Universidade de Aveiro Departamento de Biologia
Ano 2010

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor João António de Almeida Serôdio, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Doutor Henrique José de Barros Brito Queiroga, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro.

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Ácidos gordos, acoplamento trófico, cadeia trófica, clorofila, estuários, feopigmentos, *Hydrobia*, microfitobentos, migração vertical, produção primária.

resumo

Os estuários são ambientes complexos, biologicamente diversos e muito importantes no que respeita à produtividade primária. As zonas intertidais destes ecossistemas são ocupadas por organismos que possuem uma elevada capacidade de sobrevivência e adaptação face às variadas e rápidas alterações nos factores ambientais (tais como temperatura, salinidade, conteúdo hídrico, etc.). As cadeias tróficas com origem no ecossistema estuarino bentónico são essencialmente herbívoras, regulando o fluxo de energia desde o fundo sedimentar e através do ecossistema. Nas áreas estuarinas intertidais a produção primária é essencialmente suportada pelo microfitobentos (MPB). Estas comunidades de microalgas bênticas constituem uma importante fonte de matéria orgânica e são por si só a principal fonte alimentar para as populações de *Hydrobia*. Neste contexto, a interacção MPB - *Hydrobia* é um modelo-chave na investigação da cadeia trófica estuarina de origem bentónica, actuando como um importante canal de transporte de energia para os níveis tróficos superiores, especialmente se considerarmos que *Hydrobia* é uma importante presa para peixes, aves e caranguejos.
O presente estudo tem por objectivos gerais: i) a investigação do controlo ambiental (particularmente da luz e do teor em água do sedimento) e endógeno na migração vertical do MPB e ii) a identificação e potencial utilização de marcadores tróficos (pigmentos e ácidos gordos) úteis à investigação da interacção MPB – *Hydrobia* em laboratório e em condições naturais, considerando a existência de uma elevada plasticidade trófica por parte da *Hydrobia* e a elevada densidade populacional que estes organismos podem apresentar.
A primeira fase de investigação resultou na comparação do papel dos estímulos ambientais e do controlo endógeno nos padrões de comportamento migratório vertical do microfitobentos, demonstrando a existência de um controlo essencialmente endógeno na formação e desintegração do biofilme superficial. A regulação e manutenção da biomassa à superfície do sedimento são claramente controladas pela variação dos factores ambientais, em especial da luz, cuja presença é essencial à formação total do biofilme microalgal à superfície do sedimento intertidal.
Foi proposta uma nova abordagem metodológica com vista à estimativa não-destrutiva do teor de água de sedimentos intertidais vasosos , possibilitando o estudo da influência da acção do vento no conteúdo hídrico dos sedimentos e o consequente impacto da dessecação na comunidade microfitobêntica. Observou-se que a dessecação provoca efeitos limitantes não só na biomassa superficial mas também na actividade fotossintética dos biofilmes microfitobênticos, conduzindo à diminuição da produtividade primária.
No que respeita à dinâmica trófica da interacção MPB - *Hydrobia* foi estabelecido o uso do pigmento feoforbide *a,* quantificado nas partículas fecais da fauna*,* como marcador trófico que permite estimar a quantidade de biomassa de microalgas (clorofila *a*) incorporada pelos organismos animais.

resumo

Para tal foi investigada e comprovada a existência de uma relação significativa entre a concentração de feopigmentos excretados e a concentração de clorofila *a* ingerida. Estes estudos foram desenvolvidos numa primeira fase à escala diária, considerando os efeitos dos ciclos sazonais, dia-noite e maré, e depois com a validação em condições naturais, numa escala mensal. A taxa de ingestão média de indivíduos de *H. ulvae* varia ao longo do dia, com o máximo em torno dos períodos diurnos de maré baixa, o que pode estar relacionado com a disponibilidade de MPB. As taxas de ingestão (TI) de *H. ulvae* variam ainda em função da estação do ano (TI verão > TI primavera) e em função da densidade de indivíduos (> densidade, < ingestão). Verificou-se um efeito negativo na concentração de clorofila disponível após herbívoria independentemente da densidade de indivíduos.

Finalmente, a comparação dos perfis de ácidos gordos de *H. ulvae* provenientes de diferentes habitats com os perfis de potenciais fontes alimentares permitiu demonstrar que os ácidos gordos são ferramentas úteis na identificação do habitat ocupado por estes organismos. No entanto, apesar da ocupação de diferentes habitats e da integração de múltiplas fontes de produção primária na sua dieta foram sempre observados significativos níveis de ácidos gordos específicos de microalgas (em particular diatomáceas), reforçando o papel importante das comunidades de microalgas bênticas na dieta das populações de *H. ulvae*.

keywords

Chlorophyll, estuaries, fatty acids, food chains*, Hydrobia,* microphytobenthos, pheopigments, primary production, trophic coupling, vertical migration.

abstract

Estuaries are biologically diverse and form complex environments, which play an important role on the global primary productivity of aquatic environments. Intertidal areas of estuaries are inhabited by organisms with a strikingly capability to survive and to be adapted to frequent and fast changes in several environmental factors (such as temperature, salinity, water content, etc.). Grazing food chains are common in intertidal mudflats regulating the flow of nutrients and energy from the bottom throughout the estuarine ecosystem. Within intertidal estuarine areas the primary production was predominantly supported by microphytobenthos (MPB). These benthic microalgae assemblages are an important source of organic matter and are a main food source for *Hydrobia* populations. The MPB - *Hydrobia* interaction is a key model for the estuarine grazing food chain, acting as a significantly channel of energy to higher trophic levels, since *Hydrobia* is an important prey item for fish, birds and crabs.

The present work addressed: i) the environmental (namely light and sediment water content) and the endogenous control of the vertical migration by microphytobenthos, and ii) the identification and the potential use of trophic markers (pigments and fatty acids) to establish this relationship under laboratory and natural conditions, considering that *H. ulvae* showed significant trophic plasticity and that mud snails could reach extremely high densities.

The role of exogenous cues and endogenous control of the patterns of vertical migratory behavior of intertidal MPB biofilms were compared, showing that the formation and disintegration of the biofilm is endogenously-controlled. The regulation and maintenance of the microalgal biomass at the sediment surface is dependent on the variation of environmental factors, namely light, which is essential for the full formation of the MPB biofilm.

A new methodological approach was proposed to estimate the water content of muddy intertidal sediments, enabling the study of the influence of wind on the hydric level of the sediment and the consequent impact of desiccation on the MPB biomass. This investigation showed that desiccation might be responsible to cause important limiting effects on biomass and photosynthetic activity of intertidal MPB biofilms, reducing the primary productivity.

Regarding the trophic dynamics of the interaction MPB - *Hydrobia*, it was established the use of the pigment pheophorbide *a,* present on *Hydrobia ulvae* faecal pellets, as a trophic marker to estimate the amount of microalgal biomass incorporated, as chlorophyll *a*, by benthic macrofauna. A significant relationship between egested pheopigments and ingested chlorophyll *a* was investigated and validated. These studies were firstly developed on a daily scale, considering the effects of seasonal, tidal and day-night cycles, followed by a validation under natural conditions, on a monthly scale. The mean ingestion rate of *H. ulvae* individuals varied along the day, with the maximum around the diurnal low tide periods, which may be related with MPB availability. *H. ulvae* mean ingestion rate (IR) also varies seasonally (IR summer > IR spring) and depending on mud snails density (> density, < ingestion). There was a negative effect on chlorophyll concentration available after grazing, independently of *H. ulvae* density.

abstract

Finally, the comparison of fatty acid profiles of mud snails from different habitats with the ones from potential food sources allowed identifying fatty acids as a useful tool to indicate *H. ulvae* habitat. Although the occupation of different habitats and the integration of multiple primary food sources on mud snails diet, significant inputs of fatty acids specific of microalgae (namely diatoms) were always found, which reinforce the important role of MPB on the diet of *H. ulvae* populations.

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Chapter 1

General Introduction

1.1. Intertidal mudflats

Intertidal mudflats are natural ecosystems occurring in estuaries and in the adjacent sedimentary coastal areas, such as semi-enclosed areas and sheltered marine bays (Elliott et al., 1998). As estuarine components they are in the interface between terrestrial, freshwater and marine environments (McLusky and Elliott, 2004), being ecologically, but also economically important. These natural ecosystems represent a complex and dynamic environment subject to tidal influence, where resident and visiting organisms need to be adapted in order to survive to frequent and fast changes in several environmental factors (such as temperature, salinity, water level etc). Estuarine intertidal areas are amongst the most productive marine ecosystems (Underwood and Kromkamp, 1999) as their high productivity fuels carbon flow through invertebrate and bacterial food webs, supporting important trophic levels which include coastal fish, shell fisheries and coastal avifauna (Elliott et al., 1998; Perkins et al., 2010).

1.1.1. The intertidal grazing food chain

Trophic dynamics and food webs of estuarine intertidal ecosystems tend to be more complex than in other environments, partly as a consequence of the multiplicity of organic sources and of primary producers (Carlier et al., 2007). Grazing food chains are common in benthic intertidal environments, representing and regulating the flow of nutrients and energy from the bottom throughout the estuarine ecosystem and to adjacent marine and freshwater environments (Carlier et al., 2007). In estuarine intertidal mudflats the primary production is predominantly supported by benthic photoautotrophs, mainly microphytobenthos (MPB) and macrophytes (Underwood and Kromkamp, 1999). In estuaries with large intertidal areas MPB can contribute considerably (up to 50%) to total primary production and to the nutrient fluxes (Kromkamp et al., 1995; MacIntyre et al., 1996; Underwood and Kromkamp, 1999; Middleburg et al., 2000). These benthic photosynthetic organisms supply organic matter to the macrobenthic community of intertidal mudflats (Elliott et al., 1998), representing an important primary food source for higher trophic levels (herbivores or carnivores). Intertidal mudflats are mainly colonised by small molluscs, crustaceans

and polychaetes. This macrofauna community is relatively poor in diversity but have high abundances, offering dense prey populations that support estuarine predators, such as fish and birds (Piersma et al., 1993; Elliott et al., 1998; Aarnio and Mattila, 2000).

The structure of an ecosystem can be largely derived from the top-down influence, consequence of the effects of the activity of organisms in the uppermost trophic levels. The increment of grazing or predation activity in middle and upper trophic levels must conduct to a release of production on the first level (Asmus and Asmus, 1985; Morrisey, 1988; Daborn et al., 1993; Pillay et al., 2009). In this context, trophic links, which are any trophic or feeding relationship between two species in a food web (Cohen and Briand, 1984), represent an important component to understand the trophic dynamics in intertidal environments.

1.2. Microphytobenthos *versus Hydrobia*: an example of trophic coupling in the estuarine environment

Macrofauna, namely estuarine invertebrates, are known to feed on microphytobenthic biofilms (Hawkins and Hartnoll, 1983; Currin et al., 1995; Maddi et al., 2006). The trophic interaction MPB - *Hydrobia* is an important model of the grazing food chain in benthic environment, acting as a significantly channel of nutrients and energy to higher trophic levels of the complex estuarine food web, since these gastropods are common prey items for secondary consumers (Piersma et al., 1993; Aarnio and Mattila, 2000) in estuaries. Although the relationship between MPB and *Hydrobia* has been addressed in a number of studies (Blanchard et al., 2000; Hagerthey et al., 2002; Orvain et al., 2004; Haubois et al., 2005), the proportion of benthic microalgae production that is consumed by *Hydrobia,* or other estuarine grazers, is mostly unknown.

1.2.1. Microphytobenthos

MPB are a benthic photosynthetic community, including diatoms (Bacillariophyceae), euglenophytes (Euglenophyceae) and other microalgae (e.g. Chlorophyceae), as well as cyanobacteria and photosynthetic bacteria (Admiraal, 1984; MacIntyre et al., 1996;

Paterson and Hagerthey, 2001; Janousek et al., 2007). This compact association of organisms embedded in a mucus matrix of extracellular polymeric substances (EPS) colonize a wide range of habitats, including salt marshes, subtidal sediments or intertidal sand and mudflats. The biofilms developed in intertidal mudflats are often dominated by epipelic diatoms, i.e. diatom cells that are able to move trough the interstitial space (Admiraal, 1984; Brotas and Plante-Cuny, 1998; Consalvey et al., 2004).

MPB play a key role in the ecosystem function. Many studies have described the significant contribution of benthic microalgae to the primary productivity of estuarine intertidal mudflats and shallow water ecosystems (Pomeroy, 1959; Miller et al., 1996; Cahoon, 1999; Underwood and Kromkamp, 1999). Moreover, the photosynthetic activity of these active communities affects significantly the dynamics of nutrients and oxygen in the water-sediment interface (Miller et al., 1996; Bartoli et al., 2003; Tyler et al., 2003). The microphytobenthic biofilm has also an important function actively transforming the sediment properties and increasing the stabilization of the sediment by the secretion of EPS (Paterson, 1989; Yallop et al., 1994; de Brouwer et al., 2000). Both, microalgae and EPS, represent an important food source for grazers and pelagic communities (MacIntyre et al., 1996; Buffan-Dubau and Carman, 2000; Herman et al., 2000).

1.2.1.1. Vertical migration of benthic diatoms

Most organisms of estuarine ecosystems display rhythmic behaviours that are closely synchronized with tidal cycles, being vertical migration a well-documented phenomenon both in the sediment and in the water column (Palmer and Round, 1967; Palmer, 1974; Hough and Naylor, 1992; McGaw and Naylor, 1992; Laprise and Dodson, 1994). The benthic microalgae community exhibit this striking feature of vertical migration in the sediment, which consists in the movement to and from the surface of the sediment, closely synchronized with the beginning and end of the daylight period of low tide (Round and Palmer, 1966; Palmer and Round, 1967; Paterson, 1986; Serôdio et al., 1997; Consalvey et al., 2004). The vertical migratory behaviour of benthic microalgae is often cited as one of the main reasons for the success of benthic microalgae on the sedimentary environment. Vertical migration has been interpreted

as a mechanism to avoid grazing pressure (Buffan-Dubau and Carman, 2000) or resuspension into the water column (Kingston, 1999a), but also as a behavioural photo-protection to avoid exposure to potentially damaging irradiance levels (Perkins et al., 2001; Underwood, 2002; Serôdio et al., 2006).

The migratory movements are responsible for large and rapid fluctuations in the microphytobenthic biomass available in the upper layers of the intertidal sediment and represent the main factor in determining the short-term variability in microalgal photosynthetic rates (Pinckney and Zingmark, 1991; Serôdio et al., 2001). Consequently, the variability in the amount of microphytobenthic biomass available near the surface might have a significant impact in the macrofauna trophic dynamics, as grazer feed on the biofilm.

1.2.1.2. Environmental control of microphytobenthic biomass

The partially endogenous nature of the vertical migratory behaviour of MPB has been demonstrated based on the evidence that rhythmic movements can be maintained under constant conditions and in the absence of external stimulus (Palmer and Round, 1967; Serôdio et al., 1997). However, the role of environmental factors on microalgal biomass has been progressively more investigated, recognizing their ecological importance as a major controlling factor of variability in primary productivity (Barranguet et al., 1998; Serôdio et al., 2001; Saburova and Polikarpov, 2003; Consalvey et al., 2004; McLachlan et al., 2009). MPB surface biomass, and consequently production, is known to be affected by temperature (Cohn et al., 2003), physical disturbance of sediments (Hopkins, 1966) as well as wave action (Kingston, 1999a), water cover (Pinckney et al., 1994; Mitbavkar and Anil, 2004) or sub-surface nutrients (Kingston, 2002). The control of MPB production by irradiance, light intensity and quality (Paterson, 1986; Kingston, 1999b; Cohn et al., 2004; McLachlan et al., 2009), is particularly interesting considering the variability of this factor and especially because it affects the normal function of the photosynthetic system (Serôdio et al., 2005; Serôdio et al., 2006). Important limiting effects on the production and biomass of intertidal MPB might also be caused by desiccation, as well as by high temperatures or wind activity, as a consequence of the periodic exposure to air during periods of low tide (Holmes and Mahall, 1982; Lamontagne et al., 1989; Coelho et al., 2009).

1.2.2. *Hydrobia*

Hydrobiidae mudsnails are common and dominant inhabitants of European estuaries (Fish and Fish, 1974; Newell, 1979; Barnes, 1999), forming populations that can reach several thousands of individuals per square meter in intertidal areas (Sola, 1996; Barnes, 1999; Lillebø et al., 1999). The mudsnails are benthic deposit-feeders, feeding on different sources of organic matter at surface sediments (Lillebø et al., 1999; Blanchard et al., 2000; Cardoso et al., 2002). Benthic microalgae have long been recognized as the major food source of these gastropods (Levinton and Bianchi 1981, Morrissey 1988, Barnes 2001).

Aspects of feeding, behaviour and locomotion activities of Hydrobiidae have been investigated mostly in *Hydrobia ulvae* (Pennant). Several studies have been carried out research on the behavioural mechanisms, feeding, locomotion, population dynamics and dispersal even thought the main control of these processes remains partially unknown (Sola, 1996; Lillebø et al., 1999; Cardoso et al., 2002; Orvain and Sauriau, 2002; Barnes, 2003; Meireles and Queiroga, 2004; Vieira et al., 2010). The periodic behaviour of *Hydrobia ulvae* is controlled by direct exposure to physical and biological conditions closely related with day and tidal cycles. Moreover, several studies were carried out regarding the effect of sediment particle size (Forbes and Lopez, 1989), cell size and algal biomass (Forbes and Lopez, 1986; Haubois et al., 2005), food concentration (Cammen, 1989; Blanchard et al., 2000) as well as mudsnail density (Blanchard et al. 2000) on the ingestion rate of the deposit-feeder *Hydrobia ulvae*. The influence of environmental factors on bacterial ingestion rate and activity of mudsnails was also studied (Orvain and Sauriau, 2002; Pascal et al., 2008).

1.3. Trophic markers: photosynthetic pigments and fatty acids

First investigations on trophic interactions between primary producers and consumers were based on gut content analysis, direct counting of faecal pellets or in the measurements of ingestion rate or faecal production (Levinton, 1979; Whitlatch and Obrebski, 1980; Peduzzi, 1987; Forbes and Lopez, 1989). More recently, *in situ* stable isotope labelling experiments have been widely used as an important tool to study element transfer on trophic dynamics (Middleburg et al., 2000; Aberle et al., 2009;

Riera, 2010). However, natural organic markers, such as photosynthetic pigments and fatty acids have also proven to be good trophic markers on the study of interactions between producers and consumers in marine and estuarine environments (Welschmeyer et al., 1991; Desvilettes et al., 1994; Cotonnec et al., 2001; Cartaxana et al., 2003).

1.3.1. Photosynthetic pigments

Photosynthetic pigments are commonly distributed among primary producers and its use as markers has been applied to describe consumers' diets and investigate trophic interactions along the grazing food chains by several researchers (Abele-Oeschgerl et al., 1992; Kleppel, 1998; Pandolfini et al., 2000). Photosynthetic pigments, such as chlorophyll *a* or carotenoids, have been used as biomarkers (Williams and Claustre, 1991; Buffan-Dubau et al., 1996; Pandolfini et al., 2000), allowing to investigate feeding selectivity and food quality. In grazing systems, the use of photosynthetic pigments as trophic markers presume a conservation of these pigments during their passage trough the grazer gut or the knowledge of degradation rates (Pasternak and Drits, 1988; Penry and Frost, 1991; Perissinotto and Pakhomov, 1996), as degradation of pigments occurs during the ingestion and digestion of food sources (Klein et al., 1986; Lopez et al., 1988). Consequently, investigations have been carried out to evaluate the use of degradation products, like pigments, as useful markers for grazing activity (Bianchi et al., 1988; Bianchi et al., 1991; Strom, 1993; Cartaxana et al., 2003; Collos et al., 2005). The impact of grazing benthic macrofauna, such as mud snails, on intertidal MPB has been validated using pheopigments (pheophorbide and pheophytin) like natural organic markers (Cartaxana et al., 2003).

1.3.2. Fatty acids

The use of fatty acids as trophic markers in marine and estuarine environments are well established (Desvilettes et al., 1994; Kharlamenko et al., 2001; Dalsgaard et al., 2003; Hall et al., 2006; Kharlamenko et al., 2008; Iverson, 2009). Fatty acids have been used as qualitative markers to confirm and trace trophic relationships (Auel et al., 2002; Phillips et al., 2003; Shin et al., 2008), as they provide information on the dietary intake over a long period of time. In general, marine primary producers have specific

fatty acids and these are transferred into the storage lipids of higher trophic organism with unchanged or recognizable form that leads to a fatty acid pattern in the consumer that reflects the composition of its diet (Desvilettes et al., 1997; Dalsgaard et al., 2003; Shin et al., 2008). The identification of characteristic fatty acids patterns at different trophic levels allow to trace the relationship among primary producers, primary consumers and/or higher trophic organisms (Kharlamenko et al., 1995; Kharlamenko et al., 2001).

Primary producers, such as benthic microalgae, supports the basic fatty acid pattern in estuarine food chains and are the major source of polyunsaturated fatty acids (PUFAs) such as 18:2*n*-6, 18:3*n*-3 and the derivates 20:5*n*-3 and 22:6*n*-3 (Bergé and Barnathan, 2005; Rezanka and Sigler, 2009). Diatoms, the dominant group on benthic microalgae assemblages in estuarine areas, are known to be rich in 16:0, 16:1*n*-7 and 20:5*n*-3, while other microalgae groups produce in general higher amounts of 18:3*n*-3, 18:4*n*-3 and 22:6*n*-3 for example (Behrens and Kyle, 1996; Volkman et al., 1998). Frequently, ratios of these specific fatty acids have been used as dietary indicators, such as the EPA/DHA ratio (20:5*n*-3 *versus* 22:6*n*-3) or the ratio of 16:1*n*7 *versus* 16:0 (Kharlamenko et al., 2001; Auel et al., 2002; Graeve et al., 2005).

1.4. General objectives and thesis outline

The ecological relevance of estuarine areas has long been studied, as they are among the most productive ecosystems in the world. Estuarine intertidal areas, such as mudflats, may represent a large proportion of the total ecosystem area, being a significant proportion of the total primary production due to the MPB. Benthic microalgae are at the base of the grazing food chain that supports consumers and the interaction MPB - *Hydrobia* has a key role on these intertidal trophic dynamics, contributing to the beginning of carbon dissemination trough the ecosystem. Considering the significance of this link to the estuaries and adjacent environments, several objectives were addressed:

- to investigate the environmental and endogenous control, of the vertical migration by MPB;

- to explore the role of desiccation on the photosynthetic activity of MPB, establishing a new non-destructive methodology to estimate water content on the surface layers of intertidal sediments;
- to determine the relationship between ingested chlorophyll *a* and egested pheophorbide *a*, in order to establish a new non-invasive approach to measure ingestion rate of *Hydrobia ulvae*;
- to investigate the trophic interaction between MPB and *Hydrobia ulvae* under laboratory (seasonal, tidal and day-night cycles) and *in situ* (density and lunar phase) conditions, using photosynthetic pigments;
- to indicate the habitat occupied by mud snails using fatty acid markers.

The vertical migratory behaviour displayed by benthic microalgae inhabiting intertidal sediments is one of the main factors for their success. **Chapter 2** compares the role of environmental cues and endogenous control in establishing the patterns of vertical migration. A clear two-step pattern of biofilm formation was observed, starting with the accumulation of cells at the surface driven by an endogenous control. The full formation and regulation of the surface biomass was strongly depended on the presence of light as well as dependent on other exogenous factors. The absence of light inhibits the upward migration and promotes the disaggregation of the incipient biofilm. The disaggregation of migroalgal biofilm was also found to be endogenously controlled, as a spontaneous downward migration occurred shortly before the end of the low tide.

The short-term effects of desiccation (as exogenous factor) on the photosynthetic activity of intertidal MPB biofilms was studied in **Chapter 3.** A new methodological approach to estimate the water content of muddy intertidal sediments was proposed based on the non-destructive measurement of the specular reflectance in the visible spectral region, which was found to be linearly related to the water content of the uppermost 200 µm of the sediment. The new approach has considerable advantages over traditional methods, by allowing non-destructive, rapid and simultaneously monitoring of water content and other relevant parameters (e.g. biomass or photosynthetic efficiency). The process of water loss by surface sediments was shown to induce significant decreases in the photosynthetic activity and in surface microalgal

biomass. These have potentially important repercussions for the modelling of primary productivity of estuarine intertidal areas.

Considering the importance of the interaction MPB - *Hydrobia* to the trophic dynamics of estuarine grazing food chain, **Chapter 4** establishes the use of pheophorbide *a* from *H. ulvae* faecal pellets as trophic markers to estimate the amount of primary production incorporated, as chlorophyll *a*, by benthic macrofauna. The incorporation of benthic microalgae biomass by *H. ulvae* was quantified along seasonal, tidal and day-night cycles. **Chapter 5** investigates the role of macrofauna density on the consumption of MPB, determining the spatio-temporal variability of the relationship between *H. ulvae* density and benthic microalgae ingestion. *H. ulvae* density increased significantly during new moon events, while ingestion rate on microalgae decreases. It was also concluded that populations of mud snails negatively affected MPB biomass (up to 40% of chlorophyll *a* consumed).

Finally, **Chapter 6** explores the relationship between mud snails and different sources of primary productivity. The fatty acid composition of the gastropod *H. ulvae* collected in two different locations in Ria de Aveiro, Portugal, was found and compared with the fatty acid profiles of MPB and *Zostera noltti*. Fatty acid markers allowed understanding of the trophic interaction between the lower trophic levels of grazing food chain, reinforcing the role of MPB on *H. ulvae* diets but also emphasised the importance of seagrass meadows on mud snails habitats.

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Chapter 2

Endogenous *vs.* environmental control of the vertical migration by intertidal benthic microalgae

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2.1. Abstract

The vertical migratory behaviour of microphytobenthos (MPB), the biofilm-forming microalgae inhabiting estuarine intertidal sediments, is likely a significant factor for their success in this extreme and unstable environment. This work aimed to assess the relative role of endogenous and environmental control of benthic microalgae vertical migration. This was done by comparing the microalgal migratory behaviour in undisturbed sediment samples kept under constant (darkness and low light) and natural environmental conditions, by measuring the changes in the surface microalgal biomass during daytime low tide periods. The results showed that the formation of a biofilm consists of a two-step process. It starts with a relatively small accumulation of cells at the surface, starting hours before the beginning of the light period, and driven by an endogenous negative geotactic behaviour. However, the full formation of the biofilm requires the exposure to light by the time expected for the start of the photoperiod, which further promotes upward migration and accelerates the cell accumulation at the surface. In the absence of light, microalgae stop to move towards the surface and the incipient biofilm starts to disaggregate. The relative importance of the endogenously-controlled behaviour varied along the spring-neap tidal cycle, being maximum for days when low tide occurred at the middle of the day, suggesting its entrainment by the duration of light exposure on previous days. The regulation of the surface cell concentration during daytime low tides was found to be strongly dependent on exogenous factors, particularly light intensity. The spontaneous disaggregation of the biofilm shortly before the end of the low tide period (due to tidal flood or sunset), both under constant as well as natural conditions, suggested the presence of an endogenously-controlled positive geotactic behaviour.

