2001; Chapman et al. 2001) and the hormetic responses have to be taken into consideration when determining the lowest effect concentration (LOEC).

Considering results obtained exposing ephyrae of *Aurelia sp.*to CPF (Figure 16), a significant effect on both end-points was observed, but for this neurotoxic compound the differences between acute and sub-lethal responses were less marked than those observed with ES. The % AFp seemed to be at least of one order of magnitude lower than the sensitivity threshold showed by % I. This difference was only evident after 24h of exposure with a $LOEC_{AFp}$ of 0.1 mg L^{-1} and a $LOEC_{I}$ value of 1 mg L^{-1} (Table 6). Moreover, between two neurotoxic compounds tested on ephyrae of *Aurelia sp.*, CPF resulted more toxic than ES. In particular, it is evident (Table 6) from EC_{50} values for both end-point after 24h and 48h of exposure and it could be in agreement with their different mechanism to exert their effect at nervous system level (Pesando et al.2003). In addition to alteration of swimming behaviour, change in morphology, coloration (darkening) and complete disintegration of tissue were among the physical abnormalities observed in ephyrae of *Aurelia* sp. exposed to ES and CPF (Figure 15 and 16). In agreement with a recently study by Echols et al. (2016) the effects of neurotoxic compound suggested a condition of "moderate and several" stress in ephyrae of *Aurelia* sp. Overall, teratological effects in jellyfish may include discoloration, signs of dehydration and the deformation of structural morphology and loss of tissue integrity as reported by Spangemberg (1984), Neff and Anderson (1981) exposing ephyrae of *Aurelia* sp. to hydrocarbos and oil-disperdant mixture. Moreover, stressed ephyra may also exhibit irregular muscle contractions as highlited by ES and CPF with a significant ($p < 0.05$) alteration of Frequency pulsation already at the lowest concentrations of neurtoxic compounds tested.

According to the toxicity categories based on EC_{50} values established at European level, the CPF, that is classified as priority substance (since it may potentially cause deleterious effects at environmentally relevant concentrations; 2000/60/EC), can be ranked as "very toxic" (EC_{50} < 1mg* L^{-1}) to ephyrae of *Aurelia* sp. (Table 6). Only few ecotoxicological data are reported for ephyrae of jellyfish, and in particular due to the lack of knowledge about the CB and OP toxicity, in this study we compared the EC_{50} values obtained by % AFp and % I for *Aurelia sp.*with LC_{50} and EC_{50} values for other biological models (cnidarians, crustaceans, molluscs, rotifers and echinoderms) exposed to the same neurotoxic compounds. The comparison of EC_{50} obtained with *Aurelia* sp. exposed to ES with other marine invertebrates is reported in Table 7.

The % of AFp of *Aurelia sp.*ephyrae appears to be the most sensitive end-point among the considered model organisms. Considering the toxic effect of the carbamate ES, it could be hypothesized that the inhibition of the periodic contractions of *Aurelia* sp. ephyrae was due to a similar mechanisms of that highlighted in other marine organisms as crustaceans and rotifers (Faimali et al. 2006 ; Garaventa et al. 2010). Some authors showed that the ES neurotoxicity in crustaceans larvae such as *A. salina* has been related to the inhibition of the motor control of somatic muscles during development, and in particular to the inhibition of AchE activity (Charoy and Jensen, 1999; Varo et al. 2002; Rao et al. 2007). Faimali et al. (2014) in agreement with other authors (McHenry et al. 2003; Rakow and Graham, 2006; Metanoski et al. 2004; Newroth et al. 2014) have highlighted a marked swimming activity in ephyrae of this species, that did not just passively float but were also able to carry out an extremely active swimming behaviour. Moreover, a role of neuronal control has been proved to regulate the periodic contractions of *Aurelia sp.* body, in order to generate ring-vortices of the surrounding medium (Satterlie, 2002; Watanabe et al. 2009). In the aquatic environment, the CPF toxicity to marine organisms is well known and the studies reported by some authors highlighted that the general pattern of the biological effects of this neurotoxic compound (OP) on the organism with a simple anatomical and physiological structure is different from that observed for other species belonging to higher level of biological organization

(Key et al. 2013). For ephyrae of *Aurelia sp.*exposed to CPF, the higher sensitivity for both end-points was particularly evident observing EC_{50} values for % AFp and % I compared with LC_{50} and EC_{50} values of other model organism (Table 8). The % AFp and % I were found to be more sensitive compared to mortality and luminescence inhibition of *Hydra attenuata* (Cnidarian) and of *Vibrio fischeri*, respectively (Table). These results could be explained considering the level of organization of the nervous system of *Hydra* and *V. fischeri* compared to that of Scyphozoa jellyfish (Watanabe et al. 2009). In fact, some author report that the low sensitivity to OP could be due to the simple nervous system to centralize vital functions consequently leading to a lower impact when compared with organisms having specialized or very specialized systems (Demetrio et al. 2012; Aydin et al. 2011). Moreover, the % AFp and the % I values for *Aurelia sp.* (Table 6) seems to be lower than mortality value of the most sensitive marine crustaceans and echinoderms to OP compounds as *A. salina* (Varó et al.2002) and *Paracentrotus lividus* (Bellas et al. 2005). Furthermore, Varó et al. (2002) and Rao et al. (2007), have demonstrated that CPF is able to inhibit the ChE activity and the morphology and locomotors behaviour of brine shrimp of *A. salina* nauplii.Therefore, it is could hypothesize that this neurotoxic compound exerted their toxicity in ephyrae of *Aurelia* sp. with the same mechanism and also the presence of AChE in jellyfish, since it has been demonstrated its gene homology among Cnidarians, and in particular in developing and juvenile stages of hydrozoans and anemone (Falugi et al. 1998; Anctil et al. 2009; Takahashi et al. 2009). Finally, the comparison of the EC_{50} values obtained in this study exposing the ephyrae of *Aurelia sp.*to ES and CPF and the EC_{50} - LC_{50} found in literature with other marine invertebrates, indicates that jellyfish were more sensitive than other model organisms used for ecotoxicological bioassays. However, due to the lack of knowledge about the cholinergic system in jellyfish, currently we can only hypothesize the mechanism through which these compounds exert their toxicity in this new model organism and for this reason further experiments will be performed to understand the AchE activity at the neurotransmission level in ephyrae of *Aurelia* sp. and how it is affected by marine environmental pollutants. Further investigation, could be needed to provide

information in this concern since the early behavioral and morphological responses observed in epehyrae of *Aurelia* sp. exposed to neurotoxic compound, considering the classical stress syndrome (also called general adaptaon syndrome or GAS) (Selye, 1950b; Echols et al. 2016) could be relevant to understand the the first indication to a non-optimum environment condition.

5.1.2 Ostreopsis cf.avata strains

In general, most studies addressed at increasing knowledge on bloom dynamics consider different factors separately (e.g. physical forcing, cell cycle, seasonal variability) and this approach prevents to reach a global view.

Jellyfish and dinoflagellates are marine organisms with complex life cycles that combine benthic and planktonic phases, and for this reason investigating the potential interaction between such organisms requires to take into consideration their different life cycle stages. In particular, despite the relevance of the biological interactions (e.g. predation, competition) among different groups of organisms, these aspects are seldom investigated, in part due to experimental constraints. This approach has been taken into account in this study, which reports, for the first time, the different effects of vegetative cells of *O*. cf. *ovata* on ephyrae stage of *Aurelia* sp. As regard, the results highlighted that the planktonic ephyrae suffered strong negative effects in terms of swimming behavior even at 10 cells/mL, a concentration that commonly occurs in the Mediterranean Sea (e.g. Vila et al. 2001; Mangialajo et al, 2008; Asnaghi et al. 2012; Vila et al. 2012) as well as in other regions (Shears and Ross, 2009; Rhodes et al. 2011).

In particular, comparisons among other model organisms exposed to this dinoflagellate, under similar experimental conditions, highlighted that *Aurelia* sp. ephyrae seemed to be the most sensitive model organism tested so far, showing an immobility EC_{50} of 10.5 cells/mL after 24 h. Conversely, *A. salina* nauplii, one of the most common and standard model organisms used in ecotoxicology (Artoxkit, 1990) recorded an EC_{50} > 400 cells/mL and <4 cells/mL after 24 and 48 h

of exposure, respectively (Table 9). As regard, the high sensitivity of *A. salina* exposed to this harmful dinoflagelate was explained by Faimali and co-authors, (2012) how alteration mechanisms of ion homeostasis by *O.* cf. *ovata* toxins like palitoxine (PTX) (Rossini and Bigiani, 2011). Despite, have been well documented the toxicological effects of PTXs on mammalian models and recently on marine invertebrates to cause physiological, biochemical and cellular alterations on antioxidant system, biotransformation mechanisms, lysosomal membrane stability and immune defences after exposure in laboratory conditions (Hégaret and Wikfors, 2005; Gorbi et al. 2012), however there is the totally lacking of information about toxicological mechanism of these toxins on jellyfish. In this study the inhibition of pulsation observed in ephyrae could be explained with a probably mechanical disturbance by dinoflagellate cells being attached around the gelatinous body surface of ephyrae as displayed in Figure 19. In general, to date, studies addressing possible relationships among gelatinous zooplankton and microalgae are rare, although algal and jelly blooms are gaining more attention from scientists and stakeholders involved in fisheries, aquaculture or marine touristic activities (Boero, 2013; Sun et al. 2015; Wang et al. 2015). Wang et al. (2015) investigated the effect of cultures of one dinoflagellate (*Prorocentrum donghaiense*) and one diatom (*Skeletonema costatum*), both high-density bloom forming but non-toxic species, as food source for *Aurelia* sp. polyps. Polyps survived until the end of the experiment (77 days) showing reproduction through strobilation; however, the abundance of ephyrae released was lower than that produced by polyps fed on *A. salina* nauplii. Consequently, although phytoplankton can serve as a nutrient source, it may represent a lower quality food than nauplii. Other studies also speculated on the possibility that *Aurelia* sp. could ingest phytoplankton and younger ephyrae could utilize it for growth (Bamstedt et al. 2001). Positive effects, in terms of increased diameter of ephyrae, due to the presence of low concentration of the dinoflagellate *Alexandrium catenella* (25 cells/mL of both toxic and not toxic strains) was recorded by Huang et al. (2013) during the early four days of incubation. However, at the end of the experiment (8 days), ephyrae exposed to the toxic strain started to shrink and died, while not toxic culture resulted in stopping the growth. Thus,

again, phytoplankton turned out as low quality food for jellyfish. The same study (Huang et al. 2013) also states that saxitoxin and its analogs produced by *A. catenella*, species that blooms also in the Mediterranean Sea and is responsible of paralytic shellfish poisoning (Bravo et al. 2008), seem to affect this model organism (the *Aurelia* sp. ephyra). In fact, a positive correlation was detected between increased concentration of toxic strain of *A. catenella* and inhibition of ephyrae's swimming rate over 12 h of exposure. In particular, a decreased pulsation rate was shown with 50 and 100 cells/mL and almost motionless conditions, coupled with a lack of recover, were observed in ephyrae exposed to 300 cells/mL. In this study also highlights increased toxic effects due to increased densities of *O*. cf. *ovata*, but this dinoflagellate seems to be more noxious than *A. catenella*: in fact *Aurelia* sp. ephyrae showed a stronger alteration of pulsation frequency (% AFp, –81%) and higher immobility values (54 %) already from exposure to 10 cells/mL. Despite these findings, it is important to point out that higher toxicity on a specific model organism (jellyfish species in our case) caused by *O*. cf. *ovata* than *A. catenella* does not necessarily imply the same effects on other species, and this is especially true when comparing among different models (for example crustaceans, fish, mammals including humans; Ramos et al. 2010). A large variability is also expected in the concentration of toxins among strains of the same species (Bachvaroff et al. 2009, and references therein). Given all the above and the different cellular mechanism of saxitoxin and ovatoxins, these findings do not imply that *O*. cf. *ovata* is in general more harmful than *A. catenella*. Moreover, the mucilage produced by *O*. cf. *ovata*, which was naturally present in the whole culture tested, could play a relevant role affecting pulsation frequency or immobility on ephyrae, as described by other studies (Privitera et al. 2012; Giussani et al. 2015). These outcomes suggest an interesting scenario on these two bloom forming species, reporting that could reduce proliferation of the planktonic *Aurelia* sp. in the natural marine environment and that *O*. cf. *ovate* affect the frequency pulsation of ephyrae jellyfish changing their swimming behavior. Lastly, considering laboratory studies often cannot fully mimic nature, further works are necessary to

understand the ecological and toxicological implication between these two bloom forming species in the natural marine environment.

