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JELLYFISH, A THREAT OR AN OPPORTUNITY? THE NON-INDIGENOUS *BLACKFORDIA VIRGINICA* AS A POTENTIAL FOOD SOURCE FOR HUMANS AND AQUATIC ORGANISMS

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Mestrado em Biologia Marinha

Supervisors:

Drª Ester Dias;

Drª. Luísa Custódio

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Author: Mariana Anastácio Cruz[1]

Thesis supervised by: Dr. Ester Dias[2], Prof. Dr. Luísa Custódio[3]

Affiliation address: [1]Faculdade de Ciências e Tecnologia, Universidade do Algarve, 8005-139 Faro, Portugal; [2] CIIMAR Universidade do Porto, 4450-208Matosinhos, Portugal; [3] CCMAR Universidade do Algarve, 8005-139 Faro, Portugal

Corresponding author- E-mail address: marianaanastaciocruz@gmail.com

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Palavras-chave: Medusa exótica**;** caranguejo azul, Estuário do Guadiana, , composição nutricional, isotopos estáveis

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Declaração de autoria de Trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de refências incluida.

Mariana Anastácio Cruz

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ABSTRACT

Anthropogenic activites such as those producing environmental changes have promoted the proliferation and establishment of non-indigenous species (NIS) in estuaries worldwide and the Guadiana estuary (Southern-Iberian Peninsula) is no exception. The extensive human development in this estuary, including river flow regulation, favoured the colonization by several NIS such as the black sea jellyfish *Blackfordia virginica* and more recently the blue crab *Callinectes sapidus*. The seasonal occurrence of *B. virginica* blooms have become a reason of concern due to reports of considerable economic and ecological impacts to fisheries and to the local food webs. However due to jellyfish properties, they represent an opportunity as an alternative food source for humans, while contributing to control their biomass in invaded ecosystems. Therefore, the objective of this work was to evaluate the nutritional profile of this jellyfish, testing the hypothethis that *B. virginica* represents an alternative and healthy food source if it follows the same composition of other known edible jellyfish. However, they may also represent a threat to the ecosystem if consumed by other NIS. Thus, the second objective of this work was to evaluate the contribution of *B. virginica* to the diet of opportunistic consumers in the middle Guadiana estuary, testing the hypothethis that generalist predators such as the NIS blue crab *Callinectes sapidus* and the indigenous green crab *Carcinus maenas* will benefit from *B. virginica* seasonal blooms by consuming this jellyfish. For the evaluation of the nutritional profile the determination of the total lipids (modified protocol of the Bligh & Dyer method), crude protein, (elementar analysis of nitrogen), ash content (incineration), fatty acids methyl esters, (gas chromatography-mass spectrometer; GC-MS), aminoacids (high pressure liquid chromatography; HPLC reverse phase), and minerals (microwave plasma – atomic emission spectrometry; MP-AES) were made. To investigate the contribution of *B. virginica* to the diet of the selected consumers, the presence of this jellyfish was investigated in the stomach contents of both crab species, through molecular analysis (DNA-PCR). Afterwards, its contribution to these consumers' biomass was determined using carbon ($\delta^{13}C$:$^{13}C/^{12}C$) and nitrogen ($\delta^{15}N$:$^{15}N/^{14}N$) stable isotope analysis. Results suggest that *B. virginica* dry biomass is mainly composed by essential minerals, and proteins rather than lipids. *Blackfordia virginica* composition resembled other edible jellyfish. Nonetheless the presence of cadmium, which is a toxic element, was high (3 mg/Kg) which meand they have a great potential to be used as food for humans if cadmium levels decrease. In addition, molecular analysis revealed the presence of *B. virginica* only in blue crab gut contents, although the Bayesian stable isotope mixing model did not show any relevant contribution to their biomass. Therefore the use of *B. virginica* for human consumption can represent an opportunity to decrease the abundance of this species in

the ecosystem through commercial exploitation, while representing a threat if other NIS, mainly the blue crab, can take advantage of this species by consuming it.

Keywords: Exotic jellyfish; blue crab, Guadiana estuary;, nutrional composition; stable isotopes

RESUMO

Nos últimos anos as atividades antropogénicas têm promovido o aparecimento e colonização de espécies não indígenas em estuários distribuídos por todo o mundo e o estuário do Guadiana (Sul de Portugal) não é exceção. O estuário do Guadiana tem sofrido ao longo dos tempos diversos impactos antropogénicos, incluindo, a construção da barragem do Alqueva em 2002 que promoveu a regulação do caudal do rio. Com a regulação do caudal, a salinidade da água tornou-se mais alta e mais estável, principalmente na zona do meio estuário que antes da construção da barragem era caracterizada por grandes variações de salinidade. Assim, as condições de salinidade mais altas e estáveis, juntamente com o facto desta zona ser caracterizada por ter uma baixa abundância e diversidade de espécies indígenas promoveram o aparecimento e suporte de espécies marinhas não indígenas como a medusa do mar negro *Blackfordia virginica* e mais recentemente o caranguejo azul *Callinectes sapidus.* A *B. virginica* tornou-se a espécie não indígena com uma maior distribuição no estuário e os seus blooms sazonais tornaram-se um motivo de preocupação devido aos seus consideráveis impactos tanto a nível económico como ecológico. Por exemplo, após o aparecimento desta espécie, foi observado uma diminuição na abundância de todos os organismos zooplantónicos incluindo de larvas e ovos de peixes. No entanto, devido às propriedades das medusas, como o alto teor em proteínas e minerais e o baixo teor lípidos, estas podem representar uma oportunidade como fonte de alimento para a população humana e assim contribuir para o controle dos seus blooms nos ecossistemas. De facto, com o aumento da população humana e consequente redução dos "stocks" de peixe, as fontes de alimento alternativas e saudáveis nunca foram tão importantes, e as medusas poderão ser uma delas. Por outro lado, e apesar de frequentemente se pensar que as medusas não são consumidas por organismos aquáticos devido ao seu elevado teor em água, estudos recentes demonstraram que as mesmas são muitas vezes uma fonte de alimento importante na dieta de organismos aquáticos incluindo de caranguejos e espécies indígenas de peixes, muitos deles comerciais. Assim, os objetivos deste trabalho são (1) estudar o perfil nutricional da *B.virginica*, tendo como hipótese que é uma fonte alternativa e saudável de alimento para a população humana se seguir o mesmo perfil nutritional de outras espécies de medusas usadas atualmente na colinária e (2) avaliar a sua contribuição como fonte de alimento para consumidores oportunistas na zona do meio estuário do Guadiana, testando a hipótese que predadores generalistas como caranguejo azul não indígena, e o caranguejo verde indígena *Carcinus maenas* irão beneficiar dos sasonais blooms da *B. virginica*, consumindo esta medusa. O valor nutricional da *B.virginica* foi analisado através de métodos disponíveis para determinar os lípidos totais (protocolo modificado do método de Bligh & Dyer), a proteína em bruto (analises elementares de azoto), o teor de cinzas (por incineração), os ácidos gordos metils esteres (por GC-MS), os

aminoácidos (fase reversa de HPLC) e finalmente os minerais (MP-AES). Neste estudo a atividade antioxidante também foi avaliada através de quatro métodos diferentes (ABTS, DPPH, ICA, CCA). Os nossos resultados sugeriram que a biomassa seca da *B. virginica* é composta por proteínas e minerais e aminóacidos essenciais, apresentando um perfil nutricional semelhante a medusas que são consideradas comestíveis. No entanto, a preseça de cádmio, que é um elemento tóxico, foi encontrado em altas concentrações na biomassa da *B. virginica* (3mg/Kg) e assim esta espécie poderá ter um grande potencial para ser utilizada na alimentação humana se os níveis de cádmio diminuírem. Para avaliar a contribuição da *B. virginica* na dieta dos consumidores selecionados, foi utilizada uma combinação de técnicas de análise molecular nos conteúdos estomacais dos caranguejos (DNA-PCR) com análises de isótopos estáveis (SIA) de carbono ($\delta^{13}C$:$^{13}C/^{12}C$) e azoto ($\delta^{15}N$:$^{15}N/^{14}N$). Os nossos resultados revelaram a presença da *B. virginica* nos conteúdos estomacais do caranguejo azul, no entanto com uma baixa contribuição, segundo os resultados do modelo de mistura Bayesiana de isótopos estáveis. Esta baixa contribuição poderá ter sido resultado de um grande período de "turnover" dos tecidos musculares dos caranguejos, ou seja, poderá não ter havido tempo para a *B. virginica* se incorporar nos tecidos. Estes resultados podem então sugerir que a *B. virginica* poderá estar a facilitar uma segunda invasão por estar a contribuir como fonte de alimento para o caranguejo azul. Assim, o uso da *B.virginica* na alimentação humana poderá representar uma oportunidade para diminuir a abundancia desta espécie no ecossistema uma vez que poderá estar a facilitar diretamente o sucesso de invasão do caranguejo azul.

Palavras-chave: Medusa exótica**;** caranguejo azul, Estuário do Guadiana, composição nutricional, isotopos estáveis

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^{12}C carbon isotope with an atomic mass of 12

^{13}C carbon isotope with an atomic mass of 13

^{14}N nitrogen isotope with an atomic mass of 14

^{15}N nitrogen isotope with an atomic mass of 15

C:N carbon to nitrogen ratio

δ The "delta" expresses the variation of an isotopic ratio of an element R relative to the isotopic ratio of a standard R_{std} **NIS** non-indigenous species

POM particulated organic matter

SOM soil organic matter

MPB microphytobenthos

EPI epiphytes

FF filter feeders

RSA radical scavenge activity

DPPH 1-diphenyl-2picrylhydrazyl

ABTS 2,2 -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid

ICA Metal chelating activity on iron

CCA Metal chelating activity on copper

EDTA ethylenediamine tetraacetic acid

FAMEs fatty acids methyl esters

GC-MS Gas Chromatography Mass Spectrometry

HPLC High Pressure Liquid chromatography

MP-AES Microwave Plasma-Atomic Emission

PCR Polymerase Chain Reaction

HCL Hydrochloric acid

HNO3 Nitric acid

CW carapace maximum width

µl microliters

mg milligrams

SIA Stable isotopic analysis

PCR polymerase chain reaction

ME metabolized energy

TL trophic level

ETM estuarine turbidity maximum

SD standard desviation

AA Amino acids

EAA Nutritionally essential amino acids

NEAA Nutritionally non-essential amino acids

DW dry weigth

FW fresh weigth

Chapter 1 – INTRODUCTION

1. INTRODUCTION

Estuaries are among the most productive ecosystems on the planet, supporting high abundances of several species with ecological and socio-economic interest (Kokum et al. 2002). Yet, they are among the most endangered ecosystems due to a variety of direct and indirect anthropogenic disturbances such as pollution, marine and coastal construction, maritime transport, overfishing, climate change, and the introduction of non-indigenous species (NIS; Halpern et al. 2008). Non-indigenous species have been increasing in number in estuaries, especially in the brackish areas, as a consequence of intense shipping and opening of new transport routes (Paavola et al. 2005). Indeed, NIS have a higher potential to survive under extreme conditions during transportations and can adapt well to the new conditions found in the invaded areas (Theoharides and Dukes 2007). More specifically, NIS are more opportunistic species with higher ability to adapt to new habitats than indigenous species (Theoharides and Dukes 2007). Explanations to why brackish areas are normally prone to introductions of NIS include: (1) most ports worldwide are located at the river mouths, areas where the discharge of ballast water often contains euryhaline species; (2) the wide salinity gradients in brackish waters provide a greater range of opportunities for NIS to establish in the estuary; and (3) the low species richness in brackish waters provides less competition for establishing (Paavola et al. 2005). Biological invasions are considered one of the most important direct drivers of biodiversity loss due to both direct biotic interactions with the indigenous community, such as predation and competition (*e.g.*, O'Neill et al. 2015; Bohn et al. 2007) and indirect changes in habitat conditions such as turbidity and habitat structure (e.g., Crooks 2002; Gallardo et al. 2016). Thus, new trophic links can be created affecting the demography and abundance of indigenous species (David et al. 2017). It is expected that invasive species trigger distinct changes depending on their position in the food web (Pace et al. 1999). Direct predation usually occurs when invasive species occupy higher trophic levels, being the predominant mechanism by which invaders can dramatically decrease populations of indigenous species, exerting a top-down control in the food web (Bruno et al. 2005). The top-down impacts of an invasive predator may propagate both negative and positive changes in the abundance and biomass of lower trophic levels in what is often called a cascade effect (White et al. 2006). For example, the invasion of the carnivorous ctenophore comb jelly *Mneomiopsis leidyi* (Agassiz, 1865) in the Black and Caspian Sea caused dramatic

reductions in zooplankton abundance mainly in ichthyoplankton and zooplanktivorous fish populations (Katsanevakis et al. 2014). This resulted in an increase in the abundance of phytoplankton and bacterioplankton populations, triggering increases in the abundance of zooflagellate populations and causing an overall decline in water quality (Katsanevakis et al. 2014).

When NIS and indigenous species share the same resources or the same space, competition can occur. For example, the non-indigenous sessile invertebrates such as the barnacles *Amphibalanus improvises* (Darwin, 1854) and *Austrominius modestus* (Darwin, 1854) and the freshwater hydroid *Cordylophora caspia* (Pallas, 1771) have been reported to dominate benthic communities in European waters, outcompeting other indigenous sessile species for space, which resulted in an ecological displacement (Kochmann et al. 2008). Also, some invasive fish species such as the Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) in the northern Gulf of Mexico coastal areas had a strong impact on indigenous fish such as the redspotted sunfish *Lepomis miniatus* (Jordan,1877) by competitive exclusion from their preferred habitat (Martin et al. 2010).

Therefore, the effects of NIS typically tend to be negative on the diversity and abundance of species that occupy the same trophic niche due to competition for food or space (Thomsen et al. 2014). However, in some cases, they can produce positive impacts on the diversity of species at higher trophic levels. The relative trophic position states that NIS may often impact different trophic levels around them and such impacts can be negative or positive (Thomsen et al. 2014). For example, in the Amstel River (West Holand) the NIS *C. caspia* can negatively impact indigenous Bryozoans by competing for space, while it represents an important food source for local gastropods and amphipods (Roos et al. 1979). The non-indigenous crayfish *Procambarus clarkii* (Girard, 1852) in the Minho River (Northwest Portugal) competes with other invertebrates and vertebrates for food and space, however it is an important food source for higher trophic levels, such as mammals, birds, and fishes (Tablado et al. 2010; Sousa et al. 2013). Also, NIS can act as ecosystem engineers, *i.e.*, affecting other biota via alterations to the abiotic environment by creating, destroying or modifying their habitats (Crooks 2002). For example, they can change water transparency, nutrients and organic matter concentration, and can also provide habitat formation (Gallardo et al. 2016) as for example, the non-indigenous zebra mussels *Dreissena polymorpha* (Pallas, 1771) in the Great Lakes. This species, despite exerting a bottom-up control in the local food webs by filtering plankton from the water column, can increase the water transparency

and create dense shell beds which harbour relatively high densities of other small invertebrates (Ricciardi et al. 1997). Thus, NIS are often an important new resource benefitting the higher trophic levels in the recipient ecosystems, but this positive effect may be opposed or reversed not only by the decline in the abundance and diversity of indigenous species, but also if the higher trophic levels that are benefiting with the NIS occurrence include other NIS (Simberloff and Von Holle 1999; Grosholz 2005; Green et al. 2011; David et al. 2017, O'Loughlin and Green, 2017). More specifically, successful primary invaders may sometimes facilitate directly or indirectly the invasion success of secondary invaders by altering attributes of the recipient communities and propagule pressure (*i.e.,* a composite measure – quality, quantity, and frequency - of the number of invading organisms (Groom et al. 2006; Green et al. 2011)). Indeed, examples of facilitation of one invader by another are very common (Green et al. 2011). For example, the invasion of the blueback herring *Alosa aestivalis* (Mitchill, 1814), in Lake Ontario, may have been facilitated by the prior establishment of abundant prey such as zebra mussel larvae and the cladoceran *Eubosmina coregoni* (Baird, 1857) (Molloy et al. 1997; MacNeill 1998). Also, in the St. Lawrence River, the european faucet snail, *Bithynia tentaculata* (Frauenfeld, 1862), has tripled its abundance in association with the growth of dense invasive mussel populations, whose shells support rich microflora and provide the small snail with increased grazing area (Ricciardi et al. 1997) and refuge from large predators (Stewart et al. 1994). Therefore, interactions among NIS can facilitate secondary invasions and accelerate the overall rate of invasion (Simberloff and Von Holle 1999). Non-indigenous species can also show a high site-related variability in trophic position, thus impacting species communities at multiple trophic levels. For example, the crayfish *Cambarus bartonii* (Fabricius, 1798) in Powder Creek (Eastern New Zealand) is known to affect intermediate consumers and their basal resources because they are omnivorous (Usio, 2000). More specifically, crayfish, decrease the quality of the leaf material due to their shredding activites, which becomes unlikely to be attractive to other invertebrates mainly to collector-gatherers (Oligochaeta and Oribatei) and predatory chironomids (Tanypodinae). In addition crayfish also predates on this invertebrates (Usio, 2000).

1.1. Non-indigenous species in the Guadiana estuary

Aquatic invasions have been reported in estuaries worldwide, including in the Guadiana estuary (South Portugal, Southwestest Iberian Peninsula; Fig.1.1). The construction of the Alqueva dam promoted a decrease in the mean annual river discharge causing profound changes in its geological, physical, chemical, and biological conditions (Morais 2007, 2008), specifically on the downstream estuarine habitats and adjacent coastal ecosystems (Muha et al. 2013). The decrease in the river flow and the considerable shipping traffic in the Gulf of Cadiz contributed to the increase in the number of NIS in this estuary, mainly in its brackish portion (salinity values varying between 0.5 and 25 PSU; Chícharo et al. 2006a) which corresponds to the middle estuary (Foz de Odeleite to Porto Cinturão, Fig.1.1; Chícharo et al. 2006a; Montagna et al. 2013). Before the dam´s construction, the middle Guadiana estuary was characterized by a reduced number of species mainly due to marked salinity variations along the year at the estuarine turbidity maximum (ETM; Chícharo et al. 2001; Garel et al. 2009; Morais et al. 2009a). The few species present were mainly suspension and deposit-feeders that helped to minimize the water quality degradation (Chícharo et al. 2006a). After the dam's construction, freshwater inflow became the most important factor determining abiotic and biotic variability in the Guadiana estuary (Morais et al. 2008). The ETM shifted more than 10 km upstream to Guerreiros do Rio as a consequence of changes in salinity (salinity became more stable) and temperature gradients, and nutrient stoichiometry, (Chícharo et al. 2006a; Morais 2007). The flow regulation decreased the distribution and abundance of indigenous ichthyoplankton species such as european achovy *Engraulis encrasicolus* (Linnaeus, 1758) and european pilchard *Sardina pilchardus* (Walbaum, 1792), due to physical obstructions and destruction of spawning and nursery areas (Moura et al. 2003; Chícharo et al. 2006b). Thus, the number of indigenous species decreased, leaving this habitat empty for NIS to colonize and dominate the food web in this area (Chícharo et al. 2006b; Montana et al. 2013; Morais et al. 2018).

