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Bivalve culture optimisation of three autochthonous species (*Ruditapes decussatus*, *Mytilus galloprovincialis* and *Ostrea edulis*) in a central-western Mediterranean lagoon (Porto Pozzo, northern Sardinia)

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Abstract

Three autochthonous species of Bivalve Molluscs were investigated in the Porto Pozzo lagoon (northern Sardinia, central-western Mediterranean Sea). Gametogenic cycle, Condition Indexes and biochemical composition of the grooved carpet-shell, *Ruditapes decussatus*, from local natural banks were studied during the period July 2009-July 2010. All the factors examined showed a clear seasonal cycle, depending on the reproductive activity and on some environmental variables.

Growth, Condition Index and proximate composition of the Mediterranean mussel, *Mytilus galloprovincialis*, cultured in the oligotrophic lagoon of Porto Pozzo were compared with those obtained in a typical eutrophic ecosystem (the Calich lagoon) and in a mesotrophic basin (the Tortolì lagoon). The trial was carried out in long-line systems in the three lagoons considered, from April to October 2010. The results obtained confirmed a high variability of the three coastal habitats and among the mussel groups cultured in each of them, simultaneously revealing excellent performances, in term of morphometric variables and Condition Index, of the molluscs grown in the Porto Pozzo lagoon.

Finally, comparisons of the growth and survival rate of the European flat oysters *Ostrea edulis* reared using three different experimental tools were performed at two depths. The trial was carried out in the long-line system of the Porto Pozzo basin between June and October 2011. The best results in terms of survival rate and growth performances were achieved at the lower depth of -1 m, regardless of the experimental tools used.

Given the results obtained, the observation that Porto Pozzo lagoon is an excellent area for Bivalve Molluscs culture can be supported.

Chapter 1

GENERAL INTRODUCTION

1. General introduction

Nowadays, seafood plays a central role in human life. In 2008 fisheries and aquaculture activities supplied the world with about 142 million tonnes of product (Fig. 1.1), of which 115 million were for human consumption. In this regard, aquaculture productions represent about 46% of the total and China is the largest fish producing country, reaching amount up to 47 million tonnes, with nearly 15 from aquaculture activities (FAO, 2010).

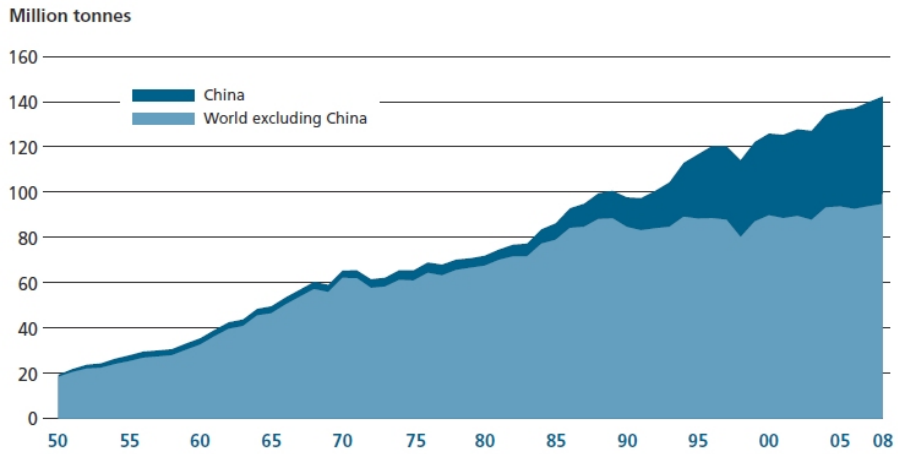

Fig. 1.1. Trend in world capture fisheries and aquaculture production between 1950 and 2008 (from FAO, 2010).

Therefore, aquaculture represents the fastest and largest growing sector in food industry (Costa-Pierce, 2002), increasing from 0.7 to 7.8 kg *per capita* quantity between 1970 and 2008. In 2008, it revealed a mean annual growth rate of 6.6%, and registers a total production of 52.5 million tonnes with an economic value of 98.4 billion US $ (FAO, 2010).

Farming activities are performed in different aquatic environments, as fresh, sea and brackish waters, but it is mainly practised in these two last realities where high-value fish, crustaceans and molluscs are cultured. In particular, 32.2% of the world aquaculture production (and about 31% in economic value) is attributable to seawater environments while brackish-water production is only 7.7%. Nevertheless, it represents up to 13% of the total economic value. In this regard, diagrams in Fig. 1.2. show the world fish production subdivided into major species groups: though more than a half of this is related to freshwater (with about 30 million tonnes), almost 25% is due to the mollusc culture industry, representing about 25% of the whole sector. At the end of

2000s, indeed, world production of molluscs reached more than 30 million tonnes, for a total economic value of 13 billion US $. In the same period and also today, the principal components of mollusc culture sector are Bivalves. Their production (from both capture and culture) increased rapidly over the last 50 years growing from 1 million tonnes in 1950 to 13.2 million tonnes in 2003 (despite 66% of that production is from China alone) (Dumbauld *et al.*, 2009). In the whole Bivalve shellfish market, oysters, venerids and mussels are the most representative. They amount to about 32%, 25% and 12% of the total, respectively, and were characterized by a mean annual rate of growth of almost 4% between 2000 and 2008 (FAO, 2010).

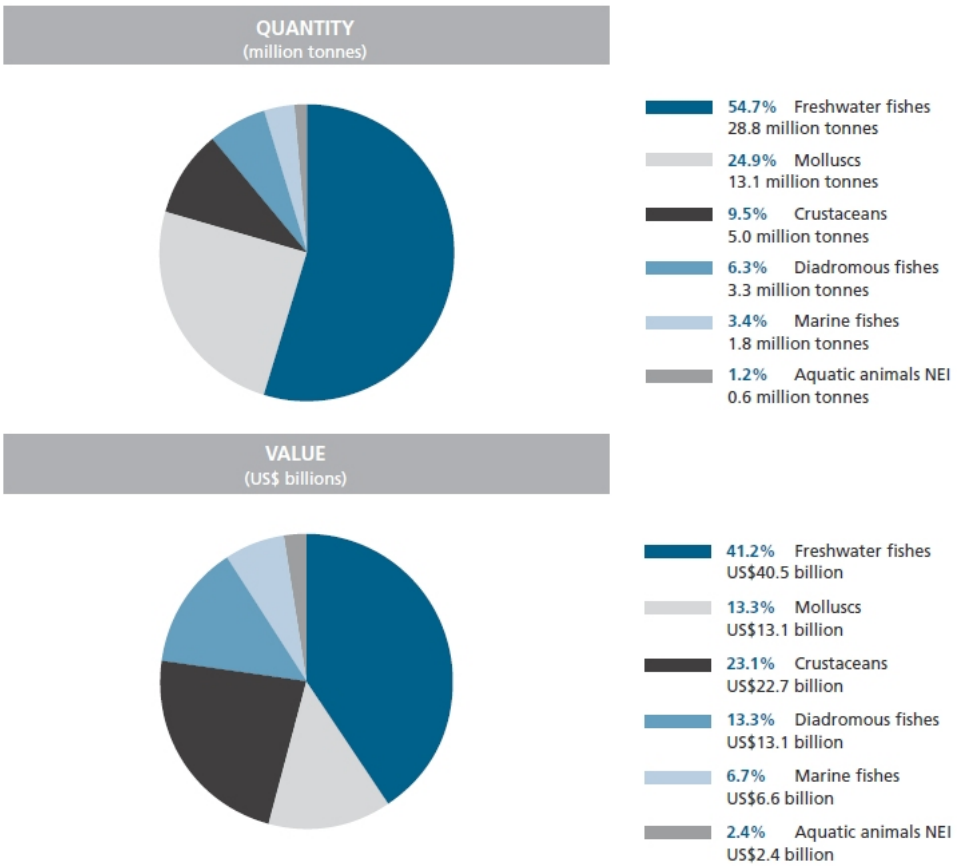

Fig. 1.2. World aquaculture production: major species groups in 2008 (from FAO, 2010).

In many countries, seafood represents a fundamental component of the human diet and its consumption is destined to increase with population growth. In general, despite the demand for finfish is predominant, the request for molluscs becomes increasingly important, in particular for Bivalves. It should be highlighted, however, that the preponderance of Bivalve production results from harvesting on wild populations in the

natural environment, thus contributing to deplete the wild stocks. A plausible solution to the overexploitation of resources, but simultaneously to supply the increasing demand from the Bivalve industry, is the hatchery culture, in particular with regard to oysters and clams.

Bivalves are ideal for culture methods because they simply require phytoplanktonic algae as food (naturally present in the water) to live, grow and reproduce. A limiting factor for their farming activities, however, can be considered an abundant, guaranteed and economical source of juveniles (spat or seed). In this regard, many farms in the world still collect spats in the natural environment and transfer them to growing facilities until they reach the commercial size. Where instead natural breeding do not exist because they are too far from the culture site, or do not well provide sufficient quantity of seed to ensure profitable production, artificial reproduction activities can be performed to produce juveniles into a hatchery. Bivalve hatcheries originated in the 1960s in Europe and United States and now are quite common in several countries. Since then, the knowledge of biology of widely reared species and the technology to produce them are continuously growing and improving (FAO, 2004).

In Italy, the national fish production is represented for 53% by fishing activities and the remaining 47% by aquaculture. In 2007, in fact, a decline in catches (-6.5% over the previous year) happened in the Mediterranean Sea and, at the same time, thanks to the shellfish industry, aquaculture production increased of 2.2%. Molluscs represented approximately 33% (about 175,000 tonnes) of the total aquaculture production, for an economic value greater than 300 million € (about 15% of total production), and registering a significant mean increase in prices of 3.4% in the period 2002-2007 (ISMEA, 2009). Among the main producer regions, Apulia, Veneto, Friuli-Venezia Giulia, Emilia Romagna, Liguria, Campania as well as Sardinia (Prioli, 2008) are included. Sardinia, with its 1,852 km of coastline and about 15,000 ha of wetlands, represented mainly by brackish coastal ponds and lagoons, and its favourable position in the Mediterranean, is one of the most suitable zone for euryhaline species breeding. As regards the island aquaculture practises, for quantity produced, the induced employment derived from such activities, and for the great economic value of marketed species, Bivalve farming can undoubtedly play an important role.

In this region, in fact, the local shellfish culture is dated to the early 80's. Actually, from 1992 to 2009, the number of companies in the sector fell by more than 20 to 15, despite the turnover of the industry amounted to more than 19 million € in 2009. As regard

mussel production (which includes the product imported and marketed in periods when the local one does not meet the demand, in particular during the summer), it represented about 75% of the total regional aquaculture production in 2002, and more than 82% in 2008. In the last twenty-year period, indeed, there has been a gradual increase of the sector, from 4,000 tonnes in 1992 to 7,900 in 2002, and up to 10,662 tonnes in 2008 (LAORE, 2009).

1.1 General description of the class Bivalvia

The phylum Mollusca is represented by a wide variety of both terrestrial and aquatic animals, and is taxonomically subdivided into 9 classes. Among these, the class of Bivalves or Lamellibranchs (*e.g.* clams, mussels, and oysters) includes predominantly only aquatic and marine forms. The organisms belonging to this class have an internal and external symmetry, are flattened shaped laterally and their soft components are even protected by a peculiar shell consisting of two hinged valves (*i.e.* a bivalve shell). Furthermore, they are characterised by particular gills (or *ctenidia*) highly developed and suited to both respiratory and feeding activities.

The main feature of Bivalves, therefore, is the shell. Each valve is composed of calcium carbonates that the animal is able to extract from the seawater and consists of three layers: the inner named nacreous, the intermediate that is the prismatic layer and constitutes the bulk of the shell, and finally the external one or periostracum. Moreover, in the dorsal part of the mollusc, specifically in the umbo, the two valves are joined together by a chitinous ligament that allows their gapping. In the opposite site, instead, the ventral margin occurs.

Bivalves do not have head but a rudimentary cephalic region only furnished with a pair of labial palps on each side. The other soft parts of the body are completely covered by a thin tissue (*i.e.* mantle or pallium), composed by two distinct layers. The mantle is organised into two lobes, connected dorsally to the shell and free at their edges, the latter often provided with glands, pigments spots, and various sensory organs in the form of tentacles, and even of eyes. Furthermore, the pallial lobes can be partially united in their posterior side to form two orifices. They can be more or less prolonged as two muscular tubes that may be extended for a greater distance beyond the shell (*i.e.* siphons). In general, besides secreting the shell, the mantle can control water flow into the body chamber thus ensuring the animal his normal nutrition and respiration activities. In particular, species equipped with siphons carry the entry of water by the

inhalant siphon and the ejection by the exhalant one. Furthermore, the pallial tissue has also a sensory function and can close the valves in response to unfavourable environmental conditions.

Inside the shell, the two mantel lobes are provided with some muscles inserted on the valvar surface and divisible into groups (*i.e.* pallial, orbicular, adductor and retractor). Among these, the adductoral muscles have the function to close the shell by their contraction thereby acting contrary to the chitinous ligament (which allows the shell gapping when the adductoral are relaxed). More precisely, these muscles are two (anterior and posterior) in dimyarian species as clams and mussels, and a single muscle is in monomyarian species as oysters.

Peculiar features of the Lamellibranchia are two pairs of gills or *ctenidia*, located on each side of the body between the mantle and the posterior part of the visceral mass. Each *ctenidium* consists of a hollow vascular axis bearing on each face a row of flattened filaments. This apparatus is used by the animal for both respiration and nutrition activities: the water which enters in the pallial cavity by the posterior part (*i.e.* the inhalant siphon) passes through the branchial filaments, and leaves by the anal orifice of the mantle or by the exhalant siphon. In this way, respiratory exchanges can take place and particles suspended in the water are carried towards the labial palps and consequently towards the mouth.

The so-called foot is a muscular projection from the ventral surface of the body. Its size and form are very variable, depending on the life habits of the animal: it is well developed in borrowing species (such as clams), but is generally reduced in those completely sedentary (such as mussels and oysters). The foot, when well developed, has a locomotory function allowing the slow dragging of the animal on and into the sediment by its successive contraction and extension. The mass of this organ is frequently occupied by a portion of the viscera, at least by a part of the digestive canal, the liver, and the gonads, and a cavity known as the byssogenous cavity in its middle part. Here, a byssal gland discharges its secretion that hardens on contact with the surrounding water and forms threads of a protein (*i.e.* conchiolin), constituting the trunk of the byssus. The animal can fix itself on a hard substrate through this structure and replace this protein filament with a new one when necessary.

As already mentioned, Bivalves can select food from the water. Food consists of micro-algae, micro-invertebrates, bacteria, detritus, and other organic material. The large gills capture particulate food, cilia on the labial palps selects particles into edible and

inedible portions, and move them into the mouth. Rejected particles are bound by mucus and moved to the mantle edge. Rapid closing of the shell valves ejects these mucoid-bound particle strings (*i.e*. pseudofeces) out of the mussel via the exhalant siphon. Once the food particles enter the mouth, they pass through the esophagus into the stomach where a combination of mechanical and chemical digestion breaks them into smaller particles. The stomach is a thin-walled sac, more o less deeply included into the foot, where the crystalline style revolves. It is a structure containing starch-digestive enzymes and is continually used and renewed. The food passes through the sorting area of the stomach and the finer particles are moved into the digestive glands. Here digestion and absorption takes place intracellulary.

Molluscs, in general, have an open circulatory system, and blood (*i.e.* hemolymph) is not completely contained within blood vessels. This apparatus is not composed by true vessels but rather by more or less dilated and spacious sinuses characterised by connective tissue without endothelium. The heart lies in the pericardium (a transparent sac), and is formed by two irregular shaped auricles and a ventricle. From this latter two aortas (anterior and posterior) is blood distributed to the entire body mass. The blood always represents an important component of the body and can constitute up to half of its weight. The hemolymph usually lacks any respiratory pigment, although some species are known to possess haemoglobin dissolved directly into the serum, and in some other species (*e.g*. certain clams) it appears bluish coloured owing to the presence of haemocyanin.

The nervous system fundamentally consists of three different pairs of cerebral, pedal, and visceral ganglia in Lamellibranchia. Tactile sensibility instead is specially localised in the most exposed parts of the body, as the mantle edges where, very often, sensory papillae or more or less well-developed tentacles are situated.

Bivalves are mainly dioecious (*i.e.* with separate sexes) but some isolated groups can be monoecious (*i.e.* hermaphroditic), and only few species present a clear sexual dimorphism. The fertilization is external. Generally, when gonads are prominent and well defined they appear paired and symmetrical (as in oysters) but they can also be less distinct and occupy the most part of the visceral mass. They can be close to the intestine, often extending thence into the foot (as in clams), and either opening into the nephridia, or through a separate pore into the mantle cavity. Furthermore, gonads are particularly evident during the breeding period of their biological cycle and practically indistinguishable in the other phases (Barnes, 1982; FAO, 2004).

1.1.1 Larval development

Bivalve eggs are usually small, ranging between 40 and 360 μm in diameter, and are generally characterised by a relatively little yolk uniformly distributed throughout the cell, and surrounded by a vitelline and a jellylike membrane.

Oocytes and spermatozoa are released in the water where fertilization and subsequent development take place. In species that brood embryos and larvae in the mantle cavity or gills, sperms pass from water to the maternal organism through the inhalant siphon, and reach the mantle cavity or the oviduct where fertilization occurs.

After a spiral cleavage of the egg, the first larval stage is formed: a ciliated *stereoblastula* for marine species, and a ciliated *coeloblastula* for brackish and freshwater species (Malakhov & Medvedeva, 1986). Moreover, a primordium of the shell gland is formed quite early by invagination in the embryo and the gastrulation process occurs immediately after. Although in marine Bivalves already the blastula stage is a transition to free living, only after the eversion of the rudimentary shell gland a trochophore-like larva forms, characterised by a broad ciliary circlet named prototroch, the main organ of locomotion. The mouth is under this structure and introduces to a blind gut, which connects the ventral wall of the trochophore forming the anus.

Subsequent metamorphosis is the transformation of trochophore into a *veliger*, a more complex larval form. It is characterised by a typical disk fringed with cilia (*i.e. velum*) as a swimming organ, an apical tuft of cilia in the centre of the *velum* with sensory function, and a translucent shell covering the rest of the larval body. More precisely, the *velum* is important for feeding as well as for locomotion: food particles are captured by its long cilia (Yonge, 1926), encased in mucus, and after moved toward the oral opening. Moreover, the tuft of cilia around the oral region seems to facilitate expulsion of excess mucus and excess food from the mouth (Waller, 1981). The buccal opening leads to the oesophagus, which continues with the stomach containing vacuoles. Finally, from this latter develops the liver, organised in two lobes and enclosing granules of food. An important difference from the adult molluscs is that the larval forms have neutral lipids as main reserve energy and not glycogen (Holland & Spencer, 1973; Holland, 1978): it seems that the changeover takes place in juveniles (*i.e.* spat) ranging between three and five months of age (Holland & Hennant, 1974). The reserve granules in the liver suggest that this organ has a storage function and most of these reserves are consumed both during the metamorphosis phases and the early period of the larval

settlement on the substrate, when the individual does not feed (Holland & Spencer, 1973). The gland of the crystalline style also begins to form rather early and the glandular cells secrete this apparatus containing digestive enzymes at the end of larval life or after settlement on the bottom.

Bivalve larvae do not posses a specific respiratory apparatus, so that oxygen and carbon dioxide are exchanged by diffusion. Clumps of cilia positioned in several portions of the body are designed for these activities. Also a real circulatory system is not present in larval forms and the transport of substances (particularly nutrients) among body regions occurs through the extensive body cavity and by muscular contractions.

In the *veliger*, swimming consists of a vertical rise followed by a passive sinking. It moves by beating the cilia of the ciliary band along the margin of the *velum*, which promotes movements to greater distances and, simultaneously, increases the food particles collection. It is important to underline that the *veliger* spends almost 10% of its energy to move with an average speed and over of 50% when speed increases (Zeuthen, 1947). Moreover, changes in salinity, temperature, and pressure affect nature and speed of *veliger* locomotion (Mileikovsky, 1973; Cragg & Gruffydd, 1975; Hidu & Haskin, 1978; Cragg, 1980).

The end of the planktonic life is characterised by further changes: by continuous addiction of material at the valval border, a new form of juvenile shell appears (Gruffydd & Beaumont, 1972; Maru, 1972; Le Pennec, 1974; Dix, 1976; Hodgson & Burke, 1988; Waller, 1991; Bellolio *et al*., 1993). Now, larva is in the *pediveliger* stage characterised by a maximum larval size and a functional foot to move, at once keeping the swimming capacity by its *velum*. The primary aim of the *pediveliger* larva is the selection and the colonization of a suitable substrate where it acquires its final arrangements in terms of nutrition and locomotion. In addiction, this larval form possesses already functional sense (*i.e.* statocysts) and locomotive organs (*i.e.* the foot). Moreover, although a real specialized respiratory system is absent, filaments on each rudimentary gill are added (Bayne, 1971, 1976), the velum reaches its maximum development and the new locomotive organ starts to function. The latter is a kind of ectodermal outgrowth on the ventral portion of the body and its primary function is to explore the substrate to allow the settlement. Indeed, the swimming larva can extend its foot but when it meets an appropriate substrate it starts to creep.

The most crucial phase in the entire life cycle is the stage where, once found a suitable substrate, the *pediveliger* released its byssus and attached itself. It is important to

underline that larvae of different species have a well-defined specificity for their appropriate substrate. If the larva does not meet the desirable substrate, it may postpone metamorphosis and even restart swimming (if the velum is not discarded). In many species, metamorphosis means a partial or even total reabsorption of some larval organs. The digestive apparatus moderately changes (Bayne, 1971), the liver gets its definitive organization, and respiration is provided by the gill apparatus, while the velum is completely broken up. On the whole, there is a general migration of those organs that survive metamorphosis (Belding, 1910; Jorgensen, 1946; Sastry, 1965; Hodgson & Burke, 1988): the mouth moving from its posterior-ventral location to the anterior-dorsal position, the foot becomes ventral rather than posterior and the posterior adductor migrates to the centre of the valve (Belding, 1910). In these conditions, the entire life pattern is modified: locomotion is provided by the foot, and capture of food by the gills and preoral lobes. In borrowing species, like clams, the foot develops further while it totally o partially reduces in the sessile ones (*i.e.* mussels and oysters), being their cells phagocytised (Hickman & Gruffydd, 1971).

Changes in shell structure correspond to the end of metamorphosis. The shell modifies from both a microstructural and mineralogical point of view, forming layers of the new structure (Wilbur, 1964). Finally, metamorphosis transforms larvae into juveniles, even causing deformity or mortality of the animal if disturbed in its sequences or blocked at any stage (Turner, 1976; Kasyanov *et al*., 1998; Shumway & Pearsons, 2006).

1.2 Regulation and production: water requirements and bivalve depuration

The areas for Bivalve production must meet the health parameters defined by the European Union Directive 91/492/EEC and adopted by Italy with D.L.n.530 of December 30, 1992 and subsequent amendments and additions. The competent authority defines the location and the boundaries of Bivalve production. The zones in which the collection of live Bivalves is authorized must be classified by the competent authority in the following three categories, according to the level of fecal contamination:

a) Class A: areas where live Bivalves may be collected for direct human consumption. The product harvested from these areas must meet the health standards for live Bivalve molluscs such as *Eschirichia coli* ≤230 UFC for 100 g of flesh and absence of *Salmonella* spp. in 25 g of flesh.

b) Class B: areas where live Bivalves may be collected, but they can be marketed for

human consumption only after treatment in a purification plant or after relaying in order to meet the health standards above referred. Live molluscs from these areas must not exceed the levels of 6,000 fecal coliforms per 100 g of flesh or 4600 *E. coli* per 100 g of flesh in 90% of the samples.

c) Class C: areas where live Bivalves may be collected, but they can be marketed only after relaying over a long period (at least two months), whether or not combined with purification, or after intensive purification, in order to meet the health standards above-referred. Live molluscs from these areas must not exceed the levels of 60,000 fecal coliforms per 100 g of edible tissue.

Depuration is a necessary technique adopted to remove microbial contaminants (such as *E. coli* and *Salmonella* spp.) and algal biotoxins from the molluscs. They are located in tanks equipped with clean, previously filtered, and disinfected seawater, to promote the resumption of their normal pumping activity. Storage water, in fact, must be characterised by specific ranges of temperature (12-20°C), salinity (>20.5 PSU) and concentrations of dissolved oxygen, because of the absolute upper and lower limits under which shellfish can carry out their biological functions. In particular, the level of oxygen is a fundamental factor to ensure the physiological activity of the animals and its amount varies with temperature (high temperature imply low concentrations of oxygen), while the oxygen requirement of Bivalves increases with temperature. The depuration phase is practised from several hours to days and normally is required by both the national and the local regulations.

When stocked in the depuration and dispatch plant (Fig. 1.3), clams are positioned in baskets and than immersed in horizontal or vertical tanks (*i.e.* bins; Fig. 1.4), square or rectangular shaped, where they remain in contact with water for about a night. Tanks are usually made of glass-reinforced plastic, high-density polyethylene or concrete sealed with epoxy resin that can come into contact with food, as well as connecting pipework and internal fittings. Within this system, treated clean seawater circulates with an adequate flow ensuring that wastes and pseudofeces are taken away.

Once the depuration process is concluded, the packing operations take place, generally in a separate area of the plant. Mesh nets or plastic bags containing a standard amount of shellfish (usually 0.5, 1 or 2 kg) are generally employed, as well as packing machines set for specific weights of product for each pack and to clean and sorting it (Fig. 1.5).

A specific label is needed to indicate all the information about the product: species, date of packing, shelf life (normally 5 days), number of the packing centre. Moreover, the

label has to be waterproof and well fixed to the pack, respecting local or international regulations.

From this moment packed product can be sale but for the transport it is better to keep shellfish at cold temperature (generally 2-10 °C), avoiding any contamination (FAO, 2004).

Fig. 1.3. Indoor mechanized Bivalve depuration plant.

Fig. 1.4. Horizontal and vertical depuration systems (bins).

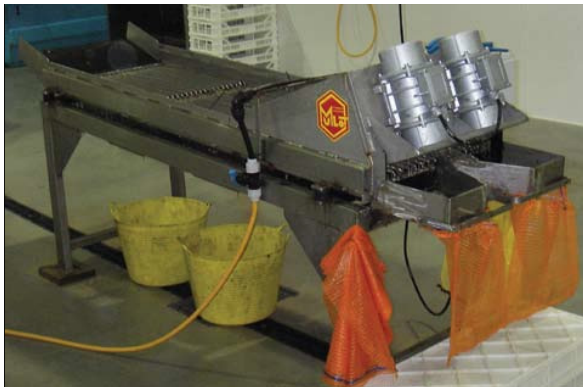

Fig. 1.5. Sorting and packing table.

1.3 Nutritional quality of Bivalves

Bivalves have a nutritional composition similar to each other. The proteins of high biological value, up about 10% by weight, represent a lower quantity compared to those found in meat and fish, but are comparable to that of eggs. Fats, present in limited quantities (between 1 and 3% by weight) consist mainly of long-chain polyunsaturated fatty acids. These lipid components are known to play an important role in reducing the risk of many degenerative diseases including atherosclerosis and coronary thrombosis. Among their characteristics, Bivalves are an excellent source of vitamin B12 and contain varying degrees of the other B vitamins. As regards minerals, iodine (essential for the proper functioning of the thyroid), iron (needed for binding oxygen in hemoglobin of red blood cells), zinc (important for growth and the immune system) and selenium (antioxidant and favoring the growth and fertility) are very well represented. In addition, the very low caloric content (between 70 and 85 grams calorie/100) and the low proportion of connective tissue in the meat, make this shellfish soft and highly digestible, consequently, Bivalves are also suitable for diets, with the exception for those suffering from gout and in some cases of hypertension. As regards cholesterol excess attributed to certain shellfish species, especially oysters and mussels, it should be noted that its "dangerousness" is greatly reduced when combining with dietary foods with low total lipid contents.

The quality features of Bivalve molluscs are primarily dependent on the quality of the aquatic environment, assuring a healthy product and a safe consumption. However, from a nutritional standpoint, other characteristics may influence the product quality (Beninger & Lucas, 1984; Karakoltsidis *et al.*, 1995). Water, protein, lipid, mineral and glycogen contents of the meat, together with minor components of hydrophilic or lipophilic nature, contribute to the nutritional value and organoleptic characteristics. Proteins, fats and carbohydrates are the basic building blocks of the living organisms. These molecules are changed by living organisms in their metabolisms, give out energy through fragmentation, and form specific synthesis products. A parameter of ecophysiological and economic relevance, especially in view of the industrial processing, is represented by the Condition Index, a measure of the apparent health and commercial quality of Bivalves (Orban *et al*., 2002). These molecules and condition index should be regularly monitored for successful Bivalves culturing activities.

Density, temperature, salinity, pH, chlorophyll *a*, organic matter, gametogenetic activities of the animals and environmental factors like streams and waves are

significant factors that can affect meat yield, biochemical composition and condition index of Bivalves (Saxby, 2002; Zrncic *et al.*, 2007; Gullian & Aguirre-Macedo, 2009).

1.4 The Porto Pozzo lagoon and its productive utilization

In Sardinia, the Porto Pozzo lagoon (Fig. 1.6) represents a typical coastal area where numerous different species of Bivalves naturally live, as well as several aquatic species. It is located in the North-eastern Sardinian coast, in the municipality of Santa Teresa Gallura, near the homonymous town. This is the most confined area of a long ria, which arose during the geological evolution of the Tertiary to Quaternary as a result of post-glacial sea rising, resulting in an invasion by seawater of ancient valleys previously carved by rivers (De Muro & Piras, 2007). The lagoon is divided into three basins: the main one, "La Peschiera", with an extension of 36 ha, "Balisgioni" covering a surface of 20 ha, and "Padula Chioca" (the smallest) of about 8 ha. Two outlets provide to its water exchanges with the sea. The mean depth is about 4 meters, although the maximum depth of 16.5 meters is reached in the largest basin (*i.e.* La Peschiera), and the a bathimetric of 6 meters is reached in the smaller one (*i.e.* Balisgioni), for a total volume of about 3 million m^3 of water. The internal water circulation is regulated by the strong winds from North, North-West, and by the tidal movements. On the contrary, following the damming of the Liscia River, freshwater inflows are rather scarce (the only tributary is a branch of the Rio Lu Bianconi). In general, the bottom is not characterized by a homogeneous texture but sandy and rocky areas are present throughout the basin. The shores are mostly sandy, and a typical marine vegetation is present especially in the southern part of the lagoon.

Fig. 1.6. The Porto Pozzo lagoon.

Due to the above-mentioned peculiarities, high abundance of fish can be found in the Porto Pozzo basin, particularly fish species as *Mugil cephalus*, *Sparus aurata*, *Dicentrarchus labrax*, *Lithognathus mormyrus*, *Diplodus sargus sargus*, *Mullus barbatus*, *Solea solea*, and shellfish as *Cerastoderma edule*, *Venus verrucosa*, *Venerupis aurea*, *Ruditapes decussatus* (Gazale & Morucci, 1991), *Ostrea edulis* and *Mytilus galloprovincialis*.

In 1996, based on an authorization from the Regional Determination No. 371 of 17/2/99, subsequently extended indefinitely by the Regional Resolution No. 75/7 of 30/12/2008, the local cooperative of fishermen ("La Peschiera") was founded. Nowadays, six members compose the cooperative and for many years their activity was limited to fishing activities and Bivalve molluscs harvesting. Because of the particular vocation of these waters, in 2007 a mussel farming system was established, whose productions, from the beginning until now, are steadily increasing from about 40 to 110 tonnes per year. The plant allows the production planning, permitting the cooperative to have product at commercial size almost throughout the year or at least during periods of increasing demand (*i.e.* summer and Christmas time). By contrast, other shellfish production (in particular clams and oysters) is highly dependent on natural populations resident in the lagoon. In fact, although the cooperative's members avoid the removal of small specimens, many illegal poachers are constantly present in the basin, causing then not only an economic damage to the fishermen, but also a structural damage to Bivalve stocks. Overall, however, production of native clams is around 300 kg per year (excluding the winter collection period) and, of course, this quantity is not sufficient to satisfy the high market demand. Finally, as regard to the oyster production, it is practically absent despite this Bivalve is autochthonous in this area. Consequently, because of the high market demand, also their product is completely imported from neighbouring plants or other Italian regions, in some cases even from abroad.

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Chapter 2

RUDITAPES DECUSSATUS

2.1 Introduction

In the mid 1920s, intensive harvesting of the grooved carpet-shell *Ruditapes decussatus* began in Spain and till today, along with France, this is the country where this activity mainly occurs. During that period, several fishing techniques were employed (also the forbidden ones) and specimens of all size were captured. Nevertheless, it was noted that the natural populations of this species showed a great recovery capacity.

Nowadays, the production of this Bivalve is basically due to a few countries, specifically Spain, France and Portugal on the Atlantic cost, and Algeria and Italy in the Mediterranean Sea (www.fao.org; Fig. 2.1). However, this species is commonly present along the eastern Atlantic Coast from Norway to Senegal, and in the South and West coast of the British Isles (Tebble, 1966; Breber, 1985).

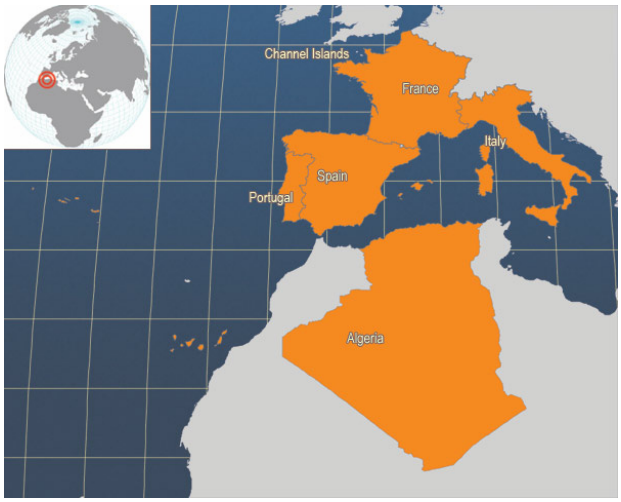


Fig. 2.1. Main producer countries of *Ruditapes decussatus* (from www.fao.org).

Overall, the global aquaculture sector recorded a slow but steady growth, and simultaneously fish production obtained by fishing activities decreased. The shellfish industry is no an exception, with a world production in 2008 assessed at values of around 13 million tonnes, compared to about 10 million tonnes produced in 2000. In the same period, farming of clams belonging the genus *Ruditapes* registered a total production of about 3 million tonnes and Italy, with over 60,000 tonnes (FAO, 2010), results the first European producer of clams and the second one worldwide. It is important to clarify, however, that most of the clam production is due to the culture of the Manila clam *Ruditapes philippinarum*, introduced into brackish lagoons of the Northern Adriatic in 1983 (Cesari & Pellizato, 1990), and not by the endemic one *R.*

decussatus. In fact, this latter is almost exclusively collected from natural beds, particularly in Sardinia where strict restrictions imposed by the Regional Government are aimed at resource management.

Before the Manila clam introduction, the Italian market was exclusively based on the harvesting of the endemic species (about 1,000 tonnes year^{-1}), whose origin was limited to natural environment especially in the Venice Lagoon (Breber, 1996) and in the Po river delta (Carrieri *et al.*, 1992). As a consequence, in several cases, the continuous and intensive fishing of this product led to a sharp decline of the wild populations (Pellizzato *et al.*, 1989), which caused a massive importation from other countries, such as Morocco, Tunisia, Turkey, France, Spain and Greece. The need to preserve this natural resource, however, have led to put particular attention on fisheries management of the wild stocks, also developing breeding techniques and natural restocking activities by hatchery production and culture on the bottom (Hamida *et al.*, 2004). Currently, in fact, shellfish aquaculture is an intensively growing industry that implements the natural production from harvesting activities and enriches the local economies (Nunes *et al.*, 2003). Actually, the grooved carpet shell has a good market demand and its price has remained high (Breber, 1985). In some countries (*e.g.* Portugal), moreover, it is commercially more important and appreciated by consumers than the Asian species (Matias *et al.*, 2009), and because it is an eurythermal, euryhaline, and highly resistant to desiccation species is particularly suitable for aquaculture production (Christophersen, 1994). In general, farming of *R. decussatus* primarily depends on the availability of the seed (juveniles) so that, until few years ago, the most of its production was based on natural recruitment. Nowadays, however, modern rearing techniques associated with manipulation methods of the gonadic cycle and spawning period of this species are widely employed. These considerable programmes had allowed the establishment of specific production schedules (according to breeders demand), the improvement of natural breeding sites efficiency, and a considerable time saving. In addition, the overall method of farming has the advantage of being well organized in separate and distinct phases interconnected among them, and each one having a specific technical solution, which permits to intervene more easily in case of any problem (*e.g.* pollution, parasitism, abrupt environmental changes, etc.).

Therefore, it is clear how fundamental a high level of knowledge on the reproduction activity can be to start a successful rising. In this regard, a number of studies demonstrated that gametogenesis in marine invertebrates is closely related and

dependent on the environmental factors, and above all water temperature (Giese, 1959; Sastry, 1975; Adiyodi & Adiyodi, 1983; Ponurovsky & Yakovlev, 1992). Further research, moreover, reported that also food availability is a key factor playing a primary role (as nutritional reserves in the mollusc) in the reproductive activity evolution of Venerids (Goulletquer *et al.*, 1988; Pérez-Camacho *et al.*, 2003). Thus, metabolic activities of these invertebrates are the consequence of the interaction among environmental conditions, food availability and gonadic cycle (Gabbott, 1983), so that the induction of sexual maturation concerns the manipulation of their physical and nutritional environment (Gallager & Mann, 1986), the so-called "broodstock conditioning".

In general, being the temperature and food availability closely related with geographic location and seasonality, Bivalve species have adopted particular adaptive strategies. Gametogenic cycles, in fact, can vary from one geographical area to another (Robert *et al.*, 1993; Laruelle *et al.*, 1994; Urrutia *et al.*, 1999; Drummond *et al.*, 2006), but also among species and among different geographical population within the same species (Avendaño & Le Pennec, 1997). It is therefore clear that collected reserves, management of the stored energy for growing, and biochemical components for the reproductive events contribute to define the proper adaptive strategy of a species (Goodman 1979). Generally, when food is abundant, glycogen, lipid and protein are accumulated as energetic reserves prior to beginning of gametogenesis, then being used to produce gametes (Giese, 1969; Bayne, 1976), and finally released during the spawning process. Widdos and Bayne (1971), for instance, reported high levels of glycogen, as well as lipids and proteins, in *Mytilus edulis* during summer, when energy needs are low; in autumn and winter, on the other hand, when energetic demand raised, glycogen registered minimum levels. This fact confirms that biochemical composition and reproductive cycle are closely related in different species of Bivalves (Beninger & Lucas, 1984; Bressan & Marin, 1985). Carbohydrates (above all glycogen) result the major resource of energy and are necessary for the development of gametes, when a moment of nutritive stress (*e.g.* in winter) occurs (Gabbott, 1975) and as structural elements (Robledo *et al.*, 1995), so that their content represents the nutritional condition of the mollusc (Uzaki *et al.*, 2003). Lipids are also an important quota of reserves when food is deficient, and constitute a large portion of oocytes (Holland, 1978) reaching their maximum level in the pre-spawning phase (Taylor & Venn, 1979). Lastly, proteins represent the largest fraction both in tissues and in oocytes, assuming the role of energy

reserve during gametogenesis (Holland, 1978; Beninger & Lucas, 1984).

