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Simão Pedro Domingues Correia

Comunidades intertidais e subtidais de trematodes: influência dos fatores ambientais na infeção de berbigões

Intertidal and subtidal trematode communities: influence of the environmental factors on cockles infection

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Rosa de Fátima Lopes de Freitas, Investigadora Auxiliar e da Doutora Luísa Virgínia de Sousa Magalhães, Investigadora em Pós-Doutoramento, ambas do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.

“And following our will and whim
we may just go where no one’s been”

Lateralus, Tool

o júri

presidente

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palavras-chave

Cerastoderma edule; Zona Intertidal; Zona Subtidal; Hidrodinamismo; Cercariae; *Himasthla elongata*; Fototropismo; Bivalve; Fatores Abióticos

resumo

O berbigão, *Cerastoderma edule*, é uma espécie de bivalve amplamente distribuída pela costa nordeste do Atlântico. Este bivalve é altamente explorado em vários países, inclusive Portugal, tendo uma alta relevância socioeconómica e um papel importante em serviços cruciais para o funcionamento do ecossistema. Este bivalve é hospedeiro de várias espécies de trematodes, os macroparasitas mais prevalentes em águas costeiras, que exibem um ciclo de vida complexo e que geralmente usam três espécies diferentes de hospedeiros. Os trematodes são capazes de modular a dinâmica populacional dos berbigões, exercendo sobre eles impactos devastadores dependendo da abundância, fase do ciclo de vida ou da combinação com fatores abióticos. No entanto, os padrões pelos quais estes parasitas se governam ainda são pouco compreendidos. Assim, esta tese teve como objetivo principal, reconhecer padrões ambientais que podem regular a população de trematodes, identificando os principais fatores implicados na variabilidade da taxa de infeção.

Num primeiro momento, foi realizada uma extensa amostragem espacial de berbigão, desde o norte de Portugal até ao sul de Marrocos, de forma a identificar fatores que influenciam, em larga escala, a comunidade dos parasitas e que permitiu analisar, pela primeira vez, berbigões a sul de 31 °N. Das 12 espécies de trematodes identificadas, várias foram observadas em todo o gradiente latitudinal demonstrando a grande capacidade de dispersão destes parasitas. Os resultados sugeriram que a temperatura foi o fator mais importante para despoletar a infeção por trematodes, com o sistema de upwelling da costa Portuguesa a funcionar como proteção contra a infeção por trematodes. No entanto, a distribuição geográfica dos hospedeiros, a posição de maré do segundo hospedeiro e a influência oceânica também pareceram ter efeito na abundância dos trematodes.

No seguimento destes resultados, avaliou-se os efeitos, em pequena escala, da posição vertical (intertidal ou subtidal) e do hidrodinamismo na taxa de infeção por trematodes. Para isso, foi recolhido berbigão em zonas intertidais e subtidais de uma área a montante e a jusante da Ria de Aveiro. Foram analisados a densidade de berbigão, a infeção por trematodes e os parâmetros físico-químicos do sedimento e da água. Para caracterizar o hidrodinamismo da Ria procedeu-se a várias simulações através do modelo Mohid. Berbigão da zona intertidal demonstrou maiores níveis de infeção, destacando a importância da posição vertical do hospedeiro no sucesso da infeção pelos trematodes, possivelmente devido a uma distância mais curta ao primeiro hospedeiro (geralmente gastrópodes intertidais) ou a uma menor capacidade das cercariae (estado de vida livre dos trematodes que emerge do primeiro hospedeiro e infeta o segundo) para encontrar o seu hospedeiro num ambiente subtidal e completar o ciclo de vida. No geral, as áreas a jusante (maior hidrodinamismo) apresentaram níveis de infeção mais altos, sugerindo uma influência positiva da dinâmica da água na qualidade ecológica destes habitats, aumentando a dispersão das cercariae promovendo, desta forma, o contacto com os seus hospedeiros e consequentemente, aumentando o sucesso da infeção. A influência da corrente da água na infeção de trematodes foi corroborada por uma experiência laboratorial.

Por último, e seguindo alguns dos resultados obtidos (principalmente a diferença de infeção entre a zona intertidal e subtidal), foi testado o efeito da luz no sucesso da transmissão pela cercaria da espécie de trematode *Himasthla elongata*. Através duma experiência laboratorial, pretendeu-se observar o efeito da luz (ausência vs. presença de luz) no comportamento das cercariae. Berbigões sob a ausência de luz demonstraram ficar mais parasitados do que os que se encontravam expostos à luz. As diferenças foram explicadas pela influência da luz na atividade natatória das cercariae e não pela presença ou capacidade filtradora dos berbigões, uma vez que, estes não demonstraram diferenças na taxa de respiração, qualquer que fosse a exposição luminosa.

Esta tese demonstrou a importância dos fatores abióticos no sucesso de transmissão dos trematodes entre hospedeiros. Os resultados deste estudo podem ser utilizados de modo a controlar e a melhor gerir as populações de berbigão, ajudando a prever áreas ou períodos de maior probabilidade de surtos de abundância de trematodes.

keywords

Cerastoderma edule; Intertidal; Subtidal; Hydrodynamics; Cercariae; *Himasthla elongata*; Phototaxis; Bivalve; Abiotic drivers

abstract

Cerastoderma edule, the edible cockle, is a dominant and widely distributed bivalve of the northeast Atlantic coast. This bivalve is a highly exploited species in many countries, displaying an important socio-economic role and playing crucial services for ecosystem functioning. This bivalve is host of several trematode species, the most prevalent clade of macroparasites in coastal waters, that display a complex life cycle commonly including three host species. Trematodes are able to modulate cockles population dynamics, exerting devastating impacts depending on abundance, life-cycle stage or combination with abiotic factors. However, the patterns by which they govern are still poorly understood. The present thesis aimed to recognize environmental patterns that could regulate trematode population dynamics, identifying the major factors implicated in infection rate variability.

In the first instance, an extensive spatial sampling of cockles was performed from north Portugal to south Morocco in order to identify large scale drivers of parasite community structure and to analyse, for the first time, cockles south of 31 °N. Most of the 12 identified trematode species were observed along the whole latitudinal gradient demonstrating the high dispersal ability of these parasites. Results suggested that temperature was the most important trigger to predict parasite infection, with coastal upwelling system operating for Portugal as a shield against trematode infection. Nonetheless, hosts geographical distribution, second intermediate host tidal position and the oceanic influence also seemed to affect trematode abundance.

Following these results, the effect of host vertical position (intertidal vs. subtidal) and hydrodynamics on trematode infection rate was assessed at a small-scale. For this, cockles were collected in intertidal and subtidal sites of downstream and upstream areas of the Ria de Aveiro coastal lagoon (Portugal). Cockle density, trematode infection and sediment and water physico-chemical features were assessed. To characterize the lagoon hydrodynamics, simulation with the numerical model Mohid were performed. Intertidal cockles displayed significantly higher individual infection than those from subtidal sites, highlighting that the host vertical position represent an important driver of trematode infection success, possibly due to shorted distance from the first intermediate host (often intertidal gastropods) or lower capacity of cercariae (trematode free-living stage emerging from the first intermediate host to infect second intermediate host) to find their suitable host in a subtidal environment and then to complete the life cycle. Overall, the downstream areas (= higher hydrodynamics) presented higher trematode abundance suggesting a positive influence of water dynamics in the ecological quality of these areas, increasing the dispersion of trematode free-living stages that promotes contact with the second intermediate host and consequent infection success. The influence of water current on trematodes infection was corroborated through experimental approach.

Lastly, and pursuing the track of some of the obtained results (namely the infection between intertidal and subtidal sites), the effect of light on cercariae transmission success, using the trematode *Himasthla elongata* was assessed. Through experimental approach, this study aimed to observe the influence of light (no light vs. light exposure) on the cercariae behaviour. Nevertheless, cockles under dark conditions presented higher levels of infection compared to cockles exposed to light. These differences were explained by the influence of light on the cercariae swimming activity, which navigation showed to be guided by the light position and not by the presence and/ or filtering capacity, since that cockles respiration rate was similar regardless light conditions.

This thesis findings demonstrated the importance of abiotic factors on the transmission success of trematode parasites. The outcomes of this study can be used for a more efficient management of cockles stocks by helping to predict areas or periods of higher probability of a trematode abundance outbreak.

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CHAPTER 1. GENERAL INTRODUCTION

1. General Introduction

1.1. Coastal water systems hydrology and biodiversity

Surpassing 70 % of total world surface area, aquatic systems display important environmental functions and are constituted mainly by saltwater ecosystems (oceans), freshwater ecosystems (rivers) and coastal zones, i.e. transitional zones between the sea and the land where the saltwater from the oceans encounters and merge with the freshwater from rivers (FAO, 1998). Due to the interaction between oceanic and riverine influences, together with the terrestrial interaction (anthropogenic activity), coastal waters, which include coastal lagoons, estuaries and bays, represent one of the most complex ecosystems (Pérez-Ruzafa et al., 2019).

Coastal systems are highly diverse systems, providing habitat for populations of several migratory and resident species (Levin et al., 2001), important in both ecological and economical aspects (Agardy et al., 2005). Moreover, these areas account for numerous essential ecological functions, such as nutrients recycling, and represent a high fraction of the total oceanic biological production (Sigman and Hain, 2012). Besides, these aquatic systems have high economic value providing many natural resources and settlement sites for humans, gathering a significant proportion of the human population (Basset et al., 2013; Dayton et al., 2005). Additionally, the modification experienced by these systems due to freshwater inputs from rivers, saltwater inputs from oceans, precipitation and evaporation balance, wind and tidal stress and water circulation, makes coastal waters highly dynamic ecosystems (Dyer, 1997; Prandle, 2009). These natural abiotic disturbances promote the appearance of horizontal and vertical abiotic gradients in the water column. Adding to these gradients the tidal and seasonal climatic variations, with consequent influence for example on water temperature and salinity, cause these ecosystems to be one of the hardest environments that inhabiting organisms have to endure. This set of abiotic variations, are then responsible for coastal communities distributional patterns in the sediment and in the water column (Bianchi, 1991; Massutí and Reñones, 2005; Kallianiotis et al., 2000).

In coastal waters, communities composition is described to be different according to bathymetric position (Carney, 2005) which might be explained by depth control over several environmental variables variation. For example, temperature and salinity, abiotic variables presented as playing highly important roles on spatial distribution of organisms by affecting individuals growth, reproduction or feeding activity (Gagnaire et al., 2006; Gosling, 2015;

Hale, 2010), are largely influenced by depth. Water density is increased by salinity but decreases with temperature. Thus, cold saltier waters, due to the higher density, sink beneath less saline and warmer waters (Kjerfve, 1979), creating a tidal gradient where salinity increases with depth and temperature decreases. Salinity positively influences biodiversity (Palmer et al., 2015; Sousa et al., 2006), however, only within a certain range of values considered optimal (Montagna et al., 2002). Similarly, higher water temperature, due to the positive influence on primary production and nutrient availability, promotes higher benthic diversity (Cochrane et al., 2009). Nonetheless, the increase of water temperature can also lead to lower levels of oxygen on the water column, which may lead to hypoxia events and consequently biodiversity loss (Villnäs et al., 2012). Indeed, in shallow lagoons or estuaries, the bottom layer of the water column is usually richer in nutrients than the air-water interface (Dobson et al., 2000).

In the case of transitional water systems, such as coastal lagoons or estuaries, it is possible to observe, as well, a horizontal abiotic gradient. The downstream areas of these systems, due to the water exchange with the ocean, display more dynamic features with higher salinity levels and less pronounced temperature shifts (e.g. Vaz et al., 2005). Moreover, as a result of the higher water flow, these areas also present higher sediment granulometry and nutrients availability on the water column (Molinaroli et al., 2009). This way, the benthic community of downstream areas, is usually characterized by higher diversity and composed mainly by suspension feeding fauna (Rosenberg et al., 1995; Thistle, 2003). On contrary, upstream areas of costal systems, as a result of freshwater inflows, show lower water dynamics and less saline waters with high variations of temperature (e.g. Vaz et al., 2005). The lower hydrodynamics increases particle deposition, reducing nutrients availability on the water column, which promotes deposit feeding organisms (Thistle, 2003). The high diversity of abiotic characteristics and the different habitats created by these gradients make costal systems hotspots of biodiversity (Watanabe et al., 2018).

1.2. *Cerastoderma edule* (Bivalvia: Cardiidae)

Among the benthic macrofauna of coastal water systems, bivalves constitute a predominant component and represent a major proportion of benthic fauna biomass (Sousa et al., 2009). This class of animals play important roles on the ecosystem and are considered keystone species, contributing for the resilience of marine environment (Taylor et al., 2018). Due to their filter-feeding habit and bioturbation activity, marine bivalves provide several essential ecological services such as carbon storage or cycling energy (Morgan et al., 2013). Moreover, in the ecological community food web, they represent a link between primary producers and the higher trophic levels (Rakotomalala et al., 2015). In the case of infaunal organisms, due to their burrowing activity, bivalves also transform the habitat and therefore they are considered ecosystem engineers (Philippart et al., 2007). Besides, bivalves are the basis of important commercial activities, representing a highly important socio-economic role (Beukema and Dekker, 2006), accounting, approximately, to 15 % of worldwide total marine species production (Wijsman et al., 2019).

The edible cockle (Figure 1), *Cerastoderma edule* (Linnaeus, 1758), is an infaunal suspension-feeder, predominant and widely distributed bivalve species that inhabits the coastal systems of the northeast Atlantic coast from Norway in the north Europe to Mauritania in the north of Africa (Dabouineau and Ponsero, 2011; Honkoop et al., 2008). Cockles have high socio-economic value due to their extensive commercial exploitation (Beukema and Dekker, 2006), with special significance in Portugal (Pereira et al., 2014).

In this species, physiological conditions are well studied (Malham et al., 2012). *C. edule* lives buried in the sediment of intertidal and subtidal areas (up to 20 meters; Riesen and Reise, 1982) and reaches its first sexual maturity around 12 mm for males and 14 mm shell length for females (Hancock and Franklin, 1972; Cardoso et al., 2009). Cockles are eurytherm and euryhaline species (Malham et al., 2012), i.e. organisms able to adapt to a wide range of temperatures and salinities, respectively. They are able to tolerate temperatures between 4 °C and 38 °C (Compton et al., 2007) however, the optimal temperature is between 20 and 23 °C (Verdelhos et al., 2015) and spawning only occurs at temperatures higher than 13 °C (Boyden, 1971). Regarding salinity, the optimal salinity for cockles is between 30 and 35 (Malham et al., 2012), nonetheless it can survive to salinities nearby 12 (Russell and Petersen, 1973). Despite its usual association to sheltered intertidal areas, *C. edule* display a higher settlement capacity and growth rate when exposed to higher water flow (Kater et al., 2006). Boyden and Russell (1972) reported that cockles were incapable to settle in still water conditions, while higher food availability on more dynamic areas promote cockles growth (Eckman, 1987). When exposed to air, cockles are still able to consume oxygen and, therefore survive for long periods (Widdows et al., 1979). However, in emersion periods over than 50 %, growth has been reported to be reduced or even discontinue if time exposure exceeds 70 % (de Montaudouin, 1996).

Figure 1. *Cerastoderma edule,* the edible cockle.

The population dynamics of cockles is controlled by abiotic factors such as temperature (Beukema and Dekker, 2005), immersion time (Kater et al., 2006), salinity (Peteiro et al., 2018) and water velocity (Kater et al., 2006). Additionally, population dynamics of cockles is also controlled by biotic factors such as parasitism (Desclaux et al., 2004; Longshaw and Malham, 2013). In fact, from protozoan to eukaryotic species, it is recognized that cockles are hosts to a wide variety of parasites and diseases (Longshaw and Malham, 2013). The presence of some of these parasites is usually innocuous, however in the case of high prevalence and abundance outbreaks, parasites can have highly devastating impacts to wild populations, fisheries or aquaculture industries (Elston, 1990; Adlard et al., 2015). There are several studies demonstrating the effect of parasites on cockles individuals or population dynamics (for a revision view Longshaw and Malham, 2013). Nevertheless, the factors that trigger pathogen abundance are still unclear. Some studies predict that in a climate change scenario, marine diseases are likely to become more frequent and severe (Burge et al., 2017; Harvell et al., 1999). Some studies have been conducted on various abiotic variables and their effect on parasite abundance and prevalence (Magalhães et al., 2018b; Thieltges and Rick, 2006; Wegeberg and Jensen, 1999) however, the results are not always consensual. For instance, an increasing cercariae emergence under increasing salinities has been reported by Lei and Poulin (2011) and Studer and Poulin (2012). Nonetheless, Koprivnikar and Poulin (2009) and Koprivnikar et al. (2014) showed the opposite pattern, i.e. decreasing cercariae emergence with increasing salinities. Besides, a higher a higher trematode cercariae infectivity has been reported under higher temperatures (Thieltges and Rick, 2006; Studer et al., 2010) and low pH (Harland et al., 2016), however a reduced survival of the cercariae has been reported for the same conditions (MacLeod and Poulin, 2015; Mouritsen; 2002).

Being the favourite hosts of several parasite species, coupled with the described ecological and economic importance and other important features such as wide and abundant distribution among different habitats and easy collection, cockles constitute a suitable model to study host-parasite interactions and parasite distributional patterns.

1.3. Trematodes as cockles parasites

In coastal water systems, a considerable proportion of the animal biological diversity is composed by parasitic species, corresponding, approximately, to 2/5 of all eukaryotic species (Dobson et al., 2008). Parasites are ubiquitous in every free-living community however, they are often neglected as a major component of biodiversity (Dobson et al., 2008; Hudson et al., 2006). They play a key structuring role in communities, providing information about the ecosystem functioning (Hudson et al., 2006; Marcogliese, 2004; Dairain et al., 2019). Parasites impose adverse impacts on their hosts phenotype and health (Marcogliese, 2004) which may result into high mortality at population scale (Curtis, 1995; Fredensborg et al., 2005; O'Connell-Milne et al., 2016). At a higher scale, parasites can also induce environmental-modifying functions on their hosts by phenotype alteration (Thomas et al., 1999). Thus, parasites are considered ecosystem engineers by interfering on the host local population and consequently affecting the value and impact of these species in the ecosystem (Thomas et al., 1999). This role is exacerbated when the host species itself is also an ecosystem engineer (Dairain et al., 2019).

As stated before, cockles are hosts of several pathogenic agents (Longshaw and Malham, 2013). Among those, the digenetic trematodes represent an important component as they are the most abundant and prevalent clade of macroparasites in coastal waters (Lauckner, 1983; Roberts et al., 2009). Trematodes have molluscs, especially bivalves, as favourite hosts (Mouritsen and Poulin, 2002) since molluscs are easily invaded by parasite free-living stages through their suspension-feeding activity.

Trematodes display a complex and heteroxenous life cycle, infecting more than one host species (Figure 2) and alternating between parasitic and free-living stages (Bartoli and

Gibson, 2007). During the different life stages, trematodes have a host species-specific and tissue-finding selection behaviour (Sukhdeo and Sukhdeo, 2004), varying between asexual multiplication and sexual reproduction phases (Whitfield, 1993). This way, the typical trematode life cycle consists on a free-living larva (miracidium) that hatches from the egg and infect the first intermediate host, most of the time a mollusc. At this stage, the parasite will transform into a sac-like mother sporocyst that will develop, according to trematode species, into a mature sporocyst or a redia, the latter being a developed form with a mouth. After, within the sporocysts or rediae, cercariae free-living stages develop by asexual multiplication. Cercariae will emerge from the first host and reach the second intermediate host, an invertebrate or vertebrate depending on the trematode species. The cercariae penetrate the host and metamorphose into a metacercariae. Their cycle is complete when the parasitized second intermediate host is predated by the final host, a vertebrate. Then, each metacercariae transforms into an adult form that will reproduce sexually and produce eggs that will be released in the environment along with the faeces of the final host (Bartoli and Gibson, 2007; Esch, 2002; Roberts et al., 2009).

