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Ciclo XXXI

Detection of trace elements in the bivalve *Ruditapes decussatus*

from Sardinian coastal lagoons: effects on food safety

and pathological findings in target organs

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Abstract

Coastal waters and lagoons are typical environments devoted to extensive aquaculture. In Italy, which annual production is nearly 133,731 tonnes, shellfish farming is the main extensive aquaculture activity with an average production of about 119,166 tonnes in 2016 and representing over 90% of total production from transitional environments. Shellfish farming, being practiced above all in these ecosystems, is by its nature a very fragile productive sector. These environments represent the most widespread model of transition systems in the Mediterranean, whose possible pollution status must be assessed both in terms of the health status of the ecosystem and of direct or indirect risk to human health.

The anthropogenic contamination of water bodies with heavy metals *via* fertilizer, industrial sewage and urban wastewater has resulted in widespread problems in aquatic organisms, but also poses a risk to consumer health. Shellfish from coastal and estuarine environments bioaccumulate toxic metals in their tissues due to their ability to concentrate inorganic contaminants several orders of magnitude above ambient levels. Since biomonitoring by using bivalve molluscs is currently considered one of the most effective approaches for assessing the degree of pollution of coastal brackish environments, the aims of this PhD thesis were: a) to detect the content of trace elements in the Grooved carpet shell *Ruditapes decussatus* collected in Sardinian coastal lagoons (Western Mediterranean Sea, Italy) and their effects on food safety; b) to evaluate the pathological findings in its target organs.

To this end, in the first experimental contribution of the PhD thesis, wild clams were collected from five different brackish areas of Sardinia devoted to extensive aquaculture. In detail, 400 clams were sampled during autumn 2016 (100 specimens from Calich, Porto Pozzo, San Teodoro and Santa Gilla lagoons). During springer 2017 instead, 100

clams were harvested from Calich, Marceddì, Porto Pozzo and Santa Gilla lagoons (400 in total).

The concentration of 16 trace elements (Al, Ag, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sn, Tl and Zn) in the tissues of the clams was quantified. On the other hand, for the year 2017 the concentration of inorganic arsenic (iAs) was also considered as the sum of AsIII and AsV. The legal limits set by European Regulations for cadmium (Cd), mercury (Hg) and lead (Pb) were never exceeded.

However, in both years, unexpectedly high values for aluminium (Al) and iron (Fe) (mean 127 and 105; 139.4 and 97.4 mg kg^{-1} wet weight, for 2016 and 2017 respectively) were found in Santa Gilla lagoon, which is close to industrial settlements and had the highest values for most of the investigated chemical elements.

Nevertheless, during spring elevated values for the same metals were recorded also in Marceddì lagoon (133.1 and 106.6 mg kg^{-1} w.w., Al and Fe respectively), characterized by intensive zootechnical and agricultural activities.

In 2016, the highest values of the metalloid arsenic (As) were reported in San Teodoro and Porto Pozzo (12.8 and 9.6 mg kg^{-1} w.w., respectively) while in 2017 the major value was found for Marceddì (3.4 mg kg^{-1} w.w.) followed by Porto Pozzo and Santa Gilla lagoons (8.2 and 3.3 mg kg^{-1} w.w., respectively). As for the concentration of inorganic arsenic (%iAs/tAs) instead, the highest values were recorded in Marceddì and Santa Gilla (2.76 and 1.94 mg kg^{-1} w.w., respectively). The clam *R. decussatus* confirmed the capacity of bivalves as suitable bioindicators of trace elements pollution.

In the second experimental contribution of the PhD thesis, was evaluated the presence of degenerative and inflammatory lesions in the clams *R. decussatus* contributing to the knowledge of histopathology in the target organs (*i.e.*, digestive gland, gills and kidney).

In 2016, a histopathological survey was performed on a total of 400 specimens of *R. decussatus* from the Sardinian coastal environments taken into consideration for metal analysis too. These biotopes are characterized by presence of: large urban and industrial settlements (Santa Gilla lagoon); urban settlement (Calich lagoon) and important fisheries and/or aquaculture activities (Porto Pozzo and San Teodoro lagoons). A total of six histopathological alterations were analysed in the digestive gland, gills and kidney following a weighted condition indices approach, including hemocytic infiltration, necrosis, parasites, loss of epithelium and lamellar fusion.

Gills show the highest prevalence of lesions than digestive gland, followed by kidney for specimens harvested in all sites. Hemocytic infiltration and loss of epithelium as well as the presence of parasites were the most common alterations. Kidney infections were more similar between lagoons.

Santa Gilla and Calich lagoons showed the major prevalence of hemocytic infiltration for all organs considered and always correlated with the presence of parasites like *Perkinsus* spp. However, an important inflammatory response as well as loss of epithelium was highlighted in the gill tissues of clams from San Teodoro lagoon. In this case, the alteration was not positively correlated with the presence of parasites.

Therefore, the use of bivalve molluscs such as the clam *R. decussatus*, based on a histopathological approach should be a powerful tool in environmental monitoring plans. It is useful for understanding directly the health status of the marine organisms and indirectly the impact which different anthropogenic activities have on Sardinian coastal environments.

To date, the shellfish farming development represents a strategic importance for the Sardinia region. The expansion of this aquaculture practice could allow a possible reconversion of the workers in the traditional fishing sector which, in the last decade,

have undergone an increasing downsizing in terms of employment. Furthermore, shellfish activities can guarantee the protection of the environment without impeding economic growth through the sustainable exploitation of fish resources.

A more efficient organization of the sector, with the predisposition of appropriate programs for the control of production quality, could therefore constitute a key role for the further commercial exploitation of some bivalve species of considerable economic importance such as the Grooved carpet shell clam *R. decussatus*. In this context, the present results should be a useful tool for the protection of producers but, above all, of consumers.

In fact, if on one hand the first ones would have useful information on compliance with the food safety criteria of bivalves (essential for the preparation of quality specifications based on the control of the characteristics of healthiness of the product), the latter would benefit from further guarantees on the quality of the molluscs purchased.

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Chapter I

General Introduction

1.1 An overview of global fish production

Nowadays, the world's population continues to grow, reaching 8.6 billion in 2030 until to increase further to 9.8 billion in 2050, according to the medium variant of UN projections (UN, 2017). This will determine an increasing in fish consumption driven also by reduce wastage, urbanisation, and the improving of fish production technology.

At present, seafood are the most traded food items in the world today, with an increase in exports equal to 245% over 1976 (FAO, 2018a). China is the main fish producer and largest exporter since 2002 followed by Norway, Viet Nam and Thailand. In 2016, the European Union, the United States of America and Japan, on the contrary, together represented the largest importers of fish and fish products in the world.

As highlighted by the latest State of World Fisheries and Aquaculture report (FAO, 2018a), the global food fish consumption increased significantly at an average annual rate of 3.2% in the period 1961-2016, exceeded that of meat from all terrestrial animals (2.8%).

The world fisheries and aquaculture production combined (excluding aquatic mammals, reptiles, seaweeds and other aquatic plants) peaked at approximately 171 million tonnes in 2016, with a first sale value estimated at about EUR 310 billion (FAO, 2018a), (Tab.1.1; Fig. 1.1). In the last few years, fish provided more than 6% of all protein intake and between 17 and 20% of animal protein consumed in 2015 by the global population. The bigger consumption is related to developing countries in addition to areas close to the sea, lakes and waterways, while the lowest levels are registered in some landlocked countries. Specifically, world *per capita* apparent fish consumption grew from an average of 9.9 kg in the 1960s to 14.4 kg in the 1990s until reaching 20.2 kg in 2015, at an average rate of about 1.5% per year (FAO, 2014; FAO, 2018a).

The Food and Agriculture Organisation (FAO) and the Organisation for Economic Co-operation and Development (OECD) showed fish *per capita* apparent consumption projections for the period 2018-2027. In 2017 was consumed 20.5 kg with further growth to about 25.3 kg within 2027. In this perspective, the increase future demand for fish products can only be maintaining through effective fish stocks management and the growth of the aquaculture sector, so mitigate the increasing demand for fishmeal by animal feeds industry too (Kristofersson & Anderson, 2006; Merino *et al.*, 2012; FAO, 2018a).

Tab. 1.1 World fisheries and aquaculture production and utilization (FAO, 2018a).

Category	2011	2012	2013	2014	2015	2016
Production[a]	*Million tonnes*					
Capture						
Inland	10.7	11.2	11.2	11.3	11.4	11.6
Marine	81.5	78.4	79.4	79.9	81.2	79.3
Total Capture	**92.2**	**89.5**	**90.6**	**91.2**	**92.7**	**90.9**
Aquaculture						
Inland	38.6	42.0	44.8	46.9	48.6	51.4
Marine	23.2	24.4	25.4	26.8	27.5	28.7
Total Aquaculture	**61.8**	**66.4**	**70.2**	**73.7**	**76.1**	**80.0**
Total world fisheries and aquaculture	**154.0**	**156.0**	**160.7**	**164.9**	**168.7**	**170.9**
Utilization[b]						
Human consumption	130.0	136.4	140.1	144.8	148.4	151.2
Non-food uses	24.0	19.6	20.6	20.0	20.3	19.7
Population (billions)[c]	7.0	7.1	7.2	7.3	7.3	7.4
Per capita apparent consumption (kg)	18.5	19.2	19.5	19.9	20.2	20.3

[a] Excludes aquatic mammals, crocodiles, alligators and caimans, seaweeds and other aquatic plants.

[b] Utilization data for 2014-2016 are provisional estimates.

[c] Source of population figures: UN, 2015e.

Fig. 1.1 Trend in global capture fisheries and aquaculture production from 1950 to 2016, (FAO, 2018b).

*Excludes aquatic mammals, crocodiles, alligators and caimans, seaweeds and other aquatic plants.
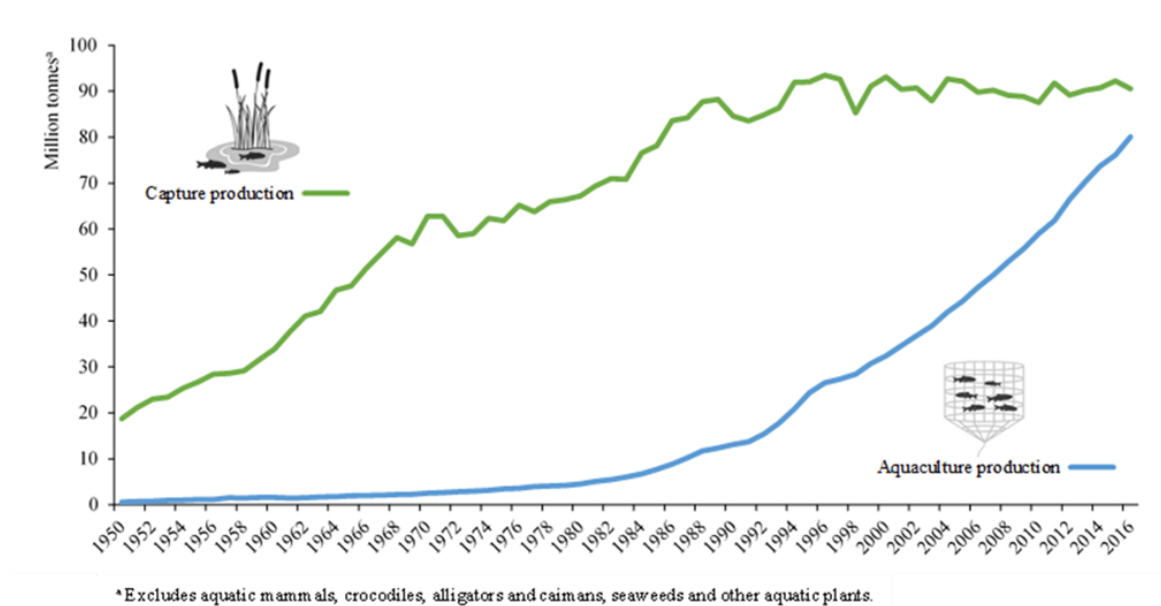
1.1.1 Production from capture fisheries

Over the last few years, the fishery sector has achieved a persistent fish production despite some marked changes in the catch trends at the country level, fishing and species areas (FAO, 2018a). However, there has been a small decrease compared to previous two years (-0.33 and -1.98% for 2014 and 2015, respectively). Global capture production recorded values of 90.9 million tonnes for the year 2016, roughly 53% of the total world fish supply (Tab. 1.1).

1.1.2 Marine capture production

The world's marine fisheries expanded continuously to a production peak of about 86 million tonnes in 1996 but have since exhibited a general declining trend. According to official statistics, world total catch stands at 79.3 million tonnes in 2016, which represent 87.2% of the global total (Tab. 1.1).

Nevertheless, it was recorded a decrease of 1.9 million tonnes compared to 2015. In 2016, the major producer countries were China, Indonesia and United States of America representing together almost 26 million tonnes of the global total (33%).

1.1.3 Inland waters capture production

The inland water catches unlike marine production showed a small constantly growing, with an increase of about 9.5% compared to 2011. Nowadays, total global production amounts to 11.6 million tonnes, representing 12.8% of total global capture fishery production (Tab. 1.1). Sixteen countries mostly Asian, produce almost 80% of the share inland fishery catch. In detail, China, India and Bangladesh were the major producer countries that together produced 4.8 million tonnes in 2016, which represent 41.4% of the global total.

1.1.4 Production from aquaculture

Despite fluctuations in supply and demand caused by the changing state of fisheries resources, the economic climate and environmental conditions, fisheries, including aquaculture, have traditionally been, and remain an important source of food, employment and revenue in many countries and communities (FAO, 2018a). The capture fishery has reached its maximum production level unlike the aquaculture growing exponentially since 1980s, with an average growth of 8.6% per year (Costa-Pierce, 2002; FAO, 2018a). Since 2000, however, world aquaculture showed most restrained annual growth rates around 5.8% in the period 2001-2016 (FAO, 2018a).

In farming activities approximately 600 aquatic species mainly finfishes (369), molluscs (109) and crustaceans (64) are cultured in different aquatic environments such as freshwater, brackish water and marine water.

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Global aquaculture production was 80 million tonnes in 2016 but raises to 110.2 million tonnes as we consider the aquatic plant, with the first sale value estimated at about EUR 208 billion (FAO, 2018a). However, a modest share of roughly 37,900 tonnes was non-food products. The total farmed food fish production included 54.1 million tonnes of finfish, 17.1 million tonnes of molluscs, 7.9 million tonnes of crustaceans and 938,500 tonnes of other aquatic animals (*e.g.*, sea cucumbers, sea urchins, etc.). The major producer in 2016 was Asia which alone comprises 89.4% of the total global (71.5 million tonnes) followed by Americas (3.3 million tonnes), Europe (3 million tonnes), Africa (2 million tonnes) and Oceania (210,000 tonnes), (Tab. 1.2).

1.1.5 Marine and coastal aquaculture

The Food and Agriculture Organisation (FAO) introduced a definition of marine aquaculture, which reduce its confusion with coastal aquaculture: "…*marine aquaculture, also known as mariculture, is practised in the sea, in a marine water environment, while coastal aquaculture is practised in completely or partially human-made structures in areas adjacent to the sea, such as coastal lagoons and gated lagoons. In coastal aquaculture with saline water, the salinity is less stable than in mariculture because of rainfall or evaporation, depending on the season and location…*" (FAO, 2018a). In 2016, the combined mariculture and coastal aquaculture production was 28.7 million tonnes, showing a constantly increase of about 19% compared to 2011. In general, molluscs and finfish were the main groups of food fish with 16.8 and 6.5 million tonnes produced, respectively (Tab. 1.2).

1.1.6 Inland aquaculture

In 2016, inland aquaculture produced about 51 million tonnes of food fish clearly greater in relation to 2011 (38.6 million tonnes). Currently, finfish are the main food fish from inland aquaculture practices accounting for around 93% (47.5 million tonnes) of total production, where Asian countries are the major producers (43.9 million tonnes), (Tab. 1.2).

Tab. 1.2 Aquaculture production of main groups by continent (FAO, 2018a).

Category	Africa	Americas	Asia	Europe	Oceania	World
Production[a]			*Thousand tonnes, live weight*			
Inland aquaculture						
Finfish	1,954	1,072	43,983	502	5	47,516
Crustacea	0	68	2,965	0	0	3,033
Molluscs	-	-	286	-	-	286
Other aquatic animals	-	1	531	-	-	531
Subtotal	1,954	1,140	47,765	502	5	51,367
Marine and coastal aquaculture						
Finfish	17	906	3,739	1,830	82	6,575
Crustacea	5	727	4,091	0	6	4,829
Molluscs	6	574	15,550	613	112	16,853
Other aquatic animals	0	-	402	0	5	407
Subtotal	28	2,207	23,781	2,443	205	28,664
All aquaculture						
Finfish	1,972	1,978	47,722	2,332	87	54,091
Crustacea	5	795	7,055	0	7	7,862
Molluscs	6	574	15,835	613	112	17,139
Other aquatic animals	0	1	933	0	5	939
Total	1,982	3,348	71,546	2,945	210	80,031

[a] Productions refer to 2016.

1.2 Aquaculture production systems

Aquaculture is rapidly expanding primarily due to the growing demand in seafood products. Nowadays, with the term "aquaculture" is defined the farming of aquatic organisms mainly fish, crustaceans and molluscs but also aquatic plants developed in confined environments that implies some forms of intervention in the rearing process to enhance the productions (FAO, 1988). In relation to the environment, it is possible to distinguish between marine and freshwater aquaculture both classifiable in warm waters and in cold waters aquaculture. One of the most important distinction, however, is that defined based on the contribution made by human which generally classified aquaculture in two production categories: intensive and extensive.

The first practice is based on the rearing of fish in inland tanks or in marine floating cages where human intervention is necessary for feed with formulation suitable for the bred species and for stocks managements (MIPAAF, 2014). The surface/volume is variable depending on the rearing phase and the typical characteristics of the system, approximately from 25 to 20,000 m^3 with a breeding density averaged 20-30 kg/m^3 (Cataudella & Bronzi, 2001).

Extensive aquaculture, on the contrary, cover quite large surface of water generally near to the coasts such as lagoons, delta rivers, estuaries, bays and lagoons (Anras *et al.*, 2010). Consequently, the breeding density (0.0025 kg/m^3) is very low as well as the yields, in the order of kilograms per hectare (kg/ha). Thus, this farming technique is based entirely or partially on the use of trophic resources and sure enough is regulated by minimal human intervention limited to simply catching aquatic organisms (Cataudella & Bronzi, 2001).

1.2.1 Extensive aquaculture in the Mediterranean

In the context of international cooperation, the Convention on Wetland (Ramsar, 1971) provides the framework for the conservation and wise use of wetlands and their resources. Europe currently has 1,100 sites (Ramsar Sites), with a total surface area of 27,941.725 hectares. However, the brackish ecosystems, also known as coastal transition areas, have always represented an important resource in fish production for local communities in the Mediterranean basin. Despite these environments greatly vary in some characteristics (*e.g.*, size, location, climate and management), they share many features such as shallowness, connections to the sea and ecological gradients (Unesco,1981; FAO, 2015). Nowadays, the Mediterranean basin counts roughly 400 coastal lagoons, covering a total surface of about 641,000 hectares. Since ancient times, several forms of aquaculture are traditionally practiced in these environments, including extensive aquaculture based on the use of trophic resources of these biotopes, moreover, contributing to the conservation of biodiversity. These environments are very fragile and sensitive ecosystems where productivity varies from a few to several hundred kilograms per hectare per year (kg/ha/year) usually linked to the lagoon typology and ecology.

In the recent decades, the strong anthropogenic pressure (*e.g.*, urbanisation, tourism, agriculture and industrial activities) took place a harmful effect on biotic and abiotic factors of these environments. For this reason, to evaluate their weight but also the possible impact on the consumer's health some lagoons have been extensively studied and monitored using suitable biological indicators (Lardicci *et al.*, 1997; Simboura & Zenetos, 2002; Bernard *et al.*, 2007; Moreno-González *et al.*, 2013; Chiesa *et al.*, 2018; Esposito *et al.*, 2018).

1.2.2 Extensive aquaculture in Italy

Italy is one of the largest brackish water areas in the Mediterranean basin. Along the coastline, around 198 coastal environments including wetlands, lagoons, coastal lakes, lagoons, saltmarsh, "*sacche*", delta areas and "*valli*" are distributed. At present, these environments cover a total surface of over 167,000 hectares and about 23% (≈40,000 hectares) are dedicated to extensive aquaculture practices. Of this, moreover, roughly 73,308 hectares (n=56) has currently recognized as Ramsar sites.

Overall, the brackish environments are distributed mainly in four geographic zones extremely diverse in morphology and pattern: the northern Adriatic, the south Adriatic, the central Tyrrhenian and Major Islands (*i.e.*, Sardinia and Sicily), (FAO, 2015). Therefore, almost all the main systems are in the Northern Italy, particularly in the Veneto, Emilia-Romagna and Friuli-Venezia Giulia regions (FAO, 2015).

To date, the extensive aquaculture represents over the 12% of national seafood production ranging from 40 kg/ha of Lesina lagoon to 319 kg/ha of Sardinia lagoons (Cataudella & Bronzi, 2001; MIPAAF, 2014). Notwithstanding these areas are very rich in biodiversity only a few species are of commercial interest. The production is exceedingly variable, and data began to be collected from 2008. FAO reports 119,166 tonnes of shellfish, 8,065 tonnes of fish and 6,500 tonnes of crustaceans in 2016 (FAO, 2018b). Thus, as can be seen from the above data, shellfish farming is the main extensive aquaculture activity practiced, representing over 90% of total production from wetlands. Among the bivalve, the main marketed species are: *Mytilus galloprovincialis* (Mediterranean mussel), *Ruditapes philippinarum* (Japanese carpet shell), *Chamelea gallina* (Striped venus) and *Crassostrea gigas* (Pacific cupped oyster).

A small amount of bivalve production is represented instead by: *R. decussatus* (Grooved carpet shell), *Ostrea edulis* (European flat oyster), *Callista chione* (Smooth callista),

Donax trunculus (Truncate donax)*, Venus verrucosa* (Warty venus), *Cerastoderma glaucum* (Olive green cockle) and *Solen marginatus* (European razor clam).

The remaining 30% regards fish and crustacean productions. Among fish, *Mugilidae* is one of the main families and includes *Chelon labrosus* (Ticklip mullet), *Liza aurata* (Golden grey mullet), *L. ramada* (Thinlip mullet), *L. saliens* (Leaping mullet) and *Mugil cephalus* (Grey mullet). The latter is greatly appreciated for its salted and dried roe (*i.e.*, "bottarga"). Other common fish species are *Sparus aurata* (Gilthead seabream), *Dicentrarchus labrax* (European seabass), *Anguilla anguilla* (European eel) and *Atherina boyeri* (Big-scale sand smelt), (Cataudella & Bronzi, 2001). Furthermore, a wide variety of crustaceans is fished as *Palaemon serratus* (Common prawn), *Carcinus aestuarii* (Mediterranean shore crab) and gastropods as *Bolinus brandaris* (Purple dye murex).

1.2.3 Extensive aquaculture in Sardinia

Sardinia has one of the most extended areas of lagoons and lagoons of Europe covering approximatively 1,900 km of coastline. At present, out of the 77 brackish environments, covering a total area of around 15,000 hectares, only 27 (5,700 ha) are used for extensive aquaculture. The central western coast ("*Oristanese*") is the richest in wetlands, covering a total surface of 6,000 hectares. In detail, the most important lagoons are located in Cabras (OR, 2,230 ha) and in S. Giovanni-Marceddì (OR, 1,600 ha), (Cataudella & Spagnolo, 2011; Fenza *et al.*, 2014).

Other major lagoons are distributed in the North-western area ("*Nurra*"), North East ("*Gallura - Baronia*"), South East ("*Ogliastra - Sarrabus - Gergei*"), South ("*Cagliaritano*") and South West ("*Sulcis - Iglesiente*").

In Sardinia, extensive aquaculture represents one of the main productive resources. It is chiefly based on the management of coastal lagoons and lagoons for production purposes, promoting an interaction between human activity and environmental conservation. Many aquatic organisms habit these waters but only a few species have a significant commercial interest. Generally, the productions range from 50 to 150 kg/ha/year, with a minimum of 25 and a maximum of 325 kg/ha/year (Fenza *et al.*, 2014).

Among the fish species, mullet, eel, seabass and gilthead seabream are the most represented. Regarding shellfish, the main species are mussels, oysters and clams.

In this context, it must be specified that Sardinia is the largest, if not the only producer in the Mediterranean basin, of endemic Mediterranean clam *R. decussatus* (Prioli, 2001). Nevertheless, bivalve production is characterized by the seasonality. Thus, the great part of the production is concentrated in spring and summer.

1.3 Introduction to Bivalve Molluscs

Among the Animal Kingdom, the phylum Mollusca is one of the largest and important groups represented by a wide range of both terrestrial and aquatic organisms. There are 73,000 to 76,000 described species of recent Mollusca and about 44,000 of these are found in the sea, which are surpassed only by the arthropods as the largest phylum of animals (Rosenberg, 2014), (Tab. 1.3).

Tab. 1.3 Estimate (±SE) of recent marine, freshwater and terrestrial Molluscan species diversity by Rosenberg, 2014.

Group	Estimate ±SE
Marine molluscs	43,600 ± 900
Stylommatophora	20,000 ± 1,500
Terrestrial operculates	4,200 ± 450
Other terrestrial pulmonates	180 ± 50
Freshwater gastropods	3,900 ± 100
Freshwater bivalves	1,200 ± 50
Total	**73,000 ± 3,000**

Generally, molluscs can be divided into six classes. The Gastropods are one of the most diverse groups of animals both in form, habit and habitat. They belong to a large taxonomic Class of invertebrates within the phylum Mollusca with more than 32,000 described living species and, they comprise over 40% of living molluscs. Bivalvia with roughly 8,000 species, is a Class of molluscs also known as Lamellibranch. It includes animals with laterally compressed bodies enclosed by two shell valves such as clams, oysters, cockles, mussels, scallops, and numerous other families that live both in marine and freshwater. The Class Cephalopoda comprises approximately 830 exclusively marine species such as octopus, squid and cuttlefish, what is more, the most advanced organisms

among the molluscs. Lastly, other important Classes are Polyplacophora and Scaphopoda that together contain nearly 1,400 species (Rosenberg, 2014).

In general, although most molluscs share a similar basic body plan, the group often shows a greatly diversity in form and habit (Morton, 1967; Gosling, 2004).

1.3.1 Global production of Bivalve Molluscs

Aquaculture continues to grow faster than other major food production sectors. In this scenario, the quantities deriving from unfed species have reached quite remarkable levels, representing almost 30% of the total farmed food fish, amounting to over 24 million tonnes (FAO, 2018a). In this regard, bivalve are the most heavily traded species, and demand has been rising in recent years. Specifically, these invertebrates harvested or farmed in the sea, lagoons and coastal lagoons reached a global total production of about 16 million tonnes in 2016 (FAO, 2018b).

China is the largest exporter of bivalves with an estimated production referred to 2016 equal to 13.2 million tonnes of live weight (FAO, 2018a). Clams, oysters, scallops and mussels have a greatly economic importance, besides representing a greatly share of global total bivalve production. Furthermore, their estimated production for 2016 is approximately equal to 5.4, 5.5, 2.7 and 2.2 million tonnes, respectively (Tab. 1.4). Thus, it is important to safeguard the health of the shellfish stocks applying a suitable management and conservation of the wild population.

1.3.2 Bivalve production in Europe

Despite China's exceptional bivalve productions followed by huge exports and significant local consumption, the European Union (EU) represents the largest single market for bivalves (FAO, 2018a).

The EU molluscs trade (*i.e.*, imports and exports) as increased over the past few years, reached 634,206 and 48,308 tonnes for imports and exports, respectively (EU, 2018), (Tab. 1.5). According to the latest FAO report, the total European bivalve production of fisheries and aquaculture combined reached 842,512 tonnes. In 2016, Spain was the main European producer reaching an extraordinary production of 230,870 tonnes, followed by France and Italy with respectable productions (160,870 and 119,166 tonnes, respectively). Other minor producers were: Netherlands, United Kingdom and Denmark (179,238 tonnes altogether). By far, as it shown in Tab. 1.6, the most bred species are mussels (548,147 tonnes), oysters (83,244 tonnes) and clams (41,511 tonnes).

Tab. 1.4 Global productions by production source (live weight, metric tonnes) of main bivalve groups for 2013-2016 (FAO, 2018b).

Group	2013	2014[a]	2015[a]	2016[a]	Average 2013-2016
Clams	5,066.113	5,205.130	5,263.148	5,385.934	5,230.081
Oysters	4,863.570	5,067.011	5,243.461	5,504.046	5,169.522
Scallops	2,597.626	2,637.329	2,639.180	2,677.606	2,637.935
Mussels	1,936.588	2,039.400	2,053.483	2,229.806	2,064.819
Total	14,463.897	14,948.870	15,199.272	15,797.392	15,102.358

[a] Utilization data for 2014-2016 are provisional estimates.

Tab. 1.5 Trade of fisheries and aquaculture products between the EU and non-UE countries in 2016 (EU, 2018).

	Imports		Exports	
	volume	value	volume	value
Pelagic fish	1,124.505	3,408.570	885,354	1,319.384
Salmonids	880,018	5,753.590	107,431	753,163
Other fish	1,906.508	6,883.792	419,173	1,382.956
Crustaceans	628,078	4,717.244	66,991	406,790
Molluscs	634,206	2,718.821	48,308	306,873
Non-food use products	843,478	911,084	338,406	552,435
Total EU-28	6,016.791	24,393.100	1,865.662	4,721.601

Note: volume in tonnes and value in thousands of EUR.

Tab. 1.6 European productions (live weight, metric tonnes) of main bivalve groups and principal producer countries for 2016, (FAO, 2018b).

Countries	Clams[a]	Mussels[b]	Oysters[c]	Others	Total
Denmark	-	45,130	145	6,276	51,551
France	725	57,960	64,969	37,216	160,870
Germany	-	44,506	-	-	44,506
Greece	-	23,360	50	722	24,132
Ireland	2	15,121	8,192	3,306	26,621
Italy	36,500	63,700	145	18,821	119,166
Netherlands	-	54,000	3,288	7,061	64,349
Portugal	2,536	832	633	5,920	9,921
Spain	1,695	215,948	1,448	11,779	230,870
UK	3	16,302	2,253	44,780	63,338
Others EU	50	11,288	2,121	33,729	47,188
Total	41,511	548,147	83,244	169,610	842,512

Utilization data for 2016 are provisional estimates and represent the productions of aquaculture and capture combined. Data might not correspond due to rounding. [a]sum of *Ruditapes philippinarum* and *R. decussatus*; [b]sum of *Mytilus edulis* and *M. galloprovincialis*; [c]sum of *Ostrea edulis* and *Crassostrea gigas*.

1.3.3 Current status of Bivalves production in Italy

According to the latest annual environmental report (ISPRA, 2017), in 2014 a total of 579 enterprises and 776 active aquaculture farms were census. About 37% were from freshwater and roughly 63% from marine or brackish water. According to the Regulation No 762/2008/CE, the Italian production in 2016 was 354,028 thousand tonnes. In detail, fish (193,610 tonnes), molluscs (139,707 tonnes) and crustaceans (20,711 tonnes) (FAO, 2018b), (Fig. 1.3). The total production of molluscs was second only to the production of fish (Fig. 1.3).

Fig. 1.3 National production by production source in the period 1950-2016 (FAO, 2018b).
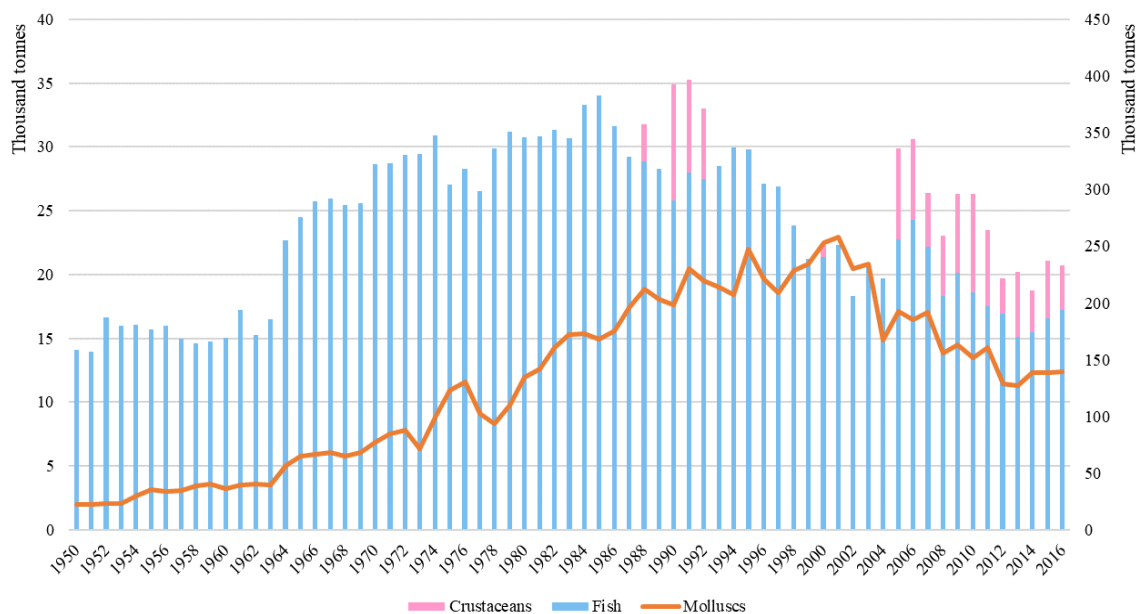
However, the Italian production reached a peak of 258,176 tonnes in the 2001, exceeding the total production of fish. Conversely, from 2002 onwards production decreased, stabilizing from 2014 to today (139,707 tonnes). Specifically, bivalve represent the main part of these productions reaching a considerable production in 2016 (Tab. 1.6), equal to 85.3% of the total mollusc productions (FAO, 2018b).

Thus, shellfish farming is currently the main production item for Italian aquaculture, chiefly based on rearing of mussels (*Mytilus galloprovincialis*), clams (*Ruditapes* spp.) and oysters (*Crassostrea gigas*), (Tab. 1.6). Although the major production is attributable to mussels, clams plays a role of primary importance, given that Italy is the first producer at European level and the second in the world (Tab. 1.7). In this context, the genus *Ruditapes* occupies a relevant place and as reported above, is represented by two different species: *R. philippinarum* (Manila clam) and *R. decussatus* (Grooved carpet shell). This latter specie is the endemic clam of the Mediterranean Sea, even if the Italian law (DM 31/01/2008) extends the name of "*Vongola verace*" to the indopacific congener *R. philippinarum*.

