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Struttura trofica delle comunità pelagiche del Mar Adriatico attraverso l'analisi degli isotopi stabili: dallo zooplancton ai piccoli pelagici

Food web structure of pelagic communities in the Adriatic Sea elucidated by stable isotope analysis: from zooplankton to small pelagic fishes

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Summary

STRUTTURA TROFICA DELLE COMUNITÀ PELAGICHE DEL MAR ADRIATICO ATTRAVERSO L'ANALISI DEGLI ISOTOPI STABILI: DALLO ZOOPLANCTON AI PICCOLI PELAGICI

(riassunto in italiano)

L'obiettivo di questo lavoro è lo studio dell'ambiente pelagico dell'Adriatico Occidentale, attraverso l'analisi della comunità zooplanctonica e delle sue relazioni trofiche con due specie di pesci pelagici (piccoli pelagici), l'acciuga *Engraulis encrasicolus* e la sardina *Sardina pilchardus*, mediante l'analisi degli isotopi stabili (di seguito indicate come AIS) di azoto e carbonio. I campioni utilizzati sono stati raccolti durante la campagna oceanografica MEDIAS 2019 GSA 17 e GSA 18, a bordo della R/V "G. Dallaporta", nel periodo di giugno-luglio 2019, coprendo la costa italiana dell'Adriatico da nord a sud. I campioni di zooplancton sono stati raccolti attraverso un retino WP2, con maglia da 200 μm. In corrispondenza di ogni retinata sono stati raccolti dati sulle variabili oceanografiche, mediante sonda CTD. I campioni di acciuga e sardina sono stati invece catturati con una rete volante scientifica monobarca. Per i campioni di zooplancton, gli animali sono stati identificati al livello tassonomico più basso possibile, contati e pesati per le stime di abbondanza e biomassa. I taxa più abbondanti sono inoltre stati messi a seccare in stufa a 60° C per essere utilizzati per le AIS. Nei campioni di pesce, il muscolo bianco di alcuni individui è stato seccato e preparato per le

AIS. I campioni così processati sono stati inviati all'Università di Palermo, per l'analisi della composizione elementare (%N e %C) e i valori di $\delta^{13}C$ e $\delta^{15}N$ dello zooplancton e dei pesci in esame. Dai risultati è emerso che la biomassa e l'abbondanza dello zooplancton diminuiscono significativamente spostandosi da Nord, verso Sud. Inoltre, le comunità zooplanctoniche delle diverse sub-aree dell'Adriatico mostrano differenze significative in composizione e abbondanza delle diverse specie tra le aree settentrionali e quelle meridionali, ma non tra campioni costieri e del largo. Tali variazioni sono state messe in relazione con le variabili oceanografiche, mostrando una correlazione con i valori di clorofilla a, salinità e ossigeno disciolto. Le AIS della comunità zooplanctonica hanno permesso di dividere i taxa esaminati raggruppando quelli con valori isotopici più vicini. Questi gruppi sono stati poi confrontati con i gruppi trofici riportati in letteratura, mostrando un certo grado di variazione. Questo fenomeno potrebbe essere dovuto alla plasticità trofica di alcuni organismi dello zooplancton, in base alla disponibilità di risorse. I risultati delle AIS hanno inoltre permesso di evidenziare la presenza di variazioni significative nel $\delta^{13}C$ dei campioni costieri e del largo nella parte centrale della GSA 17, e variazioni significative nel $\delta^{15}N$ tra i campioni costieri e del largo, nella parte settentrionale e centrale della GSA 17. Tali variazioni potrebbero essere dovute ad un diverso contributo di materia organica di origine terrestre *vs*. marina, e/o a dinamiche trofiche diverse tra le

comunità costiere e quelle del largo. Le AIS delle acciughe hanno invece permesso di evidenziare la presenza di un andamento polinomiale nel valore di $\delta^{15}N$, che aumenta fino a circa 9 cm, e poi diminuisce al diminuire della taglia, probabilmente correlato ad un aumento dell'erbivoria nei pesci più grandi. I valori di $\delta^{13}C$ invece tendono ad aumentare con la taglia, indicando un possibile spostamento degli animali di taglia maggiore per l'alimentazione verso il largo. Le AIS delle sardine hanno mostrato un andamento simile a quello delle acciughe per il valore di $\delta^{15}N$, ma non hanno evidenziato differenze significative per il valore di $\delta^{13}C$. Le AIS combinate delle due specie hanno infine permesso di mostrare come queste abbiano una posizione trofica simile, dato che i valori di $\delta^{15}N$ sono simili, ma utilizzino risorse trofiche differenti, in quanto i valori di $\delta^{13}C$ sono poco sovrapposti. Inoltre queste due specie hanno una posizione fondamentale nelle reti trofiche pelagiche, occupando una posizione intermedia tra lo zooplancton ed i predatori più grandi.

Chapter one

INTRODUCTION

1.1 Small pelagic fishes and their role in pelagic trophic webs

Small pelagic fishes (here after briefly indicated as small pelagics) are a group of epipelagic fishes that live in the photic zone, in the first 200 metres of the water column. This zone is characterized by the presence of light, which allows a large variety of phytoplanktonic species to perform photosynthetic processes. Small pelagics are usually 10-30 cm in length and show a strong schooling behaviour (Fréon and Misund, 1999). Despite their relatively small size, they represent the majority of the fish biomass in pelagic ecosystems, due to their high abundance, especially in nutrient rich upwelling regions, like eastern boundary currents (Canary, Benguela, California, and Humboldt Currents) (Fréon *et al.*, 2005). Even though they reach an high biomass, the trophic level of small pelagics (*i.e.* mesopredators) is usually dominated by only a few species; on the contrary, in lower and higher trophic levels biodiversity is higher (Bakun, 1996).

Small pelagics mainly feed on phytoplankton and micro/mesozooplankton (Fréon *et al.*, 2005). This is why they are very abundant in near-shore productive waters. They are actually present in all oceans and seas, except the Antarctic, where their trophic role is played by large euphausiids known as

krill (Fréon *et al.*, 2005; Leonori *et al.*, 2017). Due to their small size, they are a prey to many large pelagic predators, like tunas, swordfishes, small sharks, marine mammals and seabirds; indeed, they are also known as forage fish (Jarre-Teichmann and Christensen, 1998). Small pelagics are an important source of low-cost food in many countries, representing about 22% of the global finfish marine capture production in 2018 (FAO, 2020). Some of these catches are used to produce fishmeal and fish oil. However Jarre-Teichmann and Christensen (1998) reported that, at least in the four major eastern boundary current ecosystems, natural mortality of small pelagic fish is much higher than fishing mortality.

Small pelagics share some morphological features, like a fusiform and laterally compressed body and a forked tail, which makes them good swimmers (Fréon *et al.*, 2005). They also have a similar mimetic coloration, called *counter-shaded coloration*: their flanks are highly reflective, while their dorsal surface is dark; in this way, fish always appears as the same colour as the background. Some species can also change their dorsal colour controlling their melanophores (Blaxter and Hunter, 1982). Their skin is often covered by small scales and mucus, which are easily lost during manipulation. For this reason, small pelagics are considered as frail fishes, with high

mortality during the fishing activity, even though they are not actually caught (Misund and Beltestad, 1995).

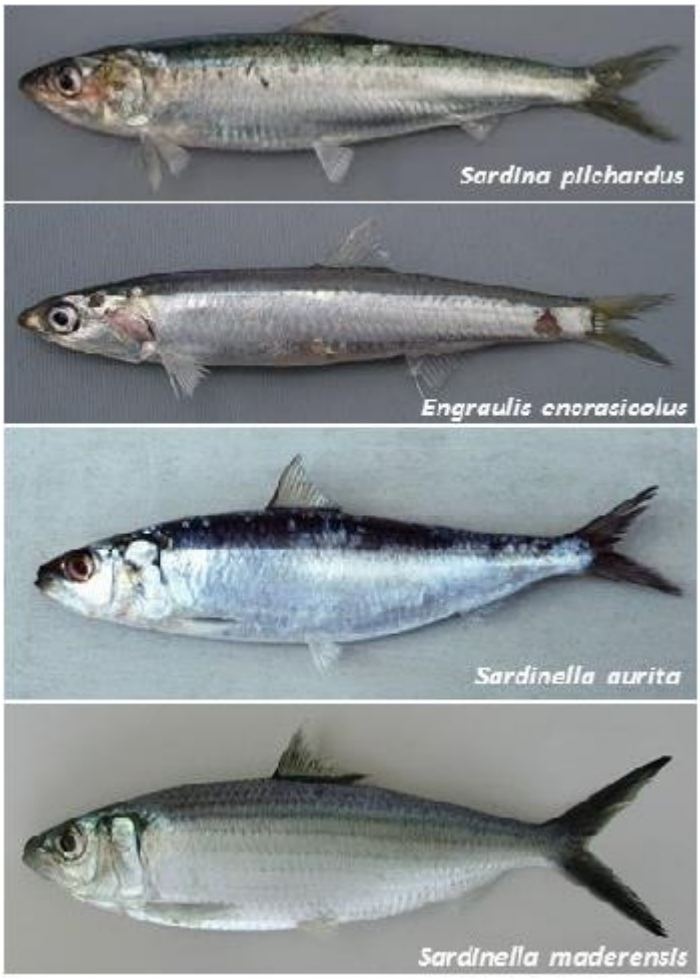

Figure 1. Examples of small pelagic fishes, that show a counter-shaded coloration (websource: http://www.inrh.ma)

Small pelagic fishes usually form large schools, which can be evolutionarily advantageous for many reasons (Fréon and Misund, 1999). The first one is the reduction of predation: in a school, small fishes are more difficult to focus on, so the predator is confused and can hesitate to attack. Moreover, preys can more easily see predators, thanks to many contributing eyes, and can

cooperate to avoid them, thanks to escape manoeuvres (Fréon and Misund, 1999). Large groups of fish can also enhance the trophic efficiency of individuals, thanks to an easier food localization. Fishes can also concentrate on the feeding activity, as they are less vulnerable to predation (Fréon and Misund, 1999). The schooling behaviour is also able to enhance the reproductive success of schooling species, since it is easier to find a partner, especially in pelagic promiscuous spawners, and reduces the chances of being eaten during spawning (Fréon and Misund, 1999). Fishes can benefit from schooling behaviour also during migrations, because, when the school needs to choose where to go, the paths chosen by single individuals are averaged in one, more precise, direction (Fréon and Misund, 1999). Some authors also suggested that schooling fishes can learn from other fishes in the same group, for example, to escape a fishing net (Fréon and Misund, 1999).

Figure 2. A large school of small pelagic fishes (websource www.scubashooters.net)

Within a school of small pelagic fishes, individuals tend to have similar size and body shape, but they can belong to different species. Fréon and Misund (1999) reported that mixed species schools are more common when there are secondary species which are less abundant, so that they cannot form a school on their own. This behaviour could lead to a phenomenon known as “school trap” (Bakun and Cury, 1999): the less abundant species might subordinate their needs to those of the dominant species, leading to a sub-optimal lifestyle and creating a negative feedback. Therefore the “school trap” mechanism could interact with overexploitation of a less abundant species, preventing a fast recovery (Bakun and Cury, 1999). The schooling behaviour could also mask the effects of stock depletion: the catch rate would not decrease in

proportion to the stock depletion, because fishes can still form fewer large schools, which are easily detected and harvested by fishermen, leading to an unexpected collapse (Fréon *et al.*, 2005).

Large schools of small pelagic fishes usually undertake diurnal vertical migrations: most species move to the upper layer of the water column at dusk, swimming with a lower level of aggregation (Fréon *et al.*, 2005). Then, before sunrise, they aggregate again and move to a deeper layer. These changes in their spatial distribution are mainly driven by distributional changes in prey and predator species, but they can be influenced by other phenomena, like changes in illumination (Neilson and Perry, 1990). Many species can even perform long range horizontal displacements, in response to environmental changes, or to move from a feeding ground to a spawning area (Fréon *et al.*, 2005).

Most pelagic fishes are pelagic spawners, with external fertilization. They usually have a very high fecundity, producing many small eggs, with a small yolk sack. This means that fish larvae need to feed soon after hatching. There is no parental care and cannibalism can be severe. Mortality rate is very high during the early life stages, but it decreases with fish growth. Small pelagics have a fast growth rate, reaching sexual maturity early, with a relatively short lifespan (Fréon *et al.*, 2005), showing a typical “r strategy”.

Despite their name, some pelagic species show a peculiar link with benthic ecosystems: some species, like herrings and capelins are demersal spawners, releasing adhesive eggs on the sea floor (Blaxter and Hunter, 1982). Moreover, many species can be found close to the seabed during daytime and can feed on or near the bottom. These species are therefore vulnerable to semi-pelagic fishing and bottom trawling (Fréon *et al.*, 2005).

Small pelagic fishes have been observed to face intense fluctuations of their biomass (Fréon *et al.*, 2005). This high instability seems to be related to environmental changes, to both medium-term (interannual fluctuation) and long-term climatic variations (interdecadal fluctuation). Therefore, small pelagics do not show a clear density-dependent recruitment (Fréon *et al.*, 2005). This phenomenon has been observed even in absence of fisheries, thanks to the analysis of scale deposition in anaerobic sediments (Baumgartner and Soutar, 1992). However, overfishing can interact with environmental changes, increasing the extent of the period of depletion of the stock. In many pelagic ecosystems it has been noted that when a dominant pelagic fish species becomes less abundant, another species can increase its biomass, creating an alternating pattern. While the dominant species responds to environmental factors, the subordinate species responds to the abundance of the dominant one (Skud (1982). The biomass of these species is indeed

influenced to different extent, by environmental changes, competition and school-trap effect. In many upwelling ecosystems the reduction of a species is also connected to a decrease in its spatial range. Since fishes are all gathered in a small area, they are more available to fisheries (Fréon *et al.*, 2005).

Due to their intermediate trophic level, small pelagic fishes can play a crucial role in many ecosystems. Regarding ecosystem control, ecologists often consider three main models: bottom-up, top-down and wasp-waist control (Fréon *et al.*, 2005). Bottom-up controlled ecosystems are regulated by environmental conditions, which can influence primary production, with a positive effect on higher trophic levels. If primary production increases, the biomass of all trophic levels should increase, due to a higher food availability. This pattern has been proposed for the first time by Hensen in 1887. In many upwelling ecosystems, environmental changes can indeed influence fish biomass, but this effect is not necessarily mediated by primary production: sometimes an increase in productivity does not correspond to an increase in fish recruitment. For example, environmental features can shape not only abundance, but also size and diversity of zooplanktonic community, which can alter small pelagics' biomass (Costalago, 2015). Top-down controlled ecosystems are instead controlled by predation. Top predators can down-regulate the abundance of small pelagics, leading to an increase of

zooplanktonic biomass. This type of control has been observed in many ecosystems, and it is very important due to its connection with fisheries, which usually target top predators (Fréon *et al.*, 2005). Since small pelagics occupy a central trophic position, they can exert a negative top-down control on plankton abundance, but also a positive bottom-up control on top predators. This is the principle of wasp-waist controlled ecosystems (Fréon *et al.*, 2005) and thus small pelagic act as top-down controllers. Environmental changes can actually influence the abundance of forage fish, but not through primary productivity. Marine ecosystems are very complex, so various forces can act together to control energy fluxes and their relative weight can be different in different systems (Fréon and Misund, 1999).

1.2 Small pelagic fishes in Mediterranean Sea and their commercial interest

In the Mediterranean basin, small pelagic fisheries provide a substantial share of marine capture landings, representing 55% of total landings (FAO, 2018b). However, most of these catches are composed by only two species: European anchovy (*Engraulis encrasicolus*, Linnaeus, 1758) and European pilchard (*Sardina pilchardus*, Walbaum, 1792). These two species combined, in 2018, represented 95% of small pelagics landings (Figure 3) (FAO, 2018a). Some

minor species are round sardinella (*Sardinella aurita*, Valenciennes, 1847) and European sprat (*Sprattus sprattus*, Linnaeus, 1758).

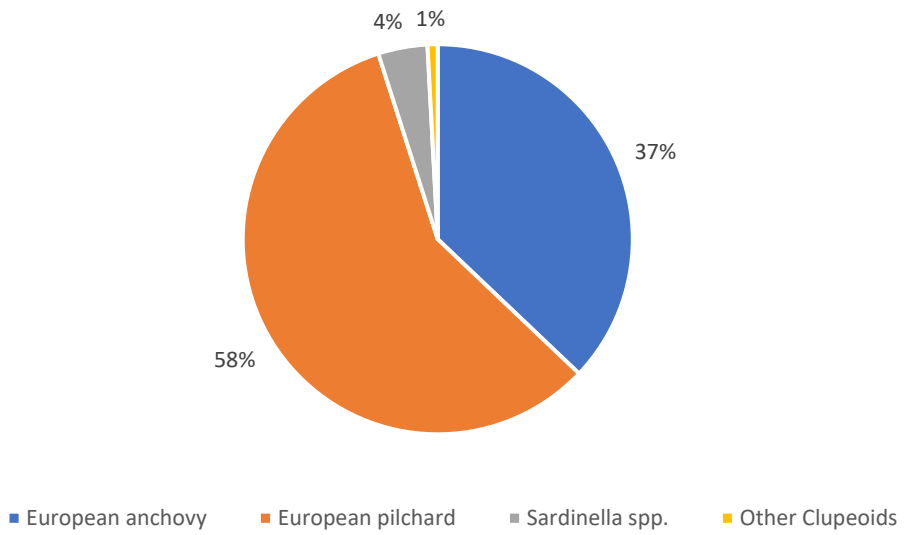

Figure 3. Landings of small pelagic species for Mediterranean countries. Data obtained by FishstatJ program.

Small pelagics are mostly caught by purse seining, a fishing technique that exploits the fish behaviour to form schools, encircling the school with long nets which are then closed beneath the fish group (Figure 4). Since small pelagics tend to be attracted by strong light sources at night, fishermen developed a purse seine technique that aggregates fish schools using a boat with a powerful artificial light, called “lampara” (Morello and Arneri, 2009).

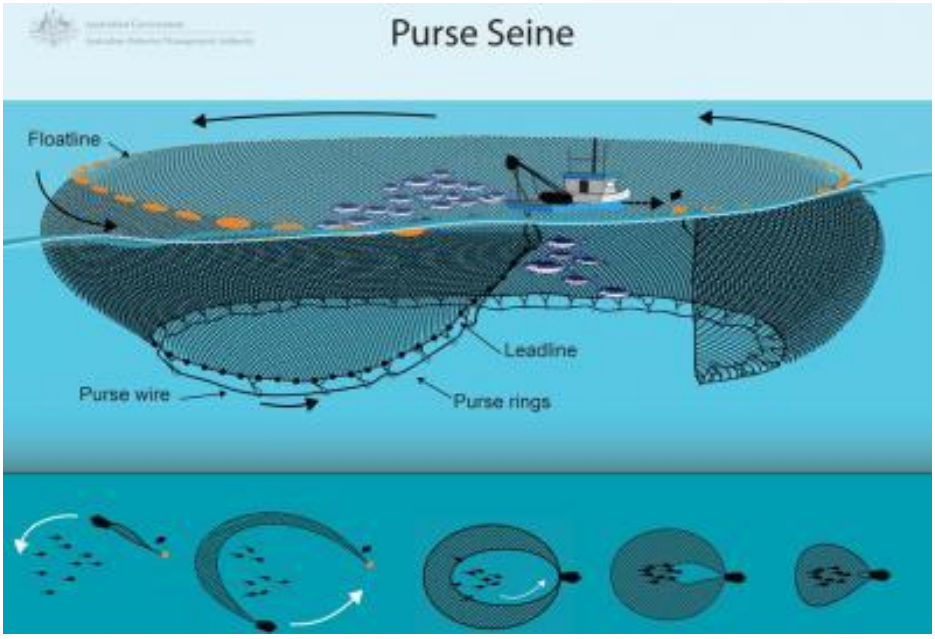

Figure 4. Purse seine scheme (websource: www.afma.gov.au)

Another important fishing method is pelagic (or midwater) trawling, which uses a bag net towed by one or two boats and it is kept open through metal diverging doors (Figure 5). This technique is considered more efficient when the school is not very large or fish are dispersed or swim in a deep layer of the water column (Fréon *et al.*, 2005).

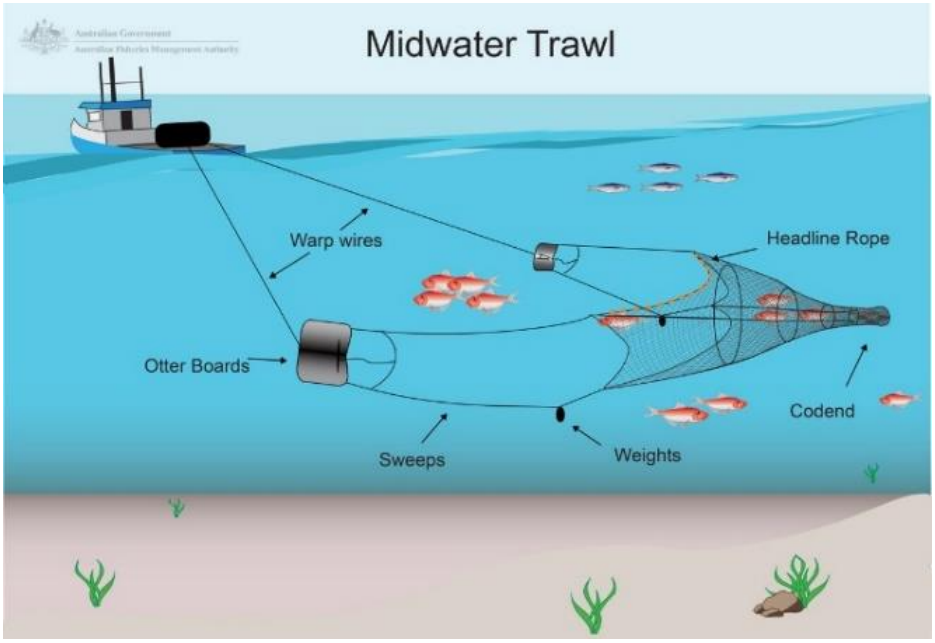

Figure 5. Midwater trawl scheme (websource: www.afma.gov.au)

Since pelagic fish tend to aggregate, fisheries developed many systems to detect fish schools. The most advanced one is acoustic detection, using soundwaves produced by low-frequency and high-frequency sonars. Pelagic trawlers can also use acoustic devices mounted on the mouth of the net to track how the school behaves. Many experienced fishermen can also track fish through visual detection of the school or of seabirds. They can also track other operating fishing vessels. Small pelagics can also be captured through semi-pelagic trawls, bottom trawls, gill-nets, beach seines, and hand lines (the latter three are often associated to small scale and artisanal fishery) (Fréon *et al.*, 2005).