5.2 Experimental activities on *Sanderia malayensis*

5.2.1 Methodological parameters investigated to develop a protocol testing with ephyrae of *S.malayensis*

The purpose of the first part of this study was to experimentally investigate the influence of different culturing and methodological parameters on the Frequency pulsation (Fp) of ephyrae of *S. malayensis* in order to develop a protocol for the use of this species of jellyfish as model organism in ecotoxicological bioassays. Due to the lack of information in the literature on this species in this study the results were compared to those reported on *Aurelia* sp by Faimali et al. (2014). The proposed model organism in this study, shows a more marked swimming activity than ephyrae of *Aurelia* sp. as reported Faimali et al. (2014) even if considering the Frequency pulsation (average of 60 puls/min for *Aurelia* sp. and 80 puls/min for *S. malayensis* in the control) under the same temperature, salinity, volume of sea water and photoperiod condition (Table 10). In addition *S.malayensis* result to be higher than *Aurelia* sp. suggesting that the volume of exposition need to take into account during the experimental set-up. Preliminary observation on ephyrae of *S.malayensis*, highlighted in according to Faimali et al. 2014, how the density of the organism in the well significantly affected the number of pulsations, probably because a decrease of the available space (more organism in each well) caused a negative larval/larval interaction, inhibiting their movement. However, the experimental results (Figure 20) showed that a significant difference on Frequency pulsation were observed in ephyrae exposed to the biggest volume tested ,10 ml of sea

water, suggesting that to have the higher number of pulsations the ephyrae should be exposed in a smaller volume (2 or 5 ml).

Temperature (Figure 21) was found to exert a drastic effect on the pulsation rate of the ephyrae of *S. malayensis*, as the results suggest after 24 hours of exposure to either low (10° C) or high temperature (30° C) conditions.

Interestingly, considering the pulsation measured in ephyrae soon after strobilation (0 days), *S. malayensis* appeared to be more affected by low than high temperature, with 100% of immobility and 52.6 ±13.5 puls/min in cold and warm temperature conditions, respectively. In contrast, ephyrae of *Aurelia* sp., as reported by Faimali and co-workers (Faimali et al. 2014), showed to be more active at lower (10°C, range value of Fp of 8.38 ± 2.12 puls/min) than higher temperature (30°C, 2.75 ± 1.64 puls/min) conditions. Thus, the inactivity at low temperatures observed in the ephyrae of *S. malayensis* could be explained by the tropical distribution of this species compared to the broader spectrum in the natural habitat of *Aurelia* sp. These finding could be explained considering that tropical species like *S. malayenis* are usually stenothermal and do not tolerate deviation from their optimal temperature range (Boero et al. 2016) In spite of the differences between these two species, both *S. malayensis* and *Aurelia* sp. preferred a temperature of 20°C, which was optimal to produce a high value of pulsations, although Faimali et al. (2014) observed a proportionally lower average value of Fp (26.88 ± 1.13 puls/min) in *Aurelia sp* than we did in *S. malayensis* (122.7 ± 30.7 puls/min). Compared to temperature, photoperiod did not significantly affect the Fp in ephyrae of *S. malayensis* (Figure 22) although individuals exposed to dark conditions showed a higher value of Fp than individuals exposed to an equal number of hours of light and dark (12 L: 12 D h). The role of light has been widely investigated in ephyrae of *Aurelia* sp., where the inhibition of Fp resulted to be strictly proportional to the increase in exposure of hours of light (Faimali et al. 2014). Faimali e co-authors suggested that the higher value of Fp observed in ephyrae of *Aurelia* sp. exposed to darkness may be explained as a warning sign of sinking to the bottom of the plate where

an increase in pulsation could have been induced by rhopalial, responsible for the transduction of light stimuli. Another possible explanation can be sought in the feeding habits of the ephyrae of *Aurelia* sp. that could increase the Fp in low-light conditions to follow the movements of zooplankton in search of food.

These hypotheses, suggested for *Aurelia* sp., can be extended to *S. malayensis*, since no information on this species, concerning these parameters, is available to date in the literature.

Considering that photoperiod and temperature are the main drivers that regulate the responses of species to seasonal cycles (pheology) in particular in terms of periodic activities such as migrations or reproduction, further investigation on the effect of these parameters on the swimming performance of ephyrae of jellyfish is needed. however, our results suggest to use a full dark condition during the exposure in toxicity tests. Further investigation should be promoted to understand the important role of this environmental factor on the model orgasm selected, however from these results it was possible to define the following parameters to be used to perform the ecotoxicological test with ephyrae of *S. malayensis* (Table 10).

5.3 Experimental activities on *Aurelia* sp. and *S.malayensis* : ecotoxicological comparison with refence toxic compounds

4.3.1 Ephyrae Test_ET

To validate the bioassay with ephyrae of *S. malayensis* and compare the sensitivity of this new model organism with *Aurelia* sp. proposed by Faimali et al. (2014) the methodological protocol, optimized after the first part of this work, was applied using two reference toxicants, Cadmium nitrate and SDS (Faimali et al. 2014).

As Figure 23 depicts, ephyrae of *S. malayensis* exposed to Cadmium nitrate showed an effect on both end-points measured. More specifically, the sub-lethal end-point (% AFp) seemed to be more sensitive than the acute one (% I) after both times of exposure in agreement with previous reports by Faimali and co-workers (Faimali et al. 2014) in *Aurelia* sp., where the same was observed but only after 24 hour of exposure. This difference in *S. malayensis* was already clear from the $LOEC_I$ at 0.5 mg*L^{-1} for immobility and from the $LOEC_{AFp}$ at 0.1 mg*L for frequency of pulsation (Table 5.3). After 48 h, the same difference in sensitivity was observed between the end-points measured, but one order of magnitude lower than those observed after 24 hours. Indeed, as reported in Table 5.3, the $LOEC_I$ for immobility was 0.05 mg*L-1 and the $LOEC_{AFp}$ for Fp was 0.01 mg*L^{-1}. The same effects were caused by a concentration of 0.05 mg*L-1 of Cadmium nitrate in ephyrae of *Aurelia* sp. after 48 h (Table 11). Comparing the EC_{50} for both species of jellyfish exposed to Cadmium nitrate, *S. malayensis* appeared to be more sensitive than *Aurelia* sp. for both end-points evaluated and for time of exposure considered in this study and also in agreement with Faimali et al. 2014 (Table 11).

As regards SDS (Figure 24), ephyrae of *S. malayensis* showed a significant effect on both end-points but, in the case of this toxic compound, the differences between acute and sub-lethal responses were more marked than those observed with Cadmium nitrate. The % AFp seemed to be at least one order of magnitude lower than the sensitivity threshold showed by % I. This difference was clear after 24 hours of exposure from the $LOEC_I$ at 5mg*L-1 for immobility and from the $LOEC_{AFp}$ at 0.5 mg*L-1 for frequency of pulsation. After 48 h of exposure the difference in sensitivity between parameters resulted to be more marked with $LOEC_I$ at 5mg*L-1 and the $LOEC_{AFp}$ at 0.1 mg*L-1 (Table 10). When comparing the EC_{50} between species of jellyfish exposed to SDS (Table 10), *S. malayensis* appeared to be more sensitive than other species of jellyfish. In particular, this difference in sensitivity was more evident in terms of the sub-lethal end-point, due to the fact that in the ephyrae of *S. malayensis* the alteration of frequency of pulsation was at least one

order of magnitude lower than that observed in the ephyrae of *Aurelia* sp. and also in Faimali et al. 2014.

However, in this thesis were not able to observe the presence of the hormetic phenomenon for *S. malayensis*, which is revealed at lower testing concentrations following the exposition to Cadmium nitrate and SDS, as was observed instead in ephyrae of *Aurelia* sp. exposed to both toxic compounds (Faimali et al. 2014), which could be important to determine the minimum effect concentration (LOEC). The comparison of EC_{50} obtained with *S. malayensis* shows that the new model organism proposed appears to be more sensitive between the two species of jellyfish, and among the considered invertebrate models exposed to the same toxic compounds.

5.3.2 Ephyra Test in semi-dynamic exposition_ ETsd

The results of the second part of this study, have led to confirm the high potentiality of ephyrae jellyfish as model organism in ecotoxicology by a semi-dynamic exposition to Cadmium nitrate. Considering that the jellyfish belong to a group of aquatic animals that use periodic contractions of their own body to generate ring-vortices of the surrounding medium in which they swim (Peng et al., 2012), but are unable to swim against a current, the bioassay was performed exposing the ephyrae to toxicant with an aeration system. In addition, the laboratory conditions during acute toxicity tests generally do not reflect the complexity of the chemical dynamic that occur in the field, thus a semi-dynamic bioassay proposed in thi study could simulate a contamination under environmental condition. The results of toxicity tests highlighted the high sensitivity of the sub-lethal end-point (Figure 24) as showed by the static expositions (Ephyra Test_ET) , but not the same effect, considering the Immobility were observed (Figure 25). In particular, for both model organisms used the immobility results to be affect by Cadmium nitrate only after 48 hours of

exposure , highlighting how *Aurelia* sp. semed to be more sensitive (in term of magnitude of response) than *S. malayensis* showed a LOEC of 0,5 and 1 mg*L-1 for and respectively.

On the contrary the same results were observed exposing the ephyrae in the static exposition (Ephyra Test) but after short exposure time (24 h).

Considering the sub-lethal end-point, the Frequency pulsation results to be more sensitive than acute end-point in accordance with the results obtained from static (Ephyra Test_ET) exposition independently from species of jellyfish considered.

In addition, the value of EC_{50} obtained from the sub-lethal end-point seemed to be comparable between static and semi-dynamic bioassays at both time of exposition and model organism used (Table 11 and 13).

However, after the first 24 hours of exposition was evident a different of sensitivity (in term of magnitude of response) between the Frequency pulsation measured in ephyrae exposd to Cadmium nitrate by semi-dynamic bioassay with a LOEC of 0.05 and 0.01 $mg*L^{-1}$ for *S.malayensis* and *Aurelia* sp. respectively, than the Ephyra Test (ET) with a LOEC of 0.1 $mg*L^{-1}$ for both species of jellyfish.This different in sensitivity between the bioassays was not evident after 48 hours of exposition. Moreover, the results of Immobility and Frequency pulsation obtained among the three repetitions of bioassay in semi-dyanamic indicated the absence of significant difference among them suggesting a good reliability of the test performance (Table 13). In conclusion this study was been an important step towards the understanding of how environmental and methodological factors, such as temperature, volume and photoperiod influenced the swimming performance of ephyrae of *S. malayenis*. The parameters defined in this study have allowed develop a preliminary bioassay with this species of jellyfish. From results of this work s, *S. malayensis* semed to be a very promising model organism in ecotoxicological studies likewise *Aurelia* sp. for the two reference toxic compounds tested. These experiments also allowed to identify a high level of sensitivity for the sub-lethal end-point suggesting that the Frequency pulsation could be considered a suitable tool for detecting the effect of contaminants independently from the condition of exposure performed.

However, further investigation to test the ephyrae jellyfish on a wider spectrum of contaminants performing different method of exposition are needed to better understanding the relation between jellyfish and pollutants in marine ecosystem.

5.4 Experimental activities on *Aurelia* sp. and *S.malayensis* : ecotoxicological comparison with emerging compounds.

In this study were used the bioassays in static and semi-dynamic exposition exposing the model organisms proposed in this thesis to emerging contaminants like microplastics. In details were evaluated the effects of polyethylene particles (different size) on MPs accumulation, immobility and Frequency pulsation in ephyrae of *S.malayensis* and *Aurelia* sp.

In addition the results of this study allowed to better understand the effects of these emerging contaminants on invertebrate species in marine ecosystem that is the aim of the European research project (JPI Oceans) "EPHEMARE" (Ecotoxicological Effect of Microplastics in Marine Ecosystem) which ISMAR is involved.

Global plastic production has consistently increased over the last few years and currently stands at about 300 million tons (Plastics Europe, 2015). Due to its production and high durability, plastic rapidly accumulates in the environment, being the most common type of marine litter worldwide (Bhattacharya et al. 2010). Since plastic debris tends to end up in waterways, aquatic habitats are mostly concerned, where several degradation processes break up plastic litter into a wide array of particle size fractions (Gewert et al. 2015), ranging from macroscopic (> 5 mm) to microscopic (< 1 μm). Microplastics (MP) include particles less than 5 mm in diameter, which can be readily ingested by biota, thus accumulating across the marine food chain (Setälä et al. 2014). Their presence is considered as an emerging threat for the marine ecosystem, more than larger plastic

items (i.e. entanglement, GESAMP, 2015). Today, microplastics are now considered global environmental contaminants of priority study among the high diversity of environmental contaminants that are in general present in aquatic ecosystems of industrialized regions (Ferreiraet al. 2016). Their environmental effects should be further investigated in the scope of several international regulations such as the European Marine Framework Directive (MSFD, 2008/56/EC).

Microplastics are able to cause adverse physical and chemical effects on the biota (e.g. Andrady, 2011; Cole et al. 2011; Oliveira et al. 2013; Wright et al. 2013; Lee et al. 2013; Luís et al. 2015; de Sá et al. 2015). In marine invertebrates, most research refers to controlled laboratory experiments (Ivar do Sul and Costa, 2014), where plastic microspheres (Ø<5 mm) are commonly used in laboratory-based feeding experiments, since they have a similar size to algal prey, the likelihood of MP ingestion is emphasized (Wright et al. 2013). Therefore, MP can be prey analogues for planktonic organisms, being handled and ingested in a similar manner (Brillant and MacDonald, 2000), as demonstrated for crustaceans, polychaetes, echinoderms (Della Torre et al. 2014; Setälä et al. 2014; Nobre et al. 2015; Batel et al. 2016;).

