UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI SCIENZE ECOLOGICHE E BIOLOGICHE

Corso di Dottorato di Ricerca in

Ecologia e gestione delle risorse biologiche - XXVI Ciclo.

Impacts of *Aurelia* sp. 1 outbreaks in a Mediterranean coastal lagoon (Varano, SE Adriatic coast)

(s.s.d. BIO/05)

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Data della discussione
10/06/2014

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Abstract

In recent decades, scientific efforts focused on observed increase of jellyfish outbreaks, particularly in coastal ecosystems, as both causes and consequences of significant alterations in zooplankton community and dynamics.

One of the most common and ubiquitous outbreak - forming species, the scyphomedusa *Aurelia* sp.1 (*sensu* Dawson, 2003), occurs worldwide throughout many coastal areas. In Mediterranean sea, it recently established in enclosed areas, like the coastal lagoon of Varano (Apulia, SE Adriatic coast).

The total absence of bibliographic data requested a broad-spectrum investigation, with the main aim of creating the basis for a general knowledge of the impacts *Aurelia* sp. outbreaks. In the framework of the VECTORS project, a two-year investigation was launched in 2011 in order to collect data about population dynamics and trophic ecology of *Aurelia* sp.1 in the coastal lagoon of Varano.

The results showed that the population dynamic of the established *Aurelia* sp.1 is regulated by the variation in physical factors as temperature and salinity. Spatial and temporal analysis of plankton community showed also that trophic cascades on food web are the result of a sudden increase in the abundance of jellyfish population. Gut content and stable isotope analysis clarified the trophic role of *Aurelia* sp.1 in the lagoon according to size, and showed that jellyfish can vary the diet over the year by an opportunistic and omnivorous foraging behavior. The study also unveiled the key role of micro-zooplankton as food source for the early jellyfish life stages.

Furthermore, morphological analyses provided a complete description based on life stages for the population resident in the coastal lagoon of Varano. Molecular data (COI barcoding) documented the first record of *Aurelia* sp.1 along the Adriatic coasts and the first undisputable evidence of its establishment in the Mediterranean. Moreover the integration of morphology/morphometrics and molecular systematics applied to *Aurelia* spp. populations from Mediterranean and North Sea, demonstrated to be a powerful tool for species identification and for determining geographical distributions of *Aurelia* spp. cryptic species.

Finally, testing natural and artificial inducers of strobilation on *Aurelia* spp. polyps, elucidated differences in the developmental patterns regulating the initiation of jellyfish outbreaks of different *Aurelia* species.

Keywords: *Aurelia* spp., plankton dynamics, trophic level, cryptic species, Varano.

Riassunto

Gli sforzi scientifici degli ultimi decenni si sono concentrati sull'aumento degli outbreaks di meduse, in particolare negli ecosistemi costieri, sia come cause che come conseguenze di alterazioni significative nella comunità e nella dinamica zooplanctonica.

La scifomedusa *Aurelia* sp.1 (*sensu* Dawson, 2003) è una delle più ubiquitarie e comuni specie capace di formare outbreaks, ed è infatti presente in molte zone costiere nel mondo. In Mediterraneo si è recentemente stabilita in aree confinate, come la laguna costiera di Varano (Puglia, costa SE Adriatica).

La totale assenza di dati bibliografici ha richiesto una indagine ad ampio spettro, con l'obiettivo principale di gettare le basi per una conoscenza generale degli impatti creati dagli outbreaks di *Aurelia* sp.. Nel 2011, nel quadro del progetto VECTORS è stata lanciata una campagna di due anni col fine di raccogliere dati sulla dinamica di popolazione ed ecologia trofica di *Aurelia* sp.1 nella laguna costiera di Varano.

I risultati hanno mostrato che la dinamica di popolazione di *Aurelia* sp.1, è regolata dalla variazione dei fattori fisici come temperatura e salinità. L'analisi spaziale e temporale della comunità planctonica ha poi evidenziato che le cascate trofiche osservate sono il risultato dell'improvviso aumento di abbondanza della popolazione di meduse. L'analisi dei contenuti stomacali e l'analisi isotopica, hanno chiarito quale sia il ruolo trofico di *Aurelia* sp.1 nella laguna in base alle dimensioni, e hanno dimostrato che le meduse tramite un comportamento di foraggiamento opportunista ed onnivoro, sono in grado di variare la dieta nel corso dell'anno. Lo studio ha anche svelato il ruolo chiave del micro-zooplancton come fonte di cibo per le prime fasi di vita delle meduse.

Inoltre per la popolazione residente nella laguna costiera di Varano, le analisi morfologiche hanno fornito una descrizione completa basata sugli stadi vitali. I dati molecolari (barcoding del gene COI) hanno fornito la prima segnalazione di *Aurelia* sp.1 lungo le coste Adriatiche così come la prima prova indiscutibile della sua presenza in Mediterraneo. Inoltre, l'integrazione di morfologia/morfometria e sistematica molecolare applicata alle popolazioni di *Aurelia* spp. provenienti dal Mar Mediterraneo e dal Mar del Nord, ha dimostrato di essere un potente strumento per l'identificazione delle specie e per la determinazione delle distribuzioni geografiche delle specie criptiche di *Aurelia* spp..

Infine, i test effettuati sui polipi di *Aurelia* spp. con induttori naturali e artificiali della strobilazione, hanno chiarito quali siano le differenze nei pattern di sviluppo che regolano l'inizio degli outbreaks di meduse nelle diverse specie di *Aurelia*.

Parole chiave: *Aureli*a spp., dinamica del plancton, livello trofico, specie criptiche, Varano.

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CHAPTER ONE:

General introduction

1. Introduction

1.1. Gelatinous zooplankton outbreaks : the origin

The term gelatinous zooplankton refers to a particular ecological group, which is characterized by soft - bodies organisms, usually transparent (Hamner et al., 1975).

Jellyfish such as cnidarians and ctenophores are the most common example of gelatinous organisms, sharing a similar trophic role in spite of sharp anatomical differences (Mills, 1995).

A consistent number of taxa throughout the metazoan tree of life share soft-bodied characteristics. The term "gelata" has been proposed to include a polyphyletic assemblage, including organisms producing extensive extracellular matrix, such radiolarians, larvaceans, salps, molluscs, chaetognaths, polychaetes and nemerteans (Haddock, 2004). Indeed, a gelatinous consistency and a planktonic life habit remains the only attributes grouping together these taxa.

Cnidarians and ctenophores are among the most primitive clade in the evolution of metazoans, for long time associated under the term Coelenterata and successively separated but brought closer on the base of morphological and molecular results (Bridge, 1995). Representatives of the phylum of Cnidaria can be found in the Ediacaran fauna from the late Precambrian period (Scrutton, 1979) and the oldest cnidarian jellyfish fossils trace back to the Middle Cambrian, before the Cambrian explosion, 530 million years ago (Cartwright et al., 2007). In light of their long fossil history, the actual persistency can be interpreted as the favorable outcome of a suite of attributes making these organisms efficient competitors in harsh environments (Richardson et al., 2009).

Together with colonial radiolarians, the metazoan cnidarians and ctenophores possess a distinctive and a well-known feature: they can occur *en masse*.

A jellyfish aggregation is any accumulation of individuals , which are named as a swarm when they occur together as a consequence of behaviour (as observed in several scyphomedusae families) (Zavodnik, 1987; Shank and Graham, 2001; Dawson and Hamner, 2009). Finally, as a consequence of species-specific life histories "bloom" and "outbreak" are synonymous of recurring high peaks of abundances, which in some cases are referred to multispecific or monospecific proliferation events, respectively (CIESM, 2001).

The sudden increase of population can be driven by hydrographic factors as converging currents or by swimming behavior that follows variation in physical factors (apparent bloom) or more often, can be the result of the seasonal life cycle (true bloom), so directly related to reproduction and growth (Graham et al., 2001; Hamner and Dawson, 2009).

Also tunicate (salps and dolioliods) can rapidly reach high abundance following asexual reproduction, but often this phenomena are carried out far from the coast and being also unpredictable, are finally rarely detected (Boero et al., 2008, Deibel, 2009; Boero et al., 2013). Instead the perception of humans of a gelatinous invasion is more often linked to near - shore blooms where the actors are cnidarians and ctenophores.

In Mediterranean basin, the early 1980's were significant years for the invasion of *Aurelia aurita* and *Pelagia noctiluca,* and contributed greatly to the public awareness first and then to the development of scientific studies on biology, ecology and distribution of one of the most common and harmful specie (Rottini - Sandrini and Avian, 1983; Malej, 1989).

Since 1980 many aspects of jellyfish biology were investigated with the aim of understand the causes and the negative effects of blooms, often looking for monofactorial explanations. But to understand the mechanisms underlying jellyfish blooms, it is necessary to come back to the origin, to the jellyfish life cycle.

Cnidarians has commonly a metagenetic life cycle where a sexual, reproducing, planktonic stage (medusa) alternates with an asexual reproducing, benthic post-larval stage (polyp or scyphistoma). The formation of the medusa is the result of a process of morphogenesis and differentiation that occurs through developmental processes named polyp strobilation in the class Scyphozoa.

Several scyphozoans have no polyp stage and are represented by holoplanktonic medusae quite common in open oceanic or deep sea waters (Lucas et al., 2012).

The metagenetic life cycle confers several advantages for an invasive potential. Benthic polyps may produce one or many ephyrae all at once, over a period of time, or at different intervals. The high potential of this animals is in effect linked to their modularity factor that is species – specific (Boero et al., 2008) and it is associated also with the type of strobilation (monodisk, polydisk). In addition, the perennial polyps can reproduce asexually not only by budding from their bases, but also by fission and by stolon budding (Arai, 1997). In unfavorable environmental

conditions, polyps can release cysts made by chitin-protein complex cuticle that are able to survive in a resting stage more than 3.2 years (Thein et al., 2012).

As end result, life - cycle and life - history adjustment confer to cnidarians and ctenophores the potential to be absent for long period and suddenly re - appear overcoming any environmental constraints (Boero, 2008). Podocyst, buds, the type of strobilation, all contribute to the ability of a species to disperse (Fig. 1.1) and reach bloom size populations (Vagelli, 2007; Arai, 2009).

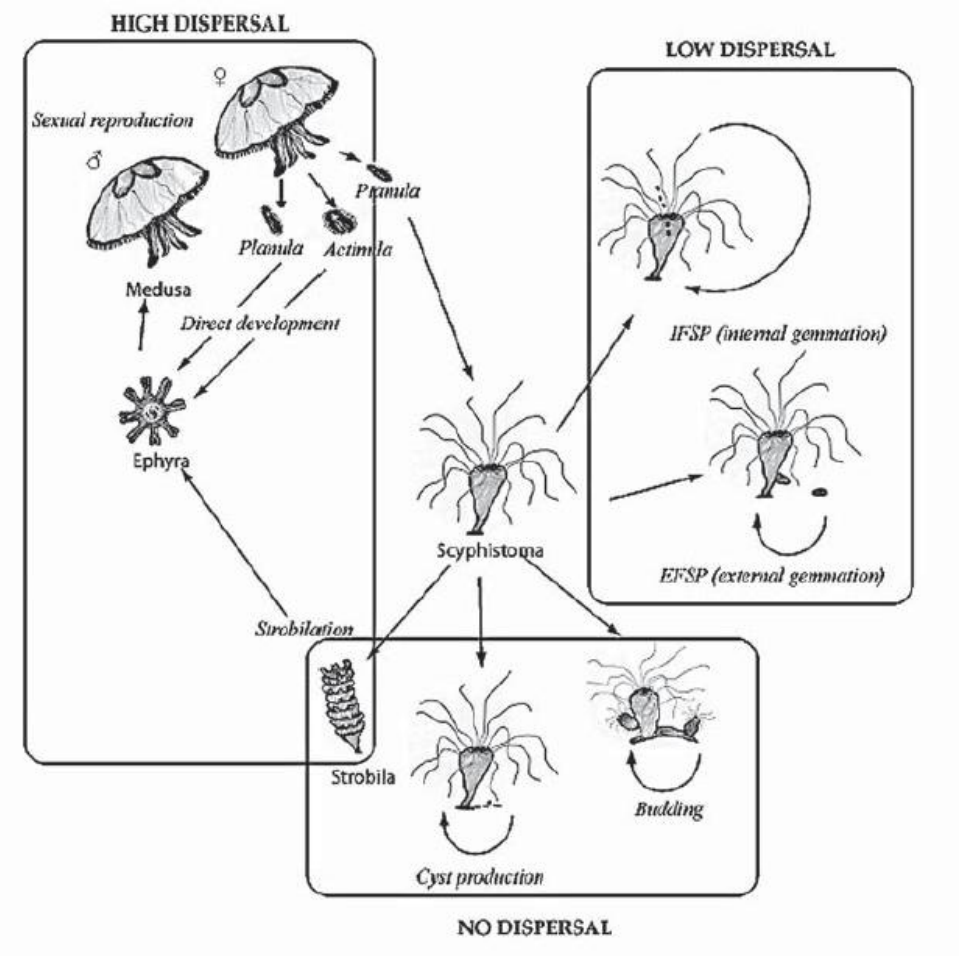

Figure 1.1. Life cycle of *Aurelia* spp. and dispersal strategies (Vagelli, 2007).

All these traits represent synapomorphic scyphozoan characters related to the potential to form *en mass* occurrence (Dawson and Hamner, 2009; Fig.1.2).

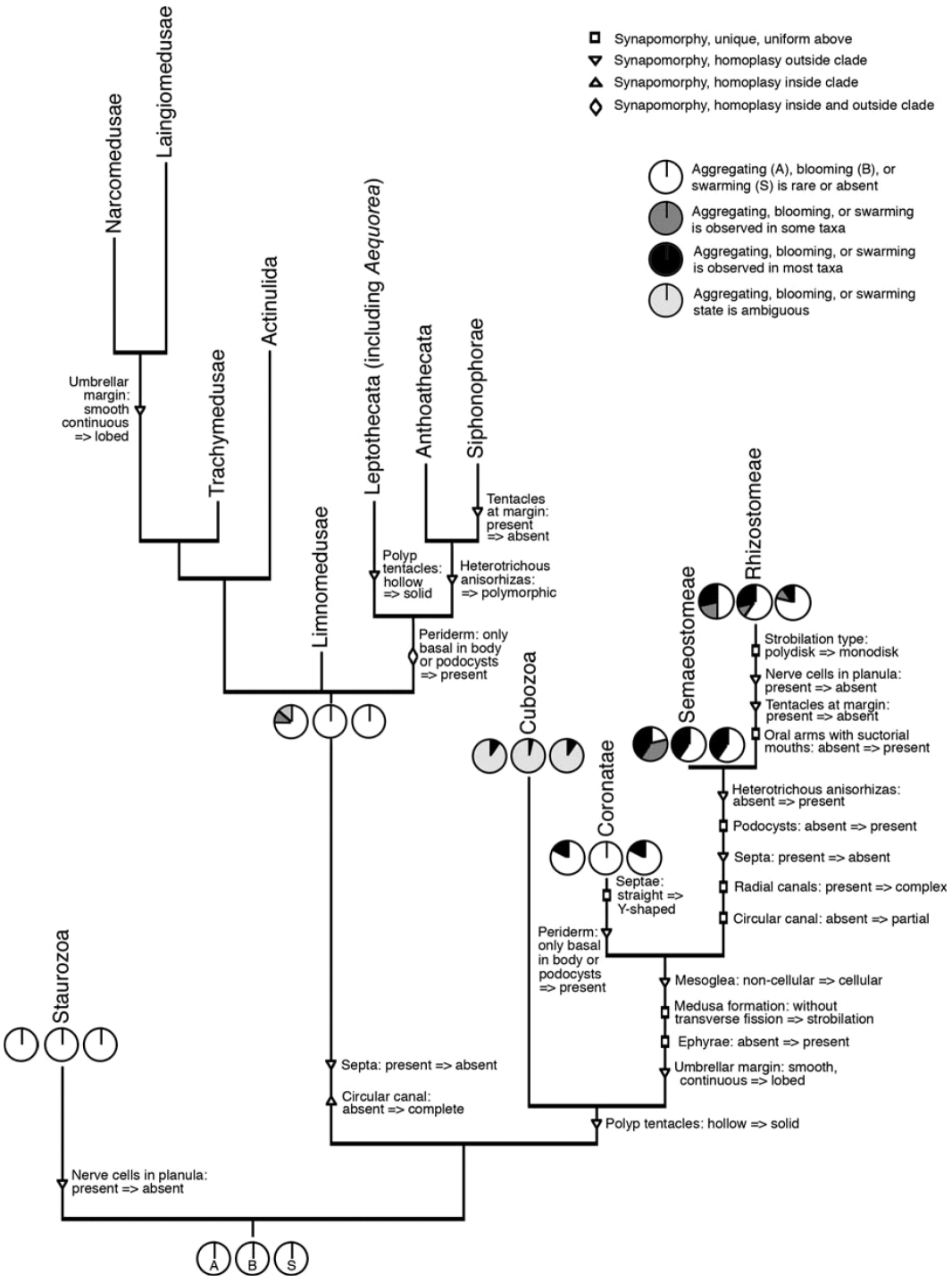

Figure 1.2 Characters potentially or causally correlated to aggregating, blooming, and swarming (Dawson and Hamner, 2009).

However natural and ecological forcings as the opportunistic behavior and the "hit - run" strategies that make jellyfish the good candidates for invading an area and outcompete other species, do not operate alone (CIESM, 2001).

Anthropogenic impacts such as eutrophication, climate change, overfishing, increase in artificial substrates into marine environments, have been proposed as external factors promoting jellyfish blooms (Arai, 1996; Mills, 2001; Richardson et al., 2009; Condon et al., 2012).

In the last decades, the intensive use of fertilizers in agricultural and farming activities have determined a strong input nutrients in ecosystems because through sewage and runoff they are delivered in the water and consequently promote phytoplankton blooms (Purcell, 2001). Since anthropogenic nutrients are rich in nitrogen and phosphorus but poor in dissolved silica, the result is an alteration of phytoplankton assemblage, where diatoms are substituted by phytoplagellates (Egge and Aksnes, 1992). A flagellate-based food chain do not lead to an elevated primary and secondary productions and as result, jellyfish are more favorite than fish (Cushing, 1989; Parson and Lalli, 2002).

As the phytoplankton die and sink out of the water column, oxygen levels can decrease rapidly leading to hypoxic or also anoxic levels where, differently from fish, both medusa and polyps stage are able to survive (Condon et al., 2001). Indeed, many studies demonstrated the ability of jellyfish to survive in despite of broad variations in different physical factors and it is therefore intuitive the reason why many studies recently addressed jellyfish as a negative outcome of climate change (Purcell, 2007; Brodeur et al., 2008; Lynam, 2010). Warming temperature may accelerate the growth of jellyfish or the rates of ephyra production (Holst, 2012) but can also favor the extension of the areal of invasive alien species (Daly Yahia et al., 2013). Habitat modification, with the creation of artificial hard substrate obviously facilitates the larval settlement and polyp colonies growth and consequently the entrance of the new invaders (Purcell, 2012, Purcell et al., 2007, Richardson et al. 2009).

Jellyfish are taking over fish, behaving as good actors in a movie where humans are the inactive viewers. Nowadays, the most accepted cause of jellyfish bloom is represented by overfishing. Intensive fisheries, fishing down the food chain, are removing the predators and the competitors of gelatinous plankton so that we are gradually assisting to a regime shift, from a fish- to a jellyfish-driven ocean (Pauly et al., 1998; CIESM, 2001).

1.2. Gelatinous zooplankton outbreaks : the consequences

The aggregations, the bloom, both apparent or true, or the swarm of gelatinous predators all cause several ecological and economical disorders. Mills (2001) distinguished “natural” from

"unnatural" blooms to give attention to the state of ecosystems that being often disturbed and over - fished switch the production of fishes over to the production of pelagic Cnidaria or Ctenophora.

Jellyfish are voracious predators, and their prey can come from a variety of size classes and taxonomic groups ranging from microzooplankton to ichthyoplankton (Sullivan, 1994; Arai, 1997; Malej et al., 2007; Purcell, 2007; Han, 2009).
During bloom events, jellyfish are able to control the flux of energy and nutrients in the ecosystem through their high consumption rates (Purcell, 1992, 1997). In some ecosystems, cnidarians can be cause of daily mortalities of early life stages in fish (Arai, 1997). A clear example is provided by the ctenophore *Mnemiopsis leidyi* that in 1980, entered in the Black sea through ballast water and led to fisheries collapse in a over - fished and eutrophic ecosystem (Shiganova, 1998).
Jellyfish can also impact phytoplankton and microbial communities. Sloppy feeding, excretion, and decaying biomass, all contribute to the release of nutrients and dissolved organic matter (DOM) to the microbial loop (Riemann et al., 2006; Titelman et al., 2006; Tinta et al., 2010). Both Condon (2011) and Tinta (2012) observed a shift in bacterial community from Alphaproteobacteria to Gammaproteobacteria and Flavobacteria, favored
by the bacteria uptake of dissolved organic matter (DOM) released by jellyfish.
The bacteria can also be ingested by cyclopoid copepods, one of the favourite jellyfish prey, further reducing the number of trophic levels from bacteria to jellyfish (Malej, 2007).

Most of time, jellyfish bloom are associated to economical damages where human - activities like fisheries, tourism and power plant, pay huge costs.
Gelatinous organisms can easily clog fisherman net causing possible economical loss and impeding the normal activity. As result, fishing industry seems to be both economical and ecologically affected.
Jellyfish can also clog seawater intake screens of power and desalination plants causing power reductions and shutdowns on a wide geographical scale (Purcell et al., 2012). In 2011, at the Orot Rabin Electric Power Station in Hadera on Israel's west coast, tonnes of jellyfish clogged up the filters. At the Torness power station (south-east coast of Scotland) both reactors

were shut down after jellyfish were found in their seawater filters at the end of last month. Jellyfish bloom had same effect for the Shimane plant in western Japan.

As demonstrated, jellyfish outbreaks often occur near coastal area or swimming areas, and some species can cause severe injuries, and in the worst case a jellyfish sting can rapidly lead to death. But there are place like the jellyfish lake in Palau where they have become an attractive and not a plague.

On the other hand, the public awareness has always associated jellyfish with their stinging ability and as expected, the idea of a tourism associated to jellyfish, is still far from the actual public's perception.

1.3. VECTORS project and PhD aims

VECTORS (Vectors of Change in Oceans and Seas Marine Life, Impact on Economic Sectors) is a multidisciplinary large-scale integrated European Project supported within the Ocean of Tomorrow call of the European Commission Seventh Framework Programme (FP 7 - OCEAN 2010). VECTORS investigates drivers, pressures and vectors that cause change in marine life like also the mechanisms of change and the impacts that they have on ecosystem structures and functioning. A total of 37 European countries facing on the three Regional Sea chosen as study areas (Mediterranean, North Sea and Baltic Sea) are involved in the understanding of drivers and pressure with the final aim of predict the future scenario.

A work package is dedicated to the evaluation of mechanisms of species outbreaks and pathways of invasions as vectors of change in marine ecosystems. The occurrence, abundance, distribution, successful traits of OFS (outbreak forming species) and IAS (invasive alien species) are investigated within the Mediterranean sea, also with reference to jellyfish outbreaks along the Italian coasts (http://www.marine-vectors.eu/default.aspx).

Within the VECTORS framework, the main topic of this PhD thesis has focused on several ecological aspects of a «jellyfish-driven» ecosystem in a Mediterranean coastal lagoon (Varano, SE Adriatic coast), recently invaded by the jellyfish *Aurelia* sp., addressing the question on what could be the most probable future scenario for gelatinous - dominated ecosystems.

To address this issue, the present thesis work provided first a description of the life cycle of *Aurelia* sp. in the Varano lagoon, to analyse how the environment can regulate seasonal patterns

of growth and abundance of the jellyfish (Chapter 2). Second, data on environmental variability and multi-trophic levels (including cascading trophic impacts) driven by *Aurelia* sp. competition and predation were integrated to evaluate the potential jellyfish impacts on plankton composition and abundance (Chapter 2). Third, data from traditional (gut content) and innovative (stable isotopes) methods were gathered to investigate jellyfish trophic ecology (Chapter 3).

In the second section, the thesis work included the analysis of the cryptic species of *Aurelia* genus from both taxonomical and physiological perspectives, in order to:

- improve the knowledge on systematic of *Aurelia* spp. cryptic species in Mediterranean, through combined morphological/morphometrics and molecular approaches (Chapter 4);
- gain better understanding of different effects of natural and artificial inducers of strobilation in *Aureli*a spp. cryptic species (Chapter 5).

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CHAPTER TWO:

Plankton dynamics in the coastal lagoon of Varano

2. Plankton dynamics in the coastal lagoon of Varano.

2.1. Abstract

In recent years, many coastal environments in the Mediterranean Sea were invaded by carnivorous gelatinous organisms like jellyfish and ctenophores. Gelatinous outbreaks can significantly alter zooplankton dynamics and size distributions, with severe impacts on trophic interactions.

A confined environment like the coastal lagoon of Varano represents a suitable habitat for invasion and establishment by the scyphomedusa *Aurelia* sp.1 which has resided there since 2000. In the framework of the VECTORS project, a two-year investigation was launched in 2011 by bi-seasonal samplings in the Varano Lagoon in 4 sectors characterized by different environmental features along the main sea-lagoon and W-E axes.

Ephyrae and adult medusae of *Aurelia* sp.1 were sampled for determination of population size structure and abundance. The role of the gelatinous top predator was investigated by analyzing spatial and temporal dynamics of the micro-, meso-, and macrozooplankton and phytoplankton community.

PERMANOVA analysis demonstrated that the factor "month" explained the abundance of nutrients, phytoplankton, zooplankton and jellyfish in the four sectors investigated.

The peaks of microzooplankton, mesozooplankton and jellyfish abundances occurred sequentially in the period considered. Release of the *Aurelia* sp.1 ephyrae occurred in spring and the peaks of jellyfish abundance were recorded in late spring and summer when the predatory control exerted on zooplankton community was high.

In May 2012, the exponential population growth of jellyfish reduced the standing stock zooplankton and restructured the zooplankton community, suggesting trophic cascades driven by jellyfish. Food limitation and high temperature were the main forces involved in the jellyfish population collapse.

The present study suggests that *Aurelia* sp., at high abundance, exerts a top - down effect on the zooplankton community and consequently may regulates the trophic interactions in the Varano Lagoon ecosystem.

2.2. Introduction

The impacts of jellyfish blooms on food webs have been long investigated in recent years due to the progressive interest of social media to the economic and social consequences (Purcell, 2012). In coastal areas impacted by humans, habitat modification with artificial substrates, eutrophication and overfishing, may have enhanced jellyfish proliferation (Purcell et al., 2007; Richardson et al., 2009) and consequent causes on trophic web. Ecosystems can be altered by these voracious predator that can feed on different organisms like microzooplankton, mesozooplankton and ichthyoplankton (Purcell, 1985; Arai, 1997; Malej et al., 2007; Brodeur et al., 2008).

In recent years, in many areas around the world were observed blooms of jellyfish like *Aurelia* sp., *Pelagia noctiluca*, *Rhizostoma* sp., *Cotylorhiza tuberculata*, and *Chrysaora* sp. In the Northern Adriatic, the frequency of blooms increased through the 1970s, 1980s, and 1990s (Brotz and Pauly, 2012). Along the Adriatic coasts, historical reports about jellyfish presence indicated *A. aurita* and *R. pulmo* were the most frequently reported scyphomedusae in the northern Adriatic over the 1785-2010 period with *Aurelia* dominance in 2004 (Di Camillo et al., 2010; Kogovšek et al., 2010; Malej et al., 2012). The moon jellyfish, *Aurelia* spp., are the jellyfish most commonly associated with bloom formation and they are now distributed in different areas along the Mediterranean coasts from the western to the Levant basin (Papathanassiou et al., 1987; Mutlu, 1994; Benović, 2000; Bonnet et al., 2012; Isinibilir, 2012).

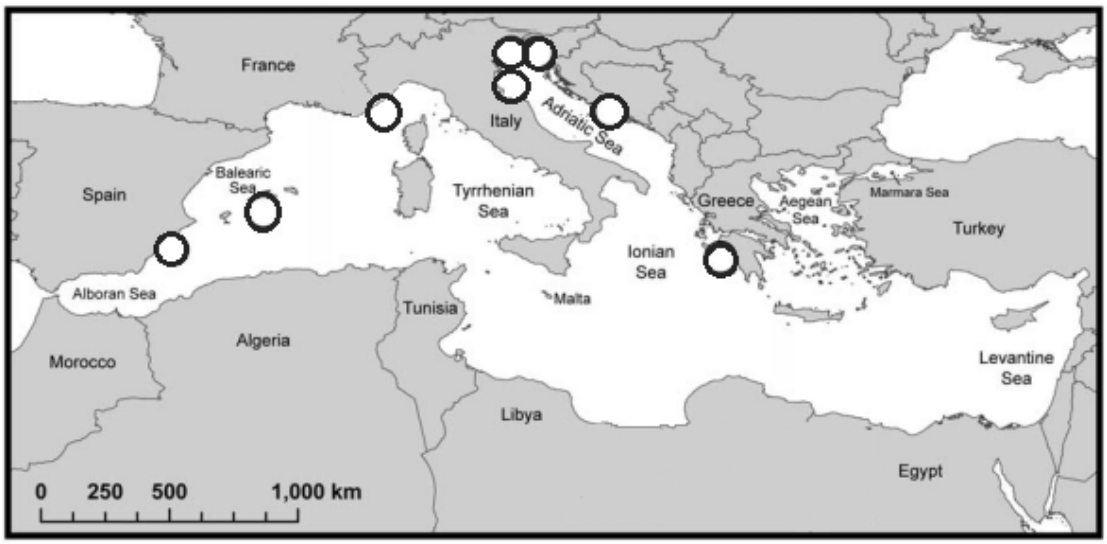


Figure 2.1. Maps of some blooming areas in Mediterranean Sea (modified from Brotz and Pauly 2012).

Although jellyfish often are associated with negative effects, there are very few scientific studies of *Aurelia* spp. impacts on the trophic web in the Mediterranean Sea. The studies have focused on enclosed environments, such as the marine lakes of Mljet island (D'Ambra et al., 2013; Malej et al., 2007). Coastal environments like bays, harbours, and lagoons are suitable environments for *Aurelia* sp. invasion. Polyps can attach on hard substrates that are easy available in these areas, as demonstrated for polyps detected in northern Adriatic harbors (Ramšak et al., 2012). Different materials like live mussels, polychaete and amphipod tubes, barnacles, rocks and stones, polystyrene, provide suitable surfaces for scyphopolyp settlement (Holst and Jarms, 2006; Miyake et al., 1997; Purcell et al., 2009).

Cnidarian polyps are very resistant to conditions that vary from hypoxia (Condon et al., 2001; Ishii and Katsukoshi, 2010) to variation in temperature, salinity and pH (Willcox et al., 2007; Liu et al., 2008; Prieto et al., 2010; Winans and Purcell, 2010; Purcell et al., 2012). Being able to adapt to different environmental situations, they are comparable to an extra-long resting stage that can survive without producing medusae for long time (Boero et al., 2008).

The timing of jellyfish appearance depends on triggers of strobilation and environmental stimuli (Spangenberg, 1968; Kroiher et al., 2000; Holst, 2012). Nevertheless, the presence of several cryptic species of *Aurelia* suggest that distribution and time of appearance can vary within species relative to the geographic area (Dawson and Jacobs, 2001) explaining different bioclimatic envelopes needed for asexual reproduction and subsequent jellyfish growth in *Aurelia* spp.

Thus, the knowledge acquired about the biology and ecology of the various species can clarify the role played in the food chain by each life stage and consequently can allow an evaluation of positive and negative effects in an jellyfish dominated ecosystem.

The objectives of this study were to provide the first description of *Aurelia* sp. life cycle in Varano lagoon, analyzing the population dynamics in the 2011 - 2013 period. Special emphasis was given to the role of environmental factors in the pattern of abundance and distribution of planktonic organisms. The potential impacts of *Aurelia* sp. outbreaks were then investigated by analyzing the variations in spatial and temporal dynamic of the plankton community correlated with jellyfish abundance.

2.3.Methods

2.3.1. Study area

Varano lagoon (Fig. 2.2) is located on the southern Adriatic coast (Apulia region, Italy) on the north side of the Gargano promontory (41.88°N; 15.75°E). The lagoon is trapezoidal and the major base is partially isolated from the southern Adriatic by a coastal barrier facing north called "isola" or "tombolo", 1 km wide, 10 km long. The lagoon has a 33 km perimeter and covers an area of 60.5 km^2. The bottom of the lake is characterized by a series of overlapping layers. The deepest one is karstic in nature, and is placed underneath a stratum of sand, which in turn is covered by a discontinuous muddy layer alternating with patches of sandstone along the shore. The depth is variable, generally increasing towards the center of basin where it reaches about 5.5 m. Two artificial channels (foce Varano and foce Capojale), located on the west and on the east side, respectively, provide water exchange with the open sea. The tidal excursion is on the order of ~30 cm (Caroppo, 2000), insufficient to remove the sands accumulated in the inlets. Tidal levels, freshwater inputs, wind strength and direction and anthropogenic activities affect hydrodynamics (Specchiulli et al., 2008b). The most frequent winds come from the north and northwest, and allow the water exchanges between lagoon and open sea that are limited to autumn and winter (Spagnoli et al., 2002). The residence time of water is approximately 1.5 years (Specchiulli et al., 2008a).

The lagoon shows a high variability of physical characteristics like salinity and temperature (Belmonte et al., 2011; Caroppo, 2000). Freshwater inputs come from natural springs located on the western (Casa Coccia, S. Nicola, Ospedale) and eastern sides (Bagno, Irchio and Muschiaturo) (Caroppo, 2000). There, urban wastewater and drainage water discharge into the lagoon through two effluents (Antonino and S. Francesco) (Specchiulli et al., 2008b).

The lake and the surrounding coastal area are exploited by mussel farming from 1960, but around 1990 the activity was gradually reduced within the lake and transferred to the nearby Adriatic Sea. The fishery is now the most important resource in the lagoon where mullets, eel, sea bass, sea bream, sand smelt and sole, are the most common fish species.

2.3.2. Sampling

A sampling campaign was launched in spring-summer 2011 in four sectors (NW, SW, SE, NE) of the Varano lagoon, each characterized by different environmental features, along the main

sea-lagoon and W-E axes (Fig. 2.2). Sampling was carried out approximately every 45 days and in daylight in order to reduce variations of chemical-physical parameters.

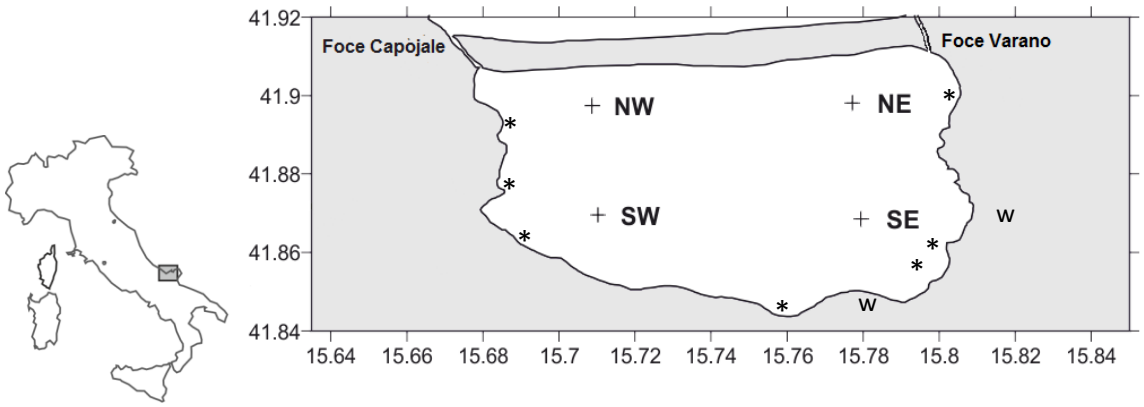


Figure 2.2. Map of the coastal lagoon of Varano Varano coastal lagoon and sampling sectors. * indicate natural springs, w indicate drainage water discharges.

In each sector, the **geographical coordinates** of the sampling stations were acquired with a GPS device, while profiles of temperature, salinity, depth, and pH were acquired using a CTD probe (HYDROMAR) deployed from the boat with the boat motor turned off.

Jellyfish were sampled at each station in 3 replicate horizontal surface tows using a 1 m diameter, 1 cm mesh plankton net equipped with a flow meter for calculation of the volume filtered. Jellyfish were enumerated and measured to nearest centimeter for determination of population size structure and abundance.

Mesozooplankton was collected by horizontal tows of a 200 µm mesh plankton net (40 cm diameter) to estimate the abundance. Triplicate transects were carried out in each sector. In April 2012, an extra net of 500 µm was added to properly sample **ephyrae, juvenile medusae** and **fish larvae** in the four sectors. Samples were fixed in situ with ethanol 100% (200 µm) or formalin (500 µm) with a final concentration of 4%.

Microzooplankton was collected by vertical tows of a 50 µm mesh plankton net (25 cm diameter) equipped with a flow meter. Samples were fixed *in situ* in ethanol and analyzed in the laboratory as soon as possible. Because microzooplankton includes organisms smaller than 50 µm (20-200 µm), from April 2012, samples also were collected in 2 L of water taken at 50 cm

depth. These samples were then filtered through 10 µm mesh and fixed in formalin with a final concentration of 2.5%. Samples from both methods were compared.

Phytoplankton and **nutrient** samples were collected using a 5-L Niskin bottle at 50 cm depth. In each sector, two replicate samples of 0.5 L were collected in dark bottles, preserved in Lugol's solution and kept in the dark for later identification.
In parallel, two replicate water samples of 1 L were collected in plastic containers and kept in the dark at -4°C until arrival at the laboratory. Nutrient samples were then partially (0.5 L) filtered through a GF/F filter and stored at –20°C. Nutrient and phytoplankton analyses were performed at the IAMC – CNR (Instituto Ambiente Marino Costiero) in Taranto.

2.3.3. Laboratory analyses

Mesozooplankton

The samples were analyzed in the laboratory for taxonomic identification (Leica MZ 12 stereoscope) that was carried out at the order level.
The filtered volume (m^3) was calculated with the following formula:

$$V = N * c * A$$

where

N = number of flow meter revolutions
c = calibration factor of flow meter
A = net mouth area (m^2)

Samples were filtered through a mesh with a smaller pore size than the net mesh size used during sampling. The sample was resuspended in filtered sea water. The sample was then poured into a beaker and mixed to homogenize before taking an aliquot. Sub-sampling by the Stempel pipette was carried out and analysis was performed in a Bogorov counting chamber . The count was repeated for additional sub-samples until 100 specimens of the most abundant taxonomic groups were reached in a sample. For samples collected by 500 µm mesh net, the entire sample was analyzed.
The abundance of each taxonomic group (ind. m^{-3}) was calculated trough the formula:

$$\text{ind. } m^{-3} = (n*k) / v$$

n= number of individuals
k= fraction of sample (volume of sample diluted/volume of samples analyzes)
v= filtered volume

Microzooplankton

Counting and species identification were performed in a glass cell of dimensions 7 x 4.5 x 0.5 cm, using an Olympus inverted microscope at magnifications of 100x and 400x. One-sixteenth of each sample was analyzed for common species, and the entire sample was analyzed for rare species. Abundance was expressed as ind/m^3 or ind L^{-1}.

Phytoplankton

Micro- and nanophytoplankton (MPP and NPP) samples were examined with an inverted microscope (Labovert FS Leitz) at a magnification of 400x. Subsamples ranging from 0.025 to 0.05 L, depending on the phytoplankton concentration, were allowed to settle for 12–24 h (Utermöhl, 1958) and identified and counted. Identification was to general group: diatoms, coccolithophorids and dinoflagellates. Phytoflagellates were included in the group “other" that contains cells that could not be identified in the previous groups.

Nutrients

Nutrients were assayed by an automated multiparameter autoanalyzer (Micromac-Lab 1000, Systea srl) based on technology called LFA (Loop Flow Analysis). Nutrient analyses for ammonia nitrogen ($N\text{-}NH_3$); nitrous nitrogen ($N\text{-}NO_2^-$); nitric nitrogen ($N\text{-}NO_3$); total nitrogen(N_{TOT}), phosphate ($P\text{-}PO_4^{3-}$); total phosphorous (P_{TOT}) and silicates ($Si\text{-}SiO_3^{2-}$) were performed according to Strickland and Parsons (1972).

2.3.4. Statistical analysis

Spatio - temporal distribution

The physical (temperature, salinity and pH) and biological data (abundance of jellyfish, ctenophores, fish eggs and larvae, meso- , micro- , and phytoplankton) were compared among the surveyed sectors (NW, SW, SE, NE) for each sampling. To assess the spatio – temporal variation, a resemblance matrix based on Euclidean (environmental data) or Bray – Curtis similarity (biological data) was calculated. Data were then analyzed using the non – parametric permutational multivariate analysis of variance (PERMANOVA) statistical test with sector and month as fixed factors in the PERMANOVA design. Statistical analyses were performed with PRIMER 6+ software (Plymouth Marine Laboratory) using a significance level of 0.05. PERMANOVA calculates a pseudo-F statistic that is directly analogous to the traditional F-

statistic for multifactorial univariate ANOVA models, but uses permutation procedures (here 4999 permutations) to obtain p-values for each term in the model (Anderson et al., 2008).

In order to illustrate the jellyfish spatio – temporal distribution, abundances of *Aurelia* sp. were visualized by plotting relative abundance in space on a map of the survey sites using the program SURFER 9. A post map of abundance was overlaid on a blanking file with mean species abundances geo-referenced to survey site locations.

Correlation with environmental factors

To test for linear relationships between variation in jellyfish abundance and environmental factors, correlation analysis using Spearman's correlation coefficients was conducted for sampling months. Similarly, effects of the environmental factors on ctenophores, ichthyoplankton, mesozooplankton, microzooplankton and phytoplankton abundances were examined by correlation analysis. Temperature and salinity in each sector were used as explanatory variables and abundance of the target group as dependent variable. The linear regression and Spearman's rank order correlation were performed with SPSS software.

In addition, to determine which of the environmental parameters (temperature, salinity, or nutrients) could best explain the pattern observed in the plankton assemblages each year, the BIO-ENV procedure in PRIMER was used (Clarke and Warwick 2001). All the data obtained from the different samplings were merged in a single table with the abundance of each taxonomic group. The environmental similarity matrices were constructed using normalized Euclidean distance whilst Bray-Curtis Index was used to construct the similarity matrix for biological data. To find the most parsimonious model explaining the variation in zooplankton data, the DISTLM analysis was carrying out with ''All specified" as selection procedure and "R^2" as selection criterion. A dbRDA graph was built from the model.

Population patterns

The Spearman's rank-order correlation coefficient was used to compare spatial abundances of mesozooplankton to spatial densities of microzooplankton and phytoplankton and that of microzooplankton to spatial densities of phytoplankton by month.

Similarly, the Spearman's rank-order correlation coefficients were tested for jellyfish – zooplankton (both meso- and microzooplankton) and ctenophore - zooplankton (both meso- and microzooplankton) spatial interactions.

2.4. Results

2.4.1. Hydrographic conditions

The lagoon showed a high variability of the physical characteristics throughout the year and the same seasonal dynamics across years sampled. The temperature and the salinity varied in the four sectors of the lagoon following the distance of the seawater input from the Adriatic Sea through the channels Capoiale and Varano and the fresh water input from the natural springs. The mean temperature oscillated between 6°C in the winter (December, February) and 31°C in summer (July) (Fig. 2.3). Surface water temperature was generally higher in summer, whereas surface – bottom difference was variable between 0.7 and 1.2°C, varying with the sector, and consequently did not create a real thermocline in the lagoon. Any significant distinction between surface and bottom was observed in the other seasons. The temperatures were similar among sectors within the same months. In spring, the CTD profiles showed that temperature decreased through the water column but finally increased in the bottom layer. In summer, temperature decreased with depth and in the autumn-winter it homogenized through the water column (Fig. 2.4).

By contrast, salinity values were more variable both in surface and bottom layers. Surface water salinity oscillated between 20 and 29 ppt meanwhile bottom values never was less than 23 ppt. The higher values were recorded in the bottom layers and the mean surface-bottom difference was particularly large in December 2011 (3.7 ppt difference) and April 2012 (4.5 ppt difference). Mean salinity values progressively increased across years (Fig. 2.3). In particular, in the last year of sampling, the earlier seasonality with high salinity oscillations was replaced by slight variations between layers and months.

The salinity values were comparable between sectors and remained stable or slightly increased through the water column depending on the season. By contrast, in the NW sectors, the increase of salinity was permanent and created a halocline around 3 m deep (Fig. 2.4). Due to its proximity to the Adriatic Sea, the high-salinity water entered the lagoon trough the Varano Channel and stratified in the bottom layer, giving the NW sector the predominant characteristics of a marine area. Unlike the NW sector, exchange with the open sea in the NE sector was

reduced because of shallowing of the Capojale Channel. Nevertheless, when north winds blew, a stronger seawater input increased salinity in all sectors; maxima of 11 and 6 ppt in the surface – bottom difference were observed in the west and east area, respectively (April 2012).

Monthly average pH values varied from 7.1 to 8.5. Generally pH values were approximately 8 or higher throughout the year, with lower values were observed in spring (May 2012, April 2013).

As conclusion, PERMANOVA analysis showed that the sectors analyzed did not explain the differences in hydrographic values ($p = 0.2265$). The effect of sampling month within the year (Mo(Ye)) was instead significant (Table 2.1; $p < 0.01$ **).