Despite the high tonnage of catches of small pelagics, they give only a small contribution to Mediterranean fishery incomes: SoMFi reported that in 2018 most of the economic income from fishing came from trawlers (Figure 6), even though they are only 8.6% of the Mediterranean fishing fleet (Figure 7). Small pelagics give a smaller income when compared to other seafood, since they usually have a lower value and they are mostly used to produce fishmeal (Fréon *et al.*, 2005).

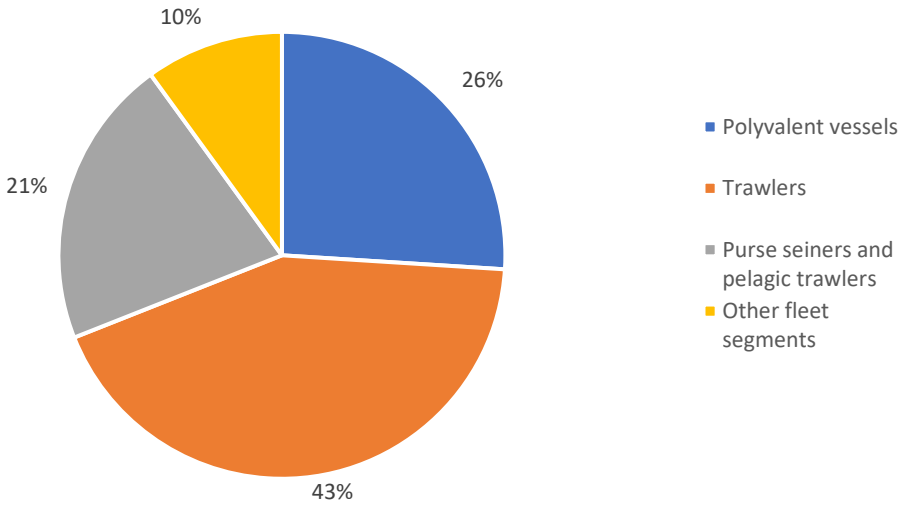

Figure 6. Percentage of total landing value by vessel group in the GFCM area of application

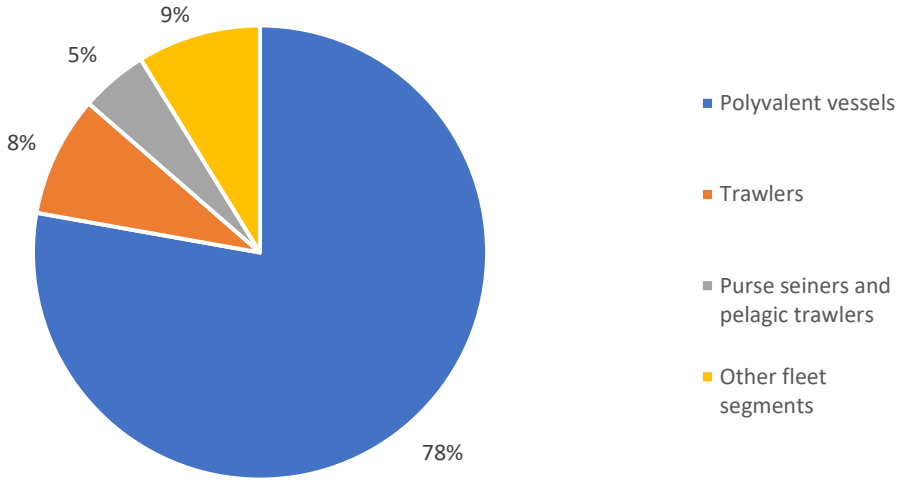

Figure 7. Percentage of fleet segments operating in the Mediterranean

Fishing activities on small pelagics can have negative direct effects on fish stocks, but can also cause indirect effects on the whole ecosystem. Small pelagics tend to fluctuate, even without any fishing pressure, but fishing activities can alter their abundance, preventing the normal stock recovery. Fishery can indeed cause a recruitment overfishing, when catches affect the

reproductive capacity of the stock (Fréon and Misund, 1999). Overfishing can interact with stock fluctuation, preventing the recovery of fish population. The central role of small pelagics in pelagic ecosystem can explain how a depletion of their stock can affect both higher and lower trophic levels (Fréon *et al.*, 2005). Fishing activities can also accidentally cause damage to non-target species, through the effects of bycatch. For example, Bonanomi *et al.*, (2016) reported a high presence of elasmobranchs in pelagic trawlers net (in particular smooth hound (*Mustelus mustelus*), spiny dogfish (*Squalus acanthias*) and common eagle ray (*Myliobatis aquila*) and a minor presence of loggerhead turtles (*Caretta caretta*), twaite shad (*Alosa fallax*) and fan mussel (*Pinna nobilis*), most of them considered threatened species (critically endangered to vulnerable). Fishermen can also accidentally lose their net, which is then carried by currents and can still catch fishes, causing the so-called ghost net phenomenon (Brown and Macfadyen, 2007).

The most common strategy to prevent overfishing and stock collapse is the adoption of management procedures, which is a set of clearly defined rules, based on stock assessment and fishery data, to define certain regulatory mechanisms (Fréon *et al.*, 2005). Stock assessments are necessary to implement effective protection measures. Small pelagics are generally estimated through acoustic surveys: the scientific echosounder mounted on a

research vessel emits soundwaves, that are reflected by fishes, and are captured by a receiver (transducer). These reflected soundwaves are then converted to an estimate of the fish biomass per length class and age thanks to biological data acquired from pelagic trawling during the survey (Fréon and Misund, 1999; Leonori *et al.*, 2012; MEDIAS, 2019). An additional method is the Daily Egg Production Method (DEPM), which uses an estimate of daily produced eggs, to assess the biomass of spawners that produced those eggs. This method is only rarely applied, and resulting biomass estimates appear to be similar to acoustic results (Lleonart and Maynou, 2003; Somarakis *et al.*, 2004).

Rules are agreed by all parties before implementation and are tested by simulations to assess how much they are reliable. In general, these procedures are defined for a period of 3-5 years and can then be modified according to additional knowledge. Regulatory mechanisms are usually calculated on a yearly basis, from all available data (Fréon *et al.*, 2005). These mechanisms are divided in five categories:

1. Fish size control: this strategy allows to directly limit growth overfishing (when fish are harvested at an average size that is smaller than the size that would produce the maximum yield per recruit) and, indirectly, recruitment overfishing, because it allows young fishes to

reach sexual maturity. In small pelagic fishes, the effects on growth overfishing are usually negligible, but it can have positive effects on recruitment overfishing. The main problem of this strategy is the low selectivity of fishing gears, which favours the dumping of small fishes. Also, it could genetically select fishes that reach maturity at a lower size.

2. Quotas (or TACs, Total Allowable Catches): this strategy allows to control fishing mortality and quotas can be regulated year by year to prevent overexploitation. However, quotas are usually based only on landings and ignore possible dumping of species for which the maximum quota was already reached.

3. Control of the overall fleet capacity: this strategy allows to affect the overall pressure on commercial fish species, but it does not make any distinction between targeted species.

4. Control of standardized effort: this strategy is similar to the previous one, but it allows to take into account eventual changes in the efficiency of fishing units. However, fishing effort can be difficult to assess and control.

5. Time or area of closure: this strategy can be very useful to prevent collateral damages on important habits, like spawning grounds or

nursery areas, but is not very effective on small pelagic species, due to their wide habitat and extend spawning season. MPAs are an example of this strategy.

A more recent approach is ecosystem-based fishery management which tries to switch from a simple single-species management to a multi species approach. This strategy considers the effects that the reduction of a single species can have on the whole ecosystem, on lower or higher trophic levels, and searches for emergent properties of multispecies assemblages (for example redundancy in small pelagic species) (Fréon *et al.*, 2005).

1.3 Adriatic Sea and its main features

The Adriatic Sea is an elongated semi-enclosed basin, with its major axis in the northwest–southeast direction, located in the central Mediterranean, between the Italian peninsula and the Balkans (Figure 8). It is 800 km long and 150-200 km wide. It has a total volume of 35000 km ^{3}, that belongs for 5% to the Northern basin, 15% to the middle basin and 80% to the Southern basin. The North Adriatic is very shallow, with an average bottom depth of 35 m and a maximum depth of 70 m. The Middle Adriatic has a maximum depth of 100 m, with the exception of the Pomo Pit (maximum depth 280 m). In these two areas the eastern part has a deeper bottom, with high and rocky

shores, while the western part is shallower, with low sandy shores. Despite the aforementioned differences, these areas share many bioecological features, so GFCM grouped them in the same Geographic Sub-Area, GSA 17. The southern part of the Adriatic Sea, the GSA 18, is much deeper, with a wide depression of 1200 m deep. Here, the Otranto channel, which is 800 m deep, acts as an exchange area for water masses with the Mediterranean Sea (Artegiani *et al.*, 1997; Marini, Bombace and Iacobone, 2017).

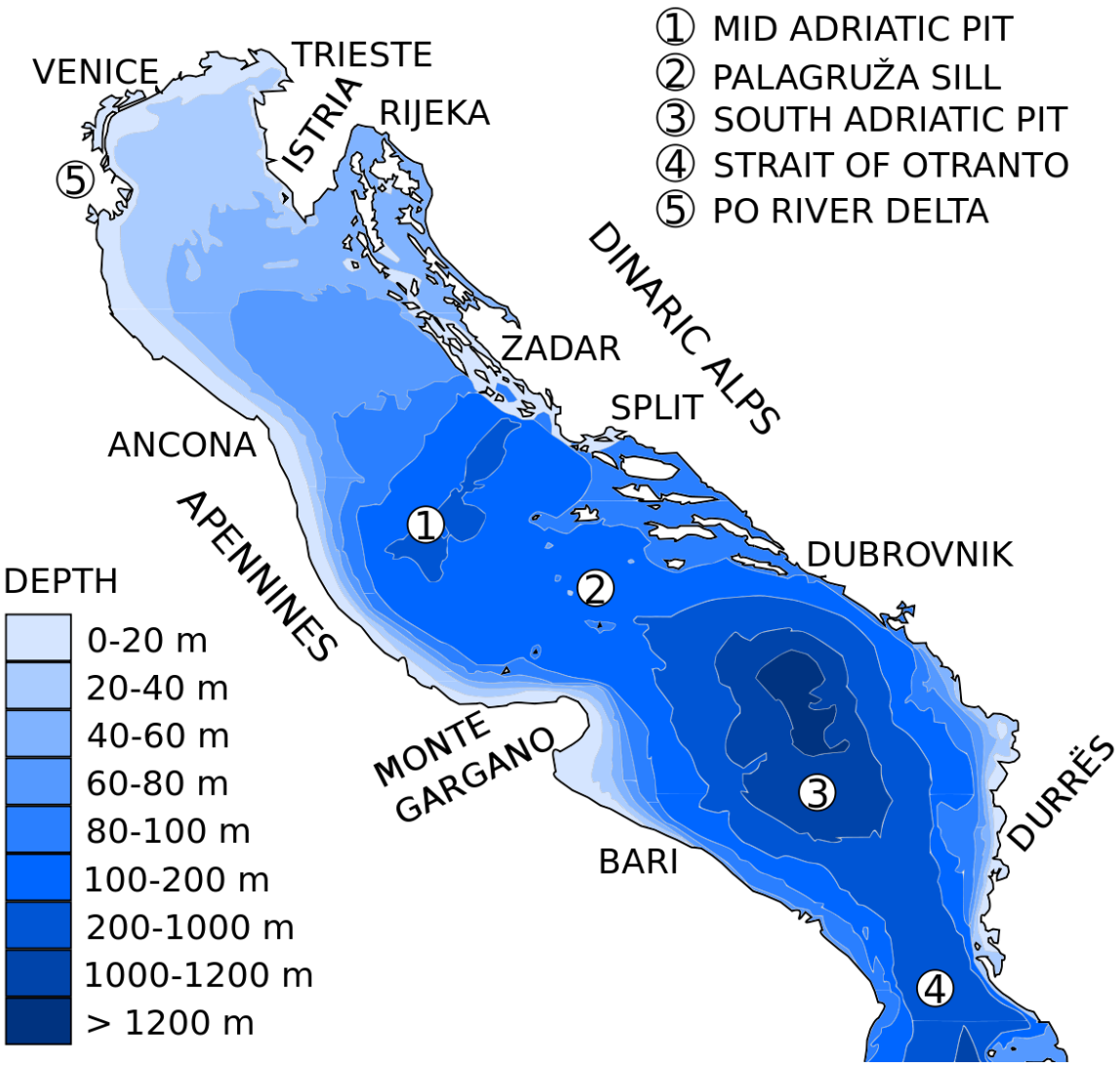

Figure 8. Bathymetric map of the Adriatic Sea. (websource: www.wikipedia.org)

The Adriatic Sea, because of high freshwater input, is very different from the rest of the Mediterranean Sea, providing 30% of Mediterranean freshwaters (~ 30% of that come from Po River). The Adriatic average salinity is 38‰, but in the northern area is lower and also more variable. Due to this input, there is a positive water balance of 90-150 km³, that is exported to the

Mediterranean. The turnover time for the whole basin is 3-4 years (Artegiani *et al.*, 1997; Marini, Bombace and Iacobone, 2017).

The Adriatic is a temperate warm sea, with wide range of surface temperature, from 6 ºC to 29 ºC. Even the temperatures of the deepest layers are, for the most part, above 10ºC. The South Adriatic is warmer than its central and northern parts during winter. In other seasons the horizontal temperature distribution is more uniform. (Artegiani *et al.*, 1997; Marini, Bombace and Iacobone, 2017).

The basin has a low tidal amplitude, limited to 30 cm in the southern part and 90 cm in the extreme North. Water circulation in the Adriatic is mainly driven by dominant winds (Bora and Scirocco) that cause a cyclonic circulation (anticlock-wise), with three closed circulation cells (one for each sub-basin) (Figure 9). The Adriatic contains three different water masses: Adriatic Surface Water (AdSW), Levantine Intermediate Water (LIW) and Adriatic Deep Water (AdDW), which branches out in Northern (NAdDW), Middle (MAdDW) and Southern (SAdDW) Adriatic Deep Water. The LIW is formed in the Levantine Basin. This water experiences a salinity decrease on its way to the Adriatic, and eventually enters the Adriatic through the eastern part of the Strait of Otranto. This water type can be seen in the intermediate layer of the South and Middle Adriatic as mLIW (modified LIW). AdDW are formed

in the Adriatic basin. The NAdDW forms in the North Adriatic and, due to its high density, it fills up the Jabuka/Pomo Pit and only occasionally spreads to the South Adriatic. The MAdDW is formed in the Jabuka/Pomo Pit area, when there is no intensive northwestward flow, (*i.e.* during the period of a low Mediterranean water inflow). The SAdDW originates in the South Adriatic Pit. Due to its high density, this water spreads into the bottom layer of the Eastern Mediterranean. Waters that leave the Adriatic basin, pass through the western part of the Otranto Channel (Artegiani *et al.*, 1997; Marini, Bombace and Iacobone, 2017).

Figure 9. Adriatic Sea water circulation. (websource: www.researchgate.net)

The Adriatic is a very productive basin, compared to the rest of the Mediterranean. Despite being only the 5% of the total Mediterranean surface area, the Adriatic sea produces about 15% of total Mediterranean landings (and 53-54% of Italian landings), with a fish production density of 1.5 tonnes/km², which is three times the Mediterranean density (Marini, Bombace

and Iacobone, 2017). This impressive feature is shaped by three main factors: river runoff, shallow depths and oceanographic structure. Rivers can indeed provide the sea with nutrients, which favour phytoplanktonic blooms, thus causing a bottom-up effect of the whole trophic chain. Rivers can also provide suspended particulate organic matter and organic detritus, that feed numerous particulate feeders and detritivores, such as bivalves (which is one of the main fisheries of the North Adriatic Sea). The wide extension of the continental plate favours a short trophic chain, which is not possible in deep basins. This can improve the efficiency of energy transfer from lower trophic levels to higher ones. Moreover, the structure of the basin allows water mixing during winter, especially in North and Middle Adriatic, transferring nutrients from sediments to the water column. However, the same condition can be responsible for water stratification, harmful algal blooms, mucilage, dystrophy and anoxia phenomena during summer (Marini, Bombace and Iacobone, 2017).

The presence of different water condition in the three Adriatic subareas determine the presence of a typically boreal ichthyofauna in North Adriatic, like *Solea spp.* and *Sprattus sprattus*, while bathyphilous and thermophilous elements can be found in the Central and Southern Adriatic Sea. In general, the number of species decreases moving from the South to the North Adriatic,

but only a few species are exclusive of a single sub-area (Marini, Bombace and Iacobone, 2017).

However, this peculiar biodiversity is threatened by well-known and emerging factors:

1. intensive fishing activity, which could overexploit target species and cause indirect damage to the ecosystem;
2. human activities along the coast, which could cause pollution and eutrophication (especially in North Adriatic, due to the presence of the Po Rivers, which contributes more than 50% of total inflow of nutrients in Adriatic);
3. alien species, whose presence and effect on local fauna are still to be well understood;
4. tropicalization, which could favour the settlement and diffusion of alien species and damage native cold-water species, like *Sprattus sprattus* (Boero *et al.*, 2009; Marini, Bombace and Iacobone, 2017).

1.4 Characterization of food webs

Characterizing food webs is a fundamental process to understand key features of an ecosystem and general energy fluxes. This has become particularly important since the rise of ecosystem-based management, which aims to

create a sustainable exploitation strategy that protects the ecosystem as a whole (Pasquaud, Lobry and Elie, 2007; da Silveira *et al.*, 2020). Trophic webs are generally described in terms of prey-predator interactions (Sheppard and Harwood, 2005), so ecologists try to understand what a fish eats (*i.e.* qualitative information) and in what proportions (*i.e.* quantitative information) (da Silveira *et al.*, 2020).

In diet evaluation analysis, stomach content analysis is considered as a standard procedure and is the most used procedure in short-term evaluations (Pasquaud, Lobry and Elie, 2007; da Silveira *et al.*, 2020). Generally, stomach content analysis is an invasive and lethal procedure because it requires the extraction of digestive trait. However non-lethal procedures can be applied to work with rare and endangered species, for example gastroscopies tubes, stomach suction and lavage, or ministration of emetic chemicals (da Silveira *et al.*, 2020). Once the digestive trait is extracted, the traditional approach is the visual identification of its content. Visual identification is the only approach that provides direct qualitative information on the trophic ecology of a species, targeting different taxonomic levels, also providing quantitative data, through the measurements of amount, weight/volume and size of the different consumed foods. Moreover, it is the only method that allows identification of different life stages of prey. However, visual identification

can be very difficult, since prey items in stomach contents are often broken and/or digested/softened/liquefied. The integrity of these preys depends on the acquiring strategies employed by consumers (for example, whether they ingest whole preys or cut and crush them to very small pieces, like crabs), metabolic and digestive features of the predator, and the resistance of preys to digestion. Also, integrity of consumed preys can be affected by fishing gears and non-lethal techniques applied to capture them and by the time among feeding, consumer death and stomach contents fixation (stopping the digestive activity). For these reasons visual identification can lead to misidentification of some taxa and underestimate of easily digested preys (like those that lack of hard structures)(Pasquaud, Lobry and Elie, 2007; da Silveira *et al.*, 2020). However, DNA techniques recently developed can overcome some of these issues. Sanger sequencing of the CO1 mitochondrial gene is the traditional approach, but has been proved to be laborious, expensive and constrained by the necessity to isolate the prey item (which also must have a well-preserved DNA). Next-Generation sequencing techniques can overcome these problems, since metabarcoding analysis allows high-throughput multispecies identification using total DNA samples, despite different damage degrees, with reduced costs and time. Nevertheless, DNA techniques cannot detect cannibalism and are unable to distinguish real prey from secondary predation (prey items captured by the prey) (da Silveira *et al.*, 2020). A similar

approach is detection of prey items with monoclonal antibodies, but has limited applications, since it is a long and expensive procedure, that requires specific antibodies for the expected prey item (Sheppard and Harwood, 2005).

Stomach content analysis, in general, is difficult to apply on small animals, with very small preys (Pasquaud, Lobry and Elie, 2007), and deep fishes, because they usually evert their stomach due to pressure change (even though intestine analysis can partially overcome this issue) (da Silveira *et al.*, 2020). Another important problem is "net feeding", *i.e.* preys consumed by fish in the net due to an artificial aggregation, which can be partially avoided through intestine analysis. Despite being one of the best methods to obtain long-term precise data on fish diet, both on a seasonal and ontogenetical basis, this kind of activity is extremely time consuming: a very large number of samples are needed to obtain significative data on a large spatio-temporal scale. In fact, stomach content analysis provides a snapshot of the species diet, and unless an intensive temporal sampling is carried out, temporal changes in a species diet cannot be appreciated (Fanelli & Cartes, 2008). Furthermore, stomach content analysis only gives information on consumed food items, thus excluding the assimilation process and data on the origin of consumed organic matter (da Silveira *et al.*, 2020).