Hence, broodstock conditioning is a fundamental phase in the productive thread providing spat outside to the natural reproductive season and allowing the subsequent seeding of molluscs in the natural environment (pre-fattening and fattening phases). For this reason, sediments for breeding activity and the surrounding area should be subjected to arrangement, monitoring constantly the main physico-chemical and biological variables of the water column (including the concentrations of nutrients) and the principal characteristics of the substrates, cleaning and delimiting them in order to improve and maximise yield and quality of the final product.

In the early 1930s, in Italy, exactly in the Goro lagoon (northern Adriatic Sea), a cooperative of fishermen (named “Cooperativa Pescatori di Goro) was the first to begin organizing themselves in a democratic management of the fish market (*i.e.* establishing the fishing quotas per household, fishing less, with constant prices and guaranteeing the reproduction of fish species). It was the expression "to cultivate the sea" that began the change of mentality, marking the transition from a traditional conception of fishery to a new one based on the management of marine wealth. Firstly, this happened just for clams, passing from a simple fishing activity to one based on aquaculture techniques. Fishermen began to manage resources in a rational way, organizing the seeding activity, moving the product from less productive areas to others more suitable to growth, and by implementing the cleaning of their facilities. Since the 60's, in fact, they have moved from fish to grow clams (Credi, 2007).

Despite aquacultural activities, however, clam harvesting from natural beds is still a fairly common practice and, in the past, this was rather indiscriminate: in fact, special attention was not paid to the instruments used, period of harvest or maximum quantities. Nowadays, by contrast, the Italian Coast Guard has issued the so-called "technical stop", which lasts two months between April and September, just in order to promote the growth and reproduction of this resource. The measures also indicate the maximum quantities of shellfish per day, the perimeter and the bathymetry of the collection area, the days and hours of fishing. Beyond that, by June 1st 2010, a new EU rules contained in the Community Regulations of the Mediterranean fisheries, prohibits the collection of shellfish with hydraulic dredges up to 0.3 miles from the coastline.

Finally, EU, national and regional regulations establish the minimum harvesting size for the genus *Ruditapes* to consumption. The EU legislation, in fact, provides that this must be 25 mm [R. (CE) 1967/2006], and the Italian government has adopted the same extent

(D.P.R. 1639-2/10/1968 and subsequent amendments). Despite this, the Sardinian government adopted a more restrictive regulation setting the minimum size to 35 mm (D.A.D.A.R.S. nr. 412-10/05/1995). Sardinia, indeed, is a region where venericulture sector plays an important role for the quantity produced, the induced employment derived from it but, above all, for the remarkable economic value of marketed species. Although some attempts of introduction of the exotic clam *R. philippinarum* were illegally made, results were much lower than expected, if compared with those recorded in other Italian areas where this species acclimated better. Currently, therefore, in Sardinia the natural beds of clams belong almost exclusively to the indigenous species *R. decussatus*. However, the ever-growing demand for a quality product from both the local market and the touristic one determines the need to enhance and increase aquacultural activities. In fact, the simple harvesting from the wild is not able to meet the increasing demand of the product and that, in some cases, may even result in an overexploitation of the natural resources.

The aim of this study, therefore, was to assess the real potential for exploitation of the autochthonous grooved carpet-shell *R. decussatus*, naturally present in the Porto Pozzo lagoon, but not yet sufficiently managed in terms of production. Presently, indeed, the considerable limitations for its reasonable use within this lagoon are mainly due to the lack of an adequate amount of information about the consistency of the natural beds. Furthermore, in addition to the harvesting activities normally carried out by the local fishermen cooperative, the clam stocks are constantly subject to withdrawal by illegal poaches.

Based on the above considerations, therefore, in order to perform a preliminary study for establishing a future successful hatchery-based production and for implementing programs to enhance and restore the natural stocks, the expected results from this study may be summarized as follows:

- Characterise the natural reproductive cycle of *R. decussatus* population in the Porto Pozzo lagoon.
- Monitor the main physic-chemical variables of the water of the lagoon and relate them with the mollusc specimens sampled.
- Detect seasonal changes of the biochemical composition of edible parts of the clams.

2.2 Materials and methods

2.2.1 The grooved carpet shell Ruditapes decussatus

2.2.1.1 General description of the species

The grooved carpet shell *Ruditapes decussatus* (Linnaeus, 1758) is a Bivalve belonging to the Veneridae family (Rafinesque, 1815) (Tab. 2.1).

Tab. 2.1. Systematic framework of *Ruditapes decussatus* (Linnaeus, 1758).

Taxon	Name	Author
Phylum	Mollusca	
Class	Bivalvia	(Linnaeus, 1758)
Subclass	Heterodonta	(Neumayr, 1884)
Infraclass	Euheterodonta	
Order	Veneroida	(H. & A. Adams, 1815)
Superfamily	Veneroidea	(Rafinesque, 1825)
Family	Veneridae	(Rafinesque, 1815)
Subfamily	Tapetinae	(Gray, 1825)
Genus	*Ruditapes*	(Chiamenti, 1990)
Species	*Ruditapes decussatus*	(Linnaeus, 1758)

The shell is oval shaped and slightly compressed on dorsal and ventral margins, with the umbo (or hinge area) well evident and distinctly anterior, trunked at the back. The external surfaces of the shell are sculptured by concentric striae and radiating lines that make evident the growth stages, whereas the inner faces are smooth and glossy white, generally with yellow or orange tints and a bluish nuance along their dorsal edge. The term "*decussatus*" means crossed, and refers to streaks arranged in a cross (Fig. 2.2).

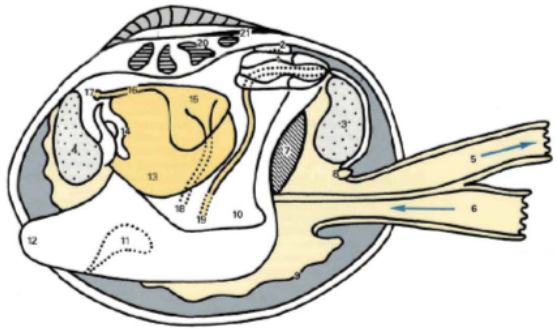
Inside the shell (Fig. 2.3), the body of the mollusc has the typical features of Bivalves with a peculiarity: the large foot and, above all, the two long and well divided siphons (that distinguishes it from the Philippine species *Ruditapes philippinarum*, whose siphons are fused at their origin; Fig. 2.4).

This Bivalve typically lives borrowed in sandy and silt-muddy bottom, inhabiting the

areas near and below the mean sea level (intertidal zone and subtidal zone, respectively), and buried 15-20 cm into the sediment. Moreover, it continuously filters surrounding water through its two siphons protruded from the substrate, picking up organic particles and phytoplanktonic cells as nourishment and to allow gas exchange between oxygen and carbon dioxide that occur with breathing.

Fig. 2.2. The shell of *Ruditapes decussatus.*

Even though cases of hermaphroditism can be found (Delgado & Pérez Camacho, 2002) especially in juvenile forms (Lucas, 1975), this clam is strictly gonochoristic and the reproduction takes place externally in the aqueous medium, mainly in summer when temperature is higher and food is abundant. Resulting larvae are freely floating for 10-15 days until once, found a suitable substrate, they settle as spat (about 0.5 mm in length) and continue their growth to adult form.



1. Heart
2. Pericardium
3. Posterior adductor muscle
4. Anterior adductor muscle
5. Exhalant siphon
6. Inhalant siphon
7. Gills
8. Anus
9. Mantle edge
10. Gonad
11. Byssus gland
12. Foot
13. Digestive gland
14. Labial palps
15. Stomach
16. Esophagus
17. Mouth
18. Gland of the crystalline style
19. Gut
20. Cardinal teeth
21. Ligament

Fig. 2.3. Anatomy of *Ruditapes decussatus* after ablation of the upper gill.

Overall, clams bear quite well the variations of chemical and physical variables of water, such as temperature, salinity, dissolved oxygen, turbidity, typical of lagoon environments or estuarine areas where they live. For this reason, their favourite sites are generally located away from zones with high hydrodynamic, and from windy areas where the substrate in which they are buried can be destabilised. Nevertheless, it is important the presence of a slight and a constant current that allows a good water exchange and the constant flow of food. For this reason, clams can live on a variety of substrates although a mixture of sand, silt and granules is the most suitable composition which allows a good oxygenation and a comfortable softness of the bottom.

In the wild, *R. decussatus* (especially at spat stage) has several natural predators, in particular crabs, some fish species (*e.g. Sparus aurata*), gastropods (*e.g. Rapana venosa*), and birds (*e.g.* herons and gulls). Beyond this, different etiologic agents (especially parasites) have been found in Italian waters, including species such as *Perkinsus* sp., *Vibrio* sp., and *Cercaria pectinata*. In addiction to the above-mentioned predatory activities, it is important to emphasize that other forms of competition can affect the stability of a clam population. Marine organisms, such as other species of Bivalves, Hydroids, Bryozoans, Serpulids, etc., being filter feeders can compete for food availability. At the same time, another form of competition can take place during the recruitment, depending on the availability of suitable substrates (Paesanti & Pellizzato, 1994; FAO, 2004).

Fig. 2.4. Anatomical difference between *Ruditapes decussatus* and *R. philippinarum*.

2.2.1.2 Biological cycle and artificial reproduction

The carpet shell *Ruditapes decussatus*, like most of other marine benthic Bivalves, is characterised by a cyclical pattern of reproduction, which can be divided into different phases: gametogenesis and vitellogenesis, spawning and fertilization, larval

development and growth. Each species evolved a number of adaptive strategies (genetic and non) to coordinate these events with the environment in order to maximize the reproductive process (Newell *et al.*, 1982). In this regard, in fact, numerous studies show that gametogenic cycle in marine invertebrates is strictly conditioned by the interaction between exogenous factors (*i.e.* temperature, salinity, light, availability of food, parasitic infestations) as well as by internal factors (Rodríguez-Moscoso & Arnaiz, 1998). Temperature is certainly one of the most important factors influencing the reproductive cycle (Sastry, 1975), defining both the starting point and the rate of gonadal development, whereas food availability can determine the extension of the reproductive process (Lubet, 1959). These two factors are subject to natural seasonal fluctuations and their variability is closely related to the energy available for growth and reproduction. In particular, reproduction requires abundant energy for providing a suitable gonadic development so that the success directly depends on ingested food or on previously stored reserves (Delgado & Pérez Camacho, 2005). In general, when food is abundant, reserves accumulated before and after gametogenesis (*i.e.* glycogen, lipid and protein) are utilized to produce gametes when metabolic demand is high (Bayne, 1976). As a consequence, therefore, gametogenesis varies from location to location depending on the geographic area considered: in adult clams from Southern Europe, for example, the cycle generally starts in March, gonads become ripe in May-June and spawning occurs in summer, following a phase of inactivity in winter (Shafee & Daoudi, 1991).

Until a few decades ago, the management of this species was exclusively linked to the availability of natural seed, but nowadays manipulation of its gonadal cycle is possible. In fact, artificial spawning techniques and larval rearing programs have been recently developed. These methods are applied in highly specialized systems, the hatcheries, where breeders (previously selected from natural beds on the basis of their appearance, size and shape) are stocked into tanks for 30-40 days at 20 °C of temperature (Fig. 2.5), and richly fed with phytoplanktonic algae. To assure the continuous availability of this nourishment for breeders and future larvae, hatcheries have to possess algal culture systems (Fig. 2.6).

The specimens selected are richly fed to maximise their gonadic maturation until they are ready to reproduction. At this phase, the release of gametes is induced by a thermic shock of the water of about 10°C (from 18 to 28°C), repeated for one or more cycles of about 30 minutes each. Generally, males emit before females and fertilization occurs in

small containers. The eggs thus obtained are counted, filtered and placed into little aquariums (about 10 l in volume), where *veliger* larvae appear after 8 days. Then they are filtered through a 100 µm mesh, daily fed with phytoplankton for the first week and subsequently every two days. At the *pediveliger* stage, clams have a diameter of about 180-220 µm, they already have the foot but the "*velum*" is still present. Indeed, they spend most of their time swimming and sometimes are fixed on the container surfaces. After about 3 weeks, the metamorphosis process is completed and the spat stage is reached (about 250 µm in size). The little mollusc can be now reared in greenhouses, fed by phytoplankton or by pumping environmental water into inland tanks, where they are placed inside small containers having a rigid mesh as bottom (*i.e.* nursery).

Fig. 2.5. Broodstock conditioning system (www.fao.org).

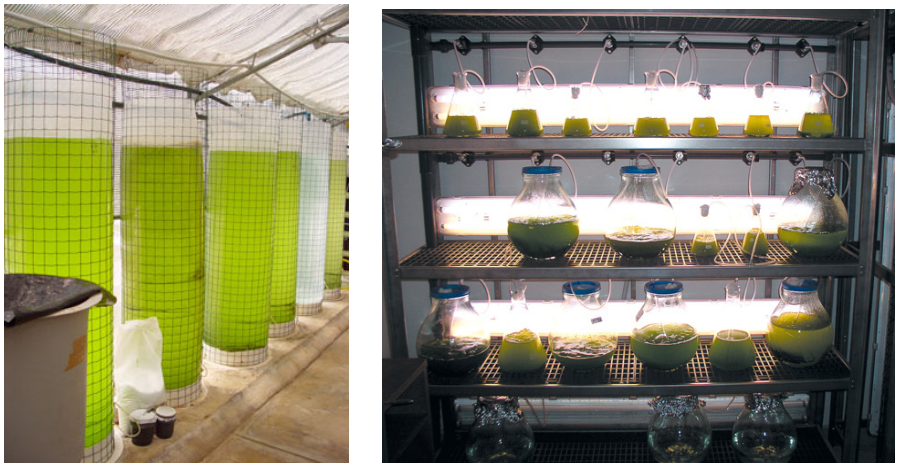


Fig. 2.6. Phytoplankton culture systems.

From this stage onwards, methods of farming may be different depending on the features of the hatchery (*e.g.* standing water, constant water flow, downwelling and upwelling forced water flow).

2.2.1.3 Production cycle and culture methods

As said above, spat can be obtained both from natural populations in the vernal period (digging them with sand by a small rank and riddling it to retain the seed) and from hatcheries. When a size of about 1 mm is reached, a new phase of rearing can start using a controlled system: the so-called pre-fattening (Fig. 2.7). Clams grow up to about 10-15 mm in 2-4 months and it would be convenient to complete their weaning period outside, pumping natural seawater or brackish water, because their maintenance into the hatchery is quite difficult for both management and for economic reasons (Fig. 2.8).

Fig. 2.7. Pre-fattening phase (www.fao.org).

Once they reached this size, depending on the preferences and the possibilities of the farmer, molluscs can be transferred to the ground (with a density of about 5,000 individuals m^{-2}) or in special facilities that allow their growth in suspension, such as net bags (pôches) or stacked baskets (at lower density) (Fig. 2.9). Moreover, if they are sown directly on the substrate, it is advisable to protect the seed from predation by plastic nets. In this way, clams are able to reach a size of 20-25 mm in about 2 months. At this phase, management regards only the preparation and maintenance of breeding substrate (*i.e.* cleaning and removal of algae or predators) or the control of the suspension systems (*i.e.* attachment and clearing of encrusting organisms or fouling).

The last procedure of production cycle is the fattening, where carpet shells grow in the bottom within the substrate. In this way, molluscs live following their natural pattern, filtering water and then feeding, until they achieve the commercial size of about 30 mm in length. According to environmental conditions and breeding, the fattening stage can be completed in a period of 12 to 28 months.

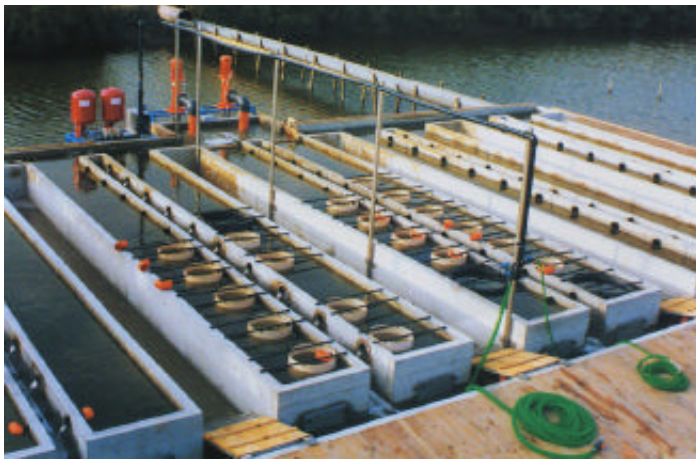


Fig. 2.8. Weaning system in upwelling.

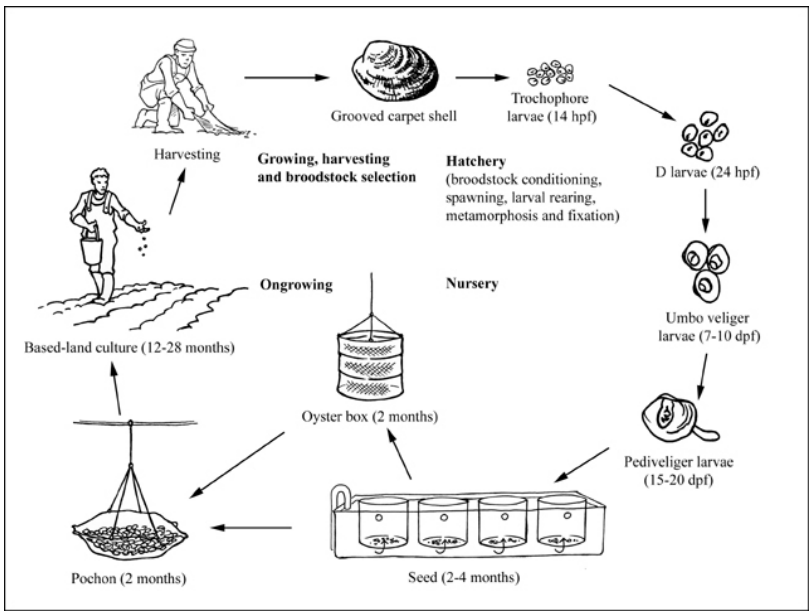


Fig. 2.9. Production cycle of *Ruditapes decussatus* (www.fao.org).

Once they have reached the commercial size, clams can be gathered in different ways depending on the type of farming. When and where it is possible, fishermen manually collect the Bivalves by walking using a rake equipped with an appropriate net, whose mesh is sized to hold the molluscs and allow the escape of sediment. Alternatively, the harvest can be made from boats (with oars or engines) furnished with an extended rake.

2.2.2 Natural banks in the Porto Pozzo lagoon

The native clam *Ruditapes decussatus* (Fig. 2.10) is a species of Bivalve mollusc commonly present in the Porto Pozzo lagoon. Its natural beds are scattered in the sandy areas of the basin, although its distribution is strongly influenced by natural phenomena,

such as environmental changes related to seasonality, food availability, reproduction cycle, and also by human harvesting pressure. Actually, the exploitation of this resource is a quite old and widespread practice in this biotope. Despite regional restrictions, in fact, the natural beds of *R. decussatus* are constantly subjected to an indiscriminate fishing by unauthorized poachers, especially in spring and summer. These people operate without regards to size or number of specimens collected, thereby causing both a direct damage to the structure of this shellfish population and a significant economic loss for the local cooperative of fishermen.

Nevertheless, an extraordinary market demand for this Bivalve species, particularly for its high quality, led the members of the cooperative to protect the natural banks and optimize the production of *R. decussatus* in the Porto Pozzo lagoon, thus laying the basis for the realization of a future plant breeding, possibly with the use of native broodstock.

Fig. 2.10. *Ruditapes decussatus* specimens from the Porto Pozzo lagoon.

2.2.3 Analytical methods

2.2.3.1 Sampling procedures and morphometric measurements

Samples of *Ruditapes decussatus* were collected fortnightly from July 2009 to July 2010. Fifteen specimens, at least 28 mm in shell length, were manually sampled by a rake with a plastic net (Fig. 2.11) at about 1 meter depth on sandy bottoms. They were immediately opened by cutting the adductor muscle to rinse the whole mollusc body and for draining it (Fig. 2.12). Each clam was individually placed in an airtight container previously marked for identification and then immersed in a 4% aqueous solution of formaldehyde to preserve it (Fig. 2.13).

Fig. 2.11. Sampling of *Ruditapes decussatus* specimens.

The samples collected were immediately transported to the laboratory where, after a thorough rinsing with deionized water, they were drained on paper towels for 5 minutes. The day after, their main morphometric characters were measured as follow: linear dimensions of the shell (as total length of anterior-posterior axis), width of the dorsal-ventral axis (from the umbo to the opposite border), and the main height (with closed valves), were measured using a 0.1 mm precision calliper (Fig. 2.14).

Fig. 2.12. Opening and draining of the samples.

Afterwards, each shell was separated from the edible portion using a scalpel and weighed in a precision balance (Fig. 2.15). The soft tissue was washed to remove the residual formalin, placed on absorbent paper to drain off for 5 minutes and finally weighed in the same way. The bodies of each *R. decussatus* specimen (390 in total) were individually kept in 4% solution of formaldehyde for the subsequent histological analyses.

Fig. 2.13. Fixation of the samples.

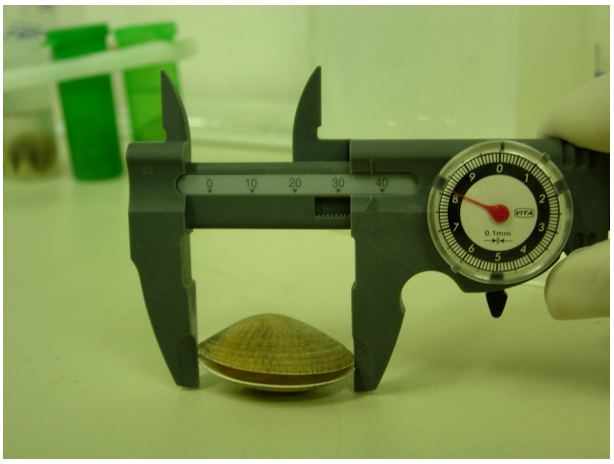


Fig. 2.14. Measurements of the linear dimensions of *Ruditapes decussatus*.

Fig. 2.15 Weighing by a precision balance.

2.2.3.2 Water analyses

Simultaneously with the molluscs, a number of physico-chemical variables of the water column were estimated in the area where clams naturally live on a fortnightly basis. Seawater temperature, pH and salinity were registered *in situ* by field instruments (*i.e.* thermometer, pH-meter and hand refractometer, respectively) (Figg. 2.16, 2.17).

Fig. 2.16. *In situ* measurements of pH and temperature.

Fig. 2.17. *In situ* measurements of the salinity.

In addition, monthly samples of the lagoon's water were collected in polyethylene containers. They were transported to a controlled temperature (5°C) in the laboratory, where concentrations of chlorophyll (Chl *a*), nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_3), and orthophosphate (PO_4^{3--}) were determined. Before performing the analyses,

water was filtered using a vacuum pump equipped with a glass microfiber filter (GF/F 0,70 µm per Chl-*a* Whatman International Ltd., Maidstone, England) or with nylon filters (0.45 µm Whatman International Ltd., Maidstone, England) for the analysis of chlorophyll and the determination of all the other compounds, respectively. All the analyses were always performed in according to the methodology APAT (2004), with the spectrophotometric method described by Strickland & Parsons (1968) and in triplicate.

2.2.3.3 Histological techniques

The edible part of the 390 specimens of *Ruditapes decussatus* collected was dissected separating the visceral mass from the other organs (*i.e.* siphons, gills, adductor muscle, etc.). In order to initiate the preliminary procedures for histological analyses, a longitudinal cut through the mollusc body was made thus obtaining two nearly identical halves containing some gonad, digestive gland and muscular tissue. Half of each clam was dehydrated using an automatic tissue processor (Pabisch, Top Processor LX 120/300; Fig. 2.18), where the samples were transferred in a series of increasing concentrations of ethyl alcohol solutions to eliminate water, then cleared with a hydrophobic agent (*i.e.* xylene) to remove the alcohol and finally replaced with molten paraffin wax.

Fig. 2.18. Automatic tissue processor "Pabisch, Top Processor LX 120/300".

In about 12 hours, the samples were ready to embedding. They were placed into molds where, by an inclusion equipment (ACM 50; Fig. 2.19), embedding material as liquid paraffin could be cast.

Fig. 2.19. Inclusion equipment "ACM 50".

After hardening, the paraffin blocks obtained (Fig. 2.20) are storable and were ready to be sectioned using the Pabisch Top Automat S-140 microtome (Fig. 2.21).

In order to obtain an exhaustive overview of the gonadal tissue, three sections 4 μm thick were cut at different depth from each paraffin block. The resulting slides were stained with Harris' hematoxylin and eosin (Leica ST 5020; Fig. 2.22) (Bancroft & Stevens, 1996) to get different colorations of gametes (deep purple), muscular tissue and reserves (deep and pale pink), and empty zones (white) (Fig. 2.23).

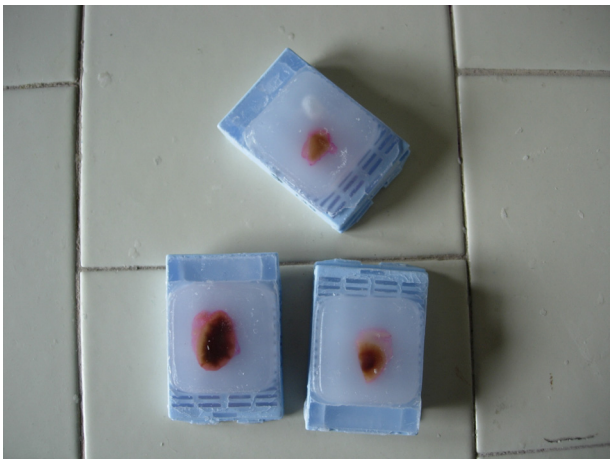


Fig. 2.20. Paraffin blocks.

For the sex and gametogenic stage determinations, each slide was examined under a light microscope (Nikon Eclipse 80i, equipped with a Nikon Plan 10X/0.25 WD 10.5 objective), randomly choosing and then photographing (by a digital camera Nikon DS-Fi1) nine fields of vision corresponding to three different depths in the body of the clam (Delgado & Pérez Camacho, 2003).

Fig. 2.21. Microtome "Pabisch Top Automat S-140".

Overall, nine images per clam were obtained, for a total amount of 3510 images (*i.e.* 3 photos × 3 slices of tissue × 390 individuals). Finally, to determine the monthly *sex ratio* [*i.e.* (number of males/total number of individual sampled) × 100) and gametogenic stage of each clam analysed, the images were digitalised.

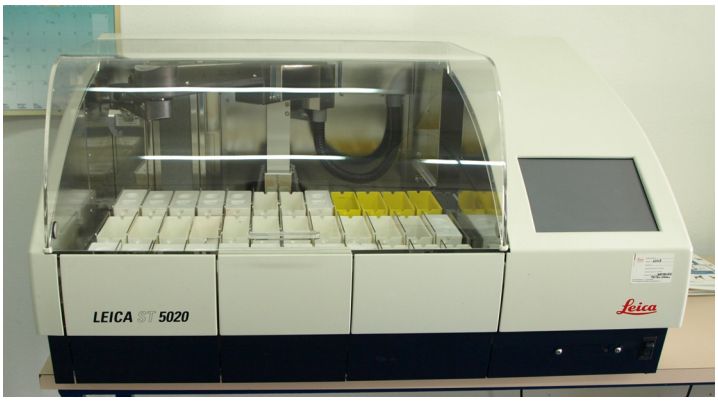


Fig. 2.22. Leica "ST 5020".

Fig. 2.23. Slides of a sample.

2.2.3.4 Image analysis and gametogenic cycle

In order to obtain an overall view of the annual gametogenic cycle of *Ruditapes decussatus* population from the Porto Pozzo lagoon, every image belonging to each clam dissected (*i.e.* 9 per sample) was analysed using a dedicated software (ImageJ; Fig. 2.24). In particular, photographs were firstly digitalised and reduced to a grey-scale colour; then, a proper macro routine was developed to calculate the percentage of area occupied by gametes (male or female) in the slice considered, and the average size of gametes found in each one.

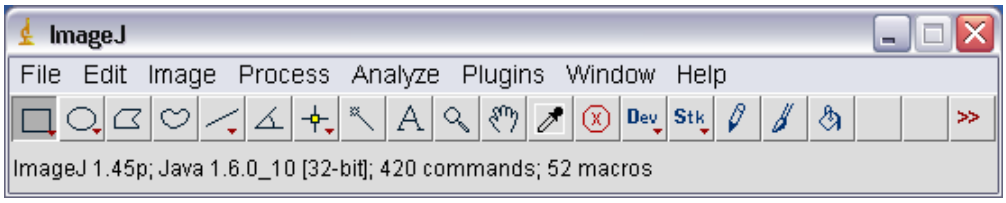


Fig. 2.24. The image software ImageJ.

Based on these two parameters, according to Xie & Burnell (1994) and Delgado & Pérez-Camacho (2003), the status of gametogenic cycle was categorised into six gonadal stages, including the inactive one where clams were sexually undifferentiated. The criteria adopted were the following:

1. Inactive stage

   The main component of the gonad is connective tissue and gametes are not distinguishable.

2. Early active stage

   Female: gonadal tissue starts to proliferate and oocytes are visible and increasing in numbers although still small. Free oocytes are not present in the lumen and their mean diameter is between 20 and 30 μm. It is no easy to distinguish the follicle boundaries.

   Male: gonad starts its proliferation and spermetogonia and spermatocytes are in the follicles. When the mollusc is more developed, spermatids can be noted but not spermatozoa.

3. Late active stage

   Female: a reduction of connective tissue is distinguishable and free oocytes appear in

the lumen, even if they are less than half of the total in the follicles. Into and along the follicle wall, new oocytes of different sizes are visible, characterised by a mean diameter between 20 and 35 μm. In general, follicles are moderately small but well evident and their walls are thick.

Male: into the follicles, spermatogonia, spermatocytes, spermatids and spermatozoa are evident. Less developed specimens do not show the predominance of a particular cell type but, when the mollusc is more developed, spermatids and spermatozoa are dominant. Furthermore, spermatozoa can form centric or elongated bands in the follicles.

4. Ripe stage

Female: most oocytes (more than half) are free in the lumen and are polygonal shaped. Half or more cells have a mean diameter equal or greater than 35 μm and while the follicles increase in size their walls thin.

Male: the principal gonadic components are mature spermatozoa, forming centric or elongate bands (named plugs) in the follicles, that have a radius greater than that of the follicles.

5. Partially spent stage

Female: free oocytes decline in number, some of these undergo lysis, and some follicles appear empty.

Male: the release of spermatozoa starts and about 20% of the follicles reveals an empty space in the centre.

6. Spent stage

Female: at least half of the follicles are empty but most of them are reduced, fused or scattered. The follicle walls are broken and just a few of free oocytes are present. In some individuals, small gametes remain among phagocytes and connective tissue.

Male: follicles are reduced, fused, scattered, and contain a spermatic mass for about 20% of its space. Phagocytes and connective tissue increase.

In a second phase, in male and female specimens with a development of the gonadal tissue easily observable using the image analysis, the gonadal occupation index (GOI) was determined acoording to Delgado & Pérez-Camacho (2003) as follows:

(area occupied by gametes / total area analysed) × 100

Finally, the values obtained from specimens of the same sampling period were averaged.

2.2.3.5 Condition index and proximate composition

Four seasonal samplings (*i.e.* summer, autumn, winter and spring) were carried out and 50 adult specimens of *Ruditapes decussatus* were collected at quarterly intervals between August 2010 and July 2011 from naturals banks into the lagoon studied. Samples were transported alive to the laboratory and stored in filtered seawater, at 20°C of temperature and salinity of 30 PSU, to facilitate the expulsion of sand and pseudofaeces, until water tanks appeared clean. After two days the shell of the clams was dried with absorbent paper, measured by a precision calliper to determine their maximum length and opened with a scalpel to facilitate the escape of intervalvar water draining them for 5 minutes. Two different groups were considered: one composed by 20 *R. decussatus* specimens for the calculation of the Condition Index and another of 30 individuals for proximate composition in relation to seasonality and reproductive cycle. For the fist analysis, each mollusc was dissected separating the shell from the edible part, then treating them individually. Proximate analyses, instead, were performed transferring the soft bodies of clams in a glass container and homogenizing them using an Ultra-Turrax tissue homogenizer at 10.000 rpm for 1 minute. The use of pooled tissue to test invertebrate fleshes composition is recommended by Giese (1966) and Giese *et al.* (1967). All analyses were executed in triplicate.

2.2.3.5.1 Condition Indexes

The Condition Index (CI) represents an eco-physiological value to estimate meat quality and yield in cultured Bivalve molluscs (Rebelo *et al.*, 2005). The two components (*i.e.* shell and edible part) of 20 specimens of *Ruditapes decussatus* were placed in previously weighted porcelain crucibles and exsiccate in a 105°C oven for 24 h. Afterwards, samples were again weighed to obtain the dry weight (AOAC, 1990) and the Condition Index was calculated according to Walne (1976):

$$CI_1 = (\text{dry flesh weight} / \text{dry shell weight}) \times 100$$

The porcelain crucibles containing the edible parts were further treated placing them in a muffle furnace at 550°C for 5 h (AOAC, 1923), weighed in order to obtain the ash content and calculate the ash free dry weight (AFDW) and a second more meaningful ratio according to Walne & Mann (1975):

$$CI_2 = (AFDW / \text{dry shell weight}) \times 100$$

All ratios were determined for each clam and data from each experimental group averaged to obtain a unique seasonal value (*i.e.* 4 values of CI_1 and 4 values for CI_2).

2.2.3.5.2 Moisture and ash content

About 1 g of homogenized tissue was weighed and located in a previously weighed porcelain container to dry it in an oven at 105°C. After 24 h, samples were cooled for 1 h in a glass dryer equipped with silica gel and weighed. Moisture was expressed as percentage of water and obtained from the difference between the initial weight of the fresh sample and its final weight (AOAC, 1990). The same porcelain containers with the sample were transferred in a muffle furnace at 550°C for 5 h to ensure complete incineration of organic matter and thus quantify the ash content (AOAC, 1923; Mortensen & Wallin, 1989). All determinations were performed in triplicate for each seasonal sampling (*i.e.* 3 replicates × 4 season).

2.2.3.5.3 Crude protein

Kjeldahl method was employed to determine crude protein (AOAC, 1992). About 1 g of fresh homogenate seasonal sample was weighed and digested with 10 ml of 96% sulphuric acid (H_2SO_4) at 400°C in presence of a catalyst in tablet (FOSS, DK) constituted by 5.5 g of potassium sulphate (K_2SO_4) and 0.5 g of copper sulphate ($CuSO_4 \times 5H_2O$). Afterwards, it was distilled and titrated with HCl 0.1 N by an analyser unit Kjeltec 2300 (FOSS, DK), and the ml of HCl added were reported. The crude protein content was calculated using the following formula:

$$\text{Crude protein (\%)} = (0.875 \times \text{ml HCl 0.1 N}) / \text{fresh sample weight}$$

All determinations were carried out in triplicate (*i.e.* 3 replicates × 4 seasons).

2.2.3.5.4 Fatty acids analysis

2.2.3.5.4.1 Total lipid

Three lipid extractions from each pooled clam sample were carried out according to the modified method of Folch *et al*. (1957). In detail, 5 g of homogenized tissue were weighed in a 50 ml glass tube and 20 ml of 2:1 dichloromethane/methanol with 0.1% of butylated hydroxytoluene (BHT) solution were added. The tube was sonicated in an Ultrasonic Bath (Branson 1510) for 5 minutes and vortexed for 1 minute. Subsequently, sample was centrifuged at 2,000 rpm for 10 minutes at 4°C (Centrifuge ALC mod. 4227R). The entire sample was vacuum-filtered in a Whatman filter n. 541 (Whatman International Ltd., Maidstone, England) and 5 ml of 0.73% NaCl were added.

The tube was centrifuged again at 2000 rpm for 10 minutes at 4°C and stopped for 30 minutes. Supernatant (*i.e.* methanol/water layer) was removed using a water aspirator and the remaining solution (*i.e.* dichloromethane extract layer) transferred in a Pyrex glass flask previously weighed. It was then positioned in a "Rotavapor Buchi 461" water bath for about 15 minutes in order to speed up the evaporation of dichloromethane extract and finally left overnight in a vacuum desiccator.

After 24 hours, the flask was first weighed to get the total quantity of fat in the sample and subsequently it was methylated adding an amount of hexane equal to 1 ml/25 mg of fat.

2.2.3.5.4.2 FAMEs

Analyses were carried out using the base-catalyzed methylation modified procedure described by FIL-IDF (1999). From each seasonal sample, 1 ml of lipid extract was collected and then transferred in a 1.5 ml vial. Afterwards, the vial was positioned under a nitrogen flow for 15 minutes to accelerate the hexane evaporation, and 0.5 ml solution of sodium methoxide 0.5 M was added to the residual fat. The vial was vortexed for 2 minutes to facilitate the separation of fatty acid from triglycerides and its methylation, just converting the fatty acid into ester. A further 1 ml of hexane was added to the sample and then vortexed for 1 minute to move the ester to hexane (*i.e.* the organic solvent), for the subsequent gaschromatographic analysis.

After 15 minutes, at least 0.5 ml of the hexane phase were taken and transferred in a new vial to analyse the fatty acid methyl esters (FAMEs) by a gaschromatograph (GC) Varian Star 3400 CX with Varian 8200 autosampler (Varian, Walnut Creek, Ca). This instrument is equipped with a Flame Ionization Detector (FID) with a capillary column

WCOT (Wall Coated Open Tubular) Fused Silica 100 m × 0.25 mm i.d., 0.25 μm film thickness, in stationary phase CP-Select CB for FAME (Varian, Walnut Creek, Ca). The temperature values of injector and FID were 255°C and 285°C, respectively.

The temperature program used was the following: 75°C for 1 minute, up to 165°C increasing of 8°C min^{-1}, held for 35 minutes, up to 210°C increasing of 5.5°C min^{-1}, held for 1 minute, up to 230°C increasing of 3°C min^{-1}, held for 11 minute. The split ratio was 1:100, and high purity helium was the carrier gas with 37 psi of pressure and a linear flow rate of 1 ml min^{-1}.

Flows of air and hydrogen were calibrated at 450 ml min^{-1} and 45 ml min^{-1}, respectively. Fatty acids from each seasonal sample were identify comparing retention times of their peaks with those of methyl ester standards (PUFA-1 and PUFA-3, Matreya Inc., Pleasant Gap, PA, USA) as well as the references samples.

Finally, the quantity of each fatty acid was expressed as percentage of total FAME present in the sample.

2.2.3.5.4.3 Indexes of lipid health

In order to assess the lipids quality (Amerio *et al.*, 1996), both the atherogenic index (AI) and the thrombogenic index (TI) were calculated (Ulbricht & Southgate, 1991):

$$AI = \frac{(C12 + 4*C14 + C16)}{(\Sigma MUFA + \Sigma PUFA\omega6 + \Sigma PUFA\omega3)}$$

$$TI = \frac{(C14 + C16 + C18)}{[0.5\ (\Sigma MUFA) + 0.5\ (\Sigma PUFA\omega6) + 3\ (\Sigma PUFA\omega3) + (\omega3/\omega6)]}$$

The first is an indicator of risk for cardiovascular diseases and the second is an indicator of the potential for blood platelets aggregation. They are more positive as their values are more close to zero.

