UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

PhD thesis

"Marine organisms model species for the assessment of biological, environmental and economic impacts on marine aquaculture in Campania"

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“First, think. Second, believe. Third, dream. And finally, dare.

If you can dream it, you can do it”

Walt Disney

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Acknowledgemetns

Thanks to my tutor, the doctor Francesco Paolo Patti. Thanks for the great opportunity that offered me, for being my "daddy in science" since the master thesis. Thank you for teaching me so many things and supporting me even when my creativity gained the upper hand…!

Thanks to the professor Aniello Anastasio, my university tutor.

Thanks to all the staff of the Stazione Zoologica Villa Dohrn-Benthic Ecology Center, in Ischia. You have all been a precious and fundamental presence, for sampling, for your technical help, for your friendship.

Thanks to the professor Yung-Che Tseng for hosting me in his beautiful Lab of Marine Organismic Physiology in Taiwan! Thanks for alla the facilities and the way we dealt with the topic, for all the different approaches we have used. Thanks for introducing me to the fascinating world of physiology. Thanks for the beautiful field trips in Matsu Island and in the south of Taiwan.

Thanks to Tzu Yen Liu for her crucial and precious support. Thanks for your friendship and assistance in anything.

Thanks to all the staff of the Lab of Marine Organismic Physiology, for all the technical and personal support. I always felt at home with you, thanks for your help, for your smiles. Thanks all for giving me one of the most beautiful experiences of my life! And thanks for your visit in Ischia and in Napoli, I'm so proud of it. What a beautiful memories!

Thanks to the doctor Alfonso Appierto, for its precious help for sampling at the Pozzuoli wholesale fish market.

Thanks to Cristoforo, owner of the mussel farm Elisea. Thank you for your collaboration, your help in sampling and your precious suggestions. A mix of passion and great technical skills… a model mussel farmer!

Thanks to the doctor Ciro Sbarra and all the staff of the ASL Napoli 2 Nord, in Bacoli, for their support in sampling.

Thanks to all my friends, the closer and the geographically distant ones. Life is absolutely better with good friends!

Thanks to my swimming friends, stroke after stroke they are always there. Thanks to be a fix and fundamental point in my life!

Finally, thanks to my unique lovely family, always present, always supporting me!

Abbreviations list

16S	large mitochondrial RNA subunit
28S	ribosomial large subunit
A.D.	Anno Domini
AAS	Atomic Absorption Spectroscopy
B.C.	Before Christ
CA	Carbonic Anhydrase
CE	European Community
COI	Cytochrome Oxidase subunit I
DNA	DeoxyriboNucleic Acid
DOC	Denominazione di Origine Controllata
DOP	Denominazione di Origine Protetta
EF1à	Elongation Factor 1à
EMA	Economically Motivated Adulterations
EU	European Union
FEAMP	Fondo Europeo per gli Affari Marittimi e la Pesca
HRM	High Resolution Melting
MPs	Micro Plastics
NHE8	Sodium 8 Hydrogen Exchanger
NKA	Na+-K+-ATPase alpha subunit
NPs	Nano Particles (NPs
OA	Ocean Acidification
PAHs	Polycyclic Aromatic Hydrocarbons
PAPM	Polyphenolic Adhesive Protein of Mussels
PCB	Poly Chlorinated Biphenyl
PCR	Polymerase Chain Reaction
PPCPs	Pharmaceuticals and Personal Care Products
PUFA	Poly Unsutered Fatty Acids
PVC	Polyvinyl Chloride
qRT PCR	quantitative Real Time Polymerase Chain Reaction
RNA	RiboNucleic Acid
SEM	Scanning Electron Microscope
STG	Specialità Tradizionali Garantite
UPLC	Ultra Performance Liquid Chromatography
WFD	Water Framework Directive

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Abstract

Campanian region has an important mussel farming tradition, since the Cuman and Greek domination around 700 A.C. through the Bourbons until today. Mussels and the linked supply chain were always been present in the socio-economic scenario of the regional coastal area. Today mussel production in Campania represents almost all the aquaculture total production, resulting one of the most productive Italian regions in this sector. In order to evaluate biological, environmental and economic impacts on marine aquaculture, mussels of the genus Mytilus were chosen as model species.

Mussels are perfect model organisms for their biological and physiological characteristics; they are bioengineering species, sedentary organisms, filter feeders, widespread all over the world, easy to keep in laboratories and important food source. Besides food source, thanks to their features mussels provide important ecosystem services as regulating the water column, supporting the food web and provisioning cultural services. Moreover, recently, are becoming source of important bioactive compounds, revealing to be organisms with a high potentiality in several applications.

Different kind of stressors, anthropogenic as well as natural stressors, can impact, negatively or positively, on mussels and consequently on the ecosystem services they provide. Aims of the thesis are the evaluation of the effects of three different stressors on mussels supply chain: food frauds, ocean acidification and the grafting operation.

Food frauds, the act of defrauding buyers of food and food ingredients for economic gain, can threaten the Campanian mussel supply chain. Unhealthy storage conditions, species substitutions, lack of label and informations on the product origin are all ordinary risks when buying mussels. The threats are both for human health than for the local economy of the honest mussel farmers. In order to investigate on the situation of the Campanian markets, *Mytilus galloprovincialis* mussels, the native species of the Mediterranean Sea, were sampled in different fish markets, local mussel farms and in their local natural environment. Mussels were genetically characterized in order to assess the species and their origins using the molecular markers COI, 16S, PAPM and 28S. The final aim was the attempt of the genetic identification of a local Campanian mussel, intended to officially recognize such local excellence product considering all its productions steps, from the seed recruitment to the marketing. Investigation on this topic has shown that Campanian local markets are not highly affected by the food frauds of species substitutions but is difficult

define the situation for the other type of fraud, the origin declaration. Just using simple molecular biology tools was not possible identify different mussel populations and define the genetic characterization of the local *Mytilus galloprovincialis* species. Further investigations are essential in order to identify a simple, fast and cheap method for mussels origin identification.

Mussels supply chain could be also affected by other kind of human impacts, not only in Campania region but on a global scale. Global changes can induce important vital alterations in aquatic systems, the increasing amounts of CO_2 in the oceans, known as the phenomenon of Ocean Acidification, affect our life via influencing the environment and our economy. Ocean acidification and other anthropogenic stressors can were already proved to cause negatively or positively changes on coastal dynamics, in marine organisms and consequently to the ecosystems services that they provide. Ocean acidification effects were tested on *Mytilus unguiculatus*, a pacific mussel widely bread in the Chinese Sea with similar anatomy and physiology to *M. galloprovincialis*. *Mytilus unguiculatus* has been exposed to different pH values (7.4 and 7.8) and then were analyzed the respective physiological parameters (O_2 consumption and NH_4^+ excretion), gene expression of NKA and NHE8 (both involved in the acid-base regulation mechanisms), free amino acids from mantle and gills with UPLC analysis, shell characteristics with AAS analysis, SEM pictures and X- ray testing. *Mytilus unguiculatus* easily survive in 7.8 pH conditions but further investigations (different life stages, longer exposure time, additional genes expression) are needed to better understand the effects of Ocean Acidification on this species and other species as the Mediterranean *Mytilus galloprovincialis*.

However an anthropogenic stressor for certain organisms could have a positive impact on the linked aquaculture economy. It is the case of the induced grafting operation on the pearl molluscs, in order to produce a pearl. Since the first decade of 1900 pearls production is an industrialized process, there are some bivalves of the pacific area widely used in this manufacturing production. Molluscs are able to produce pearls in response to a natural stressors (the famous grain of sand, a small piece of broken shell, a parasite or a small animal) causing an injury in the mantle of the damaged mollusc. In the “classical pearl molluscs” pearl production is human surgically induced, imitating what happens in nature and using a nucleus of mother-of-pearl. In a similar way the Campanian mussel *Mytilus galloprovincialis* could react producing a pearl too. The local

mussel could be a “new pearl molluscs” and pearls productions would be fully included in the well-established Campanian gemstone market. As for mussels farming activity, Campania region has an ancient coral manufacturing tradition, hosting in the southwestern coast of the Gulf of Naples the “the world’s capital of coral”.

In conclusion, mussels have been further appreciated as model organisms in different application fields; food frauds can affect negatively the Campanian market and new investigations are needed in order to evaluate their origin; mussels seem to easily survive to the predicted future acidified oceans; finally, mussels have great potentials, among their innovative applications they could become “new pearl molluscs”. In order to carry on and improve the millennial Campanian mussel farming tradition, it is crucial enhance and promote Campanian mussels as excellent local products, promoting new productions and encourage buyers to make a conscious choice, both for enhancing local mussel farmers than for preserving the environment for the future generations.

Introduction

I Aquaculture in Campania: from the origin to the current times

Aquaculture consists of aquatic animals and plants breeding, in fresh or brackish water or in the sea. The origin of aquaculture is attributed to early Chinese societies around 1000 B.C., the first records of keeping fish in captivity are found in the classic writing of the Chou Dinasty (1112 B.C. – 221 B.C.). The origin of domestication of fish may have no more than a practical expedient of having fresh food ready on the hand each day or for an ornamental use, is the case of the common carp that became a noble symbol (Nash, 2010). Lakes and sheltered docks were very useful places on which store and wait that fishes or other animals grow up, particularly during long time of bad weather conditions when fishing was not possible. In Italy, the first evidence of the use of this kind of natural or sometimes manmade structures dates back to the III century B.C., the latin author Plauto defined such natural or building pool "piscinae" or "vivarium" (Giacopini et al, 1994). There were different kind of piscinae, with hard or soft bottom, in which were bread different kind of fishes: the muddy pools were useful for flat fishes, like sole or turbot, for molluscs like murex and oysters; the sandy or rocky pools were perfect for dentex, bream and moray (Malossini, 2011). Generally, the pool for marine species were built close to the coast. In each kind of pool the seawater could enter freely and flow through lateral channels closed by bronze grates with small holes, in this way the water was not stagnant and the temperature was stable (Malossini F., 2011).

In the ancient Rome, not only fishes but also molluscs were appreciated, especially oysters. In 108 B.C. the business man Sergio Orata start to bread oysters in his house, the activity was source of good income so he moved soon his oyster farm in Lucrino Lake, near Pozzuoli. Oysters were particularly appreciated among the high social classes, were considered the "food of the owner" (De Grossi Mazzorin, 2008). For this reason there was a high demand of oyster at the market and the production was very good even the name of the lake derives from the adjectives lucrum that means profit, for its activity Sergio Orata became one of the richest men in the Roman Empire. The breeding system was probably the pergolaro consisted of some poles fixed on the bottom and ropes between them, at the ropes with other small lines were fixed the oysters. This system was still used in the Fusaro Lake, close the Lucrino Lake, in the XIX century (De Grossi Mazzorin, 2008). In Lucrino Lake and generally in the Phlegrean area, oysters production continued even in the Middle

Ages until 1538 when a strong volcanic eruption occurred changing drastically the landscape. Under the Bourbons reign, around 1750, the area was reclaimed. In the Phlegrean lakes oysters were raised again, they seem to have been imported from Taranto farms. Oyster farms were then replaced by mussel farms. The tradition of mollusc farming is well established in the Phlegrean area that even praises one of the major national purification plant for molluscs, the IRSVEM.

As regard as the history of mussel farming, unlike oysters, there are not many testimonies in Italy although today are the main Italian aquaculture product. The origin of mussel farm is attributed to an English sailor who shipwrecked on the French coasts in 1235 and hunted seabirds to survive. He used poles and a net between them, on the submerged part of the poles had taken root a lot of mussel. In such simple way, the mussel breeding began spreading throughout France and later in other European countries, in Italy too (De Grossi Mazzorin, 2008). First records of mussel farm in Italy go back to the XVI century, in Taranto in the south east of the peninsula. In this area, the production of mussels grew incessantly in the following centuries (Farella et al. 2011) and is still very productive today. Mussel farming in Campania was introduced with mussels and technicians from Taranto. The industrial mussel farming began in the 1920s, it developed slowly transferring the culture from lakes and protected areas to the open sea, improving the breeding techniques. Mussel farming increased considerably after the Second World War, it only fell sharply in the 1970s due to a cholera epidemic. In 1973 in Naples a great cholera epidemic broke out, the biggest defendants were the mussels for this reason mussel industry collapsed. Mussel farmers and politicians defended their mussels, a strong economical value was linked to mussel supply chain. In Naples people was shocked by this problem, even a song was written on this topic, "*A cozzeca*", of the Neapolitan singer De Piscopo in 1976. He wrote about a mussel asking for help because had to defend itself from the judges of the sea. The mussels said it has no fault if Naples was experiencing great health problems, the guilty were those who polluted the sea. In the following years the mussel farming economy recovered and the Campania region is today one the main Italian mussels producer.

II Aquaculture today: global and European trend

Aquatic food has always been an important source of food for humans from prehistory to contemporary age. This kind of food has a very important nutritional value. Although hard scientific proof on the nutritional benefits of aquatic food consumption is only of recent date, anecdotal evidence and observations were present also in historic times. In the 13th century, there were an interesting correlation between the longevity of the Knights Templars and their diet. Templars lived far longer compared to other people at the time, such longevity would be attributed to a special divine gift; today science attributes it to a balanced and nutritious diet, a large intake of fish, vegetables and fruit as well as strict hygienic precautions (FAO, 2017). Fish and more generally aquatic food today are considered natural superfood. Fish, shellfish and algae are all rich of proteins, healthy long-chain fatty acids, fat-soluble vitamins, minerals like iron, calcium, iodine, zinc and selenium. Seafood consumption has numerous health benefits: reduced risk of cardiac death, sustains the neurodevelopment in unborn infants, reduces probably the risk of stroke and induces a possible decrease of the risk of depression (FAO, 2016).

Today aquatic foods consumption is around 20 kg per capita, according to the increase of the global population in order to maintain at least the current consumption level, the world will require an additional 23 million tons thereof by the 2020. This additional request will have to provide by the aquaculture (FAO, 2016). Aquaculture shows a constantly world growing trend over the years, in the figure I.1 is clear that just in 15 years, from 1990 to 2015, not only finfishes but also aquatic plants, crustaceans and molluscs are increasingly present in the farms.

The biggest aquaculture producer, in term of volume, is China, with around 60% of the world aquaculture production, European Union is at the 8th position (EU Maritime affairs and Fisheries, 2016). As regard as the consumption of aquatic food in Europe, according to the EU Consumer Habits report (2016), amounts to 25,8 kg per capita, a value little bit higher than the average of the global population (FAO, 2016). The main species bread in EU are molluscs and crustaceans, they represented togheter the 43,6 % of the total EU aquacolture production; the 34,6% are freshwater fishes and the 21,8% are marine fishes (figure I.2 – EUMOFA). Shellfish are today much appreciated as well as in ancient times. The per capita shellfish consumption in 2013 was 4.9 kg, subdivided into 1.8 kg of crustaceans, 0.5 kg of cephalopods, and 2.6 kg of other mollusks. Shellfish

have a great economical value: in terms of quantity they amounted to 38% of total seafood traded in 2013, in terms of value recovered, its contribution was 63.7% (FAO 2016; Venugopal et al. 2017).

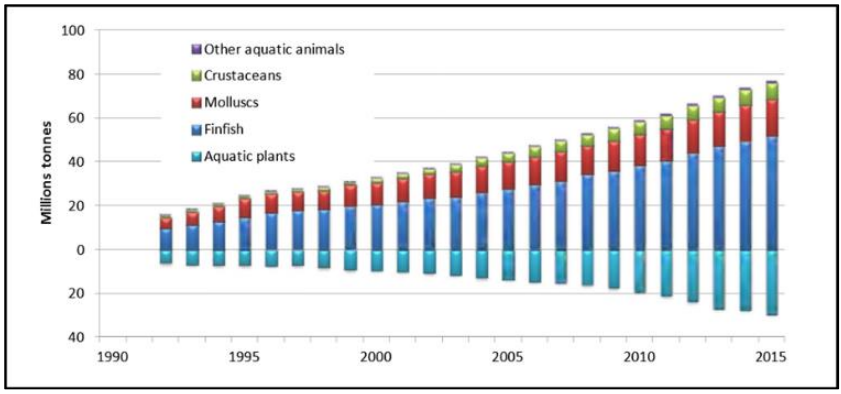

Fig. I.1 World aquaculture production of farmed aquatic animals and plants (1990-2015) (FAO, 2017).

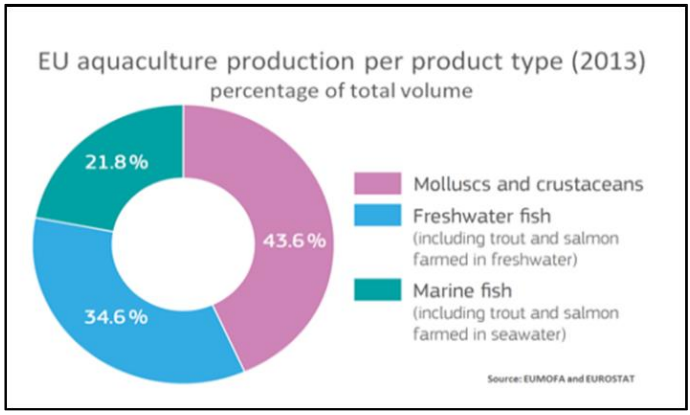

Fig. I.2 The 43,6 % of the total EU aquaculture production is constituted by molluscs and crustaceans (EUMOFA, 2016).

The shellfish species consumed in Europe are mainly oysters, mussels, king scallops, lobsters, winkles, whelks, cockles, clams, crab, and others (Venugopal et al., 2017). The major commercial bivalve species include the soft-shelled as well as hard-shelled clams, blue mussel, eastern oyster, and sea scallop. As regard as the nutritional value of shellfish, they

have higher protein contents than finfish. The reported average protein contents (g/100 g raw meat) of various shellfish vary, for example for mussels 12.6 to 13.0, for oyster 8.9 to 14.3 (USDA, 2012).

Considering the total production of molluscs and crustaceous, the main bread species are mussels. During the years 2008-2014 around 350,000 tons of mussels were been produced in Europe (figure I.3-EUMOFA), the consumption of marine mussels has increased steadily over the past decades (Grienke and others 2014).

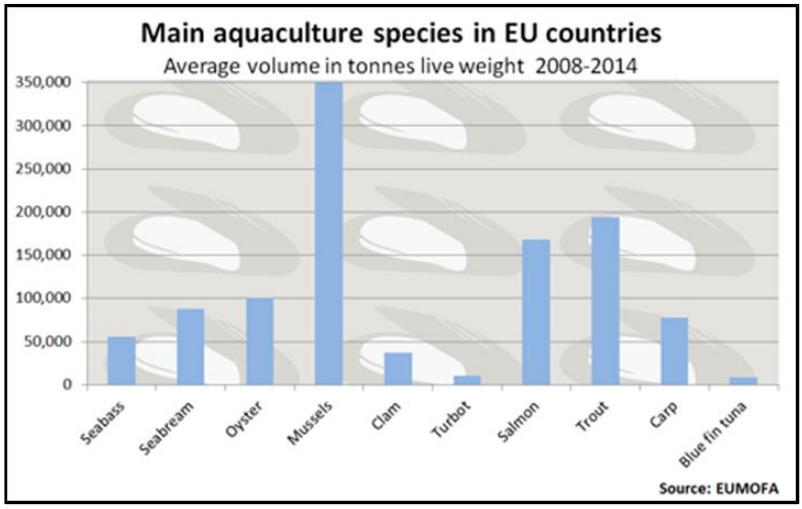

Fig. I.3 In Europe mussels are the main aquaculture species (EUMOFA, 2016).

III Aquaculture in Italy

Italy is the fourth European country for aquaculture production, contributing for the 13% of the total volume of the EU aquaculture products. Aquaculture companies are around 800 and produce 140.000 tons/year of fresh products (FEAMP Campania 2014-2020). Despite are bred around 30 species of aquatic organisms, the 97% of the Italian production is based on a few species: trout, sea bream, sea bass, mussels, clams and oysters (FEAMP Campania 2014-2020). Rather than fish culture, shellfish culture is the leading production item for Italian aquaculture. The main products are mussels (*Mytilus galloprovincialis*), Philippines clams (*Tapes philippinarum*) and small quantities of clams (*Tapes decussatus*) and oysters (*Crassostrea gigas* and *Ostrea edulis*). The companies involved in mussel or oyster farms are around 200 (Prioli, 2008), sometimes such companies associate their activity to other kind of aquaculture activities involved different species of molluscs or fishes too. The most productive regions are Puglia, Emilia Romagna, Veneto, Sardegna, Marche and Campania (Prioli, 2008).

IV Aquaculture in Campania

Mussel farming is the most important aquaculture industry in Italy. In numerical terms (number of companies and number of plants) it covers the 90% of the regional aquaculture production and about 100% in terms of production. There are 25 companies, generally small cooperatives, involved in this sector that have 37 plants for a total area of 800 ha. The 81% of mussels farms are in the Gulf of Pozzuoli, along the coasts of Baia, Capo Miseno, Arco Felice, Mount of Procida, the other 19% are along Domitio coast, Naples and Vesuvian coast. Campanian mussel farms distribution is shown in the map in figure I.4.

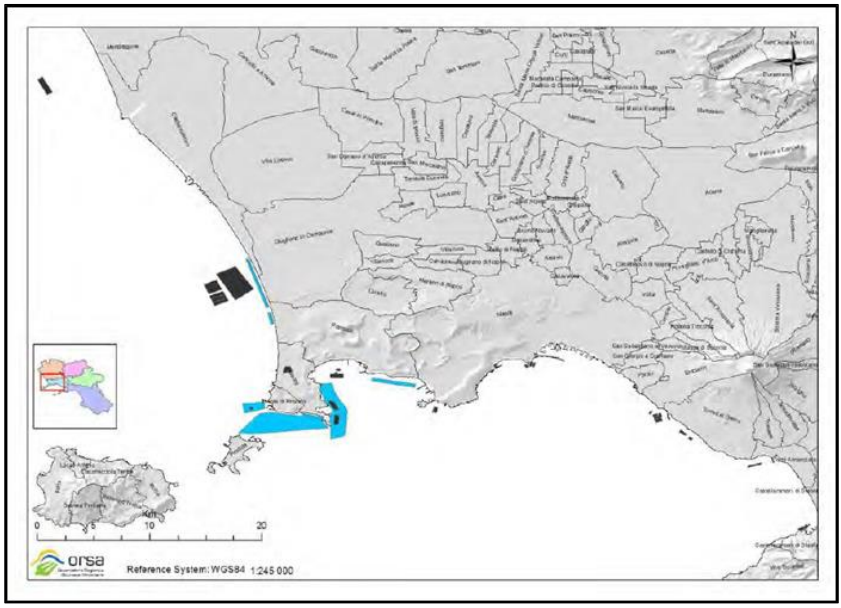

Fig. I.4 Mussels farms distribution in Campania (ORSA, 2015).

The chain of mussel farming in Campania is made up also of 50 shipping centers and 12 purification centers; one of the most famous is the IRSVEM, founded in 1980 after the law 192/1977 about the hygienic and sanitary standards for the production, trade and sale of edible lamellibranch molluscs. Finally, the total economic value of the entire mussel farm sector in Campania is around 5 million euro, with around 80 permanent employees. Although the sector is characterized by a good

production with a good stability during the years, it could better express its potential if it were organized in a better way both from a structural and from a managerial point of view (FEAMP Campania 2014-2020).

V Mussel farms: breeding systems

In Italy there are three different breeding systems for mussels: a fixed system, the monoventia longline and the Trieste longline. The first system is the oldest one, is generally used in lagoon or repaired areas. After technological improvements, the system of monoventia longline farming has become widespread and has replaces in many cases the fixed system. Most of this kind of system has appeared for about 25 years ago, but they are now the strong point of Italian mussel farming (Prioli, 2008).

For the fixed system, mussel seed were collected in nature and then it is left to grow until the minimum commercial size is reached in a prepared protected area. Mussels could stay on the bottom or fixed in some poles. The poles were made of chestnut wood, easy to work, or, in modern time, of cement or metal. Among the poles there are some ropes to which socks with mussels are hung. The sock, made generally of polypropylene, together with the mussels inside constitute an awn.

The longline system is composed of two dead anchor bodies, arranged at a variable distance from 100 to 200 meters. They are linked with one or two ropes and are kept suspended by some sequentially big floats. To the ropes are hung the awns of mussels spaced from one meter away, the socks could be long from 2 to 4 meters with a different mesh size based on the age of the mussels inside it. The entire equipment of the longline (anchor bodies, ropes, float and awns) is repeated for the whole mussel farm area. If there is one suspended rope the breeding system is called monoventia longline, if there are two or three ropes is called bi/tri ventia longline (or Trieste longline). The monoventia longline is generally used in most exposed areas and the rope is kept at a depth ranging from two to five meters, this kind of longline system is used in mussel farms along Campania coasts. The sea bottom for this kind of system ranges generally from 10 to 30 m, with calm hydrodynamic conditions besides a high trophic level.

Another essential element in a longline mussel farm is the boat, especially set up for this kind of activity. On the boat there are specific tools: to pull up the awns, to change the socks with different meshes when the mussels grow up, to pack the finished product. The first step to raising mussels is the seed collection. Young mussels of 2-3 cm constitute the seed. They could be collected in natural places or in the breeding units or could be purchased from some companies specialized in seed production. The seed is put into a first sock with small meshes; this operation is

performed manually with a polypropylene tube. The socks with the seeds are tied up to the ropes and are left to grow for 2 or 3 months. After this time, mussels are pulled up and placed in a new sock with bigger meshes. This operation is repeated at least another time, during these operations mussels were also separated by size with an electric or manual rake in order to identify different sizes to put it again in different socks. The real growth phase lasts from 8 to 12 months, during this phase sometimes the socks must be cleaned for the fouling, an important threat for mussels because it can cause annoyances in breeding and feeding, causing even the death of the animals, as well as increase the weight of the sock. To remove the fouling, the socks are pulled up on the boat and pressure washer is used. Generally, the boats have a mechanical tape to pull the socks from the water. With the same tape, when mussels are ready to be commercialized, they are not only taken out from the water but also transported to the mechanical rake. The rake can separate them by the size and, thanks to its shaker movement, can also separate mussels between them as in the sock they are naturally all attached to each other. Finally, mussels are now separated by size, there are small mussels (the seed) and young mussels, both of them will be put again in a sock, with different mesh for each different size, and returned to the sea until the adult size is reached. As regard as the adult mussels, if they are bred in good water quality (water of type A, according to the law 192/1977) they could be directly commercialized. They are packaged and labeled immediately on the boat, in different packages generally from 3 kg to 20 kg. If mussels came from worse water quality must be sent to a purification center and then they can be marketed.

VI Mussel farms threats and future perspectives for the Campanian industries

The mussel farming sector is constantly evolving and more attention is given to environmental issues in the last few years. One of the environmental impacts of mussel farming is the use of polypropylene nets, often accidentally released into the sea and found along the coast. To overcome this problem and in order to have sustainable companies, many mussels farmers started using biodegradable socks in recent years.

Technological advances are not just towards greater attention to the environment but they have also the aim to increase the productivity. For example, there are some experimental breeding of mussel and oysters together in the same factory. Oyster farming was dropped out in the past for the high production costs related to the labor. Today, thanks to new equipments, it is possible to reduce such costs particularly for the most delicate part of the procedure: the seed production. Sometimes it is possible use the same equipments of the mussel industry. This is the case of a mussel farm in Taranto that has expanded its production to the oysters. They have noticed that oysters lay down the seed on the shells of dead mussels, on the bottom. A specific machine collects the shells with the seed and with the same rake used for mussels size selection, the seed is put aside and then implanted.

Aquaculture also has some threats, first of all the water quality. Mussels are animals with high plasticity, they can adapt to rapid environmental changes, they even can survive at extreme conditions but they remain still filtering animals and fixed to a substrate. Therefore, a mussel farm area need a good water quality, without the presence of hydrocarbons and other pollutants, with fair nutrient supply and good hydrodynamic conditions.

Another notable threat for mussel market are the food frauds. Aquatic food is identified as one of the highest risk category of foods with the potential for frauds (FAO, 2018). Most frequently, food frauds can occur by substitution of species. Due to the shellfishes plasticity, sometimes is not possible strictly identify a mussel or an oyster by the morphology of their shells (Cubillo et al., 2012; Beadman et al., 2003). Moreover, they are often commercialized without shell, frozen or canned, in this case is almost impossible certainty identify a species based on its morphological characteristics. Food fraud also means the false declaration about the origin of a given species. Sometimes mussels and oysters are

passed off for local products but they came from another geographical region. The frauds as substitutions of a species can be very dangerous even from a health point of view, in the second case, when the frauds are about the origins of the products, the risk is particularly for the local business. At the Campanian fish market imported mussels are easy to find, there is a constant request throughout the year that local production cannot sustain. Initially such imports were mainly concentrated during the winter period, when the regional production as the national one is not active on the internal market; but currently imported products cover the entire annual period. Spain and Greece are two main countries involved in this business (FEAM Campania 2014-2020). Mussels as well as all the foodstuffs must be traceable, but every year tons of mussels are confiscated for mislabel or the total absence of a label. Not only adult mussels are imported (legally or illegally) from other countries but also young mussels, particularly some mussel farms take the seed from others geographical areas, from different Italian regions and/or from others European countries. In this way is getting hard to have a local product, completely produced in a specific region. In some situations, threats of this kind can however become strenghts. In recent years, there has been a growing focus on the production and enhancement of local products in the food farming economy. In Italy there are two examples of enhancement of the local product in the mussels production chain: the *Scardovari* mussel and the *Nieddittas* mussels. The *Scardovari* mussel belongs to the native Italian species *Mytilus galloprovincialis*; Scardovari is a geographical area located on the delta of the river Pò on the Adriatic Sea. Since 2013 this mussel is the first Italian mollusc certify as DOP (Designation of Protected Origin) product from the European Union Commission (figure I.5). The DOP certification was obtained for the entire production process and for the typical environmental characteristics of the place; both factors guarantee a unique product. The *Nieddittas* mussel belong to *Mytilus galloprovincialis* species too, they are bred in Sardinia in the Gulf of Oristano (figure I.5). Since 2013, the mussels and the whole supply chain have obtained a product certification.

As well as for the two certified mussels *Scardovari* and *Nieddittas*, in Campania it could be possible identify by the same way a certified product. Some mussel farms of the Phlegrean area can produce the seed, so mussels bred in that area are indigenous and the completely productive process would be fully Phlegrean or at least Campanian. A Phlegrean mussel it would be an excellent incentive to the local mussel farming

industry that is already well established, but as reported by FEAMP Campania this sector has much greater potential. According to the guidelines of FEAMP Campania for the years 2014-2020, an operational program will be implemented to support the development of aquaculture and fisheries. Campania Region have allocated 73 million euro in order to increase the competitiveness of the aquaculture sector; promote innovation, knowledge and its dissemination; expand the production offer to increase their added value; improve planning and governance for the use of the coastline; investing in professional training and operators lifelong learning; strengthen the collaborations with research centers; develop new businesses to foster new levels of employment; strengthen the regional aquaculture centers. The identification of a certified local product like a Phlegrean DOP mussel would entirely hits the FEAM Campania 2014-2002 objectives.

Fig. I.5 Logos of the DOP Scardovari mussel and the Nieddittas mussel.

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Chapter 1

Mussels as model organisms

1.1 Model organisms

The term “model organism”, despite being known for a long time in the history of science, has become ubiquitous in contemporary biological discourse in the past fifteen years, particularly since the advent of the largescale genomic sequencing (Gelfert, Ankeny, and Leonelli 2011). A model organism is a non-human species that is easily used in the laboratory in order to understand biological processes. To be elected as a model, an organism must generally have certain characteristics: it must be easy to maintain and reproduce in the laboratory, it can breed in large numbers, it must have a short life cycle, small dimensions and it must already be well studied with known knowledge about its biological, physiological and genetic characteristics. Obviously a model organism cannot be universal, the choice of a model depends on the target topic. For example, in medical or pharmaceutical studies generally related to human health, the best choice will be an animal as similar as possible to human species; in ecological or toxicological studies could be better other organisms easy to find in the environment of interest, in order to identify not only a model species but also a bio-indicator.

The first model organism in the science history could be *Pisum sativum*, the peas used in Mendel studies. The priest biologist Gregor Mendel developed the model of heredity that now bears his name by experiments on various characteristics of pea plants: height (tall vs. short); seed color (yellow vs. green); seat coat (smooth vs. wrinkled). Peas were easy to obtain, to raise in the convent garden and have a simple reproductive system, so he easily made the cross among different dominant and non-dominant character (Snustad, 2014). Among the model organisms can be found all the living form: viruses, bacteria, yeasts, plants and animals. In viruses the most used are bacteriophage; because of their ability to infect bacteria they are very useful to study the DNA structure, replications and mutations. In prokaryotes, the ubiquitously bacterium *Escherichia coli* is one of the most used model. In the eukaryotes several models are used: the yeast *Saccharomyces cerevisiae*, a unicellular organism that can reproduce asexually or sexually; the plant *Arabidopsis thaliana*, its genome has only five chromosomes so it is perfect to be a model for genome structure and higher plants development. Few chromosomes, only four, are also in the genome of the famous fruit fly *Drosophila melanogaster*. In 1933 the geneticist and biologist Thomas H. Morgan won the Nobel in Physiology or Medicin for its experiments on

the fruit fly with which he demonstrated that the genes are in the chromosomes. Finally, probably the most famous model organism is the mouse *Mus musculus*, the more easy and small animal to raise in laboratory closer to human.