In this study, the ephyrae seemed not to be able to ingest MPs (Figure 31 and 32) however results to be more evident sub-lethal effects on ephyrae swimming behaviour. In literature were found information about the ingestion of microplastics in marine crustaceans (Powell et al. 1990; Cole et al. 2013; Setälä et al. 2014, 2016; Batel et al. 2016; Gambardella et al. 2017).

In particular ,the uptake of different size, less than 1 µm, polystyrene microbeads has been documented in the gut of copepod nauplii and adults (Lee et al. 2013) and brine shrimp larvae (Bergami et al. 2016). Recently Gambardella and co-authors (2017) found that brine shrimp larvae of *Artemia* sp. and barnacle nauplii of *Amphibalanus amphitrite* ingested polystyrene beads of 0.1 µm after 24 h and 48 h of exposition since that MPs were localized in the gut. The accumulation of high density polyethylene microplastics was also demonstrated in the digestive gland of the bivalve mollusc

Mytilus edulis (Von Moos et al. 2012) and in the digestive system of other marine invertebrates (Powell et al. 1990, Cole et al. 2013; Kaposi et al. 2013; Setälä et al. 2014, 2016; Cole et al. 2015; Batel et al. 2016). In addition also in rotifer *Brachionus plicatilis* and in *Tigropius fulvus* was found ingestion of the same Mps 1-4 micron of polyethylene used in this study already at the concentration 0.1 mg*L^{-1} (data unpublished obtained within EPHEMARE project).

The no ingestion observed in this study for ephyrae of *S.malayensis* and *Aurelia* sp., could be explained with the feeding behaviour of these invertebrate marine. Indeed the jellyfish are important predators in marine ecosystem, and no filter feeders like other marine invertebrates mentioned above. In addition, the size-selective predation and the behavioural responses of the prey are two important factors in the mechanism of ephyrae jellyfish predation. Thus, the selective predation, could suggest that ephyrae are able to capture prey alive and no filter particles like microplastics (Sullivan et al. 1994; Bamstedt, 2001)

However, in this study interestingly sub-lethal effets were observed on Frequency pulsation for both species of jellyfish considered. These findings could be correlate with MPs aggregation observed around to rhopals and attached onto the gelatinous body surfaces and lappets of ephyrae (Figure 31 and 32).

The rhopalium represents the nervous system, a ephyra-specific sensory structure, where were also localized mechanoreceptor cells and they are protected by lappets, the marginal segments of the "rhopalar arms" (Nakanishi et al. 2009)Beside, the MPs attached around to this structure, could explained an alteration of pulsation in ephyrae, due to that are the electrical impulses that from the rhopalia allowed the spontaneuous contraction of "rhopalar arms" in this organism. Similar results were observed in previous experiment, exposing the ephyrae to different concentration of *Ostreopsis*. cf. *oavata* where this harmful dinoflagellate was attached with filament on ephyrae lappets causing a frequency pulsation inhibition (Giussani et al. 2016). Moreover, the pulsation were affect also by exposition to nanomaterials in ephyrae of *Aurelia* sp. (Gambardella et al.2015).

In according to Mesaric and co-authors (2015) a swimming speed alteration was observed in *Artemia* sp. nauplii exposed to nanoparticles where at the highest concentration the carbon-based nanomaterials were attached over the entire body surface of nauplii including the gills and the appendages causing a fusion together of the gill branches and swimming inhibition.

In ephyrae of *S. malayensis* and *Aurelia* sp. was observed a significant effect on Immobility and Frequency pulsation at already low MPs concentrations (0.01 and 0.1 $mg*L^{-1}$), while no lethal effects on mortality (100% of Immobility) were observed. In particular, from exposition with MPs of 1-4 µm, *Aurelia* sp. seemed to be more sensitive than *S.malayenis* since in this species of jellyfish the MPs affect only Frequency pulsation independently manner to methods of exposition, while *Aurelia* sp. showed inhibition also in Immobility (Table 14 and 15).

MPs of 4-6 µm, on the contrary seemed to be more toxic than 1-4 µm, since the microplastics affect both end-points in ephyrae of *S.malayenis* as well as in *Aurelia* sp. in both methods of exposition.

These findings, may be correlate to MPs size and to high amount of MP aggregates observed at highest concentrations tested in this study (1 and 10 $mg*L^{-1}$) exposing the ephyrae in static condition, that could affect the Frequency pulsation , resulting in mechanical disturbance. Beside, for *S.malayensis* and *Aurelia* sp. experiments, Mps aggregations were observed only the static exposition and no in semi-dynamic bioassays. However, the Frequency pulsation results significantly affect by MPs of polyethylene already at the lowest concentration tested ($0.01mg*L^{-1}$) independently manner of method of exposition in particular considering results on *Aurelia* sp. On the contrary, the immobility result to be more affect exposing the ephyrae in static condition than semi-dynamic condition. Furthermore, the MPs attached on the body surface were observed only exposing the ephyrae in static condition, probably because in semi-dynamic the aeration may be useful to prevent agglomerates and contact with ephyarae body.

As highlighted in the previous experiment (see paragraph 4.3.2) , these results suggest, that the ephyrae exposed by a semi-dynamic condition could be help by aeration to generate ring-vortices to swim reflecting a low inhibition of immobility.

Considering the plastics additive used in this study, the Waterborne-BP-3, affect significantly the Immobility and Frequency pulsation in static condition, but only sub-lethal end-point in semi-dynamic exposition (Table 14 and Table 15) both species of jellyfish used. This has been the first study to investigate the effect of Waterborborne -BP-3 on ephyrae jellyfish and seemed that this innovative model organism appear to be more sensitive than other invertebrate organisms exposed to the same toxic compound like *B.plicatilis* with a EC_{50} value of 8.12 (1.67-39.56) mg * L^{-1} after 48 hours of exposition (data unpublished derived from experiments within EPHEMARE project) and also compared with the *Mitilus galloprovincialis* and *Paracentrotus lividus* with a EC50 value of 3.47 (3.35-3.58) mg*L-1 and 3.28 (2.50-3.95) mg*L-1 respectively after 48 hours of exposition (Paredes et al.2014). The use of Waterborne- BP-3 was chosen in this study to investigate its role as plastic addictive. Indeed, microplastics may contain other chemicals incorporated during their manufacture, use and/or environmental permanence, they can act as vectors for the entry of highly concerning chemicals into organisms and food webs (Frias et al. 2010; Rochman et al. 2014; Avioet al. 2015; Wardrop et al.2016; Wu et al. 2016). Moreover, the microplastic in the sea can absorbed a series of marine chemicals and contaminants, such as heavy metals (Betts, 2008; Ashton et al. 2010), endocrine disruptors (Ng and Obbard, 2006) and persistent organic contaminants (POP, Rios et al. 2007) that may be transported across oceans polluting otherwise pristine ecosystems (Zarfl and Matthies, 2010), or be ingested by marine organisms, thus transferring toxins from the environment to biota (i.e. a ‘‘Trojan horse’’ effect) (Gregory, 1996; Thompson et al. 2004; 2005; Zarfl and Matthies, 2010). For example, this effect was demonstrated by Teuten et al. (2007) in marine polychete exposed to polyethylene micro-plastics contaminated with phenanthrene, and IPA. In the present study was not possible to find significant differences (Table 14 and 15). between the results obtained exposing ephyrae to MPs of polyethylene (LDPE 4-6 μm) "virgin" and with the same microplastics contaminated with oxybenzone at both concentration of addictive (LDEPE 4-6μm + BP-3 low and high concentration)

In particular in *S.malayensis* as well as in *Aurelia sp.* the MPs contaminated and no, affected the Frequency pulsation already at the lowest concentration tested (0.01 mg*L-1). These finding suggested that BP-3 did not to lead more toxicity of Mp of polyethylene and consequently did not to promote the "Trojan effect". These results were in according to those highlighted in *B.plicatilis* (data unpublished derived from experiments within EPHEMARE project) and with Oliveria and co-authors that reported a study about the exposition of *Pomatoschistus microps* to polyethylene microplastics contaminated with pyrene, where PAHs were able to induce acute toxicity and inhibit enzymatic activity (eg acetylcholinesterase) in fish after a brief exposure of 48 hours, while the synergistic effect of these contaminants with microplastics only manifests itself after a longer exposure period (60 hours). These findings suggest that the exposure time is one parameter that should be further taken into account for MP toxicity assessment.

In conclusion in this study was demonstrated that ephyrae jellyfish did not able to accumulate polyethylene microparticles though further investigation would be needed. However, MP exposition seemed to affect sub-lethal responses evaluated (Immobility and Frequency pulsation) at environmental and high MP concentrations. Also this emerging compounds, highlighted the high sensitivity of the behavioural response like the Frequency pulsation compared to immobility as reported in previous study of this thesis for ephyra jellyfish. These feeding were according to literature information that have demonstrated how this sub-lethal and-point as well as swimming speed alteration in other marine invertebrate is an ecologically relevant parameter to assess the effect of contaminants even at concentrations that do not cause mortality (Faimali et al. 2016). On this basis, the Frequency pulsation measured in ephyrae may be a suitable sub-lethal response to assess the effect of environmental and high concentration MP in marine ecosystem.

5.5 Experimental activities on *Aurelia* sp. and *S.malayensis* : trophic transfer of contaminants

5.6.1 Experimental set-up to perform a simplified food chain.

The first part of this study was directed to define the optimal predator/prey ratio to perform a simplified food chain where *S.malayensis* and *Aurelia* sp. and *Artemia* sp. nauplii as predators and preys respectively.

Due to the limited information regarding the *S.malayensis* the mechanism of predation and results obtained in this study were discussed following the information reported by some authors on behavioural feeding observed in ephyrae of *Aurelia* sp. Mecchianism of predationof jellyfish is characterized by selectivity, which depends on many factors such as prey size and swimming speed, predator tentacle length, width and spacing, predator swimming behavior and resulting bell-margin water flow, nematocyst types affecting penetration of prey with different vulnerability to toxin and escape abilities (Costello and Colin, 1994; Ford et al. 1997; Purcell, 1997; Sullivan et al. 1997; Suchman, 2000).

Prey selection in *Aurelia* sp. ephyrae was addressed experimentally in several laboratory studies, but the results appear to conflict. Early experiments by Delap (1907) indicated that young *A. aurita* preferred hydromedusae and only ate copepods when no alternative prey were available.

Bamstedt, (1990) saw no evidence for selective feeding: consumption of prey by both ephyrae and older medusae was proportional to prey abundance in natural, but very dense, zooplankton assemblages. On the other hand, Stoecker et al. (1987) reported selective feeding by *A. aurita* on different types of microzooplankton and found a preference for copepod nauplii over rotifers and potychaete larvae. In particular the mechanics of prey selection by *Aurelia* sp. ephyrae was widely studied by Sullivan et al. (1997), who observed that *Artemia* nauplii and rotifers selected as a prey

continue to swim after entrainment and are captured more often than copepod nauplii of equal size, which cease normal swimming ("play dead").

In addition, Sullivan and co-authors (1997), observed that capture efficiencies of ephyrae feeding on large prey (barnacle nauplii, brine shrimp, hydromedusae) were 4 to 12 times greater than for small prey types (rotifers, copepod nauplii), and further, that capture efficiencies for prey of equal sizes differed, indicating that factors in addition to size influence the predator–prey interaction. In ecological studies, prey-selection mechanisms result to be important for understanding how jellyfish predation may alter the zooplankton species composition (Costello and Colin, 1994; Purcell, 1997; Ford et al. 1997), and likewise, knowledge of the actual amounts of prey eaten by the jellyfish is crucial to understand the plankton dynamics in marine ecosystems with frequent mass occurrences of jellyfish (Olesen et al. 1995; Hansson et al. 2005;Møller and Riisgård, 2007a,b,c; Purcell, 2009). In this study was selected only type of prey (*Artemia* nauplii), to better understand the potential effect of transfer of contaminant and investigate also on prey and predators independently of each other. However, observations on ephyrae *S.malayensis* and *Aurelia* sp. behavioral predation allowed to highlight a similar capture mechanisms of prey reported by other authors, despite the ingestion rate in *S.malayensis* result to be higher than *Aurelia* sp. and in addition by direct observation (Figure 48).