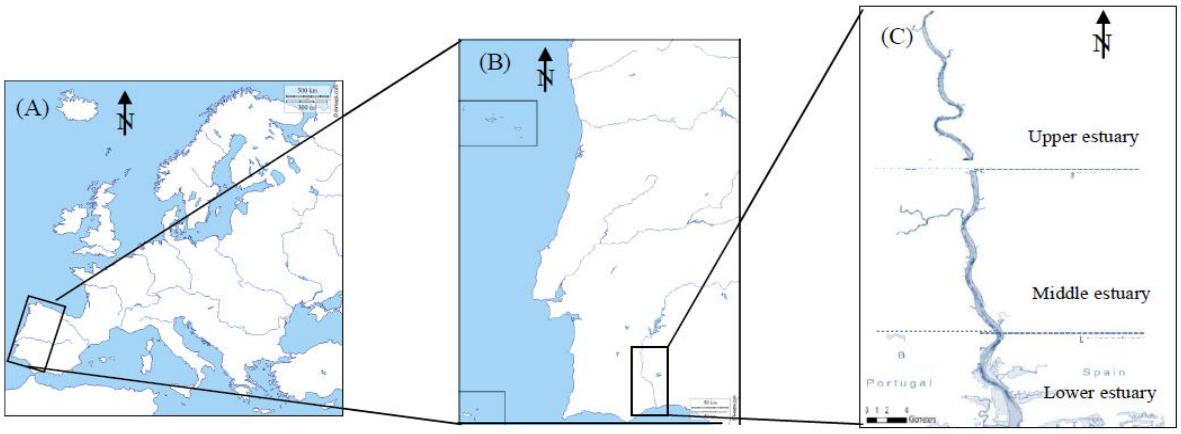


Figure 1.1 (A) Geographical context of the Guadiana estuary in Europe, northern African coast and Iberian Peninsula (B) Geographical context of the Guadiana estuary in Portugal; (C) represents the three different areas of the estuary: the upper estuary, the middle estuary and the lower estuary (Maps retrieved from D-Maps).

Currently more than 10 NIS have been identified in this estuary, which include the calanoid copepod *Acartia tonsa* (Dana, 1849; Crustacea: *Copepoda*; Mattos, 2016), the asian clam *Corbicula fluminea* (Müller, 1774; Mollusca: *Bivalvia*; Morais et al. 2009b); the oriental shrimp *Palaemon macrodactylus* (Rathbun, 1902; Crustacea: *Decapoda*; Chícharo et al. 2009), the freshwater hydroid *C. caspia* (Seyer et al. 2017), the black Sea jellyfish *Blackfordia virginica* (Mayer, 1910; Cnidaria: *Hydrozoa*; Chícharo et al. 2009), the weakfish *Cynoscion regalis* (Bloch and Schneider, 1801; Pisces: *Sciaenidae*; Morais and Teodósio 2016), and more recently the blue crab *Callinectes sapidus* (Rathbun, 1896; Morais et al. 2018). These species have the potential to cause several ecological impacts, which will vary according to their position in the food web and functional traits (Chícharo et al. 2009; Gallardo et al. 2016; Morais and Teodósio, 2016; Seyer et al. 2017).

The first trophic level in the middle estuary is composed by phytoplankton and marine plants, such as *Spartina* spp. Typically, phytoplankton abundance is higher during spring and summer months being dominated by chlorophytes (mainly *Dictyosphaerium reniforme*, *Crucigenia tetrapedia*, *Scenedesmus acutus*), unidentified *Cryptophyceae*, diatoms (*Cyclotella* sp., *Melosira* sp., *Leptocylindrus minimus*), and cyanobacteria (mainly

Microcystis spp.; Chícharo et al. 2006b), and their productivity varies according to freshwater discharges (Chícharo et al. 2006b; Mattos, 2016). An increase in the freshwater discharge increases phytoplankton diversity due to reduction in the competition for nutrients (Chícharo et al. 2006a). This favours an increase in the primary consumers abundance and diversity (Chícharo et al. 2006a; Muha et al. 2012; Mattos, 2016). The most abundant primary consumers include several copepod species such as *Acartia clausii* (Giesbrecht, 1889), *Calanipeda aquaedulcis* (Kritschagin, 1873), the invasive *A. tonsa*, and the cladocerans *Bosmina longirostris* (O.F. Müller, 1785; Chícharo et al. 2006b). The highest abundances of primary consumers are usually observed during spring/summer and autumn (Chícharo et al. 2006b; Mattos, 2016; Cruz et al. 2017). The limited distribution of *A. clausii* has been attributed to the introduction of *A. tonsa* which is an euryhaline, eurythermic, and eutrophic species with a high level of success as an invader (Mattos, 2016; Cruz et al. 2017). Along with some zooplankton species, filter- and deposit feeders also consume phytoplankton including the invasive asian clam (Boltovskoy et al. 1995; Dias et al. 2014). The asian clam is one of the 100 worst invasive species in Europe (DAISIE, 2019) and was first reported in the middle Guadiana estuary in 2000 (Chícharo et al. 2000; Pérez-Bote et al. 2008). Now it has spread to areas downstream of the Alqueva dam (Morais et al. 2009b) with the potential to compete with adult and juvenile indigenous bivalves for food and space (Pérez-Bote et al. 2008).

Secondary consumers (*i.e.*, feeding on zooplankton and other primary consumers) in the middle estuary include planktivorous fishes such as european anchovy, european pilchard, and salema *Sarpa salpa* (Linnaeus, 1758; Chícharo et al. 2006b). Despite the decrease on these species' survival after the Alqueva dam construction (Chícharo et al. 2006b), fish display substantial seasonal variations in abundance, being more abundante during spring and summer months, when the zooplanktonic productivity is usually high along the Portuguese coast and adjacent estuaries (Chícharo et al. 2006b; Faria et al. 2006). Therefore, they act as key species, preventing zooplankton community from achieving the situation of high dominance and favouring an increase on zooplankton diversity (Chícharo et al. 2006b). Although summer months have the highest ichthyoplankton abundances, sharp decreases between months may be explained by the increase in jellyfish abundance. The NIS *P. macrodactylus* and the indigenous delta prawn *Palaemon longirostris* (Milne-Edwards, 1837; caridean shrimps) along with other crustaceans such as amphipods, isopods, and barnacles also consume zooplankton (Chícharo et al. 2009).

Palaemon macrodactylus appears to be a very successful invader, able to colonise a wide geographical range with a varied range of temperature and salinity (Ashelby et al. 2004; González-Ortegón et al. 2006; Beguer et al. 2007). Thus, it has been found in several Europeans waters, including in Spain (Cuesta et al. 2004), England (Ashelby et al. 2004), and Portugal (Guadiana estuary; Chícharo et al. 2009). Like other carideans, *P. macrodactylus* is a carnivorous species, feeding mainly on animal fragments (Chícharo et al. 2009). Because both caridean shrimps (*P. macrodactylus* and *P. longirostris*) have similar diets, a dietary overlap between the NIS *P. macrodactylus* and indigenous populations of *P. longirostris* appears to occur in the Guadiana estuary (Ashelby et al. 2004; Chícharo et al. 2009). Furthermore, in this estuary *P. macrodactylus* may compete for food and habitat with the indigenous fish larvae such as early stages of european anchovy, european pilchard, common goby *Pomatoschistus* sp., (Kroyer, 1838), common sole *Solea* sp. (Quensel, 1806), common two-banded seabream *Diplodus vulgaris* (Geoffroy, 1817), and with the greater pipefish *Syngnathus* sp. (Linnaeus, 1758; Gonçalves et al. 2017). The non-indigenous hydrozoans *C. caspia* and *B. virginica* also feed on zooplankton (Chícharo et al. 2009; Morais et al. 2017a). *Cordylophora caspia* is native from the Ponto-Caspian region where it inhabits brackish and freshwater environments because it tolerates salinities from 0 to 40 PSU (Seyer et al. 2017). It was first observed in the middle area of Guadiana estuary in June 2015 where it could have been introduced by shipping activities (Seyer et al. 2017). Predation by marine or brackish invertebrates might have prevented *C. caspia* from colonizing the lower estuary, being an important food source for gastropods and amphipods (Roos et al. 1979; Seyer et al. 2017). *Cordylophora caspia* is included in Europe's 100 worst invasive species (DAISIE 2019), because it facilitates the settlement of invasive dreissenid mussels by providing additional surface area for their settlement and competes for food with larvae and juveniles of benthivorous fish (Pucherelli et al. 2016; Seyer et al. 2017). The ecological impacts in the Guadiana estuary are yet to be determined but the competition for settlement space may be relevant with other sessile invertebrates, like mussels, oysters, and bryozoans. However, the potential for competition for food with planktivorous fish species and larval phases is probably minimal since the population standing stock is low owing to the small amount of settlement habitat available despite the species broad distribution along the estuary (Seyer et al. 2017).

The non-indigenous weakfish and the blue crab along with the indigenous jellyfish (*e.g.* moon jellyfish *Aurelia aurita* (Linnaeus 1758), *Maeotias marginata* (Modeer 1791) and *Catostylus tagi* (Haeckel, 1869; Muha et al. 2016)), fish (*e.g.*, meagre *Argyrosomus regius;* Asso, 1801; Perciforme: *Sciaenidae)*), and crabs (*e.g.*, european green crab *Carcinus maenas;* Linnaeus, 1758) are expected to occupy high trophic positions in the in the middle Guadiana estuary food web. The weakfish is native to the east coast of North America and recently was detected by fishermen in several regions of the Iberian Peninsula where they could have been introduced by ballast waters (Morais and Teodósio 2016). They were captured in the Sado estuary (July 2014), Gulf of Cadiz (November 2015) and latter in the Guadiana estuary (June 2016; Morais and Teodósio 2016). It was hypothesized that individuals of established populations from Sado and Gulf of Cádiz dispersed and colonized other estuaries and regions, such as the Guadiana estuary (Morais et al. 2017b). This species uses both coastal areas and estuarine ecosystems as spawning, nursery (from spring until late summer and early autumn), and feeding areas, preying mainly on bivalves, amphipods, isopods and other small invertebrates, and also on fish species such as European anchovy (Morais and Teodósio 2016; Bloch et al. 2017; Morais et al. 2017b). Meagre is an indigenous species from the Guadiana estuary with a high economic importance at least since the 18th century (Prista 2013; Morais and Teodósio 2016). Both weakfish and meagre share several ecological characteristics, including feeding upon similar types of prey (*e.g.*, fish, penaeid and mysid shrimps, crabs, amphipods, clams, and annelids), using estuaries as nurseries during the same period, and seeking protection in holes and deep channels (Morais and Teodósio 2016). Thus, there is a high potential for competition between these species.

The blue crab is a euryhaline and eurythermal species (Beqiraj, 2010) and its native range includes the western North Atlantic Ocean from Nova Scotia to Argentina. However, during the beginning of the 20th century, its range has expanded to Africa, Asia, and Europe probably due to the increase in global temperatures (Manfrin et al. 2016; Morais et al. 2018). It was first observed in the middle Guadiana estuary on June 2017 (Morais et al. 2018). This species preys on clams, annelids (polychaetas), oysters, mussels, smaller crustaceans, freshly dead fish, plant and animal detritus, and can also prey on smaller and soft-shelled blue crabs and on indigenous crabs (Manfrin et al 2016; Mancinelli et al. 2017; Morais et al. 2018). Because it is a generalist predator, it has the potential to impact the diversity and structure of the local benthic communities. In the Guadiana estuary, the blue

crab distribution range overlaps with the distribution range of the European green crab which is present both in the lower and middle estuary (Morais et al. 2018). The competition between the blue crab and the green crab is modulated by temperature-dependent interactions (Rogers et al. 2018). At low temperatures, green crabs prey upon blue crabs, while at higher temperatures similar size blue and green crabs have similar competition capabilities but larger blue crabs can prey on green crabs (Rogers et al. 2018). Then, a significant increase of the blue crab may have severe consequences for the autochthonous ecological communities (Manfrin et al 2016; Morais et al. 2018).

1.2. The colonization of the Guadiana estuary by the jellyfish *Blackfordia virginica*

The *B. virginica* is one of the most widespread NIS in the Guadiana estuary (Fig. 1.2.; Muha et al. 2013). Normaly this species has higher abundances in the brackish zone, where there is a highly productive ETM zone (Chícharo et al. 2006a). Such productivity provides suitable conditions for medusa being their preferable habitat (Chícharo et al. 2009; Muha et al. 2013). However, lower densities of *B. virginica* species were noticed in other areas of the estuary due to their capacity to tolerate high salinity variations (Chícharo et al. 2009). The density of *B. virginica* also varies along the years: in wet years they have not been observed which suggests that freshwater pulses control the density of jellyfish populations (Marques et al. 2017).

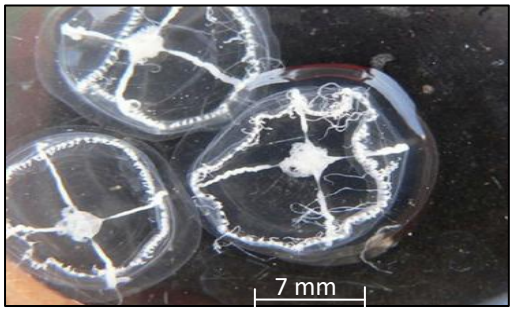


Figure 1.2 *Blackfordia virginica* jellyfish (Chícharo et al. 2009)

The ability of *B. virginica* to occur in blooms, like other cnidarians, is due to the fact they can reproduce both asexually and sexually (Fig. 1.3; Kimber et al. 2014; Purcel et al. 2007). Sexual reproduction occurs during the medusa stage with sexually mature adults releasing eggs and sperm daily into the water column (Baumsteiger et al. 2017). After fertilization and a period of growth, the eggs hatch and settle on a hard substract as planular larvae (Kimber et al. 2014). The planular larvae then form small benthic

polyps (0.5 mm), which reproduce asexually by budding to achieve a stacked colony of same-sex medusa (Kimber et al. 2014; Baumsteiger et al. 2017). These polyps release larval medusa, when environmental conditions are favourable, normally during spring and summer (Kimber et al. 2014; Baumsteiger et al. 2017). These newly released medusae are small (1 mm in diameter), but will eventually grow to *ca*. 14 mm and reach sexual maturity (Figs. 1.2 and 1.3; Kimber et al. 2014).

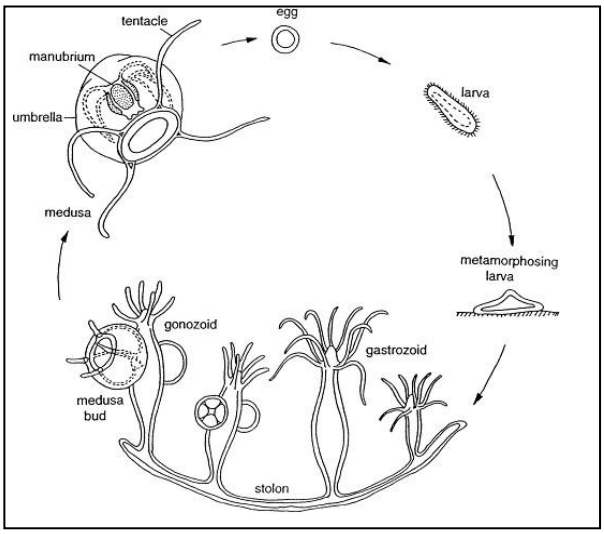


Figure 1.3 Hydrozoan life cycle (Aerne et al. 1996).

The *B. virginica* was first described by Mayer during autumn 1904 in Virginia USA, but later Theil (1935) found that this species was a common member of the hydromedusa community of the Black Sea suggesting that it is actually native from the Black Sea region (Theil, 1935). Established *B. virginica* populations can be found worldwide tolerating a wide range of temperature (16.5 to 23ºC) and salinity values (2 to 35 PSU). In Portugal they were first observed in the Mira estuary (Moore, 1987) and latter in the Guadiana estuary (June 2001; Muha et al. 2012) with abundances of 0.22 ind.m3 and a maximum density of 31.5 ind. m3 recorded in July 2008 (Chícharo et al. 2009), precisely in the middle area of the estuary, where they could have been introduced in either the medusa or the polyp stages (or both), probably by nautical activities (Chícharo et al. 2009; Freire et al. 2014). Although *B. virginica* has been reported over a wide geographic area, it is mainly restricted to scattered records within estuarine areas of temperate and tropical regions (Moore, 1987; Kimber, 2014).

This distribution pattern could be the result of repeated introductions, with medusae having limited dispersive capacity. Also, the asexual form of this hydromedusae life cycle is a reduced polyp stage with a short benthic life, which makes its records rare. Dormant stages may play an important role in the dispersal of the species, nonetheless they are unknown (Bardi and Marques 2009).

In the Guadiana estuary the abundance of *B. virginica* started to increase in years after the Alqueva dam construction due to the river discharge regulation (Chícharo et al. 2009; Muha et al. 2013; Amorim et al. 2017). According to Amorim et al. (2017), years with high freshwater discharge during winter and spring are correlated with lower jellyfish densities during the following summers in the Guadiana estuary. When salinity is lower, the polyps decrease their feeding behaviour maybe due to physiological reaction or osmotic stress, but also due to a degeneration of tentacles as observed in other studies (Holst and Jarms 2010). This affects the survival, ecophysiological performances (*i.e.*, feeding rate and swimming ability), and budding of early life stages (Amorim et al. 2017). When the salinity increases, strobilation will also increase (Amorim et al. 2017). Low water temperature values favour strobilation but decrease ephyra and medusa growth (Amorim et al. 2017). Thus, a reduction in the river discharge, after the Alqueva dam construction, resulted in an overall increase in the salinity conditions in the estuary which likely favoured the establishment of this NIS, especially during the summer months when the water temperature is higher (Muha et al. 2013). Therefore, blooms formation largely depends on the successful development of the early life stages, with temperature and salinity playing a fundamental role in the performance and survival of polyps, ephyra, and medusa stages.

The *B. virginica* is only present from early summer to latter autumn in the Guadiana estuary, at least the medusa stage (Muha et al. 2013). During the spring and summer months light intensity is higher in this estuary which promotes an increase in the abundance of *B. virginica* prey such as barnacle nauplii, copepods and their eggs (Moyle and May 2011). Also during this period, water transparency is higher which reduces the contact damage between sediments and detritus and their fragil polyps (Baumsteiger et al. 2017), leading to an increase in polyps productivity (Moyle et al. 2011).