In recent years, aquatic organisms are also used as model in many scientific publications. The fish *Danio rerio*, commonly zebrafish, is one of the most used vertebrate model for genetic and developmental biology; there is also a web based community resource, the Zebrafish Information Network (ZFIN) (http://zfin org), that implements the curation of zebrafish genetic, genomic and developmental data (Sprague et al., 2006). Zebrafish has a high reproduction rate, produce a big number of transparent embryos developing in the water outside the body of the mother and the arrangement and the function of internal organs are comparable with human ones. Moreover this model organism is particularly used for transgenesi experiments as model for cancer studies. Other important aquatic model organism are the algae, both microalgae and macro algae. Microalgae like Diatoms, unicellular eukaryotes abundant in aquatic environments, and the genus Volvox, unicellular algae but able to form spherical colonies, are both good candidate to be model organisms (Russel et al., 2017). Among macro algae *Ectocarpus siliculosus* is a good model organism for the genetics and genomics of brown algae, as it has a small nuclear genome size, sexuality, and a short life cycle (Peters et al., 2004). The red algae genus Phorphyra is another good algal model, besides is one of the most valuable marine crops in the world (Saga, 2002). Other benthic animals recently used as model organisms are the sea urchin; different species of sea urchin at larval or adult stages are used as models in environmental studies, linked to the ocean acidification (Zahn et al., 2016; Stumpp et al., 2011; Havenahand et al., 2008;) and toxicological studies, as the bioaccumulation of heavy metal (Pagano et al., 2017; Morroni et al., 2016). The increasing use of the sea urchin as a model species has led to the production of a database, SpBase (http://spbase.org/), focused on the genomic information from sea urchins and related echinoderms (Cameron et al., 2008).

1.2 Bivalves as model organism

The phylum Mollusca is one of the largest, most diverse and important group in the animal kingdom, with at least 50.000 described species and probably as many as 200.000 living species, most of which are marine (Gosling, 2015). Eight classes of molluscs are recognized. After the class of Gastropoda, Bivalvia is the second largest class with about 9200 species (Huber 2010). Over evolutionary time, they have become flattened side to side. Extant bivalves are an important component of marine and freshwater ecosystems, with more than 80% of species living in ocean habitats, and exhibiting varied ecologies beyond that they form the basis of valuable fisheries and aquaculture industries worldwide as their protein richness (Gosling, 2015).

Bivalves are shelled molluscs, the two shell valves are hinged dorsally and are secreted by two mantle lobes that cover the body organs. Adductor muscles hold the valves together, and relaxation of the ligament and contraction of these muscles open and close the shell, respectively. A series of interlocking teeth and sockets along the hinge line prevent the valves from sliding against one another. The shell, mainly composed of calcium carbonate, has several functions: it acts as a skeleton for the attachment of muscles, it protects against predators, and in burrowing species it helps to keep mud and sand out of the mantle cavity. Sessile epifaunal bivalves, such as oysters and mussels, attach themselves to hard surfaces using cement or byssal threads. Although some bivalves are deposit feeders, the majority use greatly enlarged gill surfaces to filter food particles from the surrounding water. However, because of their mode of feeding they pump large volumes of water and thus have the potential to accumulate contaminants, bacteria, viruses and toxins, frequently posing significant public health risks (Gosling, 2015).

Bivalves by their nature of benthic organisms, sessile and filter feeders are perfect model organisms to investigate the effects of pollutants and nanoparticles. According to Phillips (1977), a model organisms or indicator: should accumulate the pollutant without being killed by the levels encountered; should be sedentary in order to be representative of the area of collection; should be abundant in the study region; should be of reasonable size, giving adequate tissue for analysis; should be easy to sample and hardy enough to survive in the laboratory; should tolerate brackish water. Another notable bivalve characteristic is their longevity, they are within the long-lived animal species reaching more than 150

years, including turtles, tortoises, whales, fish and sea urchins (Philippe and Abele, 2010). The longevity allows them to be good bio-indicators over time and to be a candidate model in ageing research in order to clarify genetically if the extreme longevity is a programmed mechanism and which are the involved processes. Furthermore, for the most common species the anatomy, physiology and the genome is well known so bivalves are good model organisms for comparative studies too. In the last few years molluscs have become emerging animal model for human cancer, particularly bivalves (Walker et al., 2011; De Vico and Carella, 2015). Following the interest for bivalves in different field of studies, investigations on bivalve genomics has emerged during the last twenty years; particularly oysters (*Crassostrea sp*.), bay scallops (*Argopecten irradians*) and mussels (*Mytilus sp.*) dominate DNA databases (Saavedra and Bachère, 2006). In addition to the genome, molluscs proteome is also causing interest in recent years as integrative omic studies constitute a powerful tool in addressing the links between environmental conditions, harmful effects and associated responses in marine bivalves (Campo et al., 2012; Suàrez-Ulloa et al., 2013).

In the field of environmental research, molluscs have always been great protagonists. Since 1960s, bivalves and other molluscs have been used for biological monitoring of environmental water quality (Butler, 1966; Coughtrey and Martin, 1977; Phillips, 1977; Burns and Smith, 1981). Monitoring of polluted water by heavy metals, pesticides and hydrocarbons is still today a very important question and molluscs are the best models for these studies (Burioli et al., 2017; Cariou et al., 2017; Breitwieser et al., 2017; Faggio et al., 2018). Moreover biomonitoring programs are now very important, they are essential for the stakeholders in order to make political and managerial key choices to protect and restore the environment. In Europe, for example, Members States of the Union have been developed biomonitoring programs in order to implement the Water Framework Directive; the aims of the WFD is to prevent qualitative and quantitative deterioration of water status, improve it and ensure its sustainable use (EPC, 2000).

Finally, bivalves are useful as model organism in the recent investigations on micro plastics and nanoparticles, particularly because they are filter feeders; many studies about this topic were conducted used bivalves (Canesi et al., 2011; Bonello et al., 2018; Pittura et al., 2018).

1.3 The genus Mytilus as model organism

To the genus Mytilus belong bivalves molluscs, mostly mussels, able to form a belt along the coasts; almost all are edible species. The genus Mytilus is one of the most cosmopolitan of all marine genera, occurring in estuarine and ocean habitats, in both the subtidal and intertidal zones, occupying a diversity of substrates and distributed at higher latitudes in all the oceans and major seas of the world (Koehn, 1991). According to Phillips (1977), Campos et al. (2012) and Suàrez-Ulloa et al. (2013) marine mussels rather than freshwater species are perfect sentinel and model organisms for marine environmental programs because of their biological, physiological, ecological and economical characteristics:

- they are sedentary filter feeders, they can be representative of the collection area;
- they have a reasonable size, giving adequate tissue for analysis;
- they are easy to sample and hardy enough to survive in the laboratory;
- they should be sufficiently long-lived to allow the sampling of more than one year-class, if desired;
- they are strongly resistant to a wide variety of pollutants and environmental stress, they can generally accumulate big amount of pollutants without being killed by the levels encountered;
- they are widespread all over the world, so it could be possible to compare the same or similar species in different areas;
- they are bio engineering species;
- they have a strong economic interest associated to human consumption and aquaculture;

For the characteristics listed above, mussels have always been used in environmental biomonitoring programs. In the 1970s were done the first investigations about the responses of mussels to environmental stress and pollution, particularly as regard as heavy metals (Phillips, 1976; Bayne et al., 1979). Continuing on this path, different international programs were carry on using mussels as sentinel organism. In 1976 in the United States the "*mussel watch"* concept was evolved, the aim was using different species of mussels (genus Mytilus) and oysters (genus Ostrea and Crassostrea) in order to monitor coastal regions for their pollutant contents (Goldberg et al., 1978). A few years later, in the 1986, started the *Mussel Watch Program* today still in progress. The National Oceanic and Atmospheric Administration (NOAA) coordinate the program; the aim is

the monitoring of the spatial distributions and temporal trends of contaminant concentrations in coastal and estuarine regions of the United States (O' Connor, 1998). Bivalves from at least 150 sites were collected on an annual basis along the East, Gulf and West Coasts, including the Hawaiian Islands (Sericano et al., 1993). The *Mussel Watch Program* started in the U.S, but it soon spread all over the world; coupled with economic development, an escalation in agricultural and industrial activities, also the least developed countries were interest in monitoring their environment from Europe to Asia, from Australia to Egypt (Cantillo, 1998; Ramu et al., 2007).

On a smaller spatial scale, other biomonitoring programs have been conducted using mussels as bio indicator. The *Gulfwatch*, held from 1991 to 1997, had the purpose to evaluate the presence of metals, PAHs, PCB and pesticides in the Gulf of Maine, measuring the contaminants concentrations in *Mytilus edulis* tissues (Chase et al., 2001).

Mussels have also been very often used to evaluate the biological consequences of oil spills in marine environments. In March 1989 occurred the *Exxon Valdez* oil spill in Prince William Sound, Pacific Ocean. The oil tanker was full of oil, so spilled 41 millions of liters of crude oil in that area. *Mytilus trossulus* were sampled in 1992 and 1993 from beaches that had been oiled and used to assess the impact of this environmental disaster; were evaluated the polynuclear aromatic hydrocarbon (PAH) concentrations in mussel tissues, physiological responses as byssal thread production, condition index, clearance rate, and glycogen content (Thomas et al., 1999). In 2002 occurred one of the largest spills in the maritime history, the oil tanker *Prestige* spilled around 60,000 tons of fuel oil along the Spanish Galician coasts, Atlantic Ocean. In this case, to evaluate the extent of the spill damage, was analyzed the protein expression of *Mytilus galloprovincialis* sampled along the Galician coasts (Apraiz et al., 2009).

Recently, mussels are used as model organisms not only for classical pollutants but also for pharmaceuticals and personal care products (PPCPs), biocides, micro plastics (MPs) and nanoparticles (NPs). Particularly, in Faggio et al. (2018) the latter pollutants are investigated in mussels' digestive gland, using different approaches and analytical methods in order to elucidate both the presence and the toxic mode of action of xenobiotics both the important role of the digestive gland as a reliable target-tissue for investigating the effects of xenobiotics at cellular, biochemical, and molecular levels.

One of the most concerning contemporary pollution is the presence of micro plastic in the marine pollution, therefore there is a recently increasingly interest on the topic. In Italy, in the gulf of La Spezia, Bonello et al. (2018) have done a first evaluation of micro plastic content in benthic filter-feeders*: Mytilus galloprovincialis*, *Crassostrea gigas* and *Anomia ephippium.* This preliminary study showed low levels of micro plastic contamination, but continuous environmental monitoring is advisable. In fact, the toxicity for humans coming from micro plastics contained in bred lamellibranchs is still to be verified.

Finally, mussels of genus Mytilus could be used as a model in biomonitoring programs in order to investigate on environmental questions, but now also as emerging model organisms for cancer study as well as other species of molluscs (Carella et al., 2016; Carella et al., 2017).

1.3.1 Mytilus galloprovincialis and Mytilus unguiculatus Valenciennes

The bivalve *Mytilus galloprovincialis* Lamarck, 1819 and *Mytilus unguiculatus* Valenciennes 1858 belong to the genus Mytilus, family Mytilidae, class Bivalvia. The two different species of mussel came from different regions: Mediterranean area for *M. galloprovincialis* and western pacific area for *M. unguiculatus*. *M. galloprovincialis*, as a pure taxon, it occurs specifically in the Black and Mediterranean Seas (Daguin et al., 2001; Śmietanka et al., 2004, Kijewski et al. 2011). Outside of these areas, it has been reported from the Atlantic coastal areas of Spain, France, Great Britain and Ireland (Gosling et al., 2008; Quesada et al., 1998). The widespread geographic distribution of Mytilus in Europe may result not only from natural processes, but also from introductions and translocations as mussels have been cultured in Mediterranean Sea for hundreds of years (Smaal,2002; Kijewski et al., 2009; Kijewski et al. 2011). *M. unguiculatus* (unaccepted name as *Mytilus coruscus* Gould, 1861, corrected in World Register of Marine Species, WoRMS) is widespread and widely bread along the costs of China (Lu and Wang, 2018)

From an anatomical point of view both the two species are very similar. As other mussels species, they have the two shell valves similar in size and roughly triangular. Shell shape and colors could be very variable among mussel species. The valves are always hinged together at the anterior by means of a ligament, this area of the shell is called the umbo. The interior of the shell is white with a broad border of purple or dark blue.

This is called the pallial line and is the part of the shell along which the mantle is attached when empty shells are examined. On the inside of each valve there are two muscle scars, the attachment points for the large posterior adductor muscle and the much-reduced anterior adductor muscle. Anterior and posterior retractor muscles are also attached to the shell; these control the movement of the foot. The foot in turn secretes a byssus, a bundle of tough threads of tanned protein. These threads emerge through the ventral part of the shell and serve as mooring lines for attachment of the mussel to the substrate, and to other mussels. The presence of concentric rings on bivalve shells has been extensively used to estimates their age. The material constitutes the shell is $CaCO_3$, organized in crystals of calcite and/or aragonite embedded within an organic framework. Crystals in different shell layers may adopt different morphologies and arrange themselves in different spatial configurations, or microstructures. To form the shell bivalves use a highly cross-linked protein layer (periostracum, the outermost side) and the epithelial cells of the mantle, the organ directly responsible for shell formation. They then elaborate a matrix within this space in which mineral forms. The major components of the matrix are the polysaccharide b-chitin, a relatively hydrophobic silk protein, and a complex assemblage of hydrophilic proteins. The final stage of the process is the formation of the mineral itself within the matrix. The mineral in mature shells is made of aragonite and calcite. Finally, nacre, the innermost side of the shell, comprises uniformly thick layers of aragonite crystals separated by interlamellar layers of organic matrix (Addadi et al., 2006).

Inside the two valves of the shell there is the mantle, a two lobes tissue that completely encloses the animal within the shell. Between the mantle and the internal organs is a capacious mantle cavity. The mantle consists of connective tissue with haemolymph vessels, nerves and muscles that are particularly well developed near the mantle margins. Cilia on the inner surface of the mantle play an important role in directing particles onto the gills and in deflecting heavier material along rejection tracts towards the inhalant opening, the entry point on the mantle for incoming water. The mantle contains most of the gonad. Gametes proliferate within the mantle and are carried along ciliated channels to paired gonoducts that discharge into the mantle cavity. The mantle is not only the site of gametogenesis but is also the main site for the storage of nutrient reserves, especially glycogen. The mantle has margins that are thrown into three folds: the outer one, next to the shell, is concerned with

shell secretion; the middle one has a sensory function and the inner one, or velum, is muscular and controls water flow in the mantle cavity. A minute space that contains pallial fluid separates the mantle from the shell, except in the regions of muscle attachment.

Mussels feed and breathe by using the incoming current as a source of food and oxygen. The food is composed of a mixture of organic and inorganic particulate matter, which varies with temperature, water stratification, and hydrodynamics (Berg and Newell, 1986; Claereboudt et al. 1995; Abraham, 1998). Mussels can filter a variety of suspended particles like bacterioplankton, phytoplankton, organic detritus, microzooplankton, and mesozooplankton (Hawkins and Bayne, 1992; Lehane and Davenport, 2002). Phytoplankton and organically rich detrital particles are generally the main component of mussel nutrition (Bayne and Hawkins, 1992). Several studies have indicated that bivalve molluscs retain particles in the 2–20 µm range (Bayne and Newell, 1983), cytometric studies have showed that mussels are particularly efficient at filtering particles in the 3–5 µm range (Cartier et al., 2004). Generally, the gills follow the curvature of the shell margin with the maximum possible surface exposed to the inhalant water flow; each gill is made up of numerous W-shaped filaments and an internal skeletal rod rich in collagen strengthens each filament. Each gill terminates within a pair of triangular palps that are situated on either side of the mouth. The inner surface of each palp faces the gill, and is folded into numerous ridges and grooves that carry a complicated series of ciliary tracts. The main function of the labial palps is to continually remove material from the food tracts on the gills in order to prevent gill saturation; the filtered material is carried away from the mouth and deposit as pseudofaeces. The mouth is ciliated and leads into a narrow ciliated esophagus. Ciliary movement helps to propel material towards the stomach. The stomach is large and oval-shaped and lies completely embedded in the digestive gland, which opens into it via several ducts. The digestive gland, which is brown or black and consists of blind-ending tubules that connect to the stomach by several ciliated ducts, is the major site of intracellular digestion. The digestive gland also plays an important role in the storage of metabolic reserves, which are used as an energy source during the process of gametogenesis and during periods of physiological stress (Bayne et al. 1976).

The heart lies in the mid-dorsal region of the body, in a space called the pericardium. Heart allows the hemolymph to circulate and perform its functions: gas exchange, osmoregulation, nutrient distribution, waste

elimination and internal defence. The haemolymph contains cells called haemocytes that float in a colourless plasma.

The nervous system of bivalves is fundamentally simple. It is bilaterally symmetrical and consists of several pairs of nerves and three pairs of ganglia: cerebral, pedal and the visceral one. During the evolution of bivalves, with loss of a distinct head, most of the sense organs withdrew from the anterior end and have came to lie at the edge of the mantle.

Mussels reproduce by means of gametes released into the water, where fertilization takes place. Mussels are dioecious (with separate sexes) and iteroparous, reproducing annually and occasionally more frequently whilst continuing to grow. The gonads develop within the mantle tissue and can often be morphologically recognized, as the case of *Mytilus edulis* in which the mantle containing the gametes is typically orange in females and creamy-white in males. After the hatching, mussels have a period of planktotrophic larval development that terminates with settlement and metamorphosis onto benthic substrates. When a suitable substrate is found, the larva continues to crawl for some time, gradually ceases movement, protrudes the foot and quickly secretes a single byssal thread. As the mussel grows in length, an increasing number of attachment threads are secreted thus tethering the animal to the substrate (Bayne et al., 1983).

Mussels adhere to the substratum by mean the byssal threads, among nature's most peculiar tendons. One end of each thread inserts into the byssal retractor muscles at the base of the foot; the other end is disposed outside the animal and attached to a hard surface by an adhesive plaque (Coyne et al., 1997). Despite their rapid production and vulnerable location, byssal threads are durable and exquisitely engineered fibers. Each byssal thread resembles a shock absorber in its mechanical design: it is strong and stiff at one end and pliably elastic at the other (Waite et al., 1997). Each thread has a flexible, collagenous inner core covered by a tough, durable cuticle of polyphenolic protein (Sagert & Waite 2009). Each species of bivalves show different threads technical properties, depending on their life style and the conditions of their environment. For example, in *Mytilus californianus* distal threads are 2–3 times stiffer and 30% more extensible than those in either *M. trossulus* or *M. galloprovincialis*, which may contribute to the strong attachment strength of *M. californianus* and its ability to dominate wave-exposed shores (Bell & Gosline, 1997). In the plaques at the end of each thread, use a natural adhesive, to date there are no synthetic glues that can perform this function. It is not surprising, therefore, that over the past decade mussel

adhesive proteins are attractive targets for biomimetic technology, which entails using designs from nature to solve problems in engineering, materials science, medicine and other fields (Silverman & Roberto,2007; North et al., 2017; Jo et al., 2018).

In the figure 1.1 from Marine Bivalve Molluscs (Gosling, 2015) are shown the previously described elements of mussels, both for the outside and inside of the animal.

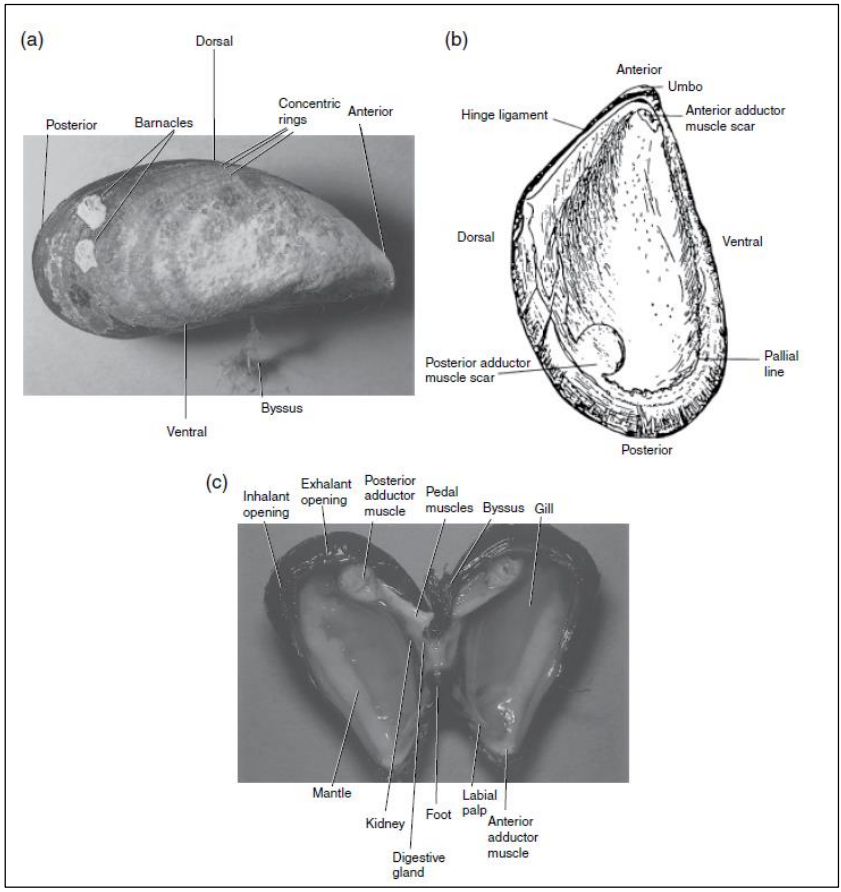

Fig. 1.1 a) the outer part of the shell; b) the inside of the shell c) the inner part of the shell with the internal organs.

Thanks to the byssus mussels adhere to the substratum, in their natural environment they generally form a belt on the rocky shores. While mussels often dominate primary space on rocky shores, they

also provide refuge and habitat for many organisms living on and amongst their shells. Interactions between organisms (competition for abiotic and biotic resources, predation, parasitism, and mutualism) are crucial for the distribution and abundance of species, but fundamental is also the role that many organisms play in the creation, modification and maintenance of habitats. These activities, known as ecosystem engineering, do not involve direct trophic interactions between species, but they are nevertheless important and common (Jones et al., 1994). The species carrying out this activity are known as ecosystem engineers or bioengineering species. Mussels, and other species of molluscs, are fully recognized as bioengineers (Borthagaray and Carranza, 2007). Different characteristics are appreciated to cover this role, particularly the ability to produce the shell and their filter feeders nature. Molluscs shell production is a very high rate process, the amount of persistent structure by mollusks is comparable to the average production of wood in temperate forests. Shells and shell aggregations introduce complexity and heterogeneity into benthic environments, providing substrata for attachment and refuges to avoid predators or physical or physiological stress (Gutierrez et al., 2003). Furthermore, mussels are filter-feeding animals. For this reason they have long been known to play a key role in the functioning of coastal benthic ecosystems mainly through the controls of pelagic primary production, bacteria populations, and the fluxes of particulate organic matter and nutrients between pelagic and benthic compartments through biodeposition (Raise, 2002; Maire et al 2007).

1.4 Mussels provide ecosystem services

An ecosystem is a dynamic complex of plant, animal, microorganism communities and the nonliving environment interacting as a functional unit. Ecosystem services are the benefits people obtain from ecosystems. These include provisioning services such as food, water, timber, and fiber; regulating services that affect climate, floods, disease, wastes, and water quality; cultural services that provide recreational, aesthetic, and spiritual benefits; and supporting services such as soil formation, photosynthesis, and nutrient cycling. The human species, while buffered against environmental changes by culture and technology, is fundamentally dependent on the flow of ecosystem services (Costanza et al., 1997; Millennium Ecosystem Assessment, 2005).

Marine ecosystems represent some of the most heavily exploited ecosystems throughout the world (Barbier, 2017). They provide: goods (fish harvests, wild plant and animal resources, raw material, genetic material, water); services (recreation and tourism, transportation, scientific and educational opportunities, food control, storm protection, pollution control, breeding and nursery habitats, shoreline stabilization and erosion control, carbon sequestration) and cultural benefits (religious significance, bequest for future generations) (Barbier, 2017). Valuing ecosystem services is sometimes difficult, due to the inadequate knowledge available to link changes in ecosystem structure and function. This connection is more straightforward for well-known goods and services, such as fish harvests and recreation. Exist a strictly economic correlation with these activities, the prices of marketed fish is the real value of fishing. Recreation and tourism values can also be determined by estimating the willingness-to-pay of visitors to unique marine habitats for specific activities, such as snorkeling or scuba diving. Most resource management decisions are most strongly influenced by ecosystem services entering markets; as a result, the non-marketed benefits, often high and sometimes more valuable than the marketed ones, are often lost or degraded (Costanza et al., 1997; Millennium Ecosystem Assessment, 2005). However, estimate the services associated with regulatory and habitat functions of marine ecosystems is often more challenging. Services such as storm protection, habitat support for offshore fisheries, and erosion or pollution control, are generally not marketed (figure 1.2). Evaluate this

kind of services requires knowledge of how the specific marine ecosystem functions and structure influence the "ecological production" of that service, such as storm protection or habitat–fishery linkages, as well as the market conditions of the final marketed commodity that is impacted by the marine ecosystem service (Barbier, 2017).

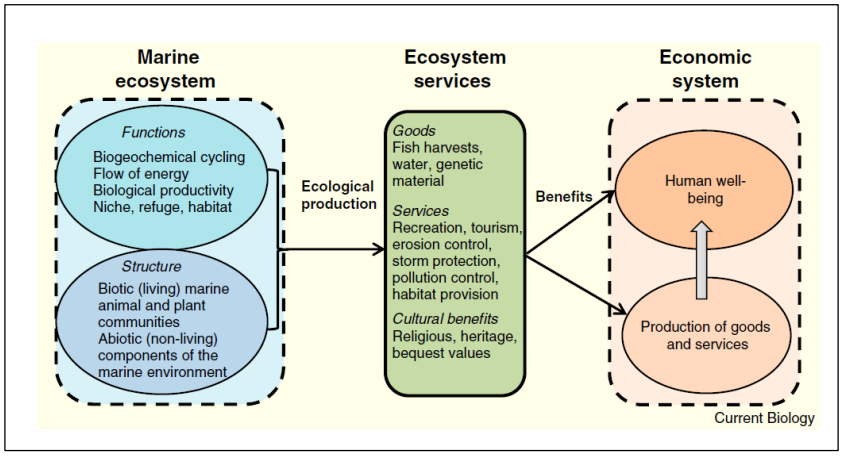

Fig. 1.2 From Barbier et al., 2017. Marine ecosystems provide ecosystem services that generate economic benefits, not only with the conventional marketed goods and services, but also with ecological functions and productions whose economic value cannot be calculated directly.

Bivalves, as bioengineer species, provides for ecosystem services valuable both directly and indirectly. Mussels and oysters are edible molluscs already have a market price; it is easy to define their value for their most intuitive ecosystem service: food supply. They also provide other types of ecosystem services: regulating, supporting, provisioning and cultural services as shown in figure 1.3. Through the process of filtering suspended matter, mussels link benthic and pelagic compartments. They can transfer energy and nutrients from the water column to the sediment, bio deposit organic matter and
excrete nutrients, purify the water, recycle and store nutrients; the effects of the energy and nutrient subsidies provided by mussels cascade through food webs and stimulate both algal and macroinvertebrate production . Moreover, mussels form structural habitat and substrate biogenic reef, protect the coastline and could be used as tools in jewelry and art and for

spiritual enhancement (Kent et al., 2016; Lemasson et al, 2017; Vaughn et al., 2017).

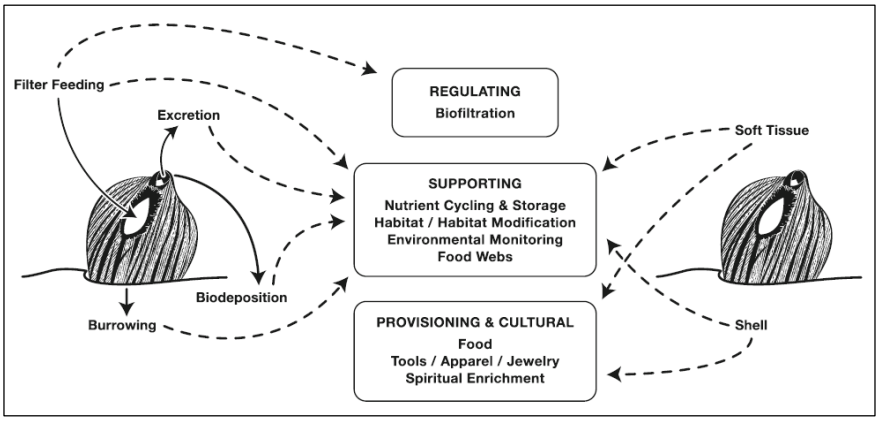

Fig. 1.3 Activities that mussels perform can be translated into ecosystem services that are beneficial to humans (Vaughn et al., 2017).

1.4.1 Threats to ecosystem services

Due to several human impacts as pollution, population growth, development and destruction of the coast and other anthropic activities, marine ecosystems have been lost or degraded worldwide over the last decades. Climate changes, overfishing, harmful algal blooms, invasive species, polluted water and oxygen depletion are all alarming threats for marine environment could affect the functioning of marine ecosystem. These changes affect marine biodiversity, altering the distribution of species, the community structure, and ecosystem functioning. The effect of any ecological change influences ecosystem services and, in turn, human well-being (Dìaz et al., 2006; Worm et al., 2006; Danovaro et al., 2008; Cardinale et al., 2012).

Threats to the marine ecosystem previously listed can lead to the loss of biodiversity. Hooper et al. (2012) assert that terrestrial and marine biodiversity loss in the 21st century could rank among the major drivers of ecosystem change. Impressive is the case of the North America freshwater mussel fauna (order Unionoida) has suffered an inordinately high recent extinction rate, and the small size and isolation of many remaining populations portends a continued diminishment of this fauna (Haag and

Williams, 2013). They have suffered one of the highest extinction and imperilment rates of any group of organisms on the planet: about 30 North American taxa have become extinct in the last 100 years and 65% of remaining species are considered endangered, threatened, or vulnerable (Haag and Williams, 2013). As healthy mussel communities occur as multispecies assemblages in which species interactions are very important, this situation negatively affect the ecosystem and ecosystems services they provide (Vaughn et al., 2008). For their important rule in ecosystem functioning, has been implemented a National Strategy for their conservation (The National Native Mussel Conservation Committee, 1998).

Like mussels, even oysters have suffered serious losses over the years: oyster reefs have been extirpated from estuarine, coastal, and nearshore environments, primarily due to over extraction for food and disease and water pollution, to the extent that 85% of reefs have been lost worldwide (Beck et al. 2011; Barbier, 2017). The loss of oyster reef represents one of the more substantial losses of an ecologically important and widespread ecosystem because these reefs, as mussels beds, would have supported the productivity of associated fishes, stabilized the cost and helped maintain water quality (Alleway & Connel, 2014).

Alien invasive species represent another important threat to the ecosystem functioning, threatening biodiversity. Alien species from all taxonomic groups affect supporting, provisioning, regulating and cultural services and interfere with human well-being (Vilà et al., 2010). Biological invasions also cause economic impacts that can be valued as financial costs, based on expert extrapolations of high-profile alien pests. An impressive example is the invasion of the ctenophore *Mnemiopsis leidyi* in the Black Sea. Following the increase of *M. leidyi*, there was a decline in the abundance and species diversity of ichthyoplankton and mesozooplankton, high fishery pressure and increasing impact of *Mnemiopsis* on the food web further induced the anchovy stock collapse (Shiganova, 1998; Oguz et al., 2008).

Finally, other two emerging trouble threatens marine ecosystems: food frauds and Ocean Acidification (OA). The first one directly affects the ecosystem services of food provisioning and it could be dangerous for the introduction of new species, the second one globally affect marine environment.

1.4.2 Food frauds

Food fraud means the act of defrauding buyers of food and food ingredients for economic gain (Johnson, 2014). According to Moore et al., 2012, there are three different type of food frauds:

- replacement (complete or partial) of a food ingredient or valuable authentic constituent with a less expensive substitute without the purchasers' knowledge. This type of fraud include other different subtypes:
  - addition, dilution, or extension of an authentic ingredient with an adulterant or mixture of adulterants;
  - false declaration of geographic, species, botanical, or varietal origin
  - false declaration of the raw material origin or production process used to manufacture an ingredient
  - false declaration of origin to evade taxes or tariffs
- addition of no authentic substance to mask inferior quality ingredient without the purchasers' knowledge
- removal of an authentic and valuable constituent without the purchasers' knowledge.

Seafood is probably the largest category of foods subject to food frauds (Roberts & Turk, 2017). However, food frauds are very common in every food sector. Some products are particularly affected by this problem, among them there are wine, meat, honey, fruit juices, coffee, tea and olive oil that is the world's most adulterated oil. Moreover, Italy is the absolute leader in the field of agri-food excellence with 818 DOP, DOC and STG products approved by European Union. For such excellences, Italian products are always objects of food frauds, on the national and abroad market (MiPAAF, 2017).

Food fraud referred also as EMA (Economically Motivated Adulteration) is both an old and modern problem (Roberts & Turk 2017; Shears 2010). Already in the Bible are reported the sins of those who altered the weights of the scales (Semeraro et al., 2011). In ancient Rome and Athens, there were laws regarding the adulteration of wines with flavours and colours (Sumar and Ismail, 1995). The phenomenon of food fraud reached its peak in '800 in England, where the first anti-fraud law was issued: the *sale of food and drug act* (Semeraro et al., 2011). In Italy the first laws in such context go back to 1888 when the law *Crispi Pagliani*

established the national health system so the public health became a State duty. Since then, in Italy each Government has always been issued laws on control of food production and food trade.

As Italy, also the others European members had its own legislation on the topic in the past. Today, following trade increasing, there is a common European management on foodstuffs and animal feed. The Regulation CE n. 178/2002 of European Parliament and Council regulates food safety and lays down procedures in the field of food safety. Aim of this legislation is to prevent fraudulent or deceptive practices, food adulteration and every other kind of behavior capable of misleading. Particularly the article 14 of the Regulation ratifies that food at risk cannot be placed on the market (food frauds are all included). Moreover, in 2004 production processes have been regulated to support the Regulation 178/2002.

However, despite the strict legislation, food frauds are always a globally important business (Shears, 2010). Particularly Italy with the significant own food production and the great quality of food products is often victim of food frauds. According with the data of FareAmbiente (2018), about three-fifths of "Made in Italy" food products on abroad shelves are false. 110 thousand jobs are overall compromised by the fake market, of which about 30% from the food market. In one year, it has been estimated about 90 billion euros subtracted to Italian companies in the food & beverage sector.