Faecal pellet analysis is similar to stomach content analysis, but its applicability is much more limited in fish ecology, since the obvious difficulties of collecting faecal pellet in wild animals. It is a non-invasive method, like direct observation of fish behaviour through underwater cameras. Direct observation can easily explain complex behaviours, like interspecies association and use of habitat, data collection is difficult and time consuming. Moreover, the use of baited traps can cause bias (da Silveira *et al.*, 2020).

Lipid biomarker analysis is an invasive, often lethal, technique that exploits some properties of lipids, that are a fundamental organic molecule, present in every cell, with different functions. Fatty acids, sterols and pigments are generally used for this kind of analysis. Organisms can acquire fatty acids by de-novo biosynthesis, or by trophic ways, where predators acquire fatty acids from preys and deposit them into their tissues with few modifications, leading to predictable assimilation patterns. This feature allows to obtain data on both short-term and long-term predation activity, and distinction among taxonomic groups (on a low distinction level), highlighting primary producers. Fatty acids analysis helps to distinguish between autochthonous and allochthonous contributions in aquatic trophic webs, to identify wild individuals and cultured escapes and to quantify the influence of human activities on

environmental pollution and fish diets. It can also reveal intraspecific trophic variations and energetic mobilizations related to ontogeny, reproduction, and migration (da Silveira *et al.*, 2020).

However, linking fatty acids contents between consumers and food sources still requires stomach content analysis, to obtain knowledge about available and accessible potential preys. Fatty acids profile of prey must be sampled to understand trophic relationships, with a large sampling effort, since fatty acids profiles can change dramatically due to environmental conditions. This analysis is quite expensive and time consuming. Moreover, consumers with the same dietary niches can present overlapped profiles, making difficult to identify and differentiate food sources, while primary and secondary consumers can show profiles highly similar to those of their preys. For this reason, fatty acids are more recommended to assess trophic relationship on lower trophic levels (Pasquaud, Lobry and Elie, 2007; da Silveira *et al.*, 2020). At last, quantitative data on fish diet can be obtained only through modelling techniques (da Silveira *et al.*, 2020).

Recently, most of the studies assessing food web structure and function, use stable isotope analyses. Since tissue growth in predators depends on chemicals in preys, they should have a similar isotopic signature. Indeed, stable isotopes are enriched along the trophic chain, due to selective metabolic

fractionation, that leads to a preferential loss of lighter isotopes during respiration (carbon) and excretion (nitrogen) (Fanelli *et al.*, 2011). Nitrogen $\delta^{15}N$ and carbon $\delta^{13}C$ are the most common isotopes measured in trophic ecology studies. Trophic enrichment of $\delta^{15}N$ is about 3‰ per trophic level on average, so it can be used to determine the trophic position of the consumers along the food web. On the contrary, $\delta^{13}C$ has a trophic enrichment below 1‰, so it can't be used to distinguish trophic level; however, it varies between different sources of organic carbon, so it can be useful to track the origin of organic matter (for example plankton or benthos). Stable isotope analyses give a time integrated picture of fish diet, which depends on the turnover rate of C and N in the chosen tissue (Hesslein, Hallard and Ramlal, 1993). A time averaged picture can be useful to understand general ecologic features in a relatively short time (especially when compared to stomach content analysis), but it can hide short-term variations (Pasquaud, Lobry and Elie, 2007; da Silveira *et al.*, 2020). Stable isotope analyses allow to evaluate the origin (for example autochthonous *vs.* allochthonous) and the relative contribution of various food sources in consumers diet, intraspecific trophic relationships as a response to ontogeny, neighbouring-linked connectivity, migration, reproduction, changes in environmental features and human influence, and complex trophic strategies, like symbiosis parasitism and detritivory. Since proportions of $\delta^{15}N$ and $\delta^{13}C$ vary on a spatio-temporal

scale, stable isotope analyses require the evaluation of an isotopic baseline of potential preys (which are in general assessed through stomach content analysis) at a given time, in a given place. Stable isotope analyses alone also fail to differentiate between two food sources with overlapping isotopic concentration and among ecological niches of species that have the same isotopic niche. However, this can be partially solved by analysis of additional stable isotopes, like $\delta^{34}S$ and $\delta^{2}H$ and through Bayesian mixing models to estimate errors concerning turnover rates and isotope ratios of putative food sources. At last, stable isotope analyses cannot directly evaluate quantitative dietary data, but it requires the application of modelling techniques (da Silveira *et al.*, 2020).

Compound specific isotope analyses evolved from traditional stable isotope analyses, since they evaluate stable isotope signatures of specific molecules, like amino acids and lipids. It is a more refined analysis, because it joins the data of isotopic enrichment with easily predictable assimilation patterns of certain molecules. This causes a lower isotopic variability, so a lower number of samples is required to obtain the same data quality, when compared to traditional stable isotope analyses. However, it still requires the evaluation of fish diet, through stomach content analysis, and isotopic baseline. This

analysis is also more expensive and time-consuming than the traditional one (da Silveira *et al.*, 2020).

1.5 Engraulis encrasicolus

Engraulis encrasicolus or European anchovy is a Clupeiformes fish belonging to Engraulidae family. Its range extends in the Eastern Atlantic Ocean, from Bergen, Norway, to East London, South Africa (perhaps reaching Durban). It is common in Mediterranean Sea, Black Sea and Azov Sea. Some stray individuals have been reported in Suez Canal and Gulf of Suez. This species has also been reported in St. Helena and Estonia (Froese and Pauly, 2019). It is mainly marine, pelagic, coastal and forming large schools, recorded down to 400 m depth off West Africa and descending in winter to 100 to 150 m depth in the Mediterranean. It is also an euryhaline species, tolerating salinities from 5 to 41‰ and, in some areas, entering lagoons, estuaries or lakes, especially in the warmer months, during the spawning season. It has a tendency to extend into more northern waters and to move into the surface layers in summer, retreating and descending to the bottom in winter (Whitehead, Nelson and Wongratana, 1988).

European anchovy has a slender, elongate body, oval in cross-section with a silver stripe along flanks, that disappears with age. The snout is pointed; the maxilla is short, with a blunt tip, reaching almost to front border of pre-

operculum, not projecting beyond the tip of the second supra-maxilla; the tip of the lower jaw reaches below the nostril. Anchovies have 27 to 43 lower gillrakers, on the hind face of the third epibranchial. The pseudobranch is longer than the eye, reaching onto the inner face of the operculum. The anal fin is short, with 13 to 15 finrays, and originates well behind the base of the last dorsal finray (Whitehead, Nelson and Wongratana, 1988). Anchovies can reach a maximum length of 20 cm, which corresponds to a 6 years lifespan (ACOM/ ICES CM, 2009) with an average length of 12-15 cm; those in tropical waters are smaller than those in northern waters. (Whitehead, Nelson and Wongratana, 1988).

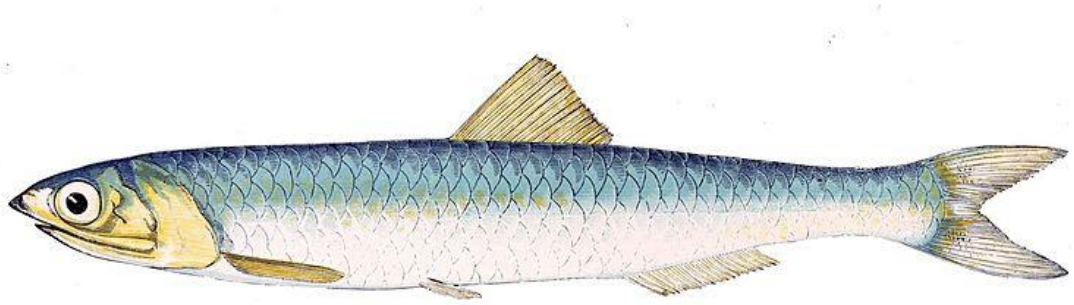

Figure 10. Engraulis encrasicuolus (websource: wikipedia.org)

European anchovy is a planktivorous species. Anchovies mainly feed during the day, since fishes detect preys mainly through visual detection (Borme, 2006). In Adriatic, studies on stomach content show how anchovy larvae feed mainly on copepod developmental stages (eggs, nauplii, meta-nauplii and

copepodites) and phytoplankton predation has been rarely reported, occurring only when mesozooplankton is limiting (Morello and Arneri, 2009). Late larvae and juvenile mainly feed on small copepods, in particular *Oncaea spp*. and *Euterpina acutifrons* (Borme, 2006). Adult anchovies can feed on *Oncaea spp*., *Euterpina acutifrons*, *Corycaeus spp.*, *Microsetella rosea*, but also bivalves, ostracods and decapod larvae. These preys are positively selected, while *Temora stylifera* and Clauso-Paracalanidae, despite being quite abundant, are poorly selected (Borme, 2006; Borme *et al.*, 2009). Some authors also reported *Penilia avirostris* as an important prey item in Northern Adriatic (Morello and Arneri, 2009).

European anchovies, like many other small pelagic fishes, are pelagic spawners, releasing gametes in the water column. This species is a serial batch spawner, having an indeterminate annual fecundity and producing multiple batches of eggs over a protracted spawning period (Morello and Arneri, 2009). Anchovies generally reach sexual maturity at the end of their first year of life, at a size of 7.5-9 cm on average in the Adriatic. Anchovy spawning takes place in the warmer months, generally between April and October, with two peaks: one between May and June and another one between August and September. Anchovy adults migrate

from the deeper overwintering waters to shallower coastal areas for spawning. Then, once spawning is completed and temperatures fall, adults return offshore, while juveniles generally remain closer to the coast until January, or until the following year when first maturity is attained (Morello and Arneri, 2009). The main spawning grounds are coastal waters of the western Adriatic between the Gulf of Trieste and the Gulf of Manfredonia. Eggs can be found in the Adriatic within 200 m depth, and are more abundant in warm waters with high zooplankton abundances. Eggs density reaches its peak in Northern Adriatic, in the Gulf of Trieste, near the Po River delta, and in the Southern Adriatic in the Gulf of Manfredonia. Eggs appear to be positively related to zooplankton abundance, both on spatial and temporal scale. Fronts, created by the clash between freshwater inputs (*e.g.* Po River) and the more saline water of Mediterranean sea, combined with limited mixing of water column and high stratification, due to mild wind action in summer, create an area where nutrients and zooplankton are concentrated (Morello and Arneri, 2009; Malavolti *et al.*, 2018).

Adriatic anchovies were thought to belong to two different species, a small silver anchovy, that occurs largely in the shallow and less saline

waters of the Northern Adriatic, and a larger bluish form that inhabits the open waters of the central basin, but genetic studies did not support this hypothesis (Ruggeri *et al.*, 2016). However, anchovies could be divided in three different clusters, implying that this species is not entirely panmictic: one in Northern Adriatic, a second one in the coastal area of North-Eastern Adriatic and a third one in the rest of the basin, which was similar to populations of the Tyrrhenian Sea. This separation is probably caused by the formation of local fronts and gyres, that retain eggs and larvae (Ruggeri *et al.*, 2016).

1.6 Sardina pilchardus

Sardina pilchardus, or European pilchard, is a Clupeiformes fish belonging to Clupeidae family. It is distributed along the eastern coasts of North Atlantic, from Iceland (rare) and North Sea, southward to Bay de Gorée, Senegal. It is also present in Mediterranean (common in the western part and in Adriatic), Sea of Marmara and Black Sea. It is a coastal pelagic species, which can be usually found at 25 to 55 m or even 100 m by day, but rises to 10 to 35 m at night (Whitehead, 1985).

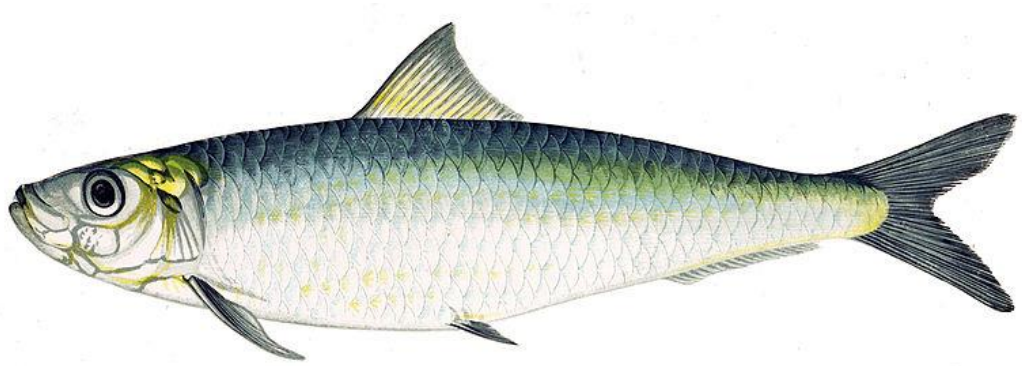

Figure 11. Sardina pilchardus (websource: wikipedia.org)

European pilchard has a subcylindrical body, with a rather rounded belly (but more compressed in juveniles). The hind margin of gill opening is smoothly rounded (without fleshy outgrowths); this species has 3 to 5 distinct bony striae radiating downward on the lower part of operculum; lower gillrakers are 44 to 106 and do not become shorter at the angle of the first gill arch, with the upper series not overlapping the lower one. Pelvic fin insertion is well behind the dorsal fin origin; the last two anal finrays are enlarged. A series of dark spots covers the upper flanks, sometimes with a second or even a third series below. This species can grow up to 25 cm, but the common length is 20 cm (Whitehead, 1985).

European pilchard is a planktivorous fish that mainly feeds during the day. Feeding activity has its peak at dusk, probably due to a higher zooplankton availability and an easier visual detection through light. Sardines are generally regarded as an omnivorous species, since many authors reported

phytoplankton as an important prey item. This is probably due to their ability to switch from particulate feeding (fish opens its mouth near prey items) to filter feeding (fish swims with its mouth open) in presence of small prey items. Moreover, filter feeding increases with fish size, since the feeding apparatus of small sardines is not enough developed (Borme, 2006; Morello and Arneri, 2009). Even in Adriatic, larval sardines are considered as entirely planktivorous (Morello and Arneri, 2009; Borme, Tirelli and Palomera, 2013), feeding mainly on small copepods like *Temora longicornis*, Clauso-Paracalanidae, *Centropages spp*., Harpacticoids and Corycaeids (Borme, Tirelli and Palomera, 2013). Adult sardines still feed mainly on calanoid copepods, but small planktonic algae, like diatoms and dinoflagellates, can become relevant on a seasonal basis (Borme, 2006; Hure and Mustać, 2020).

European pilchard, like anchovy, is a pelagic spawner, with batch fecundity (Morello and Arneri, 2009). Sardines generally reach sexual maturity at the end of their first year of life, at a size of 7-14 cm on average in the Adriatic. The spawning season goes from October to May, with generally one or two peaks. The timing and location of spawning is mainly dependent on temperature, salinity and food availability. In the Adriatic Sea, spawning has been reported to take place between 9 and 20°C and at salinities ranging from 35.2 to 38.8 PSU, with spawning peaks occurring between 11 and 16°C.

Spawning typically takes place at depths between 60 and 120 m. Although the presence of eggs has been observed in all continental shelf waters of the Adriatic Sea, two main spawning grounds, with an higher egg density, have been identified: one in Northern Adriatic, off the Dugi Otok Island, and one in Southern Adriatic, around the exterior of the mid-Dalmatian Islands and extending offshore to Palagruža; in some years, coinciding with very intense spawning, these two areas may be joined and the southern spawning area may also extend along the Italian coast down to Otranto (Morello and Arneri, 2009). In Northern Adriatic, in winter (starting as early as September) adult sardines migrate southwards from the Gulfs of Trieste and Venice and the Istrian coast towards Dugi Otok (and to a lesser extent the Kvarner region) for spawning, in search of more stable water conditions for their larvae. Then, in spring (as early as March), the spawners go back to the productive Northern Adriatic waters in search of food. In central Adriatic, adults move to offshore stable water in late Autumn; then, in early spring, adults, larvae and post-larvae turn towards the inshore waters in search of food. Another inshore migration can happen in late summer, in search of homogeneous water conditions (Morello and Arneri, 2009).

Thanks to morphological and reproductive information, Adriatic sardines where previously divided in two different sub-populations, a northern one,

and a southern one. However, works on genetic variability of the stock, show lack of heterogeneity between sardines in the whole Adriatic, thus excluding the presence of sub-populations or reproductive barriers. Moreover, these studies also excluded the presence of variability between Adriatic and Ionian samples, so Adriatic sardines belong to a much larger stock (Tinti *et al.*, 2002; Ruggeri *et al.*, 2013).

1.7 Aim of the study

Anchovies and sardines are one of the most exploited fish sources in the Mediterranean Sea. Despite being fished within biologically sustainable limits (FAO, 2018b), thanks to GFCM multiannual plans, small pelagic fish stocks can undergo strong fluctuations with a sudden collapse. In the Adriatic Sea, anchovies have already collapsed in 1987, under a strong overexploitation, and have only recently recovered (Azzali *et al.*, 2002; Morello and Arneri, 2009). However, the role of overexploitation is yet to be understood: the anchovy stock was already declining before its collapse, independently from levels of fishing effort and fishing mortality. In 1986-1987 the species showed a scarce recruitment, which could be linked with variations in environmental conditions (Santojanni *et al.*, 2003; Leonori *et al.*, 2007). Relationship between sardines and anchovies could regulate both stocks (Morello and Arneri, 2009). Klanjšček and Legović (2007) used information on stock

biomass and fishing effort and discovered that overfishing in 1985 is the best candidate for the stock collapse in 1987, while poor recruitment only speeded it up. Moreover, fishing effort in the subsequent years might have slowed down the stock recovery (Santojanni *et al.*, 2003).

Since small pelagic fishes play a crucial role in the ecosystem, due to their trophic position, their stocks must be preserved (Fréon and Misund, 1999). The most advanced approach is ecosystem-based management, that requires a profound knowledge on the main ecosystem features to adopt scientifically based management plans (Fréon *et al.*, 2005). Moreover, small pelagic stocks appear to be heavily influenced by environmental conditions, that might interact with fishing effort to determine the abundance of the stock (Fréon and Misund, 1999).

For this reason, this study aims to understand some features of the Western Adriatic pelagic ecosystem, through a characterization of the zooplanktonic community and its trophic relationship with *Engraulis encrasicolus* and *Sardina pilchardus*. The trophic features of both species which will be evaluated through stable isotope analyses.

These two fish species share the same area and the same trophic resource, so trophic characterization can be fundamental to assess if there is an actual niche overlap, that could lead to competition and consequent influence

between the two stocks. This kind of study has already been extensively done in other areas, like the Gulf of Lions (Le Bourg *et al.*, 2015), central Mediterranean (Rumolo *et al.*, 2016) and Bay of Biscay (Chouvelon *et al.*, 2014). Feeding ecology of small pelagics has also been recently investigated in Eastern Adriatic, through visual identification of gut content, reporting a dietary overlap between small pelagic species (Hure and Mustać, 2020). Stable isotope analyses have never been applied in the study area and could lead to the discovery of new information that cannot be inferred from gut content, like source of organic carbon and continental influences (Le Bourg *et al.*, 2015).

This study also searches for spatial variations in pelagic trophic webs along the western coast of the Adriatic Sea, to assess environmental factors that can determine such differences.

Chapter two

MATERIALS AND METHODS

2.1 Samples collection

Samples for this study were collected on board R/V "G. Dallaporta" (Figure 12) during the acoustic survey MEDIAS 2019 GSA 17 and GSA 18, that took place in June-July 2019, in Adriatic Sea (Leonori *et al*., 2020). The scientific survey is part of MEDIAS (MEDiterranean International Acoustic Surveys) action that coordinates the acoustic surveys performed in the Mediterranean and Black Sea to assess the biomass and spatial distribution of small pelagics in the target areas. This project is part of the EU Fisheries Data Collection Framework (DCF), where data collected are used for management decisions. Surveys are performed with an annual basis, following a common standardized methodology, which is annually reviewed and updated in the MEDIAS Handbook (MEDIAS, 2019) (http://www.medias-project.eu).

In Western Adriatic, the survey is performed by CNR-IRBIM of Ancona, that allowed the sample collection for this study. The survey also covers the Adriatic coast of Slovenia and, in 2019, Adriatic waters in front of Albania, as part of the MarE Albania project.

Figure 12. R/O "G. Dallaporta" (websource: www.ismar.cnr.it)

MEDIAS survey uses an integrated approach (Leonori *et al*., 2017) to collect:

1. acoustic data, used for small pelagics abundance and biomass estimates and their spatial distribution;

2. biological data, on both pelagic fish species and planktonic communities, like size distribution of fishes, length at age, maturity stages and sex ratio, fecundity estimates, distribution and abundance of anchovy eggs and larvae;

3. environmental data (*e.g.* temperature, salinity, chlorophyll), used to describe the oceanography of the area.

Acoustic data were collected thanks to a SIMRAD EK 60 split-beam multi-frequency echo-sounder (at 38, 70, 120 e 200 kHz frequencies), mounted on the vessel. Acoustic sampling was performed according to standard methodology, with transects perpendicular to the coastline. Transects started from 10 m depth, when possible, and reached 200 m depth, or the Adriatic Midline in shallower regions. (MEDIAS, 2019).