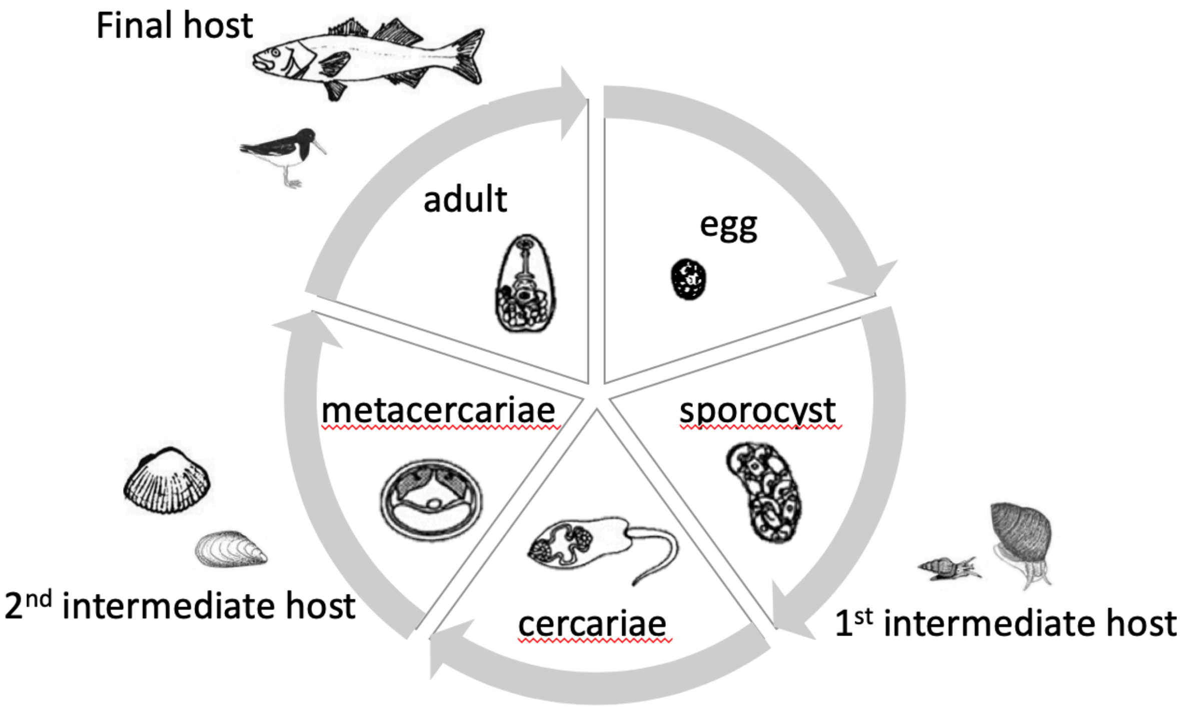

Figure 2. Schematic representation of the typical trematode life cycle. Adapted from Magalhães et al., 2015; Mouritsen et al., 1997; Thieltges et al., 2008a.

This complex life cycle shows how important free-living diversity (i.e. different host species presence) is for trematodes but so are the environmental parameters (biotic and abiotic factors (Pietrock and Marcogliese, 2003; Thieltges et al., 2008b)), especially for infective free-living larvae transmission and infection success. For example, concerning biotic factors, host population infection is dependent on the host size (Thieltges and Reise, 2007; de Montaudouin et al., 2012), the presence of the other host species (Combes, 2001) and population density (Magalhães et al., 2016; Mouritsen et al., 2003). On the other hand, abiotic factors are also important triggers of trematode infection, mainly temperature (Achiorno and Martolrelli, 2016; de Montaudouin et al., 2016), but also light, salinity, pH and hydrodynamics (de Montaudouin et al., 1998, 2016; Koprivnikar et al., 2010, 2014; Mouritsen, 2002; Studer and Poulin, 2013).

In the first intermediate host, the impacts of trematodes are more deleterious (Curtis et al., 2000). These impacts can imply perturbations on growth (Curtis et al., 2000; Sousa, 1983) and reproduction (Schulte-Oehlmann et al., 1997) by castrating and weakening the host through deprivation of the gonadal tissue and by disrupting the normal neurosecretory activity (Lauckner, 1983). Trematodes can also reduce host overall resistance to environmental stress, decreasing muscles strength and inhibiting the process of defence which results in a vulnerability of the host and the possible consequent death (Lauckner, 1983). In the second intermediate host (metacercariae stage), the pathological effects are less destructive, depending on the number of metacercariae accumulated in the host (Desclaux et al., 2004; Gam et al., 2009) as well as, on abiotic factors residing in the environment such as pollutants or other diseases (de Montaudouin et al., 2010; Paul-Pont et al., 2010).

Trematode free-living stages have developed as well behavioural responses to certain stimulus to help to identify its hosts and consequently enhance the chance of transmission (Poulin, 1998). Depending on the responses to the different stimulus, trematode free-living stages can be guided to search for the microhabitat where the host inhabits (Combes et al., 2002) or for the host species (Haas et al., 1992; Shiff and Graczyk, 1994) that can confer different advantages to the trematodes. In the case of trematodes that look for the microhabitat, these species can present phototaxis, i.e. the movement of an organism towards or away from a light stimulus and/ or geotaxis, i.e. the vertical movement of a trematode in the water column in response to a gravity force, and then rely on the chance of contact with the host to initiate infection (Combes et al., 2002). When a trematode species displays phototaxis, this species uses photoreceptors that facilitate light perception (Haas, 1992). The response of the trematode towards or away from the light is dependent on the next host habitat and responses to light (Combes et al., 1994, 2002; Haas, 1992). Geotaxis can be often misguided with phototaxis since, light intensity in the higher parts of the water column is greater than in the bottom. This way, geotaxis should be tested without the presence of a light gradient. For example, the swimming behaviour of the cercariae of trematode species *Maritrema subdolum* is attributed to positive geotaxis, due to the absence of reaction to light (Mouritsen, 2001). On the other hand, trematodes that search for the host, usually display chemotaxis behaviour, i.e. the movement of an organisms along a chemical compound concentration gradient either towards or away from the chemical stimulus. Despite, when present, the chemotaxis is the dominant behaviour, it is possible for trematodes to display responses to various stimuli (Haas et al., 1992). For example, the cercariae of *Pseudechinoparyphium echinatum*, a trematode species that present positive phototaxis (Loy et al., 2001) can reverse its course in the presence of the chemical compounds segregated by its host in the water (Haas, 1992).

1.4. Thesis objectives

Being cockles *C. edule* such an important species for local coastal communities socio-economic development, it is imperative to identify the abiotic and biotic factors or their interaction that most contribute to its population dynamics modulation and possible impacts at the ecosystem level. As described before, trematodes are the most prevalent pathogenic agent infecting this bivalve species with possible deleterious effects but which dynamics is still poorly understood. In this sense, this thesis general aim was to recognize environmental patterns that could regulate trematode parasites population dynamics, identifying and quantifying the factors (or combination of factors) involved in trematodes transmission, infection success and cockles infection rate following an integrative approach that combined large scale and small scale monitoring as well as experimental analyses.

This thesis was subdivided into three chapters with following specific objectives:

Chapter 2 – Large scale cockle trematode communities: to provide a large spatial survey of trematode communities from north Portugal to south Morocco and to identify possible environmental factors that enhance trematode abundance and prevalence.

Chapter 3 – Small scale cockle trematode communities: to provide a seasonal survey of trematode communities in the Ria de Aveiro and the influence of vertical (tidal) and horizontal position of the host in trematode communities.

Chapter 4 – Trematode behavioural response to environment: to assess the influence of light on cercariae transmission success.

CHAPTER 2. LARGE SCALE COCKLE TREMATODE COMMUNITIES

This chapter is submitted as:

Simão Correia, Luísa Magalhães, Rosa Freitas, Hocein Bazairi, Meriam Gam, Xavier de Montaudouin. Large scale patterns of trematode parasites communities infecting *Cerastoderma edule* from Portugal to Morocco. Estuarine, Coastal and Shelf Science (submission number ECSS_2019_589).

2. Large scale patterns of trematode parasites communities infecting *Cerastoderma edule* from Portugal to Morocco

2.1. Introduction

The Latitudinal Diversity Gradient describing that species richness increases from the poles to the tropics, is a widely recognized and applicable ecological pattern to many terrestrial and marine species (Hillebrand, 2004). However, the causes that determine this gradient are not fully understood, with hypotheses that go from higher migration to the tropics (Jablonski et al., 2006) and/ or lower climate variation that allows the accumulation of species (Guo and Ricklefs, 2000), to the highest opportunity of species to specialize due to the high productivity and environmental stability, characteristic of tropical habitats (Harrison and Cornell, 2008; Mittelbach et al., 2007). Nonetheless, when referring to parasitic species, the knowledge is more limited (Stephens et al., 2016), and the observation of this pattern is not evident for parasitic species with complex life cycles (Poulin and Leung, 2011).

Trematode parasites are highly dependent on the presence of their hosts (Combes, 2001) and its distributional range can only be as wide as their hosts distributional range. The edible cockle, *Cerastoderma edule*, populations are broadly distributed along the northeast Atlantic coast, inhabiting coastal water systems from the north of Europe (Norway) to the northeast of Africa (Mauritania) (Dabouineau and Ponsero, 2011; Honkoop et al., 2008). Cockles act as first and/ or second intermediate host of several trematode species (de Montaudouin et al., 2009). Due to large distributional range of this bivalve, *C. edule* is a great model to study latitudinal patterns of trematode parasites.

In this sense, the present study focused on a large scale spatial survey of trematode communities infecting *C. edule* with two different objectives. First, to provide for the first time information of trematode communities infecting cockles near to the southern boundary of this species, i.e. from the North of Portugal to the South of Morocco (ca. 2500 km). Second, to identify abiotic patterns that could influence the diversity, abundance and prevalence of trematode parasites. The tested hypotheses were 1) trematode communities follow a latitudinal diversity gradient and 2) trematode communities abundance and/ or diversity is dependent on the type of studied systems (lagoon vs. estuary vs. bay).

2.2. Material and Methods

2.2.1. Study Area

The present study was conducted in a total of seventeen semi-diurnal tidal marine systems distributed along Portugal and Morocco coastline (Figure3). Six aquatic systems were sampled in Portugal from July to October 2016: the Ria de Aveiro coastal lagoon, Óbidos coastal lagoon, Albufeira coastal lagoon, Sado estuary, Mira estuary and Ria Formosa coastal lagoon (Figure 3). In Morocco, eleven aquatic systems were sampled from November 2007 to January 2008: the Tahaddart estuary, Loukkos estuary, Merja Zerga coastal lagoon, Sebou estuary, Oum er Rbia estuary, Sidi Moussa coastal lagoon, Oualidia coastal lagoon, Souss estuary, Chbika estuary, Khnifiss coastal lagoon and Dakhla bay (Figure 3).

From each area, information on annual variation of water temperature and salinity was obtained through literature review and all the sampled aquatic systems were characterized in terms of water and sediment environmental variables. The data was gathered in Table 1 and used to analyse environmental patterns of trematode assemblage.

Throughout this chapter, it was used the term 'coastal lagoon' (referred as 1 in Table 1) when referring to coastal water bodies, connected to the ocean through one or more inlets and separated by a barrier. The lagoons vary from oligohaline to hypersaline conditions (Gooch et al., 2015). Whereas, 'estuaries' (referred as 2 in Table 1) were considered as semi-enclosed coastal water bodies, that have a connection with the open sea and within which seawater is measurably diluted with fresh water derived from land drainage (Pritchard, 1967). When in the presence of large bodies of water that enters through the coast and are intimately connected to an ocean by a wide entrance, the term 'bay' (referred as 3) was used (UN, 1982).

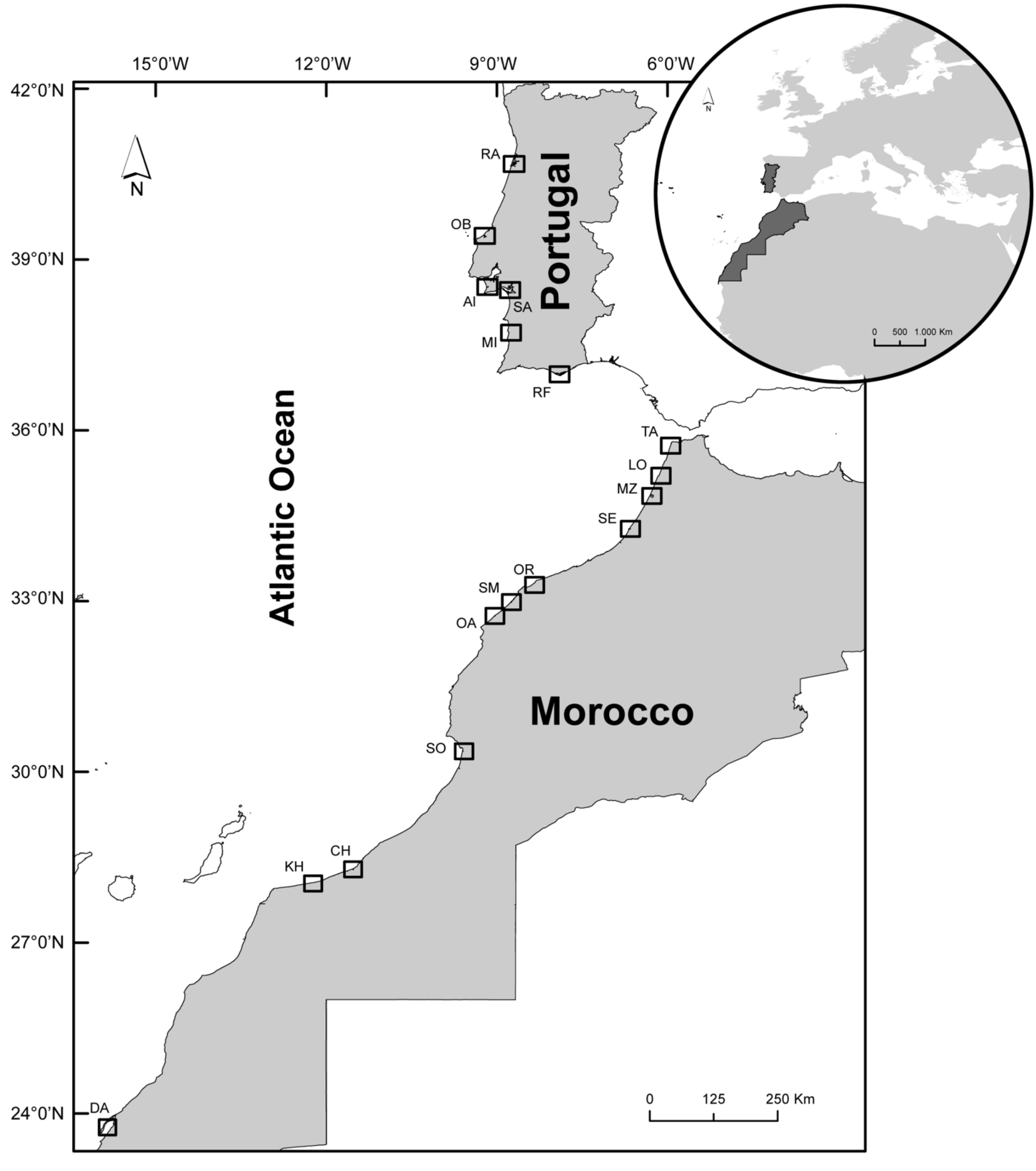

Figure 3. Study area. Geographical location of the 17 aquatic systems distributed along the Portuguese and Moroccan coastlines. Sampling sites: RA - Ria de Aveiro; OB - Óbidos lagoon; AL - Albufeira lagoon; SA - Sado estuary; MI - Mira estuary; RF - Ria Formosa; TA - Tahaddart estuary; LO - Loukkos estuary; MZ - Merja Zerga lagoon; SE - Sebou estuary; OR - Oum er Rbia estuary; SM - Sidi Moussa lagoon; OA - Oualidia lagoon; SO - Souss estuary; CH - Chbika estuary; KH - Khnifiss lagoon; DA - Dakhla bay.

Table 1. Characterization of each sampled area in terms of type of aquatic system (1 = lagoon; 2 = estuary; 3 = bay), latitude (LAT), longitude (LON), surface area, maximum annual water temperature (M Temp), minimum annual water temperature (m Temp), maximum annual water salinity (M Sal), minimum annual water salinity (m Sal) and sediment median grain-size (MGS). ND – no data.

System	Type	LAT (N)	LON (W)	Surface Area (km^2)	M Temp	m Temp	M Sal	m Sal	MGS (mm)	References
Ria Aveiro	1	40°38'	8°44'	83.0	22.0	15.0	28.0	12.0	0.277	Dias et al., 2000 Lillebø et al., 2015
Óbidos	1	39°24'	9°12'	7.0	22.0	10.0	37.0	26.0	0.392	Malhadas et al., 2009 Oliveira et al., 2006
Albufeira	1	38°30'	9°10'	1.3	22.5	11.0	36.0	31.0	0.404	Fortunato et al., 2014
Sado	2	38°28'	8°50'	240.0	21.9	13.8	36.0	10.9	0.304	Bao et al., 1999 Martins et al., 2001
Mira	2	37°43'	8°46'	16.0	22.5	12.0	35.0	27.0	0.353	Silva et al., 2006
Ria Formosa	1	36°58'	7°52'	170.0	26.0	12.0	36.5	13.0	0.326	Gamito and Erzini, 2005
Tahaddart	2	35°46'	5°42'	10.0	26.0	13.0	41.0	21.0	0.194	Achab, 2011
Loukkos	2	35°07'	06°00'	72.0	27.0	15.0	34.0	22.0	0.203	Geawhari et al., 2014
Merja Zerga	1	34°51'	06°16'	27.0	28.0	11.0	35.0	27.0	0.272	Gam et al., 2010
Sebou	2	34°16'	06°39'	17.5	30.0	16.0	35.0	12.0	0.219	Haddout et al., 2016

Table 1. (continued)

System	Type	LAT (N)	LON (W)	Surface Area (km²)	M Temp	m Temp	M Sal	m Sal	MGS (mm)	References
Oum er Rbia	2	33°28'	08°34'	1.5	25.0	15.0	35.0	30.0	0.146	Khalki and Moncef, 2007
Sidi Moussa	1	32°54'	08°49'	4.2	27.0	15.0	33.0	22.0	0.273	Maanan et al., 2004
Oualidia	1	32°45'	08°30'	3.0	21.0	16.0	36.0	28.0	0.181	Hilmi et al., 2005
Souss	2	30°21'	09°35'	16.0	25.0	19.0	39.0	35.0	0.202	Anaijar et al., 2008
Chbika	2	28°14'	11°42'	0.2	ND	ND	ND	ND	0.184	
Khnifiss	1	28°03'	12°15'	65.0	22.0	16.0	38.0	34.0	0.150	Semlali et al., 2012
Dakhla	3	23°45'	15°50'	400.0	26.0	14.0	39.5	37.0	0.120	Zidane et al., 2017

2.2.2. Field sampling and parasite identification

At each sampling area, sediment samples were collected to perform grain size analysis following the method described by Quintino et al. (1989). Silt and clay fraction (fine particles with diameter below 63 μm) were assessed by wet sieving and the remaining fractions (sand and gravel) were determined by sieving through a column of sieves with decreasing mesh sizes. The median grain size was defined in mm by calculating the mean value (P_{50}) of the cumulative frequency of each fraction (Wentworth, 1922).

Cockles were collected in the intertidal zone (with exception of Óbidos lagoon) using six quadrats (0.25 m^2 each) and by sieving the sediment though a 1 mm mesh. The number of cockles per square meter (density) was then estimated. In Óbidos lagoon, a subtidal area where the quadrat method was impossible to perform, samples were collected with a hand dredge and cockle density (d) was calculated following the equation:

$$d(ind.m^{-2}) = \frac{n}{a \times t \times l}$$

where “n” corresponds to the total number of cockles collected; “a” to the hand dredge area (m^2); “t” the trawls number (times the dredge was dragged in the sediment); and “l” the number of launches.

Shell length (SL) of each cockle was measured to the lowest mm with a calliper. From each aquatic system, according to availability, a variable number (between 11 and 65) of adult cockles (23 – 30 mm) were dissected. In Albufeira lagoon and Mira estuary, due to low abundance of cockles from this length class, twenty cockles representing the SL of each area (16 – 31 mm and 8 – 18 mm, respectively) were dissected. Dissected cockles were squeezed between two glass slides and observed under a stereomicroscope. All trematodes were identified to the species level following de Montaudouin et al. (2009) identification key. Parasite abundance (per cockle) and prevalence (percentage of infected cockles) were calculated according to Bush et al. (1997).

2.2.3. Data analysis

To test the influence of latitude (independent variable) on the variation of trematode species richness (TSR), trematode total prevalence (TTP) and trematode mean abundance (TMA) (dependent variables), regression analyses were performed using the SPSS v.25 software.

The Chbika estuary, due to the lack of abiotic information, was excluded from all the following multivariate analyses and used only on trematodes descriptive information.