2.2.4 Statistical analyses

One-way Analysis of Variance (ANOVA) was used to detect putative differences among the four groups of *Ruditapes decussatus* examined. Cochran's C test was used to check the assumption of the homogeneity of variance. When appropriate, the Student-

Newman-Keuls' (SNK; $p<0.05$) test was used for post-hoc comparisons to formulate alternative hypothesis. ANOVAs were always performed using the STATISTICA® software package.

2.3 Results

2.3.1 Hydrological and mesological variables

Measures of temperature were registered fortnightly during the entire sampling period (Fig. 2.25), revealing a gradual evolution related to the seasonal fluctuations. After the high value recorded in July 2009 (28.9°C), there was a slow decrease during the autumn and winter periods, until the minimum of 8.4°C recorded at the end of February. From this period, and throughout the spring, temperature increased, showing a peak of 30.1°C in July 2010.

As regards pH, values showed a certain degree of variability and were characterized by alternating peaks, with a maximum (8.54) and a minimum (8.08) recorded in May and October, respectively (Fig. 2.26).

The graph illustrated in Fig. 2.27 shows the salinity dynamics in the lagoon. Initial values were always more or less similar, ranging between 39 and 40 PSU from July until December 2009, and reaching a maximum of 42 PSU in September. In January 2010, however, occurred to a sudden drop of the salinity (22 PSU) was registrered, but subsequently its values increased again until the following summer (about 40 PSU).

Chlorophyll *a* concentration (Fig. 2.28) revealed large fluctuations from month to month during the trial. It showed its lowest value in October 2009 (0.19 mg l^{-1}), and the maximum in December of the same year (1.50 mg l^{-1}). In the following months, however, its values were quite variable.

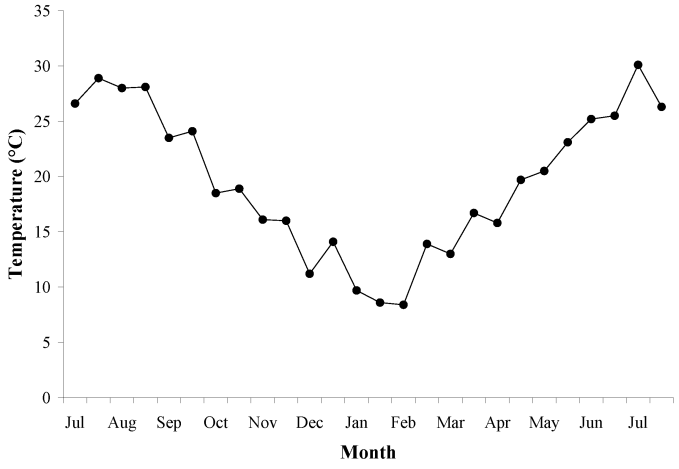


Fig. 2.25. Temperature values recorded in the Porto Pozzo lagoon during the experimental period.

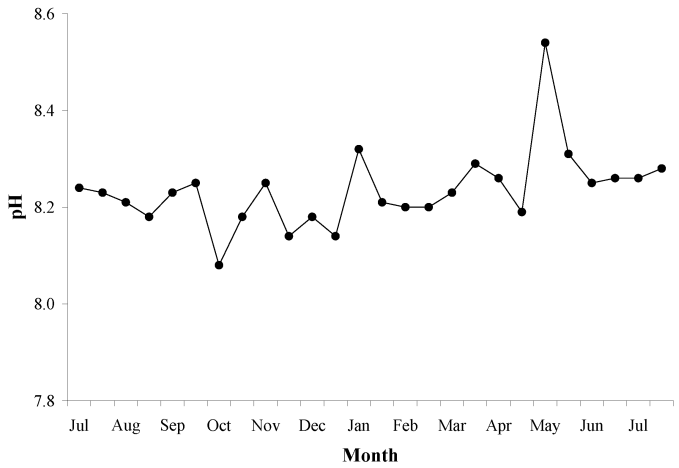


Fig. 2.26. pH values recorded in the Porto Pozzo lagoon during the experimental period.

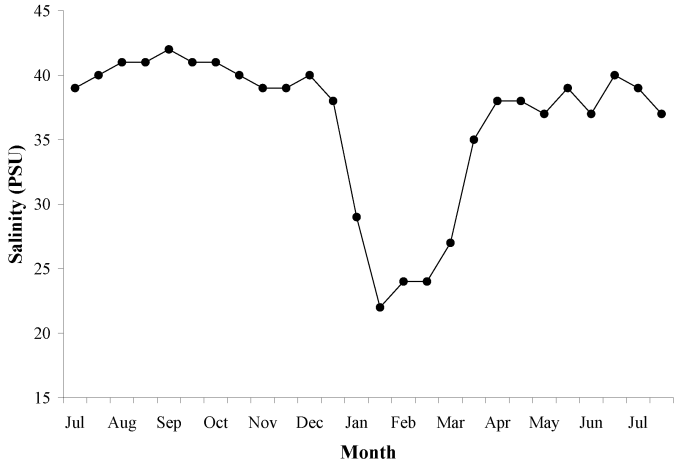


Fig. 2.27. Salinity values recorded in the Porto Pozzo lagoon during the experimental period.

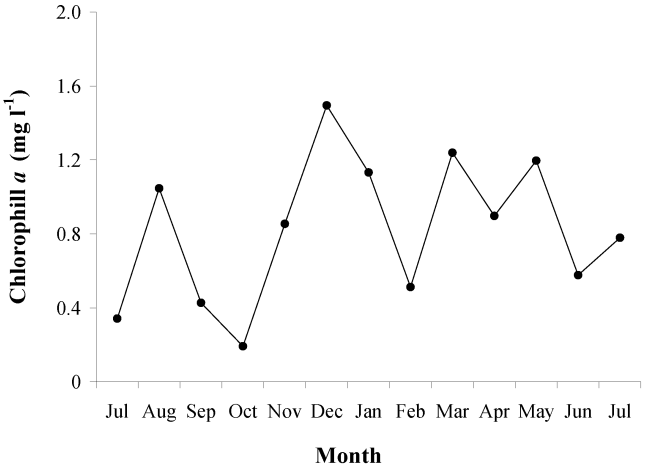


Fig. 2.28. Chlorophyll *a* concentration recorded in the Porto Pozzo lagoon during the experimental period.

As regards nutrients, they revealed different concentrations, but never reaching high values. Nitrate amount (Fig. 2.29) showed a maximum value in November (2.23 mg l^{-1}) and a minimum in June of the subsequent year (0.06 mg l^{-1}). Nitrite concentration (Fig. 2.30), instead, always revealed lower values than the former. High peaks of 0.10 µmol l^{-1} were recorded in July and November 2009, and in July 2010. In September 2009, by contrast, nitrite was absent.

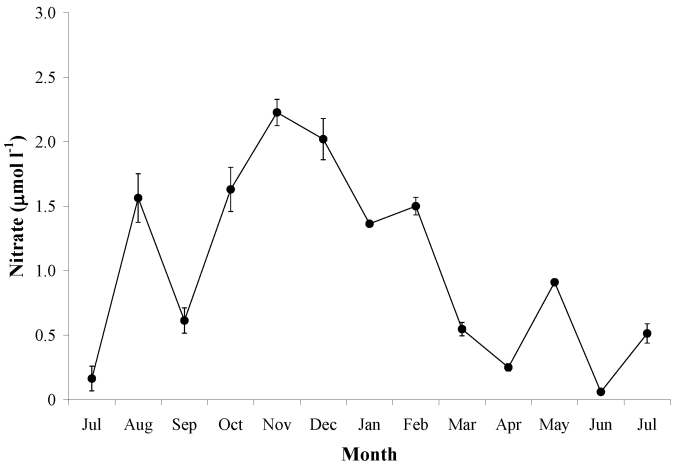


Fig. 2.29. Mean nitrate concentration (±SE) in the Porto Pozzo lagoon during the experimental period.

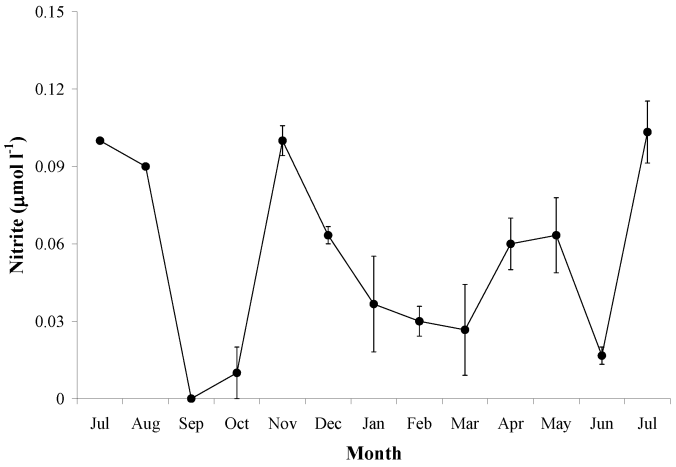


Fig. 2.30. Mean nitrite concentration (±SE) in the Porto Pozzo lagoon during the experimental period.

The presence of ammonium (Fig. 2.31) was irregular in the lagoon, and its concentration (1.53 µmol l^{-1}) was recorded in January 2010. In April ammonium was instead absent.

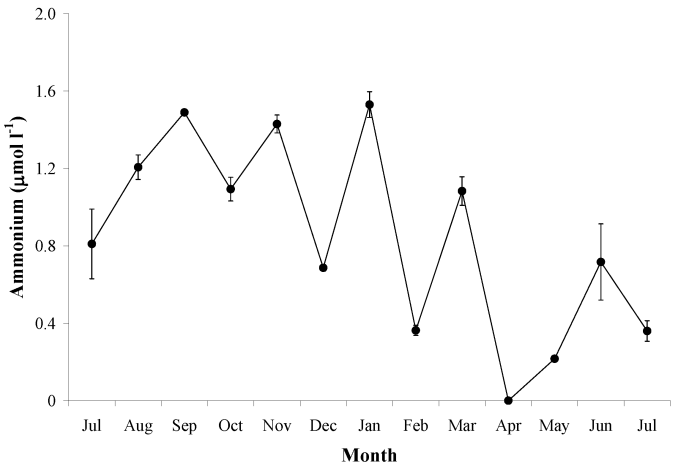


Fig. 2.31. Mean ammonium concentration (±SE) in the Porto Pozzo lagoon during the experimental period.

Finally, orthophosphate (Fig. 2.32) showed a gradual trend, decreasing from the values of 0.77 µmol l^{-1} in July 2009, to 0.01 in June 2010, and then again increasing in July of the same year (0.23 µmol l^{-1}).

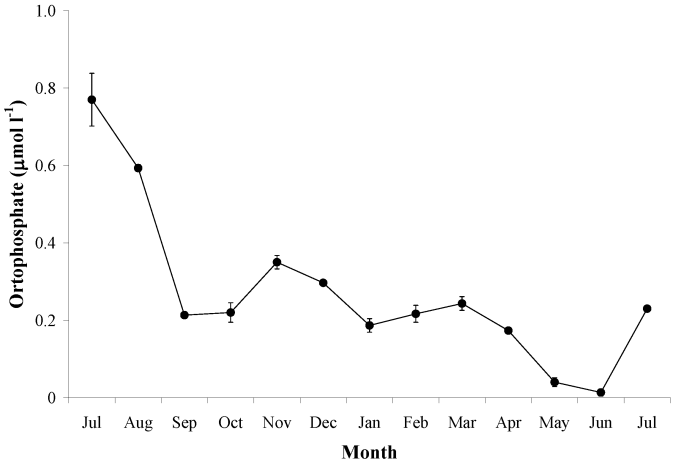


Fig. 2.32. Mean orthophosphate concentration (±SE) in the Porto Pozzo lagoon during the experimental period.

2.3.2 Sex ratio and morphometric measurements

The sex ratio (Fig. 2.33) was expressed as percentage of individual belonging to a gender on the total number of the clams monthly sampled. Molluscs for which it was not possible to determine the sex, instead, were classified as "unidentified". At the beginning of the study (July 2009), males represented more than 56% and females about

43%, while in the following month the proportion inverted, females being more than 56%. Between September and November 2009, ratio began to change due to a gradual decrease of both sexes and a dramatic appearance of undifferentiated specimens: in November, for example, males were 30%, females only 6.67% and unidentified clams 63.3%. At the end of the year, the proportion of males suddenly increased, reaching a value of about 57%, whereas females and unidentified were 13% and 30%, respectively. After a new increase of unidentified individuals (60%) and a subsequent decrease of males (33.3%) and females (6.7%) in January, the proportion between sexes gradually changed with a peak of female specimens in March (60%), and a ratio females/males more or less stable throughout the spring (*i.e.* between April and June 2010).

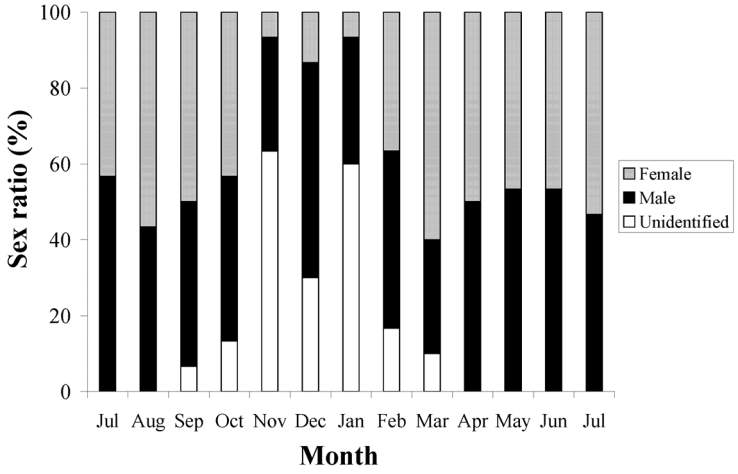


Fig. 2.33. Sex ratio (%) of *Ruditapes decussatus* specimens sampled during the experimental period.

By considering the length of the shell of all the specimens sampled (Fig. 2.34), it was evident that most of the clams were between 32 and 42 mm, independently of the gender, although males represented the majority.

For the whole sampling period each gender (female, male and undetermined) was correlated to all morphometric measurements registered. Histograms in Figs. 2.35, 2.36, 2.37 show that the three shell measures (*i.e.* length, width and height) had the same trend. In particular, males always exhibited their higher mean values in July 2009 (40.0 mm in length; 25.8 mm in width; 17.1 mm in height), November 2009 (39.6 mm in length; 25.5 mm in width; 17.1 mm in height) and January 2010 (40.1 mm in length; 26.1 mm in width; 17.0 mm in height), and smaller sizes in August 2009 (34.3 mm in

length; 22.0 mm in width; 14.6 mm in height).

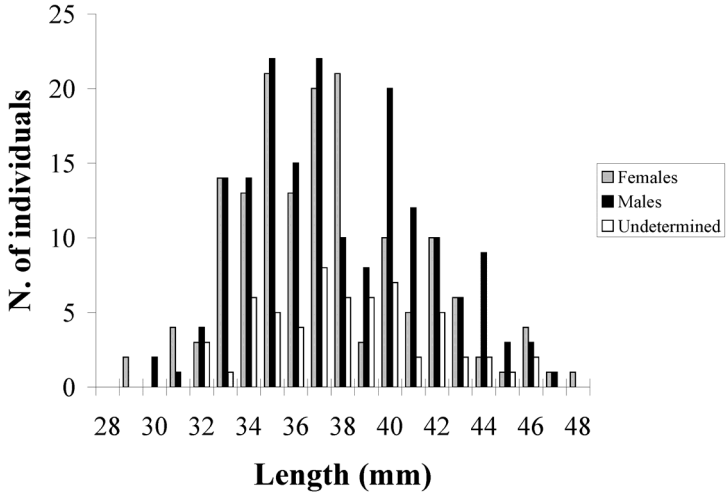


Fig. 2.34. Length-frequency distributions of the *Ruditapes decussatus* specimens examined for sex determination (n=390) throughout the experimental period.

Females, instead, revealed their mean maximum size in July 2009 (39.8 mm in length; 25.8 mm in width; 17.5 mm in height), minor mean length and width in August 2009 (33.6 and 21.9 mm, respectively) and minimum height (13.9 mm) in December 2009. Lastly, undetermined specimens showed a peak for each variable considered in March 2010 (41.2, 26.9 and 18.0 mm, in length, width and height respectively), and minimum values in September 2009 (35.2 mm in length; 22.7 mm in width; 14.9 mm in height).

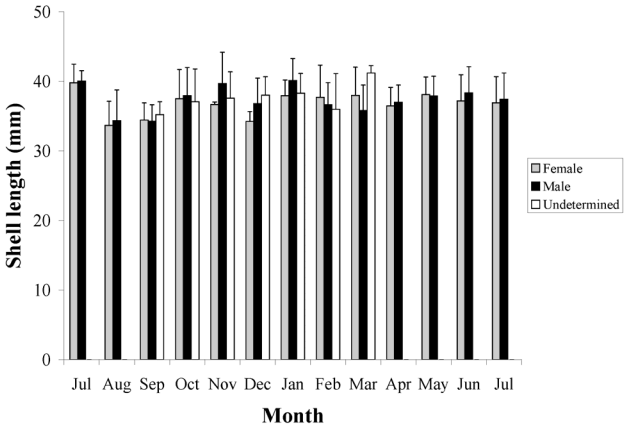


Fig. 2.35. Mean shell length (±SD) of *Ruditapes decussatus* specimens divided per sex.

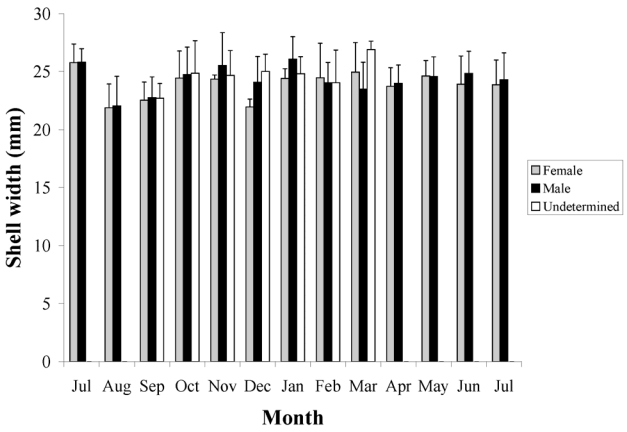


Fig. 2.36. Mean shell width (±SD) of *Ruditapes decussatus* specimens divided per sex.

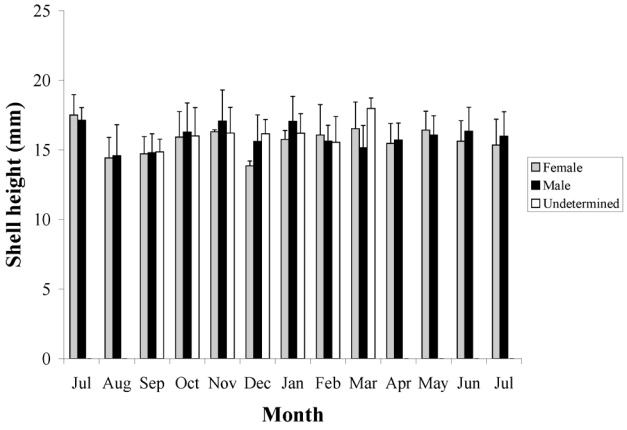


Fig. 2.37. Mean shell height (±SD) of *Ruditapes decussatus* specimens divided per sex.

As regards shell weight, males (Fig. 2.38) showed higher values in November 2009 and January 2010 (4.65 and 4.67 g, respectively) and the lower one in August 2009 (2.79 g). Their edible tissue weights (Fig. 2.39), instead, were higher in January (2.39 g), May (2.45 g) and July 2010 (2.36 g), while in August 2010 the lower value of 1.40 g was recorded.

On the other hand, females showed similar weights of both shell and edible part: the former registered its maximum in July 2009 (4.59 g) and its minimum in August and December 2009 (2.53 and 2.47 g, respectively); the latter its higher values in May 2010 (2.31 g) and its lower one in the same period of the former (1.27 g in August and 1.37 g in December).

Finally, undetermined specimens were characterised by higher shell and mollusc weight values in March 2010 (2.57 and 4.91 g, respectively) and reached mean lower values in September 2009 (2.95 and 1.43 g, respectively).

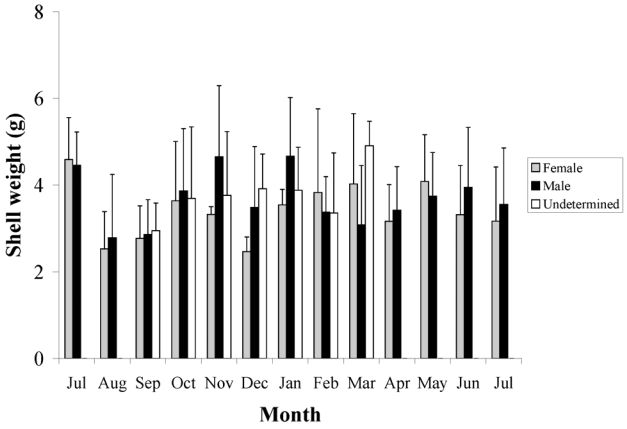


Fig. 2.38. Mean shell weight (±SD) of *Ruditapes decussatus* specimens divided per sex.

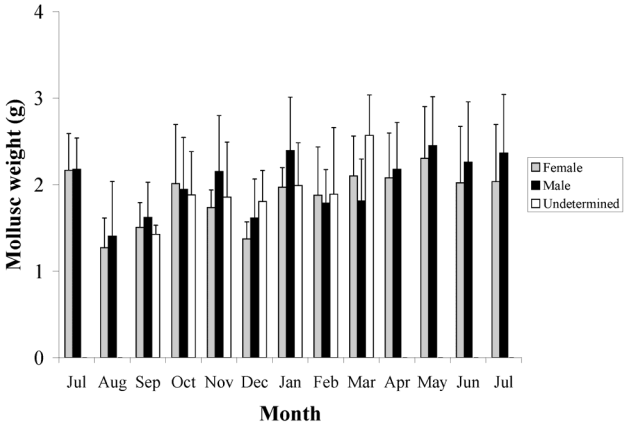


Fig. 2.39. Mean weight of soft tissue (±SD) of *Ruditapes decussatus* specimens divided per sex.

By relating total weight and shell length of the clams examined, important relationships were found. In particular, males (Fig. 2.40) showed a coefficient of determination (R^2) equal to 0.937, females (Fig. 2.41) one of 0.936 and undetermined specimens (Fig. 2.42) the highest one equal to 0.943,

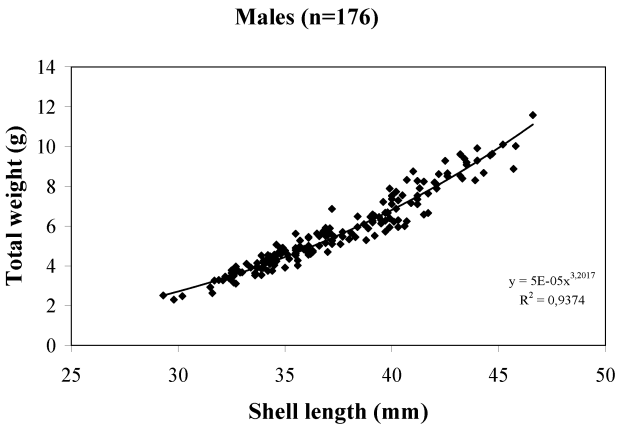


Fig. 2.40. Regression between total weight and shell length of the males of *Ruditapes decussatus* specimens examined.

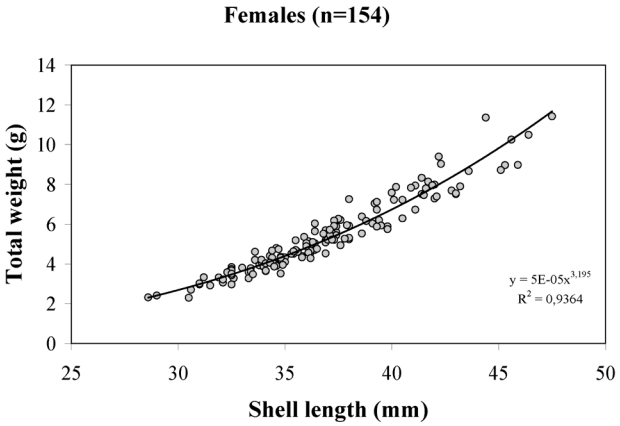


Fig. 2.41. Regression between total weight and shell length of the females *Ruditapes decussatus* specimens examined.

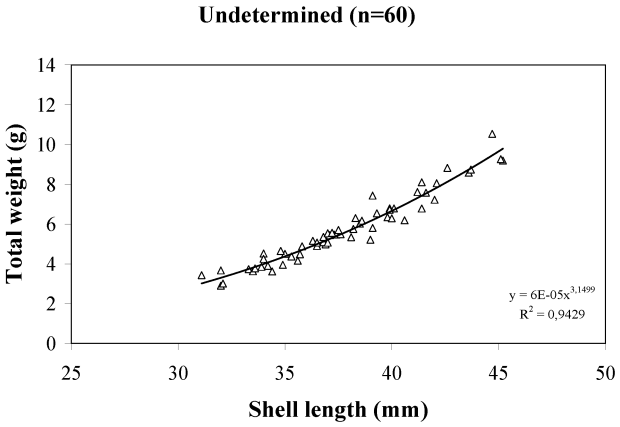


Fig. 2.42. Regression between total weight and shell length of the undetermined *Ruditapes decussatus* specimens examined.

2.3.3 Gametogenic cycle

The analysis of 3510 images of gonadal tissue (Figs. 2.43 and 2.44) not only allowed to define the sex ratio of *Ruditapes decussatus* population in the Porto Pozzo lagoon, the stage of sexual maturation of each clam collected and the relative gonadal occupation index (GOI).

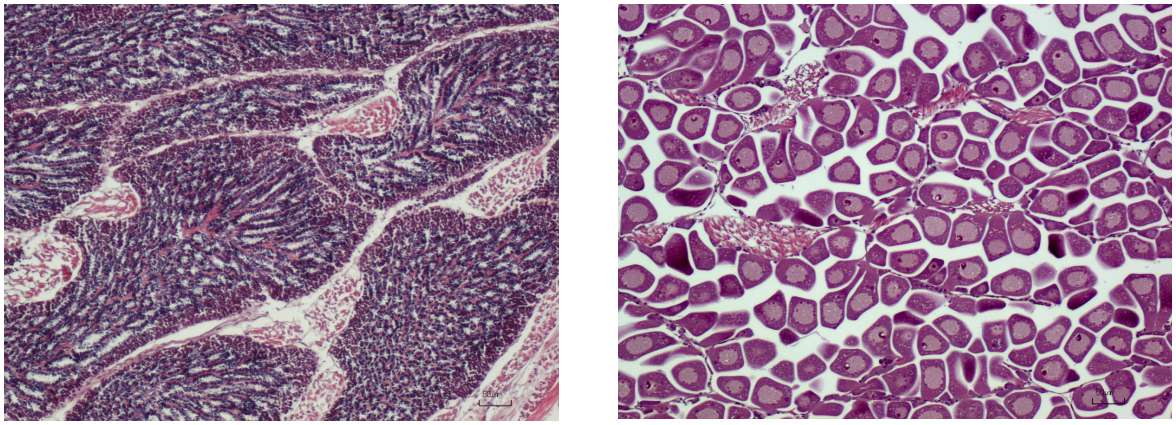


Fig. 2.43. Male (left) and female (right) gonads of *Ruditapes decussatus* in ripe stage.

In Fig. 2.45 is illustrated the trend of gametogenic cycle for male specimens during the study period. The highest GOI values occurred in summer months, while they steady decreased in autumn. New increases of the GOI were registered in spring, up to values characteristic of the summer season.

Due to the state of sexual inactivity of this species in winter months, it was not possible to calculate the GOI for the male specimens collected between November and January.

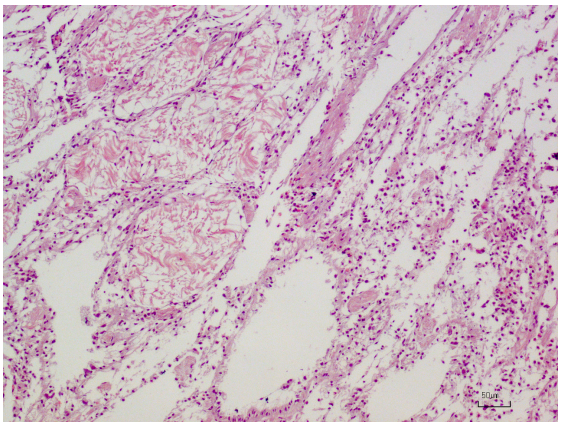


Fig. 2.44. Example of gonadal inactive stage of an undetermined *Ruditapes decussatus* specimen.

Finally, the highest gonadal occupation index value (50.73%) was recorded during the first sampling in July 2009, while the lowest was found in the second sampling in October (5.12%).

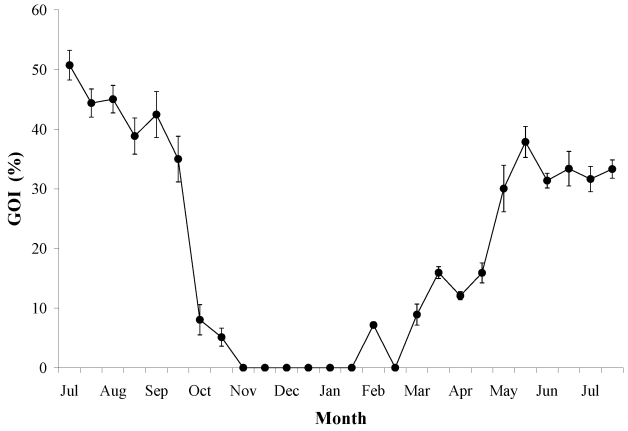


Fig. 2.45 Mean variation of the Gonadal Occupation Index (±SE) in males of *Ruditapes decussatus* during the study period.

The females showed a GOI very similar to that of males (Fig. 2.46). During the autumn and winter months (October 2009-March 2010), the index values were quite low, recording a minimum in January (0.74%). The peaks, however, were reached in summer, particularly in August 2009 (21.36%) and in June and July 2010 (22.64% and 22.92%, respectively).

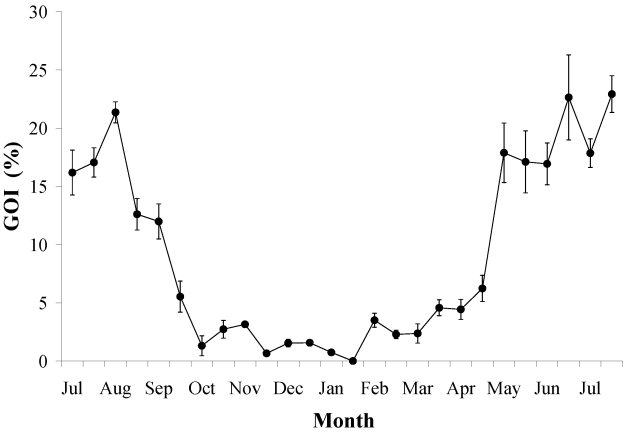


Fig. 2.46. Mean variation of the Gonadal Occupation Index (±SE) in females of *Ruditapes decussatus* during the study period.

2.3.4 Condition index and proximate composition

In order to carry out a proper assessment of meat quality of the clams present in the Porto Pozzo lagoon, adult specimens with shell length between 29.3 and 47.8 mm (mean 36.1±3.9 mm) were analysed. The two condition indexes (*i.e.* CI and CI-AFDW) shown in Fig. 2.47 were calculated. The first CI, after an initial value of 12.05% in the summer period, decreased gradually during autumn (12.03%), and showed its minimum (11.98%) in winter months. In spring, however, its values started to increase, reaching a peak of 12.08%. The second index (CI-AFDW) showed a similar tendency, but having a minimum in autumn (9.73%) and a maximum in winter (10.41%), it was more or less constant during the rest of the study period.

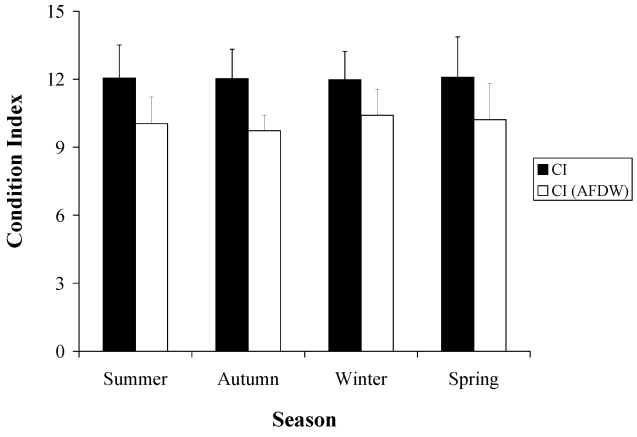


Fig. 2.47. Condition indexes of *Ruditapes decussatus* specimens during the study period.

The mean moisture content (Fig. 2.48) of pooled *Ruditapes decussatus* specimens collected during each sampling season was more or less similar in all the groups analysed. More exactly, the lowest value was recorded in summer (80.21±0.10%) and the highest one in spring (81.70±0.06%). The mean ash percentage values (Fig. 2.49) were clearly higher during summer and autumn with values of 3.27±0.03 and 3.07±0.06%, respectively. In winter and spring, instead, the pattern was more or less regular, characterized by similar values of 2.63±0.03 and 2.65±0.04%, respectively.

As regards protein content (Fig. 2.50), clams revealed a regular oscillation pattern: from the highest content found in summer (13.59±0.10%), it was observed a decrease in autumn (12.81±0.06%), a new increase in winter (13.26±0.06%), and a final small decrease in spring (13.03±0.06%).

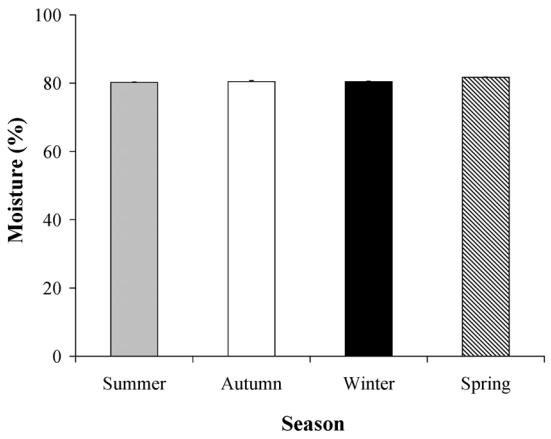


Fig. 2.48. Moisture percentage. Bars represent the mean value (±SD) of 3 replicates of 30-pooled *Ruditapes decussatus* specimens per season.

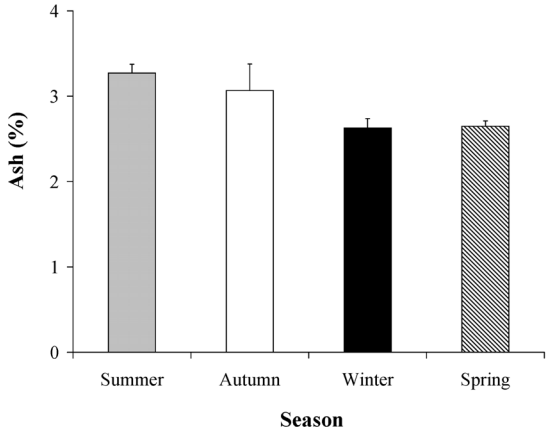


Fig. 2.49. Ash percentage. Bars represent the mean value (±SD) of 3 replicates of 30-pooled *Ruditapes decussatus* specimens per season.

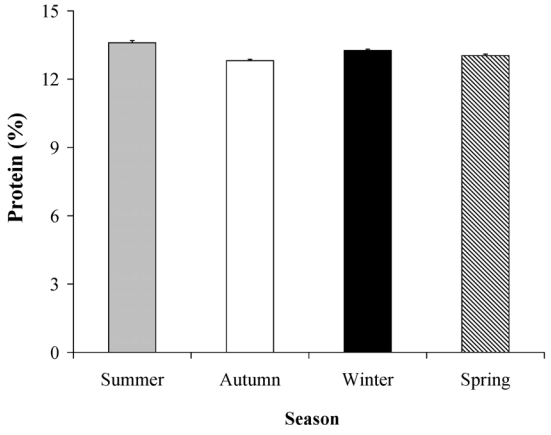


Fig. 2.50. Protein percentage. Bars represent the mean value (±SD) of 3 replicates of-30 pooled *Ruditapes decussatus* specimens per season.

Finally, the percentage of lipids observed was very low during all the study period (Fig. 2.51). In summer was recorded the maximum value of 1.28±0.03% followed by a gradual decrease in autumn (1.07±0.10%) and winter (1.01±0.13%).

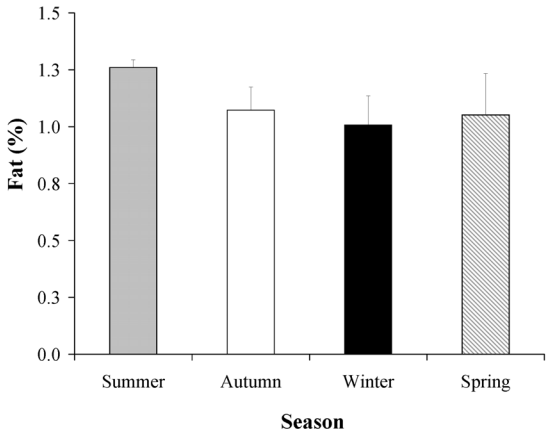


Fig. 2.51. Lipid percentage. Bars represent the mean value (±SD) of 3 replicates of 30-pooled *Ruditapes decussatus* specimens per season.

As regards the above-mentioned variables, ANOVA showed significant differences for moisture, ash and protein, among the four different *R. decussatus* groups. On the other hand, for lipid percentage and both the condition indexes considered no significant differences were found. Details of ANOVA results and Student-Newman-Keuls *post-hoc* comparisons are illustrated in Tab. 2.2.

Tab. 2.2. ANOVA results for proximate composition of the 4 groups of *Ruditapes decussatus* examined.

		Moisture			Ash			Protein			Lipid		
Source of variation	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	1.37	44.26	**0.0000**	0.30	158.47	**0.0000**	0.33	58.72	**0.0000**	0.04	2.47	0.1365
Residuals	8	0.03			0.00			0.01			0.02		
Cochran's test			0.787	p<0.05		0.566	ns		0.482	ns		0.538	ns
Transformation				none			none			none			none
SNK test				S=W=A<Sp**			W=Sp<A**<S**			A<Sp**<W**<S**			

S = Summer; A = Autumn; W = Winter; Sp = Spring; significant differences are marked in bold; *: p<0.05; **: p<0.01

		Condition index			Condition index (AFDW)		
Source of variation	df	MS	F	*p*	MS	F	*p*
Group	3	0.04	0.02	0.9967	1.67	1.16	0.3290
Residuals	76	2.14			1.44		
Cochran's test			0.373	ns		0.449	p<0.05
Transformation				none			none
SNK test							

2.3.5 Fatty acids

The fatty acids composition of the four seasonal groups of *Ruditapes decussatus* examined is shown in Tab. 2.3. Considering saturated fatty acids (SFA), the mean values were always high in all the samples considered, with a significantly higher percentage in clams collected during the summer season (30.73±0.61%) compared to those of the other periods (25.78±0.37%, 25.02±0.63% and 24.93±1.03% in autumn, winter and spring, respectively). Among these, 16:0 (13.32±0.47% in summer, 8.77±0.61% in autumn, 8.71±0.38% in winter, and 9.49±1.07% in spring) and 18:0 (6.86±0.19%, 6.59±0.22%, 6.58±0.26% and 5.54±0.39% in summer, autumn, winter and spring, respectively) predominated.