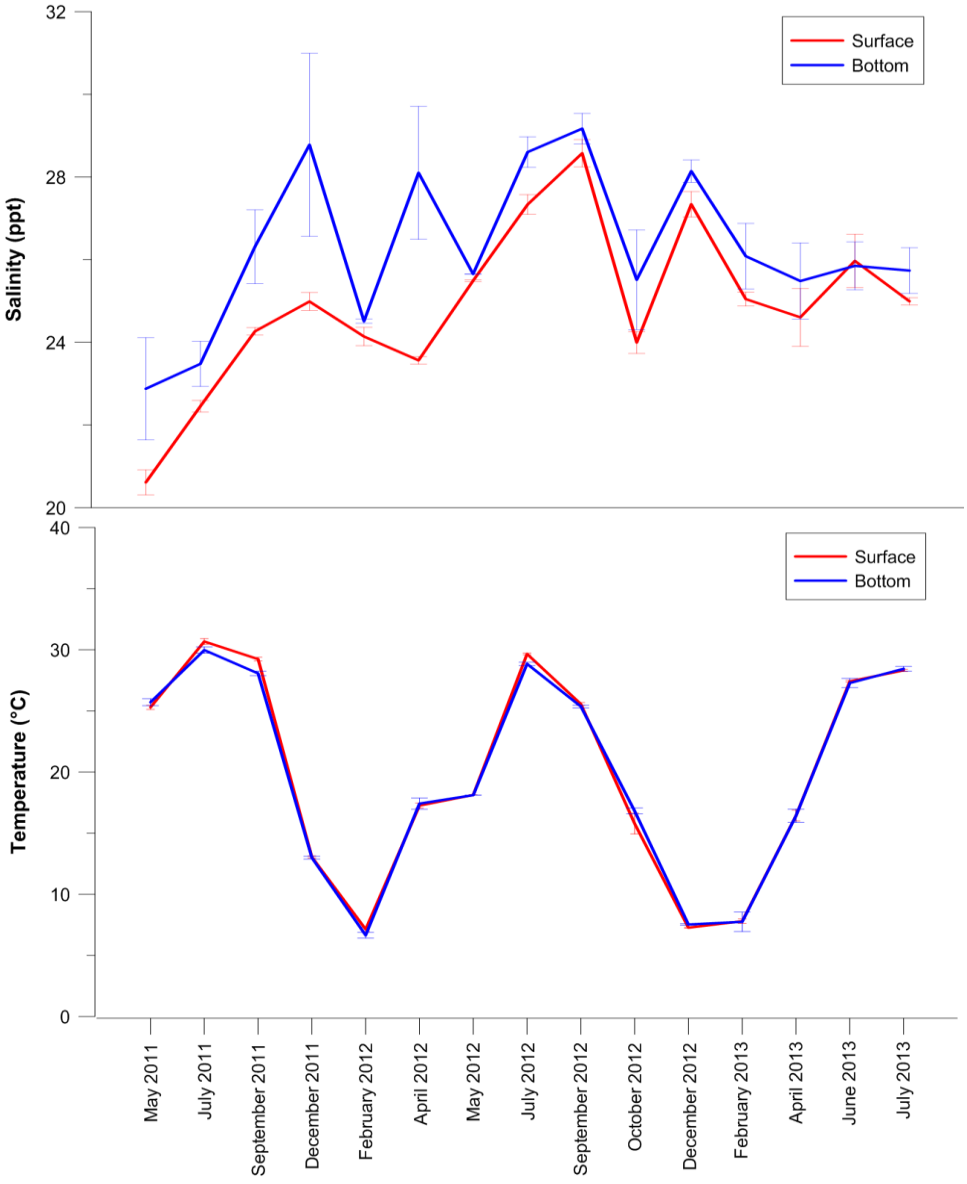
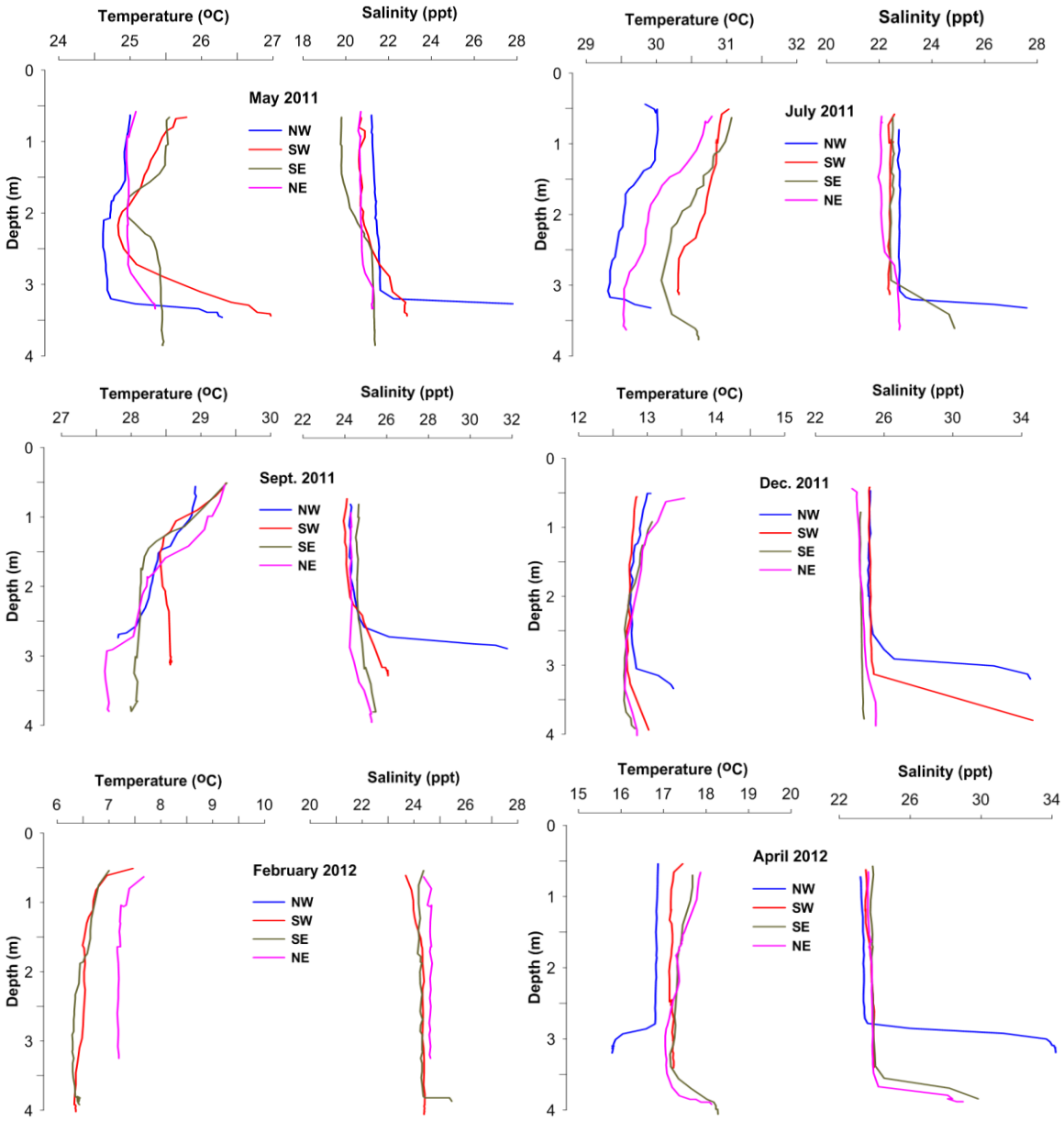


Figure 2.3. Seasonal changes in surface and bottom temperature and salinity in the coastal lagoon of Varano.

Table 2.1. Results of PERMANOVA comparison of hydrographic conditions in the coastal lagoon of Varano among sectors, months and years. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0.05$) are indicated in bold. Ye = Year; Mo(Ye) = month nested in year; Mo(Ye)xSe = interaction of Mo(Ye) and sector; Res = Residuals; Se = Sector; SexYe = interaction between sector and year.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Se	3	3.631	1.210	17.661	0.227	4729	0.172
Ye	2	11.994	5.996	87.517	**0.014**	4052	0.016
Mo(Ye)	12	69.619	5.801	84.667	**0.009**	4810	0.014
SexYe	6	3.528	0.588	8.5801	0.217	4747	0.240
Mo(Ye)xSe	31	20.68	0.667	9.7355	0.387	4996	0.251
Res	1	0.0685	0.0685				
Total	55	110					



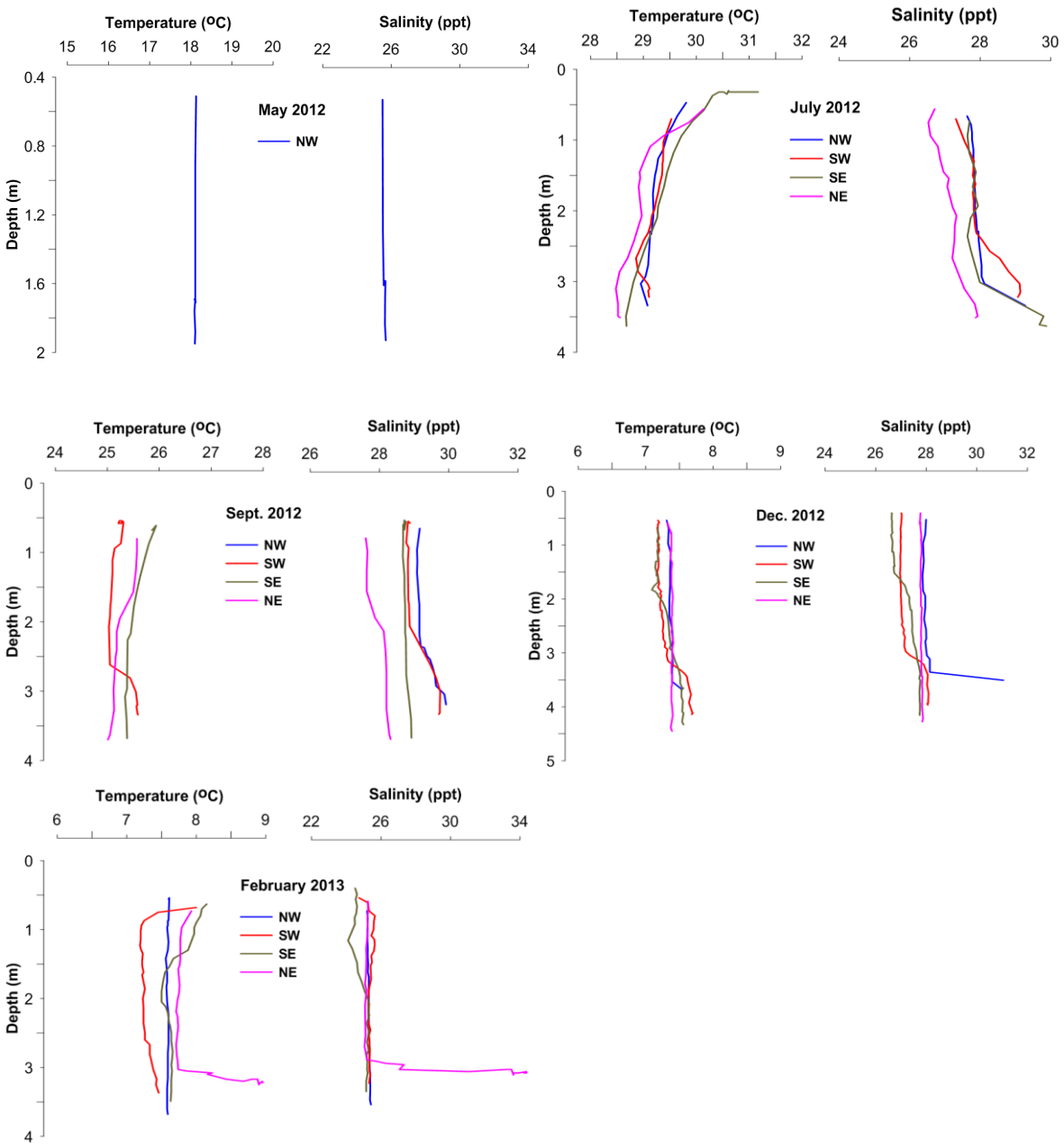


Figure 2.4. Vertical CTD profiles of temperature (in degrees Celsius) and salinity (in parts per thousand) in the four sectors (NW, SW, SE, NE) of the coastal lagoon of Varano in the period May 2011 – February 2013.

2.4.2. Population dynamics of *Aurelia* sp. 1

Aurelia sp. 1 medusae were sighted regularly in the lagoon throughout the year. From May to July 2011 the population was characterized by small medusae that grew and became sexually mature in September, when adult abundance was high (9 ind/10 m^3). After the release of gametes, most of jellyfish died, and those remaining contributed to the jellyfish population that survived cold months but slowly disappeared until April 2012 (0.2 ind/10 m^3).

Plankton data showed a small peak of ephyra production at temperature decrease in December 2011 but the maximum abundance was observed in April 2012 (131 ind/10 m^3) when the previous annual population disappeared (Fig. 2.5). A first mortality event was recorded in July 2011 when temperatures reached 30°C (Fig. 2.3), removing larger individual from the jellyfish population. In May 2012, jellyfish reached very high densities (45 ind/10 m^3 in May 2012) and in July 2012, only few individuals were found in the lagoon (Fig. 2.6). The following months were characterized by the disappearance of *Aurelia* sp. 1 medusae that extended from summer 2012 to spring 2013. In April 2013, *Aurelia* sp. 1 ephyrae again were detected in the lagoon, but with lower abundance than the previous year (5 ind/10 m^3). During summer the population grew in abundance without any apparent negative effects of temperature (28°C) as in the previous July, and medusa abundance did not change until the end of monitoring (September 2013).

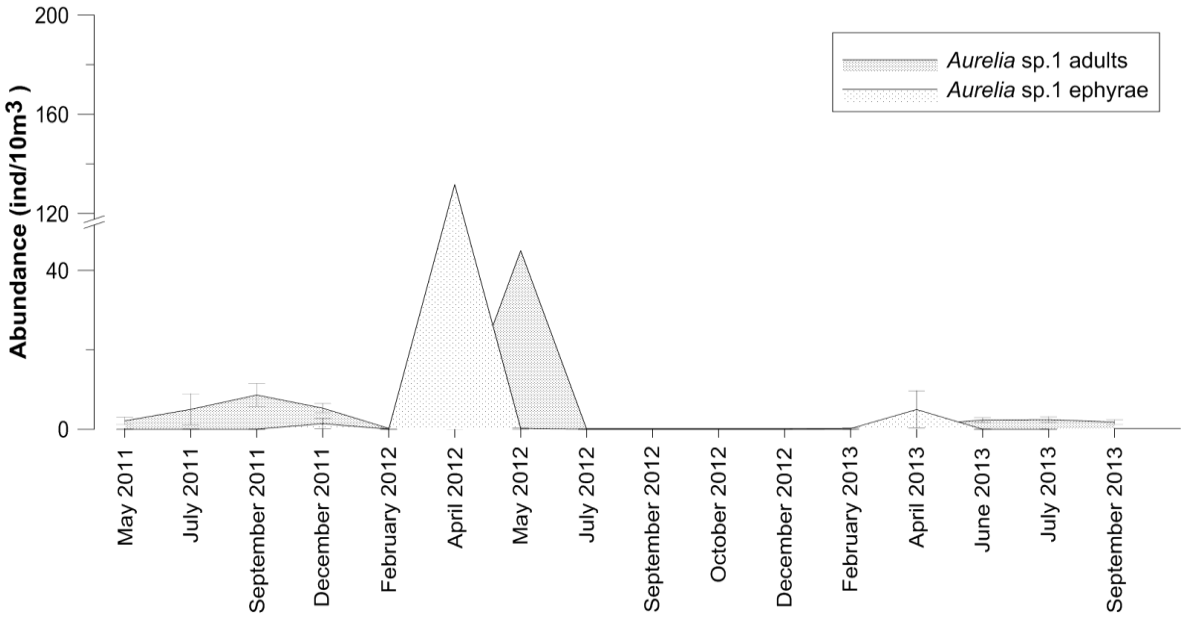


Figure 2.5. Population dynamics of *Aurelia* sp.1 ephyrae and adults in in the coastal lagoon of Varano.

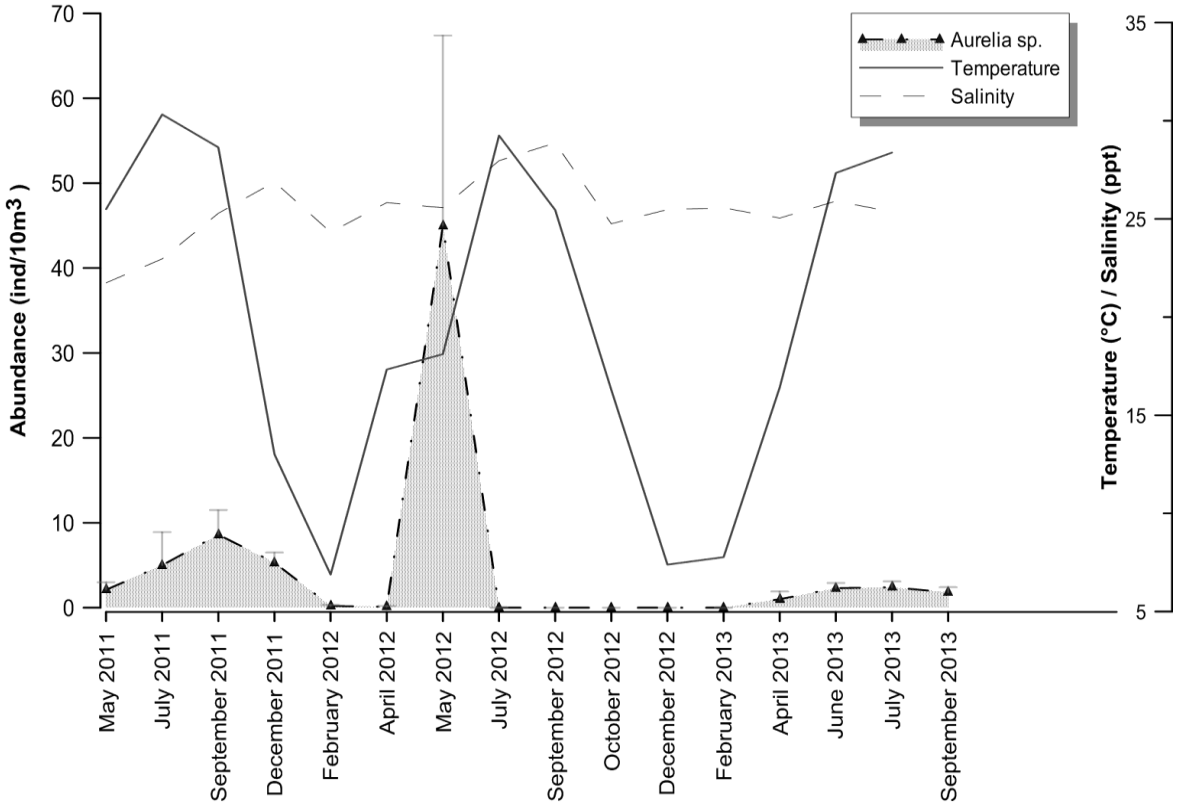


Figure 2.6. Population dynamics of *Aurelia* sp.1 and temperature/salinity trend in in the coastal lagoon of Varano.

Spearman's rank order correlation analyses of the interannual variation of monthly mean jellyfish abundances from May 2011 to July 2013 were never significantly correlated with monthly means of hydrographic parameters. A weak but not significant correlation was found for temperature ($r_s = 0.37$, $p = 0.173$) and for salinity ($r_s = - 0.182$, $p = 0.517$).

The occurrence of ephyrae in spring in the Varano lagoon is explained by the strobilation of polyps primarily caused by the achievement of a temperature optimum or more precisely by the drastic seasonal changes in hydrographic parameters.

Ephyrae are unable to swim for long distances and their size and colour is related to the time they have spent in the water column. During the sampling campaign smallest (youngest) ephyrae were found exclusively in the SW sector and metaephyrae (1 cm) were found in all the sectors, but again with the higher abundance in the same sector, leading to the hypothesis that polyps lived on hard substrates located in this area.

As consequence of strobilation, the presence of the smallest size class of medusa population (1-5 cm) was observed only in May - June (Fig. 2.8). The size frequency distributions showed the progressive shift in size classes through the summer, with an anomaly recorded in July 2011 when, as reported above, massive mortality eliminated the biggest size classes. Medusae grew during the summer and reached sexual maturity at the end of summer. Mature medusae carrying planulae were found from September 2011 to December 2011, when the bell diameter was l0 – 15 cm. The biometric relationship illustrated wet weight as a function of bell diameter (Fig. 2.7). In larger individuals, somatic growth was accompanied by the maturation of animals (gonad development) that explained higher weights observed in medusae from the autumn.

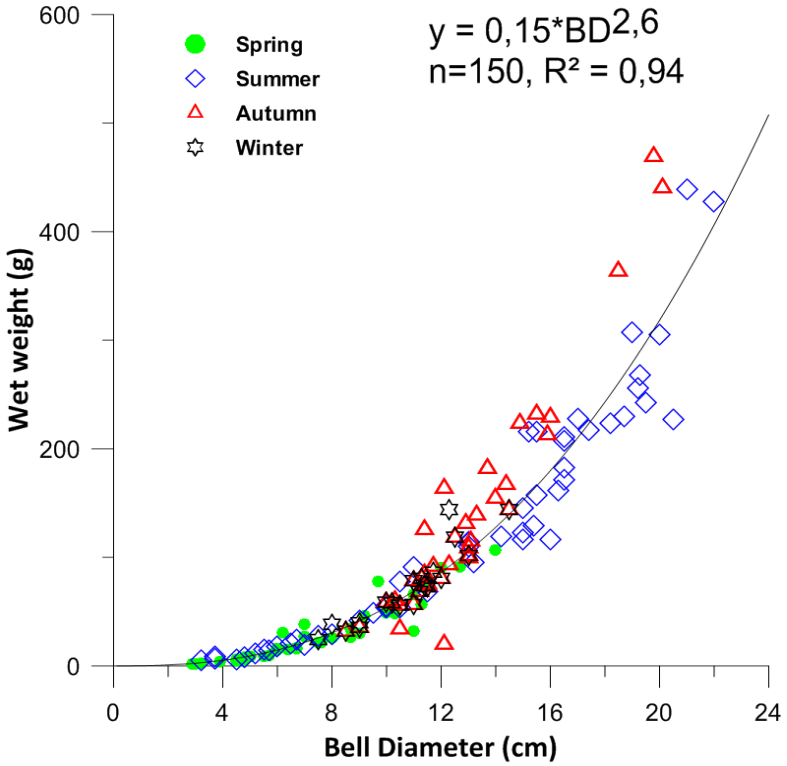


Figure 2.7. Relationship between bell diameter and wet weight of *Aurelia* sp. 1 medusae in the coastal lagoon of Varano.

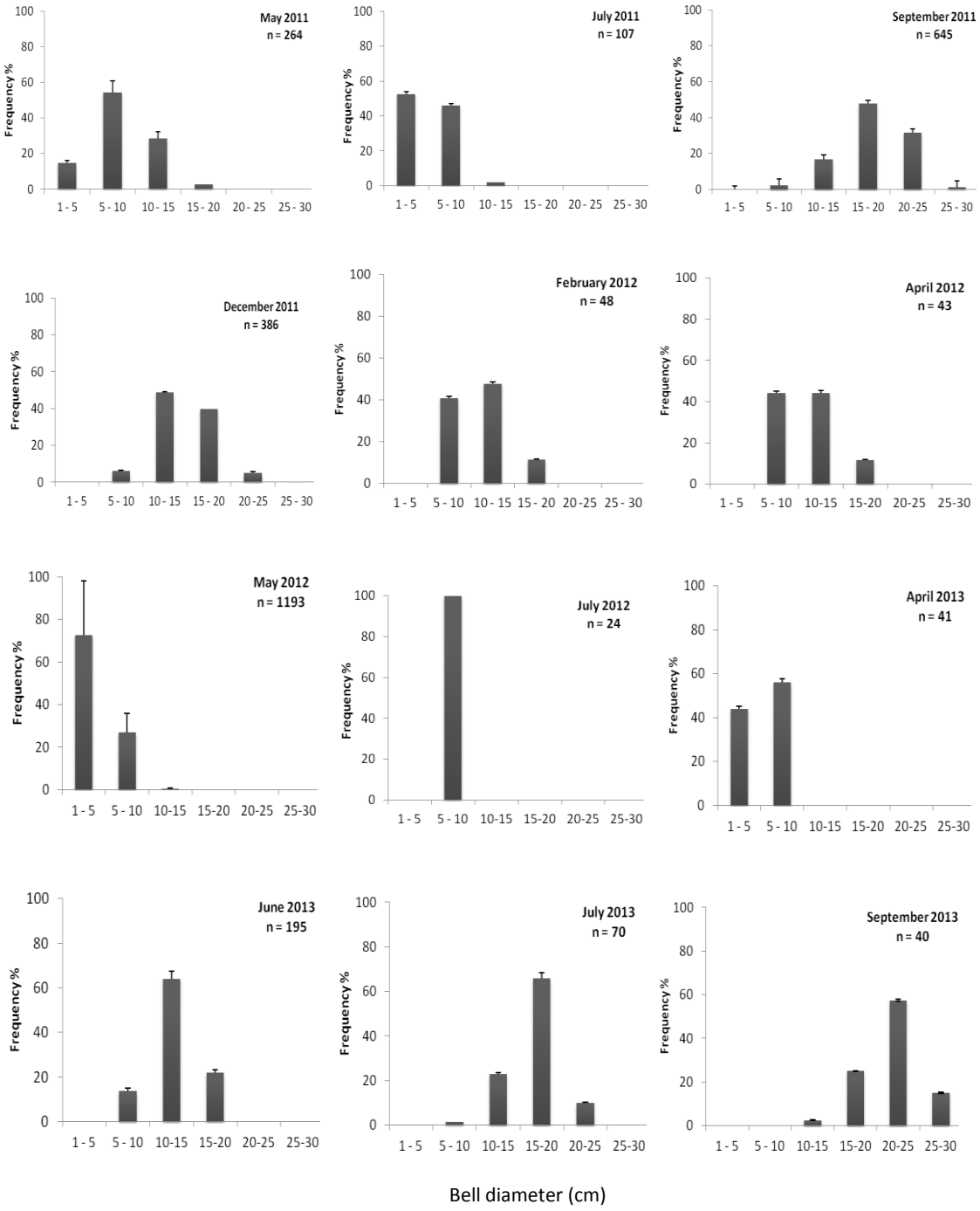


Figure 2.8. Size – class distribution of *Aurelia* sp. 1 medusae in the coastal lagoon of Varano.

The larger medusae that occurred from the winter 2011 to the spring 2012 were over wintered medusae, based on the frequency distributions of bell diameter and data on ephyra dynamics coupled with in situ observations. These results suggested that the life span of *Aurelia* sp. 1 medusae in Varano lagoon is one year.

Spatial distributions of *Aurelia* sp.1 in the four sectors of Varano lagoon varied continuously (Fig. 2.9) throughout sampling months. *Aurelia* sp.1 showed a patchy distribution and its spatial abundance was significantly related ($p < 0.05$*) to month, but not to the sector or to month x sector interaction (Table 2.2).

Table 2.2. Results of PERMANOVA comparison of *Aurelia* sp.1 medusa abundance in the coastal lagoon of Varano among sectors and months. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Mo = months; MoxSe = interaction between month and sector; Res = Residuals; Se = Sector.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Mo	7	50275	7182.1	2.1916	**0.0362**	4987	0.0404
Se	3	2616.7	872.22	0.2663	0.9366	4983	0.9302
MoxSe	21	16878	803.7	0.2452	1	4978	1
Res	32	0.000	3277.2				
Total	63	0.000					

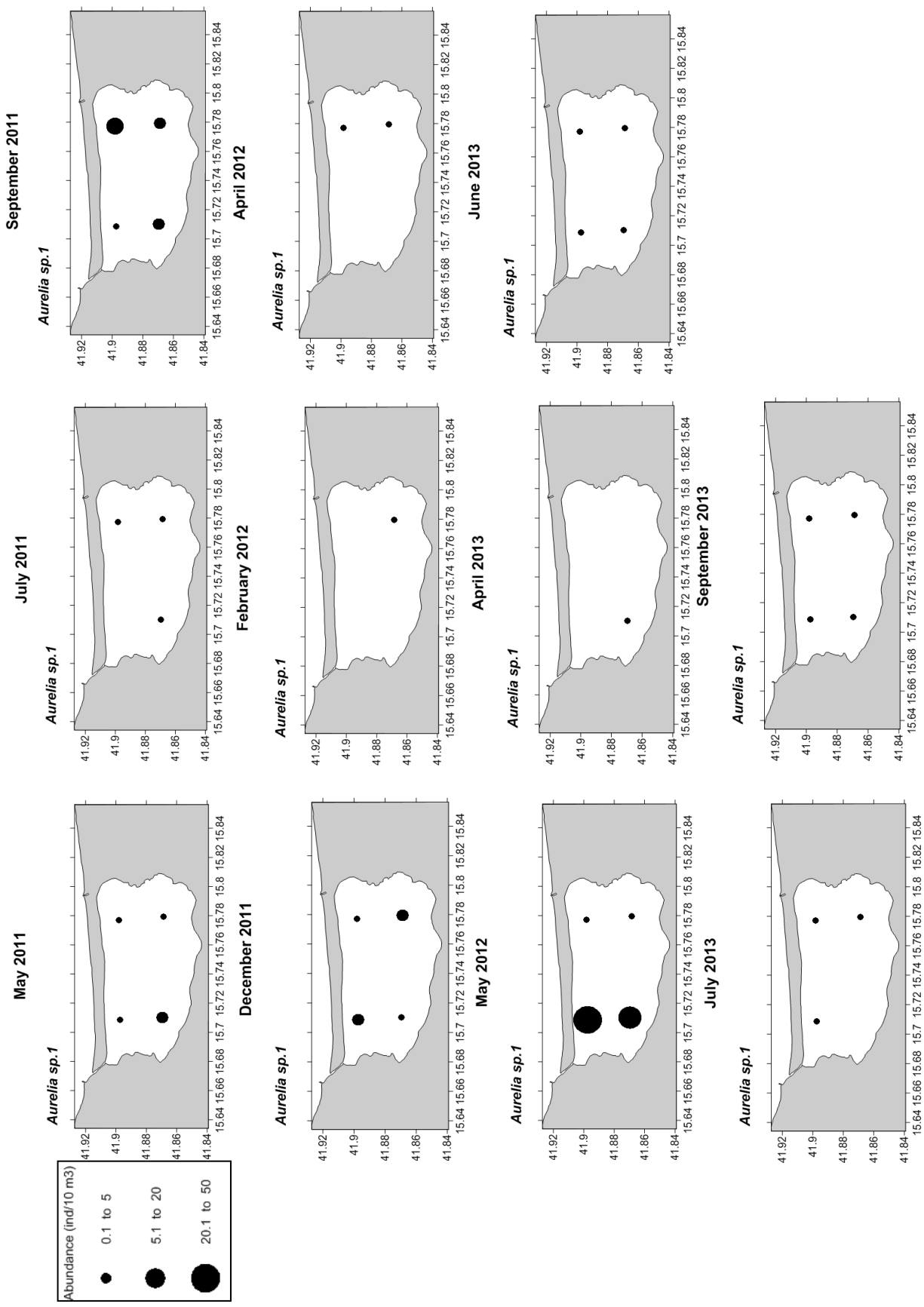


Figure 2.9. Spatio - temporal distribution of *Aurelia* sp.1 in the coastal lagoon of Varano (2011 – 2013).

2.4.3. Macrozooplankton spatio – temporal dynamics

***Pleurobrachia* sp.**

During the sampling campaign started in 2011, other species of gelatinous zooplankton were never recorded in the Varano lagoon, except for the jellyfish *Rhizostoma pulmo*, during the summer season occasionally invaded large areas of the lagoon.

The first individuals of the ctenophore *Pleurobrachia* sp. were found in December 2012 in the western sectors and represented the 1 – 5 mm size class. The peak of abundance was reached in February 2013 with 1.5 ind/10 m^3 when the ctenophores distributed in three of four sampling sectors (Fig. 2.10).

During winter months, the size distribution shifted toward the 5 -10 mm size class that also represented the end point of growth. In spring *Pleurobrachia* sp. was only found in the NW sector and in June the ctenophore had disappeared from the lagoon and was never found until the end of sampling campaign.

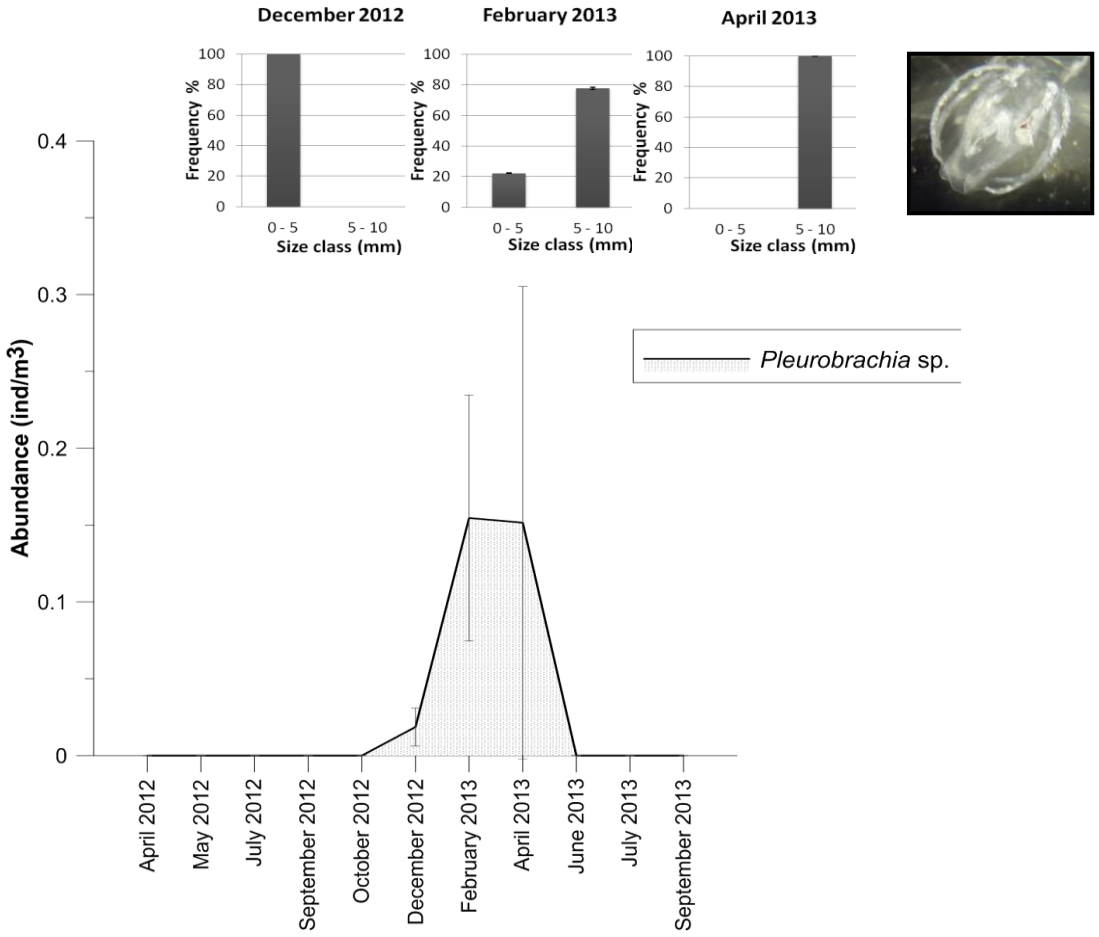


Figure 2.10. Population dynamics and size structure of *Pleurobrachia* sp. in in the coastal lagoon of Varano in the period April 2012 – September 2013. Data refer to plankton sampling with the 500 μm mesh net.

In the period December 2012 - June 2013, the abundances of the ctenophore *Pleurobrachia* sp. were significantly related to temperature among months (r_s = -0.69, p = 0.026*), unlike no effect of salinity (r_s = -0.39, p > 0.05).

In all the sampling, ctenophores were never observed in the SE sector and PERMANOVA analysis demonstrated that the spatial abundance of *Pleurobrachia* sp. was significantly affected by the month (p < 0.01 **) and by the sector (p < 0.05 *) but not from their interaction (Table 2.3).

Table 2.3. Results of PERMANOVA comparison of *Pleurobrachia sp*. abundance in in the coastal lagoon of Varano between sectors and months in the period December 2012 - June 2013. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Mo = months; MoxSe = interaction between month and sector; Res = Residuals; Se = Sector.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Mo	7	23775	3396.5	8.194	**0.005**	4989	0.000
Se	3	5775.5	1925.2	4.644	**0.029**	4988	0.011
MoxSe	21	18533	882.53	2.129	0.117	4989	0.049
Res	12	4974.2	414.52				
Total	43	51658					

Fish eggs and larvae

The highest abundances of fish eggs were observed in summer. The mean fish egg abundances were 71 ind/m^3 (± 1.67) in July 2011 and 42 ind/m^3 (± 13) in July 2012 (Fig. 2.11). Fish eggs were produced during spring (April 2012), but not in autumn and winter. The result of correlation analysis between abundance of fish eggs and physical factors showed that they were significantly correlated with temperature (r_s = 0.75, p = 0.007 **), but not with salinity (r_s = 0.27, p > 0.05).

PERMANOVA analysis demonstrated that the spatial abundance of fish eggs was significantly related to the month (p = 0.0002 ***), but not to the sector or their interaction (Table 2.4).

Table 2.4. Results of PERMANOVA comparison of fish eggs abundance in the coastal lagoon of Varano between sectors and months in the period May 2011 - February 2013. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Mo = months; MoxSe = interaction between month and sector; Res = Residuals; Se = Sector.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Mo	6	84656	14109	12.479	**0.0002**	4990	0.0002
Se	3	499.06	166.35	0.147	0.993	4984	0.9932
MoxSe	18	6099.9	388.88	0.299	1	4976	1
Res	20	22614	1130.7				
Total	47	1.1419					

Similar patterns of seasonal abundances were observed for fish larvae, but were shifted in time in respect to the abundances of fish eggs. The highest mean fish larvae abundances were recorded in April 2012 (0.33 ± 0.17) and September 2012 (0.07 ± 0.02). In 2013, abundances were very low when compared to the previous years. Unfortunately, because the 500 μm mesh net was added to the sampling in April 2012, data from 2011 were not available to compare the two years.

The abundances of fish larvae were not significantly correlated with temperature (r_s = 0.62, p > 0.05) or salinity (r_s = 0.56, p > 0.05). Therefore, the month and the sector did not explain the differences in the spatial abundance of fish larvae (Table 2.5; p > 0.05).

Table 2.5. Results of PERMANOVA comparison of fish larvae abundance in the coastal lagoon of Varano between sectors and months in the period April 2012 - June 2013. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Mo = months; MoxSe = interaction between month and sector; Res = Residuals; Se = Sector.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Mo	7	27526	3932.3	1.861	0.252	4988	0.245
Se	3	7025.1	2341.7	1.108	0.462	4984	0.432
MoxSe	21	45665	2174.5	1.029	0.549	4989	0.549
Res	4	8449.9	2112.5				
Total	35	88682					

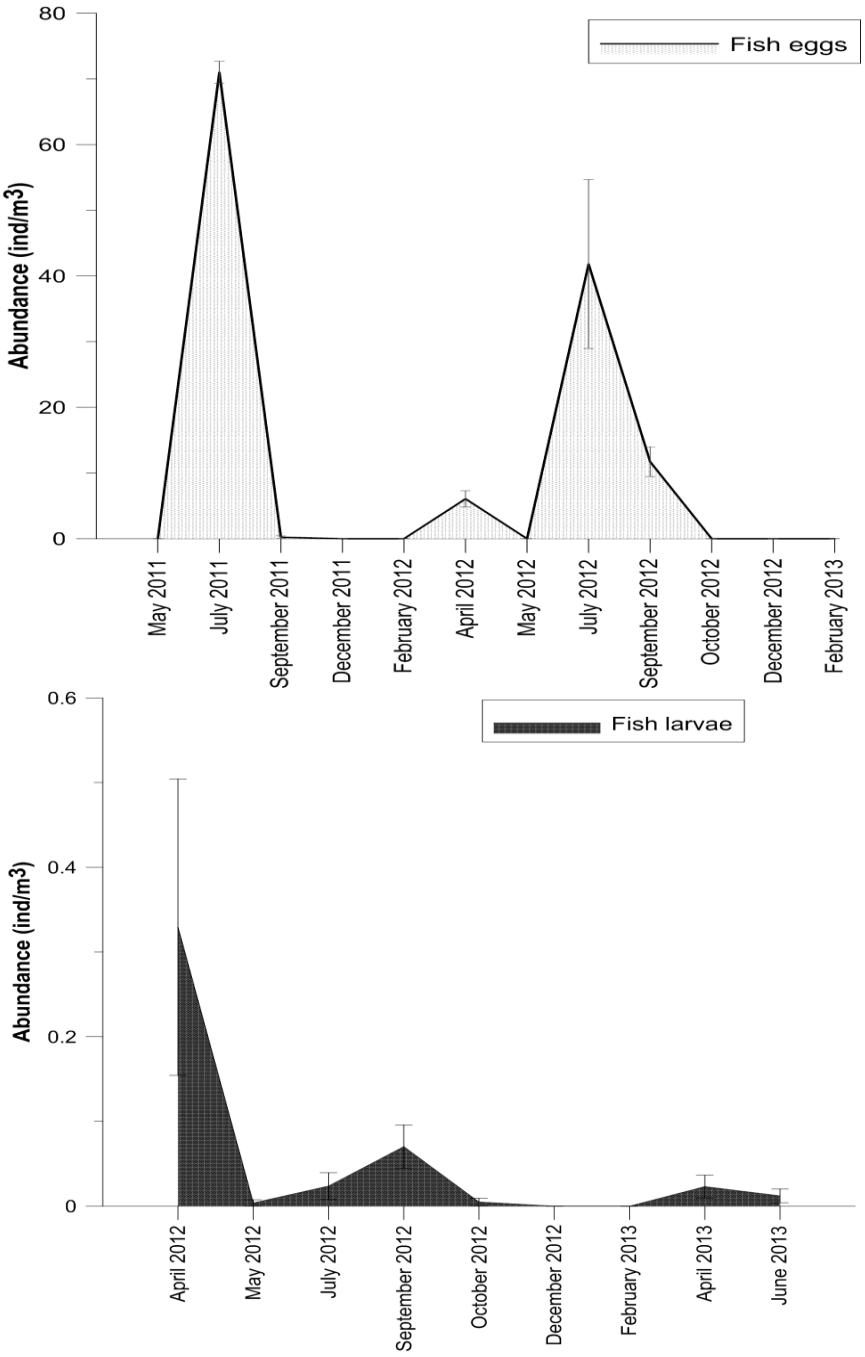


Figure 2.11. Population dynamics of fish eggs (a) and fish larvae (b) in in the coastal lagoon of Varano. Eggs and larvae were sampled with 200 μm and 500 μm mesh net, respectively.

2.4.4. Mesozooplankton spatio – temporal dynamics

During 2011-2013, mesozooplankton abundances were high in Varano Lagoon, ranging from means of 412-28,730 individuals (ind.)/m^3. The two minima were recorded in September 2012 (412 ± 94 ind/m^3) and May 2012 (452 ± 133 ind/m^3), while the peak of abundance was reached

in February 2013 (28,730 ± 9700 ind/m^3). In particular, the lowest abundance was recorded in the NW sector with 5 ind/m^3 in May 2012 and the highest in the NE sector with 70,311 ind/m^3 in February 2013 (Fig. 2.12). Of the 12 samples analyzed per month, the SE sector had the lowest mean abundances for 42% of samples. Commonly, the abundances in the other sectors were highly variable, and PERMANOVA analysis demonstrated that the spatial abundance of mesozooplankton did not change significantly with month or sector ($p > 0.05$, Table not shown).

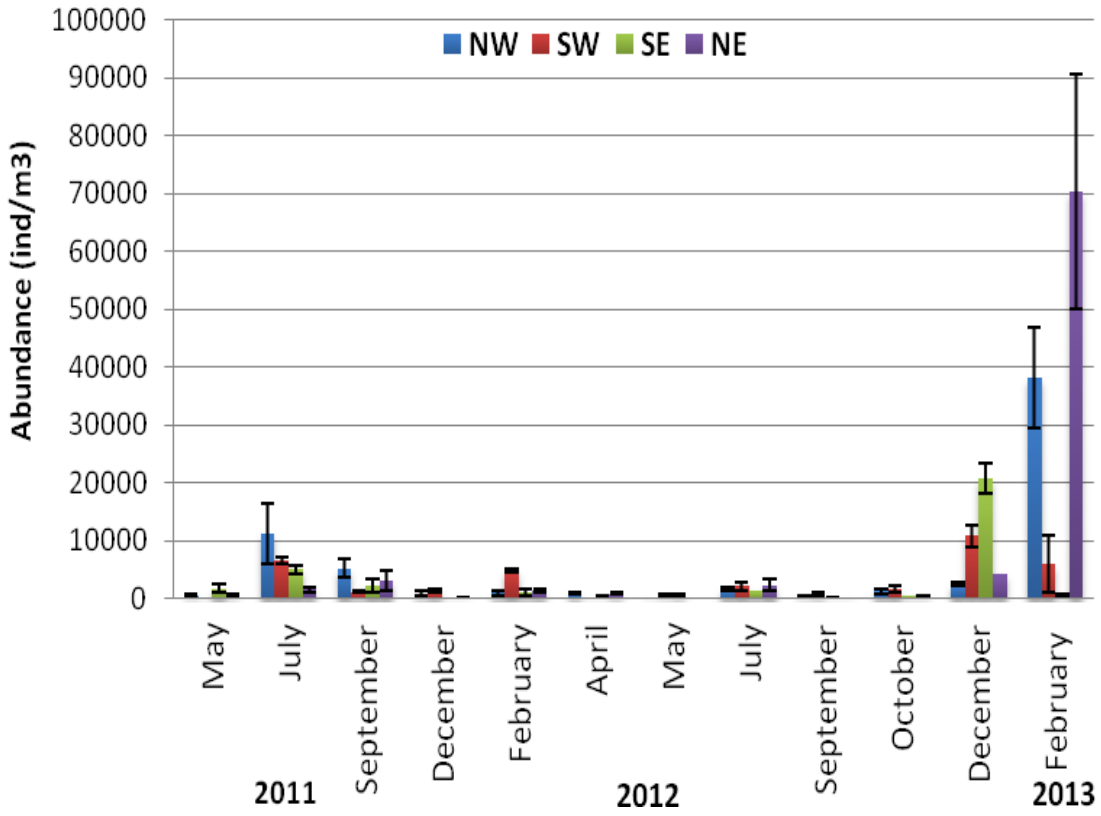


Figure 2.12. Spatio – temporal abundance of mesozooplankton.

Peaks of mesozooplankton abundance recurred in summer (July) and in winter (February) (Fig. 2.13). As result, correlation analysis of interannual variation of mean zooplankton abundance in each month from May 2011 to February 2013 with environmental factors was not significant. The linear correlation with temperature also was absent ($r_s = -0.01$, $p > 0.05$) and the negative correlation with salinity was not significant ($r_s = -0.43$, $p > 0.05$).

