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**The spread of the Atlantic blue crab *Callinectes sapidus* Rathbun  
1896 in the Mediterranean Sea: analysis of environmental and  
trophic niche and metal levels at different spatial scales**

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## Abstract

Biological invasions are globally acknowledged as one of the major causes of biodiversity loss. Considering the urgency of understanding what are the effects of biological invasions on recipient ecosystems, this research focuses on the Atlantic blue crab *Callinectes sapidus*, one of the most successful invaders of Mediterranean coastal ecosystems.

First, a general assessment of the overlap of the Grinnellian niche of the species in native and invaded ranges was carried out to verify whether the blue crab maintained the characteristics of its climatic niche when establishing in invaded areas (niche conservatism hypothesis) or, alternatively, it adapted to the specific abiotic characteristics of recipient environments (niche shift hypothesis). The results showed a low degree of niche overlap between native and invaded ranges, highlighting that a niche shift occurs and indicating that the species not only colonized the areas where the oceanographic conditions reproduced those occurring in native ranges, but was also able to adapt to novel environmental contexts occurring in the Mediterranean Sea.

Subsequently, a study of a newly recorded population within the Stagnone di Marsala, a marine coastal area in north-west Sicily (Italy), was performed. At first, an investigation of the structure and seasonal dynamics of the population within this basin was performed. Thereafter, an analysis of the isotopic niche and trophic position was conducted comparing *C. sapidus* with two autochthonous brachyurans, *Carcinus aestuarii* and *Eriphia verrucosa* in order to clarify the trophic role of the blue crab and potential interaction with other crabs. Results showed that in this marine coastal area the blue crab is much abundant in the northern part of the basin during summer, and males and females are not uniformly distributed. Moreover, no relationship among abundance of the species, and abiotic (salinity and temperature) and biotic (macrophyte biomass) factors investigated was found. The study revealed that, even if is not as abundant as in other Mediterranean area, this species is largely capable of establish and reproduce even in a habitat where environmental characteristics (*e.g.*, salinity and temperature) are remarkably different from those it experiences in its native environment as well in other areas to date invaded. Furthermore, the results of the analysis performed on the isotopic niche and trophic position of the three brachyurans species suggest that they exploit different food sources and have considerably diverse trophic habits. Specifically, the different position of the niches in the isotopic space, the negligible

overlap and the relatively different trophic positions of the three species, suggest a repartition of the niche. Moreover, a mixing model performed to elucidate which sources are the most consumed by *C. sapidus*, highlighted a general preference for animal prey, although characterized by a considerable spatial and seasonal variation likely influenced by the temporal and local availability of animal and vegetal trophic resources.

Eventually, an assessment of *Callinectes sapidus* as a biomonitor species was carried out, through the evaluation of metal concentration in blue crabs sampled in five locations across the Mediterranean Sea. Subsequently, a comparison with bivalves, the most used marine organisms for biomonitoring purposes, was performed to check whether the blue crab is able to provide reliable information on metal contamination in the environment. Results indicated that its omnivorous trophic habits may represent an important factor affecting trace metal content and, ultimately, its ability to provide information consistent with those that can be obtained from bivalves. The results emphasized that the trophic plasticity of blue crab and other invertebrate taxa proposed for biomonitoring purposes may represent an open issue in environmental toxicology. Accordingly, an advanced analysis of trophic habits through stable isotope technique may help to understand the observed patterns of variation in contaminant levels.

Overall, the findings of the present thesis provided an advanced perspective on the invasion biology and ecology of *Callinectes sapidus*, in particular addressing previously unexplored knowledge voids regarding the variation and expansion of the species, its population structure and dynamics as well its trophic role in a recently invaded marine coastal area, and the potential use of the blue crab as a bioindicator. These results are an additional step towards a deeper knowledge of this invasive species, an essential aspect in the implementation of successful management strategies for the control and mitigation of its impact on Mediterranean Sea ecosystems.

# 1. Introduction

## 1.1. Biological invasions

In the past millennia, human colonization has generally implicated the intentional or accidental introduction of animal and vegetal species outside their native ranges. Until recently, however, human-related introductions have generally involved a relatively low number of taxa, and those that were moved were generally introduced slowly, over short distances, and in small numbers. Even though this resulted in changes to the distribution of many species, due to both human-aided dispersal and human-driven modifications of ecosystems, yet species introductions have been mostly localized, sometimes involving extensions of natural distribution ranges (Hui & Richardson, 2016).

The last century has witnessed unprecedented technological advancements that allowed humans to translocate almost any species over global distances. Biogeographical barriers separating the biota of different parts of the world have been easily violated. Cultural links and emerging regional and global economies have created a wealth of complex pathways fostering species translocation (Ruiz & Carlton, 2003). The number of species moved around the world has increased dramatically, but also the abundance of translocated individuals has increased in parallel, reflecting in an increase in the number and typology of potential interactions between introduced species and recipient communities (*e.g.*, Keane & Crawley, 2002; Seebens *et al.*, 2021). In addition, an increase in the time since introduction for many species implied that many of them have been able to disperse within their adventive ranges autonomously or aided by humans or other native species (Pyšek & Richardson 2010).

A large number of species, the majority introduced only in the last two centuries, have successfully established in Europe (Hulme, 2007). Indeed, Europe can be considered a hot spot for aquatic introductions with 14,269 non-indigenous species identified at present (EASIN, <http://easin.jrc.ec.europa.eu/>, accessed April 2023). In particular, so far at least 840 (not considering microalgae, pathogen and parasites) non-indigenous species (NIS) have been reported in European seas, about 87 of which are currently considered invasive (<https://www.eea.europa.eu/ims/marine-non-indigenous-species-in>). Moreover, future global biodiversity scenarios highlight potentially dramatic increases in the introduction of species in European ecosystems (Bellard *et al.*, 2012; Roy *et al.*, 2018). Interacting effects through rising atmospheric CO<sub>2</sub> concentrations, warmer temperatures, greater nitrogen deposition, altered disturbance regimes and increased habitat fragmentation may facilitate

further introduction (Vilà *et al.*, 2010). Anthropogenic stressors (*e.g.*, pollution, overfishing, aquaculture, climate change, contaminants, and habitat loss) lead to a reduction in biodiversity and species richness, favoring species with a wide tolerance to environmental changes and determining a reduction in more sensitive species (McKenzie *et al.*, 2012; Johnston & Robert 2009). Invasive species are more tolerant to pollution and environmental stress, as they often can tolerate large abiotic and biotic variations and are generally more adaptable to fluctuations in the abiotic and structural characteristics of environments and thus better withstand anthropogenic stressors (Lenz *et al.*, 2011; Kueffer, 2017; Gelcich *et al.*, 2014; Belein *et al.*, 2016), as confirmed by a number of studies focusing on the tolerance of invasive species to anthropogenic stressors as compared with the responses of native species (Bielen *et al.*, 2016).

Despite the variety of control policies and regulations currently implemented at a national and international level, bioinvasions show no signs of saturation at a global scale, with exponential increases documented for taxa such as insects, algae and crustaceans (Seebens *et al.*, 2017), and an expected 36% rise in the next three decades (Hanley & Roberts, 2019; Pyšek *et al.*, 2020; Seebens *et al.*, 2021).

Noticeably, many introduced species are useful for human survival and wellbeing. For example, it has been estimated that 98% of food production in the USA depends on non-indigenous species of plants and animals (Pimentel, 2005). The shift from viewing introduced species as a useful component of the biota to the current perspective where the harmful effects of many introduced species is unambiguously recognized, has occurred when species introduction has been explicitly related to a number of negative effects on invaded ecosystems, even though with different trajectories in different regions (Lonsdale, 1999).

Specifically, to date the effects determined by introduced species are recognized as a significant component of global environmental change (Wallingfor *et al.*, 2020). They are acknowledged to determine fundamental and irreversible changes to the biodiversity of natural communities and ecosystems worldwide, ultimately affecting ecosystem functioning (Gallardo *et al.*, 2016), and the delivery of goods and services by terrestrial and aquatic systems (Pejchar & Mooney, 2009; see also Walsh *et al.*, 2016 for an example). In this context, it becomes crucial to understand the relationship between contaminants and invasive species, considering that they can tolerate high environmental stresses, that have a strong impact on both ecosystems and invaded trophic networks and that they may be exploited

commercially. Therefore, it is important to understand how they accumulate pollutants, including chemical contaminants such as heavy metals, in order to verify whether they may be harmful to human health. Since bioinvaders are often of commercial interest, studies on the accumulation of heavy metals on these species are of some relevance. For example, in Annabi *et al.*, (2018) an assessment of heavy metal concentrations in *Portunus segnis*, a portunid species invasive in Gulf of Gabès (Tunisia), was carried out. A comparison with other species was performed, and a relationship between metal accumulation and trophic position was highlighted clarifying the patterns of metal accumulation within the coastal food web of the Gulf of Gabès.

In the last two decades, the concept of non-native species has undergone a shift in terminology, noticeably from “introduced species” or “non-indigenous species” up to the beginning of the 1990s, to “alien species” or “invasive species” at the end of 1990s and early 2000s. The IUCN - International Union for Conservation of Nature - proposed the following definition of alien species in 1999: “*Alien species (non-native, non-indigenous, foreign, exotic) means a species, subspecies, or lower taxon, existing outside of its natural range (past or present) and its dispersal potential (i.e. outside the range it occupies naturally, or could not occupy without direct or indirect introduction, or care by humans) and includes any part, gametes or propagule of such species that might survive, and subsequently reproduce*”.

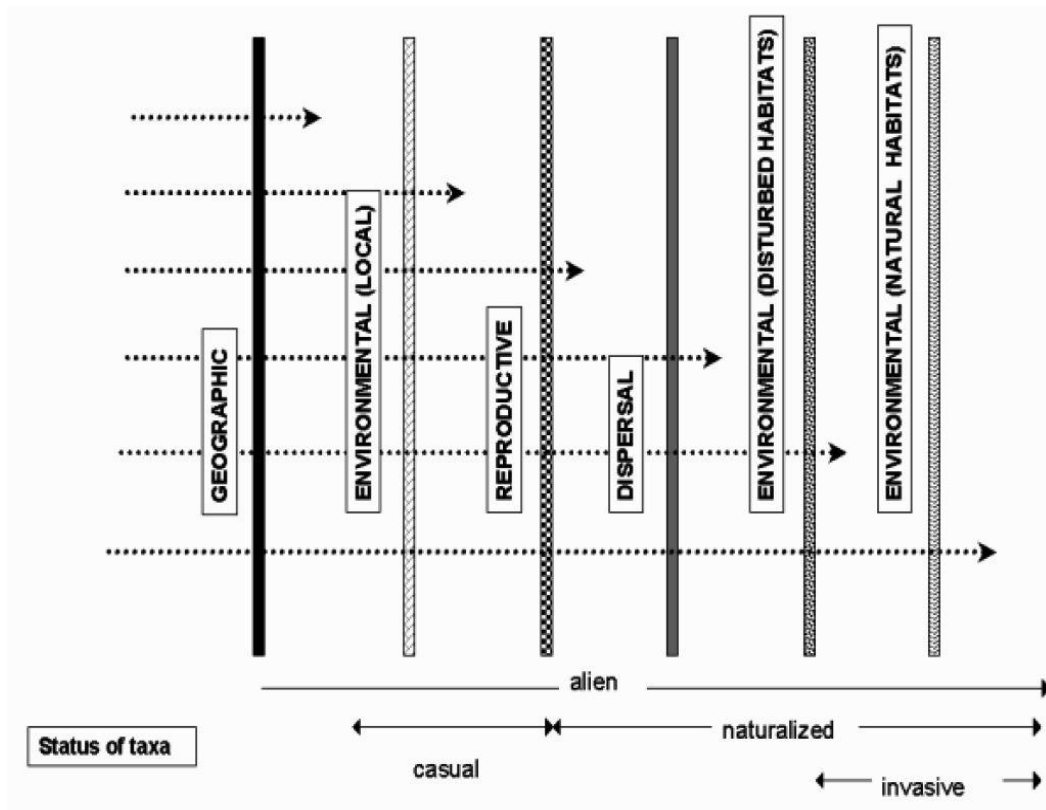
This definition has been accepted worldwide, from ecologists to policy makers. In Walther *et al.* (2009) an alien organism (and, by extension, an alien species) is defined as “*an organism occurring outside its natural past or present range and dispersal potential, whose presence and dispersal is due to intentional or unintentional human action*”. Similarly, the official US definition provided in Executive Order 13112 signed by President William Clinton on February 3<sup>rd</sup>, 1999, states that “*Alien species means, with respect to a particular ecosystem, any species, including its seeds, eggs, spores, or other biological material capable of propagating that species, that is not native to that ecosystem*”, while the European Union defines an alien species “*a species, subspecies or lower taxon occurring outside of the historically known range it occupies naturally and outside its dispersal potential as a result of direct or indirect introduction or care by humans. Includes any part, gametes or propagule that might survive and subsequently reproduce. Synonyms are non-native, non-indigenous, foreign, and exotic*” (Scalera & Zaghi, 2004).



In contrast, an intense debate has centered on the term “invasive species” (Carlton, 2002). The most widely used definitions of “invasive” include statements relating to two or more of the following: (1) the alien status of the species; (2) its ability or potential to establish and overcome various barriers to reproduction, dispersal and proliferation in the new environment; and (3) its ability or potential to cause harm to the environment and/or human health (reviewed in Richardson *et al.*, 2000a). Two main groups of definitions of “invasive species” have been provided:

1) those based on biological and ecological principles and relatively measurable criteria: *invasive species are a subset of naturalized species that produce reproductive offspring, often in very large numbers, are able to disperse considerable distances from parent populations, and thus have the potential to spread over a large area* (Richardson *et al.*, 2000a). The advantage of this definition is that “invasive” taxa can be defined, using reasonably objective criteria, according to their position along a “naturalization-invasion” continuum and compared to “alien” taxa (Richardson *et al.*, 2000a; Fig. 1); importantly, the definition implies no connotation of impact.

2) the second category of definitions may be comprised under the heading “anthropocentric”: *invasive species are those that are alien to the ecosystem under question, and whose introduction causes, or is likely to cause, economic or environmental harm to human health*. This definition has been widely adopted in policies, including such influential ones as the 1999 US Executive Order 13112, stating “*invasive species means an alien species whose introduction does or is likely to cause economic or environmental harm or harm to human health*”. The Global Invasive Species Programme (GISP) uses a similar definition of invasive alien species (1999): “*Invasive alien species are non-native organisms that cause, or have the potential to cause, harm to the environment, economies, or human health*”. The European Union defines “Invasive Alien Species” as: “*(invasive alien species) means an alien species whose introduction and/or spread threaten biological diversity*” (From Decision VI/23 of the Sixth Conference of the Parties (COP6) of the Convention on Biological Diversity in 2002).



**Figure 1.** Schematic representation of the phases between the introduction of an organism through human action, and its establishment and proliferation in natural (undisturbed) environments. The ability of a given species to overcome a series of barriers in the new environment defines its current (not necessarily ultimate) status as an alien. This scheme defines “casual”, “naturalized” and “invasive” species (adapted from Richardson *et al.*, 2000b).

Notwithstanding the open questions on the definition of the concept, invasive alien species, (IAS hereafter) are currently a central environmental issue (Galil *et al.*, 2009; Walther *et al.*, 2009; Occhipinti-Ambrogi & Galil, 2010). IAS are operatively defined as organisms whose introduction and spread outside their natural distribution range determines ecological and economic harm (Russell & Blackburn, 2017; after UNEP, 1992). They exert adverse impacts on biodiversity, ecosystem services, food security, human health and well-being (Blackburn *et al.*, 2019; Shabani *et al.*, 2020; Pyšek *et al.*, 2020; Cuthbert *et al.*, 2021).

The costs of IAS - including those related with direct impacts and their management and mitigation - have been estimated for the period 1971-2020 in US \$345 billion (Cuthbert *et al.*, 2021; but see Diagne *et al.*, 2021 for a far less conservative US \$1,288 trillion estimations over the 1970-2017 period). Noticeably, aquatic taxa accounted for only 5-9% of global total costs, despite 26% of known invaders being aquatic; moreover, only 1% of the costs were attributed to marine IAS, notwithstanding the threat they pose to recipient environments,

where their ecological impacts are intensified by habitats connectedness and the superimposition of other anthropogenic pressures including climate change (Essl *et al.*, 2015; Seebens *et al.*, 2016; Tsirintanis *et al.*, 2022).

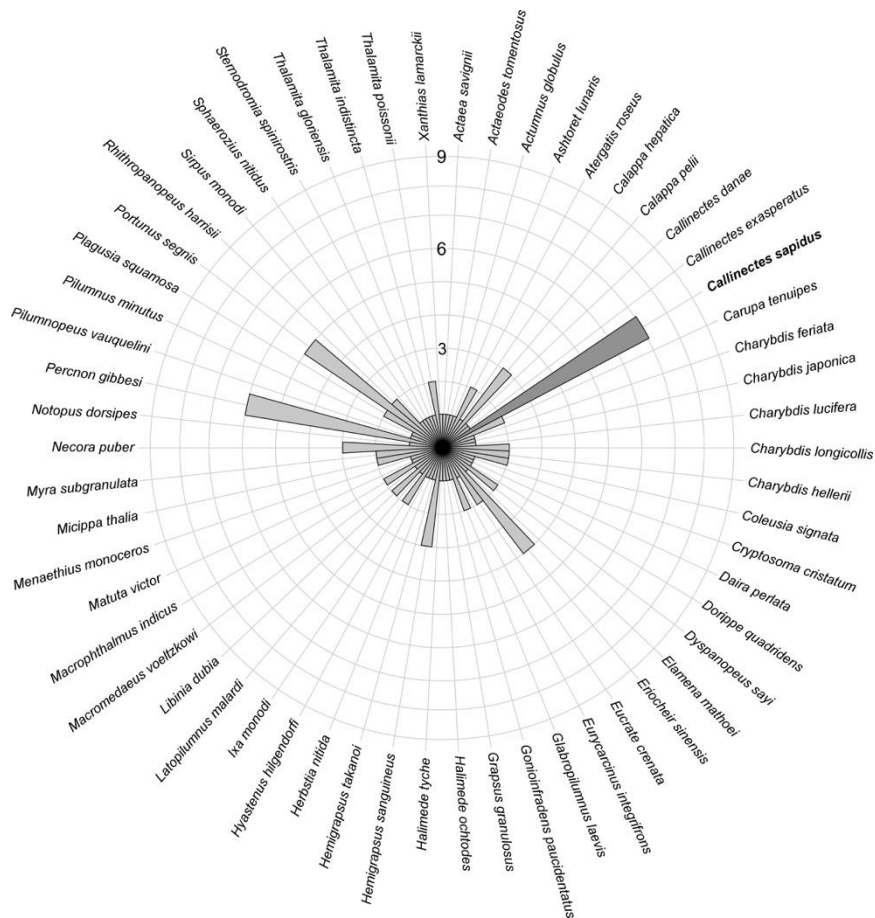
In general, marine ecosystems are experiencing the disruptive effects of invaders worldwide (Cohen *et al.*, 2019). Specifically, alarm has been raised for the Mediterranean Sea, to date recognized as one of the areas worldwide most severely affected by biological invasions, in terms of detected number of alien species and rate of introduction (Raitsos *et al.*, 2010; Zenetos *et al.*, 2012; Katsanevakis *et al.*, 2016). Changes in European reference regulations testify this growing concern: in the Water Framework Directive (WFD; 2000/60/EC) alien species were not included among the ecological quality indicators for coastal habitats (EU, 2000); conversely, in the Marine Strategy Framework Directive (MSFD; 2008/56/EC), and, more recently, in the Biodiversity Strategy to 2020 [2011/2307(INI)] IAS have been explicitly recognized as a biological pressure (MSFD descriptor D2), whose magnitude and functional effects need to be estimated for an integrated assessment of the ecological status of marine ecosystems (EU, 2010; see also Borja *et al.*, 2010).

Recently, the EU Regulation 1143/2014 (EU, 2014) imposed restrictions on a list of species known as “species of Union concern”. These are species whose potential adverse impacts across the European Union are such that concerted action across Europe is required. The list has been repeatedly updated (EU, 2016, 2017, 2018, 2019, 2022). To date the list comprises 47 animals and 41 plants; noticeably, among-animal organisms, only six crustacean taxa are included, of which only one - the Chinese mitten crab *Eriocheir sinensis* H Milne Edwards 1854 - is from marine or transitional habitats.

The Mediterranean Sea is susceptible to biological invasions principally due to the Suez Canal, aquaculture introduction and shipping. Alien algae, mollusks, crustaceans and fishes are recorded in many coastal habitats of the Mediterranean Sea, and their invasion rate is currently increasing. Crustaceans are emblematic of this invasive ongoing process: in 2008, the CIESM Atlas of Exotic Species listed 70 non-indigenous crustaceans in the Mediterranean Sea, while in subsequent years, 106 and 242 species were respectively listed by Galil (2011) and Nunes *et al.* (2014). Among other taxa, brachyurans make up a large part of the group, with 58 species recorded in invaded environments (Mancinelli *et al.*, 2017a).

## 1.2. The Atlantic blue crab *Callinectes sapidus*

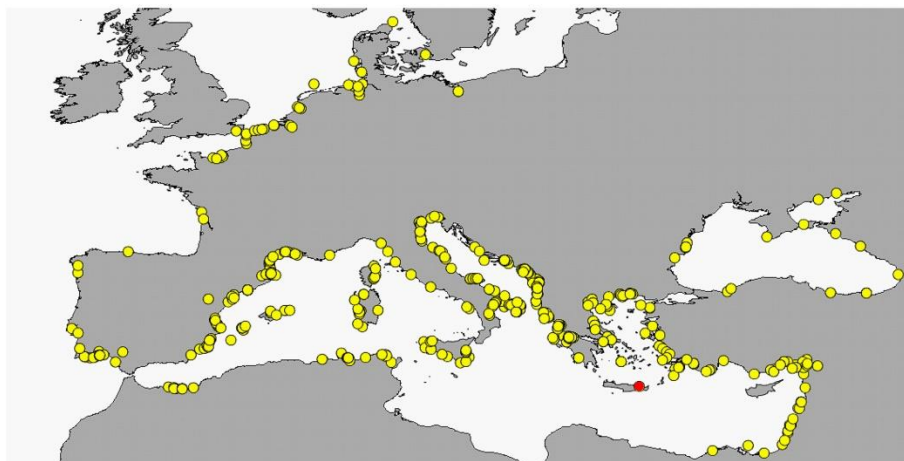
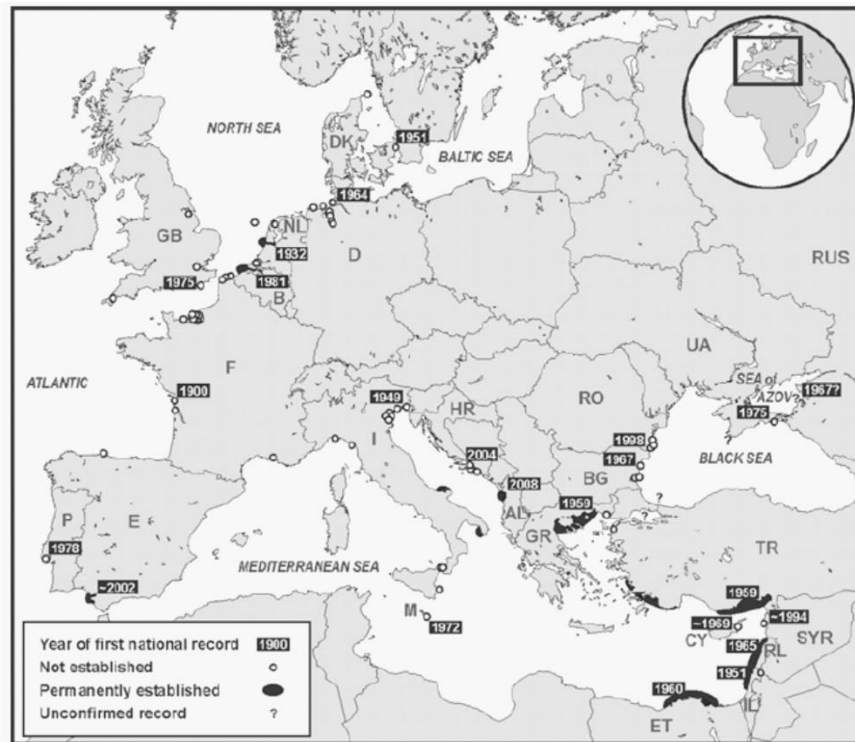
Among the most widespread non-indigenous brachyurans in the Mediterranean Sea, the Atlantic blue crab *Callinectes sapidus* Rathbun, 1896 (Portunidae) has been indicated in 2017 to occur in seven out of the nine South-European Marine Ecoregions (Fig. 2), followed by *Percnon gibbesi* Milne Edwards, 1853 (Plagusidae) in six ecoregions, and the portunid *Portunus segnis* Forkal, 1775 in five ecoregions (Mancinelli *et al.*, 2017a).



**Figure 2.** Occurrence of 58 non-indigenous brachyuran species across 9 South European Marine Ecoregions (South European Atlantic Shelf, Alboran Sea, Western Mediterranean, Adriatic Sea, Ionian Sea, Tunisian Plateau/Gulf of Sidra, Aegean Sea, Levantine Sea, and Black Sea) as of June 1st, 2016 (From Mancinelli *et al.*, 2017a).

*Callinectes sapidus* appeared for the first time along the European Atlantic coasts in 1900 (Fig. 3 top). Initially it was recorded in different estuaries along the Atlantic coasts of France, Great Britain, Spain and Portugal, then was sighted since the 1950s in the Baltic Sea and North Sea (Nehring, 2011). Regarding the Mediterranean Sea, the first records date back to

1935 in the Gulf of Thessaloniki, Greece, where an established population was observed (Nehring, 2011). Moreover, since 1940 it was recorded in Italy, Croatia, Albania, Turkey, Lebanon, Egypt and Syria. In addition, in 1975 it was observed in the Black Sea (Nehring, 2011). Records reported in Nehring (2011) refer often to single occurrences and rarely to the presence of stable populations. Furthermore, from a recent database by Mancinelli *et al.* (2021) *C. sapidus* appears to date ubiquitous in the Mediterranean Sea with an increase in the number of records. Moreover, it is worth emphasizing that the expansion of the blue crab is still ongoing along the European Atlantic coasts, as well as in the south-western Mediterranean Sea, where so far it has also been observed along the coasts of Morocco, Libya and Algeria (Mancinelli *et al.*, 2021) (Fig 3 bottom).



**Figure 3.** Temporal variation in the occurrence of *Callinectes sapidus* in European waters. Top from Nehring (2011); bottom from Mancinelli *et al.* (2021). In red: misidentification with *Portunus segnis*.

In Table 1 a summary of the biological and ecological characteristics of *C. sapidus* is provided. The species originates from the western Atlantic Ocean, and has been introduced in Europe and in the Mediterranean Sea probably by ballast waters. Interestingly, due to the similar morphologies and dimensions, *C. sapidus* has been repeatedly misidentified as *Portunus segnis* and vice versa: the first observation of *C. sapidus* in Italian waters was actually recorded as *Neptunus (Portunus) pelagicus* in Northern Adriatic (Giordani Soika, 1951), while it is likely that the two *C. sapidus* individuals episodically captured in Maltese

waters in 1972 (Nehring, 2011 and literature cited) were actually *P. segnis* (Crocetta *et al.*, 2015). More recently, juvenile *C. sapidus* repeatedly recorded in Crete Island (Katsanevakis *et al.*, 2014) was actually identified as *P. segnis* (Mancinelli *et al.*, 2022).

From an ecological point of view, the species is related with coastal areas, with a distribution including estuaries, lagoons, and other brackish habitats; its biological cycle is generally acknowledged to be strictly dependent upon brackish waters, as both the reproduction phase and the development of juvenile stages are connected with the availability of freshwaters (Fig. 4). Other aspects worth highlighting are the high trophic plasticity and omnivory of *C. sapidus* and that adult stages, in particular when they reach maximum sizes (*e.g.*, > 500 g in total wet weight), seem to be free from natural predators.

**Table 1.** Summary of the biological and ecological characteristics of *Callinectes sapidus*.

<b><i>Native geographic range</i></b>	The native range spreads from the western Atlantic Ocean, from Nova Scotia in Canada down to northern Argentina including Bermuda and the Antilles (Williams, 1974). The species is particularly common from Cape Cod (Massachusetts) to Uruguay; recently, it has been found in the Gulf of Maine, north of its historical range of Massachusetts (Johnson, 2015).
<b><i>Introduction vector</i></b>	The species was reported along the Atlantic European coast in 1900 in France (Bouvier, 1901), and in the Mediterranean Sea in 1950, reported as <i>Neptunus (Portunus) pelagicus</i> in Northern Adriatic (Giordani Soika, 1951). Larval introduction by ballast waters is considered as the main vector of introduction, and the most important factor determining the diffusion the species. The species has subsequently spread northward in the Baltic Sea and, southward, along the Atlantic coasts of Portugal and Spain; in the Mediterranean Sea, it is currently recorded in the Adriatic, Aegean, Black Sea, and throughout the Levantine sector of the basin (see Nehring, 2011; Mancinelli <i>et al.</i> , 2017a; Schubart <i>et al.</i> , 2023).
<b><i>Habitat</i></b>	The species is a bottom-dweller found in a variety of habitats ranging from fully marine waters to almost freshwater of the back bays and estuaries. The habitat ranges from the low tide line to a depth of $\approx$ 40 m. Females remain in higher salinity portions of an

estuary system, especially for egg laying. In winter, when temperatures are cold (i.e., lower than 10°C), the species migrates to deeper water and remains buried in the sediments in quiescence.

### ***Morphology***

The species is identified on the base of the presence of two large and obtuse teeth on the frontal margin which differentiate *C. sapidus* from either *C. danae* or *P. segnis*. (Castriota *et al.*, 2012).

The color of the body is generally a bright blue along the frontal area, especially along the chelipeds and the rest of the body is an olive brown color (Fig. 4). Males and females present sexual dimorphism in the shape of the abdomen; males possess a structure shaped as a long, narrow, inverted “T” named apron, whereas in females it is a wider and rounded, half-moon shape (Williams, 1974); furthermore, females show red fingers on the chelae, while males are more colorful and larger than females (Fig. 5). The fifth leg is adapted to a paddle-like shape, as is the same with other portunids.

*C. sapidus* can grow to 25 cm in carapace length (CL), with carapace width being approximately twice the length. Growth is rapid during the first summer; by the second year, maturity is reached at carapace lengths of 120-170 mm. They grow to adult size after 18 to 20 molts.

### ***Reproduction***

Spawning peaks are closely associated with the inhabited region. Females mate only once in their lifetime, after the pubertal molt; they produce 2 to 8 million eggs per spawn. When females are in their soft-shell stage, the males transfer their sperm to them for storage. The females will spawn 2 to 9 months after mating. When females are ready to spawn, they fertilize the eggs with the sperm and place them on the hairs of the appendages on their abdomen. During the incubation time, females migrate to the mouths of estuaries so that larvae may be released into high salinity waters. Larvae have a salinity requirement of at least 20 PSU, and it shows poor survival below this threshold.

### ***Development***

Seven zoeal stages and a megalopal stage are identified. Typically, development time through the seven zoeal stages is between 30 and 50 days before metamorphosis to the megalopal stage. This persists



between 6 and 58 days and the animal returns to estuaries for settlement, and eventual recruitment to adult populations. For juveniles, low salinity areas are important nursery habitats because of food availability and shelter from predation.

***Lifespan***

Average life span is of 12-24 months.

***Behaviour***

*C. sapidus* has the last pair of legs paddle shaped, in order to facilitate swimming movements in the water column. They can move from zero to 140 meters per hour, with an average of 15.5 meters per hour. This species tends to be more active during the day than in the evening. The species is generally very aggressive.

***Trophic habits***

This is an omnivore species feeding on clams, oysters, mussels and any vegetable and animal matter. This species also scavenges freshly dead organic matter and sometimes can also eat other crabs and conspecifics (Hines, 2007; Mancinelli & Vizzini, 2015).

***Trophic position***

(SeaLifeBase)

3.35 ± 0.30

***Predators***

This species is preyed by the red drum, Atlantic croaker, herons, sea turtles and humans. Parasites are very common. Barnacles, worms and leeches attach themselves to the outer carapace; isopods can live in their gills or on their abdomen, and small worms live in the muscles. Although *C. sapidus* is a host to many parasites, most of these do not affect the life of the crab. Internal parasites include viruses, dinoflagellates (*e.g.*, *Hematodinium perezii*) rickettsia-like microorganisms, microsporidians and gregarines, ciliates, nemertean, and trematodes (Messick, 1994, 1998).