Although present in lower quantities, monounsaturated fatty acids (MUFA) were also well represented, revealing their maximum level in the summer period (20.84±0.48%) than in the other seasons (12.29±0.79% in autumn, 11.12±0.55% in winter and 16.50±0.31% in spring). The 20:01 n11 was certainly the most abundant, showing a significantly higher value of 7.11±0.25% in the summer sample, a marked decrease during autumn and winter (2.58±0.03% and 2.24±0.12%, respectively), and then a reprise in spring (5.09±0.52%).

Polyunsaturated (PUFA) were the most abundant fatty acids class, showing significantly higher values in the winter sample (43.64±0.40%), followed by those harvested in spring (42.03±0.41%), winter (41.29±1.32%) and summer (33.57±0.93%), respectively. Within this class, the n-3 was the predominant one, with a maximum level in winter (36.41±0.75%) and lower percentages in the other seasons (33.84±0.25% in spring, 33.28±0.34% in autumn and 24.40±0.57% in summer). Among them, docosahexanoic acid was the most represented, with a value of 27.92±0.90% in the winter sample and of 25.71±0.04%, 23.19±0.80% and 14.94±0.42% in autumn, spring and summer samples, respectively.

Also the EPA mean content was appreciable, particularly in the vernal sample, where it recorded the value of 5.26±0.48%, followed by those registered in summer (4.11±0.29%), spring (3.65±0.13%), and autumn (2.88±0.28%), respectively.

As far as the mean levels of n-6 PUFA are concerned (9.82±0.38%, 7.96±1.01%, 7.24±0.74% and 8.13±0.54% in summer, autumn, winter and spring, respectively), they contributed to a lesser extent than the n-3. Among them, arachidonic acid was predominant and ranged between 3.98±0.16% (in summer) and 3.31±0.23% (in spring).

Accordingly to the above results, the highest n3/n6 ratio was detected in the winter sample (5.07±0.58%) and both in autumn and spring (4.22±0.48% and 4.18±0.30%, respectively), while a significant lower was found in summer (2.38±0.06%).

On the other hand, the EPA/DHA ratio was lower in the summer group (0.28±0.02%) in comparison with the clams sampled in spring (0.23±0.03%), winter (0.13±0.01%) and autumn (0.11±0.01%).

However, the sum of these two important fatty acids (EPA + DHA) exhibited the higher mean percentage in the winter sample (31.57±0.81%), followed by the autumn (28.59±0.24%), spring (28.45±0.32%) and summer ones (19.05±0.58%), with significant differences among them.

Finally, the low values detected for atherogenicity index (AI) (0.38±0.02, 0.21±0.01, 0.20±0.01 and 0.26±0.03 for summer, autumn, winter and vernal groups, respectively) and thrombogenicity index (TI) (0.25±0.01, 0.14±0.01, 0.13±0.01 and 0.14±0.01 for summer, autumn, winter and vernal groups, respectively), confirm a high polyunsaturated fatty acids content in all the four groups of *R. decussatus* examined.

As regards all the fatty acid variables considered (comprised sum, ratios and health indexes), ANOVA detected significant differences among the four samples except for 15:0, 16:1 n9, 17:0, 18:3 n3, 20:1 n9, 20:3 n6, 20:4 n3 and 22:1 n11 contents that were approximately equal in all the groups analysed (Tab. 2.3).

Details of ANOVA results and Student-Newman-Keuls *post-hoc* comparison test are illustrated in Tab. 2.4.

Tab. 2.3. Fatty acids profile (%±SD) of the 4 *Ruditapes decussatus* groups examined.

	Season			
	Summer	Autumn	Winter	Spring
14:0	1.85±0.20^{A}	0.58±0.01^{C}	0.60±0.08^{C}	1.38±0.17^{B}
15:0	2.29±0.42	3.74±0.66	3.16±0.51	3.31±0.56
16:0	13.32±0.47^{A}	8.77±0.61^{B}	8.71±0.38^{B}	9.49±1.07^{B}
16:1 n9	1.08±0.06	1.39±0.32	1.32±0.24	1.48±0.16
16:1 n7	1.99±0.20^{A}	0.68±0.08^{B}	0.82±0.10^{B}	1.76±0.34^{A}
17:0	1.46±0.06	1.25±0.31	1.34±0.05	0.97±0.07
18:0	6.86±0.19^{A}	6.59±0.22^{A}	6.58±0.26^{A}	5.54±0.39^{B}
18:1 n9	4.62±0.30^{Aa}	3.93±0.36^{Ab}	2.73±0.17^{B}	3.50±0.18^{Ab}
18:1 n7	2.87±0.17^{A}	1.09±0.19^{C}	1.26±0.19^{C}	2.03±0.28^{B}
18:3 n3	0.49±0.25	0.31±0.02	0.24±0.04	0.42±0.10
18:4 n3	0.79±0.09^{b}	1.04±0.10^{a}	0.87±0.08^{b}	0.80±0.10^{b}
20:1 n11	7.11±0.25^{A}	2.58±0.03^{C}	2.24±0.12^{C}	5.09±0.52^{B}
20:1 n9	1.12±0.03	1.16±0.14	1.13±0.01	1.03±0.02
20:1 n7	1.61±0.11^{A}	1.20±0.05^{B}	1.30±0.04^{B}	1.27±0.05^{B}
20:2 n6	2.57±0.05^{A}	1.41±0.03^{C}	1.43±0.03^{C}	2.28±0.13^{B}
20:3 n6	0.28±0.03	0.20±0.09	0.17±0.03	0.24±0.02
20:4 n6	3.98±0.16	3.70±0.60	3.35±0.25	3.31±0.23
20:3 n3	0.31±0.00^{A}	0.25±0.01^{B}	0.22±0.01^{B}	0.32±0.04^{A}
20:4 n3	0.29±0.05	0.27±0.05	0.29±0.04	0.31±0.05
22:1 n11	0.16±0.01	0.19±0.03	0.15±0.02	0.20±0.02
20:5 n3 EPA	4.11±0.29^{Ba}	2.88±0.28^{Bb}	3.65±0.13^{Ba}	5.26±0.48^{A}
21:5 n3	0.33±0.01^{B}	0.43±0.03^{Ab}	0.47±0.01^{Aa}	0.50±0.01^{Aa}
22:4 n6	2.29±0.10^{a}	2.24±0.05^{a}	2.00±0.06^{b}	1.95±0.15^{b}
24:0	2.32±0.06^{B}	2.97±0.07^{A}	2.48±0.10^{B}	2.19±0.26^{B}
22:5 n3	2.13±0.04^{Bb}	2.40±0.13^{Ba}	2.75±0.06^{Ab}	3.05±0.18^{Aa}
22:6 n3 DHA	14.94±0.42^{D}	25.71±0.04^{B}	27.92±0.90^{A}	23.19±0.80^{C}
SFA	30.73±0.61^{A}	25.78±0.37^{B}	25.02±0.63^{B}	24.93±1.03^{B}
MUFA	20.84±0.48^{A}	12.29±0.79^{Ca}	11.12±0.55^{Cb}	16.50±0.31^{B}
PUFA	33.57±0.93^{B}	41.29±1.32^{Ab}	43.64±0.40^{Aa}	42.03±0.41^{Ab}
Unidentified	14.86±0.87^{b}	20.64±1.83^{a}	20.21±0.95^{a}	16.53±1.75^{b}
∑ n3	24.40±0.57^{C}	33.28±0.34^{B}	36.41±0.75^{A}	33.84±0.25^{B}

Tab. 2.3. Continued.

	Season			
	Summer	Autumn	Winter	Spring
∑ n6	9.82±0.38^{a}	7.96±1.01^{b}	7.24±0.74^{b}	8.13±0.54^{b}
n3/n6	2.38±0.06^{B}	4.22±0.48^{A}	5.07±0.58^{A}	4.18±0.30^{A}
EPA/DHA	0.28±0.02^{Aa}	0.11±0.01^{B}	0.13±0.01^{B}	0.23±0.03^{Ab}
EPA+DHA	19.05±0.58^{C}	28.59±0.24^{B}	31.57±0.81^{A}	28.45±0.32^{B}
AI	0.38±0.02^{A}	0.21±0.01^{Bb}	0.20±0.01^{Bb}	0.26±0.03^{Ba}
TI	0.25±0.01^{A}	0.14±0.01^{B}	0.13±0.01^{B}	0.14±0.01^{B}

The fatty acids present in small percentage (*iso* 15:0; *anteiso* 15:0; 16:1 n7,*t*; *iso* 17:0; *anteiso* 17:0; 16:2 n4; 17:1 n8; *iso* 18:0; 18:1 n9,*t*; 18:2 n6; 18:2 n4; 18:3 n6 and 18:3 n9) were considered in the composite fractions but were not reported in the table.
Values are mean ±standard deviation. Values in each roe with different superscript letters are significantly different (A, B, C = $p<0.01$; a, b, c = $p<0.05$).
Abbreviation: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AI, Atherogenic Index; TI, Thrombogenic Index.

Tab. 2.4. ANOVA results for fatty acids of the 4 *Ruditapes decussatus* groups examined.

		14:0			15:0			16:0			16:1 n9		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	1.17	61.94	**0.0000**	0.33	1.11	0.4015	14.45	30.56	**0. 0001**	0.089	1.88	0. 2106
Residuals	8	0.02			0.29			0.47			0.047		
Cochran's test			0.526	ns		0.368	ns		0.614	ns		0.531	ns
Transformation				none			none			none			none
SNK test				A=W<Sp**<S**						W=A=Sp<S**			

		16:1 n7			17:0			18:0			18:1 n9		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	1.30	29.28	**0.0001**	0.13	4.80	**0.0338**	1.02	13.54	**0. 0017**	1.88	26.34	**0.0002**
Residuals	8	0.04			0.03			0.08			0.07		
Cochran's test			0.666	ns		0.899	p<0.01		0.512	ns		0.467	ns
Transformation				none			none			none			none
SNK test				A=W<Sp**=S			Sp<S*=W=A			Sp<W**=A=S			W<Sp**=A<S*

		18:1 n7			18:3 n3			18:4 n3			20:1 n11		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	2.00	45.39	**0.0000**	0.04	2.02	0.1901	0.04	4.59	**0. 0377**	15.68	180.55	**0.0000**
Residuals	8	0.04			0.02			0.01			0.09		
Cochran's test			0.438	ns		0.852	p<0.05		0.299	ns		0.784	p<0.05
Transformation				none			none			none			none
SNK test				A=W<Sp**<S**						S=Sp=W<A*			W=A<Sp**<S**

S = Summer group; A = Autumn group; W = Winter group; *: p<0.05; **: p<0.01

Tab. 2.4. Continued.

		20:1 n9			20:1 n7			20:2 n6			20:3 n6		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	0.01	1.69	**0.2463**	0.10	19.85	**0.0005**	0.12	217.28	**0.0000**	0.01	2.98	0.0967
Residuals	8	0.01			0.00			0.00			0.00		
Cochran's test			0.923	p<0.01		0.643	ns		0.738	ns		0.775	p<0.05
Transformation				none			none			ln(x+1)			none
SNK test							A=Sp=W<S**			A=W<Sp**<S**			

		20:4 n6			20:3 n3			20:4 n3			22:1 n11		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	0.30	2.37	0.1460	0.01	16.90	**0.0008**	0.00	0.43	0.7353	0.00	4.43	0.0410
Residuals	8	0.12			0.00			0.00			0.00		
Cochran's test			0.722	ns		0.755	ns		0.349	ns		0.510	ns
Transformation				none			none			none			none
SNK test							W=A<S**=Sp						

		20:5 n3 EPA			21:5 n3			22:4 n6			24:0		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	2.97	29.12	**0.0001**	0.02	53.71	**0.0000**	0.09	8.85	**0.0064**	0.35	15.74	**0.0010**
Residuals	8	0.10			0.00			0.01			0.02		
Cochran's test			0.570	ns		0.718	ns		0.583	ns		0.766	ns
Transformation				none			none			none			none
SNK test				A<W*=S<Sp**			S<W**<W*=Sp			Sp=W<A*=S			Sp=S=W<A**

S = Summer group; A = Autumn group; W = Winter group; *: p<0.05; **: p<0.01

Tab. 2.4. Continued.

		22:5 n3			22:6 n3 DHA			SFA			MUFA		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	0.02	53.71	**0.0000**	96.45	237.42	**0.0000**	23.01	46.72	**0.0000**	58.58	184.25	**0.0000**
Residuals	8	0.00			0.41			0.49			0.32		
Cochran's test			0.718	ns		0.491	ns		0.540	ns		0.497	ns
Transformation				none			none			none			none
SNK test				S<A*<W**<Sp*			S<Sp**<A**<W**			Sp=W=A<S**			W<A*<Sp**<S**

		PUFA			Unidentified			∑ n3			∑ n6		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	60.28	82.07	**0.0000**	23.89	11.86	**0.0026**	98.06	371.85	**0.0000**	3.59	7.18	**0.0117**
Residuals	8	0.73			2.01			0.26			0.50		
Cochran's test			0.586	ns		0.417	ns		0.534	ns		0.506	ns
Transformation				none			none			none			none
SNK test				S<A**=Sp<W*			S=Sp<W*=A			S<A**=Sp<W**			W=A=Sp<S*

		n3/n6			EPA/DHA			EPA + DHA		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	3	3.83	23.63	**0.0002**	0.02	49.61	**0.0000**	88.68	309.27	**0.0000**
Residuals	8	0.16			0.00			0.29		
Cochran's test			0.504	ns		0.568	ns		0.566	ns
Transformation				none			none			none
SNK test				S<Sp**=A=W			A=W<Sp**<S*			S<Sp**=A<W**

S = Summer group; A = Autumn group; W = Winter group; *: p<0.05; **: p<0.01

Tab. 2.4. Continued.

		AI			TI			
Source	df	MS	F	*p*	df	MS	F	*p*
Group	3	0.02	59.31	**0.0000**		0.01	109.58	**0.0000**
Residuals	8	0.00				0.00		
Cochran's test			0.581	ns			0.364	ns
Transformation				none				none
SNK test				W=A<Sp*<S**				

S = Summer group; A = Autumn group; W = Winter group; *: p<0.05; **: p<0.01

2.4 Discussion and conclusions

During the past few decades, numerous studies on gametogenic cycle of Bivalve molluscs have been carried out. In this scenario, Venerids and the species belonging to the genus *Ruditapes* have been widely studied, in particular *Ruditapes philippinarum*. Many of these research were carried out on wild clam populations both in their natural habitat of origin, as Japan, and in other areas of the world where this species was introduced, as the eastern Pacific, the Atlantic Coast of Africa and the European shores (Holland & Chew, 1974; Shaffee & Daouidi, 1991; Ponurovsky & Yakovlev, 1992; Toba *et al.*, 1993; Meneghetti *et al.*, 2004; Drummond *et al.*, 2006).

Conversely, literature on the native European species, the grooved carpet shell *Ruditapes decussatus*, is quite poor (but see Pérez-Camacho, 1980; Delgado & Pérez-Camacho, 2003; Ojea *et al.*, 2004) and studies performed on the Italian coasts are still scarce. In particular, no investigations of the reproductive cycle of this species have been carried out in Sardinia up to now.

Although a number of the above-mentioned studies have focused on clam gametogenic cycle in the natural environment, many of them derive from experimental trials performed in the laboratory. In this way, molluscs can be more quickly and practicably conditioned, independently from the natural patterns of the biotic and abiotic environmental variables, that can influence the biological features of the species.

In general, the gonadal cycle of marine invertebrates is strongly influenced by both endogenous and exogenous factors (Giese, 1959; Sastry, 1975; Adiyodi & Adiyodi, 1983), among which temperature is considered the most important in regulating gametes emission processes (Mann, 1979). In fact, several laboratory studies demonstrate that even slight increases of water temperature can be sufficient to stimulate factors for spawning (Gosling, 2003). At the same time, other seawater variables such as salinity, chlorophyll *a* and nutrients concentration have to be considered, as they dictate the entire biological cycle of these molluscs and can significantly affect their processes of energy acquisition and consumption (Rodriguez-Moscoso *et al*., 1992; Okumus & Stirling, 1998; Urrutia *et al.*, 1999). Over that, several Authors have demonstrated that seasonal variations of the condition index and biochemical composition can constitute a good indicator for the physiological or nutritional state of clams during the gametogenic cycle (Beninger & Lucas, 1984; Meneghetti *et al*., 2004).

In this study, the surveys carried out in the Porto Pozzo lagoon have shown that this

biotope is a typical transitional water environment, where water temperature and salinity always reveal a strong flexibility (Barnes, 1980). Both these variables, in fact, show the typical pattern of our latitudes, with relatively high values during the summer and relatively low levels in winter. By contrast, in autumn and spring water temperature reveal gradual modifications with the climatic changes typical of these seasons, while salinity values are subjected to rapid changes depending on the rainfall.

The Porto Pozzo lagoon is an oligotrophic habitat, characterised by abundant water exchanges and low intakes (*i.e.* freshwater and nutrients) from the surrounding inland. Indeed, chlorophyll *a* and major nutrients (*i.e.* phosphorus and nitrogen) concentrations were quite low throughout the whole study period and they always showed rather irregular patterns. These variables reached their highest values in late summer and in early autumn, when the increase of temperature, the intensification of human activities related to the tourist season, and the autumn rainfalls came one after the other in a short period of time. It is well reported that the amount of nutrients in water is correlated to phytoplanktonic growth, hence promoting the available food for filter-feeders (Brown & Hartwick, 1988). However, when substantial water exchanges between a lagoon and open sea occur (as in the Porto Pozzo lagoon), daily fluctuations of nutrients can originate, conditioning the performances of the resident Bivalve population (Saxby, 2002).

As regards morphometric characters, no relevant changes were observed in the clams examined during the study period, and a clear uniformity of results obtained was found. This is certainly due to the choice of collecting only specimens above 25 mm in shell length, and then to examine adult molluscs that could be sexually distinguishable. In this regards, such as in other coastal areas (Xie & Burnell, 1994), the sex ratio of clam population of the Porto Pozzo lagoon showed a male/female proportion of about 1:1 during almost the entire study period, except for the winter months when sexually undifferentiated specimens constituted a high percentage of the samples collected. However, the strong relationship between the mollusc weight and the shell length for male, female and undetermined specimens, detected also by Ojea *et al.* (2004), confirmed the correlation of these morphometric variables. Changes in weight values of these Bivalves, indeed, can be related to environmental variables dynamics, especially to the food presence (Ojea *et al.*, 2004). As a consequence, chlorophyll *a* concentration in the water column and mollusc weight followed similar patterns during the study period. Therefore, when food is abundant, clams accumulate reserves that can be used

to produce and develop the sexual products, as verified by Sastry (1979) for other mollusc species.

In this research, the highest weight of *R. decussatus* specimens was registered in July but it declined in the following month when a chlorophyll *a* peak was observed. Just in this period, the area around the lagoon is generally subject to a strong tourist presence that can contribute to an increase of nutrient inputs into the basin. From late summer onwards, values of chlorophyll *a* and nutrients showed an increase that may be related to the first autumn rainfalls. These, leaching the soil, bring a fair amount of organic matter in the Porto Pozzo lagoon. Even in winter a similar situation occurred, but in this latter case a peak of chlorophyll *a* concentration was registered in December and, later, a peak in the mollusc weight in January. From the following spring, increasing amounts of chlorophyll *a* and increasing values of water temperature positively influenced the mollusc growth, as already observed by Matias *et al*. (2009).

During the whole period of the study, the two condition indexes considered revealed excellent values for the clam population of the Porto Pozzo lagoon, registering their maximum in spring the former (CI) and in winter and spring the latter (CI-AFDW). It is interesting to note the very close correspondence between the two indexes. This fact suggests that the use of the more easily measured dry flesh weight condition index (CI) is quite adequate for *R. decussatus*, as confirmed by Beninger & Lucas (1984). It is true, however, that the exclusion of ash from the dry weight can reduce the variability of the parameter, because ash content is influenced by many factors such as salinity (Okumus & Stirling, 1998), nutritional state (Wilkins, 1967) and reproductive cycle (Beninger & Lucas, 1984) of an individual. In particular, the autumnal decrease of CI-AFDW coincides with the emptying of the gonads due to gametes emission (Breber, 1996).

Overall, both the condition indexes considered reflect the reproductive activity of *R. decussatus* in the Porto Pozzo lagoon. The variations in weigh, gonadal growth and spawning occurred almost simultaneously, especially in response to changes in water temperature. As detected for *R. philippinarum* by Mann, 1978, our results confirmed that low temperatures affect gametogenesis as well as spawning. Accordingly with previously studies on *R. decussatus* from temperate regions (Breber, 1980; Beninger & Lucas, 1984), spawning was observed when temperature values were higher in late summer. Conversely, a gradually decrease of seawater temperature, mollusc weight, condition and gonadal occupation indexes was noted during late autumn and in winter season. When environmental conditions improved in spring, also gametogenesis began

and ripe gonadal stage was reached in the following summer. This reproductive pattern is confirmed by numerous investigations on Bivalves in their natural habitat (Rodríguez-Moscoso & Arnaiz, 1998; Ojea *et al.*, 2004; Serdar & Lök, 2009), and most clams belonging to the Veneridae family seem to be summer spawners (Shafee & Daoudi, 1991). Further laboratory experiments demonstrated the influence of temperature on the reproductive behaviour of *R. decussatus*, with a direct relation between the rate of gonadal development and the increase of temperature (Delgado & Pérez-Camacho, 2007). It follows that the geographical distribution range of a species can become extremely important to define and monitor its gametogenic activities (Partridge, 1977; Beninger & Lucas, 1984; Rodriguez-Moscoso *et al.*, 1992; Robert *et al.*, 1993).

In terms of nutrient storage for supplying energy for gametogenesis, the role of protein is very important (Mathieu & Lubet, 1993) and it is generally believed that in Bivalves gametogenic cycle is closely associated to the seasonal cycle of storage and utilization of glycogen reserves. In this regard, Gabbott (1975) demonstrated that in these invertebrates the development might involve the metabolic conversion of glycogen to lipid. Accordingly, the results of the present study confirmed that seasonal variation in the biochemical composition of clams can represent a good indicator of their physiological and nutritional state during the gametogenic cycle (Beninger & Lucas, 1984; Meneghetti *et al.*, 2004). Generally, protein and lipid reserves are accumulated before gametogenesis (Gabbott & Bayne, 1973). Protein constitutes the major organic component of oocytes (Holland, 1978), and represents an energy reserve in adult molluscs, especially during gametogenesis (Beninger & Lucas, 1984). Lipid is strictly related to gamete development, increasing when the gonads are ripe (Martinez, 1991). Our results showed that the higher protein contents were found in both summer and winter periods: this might be related to the gonadal ripe stage in the former case, and to the storage function in the latter. In this regard, Galap *et al.* (1997) affirmed that protein seems to be the only alternative energetic resource in case of food scarcity. As far as the lipid content is concerned, its dependence on the sexual maturation stage is well known for *R. decussatus* (Delgado *et al.*, 2004). Lipid amount was not significantly different in the population from the Porto Pozzo lagoon throughout the study period, although its content was higher in summer when gonads are completely ripe, as already observed by Martinez (1991). Overall, therefore, it is possible to confirm that in Bivalves the fluctuation of their own biochemical components is related to their reproductive cycle

(Martinez, 1991), and *R. decussatus* obtain its energy from both protein and lipid (Albentosa *et al*., 2007). In particular, our results suggest that protein, and also lipid content, can seasonally vary together with gametogenic cycle.

The fatty acid spectrum of clam population in the Porto Pozzo lagoon is typically marine, with the dominance of palmitic acid (16:0), eicosapentaenoic (20:5 n3 EPA), and docosahexaenoic (22:6 n3 DHA) acids (Gruger *et al*., 1964; Voogt, 1972; Yamada & Hayashi, 1975; Besnard *et al*., 1989). It is generally accepted that the predominance of both these polyunsaturated fatty acids is an adaptation to the relatively low temperatures of the marine environment, contributing to the maintenance of cell membrane fluidity (Holland, 1978). Moreover, it is reported that high percentages of these compounds can be due to a bloom of phytoplankton, particularly rich in this fatty acids (Ojea *et al.*, 2004). In particular, the maximum content of DHA was found during the winter season when, after an increase of the chlorophyll *a* concentration in December, a correspondent increase in DHA percentage occurred. Marine phytoplankton is highly rich in polyunsaturated fatty acids (PUFA) 20:5 n3 and 22:6 n3, and it represents the main source for 18:1 n7, 18:1 n9, 18:2 n6 and 18:3 n3 (linoleic and linolenic acid, respectively) (Freites *et al.*, 2002). This was also confirmed by the high percentage of PUFA (n-3 in particular) found in the edible parts of clams harvested in the Porto Pozzo lagoon.

The high lipid quality of the clam flesh and its positive effect on human health are further confirmed by both the atherogenicity (AI) and thrombognicity (TI) indexes, and by the ratio between n3 and n6 PUFAs. Our results compared to those given by Ulbricht & Southgate (1991) for chicken flesh (0.30 for AI and 0.95 for TI) confirmed this assertion.

In conclusion, the present study proved that the native grooved carpet shell, *R. decussatus*, has an excellent reproductive potential in the Porto Pozzo lagoon. The autochthonous population is perfectly adapted to environmental conditions that characterize this biotope (*i.e.* sudden fluctuations of the main environmental variables, especially temperature and salinity, during winter and summer periods). As a consequence, these Bivalves can regularly perform their gametogenic cycle, resulting in a constant recruitment of juveniles. On the above-mentioned considerations, the good physiological status of the *R. decussatus* population in the Porto Pozzo lagoon is confirmed both from a nutritional standpoint and for its particular characteristics of “hardiness" (*e.g.* resistance to climatic variations, constancy of the gonadal maturation

periods, etc.). Through the current knowledge, therefore, it becomes possible to assume a responsible and sustainable exploitation of the Porto Pozzo lagoon and the employment of adult specimens of the autochthonous grooved carpet shell clam to create breeding stocks aimed at re-stocking and/or exploitation purposes.

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Chapter 3

MYTILUS GALLOPROVINCIALIS

3.1 Introduction

Mussels belonging to the genus *Mytilus* are worldwide distributed being one of the most common marine molluscs living in costal areas of both northern and southern hemispheres (Gosling, 1992b; Hilbish *et al*., 2000; Borsa *et al*., 2007). Two of them are the principal species traditionally reported: *Mytilus edulis* (Linnaeus, 1758), from the North Atlantic area and eastern coasts of North America to Europe (*i.e.* Atlantic littoral of France, The Netherlands, Germany, Great Britain, Ireland and Norway) (Riginos & Henzler, 2008; Śmietanka *et al*., 2004), and *Mytilus galloprovincialis* (Lamarck, 1819), from the Black and Mediterranean Seas (where it is recorded to be alive by about 2 million years) (Daguin & Borsa, 2000) to the Atlantic coasts of Spain, France, Great Britain and Ireland (Fuentes *et al.*, 1992; Quesada *et al.*, 1998). Farming activities of these Bivalves are also widely practiced. In particular, *M. galloprovincialis* is reared not only in the above-mentioned areas but also in China (its main producer) and South Africa (www.fao.org), where it dominates other locally farmed species (*i.e.* *Choromytilus meridionalis*, *Aulacomya ater* and *Perna perna*) (van Erkom Schurink & Griffiths, 1993).

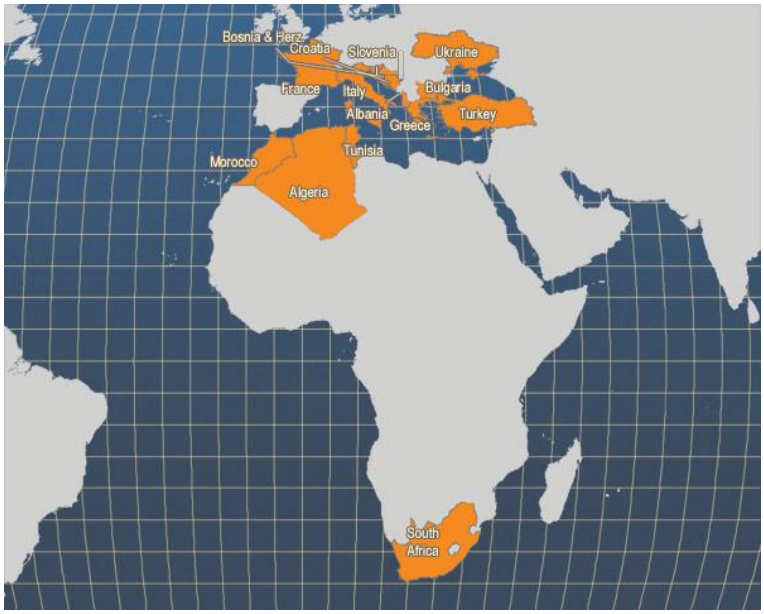

Fig. 3.1. Main producer countries of *Mytilus galloprovincialis* (from www.fao.org).

In the Mediterranean Sea, mussels are successfully reared in its southern part, where there is an abundance of sheltered and highly productive areas (*i.e.* gulfs, lagoons, coastal lakes) (*e.g.* Gulf of Gaeta and Taranto in Southern Italy; Sardinia; Biserta in

Tunisia; Ganzirri and Faro in Southern Italy). By contrast, mussels are not very common in the South-western side and their culture is unpractised probably due to the scarcity of sheltered, and shallow zones, and a lack of harvesting for consumption by local populations (Sarà *et al.*, 1998). In general, however, the ecological role of these Bivalves in the environment, with the considerable demand for human consumption, has made them a fundamental resource for aquaculture productions especially in Europe.

Mussels cultivation systems are always based on suspension growing of the animals, transplanting them from natural beds to rearing facilities, possibly all within the same geographic area. As a matter of fact, it is reported that this populations demonstrate an important genetic differentiation over both large and small spatial distances (Gosling, 1992a). Therefore, in terms of production, it is reasonable to pay attention not only to the peculiar environmental conditions of the breeding site but also to the geographic origin (and then the nature) of the grafted spats (Mallet *et al.*, 1987; Fuentes *et al.*, 1992). Production phases in this type of extensive mussel farming, consequently, depend on natural processes and so appropriate quantities of seed, knowledge of distribution, abundance, seasonal fluctuations and dynamics of settlement are the key points to maximise the production (or the status) of this resource (Appukuttan *et al.*, 1989).

Filter feeding bivalves are plankton consumers, including phytoplankton, organic detritus, bacteria and probably dissolved organic matter in the water, thus playing a significant function of energy transfer in the food chain (Navarro & Thompson, 1996). Ingested food, in turn, is related to its environmental availability, filtration activity of the animal and selection process (Richoux & Thompson, 2001) so that the mussel growth rate is strictly dependent on interactions between a set of endogenous and exogenous factors (Bayne & Newell, 1983) among which, temperature, salinity and food have received the greatest attention (Riisgard *et al.*, 1980; Sprung, 1984). Suspension-feeding activity, in fact, is quite variable depending on environmental conditions (*i.e.* temperature, salinity, seston concentration, particle quality) (Dickie *et al.*, 1984; Bayne *et al.*, 1989) so that the growth performances can differ even between very close sites in the same area (Mallet & Carver, 1989). At the same time, local factors as duration of air exposure (Seed, 1969), population density (Peterson & Beal, 1989), genotypic characteristics (Dickie *et al.*, 1984; Skidmore & Chew, 1985) and water current velocity (Grizzle & Morin, 1989) contribute to influence the rates of

biochemical processes in these marine organisms.

In the world aquaculture production, mussels represent one of the most important groups both in terms of quantities (tonnes of product) and from an economic point of view. During 2000s, this sector recorded significant production in Europe, especially in Spain where the North-western part (*i.e.* Rias Baixas) constituted the main production zone with amounts of about 250,000 tonnes year^{-1} (Figueiras *et al.*, 2002) and the Mediterranean production represented about 9% of the world mussel production (FAO, 2005). Other countries, in fact, appear among the largest producers such as Greece, with its 27,000 tonnes year^{-1} (Papoutsoglou, 2000), and particularly Italy with about 130,000 tonnes year^{-1} (Saroglia *et al.*, 2000).

According to Monfort (1999), furthermore, mussels produced in Europe are predominantly consumed within Europe, and the major internal trade occurs between neighbouring countries.

3.1.1. Suspended mussel culture techniques

Selection of sites for mussel culture represents the first step for starting an efficient production activity. Hydrodynamic stability, carrying capacity of the system, water quality (generally subject to local regulation), competition with other functions (*e.g.* recreational activities and nature conservation), are the main factor to consider (Smaal, 2002). The principles that govern any productive activity of rearing are the following:

- natural settlement of mussels in the environment concerned;
- depths from 3-12 meters with inconsistent substrate: muddy, slimy or sandy;
- areas protected by marine adversities;
- biomass or pabulum sufficient in relation to the density of the farm;
- human factor available for the demanding working conditions in the farms.

3.1.2. Mussel culture in Italy

Mussel culture in Italy has a high economic importance, considering that the value of Italian consumption in 2003 exceeded 100 million euros, and Italy's trade with foreign countries amounts annually to about 40 million € with a marked upward trend. At the same time, although not committing a large amount of personnel, the sector is a reserve of employment especially in some areas. In Italy, in the past ten years, mussel culture has lived a period characterized by alternating production developments. Despite the

production from 1990 to 2002 has been increased from 104,514 tonnes in 1990 to 138,249 tonnes in 2002, it presented some setbacks in 1994 (-11%) and in 1996 (-2%), maintaining a negative (or almost) tendency from 1991 to 1994. The causes of these trends are related to several factors such as: the adaptation to new production and hygiene regulations introduced since 1992 by the European Union; the new framework of legislation that collided with a productive structure characterized by a number of small companies with obsolete plants; the introduction of new production systems, and the subsequent occupancy of new areas (especially offshore) with environmental characteristics different from those traditionally used for mussels culture in Italy, resulting in a further effort by companies to remain competitive in the market.

All this facts led to an intensification of the international competition, in particular among those European countries that may freely circulate their product. In many cases, these countries, lacking of a significant traditional production and not having any problems of rehabilitation, could start immediately their productive activities, entering to the market with a relatively abundant product characterised by an affordable price.

Among the main causes of changes in production and commercial trades, it is worth mentioning the radical change occurred in the legislative and institutional framework. These changes were implemented through the adoption of health regulations regarding production and marketing of live bivalve molluscs laid down in Directive 91/492 of European Community and implemented in Italy by Legislative Decree No. 530 of December 30, 1992. On the basis of these regulations, the farming areas are classified in three areas according to the sanitary requirements of water: depending on the area where the mussels are reared or harvested they can be directly marketed (A zones), commercialised after a period of laying (B zones) or after a housing of at least two months associated with an intensive depuration (C zones).

3.1.2.1 Evolution of the Italian mussel culture

Fixed installations are the oldest Italian farming system and generally they were used in coastal lagoons or in repaired and sheltered costal areas. This technique consists in the arrangement of poles (made of wood, steel or concrete, having different forms) connected by cables on which are hung mesh sleeves, tubular net in plastic material (polypropylene) containing mussels (the set of the structure and mussels in Italian is named “resta”). The structures generally employed were two: rows and squares. The first consists of a long series of posts, together in groups of 3-4, perpendicular to the

coast towards the open sea. The distances between each other usually do not exceed 10-15 meters.

The classic Italian system was that of squares, born in Taranto (southern Italy) and spread to other parts of Italy. It not has undergone profound changes but, from year to year, it was possible to note transformations that have changed the original method. The boxes are made of poles placed at a distance of 5 meters apart, driven into the bottom for 1.50 to 2.50 meters in relation to the texture of the substrate and projecting the mean sea level of over 1.50 meters. Livestock farming are composed of operating units or modules, and usually have an area of 500 m^2 or multiples of these. The vertices are represented by the four corners of the operating unit whose poles are reformed from other two places in the directions of the two sides, which form a triangle. Other poles forming the box perimeter have an additional pole diagonally placed inside.

Nowadays, the union of the poles is done by means of steel or synthetic cables. The individual poles located within the square are also devoid of any reinforcement but have supported the set of strings that make up the cables, to which the mesh sleeves are hung. The mesh sleeves are secured to the cables and between a pole and the next (within 5 meters) they are 6-8, about 60-70 cm away from each other. The length of mesh sleeves varies greatly from region to region, but as a rule they must remain fixed at a distance of 0.5-2 meters from the bottom.

To the cables, other strings cross are added (called cruises) holding 9 mesh sleeves. As in all types of fixed installations, but especially in this, external mesh sleeves are more flourishing than the internal ones. Among the factors determining this, certainly the most significant are: micro-turbulences and water exchange, competition for food, chemical processes of preudofaecies decomposition.

Over the past 20 years, floating plant systems have gradually used and quickly they have become the strong point of the Italian mussel production. Moreover, suspended mussel culture exhibited higher performances in comparison to bottom and pole culture (Garen *et al.*, 2004). The evolution of this plants is referable to old floating rafts employed in Spain, consisting of a prismatic construction with strips of eucalyptus wood, separated by 50 cm, on which mesh sleeves 4-8 meters long were attached, depending on the depth. In Italy, long-line system is widely used, especially in regions such as Emilia-Romagna, Puglia, Veneto, Friuli-Venezia Giulia and Sardinia, but also elsewhere in the world, such as Sweden and New Zealand. The great advantage of this system is that the harvest of product is carried out by mechanical apparatus.

The “monoventia” system usually has a length of 60 meters. It is suspended by floats of various shapes and nature, generally plastic made. The buoys are around 150-280 litres, and these structures are interspersed between 5 and 15 meters along the suspended cable, depending on the weight they have to support and the depth in which the facility is located. The length of the Italian “monoventia” can exceed 100 meters. Mussels facilities consist of steel cables anchored to large boulders at the ends of the state-owned concessions by chains. As already mentioned, the cables are kept on the surface by buoys in galvanized iron or plastic. Cables have a variable length of about 180 meters and are located at a distance of about 10 meters apart. The mesh sleeves have a length ranging from 4 to 5 meters and are at least 40 cm from each other.

After the first experiences in 1965, special structures (fiberglass barrels) with two cables (in Italian “biventia”) were built. Affixed to their sides, were two shaped irons that allowed the establishment of two parallel cables. This system has discrete productivity benefits and it is particularly suitable for individual workers because the initial costs, the management and the manual work are significantly reduced. In addition, the system performance is extremely variable: calculating 16 mesh sleeves for cable, for a total of 32 mesh sleeves with an approximate weight of 25 kg to the sale, the total amount is about 800 kg per field (that is between buoy and buoy).

Around 1973, in the Gulf of Trieste a floating 3-cables system was used, making a further contribution to the mussel production. The third cable was intended to increase the production of a third and prevent the complete breakdown of the line in case one of the other two should break. In this case, between the buoys there was a distance of 10 meters, crossed by 3 cables where the mesh sleeves were attached (Bussani, 1983).

3.2 Materials and methods

3.2.1 Mytilus galloprovincialis

3.2.1.1 General description of the species

The Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819) belongs to the Mytilidae family (Rafinesque, 1815) (Tab. 3.1) and is native to the Mediterranean and the Black Sea.