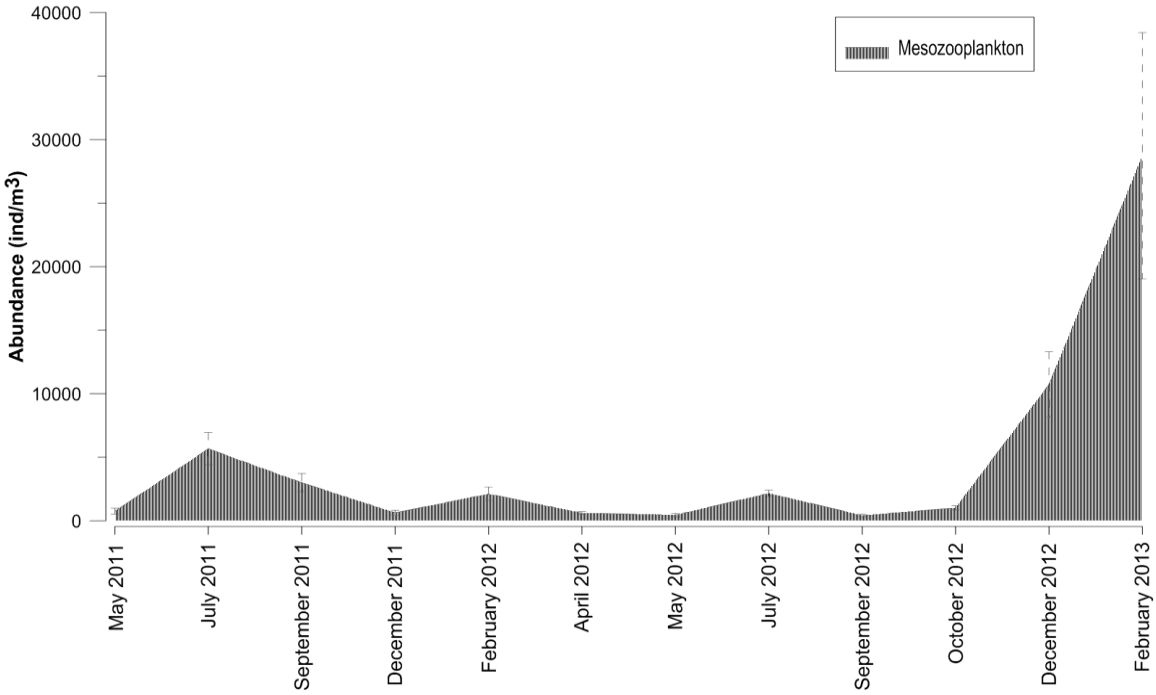


Figure 2.13. Population dynamics of mesozooplankton in in the coastal lagoon of Varano.

The zooplankton community was dominated by copepods, averaging 80% of the total mesozooplankton all year and 94% in the cold months. In particular, calanoids predominated among copepods, being present all year and characterizing entirely the zooplankton community (> 98%) in February months (Fig. 2.14). The abundance of cyclopoid copepods varied a lot during the sampling period, with mean densities ranging from 0.14 ± 212 ind/m^3 in May 2012 to 2198 ± 212 ind/m^3 in December 2012.

The contribution of benthic copepods, the harpacticoids, was insubstantial, but this was due to the horizontal tows that did not properly sampled this group.

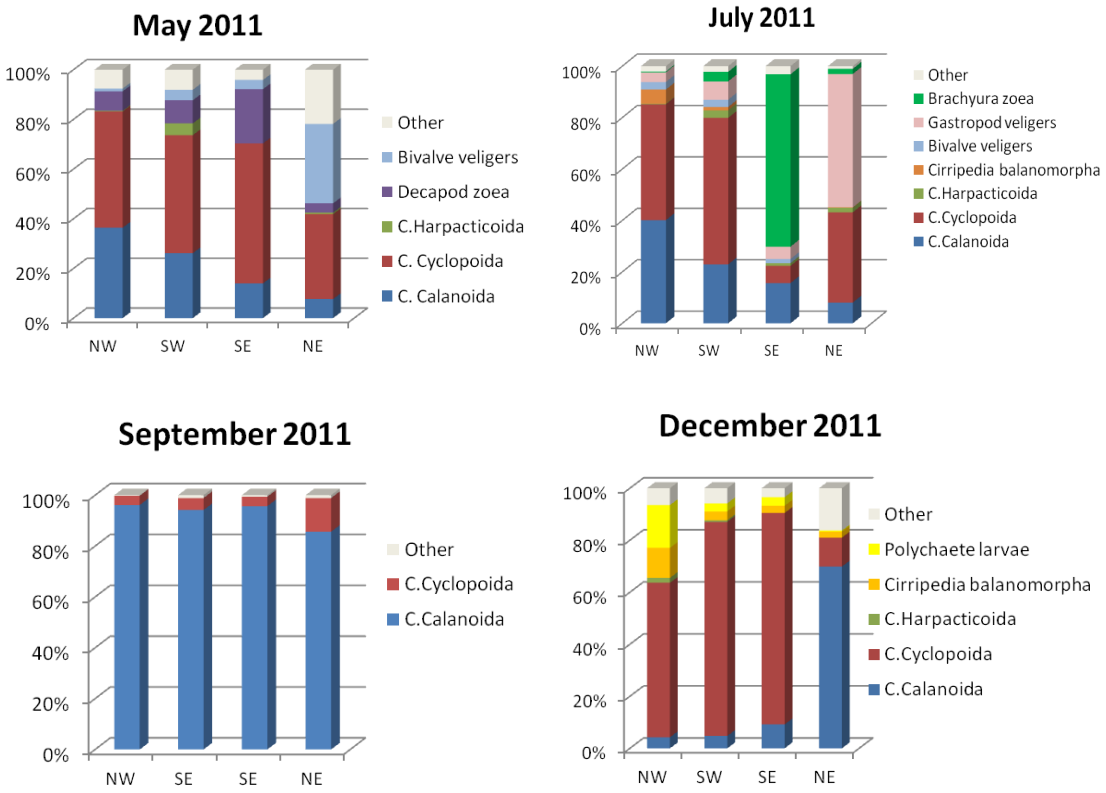
A total of 37 categories (excluding micro- and phytoplankton) of zooplankton were recognized in the samples (Appendix A). The spring season (April 2012) was the most diverse with 21 categories recognized, while the mean numbers of taxa each month were 14 and were similar in the four sectors, although some taxa showed a particular distribution related to the sectors.

The contributions of other taxa were more variable than of copepods, and depended on season and reproduction. Polychaetes (mainly Spionidae) had two peaks of abundance each year in the plankton: in spring - summer and in winter. The first corresponded to May 2011 (41 ind/m^3) and July 2012 (37 ind/m^3) while the winter peak always was in December (31 ind/ m^3 in 2011 and 25 ind/ m^3 in 2012).

Brachyura zoea were present all the year with exception of the coldest month, February, and the peak of abundance was observed in July 2011 (1010 ± 544 ind/m^3).

Less frequent taxa, such as actinotroca larvae, cladocerans, chateognaths, and appendicularians, were considered to be strictly seasonal and their highest abundances were recorded in December 2012. Cladocerans were found exclusively in February, April and December 2012 (peak of 6 ± 1.6 ind/m^3). Chaetognaths were found in both December months (peak of 7 ± 4 ind/m^3) and in May, September and October 2012. Larvae of phoronida were found in April 2012 and in both December months with a peak of 35 ± 10.6 ind/m^3 in 2012. Appendicularians were found in December 2011, April 2012 (peak of 0.68 ± 0.29 ind/m^3), and September 2012.

Although a seasonal pattern of abundances existed for several groups, variation between years monitored was observed (Fig. 2.14).



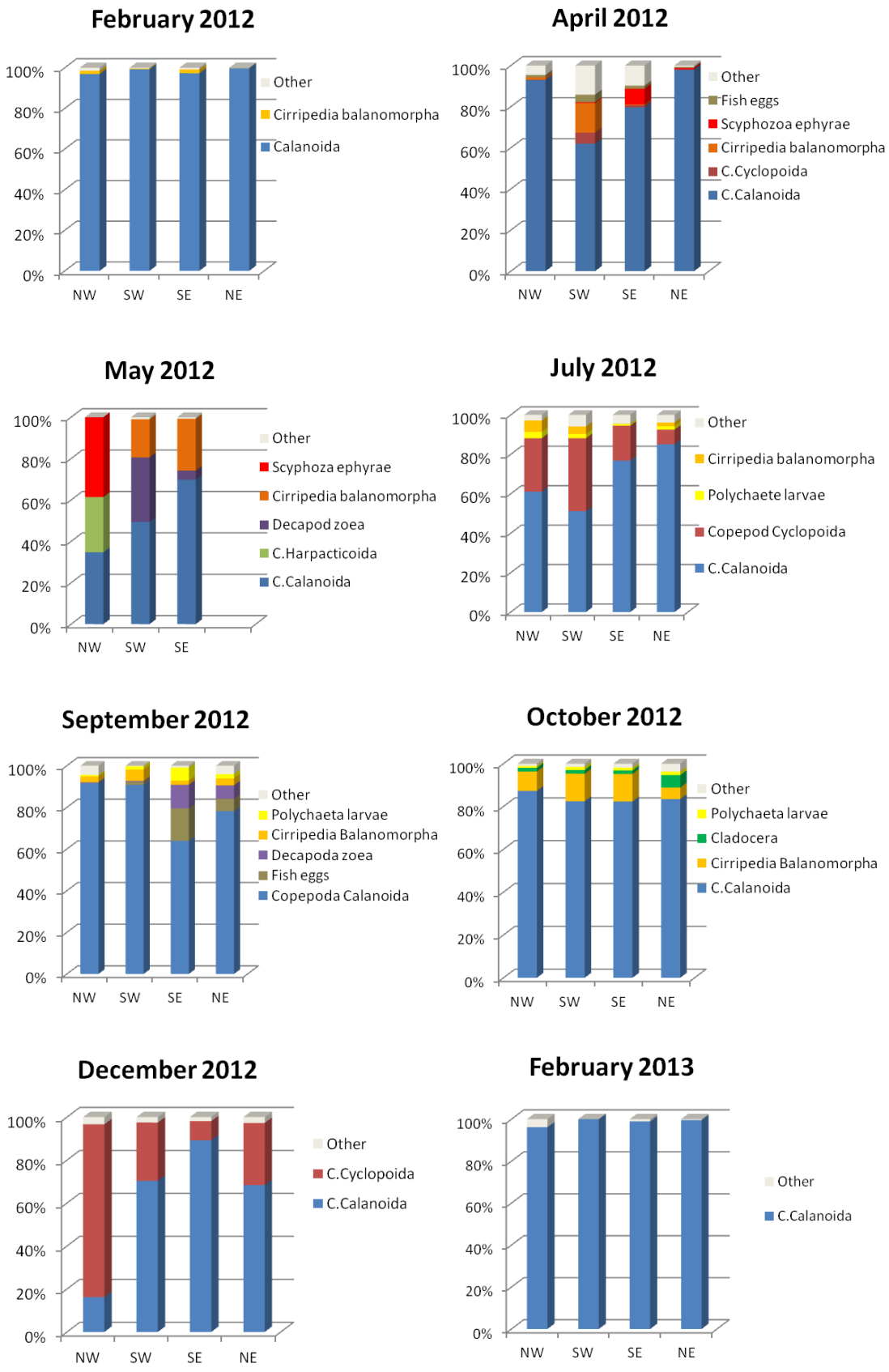


Figure 2.14. Spatio – temporal relative abundance of mesozooplankton groups in the coastal lagoon of Varano.

2.4.5. Microzooplankton spatio – temporal dynamics

Two seasonal microzooplankton peaks occurred in spring or summer (May, July) and autumn (September – October) (Fig. 2.15). The abundances recorded in the first year were highest, and the maximum was recorded in May 2011 with $800*10^3$ ind. m^3, while in 2012 and 2013 the maximum peak did not exceed $400*10^3$ ind./m^3. The lowest abundance was always recorded in the months of February with $7.7 *10^3$ ind./m^3 and $3.2 *10^3$ ind./m^3 in 2012 and 2013, respectively. The increase of microzooplankton abundance in spring – summer and the strong decrease observed in the winter months was associated with temperature. The Spearman correlation of microzooplankton with temperature was strongly significant (r_s = 0.70, p < 0.003**), while salinity showed a non-significant negative correlation (r_s = - 0.21, p > 0.05).

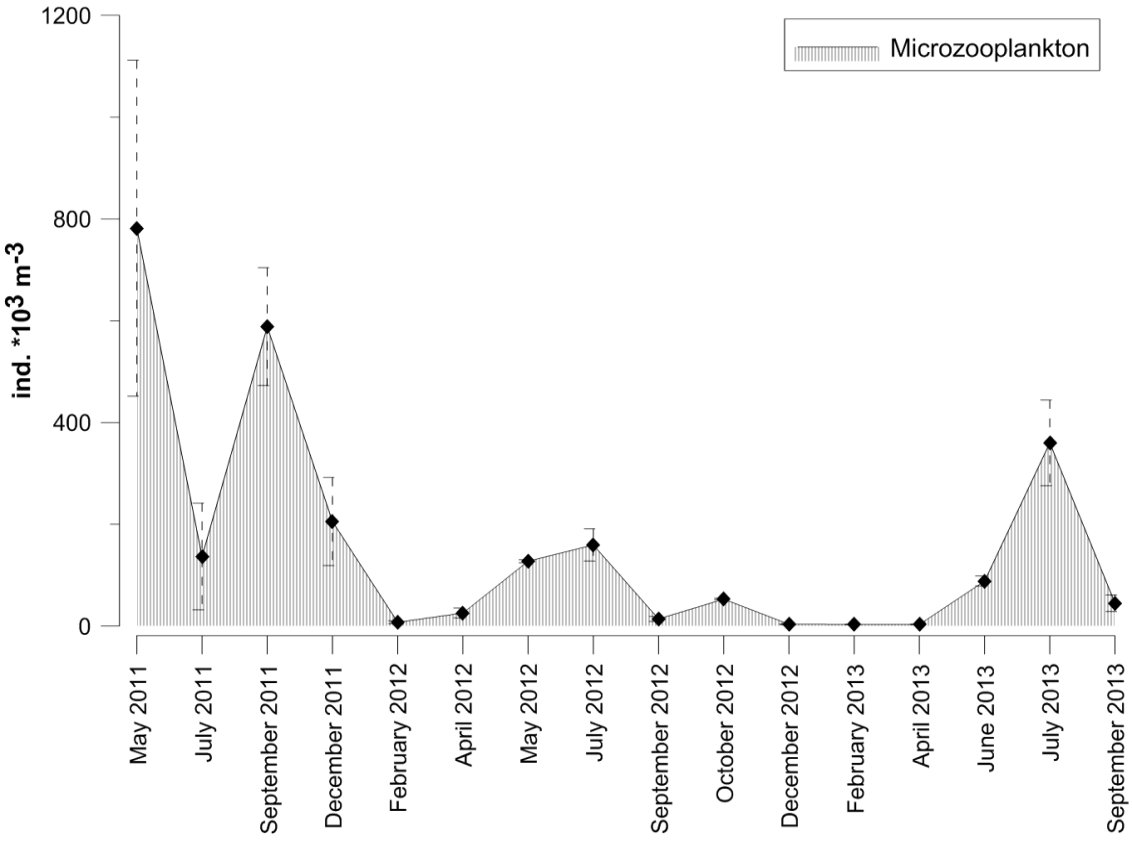


Figure 2.15. Population dynamics of microzooplankton in the coastal lagoon of Varano.

Copepod nauplii, copepodites and adults of copepods, followed the same dynamics as the annual peak in summer, generally July (Fig. 2.16). Tintinnids had a sudden increase in abundance in May (12000 ± 34597 ind./m^3) followed by a rapid decrease that was strongest in 2013. The abundances of Rotifera varied monthly, but their presence was reduced in the year of absence of

jellyfish. Polychaeta larvae were present all year and generally increased during spring season. Veligers of gastropods and bivalves gradually decreased from a huge numbers in May 2011 (234,774 and 16,831 ind./m^3). Generally, all taxa had low densities at the end of sampling campaign (Fig. 2.16).

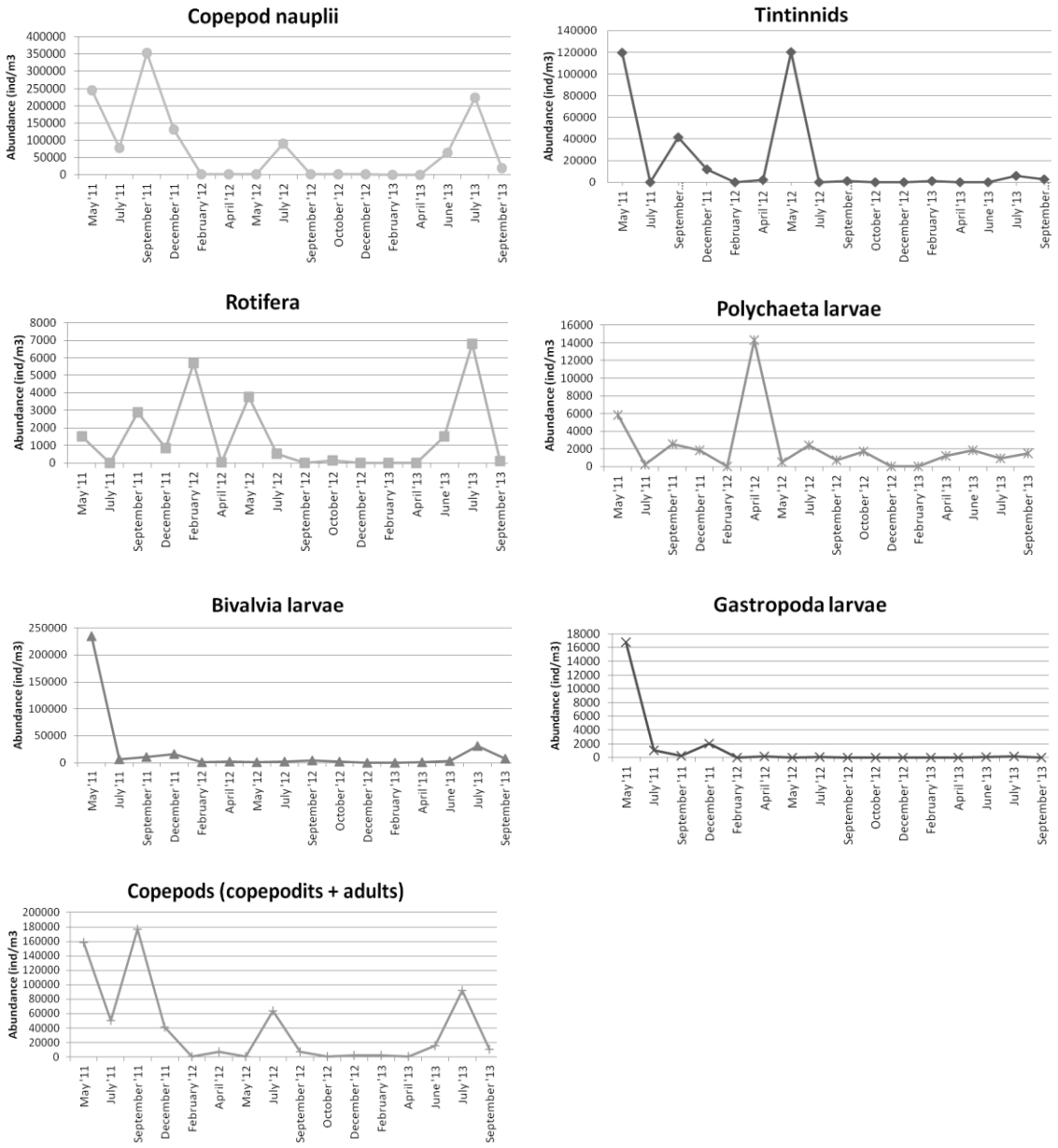


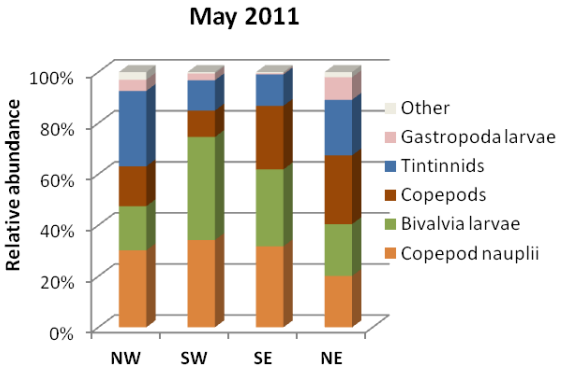
Figure 2.16. Dynamics of microzooplankton taxonomic groups in the coastal lagoon of Varano.

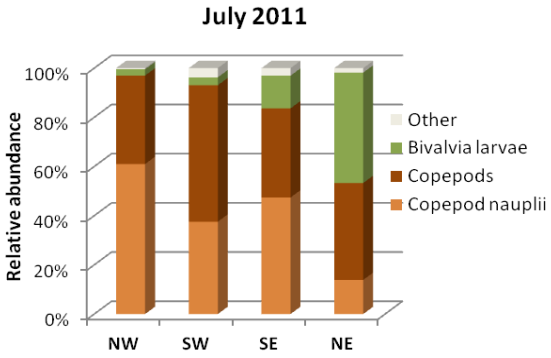
The abundance of microzooplankton in the four sectors changed across months without any spatial preference. The lowest abundance was recorded in the NE sector with 1.5 $*10^3$ ind./m^3 (Feb 2013) and the highest in the SE sector with 1721 $*10^3$ ind./m^3 in May 2011. Only the sampling month was related to the microzooplankton abundance in each sector (Table 2.6; p = 0.0002***), but month did not explain the spatial abundances of each taxonomic group (p =0.13).

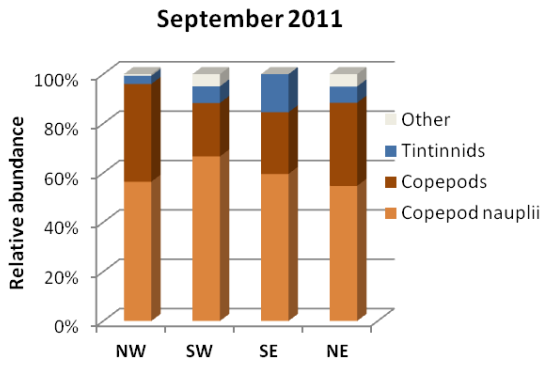
Table 2.6. Results of PERMANOVA comparison of microzooplankton abundance in the coastal lagoon of Varano between sectors and months in the period May 2011 - September 2013. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Mo = months; MoxSe = interaction between month and sector; Res = Residuals; Se = Sector.

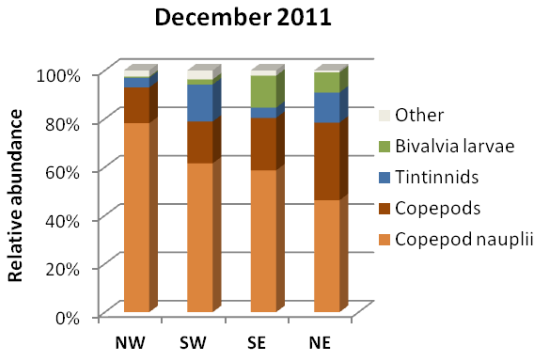
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Mo	7	42332	6047.4	4.849	**0.0002**	4985	0.0002
Se	3	1416.8	472.27	0.3787	0.873	4989	0.876
MoxSe	21	8398.5	399.93	0.3207	0.999	4976	1
Res	32	39906	1247.1				
Total	63	92688					

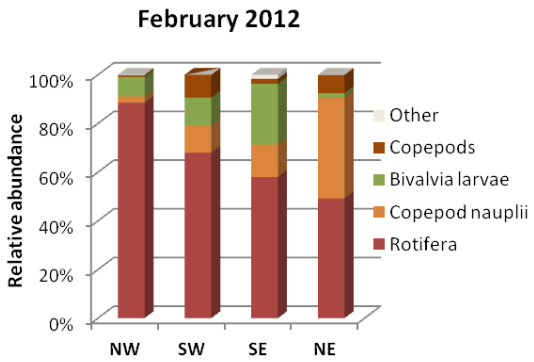
Generally, the microzooplankton community was dominated mainly by copepod nauplii and adults averaging 32 and 26% of the total microzooplankton community, and during the summer (June 2013) copepod nauplii represented the 74% the community (Fig. 2.17). Second in importance in their contributions to the microzooplankton community were Bivalvia larvae (14%), tintinnids (12%), polychaetes (10%), Rotifera (5%), and Gastropoda (0.5%) (Fig. 2.17).

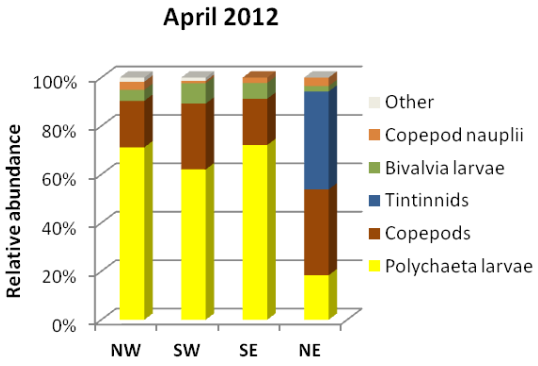

May 2011
Relative abundance
100%
80%
60%
40%
20%
0%
NW
SW
SE
NE
Other
Gastropoda larvae
Tintinnids
Copepods
Bivalvia larvae
Copepod nauplii

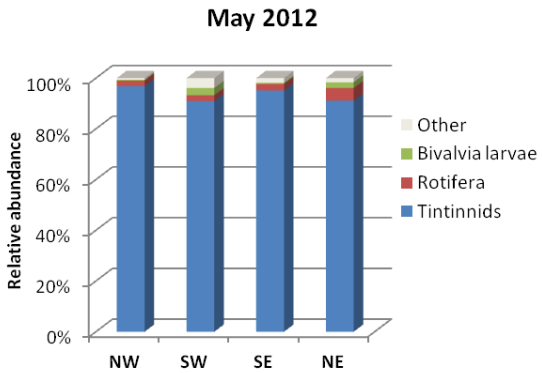

July 2011
Relative abundance
100%
80%
60%
40%
20%
0%
NW
SW
SE
NE
Other
Bivalvia larvae
Copepods
Copepod nauplii

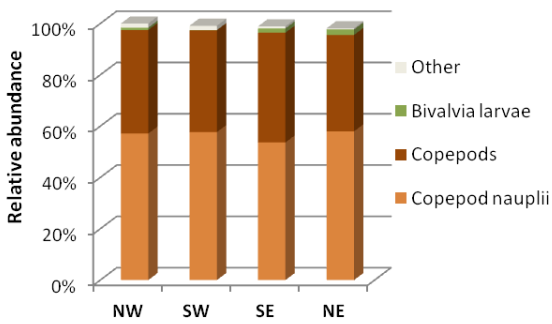

September 2011
Relative abundance
100%
80%
60%
40%
20%
0%
NW
SW
SE
NE
Other
Tintinnids
Copepods
Copepod nauplii


December 2011
Relative abundance
100%
80%
60%
40%
20%
0%
NW
SW
SE
NE
Other
Bivalvia larvae
Tintinnids
Copepods
Copepod nauplii


February 2012
Relative abundance
100%
80%
60%
40%
20%
0%
NW
SW
SE
NE
Other
Copepods
Bivalvia larvae
Copepod nauplii
Rotifera


April 2012
Relative abundance
100%
80%
60%
40%
20%
0%
NW
SW
SE
NE
Other
Copepod nauplii
Bivalvia larvae
Tintinnids
Copepods
Polychaeta larvae


May 2012
Relative abundance
100%
80%
60%
40%
20%
0%
NW
SW
SE
NE
Other
Bivalvia larvae
Rotifera
Tintinnids


July 2012
Relative abundance
100%
80%
60%
40%
20%
0%
NW
SW
SE
NE
Other
Bivalvia larvae
Copepods
Copepod nauplii

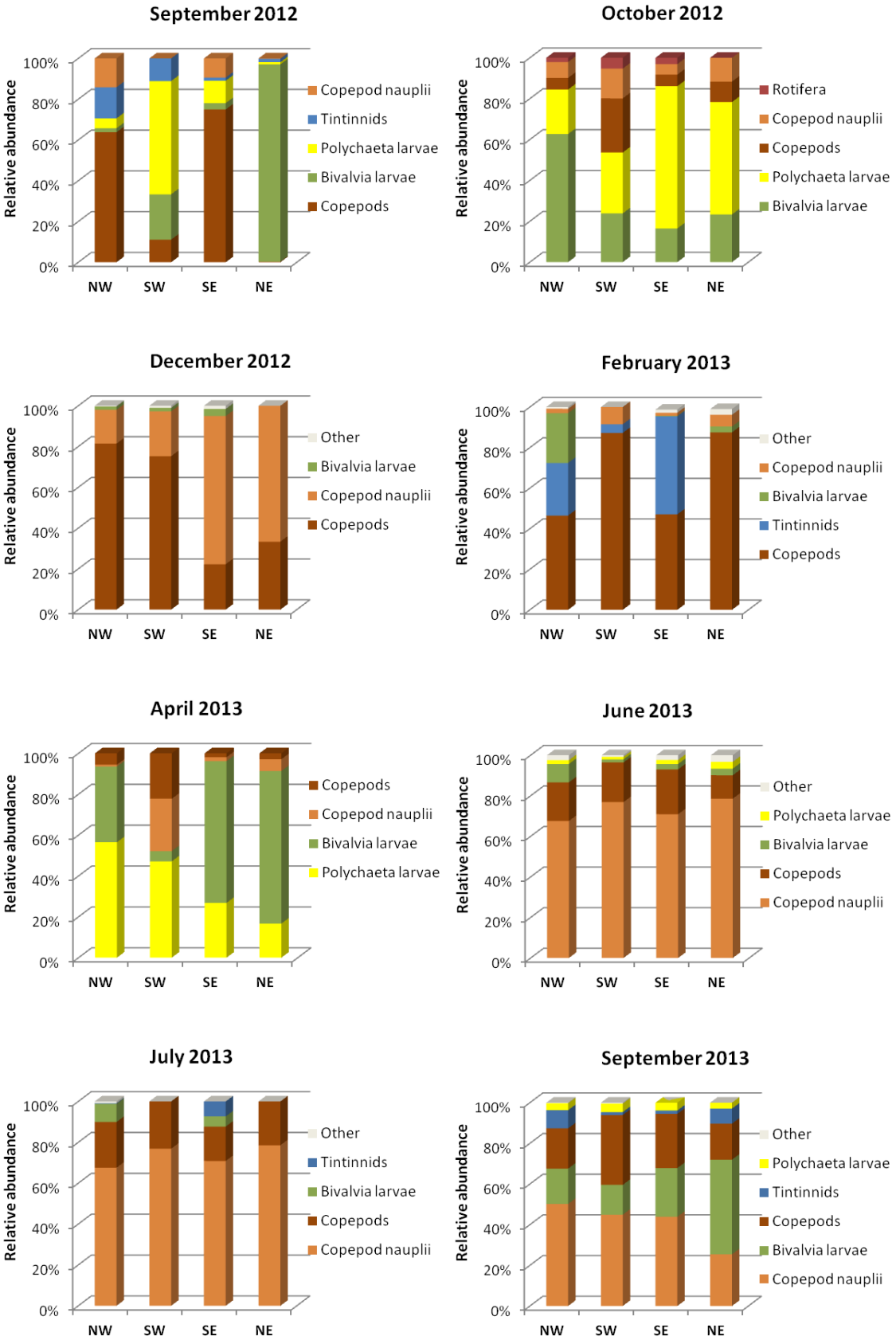


Figure 2.17. Spatio – temporal relative abundance of microzooplankton groups in the coastal lagoon of Varano.

2.4.6. Phytoplankton and nutrients

Monitoring data highlighted two seasons responsible for phytoplankton blooms in the Varano lagoon. From late spring - summer the phytoplankton increased reaching the maximum peak in July 2012 with 1467 ± 154 cell.*10^3 L^{-1} (Fig. 2.18). The abundance gradually decreased over the autumn months and in February another smaller peak of phytoplankton was observed (1014 ± 169 cell.*10^3 L^{-1}). Because the phytoplankton bloom was observed in two opposite seasons, phytoplankton abundance was not significantly correlated with temperature and salinity ($p > 0.05$). The phytoplankton had a patchy distribution in the comparison of the four sectors that was significantly explained by the factor "month" in the PERMANOVA analysis (Table 2.7; $p > 0.015$*).

The two phytoplankton peaks observed were due to rapid increases of diatoms (July) and phytoflagellates (February), two important groups in the phytoplankton community of Varano lagoon. The abundance of diatoms ranged from 0.34 (February) to 1407 cell*10^3 L^{-1} (July) and phytoflagellates ranged from 40 (December) to 1006 cell*10^3 L^{-1} (February) although they were quite abundant all year. Dinoflagellates and coccolithophores also were most abundant during the first peak in July (300 cell*10^3 L^{-1} and 3.64 cell.*10^3 L^{-1}, respectively) when they were the most conspicuous component of the community.

The relative abundance of each taxonomic group in the four sectors is shown in Figure 2.19. In spring (April – May), the phytoplankton community was represented by dinoflagellates and phytoflagellates with means of 36% and 50% of the total abundance. In summer (July – September), diatoms represented 62% of the total phytoplankton cells. In winter, diatoms still represented most of the community (57%), but gradually they were replaced by phytoflagellates, which averaged 75% of the community in winter.

Table 2.7. Results of PERMANOVA comparison of phytoplankton abundance in the coastal lagoon of Varano between sectors and months in the period April 2012 - April 2013. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Mo = months; MoxSe = interaction between month and sector; Res = Residuals; Se = Sector.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Mo	6	13992	2331.9	9.988	**0.015**	4994	0.0054
Se	3	157.63	52.544	0.225	0.927	4993	0.9104
MoxSe	18	2500	138.89	0.595	0.837	4985	0.819
Res	4	933.87	233.47				
Total	31	17549					

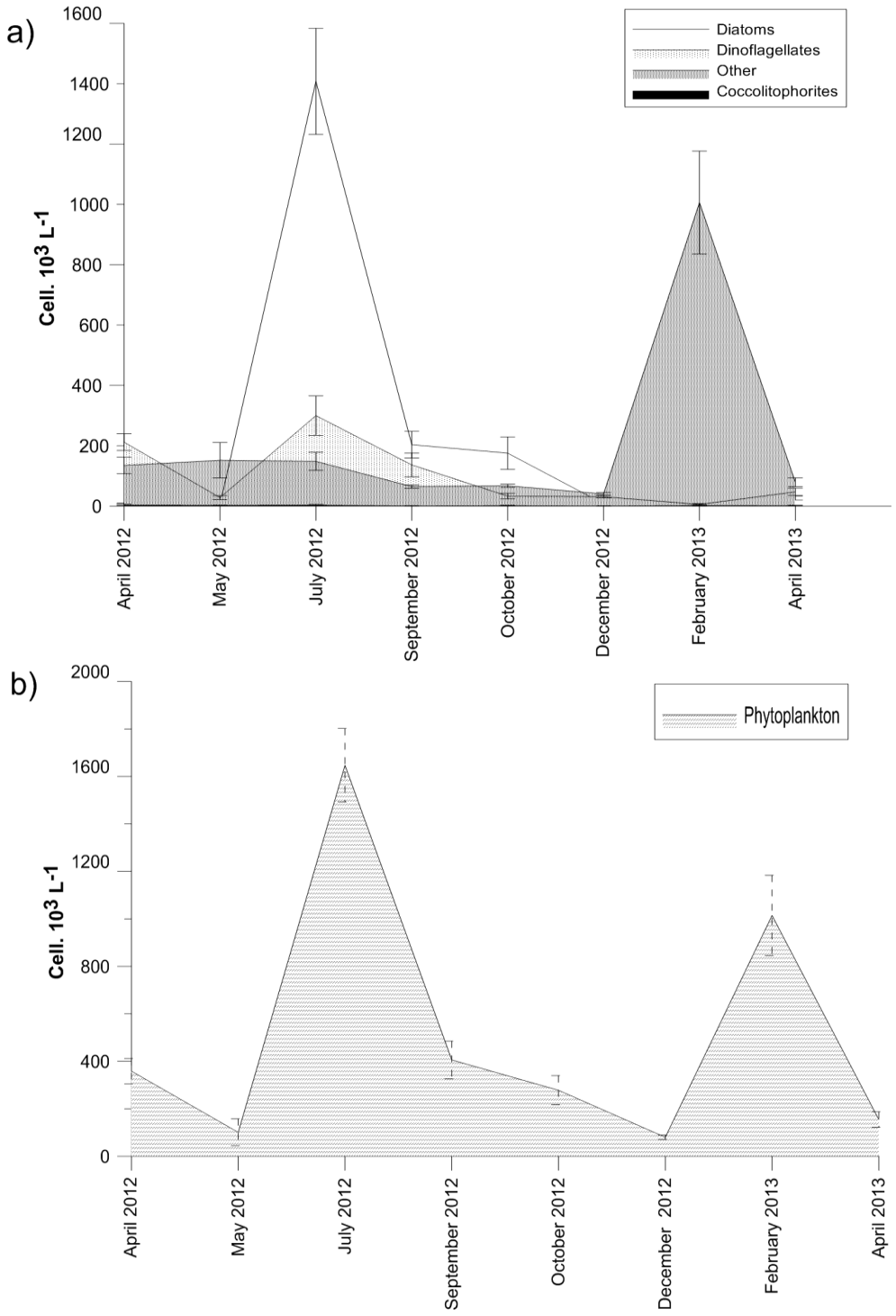


Figure 2.18. Contribution of phytoplankton density (cell. 10^3 L^{-1}) by taxonomic groups (a) to the total phytoplankton (b) of the coastal lagoon of Varano. Others refers to phytoflagellates.

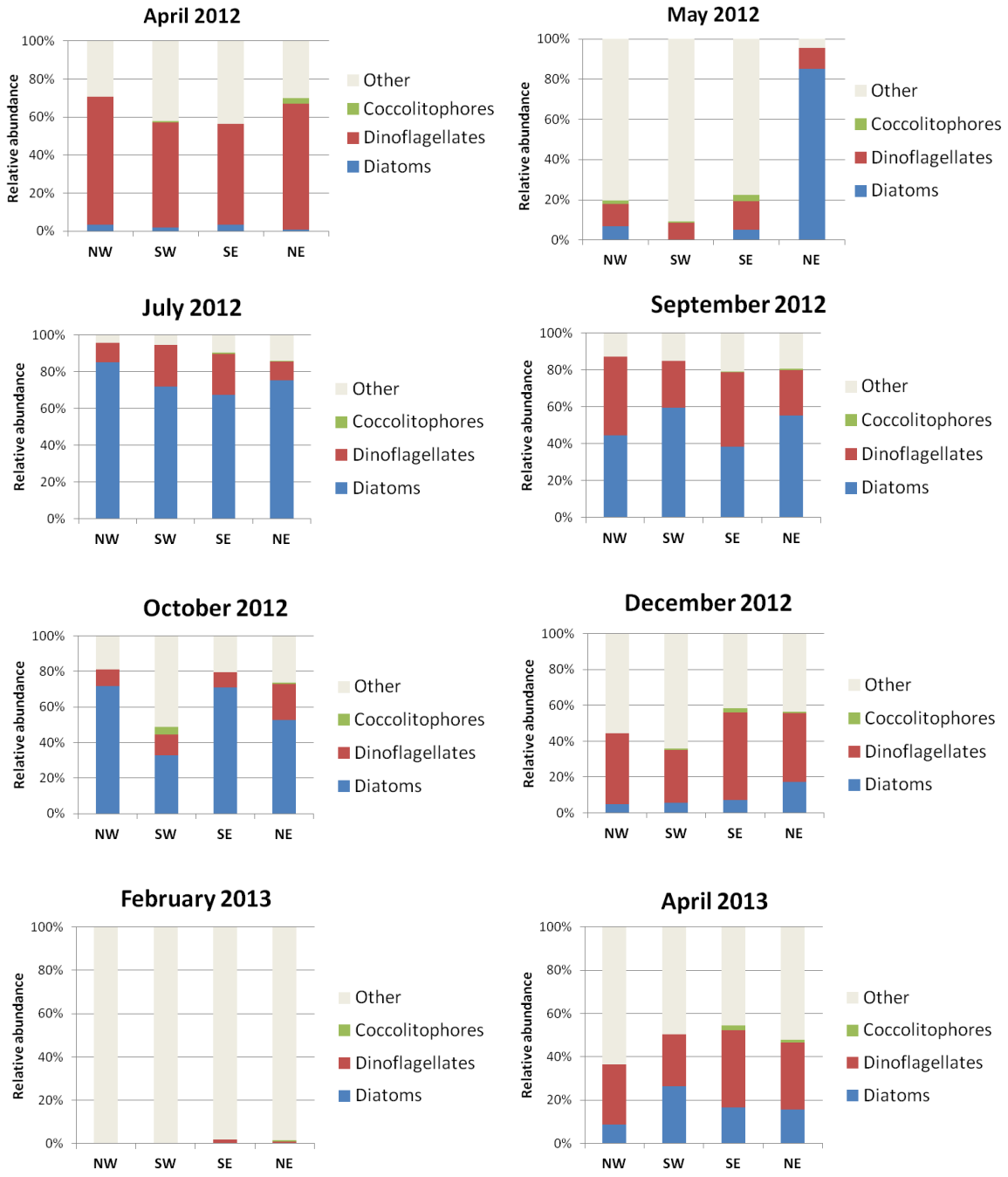


Figure 2.19. Relative abundance of phytoplankton groups in the coastal lagoon of Varano in the year 2012 – 2013.

Nutrients. The analysis of nutrients showed that ammonium (NH^{4+} -N) reached higher concentrations in summer season and in December. In particular, it ranged from the lowest (2 μM) to the highest (11 μM) values recorded in February and July, respectively (Fig. 2.20). Nitrate (NO_3^-N) was abundant only in winter (December and February) when the concentration reached 10.81 μM. The other months showed comparable values, although 2013 had higher values for the same months than in 2012. A similar trend was observed for nitrite (NO_2^-N), but because the values were recorded only in two years, no conclusions could be related to the spring–summer dynamics (Fig. 2.20). In 2012, the nitrite concentration increased only in autumn, while in 2013 the increase was in summer. However, the highest value recorded was in December (1.04 μM) and the lowest in July (0.17 μM).

PO_4^{3-}-P levels increased in summer reaching the maximum in autumn and then decreased in winter months, with concentrations ranging from 0.03 μM in April to 0.34 μM in October.

As for other nutrients, the values obtained for the silicates (SiO_3^{2-}-Si) were higher in 2013. Generally, spring was the season characterized by the lowest value (0.80 μM, April 2012), while the concentrations increased during summer with the maximum in September in both years (49 and 65 μM).

The availability of the total nitrogen and total phosphorous for phytoplankton growth generally increased during summer (Fig. 2.21). For phosphorous and silica, the months after May showed exponential increases of nutrient concentrations. A similar, but less dramatic pattern was observed for total nitrogen.

Spearman rank order correlations between environmental variables and nutrients revealed that ammonium and the total nitrogen were significantly correlated with salinity (NH^{4+} -N, $r_s = 0.68$, $p < 0.042$*; N_{tot}, $r_s = 0.65$, $p < 0.043$*).

The concentrations of nutrients then were analyzed spatially.

PERMANOVA analysis demonstrated that the spatial abundance of nutrients was significantly related to the year ($p = 0.0002$ ***), and to the factor month nested in year ($p = 0.0002$ ***), but not to the sector (Table 2.8).

As result, the concentrations recorded in the four sectors followed the same trends and the values were similar when compared among sectors. A discrepancy among the sectors was observed in ammonium concentrations in September 2013, when the concentration in the NE sector increased toward the maximum peak, values in the other sectors decreased. Similarly, an opposite pattern in total nitrogen concentrations was observed for the southern sectors in respect

to the northern in May 2012 and for the NE sector in December 2012 and July 2013. Southern sectors were responsible for the anomaly in phosphate concentration in June 2013.

Table 2.8. Results of PERMANOVA comparison of nutrients in the coastal lagoon of Varano among sectors, months and years in the period April 2012 - September 2013. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0.05$) are indicated in bold. Ye = Year; Mo(Ye) = month nested in year; Res = Residuals; Se = Sector; SexYe = interaction between sector and year.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Se	3	68.89	22.963	0.695	0,641	4993	0,646
Ye	1	1992	1992	60.333	**0,0002**	4991	0,0002
Mo(Ye)	8	7585.3	948.17	28.718	**0,0002**	4981	0,0002
SexYe	3	105.06	35.02	1.0607	0,438	4989	0,431
Res	24	792.39	33.016				
Total	39	10544					

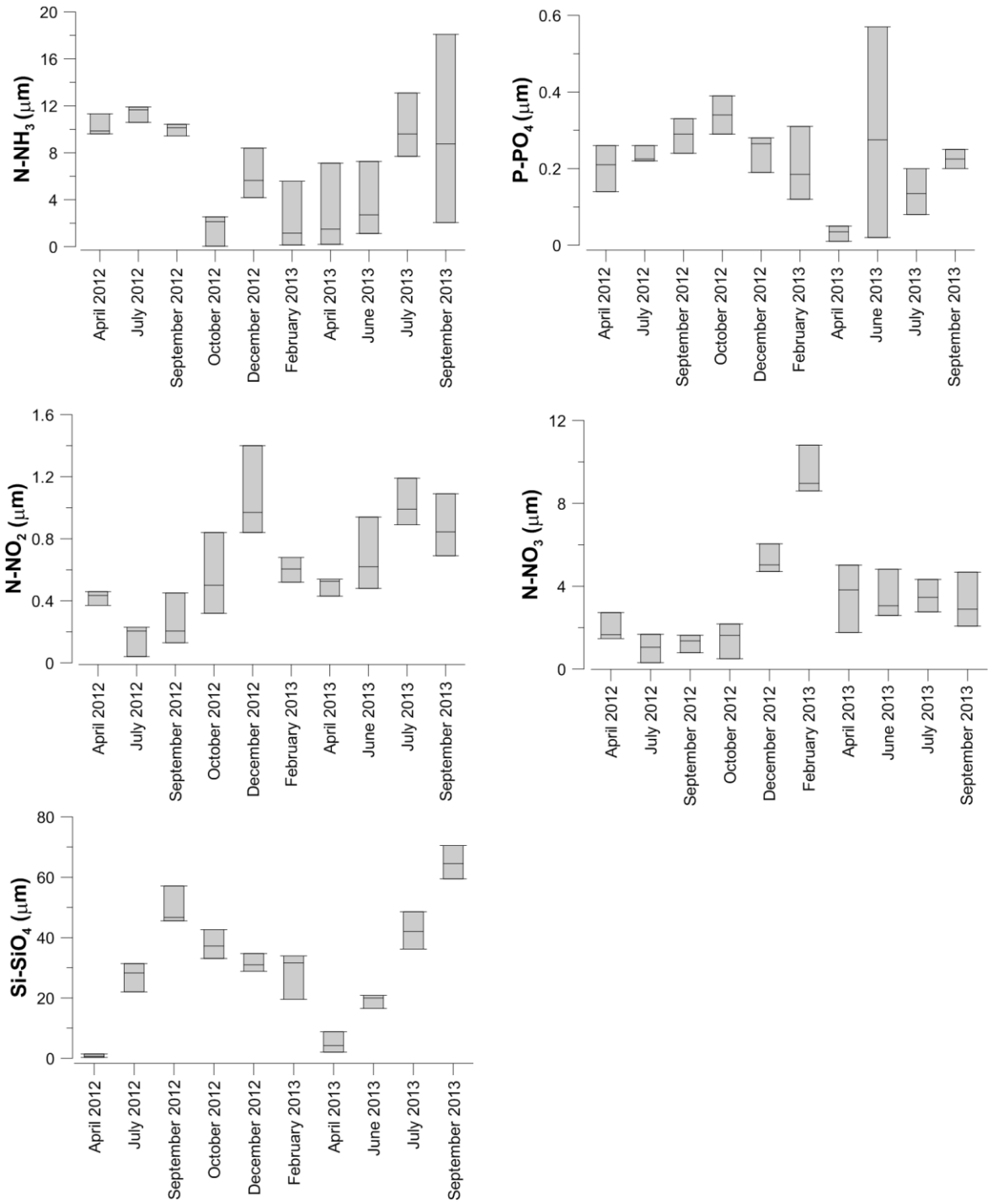


Figure 2.20. Box- and whisker plot of nutrients: a) ammonium b) phosphate c) nitrite d) nitrate e) silicate in the coastal lagoon of Varano. The centre horizontal line is the median, the top and the bottom of the box are the 25th and 75th percentiles (quartiles). In each month data of the four sectors (NW, SW, SE, NE) were averaged.