Although zooplankton is the preferential prey of *B. virginica* (Morais et al. 2017a), this species is a generalist predator feeding on invertebrates, fish eggs and larvae (Chícharo et al. 2009; Freire et al. 2014; Kimber, 2014; Morais et al. 2017a).

Surprisingly, detritus are also assimilated by this jellyfish being hypothesized that terrestrial-derived organic matter might support good physiological condition of *B. virginica* during periods of low metazooplankton abundance, through a detritus-based microbial food web (Morais et al. 2017a).

Across the world, several negative effects have been documented when *B. virginica* occurs in high densities (*i.e.*, 650 to 1700 ind.m-3; Chícharo et al. 2009; Freire et al. 2014; Kimber 2014; Morais et al. 2017a; Jaspers et al. 2018). For example, in northeast Brazil, large concentrations of these medusae were found in nurseries for mullets at the fish culture base of Itamaraca. The abundances were so high that the water inflow to these nurseries was not possible due to the gelatinous masses (Freire et al. 2014). In addition, in the Kiel Canal, high densities of this species were observed and resulted in a decreased on gobbid fish larvae (Jaspers et al. 2018).

In the Guadiana estuary, *B. virginica* represents a high risk to local zooplankton standing stocks, reducing the density of all zooplaktonic organisms, including eggs and fish larvae (Chícharo et al. 2009). Although there have been no conclusive studies on the impacts of *B. virginica* in this ecosystem, there have been many inferences on their potential impacts based on their ecological characteristics (Wintzer et al. 2013; Kimber, 2014). For example, a further spreading and abundance increase of this specie may not only result in food competition with local planktonic feeders, including indigenous pelagic fish such as the european pilchard, the european anchovy, the common sole, the grater pipefish, the common goby, and the two-banded seabream, but additionally cause direct predation on fish early life stages. Thus, it can cause not only economic losses (Chícharo et al 2009; Baumsteiger et al. 2017; Jaspers et al. 2018) but also changes in the food web structure and dynamics (Moyle et al. 2011; Carman et al. 2017).

Therefore, in estuaries where *B. virginica* is most commonly found, it is expected a profound impact as often estuaries act as nurseries for juvenile fish (Kimber et al. 2014).

1.3. *Blackfordia virginica* as a potential source of nutritional and bioactive elements

Despite the potential negative impacts in the ecosystem, jellyfish may be regarded as a new source of food and bioactive chemical compounds due to their usually high abundances and high regenerative and reproduction potential (Piraino et al. 2015). Innovative and sustainable food sources with high nutritional value have never been more important than nowadays, due to the exhaustion of several fish stocks, which

undermines food security by reducing the supply of a vital source of dietary protein (Brunner et al. 2008; Chang et al. 2015; Piraino et al. 2015).

Jellyfish have been consumed for more than 1000 years and recently became a commercial fishery (Piraino et al. 2015). The processed (dried) jellyfish was first introduced in China for human consumption and recognized as one of Asia's top food with a unique taste (Piraino et al. 2015). In recent decades, consumption of jellyfish has increased, especially in Asia, resulting in the growth of commercial jellyfish fisheries and in the development of a multi-million-dollar mariculture industry (Piraino et al. 2015). However, while jellyfish were exclusively exploited in the Eastern Asia in the past, nowadays due to worldwide migrations from China, jellyfish based products are increasingly spreading to India, Mexico, Australia, Turkey, and United States (Armani et al. 2014; Graham. et al. 2014).

The Food and Agriculture Organization of the United Nations (FAO) proposed that in order to reduce jellyfish populations they could be used as medicine or food, since their biomass could be a valuable source of bioactive compounds and essential nutrients unavailable or poorly present in products from terrestrial plants and animals (Arnani et al. 2014; Piraino et al. 2015). There are more than 200 species of Scyphozoa, about 50 species of Staurozoa, 20 species of Cubozoa, and 1000-1500 species of Hydrozoa that produce medusa, including *B. virginica* (Marques et al. 2004). According to some studies, several species of the scyphozoan jellyfish (25 to 30 species; Table 1.1) are appreciated in South- East Asia and Europe (Armani et al. 2014) not only for their texture and taste, but also because they ensure a low caloric diet, being low in fat and cholesterol (Yuferova et al. 2015, Zhu et al. 2015). Indeed, over 95% of jellyfish body weight is water, whereas the dry weight (DW) is in the range of about 3-5% of fresh weight (FW¸Piraino et al. 2015). Carbon is typically lower than 15% of DW where in non-gelatinous groups it accounts for up to 30-60% (Piraino et al. 2015; Chan et al. 2015). The organic content (%) is mainly represented by protein (5 - 30% of DW), while lipids (2 - 10% of DW) and carbohydrates (0.5 - 1.7% of DW) are minor components of the jellyfish tissues (Lucas et al. 2008; Piraino et al. 2015). However, jellyfish composition varies with species, season, and capture location (Xu, 2010). For example the proximate composition of coronate jellyfish *Atolla wyvillei* (Haeckel, 1880) from the Southern Ocean in % of FW is 0.83% protein, 0.21% lipid and 0.08% carbohydrates (Lucas 2008). In addition the large edible jellyfish, *Stomolophus nomurai* (Kishinouye, 1992) in Japan contains about 0.40% proteins, 0.02% lipids and 0.58%

carbohydrates (Huang 1988).

Table 1.1 Known edible jellyfish from Asia and Europe.

Edible jellyfish species	Comon name	Reference
Scyphozoa: Rhizostomatidae		
Rhopilema esculentum (Kishinouye, 1891)	Red jellyfish	Zhuang et al. 2009
*Rhopilema hispidum (*Vanhöffen, 1888)	Sand jellyfish	Khong et al. 2016
Rhizostoma sp.	Barrel jellyfish	
Rhizostoma pulmo (Macri, 1778)	-	Leone et al. 2015
Rhizostoma luteum (Quoy and Gaimard, 1827)	-	Prieto et al. 2018
*Acromitus hardenbergi (*Stiasny, 1934)	River jellyfish	Khong et al. 2016
*Crambione mastigophora (*Maas, 1903)	Prigi jellyfish	KitamuraandOmori 2010
Crambionella sp.	Cilacap jellyfish	KitamuraandOmori 2010
*Crambionella orsini (*Rao, 1931)	Ball jellyfish	KitamuraandOmori 2010
*Catostylus mosaicus (*Quoy and Gaimard, 1824)	Jelly blubber	
*Catostylus tagi (*Haeckel, 1869)	-	Morais et al. 2009
Labonemoides sp.	White jellyfsh	KitamuraandOmori 2010
*Lobonemoides gracilis (*Light, 1914)	-	KitamuraandOmori 2010
*Lobonemoides robustus (*Stiasny, 1920)	-	KitamuraandOmori 2010
*Lomonema smithi (*Mayer, 1910)	-	KitamuraandOmori 2010
Stomolophus nomurai (Kishinouye 1922)	Nomura's jellyfish	Huang, 1988
Stomolophus meleagris (Agassiz, 1860)	Cannonball jellyfish	Kitamura and Omori 2010
Catylorhiza tuberculata (Macrì, 1778)	Fried-egg jellyfish	Leone et al. 2015
Scyphozoa: Semaeostomeae		
Aurelia sp.	Moon jellyfish	
Aurelia aurita (Linnaeus, 1758)	-	Wakabayashi et al. 2015
Aurelia coerulea (von Lendenfeld, 1884)	-	Leone et al. 2015
Chrysaora pacifica (Goette, 1886)	Japanese sea nettle	Wakabayashi et al. 2015
Pelagia noctiluca (Forskal)	Purple jellyfish	Costa et al. 2019
Scyphozoa: Coronatae		
Periphylla periphylla (Péron and Lesueur, 1810)	Helmet jellyfish	Lucas et al. 2008

Research has shown that jellyfish proteins exhibit skin photo-protection from ultraviolet (UV) radiation, immunomodulatory, antihypertensive, anti-tumoral, antimicrobial, and antioxidant properties (Arnani et al. 2014; Piraino et al. 2015). For example, collagen is a fibrous and structural protein widely present in the animal tissues as a prevailing component of extracellular matrices in connective tissues and essential to muscle tissue, cartilage and bone (Hsieh and Rudloe, 1994). This is the main structural protein in the jellyfish dry mass (Alves and Reis, 2017). This protein is formed by three polypeptide α chains, arranged as a triple helix enfolded around each other. Each one is

composed of a set of amino acids with a repeated motif of Gly-X-Y, where X and Y are, predominantly, proline and hydroxyproline (Alves and Reis 2017). The abundance of these hydrophobic amino acids favours higher affinity to oil and better emulsifying. As a result, collagen provides natural antioxidant peptides and exerts high antioxidant effects (Zhuang et al. 2009). Therefore, these gelatinous organisms can be an important source of natural compounds with antioxidant activity (Loganayaki et al. 2011). In fact, natural antioxidants can protect the human body from free radicals including hydroxyl radical, hydrogen peroxide, singlet oxygen, and nitric oxide, which are generated in living organisms and that can result in oxidative stress, contributing to a number of health disorders, such as cellular injury and DNA degradation (Loganayaki et al. 2011; Yu et al. 2006). Therefore, molecules and/or products with antioxidant properties are of high interest for the prevention of oxidative-stress related diseases. For example, collagen from the red jellyfish *Rhopilema esculentum* (Kishinouye, 1891) can protect mice skin from the UV radiation damages alleviating the UV-inducing abnormal changes of antioxidant indicators (Piraino et al. 2015; Alves and Reis 2017).

To the best of our knowledge there is no information about the nutritional composition of *B. virginica* biomass, or its biological properties. However, if this species as a similar composition as other edible jellyfish, which includes low fat content (essential omega-3 and omega-6 unsaturated fatty acids) and rich in proteins (*e.g.*, collagen; Morais et al. 2009c) its biomass could be exploited for food and medical purposes acting also as a potential strategy to control its populations in invaded ecosystems.

1.4. *Blackfordia virginica* as a food source for aquatic consumers

Jellyfish have been considered as "trophic dead ends" in the aquatic food webs, which means that they are not susceptible to high levels of predation due to their low nutritional value (Marques et al. 2016; Hays et al. 2018). However, some authors suggested that a large number of pelagic predators may opportunistically consume gelatinous zooplankton (Avian and Rottini Sandrini, 1988; Harbison, 1993). For example, evidence from cameras deployed on the seabed suggests that dead jellyfish represent a significant component in the diet of commercially exploited lobster *Nephrops norvegicus* in Norwegian fjords (Sweetman et al. 2014; Dunlop et al. 2017). In addition, several benthic fish species in the Northwest Atlantic such as the hagfish *Myxini* sp. (Linnaeus, 1758) and grenadier *Coryphaenoides leptolepis* showed high

levels of jellyfish consumption suggesting that jellyfish blooms provide an important food source for the benthic communities (Smith et al. 2016). Furthermore, jellyfish-crab interactions have been reported and some include predation or partial predation by crabs on the jellyfish (Carman et al. 2017). This includes the spider crab *Libinia dubia* (H. Milne Edwards, 1834) in the Mississipi Sound (USA) feeding on the sea nettle *Chrysoara quinqecirrha* (Desor, 1848; Cnidaria e Scyphozoa; Phillips et al. 1969), on the cannonball jellyfish *Stomolophus meleagris* (Agassiz, 186) in Onslow Bay (USA) (Shanks and Graham, 1988; Tunberg and Reed, 2004) or on the moon jellyfish *Aurelia aurita* (Linnaeus, 1758) in Chesapeake Bay (Jachowski, 1963). Also, in a study in the island of Martha's Vineyard in Massachusetts (USA) was observed that the hydromedusae *Gonionemus* sp. was often consumed by the indigenous spider crab and by the indigenous blue crab (Carman et al. 2017).

Predators from higher trophic levels such as turtles, fish, and penguins also consume jellyfish. For example, the moon fish *Lampris sp.* (Retzius, 1799) and butterfish *Peprilus triacanthus* (Peck, 1804) prey exclusively on gelatinous zooplankton, especially during periods of massive proliferation, being a non-negligible source of energy for fishes when an alternative prey is not available (Arai 2005; Doyle et al. 2007). Likewise, different penguin species from polar to temperate habitats were observed to predate on the jellyfish (Thiebot et al. 2017). For example adélie penguins *Pygoscelis adeliae* (Hombron and Jacquinot, 1841) were observed to predate on the jellyfish *Diplulmaris antarctica* (Maas, 1908), yellow-eyed penguins *Megadyptes antipodes* (Hombron and Jacquinot, 1841) were observed to predate on the jellyfish *Aequorea forskalea* (Péron and Lesueur, 1810), magellanic penguins *Spheniscus magellanicus* (Forster, 1781) were observed to predate on the South American sea nettle *Chrysaora plocamia* (Lesson, 1830) and little penguins *Eudyptula minor* (Forster, 1781) were observed to predate on the crystal jelly *Aequorea* sp. (Péron and Lesueur, 1810) and on the jellyfish *Cyanea* sp. (Péron and Lesueur, 1810; Thiebot et al. 2017).

Therefore, gelatinous plankton can be a food source for many organisms throughout the aquatic food webs (Brodeur et al. 2016; Carman et al. 2017; Hays et al. 2018). However, the organisms that benefit with the presence of this non-indigeneous jellyfish may include several other NIS which could facilitate their colonization and establishment in a new ecosystem (Simberloff and Von Holle 1999; Marques et al. 2016). For example, in the Black sea the invasive ctenophore *Beroe ovate* (Bruguière, 1789) is known to predate on the invasive *M. leidyi* (Shiganova. et al. 2014).

In the Guadiana estuary, *B. virginica* is present in high abundances during summer months in years with low flow, typically withhigh salinity values and low nutrients' concentrations and low turbidity which are favourable conditions for *B. virginica* populations (Muha et al. 2017). When they are present, not only exert a high pressure on zooplankton and fish eggs and larvae, but also on detritus, that seems to be the most important source for jellyfish biomass increase. Therefore, during this period there is a high jellyfish production and a low abundance of both zooplankton and icthyoplankton, once that part of the ecossystem biomass is captured by jellyfish groups rather than by indigenous species (Muha et al. 2017). Nothing is know about potential predators for *B. virginica* in the Guadiana estuary, but we hypothesize that higher trophic levels, including benthic and generalistic feeders, may benefit from food resource pulses originated from the bloom events of *B. virginica*, which includes several NIS such as blue crab and indigenous species such as the green crab (Fig.1.4) thus, representing a potential threat and/or opportunity to the ecosystem.

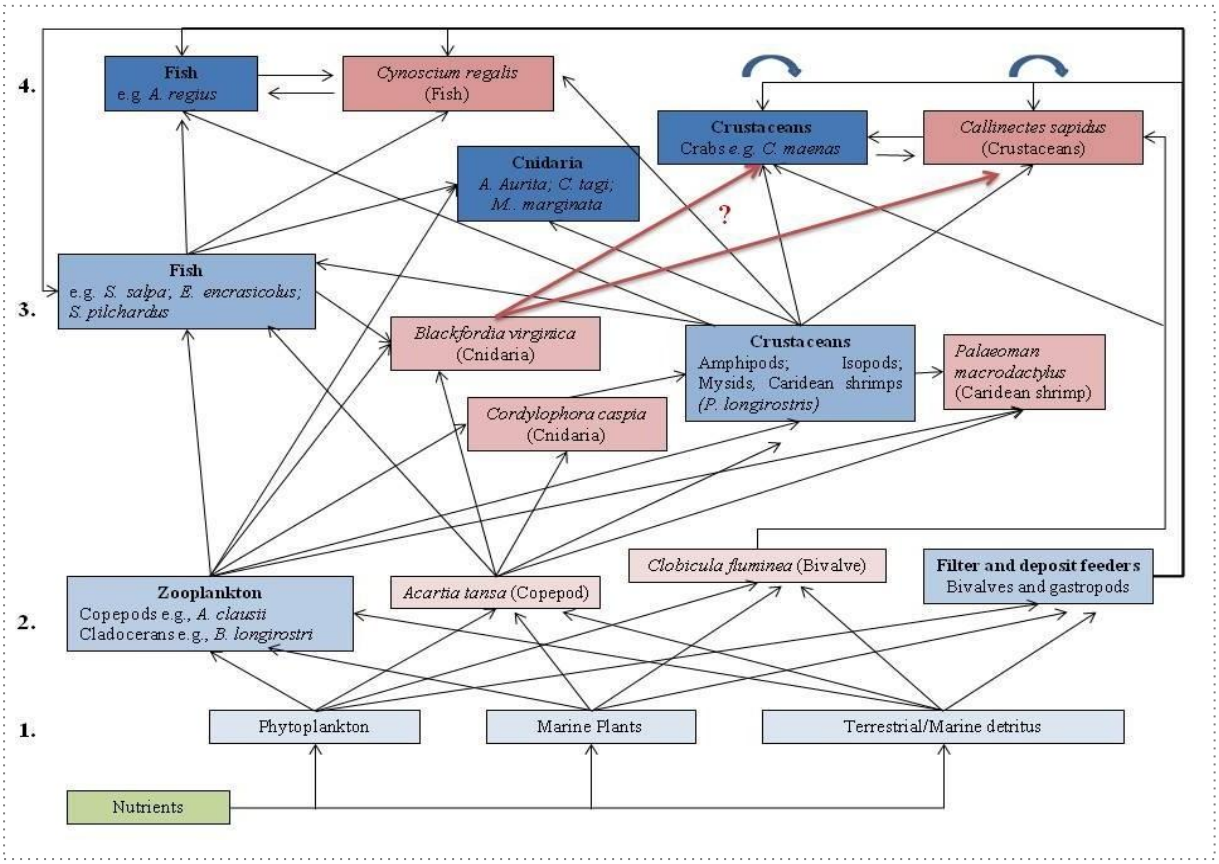


Figure 1.4 Conceptual diagram of the food web in the middle Guadiana estuary based on previous studies on the organisms feeding behavior, where narrow arrows represent predation and the dotted around the detritus fluxe on the food web once every organism consume detritus and constribute to that fluxe when die. Numbers represent the different trophic levels and the intensification in the colour represents higher trophic levels, where blue corresponds to indigenous species and red to non-indigenous species. The red arrows represent the possibility of *Blackfordia virginica* as a food source for *Carcinus maenas* and for *Callinectes sapidus.*

Chapter 2 - OBJECTIVES

2. OBJECTIVES

Jellyfish blooms are increasing worldwide and are often known for their negative economic and ecological impacts to fisheries and to the local food webs. However, they may represent an opportunity as an alternative food source and/or as a source of antioxidant products for humans, while contributing to control their biomass in the invaded ecosystems. On the other hand, they may also constitute an important source of food for aquatic consumers, especially to those that feed opportunistically. This is particularly important if those consumers are non-indigeneous species because this can facilitate their colonization and establishment in a new ecosystem.

