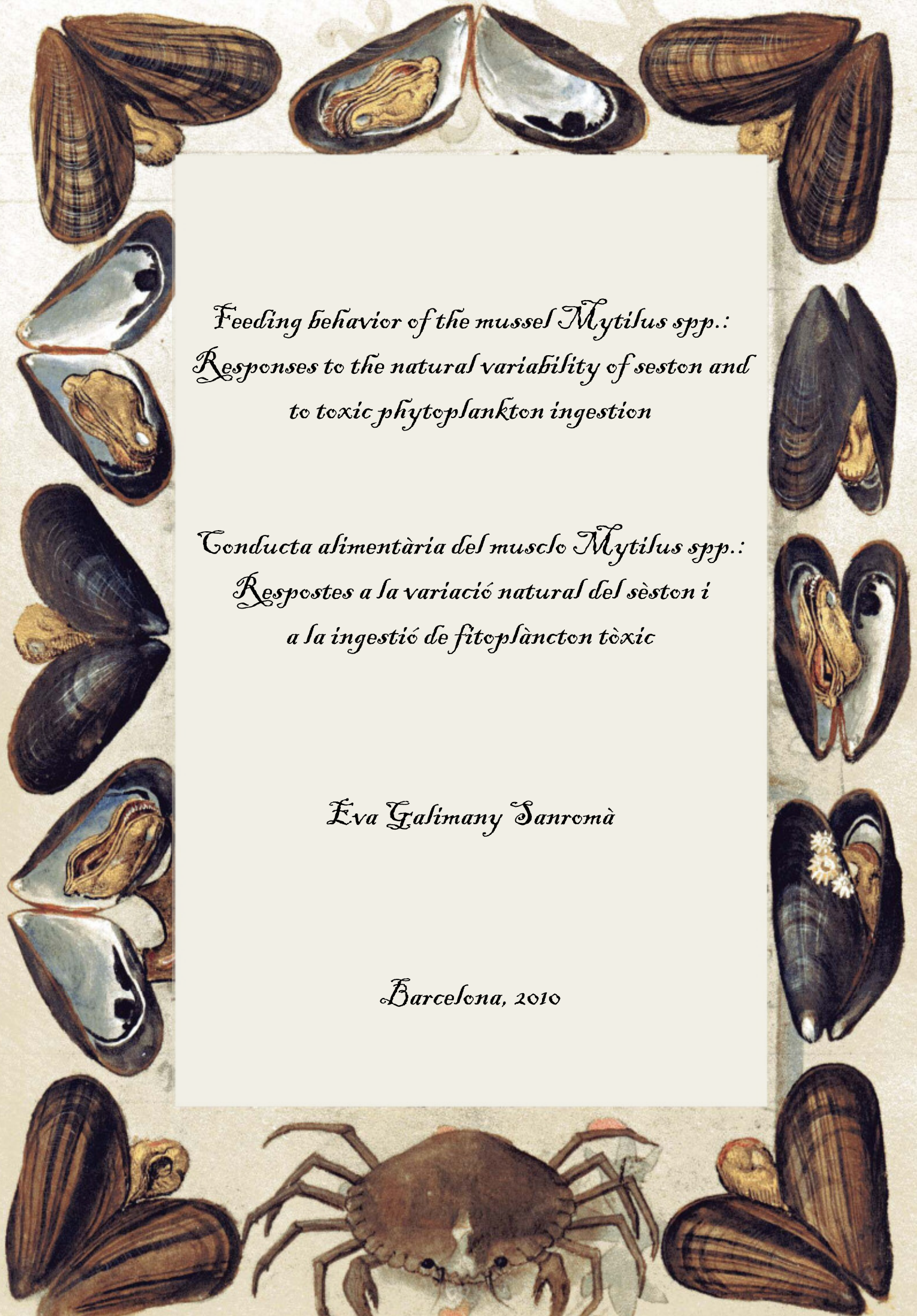

Feeding behavior of the mussel Mytilus spp.: Responses to the natural variability of seston and to toxic phytoplankton ingestion

Conducta alimentària del musclo Mytilus spp.: Respostes a la variació natural del sèston i a la ingestió de fitoplàncton tòxic

Eva Galimany Sanromà

Barcelona, 2010

Front page:

Master of Catherine of Clèves

Border of St. Ambrose framed between mussels and a crab, c. 1440

Miniature on parchment from "Book of Hours of Catherine of Clèves"

Morgan Library and Museum, MS. 917, p. 224, New York

Image obtained from the book:

Dezallier d'Argenville, A.-J. (2009). Moluscos, Conchiglie, Conchas. Taschen, 216 pp.

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Conducta alimentària del musclo *Mytilus* spp.: respostes a la variació natural del sèston i a la ingestió de fitoplàncton tòxic

Memòria presentada per

EVA GALIMANY SANROMÀ

per optar al grau de Doctor per la Universitat de Barcelona,

realitzada al IRTA St. Carles de la Ràpita.

Tesi inscrita al Departament de Biologia Cel·lular, Facultat de Biologia,

Universitat de Barcelona

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Programa de doctorat: Aqüicultura

Bienni: 2004 - 2006

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Barcelona, 2010

ACKNOWLEDGEMENTS

AGRAïMENTS

Acknowledgements

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I would like to thank,

· Montse, my PhD director, per la seva constant ajuda en tot i per tot, els seus bons consells, i que sempre hagi cregut en mi. No podria haver tingut millor directora de tesi, I'll be always grateful,

· all those aussies, specially Tim, who, with blind acceptance, introduced me to marine sciences,

· Irrintzi, Udane y su equipo de personal filtrador, por abrirme las puertas del mágico mundo de la filtración de los bivalvos,

· Richard and the MBA, for sharing his wide knowledge and helping me every time that I've needed,

· Gary, Hélène and the Milford Laboratory staff, for teaching me to trust in people and making of science and stats an enjoyable party,

· Inke, for her wise advises, perseverance and bravery in a world still not made for scientific women,

· the patience of all the people I've talked about above when I've broken lab material or messed up the labeling of samples,

· ICM-CSIC, for making me feel at home,

· Dra. Mercè Durfort, per donar-me sempre el seu suport i compartir els seus coneixements histopatològics,

· la meva família, en especial als meus pares, qui m'han ajudat sempre, en qualsevol lloc i qualsevol moment,

· last but not least, George, who has always believed in my scientific career and has helped and backed me up during all these years; and Iris, for reminding me, every single day, that science is part of our lives. We all do science, everywhere, at any time, spontaneously, since early stages of life.

The Sea Shell

BY MARIN SORESCU

I have hidden inside a sea shell
but forgotten in which.

Now daily I dive,
filtering the sea through my fingers,
to find myself.
Sometimes I think
a giant fish has swallowed me.
Looking for it everywhere I want to make sure
it will get me completely.

The sea-bed attracts me, and
I'm repelled by millions
of sea shells that all look alike.
Help, I am one of them.
If only I knew, which.

How often I've gone straight up
to one of them, saying: That's me.
Only, when I prised it open
it was empty.

TRANSLATED BY MICHAEL HAMBURGER

To George, my parents,
and scientific women, specially scientific moms.

INDEX

Index

INTRODUCTION

INTRODUCTION

1. The importance of bivalves

Bivalves are marine and freshwater molluscs which are distributed around the world. The ecological role of bivalves has been widely discussed and they can provide different ecosystem functions. For example, bivalves can improve the water quality of the ecosystems due to their filter-feeding capacity to mitigate the effects of eutrophication (Ostroumov, 2005; Coen et al., 2007; Lindahl and Kollberg, 2009). They can also contribute to the creation of habitat heterogeneity for fishes and invertebrates (Gutiérrez et al., 2003; Coen et al., 2007), and to the transfer of chemical elements and nutrients from the water and seston to other trophic levels and the sediments, linking benthic and pelagic ecosystems (Prins et al., 1998; Newell, 2004). The ecological impact of some bivalves in shallow, coastal embayments may not be solely as regulators of primary production but also secondary production (Lonsdale et al., 2009). Bivalves can also contribute to the stabilization of benthic or intertidal habitats, i.e. mussel beds (Coen et al., 2007; Dumbauld et al., 2009).

Some bivalve species also have economic roles through fisheries, aquaculture, and the ornament industry. Of all bivalves cultivated around the world, mussels are ideal for aquaculture due to their wide distribution and adaptability. Mussels are harvested for human consumption worldwide (Figueiras et al., 2002; Mortensen et al., 2006) with production of the species *Mytilus galloprovincialis* and *M. edulis* above 300.000 tons per year (FAO, 2009), mostly cultured in Spain, the leading mussel producer in Europe (Pérez Camacho et al., 1991; Keldany, 2002).

1.1 Mussels as keystone species

Mussels feed on several types of particles suspended in the water column but phytoplankton has traditionally been considered to be their main source of food (Mason, 1971). As a consequence of their filter-feeding activity, mussels can change the phytoplankton community; the filtration of bivalves has been shown to control phytoplankton growth, in what is often referred to as “top-down” processes. When not sufficiently grazed, phytoplankton populations can bloom excessively, leading to the deterioration of water quality and eutrophication. But, mussels also have the potential to promote primary production by nutrient release in the biodeposits, establishing a balance between phytoplankton grazing and release of nutrients (Asmus and Asmus, 1991). This event is especially relevant in shallow-water ecosystems, where the ratio of benthic surface area to water volume is high (Dame, 1996). The relationship established between both trophic levels is known as benthic-pelagic coupling; benthic bivalves transport particulate matter from the water column to the benthos transferring

organic nitrogen and carbon from the water column to the sediments (Verwey, 1952; Doering et al., 1986; Dame et al., 1989). Due to all these interactions, mussels are considered keystone species because they have important ecological roles maintaining water quality and participating in the cycling of nutrients from the water column to the benthos (Prins and Smaal, 1994; Ostroumov, 2005).

1.2 Mussels as sentinel species and quality programs

As a consequence of the ability of bivalves to accumulate anthropogenic pollutants and increasing industrial effluents in the estuarine and coastal environments, Goldberg (1975) proposed a "Mussel Watch" monitoring program to assess spatial and temporal chemical contamination in North America, using mussels as sentinel organisms. After such proposal, an international "Mussel Watch" program was developed in order to assess the levels of certain contaminants in bivalves collected from coastal marine waters throughout the world (International Musselwatch Committe, 1992). Sentinel monitoring species should comply with several criteria (Morgado and Bebbiano, 2005), such as being abundant, accessible, sedentary and cosmopolitan, large enough to provide sufficient tissue for biological analysis, easy to identify and available all year long. In addition, they should respond to contamination but be robust enough to be present in polluted environments and be easy to maintain in the laboratory for laboratory studies. Mussels are one of the few species that satisfy all these characteristics. In detail, the genus *Mytilus* tolerates polluted waters, wide variations in salinity and temperature, and efficiently accumulates various trace metals (Rainbow, 1995; Morgado and Bebbiano, 2005).

When consumed, bivalves can have negative impacts on human health. Due to filter-feeding, bivalves can be vectors of diseases related to the accumulation of bacteria, viruses, pesticides, biotoxins, industrial wastes, toxic metals, and petroleum derivatives from the water column, with subsequent public health concerns. Thereafter, water quality programs of shellfishing areas were also established in many countries around the world in order to protect public health (Gosling, 1992).

2. Basic knowledge on the genus *Mytilus*

2.1 Anatomy

Mussels of the genus *Mytilus* are sessile bivalve mollusks which live in sea water and estuarine habitats. They posses two shell valves with similar size and shape which protects them from predators and the environment. Figure 1 shows the gross anatomy of a mussel.

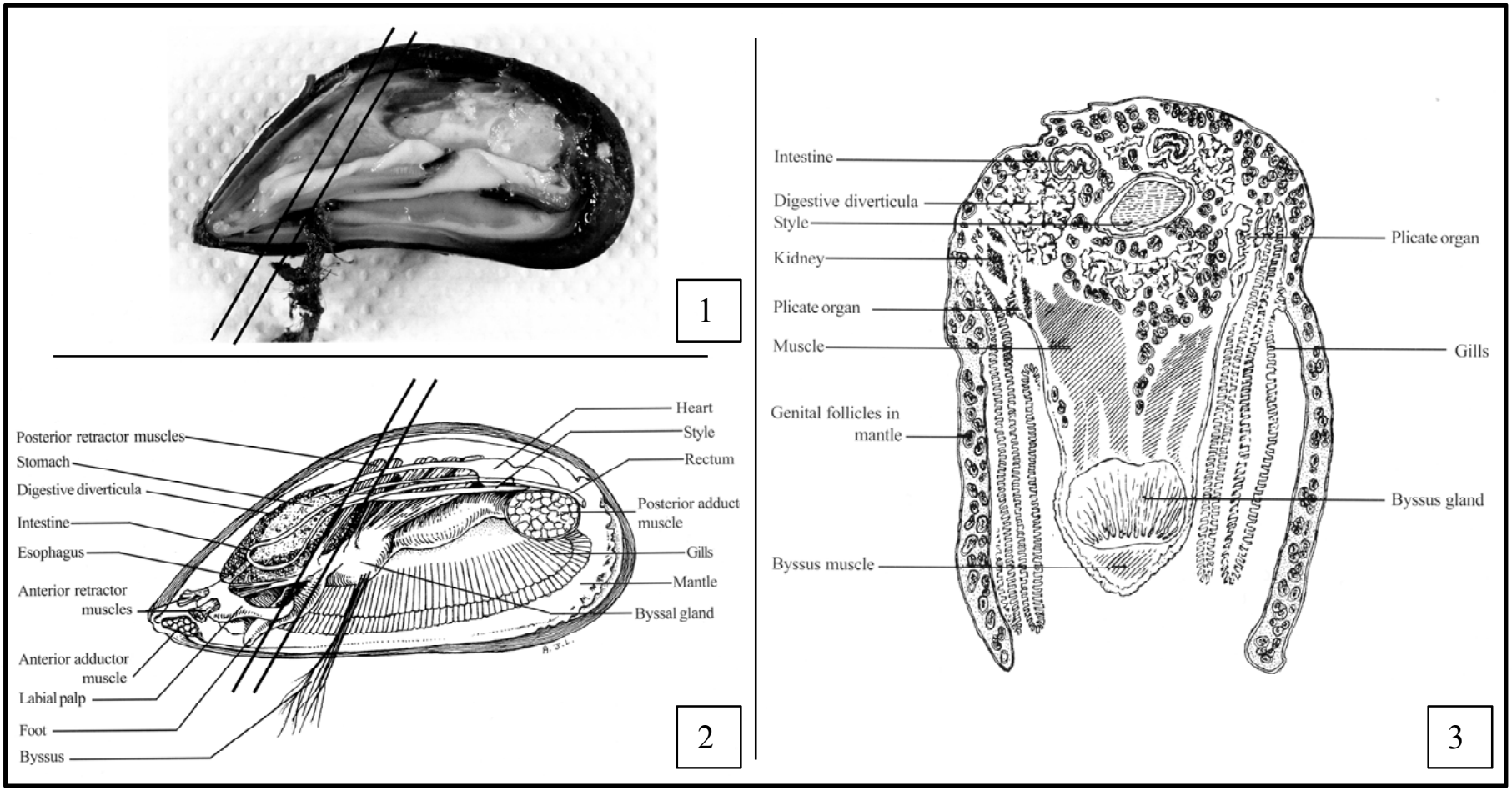

Fig. 1: Basic anatomy of *Mytilus edulis*. 1.1. Photograph of the soft part. 1.2. Illustration of the anatomy. 1.3. Illustration of a cross-section; bold parallel lines in fig. 1.1 and 1.2 show location of the cross-section. From Howard et al. (2004), illustrations by A. J. Lippson, Bozman, M.D.

The viscera of the mussels are attached to the shells by adductor muscles, the mantle edge and small points of attachment every few millimeters between the mantle and the inner shell (Bayne et al., 1979). The mantle consists of two lobes of tissue which enclose the bivalve within the shell. Between the mantle and the internal organs is the mantle cavity. In mussels, the mantle is the main site for the storage of nutrient reserves but it also contains most of the gonad. When ripe, the mantle is thick, but after the release of the gametes, it gets thin and transparent (Gosling, 2003). The gills (or ctenidia) are flat filibranch structures that are suspended from the dorsal margin of the mantle with two different physiological functions: respiration and feeding (Bayne et al., 1976; Gosling, 1992). The foot of the mussel is a long and highly mobile muscular structure which larvae and adults use to detect a suitable substrate and attach to it. Moreover, by means of the byssal gland, located in the foot, mussels secrete byssal threads for attachment to the substrate.

The alimentary canal consists of mouth, esophagus, stomach, intestine, digestive tubules and anus. The excretory organs of mussels are the pericardial glands and a pair of kidneys. The heart, in the mid-dorsal region of the body, pumps the hemolymph around the body. The circulatory system of mussels is open, which means, that the hemolymph in the sinuses bathe the tissues directly (Gosling, 2003).

2.2 Geographic distribution

The genus *Mytilus* includes the species: *M. edulis* (Linnaeus 1758), *M. trossulus* (Gould 1850), *M. galloprovincialis* (Lamark 1819), *M. chilensis* (Hupé 1854), *M. coruscus* (Gould 1861), *M. californianus* (White 1937), *M. platensis* (o'Orbigny 1846), *M. planulatus* (Lamarck 1819), and *M. desolationis* (Lamy 1936) (Gosling, 1992). Prior to the use of electrophoresis, taxonomy was based on shell morphology, a characteristic that varies with numerous environmental factors. Consequently, there is confusion in the literature concerning occurrence of the different species. Nowadays, molecular techniques allow more accuracy in mapping their distribution around the world. Figure 2 shows the worldwide distribution of four species of *Mytilus*. In detail, the species *M. galloprovincialis* and *M. edulis* occur globally, whereas *M. trossulus* and *M. californianus* are restricted to the northern hemisphere. Recent studies clarified the occurrence of *M. galloprovincialis* in the Galician Rías (N. W. Iberian Peninsula), which was thought to be *M. edulis* (Crespo et al., 1990; Sanjuan et al., 1990). Thereafter, along the European coast, the species *M. galloprovincialis* inhabits warmer waters such as the Mediterranean Sea and the coasts of Spain, France and southern Britain whereas *M. edulis* is found in higher latitudes. Both *Mytilus* species overlap and hybridize naturally in the European Atlantic coast (Beaumont et al., 2004).

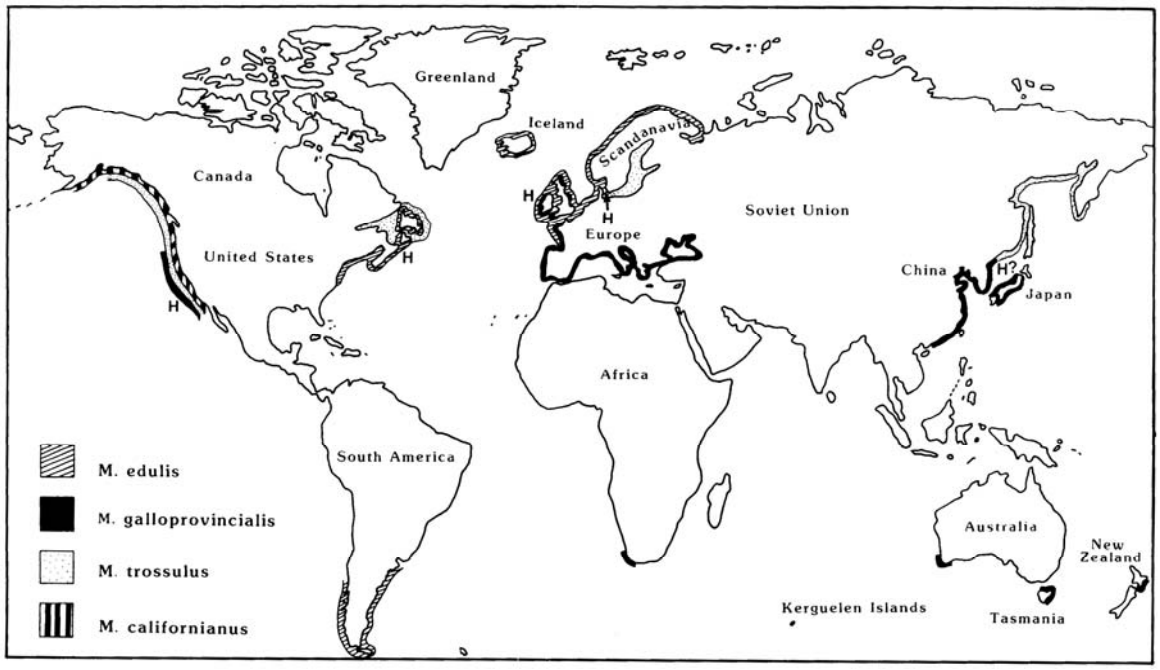

Fig. 2: Global distribution of four species of the genus *Mytilus*. H means areas of hybridization. From Gosling (1992).

Mussels of the genus *Mytilus* inhabit the low and intertidal zone of temperate seas globally. Of all the species of the genus, the blue mussel *M. edulis* has the widest distribution, from mild subtropical to Arctic regions, from high intertidal to subtidal regions, from estuarine to true marine conditions, and from sheltered to extremely wave-exposed shores (Gosling, 2003). The distribution and abundance of mussels are affected by the following physical and biological factors:

1. Physical factors: bivalve mollusks are poikilothermic animals, which mean that they produce small amounts of heat during metabolism and are thermally dependant on the environment (Dame, 1996). Thereafter, temperature might be considered as the most important physical factor that regulates the distribution of mussels; it sets limits on the spatial distribution of bivalves and affects every aspect of their biology. The different *Mytilus* species tolerate different temperature thresholds. *M. galloprovincialis* cannot survive sea water temperatures of or beyond 26ºC over extended periods of time (Anestis et al., 2007) and inhabits warmer waters than *M. edulis* (Beaumont et al., 2004). Similarly, *M. trossulus* is more thermally sensitive than *M. galloprovincialis* and heat shock proteins, which expresion increases when cells are exposed to high temperatures, are synthesized at 23ºC, a lower temperature than for *M. galloprovincialis* (Hofmann and Somero, 1996). Low temperature tolerances also vary among *Mytilus* species; whereas *M. edulis* survive winter temperatures lower than -10ºC, *M. californianus* cannot tolerate freezing conditions (Williams, 1970; Seed and Suchanek, 1992). Endogenous cellular

estress proteins in *M. edulis* vary seasonally and correlate positively with seasonal changes in both environmental temperature and thermal tolerance (Chapple et al., 1998). Water temperature is known to directly affect the physiological status of bivalves regulating feeding behavior, growth and spawning of mussels (Denis et al., 1999; Suárez et al., 2005). As an example, when temperature exceeds 25 ºC, filtration rates fall significantly in *M. galloprovincialis* and in *M. edulis* (Gonzalez and Yevich, 1976; Anestis et al., 2007).

Salinity is also a very important limiting factor in coastal and estuarine bivalves. Nevertheless, *Mytilus* spp. are considered euryhaline, and they are able to adapt to a wide range of salinities. In particular, a range between 4 and 40 ‰ has been reported for *M. edulis* (Bayne et al., 1976).

Other factors may be considered as limitations for bivalve distribution such as depth, tides, currents, type of substrate, turbidity, etc. (Dame, 1996; Gosling, 2003).

2. Biological factors: food and predators, together with pathological conditions and parasites, are probably the most important source of natural mortality in bivalves. They have the potential to affect population size structure in addition to overall abundance and local distribution patterns (Seed and Suchanek, 1992).

Of all the particles suspended in the environmental water, mussels mainly feed on phytoplankton although they can also feed on bacteria, zooplankton, detritus as well as dissolved organic material (Mason, 1971; Gosling, 2003). Potential predators of adult mussels include gastropods, starfish, sea urchins, crabs, fish, birds and humans (Gosling, 2003; Dame, 1996; Kamermans et al., 2009). Predation of bivalve larvae is mostly due to filter-feeding invertebrates, including bivalves (Dame, 1996; Gosling, 2003). There must be equilibrium for both the food source and the predators to allow mussels to survive, grow and reproduce in each inhabiting area.

Most of the pathological conditions of marine bivalves are caused by different types of infectious agents and parasites such as viruses, bacteria, fungi, trematodes, annelids, copepods and different protists (Lauckner, 1983). Some of the most common pathological conditions found in mussels include marteiliosis caused by the parasite *Marteilia refringens*, the lesions caused by the copepod *Mytilicola intestinalis* and the trematode *Proctoeces maculatus*, and disseminated neoplasia (Lauckner, 1983; Kim and Powell, 2007).

In mussels, the shell provides an excellent substrate for the settlement of fouling organisms, which can cause significant mortality due to dislodgement, as a consequence of the increase in weight and clogging. In addition, some of the epibionts are filter-feeders competing with mussels for food. Nevertheless, under conditions where food is not a limiting factor, interespecific competion by the epibionts should not significantly limit the yield of mussels

(Lesser et al., 1992). Almost 100 invertebrate species have been identified as fouling organisms on suspended mussel rope cultures (Perera et al., 1990; Hickman, 1992).

3. Feeding

3.1 Particle selection

Mussels are sessile filter-feeding bivalves which depend on the water where they are submerged for food supply. The water is forced through an inhalant siphon to get into the mussel (Fig. 3). Then, lateral cilia on the gills maintain a flow of water through the mantle cavity and gills to allow the filtration of the water. The cilia remove the large particles and trap them in a fine mucus layer, transporting them towards the labial palps, two triangular structures located on each site of the mouth. The smaller particles can either escape or are captured by the cilia. The filtered water exits the mussel through the exhalant siphon (Riisgård et al., 1996; Gosling, 2003; Ward and Shumway, 2004). The palps direct the particles retained by the gills towards the mouth, which leads into the stomach by means of a narrow esophagus.

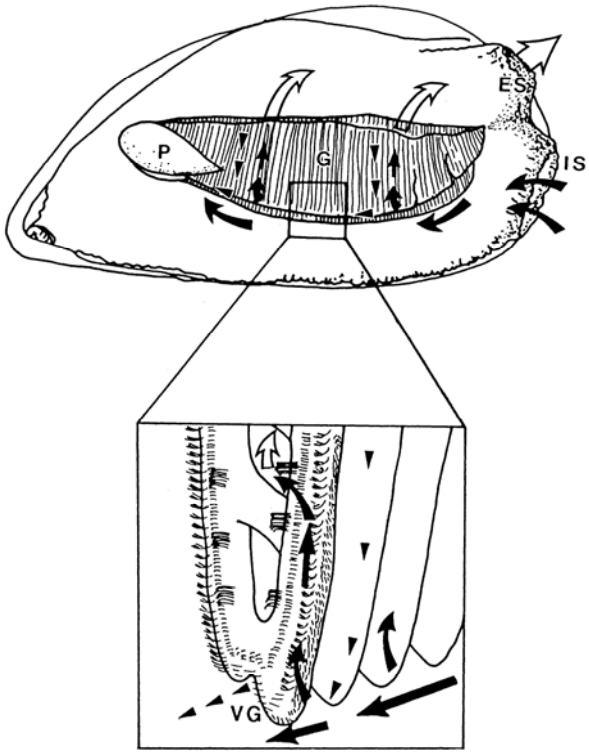

Fig. 3: Principal pathways of current flow and particle transport in *Mytilus edulis*. Water enters the bivalve via the inhlant siphon (IS). The frontal surface of the gill (G) is exposed to a postero-anterior flow at the ventral margin; the rest of the frontal surface is swept by a ventro-dorsal flow, with a progressive through component to the abfrontal region. Solid arrows indicate current flow prior to passage across gills. Water exits the gill through the exhalant siphon (ES) (open arrows). Arrowheads represent particle transport to the ventral groove of the gills (VG); (P labial palp). From Beninger and St.-Jean (1997a).

Extracellular digestion takes place in the stomach by enzymes released from the rotating crystalline style (Fig. 4). Intracellular digestion occurs immediately after the stomach in the digestive gland. The unabsorbed waste material from the digestive gland is formed into fecal pellets which are directed through the anus, towards the exhalant siphon and released (Gosling, 2003).

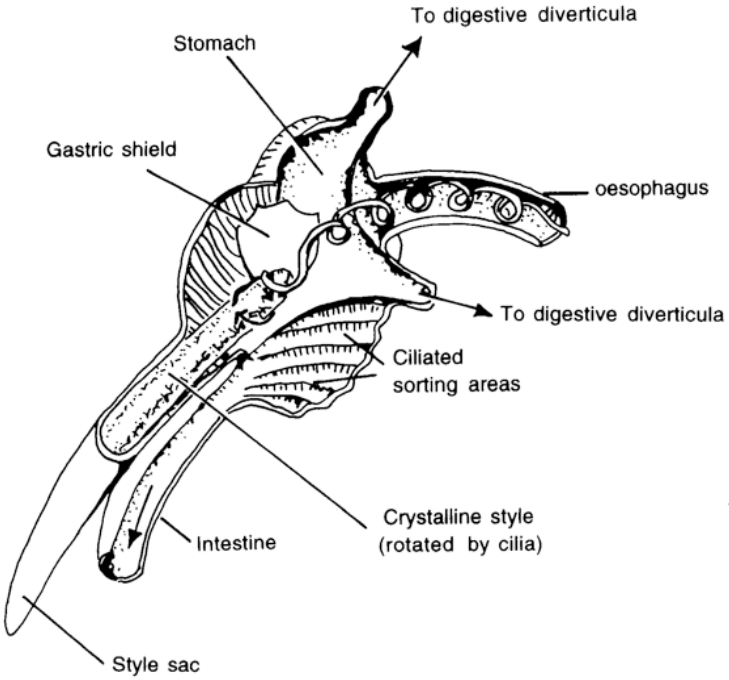

Fig. 4: Part of the digestive system of a bivalve showing rotation of the crystalline style. From Gosling (2003).

Gills act like sieves that can retain different types of particles within the size threshold of 1 to 6000 µm (Lehane and Davenport, 2006) although the retention efficiency differs according to particle size. *Mytilus edulis* can retain 1-2 µm particles with an efficiency of 50% (Jørgensen, 1975), 2 µm particles with an efficiency of 75-90%, and particles larger than 6 µm are retained with 100% efficiency (Møhlenberg and Riisgård, 1978).

However, bivalves do not ingest everything that is retained by the gills (Gosling, 2003). Pre-ingestive selection may occur on the gills and labial palps and results in the production of pseudofeces (Kiørboe and Møhlenberg, 1981; Shumway et al., 1985; Ward et al., 1998; Zemlys et al., 2003) (Fig. 5). The selection of particles can also occur in the stomach by retaining some particles longer to increase time for extracellular digestion or by directing some particles to the digestive gland (Bricelj et al., 1984; Shumway et al., 1985; Brillant and MacDonald, 2003; Ward and Shumway, 2004). The stomach can also sort and direct ingested particles directly to the intestine based on large sizes, low nutrition, high density or certain chemical properties

(Morton, 1973; Ward and Shumway, 2004). These processes are known as post-ingestive selection (Fig. 5).

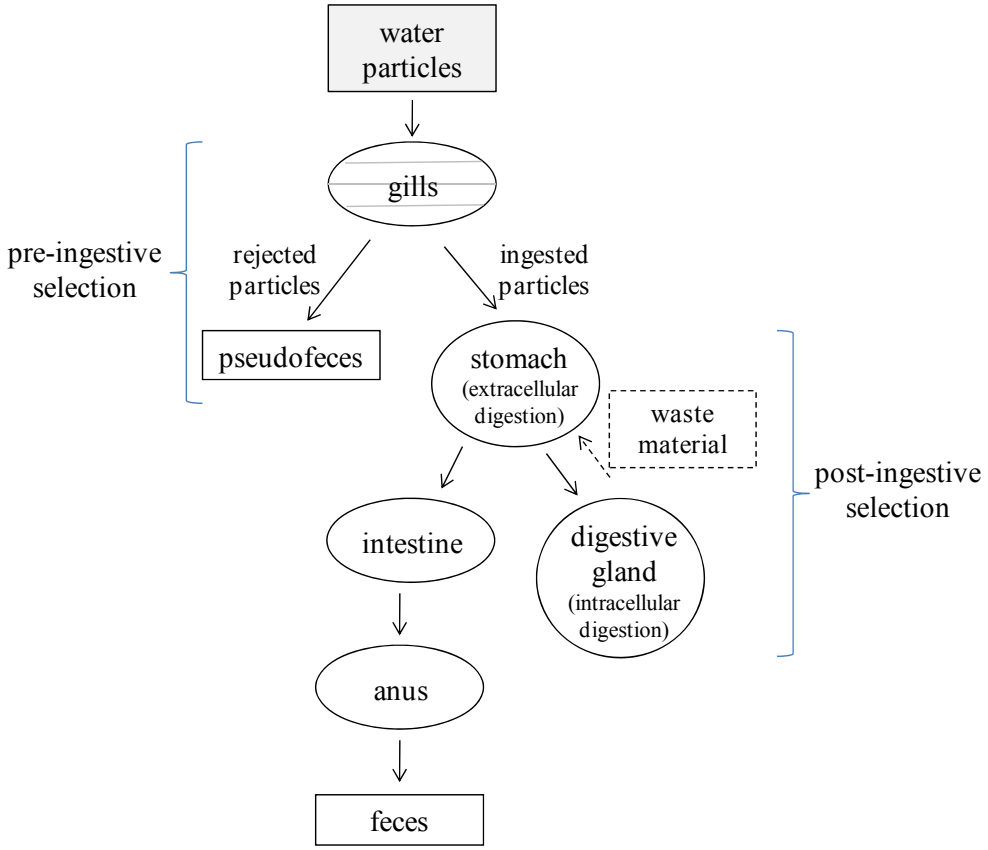

Fig. 5: Diagram of particle selection by mussels.

Food that is finally directed from the stomach towards the digestive gland (including digestive ducts and tubules) goes under intracellular digestion and the waste material is redirected to the stomach (Fig. 6).

Food that passes directly from the stomach through the intestine results in poorly digested material (Widdows et al., 1979a). Feces composed of the waste material from the digestive gland are named glandular feces; intestinal feces are produced when seston concentrations exceed the maximum digestible concentration and are rejected after deficient digestion in the intestine (Widdows et al., 1979a). The selection of particles by bivalves is a strategy to maximize the quality of the diet and to optimize the energy gain (Fig. 6).

The selection criteria of filter-feeding bivalves can be affected by different characteristics of the available food particles. Inorganic particles, such as silt or glass, silica or alumina spheres are selected and rejected as pseudofeces (Kiørboe et al., 1980; Ward and Targett, 1989; Bayne et al., 1993). Moreover, the production of pseudofeces increases along

with the inorganic content of the water (Kiørboe et al., 1980), or when the ingestive capacity of bivalves is overloaded (Beninger and St-Jean, 1997b).

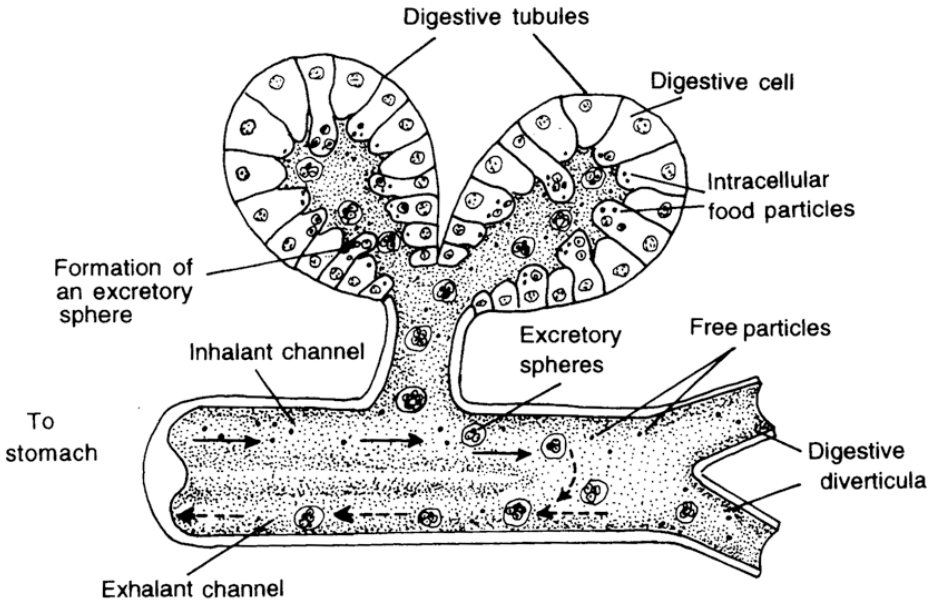

Fig. 6: Section of the digestive gland showing intracellular digestion of material coming from the stomach (solid arrows) and redirection of waste material to stomach (broken arrows). From Gosling (2003).

Pre-ingestive selection has also been reported in several other occasions. When feeding on different species of phytoplankton, diatoms are preferentially rejected over dinoflagellates and flagellates (Shumway et al., 1985; Bougrier et al., 1997). Algal size, shape, flexibility and quality can also affect the capture efficiency of particles (Bougrier et al., 1997; Defossez and Hawkins, 1997; Ward et al., 2003). As a consequence, the filtration activity of a mussel population and its selective efficiency on various taxa interacts with the phytoplankton community of the ecosystem causing changes in the species and size distributions (Asmus and Asmus, 1991; Noren et al., 1999; Dolmer, 2000; Petersen et al., 2008). Therefore, mussels have the potential to exert significant top-down control of phytoplankton (Strohmeier et al., 2008; Trottet et al., 2008).

High density mussel populations, such as farmed mussels, grazing on phytoplankton can have a top-down control effect on the communities of phytoplankton. Nevertheless, most of the ingested organic matter is rapidly recycled to the water column as inorganic nutrients, which would be expected to stimulate phytoplankton growth. As a consequence, the net effect of mussel farming on phytoplankton dynamics may increase phytoplankton turnover and overall production (Nizzoli et al., 2005).

3.2 Harmful Algal Blooms (HAB)

Among the many different phytoplankton species that mussels feed on there are some that are considered toxic. Toxic microalgal proliferations in aquatic ecosystems, referred to as Harmful Algal Blooms (HABs), were first recorded by ancient civilizations and have been observed ever since (Fogg, 2002; Landsberg, 2002). HABs appear to be increasing in geographic distribution and intensity, along with their effects and consequences on the ecosystems and their organisms (Landsberg, 2002). As an anecdote, the movie director Alfred Hitchcock witnessed an episode of HAB and how shearwaters, oceanic birds that feed on anchovies, flew inland toward the city of Capitola, California (USA), dying by the dozens as they crashed into buildings and cars. The anchovies had eaten toxic algae, which produced domoic acid, a neurotoxin. The birds in turn had eaten the fish, affecting the birds' ability to fly. This incident was used as research material for the classic thriller about a coastal town terrorized by deranged birds, *The Birds* (1963, based on the 1952 novella Daphne du Maurier).