Definitely, frauds are a serious threat for honest merchant and food industries. Food frauds can be also a risk for health consumers and for the environment, some food frauds can even be lethal as reported in South and Brisman (2013). In Italy, in 1987 the use of antifreeze in the wine killed 87 people; in China the use of melamine in baby formula involved the death of 6 babies and the illness of more than 300.000 infants.

Finally, food frauds could be a dangerous threat for the environment, this issue is particularly referred to the seafood frauds. Seafood was identified as the third-highest risk category of foods with the potential for fraud. Each seafood species it is not only used as food but has its own ecological rule. Some species are overfished or fished in a not legal area or the catches are of an illegal size. Species substitution (the most common type of seafood fraud) can occur in order to conceal the geographical origin, or to hide an illegally harvested protected species or a species from a protected area (FAO, 2018).

1.4.3 Ocean Acidification

Ocean Acidification (OA) is an emerging global problem towards which the interest of climate policy and researchers in the last decades is growing. OA is a change in ocean chemistry due to increasing concentrations of anthropogenic carbon dioxide in the atmosphere as the oceans absorb around one third of the world's CO_2 emissions (Sabine et al., 2004). In the past few decades, only half of the CO_2 released by human activity has remained in the atmosphere; of the remainder, about 30% has been taken up by the ocean and 20% by the terrestrial biosphere (Feely et al., 2004). Anthropogenic greenhouse gas emissions were increased since the pre-industrial era, driven largely by economic and population growth, and are now higher than ever. This has led to atmospheric concentrations of carbon dioxide, methane and nitrous oxide that are unprecedented in at least the last 800.000 years (IPCC, 2014) (figure 1.4).

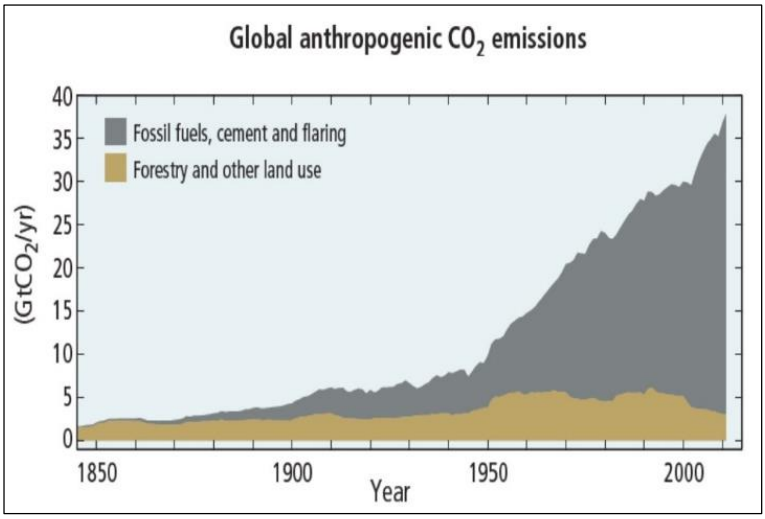

Fig. 1.4 Global anthropogenic CO_2 emissions from forestry and other land use as well as from burning of fossil fuel, cement production and flaring (IPCC, 2014).

Atmospheric CO_2 concentrations oscillated between 200 and 280 parts per million (ppm) over the 400,000 years before the industrial period (Feely et al., 2004, Collins et al., 2013). Future predictions based on business as usual emission scenarios, indicate that the oceans will continue to absorb carbon dioxide. By the end of the twenty-first century,

atmospheric CO_2 is expected to reach 800-1000 ppm, with a corresponding drop in ocean pH of 0.3–0.4 units, resulting in a pH that the oceans have not experienced for more than 20 million years (IPCC, 2014; Valenzuela et al., 2018).

Oceanic uptake of anthropogenic CO_2 has caused a wholesale in the seawater column worldwide, increasing aqueous CO_2 and decreasing pH, carbonate ion (CO_3^{2-}) concentrations, and the saturation states (Ω) of calcium carbonate minerals such as calcite (Ω_{ca}) and aragonite (Ω_{ar}) (Feely et al., 2004). As CO_2 dissolves in the surface ocean, it reacts with water to form carbonic acid (H_2CO_3), which dissociates to bicarbonate (HCO_3), carbonate ions (CO_3^{2-}) and protons (H^+). With increasing atmospheric pCO_2, the equilibrium of the carbonate system will shift to higher CO_2 and HCO_3 levels, while CO_3^{2-} concentration and pH will decrease (Cigliano et al., 2010). Altering the carbon cycle, OA affects ocean species to varying degrees. Direct physiological effects of decreased pH or increased CO_2 are readily detectable and typically include changes in survival, calcification, growth, development, reproduction and abundance (Cooley et al., 2009; Ries et al., 2009; Gattuso et al, 2015). Laboratory and field experiments revealed that ocean acidification has mostly negative impacts on the fertilization, feeding, cleavage, larva, settlement and reproductive stages of several marine calcifiers including bivalves, echinoderms, corals, crustaceans, coccolithophores and foraminifers species (Gattuso, 1998; Riebesell et al. 2000; Gazeau et al., 2007; Kurihara, 2008; Fabricius et al., 2011; Schlegel et al. 2012; Rodríguez et al., 2018).

Calcifiers organisms are thought to be among the most influenced by OA, as they generally use aragonite to build their skeleton. Under saturation of $CaCO_3$, led by the absorption of CO_2 in marine environment, could cause the formation of weak or incomplete skeletons. However, the responses to the acidified conditions are different for different species. Is the case of study of Rodolfo – Metalpa et al. (2011) in which they compare the effects of naturally acidified environment on mollusc and corals, they shown that corals net calcification decreased significantly instead molluscs (limpet and mussels) up-regulated their calcification rates, which helped counteract higher shell dissolution rates. Furthermore, in the same study, researchers have shown the responses of limpet and mussels are different. Mussels dissolved their shells more slowly than limpets, probably because their periostracum protected the shell. Protective organic layers are produced by many marine calcifiers and seem to be key in determining their relative susceptibility to dissolution owing to ocean acidification.

OA, as well other kind of human disturbances, could act as stressors for some species but as resources for other species, depending on the specific organism's responses. For example, Connel et al. (2018) have established that CO_2 enrichment had a direct positive effect on productivity of turfs, but a negligible effect on kelp. CO_2 enrichment further suppressed the abundance and feeding rate of the primary grazer of turfs (sea urchins), but had an opposite effect on the minor grazer (gastropods). Some species could adapt their self to the new conditions, as Kroeker et al. (2011) and Hall-Spencer et al. (2008) have affirmed in their studies on the benthic community of a naturally acidified seawater, in a CO_2 vents area of Ischia Island, in the Tyrrhenian Sea. Both the researchers group have showed important changes in the structure of the rocky shore and the *Posidonia oceanica* meadow community. Kroeker et al. (2011) have analyzed the fauna assemblage, showing that there is an absence of numerous taxa in extreme low pH (particularly calcifying taxa), compensated for by increased abundances of acidification-tolerant taxa (primarily crustaceans). They affirm that the trophic structure of the invertebrate community shifted to fewer trophic groups and dominance by generalists in extreme low pH, suggesting that there may be a simplification of food webs with ocean acidification. Hall-Spencer et al. (2008) also showed that along a gradients of normal pH (8.1–8.2) to lowered pH (mean 7.8–7.9, minimum 7.4–7.5), such typical rocky shore communities with abundant calcareous organisms shifted to communities lacking scleractinian corals with significant reductions in sea urchin and coralline algal abundance. Furthermore, they showed a higher sea-grass production in the area with a mean pH 7.6 where coralline algal biomass was significantly reduced and gastropod shells were dissolving. Porzio et al. (2008) also demonstrate significant loss of algal diversity and changes in macroalgal community structure in the same naturally acidified environment. Not only rocky shore and shallow water community but also biofouling and planktonic community are affected by OA. Peck et al. (2015) have shown that biofouling communities face dramatically change, with reductions in groups with hard exposed exoskeletons and domination by soft-bodied ascidians and sponges. The OA effects affect benthic communities and the whole column of water, in the ocean the main phytoplankton groups are connected with the carbon cycle exporting flux of $CaCO_3$ from the upper ocean to the deep sea. Moreover, physiology and biology of microalgae could be modified by OA (Shi et al., 2010; Wu et al., 2010; Rossoll et al., 2012) as well as zooplankton characteristics (Cripps et al., 2015)

consequently totally altering the trophic chains (Rossol et al., 2012; Branch et al., 2013). The highest part of the food pyramid is affected by ocean acidification by mean the alteration of food chain but also because the fish modify their physiology, behavior and sensitivity too. Particularly notable impacts on neurosensory and behavioral endpoints, otolith growth, mitochondrial function, and metabolic rate demonstrate an unexpected sensitivity to current-day and near-future CO_2 levels (Simpson et al., 2011; Heuer and Grosell., 2014); as well as other species, the effects of OA on fish could depend on the sensitivity of different life stages (Rodriguez-Dominguez et al., 2018).

Finally, OA affect positively or negatively marine organisms, oceans environment and its ecosystem services. Changes to biogenic habitat structure associated with OA represent a key alteration to benthic systems, with potentially large indirect effects on biodiversity (Sunday et al., 2017). Despite high variation in individual species' responses, ocean acidification generally causing a decrease in biodiversity, biomass, and trophic complexity of benthic marine communities. Future changes in ocean acidity will potentially impact the population size and dynamics, as well as the community structure of calcifiers, and will therefore have negative impacts on marine ecosystems. These outcomes suggest that a loss of biodiversity and ecosystem function is expected under extreme acidification scenarios (Kroeker et al., 2011).

In the highly complex environment of the oceans, OA is often accompanied or added to other naturally or anthropogenic factors. Climate changes led not only OA but also global warming, growing in recent period, particularly in the last thirty years. Many researcher study the synergistic effects of OA and the increase of seawater temperature (Kroeker et al., 2013; Gattuso et al., 2015; Johnson et al., 2017; Swezey et al., 2017). OA and the synergistic impacts of other anthropogenic stressors provide great potential for widespread changes to marine ecosystems (Fabry et al., 2008), causing a restructuring of ecological communities and a reduction of ecosystem function through the loss of stress-intolerant species. Political will and significant large-scale investment in clean-energy technologies are essential if we are to avoid the most damaging effects of human-induced climate change, including ocean acidification.

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Chapter 2

Sea food frauds:
a threat for mussels economy

Poster presentation at the XXVII S.It.E. (Società Italiana di Ecologia) Congress –
"La ricerca ecologica in un mondo che cambia". Napoli, 12-15 September 2017

Title: "Marine organisms model species for the assessment of biological, environmental and economic impacts on marine aquaculture in Campania"
Fioretti S., Patti F. P., Anastasio A.

2.1 Bivalves as food product

Bivalves are part of human diet since the most ancient time. Paleolithic man can still not bread any animal, they collected mussels and oysters as well as fruits and berries; in fact bivalves shells were also found in various caves in Italy, French, Spain and others coastal Mediterranean countries (Cattaneo Vietti et al., 2013). In Roman times molluscs were very appreciated, particularly oysters, widespread in the high social classes (Malossini F., 2011, J. De Grossi Mazzorin, 2015). The first evidence of molluscs breeding dates back to this period: along the Campanian coast, in the Lucrino Lake, oysters were farmed. During the medieval period, aquaculture techniques began to be improved, particularly by monks. Until the XIX century, Italian mussel farms were only along Puglia and Liguria coast. Then, thanks to technical improvements, mussel farms have become widespread in Italy and Europe. After the Second World War, mussel market increased considerably.

Today mussel market seems destined to increase as the other aquaculture productions. In Italy the sea food per capita consumption is around 26 Kg/year, it is increased compared to the previous years (2016) on which the average was around 16 kg/year with good prospects for further growth over the next two to three years (Eurispes, 2018). According to the report of FAO (2018), italian seafood consumption is above the world (20,3 kg) and european (22,5 kg) average. However, data of the New Economic Foundation and the association MedReAct highlight that fish stocks can provide 11 kg of sea food per capita annually (2016). The degree of self-sufficiency could be expressed with the 'fish dependence day': the date on the calendar when a state or a region will begin relying on fish from elsewhere because its domestic supplies have been depleted. In the year 2018, the 9 July was the "fish dependence day" in Europe (FAO, 2018). The degree of self-sufficiency of fishing decreases over the years, in just around 30 years is almost halved: in 1990 was 29 June, in 2014 was 29 March until 9 July in this year. Consequently, to compensate for the lack of natural stock, lots of seafood is imported in Europe. In Italy, most of the imported bivalve molluscs are from Spain and Greece, as reported in figure 2.1.

The causes of the depletion of seafood stocks are the increase of world seafood consumption during the years, also for the increase of global population, but particularly the illegal fishing and the overfishing. To improve sea conditions and the ecosystem service of food providing are

essential sustainable environmental actions, by both politicians and consumers. Politicians must pay more attention on environmental questions, increasing controls on fishing catches and on fishing techniques. Consumers must be more responsible for their food choices, preferring local species, in the right seasonality, favoring aquaculture products as they are of good quality not so different by the natural ones. Aquaculture is obviously a good solution to the high seafood demand; in fact is a growing economic activity and it will increase in future years. It is clear in the figure 2.2 that aquaculture (of fish, crustaceans and molluscs) has an increasing trend from 1990s until today (FAO, 2018).

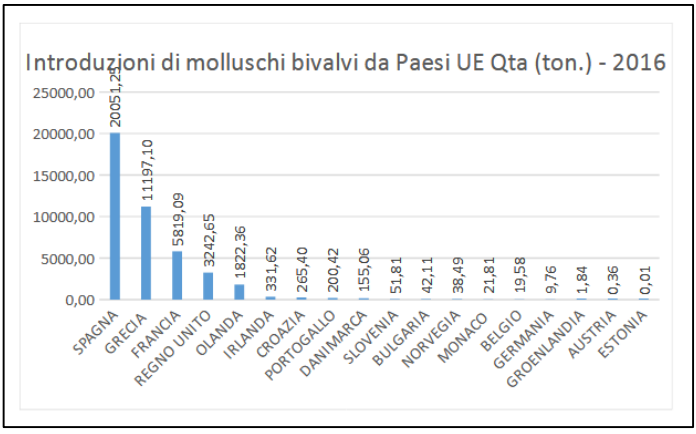

Fig. 2.1 Bivalve molluscs imported in Italy from others European countries, mainly from Spain and Greece (Molluschi Bivalvi, Attività del controllo ufficiale 2016).

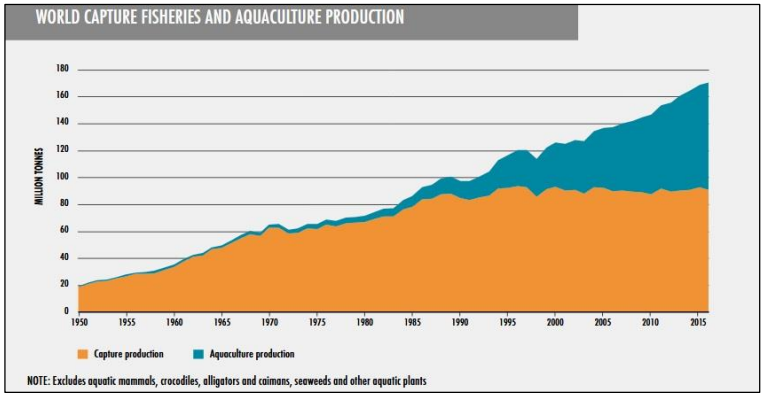

Fig. 2.2 World capture fisheries and aquaculture production (FAO, 2018)

2.1.1 Seafood labels

How can a consumer make sustainable choices? Where can the consumers find informations in order to choose the right products? How they can know about the seafood origin? The seafood species they are buying? All the answers are in the food labels, in the figure 2.4 are reported two of the mussel labels collected in our sampling. The law n. 109/92 define the regulation on labels in a general way, the law n. 104/2000/CE and n. 2065/2001/CE defines the rules for seafood products, both fresh caught seafood and that of aquaculture. According with such laws and as reported by European Union (2014), on the seafood labels the following informations must be reported:

- Prepacked and non-prepacked products:
- Commercial designation and scientific names: both the commercial and scientific names must be displayed. These names must match those on the official list drawn up and published by each EU country;
- Production method: the production method should be displayed using the following designations in particular 'caught…' or 'caught in freshwater…' or 'farmed…'. Mixed products of the same species and different production methods must display the method of production for each batch.
- Catch area/country and body of water/country of production: the catch area for fish caught at sea is the FAO area (figure 2.3), sub-area or division where the fish were caught. Farmed fish (aquaculture) must display the country of production.
- Fishing gear: wild fish must display one of the following fishing gear categories used to catch the fish: 'seines', 'trawls', 'gillnets and similar nets', 'surrounding nets and lift nets', 'hooks and lines', 'dredges', and 'pots and traps'.
- Defrosted: the label should show whether the product has been defrosted
- 'Best before' date / 'Use by' date: the date of minimum durability corresponds to the 'best before' date or 'best before end' date. For live bivalve molluscs, the 'best before' date can be replaced by the label 'these animals must be alive when sold'
- Allergens: both for prepacked and non-prepacked products, a clear reference to the name of any allergens should be included in the list

of ingredients. Where no list of ingredients exists, the presence of allergens must be indicated as follows: 'contains…'. It is not required when the food name clearly refers to allergen(s).

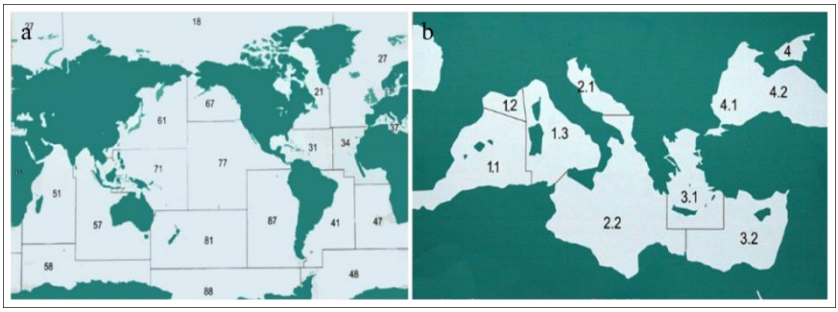

Fig. 2.3 FAO fishing Areas. The Mediterranean area is the n. 27 (figure a), which is further divided in subareas (figure b).

- Additional requirements for prepacked products. In addition to that listed above, the prepacked products labels must be reported:

- List of ingredients, not necessary for single-ingredient foods that have the same name as the ingredient;
- Quantity of ingredients, expressed as a percentage;
- Net quantity (the net weight expressed in grams or kilograms);
- Conditions for storage and use;
- Name or business name and address of the food business operator;
- Country of origin or place of provenance;
- Instructions for use (only if needed);
- Nutrition declaration;
- Packaged in a protective atmosphere (this must be included if the product was packaged in certain gases);
- 'Date of freezing' or 'Date of first freezing';
- Added water: must be shown in the list of ingredients in accordance with the requirements of the FIC Regulation.
- Added proteins of different animal;
- Formed fish (products which give the impression that they are made of a whole piece of fish but actually consist of different pieces combined using other ingredients (e.g. food additives, food enzymes) or other means, need to indicate this;
- Identification mark (the name of the country, the approval number of the establishment where production takes place and the

abbreviation EC, or its translation in other EU languages, must be shown when the product is produced in the EU);

- Date of packaging: this date must be shown for live bivalve molluscs and must comprise at least the day and the month.

Fig. 2.4 Two example of mussels labels sold at the Pozzuoli fish market, collected during our sampling. In the label are clearly reported and underlined with different colors: the commercial designation (mitili or cozze) and the scientific name (Mytilus galloprovincialis); the production country (Italy); the label 'these animals must be alive when sold' typically used for molluscs; the storage conditions; the seller and the identification marks. The other important informations, the packaging day and weight, is generally on the back of the label.

2.2 Seafood frauds

Labels should be a guarantee for consumers, unfortunately many times what is written on the label is not the truth. Despite strictly European and world regulations on seafood trade, in Italy in the 2017 the ICQRF (Central Inspectorate of the protection of Quality and Repression of Frauds of agri-food products) declared over 22,000 tons of products seized, for a total seizures value of more than 103 million euros. The act of defrauding buyers of food and food ingredients for economic gain is defined as frauds (Johnson, 2014) and is a big European and world business; its true extent is unclear but consumers are undoubtedly being cheated out of hundreds of millions of euro each year in the UK alone (Shears, 2010).

Among food frauds, seafood frauds are highly common, on the whole supply chain (Oceana 2016; Warner et al. 2013; Warner et al. 2016; FAO, 2018; Fox et al., 2018). According with Roberts & Turk (2017) seafood is probably the largest category of food involved in frauds. This has been attributed to the increasing demand and recognition of seafood as a healthy alternative to red meat, the similarity and diversity of seafood species available, the stock limitations and price pressures in the food market (Barbuto et al., 2010; Jacquet & Pauly, 2008; Jennings et al., 2016; Miller & Mariani, 2010; Mohanty et al., 2013). Seafood fraud is committed when seafood is deliberately placed on the market, for financial gain, with the intention of deceiving the customer. Seafood frauds occur in a variety of different ways, in the following list are reported the most common seafood frauds:

- replacement or species substitution (the most common fraud), where a low-value species replaces a more expensive variety for economic gain, or where a high-value species is presented as a lower-value species for tax evasion purposes;
- mislabeling of fish to conceal the geographical origin of illegally harvested species;
- marketing of counterfeit products, where brand names are fraudulently used;
- undeclared use of food additives such as water-binding agents to deceptively increase the weight of products;
- illegal use of food additives such as carbon monoxide to enhance the visual quality of fish products;
- addition of glaze water to frozen products to increase weight;

- mislabeling of ingredients, such as batter or breadcrumbs, to bulk up the weight of processed products.

Seafood frauds rates could be very high. On Italian markets, the authorities found very high rate of species substitution and mislabeling (Armani et al., 2015; Di Pinto et al., 2015; Pappalardo & Ferrito, 2015). According to Guardone et al. (2017), on Italian markets at least 22.5 % of products were mislabeled. The highest level of mislabeling was found in cephalopod-based products (43.8%), followed by crustaceans (17%) and fish (14%), with the highest rate of mislabeling in products imported from China, VietNam and Thailand. For fish fillet, as on the abroad market, a high incidence of substitutions of the most common fish fillet (sole, plaice, salmon and hake) involve pangasius fish fillet (Di Pinto et al., 2015). The lower production price of Pangasiidae has resulted in high potential for species substitution and country of origin mislabeling among cat fish products (Bosko et al., 2018). Moreover, high substitution rate close to 80% was also found in shark seafood products (Barbuto et al., 2010). On the international markets the situation is not so different from the Italian one, high species substitution rates are always very common and different species are involved. In the USA, e.g., between 60 and 94% of fishes labeled as Red Snapper were mislabeled for sale (Marko et al., 2004). In Ireland, a substitution rate of 25% was revealed among cod and haddock products, and this increased to 82% among smoked fish samples (Miller & Mariani, 2010). In Spain high value of tuna, one of the world's most traded valued and sought-after fish species, is often mislabeled with lower price tuna species (Gordoa et al., 2017). Finally, besides, the most commonly food frauds at global level involved seafood are: red snapper, cod, shark tuna, butterfish, wild Alaskan salmon, caviar, puffer fish, halibut, sole, grouper, striped bass, shrimp, clam, mussel and oyster. Moreover, food frauds are very common for processed fishery products (gutting, heading, slicing, filleting and chopping) and for canned seafood, as the morphological characteristics are no longer evident.

2.2.1 Health, economic and environmental threats

Regardless of the manner in which the fraud occurs, seafood fraud is illegal; it can affect public health, it undermines confidence in the market place and it can have serious consequences for fishery management and the fish industry, in addition to economic, social and environmental

costs (FAO, 2018). Therefore, food frauds affect the ecosystem services that seafood provides: food provisioning, human and environmental well-being.

Species substitution and use of additives could be very dangerous for human health. Some species of fish or molluscs may be potentially harmful. Some species of puffer fish (e.g. *Lagocephalus sceleratus*) contain tetrodotoxins, which are powerful neurotoxins that can cause fatalities. Eat such species without any provision can cause death resulting by muscular paralysis, respiratory depression and circulatory failure (Cohen et al., 2009; Hwang et al., 2002). Moreover, the consumption of a species rather than another can cause allergic reactions. According to Triantafyllidis et al. (2010) certain species are more allergenic than others as the case of Gadoids. For example, in Greece, the word "bakaliaros" generically identify different Gadoids species, including different species like cod, haddock and others, which are not native of Greek waters but are imported from other countries and they are labelled with the generic Greek common name. For a fish allergic consumer, it is important know the real Gadoids species since they are not equally allergenic. It is indeed important that the scientific name must be reported on the label. Furthermore, substitution of species could exist for fish not allowed to be sold as food because of high levels of toxic substances (e.g., mercury, which should be particularly avoided by pregnant/nursing women and children) in their flesh, could be mislabeled as a type of fish considered edible, presenting a health risk (Clark, 2015).

Another kind of threat for people health is the lack of origin declaration of seafood. It is noteworthy to observe that most of the fish products come from outside Europe, where controls on farming sites, pathogens and bioaccumulation of heavy metals are lacking, and there maybe polluted waters (Filonzi, et al., 2010).

Seafood frauds heavily affect the environment and the whole fish supply chain. Sometimes seafood could be a product of illegal catch. Illegal, unreported and unregulated fishing (IUU) contributes to fish frauds in that IUU fish catches are illegally marketed and laundered through the legitimate fish marketing chain. Mislabeling threatens endangered fisheries, as vulnerable species may be intentionally misbranded as another type of fish or from a sustainable fishery to benefit from high prices in niche markets (Clark et al., 2015). Mislabeling and inaccurate identification of species in fish landings or modifying the capture area also contributes to underreported exploitation of stocks and the consequent

reduction of fishery resources. This lack of control plays an important role in the threatening of fisheries sustainability despite international efforts, and can even imply the eventual extinction of the most vulnerable overexploited species (Agnew et al., 2009), contributing to the loss of biodiversity. This situation not only does undermine consumer confidence in fish and seafood products, it places honest local or domestic producers, the organic farmers and sustainable fisheries, at a disadvantage when fraudulent suppliers or importers undercut their margins through substitution, overfishing, or disobedience of fisheries regulations. Actually, seafood frauds are a threat for the ecosystems services provided by marine environment, directly linked to the humans wellbeing and the economy linked to such species and to the honest merchants besides

2.2.2 DNA barcoding and other molecular techniques for seafood frauds identification

In order to identify a food fraud, it is essential to be able to strictly recognize the examined species. The consumer might be often unable to verify whether what they are buying or eating corresponds with that stated on the label or on the menu. The identification of a fish or shellfish to species level using traditional morphological methods is difficult if not impossible also for a specialist. The morphological characteristics such as skin and heads are lost when the fish is processed, and other characteristics such as color might be unstable after freezing or cooking. The only unequivocal way to identify a species and the eventually food frauds is by mean molecular investigations.

The first seafood fraud recorded was in 1915 (figure 2.5) when *The New York Time* reported that shark meet was sold as swordfish. Most studies have been conducted since the turn of the 21st century, as shown in figure 2.4. The reason of such increase it is not because fraud did not exist before, as they are old as the humanity history; but from 1980s to today there has been a strong improvement in technical skills. Particularly from 2000, there were rapid advances in DNA-based technology, the species authentication method used by most researchers today. Protein analyses such as isoelectric focusing, high-performance liquid chromatography (HPLC), or enzymatic analysis (for example, allozymes) have been used for species identification of meats (Tepedino et al., 2001; Ukishima et al., 1991). Unfortunately, these identification methods are not suited for

commercial products processed by heating, boiling, or drying because proteins are easily degraded by these methods of preservation. On the contrary, DNA is comparatively less affected by preservation/processing conditions and the analysis thereof would yield the same result regardless of the sampled part or the growth stage of the fish (Bartlett & Davidson, 1991; Quinteiro et al., 1998). Additionally, DNA identification methods generally give better resolution than the traditional morphological or protein identification methods.

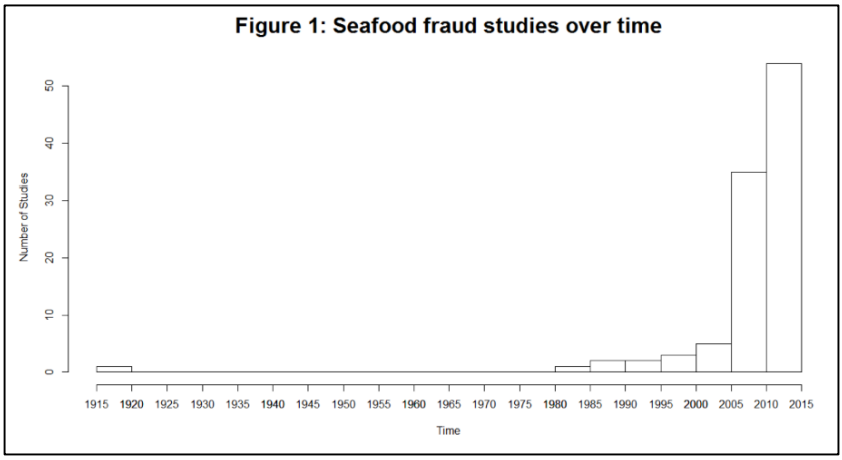

Fig. 2.5 The first seafood fraud record in 1915 and the increment in seafood frauds identification from 1980s

Genetic traceability is based on genome studies, employing different DNA markers, that is, physically identifiable locations within a chromosome called “loci” (singular “locus”). These loci may be located in expressed regions of DNA or, more often, in DNA segments with unknown coding function. DNA markers show allelic variants that result in polymorphism-producing DNA mutations. These variants can be identified by molecular techniques used to classify the genotype, and these techniques can be applied to identify individuals, populations, species, or groups of interest (Larraín et al., 2014). Whereby DNA-based technology has simplified the process of species identification and made it more cost-effective than the previous techniques (morphological identification, isoelectric focusing, etc…). In the last decade, molecular barcoding has been proposed as the favorite methodology in forensic taxonomy (Dawnay

et al., 2007). The idea of a barcode is significant in the sense that every single species is identified with its unique code. DNA barcoding is based on the sequence diversity of a short DNA region (DNA barcode) in the genome that possesses a high interspecific, but low intraspecific, variability for reliable differentiation between species (Galimberti et al., 2013). The mitochondrial reference marker gene cytochrome oxidase subunit 1 (CO1), around 650 base pair, is relatively conserved between species but at the same time has a sufficient variation to allow species differentiation. Several other markers have already been used for fish authentication as the Cytb (Cytochrome b) (Akasaki et al, 2006; Céspedes et al., 2008; Parson et al., 2000). However, **t**he mainly used barcode has always been the COI. (Hebert et al., 2003) first suggested the usage of the mitochondrial gene COI as the core of a global bio identification system for animals. Today COI is the most used barcode, widely used in various fish and shellfish identification studies such as in pangasius, tilapia, rainbow trout, sea bass, sea bream, turbot, sole, red mullet, grouper, atlantic cod, halibut, shortfin mako, european hake, european and nile perch, atlantic salmon, butterfish, swordfish, porbeagle, piked dogfish, blue shark (Di Pinto et al., 2015; Filonzi et al., 2010; Galal-Khallaf et al., 2014; Pardo et al., 2018). On different online database (Standard Sequence Library for Seafood Identification, GenBank and FISH-BOL) DNA sequences can be compared with standard sequences for fish identification.

The right species identification is very important also because the commercial name of some species can often encompass several species, varying across different countries, and even different regions. This can hinder consumer's choice since in some cases, species with different market prices can be marketed under the same commercial name (Pardo et al., 2018). For this reason, European Union labeling laws states that seafood products must be labeled with the complete scientific name of the species (i.e. genus and species, Latin binomial nomenclature).

Thanks to the genetic analysis it is also possible determine the geographical origin of seafood, this is an important point in order to manage and preventing the IUU (Illegal Unreported and Unregulated) fishing. Advanced DNA analysis has been successfully used to identify the river of origin of wild-caught salmon (Horreo et al., 2017). Population genetic studies can identify differences between populations of seafood from different geographical area. Such aim could be reach using different molecular markers and particularly with advanced DNA analysis, such as next-generation sequencing. These methods need further development and

need to become cheaper before use in routine official food control programmes. However, an international project, FishPopTrace, funded under the 7th Framework Programme of the European Union, is investigating the use of next-generation sequencing for product traceability and policy-related monitoring, control and surveillance in the fisheries sector.

The field of molecular diagnostics for authentication of seafood is a rapidly developing area. Within the sphere of DNA-based approaches, recent techniques applied in the food frauds detection are the High Resolution Melting (HRM) and the use of microsatellites (Rasmussen & Morrissey, 2008) and pyrosequencing (Abbadi et al., 2017). HRM It is an excellent tool for the identification and differentiation of closely related species or cultivars, identification of pathogens, screening of genetically modified organisms and detection of food allergens; is a post-PCR analysis method used for identifying genetic variation in nucleic acid sequences. Is a simple and fast method, based on PCR melting curve techniques and is enabled by the recent availability of improved double-stranded DNA (dsDNA)‑binding dyes along with next-generation real-time PCR instrumentation and analysis software. HRM analysis can discriminate DNA sequences based on their composition, length, GC content, or strand complementarity. It has been used to identify meat (Sakaridis et al., 2013), olive oil (Montemurro et al., 2015; Pereira et al., 2018), wine (Pereira et al., 2018) and seafood frauds, identifying different species of cod (Tomás et al., 2017;), shrimps (Fernandes et al., 2017) and mussels (Jilberto et al., 2017).

Besides DNA analysis, proteomics is reassessed compared to the past as according to different authors it can provide suitable and powerful tools to investigate main aspects of sea food quality and safety (Carrera et al., 2004; Mazzeo & Siciliano, 2016; Ortea et al., 2016).

However, molecular investigations on genomics or proteomics are the only arm to fight food frauds and are essential to improve the techniques and make them cheaper. The adoption of these methods requires technical expertise and a high level of food laboratory capacity. There is also a need to standardize and accredit methodologies (Griffiths et al., 2014; Vartak et al., 2017) and to harmonize DNA databases for confirmation of barcodes. Moreover, developing countries may need technical assistance to integrate this system into their food control structures.