Thus, the main objective of this study was to evaluate the potential of *B. virginica* to be used as a food source or as an alternative source of antioxidant products for humans and also as a food source for estuarine opportunistic consumers in the Guadiana estuary. Because edible jellyfish present high protein and essential mineral content (Costa et al. 2019), we hypothesize that *B. virginica* has a great potential to be an alternative healthy human food source. Also, because they form seasonal blooms (31.5 ind. m^3; Chícharo et al. 2009), we expect they will be consumed by opportunistic consumers such as crabs, which are generalistic consumers (Seitz et al. 2011).

To evaluate the potential of *B. virginica* as a food source for human consumption a preliminary evaluation of its nutritional composition was made, and included the determination of the crude protein, ash, amino acids, fatty acids methyl esters, and mineral contents. To evaluate the *in vitro* antioxidant properties of this species different methods were used according to Yu et al. (2006). To determine the role of *B. virginica* as a food source for opportunistic consumers in the Guadiana estuary, molecular analyses (DNA-PCR based analysis) were combined with carbon ($\delta^{13}C$: $^{13}C/^{12}C$) and nitrogen ($\delta^{15}N$: $^{15}N/^{14}N$) stable isotope analysis. The consumers selected were the non-indigeneous blue crab *C. sapidus* and also the indigenous green crab *C. maenas.* These species were chosen because they have a similar distribution, they are both abundant in this area of the estuary, and are considered opportunistic and generalistic species (Baeta et al. 2006; Rogers et al. 2018; Morais et al. 2018).

Chapter 3 – MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Study area

The Guadiana River is located in South of Portugal (South-West Europe; Fig. 3.1) and its catchment basin is the fourth largest in the Iberian Peninsula, with 67.500 km^2 (Chícharo et al. 2006a). Its estuary is approximately 80 km long, with the lower 50 km delimiting the border between Portugal and Spain, and occupies a total area of 22 km^2 (Bettencourt et al. 2003). This estuary is mesotidal with mean tidal amplitudes ranging from 1.3 to 2.6 m (with a maximum of 3.4; Garel et al. 2009). The mean depth of the estuary is 6.5 m and the maximum depth is *ca*. 18m (Garel et al. 2009). The Guadiana basin has Mediterranean climatic characteristics, with hot and dry summers (24ºC near the sea) and relatively rigorous winters (11ºC near the sea; Chícharo et al. 2006a; Garel, 2017). Data for rainfall and river discharges for the Guadiana river basin show a strong link with North Atlantic Oscillation (NAO) index patterns (Garel, 2017). A negative NAO index (dry conditions in the northern latitudes) usually results in more rainfall (in the southern latitudes of Europe), and subsequent flooding in the river basin during winter months (Garel, 2017). This estuary is listed as Wetland of International Importance and is included in the Natura 2000 Network, being an area of high ecological importance (Garel, 2017).

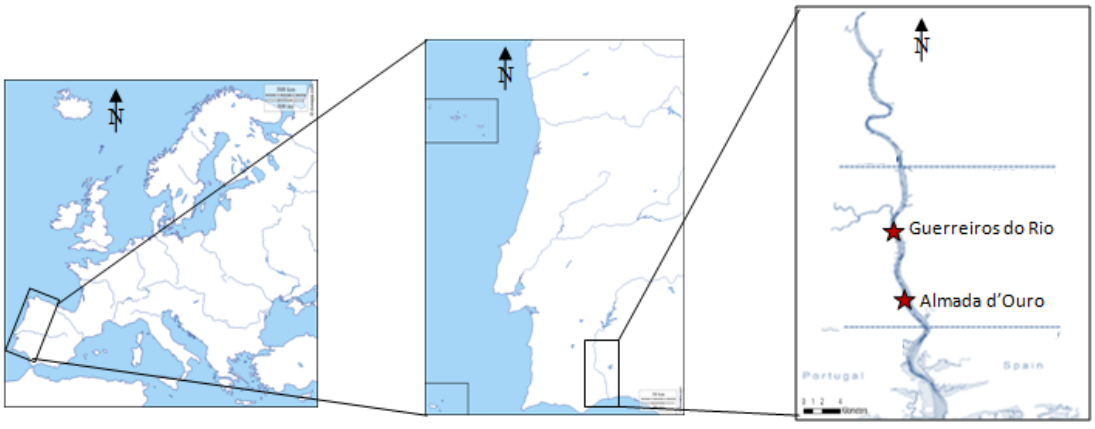


Figure 3.1 Sampling sites at Guerreiros do Rio and Almada de Ouro (adapted from D. Maps)

3.2. Field sampling

Sampling was conducted in June 2019 at the middle section of the Guadiana estuary, from Guerreiros do Rio (37° 23' 51.081" N/ 7° 26' 47.782" W) to Almada d'Ouro (37° 18'49.654" N/7° 26' 39.517" W; Fig.3.1).

Samples of *B. virginica* and other zooplankton groups were collected by a horizontal tows with a conical plankton net (200 μm mesh size, area=0.13m^2), equipped with Hydro- Bios flow meter, during 10 minutes. *Blackfordia virginica* samples were pooled into three sub-samples: one was frozen and latter freeze dried for the evaluation of the nutritional profile, another was frozen for DNA-PCR analysis, and for stable isotope analysis one sub-sample was preserved in 70% ethanol. The other zooplankton organisms were preserved in 70% ethanol for stable isotope analysis.

To investigate if *B. virginica* could represent a food source for opportunistic and generalistic consumers in the middle section of the Guadiana estuary, 12 individuals from the blue crab and five individuals from the green crab, were collected along with their potential sources, which were selected based on the available information from stomach content analysis (Table 3.1). The potential sources collected included terrestrial plants (*Eucalyptus* and *Salix* sp.) and its detritus, macroalgae (*Ulva* sp., and *Rhizoclonium riparium*), filter feeders (copepod *A. tonsa* and mysids), amphipods (*Cerapus* sp., and *Gammarus* sp.), annelids (*Autolytus* sp., and oligochaetes), isopods (*Shaeroma quadridentatum*), bivalve molluscs (Oysters), hydroid (*C. caspia*), caridean shrimps (*P. longirostris* and *P. macrodactylus*) and fish (*Solea solea*).

Detritus, terrestrial plants, and macroalgae were hand collected; mysids, annelids, ampipods and isopods were collected with a dredge; *C. caspia* and oysters were collected on the floating structures present on river platform by knife; fish, crabs, and caridean shrimps were obtained from commercial fishing operating in the area. All the organisms were kept one ice during transportation and then kept frozen at -20ºC until processing.

Table 3.1 Food items identified in the stomachs of *Callinectes sapidus* and *Carcinus maenas*.

Specie	Common name	Diet	References
Callinectes sapidus	Blue crab	M, Nc, Dc, A, C, V	Marlin and Tagatz 1968; Seitz et al. 2011
Carcinus maenas	Green crab	M, NC, Dc, C, A, V	Baeta et al. 2006

The diet column reports the preys contributing at least in 5% in weight or volume to the total stomach contents (F: Teleostei); Nc: non-decapoda crustaceans (amphipods, mysids, barnacles), Dc: Decapoda crustaceans, C: cannibalism, M: molluscs (clams, mussels, snails), A: annelids, V: vegetation (plant matter and detritus) . Data from the North Atlantic.

3.3. Laboratory analysis

3.3.1. Determination of the nutritional profile

The determination of the proximate composition and antioxidant activity of *B. virginica* was made following the standard procedures at the XtremeBio laboratory (CCMAR, Algarve University, Campus de Gambelas, Faro, Portugal).

The determination of amino acids content of *B. virginica* was made following the standard procedures at AQUAGROUP (CCMAR, Algarve University, Campus de Gambelas, Faro, Portugal).

The determination of FAMEs and minerals was made at CCMAR, Algarve University, Campus de Gambelas, Faro, Portugal.

3.3.1.1. Proximate composition

For the determination of the moisture content of the jellyfish biomass, fresh samples (three replicates, n=3) were weighed and dried in an oven at 60ºC until constant weigth (12h). After that period, samples were weighed and moisture was calculated as the difference between fresh and dry weigth.

Ash was determined by incineration of jellyfish biomass (aprox. 0.5 g, n=3) in a muffle furnace at 525°C for 5h (AOAC, 1990). The ash content was calculated as the difference between the final and initial weight of the jellyfish biomass.

The crude protein content was determined by the elemental analysis of nitrogen (N), in a combustion analyzer (n=3). The N value was then multiplied by the conversion factor specific for aquatic invertebrates (6.25) to determine the crude protein.

The total fat was determined gravimetrically by a modified protocol of the Bligh & Dyer method involving the homogenization of the dried biomass (n=3) in a mixture of chloroform, methanol and water (2:2:1) using an ultra sound bath (IKA-Werke GmbH, Staufen, Germany), as described in Pereira et al. (2013). Carbohydrates were calculated by difference, *i.e.*, equation 1: [100% - (moisture content + crude protein + ash content + total fat)]. Results are expressed as g per 100g of dry weight biomass (DW). Whenever needed, results were also expressed as g per 100g of wet weigth biomass (WW). Metabolizable energy (ME) was calculated using the specific factor for fish (FAO, 2002) according to the following equation 2: ME (Kcal) = 4.27× (g protein) + 4.11 × (g carbohydrate) + 9.02 × (g lipid). ME was expressed as kcal/100g of WW.

3.3.1.2. Amino acids content

To determinate the total amino acids contents a high pressure liquid chromatography (HPLC) with a reverse phase analytic system for amino acid (Waters ACQUITY UPLC H- Class System) was used using norvaline as an intern standard. The principle of this method is that solute hydrophobicity, *i.e. B. virginica* dry biomass (30 mg; n=2) was hydrolyzed in HCL 6M for 48 hours in vessels with a hydrogen atmosphere in order to disrupt the proteins. In order to increase thermal stability and improve chromatographic properties of compounds of interest, samples were derivatizated with Waters AccQ Fluor Reagent (6-aminoquinolyl- N-hydroxysuccinimidyl carbamate) according to AcccQTag method (Waters, Milford, USA). The mobile phase (polar) was applied to the column where the most hydrophobic compounds interacted with the column best. Therefore, the least hydrophobic eluted first and the most hydrophobic eluted last.

To quantify the amino acids concentration, a set of standards containing amino acids (Waters) were prepared, and calibration curves were generated for each amino acid using the Empwer software (Waters). Results are expressed as mg/100g DW and as percentage of total amino acid content.

3.3.1.3. Preparation and determination of fatty acid methyl esters (FAMEs)

Lipids and free fatty acids (FA) were converted to the corresponding FAME, by a

direct transesterification method using acetyl chloride/methanol, followed by extraction of the lipidic phase into hexane (Lepage and Roy, 1984). Briefly, 200 mg of dried biomass (n=3) were mixed with 1.5 ml of the derivatization solution (methanol/acetyl chloride, 20:1, v/v), and homogenized in an ultrasound-water bath for 15 min., at room temperature (RT, aprox.20ºC). Then, 1 ml of hexane was added and the samples were heated for one hour at 100°C. After cooling in an ice bath for 15 min., 1 ml of distilled water was added and the organic phase was removed and dried with anhydrous sodium sulfate. The extracts were then diluted in hexane to give an estimated fatty acid concentration of 0.1 g/ml and 1 ml of sample was filtered (0.2 nm) and transferred into Gas Chromatography (GC) vials for the determination of FAMEs profile (Pereira et al. 2012).

The FAME profile was analyzed on an Agilent Gas Chromatography with mass spectrometry detection (GC-MS; Agilent Technologies 6890 Network GC System, 5973 Inert Mass Selective Detector, CCMAR, Portugal) equipped with an Bruker SCION TQ gas chromatograph fitted with a fused silica capillary column ZB-5MS (30 m × 0.25 mm internal diameter, 0.25 µm film thickness, Agilent Tech) using nitrogen as the carrier gas (1 ml/min).

The GC-MS is a combination of two different analytical techniques. Gas chromatography is a type of chromatography in which the mobile phase is a carrier gas and the stationary phase is a capillary column in this case with fused silica. Sample is swept through the column by a stream of gas, such as nitrogen. Components in the sample are separated from each other base on volatility because some take longer to pass through the column than others. Mass spectrometry is the detector for GC. As the sample exits the end of the GC column it is fragmented by ionization and the fragments are sorted by mass to form a fragmentation patern. The fragment pattern for a given component of sample is unique and thus is an identifying characteristic of that component (Hussain and Maqbool 2014).

Therefore, vials were then injected on-column auto injector at 300ºC, and the temperature profile of the GC oven was 60°C (1 min), 30°C min^{-1} to 120°C, 4°C min^{-1} to 250°C, and 20°C min^{-1} to 300°C (4 min; Costa et al. 2019).

For the identification and quantification of FAME, the total ion mode was used. Identification of FAME were performed by comparing the retention times of biodiesel samples with an external standard (Supelco® 37 Component FAME Mix ; Sigma-Aldrich, Sintra, Portugal), and further confirmed by comparison of the MS spectra. For

quantification purposes, separate calibration curves were generated for each FAME in this standard. Assays were done in triplicate and between each three replicates was calculated the average, standard deviation and coefficient of variation. Values were expressed in terms of percentage of total FAME identified in the sample and also as concentration of μg/100g of DW.

3.3.1.4. Minerals content

Minerals were analysed by Agilent's Microwave Plasma (MP) - Atomic Emission Spectrometer (AES; MP- AES; CCMAR, Portugal). For MP, approximately 500 mg (n=3) of lyophilized jellyfish biomass were digested with 6ml of HNO3 for 30 minutes and put in a closed-vessel microwave digestion system Ethos 1 equipped with PTFE vessels.

The purpose of any AES technique is to identify elements and quantify their concentrations. The principle of this emission technique (*e.g.,* flame emission) is that the intensity of each emitted line is directly proportional to the concentration of a particular element. It envolves some steps such as sample introduction into the high temperature source, atom formation, excitation, emission, measurement of the emitted light intensity of a particular element of interest at a specified wavelength, and computation of the concentration by comparing it with that of a known concentration (Balaram et al 2014).

The mineralization was carried out by setting the following temperature program: 0-200ºC in 2 min (step 1), 200ºC held for 3 min (step 2) and 200-220ºC in 5 min (step 3) with a constant microwave power of 1000W (Costa et al 2019). Because the samples were not completely digested, maybe due to high amount of sample (it should be 250mg or less), 1.5 ml of peroxide was add to each replicated. Due to the expected very high salt concentrations, each digested pool was diluted by ultrapure water with a dilution factor of 1000. Samples were analysed in three replicates along with blacks to check for any loss or contamination. Magnesium (Mg), sodium (Na), potassium (K), calcium (Ca), iron (Fe), manganese (Mn) and zinc (Zn) were analysed by flame AES with an air-acetylene flame. Cadmium (Cd), chromium (Cr), nickel (Ni) and lead (Pb) were analysed with electrothermal atomisation (GBC graphite furnace 3000) using an auto-sampler (PAL 3000) and wavelengths are shown in Table 3.2.

Table 3.2 Minerals wavelength (nm)

Element	Wavelength nm
Fe	259.94
Ca	317.93
K	766.49
Mg	383.83
Na	588.99
Zn	213.86
Cd	214.45
Ni	231.09
Cu	324.75
As	234.98
Pb	368.35
Cr	425.43
Mn	403.08

For quantitative proposes the external calibration procedure was carried out with the help of multielemental standard solutions with concentration ranging between 0.1-50 ppm. For method validation, a linear least-square regression analysis of the calibration graphs was performed to check for the linearity between the instrumental response and the nominal concentration of each elemental standard. Values were expressed as g/100g DW (Ca, Mg, Na and K) or μg/100g DW (Fe, Mn, Zn, Cr, Pb, Ni and Cd).

3.3.2. Determination of the in vitro antioxidant properties

3.3.2.1. Preparation of the extracts

The extraction was made according to Yu et al. (2006), with some modifications. The dried biomass (100 mg) was mixed with cold distilled water (5 ml), and extracted in an ultrasonic water bath (eight times, 30 s each time, samples kept on ice between extractions). Samples were centrifuged (13 000 rpm, 20 min., 4ºC), and the supernatants were recovered, mixed, frozen, and freeze-dried for two days. Obtained dried extracts were weighed, dissolved in ice cold water at a concentration of 25 mg/ml and stored at -20 ºC, until necessary. For the assays, working concentrations of 10, 5 and 1 mg/ml

were prepared in cold water and stored at the indicated conditions.

3.3.2.2. Radical scavenging activity (RSA) on the DPPH and ABTS free radicals

The antioxidant activity is the ability to inhibit the process of oxidation. Consequently, all test systems used a stable free radical, in this case the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), providing information on the radical scavenging activity (RSA; Shalaby et al. 2012). The ABTS assay measures the relative ability of an antioxidant to scavenge the ABTS generated in aqueous phase. The radical $ABTS^{\bullet+}$ is generated by reacting a strong oxidizing agent with the ABTS. Reduction of blue-green $ABTS^{\bullet+}$ colored solution by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum (650 nm). DPPH is a stable free radical with an absorption band at 515 nm. It loses this absorption when reduced by an antioxidant (Williams et al. 1995; Shalaby et al. 2015).

The RSA on the DPPH radical and ABTS radicals were evaluated according to Brand- Williams et al. 1995 adapted to microplates (Moreno et al. 2006), as described in Rodrigues et al. (2015). Samples were tested at different concentrations (1, 5, 10 and 25 mg/ml) and each concentration had 6 replicates. For the DPPH method, 22 µl of each concentration were mixed with 200 µl of DPPH solution (120 µM in ethanol). The plate was incubated in the dark for 30 min and the absorbance was read at 517 nm on a microplate reader (EZ Read 400, Biochrom). For the ABTS method, 10 µl of each sample concentration was mixed with 190 µl of ABTS. The plate was incubated in the dark for 6 min. and the absorbance at 650nm was read on the above mentioned microplate reader. BHT was used as the positive control at 1 mg/ml, and water was used as the negative control.

For RSA using ABTS and DPPH results are expressed as percentage of antioxidant activity, calculated in relation to the negative control, according to the Eq. 3:

Equation 3 RSA(%) = [(Control absorbance – Real sample absorbance)] / [Control absorbance] × 100

3.3.2.3. Metal chelating activity on copper (CCA) and iron (ICA)

Materials with chelating activity can inhibit the oxidative damage due to reactions catalyzed by the transition metals such as iron and copper (Megías et al. 2009). More specifically, hydroxyl radical can be formed from superoxide anion and hydrogen peroxide in the presence of these transition metal ions and chelating metal ions may inhibit the formation of hydroxyl radical what is often call Fenton reaction (Kàramak et al. 2009; Zhuang et al. 2009). For example, ferrozine can quantitatively form complexes with Fe^{2+} resulting in a red colour. In the presence of chelating agents, the formation of complexes is prevented because there is less Fe^{2+} in the solution bound with ferrozine. Consequently, there is less formation of complexes and thus the colour decreases. Therefore, measurement of the rate of colour reduction estimates the chelating activity of the coexisting chelator.