Keywords: Benthic microalgae, diatoms, endogenous rhythms, entrainment, environmental cues, intertidal sediments, vertical migration.

2.2. Introduction

The intertidal areas of estuaries are often inhabited by communities of microalgae, the microphytobenthos (MPB), formed mostly by benthic diatoms, but also by species of euglenophytes and cyanobacteria (Admiraal, 1984; MacIntyre et al., 1996; Underwood and Kromkamp, 1999). These photosynthetic communities form dense and highly productive biofilms, detectable at the naked eye at the surface of the sediment during daytime low tide (Consalvey et al., 2004). The microphytobenthic biofilms play an important role in estuarine ecosystem functioning, by contributing to up to 50% of primary production (MacIntyre et al., 1996; Underwood and Kromkamp, 1999), constituting an essential food source for grazers (Herman et al., 2000; Middleburg et al., 2000), regulating the exchange of nutrients across the sediment-water interface (MacIntyre et al., 1996; Tyler et al., 2003) and promoting sediment biostabilization (Paterson, 1986; Yallop et al., 1994; de Brouwer et al., 2000).

Likely the most remarkable feature of sedimentary microalgal biofilms is the rhythmic vertical migratory behaviour displayed by many species of microalgae in the vicinity of the sediment surface, closely synchronized with solar and tidal cycles. This phenomenon is long-known and consists in the upward migration of cells to the sediment surface during diurnal low tides and the downward migration shortly before tidal flood or night (Fauvel and Bohn, 1907; Palmer and Round, 1967; Admiraal, 1984; Consalvey et al., 2004; Du et al., 2010). This rhythmic behaviour has been considered as a key adaptation to the sedimentary intertidal habitat (Serôdio and Catarino, 2000; Mitbavkar and Anil, 2004). Upward migration allows the microalgae to reach the illuminated layers of the sediment where they can carry out photosynthesis, while downward migration may allow to avoid grazers (Buffan-Dubau and Carman, 2000) and resuspension into the water column (Kingston, 1999a), search for carbon or nutrients (Barranguet et al., 1998; Kromkamp et al., 1998; Kingston, 1999a, 2002) or undergo sexual reproduction (Saburova and Polikarpov, 2003). Vertical migration has also been considered as a unique form of behavioural regulation of light exposure and absorption (Admiraal, 1984; Perkins et al., 2002; Underwood, 2002; Serôdio et al., 2005).

The vertical migratory behaviour of benthic microalgae is also of ecological importance, as the large changes in microalgal biomass associated to vertical cell

movements have been shown to represent a main factor in determining the short-term variability in biofilm-level photosynthetic rates (Pinckney and Zingmark, 1991; Guarini et al., 2000; Serôdio et al., 2001). Moreover, migratory rhythms cause not only changes in microalgal biomass but also changes in the photophysiology of the cells in the sediment photic zone, affecting the biofilm-level photosynthetic efficiency (Serôdio et al., 2005).

Several studies have reported the endogenous nature of the vertical migratory behaviour of benthic microalgae, by showing the vertical movements to continue synchronized with solar and tidal cycles for several days in the absence of external stimuli (Palmer and Round, 1967; Palmer, 1974; Serôdio et al., 1997). Nevertheless, the migratory rhythm is known not to be exclusively controlled by an internal clock, as the motility of benthic microalgae has been shown to respond to a range of exogenous cues, like temperature (Cohn et al., 2003), presence of sub-surface nutrients (Kingston, 2002), physical disturbance of the sediment (Hopkins, 1966) or wave action (Kingston, 1999a) and sediment desiccation (Coelho et al., 2009). Of particular importance is the role of light, both in terms of incident intensity (Kingston, 1999b; Serôdio et al., 2006; Kingston and Gough, 2009; McLachlan et al., 2009) as well as of spectral composition (Cohn et al., 2004; McLachlan et al., 2009), that can induce the upward migration and biofilm formation at the sediment surface or, inversely, promote downward movements and the decrease of surface biomass (Mitbavkar and Anil, 2004; Serôdio et al., 2006; McLachlan et al., 2009).

The purpose of this study was to evaluate the role of endogenous control and exogenous environmental cues in determining the patterns of vertical migration of biofilm-forming microalgae. This was carried out by comparing the migratory behaviour of replicated MPB samples kept under constant conditions (darkness and constant low light) and exposed to natural environmental conditions. The vertical migratory behaviour was studied by monitoring the microalgal biomass present in the photic zone of the sediment over periods coinciding with daytime low tide. Two optical techniques were used to non-destructively estimate microalgal surface biomass: Pulse Amplitude Modulation (PAM) fluorometry (by measuring dark fluorescence level, F_0; Table 2.1 for notation) and Spectral Reflectance Analysis (by calculating the Normalized Difference Vegetation Index, NDVI). We also investigated the entrainment

of the endogenous migratory behaviour, by comparing the hourly patterns of vertical migration under dark, low light and natural conditions along spring-neap tidal cycles. The effects of vertical migration on biofilm-level photophysiology were evaluated by monitoring changes in the maximum quantum efficiency of photosystem II (F_v/F_m) in dark and low light-exposed microphytobenthic assemblages.

2.3. Material and Methods

2.3.1. Sample collection and preparation

Samples of the surface of the sediment were collected during low tide on a muddy tidal flat at Vista Alegre (40° 35' N, 8° 41' W), Ria de Aveiro, Portugal. The sampled sediments were composed by fine grains (97% particles < 63 µm) and were dominated by epipelic diatoms (Serôdio et al., 2008). Samples were collected on different days evenly distributed along two spring-neap tidal cycles (five days per cycle), in October 2006 and March 2008. The upper 1 cm of sediment was collected using a spatula and was thoroughly mixed before being deposited on plastic trays (20 × 40 cm) and on plexiglas circular plates (10 cm internal diameter), so that a slurry of ca. 5 cm deep was obtained. In the laboratory, the sediment samples were left overnight immersed in filtered seawater and in the dark. In the next day, the overlying water was carefully removed so that all measurements were carried out in air-exposed samples. Trays (three replicates) were placed outside under natural conditions and the plates (three replicates for each treatment) were kept in the laboratory under darkness or low light (150 µmol m^{-2} s^{-1}). Outside, measurements on trays were started at the time coinciding with sunrise or the expected time of beginning of the low tide in the sampling site. In the laboratory (darkness and low light treatments), measurements were started ca. 90 min before. In all cases measurements continued throughout the period matching the daytime low tide.

2.3.2. Chlorophyll fluorescence

Variable chlorophyll (Chl) fluorescence was measured using a fluorometer composed by a computer-operated PAM-Control Unit (Walz, Effeltrich, Germany) and a WATER-EDF-Universal emitter-detector unit (Gademann Instruments GmbH, Würzburg, Germany) (Serôdio et al., 2006). A blue LED-lamp (peaking at 450 nm, half-bandwidth of 20 nm) provided measuring, actinic and saturating light, conducted by a 6-mm diameter Fluid Light Guide fiberoptics. The fiberoptics was set perpendicularly to the sediment surface, at a fixed distance of 1 mm, controlled by a micromanipulator (MM33, Märtzhäuher, Germany). Saturating pulses of 0.6 s were used to measure the minimum (F_0) and maximum (F_v/F_m) fluorescence levels (Table 2.1), and subsequent determination of F_v/F_m, the maximum quantum yield of photosystem II (PSII). F_0 was used as a proxy for the microalgal biomass in the photic zone of the sediment (*B*) (Serôdio et al., 2001) of samples kept in darkness and under low light. Samples exposed to low light were dark-adapted for 2 min before the measurement of fluorescence. Fluorescence was measured every 20 min during the daytime periods corresponding to low tide in the sampling site. On each occasion, measurements were made on three separate, non-overlapping areas of sediment.

The relationship between F_0 and *B* (μg Chl *a* g^{-1}) was determined using vertically-homogenized sediment samples of increasing Chl *a* content as described by Serôdio *et al.* (2006). The finding of a significant linear regression between the two variables (n = 40, r = 0.969; $P < 0.001$; after pooling data from the two sampling periods) allowed subsequent conversion of arbitrary F_0 signals into microalgal biomass values.

2.3.3. Spectral reflectance

The microalgal surface biomass of the samples kept under natural conditions was estimated using spectral reflectance analysis. Reflectance spectra were recorded using a USB2000 spectrometer (USB2000-VIS-NIR, grating #3, Ocean Optics, Duiven, The Netherlands), connected to a 400 μm diameter fiber optic (QP400-2-VIS/NIR-BX, Ocean Optics), covering a 350-1000 nm bandwidth with a spectral resolution of 0.38 nm.

Table 2.1 Notation used in the text.

Parameter	Explanation
a, b, c, d, e	Parameters of model (Equation 2.3) used to describe the fortnightly variation of ΔB
B	Surface microalgal biomass, given as the Chl *a* content present the photic zone of the sediment (µg Chl *a* g^{-1})
B_{max}, B_{min}	Maximum and minimum surface biomass recorded during a daytime low tide and during the previous night or high tide, respectively (µg Chl *a* g^{-1})
Chl	Chlorophyll
ΔB	Migration amplitude, maximum change in surface biomass
$\Delta\widehat{\boldsymbol{B}}$	Predicted migration amplitude, maximum change in surface biomass
E	PAR irradiance (µmol m^{-2} s^{-1})
F_0, F_m	Minimum and maximum fluorescence levels
F_v/F_m	Maximum quantum yield of PSII
NDVI	Normalized Difference Vegetation Index
PSII	Photosystem II
R_λ	Spectral reflectance at wavelength λ
SUN(t)	Solar angle at time t
t	Time of day (h)
$t_{\Delta B}$	Time of day corresponding to ΔB (h)
t_{Bmax}	Time of day corresponding to surface biomass maximum (h)
t_{eb}, t_{fl}	Time of ebb and flood at the sampling site (h)
t_{sr}, t_{ss}	Time of sunrise and sunset (h)
t_i, t_f	Time of beginning and end of daytime low tide exposure at the sampling site (h)
t_{lt}	Time of low tide at the sampling site (h)
TIDE(t)	Tidal angle at time t

A reference and dark spectra were recorded immediately before each measurement of the reflectance spectrum and spectra measurements were carried out under variable natural light conditions. Reference spectra were obtained by measuring the spectrum reflected by a surface of reflectance standard (WS-1-SL Spectralon Reference Standard, Ocean Optics) placed under identical sunlight exposure conditions. A dark reflectance spectrum was subtracted to both sample and reference spectra to account for the dark current noise of the spectrometer.

The incident solar irradiance at the time of each measurement was recorded using a PAR sensor (LI-193SA and LI-250 light meter, Li-Cor, Lincoln, Nebraska, USA). Three replicated spectra were measured every 20 min, on separate, non-overlapping areas, and the mean spectrum was smoothed using a 10-point moving average filter before used for the subsequent calculations. The fiberoptics was positioned perpendicularly to the sample surface at a distance such that a circular area of ca. 10 cm in diameter was monitored. The surface microalgal biomass was estimated using the normalized vegetation index NDVI (Rouse et al., 1973), calculated by:

$$NDVI = \frac{R_{750} - R_{675}}{R_{750} + R_{675}} \qquad \text{Equation 2.1}$$

where R_{750} and R_{675} represent the average reflectance in the intervals 749.73–750.39 nm and 674.87–675.55 nm, respectively.

2.3.4. Migration amplitude

To compare the migratory behaviour between different samples, dates or endogenous *vs.* environmental control, the pattern of vertical migration during each low tide period was characterized by calculating the maximum relative change in surface biomass, or migration amplitude, given by:

$$\Delta B = \frac{B_{max} - B_{min}}{B_{min}} \qquad \text{Equation 2.2}$$

where B_{max} is the biomass maximum recorded during the daytime low tide and B_{min} is the minimum recorded during the previous nocturnal immersion periods. Under

constant conditions, ΔB was used as an indicator of the degree of entrainment of the endogenous migratory rhythm.

2.4. Results

2.4.1. Endogenous control: darkness

Samples kept in the dark displayed a pattern of hourly variation of surface biomass that was consistently repeated throughout the whole spring-neap tidal cycles (Figure 2.1). It was characterized by an asymmetrical peak resulting from a rapid increase in microalgae concentration at the sediment surface, starting at least 2-3 h before, followed by usually slower decrease leading to virtually constant levels during the rest of the subjective photoperiod. No changes in microalgal surface biomass were observed during subjective nocturnal low tides. The migration amplitude varied along the spring-neap tidal cycle, with maximum values being observed for days when low tide occurred at the middle of the day (Figure 2.1 A), and minimum values when light exposure took place during early morning or late afternoon (Figure 2.1 C, D). Despite this fortnightly variation in migration amplitude, the maximum surface biomass was in all cases reached at times ($t_{B\max}$) close to those of the start of light exposure at the sampling site (t_i) and a significant correlation was found between $t_{B\max}$ and t_i (Figure 2.2; $r = 0.966$; $P < 0.001$).

2.4.2. Endogenous control: low light

The exposure of samples to low light at time t_i caused a marked increase in the rate of microalgae accumulation at the sediment surface, causing a marked difference between the pattern of biomass hourly variation of dark- and low light-exposed samples (Figure 2.1). The accentuated increase in surface biomass upon the start of light exposure was repeatedly observed along the spring-neap tidal cycle, and was followed by a prolonged phase (> 4 h) around the middle of the subjective low tide when microalgal biomass reached maximum values and remained approximately constant.

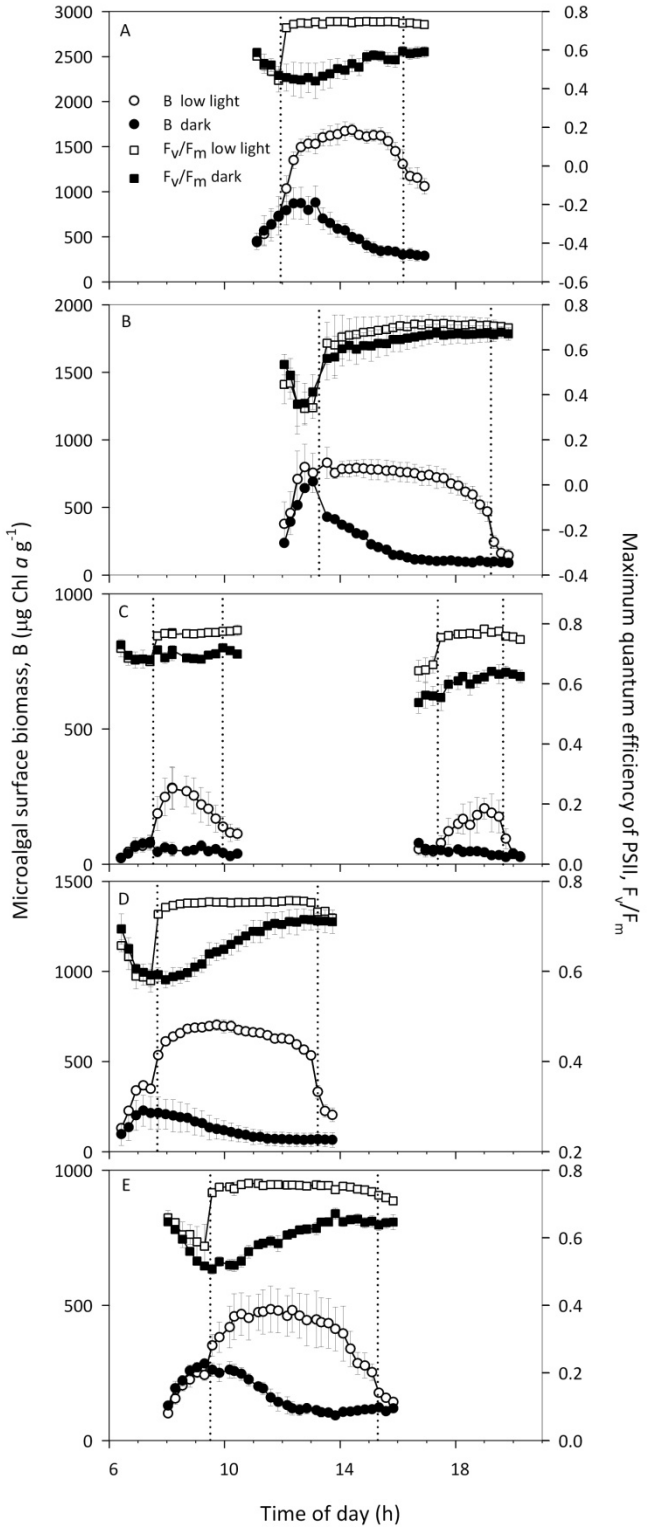

Figure 2. 1 Hourly variation of surface microalgal biomass, B (µg Chl *a* g^{-1}), and maximum quantum efficiency of PSII, F_v/F_m, in sediment samples kept in darkness and exposed to low light (150 µmol m^{-2} s^{-1}), during five days along one spring-neap tidal cycle. Vertical dotted lines define the exposure to low light. Mean values of three replicates. Error bars: 1 standard error.

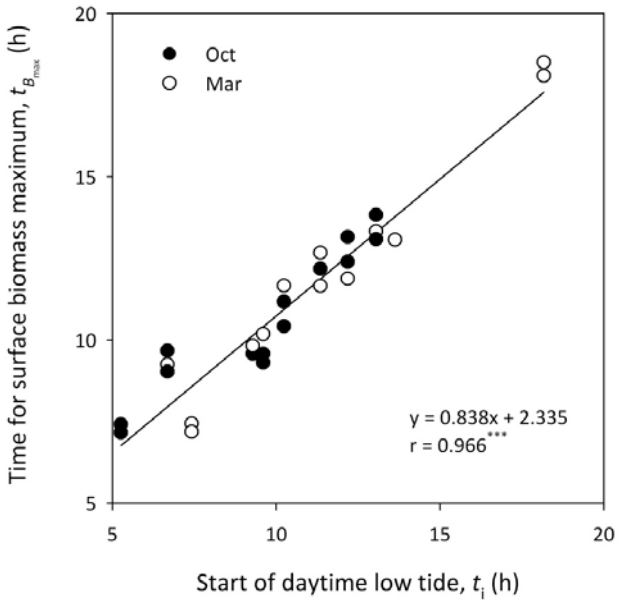

Figure 2. 2 Linear relationship between time for maximum in surface biomass, t_{Bmax}, in samples kept in the dark (hours) and t_i, the expected time for the beginning of the daytime emersion period in the sampling site (hours).

The exception were days when the period of light exposure was very short, due to low tide taking place in early morning or late afternoon (Figure 2.1 C). The rate of decrease of surface biomass was roughly symmetrical to the one of increase. As shown in Figures 2.1 A, E, a slow decrease in biomass started approximately 1-2 h before the end of subjective low tide light period. As with the samples kept in the dark, the migration amplitude under constant low light varied along the spring-neap tidal cycle, with maximum values being recorded for days when the light period occurred at the central part of the photoperiod (Figure 2.1 A).

2.4.3. Endogenous control: effects on photosynthetic efficiency

In the samples kept in the dark, F_v/F_m showed a marked variation around t_i, displaying a pattern of variation almost inversely symmetrical to the one displayed by biomass (Figure 2.1). The fast increase in surface biomass was generally followed by a steep decrease in F_v/F_m from values ca. 0.75 to 0.4-0.6, reached at times closely matching the maxima in biomass (e.g. Figure 2.1 A, D). The subsequent slow decrease in biomass

was paralleled by an identically slow return of F_v/F_m to initial levels. This inverse relationship between *B* and F_v/F_m was observed throughout the fortnightly cycle.

For the samples kept under constant low light, the pattern of variation was characterized by a fast increase of F_v/F_m upon the transition from dark to light, causing the reaching of maximum values shortly following the start of the illumination period (e.g. Figure 2.1 A, E). This increase was in all cases much faster than the one observed for surface biomass during the same periods. Whilst in the dark-adapted samples F_v/F_m took several hours to recover to initial values, in low light-exposed samples F_v/F_m remained virtually constant throughout the whole photoperiod, showing only some slight decrease when biomass started to decline (e.g. Figure 2.1 B,E).

2.4.4. Environmental control

Figure 2.3 shows the hourly variation of surface microalgal biomass and incident irradiance (PAR, µmol m^2 s^{-1}) on days distributed along the two studied spring-neap tidal cycles (October 2006: Figure 2.3 A-E and March 2008: Figure 2.3 F-J). The highest PAR levels were recorded during March, with ca.1500 µmol m^2 s^{-1} being registered in 4 of the 5 studied days. The samples exposed to natural environmental conditions exhibited in most cases a much more variable and irregular pattern of variation in surface microalgal biomass than those displayed by samples kept under darkness or low light (Figure 2.3). In most cases, the surface biomass reached values intermediate between dark- and low light-treated samples (e.g. Figure 2.3 A, H). Exceptions were related to incident PAR, with biomass values remaining below those of samples kept in the dark when solar irradiance reached very high levels (Figure 2.3 F), or reaching values higher than those of samples kept under low light, when PAR levels were low (Figure 2.3 B).

2.4.5. Variation over the spring-neap tidal cycle: entrainment of the migratory rhythm

Samples exposed to constant conditions (dark or low light) showed a well-defined fortnightly variation in migration amplitude, characterized by an increase as the time

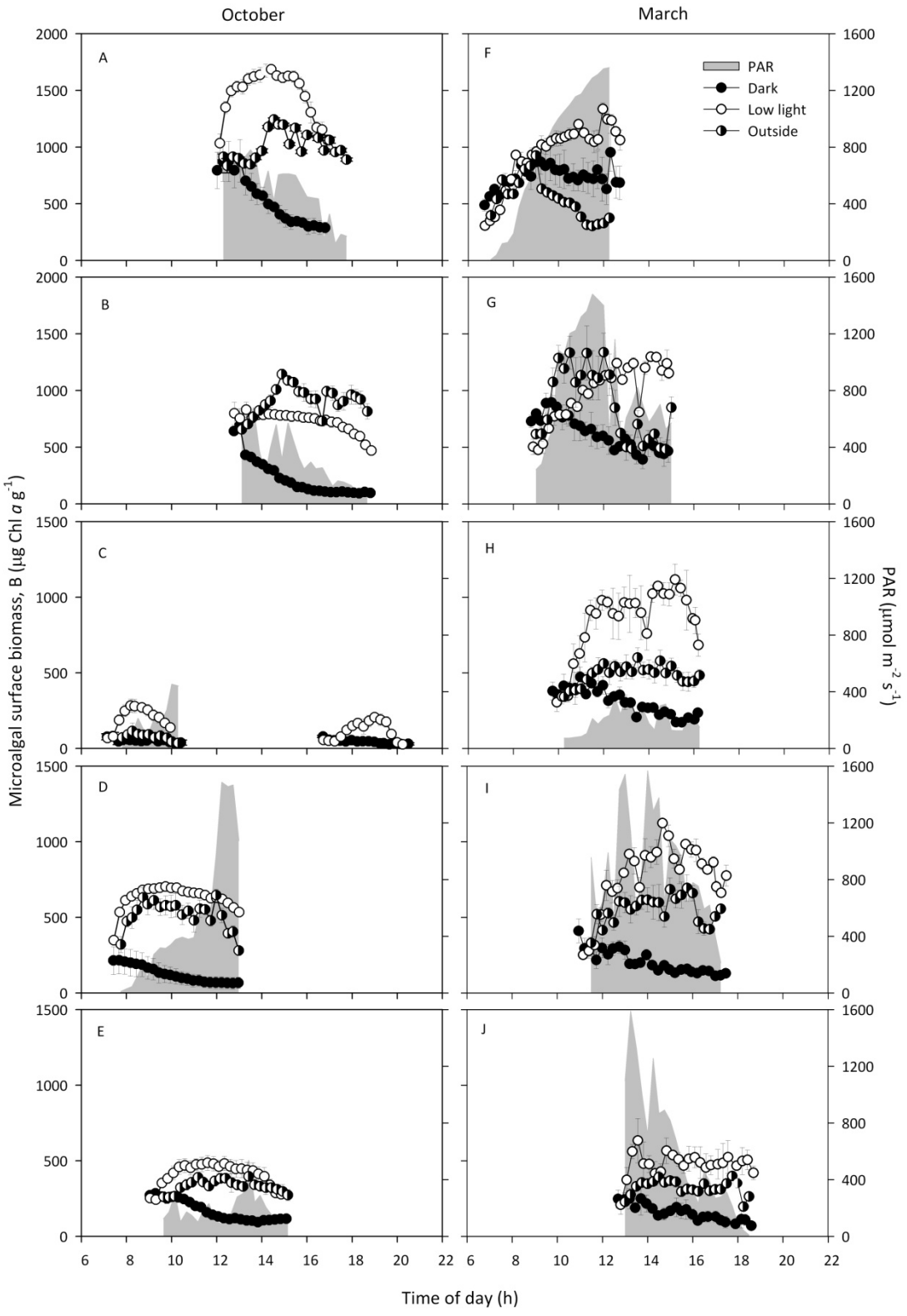

Figure 2. 3 Hourly variation of hourly surface biomass, *B* (µg Chl *a* g^{-1}) and PAR (µmol m^2 s^{-1}) for the sediment samples kept under natural conditions, during five days along two spring-neap tidal cycles (October 2006 and March 2008). Mean values of three replicates. Error bars: 1 standard error.

of low tide, t_{lt}, varied from sunrise to midday, followed by a gradual decrease as t_{lt} occurred latter in the day (Figure 2.4 A, B).

The maximum values of *ΔB* were registered around noon and the lowest values were observed during early-morning and late-afternoon low tides, on days with two daytime emersion periods. This cyclic variation of *ΔB* along the spring-neap tidal cycle could be described by a mathematical model based on basic periodic functions relating *ΔB* to the time of day of its occurrence. The model is a modification of a model previously used to describe the hourly variation of MPB surface biomass and productivity (Pinckney and Zingmark, 1991; Serôdio and Catarino, 2000):

$$\Delta\hat{B}\ (t) = \ a\ \sin[b\ SUN\ (t) + \ c] + \ d\ \cos[e\ TIDE\ (t) + \ f] \qquad \text{Equation 2.3}$$

where SUN and TIDE are sun and tidal angles, used to normalize the time of day *t* relatively to the times of start and end of daytime (times of sunrise and sunset, t_{sr} and t_{ss}) and low tide (times of ebb and flood, t_{eb} and t_{fl}) (*a, b, c, d, e* and *f* being arbitrary constants), respectively:

$$SUN\ (t) = \left[\frac{t - t_{sr}}{t_{ss} - t_{sr}}\right]\pi \qquad \text{Equation 2.4}$$

and

$$TIDE\ (t) = \left[\frac{t - t_{eb}}{t_{fl} - t_{eb}}\right]2\pi \qquad \text{Equation 2.5}$$

The model was fitted by applying a non-linear iterative fitting routine (Statistica 8.0, Statsoft, Inc., Tulsa, USA), and a significant correlation was found between model predictions and observations for samples kept in the dark and in constant low light (r = 0.859, $P < 0.001$ and r = 0.830, $P < 0.001$, respectively; Figure 2.4 A,B). In the case of samples incubated under natural conditions, the pattern of variation along the spring-neap tidal cycle was much less regular. Yet, the model could still describe a large proportion of *ΔB* variability (53%), with a significant correlation being found between model predictions and observations (Figure 2.4 C).