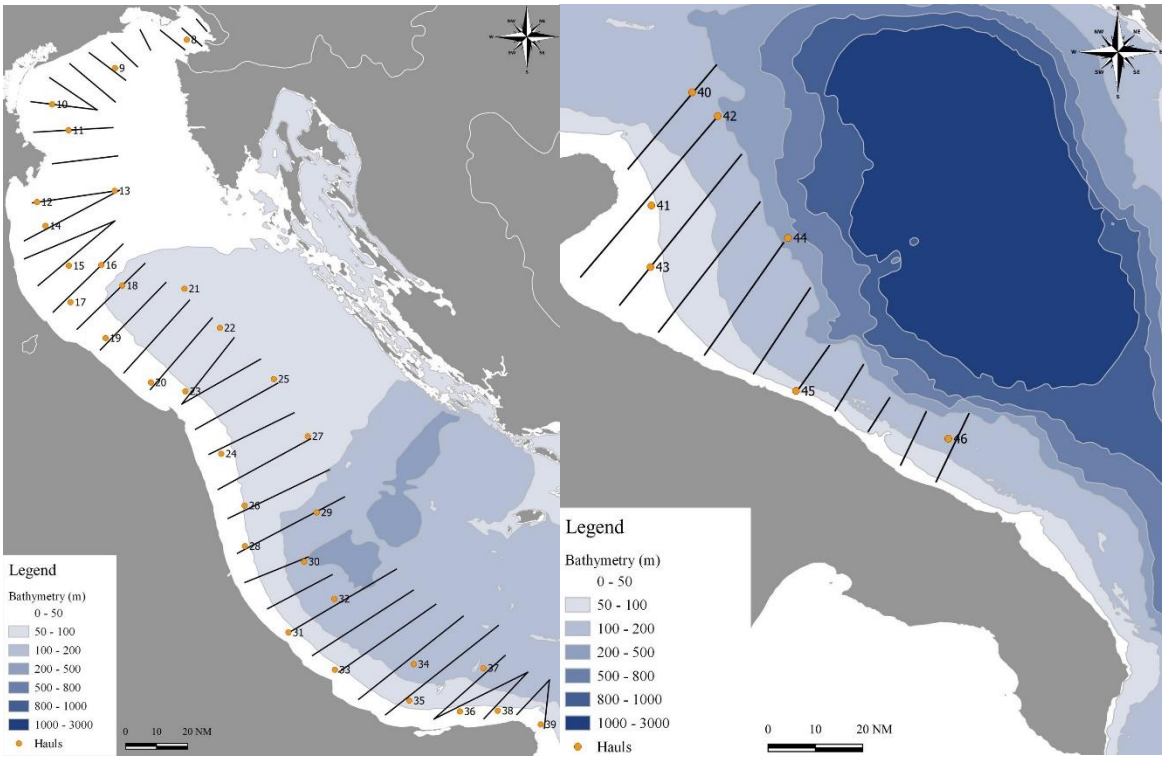

Figure 13. Map of hauls performed in 2019 during the MEDIAS survey for GSA17 (on the left) and GSA18 (on the right). Black lines represent transects for acoustic sampling

Biological data of pelagic fishes were taken through pelagic trawling, with a sampling net (10 m vertical opening and 12 m horizontal opening, with mesh

size 18 mm). The net also had a wireless SIMRAD “trawl eye” system that allowed to gather information on the correct opening of the net and on entering fishes during trawling. Hauls were performed at different times of the day, covering as evenly as possible the target area, both inshore and offshore, also considering acoustic data of fish position and aggregation (Figure 13). Hauls were at least 30 minutes long.

Figure 14. Echosounder and trawl eye tracks during the haul in the acoustic laboratory on board R/V Dallaporta (top) and fishing operations (bottom)

At the end of the haul, fishes were sorted, counted, measured and weighted.

Since sardines, anchovies and sprats were the main target, 10 individuals per

length class (0.5 cm) of each species were frozen at -20 °C for further laboratory analysis.

CNR-IRBIM generally collects zooplankton samples to determine anchovy biomass through DEPM. Therefore, during the survey, zooplankton sampling was performed, using a WP2 net (Figure 15). The net had a circular mouth of 57 cm diameter and was 2.6 m long. It had a mesh size of 200 μm and was equipped with a flowmeter MF 315 to estimate the volume of filtered water. Vertical tows were performed with a towing speed of 1 m/s, starting from three meters above the bottom, to the surface. The starting and ending values of the flowmeter were recorded, to obtain an estimate of the volume of filtered water. Sampling stations were located along acoustic sampling transects.

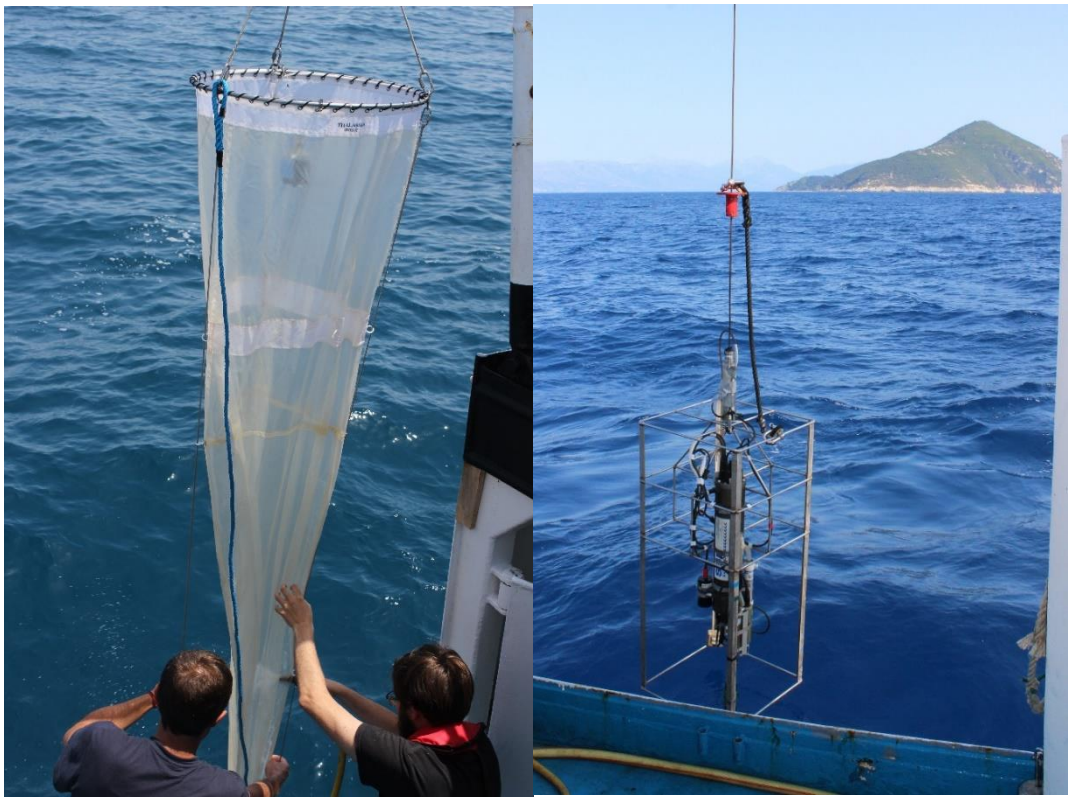

Figure 15. WP2 net (left) and CTD (right)

In general, zooplankton samples for DEPM are preserved in 4% formaldehyde solution, but formaldehyde has been proven to alter the $\delta^{13}C$ signature of samples (Fanelli *et al.*, 2010). For this reason, zooplankton samples near the fishing hauls were subsampled: half of each sample was frozen at -20 °C, to be used for this study, while the other half was preserved in formalin. Concurrently with each vertical plankton haul, a CTD cast was performed, to acquire information on the oceanographic parameters of the chosen site (Figure 15).

Once the survey was completed, a set of fishing hauls was selected, to be used for this study. The whole Western Adriatic (GSA 17 and GSA 18) has been divided in four different sub-areas, based mainly on oceanographic characteristics:

1. GSA 17 North, characterized by a low depth, up to about 60 m, and the mouth of Po River;
2. GSA 17 Central, with deeper bottoms, up to about 100 m;
3. GSA 17 South, characterized by the presence of the Pomo Pit;
4. GSA 18, characterized by the presence of the South Adriatic Pit and the Otranto Channel.

For each sub-area, hauls that were representative of variations in oceanographic characteristics (mainly depth, distance from the coast and latitude) were selected, to determine the trophic relationship between zooplankton and small pelagic fishes. As near as possible to each haul, a zooplankton sampling station was selected, to gather information on planktonic community of the area (Figure 16).

In selected hauls, for each species three individuals per cohort were chosen for muscular tissue extraction. Cohorts were determined using the length-frequency distribution.

To investigate the presence of variations in isotopic signatures in relation to ontogenetic changes, three individual per length class (0.5 cm) were also selected in each sub-area for muscular tissue extraction, from the smallest class to the largest one.

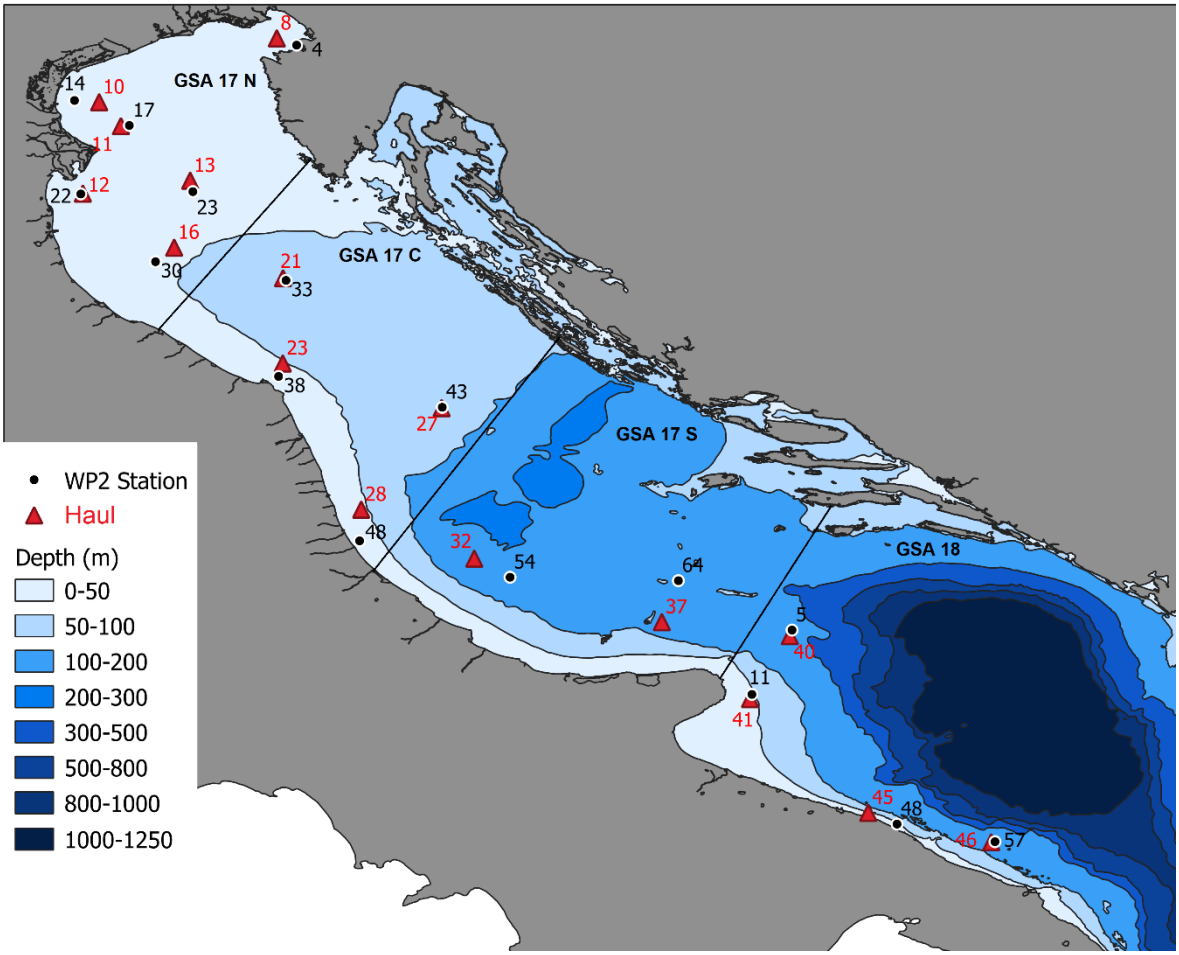

Figure 16. Selected hauls and stations

2.2 Taxonomic determination of zooplankton

Selected zooplankton samples were analysed in the laboratory to characterize the planktonic community. The same workflow was used for each sample:

1. the frozen sample was defrosted and filtered with 200 µm sieve and the obtained mass was weighted to quantify planktonic biomass;
2. the sample was placed in a Petri dish with water to perform sorting;
3. bigger animals were isolated first and placed in Petri dishes located on ice, in order to preserve the integrity of tissues;
4. isolated animals were than identified to the lowest taxonomic level possible;
5. the rest of the sample was sub-sampled to identify smaller organisms. About 10% of the sample was therefore weighted and all organisms in the sub-sample were identified to the lowest taxonomic level possible; since *Clausocalanus sp.*, *Paracalanus sp.* and *Pseudocalanus sp.* were difficult to sort, they were grouped in the "Calanus-like group";
6. identified taxa were counted and weighted with an analytical weight scale, to obtain abundance and biomass estimations;
7. eventually, identified taxa were prepared for isotope analyses.

Zooplankton sorting and identification was performed using ZEISS Stemi 2000 stereomicroscope, with 6.5-50x magnification.

Figure 17. Stereomicroscope used for sample analysis

To identify planktonic organisms, the following texts were used:

- Rose M., 1993. Faune De France. Copèpodes Pèlagiques. Federation Française de Sociètes de Sciences Naturelles. Office Central de Faunistique. Paris, pp. 374
- ICRAM, 2006. Guida al riconoscimento del plancton neritico dei mari italiani, Volume II - Zooplancton Neritico - Tavole pp. 196
- Mauchline J., 1984. Euphausiid, Stomatopod and Leptostracan Crustaceans. Key and notes for the identification of species. London, Leiden, Koln
- Tattersall W.M., Tattersall S. Tattersal, 1951. The British Mysidacea. London, 1951. pp. 267

- Costa F., Krapp T., Ruffo S., 2009. Atlante degli anfipodi mediterranei. Guida illustrata a colori. Milano, pp. 221
- Chevreux, E. & Fage, L. 1925. Faune de France. Amphipodes. 9. Lechevalier, Paris, pp. 488
- Williamson D.I. 1957. Crustacea, Decapoda: larvae I-IX

2.3 Samples preparation for isotope analyses

The most abundant taxa in each sample were prepared for stable isotope analyses. Selected taxa were oven-dried for 24 hours at 60°C. Dried samples were converted to a fine powder with a mortar and pestle. For each taxon, three replicates (when possible) were weighted, between 1.3 and 0.3 mg, and placed into tin capsules. These capsules were then placed in a numbered rack, that allowed to easily identify the corresponding position of a particular taxonomic group. Since in stations 22_17 and 38_17 it was not possible to obtain enough material of a single taxon to prepare a sample for stable isotope analyses, a bulk of the whole mesoplankton community of the station was prepared for the analyses.

Acidification of samples prior to stable isotope analyses is usually regarded as a standard procedure, since inorganic carbon could lead to an increase of $\delta^{13}C$, because it is isotopically heavier than most carbon organic origin and could reflect the isotopic signature of environmental carbon (Schlacher and

Connolly, 2014). However, for this study, no acidification was carried out, as this procedure generally reduces sample biomass, leading to too little matter available for isotope analyses. Moreover, some authors revealed negligible differences between acidified and not acidified samples (Rumolo *et al.*, 2018). To confirm this, only one sample was acidified: the chosen sample was a bulk of *Euchaeta sp.*, which is a copepod genus very abundant in Adriatic communities. This taxon was chosen because it has a calcified exoskeleton and it was abundant enough to undergo this process. Half of the sample was acidified with HCl 1M, by adding it drop by drop to the sample until bubble cessation. The other half, for the analysis of $\delta^{15}N$, was not acidified, as several studies demonstrated that the acidification procedure can alter nitrogen isotopic signature (Kolasinski, Rogers and Frouin, 2008). Then, three replicates of each sub-samples were prepared for isotope analyses.

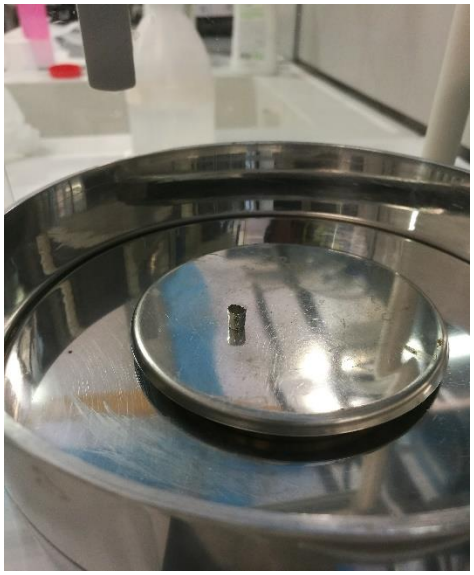

Figure 18. Tin cap on the weight scale

For fishes, a small sample of white muscle close to the dorsal fin, from selected specimens, was also oven-dried for 24 hours at 60°C, weighted, between 1.3 and 0.3 mg, and placed into tin capsules, that were then put in a numbered rack.

All instruments used to powder samples (*i.e.* mortar, pestle and tweezers) were cleaned after each sample, with deionized water, in order to avoid contaminations.

The isotope analyses were conducted at the University of Palermo, where tin capsules were automatically loaded in an elemental analyser (Thermo Flash EA 1112) for the determination of total carbon and nitrogen, and then analysed for $\delta^{13}C$ and $\delta^{15}N$ in a continuous-flow isotope-ratio mass spectrometer (Thermo Delta Plus XP). Stable isotope ratio was expressed, in relation to reference international standards (atmospheric N_2 and PeeDee Belemnite for $\delta^{15}N$ and $\delta^{13}C$ respectively), as:

$$\delta^{13}C \text{ or } \delta^{15}N : [(R_{sample}/R_{standard})-1)]*10^3$$

where $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$. Analytical precision based on standard deviations of internal standards (International Atomic Energy Agency IAEA-CH-6; IAEA-NO-3; IAEA-N-2) ranged from 0.10 to 0.19‰ for $\delta^{13}C$ and 0.02 to 0.08‰ for $\delta^{15}N$.

2.4 Data analyses

Zooplankton abundance and biomass were standardized to a constant value. The adopted constant was the volume of water filtered by the net. This value was obtained thanks to the flowmeter mounted on the net mouth. The

flowmeter provides the number of spins performed by the helix, which is then converted to the volume value thanks to this formula:

$$V(m^3) = A \cdot B \cdot C$$

where A is the number of spins, B is the number of meters travelled with each spin (given by the manufacturer) and C is the area of the net mouth (m^2). When flowmeter data were not available (due to misfunctioning), the volume was calculated as a mean value of similar nearby stations.

Zooplankton abundance was expressed as number of individuals per m^3, while zooplankton biomass was expressed as milligrams of wet weight per m^3.

First, the Shannon-Wiener diversity index of each station was calculated. Then, total biomass, total abundance and H' diversity index were tested by univariate ANOVA. These data were used to create three separate Euclidean distance resemblance matrixes on untransformed data. On each matrix, a univariate PERMANOVA (Permutational Multivariate Analysis of Variance; Anderson et al., 2008) was run on a two-way design with sub-area as a fixed factor with four levels (GSA17N, GSA17C, GSA17S and GSA18) and inshore-offshore location, as a random factor with two levels (inshore *vs.* offshore), nested within each other, in order to assess the presence and

significance of differences between stations. The choice of a nested design was due to the absence of the level inshore in the sub-area GSA17S.

Multivariate analyses were run on zooplanktonic abundance data. First, the abundance data were fourth root transformed and a resemblance matrix was obtained using the Bray-Curtis distance.

On the resemblance matrix, a nMDS (non-metric Multi-Dimensional Scaling) was carried out, to visualize the level of similarity of samples, followed by a CLUSTER analysis. To test the significance of observed differences a PERMANOVA (Anderson, 2001) was performed using the same design described for univariate analyses. In this analysis 9999 permutations were used, with permutation of residuals under a reduced model as permutation method.

Then, SIMPER analysis, which provides the percentage contribution of the different taxa to the average similarity/dissimilarity was calculated. SIMPER was calculated for each sub-area. The SIMPER analysis was conducted using Bray-Curtis similarity (with a cut-off at 60%).

All analyses were run using the software PRIMER6&PERMANOVA+ (Anderson *et al.,* 2008; Clarke and Gorley, 2006).

In order to identify the environmental drivers of the zooplanktonic community and their structure across the sampling area, biotic data were correlated to environmental data. Environmental data considered were pressure (db), temperature (°C), fluorescence (µg/l), turbidity (NTU), dissolved oxygen (expressed as ml/l and saturation percentage), salinity (PSU) and density (km/m^3). All data were collected through a CTD for each station.

Fluorescence, O_2 (ml/l), O_2 (%) and turbidity data were transformed (Log (V+1) to obtain a linear distribution in the Draftsman plot.

Environmental data were tested for collinearity by using a Draftsman plot to assess covarying variables.

Abundance data were then forth root transformed to create a Bray-Curtis resemblance matrix. This matrix was used for a DistLM (Distance based linear models) analysis, with temperature, fluorescence, turbidity, oxygen and salinity as environmental variables. DistLM was performed using step-wise as selection procedure and AIC (Akaike Information Criterion) as selection criterion.

Isotopic analyses provided the $\delta^{13}C$ and $\delta^{15}N$ values, as well as percentage of carbon and nitrogen and C/N ratio of each sample.