Different taxa of phytoplankton, including dinoflagellates, diatoms, raphidophytes, prymnesiophytes, silicoflagellates, ciliates, and cyanobacteria have the potential to be toxic to a wide variety of organisms, including bivalves (Shumway, 1990; Fogg, 2002; Landsberg, 2002). The filter-feeding activity of bivalves makes them susceptible to ingest toxic algae whenever present in the environmental water. It is known that mussels can use toxic dinoflagellates as a food source in the absence of other phytoplankton cells (Bricelj et al., 1993). Moreover, when a toxic algal species proliferates, it becomes predominant among the entire phytoplankton community of the ecosystem, becoming almost the only available food source for bivalves.

As bivalves are grown under natural conditions the biological quality of the growing water is very important. Bivalves can be affected by different types of algal toxins (Shumway, 1990; Landsberg, 2002) and their responses to the different harmful algae seem to be species-specific (Shumway et al., 2003; Hégaret et al., 2007a). In detail, several biological effects of toxic microalgae upon mussels have been described (Table 1). Moreover, toxins produced by HAB species can accumulate in bivalve tissues and affect their predators, passing the toxins to higher trophic levels, including top predators in food webs such as humans (Azanza and Taylor, 2001; Landsberg, 2002; Jester et al., 2009).

Of all bivalves consumed by humans, mussels accumulate toxins more rapidly than any other species (Shumway et al., 1990; Gosling, 2003). Therefore, to protect public health, monitoring and management programs for bivalve toxins have been implemented (Shumway et al., 1990; Rehnstam-Holm and Hernroth, 2005). HAB-related closures of shellfish harvesting can result in great economic losses for the aquaculture industry (Shumway, 1990; EUROHAB, 1998).

Effects	Microalgal specie	Bibliography
Decrease of clearance rate	*Alexandrium monilatum* *Alexandrium tamarense* *Gymnodinium aureolum* *Karenia brevis* *Prorocentrum lima*	Pate et al. (2005) Lesser and Shumway (1993) Widdows et al. (1979b) Leverone et al. (2007) Pillet and Houvenhagel (1995)
Reproductive failure	*Aureococcus anophagefferens* *Chrysochromulina polylepis*	Bricelj and Kuenstner (1989) Granmo et al. (1988)
Inhibition of byssus production	*Alexandrium tamarense* *Heterocapsa circularisquama*	Shumway et al. (1987) Matsuyama et al. (1998)
Growth suppression	*Aureococcus anophagefferens* *Chrysochromulina polylepis*	Bricelj et al. (2001) Nielsen and Strømgren (1991)
Shell valve closure	*Alexandrium tamarense* *Heterocapsa circularisquama*	Shumway and Cucci (1987) Matsuyama et al. (1998)
Mortality	*Aureococcus anophagefferens* *Gonyalaux* spp. *Gymnodinium aureolum* *Gyrodinium corsicum* *Rhizosolenia chunii*	Bricelj et al. (2001) O'Sullivan (1978) Cross and Southgate (1980) ICES (1999) Parry et al. (1989)

Table 1: Effects of different toxic microalgal species upon mussels (*Mytilus* spp.).

Recent research has reported viable cells and temporary cysts from different harmful algae in the feces of several bivalves. Thus, in addition to the noxious effects of HABs to bivalves and predators, there is a potential transport of harmful algae via relocation of bivalves (Hégaret et al., 2008).

4. The immune system

Circulating hemolymph cells, known as hemocytes, constitute the major medium for immune defense of bivalves. These ubiquitous cells are found in the open circulatory system of bivalves including heart, hemolymph vessels and variably sized sinuses localized in the major organs (Auffret, 2005). The capability of hemocytes to discriminate between self and non-self is, in part, based on the presence of lectins on the surface of the cells (Renwrantz, 1990). Nevertheless, it is suggested that the type of reaction depends upon the nature of the particles presented, i.e.: hemocytes exhibit chemotactic and chemokinetic reactions when exposed to bacterial products (Schneeweiß and Renwrantz, 1993).

Hemocytes can be divided into two main groups: basophilic and eosinophilic cells; and hemocytes may be either agranular or granular (Fig. 7).

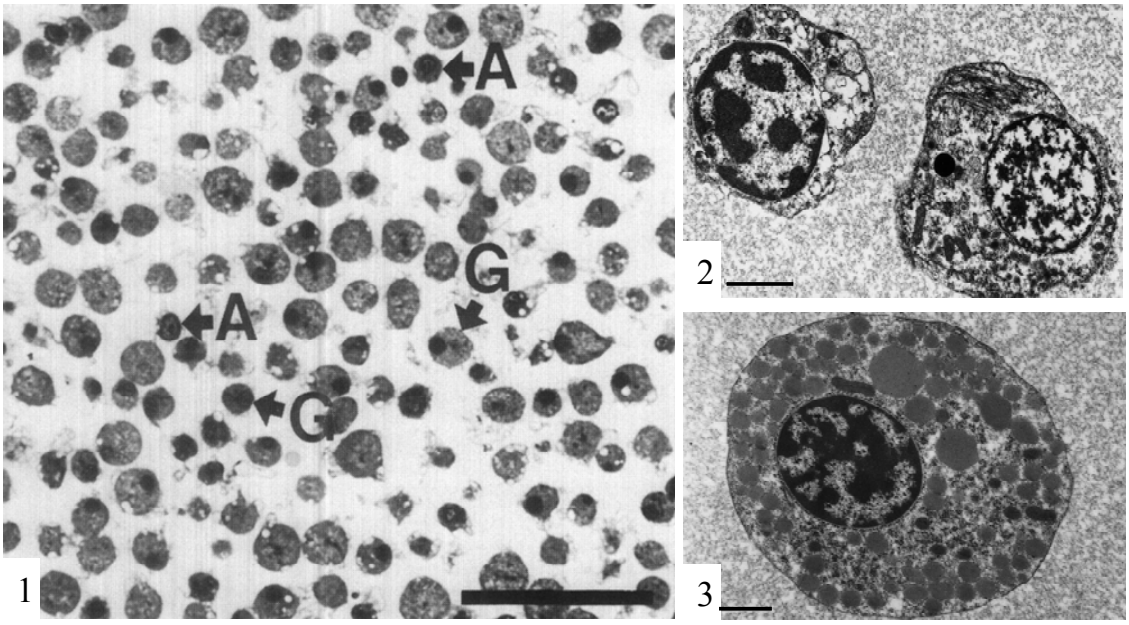

Fig. 7: 7.1. Light microscopy image of agranular (A) and granular (G) hemocytes of *Mytilus edulis*. Scale bar = 20 µm. From Rasmussen et al. (1985). 7.2 and 7.3. Electron microscopy images of two basophilic and an eosinohilic hemocyte respectively of *M. edulis*. Scale bar = 1 µm. From Pipe et al. (1997).

Basophilic hemocytes can be divided into small (4-5 µm) and large (7-9 µm) cells (Moore and Lowe, 1977; Bayne et al., 1979; Pipe, 1990a). The small basophilic hemocytes are generally spherical with a spherical nucleus and hyaline cytoplasm. The larger basophilic cells assume an irregular appearance with granules and vacuoles in the cytoplasm, which considerably vary in diameter. The eosinophilic hemocytes, or granulocytes, are often spherical and filled with spherical granules (0.5-1.0 µm) which range from neutrophilic to acidophilic in staining properties (Moore and Lowe, 1977; Pipe, 1990a). Table 2 shows a brief simple classification of the hemocytes with their basic characteristics.

Once hemocytes have recognized a non-self particle, they can either phagocytose the particles or release a range of antimicrobial molecules including reactive oxygen metabolites (Pipe, 1992; Dyrynda et al., 1998; Wootton et al., 2003a). The granules of the different types of hemocytes posses a wide range of hydrolytic enzymes, such as phosphatases, esterases, peroxidases, proteinases, glycosidases and sulphatases, showing that these granules are a type of lysosomes (Moore and Lowe, 1977; Pipe, 1990b; Carballal et al., 1997a). Nevertheless, mussel hemocytes are functionally heterogeneous as the eosinophilic cells posses the ability for phagocytosis whereas the basophilic hemocytes seem to be non or much less phagocytic (Pipe, 1990b; Carballal et al., 1997b; Pipe et al., 1997). The release of lysosomal enzymes by granular hemocytes is accompanied by degranulation of the cell (Foley and Cheng, 1977).

Type of hemocyte	Basophilic		Eosinophilic (Granular)
	small	large	
Shape	spherical	irregular	spherical
Granules	no granules	different diameters	spherical (0.5-1.0 μm)

Table 2: Classification and characteristics of the different types of hemocytes.

The immune system of invertebrates is considered non-specific (or innate), which means, that the immune system is wide-ranging providing immediate defense recognizing and responding to noxious particles and environments in a generic way. In contrast to the adaptive immune system, it does not confer long-lasting or protective immunity to the host. Nevertheless, bivalves possess adaptive strategies against non-favorable ecosystems. In accordance, when comparing the immune function of the mussel *M. edulis*, the edible cockle *Cerastoderma edule* and the razor-shell *Ensis siliqua*, mussels showed a higher level of immunological vigor probably linked to their considerable resilience to adverse environmental conditions (Wootton et al., 2003b). Mussels inhabiting contaminated areas are more susceptible to infections because of the immunosuppression caused by pollution stress (Pipe and Coles, 1995; Dyrynda et al., 1998). Nevertheless, *Mytilus* populations have been reported to survive exposure to heavy oil pollution in contrast with other bivalves (Dyrynda et al., 2000; Ordás et al., 2007). In addition, it has been reported that bivalves periodically exposed to HABs become more resistant to the toxic effects of the different microalgal toxins to which they are commonly exposed (Bricelj et al., 2005).

5. Mussel culture in the Ebro Delta

Mussel (*Mytilus galloprovincialis*) culture along the Spanish Mediterranean coast is traditional in the regions of Catalonia, Valencia and Menorca, and is now also developing in Andalusia (Ramón et al., 2005) (Fig. 8). Among these regions, the major production occurs in Catalonia, specifically in the Ebro Delta bays (Ramón et al., 2005), with an average annual mussel production of about 3000 Tn (Fig. 9). At this site, mussel culture was initiated at the beginning of the 60's and has been traditionally done ever since. Mussels are cultured in suspension from the 166 fixed rafts that are spread between the bays of Alfacs (90 farms) and Fangar (76 farms). The mussel rafts are rectangular wooden frames measuring 200 m x 15 m, from which 2-3 m long mussel ropes are suspended. They are anchored to the seabed by concrete girders, and only protrude from the surface of the water by 1 m.

Fig. 8: Map of the Spanish Mediterranean coast. Arrows and images of mussels show culture locations: 1. Ebro Delta; 2. Valencia; 3. Menorca; 4. Andalusia.

The culture cycle begins during December and January by means of hanging collector ropes to obtain natural spat. The seed mussel reach 3 cm long some months later, at around October, when they are moved to the final ropes and remain there until reaching 6 cm in length during May - June.

Despite the relatively low mussel production compared with Galicia and other world aquaculture centers, the Ebro Delta represents an important income for many families in the area. Moreover, the bays are embedded in a naturally protected area, which is the second largest wetland area in the western Mediterranean. This peculiarity makes it difficult to manage the bivalve cultivation given the protected area regulations in a widely variable environment such as this one.

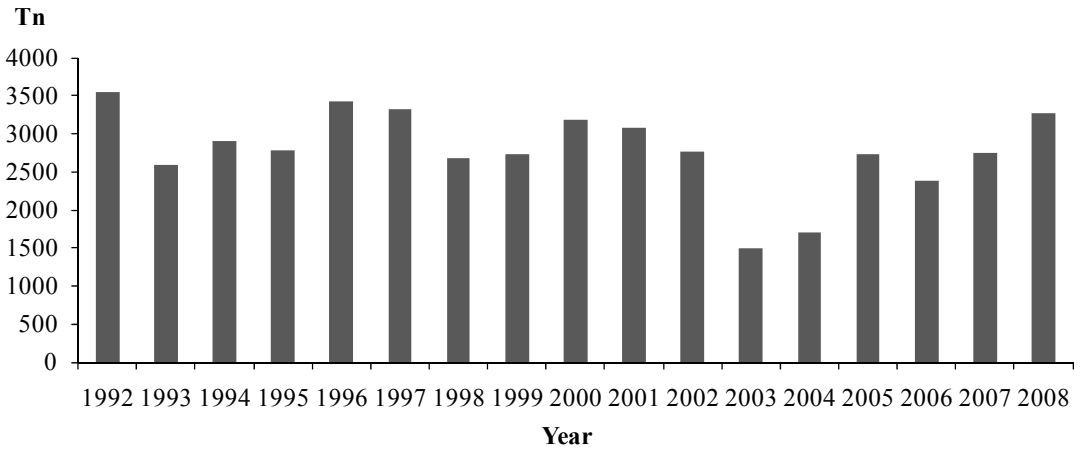

Fig. 9: Mussel production in the Ebro Delta bays (DAR, 2009).

OBJECTIVES

OBJECTIVES

The purpose of the present thesis was to study the feeding behavior of mussels in terms of selection and physiological components of absorptive balance, and to find out whether ingestion of toxic algae species modulates the immune function and causes pathological changes.

To address this problem statement, two different mussel species, *Mytilus galloprovincialis* and *M. edulis* were used. Two different experimental setups were applied; the feeding experiments were performed *in situ* on top of a mussel raft under natural conditions, and mussels were experimentally exposed to toxic algae in aquaria under controlled laboratory conditions.

Following hypotheses were formulated:

Hypothesis I: The feeding behavior of mussels in Alfacs Bay (N.W. Mediterranean Sea) depends upon the quantity and quality of seston, which may vary in time.

Hypothesis II: Mussels in Alfacs Bay (N.W. Mediterraean Sea) preferentially select some of the available suspended matter but not other.

Hypothesis III: Harmful Algal Blooms modulate the immune function of mussels and cause pathological changes.

This thesis is based on six chapters, which are referred to in the text by their Roman numeration.

The hypotheses were tested by the following aims:

1. Determine the main physiological parameters related to mussel feeding behavior by means of the biodeposition method in Alfacs Bay (Chapter I).

2. Study the phytoplankton composition, determine the dominant taxa, and detect phytoplankton peaks during four periods of a culture cycle (from October to July) in a mussel aquaculture site (Alfacs Bay) (Chapter II and III).

3. Study the selection and ingestion of available particles by mussels, including some toxic phytoplankton species, during four periods of a culture cycle in Alfacs Bay (Chapter II and III).

4. Study the immunological and histological effects of three different toxic algae: *Karlodinium veneficum*, *Prorocentrum minimum* and *Alexandrium fundyense* on mussels using experimental laboratory exposures (Chapter IV, V and VI).

5. Test possible recovery of mussels after experimental exposure to toxic *Alexandrium fundyense* (Chapter VI).

REPORT OF THE DIRECTOR

REPORT OF THE DIRECTOR

La Dra. Montserrat Ramón Herrero, directora de la tesi titulada: **"Feeding behavior of the mussel *Mytilus* spp.: responses to the natural variability of seston and to toxic phytoplankton ingestion" ("Conducta alimentària del musclo *Mytilus* spp.: respostes a la variació natural del sèston i a la ingestió de fitoplàncton tòxic")** realitzada per Eva Galimany Sanromà,

Informa de la implicació de la doctoranda en cada article científic desenvolupat per la present tesi i que cap dels articles, ni de les dades aquí presentades, han estat usades per la memòria d'una altra tesi.

Chapter I. Article: **"Feeding behavior of the mussel *Mytilus galloprovincialis* (L.) in a Mediterranean estuary: a field study"**

no publicat, la versió que es presenta en la memòria de tesi es la versió consensuada pels coautors i ja enviada a avaluar en una revista especialitzada internacional. El disseny experimental es va fer conjuntament entre la directora i la doctoranda. La doctoranda va intervenir en la construcció i posta a punt dels acuaris del sistema experimental i en el mostreig. L'asessorament del mètode de la biodeposició, usat pel càlcul dels components biològics del balanç d'absorció en bivalves correspon al Dr. Irrintzi Ibarrola, del Dpto. de Genética, Antropología Física y Fisiología Animal de la Facultad de Ciencia y Tecnología de la Universidad del País Vasco. La doctoranda va realitzar la cuantificació del contingut en sèston de l'aigua, i de les femtes i pseudofemtes del musclo, va treballar les dades, interpretar els resultats i redactar l'article, sota l'assessorament dels coautors (M. Ramón i I. Ibarrola).

Chapter II. Article: **"Pre-ingestive selection of mussels, *Mytilus galloprovincialis* (L.), grazing on natural phytoplankton in a N.W. Mediterranean estuary"**

no publicat, la versió que es presenta en la memòria de tesi es la versió revisada i consensuada pels diversos coautors i enviada a una revista especialitzada internacional. El disseny experimental es va fer conjuntament entre la directora i la doctoranda. La identificació d'espècies de fitoplancton es va dur a terme pel Dr. Delgado. La doctoranda va intervenir en la posta a punt del sistema experimental i el mostreig, la interpretació dels resultats i la redacció de l'article, assessorada pels coautors (M.Ramón y M. Delgado).

Chapter III. Article: **"First evidence of fiberglass ingestion by a marine invertebrate (*Mytilus galloprovincialis* L.) in a N.W. Mediterranean estuary"**

publicat a la revista Marine Pollution Bulletin, l'índex d'impacte de l'any 2008 fou de 2.562. El disseny experimental es va fer conjuntament entre la directora i la doctoranda. El Sr. José Manuel Fortuño, tècnic del Microscopi Electrònic de Rastreig (SEM), va realitzar el microanàlisi per dispersió d'energia de raigs X (EDS) per determinar la composició química de les fibres i la seva morfologia amb mostres prèviament preparades per la doctoranda. A més, la doctoranda va participar en el mostreig, extreure el contingut estomacal i va mesurar les fibres de vidre, analitzar les dades i redactar l'article, assessorada pels coautors (M. Ramón y M. Delgado).

Chapter IV. Article: **"The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*"**

publicat a la revista **Harmful algae**, l'índex d'impacte l'any 2008 fou de 2.688. L'experiment es va dur a terme en el Marine Biological Association, Plymouth (UK) sota la supervisió del Dr. Pipe. El disseny experimental es va fer conjuntament entre el Dr. Pipe, la directora i la doctoranda. L'anàlisi de toxines de *Karlodinium veneficum* (PLY # 103) el va dur a terme el Dr. Place en el seu laboratori de USA. La doctoranda i el Dr. Pipe es van encarregar de la recollida dels animals experimentals. La doctoranda va realitzar el muntatge i manteniment dels aquaris experimentals, la recollida de mostres, l'anàlisi de dades i la seva interpretació (excepte el de toxines), així com de la redacció de l'artícle, assesorada pels coautors (A. R. Place, M. Ramón, M. Jutson and R. K. Pipe).

Chapter V. Article: **"Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*"**

publicat a la revista **Harmful algae**, l'índex d'impacte l'any 2008 fou de 2.688. El treball experimental es va dissenyar pel Dr. Wikfors, la doctoranda i la tutora de tesi, i es va realitzar en el NOAA Milford Laboratory (USA), sota la supervisió del Dr. Wikfors, durant una estada de la doctoranda en aquest laboratori. La Dra. Hégaret va ensenyar a la doctoranda l'ús del citòmetre de flux i va col·laborar amb ella en l'anàlisi de les mostres. La doctoranda va avaluar els canvis histopatològics dels teixits dels musclos sota la supervisió de la Dra. Sunila. La doctoranda es va encarregar del manteniment dels aquaris, de dur a terme l'experiment, de l'elaboració de les preparacions histològiques, de l'anàlisi de les dades i de la seva interpretació, així com de la redacció de l'article, assessorada pels coautors (I. Sunila, H. Hegaret, M. Ramón, G. H. Wikfors).

Chapter VI. Article: **"Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: histopathology, immune responses, and recovery"**

publicat a la revista **Harmful algae**, l'índex d'impacte l'any 2008 fou de 2.688. El treball experimental es va dissenyar pel Dr. Wikfors, la doctoranda i la tutora de tesi, i es va realitzar en el NOAA Milford Laboratory (USA), sota la supervisió del Dr. Wikfors, durant una estada de la doctoranda. La Dra. Hégaret va ensenyar a la doctoranda l'ús del citòmetre de flux i va col·laborar amb ella en l'anàlisi de les mostres. La doctoranda va avaluar els canvis histopatològics dels teixits dels musclos sota la supervisió de la Dra. Sunila. La doctoranda es va encarregar del manteniment dels aquaris, de dur a terme l'experiment, de la preparació posterior de les mostres d'histologia, de l'anàlisi de les dades i de la seva interpretació, així com de la redacció de l'article, assessorada pels coautors (I. Sunila, H. Hegaret, M. Ramón, G. H. Wikfors).

RESULTS

Chapter I

Feeding behavior of the mussel *Mytilus galloprovincialis* (L.) in a Mediterranean estuary: a field study

Submitted

Feeding behavior of the mussel *Mytilus galloprovincialis* (L.) in a Mediterranean estuary: a field study.

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Key words: feeding behavior; shellfish; physiology; field experiment; Alfacs Bay; seston.

Abstract

The feeding behavior of the mussel *Mytilus galloprovincialis* was studied under field conditions on top of a mussel raft in Alfacs Bay, N.W. Mediterranean Sea. The experiments were performed in November 2006 and February, April and July 2007 using a flow-through filter feeding device. Total particulate matter (TPM), particulate organic matter (POM) and particulate inorganic matter (PIM) were calculated for the bay water, and the feces and pseudofeces of the mussels, to obtain different feeding-physiological parameters through the biodeposition method, such as the clearance rate (CR), organic ingestion rate (OIR), absorption rate (AR) and absorption efficiency (AE). The device employed for the experiments was validated before the data was analyzed. The results showed that short-term variations in food quantity and quality were similar to the long-term variations; nevertheless, we found a high organic content of the bay water throughout, with *f* (POM/TPM) values ranging from 0.51 to 0.72, which is comparable to other areas with high mussel aquaculture production. Physiological parameters characterizing both food acquisition and absorption were found to vary greatly in the short-term (days). Nevertheless, we found high CR and AE values throughout the study. As a response to the variable environment of Alfacs Bay, mussels reduced their clearance rate when seston concentrations were high instead of increasing their pseudofeces production. Absorption efficiency was positively related to the organic content of the ingested particles (i), which has been shown previously to be a main determinant of absorption efficiency. We could not determine a seasonal pattern but there was a

clear tendency for mussels to decrease their CR, OIR and AR during July. It is possible that this feeding restriction was due to the high temperature of the bay water in that month, which reached 25ºC.

Our results show that the quality of seston in Alfacs Bay is good for bivalve farming. The physiological parameters measured were high, except in July, when mussels were negatively influenced by the high water temperature. This type of study is very useful for the management of bivalve aquaculture sites because data is obtained from a field study.

1. Introduction

Mussels (*Mytilus galloprovincialis*) are cultured on the Spanish Mediterranean coast in the two Ebro Delta bays, Fangar and Alfacs, with an annual production of 3000 t per year (Ramón et al., 2005a). They are cultured in suspension from a total of 166 fixed rafts divided between the bays of Alfacs (90 farms) and Fangar (76 farms). The rafts are rectangular wooden frames measuring 200 m x 15 m, from which 2 to 3 m long mussel ropes are suspended. There is a seasonal pattern for the growth of mussels in Fangar Bay, with higher rates in spring and the lowest rates in winter and August. The growth and mortality of mussels cultured in the two delta bays are strongly affected by the high seawater temperatures reached during July and August, which cause a detention in growth and high mortalities of adults and juveniles (Ramón et al., 2005a).

Mussels (*Mytilus* spp.) are filter feeding bivalves that feed on a suspension of water particles. Areas that have bivalve cultures are characterized by high primary production that sustains their grazing pressure. Facing the Mediterranean Sea, Thau Lagoon (S.E. France) has high primary production (Gasc, 1997), with particulate organic matter (POM) values ranging from 0.1 to 1.7 mg L^{-1} (Gangnery et al., 2004). The study site, Alfacs Bay (N.E. Spain), has a chlorophyll *a* level that is one order of magnitude above the surrounding Mediterranean Sea (Delgado, 1987), and POM values ranging from 1 to 3.4 mg L^{-1} (Ramón et al., 2005b). Moreover, the POM concentration in Alfacs Bay is even higher than in the Rías Gallegas (N.W. Spain), the largest mussel producer in Europe (Pérez Camacho et al., 1991; Keldany, 2002). As an example, POM concentrations found in Ría de Arousa, the most important of the Galician Rias for mussel production, range from 0.28 to 1.08 mg L^{-1} (Navarro et al., 1991; Babarro et al., 2003). In addition to the POM, food quality expressed as POM/TPM can have a significant influence on the growth of bivalves, which gives an idea of the relative importance of the organic matter in the environment. There is a high percentage of organic content in the water of both Alfacs Bay and Ría de Arousa, and values can reach up to 50-60% (Ramón et al., 2005b; Babarro et al., 2003).

The feeding behavior of mussels has been studied by means of different

approaches. Foster-Smith (1975) calculated filtration rates from the particle removal rates from a fixed volume of suspension. For a better approach to natural conditions, other authors estimated clearance rates by monitoring the removal of suspended particles as water passed through mussels; therefore, the filtration rates could be calculated as the clearing of water particles from the environmental water (Riisgård, 1977; Bayne and Widdows, 1978; Widdows et al., 1979). The need to simulate real conditions in order to better understand the feeding behavior in the field has led researchers to design new devices. Smith and Wikfors (1998) developed an automated rearing chamber system for studies of shellfish feeding behavior, and Babarro et al. (2000) used a portable box raft experimental chamber to conduct experiments in situ on top of mussel rafts. Different portable filter feeding devices have been designed in order to better understand the feeding physiological parameters of mussels (Filgueira et al., 2006; Grizzle et al., 2006). Nowadays, there are different methods for measuring filtration rates in suspension feeding bivalves, each of which should be used according to the experimental conditions and taking into account their advantages and disadvantages (reviewed by Riisgård, 2001).

The aim of the present study is to use the biodeposition method to determine the main physiological parameters related to mussel feeding behavior in a Mediterranean estuary where bivalve aquaculture takes place (Iglesias et al., 1998). The experiments were carried out on top of a mussel raft at four different periods of the year for a more realistic approach. These results will be useful for evaluating the carrying capacity of Alfacs Bay for bivalve growth, and for production management using simulation models.

2. Materials and methods

2.1 Experimental design and animals

The filter feeding experiments were performed from November 2006 to July 2007. Four filter feeding experiments, lasting 2 hours each, were carried out per sampling period (i.e., November 2006, and February, April and July 2007), corresponding to 2 consecutive days in 2 consecutive weeks in each period, except for July, in which 3 experiments were carried out.

Mussels, *Mytilus galloprovincialis*, were collected from a mussel aquaculture farming site in Alfacs Bay (Ebro Delta) the day before each experiment. Twenty mussels per experiment were collected, and epiphytes and other encrusting organisms were removed from the shells. A little plastic hook and loop fastener was glued to one of the two shells of each individual. When the glue dried, mussels were hung back on the raft where the experiments were performed the following day. Acclimation was not necessary as the mussels were always submerged in the bay water. Fake mussels were made by collecting four of the twenty

fresh mussels, taking out the flesh and gluing the shells back together to act as controls.

2.2 Flow-through devices

Two portable filter feeding flow-through devices were designed to simulate *in vivo* conditions of mussel feeding (Fig. 1).

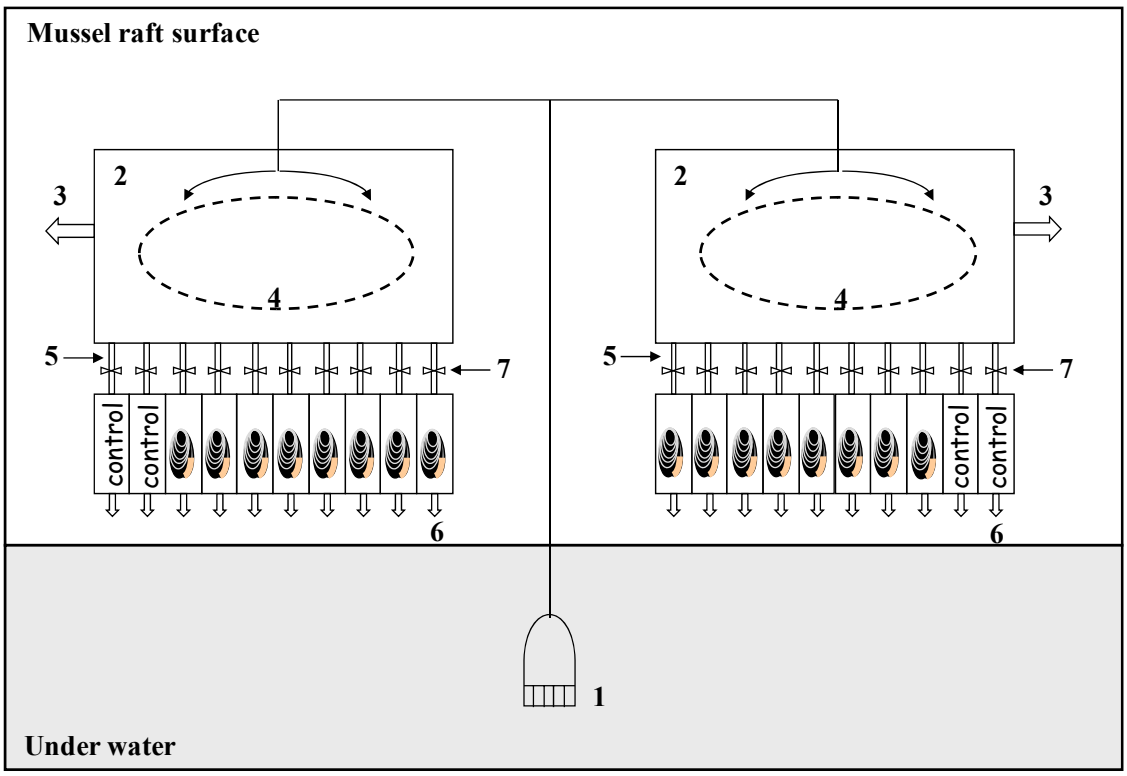

Fig. 1: Diagram of the two portable filter feeding devices. 1: underwater pump; 2: common PVC tanks; 3: common tank extra flow exit tube; 4: aeration; 5: rubber tubes; 6: aquarium extra flow exit tubes; 7: manual valves.

One portable filter feeding device consisted in a common PVC tank (length 560 mm, width 300 mm, height 150 mm) that received bay water from an underwater pump hung from the mussel raft poles. Laterally, the tank was provided with an extra flow exit tube. Aeration was provided to mix the bay water in the common tank. Ten rubber tubes emerged from the lower part of the tank. Each tube was connected to an individual PVC aquarium. The aquaria measured 45 x 180 x 60 mm (length x width x height), and each aquarium contained a single mussel. Two of the aquaria contained one fake mussel each, which were used as controls. The mussels were positioned near the flow exit tube of the aquaria and attached to the bottom with a piece of plastic hook and loop fastener. The flow of bay water from the common tank to each aquarium was regulated through a manual valve and maintained at 12 L h^{-1}. This flux was determined by previous laboratory experiments to validate the optimal geometry chamber using the flow-through chamber method, following Riisgård (1977). A flow

of 12 L h^{-1} showed a homogeneous distribution of particles between aquaria and no water recirculation occurred in any of the aquaria. These filter feeding devices were deployed in the same area that the mussel cultures are located, i.e., on a mussel raft.

2.3 Characteristics of the bay water

Bay water (1 L) from the tanks with fake mussels (acting as controls) was collected every 15 minutes, filtered separately through washed Whatman GF/C filters (25 mm diameter circles) and rinsed with ammonium formate. In the laboratory, filters with water samples were dried at 60ºC for 48 h and weighed to obtain the dry weight, which accounted for the total particulate matter (TPM). Afterwards, filters were ashed at 450ºC for 4 h before the final weighing to obtain the particulate inorganic matter (PIM). The particulate organic matter (POM) was calculated as the weight loss between TPM and PIM. The organic content of the bay water (*f*) was calculated as the fraction between POM and TPM.

2.4 Physiological feeding parameters

The individual chambers of the feeding-devices were cleaned before the collection of biodeposits for the experiments. Then, the feces and pseudofeces of each mussel were collected separately with a pipette as soon as they were produced and accumulated in each individual filter. After approximately 2 h the mussels had produced a large amount of biodeposits ending the collection period of the experiment.

Filters with samples of feces and pseudofeces produced by each individual mussel (n= 16) were processed for organic and inorganic matter as indicated for the water samples, in order to compute the total, organic and inorganic egestion and rejection rates. The physiological components

Parameter	Acronym	Units	Calculation
Clearance rate	CR	L h^{-1}	(mg inorganic matter from both feces and pseudofeces per unit of time) / (mg inorganic matter available in bay water L^{-1})
Filtration rate	FR	mg h^{-1}	CR x mg total matter available in L^{-1} bay water
Rejection rate	RR	%	[(mg total matter within pseudofeces deposited h^{-1}) / FR] x 100
Organic ingestion rate	OIR	mg h^{-1}	(CR x mg organic matter available in L^{-1} bay water) – (mg organic matter within pseudofeces deposited h^{-1})
Absorption rate	AR	mg h^{-1}	OIR - (mg organic matter within feces deposited h^{-1})
Absorption efficiency	AE	fraction	AR / OIR
Selection efficiency	SE	fraction	1 – [(organic fraction within pseudofeces) / (organic fraction within total particles available in bay water)]

Table 1: Physiological components of absorptive balance for mussels in Alfacs Bay.

of the absorptive balance (Table 1) were then calculated according to the biodeposition method (Iglesias et al, 1998).

The ingestion rates of total matter (TIR: mg/h) and organic matter (OIR: mg/h) were obtained as the difference between the filtration and rejection rates of either the total or organic matter. In order to quantify the preingestive rejection of food through pseudofeces production, we expressed rejection of food as a percentage of filtered matter. Finally, the organic content of ingested matter was calculated as OIR/TIR.

It was necessary to calculate the gut transit time (GTT) in order to compare the seston ingested with the corresponding biodeposits of the mussels, as the food digestion process takes some time. Therefore, the GTT was calculated before each experiment using a method adapted from Hawkins et al. (1996). Five mussels were placed individually in a beaker in a mixture of bay water and *Tetraselmis suecica* monoculture. The time that elapsed between the beginning of the exposure and the deposition of green colored feces was considered to be the GTT (min).

All parameters were standardized to 1 g of dried mussel flesh using the following equation:

$$Y_s = Y_e \times (1/W_e)^b$$

where Y_s is the standardized physiological rate, Y_e is the experimentally determined rate and W_e is the measured dry body mass of each mussel. We used a b value of 0.67, which has been used in previous mussel feeding studies (Bayne et al., 1989, 1993; Jones et al., 1992; Hawkins et al., 1997).

2.5 Statistical analyses

Data were checked for normality and variance homogeneity. A non-parametric test (Kolmogorov-Smirnov) was used to compare the results obtained for the TPM, POM, PIM and the organic content of the bay water (*f*) with the two filter feeding devices. The results for TPM, POM, PIM and the percentage of organic content of the bay water, gut transit time (GTT) and the in situ filter feeding parameters were compared between sampling periods using the Kruskal-Wallis one-way Analysis of Variance. The statistical software used was Statgraphics Plus (Manugistics, Inc., Rockville, MD, USA). Correlations were established between *f* and TPM, CR and TPM, and AE and i (organic content of the ingested material) using non-linear regression models and the statistical software SPSS Statistic 17.0 (SPSS Inc, Chicago).

3. Results

3.1 Bay water

The mean values of the bay water temperature (ºC) for November, February, April and July during the experiments were 18.1 ± 0.1, 10.56 ± 0.1, 16.7 ± 0.1 and 25.9 ± 0.1 respectively.

TPM, POM, PIM and the organic content of seston (*f*) of the bay water showed no significant differences between the two flow-through devices when all the samplings were analyzed together ($p>0.05$). Mean

values for the different parameters for the two devices together throughout the study period were TPM: 1.67 ± 0.09; POM: 0.99 ± 0.04; PIM: 0.68 ± 0.06, expressed as mg L^{-1}; and *f*: 0.62 ± 0.01. Nevertheless, when the two devices were compared for each sampling day and period of the study, TPM, POM and PIM showed significant differences for April and day 9 (the first sampling day of April) ($p<0.05$). When data from day 9 was not considered in the statistical analyses, the devices did not show any significant differences between days or sampling periods ($p>0.05$). Therefore, day 9 was not taken into account in the study of mussel filter feeding parameters, as the devices were not comparable on that day for unknown reasons.

	Sampling day	TPM (mg L^{-1})	POM (mg L^{-1})	PIM (mg L^{-1})	*f* (POM/TPM)
November 2006	1	1.63 ± 0.13	1.03 ± 0.05	0.60 ± 0.07	0.65 ± 0.02
	2	1.41 ± 0.13	0.88 ± 0.05	0.55 ± 0.08	0.63 ± 0.02
	3	1.30 ± 0.13	0.79 ± 0.05	0.51 ± 0.08	0.62 ± 0.02
	4	2.09 ± 0.13	1.14 ± 0.05	0.88 ± 0.08	0.54 ± 0.02
February 2007	5	2.17 ± 0.16	1.06 ± 0.05	1.11 ± 0.08	0.51 ± 0.02
	6	1.51 ± 0.16	0.85 ± 0.06	0.42 ± 0.08	0.66 ± 0.02
	7	1.21 ± 0.17	0.71 ± 0.05	0.50 ± 0.09	0.62 ± 0.02
	8	1.02 ± 0.17	0.67 ± 0.05	0.35 ± 0.08	0.67 ± 0.02
April 2007	10	1.72 ± 0.10	0.99 ± 0.05	0.73 ± 0.08	0.58 ± 0.02
	11	2.00 ± 0.18	1.27 ± 0.09	0.74 ± 0.14	0.64 ± 0.04
	12	1.71 ± 0.15	1.12 ± 0.08	0.60 ± 0.12	0.66 ± 0.03
July 2007	13	2.30 ± 0.14	1.30 ± 0.06	0.91 ± 0.09	0.58 ± 0.03
	14	2.16 ± 0.17	1.36 ± 0.07	0.80 ± 0.10	0.61 ± 0.03
	15	1.59 ± 0.18	1.15 ± 0.07	0.44 ± 0.11	0.72 ± 0.03

Table 2: Mean values (±SE) of total particulate matter (TPM), particulate organic matter (POM), particulate inorganic matter (PIM) and quality of seston (*f*) of the bay water for each sampling day.

The mean values for TPM, POM, PIM and *f* are shown in Table 2. Mean values of TPM ranged from approximately 1 to 2.3 mg L^{-1}. February showed significantly lower values of TPM, whereas April and July showed the highest seston concentration. The organic content of particles (*f*) ranged from 0.51 to 0.72 with no significant differences between sampling periods ($p>0.05$). There was a negative relationship between organic content (*f*) and total particulate matter (TPM) in the bay water throughout the entire study

period, (f = 0.827 (±0.021) e $^{-0.183\ (\pm 0.014)}$; r^2 = 0,466) ($p<0.001$); that is, the more TPM, the less organic fraction the water contained (Fig. 2).