Nevertheless, clam productions are mainly attributable to this latest specie, voluntarily introduced in the northern Adriatic lagoons since 1983 (Cesari & Pellizzato, 1990). Nowadays, this bivalve has largely colonized the Mediterranean coasts over the years. Previous studies pointed out that that this indopacific specie has almost entirely superseded the native species *R. decussatus* (Breber, 1996; Casale *et al.*, 2001; Giovanardi *et al.*, 2002). However, Rossi (1996) highlighted that there is no inhibition of growth and survival due to the allochthonous specie.

Before the introduction of *R. philippinarum*, the Italian market absorbed about 1,000 tonnes/year of *R. decussatus* clam from areas located mainly in the upper Adriatic (Cesari & Pellizzato, 1985; Breber, 1996; Turolla, 2008). Since 1999, the production (equal to about 130 tonnes) was limited only to the Major Islands (Prioli, 2001). At present, the Grooved carpet shell clam represents the real flagship of Sardinian bivalve production and due to its refinement, is preferred by consumers despite the rather high market price (20-25 €/kg), in respect to the Manila clam, whose cost is decidedly more contained (8-12 €/kg) and whose main Italian production areas are located in the North Adriatic (Parisi

et al., 2012). Notwithstanding the foregoing, in Italy, only 3,000 tonnes were produced

in 2016 and, the largest productions were recorded in Sardinia (FAO, 2018b).

The genus *Ruditapes* is subject to European, national and regional constraints on the

minimum size of capture. According to European regulations, is equal to 25 mm

(Regulation No 1967/2006/EC). To protect and optimize the management of these

resources, the Autonomous Region of Sardinia, has a more restrictive regulation and the

minimum size is fixed at 35 mm (D.A.D.A.R.S. nr 412/1995).

Tab. 1.7 *Ruditapes* spp. productions (live weight, metric tonnes) and main producer
countries for 2016, (FAO, 2018b).

Country	*R. decussatus*	*R. philippinarum*	Total
Portugal	2,344	192	2,536
Italy	3,000	33,500	36,500
Spain	432	1,263	1,695
France	8	717	725
UK	-	3	3
Total	5,784	35,675	41,459

Utilization data for 2016 are provisional estimates. Data might not correspond due to rounding.

1.4 Controls on the production and processing

1.4.1 Classification of production and relaying areas

The competent authority must fix the location and boundaries of production and relaying

areas that it classifies. It may, where appropriate, do so in cooperation with the food

business operators. The criteria for classification are present in the Regulation No

854/2004/EC and in the Regulation No 1021/2008/EC and by cross-reference with the

Regulation No 2073/2005/EC on the microbiological criteria for foodstuffs.

Giuseppe Esposito – "Detection of trace elements in the bivalve *Ruditapes decussatus* from Sardinian coastal lagoons:
effects on food safety and pathological findings in target organs" - Tesi di Dottorato in Scienze Veterinarie,
indirizzo Qualità e Sicurezza Alimentare. Università degli Studi di Sassari.

The competent authority must classify production areas from which it authorises the harvesting of live bivalve molluscs as being of one of three categories (class A, B and C) according to the level of faecal contamination (Tab. 1.8):

Class A   areas from which live bivalve molluscs may be collected for direct human consumption. Live bivalve molluscs taken from these areas must have organoleptic characteristics associated with freshness, shells free of soil, adequate response to percussion and normal amounts of intravalvular liquid (Lee *et al*., 2008). Moreover, they must meet the health standards for live bivalve molluscs laid down in Commission Regulation No 2015/2285/EC amending Annex II to Regulation No 854/2004/EC of the European Parliament and of the Council laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. They must not exceed the limits of a five-tube, three dilution Most Probable Number (MPN) test of 230 *E. coli* per 100 g of flesh and intravalvular liquid, in 80% of samples collected during the review period. The remaining 20% of samples must not exceed 700 *E. coli* per 100 g of flesh and intravalvular liquid.

Class B   areas from which live bivalve molluscs may be collected and placed on the market for human consumption only after treatment in a purification centre or after relaying to meet the above-mentioned health standards. Live bivalve molluscs from these areas must not exceed the limits of a five-tube, three dilution MPN test of 4,600 *E. coli* per 100 g of flesh and intravalvular liquid, in 90% of samples collected during the review period. The remaining 10% of samples must not exceed 46,000 *E. coli* per 100 g of flesh and intravalvular liquid.

Class C   areas from which bivalve molluscs can be collected and placed on the market

for human consumption only after long-term relaying (from two to six

months), to meet the health requirements fixed in the Regulation No

2073/2005/EC. Furthermore, the bivalves that come from these areas should

not exceed the limits of a five-tube, three dilution MPN test of 46,000 *E. coli*

per 100 g of flesh and intravalvular liquid.

Prohibited   area not classified (>46,000 *E. coli* MPN test) and as such precluded to the

catching and rearing of live bivalve molluscs, for subsequent place on the

market for human consumption.

Tab. 1.8 Summary of the criteria for the classification of the classification of production and relaying areas of bivalve molluscs.

Class	Microbiological Standard[1]	Treatments
A	*E. coli* ≤230 per 100g of flesh and intravalvular liquid[2] *E. coli* ≤700 per 100g of flesh and intravalvular liquid[2] *Salmonella* spp. absence in 25g of flesh and intravalvular liquid	None
B	*E. coli* ≤4,600 per 100g of flesh and intravalvular liquid[3] *Salmonella* spp. absence in 25g of flesh and intravalvular liquid	Purification, relaying or transformation using recognised methods
C	*E. coli* ≤46,000 per 100g of flesh and intravalvular liquid[4] *Salmonella* spp. absence in 25g of flesh and intravalvular liquid	Relaying over a long period in classified areas or transformation using recognised methods

[1]The reference method is given by ISO 16649-3. [2]Reg. No 854/2004/EC, Reg. No 853/2004/EC and Reg. No 2285/2015/EU. [3]Reg. No 1021/2008/EC. [4]Reg. No 854/2004/EC.

1.4.2 Purification of Bivalve Molluscs

Purification is a process by which bivalve molluscs are held in tanks of clean seawater under conditions which maximize the natural filtering activity which results in expulsion of intestinal contents, which enhances separation of the expelled contaminants from the bivalve, and which prevents their recontamination (Lee *et al.*, 2008). In general, the containers utilized for depuration processes have varying in dimensions and characteristics (Gosling, 2015), (Fig. 1.4a, b).

A primary requirement for avoiding recontamination during purification is the operation of a batch "all-in/all-out" system with no more bivalve molluscs being added to the system once the purification cycle has been started. This is necessary to prevent partially depurated bivalve molluscs being re-contaminated by the material excreted from freshly introduced molluscs (Lee *et al.*, 2008). In general, the purification systems can be "flow-through" or "recirculating": in the first type, clean saltwater is supplied directly from an intake point located in an area conform to the requirements for a production area suitable for depuration and taking account of tidal flows (Lee *et al.*, 2008).

The water is typically treated with chlorine dioxide and subsequently subjected to filtration with sand and activated carbon units to lower the level of faecal contamination (Gallina *et al.*, 2013). Salt water is then conveyed to apposite tanks in which bivalve molluscs are placed such that they can undertake their normal pumping activity to get rid of intravalvular sand and faecal bacteria for a period that may range from several hours to days (Lee *et al.*, 2008). However, to avoid re-contamination at the end of purification cycles, used seawater is subjected to UV disinfection to prevent introduction of shellfish pathogens or release of toxin producing phytoplankton from imported bivalve molluscs before to be discharged into the sea. The discharge point of water for used process, finally

should be located away from the intake point so that there is no chance of the contaminated discharged water being recycled (Lee *et al*., 2008).

On the other hands, in recirculating system, natural seawater for use in purification is supplied as reported above for "flow-through" systems. Bivalve molluscs are placed in one or more high density polyethylene (HDPE) tanks stacked on top of each other (2 or 3) and supplied by a common seawater source in parallel, rather than sequentially, to prevent contaminants from one tank passing to another (Lee *et al*., 2008).

The flow of disinfected water (by means of ozone and/or UV) is preferably introduced into the tank by means of a spray bar onto the surface of the water at one end, with take of via a suction bar a few centimetres off the base of the other end of the tank (to avoid taking up sedimented material). Spray bars or other cascade systems will generally provide enough aeration to keep the dissolved oxygen content above 5 mg/l, provided that the bivalve molluscs to water ratio is sufficiently low, the flow rate is correct for the system and the water temperature is not too high (Lee *et al*., 2008).

Prior to disinfection processes, additional treatments are applied to recirculated seawater to reduce concentrations of metabolic by-products from the bivalve molluscs (such as proteins and ammonia). These include protein skimmers and biofilters.

In recirculation system using ozone and/or UV, the water will then pass through the pump and UV unit back to the spray bar (Lee *et al*., 2008). To maintain purification efficiency and to replace water lost during cleaning of systems after each cycle, if seawater is re-used from one cycle to another, a proportion of seawater is replaced with new water daily. Moreover, the entire volume of seawater is replaced on a regular basis (Lee *et al*., 2008). Nowadays, recirculating system is the most widely used and effective methods in the maintenance of viability and quality of bivalve molluscs: they provide more adequate

flow and dissolved oxygen during the purification process, avoiding temperatures that are too high or too low (Lee *et al.*, 2008; Gallina, *et al.*, 2013). Purification is effective in removing only faecal bacterial contaminants as *E. coli* from bivalve molluscs (Gallina *et al.*, 2013). A depuration for ~8 hours led to a rapid decline in the concentration of *E. coli*, complying to the Food Safety Criteria of the Regulation No 2073/2005/EC (Sferlazzo *et al.*, 2018).

Generally, the decrease in numbers of *E. coli* does not correlate with the presence of naturally occurring marine *Vibrio* (*e.g.*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*) which decline is in even slower rate. Nevertheless, the adoption of shorter treatment times for bivalve molluscs with high early counts of *Vibrio* could lead to a reduction unfitted to guarantee the safety of consumers (Sferlazzo *et al.*, 2018). As currently commercially practiced, purification it is also less effective at removing protozoa and viral contaminants such as norovirus and hepatitis (Gallina *et al*., 2013).

Regarding the presence of *Salmonella* spp., previous studies have shown that the purification process is effective if it lasts 12 to 84 hours (Son & Fleet, 1980; Timoney & Abston, 1984; Manzanares *et al*., 1990; Correa *et al*., 2007; Barile *et al*., 2009).

However, purification is not consistently effective, or is ineffective, in removing other contaminants such as marine biotoxins [*e.g.*, those causing paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP) and amnsesic shellfish poisoning (ASP) or heavy metals or organic chemicals] (Lee *et al*., 2008). Furthermore, bivalve molluscs harvested from areas classified as B and C which have not submitted to purification or relaying, may be placed on the market only after being sent to a processing plant where they must be subjected to a treatment intended to inhibit the development of pathogenic microorganisms. According to Regulation No 853/2004/EC the permitted treatments are:

1. Sterilization in hermetically sealed containers (120°C for 30 minutes);

2. Heat treatment involving immersion in boiling water for the period required to raise the internal temperature of the mollusc flesh to not less than 90°C and maintenance of this minimum temperature for a period of not less than 90 seconds;

3. Cooking for three to five minutes in an enclosed space where the temperature is between 120 and 160°C and the pressure is between 2 and 5 kg/cm^2 followed by shelling and freezing of the flesh to a core temperature of -20°C;

4. Steaming under pressure in an enclosed space for the period required to raise the internal temperature of the mollusc flesh to not less than 90°C and maintenance of this minimum temperature for a period of not less than 90 seconds. A validated methodology must be used. Procedures based on the HACCP principles must be in place to verify the uniform distribution of heat.

If the competent authority decides in principle to classify a production or relaying area:

1. It must make an inventory of the sources of pollution of human or animal origin likely to be a source of contamination for the production area;

2. Examine the quantities of organic pollutants which are released during the different periods of the year, according to the seasonal variations of both human and animal populations in the catchment area, rainfall readings, waste water treatment;

3. Determine the characteristics of the circulation of pollutants by analysis of current patterns, bathymetry and the tidal cycle in the production area;

4. Establish a sampling programme of bivalve molluscs in the production area which is based on the examination of established data, and with several samples, a geographical distribution of the sampling points and a sampling frequency which must ensure that the results of the analysis are as representative as possible for the area considered.

Fig. 1.4 An example of tank utilized during purification process: (a) Multilayer system and (b) Bulk bin system, from Gosling, 2015. Cefas. © Crown Copyright.
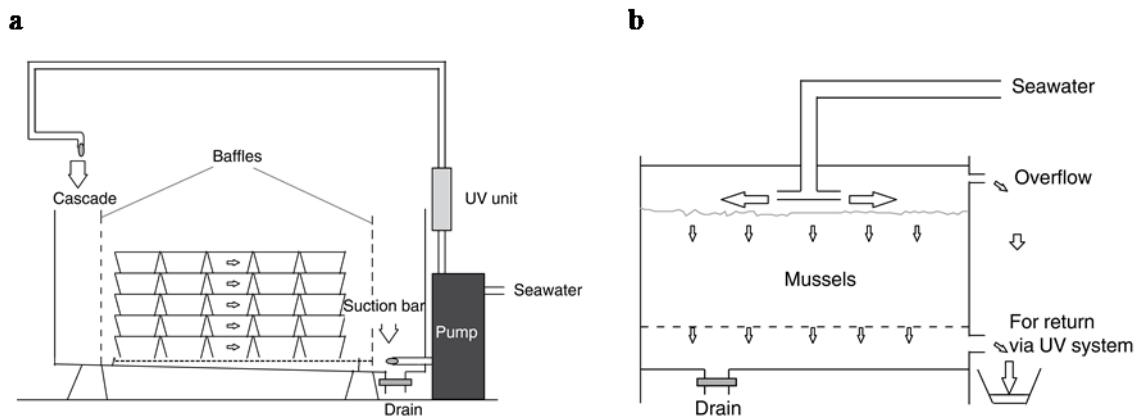
1.4.3 Monitoring of classified relaying and production areas

Classified relaying and production areas must be periodically monitored. Sampling plans must be drawn up to take place at regular intervals, or on a case-by-case basis if harvesting periods are irregular. The geographical distribution of the sampling points and the sampling frequency must ensure that the results of the analysis are as representative as possible for the area considered. The main aims of monitoring of classified relaying and production areas are:

1. *To check that there is no malpractice about the origin and destination of live bivalve molluscs*. To prevent the diffusion of exotic and non-exotic diseases affecting bivalve molluscs, the health status of the relaying and production areas must be assessed. An overall health assessment of the harvested species also for non-exotic diseases not subjected to Community measures, but having local impact, must be programmed.

2. *To evaluate the microbiological quality of live bivalve molluscs*. By means of scheduled samplings taking account of the likely variation in faecal contamination and all the available information (*e.g.*, sources of pollution, seasonal variations, bathymetry, water circulation). Microbiological monitoring aims to verify the confirmation of the sanitary status assigned during the classification (A, B, C) and consequently, to ensure the safety of the food products.

3. *To evaluate the presence of toxin-producing plankton in production and relaying waters and biotoxins in live bivalve molluscs*. Sampling plans to check for the presence of toxin-producing phytoplankton in production and relaying waters and, consequently in tissues of live bivalve molluscs must take account of possible variations in the presence of phytoplankton containing marine biotoxins. Thus, a periodic sampling to detect changes in the composition of toxic phytoplankton and their geographical distribution must comprise.

Results suggesting an accumulation of toxins in mollusc flesh must be followed by intensive sampling and periodic toxicity tests using those molluscs from the most susceptible to contamination areas. As a rule, the sampling frequency for toxin analysis in the molluscs is, on a weekly basis during the periods at which harvesting is allowed. This frequency may be reduced in specific areas, or for specific types of molluscs, if a risk assessment on toxins or phytoplankton occurrence suggests a very low risk of toxic episodes. This frequency should be increased if a risk assessment suggests that weekly samplings would not be enough. The risk assessment should be periodically reviewed to assess the risk of toxins occurring in the live bivalve molluscs from these areas. When the knowledge on toxin accumulation rates is available for a group of species growing in the same area, a species with the highest rate may be used as an indicator species.

This will allow the exploitation of all species in the group if toxin levels in the indicator species are below the regulatory limits. When toxin levels in the indicator species are above the regulatory limits, harvesting of the other species is only to be allowed if further analysis on the other species shows toxin levels below the limits. Regarding the monitoring of phytoplankton, the samples should be representative of the water column and provide information on the presence of toxic species as well as on population trends. If any changes in toxic populations that may lead to toxin accumulation are detected, the sampling frequency of molluscs should be increased, or precautionary closures of the areas are to be established until results of toxin analysis are obtained.

If the Competent Authority (CA) closes a production area because of the presence of phytoplankton or excessive levels of toxins in molluscs, at least two consecutive results below the regulatory limit separated at least 48 hours are necessary to re-open it. Besides, it may take account of information on phytoplankton trends when taking this decision. When there are robust data on the dynamic of the toxicity for a given area, and provided that recent data on decreasing trends of toxicity are available, it may decide to re-open the area with results below the regulatory limit obtained from one single sampling.

4. *To evaluate the presence of chemical contaminants in live bivalve molluscs*. Sampling plans to check for the presence of chemical contaminants must enable the detection of any overshooting of the levels laid down in Regulation No 1881/2006/EC.

   Where the results of sampling show that the health standards for molluscs are exceeded, or that there may be otherwise a risk to human health, the competent authority must close the production area concerned, preventing the harvesting of live bivalve molluscs.

However, the CA may reclassify a production area as being of Class B or C if it presents no other risk to human health. It may re-open a closed production area only if the health standards for molluscs comply once again with Community legislation.

The CA monitor classified production areas from which it has forbidden the harvesting of bivalve molluscs or subjected harvesting to special conditions, to ensure that products harmful to human health are not placed on the market. In addition to the monitoring of relaying and production zones, a control system must be set up comprising laboratory tests to verify food business operators' compliance with the requirements for the product at all stages of production, processing and distribution.

This control system is to verify that the levels of marine biotoxins and contaminants do not exceed safety limits and that the microbiological quality of the molluscs does not constitute a hazard to human health. The CA must establish and keep up to date a list of approved production and relaying areas, with details of their location and boundaries, as well as the class in which the area is classified, from which live bivalve molluscs may be taken. This list must be communicated to interested parties such as producers, gatherers and operators of purification centres and dispatch centres.

They must be immediately informed about any change of the location, boundaries or class of a production area, or its closure, be it temporary or final and act promptly where the controls indicate that a production area must be closed or reclassified or can be re-opened. To decide on the classification, opening or closure of production areas, the CA may consider the results of controls that food business operators or organisations representing food business operators have carried out. In that event, it must have designated the laboratory carrying out the analysis and, if necessary, sampling and analysis must have taken place in accordance with a protocol that the competent authority and the food business operators or organisation concerned have agreed.

1.5 Bivalves contamination and their risk as vehicles of diseases

As reported above, the main reason that makes these invertebrates potentially dangerous is enclosed in their nature of filter feeders, that is the need to continuously clear and filter the water to perform normal biological functions. These processes regulated and linearly linked to the temperature (Kittner & Riisgård, 2005 and references therein), allow them to concentrate and accumulate potentially dangerous material from the environment in which they live (Tab. 1.9). Thus, bivalves can be a potential vectors for human diseases from waterborne agents (Au, 2004; Croci *et al.*, 2005; Graczyk *et al.*, 2005; MacRae *et al.*, 2005; Martinez-Urtaza *et al.*, 2005; Quilici *et al.*, 2005; Toyofucu, 2006; Sivaperumal *et al.*, 2007; Twiner *et al.*, 2008; Etheridge, 2010; Morley, 2010; Le Roux *et al.*, 2015; Esposito *et al.*, 2018). The consumption of raw or partially cooked bivalves amplifies the risk to the consumer (Mason & McLean, 1962; Blake *et al.*, 1980; Taylor *et al.*, 2015; Hill & Dubey, 2018).

If adequately cooked, the food safety hazards are insignificant (Feldhusen, 2000). However, for some toxic compounds such as heavy metals and algal biotoxins the cooking processes have a slight influence (Domingo, 2011), (Tab. 1.9).

Generally, transmissible diseases can be infectious (*e.g.*, bacterial, viruses and parasitic protozoa) or of toxic nature (*e.g.*, chemical compounds and algal biotoxin), (Tab. 2). Nevertheless, the main hazards are present in the pre-harvest and are difficult or impossible to control by applying presently available preventive measures (Huss *et al.*, 2000).

In contrast, the hazards related to contamination, recontamination or survival of biological hazards during processing are well-defined and can be controlled by applying Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP) and a well-designed HACCP-programme (Huss *et al.*, 2000; Butt *et al.*, 2004a, b).

Tab. 1.9 The influence of cooking processes on the concentrations of inorganic and organic pollutants in seafood, modified from Domingo, 2011 and references therein.

Food	Metal	Cooking process	Main effects on metal levels
various seafood products	total and inorganic As	various procedures	↑after coking salted cod, bivalves and squid
various seafood products	Cd	steaming, baking	↓after cooking
mollusks	Cd, Pb	various processes	↓Cd bioaccessibility
fish species	Hg	various procedures	↑ 11 times higher Hg levels than those found in raw samples
fish species	Hg	deep-frying	↑Hg levels after cooking
fish and fish products	Hg	various procedures	↔no differences in Hg levels in comparison
fish species	HCB	frying, baking, charbroiling	↓in HCB levels in all processes
catfish	PCDD/Fs, co-PCBs	broiling	↓ levels in catfish
fish species	PCBs	baking, boiling, roasting, microwave, frying	↓for all the cooking procedures. The highest reduction for frying (50%)
fish and meat samples	PBDEs	broiling	↓in PBDE levels
salmon	PBDEs	various procedures	↓ 25 − 44%, depending on the procedure

Tab. 2 Main biological and chemical hazards in bivalve molluscs, adapted from Gallina *et al.*, 2013.

Thermostable chemical hazards		Thermolabile biological agents	
Biotoxins	*Chemicals*	*Bacteria and Protozoa*	*Enteric viruses*
PSP, DSP, ASP, NSP	Pb, Cd, Hg, Dioxins, PCBs, PAHs	*Salmonella, Shigella, V. parahaemolyticus, V. vulnificus, V. cholerae, Giardia, Criptosporidium, Toxoplasma*	*Norovirus, Hepatitis A, Hepatitis E*

1.5.1 Bacterial infections

The bacteria present in the water can cause foodborne infections, other poisoning and can be divided into two main groups: native and non-native flora. In detail, belong to the first group the bacteria commonly found in the aquatic environment such as *Aeromonas*, *Clostridium botulinum* (non-proteolytic types B, E, F), *Listeria monocytogenes*, *Plesiomonas shigelloides* and halophilic *Vibrio*.

Otherwise, belong to the second group all the pathogenic bacteria such as *Escherichia coli*, *Salmonella*, *Shigella*, *Campylobacter*, *Staphylococcus aureus* and *Vibrio* spp., typically deriving from human activities such as domestic sewage and land run-off. In more detail, bivalve molluscs that live in polluted brackish environment acquire pathogenic microorganism before harvest even if shellfish can also become contaminated during the storage and processing stages. Consequently, the use of monitoring programs based on faecal indicator organisms (FIOs) as surrogates for microbial pathogens, is useful to assess the microbiological quality of bivalves and the waters they inhabit (Gosling, 2015).

Also, shellfish production areas have a favourable condition (*i.e.*, low solar radiation, low temperature, low salinity, low densities of micro-predators and high levels of organic matter) for FIOs survival, abundance and distribution (Campos *et al.*, 2013; Gosling, 2015). Nevertheless, presence of microorganisms in the aquatic environment may be conditioned by environmental and hydroclimatic factors (Neogi *et al.*, 2018) as well as physical water variables (Le Saux *et al.*, 2009; Rani *et al.*, 2018).

1.5.2 Viral infections

Viruses are recognised as cause of foodborne diseases. The human outbreaks in Europe increasing as highlighted in a recent study of *Vibrio*-associated diseases caused by *Vibrio* (Le Roux *et al.*, 2015 and references therein) as well as the prevalence of human enteric viruses in shellfish (Romalde *et al.*, 2017).

Although it is very difficult to identify the etiologic agent, bacterial agents associated to human disease represent a small proportion of disease outbreaks if compared with viral agents (Gosling, 2015).

Among the enteric viruses capable of causing human disease associated with the consumption of bivalves, the viruses of Hepatitis A and E (HAV, HEV), Poliovirus, Noroviruses (NoVs), Coxsackievirus and Astrovirus should be mentioned (Gallina *et al*., 2013). However, the most frequently implicated in foodborne outbreaks linked to the consumption of bivalves are NoVs and HAV.

In general, viruses are not able to multiply in bivalves, but accumulate by a sort of bond due to ionic interactions between sulphate radicals of the mucopolysaccharides of the mollusc and to the hydrogen bonds (Gallina *et al*., 2013). If the salinity of the water decreases, the absorbing capacity of the virus increases, or the other way around (Gallina *et al*., 2013 and references therein). On the other hand, the presence of viruses in bivalves in not linked to existence of FIOs. In fact, in environments heavily contaminated by FIOs due to inhibiting action of the same, the presence of viruses results very low (Goyal *et al*., 1979).

1.5.3 Parasitic protozoa

The phylum of the Protozoa of zoonotic interest concerns, in the aquatic animal sector, essentially bivalve molluscs. *Giardia*, *Cryptosporidium* and *Toxoplasma* spp. are among the most widespread, being also particularly resistant in the external environment. They have been reported in Italy in the bivalves, even if to date, no cases of human infection due to the consumption of bivalves have been reported (Giangaspero *et al.*, 2009; Putignani *et al*., 2011; Tedde *et al*., 2013).

1.5.4 Marine phytoplankton

Microscopic planktonic algae are an important source of food for bivalve molluscs as well as for the larvae of crustaceans and finfish (FAO, 2004). There are about 5,000 known marine algal species and about 75 of these, mainly belonging to the Dinoflagellates and Diatoms classes, can produce toxic or noxious bioactive compounds (secondary metabolites or phycotoxins) that can adversely affect human, aquatic organisms or ecosystems (FAO, 2004; Landsberg, 2010). It is not clear why some micro-algal species produce toxins, probably used to compete for space, fight predation or as a defence against the overgrowth of other organisms (Botana *et al*., 1996). Massive phytoplankton proliferation is a natural phenomenon occurring cyclically when temperature and photoperiod increase simultaneously to the presence of high trophic level, giving rise to the so-called "*Harmful Algal blooms*" (HABs). Salinity is also an important factor as it determines the types of bloom species (Terlizzi & Mazzacaro, 2010). In any event, these toxic substances can find their way through levels of the food chain (*e.g.*, molluscs, crustaceans and finfish) and are ultimately consumed by humans causing a variety of gastrointestinal and neurological illnesses (FAO, 2004).

Bivalve molluscs are the most involved fish products in accumulation of the phycotoxins in their tissues during filtration. These substances, moreover, are thermostable, so cooking molluscs does not solve the problem. Nowadays, five groups of shellfish toxins have been distinguished and are classified based on their solubility (*i.e.*, hydrosoluble and fat-soluble biotoxins). Specifically, belong to the first group the amnsesic shellfish toxins causing paralytic shellfish poisoning (ASP) and the paralytic shellfish toxins causing paralytic shellfish poisoning (PSP). On the other hand, belong to the second group the diarrhoetic shellfish toxins causing diarrhoetic shellfish poisoning (DSP), azaspiracid causing azaspiracid poisoning (AZP) and the brevetoxins (NSP) causing neurologic or

neurotoxic shellfish poisoning, (FAO, 2004). The determination of toxins in bivalve molluscs is carried out by different assays (*i.e.*, in vivo, biochemical and chemical), (FAO, 2004). The permitted levels are set by the Regulation No 853/2004/EC.

Over the past few decades, as reported by the Harmful Algae Information Systems (HAIS), the frequency, intensity and global geographic distribution of HABs has increased, along with the number of toxic compounds found in the marine food chain (FAO, 2004; HAIS, 2018). Thus, according to the HAIS, the major events from 1988 to 2018 are registered for DSP (n=1,533), ASP (n=358) whereas far less for AZP and NSP (n=54). Therefore, it is necessary to set up a continuous surveillance through monitoring programs of the waters and bivalves to effectively manage the risk to consumers health.

1.5.5 Chemical compounds

The increasing worldwide contamination of environment linked to anthropogenic activity or natural chemical compounds is one of the key environmental problems facing humanity. Although most of these compounds are present at low concentrations, many of them raise considerable toxicological concerns, particularly when present as components of complex mixtures (Schwarzenbach *et al*., 2006). In this regard, water is one of the main vehicles for the transport of pollutants produced by domestic or civil (wastewater), agriculture-zootechnical and industrial activities. The knowledge about effects of pollutants in water has evolved significantly over recent years. Chemical pollution of surface water poses significant risk to the aquatic environments, with effects such as acute and chronic toxicity in aquatic organisms, accumulation of pollutants in the ecosystem, and poses a threat to human health.

Nowadays, the occurrence of priority substances (PSs) and contaminants of emerging concern (CECs) are monitored in Europe in surface water, according to the EU Directive 2013/39/EU. The procedure is based on the retrieval of ecotoxicological data on representative taxa of the aquatic environment such as algae, shellfish and fish. Thus, these aquatic organisms play a key role as biological indicators to evaluate a change in the quality of the environment due to natural or anthropic stress, through detectable variations (*i.e.*, genetic, morphological, vitality changes, etc.) of its natural state. The suitability of the species as bioindicators moreover, depends on their specific relationship to the environmental compartment (Gundacker, 2000). Within the bioindicators, it is possible to further distinguish the ecotoxicological indicators and the bioaccumulation process. An ecotoxicological indicator allow to assess environmental stresses through the study of the effects of specific substances on target organisms.

The term bioaccumulation instead, refers to the phenomenon of irreversible accumulation of a substance in the tissues of living organisms. It can occur either directly from the environment in which the organism lives or through ingestion along the trophic chains or in both ways. Specifically, in the first case the phenomenon is called bioconcentration, in the second case biomagnification. The bioconcentration is the phenomenon through which the concentrations of the substance in the tissues of the organism become progressively higher than those present in the environment from which it was absorbed. Therefore, bioconcentration factor (BCF) is defined as the equilibrium ratio between the concentration of a toxic substance in the organism and that in the surrounding. This factor varies not only based on the substance, but also is related to the species considered. In this regard, in the mussel *Unio elongatulus* subsp. *eucirrus* the BCF for cadmium (Cd) was estimated to be 2,559 while in the crayfish *Astacus leptodactylus* was equal to 88 (Varol & Sünbül, 2018).

However, a metal is bio-accumulative if its BCF value is between 1,000 and 5,000 and, very bio-accumulative if is greater than 5,000 (Costanza *et al*., 2012). On the contrary, biomagnification is the accumulation along the food chain of certain substances. Therefore, a wide array of fish bioaccumulation markers and biomarkers may be applied in order to elucidate the aquatic behaviour of environmental contaminants, as bio-concentrators to identify certain substances with low water levels and to assess exposure of aquatic organisms (van der Oost *et al*., 2003).

However, in addition to the impact of these substances on aquatic environments and consequently on the organisms that inhabit them, the potential effects that these substances may have on human health through the consumption of contaminated food must also be considered.

Therefore, the Regulation No 1881/2006/EC set maximum levels for certain contaminants in foodstuffs to protect public health and to keep contaminants at toxicologically acceptable levels.

In addition, the provisional weekly tolerable intake (PTWI) of the main forms of contaminants in food, including seafoods, are established by the European Food Safety Authority (EFSA).

1.5.5.1 Inorganic compounds

Metals like arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) are chemical compounds that exist in nature and can be found in the environment at various concentrations. However, anthropogenic activities such as industrial activities, urban settlements and agricultural-zootechnical practices can contribute to raising or spreading these pollutants. Humans can therefore be exposed to these toxic substances through the environment or by ingestion of contaminated food or water.

Giuseppe Esposito – "Detection of trace elements in the bivalve *Ruditapes decussatus* from Sardinian coastal lagoons: effects on food safety and pathological findings in target organs" - Tesi di Dottorato in Scienze Veterinarie, indirizzo Qualità e Sicurezza Alimentare. Università degli Studi di Sassari.

Because of their accumulation in the human organism, multiple effects on health, with varying degrees of severity and conditions are associated.

Data from the latest report on the RASFF (2017) indicated that of the over 3,000 notifications of irregularities received in 2017, most of them concerned fish and above all bivalve molluscs and products thereof. Although the major irregularities derived from microbiological contaminations, heavy metal contaminations were quite numerous. The most frequent reports concerned Hg (61) less than for Cd, As and Pb.

Mercury (Hg) is a metal that occurs naturally in the environments through the degassing of the earth's crust, emissions from volcanoes and evaporation from water. However, anthropogenic emissions such as coal burning, mining and other industrial activities add to the overall mercury release. Once spread, it undergoes a series of complex transformations and cycles between atmosphere, ocean and land (EFSA, 2012).

The main chemical forms in which mercury occurs in water are elemental mercury (H_0^+), inorganic forms such as mercurous (Hg_2^{2+}) and mercuric (Hg^{2+}) cations and organic compounds as methylmercury (CH_3Hg^+) and dimethylmercury [$(CH_3)_2Hg$]. Nevertheless, the occurrence of these chemical forms in the aquatic systems depends on the temperature, pH, redox potential and the concentration of inorganic and organic complex agents (Ullrich *et al*., 2001). According to several authors, the total mercury concentrations in marine ecosystems is between 0.2 and 0.5 ng/L (Cossa *et al*., 1997; Mason *et al*., 1998; Laurier *et al*., 2004) as opposed to fresh waters concentration of about 1.0 to 20 ng/L (Morel *et al*., 1998).

Furthermore, mercury undergoes a process of methylation (WHO, 1990; EFSA, 2012) mostly on sediments but also in the water column, which gives rise to compounds with higher solubility, bioavailability and toxicity to animals and humans (Stein *et al*., 1996).