2.3 Mussels threatened by seafood frauds

Fishes are the seafood products most involved in food frauds, but shellfishes and crustaceans are also threatened by food frauds. Besides species substitutions, molluscs are particularly interested by false origin declaration and bad storage conditions or a lack of depuration steps. In order to avoid frauds buying mussels, some rules must be followed; these rules are generally valid for all molluscs, not only for mussels. According with the laws n. 104/2000/CE and n. 2065/2001/CE for seafood labels, when molluscs are sold their label have to report the right species name and origin and a list of additional information, if just one of the following points is not respected, there is a fraud:

- molluscs must be bought in places authorized for sale like fish markets and supermarkets;
- they must be sold packaged, with a seal and a clear label;
- the products can also be sold in bulk but must be taken from 5 or 10 kg packages and the seller must keep the label for the next 60 days;
- at the purchase time they must not be kept submerged in water;
- at the purchase time the bivalves must be alive, with the valves intact and well closed (when open they must oppose resistance to opening);
- when opened they must have a typical smell of the species, but not unpleasant.

For the most common fraud, the species replacement, it is easy to think that it should be easier to identify different mollusc species rather than fishes, especially fillets, as molluscs have the shells. Actually, molluscs identification is difficult as well as fish fillet identification because some species are very similar and they often show a high shell plasticity. According with different authors, Mytilus species in Europe are difficult to identify using only shell characteristics because of adaptations of the shell to different environmental conditions, and also because of hybridization between pairs of taxa (Bierne et al., 2002; Gardner & Thompson, 2009; Riginos & Cunningham, 2005).

Genetic and/or molecular analysis, as well as for fish, are fundamental also for mussels identification. There are two “levels of traceability”: the first one is the species identification, the second regards the geographical origin of the examined species. Many mussels species have a great economic value (e.g. *Mytilus galloprovincialis, Mytilus edulis, Mytilus*

chilensis, Perna canaliculus), in order to avoid frauds is fundamental recognize them. From the letterature different PCR-based analysis were performed in order to distinguish mussels species: simple PCR, Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP), Forensically Informative Nucleotide Sequencing (FINS), Amplified Fragment Length Polymorphism (AFLP) (Canesi et al., 2012; Rasmussen & Morrissey, 2008; Rego et al., 2002). In all these analysis, different molecular markers were used: COI and Cytb (the two most used markers for fish identification, with the DNA barcoding), ITS1 and ITS2 (Internal Transcribed Spacer 1 and 2), 18S, 16S, the adhesive protein gene, Me 15/16 and COIII (Abbadi et al., 2017; Rasmussen and Morrissey, 2009, Rego et al., 2002). PCR-based methods associate with the variability of most of the molecular markers previously listed can be used to study the genetic diversity and population structure and consequently mussels, or other organisms, origin.

In addition to the PCR-based analysis, other DNA-based methods were tested in order to identify both mussel species than their origins. Among these techniques very useful tool are the microsatellites, according with Larraìn et al. (2014) and the HRM analysis (Jilberto et al., 2017). According with Abbadi et al. (2017) pyrosequencing also provide an alternative, simple, rapid and economical tool to detect seafood substitution frauds.

Moreover proteomics and metabolomics are new approaches useful to identify food frauds also in mussels species. Proteomics were used in López et al. (2002) in order to identify different mussels species. Rochfort et al. (2013) demonstrate that metabolomics approach is particularly suitable in the investigation on mussels provenance determination.

2.3.1 The situation in Italy and in Campania

In Italy every day police officers are involved in food inspections. Among the Italian armed forces, the most involved in seafood inspections is certainly the Guardia Costiera. Sometimes there are national control plans against seafood frauds, is the case of the operation “Tallone d’Achille” carried out between November 2015 and January 2016. Inspections were done throughout the whole fish supply chain and in different places: retail and wholesale fish market, in the ports at landing sites, in the restaurants. Most of the violations concern the traceability and

the labels (with 647 penalties on 1267), followed by sanitary conditions (125 penalties), recreational fishing (109 penalties) and illegal fishing (74 penalties) and a list of others irregularities (PNI, 2015). Another national control plan is "Labyrinth", conducted in the years 2014/2015. As the previous cited one, also in this national plan the penalties of the traceability and the labels are around the half of the total penalties, followed by sanitary conditions and illegal fishing (Relazione Controllo Pesca, 2014). Moreover according with an activity report of the Guardia di Finanza for the year 2016, around 80 kg of seafood were seized just before Christmas days, a periodof important seafood request. Three quarters of the seizured safood is represented by molluscs and crustaceans (61.947 kg); followed by fresh, chilled and frozen fish (22.946 kg); finally caviar, tuna and salmon (740 kg).

Seizure seafood are not only fishes, an important part of such products is represented by molluscs particularly bivalves. The report "Molluschi bivalvi" (2016) records tons and tons of seizured mussels in the province of Napoli, Bari, Ancona and Reggio Calabria. In the newspapers is also frequently report the seizure of molluscs in different Italian regions. For examples, in Lecce mussels and others molluscs illegally imported from Greece and without health stamp were seizured (Repubblica, 2016). In the same area, around one quintal of mussels was about to be commercialized with a false label. Such mussels came from the Taranto Small Sea, with unacceptable value of dioxin and Pcb so mussels were not edible, instead the false label declared the opposite (La gazzetta del mezzogiorno, 2018). In the area around Roma, 9 tons of mussels were seizured becouse without traceability information and no origin declaration, moreover the truck was not authorized for the transport (La Stampa, 2018). In the north of Italy are also frequently seizured mussels, as happened around the Lake Maggiore where mussels were sold without authorizations (La Stampa 2018).

Illegal mussels are found in the supermarket counters throughout Italy but some areas are particularly affected by this problem as Campania region, in which also mussels production is very high. The newspapers are unfortunately full of reports of food frauds regarding mussels in Campania. Tons and tons of mussels were seizured becouse of illegal sold (Il Mattino, 2018), from illegal mussel farms (Il Mattino, 2017; Corriere del Mezzogiorno, 2015), for a lack of traceability (Internapoli, 2018).

Food frauds cause an important image and economic damage for honest mussel farmers. The image damage is caused because such situation

can discourage potential buyers, the economic damage is caused by the adulterated product on the market, often cheaper than the legal ones.

2.4 Enhance Campanian mussels and their local market

Seafood frauds negatively affect the market, the local honest dealers and mussel farmers. One of the aims of this PhD project is enhance Campanian mussels and the linked economy, their production systems and their value on the market. Mussel farming is a well-established activity along Campanian coast, it is also an activity rooted in the history of the area; for these reasons is important enhance and safeguard mussel farming and its products by frauds. As reported by the police officers reports, the newspapers and the personal mussel farmers observations, seafood frauds that threaten this activity are the poor hygienic conditions (both for storage than for depuration), the non-authorized stores and farms and particularly the lack of labeling, so without any indication of the species or origin.

The lack of mussels origin declaration can imply that adult individuals could be sold as local species (a local species is more appreciated by the buyer), actually they are from other italian or european regions. Moreover, the non-declaration of the origin allows not honest mussel farmers to import seed from other geographical areas, or sometimes in mussels natural environment, also causing environmental damage. In addition, a false declaration of the origin and/or the absence of this information could imply health problems to people as often mussels have to be store in the sewage treatment plants before sold, depending on the water quality where they are farmed.

The other important food fraud is the species replacement; it could be easily happens with mussels for their plasticity. This kind of fraud is mostly report for fish, but also mussels are certainly affected. *Mytilus galloprovincialis* is the autochthonous Mediterranean mussel species of the genus Mytilus. Are all the mussels sold with such “name” really belonging to this species? Are the defined “local mussels” real born and raised in the same local place? Our study aim is the investigation on such questions with final goal to enhance the local campanian mussel and the linked supply chain. False origin declaration and species substitutions can affect local honest farmers and so can affect the economy; moreover can affect people health if sold conditions are not hygienically correct or if mussels came from unauthorized farms. Finally false origin declaration and species substitution can affect the local environment, e.g. if mussel seed is taken in large quantities or new species are accidentally introduced for farming.

To reach our aims, we have used genetic tools, with DNA-barcoding based technique for the species identity, and population genetics

studies to identify the origin of mussels. We have sampled them in their natural environment and from mussel farms, both from local mussels farms that from farms of other Italian and European geographical areas. We have used four different molecular markers: COI, 16S, PAPM and 28S, both for the genetic characterization of mussels species than to identify their origin. The overall project goal is the enhancement of local Campanian mussel and the local economy linked to mussel supply chain by means of a molecular technique that is fast, realible and at relative low cost.

2.4.1 Sampling area

The identification of a local mussel is possible just comparing different mussel populations, for this reason we have sampled mussels along Campanian coasts, in the Campanian mussel farms and in the local fish market. Moreover, for the market samples we have tested if the species is really that one reported in the labels, *Mytilus galloprovincialis*. The declared origin of sampled mussels indicate different geographical areas (both from different Italian regions than from European countries) as it shown in figure 2.6. In Spain the main area of mussel production is Galicia, even if mussel culture is also being recently developed in Andalusia. Samplings were carried out for two years, from 2016 to 2018.

Fig 2.6 In the map, in green are reported mussels origin of the sampling carried out at the fish markets. In red is marked the mussel farms sampling area. In yellow, the natural environmental sampling area.

Samplings at the fish market were mainly carried out at the wholesale fish market of Pozzuoli and in different local fishmarket. Mussels sampled in the markets are from different geographical areas as shown in figure 2.7 c, however all of them are from a mussel farm of the indicated area. Besides the farms of Campania area (Santa Lucia, in the Gulf of Napoli, and the Gulf of Pozzuoli), mussels are from Chioggia, Ravenna, Sabaudia, Gaeta and Spanish mussel farms. When we sampled it, the origin was clearly indicated on the label, as it shown in figure 2.4.

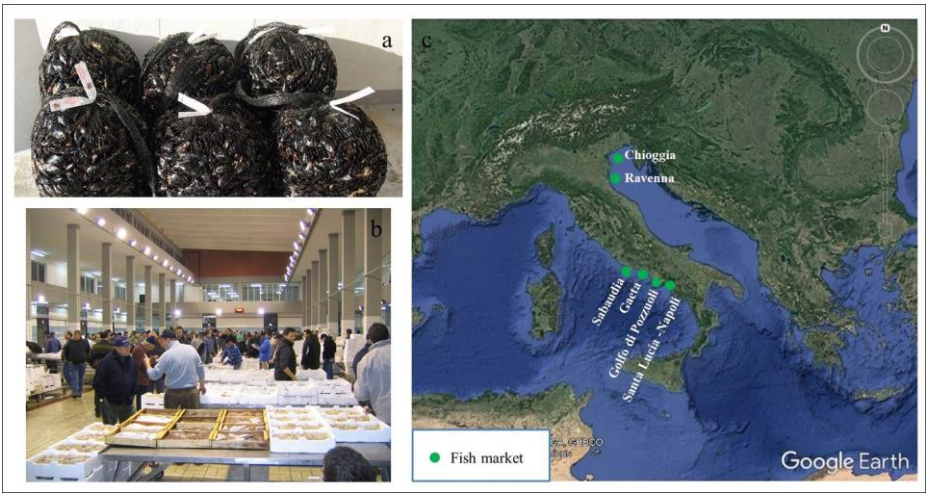

Fig. 2.7 a) mussels sold regularly in packages of 5 or 10 kg; b) the wholesale fish market of Pozzuoli; c) the map reports the origin of mussels sampled at the market, apart from the various reported Italian there is also a Spanish sample.

Samplings in the mussel farms were done in different Campanian mussels farms together with the mussel farmers, during their daily operations (figure 2.8 a, b, c and d). Mussel farms where we carried out our samplings are in the Gulf of Pozzuoli, in the Litorale Domitio e in the Gulf of Napoli (figure 2.9 b).

Fig. 2.8 The Elisea mussel farmer's daily operations. a) pull out of the water musselssacks; b) separate mussels by size; c) put the small size mussel into new socks; d) adult individuals packaged to sell.

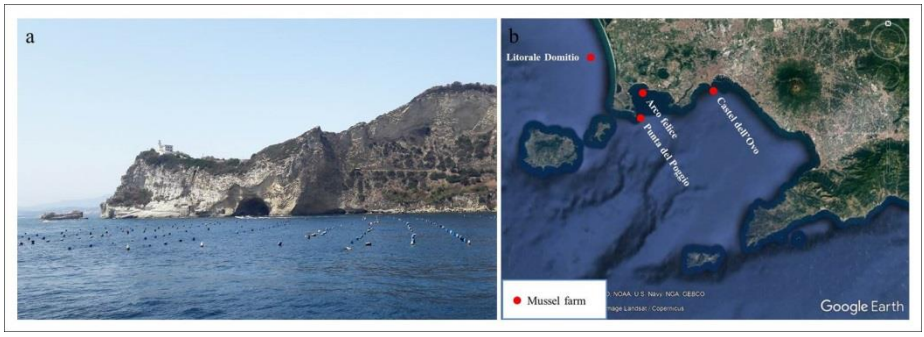

Fig. 2.9 a) mussel farms of Punta del Poggio (Capo Miseno, in the Gulf of Pozzuoli); b) Campanian mussel farms, here we have sampled local farmed mussels.

Samplings in the natural mussels environment were done in snorkeling and/or ARA. Mussels were sampled in shallow coastal waters (snorkeling) and along to the buoys ropes in deeper water, around 10 to 15 meters deep (ARA). Samples from natural environment are from different area of Ischia Island (Cartromana, Castello, Lacco Ameno, Punta Caruso, Sant'Angelo and Scannella), Capri Island (Grotta Azzurra and Porto) and from Baia, in the gulf of Pozzuoli. The previous listed sites are reported in figure 2.10 b

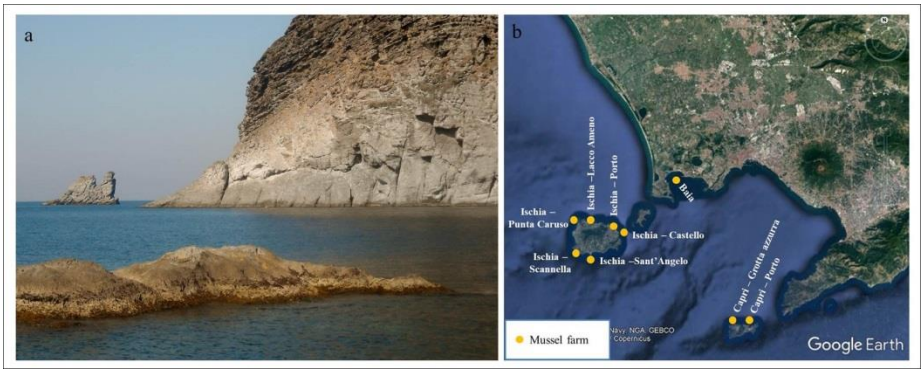

Fig 2.10 a) Scannella, an environemtal sampling site of Ischia Island; b) all the environmental sampling sites in Campania.

2.4.2 DNA extraction

The DNA was extracted from 266 individuals, mussels were sacrificed in order to extract the DNA. Live mussels were opened and a piece of mantle of around 0.1g were sampled and used for the extraction. When the mantle sample was not immediately processed, it was stored at -20°C. DNA extraction was performed both from fresh than frozen tissue.

Two different extraction methods were tested: a specific adapted protocol to molluscs and a DNA extraction kit (the E.Z.N.A. ® Mollusc DNA Kit). The specific protocol was performed using the CTAB as extraction buffer and phenol/Chloroform/Isoamyl alcohol to isolate the DNA.

The quality of extracted DNA has been checked with the electrophoresis. Both the two extraction methods resulted in a good DNA

quality, the quantity was a little bit higher for the manual protocol extraction but at the expense of a higher timing.

2.4.3 Molecular markers

The genomic extracted DNA was amplified through the Polimerase Chain Reaction (PCR) with the thermocycler Thermal Cycler C1000 Touch and the Thermal Cycler S1000. Four different molecular markers were used (COI, 16S, PAPM and 28S) (table 2.1).

Tab. 2.1 Mussels sampling and molecular markers.

Origin	Sampling site	Sampling date	Molecular markers			
			COI	16S	PAPM	28S
Baia	Natural environment	29/08/2016	✓	✓		
Capri - Grotta azzurra	Natural environment	30/04/2016	✓	✓		
Capri - Porto	Natural environment	29/04/2016	✓	✓		
Ischia - Cartromana	Natural environment	17/04/2016	✓	✓		
Ischia - Castello	Natural environment	30/05/2016	✓	✓		
Ischia - Lacco Ameno	Natural environment	15/04/2016	✓	✓		
Ischia - Porto	Natural environment	30/05/2016	✓	✓		
Ischia - Punta Caruso	Natural environment	21/06/2016	✓	✓		
Ischia - Sant'Angelo	Natural environment	16/04/2016	✓	✓		
Ischia - Scannella	Natural environment	01/07/2016	✓	✓		
Vietri	Natural environment	11/06/2016	✓	✓		
Arco Felice	Mussel farm	05/08/2016	✓	✓		
Castel dell'Ovo	Mussel farm	19/05/2016	✓	✓		
Litorale Domitio	Mussel farm	28/09/2016	✓	✓		
Punta del Poggio	Mussel farm	29/08/2016	✓	✓		
Chioggia	Fish market	18/03/2016	✓	✓		
Sabaudia	Fish market	11/03/2016	✓	✓		
Spagna	Fish market	18/03/2016	✓	✓		
Gaeta	Fish market	08/05/2018	✓	✓	✓	✓
Golfo di Pozzuoli	Fish market	09/05/2018			✓	✓
Ravenna	Fish market	10/05/2018			✓	✓
Santa Lucia (Napoli)	Fish market	10/05/2018			✓	✓

Two mithocondrial fragments COI and 16S were initially used to characterize mussels from 19 sites (11 from natural environment in the Gulf of Naples and Gulf of Pozzuoli; 4 from mussel farms in the Gulf of Naples and Gulf of Pozzuoli; 4 from fish markets best representative for the main Mediterranean commercial areas). Three individuals for each site

were analyzed with both the markers. In order to investigate about the origin and the distribution of the species among the local fish markets we added to our analysis two nuclear markers, the PAPM and the 28S. Molecular analyses with theese marker were applied just on four different sites but we increased the individual's number when possible. We have focused the carachterization with the markers PAPM and 28S on two local mussel populations, in the Gulf of Naples (site of Santa Lucia) and in the Gulf of Pozzuoli; a geographically close population, Gaeta; and finally a more distant one, in Ravenna. In the table 2.1 are summarized samples origins, sampling sites and the molecular markers used.

2.4.3.1 Molecular marker: COI

The Cytochrome c oxidase subunit I (COI) marker is a mitochondrial marker. Generally, mitochondrial DNA (mtDNA) is a useful marker to investigate genetic diversity and it has been used to analyse polymorphisms and define stocks in many bivalve marine species (Arnaud-Haond et al., 2003) also in *Mytilus galloprovincialis* (Luis et al., 2011). COI is so widely used due to its technical simplicity, abundance of selectively neutral mutations, and low rate of recombination. In 1994 Folmer et al. adfirmed that COI primers generate informative sequences for phylogenetic analyses at the species and higher taxonomic levels. Moreover, its use was even more widespread since Hebert et al. (2003) suggested the COI marker in order to identify each animal's species. Today is the most used marker in the DNA barcoding (see paragraph 2.2.2). COI was also used in phylogenetic studies of different molluscs species (Feng et al., 2011; Fernández-Pérez et al., 2017; Khatami et al., 2018).

In our research, we have characterized the sampled mussels with the COI mithocondrial marker (Folmer et al., 1994) in order to identify the species and eventually their origin. The length of the amplified COI gene region was 593 base pair (bp); primers composition and PCR conditions were reported in the table 2.2.

Table 2.2 The molecular marker COI

Primers COIF/COIR*	COIF: 5'-GGTCAACAAATCATAAAGATATT-3' COIR: 5'-TAAACTTCAGGGTGACCAAAAA-3'
PCR mix	17,9 µl of dd water, 2 µl of buffer, 2 µl of dNTPs, 0,8 µl of COI F and 0,8 µl of COI R, 0,5 µl of Taq DNA Roche, 1 µl of DNA
PCR conditions	Denaturation: 94° for 5' 35 cycles [Denaturation: 94° for 1' Annealing: 40° for 1' Elongation: 72° for 45"] Elongation: 72° for 7' Storage: 4° forever

**Folmer et al., 1994*

2.4.3.2 Molecular marker: 16S

The gene coding for the large mitochondrial RNA subunit (16S) is easily amplified by means of general primers and has been widely applied in phylogenetic studies in different groups, among molluscs too (Barucca, et al., 2004; Canapa et al., 2003; Feng et al., 2011; Fernández-Pérez et al., 2017; Khatami et al., 2018; Thollesson, 1999). Its essential function and ubiquity, have allowed the mitochondrial markers 16S to be a very common and useful markers in such kind of studies.

In our research, we have used the 16S fragment of Palumbi et al. (1996), amplifying around a 507 bp region. Primers composition and PCR conditions were reported in the table 2.3.

Table 2.3 The molecular marker 16S

Primers 16SF/16S R*	16SF: 5'-CGCCTGTTTATCAAAAACAT-3' 16SR: 5'-CCGGTCTGAACTCAGATCAGT-3'
PCR mix	12,9 µl of dd water, 2 µl of buffer, 2 µl of dNTPs, 0,8 µl of COI F and 0,8 µl of COI R, 0,5 µl of Taq DNA Roche, 1 µl of DNA
PCR conditions	Denaturation: 94° for 5' 35 cycles [Denaturation: 94° for 30" Annealing: 52° for 30" Elongation: 72° for 1'] Elongation: 72° for 7' Storage: 4° forever

** Palumbi et al., 1996*

2.4.3.3 Molecular marker: PAPM

The gene for the Polyphenolic Adhesive Protein of Mussels (PAPM) was used to identify different species of Mytilus by length polymorphism and RFLP by Santaclara et al. (2006) and more recently by Jilberto et al. (2017) using PCR coupled with HRM analysis. Moreover in May 2018, the same authors patented the method described in their research as a simple, rapid and less costly method in order to identify mussel species of the genus Mytilus, the patent disclosed can be used by companies or laboratories that serve the mussel industry and authorities in order to respond to the traceability demands, particularly, to certify-authenticate the species of the raw material used (Patent EP3315612A1). PAPM primers (Jilberto et al., 2017) and PCR conditions are reported in table 2.4, the amplified fragment is of 114 bp lenght.

Table 2.4 The molecular marker PAPM

Primers PAPMF/PAPMR*	PAPMF: 5'-GGAACAAAGCATGGACCA-3' PAPMR: 5'-GACAGCTTCTTTGCAAGTGG-3'
PCR mix	12,9 µl of dd water, 2 µl of buffer, 2 µl of dNTPs, 0,8 µl of COI F and 0,8 µl of COI R, 0,5 µl of Taq DNA Roche, 1 µl of DNA
PCR conditions	Denaturation: 94° for 5' 35 cycles [Denaturation: 94° for 30" Annealing: 52° for 30" Elongation: 72° for 1'] Elongation: 72° for 7' Storage: 4° forever

** Jilberto et al., 2017*

2.4.3.4 Molecular marker: 28S

28S ribosomal RNA is the structural ribosomal RNA (rRNA) for the large component, or large subunit (LSU) of eukaryotic cytoplasmic ribosomes, and thus one of the basic components of all eukaryotic cells. The gene coding for the 28S or LSU is often used in phylogenetic analysis, particularly the D1/D2/D3 region includes one of the most variable regions in the gene, for this reason useful in phylogenetics. In different molluscs phylogentic studies were used the nuclear marker 28S (Colgan et al, 2007; Hosoi et al., 2004; Passamaneck, Schander, & Halanych, 2004). 28S or

LSU from Hosoi et al (2004) was used in our investigations, amplifying a region of 661 bp; primers and PCR conditions are reported in table 2.5.

Table 2.5 The molecular marker 28S

Primers 28SF/28SR*	28SF: 5'-GGGTGGTAAACTCCAYCTAARGC-3' 28SR: 5'-CTRCGGACCTCCATCAGAGTTTCC-3'
PCR mix	12,9 µl of dd water, 2 µl of buffer, 2 µl of dNTPs, 0,8 µl of COI F and 0,8 µl of COI R, 0,5 µl of Taq DNA Roche, 1 µl of DNA
PCR conditions	Denaturation: 94° for 5' 35 cycles [Denaturation: 94° for 1' Annealing: 55° for 1,5' Elongation: 72° for 2,5'] Elongation: 72° for 10' Storage: 4° forever

**Hosoi et al., 2004*

2.4.4 Purification of PCR products, sequencing and molecular software analisys

All PCR products obtained were puriefied and then sequenced. The PCR products were purified with the Sigma GenElute™ PCR Clean-Up Kit following the supplier's protocol. PCR products were directly sequenced in both directions with the primers previously described used for PCR amplification (Tables 2.2, 2.3, 2.4, 2.5). The sequencing process was performed in a BigDye Terminator Cycle Sequencing (Beckman Instruments).

All sequences were corrected with Chromas, version 2.01. Then sequences were allined using BioEdit Sequence Aligment Editor (version 7.1.9), that is based on the alignment program ClustalW (Thompson, 1994). The software DnaSP - DNA Sequence Polymorphisms (Ver. 5.10.01) was used in order to evaluate the variability of the different markers and the polymorphisms carachterizing different individuals. Finally networks were obatained analysing our sequences with the software Network 5.0 Fluxus Technology Ltd.

2.4.4.1 The assessment of Mytilus galloprovincialis species

In order to identify the species of our sampled mussels, from the different sampling sites (natural environment, mussel farms and fish markets) sequences amplified with the COI and 16 markers were compared with the other sequences already available on databases. Particularly our sequences were compared on BLAST (Basic Local Alignment Search Tool), based on the algoritm MegaBLAST available on the web site http://www.ncbi.nlm.nih.gov (NCBI – National Centre of Biotechnology Information). The "query sequence", in our case nucleotides sequence, is blasted and is quickly compared to other sequences already present on the database. All the blasted sequences (57 for each markers) gained at least 98-99% of similarity with Mytilus galloprovincialis sequences of the database.

The result of this comparison demonstrate that all the mussels characterized using the universal primer COI and the large mitochondrial RNA subunit 16S all belong to the species *Mytilus galloprovincialis.* This is the species indicated on the labels of fish market sampling and the further confirmation that *M. galloprovincialis* is the autochtone local mussel species as it is always found in environmental samplings. Moreover, the assessment of that species also in mussel farms samples is the the proof that mussel *M. galloprovincialis* is the species that the farmers declare to bread.

These findings allow us to affirm that the food fraud of species substitution for *Mytilus provincialis* mussels does not occurr in Campanian fish markets. Probably is and understimated date as this kind of analysis should be done with a higher frequency and on a wide number of samples. However, the situation we have encountered almost certainly is reflected on the entire Campania market with a zero or very low incidence rates.

2.4.4.2 The attempt in local mussel origin identification

In order to identify a typical local mussel population, different from the populations of others Italian and European area, we have analysed COI, 16S, PAPM and 28S sequences polymorphisms and variability. Molecular markers, revealing polymorphisms at the DNA level, are key players in animal and plant genetics (Vignal et al., 2002). Actually, the most used markers are DNA markers as it is possible characterize different

genotypes and phylogenetic relationships in a simpler and more precise way compared to other methods (Soller & Beckmannm 1983). Moreover sequencing of DNA markers is useful in order to determine the history and evolutionary processes of a group of organisms, the genetic connectivity among populations and the phylogeography.

Polymorphisms analysis indicates the genetic variability of the examined sequences. A genetic polymorphism indicates the presence of multiple alleles of the same gene within a population. Generally are considered polymorphisms the variations (mutations, insertions and deletions, nucleotide variations) with a frequency higher than 1%. DNA polymorphisms of our sequences were measured using two parameters: the nucleotide diversity (π) and the mutations per sites (θ). π is defined as the number of nucleotide differences per site between two randomly chosen sequences in a population. The population mutation per site or mutation rate (θ) is one of the most fundamental parameters in genetics, ecology, and evolutionary biology; this parameter measures the effective size and whole-locus mutation rate of a population. In the table 2.6 are reported both π and θ values for each markers as well as the polymorphic sites.

Table 2.6 Markers variability and sequences polymorphisms

Molecular markers	COI	16S	PAPM	28S
Number of sequences	57	57	58	40
Sequence lenght (bp)	593	507	114	661
Invariable (monomorphic) sites	438	449	113	651
Variable (polymorphic) sites	155	58	1	10
Parsimony informative sites	127	51	0	6
Number of haplotypes	25	19	2	11
Nucleotide diversity (π)	0,07489	0,02607	0,00030	0,00230
Mutations per sites (θ)	0,05605	0,02444	0,00189	0,00356

In the table 2.6 are also showed the number of sequences used for each molecular markers analysis. For the markers COI and 16S the same sequences of the previous analysis were used, so a total of 57 sequences for each markers were used. The total sequences are representative of 19 different sampling site (table 2.1). For the other two markers, PAPM and 28S, were analysed just four sites but with a higher number of individuals per site compared with the other markers. The sites analyzed with the

PAPM and 28S are: Santa Lucia (in the Gulf of Naples) and the Gulfo of Pozzuoli, Gaeta and Ravenna (table 2.1). Respectively, were analysed 58 sequences amplified with PAPM marker and 40 with the 28S marker.

Based on the nucleotide variability data, a Median Joining network have been elaborated for each marker.

The COI marker has the the highest variability, showing the highest values of π and θ, identifying 155 polymorphic sites and 127 parsimony informative sites. Overall, the COI have distinguished 25 haplotypes, 8 main haplotypes and 17 smaller ones. In the figure 2.11 is represented the COI network.

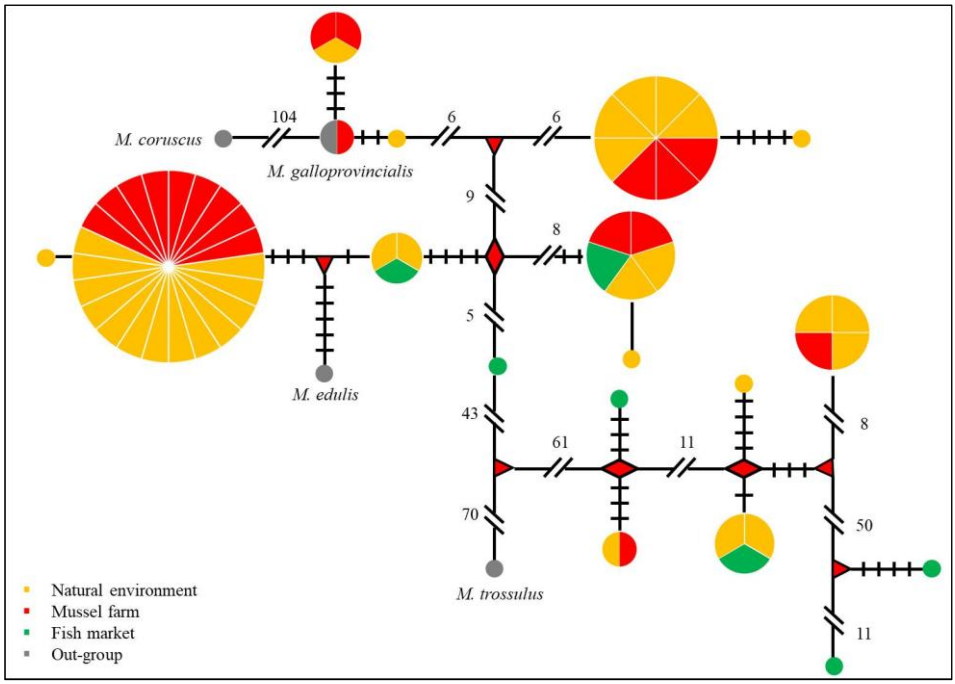

Fig. 2.11 COI network

In the network have been included 4 different out groups: *Mytilus edulis, Mytilus galloprovincialis, Mytilus trossulus and Mytilus coruscus*. As graphically evident in the network, although some main haplotypes were identified the marker COI is not able to distinguish different populations neither among the different geographical areas nor between the farming mussels and those found in the natural environment. The main

haplotypes are genetically closer to the *M. galloprovincialis* and *M. edulis* outgroups, the smaller haplotypes are all more distant; in anycase theese main haplotypes represent mostly a mix of individuals from natural environmental and mussel farms.

In conclusion, the COI nucleotide variability is very high but is not useful to our aim of separate different mussels population. However, the high nucleotide variability does not correspond to the same aminoacidic variability, such carachteristic is probably due the doubly uniparental inheritance mode of mtDNA typical of the mussel *Mytilus galloprovincialis* (Hoeh et al., 2002; Mizi et al., 2005).