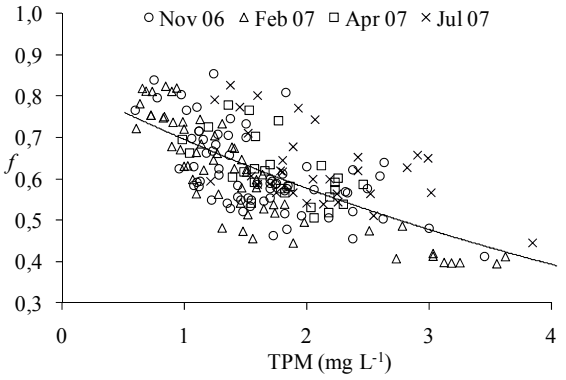

Fig. 2: Relationship between the organic content of seston (f) and total particulate matter (TPM) from the bay water during the different periods of the study (Nov: November 2006; Feb: February 2007; Apr: April 2007; Jul: July 2007). Lineal correlation shown in the graph.

3.2 Gut Transit Time (GTT)

The mean GTT values for the different periods of the study are shown in Table 3.

Sampling period	GTT (min)
November 06	71.82 ± 1.35
February 07	82.09 ± 1.30
April 07	67.29 ± 1.52
July 07	61.70 ± 1.47

Table 3: Mean values (±SE) of gut transit time (GTT) for each sampling period.

The longest time was 84 min and the shortest 59 min, obtained in February and July respectively. There were no significant differences between the different mean GTT obtained for each sampling period (November, February, April, July) ($p>0.05$).

Figure 3 shows the relationship between GTT and water temperature. The linear regression indicates that temperature explains 30% of the GTT variation ($p<0.05$).

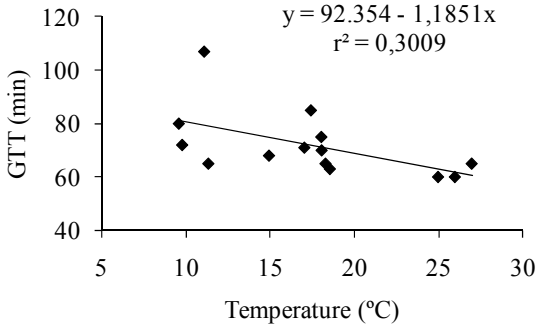

Fig. 3: Relationship between gut transit time (GTT) and temperature.

3.3 Feeding parameters

Table 4 shows values for CR, FR, RR, SE, OIR, AR and AE for a common standard sized (1g) mussel throughout the study period. There were no significant differences between the rejection rates (%RR) during the different sampling periods ($p>0.05$). Physiological parameters that characterize both food acquisition and absorption were found to change greatly in the short-term (days). For instance, whereas the total annual variation of the clearance rate ranged from a minimum of 1.06 L h^{-1} (July) to a maximum of 4.83 L h^{-1} (February), on two different days of November the CR ranged from 1.51 to 4.34 L h^{-1}. There seems to be a downward trend of

	Sampling day	CR (L h^{-1})	FR (mg h^{-1})	RR (%)	SE	OIR (mg h^{-1})	AR (mg h^{-1})	AE
November 2006	1	3.02 ± 0.38	6.21 ± 0.62	2.92 ± 2.36	0.22 ±0.18	3.01 ± 0.35	2.40 ± 0.28	0.74 ± 0.02
	2	4.34 ± 0.39	6.11 ± 0.64	4.51 ± 2.44	0.72 ± 0.10	3.58 ± 0.36	2.65 ± 0.29	0.73 ± 0.02
	3	3.21 ± 0.39	4.12 ± 0.64	1.38 ± 2.36	0.88 ± 0.10	2.47 ± 0.36	1.70 ±0.29	0.67 ± 0.02
	4	1.51 ± 0.85	3.07 ± 1.39	9.70 ± 5.27	0.75 ± 0.23	1.58 ± 0.78	1.03 ± 0.63	0.62 ± 0.05
February 2007	5	3.17 ± 0.46	7.24 ± 0.76	4.23 ± 2.89	0.77 ± 0.13	3.33 ± 0.43	2.18 ± 0.34	0.61 ± 0.03
	6	3.17 ± 0.38	4.56 ± 0.62	1.27 ± 2.36	0.83 ± 0.10	2.75 ± 0.35	2.08 ± 0.28	0.73 ± 0.02
	7	2.31 ± 0.37	2.68 ± 0.60	0	1	2.68 ± 0.60	1.09 ± 0.27	0.65 ± 0.02
	8	4.83 ± 0.41	4.99 ± 0.67	0.22 ± 2.53	0.57 ± 0.11	3.11 ± 0.38	2.50 ± 0.30	0.80 ± 0.02
April 2007	10	2.16 ± 0.37	2.33 ± 0.60	0	1	2.33 ± 0.60	1.48 ± 0.27	0.62 ± 0.02
	11	2.29 ± 0.50	4.53 ± 0.80	7.71 ± 3.04	0.60 ± 0.13	2.65 ± 0.45	1.71 ± 0.36	0.64 ± 0.03
	12	1.97 ± 0.41	3.26 ± 0.67	2.73 ± 2.53	0.73 ± 0.11	2.06 ± 0.38	1.38 ± 0.30	0.65 ± 0.02
July 2007	13	2.16 ± 0.38	4.91 ± 0.62	4.33 ± 2.35	0.65 ± 0.10	2.62 ± 0.35	1.57 ± 0.28	0.60 ± 0.02
	14	1.06 ± 0.46	2.32 ± 0.76	11.61 ± 2.89	0.49 ± 0.13	1.22 ± 0.43	0.75 ± 0.34	0.60 ± 0.03
	15	1.06 ± 0.42	1.60 ± 0.70	13.23 ± 2.31	0.60 ± 0.11	1.06 ± 0.39	0.73 ± 0.31	0.67 ± 0.02

Table 4: Mean values (±SE) of clearance rate (CR), filtration rate (FR), rejection rate (RR), selection efficiency (SE), organic ingestion rate (OIR), absorption rate (AR) and absorption efficiency (AE) for each sampling day.

the CR, OIR, AR and AE values from November and April to July (Fig. 4). Table 5 shows significant associations between the different sampling periods for CR, OIR, AR and AE. The maximum values for CR were obtained in November and February, and the mean values for these sampling periods were not significantly different ($p>0.05$). In contrast, April and July showed significantly lower values compared to November and February ($p<0.05$) and also differences between them ($p<0.05$). July was the period with the lowest CR of all (Fig. 4A). Although OIR and AR followed the same pattern, November, February and April were not significantly different ($p>0.05$), but they did differ from July ($p<0.05$), which showed lower values (Fig. 4B, 4C). Finally, AE was significantly higher in November and February, with values of around 70%, but significantly lower in April and July ($p<0.05$), with values near 63% (Fig. 4D).

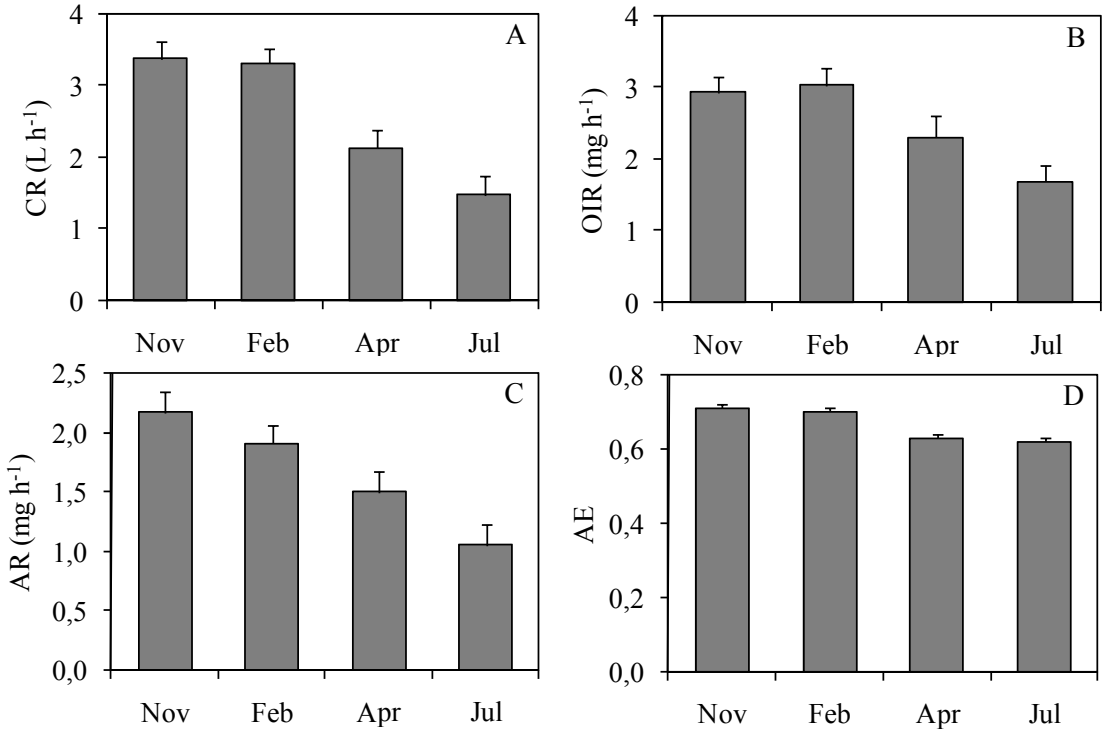

Fig. 4: Mean values for each sampling period (Nov: November 2006; Feb: February 2007; Apr: Aril 2007; Jul: July 2007) of the following feeding parameters: A. clearance rate (CR); B. organic ingestion rate (OIR); C. absorption rate (AR); D. absorption efficiency (AE). SE shown in bars.

Short-term variations in the clearance rate of mussels in Alfacs Bay appear to be related to environmental TPM fluctuations. Figure 5 shows the mean clearance rates in relation to the corresponding TPM. The clearance rate decreases exponentially with increasing food concentration according to the following equation:

$$CR = 7.600\ (\pm 2.513)\ e^{-0.722(\pm 0.188)xTPM};$$

$r^2 = 0,36$; $F = 14.710$; $p < 0.01$

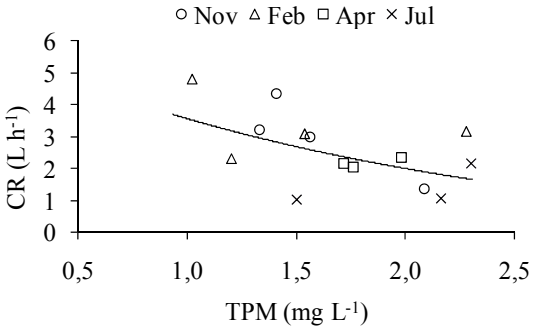

Fig. 5: Clearance rate (CR) related to total particulate matter (TPM) during the different periods of the study (Nov: November 2006; Feb: February 2007; Apr: Aril 2007; Jul: July 2007). Exponential relationship shown in the graph.

In agreement with previous authors, the absorption efficiency was found to be a positive function of the organic content of ingested material (i), described by an asymptotic exponential. Although variability of both AE and food organic content is relatively small in these experiments, we have fitted our results to asymptotic exponential curves (Fig. 6) and the following models were obtained for the different sampling periods:

November:

$AE = 0.950\ (\pm 0.143)\ (1\text{-}e^{(-2.180\ (\pm 0.706)xi)})$;

$r^2 = 0.334$, n= 46

February:

$AE = 0.950\ (\pm 0.116)\ (1\text{-}e^{(-2.432\ (\pm 0.666)xi)})$;

$r^2 = 0.586$, n= 38

April:

$AE = 0.950\ (\pm 0.662)\ (1\text{-}e^{(-1.751\ (\pm 2.339)xi)})$;

$r^2 = 0.237$, n= 22

July:

$AE = 0.825\ (\pm 0.119)\ (1\text{-}e^{(-2.196\ (\pm 0.711)xi)})$;

$r^2 = 0.228$, n= 38

Mussels had a lower capacity to absorb available food during July, which is shown by the fact that the lowest asymptotic AE value was recorded for this period.

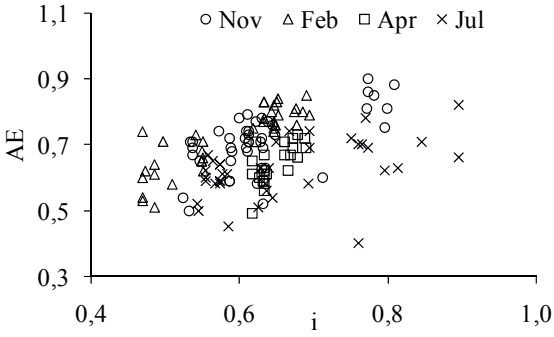

Fig. 6: Absorption efficiency (AE) related to the organic content of the ingested material (i) for each individual mussel.

Discussion

The methodology used for bivalve feeding experiments is a key issue for obtaining valuable data. Petersen et al. (2004) found significantly lower clearance rates measured with the biodeposition method compared to those measured with the flow-through and indirect methods. However, these last two methods considered shorter periods of time, when mussels were actively feeding, whereas the biodeposition method includes time periods of potential inactivity (Bougrier et al., 1998; Iglesias et al., 1998). Therefore, the biodeposition method is recommended for studying dynamic feeding responses in natural environments, as it integrates over time and enables several feeding parameters to be calculated (Pascoe et al., 2009), as we have done in this study.

	November	February	April	July
CR	———		———	———
OIR	———		———	———
AR	———		———	———
AE	———		———	———

Table 5: Summary of the Kruskal-Wallis one way Analysis of Variance among physiological determinations over time. CR: Clearance Rate; OIR: Organic Ingestion Rate; AR: Absorption Rate; AE: Absorption Efficiency. Straight lines indicate no significant differences ($p>0.05$).

Food characteristics (quantity and quality) during the experiments were found to be rather high during the study and similar to the values previously obtained in the area (Ramón et al., 2005b). Short-term variation (days) is distinctive of Alfacs Bay, and can be as large as the variability found for the different periods; it is mainly influenced by the wind regime and the shallow depth (5 m maximum). The negative relationship found for the bay water between organic content (*f*) and total particulate matter (TPM) is probably due to resuspension phenomena, as reported in other estuarine ecosystems, which would add silt and inorganic material to the water column (Hawkins et al., 1996; Velasco and Navarro, 2005). The seston characteristics from Alfacs Bay, on average, are similar to other aquaculture sites, but the minimum values of TPM and POM were higher than in Ría de Arosa, the largest mussel producer in Europe (Navarro et al., 1991; Babarro et al., 2000, 2003). Our results indicate that the seston (*f*) is of good quality with an average value of 0.62. In addition, in Alfacs Bay, *f* is also higher than reported for the Galician Rías (Babarro et al., 2000, 2003). Therefore, it is evident that Alfacs Bay is an ideal area for mussel farming in terms of food quality, and that the organic content of the seston is unlikely to be a limiting factor for this industry with the current mussel production.

The physiological parameters related to filter-feeding activities were found to change greatly in the short-term. However, the wide range of values found in the long-term for CR and OIR throughout the study has also been reported in other estuarine aquaculture sites (Babarro et al., 2000). As a response to the variable environment of Alfacs Bay in which the seston concentration is not constant, bivalves possess regulatory mechanisms. Foster-Smith (1975) proposed two mechanisms, which can occur together or separately: 1) a clearance rate reduction, and 2) an increase in pseudofeces production. Since the food rejection rate was low and constant throughout our study, the negative relationship between the clearance rate and the particle concentration becomes the main determinant for the food ingestion rate, as reported elsewhere (Widdows et al., 1979; Babarro et al., 2000). However, other authors

have obtained the opposite results, in which mussels increased the production of pseudofeces instead of regulating the clearance rate (Foster-Smith, 1975; Navarro et al., 2003). In these cases, the quality of food was low, in contrast to what we found in Alfacs Bay, and it seems that bivalves increase the rejection rates when they feed on seston with low organic content (Navarro et al., 1992; Urrutia et al., 1996; Navarro et al., 2003). Nevertheless, it is not clear whether the different strategies used to regulate the feeding behavior in relation to the seston concentration variability are species-specific or habitat-specific (Bacon et al., 1998). Absorption efficiency was positively related to the organic content of the ingested particles (i), which has been previously shown to be a main determinant of absorption efficiency (Hawkins et al., 1998). The relationship between AE and the quality of seston has also been documented for mussels in Marennes-Oléron Bay and in the Galician Rías (Navarro et al., 1991; Hawkins et al., 1996; Babarro et al., 2000), where AE is clearly dependent on food quality.

We observed a tendency for the physiological parameters characterizing both food acquisition and absorption to decrease during April and July. CR in April was lower than in the previous two sampling periods but, as OIR was not significantly different from that of November and February, this suggests an ingestion regulation phenomenon due to a high seston concentration in the bay water. July was the period with the lowest CR, OIR and AR. Although similar values of TPM were obtained during all the sampling periods, the CR of mussels decreased in July. Similarly, a decrease in the asymptotic AE value recorded for July compared with the remaining sampling periods (0.825 *vs*. 0.950) suggests that the mussels have a relatively lower capacity to absorb the available food during July. Low ingestion rates would be coupled with high GTT and high AE as an acclimation response to high seston quantity (Bayne et al., 1987). However, and although we found no significant differences between GTT during the study, these values were lowest in July and ingestion rates were also the lowest. It is known that ectothermic animals, such as bivalves, depend on the water temperature for many of their physiological processes. The inverse relationship found between GTT and temperature might indicate that this environmental parameter is responsible for restricting the functional capacity of mussels during July. The thermodependance of the CR of mussels has been previously reported (Denis et al., 1999; Babarro et al., 2000). The temperature threshold at our experiment site, despite a difference of 16ºC during the study, is in the tolerance range of *M. galloprovincialis* (Anestis et al., 2007). Nevertheless, Hofmann and Somero (1996) reported that the threshold induction temperature for stress proteins for *M. galloprovincialis* is 25ºC, and we recorded a water temperature of 25.98±0.14 during July. Therefore, temperature is probably the factor

that most influences the decrease in the feeding parameter values for mussels at the study site during July.

As a summary, we can conclude that Alfacs Bay is an ideal site for developing mussel aquaculture due to its high water quality, which seems to be stable throughout the year. Similarly, filtration activity and absorption efficiency were also high throughout the studied periods, and depend on TPM and the organic content of the ingested particles respectively. The lower feeding capacity observed during July seems to be influenced by high water temperatures, which would cause physiological stress on the mussels. This study contributes to a better understanding of in situ feeding behavior of *M. galloprovincialis* in Alfacs Bay, which is useful for further studies on the carrying capacity of the ecosystem.

Acknowledgements

This study has been supported by a grant from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) to the first author and partially financed by the RTA04-023 INIA research project. We would like to thank Xavier Mas, Xavier Leal and David Pina from ICM-CSIC for their advices and help during the construction of the flow-through devices. We would also like to acknowledge J. M. Reverté, Vanesa Castán and Esther Dámaso for their help during the field work.

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Chapter II

Pre-ingestive selection of mussels, *Mytilus galloprovincialis* (L.), grazing on natural phytoplankton in a N.W. Mediterranean estuary

Submitted

Pre-ingestive selection of mussels, *Mytilus galloprovincialis* (L.), grazing on natural phytoplankton in a N.W. Mediterranean estuary

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Key words: mussel; feeding; phytoplankton selection; Mediterranean.

Abstract

The pre-ingestive selection of mussels (*Mytilus galloprovincialis*) was investigated in Alfacs Bay, a N.W. Mediterranean estuary. For this purpose, we conducted experiments on a commercial raft at four different periods throughout a year. To evaluate the pre-ingestive selection of mussels towards the natural phytoplankton population, we identified and quantified the phytoplankton species of the bay water and the pseudofeces of mussels; we also identified and quantified the species in the stomach contents and feces of mussels as complementary information. Forty seven species of phytoplankton were identified in the bay water, where planktonic diatoms were dominant in number of cells during all the periods of study. Nevertheless, it was mostly dinoflagellates that were selected and ingested by mussels. The preferential selection of some species varied according to their relative concentration, as occurred with the diatom *Cyclotella meneghiniana*. In this particular case, *C. meneghiniana* was preferentially selected and ingested in February, but preferentially rejected in July, when it reached up to 10^5 cells L^{-1} in the bay water.

The present field study shows the preference of *M. galloprovincialis* towards some species of phytoplankton, mostly dinoflagellates, regardless of the potential toxicity of some of the species. Moreover, it shows that pre-ingestive selection of mussels in Alfacs Bay is very important in order to increase the quality of the diet. This study contributes to understanding the important role of bivalves in the ecosystems, due to their potential to change the structure of the phytoplankton communities as a consequence of their selective filter feeding behavior.

1. Introduction

Bivalve food consists of a wide variety of suspended particles such as bacteria, phytoplankton, zooplankton, detritus and dissolved organic material (Gosling, 2003). Nevertheless, phytoplankton has traditionally been thought to be their main source of food (Mason, 1971). The gills and their cilia are responsible for filtering the water, removing the particles, and then transporting them towards the labial palps (Jørgensen, 1975; Foster-Smith, 1978; Riisgård et al., 1996). In particular, *Mytilus edulis* can retain 2 µm particles with an efficiency of 75-90%, and the efficiency of retention increases along with the particle size up to 100% in particles greater than 6 µm (Møhlenberg and Riisgård, 1978).

Particle selection plays a key role in the feeding behavior of bivalves. Two types of selection have been described: 1) pre-ingestive selection, which occurs on the gills and the labial palps, results in the production of pseudofeces, formed by mucus and rejected particles (Kiørboe and Møhlenberg, 1981; Shumway et al., 1985; Ward et al., 1998; Zemlys et al., 2003), and 2) post-ingestive selection, which occurs in the stomach, by differentiating particles to digest those that are more nutritious (Bricelj et al., 1984; Shumway et al., 1985; Bayne, 1993; Wang and Fisher, 1996). In the stomach, particles can i) be retained longer to increase time for extracellular digestion, ii) be directed to the digestive gland for intracellular digestion (Bricelj et al., 1984; Brillant and MacDonald, 2003), or iii) be directed to the intestine to mix with other undigested material, and be incorporated into the feces (Gosling, 2003). The selection of particles by bivalves is a strategy to maximize the quality of the diet and optimize the energy gain.

Mussel filtration activity and its selective efficiency on various taxa interact to produce changes in the phytoplankton community of the ecosystems (Asmus and Asmus, 1991; Noren et al., 1999; Dolmer, 2000; Petersen et al., 2008). Thereafter, mussels have the potential to exert significant top-down control on phytoplankton (Strohmeier et al., 2008; Trottet et al., 2008). Despite the studies concerning primary production and bivalve-food interactions, there is scarce knowledge on the preferences of bivalves amongst all the phytoplankton species that are available in the ecosystems, including the potentially toxic species. Moreover, only few attempts have been made to find the relationship between the food source and the feeding behavior of bivalves under natural conditions (Sidari et al., 1998; Dolmer, 2000; Rouillon et al., 2005; Lehane and Davenport, 2004, 2006).

Therefore, the present study aims to characterize the phytoplankton pre-ingestive selection of mussels in a Mediterranean estuary during different periods of the year, throughout a mussel culture cycle. The feeding experiments were performed in Alfacs Bay, included in the Ebro Delta estuarine ecosystem (N.E. Spain), where bivalve aquaculture activities are traditional

(Ramón et al., 2007). Comparisons of phytoplankton composition within the bay and the mussel pseudofeces herein produced constitute a reliable method to study *in situ* interactions.

2. Materials and methods

2.1 Experimental animals

Mussels, *Mytilus galloprovincialis*, were collected in Alfacs Bay (Ebro Delta) the day before each experiment. Ten individuals per experiment were collected (36 to 87 mm shell length, according to the sampling period), and epiphytes and other encrusting organisms were removed from the shells. Once cleaned, the mussels were submerged again in the bay water in a mesh bag; thereafter, acclimation was not necessary.

2.2 Experimental design

Four feeding experiments were performed on a commercial raft during four different sampling periods, i.e., November 2006, and February, April and July 2007 (2 consecutive days of 2 consecutive weeks each period).

For the experiments, ten beakers were placed in a tray with a constant flow of natural water collected from the bay through an automatic pump. Mussels were placed individually in each 300 ml beaker covered with a 1 mm^2 mesh sealed with an elastic band to avoid the lost of biodeposits. A plastic hose released the excess water from the tray. Three samples of 100 ml each of bay water were collected at the beginning of the experiments and preserved in an additional 100 ml 1% glutaraldehyde. Feces and pseudofeces of each mussel were collected *in situ* individually with a pipette during 1 h (when the biodeposits were abundant) and preserved in 7 ml of 1% glutaraldehyde. Experimental mussels were immediately placed in cold storage and transported to the laboratory, where the stomach content was removed with a 25-gauge needle and a 1-ml syringe and preserved in 7 ml of 1% glutaraldehyde.

2.3 Phytoplankton identification and counts

Natural water, stomach contents, feces and pseudofeces were gently shaken; feces were additionally subjected to pressure with a syringe in order to disaggregate them. The samples were allowed to settle for 24h. Afterwards, we used an inverted microscope (XSB-1A) to identify and enumerate the phytoplankton organisms according to their sizes; 400x magnification was used for nanophytoplankton (ranging from 2-20 μm) and 250x and 100x were used for microphytoplankton (larger than 20 μm). Phytoplankton identification in the water, stomach, fecal and pseudofecal samples were performed to the closest taxon possible.

2.4 Selection

To analyze pre-ingestive selection of mussels towards the different phytoplankton species, we used a selection index (SI) based on Bayne et al. (1993) and defined as:

$$SI = 1 - (PS / W)$$

where PS is the percentage of each analyzed algal species in the pseudofeces and W is the percentage of each analyzed algal species in the bay water. Positive values of the SI

indicate selection and ingestion of the species whereas negative values indicate selection and rejection via pseudofeces.

2.5 Statistical analyses

Data was checked for normality and variance homogeneity. Due to the differences in cell density between water and pseudofeces, statistical comparisons were performed using the percentage of cell number, which is, the relative density values for each type of sample. Data was previously normalized using square root arcsine transformation. Species occurring in less than 5% of the samples were excluded from all the statistical analyses.

Statistical significant differences between samples, sampling days and periods were tested using Analysis of Variance (ANOVA). The statistical software used was Statgraphics Plus (Manugistics, Inc., Rockville, MD, USA).

3. Results

3.1 Alfacs bay phytoplankton composition and abundances

Forty seven species of phytoplankton were identified during the feeding experiments in the bay water. In addition, other non-identified species were categorized into 6 groups: (1) small dinoflagellates (<20μm); (2) large dinoflagellates (>20 μm); (3) dinoflagellate cysts; (4) small benthic diatoms (<30μm); (5) large benthic diatoms (>30 μm) and (6) centric diatoms. Planktonic diatoms were dominant in terms of the number of species and abundances. The 10 most common species or categories for each sampling period are shown in Table 1. The species *Prorocentrum minimum*, *Cryptomonas* spp. and small dinoflagellates were frequent throughout. Some species and groups were only common during one of the studied periods: *Lioloma pacificum*, *Gymnodinium* spp. and *Leptocylindrus minimus* were frequent in November; *Proboscia alata*, *Alexandrium minutum* and dinoflagellate cysts in February; *Thalassionema nitzschioides* and *Scrippsiella* spp. in April; and *Hermesinum adriaticum* and *Pleurosigma* spp. in July. Some species were common during three of the periods, such as *Cyclotella meneghiniana* (November, February and July), large dinoflagellates (February, April and July), *Chaetoceros* spp., and *Pseudo-nitzschia* spp. (first 3 periods). Finally, the species and groups only frequent during two periods were *Cylindrotheca closterium* (November and July) and *Prorocentrum micans* and the centric diatoms group (April and July).

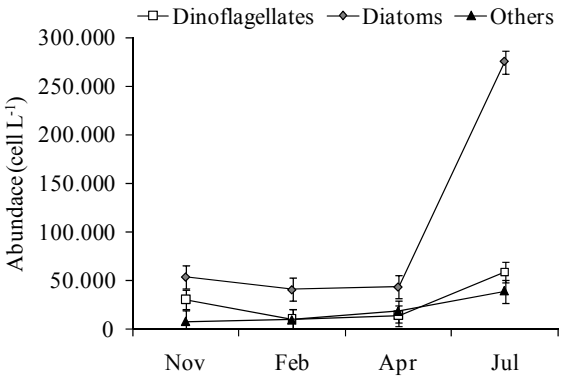

Fig. 1: Abundances expressed as cell L^{-1} of dinoflagellates, diatoms and "others" in the bay water (±SE) for each period of study (Nov: November 06; Feb: February 07; Apr: April 07; Jul: July 07).

November 2006			February 2007			April 2007			July 2007		
Species	cell L^{-1} (±SE)	Taxon group	Species	cell L^{-1} (±SE)	Taxon group	Species	cell L^{-1} (±SE)	Taxon group	Species	cell L^{-1} (±SE)	Taxon group
Pseudo-nitzschia spp.	26901±1487	DIAT	*Pseudo-nitzschia* spp.	18117±1487	DIAT	*Pseudo-nitzschia* spp.	23332±1487	DIAT	*Cyclotella meneghiniana*	200934±5578	DIAT
Prorocentrum minimum	13785±1229	DINO	*Cyclotella meneghiniana*	17293±5578	DIAT	*Cryptomonas* spp.	18391±1148	OTH	*Hermesinum adriaticum*	37932±2454	OTH
Small dinoflagellates	10156±993	DINO	*Cryptomonas* spp.	9607±1148	OTH	*Chaetoceros* spp.	12868±283	DIAT	*Cylindrotheca closterium*	36783±1476	DIAT
Lioloma pacificum	8097±236	DIAT	Small dinoflagellates	4666±993	DINO	*Thalassionema nitzschioides*	4650±116	DIAT	Small dinoflagellates	28273±993	DINO
Cryptomonas spp.	7960±1148	OTH	*Proboscia alata*	3893±432	DIAT	*Prorocentrum micans*	2997±201	DINO	*Pleurosigma* spp.	21537±1931	DIAT
Cyclotella meneghiniana	7137±5578	DIAT	*Prorocentrum minimum*	2281±1229	DINO	Small dinoflagellates	2870±993	DINO	Large dinoflagellates	19489±896	DINO
Chaetoceros spp.	5049±283	DIAT	Dinoflagellate cysts	1200±92	DINO	*Prorocentrum minimum*	2800±1229	DINO	Centric diatoms	13176±1518	DIAT
Gymnodinium spp.	3421±225	DINO	Large dinoflagellates	890±896	DINO	Centric diatoms	1667±1518	DIAT	*Prorocentrum minimum*	5490±1229	DINO
Cylindrotheca closterium	2236±1476	DIAT	*Alexandrium minutum*	860±65	DINO	Large dinoflagellates	1480±896	DINO	*Prorocentrum micans*	1880±201	DINO
Leptocylindrus minimus	2196±175	DIAT	*Chaetoceros* spp.	579±283	DIAT	*Scrippsiella* spp.	1400±54	DINO	*Cryptomonas* spp.	1372±1148	OTH

Table 1: Mean (± SE) values (cell L^{-1}) of the ten most abundant phytoplankton groups or species in Alfacs Bay water for each sampling period. The taxonomic groups (Taxon group) are indicated as dinoflagellates (DINO), diatoms (DIAT) and "others" (OTH).

From all the identified phytoplankton species, which occurred naturally during the feeding experiments, ten were potentially toxic (Table 2). The toxic species were found during the 4 periods studied at different concentrations except for *Gonyalaux verior* and the various *Dinophysis* spp. The species *Prorocentrum minimum* and *Pseudo-nitzschia* spp. were dominant and reached concentrations above 10^4 cells L^{-1} in the bay water during at least one sampling period.

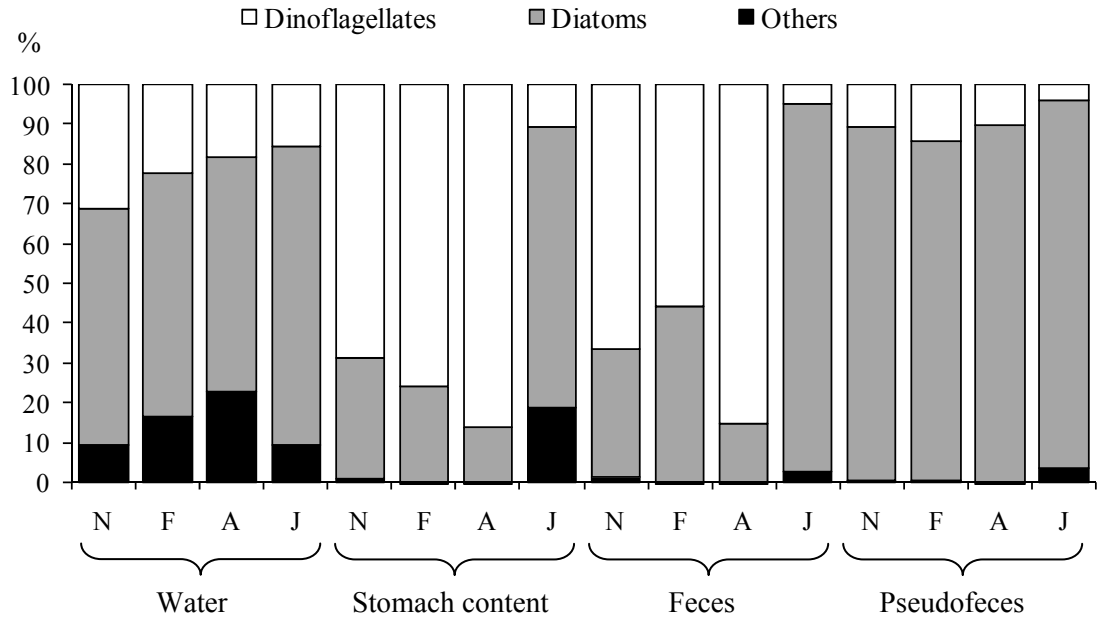

Fig. 2: Mean values of percentage of cell number of dinoflagellates, diatoms and "others" in the water, stomach contents, feces and pseudofeces of mussels for each period of study (N: November 06; F: February 07; A: April 07; J: July 07).

3.2 Phytoplankton taxonomic groups, abundances and selection

The phytoplankton species found in the natural water were grouped in order to analyze their relative importance among the pre-ingestive selection of mussels. Thereafter, they were grouped into dinoflagellates, diatoms and "others", which included 4 marine species (*Cryptomonas* spp., *Dictyocha fibula*, *D. octonaria*, and *Hermesinum adriaticum*) and the freshwater species *Scenedesmus* spp. Benthic diatoms were very scarce in all types of samples studied in comparison to planktonic diatoms. In this paper the word 'diatoms', unless specified, refers to the planktonic species.

Abundances in cell L^{-1} of the different groups of phytoplankton in the bay water are shown in Fig. 1. Diatoms were significantly more abundant during the whole study ($p<0.05$). In July, all phytoplankton groups increased compared to the rest of the periods, especially diatoms ($p<0.01$).

HAB species	Taxon group	November 2006	February 2007	April 2007	July 2007
Alexandrium minutum	DINO	+	+	+	+
Dinophysis caudata	DINO	+			+
Dinophysis rotundata	DINO			+	
Dinophysis sacculus	DINO	+	+	+	
Gonyalaux verior	DINO				++
Gymnodinium spp.	DINO	++	+	+	+
Prorocentrum micans	DINO	+	+	++	++
Prorocentrum minimum	DINO	+++	++	++	++
Prorocentrum triestinum	DINO	+	+	+	+
Pseudo-nitzschia spp.	DIAT	+++	+++	+++	++

Table 2: Potential harmful algal bloom (HAB) species in the bay water throughout the feeding experiments. Taxonomic groups (Taxon group) are indicated as DINO for dinoflagellates and DIAT for diatoms. Abundances are expressed as blank: non-present; +: present (less than 10^3 cells L^{-1}); ++: abundant (between 10^3 cells L^{-1} and 10^4 cells L^{-1}); +++: very abundant (more than 10^4 cells L^{-1}).

The samples of stomach contents, feces and pseudofeces processed to identify phytoplankton species contained unbroken and a minor part of broken cells, in contrast to the bay water, which mainly contained intact cells. Nevertheless, phytoplankton species were identifiable in all types of samples. The percentages of dinoflagellates, diatoms and "others" from the bay water and the different mussel digestive components (stomach contents, feces and pseudofeces) studied throughout the experiments are shown in Fig. 2. Accordingly, diatoms were significantly more abundant during the whole period of study in the bay water ($p<0.01$). Mean annual values (± SE) for percentage of dinoflagellates, diatoms and "others" in Alfacs Bay water were 21.92±10.22; 63.62±15.14 and 14.46±12.87 respectively. Stomach contents, however, presented a higher percentage ($p<0.01$) of dinoflagellates during most of the sampling periods (November, February and April), though diatoms were significantly more abundant during July ($p<0.01$). This was also the only period where "others" were significantly present in the stomach ($p<0.01$). Feces showed a similar pattern to stomach contents,

although the percentages of dinoflagellates and diatoms were not significantly different for the second period of study (February) ($p>0.05$). In July, the percentage of diatoms in the feces increased significantly ($p<0.01$), and became dominant. Pseudofeces presented a very high percentage of diatoms during the whole period of study ($p<0.01$), whereas dinoflagellates and "others" remained below 15% and 4%, respectively.

The selection index (SI) calculated for the different phytoplankton taxonomic groups throughout the study period are shown in Table 3. For the different sampling periods, and when considering all the samplings together, the selection index was positive for dinoflagellates and "others" but negative for diatoms; these results indicate pre-ingestive selection and ingestion of dinoflagellates and "others" but selection and rejection of diatoms.

Sampling period	Dinoflagellates	Diatoms	Others
November	0.66	-0.49	0.93
February	0.36	-0.41	0.98
April	0.44	-0.52	0.99
July	0.76	-0.23	0.60
All periods	0.56	-0.40	0.92

Table 3: Selection index (SI) for the different groups of phytoplankton at each sampling period and throughout.

3.3 Phytoplankton species, abundances and selection

The most abundant species of phytoplankton found in Alfacs Bay water throughout the feeding experiments and their relative abundances in the bay water and pseudofeces are shown in Table 4. The SI showed positive selection for some of the phytoplankton species by mussels, while others were discarded. The species preferentially selected and ingested were *Prorocentrum minimum*, small dinoflagellates, *Cryptomonas* spp., *Hermesinum adriaticum*, and *Cylindroteca closterium*. A peculiar behavior was found for *Cyclotella meneghiniana*. In February the species was preferentially selected and ingested whereas in July, the species was preferentially rejected. *Chaetoceros* spp. and *Pseudo-nitzschia* spp. were preferentially rejected overall.