The data matrix with the trematode abundance per site [abundance per trematode species x sampling site] was square root transformed and the Bray–Curtis (Legendre and Legendre, 1998) similarity calculated between sites. To identify the biological affinity groups, the similarity matrix was then analysed using a hierarchical clustering analysis tool. The affinity groups were characterized according to the mean to environmental and biological features by calculating the mean value of annual maximum and minimum water temperature, annual maximum and minimum water salinity, median grain-size, cockle density at sampling time and by calculating the rarefaction index, i.e. and estimation of the trematode species diversity through standardization of the number of samples (Gotelli and Colwell, 2001). Differences in terms of trematode abundance among the affinity groups were then tested using permutational multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER-E v.6 (Anderson et al., 2008) following unrestricted permutation of the raw data (9999 permutations) and the calculation of type III sums of squares.

Affinity groups differences were visualized through Principal Coordinates Ordination analysis (PCO) after a distance among centroids resemblance (Clarke and Warwick, 2001). The biotic and abiotic variables that were highly correlated (Spearman $\rho > |0.7|$) to samples ordination were represented as superimposed vectors in the PCO graph.

The BIOENV procedure was used to assess the biological-environmental relationship, using the Spearman correlation (Clarke and Gorley, 2006) and considering annual maximum and minimum water temperature, annual maximum and minimum water salinity, median grain-size, latitude and the type of aquatic system as environmental variables.

All multivariate analyses were performed using the PRIMER v.6 software.

2.3. Results

2.3.1. Trematode species richness, prevalence and abundance

During this study, 398 cockles were dissected, from which 280 were infected by a total of twelve trematode species. *Bucephalus minimus* and *Monorchis parvus* infecting cockles as first intermediate host, *Gymnophalus choledochus* using cockles as first and second intermediate host and nine species of trematodes at metacercariae stage, i.e. infecting cockles as second intermediate host, *Curtuteria arguinae, Diphterostomum brusinae, Himasthla continua, H. elongata, H. interrupta, H. quissetensis, Parvatrema minutum, Psilostomum brevicolle* and *Renicola roscovitus* (Table 2).

Table 2. Digenean trematode species found in Portugal and Morocco with indication of the hosts involved in their life cycle. Adapted from de Montaudouin et al. (2009).

Trematode species	**1st intermediate host**	**2nd intermediate host**	**Final host**
Bucephalus minimus	*Cerastoderma edule*	*Pomatoschistus* spp.	*Dicentrarchus labrax*
Monorchis parvus	*Cerastoderma edule*	*Cerastoderma edule*	*Diplodus* spp.
Gymnophalus choledochus	*Cerastoderma edule*	*Cerastoderma edule*	Water birds
Curtuteria arguinae	Unidentified species	*Cerastoderma edule*	Water birds (*)
Diphterostomum brusinae	*Tritia reticulata*	*Cerastoderma edule*	*Blennius, Sargus. Symphodus, Oblata*
Himasthla continua	*Peringia* spp.	*Cerastoderma edule*	Water birds
Himasthla elongata	*Littorina littorea*	*Cerastoderma edule*	Water birds
Himasthla interrupta	*Peringia* spp.	*Cerastoderma edule*	Water birds
Himasthla quissetensis	*Tritia reticulata*	*Cerastoderma edule*	Water birds
Parvatrema minutum	*Scrobicularia plana*	*Cerastoderma edule*	*Haemotopus ostralegus*
Psilostomum brevicolle	*Peringia* spp.	*Cerastoderma edule*	Water birds
Renicola roscovitus	*Littorina littorea*	*Cerastoderma edule*	Water birds

Overall, trematode species richness (TSR) increased linearly from north to south. Nevertheless, explaining only 20% of the total variation ($R^2 = 0.22$). When excluding Chbika and Sebou estuaries that displayed the lowest species richness in this study (1 trematode species each), the linear model fitted better with latitude, explaining 60% of total variation (Table 3).

Table 3. Results of the regression analyses performed to test the influence of latitude (independent variable) on trematode species richness (TSR), total trematode prevalence (TTP) and trematode mean abundance (TMA). SE: standard error. Sampling sites: Se – Sebou estuary; Ch – Chbika estuary.

	Predictors	**Estimate**	**SE**	**p - value**	**R^2**
Trematode species richness (TSR)					
	Constant	11.265	3.272	0.004	
	Latitude	-0.225	0.096	0.034	
	Model		1.164	0.034	0.217
Trematode species richness (TSR)					
(w/o Se & Ch)	Constant	14.501	2.344	<0.001	
	Latitude	-0.307	0.068	0.001	
	Model		1.164	0.001	0.608
Total Trematode Prevalence (TTP)					
	Constant	-4.158	1.454	0.013	
	Latitude	0.371	0.091	0.001	
	Latitude^2	-0.007	0.001	<0.001	
	Model		0.136	<0.001	0.821

P. minutum was the most prevalent trematode species, found in twelve out of the seventeen studied aquatic systems and spread throughout all the sampled latitudinal gradient (Figure 4). *H. elongata* was exclusively found in three aquatic systems located north of 38 °N (northern Portuguese aquatic systems) whereas, *H. continua*, displayed exclusivity to five aquatic systems located south of 35 °N and *R. roscovitus* was the rarest species, exclusively found in the Óbidos lagoon (Figure 3; Table 4). *C.* arguinae, *H. quissetensis* and *P. brevicolle* were common in several aquatic systems south of 37 °N, found in the two aquatic systems nearest to the north Atlantic Ocean – Mediterranean transition (strait of Gibraltar, 36 °N) and in several aquatic systems south of this biogeographical barrier (Figure 3).

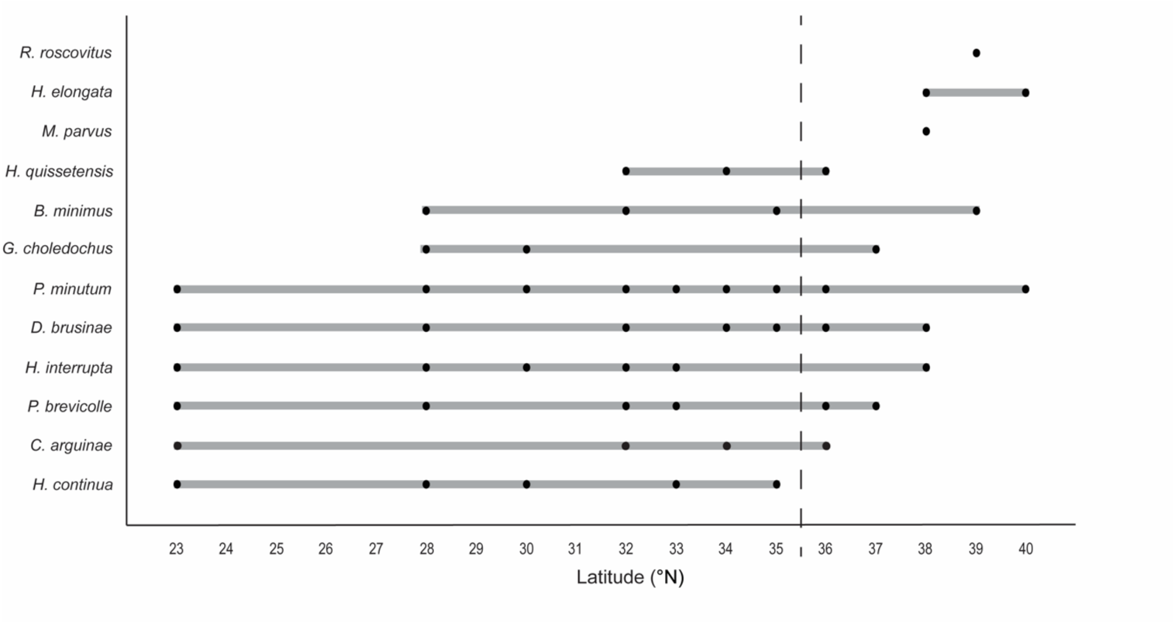

Figure 4. Latitudinal distribution of the twelve trematode species found in the present study. Dashed line: Strait of Gibraltar. Grey bar: Presumable distributional range.

Trematode total prevalence (TTP) decreased with the latitude as demonstrated by the quadratic equation that explained 80% of the TTP variety ($R^2 = 0.82$; Table 3). The Óbidos lagoon (39 °N, Portugal) showed the lowest TTP value (6%), whereas almost every aquatic system south of 36 °N presented 100% of TTP (except Loukkos estuary, 35 °N) (Table 4).

Table 4. Prevalence of trematode parasites infecting cockle populations in the aquatic systems of Portugal and Morocco. Sampling sites: RA - Ria de Aveiro; OB - Óbidos lagoon; AL - Albufeira lagoon; SA - Sado estuary; MI - Mira estuary; RF - Ria Formosa; TA - Tahaddart estuary; LO - Loukkos estuary; MZ - Merja Zerga lagoon; SE - Sebou estuary; OR - Oum er Rbia estuary; SM - Sidi Moussa lagoon; OA - Oualidia lagoon; SO - Souss estuary; CH - Chbika estuary; KH - Khnifiss lagoon and DA - Dakhla bay.

		B. minimus	***M. parvus***	***G. choledochus***	***D. brusinae***	***H. continua***	***H. elongata***	***H. interrupta***	***H. quissetensis***	***C. arguinae***	***P. brevicolle***	***P. minutum***	***R. roscovitus***	**Total Prevalence**
Portugal	RA (n=65)	0	0	0	0	0	11	0	0	0	0	26	0	35
	OB (n=35)	3	0	0	0	0	0	0	0	0	0	0	3	6
	AL (n=20)	0	40	0	20	0	10	0	0	0	0	0	0	55
	SA (n=21)	0	0	0	0	0	48	5	0	0	0	0	0	52
	MI (n=20)	0	0	50	0	0	0	0	0	0	5	0	0	55
	RF (n=25)	0	0	0	4	0	0	0	36	20	4	8	0	48
Morocco	TA (n=13)	8	0	0	15	54	0	0	0	0	0	100	0	100
	LO (n=20)	10	0	0	10	0	0	0	0	0	0	90	0	90
	MZ (n=20)	0	0	0	30	0	0	0	70	100	0	100	0	100
	SE (n=17)	0	0	0	0	0	0	0	0	0	0	100	0	100

Table 4. (continued)

		B. minimus	***M. parvus***	***G. choledochus***	***D. brusinae***	***H. continua***	***H. elongata***	***H. interrupta***	***H. quissetensis***	***C. arguinae***	***P. brevicolle***	***P. minutum***	***R. roscovitus***	**Total Prevalence**
Morocco	OR (n=20)	0	0	0	0	80	0	65	0	0	45	95	0	100
	SM (n=15)	7	0	0	47	0	0	100	100	100	13	67	0	100
	OA (n=20)	5	0	0	30	0	0	75	100	100	20	0	0	100
	SO (n=20)	0	0	5	0	10	0	60	0	0	0	95	0	100
	CH (n=11)	0	0	0	0	0	0	0	0	0	0	100	0	100
	KH (n=20)	10	0	5	85	100	0	60	0	0	45	100	0	100

Trematode mean abundance (TMA) did not follow significantly any model. Nonetheless, it was possible to observe a higher TMA in the central zone of the total extent of the sampled area in comparison to the aquatic systems located at the northern and southern edges of the total area sampled in this study. The Óbidos lagoon (39 °N, Portugal) presented the lowest TMA (0.03 ± 0.17 metacercariae.cockle^{-1}), whereas Oum er Rbia (33 °N, Morocco) presented the highest TMA (820 ± 595 metacercariae.cockle^{-1}). Overall, *P. minutum* was the most abundant species representing approximately 90 % of total metacercariae abundance, followed by *H. quissetensis* that represented 5 %. All the other species infecting cockles as second intermediate host showed low abundance, representing each less than 1.5 % of total abundance. *R. roscovitus* was the least abundant trematode, with only 1 metacercariae identified in this study (Figure 5).

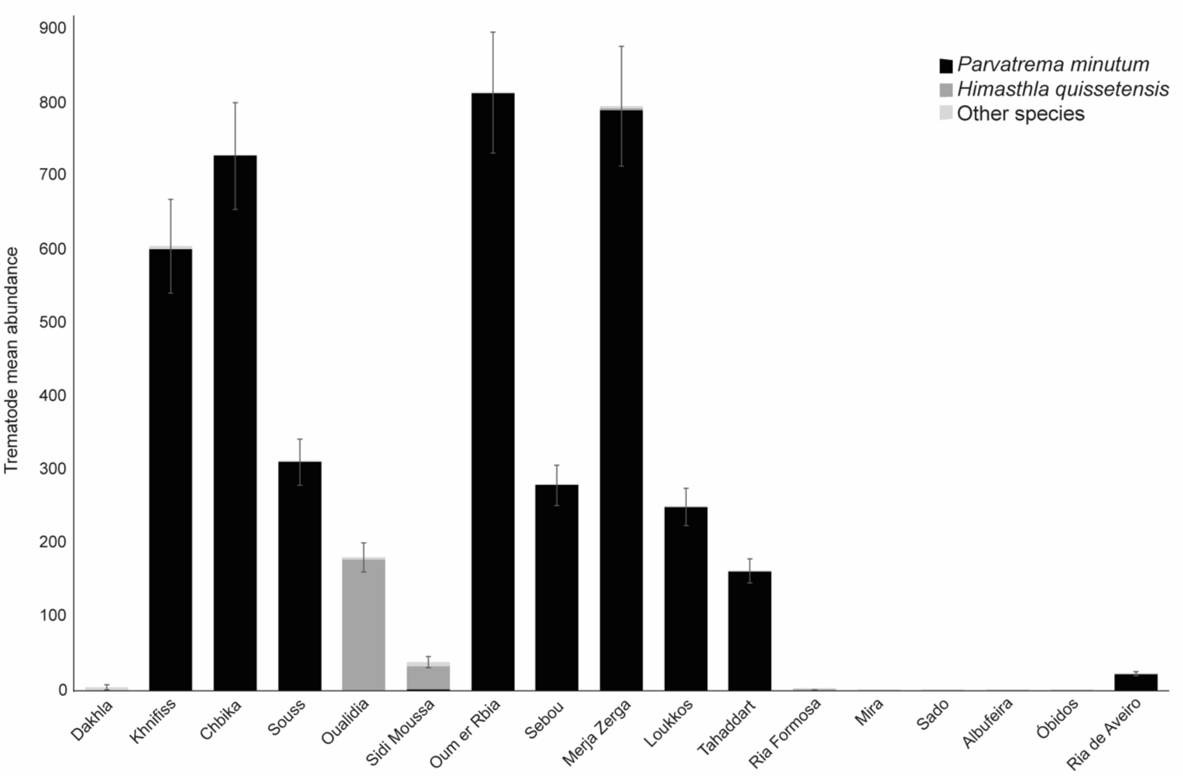

Figure 5. Trematode mean abundance per aquatic system for the two most representative species, *Parvatrema minutum* (black bar) and *Himasthla quissetensis* (dark grey bar), and the remaining cercariae (light grey bar).

2.3.2. Multivariate analysis

Cluster analysis, at a similarity distance of 70%, allowed to assemble the different areas in three affinity groups (Figure 6A): group A, subdivided into A1 composed by Tahaddart estuary, Loukkos estuary, Sebou estuary and Souss estuary and A2 composed by Merja Zerga lagoon, Oum er Rbia estuary and Khnifiss lagoon; group B, divided in B1 composed by Sidi Moussa lagoon and Oualidia lagoon and group B2, splitted into B2.1 composed by Dakhla bay and B2.2 composed by all Portuguese systems (Ria de Aveiro lagoon, Óbidos lagoon, Albufeira lagoon, Sado estuary, Mira estuary and Ria Formosa lagoon). Cockle density showed to be significantly higher in group B1 and lower in group B2.1. Sediment median grain-size was significantly higher in group B2.2 and lower in group B2.1 (Table 5). Concerning water physico-chemical characterization, all descriptors showed no significant differences among the affinity groups. Nevertheless, group A1 displayed the highest values of maximum and minimum water temperature while, group B2.2 registered the lowest values for the same variables. Maximum and minimum water salinity presented the highest values for group B2.1 and the lowest for groups B1 and B2.2, respectively (Table 5).

The obtained affinity groups were represented on the PCO ordination graph (Figure 6B). The axis 1 of the PCO explained 59% of the total variation, separating the group A, composed by the majority of the aquatic systems south of 36 °N, on the positive side of the axis, from group B2, that comprises all aquatic systems north of 36 °N and the southernmost sampled aquatic system (Dakhla bay, 23 °N), on the negative side of the axis. According to the variables that presented higher correlation values, this axis separated estuaries with higher minimum water temperature, higher cockle density, lower sediment median grain-size and lower latitude on the positive side from lagoons or bays with lower minimum water temperature, lower cockle density, higher sediment median grain-size and higher latitude on the negative side. In its turn, axis 2 described 27% of total variation dividing group B2.2 (aquatic systems north of the strait of Gibraltar, 36 °N) and group A (composed by the majority of the aquatic systems south of the strait of Gibraltar), both in the positive side of the axis, from group B1 in the negative side of the axis. Latitude presented high positive correlation to axis 2 and cockle density demonstrated a negative correlation. Species that presented high correlation (Pearson $r > |0.7|$) to samples spatial distribution were also represented in Figure 6B. *P. minutum* was correlated with group A (axis 1), while *C. arguinae*, *H. interrupta* and *P. brevicolle* were the most correlated to group B1, positioned in the negative side of the axis 2.

The BIOENV analysis showed that the combination of environmental variables that better explained the biological data were maximum water temperature, sediment median grain-size, cockle density and type of aquatic system ($r = 0.616$).

Table 5. Characterization of each affinity group in terms of maximum annual water temperature (M Temp), minimum annual water temperature (m Temp), maximum annual water salinity (M Sal), minimum annual water salinity (m Sal), sediment median grain size (MGS) and Rarefaction index for a standardized number of 10 individuals (ES(10)). Significant differences among affinity groups are represented with different letters ($p < 0.05$). Sampling sites: RA - Ria de Aveiro; OB - Óbidos lagoon; AL - Albufeira lagoon; SA - Sado estuary; MI - Mira estuary; RF - Ria Formosa; TA - Tahaddart estuary; LO - Loukkos estuary; MZ - Merja Zerga lagoon; SE - Sebou estuary; OR - Oum er Rbia estuary; SM - Sidi Moussa lagoon; OA - Oualidia lagoon; SO - Souss estuary; CH - Chbika estuary; KH - Khnifiss lagoon; DA - Dakhla bay.