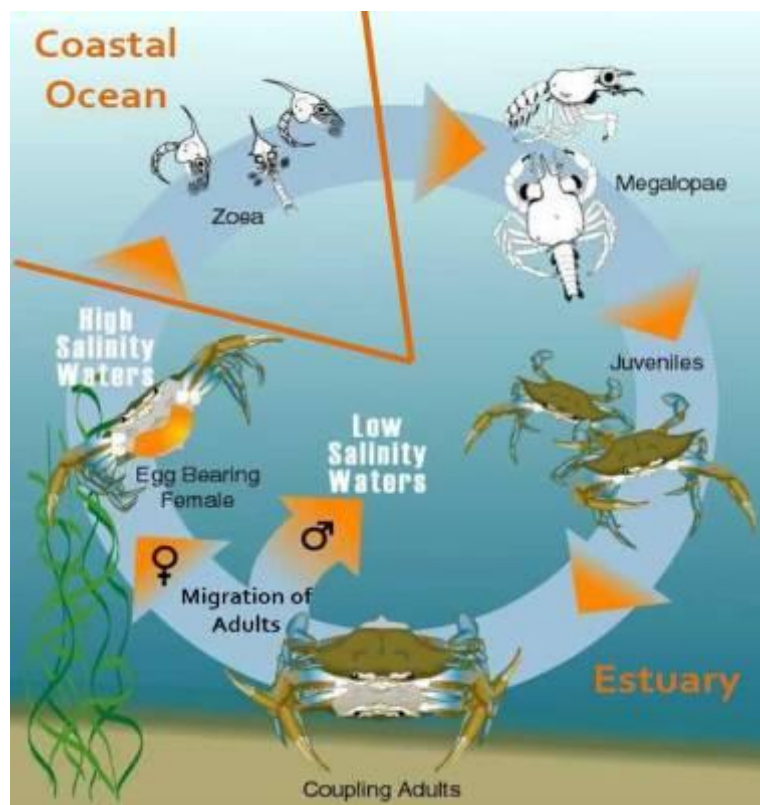
***Ecological impact***

*C. sapidus* is listed among the “worst invasive” species in the Mediterranean and it impacts both biodiversity and socioeconomics (Strefaris & Zenetos, 2006). However, to date, the species is not included in the list of invasive alien species of Union concern. In its native habitats, *C. sapidus* performs a variety of ecosystem functions and can play a major role in energy transfer within estuaries and lagoons (Mancinelli *et al.*, 2017b). This species is an omnivore, thus, its significant increase might have severe and multiple consequences

for the autochthonous ecological communities, as well as for the shellfish farms and artisanal fishery, by preying on bivalves and damaging fish nets.

***Economic importance***

*C. sapidus* is one of the most important targets of commercial and recreational fishery in its native area; in invaded habitats, a blue crab fishery should be locally promoted as a new food source and might reduce its potential negative impacts on the indigenous fauna (Mancinelli *et al.*, 2017c).



**Figure 4.** Biological cycle of *Callinectes sapidus* (modified from Kennedy & Cronin, 2007).



**Figure 5.** *Callinectes sapidus*: female (a) and male (b).

The reproductive characteristics and the tolerance to variations in water parameters of *C. sapidus* are summarized in Tables 2 and 3. The wide ranges of environmental parameters highlight the generalist nature of this species. The different duration in the spawning season, slightly narrower in invaded areas, is probably due to temperature. Since low temperature leads to a decrease in biological functions of this species, in native tropical areas with a warm season throughout all the year, the blue crab can spawn continuously and reach sexual maturity earlier than in native temperate habitats. On the contrary, the Atlantic coast of Europe and the Mediterranean Sea undergo a decline in temperature during winter season, which could slow the metabolic function and consequently the growth, sexual maturity and shorten the spawning season of the blue crab.

**Table 2.** Key reproductive characteristics of *Callinectes sapidus* in native (Kennedy & Cronin, 2007) and invaded ranges. CW = carapace width.

	<b>Native</b>	<b>Invaded</b>
<b>Spawning season</b>	All year; but spawning occurs from December until October, depending on latitude;	Eight months, with a peak between July and September (Sumer <i>et al.</i> , 2013, Turkey);
<b>Number of offspring</b>	70,000 to $6 \times 10^6$ ;	850,600 to $10^6$ (Mancinelli <i>et al.</i> , 2013, Italy); $10^6$ to $1.5 \times 10^6$ (Dulčić <i>et al.</i> , 2011, Croatia);
<b>Size at sexual or reproductive maturity (female)</b>	52-207 mm CW; 50% at 132 mm;	75.5-186.3 mm CW; 50% at 119 mm (Sumer <i>et al.</i> , 2013, Turkey);
<b>Size at sexual or reproductive maturity (male)</b>	82-227 mm CW; 50% at 107 mm;	59.6-212.7 mm CW (Sumer <i>et al.</i> , 2013, Turkey);

**Table 3.** Water parameter tolerances of *Callinectes sapidus*. Data from <http://www.cabi.org/isc/>

	Minimum	Maximum
Depth (m b.s.l.)	0	90
Salinity (PSU)	8	36.7
Temperature (°C)	3	30

## 2. Aims of the thesis

Biological invasions, as well as other anthropogenic stressors (*e.g.*, pollution, overfishing, aquaculture, climate change and habitat loss) are acknowledged to be the leading cause of biodiversity loss worldwide (Gelcich *et al.*, 2014) and one of the most effective drivers of change in the receiving ecosystem (Simberloff *et al.*, 2013).

The Mediterranean Sea is considered a hotspot for bioinvasions (Galil *et al.*, 2008), and is threatened by a number of non indigenous species currently expanding their ranges and increasing their abundances, thus having the potential to become invasive (Tsirintanis *et al.*, 2022). Considering the urgency of understanding what are the effects of biological invasions of recipient ecosystems, this thesis focused on the blue crab *Callinectes sapidus*, which is to date acknowledged as one of the most successful invader of Mediterranean coastal ecosystems.

Firstly, the environmental niche of *C. sapidus* was investigated in native and invaded habitats, by assessing the potential effects of niche conservatism and niche shift, and by measuring the overlap between native and invaded Grinnellian niches. This analysis had the aim of understand which abiotic factors (*e.g.*, salinity, temperature, chemical elements, chlorophyll) represent a constraint for the expansion of this species, and if the blue crab is able to adapt to new abiotic conditions when expanding in invaded ranges (chapter 3).

Considering that the species has recently shown to be able to colonize areas (*e.g.*, North Africa) with extreme environmental characteristics (*e.g.*, temperature and salinity) compared to what is generally assumed, the recently recorded population in Stagnone di Marsala was adopted as a case of study to verify the biological and ecological characteristics of the species under peculiar abiotic conditions. In particular, population structure, seasonal dynamics of the distribution and correlation among the presence of the blue crab and biotic (macrophytes) and abiotic (salinity and temperature) characteristics characterizing this basin were investigated (chapter 4).

Subsequently, the trophic role of the blue crab in the Stagnone di Marsala was studied. To this end, analysis of the isotopic niche and trophic position of the blue crab was carried out, and results compared with those of two autochthonous brachyurans species, in order to understand whether or not these co-occurring species partition the trophic niche in the isotopic space. Thereafter, isotope mixing models were run to investigate the trophic habits

of the crab and identify which are its most important food sources within the basin (chapter 5).

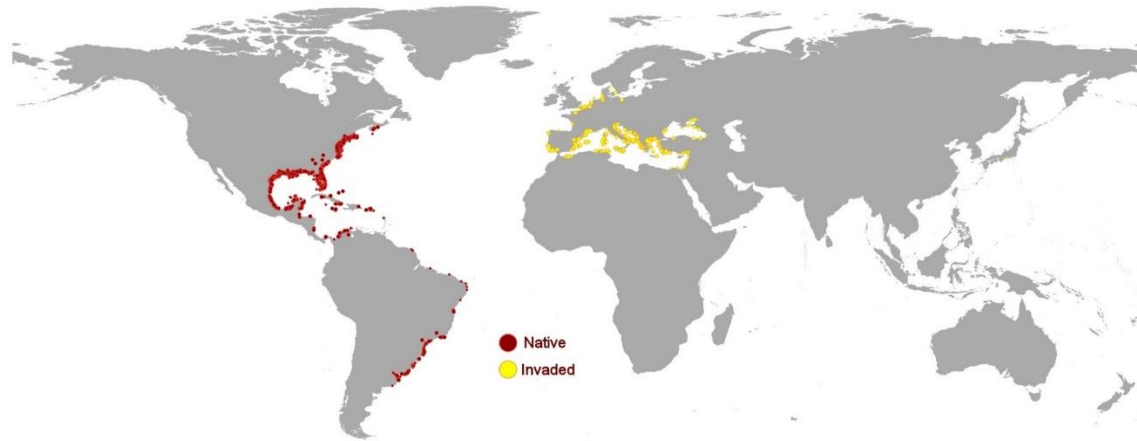
Eventually, since the blue crab is emerging as a fishery product in the Mediterranean Sea, the last chapter of the thesis was focused on the study of accumulation of heavy metals in different locations of the Mediterranean Sea. The possibility of using this species as a bioindicator was explored performing a comparison with bivalves, which are generally considered effective for bioindication purposes (chapter 6).

### **3. Comparison of native and invaded environmental niche of the Atlantic blue crab, *Callinectes sapidus*. Does a niche shift occur?**

#### **3.1. Introduction**

Throughout the centuries, humans have observed and recorded a consistent relationship between species distribution and physical environment. The environmental niche concept was defined by Grinnell in 1917 as "...the concept of the ultimate distributional unit, within which each species is held by its structural and instinctive limitations, these being subject only to exceedingly slow modification down through time" (Vandermeer 1972). Since niche is defined as a combination of factors that determine the presence, reproduction and establishment of a species, a crucial challenge for ecologists is to determine the factors that constrain a species distribution and its limit range (Soberón 2010). Drivers for species distribution could be abiotic factors, such as temperature, salinity, dissolved oxygen, soil and so on, or biotic interaction. In a framework of invasive species spread worldwide, it is of paramount importance to be aware of the degree of niche conservatism or niche shift of an invasive species established in a receiving habitat. Hence, one of the crucial steps for the development of effective control and mitigation strategies, especially within the context of climate change is to determine the degree to which the climatic niche of invasive populations resembles or differs from that of native ones (Broennimann *et al.* 2012). The blue crab *Callinectes sapidus* Rathbun, 1896, is native to the western Atlantic Ocean, from New England to Uruguay, and occurs in European waters since the 20<sup>th</sup> century (Nehring, 2011; Mancinelli, *et al.*, 2021). So far, the blue crab colonized the Mediterranean Sea, Atlantic and northern coasts of Europe, Atlantic coasts of Morocco and the Black sea (Oussellam *et al.*, 2023; Mancinelli, *et al.*, 2021) (Fig. 1). The life cycle of this species is strictly linked to salinity gradient and water temperature, since the first determines the population spatial distribution and the second determines the activity period during the year (Hines, 2007).

The aim of this study was to investigate the dynamic of environmental niche of *C. sapidus*, to verify if the invasion is related with a shift in its climatic niche or if, alternatively, the species established in European ranges maintaining its Grinnellian requirements, within a niche conservatism scenario.



**Figure 1.** Worldwide distribution of *Callinectes sapidus* (Mancinelli, *et al.*, 2021)

### 3.2. Materials and methods

A georeferenced dataset of the global occurrence of *C. sapidus*, freely available online (<https://doi.org/10.6084/m9.figshare.12896309.v2>) was used to extract bioclimatic variables from Bio-ORACLE (<https://www.bio-oracle.org>) oceanographic GIS layers. Afterwards, multivariate procedures were used to compare the native and invasive climatic niches. In particular, the original dataset was preliminarily reduced to include only records characterized by unique coordinates. This procedure reduced the original number of 40,388 entries to 21,759. Furthermore, the dataset was thinned using the R package *spThin* in order to subset the records located at a minimum distance of 10 km, matching the resolution of Bio-ORACLE layers (i.e., 5 arcminutes, approximately 9.2 km at the equator). In total, 2264 records were selected for further analysis, 1593 from native ranges and 671 from invaded areas. For both native and invaded ranges, an equal number of background data points were identified using the R package *spatialEco*. Background data were selected randomly in an area centred on each record and characterized by a radius of 200 km. Subsequently, the dataset, including occurrence records and background points, was transformed in a shapefile and superimposed to Bio-ORACLE layers to extract the corresponding environmental variables. Moreover, the Spearman Rank correlation among the extracted environmental variables was estimated, and those showing a coefficient  $> 0.75$  were excluded. The procedure reduced the original set of explanatory variables from 15 to 7 (i.e., mean chlorophyll, nitrate, pH, phosphate, salinity, silicate, and mean superficial sea temperature). Eventually, the R package *ecospat* was used to perform the comparison of the climatic niches of *C. sapidus* in native and invaded areas (Di Cola *et al.*, 2017). For this, the PCA-env



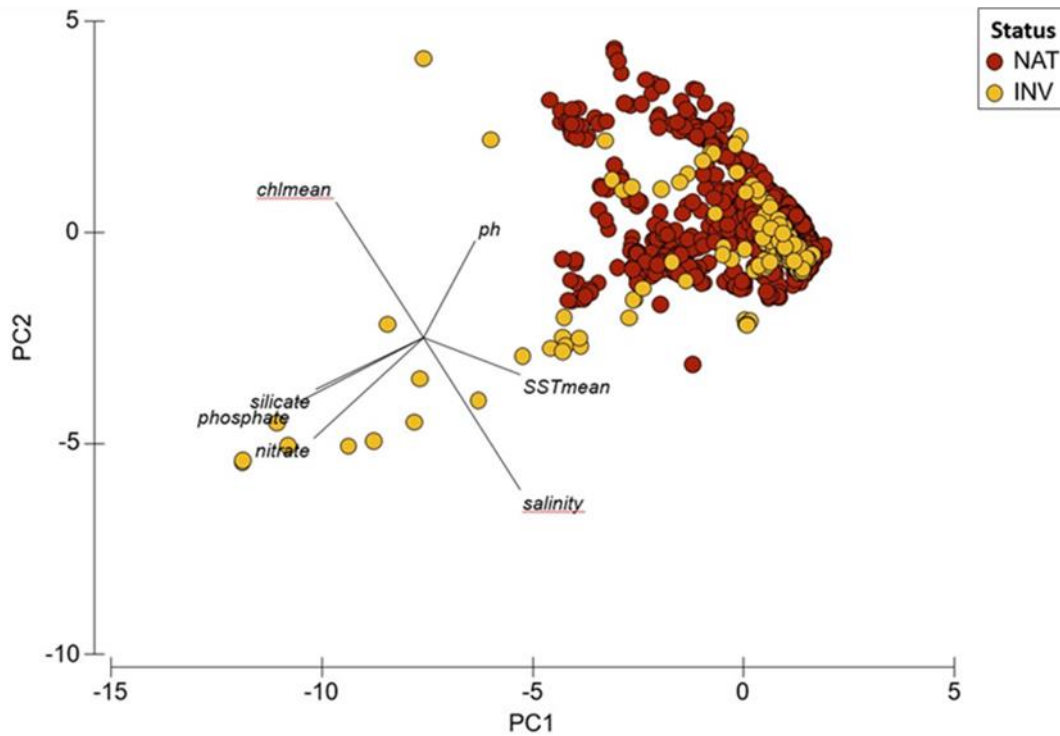
approach was adopted and the density of occurrence models were generated. Scores for niche overlap using Schoener's D metric were then estimated. These scores vary between 0, if no overlap occurs, and 1 when the overlap is complete. Moreover, the niche dynamics were analyzed through the estimation of indices representing the components into which it may be broken down. These indices are niche stability, niche expansion and niche unfilling (Datta, *et al.*, 2019; Guisan *et al.*, 2014). Hence, niche stability refers to the proportion of invaded niche intersects the native one. Niche expansion index refers to the proportion of the invaded niche which has not been yet colonized in native environment, while niche unfilling is the proportion of the native niche non overlapping with the invades one (Strubbe *et al.*, 2013; Di Cola *et al.*, 2017; Guisan *et al.*, 2014). Eventually, the equivalency and similarity between the native and invaded climatic niche was tested as proposed by Dan L. Warren (Warren *et al.*, 2008; Broennimann *et al.*, 2012).

### **3.3. Results**

#### **3.3.1. Principal Component Analysis**

As a result of the PCA analysis performed on the selected environmental variables (Fig.2), five principal components were extracted, accounting for 94.8% of the variability of the data. The first two components were used in further niche analyses and accounted for 64.4% of the variability (Tab.1). In this analysis the 45.9% of my data variability, is explained by PC1, which is related to mean sea surface temperature, phosphate, silicate, pH and nitrate. Mean temperature and pH lean on the opposite side compared to nitrate, silicate and phosphate that are those variables, which determine the wide distribution of the species in invaded niche along the PC1 axis. Salinity and mean chlorophyll, based on their orientation, are linked to both PC1 and PC2, and are the most related to PC2.

## Principal component analysis



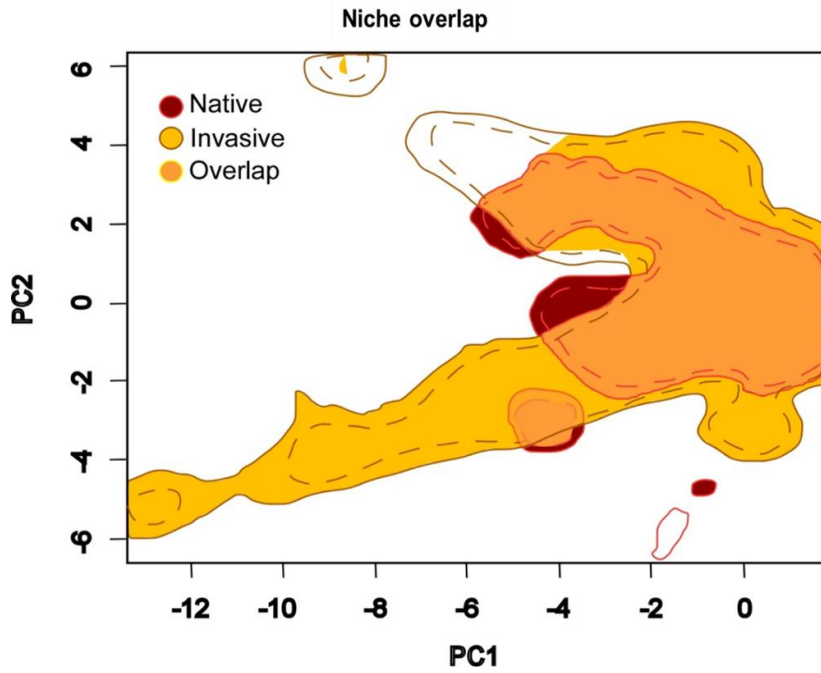
**Figure 2.** Principal Component Analysis (PCA) of geographic distribution of *C. sapidus* in native (red dots) and invaded (yellow dots) areas. The two principal components extracted from environmental variables (chlmean = mean Chlorophyll; Ph; Silicate; Phosphate; Nitrate; Salinity; SST = mean superficial surface temperature).

**Table 1.** Principal Component Analysis values.

PC	Eigenvalues	%Variation	Cum.%Variation
1	3.21	45.9	45.9
2	1.30	18.6	64.4
3	0.99	14.1	78.5
4	0.78	11.1	89.6
5	0.37	5.2	94.8

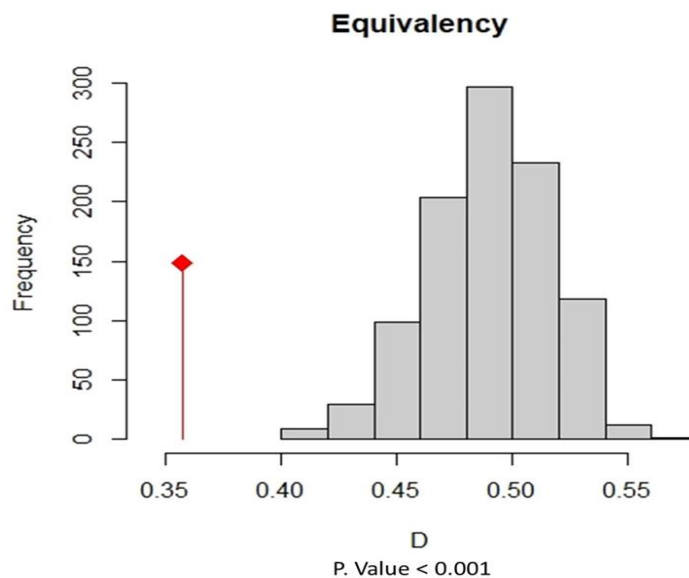
### 3.3.2. Niche Dynamic

The pairwise overlap index between the native and invasive niche of the Atlantic blue crab, estimated using Schoener's D, was 0.352. Furthermore, niche dynamics analysis showed a niche expansion index of 0.5 % and niche unfilling index of 0.9 %.



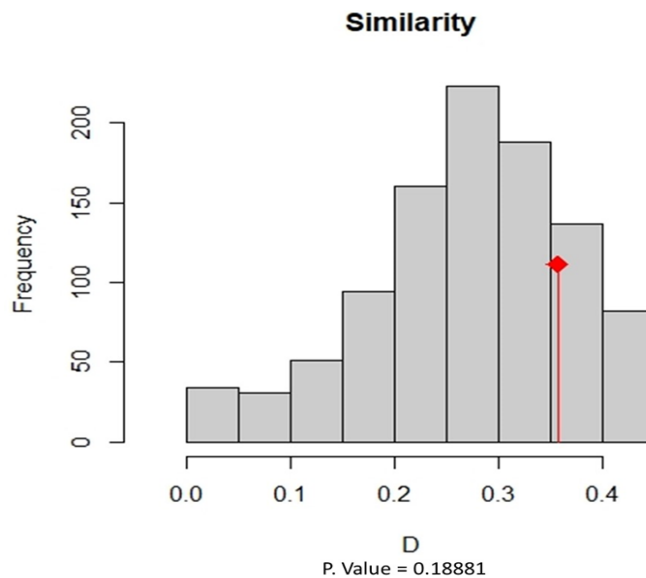
**Figure 3.** Visualization of the native and invaded niche space of *Callinectes sapidus* in Principal Component Analysis space. Red area represents the native niche (unfilled), the yellow area represents the invaded niche (expansion) and the orange area is the overlap between two niches (stable). The bold lines represent the available environment in each range (red for native and yellow for invaded).

The equivalency test indicated that the observed overlap between the native and invaded niches was significantly lower than those estimated by reallocating occurrence data randomly within the two niches range (Fig. 4).



**Figure 4.** Graphic representation of the Equivalency test. The histogram shows the observed niche overlap D between the two ranges (red bars with a diamond) and simulated niche (grey bars). Test is calculated from 100 iterations.

The similarity test showed that the observed overlap between the native and invaded niches was not significantly higher than those estimated by randomly shifting the invasive niche within invaded environmental range keeping constant the native niche (Fig. 5).



**Figure 5.** Graphic representation of the Similarity test. The histogram shows the observed niche overlap  $D$  between the two ranges (red bars with a diamond) and simulated niche (grey bars). Test is calculated from 100 iterations.

### 3.4. Discussion

A challenge for ecologists is to quantify the distribution of a species and identify which factors affect the limits range of distribution (Broennimann *et al.*, 2012). Lately, it has been highlighted how environmental conditions and their variations can affect both the future distribution of a species in its native environment and the distribution of an invasive species in the receiving environment (Fitzpatrick, *et al.*, 2008; Steiner *et al.*, 2008; Broennimann *et al.*, 2012).

In this study, the environmental niche dynamic of *C. sapidus* was analyzed to evaluate if an overlap between native and invasive niche occurs, and to subsequently evaluate the degree of niche shift experienced by this species in the process of invading European waters. To achieve this aim, species distribution modelling was used. Firstly, a principal component analysis was performed, in which starting from environmental variables, five principal components were extracted. These five components together explained more than 90% of

data variability. In this analysis the major aliquot of data variability was explained by PC1 which was related to mean sea surface temperature, pH, phosphate, silicate and nitrate. Moreover, data were more variable along both PC1 and PC2, concerning invasive niche, hence suggesting that the range of *C. sapidus* niche was wider in the receiving habitat. However, the two compared niches seemed to be more similar in terms of environmental parameters such as surface temperature and pH but different for other parameter such as salinity, mean chlorophyll, nitrate and phosphate. Nonetheless, from the analysis of the PCA emerged that the niche shift did not concern aspect such as temperature, but rather chemical aspects as phosphate, nitrate, or mean chlorophyll-*a*. High level of these components generally characterize eutrophic areas. Also, to evaluate the PCA results we have to consider that this is a eurythermal and euryhaline species, which means that is accustomed to tolerate broad ranges of temperature and salinity. Since this specie originally came from a vast area which is characterized by a wide range of temperature and salinity, regardless the variation, the species can tolerate by colonizing the invaded area, the invaded range of salinity and temperature probably fall within the native one. Besides, the reason why salinity was related to PC2, while temperature was more relevant in PC4 is probably due to the fact that the life cycle of *C. sapidus* is strongly associated to salinity gradient (Hines, 2007). This suggests that the species in native habitat probably tends to be more dependent on low salinity, while during the process of colonization it is able to amplify its range. Afterwards, the first two components that explained almost the 60% of variability, were necessarily the only two considered to subsequent analysis. The preliminary analysis carried out in the present investigation indicated a relatively low degree of overlap between native and invasive niches of the blue crab, thus suggesting a significant shift. Furthermore, concerning niche dynamics, the unfilling index referred to the portion of native niche which has not been colonized yet in invaded area. So low value of this index suggested that the shift did not concern the incapacity of the blue crab to occupy, in invaded areas, habitat with climatic features similar than those characterizing the native area. Moreover, the expanded niche index referred to the portion of the invaded area which is not yet occupied in native area.

Additionally, equivalency and similarity test results were consistent with the low degree of overlap. Indeed, the equivalency test assessed if the degree of overlap between native and invaded niches was lower of those estimated by randomizing two niches within the environmental ranges, conversely the similarity test examined if the overlap observed between actual native and invaded niche ranges was different from the overlap detected

keeping constant the native niche and shifting randomly the invasive occurrences with the range of invaded niche (Broennimann *et al.*, 2012). The significance results of equivalency test suggested that the overlap of the two niches was not higher than expected by chance. Therefore, the degree of overlap was not due to a conservatism of the niche performed by *C. sapidus* during the invasion process but was explained by chance.

Furthermore, the similarity test indicated that the overlap between native and invaded niche was not significantly different from that we would achieve between the actual native niche and a random invaded niche obtained shifting the occurrences of the blue crab within the range of values characterizing the actual invaded niche. Thus, the overlap between the two niche was due to the similarity of the climatic features of the invaded and native areas and not due to specific environmental drivers that constrain the range of *C. sapidus* occurrences.

Overall, the dynamic niche results suggested that the niche shift was due to the ability of the species to tolerate a wide range of environmental variability, colonizing a wide spectrum of habitat typologies. The expansion of the invaded niche was evident considering its spreading towards the northern coast of Africa (Mehanna *et al.*, 2019) becoming ubiquitous in the Mediterranean Sea (Mancinelli *et al.*, 2021) and across European waters from the North Sea to the Black Sea. Given the generalist nature of the species, the non-occupied portion of climatic niches could be linked to competition with other species occurring in specific areas rather than environmental constrains. The expansion of the niche occurring in invaded area may be due to several biotic and abiotic factors (*e.g.*, absence of natural predators or competitors, climate change, increasingly extreme environmental conditions) which synergically led to successful colonization by this species.

However, the complementary niche dynamics analyses explained only part of the variability observed, since it considered only the first two principal components. Therefore, this kind of analysis, to investigate such a complicate niche dynamic as could be that of the Atlantic blue crab, is not sufficient by itself to explain the reason of this shift.

### **3.5. Conclusion**

In conclusion, it is possible to claim that the overlap degree between the two niches is low. Consequently, an environmental niche shift occurs moving from native to invaded area. Nevertheless, we have to consider that principal component analysis used to perform the subsequently investigation were the first two, which explained the 65 % of total variability.

For this reason, further investigations are needed to explain the reasons of the shift and which environmental drivers might possibly lead it. Another interesting aspect to investigate is to treat the invaded areas separately, in order to verify whether the niche shift is more related to the Mediterranean Sea area or if there are differences between the niches along the east and west coasts of the Atlantic Ocean. Additional advanced approaches would include analyses implemented in the R package hypervolume - taking into consideration the multidimensional information provided by PCA procedures.

## **4. Population dynamics of *Callinectes sapidus* in a Mediterranean hyperaline coastal marine ecosystem, the Stagnone di Marsala**

### **4.1. Introduction**

The Atlantic blue crab, *Callinectes sapidus* Rathbun, 1896, native of the Atlantic coasts of America, is recognized as invasive in the Mediterranean Sea since 2006 (Streftaris & Zenetos, 2006; Nehring, 2011). *C. sapidus* is a euryhaline and eurythermal species, founded in both transitional systems and coastal environments where it lives on sandy and muddy bottoms (Hines, 2007). Recently, *C. sapidus* was found in environments with abiotic conditions, especially salinity, different than those observed in native habitats (Chairi & González-Ortegón, 2022; Kara & Chaoui, 2021; Razek, *et al.*, 2016; Chartosia *et al.*, 2018; Benabdi, *et al.*, 2019). The spread of *C. sapidus* in such different areas confirms the generalist nature of this species and suggests an environmental niche expansion while colonizing new areas. The life cycle of this species is linked to the salinity gradient, which influence the behavior of males and females, and different life stages (Hines, 2007). Adult males tend to remain in the upper part of the estuary, while females prefer higher salinity, especially during spawning season (Hines, 2007). However, the high salinity of the recently invaded areas, as those of the southern Mediterranean Sea, leads to the issue of whether brackish conditions are essential for the life cycle of the blue crab or if this species is able to grow and spread even in fully marine conditions. To address this question, we investigated the population dynamic of *C. sapidus* in a Mediterranean hyperaline coastal marine area, the Stagnone di Marsala. In more detail, aims of the study were to assess: *i*) the population distribution dynamics, across seasons and space; *ii*) the possible relationship between the blue crab and both abiotic (temperature and salinity) and biotic (macrophytes biomass) features; *iii*) the efficiency of two different sampling methods (baited traps vs gillnet) in collecting blue crab specimens.

### **4.2. Materials and Methods**

#### **3.2.1. Study area**

The Stagnone di Marsala (Fig. 1) is a semi enclosed hyperaline basin located along the north-western coast of Sicily (37.872580° N, 12.464837° E). This shallow coastal marine area with



a surface area of nearly 21 km<sup>2</sup> (Andolina *et al.*, 2022) is characterized by an average depth of 1.5 m, a salinity range of 33-48‰ and a temperature range of 10-30°C (Tomasello *et al.*, 2009). The basin is separated from the sea by a calcarenite platform (Isola Longa) and it is divided in two main sub-basins (Vizzini *et al.*, 2002). The northern one is connected to the sea by a narrow and shallow opening, named Bocca San Teodoro. This sub-basin is 400 m<sup>2</sup> wide and 0.3-0.4 m deep (Vizzini *et al.*, 2013). The southern sub-basin, instead, is connected towards the sea by a larger opening, called Bocca Grande, which is 1,200 m<sup>2</sup> wide and 1-2 m in depth (Vizzini *et al.*, 2013). These different degrees of connection with the sea result in a limited exchange of water in the central-northern part of the basin, while the southern part is more influenced by the seawater in terms of temperature and salinity (Tab. 1) (La Loggia *et al.*, 2004; Mazzola *et al.*, 2010). Due to limited openings and the absence of freshwater inputs (Sarà, *et al.*, 1999; Vizzini *et al.*, 2002), this basin is hyperhaline, with a large variation in temperature during the year, particularly in the center-northern sub-basin.

**Table 1.** Minimum (Min.), maximum (Max.) and average (mean) temperature and salinity into the Stagnone di Marsala (Mazzola *et al.*, 2010).