Since lipids can alter the values of $\delta^{13}C$ (Post *et al.*, 2007), samples with high lipid concentration can be defatted to avoid ^{13}C depletion. However, lipid extraction can alter $\delta^{15}N$ values and can complicate sample preparation: for these reasons, $\delta^{13}C$ of samples rich in lipids was normalized according to the equation

$$\delta^{13}C_{normalized} = \delta^{13}C_{untreated} - 3.32 + 0.99\ C/N_{sample}$$

which was developed as an alternative to lipid extraction (Post *et al.*, 2007). C/N ratio was used as a proxy of fat content, because their values are strongly related in animals (Post *et al.*, 2007). In particular, the normalization was applied to samples with a C/N ratio over 3, according to Post *et al.*, 2007.

Zooplanktonic samples where then used to assess the trophic web structure, by performing Hierarchical cluster analysis (Euclidean distance, average grouping methods) on the bivariate matrix of $\delta^{13}C$ and $\delta^{15}N$ mean values of each taxon. Obtained clusters were also compared with literature data on the trophic behaviour of analysed taxa. A nMDS was also performed on the same matrix.

A PERMANOVA (Anderson *et al.*, 2008) was run on the same matrix, based on a two-way design with sub-area as a fixed factor with four levels (GSA17N, GSA17C, GSA17S and GSA18) and inshore-offshore location, as a random factor with two levels (inshore vs. offshore), nested within each

other, in order to assess the presence and significance of differences between stations.

Since there were no samples in inshore area of GSA 17S, an orthogonal PERMANOVA with pairwise test could not be performed. To overcome this problem, sub-area and inshore-offshore location were combined in a single factor, and a one-way PERMANOVA with pairwise test was performed on the resemblance matrix, with sub-areaXinshore-offshore as the only factor. Finally, a CAP analysis (Canonical analysis of principal coordinates) was performed on the factor found to be significant by PERMANOVA analysis, on the same resemblance matrix, to plot the distribution of samples according to the combined factor.

The same procedure was also used to perform univariate two-way PERMANOVA and one-way PERMANOVA with pairwise test for the $\delta^{13}C$ and $\delta^{15}N$ values separately.

Isotopic signature data of sardine and anchovy were used to create a scatterplot of $\delta^{13}C$ and $\delta^{15}N$ values related to fish length, in the whole study area and for each sub-area, to assess the presence of ontogenetic changes in fish diet and differences between sub-areas.

Moreover, the variation of $\delta^{15}N$ values according to $\delta^{13}C$ was also plotted for sardine and anchovy together, to examinate trophic niche partitioning.

At last, a final scatterplot was plotted, with $\delta^{13}C$ and $\delta^{15}N$ mean values of zooplankton, divided in 4 groups thanks to cluster analysis, isotopic values of anchovies and sardines and mean values of $\delta^{13}C$ and $\delta^{15}N$ for large pelagic fishes and *Tursiops truncatus* in the study area. Data of *Scomber japonicus*, *Scomber scombrus* and *Trachurus mediterraneus* were taken from Fanelli E., Leonori I., Malavolti S. (unpub. data). Isotopic values of *Tursiops truncatus* were taken from Fortibuoni *et al.* (2013).

Chapter three

RESULTS

3.1 Zooplankton community

Zooplanktonic community was dominated by small copepods, like *Acartia sp.*, *Oncaea sp.*, *Oithona sp.* and several small unidentified calanoids, both adults and copepodites (Figure 19).

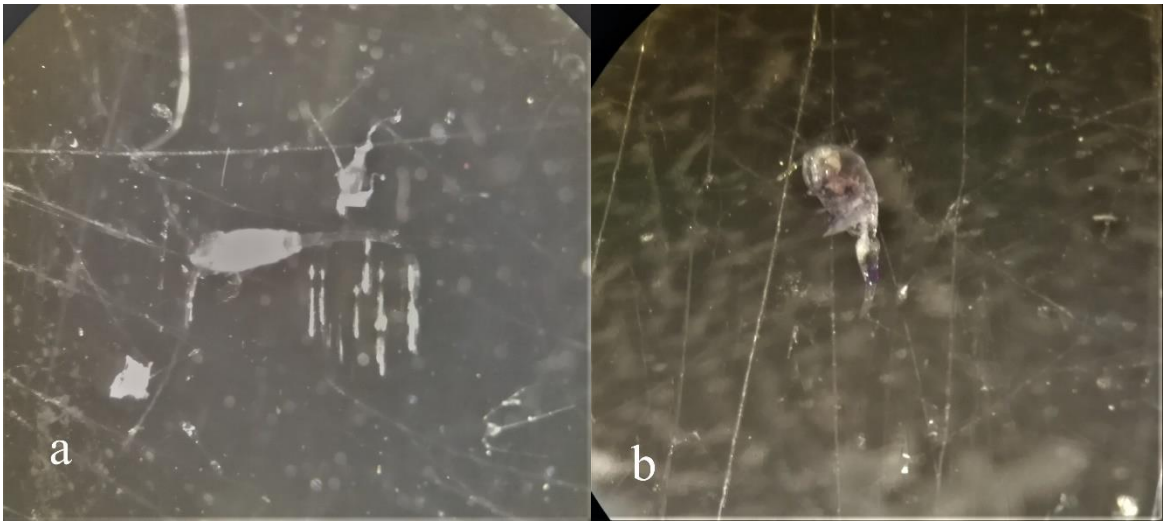

Figure 19. a) Oithona sp.; b) Oncaea sp.

Abundant large copepods were Calanoida belonging to the genera *Euchaeta*, *Calanus*, *Centropages* and *Temora* (Figure 20).

Figure 20. a) Euchaeta sp.; b) Calanus helgolandicus; c) Centropages typicus

Other common crustaceans were hyperiids, like *Lestrigonus schizogeneios* and *Phronima atlantica*, and decapod larvae, like zoeae and megalopae (Figure 21).

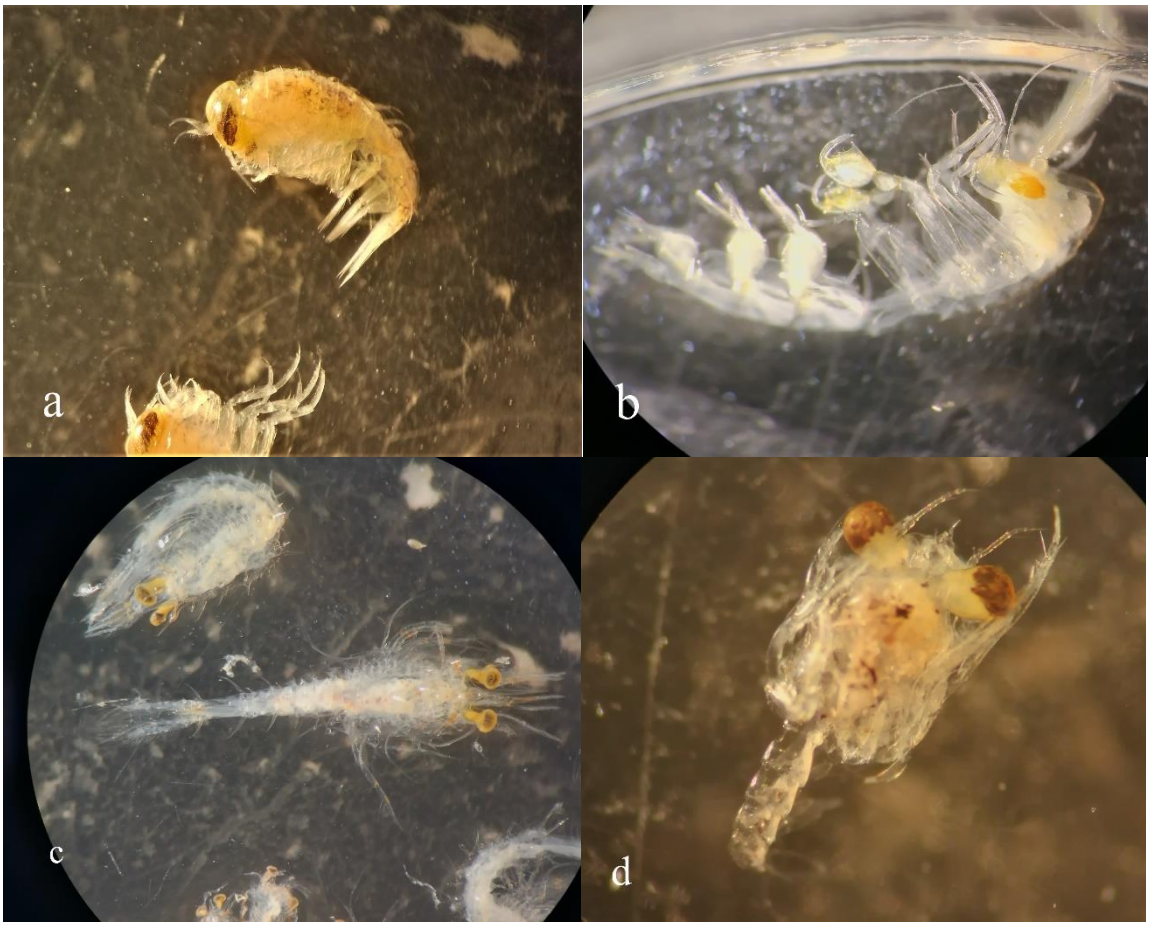

Figure 21. a) Lestrigonus schizogeneios; b) Phronima atlantica; c) Penaeidae (zoea); d) Brachyura (megalopa)

Mysids and euphausiids were also found (Figure 22).

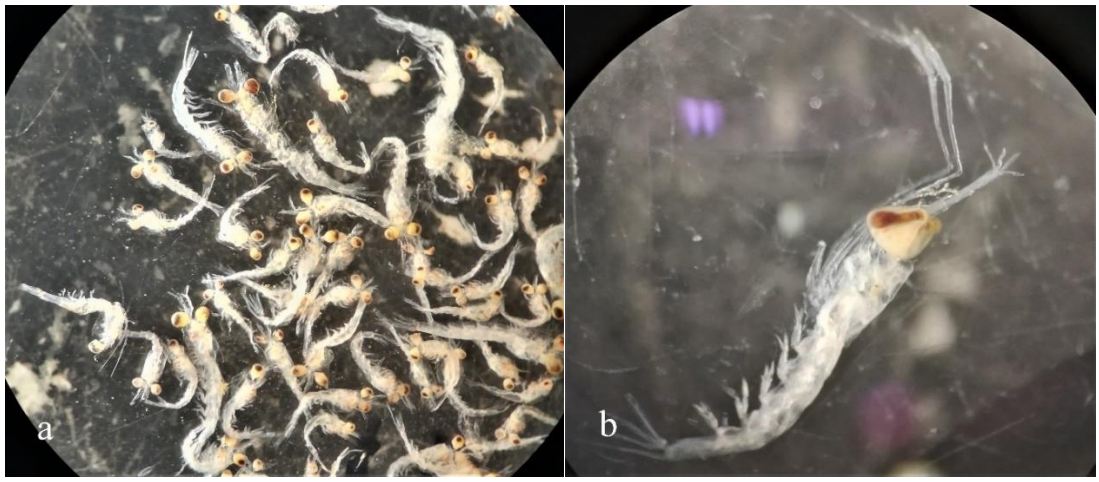

Figure 22. a) Meganyctiphanes norvegica (furcilia); b) Stylocheiron suhmi

Among non-crustaceans, molluscs were quite common, both as larvae of benthic organisms and adult Pteropoda (Figure 23). Chaetognatha were also locally abundant (Figure 23).

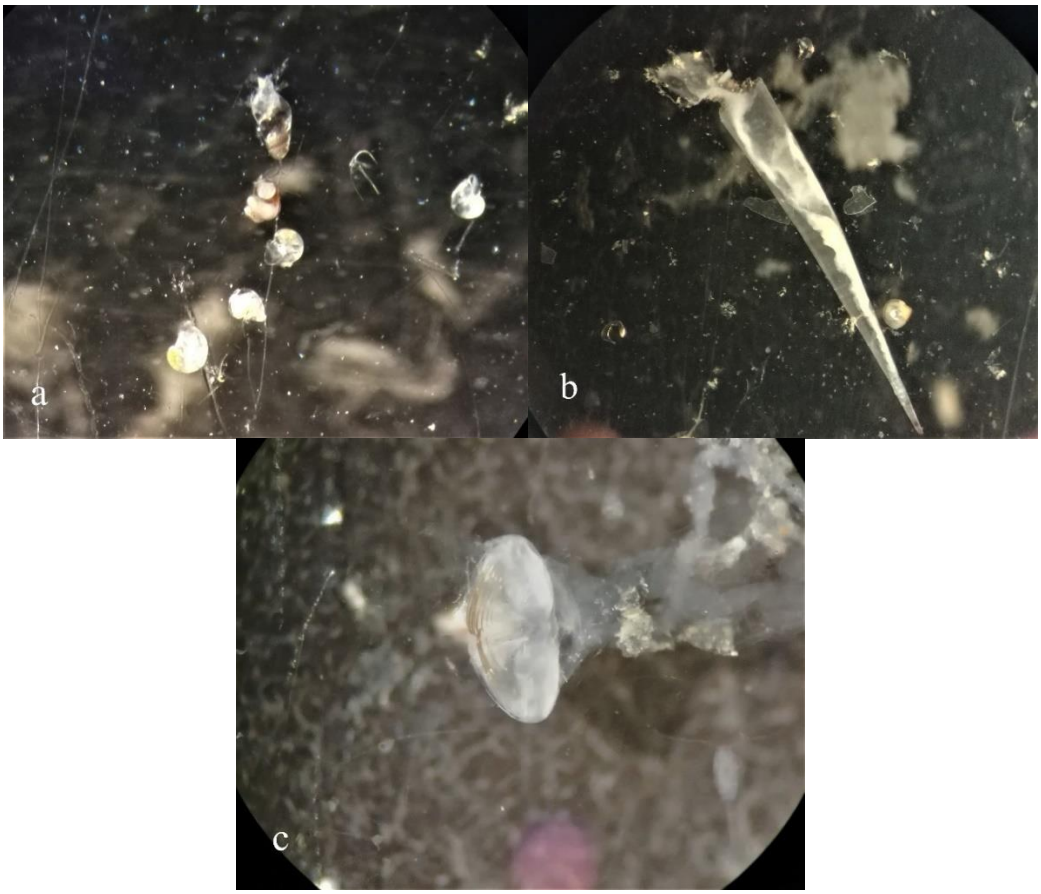

Figure 23. a) Gasteropoda larvae; c) Creseis acicula and bivalve larva; c) Chaetognata (head close-up)

Gelatinous zooplankton was represented mainly by thaliaceans and calycophorans (Figure 24). Ichthyoplankton was not very abundant, since only a few fish egg and larvae were found (Figure 24).

Figure 24. a) Thaliacea; b) fish larvae

Zooplankton abundance and biomass varied according to geographic sub-area decreasing from Northern to Southern Adriatic (Figure 25), while inshore-offshore variations were not significant. Shannon-Wiener diversity index did not show a significant variation for investigated variables (Table 1).

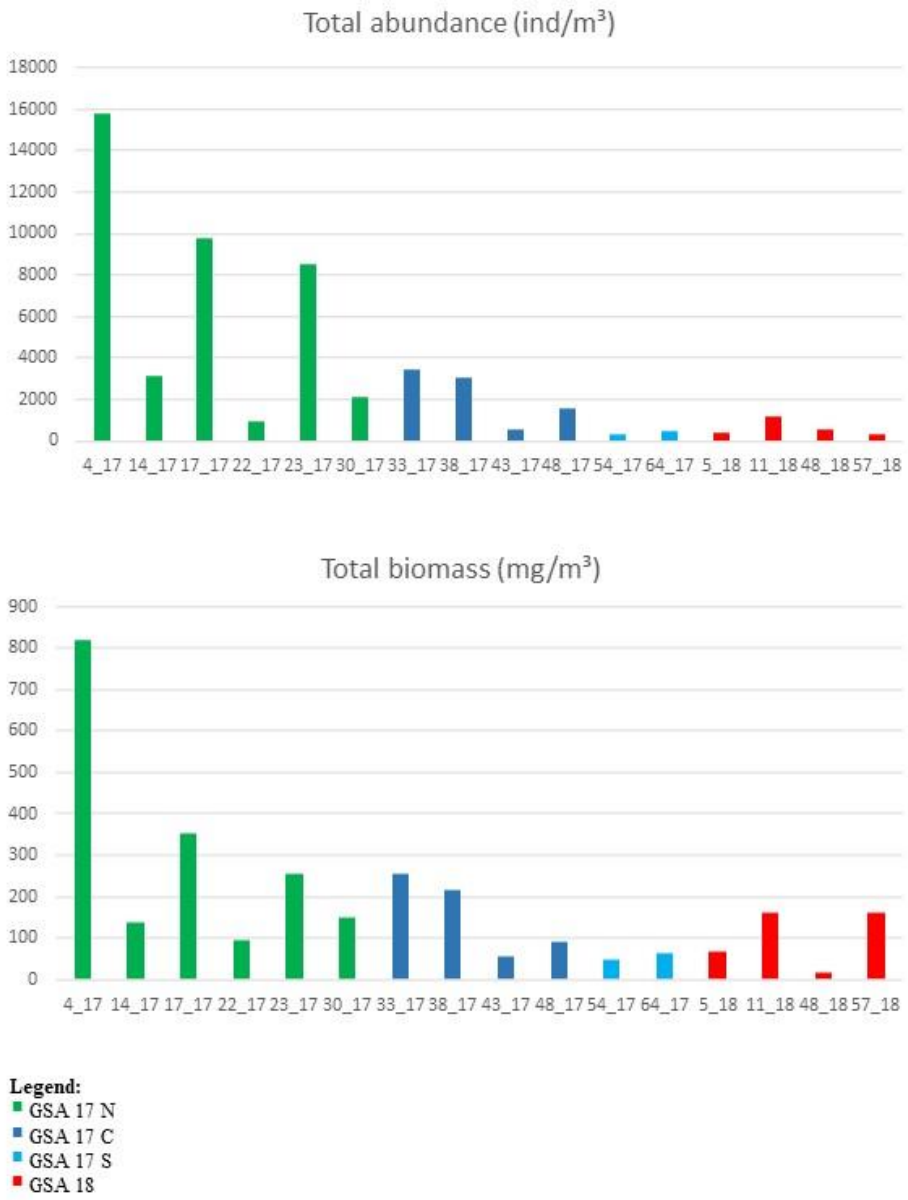

Figure 25. Total abundance (top) and total biomass (bottom) of each station.

Table 1. Permanova results of univariate analyses (left) and H' values of each station (right)

Total abundance

Source	df	MS	Pseudo-F	P(MC)
GSA	3	11.81	202.11	0.0001
In-Off	1	0.63	0.19	0.67
GSAxIn-Off	2	0.02	0.01	0.99
Residuals	9	3.26		
Total	15			

Total biomass

Source	df	MS	Pseudo-F	P(MC)
GSA	3	0.86	39.42	0.0012
In-Off	1	0.10	0.16	0.70
GSAxIn-Off	2	0.01	0.02	0.98
Residuals	9	0.65		
Total	15			

Diversity (H')

Source	df	MS	Pseudo-F	P(MC)
GSA	3	0.55	2.65	0.28
In-Off	1	0.46	1.10	0.32
GSAxIn-Off	2	0.20	0.49	0.63
Residuals	9	0.42		
Total	15			

Station	H'(loge)
4_17	2.76
14_17	2.68
17_17	2.90
22_17	0.64
23_17	2.91
30_17	3.35
33_17	3.44
38_17	2.98
43_17	3.43
48_17	3.54
54_17	3.32
64_17	3.56
5_18	3.55
11_18	3.28
48_18	3.50
57_18	3.49

In terms of abundance, copepods always had the higher percentage contribution, with a slight decrease in Southern Adriatic (Figure 26Figure 24).

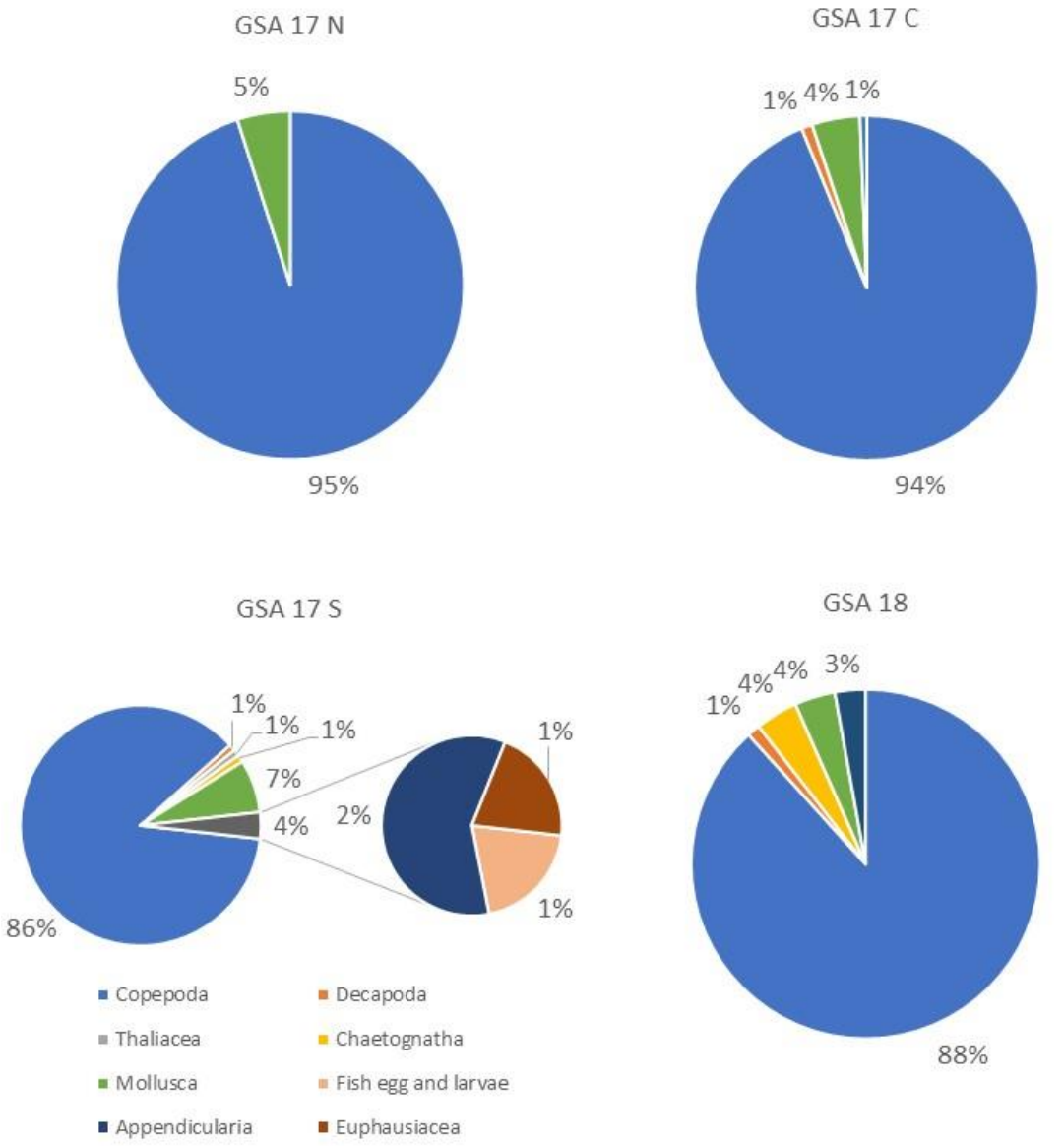

Figure 26. Mean percentage contribution in terms of abundance of major taxa in each sub-area

In terms of biomass, copepods were still dominant in every sub-area, but with much lower percentage contribution. In particular, in Southern Adriatic there is a reduction of copepods, like with abundance estimates, but a higher presence of big thaliaceans (Figure 27).