The 16S marker has showed a high variability too, but with lower values of π and θ than the previous analized marker. 16S have identified 58 polymorphic sites, 51 parsimony informative sites and 19 haplotypes. Among the haplotypes there are 8 main ones (as it is reported on the network in figure 2.12), but as for the COI the situation is very heterogeneous, the nucleotide variability reflect a mosaic distribution and there is no evidence of a separation among environment/farms/markets sites. The species *Mytilus edulis, Mytilus galloprovincialis, Mytilus trossulus and Mytilus coruscus* were also used as out groups and the main haplotypes are always phylogenetically closer to the species *M. galloprovincialis* and *M. edulis.*

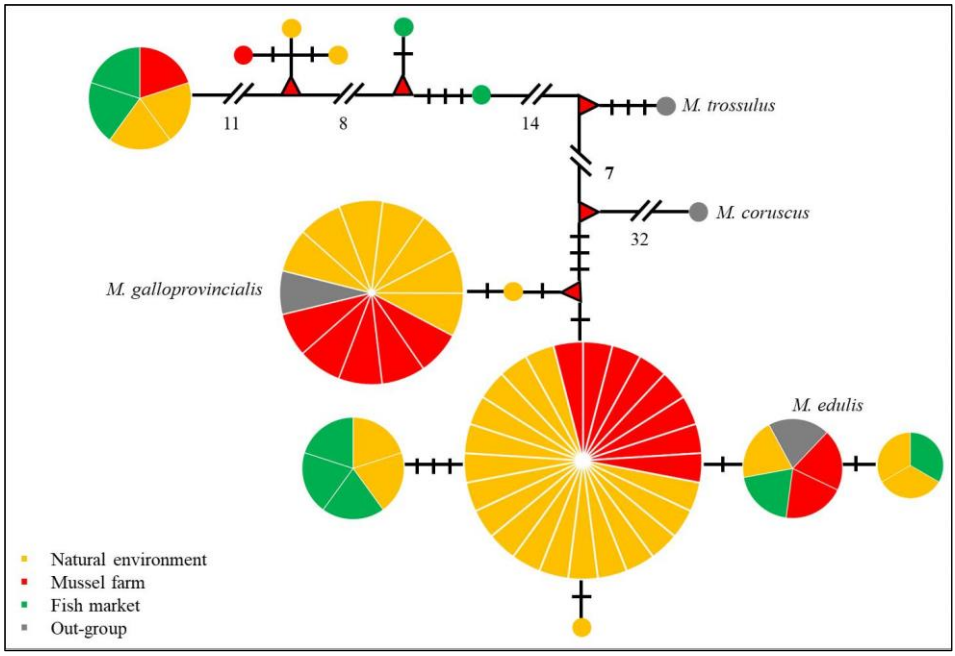

Fig. 2.12 16S network

The others two markers, the PAPM and 28S, have showed a less nucleotide variability, distinguising respectively 2 and 11 haplotypes. In the figure 2.13 is reported the PAPM network.

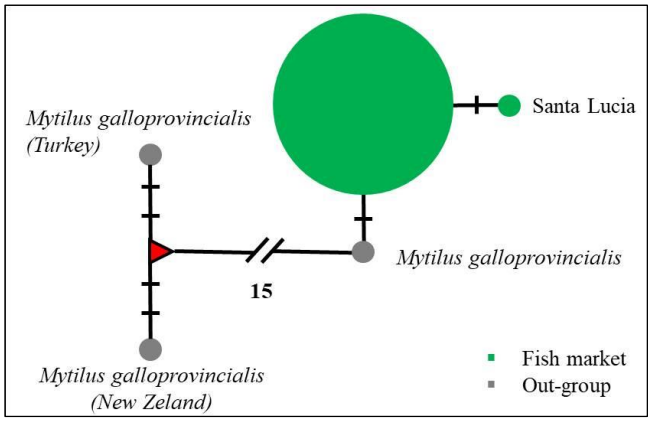

Fig. 2.13 PAPM network

All the individuals analysed and showed in the networks, both the PAPM one than the 28S, are sampled in fish markets. The PAPM nucleotide analysis shows less variability with a homogeneous distribution and the individuals (55) grouped together into one big haplotype. The PAPM network includes three out-groups: a *Mytilus galloprovincialis* individual from Turkey, one from the New Zeland and another one with an unknown origin (genbank data). *M. galloprovincialis* from Turkey and from New Zeland are each clearly seaparated by the main haplotype with 18 mutations, while one mutation separate the main haplotype from the unknown origin individual. One individual from the mussel farm in Santa Lucia, in the Gulf of Naples, is outside the main haplotype with one mutation.

The 28S network median joining analysis in figure 2.14 revealed one main haplotype (H_1) with all the individuals from the four sampling sites (Gaeta, Gulf of Pozzuoli, Santa Lucia and Ravenna). Connected to H_1 there are four smaller haplotypes respectively the h_2 (with individuals from Gaeta, Gulf of Pozzuoli and Ravenna), the h_3 (carachterised by only individuals from Gaeta and the Gulf of Pozzuoli) and the h_5 (with the individuals from Gaeta and Ravenna). The latter is the only "groupping individuals" haplotype that is connected directly to the main H_1. H_2, h_3 and h_4 are connected together and to the main haplotype separated by only one mutation. It is also evident the presence of characteristic small haplotypes/individuals from Pozzuoli (here called Pz_1 and Pz_2). A particular case is one individual from Santa Lucia (here called SL) that is separate by H_1 with two mutations and is connected to Pozzuoli (Pz_3) and h4 by means of two mutations.

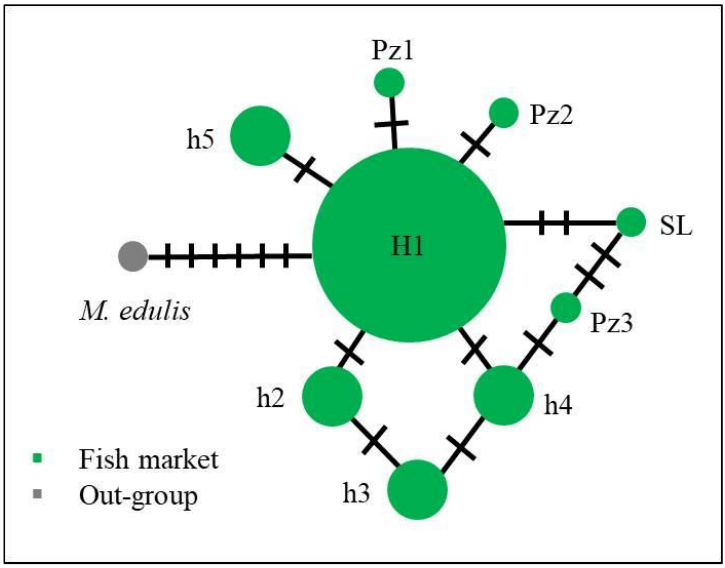

Fig. 2.14 28S network

The 28S, as the PAPM, revealed the unicity of Santa Lucia that is the only one separated by the main haplotype with two mutations. However, the 28S networks showed a more complex distribution of the individuals among the Gaeta haplotype (h3) but are also clear the connections among the different haplotypes.

Finally, the results of the PAPM and 28S analysis demonstrate that neither these markers can distinguish different Italian mussel populations. The identification of one big haplotype for both the markers demonstrate that in Italian mussel farms there is an heterogenity in mussel population, probably for the continuous interregional exchanges and for the seed recruitment, often a not practiced step in mussel farms. Moreover the seeds are not only the preferred introduction way and are often purchased also young mussels imported from other geographical sites. The result is an eterogeneus Italian mixture, the genetic identity of different Italian population were probably lost and this is more evident with the 28S analysis.

The PAPM have clearly distinguished *Mytilus galloprovincialis* individuals from different area: a mediterranean area (Turkey) and a pacific area (New Zeland). This finding demonstrates that geographically distant populations have a different genetic pool assemblage and can be

identified with this molecular marker. Even if for a few mutations, individuals from santa Lucia and the Gulf of Pozzuoli were genetically identified demonstrating that exist some differences among Italian populations. Particularly the mussels of Santa Lucia and that one of the Gulf of Pozzuoli could represent two different populations of the two areas characteristic of the Bay of Naples.

However new investigations are essential in order to have a clearly picture of the situation. Further efforts could be focused only on the Campanian mussel populations and using the markers PAPM and 28S on larger numbers of individuals. Other techniques could be applied, as the microsatellites (Diz & Presa, 2008; Gurney-Smith et al., 2017; Larraín et al., 2014) or the SNPs (Larraín et al., 2018; Xu et al., 2017) in order to try to identify if a local mussel population exist but must be considered the cost-benefit ratio (time to retrive the analysis results and economic resources for the molecular analysis). Once identified a local population or more local populations, the next step is the application of a rapid and cheap method to identify such population/s in order to evaluate potential frauds on mussels origin and consequently enhance local mussel.

2.5 Our findings, our fish market, our mussels

Considering our investigastion how is the situation for mussels in our fish market? Are really belong to the species declared in their labels? The mussel origin corresponds to that one reported on the label? Is it possible identifying a local population? All the mussels characterized with the universal primer COI and the other primer, 16S, belong to the species *Mytilus galloprovincialis*, the right species reported on the labels of sampled mussels. As regard as the other food frauds, on the product origin, and the identification of a local mussel population, further investigations are essential.

Our investigations suggest that, for mussel trade, there is no replacement food fraud in our local market. Replacement is the fraud that involves the substitution of a different species of that declared in the label product (Johnson, 2014). The sampled mussels in the mussel farms and at the fish markets were all clearly identified as *Mytilus galloprovincialis*, the authoctone mediterranen species despite were all from different origin areas (different sites of the Gulf of Naples and Gulf of Pozzuoli, Gaeta, Sabaudia, Ravenna and Spagna). All the mussels sampled in their natural environmental sites (different sites of Ischia and Capri Islands, Baia in the Gulf of Pozzuoli and in Vietri) were also genetically identified as the species *Mytilus galloprovincialis*. The same results were obtained with the characterization of the same samples with the other gene, 16S, often used in phylogenetic studies of bivalve molluscs (Barucca et al., 2004; Canapa et al., 2003; Feng et al., 2011; Fernàndez-Pérez et al., 2017; Khatami et al., 2017; Thollesson, 1998). Finally based on our sampling and the analysis of our molecular investigations using both the two genes, COI and 16S, we can adfirm that in Campanian fish markets there are no frauds on the declaration of mussels species.

However such situation could represent an understimated date, as we have genetically carachterized a very low quantity of mussels compared to the amount of mussels present on the markets. Probably species substitution is undesrtimated also referring to the whole Italian mussel market. We have analyzed just the Campanian market, mainly the Phlegrean area. In bigger markets, as in the big cities with a wider international trade, such kind of frauds could be more common. Moreover, in the national plans reports, as the Guardia Costiera “Tallone d’Achille” or “Labyrinth”, are not included DNA-based analysis for mussels. The genetic carachterization is a widespread practice just for fish, in fact the

DNA barcoding (Di Pinto et al., 2015; Filonzi et al., 2010; Galal-Khallaf et al., 2014; Hebert et al., 2003; Pardo et al., 2018) is now a common and useful practice for the fish species identification. According with the FAO report (2018) and Stawitz et al. (2017), fish traceability is the key to battle fish fraud, enforcing food safety regulations and ensuring high standards of sustainable fisheries management; traceability is also critical for ensuring the quality of fish products and minimizing health risks for consumers. This is true for all the seafood, for mussels too. So, as for fish exist the FISH-BOL (Fish barcode of life) in the same way it could be useful a "MUSSEL-BOL" in order to grouping genetic information on different mussels species, particularly the most economically important ones.

Scientific improvements are always crucial in order to identify frauds, looking for an increasingly faster and less expensive method. Recently, Jilberto et al. (2017) have applyed an easy and innovative technique to identify mussel species and hybrids, for its simplicity and usefulness their technique has resulted in a European patent. The patent EP3315612A1 (2018) provides a set of primers and method for detecting and identifying mussel species of the genus Mytilus in a rapid and less costly manner. The invention refers to a set of specific primers (PAPM primers used also in our investigations) and to the use thereof in PCR together with the High Resolution Melting (HRM) technique. The method disclosed can be used by companies or laboratories that serve the mussel industry and authorities in order to respond to the traceability demands, particularly, to certify-authenticate the species of the raw material used. This kind of analysis on mussels should be routinely used in forensic investigations in fish markets, the identification of species substitution frauds would be very easy, quickly and cheap.

The question is much more complex for the other kind of food fraud we have taking into account: the lack or false declaration about the species origin. The four molecular markers we have used (COI, 16S, PAPM and 28S) were not able to distinguish different mussel population, linked to their origin area. The markers with the high variability, the COI and the 16S have shown a mosaic distribution of the nucleotide variability with any evidence of a separation among environment/farms/markets sites and different geographical area. A similar result is obtained analizyng the other two less variables markers, the PAPM and 28S. However the two latter markers (tested on samples from Gaeta, Ravenna, Gulf of Pozzuoli and Santa Lucia in the Gulf of Naples) have identified a big main haplotype on which are represented individuals from all the different origin

sites, each one representative of a mussel farm. Such result demonstrates a heterogenity in mussel populations of the mussel farms despite located in different geographical area. An exception to this heterogeneous situation is the site of Santa Lucia. The marker PAPM has distinguished from the main big aplotype individuals from very geographycally distan area but at the same time it has distinguished a single individual in Santa Lucia. The 28S marker, even if for a few mutations, also revealed the unicity of Santa Lucia haplotype. Finally, in the heterogeneous scenario of the analysed mussel populations, the site of Santa Lucia give us hope for the existence of a local caractheristic mussel population in the Gulf of Naples. In a scenario on which the genetic identities of different Italian mussel population were probably lost, it is very important enhance and preserve local productions. The advantages of enhancing local mussels regard both the economy, encouraging local markets, and the environment health, and last but not least, the possibility to have fresh and high quality product.

Overall food frauds are an important threat for the whole mussel farming activity, both for the economy of mussel supply chain than for people health. The results of our study have shown that species substitution for mussels seems not happen in the Campania market. However, in order to preserve and enhance local market both from the species substitution than from the undeclared origin, scientific improvements are not enough. The other crucial element is the correct citizen formation. Science findings must be coupled with citizen awareness campaigns. The informations about the identification of the right species and its origin are fundamental in order to enhance local seafood and mussel economy. According with Stawitz et al. (2017) knowing such informations, consumers can make sustainable choices and prefer local products. However, citizens are often not aware of what sustainable choices are; for this reason awareness-raising project are of crucial importance. For fish there are different campaigns with the aim to raise awareness on the identification of fish species at the fish market, e.g. the Slow fish programs "*Mangiamoli giusti*" and "*Costruiamo la lisca della spesa*" (figure 2.15) and the European Commission program "*Come scegli il pesce giusto?*". Purposes of the mentioned awareness campaigns are the identification by consumers of the fish quality, the freshness, the right species (at least the most common) and sizes (not to small), the seasonality (which species are typical for each season), the identification and promotion of the less common fishes, that are products with an equal or higher nutrition value and taste of the most famous and requested ones. Such informations, in order to be more easily

accessible and quickly used, are also available on mobile apps. Apps examples are: “FishVerify”, “Applyfish”, “Che pesce sono?”. The last one was designed by the trade association Federcoopesca-Confcooperative togheter with the Ministry of Agricultural, Food and Forestry Policies. Anyway, supporting by awareness campaigns and mobile apps, consumers can make right nutrional but especially environmental sustainible choices. In this way, food frauds would occur less frequently; both frauds regard the origins of the product, as sellers would be less tempted by a high demand of non-local and out of season species, than species replacement, as buyers should be good at recognizing different fish species, at least the most common.

Fig. 2.15 Two different awareness-raising project for fish consumption.

As well as for fish, also for mussels could be very usefull give information to the consumers about the right season on which they can found local and fresh mussels at the market. *Mytilus galloprovincialis* in mussel farms became of a commercial size in around 18 months. Local mussels are not always available on the market. To increase local mussels consumpion, an app or any kind of information system, may be useful. Mussel farmers indicate when the seed is implanted (this operation places several times a year). From the implantation of the seed, just occur to calculate back the time on which adult mussels will be ready for market. In a typical Campanian mussel farm, marketable mussels are generally available two times in the year. Depending on the weather conditions, adult mussels in February have a first hatching, in around one month the very young mussels are already visible on the ropes (with the specific function of collecting the seed) and at the beginning of summer they

became young mussels of around 2 cm. The adult mussels are marketable in summer, so fresh mussels are available from June to September/October, according to the weather conditions. In September mussels can hacthing for a second time in order to have a second wave of marketable mussels aruond Easter time. So generally in Campania there are fhresh local mussels in two periods, a longer one in summer and another one in the early spring. A mobile app could make it known to consumers when these local mussels are ready on the market. As well as for fish consumption, consumers would probably change their habits preferring local products. They could even change their traditional habits on high mussel consumptions during Christamas time as during this period almost all the mussels on the market are imported, mainly from Spain.

However, increasing consumer knowledge on these dynamics it is a good input in order to direct their choices on the local product, enhancing local market and safeguard their health as in Italy there are very strict rules on hygienic conditions. It is often happened that contaminated mussels were imported from other European countries, mainly from Spain and Greece; contaminations were generally by the bacteria *Escherichia coli* and *Salmonella spp.* (Il fatto alimentare, 2017; Il Messaggero, 2018; Il Messagero, 2018; Il Giornale, 2018; Notizieora.it, 2018).

In conclusion considering the importance of mussel farming for Campanian market, in order to enhance this ancient activity and to value honest workers Campanian *Mytilus galloprovincialis* mussel could require an official recognition: a DOP or DOC recognition as the Italian "colleagues" *Scardovari* and *Nieddittas* mussels. It could be a prestigious title for honest Campanian mussel farmers, able to manage the whole production process from the seed collection. According with our findings, the Campanian mussel could be genetically identified. In this way Campanian mussel could be really be a unique mussel and the frauds on the origin about the local product could be easily identified. A strict genetic identity could be a great pride compared to the eterogeneus mixture of mussel populations in the Italian mussel farms. Moreover the evidence that haplotypes of Santa Lucia site, in the gulf of Naples, come out from the eterogeneous Italian haplotypes mixture, it could be a good base to further investigations to promote the identification of a Campanian mussel.

2.6 DNA extraction protocol for molluscs

- Cut a small piece of mantle tissue (~ 100 mg) and placed it in a 1.5 mL Eppendorf tube
- Add 700 µL of preheated CTAB* and 10 µL of proteinase K
- Pestle the soft tissue with a sterilized pestle for Eppendorf
- Vortex
- Incubate at 60°C for 1hour (mix by inversion after half hour)
- Add 30 µL of RNAase and vortex
- Incubate at 37°C for 30 min
- Add 500 µL Chloroform/Isoamyl alcohol (24:1) an mix by reversal
- Centrifugate at 14000 rpm at 15°C for 20 min
- Withdraw the supernatant and put it in a new Eppendorf tube
- Add 500 µL phenol/Chloroform/Isoamyl alcohol (25:24:1) an mix by reversal
- Centrifugate at 14000 rpm at 15°C for 5 min
- Withdraw the supernatant and put it in a new Eppendorf tube
- Add 500 µL Chloroform/Isoamyl alcohol (24:1) and shake
- Centrifugate at 14000 rpm at 15°C for 2 min
- Withdraw the supernatant and put it in a new Eppendorf tube
- Add 600 µL of cold isopropanol and mix by reversal
- Overnight at -20°C
- Centrifugate at 14000 rpm at 4°C for 30 min
- Withdraw the supernatant
- Add 600 µL of cold absolute ethanol and mix by reversal
- Centrifugate at 14000 rpm at 4°C for 5 min
- Discard the ethanol and allow drying on absorbent paper for ~ 1 hour
- Resuspend the extracted DNA in ddH2O (~ 60/80 µL)

*CTAB (Extraction buffer):
1% CTAB
1% PVP
50 mM Tris HCl (pH 8)
0,7 M NaCl
20 mM EDTA (pH 8)

2.7 Chapter 2 bibliography

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Chapter 3

Anthropogenic stressors: mussels responses to Ocean Acidification

Oral communication at the 31st ESCPB (European Society Comparative Physiology and Biology) Congress, the 9-12th of September 2018 (Porto, Portugal).

Title: "Anthropogenic stressors by mean of ocean acidification and food frauds on edible mussels species".
Fioretti S., Liu T. Z., Patti F.P., Tseng Y.-C.

3.1 Ocean Acidification: molluscs responses

Ocean Acidification, the phenomenon that led to increase the concentration of hydrogen ions (H^+) in the seawater, will change the oceans bio chemistry and the biology and physiology of oceans inhabitants (Cooly et al., 2009; Feely et al., 2004; Gattuso et al., 2015; Ries et al., 2009; Stapp et al., 2017). Since the industrial revolution, oceans have absorbed large amounts of CO_2, resulting in the acidification of the seawater. If anthropogenic CO_2 emissions will not decrease, is expected a pH decrease of 0.4 or 0,5 units by the 2100; such situation will lead the oceans pH from the actual value of 8.1 to a mean pH of 7.7 (IPCC, 2014; Valenzuela et al., 2018). Considering the pH has a logarithmic scale, this decrease corresponds to an increase of approximately 30% of H^+ in the seawater. Marine organisms react each one differently to OA, it is possible identify winners or losers organisms in these new conditions (Hall-Spencer et al., 2008; Kroeker et al., 2011; Rodolfo-Metalpa et al., 2011; Rodriguez-Dominguez et al., 2018). Moreover, the effects of OA could be also vary in the different life stages of the same organisms (Fabricius et al., 2011; Gattuso et al., 1998; Gazeau et al., 2007; Kurihara, 2008; Ramesh et al., 2017; Riebesell & Zondervan, 2000; Rodriguez-Dominguez et al., 2018; Schlegel et al., 2012). As the OA modify the carbonate biochemistry of the seawater, calcifiers organisms (molluscs, corals, echinoderms, crustaceans, algae) seem to be the main affected. The great majority of molluscs have developed shells as external calcified structures to support their living tissues, protect themselves against predators and exclude mud and sand from the mantle cavity for burrowing species (Frédéric Gazeau et al., 2013). The shell production is a ‘‘biologically controlled process’’. Shell calcification occurs in a small compartment, the extrapallial cavity, located between the calcifying outer mantle and the shell itself as represented in figure 3.1. The shell is a composite biomaterial, composed of a mineral phase (95–99 % predominantly calcium carbonate) and an organic matrix (1–5 %). Adult molluscan shells are commonly comprised of aragonite, sometimes calcite and, in certain taxa, layers of both calcite and aragonite (Addadi et al., 2006; Checa et al., 2005). In bivalves, the early shell is called the prodissoconch I. The first form of calcium carbonate produced is most likely in the form of amorphous calcium carbonate that will evolve more or less rapidly, depending on the species, to aragonite. Transformation into the motile veliger larva then occurs, and the prodissoconch I enlarge to form prodissoconch II. The final shell

(dissoconch) is produced at the end of larval development following the metamorphosis the veliger larvae into a juvenile specimen (Frédéric Gazeau et al., 2013). To form the shell, it has been hypothesized that the calcium carbonate saturation state increase in the extrapallial fluid by adding calcium and/or carbonate ions by passive transport or active pumping. The extrapallial fluid contains inorganic ions, including calcium and magnesium, and various organic compounds, including proteins, carbohydrates, and amino acids (Wilbur, 1983). Among such compounds, the metalloenzyme carbonic anhydrase (CA) have been demonstrated to play an important role also in the acid-base regulation process of vertebrates (Medakovic, 2000; X. Wang et al., 2017). CA catalyses the conversion of CO_2 to bicarbonate and vice versa ($CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+$), as illustrated in figure 3.1. Moreover molluscs produce organic compounds that help crystal synthesis (nucleation), selecting the calcium carbonate polymorph (calcite or aragonite), defining the morphology and the shape of the crystal and finally interrupting its growth (Frédéric Gazeau et al., 2013).

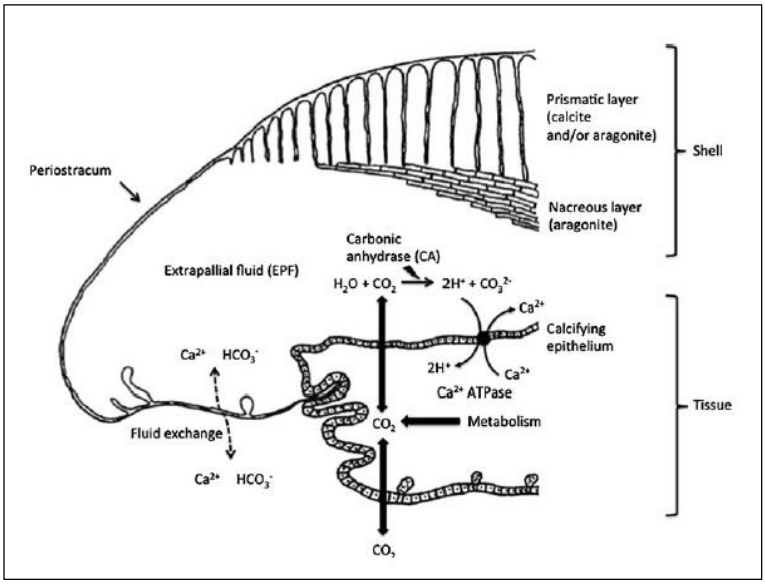

Fig. 3.1. Molluscan shell calcification process: takes place at the distal border of the shell, in the extrapallial space containing the extrapallial fluid, enclosed by the calcifying epithelium, the periostracum and the shell itself (Gazeau et al., 2013).

According with Parker et al. (2013), the calcification process is one of the ways in which OA strongly affects molluscs life. OA generally led a reduction in calcification rate with the severity depending on the $CaCO_3$ polymorph which molluscs secrete, their capacity to regulate acid-base status and the physicochemical environment of the population. A reduction in calcification rates has been shown in several molluscs: the mussel *Mytilus edulis*, the oysters *Crassostrea gigas* (Frédéric Gazeau et al., 2007) and *Crassostrea virginica*, the pearl oyster *Pinctada fucata* (Welladsen et al., 2010), the hard clam *Mercenaria mercenaria*, the soft clam *Mya arenaria*, the whelk *Urosalpinx cinerea*, the periwinkle *Littorina littorea*, the conch *Stombus alatus* and the bay scallop *Argopecten irradians* (Ries et al., 2009). However, even if directly connected to the calcification process, shell length or area might not be sufficiently accurate as indicators of the effects of OA on shelled molluscs as the organisms are potentially able to maintain a normal linear shell growth under low pH conditions. Shell dissolution might outcompete carbonate deposition consistently, resulting in thinner and lighter shells with maintained surface area (L. Parker et al., 2013). Despite it is the most common response to OA, the decrease in calcification process is not the rule. Several molluscs exhibit neutral or positive response for the calcification rate. Is the case of the limpet *Crepidula fornicata* (Ries et al., 2009) and the cephalopod mollusc *Sepia officinalis*, showing any reduction in growth or calcification of the cuttlebone under acidified conditions. Further, the effects of OA on the calcification and growth of molluscs have been found to differ across populations of the same species (Berge et al., 2006; Gazeau et al., 2007; Thomsen & Melzner, 2010) and with different physiochemical environment conditions (Thomsen et al., 2010).

In addition to the calcification process, OA strongly affects molluscs early-life history stages and associated processes such as fertilisation and larval development (Parker et al., 2013). Gametes, embryos and larvae are strongly vulnerable to environmental stressors (Przeslawski, 2004). It has been hypothesised that ocean acidification will significantly impact on the early life history stages of molluscs because such stages lack the specialised ion-regulatory epithelia required for maintenance of acid-base status (F. Melzner et al., 2009) and also deposit $CaCO_3$ shells (Ross et al., 2011). Early-life history stages have been found to be highly sensitive to ocean acidification. Impacts of ocean acidification on the embryos and larvae of molluscs include: smaller sized embryos and larvae, decreased shell thickness, increased larval development time

reduced survival, reduced metamorphosis, shell abnormalities, altered behaviour and alterations in the accumulation of heavy metals (L. Parker et al., 2013).

Molluscs responses to OA have to take in account also the synergic effects of other stressors (i.e. increased temperature, salinity fluctuations, hypoxia), their impacts will exacerbate the effects of OA (Parker et al., 2013). In the few mollusc species studied to date, most of the evidence suggests that the synergistic effect of elevated CO_2 and temperature will be greater than the effect of elevated CO_2 alone (Gattuso et al., 2015; Hale et al., 2011; Johnson et al., 2017; Kroeker et al., 2017; Matozzo et al., 2012; Swezey et al., 2017).

Finally, molluscs seem to adapt their self to the new environmental conditions. Exposure of adults to elevated CO_2 during reproductive conditioning will result in positive carry-over effects passed from adults to their offspring and increase the resilience of molluscs to ocean acidification (Parker et al., 2013).

3.1.1 Mussels responses to OA

Mussels, as well as the other shelled molluscs, response to OA in different ways. For their environmental and commercial value, mussels, more than other molluscs, are often object of OA's studies. Different species of mussels, particularly that with a high economic value, and at different life stages are take into account.

Some researchers focused their attention on hatching and larval stages, as Gazeau et al. (2010) that show important effects on both hatching and D-veliger shell growth of *Mytilus edulis*. Although their results show that blue mussel larvae are still able to develop a shell in seawater under saturated with respect to aragonite, the observed decreases of hatching rates and shell growth could lead to a significant decrease of the settlement success. Kurihara (2008) affirm that the first effects of elevated pCO_2 on the mussel *Mytilus galloprovincialis* were observed during the trochophore stage which corresponds with the onset of shell mineralization. Also in the mussel *Mytilus californianus,* Gaylord et al. (2011) demonstrate that ocean acidification markedly degrades the mechanical integrity of larval shells. In their study the larvae cultured in seawater containing CO_2 concentrations expected by the year 2100 (540 or 970 ppm) precipitated weaker, thinner and smaller shells than individuals raised under present day seawater conditions (380 ppm), and also exhibited lower tissue mass.

Other researchers as Navarro et al. (2013) focused their study on the physiological processes of juvenile of the mussels *Mytilus chilensis*. After a period of 70 days in a mesocosm system with the future predicted CO_2 ppm values, mussel show any impact on feeding activity at the beginning of the experiment, but a significant reduction in clearance rate was observed after 35 days of exposure to highly acidified seawater. Absorption rate and absorption efficiency were reduced at high pCO_2 levels. In addition, oxygen uptake significantly fell under these conditions, indicating a metabolic depression. These physiological responses of the mussels resulted in a significant reduction of energy available for growth (scope for growth) with important consequences for the aquaculture of this species during medium-term exposure to acid conditions.

Adult mussels are also affected by OA. In *Mytilus edulis* Fitzer et al., 2014) showed a reduced carbonic anhydrase protein activity and shell growth. Beyond that, at 1000 µatm pCO_2, bio mineralisation continued but with compensated metabolism of proteins and increased calcite growth. They conclude that mussel growth occurs at a cost to the structural integrity of the shell due to structural disorientation of calcite crystals. Thomsen and Melzner (2010) also demonstrate in the same species a reduced shell growth. They analysed the physiological parameters of mussels grown for two months in acidified conditions. Shell and somatic growth, calcification, oxygen consumption and NH_4 excretion rates were measured, suggesting that reduced shell growth under severe acidification is not caused by global metabolic depression but is potentially due to synergistic effects of increased cellular energy demand and nitrogen loss. The effects on the inner part of the shell were also investigated. Conclude that such part of the shell seems to be more vulnerable to OA as high body fluid pCO_2 causes low pH and low carbonate concentrations in the extra pallial fluid, which is in direct contact with the inner shell surface. Moreover, they found that low food algae concentrations and high pCO_2 values each significantly decreased shell length growth. Their findings illustrate that integrity of inner shell surfaces is tightly coupled to the animals' energy budget under conditions of CO_2 stress. It is likely that under food limited conditions, energy is allocated to more vital processes (e.g. somatic mass maintenance) instead of shell conservation. However mussels seem to adapt to the new acidified conditions as some multi-generation experiment demonstrate (Stapp et al., 2017; Thomsen et al., 2017), for example modify the ultrastructure and crystallography of the shells of the juveniles spawned from adults in high pCO_2 conditions (Fitzer

et al., 2014). Different is the situation when another kind of stressors, as the increase of temperature, is added to OA. Gazeau et al. (2014) prove that survival of *Mytilus galloprovincialis* will not be affected by a pH decrease of ~0.3 in the Mediterranean Sea but ocean warming will likely pose more serious threats to Mediterranean mussels in the coming decades.

Moreover the physiochemical environment experienced by mussels may be pivotal in determining their sensitivity to ocean acidification as showed in the study of Thomsen et al. (2010) in which a population of the blue mussel *M. edulis* that in laboratory studies have significant reductions in calcification following exposure to elevated CO_2, was able to calcify and survive in the acidified conditions. The study was conducted in in the Western Baltic Sea, where natural upwelling of CO_2 rich waters results in an increase in the CO_2 of the surface ocean for large periods of the year (>2,300 μatm). While the physiochemical environment may indeed be significant in the resilience of molluscs to ocean acidification, the rate of further increases in CO_2 may exceed tolerance thresholds of molluscs in coastal, estuarine and intertidal environments.

Sometimes OA effects are investigated together with other stressors. Adult individuals of the mussel *Mytilus coruscus* (recently accepted as *Mytilus unguiculatus*) are frequently used as model organism in studies where OA is combined with other types of stress agents. Hu et al. (2015), Wang et al., (2015) and Wu et al. (2016) have studied the combined effect of OA and temperature increasing; Sui et al. (2015) OA and hypoxia conditions; Li et al. (2015) have compared the different modes of action of pH and predator cue; Huang et al. (2018) have investigated on the effects of OA and high concentration of nano-TiO_2. These kind of studies try to emulate a real condition on which mussels have to cope (more than one stressors could act togheter), but sometimes is difficult understand which of the examined stressors is a real threat.

To better understand mussel's responses to OA, it is important further expand our knowledge on the topic. Is fundamental compare different species, also at different conditions (e.g. naturally acidified sea water and CO_2 enriched seawater) and different species life stages. Studies on which OA is associate to others anthropogenic impacts are certainly useful, but to study a single phenomenon is fundamental to understand the question and to compare the effects of the same stressors in different species. However, holistic and multi approach studies (also referred just to a single stressors) are the best solution to investigate the problem in such complex questions.

3.2 OA effects on *Mytilus unguiculatus* Valenciennes

Mytilus unguiculatus Valenciennes 1858 belong to the genus Mytilus, family Mytilidae. It was often known by its unaccepted name *Mytilus coruscus* Gould, 1861, recently corrected in its actual name by WoRMS (World Register of Marine Species). *Mytilus unguiculatus* is one of the most important economic shellfish and widely distributed in eastern coastal waters of Yellow Sea and East China Sea; it lives attached to hard substrates and forms sub tidal beds playing also an important ecological role and affecting the coastal community structure (M. Hu et al., 2015; Liao et al., 2013; Lu & Wang, 2018). *Mytilus unguiculatus* has both a great economic than ecological importance, it is interesting understand the effects of OA without the influence of others stressors on the ecology and particularly the economy linked to this mussel species. Recently studies (Hu et al., 2015; Wang et al., 2015; Li et al., 2015; Sui et al., 2016; Wu et al., 2016; Huang et al., 2016) have always investigated about the responses to OA always paired with other stressors, no investigations are about the only effects of the hypercapnia.