	<b>Min.</b>	<b>Max.</b>	<b>Mean</b>
<b>Salinity (PSU)</b>	32.8	47.1	39.9
<b>Temperature (°C)</b>	11.2	29.1	19.5

In general, the basin is characterized by sandy-muddy bottoms with few rocky patches mainly in the south. Bottoms are mostly colonized by the seagrasses *Cymodocea nodosa* (Ucria) Asherson, 1870, *Posidonia oceanica* (Linneaus) Delile, 1813, and *Caulerpa prolifera* (Forsskål) Lamouroux, 1809, which are unevenly distributed throughout the basin. *P. oceanica* meadows cover the central and southern part of the northern sub-basin. Where the hydrodynamic conditions worsen for *P. oceanica*, it is replaced by *C. nodosa* (La Loggia *et al.*, 2004).

### 3.2.2. Study sites and sample collection

For the purpose of the study, and considering the gradient of confinement of the Stagnone di Marsala, four sampling zones were identified in the study area: North (37.901727° E; 12.458296° N), Center-North (37.871882° E; 12.465895° N), Center-South (37.845351° E; 12.457651° N) and South (37.820750° E; 12.454201° N). Within each zone, a different

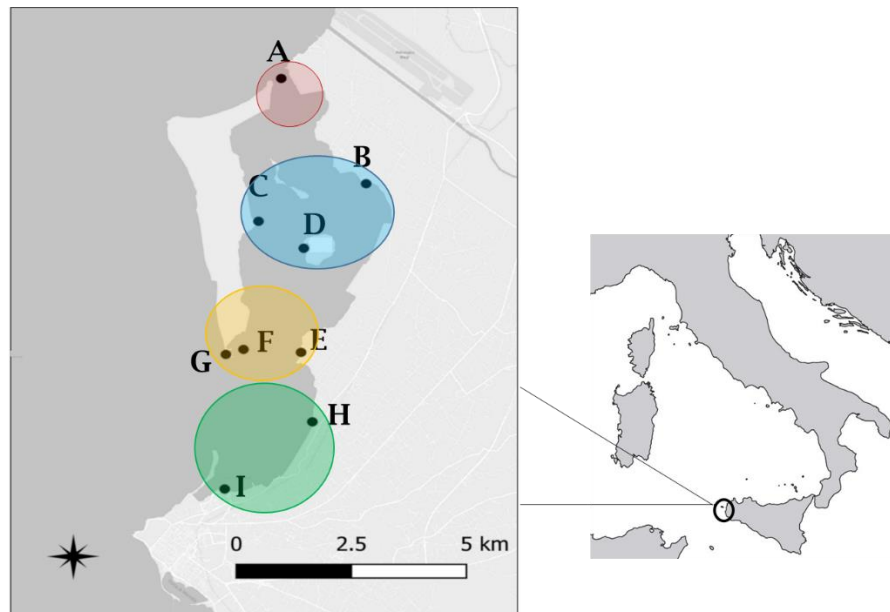
number of sites were selected, based on the habitat heterogeneity. Sites selected were one in the North zone (site A), three in the Center-North (sites B, C, D) and Center-South (sites E, F, G) zones, and two in the South zone (H, I) (Fig. 1).

Site A, which correspond to the entire North zone, is the one close to the northern narrow opening. It is characterized by sandy bottom, and a depth approximately 10-20 cm, depending on tide. Variations of salinity and temperature are relatively limited due to its proximity to the open sea and it is characterized by low macrophyte biomass. Site B, C and D in Center-North zone are the most remote to the open sea, and this leads to a wide variation in terms of salinity and temperature across the seasons, in particular at site B along the coast. Center-North sites are characterized by a muddy bottom, a depth range of 10-40 cm and an abundant coverage of macrophytes, especially during the Summer, in B and C sites where *C. nodosa* and *Laurencia sp.* are very dense. The most southern zones, Center-South and South, present a heterogeneous bottom, partially sandy and rocky. At the E, F and G sites, in Center-South zone, depth is about 45 cm, macrophytes, mainly represented by *C. prolifera* and *C. nodosa*, are abundant during the Summer and scant in other times of the year. Site I, in South zone, has a rocky sandy bottom, rough depth of 40 cm, and is featured by a high abundance of *C. prolifera*, while site H is mainly sandy and shallower than I.

Sampling activities were carried out monthly, from Summer 2021 to Winter 2023. At each sampling time, two capture methods were used to collect the blue crab: baited trap and gillnet. The traps were 60 x 30 wide, with a mesh size of 15 mm and were made of nylon. Every trap was baited with sardines and chicken waste. The gillnet was 50 m long, with a mesh size of 30 mm. Four baited traps were deployed each time, in each sampling site, at a distance of approximately 70 m each other. The traps were left overnight and withdrawn the following morning. One gillnet was deployed each time in two sites of the Center-North and the Center-South zones, respectively in sites C and D, and in sites F and G. The gillnets were left overnight and withdrawn the following morning.

Environmental parameters such as water temperature and salinity were measured with a multi-parametric probe (Hanna® HI98194) in each site and sampling occasion. Furthermore, to estimate the biomass of macrophytes characterizing each zone, during the first year of sampling (from Summer 2021 to Spring 2022) seagrasses and macroalgae were collected by hand using a 20x20 cm quadrat in three random replicates, once per season.

Captured crab specimens and macrophytes samples were put in individual labelled plastic bags and transferred to the laboratory in refrigerated cool boxes.



**Figure 1.** Sampling sites grouped by zones, into the study area of the Stagnone di Marsala. The red circle indicates the North zone with site A, the blue circle indicates the Center-North zone with location of the sites B, C and D, the yellow circle indicates the Center-South zone with sites E, F and G and the green circle indicates the South zone with sites H and I.

#### 4.2.3. Sample processing and laboratory analysis

Once in the laboratory, identification of *C. sapidus* was based on the presence of two obtuse and large teeth on the front between the inner orbital teeth (Millikin & Williams, 1984; Castriota *et al.*, 2012). Afterwards, specimens were weighed to the nearest 0.1 g and their carapace length (CL) and carapace width (CW) were measured to the nearest mm using a caliper. Carapace width was determined as the distance between the two lateral spines, while the carapace length was measured from the teeth of the frontal margin to the posterior margin of the dorsal carapace. *C. sapidus* specimens were grouped in three size classes based on carapace width (CW): small (CW < 70 mm), medium (CW 70-100 mm) and large (CW > 100 mm). Furthermore, for each specimen sex was determined by inspecting the abdomen shape. The egg masses removed from pregnant females were wet weighed and then the eggs of a pre-weighed subsample were counted using a stereomicroscope. The total number of eggs per female was estimated from the weight. Macroalgae and seagrasses were identified at the lowest taxonomic level and cleaned with distilled water to remove impurities. All the

macrophytes samples were oven-dried at 60°C for approximately 48 h and the dry weight was subsequently measured.

#### 4.2.4. Data analysis

The variability of environmental parameters such as temperature and salinity was assessed by comparing mean values between zones across seasons using one-way analysis of variance (ANOVA) and differences were analyzed using Chi-squared tests. Relative abundance of the blue crab was estimated as catch per unit effort (CPUE), *i.e.* number of crabs/number of traps deployed. CPUE was calculated separately for each capture method, season and zone, considering firstly total captures (total CPUE), then also considering captures grouped by sex (sex CPUE) and size (size CPUE). Univariate permutational analysis of variance (PERMANOVA) and pair-wise test were used to test the differences in CPUE distribution across season (factor Season, seven levels: Summer 21, Autumn 21, Winter 22, Spring 22, Summer 22, Autumn 22, Winter 23), zone (factor Zone, four levels: North, Center-North, Center-South and South) and sex (factor Sex, two levels: female and males). Moreover, PERMANOVA was used to test different CPUE distribution considering same factors of zone and season but as a third factor was considered size (factor Size, three levels: Small, Medium, Large). The analysis was run after log-transformation of the data, resembled using Bray Curtis similarity index and addition of a dummy variable.

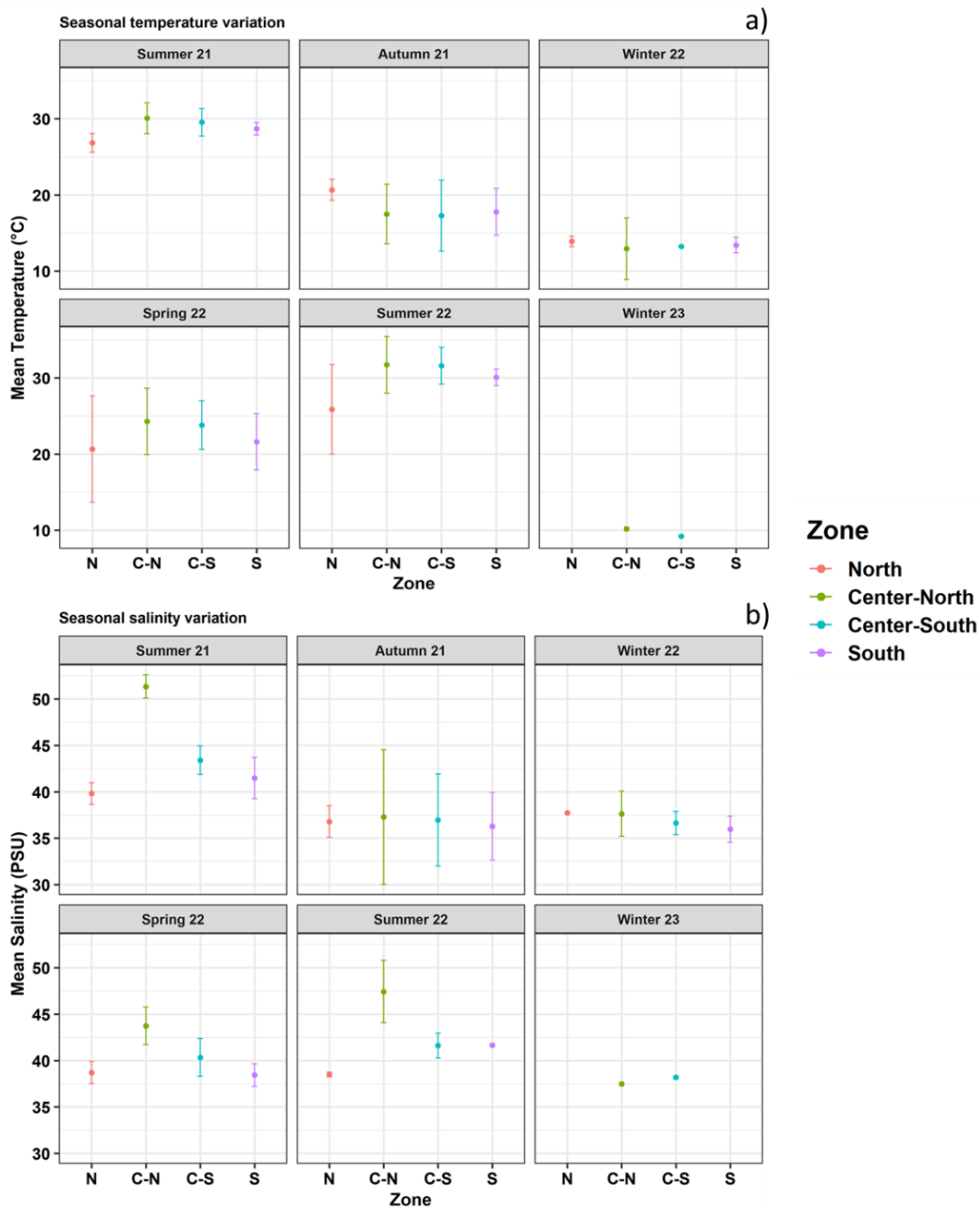
Eventually, Pearson correlation coefficient was estimated to analyze the correlation between the relative abundance of the crabs and the mean macrophytes biomass ( $\text{g/m}^2$ ), mean salinity and mean temperature, per zone in every season of sampling. PERMANOVA analysis were conducted using PRIMER (Anderson *et al.*, 2008), other analysis was performed in R.

### 4.3. Results

#### 4.3.1. Environmental variables and macrophyte biomass

Mean water temperature values varied throughout the whole sampling period in the study area, showing a similar seasonal trend among the zones (Fig. 2a). Mean values ranged from a minimum of 9.23°C in Winter 2023 in the Center-South zone, to a maximum of 31.72°C in Summer 2022 in the Center-North zone. Results of the one-way ANOVA and Chi-squared tests showed significant differences among zones between the Summer seasons of both years, Spring 2022 and Winter 2023 (Table 1).

Mean salinity showed a wider variability than temperature, among the different zones. Mean value ranged from 35.97 PSU at South zone in Winter 2022 and 51.34 PSU at the Center-North zone in Summer 2021 (Fig. 2b). The zones with the maximum and minimum values of salinity were respectively the farthest and closest to the major opening towards the sea.



**Figure 2.** Mean ( $\pm$  st. error.) water temperature (a) and salinity (b) values recorded in the four different zones of the Stagnone di Marsala at different sampling seasons. Four sampling zones: North (N); Center-North (C-N); Center-South (C-S); South (S).

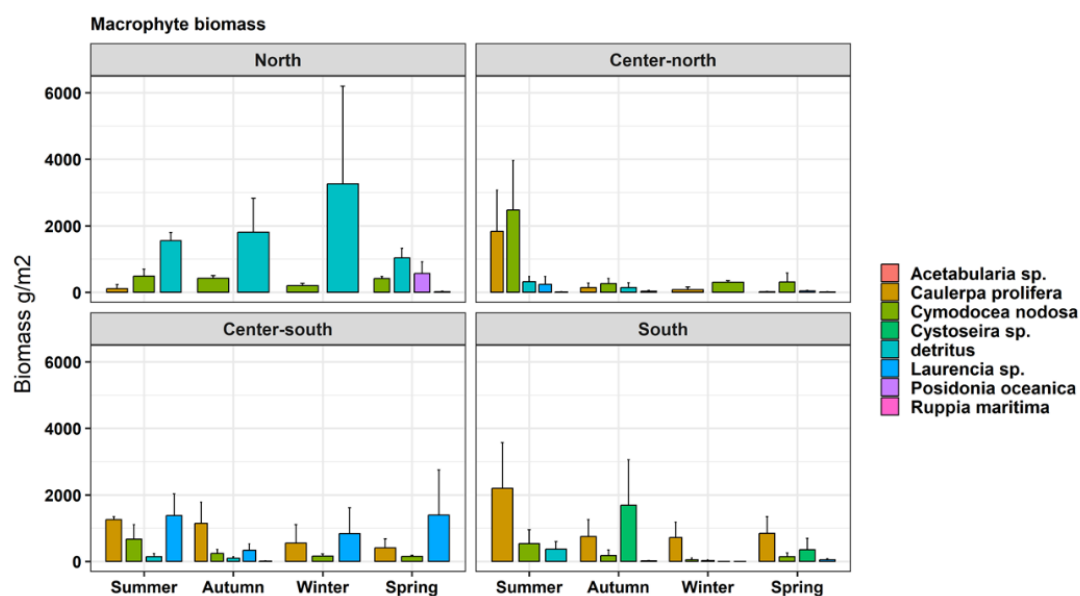
One-way ANOVA and the Chi-squared test performed on temperature and salinity showed that main differences among zones occur in Spring and Summer (Table 2). In particular, differences in salinity were significant between almost all zones, during both Summer and Spring. Temperature resulted to be significantly different during both Summer between North and any other zone.

**Table 2.** Results of the post-hoc comparisons of the One-way ANOVA performed on water temperature and salinity values recorded in the four sampling zones (North, Center-North, Center-South and South) of the Stagnone di Marsala, in all the sampling times (from Summer 2021 to Winter 2022).

Season	Zone 1 vs Zone 2		Temperature	Salinity
			<i>p-value</i>	<i>p-value</i>
<b>Summer 21</b>	North vs	Center-North	<b>&lt;0.001</b>	<b>&lt;0.001</b>
		Center-South	<b>&lt;0.001</b>	<b>&lt;0.001</b>
		South	<b>0.001</b>	<b>0.007</b>
	Center-North vs	Center-South	0.779	<b>&lt;0.001</b>
		South	0.074	<b>&lt;0.001</b>
	Center-South vs	South	0.248	
<b>Autumn 21</b>	North vs	Center-North	0.503	0.977
		Center-South	0.459	0.999
		South	0.438	1
	Center-North vs	Center-South	1	0.947
		South	1	0.963
	Center-South vs	South	0.998	0.999
<b>Winter 22</b>	North vs	Center-North	0.581	0.998
		Center-South	0.810	0.275
		South	0.872	<b>0.009</b>
	Center-North vs	Center-South	0.979	0.367
		South	0.889	<b>0.016</b>
	Center-South vs	South	0.993	0.598
<b>Spring 22</b>	North vs	Center-North	0.270	<b>&lt;0.001</b>
		Center-South	0.751	<b>0.016</b>
		South	0.866	0.599
	Center-North vs	Center-South	0.830	<b>&lt;0.001</b>
		South	0.040	<b>&lt;0.001</b>
	Center-South vs	South	0.261	<b>&lt;0.001</b>
<b>Summer 22</b>	North vs	Center-North	<b>&lt;0.001</b>	<b>&lt;0.001</b>

		Center-South	<0.002	0.001
		South	0.007	0.004
Center-North vs		Center-South	0.832	<0.001
		South	0.694	<0.001
Center-South vs		South	0.368	1.000
	<b>Winter 23</b>	Center-North vs	Center-South	<0.001
			<0.001	<0.001

Regarding the macrophytes composition, eight species were identified in the study area (Fig. 3). The species identified were *Acetabularia sp.*, *Caulerpa prolifera*, *Cymodocea nodosa*, *Cystoseira sp.*, *Laurencia sp.*, *Posidonia oceanica*, *Ruppia maritima* Linneaus 1753, and detritus. These macrophytes were not uniformly distributed across zones nor in terms of biomass ( $\text{g/m}^2$ ) either in species composition, with the only exception of the North zone, where detritus abundance dominated in Winter. The most widespread species were *Cymodocea nodosa*, *Laurencia sp.* and *Caulerpa prolifera*, especially during Spring and Summer seasons.



**Figure 3.** Mean biomass ( $\pm$  std. error) of macrophytes species collected in each study zone of the Stagnone di Marsala, across the sampling period.

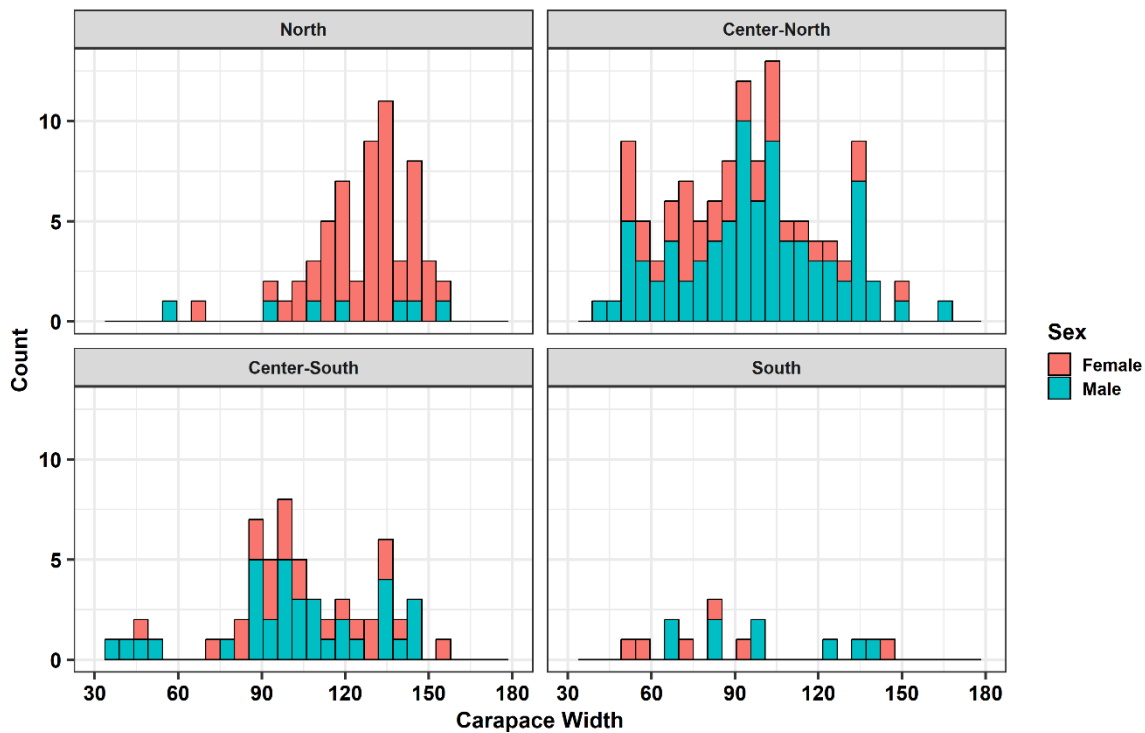
### 4.3.2. *Callinectes sapidus* distribution

A total of 252 *Callinectes sapidus* specimens were captured across all the sites and sampling seasons, 119 of which were females and 133 were males (Tab. 3). The distribution of the individuals across zones, according to carapace width is reported in Figure 4. In order to understand the structure and distribution of the population, CPUE were calculated separately by sampling method, firstly as total capture, then also by relative abundances by sex (Fig. 5 and 6) and size classes (Fig. 7 and 8).

**Table 3.** Total number of individuals and size range capture across zones during sampling period. carapace width (CW), carapace length (CL), wet weight (WW).

Sampling season	Zone	Total number of individuals	CW range (mm)	CL range (mm)	WW range (g)
Summer 21	North	11	103.9-149.5	50.19-72.8	60.79-199.3
	Center-north	0	/	/	/
	Center-south	2	142.2-147.2	70.95-74.5	241.49-306.2
	South	0	/	/	/
Autumn 21	North	5	132-155.2	63.3-79.8	136.5-231.9
	Center-north	3	126.4-134.1	64.7-67.9	141.5-223.4
	Center-south	2	86.1-89.2	43.3-43.8	39.8-43.9
	South	3	66.3-146.6	33.8-76.7	22.5-306.9
Winter 22	North	0	/	/	/
	Center-north	2	67.5-165.2	32.8-81.2	20.9-334.6
	Center-south	1	84.6	42.3	42.3
	South	0	/	/	/
Spring 22	North	18	54.6-153.2	30.3-85.5	16.3-236.8
	Center-north	14	61.9-134.5	33.8-90.5	24.1-189.7
	Center-south	10	54.2-114.8	30.2-57.3	13.3-112.8
	South	7	50.4-95.9	26.9-73.1	9-73.1
Summer 22	North	26	99.8-150.3	50.3-73.1	64.7-189.9
	Center-north	60	50-140.6	28.1-116.1	13.5-207.8
	Center-south	16	76.9-153.6	41.9-121.8	48.5-318.3
	South	5	92.7-140.4	48.4-140.4	69.5-214.0
Autumn 22	North	/	/	/	/
	Center-north	24	43-150	23-74	5.67-218.37
	Center-south	21	71.46-144	38.76-107.74	36.94-230.17
	South	/	/	/	/
Winter 23	North	/	/	/	/
	Center-north	16	49-105	26-55	7.91-86.28
	Center-south	6	38-128	20-63	4.45-127.14
	South	/	/	/	/



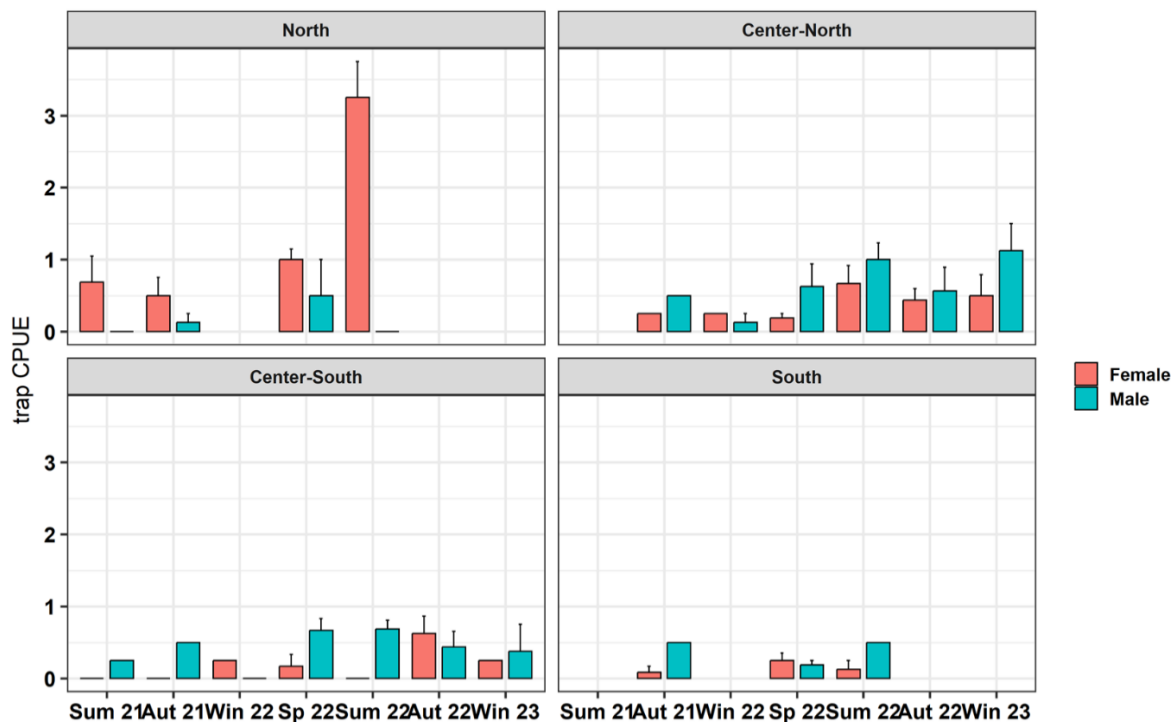


**Figure 4.** Frequency distribution of carapace width of *Callinectes sapidus* males and females across zones.

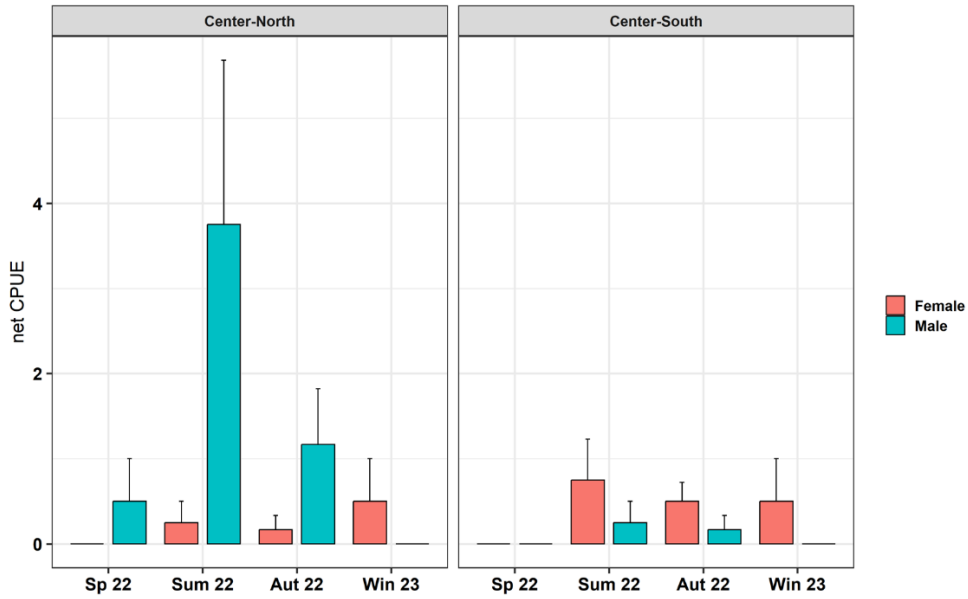
Overall, the total abundance of the species resulted non-homogeneously distributed across zones or seasons. The majority of individuals were captured during Summer 2022 at the North and Central-North zones. In particular, during Summer 2022, a total mean of  $3.25 \pm 0.71$  crabs (total CPUE) were caught by trap at the North zone and  $1.38 \pm 1.12$  at the Center-North. Whereas during Summer 2022,  $4 \pm 3.65$  specimens were captured at the Center-North zone by gillnet.

The mean abundance of the species showed a trend across seasons (not considering zones) and a significant increase from the first (2021) to the second year of sampling (2022). Taking into account only the captures performed by traps, during the first year, Summer and Autumn 2021 presented similar mean CPUE values ( $0.27 \pm 0.49$  and  $0.27 \pm 0.25$  respectively), then, during Winter 2022, CPUE were lower ( $0.13 \pm 0.14$ ) and, starting from Spring 2022, CPUE they increased again ( $0.77 \pm 0.89$ ). During the second year, in Summer 2022 the highest number of individuals were caught ( $0.94 \pm 1.58$ ), while during Autumn 2022 and Winter 2023 ( $0.52 \pm 0.44$ ;  $0.63 \pm 0.48$  respectively) the number decreases again, although the CPUE were higher if compared with the same months of the previous year.

Regarding the distribution of *C. sapidus* abundance by sex (sex CPUE), females captured by traps were overall most abundant at North and males at the Center-North zone (Fig. 5). Noteworthy, during the Summer seasons at North zone, only females were caught by traps. Two females, caught at the North zone during Summer 2021, were carrying eggs (CW respectively 119.5 and 134.6 mm, total egg weight: 18.9 and 26.2 g; estimated egg number: 1,587,742 and 1,853,617). Results of the sex CPUE by gillnet (Fig. 6) showed that the highest mean values were recorded for males in Center-North zone, during the Summer 2022. PERMANOVA carried out on sex CPUE by trap showed a significant interaction of the factors Zone and Sex, confirming that abundance of females at North zone was significantly higher than in all the other zones and the opposite result emerged for males (Tab. 4). Significant differences emerged between seasons and in particular, sex CPUE in Summer 2022 was different from all other season, Summer 21 from Spring 2022 and Winter 2022 from Winter 2023. Conversely, PERMANOVA carried out on sex CPUE by gillnet did not highlight significant differences among the factors examined (Tab. 5).



**Figure 5.** Mean CPUE ( $\pm$  std. err.) of female and male *Callinectes sapidus* caught by baited trap in all sampling zones and seasons.



**Figure 6.** Mean CPUE ( $\pm$  std. err.) of female and male *Callinectes sapidus* caught by gillnet in all sampling zones and seasons.

**Table 4.** Results of PERMANOVA and pairwise tests performed on *C. sapidus* CPUE by trap across sampling seasons and zones, considering sex.