Affinity groups	**Sampling areas**	**M Temp**	**m Temp**	**M Sal**	**m Sal**	**MGS**	**Cockle density**	**ES (10)**
A1	TA, LO, SE, SO	27.0 ± 2.2	15.8 ± 2.5	37.3 ± 3.3	22.5 ± 9.5	0.2 ± 0.0^{a}	404 ± 457^{a}	1.0
A2	MZ, OR, KH	25.0 ± 3.0	14.0 ± 2.6	36.0 ± 1.7	30.3 ± 3.5	0.2 ± 0.1^{a,b}	1643 ± 2267^{a,b}	1.3
B1	SM, OA	24.0 ± 4.2	15.5 ± 0.7	34.5 ± 2.1	25.0 ± 4.2	0.2 ± 0.1^{a,b}	6092 ± 1114^{b}	2.4
B2.1	DA	26.0	14.0	39.5	37.0	0.1^{b}	15^{c}	2.4
B2.2	RA, OB, AL, SA, MI, RF	22.8 ± 1.6	12.3 ± 1.8	34.8 ± 3.4	20.0 ± 9.0	0.3 ± 0.0^{c}	135 ± 304^{a}	1.0

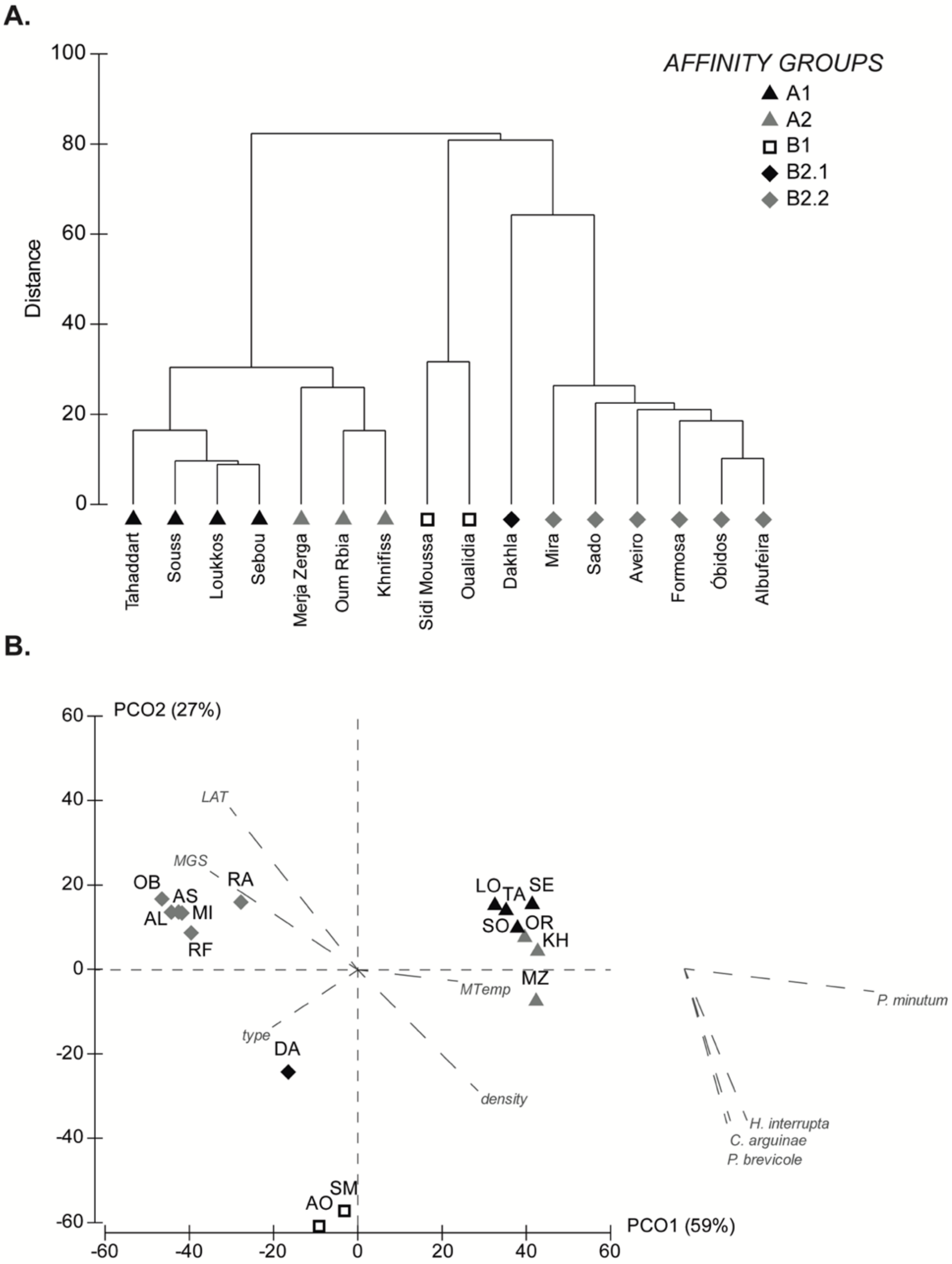

Figure 6. Cluster analysis based on trematode parasites communities in seventeen aquatic systems from Portugal and Morocco (**A**) and Principal coordinates ordination (PCO) showing the variables that better explained samples distribution (**B**). MGS: Sediment median grain-size: LAT: latitude; M Temp: Maximum annual water temperature; density: cockle density; type: type of aquatic system. Sampling sites: RA - Ria de Aveiro; OB - Óbidos lagoon; AL - Albufeira lagoon; SA - Sado estuary; MI - Mira estuary; RF - Ria Formosa; TA - Tahaddart estuary; LO - Loukkos estuary; MZ - Merja Zerga lagoon; SE - Sebou estuary; OR - Oum er Rbia estuary; SM - Sidi Moussa lagoon; OA - Oualidia lagoon; SO - Souss estuary; KH - Khnifiss lagoon; DA - Dakhla bay.

2.4. Discussion

Trematode parasites can have a significant impact on population dynamics of their hosts, implying modification on host growth (Wegeberg and Jensen, 2003) or mortalities rates (Desclaux et al., 2004). It is then of major importance to study host-parasite interactions. This study represents the first large scale survey of trematode communities infecting cockles and consequent assessment of latitudinal patterns that can drive this parasitic communities. Seventeen aquatic systems were sampled during the present work, most of them for the first time and therefore, results presented here are new and never reported before by previous authors.

There are sixteen trematode species infecting *Cerastoderma edule* in its distributional range, using this bivalve as first and/ or second intermediate host (de Montaudouin et al., 2009). In the present study, a total of 12 species were identified along the whole sampled latitudinal gradient. Nine (*Bucephalus minimus*, *Curtuteria arguinae*, *Diphterostomum brusinae*, *Gymnophallus choledochus*, *Himasthla continua*, *H. interrupta*, *H. quissetensis, Psilostomum brevicolle* and *Parvatrema minutum*) out of those 12 trematode species showed a great dispersal ability, since they were present in a wide range of the sampled latitudes, possible related to migration of birds and fish which are trematode final hosts (Feis et al., 2015). For a trematode to complete its life cycle, the three intermediate host species should be present in the same aquatic system (Bustnes and Galaktionov, 1999). Nonetheless, biogeographical barriers, such as oceans in the case of continental species, are among the most important factors contributing to isolation and prevention of species exchange among regions (Cox and Moore, 1980; Ricklefs and Schulter, 1993). In the present study, the strait of Gibraltar (36 °N), a known geographical barrier, showed some influence on trematode communities, dividing the observed trematode species in three different categories. Firstly, seven trematode species (*Monorchis parvus, Bucephalus minimus, Psilostomum brevicolle, Gymnophallus choledochus, Parvatrema minutum, Curtuteria arguinae* and *Himasthla interrupta*) with no influence of the biogeographical barrier and certainly mediated by the predominant presence of the intermediate hosts in the sampled area (*Peringia ulvae, Scrobicularia plana, Gobius* spp., among others (cf. Table 2)). Although during this study *H. continua* has been found only south of 35 °N, its previously recorded distribution (de Montaudouin et al., 2009) places this species in the same group mentioned before. In the case of *C. arguinae*, the first intermediate host is unknown (Desclaux et al., 2006) though, it was not the first record of this trematode species north of 36 °N (de Montaudouin et al., 2009), allowing to infer that its first host has also a predominant distribution. *M. parvus* during this study was only registered in the Portuguese coast, still this isolated occurrence is more likely related to the fact that trematodes in their sporocyst form, i.e. infecting their first intermediate host, usually display low prevalence (Granovitch and Johannesson, 2000; Islam et al., 2009; Tigga et al., 2014). Indeed, this species has been previously recorded along the whole studied latitudinal gradient (de Montaudouin et al., 2009). Secondly, and in contrast, two trematode species displayed a Southern distribution, namely *Diphterostomum brusinae* and *H. quissetensis*, possible mediated by the biogeographical barrier and first intermediate host distribution, *Tritia reticulata*, a southern gastropod. Thirdly, *H. elongata* and *Renicola roscovitus* displayed a Northern distribution likewise their first intermediate host *Littorina littorea*, a marine gastropod that inhabits the intertidal zone from the White Sea to Gibraltar, on the Atlantic east coast (Johannesson, 1988). Thus, this study confirms that at this latitudinal scale, the distribution of the trematode species is driven by the occurrence of the first intermediate host (de Montaudouin and Lanceleur, 2011; Thieltges, 2007; Thieltges et al., 2009), considering that final hosts have generally a wider distribution area (Magalhães et al., 2015).

Trematode species richness (TSR) and trematode total prevalence (TTP) increased towards south (from 40 °N to 23 °N). This latitudinal dependent pattern of TSR follows the same trend described for benthic macrofaunal diversity which show higher values with decreasing latitudes (Macpherson, 2002; Martins et al., 2013; Roy et al., 2004). However, this pattern was not observed in the case of Chbika and Sebou estuaries, located at 28 °N and 34 °N, respectively, that presented the lowest TSR. Chbika is not a permanent estuary ('Oued'), which might induce lower macrofaunal and parasite diversity. Conversely, the Sebou river is one of the biggest African rivers, crossing several riverside populations and then impacted by several anthropogenic activities, including agricultural activities (Perrin et al., 2014). The constant use of pesticides and fertilizers by the agricultural activities, plus the connection with untreated sewage from peripheral cities result in very pollutant loads that can contaminate the river to its estuary (Perrin et al., 2014). This presumable poor health and ecological status of this ecosystem might be inducing a low trematode parasites diversity, opposing the latitudinal trend. Indeed, trematodes, especially free-living stages, are sensitive to water disturbance (Pietrock and Marcogliese, 2003; Koprivnikar et al. 2007) and can be used as indicators of conditions decline (MacKenzie, 1999). Therefore, our results showed that such as free-living species, parasitic fauna seems to follow a latitudinal gradient of decreasing species richness from tropical to extra-tropical areas, one of the oldest recognized ecological patterns (Wright et al., 1993; Willig et al., 2003). This latitudinal pattern is usually not clear for communities of parasites with complex life cycles (Poulin and Leung, 2011; Studer et al., 2013) due to the complexity of host/ parasite interactions, but also because these cycles (and in particular infection success stage) depend on several abiotic factors, including anthropogenic stressors (Rohr et al., 2008; Altman and Byers, 2014). Among abiotic factors, temperature is one of the strongest drivers of trematode activity (propagule dispersal, survival, infection (Thieltges and Rick, 2006) and host infection levels (higher values observed during the warmer seasons (Goater, 1993; Desclaux et al., 2004)). As an example, in Arcachon bay, a synchrony was observed between the parasites emergence from the first intermediate host and the infection in the second intermediate host when water temperature was above 15 °C (de Montaudouin et al., 2016). In the case of the present study, minimum water temperature of the aquatic systems south of 36 °N is around 15°C while it is 3 °C lower north of 36 °N. This temperature difference can explain why Morocco displays higher TTP and TMA than Portugal. In fact, this study showed that abundance and prevalence of trematode communities presented higher values in ecosystems characterized by higher maximum water temperature (affinity group A). In contrast, all the northern sampled areas (Portuguese aquatic systems located north of the strait of Gibraltar) were all gathered in the same affinity group (group B2.2) characterized by the lowest trematode abundance and prevalence. The proximity of the Portuguese coast to an upwelling front and consequent occurrence of cold vertical currents (Queiroz et al., 2012) may operate as a shield against trematode infection.

Cockles from Óbidos lagoon, located 40 °N, displayed extreme values of infection, with the lowest TTP and TMA showing that tidal position of cockle is also a driver of trematode infection. The peculiarity of this site compared to all others is that cockles were sampled in subtidal position. Similar results were found by Gam et al. (2008) in Merja Zerga coastal lagoon (Morocco) where cockles living in subtidal zones presented lower trematode infection than intertidal sympatric specimens. This was interpreted as a differential distance to upstream intertidal first intermediate host. Furthermore, Óbidos lagoon presented the lowest values of water temperature (both minimum and maximum).

In the present study, the discrimination of the trematode assemblages was also explained by the cockle density, the sediment median grain-size and the type of aquatic system, some of these factors being possible cofounding. Cockles density was higher in the studied areas characterized by higher maximum temperature. This can result either from increased recruitment success (Gam et al., 2010; Magalhães et al., 2016) or from better conditions in terms of food quality and quantity. Similar to what was described for cockle density, sediment median grain-size was also correlated to water temperature described by a negative relationship (higher temperature – lower sediment particle size), explained by the decrease in the water density induced by warmer waters that can cause an oscillation in the sediment transport rate and sediment particle fall velocity with consequent influence on sediment granulometry (Tyrell, 2015). Nevertheless, the system presenting the highest cockle density (Sidi Moussa lagoon) was also the system with one of the lowest trematode abundance suggesting that when a certain density threshold is overpassed (in this case, such higher density), cercariae are diluted among second intermediate host and consequent lower mean metacercariae infection is observed, as previously highlighted by Magalhães et al. (2016).

The type of aquatic system (estuaries, lagoons or bays) showed also to be determinant for the trematode community composition. There was a clear separation between three particular areas South of 36 °N, that presented higher trematode biodiversity: Oualidia and Sidi Moussa lagoon, and Dakhla bay corresponding to group B1 and B2.1, respectively. These areas are classified as coastal lagoons (group B1) or bays (group B2.1) and therefore characterized by higher oceanic influence and hydrodynamics (Kjerfve and Magill, 1989) and lower water temperature variations, especially Dakhla bay which is located in the interaction between the Canary stream and the subtropical ridge currents (Orbi et al., 1999). Leung et al. (2009) and Mouritsen and Poulin (2005) referred that these combinations of characteristics are usually related to higher trematode diversity and abundance, which was confirmed by our results (the highest rarefaction indices were found in affinity groups B1 and B2.1).

2.5. Conclusion

Overall, in the present study, water temperature demonstrated to be the most important trigger of trematode abundance and prevalence, displaying a negative correlation with latitude. The cold waters of the Portuguese coast due to the upwelling proximity might influence negatively trematode emission from first host and consequent trematode abundance and prevalence results. Nonetheless, it is important to study the whole distributional gradient of the second intermediate host before assuming that these trematode parasites follow the same latitudinal ecological pattern as many marine and terrestrial species, increasing abundance and diversity towards the equator line. Despite this, the tidal position and the type of aquatic system also seemed to be important predictors of trematode abundance, influencing the occurrence of latitudinal outliers. Thus, it was important to study, on a small scale, the influence of host position and susceptibility to abiotic factors such as air exposure and water flow turbulence for infection by trematode parasites.

CHAPTER 3. SMALL SCALE COCKLE TREMATODE COMMUNITIES

3. Changes in the hydrodynamics associated to trematode assemblages in a bivalve host

3.1. Introduction

The structure and functioning of the ecosystem are related to links between abiotic and biotic factors (Pikitch et al., 2004). Seasonal and tidal fluctuations along with the intersection of oceanic (downstream) and riverine (upstream) influences, characteristics of coastal water systems, results in the development of abiotic gradients in both vertical, i.e. along the tidal gradient, and horizontal, i.e. along the stream gradient, scales (Carr, 1991). These gradients are responsible for communities distributional patterns in the sediment and in the water column (Bianchi et al., 1991; Ehrich et al., 2009). However, most of the studies have been focusing on the biodiversity response to environmental parameters (Pearson and Rosenberg, 1987; Xu et al., 2018) or its fluctuation towards seasonal variations (Admiraal and Peletier, 1980; Welte and Campbell, 2003). For instance, as oxygen availability reduces with depth in aquatic systems, hypoxia has been described for reducing benthic diversity (Diaz and Rosenberg, 1995; 2008). On the other hand, in the summer, due to stratification of the water column or higher temperatures, oxygen availability is reduced with a high impact on benthic diversity (Boynton and Kemp, 2000; Seitz et al., 2009).

Cockles are predominant species in coastal water systems and despite usually associated to intertidal areas, cockles are widely distributed in the water column and can be found up to 20 meters depth (Riesen and Reise, 1982). Parasites rely on hosts to survive as they represent their source of energy and the opportunity for parasites to generate offspring. Therefore, parasites spatial distributional range can only be as wide as their hosts distributional range. Notwithstanding, it is still unclear if the abiotic factors and gradients driving hosts distributional patterns, including hydrodynamics related variables and vertical/horizontal gradients, are the same that drive trematode infection.

Based on the ‘cockle-trematode’ as host-parasite system, the present work was divided into a field monitoring and a laboratory experiment. The field monitoring was conducted in the Ria de Aveiro coastal lagoon aiming to assess the effect of host vertical (intertidal vs. subtidal) and horizontal (upstream vs. downstream) distribution on the individual infection rate. The postulated hypotheses were 1) Does cockles tidal position influence the levels of individual infection by trematode parasites? 2) Is trematode infection influenced by different hydrodynamics? And 3) To what extent the small-scale factors (water temperature, pH, salinity, dissolved oxygen, redox potential, median grain-size and organic matter content) variations overlaps the effects of tidal position and hydrodynamics on cockles infection levels? Knowing the different hydrodynamic regimes characteristics of coastal systems, second postulated hypothesis was further investigated through a laboratory experiment, performed to analyse trematode infection success and individual infection on cockles exposed to two different water flows.

3.2. Material and Methods

3.2.1. Field monitoring approach

3.2.1.1. Study Area

The Ria de Aveiro is a shallow coastal lagoon located on the Northwest Atlantic coast of Portugal (40° 38'N, 8° 44'W). This lagoon is approximately 45 km long and 8.5 km wide covering a total surface of 83 km^2, with 17 km^2 of intertidal flats (Dias et al., 2000). The Ria de Aveiro is subdivided into four channels (S. Jacinto, Espinheiro, Ílhavo and Mira) and is supplied with freshwater by Antuã, Boco, Cáster, Mira and Vouga rivers (Lillebø et al., 2015). The average depth of the lagoon is about 1 m resulting on the coexistence of intertidal and subtidal zones (Dias et al., 2000).

The present study was conducted in two different seasons (summer 2018 (July) and winter 2019 (February)) during low tide in four intertidal zones and the adjacent subtidal zones (total of eight sampling sites), distributed through two geographic areas (downstream and upstream) of the S. Jacinto channel of the Ria de Aveiro (Figure 7). This channel is the longest and widest channel of the Ria de Aveiro, running parallel to the coast from the mouth of the lagoon up to Ovar (northern boundary of the Ria de Aveiro lagoon).

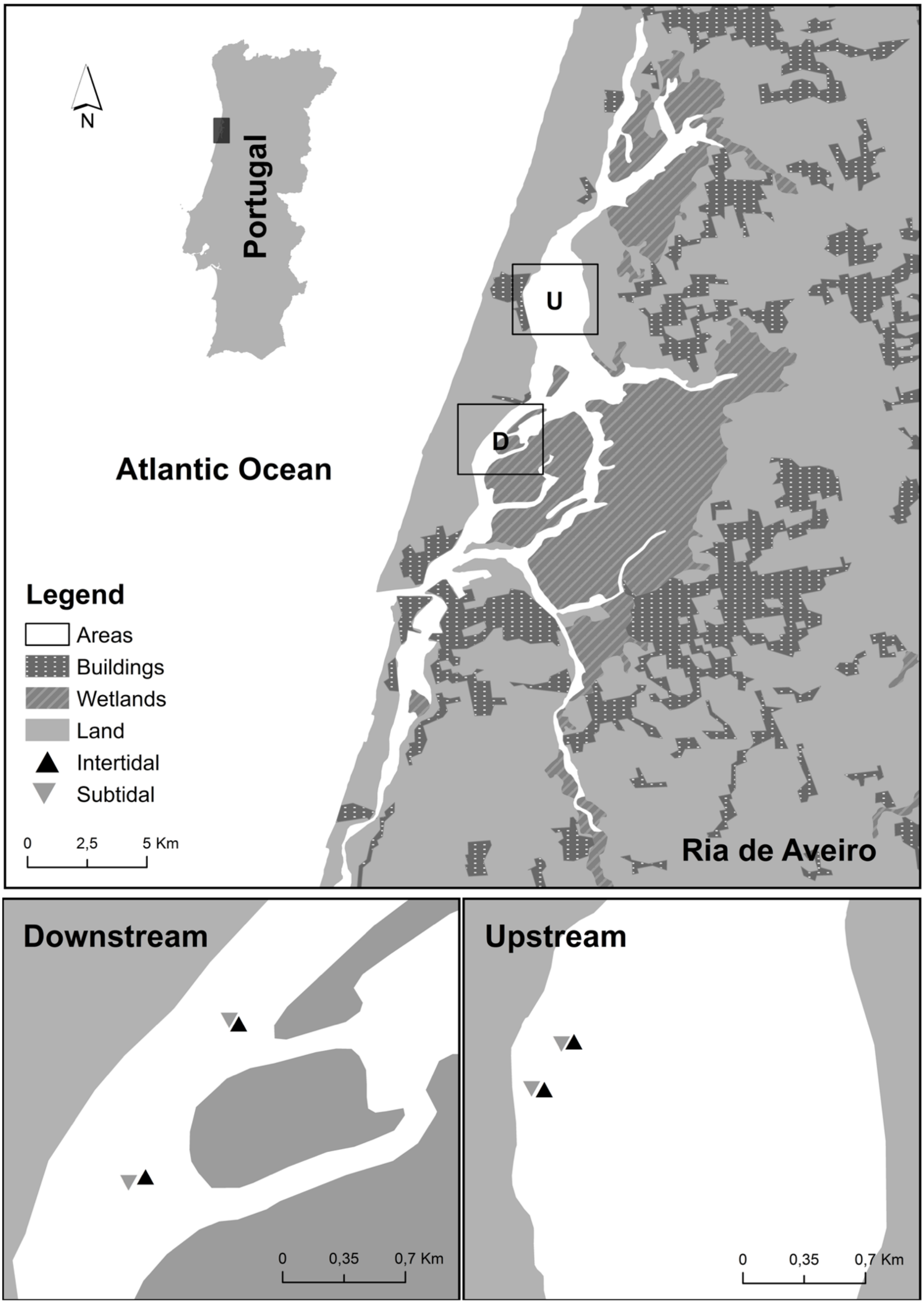

Figure 7. Study area. Geographical distribution of the studied sites. Areas: U – upstream area; D – downstream area.