Tab. 3.1 Systematic framework of *Mytilus galloprovincialis* (Lamarck, 1819)

Taxon	Name	Author
Phylum	Mollusca	
Class	Bivalvia	(Linnaeus, 1758)
Subclass	Pteriomorphia	(Beurlen, 1944)
Order	Mytiloida	(Férussac, 1822)
Family	Mytilidae	(Rafinesque, 1815)
Genus	*Mytilus*	(Linnaeus, 1758)
Species	*Mytilus galloprovincialis*	(Lamarck, 1819)

It is often confused with its congeneric, the blue mussel *Mytilus edulis* (Linnaeus, 1758), widely distributed in both European and Atlantic waters. This last is generally larger (up and over 100 mm), and shape and colour of the shell are different: in fact, *M. galloprovincialis* is characterised by an umbo more pointed and forward directed, and it is wider and less angled dorsally tending to make the basal line of the shell concave. The colour of its mantle edge is generally darker almost black or otherwise tending to violet or purple, but never yellow as in *M. edulis* (Fig. 3.2).

Internally, mussels have the typical organization of filter feeder Bivalves, with two lobes (constituting a well developed mantle) dorsally placed on two valves lining and wrapping the whole animal (Fig. 3.3). Later, the mantle edges are joined together for a short distance delimiting an opening where the water previously drawn can come out. This orifice is named exhalant opening, while that ventral one is the inhalant, from which water is drawn. Between the lobes, there is the foot composed of muscle tissue, tongue-shaped and sometimes pointed. On its basal portion extends the byssus gland,

connected with a ventral canal where proteins constituting the byssus are secreted. When the proteins come into contact with water, solidify and form the rugged strand that allows the mollusc to anchor itself to a substrate. A medium-sized mussel can form even about 150 filaments 4-5 cm long and their weight can be up to 1/3 the its weight.

Fig. 3.2. Specimens of *Mytilus galloprovincialis* (Lamarck, 1819) (left) and *Mytilus edulis* (Linnaeus, 1758) (right).

Mussels typically live in littoral and shallow sublittoral waters although they can been found occasionally in deeper water. They stay in both open marine and brackish waters, especially where water movement is considerable, fixed on different substrates (*i.e.* rocks, stones, shingles, shells, compacted mud or sand) that may represent a safe anchorage (Bayne, 1976). The continuous water flow provides not only the oxygen supply but also a constant cleaning of the mantle cavity carrying out carbon dioxide and catabolites and guaranteeing nourishment, such as organic and inorganic matter, and planktonic organisms (*i.e.* the *pabulum*). These Bivalves, in fact, can filter up to 5 l h^{-1} of water, depending on their physiological status and environmental conditions. In particular, *M. galloprovincialis* shows an *optimum* of temperature of about 8-25 °C, filtering more than 100 l day^{-1} of water having a salinity of at least 10 PSU.

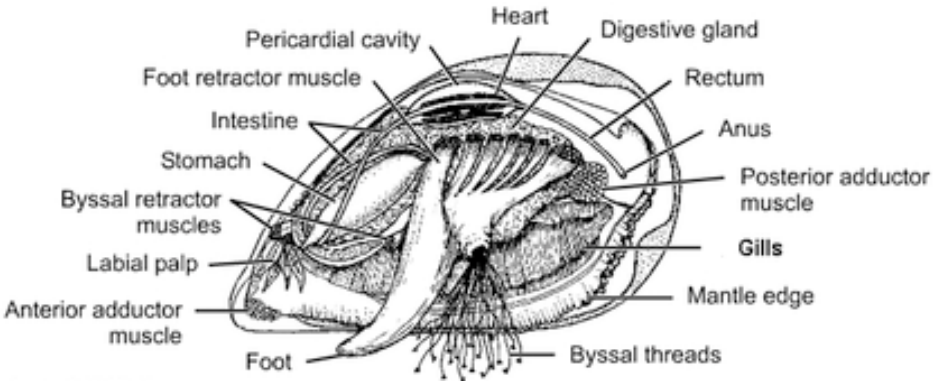

Fig. 3.3. Internal anatomy of *Mytilus* spp.

Like other Bivalves, Mytilids are gonochoristic but the sex ratio does not appear balanced: it seems that females have the edge on males and, in particular, female specimens are usually over 54% in *M. galloprovincialis*. Sexual maturity can be reached at the first year of life and spawning events occur generally between late winter and early spring although they may occur to a lesser extent during late autumn. In general, the reproductive events are due to an interaction between environmental factors (*i.e.* temperature, salinity, amount of food, sometimes even light) and endogenous factors. During the last phase of their development, gonads reach their maximum size allowing the distinction of the two sexes: in fact, female gonads are usually pink, whereas the male ones yellowish-white. Depending on the size, the quantity of eggs released in the water can range from about 100,000 for a female of 25 mm up to 8-10 million for another one of about 80 mm in length. Once fertilized, in a few hours the egg turns into a pelagic larva and, depending on water temperature, it is passively transported for a variable period of 3 weeks to several months, until a suitable substrate on which to settle and grow to adult size is met. As other Bivalves, the timing of growth and the attainment of commercial size are strictly dependent on water temperature and availability of food.

Overall, these molluscs are quite resistant to changes of both salinity and temperature values, provided that extreme situations do not be over-long (in this case, conditions of suffering may occur even leading to important mortality events). Furthermore, because of their coastal distribution, mussel beds are often subject to pollution, whose primary risk can be the presence of bacterial (*e.g. Escherichia coli*, *Salmonella* sp.) and viral pathogens (*e.g. Vibrio* spp.), particularly when mussels are eaten raw or lightly cooked (Wood, 1972). In general, however, there are no diseases that can cause serious damages for *M. galloprovincialis* cultures and the related species that occur in the natural mussel beds are those naturally living on rocky intertidal coasts, such as barnacles (*Balanus* sp.) and some algal species (*e.g. Enteromorpha* sp.). Mussels have natural predators such as crabs (*Carcinus maenas*), starfish (*Asterias rubens*), and finfish (*Diplodus* spp. and *Sparus aurata*), but fouling organisms (*e.g.* crustaceans, ascidians, worms, larvae of different animals and some species of algae) represent the main limiting factor for their growth. Actually, fouling compete with mussels for space and food, invading their shell, hindering water filtration, and at the same time, weighing down the farming structures (www.fao.org).

3.2.1.2 Production cycle

Worldwide, the rearing of *Mytilus galloprovincialis* is extensive (Fig. 3.4). In particular, this type of farming involves the employ of structures (fixed or floating) that allow the growth in suspension of these Bivalves, maximizing their exposition to water flow.

Young mussels (spat or seed) are always manually collected from the sea and it is not necessary to apply any technique of artificial reproduction due to their availability in the natural environment. Farmers can harvest the seed directly from their facilities (*e.g.* ropes, nylon nets, wooden frames, floating plastic buoys), natural beds or others plants and plan its re-immersion in water within 24 hours after retrieval. By mechanical hoppers or plastic pipes, juveniles about 20-30 mm long are placed inside particular mesh sleeves in polypropylene, with an appropriate mesh diameter and immersed in the rearing system, generally during spring and early summer. This mesh disintegrates within a few days and in the meanwhile mussels secreted new byssus attaching themselves to each other. In this way, farmers can introduce up to 1.5-1.75 kg of seed per meter of rope (www.fao.org).

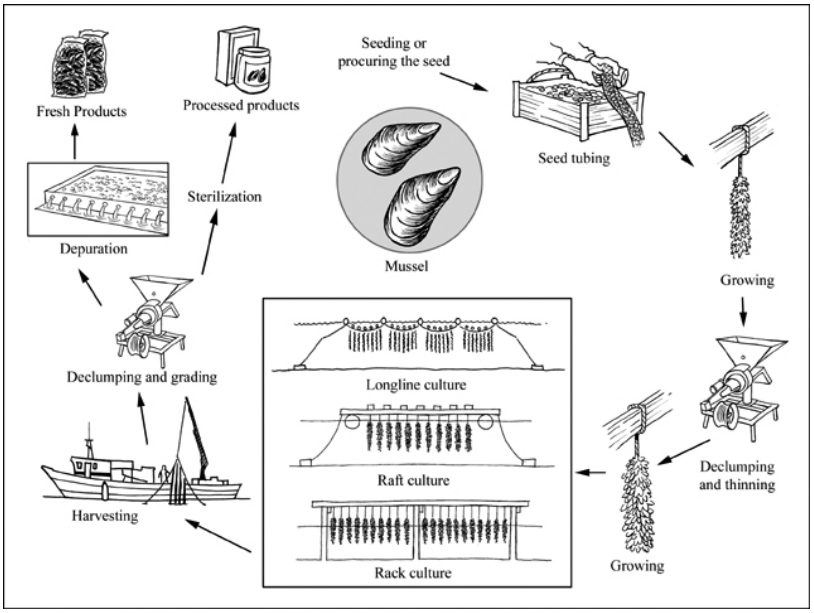

Fig. 3.4. Production cycle of *Mytilus galloprovincialis*.

As the growth of mussels gradually increases and the mesh sleeve can break away from the rearing structure, molluscs are transferred into new mesh sleeves about 2-3 months after the first dive. This implies the manual or mechanical splitting (*i.e.* declumping and thinning) of growing spat from a mesh sleeve and the subsequent relocation of the

quotes obtained in others with larger meshes. Usually, one or two reinforcements per production cycle are carried out.

The entire growing phase (*i.e.* fattening) lasts about 8-12 months, and a number of periodic maintenance operations are performed. In addition to the constant control of livestock facilities, indeed, the cleaning of the mesh sleeves is of great importance, particularly from the biofouling organisms. This last is a typical component of aquatic environment resulting from settlement and growing of sedentary and semisedentary organisms on submerged surfaces (Venugopalan & Wagh, 1990). Its excess can cause a reduction in the farmed growing molluscs and, in drastic cases, their suffocation with mortality phenomena, as well as a significant increase in weight of the mesh sleeves with a consequent detachment from the plant.

When mussels reach the commercial size (*i.e.* about 50 mm of shell length) then can be harvested but if they appear close to spawning period or just spawned the process should be postponed. Generally, the entire production cycle is programmed so that harvest can be made throughout the year, although actually the period of greatest market demand in Italy is during the summer. The product then is hoisted on board, separated and sorted by a grid of iron bars, washed of fouling and separated from undersized specimens that are relocated into new mesh sleeves for further growing. Marketable mussels, if they come from water areas classified as A, can be immediately packed in bags weighing between 2 and 30 kg. If the origin is from water zones classified as B, mussels have to be depurated prior to human consumption.

3.2.2 Study areas

3.2.2.1 Plant breeding system in the Porto Pozzo lagoon

Mollusc culture in the Porto Pozzo lagoon started in 2007, when the resident fishermen cooperative established the first *Mytilus galloprovincialis* breeding system. The installation (Fig. 3.5) was located in the central part of the lagoon (La Peschiera), where the maximum depth is 17 meters. It was a typical rows floating system (long-line), composed by 10 parallels modules each consisting of a singular rope. Each rope was about 90 meters long and up to 150 polypropylene tubular nets (5 m long) containing mussels (*i.e.* mesh sleeves) were hung on it. Nowadays, the plant is almost doubled, still organised in 10 parallel modules but each consisting of a double ropes, where 300 mesh sleeves 4 m long are positioned, thus increasing the first annual production of about 40 tonnes to more than 110. At the same time, the addition of a perimeter net around the

plant as a defence against predators (*i.e. Sparus aurata*) and the long-line system positioning along the main direction of dominant currents contributed to the improvement of the plant.

Fig. 3.5. Mollusc culture system in the Porto Pozzo lagoon.

The seeding schedule involves autochthonous juveniles collected from the local plant breeding and the integration with seed purchasing from other brackish environments. As a general rule, it comes from some breeding areas of the Adriatic coast (from the Gargano to the Po delta river) with prices ranging between 0.55 and 0.75 € kg^{-1}. Usually, the seeding process takes place between February and May (not *a priori* excluding the months of January and June), depending on weather conditions, size of the seed, and rearing facility availability at that time (*e.g.* number of buoys to carry the load). It is not easy to predict exactly the real amount of product produced but the main purpose of the fishermen cooperative is to exceed 100 tonnes of mussels for the best selling season (*i.e.* summer months).

After about 8-10 months, *M. galloprovincialis* specimens commercial-sized are harvested and subjected to a series of processes before the sale. The mesh sleeves are transported to land and molluscs are separated from each other using a declumping apparatus positioned above a pier. Then, the product can be moved to the sale point where it is processed. Mussels are subjected to phases of sorting through the vibrating screen sizes, immersed into tanks with filtered seawater regulated at 15°C and 30 PSU for at least a night for depuration, and finally packaged in plastic nets of 2 kg each for the sale.

The price of mussels from the Porto Pozzo lagoon does not vary with seasons: the retail

price, in fact, was always equal to 3.50 € kg^{-1} during the last 5 years.

3.2.2.2 The Calich lagoon

The Calich lagoon (Fig. 3.6) is a brackish basin between the geographical coordinates 40°35'25'' N and 8°16'00'' E. It is located near the town of Alghero, covering a surface of about 97 ha, with an elongated and narrow shape having a maximum length of 2650 m, which follows the coastline for about 400 m (Fatichenti *et al.*, 1978; Chessa, 1980). Its main inlet is the Rio Barca that receives the confluence of the Rio Filibertu, Rio Sassu and Rio Serra. Other tributaries are the Nurra channel (Fighera channel) and the Rio Fangal. Its communication with the sea is guaranteed by a channel about 260 m long.

Fig. 3.6. The Calich lagoon.

The Calich lagoon has a catchment area of 42,500 ha, a maximum depth of about 2 meters and is characterized by eutrophic waters. It stretches beyond the boundaries of Alghero, involving the municipalities of Sassari, Olmedo, Ittiri, Putifigari and Villanova Monteleone. The waters of the Rio Serra upstream are routed into Cuga River basin, thereby reducing the catchment area to 35,000 ha.

During the summer, due to the negligible contribution of water from the tributaries, there are significant increases of the salinity in the lagoon. In the smaller part of the basin, the amount of water is reduced to a minimum and in the past surfaced small islands. The exchange of water in this season is due to the tidal wave, although its effect is rather modest. In the winter season, however, rainfalls are abundant often leading to a dramatic lowering of the salinity. The winter rains, coinciding with strong winds and

moderate sea storms blocking the water flow towards the sea, cause the rising waters in the lagoon. The smaller part of the basin, being less deep, is the first to feel the influence of these elements. The particular position of the channel to the sea, coupled with the elongated and narrow shape of the basin, does not provide a suitable water turnover (Chessa *et al.*, 1988).

Some studies conducted in 2001 (Chessa *et al.*, 2001) showed that the Calich lagoon has a quite stable equilibrium. In fact, the benthic and planktonic communities are fairly consistent across the basin and the only potential environmental impacts for the lagoon are represented by the port of Fertilia and by the tributaries. For the above-mentioned reasons, this biotope has great potential for the production of shellfish.

Further research (Chessa *et al.*, 2005) showed that the levels of primary production in the lagoon (*i.e.* concentration of chlorophyll *a*) are very high, thus leading to high growth rates of some Bivalve molluscs (*e.g. Ruditapes decussatus*) bred in these waters (more than 2 times higher than those reported in the literature for the same species).

The Calich lagoon has been managed by a cooperative of local fishermen from several decades. So far, however, the waters of the basin have not been classified to start farming/harvesting activities of Bivalve molluscs, despite their amazing potential to shellfish culture.

3.2.2.3 The Tortolì lagoon

The Tortolì lagoon (Fig. 3.7) is located in the Central-eastern Sardinia near the city of Arbatax, with a total surface of about 250 ha. It has two mouths (one between the Rio Mannu and the Baccasara Channel and the second at the opening of the Baccassara Channel) that provide its communication with the sea. Its depth ranges between 4 and 0.8 meters, it is characterised by salinity levels of 15-35 PSU, with mean values of 30 PSU (Cannas *et al.*, 1992), and appears as a typical mesotrophic lagoon.

The basin receives several freshwater inlets, thus enriching its waters in nutrients and consequently being particularly suitable for mollusc culture. In fact, several long-line systems for mussel breeding are present in the lagoon (Salati *et al.*, 1999). The facility includes also large structures for depuration of Bivalves.

The lagoon has been managed by the "Cooperativa Pescatori Tortolì" from about 60 years, engaged in fishing and harvesting activities within the basin and at sea. More than 100 fishermen are employed in it and, therefore, it represents an important source of income for the resident community. This cooperative, in fact, is one of the most

productive in Sardinia ensuring a considerable production of molluscs, fish and derived products (*e.g.* "bottarga", eggs of grey mullets). In addition, other additional tourism and recreational services are offered, such as a farmhouse restaurant (named "ittiturismo") for the consumption of local products.

Fig. 3.7. The Tortolì lagoon.

3.2.3 Analytical methods

3.2.3.1 Breeding, morphometric measures and hydrological variables

Small specimens of the Mediterranean mussel *Mytilus galloprovincialis* from the Gulf of Taranto (Apulia, Southern Italy) were seed in mesh sleeves of 80 cm in the Porto Pozzo lagoon long-line system. At the same time, molluscs from the same location were positioned in two other different Sardinian coastal habitats: the Calich (North-western Sardinia) and the Tortolì lagoons (Central-ester Sardinia). The trial was carried out from April to October 2010, a period of the year in which these Bivalves can better express their growth performances.

From the start of the study, 60 specimens of cultured mussels were monthly collected from each experimental group and transported to the laboratory. Here, linear dimensions of the shell, as main length of the anterior-posterior axis and height (with closed valves), were determined using a 0.1 mm precision calliper. Subsequently, wet total weight of the mussels was registered by a precision balance and, after dissection of the edible tissue from the shell and their draining on paper towel for 5 minutes, wet shell weight and wet meat weight were measured. The condition index (CI) was calculated as follows:

$$CI = (\text{wet mean weight} / \text{wet total weight}) \times 100$$

During the same period, but fortnightly, the main hydrological variables (*i.e.* temperature, salinity, pH, and dissolved oxygen) were monitored in all the three sites studied (in 13, 24 and 14 stations in the Porto Pozzo, Calich and Tortolì lagoons, respectively) by a multiparametric probe (Ocean Seven 316Plus CTD; Fig. 3.8).

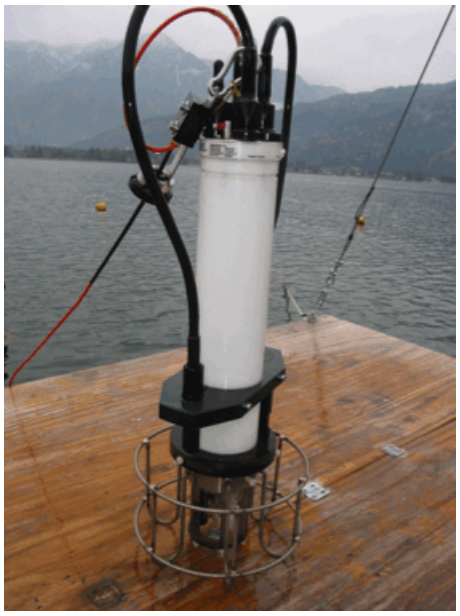

Fig. 3.8. Multiparametric probe Ocean Seven 316Plus CTD.

Contemporary, samples of water from each lagoon was collected monthly in polyethylene bottles and transported to the laboratory under controlled temperature (about 5°C) where chlorophyll *a* and seston concentrations were determined. Chlorophyll *a* was analysed vacuum-filtering a variable amount of seawater with a glass microfiber filter (GF/F, pore size 0.70 μm, Whatman International Ltd., Maidstone, England) and treating it according with APAT methodology (2004). For seston analysis, instead, the glass microfiber filters (the same type of the previous) were dried in a 105°C calibrate oven for 3 h, allowed to cool in a glass dryer equipped with silica gel and weighed. After vacuum-filtering, the filter was again dried (105°C for 3 h), cooled and weighed to obtain the amount of matter present in the known volume of the water filtered (calculated as the difference between the dry weight of the filter containing the material from the water sample and the dry weight of the clean filter).

3.2.3.2 Samples preparation and proximate composition

At the end of the trial (October 2010), 60 *Mytilus galloprovincialis* specimens from

each of the three experimental groups (hereafter PP=Porto Pozzo, C=Calich, T=Tortolì) were collected and transported to the laboratory. Then, the shells were opened using a scalpel, the intervalvar water drained and the edible parts placed on absorbent paper for 5 minutes to remove the excess of water. For each experimental group, 12 pools of 5 individuals each were randomly chosen and homogenised with an Ultra-Turrax apparatus at 10,000 rpm for 1 minute following the method reported by Giese (1966) and Giese *et al.* (1967) that recommend the use of pooled tissues to analyse invertebrate meat composition. Thus, analyses were carried out on 12 samples from each coastal lagoon (*i.e.* 12 PP, 12 C and 12 T) and all the procedures were performed in triplicate (*i.e.* 36 PP, 36 C and 36 T).

3.2.3.2.1 Moisture and ash content

About 1 g of homogenate was weighed in pre-weighed porcelain crucibles using a precision balance and then transferred in a 105°C calibrate oven for 24 h. After cooling it in a glass dryer equipped with silica gel, it was weighed to calculate the dry weight (AOAC, 1990), subtracting the initial weight of fresh homogenate from the final one. The ash content, instead, were obtained putting the same sample in a 550°C muffle furnace for 5 h (AOAC, 1923; Mortensen & Wallin, 1989), weighing it after 1 h and calculating the difference between the weight of the dry sample and that of the ashes. All analyses were performed in triplicate (*i.e.* 3 replicates × 12 pools × 3 experimental groups).

3.2.3.2.2 Crude protein

Crude protein content was determined using the Kjeldahl method (AOAC, 1992). About 0.5 g of homogenate sample were digested with 10 ml of 96% sulphuric acid (H_2SO_4) at 400°C and a catalyst in tablet (FOSS, DK) (composed by 5.5 g of K_2SO_4 and 0.5 g of $CuSO_4$ x $5H_2O$). Distillation and titration with HCl were performed using an analyser unit Kheltec 2300 (FOSS, DK) and the crude protein content calculated as:

$$\text{Crude protein (\%)} = (0.875 \times \text{ml HCl 0.1 N}) / \text{fresh sample weight}$$

All determinations were carried out in triplicate (*i.e.* 3 replicates × 12 pools × 3 experimental groups).

3.2.3.2.3 Fatty acids analysis

3.2.3.2.3.1 Total lipid

A modification of the method reported by Folch *et al.* (1957) was employed. About 5 g of homogenate were added to 20 ml of 2:1 dichloromethane/methanol (v/v) with 0.1% of butylated hydroxytoluene (BHT) solution. Consequently to a passage in an Ultrasonic Bath (Branson 1510) for 5 minutes, sample was vortexed (1 min), centrifuged at 2,000 rpm at 4°C (10 min) (Centrifuge ALC mod. 4227R), and vacuum-filtered in a Whatman filter n. 541 (Whatman International Ltd., Maidstone, England). Then, after the addition of 5 ml of 0.73% NaCl, it was centrifuged again at 2,000 rpm at 4°C (10 min) and led it stand for 30 minutes. With a water aspirator, the methanol/water layer was removed and the remaining solution (*i.e.* dichloromethane extract layer) transferred in a Pyrex glass flask previously weighed and evaporated with a "Rotavapor Buchi 461" water bath for 15 minutes. Pirex flasks were left overnight in a vacuum desiccator and subsequently weighed to estimate the total amount of fat present in the sample to evaluate the quantity of hexane (equal to 1 ml/25 mg of fat) to add for the following methylation.

3.2.3.2.3.2 FAMEs

The method used is a base-catalyzed methylation modified procedure (FIL-IDF, 1999). From each sample (*i.e.* 12 PP, 12 C, and 12 T), 1 ml of lipid extract was transferred in a vial immediately subject to a nitrogen flow for 15 minutes. Then, 0.5 ml solution of sodium methoxide 0.5 M was added and vortexed for 2 minutes. A further 1 ml of hexane was put in, vortexed again for 1 minute, stopped for 15 minutes and 0.5 ml of the hexane phase (supernatant) were taken and transferred in a new vial to analyse fatty acid methyl esters (FAMEs) by a gas chromatograph (GC) Varian Star 3400 CX with Varian 8200 autosampler (Varian, Walnut Creek, Ca).

The gas chromatograph was equipped with a Flame Ionization Detector (FID) with capillary column WCOT (Wall Coated Open Tubular) Fused Silica 100 m × 0.25 mm i.d., 0.25 μm film thickness, in stationary phase CP-Select CB for Fame (Varian, Walnut Creek, Ca). Values of injector and FID temperature were 255°C and 285°C, respectively. The temperature program used was: 75°C for 1 minute, up to 165°C increasing of 8°C min-1, held for 35 minutes, up to 210°C increasing of 5.5°C min-1, held for 1 minute, up to 230°C increasing of 3°C min-1, held for 11 minute. The split ratio was 1:100 and high purity helium was the carrier gas with 37 psi of pressure and a

linear flow rate of 1 ml min^{-1}. Flows of air and hydrogen were calibrated at 450 ml min^{-1} and 45 ml min^{-1}, respectively. Fatty acids from each seasonal sample were identify comparing retention times of their peaks with those of methyl ester standards (PUFA-1 and PUFA-3, Matreya Inc., Pleasant Gap, PA, USA).

Each fatty acid quantity was expressed as the percentage of total FAME present in the sample.

3.2.3.2.3.3 Indexes of lipid health

The atherogenic index (AI) and the thrombogenic index (TI) are indexes that allow the evaluation of the lipids quality (Amerio *et al.*, 1996). They were calculated using the following formulas (Ulbricht & Southgate, 1991):

$$AI = (C12 + 4*C14 + C16) / (\Sigma MUFA + \Sigma PUFA\omega6 + \Sigma PUFA\omega3)$$

$$TI = (C14 + C16 + C18) / [0.5\ (\Sigma MUFA) + 0.5\ (\Sigma PUFA\omega6) + 3\ (\Sigma PUFA\omega3) + (\omega3/\omega6)]$$

The AI is an indicator of risk for cardiovascular diseases while the TI is an indicator of the potential for blood platelets aggregation. They are more positive as their values are more close to zero.

3.2.4 Statistical analyses

3.2.4.1 Univariate analysis

One-way Analysis of Variance (ANOVA) was used to detect putative differences among the 3 mussel groups examined. In particular, morphometric variables (*i.e.* shell length, shell height, shell weight, mollusc weight, total weight and condition index), proximate composition (*i.e.* moisture, ash, protein and lipid) and fatty acids profile were considered. Cochran's test was used to check the assumption of the homogeneity of variance and, whenever necessary, data were transformed to log(x+1). Where data transformation did not correct violations in the assumption of homogeneous variances, an alpha-level adjustment to 0.01 was used to compensate for increased type errors (Underwood, 1997). Finally, the Student-Newman-Keuls' test (SNK; $p<0.05$) was used

for *post-hoc* comparisons to formulate alternative hypotheses. ANOVAs were always performed using the STATISTICA® software package.

3.2.4.2 Multivariate analysis

As far as proximate composition and fatty acids profile are concerned, data were treated using Principal Component Analysis (PCA), a classical method for a linear transformation of the original variables. Starting from the correlation matrix R, this method gives an approximate representation of the original data matrix onto a lower dimensional space to allow visual inspection of similarities. Details on the calculation procedures can be found in Jackson (1991). The number of principal components to retain was chosen according to the total variation accounted for (Mardia *et al.*, 1993).

In general, PCA is a multivariate statistical technique used to form a smaller number of uncorrelated variables from a large set of data. The main goal of PCA is to explain the maximum amount of variance with the fewest number of principal components. Principal Component Analysis is commonly utilized as one step in a series of analyses. This type of computational method is used to reduce the number of variables and avoid multicollinearity, or when there are too many predictors relative to the number of observations.

3.3 Results

3.3.1 Hydrological and mesological variables

During the study period, temperature values (Fig. 3.9) were similar in the three investigated lagoons (whereafter C=Calich, PP=Porto Pozzo, and T=Tortolì), showing minimum levels (*i.e.* 15.4, 17.8 and 17.6°C in PP, C and T, respectively) at the beginning of the trial (April) and maximum values in July (*i.e.* 28.4 in PP, and 27.7 and 28.6°C in C and T, respectively). Later, there was a gradual drop in all the basins investigated with values of 19.0, 20.1 and 20.8°C in PP, C and T, respectively, from August until the end of the trial.

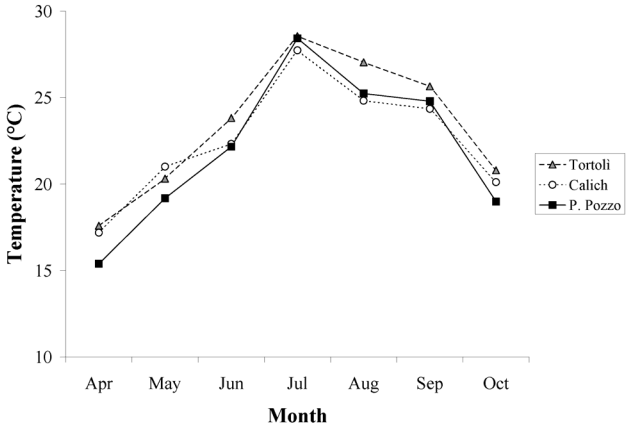

Fig. 3.9. Temperature values in the 3 sites investigated.

By contrast, salinity (Fig. 3.10) revealed different values due to the intrinsic diversity of the three coastal areas. PP waters showed the higher salinity, with a minimum of 36.8 PSU in April and a maximum of 39.5 PSU in August. The T lagoon was characterised by intermediate levels of salinity, ranging between 34.3 and 37.8 PSU (in April and September, respectively). Lastly, water of the C lagoon was the less salty, oscillating between 17.2 and 24.8 PSU in April and September, respectively.

The values of pH recorded in PP and T were quite similar (Fig. 3.11), both registering the highest value of 8.60 in June. Minimal values of 8.01 and 8.03, instead, were recorded in PP in September and in T in August. In the C lagoon values were quite different from the formers ones: the data recorded were generally higher and the maximum pH value was registered in May (8.83).

The concentration of dissolved oxygen (Fig. 3.12) in water samples from PP and T lagoons was characterized by an almost equal pattern, with highest values at the

beginning of the study (8.69 and 7.97 mg l^{-1} in PP and T, respectively). Afterwards, a slight decrease occurred in both these sites, then attending a new increase with the onset of autumn. In the C lagoon, instead, the amount of oxygen was always higher, with 12.24 mg l^{-1} in April, increasing up to 14.24 mg l^{-1} in May and then rapidly decreasing to 6.98 mg l^{-1} in October.

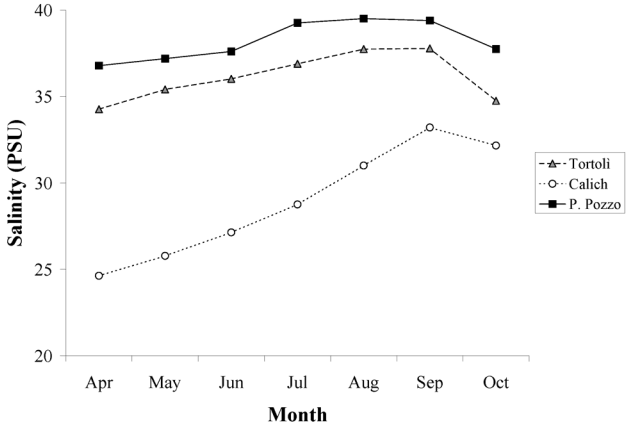

Fig. 3.10. Salinity values in the 3 sited investigated.

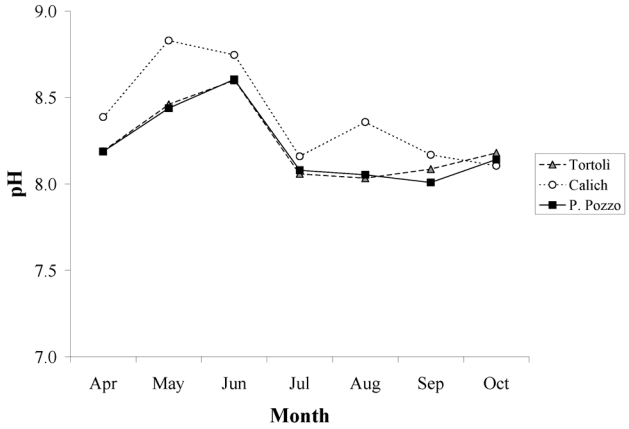

Fig. 3.11. pH in the 3 sites investigated.

Chlorophyll *a* concentrations are shown in Fig. 3.13. After an initial period characterised by quite low values, there was a sharp rise particularly in C. In fact, the concentration in this area was always the highest, reaching a maximum value of 22,1 mg l^{-1} in May, followed by a decrease during the subsequent month (5.9 mg l^{-1}), a new increment in summer (up to 20.6 mg l^{-1} in August) and a gradual reduction in autumn. In the PP lagoon chlorophyll *a* reached the lowest values, with a maximum value in August (1.0 mg l^{-1}) and a minimum in October (0.4 mg l^{-1}).

In the T lagoon, finally, chlorophyll *a* concentrations were intermediate between the two former sites, with a peack of 3.4 mg l^{-1} in May and the lowest value of 1.5 mg l^{-1} in October.

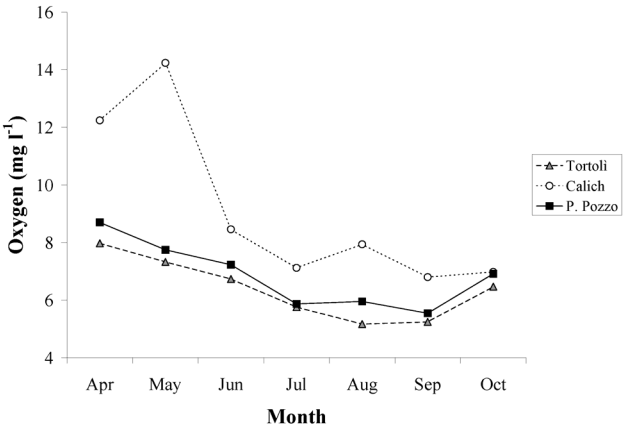

Fig. 3.12. Dissolved oxygen values in the 3 sites investigated.

As regards the seston concentration (Fig. 3.14), it revealed the highest value in the C lagoon (96 mg l^{-1}) in July and then decreased slightly until October (64.7 mg l^{-1}). In the first two months of the trial, these values were lower than those registered in the other two basins. During this period, the maximum concentration was registered in the PP lagoon (31.8 mg l^{-1}), but its peak was detected in July (36.0 mg l^{-1}). By contrast, the seston values in the T lagoon had a constant increasing trend, with concentration of 35.1 mg l^{-1} in May up to 69.8 mg l^{-1} in September, followed by a decrease in October (45.0 mg l^{-1}).

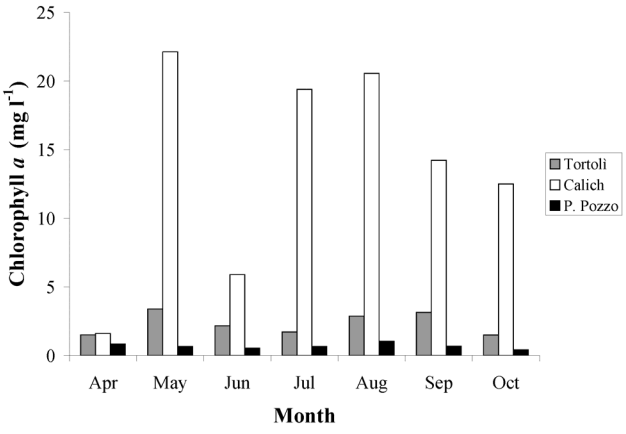

Fig. 3.13. Chlorophyll *a* concentration in the 3 sites investigated.

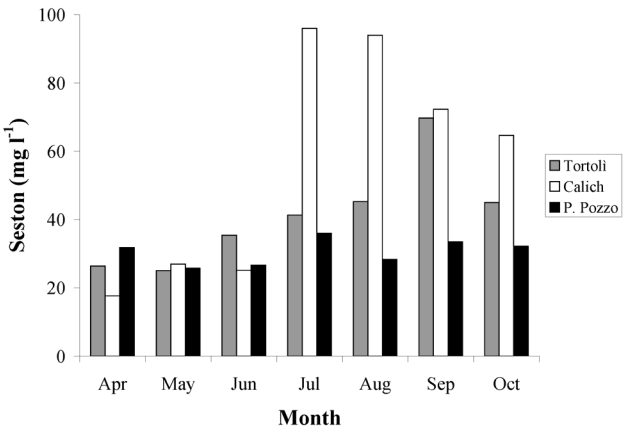

Fig. 3.14. Seston concentration in the 3 sites investigated.

3.3.2 Univariate analysis

3.3.2.1 Morphometric measurements and Condition Index

At the beginning of the trial (April 2010), before their immersion in the three investigated lagoons, 60 specimens of *Mytilus galloprovincialis* were subjected to a series of morphometric measurements, revealing the following initial mean values (±SD): shell length=42.5±3.1 mm, shell height=15.1±1.3 mm, total weight=4.3±1.0 g, mollusc weight=2.3±0.6 g, and shell weight=2.0±0.4 g. Monthly, all these variables were measured in 60 specimens from each study site. Looking at the histogram in Fig. 3.15, it is possible to note that the mean shell length of mussels from C lagoon were always higher than those from the other sites. In particular, this variable revealed an increasing trend from May to October (from 50.1±3.8 mm up to 66.2±4.7 mm). On the other hand, the mean shell length of mussels from the PP and T lagoons revealed a more or less similar trend (from 49.1±3.1 to 63.5±3.2 mm in PP samples; from 48.7±3.9 to 61.6±2.7 mm in T), with a progressive increase during the whole study period. Despite this, mussels from T lagoon showed a higher increase until August while, in September and October, they registered a slight slowdown with lower values (60.3±3.4 and 61.6±2.7 mm, respectively) than the specimens from PP (61.6±3.2 and 63.5±3.2 mm, respectively).

In terms of shell height (Fig. 3.15), molluscs from the C lagoon showed a similar pattern, increasing from 18.3±1.4 (May) to 25.6±2.3 mm (October), being always the biggest. Samples from PP, in this case, showed higher mean values (18.1±1.5 mm in May and 24.8±1.5 mm in October) than those from T (17.4±1.6 mm in May and 22.8±1.2 mm in October), except for the month of July, when both values were more or

less similar (21.5±1.6 mm and 21.6±2.0 mm, for PP and T samples, respectively).

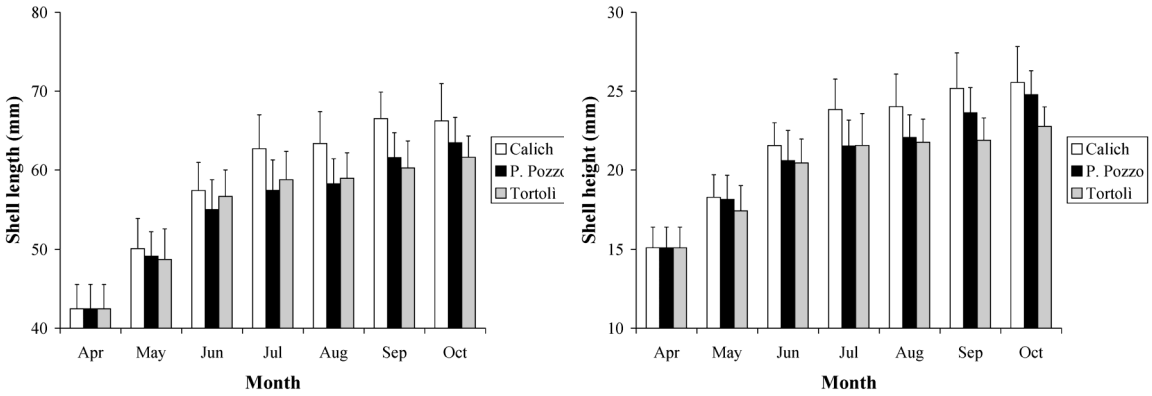

Fig. 3.15. Mean shell length and shell height of the mussels in the 3 sites investigated.