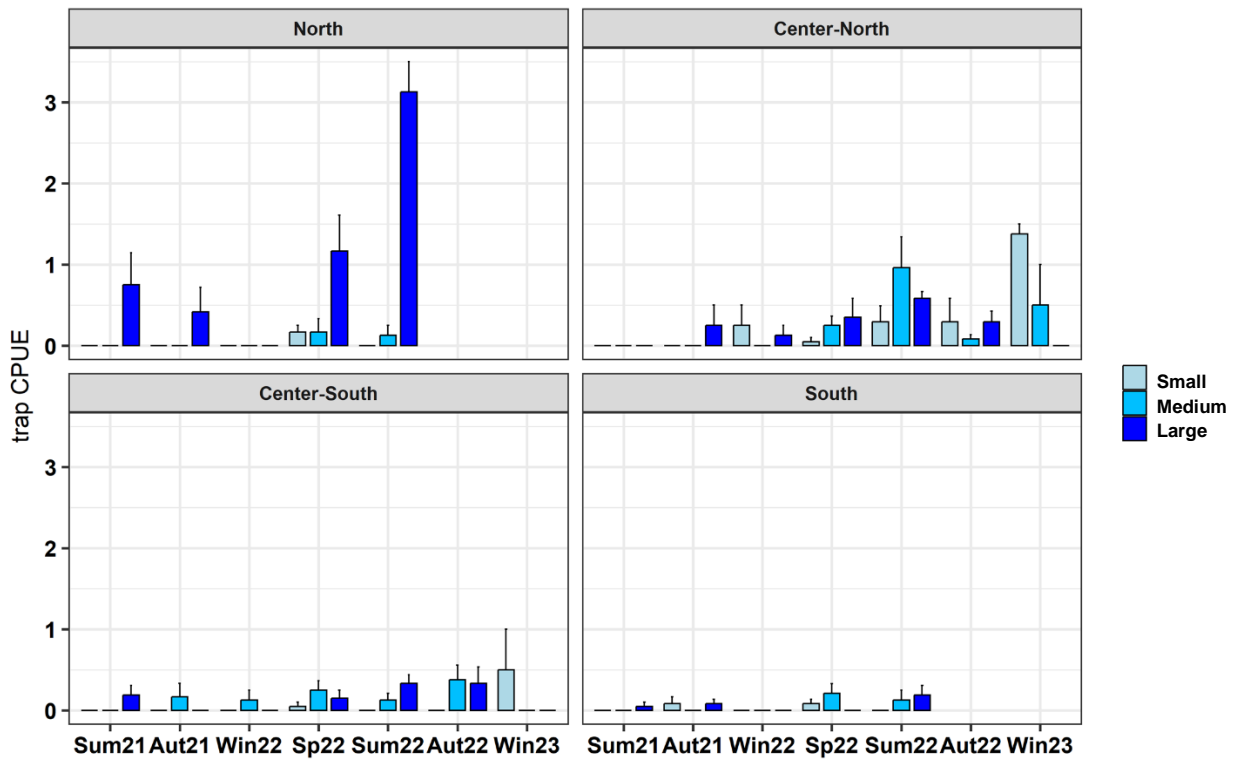
Main test	Source of variation	df	MS	Pseudo-F	P(perm)	Perms
	Season	6	195.4	2.5	<b>0.030</b>	999
	Zone	3	209.5	2.6	0.052	997
	Sex	1	130.1	1.6	0.203	998
	Season vs Zone	10	77.5	1.0	0.474	999
	Season vs Sex	6	115.2	1.5	0.200	997
	Zone vs Sex	3	1069.8	13.5	<b>0.001</b>	999
	Season vs Sex vs Zone	10	99.5	1.3	0.264	999
Pairwise tests	Between zones within sex			t	P(perm)	Perms
Female	North vs Center-North			3.3	<b>0.007</b>	999
	North vs Center-South			5.7	<b>0.001</b>	999
	North vs South			5.5	<b>0.001</b>	999
	Center-North vs Center-South			1.5	0.160	998
	Center-North vs South			1.3	0.218	999
	center-south vs South			0.9	0.377	998
Male	North vs Center-North			2.7	<b>0.016</b>	998
	North vs Center-South			2.9	<b>0.014</b>	998
	North vs South			1.8	0.112	998
	Center-North vs Center-South			0.9	0.352	998
	Center-North vs South			1.0	0.367	998
	Center-South vs South			1.2	0.262	999
Pairwise tests	Between sex within zone			t	P(perm)	Perms
North	Female vs Male			5.1	<b>0.002</b>	998
Center-North	Female vs Male			1.3	0.201	998
Center-South	Female vs Male			2.1	0.057	998
South	Female vs Male			2.4	<b>0.042</b>	997
Pairwise tests	Between seasons			t	P(perm)	Perms
	Summer 21 vs Autumn 21			0.7	0.493	999

Summer 21 vs Spring 22	2.3	<b>0.031</b>	997
Summer 21 vs Summer 22	2.7	<b>0.007</b>	999
Summer 21 vs Winter 22	1.5	1.000	959
Summer 21 vs Autumn 22	1.7	0.106	999
Summer 21 vs Winter 23	1.0	0.355	999
Autumn 21 vs Spring 22	0.7	0.497	998
Autumn 21 vs Summer 22	1.9	<b>0.074</b>	998
Autumn 21 vs Winter 22	1.3	0.244	999
Autumn 21 vs Autumn 22	0.7	0.532	999
Autumn 21 vs Winter 23	1.3	0.198	998
Spring 22 vs Summer 22	1.3	0.214	998
Spring 22 vs Winter 22	1.6	0.127	995
Spring 22 vs Autumn 22	0.6	0.540	996
Spring 22 vs Winter 23	1.2	0.238	998
Summer 22 vs Winter 22	2.8	<b>0.005</b>	998
Summer 22 vs Autumn 22	0.3	0.839	999
Summer 22 vs Winter 23	0.5	0.618	998
Winter 22 vs Autumn 22	1.8	0.097	999
Winter 22 vs Winter 23	2.6	<b>0.035</b>	998
Autumn 22 vs Winter 23	0.5	0.590	999

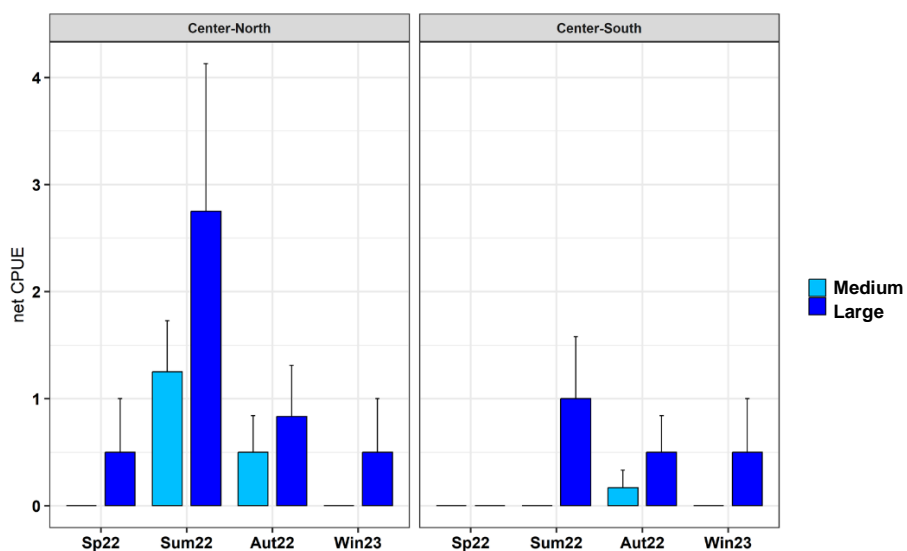
**Table 5.** Results of PERMANOVA test performed on *C. sapidus* CPUE by gillnet across sampling seasons and zones considering sex.

Main test	Source of variation	df	MS	Pseudo-F	P(perm)	Perms
	Season	3	284.96	1.3	0.295	999
	Zone	1	203.19	0.9	0.332	998
	Sex	1	30.309	0.13436	0.771	998
	Season vs Zone	3	48.701	0.2159	0.904	999
	Season vs Sex	3	211.12	0.93593	0.441	999
	Zone vs Sex	1	817.87	3.6257	0.058	999
	Season vs Zone vs Sex	3	148.31	0.65748	0.556	999

Regarding the distribution of *C. sapidus* abundance by size class (size CPUE), results highlighted that the highest values were recorded for large (CW > 100 mm) individuals at North and Center-North, for captures obtained respectively by trap (Fig. 7) and gillnet (Fig. 8). Small and medium size crabs were most abundant in Center-North and Center-South zones. No small individuals (CW < 70 mm), instead, were captured by using the gillnet (Fig. 8). PERMANOVA carried out on size CPUE by trap showed significant interactions of the factors Zone with Size, Season with Size and Season with Zone. Overall, mean relative abundance of large individuals was significantly higher than medium and small ones. Conversely, no significant differences emerged by PERMANOVA carried out on size CPUE obtained by gillnet (Tab. 7).



**Figure 7.** Mean CPUE ( $\pm$  std. err.) of *Callinectes sapidus* caught by baited trap in all sampling zones and seasons, separated by size class (small: CW<70 mm, medium: 70-100 mm, large: CW>100 mm).



**Figure 8.** Mean CPUE ( $\pm$  std. err.) of *Callinectes sapidus* caught by gillnet in all sampling zones and seasons, separated by size class available (medium: 70-100 mm, large: CW>100 mm).

**Table 6.** Results of PERMANOVA and pairwise tests performed on *C. sapidus* CPUE by trap across sampling seasons and zones, considering size class. Large (CW >100 mm), Medium (CW 70-100) and Small (CW < 70mm). Carapace width (CW).

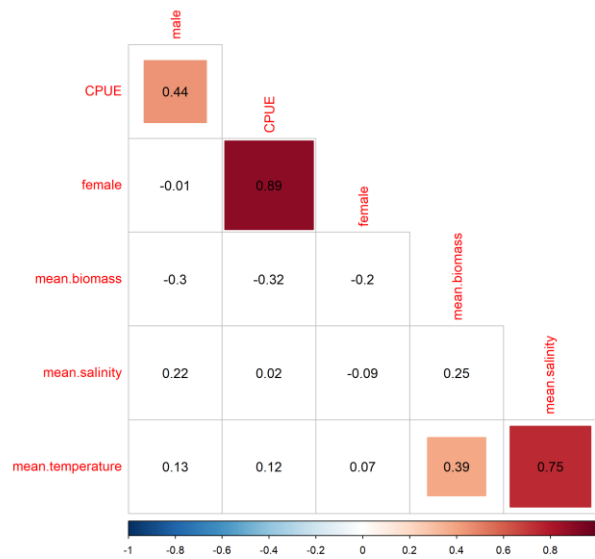
Main test	Source of variation	df	MS	Pseudo-F	P(perm)	perms
	Season	6	408.46	76.1	<b>0.001</b>	999
	Zone	3	396.53	73.8	<b>0.001</b>	998
	Size	2	298.63	55.6	<b>0.006</b>	998
	Season vs Zone	14	116.86	21.7	<b>0.010</b>	998
	Season vs Size	12	220.65	4.1	<b>0.001</b>	997
	Zone vs Size	6	325.27	60.57	<b>0.001</b>	999
	Season vs Zone vs Size	28	64949	12.1	0.294	997
Pairwise tests	Between size		t	P(perm)	Perms	
	Large vs Medium		28.3	<b>0.010</b>	999	
	Large vs Small		27.4	<b>0.004</b>	999	
	Medium vs Small		0.4	0.675	999	
Pairwise tests	Between zones within size		t	P(perm)	Perms	
Small	Center-North vs Center-South		26.2	<b>0.014</b>	999	
Large	North vs Center-North		31.9	<b>0.002</b>	999	
	North vs Center-South		41.5	<b>0.002</b>	998	
	North vs South		64.6	<b>0.001</b>	999	
	Center-North vs South		25.6	<b>0.017</b>	999	
Pairwise tests	Between size within zone		t	P(perm)	Perms	
North	Large vs Medium		48.0	<b>0.001</b>	998	
	Large vs Small		53.3	<b>0.001</b>	999	
Pairwise tests	Between seasons within size		t	P(perm)	Perms	
Small	Summer 21 vs Summer 22		28.7	<b>0.015</b>	996	
	Summer 21 vs Winter 23		74.2	<b>0.001</b>	971	
	Autumn 21 vs Winter 23		50188.0	<b>0.001</b>	997	
	Spring 22 vs Winter 23		31.8	<b>0.006</b>	970	
	Summer 22 vs Winter 23		5.2	<b>0.001</b>	997	
	Winter 22 vs Winter 23		3.9	<b>0.005</b>	999	
	Autumn 22 vs Winter 23		31.4	<b>0.008</b>	704	
Pairwise tests	Between seasons within size		t	P(perm)	Perms	
Medium	Summer 21 vs Summer 22		34.5	<b>0.002</b>	997	
	Summer 21 vs Winter 22		3.3	<b>0.003</b>	998	
	Summer 21 vs Autumn 22		25.0	<b>0.017</b>	999	
	Summer 21 vs Winter 23		22.4	<b>0.049</b>	867	
	Autumn 21 vs Summer 22		23.7	<b>0.027</b>	999	
	Autumn 21 vs Winter 22		24.0	<b>0.018</b>	998	
Pairwise tests	Between seasons within size		t	P(perm)	Perms	
Large	Summer 21 vs Winter 22		44.8	<b>0.001</b>	999	
	Autumn 21 vs Winter 22		46.6	<b>0.001</b>	998	
	Spring 22 vs Summer 22		31.2	<b>0.007</b>	999	
	Spring 22 vs Winter 22		69.0	<b>0.001</b>	999	
	Summer 22 vs Winter 22		33.1	<b>0.003</b>	999	

Pairwise tests	Winter 22 vs Winter 23	38.4	<b>0.001</b>	998
	Between size within season	t	P(perm)	Perms
Summer 21	Large vs Medium	30.4	<b>0.003</b>	<b>998</b>
	Large vs Small	30.4	<b>0.004</b>	<b>997</b>
Spring 22	Large vs Small	30.1	<b>0.009</b>	997
Summer 22	Large vs Medium	33.1	<b>0.005</b>	999
	Large vs Small	66.8	<b>0.001</b>	998
	Medium vs Small	20.9	<b>0.045</b>	999
Winter 23	Large vs Small	33.2	<b>0.026</b>	24

**Table 7.** Results of PERMANOVA test performed on *C. sapidus* CPUE by gillnet across sampling seasons and zones, considering size class.

Main test	Source of variation	df	MS	Pseudo-F	P(perm)	Perms
	Season	3	4.8	1.3	0.295	999
	Zone	1	203.19	0.9	0.332	998
	Size	1	30.309	0.13436	0.771	998
	Season vs Zone	3	48.701	0.2159	0.904	999
	Season vs Size	3	211.12	0.93593	0.441	999
	Zone vs Size	1	817.87	3.6257	0.058	999

Pearson's product-moment correlation performed to analyze the relationship between the relative abundance of *C. sapidus* (in terms of total CPUE and sex CPUE) and both abiotic (mean temperature and salinity) and biotic (mean macrophytes biomass) features of the Stagnone di Marsala, showed no significant results (Fig. 9). Figure 9 showed the value of correlation (ranging between -1 and 1) among the different factors considered (e.g. CPUE and mean temperature; female and mean salinity). Red color suggests positive correlation (0;1), while blue color suggests negative correlation (0;-1); darker is the color stronger is the correlation.



**Figure 9.** Pearson's correlation between biotic (mean macrophytes biomass) and abiotic features (temperature and salinity) and the distribution of *Callinectes sapidus* in terms of catch per unit efforts (CPUE, total number of catch per unit effort; female, female CPUE; male, male CPUE).

#### 4.4. Discussion

Coastal and estuarine environments are the most subjected to biological invasion (Ruiz *et al.*, 2000; Cilenti *et al.*, 2015). One of the most successful invaders in Mediterranean waters is *Callinectes sapidus* (Scalici *et al.*, 2022). Since its first record in Europe, in 20<sup>th</sup> century (Nehring 2011), the species was retrieved in both estuaries and lagoons that presented environmental characteristics similar to its native habitat, however, lately this species seems to show an extension of its environmental range in invaded area (Oussellam *et al.*, 2023; Scalici *et al.*, 2022; Clavero *et al.*, 2022). The life cycle of *C. sapidus* takes place under open sea conditions as well as in transitional environments such as estuaries and lagoons, and it is strictly related to salinity (Hines, 2007; Mancinelli *et al.* 2017a; Kevrekidis *et al.*, 2023). On these bases, the population of *C. sapidus* that has recently invaded the Stagnone di Marsala, represents an interesting case of study given the the peculiar environmental characteristics of this basin. The goal of this study was to assess the population dynamics of *Callinectes sapidus* within the Stagnone di Marsala, and to investigate the possible relationship between the presence of *C. sapidus* and both abiotic (temperature and salinity) and biotic (macrophytes biomass) features of the Stagnone di Marsala.



Overall, the distribution of individuals resulted to be highly heterogeneous across the different zones. With seasonal decline in temperature *C. sapidus* is known to cease activity during winter months (Hines, 2007). According to the literature (*e.g.*, Hines, 2007; Kevrekidis *et al.*, 2023; Carrozzo *et al.*, 2014), the highest number of individuals was sampled during summer seasons. Overall, the distribution of the species resulted non-homogeneous even across seasons. Considering the season, there was a clear trend in the abundance of individuals. Summer season shows the highest abundance, followed by autumn, winter and during spring the number of capture increase again. Furthermore, there was a significant increase of relative abundance from first to second years of sampling.

In general, there were large differences in the blue crab abundance between the North and South sub-basins of the Stagnone di Marsala, with the 70% of the captures occurring in the North and Center-North zones. In particular, at the North zone, almost only females were caught, while the majority of males were caught at the Center-North. Moreover, individuals with carapace width smaller than 100 mm were mainly retrieved in Centre-North and Center-South zones. The North zone is closest to the small opening towards the sea. This connection with the sea is reflected by most moderate variation of temperature and salinity across season. In contrast with other areas, here is Winter the season with the highest macrophytes biomass, mainly consisting in detritus. In this zone, nearly all specimens captured were adult females, two of which were carrying eggs during Summer 2021. Center-North zone is the farthest from the sea. Wide ranges of temperature and salinity characterize this zone, while macrophytes biomass was the poorest. Interestingly, despite the high salinity recorded, males were caught mainly in this zone. In contrast with other lagoons in the Mediterranean Sea (Dulčić *et al.*, 2011; Carrozzo *et al.*, 2014; Cilenti *et al.*, 2015), within the Stagnone di Marsala, males were present under hyperaline condition.

Indeed, in the Gulf of Mexico the presence of *C. sapidus* was reported in two hyperaline lagoons, Laguna Madre and Corpus Christi Bay (Hawley, 1964; Johnson, 1965; Plotnick, *et al.*, 1990; Ramach *et al.*, 2009). Noteworthy, in these lagoons salinity level can reach as high values as the ones recorded in the Stagnone of Marsala (more than 40 PSU). However, while in the Gulf of Mexico, a relation between increasing of salinity and decreasing of abundance of crabs were observed (Copeland, 1966), in this study a different outcome emerged. In fact, this study showed that the highest abundance of individuals were found where the salinity is higher, suggesting that, within the Stagnone di Marsala salinity is not a driving factor for the presence of the species. Despite its proximity to the sea, Center-South zone presents a range

of salinity and temperature similar to the Center-North zone, and the highest values of macrophytes biomass. Although here the relative abundance of crabs was low, this was the only zone where females and males were equally distributed. The South zone was the one with the greatest macrophytes biomass, where salinity and temperature ranges were relatively narrow, because the proximity to the open sea. This was the zone with the lowest abundance of crabs, however it must be taken into account that this area is the only one that has a pier from which several small boats depart during the whole year and from spring to the beginning of autumn is subjected to a great direct anthropic impact, being a very touristic area.

Indeed, in contrast with the information reported in literature, in this particular marine coastal area, the Atlantic blue crab did not show a relationship with salinity or temperature, and this proves the high ability of this species to colonize wider range of habitat. Furthermore, the outcomes obtained open up to issue of the necessity of brackish waters for the blue crab to complete the life cycle. Indeed, the presence of ovigerous females, juveniles and adult males suggest the capacity of the species to accomplish its life cycle even in hypersaline condition.

Eventually, since two different sampling methods were adopted, traps and gillnet, a comparison of them was performed in order to understand if there was a most efficient manner to capture this species, or if they were complementary. Traps are selective sampling methods, efficient for benthic organisms and known to be effective to capture *C. sapidus* (Aslan & Polito, 2021; Kevrekidis *et al.*, 2023). On the contrary, gillnet is a passive non-selective tool, suitable for fishing pelagic organisms, that however resulted to be effective for the blue crab (Cilenti *et al.*, 2015; Oussellam *et al.*, 2023). Based on the outcomes of this study, traps resulted to be efficient to catch females and according to the literature, are functional to capture ovigerous females as well (Cilenti *et al.*, 2015). Gillnet was found being effective to catch males and females. Nevertheless, we have to consider that, due to the morphology of the Stagnone di Marsala, it was not possible to use the gillnet method in the North and South zone. Since the northern zone of the Stagnone di Marsala, is actually the one with the highest number of females, so far we can't say if the gillnet is more efficient to capture females than traps. However, in Center-South and Center-North these two capture methods resulted to be similarly effective to catch females. On the contrary, we may say that is a useful method, even better the traps, to capture males. Indeed, Center-North zone is the zone with the most abundance of males, and gillnets resulted to be more efficient than

traps. Furthermore, while with traps all the sizes were caught, with gillnet only individuals larger than 70 mm were retrieved, probably acting as selective method on size class.

#### **4.5. Conclusions**

Although the Stagnone di Marsala showed environmental variables (temperature and salinity) remarkably different from those *C. sapidus* is used to inhabit, it was evident that in this coastal area there is a structured population. This suggests a great ability of this species to colonize a wide range of habitats. Even if abundances found were not as high as in other invaded area, the population seems to be significantly growing. This outcome highlights the importance to continue the monitoring of the population to understand if the low relative abundance of individuals was due to the particularly extreme conditions of the basin in terms of salinity and temperature, or if it was linked to the recent establishment of the population, and so the population didn't reach a stable abundance yet. The established population observed indicated that the species was able to complete its life cycle not only in transitional environments but also under fully marine and hypersaline condition, opening up to interesting future prospective by expanding the range of environments that this species is able to colonize.

## **5. Analysis of the isotopic niche of *Callinectes sapidus* in invaded marine coastal area: a comparison with autochthonous brachyurans in the Stagnone di Marsala.**

### **5.1. Introduction**

Characterizing the biotic and environmental factors determining the ecological niche of a species is crucial to clarifying the adaptive nature of organisms and their interactions. Ecological niche has been defined by Hutchinson, as an n-dimensional space represented by scenopoetic and bionomic axes. The scenopoetic axis defines a set of bioclimatic features in which a species occurs, while the bionomic axis describes what resources organisms use (Hutchinson, 1957, 1978). According with this definition, in recent years stable isotopes analysis has been used to analyze the isotopic niche that could be represented in a bivariate “ $\delta$ -space”, allowing us to evaluate the trophic features of a species (Jackson *et al.*, 2011; Newsome *et al.*, 2007). Besides, it is a highly effective approach that provides relevant information on species feeding patterns and exploitation of trophic resources within ecosystems. Moreover, stable isotopes analysis is useful for estimating the trophic level of populations in a given environment, providing a temporal and spatial trace of a consumer's diet essential to obtain information about the ecological niche of the species. In particular, stable isotopes of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) provide information respectively about the origin of the organic matter of the food sources and the trophic level. Therefore, stable isotopes analysis is deemed valuable in ecological invasion studies, as it is useful to investigate the trophic consequences of the introduction of invasive species. The Atlantic blue crab *Callinectes sapidus* Rathburn 1896, is native of the western coasts of America, from New England to Uruguay (Mancinelli *et al.*, 2021) and was introduced in Europe probably by ballast waters in 1940s (Galil, 2000). *C. sapidus* is an opportunistic omnivorous species, feeding from plant material to detritus, from polychaetes, mollusks and crustaceans to fish (Belgrad & Griffen, 2016; Mancinelli *et al.*, 2017b; Kevrekidis *et al.*, 2023).

Overall, the purpose of this study was to assess the trophic dynamics of *C. sapidus* invasion in the recently colonized coastal marine area that is the Stagnone di Marsala. In particular specific aims were:

- i)* to evaluate eventual trophic niche competition (or partitioning) among the invasive blue crab and the autochthonous brachyuran species occurring in the Stagnone di Marsala (*Carcinus aestuarii* Nardo, 1847 and *Eriphia verrucosa* Forksal, 1775), by assessing their trophic niche features (isotopic niche width and trophic diversity) and trophic position across seasons;
- ii)* to estimate the trophic pathways supporting the blue crab in the invaded basin, by investigating the contribution that available trophic resources provide to its diet across seasons.

## **5.2. Materials and methods**

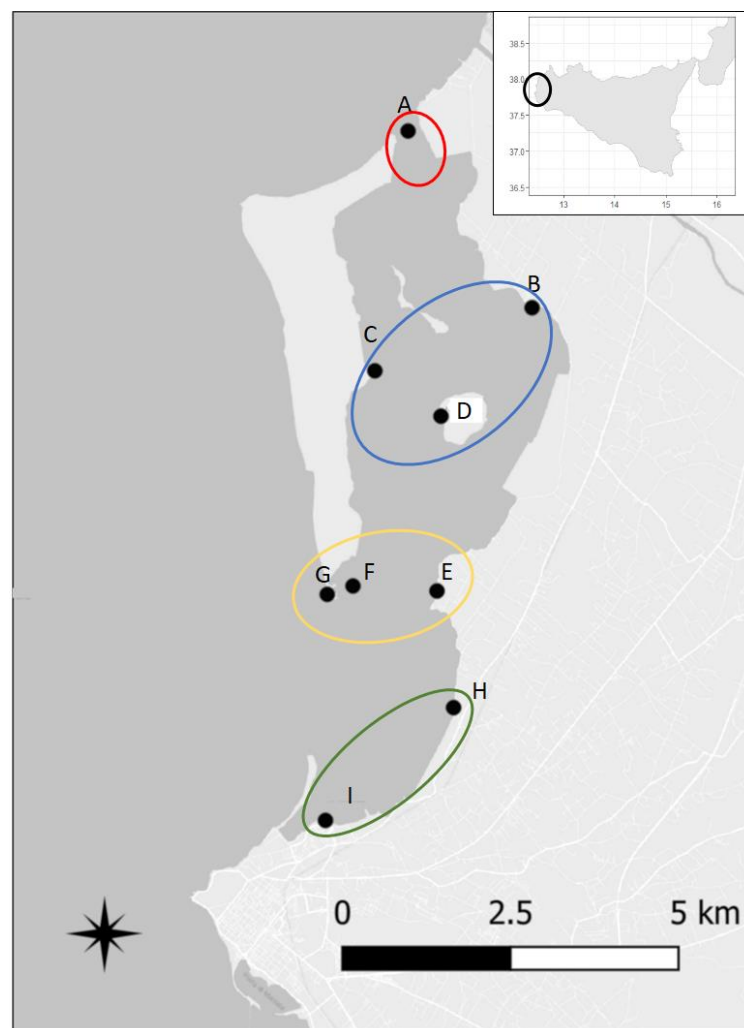
### **5.2.1. Study sites and sample collection**

The Stagnone di Marsala was chosen as the study area (see Chapter 4 for a full description) to investigate the trophic habits of the population of *Callinectes sapidus* in this recently colonized coastal marine area. Following the same experimental design presented in Chapter 4, the study area was divided in four zones with a different level of confinement, within which different sites were selected based on the habitat heterogeneity. One site (site A) was selected in the most confined zone, North zone (37.901727° E; 12.458296° N); three sites were selected in the Center-North zone (37.871882° E; 12.465895° N, where site B is located along the coast, and sites C and D are located in the central part of the zone); three sites were selected in the Center-South zone (37.845351° E; 12.457651° N, where site E is located along the coast, and sites F and G are located in the central part of the zone) and lastly two sites (sites H and I) were selected in the less confined zone, South zone (37.820750° E; 12.454201° N) (Fig. 1).

Sampling activities were performed from Summer 2021 to Winter 2023. Every month throughout the sampling period, four baited traps (60x30 cm, placed 70 m distance each other) were deployed between 6.00 a.m. and 9.00 a.m. and retrieved the day after between 5.00 p.m. and 10.00 p.m. Baited traps were useful to collect the blue crab and its benthic preys including gastropods, decapods and benthic fish, as well as the other co-occurring brachyuran species. In addition to baited traps, in each site of the Center-North and Center-South zones, a 50 m long gillnet with a mesh size of 30 mm was deployed overnight and withdrawn the following morning, to increase the chance to collect the blue crab.

Sources of organic matter, such as macrophytes and sediment (to be used as proxy for sedimentary organic matter, SOM), primary consumers (to be used as trophic baseline) and further small prey fish, were collected once per season in each site, in triplicate. Macrophytes (seagrasses and macroalgae) were gathered by hand using 20x20 cm quadrat, surficial sediment (2 cm) was collected using 3 cm Ø corers, while small fish and primary consumers were collected using a small beach seine net (1.5 x 4 m, mesh size 2 mm).

All the samples collected were stored in individual plastic bags and transferred to the laboratory in refrigerated containers. Fish were euthanized with “*Colombo Morenicol Sedation*”.



**Figure 1.** Sampling sites grouped by zones in the study area of the Stagnone di Marsala. The red circle indicates the North zone with site A, the blue circle indicates the Center-North zone with location of the sites B, C and D, the yellow circle indicates the Center-South zone with sites E, F and G and the green circle indicates the South zone with sites H and I.

### 5.2.2. Sample processing and laboratory analysis

Once in laboratory, *Callinectes sapidus* was identified based on the presence of two obtuse and large teeth on the front between the inner orbital teeth (Millikin & Williams, 1984), while the other brachyuran species were identified using Falciai & Minervi (1992). Carapace width (CW) of each brachyuran specimen was measured to the nearest mm using a caliper and sex was determined by inspecting the abdomen shape. The other invertebrate species, fish species, macroalgae and seagrasses were identified at the possible lowest taxonomic level using proper identification keys (Riedl, 1991).

For the purpose of the stable isotope analysis, the claw muscles from brachyuran individuals per each factor (season, zone, sex and size), the foot from gastropods, the entire body from shrimps and primary consumers (i.e. Isopoda), and a portion of the dorsal muscle from fish were collected with a scalpel. All the samples were opportunely cleaned with distilled water, dried at 60°C for approximately 48h and then powdered with mortar and pestle or micro mill. Similarly, sediments samples were homogenized, oven-dried and ground to fine powder.

A portion of powdered sample ( $0.8 \pm 0.1$  SD mg) destined to isotopic analyses was then wrapped in tin capsules and analyzed using an isotope ratio mass spectrometer (Thermo-Electron Delta Plus XP) coupled to an elemental analyser (Thermo-Electron Flash EA1112). Prior to analysis, part of the samples containing any carbonate component were acidified (HCl 1M) in order to remove carbonate that may affect the carbon isotopic signature determination. Carbon and nitrogen signatures were expressed with  $\delta$  notation in part per thousand related to international standards (PeeDee Belemnite for carbon and atmospheric N<sub>2</sub> for nitrogen), calculate with the formula:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$$

where X is either C or N and R is  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$ . Analytical precision based on the standard deviation of replicates of internal standards (International Atomic Energy Agency IAEA-CH-6 for  $\delta^{13}\text{C}$  and IAEA-NO-3 for  $\delta^{15}\text{N}$ ) was 0.2‰ for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

### 5.2.3. Data analysis

#### 5.2.3.1. *Callinectes sapidus*

*Callinectes sapidus* specimens were grouped in three size classes based on carapace width (CW): small (CW < 70 mm), medium (CW 70-100 mm) and large (CW > 100 mm). To test for overall differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of the blue crab among position (central and coastal), sex and size classes, a permutational analysis of variance (PERMANOVA) and relative pair-wise test was used (three fixed factor design: Position, Sex and Size). PERMANOVA was run on bivariate  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  data, on untransformed data resembled by Euclidean distance, using the software PRIMER (Anderson *et al.*, 2008).

In order to assess the isotopic niche features of *C. sapidus* across the three size classes,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  data were used to estimate the standard ellipse area (SEAc, corrected for small sample size) as proxy for isotopic niche width and the Layman metrics as descriptors of the isotopic niche (Layman *et al.*, 2007). In particular, the following Layman metrics were estimated:

- i.  $\delta^{13}\text{C}$  range (CR). It is the distance between individuals with the most enriched and the most depleted  $\delta^{13}\text{C}$  value. It provides information about the diversification of basal resources used.
- ii.  $\delta^{15}\text{N}$  range (NR). It is the distance between individuals with the most enriched and the most depleted  $\delta^{15}\text{N}$  value. This value represents the trophic length of the population.
- iii. Mean distance to centroids (CD). Provides additional information regarding niche width and species spacing. It is a measure of the average degree of trophic diversity in a population.
- iv. Mean nearest neighbour distance (NND). Provides a measure of the density and clustering of individuals within the population. Population with a large proportion of individuals characterized by similar trophic ecology will show a smaller value of NND than those where individuals exhibit more divergent trophic niche.
- v. Standard deviation of the nearest neighbour (SDNND). Returns a measure of evenness of spatial density and packing in the bi-plot space.



Standard ellipse areas and Layman metrics were calculated using the SIBER package (Jackson *et al.*, 2011) in R (R v. 4.0.2; R Core Team, 2020).

Furthermore, Bayesian mixing models (Parnell *et al.*, 2010) were used to estimate the trophic pathways supporting the invasive species *Callinectes sapidus* in the four study zones of the Stagnone di Marsala, across the seasons. The mixing model were performed on individuals captured along the coast, separately for size. Since the target species has an opportunistic feeding behavior, sources included in the model were: fishes (*Atherina boyeri* Risso, 1810; *Aphanius fasciatus* Valenciennes, 1821; *Gobius niger* Linneaus, 1758), gastropods (*Hexaplex trunculus* Linneaus, 1758), decapods (*Liocarcinus depurator* Linneaus, 1758; *Palaemon adspersus* Rathke, 1836), macroalgae (*Laurencia* sp. and *Caulerpa prolifera* (Forsskål) Lamouroux, 1809.), seagrasses (*Cymodocea nodosa* (Ucria) Asherson, 1870) and SOM. Trophic enrichment factor (TEF) used in the model were  $0.4 \pm 1\text{‰}$  for  $\delta^{13}\text{C}$  and  $3.4 \pm 1.3\text{‰}$  for  $\delta^{15}\text{N}$ , according to Post (2002). Before running each mixing model, the  $\delta^{13}\text{C}$ - $\delta^{15}\text{N}$  bi-plot of prey and target species was inspected to check that the consumer's isotope data fell into the polygon of the sources. The mixing models were run using the SIMMR package (Parnell *et al.*, 2010) in R (R v. 4.0.2; R Core Team, 2020).