This process may have different nature depending on the subjects involved and is influenced by several factors (*i.e.*, temperature, pH, etc.) that often interact. The biotic methylation is performed by both sulphate-reducing bacteria and iron-reducing bacteria (Kerin *et al.*, 2006; Slowey & Brown, 2007; Yu *et al.*, 2012; EFSA, 2012). Abiotic methylation is a pure chemical process, which is also possible when suitable methyl donors are available (Ullrich *et al.*, 2001; EFSA, 2018). However, the contribution of methylmercury to total mercury is typically less than 5% in estuarine and marine waters but can be up to 30% in fresh water (Ullrich *et al.*, 2001; EFSA, 2012). According to the latest EFSA report based on the risk for public health related to the presence of mercury and methylmercury in food, fish and other seafood products are the major food groups containing total mercury. Thus, fish meat is the dominating contributor to methylmercury dietary exposure for all age classes followed by fish products, molluscs and crustaceans. The International Agency for Research on Cancer (IARC) classified methylmercury compounds possibly carcinogenic to humans (Group 2B), while metallic mercury and inorganic mercury compounds (Group 3) as not classifiable as to its carcinogenicity to humans (IARC, 1993). The maximum assimilable dose recommended for humans is equal to PTWI of 1.3 μg/kg body weight for methylmercury (EFSA, 2012).

To protect public health, Article 2 of Council Regulation No 315/93/EEC stipulates that where necessary, maximum tolerances for specific contaminants shall be established. The current maximum levels (MLs) for mercury are laid down in the Annex, Section 3, of Commission Regulation No 1881/2006/EC. Specifically, an ML of 0.5 mg/kg wet weight applies to fishery products and muscle meat of fish (including crustaceans, excluding the brown meat of crab and excluding head and thorax meat of lobster and similar large crustaceans (Nephropidae and Palinuridae). An exception is made for muscle meat of some specific fish, and an ML of 1.0 mg/kg w. w. applies.

Other heavy metals of concern to human health through seafood consumption are cadmium, lead and arsenic. Cadmium (Cd) occurs naturally in the environment in its inorganic form because of volcanic emissions and weathering of rocks. However, anthropogenic sources such as smelting of other metals, wastewater, waste incineration and fertilizers have increased the environmental levels. In the aquatic environments is most readily absorbed in its free form (Cd^{2+}) and organisms such as fish, shellfish and crustaceans can store large quantities of this metal (EFSA, 2009a).

However, bioconcentration in fish is linked to pH value and the content of colloidal substances (John et al., 1987; ATSDR, 1999; Gallina et al., 2013).

The Rainbow trout Oncorhynchus mykiss and the Lake (=Common) whitefish Coregonus clupeaformis can accumulate from the water 0.1 and 1%, respectively (Harrison & Klaverkamp, 1989).

However, even bivalves such as Blue mussel Mytilus edulis can assimilate from 0.18 to 35% of Cd from water and 11 to 40% from phytoplankton (Wang et al., 1996; Wang & Fisher, 1997; Neff, 2002; Gallina et al., 2013).

Salinity increases the degree of complexation with chloride increases and reduces its bioaccumulation (Simpson, 1981; WHO-IPCS, 1992; EFSA, 2009a). Cadmium has no known biological function in animals and humans but mimics other divalent metals that are essential to diverse biological functions (EFSA, 2009a). The IARC classified cadmium and its compounds as carcinogenic to humans (Group 1), (IARC, 1993). It accumulates in the human body negatively affecting organs such as the liver, kidney, lung, bones, placenta, brain and the central nervous system (Castro-González & Mendez-Armenta 2008; Gosling, 2015). The Regulation No 1881/2006/EC sets several MLs for cadmium in certain foodstuffs. Specifically, an ML of 1.0 mg/kg w. w. applies to shellfish and cephalopods (without viscera).

An exception is made for crustaceans and muscle meat of some specific fish, as summarized in Tab. 2.1. The maximum assimilable dose recommended for humans is equal to PTWI of 7 µg/kg b. w. (EFSA, 2009a).

Lead (Pb) occurs naturally in the environment, but its industrial use has resulted in increased levels in soil, water and air. In the environment, inorganic lead predominates over organic lead and differs in terms of both toxicokinetics and toxicodynamics (EFSA, 2010). In the aquatic environment, lead can occur in ionic form, in organic complexes with dissolved humic materials, attached to colloidal particles such as iron oxide, or attached to solid particles of clay or dead remains of organisms (OECD, 1993; EFSA, 2010). However, the accumulation in the surface waters depends on many factors, including pH, mineral composition and amount and type of organic material.

Plants and animals may bioconcentrate lead, but lead is not biomagnified in the aquatic or terrestrial food chain so reducing the risk of lead transmission to other organisms in the food chain (Tukker *et al.*, 2001; U.S. ATSDR, 2007; EFSA, 2010). The distribution of lead within animals is often associated with their calcium turn-over. In shellfish, lead concentrations are higher in the calcium-rich shell than in the soft tissue. In teleost fish *Gillichthys mirabilis* continued accumulation rates were found for spleen and vertebrae, while a decay only for mucus-covered tissues (*i.e.*, gills, fins and intestine) due to Pb complexing with mucus (Somero *et al.*, 1977).

However, the rate of lead accumulation is related to variation in salinity and temperature, so could be significant in estuarine habitats where lead concentrations and physical variables of water are apt to vary seasonally (Somero *et al.*, 1977).

Inorganic lead compounds were classified by the IARC as probably carcinogenic to humans (Group 2A). Short-term exposure to high levels can cause brain damage, paralysis, anaemia and gastrointestinal symptoms while longer-term exposure can cause

damage to the kidneys, reproductive and immune systems in addition to effects on the nervous system (Gosling, 2015). The regulatory limit in the EU is 1.5 mg/kg w. w. for bivalve molluscs while other foodstuffs show lowest MLs (Tab. 2.2). The maximum assimilable dose recommended for humans is equal to PTWI of 25 µg/kg b. w. (EFSA, 2010).

Furthermore, a particularly dangerous metalloid is the Arsenic (As) that occurs in a broad variety of arsenic compounds, of which inorganic form (iAs) is the most toxic as compared to the organic arsenic. In the environment is released both from natural occurrence and from anthropogenic activity. However, to date most of the occurrence data in food collected in the framework of official food control are still reported as total arsenic (*i.e.*, the sum of all arsenic species) without differentiating the various arsenic species (EFSA, 2009b).

Thus, the need for speciation data is necessary to a careful risk assessment. In the marine environment is methylated and so assumed above all by crustaceans and molluscs. Therefore, seafood represents a major source of As, ranging between 5 and 250 µg g^{-1} depending on the species (Gosling, 2015). The highest concentrations of total arsenic have been found in certain crustaceans (>100 mg/kg), in marine fish (2.4-16.7 mg/kg) followed by mussels (3.5 mg/kg), (IARC, 2012). The bioaccumulation processes of As in several aquatic species, is linked to their specific sensitivity to different bioavailable heavy metals (Bonsignore *et al.*, 2018). However, arsenic organic compounds (*e.g.*, Arsenobetaine), that are of low toxicity, represent the major share of the total fraction (IPCS, 2001; Mudgal *et al*. 2010; Gosling, 2015). A recent study highlighted, that Arsenobetaine (AsB) is more efficiently assimilated and tended to be accumulated, in two deposit-feeding invertebrates (clam *Gafrarium tumidum* and polychaete *Nereis succinea*), whereas As (III) was less efficiently assimilated and more rapidly eliminated (Zhang &

Wang, 2018). Although fish products have generally low levels of inorganic arsenic, some products are excluded, namely Blue mussel *Mytilus edulis* with concentrations higher than 30 mg/kg dry weight (Sloth & Julshamn, 2008) and edible seaweed *Hizikia fusiforme* whose concentrations exceed 60 mg/kg d. w. (FSA, 2004). Arsenic and inorganic arsenic compounds were classified by the IARC as carcinogenic to humans (Group 1), (IARC, 2012). Despite different types of human exposure to arsenic, a range of adverse effects had been reported at lower exposures such as cancer of the lung and urinary bladder in addition to skin.

To date, no regulatory limits have been established for seafood. In detail, the Regulation No. 1881/2006/EC considers only an MLs for lead, cadmium, mercury and inorganic tin set for several food commodities. The maximum assimilable dose per day recommended for average consumers range from 0.13 to 0.56 µg/kg b. w. even though a risk cannot be excluded (EFSA, 2009b). Furthermore, it was also extended to feed used for animal feed (Directive 2002/32/EC) and was set the maximum content for total arsenic (Tab. 2.3).

Tab. 2.1 Cadmium maximum levels (MLs) in foodstuffs (mg/kg w. w.) according to Regulation No 1881/2006/EC.

Foodstuff	ML
1. Muscle meat of fish, excluding species listed in 2 and 3	0.05
2. Bonito (*Sarda sarda*), common two-banded seabream (*Diplodus vulgaris*), eel (*Anguilla anguilla*), grey mullet (*Mugil labrosus labrosus*), horse mackerel or scad (Trachurus spp), louvar or luvar (Luvarus imperialis), mackerel (Scomber spp), sardine (*Sardina pilchardus*), sardinops (*Sardinops* spp), tuna (*Thunnus* spp, *Euthynnus* spp, *Katsuwonus pelamis*), and wedge sole (*Dicologoglossa cuneata*)	0.10
3. Muscle meat of bullet tuna (*Auxis* spp)	0.20
4. Muscle meat of anchovy (*Engraulis* spp) and swordfish (*Xiphias gladius*)	0.30
5. Crustaceans, excluding brown meat of crab and excluding head and thorax meat of lobster and similar large crustaceans (Nephropidae and Palinuridae)	0.50
6. Bivalve molluscs	1.00
7. Cephalopods (without viscera)	1.00

Tab. 2.2 Lead maximum levels (MLs) in foodstuffs (mg/kg w. w.) according to Regulation No 1881/2006/EC.

Foodstuff	ML
1. Muscle meat of fish	0.30
2. Crustaceans, excluding brown meat of crab and excluding head and thorax meat of lobster and similar large crustaceans (Nephropidae and Palinuridae)	0.50
3. Cephalopods (without viscera)	1.00
4. Bivalve molluscs	1.50

Tab 2.3 Maximum contents for total arsenic in feed referring to a feedingstuff with a moisture content of 12%, from EFSA, 2009b.

Undesirable substance	Products intended for animal feed	Maximum Content in mg/kg relative to a feedingstuff with a moisture content of 12 %
Arsenic	Feed materials with the exception of:	2
	— meal made from grass, from dried lucerne and from dried clover, and dried sugar beet pulp and dried molasses sugar beet pulp	4
	— palm kernel expeller	4[a]
	— phosphates and calcareous marine algae	10
	— calcium carbonate	15
	— magnesium oxide	20
	— feedingstuffs obtained from the processing of fish or other marine animals	15[a]
	— seaweed meal and feed materials derived from seaweed	40[a]
	Complete feedingstuffs with the exception of	2
	— complete feedingstuffs for fish and complete feedingstuffs for fur animals	6[a]
	Complementary feedingstuffs with the exception of	4
	— mineral feedingstuffs	12

(a): Upon request of the competent authorities, the responsible operator must perform an analysis to demonstrate that the content of inorganic arsenic is lower than 2 mg/kg. This analysis is of particular importance for the seaweed species *Hizikia fusiforme*.

1.5.5.2 Organic compounds

The Persistent Organic Pollutants (POPs) are chemicals of global concern due to their chemical-physical properties (Jones & de Voogt, 1999). Specifically, POPs are characterized by a long-range transport (Tanabe *et al*., 1994), persistence in the environment, ability to bio-magnify and bioaccumulate in organisms. These toxic substances can reach potential toxicologically relevant concentrations, determining significantly harmful effects on human health as well as the environment. Therefore, since 2001, entered into force the *Stockholm Convention* based on the precautionary principle and seeks to guarantee the safe elimination of these substances, as well as reductions in their production and use (Regulation No 507/2006/EC).

Dioxins are chemical compounds composed of carbon, hydrogen, oxygen and chlorine, divided into two families: dibenzo-p-dioxins (PCDD) and dibenzo-p-furans (PCDF).

These are chlorinated aromatic hydrocarbons, mostly of anthropogenic origin (*e.g.*, incineration of solid urban waste). Their toxicity depends on the number and position of the chlorine atoms on the aromatic ring, the most toxic possess 4 chlorine atoms linked to the β carbon atoms of the aromatic ring and few or no chlorine atoms linked to the carbon atoms α of the aromatic ring. Dioxins are semi-volatile, thermostable, poorly polar, insoluble in water, highly liposoluble, extremely resistant to chemical and biological degradation. Despite being poorly water-soluble, they find in the water an excellent way of diffusion once adsorbed on the mineral and organic particles present in suspension.

The chemical-physical characteristics mentioned above, make these substances easily transportable by atmospheric currents, and, to a lesser extent, by rivers and sea currents, thus making possible the contamination of places far from the emission sources (APAT, 2006). Thus, the way of dioxins into the aquatic food chain takes place above all by the particulate that is transferred to the aquatic environment. Their nature causes them to be adsorbed to organic compounds and bioaccumulate in organisms. The amount of bioaccumulated dioxins from these organisms depends strongly, in addition to the concentration of dioxins present in the aquatic environment, on the percentage of fat content in the organism (APAT, 2006).

Dioxins are generally not detected in the different matrices as single compounds, but as complex mixtures of different congeners. However, not all congeners are toxic or are in the same way. To express the toxicity of the individual congeners, the concept of Toxic Equivalency Factor (TEF) has been introduced with respect to the toxicity of the most powerful member of this family 2, 3, 7, 8-TCDD, which is assigned a TEF of 1.0. Consequently, all other congeners have toxicity below 1.0.

Polychlorinated biphenyls (PCBs) are a series of bicyclic aromatic compounds consisting of variously chlorinated biphenyl molecules. In contrast to dioxins, PCBs have been widely used in many industrial applications.

Furthermore, some PCB congeners have chemical-physical and toxicological characteristics comparable to dioxins and furans: these are called dioxin-like PCBs (PCBdl). Other PCBs, called "non-dioxin-like PCBs", have a different mechanism of toxicity, but can also cause adverse health effects. These chemical compounds are found at low levels in many foods. However, it has been shown that prolonged exposure to these substances causes a series of adverse effects on the nervous, immune and endocrine systems, compromises reproductive function and can also cause cancer. Their persistence and the fact that they accumulate in the food chain, particularly in animal fat, continue to raise some security concerns.

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of organic compounds that have two or more fused aromatic rings. PAHs are primarily formed by incomplete combustion or pyrolysis of organic matter and during various industrial processes. Many of these compounds have toxic, mutagenic and/or carcinogenic properties and humans are exposed by various pathways. Benzo[a]pyrene can be used as a marker for the occurrence and effects of carcinogenic PAHs in food. The current MLs are laid down in the Annex, Section 6 of Commission Regulation No 1881/2006/EC setting maximum levels especially for foodstuffs containing fats and oils.

In recent years, however, some emerging organic contaminants such as microplastics, have aroused interest in the scientific community. Microplastics is a collective term used to describe a truly heterogeneous mixture of particles ranging in shape and size form (a few microns to several millimetres in diameter), (Thompson, 2015).

Microplastics originate from a variety of anthropogenic sources (*e.g.*, industrial applications). In general, these can be broadly categorized as primary (direct release of small particles) or secondary (fragmentation of larger items), (Andrady, 2003; Cole *et al*., 2011; Hidalgo-Ruz *et al*., 2012; Thompson, 2015).

This organic compound has a worldwide distribution (Barnes *et al*., 2009; Desforges *et al*., 2014; Zhao *et al*., 2014) and constituting a threat to the health of marine and freshwater ecosystems (Wright *et al*., 2013 and references therein) and consumer too (Sharma and Chatterjee, 2017). Furthermore, due to affinity with other organic compounds such a persistent organic pollutants (POPs) it makes them particularly dangerous to marine biota (Andrady, 2011). In this regard, there are no specific regulations on microplastics. Although since 2008 the EU Marine Strategy Framework included it as an aspect to be considered (MSFD, 2008/56/EC). Furthermore, following a request from the German Federal Institute for Risk Assessment (BfR), the EFSA Panel for Contaminants in the Food Chain has deliver a statement on the presence of microplastics and nanoplastics in food, with focus on seafood (EFSA, 2016).

1.6 Bivalve Molluscs as biological indicator of water pollution

Invertebrates are especially suited for monitoring of chemical stressors in the environment (Oehlmann & Schulte-Oehlmann, 2003). Therefore, these organisms defined as biological indicators, can be used to establish geographical and/or temporal variations in the bio-availabilities of heavy metals in the marine environment (Rainbow, 1995). However, the use of these indicator organisms introduces biological variables which are not present in physico-chemical studies of water or sediments (Philips, 1970). Thus, in several countries monitoring programs of coastal and estuarine areas have been

undertaken to assess the presence of chemical contaminants in the tissues of bivalves (Au, 2004; Kimbrough *et al*., 2008).

Bivalves such as *R. decussatus*, play a role of primary importance in the assessment of water contamination levels. This is related to their widespread distribution (some species are cosmopolitan), sedentary habits (sessile organisms), body size and, often, to their ecological and/or economic value.

The bivalves have developed some sub-cellular systems for the accumulation, regulation and immobilization of excess of essential and non-metallic elements (Langston *et al*., 1998). The exposure of these invertebrates to polluting elements determines their capture through mechanisms related to their ability to filter water (filter feeders). They can therefore incorporate substantial amounts of toxic metals into their organs without any apparent negative effect (Mouneyrac *et al*., 1998). The heavy metals accumulated in the edible part of the bivalve molluscs show higher concentrations in certain organs, particularly in the digestive gland or hepatopancreas, which plays an active role in their assimilation, detoxification and/or elimination (Piras *et al*., 2013).

In fact, several studies have shown that the digestive gland is the organ most involved in the metabolic and immunocompetence mechanisms of molluscs, as well as in the elimination of accumulated contaminants (Moore & Allen, 2002).

In recent decades, some of the variables that determine the accumulation of heavy metals in bivalves have been thoroughly studied. These variables include body size and sex of individuals (Cossa *et al*., 1979), season, salinity (Phillips, 1976), the organic compounds present in the water (Zamuda *et al*., 1985), the concentration of metals dissolved in water and their chemical speciation (Cobelo García *et al.*, 2003).

Among these, especially in estuarial zones, salinity seems to play a role of primary importance for the chemical speciation of some heavy metals and, consequently, for the

toxicity associated with them (Riba *et al*., 2003). These environments represent the most widespread model of transition systems in the Mediterranean, whose possible pollution status must be assessed both in terms of the health status of the ecosystem and of direct or indirect risk to human health. This also applies to certain specific contaminants, such as lead and other heavy metals, cadmium and mercury, deriving from both anthropogenic and natural origin pollution (Piras *et al*., 2013).

Therefore, marine animals living in polluted coastal waters nearness anthropized areas are more exposed to multiple toxic substances of domestic and industrial origin (Carella *et al*., 2018). Nowadays, several studies have highlighted the importance of histopathological indices and of the biomarkers integrated approach to describe the health status of bivalve molluscs and to determine environmental quality (Carella *et al*., 2018; Costa *et al*., 2013). Thus, bivalve histopathology has become an important tool in aquatic toxicology, having been implemented in many biomonitoring programmes worldwide (Cuevas *et al*., 2015).

The use of live organisms such as bivalve molluscs, to be used as bioindicators, therefore, can be a valid tool for the strengthening of monitoring plans in areas dedicated to the collection or extensive breeding of some of these species.

1.6.1 The Grooved carpet shell clam *Ruditapes decussatus*

The autochthonous clam *Ruditapes decussatus* (Linnaeus, 1758) is a marine invertebrate belonging to the Veneridae family (Rafinesque, 1815) and is classified as follows:

Phylum	*Mollusca*	
Class	Bivalvia	Linnaeus, 1758
Subclass	Heterodonta	Neumayr, 1884
Order	Veneroida	H. & A. Adams, 1815
Superfamily	Veneroidea	Rafinesque, 1815
Family	Veneridae	Rafinesque, 1815
Subfamily	Tapetinae	Gray, 1815
Genus	*Ruditapes*	Chiamenti, 1990
Specie	*Ruditapes decussatus*	Linnaeus, 1758

This bivalve mollusc is endemic to the Mediterranean Sea, also distributed along the coasts of the Atlantic, from Norway to Senegal, and along the south and west coasts of the British Isles (Tebble, 1966; Breber, 1985). It is a typically burrowing specie, found in coastal or lagoon beds, generally shallow, sandy and/or muddy (Parache, 1982). Typically, the body is characterized by an external skeleton (the shell), a mantle, a visceral mass and a muscular foot (Fig. 1.5).

1.6.2 The Shell

This organism, like all the members of the Bivalve class, has a typical bilateral symmetry and is laterally compressed with the soft body contained within a rigid shell which can reach a maximum length up to 75 mm (Gosling, 2003; FAO, 2018). It consists of two similar portions, called valves, distinct and hinged together by elastic ligaments and interlocking teeth, whose opening/closing is ensured by the presence of two strong

dorsally muscles (adductors), (Fig. 1.5). Each valve has an oval shape, owns ventrally smooth and thinner margins, more dorsally thickened. Furthermore, there is dorsally swollen part, called umbo, around which radial and concentric growth lines are found. The shell plays several functions such as the attachment of adductor muscles and mantle and represents the first defence barrier against predators or it helps to keep out exogenous substances of the mantle cavity (*e.g.*, mud and/or sand).

The external surface is rough, generally dark grey and yellowish, however, extremely variable in relation to the substrate characteristics in which the animal lives. On the contrary, the shell inner surface, is smooth and pearly white, often with yellow or bluish-purple tints. The calcium carbonate ($CaCO_3$) is the main mineral component of the shell; a small share instead represents the organic matrix (1-5% of total shell weight).

In detail, the calcium is uptaken from seawater and/or animal diet through the filtration mechanism conversely the carbonate in the animal's tissues (Gosling, 2015).

The combination of these mineral elements originates the shell in turn make up by three layers. The outer mantle fold secretes the first two layers: a thin periostracum, which covers the outer surface of the shell, composed of a fibrous insoluble protein (conchiolin) and a middle prismatic shell layer (mesostracum) contains aragonite or calcite (Kobayashi, 1969; Gosling, 2015). The inner nacreous layer is composed contrariwise only of aragonite and is secretes by the general mantle surface.

Fig. 1.5 (a) Internal anatomy of the Grooved carpet shell clam *Ruditapes decussatus*. **(b)** Internal and external features of the shell valves. Photographs modified from Manzoni, 2010.
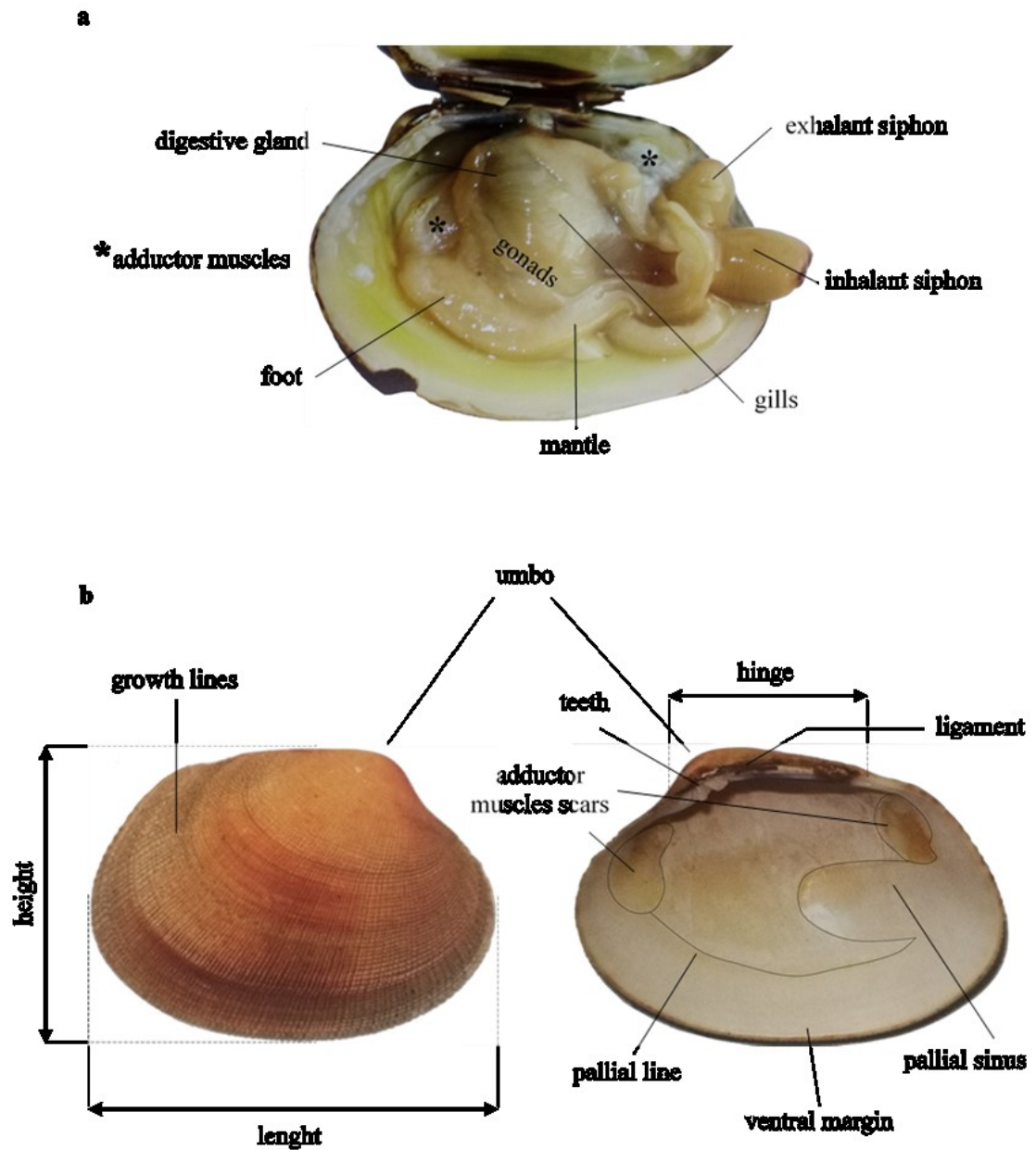
1.6.3 The Mantle

The mantle entirely wraps all organs within the shell and is attached to the inner surface by means of muscle fibres defining the line of attachment (pallial line) which in turn gives origin to a central capacious mantle cavity (pallial cavity). Anatomically, it consists in two thin and transparent lobes of connective tissue joined in dorsal position along the hinge and the cephalic hood.

The mantle may be differentiated into four distinct areas: a part near the hinge that represent the melting point of two lobes, the thin central zone, a distal zone and a thicker muscular marginal zone usually darkly pigmented and fitted with ciliated epithelium. In addition, the outer part is in turn formed of three folds: the inner fold, the middle fold and outer fold facing the inner shell surface.

The posterior mantle edges, moreover, are modified in exhalant and inhalant openings (siphons), generally darkly pigmentated, and characterized by cilia and sensory receptors at the tips. These two extensible structures are totally separated in the indigenous clam *R. decussatus*, differently to the indopacific congener *R. philippinarum*, that are fused except at the apices.

As it mentioned before, the mantle plays a key role in secreting the shell. Anyways, it performs other several important functions such as transmitting numerous external stimuli to the central nervous system (tactile sensory); initial screening of nutrition particles in directing onto the gills; contributes to energy storage (lipids and glycogen) especially during reproductive cycle; respiratory function; gametes dissemination and as well in internal defence mechanism through mucus secretioning (Blundstone, 1885; Hopkins, 1932; Elsey, 1935; Nelson, 1938; Barillè, 1994; Grizel, 2003).

1.6.3.1 Histology of the mantle

The mantle is composed mainly of muscle fibers and connective tissue crossed by numerous hemolymph vessels and nerve endings particularly well developed near the mantle edges. Columnar epithelium and cubic epithelium cover the mantle and the appearance and arrangement varies with the area considered.

Generally, it shows a pigmentated epithelial cells and it contains mucous and secretory cells localized in the subepithelial connective tissues. Furthermore, the eosinophilic granulocytes as well as the hemocytes are scattered throughout.

1.6.4 The Gills

The gills or ctenidium, flat structures usually white in colour, develop just below the mantle. These structures are suspended from the ctenidial axis that is fused along the dorsal margin of the mantle (Gosling, 2015). A cross section show the typically double V shape (or W-shaped) originated by the pair of gills (Fig. 1.6).

As it shown by the Fig. 1.6, on each side of the mollusc there are a dorsal and a ventral demibranch. In more detail, each demibranch is composed of lamella in turn divided into inner descending lamella joined directly to the visceral mass due to their branchial axis, and outer ascending lamella connected to the axis through narrow membranous bridges or fastened to the mantle (Grizel, 2003).

The lamella, moreover, are divided from each other by a narrow inter-lamellar cavity and the filaments represent the basic gill elements. In the Grooved carpet shell, a variable number of parallel, long, narrow and reflected filaments with a basic ciliary system make up each plica. Cilia on the gill filaments have different distribution beside specific functions (Atkins, 1937, 1938a, b; Owen, 1974; Owen and Mc Crae, 1976; Gosling, 2015).

Furthermore, each of these gill filaments can be distinguished into three part: frontal, intermediate and ab-frontal. Adjacent filaments are joined by interfilamental junctions. On their surface, a perforation, called ostia allows the water to enter in the gill.

Furthermore, the gills play a key role in blood hematosis, capture and subsequent uptake of nutrients from the water, especially thanks to the ciliary system and mucus cells (Grizel, 2003 and references therein).

1.6.4.1 Histology of the gills

The gills are supported mainly by muscle fibers and collagenous connective tissue, crossed by numerous hemolymph vessels. After a cross section of the lamella, it is possible to distinguish the presence of filaments, cilia and water grooves. Each filament presents a cuboidal and ciliated epithlelium rich in mucus cells. Large numbers of hemocytes and granulocytes are common along the epithelial surface. However, a longitudinal section of the gills gives a different perspective of its structures, highlighting the typical lattice formation, that contains water channels.

Fig. 1.6 The Eulamellibranch gills structures: (a) cross section of the gills. The black arrows shown the direction of water flow from inhalant (INH) and exhalant (EXH) channels. (b) Section of the filaments and (c) Transverse section showing pattern ciliation. (Modified from Gosling, 2004).
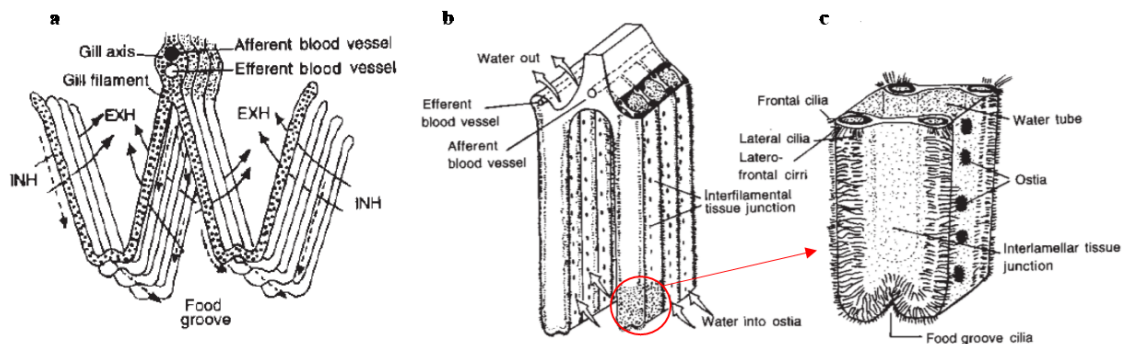
1.6.5 The Reproductive system

The reproductive system is simple and consists of paired gonadal tissue. The colour of the tissue is based on the gonads developing stages and vary from cream to white. The gonadal tissue develops in the connective tissue between the digestive gland and the muscular which encloses the mass. The gonads are crossed by branching tubules, and gametes are attached to the epithelial lining of the tubules which end in a short gonoduct (Gosling, 2015).

As most bivalves, the Grooved carpet shell clam is a dioecious specie. Notwithstanding, cases of hermaphroditism have been described, especially in the juvenile stages (Lucas, 1975; Delgado & Camacho, 2002). There is no sexual dimorphism and the only way to distinguish the sex is the histological examination. The reproductive cycle is usually annual, characterized by three phases: gametogenesis, release of gametes and reconstitution of the gonadal tissue. Fertilization is external, and the gametes are released through the siphons into the water and transported by the flow.

Generally, the spawning occurs mainly between July and October (Laurelle *et al*., 1994; Xie & Burnell, 1995). Nevertheless, in addition to endogenous factors (Gosling, 2015 and references therein), others of exogenous nature as temperature (Bayne, 1975; Chávez-Villalba *et al*., 2002), nutrients (Sastry & Blake, 1971; Navarro *et al*., 2000), salinity (Loosanoff, 1953) and photoperiod (Sastry, 1979; Fabioux *et al*., 2005) affect the reproduction activity. Detailed gametogenesis of clam *Ruditapes decussatus* has been described by several authors (Beninger & Lucas, 1984; Shafee & Daoudi, 1991; Villalba *et al*., 1993; Laurelle *et al*., 1994; Xie & Burnell, 1994; Delgado & Camacho, 2005, 2007; de Sousa *et al*., 2014). After spawning and accordingly fertilization, the first juvenile stages are planktonic (*i.e.,* trochophore larva and veliger). Subsequently, they continue

their growth in the benthic phases (*i.e.,* pediveliger and adult) until reaching the adult stage.

1.6.5.1 Histology of the gonads

The gonads development stages can be assessed through a cytologic or histological examination (De Vico & Carella, 2016). In this way, it is possible to determine a qualitative analysis of general gonads morphology. Nevertheless, the sexual maturity in relation to the sexual development of ovaries or testes can be assessed also through a quantitative examination such as the gonadosomatic index (GSI). This method expresses the gonad weight as a percentage of total body weight and so, the GSI increase during gametogenesis and decrease toward the spawning phase. Several authors reported a gonadal classification schemes for the genus *Ruditapes* (Holland & Chew, 1974; Gallois, 1977, Devauchelle, 1990; Shaffee & Daoudi, 1991). The scheme for the Grooved carpet shell *R. decussatus* as proposed by Delgado & Camacho (2005) is presented as follows:

Stage I   *Period of sexual rest*: gonadal follicles are absent and connective and muscular tissue occupies the entire zone from the digestive gland to the foot. There is no evidence of gonadal development and sex determination is not possible.

Stage II   *Initiation of gametogenesis*: follicles and gonadal acini begin to appear in female and males respectively. They increase in size and appear covered with oocytes in the growth phase in the female and with immature gametes (spermatogonia and spermatocytes) in males.

Stage III   *Advanced gametogenesis:* The follicles occupy a large part of the visceral mass. The presence of muscular and connective tissue is reduced. At the end of this stages, characterized by intense cellular growth in females, the oocytes protrude from the centre of the lumen, remaining attached to the wall via the peduncle. The abundance of free oocytes equals those attached to the wall of the follicle. In males, most of the acini were full of spermatids and spermatozoids.