The aim of this study is the investigation on the topics, considering just the hypercapnia actually caused by OA. I faced such question in collaboration with the researchers of the professor Tseng team at the Marine Research Centre, ICOB (Institute of Cellular and Organismic Biology), Academia Sinica in Taiwan (China). We have studied the effects of OA on *M. unguiculatus* with different approaches, in order to have a wider view on the topic. *M. unguiculatus* was exposed to different pCO_2 levels in aquaria and were monitored its physiological parameters, the expression of genes related to the acid-base homeostasis, free amino acid analysis with UPLC, shell composition of magnesium and calcium with flame spectrophotometer, the crystal organization of the shell with the scanning electron microscope and finally X-ray shell analysis.

3.2.1 Experimental setup

Mussels of the species *Mytilus unguiculatus* were sampled in mussel farms in Matsu island, an island belonging to the government of Taiwan but closer to the Chinese coast; it is reported in the map in figure 3.2. The typical mussel farm breeding system in Matsu island is the same

adopted in the most of Italian mussel farms, the long line system in protected areas (see Introduction, V) (figure 3.3).
In laboratory, mussels were acclimatize in a big sea water tank at room temperature (~25°) for at least one week and then transferred in some smaller experimental aquaria of 80 L to start the experiment.

Were tested 3 different pH value:

- pH 7.4 (pH value higher than the predicted one of sea water by 2100)
- pH 7.8 (the predicted value of sea water by 2100)
- pH 8.1 (control value, the actual sea water pH)

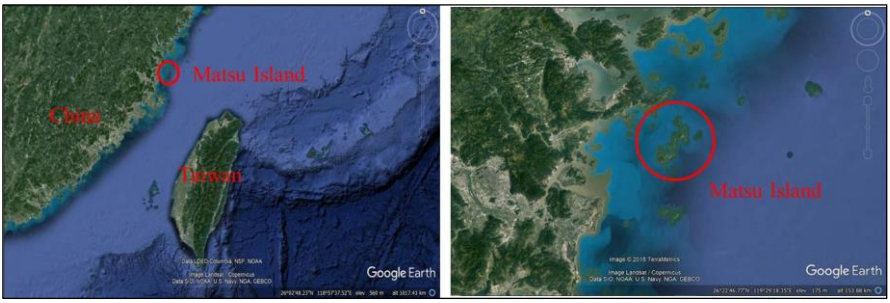

Fig. 3.2 Matsu Island (Taiwan), East China Sea.

Fig. 3.3 A mussel farm in Matsu Island.

The tanks were artificially CO_2 enriched and were continuously equilibrated with the appropriate gas mixtures using a continuous pH-stat system (pH controller MACRO pH controller, Taiwan) that controlled the addition of CO_2 into the aquaria. In each tanks there were 20 mussels. The mussels were hung individually in a small plastic net and suspended in the water, thanks to some sticks on the top of the tank (figure 3.4). This is an innovative system to breeding mussels in the aquaria, our aim was the emulation of mussels breeding condition in the farms.

Mussels by their nature adhere to a substrate and/or to others mussels, when they are manually detached they are in a stressful condition, sometimes they could die (personal observations). As physiological parameters were individually measured, in order to not cause further stress to mussels each of them had its single net on which can remain sticked. The tanks were in a continuous cycle and mussels' feeding was enriched with *Isochrysis galbana*.

Fig. 3.4 Mussel's innovative breeding system in the experimental aquaria.

The experiment time was of 15 days. Mussels were put in the experimental tanks a day before starting in order to acclimatize them. For three times during the experiment (at day 1, day 7 and day 14) mussels were sampled in order to measure physiological parameters, gene

expression, free amino acids, shell composition. In each sampling 5 mussels for each of the three tanks (pH 8.1, 7.8 and 7.4) were analysed. In total, 15 mussels were sampled each sampling day. The whole experiment has been replicated 3 times, for a total number of 135 sampled mussels.

Water parameters (pH, S and T) in the tanks were daily determined. Salinity and temperature were stable, respectively ~34‰ and 25 ± 0,5 C°. The temperature was kept stable with a thermometer in each tank.

3.2.2 Genetic characterization

Mussels sampled in the mussel farms of Matsu Island were firstly genetically characterized in order to strictly define our model species. The mitochondrial gene COI (Folmer et al., 1994) was used to identify mussels. COI is a "universal" DNA primers for polymerase chain reaction (PCR), amplifying a ~700 bp fragment of the mitochondrial cytochrome c oxidase subunit I gene. COI primers (LCO 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') generate informative sequences for phylogenetic analyses at species and higher taxonomic levels.

Three random mussels have been sacrificed. DNA for genetic characterization was extracted from a 0,1 g of fresh mantle tissue. The Wizard © Genomic DNA purification (Promega) was used for collecting DNA. The PCR condition were: a first denaturation step at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 40°C for 1 min and 72°C for 45 s and a final elongation at 72°C for 7 min. For the purification of PCR products was used the Novel Gel/PCR DNA Purification kit. Sequencing was performed by Genomics, Taiwan.

The result has shown that our mussel model organism is clearly defined as *Mytilus unguiculatus* Valenciennes 1858.

3.2.3 Physiological parameters: oxygen consumption (MO_2), ammonia (NH_4^+) excretion and O:N ratio

Several studies investigates on mussel metabolism responses towards various short term abiotic stressors, such as rapid salinity change, hypercapnia or toxin exposure (Tedengren & Kautsky, 1986; Jörn Thomsen & Melzner, 2010; Lindinger et al. 1984). Indicative metabolic

parameters are oxygen consumption, NH_4^+ excretion and O:N ratio. Ammonia and the carbon dioxide are the two main waste products of metabolism. O:N ratios are also considered a common indicator for the proportion of the three metabolic substrates, carbohydrates, lipids and proteins, used in energy metabolism (Mayzaud & Conover, 1988). Although absolute values vary for different species, regions and seasons, lower O:N ratios indicate a higher fraction of protein metabolism, whereas a higher ratio indicates an elevated turnover of carbohydrates and lipids (Mayzaud & Conover 1988).

In order to measure the previous listed physiological parameters, we have used 3 l glass chambers. A total of 4 chambers have been used simultaneously, in a 200 l water bath (figure 3.5).

Fig. 3.5 The 2 l water bath with the glass chambers inside.

The temperature of the water bath was controlled by mean a thermometer and store at the same temperature of the mussels aquaria, ~ 25°C. The chambers contained 0,2 µm filtered sea water, of the same pCO_2 value of the tested mussel. In each chamber was placed a single mussel. Each mussel was put in the chamber with its own net, without detaching it from its substrate, in order to not cause stress to the mussel. For each measurement, one chamber was used for bacterial control (figure 3.6).

Oxygen concentrations were recorded using the fibre-optic oxygen sensors OXY-4 mini multichannel fibre optic oxygen transmitter, PreSens. As mussels have not high oxygen consumption (they do not spent energy for movement), each measurement time was of 70 minutes. At the end of each measurement were sampled water aliquots in order to measure the DIC (Dissolved Inorganic Carbon), the alkalinity and the ammonium excretion. The DIC was measured with Dissolved Inorganic Carbon (DIC) Analyzer (AS-C3, Apollo SciTech, USA). The alkalinity was measured adapting the protocol of Sarazin et al., (1999). The nanodrop NanoDrop™ 2000c, USA was used. The ammonium was determined following the method of Holmes et al. (1999), using the fluorometer.

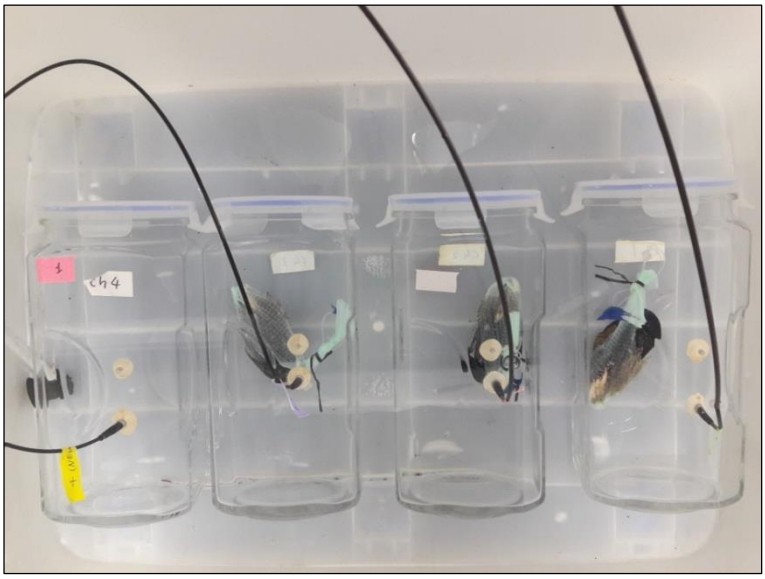

Fig. 3.6 A mussel in each chamber, one of them is empty for the bacterial control.

The results shown below are cumulative of the three mussels groups. Considering the metabolic rate (µmol O_2/Lhg), mussels exposed at a pH of 7.8 and 8.1 (the control group) have similar values in day 1 and day 14, mussels exposed to a more acid pH (7.4) increase the metabolic rate in day 14. In the middle of the experiment time, at day 7, control group show a lower metabolic rate in comparison with day 1 and day 14. On the contrary, instead, 7.8 and 7.4 mussels groups have increased their metabolic rate, particularly 7.4 group (figure 3.7).

Value of ammonia excretion (µmol NH_4^+/Lhg) are similar in day 1 and in day 14 for the mussels of control group, is slightly higher in day 7. Mussels of 7.8 group show a decreasing trend, from day 1 to day 14, without an important difference between the two extremes. Mussels of 7.4 group present a different situation, the ammonia excretion in day 1 and day 7 shows very close value; instead, in day 14 reach very high value (figure 3.8).

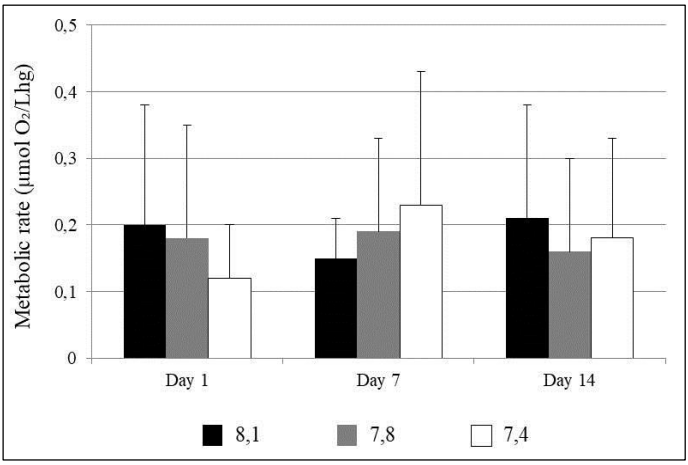

Fig. 3.7 Metabolic rate.

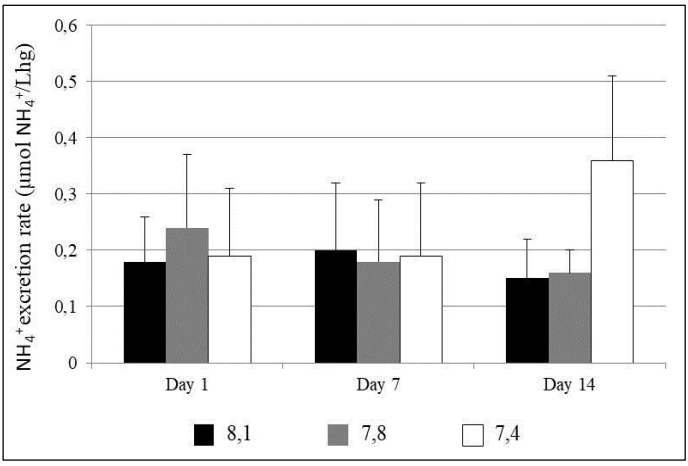

Fig. 3.8 NH_4^+ excretion.

Finally, the O:N is really high for 7.8 mussels in day 7, the ratio is 7 times higher than in day 1 and in day 14 (these two days show same values). For mussels of the group 7.4, there is a little difference between day 1 (lower) and the other two days, 7 and 14 (higher), however O:N ratio in these latter two day is just one third of the 7.8 mussels ratio in day 7. As regard as control mussels, in day 1 and day 14 they have an O:N ratio close to the highest ratio value (reach by 7.8 mussels, day 7), a lower ratio is showed in day 7 (figure 3.9).

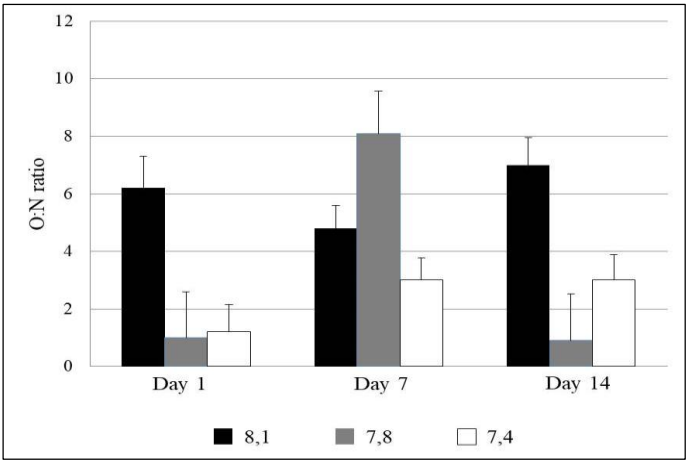

Fig. 3.9 O:N ratio.

3.2.4 Expression of NKA (Na^+-K^+-ATPase alpha subunit) and NHE8 (Sodium 8 hydrogen exchanger) genes

Physiological tolerance to elevated pCO_2 seawater is hypothesized to be connected to the ability of an organism to regulate extracellular acid-base status during exposure to hypercapnia (Hu et al., 2011). In molluscs, particularly bivalves, there are not many studies about such mechanisms and the involved genes. Instead, for fish, acid-base regulating epithelia and organs have been extensively investigated (Deigweiher et al., 2008; Grosell, 2006). These studies proposed models of acid-base regulation in specialized, mitochondria-rich cells localized in skin or gill epithelia. Besides the direct ATP-dependent extrusion of protons via V-type H^+-ATPases, these models suggest an import of HCO_3^- and an export of protons by secondary active transporters, e.g., apical Na^+/H^+ exchangers (NHE) and Na^+-dependent Cl^-/HCO_3^- exchangers of the SLC4 (Sodium

bicarbonate transporter-like protein solute transporter family) that depend on the electrochemical gradient created by the Na^+/K^+-ATPase (NKA) located in basolateral membranes (Boron, 2004; Perry & Gilmour, 2006). Considering studies on fishes, in our investigations we have choose the genes NKA and NHE, the latter in the isoform 8. Fragments of these genes were isolated from mantle gill tissues by means of RT followed by PCR. First of all RNA was extracted, cDNA was synthetize, degenerates primers for genes amplification were designed and finally the expressions of NKA and NHE was evaluated.

- RNA extraction:

Sample of tissue of ~0,1 g was taken from mantle and gills (as probably in mussels are the most affect tissues by acid-base homeostasis). Samples were store in Trizol reagent (Invitrogen, Carlsbad, CA, USA) and store at -20° until the RNA extraction. The tissues were homogenised in Trizol and the total RNA was purified following a manual protocol; DNA contamination was removed with DNase I (Roche). The concentration and integrity of mRNA was determined by spectrophotometry (NanoDrop™ 2000c, USA) and the RNA quality was checked by electrophoresis. RNA pellets were stored at−20 °C.

- primers design:

Degenerate primers were designed using PrimerQuest Tool (Integrated DNA Technologies), by using highly conserved regions of congeneric bivalves sequences obtained from GenBank. Then, primers for quantitative real-time PCR (qRT-PCR) were designed using the same software (PrimerQuest Tool, Integrated DNA Technologies). The primers are reported in the table 2.1.

- cDNA synthesis:

Reverse transcription was performed with SuperScript™ IV Reverse Transcriptase, SSIV. cDNA was eluted and stored in 90 µl DEPC water at -20°C. The amplification was performed with Taq-Polymerase (Invitrogen, Karlsruhe, Germany) at these conditions: denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 30 s, followed by a final elongation step at 72° for 7 min. PCR fragments were separated after electrophoresis in 1% agarose gels. Extraction and purification of the PCR fragments from the gel was accomplished using the Novel Gel/PCR DNA Purification. Cloning and

isolation of plasmids was performed using the ECOS ™ 101 competent cell DH5α. Plasmids were extracted using the Qiaprep Spin Miniprep Kit (Qiagen) and sequenced by the Genomics, Taiwan.

- Real Time quantitative PCR (qPCR):

cDNA was amplified using LightCycler® 480 SYBR Green I Master (Roche). According to the following conditions: 5 µL SYBR Green PCR master mix, 1 µL (10 µM) forward and reverse primers and 4 µL template cDNA in a total reaction volume of 10 µL. We selected amplicons to range from 50 to 100 bp in size. Selected genes and primer sequences are given in table 3.1.

Tab. 3.1 Primer used for qRT-PCR.

Gene name	Abbreviation	Primer sequence
Elongation Factor 1ὰ	EF1ὰ	F 5'-TTGTTGCATCTGGTACTGGTGAAT-3' R 5'-GGTGGTTCAGTGTTGTCCATCTT-3'
Sodium 8 hydrogen exchanger	NHE8	F 5'-TGGTGGAGCTACAATGCCTTTAAT-3' R 5'-TGGTCAGTATCTACTGTTTCTCCCA-3'
Na+-K+-ATPase alpha subunit	NKA	F 5'-GTGCTTGCCTCTGAATTCTGTTACT-3' R 5'-CAAAGGAGGCCAAGATAATGTTCCT-3'

qRT-PCR was performed on the LightCycler® 480 (Roche Applied Science, Mannheim, Germany) using theLightCycler® 480 SYBR Green I Master (Roche) Thermocycling was performed using the following conditions: 10 min at 95°C, 45 cycles of 15 s at 95°C, and 1 min at 60°C. Melting curve analysis demonstrated a single PCR amplicon for each reaction. Control reactions were conducted with sterile water to determine levels of background and genomic DNA contamination.

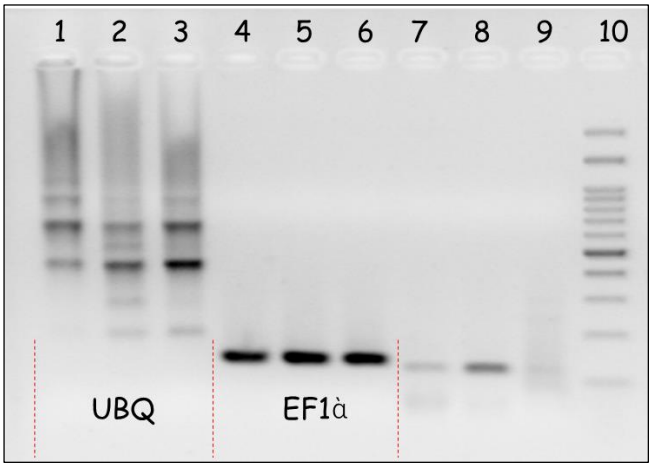

Fig. 3.10 EF1à as housekeeping gene. Two different housekeeping genes were tested in three different tissue (mantle in 1,4, 7 wells; gill in 2, 5, 8 wells and stomach in 3, 7, 9 wells). The marker ladder 100 was in the well 10. EF1à was the best expressed gene in both the three tissues.

Data were recorded as fractional cycle at an arbitrary CT-value in triplicate during the exponential phase of the reaction. The elongation factor EF1à, responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome, was identified as housekeeping gene resulting the best among vary tested genes, as ubiquitin (Fig. 3.10).

Genes expressions were evaluated at three different time during mussels exposure to the different pH values: day 1, day 7 and day 14. The values in the following graphs are the results of the cumulative data of the 3 mussels' group replicas. In the two tissues, mantle and gills, were evaluated both the two genes (NKA and NHE). In the gills NKA and NHE are both higher expressed than in mantle, moreover, among the two genes, NKA showed always higher expression level both in the mantle tissue than in the gills (Fig. 3.11 a,b,c, and d). Particularly in the mantle (fig. 3.11 a and b) the mussel group 7.8 increase NKA expression during the exposure time from day1 to day 14, following the same trend of the control group. The mussel group exposed to 7.4 pH increase NKA expression in day 7 and decrease it in day 14 (fig. 3.11 a). The NHE expression of the control group has stable values over time; both the exposed group increase their NHE expression over time. However expressions levels are not so different from the control values (fig. 3.11 b).

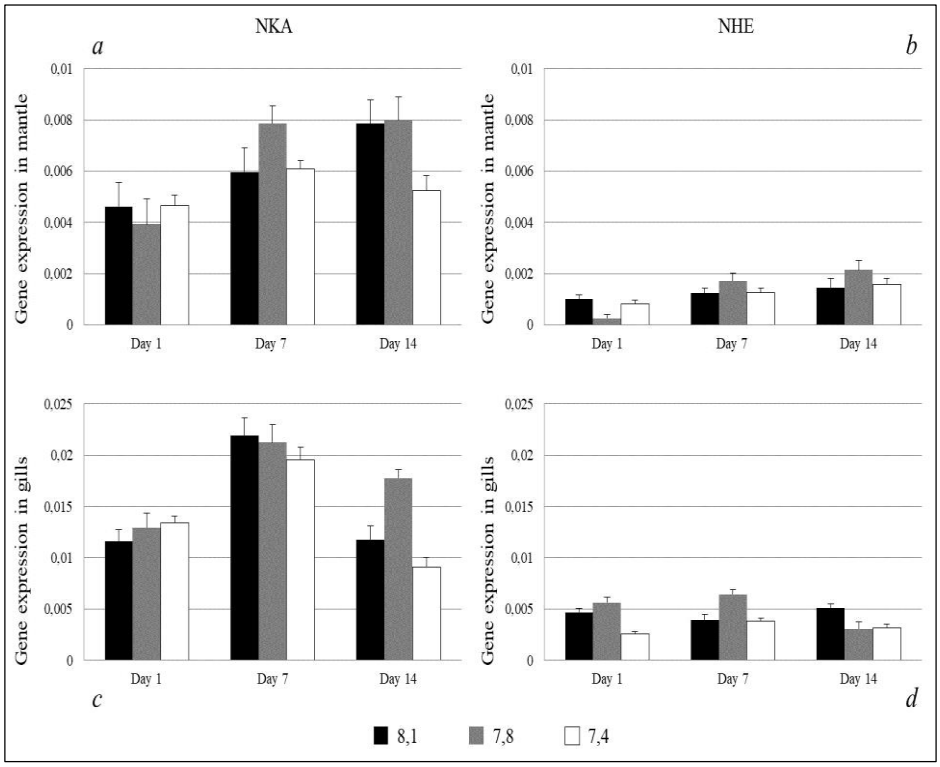

Fig. 3.11 NKA and NHE expression in the mantle and in the gills.

In the gills, NKA has the same expression pattern for the three mussel groups with an increase in the gene expression in the middle of experiment, in day 7. However, 7.8 mussel group in day 14 is the group with the higher expression value (fig. 3.11 c). NHE expression, as in the mantle, is stable for the control group (fig 3.11 d). The treated groups increase their expression just in day 7 (fig 3.11 d).

Finally, both the two genes are Na^+ exchangers showing a higher expression in the gills showing that probably gills are mainly involved in the acid base homeostasis mechanisms. Among the two genes, NKA with a higher expression in both the examined tissues is presumably mainly implicated in these mechanisms. However no impressive differenced are highlighted among the control groups and the treated groups. 7.8 and 7.4 mussels group increased their expression in day 7 and then it is back to the starting levels of day 1. Such trend for the NKA expression is the same of

the control group. Only the 7.4 group in day 14 depart from this trend, showing a under expression of NKA in day 14.

NHE expression in mantle shows the same increasing trend for all the mussels groups. NHE expression in gills increase in day 7 for treated mussels of the group 7.8 and 7.4. The expression value of the last experimental day, day 14, came back to the day 1 values. Such pattern probably indicates that treated mussels face up the new acidified conditions and then stabilizes their homeostasis balance.

3.2.5 UPLC analysis

Amino acid analysis with UPLC (Ultra Performance Liquid Chromatography) is a technique which finds many applications in sea foods analysis and related fields. The aim of our investigations using UPLC is the identification of any differences in amino acids composition among mussels exposed to high PCO_2 and the control ones, living in the current pH value. Samples of mantle and gills of 0,01 g were analysed at day 1, day 7 and day 14. In order to prepare samples for the analysis were used the AccQ Tag Ultra Derivatization Kit (Waters). The analysis was performed with ultra-performance liquid chromatography (UPLC) (ACQUITY UPLC H-class System, Waters).

The results reported in figures 3.12, 3.13 and 3.14 are cumulative of the 3 mussels groups. The mantle represents the main part of the animal body mass, all the amino acids are present in the tissues with different concentrations (fig 3.13). In the gills not all the amino acids are present; isoleucine (Ile), leucine (Leu) and phenylalanine (Phe) are totally absent; histidine (His), asparagine (Asn), serine (Ser), glutamine (Gln), arginine (Arg), threonine (Thr), proline (Pro), lysine (Lys), tyrosine (Tyr), methionine (Met), valine (Val) and norvaline (Nval) have a concentration lower of 0,5 pmol/µg.

Taurine (Tau) is reported on a different graph (fig. 3.12) as it is always present in higher concentration compared to the other amino acids (fig. 3.13 and 3.14). Considering all the treated mussel groups taurine concentration has an average of 6 pmol/µg, the other amino acid concentrations vary among 0,1 and 1,2 pmol/µg. However, Taurine concentration shows some differences among the treated groups: both in mantle than in gills, taurine concentration of 7,4 group is lower than the other groups and the two previous experimental days. The group 7.8 have no impressive differences among tissues and the exposure time (fig 3.12)

Taurine is always present at very high levels particularly in the mantle (fig. 3.12).

As regard as the other amino acids, in the mantle glycine (Gly) of 7.8 group increase in day 7 and decrease again in day 14 (fig. 3.14). In the gills, the glycine concentrations vary for 7.4 group decreasing in day 7 and increasing in day 14 (fig. 3.14). In the gills also the cysteine (Cys concentration vary during time for the 7.4 and 7.8 mussel groups, increasing in day 7 and decreasing in day 14.

Despite these small differences, there are no impressive findings on amino acid composition. All the 21 amino acids tested show no significant differences among mussels groups and/or among the exposure time. Moreover taurine is present in such large quantities as this free amino acid acts as an organic osmolyte that regulate cell volume; occur always at high levels in marine invertebrates (Thurston et al., 1981; Yin et al, 2000). Moreover, Toyohara et al. (2004) reported that taurine is a dominant osmolyte in the Mediterranean blue mussel.

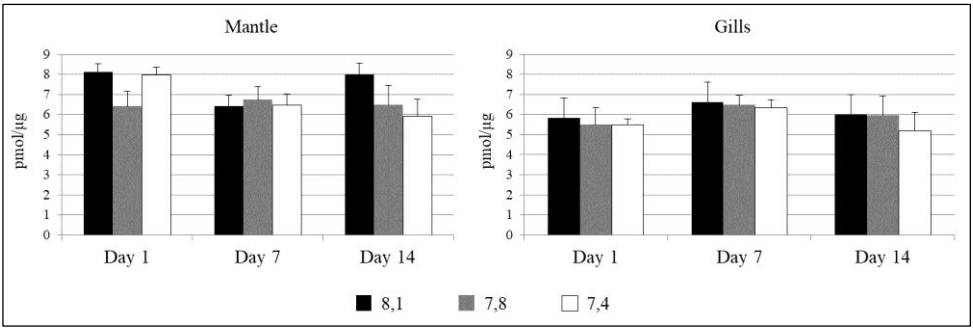

Fig. 3.12 Taurine in the mantle and in the gills tissue

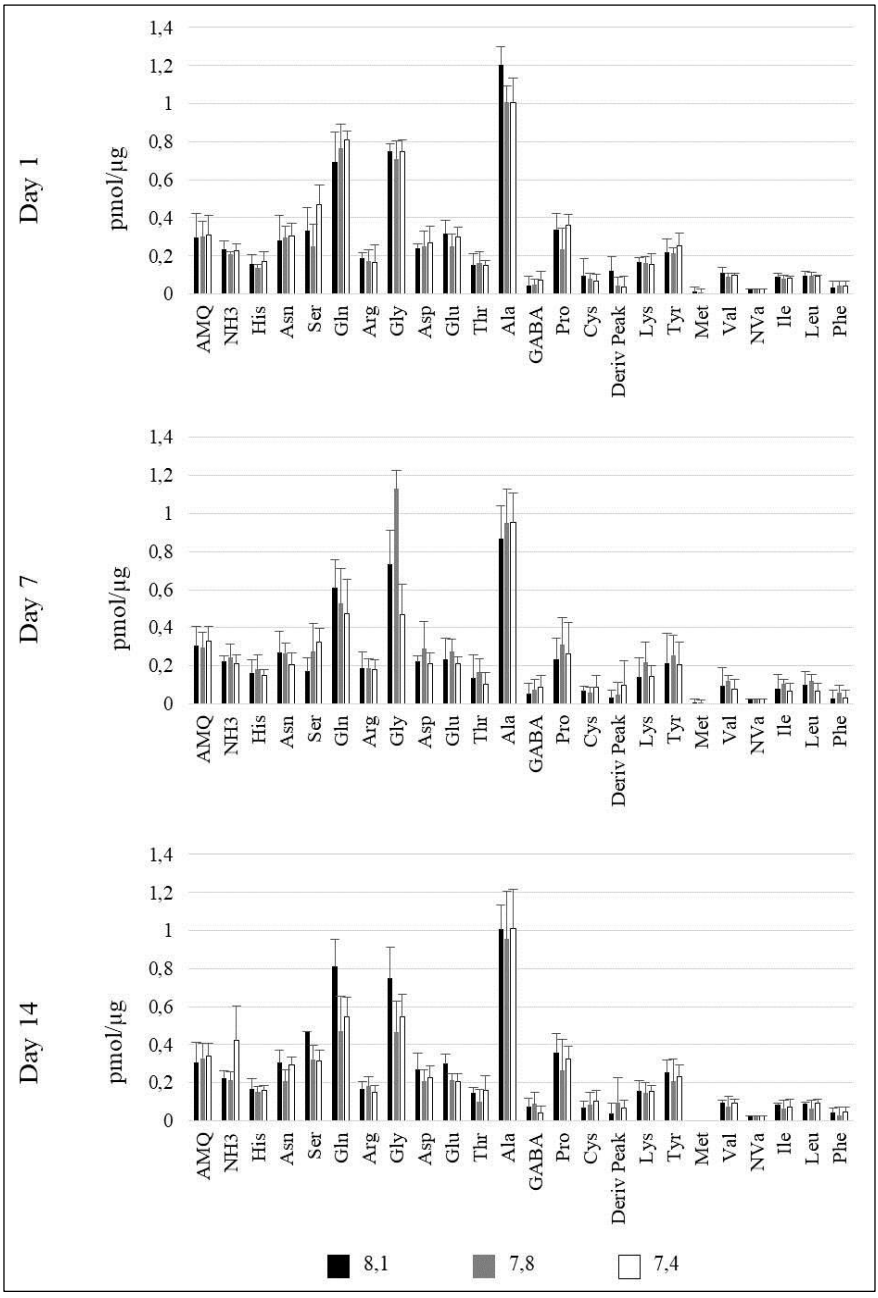

Fig. 3.13 UPLC analysis of mantle tissue.

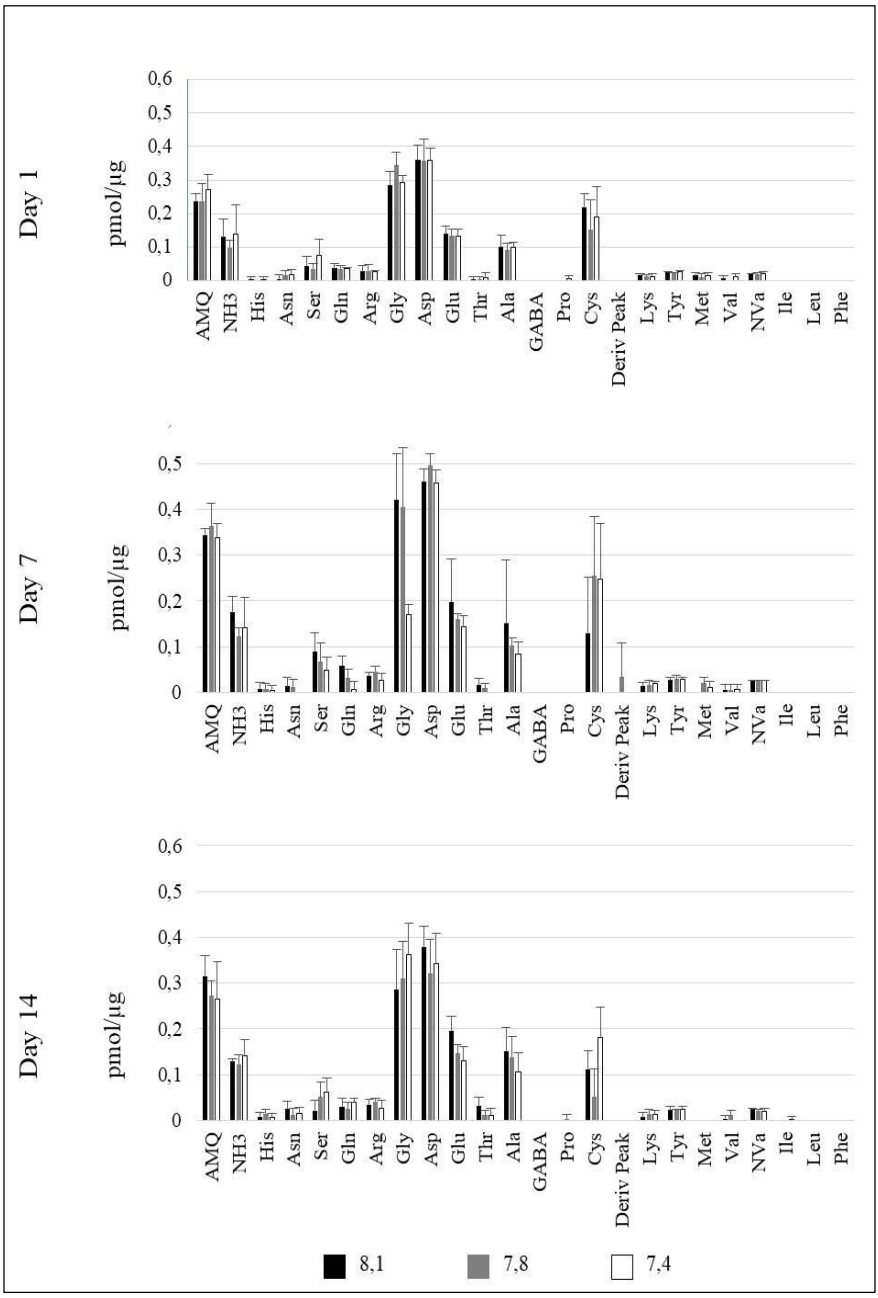

Fig. 3.14 UPLC analysis of gill tissue.