#### 5.2.3.2. Comparison between *Callinectes sapidus* and the autochthonous brachyurans

Permutational analysis of variance (PERMANOVA) and pair-wise test were used to test for differences in isotopic data obtained for the three brachyuran species, *Callinectes sapidus*, *Eriphia verrucosa* and *Carcinus aestuarii* in the different sampling zones and seasons (three fixed factor design: Season, Zone, Species). PERMANOVA was run on bivariate  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  data, on untransformed data resembled using Euclidean distance, using the software PRIMER (Anderson *et al.*, 2008).

Trophic position (TP) of the different brachyuran species was calculated using the following equation from Post (2002):

$$\text{TP} = [\delta^{15}\text{N}_c - \delta^{15}\text{N}_b] / \Delta_n + \lambda$$

where  $\delta^{15}\text{N}_c$  and  $\delta^{15}\text{N}_b$  are the isotopic signatures of the consumer and the baseline respectively,  $\Delta_n$  is the trophic enrichment factor and  $\lambda$  is the trophic position of the species used as baseline. Mean  $\delta^{15}\text{N}$  value of isopods sampled in each zone and season were used as specific baseline, having trophic position, hence  $\lambda=2$ .  $\Delta_n$  was  $3.4\text{‰}$ , in accordance with Post (2002).

To assess the trophic position and the isotopic niche features of the three brachyuran species,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  data of large (adult) individuals were used to estimate the above-mentioned TP, SEAc and Layman metrics.

### 5.3. Results

#### 5.3.1. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ spatial and temporal variations of brachyurans and sources of organic matter

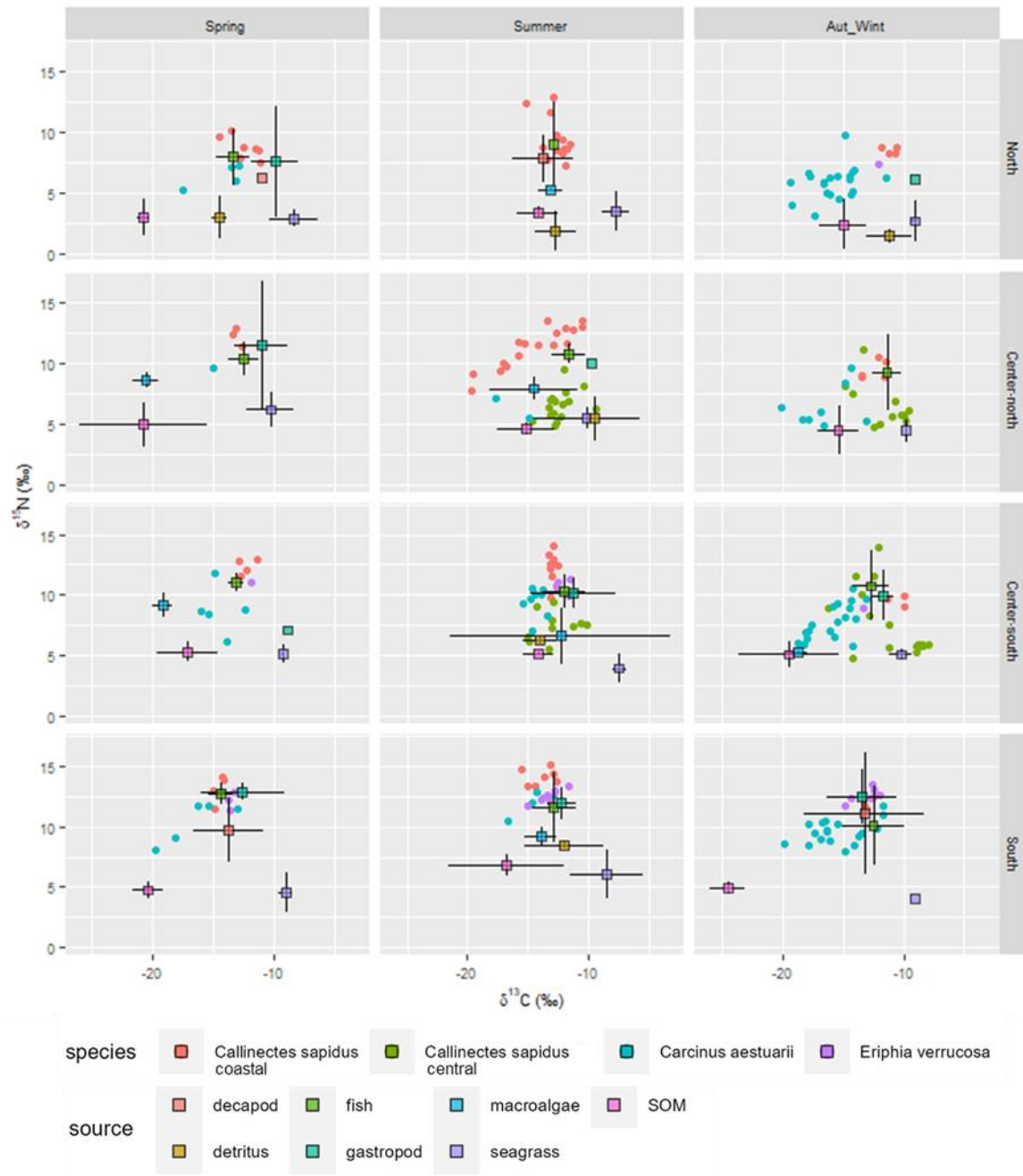
Overall, the three brachyuran species showed significant different isotopic values for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  (Fig. 1). In particular, *Callinectes sapidus* showed overall the most enriched values with ranges from -19.7 to -8‰ for  $\delta^{13}\text{C}$  and from 4.7 to 15.1 for  $\delta^{15}\text{N}$ ‰. Surprisingly, in two zones of the study area, Center-North and Center-South, *C. sapidus* showed marked intraspecific differences in isotopic values between the specimens collected along the coast (named *Callinectes sapidus* coastal) and the ones collected in central sites (named *Callinectes sapidus* central). While coastal specimens were more  $^{13}\text{C}$ -depleted and  $^{15}\text{N}$ -enriched, central specimens were markedly more  $^{13}\text{C}$ -enriched and  $^{15}\text{N}$ -depleted (Fig. 1). Hence, given the possible ecological significance it might have, I focused on such differences by considering them separately in all statistical analyses, as a new level of the factor species.

*Carcinus aestuarii* had the most depleted values with ranges from -20.1 to -11.5‰ for  $\delta^{13}\text{C}$  and from 3.1 to 12.9‰ for  $\delta^{15}\text{N}$ . *Eriphia verrucosa* had a  $\delta^{13}\text{C}$  range (from -15.1 to -11.5‰) more similar to *C. aestuarii* and a  $\delta^{15}\text{N}$  range (from 7.4 to 13.5‰) closer to *C. sapidus*.

Regarding food sources, decapods showed a mean  $\delta^{13}\text{C}$  value of  $-13 \pm 1.4$ ‰ and  $\delta^{15}\text{N}$  values of  $8.1 \pm 1.4$ ‰, with the most enriched  $\delta^{13}\text{C}$  and the most depleted  $\delta^{15}\text{N}$  in North zone in spring. Fish showed a mean  $\delta^{13}\text{C}$  value of  $12.7 \pm 1.1$ ‰ and  $\delta^{15}\text{N}$  value of  $10.6 \pm 1.5$ ‰. Gastropods showed a mean  $\delta^{13}\text{C}$  of  $-11.5 \pm 1.8$ ‰ and a mean  $\delta^{15}\text{N}$  of  $10.5 \pm 2.4$ ‰. Different fish species and gastropods showed the most depleted  $\delta^{13}\text{C}$  and enriched  $\delta^{15}\text{N}$  value in Southern zones of the basin across seasons. Detritus presented a mean  $\delta^{13}\text{C}$  of  $-12.40 \pm 1.8$ ‰ and a mean  $\delta^{15}\text{N}$  of  $4.1 \pm 2.6$ ‰. Macroalgae showed a mean  $\delta^{13}\text{C}$  of  $-15.2 \pm 4.0$ ‰ and a mean  $\delta^{15}\text{N}$  of  $7.3 \pm 1.6$ ‰, while seagrasses had a mean  $\delta^{13}\text{C}$  of  $-9.1 \pm 1.4$ ‰ and a mean  $\delta^{15}\text{N}$  of  $4.7 \pm 1.3$ ‰. SOM showed a mean  $\delta^{13}\text{C}$  of  $-17.7 \pm 3.4$ ‰ and a mean  $\delta^{15}\text{N}$  of  $4.8 \pm 1.4$ ‰.

PERMANOVA carried out on isotopic values of  $\delta^{15}\text{N}$  of target brachyuran species showed no significant differences for the interaction among factors species, season and zone, but

revealed significant differences for the interaction among species and season and among species, zone and season (Tab. 1).



**Figure 1.** Stable Isotope Analysis (SIA).  $\delta^{13}\text{C}$  vs  $\delta^{15}\text{N}$  of the three brachyurans species: *C. sapidus* coastal, *C. sapidus* central, *C. aestuarii* and *E. verrucosa*. Seasonal mean ( $\pm$ s.d) of organic matter sampled in each zone.

**Table 1.** Results of PERMANOVA test performed on bivariate  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of the brachyuran species in each sampling season and zone.

Main test	Source of variation	df	MS	Pseudo-F	Perms	P(perm)
	Season	3	11.4	2.2	<b>0.039</b>	999
	Zone	3	53.7	10.4	<b>0.001</b>	998
	Species	3	159.2	30.9	<b>0.001</b>	999
	Season x Zone	9	5.2	1.0	0.456	999
	Season x Species	6	12.1	2.4	<b>0.011</b>	999
	Zone x Species	6	4.0	0.8	0.64	998
	Season x Zone x Species	9	3.3	0.6	0.835	999
Pair-wise tests	Between seasons within species			t	Perms	P(perm)
<i>C. sapidus</i> coastal	Spring vs Autumn			4.8	0.001	998
	Spring vs Winter			3.5	0.001	999
	Summer vs Autumn			3.4	0.001	996
	Summer vs Winter			2.2	0.024	998
<i>C. sapidus</i> central	Summer vs Autumn			1.7	0.045	999
<i>C. aestuarii</i>	Spring vs Winter			2.3	0.012	995
	Summer vs Winter			2.9	0.002	998
	Autumn vs Winter			2.1	0.024	998
Pair-wise tests	Between species within seasons			t	Perms	P(perm)
Spring	<i>C. sapidus</i> coastal vs <i>C. aestuarii</i>			4.1	0.001	998
	<i>C. aestuarii</i> vs <i>E. verrucosa</i>			2.2	0.029	997
Summer	<i>C. sapidus</i> coastal vs <i>C. aestuarii</i>			4.3	0.001	998
	<i>C. sapidus</i> coastal vs <i>E. verrucosa</i>			2.0	0.042	998
	<i>C. sapidus</i> coastal vs <i>C. sapidus</i> central			7.7	0.001	999
	<i>C. aestuarii</i> vs <i>E. verrucosa</i>			3.5	0.001	999
	<i>C. aestuarii</i> vs <i>C. sapidus</i> central			3.6	0.002	998
	<i>E. verrucosa</i> vs <i>C. sapidus</i> central			3.5	0.001	999
Autumn	<i>C. sapidus</i> coastal vs <i>C. aestuarii</i>			6.1	0.001	999
	<i>C. sapidus</i> coastal vs <i>E. verrucosa</i>			2.1	0.043	999
	<i>C. aestuarii</i> vs <i>E. verrucosa</i>			2.8	0.007	998
	<i>C. aestuarii</i> vs <i>C. sapidus</i> central			4.7	0.001	998
Winter	<i>C. sapidus</i> coastal vs <i>C. aestuarii</i>			4.0377	0.001	998
Pair-wise tests	Between zones			t	Perms	P(perm)
	North vs Center-North			3.2	0.001	999
	North vs Center-South			2.0	0.033	998
	North vs South			5.7	0.001	997
	Center-North vs South			3.6	0.001	999
	Center-south vs South			3.6	0.001	999

### 5.3.2. *Callinectes sapidus* trophic features

PERMANOVA carried out on isotopic values of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of coastal and central *Callinectes sapidus*, by sex and size showed significant differences between coastal and central groups. No significant differences were detected regarding sex or the interaction with

other factors. On the contrary, significant interaction differences was found between position and size, with large size class being significantly different from small and medium size for coastal individuals (Tab. 2).

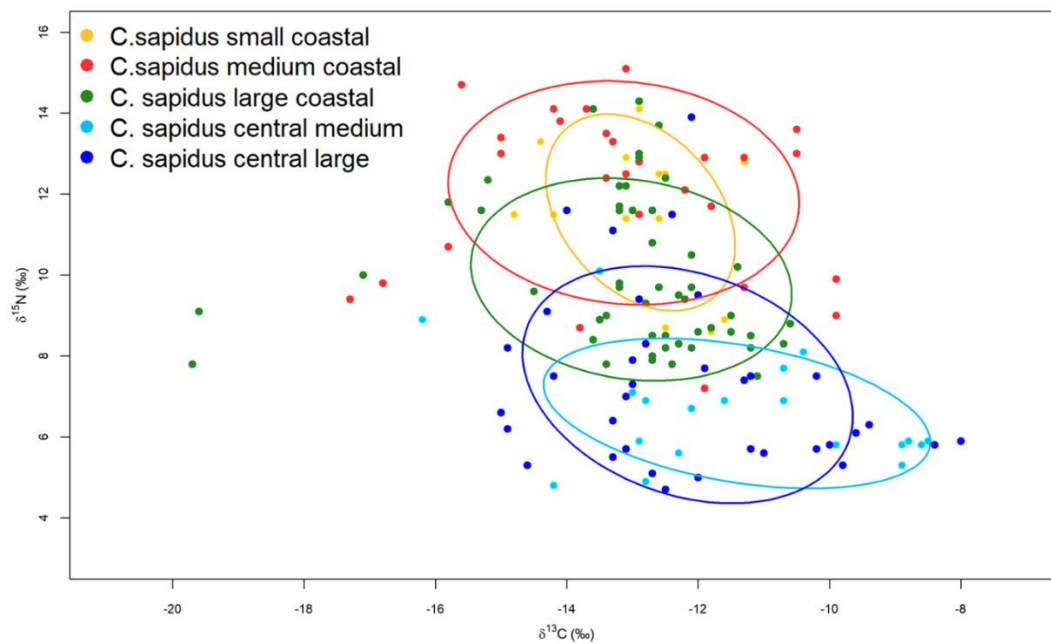
**Table 2.** Results of PERMANOVA test performed on  $\delta^{13}\text{C}$ - $\delta^{15}\text{N}$  values of *Callinectes sapidus* considering position (coastal and central), sex and size.

Main test	Source of variation	df	MS	Pseudo-F	Perms	P(perm)
	Position	1	499.17	72.85	998	<b>0.001</b>
	Sex	1	6.8	0.99	998	0.368
	Size	2	8.27	1.20	999	0.299
	Position x Sex	1	5.59	0.81	999	0.479
	Position x Size	1	54.26	7.92	999	<b>0.001</b>
	Sex x Size	2	3.69	0.53	998	0.702
	Position x Sex x Size	1	16.34	2.38	999	0.075
Pair-wise test	Between size within coastal			t	Perms	P(perm)
	Small vs large			2.03	999	<b>0.028</b>
	Small vs medium			0.58	998	0.70
	Large vs medium			3.21	999	<b>0.002</b>
Pair-wise test	Between size within central			t	Perms	P(perm)
	Large vs medium			1.12	999	0.259
Pair-wise test	Between Position within medium			t	Perms	P(perm)
	Coastal vs Central			4.86	999	<b>0.001</b>
Pair-wise test	Between Position within large			t	Perms	P(perm)
	Coastal vs Central			6.76	999	<b>0.001</b>

Standard ellipse areas (SEAc) estimated overall for the three size classes of coastal and central *C. sapidus* showed that isotopic niches were separately distributed in the isotopic space (Fig. 2). While the isotopic niches of the coastal group were displaced in a more  $^{15}\text{N}$ -enriched area of the isotopic space, central niches were shifted towards a more  $^{15}\text{N}$ -depleted area, with partial overlap mainly between niches of large individuals.

Among coastal niches, the SEAc of small individuals was entirely overlapped with the medium size's SEAc, while the SEAc of large individuals was lower along the  $\delta^{15}\text{N}$  axis (Fig. 2). Medium size individuals showed the highest value of CR, while large size individuals reported a highest value of NR among the coastal (Tab. 2).

Central *C. sapidus* showed a wide overlap between SEAc of large and medium individuals. While the large size group showed the highest CR, NR values resulted comparable between the two size classes (Fig. 2; Tab. 2).



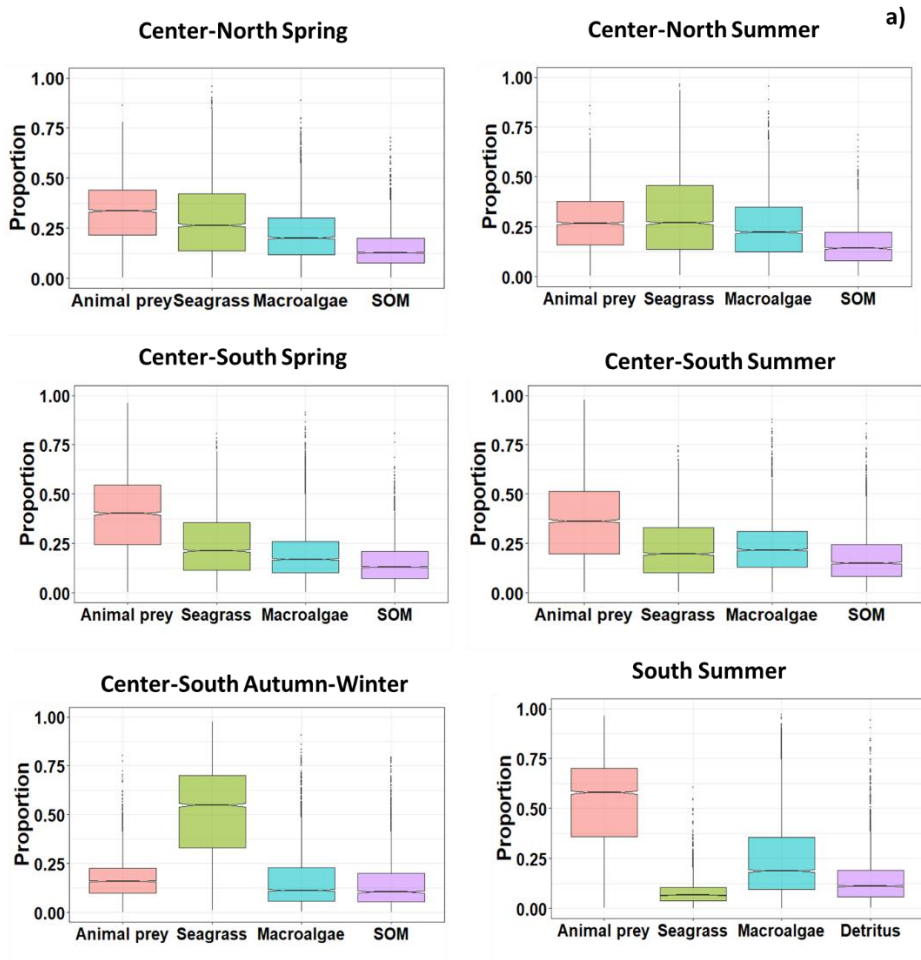
**Figure 2.** Standard Ellipse Areas (SEAc) of coastal and central *Callinectes sapidus* estimated by class size.

**Table 2.** SEAc and Layman metrics of coastal and central *C.sapidus*, *C.sapidus* estimated across size classes.

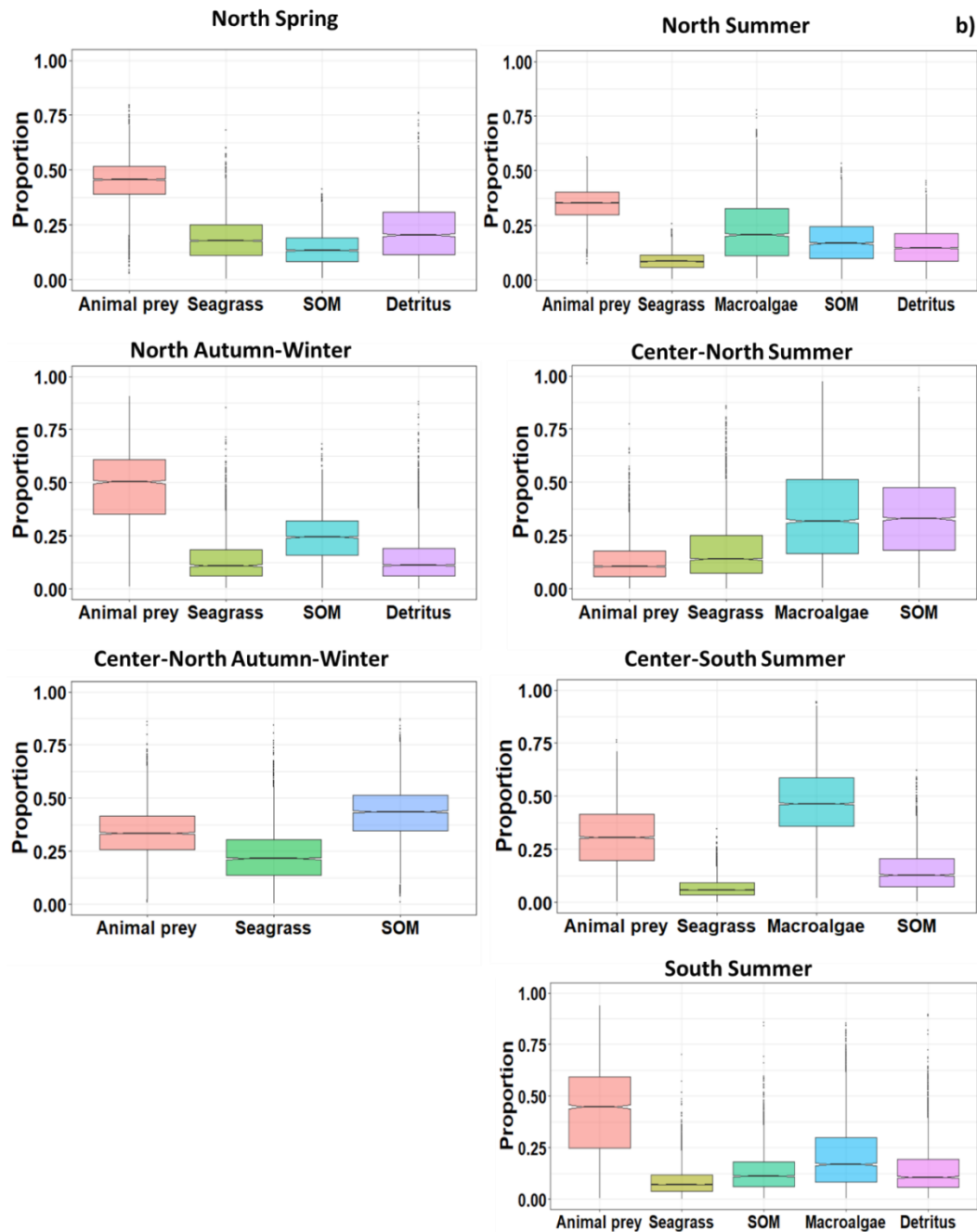
Size	Niche metric	<i>Callinectes sapidus</i> "coastal"	<i>Callinectes sapidus</i> "central"
<b>Small</b> (<70 mm)	<b>SEAc</b>	6.1	-
	<b>NR</b>	3.5	-
	<b>CR</b>	5.5	-
	<b>CD</b>	1.7	-
	<b>NND</b>	0.6	-
	<b>SDNND</b>	0.4	-
<b>Medium</b> (70-100 mm)	<b>SEAc</b>	13.1	9.2
	<b>NR</b>	7.4	7.7
	<b>CR</b>	7.9	5.3
	<b>CD</b>	2.5	2.2
	<b>NND</b>	0.8	0.8
	<b>SDNND</b>	0.6	0.8
<b>Large</b> (>100 mm)	<b>SEAc</b>	10.6	12.5
	<b>NR</b>	9.1	7.0
	<b>CR</b>	6.8	9.2
	<b>CD</b>	2.2	2.5
	<b>NND</b>	0.4	0.6

---

Since large size resulted significantly different from small and medium, but no differences were detected between small and medium size, mixing model were run separately for small and medium size together (small-medium) and large size. Results of the mixing model performed to estimate the contribution of each selected food source to the trophic pathway of small-medium and large coastal *Callinectes sapidus* are showed in Figure 3. Due to low sample size, Autumn and Winter data were grouped. Generally, food sources with higher proportion, across zones and seasons, were animal prey for both size classes. Regarding the small-medium size class, an exception was found in Center-North zone in Summer, where the contribution of all sources was comparable, and in Center-South zone in Autumn-Winter, where seagrass was the most exploited food source (Fig. 3a). Regarding the large size class, exceptions were found at the Center-North zone, where in Autumn-Winter the contribution of sedimentary organic matter (SOM) was higher than animal prey and seagrass, and during Summer where macroalgae and sedimentary organic matter (SOM) were comparable and the most exploited. In Center-South during Summer season macroalgae was the most consumed (Fig. 3b).







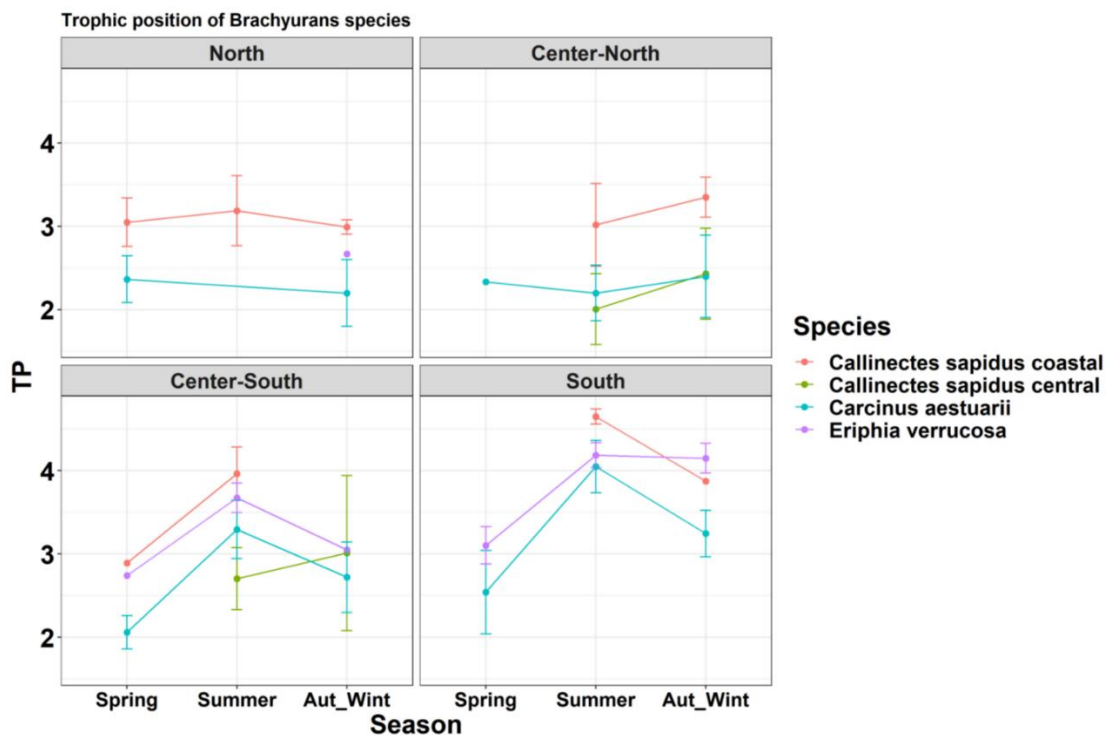
**Figure 3.** Proportional contribution of organic matter sources (animal prey, seagrass, macroalgae and SOM) to the trophic pathway of small-medium (a) and large (b) coastal *Callinectes sapidus*, across seasons and zones. Animal prey (*Hexaplex trunculus*, *Liocarcinus depurator*, *Atherina boyeri*, *Aphanius fasciatus*, *Gobius niger*, *Paleomon adspersus*); seagrass (*Cymodocea nodosa*), macroalgae (*Caulerpa prolifera*, *Laurencia* sp.) SOM (sedimentary organic matter).

### 5.3.3. Comparison of trophic features between *Callinectes sapidus* and the autochthonous brachyurans

Since all individuals of the two autochthonous brachyuran species were identified as adult stage, being their mean carapace width (respectively  $40.37 \pm 7.02$  mm for *Carcinus aestuarii*

and  $60.06 \pm 9.19$  mm for *Eriphia verrucosa*), only large class size of *Callinectes sapidus* was considered for the comparisons in this section.

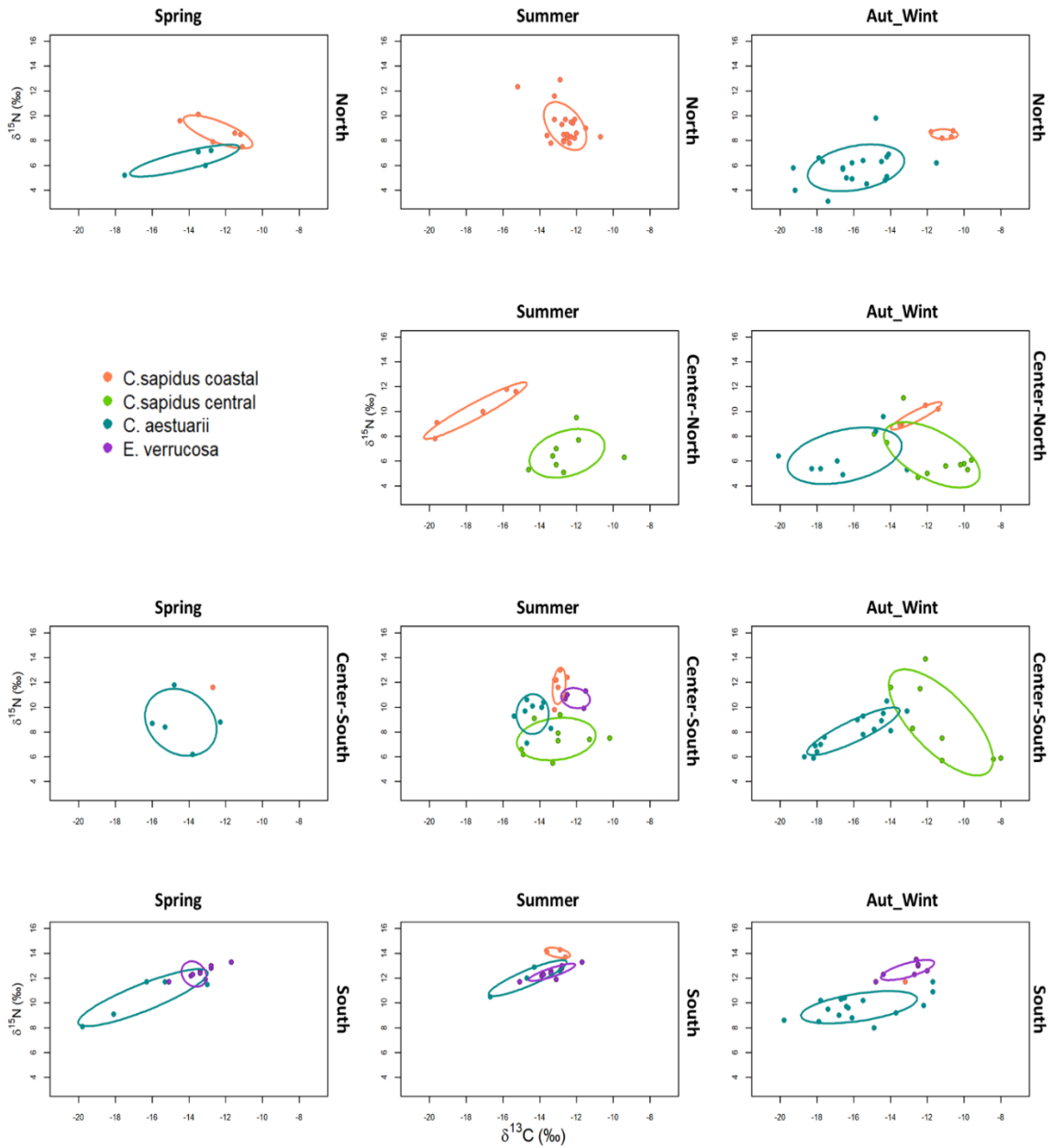
Differences in the trophic position (TP) occurred between large size of coastal and central *C. sapidus*. TP of all brachyuran species shifted during the year and among zones with a similar trend, being quite stable across seasons at North and Center-North zones, and showing a peak in summer at Center-South and South zones (Fig. 4). Despite this shift within the species, they maintain an almost constant relationship: *C. sapidus* coastal > *E. verrucosa* > *C. aestuarii* > *C. sapidus* central. The only exceptions were during during autumn-winter season, where in Center-North and Center-South zones *C. sapidus* central showed a higher position of *C. aestuarii* and in South zone where *C. sapidus* coastal showed a lower trophic position than *E. verrucosa* (Fig.4).