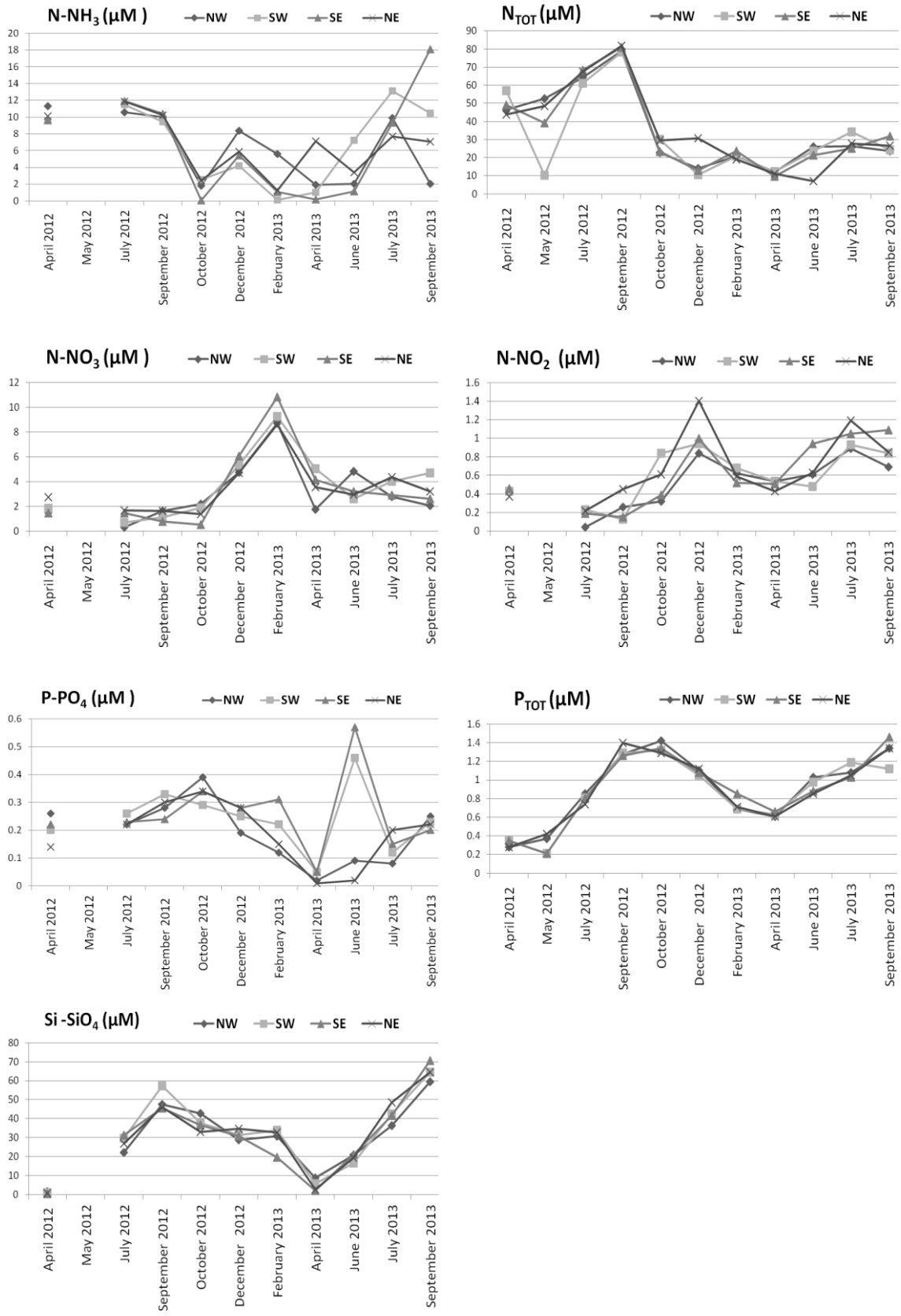


Figure 2.21. Temporal variations of nutrients concentrations in the four sectors (NW, SW, SE, NE) of the coastal lagoon of Varano.

2.4.7. Population patterns

The peaks of phytoplankton, microzooplankton and mesozooplankton occurred sequentially, suggesting prey-predator interactions (Fig. 2.22). An anomaly was observed toward the end of sampling campaign when both phytoplankton and mesozooplankton increased while microzooplankton remained low (Fig. 2.22).

Correlations between phytoplankton and mesozooplankton abundances and between micro and mesozooplankton recorded in each sector were not significant ($p > 0.05$). By contrast, phytoplankton abundance was negatively correlated to microzooplankton abundance in May 2012 ($r_s = -1$, $p < 0.01$**).

On the other hand, the spatial abundances of microzooplankton were negatively correlated to the abundances of the ctenophore *Pleurobrachia* sp. when its abundance was highest in February 2013 ($r_s = -1$, $p < 0.01$**).

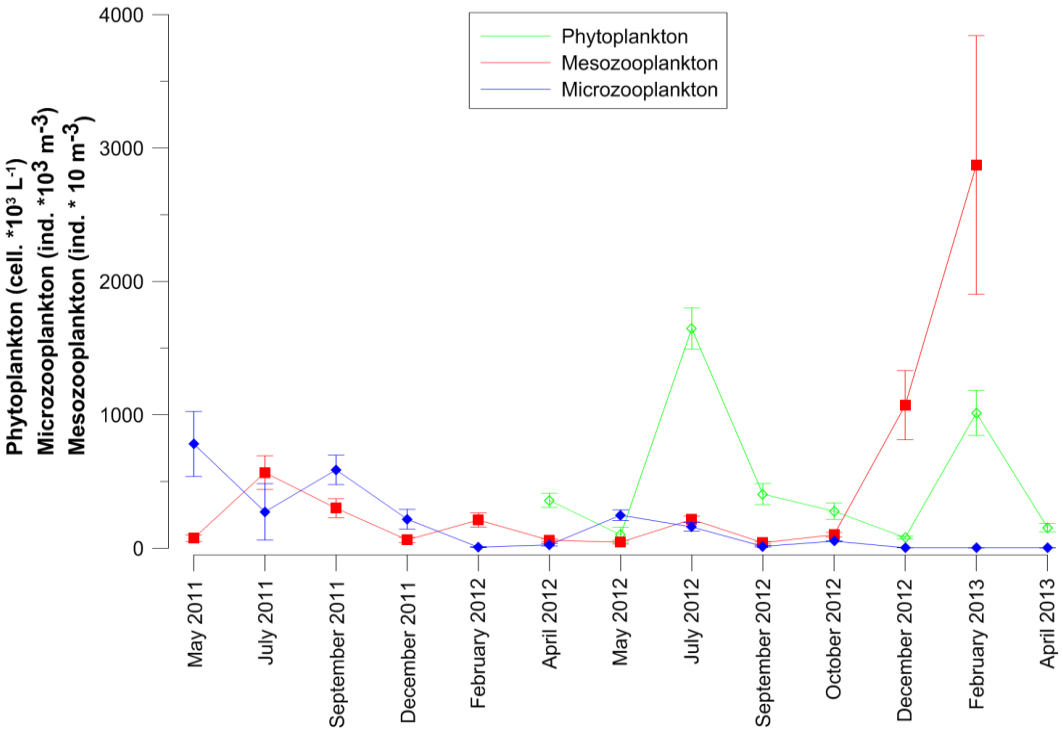


Figure 2.22. Interannual phyto- microzoo- mesozoo- plankton dynamics in the coastal lagoon of Varano.

The statistical significance of the correlation between medusa abundance and zooplankton abundance in each sector were also examined using the Spearman's rank order correlation. Only the months with jellyfish and by the availability of data for mesozooplankton (year 2011 – 2012)

were analyzed. The jellyfish abundance in each sector was significantly negatively correlated with zooplankton abundance in all the months (Table 2.9). Indeed, the correlations were significant only in July 2011 ($p < 0.01$**) and May 2012 ($p < 0.01$**) at the same time as *Aurelia* sp. 1 peaks (Fig. 2.6).

Table 2.9. Spearman correlation among the *Aurelia* sp. 1 abundance and mesozooplankton abundance in the four sectors of the coastal lagoon of Varano. "*" correlation is significant at the 0.05 level (2-tailed) and "**" correlation is significant at the 0.01 level (2-tailed).

Month	**r_s**
May 2011	-0.40
July 2011	**- 0.962****
September 2011	-0.40
December 2011	-0.40
February 2012	-0.26
April 2012	-0.10
May 2012	**-1.00****

Zooplankton assemblage and environmental factors

To determine which of the environmental parameters could explain the pattern observed in the plankton assemblages each year, the BIO-ENV procedure in PRIMER was used (Clarke and Warwick, 2001). All the data obtained from the different sampling were merged in a single table with the abundance of each taxonomic group as shown below. The environmental similarity matrices were obtained using normalized Euclidean distance whilst Bray-Curtis Index was used to construct the similarity matrix for biological data. The analysis considered data from the period April 2012 – February 2013.

The results of the analysis of the zooplankton assemblage in relation to the environmental variables using the BIOENV procedure showed that seasonal zooplankton assemblage was best correlated with temperature (ρ_s=0.36).

The dbRDA plot obtained from DISTLM analysis showed that the first dbRDA axis explained 54% of the total variation and the second axis explain 23% of the fitted variation (Fig. 2.23). In particular, temperature explained 41% of the total variation and grouped together months seasonally, confirming that the zooplankton assemblage had a seasonal dynamic.

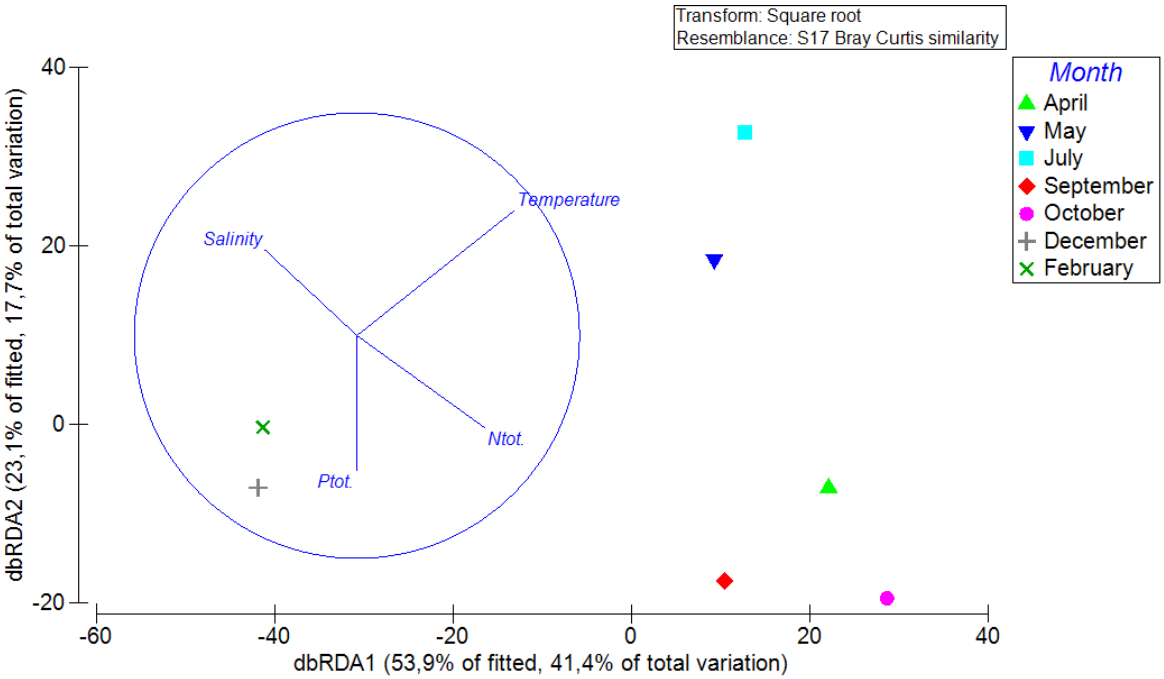


Figure 2.23. dbRDA ordination of the distLM model describing the relationships among environmental variables and the zooplankton assemblage among months of the coastal lagoon of Varano. Vectors represent the effect of each predictor variable on the two visualized axes.

Jellyfish and environmental factors

The same procedure was performed to examine the association of *Aurelia* sp. 1 abundance with environmental factors. Because it was demonstrated that *Aurelia* sp. 1 abundances were correlated with zooplankton abundances in the lagoon of Varano, the variable zooplankton abundance was added to the dataset.

The BIOENV procedure showed that monthly jellyfish abundances were best correlated with the phosphorous (ρ_s=0.54) among the environmental variables.

The dbRDA plot obtained from DISTLM analysis showed that the first dbRDA axis explained 90% of the total variation and the second axis explain 10% of the fitted variation (Fig. 2.24). In particular, phosphorous explained 72% and nitrogen explained 8% of the total variation. The two axes together explained 100% and 80% of the fitted and variation, respectively.

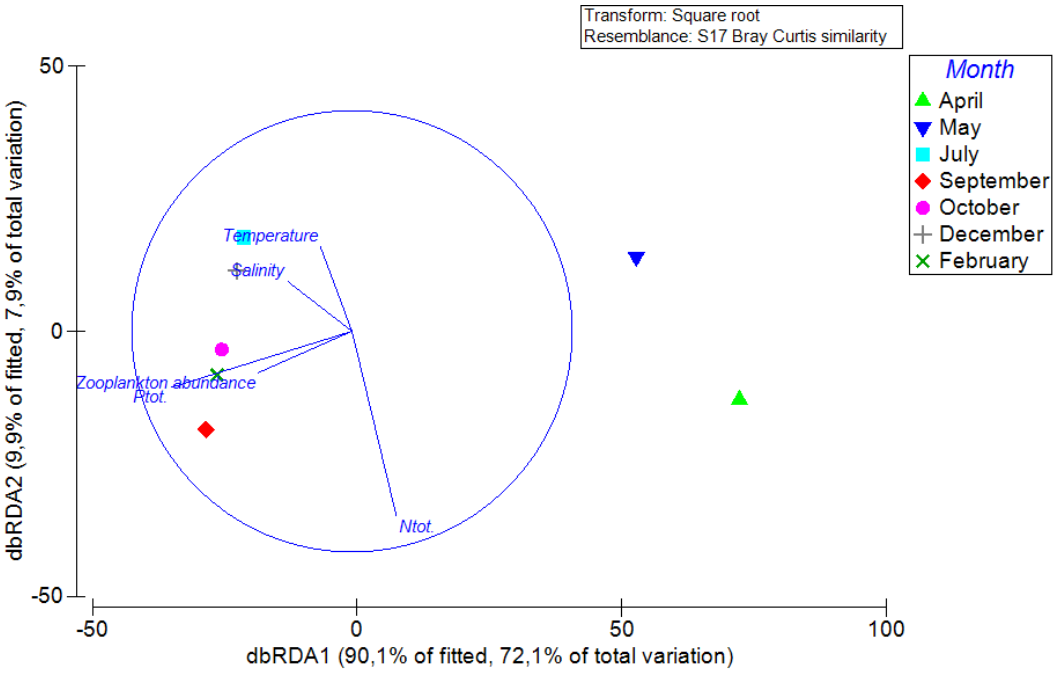


Figure 2.24. dbRDA ordination of the distLM model describing the relationship among environmental variables and jellyfish abundance among months in the coastal lagoon of Varano. Vectors represent the effect of each predictor variable on the two visualized axes.

2.5.Discussions and conclusions

In this study, the potential effects of the jellyfish *Aurelia* sp.1 were investigated through analysis of spatio - temporal variation of plankton assemblages in the four sectors of the Varano lagoon in the 2011 - 2013 period.

The month was the key factor in determining variations in environmental factors in the lagoon and it is involved in the mechanism of changes in recent years. The increased salinity in 2011-2013 in comparison to previous values (Belmonte et al., 2011; Caroppo, 2000; Spagnoli et al., 2002) indicated greater inflow of sea water from the Varano and Capojale channels, resulting from dredging activities mainly in the NW sector, that has changed the lagoon's characteristics into a marine area. The increased sea water input in April and December resulted in increased bottom salinity and the creation of a halocline, suggesting possible changes in zooplankton vertical migration (Lougee et al., 2002) and in the asexual reproduction of *Aurelia* sp. polyps (Purcell, 2007).

The strong influence of seasonality in Varano lagoon was evident from comparisons of the physical (temperature, salinity) and biological data. Comparisons among the surveyed sectors (NW, SW, SE, NE) showed small differences in temperature and salinity and excluded the

geography from explaining the patterns of plankton distribution. For phyto-, micro-, mesoplankton, and jellyfish, the spatio - temporal distributions observed were instead characteristic of the month considered. This factor seems to be crucial in the phases of the life cycle of *Aurelia* sp. 1 lagoon population, which was described here for the first time.

It was demonstrated that this invasive population, probably introduced around 2000 in the Varano lagoon on the mussel rafts, has established a population with medusae throughout the year, which were monitored from May 2011 to September 2013. Although the strobilation of *Aurelia* sp. 1 scyphopolyps occurred twice during the year, at the beginning of winter (December) and in spring (April), only the spring cohort determined the jellyfish abundance later on. Physical factors, including temperature and salinity, are involved in the control of strobilation in *Aurelia* sp. (Purcell, 2007; Holst, 2012; Liu et al., 2008; Purcell et al., 2009). The youngest ephyrae of *Aurelia* sp. 1 were found in Varano lagoon at temperature of 18° and 25 - 26 ppt of salinity. When released, ephyrae grow during summer months and the first medusae were seen in May - June. Somatic growth of the medusa is accompanied by gonad maturation that led jellyfish larger than 10 cm to be sexually mature at the end of summer. The presence of ripe medusae with planulae from September to April indicated that the production of planulae covered a long period. On the other hand, planulae were in the plankton samples only between September and December, suggesting that the recruitment period is more restricted. During those months, planulae were not homogeneously distributed in the lagoon but were found only in the SW sector, and the ephyrae appeared later in the same sector, that the presence of *Aurelia* sp. polyps may be due to hard substrate in this area. The time shift between the release of planulae and ephyrae confirmed that ephyrae cannot directly develop from the larval stage (Yasuda, 1969).

Like strobilation, also survivorship of *Aurelia* sp. polyps can be affected by variations in abiotic factors (Condon et al., 2001; Willcox et al., 2007; Winans and Purcell, 2010).

In July 2012, the population of *Aurelia* sp. 1 medusae collapsed, possibly due to high temperature and food - limitation after their outbreak in May 2012. Decreased zooplankton abundances at the spatial scale followed the two highest peaks of *Aurelia* sp. 1 medusae recorded (July 2011, May 2012). The medusa mortality event of July 2012 would have reduced jellyfish reproduction, and polyp recruitment. Also high temperatures may have affected the survivorship of polyps. However, the appearance of new ephyrae in the following year confirmed that polyps overwintered and settled successfully in the SW sector.

Phytoplankton and microzooplankton presumably were significant food sources for the growth of polyp and ephyra stages in Varano Lagoon, as demonstrated by laboratory experiments (Olesen et al., 1994; Båmstedt et al., 2001; Kamiyama, 2011; Riisgård and Madsen, 2011)

During the survey time period, the first phytoplankton bloom was observed in February and was due to the rapid increase of phytoflagellates. In summer, increasing day length, solar radiation, and nutrients, allowed for rapid growth of diatoms. The abundances of dinoflagellates varied with the seasons, but as for diatoms, the bloom occurred in summer according to previous studies (Caroppo, 2000). In both cases, the increase of phytoplankton resulted from nutrient availability. The concentrations of nutrients reported in this study reflected the oligo - mesotrophic state of the lagoon. Dinoflagellates were favoured by the input of ammonium and nitrate (Collos et al., 2007), which were very high in the lagoon in July 2012 when they bloomed. The phytoplankton analysis was not carried out to the species level, but the presence of toxic dinoflagellates was studied in the past (Caroppo, 1999). In Varano lagoon, the toxic dinoflagellate bloom (e.g. *Alexandrium* sp.) led to anoxia and consequent mortality in bottom waters (Scirocco et al., 2011). This phenomenon affected mussel survival for many years and caused most of the mussel rafts to be moved from the lagoon to the nearby Adriatic Sea (Scirocco et al., 2011). Although anoxic events may occur in Varano lagoon, the finding of reddish ephyrae that indicate a recent release (see Chapter 4) in the same sector, suggested that polyps could survive hypoxic or anoxic conditions (Ishii et al., 2008).

A negative and significant correlation between microzooplankton and phytoplankton abundance was found in May 2012, maybe due to grazing activity of microzooplankton, which abundance generally increased gradually following the phytoplankton peak.

The co - occurrence of small medusae and tintinnids always in the same period led to the hypothesis that microzooplankton play a key role in sustaining the rapid growth of *Aurelia* sp. medusae in Varano lagoon. Similarly, microzooplankton following the winter phytoplankton peak could contribute within the optimum of temperature and salinity to the ephyrae survival in the water column.

Bigger medusae of *Aurelia* sp. use mesozooplankton as food source (Riisgård and Madsen, 2011). In Varano lagoon, copepod recruitment occurred after the microzooplankton increase and their peaks coincided with that of *Aurelia* sp. 1 medusae. Medusae triggered a cascading effects on trophic web, since predating on mesozooplankton allowed the microzooplankton increase and consecutively played an indirect role on phytoplankton. In 2013, when the jellyfish population

collapsed, the mesozooplankton may have controlled microzooplankton and phytoplankton populations. In particular, this top - down effect could be one of the causes of the progressive decrease of microzooplankton in the last year that could affected the reproductive output of strobilation in the following spring. The ingestion of ciliates by copepods is an important energy pathway (Robertson, 1983; Stoecker and Sanders, 1985) and in the lagoon of Varano caused the rapid decrease of tintinnids at the end of 2012. Polyps could not take advantage from this food source in the spring 2013 and only invertebrate larvae and Rotifera were available in the late summer for ephyrae growth.

The month of December was always characterized by the inflow of sea water and by the introduction of marine plankton, like chaetognaths, larvae of phoronida, appendicularians, and fish larvae. This apparently brought in another gelatinous predator, the ctenophore *Pleurobrachia* sp. They reproduced gradually in the lagoon and reached their peak in February. The absence of ctenophores in the years of *Aurelia* sp. 1 dominance and their disappearance in June 2013 when the new generation of *Aurelia* sp. 1 appeared, suggested that other organisms with similar trophic niches could replace jellyfish when they disappear. Similarly, the ctenophore *Pleurobrachia pileus* showed the same dynamics in Izmit bay, increasing in April and May and then ceasing in June when *Aurelia* sp. increased again (Isinibilir, 2012).

Many studies evaluated the effects on fish (Purcell, 1985; Purcell and Arai, 2001; Quiñones et al., 2013) and zooplankton populations (Purcell, 1992; Barz and Hirche, 2005; Hansson, 2006; Pitt et al., 2008;), because fish can be both competitors and prey of jellyfish. Fisheries are important resources that sustain the local economy (Breber, 1982) of the Varano coastal lagoon and surrounding area. Nevertheless data about the trend of fish catches in the lagoon are very scarce and the monitoring of the biological resources in relation to the arrival of the invasive jellyfish *Aurelia* sp. 1 is missing.

From these data was not possible to extrapolate clear information on the relationship jellyfish - ichtyoplankton. A better understanding of ichtyoplankton and fish dynamics can be obtained by a pluriennal sampling and also by the investigations on trophic ecology of jellyfish (Chapter 3).

The invasive species found in Varano recently like the crustacean *Callinectes sapidus* and *Dyspanopeus sayi,* the gastropod *Rapana venosa,* the Bivalvia *Musculista senhousia* and the jellyfish *Aurelia* sp. now resident in the lagoon, indicate the extreme vulnerability of this area (Florio et al., 2008; Ungaro et al., 2012). In addition, the finding for the first time of the

ctenophore *Pleurobrachia* sp. and Siphonophora not reported in recent studies (Belmonte et al., 2011) suggest an high potential of the lagoon to be easily invaded by gelatinous predators.

As conclusion, this study showed the *Aurelia* sp. 1 can alter plankton abundance directly or indirectly. Abiotic factors and availability of food sources regulate the dynamic of *Aurelia* sp. 1 in the lagoon. Jellyfish, when abundant, can behave as keystone species in the lagoon exerting top - down control on zooplankton community.

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CHAPTER THREE:

Trophic Ecology of *Aurelia* sp. 1 in the coastal lagoon of Varano

3. Trophic ecology of *Aurelia* sp. 1 in the coastal lagoon of Varano

3.1. Abstract

The trophic role of the invasive jellyfish *Aurelia* sp. 1 has been investigated by analyzing spatial and temporal changes in diet of the medusa stage in the coastal lagoon of Varano (Apulia). In different sectors of the lagoon, an integrative sampling of jellyfish, zooplankton (including ichtyoplankton) and seston was carried out to combine gut content and isotope stable analyses with an inventory of the available zooplankton prey community. Gastric pouches of jellyfish were dissected and food item identification was integrated by calculation of prey selectivity index (Pearre, 1982). In parallel, the *Aurelia* sp. 1 jellyfish trophic position was established by means of stable isotope analysis.

Preliminary data showed seasonal variations in diet revealing that medusae ingest most of the mesozooplankton taxa present in the Varano Lagoon. The selectivity index C calculated from gastric pouches content and prey availability in the field showed null selection toward most prey items, suggesting that *Aurelia* sp. 1 medusae exhibit an opportunistic and omnivorous foraging behavior, unveiling the key role of microzooplankton as food source for the early jellyfish life stages. Furthermore, nitrogen and carbon isotopic signatures showed the trophic level of *Aurelia* sp. 1 changes with medusa size and age.

Altogether, these data suggest a) the lack of negative direct effects on ichtyoplankton and b) jellyfish may potentially outcompete fish by predating on the same food sources, leading to substantial changes in trophic links within the lagoon food web.

3.2. Introduction

Aquatic ecosystems are built on dynamic networks of interaction between abiotic and biotic compartments. Phytoplankton cells may account up to 90% the primary productivity in the oceans (Ryther, 1963), and in the classical food chain, crustacean copepods represent the link between phytoplankton and upper levels, typically represented by fish. In this framework, the knowledge of trophic relationships among species and their respective trophic levels through time is fundamental to understand the structure and organization of aquatic communities (Pitt et al., 2008a; Utne-Palm et al.; 2010; Fucks et al. 2012).

In marine communities, fish can occupy different trophic levels, and most are omnivorous with preference for animal material or carnivorous (Stergiou and Karpouzi, 2001; Pasquaud et al. 2010). However, the "normal" perception of a food web, with primary and secondary productivity mostly regulated by diatoms-crustaceans-fish interactions, has been altered in the last decades, giving space to a new vision where dinoflagellates and jellyfish are taking over (Boero et al., 2008). Overfished ecosystems increasingly showed jellyfish as substitutes of fish in their top predator role (Brodeur et al., 2002; Daskalov et al., 2007). In addition, a decreasing trend in trophic levels of fisheries landings has been estimated a rate of about 0.1 per decade (Pauly, 1998), with extreme records of fishery collapses linked to jellyfish outbreaks (Lynam et al., 2006; Lynam et al., 2010; Riisgard et al., 2012).

In spite of their increasing abundance, jellyfish are not known as a main prey item for apical predators. However, several fish species, including *Mola mola*, *Boops boops*, *Thunnus thynnus*, *Xiphias gladius* are known to feed on gelatinous prey (Mianzan, 1996; Arai, 2005; Cardona et al., 2012; Milisenda et al., 2014). Soft-bodied organism can also represent the food source for other gelatinous species, as demonstrated for ctenophores and salps (Greve and Reiners, 1988; Purcell and Cowan Jr, 1995; Finenko et al., 2001). Nevertheless, experimental evidence for a jellyfish-based diet is difficult because of their high water content and rapid digestion time (Arai et al., 2003).

As result, the scarce information available for jellyfish predator and the low nutritional value supplied by these prey have led jellyfish to be considered for long time as ''trophic dead-ends'' of the ecosystem (Sommer et al., 2002).

Given the difficulty to carry on direct observation of predator-prey interactions, for many years studies of trophic interactions in aquatic ecosystems were based on gut content analysis. Also in jellyfish, the first studies concentrated on the identification and quantification of preys found in their gastric cavities (Malej, 1989; Purcell, 1992; Spadinger and Maier, 1999). Moreover, the potential impact of jellyfish on food webs was based on laboratory experiments to assess feeding, clearance and respiration rates of adult stages (Hansson et al., 2005; Purcell et al., 2010, 1999; Riisgård and Madsen, 2010; Titelman and Hansson, 2005), whereas few studies focused on the trophic role of ephyra and polyp stages (Båmstedt et al., 2001; Kamiyama, 2011; Riisgård and Madsen, 2010). On the other hand, a complete understanding of the energy transfer between

benthic and pelagic domains (also called benthic - pelagic coupling) is achieved only by considering life cycles and life stage-specific interactions (Marcus and Boero, 1998).

The classical gut content analysis may involve direct errors in diet analyses (e.g. differences in digestion rate between food types). In particular, gut content analyses keep record of what is ingested rather than what is assimilated, providing evidence only of recent feeding. More recently, natural biomarkers such as fatty acids and stable isotopes have been used as an alternative or a complement to gut content analysis (Peterson and Fry, 1987; Iverson et al., 2004). The advantage of stable isotope analysis (SIA) is that it may integrate the diet of the animal over a much longer time period as opposed to assessing the actual diet from gut content (Peterson and Fry, 1987).

During any trophic interaction, the heavier stable isotope is retained as a molecular signature in the consumer tissue (Minagawa and Wada, 1984; Fry and Sherr, 1984; Post, 2002). Differences of 3 – 4 ‰ in the fractioning of the $^{15}N/^{14}N$ stable isotope signatures between predator and prey indicate their relative trophic level, whereas the determination of $^{13}C/^{12}C$ stable isotopic ratios (differences ranging 0.1 - 1‰) may lead to identify food sources and direct predation (Minagawa and Wada, 1984; Rau et al., 1983; Fry and Sherr, 1984; Peterson and Fry, 1987; Post, 2002).

This method has been applied only recently to investigations of trophic interactions mediated by jellyfish (Pitt et al., 2008b). In different *Aurelia* species. gut content analysis demonstrated that these jellyfish are voracious consumers of zooplankton, fish larvae and fish eggs (D'Ambra et al., 2013; Hansson et al., 2005; Hansson, 2006; Purcell, 2003). Conversely, biochemical techniques revealed trophic behavior and predation on food sources not detectable through the classic gut content method (D'Ambra et al., 2013; Ying et al., 2012).

Here the diet of *Aurelia* sp. in Varano lagoon has been investigated through a combination of both traditional and new biochemical approaches (gut content and stable isotope analyses) to detect the variability of jellyfish trophic level over a year cycle, and to correlate the impact of jellyfish predation on the observed trends in the Varano plankton dynamics and abundance (see chapter 2).

3.3. Methods

3.3.1. Gut content analysis

The trophic ecology of *Aurelia* sp. was investigated on specimens collected during the same sampling campaign (May 2011- May 2012) discussed in Chapter 2 of the present thesis. A total of 140 medusae were randomly sampled from near surface (< 1 m depth) by a hand net from the different sectors of the lagoon (Fig. 2.3). The bell diameter of medusae were measured to nearest 1 mm and medusae were then individually preserved in 4% formalin with filtered seawater. In laboratory, medusae were dissected and only the prey present in stomach, radial canals and gastric pouches were extracted and identified to order level. Prey found on oral arms or tentacles were excluded to ensure that they had not been captured in the net while towing.

The seasonality of *Aurelia* sp. diet (prey composition among months) was analysed by permutational multivariate analysis of variance (PERMANOVA) and represented by the ordination model obtained by a canonical analysis of principal coordinates (CAP) (Anderson and Willis, 2003), using the program PERMANOVA+ (Clarke and Warwick, 1995).
Finally, to test the linear relationships between variation in jellyfish bell diameter and number of prey ingested, a Pearson's correlation analysis was conducted for all sampled medusae.

The Pearre's selectivity index C (Pearre, 1982) was determined on a Chi-square analysis by 2x2 configured comparison between the average number of each prey taxon in the medusa guts and corresponding number in the water column as follow:

$$C = \pm\left[\frac{\left(\left|a_d b_e - b_d a_e\right| - \frac{n}{2}\right)^2}{abde}\right]^{\frac{1}{2}}$$

where a_d is the number of a specific prey type ingested, a_e is the number of that prey in the environment, a is the total number of that prey type, b_d is the number of all other prey ingested, b_e is the number of all other prey in the environment, b is the sum of all other prey ingested and in the environment, d is the total number of prey ingested, e is the total number of prey in the environment, and n is the sum of d and e.

Statistical significance of C was tested with the χ^2 statistic (Pearre, 1982) at a significance level of 0.05.

3.3.2. Stable isotope analysis (SIA)

Samplings were carried out from May 2011 to May 2012 to collect jellyfish across different seasons. An additional sampling for the stable isotope analyses was carried out in June 2013, when the new jellyfish population arose, following a population collapse detected in July 2012 (see Chapter 2).

At each sampling 8 *Aurelia* sp. jellyfish were randomly collected for nitrogen and carbon stable isotope analysis by hand net. In the laboratory, jellyfish were measured (bell diameter, BD), weighted (wet weight, WW), frozen in liquid nitrogen, and temporarily stored at -80°C. Mesozooplankton samples were also collected by horizontal net tows (mesh size 200 and 50 μm) and sorted out in the laboratory into major taxonomic groups.

From each sector, 2 L of water were filtered on a pre-combusted GF/F 47 mm filter (0.7mm, Whatman, 60°C, 48 hours) to collect seston samples.

All samples were finally lyophilized within a freeze-dryer and stored at -20°C until processing. Samples were oven-dried at 60°C and grinded to a fine powder using mortar and pestle. Then each sample was individually transferred into a tin capsule with a fixed amount of material (5 mg, medusa; 1 mg, zooplankton). In order to remove carbonate structures the zooplankton samples were digested with acid (1 M HCl) and dried at 60°C.

The values of the carbon stable isotopes ($\delta^{13}C$) and nitrogen stable isotopes ($\delta^{15}N$) were measured using a Thermo - Electron elemental analyser interfaced to a Thermo- Electron isotope ratio mass spectrometer.

Results were expressed in the standard ‰ unit notation:

$$\delta X(\%) = \left[\left(\frac{R_{sample}}{R_{standard}} \right) - 1 \right] * 10^3$$

where R is the ratio of either $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

These values were standardised according to the Vienna Pee Dee Belemnite standard (VPDB) for C, and to atmospheric N_2 for N.

The values of the nitrogen stable isotopes ($\delta^{15}N$) were used to estimate the relative trophic position of *Aurelia* sp. calculated on each sampling month as follows:

$$TL_{med} = \frac{\left(\delta^{15}N_{med} - \delta^{15}N_{base}\right)}{\Delta n} + TL_{base}$$

where TL_{base} is the trophic position of the organism used to estimate $\delta^{15}N_{base}$ and $\delta^{15}N_{med}$ is the measure of nitrogen stable isotope value of medusa. $TL_{base} = 1$ for primary producers; $TL_{base} = 2$ for primary consumers (Vander Zanden et al., 1997). Δn is the enrichment in $\delta^{15}N$ per trophic level, chosen to be 3.2 on the assumptions that $\delta^{15}N$ and the $\delta^{13}C$ signatures become enriched by 3.4‰ and 1.0 ‰ per trophic level, respectively (Minagawa and Wada, 1984; Fry and Sherr, 1984; Rau et al., 1983; Post, 2002). The baseline organisms were calanoid copepods and seston, according to the data availability.

Variation in stable isotope signatures was analyzed by permutational multivariate analysis of variance (PERMANOVA). A resemblance matrix based on Bray-Curtis similarity was calculated on data root - squared and the "month" was chosen as fixed factor in the PERMANOVA design.

To observe if the trophic position of *Aurelia* sp. was related to size, a Pearson's correlation analysis between $\delta^{15}N$ signature and bell diameter was carried out for the *Aurelia* sp. generation of the period 2011 - 2012. Medusae of June 2013 were not included in the analysis.

3.4.Results

3.4.1. Prey taxa composition

Overall, 13 zooplankton taxonomic categories were recognized in the stomach, radial canals and gastric pouches of *Aurelia* sp. 1 in the period May 2011 - May 2012 (Table 3.1).

The data indicated that *Aurelia* sp. 1 diet was based mainly on copepods and bivalve veligers, constituting on average 54% and 21% of the total prey (Fig. 3.1). Differently from bivalves, gastropod veligers were found in the gut content only in May and July 2011, representing in that period the second food source in order of importance (5%). Both bivalve and gastropods veligers were often observed as undigested items in the guts.

Polychaetes, cladocerans and barnacle nauplii were moderately present in the gut content of *Aurelia* sp. collected in December (~ 3%).

Other prey as fish eggs, decapod larvae, pteropods, hydromedusae appeared less frequently, contributing in total between 0 and 2.4%. Small organisms, like tintinnids and invertebrate eggs gave a significant contribution to *Aurelia* sp. 1 diet in February and May 2012. In particular Tintinnida represented the first food source in May 2012 with 54% of total abundance.

Although the diameter of medusa examined changed in time (Table 3.1), the Pearson correlation coefficient demonstrated that the number of prey found in the gut contents were not linearly correlated to the bell diameter of the medusae ($p > 0.05$).

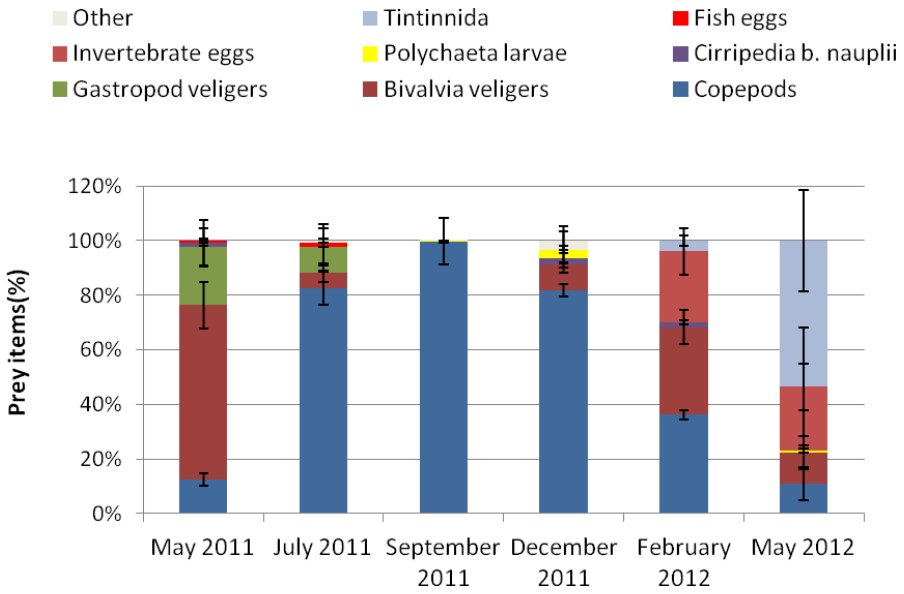

Figure 3.1. Percentage of prey items found in the guts of *Aurelia* sp.1 during the 2011 - 2012 sample period.

Table 3.1. Mean composition (%) of zooplankton taxa in the manubrium, gastric pouches and radial canals of *Aurelia* sp.1 medusae in the coastal lagoon of Varano in the period 2011 - 2012.

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	2011			**2012**		
Month	**May**	**July**	**September**	**December**	**February**	**May**
No. of medusa examined	36	28	30	16	15	15
Mean BD (mm)	93	70	129	108	95	55
Mean composition (%)						
Copepods	12.5	82.6	99.5	81.8	36.2	10.8
Bivalve veligers	63.9	5.8	0.3	9.5	32.2	11.4
Gastropod veligers	21.5	9.2	< 0.1	0	0	0
Cirripedia balanomorpha nauplii	1.3	0	< 0.1	2.3	1.8	< 0.1
Invertebrate eggs	0	0	< 0.1	0	25.9	23.3
Polychaeta larvae	<0 .1	0	< 0.1	3.2	0	0.8
Fish eggs	0.9	1.7	< 0.1	0	0	0
Tintinnida	0	0	0	0	4	53.5
Decapod larvae	0	0	< 0.1	0	0	0
Pteropods	0	0	< 0.1	0	0	0
Hydromedusae	0	0	< 0.1	0	0	< 0.1
Brachyura zoea	0	0	< 0.1	0	0	0
Cladocera	0	0	0	3.3	0	0

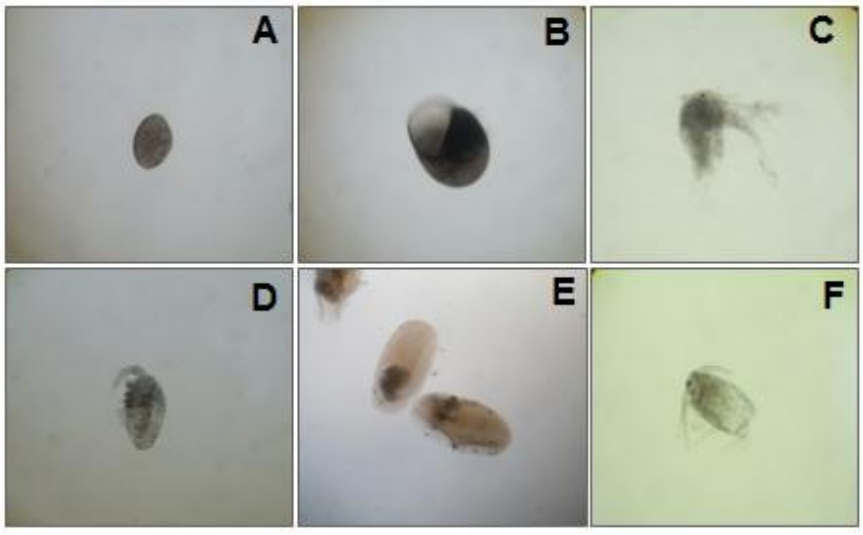

Figure 3.2. Prey items found in the guts of *Aurelia* sp.1. a) bivalve veliger, b) gastropod veliger c) cirripedia balanomorphia nauplius, d) cyclopoid copepod, e) fish eggs, f) calanoid copepod.

The composition of prey items observed in the jellyfish stomach contents varied according to the seasonal plankton availability (chapter 2, Fig. 2.18), as demonstrated by the copepod-dominated diet in September 2011, (Fig. 3.1, Tab. 3.1).

A PERMANOVA analysis on root - squared transformed data matrix supported the hypothesis that *Aurelia* sp. jellyfish diet changes over the year (Table 3.2; p = 0.0002***).

Table 3.2. Results of PERMANOVA analysis on *Aurelia* sp. 1 diets over the sampling period (May 2011-May 2012). PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0.05$) are indicated in bold. Mo = month; Se = Sector; Ye = Year; Res = Residuals.

Source	df	SS	MS	Pseudo - F	P (perm)	Unique perms	P (MC)
Mo	4	17628	4406.9	5.3774	**0.0002**	4979	**0.0002**
Res	17	13932	819.53				
Total	21	31560					

The microzooplankton represented the main source in the diet of small medusae (May 2011, May 2012). Larger medusae captured and ingested a wider number of prey taxa (Tab. 3.1), their diet shifting toward mesozooplankton prey.

The CAP analysis highlighted the ontogenetic shift in *Aurelia* sp. 1 diet from small (tintinnida and veligers) to large prey (copepods mainly) according to the sampling month considered.

The resulting ordination model (Fig. 3.3) on the first axis (CAP1) highlights a separation between samples dominated by planktonic taxa with earlier spring peak (such as mollusk veligers) and samples dominated by mesozooplankton taxa with maximum abundance in late summer (such as copepods). The second axis separates cold months samples or samples including rare food items (such as fish eggs).

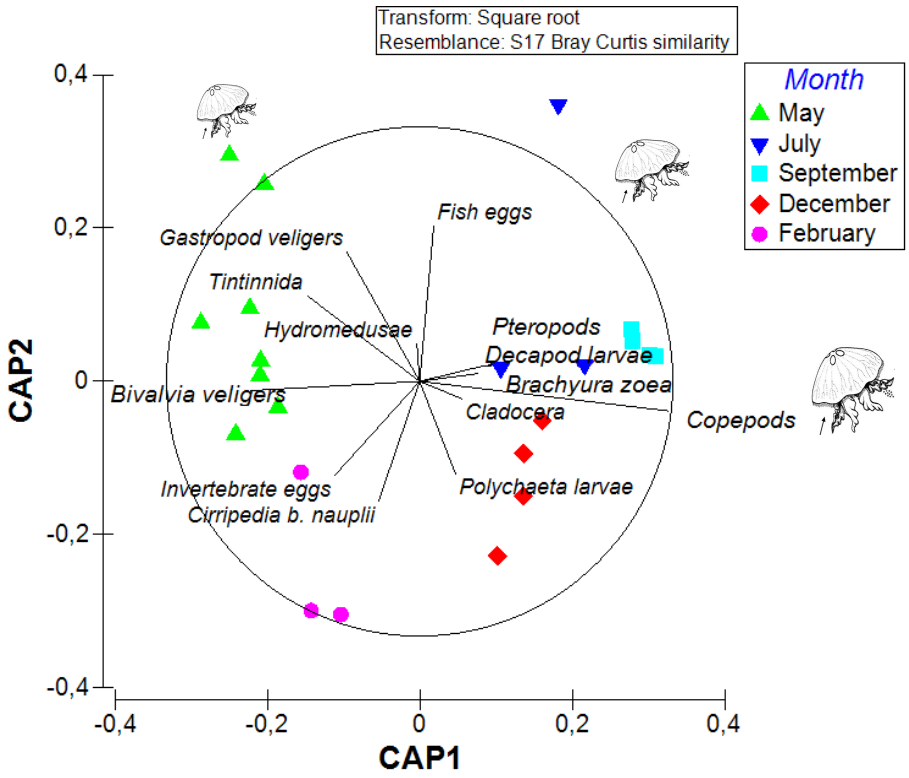

Figure 3.3. Canonical analysis of principal coordinates (CAP) bi-plot ordination of prey taxa composition.