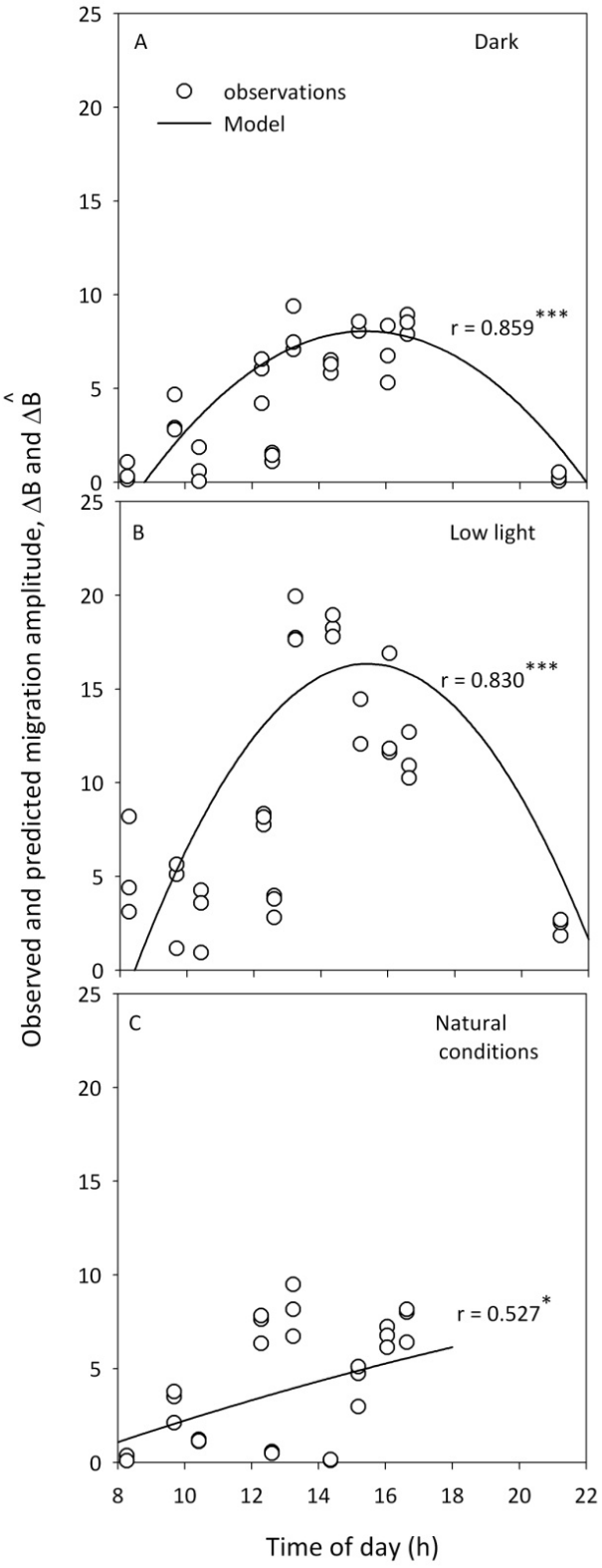

Figure 2. 4 Variation of observed and predicted migration amplitude, ΔB *and* $\Delta\widehat{\boldsymbol{B}}$, with the time of low tide at the sampling site, t_{lt}, for samples kept in the dark (A) under constant low light (B) and natural conditions (C). Significant values: ^{ns} $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

2.5. Discussion

2.5.1. Biofilm formation

Estuarine benthic microalgae inhabit an extreme and highly variable environment, which represents a major challenge for survival and requires the development of particular adaptive strategies (MacIntyre et al., 1996). Amongst the major causes of stress for microalgae living in the intertidal sedimentary environment is the periodic disturbance caused by tidal resuspension and subsequent burial in aphotic layers of the sediment (Round, 1971; Consalvey et al., 2004; Herlory et al., 2004; Chul-Hwan et al., 2006; Mitbavkar and Anil, 2006).

In this context, motility appears as a key selective advantage, enabling the cells to return to the superficial layers of the sediment and carry out photosynthesis, likely being a major factor for the MPB success (Round, 1971; Cohn and Disparti, 1994; Saburova and Polikarpov, 2003; Consalvey et al., 2004).

The results of this study showed that the upward migration at the beginning of each diurnal low tide and the formation of the microalgal biofilm consists of a two-step process, the first being fully endogenously-controlled and the second being driven by environmental factors, especially light. The initial step is an upward migration pulse starting at least 2-3 h before the daytime emersion period, and lasting until t_i, the time expected for the beginning of daytime low tide exposure. Considering the lack of physical (light, temperature) or chemical (after prolonged darkness, the upper layers becomes anoxic) gradients at the sediment-air interface, the accumulation of cells at the surface is likely driven by an endogenous negative geotactic behaviour (Mitbavkar and Anil, 2004). This allows the microalgae to reach the vicinity of the photic zone, where they are expected become exposed to light as sun rises or tide ebbs, and has an obvious adaptive value, allowing to anticipate the periods of favourable conditions for photosynthesis.

However, the results also showed that if no light is made incident upon the sediment surface by t_i, the initial surface accumulation of cells is reversed and the transient biofilm formed by then begins to disaggregate, as evidenced by the biofilms maintained in continuous darkness. The lower rate of decrease of surface biomass observed during this period, when compared with the rate of increase before t_i (as well

as when compared to the downward migration by the end of the low tide light period, see below) suggests that cell movements are not synchronized and not downward-oriented. The simplest explanation is based on the hypothesis that the negative geotaxis is "switched off" around time t_i and the observed decrease in surface biomass results from the randomly-oriented movement of cells in the upper sediment layers.

The second step of the process of biofilm formation is thus a light-driven upward migration towards the sediment surface, which is necessary for its full completion. In the presence of favourable conditions (such as low light) at the surface by time t_i, microalgae continue to accumulate in the superficial layers, reaching maximum concentrations typically within 1-2 h. This second phase seems to result from the switch from the initial negative geotaxis to a strong (although light intensity-dependent) positive phototaxis. This finding apparently contradicts the results of Round and Palmer (1966) who reported that a 2 h period of light exposure was required before the formation of a biofilm. However, this discrepancy may be due to the higher sensitivity of the fluorescence-based biomass measurement for detecting changes in microalgae in the photic zone when compared with traditional methods, such as the lens tissue technique or visual observation (Round and Palmer, 1966).

On the other hand, the results on the hourly variation of biofilm-level F_v/F_m showed that the exposure to light of the cells arriving at the sediment surface induces important changes at the physiological level. The rapid and large increase of F_v/F_m to maximum (and thereafter virtually constant) values is consistent with the well-known low light-induced dissipation of non-photochemical quenching (NPQ) that can be formed in diatoms after prolonged darkness (Arsalane et al., 1994; Olaizola et al., 1994; Muller et al., 2001; Jesus et al., 2006; Serôdio et al., 2006). NPQ induces a decrease in F_v/F_m and its build-up in diatoms in the dark has been attributed to processes leading to the formation of a proton gradient across the thylakoidal membrane, probably chlororespiration (Ting and Owens, 1993; Jakob et al., 1999; Lavaud et al., 2002). The decrease in F_v/F_m that was observed as microalgae accumulated at the surface in the dark can thus be the result of the operation of chlororespiration as cells become in contact with atmospheric oxygen, or the manifestation of an endogenous physiological rhythm affecting the transthylakoidal proton gradient (Serôdio et al., 2005). However, considering the strong parallelism

between the increase of surface biomass and the simultaneous decrease of F_v/F_m, as well as the long periods required for the build-up of significant NPQ in the dark (Jakob et al., 1999), it seems more likely that the observed sharp drop in biofilm-level F_v/F_m is caused by the accumulation in the photic zone of diatoms having a large NPQ formed during their permanence in the dark layers of the sediment in the previous night or high tide. Being this the case, the return of biofilm F_v/F_m to initial values as surface biomass decreases, would imply that it is the same cell population that undergoes the upward and downward migration. It may hypothesized that the change in the transthylakoidal proton gradient as the microalgae cells reach the illuminated layers of the sediment may play a role in the detection of light gradients and the reinforcement of their phototactic movement.

2.5.2. Biofilm regulation

Once near the surface, microalgae become directly exposed to the highly variable conditions characteristic of the intertidal habitat during low tide, which often result in high levels of solar irradiance, extreme high or low temperatures and salinities, or desiccation. The results showed that these exogenous stimuli strongly affect the patterns of microalgae vertical migration, overriding the endogenous behaviour observed under constant conditions. This flexibility in the migratory behaviour has a clear adaptive value in the context of the instability of the intertidal sedimentary environment, allowing the microalgae to quickly respond to adverse conditions by migrating downwards and seeking refuge in subsurface layers of sediment.

Although the effects of the different environmental factors could not be ascertain individually, most of the differences between the migratory patterns recorded on samples exposed to natural conditions and to constant conditions could be related to ambient light intensity. Under low ambient light, as on early morning and late afternoon, or during cloudy days, benthic microalgae accumulated at the surface, and biofilm biomass reached values comparable to those measured under constant low light in the laboratory, remaining constant over the duration of these favourable periods. On the contrary, during periods of high solar irradiance, surface biomass rapidly decreased to levels often below those measured in the laboratory, denoting a

strong negative phototactic behaviour. However, on other occasions biomass levels remained relatively high, which might be associated with the process of micro-migration, through which the cells that migrate downwards to avoid local unfavourable conditions are replaced by others coming from subsurface layers (Kromkamp et al., 1998). The clear migratory response to changes in ambient light observed in this study, characterised by an increment of surface biomass under low light (ca. 150 µmol m^2 s^{-1}) and a gradual decrease for higher levels, is in agreement with results previously obtained, described by means of biomass light-response curves generated in the laboratory (Kingston, 1999b; Serôdio et al., 2006; McLachlan et al., 2009), and illustrates well the use of motility as a form of regulating light exposure by diatoms (Cohn et al., 2001).

2.5.3. Biofilm disaggregation

The spontaneous disaggregation of the biofilm through the synchronized downward migration of the most part of its cells, anticipating tidal flood or night, is a well-known feature of the migratory behaviour of MPB (Consalvey et al., 2004). The present observation that, under constant low light conditions, surface biomass starts to decrease shortly before the expected end of the subjective low tide period, strongly suggests the presence of an endogenously-controlled positive geotactic behaviour. First, the rate of decrease observed at the end of the daytime low tide was comparable with the initial increase rate in low light samples, suggesting a downward-oriented migration and not a dispersion resulting from randomly-oriented cell movements. Second, the endogenous nature of this behaviour is supported by the consistent observation that it takes place under constant conditions, its timing being clearly determined by the proximity of incoming flood or sunset, and independent of the duration of light exposure period (Paterson, 1986; Serôdio et al., 1997). Finally, downward migration at the end of the photoperiod is likely controlled by positive geotaxis, because the strongest response is induced when the biofilm is exposed to darkness, when no light gradients are present (Consalvey et al., 2004; Mitbavkar and Anil, 2004; Cartaxana and Serôdio, 2008). The alternative hypothesis of being due to negative phototaxis is unlikely, because for diatoms negative phototaxis has always

been documented only regarding high light levels (Serôdio et al., 2006; McLachlan et al., 2009).

Considering the pattern of variation of surface biomass observed during the final phase of the photoperiod on samples exposed to constant low light, it may be hypothesized that: i) some time before the expected time for flood or sunset, cell behaviour begins to be controlled by positive geotaxis, although positive phototaxis is still the dominant process, resulting in a minor decrease in surface biomass; ii) as light declines to very low levels (or is turned off), cells become to respond only to positive geotaxis, resulting in the strong downward migration that disaggregates the biofilm. Water cover is also known to stimulate downward migration (Perkins, 1960; Kingston, 1999a) although *in situ* and laboratory observations typically show that the surface biomass is only residual at the time of tidal flooding (Pinckney et al., 1994; Mitbavkar and Anil, 2004; Serôdio et al., 2006; Du et al., 2010). Thus, the endogenously-controlled downward migration serves to anticipate the start of periods when there are no benefits in staying at the surface (flood and sunset), but the predisposition exhibited to use rapid environmental changes, such as immersion or darkening, as stimuli for downward migration may help to fine adjust the migratory behaviour.

2.5.4. Entrainment of the endogenous migratory rhythm

On the assumption that, under constant conditions the migration amplitude is an indicator of the degree of entrainment of the endogenous migratory behaviour, the successful fitting of model (Equation 2.3) to the ΔB observations strongly indicate that this entrainment is related to the variation of the timing and duration of low tide exposure periods along the spring-neap tidal cycle. In particular, the good fit of model (Equation 2.3) indicates that ΔB varies periodically over the fortnightly tidal cycle, with ΔB initially increasing with the length of light exposure period in the previous days, and starting to decrease as the light period available before sunset or tidal flood is shortened from each day to the next. The magnitude of ΔB reached each day is thus dependent on both the duration of light period until $t_{\Delta B}$ is reached as well as between $t_{\Delta B}$ and flood or sunset.

The increase of migration amplitude with the increased duration of light exposure periods in the previous days indicates that the endogenous component of the migratory behaviour gets stronger as the time of low tide advances along the day. The exposure to increasingly longer light periods as the time of low tide progresses along the spring-neap tidal cycle causes the successive re-enforcement of the endogenous behaviour, leading a larger fraction of the microalgal population undergoing endogenously-controlled vertical movements.

On the other hand, as the sunset begins to occur before the end of the low tide periods, the microalgae are induced to migrate downwards as a response to darkening. This event seems to be memorized so that on the next day downward migration anticipates the approach of night. As a result, the amplitude of the endogenous migration decreases as the light period becomes increasingly truncated by sunset and days, with increasingly shorter light periods in the afternoon succeed.

Although the best fits of the model were obtained for samples kept under constant conditions (dark and low light), a significant fit was also found in the case of samples exposed to natural conditions. This may be seen as indicating that despite the flexible and rapid migratory response to changes in environmental conditions, the endogenous component of the microalgae's behaviour is still important to the point of partially controlling migratory movements *in situ*.

These results show that the vertical migratory rhythmicity displayed by benthic microalgae colonizing intertidal sediments has a strong endogenous component that requires entrainment by solar and tidal cycles, namely by the progression of the timing of low tide throughout the spring-neap tidal cycle. Although maintaining the ability to use vertical migration to respond to the ever changing environmental conditions, the presence of basic endogenous behaviour appears as a key adaptation to the periodicity of the estuarine intertidal environment, enabling the benthic microalgae to anticipate the relevant events for their survival and thus to exploit more effectively the periods of favourable conditions.

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Chapter 3

Effects of desiccation on the photosynthetic activity of intertidal microphytobenthos biofilms as studied by optical methods

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3.1. Abstract

Due to the periodic exposure to air during periods of low tide, desiccation can be expected to cause important limiting effects on the photosynthetic activity of intertidal microphytobenthos (MPB) biofilms. This work addresses the study of the short-term effects of desiccation on MPB using a new, simple methodological approach to non-destructively estimate the water content of muddy intertidal sediments. The method is based on the non-destructive measurement of the specular reflectance in the visible spectral region, shown to be linearly related to the water content of the uppermost 200 µm of the sediment. During air exposure, water loss by the surface sediment layers was shown to induce marked decreases in both the photosynthetic activity, as measured by the maximum quantum yield of photosystem II, F_v/F_m, and the surface microalgal biomass, as estimated from the diffusive reflectance biomass index NDVI. The effects of desiccation were largely dependent on the rate of sediment de-watering. For a same level of desiccation, samples under fast desiccation (exposed to wind of 4.2 m s^{-1}) showed much larger effects on F_v/F_m and NDVI comparatively to samples under slow desiccation (maintained under still air). By showing the rapid and significant effects of desiccation on MPB biofilm functioning, the results of this study have potentially important implications for the modelling of primary productivity of estuarine intertidal areas, as desiccation and factors inducing it may result in previously unaccounted effects on photosynthetic performance and productive biomass.

Keywords: Desiccation, microphytobenthos, productive biomass, photosynthetic activity, specular reflectance.

3.2. Introduction

The intertidal areas of estuaries are characterised by a large variability in environmental conditions caused by the exposure to air and direct sunlight during low tide periods, alternating with submersion throughout high tide. During diurnal low tide, organisms living in intertidal sediments experience potentially stressful conditions that include high light intensities, extreme low or high temperatures, high salinities and wind. These variable and extreme environmental conditions are likely to affect the communities of benthic microalgae, or microphytobenthos (MPB), which form highly dense biofilms on the surface of intertidal sediments (MacIntyre et al., 1996; Underwood and Kromkamp, 1999). During low tide, the prolonged exposure to wind and direct sunlight favours the evaporation in the uppermost layers of sediment, frequently exposing the benthic microalgae to an intense process of de-watering. Desiccation may be expected to cause important limiting effects on photosynthetic activity of MPB, as shown for other photoautotrophs such as macroalgae (Bell, 1995; Matta and Chapman, 1995; Peña et al., 1999; Hunt and Denny, 2008), lichens (Veerman et al., 2007; Heber, 2008) or cyanobacterial mats (Lüttge et al., 1995; Potts, 1999; Ohad et al., 2005). Despite the predictable relevance of this factor for the primary productivity of benthic microalgal communities, and the large number of factors that affect the water content of intertidal sediments, such as topography, tidal regime, sediment type and climate factors varying with location and season, the available knowledge on the effects of desiccation on MPB is very limited (Holmes and Mahall, 1982; Lamontagne et al., 1989). This is especially true if considering that the stressful effects of other factors, such as light (Kingston, 1999; Perkins et al., 2001; Serôdio et al., 2006), temperature (Blanchard et al., 1997; Guarini et al., 1997; Cohn et al., 2003) or salinity (Admiraal, 1984; Rijstenbil, 2003; Roncarati et al., 2008) have been studied in detail. References to the impact of desiccation on MPB productivity have been made in a few descriptive studies on the photosynthetic activity under in situ conditions, when desiccation effects could not be separated from those of other co-varying factors (Lamontagne et al., 1989; Brotas et al., 2003; Serôdio et al., 2008). This lack of studies may be explained by the difficulty in relating biofilm photosynthetic activity to sediment de-watering, which demands for the quantification of the water content of the depth interval below the surface where photosynthesis can be carried

out. The traditionally used method of determining the sediment water content is based on the destructive measurement of the dry weight of sediment sections (MacIntyre and Cullen, 1995; Perkins et al., 2003; Murphy et al., 2004; Jesus et al., 2006). On intertidal muddy sediments, the photic zone is very thin, typically below 250 µm (Serôdio et al., 1997; Kromkamp et al., 1998; Serôdio et al., 2001), which poses considerable difficulties in the quantification of the water content of a matching depth interval using this method. As a result, most published values of sediment water content refer to depth intervals below the surface much larger than the photosynthetically active zone (Perkins et al., 2003; Murphy et al., 2004; Jesus et al., 2006). On the other hand, it is well recognized that intertidal estuarine areas exhibit substantial heterogeneity, both spatially and temporally, and that the characterization of this variability through isolated point samples is usually time-consuming, expensive and frequently unrepresentative (Rainey et al., 2000; Rainey et al., 2003). Considering this, recent studies have addressed the development of optical approaches, based on the measurement of diffusive reflectance, which can provide a non-invasive and non-destructive characterization of the intertidal zone (Rainey et al., 2003; Murphy et al., 2005b; Forster and Jesus, 2006; Murphy et al., 2008).

This work addresses the short-term effects of desiccation on the photosynthetic activity of intertidal MPB biofilms, based on a new, simple methodological approach to non-destructively estimate the water content of the uppermost, photosynthetically active layers of the sediment. This method is based on the measurement of the specular reflectance of visible light emitted from intact sediment samples, shown to be linearly related to the relative water content of the uppermost 200 µm. Unlike diffusive reflectance, which measures the light that after inciding on a surface is scattered in a wide range of directions and is related to the absorption characteristics of the materials, specular reflectance regards the light that is reflected in a mirror-like way and is related to the surface reflection properties (smoothness *versus* roughness). Measurements of water content can thus be obtained virtually non-invasively, enabling the simultaneous measurement of photophysiological parameters, as those based on other optical techniques such as pulse amplitude modulated (PAM) fluorometry or hyperspectral diffusive reflectance analysis. The effects of desiccation on biofilm photosynthesis were studied by simultaneously measuring changes in

relative water content and in the photosynthetic activity (using PAM fluorometry) and surface microalgal biomass (using diffusive reflectance spectral analysis).

3.3. Material and methods

3.3.1. Sampling

Undisturbed sediment samples were collected on Vista Alegre (40° 35′ N, 8° 41′ W), an intertidal mudflat in the Ria de Aveiro, west coast of Portugal. Sampling was carried out during diurnal low tide periods. Sampled sediments are composed by fine grains (97% particles < 63 μm) and are colonized by microalgal communities dominated by diatoms (Serôdio et al., 2008). The samples were collected using Plexiglas corers (1.9 and 3.6 cm internal diameter) and taken to the laboratory where they were maintained in water collected in the sampling site until measurements were carried out. All experiments were carried out in the laboratory under constant conditions of temperature (20 °C) and relative air humidity (60%).

3.3.2. Spectral reflectance

Specular and diffusive reflectance spectra were measured using a fiberoptic spectrometer (USB2000-VIS–NIR, grating #3, Ocean Optics, Duiven, The Netherlands). Spectra were recorded over the 350–1000 nm bandwidth, using a 400 μm-diameter fiberoptic (QP400-2-VIS–NIR-BX, Ocean Optics). Samples were illuminated with white light, provided by a halogen lamp (Quartzline DDL 150 W, General Electric, USA) in a fiberoptic illuminator (Olympus Highlight 3000 Cold Light Source Illuminator, Hamburg, Germany). To ensure the exposure of the sample to light in the 750 nm range (necessary for computation of the NDVI index, see below), the infrared filter present by default in the illuminator was removed. Specular and diffusive spectra were normalized to the spectrum reflected by a reference (WS-1-SL Spectralon Reference Standard, Ocean Optics). A dark reflectance spectrum measured was subtracted to both spectra to account for the dark current noise of the spectroradiometer. Sample and reference spectra were measured under a constant irradiance of 100 μmol m^{-2} s^{-1}.

Specular reflectance spectra were measured by illuminating the sediment surface with a beam of collimatedwhite light incident at a 45° angle and by measuring the spectra of the reflected light also at a 45° exit angle. The relative position of the fiberoptics used to illuminate the sample and to collect the reflected light were maintained fixed using a custom-made adaptor (Figure 3.1). Before each measurement, the distance between the sample surface and the fiberoptics was adjusted using a micromanipulator (MM33, Märtzhäuser, Germany) (Figure 3.1). This adjustment was often necessary due to the contraction of the sediment due to de-watering and of the consequent increase of the distance between the sediment surface and the fiberoptics.

Diffusive reflectance spectra were measured to estimate the surface microalgal biomass, using the biomass index NDVI (Rouse et al., 1973). For this purpose, the spectroradiometer fiberoptics was positioned perpendicularly to the sediment, at a fixed distance set to match the view field with the total area of the sample. NDVI was calculated by:

$$NDVI = \frac{R_{750} - R_{675}}{R_{750} + R_{675}} \qquad \text{Equation 3.1}$$

where R_d,750 and R_d,675 represent the average diffusive reflectance in the intervals of 749.73–750.39 nm and 674.87–675.55 nm, respectively.

3.3.3. Photosynthetic activity

The photosynthetic activity of MPB samples was measured non-destructively using PAM fluorometry (Schreiber et al., 1986). Variable chlorophyll (Chl) fluorescence was measured using a fluorometer comprising a computer-operated PAM-Control Unit (Walz, Effeltrich, Germany) and a WATER-EDF-Universal emitter–detector unit (Gademann Instruments GmbH, Würzburg, Germany).

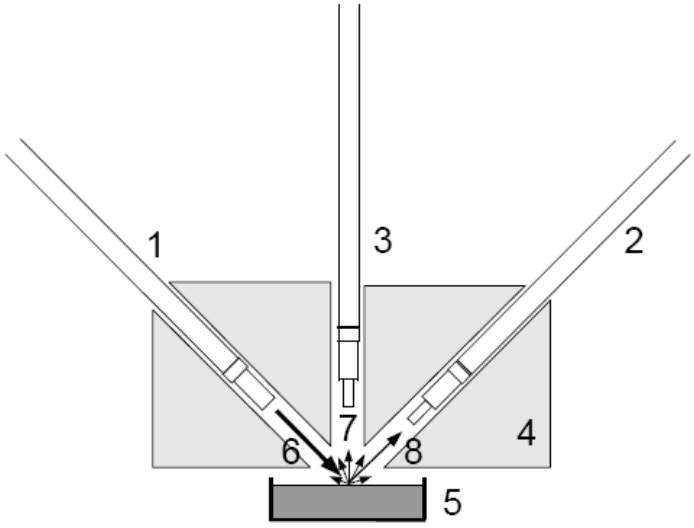

Figure 3. 1 Schematic diagram of the experimental setup used to measure specular and diffusive reflectance on undisturbed microphytobenthos biofilms. 1. Illuminator fiberoptics delivering incident white light provided by a halogen lamp. 2. Spectroradiometer fiberoptics collecting specular reflectance used to estimate the relative water content of the sample. 3. Spectroradiometer fiberoptics collecting diffusive reflectance used to calculate the biomass index NDVI. 4. Cross section of the custommade adapter used to maintain the relative positions of the fiberoptics and the sample. 5. Sediment sample, connected to a micromanipulator used to control its vertical position relatively to the fiberoptics. 6. Incident light beam. 7. Diffusive reflectance (small arrows). 8. Specular reflectance.

Measuring and saturating light were provided by a blue LED-lamp (peaking at 450 nm, half-bandwidth of 20 nm), and were delivered to the sediment sample by a 6 mm-diameter Fluid Light Guide fiberoptics bundle (Serôdio et al., 2005). The fluorometer fiberoptics was positioned perpendicularly to the sediment surface and all measurements were taken at a fixed distance of 0.1 cm, controlled by a micromanipulator (MM33, Märtzhäuser, Germany). Samples were dark-adapted for 5 min, after which minimum (F_o) and maximum (F_m) fluorescence levels were determined, and F_v/F_m, the maximum quantum yield of photosystem II (PSII) was calculated.

3.3.4. Relationship between specular reflectance and sediment water content

The rationale for the use of specular reflectance to estimate the water content of the upper layers of the sediment is based on the fact that the smooth surface of the water film present over and within the sediment particles strongly reflects light, as opposed

to the rough surface of the dry sediment, that disperses a much higher fraction of incident light beams in various directions. Therefore, changes in the relative amount of water content of the upper layers of the sediment will affect the relative surface of the sample that is covered by a highly reflective water film, and can be expected to be detected by measuring changes in specular reflectance. Because specular reflectance is not significantly affected by the spectral absorption features of the sediment particles and microalgae, but by their reflection properties that affect the dispersion of non-absorbed incident light beams, these measurements can be expected to relate mostly to the presence of water and to be largely independent from the absorption by photosynthetic pigments (which can be detected using diffusive reflectance).