**Figure 4.** TP value of the three brachyurans species in the Stagnone di Marsala. The trophic position was calculated using Trophic Enrichment Factor (TEF): Post 2002 Aut-Wint refers to Autumn- Winter season.

Isotopic niche of the three brachyurans species resulted differently distributed in space and time and well separated to each other with no remarkable overlap between the invasive and the autochthonous species (Fig. 5). Regarding the width of the isotopic niche, *C. sapidus*

showed different patterns comparing coastal and central groups. Coastal *C. sapidus* reported a lower value of SEAc than central and was located in a more  $^{15}\text{N}$ -enriched area along the  $\delta^{15}\text{N}$  axis. SEAc of *C. sapidus* coastal and *C. aestuarii* varied across zones and seasons, while SEAc of *E. verrucosa* and central *C. sapidus* did not show large variation (Fig. 5; Tab. 3). Considering the results of Layman metrics, *C. sapidus* central and *C. aestuarii* showed the highest values, while coastal *C. sapidus* presented the lowest value of all Lyman metrics and SEAc. In particular, *C. sapidus* central presented the widest SEAc and the broadest NR and CD during autumn-winter season and *C. aestuarii* showed the highest values of CR, NDD and SDNDD during spring and autumn-winter seasons (Tab. 3).



**Figure 5.** Standard Ellipse Areas (SEAc) of large coastal and central *C. sapidus*, *C. aestuarii* and *E. verrucosa* estimated by zone and season.

**Table 3.** SEAc and Layman metrics of large coastal and central *C.sapidus*, *C.aestuarii* and *E. verrucosa* calculated in every season in each zone.

Zone	Niche metric	<i>Callinectes sapidus</i> "coastal"			<i>Carcinus aestuarii</i>			<i>Eriphia verrucosa</i>			<i>Callinectes sapidus</i> "central"	
		Spring	Summer	Autumn-Winter	Spring	Summer	Autumn-Winter	Spring	Summer	Autumn-Winter	Summer	Autumn-Winter
North	SEAc	3.6	3.6	0.8	5.7		8.6					
	NR	2.6	5.1	0.6	2.0		6.7					
	CR	3.4	4.5	1.2	4.7		7.8					
	CD	1.5	1.3	0.5	1.8		2.0					
	NND	0.9	0.5	0.6	1.8		0.8					
	SDNND	0.4	0.5	0.1	1.8		0.9					
Center-North	SEAc		4.5	1.7			13.2				7.6	10.8
	NR		4.0	1.6			4.7				4.4	6.4
	CR		4.4	2.1			7.0				5.2	5.3
	CD		2.2	1.1			2.4				1.7	2.4
	NND		1.2	0.5			1.4				1.3	0.9
	SDNND		0.7	0.4			1.0				0.8	0.9
Center-South	SEAc		1.0	2.3	11.8	2.8	4.4		1.6		7.1	17.29
	NR		3.2	0.9	5.6	3.5	4.6		1.4		3.9	8.2
	CR		0.7	1.4	3.7	2.0	5.6		1.1		4.8	6.0
	CD		0.9	0.8	2.1	1.1	2.1		0.7		1.7	3.3
	NND		0.6	1.1	2.1	0.8	0.6		0.7		1.0	1.5
	SDNND		0.4	0.3	1.2	0.6	0.3		0.5		0.5	0.7
South	SEAc		0.9		9.3	3.7	6.6	1.8	0.9	1.7		
	NR		0.6		3.6	2.4	3.7	1.6	1.6	1.8		
	CR		1.0		6.8	3.8	8.1	1.2	3.4	2.8		
	CD		0.5		2.6	1.4	2.1	0.7	0.8	1.0		
	NND		0.7		1.7	1.5	0.7	0.9	0.5	0.5		
	SDNND		0.0		0.6	0.7	0.4	0.1	0.5	0.3		

## 5.4. Discussion

Since invasive species are considered as a threat worldwide (Levine, 2008; Chan *et al.*, 2019; Molnar *et al.*, 2008), understanding their feeding behaviour and strategies is the first step to know how these species can affect the recipient habitat and community (Di Muri *et al.*, 2022). This study purposed to investigate the trophic habits of the invasive Atlantic blue crab *Callinectes sapidus*. To achieve this aim, the stable isotope approach was used to investigate the isotopic niche and the trophic pathway of the organic matter that support this species in the invaded marine coastal area of the Stagnone di Marsala. In particular, the analysis of the isotopic niche was used to compare *C. sapidus*' niche with two autochthonous brachyuran species co-occurring in the same study area, *Carcinus aestuarii* and *Eriphia verrucosa*, in order to investigate trophic dynamics occurring across the basin throughout the seasons.

The  $\delta^{13}\text{C}$ - $\delta^{15}\text{N}$  bi-plot represents the isotopic space where species individuals are distributed, and was used to quantify the isotopic niche width as useful descriptor of the trophic niche, bearing in mind that isotopic and trophic niche cannot be considered the same (Jackson *et al.*, 2011). Overall, the isotopic niche descriptors showed marked differences in the trophic niche of the three brachyurans species compared. The different width of the niche of the three species, suggests a different degree of omnivory.

Noteworthy, central *C. sapidus* showed a different niche position compared to coastal individuals, located towards a more  $^{15}\text{N}$ -depleted area of the isotopic space, suggesting that different trophic pathways sustain the two groups of individuals, despite the relatively low distances (about 2 km). In fact, whilst the blue crab captured along the coast seemed to rely on higher trophic level sources, central *C. sapidus* seemed to rely on more basal sources such as detritus and macrophytes. Niche separation between coastal and central *C. sapidus* appeared to be related to the zone of the basin rather than sex or size of the individuals. This clear separation of the niches between *C. sapidus* sampled at different areas of the basin implies that, although the species is capable to cover large distances, for example for reproduction purposes (Carr *et al.*, 2004; Turner *et al.*, 2003), arguably it also displaces over a limited area for foraging (Prado *et al.*, 2022). This result confirms the high opportunistic behavior of this species, which adapts to feed on what is locally available rather than move in search of specific food resources.

According to literature sources reporting on the variability of *C. sapidus* diet (*e.g.*, Mancinelli, *et al.*, 2017a; McClintock *et al.*, 1991; Laughlin, 1982; Rosas *et al.*, 1994),

within the Stagnone di Marsala the Atlantic blue crab showed opportunistic trophic habits. Indeed, on one side the large range of carbon (CR) suggested a high variability in the food resources consumed, and on the other side the wide range of nitrogen (NR) meant that these food resources belonged to different trophic levels. *C. sapidus* central showed the highest level of trophic diversity (CD) within species. In particular, during the autumn-winter period, central *C. sapidus* was revealed to exploit a wide variety of food sources. Hypothetically, this variation in trophic diversity between summer and autumn-winter season could be due to a decrease in animal prey availability during the colder period and the ability to adapt to different resources, placed at a lower trophic level, such as plant detritus or macrophytes. Trophic position of *C. sapidus* captured along the coast denoted a fully carnivore diet, according to Mancinelli *et al.* (2016), but conversely to what they found in Parila Lagon, here *C. aestuarii* had a lower trophic position, much more similar to individuals captured in the central sites. Furthermore, *C. sapidus* central showed a trophic position consistent with those found in native areas (Abreu *et al.*, 2006; Rooker *et al.*, 2006; Akin & Winemiller, 2008), while *C. sapidus* coastal had a trophic position closer to those showed in the Mediterranean Sea (Prado *et al.*, 2022; Carrozzo *et al.*, 2014; Mancinelli *et al.*, 2016; Aslan & Polito 2021; Di Muri *et al.*, 2022). In accordance to the finding that *C. sapidus* coastal showed higher trophic position than the central individuals, the results of the mixing model overall suggested a major consumption of animal prey for small-medium and large size individuals. Nevertheless, depending on the season and area considered, there was a variation in the food consumption: in the Center-North and Center-South zones, the blue crabs fed mainly on macrophytes or sedimentary organic matter, depending on the availability of food sources. In autumn-winter season, this variation could be also due to a decrease in metabolic activities. Indeed, when temperature declines, the blue crab decreases its metabolic rates, resulting in a less aggressive behavior and reduced needs for high energy food (Hines 2007). Although, individuals of different sizes (large and small-medium) might exploit different prey, even if mainly animal sources, it should be taken into account that the size distribution was not homogeneous neither across areas nor across seasons. In fact, where small-medium sized specimens were caught, large ones were not always present. Thus, the differences in isotopic signature could be more linked to the differences in sources availability rather than ontogenetic issue. According to the literature, within the Marsala Stagnone, the blue crab engages in opportunistic behavior, proving to consume different food sources such as sedimentary organic matter or macrophytes. (McClintock *et al.*, 1991; Laughlin, 1982; Stoner & Buchanan, 1990; Rosas, *et al.*, 1994).

*C. aestuarii* inhabits Mediterranean lagoons and estuarine shallow waters and it is distributed from the Levantine basin of the Mediterranean Sea to the Suez Canal area, in the Black Sea and along the coasts of Japan (Cilenti *et al.*, 2014; Mori, 1990; Kocataş & Katağan, 2003). It is an omnivorous species (Mancinelli *et al.*, 2016; Sanchez-Salazar *et al.*, 1987; Bergmann *et al.*, 2002) and accordingly, the wide isotopic niche (large standard ellipse area and a high value of mean nearest neighbour distance, NND) found in Center-North and Center-South zones, suggest a large differentiation of food source consumed by the individuals of this species. Hence, both *C. sapidus* and *C. aestuarii* confirmed the ability to consume either macrophytes or animal food sources depending on their availability, that in the study area varied remarkably on a seasonal scale.

On the contrary, *E. verrucosa* is an intertidal crab occurring in the Mediterranean Sea and in the North-Eastern Atlantic coasts, which feeds mainly on molluscs and sea urchins (Flores & Paula, 2001; Pérez-Miguel, *et al.*, 2017). Correspondingly, based on the trophic position estimated within the Stagnone di Marsala, *E. verrucosa* has been showed to focus mainly on animal preys.

Furthermore, the SEAc (standard ellipse areas) of the three species showed different position with no or minimal overlap across all zones and seasons. The marked separation of SEAc denoted a marked partitioning of the trophic niche, probably linked to the high ability of the species, particularly *C. sapidus* and *C. estuarii* to feed on different ranges of sources, demonstrating a high level of trophic adaptability.

Looking at the trophic position, the results obtained for *C. sapidus* in the Stagnone di Marsala were generally consistent with those of the Mediterranean Sea (Carrozzo *et al.*, 2014; Mancinelli *et al.*, 2016). Indeed, the average trophic position in the Mediterranean Sea ranges between 2.2 to 4.5 (Aslan and Polito, 2021), and in this study was  $3.02 \pm 0.72$ . Different TP among species suggest a difference in resource exploitation. The lower trophic position of *C. sapidus* captured in the central sites of the basin might indicate a higher consumption of detritus, sedimentary organic matter and macrophytes. However, mixing models for this group of crabs were not run due to the lack of basic assumptions to meet the model requirements (crabs laid out of the polygon of the sources).

The spatial and temporal variation in trophic position observed in this study, apparently relates to the diversity in food availability across zones and seasons, confirming the



opportunistic nature of this species. The different position of the niche in the isotopic space, the lack of overlap and the different TP, although not always large among species, suggests the exploitation of different resources. These differences in trophic behaviour may be due to interspecific competition. On one side, interspecific competition may lead to a constraint of realized niche, since when species co-occur they have a limited access to food resources if they are competitors. On the other side, if the co-occurring competitive species are generalist omnivorous, they may exploit different food sources and consequently resulted in wide isotopic niche and a shift in trophic position, thus the niche width could be an effect of competition (Jackson M.C. *et al.*, 2012; Svanbäck & Bolnick, 2007).

## **5.5. Conclusions**

The results confirmed the highly opportunistic behaviour of the *Callinectes sapidus*, even inside a relatively small basin such as the Stagnone di Marsala. The invasive species has proven a high level of omnivory, showing a different feeding behaviour across sampling sites. Moreover, the different feeding behaviours seem to be more related to the spatial and temporal variations that characterize this basin rather than sex or size of the individuals. Furthermore, the three brachyuran species considered for the niche comparison were found to have different isotopic niche and occupy different trophic position, so they appear to use different resources avoiding competition and suggesting a different ecological role of the three species within this coastal marine area. Nevertheless, deeper investigations are necessary to fully understand the trophic niche of this invasive species, for instance considering other food sources and other possible competitors, in order to better understand its ecological role in this habitat and the possible consequence of its presence.

## **6. The Atlantic blue crab *Callinectes sapidus* as a trace metal biomonitor in Mediterranean coastal waters: does omnivory matter?**

### **6.1. Introduction**

The introduction of non-indigenous species (NIS) represents a serious threat to the integrity of marine coastal environments (Anton *et al.*, 2019; see also Solgaard Thomsen, 2020), and significant efforts are currently made to identify introduction pathways, prioritize management actions, and implement appropriate risk assessment procedures (Carboneras *et al.*, 2018; Davidson *et al.*, 2018; Roy *et al.*, 2018).

NIS impacts on the diversity of invaded communities have received considerable attention, as directly determined by biotic interactions or indirectly elicited by habitat modifications or differential tolerance to environmental conditions (Lenz *et al.*, 2011; Bates *et al.*, 2013; Thomsen *et al.*, 2014 and literature cited). Noticeably, NIS can exert far more pervasive effects and become invasive (invasive alien species - IAS - Russell & Blackburn, 2017; after UNEP, 1992), affecting biogeochemical pools and fluxes of energy and materials (including contaminants), and thereby altering the structure and functions of ecosystems (Ehrenfeld, 2010; Corrales *et al.*, 2020).

Natural ecosystems are seldom threatened by single perturbations; indeed, the superimposition of multiple biotic and abiotic stressors is almost the norm, with synergistic and only rarely additive responses from the biota (Côté *et al.*, 2016; Gunderson *et al.*, 2016; Galic *et al.*, 2018). Together with biological invasions, chemical pollution is among the anthropogenic threats (listed as an example in Gelcich *et al.*, 2014) recognized to deeply alter the functionality of coastal marine ecosystems (Johnston *et al.*, 2015). Contaminants such as trace metals can cause impairment of biological functions even at low concentrations, reducing the abundances of the most sensitive species while facilitating opportunistic or tolerant taxa (Johnston & Roberts, 2009; Mayer-Pinto *et al.*, 2010). In addition, persistent pollutants and xenobiotics can accumulate in organisms along the food chain up to top predators, including those of economic interest (Gray, 2002; Romero-Romero *et al.*, 2017; Trevizani *et al.*, 2018). Noticeably, the interaction between IAS and chemical contaminants in aquatic environments has received limited attention, mostly focused on the use of invasive

species for biomonitoring purposes (Annabi *et al.*, 2018; Mancinelli *et al.*, 2018; Tzafriri-Milo *et al.*, 2019; Squadrone *et al.*, 2020) and on the role of contaminants in facilitating invasions (McKenzie *et al.*, 2012; Kenworthy *et al.*, 2018; Osborne & Poynton, 2019). In contrast, with some notable exceptions (*e.g.*, for bivalves: Matthews *et al.*, 2015; Schaller & Planer-Friedrich, 2017) lesser efforts have been made to elucidate the influence and the functional role of IAS in the mobilization of contaminants in aquatic food webs (Flood *et al.*, 2020; see also Schiesari *et al.*, 2018).

Here, we focused on the Atlantic blue crab *Callinectes sapidus* Rathbun 1896, a portunid brachyuran originating from the western Atlantic coasts from New England to Uruguay (Millikin and Williams, 1984). The species was introduced in Europe in 1901 and appeared in 1947 in the Mediterranean Sea (but probably as early as 1935: Nehring, 2011), where it is currently included in the list of the 100 most invasive species (Streftaris and Zenetos, 2006). In the last decade, *C. sapidus* has spread almost ubiquitously in the Black Sea and in the eastern and central Mediterranean Sea, where it is currently expanding in the western and southern sectors of the basin (Cerri *et al.*, 2020; Mancinelli *et al.*, 2017a; 2021) as well as in neighbouring freshwater habitats (Scalici *et al.*, 2022).

The primal aim of the present study was to assess the concentration of trace metals in *C. sapidus* populations in different sectors of the Mediterranean Sea. The blue crab is a commercially valuable shellfish product in native areas, and represents an emergent fishery target species in invaded Mediterranean habitats (Mancinelli *et al.*, 2017c; Kevrekidis & Antoniadou, 2018; Glamuzina *et al.*, 2021). In European waters, information are available only on a reduced set of trace metals for blue crab populations from the Levantine sector of the Mediterranean Sea (*e.g.*, Türkmen *et al.*, 2006; Mutlu *et al.*, 2011; Genc & Yilmaz, 2015; but see Zotti *et al.*, 2016; Salvat-Leal *et al.*, 2020 for recent exceptions). Here, the concentrations of trace metals essential to the metabolic and physiological activities of aquatic invertebrates (*i.e.*, Cu, Fe, Mn, Ni, Sr, Zn) and of non-essential metals (*i.e.*, B, Ba, Cd, Cr, Li, Pb, V) were determined in the muscle of blue crabs from five Mediterranean coastal systems distributed over 23 degrees in longitude from Spain to Italy and Greece.

Blue crabs are benthic feeders (Hines, 2007) and their life history and behaviour place them in direct contact with environmental contaminants via the water column, sediments, and prey (Reichmuth *et al.*, 2010; Parmar *et al.*, 2016). Accordingly, *C. sapidus* has been proposed as a biomonitor species (*i.e.*, a species that, by accumulating pollutants in its tissues, may be

used to assess the bioavailability of the pollutants themselves in the surrounding habitat; (Rainbow, 1995) in estuaries and other transitional habitats (Weinstein *et al.*, 1992; Mutlu *et al.*, 2011; Salvat-Leal *et al.*, 2020). To verify the effectiveness of the species for biomonitoring in Mediterranean waters, we compared its content in trace metals with that of bivalve taxa. Species belonging to this group of molluscs have been used since the early 1970s to monitor the contamination of coastal ecosystems, as they are acknowledged to accumulate inorganic and organic pollutants from their surrounding environments, resulting more informative than other biota or non-biotic environmental matrices (Zuykov *et al.*, 2013; Beyer *et al.*, 2017). The blue crab is an opportunistic omnivore, feeding on both animal and vegetal prey; however, bivalves generally constitute an important component of its diet in native and invaded habitats (Hines, 2007; Mancinelli *et al.*, 2017a; Rogers *et al.*, 2018; Rady *et al.*, 2018). Accordingly, we assumed similar predator-prey accumulation patterns, or even biomagnification phenomena for essential and non-essential metals such as Cd, Ni, Pb, and Zn (Neff, 2002; Kouba *et al.*, 2010; Newman, 2015; Sun *et al.*, 2020). Alternatively, we hypothesized that the high trophic plasticity of the species, in turn the possibility to feed on resources characterized by different metal concentration levels, may represent a confounding factor as already suggested in native habitats (Reichmuth *et al.*, 2009; 2010).

To test these hypotheses, we measured carbon and nitrogen stable isotopes in both *C. sapidus* and bivalves, and using the latter as baseline species we calculated the trophic position of the crab across the five sampling locations included in the investigation. Stable isotope analysis (SIA hereafter) has gained in the last decades a huge popularity for the study of marine and coastal food webs (see Mancinelli & Vizzini, 2015 for a recent review), and allows for robust, regional-scale analyses of species' dietary habits (*e.g.*, Mancinelli, 2012a; Pethybridge *et al.*, 2018; Figgner *et al.*, 2019; Mancini *et al.*, 2021).

## **6.2. Materials and methods**

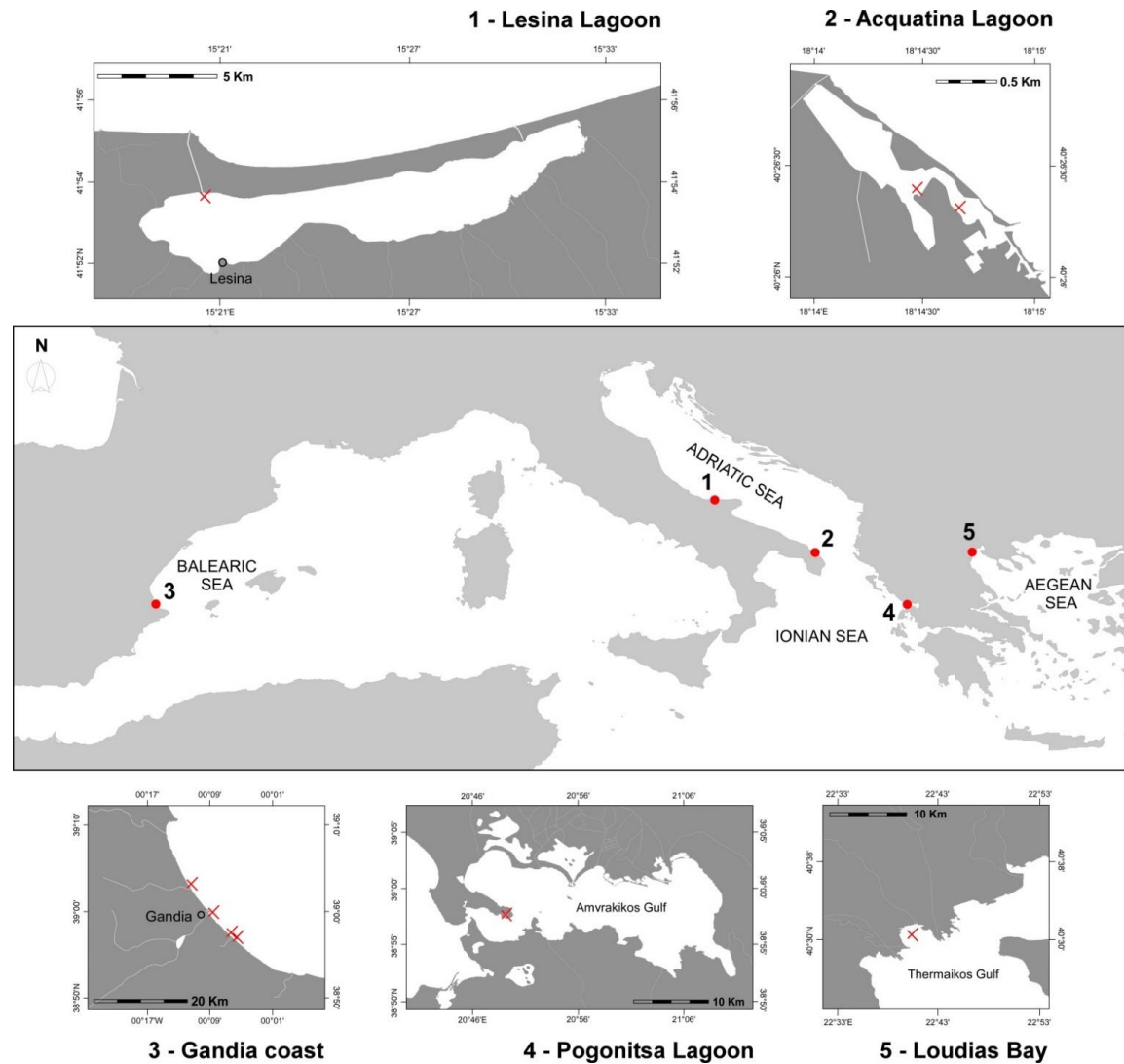
### **6.2.1. Study sites**

The study was carried out in 2016 in five Mediterranean coastal systems located over 23 degrees in longitude from Spain to Italy and Greece and characterized by established *Callinectes sapidus* populations (Fig. 1 and Tab. 1 for additional details on the systems). In brief, in Italy sampling operations were carried out in the Lesina and Acquatina lagoons (SE Italy, Fig. 1). The Lesina Lagoon is located on the northern coast of the Gargano Promontory

(41°52'57.09"N, 15°26'34.71"E); it is approximately 22 km long and 3.5 to 1.5 km wide (52 km<sup>2</sup> total area), with depths ranging between 0.5 and 1.7 m (Spagnoli & Andresini, 2018). Two channels assure continuous water exchanges with the Adriatic Sea, while perennial and intermittent freshwater inputs occur in the south-eastern area of the lagoon. The Acquatina Lagoon is located in the Salento Peninsula (40°26'34.50"N, 18°14'14.16"E); the basin is approximately 1.9 km long with a surface area of 0.45 km<sup>2</sup>; depths range between 0.5 and 1.7 m (1.2 m average depth; Mancinelli, 2012b; Pagliara & Mancinelli, 2018); the basin is connected with the Adriatic Sea by a mouth in the southward area and receives freshwater inputs in the northward area (Fig. 1).

In Spain, samples were collected in a coastal area in the southern sector of the Valencia Gulf close to the city of Gandia extending between the towns of Cullera (0°15'26.29"W, 39°8'6.18"N) and Oliva (0°4'41.29"W, 38°55'36.24"N; Fig. 1). The coastal environment is open to the Balearic Sea, characterized by a number of river mouths, and is generally low and sandy (Gadea *et al.*, 2013; Soler Blanco, 2017).

In Greece, samples were collected in the Pogonitsa Lagoon (NW Greece, 38°57'53.04"N, 20°48'52.15"E) and in Loudias Bay (NE Greece, 40°30'7.36"N, 22°40'51.33"E; Fig. 1). The Pogonitsa Lagoon is located on the Ionian Sea in the south-western sector of the Amvrakikos Gulf. The lagoon has a surface of approximately 0.45 km<sup>2</sup>, an average depth of 1.2 m, with sandy vegetated bottoms, and it is connected to the Amvrakikos Gulf by two channels (Katselis & Koutsikopoulos, 2017). Loudias Bay is situated in the inner Thermaikos Gulf (northern Aegean Sea). The bay has a surface of approximately 15 Km<sup>2</sup> and depths up to 20 m, and receives freshwater inputs from Aliakmon, Axios, and Loudias rivers (Poulos *et al.*, 2000; Catsiki & Florou, 2006).



**Figure 1.** The five coastal habitats included in the study. Crosses in the inserts indicate the sampling locations where *Callinectes sapidus* and bivalve prey (i.e. *Arcuatula senhousia* for Lesina Lagoon and *Mytilus galloprovincialis* for the remaining locations; see text and Table 1 for further details) were collected.

**Table 1.** Summary of information on abiotic characteristics and vegetation cover of the bottoms, pollution sources, and occurrence of *Callinectes sapidus* in the five sampling locations. The month of capture and sampling gears are specified.

Country /location	Abiotic and biotic conditions	Pollution sources	<i>Callinectes sapidus</i>	Sampling month	Sampling method*
Italy Lesina Lagoon 41°52'54.58"N 15°25'39.17"E	Water temperatures and salinities vary seasonally and spatially between 3 and 32°C and 5 and 51 PSU, respectively (Caroppo, Roselli & Di Leo 2018). Bottoms are muddy with extended stands of the phanerogams <i>Nanozostera noltii</i> and <i>Ruppia cirrhosa</i> ; the macroalgae <i>Valonia aegagropila</i> and <i>Gracilaria gracilis</i> are locally abundant (Mancinelli & Rossi, 2001; Specchiulli <i>et al.</i> 2016).	Chemical pollutants and nutrient inputs of agricultural and urban origin; negligible trace metals contamination with the exception of arsenic (Spagnoli & Andresini 2018 and literature cited).	First record in 2008 (Florio <i>et al.</i> , 2008); since then, the population abundance and distribution have increased considerably. and the neighbouring Varano Lagoon has been recently invaded (Cilenti <i>et al.</i> , 2015).	July	Gill nets
Italy Acquatina Lagoon 40°26'32.18"N 18°14'15.83"E	Water temperatures and salinities vary seasonally between 9 and 25°C and 20 and 38 PSU respectively (Mancinelli <i>et al.</i> 2017). Muddy-sandy bottoms with dense stands of the phanerogam <i>Cymodocea nodosa</i> ; other seagrasses of the genera <i>Ruppia</i> and <i>Zostera</i> together with macroalgae of the genera <i>Ulva</i> , <i>Dyctiota</i> , and <i>Cystoseira</i> occur occasionally (Mancinelli, 2012; Mancinelli <i>et al.</i> 2013).	Chemical pollutants and nutrient inputs of agricultural and urban origin; trace metal concentrations in sediments and fish classify the lagoon as moderately impacted (Tramati <i>et al.</i> , 2012).	First record in 2007 (Lumare <i>et al.</i> , 2009); remarkable increase in abundance in the following years (Mancinelli <i>et al.</i> 2017; Pagliara & Mancinelli, 2018).	July	Crab traps
Spain Gandia 39° 0'17.78"N 0° 8'54.97"W	Funnel-shaped estuaries of several rivers (i.e. the Serpis and Vaca Rivers in the Gandia area. the Racons and Vedat Rivers in the Oliva area. and the Júcar River in the Cullera area) characterize the area showing polyhaline, tidally-regulated regimes with water temperatures and salinities varying spatially and seasonally between 13 and 27°C and 0.5 to 38 PSU. respectively (Gadea <i>et al.</i> , 2013). Muddy bottoms with sparse <i>Cymodocea nodosa</i> and macroalgal beds at the river mouths and bare	River mouths are located in urban or highly anthropized contexts. In addition, their drainage basins experience considerable anthropogenic pressures due to urban, agricultural. and industrial activities (see <i>e.g.</i> , Kuzmanović <i>et al.</i> , 2016 for the Júcar River).	Repeated records in the Albufera Lagoon, the Gandia coastal area. and the Segura River mouth between 2014 and 2015 (González-Wangüemert & Pujol, 2016); northward expansion of the distribution range in the last five years (Fuentes <i>et al.</i> , 2019).	July-August	Fishnets/crab traps/hand nets

	sandy sediments offshore (Falco, unpublished data).				
Greece Pogonitsa Lagoon 38°57'42.29"N 20°48'55.75"E	The Amvrakikos Gulf is characterized by a number of river mouths in the northern area, with remarkable seasonal and spatial variations in water temperature and salinity (Reizopoulou & Nicolaidou 2004; Kountoura & Zacharias 2011). In contrast, the Pogonitsa Lagoon has no freshwater inputs, with a mean annual salinity of 33 PSU and water temperatures ranging between 11 and 28°C (Katselis <i>et al.</i> , 2013). Its shallow unconsolidated bottoms are generally dominated by the angiosperms <i>Zostera noltii</i> , <i>Ruppia maritima</i> , and <i>Cymodocea nodosa</i> (Christia & Papastergiadou, 2007).	The surroundings of the Pogonitsa basin are characterized by insignificant local inputs of pollutants, while in the northern Amvrakikos Gulf river inputs determine significant agriculture-related contamination (Diamantopoulou, Kalavrouziotis & Varnavas, 2018).	First records in late 2000s (Perdikaris <i>et al.</i> , 2016); since 2010 abundances have increased progressively, and to date the species is distributed ubiquitously, in particular in south-western inlets (Katselis & Koutsikopoulos, 2017).	August	Barrier traps
Greece Loudias Bay 40°30'59.37"N22°41'12.87"E	The bay is located at the mouth of the rivers Loudias, Axios, and Aliakmon (Catsiki & Florou, 2006). Accordingly, water temperatures and salinities vary both spatially and seasonally between 9.5 and 26 °C and 30.5 and 37.5 PSU, respectively (NCMR 2001). The bay is generally characterised by bare muddy/sandy sediments with macroalgal beds dominated by <i>Gracilaria sp.</i> and <i>Ulva sp.</i> located close to the river mouths (Kevrekidis, personal observation).	River inputs in the area contribute nutrients and chemical contaminants of agricultural origin (Pavlidou <i>et al.</i> , 2015). However, negligible levels of heavy metal contamination have been detected in cultivated mussels in Loudias Bay (Catsiki & Florou, 2006).	First record in 1948 in the south-western section of the Gulf of Thessaloniki (Serbetis, 1959); unconfirmed reports indicate that the crab occurred in the Gulf at least since 1935 (Nehring, 2011). To date the population is abundant and is commercially exploited (Kevrekidis & Antoniadou, 2018)	August	Fyke net

\*details on blue crab sampling procedures in Cilenti *et al.* (2015; Lesina Lagoon), Carrozzo *et al.* (2014; Acquatina Lagoon), Soler Blanco (2017; Gandia), Katselis & Koutsikopoulos (2016; Pogonitsa Lagoon), and Kevrekidis & Antoniadou (2018; Loudias Bay)



### 6.2.2. Samples collection

In the five sampling locations, blue crabs were captured in summer 2016 using crab traps or other netting devices (see Tab. 1 for additional details). After collection, specimens were transferred alive in refrigerated containers (4°C) to the laboratory, where they were sexed and had the cephalothorax width (CW hereafter) measured with a Vernier calliper to the nearest 1 mm as the distance between the two outermost lateral spines. After measurements, crabs were euthanized by thermal shock (-80°C for 10 min) to avoid artefacts on elemental and isotopic determinations (Atwood, 2013). After crab collection, several individuals of dominant bivalve taxa were collected at each sampling location by hand. *Mytilus galloprovincialis* was collected at all the locations except Lesina Lagoon, where the non-indigenous Asian date mussel *Arcuatula senhousia* has recently outnumbered other native bivalves. Specimens were transferred alive to the laboratory and kept overnight in seawater to clear gut contents; subsequently, their shell length was measured in mm using a calliper before being euthanized as already described.