Stage IV   *Reproduction period:* correspond to the maturity of most gametes. In the mature oocytes the rupture of the peduncles occurs, and the oocytes consequently occupy the follicular interior. In males, the gonadal acini mainly contain spermatozoids. Throughout this period partial spawning may occur, and it concludes with the total emission of gametes.

1.6.7 The digestive system

In general, the feeding and digestion of phytoplankton cells (microalgae), wrapped in mucus, are a continuous process in bivalves, with a well-defined rhythm (*e.g.*, *Cardium edule*), (Morton, 1970). The digestive system consists of the digestive mass that develops anteriorly, and the rectum and anus located in the posterior region. The digestive mass includes the labial palps, mouth, oesophagus, stomach, style-sac and crystalline style, digestive gland, intestinal groove, and otherwise a small intestine (midgut). Around the mouth develops triangular structures (labial palps), two on either side. Each palp is characterized by the presence of ridges, groove and ciliary tracts. Their main function is to conduct, together with the ciliary system of the gills and mantle, the food particles at the mouth, likewise promote its removal as pseudofaeces.

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Subsequently, the filtered nutrients are transported to the stomach, helped by ciliary movements into the mouth and the short oesophagus.

The stomach has a characteristic large oval shape totally set in the digestive gland, which opens into it through several ducts. A typical feature of the bivalves is the crystalline style, which originates in the style sac at the posterior end of the stomach. This rod-shaped structure is transparent and performs several important functions during the digestion process. Therefore, the rotation of the crystalline style, thanks to the ciliary complex, promotes a primary mechanical degradation (grinding against the gastric shield) of mucus food strings. Simultaneously, food particles are mixed with digestive enzymes (*e.g.*, glucanases and peptidases) released by the crystalline style. At this point, a primary extracellular digestion of the mucus-bound nutrients begins, (Nelson, 1918).

Afterwards, the finer particles are kept in suspension and carried into the digestive gland ducts. On the contrary, the larger particles move across the intestine.

The digestive gland, which visually appears brown or black, is the site of intracellular digestion and absorption (Fig. 1.7). It is involved in several functions: immune responses (Allam & Raftos, 2015), absorption and digestion of nutrients (Owen, 1955; Morton, 1970), synthesis of digestive enzymes (Ibarrola *et al.*, 1998), detoxification of xenobiotics (Petushok *et al.*, 2002) and storage of reserves (*e.g.*, carbohydrate and lipids), (Rodríguez-Moscoso & Arnaiz, 1998). Nevertheless, reserves are used main for the gametogenesis process as well as during physiological stress (De Vico & Carella, 2016).

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Fig. 1.7 (a) Eulamellibranchia schematic ducts system of the digestive diverticula. (b) A section of the digestive gland showing absorption and intracellular digestion: movement due to absorption (red arrows), particles coming from the stomach (solid arrows) and outward movement of wastes (broken arrows). Modified, from Owen, 1955.
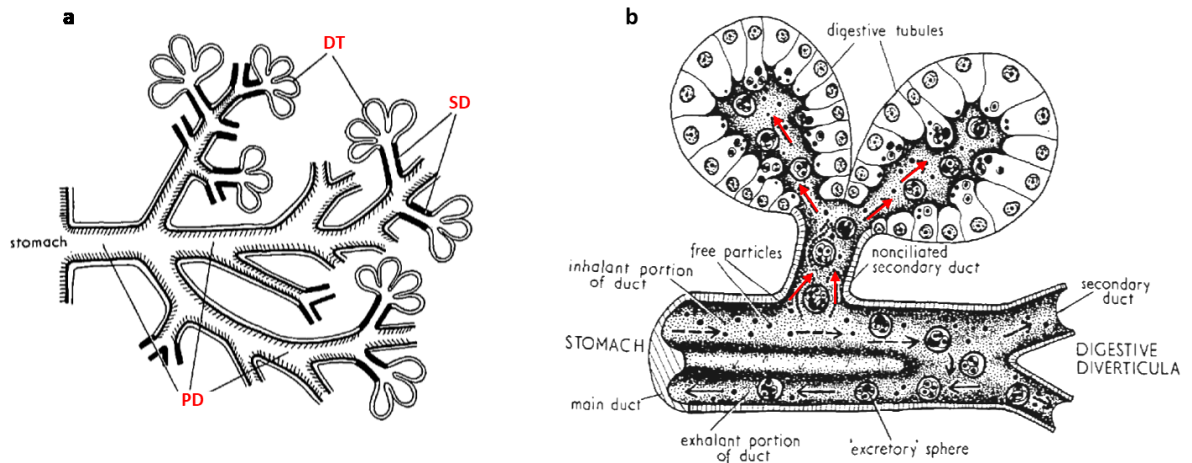
1.6.7.1 Histology of the digestive system

The labial palps have a collagenous tissue and, like the oral groove, present a ciliated columnar epithelium. The oesophagus is characterized by even layer of ciliated columnar epithelium. The apical part presents ciliated and glandular cells, with secretory granules. The appearance of the epithelium in the stomach varies.

The epithelium of the gastric shield (a chitinous sclerotized cuticle with different thickness) is characterized by tall cells with long microvilli and nucleus, muscle fibers and hemocytes. In addition, the epithelium of the sorting area is characterized by numerous grooves that divided it in epithelium of long folds and short folds.

Generally, they are characterized by ciliated cells, thick basal lamina, connective tissue and muscle fibers. The epithelium of the style sac is formed by non-secretory ciliated cells that allow the rotation of the crystalline style during digestive process.

On the contrary, the part near to the stomach is composed by tall cells with dense inclusions and vacuoles. This epithelium is composed by a basal lamina, connective tissue, muscle fibers and it is surrounded by several hemocytes. The intestine shows up with a characteristic half-moon tubular shape, is long and forms several handles. It is separated from the stomach through the typhlosoles (an infolding along the inner wall of the intestine). Generally, is composed by tall ciliated cells, some of which are glandular, and connective muscular wall.

The digestive gland is interconnected with the stomach by means of one or more primary ducts including both a ciliated and a non-ciliated region (PD) which in turn, branch into smaller, non-ciliated secondary ducts (SD) which also end in blind digestive tubules (DT), (Owen, 1955; Grizel, 2003; De Vico & Carella, 2016), (Fig. 1.8).

A cross section of a digestive tubule of *Ruditapes decussatus* during absorption phase (Morton, 1970) showing digestive cells (type A), with basal nucleus and large nucleolus, that occupy most of the tubule (Grizel, 2003) is reported in Fig. 1.8. The crypt cells or darkly staining cells (type B) are less than highly basophilic digestive cells. Furthermore, they are located at both ends and are characterized by secretory cells, flagellated cells and nest of stem cells (Grizel, 2003), (Fig. 1.8). Muscle fibers and hemocytes are distributed externally to the digestive tubule.

Fig. 1.8 (a) Schematic digestive tubule, from Owen, 1955. (b) The same cross section seen from histological examination (h&h) showing digestive cells (A) and darkly staining cells (B), modified from De Vico & Carella, 2016.
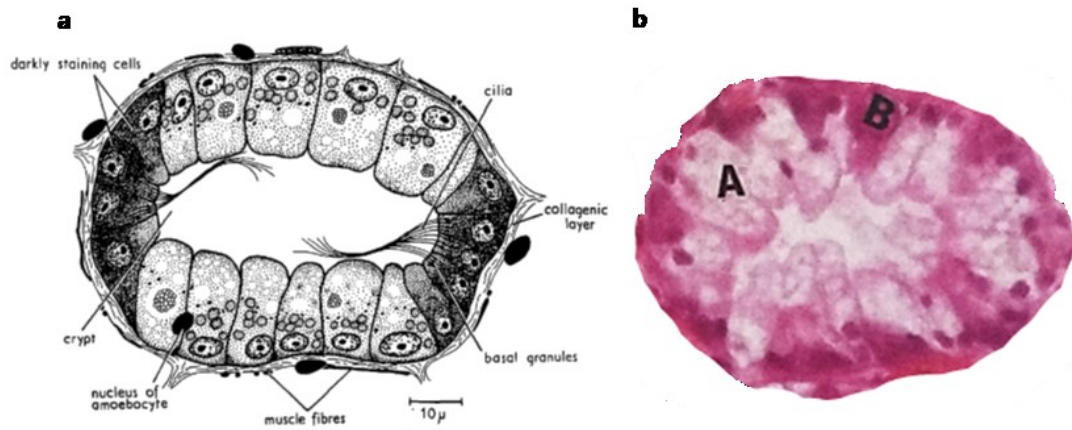
1.6.8 The cardio-vascular system

The heart, dorsally sited and enclosed by a pericardium, is composed by one ventricle and two lateral auricles. The circulatory system is of open type and is mainly made up by blood sinuses. However, the presence of capillaries provides a micro-circulation of the hemolymph.

The hemolymph contains cells called hemocytes and flows from the gills, to the heart and then proceeds through the anterior aorta which in turn divides into several arteries. Then, flows through lacunae of variable size and reaches the kidney (reno-pericardial complex) for depuration. After that, it leads to the gills through the afferent vessels and subsequently to heart with the efferent vessels.

On the other hand, the hemolymphatic system plays a key role in gas exchange, breathing process, osmoregulation, nutrient distribution, elimination of waste substances and immune defence (De Vico & Carella, 2016).

1.6.8.1 Histology of the cardio-vascular system

The ventricular wall is formed of a stratified prismatic epithelium, dense connective tissue and muscle fibers. The auricle wall is composed by a simple cubic epithelium containing pigmentary and glandular cells (Grizel, 2003), connective tissue and muscle fibers.

1.6.9 The nervous system

Bivalves do not have a central nervous system. Their nervous system is quite simple: it consists of outlying ganglia, sensory papillae, photoreceptors and cilia that have a tactile function (Lewbart, 2006; De Vico & Carella, 2016).

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Chapter II

Detection of trace elements in the bivalve *Ruditapes decussatus* from Sardinian coastal lagoons: effects on food safety

2.1 Introduction

Over the past few decades, the intensification of anthropogenic activities (*e.g.*, urban, industrial and agriculture development) has resulted in a dramatic increase of pollutants, which have strongly affected the health of aquatic ecosystems (FAO, 2009). This has been reflected in the rapid growth of a wide range of fish and shellfish diseases due to exposure to contaminants, such as inorganic compounds and xenobiotics (Au, 2004).

Aquatic organisms can accumulate polluting substances from the surrounding aquatic environment in their tissue; the extent of this accumulation is strictly related to the geographic location, species, animal size, feeding patterns, solubility and lipophilicity of the chemicals, as well as their persistence in the environment (Ahmed, 1991; Huss, 1994).

Many elements, such as selenium, iron, zinc and copper, which are present in fish and shellfish, are essential for humans at low concentrations (Reilly, 2004; Oehlenschläger, 2010; FAO, 2014). However, some of them can over-accumulate from the aquatic environment and can pose a food safety concern (FAO, 2009; EFSA 2014, 2015).

On the other hand, non-essential elements, such as cadmium, lead, mercury, and metalloids - such as arsenic - are toxic even at lower levels of exposure (Tchounwou *et al*., 2012). Moreover, the synergist action of essential and non-essential elements can occur, with negative consequences for human health (Has-Schön *et al*. 2006; Castro-González and Méndez-Armenta, 2008; FAO, 2009; Gallina *et al*., 2013).

Trace elements find different routes into the aquatic environment; bioconcentration can occur directly from water and through the uptake of suspended particles (van der Oost *et al*., 2003), and increasing levels of metals may be found in predatory species because of biomagnification (Huss, 2004). The presence of chemical compounds can be determined using widespread cosmopolitan bioindicators (Rainbow, 1995).

An organism is considered a suitable bioindicator if it can accumulate high levels of pollutants; it is sessile or constrained to a location in order to reflect local pollution; it is relevant in the food chain, abundant and widespread (Zhou *et al*., 2008). Moreover, good bioindicators should be easy to sample and identify (Rainbow and Phillips, 1993).

As shown by Burger (2006), plants, invertebrates, and fish are the main pollution bioindicators. Among invertebrates, molluscs play a primary role in assessing water contamination levels due to their ability to concentrate contaminants. Bivalve molluscs have developed some subcellular systems for the accumulation, regulation and immobilization of excessive amounts of essential and non-essential metal elements (Langston *et al*., 1998). Exposure of these invertebrates to pollutants results in the capture of these elements through mechanisms related to their ability to filter the water (filter feeders). Therefore, bivalve molluscs can bioaccumulate substantial amounts of toxic metals into their organs without any apparent negative effects (Mouneyrac *et al*., 1998).

Wetlands and coastal lagoons are very productive ecosystems but are also valuable and sensitive sites due to their unique ecological conditions (Cataudella *et al*., 2015). Thus, in moderate or heavily polluted areas that do not have enough exchange with the seas, metal concentrations found in seafood may exceed safe limits (FAO, 2014).

In the first experimental contribution part of the PhD thesis, was evaluated, for the first time, the bioaccumulation of trace metals in the autochthonous clam, *Ruditapes decussatus*, from some Sardinian brackish environments. Moreover, the compliance with maximum limits set by European Regulations for lead, mercury and cadmium (Regulation No 1881/2006/CE and amendments) were also considered.

2.2 Materials and Methods

2.2.1 Study area

The sampling areas were in a central-western part of the Mediterranean Sea, Italy (Fig. 2.1). The Sardinian brackish environments are among the most extensive in Europe. They generally have shallow water, muddy sea bottoms and moderate salinity. Among the many areas present in Sardinia, roughly 30 wetlands play an important role in fish production, covering a surface of approximately 10,000 hectares (Fenza *et al.*, 2014). Management of these wetlands is usually entrusted to cooperatives, and extensive aquaculture (carried out with traditional techniques) is a typical activity. In this study, as summarized in the Tab. 2.1, five of these biotopes were examined, namely the Porto Pozzo lagoon (pp), San Teodoro lagoon (st), (North eastern Sardinia, respectively), the Calich lagoon (ch, North western Sardinia), the Santa Gilla lagoon (sg, South Sardinia) and the Marceddì lagoon (mr, Central western Sardinia). These brackish environments will be described in detail in view of the geography of the territory and the distribution of production sites.

2.2 Lagoon characteristics

2.2.3 North western Sardinia

It is the part of Sardinia less rich in lagoons and coastal lagoons, since the different wetlands have reduced dimensions and limited significance from the point of view of fish production. The interest is more naturalistic and ornithological, since nesting and wintering habitat of migratory birds. In the peninsula of Cape Falcone there are three small lagoons: Saline, Casaraccio and Pilo. In the municipality of Alghero there is the Calich lagoon.
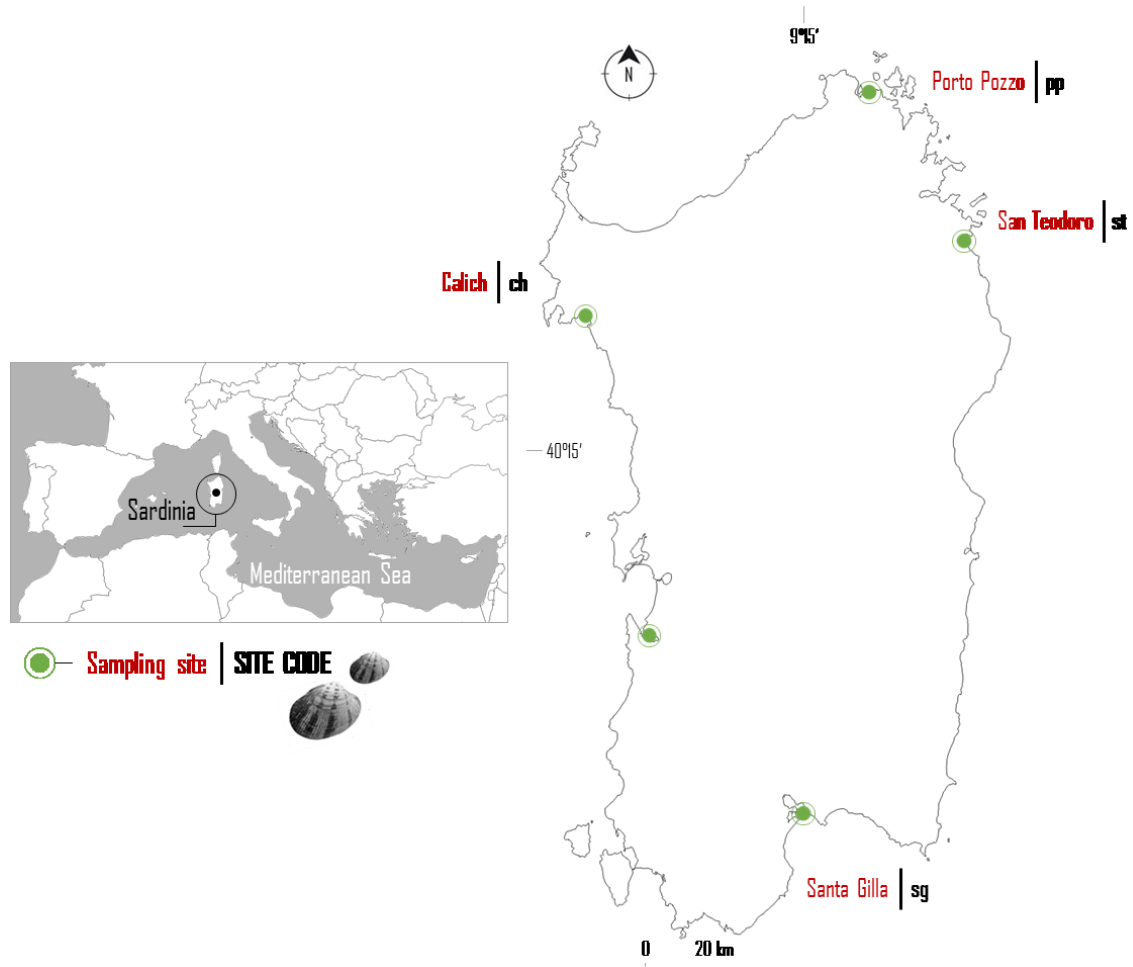
Fig. 2.1 Study area and sampling lagoons.

2.2.3.1 The Calich lagoon

The Calich lagoon (40°36′N 8°18′E) is located along the northwest coast of Sardinia behind the town of Alghero. It is a coastal wet system of 92 hectares for a length of 2,650 meters (Fig. 2.2). The Calich lagoon extends from the periphery of Alghero to the village of Fertilia. It communicates with the sea through a large canal (port of Fertilia) sixty meters wide and two meters deep and where the remains of a Roman bridge are present.

Fig. 2.2 The Calich lagoon (*), photo from www.parcodiportoconte.it.

Three waterways feed the lagoon: the Oruni channel that receives water from wastewater treatment plant of Santa Maria La Palma, the Rio Barca that brings waters from municipal sewage treatment plant located in San Marco area, and the Rio Calvia that carries water from inland cultivated territories (Fenza *et al.*, 2014). The limited supply of fresh water has favoured an increase in salinity of the lagoon with the resulting adaptation of flora and terrestrial and marine wildlife. Different selected points of the lagoon were sampled to measure salinity, temperature, pH, nitrites/nitrates, phytoplankton components and heavy metal concentrations. Collected data agree with an increase in nutrients (nitrogen and phosphorus) that could be related to the dramatic microalgae growth detected. For this reason, a better depuration of wastewaters and agricultural wastes should be considered to preserve this ecosystem (ARPAS, 2014). Although the area of the Calich lagoon is less extended and fish productive rates are lower compared to other wetlands of Sardinia, seafood production is quite varied and mullet, sea bream, sea bass, sole, eel, crabs are fished with a yield estimated at approximately 200 kg/ha/year.

2.2.4 North eastern Sardinia

In this part of the Sardinian coast (from Santa Teresa di Gallura to Orosei) are present numerous wetlands of natural beauty, which represent an ideal habitat for migratory birds. The fish production sites of major interest are the Porto Pozzo, the San Teodoro lagoon, the Sa Curcurica lagoon, the compendiun of Cedrino and Avalè-Su Petrosu.

2.2.4.1 The Porto Pozzo lagoon

The lagoon is the end of a long fjord, located in the North eastern coast, between Santa Teresa di Gallura and Palau (41°11′53.61″N 9°16′33.84″E). It covers a total surface area of 80 hectares and receives fresh water from the Rio Porto Pozzo (Fig. 2.3). Inside the lagoon there are 3 different areas called Peschiera, the deepest (10 meters) and the largest (about 36 ha), Belluscione, less deep (7 meters) and less extensive (about 12 ha) and finally a canal Padula Ciocca where the average depth is 60 cm. Bivalve molluscs is the main farming activity. Specifically, mussel farming was practiced with floating systems, long-line and the collection of bivalve molluscs including the autochthonous clam *Ruditapes decussatus*.

Fig. 2.3 The Porto Pozzo lagoon (*), photo from Fenza *et al*., 2014.

2.2.4.2 The San Teodoro lagoon

The San Teodoro lagoon (40°47′51.71″N 9°40′00.05″E) covers a surface area of 218 hectares and is located near the municipality of San Teodoro (Fig. 2.4).

This lagoon is 3.5 km long and with a depth average of 0.7 meters. Punta Sabbatino borders the lagoon in the north and the cordon dune of the Cinta beach in the east. Several tributaries feed this lagoon the main of which is the Rio San Teodoro. This lagoon is composed of two basins, the proper lagoon (200 ha) and the Pescaia basins (30 ha) that connect the lagoon to the sea. Sandy or muddy beds with granitic rock formation visible on the water surface characterize the lagoon.

The San Teodoro lagoon is comprised in two relevant areas for biodiversity (Gallura and Baronia), well known for richness in numerous vegetal and animal species.

For these reasons, the San Teodoro lagoon is considered a wetland of international importance and a place where to protect species at risk of extinction. However, the small town of San Teodoro (one of the most important tourist centres in the North-eastern coast of the Island) frequently discharges in the lagoon untreated wastewaters that potentially could affect the lagoon creating a significant impact on the environment.

These factors represent the major cause of eutrophication of waters in this lagoon (Munari & Mistri, 2007). Although this lagoon is still considered scarcely exploited for fishing, several species (mullets, eels, sea basses, sea breams and flounders) are routinely captured by the so called "*lavorieri*". In addition, there is a shellfish production of clams and oysters.

Fig. 2.4 The San Teodoro lagoon (*), photo from www.santeodoro.it.

2.2.5 Central western Sardinia

In the central part of the west coast is located the area of Oristano, one of the main Sardinians wetland, with a total area of about 6,000 hectares of water surface. Around the Gulf of Oristano there are among the most productive lagoons of the island: San Giovanni - Marceddì, Pauli Biancu Turri, Corru S'Ittiri, S'Ena Arrubia, Santa Giusta, Cabras, Mistras, Is Benas.

2.2.5.1 The Marceddì-San Giovanni lagoon

Corru s'Ittiri, San Giovanni and Marceddì are part of a lagoon system located in the southern part of the Gulf of Oristano (39°42′40.01″N 8°31′06.53″E) near Arborea Terralba, Guspini and Arbus (Fig. 2.5).

The Corru s'Ittiri lagoon covers a surface area of 120 hectares and its depth vary from 40 cm up to 2 meters. It has no natural tributaries and receives the waters that come from the agricultural land of the plain of Arborea, through irrigation canals. It is separated from the sea by a barrier, in part natural, where "lavorieri" have been built. The compendium of San Giovanni and Marceddì can be considered as a single large cove, with a total area of 1,600 hectares and depth varying from 40 cm up to 2 meters divided by means of an

artificial barrier. San Giovanni (about 700 hectares) is in the inner part of the compendium and is characterized by more fresh water (Rio Mogoro, Rio Mannu and Rio Sitzerri), while Marceddì (about 900 hectares) located in the outer part has an extensive communication with the sea. Important hydraulic works operated in the 90's and the construction of a driveway barrier, near the fishermen village of Marceddì, caused a reduction of water exchange with the sea, thus resulting in an overall lowering of the salinity of the lagoon system. The Marceddì lagoon is in an area of intensive agricultural and zootechnical activities and receives wastewaters from the surrounding watershed which can be a potential source of contaminants. The prevalent fish species are sea bream, sea bass, mullets and eels. Crab, mussels and clams are also an important economical resource. This system already identified under the Ramsar Convention Secretariat (2013) as "wetlands of international importance" is an oasis of wildlife protection and capture (Fenza *et al.,* 2014).

Fig. 2.5 The Marceddì-San Giovanni lagoon (*), photo from Fenza *et al.*, 2014.

2.2.6 South Sardinia

In the central part of the south coast is located the area of Cagliari, with a total area of about 1,830 hectares of water surface. Around the Gulf of Cagliari there are two productive ecosystems: Santa Gilla lagoon and Nora or Sant'Efisio lagoon. The latter has a small water surface of approximately 30 hectares.

2.2.6.1 The Santa Gilla lagoon

The lagoon is one of the two large wetlands located near the city of Cagliari with a surface area of about 1,800 hectares ($39°12'0''$N $09°02'0''$E), (Fig. 2.6). Located in the southern part of the Campidano plain, it is fed by the Rio Cixerri and the Flumini Mannu, while the exchange with the sea waters takes place through the opening of the "Scafa" canal. This lagoon is about 400 meters wide and nowadays is considered an ecosystem in difficulty that, despite the presence of an ever-increasing anthropic load, still maintains good production levels. Thus, the presence of significant urban and industrial settlements has involved, since the last century, profound modifications to the physical and ecological structure, with the progressive reduction of the lagoon's water surface (De Martis *et al.*, 1983).

The prevalent fish species are sea bream, sea bass, mullets and eels. Mussels and clams are also an important economical resource and a purification and dispatch centre is present.

This system is identified as a wet area to be protected: it is a special protection zone (SPA) of the European Union and a wetland of international importance under the Ramsar Convention. It is also part of the Natura 2000 ecological network.
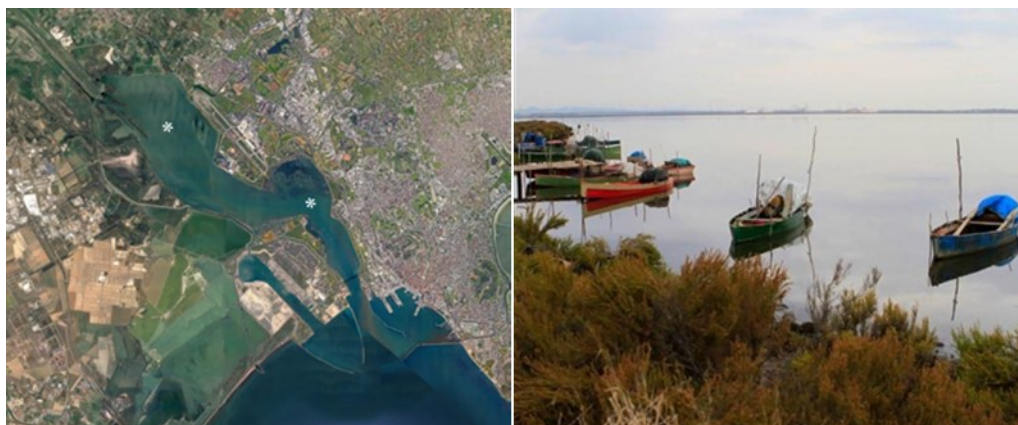
Fig. 2.6 The Santa Gilla lagoon (*), photo from www.sardegnaoggi.it.

Tab. 2.1 Geographical location, area (ha) and human activities in the Sardinian lagoons investigated.

Location	Lagoon/pond	Area (ha)	Human activities
North western	Calich	92	Near urban settlements and moderate agricultural activities. Receives water of sewage processing.
North eastern	Porto Pozzo	80	Near urban settlements and moderate agricultural activities. Receives water of sewage processing.
	San Teodoro	218	Near urban settlements. Receives water of sewage processing.
Central western	Marceddì	120	Important zootechnical - agricultural activity. Receives the waters that come from the agricultural land of the plain of Arborea, through irrigation canals.
South	Santa Gilla	1 800	Near urban and industrial settlements. Receives water of sewage processing and industrial waste, respectively.

2.2.7 Sampling procedures

Clams specimens of the species *Ruditapes decussatus* (Linnaeus, 1758), also known as the Grooved carpet shell, were collected in autumn 2016 and springer 2017. However, for the year 2017, the sampling site of San Teodoro has been replaced with the Marceddì lagoon since it was not possible to find the specimens due to important mortality, imputable probably at the high temperatures that characterized the period.

Bivalve molluscs were collected from natural banks of aquaculture facilities. Clams were manually sampled by fishermen using rakes with a plastic net at roughly a 1-meter depth on sandy bottoms. For each site, 100 adult clams were sampled (800 in total; mean±SD of total length 36±5.3 mm). In order to minimize the effect of body weight, organisms with similar weight (≈12 g) were selected. After sampling, molluscs were placed in special insulated and cooled tanks, and were then transported as quickly as possible to the laboratory. After the morphometric determination, the edible portion of each mollusc [weights ranging from 1.0 to 3.2 g, mean±SD (2.2±0.9)] was separated from the shell for subsequent chemical analysis. In detail, for each site, 10 pools consisting of 10 specimens (80 in total) were achieved and samples, subsequently were stored at -20°C until chemical analysis (Fig. 2.7).

Fig. 2.7 Example of some pool of clams used for chemical analyses.

2.2.8 Morphometric measures

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 clams 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 (*i.e.*, its edible parts) were measured (Fig. 2.8). The condition index (CI) was determined as the ratio between wet flesh weight and the sum of flesh weight and shell weight (Davenport and Chen, 1987; Peharda *et al*., 2012).

Fig. 2.8 Determination of morphometric measures.

2.2.9 Detection of trace elements

Quantification of mercury (Hg) was carried out by a Direct Mercury Analyser (DMA80, Milestone, Shelton, CT, USA), that does not require sample pre-treatment (Fig. 2.9a). Briefly, samples (≈0.12 g) were weighed in duplicate, then placed into suitable vessels and inserted into the instrument for analysis. This instrument features a circular, stainless steel, interchangeable 40-position autosampler and requires regular grade oxygen as a carrier and decomposition gas.

Samples were dried and thermally decomposed by controlled heating. Decomposition products are carried to a catalyst by oxygen flow, then sample oxidation is completed and

halogens and nitrogen/sulfur oxides are trapped. The final decomposition products pass through a mercury amalgamator which collects Hg^0. The Hg amalgamator is heated to 700 °C and the Hg^0 is released and quantified. The remaining trace elements (Al, Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, Sn, Tl and Zn) were quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS Xseries II, Thermo Scientific, Bremen, Germany) following the protocol previously described (Squadrone *et al*., 2016), (Fig. 2.9b).

Before the analysis, samples were first homogenized, then 1.5 mL H_2O_2 and 7.0 mL of HNO_3 were added to 1.0 g of samples, followed by a high-pressure microwave mineralization process (oven ETHOS 1 from Milestone, Shelton, CT, USA).

A Certified Reference Materials (Oyster Tissue- SRM 1566b from the National Institute of Standard and Technology), was processed in each analytical trial, along with blank samples. The limit of quantification (LOQ), the reference material values and the percentages of recovery obtained are shown in Tab. 2.3.
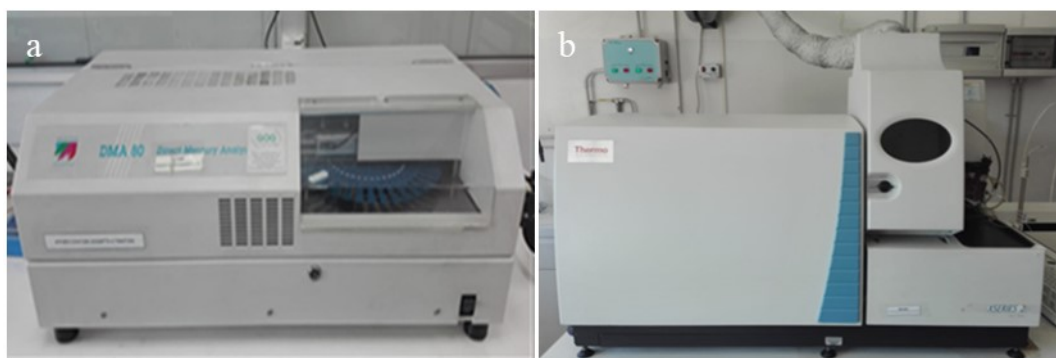
Fig. 2.9 (a) DMA 80 used for the determination of mercury (Hg). (b) ICP-MS used for the determination of trace elements.

The analytical method was validated according to ISO/IEC 17025 (general requirements for the competence of testing and calibration laboratories).

High performance liquid chromatography coupled to an inductively coupled plasma mass spectrometer (HPLC-ICP-MS), instead, was utilized to detect inorganic arsenic (iAs), intended as the sum of arsenite (As^{3+}) and arsenate (As^{5+}) following the Thermo Scientific application note n° 40741. The LOQ of this method was 0.020 mg Kg^{-1}.

Tab. 2.3 Quantification limit (mg Kg^{-1}), reference material values (oyster tissues) and percentages of recovery.

Element	LOQ	SRM 1566b	% recovery
Al	0.01	197.2 ± 6.0	92
As	0.01	7.65±0.65	101
Be	0.01	-	-
Cd	0.01	2.48±0.08	102
Ce	0.01	-	-
Co	0.01	0.371±0.009	99
Cu	0.01	71.6±1.6	99
Fe	0.01	205.8±6.8	101
Hg	0.034	0.0132±0.0013	102
La	0.01	-	-
Mn	0.01	18.5±0.2	98
Ni	0.01	1.04±0.09	96
Pb	0.01	0.308±0.009	98
Sb	0.01	-	-
Se	0.01	2.06± 0.15	110
V	0.01	0.0577 ± 0.023	104
Zn	0.01	1424±46	101

-These elements were not present in the certified material.

2.3 Statistical analyses

The analysis of descriptive statistics and graphic elaboration were carried out with $R^{®}$, version 1.0.153 (RStudio, Inc., Northern Ave, Boston, MA). The variation in the concentration of trace elements where than calculated and analysed with the following linear regression models:

$$y = site + e$$

$$y = year + e$$

where y is trace element concentrations; $site$ is the effect of sampling site (four levels in 2016: Calich, Porto Pozzo, Santa Gilla and San Teodoro; four levels in 2017: Calich, Marceddì, Porto Pozzo and Santa Gilla); $year$ is instead the effect of the month (two levels: October and April) while e express the error term.