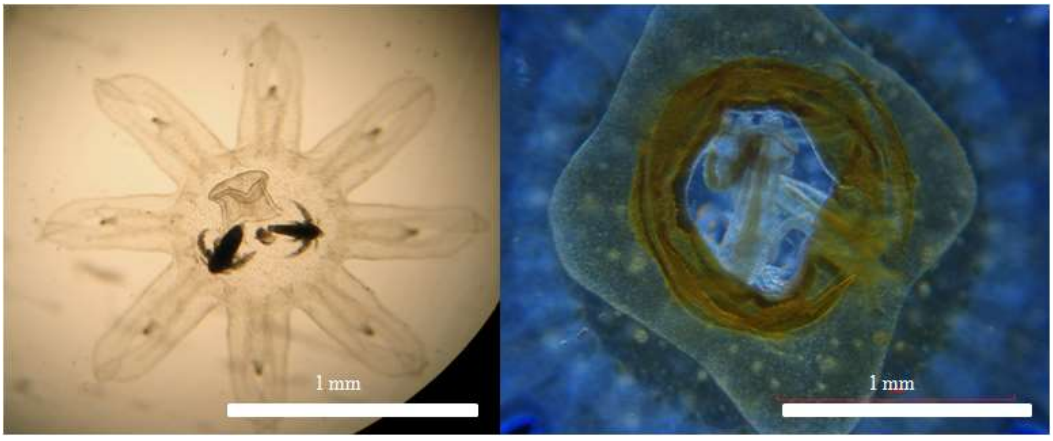

Figure 48 Ephyra of *Aurelia* sp (left) and *S.malayensis* (right with focus on mouth) observed after feeding treatment

Overall, as described by Sullivan et al. (1997) after entrainment in the flows some prey continued to swim at normal swimming speeds while being transported in the flows while others ceased any swimming activity. Prey were captured on the lappet tips, in different ways: if *Artemia* nauplii swam directly into a nematocyst-bearing surface of the ephyrae, if *Artemia* nauplii were encountered by a moving lappet tip during the recovery or power stroke, and finalli by being propelled onto ephyral manubrium (mouth) (Figure 48). In particular the active swimming by prey into the ephyrae represented the mainly mechanism predation for *Artemia* nauplii in both species of jellyfish in according with Sullivan et al. (1997). It was very difficult to compare "ingestion rate" and "Predatory performance" data with other works, because experimental condition often differ. In the first part of experiments, these approaches were used only to understand the highest number of prey eaten by ephyrae jellyfish correlate to the highest predatory performance, namely an optimal prey concentration in order to eliminate the variability of the saturated feeding condition. Overall, for both species of jellyfish the Ingestion rate seemed to be increase with the increasing availability of food in according to Pereira et al. (2014), however a decrease of prey number ingested for day by ephyrae was observed from 50 prey in *Aurelia* sp. and 100 prey in *S.malayensis.* Beside, at this concentration of prey used, was observed an excess of food in the jars, with dead prey on the bottom of jars. Probably, the ephyrae were not able to capture nauplii settled onto the bottom of tank causing an overfeeding condition. Thus, the longer the debris remains in the tank, the worse it is for the jellies' environment because it the food it can become substrate for fouling organisms as reported by Widmer, (2008). Likewise, this saturated condition can also happened when the food is fair but the water does not changed during the experiment as performed in this study. Moreover, the Ingestion rate measured in *Aurelia* sp. does not in according to literature information since that was reported at the similar temperature and salinity condition (18°C and 35‰) an average of prey ingested of 50-60 *Artemia* nuaplii for day (Bastedt et al. 1999, 2001).

However, the experiments performed by Bamstedt and co-authors, (1999, 2001) consisted of 5 ephyrae in 2L and a beginning average of concentration prey of 300 *Artemia* nauplii . In addition Bamstedt et al. (1999) observed also that the ingestion rate in ephyrae of *Aurelia* sp. highlighted a clear trend of increase with age and a strong positive effect of temperature and salinity. On the contrary in this study the temperature were maintained constant in a thermostatic room and salinity does not changed significantly during the experiment.

Thus, though, the set-up of the bioassay to perform these two simplified food chains followed the protocol proposed in this thesis in previous experiment (see section 4.3.2) exposing the ephyrae in a semi-dynamic condition, these findings suggested that parameters like volume, temperature, salinity, water changes, ephyrae age should be further taken into account to develop of bioassay through marine food web with ephyra jellyfish as model organisms.

5.6.2 Evaluation of "Cadmium-enriched diet" on ephyrae jellyfish

To evaluate the "Cadmium enriched-diet" effect, the number of prey defined after the first part of this study, was used to perform two simplified food chains (Table 16). In the second part of this work the ephyrae were fed with *Artemia* nauplii contaminated with Cadmium nitrate. In this respect the prey contamination was performed by semi-dynamic condition (with aeration) in according to Batel et al. 2015, to use the same method of exposition of ephyra jellyfish. However, *Artemia* sp. nauplii exposed to Cadmium nitrate by semi-dynamic exposition showed (Table 17) more sensitive than acute toxicity test (static condition), as observed also exposing nauplii to potassium dichromate (reference toxic compound).

Nevertheless, the results on reference compound confirmed the acceptability of the proposed test in both method and time of expositions, since the death rate of the controls was always < 10% (APAT IRSA-CNR, 2003).

Also the LC_{50} value obtained at 24 hours with potassium dichromate result to be in according to standard protocol (APAT IRSA-CNR, 2003) and literature data (Gambardella et al. 2017; Morgana

et al. 2017) considering both methods of exposure. During the prey contamination were selected the concentration of Cadmium nitrate below the LC_{50} to offer to ephyrae prey alive, considering that they probably are not able to capture inert particles, or prey dead on the bottom of tank (Sullivan et al. 1997; Hansson et al. 2005) as highlighted in the first part of this work (see 5.6.1)

Ingestion rate (%I) measured in this study (Figure 39), seemed to be no affect by prey contaminated, because the number of prey eaten by ephyrae was remained steady for all experiment both *Aurelia* sp and *S.malayensis* predators in the control and treated. However, the swimming speed alteration observed in nauplii of *Artemia* sp. contaminated and given to ephyrae could have influenced the mechanism of predation in *Aurelia* sp. and *S.malayensis*, in particular for this last species, since nauplii dead were observed in the bottom of jars at the end of experiment.

These results for *S.malayensis* were also suppurated by a significantly inhibition of %Pp at already the lowest concentration on Cadmium nitrate to which nauplii were exposed.

Biometrics and bioenergetic parameters evaluated on jellyfish, have been recorded from a lot of field studies (Henroth and Grondahl, 1983; Hansson, 1997 and Bamstedt, 1998) and from controlled tank experiments (Frandsen and Riisgard, 1997; Hansson, 1997; Ishhi and Bamstedt, 1998), however there aren't any information about their inhibition or alteration by feeding treatment with contaminated prey. Considering results on biometrics and bioenergetics parameters for *Aurelia* sp. the average diameter and AFDW reported in the control (Table 18), namely ephyrae fed with *Artemia* nauplii no contaminated, resulted to be in according to Bamstedt et al. (1999, 2001) after a week of experiment. As regard feeding treatments with contaminated prey, both *Aurelia* sp. and *S.malayensis* ephyrae showed more effect on AFDW than Disc diameter (Table 19a).

In addition, the ephyrae of *Aurelia sp.* reported a difference in sensitivity between the Disc diameter and AFDW with a LOEC of 1 and 0,1 $mg*L^{-1}$ on Cadmium nitrate to which nauplii were exposed respectively. Also the GGE% result to be significantly inhibited by prey contaminated at 0,5 $mg*L^{-1}$ of Cadmium nitrate for both species of jellyfish. Is very difficult to speculate about these

findings since there are not data in literature, and the authors that have widely discussed on importance of quality food for development growth of the zooplankton, often have investigated on temperature and salinity effect at saturated prey concentration and different types of prey, thus at laboratory condition very different compared to this study (Bamstedt, 1990; Bamstedt et al. 1997, 1999a, 1999b, 2001). Thus, this work could be useful to understand the importance of quality prey the effect of contaminated prey on behaviour and growth in ephyrae jellyfish.

For example, was highlighted that exposure by contaminated prey during early life stages can lead to sub-lethal effect, including altered behaviour and growth (Little and Finger, 1990).

In addition, Candelmo et al. (2010), reported a study about the bluefish contaminated prey with PCB and pesticides, for 4month displayed significantly reduced feeding and growth, spontaneous activity, and swimming bheaviour compared to animals fed with prey no contaminated. The alteration of swimming (pulsation) was observed also in this study for both *Aurelia* sp. and *S.malayensi*. The effect of *Artemia* nauplii contaminated on the sub-lethal end-point Frequency pulsation was observed already at the lowest concentration of Cadmium nitrate tested (Figure 44 and 45), highlighting an increasing to increase of feeding treatment (time of exposition) namely adding contaminated prey for both model organisms. In addition the sub-lethal end-point result to be more sensitive the immobility that showed a significantly effect of Cadmium nitrate only after 72 and 96 hours from first feeding treatment for *Aurelia* sp. and *S.malayensis* ephyrae respectively.

However, seemed that the Cadmium nitrate results to be more toxic by the direct exposition of ephyrae (semi-dynamic condition; Table 19 b) compared to its enriched-diet at the same metal concentrations and time of exposition (Table 19b).

Furthermore among all parameters evaluated on ephyrae, the Frequency pulsation result to be always as widely highlighted in previous experiment of this thesis, the most sensitive end-point at the same time considered (Table 19a).

It was an interesting findings because swimming activity is a fundamental feature in ephyrae jellyfish to capture food, avoids predators an maintain their orientation in the water column and the

changes in the movement behaviour can be used as suitable indicator of a stress condition for this model organism. In addition would be needed to investigate the relation of jellyfish and contaminant along the trophic chains considering their key role in marine food webs (Boero et al. 2013; Gibbons et al. 2016).

The outcomes of this investigation, for the first time have allowed to speculate the potential sub-letal effects of Cadmium enriched-diet on ephyra jellyfish by feeding treatment with contaminated prey. However, there is still a substantial lack of knowledge, regarding potential ecological effects on exposed aquatic organisms and food-webs and also the trophic behaviour of ephyrae jellyfish, suggesting further investigation to be able to develop new standard ecotoxicological tests.

6. CONCLUSION AND PERSPECTIVES

The aim of this first part of the PhD thesis was to gain a better understanding of the behavior responses of ephyrae jellyfish to contaminants in marine ecosystem. This thesis section were designed to assess the potential of ephyrae swimming activity as suitable indicators of a stress condition in marine ecosystem compared to other biological responses measured and the role of this new model organism as invertebrates model in ecotoxicological bioassays.

First experimentalactivity aimed to confirm and to strengthen the preliminary observation reported by Faimali et al. (2014) who highligthted that *Aurelia* sp. ephyrae seemed to be promising model organisms in ecotoxicological bioassay. The results were compared with those obtained on other marine invertebrates exposed to the same toxic compounds showing a high sensitivity of jellyfish in term of swimming activity alteration. In this study were also observed the morphological changes due to the exposure to ES and CPF on ephyrae jellyfish that coupled with swimming behavior could be relevant to understand the first indication to a non-optimum environment condition of this animals. An innovative study was proposed exposing the ephyrae of *Aurelia* sp. to *Ostreopsis cf. Ovate* highlighting how this harmful dinoflagellate affect the ephyrae swimming behavior,

suggesting an interesting scenario on the interaction of these two bloom forming species in the natural marine environment.

These promising results suggested to improve the ecotoxicological investigation, in order to propose a new species of jellyfishs as model organism, and a new bioassay to expose the ephyrae in semi-dynamic, with aeration to better understand the interaction between jellyfish and contaminants under similar environmental conditions.

Second experimental activity described the development of bioassay using ephyrae of *S. malayensis* showing the high sensitivity of this species of jellyfish as new model organism in ecotoxicological bioassay compared with *Aurelia* sp. The investigation proposed also allowed to perform a new toxicity test exposing the organism to semi-dynamic condition with aeration highlighting a high level of sensitivity for the sub-lethal end-point like Frequency pulsation suggesting it a suitable response tool for detecting the effect of contaminants in marine ecosystem for ephyra jellyfish.

The biological models (ephyra jellyfish) and bioassays (static and semi-dynamic) proposed , were used to investigate the effect on ephyrae jellyfish of emerging contaminants in marine ecosystem like microplastics at environmental and high concentration. The results, showed the high sensitivity of Frequency pulsation to microplastics of polyethylene of different size compared to other marine invertebrates exposed to the same MP confirming the potential of ephyrae jellyfish and the Frequency pulsation as sub-lethal response to assess the effect of contaminants in marine environment.

An interesting experimental activity was proposed to better understand the effect of transfer of contaminants through a simplified food chain, investigating on feeding behavior of ephyra jellyfish of *Aurelia* sp. and *S.malyensis* proposed as model organism in this thesis, on crustaceans *Artemia* sp. This work allowed to propose different method of investigation of trophic transfer effect of Cadmium nitrate (selected as toxicant), treated crustaceans on ephyrae jellyfish, like “ingestion rate method”, “predatory performance” and biometrics and bioenergetics parameters (Disch diameter, ash-free dry weight_AFDW and gross growth efficiency_GGE), coupled with alteration of

swimming behavior. Results showed a 100% of feeding rate and predatory performance in both control and treated jellyfish (*Aurelia* sp.and *S. malayensis*). Cadmium nitrate treated *Artemia* nauplii, once ingested, caused in ephyrae a decrease of Disch diameter and AFDW and also a decrease of GGE% and affect the frequency of pulsations. The outcomes of this investigation, for the first time allowed to speculate the potential sub-letal effects of throphic transfer of Cadmium nitrate on ephyra jellyfish by feeding treatment with contaminated prey.