3.2.1.2. Hydrodynamic characterization and particle dispersion

The numerical model Mohid, previously implemented and calibrated for the Ria de Aveiro (Vaz et al., 2007; Vaz et al., 2009) was used to characterize the lagoon hydrodynamics in the sampled periods (July 2018 and February 2019).

Mohid is a three-dimensional model that use a finite volume approach to perform the spatial discretization and solves the incompressible equations, assuming hydrostatic equilibrium, the Boussinesg and Reynolds approximations (for details see Martins et al., 2001). The Ria de Aveiro was discretized with a rectangular grid with a variable spatial resolution: 40 x 40 m in the central area and 40 x 100 m in the upper lagoon (Vaz et al., 2007; Picado et al., 2013). The bathymetric dataset available for the present study was collected in a general survey carried out in 1987/1988 by the Hydrographic Institute of Portuguese Navy (IH). This bathymetry was updated for the majority of the lagoon with recent data provided by Polis Litoral Ria de Aveiro for the main channels of the lagoon in 2011 and by the Aveiro Harbour Administration, SA in 2012, for the inlet (Figure 7).

One simulation per sampled period was performed in order to characterize the lagoon hydrodynamics at the time of the field surveys (July 2018 and February 2019). Maximum velocity (MV), root mean square (RMS) of velocity and residual velocities (RV) were computed for the entire lagoon. RMS velocity was computed through the equation:

$$RMS = \left(\frac{1}{N}\sum_{i=0}^{N} V^2\right)^{1/2}$$

where $V = \sqrt{u^2 + v^2}$ is the velocity modulus.

Residual velocity corresponds to the mean velocity of 14 days, 18 hours, 51 minutes and 24 seconds (multiple of $\frac{T_{M2} \times T_{S2}}{T_{M2} - T_{S2}}$ where $T_{M_2}$and T_{S_2} are the period of M_2 and S_2 tidal constituents, respectively).

The Lagrangian model, that is a subset of the Mohid modelling system and derives from the hydrodynamic information, was also implemented. It uses the concept of passive particles, characterized by their spatial coordinate (*x, y, z*). The major factors responsible by particle movement (u_p) are generally the mean velocity (u_M) provided by the model and a small scale random fluctuation (u_F), so that $u_p = u_M + u_F$.

Taking advantage of the Lagrangian model, two particles were released, one in the upstream and other in the downstream areas at the instant of the local high tide to guarantee the submersion of first intermediate host and the possibility of cercariae emission. Particles trajectory were tracked for 48 hours, the maximum described cercariae lifetime (Sukhdeo and Sukhdeo, 2004).

3.2.1.3. Samples collection

Sampling was performed at low tide and a total of eight sites were selected (Figure 7). Intertidal cockles (from four sampling sites) were collected using the quadrat method, i.e. sampling six quadrats of 0.25 m^2 and by sieving the sediment through a 1 mm mesh. Cockle density (per square meter) was estimated. At the four subtidal sites, close to each of the four intertidal sites, cockles were collected with a hand dredge and cockle density (d) was calculated following the equation:

$$d(ind.m^{-2}) = \frac{n}{a \times t \times l}$$

where “n” corresponds to the number of cockles collected; “a” to the hand dredge area (m^2); “t” the trawls number (times the dredge was dragged in the sediment); and “l” the number of launches.

From each site, two sediment samples were collected from the sediment surface to perform grain-size analysis (MGS) and to determine total organic matter content (TOM). Sediment from the subtidal samples were collected using a 0.05 m^2 Van Veen dredge.

Water temperature (°C), pH, salinity, dissolved oxygen (%, DO) and redox potential (mV, ORP) were measured in the nearest water column (intertidal sites) or in the sediment-water interface of the sites where cockles were collected (subtidal sites), using a handheld multiparameter probe (YSI Pro Plus Multiparameter Instrument).

3.2.1.4. Parasites identification and laboratory analysis

Shell length (SL) of all collected cockles was measured to the nearest millimetre (mm) with a calliper. Then, twenty cockles of the dominant cohort were selected according to the SL distribution, dissected, squeezed between two glass slides and observed with a stereomicroscope. All trematodes were identified to the species level following de Montaudouin et al. (2009) identification key. Abundance (per cockle) and prevalence (percentage of infected cockles) of each parasite species were calculated according to Bush et al. (1997).

Sediment grain-size analysis (MGS) was conducted by wet sieving the silt and clay fraction (fin particles, diameter < 0.063 mm) and dry sieving the remaining sediment fractions (sand and gravel) following Quintino et al. (1989) method. The median grain size was defined in Φ. This value was obtained through the mean value (P_{50}) of the cumulative frequency of each fraction ($\Phi = -\log_2(P_{50})$) (Wentworth, 1922).

Regarding TOM analysis, sediment was dried at 60 °C for 48 hours and ground to powder with a mortar and a pestle. TOM results were obtained through the difference between the dried samples (approximately 1 g) and the combusted samples (4 hours at 450 °C (Kristensen and Andersen, 1987)).

3.2.2. Experimental approach

3.2.2.1. Hosts and parasites collection

Himasthla elongata was the trematode parasite species used in this study. This parasite uses the common periwinkle, *Littorina littorea*, as first intermediate host and the cockle, *Cerastoderma edule*, as second intermediate host.

Cockles ranging from 17 to 20 mm shell length (small cockles to avoid natural infection (Mouritsen et al., 2003; Wegeberg et al., 1999)), were collected in the Mira channel of the Ria de Aveiro coastal lagoon, Portugal (40° 38’ 31.7”N, 8° 44’ 10.9”W), on March 2019. Cockles were acclimated in laboratory for a week at salinity 30 and temperature 18 °C and daily fed with Algamac Protein Plus ® at a concentration of 730 cells μL^{-1} day^{-1} as described by Pronker et al. (2015).

Infected periwinkles were collected in Texel, The Netherlands and kept in laboratory in an aquarium filled with water at salinity 35 and temperature 14 ± 1 °C and fed with *Ulva* sp. *ad libitum*. Cercariae of *H. elongata*, the model parasite used, were collected by stimulating emission from the first intermediate host by transferring the infected snails to individual containers with water at salinity 35 and approximately 24 °C. Released cercariae were individually collected with a pipette and counted under a stereomicroscope.

3.2.2.2. Experimental design

A water flow laboratory experiment was carried out in two pre-designed recirculating water mini-flumes (Figure 8) at two different current surface velocities (0.1 m s^{-1} and 0.3 m s^{-1}). Each mini-flume was composed by a main tank (1.7 x 0.2 x 0.2 m) and a reservoir (0.2 x 0.2 x 0.5 m). The water column height was approximately 0.10 m. The recirculation of the water was provided by an EHEIM 3400 universal pump located in the reservoir and entered in the flume through a PVC tube of 3.5 mm diameter. To homogenise the water flow inside the flume, the water entrance in the main tank was structured by two watercourses. Inside the flume, the water was kept at 30 salinity and at a temperature of 18 ± 1 °C. This temperature was controlled through a water bath at temperature 14 ± 1 °C where the reservoir was inserted. The water current was measured in the middle of the working section (approximately 0.85 m distance to the water entrance), with a JCD Flowatch meter.

Ten cockles per condition were transferred to the working section of the flume, a delimited area (0.15 x 0.18 m) by a 1 mm mesh size net, located 0.85 m from the water entrance (Figure 8). Subsequently, 500 cercariae of *H. elongata* were released per flume between the bifurcation of the water entrance (Figure 8). After 48 hours, the described time needed by cercariae to encyst in the second intermediate host (de Montaudouin et al., 2016), cockles were dissected, squeezed between two glass slides and the number of metacercariae of *H. elongata* identified and counted under a stereomicroscope.

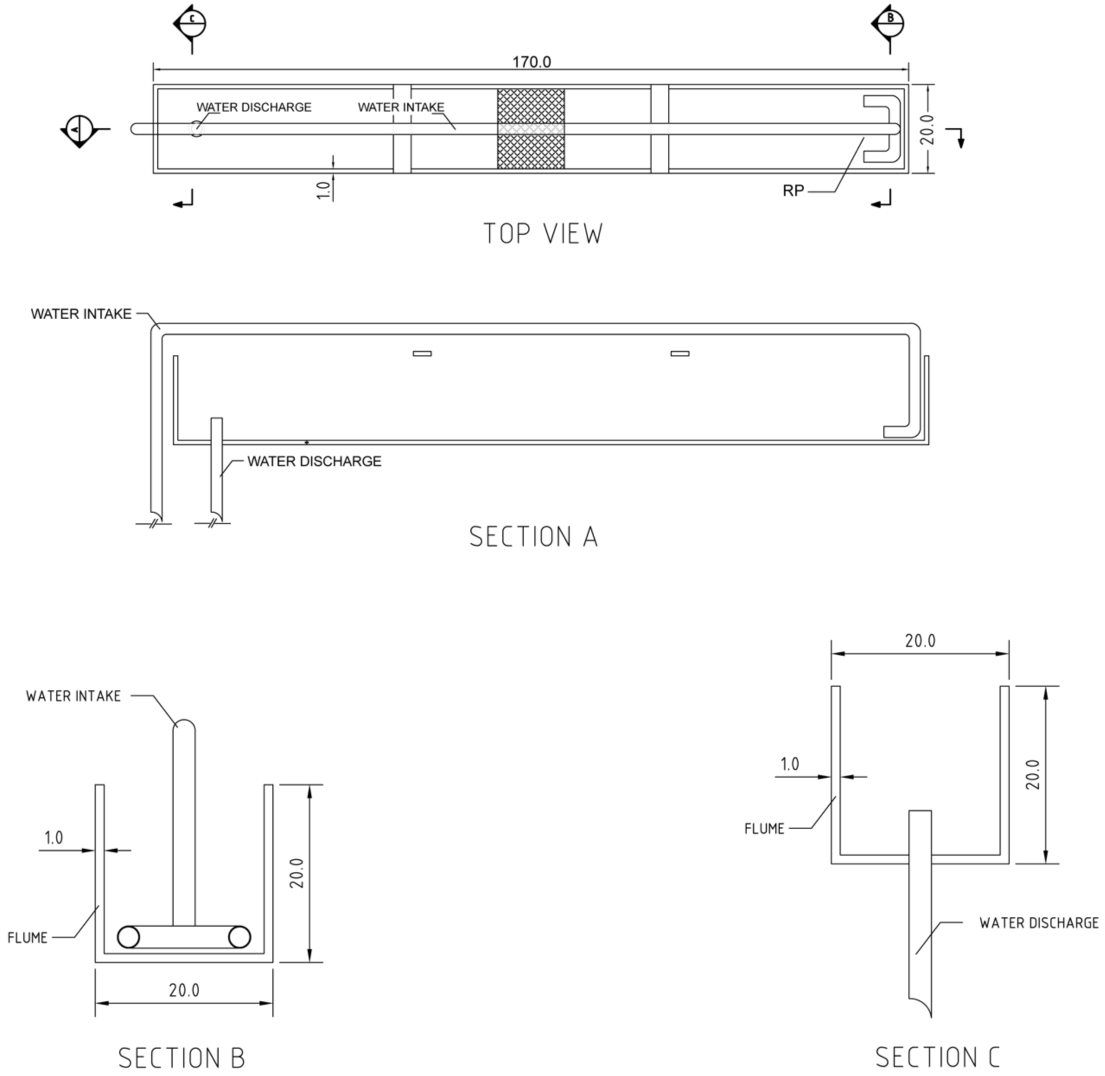

Figure 8. Schematic representation of the flume. A: Side view; B: Front view (water intake side); C: Front view (water discharge side). Grid area – working section and cockles location; RP – cercariae releasing point.

3.2.3. Data analysis

Comparisons among the hydrodynamics characteristics (MV, RV and RMS) of the downstream and upstream areas of the lagoon in each season and between seasons were performed through independent samples Student's t-tests.

In order to compare the trematode mean abundance per cockle, a three-way nested ANOVA was performed using 'season' as fixed factor (two levels: summer 2018 and winter 2019), 'sampled area' as fixed factor (two levels: downstream and upstream) nested in 'season' factor and the 'tidal position' as fixed factor (two levels: intertidal and subtidal) nested in 'sampled area' by its turn, nested in 'season'. Abundance of trematodes per cockle was $\log_{10}(y + 1)$ transformed and used as dependent variable.

Several linear regressions and a multiple linear regression were performed using the abiotic factors (water temperature, pH, salinity, DO, ORP, TOM and MGS) as independent variables and the $\log_{10}(y + 1)$ transformed mean trematode abundance per tidal position of each sampled area in each season as dependent variable. Linear regression was used to understand the isolated influence of each abiotic variable, while multiple linear regression was used to obtain the explanatory model of trematodes abundance variability among all environmental conditions.

Infection success of the laboratory experiment was tested through an independent samples Student's t-test by comparing metacercariae.cockle^{-1} in each water currents conditions (0.1 m s^{-1} vs. 0.3 m s^{-1}). Another independent samples Student's t-test was used to test cockles shell length similarity among water flow conditions.

All the performed statistical analyses have been done using the IBM SPSS Statistics software v.25, by first testing Levene's test for equality of variances and by assuming normality.

3.3. Results

3.3.1. Hydrodynamics characterization of the S. Jacinto channel

The results of the performed model simulations demonstrated a heterogeneity of the hydrodynamics along the S. Jacinto channel with increasing values of the current towards the mouth of the lagoon (Figure 9). The downstream areas of the lagoon, in both summer 2018 and winter 2019, presented higher MV (Figure 9A), RV (Figure 9B) and RMS (Figure

9C) compared to the values obtained for the upstream areas ($p > 0.001$) of the S. Jacinto channel (Table 6).

In its turn, when comparing hydrodynamics characteristics between seasons, independent samples Student's t-test showed that MV, RV and RMS displayed significant higher values in winter ($p > 0.030$) compared to summer (Table 6).

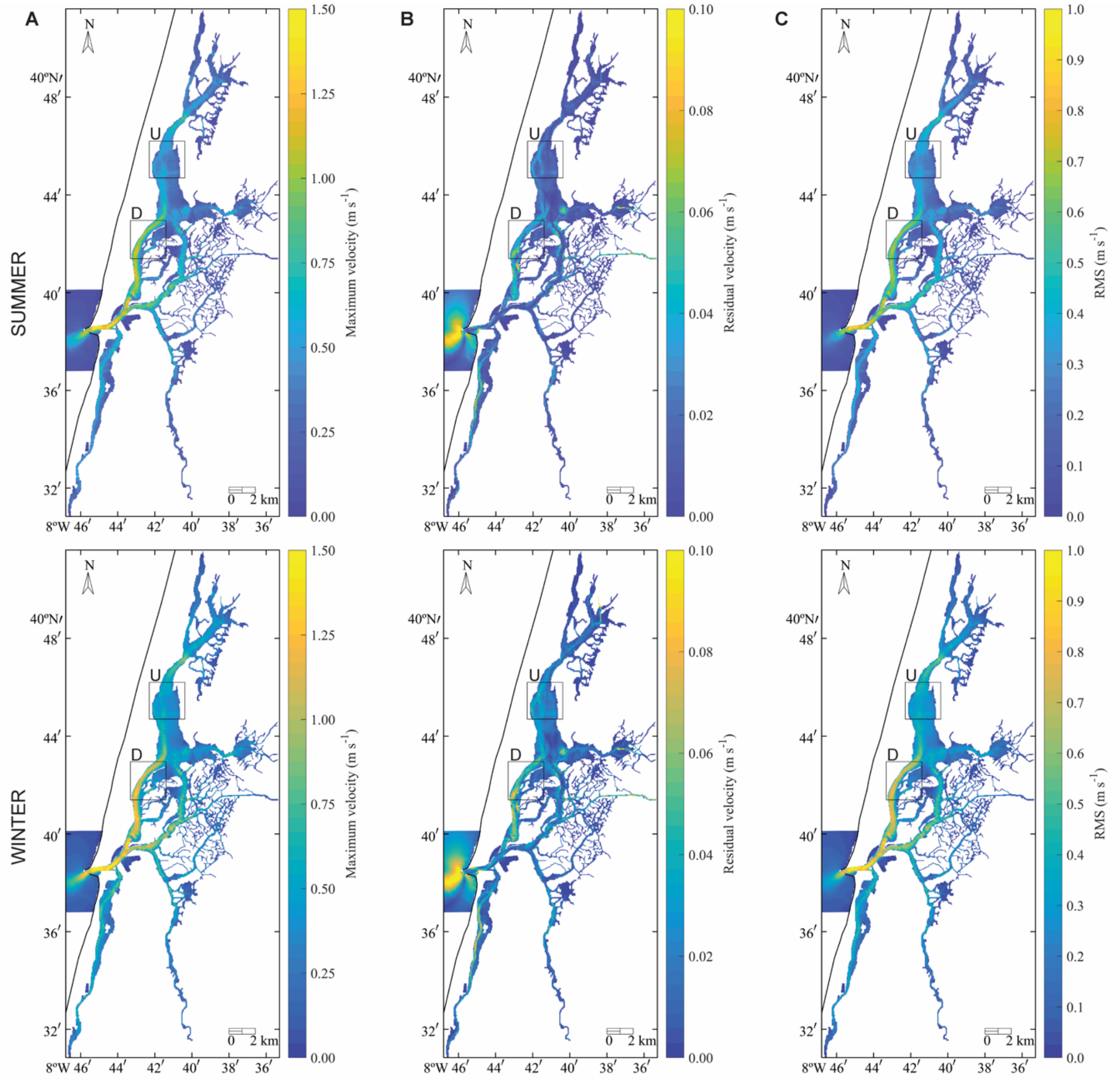

Figure 9. Hydrodynamics characteristics of the Ria de Aveiro coastal lagoon and the sampled areas referring to the periods between 16 and 17 of July 2018 (summer sampling period) and 21 and 22 of February 2019 (winter sampling period). **A**: Maximum velocity (MV); **B**: Residual velocity (RV); **C**: Root mean square velocity (RMS). Areas: U – upstream; D – downstream.

Table 6. Characterization of each sampled site in terms of the hydrodynamic factors: maximum velocity (MV), residual velocity (RV) and root mean square velocity (RMS).

	Summer 2018			Winter 2019		
	MV ($m\ s^{-1}$)	RV ($m\ s^{-1}$)	RMS ($m\ s^{-1}$)	MV ($m\ s^{-1}$)	RV ($m\ s^{-1}$)	RMS ($m\ s^{-1}$)
Intertidal site 1	0.95	0.052	0.60	1.03	0.062	0.68
Intertidal site 2	0.89	0.027	0.61	0.97	0.037	0.69
Intertidal site 3	0.49	0.032	0.30	0.51	0.038	0.33
Intertidal site 4	0.46	0.029	0.28	0.51	0.039	0.34
Subtidal site 1	0.99	0.048	0.63	1.08	0.059	0.72
Subtidal site 2	1.04	0.036	0.69	1.14	0.048	0.77
Subtidal site 3	0.50	0.031	0.29	0.53	0.037	0.32
Subtidal site 4	0.43	0.026	0.27	0.45	0.032	0.30

3.3.2. Suspended particle dispersion in the S. Jacinto channel

In the downstream area, the Lagrangian model showed that the particle followed an initial dispersion trajectory towards the mouth of the lagoon in the first hours, however, after 24 hours the particle was positioned around the release site. Between the 24 hours until 36 hours after releasing, the particle followed a rapid dispersion towards south (mouth of the lagoon) where it remained (Figure 10A).

Concerning the upstream area, the particle dispersed as well towards the south, persisting in the studied downstream area within the first 24 hours. After the 24 hours the particle dispersed into inner parts of the lagoon (Figure 10B).