### 6.2.3. Chemical analyses

Glassware and other equipment used to prepare samples for stable isotope and elemental analyses were preventively kept in diluted ultrapure HNO<sub>3</sub> 65% for 24 h, rinsed with Milli-Q water (Millipore Corp., Bedford, MA), and dried under a laminar flow hood.

For each crab, muscle tissues were removed from both claws using a ceramic scalpel, stored in falcon tubes, and randomly assigned to two groups. Samples were subsequently oven dried (40°C until constant weight) and powdered using a mortar and pestle. The first group of samples was used for carbon (C) and nitrogen (N) total content and stable isotope analyses. To this end, subsamples of known dry weight were pressed into Ultra-Pure tin capsules (Costech Analytical Technologies) and analysed using an Elemental Analyser connected with an Isotope Ratio Mass Spectrometer (Thermo Scientific Flash EA 1112 and IRMS Delta Plus XP). Concentrations of total C and N were reported as g kg<sup>-1</sup> tissue dry weight. Isotopic values were expressed in conventional δ notation (as ‰) in relation to international standards (PeeDee Belemnite and atmospheric N<sub>2</sub> for C and N, respectively) using the formula

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}})-1] \times 10^3$$

where X is  $^{13}\text{C}$  or  $^{15}\text{N}$  and R is the corresponding  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$  ratio. Analytical precision based on the standard deviation of replicates of internal standards (International Atomic Energy Agency IAEA-NO-3 for  $\delta^{15}\text{N}$  and IAEA-CH-6 for  $\delta^{13}\text{C}$ ) was 0.2 ‰ for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

The total concentration of boron, barium, cadmium, chromium, copper, iron, lithium, manganese, nickel, lead, strontium, vanadium, and zinc were determined at the University of Salento on the second group of samples by wet digestion according to Raab *et al.*, (2005). The procedure is described in detail elsewhere (Zotti *et al.*, 2016); in brief, subsamples of known dry weight were mixed with 4 ml  $\text{H}_2\text{O}_2$  and 6 ml  $\text{HNO}_3$  at  $180^\circ\text{C}$  for ten minutes using a microwave digestion system (Milestone START D). They were consequently cooled, diluted with ultrapure water to a final volume of 25 ml, filtered through Whatman No. 42 filter papers, and measured for trace metal contents using an inductively coupled plasma atomic emission spectrometer (ICP-AES; Thermo Scientific iCap 6000 Series). Results were expressed as  $\text{mg kg}^{-1}$  tissue dry weight; the minimum detection limit was  $0.001 \text{ mg kg}^{-1}$  for all measured trace metals.

For bivalves, the foot was dissected from each individual, dried, powdered, and analysed for trace metals and stable isotopes adopting procedures identical to those described for *C. sapidus* tissues.

#### 6.2.4. Statistical analysis

Values in the text are expressed as mean  $\pm$  1 SE if not otherwise specified. Morphometric and biochemical data of bivalves and blue crabs were preliminarily checked for normality (Shapiro-Wilks test) and homoscedasticity (Cochran's C-test) and transformed when required to meet the assumptions. Univariate comparisons were subsequently performed using parametric ANOVA followed by post-hoc Tukey HSD tests and Pearson product-moment correlation. Statistical significance was evaluated at  $\alpha = 0.05$ ; sequential Benjamini-Hochberg corrections (Benjamini and Hochberg, 1995) were used for multiple tests to adjust  $\alpha$  and reduce the risk of a type-I error.

Similarity matrices based on Euclidean distances were constructed with  $\log(x+1)$ -transformed and Z-scaled metal concentrations of bivalves and blue crabs. For bivalves, a Type III (partial sum of squares) permutational multivariate analysis of variance (PERMANOVA; Anderson, 2005) with 9,999 unrestricted permutation of raw data was subsequently used to test for among-

location differences in metal contents (fixed factor “location”, five levels). In addition, a Type I (sequential sum of squares) PERMANOVA with 9,999 permutations was used to verify the effect of the factor “location” on metal concentrations and isotopic ratios in *C. sapidus*, as influenced by sex (fixed factor “sex”, two levels), and the continuous covariate “body size” as estimated by individual cephalothorax widths. In general, P values for PERMANOVA tests were calculated using Monte Carlo permutations [P(MC) hereafter]. A Canonical Analysis of Principal Components (CAP) was further used to model changes in metal concentrations in individual blue crabs against their cephalothorax width across the five locations.

Lipids are depleted in  $^{13}\text{C}$  compared to proteins and carbohydrates and can significantly bias  $\delta^{13}\text{C}$  estimations when samples have a lipid content  $> 5\%$ , corresponding to a C:N ratio  $> 3.5$  (Post *et al.*, 2007). Here, the majority of blue crabs had tissues with a C:N ratio  $< 3.5$ ; conversely, bivalves from Lesina and Acquatina lagoons, and to a minor extent from Loudias Bay, showed C:N ratios exceeding 3.5 (Tab. 2). Accordingly, the  $\delta^{13}\text{C}$  of samples characterized by a C:N value  $> 3.5$  was normalized using the mathematical procedure proposed by Post *et al.* (2007).

**Table 2.** Mean ( $\pm$  1SD in brackets) C:N ratios of *Callinectes sapidus* and bivalves collected at the five study locations. Minimum - maximum ranges are included.

Location	<i>Callinectes sapidus</i>		Bivalves*	
	Mean	Range	Mean	Range
Lesina Lagoon	3.05 (0.14)	2.8 - 3.46	3.67 (0.13)	3.54 - 3.84
Acquatina Lagoon	3.21 (0.09)	3.09 - 3.39	5.58 (1.22)	3.92 - 7.69
Gandia	2.81 (0.23)	2.29 - 3.22	3.15 (0.49)	2.47 - 3.94
Pogonitsa Lagoon	2.71 (0.47)	2.11 - 3.22	2.24 (0.09)	2.14 - 2.38
Loudias Bay	2.73 (0.55)	2.14 - 4.01	6.07 (0.36)	5.61 - 6.42

\* *Arcuatula senhousia* for Lesina; *Mytilus galloprovincialis* for the remaining locations

The trophic position of *C. sapidus* at the five locations (TP hereafter) was estimated implementing a Bayesian approach using the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of the crab, while the isotopic signatures of bivalves were adopted as isotopic baselines. We used this approach as it explicitly takes into consideration individual variability and propagation of sampling error (trophic enrichment factors, and measurements of baselines and consumers) in the modelling procedure and posterior estimates of parameters (Quezada-Romegialli *et al.*, 2018).

A single-baseline Bayesian model was run with 5 parallel chains and 20,000 adaptive iterations. *M. galloprovincialis* and *A. senhousia* are filter feeders with diets mainly relying on phytoplankton and suspended particulate matter (Inoue and Yamamuro, 2000; Ezgeta-Balić *et al.*, 2014); hence, a trophic position  $\lambda = 2$  was assumed for both taxa. Given the omnivorous trophic habits of the blue crab, TP values were estimated adopting a trophic enrichment factor ( $\Delta$ ) of  $0.27 \pm 2.44$  for C and  $2.57 \pm 1.72$  for N (mean  $\pm$  1SD,  $n = 27$  and  $65$ , respectively) calculated as the average of published enrichment factors for aquatic crustaceans (including *C. sapidus*) feeding on both vegetal and animal items (Mancinelli *et al.*, 2022), and more consistent with other literature syntheses focused on crustaceans (Vanderklift & Ponsard, 2003; Mancinelli, 2012a; Carrozzo *et al.*, 2014). Pair-wise comparisons of the posterior distributions of TP estimates were performed by calculating the probability that *C. sapidus* at location  $i$  has a posterior trophic position less than or equal to that calculated at location  $k$ .

To compare the total trace metals content of bivalves and blue crabs at the different sampling sites, the metal pollution index (MPI) was calculated using the equation (Usero *et al.*, 1997):

$$MPI = (Cf_1 \times Cf_2 \dots \times Cf_k)^{1/k}$$

where  $Cf_k$  = concentration value of the  $k^{\text{th}}$  metal. When the measured content of metals was under the detection limits of the instrumentation (see previous paragraph), a concentration of  $0.0005 \text{ mg kg}^{-1}$  was used. MPI values were estimated on the whole set of analysed trace metals, and only including hazardous essential (Ni) and non-essential elements (B, Ba, Cd, Cr, Li, Pb, V). In addition, the trophic position of bivalves ( $TP_{\text{Bivalve}} = 2$ ) and those estimated for *C. sapidus* at the different locations ( $TP_{\text{Blue Crab}}$ ) were used to calculate biomagnification factors (BMF) for each trace metal according to the equation (Hoekstra *et al.*, 2003):

$$BMF_i = TTF_i / \left( \frac{TP_{\text{Blue Crab}}}{TP_{\text{Bivalve}}} \right)$$

where  $TTF_i$  is the trophic transfer factor calculated as the ratio of the concentration of each element  $i$  in *C. sapidus* to that in bivalve prey (DeForest *et al.*, 2007). Biomagnification, simple transfer, and trophic dilution occur if  $BMF > 1$ ,  $= 1$ , and  $< 1$ , respectively (Gray, 2002).

All statistical procedures were implemented in the R package (R Development Core Team, 2022). Specifically, nMDS and PERMANOVA analyses were performed using the *metaMDS* and *adonis* functions of the vegan library, respectively (Wood & Scheipl, 2020), while

*tRophicPosition* (Quezada-Romegialli *et al.*, 2019) was adopted to estimate *C. sapidus* trophic positions and to perform pairwise comparisons using the *pairwiseComparisons()* function.

### 6.3. Results

#### 6.3.1. Bivalves

Six (Gandia, Pogonitsa, and Loudias) to 10 (Lesina) bivalves were collected at each sampling location (Tab. 3); their mean shell lengths varied significantly between 22.9 and 33.4 mm (Tab. 3; 1-way ANOVA,  $F_{4,31} = 3.9$ ,  $P = 0.01$ ). Additional bivariate comparisons indicated significant size differences only between Lesina and Gandia (Tukey HSD test,  $P = 0.01$ ;  $P$  always  $> 0.05$  for the remaining comparisons).

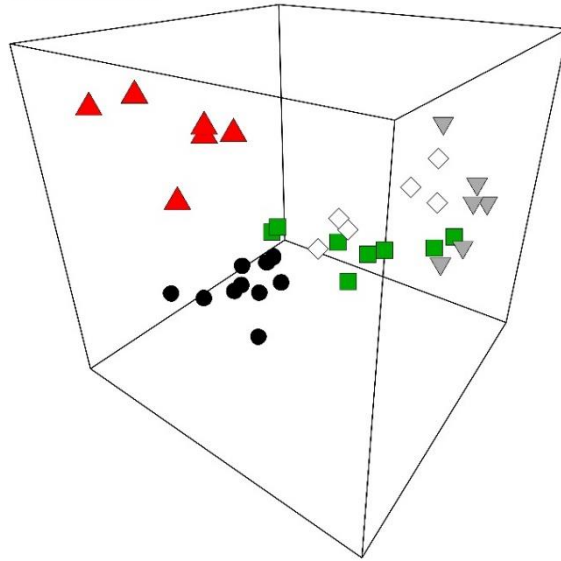
**Table 3.** Summary of information on the sex (F = females, M = males), number (in square brackets), and size (CW = mean carapace width, in mm; SE in round brackets, range in italics) of *Callinectes sapidus* specimens collected at the five study locations. Taxonomic information on the bivalve species used as baseline for chemical analyses are included (Mg = *Mytilus galloprovincialis*. As = *Arcuatula senhousia*), together with details on the number (in square brackets) and size (SL = shell length, in mm; SE in round brackets, range in italics) of the analysed specimens.

Location	Bivalves		<i>Callinectes sapidus</i>		
	Taxon	SL (mm)	Sex	CW (mm)	
Lesina Lagoon	As [10]	22.9 (1.5) 18 - 32	F [12]	152.2 (6.3)	<i>104 - 175</i>
			M [12]	131.7 (5.3)	<i>102 - 154</i>
			Total	141.9 (4.6)	<i>102 - 175</i>
Acquatina Lagoon	Mg [8]	29.1 (3.1) 18 - 39	F [10]	153.6 (8.6)	<i>101 - 183</i>
			M [10]	170.2 (4.3)	<i>143 - 196</i>
			Total	161.9 (5.1)	<i>101 - 196</i>
Gandia	Mg [6]	33.4 (0.5) 32 - 35	F [12]	141.8 (9.2)	<i>99 - 186</i>
			M [7]	97.4 (6.9)	<i>70 - 128</i>
			Total	125.5 (8.1)	<i>70 - 186</i>
Pogonitsa Lagoon	Mg [6]	26.7 (1.1) 23 - 30	F [10]	136.4 (4.7)	<i>122 - 167</i>
			M [9]	172.3 (3.8)	<i>158 - 192</i>
			Total	153.4 (5.2)	<i>122 - 192</i>
Loudias Bay	Mg [6]	29.9 (3.1) 19 - 35	F [7]	132.1 (7.9)	<i>99 - 153</i>
			M [8]	116.9 (9.5)	<i>81 - 152</i>
			Total	124.1 (6.4)	<i>81 - 153</i>

Metals content in bivalves (Fig. 2; see also Tab. 4 for mean values  $\pm$  1SE) varied significantly among locations (PERMANOVA followed by post-hoc bivariate comparisons: Pseudo-F<sub>4,30</sub> = 16.5, P(MC) = 0.0001; for pair-wise tests, maximum P(MC) = 0.03 for the comparison Gandia vs. Loudias). Univariate analyses confirmed a significant effect of the factor “location” for all trace metals (1-way ANOVAs; P always < 0.05); significantly higher concentrations were observed for most of the metals in *Arcuatula senhousia* from Lesina and in *Mytilus galloprovincialis* from Pogonitsa (Cd, Fe, Li, Pb, V and Ba, Cr, Cu, Ni, Zn respectively; see Tab. 4 for post-hoc bivariate HSD tests).

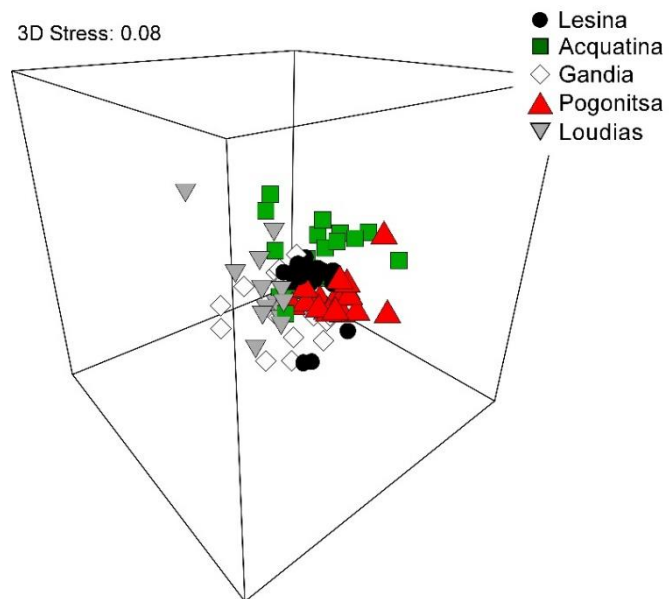
## Bivalves

3D Stress: 0.05



## *Callinectes sapidus*

3D Stress: 0.08



**Figure 2.** 3d-nMDS plots based on Euclidean distance similarity matrices of trace metal concentrations in bivalves (up) and in *Callinectes sapidus* (down) from the five sampling locations.

*M. galloprovincialis* from Acquatina showed peak concentrations only for B and Sr, with Cd and Li contents comparable to the maximum values observed in *A. senhousia* from Lesina (Tab. 4). In contrast, Loudias generally showed the lowest metal concentrations with the exception of Cd, V, and Zn, followed by Gandia; noticeably, Ni content was under the detection limits at both Loudias and Acquatina (Tab. 4).

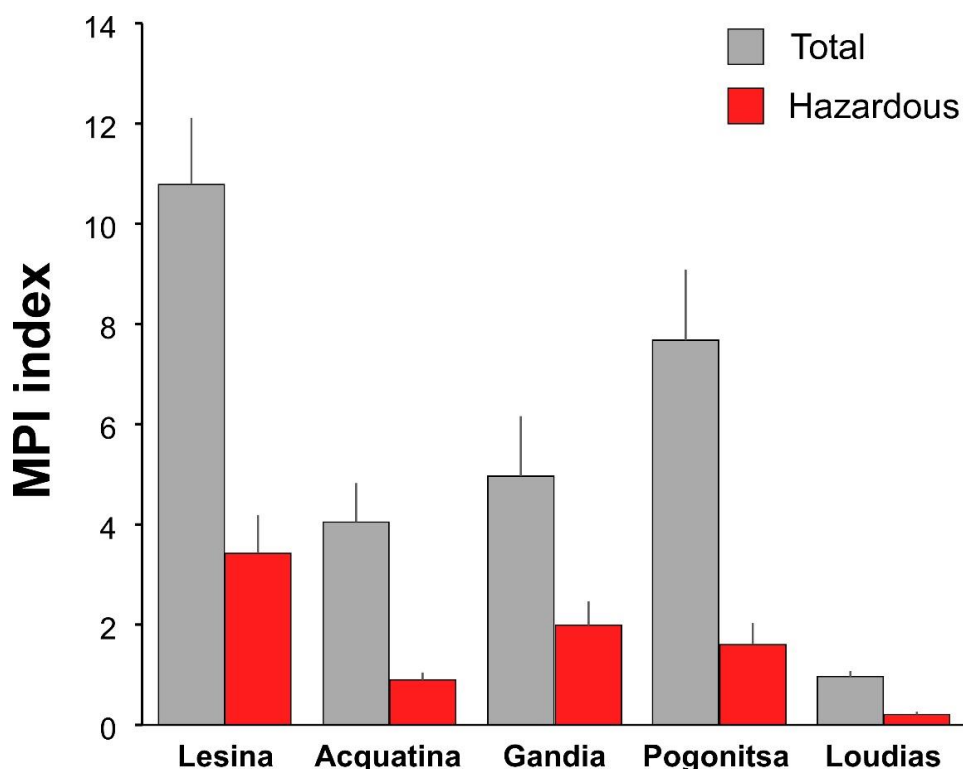
Significant among-location variations were observed in MPI values of trace metals, estimated either on the whole set (1-way ANOVA,  $F_{4,31} = 10.4$ ,  $P < 0.0001$ ). In general, Loudias showed MPI values significantly lower than those determined in the remaining locations (fig. 3; HSD tests, max  $P = 0.003$  for the comparison Loudias vs. Acquatina); Gandia, Pogonitsa, and Acquatina showed similar (HSD tests,  $P$  always  $> 0.05$ ) intermediate values ranging between 4.1 (Acquatina) and 7.7 (Pogonitsa). Lesina was characterized by the highest MPI (10.8), significantly different from values determined for all the other locations with the exception of Pogonitsa (HSD test  $P = 0.32$ ).

Similarly, significant among-location variations were observed in MPI values of hazardous trace metals (1-way ANOVA,  $F_{4,31} = 5.2$ ,  $P = 0.003$ ). Loudias and Lesina showed the lowest and highest MPI values (0.2 and 3.4, respectively; Fig. 3), with the remaining locations ranging between 0.9 and 2 (Acquatina and Gandia, respectively). Further bivariate tests indicated that Lesina differed significantly only with Acquatina and Loudias (HSD tests,  $P$  always  $< 0.05$ ).



**Table 4.** Trace metal contents expressed in ppm (mean, SE in brackets; <DL: under detection limits) in bivalves from the five sampling locations. The results of post-hoc bivariate HSD tests after Benjamini-Hochberg correction for multiple comparisons (see text for further details) are included. For each metal, the highest and lowest concentrations are indicated in red and blue, respectively.

Location Metal	Lesina (1)	Acquatina (2)	Gandia (3)	Pogonitsa (4)	Loudias (5)	HSD test
<b>B</b>	37.86 (2.34)	12.89 (6.18)	21.09 (3.2)	19.05 (2.36)	20.82 (11.04)	1 > 3 = 4 = 5 > 2
<b>Ba</b>	41.9 (3.95)	1.99 (0.86)	8.35 (2.13)	55.57 (1.14)	1.64 (0.36)	4 = 1 > 3 > 2 = 5
<b>Cd</b>	0.7 (0.05)	0.61 (0.06)	0.34 (0.08)	0.18 (0.18)	0.38 (0.14)	2 > 4 = 1 = 3 = 5
<b>Cr</b>	3.96 (0.52)	0.88 (0.22)	0.53 (0.16)	6.32 (2.11)	0.29 (0.18)	4 = 1 > 2 = 3 = 5
<b>Cu</b>	12.49 (0.98)	5.4 (0.88)	3.25 (0.72)	35.85 (6.43)	1.38 (0.26)	4 > 1 > 2 > 3 = 5
<b>Fe</b>	1012.03 (126.85)	119.64 (34.52)	112.44 (44.45)	268.69 (55.97)	43.77 (12.91)	1 > 4 > 2 = 3 > 5
<b>Li</b>	2.03 (0.19)	1.55 (0.22)	0.61 (0.17)	0.5 (0.23)	0.13 (0.08)	1 = 2 > 3 = 4 = 5
<b>Mn</b>	60.44 (5.12)	29.53 (10.39)	8.18 (2.04)	66.47 (18.82)	4.81 (0.95)	1 = 4 > 2 > 3 = 5
<b>Ni</b>	2.14 (1.26)	<DL	1.05 (0.38)	8.2 (1.36)	<DL	4 > 1 = 3 > 2 = 5
<b>Pb</b>	5.96 (0.74)	2.33 (0.8)	2.22 (0.42)	2.98 (0.53)	1.54 (0.94)	1 > 2 = 3 = 4 = 5
<b>Sr</b>	110.99 (34.65)	302.5 (117.28)	21.85 (5.41)	88.26 (14.91)	23.03 (0.93)	1 = 2 = 3 = 4 > 5
<b>V</b>	13.46 (0.64)	5.02 (0.35)	9.28 (1.41)	25.15 (4.36)	6.16 (0.37)	4 > 1 = 3 > 2 = 5
<b>Zn</b>	52.45 (2.13)	66.48 (7.23)	85.66 (18.48)	368.16 (65.59)	209.9 (67.51)	4 = 5 > 1 = 2 = 3



**Figure 3.** Metal pollution index (MPI) of bivalves from the five sampling locations. Indices estimated over the complete set of analysed trace elements and only on hazardous metals are compared.

### 6.3.2. Blue crabs

A minimum of 15 (Loudias) to a maximum of 24 (Lesina) crab specimens were analysed per location, for a total of 97 adults, 51 females and 46 males (Tab. 3). Crabs' size varied significantly among locations as well between sexes (2-way ANOVA, interaction factor location×sex,  $F_{4,87} = 7.6$ ,  $P < 0.0001$ ). Additional post-hoc comparisons indicated negligible among-location differences in the size of females (Tukey HSD tests, min  $P = 0.8$  for Lesina vs. Loudias); furthermore, males from Gandia resulted significantly smaller than females ( $P = 0.001$ ), while no significant sex-related differences were observed in the carapace width of crabs for the remaining locations (min  $P = 0.09$  for Pogonitsa).

A preliminary nMDS plot of trace metal contents in *Callinectes sapidus* did not highlighted remarkable among-location variations as those observed in bivalves (Fig. 2; see also Tab. 4 for mean values  $\pm 1SE$ ). However, a PERMANOVA followed by bivariate comparisons showed a significant spatial variation in trace metals contents in crabs [Tab. 5;  $P$  (MC) always  $< 0.05$  for all pair-wise tests]. Noticeably, no sex-related effects were observed; accordingly, the factor was discarded in further analyses.

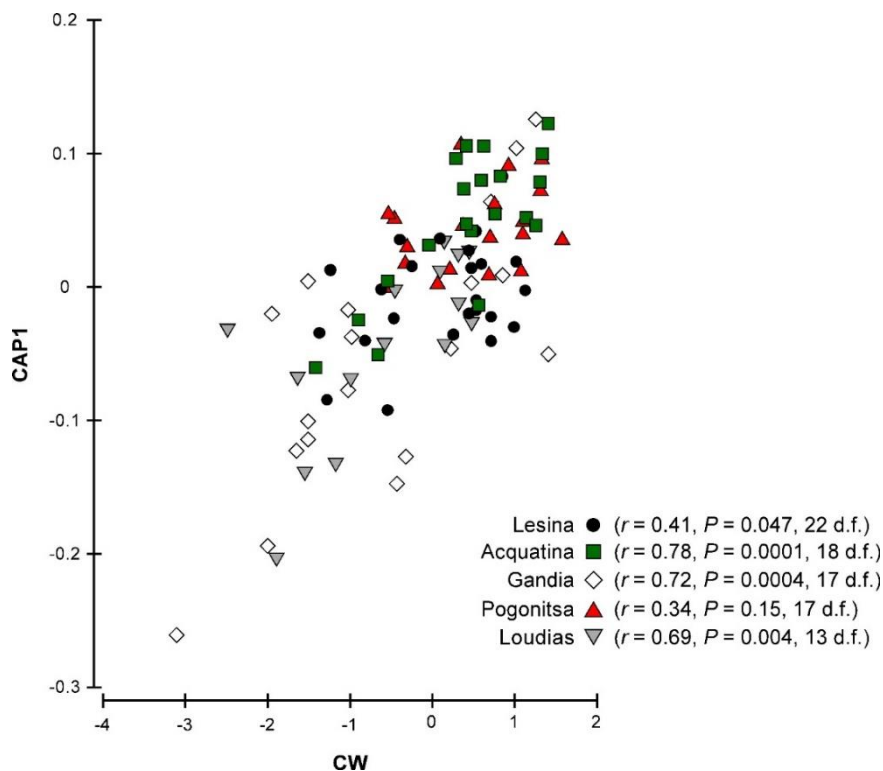
**Table 5.** Trace metal contents expressed in ppm (mean, SE in brackets; <DL: under detection limits) in *Callinectes sapidus* from the five sampling locations. The results of post-hoc bivariate HSD tests after Benjamini-Hochberg correction for multiple comparisons (see text for further details) are included.

Location Metal	Lesina (1)	Acquatina (2)	Gandia (3)	Pogonitsa (4)	Loudias (5)	HSD test
<b>B</b>	2.88 (0.77)	2.39 (0.36)	5.49 (1.93)	1.8 (0.17)	2.79 (0.49)	3 > 4 = 1 = 2 = 5
<b>Ba</b>	2.56 (0.43)	0.91 (0.16)	10.7 (2.78)	0.36 (0.05)	3.54 (0.44)	3 > 1 = 5 > 2 = 4
<b>Cd</b>	0.24 (0.04)	0.16 (0.03)	0.26 (0.09)	0.47 (0.06)	0.03 (0.01)	4 > 1 = 2 = 3 > 5
<b>Cr</b>	0.06 (0.01)	0.07 (0.01)	0.44 (0.22)	0.06 (0.03)	0.14 (0.07)	3 > 1 = 2 = 4 = 5
<b>Cu</b>	32.46 (2.58)	56.97 (5.4)	51.35 (4.48)	33.14 (2.66)	28.08 (3.13)	3 = 2 > 1 = 4 = 5
<b>Fe</b>	10.87 (0.88)	15.84 (1.89)	10.76 (3.67)	5.08 (0.59)	49.92 (35.25)	1 = 2 = 3 = 5 > 4
<b>Li</b>	0.45 (0.05)	0.29 (0.02)	0.36 (0.04)	0.29 (0.02)	0.13 (0.02)	1 > 2 = 3 = 4 > 5
<b>Mn</b>	7.23 (0.55)	1.47 (0.23)	7.89 (2.23)	1.56 (0.22)	8.22 (3.22)	1 = 3 = 5 > 2 > 4
<b>Ni</b>	0.07 (0.02)	1.07 (0.16)	<DL	0.38 (0.11)	0.11 (0.05)	2 > 4 > 1 = 3 = 5
<b>Pb</b>	0.17 (0.03)	0.53 (0.06)	0.64 (0.05)	0.22 (0.02)	0.51 (0.04)	2 = 3 = 5 > 1 = 4
<b>Sr</b>	88.85 (9.21)	94.92 (16.21)	65.24 (15.59)	34.13 (5.13)	47.81 (18.13)	1 = 2 = 3 = 4 = 5
<b>V</b>	4.05 (0.21)	3.51 (0.21)	9.35 (3.33)	3.79 (0.1)	3.68 (0.24)	3 > 1 = 2 = 4 = 5
<b>Zn</b>	176.93 (4.2)	259.84 (29.57)	212.62 (5.71)	191.7 (6.64)	137.83 (7.56)	2 = 3 = 4 > 1 > 5

**Table 6.** Summary of PERMANOVA results testing for the effects of sex and location as orthogonal fixed factors and size (carapace width, CW) as a continuous covariate on *Callinectes sapidus* trace metal contents and  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values.

Source	d.f.	Trace metals		Stable isotopes	
		Pseudo-F	P(MC)	Pseudo-F	P(MC)
CW (1)	1	5.25	<0.001	1.2	0.09
Location (2)	4	9.22	<0.001	32.08	0.001
Sex (3)	1	1.59	0.14	1.92	0.15
1 × 2	4	1.82	0.01	0.88	0.54
1 × 3	1	0.74	0.61	2.77	0.09
2 × 3	4	1.21	0.21	1.61	0.14
1 × 2 × 3	4	0.87	0.63	0.51	0.86

Noticeably, a significant effect for the interaction term “location×size” was highlighted by the PERMANOVA (Tab. 5); a Canonical Analysis of Principal Components confirmed a covariation of accumulation patterns with the size of *C. sapidus* individuals for all the location with the exception of Pogonitsa (Fig. 4).



**Figure 4.** CAP ordination plot displaying the relationship between the first principal coordinate (CAP1) estimated on trace metal contents of blue crabs and their individual cephalothorax width (CW). Data were (Log+1)-transformed before analysis. For each location, Pearson correlation coefficients, significance levels, and degrees of freedom of the relationship are included in brackets.