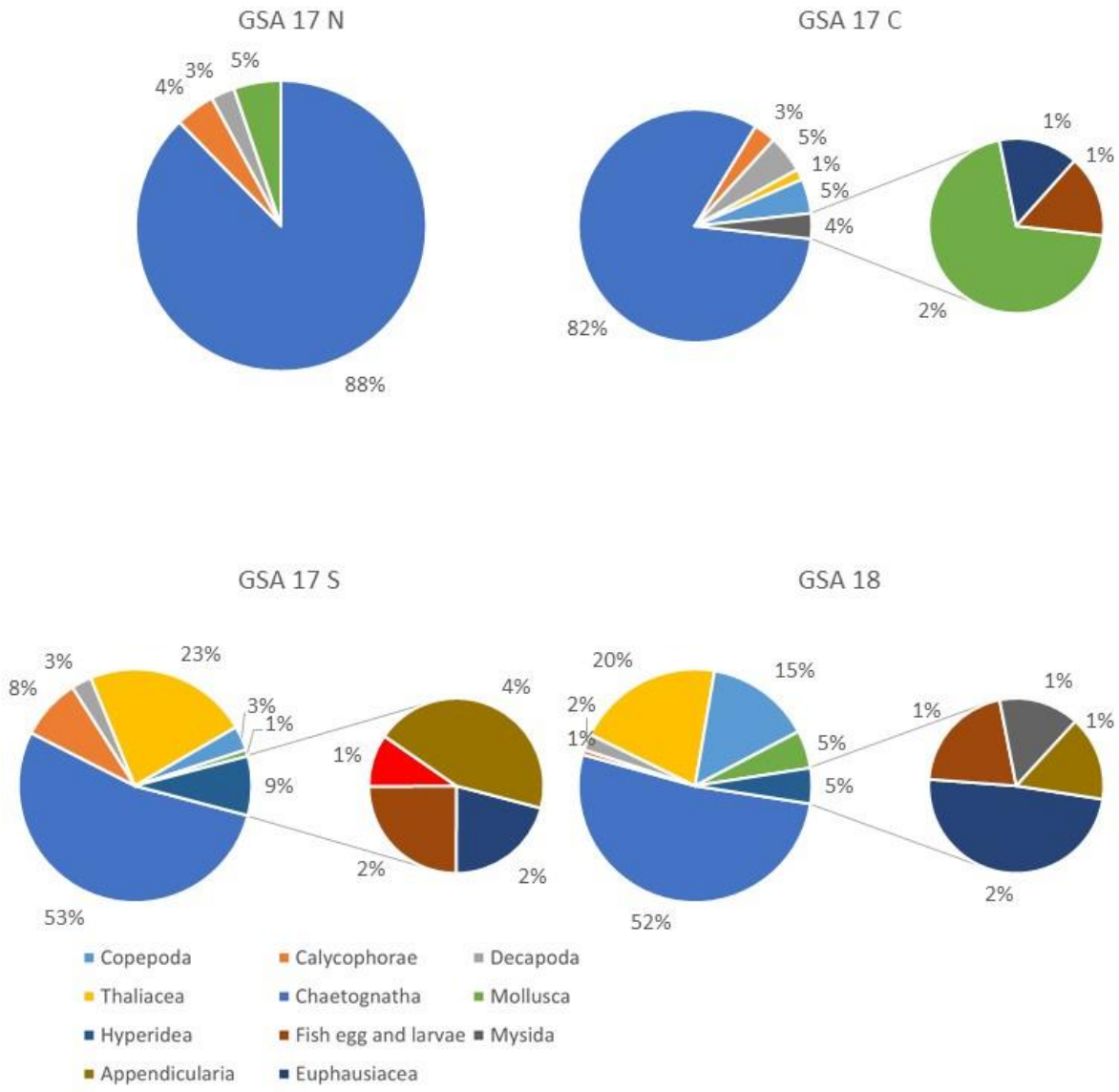

Figure 27. Mean percentage contribution in terms biomass of major taxa in each sub-area

3.2 Multivariate analyses

Cluster analyses showed a clear separation between station 22_17, directly in front of the Po delta, and all other stations. Moreover, there is another separation between northern and southern samples, both in terms of abundance and biomass: samples from GSA 17 N and GSA 17 C were

divided by those of GSA 17 S and GSA 18, except for station 43_17, which belonged to GSA 17 C, but was clustered with southern samples (Figure 28). The same trend is observed in nMDS analysis (Figure 29).

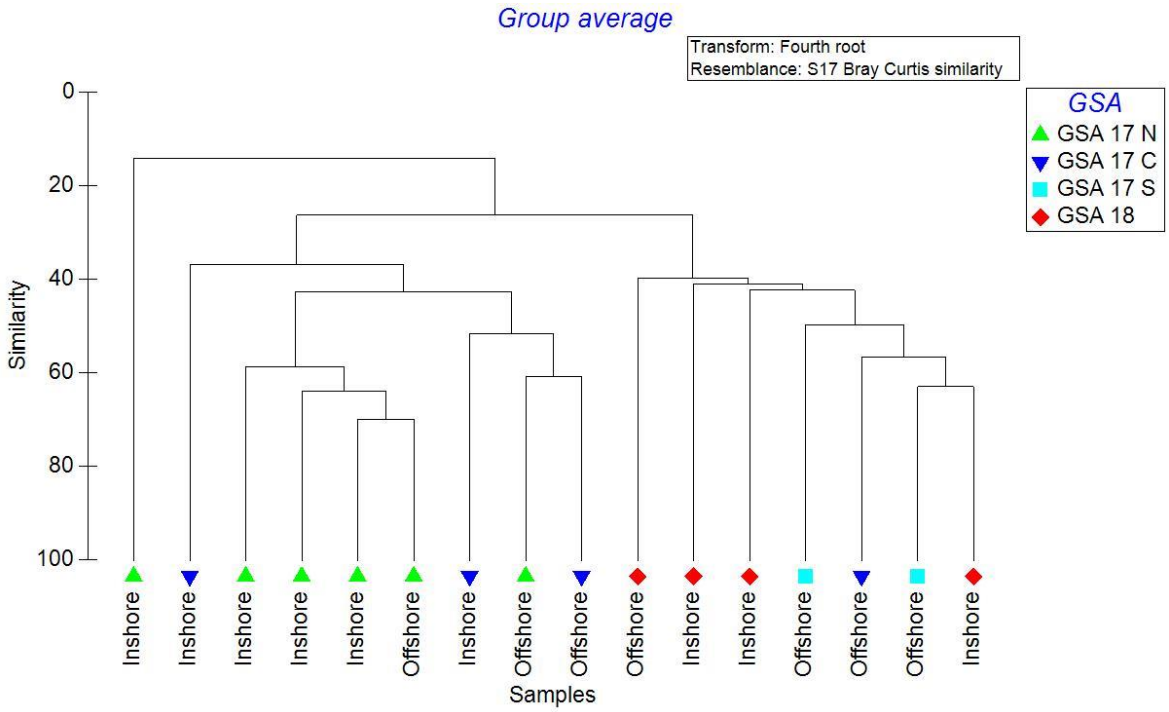

Figure 28. Cluster analysis of zooplanktonic abundance

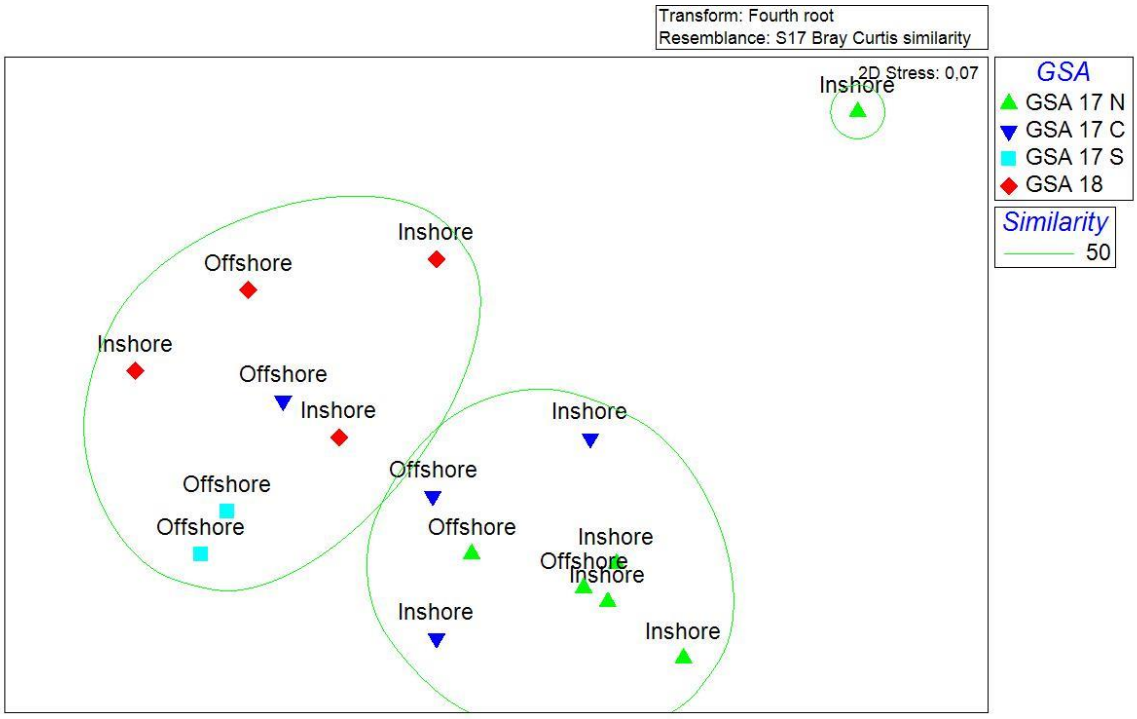

Figure 29. nMDS analysis of zooplanktonic abundance

PERMANOVA revealed that differences based on geographic sub-areas are significative, while inshore-offshore factor had no significance to sample separation (Table 2).

Table 2. PERMANOVA results of multivariate analysis on zooplanktonic abundance

Source	df	MS	Pseudo-F	P(MC)
GSA	3	2788.8	2.99	0.0291
In-offshore	1	1611.3	1.35	0.2475
GSxIn**	2	928.83	0.78	0.6511
Residuals	9	1196.3		
Total	15			

SIMPER analysis (Table 3) revealed that unidentified Calanoida had the highest contribution to similarity of samples in GSA 17 N, with 22.24% contribution, followed by *Acartia sp.* (12.98%) and *Podon sp.* (12.01%). In GSA 17 C 13.71% of similarity was explained by unidentified Calanoida,

11.3% by *Oithona sp.* and 7.48% by *Podon sp.* In GSA 17 S Calanus-like group had the highest contribution to similarity (11.48%), followed by *Oithona sp.* (11.42%) and *Euchaeta sp.* (7%). Eventually, similarity in GSA 18 was explained for 15.98% by unidentified Calanoida, 8.73% by *Oncaea sp.* and 8.47% by Chaetognatha.

Table 3. Results of SIMPER analysis per geographic sub-area, with a 60% cut-off

Group GSA 17 N		Average similarity: 53,96		
Species	Av.Abund	Av.Sim	Contrib%	Cum.%
Calanoida unid.	6.04	12	22.24	22.24
Acartia sp.	6.04	7	12.98	35.22
Podon sp.	3.37	6.48	12.01	47.23
Oithona sp.	4.94	6.29	11.65	58.88
Evadne spinifera	2.43	3.08	5.71	64.6
Group GSA 17 C		Average similarity: 51,10		
Calanoida unid.	4.76	7.01	13.71	13.71
Oithona sp.	4.47	5.77	11.3	25.01
Podon sp.	3	3.82	7.48	32.49
Bivalvia larvae	2.49	3.74	7.32	39.8
Oncaea sp.	2.73	3.65	7.14	46.94
Acartia sp.	3.2	3.56	6.97	53.91
Centropages typicus	2.18	2.8	5.47	59.38
Penilia avirostris	2.61	2.45	4.8	64.18
Group GSA 17 S		Average similarity: 63,61		
Calanus-like	3.15	7.3	11.48	11.48
Oithona sp.	3.42	7.26	11.42	22.89
Euchaeta sp.	2.06	4.45	7	29.89
Bivalvia larvae	2.12	4.38	6.88	36.78
Oncaea sp.	2.03	3.99	6.27	43.05
Calanus helgolandicus	1.61	3.67	5.77	48.81
Gasteropoda larvae	1.51	3.43	5.39	54.2
Acartia sp.	1.7	3.27	5.15	59.35
Thaliacea	1.31	2.61	4.1	63.45
Group GSA 18		Average similarity: 51,19		
Calanoida unid.	4.17	8.03	15.68	15.68
Oncaea sp.	2.45	4.47	8.73	24.41
Chaetognatha	2.13	4.33	8.47	32.88
Oithona sp.	2.04	4.02	7.86	40.74
Corycaeus sp.	1.55	2.96	5.78	46.52
Euchaeta sp.	1.62	2.91	5.68	52.2
Calanus helgolandicus	1.12	2.18	4.26	56.47
Zoea Brachyura	1.19	2.02	3.95	60.41

3.3 Planktonic community and environmental variables

Draftsman plot allowed to pair environmental variables to assess correlation (Table 4). If correlation value was over 0.7, variables were considered correlated. Oxygen (ml/l) and Oxygen (% saturation) covaried, so only

Oxygen (ml/l) was kept for further analyses. Moreover, salinity covaried with density and pressure, which were excluded. Therefore, only temperature, fluorescence, turbidity, O_2 (ml/l) and salinity were used for DistLM analysis.

Table 4. Correlation values of environmental variables

	Pressure	T	Fluo	Turbidity	O2	O2%	S	Density
Pressure								
T	-0.53							
Fluo	-0.69	0.20						
Turbidity	-0.67	0.21	0.37					
O2	0.04	-0.82	0.13	0.21				
O2%	-0.38	-0.45	0.32	0.54	0.87			
S	0.77	-0.40	-0.89	-0.38	-0.05	-0.37		
Density	0.79	-0.80	-0.69	-0.35	0.42	0.01	0.87	

DistLM analysis was run with AIC selection criterion and stepwise selection procedure. Sequential test showed how 26.89% of variance was explained by salinity, 11% by fluorescence and 8.61% by O_2, for a cumulative contribution to variance of 46.49% (Table 5).

Table 5. Results of sequential test in DistLM analysis

Variable	AIC	SS(trace)	Pseudo-F	P	Prop.	Cumul.	res.df
S	116.47	6643.1	5.15	0.001	0.27	0.27	14
Fluo	115.86	2716.6	2.30	0.032	0.11	0.38	13
O2	115.47	2126.6	1.93	0.044	0.09	0.46	12

Therefore, DistLM provided a graphic result through dbRDA (Distance-based redundancy analysis). The first axis explained 66.8% of fitted variation (variance within the linear model created during the DistLM analysis) and 31.1% of total variation (variance within the original data), while the second

axis explained 23.8% of fitted variation and 11.1% of total variation (Figure 30).

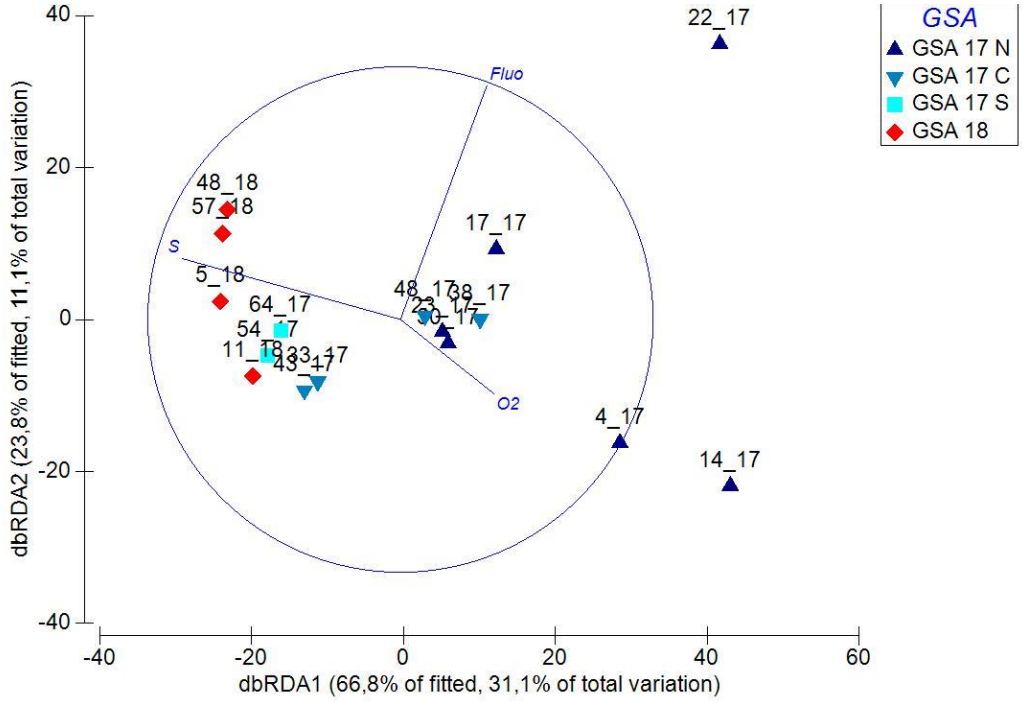

Figure 30. DbRDA of DistLM analysis of environmental variables

3.4 Stable isotope analyses on zooplankton

Stable isotope analyses provided the $\delta^{13}C$ and $\delta^{15}N$ values of 26 different taxa (Table 6).

Acidification of crustaceans was unnecessary, since the tested sample of *Euchaeta sp*. showed little difference in $\delta^{13}C$ value (-21.39±0.06 for untreated samples and -21.02±0.15 for acidified samples).

Table 6. Mean values of zooplankton samples, trophic group (TG), sub-area and number of samples analysed (N°).

Group	Code	Taxon	$\delta^{15}N \pm SD$	$\delta^{13}C \pm SD$	TG	GSA 17 N	GSA 17 C	GSA 17 S	GSA 18	N°
COP	Aca	*Acartia sp.*	7.86±0.86	-21.58±0.61	OMN	*				3
DEC	Bra	Brachyura (Zoea)	3.89±0.06	-19.17±0.07	Unk			*		2
COP	Cai	Calanoida	4.16±1.09	-20.53±0.34	Unk			*	*	3
COP	Che	*Calanus helgolandicus*	5.16±0.94	-20.83±0.40	HER	*	*	*	*	15
COP	Cli	Calanus-like	3.80±0.22	-21.28±0.48	Unk			*		2
CAL	Cal	Calycophorae	6.21±1.25	-20.05±1.28	CAR	*	*	*		9
COP	Cty	*Centropages typicus*	5.42±1.43	-21.38±0.34	OMN	*	*			4
CHA	Cha	Chaetognatha	6.19±1.40	-19.82±0.43	CAR	*	*	*	*	20
DEC	Dec	Decapoda (Zoea)	6.59±1.59	-19.81±0.24	Unk	*	*		*	5
COP	Euc	*Euchaeta sp.*	5.19±0.40	-20.91±0.23	CAR		*	*	*	16
EUP	Eup	Euphausiacea	4.69	-20.39	OMN				*	1
FIS	Fis	Fish larvae	5.09±0.53	-20.57±0.26	CAR		*	*		4
COP	Gte	*Gaetanus tenuispinus*	2.68	-20.44	Unk		*			1
MYS	Lgr	*Leptomysis gracilis*	8.14	-20.03	OMN				*	1
HYP	Lsc	*Lestrigonus schizogeneios*	8.06±3.30	-20.16±0.65	CAR			*		2
HYP	Lpu	*Lycea pulex*	4.69	-19.64	CAR				*	1
EUP	Meg	*Meganyctiphanes norvegica*	4.48±0.54	-21.18±0.57	OMN		*	*		4
COP	Nmi	*Nannocalanus minor*	3.53±0.37	-20.77±0.21	OMN		*		*	3
COP	Par	*Pareucalanus attenuatus*	4.92	-20.01	HER				*	1
DEC	Pen	Penaeidae (Zoea)	6.34±1.12	-20.54±0.41	Unk			*	*	4
HYP	Pat	*Phronima atlantica*	3.58	-20.98	CAR			*		1
HYP	Pse	*Phronima sedentaria*	5.42	-19.6	CAR				*	1
COP	Pab	*Pleuromamma abdominalis*	3.59	-21.14	HER				*	1
COP	Tst	*Temora stylifera*	5.47±1.08	-20.17±0.56	OMN		*		*	2
DEC	Thl	Thalassinidea (Zoea)	4.14	-19.92	Unk		*			1
THA	Tha	Thaliacea	3.82±0.82	-19.56±1.16	HER		*	*	*	10

Groups are Chaetognata (CHA), Copepoda (COP), Decapoda (DEC), Euphausiacea (EUP), fish larvae (FIS), Hyperiadea (HYP), Mysidacea (MYS), Thaliacea (THA)

Cluster analysis allowed to group animals according to their $\delta^{13}C$ and $\delta^{15}N$ values, but these groups do not exactly represent the expected trophic group division from literature data (Figure 31).