The outcomes of this exhaustive research indicated that ephyra jellyfish of *Aurelia* sp. and *S.malayenis* have a significant potential as model organism in ecotoxicological bioassays and their swimming behavior could be a used as suitable indicator of a stress condition for this model organism in marine ecosystem. It was an interesting findings in the jellyfish research because swimming activity is a fundamental feature in this gelatinous invertebrate to capture food, avoids predators an maintain their orientation in the water column and the changes may be affected in the development of adult stage influencing the dynamic of their blooms. Likewise, these findings confirmed the high sensitivity of ephyra jellyfish compared to other marine invertebrates exposed to a wide spectrum of contaminants suggesting their use in the ecotoxicological investigation since in this contest there is always an urgent need to identify new species for their use in the development of sensitive and reliable test methods for laboratory testing and to expand common battery bioassay.

Lastly, considering the increasingly frequent and widespread jellyfish blooms and that could be in the future of the oceans, the cause of a suppression of high-energy food chains with a possible subsequent de-evolution of the pelagic marine ecosystem back to a gelatinous dominance, (Boero et al. 2013), the results of this PhD thesis should be taken into account to further investigation.

As regard, new method of investigation should be develop to improve the research considering other life stage of jellyfish and responses at different level of biological organization, like a molecular biomarker.

The results of the first part of this PhD thesis, were used to investigate the possibility to suggest the ephyrae jellyfish as model organism to quality sea water assessment in private and public aquarium.

The experimental activity performed to aim this goal were carried out in collaboration of Acquario di Genova and described on the next section of this thesis.

7. *Aurelia* sp. and *S.malayensis* model organism for rapid assessment of seawater: A case study at Acquario di Genova (Italy).

Excellent water quality is the basis of the animal welfare in aquariums. Every aquarium routinely checks the water quality of the tanks, but it is very important also to check the quality of the raw sea water. Public aquariums have two different sources of sea water: artificial sea water and natural sea water obtained directly from the sea. The second one has a lot of advantages: there is lots available, it is low cost, low labor, it guarantees a complete chemical makeup, and it's good for invertebrates and algae growth. On the other hand it could be dangerous because of little control over pollution, nutrients, parasites, salinity changes and turbidity. In the aquariums that use natural sea water a continuous and as complete as possible monitoring of the incoming water is very important to prevent rare but extreme disasters. In these aquariums, beside chemical-physical and microbiological analyses the adding of ecotoxicological tests can be very useful even if it is often expensive or an external laboratory may be required. In addition direct observation highlighted, that among animals kept in aquarium, the ephyra jellyfish seemed to be the most sensitive organisms with other cnidarians (corals) showing mortality or swimming alteration when worse quality sea water occurred.

As regard of these findings, the last part of this thesis described the activities performed at Acquario di Genova, based on the collaboration between ISMAR-CNR and the laboratory of Tropical Department. The main objective of this experimental part has been to develop a simplified toxicity test (Ephyra Smart Test_EST) based on the ecotoxicological test in semi-dynamic condition on ephyrae jellyfish performed at ISMAR-CNR, to use in the quality seawater assessment.

7.1 Costaedutainment spa. - Acquario di Genova

The Acquario di Genova is one of the largest exhibition of aquatic biodiversity in Europe, with 71 tanks housing over 15,000 animals belonging to 400 species, against the incomparable backdrop of the Gulf of Genoa. It is located on the touristic harbor of Genova as displayed in Figure 49.

Figure 49 Acquario di Genova located on the touristic harbor of Genova

Inaugurated in 1992 for the celebrations of the 500th anniversary of Columbus's discovery of the New World, the Aquarium welcomes over one million visitors every year.

Its 27,000 square metres of exhibition space offer visitors the chance to take a trip through the seas of the world in order to admire dolphins, sharks, penguins, manatees and Antarctic animals: it is the only facility in Europe to house jellyfish, tropical fish, seals and much more, in environments that faithfully reproduce the natural habitats of the different species represented.

From years 2012. the natural seawater used to keep the animals is pumped from the tubes system situated at 200 m outside the harbor from the breakwater at 50 meters deep. The raw sea water is pumped continuously about 18 hours x day. (Figure 50a). All the water pumped is stored in big tanks for one day before using. (Figure 50b). Every day chemical physical analysis are done: pH,

nitrite, ammonium, temperature, torbidity. (in addition also the ESTsd proposed in this thesis).The new seawater is stored in a holding tank and subsequently in three different tanks before sorting the water into the aquarium. For each tank the water is pumped each 24 hours, and when one is emptied, the water is pumped from subsequently tank (Figure 50a).

Figure 50 a) System of raw sea water at Acquario di Genova. b) Big tank of sea water storage c) the last tank before its used to keep animals.

Only after the positive results of all the analysis (chemical, physical and Eco toxicological) the water is pumped in the circular tubes system that arrived directly to all the tanks of the aquarium and can be used for changing water (Figure 50c).

7.1.1 System to jellyfish keeping at Acquario di Genova

The system to keep the jellyfish at the Acquario di Genova includes 5 systems of tanks (hot/cold) with a total of 20 tanks, 46 ferplast for the polyps, 8 tanks to keep the ephyrae fora total of 12.000 liter of sea water used.

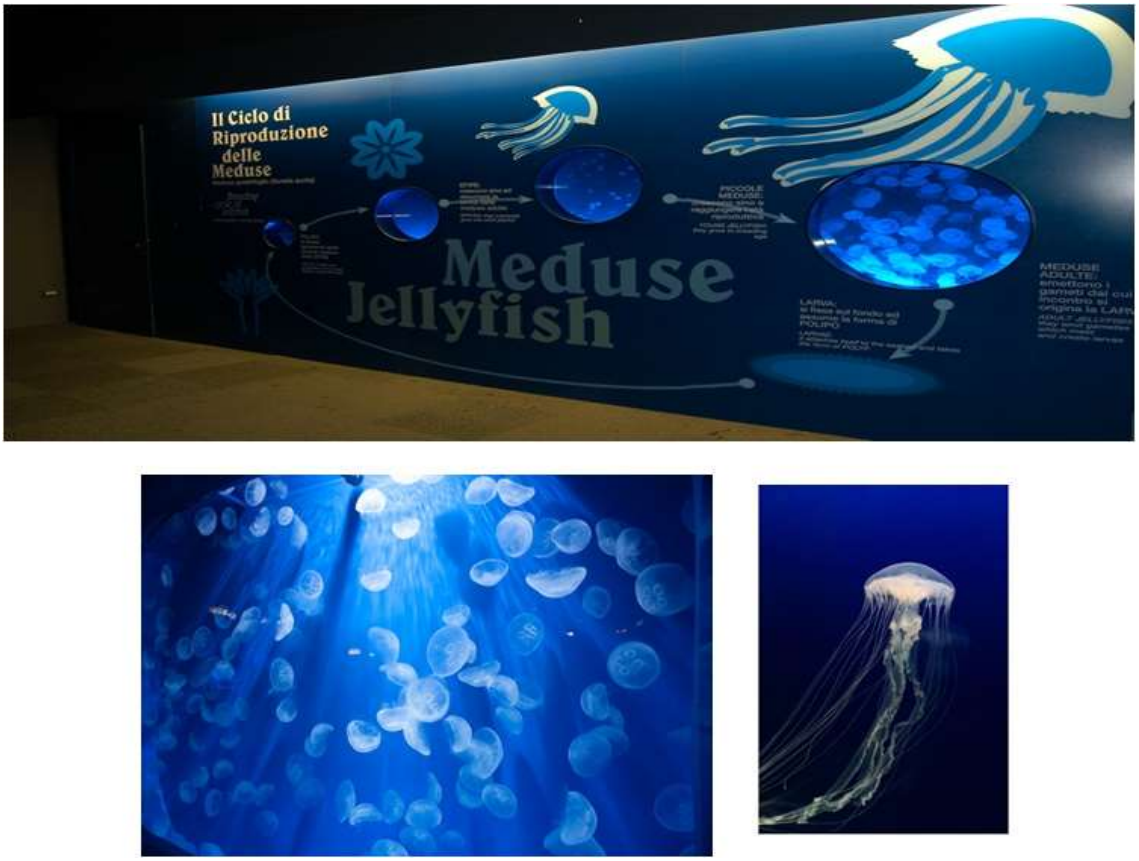

Figure 51 Jellyfish at Acquario di Genova. Below on the right, adults of Aurelia sp. and on the left adult of S.malayensis, the species of jellyfish used in this thesis.

There are different species of jellyfish reproduced: *Aurelia aurita, Chrysaora melanaster, Phylloriza punctata, Cassiopea sp., Cassiopea xamachana, Sanderia malajensis, Cothyloriza tubercolata, Mastigia papua, Phacellophora camtschatica, ,Cephea cephea.* Beside the exhibition of the jellyfish for education and entertainment purpose, the main activities in aquarium are the experimental studies on reproduction, feeding and ecotoxicological investigation in collaboration with CNR-ISMAR.

7.2 Experimetal set-up to develop a simplified toxicity test in semi-dynamic condition (Ephyra Smart Test_ESTsd).

In the first part of this study, the experimental activities were carried out at CNR-ISMAR laboratory using the ephyra stage of *Aurelia* sp. and *S. malayensis* (obtained from the Acquario di Genova), to develop a simplified toxicity test in semi-dynamic exposition (Ephyra Smart Test_ESTsd) base on the ecotoxicological test in semi-dynamic condition (ETsd) on ephyrae jellyfish performed durng this thesis, following the methodological parameters reported in Table 20. They have been defined to develop a smart toxicity test with a reasonability low level of training, and e a response time of 24 hours maximum. Thus the ESTsd should be a simple and rapid bioassay to use at laboratory not dedicated routinely to ecotoxicological investigation like the aquariums.

Table 20 Methodological parameters used to perform the Ephyra Smart Test in semi-dynamic with ephyrae of *Aurelia* sp. and *S. malayenis* defined in this study .

Test parameters	ESTsd	Notes
Organism density	10 ephyra/beaker	-
Volume	2 L	Tank available
Fp recorded	Direct observation	No tools to recorder the Fp
Ligh condition		
Exposition	With areation	-
Ephyrae age	0-7-14 days	Deily check of sweater with organism of different age
Time of exposition	3-6-24-48 hours	Rapid response

Starting from the ETsd (Table 21) performed in this thesis (see section 4.3) and following the methodological parameters reported in Table 20 the ephyrae of *Aurelia* sp. and *S.malayensis* were exposed to a reference toxic compound (Cadmium nitrate) in order to evaluate the effect of volume

(100ml and 2L) ephyrae age (0-7-14 days) and time of exposition on Immobility and Frequency pulsation.

Table 21 Methodological parameters used to perform the toxicity test in semi-dynamic condition ETsd with ephyrae of *Aurelia* sp. and *S. malayenis* defined in this thesisis (section 3.3)

Test parameters Ephyra **Test semi-dynamic_ETsd**		**References**
Organism density	10 ephyra/beaker	This study
Volume	100ml	This study
Recording light conditions	Dark	Faimali et al. 2014
End-point evalauted	SBR	Faimali et al. 2014
Photoperiod	24h Dark	This study
Condition	With aeration	This study
Ephyrae age	0 days	Faimali et al. 2014

7.2.1 Experimental activities

7.2.1.1. Model organisms: ephyrae of *Aurelia* sp. and *S. malayensis*

Ephyrae of *Aurelia* sp. and *S. malayensis* were released from the stock culture population of the Acquario di Genova laboratory. They were reared on small pieces of PVC tubes into a darkened tank of 2,5L of natural seawater with an airstone. The chemical – physical parameters were: temperature 24±1°C, salinity 31±1 ‰, ammonium less than 0,05 mg*L-1^{-1}, nitrite less than 0,05 mg*L-1^{-1} , nitrates less than 10 mg*L-1^{-1} and pH 8-8,2. Polyps were fed one time per day with enriched *Artemia salina* nauplii. One time per week the tank was cleaned and there was a 90 % of water change. First step of the original Acquario di Genova protocol to obtain strobilation and the production of ephyrae was moving polyps to another darkened incubator tank of 2,5L at 10° for *Aurelia* sp. and 18 °C for *S.malayenis* with air and the same chemical-physical conditions. During

this phase polyps were fed 3 times for week, there wasn't change of water and no control of salinity increase due to evaporation. Usually strobilation happened after 2-3-weeks. Once released by strobilation ephyrae were poured into a beaker and immediately used for the assay. For each species of jellyfish , after strobilation the ephyrae that were not use immediately for the test were poured into in a 6 L plastic tank filled with gently aerated FNSW (salinity 37‰) at 20 °C (photoperiod 12:12 light:dark) and were fed daily with nauplii of *Artemia salina* (about 40 nauplii mL^{-1}) and seawater was changed every two days. After 7 and 14 days the ephyrae were prelevated with plastic pipette from the tank and used for the test.

7.2.1.2. Toxicity tests

The assay in semi-dynamic condition for both species of jellyfish were performed exposing the model organisms by a semi-dynamic condition where the ephyrae were placed into a beaker (10 individual for each beaker); containing two different volume of test solution: 100ml as reported for ETsd and 2L following the aquariological parameters. For each concentrations (0 - 0.05- 0.1- 0.5- 1- 5 mg*$L\text{-}1^{-1}$) of $Cd(NO_3)_2$ selected, 3 replicates were prepared. For all test the controls were performed using FNSW (salinity 37‰) filtered 0,22 µm. All experiments were performed in the thermostatic room at 20 °C. After 3-6-24 and 48 hours of exposition were evaluated the Frequency pulsation for each ephyrae by direct observation. The bioassay were carried out in the same method with ephyrae of 7 and 14 days (see section 7.2.1).