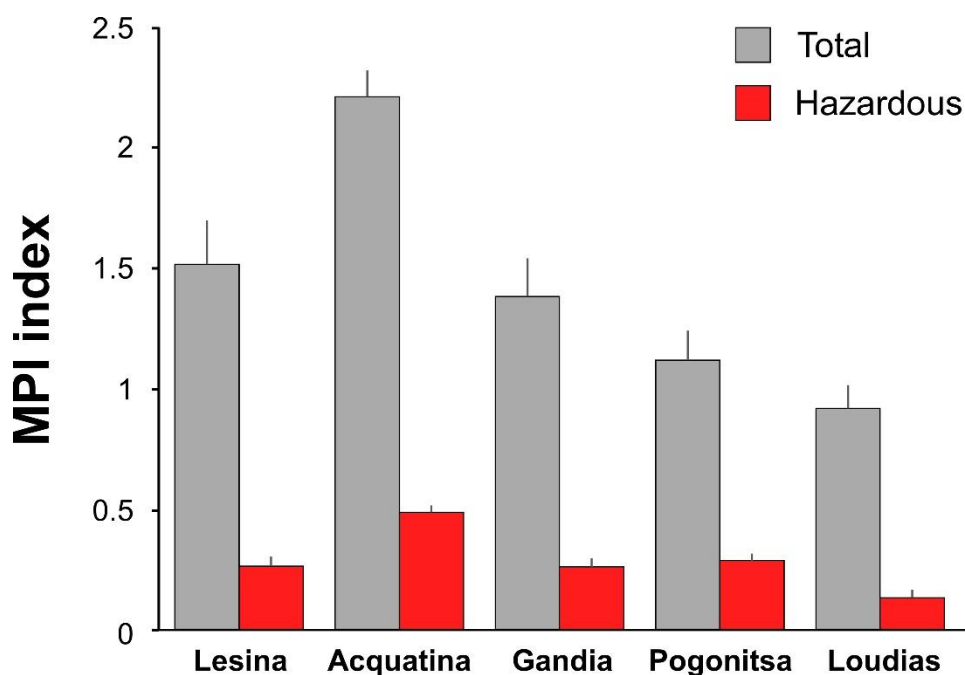
**Table 7.** Pearson correlation coefficients  $r$  of the relationship between the individual mean cephalothorax width (CW) of *Callinectes sapidus* and the respective metal concentrations at the five locations. Significant (at  $P < 0.05$  adjusted for multiple tests) relationships are reported in bold.

Location	Lesina	Acquatina	Gandia	Pogonitsa	Loudias
<b>Metal</b>					
<b>B</b>	<b>0.46*</b>	0.55**	0.33	0.19	<b>0.62*</b>
<b>Ba</b>	-0.21	0.06	0.01	-0.07	0.13
<b>Cd</b>	-0.31	-0.32	0.48	0.07	0.05
<b>Cr</b>	-0.1	0.44	0.32	0.48	-0.36
<b>Cu</b>	-0.15	-0.05	-0.1	-0.15	-0.47
<b>Fe</b>	0.13	-0.31	0.12	0.01	-0.38
<b>Li</b>	-0.22	-0.29	-0.21	<b>0.56*</b>	0.36
<b>Mn</b>	-0.04	-0.25	<b>-0.73***</b>	0.09	-0.45
<b>Ni</b>	0.24	0.36	---	-0.07	-0.24
<b>Pb</b>	-0.1	-0.35	-0.04	-0.16	<b>-0.64*</b>
<b>Sr</b>	0.11	0.43	-0.34	0.07	-0.2
<b>V</b>	-0.08	-0.01	<b>-0.49*</b>	0.21	-0.39
<b>Zn</b>	<b>0.68***</b>	-0.14	0.31	0.02	0.25

Further univariate analyses, however, indicated significant size-dependent variations only for Zn at Lesina and Pb at Loudias (Tab. 7).

In general, trace metal concentrations in *Callinectes sapidus* were lower than those determined in bivalves (Tab. 4 and 5). With the exclusion of Sr ( $F_{4,92} = 1.6$ ,  $P = 0.18$ ), the content of all metals varied significantly across the five locations (1-way ANOVAs:  $P$  always  $< 0.05$ ). Further bivariate post-hoc HSD tests indicated for crabs a pattern of trace metals accumulation markedly different from those of bivalves (Tab. 4).

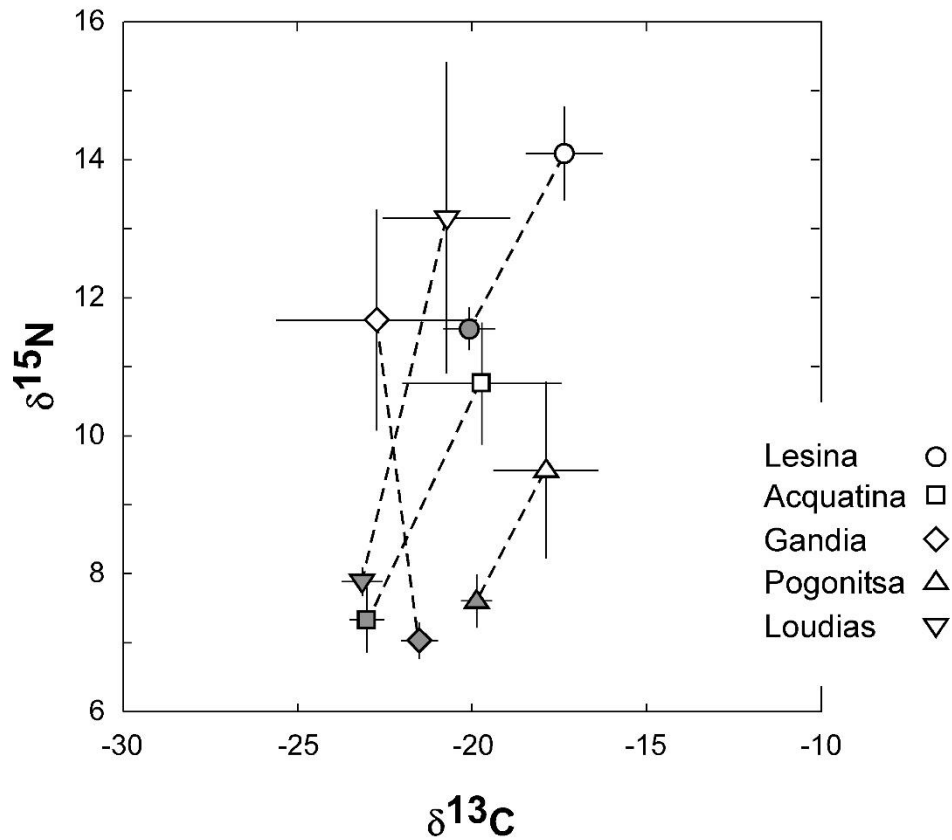
Significant among-location variations were observed in MPI values of *Callinectes sapidus*, estimated either on the whole set (1-way ANOVA,  $F_{4,92} = 11.4$ ,  $P < 0.0001$ ). In contrast with bivalves, blue crabs from Acquatina showed MPI values significantly higher than those determined in the remaining locations (Fig. 5; HSD tests, min  $P = 0.004$  for the comparison Lesina vs. Acquatina) while those from Lesina resulted significantly different from Loudias ( $P = 0.04$ ). However, Gandia, Pogonitsa, and Lesina showed similar MPI values (HSD tests,  $P$  always  $> 0.05$ ).



**Figure 5.** Metal pollution index (MPI) of *Callinectes sapidus* from the five sampling locations. Indices estimated over the complete set of analysed trace elements and only on hazardous metals are compared.

Similarly, significant among-location variations were observed in MPI values of hazardous trace metals (1-way ANOVA,  $F_{4,92} = 12.9$ ,  $P < 0.0001$ ). The variation among locations showed an almost identical pattern as that observed for the MPI indices calculated over the total set of trace metals (Fig. 5): Acquatina showed MPI values significantly higher than the remaining locations (HSD tests  $P$  always  $< 0.001$ ), whereas significant differences were observed only between Loudias and Pogonitsa ( $P = 0.03$ ).

Figure 5 shows the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of *Callinectes sapidus* and bivalves from the different locations. A preliminary PERMANOVA performed on blue crabs with “sex” and “location” as orthogonal fixed factors and “body size” (CW) as a continuous covariate indicated a significant effect only for the factor “location” (Tab. 6). Thus, in further analyses the effects of sex and body size were not considered.



**Figure 6.** Mean  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  signatures of *Callinectes sapidus* (open symbols) and bivalve prey (shaded symbols) in the five sampling locations. Whiskers = 1SD.

The trophic position of the five crab populations showed a remarkable variability, with mean values ranging between 2.7 (Pogonitsa), indicating omnivore trophic habits including both animal and vegetal items, and 4.1 (Loudias), suggesting a diet completely based on animal prey (Tab. 8A). Further bivariate tests indicated significant differences between all locations with the exclusion of the comparisons Lesina vs. Pogonitsa and Gandia vs. Loudias (Tab. 8B).

**Table 8.** (A) Median, modal, and mean posterior trophic position estimates of the five populations of *Callinectes sapidus* under analysis. Standard deviations and 95% credibility intervals are included. (B) Results of bivariate tests checking for among-population differences in posterior trophic position estimates. *P* values < 0.05 are reported in bold.

**A)**

	Mode	Median	Mean	SD	95% credibility interval
<b>Lesina</b>	2.92	2.95	2.96	0.12	2.75 - 3.21
<b>Acquatina</b>	3.36	3.35	3.36	0.16	3.09 - 3.7
<b>Gandia</b>	3.78	3.81	3.82	0.24	3.41 - 4.32
<b>Pogonitsa</b>	2.67	2.74	2.74	0.17	2.44 - 3.09
<b>Loudias</b>	4.14	4.06	4.07	0.32	3.49 - 4.78

**B)**

	Lesina	Acquatina	Gandia	Pogonitsa
<b>Lesina</b>				
<b>Acquatina</b>	<b>0.02</b>			
<b>Gandia</b>	<b>0.001</b>	<b>0.047</b>		
<b>Pogonitsa</b>	0.14	<b>0.01</b>	<b>0.0001</b>	
<b>Loudias</b>	<b>0.001</b>	<b>0.02</b>	0.26	<b>0.0001</b>

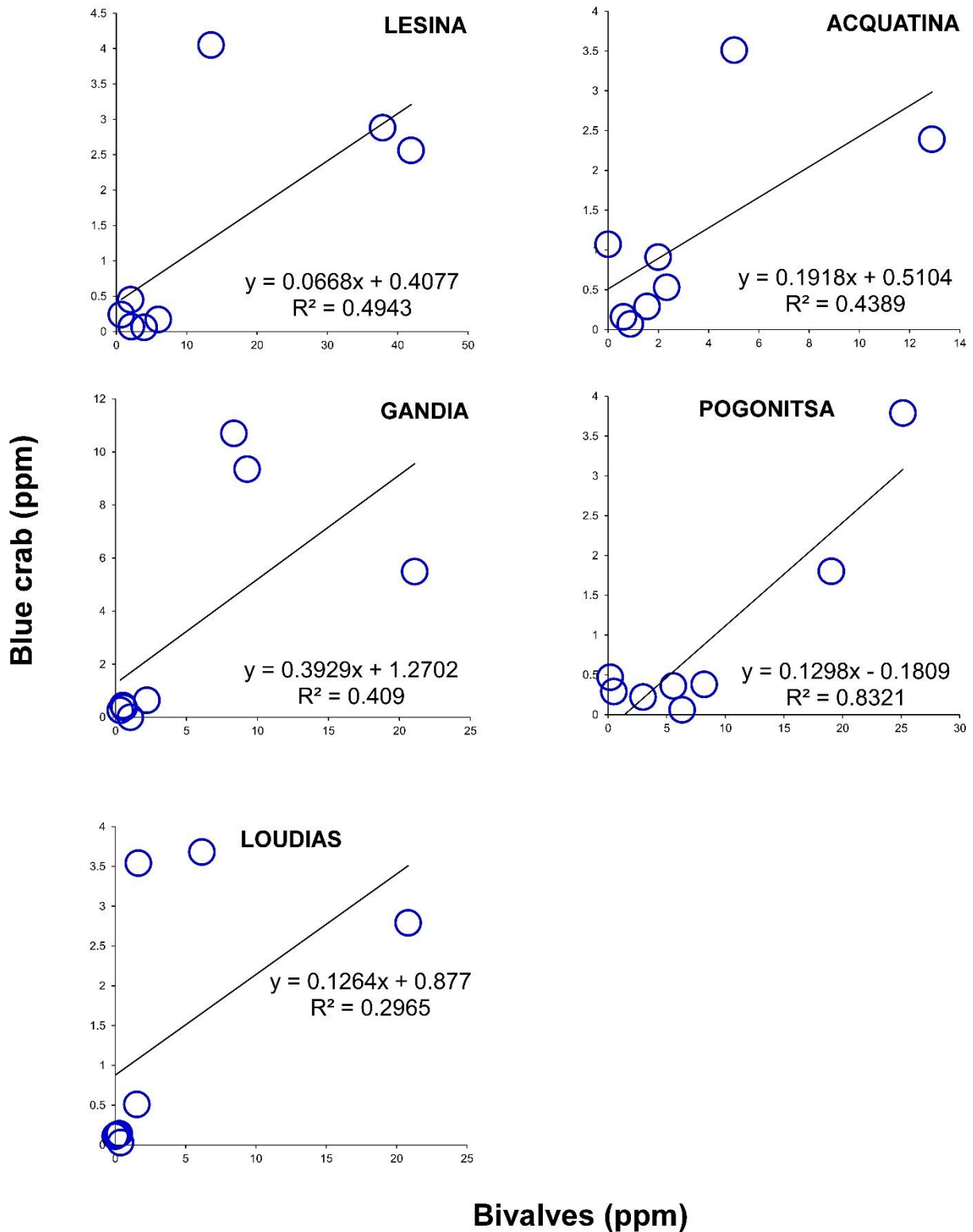
Biomagnification factors of trace metals in *C. sapidus* varied significantly both within and across locations, ranging between 0.01 (Fe at Lesina and Pogonitsa) and 10 (Cu at Loudias; Tab. 9). Weak biomagnification effects were observed in general (Overall BMF =  $0.79 \pm 0.23$ , mean  $\pm$  SE), with the majority of BMF values  $< 1$  (80%; Tab. 9). Noticeably, the most intense biomagnification effects were observed for Cu and Zn, both fundamental trace elements for the metabolic activity and physiology of both molluscs and crustaceans.

**Table 9.** Bivalves-*Callinectes sapidus* biomagnification factors of trace metals calculated at each study location. Values  $> 1$  are reported in bold.

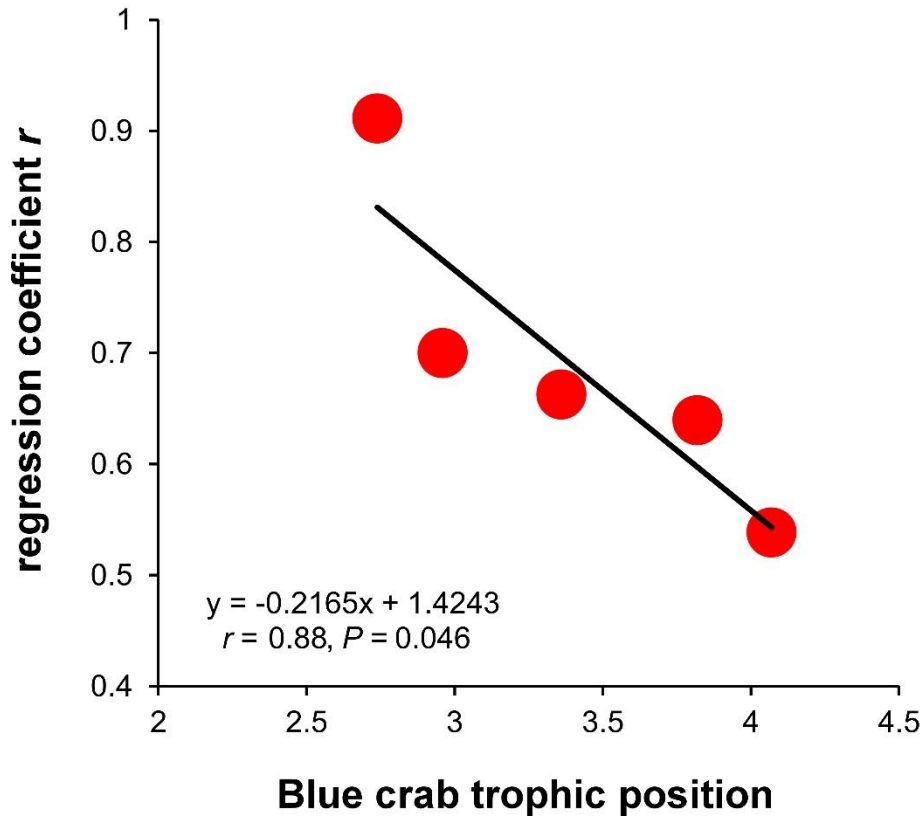
Location	Lesina	Acquatina	Gandia	Pogonitsa	Loudias
<b>Metal</b>					
<b>B</b>	0.05	0.01	0.14	0.07	0.07
<b>Ba</b>	0.04	0.27	0.54	0.05	<b>1.06</b>
<b>Cd</b>	0.23	0.16	0.4	<b>1.91</b>	0.04
<b>Cr</b>	0.01	0.05	0.43	0.01	0.24
<b>Cu</b>	<b>1.76</b>	<b>6.28</b>	<b>8.27</b>	0.67	<b>10.00</b>
<b>Fe</b>	0.01	0.08	0.05	0.01	0.56
<b>Li</b>	0.15	0.11	0.31	0.42	0.49
<b>Mn</b>	0.08	0.03	0.5	0.02	0.84
<b>Ni</b>	0.02	---	---	0.03	---
<b>Pb</b>	0.02	0.14	0.15	0.05	0.16
<b>Sr</b>	0.54	0.19	<b>1.56</b>	0.28	<b>1.02</b>
<b>V</b>	0.20	0.42	0.53	0.11	0.29
<b>Zn</b>	<b>2.28</b>	<b>2.33</b>	<b>1.30</b>	0.38	0.32

Focusing on hazardous metals, the significance of the relationship between the concentrations determined in bivalves and those determined in blue crabs (as expressed by the Pearson correlation coefficient  $r$ ) varied remarkably across sampling locations (Fig. 7). Noticeably, the degree of correlation scaled negatively with the trophic position of *C. sapidus* across the five locations (Fig. 8) indicating that the consistency between the trace element profiles of *C. sapidus* and bivalves decreased with an increase in the trophic position of the crab.





**Figure 7.** Relationships between the concentrations of hazardous trace elements determined in bivalves and in *Callinectes sapidus* in the five sampling locations.



**Figure 8.** Covariation between the trophic position of *Callinectes sapidus* and the Pearson coefficient of regression  $r$  determined between the concentrations of hazardous trace elements in bivalves and in the blue crab across the five sampling locations.

#### 6.4. Discussion

Overall, the concentration of trace metals detected in bivalves and *C. sapidus* were lower than maximum levels set from European Union and other institutions (*e.g.*, FAO, WHO, CEP) for certain contaminants in foodstuffs. In general, five sites had tolerable concentration of metals except for the Fe that showed remarkably high concentrations in Lesina lagoon. Although the concentration of metals varies across sites, Lesina and Pogonitsa showed the highest concentration, while Loudia had the lowest concentration for all metals excepted B, CD, Sr, V and Zn.

Regarding bivalves, the concentrations of Cd, Cr, Cu, and Pb were under established maximum limits (respectively 1 (7.4); 1 (7.4); 30(222.2); 1.5 (11.1)); noticeably, in Lesina lagoon Fe exceeded the limit of 100 (740.7). Generally, metal levels concentration in blue crab were lower than those detected in bivalves.

Further investigations are necessary at Lesina, but must be taken into consideration that elements such as copper (Cu), iron (Fe), manganese (Mn), selenium (Se), and zinc (Zn) are essential for bivalves and crab (White and Rainbow, 1985) as they have specific biological and physiological cellular roles. Crustaceans and bivalves can regulate the internal concentration of Zinc and copper over the environmental concentration. Since the concentration of these metals is higher than the metabolic requirements, they are probably able to store Zn and Cu. For that reason, the relation between metal concentration in the environment and metal concentration in bivalves or crustaceans may be not linear. The need for these animals to accumulate concentrations of zinc and copper to have it always available also in case of environmental scarcity must be taken into account when using these animals as biomonitors.

The most evident result of the study was that *Callinectes sapidus* and bivalves showed disjointed concentration patterns across the five Mediterranean locations included in this study, with trace element contents in crabs generally lower than in bivalves. Noticeably, a shift of approximately one level was observed in the trophic position of the blue crab across locations; the variation was negatively related with the strength of the relationship between concentration patterns of *C. sapidus* and bivalves; in addition, even though weak biomagnification effects were generally determined, maximum values were observed in locations where the blue crab exhibited the highest trophic positions.

Two mutually non-exclusive scenarios can be drawn to explain these results. The first assumes that *C. sapidus* trophic habits may have been influenced by trace metals contamination. Bivalves are important components of the crab's diet in both native and invaded habitats (Hines *et al.*, 1990; Prado *et al.*, 2020); yet, the crab is an omnivorous opportunist, whose diet comprises living and dead seagrasses, crustaceans (including conspecifics), molluscs, and fish (Ropes, 1989; Hines, 2007; Rady *et al.*, 2018); the high trophic plasticity of the crab has been corroborated by CN stable isotope studies in invaded Mediterranean systems, indicating that the species feeds on multiple trophic levels, and that trophic positions may vary substantially depending on season, location, and ontogenetic stage (Mancinelli *et al.*, 2013; 2016; 2017d; Aslan & Polito, 2021; Prado *et al.* 2022). In native estuarine habitats, blue crabs from heavy-metal contaminated sites showed an impaired predatory behaviour and a lower trophic position compared with crabs from uncontaminated locations (Reichmuth *et al.*, 2009). Interestingly, these trophic shifts ultimately reflected on peculiar bioaccumulation patterns, with metal concentrations in *C.*

*sapidus* from contaminated sites more representative of those characterizing low-level resources (*e.g.*, aquatic plants or leaf detritus) than those of prey the crab would usually consume (Reichmuth *et al.*, 2010). Consistent with this scenario, in this study the higher degree of contamination by hazardous metals was observed in bivalves from Lesina, Gandia and Pogonotza compared to Loudias and Acquatina (Fig. 1, Tab. 1), and corresponded with the lowest consistency between the concentration patterns of bivalves and *C. sapidus*, together with relatively low trophic positions and bioaccumulation effects observed in the latter. Even though some evidences are available for estuarine invertebrates and fish (Smith & Weis, 1997; Weis *et al.*, 2011; Weis & Candelmo, 2012), the interaction between organisms' trophic behaviour and the fate of contaminants in food webs is currently an open issue (Saaristo *et al.*, 2018) that deserves to be further investigated in future studies. However, in the context of the present investigation such a scenario necessarily implies that the five locations included in the study, notwithstanding their considerable environmental differences (Tab. 1), offered to *C. sapidus* similar trophic conditions in terms of *e.g.*, resource diversity and availability, and that the trophic position of the blue crab was uniquely determined by behavioural mechanisms mediated by local contamination levels. Indeed, site-specific differences in resource diversity and availability alone may have determined the observed differences in trophic position. Acquatina, Lesina, and Pogonitsa are confined systems characterized by the occurrence of submerged vegetation represented by macroalgae or seagrasses, while Gandia and Loudias are open-sea habitats characterized by benthic environments with low or negligible vegetation cover (Tab. 1). The occurrence of living and non-living vegetal resources in the lagoons, together with their associated assemblages of primary consumers, necessarily imply that in these environments *C. sapidus* may opportunistically include in its diet living or non-living primary producers characterized by contamination patterns differing from that of bivalves (among others, see Ghosn *et al.*, 2020 for an example from a Mediterranean coastal area), ultimately obscuring any bioaccumulation effect. Resource-dependent differences in ingestion and assimilation rates, coupled with variations in uptake and excretion kinetics of contaminants, may have further determined the observed differences (Jardine *et al.*, 2006). In open sea systems, conversely, the lack of primary producers and associated fauna may induce the blue crab to focus on bivalves and other animal prey located at higher trophic levels and characterized by higher levels of metal contamination. The generally higher biomagnification factors, coupled with the higher trophic positions observed in blue crabs from Gandia and Loudias indirectly support this hypothesis. Indeed, the three populations from confined lagoon habitats (*i.e.*

Acquatina, Lesina, and Pogonitsa) showed TP values significantly lower than those determined in Gandia and Loudias Bay under open-sea conditions (t-test for separate variances on mean TP values:  $t = -4.19$ ,  $P = 0.02$ , 2.99 d.f.; similar results with modal and median values).

Independently from the underlying causative mechanisms, the results of this study indicate that the omnivorous habits of *C. sapidus* can play a crucial role in generating discrepancies in concentration patterns with bivalves. Assuming the latter to provide reliable information on local contamination levels, it can be concluded that effectiveness of the blue crab as a biomonitor species is low. In blue crabs, several metallothioneins that bind trace metals have been identified (Brouwer *et al.*, 1995; Brouwer and Lee, 2007); accordingly, it has been proposed for biomonitoring of estuaries and other transitional habitats in native (Weinstein *et al.*, 1992; Sastre *et al.*, 1999; Mutlu *et al.*, 2011) and, more recently, in invaded areas (Salvat-Leal *et al.*, 2020). Our results clearly contradict this suggestion and emphasize the need to identify biomonitor animal species - in marine as well as in other environments - after a thorough scrutiny of their trophic habits. Recently, it has been emphasized how omnivory can interfere with the use of the Atlantic ghost crab *Ocypode quadrata* for monitoring micro-plastic pollution (Costa *et al.*, 2019), yet the necessity to have an advanced knowledge of the trophic ecology of the target species has long since suggested by Rainbow (1995). Noticeably, besides *C. sapidus* a number of crustaceans have been recommended for biomonitoring purposes, including other portunids (*e.g.*, *Callinectes danae*: Mutlu *et al.*, 2011; Lavradas *et al.*, 2014; *Portunus segnis*: Annabi *et al.*, 2018) the green crab *Carcinus maenas* (Pereira *et al.*, 2009; Ben-Khedher *et al.*, 2014) and the Louisiana crayfish *Procambarus clarkii* (Suárez-Serrano *et al.*, 2010; Henriques *et al.*, 2014). They are all characterized by a high degree of omnivory, and thus further studies are necessary to clarify their trophic habits, the biotic and abiotic factors influencing them, and how these reflect on the accumulation of contaminants in their tissues. In addition, here adult crabs at identical ontogenetic stages were collected from the five study locations; yet, ontogenetic variations in trophic habits (see *e.g.*, Mancinelli *et al.*, 2017b for *Callinectes sapidus*) may introduce an additional source of uncertainty that should be explicitly accounted for taken into account in future investigations (Chouvelon *et al.*, 2011; Chouvelon *et al.*, 2014).

## 6.5. Conclusions

In conclusion, invasive omnivores are to date recognized to exert significant, hard-to-be predicted threats on ecosystem functions because of their ability to directly and indirectly impact multiple trophic levels (Shea & Chesson, 2002; Romanuk *et al.*, 2009; Jackson & Britton, 2014; Pettitt-Wade *et al.*, 2015; see also Médoc *et al.*, 2018). Here, we indicated that they may represent an open issue also in environmental biomonitoring; however, we demonstrated that an in-depth comparison with “ideal” biomonitors such as bivalves and an advanced assessment of their trophic habits by stable isotope analysis can help in explaining the patterns of variation in contamination levels and, ultimately, provide more robust information for, as an example, monitoring purposes.

## 7. General conclusion

An advanced resolution of the ecological characteristics of the invasive Atlantic blue crab *Callinectes sapidus* constituted the primary theoretical conceptual mainstay of this thesis. Understanding which environmental and trophic drivers lead the colonization of invaded areas is crucial to implement effective monitoring and control actions with the aim to minimize the impact of the blue crab on the receiving communities. Eventually, since *C. sapidus* is a species of high commercial interest in native areas and is becoming a fishery target in the Mediterranean Sea, it is important to assess the quality of this species as a food resource (*e.g.*, by evaluating the accumulation of metals in the meat) and as a possible bioindicator.

On a global scale, a comparison of the environmental native and invaded niches was carried on in order to evaluate the degree of overlap between the two niches. To verify if during the invasion the blue crab undergoes a shift of the environmental niche, species distribution modelling was utilized and niche dynamic of the species investigated. Findings suggest that this generalist species performed a niche shift colonizing also habitats with different environmental features in the invaded areas. Furthermore, the outcomes indicated that the variation of the niche was positively related to nutrient concentration (phosphates, nitrates and sulfates), suggesting that *C. sapidus* occurs in more eutrophic habitats in the invaded areas.

Moreover, particular attention was given to investigate a blue crab population within the Stagnone di Marsala (western Sicily, Italy). The establishment of this population, evidenced by the presence of juveniles, adults and ovigerous females, suggests the ability of this species to colonize also marine coastal areas with extremely different environmental conditions from those of both native and previously invaded areas. Results highlighted a heterogeneous distribution of this species, independent from variations in salinity, temperature or macrophyte biomass. In general, the relative abundance of this species was lower than populations present in other Mediterranean invaded areas (*e.g.*, Torre Colimena and Acquatina, Apulia region: Mancinelli *et al.*, 2017b; Evros River: Kevrekidis *et al.*, 2023). This circumstance may be linked to extremely high salinity and temperature conditions of the Stagnone, although it must be taken into account that, in this area, the species is at an incipient stage of invasion. Indeed, the significant increase in abundance during the second year of sampling suggests that this population has not yet reached maximum abundance. Furthermore, these results testify that the blue crab is able to complete its life cycle even in

fully marine or hypersaline conditions. These outcomes indicated an expansion of the environments this species is able to colonize, highlighting new interesting perspectives on future colonization processes.

Focusing on the feeding habits of *C. sapidus*, an analysis of the isotopic niche, trophic position and food habits was carried on to investigate the trophic role of this species in the Stagnone di Marsala. Moreover, a comparison of the isotopic niche and trophic position of *C. sapidus*, *Carcinus aestuarii* and *Eriphia verrucosa*, helped to understand the relationship among the three species. In general, three brachyurans showed separated isotopic niches suggesting a repartition of the niche. This niche partition and the different trophic position occupied suggest a different exploitation of food sources by the three species. Furthermore, *C. sapidus* presented different trophic behaviour between individuals caught along the coast and in the central area of the basin. Indeed, crabs captured along the coast showed a fully carnivorous diet, with exception for the autumn-winter season, whereas in the middle of the basin exploited more vegetable food sources. These findings confirmed the opportunistic behaviour of the blue crab that is, indeed, able to feed on a wide range of food sources depending on their availability. Nevertheless, further investigations are necessary to understand how this situation will evolve, whether the three species will continue to partitioning the niche or whether their relationship will change for example with one species prevailing over the others.

Regarding the use *C. sapidus* as a bioindicator species, an assessment of the concentration of metals in the blue crab muscle was carried out across five locations in the Mediterranean Sea. Overall, it showed that the concentration of metals is no linked to sex, but depends mainly on the location, whereas body size may play a role at a local scale. No biomagnification effects as compared with bivalve prey were observed with the exception of Cu and Zn, which are fundamental elements for the physiology of crustaceans as well as molluscs. In the comparison with the “ideal” biomonitor bivalves, the blue crab showed different trends of metal concentration. Nevertheless, stable isotope analysis may be a valuable tool to clarify its behaviour as a bioindicator. Indeed, *C. sapidus* metals concentration are more similar to those of bivalves at the location where the blue crab showed a lower trophic position. Hence, this species proves to be an effective bioindicator when it feeds at low trophic levels; conversely, when its trophic habit becomes more carnivorous, the consistency with the patterns of metal accumulation showed by bivalves



decreases considerably. Consequently, due to the omnivorous feeding behaviour, the use of this species as a bioindicator represents an open issue.

Overall, the outcomes of this thesis led to an advancement in knowledge regarding the niche expansion in invaded area, and the ability of the species to colonize even extreme (*i.e.*, hypersaline) environments, providing important information to perform projection of its future distribution and to better understand the ecological role in invaded areas. Investigation at longer temporal scales is needed to clarify which are the environmental and trophic drivers that determine its presence and abundance into the Stagnone di Marsala, in order to fully understand the niche expansion that is occurring regarding this species. Eventually, having to manage the abundance and spread of this species in the Mediterranean Sea, and in order to reduce its impacts on biodiversity and invaded ecosystems, it is essential to investigate different ways to limit the further expansion of the species also through its interest as a target for fishery by turning a threat into a resource.

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