Discussion

Most of the phytoplankton species found in the natural water during the feeding experiments represented the characteristic community observed in the area throughout the year (Delgado, 1987). The total number of phytoplankton cells per liter is comparable to what previously found by Delgado (1987), who determined that the bay water has a level of chlorophyll *a* one order of magnitude above the surrounding Mediterranean Sea, and concluded that Alfacs Bay is a rich primary producer ecosystem. Some species had not been previously reported in the bay water although they were all typical species from the Mediterranean Sea (Delgado and

Sampling period	Species	Taxon group	Bay water	Pseudofeces	SI
November	*Pseudo-nitzschia* spp.	DIAT	28.39 ± 5.10	37.85 ± 2.41	-0.17
	Prorocentrum minimum	DINO	12.72 ± 10.05	4.48 ± 4.74	0.44
	Small dinoflagellates	DINO	11.23 ± 3.20	1.40 ± 1.51	0.73
February	*Pseudo-nitzschia* spp.	DIAT	23.35 ± 6.70	29.21 ± 3.16	-0.20
	Cyclotella meneghiniana	DIAT	25.14 ± 11.13	17.62 ± 5.24	0.34
	Cryptomonas spp.	OTH	16.73 ± 1.90	0.17 ± 0.85	0.98
April	*Pseudo-nitzschia* spp.	DIAT	29.34 ± 3.91	41.22 ± 1.75	-0.22
	Cryptomonas spp.	OTH	22.55 ± 1.90	0.17 ± 0.85	0.97
	Chaetoceros spp.	DIAT	17.23 ± 3.78	32.22 ± 1.69	-0.40
July	*Cyclotella meneghiniana*	DIAT	56.07 ± 7.03	76. 35 ± 3.14	-0.26
	Hermesinum adriaticum	OTH	8.67 ± 4.04	3.64 ± 1.80	0.38
	Cylindrotheca closterium	DIAT	10.40 ± 2.21	4.87 ± 0.99	0.34

Table 4: Mean percentage values (±SE) of the three most abundant phytoplankton species in the natural water for the different periods of study and their abundances in the pseudofeces of mussels. Phytoplankton species are listed in order of abundance in the bay water. The taxonomic groups (Taxon group) are indicated as dinoflagellates (DINO), diatoms (DIAT) and "others" (OTH). The selection index is indicated as SI.

Fortuño, 1991). The only freshwater species observed in the samples, *Scenedesmus* spp., is common in Alfacs Bay as it flows into the estuary from different Ebro River freshwater pipes (Delgado, 1987). Benthic diatoms were scarce in Alfacs Bay despite the fact that they can be an important source of food for bivalves in some estuarine ecosystems, when benthic diatoms may be resuspended from the sediment (Kamermans, 1994; Rouillon et al., 2005; Trottet et al., 2008).

The dominance of non-benthic diatoms *vs*. dinoflagellates in Alfacs bay water was previously reported by Delgado (1987). Mussels have a high retention efficiency of diatoms larger than 5 µm (Hildreth and Mallet, 1980); thus, this group of phytoplankton species can be positively selected and ingested by mussels. However, in this study we found preferential selection of dinoflagellates and rejection of diatoms, with abundances of such taxa in pseudofeces above 80%. This result is consistent with what found by Shumway et al. (1985), who fed one dinoflagellate, one diatom and one cryptomonad species to six different species of bivalves and found a much higher proportion of diatoms in the pseudofeces for

five of the bivalves. In a similar context, *Mytilus edulis* retained more carbon biomass from dinoflagellates than from diatoms (Trottet et al., 2008), and the mussel *Perna canaliculus* presented very high assimilation efficiency for dinoflagellates when feeding on a mixture of dinoflagellates, diatoms and flagellates (Ren et al., 2006). Nevertheless, in our study, and as an exception for the group, the diatom *Cylindrotheca closterium* was positively selected. Rouillon et al. (2005) found that dinoflagellates were a minor component in the natural water but their abundances in mussel stomach contents were significantly higher. Accordingly, we found higher abundances of dinoflagellates in the stomach contents of mussels (except for July) evidencing their selection and ingestion. But we also found high abundances of dinoflagellates in the feces, which we did not expect. We hypothesize that the diet selected by the gills and palps of mussels in Alfacs Bay was very abundant and nutritious; thus, the excess of dinoflagellate cells ingested would be released in the feces as a consequence of post-ingestive selection processes. Nevertheless, this hypothesis was not tested as it was out of the goal of the study.

Diatoms in the bay water during July were the most abundant of all periods of study; furthermore, they significantly increased in stomach and fecal samples. Such abundances could be explained by the dominance of the diatom *Cyclotella meneghiniana* in the bay water. *Cyclotella* spp. is a freshwater species; nevertheless, *C. meneghiniana* has also been recorded from the marine phytoplankton (Tomas et al., 1997). Previous studies have demonstrated that this species is selected and ingested by the freshwater mussel *Dreissena polymorpha* (Szymczak-Zyla et al., 2006; Elliot et al., 2008). In the case of *M. galloprovincialis*, our results showed that their feeding behavior varied according to the abundance of such microalgal species in the bay water. Whereas *C. meneghiniana* was selected and ingested during February, it was rejected via pseudofeces during July. This rejection might be a response of the mussels to the high concentration of *C. meneghiniana* in the water, which reached abundances of 10^5 cells L^{-1} during July. Nevertheless, we found high concentrations of such diatoms in the stomach contents of mussels revealing their ingestion. We suggest that, despite that the gills and palps selected negatively *C. meneghiniana* cells during July, the species was so abundant that mussels could not avoid the entrance of cells into the digestive system. Moreover, the high abundance of diatoms in the feces might suggest a post-ingestive selection process to reject the great amount of *C. meneghiniana* cells ingested.

The species included in the group "others" were selected and ingested by mussels. *Cryptomonas* spp. was common in the natural water throughout the period of study and the selection index showed a clear positive preferential selection towards such microalgal species. Several studies report the

preferential selection of several species of bivalves when feeding on cryptomonad flagellates (Shumway et al., 1985; Lavrentyev et al., 1995; Schlekat et al., 2002). The flagellate *Hermesinum adriaticum* is a unicellular organism with a solid siliceous skeleton rarely reported (Tiffany, 2002). The preferential ingestion of mussels for *H. adriaticum* may explain the abundance of the group "others" in the stomach contents of mussels during July, when this species became very abundant. To our knowledge, there is no previous data on the ingestion of *H. adriaticum* by any bivalve species. According to our results, although the species possesses a silica skeleton, mussels ingested *H. adriaticum*.

We identified ten different species of toxic phytoplankton in the bay water. All these species have been previously reported in the area, some of them causing administrative closures at the mussel harvesting site (Vila et al., 2001). The dinoflagellate *Prorocentrum minimum* possesses the capacity to express toxicity (Grzebyk et al., 1997) and different species of the genus *Prorocentrum* in Alfacs Bay have been associated with HAB incidents affecting shellfish cultures (Diogène et al., 2008; Fernández-Tejedor et al., 2008). The selection index obtained in our study showed positive selection of *P. minimum* by mussels. On the contrary, *Pseudo-nitzschia* spp. was rejected during the different periods of the study. *Pseudo-nitzschia* does have toxic forms, however Quijano-Scheggia et al. (2008) found no indications of toxicity within our study site. Similar results were found by Safi et al. (2007) for the pinnid bivalve *Atrina zelandica*. Thus, it seems that the pre-ingestive selection criterion of mussels in Alfacs Bay was not affected by the potential toxicity of such microalgae. It has been demonstrated that bivalves inhabiting areas commonly exposed to HAB became more resistant to their toxicity (Bricelj et al., 2005) and in the site of study, HAB incidents have increased since 1989 (Diogène et al., 2008). Further investigations relating toxic dinoflagellates and bivalve selection are needed in order to understand the mechanisms involved.

Phytoplankton characteristics are very important in relation to particle selection (Ward and Shumway, 2004). Particle size is a parameter to be considered for capture efficiency (Defossez and Hawkins, 1997). The sizes of the identified phytoplankton in this study ranged approximately between 15-100 µm. *Proboscia alata* was the only larger species, which can reach up to 300 µm in length. Even so, it has been demonstrated that the rotifer *Brachionus plicatilis*, with sizes similar to those of *Proboscia alata*, are consumed by mussels (Wong and Levinton, 2006). Algal shape and flexibility can also influence the capture efficiency (Bougrier et al., 1997). Diatoms have an external silica frustule which protects them against predators (Hamm et al., 2003). Moreover, Menden-Deuer and Lessard (2000)

demonstrated that dinoflagellates have a higher nutritional value than diatoms as their carbon to volume relationship is double in dinoflagellates. In any case, our objective was not to explain the reasons or the mechanisms of species selection, but to determine all the species available and selected within this mussel farming site.

In conclusion, we have demonstrated by means of *in situ* feeding experiments that *M. galloprovincialis* clearly selected and ingested some phytoplankton species but rejected others, mostly dinoflagellates *vs*. diatoms respectively. The selection criterion of mussels remains unknown and was out of the goals of this study. The present results reinforce the suggestion that the feeding behavior of mussels can cause changes in the phytoplankton community through selective grazing, and that these impacts depend on the phytoplankton population and their relative abundances in the bay water. This type of study contributes to the understanding of phytoplankton dynamics in ecosystems where bivalves have a key role, like shellfish farming areas.

Acknowledgements

This study has been supported by a grant from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) to the first author and partially financed by the RTA04-023 INIA research project. We would also like to acknowledge Josep Maria Reverté, Vanessa Castán and Esther Dámaso for their help during the field work. The first author would specially like to thank Dave Veilleux for his friendship and English corrections.

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Chapter III

First evidence of fiberglass ingestion by a marine invertebrate (*Mytilus galloprovincialis* L.) in a N.W. Mediterranean estuary

Marine Pollution Bulletin 58 (2009) 1334–1338

Marine Pollution Bulletin 58 (2009) 1334–1338

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

First evidence of fiberglass ingestion by a marine invertebrate (*Mytilus galloprovincialis* L.) in a N.W. Mediterranean estuary

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ARTICLE INFO

Keywords:
Alfacs Bay
Fiberglass
Filter feeding
Gut content
Mediterranean Sea
Mytilus galloprovincialis

ABSTRACT

Alfacs Bay is a N.W. Mediterranean estuary important for mussel (*Mytilus galloprovincialis*) aquaculture. During studies at the site, fiberglass particles were detected. The presence of fiberglass occurred naturally in the water throughout the study period (November 2006 to July 2007). An investigation was undertaken into its role in the feeding behavior of the local mussels. Fiberglass was present in all types of mussel samples. Rejection, which we would have expected for the whole study period, was only evident during the second season studied. To our knowledge, this is the first report of the ingestion of fiberglass by a marine organism. Our novel finding indicates the need to investigate fiberglass ingestion by marine organisms at different levels of the food web and the possible implications for human health and the health of the organisms themselves. In addition, we propose the use of mussels as sentinel organisms to monitor fiberglass contamination in marine ecosystems.

1. Introduction

Mussels are filter feeding bivalves that feed on different types of suspended particles. The retention efficiency of the particles depends on the gill structure (Gosling, 2003). It has been reported that 1–2 μm particles are retained with an efficiency of 50% (Jørgensen, 1975), while particles of 1000–6000 μm have been found in mussel stomachs (Lehane and Davenport, 2006). Thus, the gills act like sieves that can retain all kinds of particles within this size range. However, bivalves do not ingest everything that is retained by the gill (Gosling, 2003); therefore, there has to be a criteria which bivalves are based on. When mussels feed on inorganic particles, such as silt or glass, silica or aluminum spheres, pre-ingestive selection can occur and the inorganic material is rejected as pseudofeces (Kiørboe et al., 1980; Ward and Targett, 1989; Bayne et al., 1993). However, pre-ingestive selection has also been reported when mussels feed on different species of phytoplankton with a preferential rejection of diatoms vs. dinoflagellates (Shumway et al., 1985). The selection efficiency of bivalves may therefore not only depend on the type of material or the size of the retained particles (Ward and Shumway, 2004).

Fiberglass is a narrow, elongated, hard, synthetic, filament. It is usually a transparent product comprising a mixture of silica (SiO_2) with the addition of other inorganic materials to increase its durability and resistance (Manutchehr-Danai, 2009). As a result of its properties, it is a material commonly used throughout building and shipping industries and is abundant in developed areas. Fiberglass has similar chemical and physical properties to asbestos. Asbestos belongs to a group of natural minerals which can be separated into thin, long fibers composed of Si and O, as well as other inorganic materials (Manutchehr-Danai, 2009). Both fiberglass and asbestos have a fibrous character which gives them the same aerodynamic properties (IARC, 2002). Human exposure to both types of materials can cause fibrosis, lung cancer and mesothelioma by inhalation (Maxim and McConnell, 2001). Ingestion of asbestos or fiberglass can cause severe gastrointestinal disorders in humans and other mammals (Maresca et al. 1984; Hardie et al., 1994; Kærheim et al., 2005). Nevertheless, as ingestion of these materials is considered accidental and rare, little previous research has been conducted in this area.

At the study site, Alfacs Bay (N.W. Mediterranean), mussel aquaculture is based on the species *Mytilus galloprovincialis* (Ramón et al., 2007). The high primary production in Alfacs Bay makes this an ideal area in which to develop aquaculture (Delgado, 1987). Furthermore, freshwater pipes from the Ebro River flow into the estuary carrying significant amounts of dissolved inorganic nutrients (Delgado and Camp, 1987). Nevertheless, agricultural and industrial contamination has been reported at the study site and local mussels from Alfacs Bay can accumulate different types of contaminants (Martínez-Gómez et al., 2008). The sources of

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doi:10.1016/j.marpolbul.2009.04.027

these contaminants are diverse and range, among others, from the freshwater pipes of the Ebro River to trawler discards and herbicides (Mañosa et al., 2001; Arcos et al., 2002). These sources could also introduce other, as yet unstudied, noxious particles, such as fiberglass, into the bay water.

In the present study, we report the presence of fiberglass in the marine environment for the first time. Moreover, given the unknown effects of fiberglass on the feeding behavior of the suspension-feeders that inhabit these ecosystems, we have conducted research into the selection, ingestion and rejection of fiberglass by mussels.

2. Materials and methods

2.1. Experimental animals

Mussels, *M. galloprovincialis*, were collected from a mussel aquaculture farming site in Alfacs Bay (Ebro Delta) the day before each experiment. Ten individuals per experiment were collected (shell length 36–87 mm, according to the season), and epiphytes and other encrusting organisms were removed from the shells. Once cleaned, the mussels were hung back on the raft where the experiments were performed. Acclimation was not necessary as the mussels were permanently submerged in the bay water.

2.2. Experimental design

The feeding experiments were performed on a commercial raft from November 2006 to July 2007. Four feeding assays were carried out per season, i.e., November 2006, and February, April and July 2007, corresponding to 2 consecutive days of 2 consecutive weeks, in each season.

The gut transit time (GTT) was calculated at the beginning of each experiment, adapted and slightly modified from Hawkins et al. (1996). Five mussels were placed in individual beakers in a mixture of bay water and *Tetraselmis suecica* monoculture. The time elapsed between the beginning of the exposure and the deposition of green colored feces was considered the GTT (min).

Ten beakers were placed in a tray with a constant flow of natural water collected from the bay through an automatic pump. Mussels were individually placed in each beaker and covered with a 1 mm^2 mesh sealed with an elastic band. A plastic hose released any excess water from the tray back to the bay. Three 100 ml samples of bay water were collected at the beginning of the experiments and preserved in an additional 100 ml of 1% glutaraldehyde. Feces and pseudofeces from each mussel were collected *in situ* with a pipette after passing the estimated GTT and preserved in 7 ml of 1% glutaraldehyde. Experimental mussels were immediately placed in cold storage and transported to the laboratory, where their stomach contents were removed with a 25-gauge needle fitted to a 1-ml syringe and preserved in 7 ml of 1% glutaraldehyde.

2.3. Fiberglass counts and sizes

Fiberglass particles were identified, counted and measured from the natural water, stomach contents, feces and pseudofeces with an inverted microscope (XSB-1A) using 100× magnification. All fiberglass particles present were counted for each analyzed sample. For the bay water, a 50 ml aliquot was allowed to settle for 24 h. Results were expressed as number l^{-1}. All samples of stomach contents, feces and pseudofeces samples were gently shaken; additionally, feces were also subjected to pressure with a syringe in order to disaggregate them. Thereafter, a 1 ml aliquot of each mussel component sample was allowed to settle for 24 h in 9 ml of filtered sea water. Results were expressed as number ml^{-1}.

2.4. Scanning electron microscopy (SEM)

The fiberglass particles were characterized with a Hitachi S-3500 N Scanning electron microscope (SEM) located at the Institut de Ciències del Mar (CSIC), with an acceleration voltage of 15 kV, and were observed with a BSE detector. X-ray microanalysis was performed using an energy dispersive spectrometer Bruker AXS Microanalysis Gmbh. The chemical composition was obtained for both standard commercial fiberglass and the fiberglass obtained from the stomach contents of the mussels. Neither type of fiberglass was coated with any material.

2.5. Statistical analyses

Data were checked for normality and variance homogeneity. Due to the differences in sample density between water, stomach contents, fecal and pseudofecal samples, statistical comparisons were performed using the percentage of particle number, which is, the relative density values for each type of sample. Data were previously normalized using square root arcsine transformation.

Statistical significant differences in fiberglass counts between samples, samplings and seasons, and between fiberglass sizes in the different types of samples were tested using Multiple Analysis of Variance (MANOVA). The statistical software used was Statgraphics Plus (Manugistics, Inc., Rockville, MD, USA).

3. Results

3.1. Fiberglass counts and sizes

Fiberglass was found in the bay water, and in the stomach, feces and pseudofeces of mussels (Fig. 1). There were no significant differences between the sizes of fiberglass from the different types of samples ($p > 0.05$). Average lengths and widths of fiberglass were 280.09 ± 301.69 μm and 7.62 ± 1.27 μm, respectively. Though the lengths counted were very variable (minimum of 28.12 μm and maximum of 1908.57 μm), the distribution pattern showed a high abundance of shorter fiberglass (<200 μm), with a mode value of 65.83 μm (Fig. 2).

The abundance of fiberglass in the bay water in relation to the natural phytoplankton was below 1% (Fig. 3). There were no significant differences in relative fiberglass amounts between types of samples for November, April and July ($p > 0.05$). For February, however, though there were no significant differences for water,

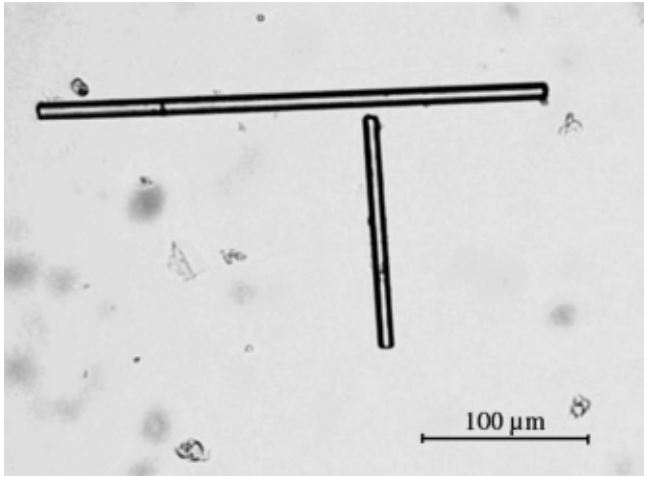

Fig. 1. Optical inverted microscope image of two fibers of fiberglass in the stomach contents of mussels. Scale bar shown in image.

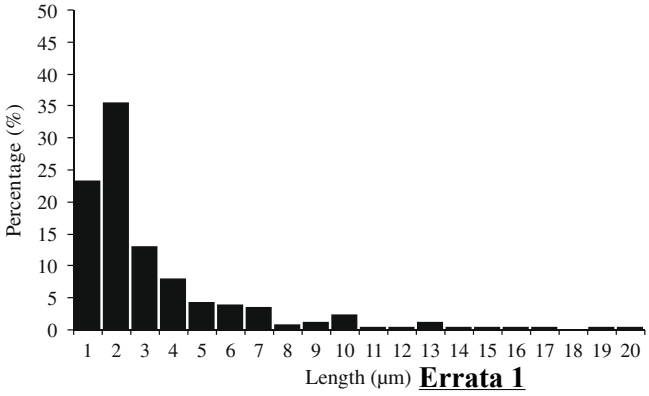

Fig. 2. Length-frequency distribution of the fiberglass found in the different types of samples ($N = 260$).

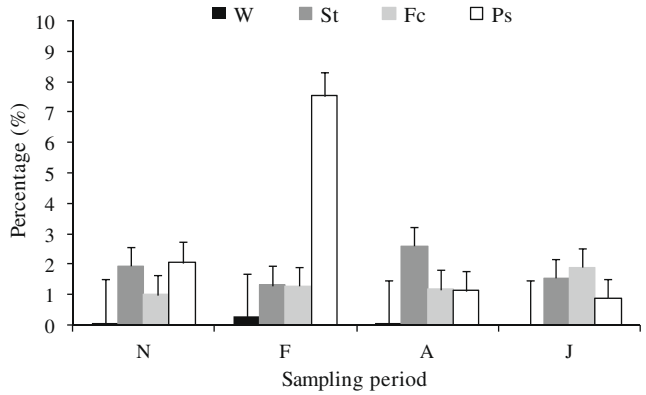

Fig. 3. Mean values for each sampling period (N: November 2006; F: February 2007; A: April 2007; J: July 2007) of the percentage of fiberglass among the phytoplanktonic community in the bay water (W) and stomach contents (St), feces (Fc) and pseudofeces (Ps) of mussels.

stomach contents and feces ($p > 0.05$), fiberglass abundances in the pseudofeces showed a significant increase in relation to the other samplings ($p < 0.05$).

3.2. Scanning electron microscopy (SEM)

X-ray microanalysis of the fiberglass found in our samples and commercial fiberglass revealed the same composition with a predominance of Si and O (Fig. 4). Only a small quantity of Mg appeared in the fiberglass found in water and stomach contents, feces and pseudofeces. Concoidal fractures of the fiber ends characterized the fiberglass found in the different types of samples (Fig. 5).

4. Discussion

The chemical composition of commercial fiberglass and fiberglass found in the mussel feeding experiments analyzed by X-ray microanalyses revealed the uniformity of the samples. Our results show the predominance of Si and O followed by Al and Ca, confirming findings reported elsewhere (Kudryavtsev et al., 2000). The concoidal fractures on fiber ends found in our fiberglass samples are a characteristic used by laboratories to identity fibers made from this material (Friedman, 2009). We can therefore confirm the presence of fiberglass in Alfacs Bay water and the stomach contents, feces and pseudofeces of the local mussels. The X-ray microanalyses give a qualitative result of the chemical composition of the materials studied. However, although Mg did not appear in the commercial fiberglass, it did appear in our samples. We hypothesize that the presence of Mg in the fiberglass from the bay water and mussel component samples may have originated from the bay water.

The lengths of the fiberglass in the different types of samples studied are within the threshold of mussel ingested particles. As Lehane and Davenport (2006) previously reported, amphipods in the stomach contents of mussels had sizes of up to 6000 μm. Nevertheless, the rejection of inorganic particles on the basis of their size has been previously reported and larger particles would be rejected as pseudofeces (Ward and Targett, 1989). The fiberglass found in all types of samples in our experiments varied greatly in length; the mussels from Alfacs Bay did not, therefore, reject the fiberglass on the basis of its length. Laboratory experiments also suggest that bivalves feeding on inorganic spheres reject material via their pseudofeces (Kiørboe et al. 1980; Ward and Targett 1989; Bayne et al. 1993). However, Defossez and Hawkins (1997) reported that there is no definitive proof that direct selection of organic vs. inorganic particles occurs in bivalves. Nevertheless, the same authors also reported that SiO_2 particles with diameters of more than 7.5 μm were preferentially rejected as pseudofeces. In accordance, we would have expected to find greater abundances of fiberglass in the pseudofeces of the mussels studied. Nevertheless, fiberglass was only clearly rejected in the February samples. Kiørboe and Møhlenberg (1981) suggest that the selection efficiency of bivalves should be interpreted in the context of the particle composition and concentration of the water in their natural habitats. This finding may explain why mussels only rejected fiberglass in February but not throughout the study period.

Unexpectedly, we found fiberglass in stomachs, fecal and pseudofecal samples for the whole period of study. We do not know why mussels ingested fiberglass but, the reason does not seem to be related to size, it could, however, be related to other factors. In this sense, the major chemical component found in the fiberglass was Si, which is excreted by mussels and is usually dependant on the amount of diatoms ingested (Asmus et al., 1990). Moreover, SiO_2 can be destroyed in alkaline environments (Fernández, 2003) but Griscom et al. (2002) reported the acidity of the stomach juice of *Mytilus edulis* at a pH of 5.6. Another major component of the fiberglass is aluminum, which is considered toxic to bivalves (Kadar et al., 2002). Si and Al are therefore unlikely to explain why mussels selected and ingested the fiberglass. The results of our analysis showed that calcium was the third main component of the fiberglass. The shells of bivalves are formed by the deposition of crystals of calcium carbonate in an organic matrix. Calcium for shell growth is obtained from either their diet or from sea water (Gosling, 2003). Thus, calcium from the fiberglass could be of use to mussels. Nevertheless, ascertaining why mussels ingested fiberglass is beyond the range of this study and cannot be explained by our results.

The main origins of fiberglass in the bay water are uncertain, as it is a common material used throughout building and shipping industries. The presence of fiberglass in N.W. Mediterranean coastal waters has also been identified by the Harmful Phytoplankton Monitoring Program (Delgado, unpublished results). In our study, the quantity of fiberglass found in the stomach contents and feces did not vary significantly over the year. Our results therefore suggest that all the ingested fiberglass was excreted in feces, as previously reported for humans (WSDH, 2006). The noxious effects of fiberglass ingestion in organisms are poorly understood, although particles of fiberglass longer than 15 μm cannot be engulfed by mammalian macrophages (Bernstein, 2007). Gastrointestinal and pancreatic cancer have been reported in humans after exposure to asbestos in drinking water (Maresca et al. 1984; Kærheim et al., 2005), and sclerosing encapsulating peritonitis has been

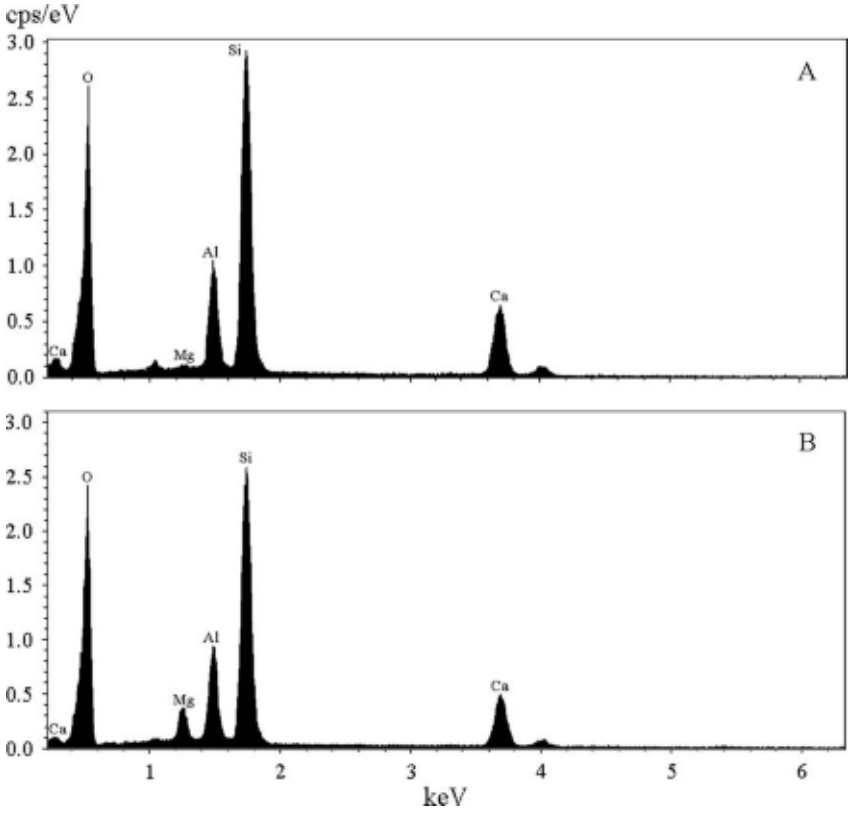

Fig. 4. X-ray microanalysis of commercial fiberglass (A) and fiberglass from the mussel samples (B).

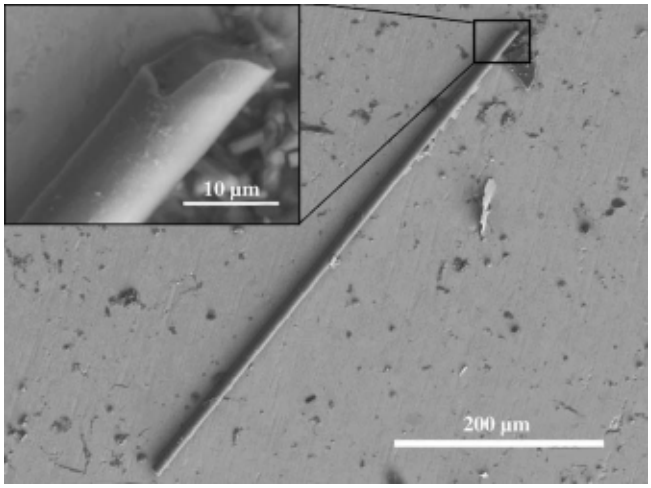

Fig. 5. Scanning electron microscope image of a fiberglass in feces and detail of the concoidal end. Scale bars shown in image.

reported in the case of a dog which had a history of fiberglass ingestion (Hardie et al., 1994). Nevertheless, these effects occurred after long periods of ingesting the inorganic materials and little is known about short term effects or occasional ingestion. The relative lack of these types of studies may be related to the fact that fiberglass ingestion by humans is very rare and could only occur accidentally when drinking contaminated water (Kærheim et al., 2005) or with food products, such as talc-coated rice (Maresca et al. 1984) or mussels, as the main potential vectors.

To our knowledge, this is the first report of the introduction of fiberglass in the marine food webs. Thus, prey–predator relationships should be studied along with their possible implications for human health and the health of the organisms themselves. Moreover, we propose the use of mussels as indicators of fiberglass contamination in marine ecosystems. As a precaution, in the case of commercial bivalves, we would advise the Water Quality Monitoring Programme to be aware of a new, potentially-harmful particle in shellfish-growing areas.

Acknowledgements

This study was supported by a grant from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) awarded to the first author and partially financed by research project RTA04-023 INIA. We would like to acknowledge José Manuel Fortuño for the SEM analyses, Dr. Pere Abelló, Dr. David M. Bernstein, Dr. Raimon Guitart, Dr. Salvador Martínez, and Dr. Joaquim M. Nogués for their interest and helpful advice, Dr. Richard K. Pipe and Dr. Gary H. Wikfors for their language revisions and Josep M. Reverté, Vanessa Castán and Esther Dámaso for their assistance during the field work.

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Errata 1

X axis of figure 2 is "Length x 100 (μm)"

Chapter IV

The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*

Harmful Algae 7 (2008) 91–98

Available online at www.sciencedirect.com
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The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*

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Received 27 March 2007; received in revised form 15 April 2007; accepted 22 May 2007

Abstract

The effects of exposure to the type species for *Karlodinium veneficum* (PLY # 103) on immune function and histopathology in the blue mussel *Mytilus edulis* were investigated. Mussels from Whitsand Bay, Cornwall (UK) were exposed to *K. veneficum* (PLY # 103) for 3 and 6 days. Assays for immune function included total and differential cells counts, phagocytosis and release of extra cellular reactive oxygen species. Histology was carried out on digestive gland and mantle tissues. The toxin cell quota for *K. veneficum* (PLY # 103) was measured by liquid chromatography–mass spectrometry detecting two separable toxins KvTx1 (11.6 ± 5.4 ng/ml) and KvTx2 (47.7 ± 4.2 ng/ml). There were significant effects of *K. veneficum* exposure with increasing phagocytosis and release of reactive oxygen species following 6 days exposure. There were no significant effects on total cell counts. However, differential cell counts did show significant effects after 3 days exposure to the toxic alga. All mussels produced faeces but not pseudofaeces indicating that algae were not rejected prior to ingestion. Digestive glands showed ingestion of the algae and hemocyte infiltration after 3 days of exposure, whereas mantle tissue did not show differences between treatments. As the effects of *K. veneficum* were not observed in the mantle tissue it can be hypothesized that the algal concentration was not high enough, or exposure long enough, to affect all the tissues. Despite being in culture for more than 50 years the original *K. veneficum* isolate obtained by Mary Parke still showed toxic effects on mussels.

Keywords: Blue mussel; Harmful algal blooms; Histopathology; Immunology; *Karlodinium veneficum*

1. Introduction

The dinoflagellate genus *Gymnodinium* forms a large group of unarmoured species which was recently subdivided into four genera, *Karenia* (Hansen and Moestrup), *Karlodinium* (Larsen), *Akashiwo* (Hansen and Moestrup), with the description of *Gymnodinium* emended (Daugbjerg et al., 2000). *Karlodinium* and *Karenia* contain several species with the potential for forming large toxic blooms which have been responsible for killing fish and molluscs, resulting in huge economic losses (Anderson et al., 2000).

Gymnodinium veneficum was first isolated from a region near Plymouth Sound, UK in 1950, by Parke and subsequently described and named by Ballantine

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doi:10.1016/j.hal.2007.05.004

(1956). The strain, PLY # 103, was described to produce a powerful toxin which was lethal to nearly all marine organisms tested (Abbott and Ballantine, 1957; Place et al., 2005). The bivalve *Lasaea rubra* was shown not to filter a suspension of *G. veneficum* (Ballantine and Morton, 1956). A recent re-examination of the PLY # 103 strain, using light and electron microscopy and partial LSU rDNA, found it to be identical to *K. micrum* and thus necessitating a change of name for both *G. veneficum* and *K. micrum* to *K. veneficum* (Bergholtz et al., 2005). Laboratory experiments have shown this organism causes mortality in juvenile cod and reduced growth in mussels (Nielsen and Strømgren, 1991; Nielsen, 1993). Despite being in culture for more than 50 years PLY # 103 was shown to still produce toxins which were similar to karlotoxins described for North American isolates of *K. veneficum* (Kempton et al., 2002; Place et al., 2005; Deeds et al., 2006).

It has been shown that filter feeding bivalves, including blue mussels *Mytilus edulis*, are affected when exposed to toxic microalgae presenting: growth rate reduction, production of mucus, lesions in different tissues, clearance-rate reduction, immune responses and high mortality rates (Shumway and Cucci, 1987; Nielsen and Strømgren, 1991; Smolowitz and Shumway, 1997; Keppler et al., 2005; Wikfors, 2005). The effects can be of great importance, not only for natural communities but also for aquaculture facilities (Shumway, 1990; Anderson et al., 2000).

The present study investigated whether exposure to *K. veneficum* (PLY # 103) would show adverse effects, especially on immune function and histopathology in *M. edulis*.

2. Materials and methods

Mussels, *M. edulis* (40–55 mm shell length) were collected at low tide from Whitsand Bay, southeast Cornwall, UK, an exposed open ocean site. They were immediately transported to the laboratory where epiphytes and other encrusting organisms were removed from the shells. A hundred mussels were placed in each of two experimental tanks, containing 8 l of filtered sea water at the same temperature as the sampling site, and left for 24 h to acclimate. The mussels were then exposed to either *K. veneficum* or *Dunaliella primolecta* (PLY # 81). Cell diameters averages were 11.4 ± 0.02 μm for PLY # 103 and 5.9 ± 0.05 μm for PLY # 81. Mussels were removed on day 0, before exposure to the algae, days 3 and 6 of exposure, 30 each time, and used for hemocyte counts, immune assays and histology.

2.1. Algal cultures

The algal cultures (PLY # 103 and PLY # 81) used for the exposure experiments were maintained in Erd-Schreiber medium (Bruce et al., 1940) at 15 °C. Both culture cell concentrations were measured with an improved Neubauer hemocytometer and adjusted to give a final concentration of 6.25×10^4 cell/l daily in each tank. Seawater in experimental tanks was changed daily and the tanks rinsed thoroughly to avoid build up of mussel biodeposits. The algae were added daily to each tank after cleaning. After removing the mussels for sampling on day 3, the algal concentration was adjusted to the remaining number of bivalves so they were exposed to the same concentration throughout the experiment.

2.2. Toxin measurement

Karlotoxin (KvTx) concentrations were measured by liquid chromatography–mass spectrometry (LC–MS) (Bachvaroff et al., 2007) using the fact that karlotoxin binds to teflon (PTFE) filters quantitatively. Aliquots (2 and 10 ml) of the cultures were filtered on 13 mm PTFE syringe filters (Whatman, 0.2 mm pore size). The filtrate was discarded and the filters eluted with 1 ml HPLC grade methanol into glass test tubes containing 2 ml dH_2O. Toxin samples were injected onto a C8 (LiChrosphere 125 mm × 4 mm × 5 mm bead size RP-8, Waters Corp.) column and subjected to a 1 ml/min 10–95% methanol: water gradient over 25 min using an HP/Agilent 1100 HPLC. Toxin peaks were detected at 225 or 235 nm as appropriate for KvTx1 or KvTx2 toxins, respectively. A portion of the mobile phase (1/3 to 1/6) was then passed to the electro-spray nozzle of the MS (Agilent G1956A SL or VL) for ionization. A 1% formic acid in methanol solution (0.1 ml/min) was added to provide appropriate pH conditions for positive mode ionization. Peaks previously determined to have hemolytic activity were quantified as a mass (pg) based on calibration curves determined with pure karlotoxin standards.

2.3. Hemocyte counts

Hemolymph samples were withdrawn from the posterior adductor muscle into an equal volume of Baker's formol calcium, containing 2% NaCl. Total cell counts were measured with a Neubauer hemocytometer. Differential cells counts were prepared using a Shandon cytocentrifuge with 200 μl of hemolymph. Cells were post fixed with methanol and stained using Wright's

stain (Parry and Pipe, 2004). After air drying, the samples were mounted with Canada balsam. Eosinophilic and basophilic cells were identified (Friebel and Renwrantz, 1996) and relative numbers were calculated by counting 200 blood cells per sample. The counts were carried out for five mussels from each treatment.

2.4. Immune assays

2.4.1. Detection of extracellular superoxide anion

Hemolymph from five mussels per treatment was extracted from the posterior adductor muscle into an equal volume of Tris-buffered saline (TBS), pH 7.6 containing 2% of sodium chloride. Six 100 μl aliquots of each hemolymph sample were pipetted into microplate wells. An equal volume of cytochrome-*C* solution (80 μM ferricytochrome-*C* in TBS containing 2% sodium chloride) was added to three wells. Cytochrome-*C* solution containing 300 units/ml of superoxide dismutase (SOD) was pipetted into the other three wells (100 μl). The cytochrome-*C* solutions (with and without SOD) were also aliquoted into wells without blood cells and, in addition, cells in only buffer were used as controls.