Furthermore, a multiple comparison of means was performed with Tukey's test (95% family-wise confidence level). Results were considered statistically significant at p values of < 0.05. However, to investigate significant differences in morphometric measures and CI between months was performed using the model: $y = site + year + e$.

2.4. Results and Discussion

2.4.1 Morphometric measurements and Condition Index

Before the realization of the chemical analysis pools, 100 specimens of *Ruditapes decussatus*, from each considered site were subjected to a series of morphometric measurements. Statistical evaluation demonstrated a high significant difference between the years for ch, pp and sg lagoons (Tab. 2.3).

Looking at the boxplot in Fig. 3.1 relatively to the 2016 year, it is possible to note that the all morphometric measures except for the condition index (CI), of clams from San Teodoro lagoon (st) were always higher than those from the other sites. In particular, the following mean values (±SD) were highlighted: shell length=43.7±2.0 mm, shell height=31.0±1.4 mm, total weight=14.8±2.0 g, mollusc weight=3.3±0.7 g, and shell weight=6.8±0.9 g. As above mentioned, (see paragraph 2.2.1, Materials and Methods section), this sampling site has been replaced with Marceddì lagoon for the year 2017. Thus, it was not possible to compare it with the data obtained in the sampling campaign of 2017.

Conversely, in 2017, the Calich lagoon (ch) showed morphometric measures higher than those from the other lagoons. In detail, the following mean values (±SD) were pointed out: shell length=43.7±2.0 mm, shell height=31.0±1.4 mm, total weight=14.8±2.0 g, mollusc weight=3.3±0.7 g, and shell weight=6.8±0.9 g (Fig. 3.2).

However, in 2017, mollusc weight and condition index resulted more higher in Santa Gilla lagoon (3.2±0.8 and 35.5±3.8, respectively).

In general, this variable revealed a moderate increment from October to April for ch and sg sites, while minimum values compared to 2016 was registered in 2017 for pp lagoons. All values (mean±SD) are showed in the Tab. 2.3.

Tab. 2.3 Morphometric measures and CI for all sites considered. Comparison between the years for ch, pp and sg lagoons.

Site	Year	Total weight (g)	Shell weight (g)	Mollusc weight (g)	Height (mm)	Length (mm)	CI
ch	2016	10.9±2.4	5.0±1.2	2.1±0.8	28.0±2.1	39.4±2.9	28.9±7.5
	2017	13.5±4.1***	6.3±1.9***	3.2±1.2***	29.6±3.2***	41.1±4.6**	33.3±4.9***
pp	2016	5.9±2.0	2.5±0.9	1.3±0.4	23.7±2.1	32.8±3.2	35.4±3.9
	2017	4.4±1.4***	1.9±0.6***	1.1±0.3***	20.7±2.0***	29.0±2.7***	37.0±5.8*
sg	2016	5.8±1.0	2.8±0.5	1.4±0.2	22.9±1.3	30.2±1.5	33.8±3.5
	2017	12.2±2.6***	5.9±1.3***	3.2±0.8***	28.7±2.3***	37.8±2.9***	35.5±3.8***
st	2016	14.88±2.0	6.8±0.9	3.3±0.7	31.0±1.4	43.7±2.0	32.2±3.7
mr	2017	7.4±2.5	3.3±1.2	2.1±0.7	24.6±2.9	34.0±3.1	39.3±2.6

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Fig. 3.1 Comparison between morphometric measurements and condition index (CI) relative to the lagoons considered for 2016.
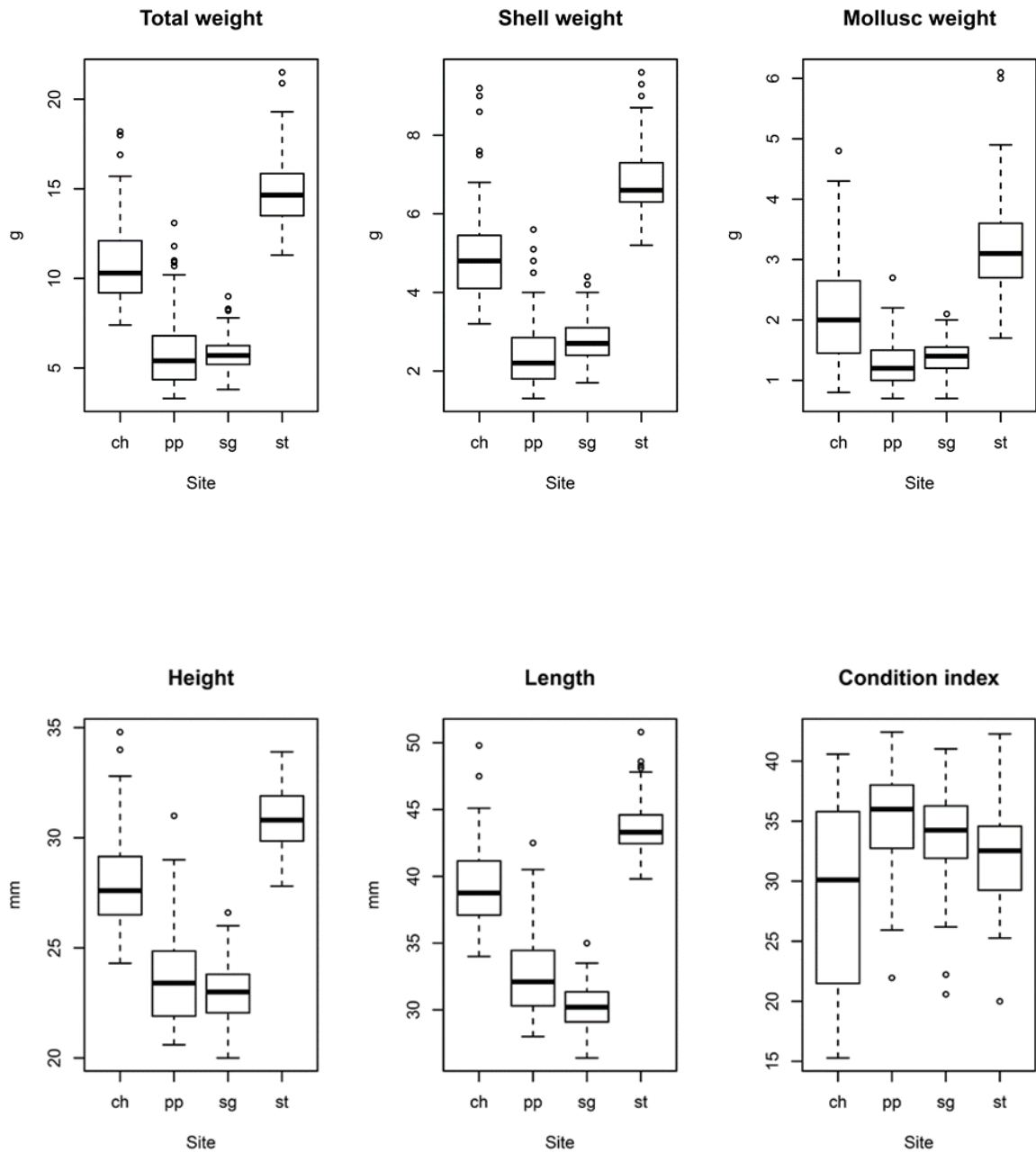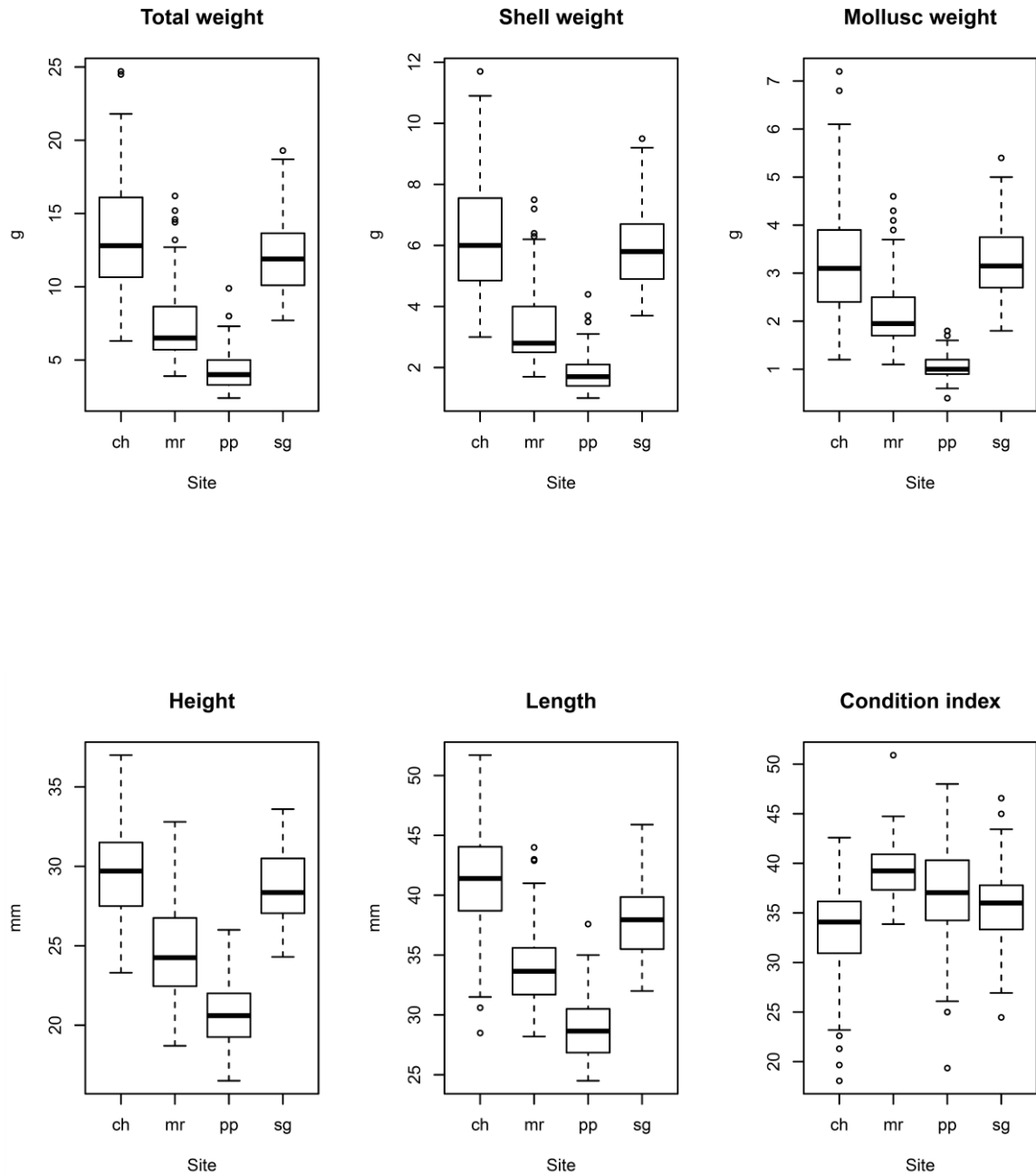
Fig. 3.2 Comparison between morphometric measurements and condition index (CI) relative to the lagoons considered for 2017.

2.4.2 Trace elements concentration in *Ruditapes decussatus*

Metal concentrations are shown in Tab. 2.4 and 2.6; results were expressed as minimum value, maximum value and mean value ± standard deviation (SD). Results were also graphically represented in Fig. 3.3 (non-essential trace elements) and Fig. 3.4 (essential trace elements) relatively to the three sites considered for both years (*i.e.*, ch, pp and sg). Conversely, the metal concentrations in clams from San Teodoro and Marceddì lagoons are showed in Figs. 3.5 and 3.6, respectively. Thallium concentrations were below the LOQ in all samples and years.

Statistical evaluation demonstrated a high significant difference between the lagoons in both years (*p* < 0.001). Results of Tukey's test are shown in the Tab. 2.5, 2.7 and 2.8. Considering the total metal values in the clams for each site, intended as the sum of all metals, the values were measured in the following decreasing trend in 2016: Santa Gilla (281.6 mg kg^{-1} w. w.) > San Teodoro (179.0 mg kg^{-1} w. w.) > Calich (173.1 mg kg^{-1} w. w.) > Porto Pozzo (81.2 mg kg^{-1} w. w.). In contrast, the following similar trend was determined for 2017: Santa Gilla (290.9 mg kg^{-1} w. w.) > Marceddì (284.3 mg kg^{-1} w. w.) > Porto Pozzo (227.5 mg kg^{-1} w. w.) > Calich (90.2 mg kg^{-1} w. w.). Thus, it is immediately evident that the Santa Gilla lagoon, in south Sardinia, which is in the proximity of industrial settlements, appeared to be more affected than the other lagoons by metals. Indeed, in 2016, high values were recorded in Santa Gilla for Al, Co, Cu, Fe, Hg, Ni and Se, while highest values for Ag, As, Cd, Cr, Mn, Pb and Sn in San Teodoro were found (Tab. 2.4). The Zn concentration, moreover, results higher than those other sites in Calich lagoon. Instead for the 2017, the highest concentrations of most elements were found in Santa Gilla (Tab. 2.6), except for Cd, Cr, Fe and Pb, (highest values in Marceddì) and As, Sn and Zn (highest value in Porto Pozzo).

Tab. 2.4 Concentrations of trace elements in clams (*R. decussatus*) from Sardinian lagoons (mg Kg^{-1} wet weight) in the year 2016.

Site	n	Descriptive statistics	Ag	Al	As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sn	Tl	Zn
Calich	100	Min. Value		27.0	1.6	0.012	0.110	0.040	1.0	27.0	0.009	1.7	0.350	0.036	0.360			22.0
		Max. Value	< LOQ	159.0	2.9	0.027	0.150	0.140	40.0	103.0	0.011	6.2	0.720	0.093	0.650	< LOQ	< LOQ	81.0
		Mean		63.9	1.9	0.019	0.128	0.073	6.0	57.8	0.010	2.8	0.478	0.056	0.441			39.5
		S.D.		45.4	0.425	0.005	0.013	0.030	13.7	25.7	0.0005	1.5	0.132	0.023	0.094			24.7
Porto Pozzo	100	Min. Value	0.011	14.0	8.8	0.014	0.100	0.040	1.1	19.0	0.013	0.770	0.480	0.092	1.1	0.010		18.0
		Max. Value	0.095	23.0	11.0	0.026	0.140	0.110	18.0	34.0	0.015	1.1	0.680	0.260	1.5	0.015	< LOQ	26.0
		Mean	0.035	17.5	9.6	0.017	0.113	0.063	3.4	25.3	0.014	0.937	0.591	0.142	1.4	0.011		22.1
		S.D.	0.026	2.9	0.783	0.004	0.015	0.024	5.9	5.5	0.009	0.133	0.074	0.052	0.138	0.002		2.9
Santa Gilla	100	Min. Value	0.024	43.0	3.1	0.054	0.130	0.080	0.79	44.0	0.065	2.3	0.180	0.100	1.3	0.006		1.0
		Max. Value	0.106	214.0	8.3	0.100	0.890	0.250	48.8	199.0	0.069	8.2	1.1	0.370	1.8	0.021	< LOQ	60.0
		Mean	0.061	127.0	4.1	0.077	0.252	0.148	9.5	105.0	0.067	4.3	0.753	0.253	1.6	0.012		28.4
		S.D.	0.024	60.7	1.7	0.019	0.258	0.056	170.0	49.3	0.002	2.0	0.285	0.085	0.155	0.005		18.5
San Teodoro	100	Min. Value	0.064	24.0	5.2	0.100	0.190	0.110	0.49	19.0	0.014	1.9	0.140	0.130	0.700	0.006		1.0
		Max. Value	0.210	140.0	22.0	0.790	0.230	0.220	1.4	118.0	0.016	18.0	0.680	3.8	0.870	0.019	< LOQ	38.0
		Mean	0.135	70.4	12.8	0.273	0.207	0.156	1.1	57.6	0.015	9.8	0.503	0.893	0.773	0.013		24.4
		S.D.	0.059	42.6	7.5	0.236	0.015	0.039	0.28	42.2	0.0004	8.0	0.158	1.2	0.053	0.003		11.3

< LOQ: under Limit of Quantification.

SD: standard deviation.

Tab. 2.5 ANOVA (Tukey post hoc test) results for trace elements (mg Kg^{-1} wet weight) variations in clams (*R. decussatus*) among the brackish environments investigated in the year 2016.

Sites	Ag	Al	As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sn	Tl	Zn
pp-ch	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.
sg-ch	*	*	n.s.	n.s.	n.s.	**	n.s.	n.s.	***	n.s.	*	n.s.	***	n.s.	**	n.s.
st-ch	***	n.s.	***	**	n.s.	**	n.s.	n.s.	***	*	n.s.	n.s.	***	n.s.	**	n.s.
sg-pp	n.s.	***	*	n.s.	n.s.	**	n.s.	**	***	n.s.	n.s.	n.s.	**	n.s.	**	n.s.
st-pp	***	n.s.	n.s.	**	n.s.	***	n.s.	n.s.	***	**	n.s.	n.s.	***	n.s.	**	n.s.
st-sg	**	n.s.	**	*	n.s.	n.s.	n.s.	n.s.	***	n.s.	*	n.s.	***	n.s.	n.s.	n.s.

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. not significative.

Tab. 2.6 Concentrations of trace elements in clams (*R. decussatus*) from Sardinian lagoons (mg Kg^{-1} wet weight) in the year 2017.

Site	n	Descriptive statistics	Ag	Al	As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sn	Tl	Zn
Calich	100	Min. Value		21.0	0.900	0.011	0.066	0.036	0.510	18.0	0.005	0.810	0.360	0.019	0.190			17.0
		Max. Value	<LOQ	38.0	1.4	0.022	0.120	0.084	0.990	43.0	0.011	1.7	0.750	0.100	0.360	<LOQ	<LOQ	49.0
		Mean		28.5	1.1	0.020	0.084	0.055	0.755	28.9	0.010	1.2	0.563	0.048	0.250			28.8
		SD		6.8	0.169	0.003	0.017	0.017	0.166	8.8	0.002	0.342	0.111	0.025	0.061			11.6
Marceddì	100	Min. Value		78.0	2.9	0.028	0.100	0.140	1.3	60.0	0.007	1.8	0.820	0.250	0.570	0.009		31.0
		Max. Value	<LOQ	188.0	4.1	0.480	0.130	0.300	24.0	145.0	0.011	2.8	1.1	1.6	0.680	0.017	<LOQ	47.0
		Mean		133.1	3.4	0.165	0.116	0.247	6.4	106.6	0.010	2.3	0.921	0.728	0.636	0.014		36.3
		SD		41.3	0.385	0.149	0.009	0.054	9.2	26.1	0.001	0.290	0.092	0.508	0.041	0.002		6.2
Porto Pozzo	100	Min. Value	0.055	70.0	7.0	0.021	0.049	0.070	0.700	54.6	0.013	0.780	0.330	0.080	0.450	0.014		18.0
		Max. Value	0.018	139.0	10.5	0.160	0.187	0.520	2.0	81.9	0.018	1.9	1.3	0.300	1.4	0.038	<LOQ	160.0
		Mean	0.032	101.1	8.2	0.061	0.084	0.153	1.0	68.0	0.015	1.1	0.497	0.176	0.696	0.019		46.3
		SD	0.013	24.7	1.090	0.052	0.044	0.152	0.510	11.3	0.002	0.439	0.321	0.088	0.353	0.007		47.6
Santa Gilla	100	Min. Value	0.000	94.0	2.5	0.000	0.000	0.000	1.4	70.0	0.035	2.6	0.000	0.000	0.000	0.000		18.0
		Max. Value	0.120	221.0	4.4	0.057	0.280	0.290	39.0	138.0	0.037	3.9	1.7	0.470	1.2	0.019	<LOQ	33.0
		Mean	0.069	139.4	3.3	0.043	0.198	0.186	13.8	97.4	0.036	3.2	1.1	0.326	0.897	0.010		24.5
		SD	0.039	48.2	0.557	0.018	0.085	0.095	13.1	25.1	0.001	0.526	0.517	0.145	0.378	0.005		4.8

<LOQ: under Limit of Quantification.

SD: standard deviation.

Tab. 2.7 ANOVA (Tukey post hoc test) results for trace elements (mg Kg^{-1} wet weight) variations in clams (*R. decussatus*) among the brackish environments investigated in the year 2017.

Sites	Ag	Al	As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sn	Tl	Zn
mr-ch	*n.s.*	***	***	**	*n.s.*	**	*n.s.*	***	*n.s.*	***	*n.s.*	***	*	*n.s.*	**	*n.s.*
pp-ch	*n.s.*	**	***	*n.s.*	*n.s.*	*n.s.*	*n.s.*	**	***	*n.s.*	*n.s.*	*n.s.*	*	**	***	*n.s.*
sg-ch	***	***	***	*n.s.*	***	*n.s.*	*	***	***	***	*	*n.s.*	***	*n.s.*	*n.s.*	*n.s.*
pp-mr	*	*n.s.*	***	*n.s.*	*n.s.*	*n.s.*	*n.s.*	**	***	***	*n.s.*	**	*n.s.*	*n.s.*	*n.s.*	*n.s.*
sg-mr	***	*n.s.*	*n.s.*	*	*	*n.s.*	*n.s.*	*n.s.*	***	**	*n.s.*	*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
sg-pp	**	*n.s.*	***	*n.s.*	***	*n.s.*	*	*	***	***	**	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; *n.s.* not significative.

Tab. 2.8 ANOVA (Tukey post hoc test) results for trace elements (mg Kg^{-1} wet weight) variations in clams (*R. decussatus*) between the years 2016 and 2017.

Sites	Ag	Al	As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sn	Tl	Zn
Calich 2016 *vs* 2017	***	*	***	*n.s.*	***	*n.s.*	*n.s.*	**	*n.s.*	*	*n.s.*	*n.s.*	***	*n.s.*	*n.s.*	*n.s.*
Porto Pozzo 2016 *vs* 2017	*n.s.*	***	*	*	*n.s.*	*n.s.*	*n.s.*	***	*n.s.*	*n.s.*	*n.s.*	*n.s.*	***	*	**	*n.s.*
Santa Gilla 2016 *vs* 2017	*n.s.*	*n.s.*	*n.s.*	**	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	***	*n.s.*	*n.s.*	*n.s.*

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; *n.s.* not significative.

2.4.2.1 Nonessential trace elements

This group of trace elements, which are toxic for living organisms, even at low concentrations includes metals such as aluminium, arsenic, cadmium, lead, mercury, silver and tin, with no recognized biological functions.

2.4.2.2 Cadmium, lead and mercury

Maximum limits are set by European Regulations (1881/2006/CE and amendments) for these three toxic elements in shellfish, while there are still no limits established by EU for other metals in food. In 2016, the highest level for Hg was recorded in Santa Gilla lagoon (0.067 mg kg^{-1} w. w.) while higher concentrations for Cd and Pb in San Teodoro lagoon were found (0.273 and 0.893 mg kg^{-1} w. w., respectively). As for 2017, however, Santa Gilla has always showed the highest concentrations for Hg (0.036 mg kg^{-1} w. w.), while high values of Cd and Pb were found, instead, at Marceddì lagoon (0.165 and 0.728 mg kg^{-1} w. w., respectively).

Nevertheless, the concentration of these trace elements in the clam's tissues were well below the legal limits, correspond to 1.0 mg kg^{-1} w. w. for cadmium (Cd); 0.5 mg kg^{-1} w. w. for mercury (Hg) and 1.5 mg kg^{-1} w. w. for lead (Pb). Moreover, cadmium concentrations were lower than those registered in the genus *Ruditapes* from other part of the world, such as coastal areas of China (Ruan *et al.*, 2008; Li and Gao, 2014) and the Atlantic coast of Spain (Usero *et al.*, 1997), but comparable to those observed by Velez and co-authors (2015) in Portugal, Ria de Averia Lagoon. Similar considerations can be drawn for mercury and lead, where values in Sardinian lagoons were in the range of the cited references. The Calich lagoon registered the lowest values for these three toxic elements in both years, except for Cd in 2016, where the lowest concentration was recorded in Porto Pozzo lagoon (Tab. 2.4 and 2.5; Figs. 3.3, 3.5 and 3.6).

A provisional tolerable weekly intake (PTWI) of 7 µg kg^{-1} body weight (b. w.) was set for cadmium (EFSA, 2009), corresponding to 0.49 mg of Cd in one week for a human adult weighing 70 kg. Consequently, an adult would have to have an intake of at least 1 kg of clams per day from Marceddì or San Teodoro to exceed this PTWI.

2.4.2.3 Aluminium and arsenic

To our knowledge, this is the first investigation concerning aluminium levels in the studied species; moreover, data on Al concentrations in bivalve molluscs are scarce worldwide. We found very different levels of Al in the examined lagoons, with the highest value for 2016 and 2017 years, in the Santa Gilla lagoon (mean 127.0 and 139.4 mg kg^{-1} w. w., respectively). Conversely, in 2016, the lowest value was registered in the Porto Pozzo lagoon (mean 17.5 mg kg^{-1} w. w.), while in 2017, the lowest value was highlighted for Calich lagoon (mean 28.5 mg kg^{-1} w. w.). In a previous investigation, we analysed Al levels in mussels and oysters from a North-western Mediterranean mariculture (Squadrone *et al*., 2016), and we found mean values of 162 mg kg^{-1} and 42 mg kg^{-1} w. w., respectively.

The tolerable weekly intake (TWI) for aluminium was recommended at 1 mg Al kg^{-1} b. w. (EFSA, 2008), which correspond to 70 mg of aluminium a week in an adult weighing 70 kg. As a result, the PTWI would be exceeded by an adult consuming over 502 g of clams in one week from Santa Gilla, over 526 g of clams from Marceddì or, over 692 g from Porto Pozzo; while for a child weighing 25 kg, the PTWI would be exceeded by consuming over 179, 188 and 247 g of clams in one week respectively for the above mentioned lagoons or lagoons.

Giuseppe Esposito – "Detection of trace elements in the bivalve *Ruditapes decussatus* from Sardinian coastal lagoons: effects on food safety and pathological findings in target organs" - Tesi di Dottorato in Scienze Veterinarie, indirizzo Qualità e Sicurezza Alimentare. Università degli Studi di Sassari.

The considerably high level of Al observed in clams from Santa Gilla could be due to the presence of chemical settlements surrounding this lagoon, such as a fluorochemicals plant that also produces aluminium fluoride.

The Sardinian Regional Agency of Environmental Protection (ARPAS) has already subjected the area to environmental characterization (ARPAS, 2017); soil pollution was excluded but the presence of metals and metalloids in ground water was signalled. In May 2017, Santa Gilla sediments were analysed to detect the presence of contaminants such as metals. Aluminium levels were found in the range of 8.045 to 34.306 mg kg^{-1} (mean value 24.486 mg kg^{-1}). There are no limits or environmental quality standards set for Al in sediments, but these levels were higher (twice as high) than those observed in Tuscany marine sediments (Battuello *et al*., 2018, mean 11.302 mg kg^{-1}) and those detected in the Venice Lagoon sediments (Botter, 2012; mean 10.122 mg kg^{-1}).

Arsenic is a metalloid that is toxic in its inorganic forms; shellfish such as molluscs and crustaceans usually have high levels of total arsenic (tAs), with a low percentage of inorganic arsenic (EFSA, 2009; 2014).

In 2016, arsenic was found at values ranging from 1.9 to 12.8 mg kg^{-1} in clam tissues from the Calich and San Teodoro lagoons, respectively. In contrast, in 2017, arsenic was found at values ranging from 1.1 to 8.2 mg kg^{-1} in clams from Calich lagoon and Porto Pozzo lagoon, respectively. These values were comparable to those observed by Bogdanović and co-authors (2014) with other species of shellfish (*Mytilus galloprovincialis*, *Ostrea edulis*, *Chlamis varia* and *Venus verrucosa*) from breeding and harvesting areas along the eastern Adriatic Coast (Croatia).

Similar values in clam *Ruditapes decussatus*, moreover, were observed by Velez and co-authors (2015) in from weakly contaminated Portugal lagoons and from Usero and

coauthors (1997) in Spanish lagoons. However, the concentrations of As found in clams from marine contaminated environments (Koch *et al*. 2007) were up to 228 mg kg^{-1}.

In bivalve molluscs, arsenic is mostly composed of the nontoxic organic forms of arsenobetaine and arsenosugars, with total arsenic concentrations between 3.5 to 10.4 mg kg^{-1} (Edmonds and Francesconi, 1993, 1997).

Inorganic As (iAs) in clams was found in the range of 0 to 8% in different investigations (Edmonds and Francesconi, 1993; Munoz *et al*., 2000; Devesa *et al*., 2001; Suner *et al*., 2002). Finally, the EFSA Scientific Report (2014), which estimated the dietary exposure to inorganic arsenic in the European population, reported a concentration of 0.047-0.055 mg kg^{-1} of iAs in clams, with a percentage of iAs varying from 0.1% to 6% in shellfish. Regarding the results obtained in 2017, it was possible to highlight different iAs concentrations (sum of III and V) between the brackish environments considered (Tab. 2.9). In more detail, concentrations in *R. decussatus* tissues of iAs ranging from 0.014 mg kg^{-1} (1.27 %iAs/tAs) from the Calich lagoon to 0.120 mg kg^{-1} (1.46 %iAs/tAs) from the Porto Pozzo lagoon. However, the highest value as %iAs/tAs was found in Marceddì lagoon (2.76 %), (Tab. 2.9). However, statistical evaluation showed that the differences between sites were not statistically significant ($p < 0.392$).

In 2010, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) withdrew the iAs PTWI of 15 µg kg^{-1} body weight, as it was deemed to be no longer appropriate, and a benchmark dose lower confidence limit for a 0.5 % increased incidence of lung cancer (BMDL0.5) of 3.0 µg kg^{-1} body weight per day was set (WHO, 2011b). In this scenario, for an adult weighing 70 kg it not advisable to consume more than 300 g per day of clams from Marceddì lagoon.

Tab. 2.9 Arsenic speciation for the lagoons studied in 2017. The data are expressed as mg kg^{-1} w. w.

Site	tAs	As species					As species % of tAs			
		ASBE	DMA	MMA	iAs (III+V)	Sum of As	%iAs/tAs	%AsB/tAs	%DMA+ MMA/tAs	%Tot
Porto Pozzo	8.20	6.90	0.60	0.000	0.120	7.60	1.46	84.15	0.60	86.21
Marceddì	3.40	2.70	0.52	0.000	0.094	3.30	2.76	79.41	0.52	82.70
Santa Gilla	3.30	2.30	0.84	0.000	0.064	3.20	1.94	69.70	0.84	72.48
Calich	1.10	0.61	0.14	0.000	0.014	0.76	1.27	55.45	0.14	56.87

tAs: total arsenic.
Organic species: ASBE Arsenobetaine; DMA Dimethylarsenic acid; MMA Monomethylarsonic acid.
Inorganic species: iAs (sum of III+V).

2.4.2.4 Silver and tin

In the Calich lagoon, which appears to have a less contaminated environment, both silver (Ag) and tin (Sn) had levels below the limit of quantitation of 0.010 mg kg^{-1} in clams for both years.

In 2016, in the other three environments, silver was registered as having values from 0.035 to 0.135 mg kg^{-1} (Tab. 2.4; Fig. 3.3, 3.5); while tin was found in concentrations from 0.011 to 0.013 mg kg^{-1}. In 2017, levels below the limit of quantitation for silver was also registered for Marceddì lagoon (Fig. 3.6). Contrariwise, in the same year, silver values ranging from 0.032 to 0.069 mg kg^{-1} in the other sites (Tab. 2.6; Fig. 3.3).

To our knowledge, there are no data regarding the levels of silver and tin in *R. decussatus*, but these values were in the range of concentrations found in other bivalves (Squadrone *et al*., 2015) and do not constitute concern.

2.4.2.5 Essential trace elements

All living organisms require essential trace elements, such as cobalt, chromium, copper, iron, manganese, nickel, selenium and zinc, which are involved in important metabolic processes. However, excessive levels of essential metals can be harmful for humans.

2.4.2.6 Cobalt and chromium

Cobalt (Co), as a component of vitamin B12, is an essential element that has important functions in human metabolism. In 2016, Co was recorded in Sardinian clams from 0.113 mg kg^{-1} (Porto Pozzo lagoon) to 0.252 mg kg^{-1} (Santa Gilla lagoon). In 2017, moreover, the concentrations of this element ranging from 0.084 mg kg^{-1} (Calich lagoon and Porto Pozzo lagoon) to 0.198 mg kg^{-1} (Santa Gilla lagoon). These concentrations are comparable to those recorded in other bivalves (Squadrone *et al*., 2016).

Chromium (Cr) is another essential element, and its deficiency causes imbalances in the metabolism of glucose, fats and proteins (EFSA 2014). We found the highest level of Cr in the San Teodoro (0.156 mg kg^{-1}) and Marceddì (0.156 mg kg^{-1}) for year 2016 and 2017, respectively. A similar concentration has been observed in the same species by other investigators (Usero *et al*., 1997; Li and Gao 2014); the lowest level was instead measured in 2017 in the Calich lagoon (0.055 mg kg^{-1}).

2.4.2.7 Copper, iron and manganese

Copper (Cu) is an essential trace element for normal cellular activity, as it is involved in many metabolic functions forming part of proteins and metal-enzymes. Copper values have different concentrations in clams between sites and years (Figs. 3.4, 3.5 and 3.6; Tab. 2.4 and 2.6).

The lowest concentrations in 2016 were reported for San Teodoro (1.1 mg kg^{-1}), while in 2017 for Calich and Porto Pozzo (0.755 and 1.1 mg kg^{-1}, respectively). These concentrations are similar to those found in *R. decussatus* from other locations (Usero *et al*., 1997; Li and Gao, 2014; Velez *et al*., 2015), while in Santa Gilla clams, the Cu value was higher in both years (9.5 and 13.8 mg kg^{-1}, respectively).

EFSA (2015) proposed a copper Tolerable Upper Intake level (UL) of 5 mg day for adults, correspond to an intake of 362.3 kg day $^{-1}$ of clams from Santa Gilla lagoon.

Iron (Fe) is an essential element for humans as it is involved in several metabolic processes, such as oxygen transport and electron transport (Abbaspour *et al*., 2014).

Iron concentrations in clams from our study were similar to values registered by Usero and co-authors (1997) in the same species and ranged from 25.3 mg kg^{-1} in Porto Pozzo clams to 105.0 mg kg^{-1} in Santa Gilla clams in 2016 and from 28.9 mg kg^{-1} in Calich clams to 106.6 mg kg^{-1} in Marceddì clams in 2017 (Tab. 2.4, 2.6 and 2.6; Figs. 3.4, 3.5 and 3.6).

