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INFLUENCE OF JELLYFISH BIOMASS ON BACTERIAL DYNAMICS IN THE LAKE OF VARANO

Tutor:
Prof. Daniela Prevedelli
Co-tutor:
Prof. Stefano Piraino

PhD-Candidate:
ROSA CAPRIOLI

Coordinator:
Prof. Maria Giovanna Vezzalini

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There is a pleasure in the pathless woods, there is a rapture on the lonely shore; there is society, where none intrudes.

By the deep sea and music its roar:

I love not man the less, but the nature more.

Lord Byron

Abstract

Jellyfish blooms were increasingly reported in the last years and interpreted as the direct effect of multiple drivers of change in marine ecosystems, including global warming. Much attention has been paid to the mechanisms of bloom formation, the predatory impact of jellyfish on food webs and ecosystem processes, and the jellyfish-related hazards for human activities in coastal areas. Conversely, much less information is available on the fate of decaying gelatinous biomasses (*jelly-falls*) following blooms events. The rapid decay of jellyfish bodies after the completion of generally seasonal or annual life cycles leads to the rapid and massive release of nutrients in the water column. This event may produce an important trophic shift in the marine food webs, related to qualitative/quantitative substantial changes in the microbiome, with consequences for the overall functioning of aquatic ecosystems.

The aim of this thesis was to evaluate the impact of jellyfish biomass produced by blooms of the scyphozoan *Aurelia* sp. on the dynamics of different microbial populations in the marine coastal lagoon of Varano (Southern Adriatic coast, Italy).

The thesis project was divided into three sections.

The first section was dedicated to the **quantitative** analysis of changes in relative abundances of the microbial community due to jellyfish biomasses, by traditional microbiological methods. The second section dealt with the **qualitative** analysis of the microbial community, which has been carried out using a combination of traditional and modern methods: a standard selective isolation and culturing methodology, a PCR-based molecular barcoding of the main taxonomic units, and a Next Generation Sequencing metagenomic analysis.

The third section explored a **blue biotech approach** to the antibacterial activity of compounds isolated from the jellyfish *Aurelia* sp.

Qualitative and quantitative studies on the bacterial community dynamics were carried out using water samples collected from different areas of the lake of Varano over one year. Such areas were characterized by either the presence or absence of live jellyfish and jelly-falls. Microbiological analysis also included the research of the classical microbial pollution indicators for the assessment of the water quality. In addition to these indicators, others microbiological parameters were considered. In particular we examined the density of culturable bacteria at 37°C (including human potential pathogens), vibrios and heterotrophic culturable bacteria at 22°C. In presence of

jellyfish, the abundance of some microbial pollution indicators, heterotrophic bacteria and vibrios was higher than in the control samples (absence of jellyfish). Despite of this, when the jellyfish bloom almost disappeared (September), most of the examined parameters showed the highest values, probably due to an enrichment of organic matter from the decaying gelatinous biomasses (jelly-falls).

The total bacterioplankton counting was also carried out by DAPI staining and observation of water samples under an epifluorescence microscope. For the calculation of the biomass, the bacteria were divided into three different size classes: small, medium and large. The biovolume was converted into biomass, assuming a carbon content of 310 fg C μm^{-3}. In May, coincident with the peak of jellyfish bloom, the bacterial biomass and abundance were higher in the zone with *Aurelia* sp. than in the control zone. The highest values of bacterial biomass and abundance were recorded in September simultaneously with the jellyfish decaying. Over the year, large size bacteria were more abundant than medium and small bacteria.

The studies on the diversity of the culturable heterotrophic were conducted at the *Morska Biološka Postaja (Piran-Slovenija) laboratories.* The taxonomic identification of the culturable heterotrophic isolates was also corroborated by a standard 16S rDNA sequencing.

The standard qualitative analysis showed that γ-proteobacteria represented the main component of the bacterial community in the lake of Varano during the jellyfish bloom and that the *Vibrionaceae* family was a key component. However, the microbial diversity resulting by a standard barcoding approach is usually low because only a minority of marine bacteria can be isolated and cultured. Therefore, the analysis of microbiome diversity of the Varano lake was also attempted for the first time through the application of a metagenomic (Next Generation Sequencing) Illumina-based protocol targeting the universally conserved 16S ribosomal RNA gene. The development of the metagenomic protocol and the sequencing analysis were carried out by the Institute of Biomembranes and Bioenergetics (CNR). In presence of jellyfish a significant increase of the *Halomonadaceae*, *Shewanellaceae*, *Comamonadaceae*, *Simkaniaceae*, *Micrococcaceae* families has been recorded. The family of *Oceanospirillaceae* also slightly increased.

To analyze the potential causal mechanisms regulating jellyfish impacts on the bacterial community, we also investigated the occurrence of bioactive metabolites with antimicrobial activity contained in different components of *Aurelia* sp. (tentacles, main

body and exudates). In each examined compartment an antibacterial lysozyme-like activity was detected. In the mucus, in the jellyfish body and especially in tentacles homogenate, a significant lysozyme-like activity has been found. Isolation and characterization of the involved metabolites could lead to the development of new biological approaches in the preparation of antibiotics

Section 1: Quantitative Analysis

1. Introduction

1.1. Changes of the structure and of the functioning of Mediterranean marine ecosystems.

After its latest report on Climate Change, the Intergovernmental Panel on Climate Change (IPCC) has declared: “Warming in the climate system is unequivocal and since 1950 many changes have been observed throughout the climate system that are unprecedented over decades to millennia” (IPCC, 2007). Most of the warming since the middle of the 20th century is very likely (subjective probability of >90%) to be due to the human-induced increase of atmospheric greenhouse gas concentrations (IPCC, 2007). Climate warming observed over the past several decades is consistently associated with changes of the hydrological cycle and hydrological systems such as changing precipitation patterns, widespread melting of snow and ice, rising sea levels, increasing evaporation (Huntington, 2006), more cases of hurricanes of great intensity and the arrival of spring in advance (Solomon et al., 2007; IPCC, 2007; Hoegh-Guldberg and Bruno, 2010).

Even marine ecosystems are strongly affected by climate change but in some case the response to climate change also depends from interactions with other human-induced changes in the marine environment.

The enclosed Mediterranean basin is the result of a complex geological and biological history and could be considerated as a miniature ocean (Lejeusne et al., 2010) where the effects of climate change are likely to be more apparent earlier than in other more open oceans (Coll et al., 2010). Data recorded in the Mediterranean Sea show that sea temperature is increasing and extreme climatic events are becoming more and more frequent. Climate change prediction models suggest by 2100 an increase of 0.7-4.6 °C in sea surface temperature, a 2-28%reduction of precipitation levels and an increase of extreme weather events. From the hydrological point of view, the Mediterranean Sea is divided in two deep basins: the western and the eastern basin. Each sub-basin has distinct water masses and is characterized by a homogeneous deep-water layer that, below 250 m depth does not get colder than 12-13 °C. In the recent years, this general pattern has been disrupted probably due to climate change and a general rise in temperature has been recorded (UNEP-MAP RAC/SPA 2010).

These phenomena may strongly influence some biotic and abiotic patterns (planktonic and larval dispersal, nutrient cycles, etc.) affecting ecosystems at several ecological levels. Some unexpected effects of thermohaline circulation (termed Eastern Mediterranean Transient, EMT) have been documented to drastically change the hydrology of deep eastern Mediterranean, influencing temperature, salinity, stratification and circulation of water masses. EMT also affected the carbon and nitrogen cycle, negatively impacting on deep-sea biota. The increase of water temperature may affect the organisms, causing several stresses and frequently bringing some physiological adaptations. In some cases, when stress exceedes tolerance threshold, it may determine alteration in life cycle or in the distribution pattern of the species, as well as a higher susceptibility to diseases and mass mortalities. In Mediterranean Sea, a direct consequence of warming trend is a simultaneous increase in the abundance of thermotolerant species and the disappearance or rarefaction of 'cold' stenothermal species (Lejeusne et al., 2010).

The stability of ecological communities largely depends on the strength of interactions between predators and their prey. Effects of climate change on ecosystem functioning affect all biological levels. The combination of thermal stress and food shortage resulting in mass mortality events induces changes in fluxes of matter and energy in trophic webs. For example in the 1990s, the EMT uplifted nutrient-rich waters, resulting in enhanced primary productivity in the euphotic zone, therefore increasing organic matter flux to the seabed, which impacted the deep-sea biota through cascading effects. The density, activity and biomass of bacteria and meiofauna were dramatically reduced, converting the eastern Mediterranean large deep water transient in a monoculture of certain opportunistic species of polychaete *Myriochele fragilis* (Sardà et al., 2004).

The arrival of new key species, could alter competition patterns between native species, and or transform current ecosystems into new ones. Invasive species therefore already play a key role in the 'revolution' underway in the Mediterranean. Mediterranean marine ecosystems are certainly heading towards a climate-induced revolution in their functioning. Deep-sea ecosystems, traditionally considered to be stable unchanging environments, seem to respond quickly to climate change (Danovaro et al. 2001)

Climate change is influencing the boundaries of biogeographic regions with some warm water species extending their ranges and colonizing new regions where they were previously absent. Observed changes involve both indigenous species subjected to a process named ‘meridionalization’ and non-indigenous species, subjected to a process defined as ‘tropicalization’ (Boero et al., 2008). Meridionalization (Bianchi, 2007) is the northward widening of the distribution of species of warm water affinity that usually thrive in the southern, and warmer, part of the basin (i.e.meridional species).

Tropicalization is the establishment of tropical species that were previously absent from the basin, for example the increasing introduction of nonindigenous Indo-Pacific species in the Mediterranean through Lessepsian migrations (Galil, 1993).

Seawater temperature rise is also increasing the risk of extinction of cold-water species in the Northern Adriatic Sea, one of the most vulnerable Mediterranean areas to climate change. Climate change in the Mediterranean also promotes epidemiological outbreaks, since most pathogens are temperature sensitive. The coral-bleaching bacterium *Vibrio shiloi* was involved in the mass mortalities of the coral *Oculina patagonica* (Kushmaro et al., 1998). Studies show that the spread of large amorphous aggregates (mucilage) in the Mediterranean Sea is linked to climate-driven sea surface warming. The mucilage contains several species of pathogenic bacteria and viruses and could contribute to their spread in different regions (Danovaro et al., 2009). On the basis of a moderate climate change scenario, Perez et al. (2010) hypothesized an extinction of 15-37% of the species occupying the North-Western Mediterranean Sea by 2050.

1.2. Bacteria abundance in sea water

In natural environments many different types of microorganisms coexist and closely interact with each other and with the physical and chemical environment around them. Scientists adopted the term "microbial community" to define these groups of microorganisms, bacteria, viruses, algae and protozoa, their functions and their interactions. The extreme diversity of their metabolic activities and their strong ability to adapt mean that they can even change the characteristics of the surrounding environment. Bacteria represent a significant part of the planktonic biomass and play a key role in most of marine ecosystem processes, such as in food webs and cycling of biogenic elements or the degradation of anthropogenic xenobiotics (Fuhrman 1992; Azam 1998).

Many studies were conducted on microbial distribution and abundance. Microbial density is extremely variable not only along from coastal waters to open sea, but also along the water column and between water and sediments. Abundant in the photic zone, the number of bacteria decreases rapidly with increasing depth, but at the level of the seabed, their density undergoes a considerable increase, reaching very high values.

The bacterial abundance ranges from 10^6 to 10^9 cells $l^{-1}$and it is different on the different examined areas: Arctic environment (Kuosa and Kaartokallio 2006), temperate (Kirschner and Velimirov 1996, Gonzalez et al., 2003) and tropical zones (Bettarel et al., 2006). Furthermore, the abundance of bacterioplankton has always been considered strongly important from primary production because the function of the bacterial component within the microbial loop is to convert the DOM (dissolved organic matter), produced by phytoplankton, in POM (particulate organic matter).

The importance of the Microbial loop

The term Microbial Loop coined by Azam et al., (1983) describes the role that microbes play in the marine ecosystem carbon cycle. Marine bacteria are the base of the food web in most oceanic environments and they improve the trophic efficiency of marine food webs and important aquatic processes (Ducklow et al., 1986). A large portion of the organic matter, synthesized by primary producers, becomes dissolved organic carbon (DOC) and is taken up almost exclusively by heterotrophic bacteria. Most of the organic carbon available to consumers in the ocean is used and respired by bacteria (Robinson and Williams, 2005) and a portion is assimilated and re-introduced into the classical food chain. (Azam and Malfatti 2007)(Fig.1).

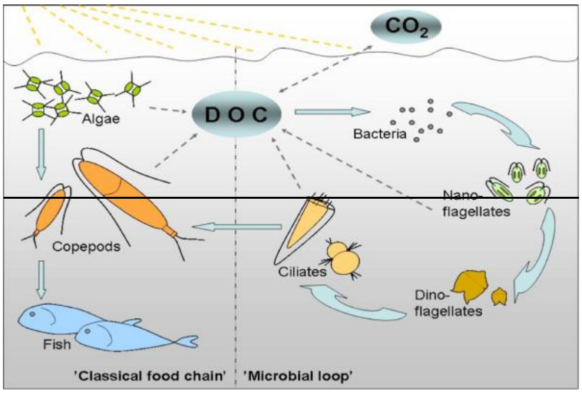

Fig. 1 Microbial food web. (Picture credit: Fenchel, 2008).

Seawater bioassay experiments demonstrate that Heterotrophic bacteria process about half of net primary production and thereby play a dominant role in the microbial carbon pump (MCP) by altering and transforming labile forms of organic matter into refractory forms that persist in the ocean (Benner and Herndl, 2011). They play a critical role in either mitigating or exacerbating the effects of climate change. The phenotypic plasticity of microbes and their ability to evolve rapidly also means that they can rapidly shift their metabolic capabilities, host range, function and community dynamics in response to changing environmental conditions (Webster and Bourne, 2012).

1.2.1 Limiting factors for marine bacteria

There are important links between environmental characteristics and bacteria. Many studies have shown that the bacterial density is significantly affected by biotic and abiotic factors such as temperature (Pomeroy and Wiebe 2001), dissolved oxygen, nutrients (such as N and P), availability of organic substances, solar radiation and grazing (made either from protozoa to viruses)(Kroer 1993).

These characteristics and the interaction with viruses and protozoa, affect the productivity and the functioning of aquatic systems because they alter the community structure of phytoplankton and bacterioplankton which are important for the transfer of energy from the microbial loop to the higher trophic levels.

Effect of temperature and nutrients on sea water bacteria abundance

Many experiments were conducted to understand the influence of abiotic factors such as temperature on the bacterial abundance. Pomeroy and Wiebe (2001) calculated that more than 50% of the variability of heterotrophic bacterial growth, increasing from 0 to 30°C, depends from temperature. Mesocosm experiments showed that increases of temperature drove direct increases of more heterotrophic than autotrophic processes (Keller et al. 1999). Conversely, it seems that in open ocean food resources play a major role with respect to temperature in influencing the bacterial density (Gasol et al. 2002). In surface waters, where light is always available, nutrient availability (carbon, nitrogen and phosphorus) is the most limiting factor for bacterial growth. In the Mediterranean Sea, Herndl et al. (2000) calculated C: N: P ratios for phytoplankton and bacterioplankton, respectively 106:16:1 and 50:10:1. Compared to phytoplankton, bacterioplankton productivity and growth are more limited by the concentrations of N and P in the Mediterranean Sea. In these conditions, large amounts of photosynthetically fixed carbon is released in the environment, without a proportionally efficient assimilation by the bacteria (Obernosterer and Herndl 1995). However, bacterial growth often depends on phytoplankton growth (Robarts et al. 1994; Hepinstall and Fuller 1994; Fitter and Hillebrand, 2009). This correlation is due to the dependence of bacterioplankton on algal exudates as a source of energy (Cole et al. 1988, Sundh 1992). In environments where bacterial production is not dependent on algal production, heterotrophic bacteria compete with primary producers for inorganic nutrients (Grover, 2000, Franken et al., 2005). N and P co-limitation in the bacterial community results in increased competition between the heterotrophic and autotrophic components of the surface communities (Mills et al. 2008)

Effect of organic matter availability on sea water bacteria abundance

The microbial community uses the dissolved organic carbon and the particulate organic carbon as a suitable source of food. The chemical composition of the substrate affects the efficiency of growth of bacteria (Connolly et al. 1992). A major source of labile organic molecules for the growth of heterotrophic bacterioplankton is represented by the dissolved primary production (DPP) (Norman et al., 1995), composed of easily degradable molecules (Amon et al. 2001). Indeed, in presence of amino acids the bacterial growth reaches values of 70%, while in the presence of esters, alcohols and organic acids is approximately 40%, and finally the lowest values, about 20% are found in the presence of DOC (dissolved organic carbon) (Meyer et al. 1987; Hopkinson et al. 1989, Griffith et al. 1990, Connolly et al. 1992). Jørgensen et al. (1993) observed that dissolved aminoacids (DOC labile components) may constitute 42-91% of the total demand for carbon by bacteria.

Jellyfish bloom: Most probable causes, effects and influence on bacterial dynamics

The functioning of marine ecosystems is affected by the recurrent cycles of abundances (blooms, increase in the population size) of key species. In temperate seas like the Mediterranean, the seasonal phytoplankton bloom is determined by a peak of abundance of planktonic protists (usually diatoms or dinoflagellates). The phytoplankton pulse is followed by a zooplankton bloom that sustains the rest of the food web. The phytoplankton is the backbone of marine production and sustains also our exploitation of marine resources through fisheries (Boero, 2013). The gelatinous plankton has an indisputable impact on marine food webs as it exploits the same resources of fish. Massive increases of gelatinous filter-feeders and/or carnivores are frequently observed, and explanations of these blooms alternatively invoke ecosystem variability, climate change, unspecified anthropogenic perturbation or the removal of top predators from trophic networks. (Boero et al., 2008).

During the past several decades, a high number of gelatinous zooplankton species has been reported in many estuarine and coastal ecosystems (Purcell et al. 2007; Condon et al., 2012)(Fig. 2). Jellyfish populations play an important role in ecosystem diversity, in the ecosystem functionand in biogeochemical cycling. Bloom populations could potentially alter trophic pathways increasing the conversion of primary and secondary production into gelatinous biomass (Condon and Steinberg 2008; Pitt et al. 2009; Lucas et al, 2011). Such gelatinous mass may limit carbon bioavailability to higher trophic levels and promote a microbially dominated food web through the release of labile organic matter (Condon et al., 2011).

Fig. 2 A bloom of moon jellyfish (*Aurelia* sp.) near Denmark. Photo credit: Casper Tybjerg, http://www.ttf.dk

The rapid decay of gelatinous biomass and the substantial release of nutrients in the water column represent two important events in the trophic web and energy flow.

Jellyfalls decomposition may produce a strong increase in oxygen demand, an increased regeneration rates of nutrients and some important qualitative and quantitative changes in the microbial communities.

In particular, the biomass of jellyfish generates large amount of colloidal and dissolved organic matter (jelly-DOM)(Hansson et al., 1995), which is released to the water and which may be used by the ambient bacterioplankton (Carlson, 2002; Condon et al., 2008). It can stimulate the bacterial growth and productivity and enzymatic activities (Riemann et al., 2006; Titelman et al. 2006). Bacterial use of jelly-DOM could lead to the repackaging of gelatinous organic carbon and its reincorporation into the food web (Condon, 2011). An experimental study was carried out to analyze the effects of scyphomedusa *Aurelia* sp. on the microbial planktonic assemblage in the marine lake of Mljet Island (Big Lake, Southern Adriatic). The addition of *Aurelia* sp. jellyfish to experimental in situ enclosures revealed that bacterial productivity was about twice as high in the presence of *Aurelia* sp., leading to a consistently higher bacterial biomass (Turk et al. 2008, Tinta et al., 2012).

Attrill (2007) reported a significant correlation of jellyfish frequency in the North Sea from 1971 to 1995 with decreased pH (from 8.3 to 8.1) of surface waters. It is very important to understand the possible linkages between jellyfish blooms and ocean acidification. It is also important to determine whether ongoing changes in the seawater inorganic carbon system will exacerbate problematic increases in jellyfish that have been associated with climate change, overfishing, eutrophication, and factors like increased space for polyps and transport of non-indigenous species through ballast water (Fig.3).

Overfishing may not always be the sole or even the primary threat, but in conjunction with other stresses it can be a serious one. It is possible to identify two main types of overfishing, the first type is the intensive fishing of a targeted species that leads to marked declines in catch per unit effort and size of individuals captured. In the second type, known as assemblage or ecosystem overfishing (Murawski 2000), overfishing of an assemblage is demonstrated when catches proceed beyond the asymptotic maximum of a plateau-type curve or decline below the asymptotic or plateau level. Global overfishing is removing top predators from the oceans. Fish larvae compete with jellyfish in eating crustacean zooplankton and if the adult populations are large, the number of produced larvae and juveniles might overwhelm the gelatinous plankton, out competing it. A lower abundance of fish, however, might release jellyfish from their competition and, if the jellyfish populations increase, a vicious circle starts, since they predate on their competitors, further reducing the resilience of the fish populations already impacted by overfishing (Boero 2013).