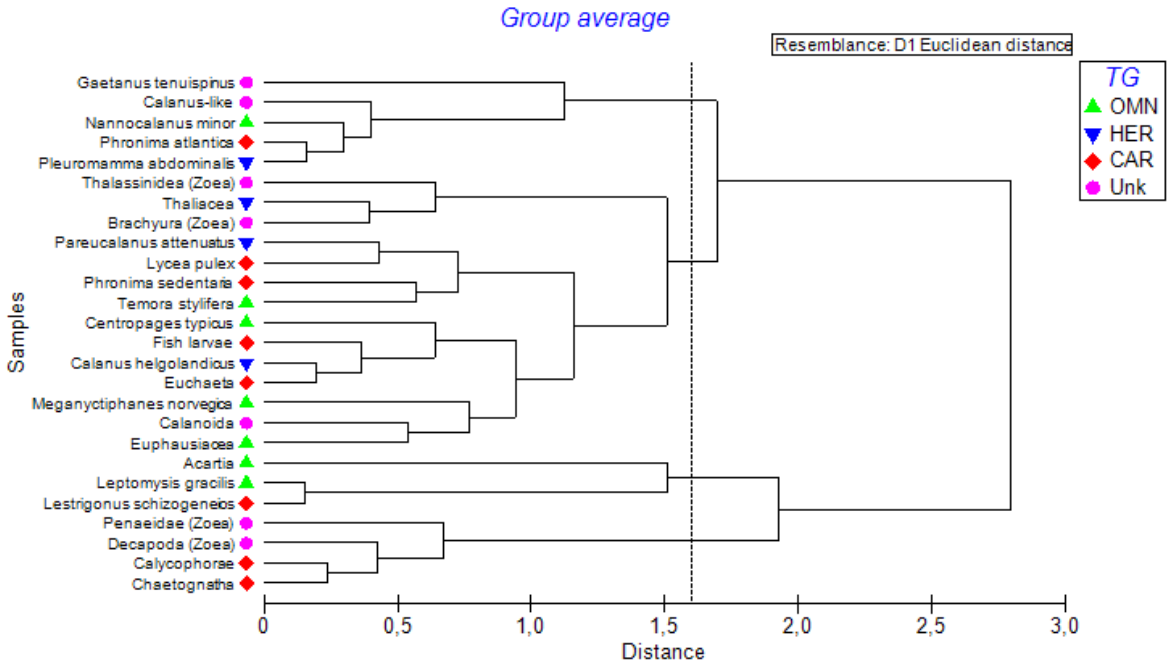

Figure 31. Cluster analysis on the bivariate matrix of $\delta^{13}C$ and $\delta^{15}N$. OMN are omnivores, HER are herbivores and primary consumers, CAR are carnivores, Unk are taxa with unknown trophic group. The dashed line is placed at 1.6 distance

The same groups could also be observed through nMDS analysis (Figure 32).

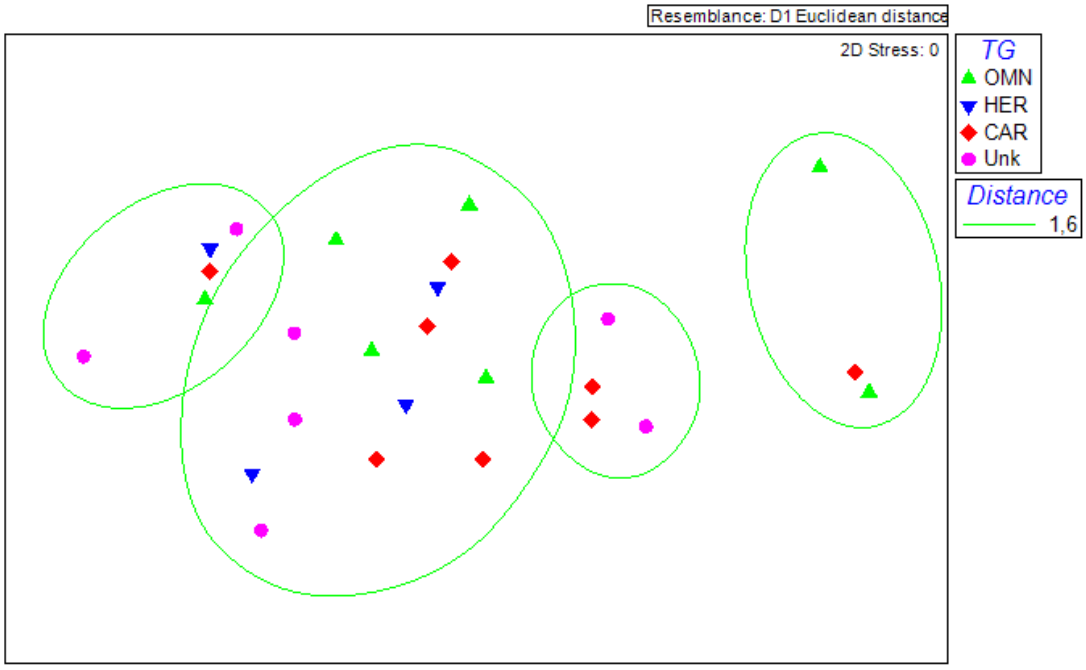

Figure 32. NMDS analysis on the bivariate matrix of $\delta^{13}C$ and $\delta^{15}N$. OMN are omnivores, HER are herbivores and primary consumers, CAR are carnivores, Unk are taxa with unknown trophic group

Two-way PERMANOVA on the multivariate matrix of $\delta^{13}C$ and $\delta^{15}N$ showed a significant separation according to inshore-offshore location within a single sub-area (Pseudo-F=3,55; P=0,0047). One-way PERMANOVA on the same matrix, with pairwise test, allowed to test in which sub-area the variance of isotopic signature was significant. GSA 17 N and GSA 18 did not show a significant variation between inshore and offshore samples, while the difference was significant in GSA 17 C (Table 7).

Table 7. Results of pairwise test from the one-way PERMANOVA of $\delta^{13}C$ and $\delta^{15}N$ matrix

Groups	t	P(perm)
GSA 17 NIn, GSA 17 NOff	0,88	0,4082
GSA 17 COff, GSA 17 CIn	38,69	0,0013
GSA 18Off, GSA 18In	10,96	0,2823

Two-way PERMANOVA on the univariate matrix of $\delta^{13}C$ showed similar results, with a significant difference between inshore and offshore samples within the same area (Pseudo-F=4,04; P(perm)=0,0124). One-way PERMANOVA, with pairwise test, also gave similar results, confirming the presence of a significant variation within GSA 17 C (Table 8).

Table 8. Results of pairwise test from the one-way PERMANOVA of $\delta^{13}C$ matrix

Groups	t	P(perm)
GSA 17 NIn, GSA 17 NOff	0,58	0,5693
GSA 17 COff, GSA 17 CIn	53,99	0,0004
GSA 18Off, GSA 18In	0,50	0,6272

Two-way PERMANOVA on the univariate matrix of $\delta^{15}N$ also confirmed the presence of significant differences in samples within the same area (Pseudo-F=3,20; P(perm)=0,0272), but the pairwise test on one-way PERMANOVA had different results from $\delta^{13}C$: both GSA 17 N and GSA 17 C showed a significant variation between inshore and offshore samples within the same sub-area (Table 9).

Table 9. Results of the pairwise test from the one-way PERMANOVA of $\delta^{15}N$ matrix

Groups	t	P(perm)
GSA 17 NIn, GSA 17 NOff	21,93	0,0396
GSA 17 COff, GSA 17 CIn	30,14	0,0067
GSA 18Off, GSA 18In	11,66	0,2515

CAP analysis was also performed to assess the distribution of samples according to inshore and offshore locations of each area (Figure 33).

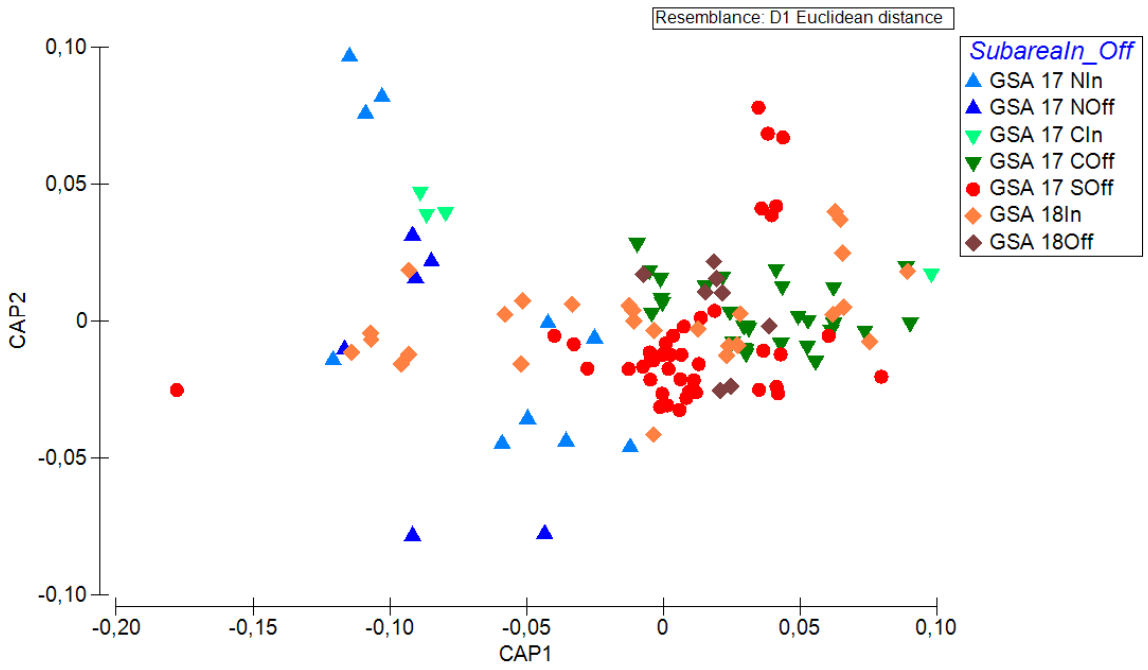

Figure 33. Result of CAP analysis on the multivariate matrix of $\delta^{13}C$ and $\delta^{15}N$

3.5 Stable isotope analyses on anchovy and sardine

A total of 189 samples were analysed for anchovies. $\delta^{15}N$ values ranged from 6.60‰ to 12.00‰, with a mean value of 9.12‰ ± 1.31‰, while $\delta^{13}C$ ranged from -20.92‰ to -18.07‰, with a mean value of -19.00‰ ± 0.51‰.

$\delta^{13}C$ and $\delta^{15}N$ data were plotted with fish length, to analyse ontogenetic variations in isotopic signatures (Figure 34).

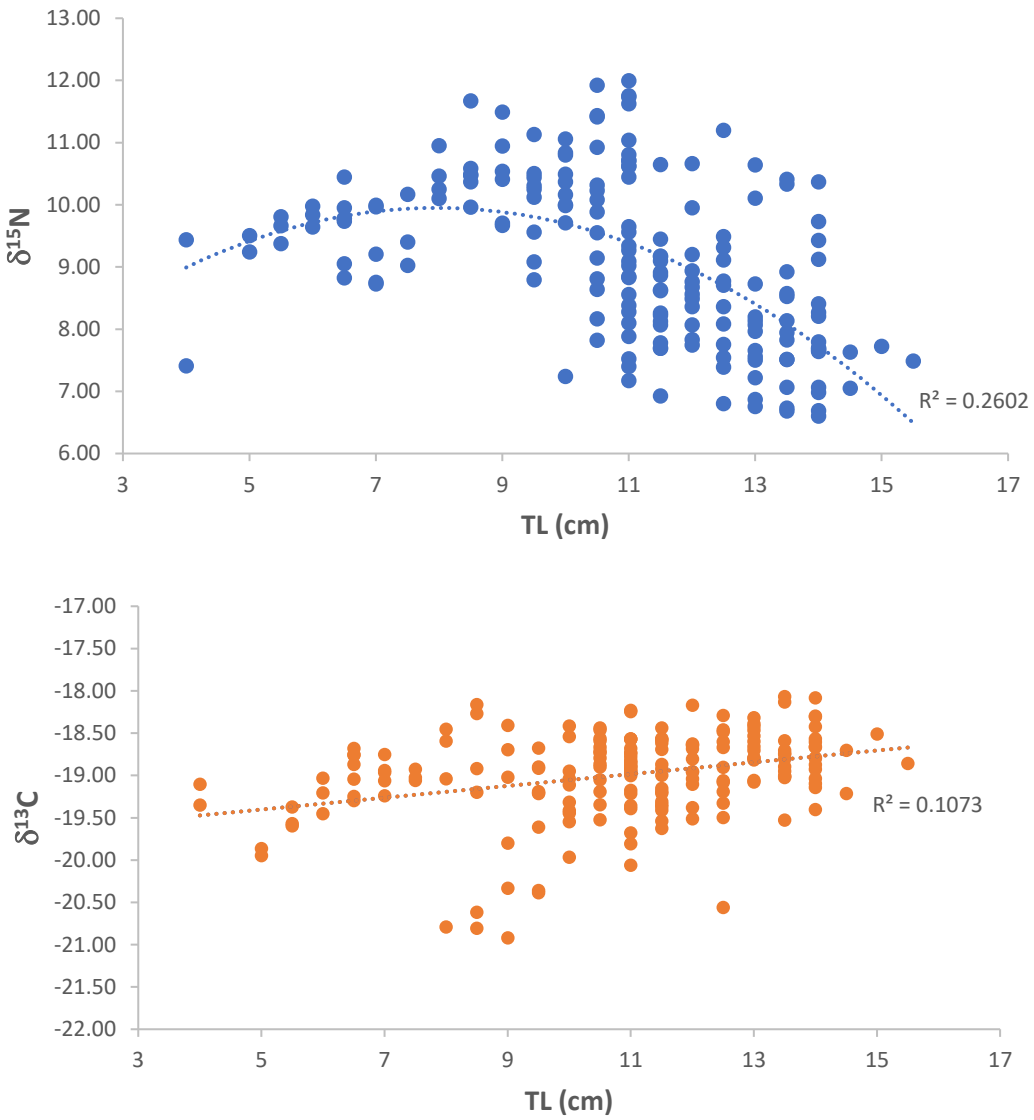

Figure 34. Scatterplots of $\delta^{15}N$ (top) and $\delta^{13}C$ (bottom) values related to fish length for anchovies in the whole study area

The scatterplot of $\delta^{15}N$ against fish size showed a peculiar trend, stronger in GSA 17 S, where $\delta^{15}N$ tended to increase up to about 9 cm, and then decrease with increasing length. Moreover, in GSA 17 N anchovies appeared to have a

higher $\delta^{15}N$ value when compared to fishes of the same size classes from other sub-areas (Figure 35).

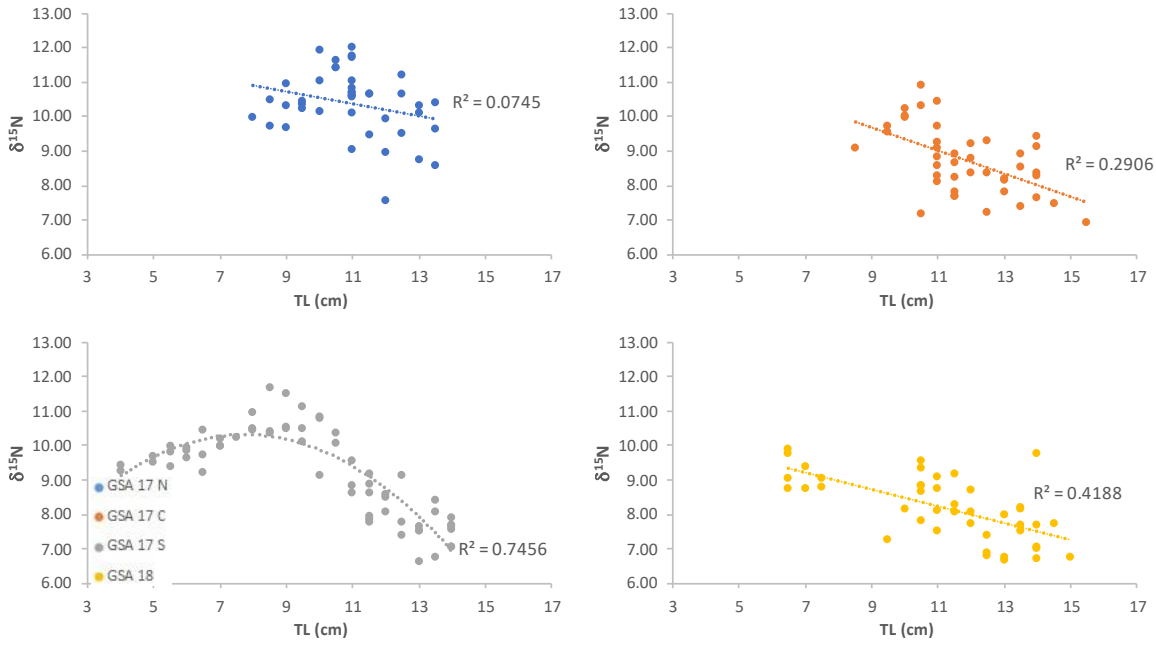

Figure 35. Scatterplot of $\delta^{15}N$ related to fish length for anchovies: top-left for GSA 17 N, top-right for GSA 17 C, bottom-left for GSA 17 S, bottom-right for GSA 18

The $\delta^{13}C$ scatterplot instead revealed a different pattern: $\delta^{13}C$ appeared to increase with size, especially in GSA 17 N (Figure 36).

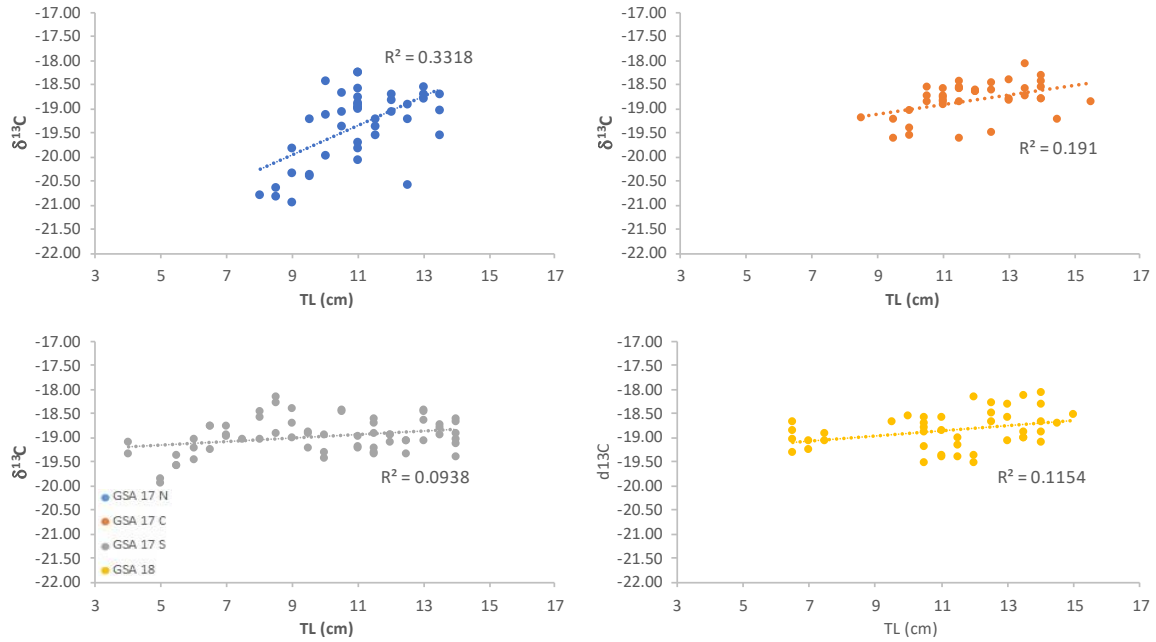

Figure 36.Scatterplot of $\delta^{13}C$ related to fish length for anchovies: top-left for GSA 17 N, top-right for GSA 17 C, bottom-left for GSA 17 S, bottom-right for GSA 18

A total of 138 samples were analysed for sardines. $\delta^{15}N$ values ranged from 6.31‰ to 10.94‰, with a mean value of 8.91‰ ± 0.94‰, while $\delta^{13}C$ ranged from -22.42‰ to -18.69‰, with a mean value of -19.86‰ ± 0.76‰.

Isotopic data were plotted against fish length to analyse the presence of ontogenetic shifts in fish diet (Figure 37).