Metal chelating activity on iron was determined by measuring the formation of the Fe^{2+} - ferrozine complex according to Megías et al. (2009), with some modifications. Briefly, samples (30 µL at 1, 5, 10 and 25 mg/ml) were mixed in 96-well microplates with 200 µl of distilled water and 30 µl of an iron (II) chloride solution (0.1 mg/ml in water), and incubated for 30 min at room temperature (RT).. Then, 12.5 µl of ferrozine solution (40 mM in water) was added, and change in colour was measured in a microplate reader at 562 nm (Biochrom EZ Read 400). CCA was determined according to Megías et al. (2009) and according to Rodrigues et al. (2015). Briefly, samples (30 µl at 1, 5, 10 and 25mg/ml) were mixed with 200 µl of 50 mM Na acetate buffer (pH 6), 6 µl of 4 mM PV in the same buffer and 100 µl of copper (II) sulfate pentahydrate in 96-well microplates. The change in colour was measured at 632 nm using a microplate reader (Biochrom EZ Read 400).

Results were expressed as percentage of antioxidant activity and were calculated using the same Eq. 3 but in relation to a negative control that uses the synthetic metal chelator ethylenediamine tetraacetic acid (EDTA; 1 mg/ml).

3.3.3. *Blackfordia virginica* as a potential food source for crabs

3.3.3.1. Molecular analysis

Because it is difficult to identify soft bodied organisms as jellyfish in the stomach contents of consumers (Lin et al. 2014), a DNA-PCR based analysis was conducted to the stomach contents of the green crab and the blue crab in order to verify if crabs were eating *B. virginica*. Thus, the growing availability of molecular genetic methods and data has fostered the use of DNA-PCR based techniques for the identification of prey items from stomach using the presence/absence of diagnostic PCR products on agarose gels (Gorokhova et al. 2006) and sequencing the PCR fragments corresponding to the potential prey items. Compared to other methodologies, DNA-based prey assays can be developed faster, allow simultaneous screening for multiple prey items and offer a greater taxonomic prey resolution, although DNA prey detectability may span shorter periods of time (Hernandez et al. 2018). Crabs were identified to the species level [blue crab (Fig.3.2a) and green crab (Fig.3.2b)] enumerated, and sexed after examination of the shape of the abdomen. For each specimen the carapace maximum width (CW) was measured as the distance (in mm) between the two outermost lateral spine tips. Blue crabs specimens with a CW < 94 mm were classified as juveniles (Mancinelli et al. 2013), while green crabs with CW < 40 mm were classified as juveniles (Klein Breteler, 1976).

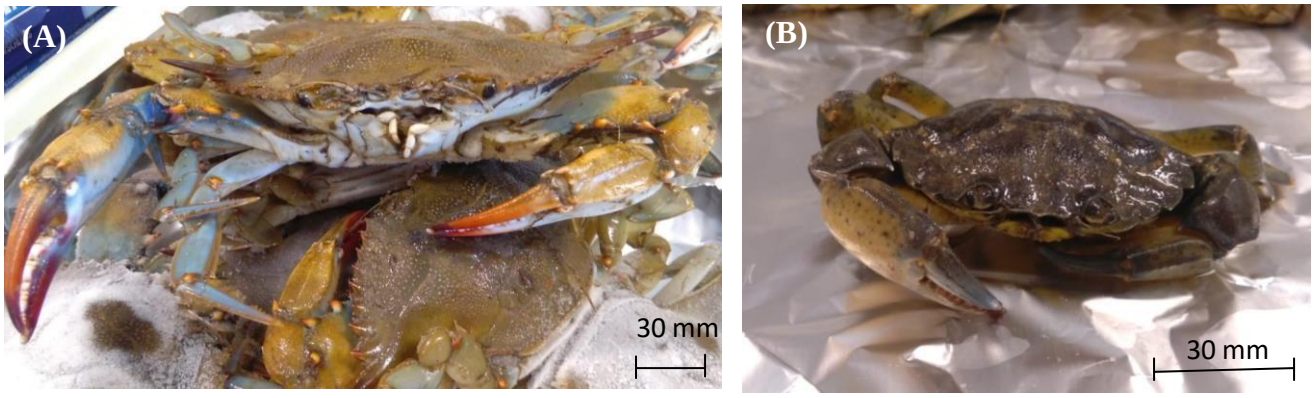


Figure 3.2 (A) Blue crab (*Callinectes sapidus)* and (B) Green crab (*Carcinus maenas*)

Genomic DNA was extracted from 25mg (WW) of *B. virginica* and 25mg (WW) from each sample of crabs gut contents (12 blue crabs and five green crabs, Fig.3.3) using a Qiagen K– QIAmp DNA mini kit. Briefly, the 25mg of stomach content of each individual was collected into a 1.5ml microcentrifuge tube and 180 ul of the Buffer ATL (for cell lysis) was added. Then, 20 µl proteinase K was added in each tube, mixed with vortex and incubated at 56ºC for 3h until the tissue was completely lysed. After 3h, tubes were centrifugated in order to remove drops from the inside of the lid, 200 µl of Buffer AL (for cell lysis) was added to each tube which was mixed by vortexing for 15s and incubated at 70ºC for 10min. Then, 200 µl of absolute etanol was added to each sample that was vortexed for 15s and centrifuged. This mixture was applied to the QIAmp Mini spin column without wetting the rim which was centrifuged at 8000 rpm for 1 min. The QIAmp Mini spin columns were placed into a clean 2 ml collection tube and the tube containing the filtrate was discarded. Then, 500 µl of Buffer AW1 (for DNA purification) was added to the QIAmp Mini spin column and centrifuged at 8000 rpm for 1 min. The columns were placed in clean 2 ml collection tubes and the collection tube containing the filtrate was discarded again. Then, 500 µl of Buffer AW2 (for DNA purification) was added to the spin column and centrifuged at full spead (14 000 rpm) for 3 min. Finally, the QIAmp Mini spin columns were placed in clean 1.5 ml microcentrifuge tube, the collection tube containing the filtrate were discarded. Then, 200 µl of Buffer AE (for DNA elution) was added to the spin column, which were incubated at RT for 1 min and centrifugated at 8000 rpm for 1 min.

Figure 3.3 Stomach from the blue crab for DNA-PCR analysis

After DNA extraction with the QIAamp® DNA Mini Kit (Qiagen), amplification of the three markers (ITS, 16S and COI; Table 3.3) was done by polymerase chain reaction (PCR) using an Applied Biosystems 2720 Thermal Cycler. *Blackfordia virginica* specific primers were mixed with ultrapure water and vortexed to make the stock solution in order to prepare the PCR that was performed in a catalytic reaction. All PCR amplifications were performed in a total volume of 25 µL containing 10 nanograms (ng) of extracted DNA, 0.2 µM of forward and reverse primer, 1U Taq polymerase (DreamTaq DNA Polymerase, Thermo Fisher Scientific), 0.4 mM of Magnesium chloride (MgCl2).The same cycling protocol was used for ITSF, 16s and COI: 95°C for 7 minutes followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, followed by an extension of 72°C for 5 minutes, and a 10°C holding temperature.

In order to verify the quality of the products of PCR amplification, an aliquot of 15 µl per sample was loaded on agarose gel 1.5%. Gel was visualized under UV light, photographed on a Geldoc XR+ system (Bio-Rad) and the amplification of the target DNA was possible to observe as bands with the same molecular weigth as *B. virginica* genomic DNA.

It was made a new PCR amplification of 18 DNA extractions for both primers 16S and COI that was visualized on the gel in order to verify if both primers in fact amplified for *B. virginica*. Products that were positively amplified were directly sequenced. The DNA sequencing was done at CCMAR's Sequencing Platform, with an Applied Biosystems 3130*xl* Genetic Analyzer, BigDye®Terminatorv3.1 chemistry and POP7 polymer.

Table 3.3 List of primers used to identify DNA from *Blackfordia virginica* in blue crab *Callinectes sapidus* and in the green crab *Carcinus maenas* stomach contents (Harrison et al. 2013).

Primer name	Loci	Sequence	Citation
jlITS1F	ITS1	5' 0GGTTTCCGTAGGTGAACCTGCGGAAGGATC0 3'	Dawson and Jacobs, 2001
jlITS1R	ITS1	5'0CGCACGAGCCGAGTGATCCACCTTAGAAG0 3'	Dawson and Jacobs, 2001
16s.Cunningham.F.1mod	16S	5' 0ACGGAATGAACTCAAATCATGTAAG0 3'	Bridge et al. 1995
16s.Cunningham.R.2	16S	5' 0TCGACTGTTTACCAAAAACATA0 3'	Bridge et al. 1996
dgLCOI490	COI	5' 0GGTCAACAAATCATAAAGAYATYGG0 3'	Folmer et al. 1994
dgHCO2198	COI	5' 0TAAACTTCAGGGTGACCAAARAAYCA0 3'	Folmer et al. 1995

3.3.3.2 Stable isotope analysis

To quantify the relative importance of *B. virginica* to the selected consumers biomass, carbon ($\delta^{13}C$: $^{13}C/^{12}C$) and nitrogen ($\delta^{15}N$: $^{15}N/^{14}N$) stable isotope analysis was conducted. For consumers, the $\delta^{13}C$ and $\delta^{15}N$ composition of tissues is a time-integrated signal of the food sources that were incorporated into a consumer's structural components and energy reserves (Peterson and Fry 1987). Thus, the stable isotope ratios of a consumer reflects its diet, demonstrating an average trophic fractionation (*i.e.*, the difference between the consumer and its diet) of +0.4‰ $\delta^{13}C$ and +3.2‰ $\delta^{15}N$ per trophic level (Vander Zanden and Rasmussen 2001), although with some variability around these means (Caut et al. 2009).

Macroalgae, terrestrial plants and its detritus, were cleaned with ultra pure water to remove epiphytes, dried at 60 ºC for 48h, and ground to a fine powder with a mixer mill for stable isotope analysis.

Crabs' muscle was removed from the claws. Then samples were dried at 60ºC in an oven for 72 h and homogenized with a lab scope (Fig. 3.4).

Figure 3.4 Dry muscle after being ground to a fine powder with a measuring scoop.

In relation to fish species, the animals were measured (± 0.01 mm) and weighted (± 0.1 g). After scales and fines were removed, muscle samples were taken from the left side of fish back to the dorsal fin. Muscle was dry (60ºC) for 72h Decapod crustaceans (caridean shrimps *P. longirostris, P. macrodactylus)* the muscle was removed and samples were dried (60ºC) for 48h. Small non-decapods crustaceans (*i.e.*, caprellid amphipods, gammarid amphipods, isopods, barnacles, mysids) and annelids, pools of

the same taxa were made in order to obtain enough sample for SIA. Samples were dried (60ºC) for for 48h *Cordylophora caspia* samples were washed with ultra-pure water, and dried (60ºC) for 48h. All the above samples were ground to a fine powder using a laboratory scoop.

Zooplankton samples, including *B. virginica* were sorted, identified and grouped by the lowest taxonomic level feasible. After that they were loaded directly into the tin capsules and dried (60ºC) for 48h.

After being prepared, samples were kept in the desiccators to avoid humidity, until analysis.

Stable isotope ratio analysis was performed at the Centro de Recursos em Isótopos Estáveis - Stable Isotopes and Instrumental Analysis Facility, at the Faculdade de Ciências, Universidade de Lisboa - Portugal. The $\delta^{13}C$ and $\delta^{15}N$ in the samples were determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston and Owens, 1983), on a Sercon Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a Euro EA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas- combustion. Delta Calculation was performed according to Equation 4: $\delta = [(R_{sample} - R_{standard})/R_{standard}]*1000$, where R is the ratio between the heavier isotope and the lighter one. The $\delta^{15}N_{Air}$ values are referred to air and $\delta^{13}C_{VPDB}$ values are referred to PDB (Pee Dee Belemnite). The reference materials used were USGS-25, USGS-35, BCR-657 and IAEA- CH7 (Coleman and Meier-Augenstein, 2014); the laboratory standard used was Wheat Flour Standard OAS/Isotope (Elemental Microanalysis, UK). Uncertainty of the isotope ratio analysis, calculated using values from 6 to 9 replicates of laboratory standard interspersed among samples in every batch of analysis, was ≤ 0.1‰. The major mass signals of N and C were used to calculate total N and C abundances, using Wheat Flour Standard OAS (Elemental Microanalysis, UK, with 1.47% N, 39.53% C, for plant material, and with 13.32% N, 46.5% C for animal material) as elemental composition reference materials.

3.4. Data analysis

3.4.1. Stable isotope analysis

The relative contribution of *B. virginica* to the diet of the selected consumers was determined using the Bayesian isotope mixing model SIAR (Stable Isotope Analysis in R; R Development Core Team, 2018). The model allows each of the sources and the

trophic enrichment factor (TEF; or trophic fractionation) to be assigned as a normal distribution (Parnell et al. 2010). SIAR will produce a range of feasible solutions to the mixing problem to which are assigned credibility intervals (CIs; in this study, 95 % CI; Parnell et al. 2010).

The potential sources for crabs were identified using graphical analysis, by comparing their $\delta^{13}C$ and $\delta^{15}N$ values to each potential source $\delta^{13}C$ and δ^{15} values, after adjusting for one trophic level using the TEF estimates from Vander Zanden and Rasmussen (2001). However, because crabs are omnivorous (Baeta et al. 2006; Seitz et al. 2011), different TEF values were assigned according to the origin of the OM source (plant *vs*. animal); Vander Zanden and Rasmussen, 2001).

Some animals were grouped by taxonomic level or functional feeding group: annelids (*Autolytus* sp., and oligochaetes), amphipods (*Cerapus* sp., and *Gammarus* sp.), isopods (*Shaeroma quadridentatum*), copepods (*A. tonsa*) and mysids were grouped as filter feeders (FF), oysters and *C. fluminea* were grouped as bivalves. Because it was not possible to collect *C. fluminea* in the Guadiana estuary, the $\delta^{13}C$ and $\delta^{15}N$ values used in this study are those obtained by Aramendía et al. (2019) for the Guadiana estuary. Terrestrial plants included samples from *Eucalyptus* and *Salix* sp. These groupings were done in order to avoid additional error in the dual stable isotope mixing model by adding multiple sources with similar stable isotope ratios.

Consumers and animal prey $\delta^{13}C$ values were corrected for lipid content because lipids are depleted in ^{13}C when compared to protein and carbohydrates which usually results in an inverse relationship between C:N and $\delta^{13}C$ in muscle tissues for aquatic animals (DeNiro and Epstein 1977). Consumers muscle tissue were corrected for lipid content using the mass balance correction for fish proposed by Hoffman and Sutton (2010; Eq 6). Zooplankton tissue were also corrected for lipid content using the mass balance correction model proposed for zooplankton by Smyntek et al. (2007; Eq.5) and $\delta^{13}C$ and $\delta^{15}N$ values were corrected for ethanol preservation (+0.4‰ $\delta^{13}C$, +0.6‰ $\delta^{15}N$; Feuchtmayer and Grey, 2003).

Chapter 4 - **RESULTS**

4. RESULTS

4.1. Nutritional profile of *Blackfordia virginica*

4.1.1. Proximate composition

The proximate composition including the moisture content, the ash content, crude protein, total lipids, carbohydrates, and metabolizable energy of *B. virginica* is depicted in Table 4.1, in relation to DW and WW. The full body of this jellyfish (1.25 ± 0.25cm diameter) is mainly composed by water (98.69%). The DW corresponds to 1.30 % and is mainly composed by carbohydrates, ash ,and proteins. The total lipids were almost non detectable, resulting in a low energetic value (Table 4.1)

Table 4.1 Proximate composition, including moisture (%), nutrients (carbohydrates, ash, crude protein, total lipids; g/100 g of wet weight, (WW) and dry weight, (DW)) and energetic value (kcal/100 g of WW and DW) of the body wall of *Blackforida virginica* jellyfish. Values are expressed as mean ± standard error, n=3

	g/100g WW	g/100g DW
Moisture (%)	98.69 ± 0.06	-
Ash	0.40 ± 0.02	30.57 ± 1.55
Crude protein	0.10 ± 0.08	7.62 ± 0.62
Total fat	0.00 ± 0.00	0.01 ± 0.00
Carbohydrates	0.81 ± 0.18	62.30 ± 14.02
ME (Kcal)	4.12 ± 0.73	288.68 ± 55.19

4.1.2. Amino acids (AA) composition

The AA composition results are summarized on Table 4.2. The essential AA (EAA) histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr) and valine (Val) were identified in the dry samples, representing 29% of the total AA detected. Tryptophan (Try) was not detected in the jellyfish samples. Non-essential amino acids (NEAAs) represented 71% of the total amino acids. The most abundant amino acid in *B. virginica* was glutamic acid + glutamine (Glx), followed by glicine (Gly), alanine (Ala), aspartic acid + asparagine (Asx), proline (Pro) and tyrosine (Tyr; Table 4.2). Cysteine (Cys) was almost non detectable (Table 4.2).

Table 4.2 Amino acid profile of *Blackfordia virginica* dry biomass. Data are expressed as mean of two replicates as mg/100g of dry weigth (DW) ± standard deviation (SD) and as percentage of total amino acids (n=2).

Amino acids	**AA**	***B. virginica***	
		mg/100g	%
Glutamic acid + Glutamine	Glx	821 ± 8.40	16.84
Glycine	Gly	471 ± 10.20	9.66
Alanine	Ala	469 ± 4.80	9.63
Aspartic acid + Asparagine	Asx	432 ± 11.30	8.86
Arginine	Arg	353 ± 8.50	7.25
Proline	Pro	326 ± 6.00	6.70
Tyrosine	Tyr	300 ± 2.80	6.15
Serine	Ser	245 ± 4.30	5.04
Taurine	Tau	48.0 ± 0.10	0.98
Cystine	Cys	3.6 ± 0.10	0.07
Total non-essential AA	**ΣNEAA**	**3471.6 ± 0.56**	**71.18**
Lysine	Lys	281 ± 3.00	5.77
Leucine	Leu	265 ± 11.60	5.43
Valine	Val	265 ± 11.60	5.43
Threonine	Thr	227 ± 7.00	4.67
Isoleucine	Ile	158 ± 1.20	3.24
Phenylalanine	Phe	129 ± 0.80	2.66
Methionine	Met	68 ± 2.10	1.40
Histidine	His	10.2 ± 0.10	0.21
Total essential AA	**ΣEAA**	**1405.6 ± 0.32**	**28.82**
EAA/NEAA			0.41
EAA/TAA			0.30
LYS/ARG			0.79

4.1.3. FAMEs composition

The GC-MS analyses allowed to determine 15 FAMEs in dried samples (n=3) from *B. virginica* (Table 4.3). The GC-MS method showed good linearity for the calibration curve of all elements, with coefficients of correlation around 1.