7.2.1.3 Data processing and statistical analysis

The EC_{50} values from Immobility and Alteration of Frequency pulsation and related 95% Confidence Limits (CL) were calculated using Trimmed Spearman–Karber analysis (Finney, 1978) after 24 and 48 h. Significant differences between controls and treated samples were determined using one-way analysis of variance (ANOVA) followed by Tukey test. When data failed to meet the

assumption of normality, non parametric Kruskal Wallis test and Mann Whitney test were used to compare individual treatments. For AFp statistical analysis were performed using Frequency pulsation data. The LOEC values for Immobility and Alteration of Frequency of pulsation were deducted by ANOVA results. Data were considered significantly different when $p<0.05$. SPSS statistical software (Statistical Package for the Social Sciences, Version 20) was used for data analysis.

7.2.1.4 Influence of volume, ephyrae age and time of exposition on Frequency pulsation

The statistical analysis on parameters evaluated, time of exposition, ephyrae age and volume of sea water was carried out by one-way ANOVA on Frequency pulsations measured in ephyrae of *Aurelia* sp. and *S.malayensis* (Figure 10.4) exposed to FNSW 0,22 µm (control).

Considering ephyrae of *Aurelia* sp. the Kruskal – Wallis analysis pointed out a significant ($P< 0,05$) effect among the time considered, age and volume as reported in Figure 52.

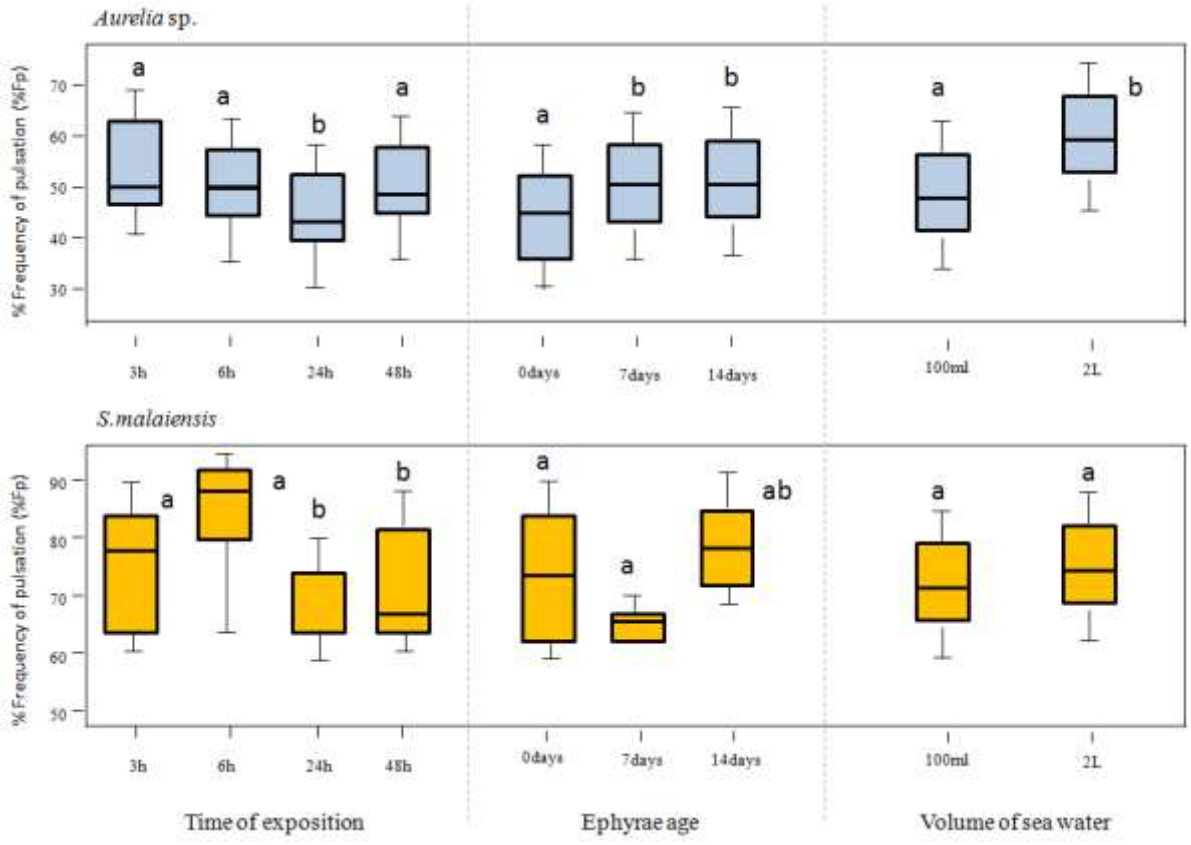

Figure 52 Frequency of pulsation (Fp) made by ephyrae of of *Aurelia* sp. and *S.malayensis* of different age, exposed to different volume of sea water after 3,6,24 and 48 hours of exposition. The horizontal line in the box shows the median and the whiskers show the range. The letter a, b indicate significantly different groups with Kruskal-Wallis post-hoc test (P <0.05). Any groups sharing the same letter are not significantly different

On the contrary in ephyrae of *S.malayensis* Frequency pulsation result to be influenced only by time of exposure and age and not by volume of sea water, in addition a significant (P< 0,05) effect among the treatment were also observed (Figure 52).

Also the two-way ANOVA weas run. In the Figure 52 the Frequency pulsation measured in ephyrae of *Aurelia* sp. seemed to be significantly influenced (p< 0.05) by different parameters measured in the first 24 hours compared to the results obtained in the second 24 hours of exposition for both species of jellyfish. Indeed in *Aurelia* sp. In addition, there was statistically significant interaction between the volume and ephyrae age in the first 24 hours of exposition.

The Frequency pulsation from the 24 hour of exposition seemed not to be influenced by volume and ephyrae age, in particular at 48 hours the pulsation were independently from all parameters investigated.

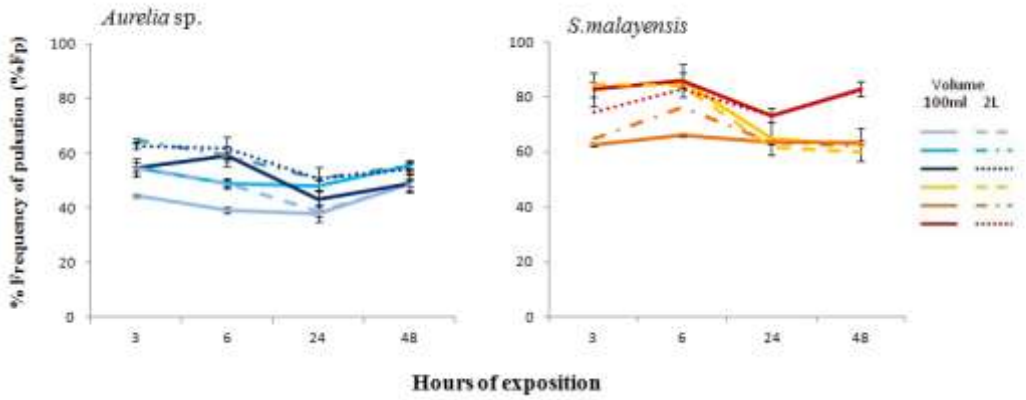

Figure 53. Percentage of Frequency pulsation (% Fp) measured in *Aurelia* sp.(different blue bars) and *S.malayensis* (different orange bars) ephyrae of 0-7 and 14 days old, after 3-6-24 and 48 hours of exposition in 100ml (continuous lines) and 2L (dotted lines) of sea water. (M ±SE, n = 3).

The same results were observed also in ephyrae of *S. malayensis* (Figure 54) however in the second 24 hours the pulsations were significantly higher ($p < 0.05$) than those observed in ephyrae of 0 and 7 days. Considering results on Frequency pulsation measured after 3 and 6 hours of exposition (Figure 54) in *Aurelia* sp. there was a statistically significantly interaction ($p<0.05$) between the volume and ephyra age, while at 24 and 48 hours the Frequency pulsation result to be significantly affect ($p<0.05$) only in ephyrae of 14 days.

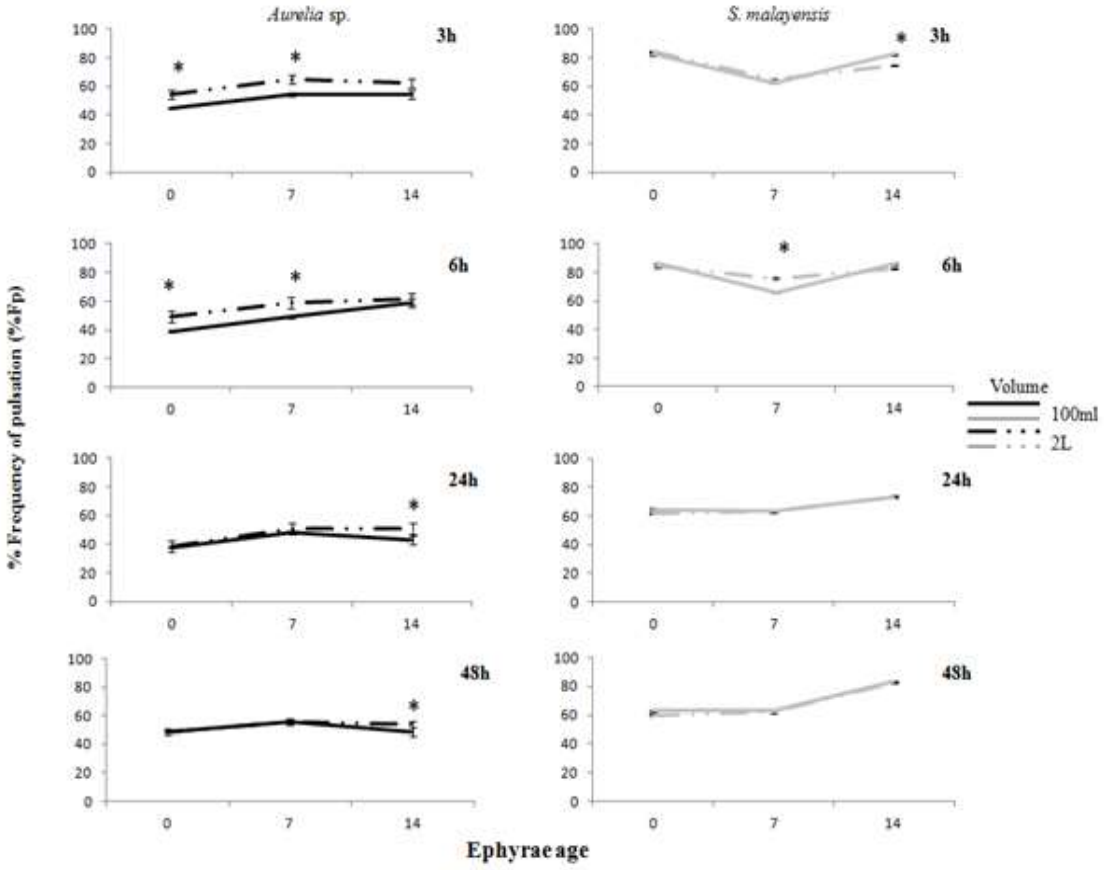

Figure 54. Percentage of Frequency pulsation (% Fp) measured in *Aurelia* sp.(black lines) and *S.malayensis* (grey lines) ephyrae of 0-7 and 14 days old, after 3-6-24 and 48 hours of exposition in 100ml (continuous lines) and 2L (dotted lines) of sea water. (M ±SE, n = 3). *= $p < 0.05$

In *S.malayensis*, significantly difference between the age and volume were observed at 3 hours for ephyrae of 14 days and at 6 hours of exposition for ephyrae of 7 days (Figure 54). In addition was also evident that at 24 and 48 hour of exposition the Frequency pulsation result to be independently from ephayre age and volume of seawater. This finding suggested that the most important parameter to take in account to develop the new bioassay seemed to be the time of exposition, thus considering the 24 hours of exposition to delete the siginificantly effect of the volume, is possible to develop a protocol suggesting the use of ephyrae of *Aurelia* sp. up to the age of 7 days or ephyrae of *S.malayensis* independently of organism age.

7.2.1.5 Effect of Cadmium nitrate on Frequency pulsation and Immobility : Influence of volume, ephyrae age and time of exposition

In Figure 55 were reported the value of EC_{50} (± standard error) from Alteration of Frequency pulsation (EC50 from Immobility was not possible to calculate) measured at 3-6-24 and 48 hours to compare the results from exposition of *Aurelia* sp. and *S.malayensis* ephyrae of different age to reference toxic compound (Cadmium nitrate) at different volume (100ml and 2L). In Table 21 and were also reported the value of the LOEC for both end-point evaluated (Immobility and Frequency pulsation).

Considering the trend of EC_{50} values obtained from Alteration of Frequency pulsation (Figure 55) exposed to Cadmium nitrate seemed to be that the effect of metal decrease with increasing of ephyrae age regardless of time and volume of exposition in *Aurelia* sp. and from 6 hours of exposition considering results of *S.malayensis.* In addition at 24 hours of exposition the EC_{50} values were comparable both species of jellyfish used.