As regards the mean shell weight (Fig. 3.16) the PP samples revealed the highest values at the final sampling of the trial with 8.5±1.3 g. Actually, also in the first two months (*i.e.* May and June) it showed the highest values (3.3±0.6 g and 5.0±1.1 g, respectively) than those from the other two sites (2.8±0.6 and 4.4±0.7 g in T; 3.2±0.7 and 4.7±0.7 g in C). General trends indicate a constant growth for mussels reared in the PP lagoon, but a first increment followed by a steady state for both the other two mussels groups during the summer period.

By considering the histogram in Fig. 3.16, mean mollusc weight (*i.e.* its edible parts) showed a progress similar to the previous variable, with a constant growth until July (12.0±2.7 g) and a subsequent decrease in August (10.0±2.3 g), followed by a slower recovery in the two following months (12.4±3.4 and 13.1±3.8 g, in September and October, respectively) for specimens cultured in the C waters. Also the data recorded in PP and T lagoons showed a steady increase (even if modest than the former), with the first ever bigger that the second in the early months of the study (*i.e.* May, June and July). However, from August to the end of the trial, samples from PP revealed a clear acceleration compared to those from T, going from a mollusc weight of 5.7±1.0 in August to 9.0±1.2 g in October the first and from 5.7±1.3 in August to 6.5±1.3 g in October the last.

Looking at Fig. 3.17, mussels from the C lagoon reached the maximum values of mean total weight in all the months considered (from 8.4±2.1 g in May to 21.3±5.1 g in October), with a constant increase except for the summer period (17.0±3.5 g in August). Total weights of the mussels from PP and T were more or less similar in May (6.5±1.2

and 6.3±1.5 g, respectively) but, despite the T samples slightly showed higher values in June and July (10.2±1.8 and 12.2±2.3 g in T, respectively; 10.1±2.2 and 12.0±1.9 g in PP, respectively), molluscs from PP revealed a stronger and rapid growth in September and October (15.1±2.0 and 17.4±2.2 g in PP, respectively; 11.2±1.6 and 13.0±1.9 g in T, respectively).

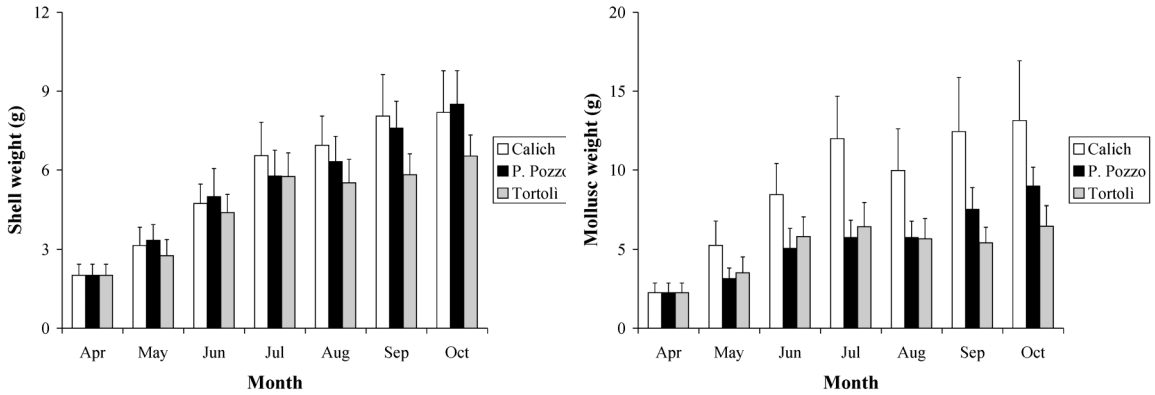

Fig. 3.16. Mean shell weight and mollusc weight of the mussels in the 3 sites investigated.

Condition index values are reported in Fig. 3.17. During the whole study period, *Mytilus galloprovincialis* specimens from the C lagoon showed mean values higher than those from the two other groups.

Also at the end of the trial, they were characterised by higher condition index, registering a mean value of 60.9±5.3%, while mussels cultured in PP and T lagoons reached percentages of 51.4±3.9 and 49.4±4.4, respectively.

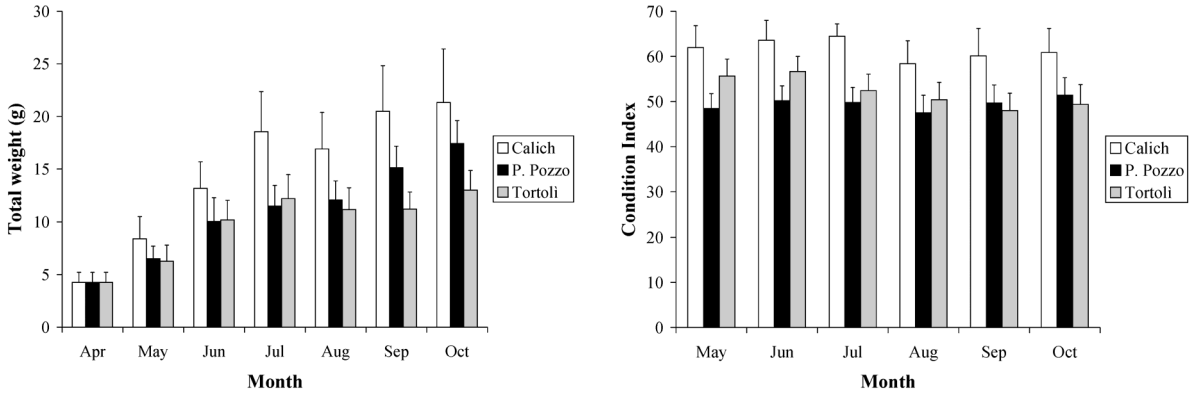

Fig. 3.17. Mean total weight and condition index of the mussels in the 3 sites investigated.

As far morphometric variables and condition index are concerned, detail of ANOVA results and Student-Newman-Keuls *post-hoc* comparison test fro the three mussel groups examined are reported in Tab. 3.2.

Tab. 3.2. ANOVA results for morphometric variables and condition index of the 3 groups of *Mytilus galloprovincialis* examined.

		Shell length			Shell height			Shell weight		
Source of variation	df	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	323.47	24.29	**0.0000**	124.30	40.83	**0.0000**	67.34	41.72	**0.0000**
Residuals	177	13.32			3.04			1.61		
Cochran's test			0.561	p<0.01		0.578	p<0.01		0.526	p<0.01
Transformation				none			none			none
SNK test				T<PP**<C**			T<PP**<C**			T<C**=PP

		Mollusc weight			Total weight			Condition Index		
Source of variation	df	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	682.74	117.69	**0.0000**	1044.64	91.45	**0.0000**	2256.07	108.44	**0.0000**
Residuals	177	5.80			11.42			20.81		
Cochran's test			0.822	p<0.01		0.766	p<0.01		0.455	ns
Transformation				none			none			
SNK test				T<PP**<C**			T<PP**<C**			T<PP*<C**

C = Calich; PP = Porto Pozzo; T = Tortolì; significant differences are marked in bold, *: p<0.05; **: p<0.01

3.3.2.2 Proximate composition

At the end of the trial, the *Mytilus galloprovincialis* specimens from the three sites investigated significantly differed for all the variables considered. Water content (Fig. 3.18) of the mussels cultured in the PP lagoon (85.19±0.35%) was significantly higher than both those from T (82.78±0.95) and C (80.15±1.22).

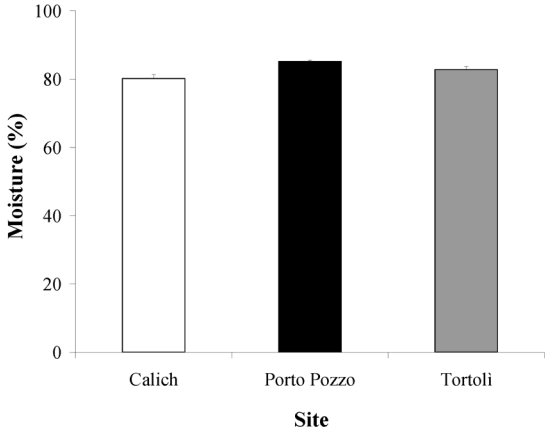

Fig. 3.18. Moisture percentage (±SD) of *Mytilus galloprovincialis* in the 3 sited investigated.

Similar differences were also found for mean ash percentage (Fig. 3.19). Mussels grown in the PP lagoon, in fact, revealed a higher value (2.54±0.06%) than those from the other basins (2.46±0.13% and 2.10±0.09%, in T and C, respectively).

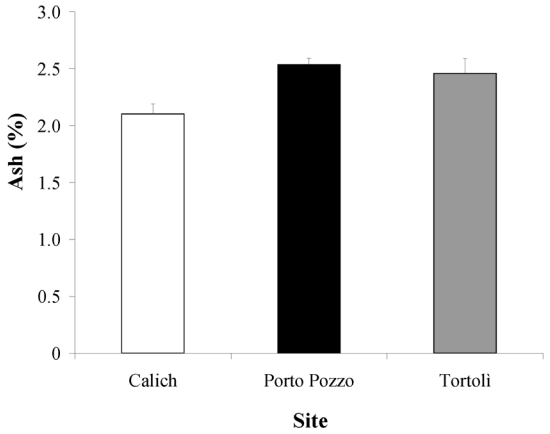

Fig. 3.19. Ash percentage (±SD) of *Mytilus galloprovincialis* in the 3 sites investigated.

By considering crude protein content (Fig. 3.20), molluscs cultured in the C lagoon were characterised by a higher percentage (12.54±0.61%) than those raised in PP (9.67±0.45) and T (9.33±0.61%).

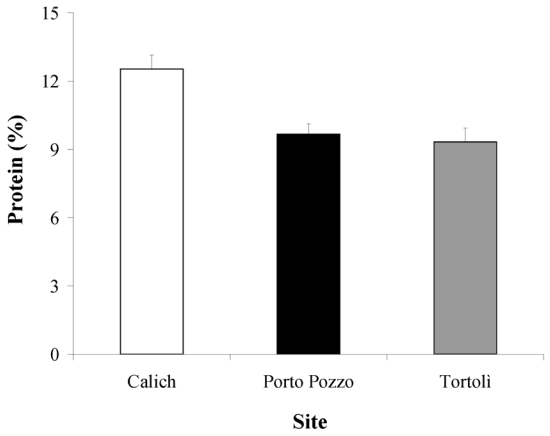

Fig. 3.20. Protein percentage (±SD) of *Mytilus galloprovincialis* in the 3 sites investigated.

On the other hand, lipid content (Fig. 3.21) exhibited an opposite pattern if compared to the previous variables. The specimens reared in the T and C lagoons had similar high percentages (2.10±±0.15% and 2.05±0.18%, respectively) whereas mussels grown in the PP lagoon revealed the lowest value of 1.37±0.09%.

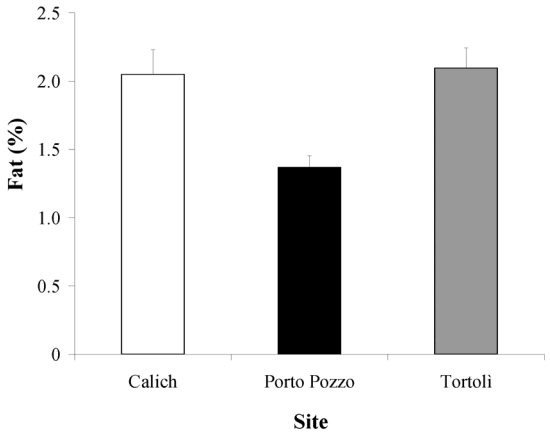

Fig. 3.21. Fat percentage (±SD) of *Mytilus galloprovincialis* in the 3 sites investigated.

Finally, as far as proximate composition is concerned, details of ANOVA results and Student-Newman-Keuls *post-hoc* comparison test are illustrated in Tab. 3.3.

Tab. 3.3. ANOVA results for proximate composition of the 3 group of *Mytilus galloprovincialis* examined.

	Moisture				Ash				Protein				Lipid			
Source of variation	df	MS	F	*p*	df	MS	F	*p*	df	MS	F	*p*	df	MS	F	*p*
Group	2	228.89	274.15	**0.0000**	2	1.92	204.56	**0.0000**	2	111.56	355.12	**0.0000**	2	2.00	96.62	**0.0000**
Residuals	105	0.83			105	0.01			105	0.31			33	0.02		
Cochran's test			0.594	p<0.01			0.610	p<0.01			0.395	ns			0.528	ns
Transformation				none				none				none				none
SNK test				C<T**<PP**				C<T**<PP**				T<PP**<C**				PP<C**=T

C = Calich; PP = Porto Pozzo; T = Tortolì; significant differences are marked in bold; *: p<0.05; **: p<0.01

3.3.2.3 Fatty acids

The fatty acids profile of mussels cultured in the lagoons of Calich, Porto Pozzo and Tortolì during the trial is illustrated in Tab. 3.4. The three groups examined showed high mean levels of saturated fatty acids (SFA), and especially the samples from the T lagoon showed significantly higher values (31.25±1.12%) than those from C (29.31±1.28%) and PP (28.55±2.17%). Among this compounds, 16:0 (18.83±1.56% in C, 16.91±0.87% in T, and 15.02±1.78% in PP) and 18:0 (4.33±0.63% in PP, 4.21±0.65% in C, and 3.68±0.25% in T) were predominant, although *Mytilus galloprovincialis* specimens cultured in the T lagoon also had a high level of the 14:0 (7.36±0.51%).

Even though to a lesser extent, mean percentages of monounsaturated acids (MUFA) were fairly good, showing higher values in the mussels from T (29.54±1.00%) than those from the others basins (19.72±2.23% and 18.51±1.96% in C and PP, respectively). In this fraction, 16:1 n7 predominated, principally in molluscs reared in the T lagoon (18.98±1.19%) and then in samples from C and PP (7.06±2.67% and 5.23±1.43, respectively). In general, among MUFAs the n-7 class was the predominant.

As far as polyunsaturated fatty acids (PUFA) is concerned, they absolutely represented the most abundant class, revealing significantly different values of 42.62±3.05%, 39.76±2.56% and 33.10±1.69% in mussels grown in the lagoons of C, PP and T respectively. Among these, the mean levels of total n-3 PUFA were the highest and were characterized by significant differences in all the sample analysed: 37.36±2.87%, 32.33±2.10% and 25.68±1.44% in *M. galloprovincialis* specimens cultured in C, PP and T, respectively.

On the other hand, the mean percentages of n-6 PUFA were fair lower than those of n-3, with a maximum level (7.04±0.70%) in mussels grown in the PP lagoon and with significantly lower values in those from T (6.00±0.69%) and C (4.26±0.24%).

As a consequence, the highest n3/n6 ratio was detected in the molluscs cultured in the C lagoon (8.78±0.55%), while significantly lower values were detected in the samples from the PP (4.62±0.37%) and T (4.34±0.67%) lagoons.

Among the n-3 series, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids were the most abundant: the former registering its maximum value of 15.28±1.33% in the mussels from the C lagoon (13.21±0.76% in T and 10.96±1.04% in PP samples, respectively) and the latter amounting to 15.58±1.17% in the molluscs reared in the PP lagoon (15.28±1.44% and 7.96±0.78% in C and T, respectively).

The ratio EPA/DHA was higher in the mussel group reared in T (1.67±0.11%) in comparison with those from C (1.01±0.10%) and PP (0.70±0.05%). The sum of these two important fatty acids (EPA + DHA) exhibited the higher level in the C group (30.57±2.30%), followed by the PP (26.53±2.05%) and T (21.18±1.45%) ones, with significant differences among them.

Finally, the low values of the atherogenic index (AI) (0.41±0.05%, 0.45±0.03% and 0.76±0.05% in PP, C and T, respectively) and of the thrombogenic index (TI) (0.19±0.02%, 0.19±0.02% and 0.28±0.02% in C, PP and T, respectively) observed confirm a very high polyunsaturated fatty acids (PUFA) content in all the three *M. galloprovincialis* groups examined.

As regards all the above-mentioned fatty acids (comprised sums, ratios and health indexes), ANOVA showed significant differences among the three different mussel groups except for the 18:4 n3 and 20:4 n3 mean contents (Tab. 3.4).

Details of ANOVA results and Student-Newman-Keuls *post-hoc* comparison test are illustrated in Tab. 3.5.

Tab. 3.4. Fatty acids profile of the 3 groups of *Mytilus galloprovincialis* examined.

	Group		
	Calich	Porto Pozzo	Tortolì
14:0	2.12±0.37^{B}	2.16±0.29^{B}	7.36±0.51^{A}
15:0	1.18±0.66^{B}	3.59±1.07^{A}	1.22±0.42^{B}
16:0	18.83±1.56^{A}	15.02±1.78^{C}	16.91±0.87^{B}
16:1 n9	0.64±0.19^{B}	1.52±0.48^{A}	0.61±0.10^{B}
16:1 n7	7.06±2.67^{Ba}	5.23±1.43^{Bb}	18.98±1.19^{A}
17:0	0.66±0.11^{B}	0.85±0.14^{A}	0.29±0.07^{C}
16:2 n4	0.19±0.07^{B}	0.13±0.03^{B}	0.49±0.06^{A}
18:0	4.21±0.65^{a}	4.33±0.63^{a}	3.68±0.25^{b}
18:1 n9	2.11±0.58^{B}	2.66±0.44^{A}	1.98±0.26^{B}
18:1 n7	2.87±0.34^{A}	1.85±0.19^{B}	3.05±0.96^{A}
18:2 n6	1.40±0.16^{b}	1.94±0.27^{a}	1.80±0.58^{a}
18:3 n6	0.18±0.06^{Ba}	0.11±0.05^{Bb}	0.46±0.04^{A}
18:3 n3	2.44±0.41^{A}	1.75±0.85^{B}	1.07±0.06^{C}
18:4 n3	2.05±0.26	2.03±0.32	1.89±0.20
20:1 n11	2.21±0.23^{B}	2.88±0.53^{A}	1.28±0.17^{C}
20:1 n9	2.23±0.22^{B}	2.82±0.18^{A}	1.24±0.16^{C}
20:1 n7	1.74±0.21^{A}	0.93±0.11^{C}	1.22±0.15^{B}
20:2 n6	0.47±0.05^{B}	0.65±0.06^{A}	0.47±0.04^{B}
20:3 n6	0.16±0.04^{B}	0.14±0.04^{B}	0.30±0.02^{A}
20:4 n6	1.87±0.16^{C}	3.66±0.44^{A}	2.73±0.21^{B}
20:3 n3	0.13±0.02^{B}	0.15±0.03^{A}	0.09±0.01^{C}
20:4 n3	0.34±0.04	0.30±0.04	0.32±0.05
22:1 n11	0.19±0.06^{a}	0.12±0.03^{b}	0.17±0.07^{a}
20:5 n3 EPA	15.28±1.33^{A}	10.96±1.04^{C}	13.21±0.76^{B}
21:5 n3	0.50±0.07^{A}	0.36±0.05^{Bb}	0.42±0.02^{Ba}
22:4 n6	0.18±0.02^{Bb}	0.55±0.11^{A}	0.24±0.03^{Ba}
24:0	0.79±0.10^{B}	1.27±0.15^{A}	0.87±0.10^{B}
22:5 n3	1.33±0.11^{Aa}	1.21±0.16^{Ab}	0.72±0.06^{B}
22:6 n3 DHA	15.28±1.44^{A}	15.58±1.17^{A}	7.96±0.78^{B}
SFA	29.31±1.28^{B}	28.55±2.17^{B}	31.25±1.12^{A}
MUFA	19.72±2.23^{B}	18.51±1.96^{B}	29.54±1.00^{A}

Tab. 3.4. Continued.

	Group		
	Calich	Porto Pozzo	Tortolì
PUFA	42.62±3.05^{A}	39.76±2.56^{B}	33.10±1.69^{C}
Unidentified	8.35±0.83^{B}	13.18±2.04^{A}	6.11±0.48^{C}
∑ n3	37.36±2.87^{A}	32.33±2.10^{B}	25.68±1.44^{C}
∑ n6	4.26±0.24^{C}	7.04±0.70^{A}	6.00±0.69^{B}
n3/n6	8.78±0.55^{A}	4.62±0.37^{B}	4.34±0.67^{B}
EPA/DHA	1.01±0.10^{B}	0.70±0.05^{C}	1.67±0.11^{A}
EPA+DHA	30.57±2.30^{A}	26.53±2.05^{B}	21.18±1.45^{C}
AI	0.45±0.03^{B}	0.41±0.05^{B}	0.76±0.05^{A}
TI	0.19±0.02^{B}	0.19±0.02^{B}	0.28±0.02^{A}

The fatty acids present in small percentage (14:1 c9; *iso* 15:0; *anteiso* 15:0; *iso* 16:0; 16:1 n7,*t*; 16:1 n6; *iso* 17:0; *anteiso* 17:0; 17:1 n8; 16:3 n4; *iso* 18:0; 18:1 n9,*t*; 18:2 n4; 18:3 n9; 18:3 n4 and 22:0) were considered in the composite fractions but were not reported in the table.
Values are mean ±standard deviation. Values in each roe with different superscript letters are significantly different (A, B, C = $p<0.01$; a, b, c = $p<0.05$).
Abbreviation: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AI, Atherogenic Index; TI, Thrombogenic Index.

Tab. 3.5. ANOVA results for fatty acids of the 3 *Mytilus galloprovincialis* groups examined.

		14:0			15:0			16:0			16:1 n9		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	108.98	681.64	**0.0000**	2.18	39.65	**0.0000**	43.51	20.60	**0.0000**	3.18	34.74	**0.0000**
Residuals	33	0.16			0.05			2.11			0.09		
Cochran's test			0.535	ns		0.495	ns		0.497	ns		0.837	p<0.01
Transformation				none			ln(x+1)			none			none
SNK test				C=PP<T**			C=T<PP**			PP<T**<C**			T=C<PP**

		16:1 n7			17:0			16:2 n4			18:0		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	669.19	189.63	**0.0000**	0.94	79.97	**0.0000**	0.45	141.24	**0.0000**	1.44	4.91	**0.0136**
Residuals	33	3.53			0.01			0.00			0.29		
Cochran's test			0.674	p<0.01		0.537	ns		0.554	ns		0.486	ns
Transformation				none			none			none			none
SNK test				PP<C*<T**			T<C**<PP**			PP<C*<T**			T<C*=PP

		18:1 n9			18:1 n7			18:2 n6			18:3 n6		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	1.62	8.10	**0.0014**	4.97	13.74	**0.0000**	0.94	6.40	**0.0045**	0.43	168.17	**0.0000**
Residuals	33	0.20			0.36			0.15			0.00		
Cochran's test			0.565	ns		0.858	p<0.01		0.779	p<0.01		0.458	ns
Transformation				none			none			none			none
SNK test				T=C<PP**			PP<**C=T			C<T*=PP			PP<C*<T**

C = Calich; PP = Porto Pozzo; T = Tortolì; *: p<0.05; **: p<0.01

Tab. 3.5. Continued.

		18:3 n3			18:4 n3			20:1 n11			20:1 n9		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	5.61	18.83	**0.0000**	0.09	1.32	0.2819	7.75	64,12	**0.0000**	7.60	108.44	**0.0000**
Residuals	33	0.30			0.07			0.12			0.03		
Cochran's test			0.806	p<0.01		0.499	ns		0.770	p<0.01		0.465	ns
Transformation				none			none			none			none
SNK test				T<PP**<C**						PP<T*=C			T<C**<PP**

		20:1 n7			20:2 n6			20:3 n6			20:4 n6		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	1.99	75.51	**0.0000**	0.12	43.94	**0.000**	0.09	87.22	**0.0000**	4.55	1963.50	**0.0000**
Residuals	33	0.03			0.00			0.00			0.00		
Cochran's test			0.567	ns		0.434	ns		0.443	ns		0.464	ns
Transformation				none			none			none			ln(x+1)
SNK test				PP<T**<C**			T=C<PP**			PP=C<T**			PP<C**<T**

		20:3 n3			20:4 n3			22:1 n11			20:5 n3 EPA		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	50.42	789.44	**0.0000**	0.00	2.24	0.1219	7.75	64.12	**0.0000**	56.26	49.15	**0.000**
Residuals	33	0.06			0.00			0.12			1.14		
Cochran's test			0.997	p<0.01		0.385	ns		0.464	p<0.01		0.517	ns
Transformation				none			none			none			none
SNK test				T=C<PP**						T<C**<PP**			PP<T**<C**

C = Calich; PP = Porto Pozzo; T = Tortolì; *: p<0.05; **: p<0.01

Tab. 3.5. Continued.

		21:5 n3			22:4 n6			24:0			22:5 n3		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	0.07	27.31	**0.0000**	0.46	99.69	**0.000**	0.82	55.03	**0.000**	1.26	95.48	**0.0000**
Residuals	33	0.00			0.00			0.01			0.01		
Cochran's test			0.663	p<0.01		0.884	p<0.01		0.551	ns		0.663	p<0.05
Transformation				none			none			none			none
SNK test				PP<T*<C**			PP<C**<T**			C=T<PP**			T<PP**<C**

		22:6 n3 DHA			SFA			MUFA			PUFA		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	223.07	164.78	**0.0000**	23.29	9.20	**0.0007**	439.18	134.16	**0.0000**	286.10	45.80	**0.0000**
Residuals	33	1.35			2.53			3.27			6.25		
Cochran's test			0.512	ns		0.621	p<0.05		0.507	ns		0.497	ns
Transformation				none			none			none			none
SNK test				T<C**=PP			PP=C<T**			PP=C<T*			T<PP**<C**

		Unidentified			∑ n3			∑ n6			n3/n6		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	156.68	92.19	**0.0000**	411.54	83.51	**0.0000**	23.68	70.14	**0.0000**	74.08	251.55	**0.0000**
Residuals	33	1.70			4.93			0.34			0.29		
Cochran's test			0.818	p<0.01		0.560	ns		0.479	ns		0.510	ns
Transformation				none			none			none			none
SNK test				T<C**<PP**			T<PP**<C**			C<T**<PP**			T=PP<C**

C = Calich; PP = Porto Pozzo; T = Tortolì; *: p<0.05; **: p<0.01

Tab. 3.5. Continued.

		EPA/DHA			EPA + DHA			AI			TI		
Source	df	MS	F	*p*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Group	2	2.92	332.51	**0.0000**	266.17	68.64	**0.0000**	0.44	217.30	**0.0000**	0.03	69.31	**0.000**
Residuals	33	0.01			3.88			0.00			0.00		
Cochran's test			0.509	ns		0.456	ns		0.453	ns		0.409	ns
Transformation				none			none			none			none
SNK test				PP<C**<T**			T<PP**<C**			PP=C<T**			PP=C<T**

C = Calich pool; PP = Porto Pozzo pool; T = Tortolì pool; *: p<0.05; **: p<0.01

3.3.3 Multivariate analysis

Principal Component Analysis (PCA) was carried out for 2 different datasets (*i.e.* proximate composition and fatty acid profiles) in order to assess which variables make the greatest contribution to the differentiation of the 3 mussels groups examined. In detail, fatty acids were divided in 2 main categories: 1) saturated and monounsaturated, and 2) polyunsaturated.

3.3.3.1 Proximate composition

The PCA scatterplot illustrated in Fig. 3.22 shows a clear separation between the three mussel groups. The first component accounted for 75.69% of the total variation (as shown in Tab. 3.6), which also displays that the main source of difference among the 3 mussel groups was due to all the 4 variables for the first principal component: moisture and ash with a positive sign of association, and protein and lipid with a negative sign.

3.3.3.2 Fatty acids

3.3.3.2.1 Saturated and monounsaturated fatty acids

The PCA scatterplot illustrated in Fig. 3.23 shows that all the 3 mussel groups are well separated from each other. The first 2 components accounted for 70.64% of the total variation (as reported in Tab. 3.7), which also shows that for the first principal component all variables considered contributed to discrimination: 14:0, 16:0, 16:1 n7, 18:1 n7, 20:1 n7, and 22:1 n11 with a positive sign of association, and 15:0, 16:1 n9, 17:0, 18:0, 18:1 n9, 20:1 n11, 20:1 n9, and 24:0 with a negative one.

3.3.3.2.2 Polyunsaturated fatty acids

The scatterplot illustrated in Fig. 3.24 also shows a clear separation of *Mytilus galloprovincialis* groups from the three lagoons examined.

The first 2 components accounted for 71.80% of the total variation (as illustrated in Tab. 3.8), that also shows that for the first principal component the variables 16:2 n4, 18:3 n6, 20:3 n6, and 20:5 n3 EPA, all with a positive sign of association, had a major influence. For the same principal component, also 18:3 n3, 18:4 n3, 20:2 n6, 20:4 n6, 20:3 n3, 22:4 n6, 22:5 n3, and 22:6 n3 DHA contributed in discriminating the 3 mussel groups, but with a negative sign.

For the second principal component, instead, 18:2 n6 significantly contributed with a positive sign of association, and 20:4 n3 and 21:5 n3 with a negative one.

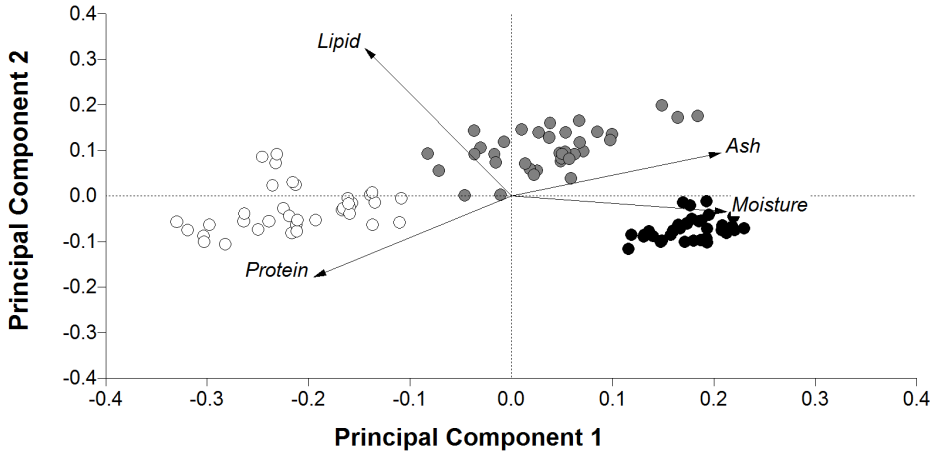

Fig. 3.22. Projection of linear principal component scores along the first 2 eigenvector axes, as a function of group provenience, considering proximate composition (white, grey, and black dots represent mussels from Calich, Tortolì and Porto Pozzo, respectively).

Tab. 3.6. Correlation coefficients between the first 4 Principal Components and the original variables, associated eigenvalues and cumulative percentage of explained variance, as regards the 3 mussel groups (significant values are in blod).

Variables	Components			
	I	II	III	IV
Moisture	**0.56**	-0.09	**0.36**	**0.75**
Ash	**0.54**	**0.25**	**-0.81**	0.01
Protein	**-0.51**	**-0.47**	**-0.47**	**0.55**
Lipid	**-0.38**	**0.85**	0.01	**0.38**
Eigenvalues	3.04	0.78	0.11	0.08
Cumulative %	**75.69**	95.36	98.02	100.00

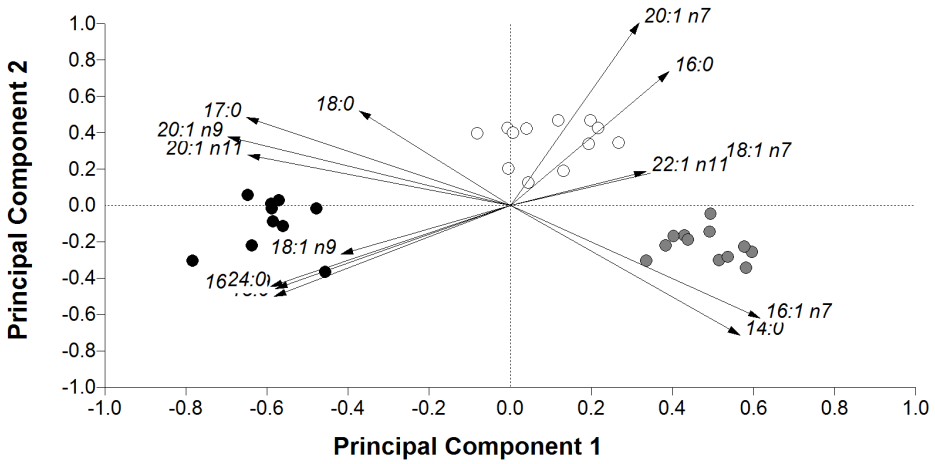

Fig. 3.23. Projection of linear principal component scores along the first 2 eigenvector axes, as a function of group provenience, considering saturated and monounsaturated fatty acids (white, grey, and black dots represent mussels from Calich, Tortolì and Porto Pozzo, respectively).

Tab. 3.7. Correlation coefficients between the first 4 Principal Components and the original variables, associated eigenvalues and cumulative percentage of explained variance, as regards the 3 mussel groups (significant values are in blod).

Variables	Components			
	I	II	III	IV
14:0	**0.28**	**-0.36**	**-0.17**	0.02
15:0	**-0.29**	**-0.25**	**0.23**	**-0.19**
16:0	**0.20**	**0.37**	**0.28**	**0.25**
16:1 n9	**-0.29**	**-0.23**	**0.22**	**-0.20**
16:1 n7	**0.31**	**-0.31**	-0.07	0.07
17:0	**-0.33**	**0.24**	-0.04	**-0.18**
18:0	**-0.19**	**0.26**	**-0.46**	**-0.18**
18:1 n9	**-0.21**	**-0.13**	**0.19**	**0.71**
18:1 n7	**0.26**	**0.13**	**-0.20**	**-0.20**
20:1 n11	**-0.32**	**0.14**	0.07	**0.20**
20:1 n9	**-0.35**	**0.19**	0.02	-0.00
20:1 n7	**0.16**	**0.50**	-0.02	0.08
22:1 n11	**0.17**	0.09	**0.67**	**-0.42**
24:0	**-0.30**	**-0.22**	**-0.19**	**-0.15**
Eigenvalues	7.18	2.71	1.04	0.84
Cumulative %	51.29	**70.64**	78.06	84.06

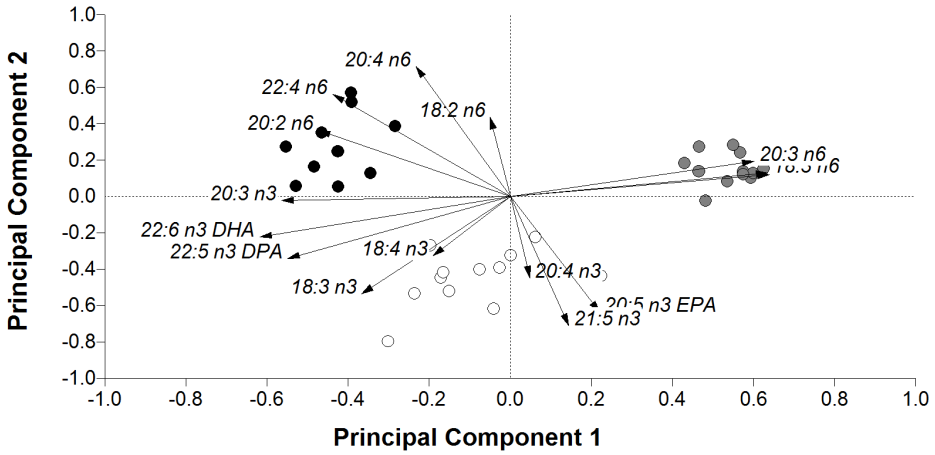

Fig. 3.24. Projection of linear principal component scores along the first 2 eigenvector axes, as a function of group provenience, considering polyunsaturated fatty acids (white, grey, and black dots represent mussels from Calich, Tortolì and Porto Pozzo, respectively).

Tab. 3.8. Correlation coefficients between the first 4 Principal Components and the original variables, associated eigenvalues and cumulative percentage of explained variance, as regards the 3 mussel groups (significant values are in blod).

Variables	Components			
	I	II	III	IV
16:2 n4	**0.38**	0.07	**0.15**	-0.02
18:2 n6	-0.03	**0.26**	**0.39**	**0.58**
18:3 n6	**0.37**	0.08	0.06	-0.09
18:3 n3	**-0.25**	**-0.32**	0.02	0.01
18:4 n3	**-0.11**	**-0.19**	**0.58**	**-0.45**
20:2 n6	**-0.28**	**0.21**	**0.30**	0.10
20:3 n6	**0.35**	**0.11**	**0.11**	**0.16**
20:4 n6	**-0.14**	**0.42**	0.07	**0.14**
20:3 n3	**-0.33**	-0.01	**0.17**	**-0.27**
20:4 n3	0.03	**-0.26**	**0.51**	**0.19**
20:5 n3 EPA	**0.13**	**-0.38**	-0.07	**0.40**
21:5 n3	0.09	**-0.42**	**0.16**	**0.19**
22:4 n6	**-0.26**	**0.33**	0.05	0.07
22:5 n3	**-0.32**	**-0.20**	**-0.21**	**0.23**
22:6 n3 DHA	**-0.36**	**-0.13**	**-0.10**	**0.20**
Eigenvalues	6.35	4.42	1.44	0.88
Cumulative %	42.35	**71.80**	81.42	87.31

3.4 Discussion and conclusions

The trophic status of the 3 lagoons studied and their different water exchanges with the sea imply that, from an ecological point of view, they are quite different from each other. In fact, the Calich lagoon is a typical eutrophic environment, the Porto Pozzo basin is an oligotrophic basin, while the lagoon of Tortolì represents a mesotrophic ecosystem. Such diversity was widely confirmed by the environmental variables recorded during the study period. In particular, salinity and chlorophyll *a* concentration were the most representative: the former, indeed, was always higher in the Porto Pozzo and lower in the Calich lagoon; the latter was maximum in the Calich basin and always lower in the Porto Pozzo one. By contrast, in the Tortolì lagoon the values recorded for these variables were always intermediate.

Differently than expected, the results obtained for morphometric variables and Condition Index of the mussels cultured were extremely positive in the Porto Pozzo lagoon. In fact, although the values registered for the molluscs grown in the Calich basin were always higher than the other (except for the shell weight that was higher in mussels reared in the Porto Pozzo lagoon), specimens from Porto Pozzo showed final values higher than those from Tortolì. In this regard, it is important to emphasize that, during the trial, water temperature was rather high during late summer, and significant phenomena of mortality occurred, especially in the Tortolì lagoon. It follows, therefore, that the occurrence of suffering conditions had certainly caused a decline in the performance of the Bivalves reared in this basin. Overall, the Condition Index values well reflect the diversity of the 3 experimental groups examined, because they basically result from the complex interaction of several factors including food, temperature and salinity (Okumuş & Stirling, 1998).