The notations followed conventional nomenclature (IUPAC-IUB): in the format X:Y (n-z), where X refers to the chain length (number of carbon atoms, including the carboxylic acid or alpha carbon), Y refers to the number of carbon-carbon double bonds and z refers to the position of the first carbon-carbon double bond in the molecule relative to the terminal methyl group (carbon number 1 in the n-z system, *i.e.*, omega carbon) .

Saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated fatty acids (PUFAs) represented 77%, 21%, and 2.0% of total FA, respectively (Table 4.3). The predominant

fatty acids in *B. virginica* DW were methyl decanoate or capric acid (20%), methyl tetradecanoate or myristic acid (17%), methyl dodecanoate or lauric acid (14%), methyl palmitate or palmitic acid (14%) and methyl oleate or oleic acid (10%).

Table 4.3 Fatty acid methyl esters determined in *Blackfordia virginica* dry biomass. Data is reported on a dry weigth (DW) basis, as average Gas Chromatography with mass spectrometry detection (GC-MS) peak area percent ±standard deviation (n = 3). Results are also expressed in µg/100g DW ± standard deviation (n = 3)

Common name	Structure	Fatty acid	µg/100g DW	%
Methyl decanoate	C11H22O2	C11:0	1198.18 ± 2.53	19.98 ± 0.02
Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6	C12:0	40.58 ± 5.54	0.68 ± 0.05
Methyl dodecanoate	C13H26O2	C13:0	899.33 ± 0.78	14.99 ± 0.01
Methyl tetradecanoate	C15H30O2	C15:0	998.51 ± 0.73	16.65 ± 0.01
Methyl palmitate	C17H34O2	C17:0	887.83 ± 0.76	14.80 ± 0.01
Methyl heptadecanoate	C18H36O2	C18:0	19.52 ± 0.38	0.32 ± 0.01
Methyl stearate	C19H38O2	C19:0	421.67 ± 12.30	7.03 ± 0.12
Methyl arachidate	C21H42O2	C21:0	99.82 ± 0.18	1.66 ± 0.00
Tricosanoic acid	C23H46O2	C23:0	50.50 ± 0.37	0.84 ± 0.01
Saturated fatty acids	**SFAs**		**4616.21**	**76.98**
Methyl Cis-9-Tetradecenoate	C15H28O2	C15:1 n-9	393.73 ± 10.15	6.56 ± 0.10
Methyl Palmitoleate	C17H32O2	C36:1 n-9	200.01 ± 0.00	3.34 ± 0.00
Methyl oleate	C19H36O2	C19:1 n-9	666.74 ± 0.02	11.12 ± 0.00
Mono unsaturated fatty acids	**MUFAs**		**1260.46**	**21.02**
Methyl linolenate	C19H32O2	C19:3 n-3	100.36 ± 0.21	1.67 ± 0.00
Squalene	C30H50	C30:6 n-2	19.46 ± 22.31	0.32 ± 0.22
Polyunsaturated fatty acid	**PUFAs**		**119.82**	**1.99**

4.1.4. Mineral composition

The contents of four major elements (Na, Mg, K and Ca), five essential trace elements (Fe, Cu, Zn, Mn and Se) and five potentially toxic elements (Cr, Ni, As, Cd and Pb) were evaluated by MP-AES in whole jellyfish, on a DW basis (Table 4.4). The MP-AES method showed good linearity for all the elements, with coefficients of correlation of 0.999. Major element signatures appeared to bioaccumulate in jellyfish body by the decreasing order Na followed by Mg, K and Ca. As a result, Na was detected in the highest levels (728 mg/100g DW), while Ca was the less abundant element (17.44 mg/100g DW) (Table 4.4).

The essential trace elements were found in the decreasing order Fe followed by Zn, Mn, Cu and Se. Fe was characterized by a behaviour similar to major elements in therms of quantity (1208 µg/100g DW) and Se was not identified (Table 4.4). Nonessential/toxic

trace elements were reported in the decreasing order of Cd followed by Ni, Cr, As and Pb, where Pb was not identified (Table 4.4).

Table 4.4 Elemental signatures of *Blackfordia virginica* dry biomass revealed by microwave plasma – atomic emission spectrometry (MP-AES). Contents of major elements (mg/100g) and trace elements (µg/100g) are expressed as mean ± SD (n = 3) on a dry weigth (DW) basis.

Mineral	Symbol	*B. virginica*
Essential major elements (mg/100g DW)		
Sodium	**Na**	728.00 ± 5.35
Magnesium	**Mg**	76.19 ± 3.29
Potassium	**K**	56.93 ± 8.87
Calcium	**Ca**	17.44 ± 0.23
Essential trace elements (µg/100g DW)		
Iron	**Fe**	1208.00 ± 212.84
Zink	**Zn**	110.86 ± 16.39
Manganese	**Mn**	45.42 ± 4.33
Copper	**Cu**	26.34 ± 1.53
Selenium	**Se**	n.d.
Nonessential trace elements (µg/100g DW)		
Cadmium	**Cd**	337.71 ± 9.16
Nickel	**Ni**	68.56 ± 6.55
Chromium	**Cr**	4.08 ± 0.58
Arsenic	**As**	2.31 ± 0.04
Lead	**Pb**	n.d.

Note: n.d.: non detected

4.2. Protein extracts and antioxidant activity

The *in vitro* antioxidant properties of the *B. virginica's* extract enriched in proteins was assessed by four complementary mehods including their ability to scavenge the free radicals ABTS and DPPH and to chelate the transition metals iron and cooper and results are summarized in Table 4.5.

The extract only exhibited moderate activity towards the ABTS radical (57.47%), at the highest concentration tested (25 mg/ml; Table 4.5). No activity was detected in the other assays (Table 4.5).

Table 4.5 Radical scavenging activity towards DPPH and ABTS radicals, and metal chelating activity towards iron (ICA) and copper (CCA) of an aqueous extract enriched in proteins for *Blackfordia virginica* dry biomass.

Samples	**Concentration (mg/ml)**	**Antioxidant activity (%)**			
		DPPH	ABTS	ICA	CCA
Extract	1	na	na	na	na
	5	na	26.84 ± 2.70	na	na
	10	na	34.64 ± 3.50	na	na
	25	na	57.47 ± 3.13	na	na
EDTA*				84.4 ± 4.88	91.67 ± 2.44
BHT*		39.78 ± 2.64	95.9 ± 0.30		

*=positive control, na= no activity.

4.3. *Blackfordia virginica* as a potential food source for aquatic organisms

4.3.1. Molecular analysis

A total of 17 crab's stomach contents (blue crab= 12; green crab= 5) were inspected for the presence of *B. virginica* using molecular analyses (Table 4.6). Blue crabs had a carapace maximum width (CW) between 69.2 mm and 150.7 mm and weigth between 84.22 g and 169.47 g and green crabs had a carapace maximum width (CW) between 58.7 mm and 70.5 mm and weigth between 45.53 g and 75.1 g (Table 4.6).

Table 4.6 Species, individual (n) , carapace maximum width (CW, mm), weight (g), and sex of crabs *Callinectes sapidus* and *Carcinus maenas*, collected in middle Guadiana estuary for molecular gut content analysis.

Species	n	CW (mm)	Weigth (g)	Sex
Callinectes sapidus (Blue crab, n=12)	1	150.70	169.47	M
	2	69.20*	134.53	F
	3	82.70*	85.74	F
	4	125.80	121.72	M
	5	120.00	79.68	F
	6	114.80	75.26	F
	7	81.20*	84.22	M
	8	102.80	84.61	M
	9	123.10	89.17	F
	10	116.80	85.92	M
	11	120.50	97.48	M
	12	143.30	102.83	F
Carcinus maenas (Green crab, n=5)	1	65.00	64.08	M
	2	70.50	75.10	M
	3	58.70	45.53	M
	4	60.00	48.25	M
	5	59.80	47.82	M

M and F, Male and Female; * juvenile

The DNA samples from *B. virginica* were positively amplified for the primer pairs ITS1 and 16S and therefore they were suitable as positive controls for *B. virginica* presence in crabs' gut contents (Fig.4.1 (A)).

Among the gut contents of the 17 crabs analyzed, only 8 were positively amplified, where five corresponded to ITS1 for the blue crab, two corresponded to ITS1 for the green crab, and one corresponded to 16S for the green crab (Fig.4.1 (A)).

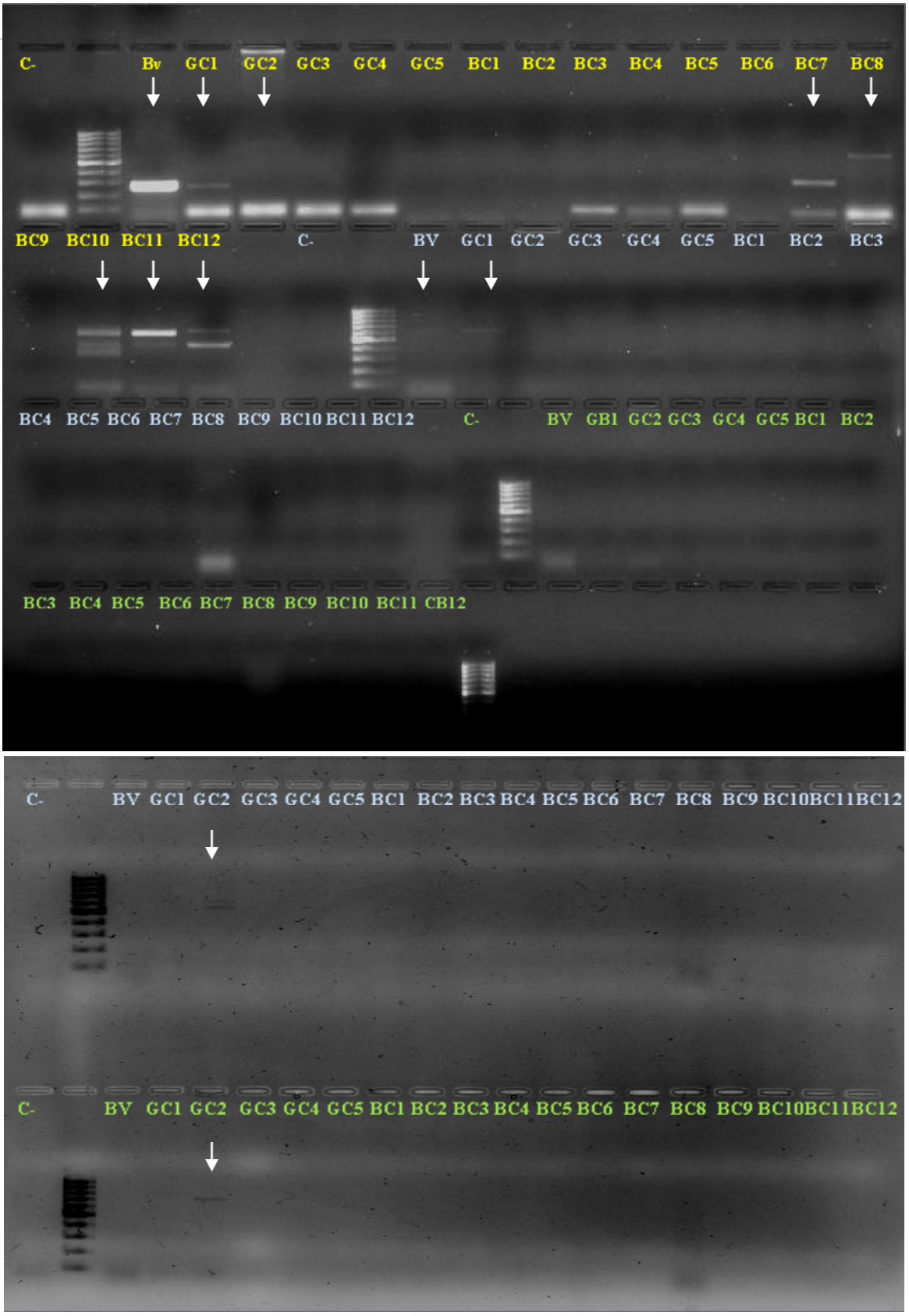


Figure 4.1 (A) Results of PCR amplification (in agarose gel, observed under UV light) with primers pairs ITS1 (yellow), 16S (blue) and COI (green) of DNA samples from *Blackforida virginica* (Bv) and from the gut contents of 12 *Callinectes sapidus* (1-12 BC) and of five *Carcinus maenas* (1-5 GC). C- is the negative control. Arrows represent crabs that were positively amplified; (B) Results of PCR amplification (in agarose gel, observed under UV light) with pairs 16S (blue) and COI (green) of DNA samples from *B. virginica* (Bv) and the gut contents of 12 *C. sapidus* (1-12 BC) and of five *C. maenas* (1-5 GC). C- is the negative control. Arrows represent crabs that were positively amplified.

All the individuals stomach which DNA samples were positively amplified for *B. virginica* were sequenced, but only three blue crabs showed results for sequenciation. One blue crab (BC12 ITS1) showed positive results for fish (*Brevoortia tyrannu*) with 85.21% of identity (Table S.1) and two blue crabs (BC7 ITS1 and BC10 ITS1) showed positive results for *B. virginica* DNA with 100% of identity (Table S.1), meaning that the DNA fragments that were positively amplified from both blue crabs stomach had similar nucleotides sequences to *B. virginica*.

The PCR amplification with both *B. virginica* primers 16S and COI was successful for one green crab stomach (GC2; Fig.4.2 (B)). Sequenciation results shows that only 16S primer worked, with 94.5% identity for *Carcinus maenas* (Table S.1).

4.3.2. Stable isotope analysis

The average (± SD) $\delta^{13}C$ values of the blue crab (-24.9 ± 0.6‰) were lower than those from the green crab (-21.6 ± 0.6‰; Table S2), suggesting that the green crab assimilated ^{13}C-enriched sources such as isopods, while the blue crab relied on more ^{13}C- depleted sources such as amphipods, annelids, or filter feeding organisms (Fig. 4.2). The $\delta^{15}N$ values were similar between species (*C. sapidus*= 15.7 ± 0.2‰; *C. maenas*= 15.3 ± 0.5‰) suggesting these species occupy the same trophic level in this ecosystem (Fig. 4.2). *Blackfordia virginica* does not seems to be a relevant source for crab's biomass because they were too ^{15}N-enriched ($\delta^{15}N$= 15.7 ± 0.4‰) when compared to the $\delta^{15}N$ values of crabs, ($\delta^{15}N_{C.sapidus}$ = 15.7 ± 0.6‰; $\delta^{15}N_{C.maenas}$ = 15.3 ± 0.5‰).

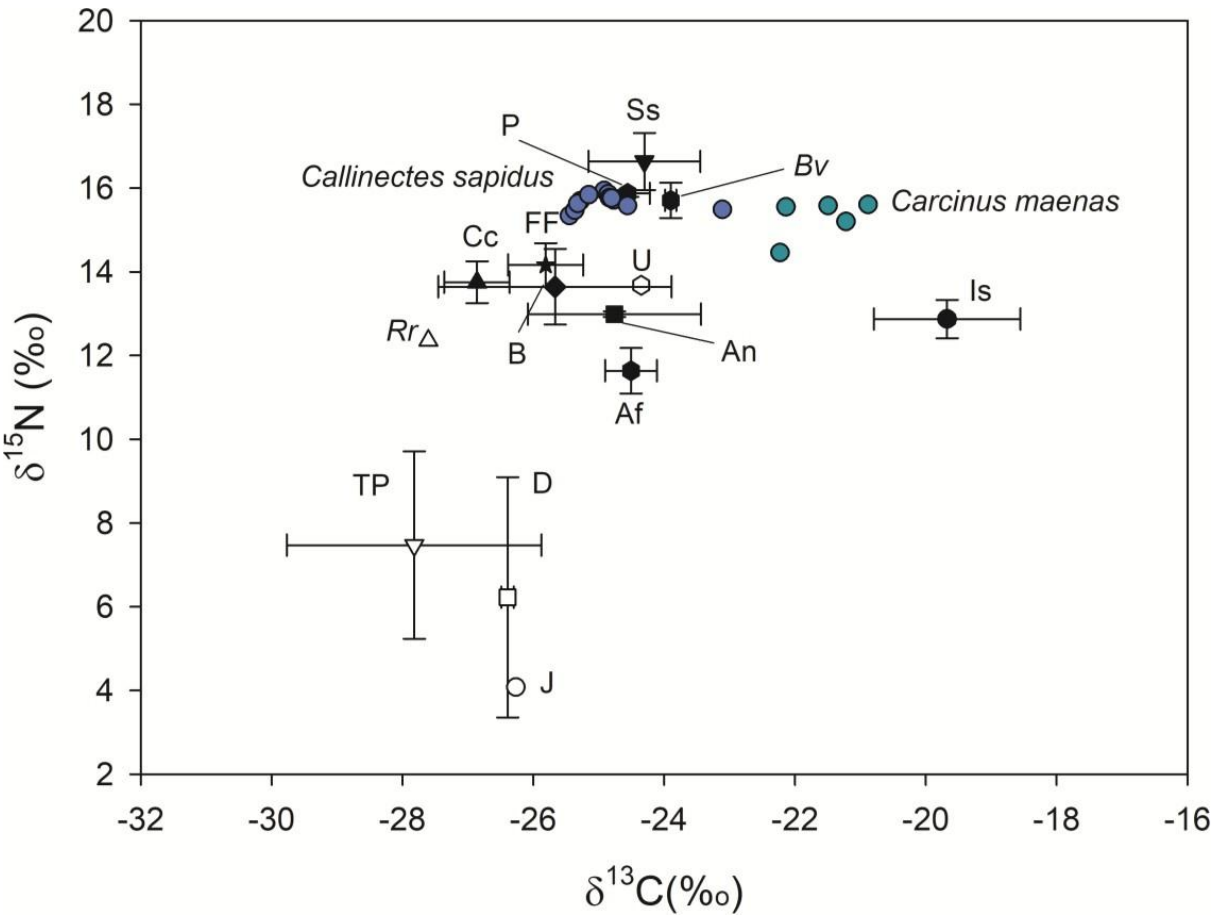
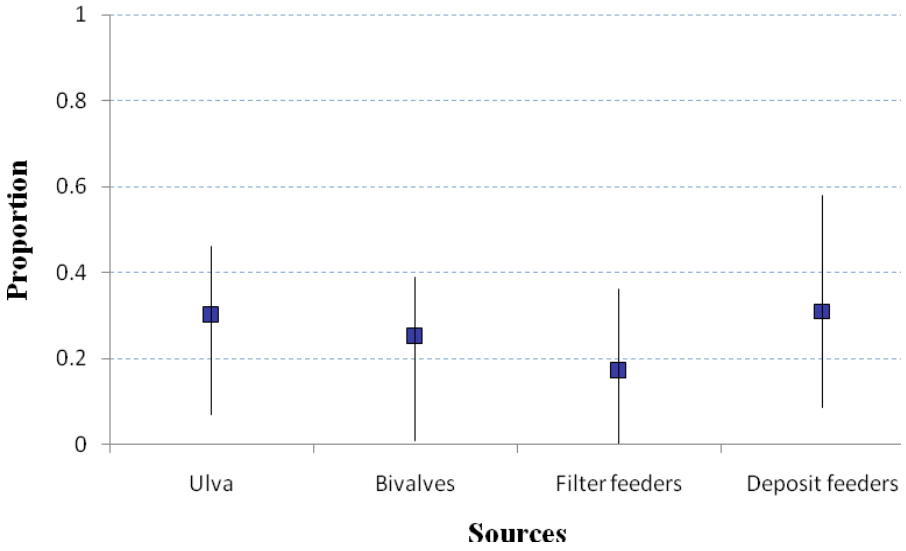


Figure 4.2 Stable isotope ratios of *Callinectes sapidus* and *Carcinus maenas* and average (± SD) stable isotope ratios of their potential food sources collected in the Guadiana estuary in June 2019, which included *Juncus* sp. (J), detritus (D), terrestrial plants (TP), amphipods (An), annelids (An), isopodes (Is), bivalves (B), *R. riparium* (Rr), *Cordylophora caspia* (Cc), filter feeders (FF), *Ulva* sp. (U*)*, *Blackfordia virginica* (Bv) , *Palaemon* spp. (P), *Solea solea* (Ss). Consumers values are not adjusted for trophic level fractionation.