The sub-lethal end-point measured in ephyrae of *Aurelia* sp. results to be more sensitive than Immobility, as widely highlighted in previous studies of this thesis, independently of time considered and ephyrae age.

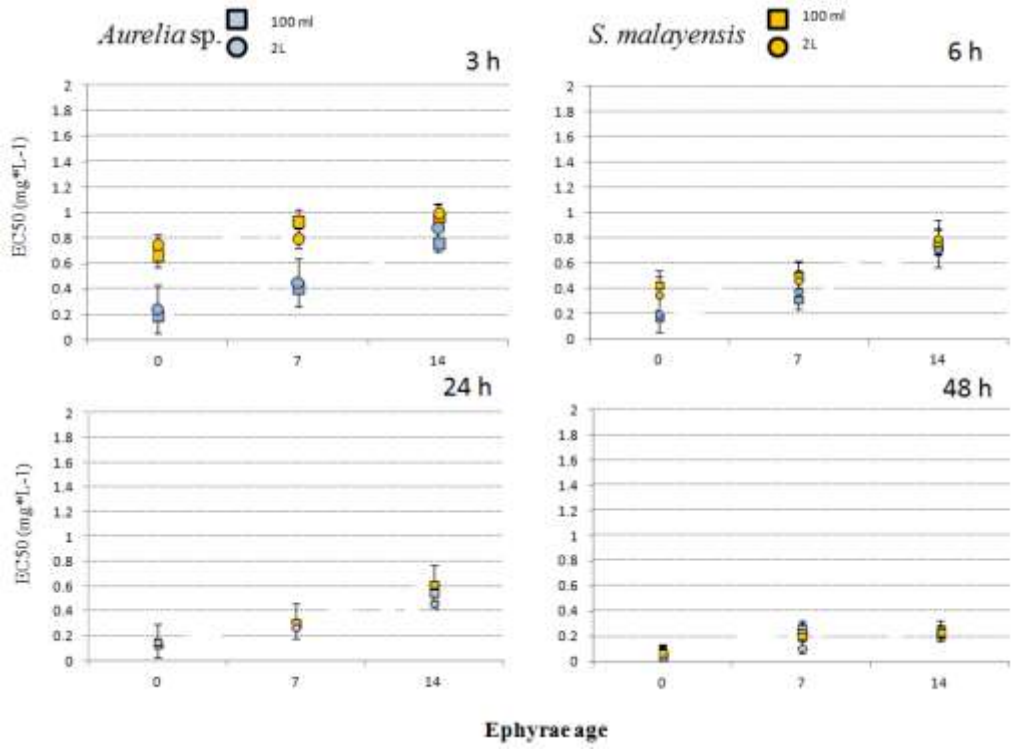

Figure 55. EC_{50} values (± confidence limits) reported from Alteration of Frequency pulsation (% AFp) measured in *Aurelia* sp.(black lines) and *S.malayensis* (grey lines) ephyrae of 0-7 and 14 days old, after 3-6-24 and 48 hours of exposition in 100ml (continuous lines) and 2L (dotted lines) to different concentration of Cadmium nitrate (EC50± c.l.).

Indeed from LOEC value reported in Table 21 showed that the lowest concentration tested of Cadmium nitrate which affect significantly the Frequency pulsation was 0.05 mg*L^{-1} already after 6 hours of exposition in ephyrae of 0 and 7 days, the Immobility does not reach even after 48 hours of exposition (LOEC 0,5mg*L^{-1}) considering the same ephyrae age.

The sensitivity of Frequency pulsation decrease with increasing of ephyrae age after the 6 hours of exposition independently of volume.

Only after 48 hours of exposition the Cadmium nitrate affect significantly ($p < 0.05$) at the lowest concentration tested with a LOEC of 0.05 mg*L-1-1, independently of volume and ephyrae age.

Also the sensitivity of Immobility decrease with increasing of ephyrae age independently of volume. Only ephyra of 0 days showed the lowest concentration (0.5 mg*L-1-1) with a significative effect ($p < 0.05$) on Immobility after 48 hours of exposition.

Also in *S.malayensis,* the sub-lethal end-point results to be more sensitive than Immobility. Indeed from LOEC value reported in Table 22 showed the lowest concentration tested of Cadmium nitrate that affect significantly the Frequency pulsation was 0.05 mg*L-1-1 after 24 hours of exposition independently of volume and ephyrae age, while Cadmium nitrate does not affect significantly the Immobility independently of time considered, ephyrae age and volume.

Table 21. 3-6-24- and 48-h LOEC values with 95% confidence limits derived from % I and % AFp evaluaeted in this study on *Aurelia* sp. ephyrae of different age exposed to Cadmium nitrate at different volume (100ml and 2L).

	Method of exposition			
	ETsd		ESTsd	
End-point Ephyrae age	Immobility	Alteration of Frequency pulsation	Immobility	Alteration of Frequency pulsation
0 days	3h-LOEC=nc 6h-LOEC=1 24h-LOEC=1 48h-LOEC=0.5	3h-LOEC=0.1 6h-LOEC=0.05 24h-LOEC=0.05 48h-LOEC=0.05	3h-LOEC=nc 6h-LOEC=1 24h-LOEC=1 48h-LOEC=0.5	3h-LOEC=0.1 6h-LOEC=0.05 24h-LOEC=0.05 48h-LOEC=0.05
7 days	3h-LOEC=nc 6h-LOEC=1 24h-LOEC=1 48h-LOEC=1	3h-LOEC=0.1 6h-LOEC=0.05 24h-LOEC=0.05 48h-LOEC=0.05	3h-LOEC=nc 6h-LOEC=1 24h-LOEC=1 48h-LOEC=1	3h-LOEC=0.1 6h-LOEC=0.05 24h-LOEC=0.05 48h-LOEC=0.05
14days	3h-LOEC=nc 6h-LOEC=nc 24h-LOEC=nc 48h-LOEC=1	3h-LOEC=0.1 6h-LOEC=0.1 24h-LOEC=0.1 48h-LOEC=0.05	3h-LOEC=nc 6h-LOEC=nc 24h-LOEC=nc 48h-LOEC=1	3h-LOEC=0.1 6h-LOEC=0.1 24h-LOEC=0.1 48h-LOEC=0.05

In the first 24 hours of exposition *S.malayensis* seemed to be more sensitive than *Aurelia* sp. since the lowest concentration tested (0.05 mg*L-1-1) of Cadmium nitrate that showed a significantly affect ($p<0.05$) on Frequency pulsation was only after 24 hours of exposition compared to the 6 hours in *Aurelia* sp. independently of ephyrae age and volume. Thus, to have a response at shorter time of exposition with Cadmium nitrate , is possible to observed a Frequency pulsation at 24 hours since the metal caused a significantly ($p< 0.05$) effect at the lowest concentration tested, 0,05 mg*L-1-1 using ephyrae of 0 and 7 days of *Aurelia* sp. and independently of age with ephyrae of *S.malayensis.*

Table 22 3-6-24- and 48-h LOEC values with 95% confidence limits derived from % I and % AFp evaluaeted in this study on *S.malayensis* ephyrae of different age exposed to Cadmium nitrate at different volume (100ml and 2L).

	Method of exposition			
	ETsd		ESTsd	
End-point Ephyrae age	**Immobility**	**Alteration of Frequency pulsation**	**Immobility**	**Alteration of Frequency pulsation**
0 days	3h-LOEC=nc 6h-LOEC=nc 24h-LOEC=nc 48h-LOEC=1	3h-LOEC=0.1 6h-LOEC=0.1 24h-LOEC=0.05 48h-LOEC=0.05	3h-LOEC=nc 6h-LOEC=nc 24h-LOEC=nc 48h-LOEC=1	3h-LOEC=0.1 6h-LOEC=0.1 24h-LOEC=0.05 48h-LOEC=0.05
7 days	3h-LOEC=nc 6h-LOEC=nc 24h-LOEC=nc 48h-LOEC=nc	3h-LOEC=0.1 6h-LOEC=0.1 24h-LOEC=0.05 48h-LOEC=0.05	3h-LOEC=nc 6h-LOEC=nc 24h-LOEC=nc 48h-LOEC=nc	3h-LOEC=0.1 6h-LOEC=0.1 24h-LOEC=0.05 48h-LOEC=0.05
14days	3h-LOEC=nc 6h-LOEC=nc 24h-LOEC=nc 48h-LOEC=nc	3h-LOEC=0.1 6h-LOEC=0.1 24h-LOEC=0.05 48h-LOEC=0.05	3h-LOEC=nc 6h-LOEC=nc 24h-LOEC=nc 48h-LOEC=nc	3h-LOEC=0.1 6h-LOEC=0.1 24h-LOEC=0.05 48h-LOEC=0.05

7.2.2 Ephyra Smart Test _ESTsd in semi-dynamic condition

Considering the results obtained from experimental activities and the parameters reported for the aquariological protocol were developed two bioassay to suggest the ephyrae of *Aurelia* sp. and *S.malayensis* as model organisms to monitor the quality of seawater at Acquario di Genova.

The parameters were reported in the table below (Table 23).

Table 23 Aquariological protocol to perform the Ephyrae Smart Test in semi-dynamic exposition with ephyrae of *Aurelia* sp. and *S. malayenis*

Test parameters	*Aurelia* sp.	*S.malayensis*
Organism	10 /beaker	10 /beaker
Ephyra age	0-7 days	0-7-14 days
End-point	Frequency pulsation	Frequency pulsation
Fp recorded	Direct observation	Direct observation
Time of exposition	24h	24h

7.3 Application of Ephyra Smart Test_ESTsd to quality seawater assessment at Aquarium of Genoa

7.3.1 Monitoring of natural seawater from the sea to keep the animals in the aquarium.

The ESTsd was proposed in order to suggest at the Aquarium of Genova a new bioassay using ephyrae jellyfish to detect toxic seawater during the routinely procedure of new seawater intake and reinforced the chemical-physical and microbiological analyses performed every days. The Ephyra Smart Test was performing in Aquarium as displayed in Figure 56, following the parameters reported in Table 23.

The toxicity threshold as defined as reported in Figure 56 , as percentage of alteration of Frequency pulsation and Immobility measured in ephyrae.

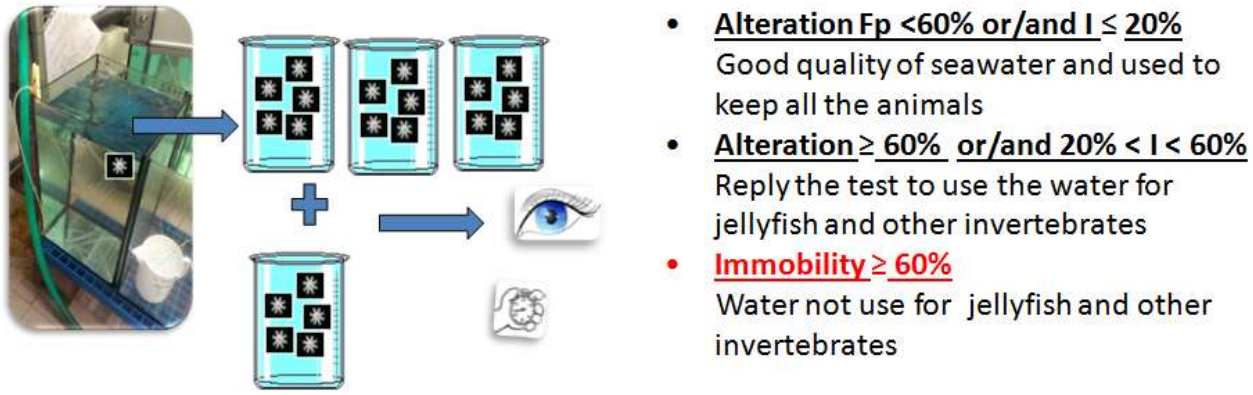

Figure 56. Ephyra Smart Test ESTsd used at Acquario di Genova to quality natural sea water assessment

Calibration of this bioassay was performed also exposing other marine models organisms common used in ecotoxicology at the same seawater samples, when the alteration of swimming behavior measured in ephyrae occurred. An example of comparison was reported in the following section (7.3.2).

7.3.2 Ecotoxicological evaluation of seawater samples from Genova harbor (incoming water in cooling systems) and from drains cooling systems and tanks.

Finally, to compare the sensitivity and relevance of new bioassay, in the last part of this study were reported the analysis performed on real sample from general rain runoff into the Acquario di

Genova like intake water in cooling systems and from drains cooling systems and tanks. The ecotoxicological tests performed at ISMAR-CNR , were carried out on *Amphibalanus amphitrite* nauplii and *Artemia franciscana* Instar I larvae (following the D. Lgs Italian low 152/06) that were selected since they are an established model species in ecotoxicological studies (Costa et al. 2016; Huang et al. 2016; Libralato, 2014; Manfra et al. 2015; Gambardella et al. 2017). The toxicity test with *A. amphitrite* was performed following the standard protocol UNICHIM 2245 (2011) and for *A. franciscana* APAT-IRSA-CNR 8060 (2003) as described in the section below. In addition was performed a ET (Ephyra Test) on ephyra jellyfish following the protocol proposed by Faimali et al. 2014 for *Aurelia* sp. or in this thesis for *S.malayensis.* At the Aquarium of Genoa was also performed the ESTsd exposing the ephyrae to the same sea water samples. This activity was extrapolated from periodic technical reports reported by ISMAR-CNR to Aquario of Genoa.