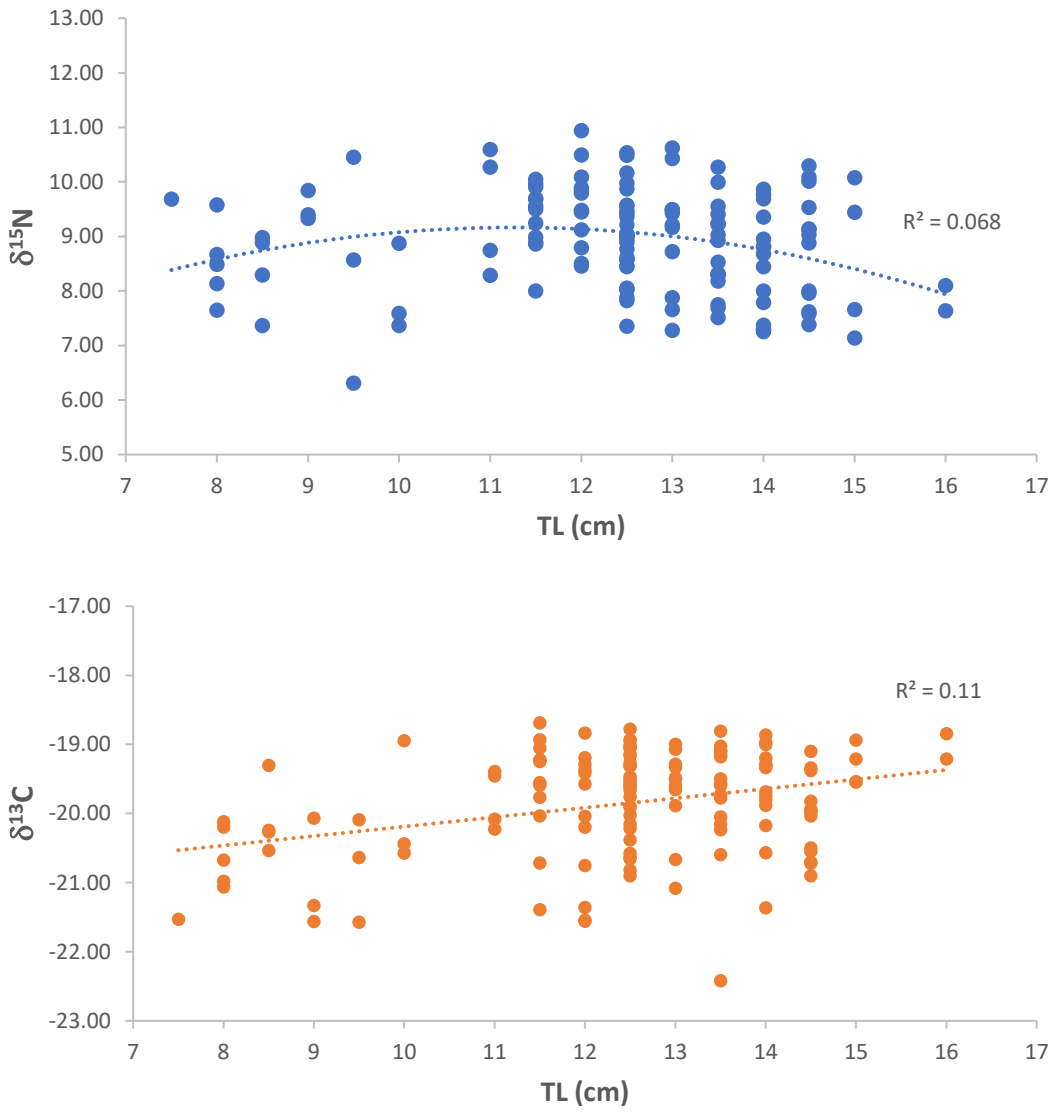

Figure 37. Scatterplots of $\delta^{15}N$ (top) and $\delta^{13}C$ (bottom) values related to fish length for sardines in the whole study area

The $\delta^{15}N$ scatterplot did not show a clear trend of nitrogen related to fish length, apart from a slight decrease in GSA 17 S (Figure 38).

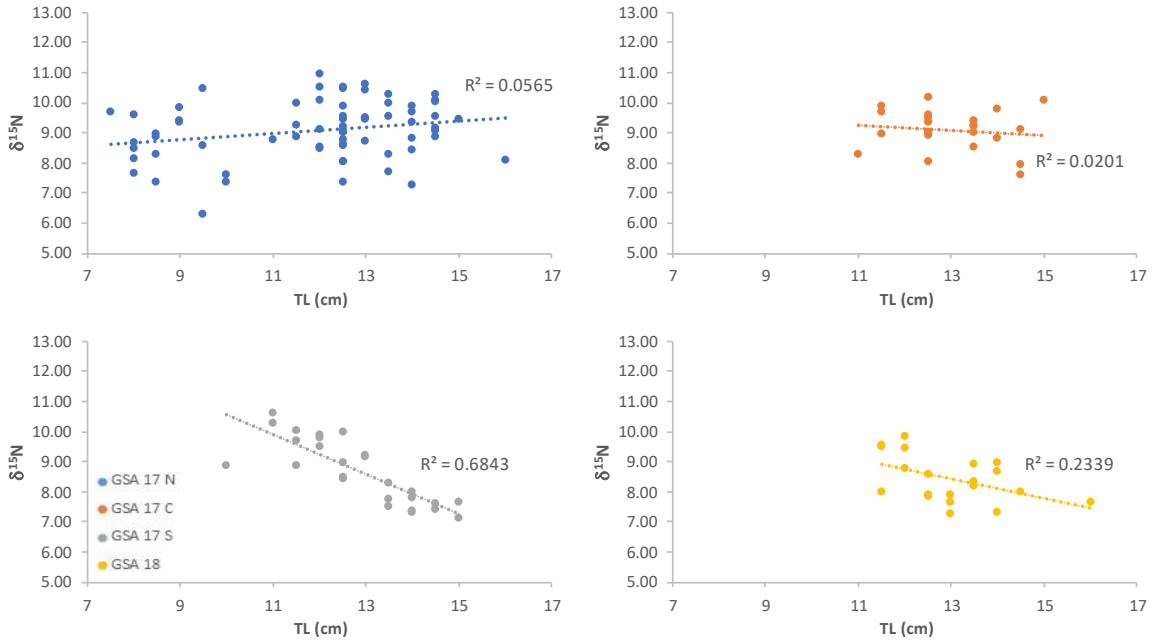

Figure 38. Scatterplot of $\delta^{15}N$ related to fish length for sardines: top-left for GSA 17 N, top-right for GSA 17 C, bottom-left for GSA 17 S, bottom-right for GSA 18

The $\delta^{13}C$ scatterplot also did not show a clear variation of carbon according to fish length (Figure 39).

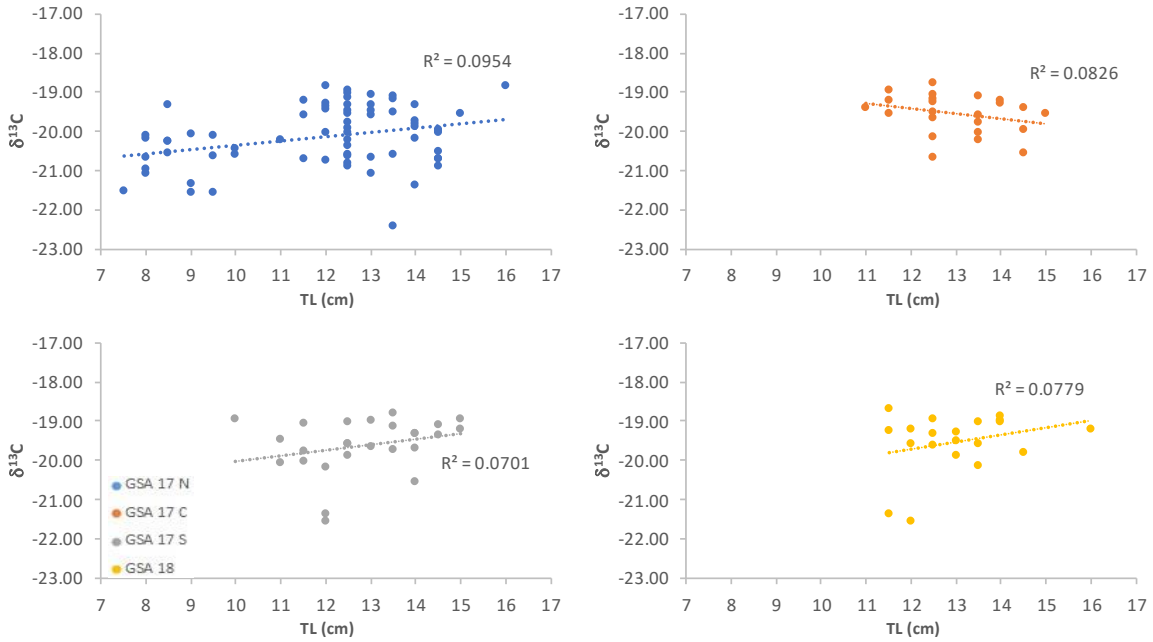

Figure 39.Scatterplot of $\delta^{13}C$ related to fish length for sardines: top-left for GSA 17 N, top-right for GSA 17 C, bottom-left for GSA 17 S, bottom-right for GSA 18

The scatterplot of $\delta^{13}C$ and $\delta^{15}N$ for both anchovies and sardines allowed to see a certain degree of separation in the isotopic signature of these species (Figure 40).

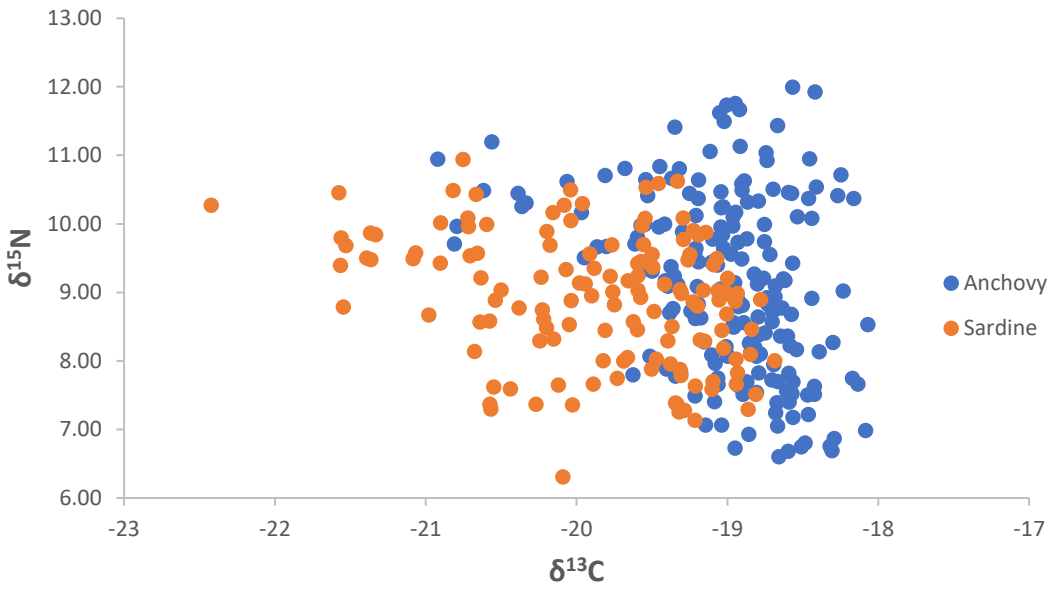

Figure 40. Scatterplot of $\delta^{13}C$ and $\delta^{15}N$ data of anchovy and sardine

At last, a final scatterplot was plotted, with $\delta^{13}C$ and $\delta^{15}N$ mean values of zooplankton, divided in 4 groups thanks to cluster analysis, isotopic values of anchovies and sardines and mean values $\delta^{13}C$ and $\delta^{15}N$ for large pelagic fishes and *Tursiops truncatus* as apex predator of the pelagic food web (Figure 41).

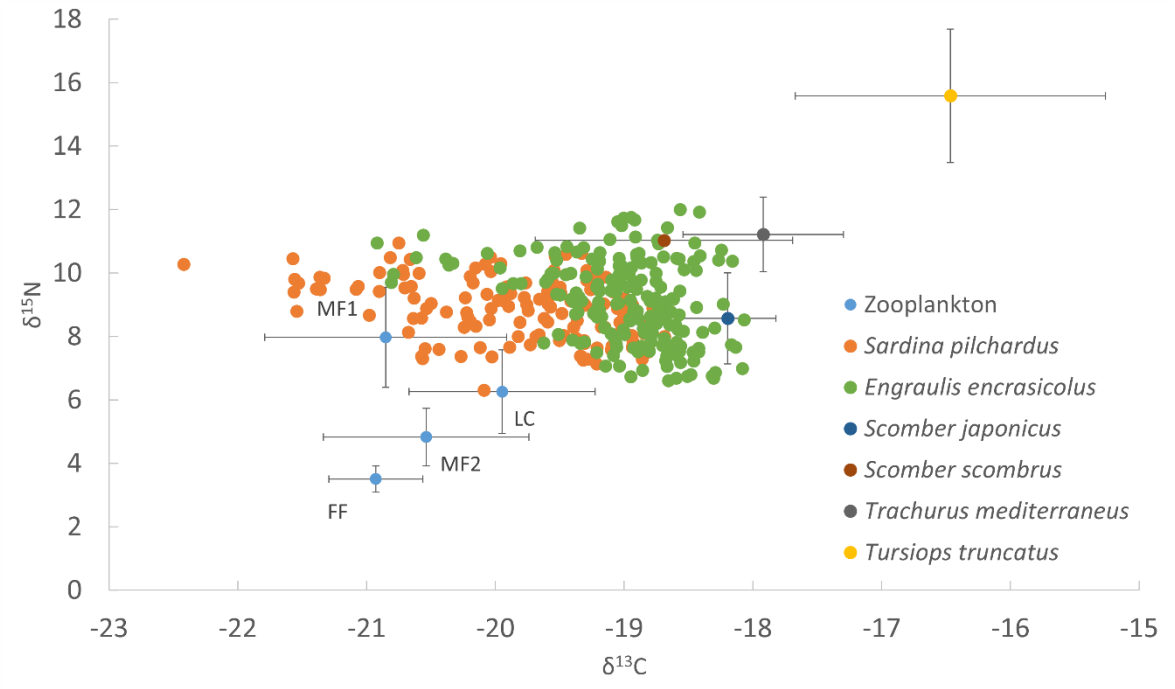

Figure 41. Scatterplot of isotopic data for zooplankton, sardine, anchovy, large pelagic fishes and Tursiops truncatus

Chapter four

DISCUSSION

4.1 Mesoscale variations in zooplankton biomass, abundance and composition

Zooplankton biomass and abundance were higher in GSA 17 N and slowly decreased moving towards Southern Adriatic (Figure 25). This trend was also observed by Fonda Umani (1996) and could be explained by the influence of Po River, which can determine a high nutrient input in the Northern Adriatic, that favours primary production and therefore zooplankton production. Indeed, in May 2019, the Northern Adriatic had a relatively high chlorophyll concentration just in front of the Po delta, that might have fuelled zooplankton production in the months of the survey (Figure 42).

Figure 42. Monthly averaged map of satellite-derived (Sensor MODIS Aqua) Chlorophyll a concentration (mg/m3) in May 2019.

Cluster analysis divided samples in three main clusters: station 22_17, located directly in front of the Po delta, GSA 17 N and GSA 17 C stations (with the exception of station 43_17, clustered with southern samples), and GSA 17 S and GSA 18. This division is consistent with the results of DistLM analysis, that showed how station 22_17 was highly influenced by the Po River, being characterised by low salinity and high fluorescence. GSA 17 N and GSA 17 C station are also similar due to similar environmental conditions, with high O_2 concentration and low salinity. GSA 17 S and GSA 18 were also clustered together, and their similarity is mostly due to high salinity and lower oxygen content. Environmental variables also explained similarity of station 43_17 to

southern samples. This similarity is probably related to its offshore location, that offers environmental condition similar to those of GSA 17 S and GSA 18. These results are supported by Fonda Umani (1996), that identified a clear distinction in zooplanktonic communities collected in offshore location of Northern and Central-Southern Adriatic: the Northern Adriatic is characterized by neritic communities, with moderate biomass, while the Central and the Southern Adriatic Sea are characterized by an "oceanic" community, with a higher presence of carnivorous zooplankton.

Such differences are also confirmed by the results of SIMPER analysis: even though similarity within sub-area is mainly driven by small unidentified copepods, *Acartia sp.* and *Podon sp.* characterized GSA 17 N and GSA 17 C, which are considered typically neritic genera (Fonda Umani, 1996), while GSA 17 S and GSA 18 are characterized by *Euchaeta sp.*, a more oceanic carnivorous genus (Razouls et al., 2021), and Chaetognatha, a phylum of carnivorous animals that can be found in both coastal and open waters (Terazaki, 2000).

4.2 Food web structure of zooplankton communities

The trophic groups highlighted by cluster analysis slightly differed from those obtained from literature in some cases: larger carnivores, like chaetognathans and calycophorans are grouped together, with zoeae of Penaeidae and

unidentified decapods. This group is also related to a first group of mixed feeders, with high $\delta^{15}N$ value. The copepod *Gaetanus tenuispinus* and Calanus-like group were sorted together with *Nannocalanus minor*, *Phronima atlantica* and *Pleuromamma abdominalis*, that showed the lowest $\delta^{15}N$ value, similar to that of filter feeders (Rumolo *et al.*, 2016). According to literature, *Phronima atlantica* should be a carnivorous species, feeding on salp tissue (Madin and Harbison, 1977). However, Elder and Seibel (2015) also reported feeding on host mucus, which could lower their trophic position, being more similar to the basal source, *i.e.* the particulate organic matter or POM (Fanelli et al., 2011). At last, the second group of mixed feeders encompassed both herbivore, omnivore and carnivorous taxa, which could be caused by some degree of trophic plasticity (Fanelli, Cartes and Papiol, 2011). Zoeae of Thalassinidea and Brachyura were also placed in this group, close to thaliaceans, that are herbivorous filter feeders (Madin, 1974).

Overall, stable isotope values of zooplankton did not show significant variations among sub-areas. However, $\delta^{13}C$ differed in inshore *vs.* offshore samples of GSA 17 C, while $\delta^{15}N$ did in inshore *vs.* offshore samples of GSA 17 N and GSA 17 C. The presence of differences in isotopic signature of zooplankton between inshore and offshore locations has already been reported by other authors (Bode, Carrera and Lens, 2003; Chouvelon *et al.*, 2014) and

it could be linked to the different contribution of terrestrial *vs.* marine sources of nitrogen and carbon moving from inshore to offshore waters, and/or to different trophic dynamics between costal and oceanic food webs.

4.3 Mesoscale variations in the trophic level and food source of small pelagic

The peculiar $\delta^{15}N$ trend observed in anchovy with size, where values increased with fish size, and then decreased, was also observed for sardine in Galicia (Bode, Carrera and Lens, 2003), where it was interpreted as a dietary shift to phytoplankton consumption in the larger fishes. Although anchovy is generally considered as a more zoophagous species than sardine (Borme, 2006; Chouvelon *et al.*, 2014), so this result is quite unexpected. This trend was observed in the co-generic *Engraulis capensis* in South Africa (King and Macleod, 1976), suggesting also for this species a shift from zooplankton to phytoplankton according to resource availability (Rumolo et al., 2016). Moreover, $\delta^{13}C$ followed an opposite trend, with increasing values with size, which could be determined by the usage of different feeding areas as fish size increases (Le Bourg *et al.*, 2015).

Sardine showed a decrease of $\delta^{15}N$ values with the increase of fish size in GSA 17 S, like anchovy, but there was not a clear variation of $\delta^{13}C$ which

could be interpreted as the absence of different feeding grounds for small and large fishes.

The combined $\delta^{15}N$ and $\delta^{13}C$ values of sardine and anchovy (Figure 40) showed how these fishes have a similar trophic position, as $\delta^{15}N$ values are similar, but there is some degree of separation in $\delta^{13}C$ values, meaning that the two species minimize dietary overlap by recurring to different carbon sources (Le Bourg *et al.*, 2015).

Finally, the combined scatterplot of $\delta^{15}N$ and $\delta^{13}C$ values for zooplankton, small and large pelagic fishes and dolphin (*i.e. Tursiops truncatus*) allowed to visualize the expected increase of $\delta^{15}N$ and $\delta^{13}C$ values with each trophic level, stressing the central role of small pelagic fishes in pelagic ecosystems, as mesopredators and top-down controllers, being located between zooplankton and larger predators.

Chapter five

CONCLUSIONS

This study represents the first application of the stable isotope approach to the analysis of the pelagic trophic web in the study area.

The results unveiled the presence of significant differences in zooplankton community and linked them to some environmental variables, evidencing a strong separation between offshore and inshore communities. Significant differences were also found in the isotopic composition of the zooplanktonic communities, although differences are more related to $\delta^{15}N$ or $\delta^{13}C$ and are possibly linked to the local oceanographic conditions of the sub-areas explored, together to the different contribution of terrestrial or marine food sources to inshore vs. offshore communities.

Moreover, differences in the isotopic signature of sardine and anchovy were also found, with trends more similar to those observed in oceanic areas (*i.e.* the Atlantic coasts) than in the southern Mediterranean. Further analysis on the potential variations at a larger time-interval of phyto- and zooplanktonic communities may help to better explain the driving factors of such differences.

At last, the analysis of the food web confirmed the importance of small pelagics as top-down controllers of pelagic food web. However further

analyses through Bayesian mixing models could link sardine and anchovy to specific prey groups, allowing to depict a more precise picture of the trophic web and explain trophic partitioning between the two species.

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