To test the possibility of using specular reflectance to monitor changes in the relative water content of the upper layers of the sediment, the two parameters were measured in undisturbed sediment samples of varying water content. Samples were maintained in the Plexiglass corers (1.9 cm internal diameter) and exposed to air during increasing periods of time in order to obtain a gradient in sample water content. Randomly chosen samples (n = 6) were sectioned with minimal disturbance into 1 cm-deep Plexiglass rings with the same diameter of sampling corers, and specular reflectance was measured in each sample. Sediment samples were then frozen in liquid nitrogen and stored in the dark at −80 °C until further processing. Frozen samples were sectioned using a microtome (R. Jung, Heidelberg, Germany) in three 200 µm-deep sections: 0–200, 200–400 and 400–600 µm. Each section was immediately weighted (wet weight, W_w) and subsequently dried to constant weight (dry weight, W_d). The sediment relative water content (WC, %) for each section was calculated by (Murphy et al., 2004):

$$WC = \frac{W_w - W_d}{W_w \times 100} \qquad \text{Equation 3.2}$$

Values of WC measured for different desiccation levels were compared to specular reflectance data using linear regression analysis (Zar, 1996) in order to identify the wavelengths (or wavelength bands) that result in a stronger correlation and in a better predictive ability of the sediment water content.

3.3.5. Effects of desiccation on surface water content as estimated from specular reflectance

To characterise the patterns of variation in the water content of the uppermost layers of the sediment during desiccation, specular reflectance was measured continuously over a period of several hours on samples exposed to two different experimental conditions, expected to induce markedly different rates of desiccation: absence of wind and under a wind field of 4.2 m s^{-1} (15 km h^{-1}), created by a fan. Sediment corers were sectioned with minimum disturbance into 1 cm-deep Plexiglass rings and 3.6 cm of internal diameter, and were maintained in Petri dishes and kept exposed to air during the whole duration of the experiment. Wind speed near the surface of the samples was measured using a portable wind speed meter (Xplorer 2, Skywatch, JDC Electronic SA, Switzerland). Specular reflectance was recorded continuously at regular intervals of 15 min.

3.3.6. Effect of desiccation on photosynthetic activity and surface biomass

The effects of desiccation on MPB biofilms photosynthetic activity and surface biomass were studied by following changes in maximum efficiency of photosystem II (F_v/F_m) and on NDVI, respectively, on samples exposed to the two different wind conditions described above. Experiments were carried out during periods coinciding with diurnal low tide, and microalgal biomass was let to accumulate at the surface (monitored continuously by NDVI) before the start of the measurements.

3.4. Results

3.4.1. Relationship between specular reflectance and water content

Significant correlations were found between the specular reflectance and the water content of the uppermost 200 µm sediment layers (WC_{200}). The correlation coefficient reached significant levels ($P < 0.05$) for all wavelengths of the visible spectrum (400–

700 nm), varying from $r^2 = 0.583$ ($P < 0.001$) to $r^2 = 0.737$ ($P < 0.001$), for 690 nm and 520 nm, respectively. The strongest correlations were found in the yellow region of the spectrum, with the highest value being found for 520 nm ($R_{s,520}$; $r^2 = 0.737$, $P < 0.001$; Figure 3.2). Specular reflectance at this wavelength was also found to correlate significantly with the water content of the 0–400 µm and 0–600 µm depth intervals ($r^2 = 0.696$, $P < 0.001$; $r^2 = 0.697$, $P < 0.001$; respectively). Significant correlations were also established when using specular reflectance integrated over spectral regions, the strongest correlation being found for the 500–530 nm band ($r^2 = 0.745$, $P < 0.001$). A significant, although weaker, correlation was also found when integrating over the entire visible spectrum (400–700 nm; $r^2 = 0.728$, $P < 0.001$).

Considering the stronger correlation with WC_{200} and the simplest calculation of $R_{s,520}$, the effects of desiccation on MPB were studied estimating the surface sediment water content from $R_{s,520}$, using (from the linear regression equation in Figure 3.2):

$$WC_{200} = 76.92\, R_{s,520} + 4.31 \qquad \text{Equation 3.3}$$

3.4.2. Effects of desiccation on surface water content

The continuous measurement of specular reflectance on undisturbed sediment samples showed a well-defined pattern of decrease in surface water content from ca. 70% to minimum values around 10% (Figure 3.3). The pattern and overall rate of decrease of $R_{s,520}$ were clearly dependent on air movement. In the absence of wind, surface water content decreased slowly at an approximately constant rate, taking up to 10 h to decrease to values near 30%.

The pattern of decrease typically showed an initial phase of high variability, followed by a phase of much smoother variation after WC_{200} decreased below 40–50%. The initial noisy phase corresponded to the period when the gradually decreasing water level became in contact with the sediment particles, causing an increase in light scattering and rapid changes in the direction of light reflection by the irregularities of the sediment surface.

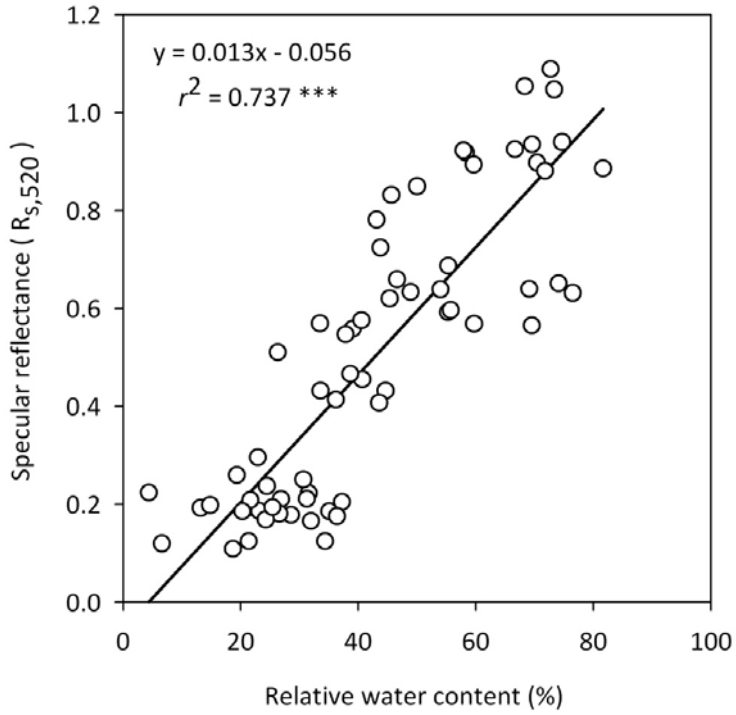

Figure 3. 2 Linear relationship between specular reflectance at 520 nm ($R_{s,520}$) and the relative water content (WC) in the uppermost 200 µm of intertidal sediments colonized by microphytobenthos. Significant values: ^{ns} $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

In contrast, samples exposed to wind showed a much higher rate of decrease in their surface water content, with minimum values being reached after 3 h of desiccation (Figure 3.3). The pattern of variation was characterised by an initial phase of rapid decrease (0–2 h) followed by a phase of constant minimum values (3–5 h), and by a slight increase in reflectance values, corresponding to the naked eye observation of the whitening of the dried sediment. Likely due to the initial rapidity of the decrease in the water content of the upper layers of the sediment, a very well-defined, negative exponential-like pattern of variation was displayed during the whole desiccation period.

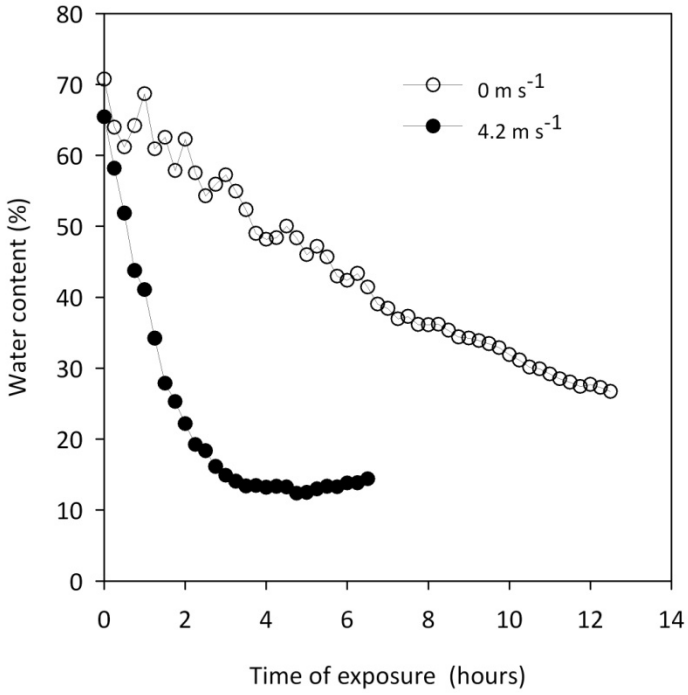

Figure 3. 3 Example of the continuous monitoring of the relative water content (WC) of the uppermost 200 µm layer of an intertidal sediment sample, exposed to wind speeds of 0 and 4.2 m s^{-1}, as estimated from changes in specular reflection.

3.4.3. Effects of desiccation on photosynthetic activity and surface biomass

The water loss by the surficial layers of the sediment induced marked changes in both the photosynthetic activity and surface microalgal biomass. The effects of desiccation were markedly dependent on wind exposure (Figure 3.4 A).

On wind-exposed samples, F_v/F_m decreased gradually following a well-defined non-linear pattern, from 0.72 to 0.65 (−9.7%) after 2 h, when WC_{200} was reduced to 40%, and to 0.54 (−25.0%) after 3 h, when WC_{200} reached ca. 25%. On samples not exposed to wind, no appreciable effects were detected on F_v/F_m even when water content reached values around 40–45%, after 3.5 h of air exposure (Figure 3.4 B). Surface microalgal biomass also varied significantly with the induced rate of water loss, with wind-exposed samples showing an accentuated decrease in NDVI, mainly after WC_{200} decreased below 35–40% (Figure 3.4 C). In contrast, NDVI measured on samples under still air continued to increase, as expected from stimulation with low light during low tide periods.

However, the observed effects were dependent not only on the water content reached at each moment, but to a large extent also on the rate of desiccation, with wind-exposed samples showing stronger effects for the same level of de-watering. This can be highlighted by comparing the relationship of F_v/F_m and NDVI to water content in wind-exposed and control samples (Figure 3.5): for the same water content of 40%, F_v/F_m decreased by 7.1% (from 0.72 to 0.65) in wind exposed samples, while remaining virtually constant (from 0.72 to 0.73) in samples under still air-conditions (Figure 3.5A.). A similar response pattern was observed for surface microalgal biomass, with NDVI decreasing by more than 20% in samples under fast desiccation while increasing (15%) in the samples under slow desiccation (Figure 3.5 B).

3.5. Discussion

3.5.1. Methodological aspects

The method for estimating the water content of the superficial layers of sediment proposed in this study presents considerable advantages relatively to the traditional method based on the determination of the dry weight of sediment vertical sections. A main advantage is that, by allowing performing rapid and non-destructive measurements, the technique permits, to our best knowledge for the first time, to monitor the same samples repeatedly and simultaneously with other parameters of interest. Also, measurements may be taken virtually non-invasively, as none of the environmental factors affecting MPB biofilms are altered during the measuring process. In particular, light intensity may be set to match the ambient irradiance field prevailing before the measurements are made, thus minimizing the induction of migratory responses to either low or high light (Cohn et al., 2004; Serôdio et al., 2006). Also temperature is not affected as a cold light source is employed.

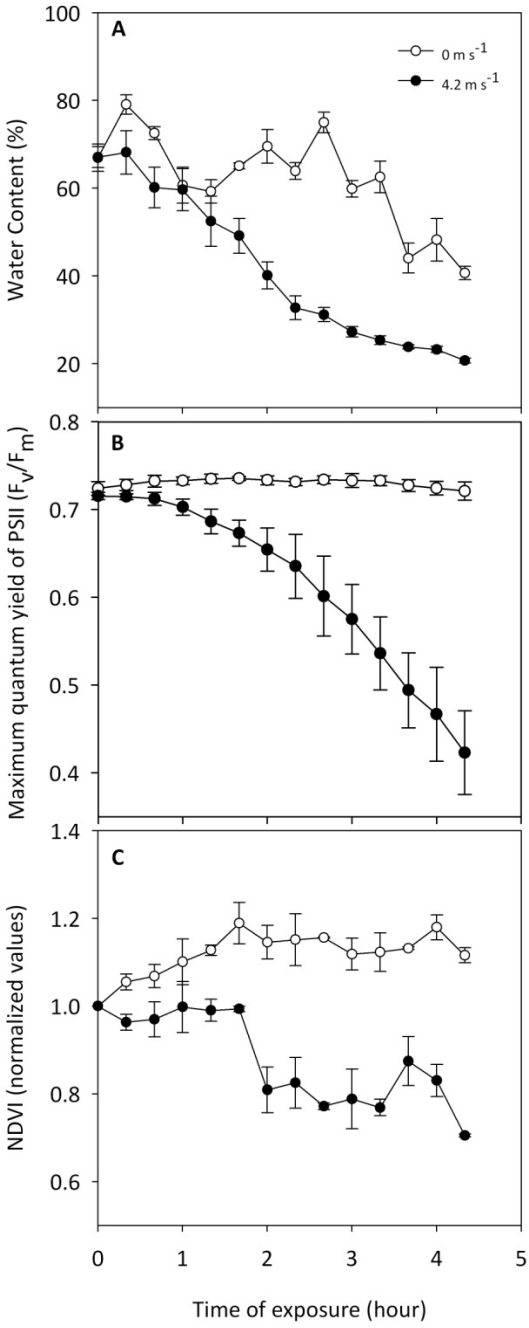

Figure 3. 4 Effects of desiccation on the photosynthetic activity and surface microalgal biomass of a microphytobenthos biofilm. Simultaneous variation of relative water content of the uppermost 200 µm (WC, A), maximum quantum yield (F_v/F_m, B) and surface Chl *a* (NDVI, C) during a simulated low tide exposure to wind speeds of 0 and 4.2 m s^{-1}. NDVI values normalized to the first measurement. Mean values of three replicates. Error bars: 1 standard error.

Of particular importance is the possibility to estimate the water content of the sediment layers near the surface that closely matches the photic zone, where photosynthetic activity takes place. As such, this method is expected to increase the capacity to detect changes in water content, and the resulting physiological or behavioural effects.

As sediment sectioning is considerably difficult to carry out if such small depth intervals are aimed, the traditional method relies on the measurement of the water content of much thicker sediment sections (Flemming and Delafontaine, 2000; Perkins et al., 2003; Jesus et al., 2006). As a result, the vertical distribution of water content is integrated over a large depth interval and minor changes near the photic zone may pass unnoticed. Furthermore, because desiccation is likely to result underestimated, detected effects on MPB physiology or biomass may be thought to correspond to unrealistically high levels of water content.

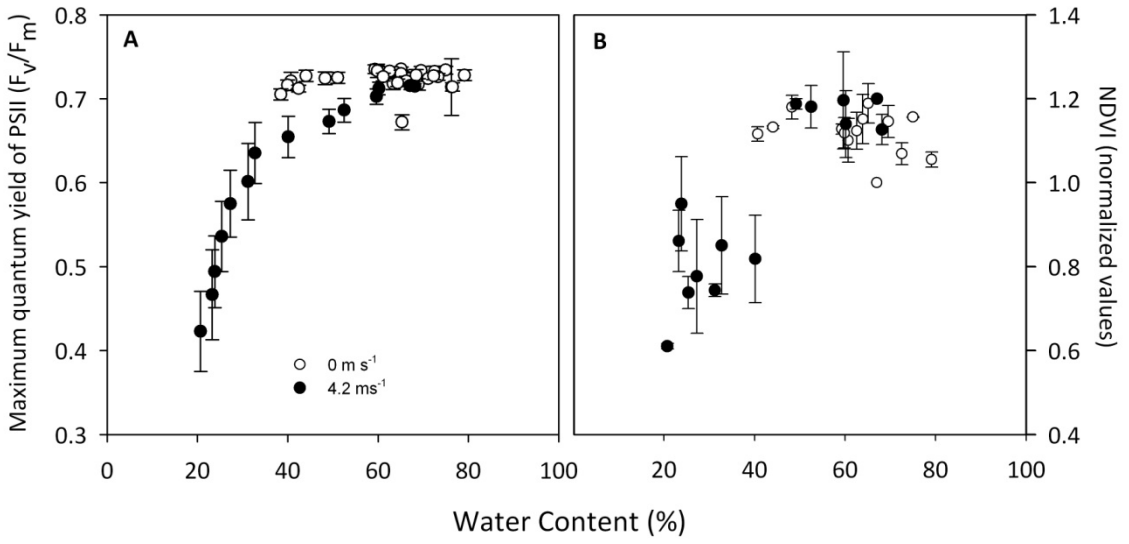

Figure 3. 5 Relationship between photosynthetic activity (F_v/F_m, A) and surface microalgal biomass (NDVI, B) and the relative water content of the uppermost 200 µm of sediment (WC) for microphytobenthos biofilms exposed to wind speeds of 0 and 4.2 ms^{-1}. Mean values of three replicates. Error bars: 1 standard error.

Another consequence of avoiding sediment sectioning is the reduction of sample replication, as the sectioning of thin layers near the sediment surface typically results in a large variability in the amount of sediment actually collected due to the irregularities of the surface microtopography. The large variability in the effective thickness of sediment sections is likely to be the main cause for the data dispersion observed in the calibration curve of the specular reflectance versus water content relationship (Figure 3.2), as when each undisturbed sample was monitored continuously a well-defined pattern was always observed (Figure 3.3). It should be noted that, due to the dependence of the specular reflectance on the light dispersion

properties of the surface sediment, the relationship between R_S and water content is likely to vary with the sediment's granulometry and new calibration curves should be constructed for sediments different from the one used in this study.

The main disadvantage of this method is the dependence of the specular reflectance signal on irregularities of the sediment surface that affect light scattering and therefore the measured signal independently of changes in water content. However, this effect seems limited to the initial phase of sample desiccation and to significantly confound the detection of an overall trend only when samples are exposed to very low desiccation rates. On samples exposed to a wind velocity of 4.2 m s^{-1}, which can be considered as reasonably low when compared to the range of in situ conditions, the detection and characterization of the patterns of water loss was not compromised. The observed negative exponential-like pattern, and its dependency on wind velocity, was very similar to the ones registered by Gao et al. (1998) on the decay in water content of wind-exposed *Nostoc flagelliforme* colonies.

This shortcoming could be overcome by using diffusive reflectance indices based on water absorption peaks, which have been successfully used in estimating water content in plant leaves (Peñuelas et al., 1997; Zarco-Tejada et al., 2003) and soil moisture (Weidong et al., 2003). However, for intertidal sediments the water absorption maxima in the 400–900 nm range could not be detected in diffusive reflectance spectra, even when performing first- or second-derivative analysis (Murphy et al., 2005a). As such, this approach would likely require the use of spectroradiometers detecting the 1000–3000 nm wavelength band, expensive equipment not usually available in estuarine and marine biology laboratories. In this regard, another major advantage of the proposed method is that it can be applied using much simple and inexpensive equipment. In fact, the correlation found between specular reflectance integrated over the 400–700 nm range opens the possibility to use a simple PAR sensor instead of a visible light spectroradiometer to monitor the surface sediment water content.

Another potential limitation of this method is the impossibility of distinguishing changes in the water content of the sediment and of the water contained in the microalgal cells. The problem with this is the possibility that changes in the microalgal concentration in the upper layers (e.g. due to vertical migration) may affect the

specular reflectance properties of the sediment surface and confound the estimation of the water content, like in the case of the simultaneous decrease in water content and microalgal biomass shown in this study (*see* also below). Nevertheless, in this respect the proposed method is not worst than the traditional method of dry weight determination, that also only quantifies the total water content, independently of its origin.

3.5.2. Effects of desiccation

This study has shown that the water loss of the uppermost layers of the sediment, resulting from the exposure to conditions that occur frequently in the intertidal environment, induces a marked decrease in photosynthetic activity of MPB biofilms. The observed decline in F_v/F_m is expected from the known effects of salinity and water stress on photosynthetic activity (Munns, 2002; Sudhir and Murthy, 2004) and concurs with the effects of desiccation in the net photosynthetic rates of intertidal sediments (Holmes and Mahall, 1982) and in other photoautotrophs, such as cyanobacteria (Potts, 1999; Ohad et al., 2005) or macroalgae (Matta and Chapman, 1995; Peña et al., 1999; Hunt and Denny, 2008). The high levels of osmotic and salt stress occurring on intertidal flats as a result of desiccation have been shown to stimulate the production of reactive oxygen species on benthic diatoms (Rijstenbil, 2003; Roncarati et al., 2008). This may be expected to result in oxidative damage to the photosynthetic apparatus, causing a decrease in F_v/F_m through the inactivation of photosystem II protein D1 (Nishiyama et al., 2006).

Of importance is the finding that the effects of desiccation on photosynthetic activity were largely depending on the rate of sediment water loss. Under slow desiccation, in the absence of wind, a decrease of water content to ca. 40% hardly induced any effects on F_v/F_m, an indication that up to this level, sediment water loss did not result in a significant degree of cell dehydration. In contrast, under wind exposure, effects on F_v/F_m were observed readily from the start of air exposure.

A possible explanation for the rate dependency of the effects of desiccation may be the production of exopolysaccharides (EPS) by the diatoms and/or bacteria under slow desiccation that could effectively reduce the rate of water loss and delay the effects of

sediment desiccation on microalgal dehydration. Salt stress has been shown to stimulate the production of EPS on bacteria (Ophir and Gutnick, 1994) and cyanobacteria (Chen et al., 2006), but also on diatoms (Abdullahi et al., 2006). On the other hand, due to its capability to retain water, EPS have a known protective role against desiccation of soil microorganisms (Roberson and Firestone, 1992; Ohad et al., 2005). It may thus be hypothesized that samples under slow desiccation had sufficient time to produce protective amounts of EPS while the faster desiccation caused by wind exposure impeded the production of significant amounts of EPS and consequent delay of de-watering effects.

However, the different results observed for slow and fast desiccation may also be related to the changes in microalgal biomass in the uppermost sediment layers. As with F_v/F_m, also the changes in surface microalgal biomass varied markedly with the rate of desiccation, with wind-exposed samples showing a steep decrease as opposed to the increase observed on non-exposed samples. It may be argued that this decline in surface biomass is due to a downward migration of motile diatoms, which could contribute to prolong their maintenance in a wetter microenvironment, and thus help to explain the relatively smaller effects showed on photosynthetic activity. A similarly protective migratory behaviour is displayed by benthic diatoms in relation to high light, allowing individual cells to escape otherwise photodamaging levels at the sediment surface (Serôdio et al., 2006). However, the question remains open if the decrease in surface biomass is due to active downward migration, or to simple passive transport following the descending of the water level between the sediment particles.

3.5.3. Consequences for the modelling of microphytobenthos productivity

The modelling of the primary productivity of MPB has assumed the coincidence of surface biomass and productivity with solar zenith (Pinckney and Zingmark, 1991; Guarini et al., 2000; Serôdio and Catarino, 2000). On the other hand, MPB productivity has been shown to be largely determined by microalgal biomass exposed to light (Serôdio et al., 2001). The results of this study clearly show that the sediment desiccation induces important short-term effects on both photosynthetic activity and

on photosynthetically active microalgal biomass. Although the results of this study alone cannot be used to predict the magnitude of the changes in microphytobenthic biomass, they indicate that the effects of desiccation, even moderate, should be considered together with those of light and temperature in the modelling of productivity. This requires the detailed characterisation of the patterns of sediment dehydration during low tide periods, and the complex relationship of surface sediment water content and key environmental variables like air temperature, solar irradiance, wind velocity or air humidity. The simple and non-destructive method proposed in this study may provide a practical and effective way to accomplish this objective.

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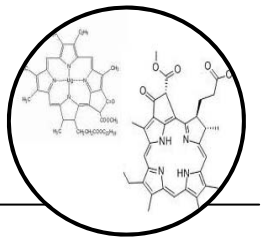
Chapter 4



Pheophorbide *a* in *Hydrobia ulvae* faecal pellets as a measure of microphytobenthos ingestion: variation over season, day-night and tidal cycles

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4.1. Abstract

Microphytobenthos (MPB) are an important food source for *Hydrobia ulvae* individuals in estuaries. The MPB – *H. ulvae* trophic interaction is one of the main channels of matter transfer to higher trophic levels in estuarine benthic environments. This work addressed the establishment of a new non-invasive approach to evaluate grazing activity of *H. ulvae* on MPB. The effects of seasonal, tidal and day-night cycles on ingestion rates of *H. ulvae* (using ^{14}C-labeled MPB) and egested pheopigments *a* (using HPLC pigment analysis) were also investigated. *H. ulvae* ingestion rate was found to vary significantly over season, day-night cycle and tidal cover, being higher in summer and during diurnal low tide periods, which was possibly related to higher growth rates of *H. ulvae* in summer, as well as to the increase of surface MPB biomass during diurnal low tides. Despite this high variability, a strong relationship was found between ingested Chl *a* and egested pheophorbide *a*, allowing the estimation of ingestion rate from the amount of pheophorbide *a* egested on *H. ulvae* faecal pellets. This new non-invasive methodology may allow to improve long-term studies of consumption rates and the evaluation of grazing of *H. ulvae* on MPB, eliminating inter-individual variability and reducing the need of replication.

Keywords: Pheophorbide *a*/chlorophyll *a* ratio, ingestion rate, grazing, microphytobenthos, *H. ulvae*

4.2. Introduction

The mud snail *Hydrobia ulvae* (Pennant) is one of the most abundant deposit-feeder species in intertidal mudflats of the North Atlantic coast, forming large populations that can reach densities of up to 10^5 individuals m^{-2} (Barnes, 1999). Such high densities may result in heavy grazing pressure on one of the main benthic primary producers of estuarine habitats, the microphytobenthos (MPB). The MPB consists of photosynthetic microalgae and cyanobacteria that accumulate in the surface layers of intertidal sediments, forming dense and highly productive biofilms (Underwood and Kromkamp, 1999). These highly productive microphytobenthic biofilms are particularly well recognized as the main source of food for *H. ulvae* populations (Bianchi and Levinton, 1984; Blanchard et al., 2000; Haubois et al., 2005; Pascal et al., 2008). Intense grazing pressure has been found to cause a significant top-down control on the MPB biomass at the surface of sediments in intertidal areas (Cariou-Le Gall and Blanchard, 1995; MacIntyre et al., 1996; Underwood and Kromkamp, 1999; Novak et al., 2001; Hagerthey et al., 2002; Hillebrand and Kahlert, 2002). This trophic link is one of the main channels of matter transfer to higher trophic levels of the estuarine food webs, since *H. ulvae* is an important prey item for fish, birds and other estuarine invertebrates (Piersma et al., 1993; Aarnio and Mattila, 2000).