Most copepods found in the jellyfish stomach belong to the order Cyclopoida while Copepoda Calanoida and Harpacticoida were less frequently recorded. To determine if the *Aurelia* sp. 1 were a "selective feeder" the proportion of prey found in the stomach contents were compared with the fractions of prey found in the plankton by comparing 2 x 2 based on the method of χ^2 (Pearre's Selectivity Index).

The selectivity index C suggested a null selection towards most of preys in the period considered (Fig.3.4). *Aurelia* sp. 1 jellyfish in the Varano lagoon almost always exhibited a positive selection for small cyclopoid copepods, with the highest selectivity value (C=0.65) in September 2011, in agreement with the data about prey composition (Fig. 3.1).

Harpacticoid copepods were positively selected in May 2011 (C = 0.19) and February 2012 (C = 0.11) while polychaeta larvae were negatively selected in May 2012 (C = - 0.3).

In February 2012, medusae selected positively invertebrate eggs (C = 0.50) and tintinnids (C = 0.11). In May 2012, the Pearre's Index for invertebrate eggs reached a value of 0.87.

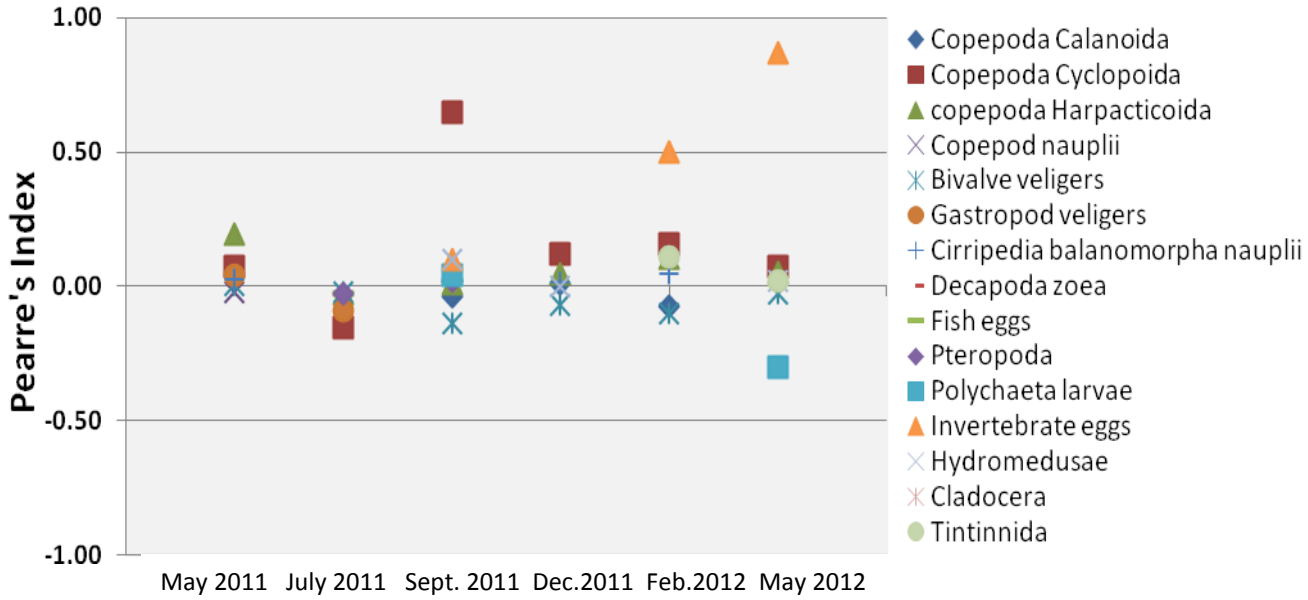

Figure 3.4. Plot of Pearre's selectivity index (C) calculated for all the prey. Positive and negative values indicate selection for the prey group. Only signififcant C values were plotted ($p < 0.05$, by χ^2 analysis).

Table 3.3. Pearre's selectivity index (C) for *Aurelia* sp.1 prey. Positive values indicate selectivity and negative values avoidance. Significant p values ($p < 0.05$) are indicated in bold.

Month	May 2011	July 2011	September 2011	December 2011	February 2012	May 2012
Copepoda Calanoida	0.00	-0.01	**-0.04**	**0.01**	**-0.07**	0.01
Copepoda Cyclopoida	**0.07**	**-0.15**	**0.65**	**0.12**	**0.16**	**0.08**
Copepoda Harpacticoida	**0.19**	**-0.03**	**0.01**	**0.05**	**0.11**	**0.06**
Copepod nauplii	**-0.02**					
Bivalve veligers	**0.01**	**-0.02**	**-0.14**	**-0.07**	**-0.10**	**-0.03**
Gastropod veligers	**0.04**	**-0.09**	-0.01			
Cirripedia balanomorpha nauplii	**0.03**		-0.01	**0.02**	**0.05**	-0.01
Decapoda zoea		-0.01	0.04			
Fish eggs		**-0.03**	**0.02**			
Pteropoda			**0.05**			
Polychaeta larvae			**0.04**	-0.01		**-0.30**
Invertebrate eggs			**0.10**		**0.50**	**0.87**
Hydromedusae			**0.10**	**0.00**		**0.02**
Cladocera						
Tintinnida					**0.11**	**0.02**

3.4.2. Isotopic fingerprint

Jellyfish. The present study showed that monthly (seasonal) differences constituted the main factor of isotopic variation in *Aurelia* sp. 1 in Varano lagoon. Variation in both $\delta^{13}C$ and $\delta^{15}N$ can be observed among the different sampling periods (Fig. 3.5).

The lowest $\delta^{15}N$ signatures were observed in May (12.38 ± 0.41) and June (12.83 ± 0.10). The values progressively increased toward winter months and the highest $\delta^{15}N$ signature was observed in February (16.18 ± 0.07).

The temporal variation in $\delta^{13}C$ signatures was rapidly detected in *Aurelia* sp. 1 tissues. The $\delta^{13}C$ values ranged from -22.90 ± 0.10 ‰ in December 2011 to 24.91± 0.19 ‰ in June 2013.

Significant differences in stable isotope signatures among months were demonstrated by Permutational ANOVA (Table 3.4; p = 0.0002***).

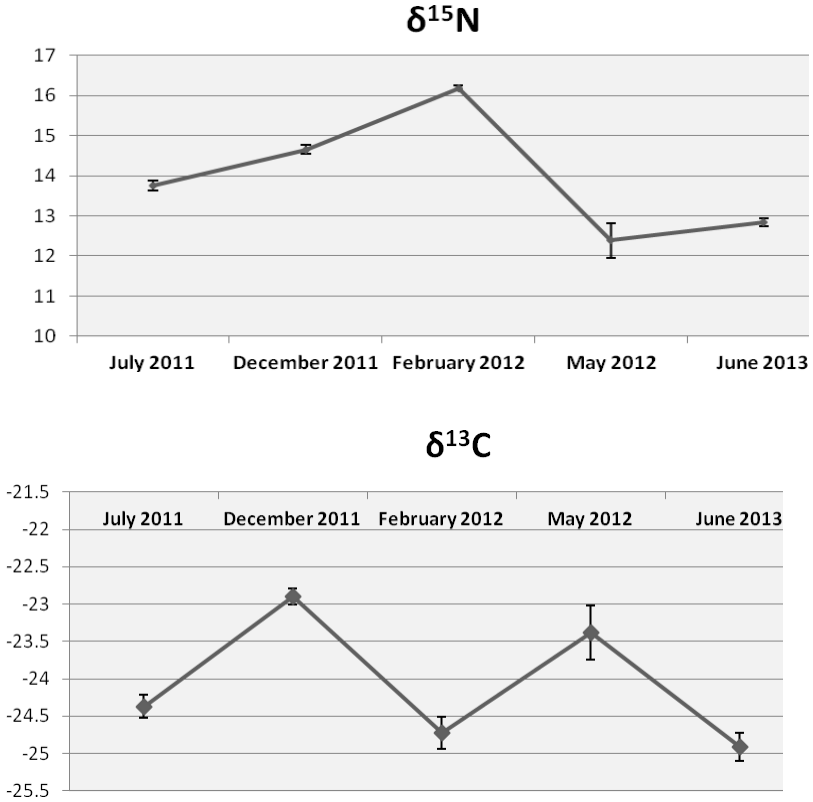

Figure 3.5. Variation of $\delta^{15}N$ and $\delta^{13}C$ in *Aurelia* sp. 1 tissues (mean number ± SD).

Table 3.2. PERMANOVA analysis of $\delta^{15}N$ and $\delta^{13}C$ stable isotopes in *Aurelia* sp.1. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0,05$) are indicated in bold. Mo = month; Res = Residuals.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Mo	4	78,705	19,676	32,548	**0,0002**	4991	0,0002
Res	34	20,554	0,60453				
Total	38	99,259					

Prey. Several groups were identified in mesozooplankton samples, but the scarcity of material limited the number of replicates and, consequently, the accuracy and resolution of results for mesozooplankton prey signatures (Fig. 3.6) Isotopic signatures of seston showed minor variation for $\delta^{15}N$ (range from 9.18 ‰ to 9.47‰), and more variation for $\delta^{13}C$ ranging between -24.97 ‰ and -26.96 ‰. Microzooplankton had a $\delta^{15}N$ signature of 12.33 ‰ and a $\delta^{13}C$ signature of -26.05 ‰. Mesozooplankton (calanoid copepods) showed higher values than microzooplankton for $\delta^{15}N$ (12.19 ‰) and lower for $\delta^{13}C$ (30.34 ‰). Jellyfish were placed at the highest trophic level, with an average $\delta^{15}N$ of 14‰.

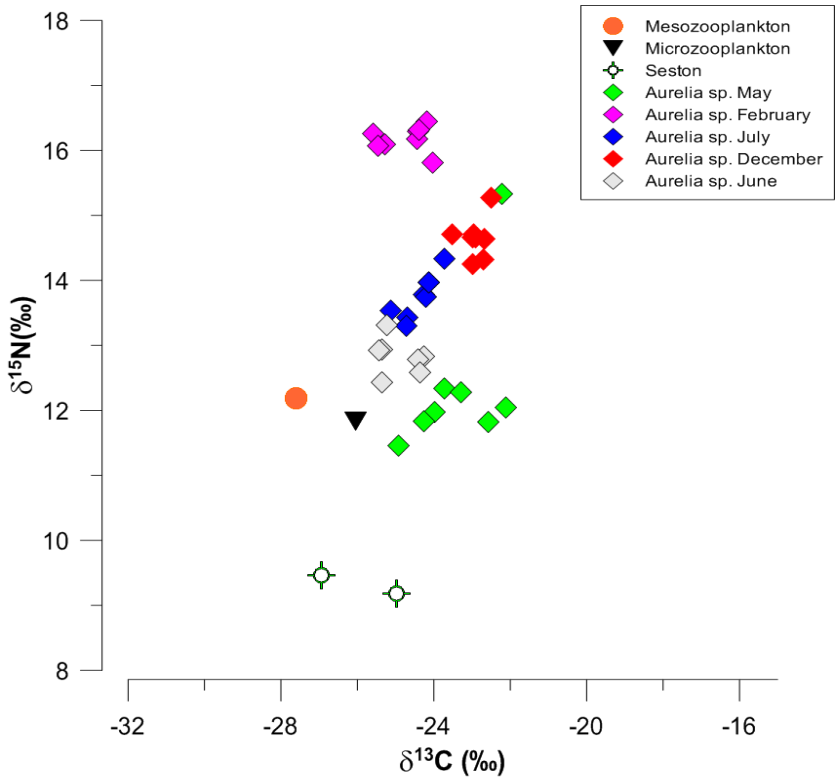

Figure 3.6. Biplot of $\delta^{13}C$ and $\delta^{15}N$ signatures of seston, microzooplankton, copepods and jellyfish.

The values of the nitrogen stable isotopes ($\delta^{15}N$) were used to estimate the relative trophic position of *Aureli*a sp. 1 calculated from calanoid copepods and seston as baseline organism (Fig. 3.7).

The results showed that *Aurelia* sp. 1 occupied a lower trophic position (TP) in late spring - early summer, comparable to an herbivourous predator (1.91 ± 0.13).

The jellyfish diet shift to omnivorous in summer when TP was 2.44 ± 0.03. Progressively in winter the jellyfish occupied higher trophic levels, typical of carnivorous diet with the maximum observed for February 2012 (3.25 ± 0.02).

A strong decrease in TP is observed from February to May (Fig. 3.7). However, as discussed in chapter 2, the population of May represented a new generation of medusae liberated trough strobilation of polyps in spring. Pearson's correlation coefficients calculated between $\delta^{15}N$ and bell diameter (BD) showed that the trophic level of *Aurelia* sp. 1 varied significantly according to medusa growth ($r = 0.60$, $p < 0.001$**).

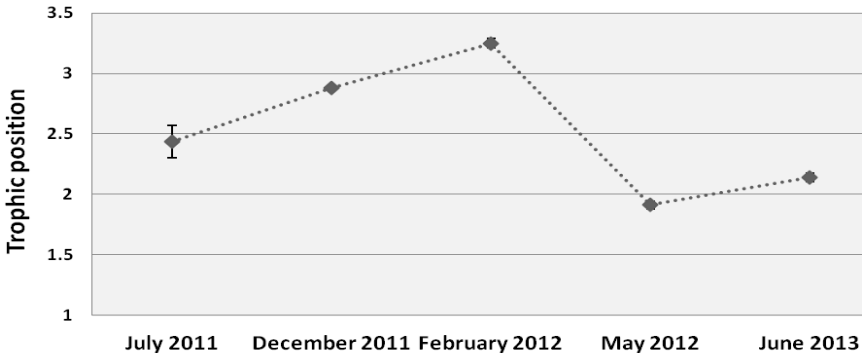

Figure 3.7. Variation of *Aurelia* sp. 1 trophic position (mean number ± SD).

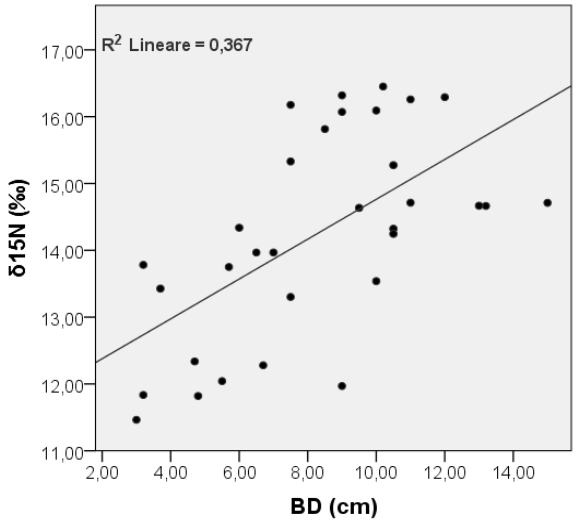

Figure 3.8. Pearson's correlation between $\delta^{15}N$ and bell diameter (BD) in *Aureli*a sp.1.

3.5.Discussions and conclusions

The scyphomedusa *Aurelia* sp. is one of the most studied jellyfish taxon and represents an important predator of zooplankton and icthyoplankton in marine and coastal waters (Moller, 1980; Barz and Hirche, 2005; Ishii and Tanaka, 2001; Purcell, 2003).

In the present work, investigation on composition and abundance of preys found in the stomach contents of *Aurelia* sp.1 allowed to identify seasonal variations and mechanism of prey selection. Jellyfish predate on food sources of different sizes in the Varano lagoon, from micro- to mesozooplankton, selecting prey as a consequence of differential prey vulnerability (Suchman and Sullivan, 2000). The variety of food items included in the jellyfish diet is determined by the combination of the natural variability of prey abundance in the water column and the life history of *Aurelia* sp.. In the Varano lagoon, polyp strobilation occurs in April, and the first size class (1 - 5 cm) is always observed in May (Chapter 2). Diet composition revealed that small medusae base their diet on small prey. In May 2011 most of prey found in the gut content were invertebrate larvae as bivalve and gastropods veligers. In the following year, when the gastropods larvae abundance strongly decreased and bivalves abundance was also lower (Chapter 2, Fig. 2.20), other prey as tintinnids and invertebrate eggs were positively selected by *Aurelia* sp.. The high percentage of bivalve (64 %) and gastropod veligers (21 %) in the guts and the observation that most were undigested might be the result of very low digestion rates as documented for another jellyfish species, *Chrysaora quinquecirrha* (Purcell et al., 1991). The aforementioned study also concluded that 99% of ingested bivalve larvae remained undigested and then egested, and that 98% of them survived following egestion. This evidence suggests that in Varano lagoon jellyfish could capture and release veligers far from the site of ingestion thus favoring the larval dispersion. On the other hand, proofs of egestion were not documented for any *Aurelia* spp. , so the Varano medusae could be really able to digest shelled veligers in a longer time.

Significant results come from the finding of tintinnid ciliates in the gut content of *Aurelia* sp. Their recurrent dynamic in springtime confirmed the hypothesis that microzooplankton play a key role in sustaining the rapid growth of medusae in Varano lagoon.

Oppositely, copepods represented on average 70% of mesozooplankton community in May 2011 and 60% in May 2012, but only a small percentage contribute to *Aurelia* sp. 1 diet in the spring period (12.5 and 10.8 %). A poor nutritional quality offered by adult calanoid copepods has been suggested by Båmstedt et al. (2001) as explanation of the limited growth rate (4 - 9 % d^{-1})

observed in *A. aurita* ephyrae compared to other prey items like phytoplankton and POM (7 - 10 % d^{-1}).

Pearre's selectivity index demonstrated that in Varano lagoon calanoid copepods were preyed in proportion to their abundance over the year. In effect, calanoid copepods can avoid ingestion by medusae through advanced mechanosensory and escape capabilities (Suchman and Sullivan, 2000). More often, small calanoid and cyclopoid copepods are known to contribute substantially to *Aurelia* spp. diets (Ishii and Tanaka, 2001; Turk et al., 2008; D'Ambra et al., 2013). Indeed, in the Varano lagoon, small cyclopoid copepods were positively selected by jellyfish over the year sampling, with exception of July 2011, when abnormally high sea surface water temperature had occurred.

The increase in size corresponds for jellyfish to a different energetic requirement but also facilitates encounter, holding and ingestion of larger prey (Båmstedt et al., 2001). This resulted in a higher number of prey categories found within the 10 - 15 size class of *Aurelia* sp. 1 population in September. In the same month, a positive selection towards cyclopoid copepods was significantly high.

A positive selectivity index recorded for harpacticoids, a benthic copepod taxon exhibiting vertical migration in the water column (Walters and Bell, 1986), can indicates that the jellyfish might feed on this group at night, also favored by the shallow depth of the Varano lagoon. Conversely, an alternative hypothesis may be related to the horizontal tows at subsurface levels made for zooplankton sampling from the water column in daytime, when harpacticoids are restricted to the benthic domain. Therefore, the hypothesis of a positive selection for harpacticoids needs to be reevaluated through stratified and nocturnal sampling, including in the proximity of bottom layers. Also, the high C index for invertebrate eggs might be biased by the absence of eggs in the zooplankton samples collected by sub-surface tows. Eggs are usually be concentrated in the neuston near the surface proximity, where jellyfish can occasionally feed and from where jellyfish are sampled by hand- nets.

Many ecological studies concerning jellyfish impacts addressed the trophic interaction with fish and the consequence on the food web (Brodeur et al., 2008; Purcell and Arai, 2001; Quiñones et al., 2013; Sabatés et al., 2010; Titelman and Hansson, 2005). These studies followed the record of up to 68 Atlantic herring larvae eaten by a single, 4 cm-sized *Aurelia aurita* jellyfish (Möller, 1980).

In Varano lagoon, apparently fish eggs were not selected by jellyfish. However, the most common fish species, *Atherina boyeri*, reproduce by massive and coordinated spawing of eggs and sperms. Following fertilization, embryos rapidly sink to the bottom, where they adhere to the substrate by maternal specialized adhesive filaments. This strategy may prevent this fish to suffer substantial predation from jellyfish. Also fish larvae were never observed in the gut content of medusae, indicating the absence of direct predation on ichthyoplankton. In fact, it is known that fish larvae can perceive predator and consequently escape. The larval size, the nutritional state and the oxygen condition are involved in the success of escape reactions (Bailey and Batty, 1983; Purcell et al., 1987; Nakayama et al., 2003; Shoji, 2008).

The association of gut content analysis with the comparison of the isotopic signature of a consumer with its potential sources of prey, provides additional information about the relative contribution of different sources of prey to the diet of the consumer (Pitt et al., 2008b). More precisely, the use of trophic levels allow the capture of complex interactions and trophic omnivory that are prevalent in many ecosystems (Paine, 1988, Vander Zanden and Rasmussen, 1999).

In *Aurelia* sp.1, the lowest $\delta^{15}N$ signatures were observed in May and June when the zooplankton community was represented mainly by invertebrate larvae and tintinnids and the jellyfish population was represented by young (1-2 months aged) medusae. The trophic level calculated on nitrogen stable isotope ratio for prey demonstrated an herbivorous behaviour and confirmed that phytoplankton and microzooplankton represent a key resource in the early stage of *Aurelia* sp. medusae in the Varano lagoon.

However, the increase during winter months and the highest $\delta^{15}N$ signature in February indicates a progressive shift in the moon jellyfish diet. In December 2011, more than 80% of the mesozooplankton community was represented by cyclopoids copepod (Chapter 2, Fig. 2.18) whereas in February 2012 calanoid copepods represented up to 98% of total mesozooplankton abundance. The increase in $\delta^{15}N$ in December can be interpreted as result of the selective predation on cyclopoids, as demonstrated by gut content analysis where copepods represented 82% of prey taxa abundance. In February 2012, *Aurelia* sp. 1 moved to a higher trophic level behaving as top carnivorous predator. Calanoid copepods and invertebrate eggs may contribute equally to the increase in $\delta^{15}N$. The successive decrease in TL from February to May was only

the consequence of two different jellyfish generations taken in account, which show different bell size.

The present results confirmed the importance of size in the jellyfish - copepods interactions (Suchman and Sullivan, 2000) and suggested that null selection for calanoids in February was only due to the high natural abundances of these prey and on the ability of jellyfish to alternate the taxa preyed. Finally, the enrichment in $\delta^{13}C$ signatures in *Aureli*a sp. 1 tissues reflected the reduction in the contribution of terrestrial inputs to the jellyfish diet and the increased contribution of marine sources due to the strenght of sea water inflow (see Chapter 2, April and December). Contrarely, the low $\delta^{13}C$ signatures at the begin of summer and winter reflected the contributions of terrestrial inputs in the jellyfish diet.

The present results led to conclusion that *Aurelia* sp. 1 behaves as a generalist, omnivorous species in Varano lagoon, feeding on zooplankton prey mainly in a non-selective way.

The results from gut content analysis highlighted that ontogenetic shift in *Aurelia* sp. diet can allow the co-existence during spring of two jellyfish generations: a population of newly liberated, small jellyfish and an overwintering cohort from the previous year. Biochemical analysis unveiled the importance of microzooplankton and phytoplankton in the diet of the earliest life stages of jellyfish, calling for further application of integrated methodologies towards a better understanding of jellyfish bloom dynamics in coastal waters.

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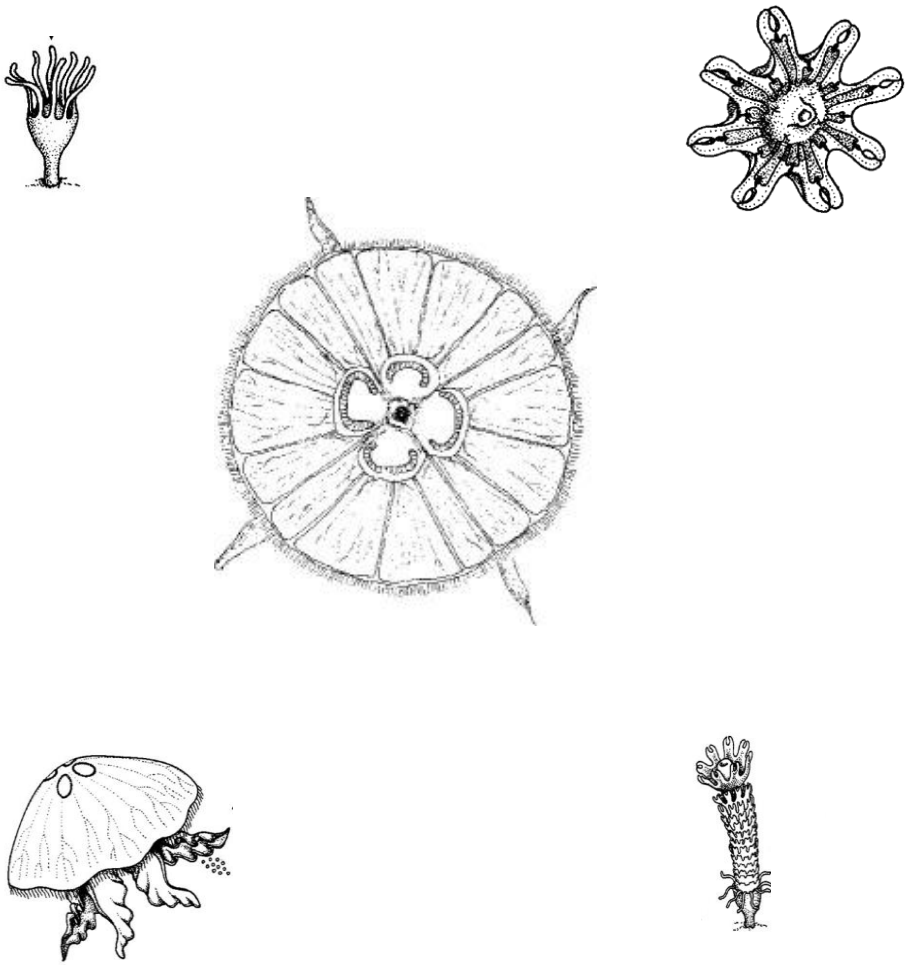
CHAPTER FOUR:

Aurelia **spp. cryptic species in the Mediterranean basin**



4. Chapter 4 : *Aurelia* spp. cryptic species in the Mediterranean basin.

4.1. Abstract

The moon jellyfish has been considered one of the most popular example of cosmopolitan geographical distribution (Kramp, 1968; Arai 1997) and only two to three taxa, *Aurelia aurita, A.limbata, A.labiata*, were recognized as valid species until the advent of molecular tools, supporting evidence of *Aurelia* as a wide cryptic species complex (Dawson and Jacobs, 2001; Shroth et al 2002)..

In the present study, the morphology and morphometrics of polyp, ephyra and medusa stages were investigated in different moon jelly populations sampled in different habitats and areas of the Mediterranean Sea. Morphological and developmental observations from each sampled population were coupled to DNA barcoding (mitochondrial cytochrome C oxidase subunit I, COI) to achieve a double, integrated identification of the Mediterranean *Aurelia* spp..

The integration of classical taxonomy and morphometrics provided insights for appropriate selection and measurement of morphological characters for both intraspecific and interspecific analyses of morphological variation. Supported by DNA barcoding, these analyses demonstrated the occurrence in the Mediterranean Sea of three cryptic species of *Aurelia* (sp.1, sp.5, sp.8) and, conversely, the absence of any of the three currently historically accepted *Aurelia* species (*A.aurita, A.limbata, A.labiata*). This is the first record of the invasive alien *Aurelia* sp.1 in the Adriatic Sea and its first morphometric description for the Mediterranean Sea.

Overall, this study represents the first attempt based on integrated morphological/morphometric and molecular approaches to reconstruct the systematics and patterns of geographical distribution of the genus *Aurelia* in the Mediterranean basin.

4.2. Introduction

Taxonomy is the science of describing, naming and classifying living things and represents the first step to understand the biodiversity. This concept has been often underestimated in the last years and taxonomy has progressively lost its leading role for reasons linked to commercial or political treaties (Agnarsson and Kuntner, 2007; Boero, 2001a, 2010). The World Register of Marine Species (WoRMS) currently recognizes the presence of 221.366 species (March 2014) although near 91% of species in the ocean still await formal description (Mora et al., 2011).

Taxonomy must also face up to the "species problem" (Guerra-García et al., 2008) especially in the context of cryptic species which are surprisingly spread across taxa and biomes (Pfenninger and Schwenk 2007). Cryptic or sibling species are two or more distinct but morphologically similar species often classified as a single species. In this prospective, the application of DNA barcoding (Hebert et al., 2003) represents a new frontier. The standard DNA barcode region, a region of approximately 648-655 bp of the mitochondrial gene cytochrome c oxidase I (COI) was demonstrated to be useful for species identification although its efficiency is reduced when intraspecific variation and genetic differentiation between sister species overlap, confounding true species boundaries (Avise, 2004; Meyer and Paulay 2005). Nevertheless, the recognition of cryptic species since the advent of DNA barcoding has increased exponentially over the past 20 years, suggesting that molecular data should be routinely incorporated in diversity and conservation research (Bickford et al., 2007).

DNA barcoding has been applied for identification and recognition of most taxa of marine metazoans, especially zooplankton (Bucklin et al 2007, 2010a, 2010b). In contrast with commercial fish species and other key zooplankton taxa (e.g. crustaceans), only 6% of the cnidarian species diversity (i.e. jellyfish and polyps) have been investigated by a combination of barcodes and traditional methods (Bucklin et al., 2011). In this group, most species descriptions are primarily based on morphological characters (Linnaeus, 1758; Mayer, 1910) and only recently COI sequences have revealed their potential for systematics and phylogenetic studies (Dawson and Jacobs, 2001; Dawson, 2005).

Traditional morphological studies often led to contrasting results, identifying separate but highly similar morphospecies with disjunct geographical ranges, or single taxa with wide or cosmopolitan distribution. In scyphomedusae, the moon jelly *Aurelia aurita* has been long considered as an example of widely distributed species (from Pole to Pole) with broad ranges of temperature and salinity tolerance (Papathanassiou et al., 1987; Russell, 1970). The first attempt to understand species diversity within the Aureliinae subfamily goes back to Mayer (1910), who recognized 13 distinct local varieties, grouped into 3 well-defined morphotypes *(Aurelia aurita, A. labiata, A. solida)*. Until the end of XXth century, only two valid species were recognized: *A. limbata,* a polar species, and *A. aurita,* an ubiquitous species present in nearshore waters circumglobally between about 70 °N and 40 °S (Kramp, 1968; Russell, 1970, Arai, 1997). A third valid species, *A. labiata,* was then redescribed as native to Pacific North America (Wrobel and Mills, 1998; Gershwin, 2001). More recently, mithocondrial (COI, 16S) and nuclear (ITS-1)

DNA sequences pointed out at least nine to thirteen distinct clades of *Aurelia* should be considered as different species exhibiting different levels of local adaptations (Dawson and Jacobs, 2001; Dawson and Martin, 2001; Schroth et al., 2002; Dawson, 2003).

In this framework, adaptive polymorphism, i.e. expression of different phenotypes, can facilitate species diversification by enhancing ecological success over a wide geographical range, especially for species with complex, bentho-pelagic or pelage-benthic, life histories.

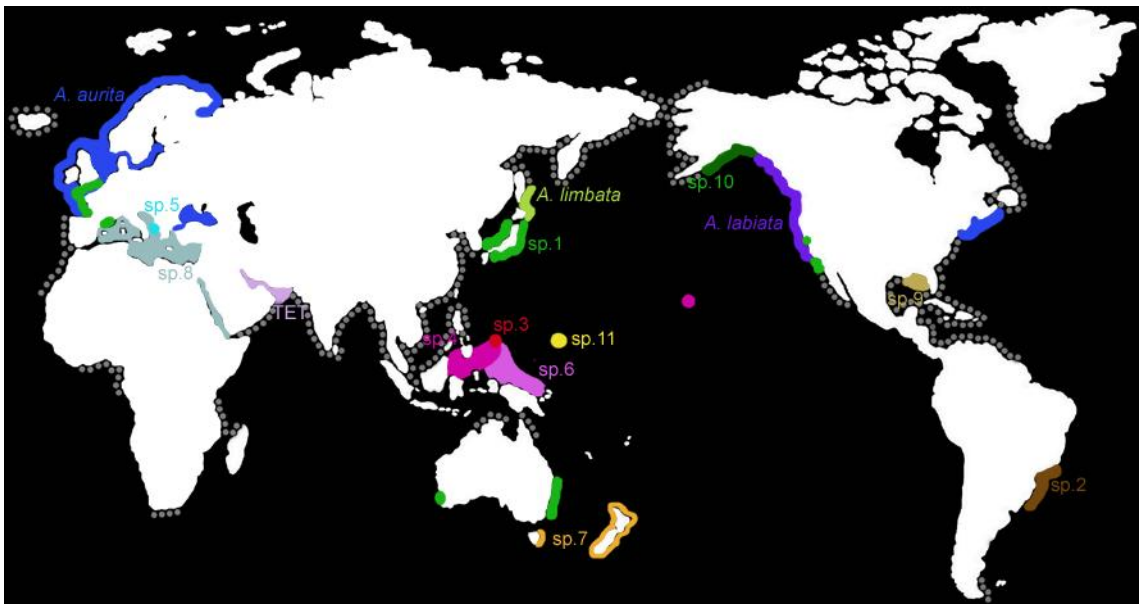


Figure 4.1. Global distribution of *Aurelia* cryptic species (Source: http://thescyphozoan.ucmerced.edu/).

The hard challenge of modern taxonomists is to interpret the work of nineteenth-century systematics deconstructing often inadequate species descriptions. For taxa with broad geographical distribution, such as the *Aurelia* clade, an integrated approach between morphological and molecular methods (Godfray, 2002; Dawson, 2003) is essential to reconstruct the evolutionary diversification and phylogeny of extant species. Unfortunately, even recent papers still perpetuate unsubstantiated identification of moon jellyfish as *Aurelia aurita*, based on approximate "parataxonomical" analysis (e.g. see Ben Faleh et al., 2009; Tomaru et al., 2014)

Such an integrated approach makes sense especially in the present scenario of increasing records of gelatinous blooms (Brotz et al,. 2012). In the Mediterranean sea, reports of *Aurelia* spp. outbreaks are becoming more and more frequent not only in coastal lagoons (Varano, Italy; Etang de Thau, France; Bizerte, Tunisia) and marinas (Empuriabrava),) but also in nearshore

open waters (South Adriatic Sea, Gulf of Tunis, Ligurian Sea) as documented by ongoing research projects of citizen science (e.g. MED-JELLYRISK 2012-2015, see http:/www.jellyrisk.eu).

Focusing on the Adriatic basin, old jellyfish reports indicate *A. aurita* and *Rhizostoma pulmo* as the most frequently reported scyphomedusae in the northern Adriatic over the 1785-2010 period, with *Aurelia* dominance in the 2004 year (Di Camillo et al., 2010; Kogovšek et al., 2010; Malej et al., 2012).

Molecular analysis of *Aurelia* in the Mediterranean led so far to the undisputable identification of *Aurelia* sp.8 (Dawson et al., 2005, Ramšak et al., 2012) and *Aurelia* sp. 5 (Dawson and Jacobs 2001). More questionably, the 16S and ITS-1 analyses by Schroth et al (2002) included sequences obtained from specimens from Perpignan, Western Mediterranean, referable to a third species, *Aurelia* sp.1, known to be the *Aurelia* species with the broadest known distribution so far. Nevertheless, molecular data from Mediterranean specimens were never paralleled by morphological analyses of voucher populations.

The present study addressed the taxonomic confusion surrounding the *Aurelia* species in the Mediterranean and pointed out relevant morphological characters in the three life stages (polyp, ephyra, medusa) to discriminate *Aurelia* spp. species.

4.3. Methods

4.3.1. Study areas and samples collection

Morphological species identifications were carried out on *Aurelia* spp. Mediterranean populations from four localities: Empuriabrava (Balearic Sea), Mljet lakes (Adriatic sea), Piran (Adriatic sea), Varano (Adriatic sea). In laboratory, polyps and ephyrae were reared at 14°C and fed daily with *Artemia salina* nauplii. Medusae were collected by hand net then fixed in 4% formalin sea water solution and finally destined to morphological analyses.

Additional *Aurelia* specimens were sampled for genetic analyses across the Mediterranean and the North East Atlantic and the North Sea. A total of 54 specimens were collected from 6 Mediterranean localities: Varano and Mljet Lakes (western and eastern Adriatic sea), Porto Cesareo (Ionian Sea), Empuriabrava (Balearic Sea), Bizerte Bay and Bizerte lagoon (southern Mediterranean Sea); 3 localities in the North East Atlantic (Southampton, Oban, Orkney) and one from the North Sea (St Andrews).

Details on samples and their origins are provided in Figure 4.2 and Table B-I (Appendix B). Samples were taken by hand-net and a little piece (4-5 cm) of bell margin or oral arm was preserved in 95% ethanol at -20°C until DNA extraction.

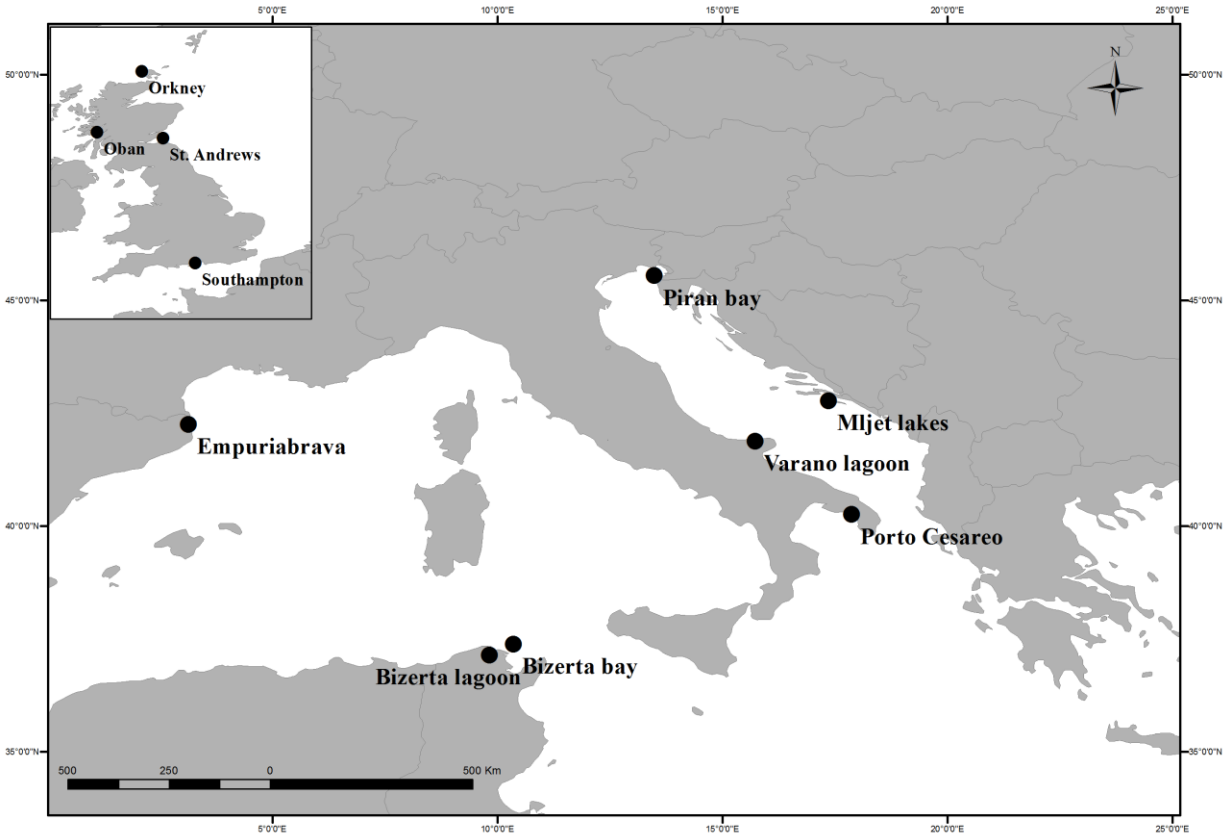


Figure 4.2. Locations of *Aurelia* spp. collection in the Mediterranean and northern European coasts.

4.3.2. Molecular species-level identification

Total genomic DNA was extracted from the ethanol preserved tissues, following a CTAB-phenol-chloroform based protocol (Dawson et al., 1998; Dawson and Jacobs, 2001). Mitochondrial cytochrome C oxidase subunit I (COI) was amplified using the primers LCOjf (Dawson, 2005) and HCO2198 (Folmer et al., 1994) in a Eppendorf Mastercycler Gradient thermal cycler using the following profile: 94°C for 4 min, 51°C for 2 min, 72°C for 2 min, 94°C for 4 min, 51°C for 2 min, 72°C for 2 min; 33 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 60 sec; final extension at 72°C for 5 min and refrigeration at 4°C. Size and quality of the PCR products were examined on 1.5% agarose gels stained with GelRed™ and then purified with DE-001 GEL/PCR extraction and purification kit (Fisher Molecular Biology). The purified

products were used as template DNA for cycle sequencing reactions performed by Macrogen (Korea - http://www.macrogen.com). Both DNA strands were sequenced.

The data set included a total of 54 sequences of *Aurelia* spp. Sequences from GenBank belonging to *Aurelia* sp. 1 to *Aurelia* sp. 10, *A. aurita*, *A. labiata* and *A. limbata* were also included in the analyses and used as references.

Sequences were viewed with 4Peaks (http://nucleobytes.com/index.php/4peaks) and contigs were assembled using Cap3 (Huang and Madan, 1999). The identity of the sequences was confirmed by matching them against the Nucleotide collection (nr/nt) database of NCBI, using the BLASTN search algorithm. Alignments were made with CLUSTALX version 2.0 (Thompson et al., 1997) using 10 as gap open and 0.1/0.2 as pairwise and multiple extension penalties. The alignment was edited using MacClade 4.08a (Maddison and Maddison, 2005) and its correctness was verified at the amino acid level using the Coelenterate mitochondrial code in MEGA 5.2 (Tamura et al., 2011), in order to ensure that no gaps or stop codons were present in the alignment.

For distance analyses, specimens were firstly grouped according to their sampling locality and pairwise intra-groups and inter-groups distance matrices were generated with MEGA 5.2 using the Kimura 2 parameters (K2P) model. Once determined the membership of each specimen to a certain species on the base of intra-group and inter-group genetic distances, within and between species genetic distances were evaluated using the same methods.

Neighbor-joining (NJ) and Maximum Likelihood (ML) trees of original and reference GenBank sequences were constructed based on the K2P model in MEGA 5.2. Bootstrap values were calculated using 1,000 iterations.

4.3.3. Morphological comparison and morphometrics

For each population, 30 polyps, 6 ephyrae and 10 jellyfish were subjected to morphometric measurements and comparisons (Dawson, 2003; Straehler-Pohl et al., 2011). Abbreviations of morphometric variables used in this study for each stage are given in Figure 4.3. Observations on polyp and ephyra morphology and development of gastric system from ephyrae to small medusa were carried out by transferring randomly selected live polyps/ephyrae on a microscope depression slide and taking pictures with a Nikon Coolpix 990 camera mounted on Leica MZ 12 stereoscope. Measurements and incorporation of scale bars were made through the graphic software ImageJ.

For the jellyfish stage, due to the effects of formaldehyde preservation, features relative to tissue colour and total wet mass (features f14, f15, f16, f17; and f4, respectively as in Dawson, 2003) were not considered. Medusae from Mljet were not reproductively mature and the measures relative to the sex and subgenital pore were not considered in the total comparison (f11, f12, f13), as made in for the Empuriabrava – Varano comparison. The difficulty of finding jellyfish excluded the Piran population from the analysis of the medusa stage.

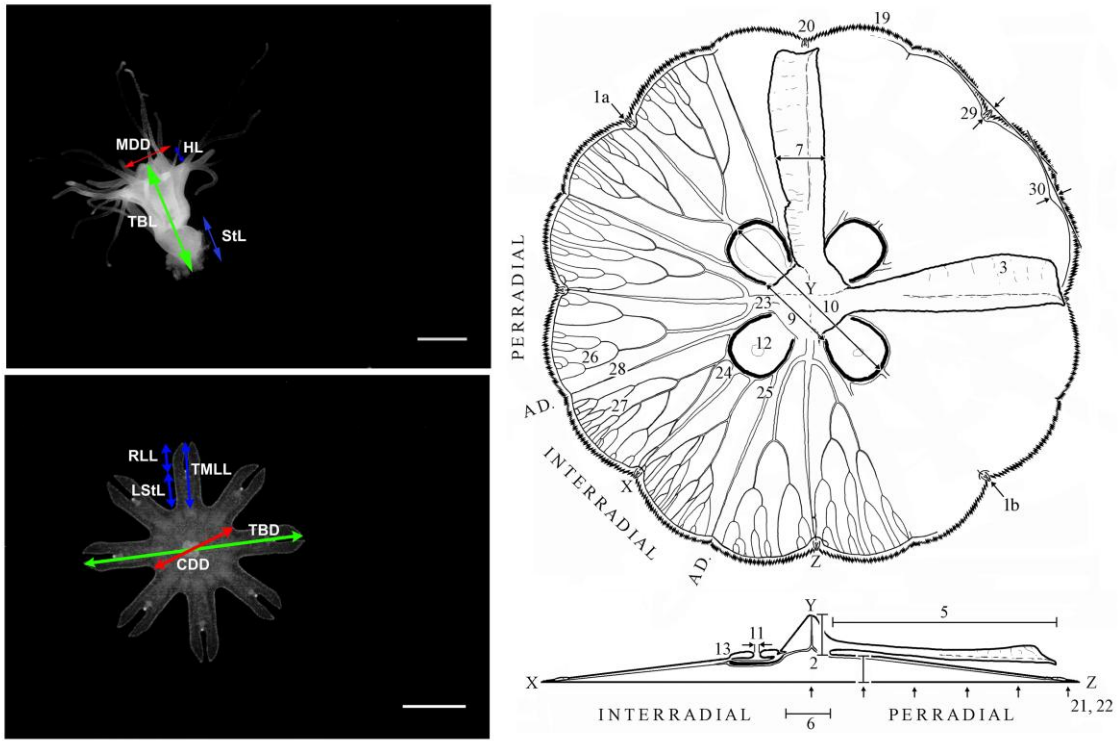


Figure 4.3. Morphometric measures analyzed in three life stages of *Aurelia* spp. a) polyp stage : CDD, central disc diameter; HL, hypostome length; StL, stalk length; b) ephyra stage: LStL, lappet stem length; MDD, mouth disc diameter; RLL, rhopalial lappet length; TBD, total body diameter; TBL, total body length; TMLL, total marginal lappet length; c) medusa stage: f1, bell diameter (mm from 1a to1b); f2, manubrium depth (mm); f3,folding of the oral arm (0–2, half-point intervals); f5, oral arm length (mm); f6, manubrium width (mm); f7, oral arm width (mm); f8, gastric pouch shape; f9, proximal gastric diameter (PGD, mm); f10, distal gastric diameter (DGD, mm); f11, subgenital pore diameter (mm); f12, subgenital pore position (central, inside, overlapping, outside); f13, subgenital pore thickening (0–2, half-point intervals); f19, number of lobes; f20, number of rhopalia; f21, bell shape; f22, bell thickness; f23, perradial origins (qtr -1); f24, interradial origins (qtr -1); f25, adradial origins (qtr -1); f26, perradial anastomoses (qtr -1); f27, interradial anastomoses (qtr -1); f28, adradial anastomoses (qtr -1); f29, rhopalar indent (mm); f30, non-rhopalar indent (mm). Scale bar = 1 mm. Drawings of medusa stage from Dawson 2003, modified.