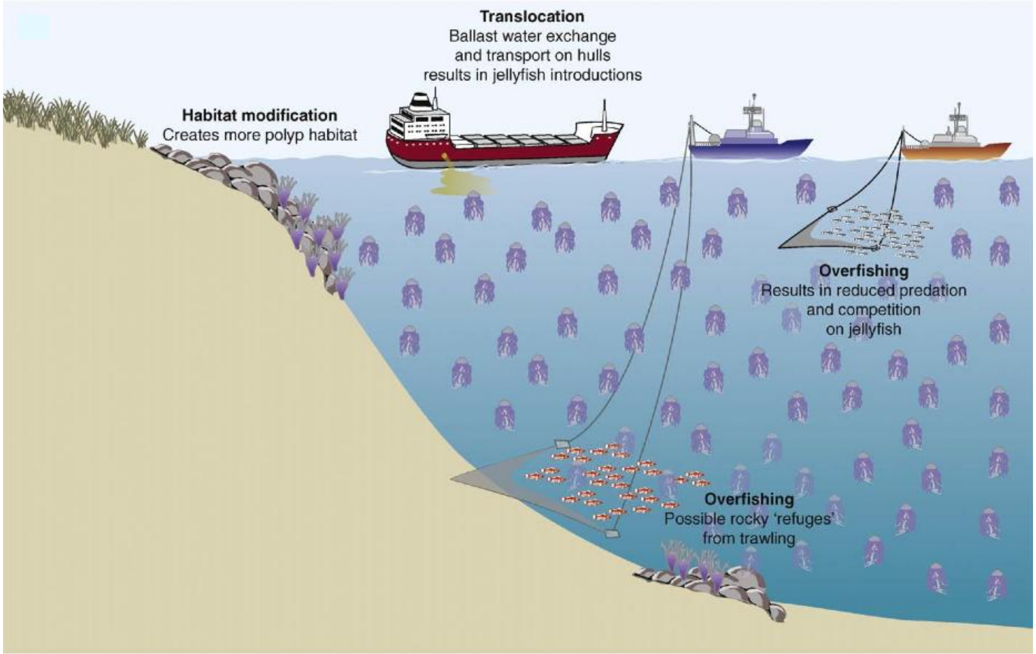

Fig. 3 Some mechanisms boosting jellyfish outbreaks. (Picture credit: Richardson et al., 2009)

Intensive trawling of the seafloor might further contribute to jellyfish blooms, as large numbers of potential competitors and predators of jellyfish are removed from heavily trawled soft-bottom habitats, while any nearby hard substrate (e.g. rocky outcrops), which is difficult to trawl, could possibly afford jellyfish polyps refuges.

Eutrophication is one of the major global pollution problems (Howarth 2008). In addition to increased nutrients, eutrophication is associated with altered nutrient ratios and increased turbidity. Most simply, increased nutrients often lead to greater biomass at all trophic levels. More food for polyps and jellies increases growth, asexual reproduction, and sexual reproduction rates. Eutrophication also causes complex changes in the food web. Accumulating evidence suggests that high N:P ratios shift the phytoplankton community from a diatom a flagellate-based food web, which may favor jellyfish blooms (Purcell et al. 2007). Eutrophication is also connected with size reduction of the zooplankton community, which may be detrimental to fish predators that prefer large zooplankton, thereby benefiting jellies that consume both small and large zooplankton. Jellies may contribute to eutrophication and be able to use it directly because they excrete and take up nutrients (ammonium and phosphate) and DOM (Pitt et al. 2009). In Chesapeake Bay (USA), ctenophores excreted high percentages (25.2% and 18.3%) of their body C and N in mucus and contributed up to 18% and 28% per day to labile DOM (Condon et al. 2010, Purcell 2013). The addition of excessive nutrients from fertilizer into coastal waters can alter pelagic communities. Nutrients of the coastal zone are rich in nitrogen and phosphorus but poor in silica. In these conditions, the silica deficiency hypothesis suggests that non-siliceous phytoplankton, such as flagellates, proliferate and replace diatoms (Harashima et al. 2006) resulting in a reduction in the size of primary and secondary producers. Cushing (1989) hypothesised that such a food web supports fewer fish, marine mammals, turtles and seabirds because of the smaller average food size and longer food chain, and is more favourable for jellyfish for their ability to feed on a range of prey, including protists (Malej et al. 2007) such as flagellates (Colin et al. 2005). Large phytoplankton blooms can sometimes sink to the seafloor, where their bacterial degradation can cause localized hypoxia (Diaz and Rosenburg, 2008). Coastal eutrophication encourages phytoplankton blooms that can ultimately lead to jellyfish outbreaks (Purcell et al. 2001).

Another probable cause of increase in jellyfish bloom is the translocations of species to new marine areas, commonly caused by the exchange of ballast water (containing organisms) between regions and the transport of fouling biota (e.g. polyps) on ship hulls (Graham and Bayha, 2007; Ghabooli et al 2013). Some jellyfish, especially ctenophores, are robust to ballast water exchange, and have often increased in abundance once moved to new areas (Daskalov et al., 2007). Finally, cnidarians polyps require a hard substrate for attachment; hence, an increase in the habitats modification and consequently the increased presence of habitat suitable for polyps, could theoretically lead them to proliferate (Graham 2001; Lo et al., 2008; Duarte et al 2012).

1.3. Phylum Cnidaria

Cnidaria, the phylum of the “stinging creatures”, includes more than 9000 species and takes its name from specialized cells, the cnidocytes or stinging cells (from the greek Knide, nettle) containing special sub-cellular organelles, the cnidocysts or cnidae (Brusca & Brusca, 1990). The possession of cnidocytes, is the defining characteristic of the phylum (Hessinger and Lenhoff 1988). All cnidarians are carnivorous, with the cnidocytes on the tentacles actively involved in prey capture. Cnidocysts can inject a venom when the animal comes into contact with a prey or with an enemy. Cnidarians are animals with radial symmetry of the body or biradial symmetric and it is believed that this is a feature within the ancestral eumetazoa. Cnidarians are not completely radial, in fact, shortly after the beginning of their life show significant signs of bilaterality (Boero et al, 2007; Boero & Piraino, 2010; Piraino et al., 2011).

The cnidarian bauplan is typically characterized by epithelial cell layers embracing a thick extracellular matrix (mesoglea) mainly composed of fiber proteins like collagen and proteoglycans. The body includes a single sac-like cavity, the coelenteron, which is in contact with the surrounding water through the mouth. The coelenteron (also termed the gastrovascular cavity) serves for gas exchange and digestion. Cnidarians have a complex life cycles, including a planktonic larval stage (the planula), a benthic secondary post-larval stage (the polyp) which may originate single or multiple copies of the planktonic adult stage (the medusa) (Fig. 4).

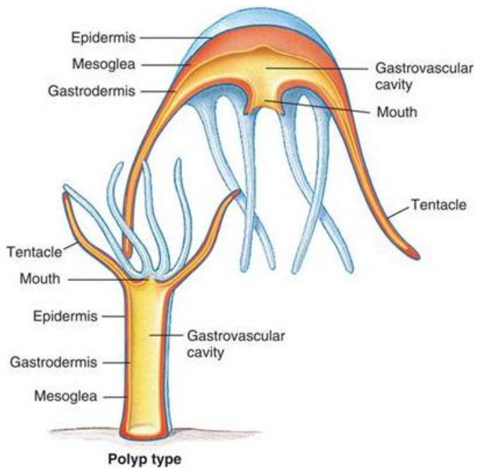

Fig. 4 Two different morphs of cnidarians: polyp and medusa. The short living larval stage is not represented.

Polyps represent the benthic phase, they can be individual or organized into colonies. Medusae (or jellyfish) represent the pelagic phase, which may swim by muscular contraction of their bell-shaped body or simply drift along with the currents. All cnidarians possess sensory cells or more specialized organs, like the compound eye found in cubozoan jellyfish (Pearse and Pearse 1978). Cnidarians possess polytrophic foraging strategies including endocytosis, absorption of dissolved organic matter and utilization of assimilates from symbiotic microalgae (zooxanthellae) (Schlichter 1982; Shick 1991). The Phylum of Cnidaria is currently divided into two subphyla: Anthozoaria represented by the class Anthozoa and the subphyla of Medusozoa, represented by classes Cubozoa Scyphozoa and Hydrozoa of the superclass (Argano *et al* 2007). According to Marques & Collins 2004, the Staurozoa, formerly considered as the order Stauromedusae of Scyphozoa, should be considered for their peculiarities a separate fifth class.

Class Scyphozoa

The Scyphozoa are approximately made up of 4 orders, 2 suborders, 24 families, 11 subfamilies, 71 genera and over 220 species. Members from this class, inhabit all the oceans and seas of the world. The majority of the species live in shallow coastal waters, though there are species have been found in deep seas (Hale, 1999).

The Scyphozoa are characterized by a dominant jellyfish phase (Fig. 5), but in the life cycle of several species a polyp stage is common.

Jellyfish occur in a wide variety of sizes, shapes and colors. They are 97 percent water and are semi-transparent or glassy and bell-shaped. Regardless of their size or shape, most jellyfish are very fragile, often containing less than 5% solid organic matter (Arai,1997).

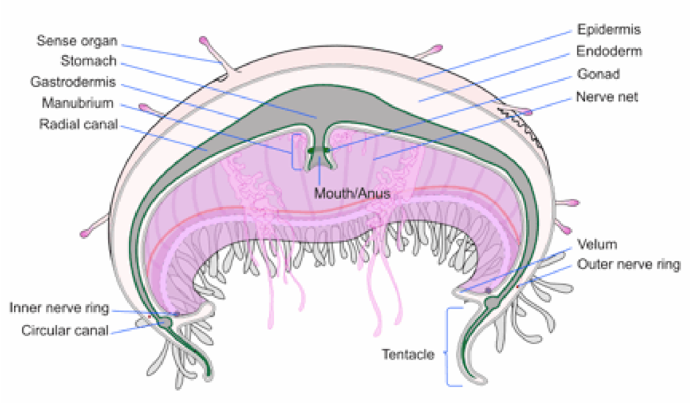

Fig. 5 Jellyfish morphology

1.3.1 *Aurelia* sp.

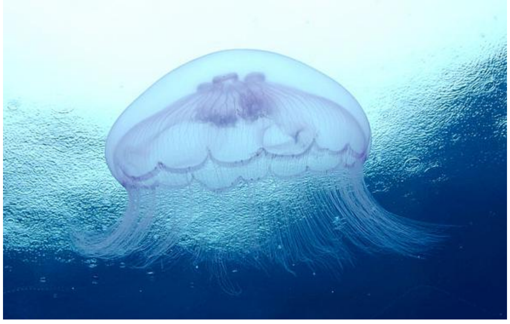

Fig. 6 *Aurelia* sp. the moon jellyfish

Jellyfish of genus Aurelia (Fig. 6) are very common and abundant in all the oceans of the northern hemisphere. The umbrella can measure 30-40 cm, has tentacles on the margin, the body is disk-shaped, whitish, with gonads arranged in four circles always visible. The handle has four long oral arms. Many radial canals connect the center with the periphery of the umbrella. *Aurelia* sp., like all cnidarians, have cnidocysts but the venom is harmless to humans and so that this jellyfish can be touched with impunity. The life cycle of *Aurelia* can exemplify the cycle of many jellyfish. The life cycle of a jellyfish is complex and involves an alteration of generations in which the animal passes through two different body forms, medusae and polyp, reproduce both sexually and asexually, and go through five life stages: egg, planula, polyp, ephyra and medusae. The dominant and conspicuous medusae is the familiar form, while the smaller polyp form is restricted to the larval stage. Jellyfish are either male or female and reproduce sexually. The reproductive organs (gonads) develop in the lining of the gut. During the reproduction, the male releases sperm through its mouth into the water column. The sperm swims into the mouth of the female where fertilization occurs. Early embryonic development begins either inside the female or in brood pouches along the oral arms. Small swimming larvae (planula) leave the mouth or brood pouches and enter the water column. The larvae then seek a shaded surface and attach to the bottom, forming polyps. Polyps of some species propagate asexually, forming colonies (if the progeny remain attached to one another) or clones (if the progeny separate). A polyp can live for several years, producing clone polyps by budding, and eventually beginning a process called strobilation that will transform a single polyp into several organisms (Ruppert, 1994). Strobilation is an asexual reproductive process involving the production of a different morph. When strobilation begins, horizontal grooves appear along the length of the polyp. The grooves deepen until the polyp is transformed into a column of multiple organisms. These organisms, stacked one on top of the other, pop off the top of the column and become a free-swimming, immature medusae called ephyra. When the ephyra first breaks off the polyp, the bell is star-shaped with eight or more points. As the ephyra grows, these points grow together until the animal assumes the adult medusae form and the process starts over again.

In a few weeks, an ephyra will grow into an adult medusa, thus completing the complex life cycle (Arai, 1997). Each polyp can also produce hundreds of small jellyfish. When the jellyfish disappear, they are usually on the bottom of the sea in the form of polyps. One polyp can be able to produces millions of jellyfish (Fig.7).

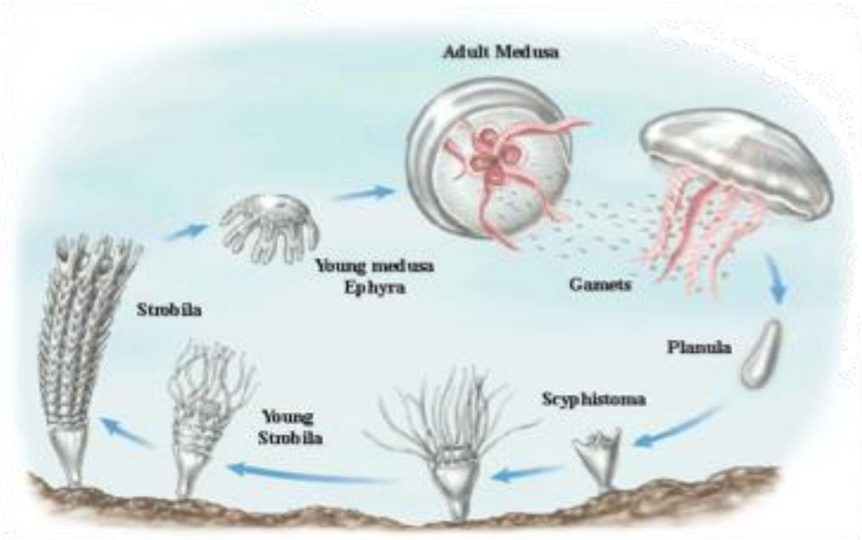

Fig. 7 *Aurelia* sp. life cycle

2. Materials and Methods

2.1. Area of study: Lake of Varano

Varano lagoon is the largest lake in southern Italy. It is situated in the southern Adriatic coast (Apulia, Italy) on the north side of the Gargano promontory. This lagoon is characterized by brackish water and it extends over a 60.5 Km^2 area, with a perimeter of 33 Km. The average depth is 4 m, with maximum values of 5 meters in the central zone. Separated from the Adriatic sea by a tongue of sand 10 km long, it is connected with open sea through two channels, respectively called Foce Varano and Foce Capoiale (Fig.8).

Salinity values are relatively stable for a lagoon, never dropping below 20 psu. Temperature extremes are 5°C and 30°C. The hydrological system consists of the lagoon, two channels to the sea, some freshwater inputs from a small catchment basin drained by the S. Antonino and S. Francesco torrents and the Muschiaturo drainage pumping station, and several stream springs (Bagno, Irchio, Ospedale, S.Nicola, etc.).

The lagoon and the neighboring coastal area are exploited by mussel farming and although such activity has been recently reduced within the lagoon, fishery is now the most important resource. According to the climatic station located in near Cagnano Varano (urban centre), the most rainy season is winter, running from November to February, with a precipitation average of 848.9 mm year^{-1}. North-North-Western winds are very frequent in this area, above all during the winter season, helping sea water inputs into the lagoon.

Fig. 8 Varano Lake

Hydrological investigations on the water balance of the lagoon (Villani et al., 2000; Spagnoli et al., 2002) estimated a freshwater input of approximately 87,000 m^3 d^{-1} with an organic content mostly originating from urban wastewater, oil mills and farming activities, with an organic content mostly originating from urban and agricultural runoff, fishfarming and zoo-technique activities. Due to the low tide excursion and reduced exchange with the adjacent coastal area, the water time residence is very long and it is estimated to about 1.5 years (Specchiulli et al., 2008; Belmonte et al., 2011).

The trophic condition of the system is characterized by the relatively low phosphorus and nitrogen concentrations (0.16 and 4.60 mmol m^{-3}, respectively), with these values comparable to those typical of oligo-mesotrophic waters.

Varano lagoon is subject to phytoplankton blooms. For several years dark red dinoflagellate blooms appeared during the summer causing general mortality in the fauna and forcing the mussel farmers to remove their stock from June to September. During the winter, the water would clear and become highly transparent. This cycle ceased about six years ago and since then the waters show green throughout the year. The bloom is a mixture of diatoms, dinoflagellates, etc. but certain harmful elements prevail at times as witnessed by the episodes of mortality in the benthos. During the same period there has been an extraordinary population explosion of jellyfish belonging to the scyphozoan taxon *Aurelia* sp. strictly confined to the lagoon and not extending into the neighbouring sea.

The benthos is negatively influenced by the blooms. During the summer the animal benthos disappears due probably to anoxia and re-colonises the area in autumn after the temperature has cooled. Representative species of the benthos are the bivalves *Loripes lacteus, Gastrana fragilis, Mytilaster minimus, Cerastoderma glaucum.* Seagrasses are present only along a strip close to the northern shore (Manini et al., 2005).

In summer time, in recent years, have been recorded presumably due to the increased anthropogenic loading of the lagoon and to aquaculture activities (Tolomio et al., 1990). Large nutrient inputs affect the Varano lagoon as sewage effluents (Muschiaturo, Antonino and Fosso S.Francesco) coming from nearby towns and from surrounding soils which are intensively fertilized. Inorganic nitrogen concentrations are typically higher in winter. In the summer - autumn period, maximum concentrations of phosphorus are recorded (Tolomio et al., 1990; Caroppo, 2000).

2.2. Sampling

Impacts of blooms are likely to be particularly severe in closed or intermittently-closed waterways with limited flushing with the open sea.

Sampling was conducted in the Varano lagoon in two zones: **M (medusa zone)**, with presence of *Aurelia* sp. jellyfish and **C** (**control zone**), without jellyfish. For microbiological analyses, one-liter water samples were collected in triplicate for one year from February 2012 to December 2012 to a depth of about 50 inches from the surface using a Niskin bottle (General Oceanics , Inc.). The samples were transported on ice and processed for enumeration and isolation of bacteria within 4 h of sampling. For the nutrients analysis additional water samples were collected for each replica with a Niskin of 1L. For some nutrients (NH3-N, NO2-N NO3-N, Si- SiO2, P- P04) 500 ml of each sample were filtered with Whatman GF/F 47mm filters (that retains particles from 0.7µm) and immediately stored at -20°C in polyethylene bottle and analyzed. For the analysis of the content of total nitrogen and total phosphorus, the remaining 500 ml of each sample were not filtered. Abiotic parameters like temperature and salinity concentration were measured in situ using a multiparametric probe 'Ocean Seven 401' (Jolzonant, Italy). To assess the jellyfish population density in the lake of Varano, individuals of *Aurelia* sp. were collected using a net with diameter of 1 m and mesh of 1 cm (Fig. 9). In the middle of net was put a device for flow measurement. The net was linked at the boat and dragged for 2 minutes. This operation was replicated for 5 times in each zone (Jellyfish and Control). The jellyfish in the net were counted e subsequently, in the laboratory, the density of population for both zones was calculated.

Fig. 9 Net with 1cm mesh

2.3. Microbiological analysis

Total bacterial abundance

In 2000, a population of the invasive jellyfish *Aurelia sp*. entered the coastal sound of Varano (Apulia), turning into a key component of the present-day lagoon biota. The impact of jellyfish biomass on the Varano bacterioplankton dynamics was investigated on seawater samples collected in different lagoon areas (M-zone and C-zone, according to the presence or absence of jellyfish respectively).

Bacterioplankton counts were performed using a Zeiss Standard Axioplan microscope equipped with an halogen lA (Hg 100) light. Duplicate slides were prepared from each sample by filtering 1ml of seawater through a Millipore filter (0.2□m pore) and using DAPI (4,6-diamidino-2 phenylindole) as fluorochrome (Porter and Feig, 1980). DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA. It can pass through an intact cell membrane so that it can be used to stain both live and fixed cells.

DAPI is a popular nuclear counter stain for use in multicolor fluorescent techniques. Its blue fluorescence stands out in vivid contrast to green, yellow, or red fluorescent probes of other structures (Fig 10).

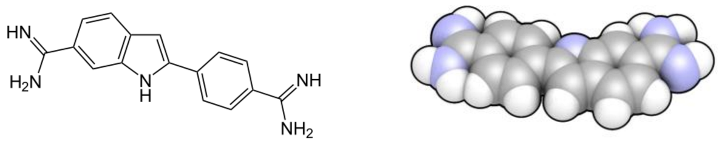

Fig 10. Molecula of DAPI

DAPI bound to a DNA double helix is excited by ultraviolet light (the maximum absorption is at a wavelength of 358 nm) and emits light blue (the maximum emission is at 461 nm) (Fig 11).

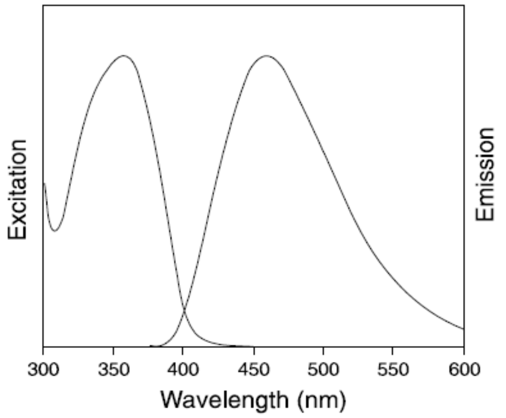

Fig 11. Fluorescence excitation and emission profiles of DAPI bound to dsDNA (https://www.ipmc.cnrs.fr/fichiers/recherche/microscopie/Sondes/Dapi.pdf).

At least 10 microscopic fields were counted for each preparation at 1000x magnification.

Culturable heterotrophic bacteria abundance

For enumeration of culturable heterotrophic bacteria, in laboratory, water samples were diluted with filtered (0.22 µm) seawater. 0.1 ml of test sample of serial dilutions of 1:10 of each sample was plated into a solid media, Marine Agar 2216E (Difco), previously prepared (Fig 9). The plates were incubated at 22°C for over 7 days, then the colonies were counted according to the colony forming unit (CFU) method. The colony count was made with a colony counter PBI and the obtained values multiplied by the dilution factor.

Abundance of human potential pathogens bacteria (PCA -Plate Count Agar)

Counts of culturable bacteria at 37 °C (including human potential pathogens) in seawater samples were determined by the spread plate method, using Bacto Plate Count Agar medium (seeding with 0.1 ml of sample). The plates were incubated at 37 °C for 48 hrs.

Vibrios abundance

In the laboratory, for the search of marine vibrios 1, 5 and 10 ml water samples were filtered on 0.45 µm pore size membrane filters. Filter disks were aseptically placed onto thiosulphate-citrate-bile-salt-agar (TCBS) plus 2% NaCl (24–48 h at 20–25°C and 35°). After incubation at 30°C for 24hrs, the grown colonies were counted according to the colony/forming units (CFU) method. Mean values for three replicate samples were determined and expressed as CFU/ml and than appropriately multiplied by the dilution factor.