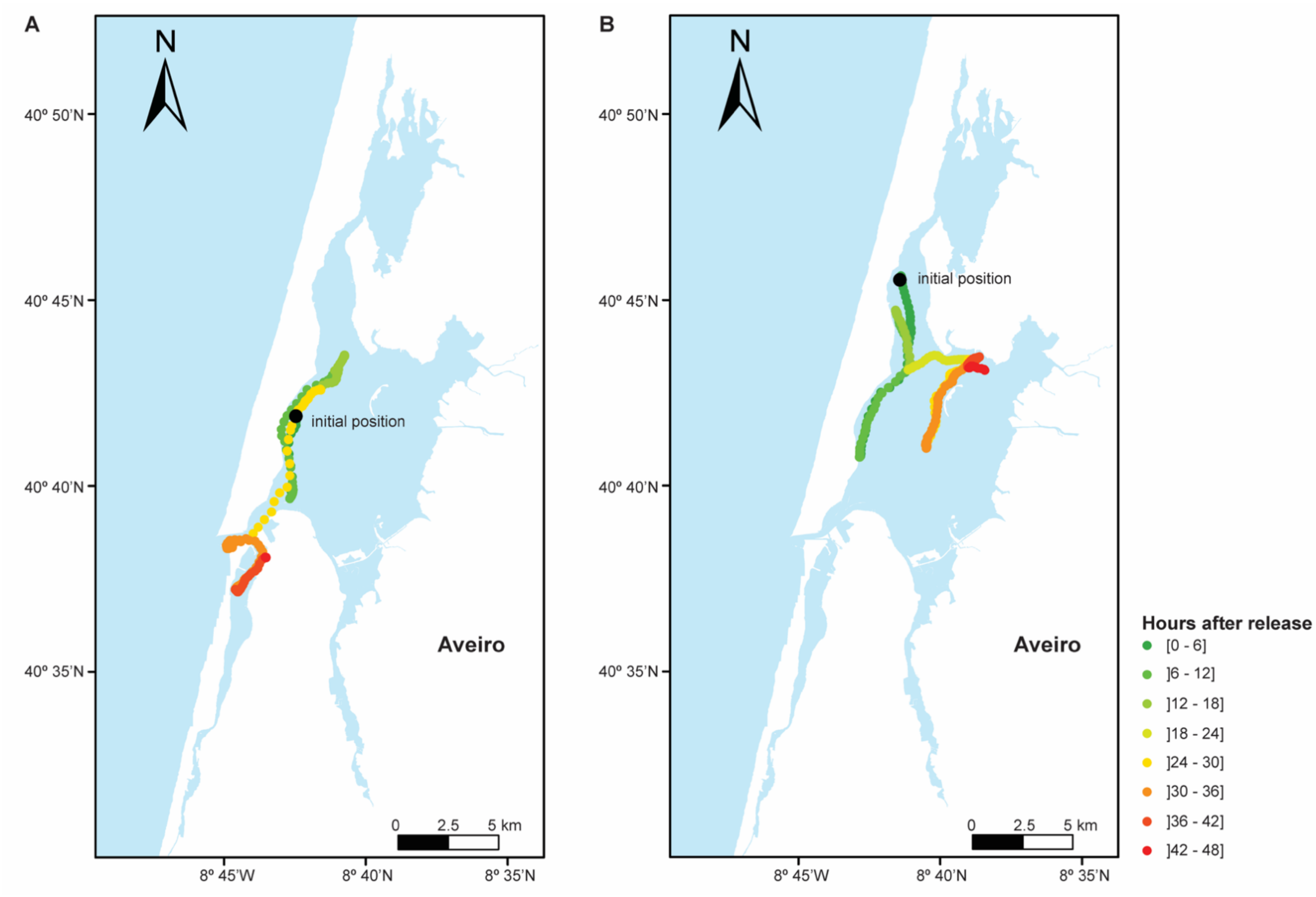

Figure 10. Lagrangian model for the dispersion of a particle. **A**: Particle release in studied downstream area **B**: Particle release in the studied upstream area.

3.3.3. Trematode community

During this study, out of 320 dissected cockles, 115 were infected by 8 trematode species of five different families (Table 7). Three species infecting cockles as first intermediate host, *Bucephalus minimus* (Prevalence (P) = 1.9 %), *Gymnophallus choledochus* (P = 0.6 %) and *Monorchis parvus* (P = 1.3 %) and five species infecting cockles as second intermediate host, *Himasthla continua* (P = 8.1 %), *H. elongata* (P = 20.9 %), *H. interrupta* (P = 12.2 %), *H. quissetensis* (P = 1.3 %) and *Parvatrema minutum* (P = 19.7 %). Overall, trematodes were absent at four sites (subtidal site 1 and 4 in the summer and subtidal sites 3 and 4 in the winter) while intertidal sites 1 and 2 in winter presented the highest trematode species richness both with six different species. Trematode mean abundance ranged between 0 and 477.1 metacercariae.cockle^{-1}, a maximum reached in intertidal site 1 of the winter season. *P. minutum* showed to be the most abundant species representing approximately 97 % of the total abundance, while *H. elongata* and *H. interrupta* represented each 1.2 %. *H. quissetensis* was the rarest species infecting only four cockles and representing less than 0.1 % of the total abundance.

Table 7. List of the digenean trematode species found in the eight sampling sites of the present study with indication of the hosts involved in their life cycle. Adapted from de Montaudouin et al. (2009).

Trematode species	**Family**	**1st intermediate host**	**2nd intermediate host**	**Final host**
Bucephalus minimus	Bucephalidae	*Cerastoderma edule*	*Pomatoschistus* spp.	*Dicentrarchus labrax*
Gymnophallus choledochus	Gymnophallidae	*Cerastoderma edule*	*Cerastoderma edule*	Water birds
Monorchis parvus	Monorchiidae	*Cerastoderma edule*	*Cerastoderma edule*	*Diplodus* spp.
Himasthla continua	Echinostomatidae	*Peringia* spp.	*Cerastoderma edule*	Water birds
Himasthla elongata	Echinostomatidae	*Littorina littorea*	*Cerastoderma edule*	Water birds
Himasthla interrupta	Echinostomatidae	*Peringia* spp.	*Cerastoderma edule*	Water birds
Himasthla quissetensis	Echinostomatidae	*Tritia reticulata*	*Cerastoderma edule*	Water birds
Parvatrema minutum	Gymnophallidae	*Scrobicularia plana*	*Cerastoderma edule*	*Haemotopus ostralegus*

Trematode mean abundance was not significantly different between seasons (*p* = 0.66). Conversely, 'sampled area', the nested factor within each season, showed a significant difference in terms of trematode mean abundance ($p < 0.001$). Trematode mean abundance of cockles living in the different tidal positions (intertidal vs. subtidal) was significantly different ($p < 0.001$) within each sampled area nested in each season, except in upstream area sampled in the summer ($p = 0.601$) (Figure 11; Table 8).

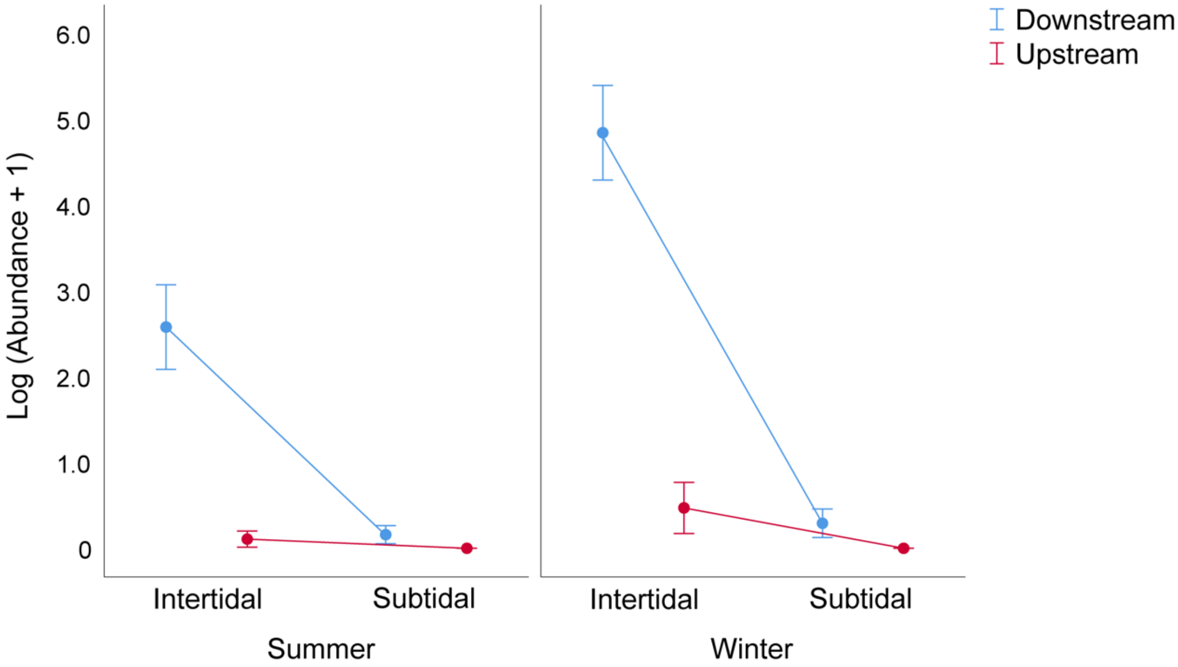

Figure 11. Mean values (± standard deviation) of trematode mean abundance per cockle in each tidal position of each area (downstream vs. upstream) and each sampled season (summer vs. winter).

Table 8. Three-way nested ANOVA results for $\log_{10}$ (y + 1) transformed metacercariae abundance per cockle (dependent variable). Season (summer 2018 vs. winter 2019) used as fixed factor, sampled area (upstream vs. downstream) and tidal position (intertidal vs. subtidal) used as nested factors. Seasons: S – summer; W – winter; Areas: D – downstream; U – upstream. MS: mean squares; df: degrees of freedom.

Random factor	**Fixed factor**	**df**	**MS**	**F**	***p-value***
	Season	1	7.19	0.27	0.657
Area	S	1	69.16	27.45	**< 0.001**
Area	W	1	217.44	86.30	**< 0.001**
Tide (D)	S	1	117.02	140.27	**< 0.001**
Tide (D)	W	1	414.25	496.57	**< 0.001**
Tide (U)	S	1	0.23	0.27	0.601
Tide (U)	W	1	4.43	5.31	0.022

3.3.4. Influence of other environmental variables on trematode abundance

Individually analysed, salinity, pH and granulometry were the only environmental parameters that showed significant correlation with trematode mean abundance (Figure 12). These three parameters displayed positive correlations to trematodes mean abundance, explaining 34 % (*p* = 0.018), 28.5 % (*p* = 0.033) and 25 % (*p* = 0.050) of the observed variation, respectively.

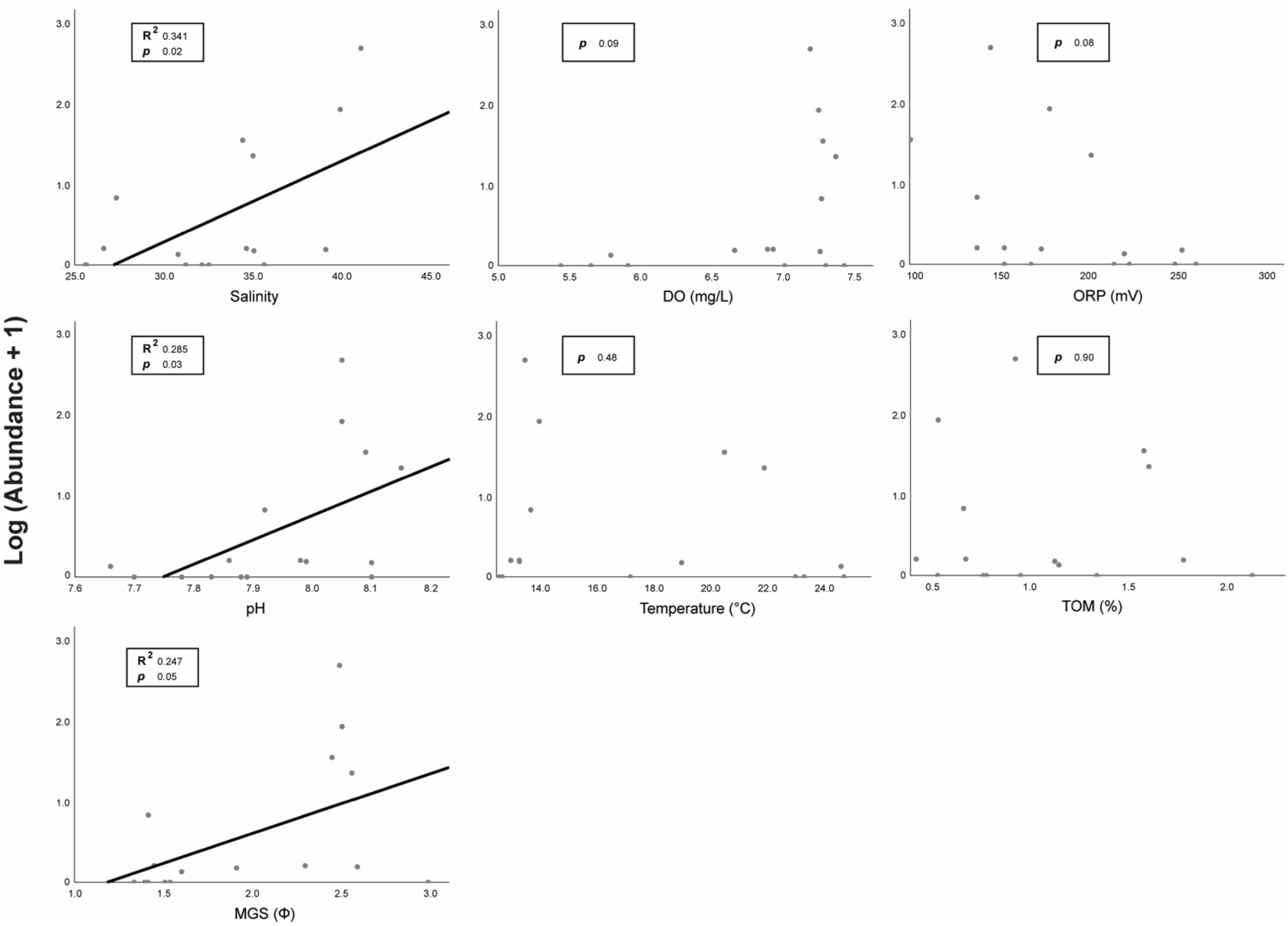

Figure 12. Linear regression of the $\log_{10}$ (y + 1) transformed mean metacercariae abundance for each studied abiotic factor. Equation line represented in the significant models. Season: S – summer; W – winter; Areas: U – upstream; D – downstream; Tidal position: I – intertidal; S – subtidal.

The multiple linear regression analysis allowed to identify salinity and redox potential (ORP) as the significant main predictors of trematode abundance per cockle. Salinity presented a significant positive correlation (p = 0.004) while, ORP displayed a significant negative correlation (p = 0.015), which model explained 59 % of the total variation of trematode abundance per cockles (Table 9):

$$\log_{10}(abundance + 1) = -1.284 + 0.106\ Salinity - 0.009\ ORP$$

Table 9. Results of the multiple linear regression model used to find the predictors salinity and redox potential (ORP) of trematodes metacercariae abundance per cockle (dependent variable, $\log_{10}(y + 1)$ transformed). SE: standard error.

	Constant	**Salinity**	**ORP**	**Model**
Estimate	-1.284	0.106	- 0.009	
SE	1.137	0.031	0.003	0.579
p-value	0.279	0.004	0.015	0.003
R^2				0.589

3.3.5. Laboratory experiment

Cockles used to perform the experiment presented similar shell lengths ($p > 0.05$). Cercariae infection success was significantly different according to different water current conditions ($p = 0.008$). The results obtained showed that cockles exposed to higher current values (0.3 m s^{-1}) presented higher infection levels (mean infection = 13.50 ± 5.78 metacercariae.cockle^{-1}) compared to cockles exposed to lower water velocities (0.1 m s^{-1}) that presented 7.40 ± 2.80 metacercariae.cockle^{-1}.

3.4. Discussion

In the sampled sites and areas of the present study, eight trematode species were identified infecting cockles *Cerastoderma edule*. This trematode diversity found in the Ria de Aveiro coastal lagoon is similar to results previously obtained within cockles distributional range. For instance, nine trematode species were found in Merja Zerga, Morocco (Gam et al., 2008) and eight trematode species were identified in south coast of Ireland (Fermer et al. 2010). When these results are compared to previous studies conducted at the Ria de Aveiro, the number of trematodes was lower than the eleven species found by Freitas et al. (2014) and by Russell-Pinto et al. (2006) however, equivalent (8 trematode species) to a more recent study performed by Magalhães et al. (2018a). On the other hand, trematode diversity found in the present study was lower when compared to Arcachon bay, France presenting thirteen trematode species (de Montaudouin et al., 2009).

As previously reported in other studies from the Ria de Aveiro (Freitas et al., 2014; Magalhães et al., 2018a), Arcachon bay (de Montaudouin et al., 2009) and Exe estuary in England (Goater, 1993), *Parvatrema minutum* was the most abundant species. This trematode uses the bivalve *Scrobicularia plana* as first intermediate host which is a broadly distributed and abundant species in European and Mediterranean coastal systems (Green, 1957). By inhabiting a wide range of environments, from muddy to sandy beaches (Verdelhos et al., 2005), *S. plana* promotes the abundance and presence of the trematode *P. minutum* in highly contrasting environments (Fermer et al., 2010).

This study demonstrated no evidences of seasonality in what concerns trematode species richness and metacercariae abundance per cockle. Trematode cercariae emission from the first intermediate host is usually linked to water temperature (Goater, 1993; Desclaux et al., 2004). Particularly for the Arcachon bay, emergence of parasites from the first intermediate host occurred in periods of water temperature above 15 °C (de Montaudouin et al., 2016). Thus, a higher number of cercariae present in the water column when temperature is higher, increases the chance of contact between cercariae and the second intermediate host, leading to higher infection levels (Thieltges and Rick, 2006) and consequent seasonality expectation. Nonetheless, this lack of seasonality observed for the Ria de Aveiro has been previously reported by Magalhães et al. (2018a) as a result of a general low trematode abundance and homogeneity of trematode communities. In fact, trematodes mean abundance obtained in the present study was low when compared to other surveys (e.g. > 5000 metacercariae.cockle^{-1}, Fermer et al., 2010) that may prevent any seasonal pattern observation. At the same time, some extreme weather events could have contributed for seasonality absence. July 2018 (summer sampling period) was the coldest since 2000 and followed a month characterized by heavy rains (IPMA, 2018). This high precipitation may have influenced a decrease in the salinity of the sampled areas and consequently, a decrease in cercariae emission (Koprivnikar et al., 2010). Conversely, February 2019 (winter sampling period) presented the warmest temperatures since 1931 and low precipitations (IPMA, 2019) which could mean an increase in cercariae emission during this season and a consequent levelling of trematode mean abundance compared to warmer seasons.

Overall, the prevalence and abundance of trematodes was higher in intertidal than subtidal cockles. The prevalence of some digenean trematode species have been previously recorded to differ along with the tidal position of the host (e.g. Gam et al., 2008). One of the factors than can explain this variation is the light exposure. In intertidal areas, due to the tidal variations from being above the water at low tides or under the water at high tides, anyhow covered by a less deep water column (with organisms living closer to water surface), the light exposure and intensity are necessarily greater than in subtidal areas. Accordingly, in a laboratory study performed by Kennedy (1979), light intensity showed a positive effect on the duration and activation of the swimming capacity of cercariae of the digenean trematode *Bunodera mediovitellata*, known to infect the pea cockle as first intermediate host and an insect larva as second intermediate host (*Limnephilus* sp.). Beyond favourable environmental conditions for the transmission to occur, a trematode rely on the presence of all its hosts in order to complete the life cycle (Bustnes and Galaktionov, 1999). Therefore, the presence and density of the first intermediate host and the final host, can influence the diversity and abundance of trematode parasites (Thieltges and Reise, 2006; Hechinger and Lafferty, 2005) and is another factor that may explain differences between intertidal and subtidal sites. In the present study, the biodiversity and/ or abundance of water birds was not assessed. Nevertheless, it has already been reported 173 different species of water birds in the Ria de Aveiro coastal lagoon (Borrego, 1996) being considered a hotspot of biodiversity and a habitat protected by the EU Birds Directive (79/409/CEE). Therefore, if on the one hand cockles from subtidal sites are more exposed to infection by free-living propagules of trematodes (with the transmission occurring exclusively through the water) than cockles inhabiting subtidal sites, on the other hand, from the parasite perspective to complete its life cycle, intertidal cockles are more attractive and advantageous hosts since they are likely to be predated by water birds, the final host of several trematode species (de Montaudouin et al., 2009) including *G. choledochus, H. continua, H. elongata, H. interrupta, H. quissetensis* and *P. minutum* found in this study (cf. Table 7).