4.3.4. Data analyses

In order to remove any effect due to allometric growth the morphometric variables were adjusted through regressions analysis. Consequently, the morphometric dimensions were plotted against

TBL (total body length) in polyps and TBD (total body diameter) and TMLL (total marginal lappet length) in ephyrae. In the medusa stage, morphometric measures were plotted against f1 (bell diameter) for the features showing significant correlation with f1 supported by the power of test equal to or greater than 0.8.

For this reason, the features associated to origins (f23, f24, f25) and anastomoses of canals (f26, f27, f28) that did not change with the size were considered without any transformations trough regression analysis.

Statistical analyses were performed with PRIMER 6+ software (Plymouth Marine Laboratory) using a significant level of 0.05 (Clarke and Warwick, 2001). A resemblance matrix based on Euclidean distance was calculated on normalized data in each life stage. All data were analyzed using permutational multivariate analysis of variance (PERMANOVA) statistical test with location as fixed factor (4 levels) in the PERMANOVA design (Anderson, 2001). Subsequent pair – wise test performed on each character (f) in the medusa stage, identified significant characters among populations.

In all the life stages, one-way similarity percentage analysis (SIMPER) routine was also used to determine which characters contribute most to the dissimilarity between *Aurelia* spp. populations. Finally, a constrained canonical analyses of principal coordinates (CAP) was used to find the axes in the principal coordinate space that best discriminate among the a priori groups (Anderson and Willis, 2003).

4.4 Results

4.4.1 Molecular species-level identification

DNA sequences of a 655 bp long fragment of the mitochondrial cytochrome oxidase subunit I (COI) were obtained from 54 *Aurelia* spp. specimens Kimura 2 parameters genetic distances for pairwise comparisons across the entire dataset ranged widely from 0.0 to 0.27 with a mean of 0.16 (Table 4.1). Grouping the samples according to their geographic origin, the average genetic distances between all sequence pairs within groups was 0.004 (from 0.001 in Bizerte Bay to 0.006 in Porto Cesareo), whereas the average difference between groups was 0.18 (from 0.003 between Bizerte Bay and Bizerte Lagoon to 0.25 between Varano and UK specimens) (See Table 4.1 for details about K2P sequence divergences).

Specimens from the Varano Lake and Empuriabrava belong to the same molecular clade (K2P between groups distance= 0.006); separately, specimens from Bizerte Bay, Bizerte lagoon and Porto Cesareo (K2P average between groups distance=0.004) group together. The specimens from Mljet Lake form a third, distinct group (K2P within group distance = 0.002). Finally, all specimens from the NE Atlantic and North Sea group together within a fourth, distinct molecular clade (K2P between groups distance= 0.005). So, the 54 sequences of *Aurelia* spp. collected across the Mediterranean Sea and North sea comprise at least four genetically distinct species of *Aurelia*.

The Neighbor-Joining and Maximum likelihood analyses, including COI fragment sequences from 13 known molecular *Aurelia* species available in GenBank, yielded unrooted trees with the same topology (Figure 4.4 shows the ML Tree; NJ tree not shown) and confirmed the monophyly of the individuals belonging to the previously identified clades, with high bootstrap support values (99%) for each cluster. The main phylogroups found were: *Aurelia* sp. 2 and *Aurelia* sp. 9 from the west Atlantic Ocean, *Aurelia* sp. 10 and *A. limbata* from northern Pacific Ocean, and the Pacific species *Aurelia* sp. 3, 4, 6, 7 and *A. labiata* that constituted another unresolved paraphyletic group. The remaining four phylogroups contained sequences from the present study. In particular, In particular, the specimens collected in the Mljet Lake confirmed to belong uniquely to *Aurelia* sp. 5; the specimens from Bizerte (Bay and lagoon) and Porto Cesareo were recognized as *Aurelia* sp. 8; the specimens from the Varano Lake and Empuriabrava unambiguously clustered together with the *Aurelia* sp. 1 sequences from Australia, California and Japan; finally, all specimens collected from the NE Atlantic and North Sea clustered with sequences from GenBank deposited sequences of *Aurelia aurita* from the Irish sea and the Bosphorus.

Figure 4.4. Maximum likelihood tree. Phylogenetic relationships within *Aurelia* spp. derived by Maximum likelihood analyses performed with Kimura 2-parameters method. For graphic reasons, the specimens' names have been omitted. Numbers at nodes are the bootstrap supporting values.

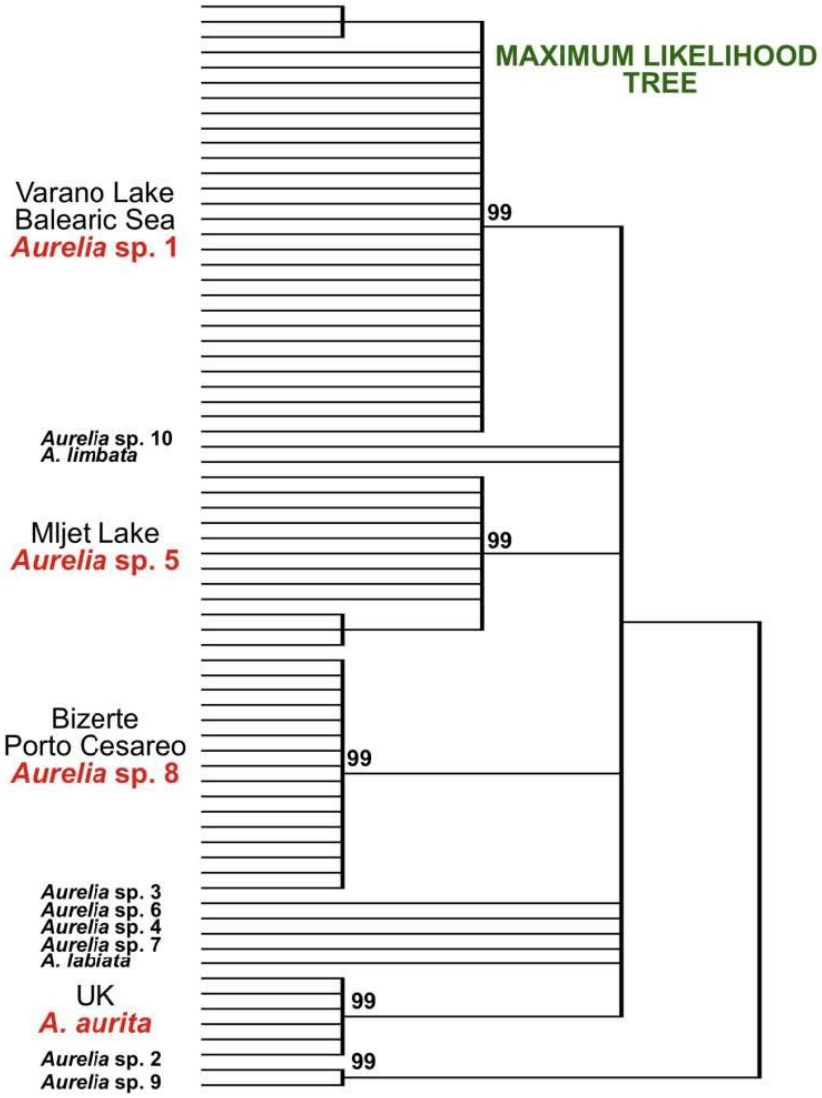


Table 4.1. Kimura 2-Parameters genetic distances within and between sampling localities and species.
a) Number of base substitutions per site from averaging over all sequence pairs within each sampling locality group. The second column shows the standard error estimates obtained by a bootstrap procedure (1000 replicates).
b) Number of base substitutions per site from averaging over all sequence pairs between sampling localities groups. Below the diagonal: K2P genetic distances between groups; above the diagonal: standard error estimates obtained by a bootstrap procedure (1000 replicates).

a)

Locality	Within-group mean genetic distances	Standard error estimates
Varano Lake	0.006	0.002
Mljet Lake	0.002	0.001
Empuriabrava	0.003	0.002
Porto Cesareo	0.006	0.002
UK	0.005	0.002
Bizerte Bay	0.001	0.001
Bizerte Lagoon	0.005	0.001

b)

Locality	Varano	Mljet	Empuriabrava	Porto Cesareo	UK	Bizerte Bay	Bizerte Lagoon
Varano Lake		0.028	0.002	0.025	0.029	0.025	0.025
Mljet Lake	0.240		0.028	0.027	0.028	0.027	0.027
Empuriabrava	0.007	0.236		0.025	0.028	0.025	0.025
Porto Cesareo	0.211	0.232	0.208		0.028	0.001	0.001
UK	0.254	0.239	0.250	0.248		0.027	0.028
Bizerte Bay	0.211	0.226	0.207	0.004	0.245		0.001
Bizerte Lagoon	0.213	0.232	0.209	0.006	0.249	0.003	

4.4.2 Morphological identification: polyp stage

Observations made on the morphology of the body structures showed that completed scyphistoma hold at least 16 tentacles variable between 16 and 22 in all the populations. The tentacles displayed the same shape except for Mjet polyps, showing a thickening in the their distalmost tip. Tentacles branching was rarely observed in all populations. The mouth appeared cruciform in Empuriabrava, Piran and Varano, dome-shaped in Mljet polyps. Polyp colour was not considered due to food-derived pigmentation in laboratory cultures.

In the studied polyps slight variations in morphology were accompanied by stronger variation in standard morphometric parameters (Tab 4.2), which allow to distinguishing polyps of different populations. Polyp length (TBL) oscillates between 1 and 3 mm. The mouth disc diameter (MDD) was the characteristic that showed the strongest variation among populations. MDD represented more than half-body length in Mljet, Piran and Empuriabrava, while the mouth

diameter exceeds the total length in Varano (Table 4.2), therefore explaining the observed squat shape in polyps (Figure 4.5).

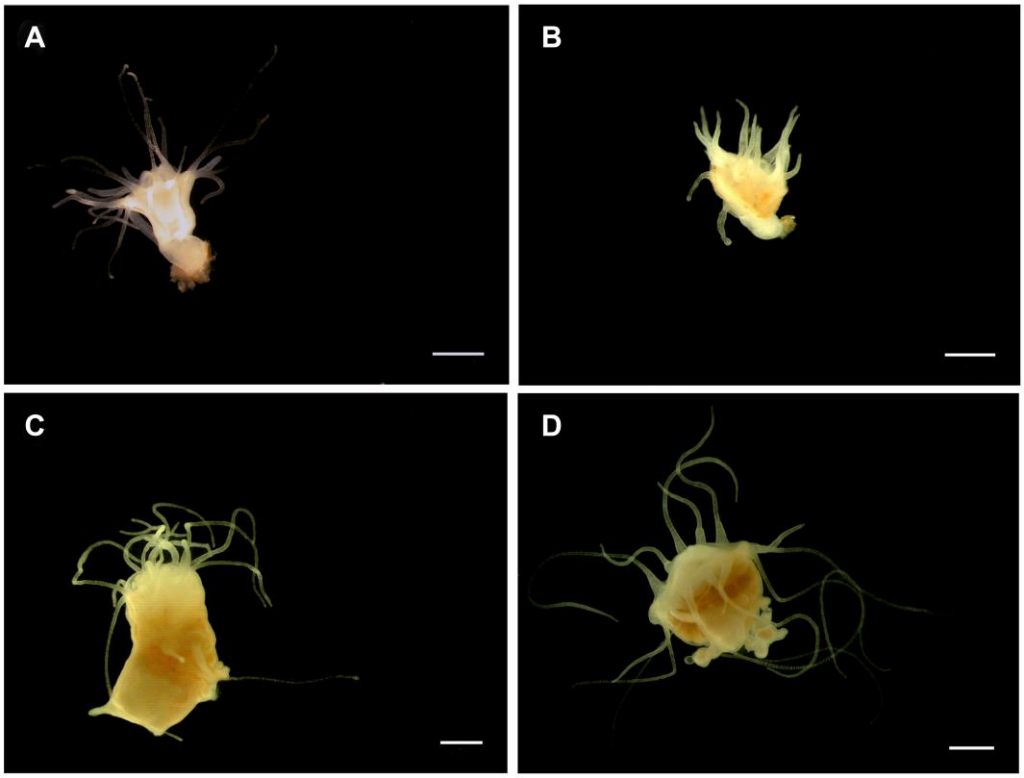


Figure 4.5. Morphology of *Aurelia* spp. polyps. a) Empuriabrava b) Mljet c) Piran d) Varano. Scale bar = 1 mm.

Exception made for Varano population, hypostome length (HL) and the stalk length (StL) played the predominant role in discriminating the other populations (Table 4.4) In Varano and Piran, stalk length represented the lower portion of the entire body length while higher values were reached in Empuriabrava and Mljet. In polyps from Empuriabrava the length of hypostome (HL) was sensibly lower than in Mljet, Piran and Varano (Table 4.2).

PERMANOVA statistical analysis demonstrated that in the pair – test comparison the four populations were significant different in terms of morphometrics of polyp stage (Table 4.3 b). Plot of the principal coordinates from the constrained CAP analysis displaced scyphopolyp populations through the variables MDD and StL and showed the population of Varano as the most dissimilar (Fig. 4.6).

Table 4.2. Morphometrics values observed in the polyp and in the ephyra stage of *Aurelia* spp. separated by location.

Life stage	Measure	Empuriabrava (*Aurelia* sp.1)	Mljet (*Aurelia* sp.5)	Piran (*Aurelia* sp.8)	Varano (*Aurelia* sp.1)
Polyp	TBL (mm)	1,6 ± 0,1	2,2 ± 0,1	2,9 ± 0,1	2 ± 0,2
	MDD/TBL (%)	59,2 ± 11,9	77 ± 4,6	59,7 ± 3,6	134 ± 3
	HL/TBL (%)	11,6 ± 1,5	18,8 ± 2,2	15,7 ± 1,4	16,7± 0,8
	StL/TBL (%)	22,7± 0,9	17,1 ± 1,8	7,21 ± 1,1	8,45± 1,5
Ephyra	TBD (mm)	3,4 ± 0,1	3,5 ± 0,2	2,8 ± 0,1	4 ± 0,3
	CDD/TBD (%)	42,2 ± 0,5	40,3 ± 0,3	39.6 ± 0,8	45,7 ± 0,7
	LStL/TMLL (%)	58 ± 0,7	53,7 ± 0,7	60 ± 1,5	62 ± 1,8
	RLL/TMLL(%)	42 ± 0,6	46,3 ± 0,7	40,1 ± 1,5	37,9 ± 1,8
	LStL/TBD (%)	17,3 ± 0,3	16,8 ± 0,5	18,7 ± 0,8	17,2 ± 0,5
	RLL/TBD (%)	12,5 ± 0,3	14,3 ± 0,4	12,2 ± 0,4	10,4± 0,6
	TMLL/TBD (%)	30,3 ± 0.5	31 ± 0,8	30.9 ± 0,7	27,6 ± 0,5

Results expressed as mean of values recorded. Each population comprised 30 polyps and 6 ephyrae at time 0.

Table 4.3. Results from PERMANOVA test on morphometrics data in *Aurelia* spp. polyps and ephyrae.

a) Main test

Polyp stage.							
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**	**P(MC)**
Po	3	111.89	37.296	17.65	**0.0002**	4982	**0.0002**
Res	116	245.11	2.113				
Total	119	357					
Ephyra stage.							
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**	**P(MC)**
Po	3	57.761	19.254	4.799	**0.0002**	4987	**0.001**
Res	20	80.239	4.012				
Total	23	138					

b) Pair - wise test

PAIR – WISE TEST									
Pairwise comparison for location					**Pairwise comparison for location**				
Stage	**Comparison**	**t**	**P (perm)**	**P (MC)**	**Stage**	**Comparison**	**t**	**P (perm)**	**P (MC)**
Polyp	V vs. M	3.6127	**0.0002**	0.0002	**Ephyra**	V vs. M	2.0556	**0.0042**	0.0134
	V vs. P	4.4311	**0.0002**	0.0002		V vs. P	2.8543	**0.0016**	0.0054
	V vs. E	6,0335	**0.0002**	0.0002		V vs. E	2,3198	**0.003**	0.0074
	M vs. P	2,9232	**0.0002**	0.001		M vs. P	1.7701	**0.0142**	0,0532
	M vs. E	2.5923	**0.0004**	0.0016		M vs. E	1.1547	0.2262	0,285
	P vs. E	5.4221	**0.0002**	0.0002		P vs. E	2.234	**0.0022**	0.026

Permutational multivariate analysis of variance calculated on resemblance matrix based on Euclidean. (E= Empuriabrava, M= Mljet, P= Piran, V= Varano). PERMANOVA and pair-wise tests were run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0,05$) are indicated in bold. Po = Population; Res = Residuals.

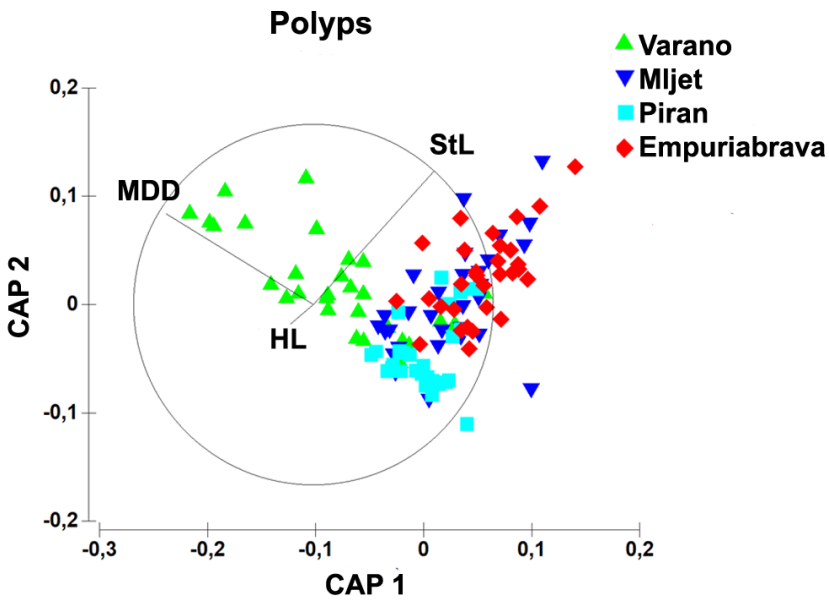


Figure 4.6. Canonical analysis of principal coordinates (CAP) bi-plot ordination (based upon a Euclidean distance similarity matrix) illustrating morphological differences between locations in *Aurelia* spp. polyps. Correlation with canonical axes are only shown when the Pearson's correlation coefficient > 0.5. The length of each vector line is proportional to the strength of the correlation.

Asexual reproduction. The asexual reproduction was guaranteed by different modalities and survival strategies. As common feature, new polyps were produced by budding from the parental polyps or from the stolone (Fig. 4.7 a). Except for these common strategies, polyps could also release small buds that did not present any tissue connection with the parental polyp. Internal and external (Fig.4.7 b) free swimming propagules (Vagelli, 2007) were observed in Varano and Mljet populations although internal modality was more often found in Mljet population (Fig. 4.7 d arrow). These buds showed a slight rotatory movement and after some days they settled at bottom and produced a new small polyp.

Sometimes pedal stolones came out from the stalk, and after the connection with the substrate, originated another polyp away from the native. A particular budding modality was observed in Mljet polyps due to thickening in the distal tentacle portion; in some case this attached to the bottom and could led to a formation of a new polyp; alternatively the polyp was originated before attachment (Fig. 4.7 c arrow). Rarely, podocysts were observed at base of pedal disc or along the stolone in both populations. Polydisc strobilation was observed in all the populations studied with strobilae producing a maximum number of disc of 15 (Mljet), 20 (Piran), 17 (Varano). Data about strobilation production were not available for Empuriabrava.

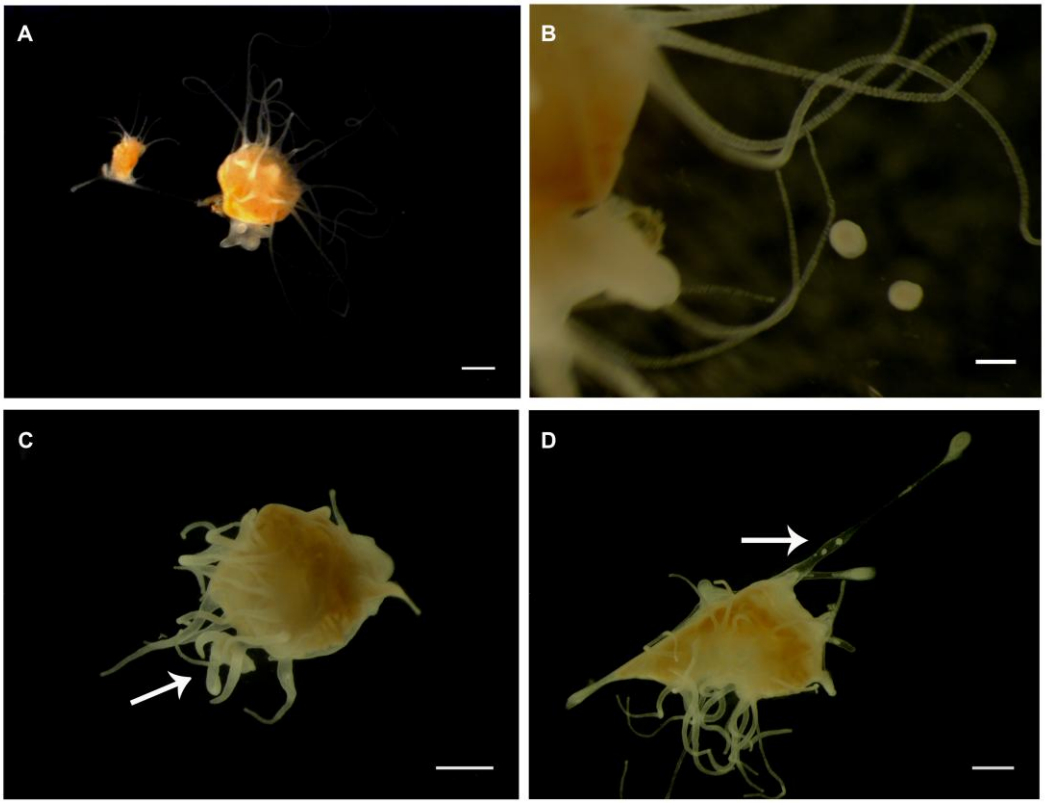


Figure 4.7 (a-d). Asexual reproduction strategies in *Aurelia* spp. **a,** directed budded polyp (Varano); **b,** free swimming buds (Varano); **c,** polyp budded on tentacle (Mljet); **d,** internal free propagules (Mljet). Scale bar = 1 mm (a,b,c), 0,5 mm (d).

4.4.3 Morphological identification: ephyra stage

The new released ephyrae had a similar diameter ranging from 3 mm to 4 mm (Table 4.2). The CDD/TBD ratio reached the maximum value (46%) in Varano and it was always higher in respect to values observed in the other three populations that explained a central disc diameter ratio around 40% (Table 4.2). This ratio contributed greatly only to the dissimilarity observed between Varano and Piran (Table 4.4). Indeed the morphometrics characteristics associated with lappets revealed to be more useful in the population comparison. The proportion of rhopalial lappet length (RLL) and lappet stem length (LStL) against the total marginal lappet length (TMLL) and the proportion of the TMLL on the total body diameter (TBD) accounted for the main differences between populations. In ephyrae of Varano and Mljet, RLL ans LStL gave an opposite contribute in the total length of lappets (TMLL) (Table 4.2). A secondary role was instead played by the other proportions that considered separately the effects of the TMLL components (RLL and LStL) on the TBD (Table 4.4). As a result the lappet shape represented the most distinctive morphological characteristic in discriminate the populations. The

morphometric differences observed were statistically significant in the PERMANOVA test at exception of the pair – wise comparison between Empuriabrava and Mljet population (Table 4. 3).

The CAP analysis was helpful in discriminating the four populations according to the two canonical axes that through the central disc diameter (CDD/TBD) on the first axis and the lappet evaluation (TMLL/TBD) on the second axis easily differentiate the population at ephyra stage (Fig. 4.8).

Lappets shape and color were variable in the populations. In Empuriabrava and Varano the lappets resembled a bread knife - like form, with the margins and the tip of lappets more rounded than other *Aurelia* spp. populations where the lappets exhibited a lancet-like shape (Fig. 4.9, stage 0). The ephyrae dark orange-brownish observed in Varano and Empuriabrava or the ephyrae milky/transparent in Mljet and Piran, could led to associate the populations by colour, but for the reliability of this characteristic we can only consider it useful but not stronger for the identification at ephyra stage.

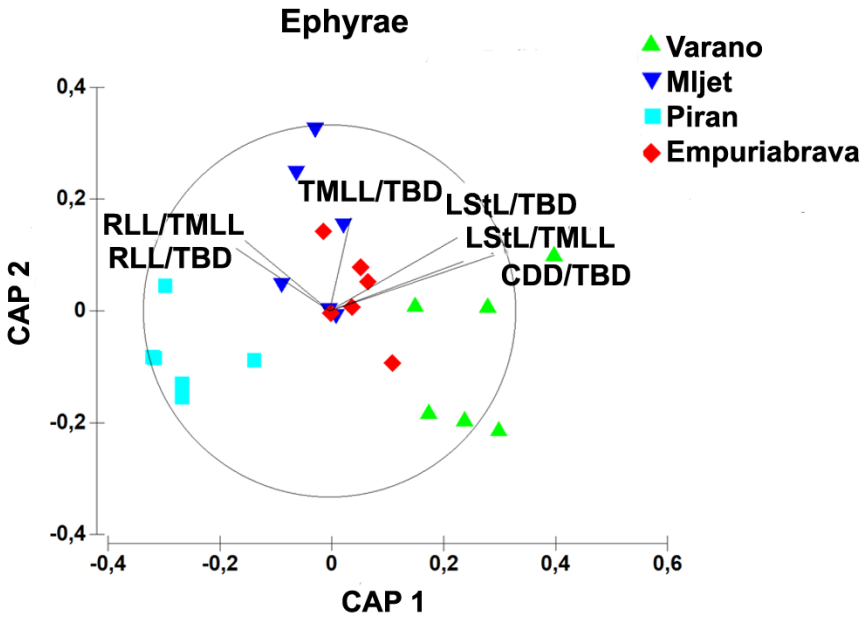


Figure 4.8. Canonical analysis of principal coordinates (CAP) bi-plot ordination (based upon a Euclidean distance similarity matrix) illustrating morphological differences between locations in *Aurelia* spp. ephyrae. Correlation with canonical axes are only shown when the Pearson's correlation coefficient > 0.5. The length of each vector line is proportional to the strength of the correlation.

Table 4.4. List of morphometric parameters in decreasing orders of their importance in the characterization of the morphology of *Aurelia* spp. polyps and ephyrae.
Analysis was performed using the SIMPER routine in PRIMER v.6. on a similarity matrix based on Euclidean distance and a cut-off set at the point where the cumulative dissimilarity reached 80%. See Fig. 3 for abbreviations of morphometric parameters. Contribution (%) describe the contribution of each variable to the dissimilarity.

Morphometric parameter	Contribution %	Cumulative %
Polyp		
Varano & Mljet (average squared distance= 8.21)		
MDD	41.95	41.95
HL	33.34	75.29
StL	24.71	100.00
Varano & Piran (average squared distance= 6.85)		
MDD	66.83	66.83
HL	24.21	91.04
Mljet & Piran (average squared distance= 5.58)		
HL	47.96	47.96
StL	42.60	90.56
Varano & Empuriabrava (average squared distance= 8.70)		
MDD	49.58	49.58
StL	34.69	84.27
Mljet & Empuriabrava (average squared distance= 5.03)		
HL	51.44	51.44
StL	40.14	91.58
Piran & Empuriabrava (average squared distance= 5.06)		
StL	69.01	69.01
HL	22.86	91.88
Ephyra		
Varano & Mljet (average squared distance= 9.83)		
RLL/TMLL	26.85	26.85
TMLL/TBD	24.27	51.12
RLL/TBD	20.30	71.42
CDD/TBD	13.62	85.04
Varano & Piran (average squared distance= 24.94)		
CDD/TBD	24.30	24.30
LStL/TMLL	20.87	45.17
LStL/TBD	19.01	64.18
RLL/TBD	16.51	80.69
Mljet & Piran (average squared distance= 17.77)		
LStL/TBD	19.63	19.63
LStL/TMLL	19.13	38.75
CDD/TBD	17.88	56.63
TMLL/TBD	15.87	72.50
RLL/TBD	13.77	86.28
Varano & Empuriabrava (average squared distance= 5.08)		
TMLL/TBD	30.70	30.70
CDD/TBD	23.22	53.92
RLL/TBD	16.02	69.94
RLL/TMLL	11.49	81.44
Mljet & Empuriabrava (average squared distance= 4.92)		
TMLL/TBD	35.07	35.07
RLL/TMLL	29.24	64.31
RLL/TBD	11.82	76.14
CDD/TBD	10.80	86.94
Piran & Empuriabrava (average squared distance= 16.08)		
LStL/TBD	24.12	24.12
LStL/TMLL	19.18	43.30
RLL/TBD	16.28	59.58
CDD/TBD	13.73	73.31
RLL/TMLL	13.57	86.89

Development of gastric system in Aurelia spp. ephyrae.

Ephyrae just released were dark orange-brownish in colour in Varano and Empuriabrava, milky/transparent in Mljet and Piran. At time 0, rhopalial canals were spade-like to minimally forked in each population (Fig. 4.9). Velar canals were not still well developed to associate an appropriate shape in this stage but all appeared unforked. Statocysts were very bright yellow and the lappets were short rounded to oval knife form in Varano, with lancet-like shape in Empuriabrava, Mljet and Piran.

The mouth was cruciform in all the populations meanwhile the number of gastric filaments differed. No gastric filaments were visible in Varano and Empuriabrava, instead in Piran and Mljet they were already developed (1 and 1-2 per quadrant, respectively).

After 1 week of development, rhopalial canals maintained the shape previous described (time 0) in each population. Velar canals became visible and appeared to be spade-like in all populations, while assumed a rhombic shape in Empuriabrava. The shape of lappet changed in ephyrae of Empuriabrava transforming into round to oval – spoon like. In Mljet and Piran it became pointed spoon – like and in Varano a bread – knife like shape was observed. Generally, slightly rounded lips developed from the cross-shaped mouth and the gastric filaments fixed at 2 per quadrant.

The main differentiation in the structures occurred between the first and the second week. The colour of ephyrae fade and became transparent. The only structures that maintained their shape were the rhopalial canals, meanwhile velar canals elongated and made side branches (Fig. 4.9 c, h, m, r). These changes led to the appearance of velar lappets (pointed in Empuriabrava) between the marginal lappets. Also the shape of lappets changed in all populations toward one more rounded: we distinguished round to oval spoon - like shape in Empuriabrava, Piran and Varano, pointed spoon – like shape in Mljet. The manubrium protruded and slight sign of oral arms formation from the manubrium were observed in each population, at exception of Mljet. The number of gastric filaments increased again reaching the number of 4-8 per quadrant (Fig. 4.9).

In the third week other changes involved the development of lappets that changed in a round spoon – like shape in each populations. In all the populations rhopalial canals became slight forked meanwhile velar canals continued to grow to join the ring canal. In Empuriabrava and Varano, the velar canals grew more quickly and in Empuriabrava the ring canal was almost completed before that rhopalial canals extended the secondary canals (Fig. 4.9 d) In Mljet and Piran, the growth of velar canals was slowly than lateral extension of rhopalial canals. In all the populations, the oral arms continued their extension from the manubrium and the gastric

filaments increased in number (more than 8 per quadrant) and consolidated. Tentacular bulbs appeared in each population but not still in Empuriabrava. In the last week of observations, the shape of lappets and rhopalial canals did not vary. In Empuriabrava and Varano, secondary canals originated from rhopalial canals, fused with velar canals and finally complete the ring canal. Same pattern was observed for Mljet and Piran, but in the fourth week was just launched . In the fourth week tentacular bulbs appeared for the first time also in Empuriabrava. In Mljet and Varano tentacles began to grow out from the bulbs toward distal position. The extension of oral arms was realized keeping their connected at the base of manubrium. The gastric filaments fused and sketched preliminary gastric sinus in Mljet and Varano.

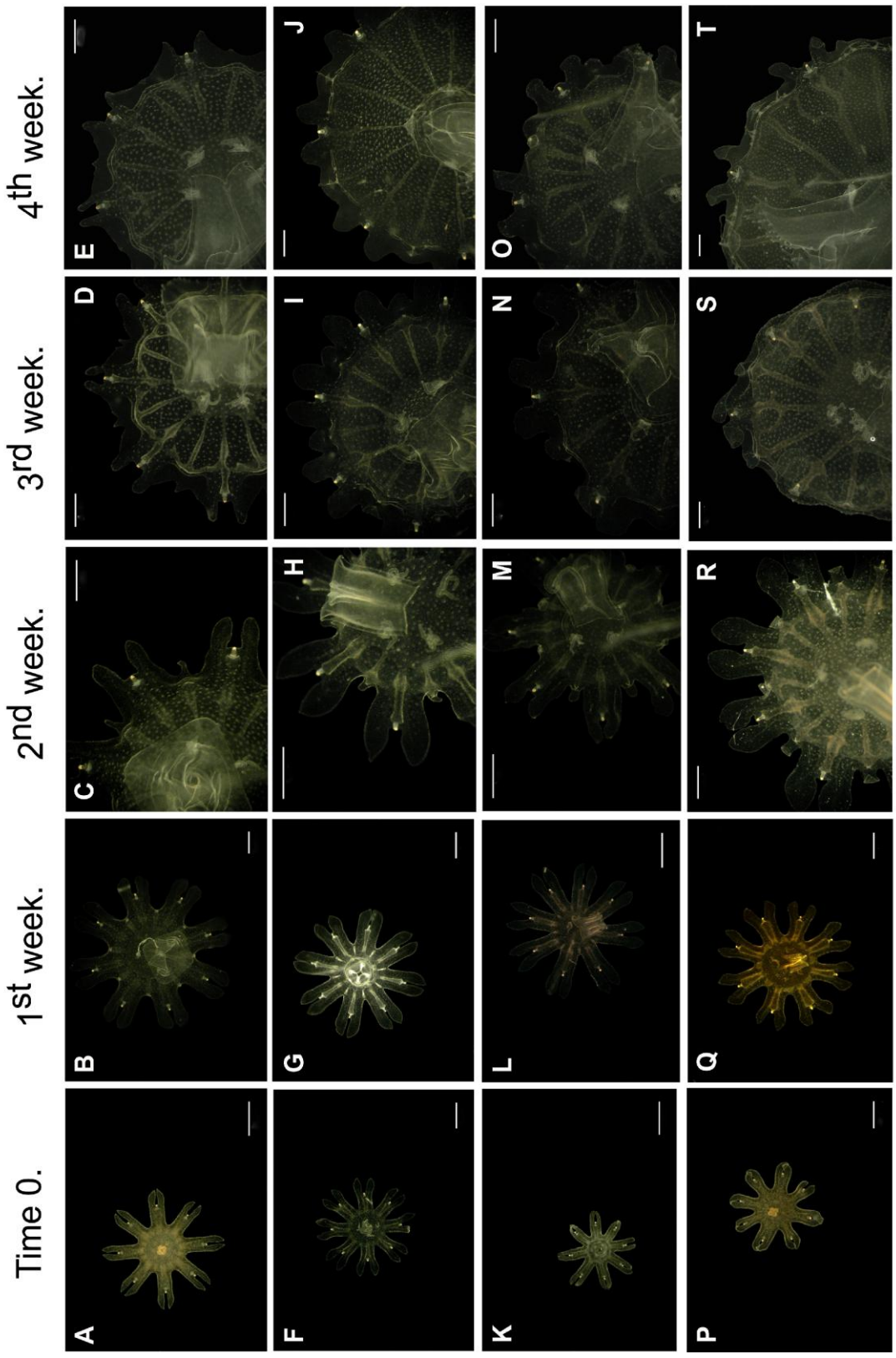


Figure 4.9. Development of gastric system in *Aurelia* spp. ephyrae from four Mediterranean locations. (**a - e**) Empuriabrava, (**f - j**) Mljet**,** (**k - o**) Piran, (**p – t**)Varano. Scale bar= 1 mm.

4.4.4 Morphological identification: medusa stage

Linking morphological descriptions to molecular species consistently revealed notable morphological differences among closely related species (Table 4.5a).

The characters related to perradial, interradial and adradial (f23, f24, f25) origins and that related to oral arms (f5) did not significantly varied among populations. As common feature, 5-7 canals depart from each gastrogenital sinus (2 adradials and 3-5 interradials). When the medusa was placed with the exumbrella surface down, the oral arms extended for 2/5 to 3/8 of bell diameter length, little shorter than umbrella - radius.

Inter - specific variation. On the total of 20 characters included in the study, 12 contributed significantly to differentiate morphologically *Aurelia* sp.1 from *Aurelia* sp. 5 (Fig. 4.10). PERMANOVA pair – wise comparison showed which morphological traits discriminated species (Table 4.5 b).

Only for perradial and adradial anastomoses (f 26,f 28) we found a discrepancy in the inter - specific comparison when the two populations of *Aurelia* sp.1 were compared with the population of *Aurelia* sp.5.. In Varano and Mljet the pattern observed for these two characters did not vary significantly; here the adradial canals were often unbranched and unusually they connected to the canals mesh both in the distal or prossimal position.

SIMPER analyses explained the contribute of each character to the dissimilarity observed (Table 4.6). Morphometrics of the manubrium, oral arms, gastric diameter, interradial anastomoses, rhopalar and not-rhopalar identations revealed that the populations compared belong to different species.

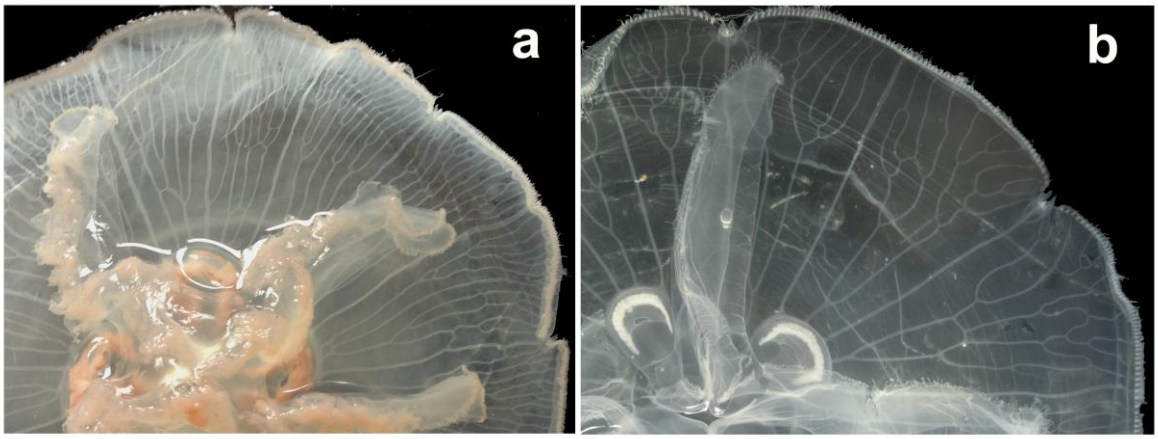


Figure 4.10. Image illustrating inter – specific morphological differences of anastomoses and bell indentations. a) *Aurelia* sp.1 (Varano); b) *Aurelia* sp.5 (Mljet).

Intra - specific variation. In the intra - specific comparison, the characters f 11, f 12, f 13, observable only in mature individuals, were also included. As result, on the total of 23 characters analyzed, only 9 discriminated Empuriabrava from Varano, the two population of *Aurelia* sp.1 (Fig. 4.11).

These characters considered the gonads, bell and the anastomoses and provided a comparable contribute to the dissimilarity observed (Table 4.7). The intra - specific variation was then confirmed by PERMANOVA test (Table 4.5 b)

In Empuriabrava a surprendent high anastomose of canals, accompanied by a maturity of individuals, was observed in smaller medusa diversely from the observations made for Varano. As result, the number of interradial anastomoses (f 26), the size and thickness (f 11,f 13) of subgenital pore were the traits that mostly contribute in the intra – specific variation of *Aurelia* sp. 1 as shown by SIMPER analysis (Table 4.7).

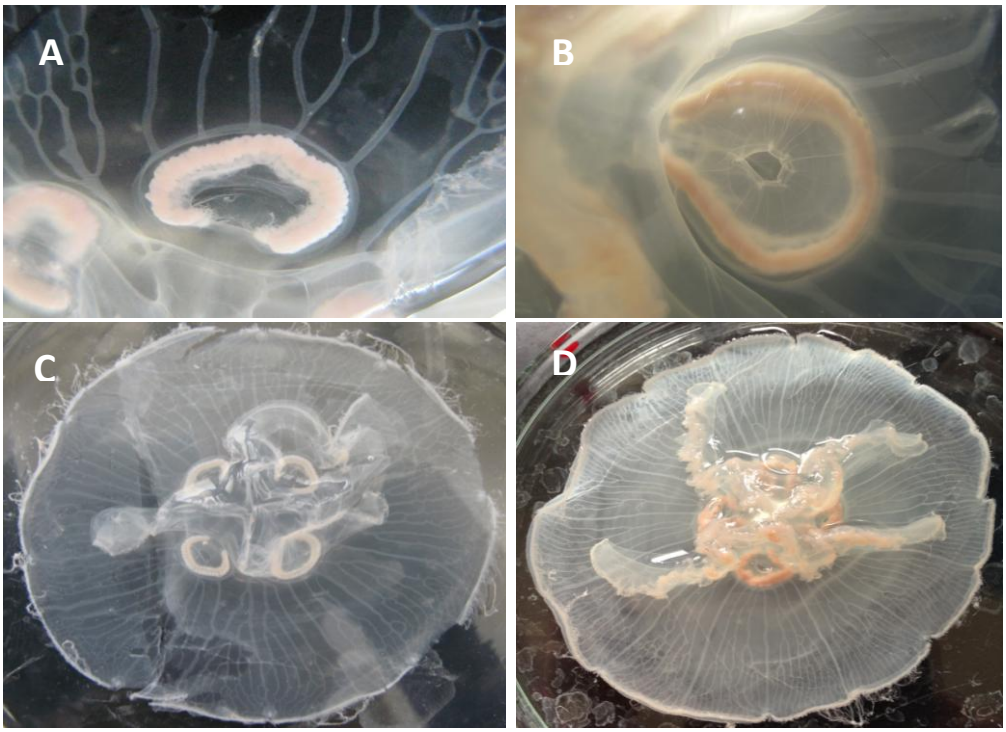


Figure 4.11. Intra – specific variation of morphological characters in *Aurelia* sp.1. Gonad pouch and subgenital pore in a) Empuriabrava; b)Varano; canal anastomoses in c) Empuriabrava, d) Varano.

Table 4.5. Results of PERMANOVA test on morphometrics in the medusa stage of *Aurelia* spp.

a) Main - test

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Po	2	156.21	78.106	6.0123	**0.0002**	4979	**0.0002**
Res	24	311.79	12.991				
Total	26	468					

b) Pair - wise test

Character (f)	Comparison	t	Unique perms	P (perm)	P(MC)
f2	Empuriabrava - Mljet	3.3852	4370	0.0004	0.0028
	Mljet - Varano	5.9794	4816	0.0002	0.0002
f3	Empuriabrava - Mljet	4.5567	4112	0.0004	0.0008
	Mljet - Varano	3.5455	4813	0.004	0.0034
f6	Empuriabrava - Mljet	2.7876	4382	0.0136	0.0144
	Mljet - Varano	4.1107	4830	0.0012	0.0006
f9	Mljet - Varano	3.322	4816	0.0046	0.0046
	Mljet - Empuriabrava	6.0661	4369	0.0002	0.0002
f10	Mljet - Varano	6.6359	4813	0.0002	0.0002
	Mljet - Empuriabrava	12.071	4357	0.0002	0.0002
	Empuriabrava - Varano	3.3934	4347	0.0002	0.0002
f11	Empuriabrava - Varano	3.7649	4393	0.0026	0.0022
f12	Empuriabrava - Varano	2.853	4322	0.0108	0.0114
f13	Empuriabrava - Varano	3.9618	4352	0.002	0.001
f21	Mljet - Empuriabrava	2.6014	4374	0.0212	0.0204
	Empuriabrava - Varano	2.9636	4392	0.0056	0.0094
f22	Mljet - Empuriabrava	2.3318	3453	0.0186	0.0376
	Empuriabrava - Varano	2.5673	4364	0.0022	0.0234
f26	Empuriabrava - Mljet	5.1789	2508	0.0004	0.0006
	Empuriabrava - Varano	3.4442	1029	0.0036	0.0044
f27	Empuriabrava - Varano	2.5579	980	0.0184	0.0236
	Empuriabrava - Mljet	4.1507	1737	0.001	0.001
	Mljet - Varano	2.1957	783	0.0462	0.0396
f28	Empuriabrava - Mljet	3.7873	183	0.0018	0.0026
	Empuriabrava - Varano	2.1907	305	0.0464	0.045
f29	Empuriabrava - Mljet	3.9617	4352	0.0024	0.0026
	Mljet - Varano	3.7313	4793	0.0022	0.0028
f30	Empuriabrava - Mljet	3.867	4391	0.0008	0.0022
	Mljet - Varano	3.4587	4811	0.002	0.0034

Pair - wise tests are shown for significant characters (f). P-value based on PERMANOVA (perm) or Monte Carlo (MC) methods depending upon the number of unique permutations. Items in bold represent significant comparisons. Po = Population; Res = Residuals. The characters f11, f12 and f13 were considered only in the comparison Varano – Empuriabrava.