Microbial pollution indicators: coliforms and intestinal enterococci

In order to evaluate the degree of water pollution in Varano lake, some indicators such as fecal and total coliforms and intestinal enterococci were determined. Fecal coliforms, total coliforms and intestinal enterococci were determined by the most probable number method (MPN) (American Public Health Association 1992) using liquid media and the standard five-tube method of ten-fold dilution. The most probable number (MPN) of bacteria present can be estimated then from the number of tubes inoculated and the number of positive tubes obtained in the confirmatory test, using specially deviced statistical tables. For total and fecal coliforms determination, lactose broth and brilliant green-lactose broth were used as cultural media in the presumptive and confirmative test, respectively. For intestinal enterococci, the confirmative probe were performed using Ethyl violet azide.

Detection of total and fecal coliforms

Coliforms bacteria can serve as indicators of fecal contamination. They are not themselves pathogenic but are common symbionts of the digestive trait of animals, and thus abundant in feces. Coliform are Gram-negative aerobic or facultative anaerobes, nonspore-forming, rod shaped bacteria that ferment lactose with acid and gas production. *Escherichia coli*, the most abundant bacterium of the human colon, is the most important indicator of human fecal contamination. The research of coliforms was based on the development of such bacteria culture at optimum incubation temperature and in a suitable liquid culture medium to the base of lactose, as well as for water. The presence of coliforms have been highlighted on the basis of lactose fermentation with gas production.

We proceeded with the MPN (Most Probable Number) calculation by two tests:

1. Presumptive evidence
2. Confirmatory test

Presumptive evidence

For each sample the following procedure was utilized:

5 tubes of double strength lactose broth were incubated with 10 ml sample;

5 tubes of lactose broth at a normal concentration were incubated with 1 ml sample;

5 tubes of lactose broth at a normal concentration were incubated with 0.1 ml sample

After 48hrs of incubation at 37°C, the tubes showing gas production were subjected to confirmatory test.

Confirmatory test

Tubes positive in the presumptive test were employed for the confirmatory test. In these cases:

For the detection of total coliforms a test tube containing 10 ml of lactose broth with bright green bile and Durham tube was incubated with 1 ml of positive broth culture at 37°C for 48hrs.

For the detection of fecal coliforms test tube containing 10 ml of lactose broth with bright green bile and Durham tube was incubated at 44°C for 48hrs for the detection of fecal coliforms.

The research can be considered positive for total and fecal coliforms if the formation of gas appears in tubes containing lactose broth with bright green bile.

Expression of results: the most probable number (MPN) is calculated by considering the number of positive tubes at the end of the confirmatory test.

The number of positive tubes obtained provides a number (N), reported in Standard Methods. The MPN value is calculated using the following formula:

N= MPN/100 ml of water

where:

N= Most probable number.

Detection of Intestinal Enterococci

Quantitative research of fecal streptococci in the samples was based on the development, in a suitable culture medium based liquid broth azide, of these bacteria.

We proceeded with the MPN (Most Probable Number) calculation by two tests:

1. Presumptive evidence
2. Confirmatory test

Presumptive evidence

For each sample the following procedure was utilized:

5 tubes of double strength azide broth were incubated with 10 ml sample;

5 tubes of azide broth at a normal concentration were incubated with 1 ml sample;

5 tubes of azide broth at a normal concentration were incubated with 0.1 ml sample;

All tubes were incubated at 37°C for 48hrs and subsequently examined the development of turbidity was considered as sign of positivity.

Confirmatory test

From each tube resulted positive from the presumptive research, 1 ml of broth was picked and seeded in a test tube containing 10 ml of Ethyl Violet Azide Broth (EVA) and then incubated at 37°C.

The test was considered positive when the formation of a violet deposit was revealed.

Expression of results: the most probable number (MPN) is calculated by considering the number of tubes positive at the end of the confirmatory test. The number of positive tubes obtained provides a number (N), reported in Standard Methods. The MPN value is calculated using the following formula:

N= MPN/100 ml of water.

Summary table: microbiological analysis

Microoraganism	Solid Media	Liquid Media	Incubation Temperature	Incubation Time	Positivity
Heterotrophic bacteria	*Marine Agar*		22°C	24hrs	Presence of Colonies
Human potential pathogens	*PCA*		37°C	24hrs	Presence of Colonies
Vibrios	*TCBS*		30°C	48hrs	Presence of Colonies
		Presumptive evidence			
Total coliforms		*Lactose broth*	37°C	48hrs	Gas production
		Confirmatory test			
		Bright green bile	37°C	48hrs	Gas production
Fecal coliforms		**Presumptive evidence**			
		Lactose broth	37°C	48hrs	Gas production
		Confirmatory test			
		Bright green bile	44°C	48hrs	Gas production
Intestinal enterococci		**Presumptive evidence**			
		Azide broth	37°C	48hrs	Turbidity
		Confirmatory test			
		Ethyl Violet Azide Broth	37°C	48hrs	Formation of a violet deposit

2.4. Analysis of nutrients

The methodologies used for the nutrients analysis have been adapted at the Systea Srl’s methodologies since we used their Autoanalizer laboratory multi Micromac Lab1000 based on technology called LFA (Loop Flow Analysis). LFA are based on methodologies published by APAT "Manuali e linee guida 29/2003” dell’IRSA-CNR “Metodi analitici per le acque” (ISBN 88-448-0083-7).

Orthophosphate in water and sea water

For the determination of phosphate a reaction was used: the orthophosphate ion reacts in an acidic environment with molybdate to form phosphomolybdate, which in turn was reduced with ascorbic acid into blue of molybdenum.
The complex was read at 880 nm. The antimony has been used to increase the sensitivity .

Ammonia in water and sea water

For the determination of ammonia the Berthelot reaction was used in which, from the reaction between ammonia, phenol alkaline, hypochlorite (chlorine source) and nitroprusside (as catalyst in an alkaline environment). It forms a complex emerald green. The complex was read at 630 nm.

Nitrate in water and sea water

Nitrates present in the sample were reduced to nitrite in a column of cadmium with copper. The nitrites present in the sample and those formed by the reduction reacted with sulfanilamide and naftiletilendiamina in acid environment. The colored complex formed was read at 550 nm .

Nitrite in water and sea water

The method used for the determination of nitrite has been that one in which the nitrites present in the sample, react with sulfanilamide and naftiletilendiamina in acid environment. The colored complex that is formed was read at 550 nm.

Silicates in water and sea water

For the determination of the silicates was used the method that provides the photometric measurement of the reduction product (molybdenum blue). This product was formed in presence of ascorbic acid, when the silicon ion reacts in an acid environment, with the ammonium molybdate. The complex was read at 815 nm. The oxalic acid was added to eliminate the interference of phosphates.

Total nitrogen and total phosphorus in water and sea water

For the determination of Total Nitrogen and Total Phosphorus was used a procedure that involves the digestion of the sample of water with a solution of potassium persulfate, boric acid and sodium hydroxide. This mixture 3.75M was autoclaved for 35 minutes.

Determination of total nitrogen

A certain amount of the digested sample was diluted 1:1 with Imidazole Buffer pH 8 . Before proceeding with the analysis for the determination of nitrates it is important to verify that the pH of the diluted samples was greater than 7.

Determination of total phosphorus

A certain amount of the digested sample was brought to pH less than 3 for the determination of Phosphate.

2.5. Regression analysis

Regression analysis is a statistical tool for the investigation of relationships between variables (Sykes, 2000). In ecology, correlations between variables are widely used because they could be easily understood. The correlation coefficient, "r", expresses the level of correlation between two variables and how they vary at the same time. The correlation can be both positive and negative, depending on the value assumed by "r". This value is normally between -1 (maximum negative correlation) and +1 (maximum positive correlation). According to the r-value, the relationship between the two assessed variables can be different (Tab.I). It is no possible to directly compare two correlation coefficients, so the coefficient of determination "r^2" is commonly used. This coefficient describes how much the variation of one variable could be statistically explained by the other variable.

Value	**Correlation**	**Relationship**
< 0.2	Weak	Almost non existent
0.2 – 0.4	Small	Little relationship
0.4 – 0.7	Moderate	Good relationship
0.7 – 0.9	High	Definite
0.9 – 1	Very high	Very reliable

Tab. I Interpretation of "r" values

In this work the regression analysis was performed to evaluate the positive correlation between microorganisms and the quantity of nutrients and between microorganisms and abiotic parameters (temperature and salinity) in the lake throughtout the year. Moreover the regression analysis was also used to evaluate the positive correlation between microorganisms and some of the abiotic factors such as temperature and salinity.

3. Results

3.1. Density of jellyfish population

Results reported in Tab. II showed that the jellyfish bloom was recorded in May when the highest value of density were of 12,39 ind/m^3. The abundance of jellyfish was so hight that also in the Control zone were present some individuals. In February jellyfish were present with a low density in M-zone. In September and in December there were not live jellyfish in the lake, but in September there were a great amount of jellyfalls. Jelly-falls are a source of organic matter arising from corpses of jellyfish that sink through the water column (remineralizing as dissolved organic/ inorganic components), causing an accumulation of jelly-POM at the seabed. (Lebrato et al., 2012).

	M-Zone	**C-Zone**
February	0.03 ind/m^3	0
May	12.39 ind/m^3	0.03 ind/m^3
September	Jellyfalls	Jellyfalls
December	0	0

Tab. II. Density of jellyfish population in the Jellyfish zone (M) and in the Control zone (C)

3.2 Abundance of potential human pathogens at 37°C

The highest values of density of culturable bacteria at 37°C were observed in September and December with mean values of 3.7 x 10^3 CFU/ml and 4.5 x 103 CFU/ml, respectively (Fig. 12). In these months jellyfish were apparently absent in the water column. The water column in September, is rich of degradation products from the jellyfish corpses (jelly-falls).

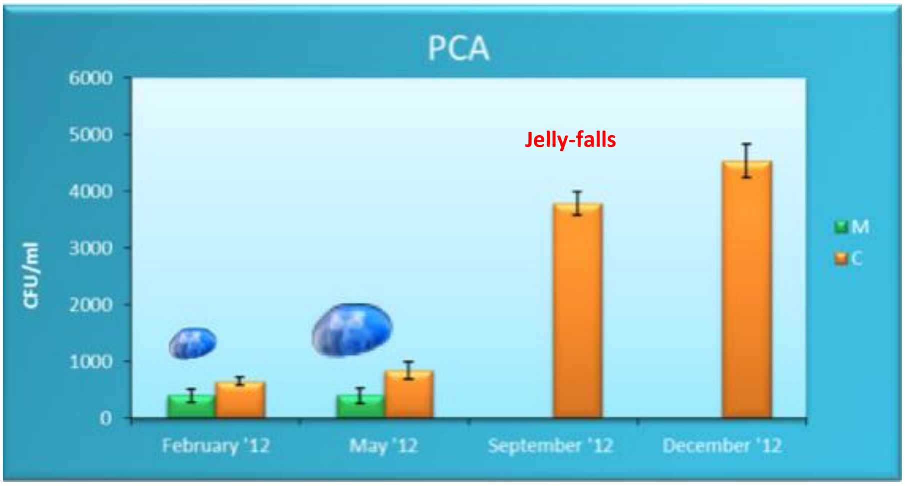

Fig. 12. Average abundance of culturable bacteria at 37°C in the M (presence of jellyfish) and in the C (control) throughout the year

Abundance of heterotrophic bacteria at 22°C

The highest values of culturable heterotrophic bacteria were observed in February, in the M-zone, when jellyfish density reached a value of 0.03 ind/m^3, and in the control area C-zone where jellyfish were absent. The average values recorded were of 3.2 x 10^4 CFU/ml in the M-zone and 3.9 x 10^4 CFU/ml in C-zone (Figure 13). Also in September, during the decay of *Aurelia* sp., values were observed in bacterial density with a high average value of 2.4 x 10^4 CFU/ml.

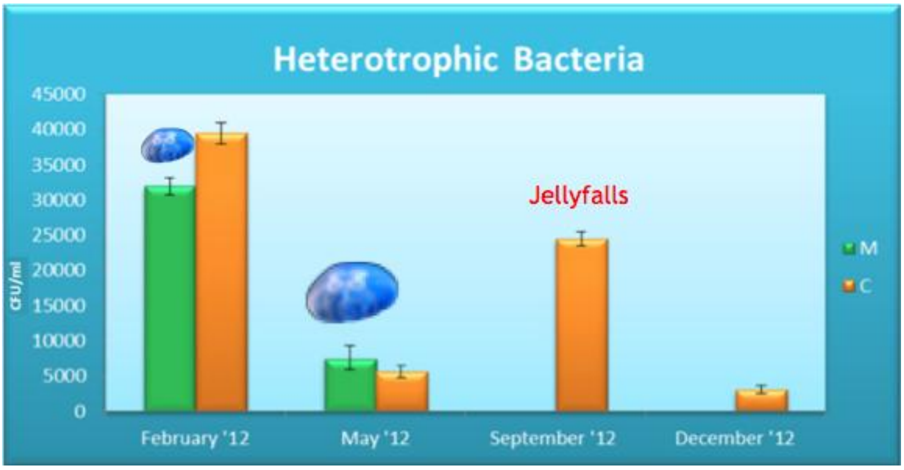

Fig. 13. Average abundance of heterotrophic bacteria at 22°C in in the M (presence of jellyfish) and in the C (control) throughout the year

Abundance of vibrios

Results obtained showed that in May, during the period of bloom of *Aurelia* sp. (12.39 individuals/m^3), the abundance of vibrios was considerable in both zones M and C with mean values of 57 and 28 CFU/ml, respectively (Fig. 14). The abundance of vibrios in the water was highest in September (506 CFU/ml) when decaying bodies of dead jellyfish (jelly-falls) were observed in the lagoon.

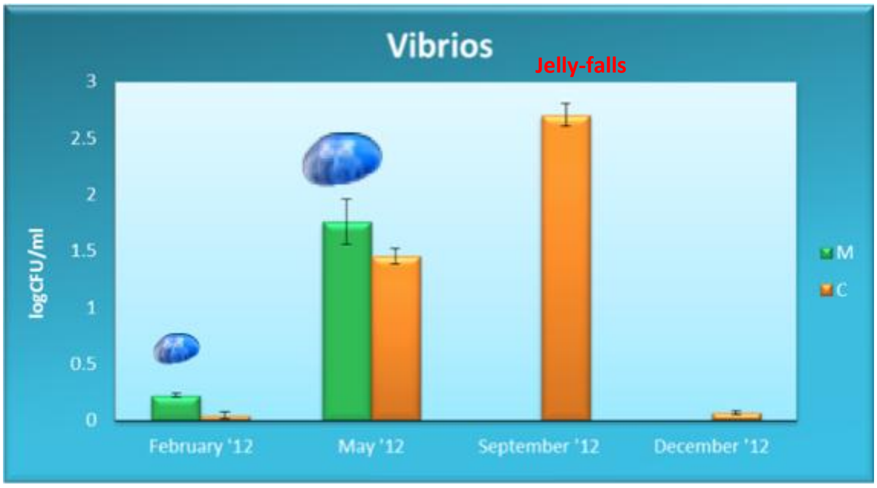

Fig. 14 Average abundance of vibrios in the M (presence of jellyfish) and in the C (control) throughout the year

Abundance of total and fecal coliforms

Fecal contamination indicators (total (CT) and fecal coliforms (CF)) were abundant in the M-zone in February (558 MPN/100 ml) The total coliforms reached a value of 22 MPN/100ml in September and amounted to 86 MPN/100ml in December (Fig. 15). The fecal coliforms instead, reached a value of 2 MPN/100 ml MPN/100 ml in September and 67 MPN/100ml in December (Fig. 16).

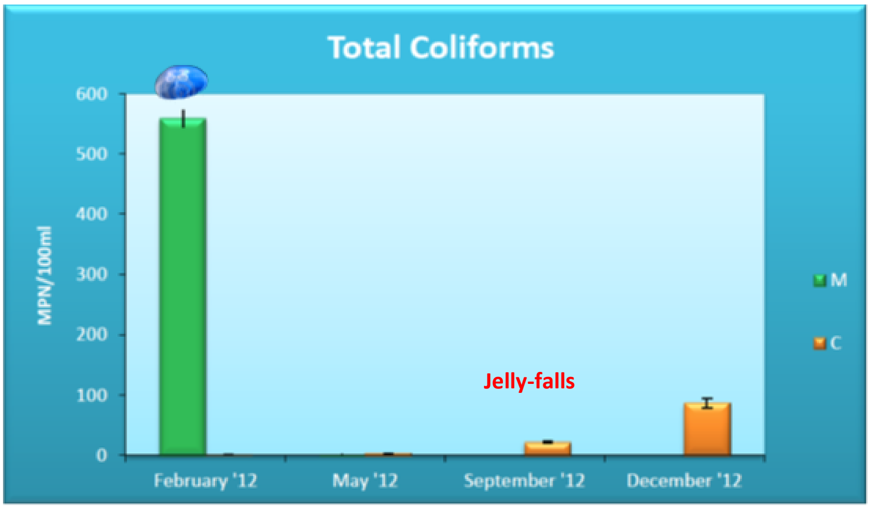

Fig. 15. Average abundance of total coliforms in the M (presence of jellyfish) and in the C (control) throughout the year

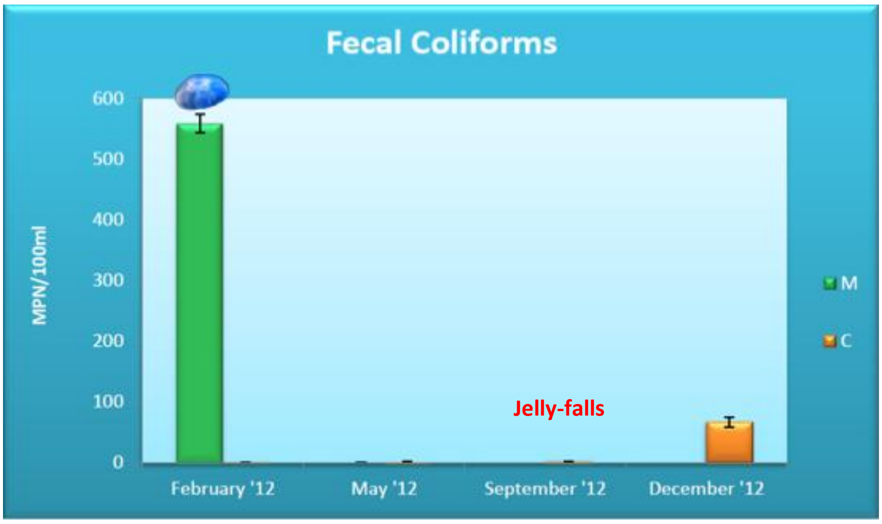

Fig. 16. Average abundance of total coliforms in the M (presence of jellyfish) and in the C (control) throughout the year

Abundance of intestinal enterococci

Results showed that the values of density of intestinal enterococci remained low (around 2 MPN/100 ml) for all year except in December, when recorded values were of 57 MPN/100ml (Fig. 17).

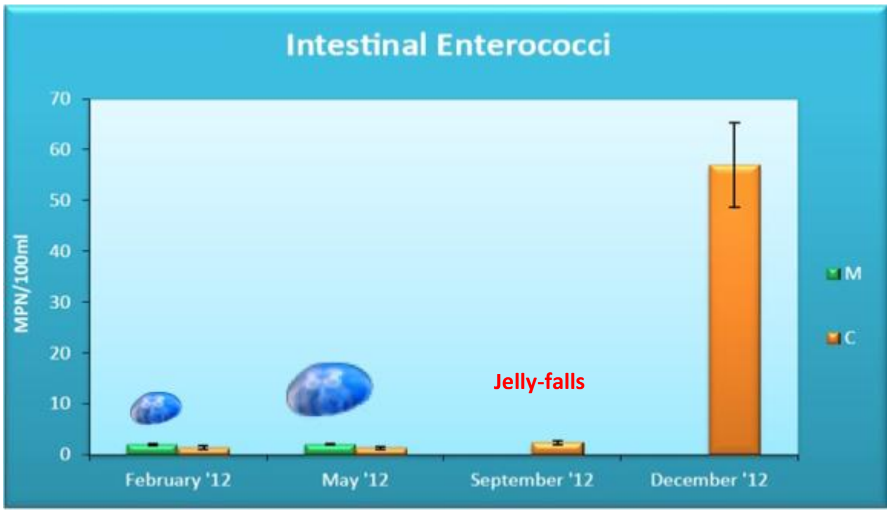

Fig. 17. Average abundance of intestinal enterococci in the M (presence of jellyfish) and in the C (control) throughout the year

Summary table: bacteria concentration

		February	May	September	December
	Zone	mean (± s.d.)	mean ±s.d.)	mean (± s.d.)	mean, (±s.d.)
Heterotrophic bacteria	*M*	31950 (± 3751.3)	7654 (± 4040.1)	n.d	n.d
	C	39450 (± 4872.3)	5623.33 (± 4442.1)	24550 (± 2865.7)	3110 (± 684.1)
Human potential phatogens	*M*	392 (± 118.1)	400 (± 140.7)	n.d	n.d
	C	656.6 (± 66.1)	843.33 (± 158.1)	3790 (± 206.4)	4533.33 (± 298.4)
Vibrios	*M*	0.22 (± 0.01)	1.76 (± 0.2)	n.d	n.d
	C	0.04 (± 0.03)	1.45 (± 0.07)	2.70 (± 0.1)	0.06 (± 0.1)
Total coliforms	*M*	558.33 (± 15.1)	1.67 (± 0.7)	n.d	n.d
	C	0.67 (± 0.01)	3 (± 0.4)	22 (± 1.2)	86 (± 8.1)
Fecal coliforms	*M*	558.33 (± 15.1)	0.67 (± 0.3)	n.d	n.d
	C	0.67 (± 0.01)	1.67 (± 0.2)	2 (± 0.4)	67 (± 7.6)
Intestinal enterococci	*M*	2 (± 0.2)	2 (± 0.1)	n.d	n.d
	C	1.33 (± 0.4)	1.33 (± 0.3)	2.33 (± 0.4)	57 (± 0.4)

3.3 Analysis of nutrients

Ammonia

The concentration of ammonia showed wide variability during the whole year, with the highest value 11.32 μM recorded in May, in presence of jellyfish bloom and 9.61 μM in the control zone. The lowest values,1.25 μM and 0.13 μM were recorded in February in both zones respectively (Fig. 18).