The dual-stable isotope mixing model (95% CI) indicates that deposit feeders (annelids, amphipods, and isopods) were the main sources assimilated by the blue crab (9-58%), followed by *Ulva* sp. (7-46%), bivalves (1-40%), and filter feeders in the water column (0-36%; Fig. 4.3). The most important group of preys assimilated by the green crab were the amphipods (17-68%), followed by isopods (27-57%), and detritus (0-34%; Fig. 4.3).

A- Blue crab



B- Green crab

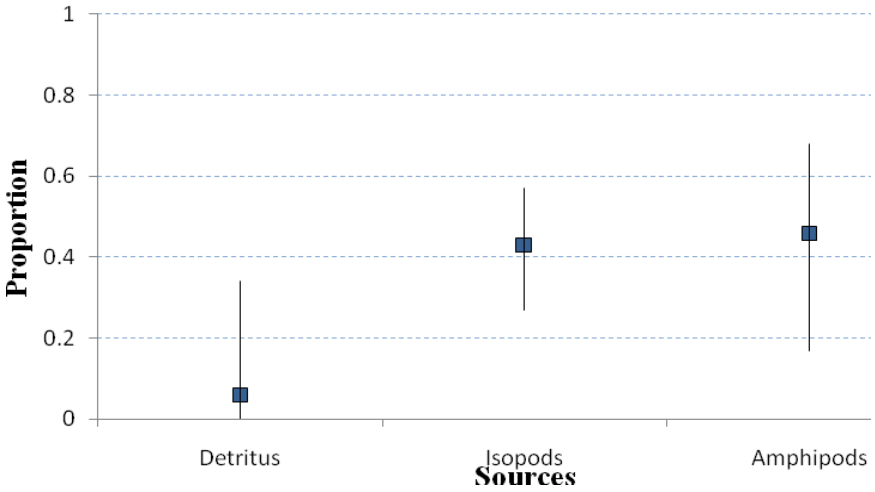


Figure 4.3 Proportion of each food source to the blue crab (A) and to the green crab (B) collected in the middle Guadiana estuary. Closed squares indicate the most likely value (mode) and lines indicate the 95% Bayesian credibility intervals

Chapter 5 - **DISCUSSION**

5. DISCUSSION

This study reports for the first time the nutritional profile and *in vitro* antioxidant properties of the hydroid *B. virginica*, which is commonly found in the Guadiana estuary during spring and summer months. Our results from nutritional analysis revealed that this jellyfish is rich in carbohydrates, minerals, and proteins. However, the high cadmium levels on its biomass may compromise the use of this jellyfish as a safety food source.

This study also identifies, for the first time, crabs as potential predators of *B. virginica* jellyfish in the middle Guadiana estuary. Our molecular results showed that the non-indigenous *B. virginica* is being consumed by the non-indigenous blue crab. However, the results from stable isotope analysis were not conclusive regarding the importance of this jellyfish to crabs' biomass. The nutritional profile, *in vitro* antioxidant properties, and *B. virginica* as a potential facilitator for the establishment of other non-indigeneous species in the middle Guadiana estuary, will be discussed below.

5.1. Nutritional profile

As found in most jellyfish species, the moisture content present in the fresh biomass of *B. virginica* was high (98.7%). For example the moisture content of edible jellyfish, such as *Aurelia coerulea*, the fried-egg *Catylorhiza tuberculata* and the barrel jellyfish were found to be 97.2%, 91.69%, and 94.5% respectively (Leone et al. 2019) which are similar to the value reported in this work for *B. virginica*. According to Lucas (2008), the moisture content in jellyfish wet weight (WW) can range from 89.47% to 98.88% corresponding to 1.12 to 10.53% of dry weigth (DW *i.e.* without water), which is in accordance to the values obtained in this work. Although soft-bodied invertebrates commonly contain a high content of water, the moisture content of jellyfish is higher than those reported in volador, pota and octopus (78.54 - 82.62% WW; Capillas et al. 2003), as well as in cuttlefish and squids (78.3- 81.2% WW; Zlatanos et al. 2006).

Jellyfish DW is typically composed by nutrients (Costa et al. 2019), which are the substances that after ingestion, digestion, absorption, and assimilation become part of the cells and maintain cellular activities (Yuan et al. 2014). Those nutrients include proteins, lipids, ash, minerals, and carbohydrates (Yuan et al. 2014). *Blackfordia*

virginica had high levels of carbohydrates (0.8 % WW; 62.3 % DW), followed by ash (0.4% WW; 30.7% DW), crude protein (0.1% WW; 7.7% DW) and total lipids (0.0% WW; 0.01% DW) which trend is similar, for example, to the large nomura's jellyfish that is often caught in Japan (Huang, 1988), and as other potential edible jellyfish such as moon jellyfish and the Japanese sea nettle *Chrysaora pacifica* (Goette, 1886) in Tokyo Bay (Wakabayashi et al. 2015). The high carbohydrates contents in *B. virginica* can be due to the presence of neutral sugars (*e.g.*, glucose and galactose) in mesoglea that were not determined in this study. However, a study from Kimura et al. (1983) revealed that in jellyfish tissues, a trace amount of carbohydrates, in the form of sugar, is bound to protein as glycoproteins (Kimura et al. 1983). More specifically, glucose and galactose were joined to the polypeptide backbone of the collagen (Kimura et al. 1983). Carbohydrates play an important role in nature because are the main energy source for animals (Abdullah et al. 2015). Furthermore, carbohydrates serve to prevent excessive protein breakdown, loss of minerals, and helps to metabolize fats and proteins (Winarno et al. 2008; Abdullah et al. 2015).

The ash contents in *B. virginica* is higher than in *C. tagi* (18.85 g/100g DW; Morais et al. 2009c), but in agreement with published values for other jellyfish species, such as the river jellyfish *Acromitus hardenbergi* (Stiasny, 1934; 48.42 ± 0.27 g/100g DW), the sand jellyfish *Rhopilema hispidum* (Vanhoffen, 1888; 57.15 ± 0.51 g/100g DW), and the red jellyfish (33.22 ± 0.53; Khong et al. 2016). Ash is a waste product of combustion of organic substances in an organic material and thus, it is a measure of the total amount of minerals (*i.e.*, inorganic elements) present in a biomass. The high ash contents observed in jellyfish are most probably related to the saline environment in which they grow and also due to their ability to retain minerals (Barreira et al. 2017). The majority of the medusa body is composed by mesoglea, a very well hydrated extracellular matrix enveloped by two thin layers of tissue: ectodermal and endodermal (Wright and Purcell 1997). Jellyfish are osmoconformers, therefore the mesogleal extracellular fluid is in osmolar balance with the surrounding seawater (Mills andVogt 1984, Wright and Purcell 1997), mainly in the bell (Mills, 1984). The bell of jellyfish works as a buffer, retaining and controlling their ionic composition and thus giving jellyfish the capacity to float (*i.e.*, mechanism of bouyounce control; Robertson 1949 in Khong et al. 2016). As a result, jellyfish can be a valuable source of essential minerals (Costa et al. 2019).

The screening of the major and trace minerals present in *B. virginica* provided further insights on the nutritional value of this species. Jellyfish bioaccumulates and transfers essential minerals and trace elements from lower trophic levels to higher trophic levels, having a key role in balancing any potential nutritional shortfall of the food chain (Munõz et al. 2015). The same applies to non-essential and potentially toxic elements, which could instead represent not only a threat for the aquatic ecosystems but also for human consumers (Templeman et al. 2010; Munõz et al. 2015). According to the results obtained during this study, *B. virginica* exhibits the same trend for the major elements as the purple jellyfish *Pelagia noctiluca* (Goette, 1886; Costa et al. 2019) and *C. tagi* from the Portuguese coast (Morais et al. 2009c), and as the moon jellyfish and the Japanese sea nettle from Tokyo Bay (Wakabayaski et al. 2015), showing highercontents in Na and lower contents in K when compared tothe other major elements. Although Na is an essential nutrient, its consumption in excess is linked to several human pathologies including hypertension and cardiovascular diseases (Kotchen et al. 2013). Therefore, the World Health Organization (WHO) recommends that the daily intake of sodium should not exced 2000 mg. Considering *B. viriginica* biomass, a consumption of 100g of dry tissue would represent an intake of 728 mg of Na. Thus, care must be taken so that the maximum allowed daily recommended by WHO is not exceeded.

Comparing with other jellyfish species, *B. virginica* presented lower amounts of major elements. For example, Mg in the bell from *C. tagi* and from the purple jellyfish reached 328 mg/100 g DW and 692 mg/100 g DW, respectively, which are higher values when compared to those from *B. virginica* (76.16 mg/100g DW). The Ca contents were 1026 mg/100g for *C. tagi*, whereas for the purple jellyfish were 215 mg/100g, which were also higher values when compared to *B. virginica* biomass (17.44 mg/100g DW; Prieto et al. 2018).

Trace elements, such as Fe, Cu, Zn, Mn and Se, are essential for the metabolism of aquatic vertebrate and invertebrates as they constitute a variety of metalloproteins and antioxidant enzymes, and play a key role in cellular detoxification activity (Sunda et al. 1998). However, these elements can become toxic at high concentrations, leading to damaging oxidative processes (Sunda et al. 1998). *Blackfordia virginica* had lower values of trace elements mainly Cu, Fe, and Mn (Cu: 26.34 μg/100g; Fe: 12086 μg/100g and Mn: 45.43 μg/100g) when compared to those from the potential edible *C. tagi* (Cu*: 564* μg/100g DW; Fe: 7064 μg/100g DW; Mn: 272 μg/100g DW; Morais et al.

2009c) but higher values of trace elements when compared for instance to those from the edible moon jellyfish and japanese sea nettle. For example the estimates for Cu, Fe and Mn contents in the moon jellyfish were 13.60 μg/100g DW, 59.96 μg/100g DW, and 15.96 μg/100g DW respectively, and the estimates for Cu, Fe, and Mn contents in the Japanese sea nettle were 21.00 μg/100g, 142.70 μg/100g ,and 11.66 μg/100g respectively (Leone et al. 2015). On the other hand for the purple jellyfish the Fe and Mn values reported (1465 μg/100g and 49.7 μg/100g, respectively) are similar to those found for *B. virginica* in this study (Costa et al. 2019).

According to the results from this study, it can be hypothesized that *B. virginica* biomass could be considered as a source of natural food supplements not only for humans but also for aquatic organisms (Costa et al. 2019). For example, other jellyfish species such the moon jellyfish and the Japanese sea nettle have already been demonstrated to support the growth and survival of some species such as the fish gilt-head bream *Sparus aurata* (Linnaeus, 1758) owing the metal profile (Wakabayashi et al. 2015; Marques et al. 2016).

Nonessential and potentially toxic elements such as Cr, As, Ni, Pb, and Cd, typically come from anthropogenic activities, and have a negative impact in the aquatic environment (Delgado et al. 2010). In general, considering the Commission Regulation (EU) N. 744/2012 setting the limits of heavy metals in animal feeds and the Commision Regulation (EC) N. 629/2008, amending the Regulation (EC) N. 1881/2006 fixing the maximum levels of heavy metals in food supplements, the mean concentration levels of toxic metals (Cr, Ni, As, Pb) in the investigated samples were bellow the registed values for As (10mg/Kg: 0.0231 mg/Kg for *B.virginica*) and Pb (5mg/Kg: non detected in B. virginica), while for Cd the values found in this study (3.3 mg/Kg) were three times higher than those recommended (1.0 mg/Kg).

In the Guadiana estuary mine related processes, which have been developed in the Iberian Pyrite Belt, along with urban waters and industrial effluents increased metal trace and toxic elements such as Fe, Mn, Al, As, Cr, Ni, Pb, Zn, and Cd in the ecosystem (Delgado et al. 2010). Metal contamination was detected in water, sediments, and aquatic organisms (*e.g.*, asian clam) owing to the impact of the acid mine drainage from the Minas of São Domingos located in the upper part of the estuary (Bebiano, 2010 in Moura et al. 2010; Company et al. 2008). Indeed, mine waters are the principal source of metal pollution because the metals are transported into the river flow and then dissolved (*e.g.*, Cd and Cu) or adsorbed to the suspended particulate matter (*e.g.*, Fe, Pb,

Cu, and Zn). These metals levels are particularly high in Guadiana sediments since 1999, mainly Cd which is the element with the highest increase in the whole estuary, with mean values as high as 1.94 ppm (Bebiano in Moura et al. 2010; Delgado et al. 2010). Comparing values of Cd in the Guadiana basin (1.94 μg/g) and in *B. virginica* tissue (3.37 μg/g), it can be hypothethized that *B. virginica* bioaccumulated Cd from the water in the dissolved form, which may vary according to the season. Indeed, studies from Duysak et al. (2013) and Munõz et al. (2015) reported that different species of jellyfish can bioacumulate toxic metals, in varying degrees according to the species, reflecting a time-integrated measure of their levels in the water and therefore, jellyfish can be useful bioindicators of coastal environments. Further studies envolving the assessment of the chemical composition of *B. virginica* along the seasons/years are needed to investigate their use as biomonitors.

The crude protein value obtained in this study (7.62 g/100g DW), is comparable with those reported in previous studies for other edible jellyfish species: moon jellyfish (3.49 g/100 g DW) and Japanese sea nettle (7.53 g/100 g DW; Wakabayashi et al 2015), purple jellyfish (12.09 – 23.53 g/100 g DW) in the Mediterranean sea and the barrel jellyfish (10.00 g/100 g DW) from the coasts of the east Atlantic Ocean and Alboran Sea (Prieto et al. 2018). In addition, Gorbatenko et al. (2009) investigated the composition of large jellyfish on the West Kamchatka shelf and found a protein content range from 7.1 to 14.6 g/100 g DW. The peculiar protein fraction can be due to the dominant structural collagen that is distributed throughout the mesoglea (about 60%) and is used to retain a large amount of water, thus making such marine invertebrates potential suitable for food/feed purposes (Leone et al. 2015).

Amino acids (AA) are used for example in the synthesis of protein, regulation of hormone secretion, gene expression, and cell signalling (Wu 2009). Amino acids are traditionally classified as as nutritionally essential (*i.e.*, indispensable; EAA) or non-essential (*i.e.*, dispensable; NEAA) for animals and humans (Wu et al. 2012). Nutritionally, essential AA are defined as either those AA whose carbon skeletons cannot be synthesized *de novo* in animal cells or those that normally are insufficiently synthesized *de novo* by the animal organism relative to its needs for maintenance, growth, development, and health and therefore must be provided in the diet to meet physiological requirements (Wu et al. 2012). In contrast, NEAA are those AA which can be synthesized *de novo* in adequate amounts by the animal organism to meet the

requirements for maintenance, growth, development, and health and therefore, do not need to be provided in the diet (Wu et al. 2012).

Blackfordia virginica contained all the EAA with exception of tryptophan, since generally this AA is destroyed during the hydrolysis process with hydrochloric acid (Molmir-Perl and Khalifa 1993). Such results are in accordance with previously studies for edible jellyfish such as for the dry biomass of *C.tagi* (Morais et al. 2009c), for protein samples of the barrel jellyfish (Leone et al. 2015), for collagen peptides derived from the fried-egg jellyfish umbrella (Zhuang et al. 2010) and for gonads (Yu et al. 2014) and total proteins profiles from the purple jellyfish *(*Krishnan et al. 2013).

The percentage of EAAs in *B. viriginica* out of the total AAs (28.82%) was lower than those recorded for the edibles fried-egg jellyfish (51.0%) and barrel jellyfish (50.6%), but comparable with those described for the edible moon jellyfish (31.4%: Leone et al. 2015), and with those recorded in other high value Asiatic and European seafood, as for example with seacocumber (31.02%) and with the bivalve abalone (32.94%; Usydus et al. 2009). When looking for amino acids amount (mg/100g DW), the EEAs contents in the dry biomass of *B. virginica* (1405.60 mg/100 g DW) were higher than those recorded for the above mentioned species with exception for the moon jellyfish that presented comparable values (1402 mg/100g DW; Leone et al. 2015).

The most abundant amino acid found in the *B. virginica* was Glutamic acid + Glutamine (Glx) followed by Glycine (Gly), Alanine (Ala) , Aspartic acid + Asparagine (Asx), Proline (Pro), Tyrosine (Tyr) and Lysine (Lys). Glutamic acid is the crucial AA entering into a variety of transamination metabolic reactions to produce other NEAA (McClelland and Montoya 2002, Chikaraishi et al. 2009, Hannides et al. 2009), and the abundance of Gly is of particular importance, since there is evidence that the intake of food rich in this AA can contribute for the reduction of total cholesterol levels in serum (Ikeda et al. 1993). Furthermore, Gly is the fixed constituent of collagen-typical repeating triplets with a repeating X-Y-Gly sequence, where X and Y can be any amino acid, although Pro and Hydroxyproline (Hyp) residues are the most common triplet in collagen (Gomez-Guillen et al. 2010). Indeed Cheng et al. (2017) found that Gly was the most abundant amino acid in the red jellyfish collagen (Cheng et al. 2017), where the single AAs profile (Gly followed by Ala, Glx, Arg, Pro, Asx, and Lys) was similar to the profile found in *B. virginica* dry biomass suggesting that this jellyfish can be a potential source of collagen. The single AAs profile in *B. virginica* is also comparable to those present in the dry biomass of the purple jellyfish (Gly followed by Glx, Asx,

Arg, Lys, Pro), the barrel jellyfish (Glx followed by Gly, Asx, Lys; Kogovsek et al. 2014) and *C.tagi* (Glx followed by Asx, Gly, Pro, Lys*;* Morais et al. 2009c), suggesting that the main protein in jellyfish dry biomass corresponds to collagen (Leone et al. 2015). The ratio values between total EEA and total amino acids (0.3) and between EEA and NEAA (0.7) means that *B. virginica* proteins are of good quality according with FAO. In addition, *B. virginica* has a low lysine/arginine ration (0.8%), lower than the ones described for example for the moon jellyfish, fried-egg jellyfish and for the barrel jellyfish (Leone et al. 2015). Low lysine/arginine ratios are usually linked to hypocholesterolemic effects, thus suggesting that *B. virginica* could be useful for people with hyperlipidemia disorders (Bordbar et al. 2011).