The optical density (OD) was read immediately and every 30 s for 20 min using a 550 nm filter and a kinetics package. Results are expressed in optical density per milligram hemocyte protein which was analyzed using a bicinchoninic acid (BCA) protein assay (Pierce Chem. Co).

2.4.2. Phagocytosis

Hemolymph from five mussels per treatment was withdrawn from the posterior adductor muscle into an equal volume of TBS, pH 7.6 containing 2% sodium chloride. Aliquots of 50 μl of each hemolymph sample were pipetted into four replicate wells of one plate and one well of another for the protein assay. After 10 min, an equal volume of neutral red-stained zymosan suspension was added (Pipe et al., 1995). Hemocytes fixed with Baker's formol calcium containing 2% sodium chloride were aliquoted and zymosan added to be used as blanks. Zymosan in buffer only was used as negative control (same volumes as samples). After incubating the plates for 30 min at 15 °C, 100 μl of Baker's formol calcium was added to each well to stop the reaction. The plate was spun at 70 g for 5 min, the supernatant discarded and the cells re-suspended in 100 μl of TBS buffer. This washing procedure was repeated until there was no evidence of zymosan remaining in the wells of the negative controls (~6 washes). Just before the last spin, 50 μl of standard zymosan suspensions of known particle concentrations were pipetted into duplicate wells, using serial dilutions of the zymosan from a stock suspension, 5.0×10^6 particles per well and 50 μl TBS added to each well to provide a standard curve. Finally, the neutral red was solubilised by adding 100 μl of 1% acetic acid in 50% ethanol to each well and incubating for 30 min. After shaking the plate, the OD was read with a 550 nm filter and results expressed as particles of zymosan phagocytosed per milligram hemocyte protein. Hemocyte protein was analyzed using a BCA protein assay (Pierce Chem. Co).

2.5. Histology

Digestive gland and mantle tissues were dissected from 10 individuals on day 0, before exposure to the algae, and on days 3 and 6 after the exposure and fixed in Baker's formol calcium for 48 h. The tissues were then rinsed in tap water and transferred to 70% alcohol. Samples were dehydrated and embedded in paraffin. After processing, sections were cut (5 μm thickness), stained using a hematoxylin–eosin staining procedure and examined under a light microscope. The thickness of the digestive gland tubular epithelium was measured for five mussels from each treatment. The measurements involved four readings from each of 10 tubules chosen for each individual using the Image-Pro Plus image analysis software package (Media Cybernetics, L.P.).

2.6. Statistical analysis

Results of hemocyte counts, immune assays and digestive gland tubule thickness were analyzed using Statistica 98 Edition (Stat Soft Inc., 1998). Pair-wise comparisons between tanks at each experimental time were done using *t*-test considering that each mussel was a unit of replication. Results were significant with a probability (*P*) value of <0.05.

3. Results

3.1. Algal toxicity

Two major toxic peaks (KvTx1 and KvTx2) were obtained upon methanol gradient elution for PLY # 103. Both were hemolytic to rainbow trout erythrocytes and found in nearly equivalent cell quotas (0.93 pg/cell versus 1.25 pg/cell) (unpublished data). The UV spectra of the two toxins differed with KvTx1 having a peak at 225 nm while KvTx2 had a UV absorption maximum at

Table 1
Percentage of eosinophilic (eos) and basophilic (bas) cells throughout the experiment for both toxic (T) and non-toxic (NT) treatments

	D0	D3	D6
Eos T		29.85 ± 6.69	51.57 ± 2.41
Bas T		70.15 ± 6.69	48.43 ± 2.41
Eos NT	60.13 ± 2.54	58.09 ± 3.22	61.73 ± 5.50
Bas NT	39.87 ± 2.54	41.91 ± 3.22	38.27 ± 5.50

Numbers are expressed in mean ± S.E.

235 nm. LC/MS analysis of the two peaks found masses of 1208.8d and 1267.8d for KvTx1 versus 1242.7d and 1301.8d for KvTx2. The concentration of the two toxins measured for the *K. veneficum* culture (4.0×10^6 cell/l) was KvTX1 11.6 ± 5.4 ng/ml and KvTX2 47.7 ± 4.2 ng/ml. After dilution in the tank for feeding to the mussels, the concentrations were KvTX1 $< 0.4 \pm 0.0$ ng/ml and KvTxX2 $< 2.7 \pm 0.0$ ng/ml.

3.2. Hemocyte counts

There were no significant differences between treatments for the total blood cell counts ($P > 0.05$); however, there were significant differences for the differential blood cell percentages between treatments ($P < 0.05$) on day 3 but not on day 6 (Table 1). On day 0, prior to algal exposure, the ratio of eosinophilic to basophilic cells was 60:40. This ratio did not vary with time for the mussels exposed to the non-toxic alga. On day 3, the mussels exposed to toxic algae did show significant differences with eosinophilic cells decreasing from 60 to 30% and basophilic cells increasing from 40 to 70%. These values changed again on day 6 of the experiment and both cell types reached a final concentration of 50% of the total hemolymph content.

3.3. Immune assays

The cytochrome-*C* reduction assay to detect extracellular superoxide anion showed significant differences between treatments ($P < 0.05$) at both times. Blood cells from mussels fed with the toxic algae showed an increase in the release of reactive oxygen species on both days 3 and 6 in comparison with the mussels fed with the non-toxic alga (Fig. 1).

Phagocytosis of zymosan showed significant differences between treatments on day 6 ($P < 0.05$) but not on day 3 (Fig. 2). Hemocytes from mussels exposed to the toxic algae showed significantly more phagocystosis compared with both pre-exposed mussels and those exposed to the non-toxic algae.

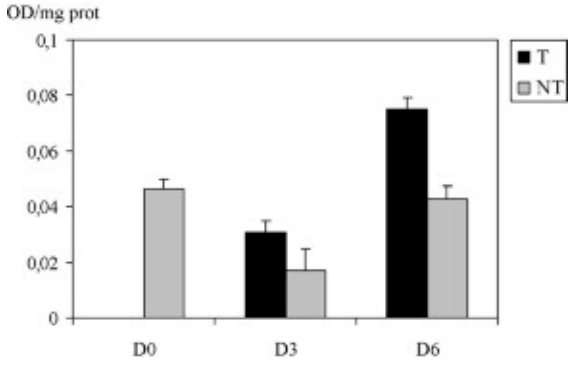

Fig. 1. Detection of extra cellular superoxide anion in mussel hemocytes sampled on days 0, 3 and 6 of the experiment (T: toxic; NT: non-toxic). Error bars are 1 S.E.M. Significant differences were found between time ($P < 0.05$) and treatment ($P < 0.05$).

3.4. Histology

The mantle tissues contained ripe gametes in all cases and no differences were detected between treatments, gender or time. Hemocyte infiltration and oocyte atresia were not observed in the mantle tissues of any of the samples examined. The digestive gland, however, did show histological differences with time and treatments. Hemocyte infiltration appeared on days 3 and 6 in mussels exposed to the *K. veneficum* whereas the mussels exposed to the non-toxic algae, remained the same throughout the experiment (Photo 1). The lumina of the digestive glands tubules were full in both treatments throughout the exposures showing that the algae were not rejected prior to ingestion. Intact *K. veneficum* cells were present in the digestive gland tubules on days 3 and 6 (Photo 2) but intact cells of *D. primolecta* were not apparent in the tubules. Measurements of the digestive tubule thickness did not show

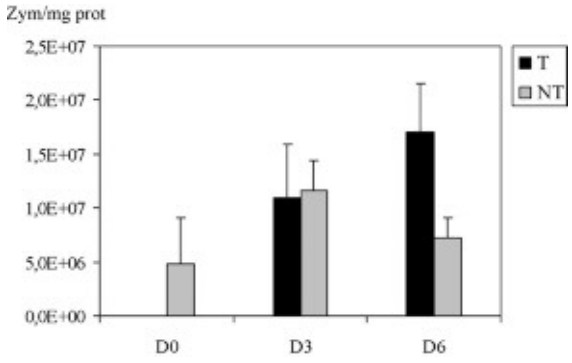

Fig. 2. Phagocytosis of zymosan by mussel blood cells sampled on days 0, 3 and 6 of the experiment (T: toxic; NT: non-toxic). Error bars are 1 S.E.M. Significant differences between treatments ($P < 0.05$) were found on day 6.

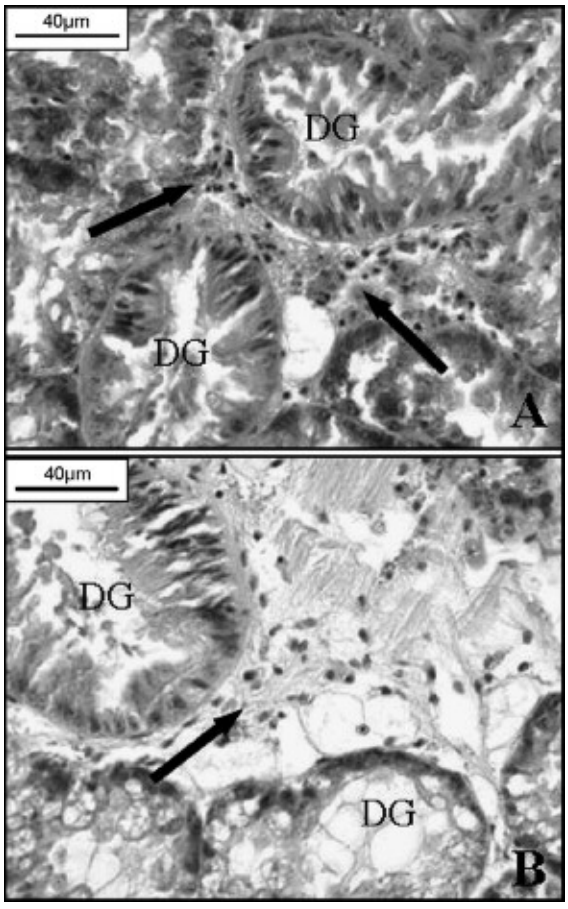

Photo 1. Optical micrographs of digestive gland tissues sampled on days 3 (A) and 6 (B), from mussels exposed to the toxic algae. Arrows show hemocyte infiltration. DG: digestive gland.

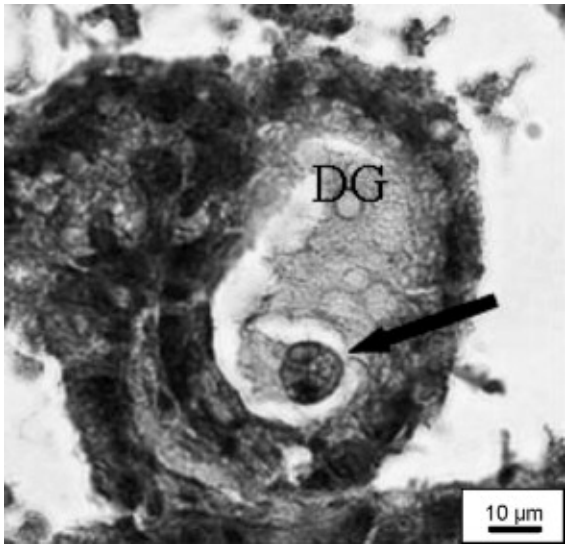

Photo 2. Optical micrograph of a digestive gland containing a cell of *Karlodinium veneficum* shown by an arrow. DG: digestive gland.

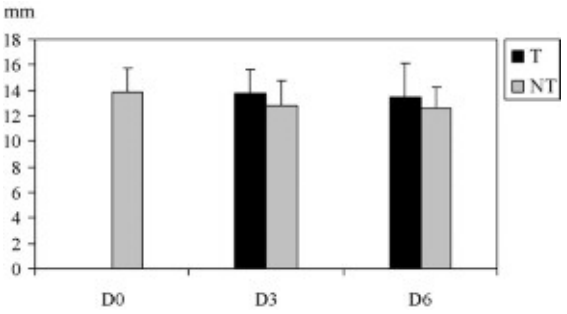

Fig. 3. Digestive gland tubule thickness (mm). Error bars are 1 S.E.M. No significant differences were found (T: toxic; NT: non-toxic).

differences with treatment at both times ($P < 0.05$) (Fig. 3).

4. Discussion

This study demonstrates that exposure to pre-bloom concentrations of PLY # 103, for up to 6 days, results in measurable effects on the blue mussel, *M. edulis*. During bloom conditions of *K. veneficum* cell densities can reach 10–100 times those used in this experiment (Fensin, 2004; Goshorn et al., 2004).

Differential blood cells counts showed differences with treatments and time, with circulating eosinophilic cells decreasing in the *K. veneficum* exposed mussels. Eosinophilic hemocytes include only granular cells whereas the basophilic hemocytes include both agranular and granular cells with small granules (Pipe et al., 1997). The basophilic blood cells are less phagocytic than the eosinophilic cells (Carballal et al., 1997; Pipe et al., 1997; Hine, 1999). It appears that the eosinophilic cells were stimulated by the presence of the toxic algae resulting in a rapid decline from circulation, possibly due to movement into the tissues to phagocytose damaged cells. In support of this hypothesis, larger eosinophilic cells were observed on days 3 and 6 in the mussels exposed to the toxic algae, the cells appeared to be granular hemocytes containing phagocytosed material. By day 6 of the study the baseline levels of eosinophilic and basophilic hemocytes had almost recovered. This result is similar to the findings of Hégaret and Wikfors (2005a) who exposed *Crassostrea virginica* and *Argopecten irradians* to the toxic alga *Prorocentrum minimum*. They observed that the percentage of granulocytes decreased at the very beginning of their exposure but increased after a few days.

The results from the phagocytosis assays support the differential blood cells counts, as there was a significant increase in phagocytosis on day 6 in mussels exposed to

the toxic algae compared with pre-exposure and controls. On day 3 of the study there was also an increase in phagocytosis in mussels exposed to the toxic algae, despite the reduced numbers of eosinophilic hemocytes; however, it should be noted that there was also an increase in phagocytosis in the controls which may reflect a tank effect. Hégaret and Wikfors (2005b) also found an increase in phagocytosis when exposing *C. virginica* to toxic *P. minimum*.

The release of reactive oxygen species by blood cells from mussels fed with the toxic algae was higher on both days 3 and 6 compared with mussels fed on the non-toxic algae. In general, exposure to low concentrations of chemical contaminants has resulted in an enhanced response for release of reactive oxygen species in bivalve hemocytes (Pipe and Coles, 1995; Dyrynda et al., 1998, 2000). With increasing levels of pollutant exposure there has tended to be an inhibition of reactive oxygen release (Pipe and Coles, 1995; Pipe et al., 1999). The decrease in values for release of reactive oxygen species for both toxic and non-toxic treatments on day 3 compared with pre-exposure levels could again reflect a tank effect, although it should be noted that the toxin exposed mussels did show a significant increase compared with the controls.

Wootton et al. (2003) recently compared immune function in *M. edulis* with two other bivalve molluscs (the edible cockle, *Cerastoderma edule*, and the razor-shell, *Ensis siliqua*). *M. edulis* hemocytes were much more active in phagocytosis and superoxide generation than hemocytes from the other two species. Although the authors were not exposing the bivalves to toxic algae, it can be suggested that the enhanced levels of phagocytosis and release of reactive oxygen species in the present study indicate a stimulation of immune response in the mussels following exposure to the toxic algae.

Digestive gland tissues showed hemocyte infiltration following toxic algal exposure in mussels sampled on both days 3 and 6. Similar results were found when exposing bivalves to toxic *P. minimum* (Wikfors and Smolowitz, 1995). The karlotoxins produced by *K. veneficum* act by depolarization of cell membranes and are able to lyse rainbow trout erythrocytes and cause extensive gill damage in exposed fish (Abbott and Ballantine, 1957; Deeds et al., 2006). Hemocyte infiltration was generally associated with the connective tissues surrounding the tubules and could be related to phagocytosis of tissues damaged by the toxin. Interestingly, despite the cytotoxic effects of *K. veneficum*, histology revealed that the digestive gland tubules were full throughout the exposure, indicating that feeding was not inhibited by the toxic algae. Feeding on toxic algae by other bivalves resulted in a decrease of digestive gland wall thickness, dilation of the digestive gland tubules and no evidence of algae within the tubule lumen (Bricelj et al., 2004; Pearce et al., 2005). In the present study, there were no differences observed in the digestive tubules between treatments or over time, indicating that all mussels were able to continue feeding at this algal density with no apparent direct effect on the digestive cells. In addition, faeces were found each day throughout the experiment without any evidence of pseudofaeces, suggesting that the mussels were able to filter and digest both the toxic and non-toxic algae. Further experiments at higher algal densities will be necessary to determine whether similar findings are found under bloom conditions.

The mantle tissue showed ripe gametes in all cases and no differences were detected between treatments or time. The fact that the reproductive tissue did not appear to be affected by the *K. veneficum* may be due the relatively short exposure time together with the low concentration of the toxic algae ingested. Karlotoxins exhibit very steep dose response curves to hemolysis or fish death (Deeds et al., 2006). Notwithstanding, the results are in accord with those found by Franchini et al. (2003) who localized yessotoxins in hemocytes and digestive glands of *M. galloprovincialis* but did not detect toxin in the gonads.

5. Conclusion

Mussels ingested and digested the toxic algae as the digestive glands tubules were full throughout the experiment, faeces were found daily and the toxin levels in the experimental tank decreased with addition of the mussels. These results indicate that *M. edulis* did not reject or avoid the toxic algae by closing down and stopping filtration. The karlotoxins seemed to affect the mussel tissues and cells that were in first contact with the algae including the hemocytes and digestive gland cells. Observations on gill tissues are highly recommended for further studies. There was no observed effect on the mantle tissues, possibly due to the low algal concentration and the short exposure time. The differential blood cells counts varied with time due to stimulation of the immune system by the toxins and movement of the granular eosinophilic cells from circulation into the tissues to phagocytose damaged cells. This result is supported by the increased capacity of the hemocytes to phagocytose zymosan in the toxin exposed mussels. The blood cells from mussels exposed to the toxic algae also showed an increase in the release

of reactive oxygen species. A possible tank effect was observed on day 3, suggesting that the acclimation period should be increased for future experiments.

The possibility cannot be dismissed that a difference in the two tanks used, not related to the experimental *K. veneficum* exposure, could be confounding results attributed to *Karlodinium*. Nevertheless, immunological measurements and histological data are consistent with the sequence of hemocyte proliferation and then infiltration into tissues suggesting that the observed effects did result from exposure to the toxic algae. Although faeces where not examined for the presence of intact *K. veneficum* cells, it is clear that *M. edulis* were affected by the toxic algae due to different responses obtained between treatments. Longer exposure experiments would be of interest to see whether the effects increase with time or whether the mussels may be able to adapt over a longer time period. Similarly, exposure to bloom densities of *K. veneficum* may have more immediate and pervasive affects.

Acknowledgements

This study has been supported by a grant from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) to the first author and partially financed by the RTA04-023 INIA research project. This work was funded in part by grants from National Oceanic and Atmospheric Administration Coastal Ocean Program under ECOHAB award # NA04NOS4780276 to University of Maryland Biotechnology Institute, and Grant # U50/CCU 323376, Centers for Disease Control and Prevention and the Maryland Department of Health and Mental Hygiene. This is contribution # 07-170 from the UMBI Center of Marine Biotechnology, and # 219 from the Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) program.[SS]

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Chapter V

Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*

Harmful Algae 7 (2008) 630–638

Available online at www.sciencedirect.com
ScienceDirect
Harmful Algae 7 (2008) 630–638
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Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*

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Received 26 November 2007; received in revised form 28 December 2007; accepted 9 January 2008

Abstract

The harmful dinoflagellate *Prorocentrum minimum* has different effects upon various species of grazing bivalves, and these effects also vary with life-history stage. Possible effects of this dinoflagellate upon mussels have not been reported; therefore, experiments exposing adult blue mussels, *Mytilus edulis*, to *P. minimum* were conducted. Mussels were exposed to cultures of toxic *P. minimum* or benign *Rhodomonas* sp. in glass aquaria. After a short period of acclimation, samples were collected on day 0 (before the exposure) and after 3, 6, and 9 days of continuous-exposure experiment. Hemolymph was extracted for flow-cytometric analyses of hemocyte, immune-response functions, and soft tissues were excised for histopathology. Mussels responded to *P. minimum* exposure with diapedesis of hemocytes into the intestine, presumably to isolate *P. minimum* cells within the gut, thereby minimizing damage to other tissues. This immune response appeared to have been sustained throughout the 9-day exposure period, as circulating hemocytes retained hematological and functional properties. Bacteria proliferated in the intestines of the *P. minimum*-exposed mussels. Hemocytes within the intestine appeared to be either overwhelmed by the large number of bacteria or fully occupied in the encapsulating response to *P. minimum* cells; when hemocytes reached the intestine lumina, they underwent apoptosis and bacterial degradation. This experiment demonstrated that *M. edulis* is affected by ingestion of toxic *P. minimum*; however, the specific responses observed in the blue mussel differed from those reported for other bivalve species. This finding highlights the need to study effects of HABs on different bivalve species, rather than inferring that results from one species reflect the exposure responses of all bivalves.

Keywords: Harmful algal blooms; Hemocyte; Histopathology; Immunology; *Mytilus edulis*; *Prorocentrum minimum*

1. Introduction

Mussels (*Mytilus* sp.) are suspension-feeding bivalves that are harvested for human consumption around the world (Figueiras et al., 2002; Mortensen et al., 2006). As mussels, whether from wild fisheries or aquaculture, are grown and harvested under natural conditions, chemical and biological quality of growing waters is very important for survival and growth, hence quantity and quality of the yield. Mussels can be affected by different types of toxins, from both chemical (Dyrynda et al., 2000; Parry and Pipe, 2004) and biological sources (Galimany et al., 2008).

Biological effects of toxic microalgae upon bivalve mollusks can include mortality (Wikfors and Smolowitz, 1993), tissue damage (Pearce et al., 2005), cellular dysfunction (Hégaret and Wikfors, 2005), and reproductive failure (Granmo et al., 1988). In addition, edible tissues can become contaminated with chemical or biological toxins, rendering them unfit for human consumption (EUROHAB, 1998; Heil et al., 2005). Harmful algal blooms (HABs) appear to be increasing in geographic distribution and intensity (Hallegraeff, 2003). This has led to a growing concern about effects of HABs upon shellfish resources, in terms of both seafood safety and production efficiency.

Inimical effects of HABs upon grazing animals have raised questions about the evolutionary and ecological relevance of toxic or noxious properties in these microorganisms. Do toxins

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doi:10.1016/j.hal.2008.01.001

or harmful properties of HABs confer protection from grazing? It is very common for forage species of land plants to protect themselves from grazing by producing noxious properties or toxicity (Agrawal et al., 1999; Agrawal, 2007). In terrestrial ecosystems, it is not uncommon for grazing species to have developed tolerance to toxins from specific forage species (De Mazancourt et al., 2001; Karban and Agrawal, 2002). Recently, growing evidence for similar, grazing-deterrent functions of toxins in marine dinoflagellates have been reported (Selander et al., 2006). As in terrestrial ecosystems, susceptibility of grazers, including bivalve mollusks, to specific grazing-deterrent chemicals produced by HAB taxa appear to be species-specific (Landsberg, 2002).

The microalgal species *Prorocentrum minimum* (Pavillard) Schiller is a dinoflagellate with the capacity to express toxicity (Grzebyk et al., 1997; Wikfors, 2005) that is now considered a "HAB" species (Heil et al., 2005). Toxicity of *P. minimum* varies among different strains studied (Grzebyk et al., 1997; Denardou-Queneherve et al., 1999) and has been found to fluctuate according to growth phase, i.e., stationary-phase populations are more toxic than actively growing populations (Grzebyk et al., 1997). Despite this variability, *P. minimum* has been demonstrated to be toxic to molluscan shellfish, causing a wide diversity of symptoms, including pseudofeces production in several species of oyster and clam (Hégaret et al., 2007), tissue damage and developmental abnormalities in young stages of the Eastern oyster, *Crassostrea virginica* (Wikfors and Smolowitz, 1995a), changes in immune parameters of Eastern oysters and bay scallops, *Argopecten irradians irradians* (Hégaret and Wikfors, 2005) and mortality (Shumway and Cucci, 1987; Shumway, 1990; Luckenbach et al., 1993). Most studies of *P. minimum* effects upon mollusks reported to date have been with oysters and clams, but not with mussels, although it has been demonstrated that mussels can accumulate toxicity (Denardou-Queneherve et al., 1999). Mussels are known to have unusual tolerances to microalgal biotoxins (Wootton et al., 2003), such as saxitoxin, that cause clear biological effects in other molluscan species (Landsberg, 2002; Heil et al., 2005). Thus, possible effects of *P. minimum* upon mussels cannot be deduced from findings with other molluscan species.

The present study, therefore, investigated the effects of *P. minimum* upon adult blue mussels, *Mytilus edulis*, under experimental conditions, focusing on immune functions and histopathology, both of which have been shown to respond to *P. minimum* exposure in other molluscan species.

2. Materials and methods

2.1. Experimental animals

Mussels, *M. edulis* (47.6–73.9 mm shell length) for this experiment were collected from Westcott Cove, Stamford, CT, USA, from an intertidal beach on the north shore of Long Island Sound in June of 2007. Mussels were acclimated for 4 days before the experiment, the first 3 days in the experimental tanks with filtered seawater, and the fourth day fed with *Rhodomonas* sp. (RHODO, see below) at a concentration of 1×10^4 cells ml^{-1}.

2.2. Algal cultures

The *P. minimum* strain used for the experiment was obtained from the Milford Microalgal Culture Collection, strain JA-98-01 (isolated from the Choptank, River, Chesapeake Bay, MD, USA). In addition, the RHODO strain of the cryptophyte *Rhodomonas* sp. was used as a non-toxic, control alga.

The microalgae were cultured in 20-l glass carboy assemblies using aseptic technique (Ukeles, 1973). Cultures were harvested semi-continuously to maintain consistency in culture quality over the course of the study and were harvested in late-log or early-stationary phase. Cultures of *P. minimum* were grown in EDL7 medium, a modified version of the enriched-seawater E-medium (Ukeles, 1973) that contains L-1 trace metals (Guillard and Hargraves, 1993), double the EDTA of the standard E formulation, KNO_3 rather than $NaNO_3$, and 10 ml l^{-1} soil extract. *Rhodomonas* sp. was cultured in E-medium. Both cultures were maintained at 20 °C with 24-h light. Algal cell densities were determined by hemocytometer counts with a light microscope.

The toxicity of the *P. minimum* culture used in the mussel-exposure experiment was tested with a scallop bioassay (Rosetta and McManus, 2003). Five northern bay scallops, *A. irradians irradians*, were placed in each of twelve 1-l beakers; the following 4 treatments were tested in triplicate beakers: (1) filtered seawater, (2) filtered seawater diluted with distilled water to equal salinity of algal treatments, (3) *Rhodomonas* sp. at 1.9×10^5 cells ml^{-1}, and (4) *P. minimum* at the same cell density. Observations of scallop activity and mortality were made periodically for 20 h. Dissolved oxygen, pH and salinity were measured at the beginning and at the end of the exposure.

2.3. Experimental design

The main experiment tested the effects of cultured *P. minimum*, upon the immunology and histopathology of mussels, *M. edulis*. Two-hundred and seventy (270) mussels were distributed randomly into six 20-l glass-aquaria, i.e., 45 mussels per aquarium. Three replicates of each treatment were done in this experiment: *Rhodomonas* sp. or *P. minimum*, each at 1×10^4 cells ml^{-1}, were given with a regime of 16 feedings per day, every 90 min (285 ml per day), using the cover of a rearing-chamber system incorporating computer-automated valves to add microalgal culture as programmed (Smith and Wikfors, 1998).

Samples of mussels were collected on day 0, before exposures, and after 3, 6 and 9 days of exposure to the experimental, microalgal treatments. At each sampling time, mussels were removed from the basins and analyzed for hemocyte parameters, stomach contents, and histopathology. Feces and pseudofeces were also examined microscopically throughout the experiment.

2.3.1. Stomach contents, feces and pseudofeces

Stomach contents of mussels were removed with a 25-gauge needle and a 1-ml syringe. Samples were preserved in 300 μl of 1% glutaraldehyde and observed with a light microscope. Feces and pseudofeces were collected from the tanks with a pipette and observed directly with a light microscope.

2.3.2. Immunological analysis—Hemocytes

Hemolymph was withdrawn with a 21-gauge needle and a 1-ml syringe from the adductor muscle of each individual mussel and stored temporarily in an Eppendorf microcentrifuge tube on ice. Analyses of hemocyte morphology and function were done on hemolymph extracted from each individual mussel.

Procedures for characterization of the hemocytes, cell density of circulating hemocytes (cells ml^{-1}), size, and internal complexity of the hemocytes, and immunological functions (listed below) were adapted from Delaporte et al. (2003), Hégaret et al. (2003a,b), Lambert et al. (2003), Soudant et al. (2004) and Buggé et al. (2007). Hemocyte apoptosis was also assessed according to a protocol adapted from Goedken et al. (2005a). For the hemocyte analyses, a FACScan flow-cytometer (BD Biosciences, San Jose, CA) was used.

The five functional hemocyte parameters measured were:

(a) hemocyte mortality, as percentage of dead hemocytes,
(b) phagocytosis of fluorescent microbeads by hemocytes, which simulates the engulfment of non-self particles,
(c) respiratory-burst response in hemocytes, that measures reactive oxygen species' potential to kill non-self particles previously engulfed,
(d) percentage of adhering hemocytes,
(e) percentage of apoptotic hemocytes.

2.3.3. Histopathology

A 4-mm cross-section of each mussel, including digestive diverticulum, gills, mantle, kidneys, plicate membranes, and the byssus gland, was dissected and fixed in Davidson's fixative for 48 h at 4 °C. The tissues were then rinsed in 50% ethanol in filtered seawater, and transferred to 70% ethanol. Samples were dehydrated and embedded in paraffin. After processing, 5-μm sections were stained using a hematoxylin-eosin staining procedure (Howard et al., 2004) and examined under a light microscope. Blocks containing four mussels with bacteria subsequently were resectioned and stained with Gram-stain according to Howard et al. (2004).

2.4. Statistical analysis

Results for the hemocyte assays and total number of pathological changes were analyzed statistically using correlation analysis and multifactor analysis of variance (MANOVA) to assess effects of experimental treatments upon the response variables. Results for single pathological changes (bacteria in the intestine, migration of hemocytes to the stomach and intestine, and hemocytes around gonadal follicles) and stomach contents were analyzed using Chi-square tests for each sampling time. The statistical software used was Statgraphics Plus (Manugistics, Inc., Rockville, MD, USA).

3. Results

3.1. Prorocentrum minimum toxicity

At the beginning of the bay-scallop exposure, the media had pH values in the range of 7.5–8.0, oxygen saturation ranged between 92.3 and 94.4%, and salinity was 25‰ in the filtered seawater beakers but 13‰ in all other containers. After 4 h of exposure, all scallops were open and alive. 20 h after beginning the exposures, all scallops exposed to *P. minimum* were dead, and dissolved oxygen had decreased to a mean value of 35% in these beakers. One third of the scallops exposed to *Rhodomonas* sp. also died, but pH, oxygen saturation, and salinity of the alga media tested remained constant. We note that lower salinity in beakers containing algal cultures probably stressed scallops, but we chose to maintain the salinity used to culture the algae, rather than add salt or brine to increase salinity to that of the source water for the scallops. We interpret the finding of 100% mortality in scallops exposed to *P. minimum*, compared to 33% mortality in *Rhodomonas* sp.-exposed scallops, as an indication that the *P. minimum* culture was bioactive against a bivalve model at the time it was used—an important consideration in light of previous findings of fluctuating toxicity in *P. minimum* cultures.

3.2. Stomach contents, feces and pseudofeces

Cells of the alga provided, either *P. minimum* or *Rhodomonas* sp., were present in the stomach, feces and pseudofeces of the exposed mussels. After 3 days of exposure to *P. minimum* two of the mussels exposed to the toxic algae had large quantities of bacteria included in the stomach contents ($p > 0.05$). This observation differed significantly between treatments at the end of the experiment ($p < 0.05$). High numbers of hemocytes were observed in the feces of *P. minimum*-exposed mussels, usually forming aggregations around *P. minimum* cells.

3.3. Immunological analyses

Results from both hemocyte characterization and immunological functions are shown in Table 1. Main effects and interactions were not significantly different for most parameters studied when analyzed with the MANOVA. The only hemocyte characterization that showed significant differences throughout the experiment was internal complexity of hemocytes ($p < 0.05$) (Fig. 1). Neither cell density of the hemocytes nor cell size varied with time or treatment. The immunological functions also did not show significant differences throughout the experiment.

3.4. Histopathology

Pathological changes found in the *M. edulis* tissues in the day-0 sample consisted of focal inflammatory responses (prevalence in the day-0 sample 2/30, 7%; in the entire experiment 33/210, 15%), phagocytes in follicles (7%/1%), kidney stones (3%/2%), and ceroid (3%/13%). Parasites present

Table 1
Mean values (±standard error) for hemocyte characterization and immune functions for mussels exposed to both algae throughout the experiment

	D0		D3		D6		D9	
	Rhodomonas sp.	*P. minimum*	*Rhodomonas* sp.	*P. minimum*	*Rhodomonas* sp.	*P. minimum*	*Rhodomonas* sp.	*P. minimum*
Hemocyte characterization								
Cell density (cells ml^{-1})	$1.05 \pm 0.17 \times 10^6$	$1.54 \pm 0.17 \times 10^6$	$1.07 \pm 0.010 \times 10^6$	$9.80 \pm 1.00 \times 10^5$	$7.89 \pm 0.71 \times 10^5$	$6.41 \pm 0.68 \times 10^5$	$8.52 \pm 0.89 \times 10^6$	$7.33 \pm 0.89 \times 10^6$
Size	278 ± 7.24	293 ± 7.24	285 ± 4.80	282 ± 4.88	315 ± 5.84	298 ± 5.64	229 ± 5.12	229 ± 5.12
Internal complexity	46.2 ± 3.36	52.3 ± 3.24	50.9 ± 2.28	51.8 ± 2.32	60.4 ± 3.08	49.7 ± 2.91	39.1 ± 2.18	39.0 ± 2.18
Hemocyte functions								
Phagocytosis (%)	28.93 ± 2.01	22.77 ± 2.01	49.34 ± 2.83	53.82 ± 2.88	37.51 ± 2.02	36.70 ± 1.95	48.48 ± 2.56	47.19 ± 2.56
Respiratory burst	127 ± 13.99	107 ± 13.99	173 ± 21.13	164 ± 21.49	96 ± 15.99	105 ± 15.43	90 ± 13.12	56 ± 13.12
Adhesion (%)	83.2 ± 2.31	83.8 ± 2.31	80.9 ± 2.33	81.4 ± 2.33	90.7 ± 2.32	84.9 ± 2.24	72.5 ± 2.63	68.3 ± 2.68
Mortality (%)	1.79 ± 0.27	2.06 ± 0.27	1.32 ± 0.15	1.69 ± 0.15	2.57 ± 0.21	2.33 ± 0.20	1.90 ± 0.18	1.11 ± 0.18
Apoptosis (%)			3.08 ± 0.44	2.60 ± 0.42	3.03 ± 0.40	3.05 ± 0.40	3.02 ± 0.40	3.58 ± 0.41

Size and complexity of hemocytes, as well as their production of ROS (respiratory burst) are presented as arbitrary units. Percentage values were Arcsin(sqrt) transformed for analyses.

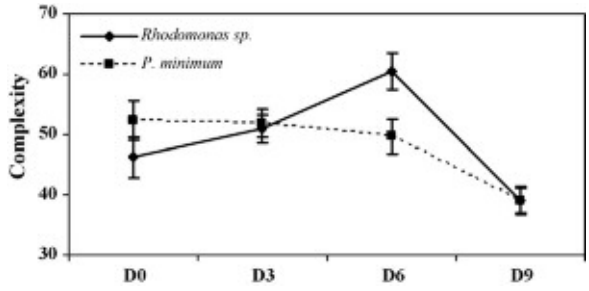

Fig. 1. Hemocyte complexity throughout the experiment. Black line: mussels exposed to *Rhodomonas* sp., dotted line: mussels exposed to the toxic dinoflagellate *P. minimum*. S.E. shown in graph.

were trematodes *Proctoeces maculatus* (3%/3%), and trematodes from the family Gymnophallidae (50%/57%). The ciliate *Ancistrum mytili* was present on the gills at a low prevalence (0%/1%). The presence of focal inflammatory responses in the digestive diverticula or the connective tissue of the mantle, and pearls (0%/2%) were associated with Gymnophallidaes. Ceroidosis occurred with a prevalence of 11% in *P. minimum*-exposed mussels, and 19% in *Rhodomonas* sp.-exposed mussels. There was a 2% prevalence of abscesses in the mussels: two cases occurred in *P. minimum*-exposed mussels and three in *Rhodomonas* sp.-exposed mussels. The above pathological changes occurred independently of the algal exposure or time, and are considered to be background pathology, or ''noise'' in this experiment.

Mussels exposed to the harmful dinoflagellate *P. minimum* showed significant pathological differences compared to those exposed to the non-toxic *Rhodomonas* sp. (Figs. 2 and 3). *P. minimum*-exposed mussels had significantly more cases of elevated quantities of bacteria in the stomach and intestine ($p < 0.01$), and migration of hemocytes into the stomach and intestine ($p < 0.01$). Sixty percent of mussels exposed to *P. minimum* showed abnormally high numbers of bacteria in the alimentary canal, starting in the first sampling following initial exposure. Gram-negative, rod-shaped bacteria appeared as large, grey colonies in hematoxylin-eosin stained sections and red colonies in Gram-stained sections in the lumina of the stomachs and intestines (Fig. 4) forming a biofilm in the middle of the alimentary canal. Cilia in the intestine epithelium were surrounded by bacteria, and in some areas appeared to be beating asyncronously while caught in the biofilm. During the

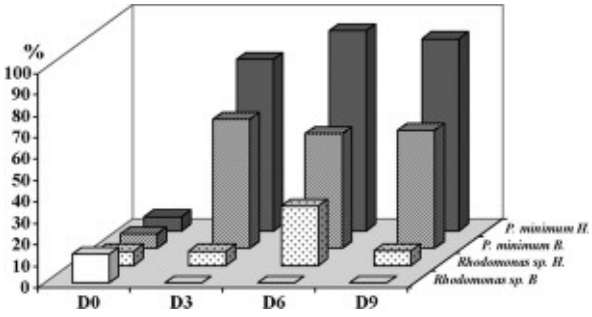

Fig. 2. Percentage of mussels showing bacteria (B) in the intestine or hemocytes (H) in the stomach and intestine during exposure to *P. minimum* and the control algae *Rhodomonas* sp. throughout the experiment.