The trophic relationship between MPB and mud snails has been mostly investigated through the measurement of the ingestion rate of mud snails on MPB, based on the use of ^{14}C-labelled microalgae (Forbes and Lopez, 1989; Blanchard et al., 2000; Haubois et al., 2005). The effects of mud snail density (Blanchard et al., 2000) as well as the effect of cell size (Haubois et al., 2005) have been considered in the estimation of the energy flow and grazing rates. Nevertheless, to our knowledge, the carbon flow through the link MPB/*H. ulvae* has been quantified only for diurnal low tide periods, and its variability along day/night and tidal cycles has never been characterized. Several studies have used the pheopigments content of intertidal sediments as an index of grazing activity by zoobenthos (Brotas and Plante-Cuny, 1998; Lucas and Holligan, 1999; Cartaxana et al., 2003), based on the finding that pheophorbide *a* and pheophytin *a* are the main degradation products of chlorophyll (Chl) *a* found in the sediments (Bianchi et al., 1988; Abele-Oeschger and Theede, 1991; Buffan-Dubau et al., 1996) as well as in the water column (Klein et al., 1986; Strom, 1993). The

breakdown of Chl *a* during digestion by mud snails is known to result in the accumulation of those degradation products in their faecal pellets (Cartaxana et al., 2003), however, the relationship between the ingested Chl *a* and its degradation products has never been investigated.

The present work addresses the development of a new non-invasive methodology to evaluate grazing activity of *H. ulvae* on MPB, based on the relationship between the ingested Chl *a* (as proxy for MPB biomass) and its degradation products, pheophorbide *a* and pheophytin *a.* The ingestion rate of mud snails on microalgae was estimated through the incorporation of benthic microalgae labelled with ^{14}C by *H. ulvae* and pigment content of *H. ulvae* faecal pellets was quantified by HPLC. The relationship between egested pheophorbide *a* and ingested Chl *a* was characterized by the ratio pheophorbide *a*/ Chl *a*. The work also investigated how *H. ulvae* ingestion and egestion rates vary over periods of 24 h, covering different situations along the day-night and tidal cycles, on spring (April) and summer (July).

4.3. Material and Methods

4.3.1. Sampling

*S*amples of the sediment surface containing MPB and *H. ulvae* were collected from intertidal mudflats in the Ria de Aveiro (40° 38’ N, 8° 44’ W), a shallow coastal lagoon located on the north west coast of Portugal, during the low tide prior to the experiments. The sediment surface (approximately the top 2 mm) was scraped using a spatula, stored and transported to the laboratory. The sediment samples were sieved through a 1 mm mesh to separate snails from sediment. *H. ulvae* individuals were measured under a stereoscopic microscope and selected according to the shell height (apex to aperture). Only individuals considered as adults (Haubois et al., 2002) were used. Selected mud snails were kept in filtered seawater at 20° C and submitted to a starvation period of 24 h before the beginning of the experiments. The sediment was stored until further processing.

4.3.2. Measurement of Chl *a* ingestion rate

Measurements of Chl *a* ingestion rate in *H. ulvae* were made in experimental controlled microcosms (75 cm^2 culture flask) based on a protocol adapted from Blanchard et al. (2000). This protocol assumes that: i) labelled microalgae accumulate in the gut of the snails at a constant rate during the experimental period, ii) egestion does not take place during the incubation period when the snails are feeding (Barnes, 2001), iii) labelling is a conservative process, i. e., the radioactivity contained in the microalgae does not change with time, and iv) labelled and unlabelled microalgae are grazed at the same. Briefly, the protocol consists in adding ^{14}C-labelled microalgae to the sediment and then recording the radioactivity incorporated by the snails. The epipelic benthic microalgae were isolated from the sediment using the lens tissue method (Eaton and Moss, 1966) and a microalgal suspension was made by rinsing the lens tissue in natural seawater. This suspension was labelled by adding a volume of 40 to 50 mCi mmol of $NaH^{14}CO_3$ stock solution (PerkinElmer) in order to obtain a final concentration of 0.4 µCi ml^{-1}, and left to incubate for 2 h under saturating light at 20° C. The suspension was stirred during incubation in order to ensure homogeneous labelling. Following incubation the suspension was centrifuged (3 min at 1500 rpm min^{-1}), the supernatant was discarded and the remaining was rinsed in filtered seawater and centrifuged twice, in order to obtain a concentrated suspension of labelled microalgae free of non-assimilated $NaH^{14}CO_3$. This suspension was then added to the sediment that was left from the isolation of the microalgae, which had been diluted with filtered sea water (1:1, v / v). The final suspension of sediment and labelled microalgae was then homogenised with a glass rod and 10 ml were introduced to each microcosm, together with 8 snails that were left to feed for 2 h at 20º C. The density of snails in each microcosm (8 ind 75 cm^{-2}) was chosen such that it remained below the density threshold that results in a decrease of ingestion rate (Blanchard et al., 2000). Feeding was interrupted by adding freshwater and the snails were sieved from the sediment (with a 1 mm mesh sieve), rinsed with filtered seawater and placed in defecating chambers (*see* section 4.3.3.). After defecation, the radioactivity remaining in the snails was measured by pooling all 8 snails from each microcosm and solubilising the soft tissues by the addition of 180 µl of Soluene-350 (Packcard) tissue solubilizer for 72 h at 50º C, in a Selecta Frigiterm-10 water bath. A volume of 1.8 ml

Hionic-fluor (Packcard) scintillation cocktail was then added to the solution and radioactivity was measured in a Beckman LS 6000 IC liquid-scintillation counter. The above procedure was repeated in four experiments made within two different 24 h periods, one in April (spring) and the other in July (summer) 2009. The four experiments within each 24 h period coincided with four different combinations of the day and tidal phases (day/high water, day/low water, night/high water and night/low water) to which the snails would be subjected if they remain in the field. In each experiment 5 microcosms were used.

Ingestion rates (*IR*, µg Chl *a* ind^{-1} h^{-1}) of *H. ulvae* were calculated from the total radioactivity ingested by each snail (*IRad*, dpm ind^{-1} h^{-1}) using the equation:

$$IR = IRad\ x\ CR \qquad \text{Equation 4.1}$$

where *CR* (µg Chl *a* ind $^{-1}$ h^{-1}) is a conversion factor to transform radioactivity counts into Chl *a* biomass. *CR* was calculated from measurements of radioactivity and Chl *a* made in a 1 ml suspension of sediment set aside before inoculation of the microcosms. Radioactivity was read in the Beckman LS 6000 IC liquid-scintillation counter, after addition of 1 ml Hionic-fluor (Packcard) scintillation cocktail. Chl *a* was extracted in 90% aqueous acetone and quantified spectrophotometrically (Genesys 6, Thermo Spectronic, Whalthman, USA) following the method of Lorenzen (1967).

4.3.3. HPLC pigment analysis of faecal pellets

In order to collect the faecal pellets the mud snails were placed in defecating chambers without food after being removed from the microcosms. The defecating chambers consisted of plastic flasks where a bottom partition was created with a 500 µm net, that ensured a separation between individuals and faecal pellets. The snails were left to defecate for 48 h and the faecal pellets were then retrieved from the bottom compartment, frozen in liquid nitrogen and stored at -80° C until freeze-drying. After freeze-drying the pellets were weighted and pigments were extracted in 95% cold buffered methanol (2% ammonium acetate) during 15 min, with 30 s sonication (Brotas and Plante-Cuny, 1998). The solution was then filtered using 0.2 µm-pore

filters (Fluoropore PTFE filter membranes) and extracts were immediately injected in a Shimadzu HPLC with photodiode array (SPD-M10ADVP) and fluorescence (RF-10AXL) detectors. Chromatographic separation was achieved using a C18 column for reverse phase chromatography (Supelcosil, 25 cm length, 4.6 mm diameter, 5 µm particles) and a 35 min elution programme. The solvent gradient followed Kraay et al. (1992), with a flow rate of 0.6 ml min^{-1} and an injection volume of 100 µl. Pigments were identified from absorbance spectra and retention times, and concentrations calculated from signals in the photodiode array and fluorescence detectors. Identification and calibration of the HPLC peaks were done using commercial standards: Chl *a* standard from Sigma-Aldrich and pheophytin and pheophorbide *a* standards from DHI (Institute for Water and Environment, Hørsholm, Denmark).

4.3.4. Statistical analysis

The existence of a linear relationship between pheophopigments and Chl *a* was tested by linear regression analysis and regression equations (slope and intercept) were compared by Analysis of Covariance (ANCOVA). A 3-way ANOVA was carried out to test the effect of season (April and July), tidal stage (low tide, LT and high tide, HT) and day-night cycle (day and night) on the *H. ulvae* ingestion rate and on the egestion of pheopgiments. Prior to analysis, assumptions were verified and data transformed whenever necessary. However, variances in *H. ulvae* ingestion rate were heterogeneous and this condition could not be corrected by any of the usual transformations. Therefore, the ANOVAs were performed on the ranks of the observations (Zar, 1996). This procedure homogenized variances and corresponds to a nonparametric ANOVA. The Tukey and Student-Newman-Keuls (SNK) test were used as a post-hoc comparison of the main effects, in situations where significant differences were found. A 1-way ANOVA was performed to test the effect of season in the amount of Chl *a* available and to test differences in *H. ulvae* ingestion rate between microcosms on identical conditions. All statistical analysis was carried out following Zar (1996) and using STATISTICA v.8 (StatSoft Inc, USA).

4.4. Results

4.4.1. Variation of *Hydrobia ulvae* ingestion rate

The amount of ^{14}C accumulated in labelled microalgae was monitored during the experiments, confirming that it did not vary significantly in the gut of *H. ulvae* over 2 h (data not shown). Mud snails ingested benthic microalgae at a rate that did not change significantly between microcosms on identical conditions (e.g. ANOVA, April LT day: P = 0.933 and April HT night: P = 0.995).

Figure 4.1 shows the variation of ingestion rate over the periods monitored around diurnal and nocturnal tides in April and July. A significant effect of season on *H. ulvae* ingestion rate was observed (Table 4.1). Mean ingestion rate increased significantly from April to July (Figure 4.1), despite the fact that the Chl *a* content available in the sediment in April and July were not significantly different (10.93 ± 1.65 and 14.06 ± 4.60 µg Chl *a* g dry, respectively; one-way ANOVA, P = 0.529). The effects of day-night cycle suggested that the ingestion of microalgae by mud snails depended on the time of day, being higher during daytime than during night (Table 4.1, Figure 4.1). This was confirmed by the results of the SNK test (Figure 4.1). Regarding the effects of tidal cycle, significant differences were found between the *H. ulvae* ingestion rate in low and high tide (Table 4.1). It was not observed a significant interaction between the three factors or in the interaction between tide and day-night cycles (Table 4.1). However, the SNK test showed that there were no differences between low and high tide when considering day *vs.* night, as well as April *vs.* July. Significant differences between the ingestion rate in low tide and high tide were only considering each period of the day separately, being always higher during daytime low tide periods (Figure 4.1).

4.4.2. Variation of egested pheopigments

Figure 4.2 shows the variation of egested pheopigments per *H. ulvae* adult. Pheophorbide *a* content (µg Pheophorbide *a* ind^{-1}, Figure 4.2 A) was always higher than pheophytin *a* (ng Pheophytin *a* ind^{-1}, Figure 4.2 B) in *H. ulvae* faecal pellets.

C4. Pheophorbide *a* as measure of MPB consumption

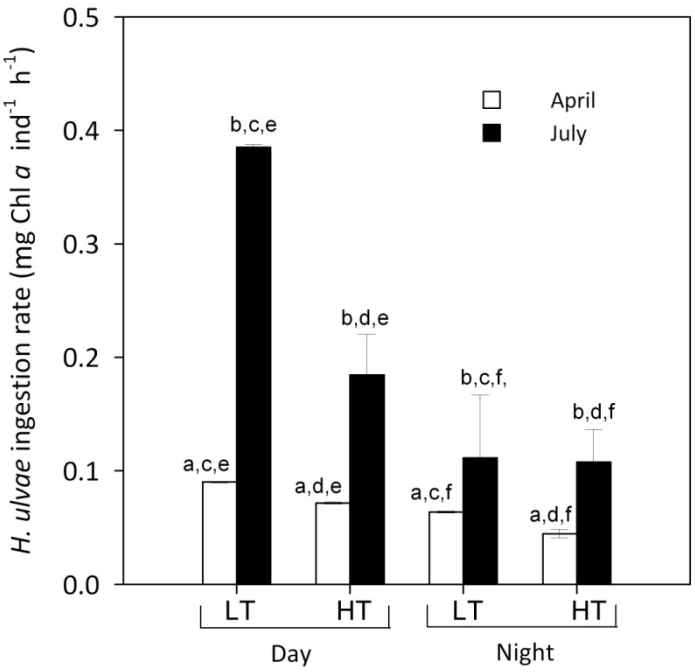

Figure 4. 1 Individual mean ingestion rate of *Hydrobia ulvae* for different tidal situations and time of day over a period of 24 h, in April (white) and July (black). Mean values of three replicates. Error bars: 1 standard error. LT and HT represent low tide and high tide, respectively. Different pairs of letters were used to indicate significant differences by SKN test for the main effects: a and b represent differences between season, c and d between tides and e and f between day-night ($p < 0.05$).

Table 4.1 Statistical results of the non-parametric ANOVA and 3-way ANOVA on the effects of seasonal (*S), tidal (*T) and day-night cycles (*D/N) on the *H. ulvae* ingestion rate (Chl *a*) and on the egestion of pheopigments (pheophorbide *a* and pheophytin *a*).

*Source of variation**	*Ingestion rate (Chl* a*)*		*Pheophorbide a*		*Pheophytin a*	
	df	F	df	F	df	F
Effect						
S	1	115.883^{***}	1	67.586^{***}	1	35.99^{***}
T	1	9.460^{**}	1	7.454*	1	0.020^{ns}
D/N	1	53.984^{***}	1	61.749^{***}	1	9.850^{**}
Interaction						
S x T	1	2.365^{ns}	1	15.130^{***}	1	1.780^{ns}
S x D/N	1	0.117^{ns}	1	21.092^{***}	1	0.460^{ns}
T x D/N	1	3.533^{ns}	1	5.693^{*}	1	0.740^{ns}
S x T x D/N	1	0.119^{ns}	1	21.186^{***}	1	57.90^{***}

Note: df, Degrees of freedom; Significant values: ^{ns} $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

ANOVA detected a significant effect of season and of the day-night cycle on the production of both pheopigments (Table 4.1), while tidal cycle was found to affect significantly only the production of pheophorbide *a* (Table 4.1). Moreover, a significant interaction between season, tide and day-night cycle was observed for the egestion of pheopigments by mud snails (Table 4.1). The egestion of pheophorbide *a* during the day in July was significantly higher under low tide than under high tide, while in April those differences not occurred (Figure 4.2 A, Tukey's test). During the night, the egestion of pheophorbide *a* did not vary with season or tide (Figure 4.2 A, Tukey's test).

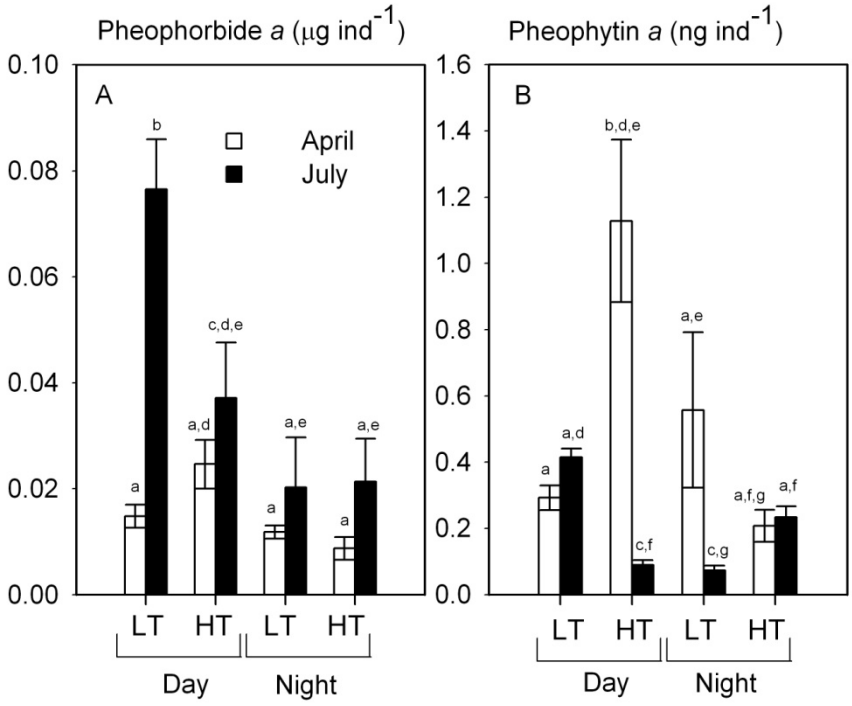

Figure 4. 2 Egested pheophorbide *a* (A) and pheophytin *a* (B) by *Hydrobia ulvae* individuals for different tidal situations and time of day over a period of 24 h in April (white) and July (black). Mean values of three replicates. Error bars: 1 standard error. LT and HT represent low tide and high tide, respectively. Columns with different letters indicate significant differences by Tukey's multiple comparison test for the interaction between season*tide*day-night ($P < 0.05$). Means having at least one letter in common are not significantly different.

The egestion of pheophytin *a* during diurnal low tide as well as under nocturnal high tide did not shown significant differences conditioned by the season (Figure 4.2 B). However, under diurnal high tide the levels of pheophytin *a* egested were relatively higher in April and lower in July (Figure 4.2 B), when compared to other situations.

4.4.3. Relationship between egested pheopigments and ingested Chl *a*

Ingested Chl *a* was found to vary linearly with egested pheophorbide *a* (Figure 4.3 A; r^2 = 0.937, P < 0.001), but not with egested pheophytin *a* (Figure 4.3 B; r^2 = 0.011, P = 0.628). Nevertheless, a significant correlation was found when considering the whole pheopigment content (pheophorbide *a* plus pheophytin *a*) (data not shown; r^2 = 0.934, P < 0.001).

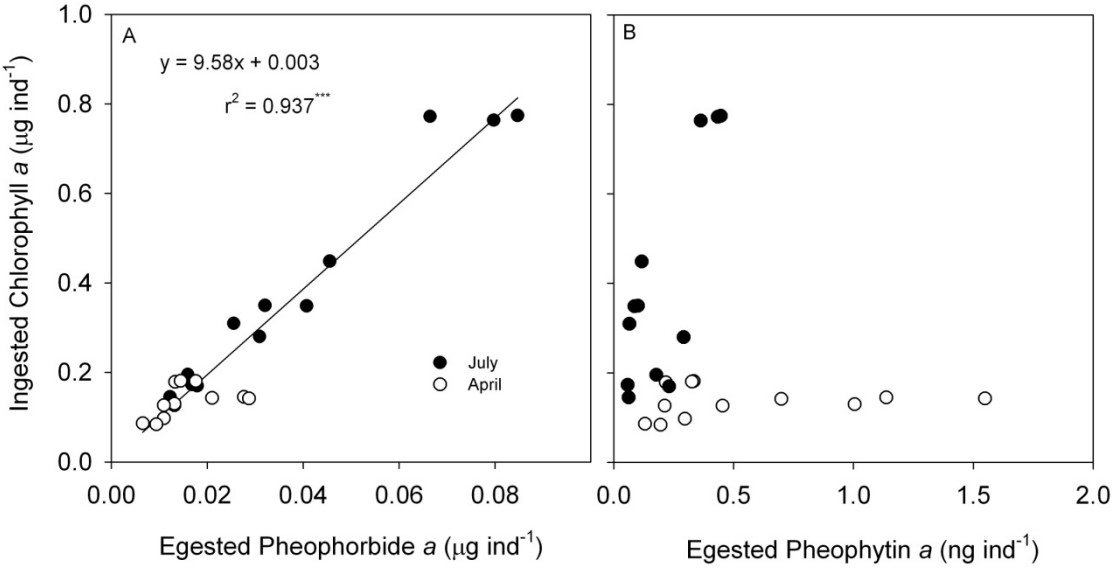

Figure 4. 3 Relationship between egested pheophorbide *a* (A) and pheophytin *a* (B) and the previously ingested chlorophyll *a* by *Hydrobia ulvae* individuals, in April (white) and July (black).

The relationship between pheophorbide *a* and Chl *a* did not vary substantially from April to July, as no significant differences were found between the slopes or the intercepts of the linear regression equations (ANCOVA, P = 0.837 and P = 0.418, respectively). Regarding the relationship between pheophytin *a* and Chl *a*, significant differences were found between intercepts (ANCOVA, P < 0.05), but not between slopes (ANCOVA, P = 0.556). Considering the stronger correlation found between egested pheophorbide *a* and ingested Chl *a*, the ingestion rate by *H. ulvae* adults could be determined estimating the ingested Chl *a* using the equation 4.2 from the linear regression equation of Figure 4.3 A.

$$Chl\ a\ (\mu g\ ind^{-1}) = 9.58\ Pheophorbide\ (\mu g\ ind^{-1}) + 0.003 \qquad \text{Equation 4.2}$$

4.5. Discussion

4.5.1. Relationship between egested pheopigments and ingested Chl *a*

The major aim of this study was to investigate the possibility of establishing a new non-invasive methodology to allow the estimation of the ingestion rate as a Chl *a* flux from benthic microalgae to *H. ulvae,* based on the relationship between ingested Chl *a* and egested degradation products. Pheopigments appeared as the main degradation products of Chl *a* (Buffan-Dubau et al., 1996; Cartaxana et al., 2003). Clear peaks of pheophorbide *a* and pheophytin *a* were detected in the HPLC analysis of *H. ulvae* faecal pellets as already described by Cartaxana et al. (2003), in contrast with the absence of pheopigments reported by Ford & Honeywill (2002) for mud snails from the Eden Estuary. Pheophorbide *a* was found in high concentrations in faecal pellets, being significantly correlated with the amount of microalgae (as Chl *a*) previously ingested by the same individuals. This suggests a close link between the pheophorbide *a* produced by *H. ulvae* adults and the ingested Chl *a*, allowing establishing a direct relation between them. This relationship can thus be used to estimate non-invasively the Chl *a* ingested by snails (*see* section 4.5.2).

Contrary to pheophorbide *a*, that was the major pheopigment found in faecal pellets of *H. ulvae*, formed by removal of both the Mg atom and the phytol chain from the Chl *a* molecule, pheophythin *a* showed no correlation to ingested Chl *a*. The extent of pigment digestion should vary as a function of the composition of the grazed community as well as the gut chemistry and gut residence time of the grazer (Penry and Frost, 1991; Cartaxana et al., 2003). The latter authors have shown a 16x higher accumulation of pheophorbide *a* in relation to pheophythin *a* in faecal pellets of *H. ulvae* feeding on MPB.

4.5.2. Pheopigments as consumption markers: methodological aspects

Photosynthetic pigments have been widely used to provide useful information as taxonomic biomarkers and, more recently, as grazing markers in planktonic and benthic photosynthetic communities (Roy et al., 1996; Barranguet et al., 1997; Brotas and Plante-Cuny, 2003; Cartaxana et al., 2003). The results of the present work support

the use of pheophorbide *a* content of *H. ulvae* faecal pellets as a potential marker for ingested Chl *a* (*see* Equation 4.2). The methodology proposed in this study for estimating the MPB biomass ingested by mud snails has several advantages over the methodologies based on the use of pre-labelled food sources. One main advantage is that it allows estimates to be made non-invasively, avoiding the sacrifice of the animals. This eliminates inter-individual variability, reducing the need of replication, and thus contributing to improve long-term studies of consumption rates, something impossible to achieve using labelling techniques. Also, the traditional label approach implies that the label should be homogenously distributed within the available food and the specific activity should remain constant over the experiment (Lopez and Cheng, 1983). This requires the continuous control of the application, impeding long-term monitoring in the field. Moreover, the new method allows the characterization of the MPB consumption in natural conditions, allowing a better assess of net primary production by considering the losses due to grazing pressure.

A potential disadvantage of the proposed methodology is related with the possible pheopigment conversion into colourless products, thus not possible to quantify by HPLC. This limitation, however, is shared with the all methods based on the use of pheopigments as grazing markers (Buffan-Dubau et al., 1996; Cartaxana et al., 2003). Also the hypotheses that mud snails re-ingest their own faecal pellets (Lopez-Figueroa and Niell, 1988) might contribute to the degradation of pheophorbide *a* to colourless residues (Cartaxana et al., 2003). The application of a previous starvation period was also necessary to assure that all pheopigments accumulated in faecal pellets resulted from the degradation of the labelled Chl *a*. However, this approach would likely require the test under natural conditions, addressing the effects of non-starvation and re-ingestion of own pellets. Preliminary results of the application of the proposed methodology under natural conditions have shown that under non-limiting food (opposite to starvation) *H. ulvae* faecal pellets contain similar levels of pheophorbide *a* and higher values of Chl *a* (Coelho et al*. unpublished*). This suggests the incomplete digestion of ingested microalgae.

The constancy of the relationship between pheophorbide *a* and Chl *a* over the range of different combinations of seasonal, tidal and day-night cycles strongly suggests the general applicability of the method for studying the trophic link between primary

producers and consumers in aquatic environments. Furthermore, the important role that MPB and *H. ulvae* play in estuarine ecosystems, as well as the relevance of this link in the structure of benthic food chains, reinforces the potential applicability of the results of this study.

4.5.3. Daily variation of *Hydrobia ulvae* ingestion rate

The linear accumulation of labelled benthic microalgae by *H. ulvae* adults has been previously recorded over periods of 2 h (Blanchard et al., 2000; Haubois et al., 2005) and similar results were registered regarding the accumulation of bacteria by *H. ulvae* (Pascal et al., 2008). The absence of egestion over the 2 h of feeding may be associated with the complexity of the digestive tracts of molluscs, which allows the partitioning of food particles within the gut. Nutritious particles from microalgae or bacteria are usually diverted to the digestive gland, undergoing intracellular digestion (Pascal et al., 2008) with higher residence times (Kofoed et al., 1989; Pascal et al., 2008).

The individual ingestion rate of *H. ulvae* adults varied from 0.044 to 0.090 µg Chl *a* ind^{-1} h^{-1} (from HT to LT) in April and from 0.107 to 0.385 µg Chl *a* ind^{-1} h^{-1} (from HT to LT) in July. Considering a C/Chl a ratio of 40 (de Jonge 1980), this is equivalent to 1.6 to 3.6 µg C ind^{-1} h^{-1} in April and to 4.3 to 15.4 µg C ind^{-1} h^{-1} in July. As such, the results of the present study on the ingestion rate of MPB during night periods (1.6 and 4.3 µg C ind^{-1} h^{-1} in April and July, respectively) are consistent with those previously reported to the ingestion rate of benthic microalgae (mostly diatoms) (Forbes and Lopez, 1989; Haubois et al., 2005; Pascal et al., 2008). However, the values found for diurnal simulations were higher than the ones described in the literature, reaching almost three times more in summer (3.6 and 15.4 µg C ind^{-1} h^{-1} in April and July respectively). The remarkable high ingestion rates of microalgae by mud snails in July might be associated with the life cycle of *H. ulvae*. Maximum growth rate have been found to occur during the summer in European estuaries (Sola, 1996; Haubois et al., 2002) and some studies have already shown the positive relationship between growth rate and ingestion rate for other *Hydrobia* species (Bianchi and Levinton, 1984) as well as for other molluscs (Sommer et al., 1999).