7.3.2.1 Sea water samples

The water samples analysed (in this thesis were identified with abbreviations of the withdrawal points) were described below and the parameters were reported in Table 24.

Table 24 Chemical parameters reported for samples taken at the Genoa Aquarium (the analyzes were carried out by the Genoa Acquario chemical laboratory).

Sample	**Salinity (ppt)**	**Temperature (°C)**	**pH**	**Residula chlorine (mg*L-1-1)**	**N-NH_4 (mg*L-1-1)**	**N_NO_2**	**NO_3**	**PO_4**
Point D	37.1	24.6	7.90	-	0.4	0.01	< 1	0.5
Point A	38.3	29.4	7.89	0.10	0.2	0.01	< 1	0.1
Point D3	38.2	33.7	7.83	-	0.2	Ils	< 1	0.1
Point D4	34.2	26.8	7.75	-	0.25	0.01	< 1	4.7
Point N2	38.4	25.8	7.67	-	0.1	0.03	< 1	0.2

7.3.2.2 Toxicity tests with other invertebrates model

A. amphitrite

Nauplii of *A. amphitrite* were obtained from laboratory cultures of adult brood stock at CNR ISMAR (Genoa, Italy) according to the method described by Piazza et al. (2016). Twenty to thirty adult barnacles were reared in 700 mL beakers containing aerated 0.45 μm FSW at 20±1 °C, with a 16:8 h light:dark cycle. They were fed every other day with 50–100 mL of Artemia salina at a density of 20 larvae mL-1, and 200–400 mL of Tetraselmis suecica at a concentration of 2 ×106 cells mL-1. Seawater was changed three times a week, and barnacles were periodically rinsed with clean water to remove epibionts or debris. Nauplii were collected and maintained in 500 mL gently aerated beakers with 0.22 μm FSW in a final concentration of 10–15 larvae mL-1, until they were used for toxicity tests.

A. franciscana

Certified dehydrated cysts of *A. franciscana* were purchased from the company MicroBioTests Inc. (Belgium) and used for the experiments (Batch n. AF/F2015). Instar I stage larvae were obtained as described by Garaventa et al. (2010), by incubating 500 mg of cysts for 24 h at 28 °C under light source (3000–4000 lx) and continuous aeration of the cyst suspension in seawater (37% salinity). The newly hatched larvae were separated from non hatched cysts based on their phototaxis and then transferred with a Pasteur pipette into a beaker containing 0.22 μm FSW in a final concentration of 15–20 larvae mL-1.

Acute Toxicity test

Organisms were transferred from the beakers into each well of 24 multi-well plates containing 1 mL of different MP concentrations using a small 80 μm mesh filter. They were incubated in the dark, for 24 and 48 h, at 20 °C for *A. amphitrite* nauplii and at 25 °C for *A. franciscana* larvae according

to Gambardella et al. (2015a). After exposure, mortality analysis was performed under a stereomicroscope: completely motionless larvae were counted as dead organisms, and the percentage of mortality was compared to the controls. Organisms that do not change their own barycentre position and do not move their appendages in 5 s are referred to as 'motionless' (Garaventa et al. 2010). Swimming Speed Alteration (SSA) – a sub-lethal behavioral endpoint – was also evaluated. The Swimming Behavioral Recorder System (e-magine IT, Genoa, Italy) was used to track swimming paths as described in detail in Faimali et al. (2006). Briefly, swimming behavior was monitored in dark conditions, under infrared light, for three seconds.

The resulting digital images were analyzed using an advanced image processing software to reconstruct individual swimming paths and measure the average swimming speed (mm/s) for each test population organism (10–20 organisms). Data were expressed as percentages of swimming speed alteration (SSA) normalized to controls' swimming speed (S), as follows:

$$SSA\ (\%) = [(S\ Treated\ Control - S\ Control)/\ S\ Control \times 100].$$

Where the S Treated Control is the swimming speed registered in organism exposed to water sample and S Control is the control.

7.3.2.3 Toxicity tests with *Aurelia* sp. *and S.malayensis ephyrae*

Ephyra Test _ET

The Ephyra Test with *Aurelia* sp. and *S. malayensis*, were prepared using ephyrae collected immediately after strobilation (0 days old ephyrae) organisms were placed individually into a multi-well plate containing 2 ml of water sample analysed (one individual for each well). For each sample, 3 replicates with 8 ephyrae (one ephyrae in each well) were prepared.

Plates were covered with 1 layer of transparent film to prevent evaporation and were kept in the thermostatic room at 20 °C in dark conditions, after 24 and 48 h both the acute end-point and the sub-lethal one were evaluated using the SBR set to record in dark condition for 1 min as described by Faimali et al. 2014 and in the introduction of this thesis (1.3.4.1)

Ephyra Smart Test_ESTsd

The Ephyra Smart Test semi-dynamic condition for both species of jellyfish were performed exposing the model organisms into a beaker (10 individual for each beaker); containing a 2 L of sea water sample. For each sample, 3 replicates were prepared. For all test the controls were performed using the natural seawater to keep the jellyfish in aquarium. After 24 and 48 hours of exposition were evaluated the Immobility and Frequency pulsation for each ephyrae by direct observation (see section 7.2.2).

7.3.2.4 Results and discussion

Results of Immobility ,Swimming speed alteration and Alteration of Frequency pulsation were reported in Figure 10.9. All test performed confirmed the acceptability of the proposed test organism since the death rate of the controls was always < 10% (APAT IRSA-CNR, 2003 for crustaceans and Faimali et al. 2014 for jellyfish).

Among the tests performed the swimming behavior on crustaceans and jellyfish confirmed to be suitable and accurate end-point to evaluate marine environmental health and quality sea water assessment independently of organism considered.

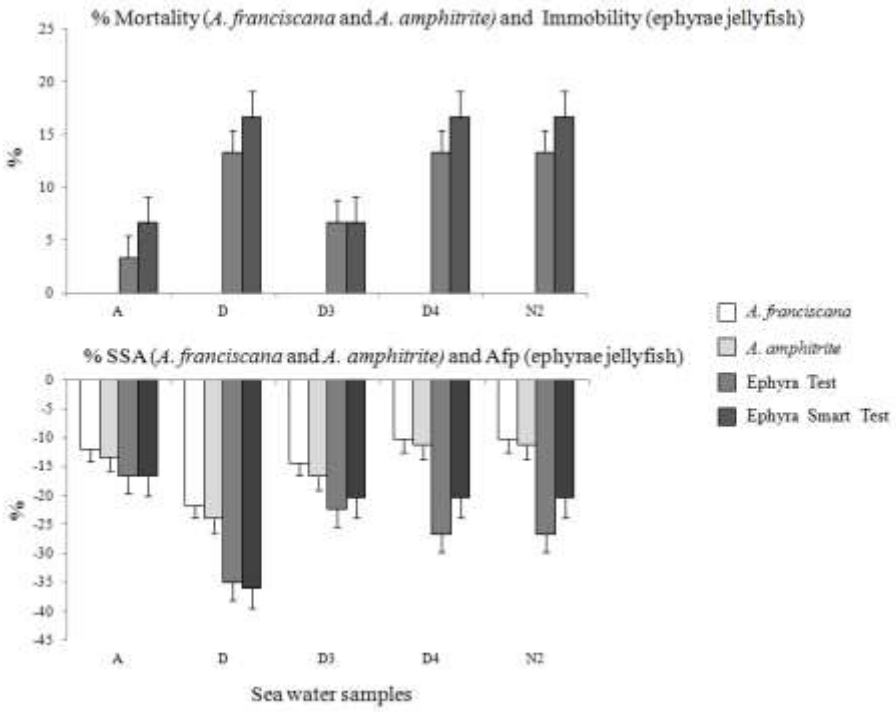

Figure 57 Mortality and, Swimming Speed Alteration (%SSA) evaluated on *A.franciscana* and *A.amphitrite*; Immobility and Alteration of Frequency of pulsation (% AFp) measured on ephyrae jellyfish after 24 hours of exposition to sea water samples from Aquario of Genoa. Ephyrae were exposed to samples by Ephyra Test and Ephyra Smart Test after. (M ±SE, n = 3).

As regards the crustaceans, the brine shrimp *Artemia* sp. (Alyuruk et al. 2013; Anufriieva and Shadrin, 2014; Costa et al. 2015b; Gambardella et al. 2014; Garaventa et al. 2010; Huangmet al. 2016; Kokkali et al. 2011; Manfra et al. 2016; Mesari_c et al. 2015; Venkateswara Rao et al. 2007) and the barnacle *Amphibalanus amphitrite* (Amsler et al. 2006; Costa et al. 2015b; Faimali et al. 2006; Gambardella et al. 2015; Mesari_c et al. 2013; Piazza et al. 2014, 2016; Wu et al. 1997) seemed to be the organisms most used as a model for ecotoxicological studies that investigate the effects of different contaminants, including some emerging ones, on the swimming behavior.

These species are particularly suitable for the analysis of the swimming speed behavior because their larval stages spend most of the time swimming actively. Alyuruk et al. (2013) and Venkateswara Rao et al. (2007), in addition to speed, also measured swimming path alteration in the organisms exposed to contaminants (booster biocides and organophosphates) as an additional

ecotoxicological end-point. However ephyra jellyfish result to be more sensitive than crustaceans for both end-points and bioassays considered. In addition, results by The Ephyra Smart Test seemed to be comparable with Ephyra Test . These feedings seemed to confirm the high sensitivity of new biological model proposed (ephyrae jellyfish) as widely highlighted in this thesis exposing the organisms to a wide range of toxic compounds and the ecological relevance of the Frequency pulsation to assess the quality of seawater when it does not caused mortality.

7.4 Conclusion and perspectives

In terms of operations at Acquario di Genova, the results of this experimental activity suggested that the ESTsd could be the best option available to date as an early warning indicator of a potential toxicity effect of the new sea water on marine life and used in conjunction with other ecotoxicological test a good tools to quality sea water assessment. The proposed bioassay results to be simple, reliable and relatively inexpensive using the animal keeping in the aquarium. The in-house protocol developed at Acquario di Genova for a rapid screening of seawater toxicity using ephyrae jellyfish is in addition available for other users to reproduce or adapt. However, further calibration could also be carried out for other kind of contaminants, that can determine any potential harmful input in the Acquario system.

Further optimizations, it would be developed small sentinel tanks like displayed in Figure 58, where the swimming behavior of ephyra jellyfish (keeping in this tank) could be continuously monitored by an automatic image analysis system to generate an alarm signal in an “online” operation regime when than alteration occurred, before the natural seawater comes used to keeping the animals.

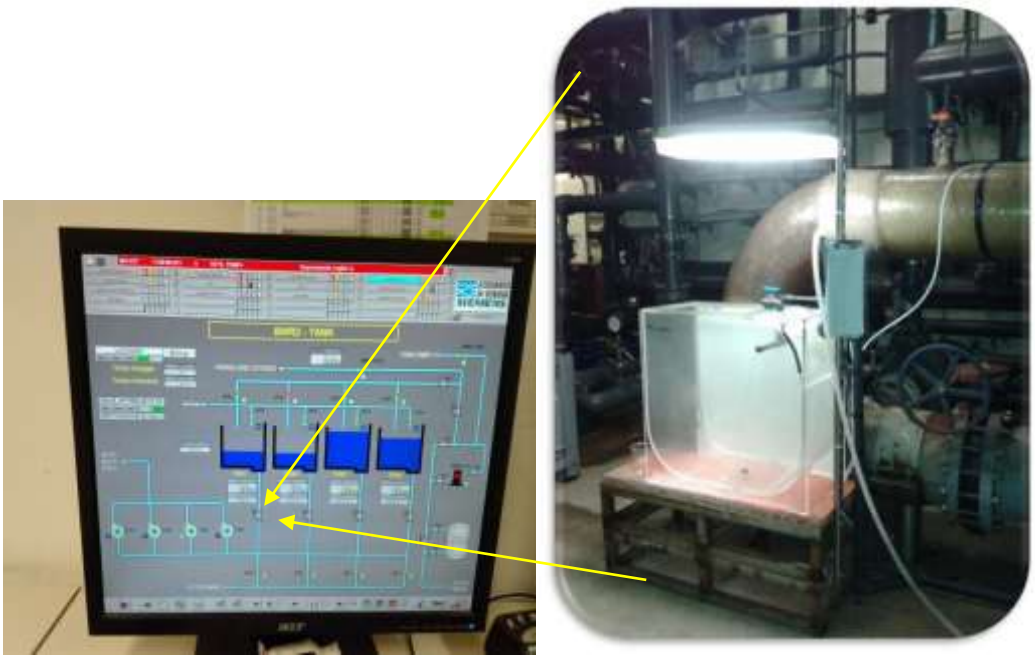

Figure 58 A sentinel tank (Krisel) to monitor by an automatic image analysis system the swimming pulsation of ephyrae jellyfish to generate an alarm signal in an “online” operation regime before the natural seawater comes to use in the Acquario to keep animals.

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