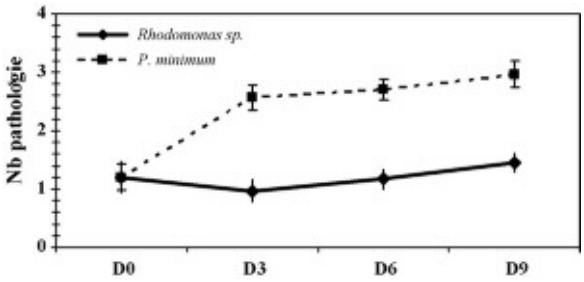

Fig. 3. Total pathological changes per algal exposure throughout the experiment. Numbers refer to the sum of observations/individual expressed as mean ± S.E.

following samplings, the percentages of mussels with gut-bacterial proliferation remained near 54%. None of the mussels exposed to *Rhodomonas* sp. showed this anomaly.

Mussels exposed to *P. minimum* also had hemocytes in the stomachs and intestines (Figs. 2 and 5). Healthy hemocytes surrounded the intestine and stomach epithelia, and were seen migrating through epithelia into the lumina of the alimentary canal (diapedesis). The prevalence of mussels with this anomaly reached 80% in the first sampling after exposure and increased to 93% after six days and 90% nine days after the beginning of the experiment (Fig. 2). Once in the alimentary canal lumina, hemocytes engulfed some bacteria, but were generally overwhelmed and instead succumbed to bacterial degradation (Fig. 6). Some hemocytes died by apoptosis, distinguished by marginal, crescent-shaped, condensed chromatin inside hemocyte nuclei, but some hemocytes also were necrotic. Hemocyte nuclei and remnants of hemocytes were observed within the bacterial mass during degradation. This condition was also present in some individuals from the control tanks, but at significantly lower prevalences (Fig. 2).

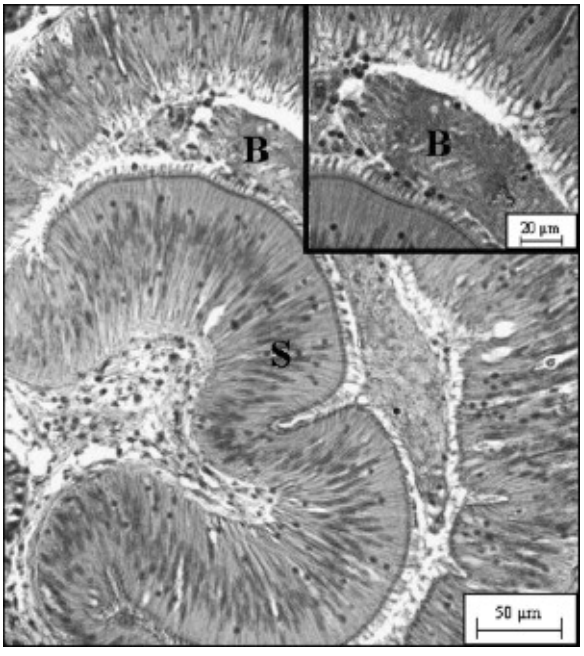

Fig. 4. Bacteria in the intestine of a mussel *Mytilus edulis* exposed to *P. minimum*. Hematoxylin-eosin stained paraffin section. B: large colony of bacteria S: intestine epithelium or typhlosole.

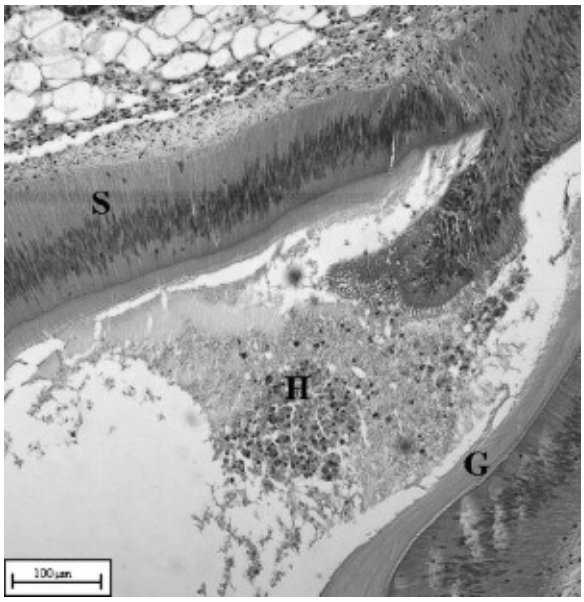

Fig. 5. Hemocytes in the stomach of a mussel *Mytilus edulis* exposed to *P. minimum*. Hematoxylin-eosin stained paraffin section. G: gastric shield, H: hemocytes, S: stomach epithelium.

After 9 days of exposure to *P. minimum*, but not after an exposure to *Rhodomonas* sp., some mussels had aggregations of hemocytes in the connective tissue between the gonadal follicles ($p < 0.05$) (Fig. 7). This pathological change appeared also in one mussel after 3 days of exposure to *P. minimum*, but this observation was not statistically significant ($p > 0.05$).

The total number of pathological changes increased in the *M. edulis* exposed to *P. minimum* during the experiment (Fig. 3). Significant differences were found between the algae ($p < 0.01$), time ($p < 0.01$) and the interaction of these two factors ($p < 0.01$).

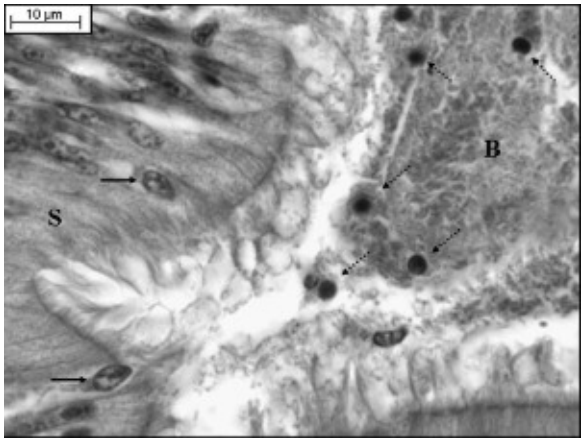

Fig. 6. Diapedesis of hemocytes into the intestine of a mussel *Mytilus edulis* exposed to *P. minimum*. After diapedesis hemocytes migrate to the bacterial mass in the lumen of the intestine, but appear to go through apoptosis and end up being degraded by the bacteria. Hematoxylin-eosin stained paraffin section S: intestine epithelium, B: bacteria. Arrows point to hemocytes in diapedesis, broken arrows to apoptotic hemocytes.

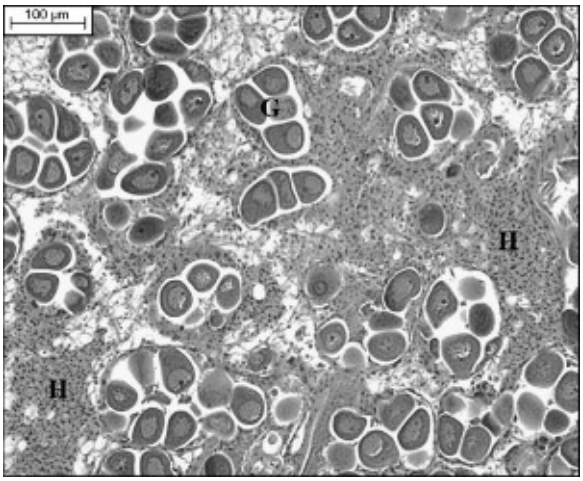

Fig. 7. Aggregations of hemocytes in connective tissue of a mussel, *Mytilus edulis* exposed to *P. minimum*. Hematoxylin-eosin stained paraffin section. G: gonadal follicle, H: hemocytes.

4. Discussion

The scallop bioassay to test *P. minimum* toxicity showed mortality in all scallops exposed; thus it can be concluded that the algal culture was toxic. Although this method does not quantify the toxicity in any way, it showed harmful effects of the dinoflagellate upon bivalves as anticipated. These findings agree with those of Hégaret and Wikfors (2005) who also found 100% mortality of scallops exposed to the same *P. minimum* strain. One third of the scallops exposed to *Rhodomonas* sp. also died by the end of the bioassay; this may be a consequence of ammonia accumulation resulting from metabolism of scallops in the beakers (Widman et al., 2008).

Histology revealed that the immune response of mussels to *P. minimum* was a massive migration of hemocytes into the stomach and intestine (diapedesis) to protect the tissues from exposure to the toxic algae. Similar results were found by Hégaret et al. (in preparation) when Manila clams were exposed to *P. minimum*. Hemocytes appear to encapsulate the toxic cells in the alimentary canal and form hemocyte-*P. minimum* aggregates to remove *P. minimum* cells from the bivalves, thereby minimizing contact with other tissues. Histological sections of bay scallops after exposure to *P. minimum* (Wikfors and Smolowitz, 1993) showed increased numbers of hemocytes in the connective tissue surrounding digestive diverticula and other pathological changes, in contrast to findings of the present experiment. It has been suggested that the site or tissue within the animal where *P. minimum* is digested may play a role in determining response to this dinoflagellate (Wikfors and Smolowitz, 1995b).

The immunological analyses done for the hemocytes showed different results than those found for oysters and scallops (Hégaret and Wikfors, 2005), wherein hemocyte numbers and functions were affected by the toxic-algal exposure. In the present study, the immune parameters analyzed did not show significant differences throughout the experiment. The hemocytes did not phagocytose *P. minimum* but seemed to encapsulate it. Phagocytosis is a process that can be linked to a subsequent respiratory-burst response (Pipe, 1992; Carballal et al., 1997a). In the present study, neither the phagocytic capacity of the hemocytes nor the respiratory-burst response showed differences throughout the experiment. Adhesion of circulating hemocytes remained above 80% throughout the experiment; this hemocyte characteristic did not diminish, and *P. minimum* did not affect this function in the circulating hemocytes (Chen and Bayne, 1995). This observation is consistent with the effective adherence and aggregation of the hemocytes within the digestive system. Neither hemocyte mortality nor apoptosis showed significant differences with flow-cytometry throughout the experiment.

The mean number of circulating hemocytes did not vary throughout the exposure, although many hemocytes were removed from circulation into and through the alimentary canal. The explanation for this phenomenon is that new hemocytes were produced to replace those responding to the presence of *P. minimum* and bacteria in the intestine and stomach. The site of hematopoiesis in bivalves is not known (Bachère et al., 2004); data from cytograms or histological samples did not reveal a specific anatomical location where the hemocytes were dividing.

The complexity of the hemocytes was the only hemocyte characteristic that showed significant differences between the algal exposures and time. Complexity of hemocytes in mussels exposed to *P. minimum* showed a progressive decrease from the beginning of the experiment until day 6. This decline in hemocyte complexity could indicate degranulation, as part of the immune response (Carballal et al., 1997b), or a consequence of dilution of the existing granules as cells divided faster than they created new granules. We favor the second hypothesis, as none of the other immune characteristics were altered, but hemocytes apparently were dividing quickly to maintain the number of circulating hemocytes. On day 9 of the experiment, hemocyte complexity from both treatments decreased and reached the same value, suggesting a possible general response to deteriorating conditions in the aquaria.

Elevated concentrations of bacteria were observed in intestines and stomachs of mussels exposed to *P. minimum*. As bacteria are too small to be efficiently filtered by the mussels (Jørgensen, 1975; Riisgård et al., 1996), and considering the different results found for each algal exposure, we hypothesize that there was bacterial growth in the alimentary canal. Although the *P. minimum* culture was not bacteria-free, the numbers of bacteria relative to *P. minimum* cells were not sufficiently high to account for the mass in the alimentary canal. Alternately, it is possible that these bacteria were normal fauna within the intestine that proliferated abnormally because digestive functions of the mussels were impaired. Bacteria in the *P. minimum* culture were indistinguishable, morphologically and according to Gram-stain, from each other; therefore, the origin of these bacteria remains unknown. Within the intestines of mussels exposed to *P. minimum*, hemocytes eventually were degraded by bacteria and died by both apoptosic and necrotic processes.

Chemical toxins can suppress immune function in bivalves (Gagnaire et al., 2004; Ordás et al., 2007) but potential

pathogens or parasites can activate hemocyte responses (Cáceres-Martínez et al., 2000; Villalba et al., 2004). In our study, the lack of immunosuppression, but rather activation of a protective immune response in mussels exposed to *P. minimum*, suggests that the mussels perceive this dinoflagellate as a potential invader, rather than experiencing physiological impairment from a chemical toxin in *P. minimum*. It is noteworthy that the mussels did not have the same response to *Rhodomonas* sp. cells within the digestive system. Mussels thus appear to perceive *P. minimum* as not only non-self, but also a threat, and activate internal defense mechanisms: recognition, migration, diapedesis, and isolation by encapsulation. Several authors have reported the close phylogenetic relationship between protozoan parasites from the Family *Perkinsidae* and dinoflagellates (Goggin and Barker, 1993; Leander and Keeling, 2004). We hypothesize that mussels could recognize *P. minimum* as a threat because it is similar to *P. marinus* in some chemical or cell-surface factor. This is supported by the findings of Bushek et al. (2002) who found nearly identical reciprocal labeling of epitopes on *P. marinus* and seven parasitic dinoflagellates. No cryptophytes have been identified as bivalve parasites, thus, this difference in phylogeny between *P. minimum* and *Rhodomonas* sp. could explain the different response of the mussels to these two phytoplankters, i.e., there has been no evolutionary selective pressure for an immune response to cryptophytes, but there has been for perkinsus-like protists. As the innate-immune system of invertebrates is non-adaptive, i.e., there is no antibody ‘‘memory’’ of prior exposures to a specific pathogen or parasite, the only mechanism by which recognition of threatening non-self organisms can be improved is by selective survival of high-responding individuals in a population experiencing disease pressure (Janeway et al., 2004). Thus, the findings of this study suggest the hypothesis that blue mussels may have evolved an effective immune response to *P. minimum*, through survival of individuals in populations experiencing *P. minimum* blooms, or of individuals surviving *Perkinsus* species with similar immune-recognition characteristics (Medzhitov and Janeway, 2002).

The immune response activated in *P. minimum*-exposed mussels was, however, different from what has been observed in oysters exposed to *Perkinsus marinus*, wherein phagocytosis occurs (Goedken et al., 2005b). The immune response observed in this experiment is much more similar to the clam hemocyte response to QPX (Quahog Parasite Unknown, Tharaustochytridae, Labyrinthulomycota) than the oyster hemocyte response to *Perkinsus marinus*. Hemocytes of hard clams (*Mercenaria mercenaria*) are unable to engulf the QPX parasite but encapsulate the parasite to isolate it (Smolowitz et al., 1998). Small particles, such as *Perkinsus marinus* (2 μm), can be engulfed by bivalve hemocytes, but large particles are isolated by encapsulation. *P. minimum* has a similar size to QPX (thalli and sporangia ∼20 μm), which could explain the similarity of immune responses between mussels to *P. minimum* and clams to QPX. Indeed, phagocytosis of bacterial and viral particles, but aggregation and encapsulation around larger intrusions, are general characteristics of the innate immune response in bivalves (Bayne, 1983). Recent experiments investigating *in vitro* interactions between *P. minimum* cells and hemocytes of bay scallops and quahogs yielded similar aggregation and encapsulation responses of the hemocytes (Hégaret et al., in preparation).

5. Conclusions

This is the first study that has combined immune and histopathological techniques to determine the effects of the harmful dinoflagellate *P. minimum* upon the blue mussel, *M. edulis*. Results showed the importance of using different analyses to achieve a more-complete understanding of the effects of a HAB species upon a bivalve. Mussel hemocyte responses to *P. minimum* were diapedesis, aggregation and encapsulation; these responses were activated within the mussel alimentary canal. No putative microalgal toxins affected the hemocytes in circulation because the first line of defense occurred outside the epithelial barriers in the stomach and intestine. Healthy hemocytes left circulation and became impaired in the intestine when encountering *P. minimum*. Bacteria proliferated in the stomach and intestine as hemocytes were responding to the *P. minimum* ingested and were unable to control the bacterial growth.

The close phylogenetic relationship between *Perkinsus* and some dinoflagellates suggest that the genera may share cell-surface factors that make them recognizable to the bivalve host as non-self and a potential threat. But because of the large size of *P. minimum* relative to hemocytes, the aggregation and encapsulation response is more effective than phagocytosis.

Acknowledgements

This study has been supported by a grant from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) to the first author and partially financed by the RTA04-023 INIA research project. The following Milford Laboratory staff provided much-appreciated technical assistance: Jennifer H. Alix, April N. Croxton, Mark S. Dixon, Diane Kapareiko, John Karolus, Yaqin Li, Shannon L. Meseck, and Barry C. Smith.[SES]

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Chapter VI

Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: histopathology, immune responses, and recovery

Harmful Algae 7 (2008) 702–711

Available online at www.sciencedirect.com
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Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: Histopathology, immune responses, and recovery

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Received 10 February 2008; received in revised form 18 February 2008; accepted 18 February 2008

Abstract

Mussels (*Mytilus edulis*) were exposed to cultures of the toxic dinoflagellate *Alexandrium fundyense* or the non-toxic alga *Rhodomonas* sp. to evaluate the effects of the harmful alga on the mussels and to study recovery after discontinuation of the *A. fundyense* exposure. Mussels were exposed for 9 days to the different algae and then all were fed *Rhodomonas* sp. for 6 more days. Samples of hemolymph for hemocyte analyses and tissues for histology were collected before the exposure and periodically during exposure and recovery periods.

Mussels filtered and ingested both microalgal cultures, producing fecal pellets containing degraded, partially degraded, and intact cells of both algae. Mussels exposed to *A. fundyense* had an inflammatory response consisting of degranulation and diapedesis of hemocytes into the alimentary canal and, as the exposure continued, hemocyte migration into the connective tissue between the gonadal follicles. Evidence of lipid peroxidation, similar to the detoxification pathway described for various xenobiotics, was found; insoluble lipofuchsin granules formed (ceroidosis), and hemocytes carried the granules to the alimentary canal, thus eliminating putative dinoflagellate toxins in feces. As the number of circulating hemocytes in *A. fundyense*-exposed mussels became depleted, mussels were immunocompromised, and pathological changes followed, i.e., increased prevalences of ceroidosis and trematodes after 9 days of exposure. Moreover, the total number of pathological changes increased from the beginning of the exposure until the last day (day 9). After 6 days of the exposure, mussels in one of the three tanks exposed to *A. fundyense* mass spawned; these mussels showed more severe effects of the toxic algae than non-spawning mussels exposed to *A. fundyense*.

No significant differences were found between the two treatments during the recovery period, indicating rapid homeostatic processes in tissues and circulating hemocytes.

Keywords: *Alexandrium fundyense*; Harmful algal blooms; Hemocyte; Histopathology; Immunology; *Mytilus edulis*

1. Introduction

Toxic or otherwise inimical microalgal proliferations in aquatic ecosystems, referred to as harmful algal blooms (HABs), were first recorded by ancient civilizations and have been observed ever since (Fogg, 2002; Landsberg, 2002). These HABs, caused by a wide variety of microalgal species, can affect many sympatric marine organisms (Shumway, 1990; Fogg, 2002; Landsberg, 2002). HABs are increasingly impacting seafood production, especially aquaculture. As bivalve mollusks are filter-feeding animals that can bio-accumulate toxins and other compounds, toxins produced by HAB species can accumulate in bivalve tissues and affect their predators, including top predators in food webs such as humans (Azanza and Taylor, 2001; Landsberg, 2002). To protect public health, monitoring and management programs for bivalve toxins have been implemented (Shumway et al., 1990; Rehnstam-Holm and Hernroth, 2005); HAB-related closures to shellfish harvest can result in great economic losses to the aquaculture industry (Shumway, 1990; Whyte, 1997; EUROHAB, 1998). Moreover,

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doi:10.1016/j.hal.2008.02.006

of all cultured bivalves, mussels accumulate Paralytic Shellfish Poisoning (PSP) toxins most rapidly and to a greater degree compared to other harvested shellfish (Shumway et al., 1990; Gosling, 2003).

In addition to accumulating and passing toxins to higher trophic levels, bivalves themselves can be affected by HAB events. For example, it has been shown that toxic algae can produce histopathological lesions and impair immune responses in commercially harvested bivalve species exposed to them (Wikfors and Smolowitz, 1995; Wikfors et al., 2000; Hégaret and Wikfors, 2005a,b; Pearce et al., 2005; Hégaret et al., 2007a,b; da Silva et al., 2008; Galimany et al., 2008a,b).

The dinoflagellate *Alexandrium fundyense* is a HAB species that produces a group of neurotoxins called saxitoxins (Anderson et al., 1990). These toxins affect many marine organisms and, through dietary consumption, can cause a syndrome known as PSP which impairs sodium-channel signal transmission between neurons (Daranas et al., 2001; Landsberg, 2002; Bricelj et al., 2005). Blooms of this dinoflagellate and trophic transfer of toxins can lead to effects upon finfish, including erratic swimming and mortality (Samson, 2002; Martin et al., 2006; Sephton et al., 2007) and on shellfish, reducing clearance rate, growth rate of soft tissues, and condition index (Bricelj et al., 1993; Landsberg, 2002). Nevertheless, when no other type of alga is available, *A. fundyense* can be used as a food source by mussels despite its toxicity (Bricelj et al., 1993).

This study investigated the effects of *A. fundyense* exposure upon blue mussels, *Mytilus edulis*, under experimental conditions and the recovery of the mussels after the exposure was terminated, with a focus on histopathology and hemocyte immune functions of exposed mussels.

2. Materials and methods

2.1. Experimental animals

Mussels, *Mytilus edulis* (44.1–77.2 mm shell length), were collected from Westcott Cove, Stamford, Connecticut, USA from an intertidal beach on the north shore of Long Island Sound in May of 2007. Mussels were cleaned of fouling organisms and acclimated for 4 days before the experiment, the first 3 days in the experimental tanks with filtered sea water and the fourth day fed with *Rhodomonas* sp. (RHODO, see below) at a concentration of 1×10^4 cells ml^{-1}.

2.2. Algal cultures

The harmful algal species tested in this study was the BF2 strain of the dinoflagellate *A. fundyense* (Balech), obtained from the Milford Microalgal Culture Collection (isolated from the Gulf of Maine, U.S.A.). In addition, a non-toxic cryptophyte, the RHODO strain of *Rhodomonas* sp., was used as a non-toxic, control alga.

The microalgae were cultured in 20-L glass carboy assemblies using aseptic technique (Ukeles, 1973). Cultures were harvested semi-continuously to maintain consistency in culture quality over the course of the study and were harvested in late-log or early-stationary phase. *A. fundyense* was grown in F/2-enriched (Guillard and Ryther, 1962; Guillard, 1975), filtered seawater from Milford Harbor, and *Rhodomonas sp.* was cultured in E-medium (Ukeles, 1973). Both cultures were maintained at 20 °C with 24 h light. Algal-cell densities were determined by hemocytometer counts with the light microscope.

To determine PSP toxicity in mussels, the mouse bioassay was performed according to APHA (1970), using mussels exposed for 8 and 9 days to *A. fundyense*. Briefly, 100 g of mussel meat was collected per replicate sample and homogenized, and an equal volume of 0.18N HCl was added to the homogenate. Sample pH was adjusted to 3, and the mixture was brought to boil at 100 °C for 5 min. Then the extract was centrifuged for 5 min at 3000 rpm and decanted. Mice (ICR females, 17–23 g) were inoculated with 1 ml of this extract intraperitoneally and observed for symptoms and mortality for 60 min. A standard PSP toxin reference solution (diluted to 0.33 μg/ml H_2O pH 3) served as a positive control. The conversion factor (CF) was determined by injecting 5 mice IP with a dilution of the reference solution that produces a median death time of 5–7 min. The death time was converted to mouse units (MU) using Sommer's tables. Mice that survived the 60 min time period were given a MU value of <0.875. Calculated MU values were converted to corrected mouse unit, CMU, with a weight-correction factor, and median value MCMU was calculated to represent the true median death time of the group. Concentration of the toxin was determined by the following formula: μg toxin/100 g meat = MCMU × CF × dilution factor × 200.

Toxins within *A. fundyense* cells harvested from cultures used for the exposure experiment were analyzed by HPLC. An HPLC-UV-FL (Alliance 2695, Waters) was used to determine the toxic PSP compounds in *A. fundyense* using Lawrence's methodology (Lawrence et al., 1995).

2.3. Experimental design

Six hundred (600) mussels were distributed randomly into six 20-L glass aquaria, i.e. 100 animals per aquarium. Triplicates of each of two treatments were done in this experiment: mussels were fed either *A. fundyense* or *Rhodomonas* sp., each at 4×10^3 cells ml^{-1} during the first 9 days of the experiment, and all mussels were fed *Rhodomonas* sp. during the last 6 days to test recovery. All mussels were given a regime of 16 feedings per day, one every 90 min, using computer-automated valves attached to the cover of a rearing-chamber system (Smith and Wikfors, 1998).

Samples of mussels were collected on day 0 (before exposures), after 3, 7 and 9 days of exposure to the experimental microalgal treatments, and on days 12 and 15 to study the recovery process. At each sampling time, 60 mussels were removed from the aquaria (10 from each, individual aquarium) and analyzed for stomach contents, histopathology, and hemocyte parameters. Feces and pseudofeces also were examined microscopically throughout the experiment.

2.3.1. Stomach contents, feces, and pseudofeces

Stomach contents of mussels were removed with a 25-gauge needle and a 1-ml syringe. Samples were preserved in 300 μl of 1% glutaraldehyde and observed with a light microscope. Feces and pseudofeces were removed from each aquarium with a pipette and observed directly with a light microscope.

2.3.2. Histopathology

A 4-mm cross-section of each mussel, including digestive diverticula, gills, mantle, kidneys, plicate membranes, and the byssus gland, was dissected and fixed in Davidson's fixative for 48 h at 4 °C. The section then was rinsed in 50% ethanol in filtered seawater and transferred to 70% ethanol. Samples were dehydrated and embedded in paraffin. After processing, 5-μm sections were stained using a hematoxylin–eosin staining procedure (Howard et al., 2004) and examined under a light microscope.

2.3.3. Hemocyte analyses

Hemolymph was withdrawn with a 21-gauge needle and a 1-ml syringe from the adductor muscle of each individual mussel and stored temporarily in an Eppendorf microcentrifuge tube on ice. Analyses of hemocyte morphology and function were done on hemolymph extracted from each individual mussel.

Procedures for characterization of the hemocytes, cell density of circulating hemocytes (cells ml^{-1}), size and internal complexity of the hemocytes, and immunological functions (listed below) were adapted from Delaporte et al. (2003), Hégaret et al. (2003a,b), Lambert et al. (2003), Soudant et al. (2004) and Buggé et al. (2007). Hemocyte apoptosis also was assessed according to a protocol adapted from Goedken et al. (2005). For the hemocyte analyses, a FACScan flow-cytometer (BD Biosciences, San Jose, CA) was used.

The five functional hemocyte parameters measured were:

(a) hemocyte mortality, as percentage of dead hemocytes
(b) phagocytosis of fluorescent beads by hemocytes, which simulates the engulfment of non-self particles
(c) respiratory-burst response in hemocytes, that measures reactive oxygen species' potential to kill non-self particles previously engulfed by hemocytes
(d) percentage of adhering hemocytes
(e) percentage of apoptotic hemocytes

2.4. Statistical analysis

Results of the hemocyte assays were analyzed statistically using one-way analysis of variance (ANOVA) and multifactor analysis of variance (multifactor ANOVA) to assess effects of experimental treatments upon the response variables. The total number of pathological changes was analyzed statistically using multifactor analysis of variance (multifactor ANOVA). Results for individual pathological conditions (migration of hemocytes to the stomach and intestine, red granules in the digestive gland, degenerated digestive gland, presence of blue mucus-secretory cells in gills, abnormal gonads, hemocytes infiltrating gonadal follicles and ceroidosis) were analyzed using Chi-square tests for each sampling time, with even distribution of each condition in each category as the null hypothesis. The statistical software used was Statgraphics Plus (Manugistics, Inc., Rockville, MD, USA).

3. Results

3.1. Experimental animals

An unexpected variable was introduced into the experiment when, on the 6th day of the exposure, mussels in one of the three aquaria exposed to *A. fundyense* mass spawned; sperm clouded the water and oocytes precipitated at the bottom of the aquarium. The mussels apparently remained functional as they were open and filtering. On the 7th day, before sampling, the aquarium was still cloudy, and mussels continued to spawn. Only four dead mussels were removed from this aquarium and discarded, but most were paralyzed, unable to close the valves when handled. To minimize effects of the mass spawning upon this population of mussels, the water was removed and the aquarium was rinsed and cleaned and refilled with filtered seawater and *A. fundyense* at a concentration of 4×10^3 cells ml^{-1}, as in the initial conditions. Spawning did not recur after the 7th day of the experiment nor in any other aquarium.

On the 9th day of the experiment, mussels in the aquarium that had spawned previously still were paralyzed but all alive. Paralysis disappeared during the recovery period when these mussels were fed the non-toxic alga *Rhodomonas* sp.

3.2. Mouse bioassay for mussel meats and algal-cultures toxicity tests

The results from the mouse bioassay for PSP in *A. fundyense*-exposed mussels were below the detection limit in both samples studied. The detection limit was between 36.5 and 38.3 μg toxin/100 g meat. HPLC analysis of the *A. fundyense* culture revealed the presence of PSP toxins in the algal cells, with the following components (units are pg $cell^{-1}$): GTX1,4 0.42 ± 0.02; NEO 0.70 ± 0.07; GTX2,3 0.13 ± 0.01; B-1 $0.15 \pm <0.00$; STX $0.16 \pm <0.00$; and C1,2 0.85 ± 0.06. Total toxicity was estimated according to Parkhill and Cembella (1999) as 0.43 pg STXeq $cell^{-1}$.

3.3. Experimental results

3.3.1. Stomach contents, feces, and pseudofeces

Either *A. fundyense* or *Rhodomonas* sp. cells fed were present in the stomach, feces and pseudofeces of the exposed mussels. *A. fundyense* was found mostly as temporary cysts in all cases (Fig. 1). Fecal pellets from mussels fed *Rhodomonas* sp. consisted mainly of *Rhodomonas* cell fragments showing characteristic red and orange fluorescence of chlorophyll and phycoerythrin pigments under blue-light excitation fluorescence microscopy. Fecal pellets from *A. fundyense*-exposed mussels were composed of masses of brown, non-fluorescent material with *A. fundyense* resting cysts and some fragmented cells embedded.

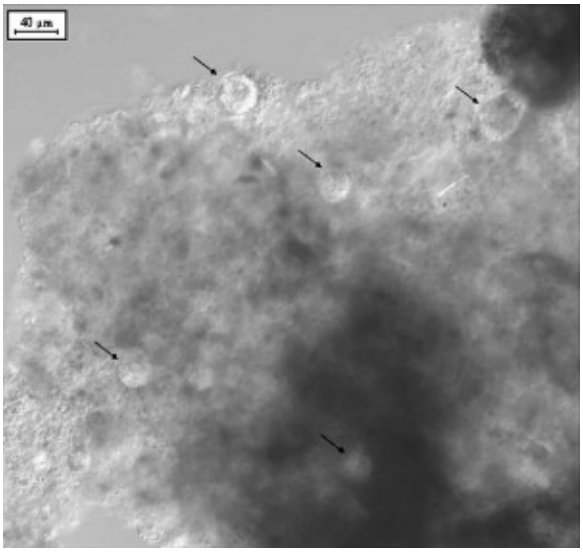

Fig. 1. Diffusion-interference contrast photomicrograph of fecal pellets from mussels, *Mytilus edulis*, feeding on *Alexandrium fundyense*. Arrows point to *A. fundyense* temporary cysts.

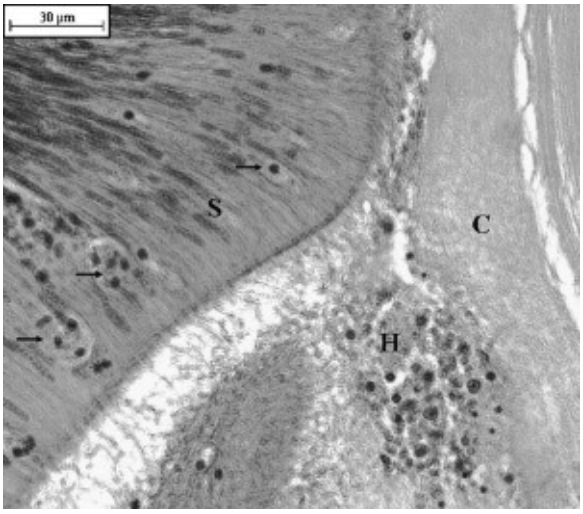

Fig. 2. Hemocytes in stomach of a mussel exposed to *A. fundyense*. Hematoxylen–eosin stained section. Arrows point to hemocytes in diapedesis. C: gastric shield, H: hemocytes, S: stomach epithelium.

3.3.2. Histopathology

Some pathological conditions observed in the mussels were present in the tissues throughout the entire experiment, including the time-0 sample, independently of the algal exposure or time, and are considered background pathology, or ''noise''. Pathological conditions found in the *M. edulis* tissues consisted of focal inflammatory responses (prevalence in the entire experiment 148/322, 46%) and kidney stones (0.9%). Parasites, the trematodes *Proctoeces maculatus* (1%) and the ciliate *Ancistrum mytili* on the gills (2%), were present at low prevalences. There was a 3% prevalence of abscesses in the mussels.

Mussels exposed to the toxic *A. fundyense* showed a significant increase in pathological conditions from those exposed to the non-toxic alga. After 3 days of exposure, five mussels exposed to *A. fundyense* and one mussel exposed to *Rhodomonas* sp. showed hemocytes in the stomach and intestine. The prevalence of mussels with hemocytes in the stomach and intestine became significantly higher after 7 and 9 days of exposure to *A. fundyense* in comparison to mussels exposed to *Rhodomonas* sp. (ANOVA, $p < 0.05$) (Fig. 2). During the recovery period, there were no significant differences ($p > 0.05$) between mussels from the two previous treatments; all treatments included mussels with hemocytes in the stomach and intestine, but at very low prevalence. The prevalence of mussels with infiltration of hemocytes into the connective tissue between gonadal follicles increased significantly after 9 days of exposure to *A. fundyense* in comparison to mussels exposed to the non-toxic alga ($p < 0.05$). This pathological change also was present during the recovery period, but decreased with no statistical differences ($p > 0.05$) between *Rhodomonas* sp.-exposed and *A. fundyense*.-exposed mussels. Ceroidosis and trematodes from the family Gymnophallidae were found in both treatments, including the time-0 sample, but the prevalences increased significantly only in the *A. fundyense*-exposed mussels after 9 days of exposure ($p < 0.05$). The presence of focal, inflammatory responses in the digestive diverticula or the connective tissue of the mantle, and pearls (4% of total prevalence), were associated with parasitic Gymnophallidaes (Fig. 3), this increasing significantly only in the *A. fundyense*-exposed mussels after 9 days of exposure.

Mussels exposed to *A. fundyense* from the aquarium in which mussels spawned showed a different pathological pattern than the mussels from the other two replicates exposed to the toxic alga, but without spawning. In addition to the pathological changes described above, abnormal gonads appeared starting on day 7, the first sampling after the mass spawning, and lasted until the end of the experiment, including the recovery period (day 15), with a significantly higher prevalence ($p < 0.05$) compared to non-spawned mussels from the other two *A.*

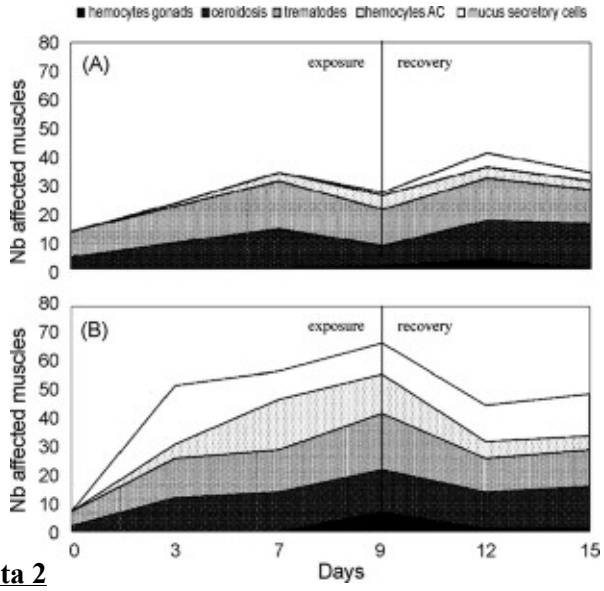

Fig. 3. Pathological conditions in mussels exposed to *Rhodomonas* sp. (A) and *Alexandrium fundyense* (B) throughout the experiment. AC: alimentary canal.

Errata 2

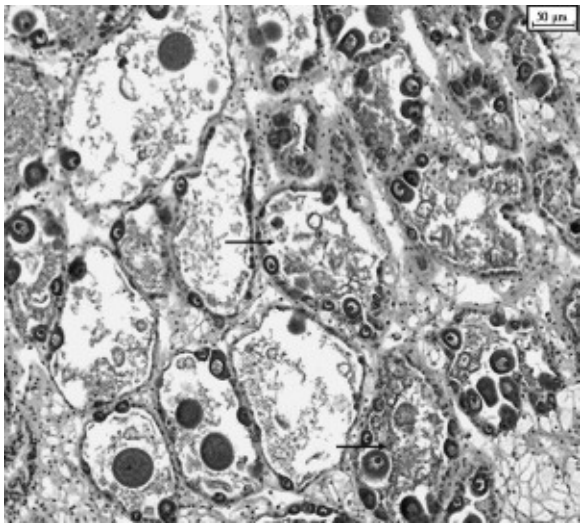

Fig. 4. Section of the gonad of a female mussel exposed to *Alexandrium fundyense*. Arrows show cell debris and remnants of ova within the lumina of follicles.

fundyense-exposed populations. Ova were degenerating, and only a few healthy-looking, early stages of gametocytes were present in the germinal epithelium. Instead there was cell debris in the lumina of the gonadal follicles (Fig. 4).

Red granules were present in the digestive tubules of *A. fundyense*-exposed mussels on days 7 and 9 with significantly higher prevalence compared to *Rhodomonas* sp.-exposed mussels ($p < 0.01$) (Fig. 5). Degeneration of the digestive gland was found in only one mussel on day 7 in the *A. fundyense*-exposed and spawned population, but this condition reached 60% prevalence on day 9 in this individual aquarium ($p < 0.01$) significantly higher than the other, non-spawned, *A. fundyense*-exposed aquaria (Fig. 5). The prevalence of the pathological condition described above in non-spawned mussels exposed to *A. fundyense* was significantly lower than in mussels in the population where spawning occurred (ANOVA, $p < 0.01$).