Natural seasonal changes in gastropod physiology or sediment characteristics might be responsible for some of the variability found regarding the ingestion rates. Our results showed that *H. ulvae* ingestion rates were always higher during the day, indicating an important role of the day-night cycle in the ingestion activity of mud snails. This is in accordance with the increase in *H. ulvae* crawling activity with light (Orvain and Sauriau, 2002). On the other hand, it contrasts with the results found by Pascal et al. (2008) that showed similar ingestion rates under light and dark. This work provides essential new information regarding the effects of light (day) on the ingestion rate of *H. ulvae*. However, we should point out that considering the daylight time, higher values of ingested Chl *a* were found during low tide, as significantly differences were established by the effect of tide. This might suggest that an increase of grazing by *H. ulvae* adults under natural conditions could be synchronized with the increase of MPB biomass at the sediment surface during diurnal low tides.

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Chapter 5

Spatio-temporal variability of *Hydrobia ulvae* density and ingestion rate under natural conditions as estimated from pheophorbide *a* content of faecal pellets

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In preparation

5.1. Abstract

In estuarine environments, grazing by the mud snail *Hydrobia ulvae* has both direct and indirect effects on microphytobenthic biomass. The relationship between *H. ulvae* density and ingestion rate of benthic microalgae was never investigated under natural conditions. The present work aimed to the changes on *H. ulvae* density and ingestion of microphytobenthic biomass (Chl *a*) under natural conditions in Ria de Aveiro, based on the non-invasive estimation of mud snail ingestion rates using the amount of pheophorbide *a* accumulated on faecal pellets. Snail density and ingestion rate were estimated over different spatial (lower, middle and upper tidal flat) and temporal scales (month and nested lunar phase). Significant variability of *H. ulvae* density was found between months, but also between full and new moon, suggesting a behavioural strategy to avoid predators, as these are mainly depended on light to find their preys. The increase of snail density led to a significant decrease of the ingestion rate of *H. ulvae*. Intraspecific competition for space and food may affect the growth rate of snails and their reproductive potential. The increase on top-down control caused by the increase of grazer density might negatively affect the benthic microphytobenthic production in estuaries: directly by consumption and indirectly through increased bioturbation and consequent significant microalgae resuspension.

Keywords: Chlorophyll *a,* density*, Hydrobia ulvae,* ingestion rate*,* microphytobenthos**,** pheophorbide *a*.

5.2. Introduction

Top-down control by grazers is known to cause important and diverse effects on marine and intertidal environments (Hawkins and Hartnoll, 1983; Anderson and Underwood, 1997; Kelaher et al., 2003; Pillay et al., 2009). Both direct and indirect consequences of grazing on marine intertidal ecosystems have been investigated on mud snails (Morrisey, 1988; Dunn et al., 1999; Hagerthey et al., 2002; Kelaher et al., 2003). These gastropods have been reported to affect directly the community structure and density of other macrofauna (Kelaher et al., 2003; Pillay et al., 2009), sediment stabilization (Andersen et al., 2002; Orvain and Sauriau, 2002) and to have an important role in the regulation of benthic primary productivity (Novak et al., 2001). Indirectly, gastropod grazers can also increase microalgal resuspension due to bioturbation (Andersen, 2001; Andersen et al., 2002; Orvain et al., 2004). *Hydrobia ulvae* and microphytobenthos (MPB) represent an important trophic interaction between community components within benthic food chains. *H. ulvae* are among the most abundant grazers in North Atlantic coasts (Newell, 1979; Barnes, 1999) and benthic microalgae have been widely reported as their main food source (Miller et al., 1996). Although some studies have highlighted the stimulatory effects of grazing on benthic microalgae biomass (Fenchel and Kofoed, 1976; McClatchie et al., 1982), others reported negative effects of gastropods on MPB biomass and productivity (Morrisey, 1988; Blanchard et al., 2000; Hagerthey et al., 2002; Orvain et al., 2004) as well as on the diversity of the microalgal community (Cuker, 1983).

Microalgal surface biomass is highly variable over spatial and temporal scales, being spatially depended on sediment characteristics (Serôdio and Catarino, 2000) and temporally on vertical migration of diatoms as well as on seasonal and tidal cycles (Pinckney and Zingmark, 1991; Serôdio et al., 2001; Consalvey et al., 2004). The top-down control by grazers is conditioned by grazer density, type or species (Morrisey, 1988; Smith et al., 1996; Anderson, 1999; Pillay et al., 2009). The gastropod density is directly depended on growth rate and indirectly affected by several other factors, such as salinity, temperature, sediment grain size, and organic content (Evans et al., 1999). High grazer densities have also been reported to have an important impact by increasing the competition for resources (Levinton, 1979; Blanchard et al., 2000; Barnes, 2001). Levinton (1979) suggested that mud snails could experience

interference competition during feeding activity, showing that egestion rate of *Hydrobia ventrosa* (as a measure of feeding activity) could fell by 50% with density increase. More recently, Barnes (2001) suggested that these findings could be an artefact of coprophagy, as no negative effects of density occurred in egestion rates of *H. ulvae*. However, to our knowledge, no investigations were carried out relating mud snail density and ingestion of MPB in natural conditions, despite the fact that Blanchard et al. (2000) showed a decrease in ingestion rate with density increase under laboratory conditions.

The present work addressed the issue of spatio-temporal variations on *H. ulvae* density and snail ingestion rate, during 4 months and under natural conditions. The effects of lunar phase (full *vs.* new moon) on *H. ulvae* density and benthic microalgae ingestion were also analysed, as well as MPB spatial variation in order to understand what fraction of productive biomass was consumed by mud snails. The research was based on the non-invasive estimation of the ingestion rate of *H. ulvae* using the amount of pheophorbide *a* accumulated in mud snail faecal pellets (Chapter 4).

5.3. Material and methods

5.3.1. Study site and sampling

Samples were collected on an intertidal flat near Gafanha da Encarnação (40° 38´ N, 8° 44´ W), located in the Ria de Aveiro, a mesotidal estuary on the west coast of Portugal. Ria de Aveiro has a very complex geometry characterised by the existence of considerable intertidal areas, distributed along narrow channels and subjected to semidiurnal tides (Dias et al., 2000). The sediment containing *H. ulvae* was collected to a depth of 4 cm using Plexiglass corers (Ø = 12.6 cm), corresponding to a surface area of 125 cm^2 and a volume of ca. 500 cm^3. The samples were collected in three different areas within the tidal flat (upper, middle and lower) (Dyer et al., 2000) every 15 days during a period of 4 months (from May to August 2009), coinciding with full and new moon events (spring tides). Sediment samples were also collected in August 2009 to determine the microalgal biomass. Three replicated Plexiglass corers (Ø = 3.6 cm) were taken in each area. *H. ulvae* samples were taken ca. 2 h after the expected time of the

beginning of the low tide, while sediment samples were collect at the time corresponding to the beginning of low tide in order to match the biomass available at the beginning of the expected feeding time.

Once in the laboratory, the sediment was sieved through a 1 mm mesh to separate the snails from the sediment. The shell height of the snails (apex to aperture) was measured under a stereoscopic microscope (to the nearest 0.1 mm) and the individuals corresponding to adults in the range of 4 to 6 mm (Haubois et al., 2002) were separated and counted to determine the number of individuals per square meter (density). Snails were then placed in defecating chambers with natural seawater but without food for 48 h at 20° C. The defecating chambers were made of plastic bottles having a horizontal barrier made of a 500 µm mesh, which allowed the collection of faecal pellets and prevented their re-ingestion (Chapter 4). The faecal pellets were collected after 48 h and frozen in liquid nitrogen, and immediately stored at -80ºC and later freeze-dried to further pigment analysis. The sediment corers collected to determine the microphytobenthic biomass were sectioned into 2 mm deep sections and Chl *a* was extracted in 90% aqueous acetone and quantified spectrophotometrically following the method of Lorenzen (1967). Chl *a* per unit of area was then calculated following van Leeuwe & Consalvey (2005).

5.3.2. HPLC pigment analysis

Freezed-dried faecal pellets were weighed and extracted in 95% cold buffered methanol (2% ammonium acetate) during 15 min, with 30 s sonication. The solution was then filtered using 0.2 µm-pore filters (Fluoropore PTFE filter membranes) and extracts were immediately injected in a Shimadzu HPLC with photodiode array (SPD-M10ADVP) and fluorescence (RF-10AXL) detectors. Chromatographic separation was achieved using a C18 column for reverse phase chromatography (Supelcosil, 25 cm length, 4.6 mm diameter, 5 µm particles) and a 35 min elution programme. The solvent gradient followed Kraay et al. (1992), with a flow rate of 0.6 ml min^{-1} and an injection volume of 100 µl. Pheophorbides *a* were identified from absorbance spectra and retention times, and concentrations calculated from signals in the photodiode array and fluorescence detectors. Identification and calibration of the HPLC peaks were done

using commercial standards from DHI (Institute for Water and Environment, Hørsholm, Denmark).

5.3.3. Chl *a* ingested by *Hydrobia ulvae*

Chl *a* ingested (µg ind^{-1}) by *H. ulvae* was estimated from the pheophorbide *a* content of faecal pellets (µg ind^{-1}), using the relationship previously established between the pheophorbide *a* content of faecal pellets and the microalgal biomass ingested on the field during the 2 h prior to sampling (Chapter 4):

$$Chl\ a\ (\mu g\ ind^{-1}) = 9.58\ Pheophorbide\ (\mu g\ ind^{-1}) + 0.003 \qquad \text{Equation 5.1}$$

5.3.4. Statistical analysis

Spatial (low, middle and upper tidal flat) and temporal (month), as well as lunar phase (new and full moon) nested within month, variability in *H. ulvae* densities and ingested Chl *a* were tested using a nested ANOVA design (Zar, 1996). The differences between the concentration of Chl *a* as food were tested using two-way ANOVA, regarding the effects of sampling area and lunar phase. ANOVA assumptions were verified and data were transformed whenever necessary, using a square root transformation. When significant differences were found, the Tukey's HSD test was used as a post-hoc comparison of the main effects. All statistical analysis was carried out following Zar (1996).

5.4. Results

5.4.1. Variation of *Hydrobia ulvae* density

Figure 5.1 shows the variation of *H. ulvae* density throughout the study period. In July, mean density reached the minimal values (1872 ± 71 ind m^{-2}), while the highest mean densities were registered in August (3754 ± 167 ind m^{-2}). *H. ulvae* density was not found to vary with the position along the mudflat (Nested ANOVA: F = 0.444, P = 0.650), but vary with the time of year (months; Nested ANOVA: F = 17.367, $P < 0.001$) and with nested lunar phase (Nested ANOVA: F = 40.278, $P < 0.0001$). For the effect of

month on snail density, the post-hoc tests showed that differences occurred between May and all the other months (Tukey's HSD, $P < 0.05$). Densities at new moon were significantly higher than at full moon from June to August, while in May there were not statistically significant differences between lunar phases (Figure 5.1).

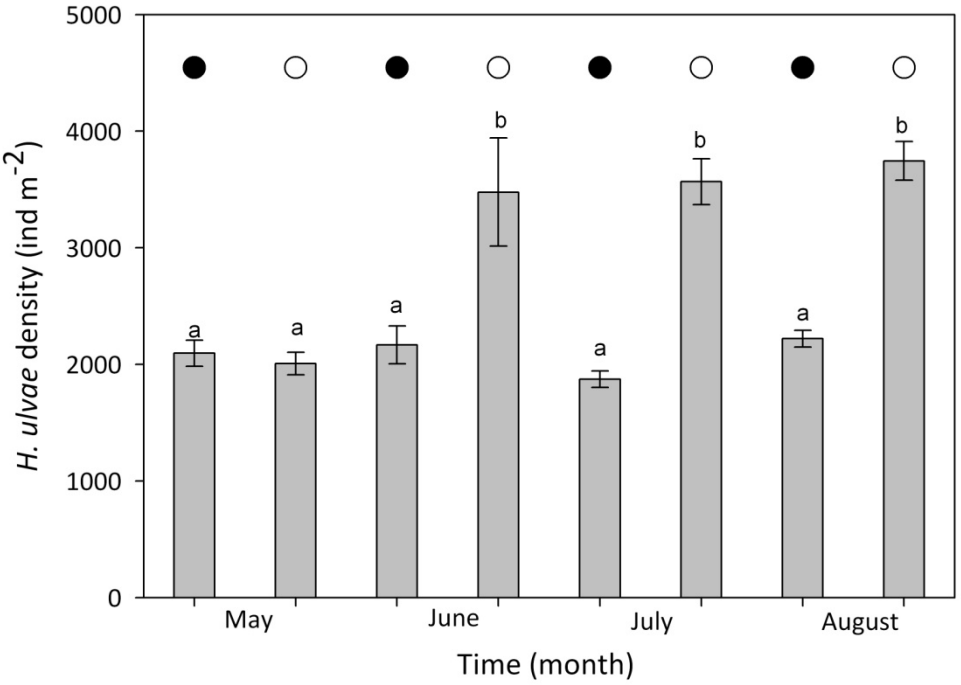

Figure 5. 1 Variation of *Hydrobia ulvae* density (ind m^{-2}) over 4 months synchronized with spring-tides in Ria de Aveiro, Portugal. The open (○) and close (●) circles at the top of the plot represent new and full moon, respectively. Bars represent one standard error. Different letters indicate significant differences (Tukey's HSD, $P < 0.05$).

5.4.2. Variation of *Hydrobia ulvae* ingestion rate

Figure 5.2 shows the monthly variation of *H. ulvae* ingestion rate (µg Chl *a* ind^{-1} h^{-1}) as estimated from pheophorbide *a* content (µg pheophorbide *a* ind^{-1}) of faecal pellets. A considerable variation was found on ingestion rate between *H. ulvae* individuals, as shown by the high standard deviation in most of the treatments. Mud snails ingestion rate was not significantly affect by the occupation of different areas on mudflat, as no significant differences were found between *H. ulvae* individuals from upper, middle and lower tidal flat (Nested ANOVA, F = 0.057, $P = 0.945$). However, statistical analysis showed a significant effect of month (Nested ANOVA, F = 5.567, $P < 0.05$) and of moon (Nested ANOVA, F = 7.004, $P < 0.01$) on the ingestion rate of *H. ulvae* on MPB. The

highest values of individual mean ingestion rate (0.236 ± 0.084 µg Chl *a* ind^{-1} h^{-1}) were found on August under full moon, while *H. ulvae* individuals collected in July during new moon registered the smallest values of mean Chl *a* ingested by mud snails per hour (0.031 ± 0.009 µg Chl *a* ind^{-1} h^{-1}) (Tukey's HSD, Figure 5.2)

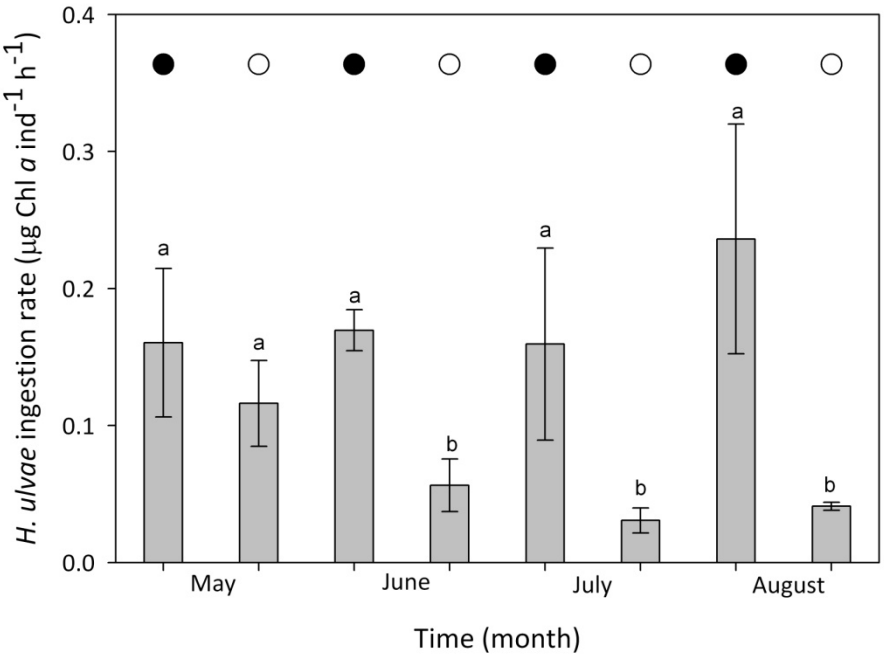

Figure 5. 2 Variation of ingestion rate by *Hydrobia ulvae* (µg Chl *a* ind^{-1} h^{-1}) over 4 months synchronized with spring-tides in Ria de Aveiro, Portugal. The open (○) and close (●) circles at the top of the plot represent new and full moon respectively. Bars represent one standard error. Different letters indicate significant differences (Tukey's HSD, $P < 0.05$).

5.4.3. Relationship between ingestion rate and snail density

The ingestion rate of *H. ulvae* adults varied from 0.018 µg Chl *a* ind^{-1} h^{-1} at new moon to 0.376 µg Chl *a* ind^{-1} h^{-1} at full moon, while densities varied from ca. 3700 to 2100 ind m^{-2} (Figure 5.3). When considering the data obtained in all sampling occasions, the ingestion of Chl *a* per snail per hour was found to decrease markedly with snail density. A doubling of snail density resulted in a decrease of ca. 70% of the ingestion rate of mud snails on MPB. Despite the large data dispersion observed for low snail densities, the variation of ingestion rate with density could be described by a first-order negative exponential curve (Figure 5.3) ($r = 0.745$, $n = 24$, $P < 0.001$):

$$Chl\ \hat{a} = 1.54e^{-0.0008density}$$ Equation 5.2

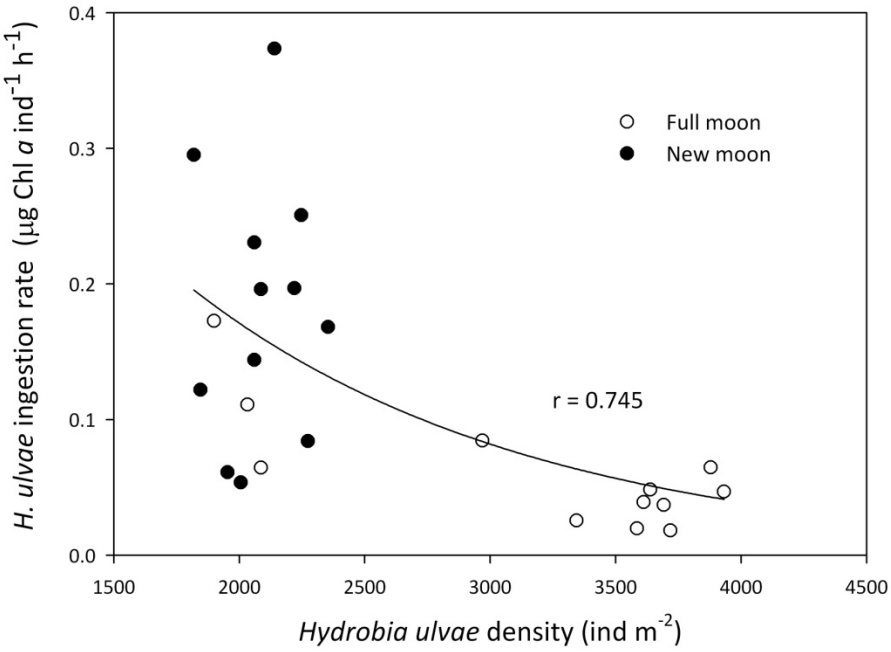

Figure 5. 3 Decrease of ingested Chl *a* (µg Chl *a* ind^{-1} h^{-1}) through a range of *Hydrobia ulvae* densities (ind m^{-2}) under natural conditions, fitted by Equation 5.3.

5.4.4. Microphytobenthos biomass (Chl *a*) available as food source

Chl *a* available for ingestion during August 2009 varied between 44.42 mg m^{-2} in the upper tidal flat and 3.48 mg m^{-2} in the middle zone at new moon, and from 38.82 mg m^{-2} in the upper zone to 2.24 mg m^{-2} in the middle tidal flat at full moon (Figure 5.4). The results of the two-way ANOVA showed that sediment Chl *a* content varied substantially between sampling areas (two-way ANOVA, $P < 0.05$), but not between lunar phase (two-way ANOVA, $P = 0.575$). Within sampling areas there were no significant differences between middle and lower tidal flat (Tukey's test, $P > 0.05$), but there were significant differences between upper and middle as well as between upper and lower tidal flat (Tukey's test, $P < 0.05$) (Figure 5.4). Figure 5.5 displays the percentage of Chl *a* ingested during ca. 2h by the snails relative to the total Chl *a* available (100%, mg m^{-2}). Ingested Chl *a* by mud snails usually represented less then 10% of the total Chl *a* at the sediment surface during diurnal low tides, for full and new moon. However, during full moon in middle tidal flat the ingested Chl *a* was almost 40% of the total Chl *a* available.

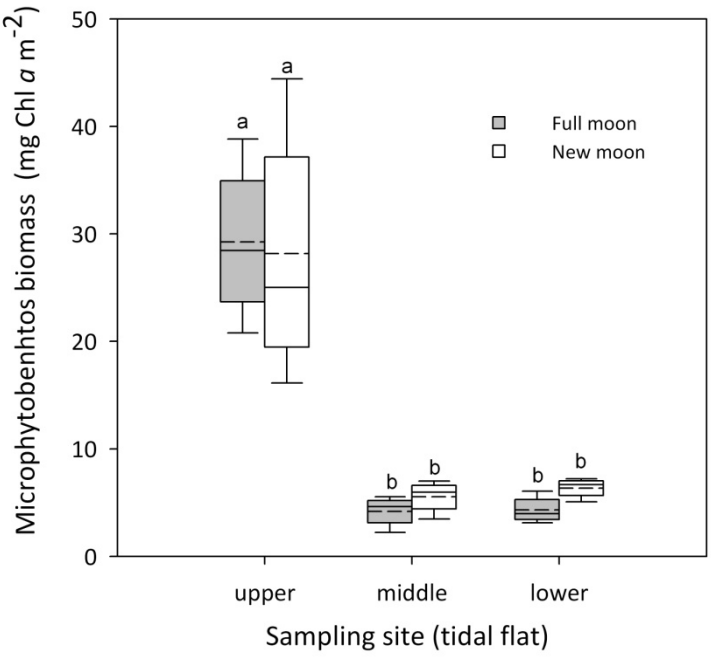

Figure 5. 4 Variation of intertidal sediment microphytobenthic biomass (mg Chl *a* m^{-2}) for different sampling areas (upper, middle and lower tidal flat), at full and new moon. Bars represent one standard error. Simple and dotted horizontal lines inside the boxes represent the median and the mean, respectively. Different letters indicate significant differences (Tukey's HSD, $P < 0.05$).

5.5. Discussion

5.5.1. Short-term changes on *Hydrobia ulvae* density

Density of macrofauna on intertidal environments is an important controlling factor of the abundance of benthic primary producers (Morrisey, 1988), influencing sediment stability (Andersen et al., 2002; Lelieveld et al., 2004) and community structure (Kelaher et al., 2003; Pillay et al., 2009). *H. ulvae* is one of the most abundant species on European estuaries, reaching extremely high densities (Newell, 1979; Sola, 1996; Barnes, 1999; Lillebø et al., 1999; Haubois et al., 2004).

Densities found in the present work (*i.e.,* up to 4000 inv m^2) were apparently lower than those reported in the literature, which can be explained by the fact that they represent the adult individuals ranging from 4 to 6 mm. Considering this, our findings are in agreement with the densities found by Haubois et al. (2004) for *H. ulvae* adults from Marennes-Oléron Bay (France).

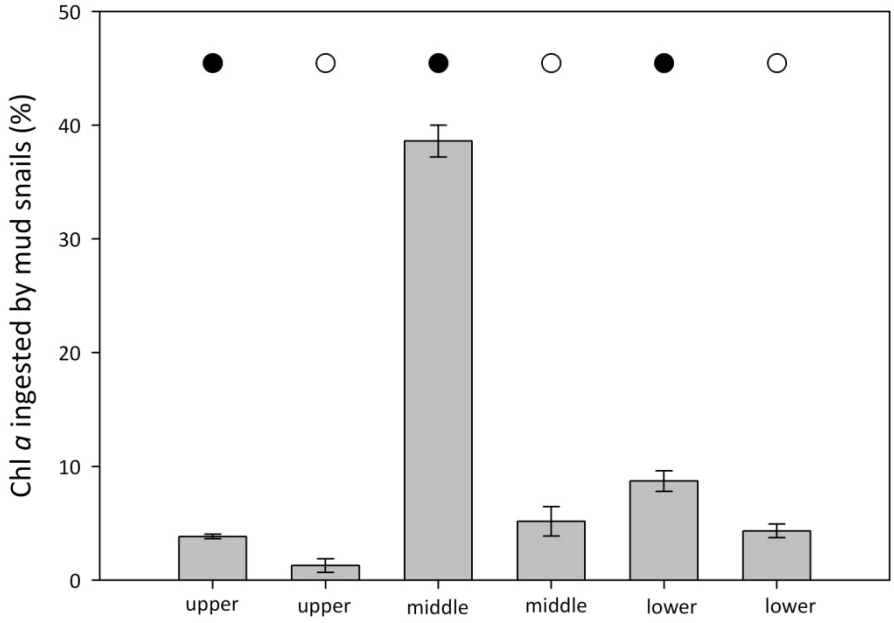

Figure 5. 5 Chl *a* ingested (%) by *Hydrobia ulvae* relatively to the total Chl *a* available (100%) for each sampling areas. The open (○) and close (●) circles at the top of the plot represent new and full moon, respectively. Bars represent one standard error.

H. ulvae density changed throughout the four months of the study showing the same pattern of variation at the three sampling areas, with *H. ulvae* density being frequently higher during new moon than during full moons. To our knowledge, the present study might constitute the first description of short-term changes in *H. ulvae* density during spring tides. In fact, some authors have reported an influence of moon phases on fish populations (Luecke and Wurtsbaugh, 1993; Gaudreau and Boisclair, 2000) or herbivorous zooplankton (Morgado et al., 2006), with densities being often lower during full moons than during new moons.

H. ulvae predators include birds and large number of fishes, such as the flounder *Platichthys flesus* (Aarnio and Mattila, 2000). Flounder is a visual hunter (Mattila and Bonsdorff, 1998), which would take advantage of the brighter nights, such as the ones during full moons. Moreover, prey animals commonly change their behaviour, during intermittent periods, in response to stimuli signalling higher predation risk (Lima, 1998). The reduction of activity or the shift to safer habitats, decreasing encounter rates with predators, are frequent behavioural responses to the increase of predation risk (Lima and Dill, 1990; Lima, 1998). In general, non-lethal effects of predator on prey

populations can be understood in terms of choices that affect the use of space by animals, determining the density and dispersion of prey over large areas (Lima, 1998).