The recommended dietary intake of Fe proposed by the US Institute of Medicine, Food and Nutrition Board, (2001) was 40 mg day^{-1} in adults. Marceddì clams were found to contain an average level of about 107 mg kg^{-1} similar to the value recorded in Santa Gilla clams (105 mg kg^{-1}); therefore, consuming more than 374 g of these clams would exceed this recommended value in adults.

Moreover, the amount of Fe, that most of the people need for good health according to the proposed EFSA Population Reference Intake (PRI) is lower and correspond to 11 mg day^{-1} (EFSA, 2015).

Manganese (Mn) is another essential element that is a cofactor of several enzymes involved in cellular metabolism and defence mechanisms against oxidative stress, such as superoxide dismutase (SOD).

A UL of 11 mg of manganese per day for adults of 70 kg has been suggested (ATSDR, 2012), while EFSA (2013) proposed an Adequate Intake (AI) based on observed mean manganese intakes from mixed diets in the EU, correspond to 3 mg day^{-1} for adults.

Mn levels in clams from Sardinian lagoons (Tab. 2.4, 2.6) were comparable to previously reported data in the same species (Usero *et al*. 1997), with the exception, in 2016, of Santa Gilla and San Teodoro clams where a concentration of 4.3 and 9.8 mg kg^{-1} were detected, respectively.

2.4.2.8 Nickel, selenium and zinc

Nickel (Ni) nickel is a ubiquitous element, which has been proposed as a coenzyme for the metabolism of glucose, some hormones and lipids. A tolerable daily intake of 2.8 μg Ni/kg body weight (b. w.) was set (EFSA, 2015) correspond to 0.20 mg Kg^{-1} per day for an adult weighting 70 kg.

We found Ni concentrations ranging from 0.478 mg kg^{-1} (clams from Calich in 2016) to 1.1 mg kg^{-1} (clams from Santa Gilla in 2017), concentrations in the order of those observed in the same species from Portuguese and Spanish marine environments (Usero *et al*., 1997; Velez et al., 2015).

Selenium (Se) exerts most of its biological effects as a cofactor of at least 30 seleno-proteins, including glutathione peroxidase (GPx), which has the important function of protecting the cell from oxidative damage.

The lowest Se values were found in clams from Calich lagoon in both years (0.25 and 0.441 mg kg^{-1}, respectively) which were comparable to concentrations registered in *R. decussatus* from a Spanish environment (Usero *et al*., 1997).

However, the highest Se values were recorded in 2016, in Santa Gilla and Porto Pozzo

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clams (1.6 and 1.4 mg kg^{-1}, respectively); these lagoons appeared to be the most affected by industrial and anthropogenic contamination and selenium values probably reflect its involvement as cofactor in antioxidant enzymes able to prevent cell oxidative damage induced by heavy metal toxicity. It is interesting to note that the highest values of mercury (Tab. 2.4, 2.6) correspond to the highest values of selenium in clams (Santa Gilla and Porto Pozzo), according to the well-known correlation between these two elements, which suggests the protective role of selenium against methylmercury. In fact, several investigations have assessed the role of selenium in counteracting mercury toxicity and showed that increasing concentrations of Se in the diet increasingly counteracts methylmercury toxicity (Ralston *et al*., 2008; Olmedo *et al*., 2013; Squadrone *et al*., 2015).

Zinc (Zn) is a trace element essential for the correct functioning of numerous enzymes, for body growth and development and, at the immune level, zinc influences the reactivity of lymphocytes and is usually suggested for the therapy of immune deficiencies.

The UL proposed for zinc in adults is 25 mg per day (EFSA, 2014).

Zinc levels were found in the range 22.1-39.5 mg kg^{-1} in clams from the studied lagoons in 2016, while in the 2017 have reached much higher concentrations ranging from 24.5 to 46.3 mg kg^{-1}. These values are greater than those registered in *R. decussatus* from different locations such as Spain, Portugal, and China (Usero *et al*., 1997; Ruan *et al*., 2008; Li and Gao, 2014; Velez *et al*., 2015).

Fig. 3.3 Comparison of non-essential metals (mg kg^{-1} w. w.) between sites and years.
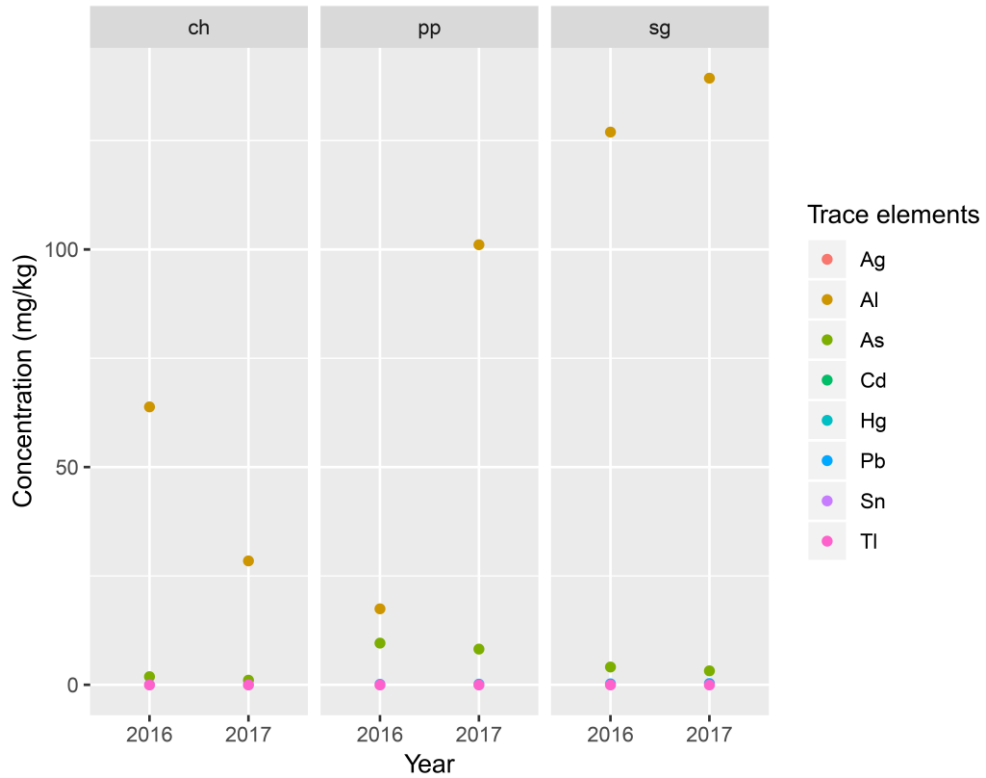

Fig. 3.4 Comparison of essential metals (mg kg^{-1} w. w.) between sites and years.
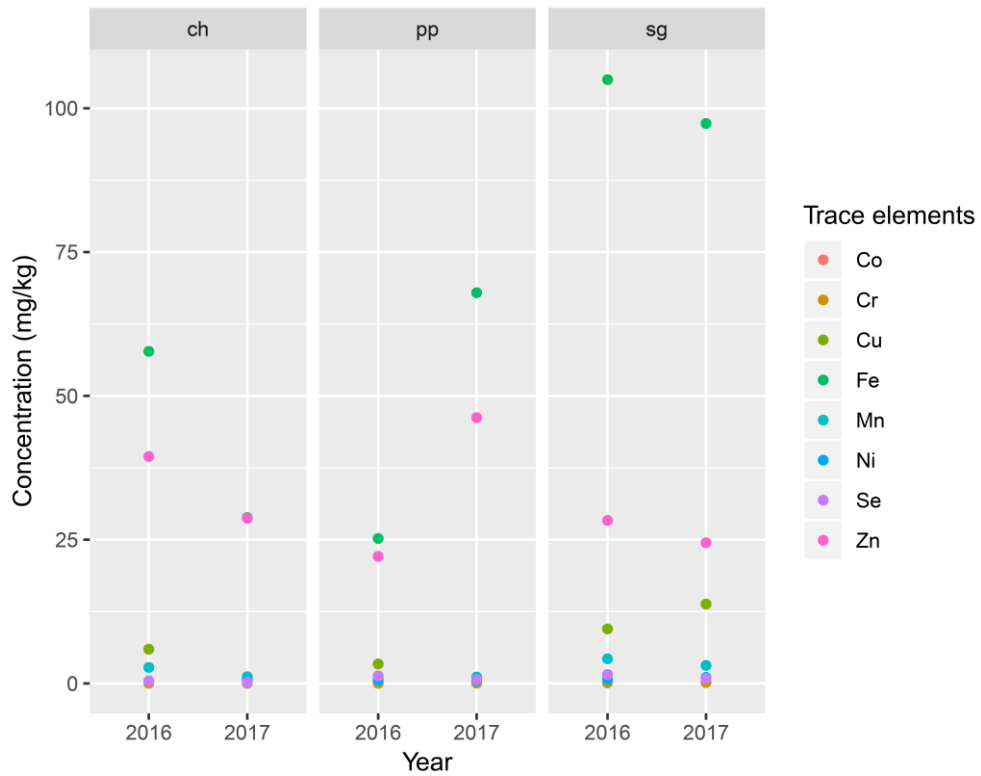
Fig. 3.5 Essential and non-essential metals (mg kg^{-1} w. w.) of San Teodoro lagoon in 2016.
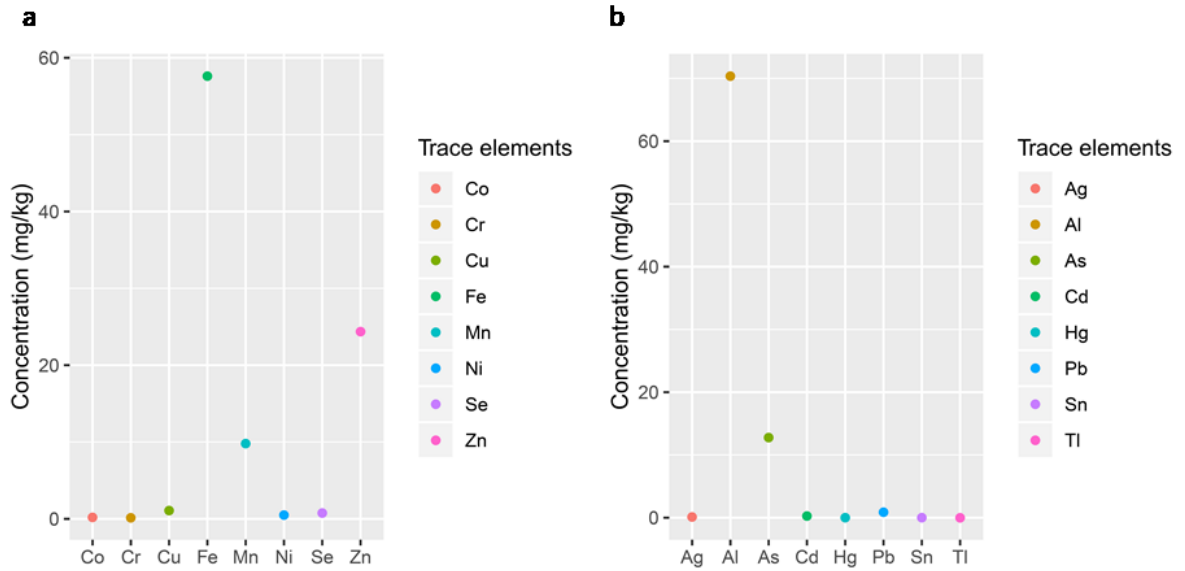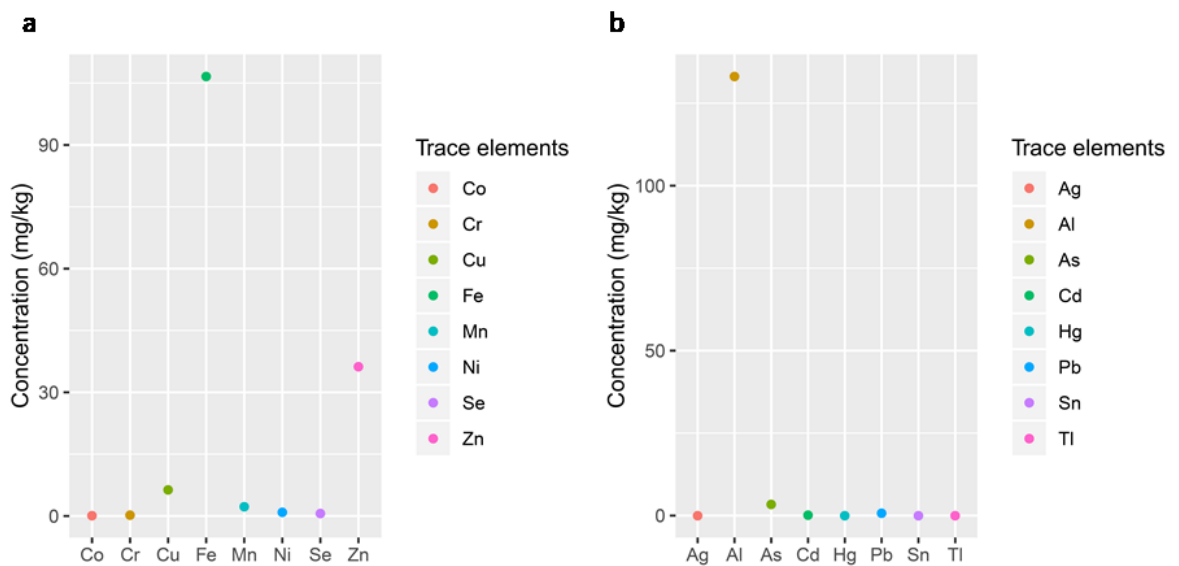

Fig. 3.6 Essential (a) and non-essential (b) metals (mg kg^{-1} w. w.) of Marceddì lagoon in 2017.

2.4.2.9 Metals levels in clams from different countries

In Tab. 3 is reported the mean concentrations of trace elements in some clams from different countries to allow a comparison with our data (Tab. 2.4 and 2.6) regarding *Ruditapes decussatus* in Sardinian lagoons.

Differences are surely due to different geographic locations, different environments and differently anthropogenic affected ecosystems, as well as to natural differences in clams' species and genera. However, a literature comparison could help in assess the risk of consumption of clams from our study in respect to other locations.

In a recent monitoring Chiesa and co-authors (2018) analysed metals levels (As, Cd, Cr, Hg, Ni, Pb and Zn) in the clam *R. philippinarum* from and highly industrial and anthropogenic impacted area, the Tagus estuary (Portugal). They found different concentrations of some considered elements than those we detected in *R. decussatus* from Sardinian lagoons. Arsenic concentrations found on our clams were lower for Calich and Santa Gilla lagoons for 2016. However, higher values of As were found for Porto Pozzo and San Teodoro lagoons compared to those found by Chiesa *et al*., (2018). In 2017, however, lowest values of As were recorded in all the considered sites with the exception of Porto Pozzo lagoon. We found similar concentration for Cd in all sites and for both years except for Marceddì lagoon which reported higher values for the year 2017. Cr, Ni and Pb elements were lower in all sites and both years, if compared with those detected in Tagus estuary. Instead the concentrations of Hg and Zn that we found were comparable in both years with those found from Chiesa and co-authors (2018). However, higher concentrations of Zn were recorded in the Calich lagoon in 2016 and Porto Pozzo and Marceddì for 2017.

Gedik and Ozturk (2018) analyzed instead metal levels (As, Cd, Cu, Pb and Zn) in the Venus clam *Chamelea gallina* from different locations along the Black Sea coast of Turkey, Bulgaria, and Crimea. Generally, they found similar concentrations of As, Cd, Cu, Pb and Zn in comparison to our data. However, we found higher concentrations of As in Porto Pozzo in both years and in San Teodoro lagoon relatively to 2017. Simultaneously, Cu concentration resulted in ours clams.

Clams available in the Italian market (*R. philippinarum*, *R. decussatus*, *Meretrix meretrix*, *M. lyrat*) from five different FAO areas, were analysed for As, Cd, Cr, Hg, Ni and Pb concentrations (Chiesa *et al*., 2018). Cd, Cr, Hg, Ni and Pb mean values were in the range of concentrations we recorded in *R. decussatus* from Sardinian lagoons, while As was higher.

Trace elements (Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Se, Zn) were also investigated in the lokan clam *Polymesoda expansa* from Singapore mangroves (Estrada *et al*., 2017).

On average, values of As, Co, Cu and Zn were lower in comparison to our results, while Cd, Cr, Fe, Mn, Ni and Se concentrations were found similar to *R. decussatus* in some lagoons (this study).

Li and co-authors (2015) analysed concentration of As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se and Zn in the clam *Mactra veneriformis* from Bohai Bay, a semi-enclosed coastal water body of the Northwest Pacific Ocean (China), surrounded by highly industrialized areas. As, Cu, Pb, Ni, Se and Zn were found in values comparable to those we found, while Co, Cr and Mn were recorded at higher concentrations. However, for this latter compound (Mn) higher concentration was detected in San Teodoro lagoon. Metals concentrations were also detected in the Venus clam *Chamelea gallina* from Egyptian Mediterranean coastal fisheries (El-Wazzan *et al*., 2014) and Cd, Cr, Fe and Pb were on average higher in comparison to our study.

Saaedi (2012) studied trace elements levels in the clam *Amiantis umbonella* from the Northern coast of the Persian Gulf (Saudi Arabia). Cd, Co, Mn and Pb levels were found higher than in *R. decussatus* from Sardinia lagoons, while for Ag, As, Cr, Cu, Fe, Ni and Zn comparable concentrations were registered.

Obirikorang and co-authors (2009) analysed concentrations of Fe, Hg Mn and Zn in the clam *Galatea paradoxa* from the Volta estuary (Ghana). The levels recorded for Fe, Mn and Zn were higher than those we found in *R. decussatus* from the Sardinian lagoons.

The trace element aluminium was scarcely investigated in clams, and in our knowledge two recent studies evaluated Al level, Saaedi (2012), that found in *A. umbonella* a mean concentration similar to value from *R. decussatus* from Porto Pozzo lagoon in 2016 (17.5 mg kg^{-1}) but lower than in Santa Gilla and Marceddì lagoons, and Estrada and co-authors (2017) that recorded in *P. expansa* a mean Al concentration comparable to value from *R. decussatus* from Porto Pozzo lagoon (101 mg Kg^{-1}) but still lower than in Santa Gilla (roughly 139 mg kg^{-1}) in the same year.

Although the above discussed data relate to the metal burden of different species of clams coming from different geographical areas, we can confirm that the Al values found in *R. decussatus* from Santa Gilla lagoon was higher that levels found in studies regarding clams.

Tab. 3 Metals concentrations in clams from different countries (mg Kg⁻¹).

Locations		Italy, fish market, different FAO zones	Singapore, Mangrove	Black Sea	Ghana, Volta estuary	Portugal, Tagus estuary	China, Bohai Bay	Saudi Arabia, Persian Gulf	Mediterranean coast of Egypt
References		Chiesa *et al* ., 2018	Estrada *et al* ., 2017	Gedik & Ozturk 2018	Obirikorang *et al* ., 2009	Chiesa *et al* ., 2018	Li *et al* ., 2015	Saaedi, 2012	El-Wazzan, 2014
Clam species		*Ruditapes decussatus, R. philippinarum, Meretrix meretrix, Meretrix lyrat*	*Polymesoda expansa*	*Chamelea gallina*	*Galatea paradoxa*	*R. philippinarum*	*Mactra veneriformis*	*Amiantis umbonella*	*Chamelea gallina*
	Ag	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	0.05	*n.d.*
	Al	*n.d.*	90	*n.d.*	*n.d.*	*n.d.*	*n.d.*	16	*n.d.*
T	As	4.9	1.6	0.68-3.57	*n.d.*	6.4	1.8	3.3	*n.d.*
r	Cd	0.31	0.075	0.17-1.03	*n.d.*	0.078	0.4	0.3	0.43
a	Co	*n.d.*	0.09	*n.d.*	*n.d.*	*n.d.*	0.64	1.6	*n.d.*
c	Cr	0.16	0.24	*n.d.*	*n.d.*	3.6	1.3	0.15	0.94
e	Cu	*n.d.*	2.5	1.59-7.19	*n.d.*	*n.d.*	1.8	1	2.2
e	Fe	*n.d.*	86	*n.d.*	71-539	*n.d.*	*n.d.*	75	185
l	Hg	0.05	*n.d.*	*n.d.*	0.028-0.074	0.06	*n.d.*	*n.d.*	0.011
e	Mn	*n.d.*	8	*n.d.*	49-867	*n.d.*	29	60	5
m									
e	Ni	1.2	0.57	*n.d.*	*n.d.*	1.8	1.1	1.8	*n.d.*
n	Pb	1.2	*n.d.*	0.09-1.35	*n.d.*	1.3	0.37	1.3	1.4
t	Se	*n.d.*	0.43	*n.d.*	*n.d.*	*n.d.*	1.4	*n.d.*	*n.d.*
s	Sn	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
	Tl	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
	Zn	*n.d.*	21	9.01-30.2	13-49	21	16	11	12

n.d. : not determined. Concentrations are expressed as mean or range values.

2.4.3 Conclusions

Coastal lagoons, due to their chemical-physical characteristics, their hydrodynamics and especially for their trophic conditions and food sources, are suitable for the existence of edible aquatic species. Extensive aquaculture activities, shellfish, professional and sport fishing are relevant in such environments. However, these areas are often characterized by a widespread pre-existing chemical contamination of sediments caused by emissions and discharges into the atmosphere and into the water by industries. Contamination of sediments can be transferred to aquatic organisms - such as shellfish - with consequent risk to human health. This, to our knowledge, is the first investigation performed in Sardinian lagoons and in Italy regarding trace elements in the clam *Ruditapes decussatus,* a traditional resource for fishing activity in Sardinia. Here, we confirmed that this shellfish is an important source of essential elements for the human diet, however some of them - such as iron - could be found in higher concentrations than the recommended reference values if Santa Gilla clams are consumed in large amounts. Moreover, concentrations of aluminium in this lagoon, which is close to industrial settlements, could constitute a potential risk, particularly for frequent consumers of clams, as the PTWI is exceeded.

The clams, *R. decussatus,* were confirmed to be a suitable tool for biomonitoring of trace elements in the environment; further studies are necessary to monitor the presence of inorganic contaminants, such as aluminium, in Sardinian lagoons, in order to protect these ecosystems and the human health.

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Chapter III

Histological indices and inflammatory responses in target organs

3. Introduction

Coastal lagoons are transitional water systems (Directive 2000/60/EC) showing high geomorphologic, hydrographic and biodiversity variability (Pérez-Ruzafa *et al*., 2007). Thus, due to their nature are sensitive and breakable ecosystems (Barnes, 1980; FAO, 2015). Contamination sources arising from human activities (*i.e.*, discharges from sewage works often containing industrial wastes, discharges from animal rearing and agriculture, urban surface water run-off, etc.) affect these aquatic environments in different ways.

Aquatic organisms that inhabit polluted waters can accumulate toxic substances such as inorganic and organic compounds in their tissues (Connell, 1988; van der Oost *et al*., 1988; Andral *et al*., 2004). Therefore, living organisms act as bioindicators are utilized to screen the health status of the environment (Parmar *et al*., 2015). As shown by Burger (2006), plants followed by invertebrates and then fish represent the major ecological indicators as a measure of stressors and contaminants.

Many monitoring programs have been developed worldwide to assess the status and trends of contamination of coastal waters (Wade *et al*., 1988 a, b,1989, 1990; Sericano *et al*., 1990). An example is given by the Mussel Watch Program (MWP) of U.S. National Oceanic and Atmospheric Administration (NOAA). The Program began in 1986 and is one of the longest running, continuous monitoring programs of coastal waters, including Great Lakes. It is based on yearly collection and analysis of oysters and mussels for monitoring a suite of trace metals and organic contaminants such as DDT, PCBs and PAHs as well as new and emerging contaminants (Kimbrough *et al*., 2008).

At European level, the Water Framework Directive (WFD) establish a framework for the protection of surface waters (*i.e.*, inland waters, transitional waters, coastal waters and groundwater), (2000/60/EC).

However, roughly eighteen years since it was adopted, moreover showing many problems and delays in its implementation, the WFD has not delivered its main objectives (Voulvoulis *et al*., 2017).

On the other hands, the European Union Marine Strategy Framework Directive (MSFD) is a framework for community action with the goal achieving of Good Environmental Status (GES) in European marine environments by 2020 (2008/56/EC).

Among the qualitative descriptors for determining GES reported in the Annex I [referred to in Articles 3(5), 9(1), 9(3) and 24] three are directly related to marine organisms (GES descriptor 3, 8, 9). Thus, the use of biological effects tools is essential to meet the challenges outlined by the MSFD. Therefore, for undertaking assessments of GES across European marine regions, some authors have discussed the combined approach of monitoring contaminant levels, alongside biological effect measurements relating to the effect of pollutants (Lyons *et al*., 2010).

In Europe, moreover, the use of benthic indicators such as bivalves has been widely discussed in relation to WFD and MSFD (Van Hoey *et al*., 2010).

Bivalves are sessile, and filter-feeder organisms able to accumulate and concentrate particles from water (Bebianno, 1995; Mouneyrac *et al*., 1998) as well as many pathogens. Chemical, physical and biological stressors have negative effects on its health status; thus, depending on the degree of exposure (*i.e.*, acute or chronic), determine reversible and irreversible cellular lesions resulting in diseases (Carella *et al*., 2015), (Fig. 1.1). Therefore, the etiologic agents may have exogenous (*i.e.*, physical, chemical, biological) or endogenous (*i.e.*, immunologic genetic defect) nature (Carella *et al*., 2015).
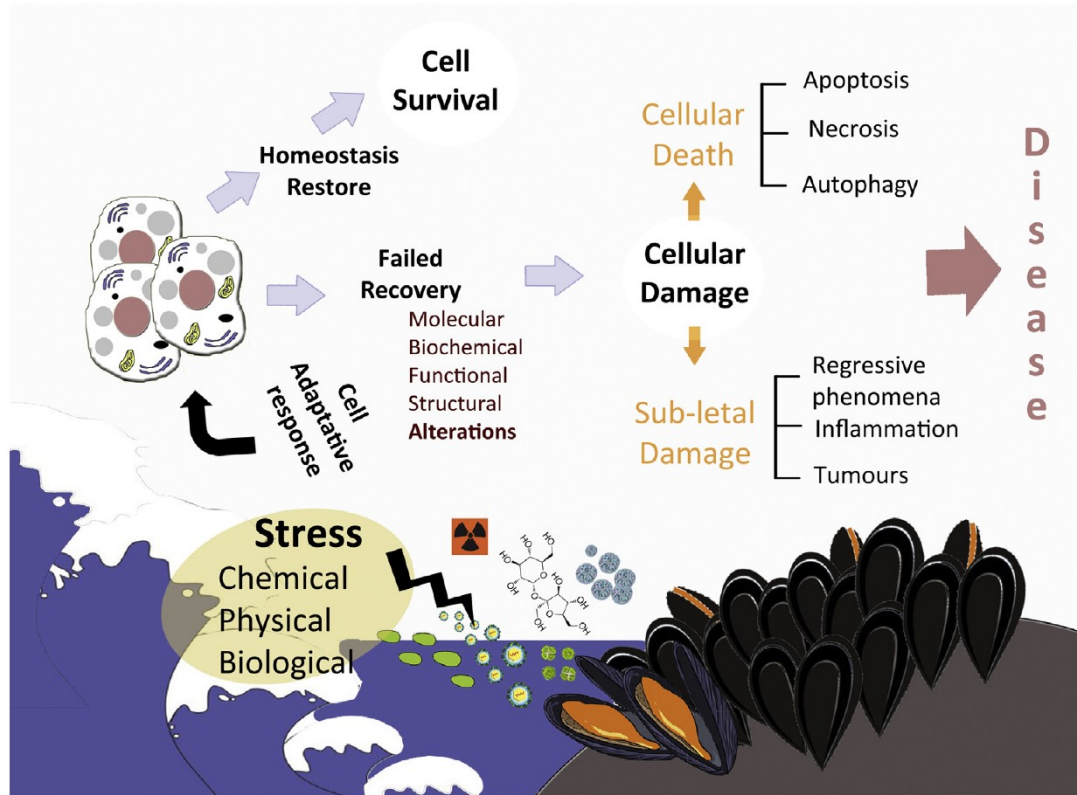
Fig. 1.1 Cellular response and reactions to external stress stimuli, from Carella *et al*., 2015.

Bivalves possess only innate immunity with remarkably effective capabilities both humoral and cellular defence mechanisms (Canesi *et al*., 2002). The humoral responses include soluble lectins, hydrolytic enzymes, antimicrobial peptides and protease inhibitors (Cheng, 1996; Canesi *et al*., 2006).

The hemocytes as macrophages in vertebrates, are the cells involved in the immune response (Galloway and Depledge, 2001) in invertebrates as well as in physiological functions (*i.e.*, digestion and nutrient transport), (Cheng, 1981; Carella *et al*., 2015). There are two distinct hemolymph cells, namely granulocytes and hyalinocytes (agranulocytes), (Tiscar and Mosca, 2004; Martin *et al*., 2007; Carella *et al*., 2015). The first cells are larger (10-15 μm), have granular cytoplasm and a low nucleus-to-cytoplasm ratio (De

Vico and Carella, 2012). Conversely, hyalinocytes are smaller (6-7 μm) with agranular-hyaline cytoplasm and a high nucleus-to-cytoplasm ratio (De Vico and Carella, 2012). Both cells have phagocytic capability, but granulocytes are the primary cells involved in the cellular response to infection (Chu, 2000; Carella *et al.*, 2015) showing the highest phagocytic capacity (De Vico and Carella, 2012). Phagocytosis process was described by several authors: according to Cheng (1983, 1996) and Renwrantz (1990) it can be divided in: chemotaxis, recognition or attachment, internalisation or endocytosis and degradation. Cellular degradation of internalized material, moreover, occurs in secondary phagosomes of the clam hemocytes (López *et al.*, 1997).

Furthermore, as highlighted by Carella and co-authors (2015), this defensive response shows three basic histotypes: (1) *Infiltrative/focal hemocytosis*, (2) the *Nodulation* and (3) the *Encapsulation*-like response, analogous to granuloma formation in vertebrates. In general, nodulation is the response to numerous small particles while encapsulation is the response to larger foreign bodies that cannot be ingested by single phagocytes (Galloway and Depledge, 2001). However, a non-immunological response named *Nacrezation* was observed in the BRD (Brown Ring Disease) in clams belonging to genus *Ruditapes* (Paillard, 2004; De Vico and Carella, 2012; Carella *et al.*, 2015). Through this mechanism clams can recover from the disease by covering the organic deposit by shell secretions (Paillard, 2017).

Therefore, hemocytes infiltration can be used as biomarkers of environmental quality (Cajaraville *et al.*, 1996). The chemical-physical variables of water such as temperature can influence the total number of hemocytes, their phagocytic activity and lysozyme (Matozzo and Marin, 2001). In Mediterranean mussel *Mytilus galloprovincialis* hemocytes have a seasonal variation positively correlated with water temperature, but not associated with size or weight of mussels (Carballal *et al.*, 1998).

Besides, in Manila clams *R. philippinarum* and in Blue mussels *Mytilus edulis,* the high levels of salinity may lead to an increase in hemocytes as opposed to oysters in which increase at low salinity (De Vico and Carella, 2016). Moreover, the pH may impact the physiological condition and the functionality of the hemocytes and could have a significant effect on cellular signalling pathways (Calder-Potts *et al*., 2008). The abovementioned stress factors can also act in synergy (Gagnaire *et al*., 2006; Monari *et al*., 2007; De Vico and Carella, 2016).

Furthermore, a change in the number of hemocytes can occur in the presence of parasites and/or pathogens. However, bacteria can evade host recognition through different strategies (Canesi *et al*., 2002; Soudant *et al*., 2013), for example, becoming intracellular (Hine, 1991; Morga *et al*., 2009; Carella *et al*., 2015). An inflammatory response characterized by phagocytosis and/or encapsulation was observed in clams infected with the protozoan *Perkinsus* spp. (Chagot *et al*., 1987). Thus, clams parasitized by *Perkinsus* spp. show a variable degree of hemocytic encapsulation, while *Marteilioides-like* organism and *Cercaria tapidis*, that infected the gonad did not evoke any significant host reaction (Lee *et al*., 2001). However, significant increase of hemocytes in the hemolymph was detected after infestation by *Marteilia refringens* (Carballal *et al*., 1998).

Clams infected by pathogen *Vibrio tapetis*, instead, showed significant increase in hemocyte counts and lysozyme activity in the hemolymph, but particularly in the extrapallial fluid (Allam *et al*., 2000).

Furthermore, some chemicals such as PCBs and heavy metals can cause adverse effects on immune functions (Galloway and Depledge, 2001, and references therein) with effects on susceptibility to infection (Pipe and Coles, 1995; Cole *et al.*, 1995). Moreover, they can modulate endocrine functions (*i.e.*, reproduction and fertility) with serious consequence at the population level (De Vico and Carella, 2016).

Nowadays, the application of histological and cytological alterations in aquatic organisms (*i.e.*, fish and bivalves) have been employed as biomarkers in pollution monitoring (Au, 2004). Therefore, bivalve histopathology is an acknowledged tool to assess quality of coastal ecosystems through surveying the health status of these benthic invertebrates (Costa *et al.*, 2013).

The bivalves have developed some sub-cellular systems for the accumulation, regulation and immobilization of excess pollutants, including essential and non-metallic elements (Langston *et al.*, 1998). The exposure of these invertebrates to polluting elements determines their capture through mechanisms related to their ability to filter water. They can therefore incorporate substantial amounts of toxic substances into their organs without any apparent negative effect (Mouneyrac *et al.*, 1998). Bivalve molluscs show higher concentrations in certain organs, particularly in the digestive gland (Piras *et al.*, 2013). In fact, several studies have shown that the digestive gland is the organ most involved in the metabolic and immunocompetence mechanisms of molluscs, as well as in the elimination of accumulated contaminants (Moore and Allen, 2002). Thus, the evaluation of the alterations affecting target organs (*i.e.*, digestive gland, gills and kidney) as a reflection of the quality of the environments is particularly recommended in biomonitoring programs (Bustamante and Miramand, 2005; Costa *et al.*, 2013; Cuevas *et al.*, 2015; Chalghmi *et al.*, 2016).

Nowadays, the use of bivalves is currently considered one of the most effective approaches for assessing the degree of pollution of brackish environments. Given the economic importance of the clam *R. decussatus*, the second part of the thesis aimed to evaluate inflammatory responses in its target organs as indicators of environmental stress on the health status of these organisms from Sardinian coastal environments.