Blue, mucus-secretory cells were found in gills of mussels exposed to *A. fundyense* from day 3 to day 9 of the exposure, but not in *Rhodomonas* sp.-exposed mussels (Chi square, $p < 0.05$). During the recovery period, however, the prevalence of blue, mucus-secretory cells in mussels from the aquarium where spawning occurred decreased significantly in comparison to non-spawned *A. fundyense*-exposed mussels (Chi square, $p < 0.01$), which had no significant difference from mussels exposed to *Rhodomonas* sp. (Chi square, $p > 0.05$).

The interaction between the two factors, algae and time, caused highly significant differences in the total number of pathological conditions present in mussels throughout the experiment (MANOVA, $p < 0.01$). There was a temporal increase in the total number of pathological conditions in the *M. edulis* exposed to *A. fundyense* until day 9, but during the recovery period, days 12 and 15 of the experiment, the total number of pathological conditions in the mussels exposed previously to the toxic alga decreased, with no significant differences between the two algal treatments (Fig. 6).

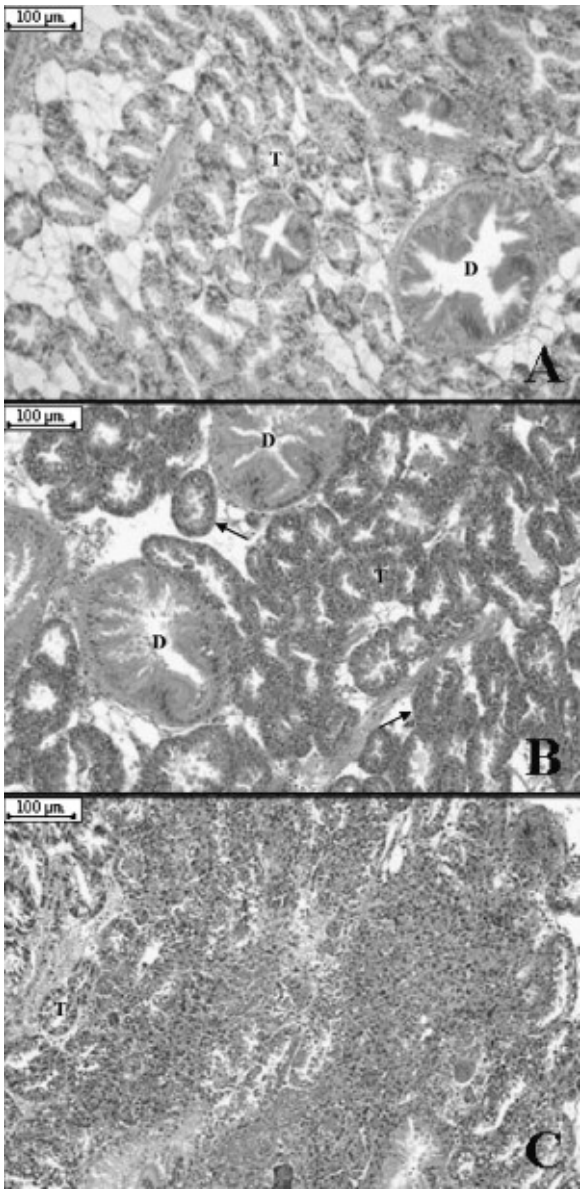

Fig. 5. Section of the digestive gland of a mussel exposed to *Alexandrium fundyense* that spawned during the experiment. (A) After 3 days of exposure; no lesions were observed; (B) after 7 days of exposure; arrow points to red granules in the digestive gland; (C) after 9 days of exposure; digestive gland degenerated (D: digestive duct, T digestive tubule).

3.3.3. Immunological analysis—hemocytes

Flow-cytometric analysis of mussel hemocytes revealed two distinct classes of hemocytes: highly granular cells, corresponding to eosinophilic hemocytes seen in histological preparations, and less-granular hemocytes corresponding to basophilic cells in histology (Fig. 7). Aggregates of hemocytes also were also found but the percentage was low throughout the experiment.

Multifactor analysis of variance showed significant differences between hemocytes sampled from the mussels exposed to *A. fundyense* or *Rhodomonas* sp. The hemolymph parameters that varied between treatments from day 0 to day 9 were the size of the basophilic hemocytes (ANOVA, $p < 0.05$) and the

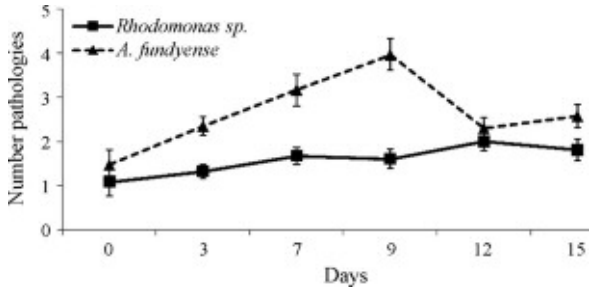

Fig. 6. Total pathological changes in mussels exposed two algal treatments throughout the experiment. Numbers refer to the sum of observations/individual expressed as mean ± S.E.

complexity of the eosinophilic hemocytes (ANOVA, $p < 0.05$), showing lower values in the *A. fundyense*-exposed mussels in both cases at the end of the exposure (day 9), but increasing at the end of the recovery period with no significant differences between algal treatments ($p > 0.05$).

One-way analyses of variance showed differences in morphology of hemocytes sampled from mussels exposed to *A. fundyense* or *Rhodomonas* sp. after 7 days of exposure. The size of the hemocytes was significantly larger in mussels exposed to the non-toxic-algal treatment (ANOVA, $p < 0.05$). Many more differences were found after 9 days of the exposure. Mean complexity values for the two types of hemocytes were higher in the *Rhodomonas* sp.-exposed mussels (ANOVA, $p < 0.05$). The size of the basophilic hemocytes, and the number of eosinophilic hemocytes, were also higher in mussels exposed to the non-toxic-algal treatment (ANOVA, $p < 0.05$) (Table 1). The total numbers of hemocytes, including dead hemocytes, were significantly higher in the *Rhodomonas* sp.-exposed mussels (ANOVA, $p < 0.05$).

Hemocytes sampled from mussels in the aquarium where spawning occurred (tank 2) showed a higher percentage of apoptotic cells than hemocytes sampled from the *A. fundyense*

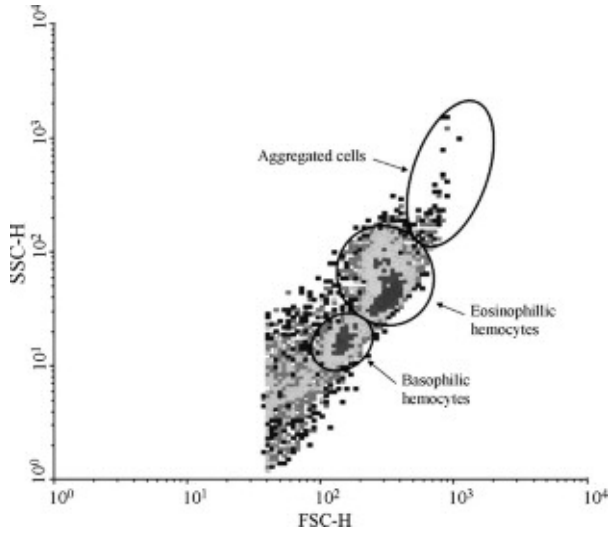

Fig. 7. Biplot of forward scatter (FSC = size) and side scatter (SSC = internal complexity) of mussel hemocytes by flow-cytometry. Three population of hemocytes are apparent: basophilic, eosinophilic, and aggregated hemocytes.

Table 1
Mean values for hemocyte characterization for mussels exposed to both algae after 9 days of exposure (±S.E.)

	Rhodomonas sp.	*A. fundyense*
Size basophilic*	1.829 ± 51	1.600 ± 50
Size eosinophilic	3.131 ± 62	3.034 ± 61
Size aggregates	6.192 ± 152	5.972 ± 149
Number basophilic	441 ± 59	434 ± 58
Number eosinophilic*	2.470 ± 262	1.576 ± 258
Number aggregates	100 ± 11	116 ± 11
Complexity basophilic*	208 ± 7	186 ± 6
Complexity eosinophilic*	612 ± 33	460 ± 33
Complexity aggregates*	2.638 ± 182	2.046 ± 179

Size and complexity of hemocytes are presented as arbitrary units; numbers of hemocytes are presented for 30 s of acquisition of the sample into the flow-cytometer (* shows significant values, ANOVA).

aquaria where there was no spawning (ANOVA, $p < 0.05$). Conversely, mean values (±S.E.) of hemocyte ROS (arbitrary units) in samples from the three *A. fundyense*-exposed mussel populations during the 9 days of exposure were relatively similar: replicate 1, 92.59 ± 12.55; replicate 2, 117.05 ± 11.82; and replicate 3, 113.97 ± 11.37. Mean percentages of highly phagocytic hemocytes were: replicate 1, 63.32 ± 2.50; replicate 2, 59.90 ± 2.45; and replicate 3, 62.41 ± 2.18. No other differences were found between the hemocytes at this sampling date, nor for any other sampling day.

The two sampling days of the recovery period showed no immunological differences between the *A. fundyense*-exposed and *Rhodomonas* sp.-exposed mussels.

4. Discussion

HPLC analysis showed that the *A. fundyense* culture used contained saxitoxins, but in very modest amounts. According to Bricelj et al. (1991), cell toxicity in *Alexandrium* sp. from the east coast of North America can range from undetectable levels to more than 100 pg STXeq cell^{-1}. The *A. fundyense* strain used in the experiment, despite its low toxicity (0.43 pg STXeq cell^{-1}) leading to toxin accumulation in mussels below the detection limit of the mouse bioassay, did affect the immune status of the mussels. In addition, paralysis of mussels in the population where spawning occurred clearly indicated neurological effects of PSP toxins. Mussels ingested and partially digested *A. fundyense* as seen in the stomach contents and feces, although most *A. fundyense* cells appear to have transformed into temporary cysts during ingestion. It is known that PSP toxins mostly accumulate in the viscera of mussels (Bricelj et al., 1990; Bricelj and Shumway, 1998; Kwong et al., 2006), causing cellular damage in the digestive gland (Widdows et al., 1979). Moreover, toxin assimilation efficiency can be regulated by the probability of contact between the toxins and walls of the digestive system (Moroño et al., 2001).

Flow-cytometric analysis of mussel hemocytes revealed two classes of hemocytes, categorized according to Pipe et al. (1997) as: basophilic (smaller and less complex hemocytes that include agranular and granular cells with small granules), and

eosinophilic (with larger granules and more internal complexity); hemocyte aggregates were also observed.

Many biological effects on the *A. fundyense*-exposed mussels were found. Firstly, an inflammatory response consisting of degranulation and diapedesis of hemocytes was observed. Eosinophilic hemocytes in the *A. fundyense*-exposed mussels degranulated within the semi-open vascular system. Hemocytes in bivalves have been shown to release granules (a.k.a. lysosomes) into the serum in response to various chemical or cellular stimuli as part of a defense mechanism (Pipe, 1990). Enzymes released extracellularly from lysosomes can cause partial degradation of materials in the serum, thereby enhancing endocytosis by hemocytes (Mohandas and Cheng, 1985; Mohandas et al., 1985). Moreover, migration of hemocytes by diapedesis from the semi-open vascular system into the stomach and intestine, as seen in *A. fundyense*-exposed mussels in the present study, has been described as part of defense mechanism in bivalves (Stauber, 1950). The lower number of eosinophilic hemocytes found in the adductor-muscle sinus of the *A. fundyense*-exposed mussels supports this interpretation. The number, size and complexity, of the circulating hemocytes in the *Rhodomonas* sp.-exposed mussels were significantly higher during the exposure, indicating that hemocytes were neither altered nor degranulated in these mussels.

As the number of circulating hemocytes became depleted by diapedesis into the alimentary canal in the *A. fundyense*-exposed mussels, the total number of pathological changes increased in these mussels from the beginning of the exposure to day 9. We hypothesize that a decrease in circulating hemocytes over time degraded the capacity of *A. fundyense*-exposed mussels to respond to the presence of trematodes, the prevalence of which increased after 9 days of exposure. The trematodes (Gymnophallidae) appeared to behave as opportunistic pathogens, taking advantage of the weakened immune status of the host.

As part of the inflammatory response, hemocytes also were found in the connective tissue between the gonadal follicles in *A. fundyense*-exposed mussels. This observation is consistent with the findings of fewer hemocytes in circulating hemolymph of *A. fundyense*-exposed mussels. It is well known that parasites, pollution (Aarab et al., 2006) or toxicity (Aarab et al., 2004) can have deleterious effects upon the reproductive cycle of a bivalve (Gosling, 2003). As gametogenesis is an energy-demanding process, we hypothesize that hemocytes were migrating into the gonadal follicles to reabsorb gametocytes, thereby re-directing energy to defense mechanisms against the toxic algae being ingested.

Ceroidosis also increased in mussels after 9 days of exposure to *A. fundyense*. Ceroidosis, accumulation of lipofuchsin pigment (ceroid) that appears yellow to brown in hematoxylin–eosin sections, is associated with disturbances in lipid metabolism in several pathological conditions caused by nutritional deficiencies, toxicity, or disease in many animal phyla, including mollusks (Wood and Yasutake, 1956). Thus, the increase of ceroidosis in tissues of *A. fundyense*-exposed mussels may be attributable to lipid peroxidation generated by ingestion of the harmful alga.

Pathological changes listed above (inflammatory response consisting of degranulation, diapedesis, and migration of hemocytes to the connective tissue between follicles, and increase in prevalences of parasites, ceroid, and blue, mucus-secretory cells), together suggest a non-specific response to a toxin. Similar changes have been described in bivalves after exposure to various xenobiotics, after sampling from polluted sites, or even after exposure to microbial pathogens. For example, Farley (1988) described diapedesis of hemocytes into the alimentary canal in oysters collected from a heavy-metal-contaminated site, and Mohandas et al. (1985) described degranulation of clam hemocytes after stimulation with *Bacillus megaterium*. Sunila (1984) reported migration of mussel hemocytes into the connective tissue between follicles after exposures to copper and cadmium. According to NAS (1980), parasite burden may increase in bivalves under pollution stress. George and Viarengo (1985) report the increase in ceroid (lipofuchsin) in mussels after a heavy-metal exposure, and Axiak et al. (1988) reported an increase in mucus cells in clam gills after exposure to petroleum hydrocarbons. In fact, the response found in the present experiment is quite similar to the detoxification pathway described by Viarengo (1985) for metal exposure.

The authors of the present study hypothesize that, after infiltration of *A. fundyense* toxins from the alimentary canal into tissues and hemolymph, lysosomes in the hemocytes were activated, and lipid peroxidation began (Valavanidis et al., 2006; Alves de Almeida et al., 2007). As a consequence, insoluble lipofuchsin granules were formed (ceroidosis). Finally, hemocytes migrated by diapedesis across the stomach and intestine epithelia, carrying the toxin, bound to lipofuchsin granules, to the alimentary canal for elimination in feces. This detoxification pathway has been described for mussels exposed to different stresses (Alves de Almeida et al., 2007). Moreover, Estrada et al. (2007) found an increase in lipid peroxidation when exposing lions-paw scallops, *Nodipecten subnodosus*, to the PSP-producing dinoflagellate *Gymnodinium catenatum*.

The mussels in the aquarium receiving *A. fundyense* that mass spawned experienced more pathological changes, in addition to those described above. Superimposed on the reproductive cycle is a nutrient-storage cycle involving the accumulation of nutrients in the mantle which are mobilized for gametogenesis (Gabbot, 1976). After spawning, the physiological status of mussels can be affected, i.e. there can be a loss of body weight and immuno-depression, arising from the lowered energy status (Hendriks et al., 2003; Delaporte et al., 2006). It appears, thus, that spawned mussels exposed to *A. fundyense* lost the ability to maintain digestive-gland homeostasis (Fig. 5). The red granules observed in the digestive glands probably were remains of ova that the mussels had ingested. As the exposure continued, degraded digestive glands were observed in these mussels. Nevertheless, attributing causes of these pathological changes to *A. fundyense*, spawning, or the combination is not possible as no mussels exposed to *Rhodomonas* sp. spawned to provide a comparison. Immediately after the spawning events, mussels had paralyzed adductor muscles and were unable to close the valves. This observation

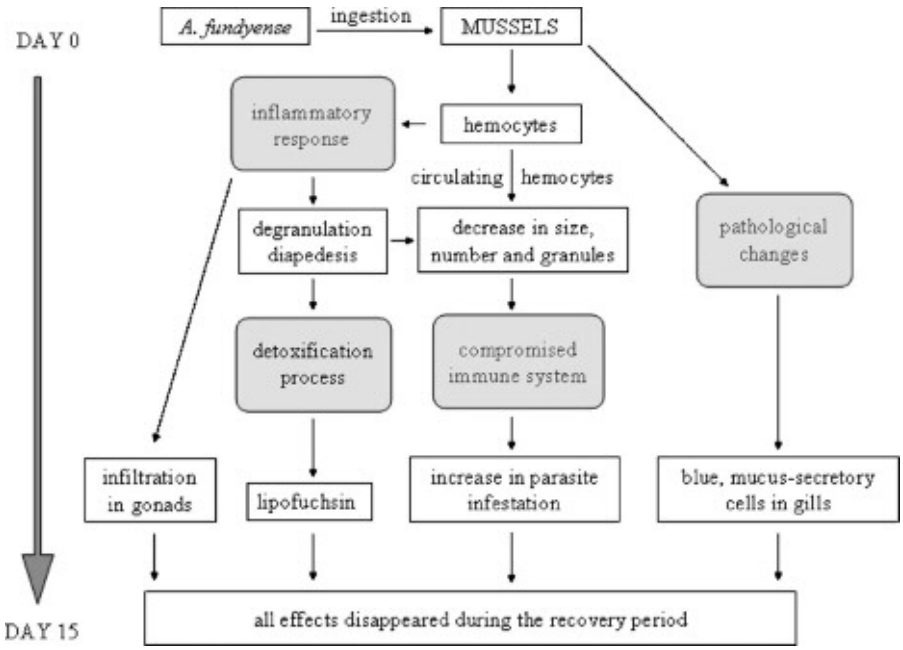

Fig. 8. A diagram summarizing mussel responses to *Alexandrium fundyens*e exposure.

confirms the findings of Hégaret et al. (2007a), who reported paralysis in oysters exposed to the same toxic-algal species. PSP toxins have an effect on the sodium-channel function in neurons (Bricelj et al., 2005), thus paralyzing the adductor muscle. After the mass spawning, combined with the toxic exposure, mussels apparently were physiologically less capable to resist the toxin effects because of a low energy status (Bricelj et al., 1993). After 9 days of exposure, the higher apoptosis values found in the hemocytes from mussels in the *A. fundyense*-treated aquarium in which spawning occurred could be related to the lowered energy status as well, but this cannot be confirmed from the present results.

Nearly all effects described above disappeared within a few days when *A. fundyense* was removed and all mussels fed on *Rhodomonas* sp. As an exception, abnormal gonads were found from day 7 until the end of the experiment, including the recovery period. The gametocytes lost structure and were partially reabsorbed. Remaining gametocytes (gamètes de réserves, Lubet, 1957), however, were found until the last sampling. Although PSP toxins concentrate in the digestive gland more so than in other tissues (Bricelj et al., 1990; Bricelj and Shumway, 1998), elimination of toxins is much faster in this organ (Kwong et al., 2006), as function of the digestive gland is vital for the survival of the bivalve. Thus, toxins may persist longer in other tissues, i.e., gonadal follicles, and the detoxification period in the present experiment was not long enough for full recovery.

Mussels accumulate PSP toxins much faster and reach higher concentrations than other bivalves (Shumway et al., 1990). This observation, along with the absence of recorded *A. fundyense* blooms at the site where mussels used in this study were collected, may have rendered the mussels especially susceptible to this HAB exposure. The effects of the toxic alga *A. fundyense* in this experiment were shown to be dependent upon the physiological status of mussels, i.e., effects were more severe and pronounced in spawned mussels. Nevertheless, when ingesting non-toxic algae after the *A. fundyense* exposure, mussels showed a high capacity for recovery, losing nearly all effects almost immediately.

5. Conclusion

Despite the low toxicity of the *A. fundyense* strain used in this experiment, mussels were affected by exposure to this strain, as summarized in Fig. 8. Mussels ingested and partially digested toxic *A. fundyense*, but damage to the digestive system and pathological changes occurred. The biochemical process of lipid peroxidation was used as the detoxification pathway. Recovery from exposure to this toxic alga was rapid, although spawned mussels were not able to completely recover, directing energy to repairing the digestive gland but not the gonads.

Several authors have reported immune and pathological changes when bivalves are exposed to HABs. It has been demonstrated that mussels are affected by PSP toxins, but our findings contribute to a new view of the effects and detoxification pathways. This work demonstrates the effects of the toxic alga *A. fundyense* on mussels *M. edulis* and the importance of the physiological status of the mussels in the degree of injury and recovery.

Acknowledgements

This study has been supported by a grant from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) to the first author and partially financed by the RTA04-023 INIA research project. The following Milford Laboratory staff provided much-appreciated technical assistance: Jennifer

H. Alix, April N. Croxton, Mark S. Dixon, Diane Kapareiko, Yaqin Li, Shannon L. Meseck, and Barry C. Smith.

We want to thank John Karolus and Joseph DeCrescenzo from the Bureau of Aquaculture, Milford (USA) for the mouse bioassays and Pablo de la Iglesia and Jorge Diogene from the IRTA, St. Carles de la Ràpita (Spain) for the HPLC toxin quantification.[SS]

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Errata 2

Y axis of figure 3 is "Nb affected mussels"

DISCUSSION

DISCUSSION

1. The ecological role of mussels

The ecological role of mussels in the marine ecosystem is summarized in figure 10. Mussels feed on the suspended particulate matter of the seawater, mostly phytoplankton. Mussels, therefore, can cause changes in the phytoplankton community through differential grazing and preferential selection of some species, rejecting others in pseudofeces. Bivalve feeding, thus, may be an important mechanism controlling phytoplankton dynamics in estuarine or shallow ecosystems, by altering both quantity of cells and relative abundances of the phytoplankton species (Cloern, 1982; Asmus and Asmus, 1991; Prins et al., 1998; Norén et al., 1999). Concurrently, bivalve populations are controlled by predators, which may ultimately be responsible for variability in primary production (Thompson et al., 2008).

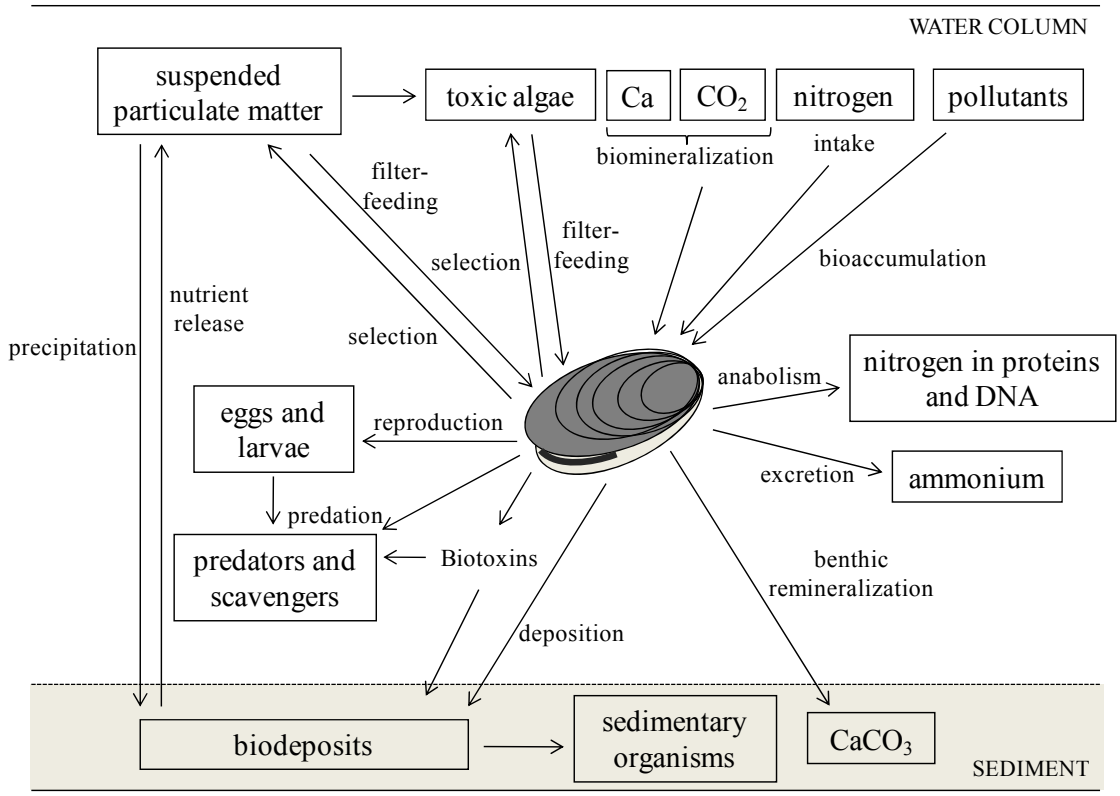

Fig. 10: Synthesis of the ecological role of mussels in the ecosystems.

Within the particulate matter suspended in the water, some phytoplankton species naturally occurring in the ecosystems can be toxic. Mussels can ingest toxic algae and accumulate microalgal biotoxins in tissues, subsequently transferring toxins to higher trophic levels (predators and scavengers) (Shumway, 1990; Landsberg, 2002). Microalgal biotoxins can

also be deposited within cysts in the feces of grazing bivalves from the suspended matter to the benthos (Harper et al., 2002; Hégaret et al., 2008). Microalgal biotoxins can also affect physiological processes of mussels, such as reducing filtration activity (Widdows et al., 1979b; Lesser and Shumway, 1993; Matsuyama et al., 1997).

The role of mussels in the cycling of nutrients in the ecosystems is well known. Mussels can assimilate a number of compounds from the water column, such as calcium and nitrogen, and transfer them to different trophic levels, both pelagic and benthic, by means of different processes, i.e.: anabolism, excretion, benthic mineralization, and biodeposition (Fig. 10). In areas with mussel farming, where bivalves can become the dominant grazer in the ecosystem, the amount of biodeposition can play an important role in the cycling of nutrients. Biodeposits can accumulate and enrich the sediments, but biodeposits can also be resuspended, releasing nutrients back into the water column (Prins and Smaal, 1994; Prins et al., 1998; Newell, 2004). Mussels, therefore, can also increase phytoplankton turnover through nutrient regeneration (Asmus and Asmus, 1991; Prins et al, 1998; Smaal et al, 2001).

As a consequence of the ecological and economic roles of bivalves, and despite the large amount of research done in such species, there is still a need to investigate *in situ* bivalve-plankton interactions within the environment as well as the effects of harmful algal blooms, which are becoming more frequent and widespread.

2. Validation of methods

The problem statement of the thesis, "to study the feeding behavior of mussels, in terms of selection and physiological components of absorptive balance, and to find out whether ingestion of toxic algae species modulates the immune function and causes pathological changes", was addressed by two different experimental setups and by studying two different *Mytilus* species. The feeding-behavior experiments were performed *in situ* on a mussel raft under natural conditions (Chapter I, II and III), and mussels were experimentally exposed to toxic algae in aquaria under controlled laboratory conditions (Chapter IV, V and VI). The mussel species studied in the present thesis represented model species at each experimental site in different areas of their geographical distribution (Gosling 2003); that is, *Mytilus galloprovincialis* was used for the Mediterranean studies in Alfacs Bay (Spain) (Chapter I, II and III); whereas, *M. edulis* was used for the studies performed in two different Atlantic estuaries; Plymouth Sound (UK) on the east side of the Atlantic Ocean (Chapter IV), and Long Island Sound (USA) on the west side of the Atlantic (Chapter V and VI). When the aim of a study, as in Chapters I, II and III of the present thesis, was to approximate real values for

feeding rates in bivalves in the natural environment, field setups using natural seston are recommended as optimal experimental designs (Velasco and Navarro, 2005). Nevertheless, the impossibility to control all environmental variables in the field known to affect the physiological status of bivalves (Walne, 1972; Bernard, 1983; Denis et al., 1999) can lead to the misinterpretation of the results when testing the effects of a single variable upon bivalves, i.e., immune and pathological changes caused by the ingestion of toxic algae. In the latter case, controlled laboratory experiments were determined to be a better approach. Bignell et al. (2008) recommended the collection of health parameters when sampling mussels in the field to dissociate markers of underlying health or condition from those associated with exposures to contaminants. In this thesis, it would have been ideal to compare the responses in mussels studied in the laboratory with those in the field during a natural HAB event; however, no such opportunity arose during this study.

2.1 The *in situ* feeding experiments

The characteristics of seston in the Mediterranean field site, Alfacs Bay, were evaluated in terms of quantity and species composition in relation to mussel feeding behavior (Chapters I and II). Alfacs Bay was shown to be a suitable site for mussel farming because the water quality (*f*), defined as POM/TPM, was high and constant throughout the study period (Chapter I). Table 3 shows seston characteristics from different mussel aquaculture sites in Spain, France, The Netherlands, and Australia. Maximum TPM values were recorded in Brisbane Water (Australia), where the inorganic content of seston was also high. Nevertheless, the water quality in this location might be low as the fraction POM/TPM would result in lower values than in Alfacs Bay, and the inorganic fraction of the seston is the highest of the reported sites in the table. The maximum value for water quality (*f*) was recorded in Thau Lagoon (France). This site also had very low values of *f*, offering a wide variability in water quality attributable to sediment-resuspension phenomena.

Seston characteristics are a key component when studying the feeding physiology of bivalves. As an example, filtration activity and absorption efficiency of mussels depend, among others, upon TPM and organic content of ingested particles, respectively, as observed in several field studies (Widdows et al., 1979a; Navarro et al., 1991; Hawkins et al., 1996; Babarro et al., 2000). In Alfacs Bay, an estuary where short-term variations in seston quantity were quite large, mussels regulated filtration activity in response (Chapter I). As a consequence, the amount of biodeposits released into Alfacs Bay should be lower than in areas where bivalves increase pseudofeces production in response to increased seston quantity, i.e. Marennes-Oléron (Urrutia et al., 1996). The use of one or the other regulatory mechanism, either clearance rate reduction or increase in pseudofeces production, as a response to variability in seston concentration

(Foster-Smith, 1975), may be important in relation to the role of mussels in the ecosystems, especially at sites with high bivalve population densities, i.e. mussel-farming areas.

Location	TPM (mg L^{-1})	POM (mg L^{-1})	PIM (mg L^{-1})	*f*	Bibliography
Alfacs Bay (Spain)	2.30 - 1.02	1.36 – 0.67	1.11 – 0.35	0.72 – 0.51	Present thesis (Chapter I)
Ría Arousa (Spain)	2.56 - 0.49	1.00 – 0.28	1.56 – 0.20	0.60 – 0.29	Babarro et al. (2003)
Ría Arousa (Spain)	2.71 – 0.74	1.08 – 0.35		0.54 – 0.35	Navarro et al. (1991)
Thau Lagoon (France)		1.7 – 0.1		0.88 – 0.12	Gangnery et al. (2004)
Marennes-Oléron (France)				0.28 – 0.08	Hawkins et al. (1996)
Marennes-Oléron (France)				0.33 – 0.03	Grant and Bacher (1998)
Oosterschelde (Netherlands)				0.20 – 0.15	Scholten and Smaal (1998)
Brisbane Water (Australia)	6.95 – 3.54	1.87 – 0.97	5.08 – 2.57		Paterson et al. (2003)

Table 3: Seston characteristics from different mussel aquaculture sites, indicated as TPM: total particulate matter; POM: particulate organic matter; PIM: particulate inorganic matter; *f*: organic fraction of seston expressed as POM/TPM for each location. The values correspond to maximum and minimum values reported.

Bivalves feed on the seston particles suspended in the water column. Grazing pressure, however, varies between available particles, with some selected but others not. The objective of preferential particle selection by bivalves is to improve the quality of ingested material. Chapter II of the present study focused on the selection of particles from natural seston from an ecological point of view, concluding that the pre-ingestive selection of phytoplankton by mussels in Alfacs Bay varied according to the composition and the relative abundance of different phytoplankton species within the available seston, as observed in other studies (Kiørboe and Møhlenberg 1981; Bougrier et al., 1997). Results in Chapter II also showed that mussels preferentially ingested dinoflagellates, in agreement with previous studies (Shumway et al, 1985; Sidari et al., 1998), even when these taxa were not dominant in the ecosystem. Rouillon et al. (2005) also found an increase in dinoflagellates within the stomachs of mussels compared to the concentration in the seawater and offered two possible explanations: 1) mussels

could not digest the dinoflagellates, or 2) mussels preferentially ingested such taxa of microalgae. Results in Chapter II of the present study support the conclusion that mussels preferentially ingested dinoflagellates among other taxa of phytoplankton, because of high dinoflagellate abundances in the stomachs but low abundances in the pseudofeces. In contrast, most diatoms, which were dominant in the seston, were rejected as pseudofeces. As a complement to Chapter II, table 4 shows the list of the forty seven phytoplankton species identified in Alfacs Bay water during the feeding experiments. Apart from phytoplankton, other types of particles were also found in the stomach contents of mussels, including some not reported in Chapter II because of their relative low abundances, i.e., cocolitophores, nanoflagellates and fungi spores. Zooplankton was also found in the different types of mussel samples, that is, stomach contents, feces and pseudofeces. Nevertheless, the water sampling was not appropriate for evaluating the relative abundances of such taxa and to study pre-ingestive selection of these particles was not a goal of the study. The summary conclusion, therefore, is that mussels ingested different types of zooplankton (tintinnids, rotifers, copepods, and bivalve larvae) as reported in other studies (Lehane and Davenport, 2002; 2006), but selection indexes could not be calculated in Chapter II.

The trophic capacity of an ecosystem depends on the food sources (phytoplankton and partulate matter), the water renewal rate, the ecophisiology of the cultured specie and the culture type and strategy (Carver and Mallet, 1990). Several mathematical models have been developed to estimate the carrying-capacity of the ecosystems, based on the factors in which it depends. Thereafter, the studies herein performed (Chapters I and II) may be useful, among with other data, to estimate the carrying capacity of Alfacs Bay.

As summarized in Fig. 10, pollutants are also suspended in the water column with different types of origins, chemistries and properties. The fiberglass found in Alfacs Bay water (Chapter III) should be considered a new contaminant in marine ecosystems because fiberglass is recognized as an environmental pollutant and inhalation hazard when present as dust in the air (IARC, 2002). Because fiberglass was also found in the stomach contents of mussels, there was evidence of the transfer of such particles in the marine food web. Some studies have reported serious consequences, such as stomach cancer or peritonitis, when humans and a dog ingested fiberglass (Maresca et al., 1984; Hardie et al., 1994; Kærheim et al., 2005), but nothing is known about possible effects on marine food-webs. Santschi et al. (1982) reported that benthic suspension feeders can accelerate the flux of particles and trace metals from the water column to the sediment. The present finding of fiberglass in the biodeposits of mussels indicates that these bivalves can be vectors transporting fiberglass from the pelagic to the benthic environment, making the noxious particles available to the deposit-feeding organisms.

2.2 The HAB experiments

The increasing frequency and persistence of HABs in the natural environment and the accumulation of biotoxins in mussels lends importance to investigation into possible effects of HAB taxa and associated toxins upon bivalves. Mussels had been reported to be highly resistant

Dinoflagellates	Diatoms	Others
	Species	
Alexandrium minutum	*Asteromphalus heptactis*	*Cryptomonas* spp.
Alexandrium spp.	*Bacillaria paxillifer*	*Dictyocha fibula*
Ceratium furca	*Bacteriastrum delicatulum*	*Dictyocha octonaria*
Dinophysis caudata	*Bellerochea malleus*	*Hermesinum adriaticum*
Dinophysis rotundata	*Cerataulina pelagica*	
Dinophysis sacculus	*Chaetoceros* spp.	
Gonyaulax verior	*Coscinodiscus* spp.	
Gonyalaux spp.	*Cyclotella meneghiniana*	
Gymnodinium spp.	*Cylindrotheca closterium*	
Heterocapsa niei	*Grammatophora marina*	
Peridinium quinquecorne	*Guinardia flaccida*	
Prorocentrum micans	*Guinardia striata*	
Prorocentrum minimum	*Leptocylindrus minimus*	
Prorocentrum triestinum	*Lioloma pacificum*	
Protoceratium reticulatum	*Paralia sulcata*	
Protoperidinium diabolus	*Pleurosigma* spp.	
Protoperidinium spp.	*Proboscia alata*	
Pyrophacus horologium	*Pseudo-nitzschia* spp.	
Scrippsiella trochoidea	*Rhizosolenia* spp.	
Scrippsiella spp.	*Striatella unipunctata*	
	Synedra spp.	
	Thalassionema nitzschioides	
	Thalassiosira spp.	

Table 4: Species of phytoplankton found in natural seawater of Alfacs Bay in November 2006 and February, April and July 2007 (M. Delgado personal communication, ICM-CSIC, 2009).

to phytoplankton toxins, which led to speculation that mussels, unless exposed to high concentrations of HABs, were only vectors passing the toxins to higher trophic levels (Twarog et al., 1972; Bricelj et al., 1990; Marsden and Shumway, 1991). Reports of histopathological changes in bivalves after HAB exposure are scarce (Wikfors and Smolowitz, 1993, 1995), but several have demonstrated pathological changes in the digestive gland associated with HAB

ingestion. Nevertheless, Smolowitz and Shumway (1997) could not relate histopathological changes in the digestive tubules of mussels with a HAB exposure.

	Karlodinium veneficum (Chapter IV)	*Prorocentrum minimum* (Chapter V)	*Alexandrium fundyense* (Chapter VI)
Hemocytes			
Internal complexity	non-studied	decreased	decreased
Size	non-studied	maintained	decreased
Total number	maintained	maintained	decreased
Immune assays			
Reactive oxygen species	increased	maintained	maintained
Phagocytosis	increased	maintained	maintained
Histopathology			
Mantle tissue	maintained	hemocytes in connective tissue between gonadal follicles	hemocytes in connective tissue between gonadal follicles
Digestive gland	hemocytes infiltration between digestive tubules	- diapedesis - bacteria in stomach and intestine	diapedesis

Table 5 shows the main immunological and pathological changes observed in mussels after exposure to the HAB cultures: *Karlodinium veneficum*, *Prorocentrum minimum* and *Alexandrium fundyense*.