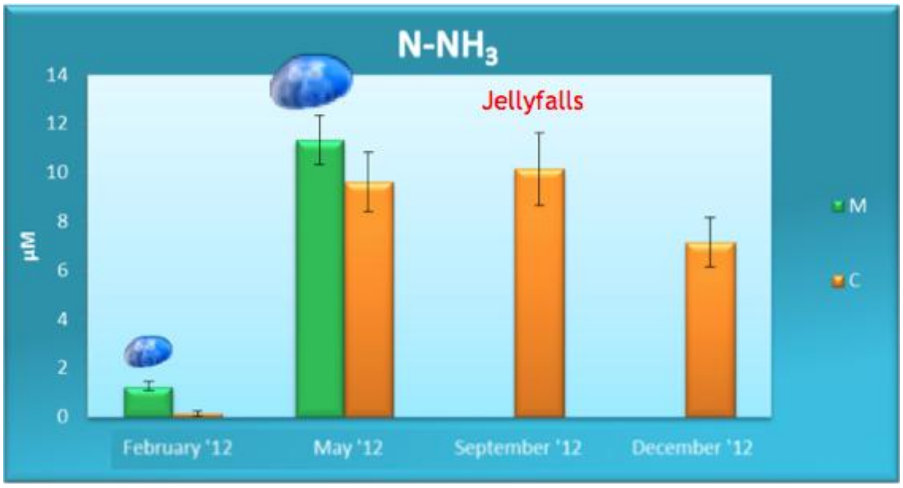

Fig. 18. Concentration of Ammonia (μM) in the M (presence of jellyfish) and in the C (control) throughout the year

Nitrite

Nitrites are the less oxidized form of nitrogen. The higher values were recorded in December (1.12 μM) when the jellyfish were absent. In May, during the period of bloom of jellyfish values of nitrite seem to be similar in both areas. The recorded values were 0.44 μM in the M-zone and 0.46 μM in C- zone (Fig. 19).

Fig. 19. Concentration of Nitrites (μM) in the M (presence of jellyfish) and in the C (control) throughout the year

A significant positive correlation was between intestinal enterococci and nitrites densities was observed (Fig. 20). The value of R obtained is 0.89386799920

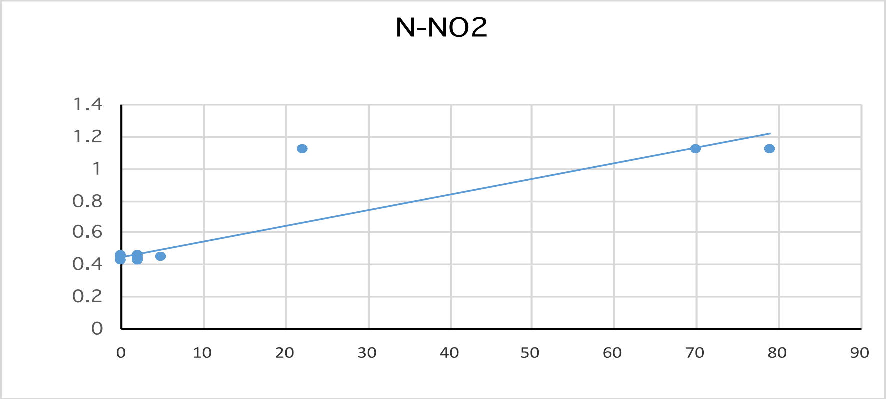

Fig. 20. Correlation between intestinal enterococci density and nitrites concentration throughout the year

Nitrate

Results shown that the maximum concentrations of nitrates were recorded in February in both zones with values of 8.6 μM M-zone and 9.24 μM in C-zone respectively. The minimum concentrations however, were recorded in May, with values of 1.49 μM in presence of jellyfish and 1.47 μM in the control zone (Fig. 21).

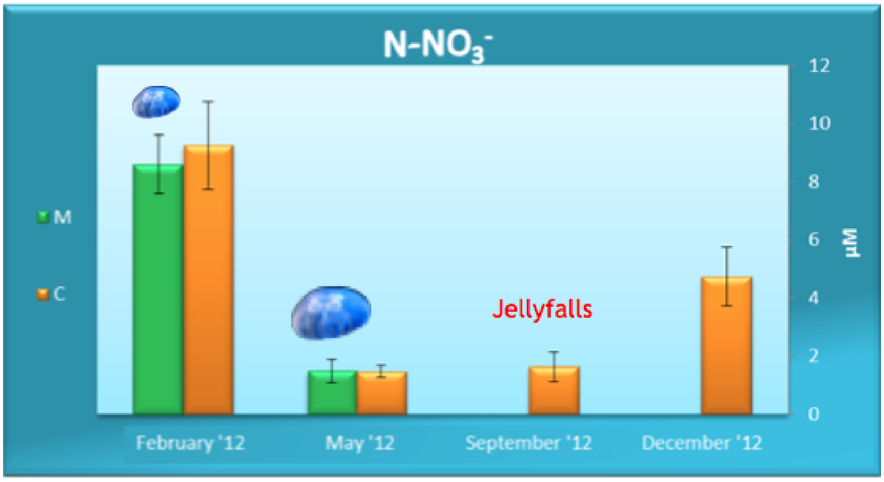

Fig. 21. Concentration of Nitrate (µM) in the M (presence of jellyfish) and in the C (control) throughout the year

Total nitrogen

The highest value of total nitrogen concentration (80.41 µM) was observed in September during the period of jelly-falls occurrence. The lowest concentrations were recorded in February in both areas (19.08 µM for the M-zone and 20.79 µM in C-zone) (Fig.22).

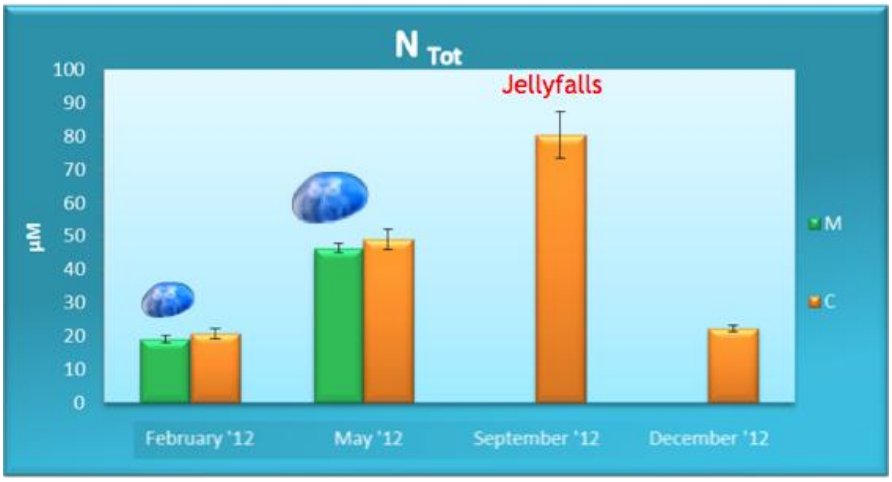

Fig. 22. Concentration of total ammonium (µM) in M (presence of jellyfish) and C (control) throughout the year

Phosphate

The concentration of phosphate was constant throughout the year with values between 0.15 µM e 0.29□µM (Fig. 23).

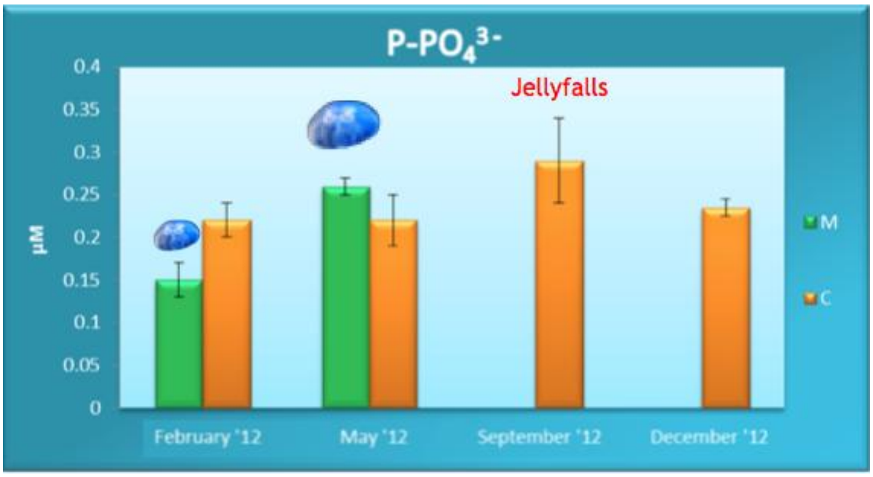

Fig. 23. Concentration of Phosphate (µM) in the M (presence of jellyfish) and C (control) throughout the year

A significant positive correlation between vibrios and phosphate densities was observed (Fig. 24). The value of R obtained is 0.6618156843

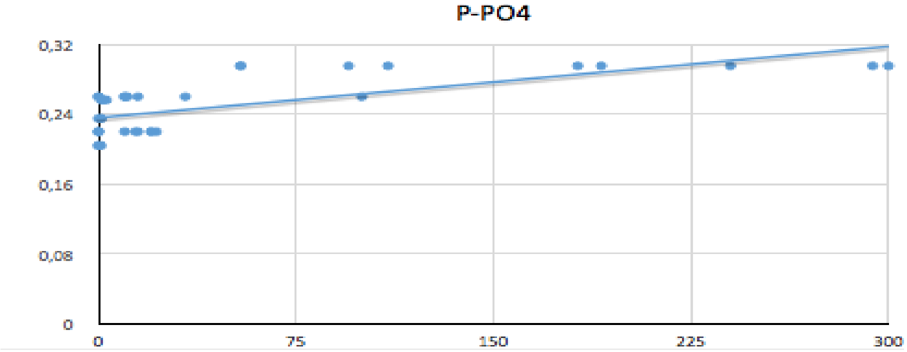

Fig. 24. Correlation between vibrios density and phosphate concentration throughout the year

Total phosphorus

The highest value of total phosphorus (1.34µM) was observed in September. The minimum concentrations however, were recorded in May, during the bloom of jellyfish, in both areas examined (0.28□µM in M-zone and 0.35 µM in C-zone) (Fig. 25).

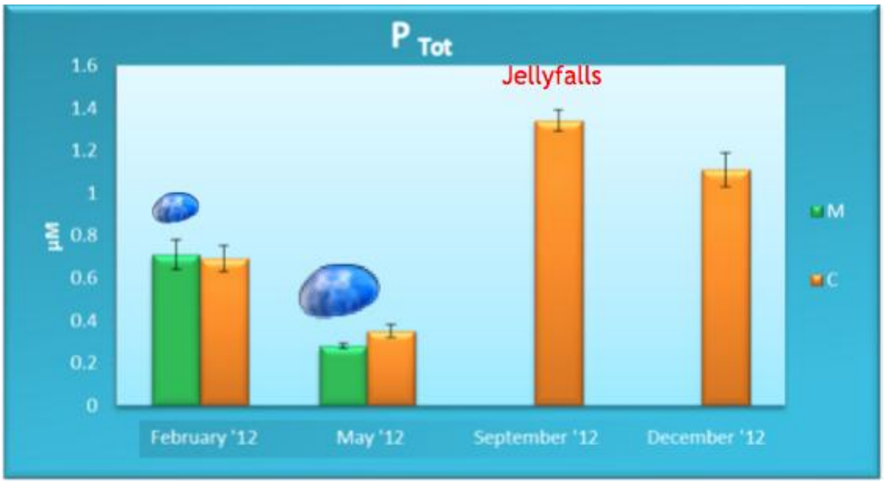

Fig. 25. Concentration of total phosphorous (µM) in the M (presence of jellyfish) and in the C (control) throughout the year

A significant positive correlation between vibrios density and total phosphorus concentration was observed (Fig. 26). The value of R obtained is 0.60819404798

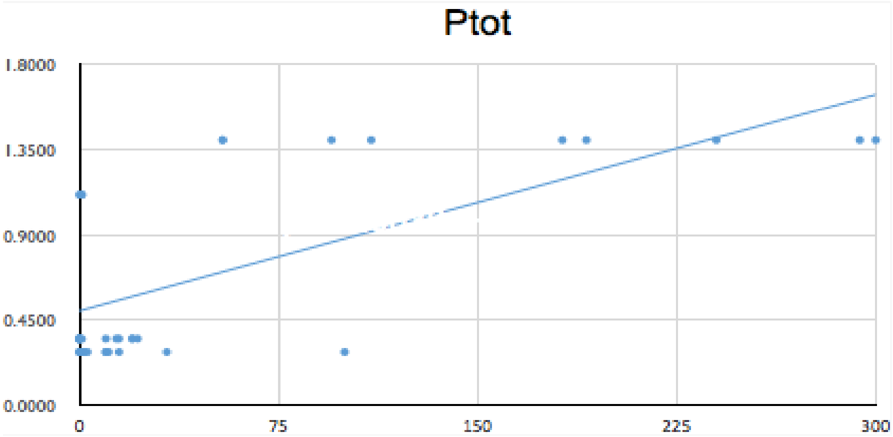

Fig. 26. Correlation between intestinal enterococci and total phosphorus throughout the year

3.4. Temperature and Salinity

During all year the waters of Lake of Varano have a high variability especially regards the parameters of temperature and salinity. The variability of the abiotic conditions in the basin can be linked to meteorological variability, the low depth of the basin and the low water exchange. There are not positive correlation between temperature and salinity factors and density of microorganisms.

Temperature

The maximum value of temperature recorded during the year 2012 in the Varano lake, was in July (29.25°C). The minimum temperature instead was recorded in February (6.88°C) (Fig. 27).

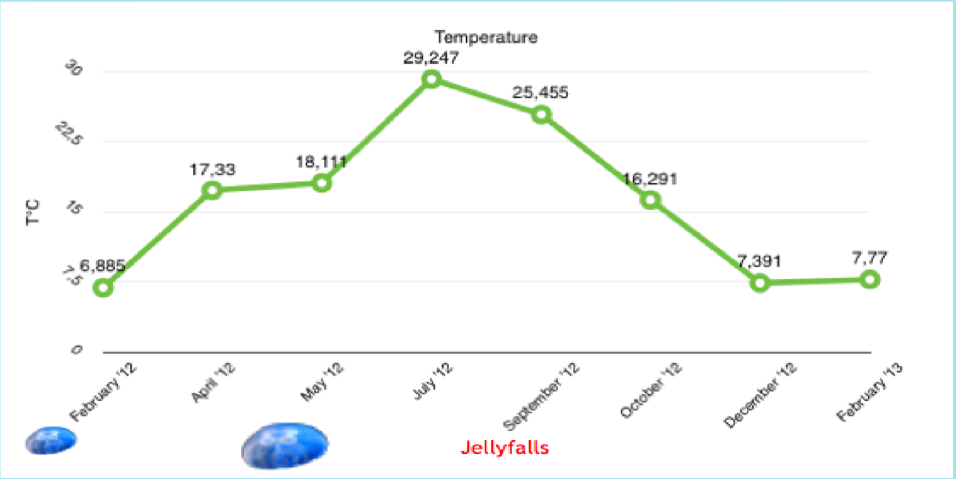

Fig. 27. Temperature in the M (presence of jellyfish) and in the C (control) during throughout the year

Salinity

The maximum value of salinity in the lake during the year 2012 was recorded in September (28.87‰). The minimum value instead, was recorded in February (24.33‰) (Fig. 28).

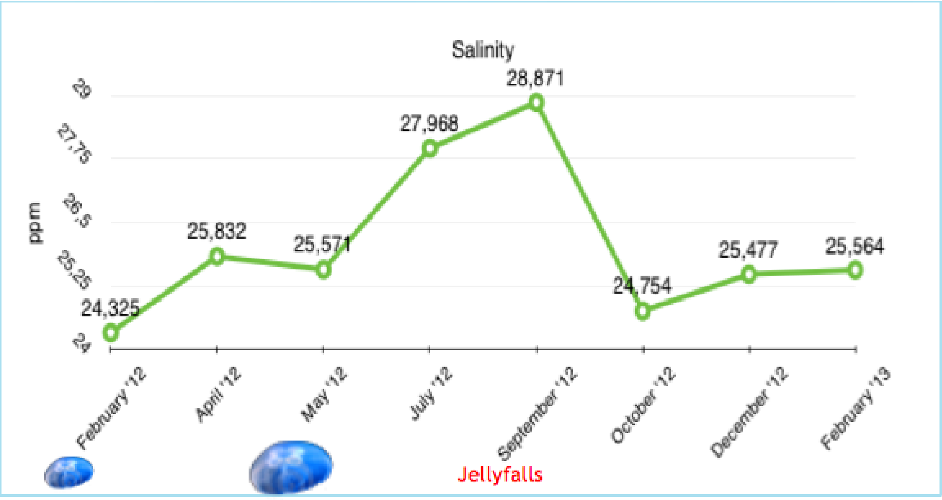

Fig. 28. Salinity in the M (presence of jellyfish) and in theC (control) during throughout the year

Summary table: nutrients concentration

		February	**May**	**September**	**December**
	Zone	**mean (± s.d.)**	**mean (± s.d.)**	**mean (± s.d.)**	**mean, (± s.d.)**
NH3	***M***	1.25 ± 0.2	11.32 ± 1	0	0
	C	0.13 ± 0.1	9.61 ± 1.2	10.13 ± 1.5	7.125 ± 1
NO2$^-$	***M***	0.59 ± 0.02	0.44 ± 0.01	0	0
	C	0.68 ± 0.02	0.46 ± 0.03	0.355 ± 0.05	1.12 ± 0.01
NO3$^-$	***M***	8.6 ± 1	1.49 ± 0.4	0	0
	C	9.24 ± 1.5	1.47 ± 0.2	1.625 ± 0.5	4.73 ± 1
Ntot	***M***	19.08 ± 1	46.33 ± 1.4	0	0
	C	20.79 ± 1.5	48.98 ± 3	80.415 ± 7.1	22.28 ± 1.2
PO4^{3-}	***M***	0.15 ± 0.02	0.26 ± 0.01	0	0
	C	0.22 ± 0.02	0.22 ± 0.03	0.29 ± 0.05	0.235 ± 0.01
Ptot	***M***	0.71 ± 0.07	0.28 ± 0.01	0	0
	C	0.69 ± 0.06	0.35± 0.03	1.34 ± 0.05	1.11 ± 0.08

4. Discussions and Conclusions

This work is aimed to describe the seasonal pattern of bacterial abundance and some abiotic parameters in the Lake of Varano, as well as to estimate the potential influence of a seasonal jellyfish bloom and the subsequent decay of jellyfish biomasses on the above mentioned parameters. Knowledge of the bacterial density is usually the first step in the study of ecological and biogeochemical processes involving the microbial component of marine ecosystems. Throughout the year 2012, the coastal sound of Varano was subjected to strong seasonal temperature and salinity fluctuations typical of confined coastal environments (Guelorget and Perthuisot 1983), as already reported in previous studies carried out in the Varano lagoon (Marolla, 1980; Caroppo 2000). Increases in temperature usually are responsible for the increase in bacterial abundance, biomass and enzymatic activity (Duyl and Kop, 1990). However, from obtained data and from the regression analysis, it was possible observed that the overall structure of the microbial community in the lake of Varano, does not appear to be significantly modified by changes in temperature and salinity values. In spite of high agricultural and domestic pollution potential of the surrounding terrestrial areas, the densities of the microbial pollution indicators indicate that the water quality of the Varano lagoon remains almost unchanged since several years (Caroppo et al., 2002). In general, the recorded bacterial densities are comparable with those observed in other lagoonal environments and in previous studies on the Varano lagoon (Caroppo et al., 2002). The TC, FC, IE concentrations remained constant throughout the year, except in February, when in the jellyfish area significant increase in bacterial density were recorded (Tab. III). Such variation has also been observed in the Varano lagoon in summer, possibly as the result of a combination of higher water temperature and trophic load, mainly due to the increase of touristic and recreational activities. However, the decay of a large amount of gelatinous biomass from jellyfalls can be considered as an additional input of particulate and dissolved organic matter that could contribute to the bacterial growth observed in summer. Exudates (mucus) from jellyfish blooms (Arai, 1997) might also influence the development of the microbial community. The highest heterotrophic bacteria abundance was observed with low jellyfish density (Tab. I and Tab. II). Conversely high jellyfish density may lead to high amount of mucus and other exudates in the water column, which, may limit the total bacterial abundance in the water column by the possible occurrence of biogenic anti-bacterial compounds. By contrast, less sensitive procariotic groups, such as *Vibrio* spp., might be favoured by the increased availability of mucopolysaccarids and glycoproteins from jellyfish tissues. The highest vibrios abundance was indeed observed in coincidence with high jellyfish abudance (Tab II). When the jellyfish are alive, the influence on the microbial compartment is restricted to the areas with jellyfish. But when jellyfish die, the release of nutrients and organic carbon from jellyfalls scatter across the lagoon. .