3.1 Materials and Methods

3.1.1 Description of the study area and sampling procedures

Four different coastal areas of Sardinia (central-western Mediterranean Sea) (Fig. 1.2), in which clams were also sampled for the detection of trace elements, were considered: Calich (ch, North-western Sardinia), Porto Pozzo, San Teodoro (pp, st respectively; north-eastern Sardinia) and Santa Gilla (sg, South Sardinia), (for a detailed description of the four areas, see chapter II, Materials and Methods section). The selection of the harvest points together with all field activities, have been planned by using the experience and collaboration of the fishermen's cooperatives that manage each aquaculture facilities. In October 2016, with the help of local operators and by means of special sampling systems (rakes called "*rasche*"), 100 adult specimens of *Ruditapes decussatus* were manually collected from each site (400 in total). The clams were transported alive in refrigerated and insulated containers at the Pathological Anatomy Laboratory of the Department of Veterinary Medicine of Sassari. Subsequently, bivalves were processed after species recognition and morphometric measures determination. Specimens from site "ch" measured (mean ± SD) 39.83 ± 2.47 mm (TL, total length) and weighed 2.84 ± 0.55 g wet weight (ST, soft tissue), those from sites "pp" and "st", respectively TL = 32.82 ± 3.15 mm; ST = 1.31 ± 0.36 g w. w. and TL = 43.62 ± 3.21 mm; ST = 1.95 ± 0.39 g w. w., while those coming from the site "sg" TL = 30.31 ± 1.7 mm; ST = 1.4 ± 0.22 g w. w.
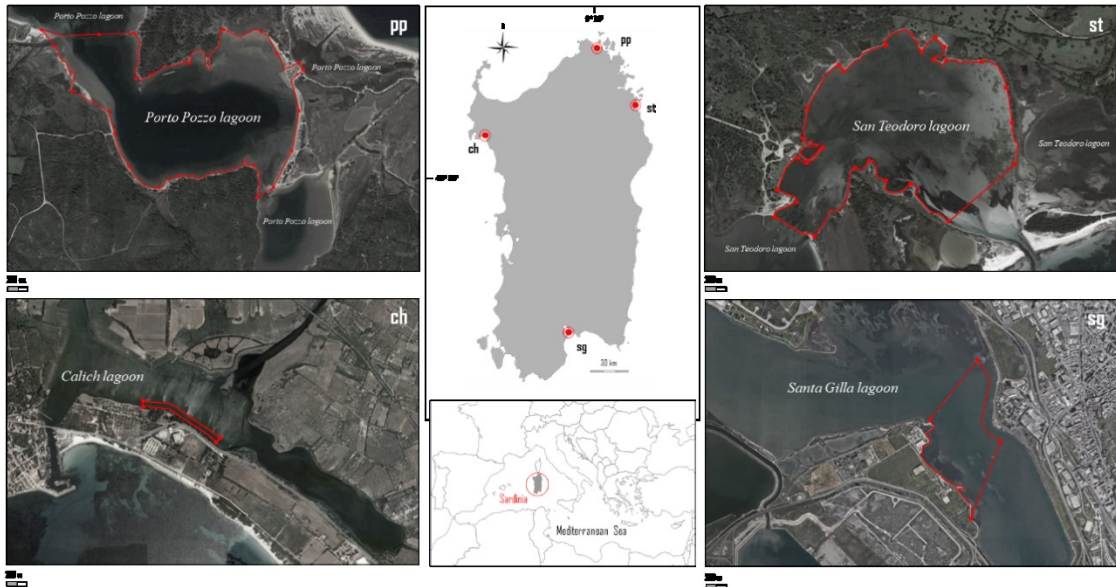
Fig. 1.2 Study area and sampling locations.

3.1.2 Necropsy

Clams were disposed in lateral side and the first incision was made with a scalpel on the anterior adductor muscle, being careful to avoid damaging of visceral mass. Then, the cut was continued from the posterior region. The second incision was made from the posterior adductor muscle. At this point, the shell was opened showing all the visceral organs (Fig. 1.3).
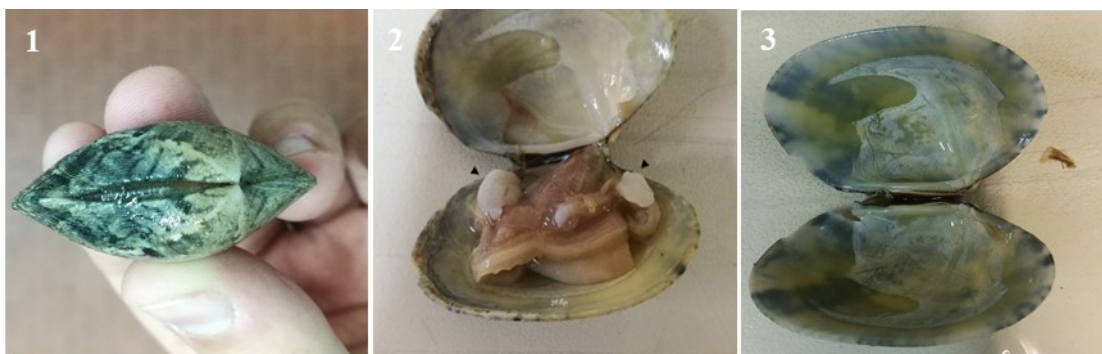

Fig. 1.3 Cutting phases of *Ruditapes decussatus* specimen. The two adductor muscles of white colour in evidence (2, black arrows).

3.1.3 Histopathology

Tissues of each clams, previously separated to the shell, were fixed in 10% neutral formalin for 48h (Fig. 1.4a). Subsequently (after fixation process) with a scalpel a longitudinal cut was made; thus, the animal was divided into two perfectly identical parts (Fig. 1.4b). One side (external) was used for the gills analysis, while the other (internal) for the digestive gland and kidney evaluation.
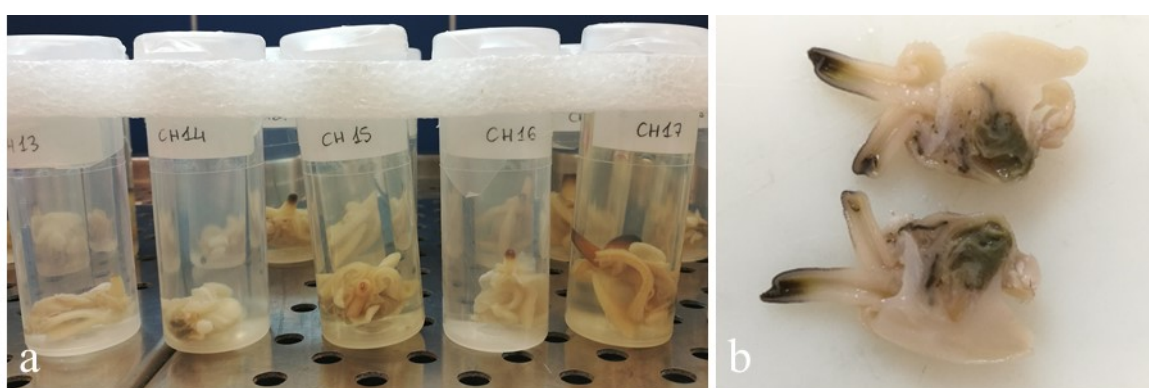

Fig. 1.4 (a) Samples fixed in 10% neutral formalin. (b) Longitudinal cut of clam tissues.

Tissue sections were dehydrated with increasing concentrations of alcohol and xylene in an automatic tissue processor (HISTO-PRO 200) and paraffin embedded. The protocol proposed by Mazzi (1977) was used according to the histological procedures:

○ *Fixation*: samples were treated in a liquid fixing agent. Formaldehyde commercially available in solution of 40% was buffered with CaCO3 and 10% neutral formalin was used to fix the tissues. This fixative penetrate tissue causing chemical and physical changes increasing their hardness. Specimens were placed in labelled cassettes (small perforated baskets) to separate them from other

specimens. To prevent the autolysis and degradation of the tissue, the duration of the processing schedule was 48 h.

o *Dehydration*: samples were immersed in a series of ethanol solutions of increasing concentration to remove the water and to allow melted paraffin to infiltrate the tissue: 50% ethanol (2h), 70% ethanol (2h), 90% ethanol (2h), 100% ethanol (2h) and 100% ethanol (2h).

o *Clearing*: this process provides the use of xylene, an intermediate solvent that is miscible with both ethanol and paraffin wax. This solvent has an important role to remove also a substantial amount of fat from the tissue. When ethanol has been entirely replaced by xylene, the tissue has a translucent appearance, so this is called clearing agent. Two clearing sequence were made: xylene (2h) and xylene (2h).

o *Wax infiltration*: in this phase tissue cassettes were dipped in 3 steps of paraffin, a mixture of hydrocarbons with a melting point at 60°C. At the end of this process the paraffin entirely infiltrated the tissue and replaced xylene. Three wax infiltration were made: wax infiltration (2h), wax infiltration (2h) and wax infiltration (2h).

o *Embedding or blocking out*: the tissue is embedded within a block of paraffin, using an embedding centre (ACM 50; Fig. 1.5a), where a mould is filled with molten wax and the specimen placed into it (Fig. 1.5b). It is very important to orientate the specimens to obtain a well oriented plane of section. (Fig. 1.5c).
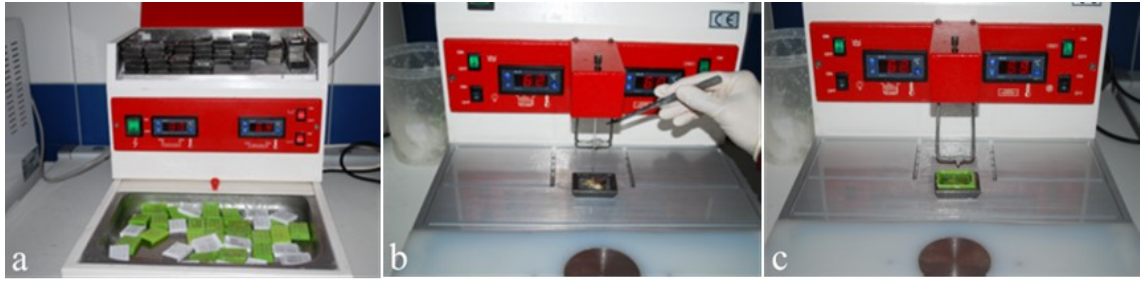
Fig. 1.5 (a) Embedding centre (ACM 50). (b) Tissue into a mould filled with molten paraffin. (c) Tissue sample well oriented.

o   Cut and stained: the paraffin blocks were removed from the mould and were cut with the microtome (Leica RM 2245) (Fig. 1.6a). Sections of 3μm were obtained and were stained in an automatic multistainer (ST5020, Leica Biosystems) (Fig. 1.6b) with Hematoxylin and Eosin (HE) according to a standard method (Mazzi 1977). Sections were mounted on a glass slide and then evaluated at light microscopy (Nikon Eclipse 80i).
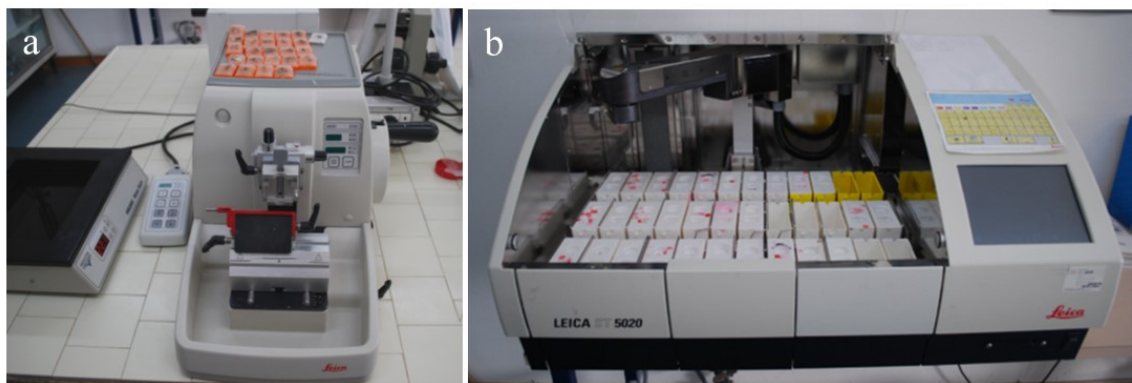

Fig. 1.6 (a) Microtome and paraffin blocks. (b) Automatic multistainer.

The histological protocols used for staining sections are reported below:

Haematoxylin and eosin (H&E)

1. Slides were deparaffinized and rehydrated through graded alcohols

2. Harris' hematoxylin (3ʹ)

3. Washed in running tap water (5ʹ×2)

4. Rinsed in distilled water

5. Eosin (2ʹ)

6. Rinsed in distilled water

7. Dehydrated, cleared and mounted

3.1.4 Histopathological indices

The histopathological indices (*Ih*) for each subject were calculated according to the method proposed by Costa *et al*., (2013) for the species under investigation (*R. decussatus*) through the following formula:

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

where *Ih* is the histopathological index for the individual *h*; w_j the weight of the *j*th histopathological alteration; a_{jh} the distribution value selected for the *jh* alteration and M_j is the attributable maximum (weight×maximum distribution) for the jth alteration. According to Costa and co-authors (2013) the denominator of the equation standardises the indices to a value between 0 and 1, permitting comparisons between different conditions such as different organs, sampling lagoons. Briefly, the histopathological assessment considered the weight (*w*) of the alteration to which a value of between 1 (minimal significance) and 3 (highest severity) and distribution (*a*), ranging from 1 (focal)

to 3 (diffuse) were attributed. For each clam, moreover, the histopathological index was estimated based on organ and type of alteration considered. The alterations considered within each organ are summarised in Tab. 1.1.

Tab. 1.1 Alterations considered for each organ and their importance weight (*w*).

Organ	Alteration	*w*
Digestive gland	Hemocytic infiltration	1
	Necrosis	3
	Perkinsus spp.	1
	Other parasites	1
Gills	Hemocytic infiltration	1
	Necrosis	3
	Loss of epithelium	2
	Lamellar fusion	1
	Perkinsus spp.	1
	Other parasites	1
Kidney	Hemocytic infiltration	1
	Necrosis	3
	Perkinsus spp.	1
	Other parasites	1

3.1.5 Statistical analyses

Statistical evaluations were performed using the software STATA/IC 11.2 (StataCorp LP, USA). The normality of data was verified through the Shapiro-Wilk test. The rank-based non-parametric Kruskal-Wallis and Dunn's multiple pairwise tests were used to compare sampling sites and response variables, respectively. Correlation analyses were attained through Spearman's rank correlation coefficient. Results were considered statistically significant at *p* values of < 0.05.

3.2 Results and Discussion

3.2.1 Morphometric measures

Overall, four hundred adult Grooved carpet shell clams *Ruditapes decussatus* belonging to the Veneridae family were sampled during October 2016. Below, in detail for each site the main morphometric data are reported:

3.2.1 Calich lagoon

Overall, 100 specimens were examined*:* (TL=39.83±2.47 mm; ST=2.84±0.55 g), of which 38 were females (TL=39.87±2.83 mm; ST=2.47±0.56 g; Figs. 2.9 & 3.0) and 31 were males (TL=40.07±2.51 mm; ST=2.84±0.54 g; Figs. 3.1 & 3.2).
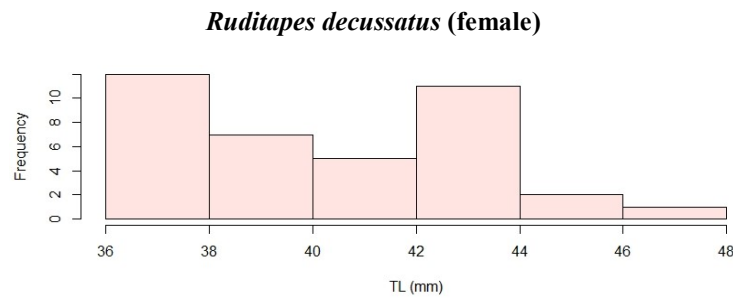

Fig. 2.9 Distribution of length size (LT) classes of *R. decussatus* at the Calich lagoon.
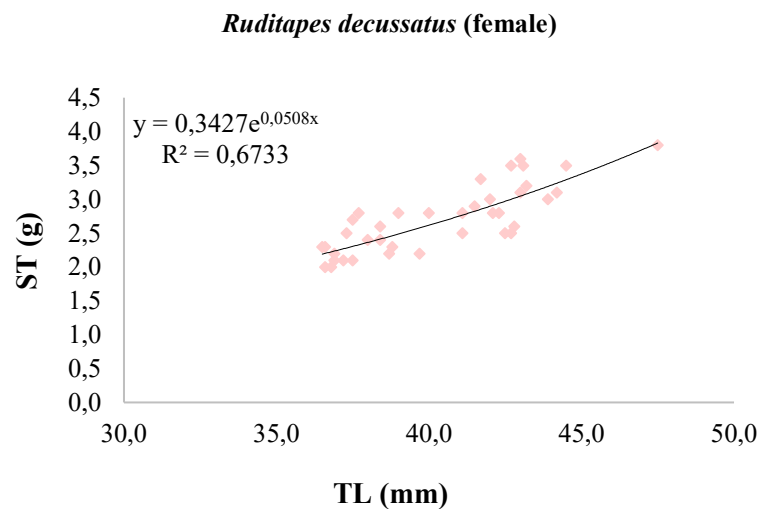
Fig. 3.0 Length-soft tissue (TL-ST) relationship of *R. decussatus* at the Calich lagoon.
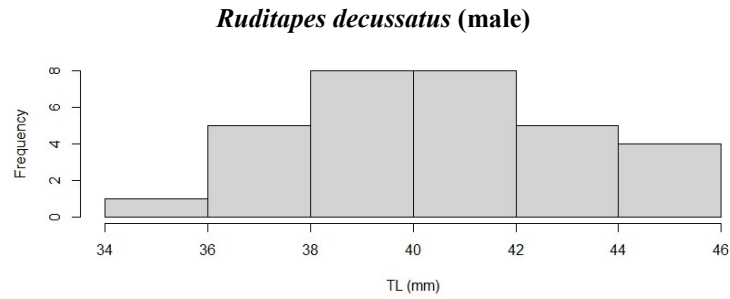
Ruditapes decussatus (male)

Fig. 3.1 Distribution of length size (LT) classes of *R. decussatus* at the Calich lagoon.

Ruditapes decussatus (male)

$y = 0{,}3381e^{0{,}0494x}$
$R^2 = 0{,}7548$
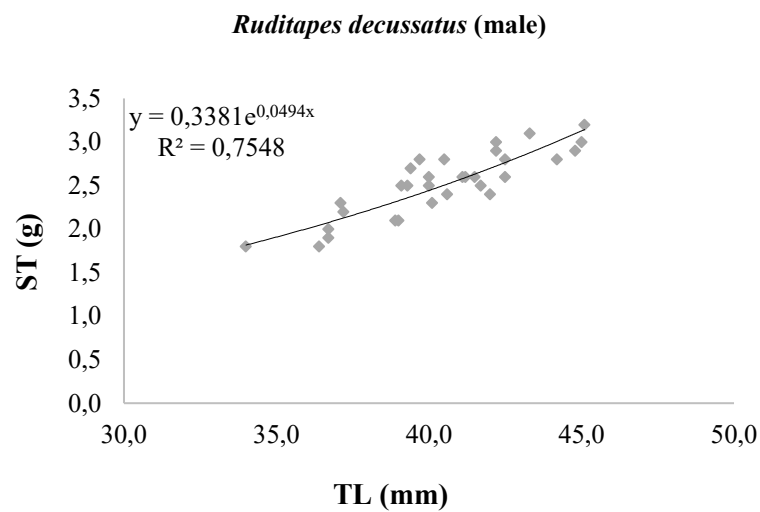
Fig. 3.2 Length-soft tissue (TL-ST) relationship of *R. decussatus* at the Calich lagoon.

3.2.2 Porto Pozzo lagoon

Overall, 100 specimens were examined: (TL=32.82±3.15 mm; ST=1.31±0.36 g), of which 52 were females (TL=32.76±1.31 mm; ST=2.99±0.34 g; Figs. 3.3 & 3.4) and 40 were males (TL=32.8±4.27 mm; ST=1.30±0.0.37 g; Figs. 3.5 & 3.6).
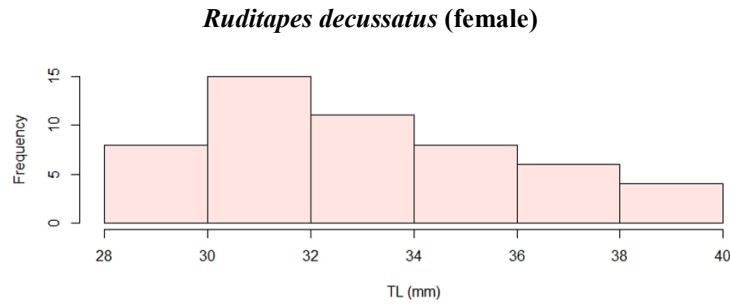
***Ruditapes decussatus* (female)**

Fig. 3.3 Distribution of length size (LT) classes of *R. decussatus* at the Porto Pozzo lagoon.

***Ruditapes decussatus* (female)**

$$y = 0,1032e^{0,0766x}$$
$$R^2 = 0,8005$$

Fig. 3.4 Length-soft tissue (TL-ST) relationship of *R. decussatus* at the Porto Pozzo lagoon.

***Ruditapes decussatus* (male)**

Fig. 3.5 Distribution of length size (LT) classes of *R. decussatus* at the Porto Pozzo lagoon.

Ruditapes decussatus (male)

$$y = 0,1271e^{0,0704x}$$
$$R^2 = 0,8542$$
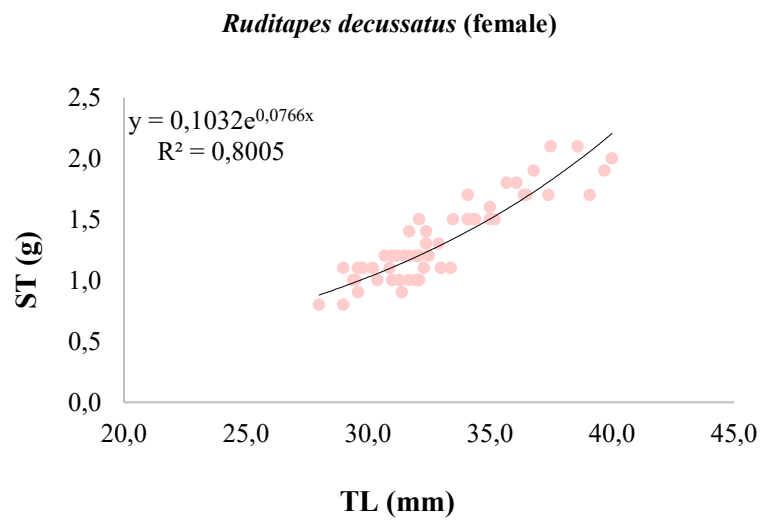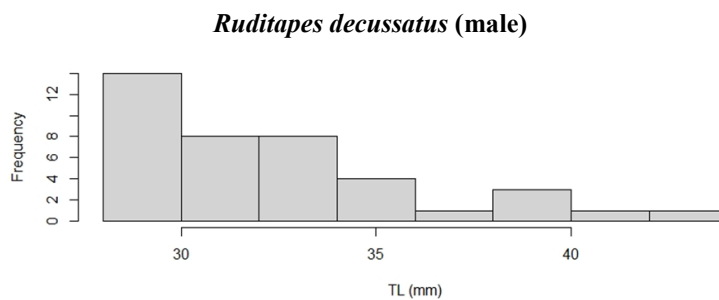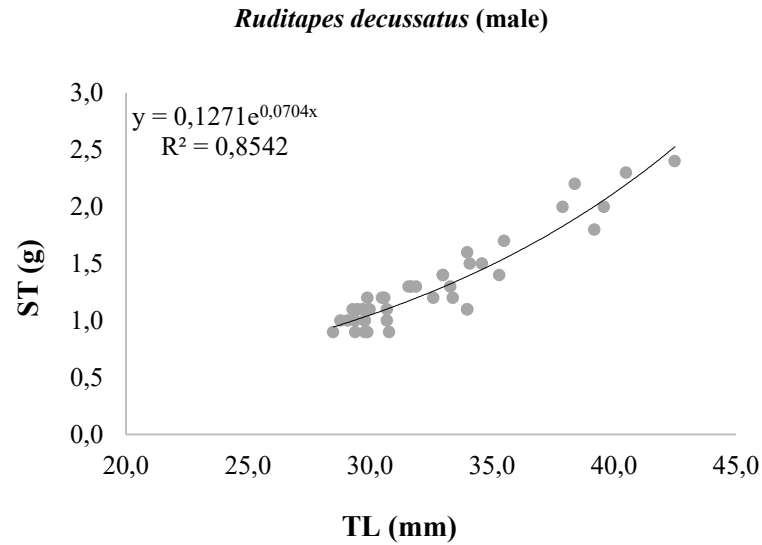
Fig. 3.6 Length-soft tissue (TL-ST) relationship of *R. decussatus* at the Porto Pozzo lagoon.

3.2.3 San Teodoro lagoon

In total, 100 specimens were examined: (TL=43.62±3.21 mm; ST=1.95±0.39 g), of which 48 were females (TL=43.66±3.21 mm; ST=1.92±0.39 g; Figs. 3.7 & 3.8) and 38 were males (TL=40.6±3.2 mm; ST=1.8±0.4 g; Figs. 3.9 & 4).
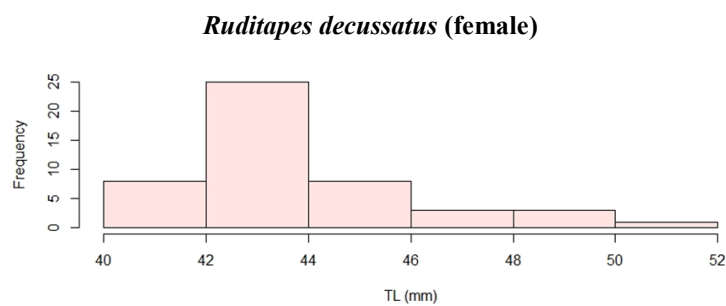
Ruditapes decussatus (female)

Fig. 3.7 Distribution of length size (LT) classes of *R. decussatus* at the San Teodoro lagoon.

***Ruditapes decussatus* (female)**

$$y = 1{,}0741e^{0,025x}$$
$$R^2 = 0{,}6226$$

Fig. 3.8 Length-soft tissue (TL-ST) relationship of *R. decussatus* at the San Teodoro lagoon.

***Ruditapes decussatus* (male)**

Fig. 3.9 Distribution of length size (LT) classes of *R. decussatus* at the San Teodoro lagoon.

***Ruditapes decussatus* (male)**

$$y = 0{,}1604e^{0,0685x}$$
$$R^2 = 0{,}7492$$
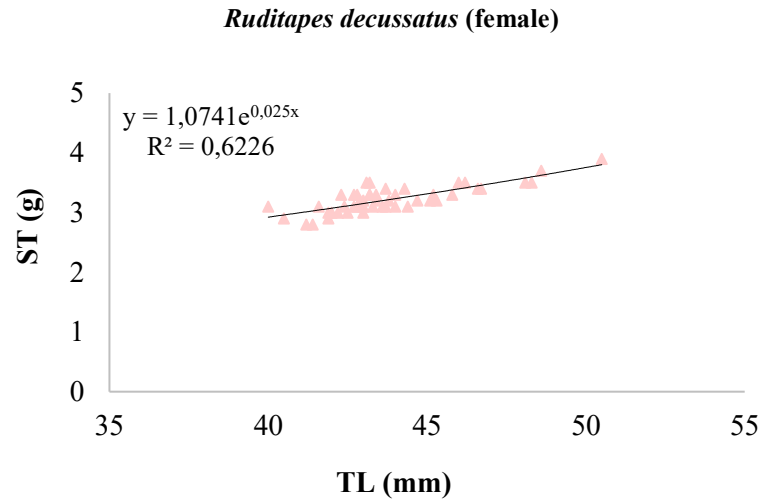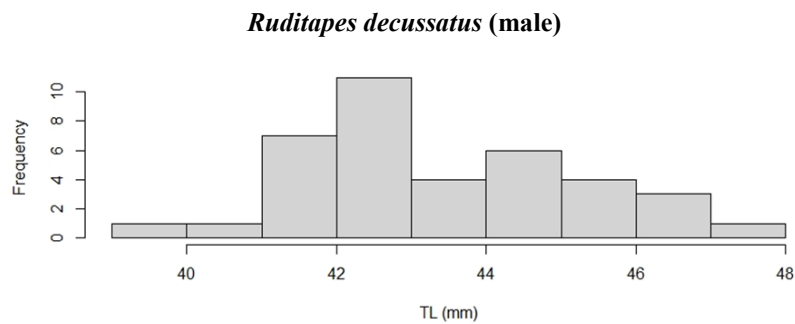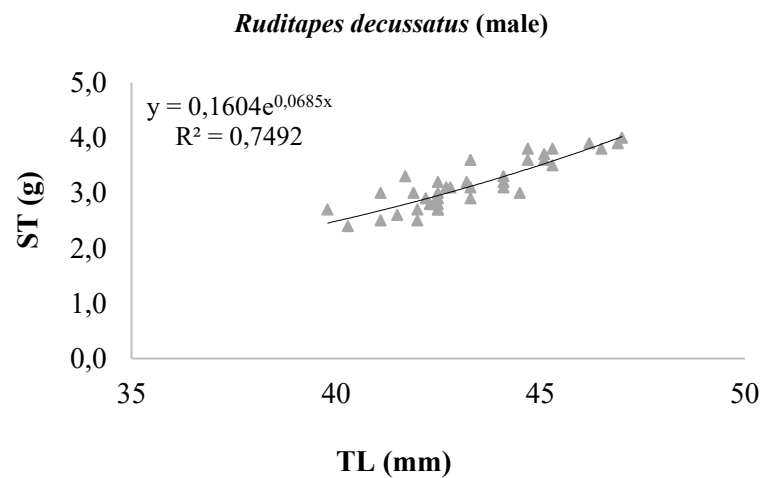
Fig. 4 Length-soft tissue (TL-ST) relationship of *R. decussatus* at the San Teodoro lagoon.

3.2.4 Santa Gilla lagoon

One-hundred specimens were examined: (TL=30.31±1.7 mm; ST=1.4±0.22 g), of which 51 were females (TL=30.35±1.75 mm; ST=1.4±0.21 g; Figs. 4.1 & 4.2) and 39 were males (TL=30.31±3.31 mm; ST=1.4±0.24 g; Figs. 4.3 & 4.4).
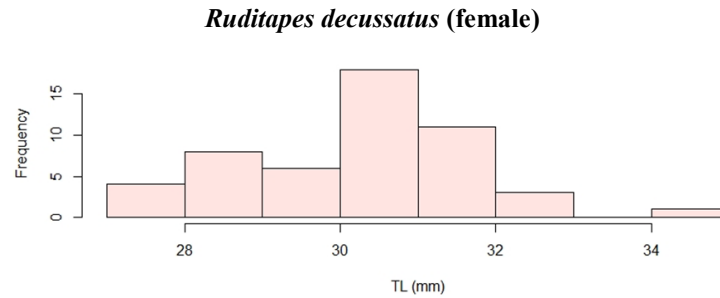
Ruditapes decussatus (female)

Fig. 4.1 Distribution of length size (LT) classes of *R. decussatus* at the Santa Gilla lagoon.

Ruditapes decussatus (female)

$$y = 0{,}1131e^{0{,}0824x}$$
$$R^2 = 0{,}6583$$

Fig. 4.2 Length-soft tissue (TL-ST) relationship of *R. decussatus* at the Santa Gilla lagoon.

***Ruditapes decussatus* (male)**

Fig. 4.3 Distribution of length size (LT) classes of *R. decussatus* at the Santa Gilla lagoon.

***Ruditapes decussatus* (male)**

$$y = 0{,}3964e^{0{,}0425x}$$
$$R^2 = 0{,}6536$$

Fig. 4.4 Length-soft tissue (TL-ST) relationship of *R. decussatus* at the Santa Gilla lagoon.

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indirizzo Qualità e Sicurezza Alimentare. Università degli Studi di Sassari.
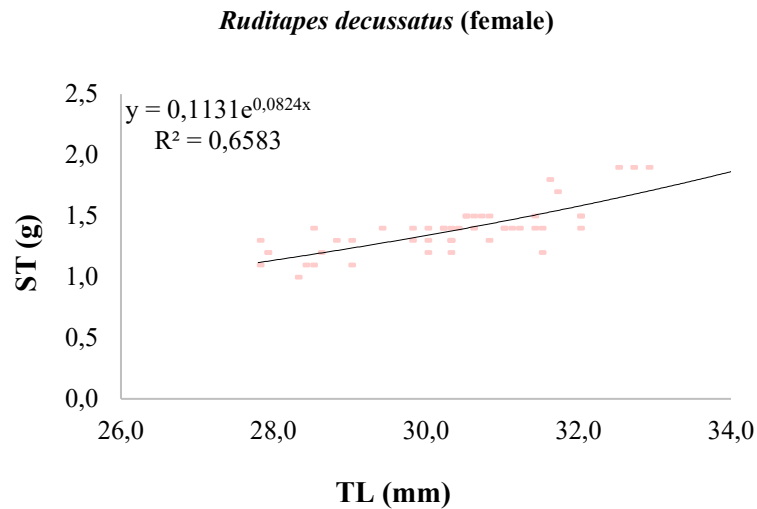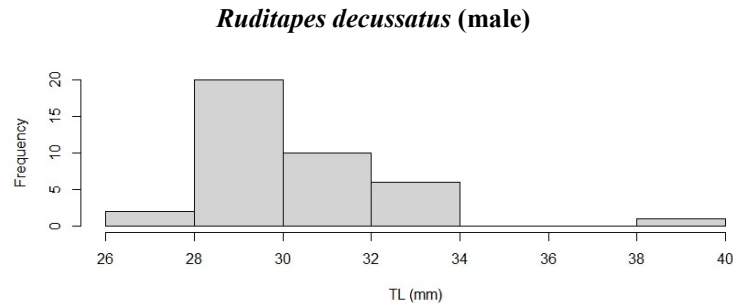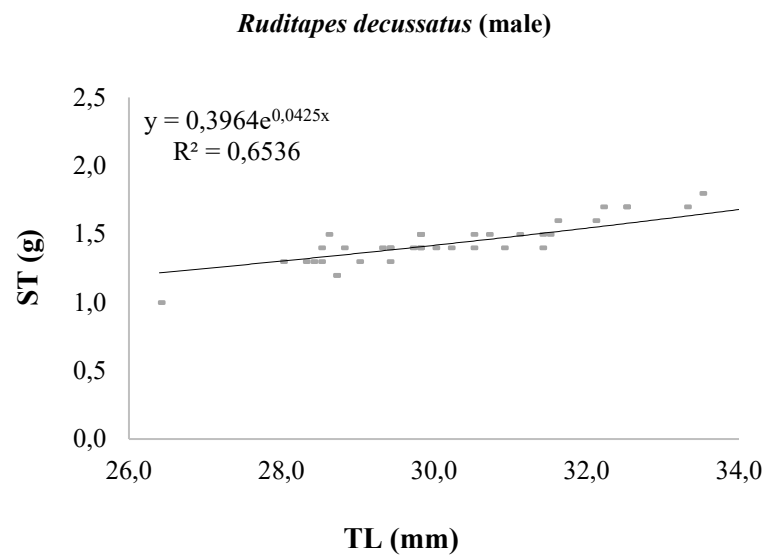
3.2.5 Digestive gland histopathology

Overall, clams collected at Porto Pozzo (pp) and San Teodoro (st) lagoons have shown minimal histopathological lesions in the digestive gland, in terms of hemocytic infiltrate and necrosis of the digestive tubules or the intertubular tissue (Tab. 1.2-1.6). On the contrary, the specimens collected in the Calich lagoon (ch) have reported the highest prevalence of alteration, followed by those from Santa Gilla (sg) lagoon.