Plots of the principal coordinates from the constrained CAP analysis showed a net separation among population (Fig. 4.12). The distal gastric diameter (f10) played the prominent rule in the displacement of the populations along the canonical axis 1. Secondly, the manubrium depth (f2) separated the populations along the canonical axis 2. Particularly, the rhopal- and non rhopal-indentations characterized the individuals of *Aurelia* sp.5 that were grouped together in the CAP representation (Fig. 4.12).

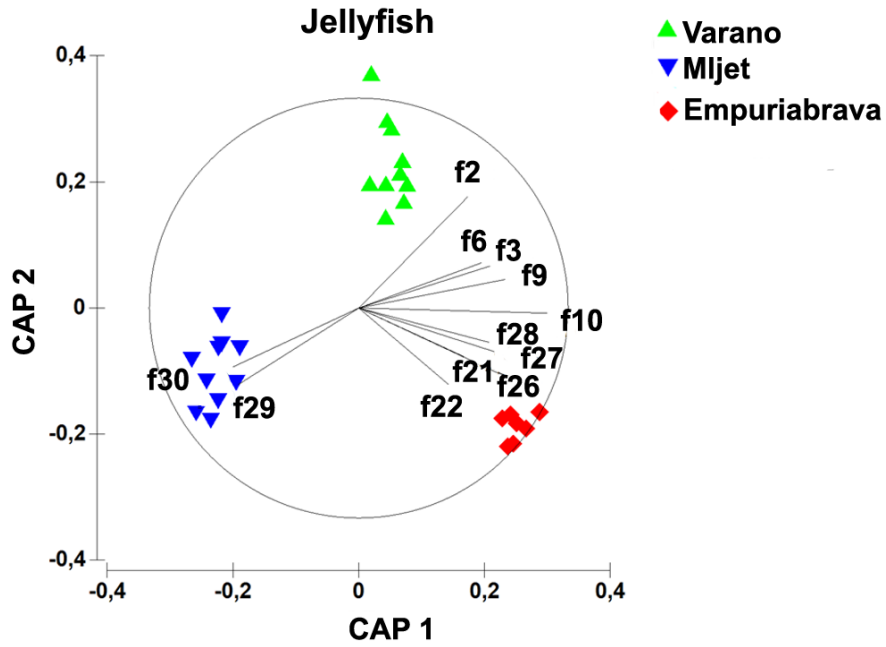


Figure 4.12. Canonical analysis of principal coordinates (CAP) bi-plot ordination (based upon a Euclidean distance similarity matrix) illustrating morphological differences in *Aurelia* spp. medusae. Correlation with canonical axes are only shown when the Pearson's correlation coefficient > 0.5. The length of each vector line is proportional to the strength of the correlation.

Table 4.6. List of morphometric parameters in decreasing orders of their importance in the characterization of the inter - specific variation in *Aurelia* spp. medusae.
Analysis was performed using the SIMPER routine in PRIMER v.6. on a similarity matrix based on Euclidean distance and a cut-off set at the point where the cumulative dissimilarity reached 80%. See Fig. 3 for abbreviations of morphometric parameters. Contribution (%) describe the contribution of each variable to the dissimilarity.

Groups Varano & Mljet						
Average squared distance = 34.46						
	Group Varano	**Group Mljet**				
Variable	**Av.Value**	**Av.Value**	**Av.Sq.Dist**	**Sq.Dist/SD**	**Contrib%**	**Cum.%**
f29	-0.586	0.849	3.39	0.92	9.31	9.31
f2	0.773	-0.872	3.39	1.26	9.29	18.60
f30	-0.494	0.839	3.11	0.80	8.54	27.14
f9	0.343	-0.876	2.7	1.00	7.40	34.54
f3	0.406	-0.834	2.64	0.95	7.25	41.79
f7	0.285	-0.435	2.58	0.88	7.09	48.88
f5	0.275	-0.129	2.37	0.69	6.49	55.37
f6	0.411	-0.788	2.21	0.97	6.05	61.42
f24	9.23E-2	-0.306	2.19	0.85	6.00	67.41
f23	-0.138	0.243	1.98	0.67	5.42	72.84
f19	-0.192	-0.198	1.85	0.90	5.09	77.92
f10	0.203	-1.03	1.84	1.06	5.04	82.96
Groups Mljet & Empuriabrava						
Average squared distance = 54.16						
	Group Mljet	**Group Empuriabrava**				
Variable	**Av.Value**	**Av.Value**	**Av.Sq.Dist**	**Sq.Dist/SD**	**Contrib%**	**Cum.%**
f10	-1.03	1.18	5.2	1.96	9.59	9.59
f26	-0.623	1.18	4.25	1.15	7.84	17.43
f27	-0.648	1.04	4.24	0.89	7.82	25.26
f28	-0.637	0.961	4	0.85	7.39	32.65
f22	-0.312	0.912	3.76	0.61	6.95	39.60
f21	-0.463	0.874	3.65	0.97	6.74	46.34
f6	-0.788	0.539	3.52	0.96	6.51	52.85
f9	-0.876	0.761	3.17	1.35	5.85	58.70
f3	-0.834	0.612	2.87	0.90	5.31	64.00
f24	-0.306	0.306	2.85	0.79	5.26	69.26
f30	0.839	-0.494	2.57	0.79	4.75	74.01
f23	0.243	-0.151	2.4	0.62	4.43	78.44
f25	-0.409	0.38	2.18	0.63	4.03	82.46

Table 4.7. List of morphometric parameters in decreasing orders of their importance in the characterization of intra - specific variation of *Aurelia* sp. 1 medusae. The characters f11, f12, f13 were included in this comparison.
Analysis was performed using the SIMPER routine in PRIMER v.6. on a similarity matrix based on Euclidean distance and a cut-off set at the point where the cumulative dissimilarity reached 80%. See Fig. 3 for abbreviations of morphometric parameters. Contribution (%) describe the contribution of each variable to the dissimilarity.

Groups Varano & Empuriabrava **Average squared distance = 47,77**						
	Group Varano	Group Empuriabrava				
Variable	**Av.Value**	**Av.Value**	**Av.Sq.Dist**	**Sq.Dist/SD**	**Contrib%**	**Cum.%**
f11	-0,566	0,808	2,86	0,98	5,99	5,99
f26	-0,539	0,771	2,82	0,96	5,91	11,90
f13	0,58	-0,829	2,82	1,15	5,89	17,79
f21	-0,493	0,705	2,76	0,78	5,78	23,57
f10	-0,535	0,764	2,75	0,93	5,75	29,32
f22	-0,448	0,641	2,74	0,60	5,73	35,05
f12	-0,481	0,688	2,72	1,06	5,69	40,74
f27	-0,447	0,639	2,62	0,73	5,48	46,22
f28	-0,4	0,571	2,49	0,72	5,21	51,44
f24	-0,149	0,214	2,18	0,50	4,56	56,00
f6	-6,52E-2	9,31E-2	2,16	0,91	4,53	60,52
f2	0,28	-0,4	2,14	0,95	4,48	65,01
f19	-0,281	0,402	2,04	0,77	4,26	69,27
f23	6,4E-3	-9,14E-3	1,98	0,68	4,14	73,41
f25	-8,07E-2	0,115	1,94	0,76	4,07	77,47
f5	0,215	-0,307	1,9	0,71	3,97	81,44

4.5. Discussions and conclusions

In systematics of Cnidaria, most miss-classification errors were historically related to a wrong association of the species according to life stage considered (Kölliker, 1853; Allman, 1875). In systematics of scyphozoan, the medusa stage has been considered for long time the stage key in most of the papers, despite the fact of a metagenetic life cycle (Mayer, 1910; Kramp, 1961, 1968; Russell, 1970). In the optic of increasing discovery of new cryptic species in all the marine taxa and also in Cnidaria (Holland et al., 2004; Moura et al., 2007; Pfenninger and Schwenk, 2007), studies on systematics can be facilitated by the recent development of morpho - molecular methods helpful in discriminating sibling species (Dawson, 2003).

In the present study morphology, morphometrics and development stages were compared between *Aurelia* spp. collected from different locations in the Mediterranean and North sea. With the support of COI data, our attempt to make morphological comparison based on literature and observations pointed out significant intra- and inter-specific differences.

It is recognized that *Aurelia* sp.1 has a world-wide extension but in the Mediterranean sea its presence was only previously recorded in the south of France (Scroth et al., 2002) and *Aurelia*

sp.8 was for long time considered the only Mediterranean species existing of *Aurelia* sp. with the exception of *Aurelia* sp. 5, the Messinian relict inhabiting the Mljet lakes (Dawson and Jacobs, 2001; Dawson et al., 2005; Ramsak et al., 2012).

The molecular analyses here performed revealed *Aurelia* sp.1 as invaders of new habitats in the Mediterranean (Varano lagoon and the Empuriabrava's harbour). Our data also confirmed that *Aurelia* sp.8 remains the more widespread species in Mediterranean from the North Adriatic until the Tunisian coasts. Unluckily, the morphology and morphometrics in *Aurelia* sp.8 and *Aurelia* sp.5 were not available before the present study and in *Aureli*a sp.1 only the medusa stage has been recently investigated (Dawson, 2003).

Morphological studies highlighted a particular squat shape in Varano scyphopolyps, rather atypical in respect to the other *Aurelia* spp. described (Uchida and Nagao 1963; Gershwin, 2001) meanwhile reproduction strategies observed in all the populations studied were comparable to that observed in all Aurelinaae species (Vagelli, 2007; Gershwin, 2001; Gilchrist, 1937; Straehler-Pohl et al., 2011). At this stage, the morphometrics can be useful but also questionable due to the perennial life-span of polyp stage which overcomes environmental constraints and responds more quickly to changes with morphological adaptations of their structures. The intra - specific high polymorphism in polyps of *Aurelia* sp.1 could be the consequence of variation in physical factors that occurs in two different habitats: a highly variable, coastal lagoon (Varano) and a marine harbour (Empuriabrava). Furthermore, the high MDD in polyps of Varano may reflect the trophic condition of the lagoon and the requirement of a larger mouth to ensure the ingestion of more preys.

Contrarily from polyp stage, the life of ephyra is very short and we could suppose that morphometrics could be less unaltered across geographic ranges. This is the case of *Aurelia* sp.5 from Mljet, that exhibit comparable morphometrics of *Aurelia aurita* described by Strahler-Pohl (Straehler-Pohl & Jarms 2010) and consequently validates the hypothesis made by Dawson of a contact between the two species in the Plyocene (Dawson and Jacobs, 2001). Some characters are inherited in the transition from polyp to ephyra stage and this is clearly demonstrated considering the highest values recorded for the MDD and CDD in polyp and in ephyrae of Varano *Aurelia* sp.1, respectively. In all the cryptic species here investigated, the newly released ephyrae showed a diameter analogous to *Aurelia aurita* (Straehler-Pohl and Jarms 2010, Yasuda 1979; Gröndahl and Hernroth, 1987) and *Aurelia limbata* (Straehler-Pohl and Jarms 2010, Uchida and Nagao, 1963).

Differently, the shape of the rhopalial lappets and the relative proportions are relevant for species identification (Straehler-Pohl and Jarms, 2010, Gröndahl and Hernroth, 1987) and their usefulness is reflected in the differences found across *Aurelia* cryptic species here considered. The shape of lappet and proportion of RLL and LStL on the TMLL at time 0, clustered together *Aurelia* sp.1 and sp.8 giving evidence of a morphological (Straehler - Pohl and Jarms, 2010) and genetic affinity with *Aurelia limbata* (Fig. 4.3). However, dissimilarity in other characters separated these cryptic species from the specie taxonomically recognized *A.limbata.*

Morphology of lappets and morphometrics on ephyra stage also demonstrated that *Aurelia* sp. 5 is morphological close to *Aurelia aurita* (Straehler - Pohl and Jarms, 2010) although genetic distance indicates this as a separate species.

Being the development of gastric system linked to the potential of each species to bloom, (Dawson and Hamner, 2009) then its analysis from the ephyra stage can provide new insight at species level. In despite of differences on morphometric measures observed between cryptic species, in the first two weeks the development of gastric system in all the four populations corresponded to the description made by Straehler-Pohl et al. in 2011 for *Aurelia limbata*. Subsequently, the pattern changed and in each species/population the development proceeded as a transition of that observed in *A.aurita* and *A.limbata,* with slight variations given by the species considered.

The external environment acts continuously in the development of characters and a proof is given by morpho-genetic variability that we observe in *Aurelia* spp. and that also explains the polymorphism in *Aurelia* sp.1 that for some authors descends from a single population but with a dispersal time and a origin quite dubious (Ki et al., 2008). On the other hand, most of morphological traits analyzed in the medusa stage varied but some remain conserved more intra-species than inter-species.

In late stage of development, the description made for *A.limbata* (Brandt, 1838; Uchida, 1934) argued about a higher number of interradial origins, 16 marginal lobes, and bluish umbrella that we did not observed in any population. Nevertheless, in *A.limbata* the gastric system was well developed, creating a mesh net with a high number of anastomoses that we also observed in medusae from Empuriabrava.

COI sequences clustered together individuals of *Aurelia* sp.1 from the Varano Lake and Empuriabrava with the *Aurelia* sp. 1 sequences from Australia, California and Japan.

Both *A.limbata* and *Aurelia* sp.1 are distributed in this area of Pacific Ocean (Dawson and Jacobs, 2001). Old literature data of the nominal species *A. japonica* could be morphologically associated to the two populations of *Aurelia* sp.1 here studied but few characters are considered in the old description (Kishinouye, 1891). The specimens from Bizerte (both bay and lagoon) and Porto Cesareo were recognized as *Aurelia* sp.8, confirming the occurrence of this species across several areas in the Mediterranean, from the Levantine Sea, to the Adriatic Sea (Dawson et al., 2005; Ramsak et al 2012), the Ionian Sea and the Tunisian coasts. Indeed *Aurelia* sp.8 is considered a lessepsian immigrant occurring on both sides of the Suez Canal (Dawson et al., 2005); its origin could be traced in the two old nominal species, *A.maldivensis* (Bigelow, 1904) and *A.solida* (Browne, 1905).

The absence of objective morphological keys led to misleading taxonomic classifications where each species became a tile easy-fitting in the mosaic of species. As conclusion of this study, we can suggest that cryptic species here investigated may have evolved as different lineages both from *Aurelia aurita and Aurelia limbata.*

The physiological/morphological constraints that *Aurelia* sp.5 shares with *Aurelia aurita* could explain why *Aurelia* sp.5 remain confined to Mljet Lake, even though there is no physical barrier to prevent a wider distribution (Ramsak *et al.* 2012). Here we demonstrated for the first time that two characters, rhopal- and non rhopal- indentations, can be selected to rapidly identify *Aurelia* sp.5.

The combined approach here tested should be applied to the other molecular species in order to reveal key morphological characters that are kept within the same molecular species and that allow species - identifications. A successive step could be to investigate epigenetics processes involved in the adaptation and phenotypic plasticity (Newman and Müller, 2000). In this direction old morphospecies descriptions (Mayer, 1910; Kramp, 1961,1968; Russell, 1970) can regain values or can help in the systematic revision as already demonstrated in other scyphozoans (Gershwin and Collins, 2002).

Both morphological and ecological divergences inevitably lead to sibling species (Knowlton, 1993) and in the open marine environment an efficient tool of research and validation could be obtained only by a multi disciplinary approach that need to be standardized soon.

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CHAPTER FIVE:

Strobilation inducers in *Aurelia* spp.

Chapter 5: Strobilation inducers in *Aurelia* spp.

5.1. Abstract

Medusa production in the Class Scyphozoa is performed by the production of discs by transverse fissions of the polyp body column until young medusae (ephyrae) are released in the water. This asexual reproduction (strobilation) was investigated in three populations of the moon jellyfish *Aurelia* spp. from the Adriatic coast: Piran, Mljet and Varano. Natural (decrease in temperature) and artificial inducers (hydrogen peroxide and indomethacin) of strobilation were tested on well-fed polyps held at 21°C and checked for 117 days. Hydrogen peroxide did not induce strobilation in any of the populations tested. The effects of the other inducers differed by population. For the indomethacin treatment, higher percentages of polyps strobilated in the lowest concentration (20 μm) for Piran and Mljet populations, but Varano polyps responded conversely. High indomethacin concentration (20 μm) also reduced the survival rate of polyps. Decreased temperature induced more Piran polyps to strobilate than Varano polyps, but no effect was observed for Mljet polyps. Although the most ephyrae per polyp were released in all populations with the natural inducer, we demonstrated that indomethacin was the best inducer to obtain high percentages of strobilating polyps (83%) quickly (10 days) to obtain ephyrae. This work represented the first step in the research of possible triggers and pathways involved in the strobilation of *Aurelia* spp. polyps.

5.2. Introduction

In the metagenetic life cycle of *Aurelia* spp., the scyphistoma is considered equivalent to a long resting stage (Boero et al., 2008) that reproduces continuously throughout years by budding. Ephyrae are produced by the scyphistoma through strobilation, an asexual reproduction that involves anatomical changes in the polyp structures (Arai, 1997). Following particular and species-specific stimuli, at particular times of the year budding ceases, a scyphistoma loses its tentacles and forms stacked segments that subsequently differentiate in sequential order. These gradually transform into ephyrae that, when mature, are released from the unaltered proximal portion of the strobila through a process of transverse fission (strobilation), changing from the asexual to the sexual generation (Spangenberg, 1968).

When all the ephyrae are released, the scyphistoma regenerates tentacles and a new proboscis and is then capable of strobilating again.

Consequently, the term "strobilation" is associated to two separate processes: segmentation and metamorphosis (Spangenberg, 1968). During this metamorphosis differential destruction occurs of tentacles, septal muscles, and atrichous polyspiras nematocysts; meanwhile, new structures develop (rhopalia, lappets, manubria of non-terminal segments, gastric filaments, etc.) (Spangenberg, 1965). The formation of the strobila ends when the ephyral segments, with lappets and rhopalia (originating at the base of interradial lobes) on the margin, and the basal polyp, with the new ring of tentacles, are completely developed. The first ephyra of the strobila uses the mouth of the polyp from which it originated, but the other ephyrae and the basal polyp at the end of the strobila each develop a new mouth (Arai, 1997). In addition to the disappearance of the tentacles, other morphological signals of the start of the strobilation in polyps are colour changes and elongation of the calyx.

The metamorphosis of jellyfish caused by the segmentation process is different from metamorphosis of higher animals. Through segmentation, a polyp may give rise to many new organisms, and this is generally not possible in higher organisms during metamorphosis. The high potential of this animals is in effect linked due to their modularity factor that is species – specific (Boero et al., 2008) and it is associated also with the type of strobilation. This depends upon the size and shape of the scyphistoma head or column. When the head is shallow and the stalk long, strobilation is usually monodisc, resulting in one, sometimes two ephyrae (Berrill, 1949) as observed in *Cotylorhiza tuberculata* (Kikinger, 1992) and *Sanderia malayensis* (Uchida and Sugiura, 1978). Instead in *Aurelia* spp., *Chrysaora* spp. and Rhizostomatidae, polydisk strobilation is always observed (Dawson and Hamner, 2009) with a number or ephyrae released varying with species.

Therefore it is easy to understand how species with polydisk strobilation form blooms in several locations around the word (Dong et al., 2010; Kogovšek et al., 2010; Robinson and Graham, 2013). Depending on the species, a single polyp may produce one or many ephyrae all at once, over a period of time, or at different intervals. The strobilation is in effect controlled by endogenous factors (Spangenberg, 1965) but other factors operate synergistically to activate this process.

In polyps of *Mastigias papua* (Sugiura 1965), *Cephea cephea* (Sugiura, 1966), and *Rhopilema verrilli* (Calder, 1973), strobilation could be induced by raising the temperature. Also in

Rhopilema nomadica the strobilation process, rather than the number of the ephyrae on each polyp, is controlled by the water temperature (Lotan et al., 2004). An increase in temperature favours the production of strobilae and the formation of ephyrae in *Aurelia* spp. (Purcell et al., 1999, 2007, 2012; Widmer, 2005; Liu et al., 2008; Holst, 2012). Other physical factors like salinity and light, affect the number of ephyrae produced, the percentage of ephyrae of the total asexual reproduction, and the delay before ephyra production (Purcell et al., 2009). On the other hand, the variation of temperature also promotes strobilation and polyps also strobilate when temperature is lowered (Kroiher et al., 2000; Di Camillo et al., 2010;).

In the present work, the scyphopolyps of *Aureli*a spp. were from locations along the Adriatic coasts. In these environments, scyphopolyps strobilated only in colder months from November to February, with maximum proportions (up to 82% of polyps) in November at temperatures and salinities around 15°C and 35 psu, respectively (Malej et al., 2012). Therefore, in the northern Adriatic *Aurelia* medusae were generally observed from February until June. Rarely did medusae already appear in January, and in some recent years they were seen until end of July. In the central Adriatic, polyps attached to a ship wreck began to strobilate between the end of November and the beginning of December when temperature was lower than 15°C and strobilation was observed until February (Di Camillo et al., 2010). A completely different situation was observed in the marine lake of Veliko Jezero in the Adriatic Mljet island where medusae persist all year and are especially observed in August-December (Kogovšek et al., 2012), while in the open Adriatic Sea adults have already disappeared. Differently from the other areas, in the coastal lagoon of Varano strobilation take place in April with temperatures and salinities around 18° and 25 -and 26 ppt (Chapter 2).

The time and rate of strobilation are also influenced by chemical factors including iodinated compounds and polypeptides (Arai, 1997). The neck-inducing factor (NIF) is a large naturally produced polypeptide that is involved in the initiation of strobilation in *Chrysaora quinquecirrha* (Loeb, 1974). When NIF is released by one polyp into sea water, it induces formation of the first circular groove below the tentacles (the 'neck') in neighbouring scyphistomae (Loeb, 1974). Others chemicals initiate strobilation, such as the ionic iodide for which Spangenberg (1967) found that reduced compounds are effective in inducing strobilation in *Aurelia* sp. maintained in laboratory culture. Iodine is as effective in initiating strobilation as iodide, and iodine dilution of $1{:}10^7$ and $1{:}10^8$ of iodine produced 100% strobilation within a two-week period, usually within

five days. Unconditioned polyps kept for several months in seawater containing the natural amount of iodide began to strobilate by application of elemental iodine or hydrogen peroxide for 24 hours (Berking et al., 2005). The authors suggested that iodine was naturally involved in the induction of strobilation and iodide ions combined with tyrosine into a reactive oxygen species (ROS) pathway that is common in other marine invertebrates. Nevertheless, subsequent attempts to induce strobilation by the addition of potassium iodide were not successful and no strobilation was observed in *Aurelia* sp. in other experimental conditions (Willcox et al., 2007).

Recently indomethacin, a molecule used as an prostaglandin synthesis inhibitor, was discovered to induce the beginning of strobilation in *Aurelia* sp. 1 polyps in a dose-dependent way (Kuniyoshi et al., 2012), but it was not involved in the continuation of strobilation. The release of ephyrae occurred after 9 to 14 days depending of the dose of indomethacin that was tested with different concentrations between 2.5 μM and 20 μM and at 25 °C. The target receptor molecule of indomethacin has not been identified yet, and the main step for the molecular mechanism of jellyfish metamorphosis has yet to be clarified (Kuniyoshi et al., 2012). However, a recent work by Fuchs et al. (2014) shed light on the molecular framework controlling the polyp-to medusa transition in *Aurelia aurita* (strains from North European seas). Based on transcriptome sequencing from polyp, strobila and ephyra stages, a diffusible protein (CL390) has been discovered to be strongly upregulated in the scyphistoma stages preceding strobilation by short-term cooling temperature. Analysis of CL390 expression by real-time PCR showed that as soon as a concentration 15 times higher than the background level is reached, strobilation occurs. The increase of concentration of CL390 is achieved by 9 days of polyp incubation at 10 °C, indicating a temperature-dependent timing of polyp strobilation. This CL390 protein plays a role as a metamorphic signal through its fragmentation into small peptides: one of these peptides (with sequence WSRRRWL) can induce polyp strobilation within 72h. Indomethacin is a synthetic molecule including in its central part a molecular structure very similar to the CL390-contained peptide WSRRRWL. Therefore, indomethacin activates strobilation because of its similarity with the natural strobilation inducer CL390 (Fuchs et al., 2014).

In the present study, strobilation inducers were tested under controlled laboratory conditions in three *Aurelia* spp. population (Varano, Mljet, Piran) from the Adriatic sea previously identified as different species (sp.1, sp.5, sp.8, Chapter 4). Mechanisms involved in the strobilation and the different responses to inducers are not still well know and background in the context of cryptic

species is missing. In the present chapter helpful fundaments to produce a validated protocol to induce strobilation in *Aurelia* spp. cryptic species are provided.

5.3. Methods

5.3.1. Location of *Aurelia* spp. collection

Aurelia spp. scyphopolyps were collected from three different locations along the Adriatic coasts by scuba diving (Mljet, Croatia; Piran, Slovenia) or obtained in the laboratory following settlement and metamorphosis of competent planulae released from mature jellyfish in experimental aquaria (Varano, Italy).

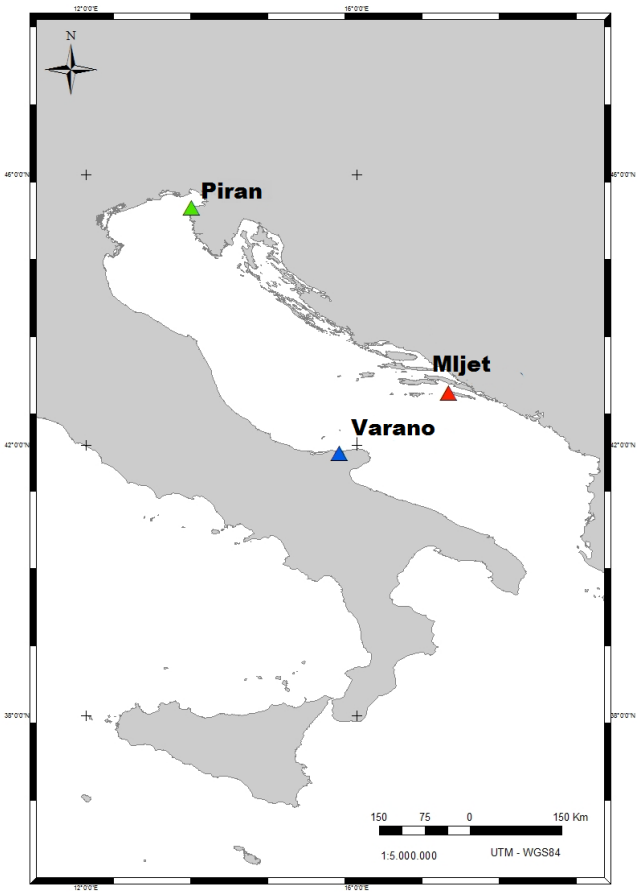

Fig. 5.1. Sampling locations of *Aurelia* spp. scyphopolyps.

Mljet is the most southerly and easterly of the larger Adriatic islands of the Dalmatia region of Croatia and is separated from the Pelješac peninsula by the Mljet Channel. Veliko Jezero ("Big Lake") is a karstic depression with an area of 145 hectares and with a maximum depth of 46 meters (Benović et al., 2000). It is connected with the open sea by shallow (2.5 to 4 m depth) 30

meters long channel. This lake is divided into two > 40 m deep depressions separated by a 15 m deep reef. The hydrography is characterized by large temperature fluctuations in the surface layer (between 8 and 28 °C), which allow a pronounced seasonal stratification, whereas salinity does not show large variation (Buljan and Špan, 1976). In summer, the thermocline separates the water column in a colder (10-12 °C) and more saline (37.5-38.5) layer below ca. 15 m from the upper layer with a large variation in physical parameters (Buljan and Špan, 1976).

Piran Bay is in the northern part of the Adriatic Sea and is part of the southernmost tip of the Gulf of Trieste. Here the Adriatic Sea presents characteristics of a shallow sea with large temperature and salinity variations and seasonally strong vertical stratification. The Piran bay is semi-enclosed and shallow with mean depth of 13 m in the inner part of the bay. It is 7 km long and 5 km wide and characterized by weak tidal and wind-driven currents mainly from the Northeast (Bora) (Grego et al., 2009).

The lagoon of Varano was previously described in Chapter 2.

5.3.2. Protocol tested on *Aurelia* spp. polyps.

Polyps of *Aurelia* spp. from the Adriatic coasts (Varano, Mljet and Piran) were cultured in filtered sea water and acclimated at 21°C from November 2012 to February 2013.

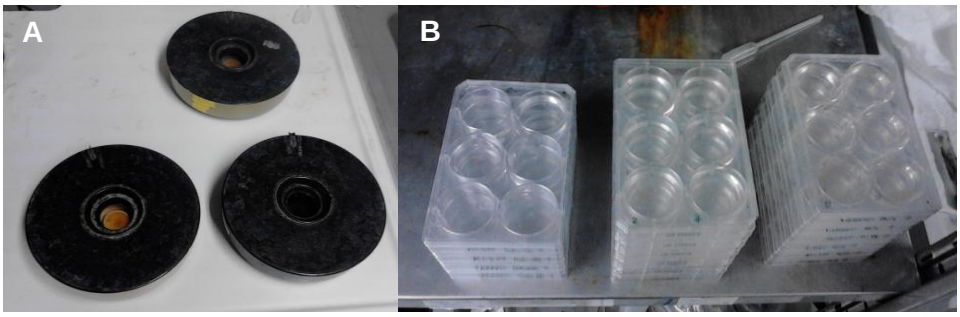

Figure 5.2: Materials used for the experiments. a) *Artemia salina* cysts in special containers for hatching (24 - 48 hours); b) *Aurelia* spp. polyps placed in 6-multiwell plates.

On February 4, the polyps were divided into 3 treatment groups and 1 control group (Table 5.1). For each group, 12 polyps of each population were placed individually in wells of 6-multiwell plates with 10 ml of the test solution (Fig. 5.2). In the first and the second group, artificial inducers of strobilation (indomethacin and hydrogen peroxide) were tested for 1 week of

treatment. The third group of polyps was moved from 21°C to a 14° C thermostatic chamber and monitored for 117 days. After 1 week, the test substances were replaced with filtered seawater and polyps monitored for 30 days. The control group was maintained in filtered sea water at 21°C for 117 days.

In each group, after the polyps fed on *Artemia salina* for 2-3 hours (Fig. 5.2), the wells were cleaned with swabs; seawater and uneaten food were discarded and replaced with the filtered solution containing the tested solution (indomethacin/ hydrogen peroxide) or filtered sea water, as above. The feeding protocol was repeated three times per week.

The survival of polyps, the presence of strobilae, the number of ephyrae released and malformed and the day of release was checked for each polyp daily at the stereoscope throughout the experiment. Buds were not counted. Ephyrae and buds that had detached from the initial polyps were removed.

Survival rates of polyps were calculated following this formula:

Survival = (live polyps/polyps total) * (days lived/days of experiment)

The ephyrae production (n° ephyrae polyp $^{-1}$) was calculated by the sum of the number of ephyrae produced and divided by the number of strobilating polyps for each well.

Table 5.1: Experimental protocol adopted to test strobilation inducers in *Aurelia* spp.

Initial temperature	Treatment	Experiment temperature	Concentration	Total Treatment Time/Monitoring time
21°	Indomethacin	21°	10 μM 20 μM	1 week/ 30 days
21°	Hydrogen peroxide	21°	20 μM 500 μM	1 week/ 30 days
21°	Decrease of temperature	14°	-	117/117 days
21°	No substance - Control	21°	-	117/117 days

Second treatment with indomethacin.

From 5 to 11 June 2013, 12 *Aurelia* spp. polyps from each population that survived the treatment with 10 μM indomethacin in the previous experiment were subjected to another treatment with 10 μM indomethacin in order to observe the ability to react again to the artificial inducer. The delay from the first and the second treatment was 1 week. The monitoring time was 30 days.

5.3.3. Statistical analysis

Statistical analyses were performed with PRIMER 6+ software (Plymouth Marine Laboratory) using a significance level of 0.05 (Clarke and Warwick, 1995). Data were normalized and a resemblance matrix based on Euclidean distance was calculated.

Three-way analysis of permutational multivariate analysis of variance (PERMANOVA) was used to test for differences between treatments, population and plate. The PERMANOVA design involved 2 fixed factors (population, "Po", and treatment,"Tr") with 3 and 6 levels, respectively. The factor plate "Pl", was considered random and nested in the factor treatment.

Finally, a constrained canonical analysis of principal coordinates (CAP) was used to find the axes in the principal coordinate space that best discriminated among the a priori groups of treatments (Anderson and Willis, 2003).

5.4. Results

5.4.1. Polyp survival and strobilation

The effects of the inducers differed among the different populations (Fig. 5.3, 5.4) with the exception of hydrogen peroxide that did not induce strobilation in any tested polyps. In general, the highest survival was recorded for Varano polyps, followed by Piran and Mljet polyps. In particular, for Varano and Piran polyps, survival was higher than 85% in both hydrogen peroxide treatments (20 μM and 500 μM), in the natural inducer treatment, in the control and in the lower indomethacin concentration. In the higher indomethacin concentration, only a small percentage of polyps survived in Mljet (9.62%) and no polyps survived until the end of the experiment in Piran. By contrast, Varano polyps showed lower survival (48.69%) only during treatment with 20 μM of indomethacin (Fig. 5.4 a). Survival of polyps differed significantly among treatments and between populations (Table 5.3). The interactions between population and treatment were statistically significant for survival ($p < 0.001$ ***) and strobilation ($p < 0.01$ **).

Both concentrations of indomethacin induced polyps to strobilate, but opposite trends were observed in Varano and Mljet polyps. At the lower indomethacin concentration (10 μM), the percentage of strobilating polyp was higher for Mljet (58.3%), while 83.3% of Varano polyps strobilated with 20 μM indomethacin. Piran polyps strobilated only with 10 μM of indomethacin because all polyps died after two days of treatment with the higher concentration .

The decrease of temperature induced a higher percentage polyps to strobilate for Piran (33.3%) than Varano (8.3%), meanwhile, no Mljet polyps strobilated (Table 5.2, Fig. 5.4 b). Strobilation by polyps differed significantly among treatments and between populations (Table 5.4). The interactions between population and treatment were statistically significant for survival ($p < 0.001$ ***) and strobilation ($p < 0.01$ **).

The ephyrae production per polyp was higher in the 20 μM indomethacin treatment for Varano, but almost no difference between indomethacin treatments was observed in Mljet polyps. High numbers of ephyrae polyp^{-1} were recorded in the controls of Varano and Piran after polyps were moved to a lower temperature (11 and 10 ephyrae per polyp, respectively) (Table 5.2, Fig. 5.4 c). Twice the maximum number of ephyrae per strobila occurred in the lower concentration of indomethacin (10 μM) compared to the higher (20 μM) for Mljet polyps, meanwhile the concentrations tested did not affect this response for Varano polyps. The most ephyrae per strobila occurred in the control for Varano polyps. Similarly, the natural inducer led to the higher ephyra production by Piran polyps than did treatment with indomethacin (Table 5.2, Fig. 5.4 d).

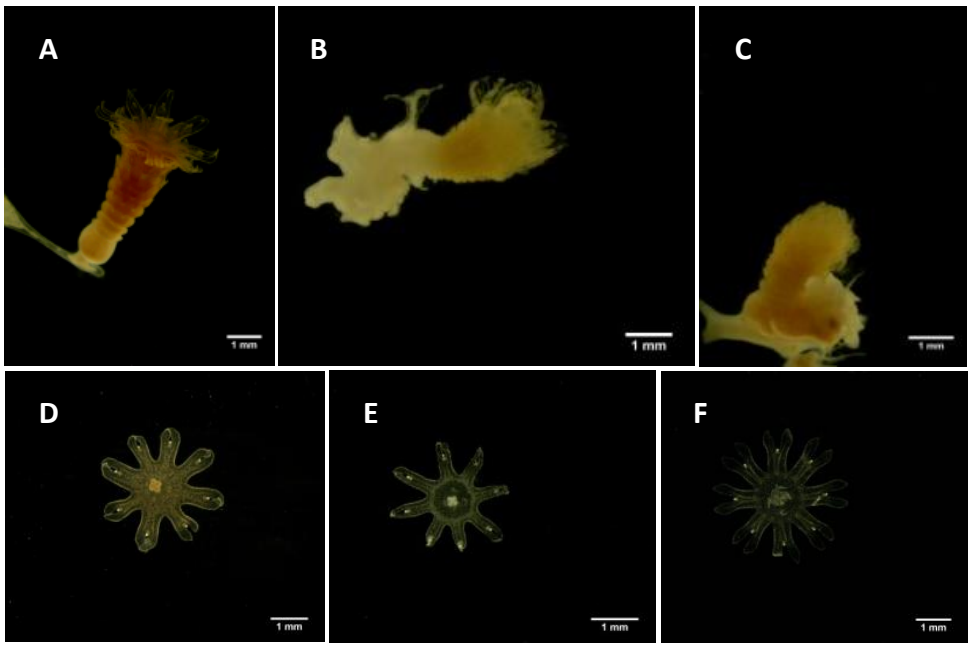

Fig. 5.3. Strobilae and ephyrae of *Aurelia* spp. populations. a-d= Varano, b-e = Mljet, c-f = Piran.

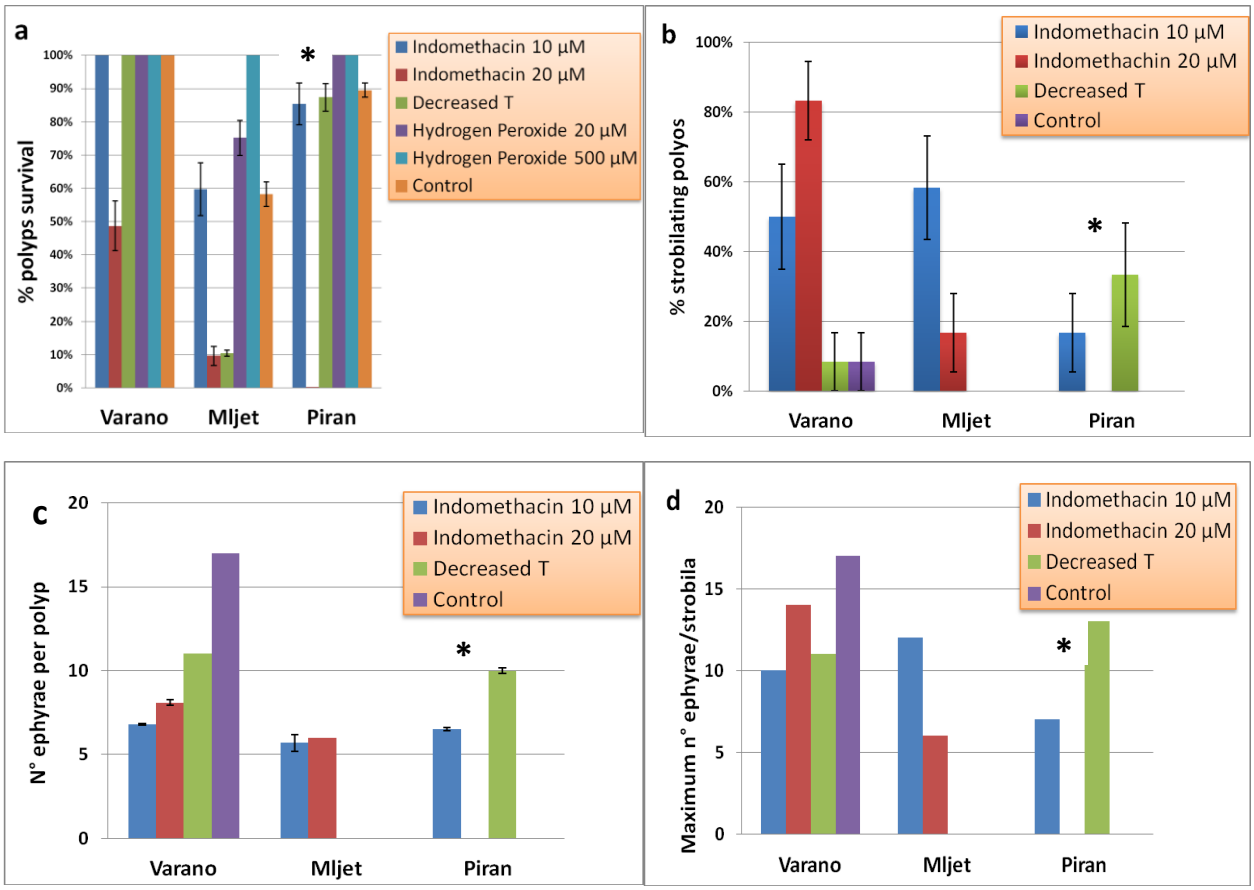

Figure 5.4. Polyps survival and strobilation under strobilation induction in *Aurelia* spp. populations (Varano, Mljet, and Piran). a) percentages of polyps survival; b) percentages of strobilating polyps; c) number of ephyrae produced per polyp; c) maximum number of ephyrae produced per strobila. Data are Mean % ± SD). Asterisks (*) indicate death of polyps.

Table 5.2. Percentages of strobilating polyps, number of ephyrae produced per polyp and maximum number of ephyrae produced per strobila, obtained during the experiment of strobilation induction in *Aurelia* spp. populations (Varano, Mljet, and Piran). (mean percentage or mean number ± SD; SD= ± percentage if > 0 or (0) when SD was 0).

Populations	Treatments			
	Indomethacin 10 µM	Indomethacin 20 µM	Decreased temperature	Control
Strobilating polyps				
Varano	50 ± 15.1%	83.3 ± 11.2%	8.3 ± 8.3%	8.3 ± 8%
Mljet	58.3 ± 14.9%	16.6 ± 11.2%	0%	0%
Piran	16.6 ± 11.2%	0%	33.3 ± 14.9%	0%
Ephyrae per polyp				
Varano	6.8 ± 0.1	8.1 ± 0.2	11 (0)	17 (0)
Mljet	5.7 ± 0.5	6 ± (0)	-	-
Piran	6.5 ± 0.1	-	10 ± 0.2	-
Maximum number of ephyrae				
Varano	10	14	11	17
Mljet	12	6	-	-
Piran	7	-	13	-

Table 5.3. Results of three-way PERMANOVA for the survival of *Aurelia* spp. polyps from three populations. The test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0,05$) are indicated in bold. The test revealed differences among Po = Population; Pl = Plate; Res = Residuals; Tr = Treatment.

Source	df	SS	MS	Pseudo-F	P(perm)	perms	P(MC)
Po	2	1.3449	0.6725	56.232	**0.0002**	4986	**0.0002**
Tr	5	9.2929	1.8586	263.6	**0.0004**	2433	**0.0002**
Pl(Tr)	6	0.0423	0.0705	0.1810	0.9808	4989	0.981
PoxTr	10	2.7222	0.2722	22.763	**0.0002**	4981	**0.0002**
PoxPl(Tr)	12	0.1435	0.0196	0.3070	0.9882	4983	0.9856
Res	180	7.0104	0.0389				
Total	215	20.556					

Table 5.4. Results of three-way PERMANOVA in the comparison of the presence/absence of strobilation in *Aurelia* spp. polyps from three populations. Test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0,05$) are indicated in bold. Po = Population; Pl = Plate; Res = Residuals; Tr = Treatment.

Source	df	SS	MS	Pseudo-F	P(perm)	perms	P(MC)
Po	2	0.9537	0.4768	6.0588	**0.016**	2813	**0.015**
Tr	5	5.9259	1.1852	128	**0.0016**	60	**0.0002**
Pl(Tr)	6	0.0556	0.0093	0.1136	0.9968	525	0.9948
PoxTr	10	6.1019	0.6102	7.7529	**0.0016**	3992	**0.0006**
PoxPl(Tr)	12	0.9444	0.0787	0.9659	0.4868	641	0.4838
Res	180	14.667	0.0815				
Total	215	28.648					

5.4.2. Ephyrae production and malformation

When strobilation was observed, no differences among populations ($p > 0.05$) were recorded for the variables studied. Differences between the 2 plates for each population in each treatment were also not significant (Table 5.6). Higher indomethacin concentration corresponded with production of malformed ephyrae in Varano (12.35%) and in Mljet (8.3%). Similar observations were not possible for Piran due to the death of the polyps. Malformed ephrae (40%) were produced in the decreased temperature treatment, which was much higher than in treatments with indomethacin (Table 5.5, Fig. 5.5 a).