The total lipids in *B. virginica* (0.01 g/100g DW) were almost not detected. Indeed, they were present in low quantities than those reported for the moon jellyfish from Japan (0.43 g/100g DW; Wakabayashi et al. 2015) and for the barrel jellyfish from Spain (0.94 g/100g DW; Prieto et al. 2018), resulting in low energetic value (288.68 kcal/100 g DW), lower than those reported for several fish species (Norwegian Food Safety Authority 2014). According to Joseph (1979), jellyfish contain no visible lipid deposits, except in relatively well-developed gonads during the reproductive cycle, which can explain the low lipids content in *B. virginica* once they were collected at the beginning of their annual bloom. However, they can be also structural elements of jellyfish cell membranes (Zhu et al. 2015). Overall, *B. virginica* has an adequate protein/lipid ratio (23:1), which is of particular interest from a nutritional point of view, since proteins are valuable nitrogen and amino acid sources for the human body.

As for FAMEs profile of *B. virginica*, our results are in accordance with some studies reported for other potential edible jellyfish species, such as the purple jellyfish (Costa et al. 2019). Saturated fatty acids have all or predominantly single bondes in their chains, and accounted for two third of the total FA (55% - 75%), followed by MUFAs that have one double bound in the fatty chain with all of the remainder carbon atoms being singled bonded, and PUFAs that have more than one double bound. However, most jellyfish studies reported a different trend, with PUFAs being present in higher amounts as for example the moon jellyfish , the fried-egg jellyfish and the barrel jellyfish (Leone et al. 2015; Prieto et al. 2018). That could be to the fact that these species are known to have microalgal symbionts which are an important and significant source of essential ω-3 fatty acids (Leone et al. 2015).

The composition of the 15 fatty acids identified in *B. virginica* is similar to those found in the purple jellyfish (Costa et al. 2019), in the moon jellyfish, in the fried-egg jellyfish and in the barrel jellyfish (Leone et al. 2015), however with different trends. While in *B. virginica*, the saturated fatty acids consisted mostly of capric and myristic acid followed by lauric and palmitic acid, the above mentioned species contained mostly palmitic and stearic acids followed by lauric and arachidic acids. Indeed, palmitic, stearic, myristic, and lauric acids arethe most common FA in animal tissues. Palmitic and stearic acids are universally found in natural fats. Lauric acid is specifically abundant in copra and palmist oils (Legrand, 2010) and is recognized for its antiviral and antibacterial properties (German and Dillard, 2010). Myristic acid and short-chain FA (including capric acid) represent each about 10% of FA in milk fat where capric acid has antiviral activity agains HIV (German and Dillard, 2010). Among MUFA, oleic acid was the prevalent FA not only in *B. virginica* but also in the moon jellyfish, in the fried-egg jellyfish and in the barrel jellyfish, however myristoleic acid was only detected in *B. virginica* (Leone et al. 2015; Wakabayaski et al. 2015). Oleic acid (18:1(n-9) is the precursor of all (n-3) and (n-6) PUFA, and is essential to heterotrophic organisms (Legrand, 2010). It is the most common MUFA in human cells, and is incorporated into cell membrane phospholipids, where it is important for proper membrane fluidity, being the major energy source for cells (Lopes et al. 2010). In relation to PUFAs ω-3 FA, linolenic acid was the major component together with squalene in *B. virginica* samples. In the three jellyfish reported above, the linoleic acid ω-6 was the major component, together with the ω-3 eicosatetraenoic acid, docosahexaenoic acid and the eicosapentaenoic and arachidonic acids (Leone et al. 2015; Wakabayaski et al. 2015). The ω-3 types of FA are involved in a number of biological processes (*e.g.*, growth, development, tissue and cell homeostasis) and have a variety of health benefits including hypo-triglyceridemic, anti-inflammatory, antihypertensive, anticancer, antioxidant, antidepressive, antiaging, and antiarthritis effects (Leone et al. 2015). Differences in FA composition between jellyfish species might be related to specific requirements both to physiological adaptation to different habitats and to evolutionary constrains (Dalsgaard et al. 2003).

5.2. *In vitro* antioxidant properties

Oxidative stress is considered a main environmental risk factor for the development of several forms of pathologies, as for example neurological and skin disorders,

diabetes, and obesity (Shaw et al. 2014; Conti et al. 2016). In this regard, one approach to prevent age related diseases is the use of antioxidants that protect the organism from excessive ROS production, such as the superoxide or hydroxyl radicals (Conti et al. 2016). To the best of our aknowledge this is the first preliminary report on the *B. virginica* antioxidant activity.

In this work, an aqueous extract was prepared from the dried biomass from *B. virginica* and evaluated for *in vitro* antioxidant properties, by four methods. Given the different response of antioxidants to different radicals or oxidant sources, a single assay is generally not enough to assess the antioxidant activity of target samples (Custódio et al. 2012). The extraction method was based on Yu et al. (2006), which allowed the extraction of bioactive proteins from the red jellyfish (Yu et al. 2006). In this work, the extract presented a moderate activity on the ABTS method (57% at the concentration of 25 mg/ml), but was innefective in the other assays. This can be because ABTS radical scavenging method it is one of the more effective in aquose solution (Leone et al. 2019). Values from *B. virginica* can not be compared with those ones for the red jellyfish (Yu et al. 2006), because *B. virginica* samples required higher amount of extract concentrations to reach those values. For example proteins from the red jellyfish had a scavenging effect on hydroxyl radical of 10.6% with a concentration of 13μg/ml, reaching 69% at a concentration of 65.1 μg/ml.

Overall, because in the present study results for antioxidant activity were not relevant, the chemical analysis of the extract was not made, but further studies are needed. Indeed, increasing scientific evidence demonstrates that peptides with antioxidant properties can be obtained from marine vertebrates and invertebrates proteins, hydrolysed proteins, and seafood by-products, with peptides exhibiting higher antioxidant activities than proteins (Domenico et al. 2019).

Owing to the preliminary composition data of *B. virginica* herein discussed may be a valuable starting point of acknowledging its nutritional properties for food. It has been widely discussed the relevant value of some jellyfish species for both aquatic organisms and human consumption.

But before getting into any pratical application of *B. virginica*, other *in vitro* studies will be necessary for developing the most suitable procedures for biomass processing. Then, if the applications turned out to be realistically feasible, further research will be required to investigate *in vivo* toxicity and effectiveness for food supplements.

5.3. *Blackfordia virginica* as a potential food source for aquatic animals

The relevance of *B. virginica* as food for opportunistic aquatic consumers in the middle Guadiana estuary was investigated in this study combining molecular techniques (*i.e.*, DNA-PCR approach) with stable isotope analysis (SIA). The use of molecular techniques allowed to identify the presence or absence of *B. virginica* in the gut contents of wild crabs. When assessing dietary composition based on gut content analysis, soft bodied organisms are usually difficult to identify, especially jellyfish, because they are digested very rapidly and preservative methods may destroy or shrink gelatinous material (Arai, 2005). Therefore, development of PCR-based techniques allows the identification of prey remains even those partially digested (Symondson, 2002). Comparing the two methods, SIA allow identifying the most important preys that were assimilated by the organisms's tissues that maybe are not evident based on the DNA- PCR approach. However, DNA-PCR approach provides more specific trophic (predator-prey) interaction information that wouldn't be possible from SIA alone (Carreon et al. 2010). In this study PCR-based techniques allowed detecting the DNA of the *B. virginica* in two blue crabs' stomach contents. *Blackfordia virginica* was used as positive control, and was only positivelly amplified by the specific primers ITS1. The consistent negative amplification of both *B. virginica* and crabs samples for primers 16S and COI, with the exception of one green crab (GC2), suggests a deficient methodological procedure. Harrison et al. (2013) reported that the primers that were used in this study are able to amplify the genomic DNA of *B. virginica*. Therefore, an optimization on the PCR reaction would be necessary to enable the amplification using the 16S and COI fragments.

Nonetheless, during this study, it was possible to confirm that at least the non-indigenous blue crab preys on *B. virginica*. Because the sampling occurred at the beggining of the jellyfish annual bloom (sampling occurred in June 2019, and the bloom started in May 2019) it is possible that if sampling had occurred later in the summer or early autumn, the proportion of crabs with *B. virginica* in their stomachs would increase. Most likely due to the sampling design, combined with the low number of individuals analyzed, the quantification analysis of the prey assimilated by these crab species, using stable isotopes, showed no importance of *B. virginica* to their biomass. In fact, the most important preys contributing to these species' biomass were deposit

feeders such as amphipods, annelids, and isopods.

A critical assumption to the SIAR mixing model is that both sources and consumers are sampled on temporal scales that reflect the relative incorporation rates of the elements and the turnover rates of tissues (Layman et al. 2012). Vedral (2012) estimated that the half-life muscle tissue turnover in the blue crab was 83 days. Thus, in order to quantify the real importance of *B. virginica* to crabs' tissue biomass, crabs should have been sampled at least at the end of the jellyfish bloom period (autumn). Another critical assumption to conduct a proper stable isotope analysis is that the isotope fractionation values used reflect those from consumers' tissues (Layman et al. 2012). However, fractionation values can vary with the type of diet (plants *vs* animals), species, or life cycle stage (Vander Zanden and Rasmussen 2001; Caut et al. 2009). In fact, laboratory experiments with juvenile blue crabs indicated that fractionation can vary according to the type of diet, being higher in plant-derived diets when compared to animal- derived diets (Fantle et al. 1999).

Notwithstanding, this study provided evidences that jellyfish are not 'dead ends' in the Guadiana estuary food web, as they are predated at least by the blue crab. Blue crab is an opportunistic and generalistic feeder exhibiting a high trophic flexibility (trophic level varying between 2.8-4.3) in both native and invaded ecosystems (Mancinelli et al. 2017) and dietary flexibility has been acknowledged as key aspect for the success of invasive species (Dias et al. 2014; Mancinelly et al. 2017). Previous studies reported that the blue crab prey frequently on bivalves, amphipods, polychaetas, crustaceans, detritus, and plant matter (Seitz et al 2011; Lipius et al. 2016), but commonly adapts its diet to the food resources available in its environment (Mancinelli et al. 2017). During early summer to latter autumn, *B. virginica* becomes one of the most widely spread species reaching high densities in the middle estuary (31.5 ind. m^3; Chícharo et al. 2009; Muha et al. 2013), which coincides with the area inhabited by the blue crab (Morais et al. 2018). Therefore, it is expected that the availability of *B. virginica* medusa during bloom periods in the water column, and their vertical movements, will benefit these benthic and generalistics predators. In addition, *Blackforida virginica* individuals have low size, usually exist in high numbers and have slow mobility, and thus can be easily preyed (Heeger et al. 1992).

Although fish were not targeted during this study, they may also benefit from this occasional resource. Studies conducted on the Thau lagoon (NW Mediterranean) found that the gilthead seabream, the european eel *Anguilla Anguilla* (Linnaeus, 1758), the

golden mullet *Liza aurata* (Risso, 1810), and the salema can feed on species from the genus *Aurelia* (Marques et al. 2016; 2019). Despite their high water content, medusae may provide enough energy to sustain the standard metabolism of fish. Indeed, Marques et al. (2019) suggested that even though a large amount of jellyfish consumption is needed to meet their energy requirements, the rapid digestion and gut clearance rates allow the fish to increase its ingestion rates with medusae being a non-negible food source for these commercially important fish species (Marques et al. 2019).

Therefore, although it was not possible to quantify the importance of *B. virginica* to crabs' biomass, it was possible to confirm that it is consumed at least by the blue crab. The number of studies showing that successful primary invaders can facilitate directly or indirectly the invasion success of secondary invaders are increasingly common (*e.g.*, Green et al. 2011). In this case the non-indigenous *B.virginica* may act as a facilitator for the establishment and invasive behavior of the blue crab in the Guadiana estuary by contributing as prey to its the diet. In Massachusetts (USA), Carman et al. (2017) reported, for the first time, the consumption of the toxic clinging jellyfish *Gonionemus* sp. (Cnidaria, Hydrozoa) by indigenous spider crabs and occasionally by the blue crab (Carman et al. 2017). They suggested that jellyfish has the potential to favour indirectly the invasive green crab populations by inducing mortality in a native competitor, in this case the native spider crab (Carman et al. 2017).

However, because only few individuals of both blue crab and green crab were analyzed in this study and because crabs may alter their diet along their development (Lipcius et al. 2007; Seitz et al. 2011), additional studies are needed, involving more crabs from different ontogenetic stages, but also including other indigenous generalist consumers such as fish, to confirm the actual importance of *B. virginica* as a source of food to the consumers in the middle portion of the Guadiana estuary. Its potential as food not only for the blue crab but also for other NIS poses a serious threat to the conservation of the Guadiana estuary. This ecosystem is already colonized by several NIS, and due to other human-induced modifications, such as those related with the Alqueva dam's construction, several brackish habitats,once occupied by indigeneous species, are now available for NIS

Therefore, if further studies confirm that *B. virginica* might be sustaining other NIS rather than indigenous species, one way to control its blooms during summer months might be using this jellyfish as a human food source if cadmium levels decrease. However, there are important aspects to consider when exploring NIS as a resource

(Morais et al. 2018). For example fishing pressure must efficiently reduce the NIS population size and growth without affecting other native species (Morais et al. 2018); public must be made aware of the putative negative impacts of NIS and that introductions into non-invaded areas are not permited (Morais et al. 2018) and the fishery of an invasive species can never be managed to make it sustainable, which disables local communities from obtaining a long-term financial revenue (Morais et al. 2018).

5.4. Future perspectives

Further studies envolving the determination of collagen and the total protein in the aquose extract of *B. virginica* dry biomass are need. If the aquose extract is rich in proteins then enzymathic hydrolysis of *B. virginica* proteins can be made and retested for antioxidant activities. Indeed, Zhuang et al. (2009) reported that jellyfish peptides exibit higher antioxidant activity than proteins, such as those from the red jellyfish. In addition, Domenico et al. (2019) reported that enzymatic hydrolysis of jellyfish proteins is the most efficient method to produce homogenous bioactive peptides.

Furthermore, in order to have the real contribution of *B. virginica* to the diet of both green crabs and blue crabs, further studies are needed envolving a higher number of individuals, including individuals indifferent life- cycle stages, and crab's sampling must reflect their tissues turnover, *i.e.,* crabs must be sampled after *B. virginica* annual blooms in order to analyse its potential assimilation. Furthermore, in order to understand the role of *B. virginica* in the middle Guadiana estuary food web, studies including other potential predators as well as species sharing the same trophic niche, are mandatory.

Chapter 6- **CONCLUSION**

5. CONCLUSION

Blackfordia virginica showed a nutritional profile similar to those found in edible jellyfish, such as in the moon jellyfish from Japan and *C. tagi* from Portuguese coast, however with higher concentrations of cadmium. Thus, *B. virginica* showed a high potential to be used as food for human but only if cadmium levels decrease :

- The moisture, carbohydrates, ash, and proteins levels are high and presented adequated amounts of most of the essential amino acids, coupled with low lysine to arginine ratios;
- The total lipis were almost non detectable, resulting in low energetic values, where the fatty acid profiles were characterized by high levels of saturated fatty acids mainly C11:0;
- It also presented high content of metals, mainly the essential Na, the essential trace metal Fe, and Zn. However it also presented high contents of the toxic metal Cd. This compound is efficiently retained in the human body, accumulating, being primarily toxic to the kidney.

Furthermore, this study showed that *B. virginica* is preyed by the non-indigeneous blue crab *Callinectes sapidus*, which may represent an additional threat to the ecosystem. Due to the fact they form blooms during the summer and autumn, they may constitute a highly abundant seasonal resource favouring the establishment of this and other generalist non-indigeneous species at the middle portion of the Guadiana estuary. However, they also may constitute a highly abundant food source for other generalist indigenous consumerss. Thus, further studies are needed to quantify the importance of jellyfish to the blue crab diet and also their role in the estuarine food web.

In conclusion, this study shows some evidences that *B. virginica* may represent a threat to the aquatic ecosystems by favouring the non-indigenous blue crab, while simultaneously may provide an opportunity for managment through commercial exploitation as food for humans.

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7. Supplementary material

Table 0.1 Sequentions results that work out for positive amplifications, with max score, total score, query cover, E value, percentage of identification and accession number.

Samples	Description	Max score	Total Score	Query Cover	E value	% Identity	Acession number
BV ITS[a]	Blackfordia rginica internal anscribed spacer 1, rtial sequence	427	427	96%	9,00E-116	99.1%	MH460960.1
	Blackfordia rginica internal rtial sequence	63.9	118	24%	3,00E-06	100%	MH460960.1
BC10 ITS [a]	Blackfordia rginica internal anscribed spacer 1, rtial sequence	56.5	56.5	7%	8,00E-04	100%	MH460960.1
BC12 ITS[a]	Brevoortia rannus group A one 5 internal anscribed spacer 1 d 5.8S ribosomal quence	355	355	84%	8,00E-94	85.2%	FJ195916.1
GC2 16s[b]	Carcinus maenas mitochondrial 16S rRNA gene (partial), tRNA-Leu gene and NADH1 gene (partial), specimen voucher SMF<DEU>:32757	758	758	90%	0.0	94.51%	FM208763.1

[a] Sequencion results of the first PCR amplification; [b] Sequencion results of the second PCR amplification for primers 16S and COI

Table S.2 Values of $\delta^{13}C$ and $\delta^{15}N$ from *C.sapidus* and *C.maenas* and their potential preys in the Guadiana estuary. Samples were grouped by species and results are expressed as mean ± SD

Group	**$\delta^{15}N$ Mean ± SD**	**$\delta^{13}C$ Mean ± SD**
Predators		
C.sapidus	15.67±0.62	-24.86±0.62
C.maenas	15.27±0.58	-21.59±0.58
Preys		
Filter feeders	14.16±0.54	-25.82±0.57
Bivalvia	13.64±0.90	-25.70±1.78
Annelida	12.99±0.07	-24.80±1.32
Amphipods	11.64±0.54	-24.51±0.40
Isopods	12,90±0,46	-19,67±1.12
B.virginica	15,70±0,42	-23,90±0.09
Paaemon sp.	15,87±0,08	-24,56±0.34
S.solea	16,64±0,68	-24,30±0.85
Detritus	6.20±2.90	-26.40±0.10
Terrestrial plants	7.5±2.23	-27.80±1.95