Normally, the digestive gland of *Ruditapes decussatus* is formed by digestive tubules which are characterized by digestive cells and darkly staining cells located at both ends also characterized by secretory, flagellated and nest of stem cells (Grizel, 2003), (Fig.4.5). Hemocytic infiltration was observed in clams from all sites, from 10% (pp, Porto Pozzo lagoon) to 40% (ch, Calich lagoon) of the specimens.

Our results are in accordance with those by Cuevas and co-authors (2015) in Mediterranean mussels *Mytilus galloprovincialis* sampled along different environments from Basque coast. Specifically, they reported inflammation-related alterations ranging from 15 to 80% typically associated with deteriorated tissue or infections. Similar data have also been found by Costa and co-authors (2013) in the digestive gland of clams belonging to the genus *Ruditapes* from coastal ecosystems of the Southern Portuguese coast, affected by different sources of pollution. Furthermore, the presence of hemocyte infiltrate for ours sites was always correlated to the presence of the protozoan *Perkinsus* spp. ($p < 0.001$). Infection by *Perkinsus* spp. was recurrent and was observed from all site, especially in the clams from Calich lagoon (prevalence of 37%), (Fig. 4.5).

This protozoan determined a strong host inflammatory response, with an infiltration of numerous hemocytes into the surrounding tissue characterized by phagocytosis and/or encapsulation (Chagot *et al*., 1987; Cremonte *et al*., 2005). The trophozoites of *Perkinsus* spp. were observed mainly in clusters in the connective tissue as well as in the mantle and

gills (see later). These cyst-like structures presented the typical "signet-ring" shape with a large eccentric vacuole that displaces the nucleus to the periphery of the cell (Cremonte *et al.*, 2005). In general, it was observed that hemocytes enclosing the trophozoite stages in a capsule within many different immune reactions, take place (*i.e.*, formation of reactive oxygen and melanin synthesis), (Carella *et al.*, 2015).

Furthermore, a low prevalence (ranging from 0 to 2%) of different other than *Perkinsus* spp. parasites in the digestive gland was highlighted.

As in vertebrates, necrosis in mollusc tissues can have a focal and diffused distribution and according to the morphology, it can be divided in two main categories (*i.e.*, coagulative and colliquative). In general, it can be accompanied by morphological modifications of the nucleus (De Vico and Carella, 2016). This condition was observed in 6% of clams collected from Santa Gilla lagoon and according to previous authors (Villalba *et al.*, 1995; Costa *et al.*, 2013), was diagnosed by the presence of enlarged eosinophilic cell with scant cytoplasm without nucleus.

Costa and co-authors (2013) showed a limited severe necrosis of tubules of intertubular tissue usually linked to a fibrosis alteration. On the contrary, Cuevas and co-authors (2015) reported the presence of necrosis associated with severe atrophy of digestive tubules, intertubular fibrosis (*i.e.*, digestive tubule necrosis: 10-75%; intertubular necrosis: 10-60%). However, despite Cuevas and co-authors (2015) found the greater frequency of necrosis in autumn, significantly lower percentages were reported in our study (1-6%).

In conclusion, the non-parametric analysis of variance showed statistically significant differences for Calich, Porto Pozzo and San Teodoro lagoons in terms of hemocytic infiltration ($p < 0.05$; ch ≠ pp and st ≠ ch). Statistically significant differences between the considered sites was also reported for the presence of *Perkinsus* spp. ($p < 0.05$; sg ≠

ch, pp ≠ ch and st ≠ ch). No statistically significant differences were observed for necrosis and the presence of parasite in clams from all sites investigated.

3.2.6 Gills histopathology

Lesions in gill epithelia were frequent with different degrees of severity in clams sampled from all sites, ranging from about 33% (ch, Calich lagoon) up to 46% (pp, Porto Pozzo lagoon), (Tab. 1.2-1.6). The non-parametric analysis of variance showed statistically significant differences for all sites and considered alterations, except for necrosis ($p < 0.05$).

Normally, the gills or ctenidium are flat structures usually white in colour and developed just below the mantle. These structures are suspended from the ctenidial axis that is fused along the dorsal margin of the mantle (Gosling, 2015). The gills are supported mainly by muscle fibers and connective tissue, crossed by numerous hemolymph vessels. Each gill filament presents a cuboidal and ciliated epithelium rich in mucus cells. Large numbers of hemocytes, mostly hyalinocytes, are common scattered along the undamaged epithelial surface. One of the most recurrent alteration, as for the digestive gland tissue consisted of gills inflammation, revealed by different types of hemocyte infiltration.

In our study this alteration was always present in all sites with the following trend: 91% for Porto Pozzo and Santa Gilla, 82% for Calich and 71% in San Teodoro lagoons. Mostly the hemocytic infiltration was always correlated to pathogenic microorganisms like *Perkinsus* spp., except for the San Teodoro lagoon where, on the contrary, flogosis was related to necrosis, loss of the gill's epithelium and lamellar fusion ($p < 0.001$).

Perkinsus spp. has been responsible for mass mortalities worldwide, with a significant aquaculture impact resulting in severe economic losses. In the North-eastern Atlantic and Mediterranean Sea, it was the most important pathogen of the clams of the genus

Ruditapes, (Ruano *et al*., 2015 and references therein). The pathology named *Perkinsosis* damages the main bivalve defence mechanisms (*i.e.*, phagocytosis and ROS production), (Queiroga *et al*., 2013) as well as the spawning frequency reducing egg production (Park *et al*., 2006). Lesions were found in the connective tissue of gills (Ngo and Choi, 2004; Pretto *et al*., 2014) causing necrosis too (Choi and Waki, 2016). Multicellular stages resulting from vegetative multiplication (putative schizonts), with numerous daughter cells arranged in a "*rosette*" (Ruano *et al*., 2015) have been pointed out. Generally, bivalve molluscs with parasitized gills revealed an important host immune response: in our study, the examination of the histological sections revealed an occurrence of *Perkinsus* spp. which affected the connective gill tissues, typically characterized by encapsulation through severe hemocytic infiltration, especially granulocytes. It was detected in clams from all the sites and the specimens collected from Calich lagoon (ch) have reported the highest incidence (68%), followed by those from Porto Pozzo and Santa Gilla lagoons (both 23%).

Our results can be compared with those found by Costa and co-authors (2013) in clams belonging to Veneridae family reporting high prevalence (over 50%) in gills and digestive gland. Moreover, Ngo and Choi (2004) reported an infection prevalence of *Perkinsus* spp. in October, up to 36%. These data are in accordance with our results related to the same period. Furthermore, a high prevalence of other parasites, different from *Perkinsus* spp., in the gills was highlighted, ranging from 8% (ch, Calich lagoon) to 86% (pp, Porto Pozzo lagoon) and always associated to hemocytic infiltration. A similar situation was observed by Carballal and co-authors (2001) in parasitized Common edible cockle *Cerastoderma edule* from natural beds of Galician estuaries.

The necrosis of gill tissues was less frequent and less severe in animals collected from Santa Gilla lagoon (7% of prevalence) and, especially for Calich lagoon (1%).

Conversely, the other two sites reported a highest prevalence of necrosis ranging from 12% to 19% (San Teodoro and Porto Pozzo lagoons, respectively).

Furthermore, other two types of lesions (loss of epithelium and lamellar fusion) were considered for the evaluation of gills. In detail, the clams from San Teodoro lagoon reported the highest prevalence of these lesions: 64% for loss of epithelium and 53% for lamellar fusion. On the contrary, the site with the lowest values was Santa Gilla (32% and 6%, respectively). As mentioned above, for this lagoon (San Teodoro) the non-parametric statistical analysis has shown strictly correlation between hemocytic infiltration and gills alterations but not with the presence of pathogenic microorganisms as highlighted for all other lagoons. A recent study conducted in different Tunis lagoons located near to intense anthropogenic activities such as chemical industrial areas, reported diffuse inflammation due to ciliary erosion, fusion of lamellae and in general epithelium alteration in clams *R. decussatus* (Chalghmi *et al.*, 2016).

Chalghmi and co-authors (2016) established a possible positive correlation between trace elements accumulation (*i.e.*, Cd, Pb, Hg, Cu and Zn) and the histopathological lesions in gills. It is interesting to note that similar values for the same heavy metals, except for Cu, were detected in our study in San Teodoro lagoon, which reported the highest incidence of gill alterations (see Tab. 2.4 in Chapter II, Results and Discussion section).

Furthermore, previous studies (Martín-Díaz *et al.*, 2005; 2008) have assessed the role of heavy metals involved in morphological changes and alterations charged to the gills of these benthic organisms.

3.2.7 Kidney histopathology

Overall, kidney lesions were less frequent. The clams with the major incidence of alterations were found in Santa Gilla lagoon, followed by Calich lagoon (Tab. 1.2-1.6). The non-parametric analysis of variance showed not statistically significant differences for all sites and alterations considered.

Normally, the kidneys are enclosed by a collagenous connective tissue which contains muscle fibres, hemolymphatic sinuses with scattered hemocytes. Kidney is characterized by nephridial tubules with regular size cells and basal nucleus (Fig. 4.8). The sub-epithelium is formed of a layer of muscle and connective fibers and of open-weave connective tissue (Grizel, 2003). An inflammation-related response through hemocytic infiltrations was observed in Santa Gilla and Porto Pozzo lagoons which showed the high prevalence (39% and 32%, respectively), followed by Calich and San Teodoro lagoons (27% and 16%, respectively). Flogosis was generally associated to parasites in kidney.

Moreover, necrosis was detected only in Santa Gilla lagoon (7%). Regarding parasites, the highest prevalence was observed in clams from Porto Pozzo lagoon (28%) followed by Santa Gilla lagoon (25%), (Fig. 4.9). The presence of *Perkinsus* spp. ranged from 0% (San Teodoro lagoon) to 7% (Porto Pozzo lagoon), except for clams from Calich lagoon which reported the highest prevalence (22%), (Fig. 4.8). Infections in clam's kidney was reported by different authors (Sagristà *et al*., 1995; Ngo and Choi, 2004) even if lesions in kidney of bivalves are poorly investigated.

However, our data agree with other studies that considered the gills to be the major target organ where found pathological lesions (McLaughlin and Faisal, 2014).

Giuseppe Esposito – "Detection of trace elements in the bivalve *Ruditapes decussatus* from Sardinian coastal lagoons:
effects on food safety and pathological findings in target organs" - Tesi di Dottorato in Scienze Veterinarie,
indirizzo Qualità e Sicurezza Alimentare. Università degli Studi di Sassari.

Fig. 4.5 The digestive gland of *Ruditapes decussatus* collected from the Sardinian brackish environments. (A) Normal structure of the digestive gland of a clam sampled from Porto Pozzo lagoon (pp) with digestive tubules (dt) characterized by a single layer of ciliated eosinophilic epithelial cells (ec) and apical basophilic cells. The tubule lumen (tl) is narrow or almost occluded. The intertubular tissue (it) comprised of hemocytes mainly hyalinocytes (agranulocytes). (B) *Perkinsus* spp. trophozoites (pk) around digestive tubules (dt) of clam sampled from Calich lagoon (ch) with a strong host inflammatory response, with an infiltration of numerous hemocytes (hi) mostly granulocytes characterized by typical *encapsulation*-like response (white arrows). HE stain, scale bars: 10 μm.

Fig. 4.6 The gills of *Ruditapes decussatus* collected from the Sardinian brackish environments. (A) Normal structure of the gills of a clam sampled from Calich lagoon (ch) with gills lamellae (gl) which are characterized by a ciliated epithelium (c). The gills are supported mainly by muscle fibers and connective tissue, crossed by numerous hemolymph sinuses (hs). Large numbers of hemocytes, mostly hyalinocytes (h), are common scattered along the undamaged epithelial surface. (B) *Perkinsus* spp. trophozoites (pk) in gills of clam sampled from Calich lagoon (ch), with an infiltration of numerous hemocytes (hi) mostly granulocytes (g). (C) Parasite (p, white arrow) in gill lamella characterized by hemocytic infiltrate (hi). HE stain, scale bars: 10 μm.
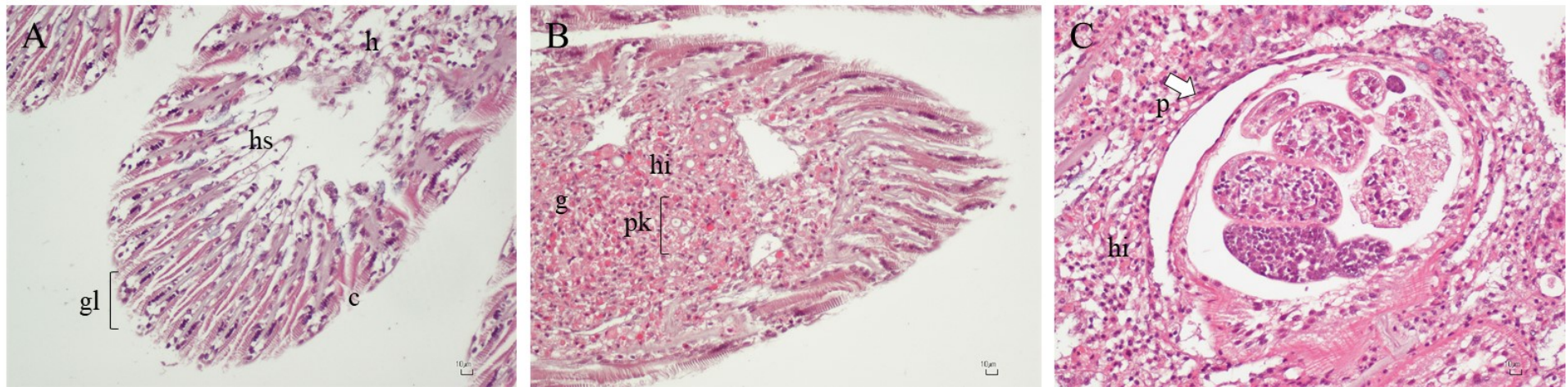
Fig. 4.7 The gills of *Ruditapes decussatus* collected from the Sardinian brackish environments. (A) The gills of clam collected from San Teodoro lagoon (st) characterized by high loss of epithelium (le). HE stain, scale bars: 100 µm. The area circumscribed by the dotted line is represented by the figure (B). In detail the supporting cartilage (ct) of gills filaments without epithelium. Large numbers of hemocytes (hi) are scattered along the epithelial surface. HE stain, scale bars: 100 µm. Inset: detail of the supporting cartilage (white arrow). Masson's Trichrome stain, scale bars: 10 µm.
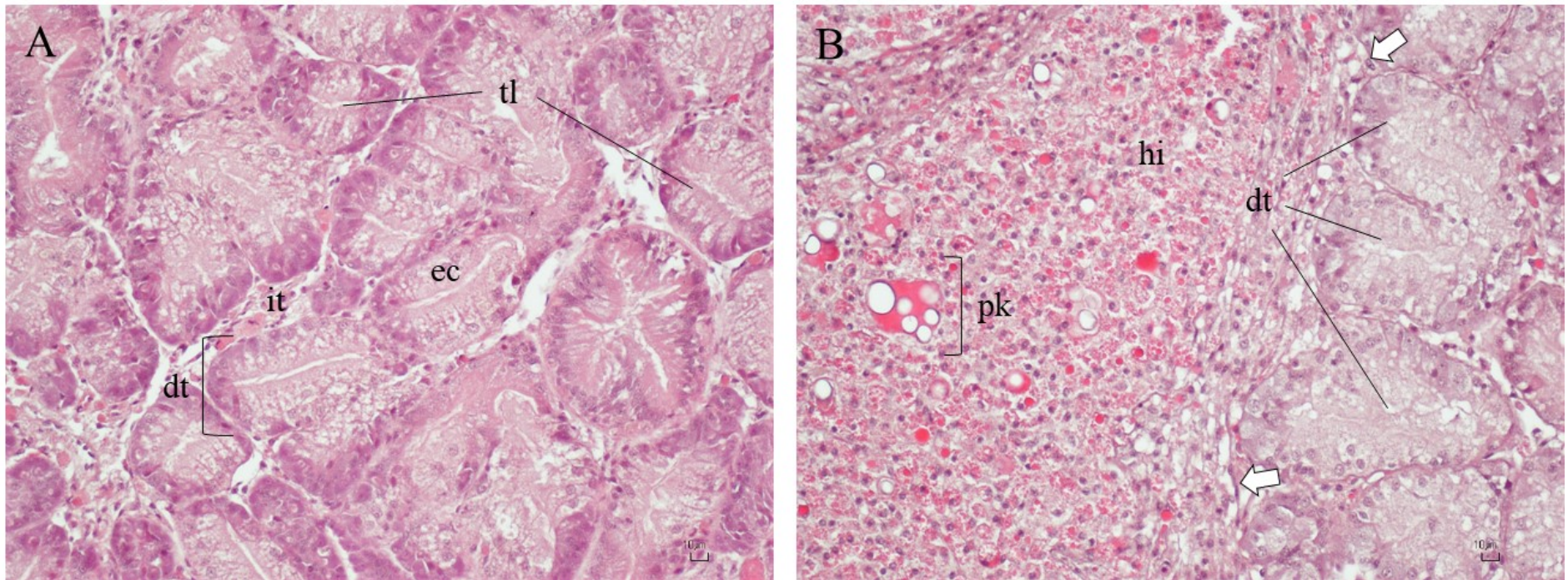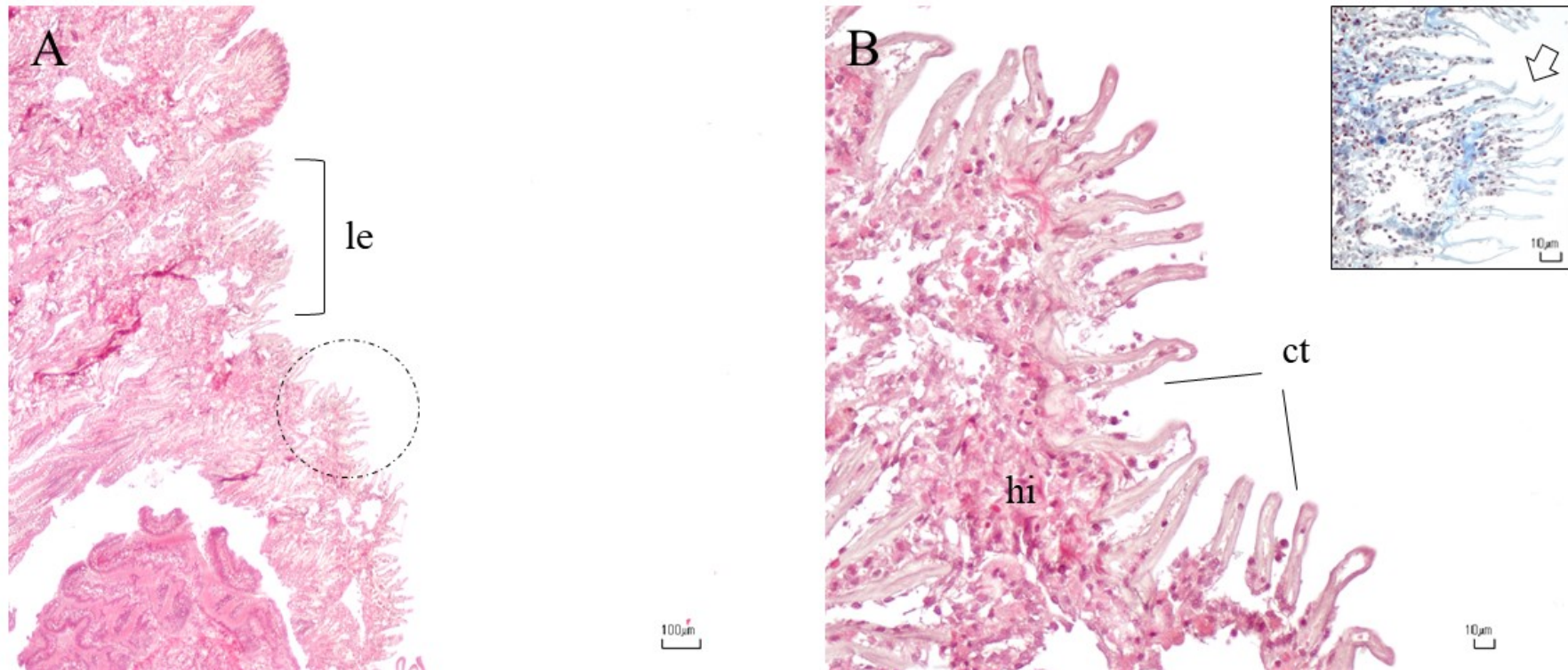
Fig. 4.8 The kidney of *Ruditapes decussatus* collected from the Sardinian brackish environments. (A) Normal structure of the kidney of a clam sampled from Calich lagoon (ch) shows a collagenous connective tissue which contains muscle fibres, hemolymphatic sinuses (hs) with scattered hemocytes (h, solid black arrow). Kidney is characterized by nephridial tubules (nt) with regular size cells and basal nucleus. (B) *Perkinsus* spp. trophozoites (pk) in kidney of clam sampled from Santa Gilla lagoon (sg), with an infiltration of numerous hemocytes (hi), showing the characteristic *encapsulation*-like response (white arrow and dashed line). HE stain, scale bars: 10 µm.
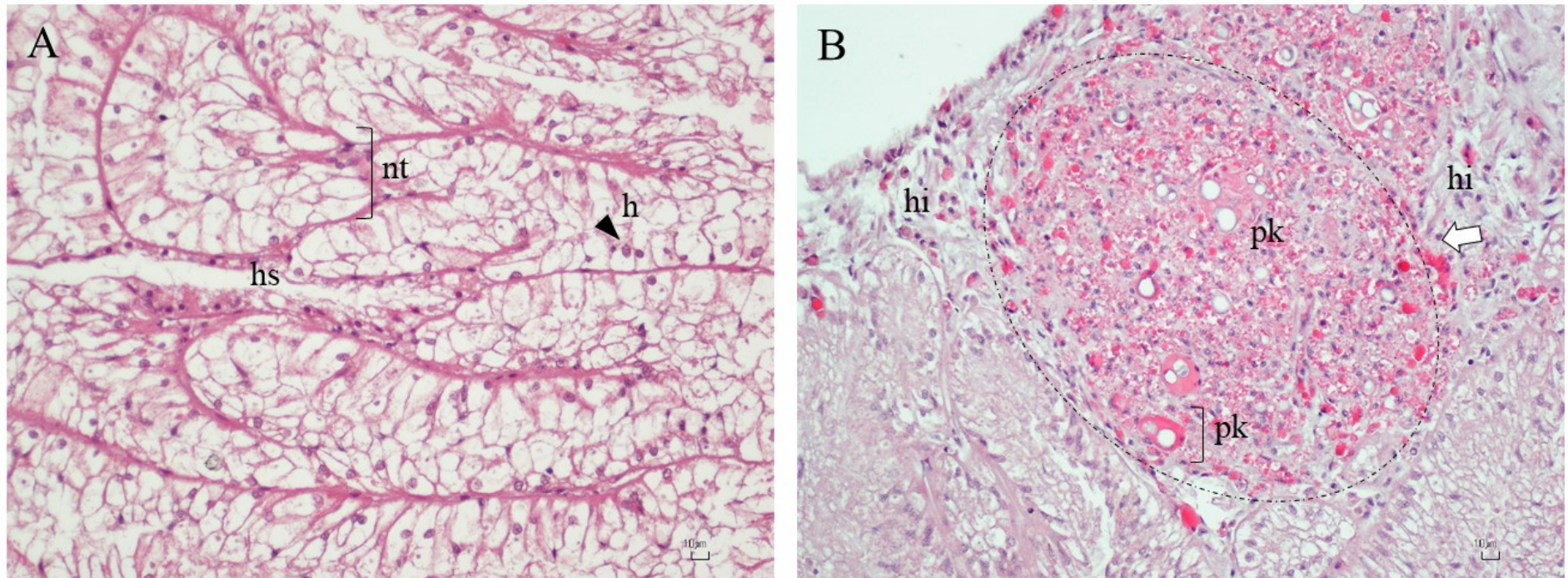
Fig. 4.9 The kidney of *Ruditapes decussatus* collected from the Sardinian brackish environments. (C) Parasite (p, white arrow) in kidney of clam sampled from Calich lagoon (ch). HE stain, scale bars: 10 μm.
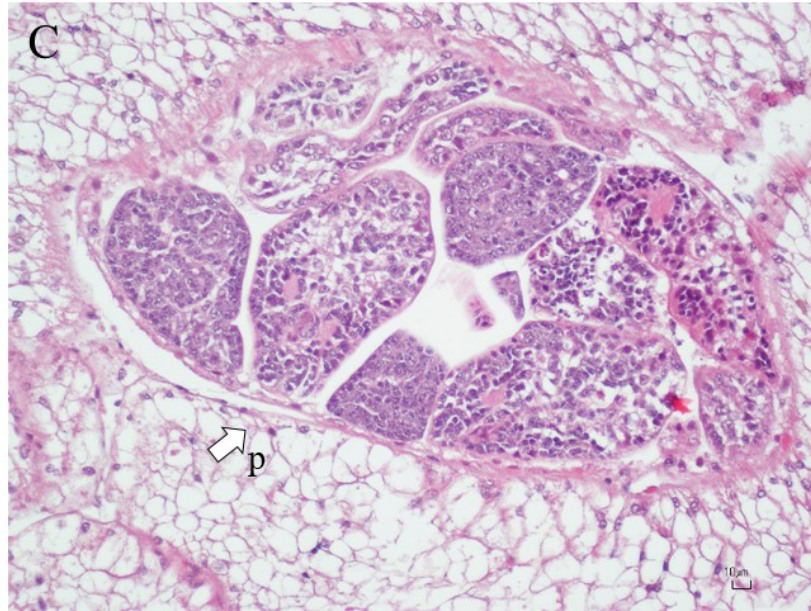
Tab. 1.2 Prevalence of hemocytes infiltration and distribution in clams *Ruditapes decussatus*.

Lagoon	Organ	n_tot	n	%n/tot	focal	multi-focal	diffuse
			5	19.2	x	-	-
	Digestive gland	26	6	23.1	-	-	x
			15	57.7	-	x	
			7	7.7	-	-	-
sg	Gills	91	34	37.4	-	-	x
			50	54.9	-	x	-
			7	18.0	x	-	-
	Kidney	39	9	23.0	-	-	x
			23	59.0	-	x	-
			2	14.3	-	-	x
	Digestive gland	14	12	85.7	-	x	-
			2	2.8	x	-	-
st	Gills	71	24	33.8	-	x	-
			45	63.4	-	-	x
			2	12.5	x	-	-
	Kidney	16	3	18.7	-	x	-
			11	68.7	-	-	x
			2	20.0	x	-	-
	Digestive gland	10	2	20.0	-	-	x
			6	60.0	-	x	-
			8	8.80	-	-	x
pp	Gills	91	12	13.2	x	-	-
			71	78.0	-	x	-
			2	6.2	-	-	x
	Kidney	32	12	37.5	x	-	-
			18	56.2	-	x	-
			8	20.0	x	-	-
	Digestive gland	40	9	22.5	-	-	x
			23	57.5	-	x	-
			5	6.1	x	-	-
ch	Gills	82	33	40.2	-	-	x
			44	53.7	-	x	-
			3	11.1	-	-	x
	Kidney	27	6	22.2	x	-	-
			18	66.7	-	x	-

w= weight; d=distribution.

Tab. 1.3 Prevalence of necrosis and distribution in clams *Ruditapes decussatus*.

Lagoon	Organ	n	$n\_$tot	%n/$n\_$tot	d focal	multi-focal	diffuse
	Digestive gland	3	6	50.0	x	-	-
		3		50.0	-	x	-
sg	Gills	3	7	42.9	-	x	-
		4		57.1	x	-	-
	Kidney	3	7	42.8	-	x	-
		4		57.2	x	-	-
	Digestive gland	-	-	-	-	-	-
st	Gills	3	12	25.0	x	-	-
		4		33.3	-	-	x
		5		41.7	-	x	-
	Kidney	-	-	-	-	-	-
	Digestive gland	1	1	100.0	x	-	-
pp	Gills	1	19	5.3	-	-	x
		5		26.3	-	x	-
		13		68.4	x	-	-
	Kidney	-	-	-	-	-	-
	Digestive gland	-	-	-	-	-	-
ch	Gills	1	1	100.0	-	x	-
	Kidney	-	-	-	-	-	-

w= weight; d=distribution.

Tab. 1.4 Prevalence of *Perkinsus* spp. and distribution in clams *Ruditapes decussatus*.

Lagoon	Organ	*n*	*n*_tot	%*n*/*n*_tot	*d*		
					focal	multi-focal	diffuse
	Digestive gland	3	6	50.0	-	x	-
		3		50.0	-	-	x
sg	Gills	3	23	13.1	x	-	-
		5		21.7	-	-	x
		15		65.2	-	x	-
	Kidney	1	5	20.0	-	x	-
		1		20.0	-	-	x
		3		60.0	x	-	-
	Digestive gland	1	1	100.0	-	x	-
st	Gills	1	18	5.6	-	-	x
		17		94.4	-	x	-
	Kidney	-	-	-	-	-	-
	Digestive gland	4	4	100.0	-	x	-
	Gills	2	23	8.7	x	-	-
pp		4		17.4	-	-	x
		17		73.9	-	x	-
	Kidney	1	7	14.3	-	-	x
		2		28.6	x	-	-
		4		57.1	-	x	-
	Digestive gland	5	37	13.5	x	-	-
		10		27.0	-	-	x
		22		59.5	-	x	-
ch	Gills	1	68	1.5	x	-	-
		30		44.1	-	-	x
		37		54.4	-	x	-
	Kidney	4	22	18.2	-	-	x
		7		31.8	x	-	-
		11		50.0	-	x	-

w= weight; d=distribution.

Tab. 1.5 Prevalence of other parasites and distribution in clams *Ruditapes decussatus*.

Lagoon	Organ	*n*	*n*_ tot	%*n* /*n*_ tot	*d*		
					focal	multi-focal	diffuse
	Digestive gland	1	2	50.0	x	-	-
		1		50.0	-	x	-
	Gills	9	65	13.8	x	-	-
sg		17		26.2	-	-	x
		39		60.0	-	x	-
	Kidney	3	25	12.0	x	-	-
		10		40.0	-	x	-
		12		48.0	-	-	x
	Digestive gland	-	-	-	-	-	-
	Gills	13	58	22.4	-	-	x
st		45		77.6	-	x	-
	Kidney	2	4	50.0	x	-	-
		2		50.0	-	x	-
	Digestive gland	-	-	-	-	-	-
	Gills	4	86	4.6	x	-	-
pp		20		23.3	-	-	x
		62		72.1	-	x	-
	Kidney	3	28	10.7	-	-	x
		6		21.4	x	-	-
		12		42.9	-	x	-
	Digestive gland	-	-	-	-	-	-
	Gills	1	8	12.5	-	-	x
ch		7		87.5	-	x	-
	Kidney	1	4	25.0	x	-	-
		1		25.0	-	-	x
		2		50.0	-	x	-

w= weight; d=distribution.

Tab. 1.6 Prevalence of loss of epithelium and lamellar fusion and distribution in gills of clams *Ruditapes decussatus*.

Lagoon	*n*	*n_* tot	%*n*/*n_* tot	*d*		
				focal	multi-focal	diffuse
	3		9.4	-	-	x
sg	6	32	18.7	x	-	-
	14		43.7	-	x	-
	15		23.5	x	-	-
st	23	64	35.9	-	x	-
	26		40.6	-	-	x
	6		12.5	-	-	x
pp	17	48	35.4	x	-	-
	25		52.1	-	x	-
	4		11.4	-	-	x
ch	7	35	20.0	x	-	-
	24		68.6	-	x	-
				-	-	-
	1		16.7	-	-	x
sg	1	6	16.7	-	x	-
	4		66.6	x	-	-
	4		7.5	x	-	-
st	21	53	39.6	-	x	-
	28		52.8	-	-	x
	1		10.0	x	-	-
pp	1	10	10.0	-	-	x
	8		80.0	-	x	-
	1		16.7	-	-	x
ch		6				
	5		83.3	-	x	-

w= weight; d=distribution.

3.3 Conclusion

The use of histopathological indices allowed us to differentiate the different sampling lagoons in a more sensitive way. Clams from different Sardinian coastal environments characterized by different anthropogenic impact, have constantly reported injuries in all the considered organs with different degrees of severity. However, the recurrent presence of parasites such as *Perkinsus* spp. could mask or amplify the effects of polluting substance released in environment.

Moreover, our findings revealed that clams collected from San Teodoro lagoon reported the highest prevalence of gill lesions not linked to the presence of parasites like *Perkinsus* spp. As highlighted by several authors, an occurrence of various histopathological alterations in gills confirmed the possible metal contamination impact on clam's health as well as the consumers.

However, our results demonstrate that *R. decussatus* is a suitable biological indicator sensitive to environmental stressors. Thus, histopathological studies on this specie could be done in monitoring programs in compliance with the EU Marine Strategies Framework Directive (MSFD).

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