It seems that the greatest concentration of jellyfish can be found in the proximity of the west side which is first flanked by the north-west coastal currents. This is the most populated and productive area of the lagoon (Specchiulli et al., 2008). It seems that if the jellyfish cannot be found in the east area it is because they swim against the current towards west. When jellyfish die, instead, the DOM and other nutrients from jellyfalls are carried eastward. This could explain what happens in September in the control area. The seasonal dynamics and concentrations of nutrients in the lagoon were generally in agreement with previous investigations (Tolomio et al., 1990; Caroppo, 2000; Specchiulli et al. 2008). The highest values of ammonia concentrations were observed in May and September (Tab. IV) e in the jellyfish bloom area (M zone, Fig. 17), whereas the highest values of nitrites and nitrates were recorded in winter in absence or low presence of jellyfish (Tab. II and Tab. IV). Higher concentrations of nitrates in winter can be related to terrestrial runoff during seasonal rain precipitations (Specchiulli, 2008). However, the assimilation of NO3- by heterotrophic bacteria is usually low due to the high energy cost to reduce NO3- in the oxidation state of nitrogen within amino acids (Specchiulli, 2008). So, the bacterial density should be slightly influenced by enviromental nitrate concentrations. A significant correlation was found only between Intestinal Enterococci density and N-NO2. Particularly, high concentrations of nitrites and Intestinal enterococci in December water samples revealed recent contamination from the land. The levels of P-PO4- were very low and relatively constant (Tab. IV) it with Ptot concentration reaching a peak in September. As expected, significant correlation was observed between vibrios abundance and P salts concentrations required for DNA synthesis. Caroppo (2002) suggested that in the lagoon of Varano the content of inorganic phosphorus in the water is kept low by the high biomass of bacteria and jellyfish as the standing stock of phosphorus is retained in its organic form by the microbial and metazoan planktonic communities. Similar conclusions were reached by Sorokin et al., (1996) for the lagoon of Comacchio. Enrichment experiments showed that the phosphorus may be the main factor that limits the bacterial growth in the Mediterranean Sea (Zweifel et al., 1993; Thingstad et al., 1998 ; Legovic et al., 1994). In general, our data corroborate the hypothesis that the Varano lagoon represent a high productivity habitat (Tolomio et al., 1990), similarly to other coastal brackish environments. Due to its high water content, gelatinous macrozooplankton in the past has often been considered as a poor source food from a nutritional point of view due to the high water content, energetically a trophic dead end (Sommer et al. 2002). More recently, jellyfish blooms were assumed to be a low-value biomass not readily consumed by higher trophic levels, representing a sink of carbon, directly leading toward bacterial production (Condon et al. 2011). However, the jellyfish predation by fish and other marine animals, such as sunfish and some species of turtles *Caretta caretta* included (Arai, 2005; Houghton et al., 2006) indicates that these organisms are good sources of organic matter also for predators. Evidence that jellyfish biomass does not represent a trophic dead end came also from observations of dead biomass of the giant jellyfish *Nemopilema nomurai*, which is consumed by macrobenthic scavengers more rapidly than decomposed by bacteria (Yamamoto et al. 2008). Recent studies have shown that jellyfalls are important sources of carbon for the microorganisms. The total organic content of jellyfish, in fact, generally consists of carbohydrates (7 ± 5%), lipids (22 ± 12%), and especially proteins (72 ± 14%) (Billett et al., 2006; Yamamoto et al., 2008; Pitt et al., 2009, Tinta et al., 2010). It is well known that the bacteria are capable of hydrolyze and use almost all natural polymers by means of extracellular enzymes. The turnover rate of the soluble proteins by bacteria in seawater is a few hours (Hollibaugh & Azam, 1983; Hoppe et al., 1988) and similar to the turnover rate of free amino acids in solution (Keil & Kirchman, 1993). Final products of protein degradation contribute to the pool of dissolved inorganic and organic nitrogen (Kremer, 1977). Indeed in our study, an increase of total nitrogen has been found just in September, during the decay of the bloom of jellyfish. Large quantities of nutrients are presumably released in the surrounding environment by the decomposition of jellyfish. potentially leading to substantial changes in the quality and quantity of available substrates for microbial respiration. Therefore, the composition and activity of the bacterial community may be severely influenced (Martinez et al., 1996; Pinhassi et al., 1999. Schafer et al., 2001; Carlson et al., 2002). In a recent study conducted in experimental enclosures in the northern Adriatic Sea, Tinta et al. (2010) observed an increase in bacterial biomass and growth rates by the decomposition of jellyfish biomass releasing large amounts of nutrients. The present observations may corroborate the suggestion that jellyfish blooms can act as drivers of changes in the microbial components of marine and coastal ecosystems. By the occurrence of a persistent large population of *Aurelia* sp. jellyfish, the Varano lagoon may represent a natural experimental macrocosm where to trace mechanisms and processes influencing the functioning of coastal marine ecosystems. Quantitative and qualitative analysis of the microbial communities may drive information to clarify the role of outbreaks of gelatinous biomasses as source of carbon, through the microbial loop, in marine food webs. Jellyfish blooms may therefore result as beneficial for the efficiency of ecosystem processes and the provision of fundamental ecosystem services, as key knowledge for the implementation of best practices for the management of coastal habitats and their biological resources.

Section 2: Qualitative Analysis

1. Introduction

Microbial engines drive the biogeochemistry of any ecological system. In aquatic systems, the largest fraction of the organic C cycles through the microbial food web. Mass concentrations of decaying biomasses and exudates (as colloidal and dissolved organic matter, or *jelly-DOM*) from jellyfish blooms may influence nutrients and DOM pathways, and regulate dynamics of microbial communities in coastal waters. In fact, heterotrophic bacteria are known to efficiently decompose organic matter and regenerate minerals in all ecosystems, their abundance representing an index of heterotrophic activity (Overbeck and Chrost, 1990; Henssen and Tranvik, 1998). Condon et al., 2010 have demonstrated that the jelly-DOM can favor the rapid growth and the dominance of specific bacterial phylogenetic groups that were rare in ambient waters. It implies that jelly-DOM, channeled through a small component of the in situ microbial assemblage, induce large changes in community composition. Also changes in bacterial and nutrient dynamics, due to the addition of jellyfish biomass, can lead a shift in the bacterial community structure. Metabolic activity is also strongly associated with bacterial cell sizes and usually more active bacteria are larger than less active bacteria (Del Giorgio et al., 1996; Gasol et al., 1995). A reduction in cell size is possible, being interpreted as an adaptive mechanism to overcome heterotrophic starvation. Microbial cell size has many implications for population growth rates and for influencing the paths and efficiency of carbon flow through the microbial food webs (Legendre and Lefevre 1995; Verity and Smetacek 1996). The size of heterotrophic bacterial cells is a key factor controlling interactions between bacteria and their protistan predators due to a size-selective grazing (Gonzalez et al., 1990; Hahn et al., 2000; Jugnia et al., 2000; Harsha et al. 2007). As competitors, large heterotrophic bacteria limit the development of small ones. Condon et al., (2010) report that jellyfish release substantial quantities of extremely labile C-rich DOM, which is quickly metabolized by bacterioplankton at uptake rates two to six times that of bulk DOM pools. The jellyfish blooms and jelly-falls are two sources of different organic composts (as proteins, lipids, carboidrates etc.) that can influence the abundance and the diversity of bacterial community. Different taxonomic groups have different metabolic patterns, and according to their functional diversity they may use the organic compounds issued by the jellyfish. This could result in a change in the bacterial community, to larger bacteria which are more metabolically active. Coupling bacterioplankton and jellyfish dynamics with environmental parameters can contribute to highlight changes in trophic pathways and to unravel the impact of jellyfish blooms on the ecology of coastal zones. Natural bacterial assemblages display different metabolic levels and vital states. For a long time it has been assumed that all bacteria stained using fluorochromes were alive (Kepner and Pratt. 1994; Luna et al., 2002) but now it is universally recognized that only a portion of aquatic bacteria is alive and actively growing, while a large fraction is dormant or dead (Del Giorgio et al., 1997; Sherr et al., 1999). The quantification of the fraction actually responsible for bacterial activity (C production and enzymatic activities) is of primary relevance for addressing important ecological issues such as organic matter degradation rates and nutrient cycling, as well as the factors controlling these processes (Joux and LeBaron, 1997). Bacterioplankton constitutes a potentially important food source that, in terms of total biomass, is in the same range as phytoplankton at least is in oligotrophic systems (Simon et al. 1992).

1. Biological diversity

1.1 Molecular approaches of DNA extraction: Analysis of the 16S rRNA gene sequences and Metagenomic

The biological diversity is a term used to define the whole variability among living organisms (Wilson, 1988), namely the variety of life on the Earth (Takacs, 1996). The biological diversity can be expressed in three different approaches: the ecosystemic diversity, the species diversity and the genetic diversity. The first one covers the variety of all the habitats. The diversity of the species refers to the evaluation of the number of species living on Earth. The latter measures the total quantity of genetic variety on Earth. The biological diversity also represents an important foundation and result for the evolution. In fact, the diversity represents the way and the complex web of interactions through which living organisms create life. These sets of interactions between the ecosystem elements and the biotope produce a sort of flow and transfer of energy. The biodiversity covers an important role in the ecology, since it guarantees the ongoing and constant characteristics of the ecosystems of being self-adjusting and self-balancing. The diversity is not only defined by the quantity of living species, but it also covers the variety and the variability of the genetic heritage of the diversity itself. Diversity also covers the interconnections between species and communities and how they do affect the whole ecosystem. The conservation of each species is strictly connected to the conservation of other species being these microbial, animal or vegetable and to their impact on the environment. Nowadays, the importance of biological diversity and that of its conservation is increasingly being recognized. In 1992, the Convention on Biological Diversity (CBD) was adopted at the Earth Summit in Rio de Janeiro. As the first global treaty to provide a legal framework for biodiversity conservation, the Convention established some goals among which the conservation of biological diversity, the sustainable use of its components, the fair and equitable sharing of the benefits arising from the use of genetic resources. Furthermore, the Convention encouraged national strategies and action plans to conserve, protect and enhance biological diversity. The Convention also aimed to highlight the causes of a potential loss of biological diversity and how it could affect national ecological, social, scientific, economic and cultural environment. Despite the study of the biodiversity appears increasingly necessary for assessing and monitoring the aquatic environments, much about the microbial communities still remains unexplored or in same cases under-explored in term of abundance and distribution (Kisand et al., 2012; Zinger et al., 2012). The oceans represent the largest ecosystem on earth and 90% of its biomass is microbial. The diversity of microbial life in the oceans is extremely high and spans all known groups of Bacteria, Archaea and microbial Eukarya. Due to the importance of microbial diversity, which plays a key role in biogeochemical cycles in marine ecosystems (Azam and Malfatti, 2007), is really important to investigate the origin and the maintenance of microbial diversity, its role in the structure and its function in marine ecosystems (Tinta et al., 2012). Microbes predominate over the global cycling of nutrients and the production of greenhouse gases, which both act to regulate Earth’s climate. The removal of harmful chemicals such as excess nitrogen fertilizers from aquatic environments is largely mediated by microbial processes. Microorganisms drive bioremediation and waste treatment strategies (Green et al., 2010; Lal et al., 2010; Kostka et al., 2011) and serve as a promising source for sustainable or renewable bioenergy in the form of biogas, bioethanol, biodiesel, and microbial fuel cells (Lovely 2006; Gírio et al., 2010). Over the past two decades the microbial ecology has made significant progress in molecular studies. During the second half of the 20th century, molecular developments provided a systematic way to relate all living organisms through DNA sequence comparisons, initially using the small-subunit ribosomal RNA gene 16S rDNA. The development of ribosomal-RNA-based phylogenetic surveys in the 1980s led to less biased assessments of the distribution of uncultivated bacterial, archaeal and protistan phylotypes in natural populations (Tinta et al., 2012). Due to adoption of the new molecular techniques, the number of newly recognized bacterial and archaeal phylogenetic divisions has increased markedly. These and other results from cultivation-independent surveys have fundamentally changed the perspective of microbial phylogeny, evolution and ecology. The cultivation of resident microorganisms is a valuable part to describe microbial processes in the environment, but it is not enough on its own. In traditional microbiology, isolation and growth as a pure culture are fundamental to the study and characterization of microorganisms. A collection of targeted DNA sequences, such as the 16S rRNA gene, most often derived from PCR amplification and subsequent cloning into a vector, are often used in surveys of microbial diversity from environmental samples (Allen et al., 2005). However, most species of microbial communities carried out some metabolic reactions and the growth of microorganisms in the laboratory sometimes leads to the loss of genetic diversity and therefore of the metabolic capabilities of a given community. Traditional cultivation methods have failed to ecologically grow more relevant microorganisms in the laboratory, leading to a predominance of weed-like species in the world’s culture collections (Prakash et al., 2013). Furthermore, for a long time the difficulties in collecting representative samples in particular niches (e.g. deep sea) and the experimental limitations related to the characterization of the uncultivable microbes could have represented two possible causes of failure of this technique (Kisand et al. 2012). This view has recently changed with the development of culture-independent approaches, such as the metagenomic one. This new molecular technique allows the identification and at least partial classification of bacterial species from their DNA sequences, without the need to isolate and grow up these species in the laboratory (Riesenfeld et al., 2004; Handelsman, 2005; Allen et al., 2005). This genomics technology on a huge scale enables a survey of the different microorganisms present in a specific environment, such as water or soil, to be carried out. By integrating such a qualitative inventory with information about biological functions within the community, the structure of microbial communities can potentially be probed. Metagenomics could also unlock the massive uncultured microbial diversity present in the environment to provide new molecules for therapeutic and biotechnological applications.

Metagenomics is experiencing an explosive improvement from the advent of the high-throughput Next-Generation Sequencing (NGS) technologies which allow an unprecedented large-scale identification biological communities, through the production of an enormous amount of genetic data (Bourlat et al. 2013; Manzari et al., 2014 unpublished). The integration of metagenomics, cultivation-based studies and environmental surveys can lead to a clear understanding in terms of the intersection of genes, organisms and the environment. More specifically, the integration of cultivation-dependent and cultivation-independent approaches partly bridges the gap between genomics, population genetics, biochemistry, physiology, biogeochemistry and ecology. Such approaches, that combine cultivation and metagenomic perspectives, will undoubtedly be more common in the future through collaborative studies in microbiology (Fig. 34).

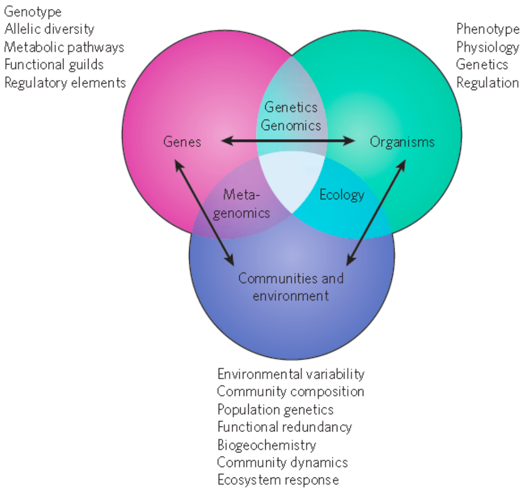

Fig. 29. DeLong, 2009. The intersection of traditional disciplines and metagenomics.

The pink, green and blue regions represent the fundamental elements of study: genes, organisms and the environment. The intersections between the elements show the disciplinary overlaps: genetics/genomics, metagenomics and ecology. The pale blue area in the middle identifies the ‘sweet spot’ in which information from cultured-based studies, environmental studies and metagenomics can be integrated and modelled.

2. *Materials and Methods*

2.1. Bacterial biomass and cell size estimation

Bacterioplankton counts were performed using a Zeiss Standard Axioplan microscope equipped with an halogen lA (Hg 100) light. Duplicate slides were prepared from each sample by filtering 1ml of seawater through a Millipore filter (0.2□m pore) and using DAPI (4,6-diamidino-2 phenylindole) as fluorochrome (Porter and Feig, 1980). Cell size of bacterioplankton was estimated by epifluorescence microscopy using microphotographs. Each cell size was determined after projection on a screen and at least 60 cells per filter were measured manually. Bacterioplankton cells were divided into three size classes: small, medium and large (<0.065, 0.065–0.320 and 0.320–0.780 □m3) (Danovaro et al., 1998). Bacterioplankton biomass can be estimated from the cellular carbon content (CC) by applying linear or allometric conversion factors (Kepner & Pratt 1994; Massana et al. 1997; Posch et al., 2001) Total bacterial biovolume (mm^3 l^{-1}) may be calculated as the product of bacterial abundance (cells l^{-1}) and mean cell volume (μm^3), wherease a total bacterial biomass (μg C l^{-1}) can be obtained from bacterial abundance multiplied by CC. Bacterioplankton biovolume was converted into biomass assuming a carbon content of 310 fg C□m^3 (Fry, 1990).

2.2. Bacterial community identification

Swarms or blooms of different jellyfish species may influence the bacterial community composition, especially in the enclosed areas, as the Varano lake (Italy). In this lagoon seawater samples were collected in May in two different areas (M-zone and C-zone, according to the presence or absence of jellyfish respectively), during the jellyfish bloom. Bacterial community structure was followed using 16S rRNA sequence analysis of bacterial colonies isolated from Marine Agar. 16S rRNA sequences of bacterial isolates reveal the heterotrophic culturable bacterial community diversity. Results obtained using this technique have been compared with the results obtained using a Metagenomic approach.

DNA Extraction and PCR of 16SrRNA genes from bacteria isolates

PCR analysis of 16S rRNA was used to track changes in bacterial community composition from isolation of pure cultures. For this purpose an aliquot of 100□l of seawater sample was spread on Marine Agar media and incubated *in situ* temperature. Colonies with different morphologies and pigmentation were picked up, isolated and subjected to identification (Fig. 30).

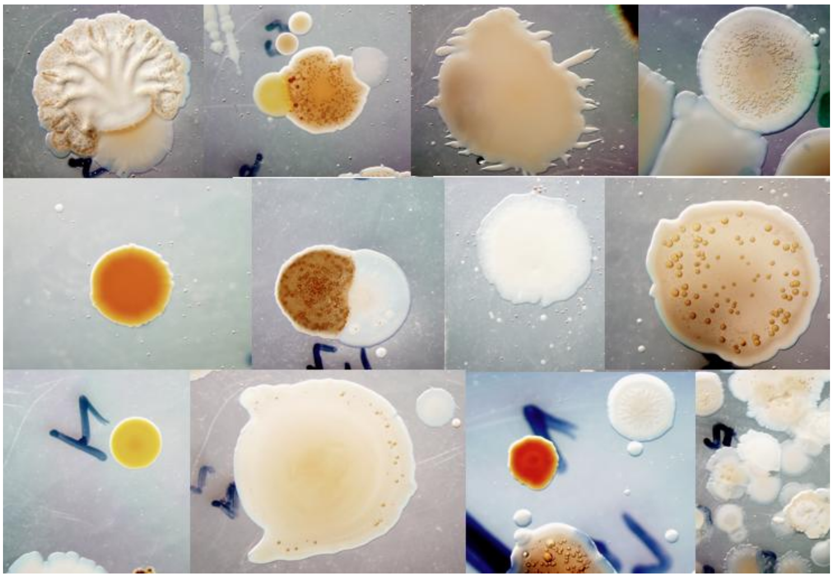

Fig 30. Some types of colonies with different morphology

In detail, after being cleaned "by streaking" three times, a single colony of each isolate was inoculated into liquid Marine Agar media and incubated in the dark at 22°C. Bacterial DNA was extracted using the 5% Chelex solution protocol. Bacterial 16S rRNA genes were amplified using universal primers 27F and 1492R. The PCR reaction mix (50□l) contained 1x reaction buffer (Tris KCl-$MgCl_2$), 2 mM $MgCl_2$, 0.2 mM dNTP, 1mM of each primer, Taq polymerase (5 U □l^{1}, Fermentas) and 2□l of DNA (50–100ng). The PCR touchdown protocol of Don et al., (1991) was used entailing initial denaturation at 94 °C for 5 min, followed by a thermal cycler program: denaturation for 1 min at 94 °C, primer annealing for 1 min at an initial 65 °C, decreasing 1 °C every two cycles to a final 50 °C, primer extension for 3 min at 72 °C. Ten touchdown cycles were run followed by 20 standard cycles (denaturation for 1 min at 94 °C, primer annealing for 1 min at 55 °C, primer extension for 3 min at 72 °C). The last cycle was followed with a 10 min final incubation at an annealing temperature of 72 °C. The size and quality of PCR products was confirmed by agarose gel electrophoresis (Fig. 31). PCR products were analyzed by electrophoresis. Electrophoresis was run at 200V (35–40 mA) for 1h using 1X TAE running buffer at 60°C. Gels were visualized using a UV transilluminator. Using Image J software the presence, position and relative brightness bands were visually detected.

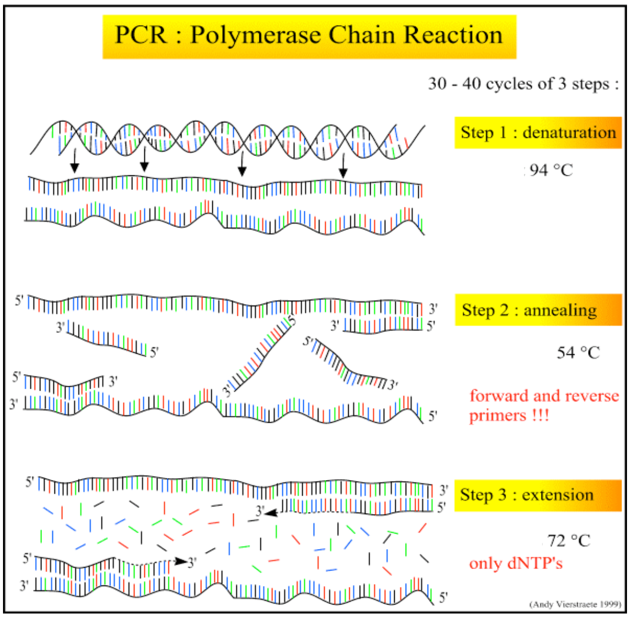

Fig 31. The steps of PCR amplification (Picture: Andy Vierstraete, 1999)

All PCR products were purified and sequenced on both strands by Macrogen Inc. (Seoul, Korea) using the PCR primers (Fig. 32).

Fig. 32 Standard PCR-based amplification of the 16S rRNA gene

The quality of sequences was controlled by removing traces of sequencing primer using the DNA baser (www.DNAbaser.com). Ambiguous base at the end of the sequences were also trimmed away. Database searches for sequence taxonomic identities and phylogenetic analysis were done using the genome Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov.blast) at the National Centre for Biotechnology Information (NCBI) (Benson et al., 2008).

3. Results

3.1 Bacterioplankton biomass and cell size distribution

The bacterial biomass in the lake of Varano was more markedly influenced by the presence of jelly-falls than by the seasonal oscillations of temperature and salinity. The bacterial biomass was higher in the zone with *Aurelia* sp. than in the control zone. In May, during the peak of jellyfish bloom the highest value of bacterial biomass recorded was 235.583 µgC l^{-1}. In September, in coincidence with jellyfish decaying (jelly-falls), the highest value of bacterial biomass recorded was 1404.03 µgC l^{-1} (Fig. 39).