The present work aimed to highlight that the prevalence of a given parasite may not only be related to final host presence and mobility (Feis et al., 2015) but is also dependent on the environment to which the parasite must quickly adapt to ensure its transfer among hosts. Concerning the different sampled areas of the Ria de Aveiro, the downstream area, which was near to the mouth of the lagoon, presented higher prevalence and abundance of trematodes than the upstream area. These type of outer areas of transitional ecosystems, such as coastal lagoons, are usually characterized by higher hydrodynamics and lower variation of salinity and temperature, due to higher oceanic proximity and influence, resulting into higher abundance of trematodes (e.g. Arcachon bay (de Montaudouin and Lanceleur, 2011), Merja Zerga lagoon (Gam et al., 2009), Northern Wadden Sea, Germany (Thieltges and Reise, 2007)). Conversely, more pronounced variations of temperature and salinity characteristic of the inner areas in addition to lower hydrodynamics are likely to result into lower parasite abundance (Magalhães et al., 2018a). Indeed, present results showed that the hydrodynamics can be a preponderant environmental factor in promoting trematode parasites abundance. It has been described that the maximum speed of a trematode cercariae from the Echinostomatidae family (family of 4 out of the 8 trematode species found in this study (cf. Table 7)) is approximately 3.6 mm.s^{-1} (Meyrowitsch et al., 1991). This maximum speed value is low in comparison to the Ria de Aveiro current values presented by the Mohid model performed for the sampling periods of this study (lowest mean residual velocity registered = 28.5 mm.s^{-1} (cf. Table 6)), which might imply that cercariae are not able to swim against the water current (planktonic life style) and may tend to drift in the water column and then infecting cockles populations that were not necessarily in direct contact with first host populations. These results are corroborated by the laboratory experiment in which cockles exposed to higher current velocity presented higher mean metacercariae infection than cockles exposed to lower water flow. Due to the presumed incapacity of trematodes to oppose the velocities of the water inside the flume, cercariae would remain in the water column for a longer period, being constantly resuspended, increasing the number of parasite-host interactions and thus enhancing the chance to be filtrated by cockles. In the case of the flume exposed to lower water flow, cercariae would not have as much scattering power, eventually sinking and decreasing infection levels. Convergent results showed a hydrodynamics influence on zooplankton demonstrated by the positive correlation between its abundance in areas of higher water flow (Harvey et al., 2001; Pinca and Dallot, 1997). Indeed, in the Lagrangian model applied, a released particle remained in the downstream studied area for at least the first 24 hours corresponding to the infective period of many trematode species (Sukhdeo and Sukhdeo, 2004) whereas, in the upstream studied area, prior completing 12 hours the particle was already far from the release area and within the first 24 hours, the particle displayed to be located in the downstream area. Despite the problems associated to this model, namely the failure to consider potential specific cercariae behaviour, these results demonstrated the higher prevalence of suspended particles under more dynamic areas which may explain higher infection levels found in these areas. Due to the described particles sedimentation prevention, these hydrodynamic areas are also characterized by having higher food availability (Iglesias et al., 1992), increasing bivalves feeding activity (Iglesias et al., 1992; Navarro, 1988) and growth (Coosen et al., 1994; Rueda et al., 2005) that consequently might increase the parasitism since cockles are infected by trematode parasites through their feeding activity (Bartoli and Gibson, 2007).

Usually, the greater hydrodynamics of downstream areas promote important gases exchanges in the air and water interface. These exchanges regulate the concentration of important constituents in the water, such as oxygen, improving its quality (Broecker et al., 1980; Thomann and Mueller, 1987; Wanninkhof et al., 1991). In the studied area, the redox potential, that is often associated to oxygen availability in the ecosystem (Stein and Hook, 2005; ZoBell, 1946), showed a negative correlation with trematode abundance. These results seem to contradict previous assumptions, since downstream area (that displayed higher hydrodynamics thus, higher oxygen availability but surprisingly lower redox potential) presented higher trematode mean abundance. Nevertheless, redox potential is also recurrently reported as shifting with sediment granulometry due to a lower penetration of oxygen and higher microbial oxygen uptake in habitats presenting fine sediments (Flenchel et al., 1998; Florek and Rowe, 1983). In fact, in this study, sediment granulometry (when considered individually), namely the sediment fines content, displayed a significant positive correlation to trematode abundance, which in turn explains the significant presence of redox potential in the explanatory model of trematode abundance. In addition, fine sediments are favourite habitats of *S. plana* (Green, 1957), first host of the most abundant trematode species in this study, *P. minutum*. A higher host abundance increases parasite prevalence that by its turn, benefits parasite transmission (Thieltges et al., 2011). Individually analysed as well, salinity (also significant in trematode abundance explanatory model) and pH emerged as explanatory variables of the differences found in terms of trematode abundance, presenting significant positive correlations. These results come from the positive relationship between higher salinity and pH values in downstream areas of transitional systems, as the Ria de Aveiro, resulting mainly from the oceanic proximity (Lee et al., 2006; Millero et al., 1998). Besides, salinity can have a positive influence on trematodes abundance in the second intermediate host due to higher cercariae emergence from the first intermediate host (Lei and Poulin, 2011; Studer and Poulin, 2012). The same for pH conditions, studies reported some evidences of higher cercariae longevity under normal pH values (approximately 8.1) (MacLeod and Poulin, 2015) which can lead to higher infection success in the second intermediate host.

3.5. Conclusion

This study highlighted the importance of second intermediate host vertical and horizontal position on trematode abundance. Higher hydrodynamics showed to benefit trematode abundance, supporting settlement and increasing quality status of the habitat. Concerning the tidal position, intertidal cockles displayed greater trematode abundance. This result could be consequence of higher proximity to the other hosts (i.e. first intermediate host) or trematode behaviour adapted to enhance the conditions of transmission to the final host and complete their life cycle. This study clearly demonstrated that hosts presence on the ecosystem is not the only important factor for trematode species to complete their life cycle, showing to be also dependent on the habitat that their hosts inhabit. Nonetheless, this study was not able to clarify if the differences observed were due to the habitat influence on the host or on the parasite itself. Namely, are cercariae pressure along the whole vertical and horizontal gradient equal but habitat conditions change host susceptibility? Or the host susceptibility is similar in all studied habitats, however, cercariae demonstrate a preference for a certain habitat over another? Therefore, it is important to understand the influence of abiotic factors on trematode infection as well as on cercariae behaviour, considering that cercariae responses to environmental factors might also influence infection levels and in turn disguise abiotic patterns.

CHAPTER 4. TREMATODE BEHAVIOURAL RESPONSE TO ENVIRONMENT

4. The effect of light on the trematode *Himasthla elongata* – From cercariae behaviour to infection success

4.1. Introduction

Light is a fundamental resource in every ecosystem functioning. For instance, light is essential for photoautotrophic organisms (Flöder et al., 2002) promoting photosynthesis that represent the basis of the carbon and nutrient cycles (Bengtsson et al., 2018; Romani et al., 2014). Nonetheless, light displays high temporal and spatial variability and complex patterns due to atmospheric conditions and daily or seasonal changes of light intensity (Litchman, 1998). Therefore, more lighted influenced environments are generally characterized by having higher productivity (Lohrenz et al., 2008) and consequently higher abundance and diversity of organisms (Costello and Chaudhary, 2017). Besides, light can also impact the behavioural responses of several organisms (Brewer et al., 1999).

In the marine environment and specifically for bivalves, light availability and intensity is also fundamental and determining (Koehl, 2007). For example, bivalves shell growth is improved by light (Richardson et al., 1980) while, bivalves higher activity periods occur preferentially under dark conditions (Richardson et al., 1993). Bivalves are favourite hosts of several digenetic trematode species, a clade of macroparasites with complex life cycles that imply the presence of free-living stages (Bartoli and Gibson, 2007; Esch et al., 2002). These free-living stages of a trematode life cycle are highly influenced by abiotic conditions of the surrounding environment, from which light can also be determinant. Many cercariae (the free-living stage that emerge from the first intermediate host and infect the second intermediate host where it metamorphoses into metacercariae) have demonstrated to be photosensitive, shifting towards or away from light, which might be related to the habitat in which its host inhabits (Combes et al., 1994). Trematodes that are photo sensitive have developed photoreceptors to help facilitate light perception by the cercariae (Haas, 1992). However, not all trematode species that respond to a light stimulus have developed these photoreceptors (McCarthy et al., 2002). Combes et al. (1994) reported that when the host of the trematode inhabits in the bottom of the water column (benthic organism), cercaria usually remains near the sediment and usually responds negatively to the light stimulus, moving away from it. Nonetheless, less is known about the behaviour of the cercariae stages of trematode species infecting cockles as second intermediate host.

Knowing that the light:dark cycle is one of the major drivers of life on Earth, the present study aimed to assess, through experimental approach, , the influence of light (no light vs. light exposure) on the cercariae behaviour, cercariae infection success and cockle susceptibility to infection. The postulated hypotheses were 1) Since the intermediate host, *C. edule,* is an infaunal bivalve, do cercariae of *Himasthla elongata* (trematode parasite) present a negative phototactic behaviour? And 2) Will light exposure result into different infection rates though influence on cockles susceptibility to infection?

4.2. Material and Methods

4.2.1. Living organisms collection and maintenance

The model parasite used in this work, *Himasthla elongata*, is a trematode species from the Echinostomatidae family. *H. elongata* uses the common periwinkle, *Littorina littorea*, as first intermediate host, where a mature redia, give rise to cercariae through asexual multiplication. Cercariae, a free-living larval stage, display a short lifespan (< 48 h) during which they have to ensure host-to-host transmission. In the second intermediate host, the edible cockle *Cerastoderma edule*, *H. elongata* settles as metacercariae. The cycle is accomplished when the infected second intermediate host is predated by the final host, water birds, where the trematode transforms into adult stage and reproduces sexually.

L. littorea infected snails, previously collected in Texel, The Netherlands, were kept in laboratory in an aquarium with artificial seawater (salinity 35), at a temperature of 14 ± 1 °C and with a natural photoperiod (12:12 h light/dark). During the maintenance period, *L. littorea* individuals were fed with *Ulva* sp. *ad libitum*. To obtain cercariae of the trematode *H. elongata*, infected snails were transferred to individual containers with artificial seawater (salinity 35) and exposed to a temperature boost (approximately 24 °C) and constant illumination. Cercariae were then counted under a stereomicroscope and collected with a micropipette.

Individuals of *Cerastoderma edule*, the second intermediate host, ranging from 13 to 19 mm shell length, were collected in the Mira channel of the Ria de Aveiro coastal lagoon, Portugal (40° 38’ 31.7”N, 8° 44’ 10.9”W). Cockles were transported to the laboratory and acclimated for one week at controlled conditions of salinity (30), temperature (18 °C) and photoperiod (12:12 h light/dark) and were fed with Algamac Protein Plus ® by

Aquafauna, a heterotrophic and phototrophic species mixture, at a concentration of 730 cells μL^{-1} day^{-1} as described by Pronker et al. (2015).

4.2.2. Cercariae behaviour experiment

A pre-design oval shape aquarium (50 x 30 x 5 cm, 10 cm channel width) was used to create a light gradient (Figure 13). Half of the aquarium was made of a transparent acrylic material and exposed to a horizontal light source, while the other half was in a black opaque acrylic. Light was supplied by a 7-volt, 1.5 watts LED bulb with a 24 h photoperiod. Maximum luminosity obtained was 50 μmol m^{-2} s^{-1}. The aquarium was filled with water at 35 salinity and maintained at 18 °C.

Per each experiment (same experiment performed in duplicate), a total of 1000 cercariae were released in two different releasing points (Figure 13). After 4 hours, cercariae were assigned to 8 different areas of similar volume (Figure 13B) and counted through image analysis. The percentage of cercariae present in each area was then calculated. Area 1 was exposed to maximum luminosity, while area 8 had no light exposure. The remaining six areas had an equal luminosity two-by-two. Area 2 and 3 were exposed to a light intensity of 35 μmol m^{-2} s^{-1}, area 4 and 5 had 23 μmol m^{-2} s^{-1} of luminosity and areas 6 and 7 had a total luminosity of 6 μmol m^{-2} s^{-1}. Luminosity was measured with an Apogee handheld MQ-200 Quantum meter with a separate sensor.

A previous study performed with a trematode parasite of the same family revealed that cercariae mobility tend to decrease severely after 12 hours (de Montaudouin et al., 2016). Therefore, in the present study, a total 10 h of lifespan (6 h emission + 4 h exposure) was selected to ensure cercariae mobility until the end of the experiment.

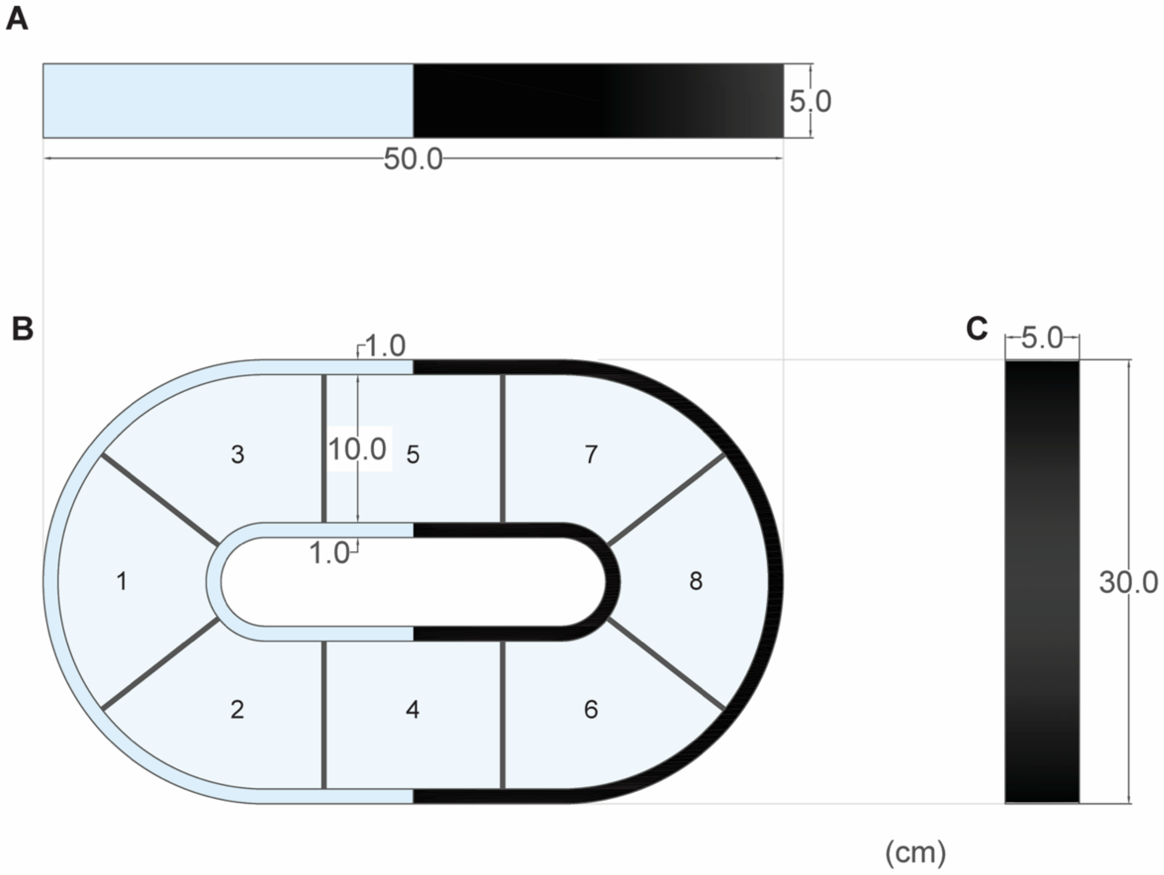

Figure 13. Schematic representation of cercariae light behaviour aquarium subdivided in the 8 areas of light gradient. A: Side View; B: Top view; C: Front view. Cercariae released in areas 4 and 5

4.2.3. Cercariae infection success

After cockles acclimation period, an experiment using twenty-four replicates was performed in duplicate. Cockles individuals were placed separately into 50 ml glass flasks at two different conditions (light vs. no light exposure). Twelve cockles were exposed to a vertical light source supplied by a 7-volt, 1.5 watts LED bulb and a light intensity of 50 μmol m^{-2} s^{-1}. The flasks of the other twelve cockles were covered in aluminium foil and had no light exposure. During this experiment, flasks water was maintained at 18 °C and 30 salinity.

Subsequently, a total of 600 cercariae, 25 per flask, were collected and used to individually infect each cockle (Figure 14). Experiments lasted for 48 h, the necessary time to *H. elongata* cercariae to encyst (de Montaudouin et al., 2016), and then *H. elongata* metacercariae were counted under a stereomicroscope by squeezing cockles flesh between two glass slides.

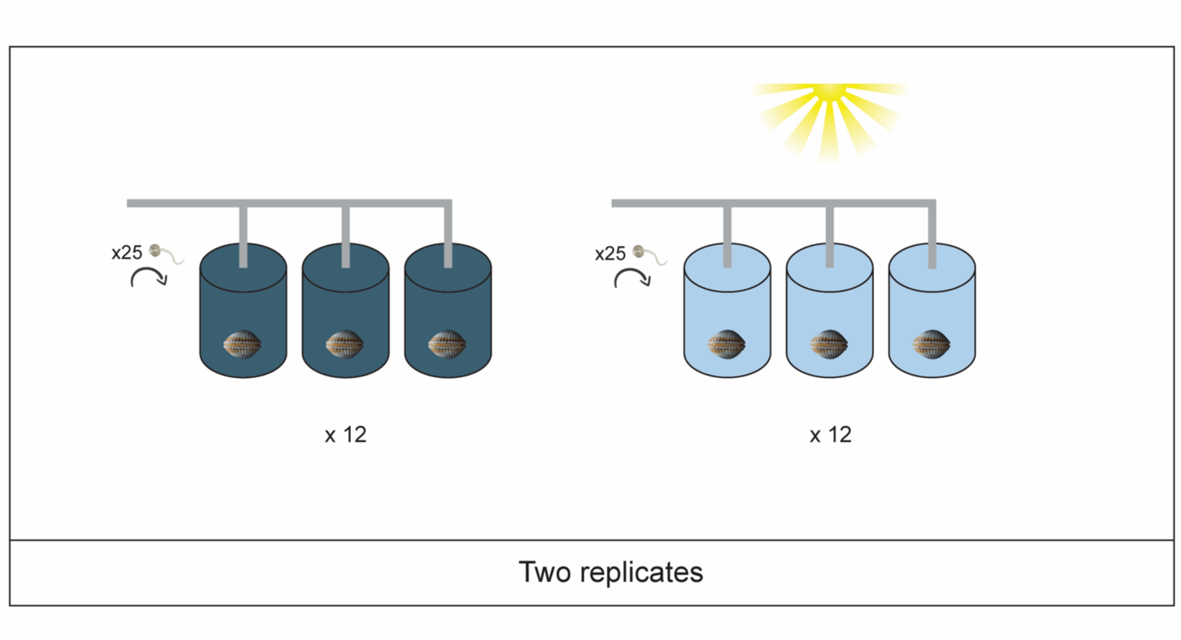

Figure 14. Schematic representation of the cercariae infection success experiment

4.2.4. Cockles respiration rate

Cockles respiration rate was measured through simple static respirometry. Twelve cockles of similar shell length (between 15 and 17 mm) were collected and individually placed in respirometric chambers. All chambers were filled to their maximum capacity with artificial seawater at 30 salinity, to avoid formation of bubbles. In order to allow cockles acclimation to the respirometric chamber conditions, individuals were left for 1 h inside the respirometric chamber to avoid manipulation influence on the results. Six of these chambers were covered in aluminium foil, preventing the entrance of light, and the other six were exposed to natural light condition. In addition, four other respirometric chambers (two covered in aluminium foil and two uncovered) were used as control, i.e. no organisms to account for background oxygen loss. Each chamber was equipped with an oxygen sensor spot glued from the inside. Oxygen concentration was measured twice, in equal time intervals (1h), by contacting the oxygen sensor with a multi-channel optic fiber oxygen meter (Multi channel oxygen meter, PreSens, GmbH, Regensburg, Germany) and reading the concentration value after stabilization in the PreSens Measurement Studio software.