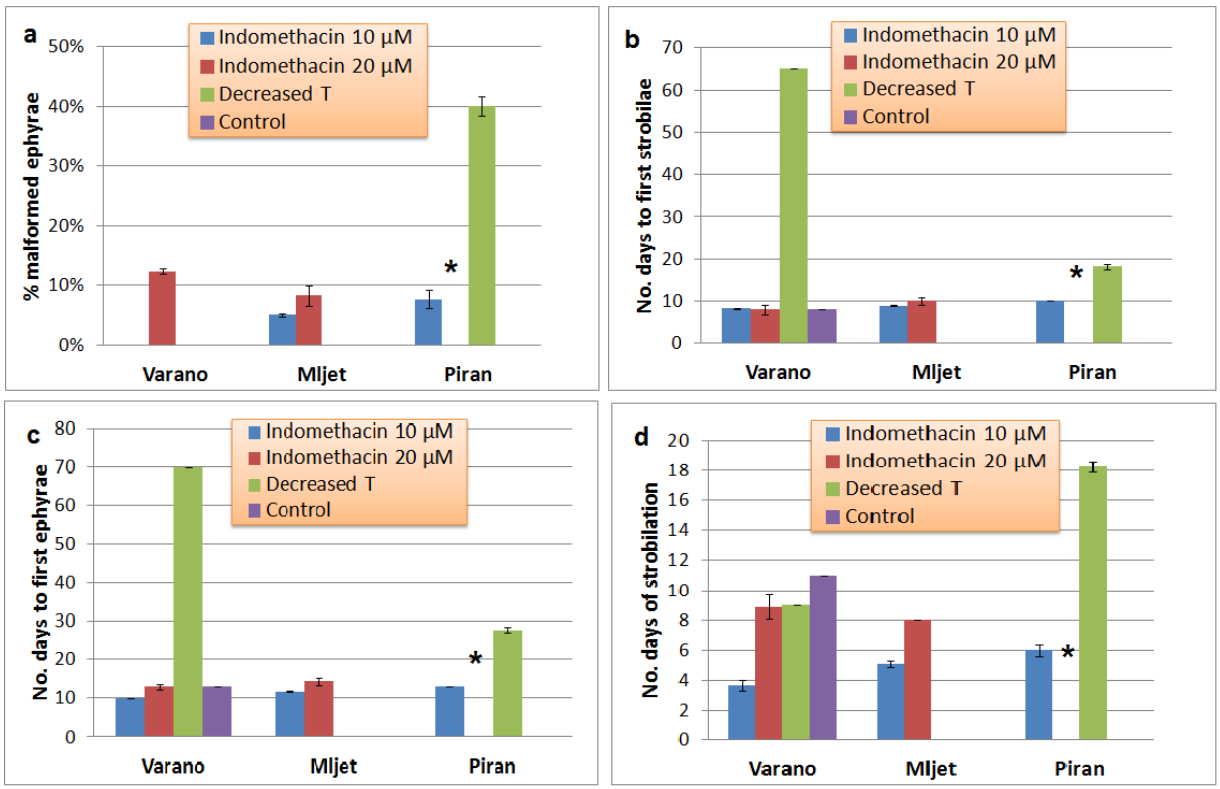

Figure 5.5. Ephyrae production and malformation under strobilation induction in *Aurelia* spp. populations (Varano, Mljet, and Piran). a) percentage of ephyra malformation in the treatments with indomethacin; b) number of days to the first strobilae; c) number of days to the first ephyrae; e) number of days of the strobilation period. Data are Mean number % ± SD. Asterisks (*) indicate death of polyps.

The PERMANOVA test showed that the strobilation inducer (treatment) significantly affected ($p < 0.001$ ***) the time required to release ephyrae, the number of ephyrae released and their malformation (Table 5.6).

The first discs of the strobilae appeared earlier when the concentration of indomethacin was higher for Varano and lower for Mljet polyps (Fig. 5.5 b). More time was needed for the natural inducer (decrease of temperature), for which the first strobila appeared after 65 days for Varano polyps and after about 18 days for Piran. Reduction of temperature did not cause strobilation in the Mljet population (Table 5.5, Fig. 5.5 b).

In Varano and Mljet polyps, ephyrae were released more quickly (10 and 11.7 days, respectively) in the lower concentration of indomethacin than in the higher concentration (13 and 14.5 days, respectively). Following the decrease of temperature, Varano polyps needed twice the time as Piran polyps in the transition from the strobila formation to release the first ephyrae (Table 5.5, Fig. 5.5 c). The period of strobilation was shorter for the lower concentration of

indomethacin (about 4-5 days) than for the higher concentration (about 8-9 days). In decreased temperature, Piran polyps showed a long strobilation period (about 18 days), double that observed in Varano (Table 5.5, Fig 5.5 d).

Table 5.5: Percentage of ephyra malformation, number of days to the first strobilae, to the first ephyrae and of the strobilation period, obtained during the experiment of strobilation induction in *Aurelia* spp. populations. (Mean percentage or mean number ± SD; SD= ± number or percentage if > 0 or (0) when SD is 0).

Populations	Treatments			
	Indomethacin 10 µM	Indomethacin 20 µM	Decreased temperature	Control
Ephyrae malformation				
Varano	0 (0%)	12.35 ± 0.47%	0 (0%)	0 (0%)
Mljet	5 ± 0.33%	8.3 ± 1.7%	-	-
Piran	7.69 ± 1.57%	-	40 ± 1.56%	-
Number of days to the first strobilae				
Varano	8.3 ± 0.15	8 ± 1.09	65 (0)	8 (0)
Mljet	8.9 ± 0.11	8 ± 0.82	-	-
Piran	10 (0)	-	18.25 ± 0.64	-
Number of days to the first ephyrae				
Varano	10 (0)	13 ± 0.76	70 (0)	13 (0)
Mljet	11.7 ± 0.15	14.5 ± 1.02	-	-
Piran	13 (0)	-	27.75 ± 0.64	-
Number of days of the strobilation period				
Varano	3.66 ± 0.35	8.9 ± 0.83	9 (0)	11 (0)
Mljet	5.11 ± 0.23	8 (0)	-	-
Piran	6 ± 0.41	-	18.25 ± 0.36	-

Table 5.6. Results of three-way PERMANOVA in the comparison of number of ephyrae released, number of days to the first strobilae, number of days to the first ephyrae and number of days of the strobilation period in polyps from three *Aurelia* spp. populations. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0,05$) are indicated in bold. Po = Population; Pl = Plate; Res = Residuals; Tr = Treatment.

Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**	**P(MC)**
Po	2	17.579	8.7894	6.4056	0.0878	4991	0.0058
Tr	3	70.382	23.461	20.944	**0.0002**	630	0.0002
Pl(Tr)	3	2.5561	0.85203	0.53987	0.7924	4982	0.8098
PoxTr**	2	20.698	10.349	7.5075	0.0652	4991	0.0036
PoxPl(Tr)**	2	2.6565	1.3283	0.84161	0.5162	4984	0.5202
Res	20	31.565	1.5782				
Total	32	160					

Canonical analysis of principal coordinates (CAP) indicated that the relative distinctiveness about strobilation process differed with the treatment. The displacement of treatment along the canonical axis 1 was realized through the variable "strobilation period" that explained a correlation value of 0.9 and grouped the indomethacin treatments together. The variable "ephyrae released" (ephyrae per polyp) separated the treatments along the canonical axis 2 with a correlation value of 0.68 (Fig. 5.6). As demonstrated in Table 5.4, the number of ephyrae per polyp was higher in the treatments with the natural inducer.

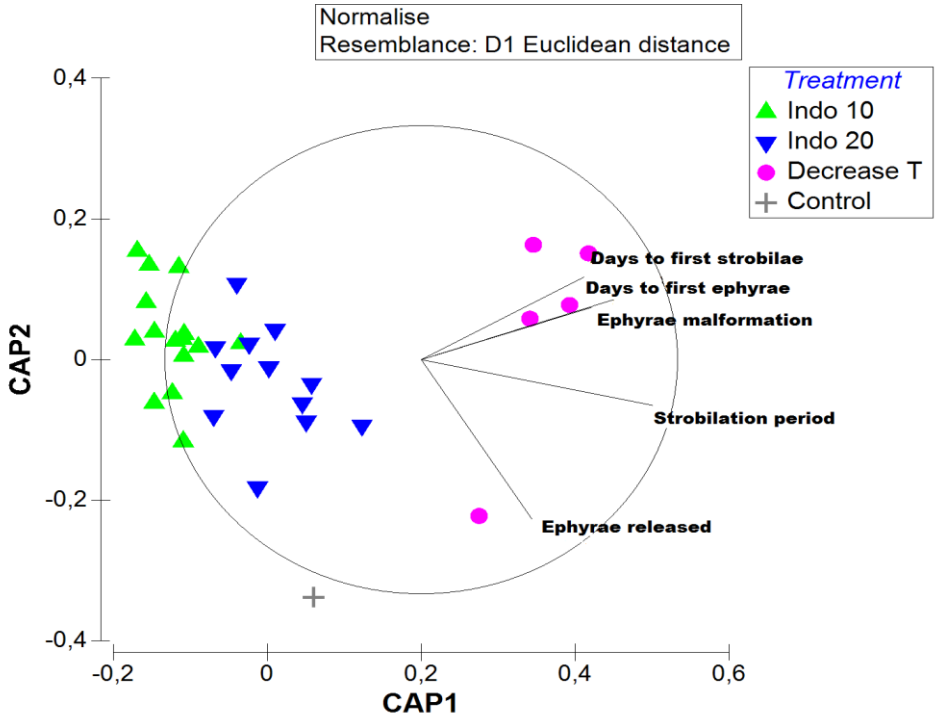

Fig. 5.6. Canonical analysis of principal coordinates (CAP) examining the effects of treatment on the variables involved in the strobilation process of three *Aurelia* spp. populations (Varano, Mljet, and Piran). Correlation with canonical axes are only shown when the Pearson's correlation coefficient > 0.2. The length of each vector line is proportional to the strength of the correlation.

The malformations of ephyrae especially modified the number of marginal lappets, and sometimes also the number of rhopalia, fusion of marginal lappets and fusion of the central discs in all populations. In the indomethacin treatments, the number of marginal lappets varied among two, three, six and seven in comparison to the normal number (8). In some cases, marginal lappets fused with rhopalia (Fig. 5.7). The main malformations for the natural inducer treatment were only in the number of marginal lappets that affected only Piran population: seven and fourteen (Fig. 5.8) were observed.

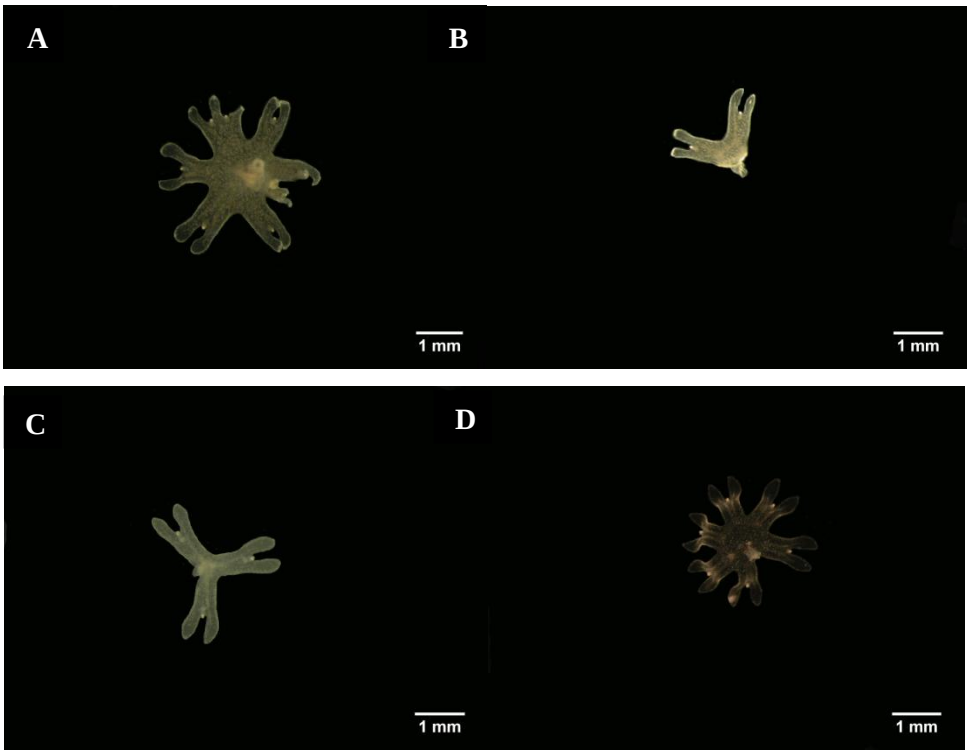

Figure 5.7. Examples of *Aurelia* spp. ephyrae malformed with a treatment of 20 μM indomethacin in Varano and. A) ephyra with one very short marginal lappet and two marginal lappets fused with two rhopalia (Varano); B) ephyra with only two marginal lappets (Varano); C) ephyra with only three marginal lappets (Mljet); D) ephyra with six marginal lappets (Mljet).

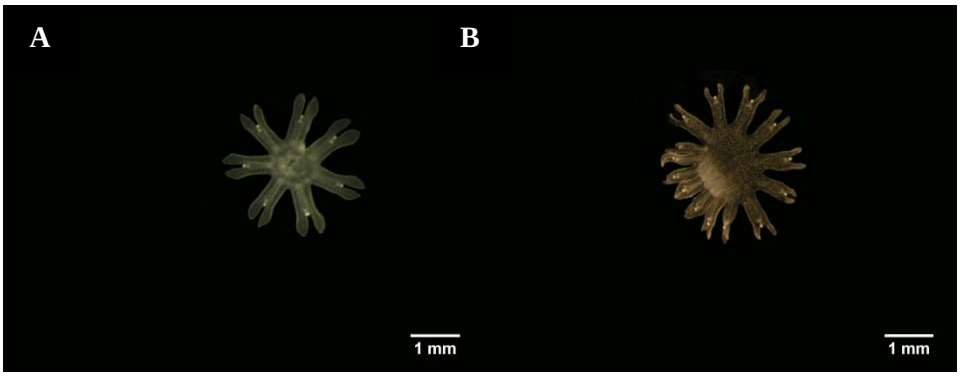

Figure 5.8. Examples of *Aurelia* spp. ephyrae malformed after decrease of temperature in the Piran population. A) ephyra with seven marginal lappets; B) ephyra with fourteen marginal lappets.

5.4.3. Second treatment with indomethacin: Polyps survival and strobilation

Another test was performed on polyps that survived the first indomethacin treatment to observe their ability to respond again to a second treatment with 10 μM indomethacin after 1 week from the first. To avoid side - effects of higher indomethacin concentration, we selected only the lower concentration for the second treatment. The results of the two initial treatments (10 μM) of indomethacin were compared to those from the second treatment of 10 μM indomethacin. In the

controls populations of Varano and Piran, the percentage of survival was similar to the first treatment with indomethacin. In Mljet, the survival in controls was lower than the first treatment with indomethacin (Fig. 5.9 a). Commonly, the second treatment with indomethacin reduced survival of polyps in all the population tested. The strongest effect was for Mljet polyps, of which only 2.1% survived the second treatment (Fig. 5.9 a). PERMANOVA test was performed to extrapolate information about the factor "population". It was demonstrated that the responses observed (Fig. 5.9) in the second contact with the indomethacin is a prerogative of the population considered being significant ($p < 0.01$**) different in one - way PERMANOVA test (Table 5.8). In controls populations, strobilation was not observed. Production of strobilating polyps was higher in Piran (50%) and in Varano polyps (75%) in the second treatment than in the previous treatment, meanwhile an opposite was observed for Mljet polyps (Table 5.7, Fig. 5.9 b). In all populations, more ephyrae were produced per polyp with the second treatment than the ephyrae produced with the first, for Piran polyps, the number almost doubled (Table 5.7, Fig. 5.9 c). The maximum number of ephyrae per strobila was higher afterthe second treatment for Varano and Piran polyps, in particular it tripled for Piran, but there was no difference for Mljet (Table 5.7, Fig. 5.9 d).

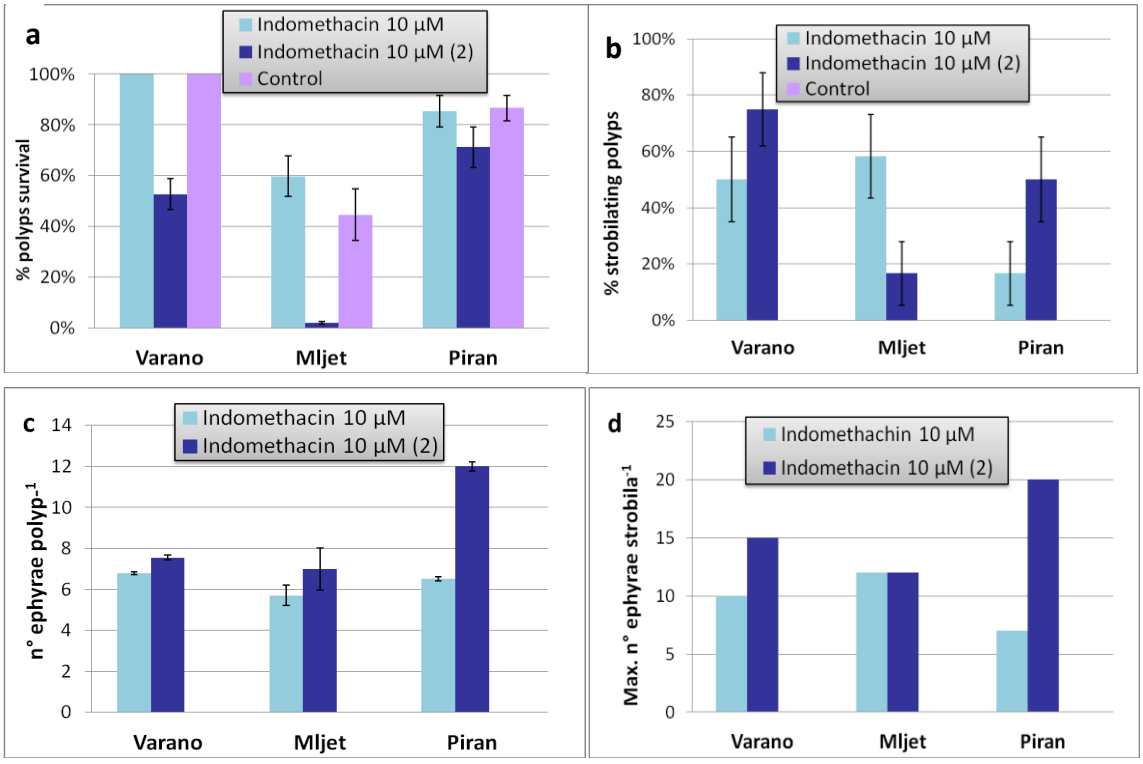

Figure 5.9. Polyps survival and strobilation under strobilation induction with the second treatment with indomethacin in *Aurelia* spp. populations (Varano, Mljet, and Piran). a) percentages of polyps survival; b) percentages of strobilating polyps; b) number of ephyrae produced per polyp; c) maximum number of ephyrae produced per strobila. Data are Mean ± SD.

Table 5.7. Percentages of strobilating polyps, number of ephyrae produced per polyp and maximum number of ephyrae produced per strobila, in the first and second induction of strobilation with indomethacin in *Aurelia* spp. population (mean percentage or mean number ± SD; SD= ± number/percentage if > 0 or (0) when SD is 0).

Populations	Treatments		
	Indomethacin 10 μM	Indomethacin 10 μM (2)	Control
Strobilating polyps production			
Varano	50 ± 15.07%	75 ± 13.05%	0 (0%)
Mljet	58.3 ± 14.86%	16.6 ± 11.24%	0 (0%)
Piran	16.6 ± 11.23%	50 ± 15.07%	0 (0%)
Ephyrae/polyp production			
Varano	6.8 ± 0.07%	7.55 ± 0.11	-
Mljet	5.7 ± 0.5	7 ± 1.02	-
Piran	6.5 ± 0.1	12 ± 0.23	-
Maximum number of ephyrae production			
Varano	10	15	-
Mljet	12	12	-
Piran	7	20	-

Table 5.8. Results of one - way PERMANOVA comparing percentage of ephyra malformation, ephyrae released, number of days to the first strobilae, number of days to the first ephyrae and number of days of the strobilation period in the second induction of strobilation with indomethacin in *Aurelia* spp. populations.
Test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0,05$) are indicated in bold. Po = Population; Res = Residuals.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Po	2	25,539	12,769	3,2826	**0,0084**	4965	**0,0138**
Res	14	54,461	3,8901				
Total	16	80					

5.4.4. Second treatment with indomethacin*:* Ephyrae production and malformation.

Ephyra malformation was considerably enhanced in the second treatment. The PERMANOVA test showed that the first and second treatments of indomethacin affected the strobilation process differently ($p < 0.01$ **) and the factor "population" did not play a significant role in the differences observed (Table 5.9).

In Varano a high percentage of malformed ephyrae that were not recorded in the first induction was observed in the following treatment (45.59%) (Table 5.10, Fig. 5.10 a).

The first strobilae appeared more quickly in the second induction than in the first. Again, strobila formation in Piran polyps was slower than in the other two populations (Table 5.10, Fig. 5.10 b). Similarly, the days requested for the release of ephyraewas longer for Piran polyps (Fig. 5.10 c). After the second treatment with 10 μM of indomethacin, the strobilation period was shorter in Mljet but longer in Varano and Piran than in the the first treatment (Table 5.10, Fig. 5.10 d).

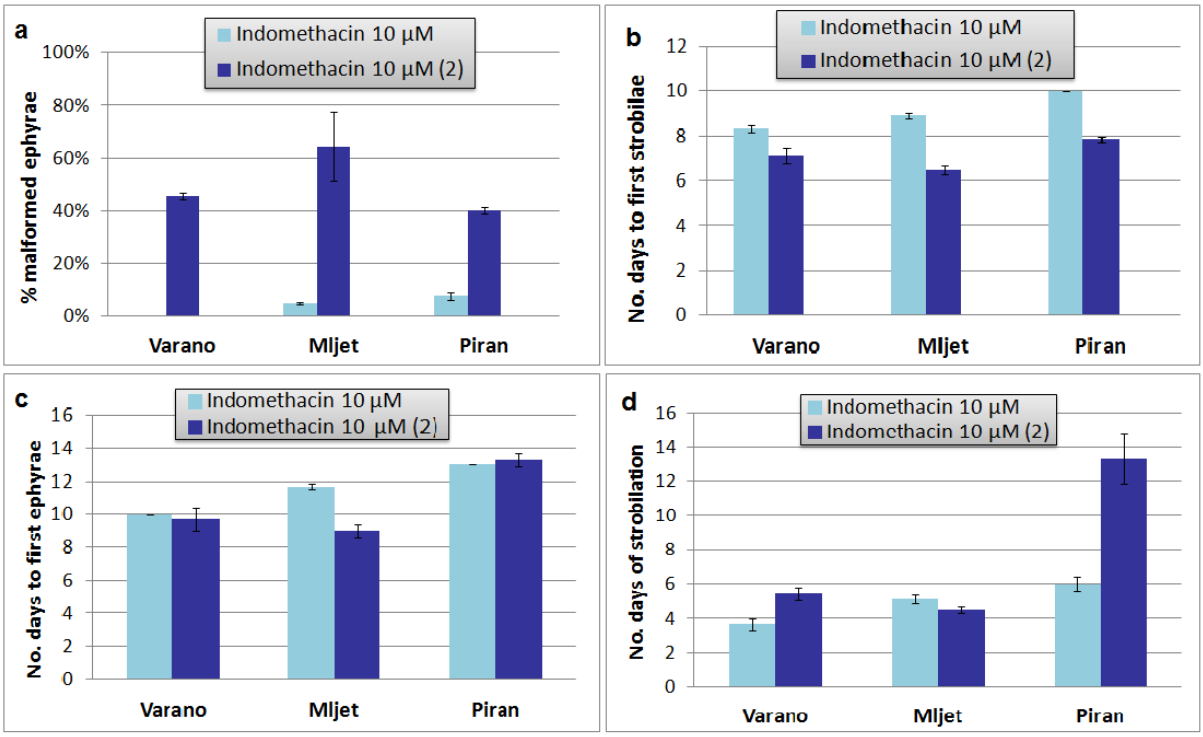

Figure 5.10. Ephyrae production and malformation under strobilation induction with the second treatment with indomethacin in *Aurelia* spp. populations (Varano, Mljet, and Piran). a) percentage of ephyra malformation in the treatment with indomethacin; b) number of days to the first strobilae; c) number of days to the first ephyrae; d) number of days of the strobilation period. Data are Mean ± SD.

Table 5.9. PERMANOVA comparison of percentage of ephyra malformation, ephyrae released, number of days to the first strobilae, number of days to the first ephyrae and strobilation period between the first and the second induction of strobilation with indomethacin in *Aurelia* spp. populations. PERMANOVA main test was run with 4999 unrestricted permutations of raw data. Monte Carlo test was performed. Significant p values ($p < 0.05$) are indicated in bold. Po = Population; Res = Residuals; Tr = Treatment.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Po	2	7.3847	3.6923	2.2127	0,0734	3848	0,1386
Tr	2	49.941	24.97	14.964	**0,0042**	3869	**0,0008**
Res	4	6.6748	1.6687				
Total	8	64					

Table 5.10. Percentage of ephyra malformation, number of days to the first strobilae, number of days to the first ephyrae and number of days of the strobilation period, in the first and second induction of strobilation with indomethacin in *Aurelia* spp populations (mean percentage or mean number ± SD); SD= ± number/percentage if > 0 or (0) when SD is 0).

Populations	Treatments		
	Indomethacin 10 μM	Indomethacin 10 μM (2)	Control
Ephyrae malformation			
Varano	0 (0)%	45.59 ± 1.33%	-
Mljet	5 ± 0.33%	64.29 ± 13.12%	-
Piran	7.69 ± 1.57%	40.28 ± 1.23%	-
Number of days to the first strobilae			
Varano	8.3 ± 0.15	7.11 ± 0.34	-
Mljet	8.9 ± 0.11	6.5 ± 0.2	-
Piran	10 (0)	7.83 ± 0.11	-
Number of days to the first ephyrae			
Varano	10 (0)	9.7 ± 0.69	-
Mljet	11.7 ± 0.15	9 ± 0.41	-
Piran	13 (0)	13.3 ± 0.43	-
Number of days of the strobilation period			
Varano	3.66 ± 0.35	5.44 ± 0.36	-
Mljet	5.11 ± 0.23	4.5 ± 0.2	-
Piran	6 ± 0.41	13.3 ± 1.46	-

The high percentages of malformation (more than 40% in all populations) recorded during the second treatment of indomethacin 10 μM were more homogeneous in terms of the structures modified. In this case, malformations in ephyrae involved mainly the number of marginal lappets and ephyrae with seven (Mljet and Piran), nine (Mljet), eleven and twelve (Varano), and fourteen (Piran) marginal lappets appeared (Fig. 5.11).

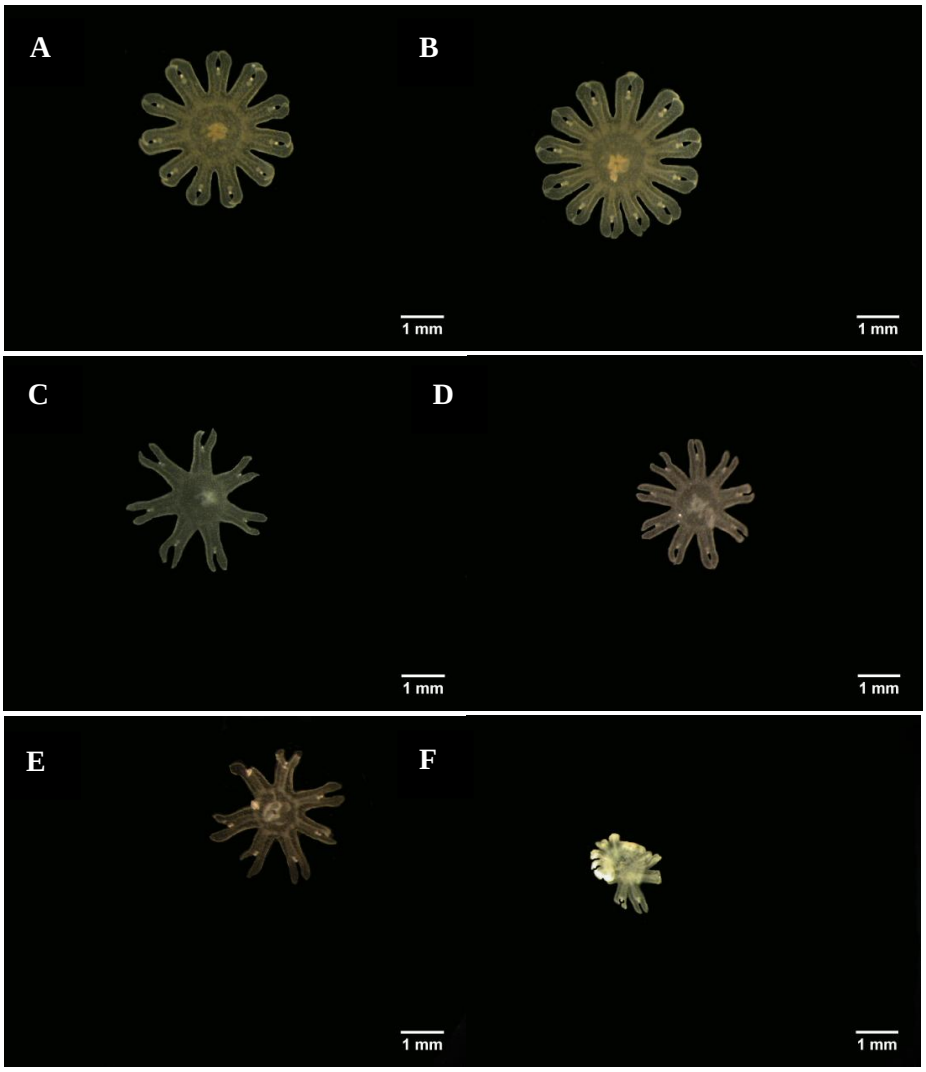

Figure 5.11. Examples of ephyrae malformed in the second treatment with 10 μM indomethacin in Varano, Mljet and Piran population. A) ephyra with eleven marginal lappets (Varano); B) ephyra with twelve marginal lappets (Varano); C) ephyra with seven marginal lappets (Mljet); D) ephyra with nine marginal lappets (Mljet); E) ephyra with seven marginal lappets (Piran); F) ephyra with in total fourteen marginal lappets and with eleven short, small lappets (Piran).

5.5. Discussions and conclusions

Experimental data on strobilation inducers in *Aurelia* spp. polyps from three populations (Varano, Mljet, and Piran) showed that responses differed among populations and treaments in terms of survival percentage, polyps strobilating, ephyrae production and times. The treatment with hydrogen peroxide did not produce strobilation in any population tested in both concentrations (20 and 500 μM). According to Berking et al. (2005), a concentration of 1 nM of hydrogen peroxide is effective to induce strobilation in 24 hours in *Aurelia aurita* polyps kept at

20° C. In the present study higher concentrations of hydrogen peroxide were tested at 21°C for longer time but no effect was observed.

In Varano, polyps strobilated in all remaining treatments (indomethacin 10 and 20 μM, decrease of temperature, and control) and the number of ephyrae released was fairly high and constant. These results pointed out a high capacity of Varano polyps to strobilate through treatments with both chemical and natural inducers.

In Mljet, strobilation was observed for both indomethacin treatments, but many strobilating polyps were observed in the lowest concentration and no effects due to decrease of temperature were recorded. Since populations showed different responses to inducers, a hypothesis could be that the monitoring time (117 days) was insufficient to observe strobilation in this population. On the other hand, ephyrae in Mljet persist all year (Kogovšek et al., 2012) and an alternative explanation could be that the polyps are adapted to live at constant temperatures in the Mljet marine lakes; therefore, strobilation in response to temperature decrease did not develop.

Unlike Mljet, Piran polyps released many ephyrae when temperature was lowered. In nature Piran polyps release ephyrae only in colder months from December to February (Malej et al., 2012). Results from in the present experiment agree with their observations and it is probably an adaptation to local conditions that led polyps also to strobilate in February in the laboratory. The high death recorded in this population at the high concentration of indomethacin contrasted with the high number of ephyrae in the lower concentration. The hypothesis that polyps can resist until a threshold level of substance probably within the 10 – 20 μM range should be investigated further at population – species level. Higher indomethacin also affected the survival of the other populations; this consequence suggests that a test with a lower concentration of this substance can minimize the risk of death in individuals. In particular, in Varano only a low percentage of polyps tested suffered in the higher concentration of indomethacin; for the remaining treatments polyps proved to be very resistant to various stimuli that received. The phenotypic plasticity of *Aurelia* sp.1 (Varano) and the highly variable environment considered (see Chapter 2) may explain the resistance of polyps to environmental stress.

In contrast, Mljet polyps were susceptible to temperature decrease that led to the death for many individuals of this population. A possible explanation could be also related to the physical characteristics of their natural environment, specifically, that the polyps were found below the thermocline in Mljet lake where temperature is around 14°C (Kogovšek *et al.* 2012). As result,

the adaptations to different abiotic conditions like the temperature may not occur in this population.

Although indomethacin has the potential to induce strobilation (Kuniyoshi et al., 2012), the side effects due to the concentration tested were remarkable and are here considered for the first time. The absence of malformation in the controls suggests that the prevalence of malformations was caused either by chemical or natural inducers seems to depend on the strobilation trigger and on the different behaviors of populations.

The rates of ephyrae malformation were in most of case due to the artificial inducer and only in Piran the natural inducer accounted for a substantial percentage of malformation. As previously discussed, the lower concentration of indomethacin (10 μM) could minimize the risks of both death and ephyrae malformations in *Aurelia* spp. These negative effects seem to affect the three populations in different ways.

The times of first strobilae formation and ephyrae release in the indomethacin treatments were shorter than in the natural inducer (temperature decrease). In general, polyps in indomethacin became strobilae in a maximum of 10 days, as compared to the maximum of 65 days of the natural inducer. A lower concentration of indomethacin was effective in ephyrae release and the strobilation period that was much longer in the natural treatment (Piran, >18 days).

The inverse correlation found between indomethacin concentration and time at ephyrae release (Kuniyoshi et al., 2012) was not found in the present study.

The successive treatment of strobilation induction with indomethacin increased the strobilation ability of *Aurelia* spp. polyps, especially of Varano and Piran populations. On the other hand, although more ephyrae per polyp were released in all the populations, mostly were malformed.

In conclusion of the present study, it was demonstrated that in the *Aurelia* sp. population from Piran (*Aurelia* sp.8), strobilation was favored by a decrease of temperature (as in Miyake et al., 2002; Di Camillo et al., 2010; Malej et al., 2012). In the northern Adriatic Sea, *Aurelia* sp. polyps strobilated in colder months from November to February around 15°C and 35 psu (Malej et al., 2012), which matched with the observation of strobilation in the laboratory (March) for the Piran population.

The scyphopolyps from Varano in the temperature decrease treatment released ephyrae in May, which confirmed the previous observation that ephyrae are found in the water column of the coastal lagoon only in spring, between April and May (pers. obs., see Chapter 2).

The molecular mechanism and the pathway at the foundation of strobilation induction in *Aurelia* spp. polyps have been actually elucidated (Fuchs et al., 2014), but the response differences revealed in this work could clarify how in the three *Aurelia* species that coexist along the Adriatic coasts (Chapter 4) the role of epigenetic factors is fundamental to explain the patterns observed.

Tests of strobilation inducers in *Aurelia* spp. can help create a validated protocol for observing strobilation in polyps and in particular this preliminary study demonstrated that indomethacin is the best inducer to obtain high percentages of strobilating polyps (83.3%) in a brief amount of time (10 days).

Future research on signaling cascades activated by endogenous or environmental factors will provide information on molecular mechanisms through new findings about this asexual process in the *Aurelia* cryptic species.

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6. General conclusions

In the Mediterranean sea, reports of native or alien jellyfish invasion, are rapidly increasing and *Aurelia* jellyfish outbreaks are frequently reported from several areas of Mediterranean sea.

In this PhD thesis, the occurrence of the non-indigenous *Aurelia* sp. 1 has been demonstrated in the Varano lagoon, along the Southern Adriatic coast, providing the first undisputable evidence of the establishment of this alien species in the Mediterranean Sea. For the first time, the life cycle of *Aurelia* sp. 1 has been reconstructed, highlighting environmental conditions that operate as trigger of jellyfish output but also as limiting factors for jellyfish dynamics.

Analyses of nutrients, zooplankton, and phytoplankton dynamics, coupled with investigations on jellyfish trophic ecology, demonstrated that *Aurelia* sp. 1 can predate on different food sources, affecting the food web at different trophic levels.

Nevertheless, the predatory control of jellyfish on the plankton community is density - dependent and cascading effects on trophic web could be detected only at high jellyfish abundance. The same analyses also led to exclude a negative impact on ichtyoplankton.

The classification of different *Aurelia* spp. cryptic species was made possible by the adoption of an integrated approach combining data gathered from morphological and morphometrics analyses with molecular data based on COI sequences. Species collected from different areas in Mediterranean were identified, showing the ability of *Aurelia* sp.1 as successful invader of enclosed coastal habitats, the Varano lagoon (Italy) and the marina of Empuriabrava, (Spain).

The molecular analyses also demonstrated the spread of a second alien species, *Aurelia* sp. 8, a Lessepsian immigrant in the Mediterranean Sea through the Suez Canal. A third species was confirmed to have a restricted distribution in the marine lake of Mljet in Croatia.

In addition, morphology/morphometrics provided the first and complete taxonomic description of cryptic species in Mediterranean focused on the three life stages (polyp, ephyra, medusa).

Finally, studies carried out in laboratory elucidated some specie – specific developmental pattern underlying the process of strobilation, suggesting that ecophysiological studies on epigenetic adaptations may help discriminating among cryptic species.

These results contribute to improve our general understanding of mechanisms fostering jellyfish blooms, supporting prediction of future scenarios of the Mediterranean ecosystem changes.

Acknowledgments

The research was supported by VECTORS (Vectors of Change in Oceans and Seas Marine Life, Impact on Economic Sectors) within the Ocean of Tomorrow call of the European Commission Seventh Framework Programme (FP 7 - OCEAN 2010).

I would like to express my profound gratitude to Prof. Stefano Piraino (University of Salento) for his precious contribution through supervision of this PhD research. He supported my research interests, he connected me with people and resources and guided me from initial proposition to its completion. Thank you for having brought out the best of me.

I kindly acknowledge Dr. Roberta Cimmaruta and the Tuscia University of Viterbo for the opportunity provided to me to achieve this academic goal.

I would like to express my most sincere gratitude to Prof. Davor Lucic and Dr. Jakica Nijre from the University of Dubrovnik for the analyses carried out on microzooplankton. I also thank Dr. Giorgio Alabiso and Dr. Carmela Caroppo (IAMC - CNR, Taranto) for the analyses of nutrients and phytoplankton.
I am grateful to Dr. Salvatrice Vizzini and Prof. A Mazzola (University of Palermo) for the help gave me during isotope analyses.

Collecting samples for over two years in the lagoon of Varano required the help of many people that I would like to thank: Rosa Caprioli, Giorgio Aglieri, Giacomo Milisenda, Noyan Yilmaz, Mar Bosh Belmar, Luis Martell and the fisherman Antonio Coccia for its assistance in field collection.
I would also like to thank Prof. Genuario Belmonte and Dott. Giuseppe Alfonso for the materials provided and for their precious advices in the study of plankton.

I thank Ivona Onofri, Sonia Gueron and Melissa Acevedo for providing *Aurelia* spp. samples from Mediterranean locations and Veronica Fuentes for its hospitality at the ICM - CSIC (Barcelona, Spain) at the beginning of this PhD.
I thank also Francesco De Leo, who provided the maps for the chapters 4 and 5.

I'd like to convey my thanks to Michael N Dawson for its encouragement and interest, and also to Jennifer Purcell who provided valuable feedback on the thesis writing.

A special *thank you* to all colleagues and friends who supported me during these years and who have shared this journey with me, giving support even through the darkest days: Adriana, Anna, Anna Lucia, Antonella, Federica, Giacomo, Mar, M.Teresa, Lucia, Luis, and Rosa.

Lastly to my family. Thank you all for everything, for your patience and counsel over the years. Without your support and encouragement I don't know where I would be or what I would be doing. Thank you.

And last, but very far from least, thanks to Fabio. He believes in me more than I believe in myself. Thanks for gave me the strength during the years of my studies.

Appendix A

TAXA	CATEGORIES
CRUSTACEA	Copepoda Calanoida
	Copepoda Cyclopoida
	Copepoda Harpacticoida
	Copepoda nauplii
	Copepoda Poecilostomatoida
	Brachyura Zoea
	Brachyura megalopa
	Palinura megalopa
	Caridea zoea
	Decapoda natantia zoea
	Cirripedia balanomorpha nauplii
	Cirripedia balanomorpha larvae (cypris)
	Cladocera
	Euphasiacea larvae
	Ostracoda larvae
	Amphipoda
	Mysidiacea
MOLLUSCA	Bivalvia larvae
	Gastropoda larvae
ANELLIDA	Polychaeta Spionidae larvae
	Polychaeta Spionidae juvaniles
	Polychaeta Nereidae larvae
	Polychaeta Sabellaridae larvae
	Polychaeta Phyllodocida larvae
SCYPHOZOA	*Aurelia* sp. ephyrae
	Aurelia sp. planulae
	Hydrozoa medusae
SIPHONOPHORA	*Lensia* sp.
CTENOPHORA	*Pleurobrachia* sp.
OTHER INVERTEBRATES	Invertebrate eggs
	Phoronida actinotrocha
	Chaetognatha
	Appendicularia
	Insect larvae
	Chirinomidae
	Nematoda
OSTEICHTHYES	Fish eggs
	Fish larvae
MICROZOOPLANKTON/PHYTOPLANKTON	Diatoms Bacillariophyceae *Nitzschia* sp.
	Diatoms (Centrales)
	Dinoflagellates (*Ceratium* sp.*)*
	Dinoflagellates (*Noctiluca scintillans*)
	Tintinnida

Appendix B

Table B - I. Previous taxonomic identification, collection locality, collection ID and GeneBank accession numbers of the sequences. n= number of samples.

Taxon	Collection locality	Specimen collection no.	Genebank accession no.	n
Aurelia sp.	Varano Lake (Italy) - Adriatic Sea	NSAM00011Q-S	XXXXXX	9
Aurelia sp.	Mljet Lake (Croatia) - Adriatic Sea	NSAM00022G-Q	XXXXXX	11
Aurelia sp.	Empuriabrava (Spain) - Balearic Sea	NSAM00033U,W-Z NSAM00034A,D-L	XXXXXX	15
Aurelia sp.	Porto Cesareo (Italy) - Ionian Sea	NSAM00034Y,Z NSAM00035A,B	XXXXXX	4
Aurelia sp.	Oban (Scotland) - North Atlantic Ocean	NSAM00035D	XXXXXX	1
Aurelia sp.	Orkney (Scotland) - North Sea	NSAM00035E	XXXXXX	1
Aurelia sp.	St Andrews (Scotland) - North Sea	NSAM00035F	XXXXXX	1
Aurelia sp.	Southampton (England) - English Channel	NSAM00035G	XXXXXX	1
Aurelia sp.	Bizerte bay (Tunisia) - Mediterranean Sea	NSAM00035K,L,Q	XXXXXX	3
Aurelia sp.	Bizerte lagoon (Tunisia) - Mediterranean Sea	NSAM00035R-V,X-Z	XXXXXX	8
Aurelia sp.1	Tokyo bay (Japan) - North Pacific Ocean	Blast	AY903203	1
Aurelia sp.1	Sakata bay (Japan) - North Pacific Ocean	Blast	AY903186	1
Aurelia sp.1	Sidney (Australia) - South Pacific Ocean	Blast	AY903181	1
Aurelia sp.1	Perth (Australia) - Indian Ocean	Blast	AY903178	1
Aurelia sp.1	San Diego (California, USA) - North Pacific Ocean	Blast	AY903089	1
Aurelia sp.2	Sao Paulo (Brazil) – Southwest Atlantic Ocean	Blast	AY903119	1
Aurelia sp.3	Malakal (Palau) – Pacific Ocean	Blast	AY903096	1
Aurelia sp.4	Oahu (Hawaii) – Pacific Ocean	Blast	AY903136	1
Aurelia sp.5	Mljet Lake (Croatia) - Adriatic Sea	Blast	AY903123	1
Aurelia sp.6	New Britain (Papua New Guinea) –Pacific Ocean	Blast	AY903129	1
Aurelia sp.7	Huon Estuary (Tasmania, Australia) – Tasman Sea	Blast	AY903138	1
Aurelia sp.8	Bay of Ston (Croatia) - Adriatic Sea	Blast	AY903134	1
Aurelia sp.9	Gulf of Mexico	Blast	AY903172	1
Aurelia sp.10	Kachemak Bay (Alaska, USA) – Gulf of Alaska	Blast	AY903067	1
Aurelia aurita	Anglesey (Wales, UK) – Irish Sea	Blast	AY903208	1
Aurelia aurita	Bosphorus (Turkey)	Blast	AY903117	1
Aurelia limbata	Hokkaido (Japan) – Sea of Japan	Blast	AY903189	1
Aurelia labiata	Tomales bay (California, USA) - North Pacific Ocean	Blast	AY903076	1

List of associated posters/publications

Publications

- Piraino S., Aglieri G., Martell L., Mazzoldi C., Melli V., Milisenda G., **Scorrano S**., and Boero F., 2014. *Pelagia benovici* sp. nov. (Cnidaria, Scyphozoa): a new jellyfish in the Mediterranean Sea. Zootaxa 3794 (3): 455 - 468.

- Manzari C., Fosso B., Marzano M., Annese A., Intranuovo M., D'Erchia AM, Gissi C., Picardi E., Santamaria M., Caprioli R., **Scorrano S**., Stabili L., Piraino S., Pesole G. (submitted to Biological Invasion journal). Analysis of the aquatic microbiome diversity under the influence of an invasive alien species through the application of an Illumina-based protocol.

- **Scorrano** S., Aglieri G., Dawson M.N., Piraino S. (in prep). *Aurelia* spp. in the Mediterranean and North Sea: molecular and morphological investigations.

Posters

- **Scorrano** S., Nijre J., Lucic D., Boero F., Piraino S., 2013. Consequences of a gelatinous dominated ecosystem: preliminary observations from a Mediterranean lagoon. VI EUROLAG & VII LAGUNET Conference, Lecce, 16-19 dicembre, (Atti VI EUROLAG & VII LAGUNET Conference). Pag.84.

- Stabili L., Caprioli R., Kos Kramar M., **Scorrano** S., Boero F., Turk V., Piraino S., 2013. Jellyfish and the bacterial community of Varano lake. First EMBO Conference on Aquatic Microbial Ecology – SAME13. Stresa, Italy, 8-13 September 2013.

- **Scorrano** S., Chirizzi C., Caprioli R., Purcell J.E, and Piraino S., 2013. Test of strobilation inducers on *Aurelia* spp: towards a validated protocol. Atti del XXIII Congresso della S.It.E. pag. 155.

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- **Scorrano** S., Nijre J., Lucic D., Piraino S., 2013. Trophic cascades driven by *Aurelia* sp.1 in a Mediterranean lagoon. International Jellyfish Bloom Symposium. Hiroshima, 5 - 7 June 2013. Pag. 110.

- Caprioli R., Lala P., **Scorrano** S., Piraino S., Stabili L., 2012. Antibacterial activity of extracts from the jellyfish *Aurelia* sp. Annual VECTORS Meeting, Piran.

- **Scorrano** S., Aglieri G., Caprioli R., Durante G., Milisenda G., Piraino S.,2012. Jellyfish invasions along Adriatic coasts: trophic impact of a resident Aurelia sp. population in a coastal lagoon. Annual VECTORS Meeting, Piran.

- **Scorrano** S., Aglieri G., Caprioli R., Denitto F., Milisenda G., Piraino S.,2011. Preliminary observations on diet and population dynamics of the moon jellyfish *Aurelia* cf. *aurita* in the Varano coastal lagoon. Atti del XXI congresso S.It.E. Pag. 163.