3.2.6 Shell contents of Ca^{2+} and Mg^{+}

In order to identify any differences in the shell composition, we have analysed the shell contents of calcium (Ca^{2+}) and magnesium (Mg^{+}) with Atomic Absorption Spectroscopy (AAS). Calcium is the major component of the shell, whether it is in the form of calcite or aragonite. Amorphous calcium carbonate is produced within an intracellular compartment and transported to the site of calcification, where it can either remain amorphous or crystallize into aragonite or calcite as dictated by the presence of matrix proteins (Frédéric Gazeau et al., 2013; Jacob et al., 2008; Weiss et al., 2002). Magnesium is fundamental to regulate the process, may also functioning to prevent uncontrolled crystallization (McCo et al., 2018).

To evaluate the concentrations of calcium and magnesium, we have collect the shell of the already sacrificed mussels sampled in day 1, day 7 and day 14. The shells have been well cleaned of any animal residue and were left to dry. A 0,1 g sample was taken from the oldest part of the shell, near the umbone. Shell fragments were dissolved in HNO_3. The flame spectrophotometer was the Polarized Zeeman Atomic Absorption Spectrophotometer Z-5000 (Hitachi, Japan).

Results, as for the other parameters, are cumulative of the 3 replicates of the whole experiment. Calcium concentration is obviously much higher than magnesium concentration: an average of 6000 ppm for calcium and 5 ppm for the magnesium. As regard as calcium, the concentration in the control mussels group is stable during time. The 7.8 mussel shell has a slightly higher calcium concentration in day 14. The 7.4 mussels present a decreasing trend, with very high concentrations in day 1, the highest calcium concentration also among the other two pH mussel groups (Fig. 3.15).

Magnesium concentration in the control group is quite stable. The 7.8 group shows the same concentration in day 1 and day 7, increasing in day 14. Finally, magnesium concentration of 7.4 mussels, as the control group, is stable for the whole experimental time (Fig. 3.15).

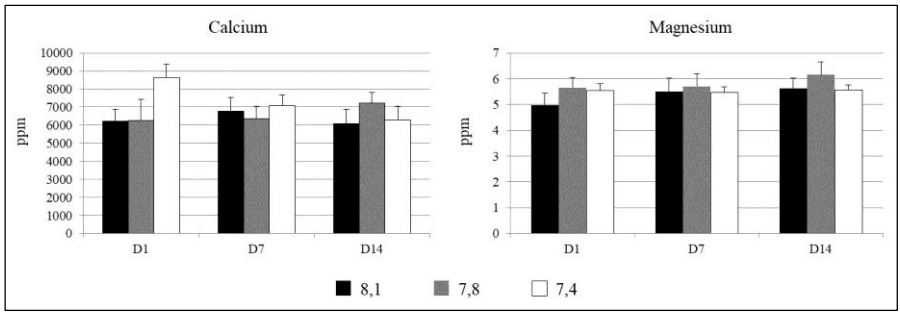

Fig. 3.15 Calcium and magnesium shells contents.

3.2.7 Shell structure

To investigate differences in the structure of the shell, focusing on the organization of carbonate layers, we have used a Scanning Electron Microscope (SEM). Small shell apical fragments have been isolated from the shell, cleaned with ethanol and let it dry. The fragments were around 2-3 mm long. All samples were mounted on SEM specimen stubs in the direction of the thickness, in order to observe the shells layers among the inner and outer part of the shell. Shell fragment were coated with gold and observed with a scanning electron microscope operated at 5.0 kV. Pictures were taken at 5000X and 10000X magnifications focusing on the calcite layer.

SEM observations were done on 3 random mussels for each pH group at day 14; moreover, from each mussels were take 3 shell fragments. SEM pictures reveal differences in calcite blocks organization. In the 8.1 shells, it seems that calcite blocks are well defined and closer together, in addition they seem follow a thinner trend, from 8.1 to 7.4 group (Fig. 3.16).

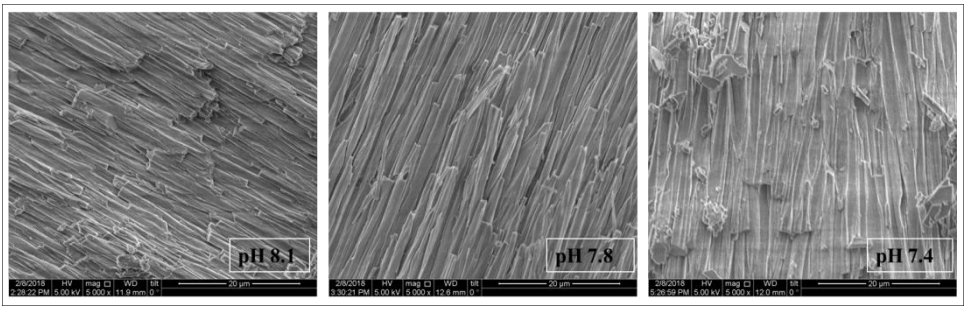

Fig. 3.16 Calcium and magnesium shells contents.

3.2.8 X-ray testing

X-rays are mainly used in medical imaging for human beings, but X-ray technology has been also developed for non-destructive testing of materials and objects, where the aim is to analyse elements undetectable to the naked eye. Non-destructive testing with X-rays, called X-ray testing, is used in many applications such as: baggage screening, automotive parts inspection, welding quality control and food product analysis as reported in Haff & Toyofuku (2008) and Mery et al. (2011), particularly for fishes identification. We have used X-ray testing in order to identify any structural shell differences among mussels exposed to different pCO_2. In order to take images, the X-ray source generates X-ray photons which irradiate the mussel shell. The shells absorbs energy according to the principle of differential absorption and then modify the expected radiation received by the X-ray detector. In these experiments, we used the X-ray source and the flat panel detector. The X-ray source, the mussel shell exposed to X-rays, and the flat panel detector are enclosed in a lead cabinet that provides enough radiation attenuation and prevents access to the X-ray beam.

We have taken an X-ray picture for each mussel, for a total amount of 135 mussels (45 mussels per group). Animals were already scarified for the other analysis (RNA extraction for gene expression and UPLC) the shells have been well cleaned and left to dry before the X-ray testing. Moreover we have taken some X-ray picture of the mussels shells that had been in the aquarium for a long time; e.g. mussels exposed at 7.4 pH for 100 days; mussels exposed at 7.8 pH more than 80 days. In the figure 3.17, are compared some mussels of the three different pH groups (7.8, 7.4 and the control 8.1) sampled in the different experimental time (day1, day 7, day 14). The focus is on the shells growth lines, particularly that ones on

the distal point of the shell, opposite at the umbone. In mussels of 7.4 pH groups, focusing on day 7 and particularly on day 14 growth lines are less marked.

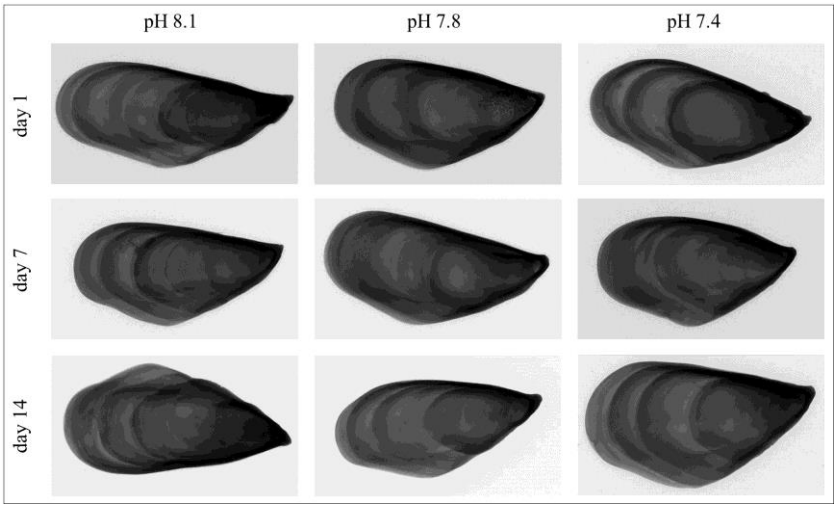

Fig. 3.17 Comparison between the three different pH mussel groups (7.8, 7.4 and the control 8.1) sampled in the different experimental time (day1, day 7, day 14).

In the picture 3.18 are compared mussels exposed to different pH, but just that one's sampled in day 14, the longer experimental time and the day on which on 7.4 mussels present faded growth lines. 7.4 growth lines compared to the control group but also compared to the 7.8 pH group, are less pronounced. Finally, in the figure 3.19 there are mussels exposed for a time much more longer than the experiment set time. The 7.4 shell growth lines are always less defined and visible compared to the 7.8 shell growth lines, also in the comparison between the oldest mussels (80 days for 7,8 mussel and 105 days for 7.4 mussel).

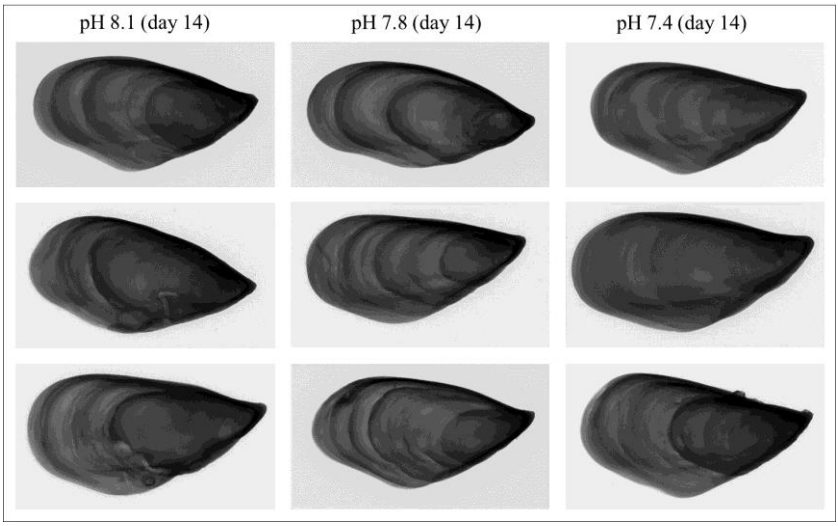

Fig. 3.18 Comparison between the three different pH mussel groups (7.8, 7.4 and the control 8.1) all sampled in day 14.

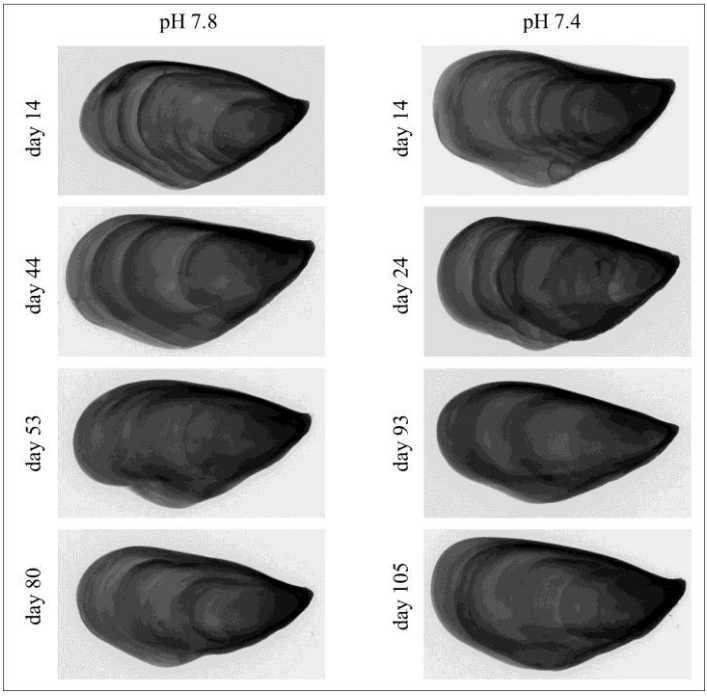

Fig. 3.19 M. unguiculatus under a longer time of pCO_2 exposure.

3.2.9 Statistics

Values are presented as the mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) was performed. Each 15 days experiment were replicate with three different mussels groups. The results (for the metabolic rate, the NH_4^+ expression, the O:N ratio, the genes expression, the UPLC analysis, the Ca^{2+} and Mg^{2+} shell content) were analysed together.

3.3 OA effects on *Mytilus unguiculatus* Valenciennes: a global overview

Mytilus unguiculatus, as well as other Mytilus species, is affected by the incoming and growing environmental problem of OA. The aim of our investigations is to define the mussel *Mytilus unguiculatus* as winner or loser to the new "acidified" environmental conditions. Mytilus genus includes all species of crucial importance for its environmental and economic ecosystem services that provide. *M. unguiculatus* Valenciennes 1858 is widespread along the costs of China where is extensively bred (Lu & Wang, 2018). Any negative effects of OA on *M. unguiculatus* would be very harmful to the environment and especially to the economy linked to this species.

The first step in our study was been certainty ascertain to which species our mussels belong. In order to better understand their physiology and ecology and in order to compare our findings with other previous or next investigations. For this reason, we have clearly identified *M. unguiculatus* Valenciennes 1858 using the universal mitochondrial marker cytochrome c oxidase subunit I gene (COI) (Folmer et al., 1994).

The evaluation of physiological parameters (metabolic rate, ammonia excretion and O:N ratio) show a different response between the two experimental mussels group exposed to the different pCO_2 (7.4 pH and 7.8 pH). The 7.4 mussels group has a high NH_4^+ excretion in day 14, they are the only mussels group to present such high value, indicating a stressful condition for them. The 7.8 mussels showing a decreasing trend in NH_4^+ excretion from day 1 to day 14, seems to adapt their self to the acidified seawater. Another important difference among the two mussels groups is found in the O:N ratio: 7.8 mussels group shows an high value in day 14; a value of one third higher that at the beginning (day1) and at the end (day 14) of the experiment. Instead, 7.4 mussels group has no high O:N ratio. Although absolute values vary for different species, regions and seasons, lower O:N ratios indicate a higher fraction of protein metabolism, whereas a higher ratio indicates an elevated turnover of carbohydrates and lipids (Mayzaud & Conover, 1988). According to Lindinger et al. 1984; (Michaelidis et al., 2005; Tedengren & Kautsky, 1986; Thomsen & Melzner, 2010), mussels generally respond to OA with a decreased O:N ratio, as a result of either decreased metabolism, increased NH_4^+ excretion rates or a combination of both. In our results, a decreased O:N ratio of 7.4 mussels is showed together with an high NH_4^+ excretion value, supporting

the previous thesis and showing a stress conditions of this mussel group. As regard as 7.8 mussel group, they face the situation differently: they have NH_4^+ excretion value similar to the control mussels for all the experimental days and, opposite to 7.4 mussels, they increased the O:N ratio, but just in day 7 and then became again very low, similar to the 7.4 mussels, both in day 1 and in day 14. O:N ratio both of 7.4 and 7.8 mussels, except that in day 7, is always lower than the control group. Considering our data, mussels have to change their metabolism, particularly, according to Thomsen & Melzner (2010) the decreased O:N ratios at the highest seawater pCO_2 indicate enhanced protein metabolism which may contribute to intracellular pH regulation, the only exception regard 7.8 mussels in day 7.

The expression of the gene NKA (Na^+-K^+-ATPase alpha subunit) and the gene NHE in the isoform 8 (sodium 8 hydrogen exchanger) was been evaluated in two tissues: mantle and gills. In the gills both the two genes show higher expression levels than in the mantle, such findings indicate that among the two tissues gills are probably mainly involved in the acid base homeostasis. Moreover, the gene NKA is highly expressed than the NHE in the two examined tissues, it is presumably mainly involved in such mechanisms. As regard as the individuals, the most affected by the new acidified condition are mussels of 7.4 group, as they decrease their genes expressions, particularly the NKA one. Instead, 7.8 mussel show not a different trend from the control group. Mussels of each group, also the control one, increase the NKA and NHE expressions in day 7 and then at day 14 the expression levels go back to the day 1 levels or at least remain stable. This expression pattern is probably due to the adaptation of mussels to the new aquaria conditions, they face up the new conditions and then stabilizes their homeostasis balance.

The UPLC analysis reveals no impressive differences in amino acids compositions. The taurine, the most abundant amino acid, vary a little among the mussels group and during the three days, but there are no important differences as well as for the other amino acids.

The shell contents of Ca^{2+} and Mg^+ reveals that 7.4 mussels shells have a lower calcium content, decreasing from day 1 to day 14, this indicate that are the most affected mussels in calcification process. As regard as magnesium shell content, in the 7.8 mussels shells, it is a little most abundant compared to the others group. Parker et al. (2013) affirm that based on to the magnesium shell content, molluscs have a different sensitivity to OA. Probably the slightly increase of Mg^{2+} in the 7.8 shell

could help 7.8 mussel to face the acidified sea water condition as, according to Addadi et al. (2006) one of the magnesium function is to prevent uncontrolled crystallization. Moreover, to strengthen our findings, conducted a meta-analysis of all marine species (not just molluscs) found that the effects of OA on calcification varied with different mineral forms of $CaCO_3$. They found that organisms with aragonite and low-Mg calcite shells and skeletons were negatively affected whilst those with high-Mg calcite, the most soluble form of $CaCO_3$, were not.

In addition to the highlighted differences in the shell contents of calcium and magnesium, exposed mussels to different pCO_2 show also differences in the shell structure. SEM images reveal a different calcite blocks organization among the different mussels group. In the 8.1 shells, it seems that calcite blocks are well defined and closer together, they seem to became thickness and with a less compacts organization going towards 7.4 mussels shells. X-ray pictures also reveal differences in the shell of exposed pCO_2 mussels. X-rays demonstrate that the most affected shells belong to the 7.4 mussels group, on which shell growth lines, particularly that ones on the distal part of the shells, are not so marked as in the other two shells groups. After 14 days such difference became evident, but is strongly marked after longer exposure time, as until 80 to 100 days. In such longer pCO_2 exposition time, also 7.8 mussels seem to have less defined shell growth lines. Such findings, together with SEM and the AAS analysis, indicate that under a CO_2 enriched scenario mussels cannot form a perfect shell. Probably, as suggested by Melzner et al. (2011) and our UPLC data, mussels in a stressful condition allocate their energy to more vital processes (e.g. somatic mass maintenance) instead of shell conservation. Fitzer et al. (2014) also affirm that under elevated pCO_2 bio mineralisation continued but with a compensated metabolism of proteins and increased calcite growth, so mussel growth occurs at a cost to the structural integrity of the shell due to the structural disorientation of calcite crystals.

In conclusion, our findings on the effects of OA on *Mytilus unguiculatus* demonstrate this species, at adult stage, can easily survive in the predicted pH of 7.7/ 7.8 by the 2100 (IPCC, 2014; Valenzuela et al., 2018). Mussels of 7.8 group can easily maintain their physiological functions. However, according to Melzner (2011) energy invested in these mechanisms are probably subtracted to the shell formation. The situation is different for *M. unguiculatus* exposed to higher pCO_2. 7.4 mussels seem to live in a more stressful condition. After the middle time of our experiment,

is harder for them restore their acid-basis homeostasis.7.4 mussels probably need more energy to maintain vital function; their shells, in fact, are more affected by acidified sea water.

Our experimental time was not so long, investigations on longer time scale could be interesting in order to understand if metabolic parameters and genes expression remain stable, particularly for 7.8 mussels group, and in order to understand if the shells can be further affected, both for the structure than the minerals contents.

Despite the shell seems to be affected over a long period, on the basis of our findings we can affirm that adult mussel of the species *M. unguiculatus* can survive in the future acidified oceans and the ecosystem services that provide seem to be not threatened. They can continue to contribute to the environment functioning as bioengineering species as there are no evidence of less filtration rate, or reduced adhesion ability to the substrate, as instead find by Donnell et al. (2013) and Zaho et al. (2017). These skills are also fundamental for providing the ecosystem service of food providing, moreover, according with our UPLC data their amino acids compositions not have impressive differences under pCO_2, as a consequence the taste will be unchanged. In addition their shells, fundamental in defending the animal from any predators, seem to have not impressive impacts, at least that one's of 7.8 mussels.

However further investigations on the effects of OA on *Mytilus unguiculatus* are essential in order to better understand the question. Exposure time to acidified conditions should certainly be increased. Different life stages have to be tested, embryos and juveniles are probably the most affected by OA as shown by studies on different mussels species (Gazeau et al., 2013; Kurihara, 2008; Navarro et al., 2013; Parker et al., 2013). The expression of more than two genes involved in the acid-base homeostasis must be evaluated. An interesting gene to investigated is for example the Carbonic Anhydrase (CA); Fitzer et al.(2014), in the mussel *Mytilus edulis* showed a reduced carbonic anhydrase protein activity. Others interesting genes probably involved in such mechanisms are the Sodium-driven Chloride/Bicarbonate Exchanger (NDCBE) and the Sodium bicarbonate transporter-like protein (SLC). Finally, further investigations with trans generational experiments could help scientific community to understand the mussel ability to adapt to the new acidified conditions. About this topic Thomsen et al. (2017) performed a three generations experiments on *Mytilus edulis*, demonstrating a probably long-term adaptation potential in this key bivalve species. Parker et al. (2015)

also demonstrate on the F1 generation of the wild-type oyster *Saccostrea glomerata* an improved capability to compensate for extracellular acidosis under elevated CO_2.

To increase investigations on OA and its effects on marine ecosystems are fundamental not only on the species *Mytilus unguiculatus* but also for the other mussels and bivalves species, the comparison of the same mechanisms or characters among different species could help in the knowledge of the topic. One of our future aim is compare the effects of OA to the Mediterranean mussel *Mytilus galloprovincialis*, a species with the same ecological and economic importance of *M. unguiculatus*. A comparison among these two species is done also by Zhang et al. (2014), considering temperature as stressors. They investigated about the caspase-8 activity in gill and tissues haemocytes of *M. unguiculatus* and *M. galloprovincialis*, finding that in both the species it plays an important role in response to temperature stress and in determining cellular thermal tolerance limits. The two species seem to be very similar, but they do not necessarily react in the same way to the OA effects. The shell of the Mediterranean mussel *M. galloprovincialis* looks more fragile and thin than the congeneric pacific mussel, probably OA could cause a greater impact. However different studies were done about *M. galloprovincialis* responses to OA. Kurihara (2008) has considered the trochophore stage, corresponding to the onset of shell mineralization, and has affirmed that OA has important effects on this species, at this stage. At the adult stage, Gazeau et al. (2014) has proved the effects of OA and increase of temperature. They affirmed that the survival of *M. galloprovincialis* will not be affected by a pH decrease of ~0.3 in the Mediterranean Sea but ocean warming will likely pose more serious threats to Mediterranean mussels in the coming decades. Freitas et al. (2017) also investigated on the effects of OA and another stressor, the salinity changes. They have demonstrated that *M. galloprovincialis* oxidative status and metabolic capacity were negatively affected by the two stressors, with alterations that may lead to physiological impairments namely on mussels reproductive output, growth performance and resistance to disease, with ecological and economic implications.

In conclusion, the same confused and discordant situation about OA effects on *M. unguiculatus* and others mussels species, it is also found in *M. galloprovincialis*. According with Wenguang & Maoxian (2012), different species respond differently to the seawater acidification; this statement is true above all for the bivalves. Impressive is the study on the

calcification process of Ries et al. (2009) on which they exposed to vary pCO_2 values 18 calcifying benthic species (including crustacea, cnidaria, echinoidea, rhodophyta, chlorophyta, gastropoda, bivalvia, annelida). Each species demonstrate a reduced or increased rates of net calcification under elevated pCO_2. The only species showed no response at all was the blue mussel *Mytilus edulis* (Fig. 3.20). These varied responses may reflect differences amongst organisms in their ability to regulate pH at the site of calcification, in the extent to which their outer shell layer is protected by an organic covering, in the solubility of their shell or skeletal mineral, and in the extent to which they utilize photosynthesis. Whatever the specific mechanism(s) involved, the results of Ries et al. (2009) suggest that the impact of elevated atmospheric pCO_2 on marine calcification is more varied than previously thought. The variety of responses to OA is not just about the calcification process, it can concern all the vital processes of marine organisms. Further studies are needed to demonstrate the exact mussels response mechanisms to OA in order to evaluate the real adaptability of such organisms to the incoming acidified conditions and any related changes to the ecosystem services they provide.

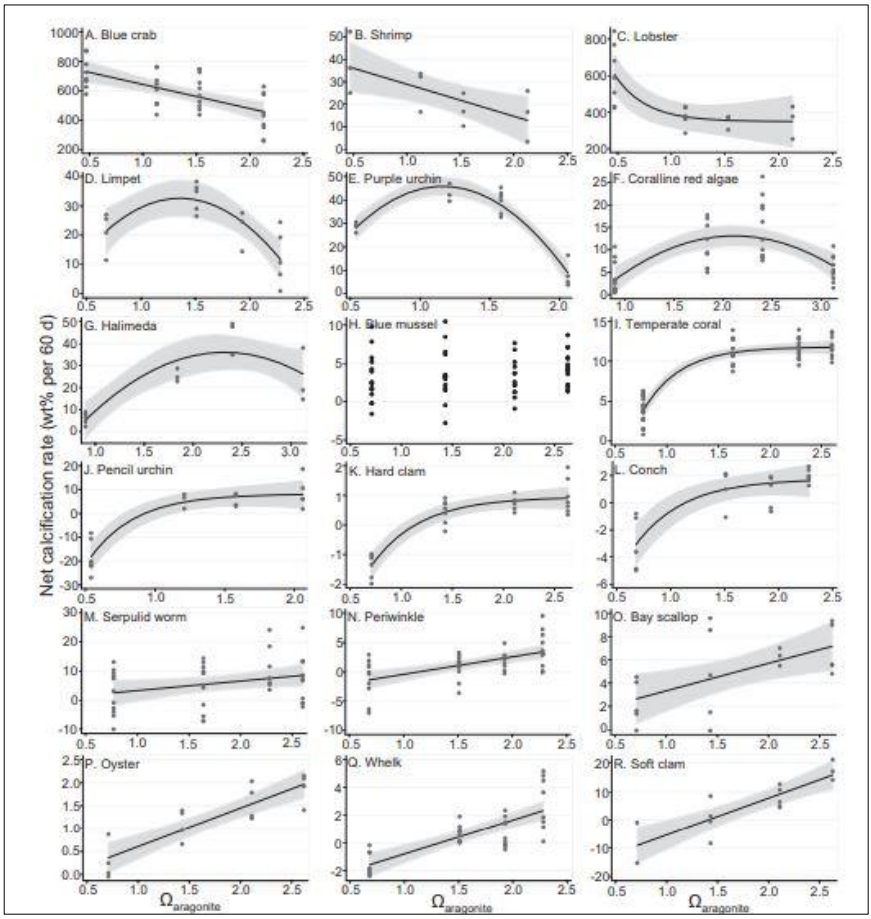

Fig. 3.20 The results of the study of Ries et al. (2009) on the net calcification rate of 18 calcifying benthic species. The blu mussel M. edulis is the unique species among them with no response.

3.4 RNA extraction protocol

Isolation of total RNA (Invitrogen TRIZOL reagent)

[Phase separation & EtOH wash]

- Tissue 50-100 mg/1 ml TRIZOL (sample/TRIZOL 1/10)
- Homogenize sample 1 min
- Incubate 5 min at RT (complete dissociation of nucleoprotein) or -20°C O/N
- Add Chloroform (0,2 ml/1 ml TRIZOL)
- Shake vigorously 15s and then incubate 3 min at RT
- 12000g 15 min at 4°C
- Take upper aqueous phase to new tube (~50% of TRIZOL vol.)
- Add equal vol. of Isopropanolo, mix
- 10 min at RT for precipitation or -20°C storage
- 12000g 10 min 4°C
- 1 ml 75% EtOH (ice cold) wash, vortex, 7500g 10 min at 4°C
- Remove EtOH, add new EtOH, sample can be stored at -20°C for 1 year

[DNase I treatment]

- Remove EtOh, 37°C dry RNA pellet (do not completely dry)
- Add 18 µl DEPC-H_2O (prewarm at 60°C), pipetting, 60°C, 30 min incubation
- Add 1 µl DNase I (Roche) and 2 µl DNase I Buffer, then incubate at 37°C for 30 min
- 75°C, 10 min inactivation
- Nanodrop Q/C (5x dilution, RNAse free water, No DEPC), -80°C storage

[cDNA synthesis – (SuperScript™ IV Reverse Transcriptase, SSIV]

- Add: 5,5 µl of 2,5 µg total RNA + DEPC; 0,5 µl 50 µM Oligo(dT)$_{20}$ Primer; 0,5 µl 10mM dNTP mix
- 65°C for 5 min and fast on ice for 1 min
- Add: 2 µl 5X SSIV BF (vortex); 0,5 µl 100 nM DTT; 0,5 µl RNase Out (40 U/µl); 0,5 µl SSIV RT (200U/µl)
- 55°C, 10 min incubation
- 80°C, 10 minute inactivation
- Finally add 90 µl water or EB Buffer

3.5 Amino acid analysis

- 500 µl 100% ethanol
- 5 µl 2,5 mM norvaline
- Homogenize 1 min
- Transfer to 15 mL tube and add 100% ethanol to 2,5 mL
- Centrifuge at 4,300g for 10 min
- Take 2 mL supernatant and dry down under vacuum (2-3hr)
- Reconstitute dried sample in 100 µl of 8 mM HCl
- Filter the sample (Milliex-GN 0,2 µm cutoff: Millipore)
- Take 10 µl aliquot for amino acid derivatization (AccQ Tag Ultra Derivatization Kit: Waters)

Amino acid derivatization (AccQ Tag Ultra Derivatization Kit: Waters)

- Reconstitute AccQ Tag Ultra Reagent Powder
- Preheat a heating block to 55°C
- Tap vial 2A (AccQ Tag Ultra Reagent Powder) to ensure all reagent is at the bottom of vial
- Draw 1 mL of AccQ Tag Ultra Reagent Powder from vial 2B
- Transfer to vial 2A
- Vortex for 10 seconds
- Heat on top of heating block, vortex occasionally, until the powder dissolves. Do not heat longer than 10 min

Preparing 100pmol/µl calibration standard for protein and peptide hydrolysates (Final concentration after derivatization will be 10 pmol/µl

- Transfer the Amino Acid Hydrolystae Standard (P/N WAT088122) from the supplied ampule to a vial appropriate for storage in a freezer
- Combine 40 µl Amino Acid Hydrolysate Standard, 40 µl 2,5 mM norvaline (in 0,1N HCl) and 920 µl H_2O

Derivatizing standards and samples

- Mix following materials in a vial or tube: 70 µl AccQ Tag Ultra Borate buffer (Vial 1), 10 µl sample and 20 µl AccQ Tag Reagent
- Cap and vortex for several seconds
- Let stand for one minute at room temperature
- Heat in heating block for 10 min at 55°C
- Remove from heating block with forceps and place in instrument for analysis.

The resulting standard has a concentration of 10pmol/µl

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Chapter 4

A biotechnological application: can *Mytilus galloprovincialis* produce a pearl?

Patent application: “Pearls production in Mediterranean edible bivalves”.
Authors: Sara Fioretti and Francesco Paolo Patti

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Chapter 5

Mussels market in Campania: anchor in the history and exiting future perspectives

5.1 Mussels in the Bourbons kitchen

In Campania mussels have always been present, already several centuries before Christ. They have always been an important source of food and have had great economic importance for Campanian populations. The first historical data of mussels presence in Campania, probably the first record for the whole Italian area, date back to the Cumans. They came from the island of Eubea at the beginning of VIII century B.C. and settled the first west Greek colony, Pithecusa, in the current island of Ischia, precisely in the area of Lacco Ameno (Buchner, 2004). From this colony, a part of the population moved to the mainland in front of Ischia. There they settled the city of Cuma, one of the most brilliant and important city of that historical period. Cuma is in the Phlegrean area among Pozzuoli and Bacoli. Mussel settlements were already present but Cumans probably started to bread them. Bread and/or fished, mussels became so important for Cumans economy that were engraved on their coins (La Repubblica, 2018), showed in figure 5.1. Coins are a fertile source of information for the ancient history (Howgego, 1995); on coins were represented gods, kings, later emperors or animals or, as in this case, food. Anyway if a symbol was engraved on a coin is a fundamental index of its importance for the local past economy.

Fig. 5.1 Cuman coins. On the right coin, is engraved a mussel. This is a symbol of the economic importance of mussels for Cumans, Campanian population af around 700 b.C.

After the brilliant Greek period, Campania hosted the main important and rich roman cities. As for Cumans and Greeks, the Phlegrean

area was a strategical place also for Romans: close to the sea, full of bays and brackish lakes and not too far from Rome. For example in the gulf of Pozzuoli the city of Baia (actually has the same name) was a luxury and very rich city, as proved by the private villas with the wonderful mosaics and the public buildings that can be visited in diving excursions in one of the most beautiful underwater archaeological parks in the world. There are no historical findings on mussels of roman period, but surely mussels together with oysters were present in those kitchens. Oysters were very appreciated among the high social classes (De Grossi Mazzorin, 2008); so oyster and other fish species were widely bread in the Phlegrean area. Especially the lake of Lucrino and Fusaro were perfect place on which bread oysters (Giacopini et al., 1994; Malossini, 2011; De Grossi Mazzorin, 2008). Considering the importance that the mussels had in previous centuries, they were surely bread in large quantities as well as oysters.