As far as proximate composition is concerned, the mussels cultured in the Porto Pozzo lagoon showed mean percentages of moisture and ash significantly higher than those from the other two basins. Conversely, their protein content was intermediate (while the higher value was detected in the samples from Tortolì) and the lipid one was the lowest. This fact further confirms the different environmental conditions characteristic of the 3 investigated ecosystems. The typical sessile lifestyle of mussels, in fact, involves that their biochemical composition strictly depends on the local food resources, represented by the phytoplanktonic compartment. In turn, the phytoplankton population depend on the climate condition, and on its local and seasonal variability (Orban *et al.*, 2002).

In general, however, molluscs from all the three sites were characterised by rather low

lipid contents. Actually, the fatty acid composition results from a complicated mechanism dependent on external factors such as environmental conditions (temperature, salinity, etc.), quantity and quality of food, sexual differences (Joseph, 1989; Galap *et al*., 1999), geographical and temporal aspects (Brazão *et al*., 2003). Accordingly to Freites *et al*. (2002), PUFAs were the most abundant class in all the samples examined (as in other marine Bivalves), especially in the mussels from the Calich lagoon. Among those, the n-3 polyunsaturated fatty acids predominated, and the 22:6 n3 (DHA) was the most abundant. In particular, the molluscs grown in the Porto Pozzo lagoon showed the highest percentage (similarly to the Calich ones) and those from Tortolì the lowest one. A number of Authors (Kayama *et al*., 1989; Pazos *et al*., 1996) reported that a specific phytoplanktonic group (*i.e.* the dinoflagellates) is particularly rich in this fatty acid, then the different content would be related to this component. The second more abundant n-3 fatty acid was the eicosapentaenoic (20:5 n3 EPA), which registered its maximum level in mussels cultured in the Calich lagoon and its minimum in those from Porto Pozzo. The above-mentioned authors (Kayama *et al*., 1989; Pazos *et al*., 1996) also affirm that EPA is generally present with high concentration in the phytoplanktonic group of the diatoms. Thus, the difference observed in the two fatty acids levels among *Mytilus galloprovincialis* specimens from Calich, Porto Pozzo and Tortolì may be related to the type of food they have ingested.

Concerning the saturated and monounsaturated fatty acids, although they appeared in less percentage with respect to the above, palmitic acid (16:0) and 16:1 n7 were well represented. The former was particularly abundant in the specimens reared in the Calich lagoon and the latter in those cultured in the Tortolì basin.

In general, our results were similar to those observed by Orban *et al.* (2002) in two Italian coastal areas. Even then, the polyunsaturated fatty acid represented the principal fraction, followed by the saturated and the monounsaturated ones, and EPA, DHA, palmitic acid, and 16:1 n7 showed the most abundant percentage.

Finally, the high n3/n6 ratio detected in all the groups analysed (especially in that from the Calich lagoon) and the low value of the atherogenicity and thrombogenicity indexes (for which molluscs cultured in the Porto Pozzo lagoon revealed the minimum values) confirm the positive nutritional features of this product. The consumption of food particularly rich in n3 PUFA, indeed, is highly recommended by some dietary guidelines (Simopoulos, 2003), especially in industrialised countries where large consumption on food rich in n6 PUFA occurs.

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Chapter 4

OSTREA EDULIS

4.1 Introduction

Oysters have been studied through more than 500 classified fossil species and, although not all conform to the same geological era, is in the Cretaceous that had the most favourable period for their development, distributing everywhere and reaching 264 species described (Ranson, 1951). About the species existing today (about a hundred), they have made their appearance at the beginning of the Quaternary, and their worldwide occurrence is a function of temperature, as this is undoubtedly the key parameter in their distribution. Therefore, since there are fundamentally two basic climatic zones of the world (a tropical area and a subtropical one) this leads to the existence of two different groups of species, which are divided in these two zones. The oysters from the sub-tropical area may extend to more or less temperate zones, where the warm water currents invade the cooler coast. On the other hand, some species are cosmopolitan, because they live at the same latitude in all oceans. This implies that there are the same conditions of temperature and salinity in different areas or the limits of variation of these parameters are close.

At the moment, the main species of existing oysters belong to the genera *Crassostrea* and *Ostrea*. The genus *Crassostrea* includes many species living in marine environment and/or estuarine intertidal areas. Among these, rather interesting and subject for breeding or exploitation, it is worth mentioning *Crassostrea gigas*, called Japanese or Pacific oyster, imported in Europe by France and in Italy since the 1960s. Instead, the species belonging to the genus *Ostrea* which arouses the greatest interest is *Ostrea edulis* (Linnaeus, 1758), the native European flat oyster.

This species naturally lives from the Fjords of Norway to Morocco (North eastern Atlantic coasts) and in the Mediterranean Sea (Jaziri, 1990) as far as the Black Sea coasts (Alcaraz & Dominguez, 1985). It is also found in South-Africa (FAO, 2006), North-eastern America (from Maine to Rhode Island), Canada, Nova Scotia, New Brunswick and British Columbia, probably imported from population whose ancestor were from Netherlands (Vercaemer *et al.*, 2006) (Fig. 4.1).

Oysters have always been a food for humans so that already Romans built structures to facilitate their settlement and consume them and, during their occupation on mainland Britain, they not only exploited native stocks but also exported oysters back to Rome (Edwards, 1997). In France, in the 1600s, spat was harvested from rocks and sown in brackish ponds along the Atlantic coasts. During the 1700s and 1800s, overfishing, failed recruitments, destruction of natural banks, and cold temperatures contributed to a

dramatic decline in populations of *O. edulis* and so began to be used the first spat collectors in order to retrieve the natural resource. In the mid 1800s, in South-western France the tile (roof tiles covered with a lime and sand) and wooden boxes techniques to grow juveniles were firstly used, and during the beginning of 1900s, off-bottom culture started along the Mediterranean coasts. Oysters were cemented individually onto steel poles within areas characterized by shallow waters (3-4 m).

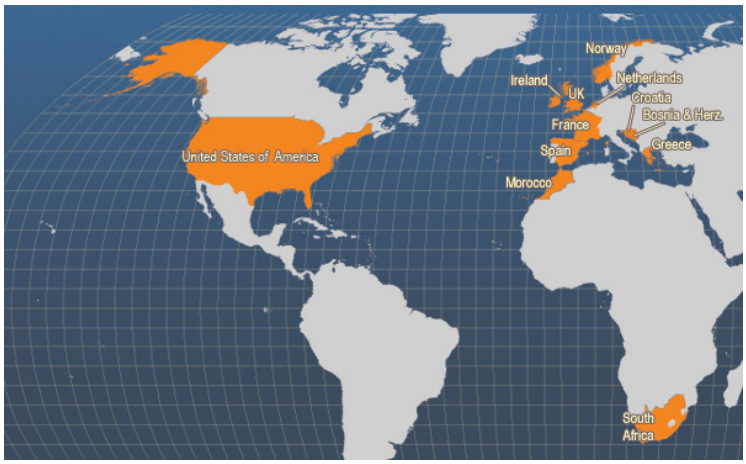

Fig. 4.1. Main producer countries of *Ostrea edulis* (from www.fao.org).

During the middle of the nineteenth century, *O. edulis* populations declined due to the overexploitation on fishing grounds (Smith *et al.*, 2006) and, by the end of the century and the beginning of the next, the decline in population size was attributed not only to overfishing, but also to habitat loss (Laing *et al.*, 2006) and diseases (Montes *et al.*, 1991; van Banning, 1991; Beaumont *et al.*, 2002). Between the 1930s and 1940s, several cold winters caused mass mortalities throughout *O. edulis* populations (Orton, 1940), and many of the wild European populations became scarce (Kennedy & Roberts, 1999). Moreover, further factors as predation, competition (Korringa, 1952a; MacKenzie, 1970), and reduced water quality (Rothschild *et al.*, 1994; Kennedy & Roberts, 1999), contributing to the decline of *O. edulis* fisheries in these periods.

Around the 1960, oyster industry showed a dramatic decline in almost all European traditional rearing areas, by a succession of diseases epidemics due to two parasites: *Bonamia ostreae* (causing the bonamiasis) (Montes, 1987; McArdle *et al.*, 1991) and *Marteilia refrigens* (Balouet & Chastel, 1979). Consequently, a consequential shift to the culture of oysters belonging to the genus *Crassostrea* (*Crassostrea gigas* in particular) occurred, as this species was not susceptible of bonamiasis and showed

faster growth rate than *O. edulis*.

Despite *O. edulis* production remained relatively low until the end of 1990s, around the 2000s more stability occurred (about 6000-7000 tonnes). Ireland, UK and Croatia produced more that 200 tonnes in 2004, so that European flat oyster constituted about 0.11% of the total global production of all the farmed species. In 2005, 51% of oyster production was in Spain (2,575 tonnes) and 30% in France (1,500 tonnes). The majority of the world production (about 96%) was due to the imported Pacific oyster *C. gigas* (FAO, 2004).

Oysters are typically consumed fresh, distributed to local markets (limited areas where it is reared), and their price is substantially high. Depending on the size and the local availability, prices of *O. edulis* are about 13 US $ kg^{-1} in France, then 3-5 times greater than those of the Pacific oyster (*C. gigas*). The total value of farmed *O. edulis* production was US $ 20.3 million in 2004. It is therefore understandable why today this resource occupies an economic niche, considering it as luxury seafood for a particular typology of consumers.

4.1.1. History and evolution of oyster culture

Oyster farming is a very ancient activity and dates back to 4,000 years ago, when Japanese, Greeks and Romans practiced it with amazing results. In China, it is dated to 460 B.C. and started by using interlocking blocks of stone with oyster shells (Ranson, 1951). Even in Europe this kind of culture is very old, dating back to at least 2,000 years, and it was practiced in bays or coves. In particular, some Latin writers have handed down numerous documents concerning the development of oyster culture in Italy, where it assumed considerable importance in the Roman epoch. This was further confirmed by a number of archaeological discoveries such as some funeral containers in Rome, on whose outer walls drawings in relief depicting parks with buildings and structures of this typical farming activity can be clearly distinguished.

It is recognized in the Roman Sergio Orata the "father" of the European oyster culture (140-91 B.C.). Plinio il Vecchio reported that in 160 B.C. Sergio Orata organized the first oyster park in the Bay of Naples, designing collection systems and developing the production in two ways: first, creating zones of fattening, and then designing systems for uptake of seed in lakes and bays. These were optimal areas for both fattening and installation of collectors for the settlement of juvenile specimens. The oysters produced in Italy, however, were not sufficient for the consumption that the Romans did, so they

imported them from all the European coasts, where oyster beds were. In addition, some poets of the fourth century wrote about the finest oysters at that time, classifying them as groups of quality. Thus, they appreciated the oysters from Bordeaux, those from Marseilles, Port-Vendres, Saintonge, the Seine and the Calvados, the ones from Brittany, Poitou, those of Scotland, or those of Byzantium and Arcade in Spain.

Throughout the middle Ages, oyster breeding continued to be practiced. According to a writer of the sixteenth century (Petrus Gyllius), it seems that in 1040 there were attempts to harvesting them in a system inherited from the Byzantine era in the West coast of Schleswig. Subsequent documents report that in all areas where oysters naturally lived and where the fishing industry was present, they were transplanted and followed, that is raised. Among countries that adopted these techniques was England, where since 1700 this type of farming was practiced. Apparently, in London were consumed increasing quantities of oysters, therefore it became necessary to have reserves of these molluscs in the vicinity, so that they were purchased abroad and deposited in suitable areas of the coast, like in the mouth of the Thames.

In Italy, the farmers of oysters near Naples practiced the harvesting of larvae on collectors and grew oysters as their ancestors. This rebirth of farms occurred also in the Gulf of Taranto in 1890, the year in which tests were also made by Austrians in the Adriatic Sea, starting breeding from traditional techniques of collecting larvae in the estuary of Grado. Other countries that had successful experiences of this kind were Norway, in lakes where the cultivation of *O. edulis* was carried out with very positive results, and in the Netherlands where breeding have been successful. Furthermore, in Germany, the attempt to restore the natural beds of oysters began in 1753, on the coast of Pomerania, trying again in 1830 and in 1843: on all occasions the result was a failure, since the difficulties of the coast and water temperature did not allow the development of this sector.

In France, the practice of oyster culture had a significant improvement, particularly in Marennes, where oysters were deposited in the cavities of rocks or in the reserves for fattening. Given the increase in the consumption of oysters in France (and especially in Paris), the supply provided by Britain threatened the extinction of the natural beds. Due to this fact, the responsible authorities of the Ministry of Agriculture commissioned researchers as Quatrefages and Coste to deal with the recovery of oyster beds in the French coasts. In 1857, under the impulse of Coste, the modern French oyster farming began, putting into practice the methodologies used by the Italians, inherited by the

Romans and probably originating from China. Thus, it can be said that although oyster farming in Europe was born in Italy, France, was the precursor country for this activity and exported the rearing technique to a number of nations like England, Holland, Spain, Norway, Denmark, Germany, USA, Canada, Australia, India and Argentina.

4.1.2. Oyster culture methods

In Europe, cultivation of the native oyster *Ostrea edulis* is not so common as the Pacific species *Crassostrea gigas*, because of its slower growth, its lower survival rate and the higher cost of the seed. It is also true that the European flat oyster is more susceptible to some pathogens, such as *Bonamia ostreae* (Montes, 1987), first recorded in the United Kingdom in 1982. This protozoan was described for the first time in French oyster farms affected by high mortality (Comps *et al.*, 1980) and, subsequently, spread in most of the culture systems of Britain (Tigé *et al.*, 1980). Later, *B. ostreae* was detected in other areas, such as Spain (Montes & Melendez, 1987), Holland (Van Banning, 1982), England (Hudson & Hill, 1991) and Ireland (Mc Ardle *et al.*, 1991), while in Italy its presence was found in a natural bed of the southern Adriatic (Tiscar *et al.*, 1991) but not in its northern part (Ceschia & Zentilin, 1990). It is possible that this pathogen was introduced in France as a result of oysters' imports from the United States. Bonamiosis manifests itself through non-specific lesions on one or more of the mollusc gills, which appear to be perforated or with cuts surrounded by typical whitish streaks. The disease can be diagnosed, as well as conventional histological or cytological methods, also with immunological systems using specific monoclonal antibodies (Boulo *et al.*, 1989). Sensitivity to infection of other species of molluscs is variable: it was verified, in fact, that *C. gigas*, *Mytilus edulis*, *Ruditapes decussatus* and *R. philippinarum* placed in areas where bonamiosis was present did no contract the disease (Grizel *et al.*, 1988). Overall, in spite of this, *O. edulis* always raises a higher market prize.

In general, oyster farming involves four basic operations: spat collection/artificial reproduction, nursery, grow-out and harvest. The first phase (*i.e.* the retrieval of juveniles) can be generally done in two ways: by collecting wild spat in the environment or by artificial reproduction in hatcheries. In the case of natural recruitment, the peculiarity to settle on different substrates is exploited by the farmers, who prepared the so-called "collectors" in areas naturally rich of juvenile oysters. In this way, fishermen remain tied to natural reproductive cycles of the species, closely related to the features of the site concerned and that certainly do not provide any

regularity. The choice of collectors, moreover, is principally correlated to the suitability of available materials, cost, and the type of culture system employed.

Hatchery rearing activities are not as reliable for European native oysters as for Pacific one. In effect, good survival rates (up to 90%) are possible with some lots of larvae. Nevertheless this result can be much lower (less than 50%) with others, certainly depending on the selection of the appropriate broodstock and on a balanced diet (Millican & Helm, 1994; Berntsson *et al.*, 1997), techniques for hatchery rearing, however, are well established and widely described in the literature (Utting & Millican, 1997; Seafish, 2002; Spencer, 2002). Generally, spat from hatcheries can be started to the pre-fattening phase when it reaches a size of 4-5 mm in shell length. It should be noted that the larger is its size, the higher is its prize, but survival rates and tolerance to handling will be greater. The purpose of hatcheries, therefore, is to continually get seed in controlled systems interrupting the reproductive seasonality of the species, so ensuring the continuity of the production process and stabilizing the supply of oysters for the market.

In order to produce marketable oysters (grow-out system), different structures can be used, and the principal methods can be summarised as bottom culture, racks, rafts, trays and stakes. The system, furthermore, is determined not only by the Bivalve biology, but also by environmental factors and the cost of material. Temperature and food availability, in fact, are fundamental parameters in physiological and biochemical regulations of oysters (Calow, 1977; Malouf & Breese, 1977; Newell *et al.*, 1977; Mann, 1979; Newell & Branch, 1980), determining their biology and their geographical distribution (Wilson, 1981).

Bottom culture is practised in areas where the sea floor is stable enough to support some kind of farming structures and where siltation is flat. This method is very old, born in Hong Kong's Bay at least 150 years ago (Bromhall, 1958; Morton, 1975), and practised in Thailand (Bromanonda, 1978), Mexico (Lizarraga, 1974), Philippines (Young & Serna, 1982) and Brazil (Akaboshi & Bastos, 1977). Facilities employed are generally angular rocks, concrete tubes, cement pieces or bars, tiles, old oyster shells, placed or planted directly in the bottom. Usually, these tools are initially positioned in the upper intertidal zone to collect spat and then transferred to deeper bottoms in order to allow the grow-out until the market size is reached. In this way, oysters are harvested after 4-6 years depending on market demand.

Stake culture is common in the Philippines (Blanco, 1956). Structures typically used are

bamboo stakes about 60 cm long, stuck into the soft bottom in the intertidal zone, where oyster shells are strung on galvanized wire and then hung on the tip of the stake. A period of 9-12 months is required to produce a marketable oyster, depending on species and settling time. A modification of this technique (*i.e.* an intermediate solution between stake and rack culture) is the lattice method (Fig. 4.2). The lattices constitutes of bamboo poles (5-9 cm in diameter), organized in the form of an inverted "V", secured together with galvanized rope (Ablan, 1955), and active for 1-2 years. Each structure is positioned in depths of less than 1 m at low tide and it can also be suspended from floats in deeper water.

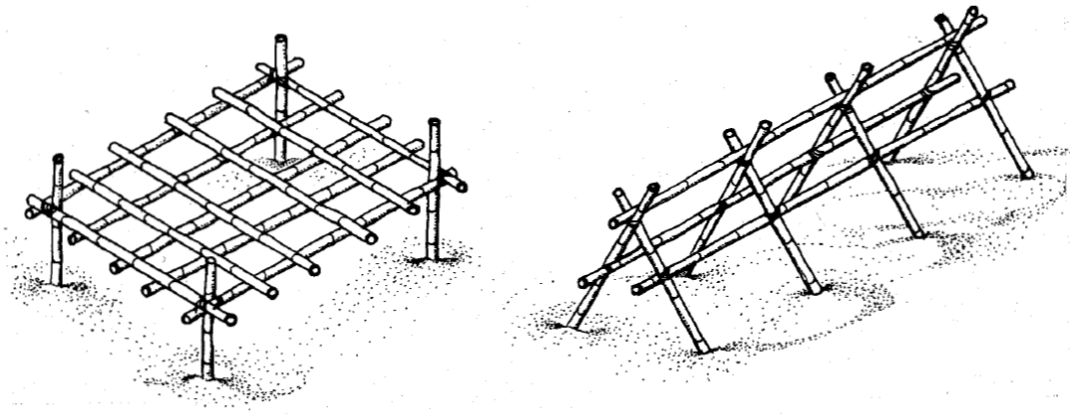

Fig. 4.2. Examples of lattice culture method.

The main advantages of the rack method are the reduced attachment of fouling organisms, the minor predation obtained raising the crop from the bottom, intensifying production by using more vertical space and decreasing the costs. A first commercially viable system was developed in Cuba (Nikolic *et al*., 1976), where collectors were constituted by branches of mangroves suspended from horizontal supports, previously fixed below the low tide level, and whose height was corrected seasonally, to make sure that they were in the lower 30-40 cm of the intertidal zone. After 5-6 months from the first set, harvesting could begin and undersized oysters transferred to the intertidal zone or selectively harvested when market size was reached. Each collector could produce up to 5 kg of marketable oysters (about 370 specimens). Similar methods were used in the Philippines (Young & Serna, 1982), North-western Australia (Bryson, 1977), Malaysia (Chin & Lim, 1975), and west coast of Java (Fatuchri, 1976).

Finally, raft and long-line culture are nowadays the most used methods. They are based

on suspension culture system and are not bound by the presence of shallow bottoms to allow management activities. Japanese developed the first one and other countries, due to the fast growth rates and the high productivity soon adopted it, although little modifications of the technique were made outside Japan. Rafts are usually simple and the greatest cost is generally due to the floats. The raft system consists of frames and floats of different material; it is anchored at depths of about 6-10 m, and is employed in different countries (Watters & Martinez, 1976; Ng, 1979; Kamara, 1982). Also the long-line method was born in Japan, to extend the oyster farming to more open waters, given the growing pollution and overcrowding near the shoreline (Imai, 1971). It is characterised by cheap construction materials and it is easy to maintain. A sequence of buoys are secured together by synthetic ropes, positioned parallel to the dominant winds, and anchored by concrete blocks on the bottom (Fig. 4.3).

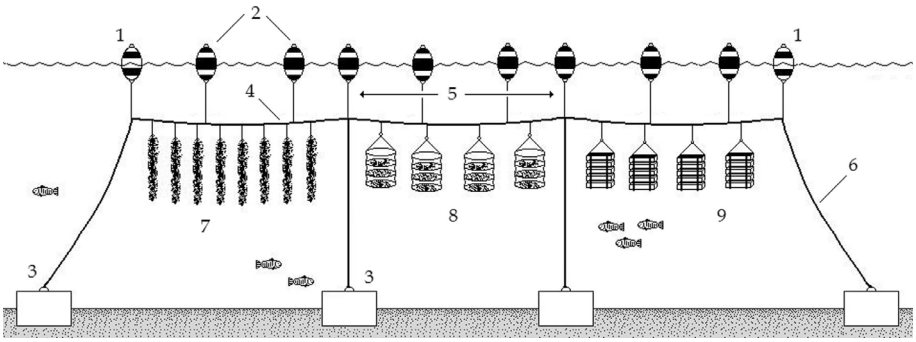

Fig. 4.3. Long line culture system.

4.1.3. Oyster culture in Italy

In Italy, oysters represent only a small fraction of total shellfish production. The quantities produced are referred both to *Ostrea edulis* and *Crassostrea gigas*, although the latter represents the main resource for trade. The major intensity behind the activities of breeding occurred in late 1800 and early 1900, when the fishermen of the northern Adriatic (especially along the coasts of Istria and Trieste), using the French experience, rationalized its availability through the installation of oak piles driven into the bottom, placing partitions to suspend the nets and collect oysters. About forty years later, oyster farming was reborn in the same area by a local cooperative operating in the Grado lagoon (*i.e.* the "Compagnia Triestina di Ostricoltura"), while, in winter, the fleet of Grado fished on rich natural beds in the Gulf of Trieste. Over the years, however, overexploitation of natural resources led to the cessation of these activities, facilitating

the transition to mussel culture (Schiavo, 2011).

As a consequence, a peculiarity worthy of note is that most of the companies engaged in the oyster farming also include mussel culture activities so that, in 2005, only a company sited in Tuscany was exclusively devoted to oyster culture. In the same year, the Italian production recorded quantity of 35 tonnes of *C. gigas*, having an average price of 2.30 € kg^{-1}. In general, the shellfish industry has a great dynamic and is always able to address the difficulties that often faced. The market, in fact, seems to still have room for expansion, especially for oysters, for which Italy depends almost exclusively from abroad. In 2005, oyster-producing regions in this country were few and among them Tuscany (with an annual production of 30 tonnes), Sardinia (with quantities equal to 4 tonnes), and Veneto (with only 1 tonnes of product per year). Moreover, although the average price of this product is around 2.30 € kg^{-1}, Sardinia records the highest prices, amounting to less than 4 € kg^{-1} (Prioli, 2008).

Due to the growing demand for oysters from the domestic market, the need for continuous imports from France (with production of over 100,000 tonnes $year^{-1}$ of *C. gigas* and 10,000 tonnes $year^{-1}$ of *O. edulis*), and to encourage a diversification of crops as an alternative source of income for mussel and clam farming, in the first 1990s a consortium of Venetian fishermen (*i.e.* the "Consorzio Cooperative Pescatori del Polesine") brought an experimental culture of the Pacific cupped oyster *C. gigas*. They chose the latter for its faster growth and also because in the northern Adriatic lagoons the wide variation in temperature, salinity and dissolved oxygen levels are more tolerated by this species than by the native one. Moreover, *C. gigas* can reach the market size of 8-9 cm and 80-100 g in weight about 8-10 months before *O. edulis*. The results obtained were excellent, with such rapid growth that they could pick up the product to commercial size after only 12 months from the sowing and a mortality rate quite low, approximately 20% of the product sown (Rossetti *et al.*, 1992).

In the same period, in Sardinia the domestic demand for shellfish was around 700 tonnes of product, especially due to a lack of production of clams (*Ruditapes* spp., 430 tonnes) and oysters to a lesser extent (*O. edulis* and *C. gigas*, 150 tonnes). In this island, oysters farming activities were conducted during the mid-80s only in the lagoon of San Giovanni (central-western Sardinia) and they were mostly carried out for experimental purposes. From the point of view of production, these results showed no particular importance, but demonstrated how this type of activity could promote the development of productive brackish environments (Ingle *et al.*, 1993). From 1990s onwards,

however, the Sardinian production of oysters is almost doubled, increasing from 3 to 6 tonnes between 1992 and 2008, although in 2002 there was a significant decline (1.30 tonnes). The sector, therefore, is actually expanding even if the supply of seed is limited due to its import from the Italian peninsula and from abroad (LAORE, 2009).

4.2 Materials and methods

4.2.1 Ostrea edulis

4.2.1.1 General description of the species

The European flat oyster *Ostrea edulis* (Linnaeus, 1758) belongs to the Ostreidae family (Rafinesque, 1815) and is native of Europe (Tab. 4.1). Its shell is oval shaped, whose left valve is typically cupped, the right usually flat and, for this reason, called inequivalve. Externally, it appears irregular, rough and scaly, off-white, greyish and consists of a series of calcareous layers. Furthermore, this Bivalve lives with its left valve fixed to the substratum and right valve sitting inside the first one and acting as a cover. Inner surfaces of the shell are smooth, pearly, off-white or bluish-grey. Finally, oysters are characterized by a single central adductor muscle that permits to close their valves (*i.e.* monomyarian species).

Internally (Fig. 4.4), soft body of *O. edulis* appears creamy-beige in colour, with a texture from tender to firm. The mouth is located near the umbonal end and is surrounded by labial pals and the gills are along the ventral parte of the body and appear beige in colour. Gonads are well enlarged when mature. When an individual reaches a shell length of about 10-12 cm, it can be considered adult, although it can be grow up to 20 cm and live up to 20 years.

Tab. 4.1 Systematic framework of *Ostrea edulis* (Linnaeus, 1758).

Taxon	Name	Author
Phylum	Mollusca	
Class	Bivalvia	(Linnaeus, 1758)
Subclass	Pteriomorphia	(Beurlen, 1944)
Order	Ostreoida	(Férussac, 1822)
Superfamily	Ostreoidea	(Rafinesque, 1815)
Family	Ostreidae	(Rafinesque, 1815)
Genus	*Ostrea*	(Linnaeus, 1758)
Species	*Ostrea edulis*	(Linnaeus, 1758)

Ostrea edulis is similar to other species widely cultivated in many regions of the world, like the Pacific cupped oyster *Crassostrea gigas* (Thunberg, 1793) (Fig. 4.5). This latter, however, has a more elongated, distorted and irregular shell and, above all, is characterized by a different sexuality.

The European flat oyster is a protandric hermaphrodite (Mirella da Silva *et al.*, 2005) and shows an alternation of sexuality within one spawning season: early in the reproductive period it is male, but when it reaches the sexual maturity can alternate between the female and male stages for the rest of its life (Laing *et al.*, 2005). Males are mature after about one year of age when they release sperms into the water depending on temperature values (with a minimum of 14-16°C) (Walne, 1979; Goulletquer, 2004). Females collect sperms by using their feeding and respiration system (Laing *et al.*, 2005).

The ovogenesys can produce up to 1 million of eggs per spawning event, releasing them from the gonad and retaining them in the mantle cavity where they can be fertilized by externally released sperms (*i.e.* larviparous species). After an incubation of about 8-10 days, when larvae develop a formed shell, a digestive system and the ciliated swimming and feeding organ (*i.e.* the vellum) reaching about 160 μm in size, they are released to the open water where live as pelagic stage (8-10 days) feeding on phytoplankton for 2 to 3 weeks (Korringa, 1941; Korringa, 1952; Eklund *et al.*, 1977; Laing *et al.*, 2005) before settlement. The amount of larvae released into the seawater is correlated to the parent size, ranging between 1.1 and 1.5 millions for oyster 4-7 years old (Walne, 1979).

Fig. 4.4. Soft body of *Ostrea edulis* (AM: adductor muscle; G: gills; GO: gonad; M: mantle; IC: inhalant chamber; EC: exhalant chamber).

By contrast, *Crassostrea gigas* inverts its sex after one spawning season and releases its gametes (eggs or sperms) into the environment at one time or in small amounts over a long period (*i.e.* oviparous species). Thus, fertilization occurs externally and the resulting larvae develop in the seawater.

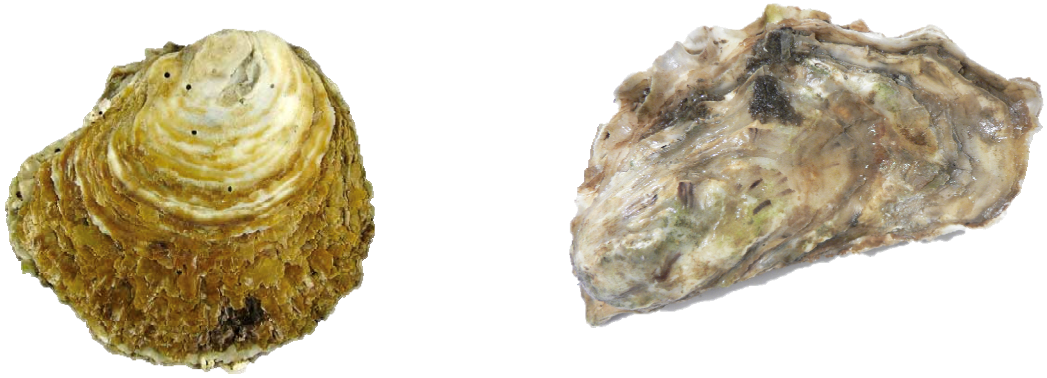

Fig. 4.5. Shells of *Ostrea edulis* (on the left) and *Crassostrea gigas* (on the right).

During the larval stage, therefore, life is typically planktonic and as metamorphosis progresses oyster moves with an extensible foot in search of a suitable substrate. When it is found, the oyster attaches itself by a byssus formation and then by cementation (with a physiological and morphological metamorphosis during 3 to 4 days) and starts its sessile life as juveniles, becoming spat (Laing *et al.*, 2005). From this event, the growth is quite quick for about 18 months, then stabilises remaining constant at about 20 g of fresh weight per year and finally slows down after five years (Jackson, 2003; Laing *et al.*, 2005). Depending on environmental conditions, these Bivalves can achieve the marketable size of 7 cm in shell length in 4-5 years and live in natural beds up to 20 years growing to 20 cm of size (Goulletquer, 2004).

Ostrea edulis is a typical filter feeder, filtering phytoplankton, copepod larvae, protozoans and detritus as food. Since it is a sessile organism, and then lives fixed to a hard substrate, its feeding depends entirely on the resources naturally present in the surrounding environment. As a matter of fact, food is pumped in with the seawater and removed by the gills (Laing *et al.*, 2005), filtering even up to 25 l hr^{-1}, depending on animal size and temperature (Korringa, 1952).

This species is typical of coastland, estuarine and marine environments and sheltered areas, preferring hard substrates as rocks or artificial structures but also muddy sand, muddy gravel with shells, and hard silt. It lives in brackish and marine seawater, having

an optimum of salinity rounding between 17 and 26 PSU (Blanco *et al.*, 1951), up to 40 meters deep.

Oysters are prey of several organisms, including fish, crabs, snails, starfish and flatworms but also of boring sponges, seaworms, molluscs, pea crabs and fouling in general, that can be cause irritation problems or compete for food. With regard to disease, the protist *Bonamia ostrae* is one of the most dangerous pathogens: in 1920 it caused massive mortality events among flat oyster populations (Mirella da Silva *et al.*, 2005), then reintroduced to Europe where the disease was transferred to other established populations.

4.2.1.2 Production cycle

Oyster spat can be obtained by both wild stocks and hatchery production (Fig. 4.6). Like in other Bivalves, sexual maturation and subsequent reproduction is got modifying temperature of water (increasing it) and administering phytoplankton *ad libitum*, imitating the natural reproductive cycle. Compared to conditioning of other species (*e.g. Ruditapes decussatus*), fertilization of *O. edulis* specimens is more difficult due to a lower larval survival rate, so that a period of incubation is necessary. In general, spat is cultured using traditional techniques for Bivalve, in nursery stages and, when size of 5-6 mm is reached, it can be moved to open water to grow up.

Natural spat harvesting, instead, is based on the employment of collectors. Some examples are mussel shells sown in density of about 30-60 m^3 ha^{-1} (Netherland), or tubular nets containing mussel shells (about 600) suspended under steel frames in shallow waters (France). Recently, PVC dishes are used in intertidal areas.

Therefore, seed can be transferred to the growing or fattening area, although it is not always necessary depending on the facility because the seeding area can also become the growing and fattening area. Breeding methods are generally categorized into “on-bottom” and “off-bottom”, having each its advantages and disadvantages. Thus, it should be better to choose the most suitable method for the selected site and for the specific financial possibility.

On-bottom techniques require that oysters are seeded directly in sub-tidal or inter-tidal grounds with a stable, non-shifting bottom (Quayle, 1980), at the density of about 50-100 kg ha^{-1}. Generally, seeding is carried out in the period between May and June, when the molluscs are about 1 cm long (1 year old), and here they reach the marketable size. Traditionally, cotton nets or steel frames are used to preserve the culture from predators.

The on-bottom method certainly is the simplest and cheapest one but mortality, stock loss caused by predation, siltation events are highest and also harvesting is difficult. In general, moreover, the main disadvantages are the restriction to shallow water with stable bottom and a summary production per unit area.

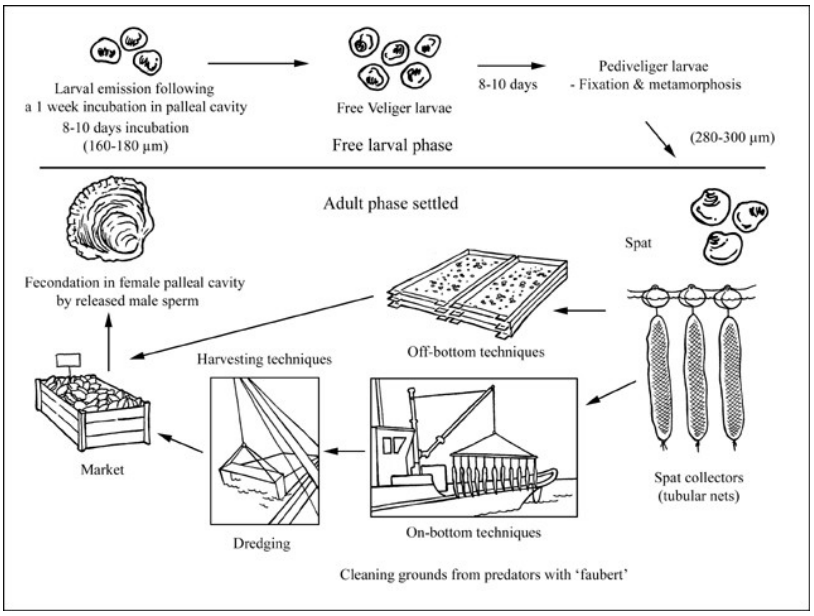

Fig. 4.6. Production cycle of *Ostrea edulis*.

On the other hand, off-bottom techniques involve that oysters are cultured in suspension. This method is certainly more expensive than the first one and requires more maintenance but it is compensated by the rapid growth and high quality of the cultured oysters. The technique consists of using floating structures, rafts, long-line systems, suspended ropes, lanterns or plastic baskets pending from a raft/rope, where oysters are located in. Product is thinned out as it grows.

Harvesting should be programmed when oysters are in their best conditions, with full and creamy meat. From on-bottom cultures, molluscs can be dredged or handily collected, whereas in the off-bottom ones handily-picked. Finally, before marketed, they are temporarily stored in clean water and subjected to depuration procedures as all other Bivalve molluscs.

4.2.2 Field methods and experimental design

The field activities started in June 2011 in the Porto Pozzo lagoon at the long-line system of "La Peschiera" a r.l., and finished in October of the same year.

At the beginning of the trial, small specimens of the European flat oyster *Ostrea edulis* from natural banks (Adriatic Sea) were immediately immersed into the water of the lagoon for few days to allow acclimatization. Later, 540 individuals were randomly chosen and divided in 3 equal groups (180 individuals each) subsequently assigned to 3 different farming tools: pôches (Fig. 4.7), Australian baskets (Fig. 4.8) and stackable trays (Fig. 4.9). In detail, the pôches having a 17 mm mesh size were made of high-density polyethylene net, rectangular shaped, measuring 100 × 50.5 cm (length × width). The Australian baskets, a modular system used in shellfish culture, were cylindrical (65 cm in length and 20 cm in width), provided with end caps to allow their opening and closing, with a mesh of 20 mm. The stackable trays, commonly used in Italian long-line cultures, were composed of 3 stackable containers round shaped, made of rigid plastic divided into four compartments, Each module was 40 cm in diameter and 8 cm in height, with a 18 mm mesh size.

Fig. 4.7. Pôche used in the trial.

Three replicates of each farming tool were positioned at 2 different depths (-1 and -5 meters) in the water column, for a total of 18 oyster containers (9 per depth). In detail, 30 oysters were positioned inside each experimental unit, thus involving a total of 540 specimens (*i.e.* 30 oysters × 3 replicates × 3 farming tools × 2 depths).

Before the trial, main length and width (in mm) of all the oysters were measured using a 0.1 mm precision calliper (Fig. 4.10), and wet total weight of each individual was registered with a precision balance. These above-mentioned morphometric variables were recorded monthly, as well as the survival of molluscs in each experimental group. During monthly samplings, furthermore, main physico-chemical variables of the water

column (*i.e.* temperature, salinity and pH) were registered *in situ* by field instruments. Water samples were also collected in polyethylene bottles and immediately transported to the laboratory under controlled temperature (about 5°C) to evaluate chlorophyll *a* and seston (by spectrophotometric and gravimetric method, respectively) (APAT, 2004).

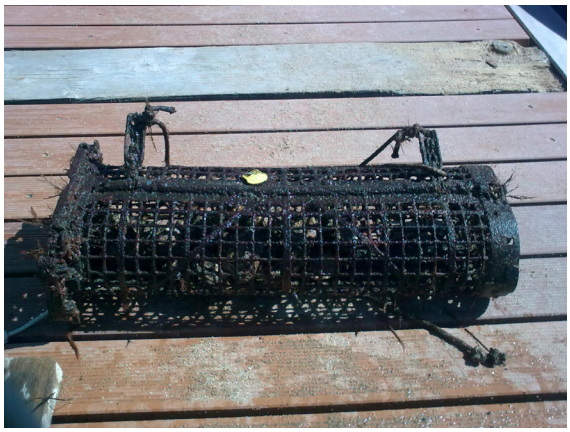

Fig. 4.8. Basket used in the trial.