On this context, we suggest that the pattern described on this work for *H. ulvae* might be relate with predation. Lower *H. ulvae* densities under full moon may suggest: i) the presence of a behavioural strategy to avoid predators or ii) the occurrence of higher predation rates that decrease density of preys, as the optimal strategy to predators would be increase their activity under brighter nights.

Nevertheless, the fact that in May no differences were found between full and new moon indicates that the influence of the moon cycle might be more complex and that this issue needs further investigation.

5.5.2. Variation of egested pheophorbide *a* and ingestion rate

Some authors have investigated the relationship between the egestion rate and snail density. Using faecal pellets production as a measure of feeding activity, Levinton (1979) has shown that egestion rates were significantly affected by the increase of density, while Barnes (2001) did not observed any effect. Our results were in agreement with those of Levinton (1979), as a significant increase in pheophorbide *a* content of *H. ulvae* faecal pellets was found during the occurrence of lower densities. The higher levels of pheophorbide *a* egested per individual are not be related with higher egestion rates but associated with the increase of MPB ingestion (Chapter 4). The existence of clear pheophorbide *a* peaks in mud snail faecal pellets detected by HPLC exclude the possibility that the decrease of egestion rate was an artefact of coprophagy (Barnes, 2001). The re-ingestion of their own faecal pellets has been reported for *H. ulvae* (Lopez-Figueroa and Niell, 1988), however the re-ingestion should intensify the degradation of pheopigments and consequently decreased the amount of these in new faecal pellets.

5.5.3. *Hydrobia ulvae* ingestion rate *vs.* density: ecological implications

The lowest mean ingestion rate of mud snails on MPB (<< 0.1 µg Chl *a* ind^{-1} h^{-1}) were recorded during new moon and mainly for high densities (> 3000 snails m^2). Large data dispersion was found for the lower densities, which might have been related with

highly variable ingestion rates already described for *H. ulvae* individuals under non-limiting food (Blanchard et al., 2000).

The observed inverse relationship between ingestion rate and mud snail density extends the results of the behavioural model of Blanchard et al. (2000) to natural conditions. The decrease of the ingestion rate of *H. ulvae* on benthic microalgae could be related with intraspecific competition for space and/or food (Lopez-Figueroa and Niell, 1988; Barnes and Hughes, 1999; Blanchard et al., 2000). Intraspecific food competition leads to lower levels of ingestion by individuals even if the increase of snails per square meter contributes to the increment of global grazer pressure on microalgal biomass (Pillay et al., 2009).

MPB biomass available at the sediment surface during low tide periods is significantly higher in the upper mudflat, even thought the ingestion rate of *H. ulvae* is the same along the mudflat. It indicates that to the range of Chl *a* found *in situ* with our study there was not observed an increase of the ingestion rate of mud snails with increasing MPB biomass (Haubois et al., 2005), even if we observed that at upper tidal flat MPB biomass was one order of magnitude higher than at middle or lower tidal flat. Despite these significant differences between areas, the observed maximum MPB biomass remained lower than the levels usually described in the literature to the occurrence of a microphytobenthic biofilms at the sediment surface during low tide (Brotas et al., 1995; Jesus et al., 2006). It should be associated with changes on the migration amplitude of benthic microalgae along the spring-neap tidal cycle, leading to the occurrence of lower microalgal biomass during spring tides in Ria de Aveiro (Chapter 2). Moreover, independently of the observed MPB biomass, *H. ulvae* ingestion rate did not reached any saturation level.

Results showed that dense *H. ulvae* populations negatively affected benthic microalgal biomass, consuming in average 10% of the MPB biomass available in intertidal areas of Ria de Aveiro. However, on food-poor areas, such as the middle tidal flat, even small densities (new moon) of *H. ulvae* may strongly affect (up to 40%) the concentration of microphytobenthic biomass at the surface of the sediment. The negative effect of mud snail density coincides with the findings of Morrisey (1988) on *H. ulvae* and Pillay et al. (2009) on *Assiminea globulus.* Both have shown that higher density of gastropods lead to a significant reduction on microalgal biomass. The increase of grazer density might

work as a stronger impulse to the increase of top-down control, resulting in significant reductions on the benthic microphytobenthic production in estuaries. MPB biomass could also be affected by the increment of bioturbation depended on snails activity, which should naturally be intensified with increasing densities and erosion rate (Andersen et al., 2002; Orvain et al., 2004).

The decrease of ingestion rate and food shortage leads indirectly to a significant decline on *H. ulvae* growth rate (Morrisey, 1987) and to a decrease of reproductive output (Barnes and Gandolfi, 1998). In fact, Morrisey (1987) found that the growth rate in mud snails decreased rapidly above 3000 ind m^{-2}, which is supported by the significant decrease on *H. ulvae* ingestion rate under identical density found on the present work.

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Chapter 6

Fatty acid profiles indicate the habitat of mud snails *Hydrobia ulvae* within the same estuary: mudflats *vs.* seagrass meadows

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6.1. Abstract

Mud snails *Hydrobia ulvae* are able to occupy different habitats on complex estuarine ecosystems. In order to determine if fatty acid profiles displayed by mud snails can be used to identify the habitat that they occupy within the same estuary, fatty acids of *H. ulvae* from one mudflat and one seagrass meadow in Ria de Aveiro (Portugal) were analysed and compared to those displayed by microphytobenthos (MPB), the green leaves (epiphyte-free) of *Zostera noltii*, as well as those exhibited by the epiphytic community colonizing this seagrass. MPB and epiphytic diatom-dominated samples displayed characteristic fatty acids, such as 16:1*n*-7 and 20:5*n*-3, while 18:2*n*-6 and 18:3*n*-3 were the dominant fatty acids in the green leaves of *Z. noltti.* Significant differences between the fatty acid profiles of *H. ulvae* specimens sampled in the mudflat and the seagrass meadow could be identified, with those from the mudflat displaying higher levels of fatty acids known to be characteristic of MPB. This result points towards the well known existence of grazing activity on MPB by mud snails. Considering the fatty acid profiles displayed by *H. ulvae* inhabiting the seagrass meadows, there is no evidence of direct bioaccumulation of the two most abundant polyunsaturated fatty acids of *Z. noltii* (18:2*n*-6 and 18:3*n*-3) in the mud snails, which probably indicates that either these compounds can be metabolized to produce energy, used as precursors for the synthesis of essential fatty acids, or that the snails do not consume seagrass leaves at all. Moreover, the fatty acid profiles of mud snails inhabiting the seagrass meadows revealed the existence of substantial inputs from microalgae, suggesting that the epiphytic community colonising the leaves of *Z. noltii* display an important role on the diet of these organisms. This assumption is supported by the high levels of 20:5*n*-3 and 22:6*n*-3 recorded in mud snails sampled from seagrass meadows. In conclusion, fatty acid analyses of *H. ulvae* can be successfully used to identify the habitat occupied by these organisms within the same estuary (e.g. mudflats and seagrass meadows) and reveal the existence of contrasting dietary regimes.

Keywords: Fatty acids, *Hydrobia ulvae*, microphytobenthos, mudflat, seagrass meadow, *Zostera noltii*.

6.2. Introduction

Estuaries contain a spatially complex diversity of habitats, which contribute to a highly dynamic food environment. Grazing-benthic food chains are common in intertidal habitats, representing and regulating the flow of nutrients and energy from the bottom and through up the estuarine ecosystem (Carlier et al., 2007). The mud snail *Hydrobia ulvae* (Pennant) is an important primary consumer in temperate European estuarine ecosystems (Newell, 1965; Riera, 2010) and can be found abundantly in a variety of intertidal habitats, from mudflats to seagrass meadows (Newell, 1965; Lillebø et al., 1999; Riera, 2010). The population structure of these mud snails is influenced by large-scale movements of individuals (Haubois et al., 2002; Haubois et al., 2004), which results in the snails experiencing a wide range of habitats and allows them to explore several food sources. Benthic primary productivity in estuarine habitats is generally dominated by microphytobenthos (MPB) production (Underwood and Kromkamp, 1999), even though these environments may also contain a multiplicity of other primary producers (e.g. macroalgae, macrophytes). Muddy sediments usually include digestible and nutritive food sources, such as benthic microalgae or bacterial communities, while seagrass meadows are dominated by less digestible marine vascular plants (Pascal et al., 2008). The existence of a complex trophic link between *H. ulvae* and MPB in mudflats has already been indicated (Fenchel et al., 1975; Morrisey, 1988; Haubois et al., 2002), with mud snails density and sediment chlorophyll *a* content playing a major role on *H. ulvae* ingestion rates (Blanchard et al., 2000; Haubois et al., 2005). *Zostera noltii* is a widely distributed seagrass in estuarine ecosystems (Duarte, 1989; Plus et al., 2001), forming highly structured intertidal habitats and providing trophic resources and refuge areas. These marine plants have an important ecological function on areas inhabited by *H.ulvae,* as their meadows provide a greater environmental stability (Lillebø et al., 1999; Cardoso et al., 2008). Its direct role in the dietary regime of estuarine invertebrates is somehow limited, as recognized for marine vascular plants in general (Kharlamenko et al., 2001). However, *Z. noltii*'s epiphytic community constitutes an important food source, as epiphytes are potentially important primary producers for herbivores in seagrass meadows (Lebreton et al. 2009).

Fatty acids have been widely used as qualitative markers to confirm and trace trophic relationships in marine and estuarine environments (Phillips et al., 2003; Shin et al., 2008; Auel et al., 2002; Dalsgaard et al., 2003). These compounds display great structural diversity and substantial taxonomic specificity. In general, marine primary producers have characteristic fatty acids and these are transferred into the storage lipids of higher trophic organism with unchanged or recognizable forms (Dalsgaard et al., 2003; Shin et al., 2008). The identification of characteristic fatty acid patterns at different trophic levels allows researchers to trace the relationship between primary producers, primary consumers and/or higher trophic organisms (Biandolino et al., 2008). The present work tests if fatty acid profiles can be used to identify the habitat (mudflat *vs*. seagrass meadows) occupied by *H. ulvae* in Ria de Aveiro, Portugal, and if they can reveal the existence of contrasting dietary regimes when these gastropods colonise different habitats within the same estuary.

6.3. Material and methods

6.3.1. Sampling

H. ulvae specimens were collected in Ria de Aveiro, a shallow coastal lagoon located on the north west coast of Portugal (Dias et al., 2000). This coastal lagoon has a very complex geometry and is characterised by the existence of significant intertidal zones, namely mudflats and salt marshes, distributed along narrow channels (Dias et al., 2000). The sampling sites were a seagrass meadow located in the east margin of Canal de Mira at Gafanha da Encarnação (40º 38' N, 8º 44' W) and a mudflat in the west margin of Canal de Ílhavo, near Vista Alegre VA (40º 35' N, 8º41' W). All *H. ulvae* specimens were randomly collected in an area corresponding to 10 m^2. Snails were immediately separated from the sediment by sieving (still in the field) and were carefully scraped from the leaves in seagrass meadow. Mud snails collected in the mudflat were either buried or crawling over the mud, while in the seagrass meadow only the specimens located over the green leaves of *Z. noltii* were sampled. Both samples were rapidly transported to the laboratory, with mud snails always being kept submerged in water collected at each sampling location. *H. ulvae* from both sampling

sites were further separated according to shell height (the maximum distance from apex to aperture), which was determined under a stereoscopic microscope to the nearest 0.1 mm (Mouritsen and Thomas Jensen, 1994; Grudemo and Johannesson, 1999). Only specimens corresponding to adult size (> 4 mm) were selected for the present study (Haubois et al., 2002), with six samples of ca. 50 individuals each being prepared for fatty acid analysis. Snails gut was not removed, as during the time elapsed from sampling until freezing all ingested material was egested by the snails (through fecal pellets). At the time of freezing, no fecal pellets were recorded on any of the selected specimens, which leaded us to assume that the amount of ingested food still present in the gut was neglectable.

MPB samples were collected on the mudflat using sediment corers (Ø 6.8 mm). Later, in the laboratory, benthic microalgae were separated from the mud using the lens tissue method (Eaton and Moss, 1966): four pieces of lens tissue were placed on the air-exposed surface of the sediment during the expected time of diurnal low tide; the upper two pieces of lens tissue were removed after approximately 1 h; benthic microalgae which had migrated through the lens tissue were ressuspended on artificial sea water (prepared using freshwater purified by a reverse osmosis unit and mixed with Pro-Reef® salt produced by Tropic Marine®). Three replicate samples were used for determination of the relative abundance of major taxonomic groups (fixed in 1% v/v formaldehyde) by counting a minimum of 400 cells, while other six samples were concentrated for fatty acid analysis. Six samples of *Z. noltii* were collected on the seagrass meadow referred above for fatty acid analysis. Only the green leaves of *Z. noltii* were considered for the present study, as the majority of mud snails observed in the seagrass meadow were clustered in their surface. All green leaves were carefully cleared of epiphytes or other particles by rinsing the leaf blade with artificial sea water (see above for preparation details) and carefully scraping their surface with a razor blade (Kharlamenko et al., 2001). Green leaves were also cut into small pieces to enable a more efficient fatty acid extraction. Nine samples of the epiphytic community colonizing *Z. noltii* were collected by scraping the surface of their green leaves, with six samples being used for fatty acid analyse and the other three for the determination of the relative abundance of major taxonomic groups. This determination followed the same procedure described above for MPB samples. All collected samples (snails, MPB,

green leaves of *Z. noltii* and *Z. noltii* epiphytes) were weighted and freeze-dried prior to fatty acid analysis.

6.3.2. Fatty acid analysis

Fatty acid extraction and preparation of methyl esters were carried out according to Lepage and Roy (1986) modified by Cohen et al. (1988). Freeze-dried samples (100 mg) were transmethylated with 5 ml of methanol/acetyl chloride (95:5 v/v). The mixture was sealed in a light-protected Teflon-lined vial under nitrogen atmosphere and heated at 80°C for 1 h. The vial contents were then cooled, diluted with 1 ml water and extracted with 2 ml of n-heptane. The heptane layer was dried over Na2SO4, evaporated to dryness under a nitrogen atmosphere and redissolved in heptane, which contained the methyl esters. The methyl esters were then analyzed by gas-liquid chromatography, on a VARIAN (Palo Alto, USA) 3800 gas-liquid chromatograph (Volkman et al.), equipped with a flame ionization detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 µm) Supelcowax 10 (SUPELCO, Bellafonte PA, USA) with helium as carrier gas at a flow rate of 1.3 ml min^{-1}. The column temperature was programmed at an initial temperature of 200°C for 10 min, then increased at 4°C min^{-1} to 240°C and held there for 16 min. Injector and detector temperatures were 250 and 280°C, respectively, and split ratio was 1:100. Peak identification was carried out using known standards (Nu-Chek-Prep, Elysian, USA). Peak areas were determined using Varian software.

6.3.3. Data Analysis

The percentage of individual fatty acids and fatty acid ratios present in mud snails sampled from the mud flat and the seagrass meadow, as well as in food sources (MPB, green leaves of *Z. noltii* and epiphyte community) were compared using the Student's *t*-test and ANOVA, respectively. Both analyses were performed using STATISTICA v8, StatSoft Inc, USA. Samples were also analysed using multidimensional scaling (MDS) ordination on the Bray-Curtis similarity index, described by Clarke and Gorley (2006). The MDS stress value indicates the level of an appropriate representation of the multidimensional distances. Data were fourth root transformed prior to analysis and in

order to validate our interpretation of the MDS we performed an analysis of similarity (ANOSIM), built on a simple non-parametric permutation procedure, and applied to the similarity matrix underlying the ordination of the samples. This procedure allows the identification of differences in the fatty acid profiles displayed by mud snails from both habitats and by MPB, green leaves of *Z. noltii* and *Z. noltii* epiphytes. Similarity percentages (SIMPER) were also explored to examine the similarity within the fatty acids of: i) *H. ulvae* from both sampling sites, ii) MPB, iii) the green leaves of *Z. noltii*, and iv) the epiphyte community of *Z. noltii*. All multivariate analyses were performed using PRIMER v6 with PERMANOVA add-on (Primer-E Ltd, Plymouth, UK).

6.4. Results

6.4.1. Taxonomic composition of MPB

The microphytobenthic assemblage from the mudflat was clearly dominated by diatoms (74.2 ± 3.3%). The relative abundance of euglenophytes was 23.2 ± 1.6%, while cyanobacteria and other benthic microalgae accounted for less than 5% of the microphytobenthic assemblage. The epiphytic algae community of the green leaves of *Z. noltii* was dominated by diatoms (91.2 ± 1.3%), with other benthic microalgae and cyanobacteria also being observed (3.5 ± 0.2 and 5.3 ± 1.2 %, respectively).

6.4.2. Fatty acid composition of food sources

MPB and green leaves of *Z. noltii* exhibited substantial variations on their fatty acid profiles, while the epiphytic algae of *Z. noltii* showed several similarities with the profile found for MPB (Table 1). The main differences in MPB fatty acid profiles, when compared with the green leaves of *Z. noltii*, were a significantly higher content of 16:1*n*-7 (palmitoleate), 20:5*n*-3 (eicosapentaenoic acid, EPA) and 22:6*n*-3 (docosahexaenoic acid, DHA) and a significantly lower content of 18:2*n*-6 (linoleic acid, LA) and 18:3*n*-3 (alpha-linolenic acid, ALA) (*Tukey's test*, $P < 0.05$, Fig. 1A). The same significant differences were found for 16:1*n*-7, ALA and EPA when epiphytes were compared with the green leaves of *Z. noltii*. Moreover, the epiphytic community also exhibited significant differences in the levels of LA and DHA when compared with MPB

and *Z. noltii* (*Tukey's test*, $P < 0.05$, Fig. 1A). Together, the fatty acids 16:1*n*-7, EPA and DHA comprised 46.44 ± 0.98% and 49.25 ± 1.05% of the total pool of fatty acids recorded for MPB and for the epiphytes of *Z.noltii* (respectively), while the sum of LA and ALA represented 74.19 ± 1.74% of the total pool of fatty acids detected in the green leaves of *Z. noltii*. Saturated fatty acids (SFAs) were predominant in both MPB and *Z. noltii*, with their percentage of total SFAs (29.5 ± 1.5 and 30.17 ± 1.9%, respectively) not being significantly different (*Tukey's test,* $P > 0.05$, Fig. 2A). The epiphytes from the green leaves of the seagrass showed a significantly lower content of SFAs when compared with both MPB and *Z. noltii* (*Tukey's test,* $P < 0.05$, Fig. 2A). Monounsaturated fatty acids (MUFAs) varied from 3.05 ± 0.9% in *Z. noltii* to 37.56 ± 0.5% in MPB samples, showing a statistically significant difference (*Tukey's test,* $P < 0.05$, Fig. 2A). A significant difference was also found between *Z. noltii* and its epiphytic community (*Tukey's test,* $P < 0.05$, Fig. 2A). Significant differences were also recorded between the percentage of polyunsaturated fatty acids (PUFAs) present in MPB and in the green leaves of *Z. noltii*, *as* well as in their epiphytes (*Tukey's test,* $P < 0.05$, Fig. 2A). Both MPB and *Z. noltii* epiphytes showed significantly higher levels of highly unsaturated fatty acids (HUFAs) when compared with *Z. noltii* (*Tukey's test,* $P < 0.05$, Fig. 2A). PUFAs displayed higher values in *Z. noltii* samples, when compared with those from MPB and *Z. noltii* epiphytes, while HUFAs displayed an inverse pattern, with higher levels being recorded in MPB and in the samples of *Z. noltii* epiphytes.

A significant difference in the fatty acid profiles of MPB, green leaves of *Z. noltii* and their epiphytes was also evidenced by the analysis of similarity (ANOSIM, R = 1, $P = 0.001$) and sustained by the MDS plot (stress value = 0.01, representative of a clear separation) (Fig.3A). Although the food sources available at both habitats occupied different regions on the MDS plot, samples from MPB and *Z. noltii* epiphytes are displayed closer to each other.

6.4.3. Fatty acid composition of mud snails

The fatty acid composition (% of total fatty acids) of the mud snail *H. ulvae* from both estuarine habitats is listed in Table 2. All specimens of *H. ulvae* exhibited high levels of

EPA and 20:0, with EPA showing statistically significant differences between sites (t = 17.08, P < 0.001).

Table 6.1 Fatty acid composition (% of total fatty acids) of MPB, green leaves (epiphyte-free) of *Zostera noltii* and epiphytes of *Z. noltii* from a mudflat and a seagrass meadow (respectively) in Ria de Aveiro, Portugal (mean ± SD n = 6).

Fatty acid	MPB	*Z. noltii*	Epiphytes *of Z. noltii*
14:0	3.42 ± 0.33[a]	0.80 ± 0.23[b]	4.92 ± 0.41
16:0	17.68 ± 0.97[a]	12.99 ± 1.74[b]	16.07 ± 1.53 [a]
16:1*n*-7	21.75 ± 0.58[a]	0.24 ± 0.15[b]	23.87 ± 4.27 [a]
16:3*n*-4	1.65 ± 0.28[a]	2.28 ± 0.15[b]	1.22 ± 0.49 [a]
17:0	2.75 ± 0.16[a]	0.51 ± 0.12[a]	n.d.
18:0	2.31 ± 0.10[a]	2.64 ± 0.11[b]	0.81 ± 0.07 [c]
18:1*n*-9	2.89 ± 0.07[a]	0.62 ± 0.05[b]	4.65 ± 1.13 [c]
18:1*n*-7	3.04 ± 0.28[a]	1.17 ± 0.03[b]	3.22 ± 0.30 [a]
18:2*n*-6	0.85 ± 0.43[a]	14.82 ± 0.30[b]	2.12 ± 0.38 [c]
18:3*n*-3	1.51 ± 0.14[a]	46.38 ± 1.65[b]	4.65 ± 1.05 [a]
18:4*n*-3	1.36 ± 0.02 [a]	n.d.	2.59 ± 0.26 [a]
20:0	n.d.	2.65 ± 0.77	n.d.
20:1*n*-9	2.68 ± 0.21	n.d.	1.25 ± 0.52
20:4*n*-6	2.06 ± 0.43[a]	0.49 ± 0.12[b]	0.52 ± 0.05 [b]
20:5*n*-3	13.37 ± 0.81[a]	0.17 ± 0.09[b]	18.39 ± 3.46 [a]
22:0	n.d.	4.86 ± 0.06	n.d.
22:6*n*-3	11.32 ± 0.89[a]	0.69 ± 0.03[b]	7.00 ± 1.29 [c]
24:0	2.89 ± 0.45[a]	5.71 ± 0.48[b]	n.d.
24:1*n*-9	7.21 ± 0.54[a]	1.03 ± 0.72[b]	n.d.
Others	1.27	1.96	8.73

Note: Only the fatty acids whose content exceeds at least 1% in MPB or *Z. noltii* are displayed. n.d. – not detected. Different letters within the same fatty acid (row) represent significant differences (Tukey's test, P < 0.05).

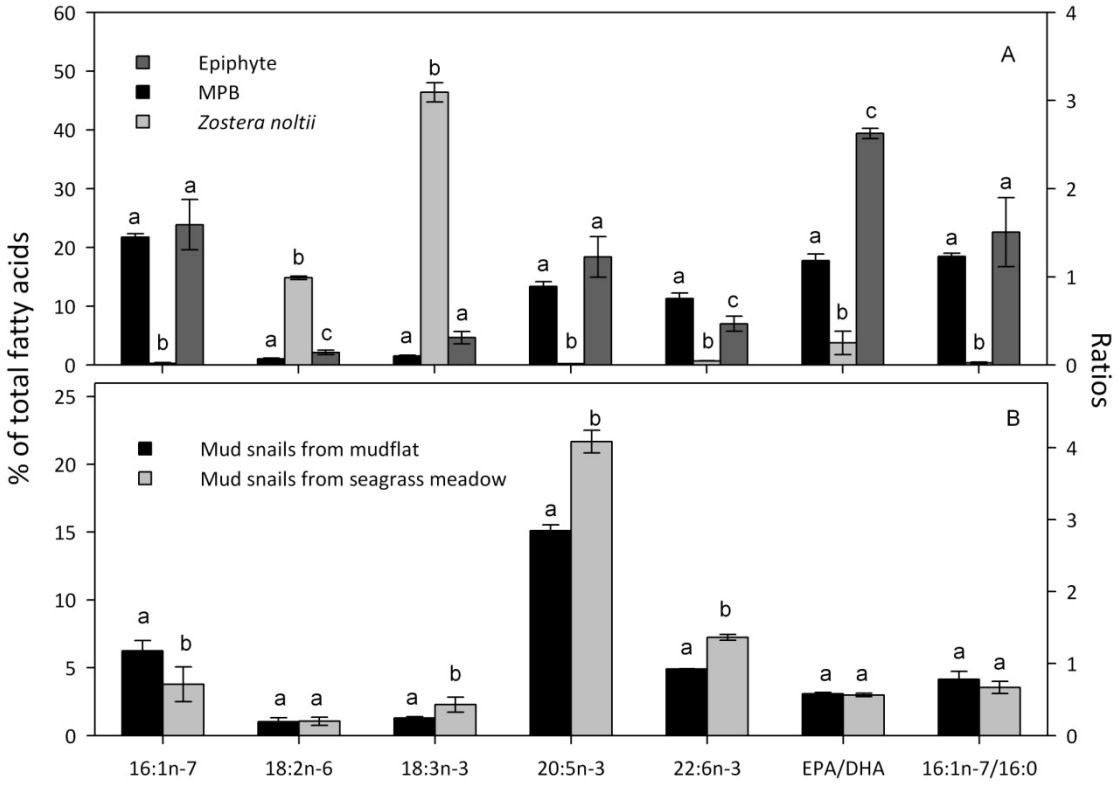

Figure 6. 1 Content of specific individual fatty acid markers (% of total FAs) and ratios in both microalgae communities (MPB and epiphytes of *Z.noltii*) and in green leaves (epiphyte-free) of *Zostera noltii* (*Zostera*) (A) and in *Hydrobia ulvae* individuals from the mudflat or the seagrass meadow (B). Values are mean ± SD (n = 6). Different letters within the same fatty acid or ratio represent significant differences (Tukey's test, $P < 0.05$ in plot A and Student's *t*-test, $P < 0.01$ in plot B).

Mud snails colonising the mudflat displayed significantly higher levels of 20:1*n*-9 (eicosenoic acid) ($t = 6.80$, $P < 0.001$) and 20:4*n*-6 (arachidonic acid, AA,) ($t = -4.26$, $P < 0.01$) than those inhabiting the seagrass meadow. However, individuals from the seagrass meadow displayed significantly higher levels of 22:5*n*-3 (docosapentaenoic acid, DPA) ($t = -17.08$, $P < 0.001$). The SFAs and MUFAs were significantly higher in mud snails from the mudflat (SFAs: $t = -6.05$, $P < 0.001$, MUFAs: $t = 10.04$ $P < 0.001$, Fig. 2B). The major SFAs registered, in increasing order, were 16:0 and 18:0. MUFAs varied between 32.03 ± 1.9% and 21.13 ± 1.8% of total fatty acids in mud snails living on the mudflat and the seagrass meadow, respectively. PUFAs were characterized by lower levels than MUFAs, varying from 2.34 ± 0.3% (mudflat) to 6.65 ± 0.9% (*Z. noltii* meadow), while the percentage of HUFAs reached 46.14 ± 1.7% in mud snails from the seagrass meadow and 32.32 ± 0.5% in those from the mudflat.