Bivalves production continued even in the Middle Ages (De Grossi Mazzorin, 2008). Natural disasters such as eruptions and earthquakes have often changed the territory and destroyed local activities. For this reason there are no information and records for some historical periods. In the sixteenth century, under the Bourbon reign, there are again records on Phlegrean area that was once more intensively used for oysters and particularly for mussels farming. Mussel farms were also extended to Naples, since then mussels became a widespread and appreciated food until today. Many typical Campanian dishes are made with mussels. Some of these traditional recipes have ancient origins. In Naples, in our modern time, on the Holy Thursday before Easter, the typical tradition dish is the mussel soup with Neapolitan pepper sauce. This tradition dates back to the Bourbons dynasty, particularly is linked to the king Ferdinand I; he was monarch in Naples from January 1751 until January 1825. Ferdinand I of Bourbon was very greedy for fishes, sea food and specially mussels, which he used to fish in the waters around the castle. Afterwards he also invented a new recipe for cooking them. As suggested by the warning from Gregory Maria Rocco, who was a Dominican friar very popular and loved by the people and the royals of Naples, especially after his hard work to help poor people and to fight human vices, the monarch Ferdinand I decided to limit his excessive gluttony during the Saint week. To not give up on his favourite food, the monarch cunningly ordered his chefs to cook mussels in a less sumptuous way. So on the Holy Thursday, before going to the main Naples streets, the chef served him a simple mussels soup with tomatoes

and a strong peppers sauce. The recipe was such a great success that even the bourgeoisie and the people started cooking it (Bracale, 2017; Granello, 2018).

This historical excursus shows mussel culture is highly rooted in the Campanian population, from Cumans through the Bourbons until today. Mussels were constantly present in the Campania history, particularly for people living along the coasts, especially the Phlegrean ones. Mussels are always present in the landscape and in the economy of the coastal area of Campania region.

5.2 Mussels in our kitchen

In modern time as in the past, mussels are still an appreciated food in Campania. Actually, all the world appreciates mussels as are one of the main bread aquatic food (FAO, 2017) and their consumption has increased steadily over the past decades (Grienke et al., 2014). In Europe Mussels are among the main produced and consumed shellfish (EUMOFA, 2016; (Venugopal & Gopakumar, 2017). As mussels trend, generally seafood consumption is increasing over the time, the causes are the world population increase and the awareness of the healthy nutritional properties of seafood. The world population increases continuously, from 3 billion of people in 1950 to the actually 7,2 billion to the predicted 12 billion in 2100 (Gerland et al, 2014). On the other hand, seafood nutritional values are more and more appreciated. According with the Mediterranean Diet, declared as an Intangible Cultural Heritage of Humanity by UNESCO (2010), seafood should be eaten 2-3 times a week. NOAA (2018) declared seafood as a high-protein food that is low in calories, total fat, and saturated fat. Seafood is a complete protein source and it contains essential amino acids enough to assure healthy growth and optimal embryo development. A serving of the most common fish and shellfish provides about 30-40% of the average daily recommended amount of protein. The protein in seafood is easier to digest because seafood has less connective tissue than red meats and poultry. Moreover, seafood is generally considered to be low in total fat and saturated fat. Most fish and shellfish contain less than 5 percent total fat and even the fattiest fish have no more than 15 percent fat. A large proportion of the fat in seafood is polyunsaturated, including omega-3 fatty acids, which have added health benefits. Seafood is also a natural source of B-complex vitamins, vitamin D and vitamin A. B-complex vitamins have been associated with healthy development of the nervous system. Vitamin A is needed for healthy vision as well as for healthy skin, while vitamin D is essential in bone development. Seafood, particularly molluscs, is also a good source of minerals such as selenium, zinc, iodine and iron. Selenium is a potent antioxidant against cell damage and may help to counter the negative effects of mercury. Zinc is needed for cell growth and immune system health. Iodine helps maintain thyroid gland function, while iron is important in red blood cell production. Finally, heat seafood can reduce the risk of cardiovascular disease, help the protection against heart attack and sudden death, decrease the risk of heart arrhythmias, decrease blood

triglyceride levels Increases HDL (good) cholesterol, improve circulation, contributes to neurological development in infants, contributes to vision development and nerve growth in the retina and help to build muscles and tissues. Obviously shellfishes are included in seafood, among shellfishes mussels confirm to be an excellent healthy food (Cameron et al., 2009; Venugopal & Gopakumar, 2017). As reported in the figure 5.2, mussels have very high proteins contents (24,0 g/100 g, w. wt.) and Amino Acid Score; low quantity of crude fat and cholesterol, high contents of PUFA (Poly Unsutered Fatty Acids), both when mussels are cooked under moist heat that when are eaten as raw edible portions.

	Source A (samples cooked under moist heat)					Source B (raw edible portions)				
Shellfish	I	II	III	IV	V	I	II	III	IV	V
Shrimp, mixed	20.9	113	1.1	195	211	22.8	–	1.7	211	590
Prawn (cold)[a]	15.4	–	0.9	143	–	–	–	–	–	–
Prawn (warm)[a]	17.6.	–	0.7	–	–	–	–	–	–	–
Oyster, mixed	18.8	107	3.6	90	1480	11.4	–	3.4	79	1056
Oyster, eastern	14.0	107	4.9	105	1345					
Squid, mixed species	15.6	108	2.0	221	549	17.9	–	1.4	233	524
Mussel, blue	24.0	107	4.5	56	866	23.8	–		56	1212
Lobster, northern	21.0	113	0.6	72	866	19.0	–	0.9	146	–
Crab, Dungeness	19.0	113	1.1	65	300	22.3	–	1.2	76	407
Crab, blue	20.2	107	1.8	100	549	–	–	–	–	–
Crab, Alaska king	19.4	113	1.5	53	636		–	–	–	–
Crab, white meat, cooked[a]	20.5	–	0.3	66	80	–	–	–	–	–
Scallop, mixed species, raw	17.0	107	0.8	33	300		–	–	–	222
Scallop, bay and sea	23.2	–	1.4	53	196	20.5	–			–
Clam, mixed species	25.5	107	1.9	67	396	–	–	–		–
Crayfish, mixed	16.6	113	1.2	133	194	16.8	–	1.2		367
Cuttlefish	32.5	107	1.4	224	224	32.4	–	1.4	224	268

[a] Prawn (cold water), *P. borealis*, cooked; prawn (warm water), *P. vannamei*, raw, Department of Health, UK (2013).

Fig. 5.2 Nutritional values of shellfish (modified from Venugopal & Gopakumar, 2017). Both samples cooked under moist heat than raw edible portions show high and healthy nutritional value. The columns I, II, III, IV and V report respectively protein, Amino Acid Score, crude fat, cholesterol and total PUFA values. Values are given as g/100 g, w. wt. for protein and fat; and mg/100 g, w. wt. for cholesterol and PUFA.

For its nutrition values, seafood enters fully in the Mediterranean Diet that together with physical activity has been reported to be consistently beneficial with respect to chronic diseases and longevity (Bach-Faig et al., 2011; Dinu et al., 2018). Actually, in Italy the sea food per capita consumption is around 26 Kg/year (Eurispes, 2018). Considering the increasing of population and the higher seafood consumption, in order to cover the high sea food demand, aquaculture sector is set to increase. In Italy aquaculture is an already well established sector, is the fourth European country for aquaculture production, contributing for the 13% of the total volume of the EU aquaculture

products and mussels together with clam, oysters and fish as trout, sea bream and sea bass, are the main aquaculture species (FEAMP Campania 2014-2020). Campania is among the most productive Italian regions, with Puglia, Emilia Romagna, Veneto, Sardegna and Marche (Prioli, 2008). In numerical terms (number of companies and number of plants), mussel farming is the most important aquaculture industry in Italy covering the 90% of the regional aquaculture production. Phlegrean area since the past to today demonstrate to be at the forefront in mussels farming, actually accoutering the 81% of the Campanian mussel farms.

5.2.1 Campanian Mytilus galloprovincialis mussel as an excellent product

Mussel farming for Campanian economy and society is a very important activity. Considering its importance from an historical and social point of view, particularly for the Phlegrean area, the Campania region should make an effort to enhance this product. A Campanian or at least a Phlegrean Mussels should be officially recognized by political institutions in order two reach two important aims: the promotion of the excellent product and the enhancement of the honest mussel farmers.

In order to be officially recognized, the Campanian or Phlegrean Mussel have to born and grow up in the local area, the mussel farmer must be able to follow the whole production process from the seed recruitment until the sale. However such mussel must to be genetically identified, it has to belong to a Campanian/Phlegrean mussel population. This necessity also derives from the need to protect such unique mussel from food frauds. In order to identify and protect the local mussel by eventually frauds, it is important the strictly identification of the species and the origin of our product. For this reason is crucial the genetically identification of the Campanian or Phlegrean mussel populations.

The most common food fraud is the replacement (Johnson, 2014), according with our findings on the topic, showed in chapter 2, mussels in Campania markets seem to be not so affected by this problem. In our research, we have sampled mussels in different fish market and characterized them with two molecular markers (the universal marker COI and the other mitochondrial marker 16S). Both of them demonstrated that mussels were all belong to the species *Mytilus galloprovincialis*, the native species of Campanian coasts. More difficult is the identification of a local population of *Mytilus galloprovincialis*. In our investigations we have

tested two other molecular markers: the nuclear markers PAPM and 28S. These two markers are also unable to distinguish different Italian populations, showing a heterogeneous situation among the Italian musseò farms. However they both can distinguish individual of a musselfarms in the Gulf of Napoli, in the site of Santa Lucia. This leads to hypothesis of a local Campanian population on which invest further research efforts.

Identify a typical local *Mytilus galloprovincialis* mussel uniquely of the Campania region enhance the product itself and could enhance the whole local economy linked to the mussel supply chain. Some Campanian mussel farmers are able to produce marketable mussels from the seed production, some other farmers just make mussels grow up. The phase of seed recruitment involves additional cost and experience, instead, buying the seed, often on abroad countries, is more practical and faster. In this regard, the institutional recognition of a local mussel could enhance the work of these mussels farmers, increasing the economy linked to such process. Seed recruitment would be an additional mussel farming phase, it means more personal and more time invested.

An official recognition of the local Mussel with the aim of enhance the work of the honest mussel farmers it would be useful to preserve the environment too. Some of them take the seed indiscriminately from the coasts, leaving the natural environment without seed. Mussels have their important for the environment as well as food product. In addition to providing food, among their ecosystem services there are the regulation of water columns, the support to entire food web and cultural services. Mussels act as bioengineering species, they form a substrate for the other benthonic organisms; they can transfer energy and nutrients from the water column to the sediment, bio deposit organic matter and excrete nutrients, purify the water, recycle and store nutrients; they stimulate both algal and macroinvertebrate production (Kent et al., 2017; Lemasson et al. 2017; Sunday et al., 2017). If mussel seed is depleted, no more mussels will be in their natural ecosystems and the ecosystem services linked to their characteristics will be no more available. Actually, always low quantity of mussels is present along our coasts (personal observations).

However, in order to enhance the suggested Campanian/Phlegrean mussel, is important disclose the right information to the buyers. People have to know that local product, more generally the Italian product, is a quality assurance. Italian legislation is very strict in quality certifications. Contaminated mussels are often imported in Italy, for example sometimes in Spanish and Greek mussels *Escherichia coli* and *Salmonella spp.* were

found (Il fatto alimentare, 2017; Il Messaggero, 2018; Il Messagero, 2018; Il Giornale, 2018; Notizieora.it, 2018). Moreover in Campania there are also 12 purification centers, so mussels do not have to travel long distances from the sea plant to the purification centre and finally to the market. People should understand that eat a local mussels is synonymous with taste and freshness and with best nutritional values as local product is "at zero kilometre", not need long journeys to get to the table. So, local products are more tasty, fresh and healthy. In addition, the most environmentally conscious buyers should choose the local product for two reasons: the "zero kilometre" products do not increase the use of fuels for travel and the local mussel production make sure that the seed is not indiscriminately collected, without depleting the environment. Finally, people have to know that as for the other seafood products, particularly for fish, but also for fruits and vegetables, mussels have their seasonality too. There are some periods of the year on which mussels are ready to be eaten, other periods on which there are no marketable local products. Generally in Campania we have local mussels from June to September/October (it depends on marine weather conditions) and in March/April (for Easter period, in order to honour the traditional Bourbon soup). With the aim of inform people on the right period on which they can found local products on the market, could be very useful the app we have proposed in chapter 2 (paragraph 2.5). The app should be managed by mussel farmers, it should take into account the whole production process and advise the buyers when their product is ready on the market, it would also be a nice way to make people participate in such kind of activities.

Definitely, Campanian or Phlegrean mussels produced from the seed recruitment, have the right to be officially recognized as unique products. Equally with others "Made in Italy" products, such kind of mussels could be proclaimed as products with a protected or certified origin (DOP or DOC certification), as the colleagues *Scardovari* and *Nieddittas* mussels. We hope our investigations can be the premise and the basis to start this bureaucratic process together with the interested Campanian mussel farmers.

2.2.2 Will mussels still exist in our future kitchen?

Mussels as well as other marine organisms could be seriously threatened by different human induced risks. The temperature increase, the

presence of plastics and micro plastics, the ocean acidification, are all dire threats for the environment (including paradoxically human life) and our economy, as economy and ecology are inescapable linked to each other.

Following the very rapid increase of the population (Gerland et al, 2014) and the CO_2 emissions amount since the industrial revolution (IPCC, 2014), oceans have absorbed too much CO_2 and they will continue to do so if emissions will always be so high (Feely et al., 2004). The result is a change in ocean carbon chemistry with a corresponding drop in ocean pH of 0.3 - 0.4 units (from 8.1 pH, the actually value, to 7.8 pH) (Feely et al., 2004; IPCC, 2014; Cooly et al., 2009; Gattuso et al., 2015; Ries et al., 2009). This phenomenon, the Ocean Acidification, will affect marine ecosystems, particularly marine calcifiers could be the main affected, as their calcification process necessity for their physiological requirements (Cooley et al., 2009; Ries et al., 2009; Gattuso et al, 2015). Mussels are among marine calcifiers, they can be affected by this problem at different vital stages ((Fitzer et al., 2014; Gazeau et al., 2010; Kurihara, 2008; Melzner et al., 2011; Navarro et al., 2013; Thomsen & Melzner, 2010). However, among calcifiers species, mussels are the unique that have a neutral response to OA as it shown in Ries et al., (2009). So, in order to have a better understanding of the situation and considering that different species respond differently to the seawater acidification (Wenguang & Maoxian, 2012), new investigations are needed

We have investigated on the responses of adult individuals of *Mytilus unguiculatus* when exposed to a short period in acidified conditions, testing two different pH values: 7.4 pH and the future predicted 7.8 pH. On the effects of OA on this species there are still low findings, despite its important commercial value. Our investigations are shown in the chapter 3. We have tested *M. unguiculatus* physiological responses (oxygen consumption and ammonia excretion) and we have evaluated the expressions of two genes related to the acid base homeostasis (NKA and NHE). We have analysed the shells (the Mg^{2+} and Ca^{2+} contents and its structure at SEM and with x-ray testing). Moreover we have analysed the amino acids composition, as in quality of food product *M. unguiculatus* could change its protein assemblage under acidified conditions; this could result in changing of the mussels taste. Our findings demonstrate that *M. unguiculatus* at the adult stages can easily survive in the future acidified conditions. OA does not compromise its ecosystem service of food providing.

Obviously new investigations are fundamental in order to understand the question and really declare safe by OA the mussels market. Moreover, investigations on young and larval stages and on reproduction are essential, as they seems to be more affected by OA than adult stages (Gazeau et al., 2013; Kurihara, 2008; Navarro et al., 2013; Parker et al., 2013). However, on the one hand these delicate phases seem to be more disturbed and so would put at risk mussels existence; on the other hand different mussels species have also shown a good adaptation ability through different generations (Parker et al., 2015; Thomsen et al., 2017).

What would happen with our Mediterranean mussel, *Mytilus galloprovincialis*, exposed to acidified conditions? Probably our mussel will also take front to acidification conditions like a winning warrior as the congeneric *Mytilus unguiculatus*. However, probably all the mussels genus will able to adapt their self to the new ocean conditions. Taking into account that mussels are very strong organisms, they always face to different environmental conditions as their natural environment, the coastline, commonly have temperatures and salinity changes and frequently hypoxic conditions. Thanks to their high plasticity nature, mussels will probably survive to OA and continuing to be present in our kitchens.

However, although mussel will still be present as food source, mussel farms economy probably have negative effects for mussels growth time under acidified conditions. As according with our findings (in chapter 3) and with other authors researches as in Melzner (2011) mussels invest a lot of energy to maintain the acid-base homeostasis in the acidified conditions; such energies are subtracted to the shell growth. Consequently, the shells grow up more slowly and are even more fragile, easily attacked by any predators. So, mussel farms have to consider longer times to reach the adult marketable individual and a high risk of mussel predation.

In conclusion, mussels will probably be always present in the future acidified seas but they will provide sure a better food provisioning service with the actual oceans conditions rather than the future predicted ones. The solution would be to keep the current conditions as stable as possible. A political effort is needed together with citizens' daily actions. As well as to counter frauds, people awareness on the topic is the best solution. People have to know exactly the question and promise to take daily actions for the environment in order to reduce CO_2 emissions.

5.3 The interesting mussels perspectives outside the kitchen

Mussels provide ecosystem services as food provisioning and regulation of water columns but they can also provide cultural services (Kent et al., 2016; Lemasson et al, 2017; Vaughn et al., 2017). Among the cultural/social services, mussels are often used as model organism in scientific researchers, thanks to their biological and physiological characteristics. Several biomonitoring programs, e.g. the *Mussel Watch Program*, have used this cosmopolitan organism in order to monitor coastal regions for their pollutant contents (Cantillo, 1998; Goldberg, 1986; O' Connor, 1998; Ramu et al., 2007; Sericano et al., 1993); such programs are also important for the politic managing of environmental questions. Since 1960s mussels were used as environmental bio indicator for classical pollutants as metals, PAHs, PCB and pesticides (Apraiz et al., 2009; Bayne et al., 1979; Chase et al., 2001; Phillips, 1976; Thomas, et al., 1999). Today mussels still persist to be used as model species with "modern" pollutants like pharmaceuticals and personal care products, biocides, micro plastics and nanoparticles (Bonello et al., 2018; Faggio, et al., 2018) and also in cancer studies (Carella et al., 2017; De Vico & Carella, 2015)

Apart from these classic but essential bio indicator uses, mussels can provide interesting and unconventional applications in different application fields.

5.3.1 Mussels as pharmaceuticals, functional foods, food ingredients and much else

Mussels contain many functional molecules that once isolated can be used in many practical applications in different fields. Currently, many research groups are focusing their investigations on the evaluation of the bioactive potential of extracts, hydrolysates, or purified components derived from whole mussel meat, single organs, cell compartments, or blood (Grienke et al., 2014). Researches on this topic are mainly on the two most abundant mussel genera Mytilus and Perna. The main investigated compounds are primary metabolites of the proteins/peptides/amino acids group and lipids group (Grienke et al., 2014). As regard the first group, bioactive peptides derived from marine

mussels contain 5 - 40 amino acid residues. Depending on the amino acid sequence and structural properties, major biological effects of mussel peptides include antimicrobial, antihypertensive, antioxidant and anticoagulant activities (Jung et al., 2005; Löfgren et al., 2008; Jung et al., 2005). These kind of metabolites have antifungal, antibacterial, or antiviral effects; they have been proposed in aquaculture as natural antimicrobial agents for the treatment of infectious diseases in marine species (Balseiro et al., 2011) and as antimicrobial food additive for human consumption (Grienke et al., 2014). Other peptides have antihypertensive, antioxidant and radical scavenging capacity that allow them to find further applications in food preservation as an alternative to synthetic additives or in pharmaceutical industries to avoid lipid peroxidation (Jung et al., 2005; Rajapakse et al., 2005). Thanks to the antioxidant properties of similar peptides, it could possible synthetize antiaging products, as already produced by snails mucus (Greistorfer et al., 2017). Another important finding on mussel peptides is reported in Kim et al. (2012). They have isolated an anticancer peptide in *Mytilus coruscus* (actually *M. unguiculatus* Valenciennes). This peptide effectively induced cell death on prostate, breast and lung human cancer cells but not on normal liver cells.

The other group of mussel metabolites, the lipids, have a high potential for the commercial development of health beneficial functional foods or dietary supplements. Several anti-inflammatory and anti-arthritic dietary supplements, which contain mussel lipids, are still commercially available (e.g. Seatone® and Lyprinol®).

Moreover, several studies are focusing on the molecules and mechanisms on which mussels can attach their self to the substratum in order to mimic their adhesion mechanism and apply it in biomedical ((Jo et al., 2018; Forooshani & Lee, 2017; Li et al., 2015; Wang et al., 2015). This mechanism is a big draw for materials science also because it happens in the water. Han et al. (2017) fabricate adhesive hydrogels attractive biomaterials for various applications, such as electronic skin, wound dressing, and wearable devices. However, fabricating a hydrogel with both adequate adhesiveness and good mechanical properties remained a challenge until today. Han and colleagues, inspired by mussels, have created an excellent dressing hydrogel.

However many functional molecules are synthesized from mussels, some of these are summarized in the figure 5.3. Among mussels species, there is also *Mytilus galloprovincialis* providing different bioactive molecules. In the list there are mainly Mytilins, peptides with antimicrobial

activities (Balseiro et al., 2011; Chandran et al, 2009; Mitta et al., 2000; Mitta et al., 2008), and anti-inflammatory peptide (Badiu et al., 2010).

Overview on bioactive proteins, peptides, and amino acids derived from marine mussels.

Biological activity - name of bioactive protein/peptide	Sequence and molecular weight	Mussel species	Origin/product
Antioxidant			
Mussel-derived radical scavenging peptide (MRSP)	HFGDPFH (962 Da)	ME	Fermented sauce
N.g.	LVGDEQAVPAVCVP (1.59 kDa)	MC	*In vitro* gastrointestinal digest
N.g.	FGHPY (620 Da)	ME	Fermented sauce
Antimicrobial			
Mytilus defensin A	GFGCPNDYCHRHCKSIPGRXGGYCGGXHRLRCTCYR (~4 kDa)	ME	Blood
Mytilus defensin B	GFGCPNDYPCHRHCKSIPGRYGGYCGGXHRLRCTC– (~4 kDa)	ME	Blood
MGD-1	GFGCPNNYQCHRHCKSIPGRCGGYCGGWHRLRCTCYRCG (4.4 kDa)	MG	Hemocytes
MGD-2	GFGCPNNYACHQHCKSIRGYCGGYCASWFRLRCTCYRCG (4.4 kDa)	MG	Hemocytes
Mytilin A	GCASRCKAKCAGRRCKGWASASFRGRCYCKCFRC (~4 kDa)	ME, MG	Hemocytes
Mytilin B	SCASRCKGHCRARRCGYYVSVLYRGRCYCKCLRC (4.0 kDa)	ME, MG	Hemocytes
Mytilin C	SCASRCKSRCRARRCRYYVSVRYGGFCYCRC- (4.2 kDa)	MG	Hemocytes
Mytilin D	GCASRCKAKCAGRRCKGWASASFRRRCYCKCFRC (3.9 kDa)	MG	Hemocytes
Mytilin G1	VVTCGSLCKAHCTFRKCGYFMSVLYHGRCYCRCLLC (~4 kDa)	MG	Hemocytes
Myticin A	HSHACTSYWCGKFCGTASCTHYLCRVLHPGKMCACVHCSR (4.5 kDa)	MG	Hemocytes
Myticin B	HPHVCTSYYCSKFCGTAGCTRYGCRNLHRGKLCFCLHCSR (4.6 kDa)	MG	Hemocytes
Myticin C	QSVACRSYYCSKFCGSAGCSLYGCYLLHPGKICYCLHCSR (4.4 kDa)	MG	Hemocytes
N.g.	N.g. (9.7 kDa)	PV	Gill homogenate
Anti-inflammatory			
	15 Essential and non-essential amino acids	MG	Proteic extract
Antihypertensive			
N.g.	EVMAGNLYPG (Partial sequence – N-terminal region; 6.5 kDa)	ME	Fermented sauce
Antifungal			
Mytimycin	DCCRKPFRKHCWDCTAGTPYYGYSTRNIFGCTC– (6.5 kDa)	ME	Blood
Anticoagulant			
N.g.	EADIDGDGQVNYEEFVAMMTSK (2.5 kDa)	ME	Edible part
Anti-thrombin			
Pernin	Protein composed of 497 amino acids (60 kDa)	PC	Cell-free haemolymph
Adhesive for surgical applications			
	Adhesive protein	ME	Byssus

ME, *M. edulis*; MC, *M. coruscus*; MG, *M. galloprovincialis*; PC, *P. canaliculus*; PV, *P. viridis*; N.g., *not given.*

Fig. 5.3 Mytilus galloprovincialis provides antimicrobial (mainly Myticins) and anti-inflammatory bioactive metabolites (Modified by Grienke et al., 2014).

In addition, from the mussel *M. galloprovincialis* was recently extracted an anti-cancer lectine, the MytiLec (Hasan et al., 2015; Terada et al., 2016). It has a cytotoxic activity against cancer cell lines, as demonstrate by Hasan et al. (2015) MytiLec initiates programmed cell death of Burkitt's lymphoma cells through multiple pathways.

In conclusion metabolites from marine mussels of the genus Mytilus have shown promising results in different application fields. Hence, they represent invaluable sources for the development of functional foods, food ingredients, pharmaceuticals, anti-cancer therapy. Further advances in processing and analytical technologies, as well as a more interdisciplinary research focus, are expected to promote a straightforward and targeted development of health beneficial products in the near future (Grienke et al., 2014). *Mytilus galloprovincialis*, the local Campanian mussel, also have such remarkable potentiality.

5.3.2 Campanian pearls

Adding to the already numerous resources that *Mytilus galloprovincialis* provides, we have proposed it as a new potential pearl mollusc (see Chapter 4). Most molluscs can react to a stress stimulus forming a pearl, the unique gemstone made by animals. Pearls have fascinated man so much that there are many romantic legends about their formation as the entry in the mollusc body of God or angels tears or the heaven dew. Actually, the pearl came from a mistake: a pearl forms when the shell-formation pathway is induced in the wrong part of the animal (McDougall et al., 2013). Its formation starts when the mantle of mollusc is damaged and one or more epithelial cells from the upper epithelial layer of the mantle are transferred into the connective tissue. So the stress agent could be a piece of broken shell, lumps of conchiolin, small stone, the frequently mentioned "grain of sand", a parasite or a small animal as a crab or another mollusc that can enter into the shell. After the injury, the epithelial mantle cells multiply and form a closed cyst called the pearl-sac (Arnaud-Haond et al., 2007). The forming pearl has a similar shell structure, but the layers are in reverse order and concentric (Jacob et al., 2008). It is well known that gastropods and a many bivalves can implement this protection mechanism even that in some species the

process is human-induced. Using some species of molluscs, the "classical pearl molluscs", pearl production began a real industrialized process. The mainly used molluscs are in the Pacific area, chiefly oysters of the Pteriidae family (*Pinctada fucata*, *Pinctada maxima*, *Pinctada margaritifera*, *Pinctada mazatlantica* and *Pteria penguin)* (Gervis and Sims, 1992; Haws, 2012; Southgate and Lucas, 2011) and some mussels species as *Margaritifera margaritifera* and *Mytilus coruscus*.

In the industrial pearls production, pearls formations it happens with a surgically implantation of a mother of pearl nucleus. When the nucleus is inserted inside the molluscs, opening the valves with special pliers, an injury is also caused to the mollusc body. In this way pearl formation can start, around the nucleus. The nucleus could have a spherical or non-regular shape in order to produce different kind of pearls, however the surgically operation known as grafting is always carried out by an expert technician.

We have demonstrated in Chapter 4 that also the Mediterranean mussel *Mytilus galloprovincialis,* can react to a surgically injury producing a pearl. We have tested a different grafting operation, much more easy than the classical one and with a very low rate of mortality and rejections, the opposite situation to classical grafting. We have drilled the shell of adult mussels, insert a nucleus and close the hole with a mouldable thermoplastic material, perfectly adherent to the shell. Holing the shell the mantle is damaged, and around the nucleus, after six month, is formed a first pearl stage.

New investigations are needed in order to improve the whole operation, particularly the pearl quality as we have used a plastic nucleus with the same inert material used to cover the shell hole. New experimental tests have to perform with mother of pearls nucleus and/or with fragment of donor mussel tissue, as in the classical grafting in the classical pearl mollusc.

However, we have demonstrated that *Mytilus galloprovincialis* could be a new potential pearl mollusc. Pearls quality has to be improved: more spherical and more lustring pearls are, higher is their value. Nevertheless, if pearls have not a perfect shape can be appreciated in cosmetics and pharmaceuticals or in jewellery too as there are beautiful irregular shape pearls. In addition pearls, as well as the shells, can be manufactured. In this manufacturing art, Campania region have a strong tradition, particularly related to corals. Pearls and coral are two unique gemstone of animal origin; the first one is produced by molluscs as

described above and in chapter 4, the second one is made by the skeleton of animals of the genus Corallium, phylum Cnidaria. In the Mediterranean Sea the typical coral gem stone is represented by the species *Corallium rubrum* (Linneus, 1758) with its charming and fascinating red colour, a fascinating attraction for man for over 6000 years. In Campania corals manufacturing tradition places its root in the most ancient history and today is still a fine flagship; on the southwestern coast of the Gulf of Naples there is "the world's capital of coral" (Torntore, 2004), the country Torre del Greco, hosting also a coral museum “Il museo del corallo – Collezione Liverino”. Actually, as in the past, the Italian industry centred in Torre del Greco is one of the three most important locations in the world for the production of coral beads and other beautiful coral jewels (Liverino 1989a, 1989b). Data for coral market are impressive: in the late 1990s, Italy accounted for 90% of red coral commerce and the production of coral objects worldwide (Cattaneo-Vietti & Cicogna, 1993). Torre del Greco is always been on the front line of the coral market, the eighty percent of coral objects and beads made in "the world's capital of coral" are exported throughout the world from over 320 active businesses and workshops (Torntore 2002).

Generally companies working corals are able to work also with pearls and molluscs shells, producing real art works as cameos. Manufacturing *Mytilus galloprovincialis* pearls could surely be a source of new income for such companies. Mussel pearls of a non-premium quality could become equally beautiful jewels thanks to their manufacturing capabilities. Moreover, *Mytilus galloprovincialis* pearls can have an added value as a local production; another unique Campanian gem stone like corals.

5.4 The end of this project but not of the story… *Mytilus galloprovincialis*: a positive Campanian aquaculture model

With the investigations and observations showed in this PhD thesis we have validated mussels of the genus Mytilus as excellent model organisms and the species *Mytilus galloprovincialis* a very good model for the assessment of different kind of impacts on marine aquaculture in Campania. The mussel *Mytilus galloprovincialis* have an important economic and ecological value for Campania region. As all the other mussel species, *M. galloprovincialis* mussels are fundamental for the ecosystem services that provide: the regulation of water columns, their role of bioengineering species, the food web support, their essence of model organism and as source of bioactive compounds, besides being an important food source.

As food source, considering the whole mussel supply chain, *Mytilus galloprovincialis* mussels represent a big direct income for the local society. In Italy mussel farming is mainly based on *M. galloprovincialis* and is the most important national aquaculture industry; in Campania it covers the 90% of the regional aquaculture production. Mussel farming is an aquaculture activity always present in Campania, from the Cumans through the Bourbons until our modern time. The fact that mussel farming is an activity so well rooted in the society can take advantage of a millennial experience and, at the same time, it is an excellent basis for prosperous present and future activities.

However, various human impacts can affect negatively or positively the Campanian *M. galloprovincialis* supply chain state.

Anthropogenic stressors as the global question of Ocean Acidifications can negatively affect mussels and their linked activities. Nevertheless, we have demonstrated (chapter 3) that a congeneric species of the Campanian mussel, *M. unguiculatus*, can easily survive in the future predicted acidified sea conditions. However it would be interesting to evaluate the specifically effects of OA on *M. galloprovincialis* and compare the responses of the two mussels species.

Another anthropogenic stressor is the complex question of food frauds, it can seriously impact local mussels economy. According with our investigations (chapter 2), in Campania there are no species substitution on the market but on mussels origins, in the mussel farms too, there is still a lot of work to do.

In order to preserve the local Campanian mussels, both political than citizens actions are crucial as well as new scientific investigations. New researches are fundamental in OA questions, in order to better understand the effects on mussels, especially on *M. galloprovincialis*. New findings on the other examined question, food frauds, are crucial in order to find an easier and cheaper method to investigate both in species substitutions than for the undeclared origin. Anyway, the immediate measure for the two questions is the awareness of people. People have to know the right actions to induce less CO_2 emissions (in order to reduce or at least keep stable OA) and the right conscious food choices, preferring and enhancing the local products. In this sense with our work based on the genetically characterization of local mussel (chapter 2), we have laid the groundwork for an official recognition of a good quality and excellence product: the Campanian or Phlegrean mussel.

However, not all the anthropogenic stressors are harmful for mussel supply chain. This is the case of the induced grafting operation in *Mytilus galloprovincialis* mussels in order to produce a pearl (chapter 4). If such production will take root, Campania could have its own pearl production in a non-conventional pearl mollusc. Moreover pearl production in a local certified Campanian or Phlegrean mussels, it would be a great pride for the local economy, particularly for the well-established manufacturing corals market of the area.

Finally, with our investigations we have highlighted the importance of mussel supply chain in Campania and the crucial rule as well as the exceptional potentiality of *Mytilus galloprovincialis* on which such activity is based on. All our efforts ultimately aim to enhance the charming and ancient activity of Campanian mussel farming, both defending and preserving its products than proposing new developments. Our intentions fall perfectly in the guidelines FEAMP Campania for the years 2014-2020, planning the allocation of 73 million euro in order to strengthen and relaunch the fisheries and aquaculture sector.

5.5 Chapter 5 bibliography

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