Fig. 4.9. Stackable trays used in the trial.

Fig. 4.10. Measurement of morphometric characters using a precision calliper.

4.2.3 Data processing and statistical analyses

Starting from all the data collected during the trial, a chi-square test (χ^2) was performed to test the null hypothesis that there were no differences in survival rates due to depth (*i.e.* -1 m and -5 m), experimental tool (*i.e.* pôche, Australian basket and stackable tray), and interaction between depth and tools.

A two-way Analysis of Variance (ANOVA) was performed to test for differences in the 3 morphometric variables analysed (*i.e.* shell length, shell width and total weight) among experimental tools and depths of rearing. Both the factors "Tool" (3 levels) and "Depth" (2 levels) were considered as fixed. The Cochran's C test was used to check the assumption of the homogeneity of variances. Finally, *post-hoc* multiple comparisons were performed using the Student-Newman-Keuls (SNK) test (Underwood, 1997). ANOVA were always performed using the STATISTICA® software package.

4.3 Results

4.3.1 Hydrological and mesological variables

During the trial, water temperature (Fig. 4.11) gradually increased in the Porto Pozzo lagoon from 21.7°C in June to 26°C in October. Similarly, salinity values (Fig. 4.12) showed a regular increment from the start of the experiment (39 PSU) to September (44 PSU), although a slight decrease was recorded in the following month of October (42 PSU).

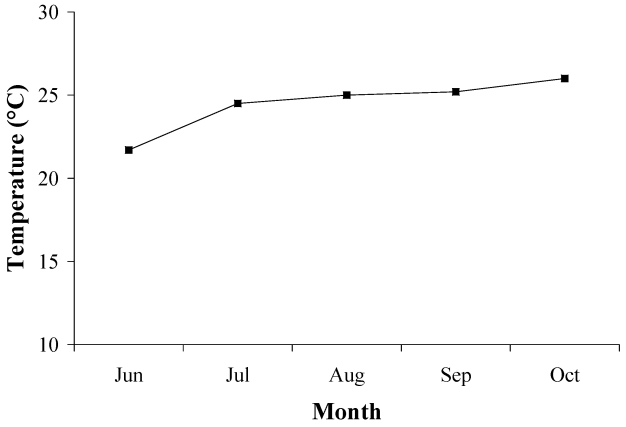

Fig. 4.11. Temperature values in the Porto Pozzo lagoon during the study period.

Moreover, the pH values registered during the experimental period showed an increasing trend (Fig. 4.13), from a minimum of 8.00 in June up to a maximum of 8.40 in the last sampling period.

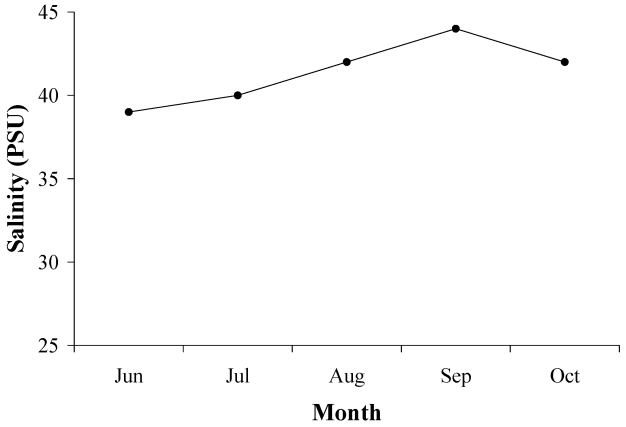

Fig. 4.12. Salinity values in the Porto Pozzo lagoon during the study period.

Chlorophyll *a* concentration (Fig. 4.14) varied every month, depending on the population dynamics of the phytoplankton. In the period between June and September, it revealed a continuous increase from a value of 0.54 mg l^{-1} to 0.96 mg l^{-1}. Nevertheless, the chlorophyll concentration decreased sharply in October, with a minimum of 0.20 mg l^{-1}.

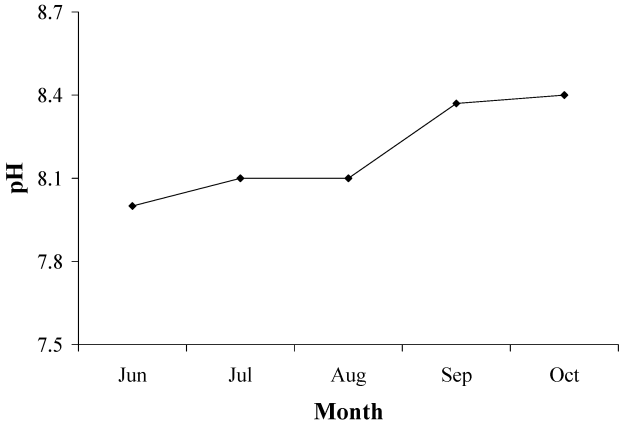

Fig. 4.13. pH values in the Porto Pozzo lagoon during the study period.

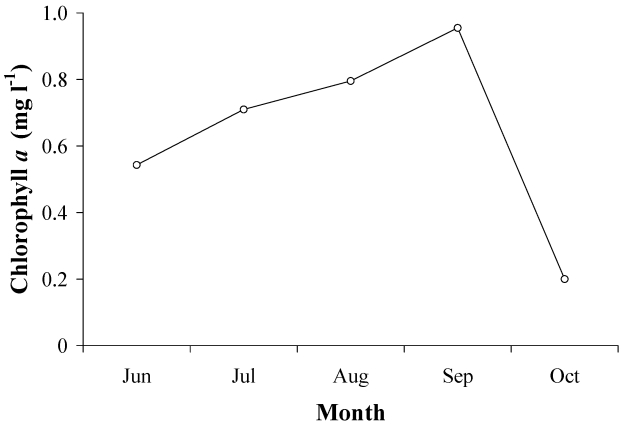

Fig. 4.14. Chlorophyll *a* concentration in the Porto Pozzo lagoon during the study period.

Finally, the amount of seston measured in Porto Pozzo lagoon (Fig. 4.15) showed a markable increment after a month from the start of the experiment (from 26.70 to 34.20 mg l^{-1}). Subsequently, steady values of the seston concentration were observed for the remaining period of the trial, ranging between 32.40 and 33.30 mg l^{-1}.

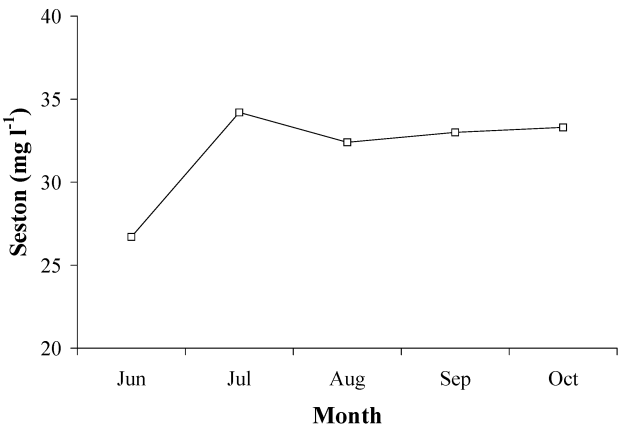

Fig. 4.15. Seston concentration in the Porto Pozzo lagoon during the study period.

4.3.2 Survival rates and morphometric variables

The number of *Ostrea edulis* specimens from each experimental group reared at both depths was counted monthly, registering the total amount of dead individuals in order to calculate the survival rate (expressed as %). At the depth level of 1 m (Fig. 4.16), the oysters cultured into stackable trays showed the highest survival percentage in July and August (95.6±1.2 and 86.7±2.6%, respectively) whereas, in the same period, those growing into baskets revealed lower values (86.7±1.0 and 82.2±1.5%, respectively), as well as the oysters placed inside pôches during August (82.2±1.5%). On the other hand, the maximum survival percentage was recorded in October in the pôches (76.7±0.0%) and the minimum in the stackable trays (73.3±4.6%).

As regards the second depth considered (*i.e.* 5 m; Fig. 4.16), survival rates into the pôches were always the highest, registering values equal to 95.6±1.5% in July, 86.7±1.0% in August, 81.1±1.5% in September, and 76.7±3.0% in October. The Australian baskets, instead, always showed the lowest percentage of survivors (91.1±0.6% in July, 78.9±1.5% in August, 73.3±1.0% in September, and 73.3±1.0% in October), while in the stackable trays was observed a final survival rate of 75.6±2.5%.

In this case, the Chi-square test was not performed because the survival rates were equal at both the depths considered (*i.e.* 203 individuals each).

By comparing the survival rates recorded in the same type of rearing tool at both depths (Fig. 4.17), oysters in the pôches at -5 m depth revealed a higher percentage than the ones placed at -1 m throughout the entire trial period, although the final value of 76.7±3.0% was the same in both the cases.

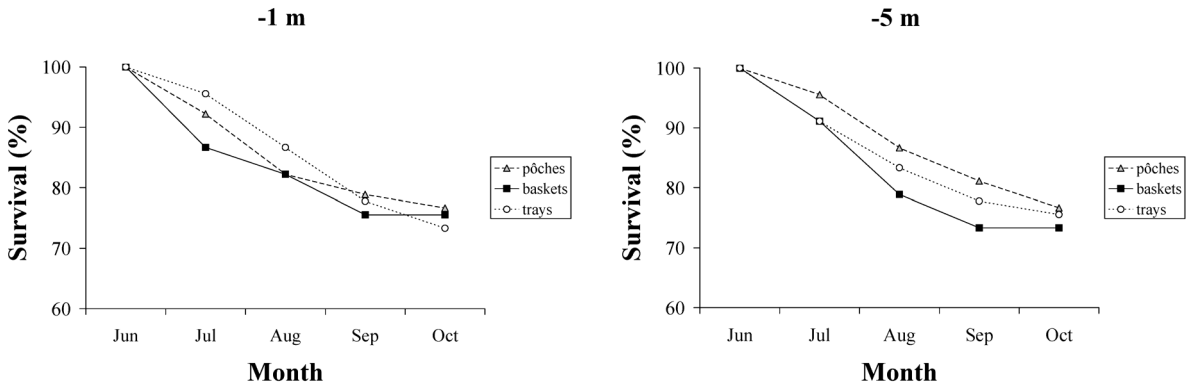

Fig. 4.16. Survival rate of the oysters reared at the depth of -1 m and -5 m.

By contrast, oysters within Australian baskets, registered higher percentages at -5 than at -1 m (*i.e.* 91.1±0.6 and 86.7±1.0%, respectively) in July but in August there was a reversal.

The survival rate in baskets at -1 m was superior than at -5 m (82.2±1.5 and 78.9±1.5%, respectively), constantly increasing until the end of the test (75.6±1.5% vs. 73.3±1.0%). Finally, stackable trays at both depths showed a similar trend during the experiment. In the early phase, trays at -1 m showed a higher percentage of survival but in September they had the same rate of those at 5 m (77.8±4.2% the first and 77.8±1.5 the latter), then becoming lower in October (73.3±4.6% at -1 m and 75.6±2.5% at -5 m).

The Chi-square test revealed no significant differences in survival rate among the three tools experimented (χ^2=0.318; p=0.853) and the interaction between tools and depths (χ^2=0.556; p=0.990).

Morphometric measurements of the three experimental groups considered were first represented as shell length-month relationships at -1 m and -5 m deep. Regarding to the depth of -1 m (Fig. 4.18), oysters in stackable trays were the longest with a value of 64.4±3.0 mm at the begin of the trial and, after a first period of intense growth (up to 76.9±3.0 mm in July), they exhibited constant values for two months and then increased up to 82.6±2.6 mm in October.

Despite in June they were slightly smaller (63.7±3.2 mm in the pôches and 61.9±0.1 mm in the baskets, respectively), also the oysters of the other two groups showed a dramatic increase during the first period, reaching values of 83.3±4.5 mm (pôches) and 82.4±2.5 mm (baskets) in October.

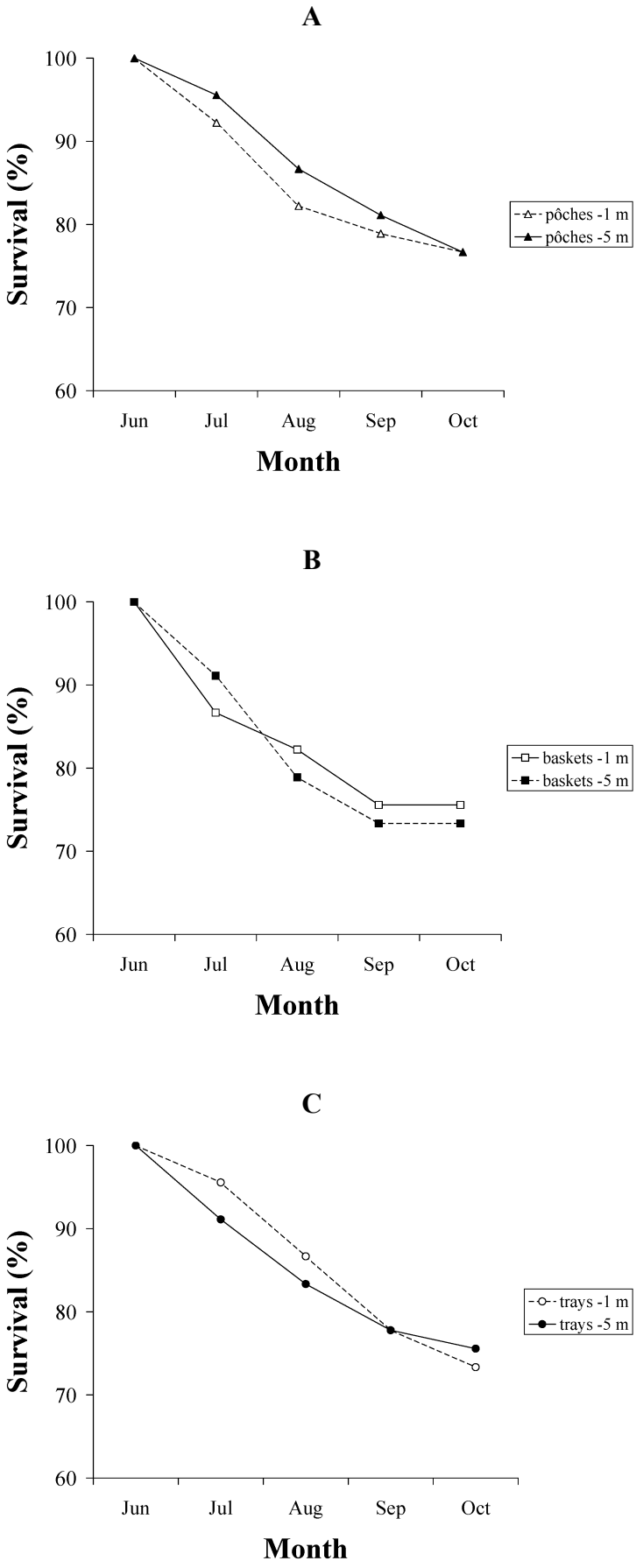

Fig. 4.17. Survival rate of the oysters reared in pôches (A), baskets (B) and trays (C).

With regard to the specimens reared at 5 m (Fig. 4.18), those from stackable trays always showed higher values, with a rapid growth between June and July (from 63.2±1.9 to 77.5±1.9 mm, respectively) and a steady state in the following months, and up to 80.9±2.0 mm in October. Oysters in the baskets, instead, revealed the lowest value of 76.4±3.3 mm in the final sampling of October.

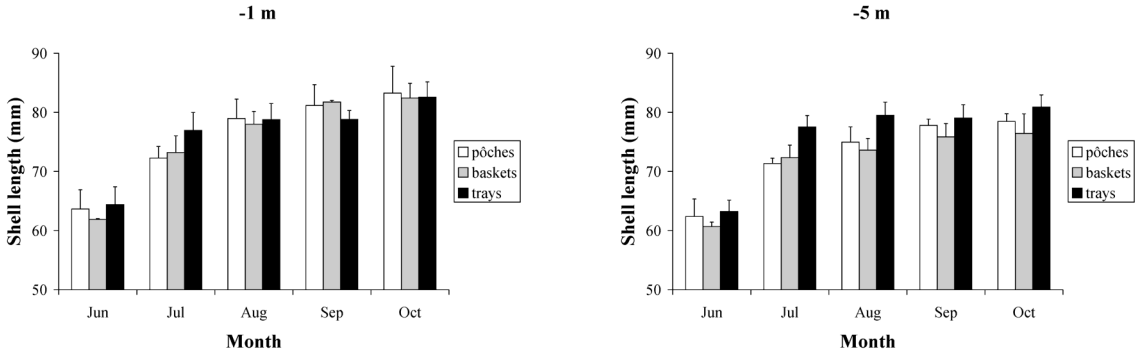

Fig. 4.18. Mean shell length (±SD) of the oysters reared at the depth of -1 m and -5 m.

The shell width measure at the depth of 1 m is shown in Fig. 4.19 The oysters cultured in stackable trays exhibited the highest values for each sampling period (from 62.9±1.7 mm in June up to 73.9±1.5 in October). Molluscs in pôches and baskets, instead, showed a similar trend during the entire study period. By contrast, although among all the oysters reared at -5 m those in baskets had the highest initial shell width (60.7±2.6 mm in June), at the end of the trial were the specimens in trays that showed the best increment (73.9±1.5 mm in October).

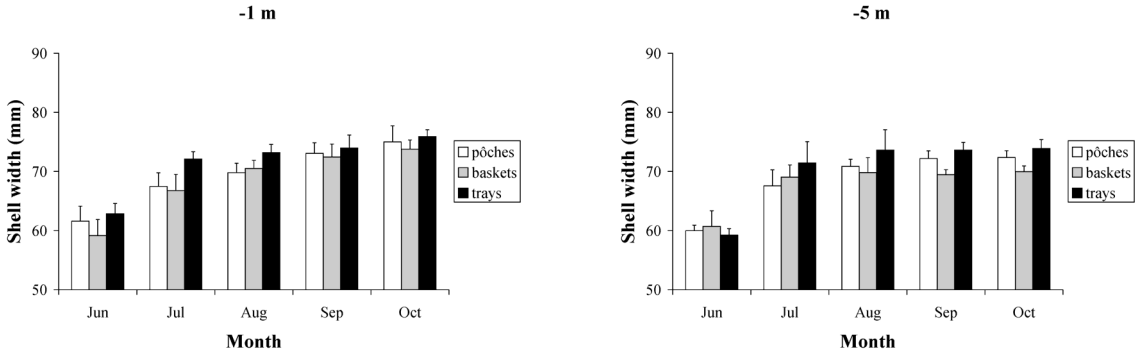

Fig. 4.19. Mean shell width (±SD) of the oysters reared at the depth of -1 m and -5 m.

Lastly, total weight of oysters from the three experimental groups reared at 1 m depth was considered (Fig. 4.20).

Contrary to what is written above for shell width, despite molluscs in stackable trays at the depth of 1 m weighed 55.0±6.1 g and the ones in pôches and baskets 51.6±4.5 and 43.7±3.8 g respectively, the situation reversed in the final sampling period, when the former were lighter (89.9±3.7 g) than those in pôches (94.7±7.7 g) and equal to those in baskets (89.9±2.6 g). Total weights registered at 5 m of depth, instead, confirmed the greatest value of 82.4±10.4 g of the oyster grown in trays in comparison with those in baskets and pôches (81.2±8.7 and 78.8±2.7 g, respectively).

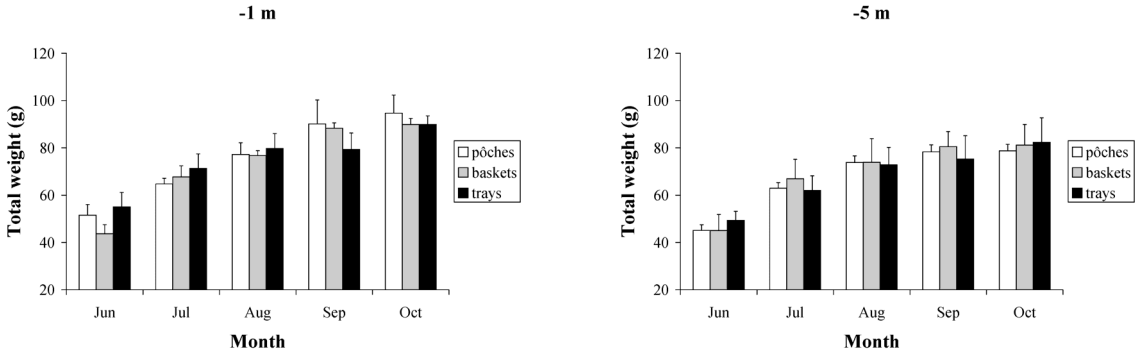

Fig. 4.20. Mean total weight (±SD) of the oysters reared at the depth of -1 m and -5 m.

In Fig. 4.21 are illustrated the relationships between the morphometric variables considered in a single system of growth at both depths investigated. The first histogram shows that the shell lengths values of oysters in the pôches positioned at -1 m were always higher (63.7±3.2 mm in June and 83.3±4.5 mm in October) than those at -5 m (62.4±3.0 mm in June and 78.5±1.3 mm in October). The same trend was observed for oysters in baskets (82.4±2.5 mm at -1 m and 76.4±3.3 mm at -5 m) and in stackable trays (82.6±2.6 mm at -1 m and 80.9±2.0 mm at -5 m, in October) during the last sampling time.

The shell width values showed a similar tendency (Fig. 4.22), with a final growth of 75.0±2.7 mm of oysters in the pôches positioned at 1 m deep that were slightly bigger than the ones at -5 m (72.4±1.2 mm). By contrast, the molluscs reared in baskets at -1 m that were smaller at the begin of the trial (59.2±2.7 mm) with respect to those at -5 m (60.7±2.6 mm), starting from they overcame the latter, reaching a final mean shell width of 73.8±1.5 mm in October, while those at -5 m measured 70.0±1.0 mm. By considering the last diagram illustrated in Fig. 4.22, it is confirmed that, at the end of the trial, also the oysters cultured in stackable trays were characterized by higher values of shell width when positioned at the depth of 1 m (75.9±1.1 mm and 73.9±1.5 mm at -1 m and -5 m,

respectively).

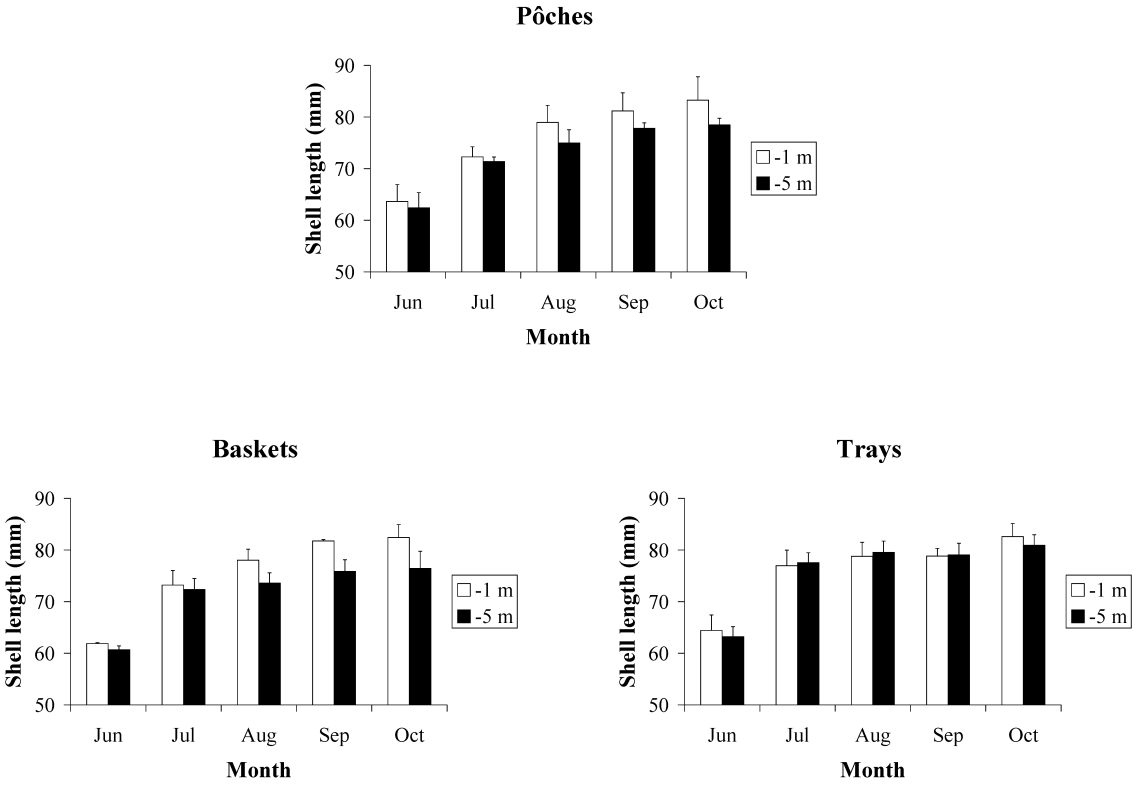

Fig. 4.21. Mean shell length (±SD) of the oysters reared in pôches, baskets and trays.

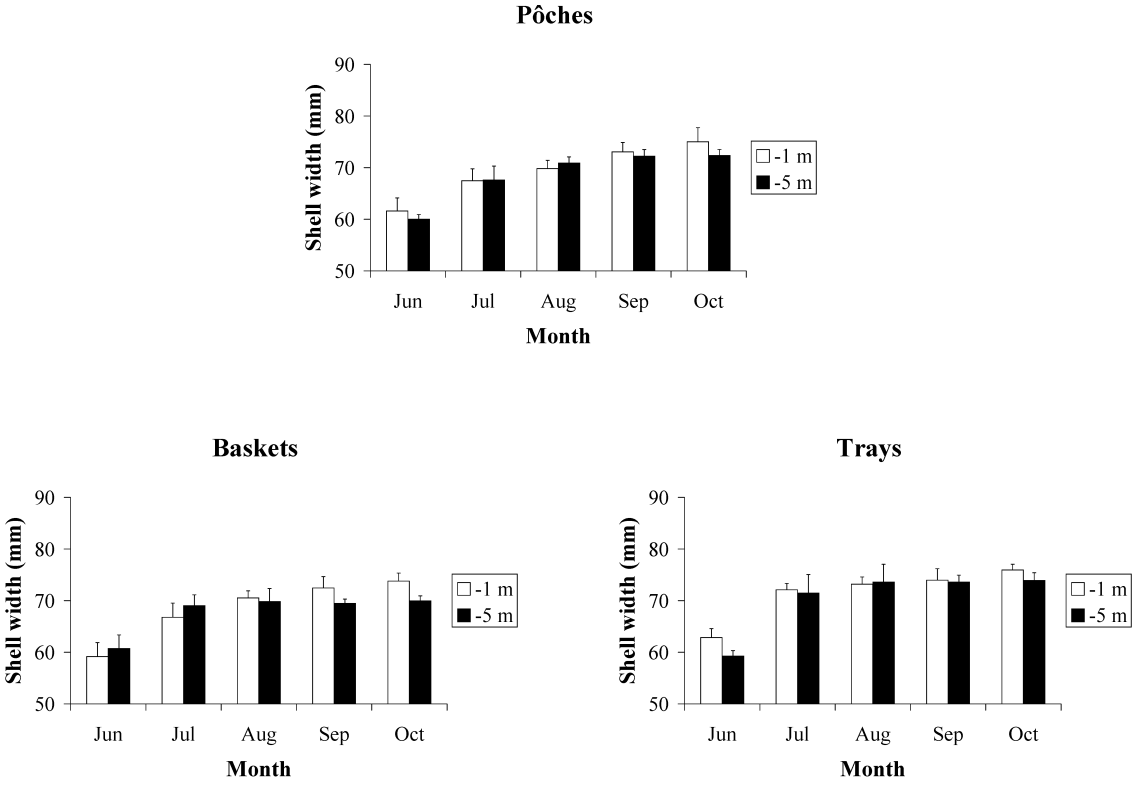

Fig. 4.22. Mean shell width (±SD) of the oysters reared in pôches, baskets and trays.

Histograms shown in Fig. 4.23 prove evidence that how total weight of oysters from each experimental group changed between the two depths considered. About pôches, data revealed that specimens were always heavier at the depth of 1 m than at -5 m, reaching 94.7±7.7 g and 78.8±2.7 g in October, respectively. Although lighter at the start of the trial (43.7±3.8 g at -1 m and 45.1±6.7 at -5 m), the oysters in baskets were heavier at -1 m than at -5 m in the sampling of October (89.9±2.6 and 81.2±8.7 g at 1 and 5 m, respectively). Similarly, the specimens in trays always exhibited higher values at 1 m of depth in all the months considered, up to 89.9±3.7 g in October.

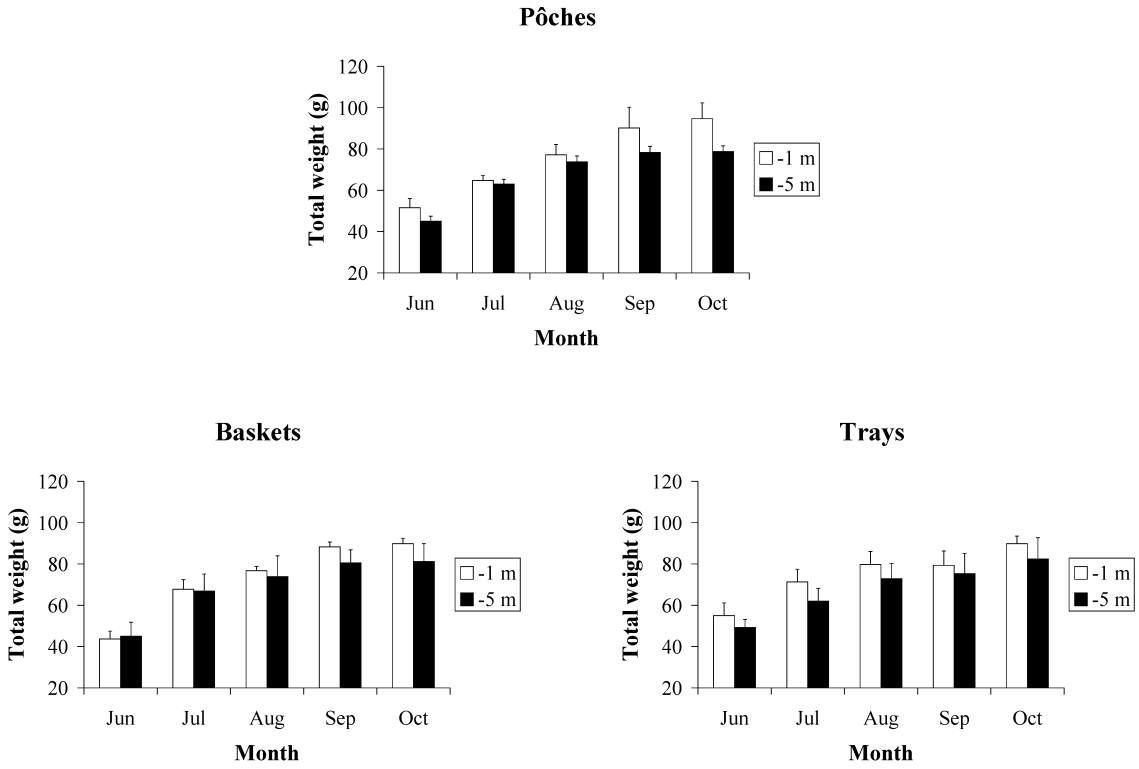

Fig. 4.23. Total weight (±SD) of the oysters reared in pôches, baskets and trays.

Looking at individual morphological variables, ANOVA performed on shell length (Tab. 4.2) revealed not-significant differences for the factor "Tool", significant results for the factor "Depth" ($F=20.91$; $p<0.01$) and any significant differences for the interaction "Tool × Depth". In particular, SNK test evidenced that shell lengths were greater in oysters reared in pôches and Australian baskets at 1 m of depth than at -5 m (Tab. 4.3). About shell width, the results of ANOVA evidenced significant differences for both the factor "Tool" and "Depth" ($F=5.10$; $p<0.01$ and $F=13.19$; $p<0.01$, respectively) but any significant results for the interaction "Tool × Depth". The

subsequent SNK test, indeed, revealed that shell widths were higher only in oysters reared in baskets at 1 m of depth. Finally, as far as total weight is concerned, ANOVA revealed not-significant differences for the factor "Tool", results significantly different for the factor "Depth" ($F=24.33$; $p<0.01$) and not-significant differences for the interaction "Tool × Depth". The SNK test showed that the total weight of oysters cultivated in all tools employed was superior in molluscs positioned at the lower depth (-1 m).

Tab. 4.2. Results of two-way ANOVA on morphological variables of *Ostrea edulis*, depending on Tool and Depth.

		Shell length			Shell width			Total weight		
Source of variation	df	MS	F	*p*	MS	F	*p*	MS	F	*p*
Tool (To)	2	81.89	2.22	0.1115	139.94	5.10	**0.0071**	21.60	0.10	0.9031
Depth (De)	1	770.87	20.91	**0.0000**	362.10	13.19	**0.0004**	5152.05	24.33	**0.0000**
To × De	2	74.62	2.02	0.1352	12.51	0.46	0.6347	309.60	1.46	0.2346
Residuals	174	36.87			27.45			211.78		
Cochran's test			0.227	ns		0.200	ns		0.210	ns
Transformation				none			none			none

significant differences at p<0.01 are marked in bold

Tab. 4.3. SNK test on the interaction of terms Tools x Depths for *Ostrea edulis* morphometric characters.

Tools	Depths	Shell length	Shell width	Total weight
Pôches	-1 m	-1 m > -5 m**	-1 m = -5 m	-1 m > -5 m**
Baskets	-5 m	-1 m > -5 m**	-1 m > -5 m**	-1 m > -5 m*
Trays		-1 m = -5 m	-1 m = -5 m	-1 m > -5 m*
		SE = 1.11	SE = 0.96	SE = 2.66

*: p<0.05; **: p<0.01

4.4 Discussion and conclusions

On the whole, the final survival rate of *Ostrea edulis* specimens from all the experimental groups reared at both the depths considered was always high and similar, confirming the excellent potential of the Porto Pozzo lagoon, in which, however, natural beds of this Bivalve species are widely found.

It is well know how much environmental factors can have relevant effects on the biology of this species (Newell *et al.*, 1977; Mann, 1979). Oysters usually live in estuarine brackish waters, where wide variations of salinity and temperature can occur. In fact, they are well adapted to tolerate low salinities down to 23 PSU, although prefer more fully saline conditions (>30 PSU) (Laing *et al.*, 2005), and can tolerate changes in salinity from 35 to 15 PSU with no effects on their feeding behaviour (Chanley, 1958). Nevertheless, Korringa (1952b) demonstrated that prolonged low salinity levels seemed to inhibit feeding activity. As far as temperature is concerned, there is no a well-defined value that governs the optimum growth rate of *O. edulis* because its wide geographical range of distribution. At our latitude, however, the temperature range of 17.5-30°C described by Davis & Calabrese (1969) seems to be the acceptable. Other studies showed that the combination of low salinity and high temperature can cause marked mortality, finding lower daily mortality rates of *O. edulis* spat when low salinity was combined with 5°C of temperature than 10°C (Rödström & Jonsson, 2000). Data recorded in the Porto Pozzo lagoon during the trial partly agreed with the above, in particular for those temperature values (ranging between about 22 and 26°C), while the salinity recorded was always higher (*i.e.* 39-42 PSU).

Also the amount of available food is a key element for Bivalve growth (Calow, 1977; Wilson, 1987; Bacher & Baud, 1992; Sims, 1993) and it varies with site or depth (Lodeiros *et al.*, 1998). In this regard, phytoplanktonic cells (whose amount was calculated as chlorophyll *a* concentration) typically tend to localize in the upper layer of the water column, where the penetration of sunlight promotes their photosynthetic activities. According to these considerations, shell length, shell width and total weight of the oysters cultured in the Porto Pozzo lagoon revealed a similar good trend for all experimental tools used. Significant differences, instead, were found between the two depths considered, where oysters superficially reared (-1 m) were characterised by higher values than those grown at -5 m. Probably, this may be due to the greater availability of food, and then chlorophyll *a*, that is usually present in the upper water layer.

In general, data reported for the Porto Pozzo lagoon were comparable with those from similar studies carried out in other Mediterranean basins. In particular, survival rates estimated for *O. edulis* in the Porto Pozzo lagoon were lower that those observed in the Adriatic Sea and in the Taranto Sea, though as in our study no significant differences in survival rates between experimental rearing conditions were observed (Zrnčić *et al.*, 2007; Carlucci *et al.*, 2010). On the other hand, survival rates were higher than those valued in the Mar Menor by Cano & Rocamora (1996).

By comparing the morphometric variables of the *O. edulis* specimens reared in the investigated lagoon, the only considerable distinction found was a higher growth rate registered in samples positioned at the lower depth. Similar results were obtained by Carlucci *et al*. (2010), who found differences between the different breeding depths considered. Nevertheless, these Authors attributed this diversity to different breeding densities, as also reported by Cano & Rocamora (1996). Zrnčić *et al.* (2007), however, demonstrated that oysters farmed at intermediate depths (about 3-5 m) showed the greatest growth values, unlike what we found in the Porto Pozzo basin. An increase of over 20 mm in shell length reached during the present study was observed not only in the some above-mentioned areas (*i.e.* Adriatic Sea) but also in in Malta (Agius *et al.*, 1978) and in the Galician coast (Mirella da Silva *et al.*, 2005). The main difference is that data reported from the other Mediterranean sites were found for at least a trialof 1 year, while those from Porto Pozzo concern only 5 months. However, it is important to point out that the specimens used in all these studies were not juveniles but adults, and therefore more able to filter (*i.e.* feed), as reported by Klaveness (1990).

Finally, commercial size of about 80 mm in length and 50-60 g in weight (Webber & Riordan, 1976), were largely achieved by the oysters reared in the Porto Pozzo lagoon during the present study, as found for the other aforementioned Mediterranean sites, confirming the excellent suitability of this basin for shellfish culture activities.

A similar investigation was previously carried out in another Sardinian lagoon (Pais *et al.*, 2007). Specifically, specimens of *O. edulis* were suspended cultured in the Calich lagoon (North western Sardinia) between March 2004 and March 2005, positioning them within a single kind of tool (*i.e.* lantern net) and at two different stations in the basin. The same morphological variables were considered and significant differences between experimental groups were found. In that case, differences were related to possible mechanical and chemical effects of water renewal by coastal waters, which may have influenced the food supplies at the two sites considered.

Given the results obtained during the whole study period, the observation that the Porto Pozzo lagoon is an excellent area for rearing *O. edulis* can be supported: the best results were achieved at the lower depth considered (-1 m), regardless of the experimental tools used. According to Bacher & Baud (1992) abundance of suspended natural food in waters and temperature are important factors influencing growth, although different conditions for this last factor were noted in relation to those reported by other Authors. Oysters cultured in the Porto Pozzo basin, in fact, showed a good condition throughout all the rearing period, even when water temperatures reached the value of 26°C. This is inconsistent with the observations reported by Azouz (1971) and Agius *et al.* (1978), who reported catastrophic mortalities in oyster cultures at temperature of 25-26°C in Tunisia and Malta, respectively. In addition, the Porto Pozzo lagoon seems a very suitable environment for the transfer of specimens from other coastal areas. The low mortality observed, in fact, was certainly due to the normal needs of oysters to adapt to new habitats, as found in similar situations (Pérez Camacho & Román, 1985; Utting, 1988; Cano & Rocamora, 1996).

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