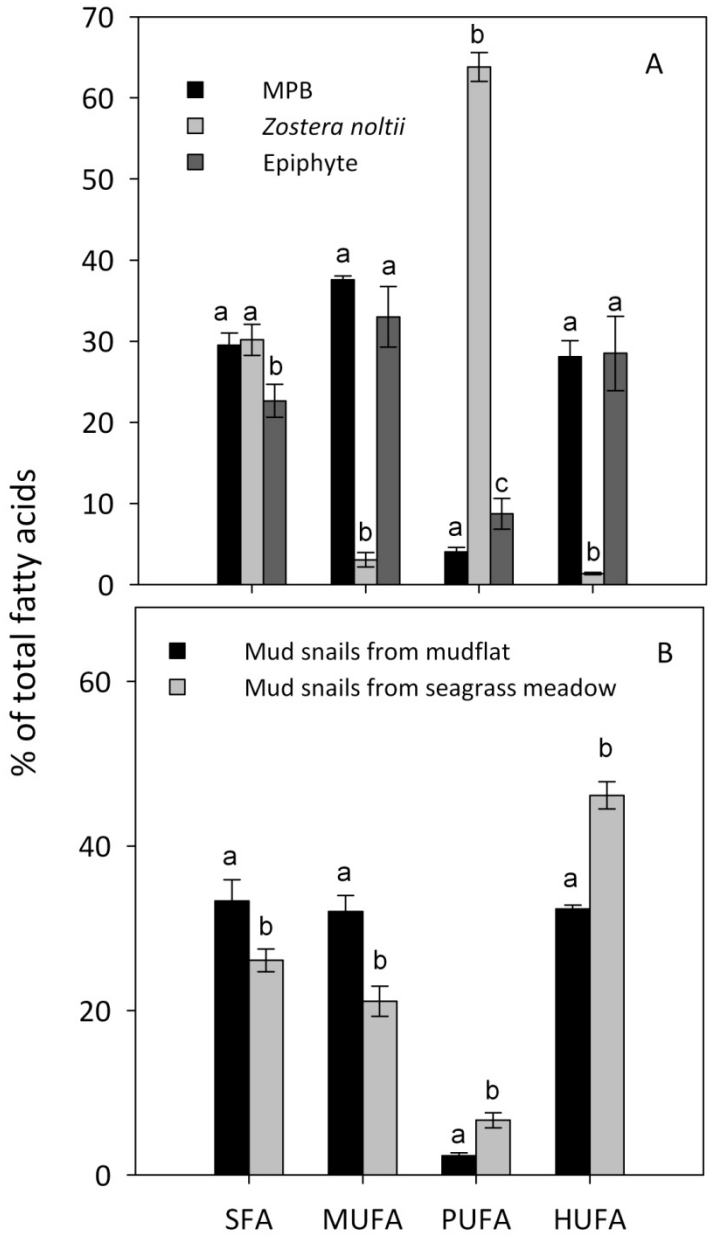

Figure 6. 2 Content of major classes of FAs in the profile of both microalgae communities (MPB and epiphytes of *Z.noltii*) and in the green leaves (epiphyte-free) of *Zostera noltii* (*Zostera*) (A) and in *Hydrobia ulvae* individuals from the mudflat or the seagrass meadow (B). Values are mean ± SD (n = 6). Different letters within the same fatty acid class means significant differences (Tukey's test, P < 0.05 in plot A and Student's *t*-test, *P* < 0.001 in plot B). SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; HUFA – highly unsaturated fatty acids.

Both groups of unsaturated fatty acids exhibited statistically significant differences between habitats, being significantly lower in *H. ulvae* individuals from the mudflat (PUFAs: t = -10.86, P < 0.001 and HUFAs: t = -19.44, P < 0.001, Fig. 2B). The mean values of *iso*- and *anteiso* branched fatty acids, such as *iso*- and *anteiso*17:0,

characteristic of marine bacteria, were always very low or not detectable. The level of 18:1*n*-7 (vaccenic acid) was always significantly higher in mud snails inhabiting *Zostera* meadows (4.67 ± 0.88%) than in those occurring in the mudflat (5.95 ± 0.22%) (t = 3.43, $P < 0.01$).

Table 6.2. Fatty acid composition (% of total fatty acids) of *Hydrobia ulvae* from a mudflat and a seagrass meadow in Ria de Aveiro, Portugal (mean ± SD n = 6).

Fatty acid	*H. ulvae* mudflat	*H. ulvae* seagrass meadow
16:0	8.14 ± 1.76[a]	5.68 ± 1.85[b]
16:1*n*-7	6.25 ± 0.75[a]	3.78 ± 1.28[b]
17:0	2.92 ± 0.23	n.d.
18:0	8.64 ± 1.05[a]	5.35 ± 1.17[b]
18:1*n*-7	5.94 ± 0.22[a]	4.67 ± 0.88[b]
18:2*n*-6	1.04 ± 0.29[a]	1.05 ± 0.30[a]
18:3*n*-3	1.30 ± 0.09[a]	2.28 ± 0.56[b]
20:0	10.07 ± 3.78[a]	11.52 ± 3.28[a]
20:1*n*-9	8.97 ± 0.30[a]	7.24 ± 0.55[b]
20:2*n*-6	n.d.	1.71 ± 0.04
20:3*n*-6	n.d.	1.60 ± 0.63
20:4*n*-6	6.35 ± 0.76[a]	7.87 ± 0.43[b]
20:5*n*-3	15.10 ± 0.44[a]	21.67 ± 0.84[b]
22:1*n*-9	4.68 ± 1.78[a]	1.88 ± 0.19[b]
22:5*n*-3	5.96 ± 0.03[a]	8.37 ± 0.89[b]
22:6*n*-3	4.90 ± 0.02[a]	7.24 ± 0.21[b]
24:0	1.83 ± 0.06[a]	3.09 ± 0.30[b]
24:1*n*-9	4.97 ± 0.16[a]	2.61 ± 0.87[b]
Others	2.91	2.38

Note: Only fatty acids whose content exceeds at least 1% in *Hydrobia ulvae* from mudflat or seagrass meadow are displayed. n.d. – not detected. Different letters within the same fatty acid (row) represent significant differences (Student's t-test, $P < 0.05$).

C6. FA profiles indicate the habitat of *H. ulvae*

The content of fatty acids considered to be good trophic markers was compared between sites and also between MPB and green leaves of *Z. noltii,* as well as between these and *Z. noltii* epiphytes. The percentage of fatty acids known to be characteristic of diatoms (16:1*n*-7 and EPA) was significantly higher in MPB and also in samples from *Z. noltii* epiphytes (Fig.1A), while significant levels of LA and ALA were normally found on samples from the green leaves of *Z. noltii* (see section 3.2.). Distinct differences between sampling areas were detected for the fatty acids characteristic of both microalgae communities (benthic and epiphytic: EPA: $t = 17.06$, $P < 0.001$ and 16:1*n*-7: $t = -4.02$, $P < 0.01$) (Fig.1B). Regardless of the sampled habitat, mud snails always displayed lower levels of fatty acids known to be characteristic of *Z. noltii* (Fig. 1B). The statistical analysis on the contribution of ALA to the total fatty acids of *H. ulvae* between sites did not show a considerable difference between the mudflat and the seagrass meadow ($t = 0.09$, $P = 0.931$). On the other hand, LA was significantly higher in mud snails from the seagrass meadow ($t = 4.24$, $P < 0.01$). Mud snails from both locations exhibited higher levels of palmitic acid than 16:1*n*-7, contributing to low 16:1*n*-7/16:0 ratios. Both16:1*n*-7/16:0 and EPA/DHA ratios did not differ significantly between sampled habitats (16:1*n*-7/16:0: $t = 2.03$, $P = 0.07$ and EPA/DHA: $t = -1.38$, $P = 0.197$) (Fig.1B). The fatty acid 22:6*n*-3 was also recorded in significantly different levels in *H. ulvae* collected in both habitats ($t = 27.12$, $P < 0.001$).

Considering the whole fatty acid profile of sampled mud snails, there was a significant difference in those inhabiting the mudflat and those from the seagrass meadow (ANOSIM, R = 0.967, $P = 0.002$). A high degree of within group similarity was displayed for each of the estuarine habitats (SIMPER: 99% and 97% similarity, for mudflat and seagrass meadow, respectively). This is reinforced by the MDS analysis, which also showed a high degree of within group similarity and a distinct separation between *H. ulvae* individuals from each habitat (Fig. 3B). The stress value for the multidimensional scaling was 0.01, which is representative of a clear separation between sites.

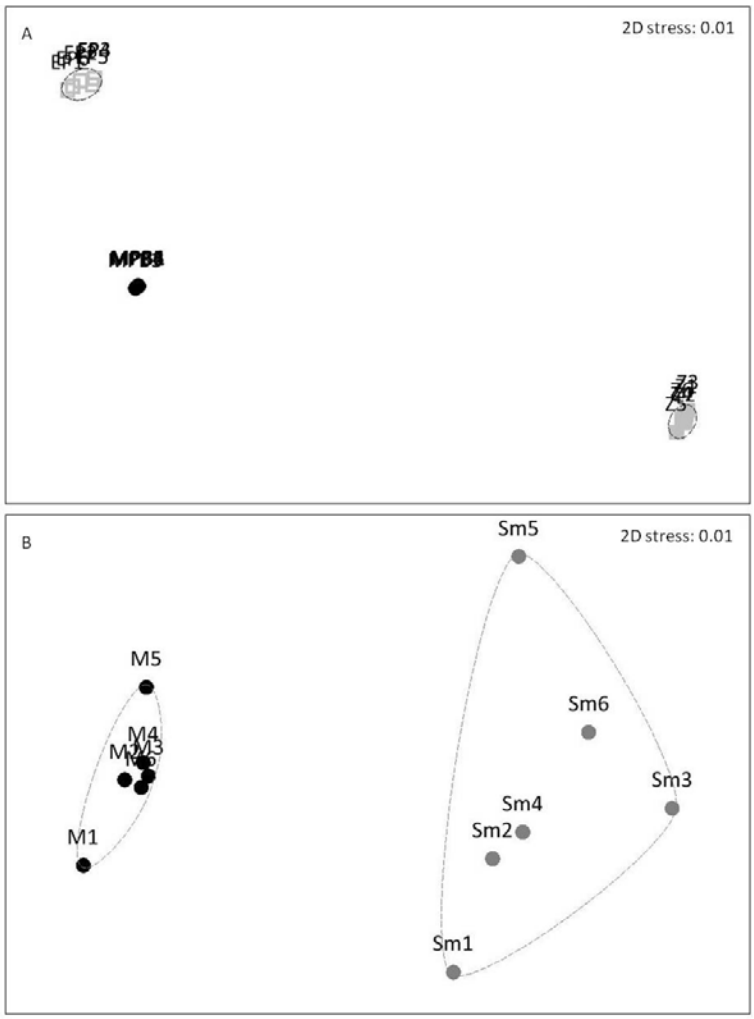

Figure 6. 3 MDS plot of the fatty acid profiles of microphytobenthos (MPB), green leaves (epiphyte-free) of *Zostera noltii* (Z) and epiphytes (EP) (A) and of *Hydrobia ulvae* individuals from the mudflat (M) or the seagrass meadow (Sm) (B). Dotted lines represent 95% of similarity (SIMPER). Dark and gray colours represent the mudflat and seagrass meadow habitat, respectively, on both plots (A and B).

6.5. Discussion

6.5.1. Fatty acid composition of food sources

Marine and estuarine primary producers biosynthesise an important structural diversity of fatty acids (Dalsgaard et al., 2003), as demonstrated by the diversity of fatty acids recorded in the present work in both microalgae communities (MPB and epiphytes) and in the green leaves of *Z. noltii*. The existence of specific fatty acids or ratios associated with particular groups of primary producers allows us to use them as fatty acid markers along trophic links. The main primary producers in the benthic intertidal community of Ria de Aveiro (Portugal), MPB and *Z. noltii*, exhibited marked

differences in their fatty acid profiles. Moreover, the epiphytic community of the seagrass *Z. noltii* also showed a distinct fatty acid profile, namely when compared with *Z. noltti*. This aspect was emphasized by the distinct distribution exhibited by the samples through the application of the multidimensional scaling analysis. The main fatty acids found in MPB and in *Z. noltii* epiphytes profiles were the SFA 16:0, the MUFA 16:1*n*-7 and the HUFA EPA, which are known to be typically associated with microphytobenthic assemblages (Kharlamenko et al., 2008) and the epiphytic community of *Zostera spp.* (Kharlamenko et al. 2001, Jaschinski et al. 2008). The fatty acids 16:1*n*-7 and EPA are biosynthesised by diatoms (Behrens and Kyle, 1996; Volkman et al., 1998; Kharlamenko et al., 2001; Dalsgaard et al., 2003) and have been frequently used as benthic diatom markers in food chains studies (Kharlamenko et al., 1995; Bachok et al., 2003). The relevance of diatoms in the taxonomic composition of MPB and epiphytes of *Z. noltii* was highlighted in the present study by the ratio 16:1*n*-7/16:0 being greater than 1, as this is commonly considered as an indicator of diatoms dominance (Napolitano et al., 1997; Kharlamenko et al., 2001).

Although the levels and ratios of fatty acids recorded in both benthic and epiphytic microalgal samples generally agree with the taxonomic composition determined in this study (ca. 74% and 91% of diatoms), the content of DHA is somehow puzzling. This highly unsaturated fatty acid is a typical marker of dinoflagellates (Kharlamenko et al., 2001; Bachok et al., 2003; Dalsgaard et al., 2003). However, this group of microalgae was not recorded in the taxonomic analysis of MPB or *Z. noltii* epiphytes. HUFAs, such as EPA or DHA, are often biosynthesized *de novo* by primary producers (Veloza et al., 2006) and Moreno et al. (1979) already provided evidence that the diatom *Phaeodactylum tricornutum* is able to synthesized DHA *de novo*. This ability displayed by some diatoms can explain the high levels of DHA recorded especially in MPB.

The fatty acid profiles of *Z. noltii* displayed high levels of specific fatty acids, such as LA and ALA, which are frequently associated with the green leaves of these marine plants (Canuel et al., 1997; Kharlamenko et al., 2001; Sanina et al., 2008). Our data are in good accordance with the results published by Kharlamenko et al. (2001), who have showed that the sum of LA, ALA and palmitc acid comprised up to 80% of the total fatty acids in the green leaves of the seagrass *Zostera marina*. These fatty acids are highly relevant in trophic relations, as both LA and ALA can be used as precursors for

the synthesis of essential fatty acids by heterotrophic organisms (Dalsgaard et al., 2003; Veloza et al., 2006).

6.5.2. Fatty acid composition of *Hydrobia ulvae*

The fatty acid profiles of mud snails showed an extensive contribution of SFAs, MUFAs and HUFAs, either in specimens collected in the mudflat or the seagrass meadow, with PUFAs being the less abundant group of fatty acids present in *H. ulvae* from both locations. The comparison of mud snails' fatty acid profiles among sites indicates that diatom-dominated MPB communities were clearly the major food source consumed by *H. ulvae* in the mudflat. The assumption that MPB assemblages represent an important nutritional component in *H. ulvae* diet has already been suggested in several studies (Fenchel et al., 1975; Blanchard et al., 2000; Haubois et al., 2005). However, to our knowledge, the present work is the first study providing analytical evidence of this trophic relationship based on fatty acid profiles.

Several gastropod species are known to feed by grazing the leaves of seagrasses (Stephenson et al., 1986; Kharlamenko et al., 2001), even though our study provided no evidence of direct bioaccumulation of fatty acids known to be characteristic signatures of *Z. noltii* in the mud snails. The hypothesis of bioconversion by *H. ulvae*, with precursors such as ALA, characteristic of *Z. noltii*, being elongated and desaturated to EPA or DHA, cannot be discarded, as it has already been showed for other organisms (Dalsgaard et al., 2003; Veloza et al., 2006). Nevertheless, bioconversion is usually characterised to be a slow process and commonly does not meet the metabolic demands of consumers (Veloza et al., 2006), being unlikely that bioconversion can explain the significant levels of EPA and DHA recorded for *H. ulvae* sampled in the seagrass meadow. Moreover, the absence of evidence pointing towards a direct bioaccumulation of specific fatty acids from *Z. noltii* on mud snails also suggests that they are unable to incorporate such compounds or that the mud snails did not eat the leaves at all. The fatty acid profiles recorded should reflect the voluntary or involuntary ingestion of components of the epiphytic community associated with the seagrass (Philippart, 1995; Kharlamenko et al., 2001). The remarkable levels of EPA measured in the mud snails from this habitat should be in

fact connected to the ingestion of the epiphytic diatoms, as supported by the fatty acid profile of epiphytes from *Z. noltii*.

The high values observed for the EPA/DHA ratio in mud snails from both habitats are characteristic of marine invertebrates, namely molluscs, and provide strong evidence of what group of primary producers was the main food source of surveyed specimens (Biandolino et al., 2008). During the present study, it was possible to separate specimens from both sites according to EPA/DHA ratios. The specimens of *H. ulvae* collected from the mudflat displayed higher EPA/DHA ratios than the ones from the seagrass meadow, clearly revealing the higher contribution of MPB to the levels of EPA recorded in the mud snails from this habitat.

In general, *H. ulvae* specimens collected from both habitats contained reduced levels of fatty acids known to be characteristic of marine or sediment bacteria, such as *iso*- or *anteiso* 17:0 (Kharlamenko et al., 2001). Nevertheless, significant levels of 18:1*n*-7 (vaccenic acid), often associated with bacteria inhabiting marine sediments, were found on mud snails not only from the mudflat, but also from the seagrass meadow. Considering that neither MPB nor *Z. noltii* samples contained significant levels of these fatty acids, it seems that these gastropods ingest (voluntarily or involuntarily) significant numbers of bacteria during their feeding activity. The role that bacteria may play in the feeding ecology of marine organisms has already been clearly demonstrated (Kharlamenko et al., 1995; Pascal et al., 2008). Gastropods inhabiting estuarine habitats can ingest bacteria by grazing upon the bacteria-rich epiphyte community (Kirchmanl et al., 1984) (e.g. covering the leaves of seagrass) or simply from assimilated sediment (Pascal et al., 2008).

6.5.3. Fatty acid as markers to differentiate estuarine habitats

A “perfect trophic marker” can be described as a compound that is not selectively processed during food uptake, is metabolically stable, and contributes to a transfer from one trophic level to the next in a qualitative manner (Dalsgaard et al., 2003). In several situations, fatty acid markers are incorporated into higher trophic levels in a conservative way, providing useful information on trophic relationships (Dalsgaard et al., 2003; Iverson, 2009). Fatty acid markers are used to provide information on the

dietary intake leading to the sequestering of stored lipids over time (Auel et al., 2002; Dalsgaard et al., 2003), contrasting with traditional analyses of simply monitoring gut content. However, this approach also has some limitations, as no individual fatty acid can be unequivocally assigned to a given species, and its dynamic is always linked with the metabolic and reproductive condition of the consumer (Dalsgaard et al., 2003). Despite such limitations, the fatty acid profiles exhibited by *H. ulvae* in the present study allowed us to clearly differentiate the estuarine habitats that they were occupying at the time of sampling. Within the same estuary, small sized motile organisms which are able to perform large-scale spatial movements (Haubois et al., 2002; Haubois et al., 2004) and display a considerable trophic plasticity (Riera, 2010) (such as mud snails) can still present diagnosing fatty acid signatures. This feature allowed us to link the fatty acid profiles of *H. ulvae* to the main energy sources present in each sampled habitat and identify the existence of contrasting dietary regimes. The use of fatty acid profiles will certainly contribute to a better understanding on the role played by motility in the trophic ecology of estuarine organisms at a small spatial scale.

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Chapter 7

Concluding remarks and research perspectives

Grazer-MPB interactions have long been investigated on estuarine systems, however several questions still remain. This last chapter synthesizes the main conclusions of the present work.

MPB living in intertidal mudflats undergo vertical migratory movements within the sediment in close synchrony with the beginning and end of the daylight periods of low tide (Fauvel and Bohn, 1907; Palmer and Round, 1967; Serôdio et al., 1997; Consalvey et al., 2004). Changes in cell concentration in the upper layers of the sediment largely affect the benthic primary productivity and might have important impacts in upper trophic levels of estuarine environment.

MPB biomass available as food source by *Hydrobia* is strongly controlled by endogenous but also by environmental factors, which together determine the migratory behaviour of benthic microalgae. The results of this work showed that the formation and disaggregation of microphytobenthic biofilms has a strong endogenous component that requires entrainment by solar and tidal cycles, specifically by the progression of the timing of low tide throughout the spring-neap tidal cycle (**Chapter 2**). The initial upward migration starts ca. 2-3 h before the daytime low tide exposure and lasts until the time expected for the beginning of diurnal low tide, being the accumulation of cells at the surface likely driven by an endogenous negative geotactic behaviour (Mitbavkar and Anil, 2004). This allows buried cells to reach the photic zone, which can be seen a successful strategy that allows anticipating the periods of favourable conditions for photosynthesis. Moreover, it was found that the full formation of the microalgal biofilm at the sediment surface is strongly depended on light availability, as the maintenance of darkness at the expected low tide time induced downward migration and biofilm disintegration. Downward migration at the end of the daytime low tide exposure is likely controlled by an endogenous positive geotaxis, enabling the anticipation of the start of periods when there are no benefits in staying at the surface, such as flood and sunset.

At the surface, microalgae are directly exposed to fluctuations of environmental factors, that have an important role in the regulation of biomass and consequently in the stabilization of the biofilm at the sediment surface. Main exogenous cues appear to be the solar radiation, which is necessary for the full formation and to the integrity

of surface biofilm, and the water content in the surface layers of sediments, which poses remarkable stressful effects on microalgal biomass and decrease photosynthetic efficiency. Moreover, the effects of desiccation on photosynthetic efficiency were depended on the rate of sediment water loss, increasing considerably with wind pressure. Desiccation should be considered in the estimation of primary productivity of estuarine environments, since even moderate water losses were found to induce significant variations on productive biomass levels (**Chapter 2 and 3**).

Recent studies have addressed the development of non-invasive approaches (Cartaxana et al., 2003; Murphy et al., 2008; Serôdio et al., 2009). The use of these non-destructive methodologies to investigate the role of water content on MPB variations and *H. ulvae* ingestion rate is particularly useful for studies in estuarine intertidal areas, since they improve the measurements and allow the simultaneous monitoring of other biological or environmental parameters. Both methods proposed in this work (**Chapter 3 and 4**) may provide a practical and effective way to: i) characterise the patterns of sediment dehydration and the complex relationship with key environmental variables (e.g. air temperature, wind velocity or air humidity) and ii) investigate *in situ* the ingestion rate of macrofauna.

The strong relationship found in this study between egested pheophorbide *a* and ingested chlorophyll *a* reinforced the use of pheopigments as trophic markers on grazer-MPB interactions, enabling to understand that ingestion of MPB by *H. ulvae* is significantly higher during summer and depends on the presence of light and of low tide (**Chapter 4**). It suggests that higher ingestion rates are possibly related to the life cycle of *H. ulvae* and higher growth rates that were found in summer (Cardoso et al., 2002), but also to the increase of surface MPB biomass during diurnal low tides. The non-invasive estimation of the ingestion rate of *H. ulvae* also improved the study of the complex mechanism between snails density and MPB ingestion under natural conditions, allowing to further understand both direct and indirect effects of consumption. Important variations on mud snails density were found for the first time among spring tides, with higher densities being observed during new moon. It suggests

a behavioural strategy to avoid predators, since many predators have been described as light dependent on prey detection (**Chapter 5**).

H. ulvae density plays a crucial role on the regulation of MPB biomass at the surface of the intertidal sediments. Mud snail densities affect the consumption of MPB, leading to the reduction of productive biomass (up to 40%) by top-down control, even though this contributes to the carbon dissemination through the estuarine food web (**Chapter 5**). Moreover, MPB production could also be affected by the increment of bioturbation, which is depended on snail activity. It should be intensified with increasing densities and erosion rate (Orvain et al., 2004).

The considerable decrease on *H. ulvae* ingestion rate may also indirectly affect the growth rate of snails, and of the population, as well as their reproductive potential (Morrisey, 1987; Barnes and Gandolfi, 1998). The occurrence of a significant decrease on growth rates results on smaller snails and consequently more exposed to predation, as Dekinga and Piersma (1993) found that individuals small than 4 mm shell height were mostly predated.

The use of fatty acid profiles was also found to be an important tool to investigate the grazer-MPB link (**Chapter 6**), as specific fatty acids are incorporated into higher trophic levels in a conservative way (Iverson, 2009). Considering the high level of trophic plasticity of *H. ulvae* (Riera, 2010), the current approach provides a qualitative characterization of fatty acid on different primary food sources and also on *H. ulvae* individuals from mudflats and seagrass meadows. These habitats are preferentially occupied by mud snails and each offered different food sources. The analyse of fatty acid profiles allowed to indicate the estuarine habitat that they were occupying, linking the fatty acid profiles of *H. ulvae* to the specific fatty acids of the main food sources present in each habitat. Despite the existence of differences on fatty acid profiles of mud snails from both habitats, the study reinforced the role of benthic microalgae on *H. ulvae* diets, since even mud snails from seagrass meadows showed considerable inputs of a diet rich on microalgae, namely diatoms.

Future

The implementation of this results and associated methodologies to ecological models for the Ria de Aveiro and other estuaries, will be the main challenge of the further work. The integration of desiccation effects and the non-destructive *in situ* monitoring of the patterns of sediment dehydration under low tide should improve the accuracy of estimations in primary productivity models. Furthermore, research on other environmental factors (e.g. wind, temperature) and its direct or indirect role on microphytobenthic biomass variations should be conducted to complement the effects of the major factors already studied.

This work opened doors for the application of non-destructive methodologies, based on optical methods or trophic markers, on important links of the complex estuarine food webs,that are expected to improve the research on primary productivity and consumption. The application of pheopigments as a measure of ingested primary production by macrofauna should be applied to future research, addressing the challenging task of understanding the role of bottom-up effect, particularly on consumers, but also on the control of the estuarine ecosystem structure.

Additionally, the investigation of fatty acids of microalgal communities has been improved on the last years although just a few investigations have been performed on MPB. The investigation of fatty acid production by MPB, namely the essentials EPA and DHA, which are economical but also ecological important must be improved. Several questions should be addressed: What fatty acid composition was characteristic of the microphytobenthic community? How do seasonal changes affect the fatty acid profiles? Or did seasonal variations markedly affect the production of essential EPA and DHA? Were environmental fluctuations (e.g. temperature, salinity etc) involved in the control of fatty acid composition in the MPB assemblages?

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