Total bacterioplancton biomass	
February 2012 M-zone	178.9325223 µgC l^{-1}
February 2012 C-zone	148.7668291 µgC l^{-1}
May 2012 M-zone	235.5838706 µgC l^{-1}
May 2012 C-zone	126.4838858 µgC l^{-1}
September 2012 C-zone	1404.03797 µgC l^{-1}
December 2012 C-zone	16.70580187 µgC l^{-1}

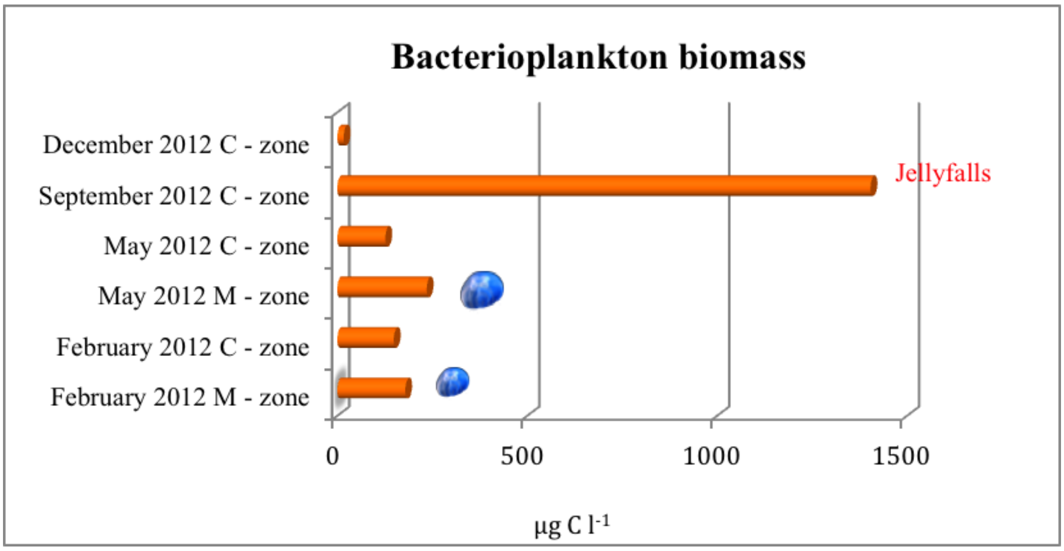

Fig 33. Total bacterioplankton biomass throughout the year

Moreover, in May, during the jellyfish bloom in the M-zone, the large size bacteria prevailed on the others classes. The abundance of large size bacteria was generally higher than medium and small size cells, with a peak in September (Fig. 34). Parallely, the peak of large size bacteria (783.144 µgC l^{-1}) was recorded in September when the jellyfish bloom almost disappeared, but releasing in the water column high quantities of decaying gelatinous organic matter. In presence of jellyfish, the highest value of large size was recorded in May (130.02 µgC l^{-1}).

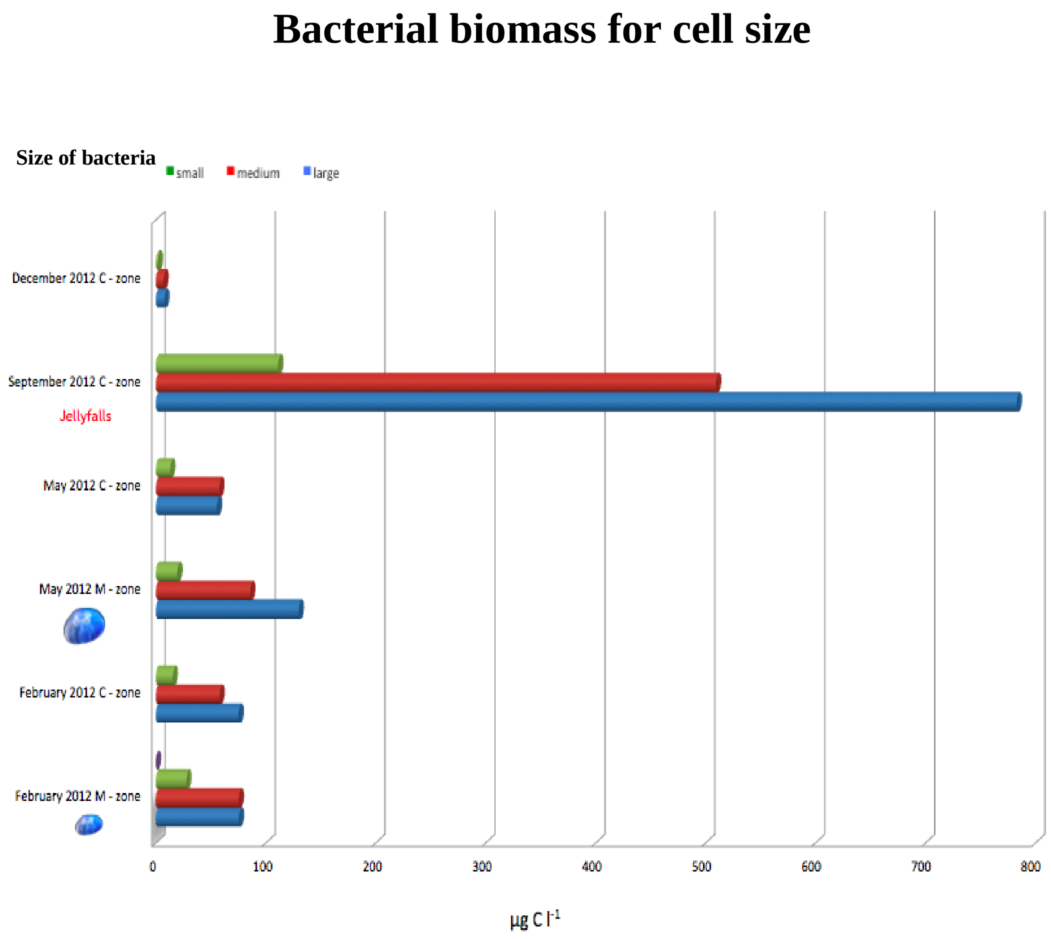

Fig. 34 Variation of bacterial size, biomass and abundance during the whole year

Bacterial biomass for cell size

	μg C l^{-1} of large size bacteria	μg C l^{-1} of medium size bacteria	μg C l^{-1} of small size bacteria	Density of jellyfish
February 2012 M - zone	75.5660665800458	75.51625262	27.85020312	0,03 ind/m^3
February 2012 C - zone	75.3000929393651	57.92011582	15.54662036	0
May 2012 M - zone	130.016781352722	85.85984636	19.70724285	12,39 ind/m^3
May 2012 C - zone	55.6032672156262	57.71564943	13.1649692	0,03 ind/m^3
September 2012 C - zone	783.144608670791	509.5623119	111.3310489	Jelly-falls
December 2012 C - zone	7.76495267653775	7.032040088	1.908809106	0

3.2 Bacterial diversity analysis

3.2.1 Standard PCR-based amplification of the 16S rRNA gene

By the 16S standard barcoding method, four classes of microorganisms were found in the lake of Varano in the jellyfish and control zones (Tab. III). The main bacterial community in the Varano lake was represented by the culturable species of γ-Proteobacteria during the jellyfish bloom.

Classes	**Number of isolates**	**Number of isolates**
	M-zone	**C-zone**
Gammaproteobacteria	10	9
Alphaproteobacteria	3	0
Actinobacteria	2	0
Bacilli	1	2

Table III. Classes of microorganisms found in both zones: Jellyfish zone and Control zone

Among γ Protobacteria, the *Vibrionaceae* represented the main family dominating the bacterial community in presence of jellyfish. In absence of jellyfish instead, the *Shewanellaceae* family was the more abundant (Tab.IV) (Fig.35).

Families of γ Protobacteria	**Number of isolates**	**Number of isolates**
	M-zone	**C-zone**
Micrococcaceae	2	0
Shewanellaceae	3	3
Pseudoalteromonadaceae	2	0
Vibrionaceae	4	3
Oceanospirillales	1	1

Table. IV Families of γ Protobacteria found in both zones: Jellyfish zone and control zone

Families of γ-Proteobacteria in the lake of Varano

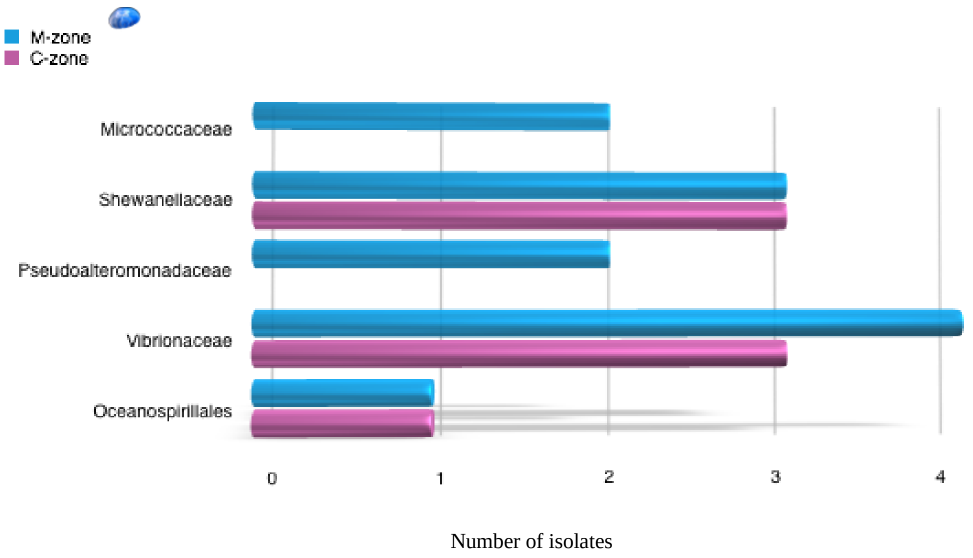

Fig 35. Families of γ-Proteobacteria found during the jellyfish bloom in May, in the lake of Varano

3.3. Sequences of γ Protobacteria found in the lake of Varano

Alignments between the sequence of bacteria found in the lake of Varano and the most similar bacteria (for percentage of sequences identity) found in GenBank

Family of *Micrococcaceae*

Nesterenkonia sp. N6(2011) 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|JF937438.1|Length: 1339Number of Matches: 1

Range 1: 63 to 1334GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	**Expect**	**Identities**	**Gaps**	**Strand**
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Family of *Vibrionaceae*

Vibrio sp. S3027 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|FJ457489.1|Length: 1375Number of Matches: 1

Range 1: 8 to 1374 GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

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Family of *Shewanellaceae*

Shewanella colwelliana strain FA122 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|JQ083323.1| Length: 1452 Number of Matches: 1

Range 1: 17 to 1366 GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

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2298 bits(1244)	0.0	1318/1350(98%)	19/1350(1%)	Plus/Plus

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Family of *Pseudoalteromonadaceae*

Pseudoalteromonas marina strain S185 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|FJ457121.1|Length: 1392Number of Matches: 1

Range 1: 123 to 1392GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

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Family of *Oceanospirillales*

Oceaniserpentilla haliotis strain DSM 19503 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_042641.1|Length: 1475Number of Matches: 1

Range 1: 31 to 1415GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	**Expect**	**Identities**	**Gaps**	**Strand**
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| ||| Sbjct

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3.4 Bacterial diversity recorded by a Metagenomic approach

The analysis of microbiome diversity in Varano lake was also attempted for the first time through the application of a metagenomic (Next Generation Sequencing) Illumina-based protocol targeting the universally conserved 16S ribosomal RNA gene. In presence of jellyfish, more specifically, during the jellyfish bloom, 75 families, 156 genus and 408 species of bacteria were found. In the control zone instead (absence of jellyfish) 81 families, 171 genus and 452 species were found.

Statistically, significant differences were observed in 6 families and 9 genera, between the jellyfish zone and the control zone. A significant increase of the *Halomonadaceae*, *Shewanellaceae*, *Comamonadaceae*, *Simkaniaceae*, *Micrococcaceae* families has been recorded (Tab. V). The family of *Oceanospirillaceae* also slightly increased.

Families	**C-zone**	**M-zone**	**pval**
Comamonadaceae	4,56E+04	1,93E+04	2,78E-13
Halomonadaceae	3,83E+04	8,21E+04	4,60E-17
Micrococcaceae	4,88E+01	9,69E+02	3,96E-03
Oceanospirillaceae	2,53E+03	5,96E+02	6,00E-03
Shewanellaceae	1,33E+05	2,76E+05	3,40E-16
Simkaniaceae	7,39E+02	6,67E+03	4,22E-06

Genera	**C-zone**	**M-zone**	**pval**
Diaphorobacter	3,27E+04	1,33E+04	2,18E-13
Halomonas	2,74E+04	6,00E+04	1,59E-16
Marinovum	1,01E+05	6,82E+04	4,71E-07
Micrococcus	0,00E+00	3,54E+02	3,07E-04

Nitrincola	1,21E+03	1,18E+02	1,44E-03
Paracoccus	6,33E+00	7,50E+03	2,32E-26
Shewanella	9,66E+04	2,04E+05	3,94E-16
Simkania	3,34E+02	3,27E+03	1,73E-06
Synechococcus	1,95E+05	3,44E+05	1,37E-21

Tab. V Bacterial population observed in the jellyfish zone and in the control zone

4. Discussions and Conclusions

A significant increase of bacterial biomass was recorded in September, probably due to enrichment of particulated and dissolved organic matter derived from decaying bodies of dead jellyfish (jelly-falls). In the lake of Varano, the quantity of large size bacteria was higher than the other two sizes of bacteria throughout the year. The Varano lagoon is a semi-enclosed basin where the physico-chemical characteristics are influenced by the different localization of sewage, terrestrial inputs, mussel culture, other anthropogenic activities and a reduced hydrodynamic circulation. Results on bacterial community structure showed that culturable species of γ-proteobacteria represented the main component of the bacterial community in the lake of Varano during the jellyfish bloom. Among γ-proteobacteria, the *Vibrionaceae* family was a key component, as confirmed from both DNA analysis and growth assays during the *Aurelia sp*. bloom.

Our results showed that the inventory of families of the γ -Proteobacteria recorded by the metagenomic approach were partially consistent with those found by the standard PCR-based amplification of the 16S rRNA gene. In presence of jellyfish, the families of *Halomonadaceae* and *Shewanellaceae* showed a significant increase, followed by the *Comamonadaceae, Simkaniaceae, Micrococcacea*, and less markedly by *Oceanospirillaceae*. All these families exert a conspicuous metabolic activity presumably related to the utilization of the jellyfish-related biomasses and compounds, which may also explain the predominance of large-sized, metabolically active bacteria. Bacteria of *Comamonadaceae*, family for example, use as its substrate a wide variety of organic acids, including aminoacids, which are the main component of jellyfish organic matter. Bacteria of *Shewanellaceae* family, can actively degrade organic matter, including the gelatinous matter deriving from jellyfalls because bacteria of this family produce a range of hydrolytic enzymes, i.e. proteinases, lipases, amylases, agarases, and alginases. Similarly, the *Micrococcaceae* family is composed by bacteria with metabolism being either respiratory and/or fermentative with carbohydrates and/or amino acids used as carbon and energy sources. Natural bacterial assemblages in aquatic environments are heterogeneous, being represented by cells with different morphologies, sizes and metabolic activities. These features, in turn, may affect the protozoan grazing rates on bacteria (Koton et al., 2002). Bianchi (1980) showed that the bacterial concentration in the water column decreases with depth and increases at the sea bottom in coastal waters. Bacterial abundance is also related to the organic matter concentration and to hydrological phenomena such as the interface of water masses with different densities (La Ferla and Crisafi 1991; Bianchi and Garcin 1993). The number of bacterial cells is usually high where waters are not deep and where there is a large number of organisms; under these conditions, in fact, leaves, plants and animal residues decay and settle on the bottom, representing a good nutritional substrate for heterotrophic bacteria and generally promoting bacterial growth.

In aquatic ecosystems, the flux of organic matter to the bottom sediments depends on the primary productivity at the ocean surface and on the water depth. In natural bacterioplankton assemblages, only a fraction of the total cell count is active, and, therefore, rates of bacterial production should be more strongly correlated to the number of active cells than to the total number of bacteria (Del Giorgio, 1997). The amount of bacterial biomass carbon produced per unit of carbon substrate utilized (i.e., the bacterial growth efficiency BGE) determines to what extent bacterial metabolism results in biomass that can be utilized in the food chain and to what extent biomass is directly dissipated through the mineralization of organic matter (Eiler, 2003). Coupling bacterioplankton and jellyfish dynamics with environmental parameters can contribute to highlight changes in trophic pathways and to unravel the impact of jellyfish blooms on the ecology of coastal zones. In aquatic habitats, the most common bacteria are Gram negative. The majority of the isolates belong to the genera *Pseudomonas*, *Vibrio* and *Flavobacterium*. A higher percentage of Gram positive bacteria instead, is found in sediments (Stolp, 1988). However, the microbial diversity resulting from a standard barcoding approach is usually low because only a minority of marine bacteria can be isolated and cultured. It is difficult to determine how an organism’s behavior changes without the ability to survey the metabolic activity of all community members simultaneously and being the change the result of cooperative and competitive interactions. The formation of microbial consortia presumably results in an optimization that is not achieved through solitary existence and might provide a high degree of plasticity to respond to environmental perturbation. It is now possible to design experiments that integrate genomics, gene expression and proteomics in an environmental context to determine how roles are distributed among different members of populations and communities. (Palleroni, 1997; Gest, 2001; Rappe et al., 2002; Keller and Zengler, 2004; Stevenson et al., 2004; Giovannoni and Stingl, 2007). Cultivation based approaches can observe rare microorganisms that are undetected by molecular methods and allow the verification of the hypotheses of metabolic potential determined by the metagenomic data (Leadbetter 2003; Giovannoni and Stingl 2007; Bent and Forney 2008; Zengler 2009; Green et al. 2010). Cultivation and purification also provide new genome sequences that assist in designing of better primers and probes for the refinement of molecular detection methods (Prakash et al. 2013). Approaches based on cultivation therefore, not only provide reference strains for the study of physiology, genetics, pathogenicity and adaptation but also widen the borders of basic research and gives new organisms for novel metabolites, enzymes of industrial application. It is important to emphasize that despite the significant progress in transcriptomics and proteomics, the physiology and the metabolism of specific microbial groups in complex ecosystems cannot be determined based solely on omics data. Thus, it becomes increasingly important to cultivate and preserve representative organisms in the face of ever expanding sequence-based estimates of microbial diversity. Studies of pure cultures serve as the backbone of molecular biology, microbial physiology and the biotechnological revolution. It is evident that using only isolation and characterization of novel microbes is not enough, but preservation of the isolated strains without changes in phenotypic and genotypic features is mandatory for future reference, research and new discoveries in the microbiology (Prakash et al. 2012). In conclusion, it is possible to affirm that further studies are needed to confirm our results and compare any changes in the microbial community within different Mediterranean areas where jellyfish blooms occur.

Section 3: Blue Biotech Approach

Introduction

1. Active Biological substances isolated from Cnidaria nematocysts

Marine invertebrates represent a source of bioactive compounds. Many groups of invertebrates indeed produce specific substances that serve them as (1) defensive agents against predators, parasites and infections or (2) chemicals for intraspecific and interspecific communication. Thus, the chemical diversity of the substances produced by marine invertebrates is an unlimited resource of new biologically active molecules (Aneiros and Garateix, 2004). Some of these compounds have interesting properties which could potentially be exploited in pharmacology. Bioactive compounds are also present in the nematocysts of cnidarians and include proteins (neurotoxins, toxins that form pore or cytolysins, phospholipases and protease inhibitors), glycoproteins, steroids, bioactive amines, carbohydrates (Watters, 2005) and secondary metabolites with both toxic and biomedical properties. However, in some cases the mechanism of action of these biotoxins is not widely known (Marino et al., 2004). Many of the toxic effects seem to result from the alteration of the permeability of the membrane. The cytolytic toxins isolated from several species of jellyfish and siphonophora are of the considerable interest (Calton and Burnett, 1988). Toxins extracted from jellyfish are highly unstable so they are less biochemically characterized than those of anthozoans. In recent years a considerable progress has been made by several researchers through the research on cytolysins isolated from the nematocysts of sea anemones. Hemolysins also represent an important set of bioactive molecules isolated from cnidocysts. These polypeptides are basic and without cysteine. Many hemolysins are inhibited by the membrane lipids such as phosphatidylcholine, sphingomyelin and cholesterol. A protein with hemolytic properties present in the nematocyst extract of the jellyfish *Carybdea alata* was isolated and characterized. The hemolytic activity is reduced or lost after being exposed to proteolytic enzymes such as trypsin, collagenase and papain (Chung et al., 2001). Another hemolysin isolated from the nematocysts of *Rhizostoma pulmo* is the rhizolysin. The rhizolysin has the maximum activity at pH 6.75 and is completely inhibited by sucrose, cholesterol and sphingomyelin (Cariello et al., 1988). A mechanism of hemolysis is represented by the permeabilization of the membrane through the lipid peroxidation. The consequences of the peroxidative process on lipid membranes include the loss of polyunsaturated fatty acids, altered ion transport and the generation of cytotoxic metabolites.

Rats treated with the crude extract of *Pelagia noctiluca*, demonstrated a strong inflammation characterized by lipid peroxidation: the poison promotes the expression of the enzyme nitric oxide synthase and the activation of nuclear enzymes (Marino et al., 2009)

1.1. Active Biological substances isolated from body and tentacles

Biologically active substances were also isolated from the tentacles and from the body of some Scyphozoa. The extract of the tentacles of *Aurelia aurita*, for example, showed phospholipase activity (Radwan et al., 2001) and causes pharmacological and toxicological effects such as neurotoxicity, myotoxicity and hemolysis. The enzymes contained in the extracts of *A. aurita* tentacles are also active on cell cycle of tumor cells, resulting in the inhibition of cell growth. From the tentacles of *Rhopilema nomadica* has been purified a toxin that shows activity of phospholipase A2, very similar to the toxin isolated from the venom of some vertebrates (reptiles) and invertebrates (hymenoptera)(Lotan et al., 1996). The antioxidant activity of a protein extracted from the tentacles of the jellyfish *Rhopilema esculentum* was tested against diseases caused by an excess of free radicals (DNA degradation, aging and cell damage). This protein is now used as an additive in food and pharmaceutical industries. Although less investigated in comparison to the tentacles also the body of some cnidarians constitutes a reserve of biologically active substances (Huahua et al., 2005).

1.2. Biologically active substances isolated from the mucus

The mucus has a variety of specialized functions in the animal kingdom (Koch et al., 1991). Invertebrates use the mucus as a surface of outer coating to reduce friction and the hydrodynamic resistance and to limit the loss of water. The mucus is also important for locomotion, it is used to trap prey, to anchor and protect the eggs. It can also be a barrier against infection (Davies and Viney, 1998). The mucus is essentially a lubricant of aqueous base: its main components are the mucin and water. The mucin is a glycoprotein that forms the macromolecular matrix of the mucus determining its properties. The mucin is characterized by a high molecular weight, by a high carbohydrate content (equal to 50-90%) and tandem repeats (TR) of proline, serine and threonine O-glycosylation (Callaghan e Voynow, 2006).