Afterwards, respiration rate was calculated as the difference between 2 reading points (separated by a period of two hours) and expressed in oxygen consumption (ppmO_2) per grams of cockle dry weight per hour. Cockles dry weight was measured following Gam et al. (2009) procedure.

4.2.5. Data analysis

Due to the low number of replicates, data of cercariae behaviour experiment (4.2.2.) was only used as descriptive information of the trematode free-living propagules responses to light.

For both cercariae infection success and cockles respiration rate experiments, an independent samples Student's t-test was performed to assess cockle shell length differences among conditions. Independent samples Student's t-test was also used to compare mean cockle respiration rate and mean infection success between conditions (light vs. no light).

All the performed statistical analyses of the present work have been done using the IBM SPSS Statistics software v.25 and by testing the homogeneity of variance first and assuming normality.

4.3. Results

In cercariae behaviour experiment, 82.5 ± 9.2 % of released cercariae were found after the 4 h. From those, 80.3 ± 2.1 % were detected in the area 1, corresponding to the highest luminosity (50 μmol m^{-2} s^{-1}). Area 8, without light exposure, was identified as the area with the lowest presence of cercariae (3.0 ± 0.9 % of total counted cercariae). The remaining cercariae were found in the areas between maximum and the absence of light. Areas 2 and 3 presented 9.3 ± 0.8 % of cercariae, while in the areas 4 and 5 a percentage of 3.8 ± 0.4 % of the total found cercariae were counted. In the areas 6 and 7, the areas closer to the absence of light, cercariae found corresponded to 3.8 ± 0.8 % (Figure 15).

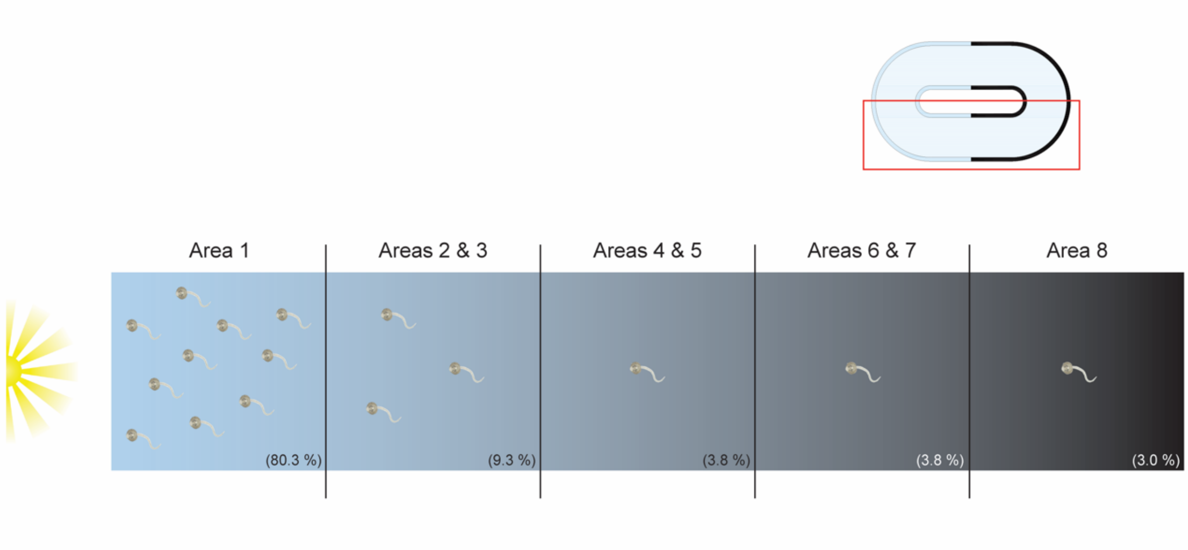

Figure 15. Schematic representation of the proportion of cercariae found in each area of the cercariae light behaviour aquarium.

Shell length of cockles used in the cercariae infection success experiment and in the respiration rate analysis was not significantly different between conditions ($p > 0.05$) (Table 10). Cercariae infection success presented significantly higher values ($p = 0.004$) in cockles with no light exposure (mean infection = 14.29 ± 4.14 metacercariae.cockle^{-1}) compared to cockles exposed to light (mean infection = 10.16 ± 4.34 metacercariae.cockle^{-1}). On the other hand, cockles respiration rate was not significantly different between light conditions (Student's t-test $p = 0.103$) (Table 10).

Table 10. Independent samples Student's t-test results for cockles respiration rate and cercariae infection success experiments. df: degrees of freedom.

	t	df	*p-value*
Cockles respiration rate			
Shell length	0.447	7.353	0.668
Mean respiration rate	- 1.797	9.359	0.105
Cercariae infection success			
Shell length	0.784	36.876	0.438
Mean infection*	3.073	37.167	**0.004**

4.4. Discussion

Several studies have been showing evidences of a strong relationship between the phototactic behaviour of parasite free-living stages and their hosts living habitat. As an example, the miracidium of *Bunodera mediovitellata* that infects the benthic bivalve *Pisidium carsenatum* as first intermediate host, presented a negative phototactic behaviour (Kennedy, 1979). Similarly, *Microphalus similis* cercariae showed a preference for darker areas where, the second intermediate host, *Carcinus maenas*, due to its movement to under rocks, can be found (McCarthy et al., 2002). On the other hand, cercariae of *Schistosoma mansoni* (Kuntz, 1947), the miracidium of several *Schistosoma* species (Chernin and Dunavan, 1962; Takahashi et al., 1961) and cercariae of *Euhaplorchis* sp. (Smith and Cohen, 2012) display a positive phototactic behaviour. In common, these latter species, present an intermediate host that live near the water column surface, suggesting that trematode free-living stages presenting a positive phototaxis, use light as a guide system to occupy higher positions in the water column.

Following the previously described evidences and conversely to what was expected, this study showed a positive phototactic behaviour of *H. elongata* cercariae, displaying movements towards light when exposed to a horizontal light source. Combes et al. (1994) verified that in the case of cercariae of parasite species which next host is a bottom dwelling, these larvae would remain near the bottom of the water column in order to increase the chance of infecting its second intermediate host. Thereby, since that the second intermediate host of the trematode species under study, the edible cockle, is a benthic organism that lives burrowed in the sediment of coastal waters (Boyden and Russel, 1972), it was expected that cercariae of *H. elongata* would demonstrate negative phototaxis.

Nevertheless, the position of cercariae in the water column is not only dependent on phototaxis, but rely as well on other exogenous cues, for example geotactic behaviour, i.e. response of the organism to the force of gravity (Mouritsen, 2001). As an example, the cercariae of *Bunodera mediovitellata* infects the bottom dwelling larva of caddisfly as second intermediate host. Nonetheless, similarly to what was presently found to *H. elongata* cercariae, this species presented a positive phototactic behaviour. This response was observed horizontally, while vertically, due to a positive geotactic behaviour or an inefficient vertical swim, cercariae of *B. mediovitellata* remained near the bottom (Kennedy, 1979). This behaviour pattern allows this trematode species to establish in a water column with higher light incidence, i.e. lower water column height, increasing the transmission to the second host. In the present study, only the horizontal response of cercariae to light was tested however, de Montaudouin et al. (1998) and Werding (1969) experimentally observed that cercariae of *H. elongata* remains closer to the bottom of the aquarium, implying a possible positive geotactic behaviour. To complete its life cycle, *H. elongata* has to infect the second intermediate host, i.e. cockles, that inhabit throughout the whole tidal range, from intertidal to subtidal areas (Riesen and Reise, 1982), which in turn has to be predated by the final host, water birds. Therefore, we can hypothesize that this parasite tends to pair a positive phototaxis and geotaxis response (likewise *B. mediovitellata*) in order to promote the infection of upstream cockles in the water column (intertidal position), where cockles are simultaneously more susceptible to be predated by the final host. Accordingly, higher infection levels of cockles of intertidal areas compared to subtidal ones have already been described in the literature (Correia et al., in prep; Gam et al., 2008).

The present study showed that cockles not exposed to light, displayed higher mean infection levels compared to cockles under a vertical light source, despite the previously mentioned positive phototactic behaviour of *H. elongata* cercariae. Cercariae of Echinostomatidae family (family of the studied trematode species) are passive invaders, i.e. cercariae infect the second intermediate host by being ingested through their feeding activity (Galaktionov and Dobrovolskij, 2003). However, during the present study, cockle respiration rate showed no significant differences according to light tested conditions (light vs. no light). Additionally, experiences were performed in small containers with a short water column height, leading authors to infer that recorded infection differences are rather dependent on cercariae behaviour towards distinct light conditions than on cockles individual activity or geotaxis. Indeed, when exposed to a light source, cercariae locomotive movements can be enhanced (Haas, 1992) which could lead to a rise in the water column.

Consequently, these vertical movements decrease the chance of contact and depart cercariae from the feeding area of the second intermediate host. On the other hand, in the absence of a light stimulus that rises cercariae in the water column, cercariae swims irregularly, slowly descending in the water column (Feiler and Haas, 1988; Haas et al., 1990). This way, the cercariae gets closer to the feeding area of its host, increasing the chance of contact between trematode cercariae and second intermediate host. When in close contact with the host, the described passive behaviour of cercariae shifts and starts an inciting active infection (Galaktionov and Dobrovolskij, 2003) increasing infection success probabilities thus, explaining the results presented here.

Trematodes free-living stages display a short lifespan (24 hours or less) in which they have to identify and promptly infect the host while using their energy resources efficiently (Holliman, 1961). As a result, the emergence of cercariae from the first intermediate host is linked to the time in which their second intermediate host is more active and susceptible to be parasitized (Lewis et al., 1989). In the instance of hosts that are active during the day and the night period, emergence from the first intermediate host matches periods that display higher advantages to the trematode life cycle completion. As an example, Lewis et al. (1989) showed that *Proterometra macrostoma*, a trematode species that infects the sun-fish as second intermediate host (an active feeder during the day and the night), presented a nocturnal emergence in order to decrease its predation by diurnal non-hosts feeding organisms. In the case of the studied trematode species, *H. elongata*, its first intermediate host is the common periwinkle, *Littorina littorea*, an intertidal gastropod that is mainly found in the supralittoral zone of rocky shores (Benson, 2008). Due to the higher tidal position of this host, compared to the second intermediate host (cockles), for a successful infection, cercariae would require moving downward in the water column. This kind of active cercariae motile actions rapidly consumes energy reserves (Shostak and Esch, 1990). Thereby, a higher cercariae emission during the night period would benefit this species in two different ways: i) Firstly, the poor movements of cercariae in the absence of light descends cercariae in the water column, infecting cockles populations living on the surroundings of the first intermediate host populations; ii) Secondly, in the case of cercariae could not successfully infect host during the “first night period”, the exposure to light would incite the movement of cercariae. This would perhaps ascend cercariae away from the sediment and the bottom of the water column decreasing a possible predation by host or non-host filter-feeding organisms located in unprivileged areas to continue the cycle. Besides, the attraction of cercariae by light could help cercariae to disperse throughout the aquatic system, inserting in more lighted areas, closer to the intertidal zone, until total depletion of the energy resources (Pechenik and Fried, 1995) or by changing for a positive geotaxis behaviour. Prokofiev (2002) has demonstrated that some cercariae can change geotactic behaviour after some hours, demonstrating a positive geotaxis after an initial dispersal phase. In either way, the cercariae would fall to the bottom of the water column where it would depend on a possible contact or predation by its host during the downward movement of the cercariae before dying.

Beyond previously mentioned geotaxis and phototaxis, trematode cercariae can reveal attraction to chemical compounds that can help to identify and infect their hosts. For example, the cercariae of *Schistosoma mansoni*, that presents a positive phototaxis, can change the swimming orientation in the presence of amino acids excreted by the human skin (Granzer and Haas, 1986; Shiff and Graczyk, 1994) demonstrating that chemotaxis can overlap the effects of the other two discussed taxis responses. *Himasthla* species have not been reported to exhibit chemotactic behaviour opposing other species of the Echinostomatidae family (McCarthy, 1990). This type of response was not studied in the present work and considered excluded from the set of explanatory variables, since, as previously stated, cercariae were inserted in a restricted area (approximately 50 ml) with no possibility of host avoidance and/or demonstration of attraction by the host presence.

4.5. Conclusion

Trematodes are organisms with life cycle fluctuating between parasitic and free-living stages (Bartoli and Gibson, 2007; Esch et al., 2002). The non-parasitic stages, miracidia or cercariae, have a short lifespan in which they have to ensure host-to-host transmission (de Montaudouin et al., 2016). Due to this short life period, a successful transmission depends on their ability to quickly find an appropriate host (Sukhdeo and Sukhdeo, 2004). Therefore, trematodes larval stages have developed species-specific behaviour, such as phototaxis, i.e. movement of an organism in response to light stimulus, that enhance the chance of infection (Isseroff and Cable, 1968; Zimmer et al., 2009). *Himasthla elongata* is a predominant trematode species in coastal waters that have been described as causing mortalities in juvenile cockles (Lauckner, 1987). When exposed to a light stimulus, cercariae of *H. elongata* demonstrated a positive phototactic behaviour, moving towards light. However, higher infection levels were observed experimentally in cockles exposed to absence of light, despite no differences found on cockles activity. These results suggest that cercariae are influenced by abiotic factors, in this case light, rather than host presence, in order to find the most suitable habitat. The present study provided the first description of *H. elongata* behavioural responses to light which can have particular relevance in the prediction of trematode infection rates variation and possible mortality outbreaks in certain locations and/ or time periods.

CHAPTER 5. FINAL REMARKS AND PERSPECTIVES

5.1. Final remarks

This thesis was focused on the study of host-parasite relationship using a bivalve species, *Cerastoderma edule*, as a model host, and its infection by trematode parasites. As discussed in several moments along the thesis, bivalves are in fact important constituents of the natural environment (Sousa et al., 2009; Taylor et al., 2018), providing several ecosystem services and thus contributing for the resilience of coastal water systems (Morgan et al., 2013; Rakotomalala et al., 2015). Trematode parasites are also important because they represent the most prevalent clade of macroparasites of coastal waters (Lauckner, 1983; Roberts et al., 2009) and are able to control the population dynamics of their hosts (Wegeberg and Jensen, 2003) and cause mortalities outbreaks (Desclaux et al., 2004). Following the described critical role of these two organism clades in the ecological communities, it becomes evident the urgent demand to deep the knowledge about their interaction, recognizing the variables that can control it and then be able to predict infection outbreaks and thereby to support bivalve resources management and bivalves production policy makers decisions. This thesis significantly contributed to the knowledge in this field.

The large scale study included in this thesis demonstrated that latitude was apparently identified as the most important determinant factor for trematode prevalence and abundance in cockles. However, this trematode latitudinal gradient was more related to temperature, that increased towards the south, than to latitude itself. The type of aquatic system, namely coastal lagoons or bays, also demonstrated to have an impact on trematode abundance, correlated with the oceanic influence. More oceanic influenced systems display more buffered temperature and salinity variations and higher hydrodynamics that increase the quality status of the ecosystem and trematode abundance. Nevertheless, it was highlighted that the occurrence of trematode species is only possible when all hosts of that species are present in the ecosystem, the first intermediate one appearing as a limiting factor. Furthermore, the obtained results highlighted the ubiquity of trematode parasites in the different aquatic systems and alert to a possible change on the trematode fauna composition and abundance in cockle populations driven by thermal modification mediated by oceanographic global circulation. Besides global temperature monitoring, it is then imperative to incorporate trematode communities assessment in ecological studies, due to their highly integrative significance, to better predict potential negative impact on host populations and communities sustainability. Despite the temporal gap among some of the samples, we believe that the present findings are still valuable and of high impact not only because of the interest in trematode communities knowledge improvement in each sampled area and each country but also because at such a spatial scale, the temporal scale is less important since that trematode spatial composition is rather stable along time (de Montaudouin et al., 2012). As an illustration, in the Ria de Aveiro coastal lagoon, one of the sampled systems, diversity of trematode was equivalent after 6 years (Freitas et al., 2014; Russell-Pinto et al., 2006).

The small scale study, using the Ria de Aveiro coastal lagoon (Portugal) as a natural laboratory, showed the influence of host vertical, i.e. position in the water column, and horizontal position, i.e. distribution on the water stream, in trematodes prevalence and abundance. Cockles occurring in the intertidal position presented higher trematodes prevalence and abundance probably due to life cycle facilitation (more favourable conditions for free-living stages transmission and proximity to the other hosts). Nevertheless, host-parasite interactions are complex and dependent on multifactorial processes occurring in the environment. In more sheltered habitats (inner part of the lagoon), that display lower hydrodynamics, trematode abundance was lower compared to areas with more oceanic influence (outer part of the lagoon) hence, higher hydrodynamics. The exchanges in the air-water interface promoted by the water dynamics leads to an improvement of the water quality. On one hand, this might promote higher diversity and abundance of hosts on areas of higher hydrodynamics, increasing the probabilities of trematode life cycle completion while, display better ecological status as trematode have been described to be sensitive species to environmental quality. Besides, due to the planktonic lifestyle of the free-living stages of trematodes, hydrodynamics increases the dispersion capacity by trematodes cercariae, increasing the chance and the times of contact between host and parasite leading to a higher infection success in that areas.

Supported by an experimental approach, this thesis showed that more than the known universal patterns of species distribution, the behaviour and responses of the free-living stages of trematodes (in this specific case, the cercariae of trematodes) depend on external cues, such as the influence of light, that are paramount for the infection success and consequent life cycle completion. In fact, cercariae of *H. elongata*, by displaying a positive horizontal phototaxis might be enhancing its dispersion in the most suitable habitat where its second intermediate host resides. Additionally, the higher infection levels observed in cockles with no light exposure, suggested that the light might incite the movement of cercariae, leading to a consequent rise in the water column, decreasing the chances to be ingested and consequently infect cockles. Therefore, in a natural environment, emission of cercariae may be higher during the night with trematodes using this period to try to infect peripheral populations of the second intermediate host with a greater energy saving. In the case of unsuccessful infection during this period, cercariae would use their resources to relocate in more lighted areas (lower water column) using light as a guide system. Furthermore, this study highlighted that trematode responses are not only dependent on the presence of the second intermediate host, as it is widely distributed along the whole tidal gradient, but on the complete life cycle with external stimuli guiding trematodes behaviour towards areas where the second intermediate host is more likely to be predated and consequently transmit the parasite to its final host.

Overall, this thesis clearly demonstrated that patterns that regulate the activity of parasites with complex life cycles at a large scale may often not be the same as those that regulate wildlife and are much more multifactorial and always dependent on the presence of all their hosts in the ecosystem. Conversely, at a small scale, these parasites seem to follow the same pattern of their hosts, influenced by higher ecological status. The results of this thesis can be used as a basis for management of cockle stocks in protected areas by demonstrating the impact of environmental factors on trematode parasites dynamics that can help to predict prevalence and abundance outbreaks. Moreover, due to the socio-economic importance of this bivalve species, this thesis outcomes can make an important contribution on cockle production and aquaculture improvement, helping in choosing sites or condition facilities that represent a reduced probability of trematode abundance outbreak and consequent lower host mortality. It is then important to continue these studies because only when we fully understand how each abiotic variable (e.g. salinity, temperature, depth, hydrodynamics) affect the population dynamics of these parasites and which are the most important, we will be able to help in natural cockles stocks management and diseases outbreak episodes prediction.

5.2. Future perspectives

Parasites are often neglected environmental components with most of the studies focusing on the effects of parasites on their hosts or on the interaction between hosts and abiotic variables and its influence on parasite abundance. Nonetheless, as demonstrated in the present thesis, parasites abundance can depend also on the free-living stages of parasites with complex life cycles, in this case trematodes, response to environmental signals. Trematode cercariae reacts to several cues such as gravity, temperature, water current or chemical compounds. Following the present thesis results, it would be interesting to understand cercaria of *Himasthla elongata* response to other non-studied cues (e.g. gravity) to try to predict and comprehend the patterns developed by this free-living stage of trematodes to enhance its infection success.

Additionally, trematode parasites using cockles as their second intermediate host display two major life cycles. The first, where the studied *H. elongata* is included, are trematodes that present water birds as final host. While the second, includes trematode species that display fishes as the final host. Assessing the behavioural responses of species displaying this type of life cycle would help to justify some results of this thesis likewise, trematodes are moved to find the most suitable habitat to complete its life cycle rather than host presence only.

Finally, gathering the previously obtained results for both species, it would be interesting to create a cercariae dispersion map of the Ria de Aveiro based on the Lagrangian model where it would be possible to identify areas of cockles greater probability to infection by the different trematodes species along with a constant monitoring for model validation.

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