Some cnidarians have mucus cells. The epidermis of these marine invertebrates, in fact, is very rich in glandular cells important for the production of mucus. It has been proven that the epithelial mucus cells secrete mucopolysaccharides unpleasant able to ward off predators (Brusca and Brusca, 1990). In Scyphozoa like *Aurelia* sp., mucus cells are localized within the gastric filaments, which are located along the inner margin of the gonads (Fig. 36) (Borradaile et al., 1967).

In addition to Scyphozoa, also corals produce mucus secreted as a insoluble hydrated glycoprotein and which forms a viscoelastic gel (Meikle et al., 1988).

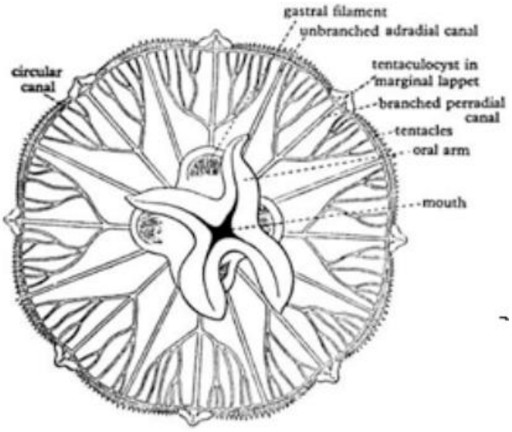

Fig. 36 Disposition of gastric filaments in *Aurelia* sp.

1.3. Antimicrobial peptides

The endogenous antimicrobial peptides (AMPs) are among the most important effectors of invertebrate innate immunity. Living in an environment rich in microbes, marine invertebrates use antimicrobial peptides as a first line of defense against potential pathogens. A wide variety of antimicrobial peptides have been extracted from sponges, annelids, mollusks, crustaceans, tunicates and cnidarians. The AMPs are a largely unexplored resource representing the starting point for the development of new antibiotic with a natural broad-spectrum of action (Ovchennikova et al., 2006). The development of resistance to the common antibiotics used to treat bacterial infections by microorganisms, have contributed to the birth of antimicrobial pharmacology with the employment of marine natural products (Mayer and Hamann, 2004). Antimicrobial peptides contain less than 150-200 amino acids, have a molecular weight below 25-30 kDa and include small proteins with structure α-helix and β-sheet, lipopeptides and peptides (Epand and Vogel, 1999). Most antimicrobial peptides are cationic and have a positive charge at physiological pH due to their higher content in lysine and arginine rather than in aspartic acid and glutamic acid which are negative amino acids. The amphipathic structure seems to be essential for the antimicrobial activity (Bulet et al., 2004). The importance of antimicrobial peptides is their ability to function without a high specificity or memory; moreover, their small size makes them easy to be synthesized (Relf et al., 1999). These peptides, therefore, are considered antibacterial agents for their broad spectrum of action, for their highly selective toxicity against prokaryotic and for the difficulty of bacteria to develop resistance towards them (Hancock and Scott, 2000). Due to their selectivity and their lytic action against pathogens, antimicrobial peptides are used not only as antibacterial agents, but also as anticancer and as compounds for the treatment of many other diseases (Ellerby et al., 2008). The first antimicrobial peptide isolated from the mesoglea of jellyfish *Aurelia aurita* is aurelin. The aurelin, consisting of 40 amino acid residues, is active against Gram positive and Gram negative bacteria. Aurelin has a molecular weight of 4296.95Da, has six cysteines that form three intramolecular disulfide bridges, but has no structural homology with other antimicrobial peptides previously identified (Ovchennikova et al., 2006). The primary structure of aurelin has the same structural features of defensins of animals. Aurelin presents the lysine in the same position of the toxins SHK extracted from the sea anemones *Stichodactyla helianthus,* in which the lysine residues and tyrosine play a key role in blocking the channels of the voltage-dependent K^+ (Gasparini et al., 1998). The structural homology of aurelin and toxins of sea anemones suggests that also antimicrobial peptides purified from jellyfish possess the ability to block voltage-dependent channels. The AMPs and toxins, therefore, have a common origin, a similar molecular structure and common chemical characteristics, such as resistance to enzymatic hydrolysis and amphipathicity. The way in which these molecules are involved in the defense mechanism is related to their accumulation into specialized cells and by the release of these molecules into the extracellular environment (Ovchinnikova et al., 2006).

Lysozyme

Among the antimicrobial peptides, the lysozyme is the best known and characterized lytic agent. The study on lysozyme a new enzymatic approach in the treatment of bacterial infections has been introduced (Bennett and Chung, 2001). Lysozyme is used in experimental studies aimed at developing a strategy to treat microbial infections using lytic enzymes. Furthermore, the lysozyme is also used as a drug as antimicrobial agent and as an immunostimulant in the veterinary field (Sava, 1996). Lysozyme is a small enzyme consisting of 129 amino acids and has a secondary structure with predominance of β-pleated sheet structures compared to the α-helix structures. It works as a glucosidase damaging the wall of Gram-positive bacteria, by hydrolyzing the beta 1,4 glycosidic bond between the C1 of N- acetylmuramic (NAM) and the C4 N-acetylglucosamine (NAG), the main components of the peptidoglycan (Stryer , 1996) (Fig. 37).

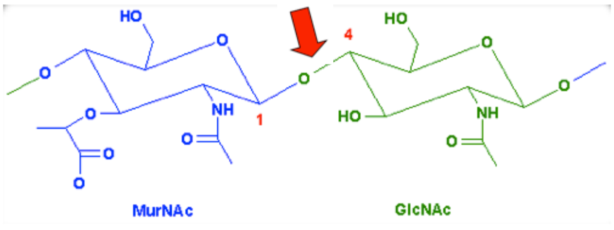

Fig. 37 The action of Lysozyme

Lysozyme is abundant in a number of secretions, such as tears, saliva, mucus, human and equine milk, and in many others marine invertebrates. It is also present in large amounts in egg white (Canicattì, 1990).

The function of the cell wall of the bacteria is to confer a mechanical support, so that a bacterial cell-free wall or with the damaged wall, bursts due to the high osmotic pressure inside the cell itself (Stabili and Pagliara, 1994). Lysozyme break the chains of carbohydrates that form the peptidoglycan. It destroy the structural integrity of the cell wall causing the osmotic lysis of the bacteria. Binding to the bacterial surface, also reduces the negative charge surface, making easier the phagocytosis of bacterium (Stryer, 1996). Recent studies have demonstrated the effectiveness of lysozyme in the treatment of oral diseases, ophthalmic infections and bacterial infections of the skin (Sava, 1996).

2. Materials and Methods

2.1. Animal collection

Specimens of *Aurelia* sp. (Cnidaria: Scyphozoa) were collected in the lake of Varano using net with mesh of 1 cm (Fig. 46). Immediately after sampling, the animals were transported to the laboratory in refrigerated containers. The jellyfish had a diameter ranging from 4 to 20 cm.

2.2. Antibacterial activity assay: samples preparation

Mucus

In order to get relatively uncontaminated mucus, the seawater in excess on the jellyfish bodies was removed blotting the body surface with an absorbent paper. The animals were washed with a sterile saline solution and then placed in sterilized Petri dishes. In these conditions they secreted mucus (Fig.47), which was immediately collected using a sterile Pasteur pipette. For the assays of lysozyme-like activity mucus from different individuals was pooled and stored at -80°C until use.

Homogenate of tentacles and umbrella

The tentacles of each individual were detached from the body of the animals, unified and successively homogenized with a Politron KINEMATICA GmbH TYP PT 10/35 using a PT 20. For the homogenate of jellyfish bodies instead, jellyfish were devoid of tentacles. They were unified, and then homogenized with a Politron KINEMATICA GmbH TYP PT 10/35 using a PT 20.

2.3. Lysozyme like activity assay

The presence of lysozyme-like activity in the mucus, umbrella and in the tentacles of *Aurelia* sp. was highlighted using a test of lysis of *Micrococcus luteus* cell wall.

To detect lysozyme activity, inoculated Petri dishes were used as standard assay, 700 µl of 5 mg/ml of dried *Micrococcus luteus* cell walls (Sigma) were diluted in 7 ml of 0.05 M PB-agarose (1.2%) (pH 5.0) then spread on a Petri dish. Four wells of 6.3mm diameters were sunk in the agarose gel and each filled with 30 µl of the different sample. The diameter of the cleared zone of the four replicates was recorded after overnight incubation at 37 °C and compared with those of reference samples represented by hen-egg-white lysozyme (Merck, Darmstadt, Germany).

Effect of pH on the lysozyme-like activity

The pH effect on the lysozyme-like activity was tested by dialyzing the mucus in PB 0.05 M, ionic strength, I=0.175, adjusted at pH 4, 5, 6, 7, 8, and by dissolving agarose in PB at the same I- and pH-values.

In the prepared plates at different pH values, 30 µl of the sample were placed in each well. The plates were incubated overnight at 37°C and the enzyme activity was evaluated by measuring the diameters of lysis around each well.

Effect of Ionic strength on the lysozyme-like activity

The ionic strength effect on the lysozyme-like activity was tested in PB 0.05 M (pH 6.0 in the case of tentacles and umbrella homogenate; pH 4.0 in the case of mucus), adjusted at I=0.0175, 0.175, and 1.75. Agarose was dissolved in PB at the same I-values.

Effect of Temperature on the lysozyme-like activity

The temperature effect was tested with incubations of samples (in PB, at pH 6.0, and I=0.175) at 5, 15, 22, and 37 °C. Dose–response curve of lysozyme-like activity was obtained employing Petri dish assays of samples (20, 30, 40, 50, 60, 80, and 100 µl of mucus).

Dose - Response curve

A dose-response curve was made by the standard Petri dish assay filling the wells with the. 20, 30, 40, 50, 60, 80, 100 µl of each sample. The plates were incubated overnight at 37°C and the enzyme activity was evaluated by measuring the diameters of lysis around each well.

3. Results

3.1 Lysozyme like-activity in the mucus of *Aurelia* sp.

Mucus of *Aurelia* sp.showed a natural lysozyme like activity. By the standard assay on Petri dishes a diameter of lysis of 3.1 ± 0.5 mm corresponding to 0.41 mg/ml of hen egg-white lysozyme was observed (Fig 38).

Fig. 38 Lysozyme-like activity in the mucus. The arrow indicates the diameter of lysis around each well in which mucus was loaded.

The lysozyme-like activity was strongly affected by the pH of the sample. In particular, the minimum diameter of lysis was 0.6 ± 0.03 mm at pH 8.0. The maximum activity instead, was observed at pH 4.0 with a diameter of lysis of 3.1 ± 0.5 mm (Fig 39).

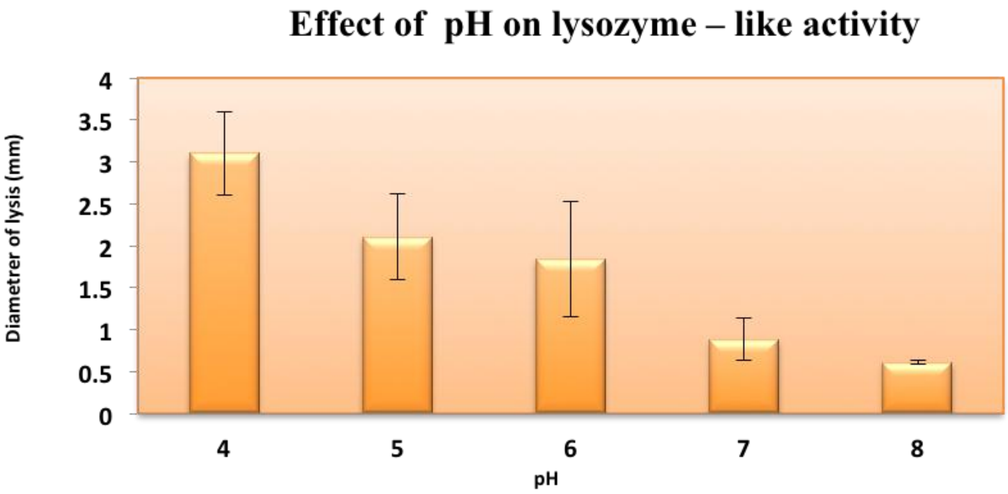

Fig. 39 Effect of pH on lysozyme – like activity in the mucus of *Aurelia* sp.

As far as the assays on the ionic strength (I) is concerned, the diameter of the lysis was 2.3 ± 0.5 mm when (I) was equal to 0,0175. When ionic strength measured 1.75, the diameter was reduced to 1.1 ± 0.3mm. The maximum activity was obtained with a value of ionic strength equal to 0.175 at pH 4.0. In these conditions, the diameter of lysis was equal to 3.1 ± 0.4 mm (Fig. 40).

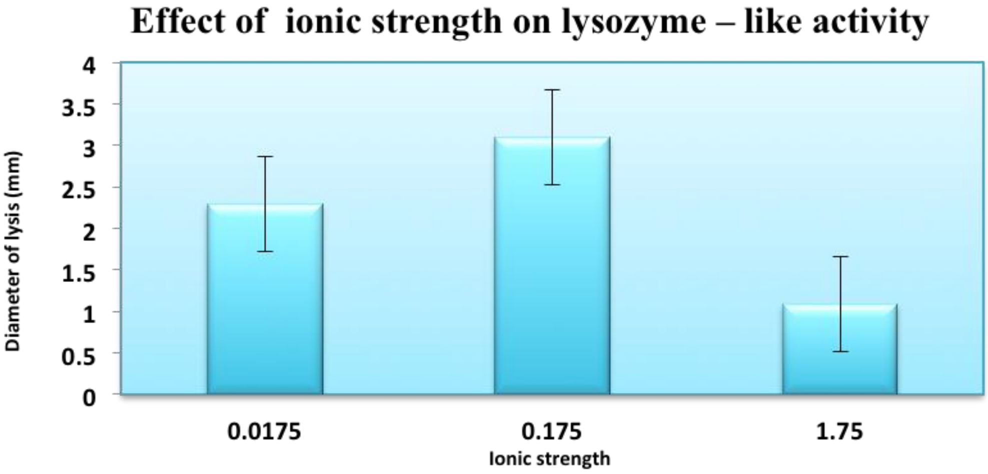

Fig. 40 Effect of ionic strength on lysozyme – like activity in the mucus of *Aurelia* sp.

The effect of temperature on the lysozyme-like activity was also evaluated. At the temperature of 5°C, the diameter of lysis was 0.4 ± 0.002 mm. At 15°C the diameter of lysis was 0.9 ± 0.2 mm and at 22°C was 2.0 ± 0.5 mm. When the test was conducted at a temperature of incubation of 37°C, the lysozyme-like activity of the mucus was the highest measured. At this temperature the diameter of lysis was 3.1 ± 0.7 mm (Fig. 41).

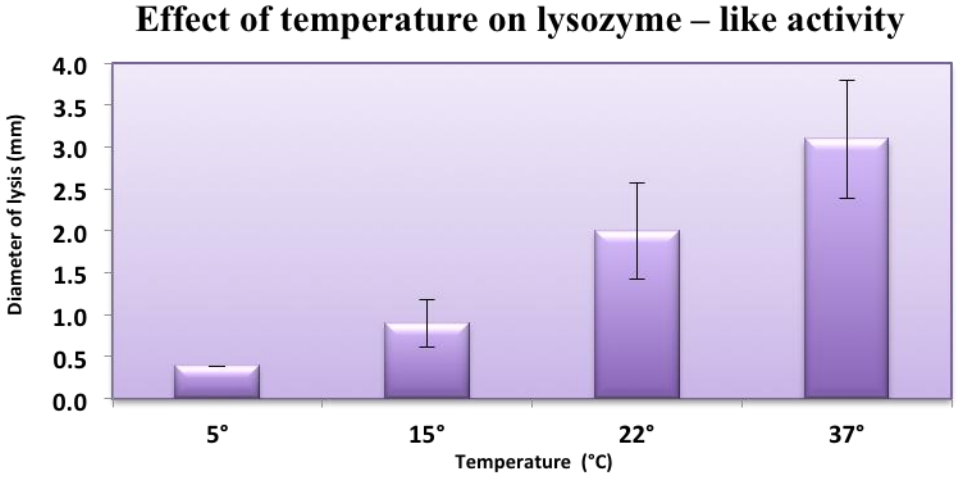

Fig. 41 Effect of temperature on lysozyme – like activity in the mucus of *Aurelia* sp.

A dose–response correlation was obtained when increasing amounts of mucus were plotted against the respective lysis area diameters (Tab. VI and Fig. 53). The diameter of the lysis area was positively correlated with the sample volume.

Dose - response curve

Mucus concentration	**Diameter of lysis**
20µl	1.7 ± 0.35 mm
30µl	2.7 ± 0.33 mm
40µl	3.1 ± 0.35 mm
50µl	4.7 ± 0.35 mm
60µl	5.2 ± 0.32 mm
80µl	5.7 ± 0.35 mm
100µl	6.7 ± 0.35 mm

Tab. VI Dose–response correlation of lysozyme-like activity of Aurelia sp.mucus. Wells in the Petri dish were filled with different concentrations of mucus (from 20 to 100 µl). Values are given as means±Standard Deviation

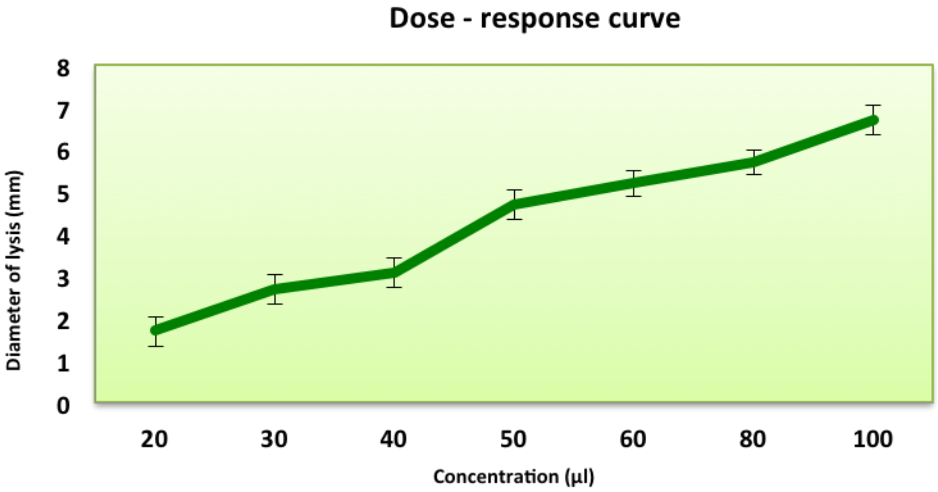

Fig. 42 Dose - response curve of lysozyme – like activity in the mucus of *Aurelia* sp.

3.2 Lysozyme like-activity in the tentacles of *Aurelia* sp.

The homogenate *Aurelia* sp. tentacles showed a lysozyme-like activity. By the standard assay on Petri dishes a diameter of lysis of 5.6 ± 0.48 mm corresponding to 0.76 mg/ml of hen egg-white lysozyme was observed (Fig 43).

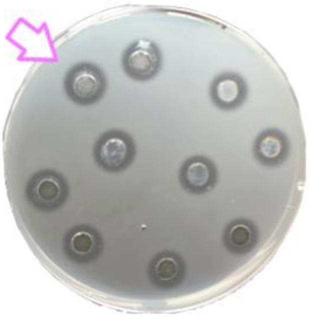

Fig. 43 Lysozyme-like activity in the tentacles of *Aurelia* sp. The arrow indicates diameter of lysis around each well in which the homogenate of tentacles was loaded.

Also in the homogenate of tentacles the lysozyme-like activity was strongly influenced by pH of sample. In particular, the minimum diameter of lysis was 0.6 ± 0.03 mm at pH 8.0.The highest activity instead was observed at pH 6.0 (diameter of lysis = 5.6 ± 0.48 (Fig 44).

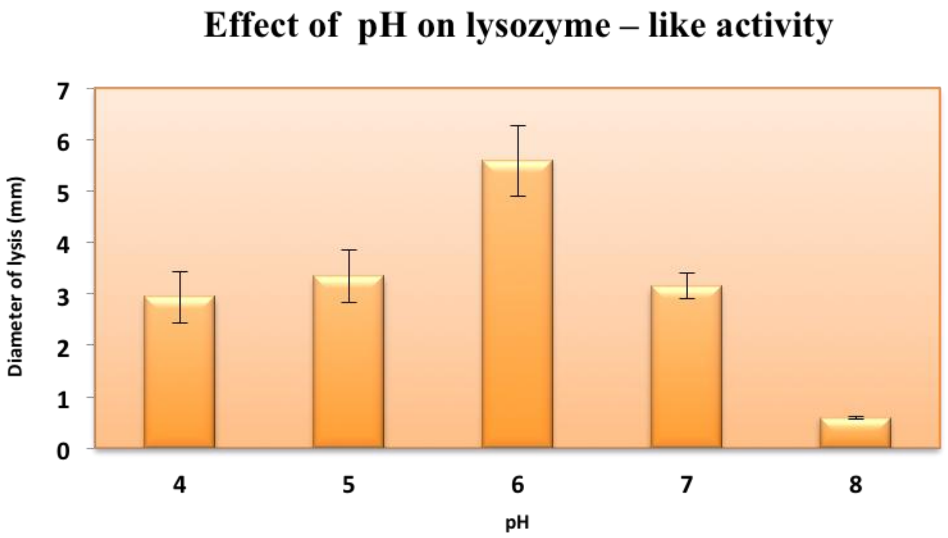

Fig. 44 Effect of pH on lysozyme – like activity in the homogenate of *Aurelia* sp. tentacles

The lytic activity varied after dialysis against phosphate buffer at different ionic strength (I) values. In particular, the diameter of the lysis was 3.3 ± 0.47 mm when (I) was 0.0175. When the ionic strength was 1.75 the diameter was reduced to 2.1 ± 0.42 mm. The highest activity was obtained with a value of ionic strength of 0.175 at pH 6.0. In these conditions, the diameter of lysis was 5.6 ± 0.51 mm (Fig. 45)

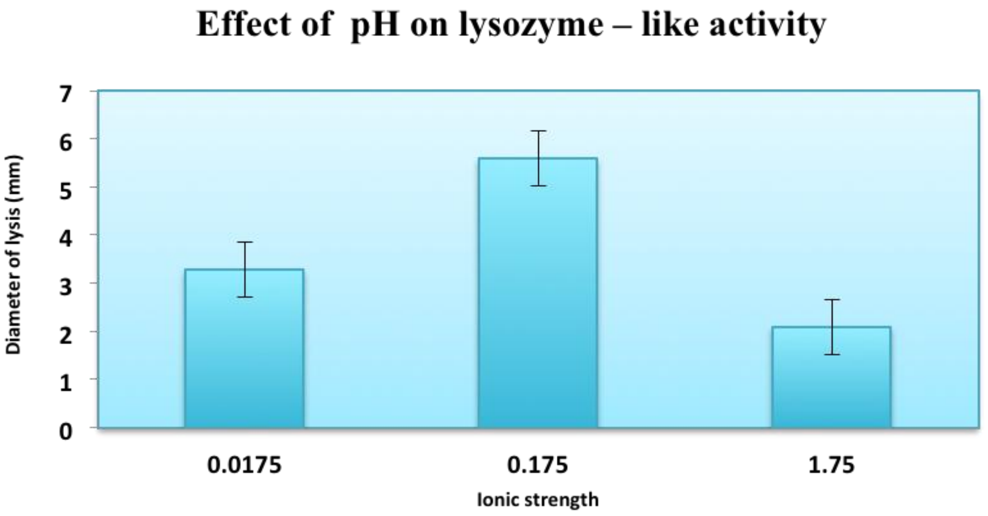

Fig. 45 Effect of ionic strength on lysozyme – like activity in the homogenate of *Aurelia* sp. tentacles

The effect of incubation temperature on the lysozyme-like activity was also investigated. At 5°C, the diameter of lysis was 0.4 ± 0.07 mm. At 15°C the diameter of lysis was 1.1 ± 0.2 mm and at 22°C was 3.5 ± 0.42 mm. The largest diameter of lysis (5.6 ± 0.48 mm) was recorded at 37 °C (Fig. 46).

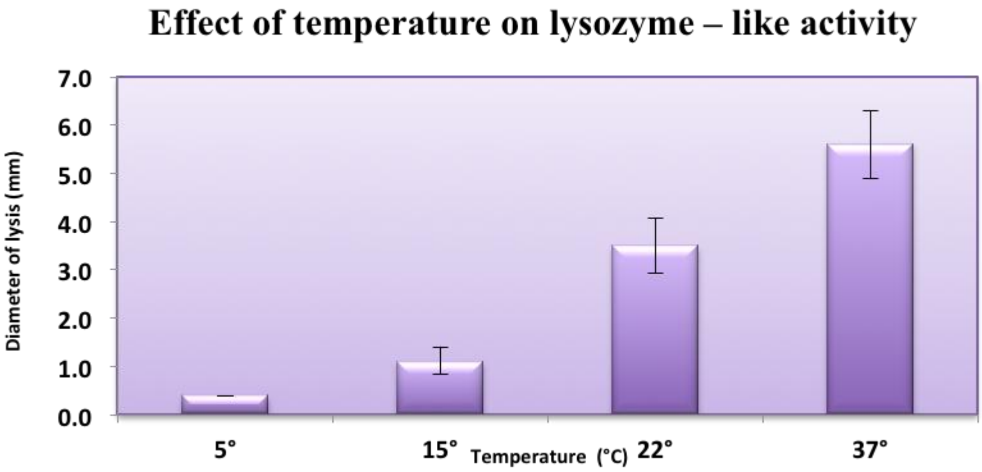

Fig. 46 Effect of temperature on lysozyme – like activity in the homogenate of *Aurelia* sp. tentacles

A dose-response effect was evidenced when increasing volumes of homogenate were employed. The obtained data (Fig. 58) showed that the diameter of the lysis area was positively correlated with the sample volume.

Dose - response curve

Homogenate of tentacles	Diameter of lysis
20µl	3.7 ± 0.35 mm
30µl	4.7 ± 0.33 mm
40µl	5.6 ± 0.35 mm
50µl	6.7 ± 0.31 mm
60µl	7.2 ± 0.31 mm
80µl	7.7 ± 0.33 mm
100µl	8.2 ± 0.34 mm

Tab. VII Homogenate of tentacles concentration and respective diameters of lysi

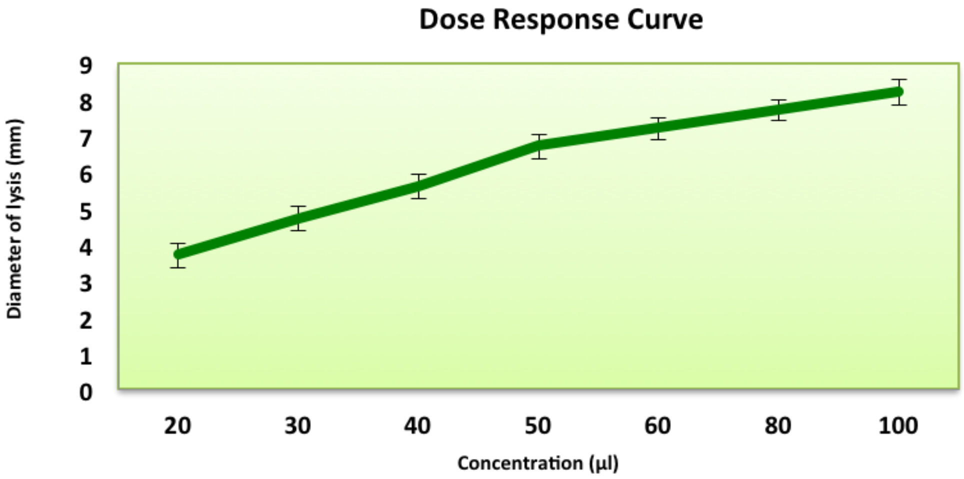

Fig. 47 Dose-response curve of lysozyme – like activity in the homogenate of tentacles of *Aurelia* sp.

3.3 Lysozyme like-activity in the umbrella of *Aurelia* sp.

The umbrella of *Aurelia* sp. exert a lysozyme-like activity. By the standard assay on Petri dishes a diameter of lysis of 4.0 ± 0.49 mm corresponding to 0.54 mg/ml of hen egg-white lysozyme was observed (Fig. 48).

Fig. 48 Lysozyme-like activity in the umbrella of *Aurelia* sp. The arrow indicates diameter of lysis around each well in which homogenate of umbrella was loaded.

The lysozyme-like activity of homogenate of umbrella was strictly affected by pH, ionic strength and temperature of incubation. In particular, the lowest diameter of lysis was

0.6 ± 0.02 mm at pH 8.0. The highest activity was observed at pH 4.0, with the diameter of lysis of 4.0 ± 0.48 mm (Fig. 49).

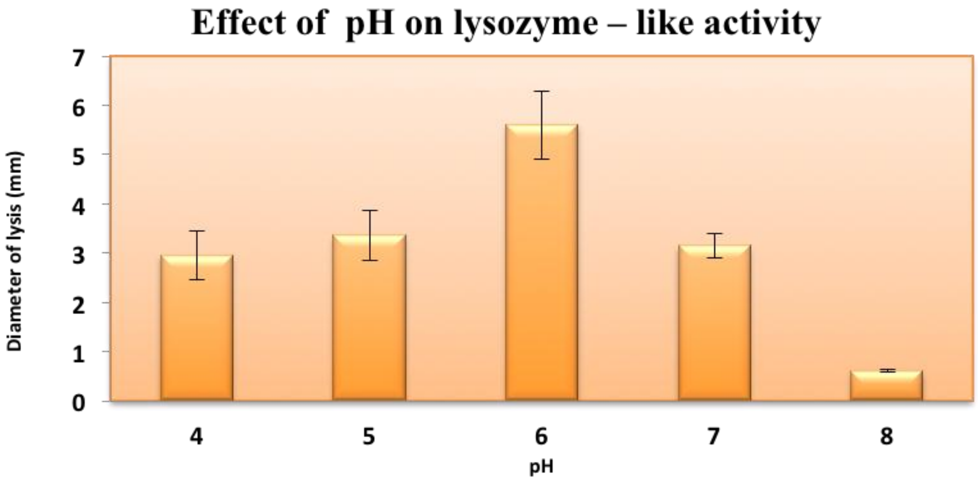

Fig. 49 Effect of pH on lysozyme – like activity in the homogenate of *Aurelia* sp. umbrella

The diameter of the lysis was 2.3 ± 0.44 mm when the ionic strength (I) was 0.0175. When ionic strength was 1.75 the diameter was reduced to 1.7 ± 0.39 mm. The maximum activity was obtained when the value of the ionic strength was equal to 0.175. (pH 6.0). In these conditions, the diameter of lysis was 4,0 ± 0,47 mm (Fig. 50).

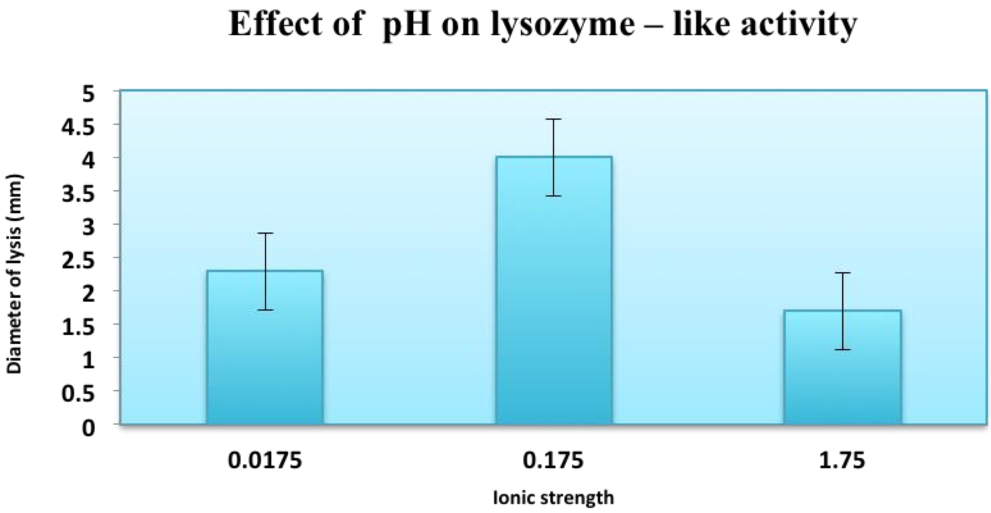

Fig. 50 Effect of ionic strength on lysozyme – like activity in the homogenate of *Aurelia* sp. umbrella

At an incubation temperature of 5°C the diameter of lysis was 0.4 ± 0.07 mm. At 15°C the diameter of lysis was 1.0 ± 0.19 mm and at 22°C was 2.5 ± 0.39 mm. The largest diameters of lysis was recorded at 37 °(4.0 ± 0.46 mm (Fig. 51).

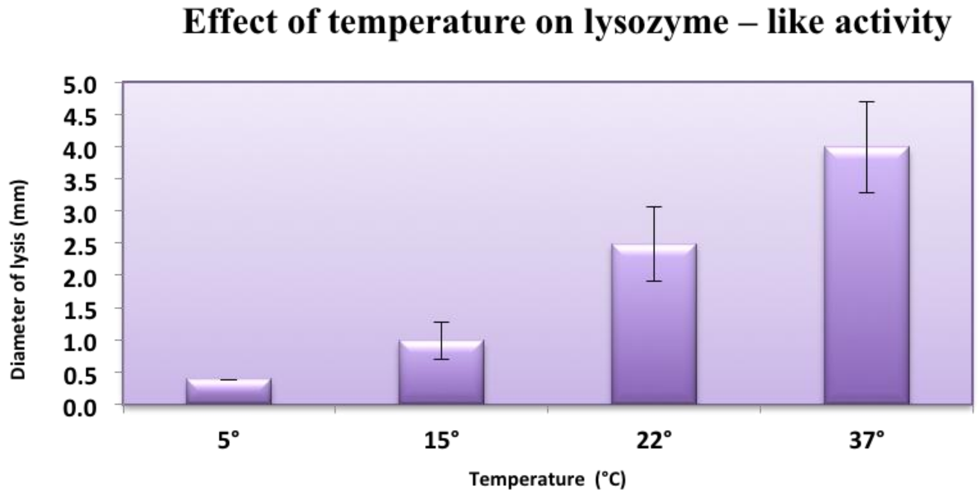

Fig. 51 Effect of temperature on lysozyme – like activity in the homogenate of *Aurelia* sp umbrella

A dose–response correlation was obtained when increasing amounts of homogenate were plotted against the respective lysis area diameters (Tab. VIII and Fig. 52) The diameter of the lysis area was positively correlated with the sample volume.

Dose - response curve

Homogenate of umbrella	**Diameter of lysis**
20µl	2.5 ± 0.34 mm
30µl	3.5 ± 0.32 mm
40µl	4.0 ± 0.31 mm
50µl	5.7 ± 0.33 mm
60µl	6.2 ± 0.32 mm
80µl	6.7 ± 0.34 mm
100µl	7.2 ± 0.31 mm

Tab. VIII Homogenate of tentacles concentration and respective diameters of lysis

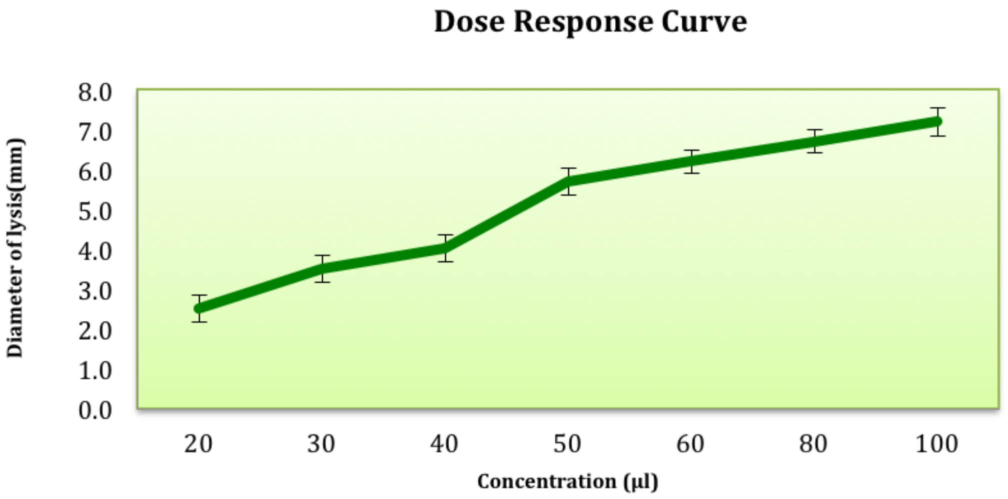

Fig. 52 Dose-response curve of lysozyme – like activity in the homogenate of umbrella of *Aurelia* sp.

SUMMARY TABLES

pH Dependence

	Max activity	Diameter of lysis
Mucus	4	3.1 ± 0.5
Homogenate of tentacles	6	4.0 ± 0.49
Homogenate of body	6	5.6 ± 0.48

Ionic strength dependence

	Max activity	Diameter of lysis
Mucus	I = 0.175	3.1±0.4
Homogenate of tentacles	I = 0.175	4.0±0.47
Homogenate of body	I = 0.175	3.1±0.4

Temperature dependence

	Max activity	**Diameter of lysis**
Mucus	37°C	3.1 ± 0.7
Homogenate of tentacles	37°C	5.6 ± 0.48
Homogenate of body	37°C	5.6 ± 0.48

Summary tables on the maximum activities into the three different compartments

4. Discussions and Conclusions

Our results suggest that the jellyfish *Aurelia* sp. may use the bioactive compounds present in the extracts of its tissues and mucus as a means of chemical defense against bacterial infections. .

The aim of this part of the study was to characterize the antibacterial activity of lysozyme in the mucus and in the homogenate of the tentacles and in the homogenate of umbrella of the jellyfish *Aurelia* sp. The results obtained (summary tables) showed that all different samples analyzed were able to lyse the cell wall of the bacterium *Micrococcus luteus* in vitro.

These results prove that the homogenate of tentacles has a lysozyme-like activity higher than that of umbrella and mucus. Since the optimum of activity in mucus was obtained at a different pH compared to the pH of the homogenate of tentacles and umbrella, it can be assumed that the effectors responsible for lysozyme-like activity were different in these three compartments. In order to prepare extracts of the tentacles and of umbrella, gastric filaments have been removed. In the gastric filaments there are cells delegated to the mucus production. Therefore, it can be assumed that mucus cells produce different antibacterial substances if compared to those produced by the cells contained in the tentacles and umbrella.

The characteristics of lysozyme found in the tentacles and in the umbrella of *Aurelia* sp. are similar to those of lysozyme present in the extract of the nematocysts of *Actinia equina*, which recorded the optimum activity at pH 6, with the ionic strength equal to 0.175 and at a temperature of incubation of 37°C. The maximum diameter of lysis observed in *A. equina* in an other study (Lala, 2008, unpublished), is equal to 4.75 ± 0.7 mm, which is very close to the diameter of lysis produced by the lysozyme-like activity in the homogenate of umbrella of *Aurelia* sp.

The presence of lysozyme in all compartments of the scyphozoa analyzed, does not exclude the cooperation with other "defensive factors" such as aurelin. A similar mechanism also could exist in some insects (Hoffman and Hoffman, 1990).

Corals use the mucus to fight against microbial infections. It has been shown that the mucus of corals is able to accommodate a large number of bacteria (Ducklow and Mitchell, 1979), as for example *Vibrio alginolyticus* which is able to destroy the layer of mucus (Phillips, 1963). The mucus, therefore, has not only a passive role in the protection of corals from the bacterial infection, but actively contributes to the lysis of the bacteria.

Lysozyme is the best characterized antimicrobial peptide in marine invertebrates and represents an innate response to bacterial infections (Bulet et al., 2004). The lysozyme hydrolyzes the bond β-1,4 glycosidic of peptidoglycan of bacterial cell wall of Gram-positive. Noller and Hartsell (1961), have also demonstrated that this enzyme is also active against Gram-negative bacteria. Currently, lysozyme is attracting the interest of researchers for its potential applications in the pharmaceutical and aquaculture. The control of bacterial infections is currently one of the main problems of aquaculture on industrial scale. This is especially due to the high treatment costs and to the environmental impact of chemotherapy against infections. Therefore, the use of drugs is an essential component of modern aquaculture in order to ensure the health and the welfare of animals and for the management of the farm. Despite this, the number of veterinary medicines authorized for being used in the aquaculture in Italy, is quite low. This also due to some of EU regulations which interfere, also economically, in the development and the registration of medicines by the pharmaceutical industry. Furthermore, since the aquaculture field still represents a small portion of the market it also represents a risky market. As a result, it does not attract a sufficient quantity of investors ready to properly support the research whose return of investment, such as marketing authorization and registration, is not guaranteed. Get proper medicines able to treat pathologies and specific diseases is necessary to guarantee the health of animals and also to encourage a safer and responsible use of medicines themselves.

Therefore, in order to improve the therapeutic effectiveness in aquaculture, new formulations prepared by the association of lysozyme to vaccines obtained from *Vibrio anguillarum* and *Pasteurella piscida* should be developed. Currently lysozyme is also used for pharmaceutical preparations. The therapeutic effectiveness of lysozyme is based on its ability to control the growth of bacteria but also to modulate the immune responses of the host. Moreover, the treatment with lysozyme leads to a regression in the growth of some tumor cells (Labro, 1997). Lysozyme can be also used in the treatment of a wide range of infections in humans. Its properties allow to use lysozyme in the treatment of gastrointestinal infections, bacterial infections, pediatric infections, ophthalmic and oral infections. Some interesting studies conducted by Kreuser et al., (1992) showed how lysozyme is able to control and limit the infections of upper alimentary tract if used it as a chewing gum. Furthermore, since it has not any toxic effect on humans, the lysozyme is a good candidate in the use of epidermal and cosmetic formulations. Results of this study showed the presence of lysozyme-like activity in all extracts of *Aurelia* sp. Such results encourage the potential use of the jellyfish for lysozyme-based preparations in pharmaceutical research.

A large number of biologically active substances has also been found in other marine organisms such as sponges, sea squirts, sea anemones, corals and other Scyphozoa (Aneiros and Garateix, 2004). Researchers are interested in these substances for the uniqueness of their structure and for the pharmacological properties they own (Monastyrnaya et al., 2002). For example, sponges are a source of bioactive metabolites such as alkaloids, macrolides and polyethers equipped with cytotoxic and antimicrobial activity (Aneiros and Garateix, 2004).

Peptides with antimicrobial activity have been extracted and purified from the marine sponge *Discodermia kiiensis* (Matsunaga et al., 1985). The synthesis of antimicrobial peptides is an efficient mechanism used by marine invertebrates as a first line of defense against pathogens. Other molecules with antibacterial properties have been discovered and characterized in annelids, molluscs, in chelicerates, crustaceans, tunicates and as well as in cnidarians (Ovchennikova et al., 2006). In particular, peptides with antimicrobial activity have been isolated from *Mytilus edulis* (Charlet et al., 1996; Mitta et al., 2000), skin and mucus from the sea hare *Dolabella auricularia* (Iijima et al., 2003), from the crab *Tachypleus tridentatus* and from the lobster *Homarus gammarus* (Hauton et al., 2006).

The importance of antimicrobial peptides is related to the possibility to use them as a lead compound for the development of broad range of antibiotics. Unfortunately, these substances represent a resource still little explored (Ovchennikova et al., 2006). In the last years, aurelin has been purified from the mesoglea of jellyfish *Aurelia aurita*. Aurelin is the first antimicrobial peptide isolated from cnidarians. Aurelin is active against Gram-positive and Gram-negative bacteria. The structure of aurelin is similar to other structures of defence isolated from many other animal species such as drosomicine of insects and the mitiline mussels (Ovchennikova et al., 2006).

However, aurelin has no structural homology with other previously identified antimicrobial peptides, including peptides isolated from crabs rich in cysteine (Saito et al., 1995) and peptides from shrimps rich in cysteine and proline (Destoumieux et al., 1997).

However the distribution of cysteine residues makes aurelin similar to K^+ channel-blocking toxins of sea anemones *Stichodactyla helianthus* (Ovchinnikova et al., 2006).

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-A-

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