Chapters IV, V and VI of this thesis, therefore, investigated the effects of three HAB species: *Karlodinium veneficum*, *Prorocentrum minimum,* and *Alexandrium fundyense*, upon mussels under laboratory conditions. The approach was holistic; the behavior of mussels during the experiments, feces and pseudofeces production, stomach contents, immunological parameters, and histopathology were examined. As a consequence of the comprehensive approach, mussels were demonstrated to be immunologically and histopathologically affected by HAB exposures, despite low levels of toxicity. As a common response in all tested HAB exposures (Chapters IV, V and VI), the HAB first affected the mussel hemocytes and digestive gland. It was reported previously that algal toxins mainly accumulate in the digestive gland of bivalves (Yasumoto et al., 1978, Murata et al., 1982; Bricelj et al., 1990); therefore, this tissue was expected to be affected by the toxins. The digestive glands of mussels showed infiltration of hemocytes between or within the digestive tubules. Infiltration of hemocytes into the

connective tissue between gonadal follicles was not observed in mussels exposed to *K. veneficum* (Chapter IV), possibly because of the low algal concentration and the short exposure time.

Oysters and soft-shell clams have been reported to show paralysis of the abductor mussel after exposure to *Alexandrium* spp. (Connell et al., 2007; Hégaret et al., 2007b). In Chapter VI, mussels showing muscle paralyses were only those that had spawned, suggesting that the physiological status of mussels is very important for the degree of injury and recovery after exposure to toxic algae. If the concentration of the paralytic-shellfish toxins had been higher in the *A. fundyense* culture used, the same effect might have been observed in all mussels.

The global response and total immunological and pathological effects of the HAB taxa in mussels varied for each of the three different species. The hemocytes of mussels exposed to *K. veneficum* increased reactive oxygen species and phagocytosis; whereas, hemocytes in mussels exposed to the other two HAB species showed no significant differences from controls. Hégaret and Wikfors (2005) found an increase in phagocytosis when oysters (*Crassostrea virginica*) were exposed to *Prorocentrum minimum*, similar to what was found in Chapter IV for *K. veneficum* in mussels, but in contrast to what found in Chapter V for *P. minimum*. When ingesting *P. minimum* the response of mussels was similar to that observed in clams with a *Perkinsus*-infection (Chapter V); whereas, ingestion of *A. fundyense* induced the biochemical process of lipid peroxidation as a detoxification pathway (Chapter VI). The results of Chapters IV, V and VI, therefore, and comparisons with previous literature verify the hypothesis that the responses of bivalves to the different harmful algae seem to be species-specific (Shumway et al., 2003; Hégaret et al., 2007a).

3. Benefits of research

The research presented in this thesis offers potential practical applications for different stakeholders:

1. The feeding experiments performed *in situ* on a mussel raft (Chapters I and II) will benefit the scientific community and mussel producers to have a better understanding of the phytoplankton ingestion preferences of mussels and of the physiology involved in mussel feeding behavior in the field. The protocol of using devices designed to distribute sea water from the bay into individual aquaria containing mussels, and then measuring the physiological processes of feeding with the biodeposition method can be used as the first step in calculating the carrying-capacity of the ecosystems to maximize the aquaculture potential of sites. In detail, the feeding experiments in Alfacs Bay will help the Catalan Government to optimize the mussel

culture management of the area. The devices can be adjusted for other bivalve species, thus helping bivalve producers to diversify and increase their aquaculture production.

2. Studies of the immunomodulatory and pathological changes in mussels after an exposure to HAB species (Chapters IV, V and VI) increase basic knowledge about the responses of an organism previously considered to be simply an inert vector of toxins to higher trophic levels. Such studies, wherein a keystone species was used as the test organism, will help natural resource managers to estimate the effects of increasing HABs on marine ecosystems. This study also will help mussel industry representatives and regulators to estimate the potential impact of increasing HABs on mussel production.

3. The recovery study after an experimental exposure to *Alexandrium* spp. (Chapter VI) may benefit public health officials to determine shellfishing closure periods after a bloom.

4. The novel finding of fiberglass ingestion by mussels (Chapter III) indicates the need to investigate the presence of such material worldwide, possible direct ingestion by other marine organisms, and trophic transfer at different levels in the marine food web. Possible implications for public health, and the health of the organisms themselves, should be evaluated. As a precaution in the case of commercial bivalves, we advise the Water Quality Monitoring Programme to be aware of a new, potentially-harmful particles in shellfish-growing areas.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

The hypotheses formulated in the present thesis were tested by means of experiments, and the following conclusions were obtained:

1. Hypothesis I: The feeding behavior of mussels in Alfacs Bay (N.W. Mediterranean Sea) depends upon the quantity and quality of seston, which may vary in time.

Conclusions:

1.1 Alfacs Bay has a high water quality for bivalve culture, similar to known, productive global aquaculture estuaries, despite that the short-term variations (days) in seston quantity and quality were similar to the long-term variations (months), a characteristic of the area.

1.2 Filtration activity and absorption efficiency of mussels in Alfacs Bay are high throughout a mussel-culture cycle and depend on TPM and the organic content of the ingested particles, respectively.

1.3 Mussels in Alfacs Bay reduced their clearance rate when seston concentrations were high instead of increasing their pseudofeces production.

2. Hypothesis II: Mussels in Alfacs Bay (N.W. Mediterraean Sea) preferentially select some of the available suspended matter but not other.

Conclusions:

2.1. Mussels select and ingest dinoflagellate cells, even when diatoms are dominant in the ecosystem. The feeding behavior of mussels can cause changes in the phytoplankton community through differential grazing.

2.2 The pre-ingestive selection of phytoplankton by mussels in Alfacs Bay varied according to the composition and the relative abundance of different phytoplankton species within the available seston.

2.3. Mussels in Alfacs Bay ingested fiberglass, a toxic, anthropogenic compound, and released in the biodeposits. Moreover, we report the introduction of fiberglass into marine food webs.

3. Hypothesis III: Harmful Algal Blooms modulate the immune function of mussels and cause pathological changes.

Conclusions:

3.1. The ingestion of HAB cells causes pathological changes first in the mussel tissues and cells that are in immediate contact with the algae, that is, hemocytes and digestive gland.

3.2. The effects of the HAB taxa upon mussels are species-specific:

3.2.1. *Karlodinium veneficum*: mussels increased phagocytic capacity and reactive oxygen species of hemocytes, and hemocyte infiltration occurred in the digestive gland;

3.2.2. *Prorocentrum minimum*: mussels developed diapedesis, aggregation and encapsulation of hemocytes in the alimentary canal, and proliferation of bacteria in the stomach and intestine;

3.2.3. *Alexandrium fundyense*: mussels produced an inflammatory response with degranulation, diapedesis, and infiltration of hemocytes into gonads, with subsequent increase in opportunistic parasite infestations.

3.3. A holistic approach including, immune and histopathological techniques is necessary to detect effects of HAB species in bivalves.

RESUM DE LA TESI

(Summary in catalan)

RESUM DE LA TESI

1. INTRODUCCIÓ

1.1 La importància dels bivalves

Els bivalves són mol·luscs marins i d'aigua dolça que es distribueixen arreu del món. Tenen un paper ecològic molt important en els ecosistemes com per exemple la millora de la qualitat de l'aigua en ecosistemes eutròfics (Ostroumov, 2005; Coen et al., 2007; Lindahl i Kollberg, 2009), la capacitat de crear hàbitats heterogenis per peixos i invertebrats (Gutiérrez et al., 2003; Coen et al., 2007), la transferència d'elements químics i nutrients entre diversos nivells tròfics (Prins et al., 1998; Newell, 2004), i la contribució a l'estabilització d'hàbitats bentònics i intermareals (Coen et al., 2007; Dumbauld et al., 2009).

Alguns bivalves també tenen importància econòmica com a consum o en la indústria ornamental. De tots els bivalves cultivats arreu del món, les espècies de musclo *Mytilus galloprovincialis* i *M. edulis* tenen una producció superior a 300.000 tones anuals (FAO, 2009), la majoria cultivades a Espanya, productor líder d'Europa (Pérez Camacho et al., 1991; Keldany, 2002).

1.1.1 Els musclos com a espècies clau

Degut a la seva capacitat de filtració, els musclos poden controlar el creixement de les poblacions de fitoplàncton en el què es coneix com a processos "top-down", evitant així una eutrofització de l'ecosistema. Però alhora poden promoure la producció primària mitjançant l'alliberament de nutrients en els biodipòsits, establint un balanç entre la predació de fitoplàncton i l'alliberament de nutrients (Asmus i Asmus, 1991). Aquest fenomen és d'especial importància en ecosistemes amb aigua poc profunda, on la proporció de superfície bentònica respecte el volum d'aigua és elevat (Dame, 1996). La relació que s'estableix entre els dos nivells tròfics es coneix com a acoblament bentopelàgic (Verwey, 1952; Doering et al., 1986; Dame et al., 1989). Així doncs, els musclos es consideren espècies clau dels ecosistemes pel seu important paper en el manteniment de la qualitat de l'aigua, i la seva participació en la circulació de nutrients de la columna d'aigua cap al bentos (Prins i Smaal, 1994; Ostroumov, 2005).

1.1.2 Els musclos com espècie sentinella i programes de qualitat

Com a conseqüència de l'acumulació de contaminants antropogènics en bivalves, Goldberg (1975) va proposar el programa de seguiment anomenat "Mussel Watch" per assessorar la contaminació química de la costa i estuaris de Nord Amèrica, usant els musclos com espècies sentinella. Aquest tipus de programes es van establir progressivament en molts

altres països arreu del món per protegir la salut pública internacional (Gosling, 1992; International Musselwatch Committe, 1992).

El consum de bivalves pot tenir efectes nocius pels humans ja que aquests poden actuar com a vectors de malalties relacionades amb l'acumulació de bacteris, diversos tipus de toxines, etc. Per protegir la salut pública, també es van desenvolupar programes d'estudi i seguiment de la qualitat de l'aigua de les zones de cultiu arreu del món (Gosling, 1992).

1.2 Coneixements bàsics del gènere *Mytilus*

1.2.1 Anatomia

Els musclos del gènere *Mytilus* són mol·luscs bivalves sèssils que habiten aigua tant salada com salobre. La seva anatomia interna bàsica es mostra en la figura 1. Els musclos tenen dues valves on s'hi insereixen les visceres mitjançant els músculs adductors, el marge del mantell, i petits punts de fixació entre el mantell i la closca (Bayne et al., 1979). El mantell, que consisteix en dos lòbuls de teixit que engloba l'organisme dintre de la closca, conté la gònada i és el lloc principal on s'emmagatzemen reserves nutritives. Les brànquies són estructures filiformes suspeses del marge dorsal del mantell amb dues funcions diferents, respiració i alimentació (Bayne et al., 1976; Gosling, 1992). El peu és una estructura llarga i musculosa que s'usa per localitzar un substrat on adherir-se a través del bissus, secretat per la glàndula del bissus. El canal alimentari consisteix en boca, esòfag, estómac, glàndula digestiva, intestí, i anus. Finalment, el cor distribueix l'hemolimfa per tot el cos del musclo mitjançant un sistema circulatori obert (Gosling, 2003).

1.2.2 Distribució geogràfica

Els musclos del gènere *Mytilus* habiten en tots els mars temperats distribuint-se des del intermareal al sublitoral. La classificació de les diverses espècies del gènere *Mytilus*, prèviament a l'ús de l'electroforesi, es basava en morfologia de la closca, altament variable amb diversos factors ambientals. Actualment, tècniques mol·leculars ens permeten conèixer la seva distribució arreu del món (Gosling, 1992). La figura 2 mostra un mapa de distribució mundial de quatre espècies del gènere *Mytilus*. Al llarg de la costa Atlàntica Europea, l'espècie *M. galloprovincialis* habita les zones més càlides però existeix una àrea de solapament amb l'espècie *M. edulis*, on les dues espècies s'hibriden de forma natural (Beaumont et al., 2004).

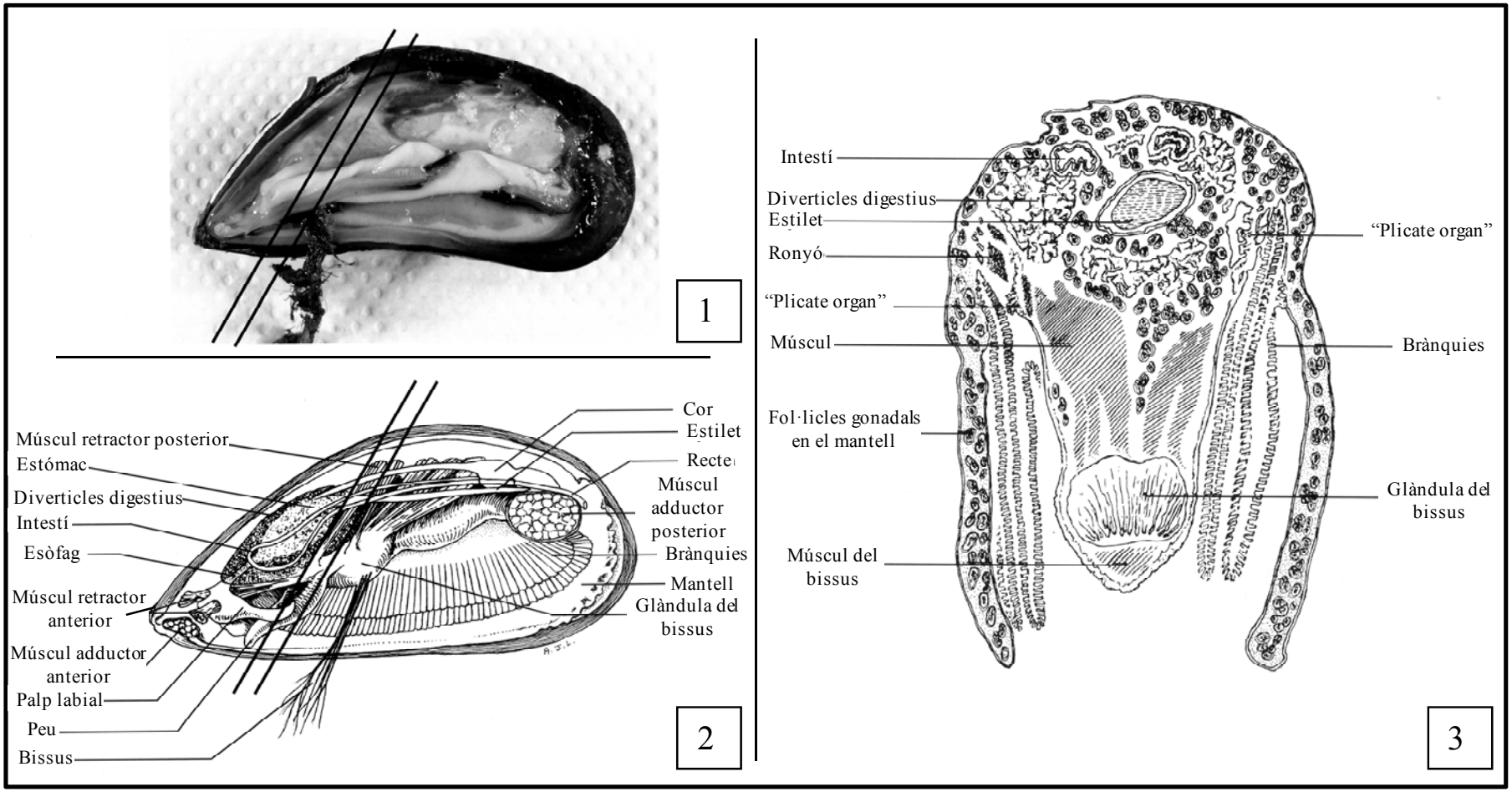

Fig. 1: Anatomia bàsica de *Mytilus edulis*. 1.1. Fotografia de les parts toves. 1.2. Il·lustració de l'anatomia. 1.3. Il·lustració d'una secció transversal; les línies paral·leles de la fig.. 1.1 i 1.2 mostren la localització de la secció transversal. De Howard et al. (2004), il·lustracions de A. J. Lippson, Bozman, M.D.

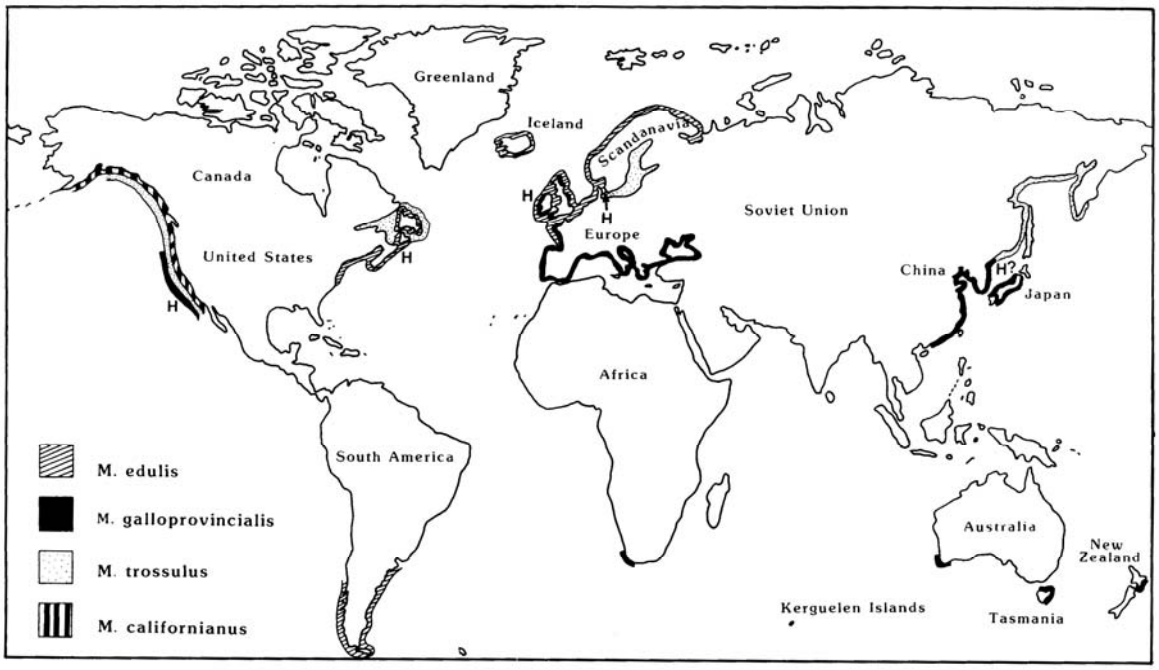

Fig. 2: Distribució mundial de quatre espècies del gènere *Mytilus*. H significa àrees d'hibridació. Obtingut de Gosling (1992).

La distribució i abundància dels bivalves es veuen afectades pels següents factors físics i biològics:

1. Factors físics: els bivalves són organismes poikiloterms (Dame, 1996), per això, la temperatura es considera el factor físic més important que regula la distribució dels musclos. Les diverses espècies del gènere *Mytilus* toleren diferents rangs de temperatura. *M. galloprovincialis* no pot sobreviure a temperatures de l'aigua de 26 ºC o superiors durant llargs períodes de temps (Anestis et al., 2007) i habita aigües més temperades que *M. edulis* (Beaumont et al., 2004). La temperatura de l'aigua també afecta l'estat fisiològic dels bivalves influint, entre d'altres, en el seu comportament alimentari, creixement, i període de posta (Denis et al., 1999; Suárez et al., 2005). La salinitat és un altre factor físic limitant important en bivalves de zones costaneres o estuaris. En el cas de *Mytilus* spp., però, com que són espècies eurihalines i s'adapten a un ampli ventall de salinitats, aquest factor no és determinant (Bayne et al., 1976). Altres factors són també considerats limitants en la distribució dels bivalves com la fondària, marees, corrents, tipus de substrat, etc (Dame, 1996; Gosling, 2003).

2. Factors biològics: l'aliment, els predadors, les condicions patològiques, i els paràsits són probablement els factors biològics limitants més importants en les poblacions de bivalves (Seed i Suchanek, 1992). En el cas dels musclos, les closques esdevenen un substrat excel·lent per l'assentament d'organismes bentònics, que poden causar competència per l'aliment, i mortalitat degut al despreniment del musclo per sobrepès o impedir que obrin les valves (Perera et al., 1990; Hickman, 1992).

1.3 Alimentació

1.3.1 Selecció de partícules

Els musclos són bivalves sèssils filtradors que depenen de l'aigua on viuen pel subministrament del seu aliment (Gosling, 2003). L'aigua entra en el musclo pel sifó inhalant; els cilis laterals de les brànquies mantenen un flux d'aigua que permet la seva filtració (Fig. 3). Els cilis retenen les partícules més grans i les transporten cap als palps labials, situats a ambdos costats de la boca; les partícules petites es poden retenir o escapar. L'aigua filtrada s'allibera al medi pel sifó exhalant (Riisgård et al., 1996; Gosling, 2003; Ward i Shumway, 2004). Les partícules retingudes arriben finalment a l'estómac, on ocorre la digestió extracel·lular. A continuació, en la glàndula digestiva, es dóna la digestió intracel·lular. El material no absorbit s'elimina per les femtes (Gosling, 2003).

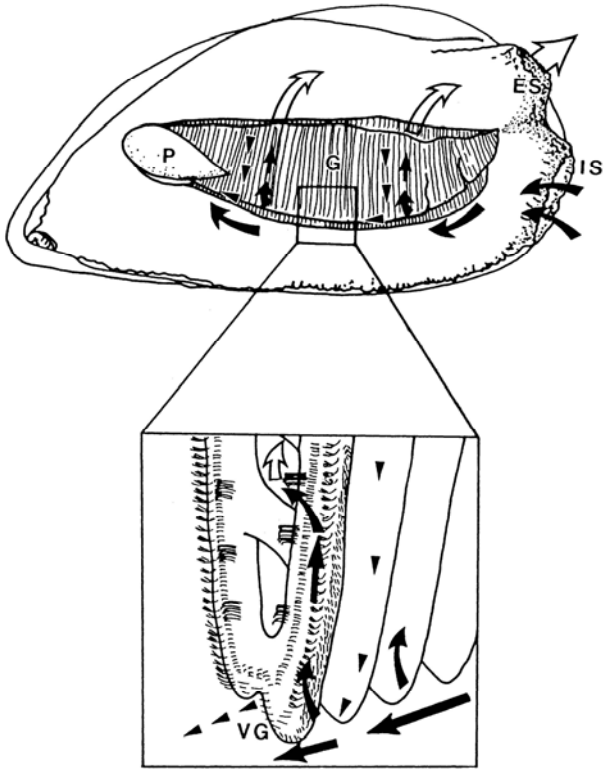

Fig. 3: Principals vies de corrent i transport de partícules en *Mytilus edulis*. ES: sifó exhalant; IS: sifó inhalant; G: brànquia; P: palp labial; VG: solc ventral branquial. Les fletxes negres indiquen la corrent d'aigua abans de travessar les brànquies; les blanques indiquen la direcció de l'aigua cap al sifó exhalant. Els caps de fletxa indiquen el transport de partícules cap al solc ventral de les brànquies. De Beninger i St.-Jean (1997a).

Les brànquies retenen partícules d'un rang de talles de 1 a 6000 μm (Lehane i Davenport, 2006) amb diverses eficiències d'absorció. Però no tot el que es retè a les brànquies és ingerit. La selecció pre-ingestiva succeeix en les brànquies i els palps labials i el material és

rebutjat en forma de pseudofemtes (Kiørboe i Møhlenberg, 1981; Shumway et al., 1985; Ward et al., 1998; Zemlys et al., 2003) (Fig. 4). La selecció post-ingestiva es dóna a l'estómac, que pot retenir les partícules per incrementar el temps de digestió o dirigir-les a la glàndula digestiva (Bricelj et al., 1984; Shumway et al., 1985; Brillant and MacDonald, 2003; Ward i Shumway, 2004). L'aliment que passa directament de l'estómac a l'intestí esdevé material poc digerit que s'elimina en forma de femtes anomenades intestinals. El material de rebuig de la glàndula digestiva retorna a l'estómac i les femtes resultants s'anomenen glandulars (Widdows et al., 1979a). La selecció de partícules per part dels bivalves és una estratègia per maximitzar la qualitat de la dieta i optimitzar-ne el guany energètic.

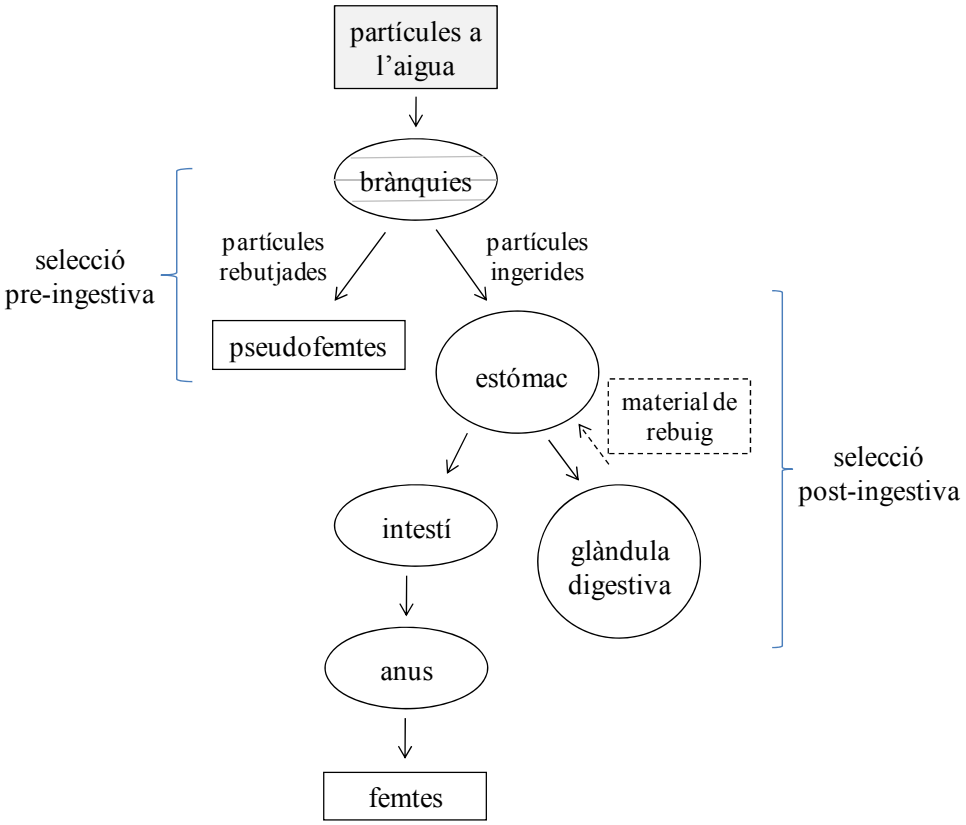

Fig. 4: Diagrama de la selecció de partícules pels musclos.

1.3.2 Proliferacions d'algues tòxiques (HAB)

De totes les espècies de fitoplàncton de les quals els musclos s'alimenten n'hi ha que es consideren tòxiques. Les proliferacions d'aquestes micro algues en els ecosistemes aquàtics, conegudes com "Harmful Algal Blooms" (HAB), es coneixen des de civilitzacions antigues (Fogg, 2002; Landsberg, 2002). Com que els musclos viuen i es cultiven en condicions naturals, la qualitat biològica de l'aigua esdevé de gran importància ja que aquests organismes són susceptibles d'ingerir les algues tòxiques quan són presents en el medi. Les respostes dels

bivalves a l'afectació de les diverses toxines sembla ser específica per cada associació alga-bivalve (Shumway et al., 2003; Hégaret et al., 2007a). En el cas del musclo es coneixen diversos efectes causats per algues tòxiques, resumits en la taula 1.

Efectes	Espècies de microalgues	Bibliografia
Minva de la taxa de filtració	*Alexandrium monilatum* *Alexandrium tamarense* *Gymnodinium aureolum* *Karenia brevis* *Prorocentrum lima*	Pate et al. (2005) Lesser i Shumway (1993) Widdows et al. (1979b) Leverone et al. (2007) Pillet i Houvenhagel (1995)
Fracàs reproductiu	*Aureococcus anophagefferens* *Chrysochromulina polylepis*	Bricelj i Kuenstner (1989) Granmo et al. (1988)
Inhibició de la producció de bissus	*Alexandrium tamarense* *Heterocapsa circularisquama*	Shumway et al. (1987) Matsuyama et al. (1998)
Supressió del creixement	*Aureococcus anophagefferens* *Chrysochromulina polylepis*	Bricelj et al. (2001) Nielsen i Strømgren (1991)
Tancament valvar	*Alexandrium tamarense* *Heterocapsa circularisquama*	Shumway i Cucci (1987) Matsuyama et al. (1998)
Mortalitat	*Aureococcus anophagefferens* *Gonyalaux* spp. *Gymnodinium aureolum* *Gyrodinium corsicum* *Rhizosolenia chunii*	Bricelj et al. (2001) O'Sullivan (1978) Cross i Southgate (1980) ICES (1999) Parry et al. (1989)

Taula 1: Efectes de diferents espècies de microalgues tòxiques en musclos (*Mytilus* spp.).

Les diverses toxines produïdes per les espècies de fitoplàncton es poden acumular en els bivalves i, en conseqüència, als seus depredadors, passant així fins als nivells mes alts de la cadena alimentaria (Azanza i Taylor, 2001; Landsberg, 2002). Per això, s'han desenvolupat programes de seguiment i maneig de les zones de cultiu de bivalves per protegir la salut pública (Shumway et al., 1990; Rehnstam-Holm i Hernroth, 2005). Les prohibicions de l'extracció de bivalves degudes a la proliferació d'algues tòxiques en el medi poden causar importants pèrdues econòmiques en la industria aqüícola (Shumway, 1990; EUROHAB, 1998).

1.4 El sistema immunològic

En els bivalves, les cèl·lules circulants de l'hemolimfa, els hemòcits, constitueixen el medi principal de la seva defensa immunitària. Aquestes cèl·lules ubiqües es troben en el sistema circulatori dels bivalves incloent el cor, els vasos de l'hemolimfa, i els sinus de diverses mides localitzats en la majoria d'òrgans (Auffret, 2005). Els hemòcits es divideixen

principalment en dos grups: basofílics i eosinofílics, també classificats com a hemòcits agranulars i granulars, respectivament (Fig. 5).

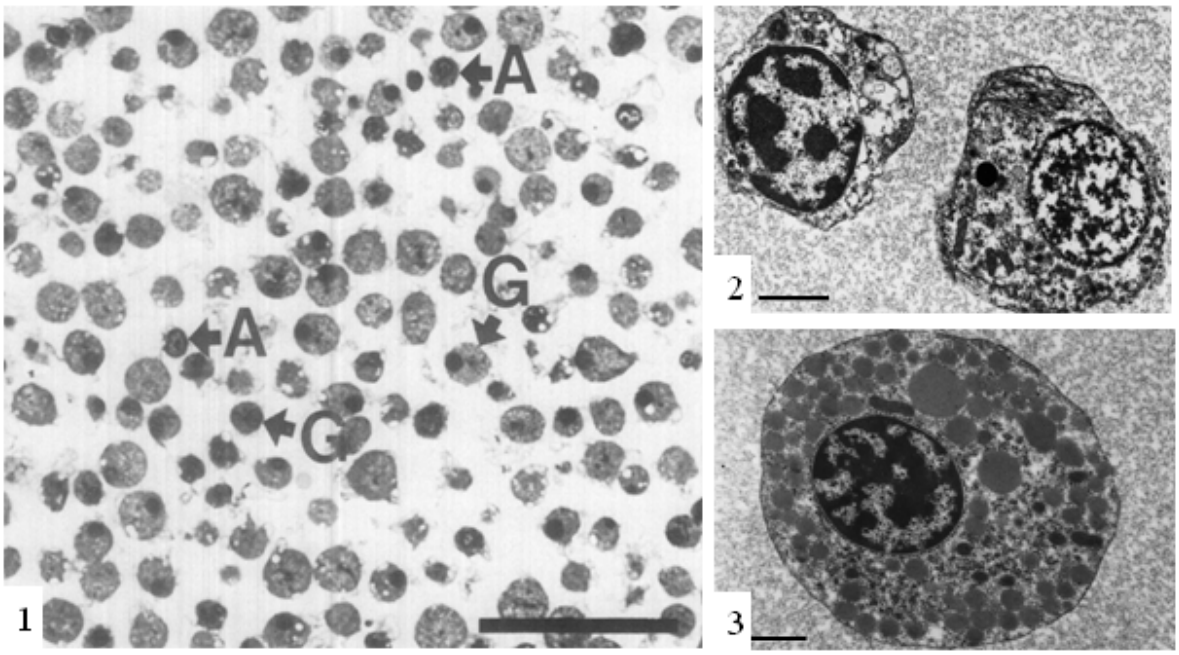

Fig. 5: 1. Imatge de microscòpia òptica d'hemòcits agranulars (A) i granulars (G) de *Mytilus edulis*. Barra d'escala = 20 μm. De Rasmussen et al. (1985). 2 i 3. Imatges de microscòpia òptica d' hemòcits basofílics i eosinofílics respectivaments de *M. edulis*. Barra d'escala = 1 μm. De Pipe et al. (1997).

Els hemòcits basofílics es divideixen en cèl·lules petites (4-5 μm) i grans (7-9 μm) (Moore i Lowe, 1977; Bayne et al., 1979). Els hemòcits basofílics petits són generalment esfèrics amb un nucli també esfèric i un citoplasma hialí. Per altra banda, els basofílics grans tenen una aparença irregular amb gràsnuls i vacúols en el citoplasma de diversos diàmetres. Els hemòcits eosinofílics, o granulòcits, són sovint esfèrics amb gràsnuls també esfèrics (0.5-1.0 μm) que segons les seves propietats de tinció varien de neutròfils a acidòfils (Moore i Lowe, 1977). La taula 2 mostra un senzill esquema de la classificació dels hemòcits.

Tipus d'hemòcit	Basofílic (Agranular)		Eosinofílic (Granular)
	petit	gran	
Forma	esfèrica	irregular	esfèrica
Gràsnuls	no existents	diferent diàmetres	esfèrics (0.5-1.0 μm)

Taula 2: Classificació i característiques bàsiques dels diversos tipus d'hemòcits.

Quan els hemòcits reconeixen una partícula estranya o nociva, la poden fagocitar o alliberar espècies reactives de l'oxigen (Pipe, 1992; Dyrynda et al., 1998; Wootton et al., 2003a). Els gränuls dels diversos tipus d'hemòcits tenen un ampli ventall d'enzims hidrolítics actuant de lisosomes (Moore i Lowe, 1977; Pipe, 1990b; Carballal et al., 1997a). Tot i això, sembla que els hemòcits eosinofílics tenen l'habilitat de fagocitar mentre que els hemòcits basofílics són molt menys fagocítics o no ho són gens (Pipe, 1990b; Carballal et a., 1997b).

El sistema immune dels invertebrats es considera no-específic (o innat), és a dir, que és d'ampli espectre, i reconeix i respon a partícules nocives i a l'ambient d'una manera genèrica, proveint una defensa immediata. Tot i que aquest tipus de sistema immune no confereix immunitat a llarg temps, els bivalves tenen estratègies adaptatives davant els ecosistemes menys favorables (Dyrynda et al., 1998; Wootton et al., 2003b; Bricelj et al., 2005).

1.5 El cultiu de musclo al Delta de l'Ebre

El cultiu de musclo (*Mytilus galloprovincialis*) en la costa Mediterrànea es desenvolupa actualment a Catalunya, València, Menorca i Andalusia (Ramón et al., 2005) (Fig. 8).

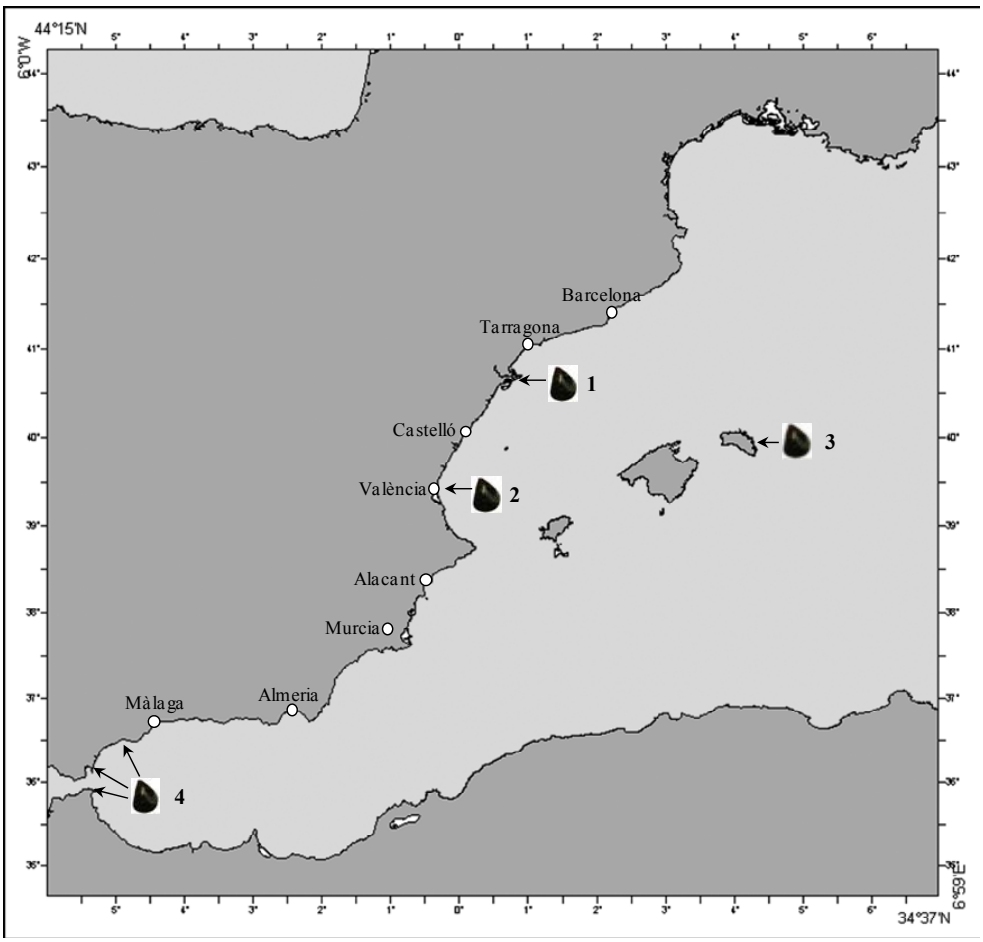

Fig. 6: Mapa de la costa espanyola Mediterrània. Les fletxes i imatges de musclo mostren la localització dels cultius: 1. Delta de l'Ebre; 2. València; 3. Menorca; 4. Andalusia.

Al Delta de l'Ebre, on se centra la producció de musclo de Catalunya, el cultiu es va iniciar als anys '60 i actualment hi ha 166 muscleres repartides entre les dues badies. Cada musclera és una estructura rectangular de fusta (200 m x 15 m) d'on hi pengen cordes d'uns 2-3 m de llargada. El cicle de cultiu s'inicia entre desembre i gener usant cordes col·lectores per l'obtenció natural de llavor. Quan aquesta assoleix els 3 cm de llongitud, cap a l'octubre, s'encorda a les cordes d'engreix, on hi roman fins maig-juny, assolint uns 6 cm de longitud.

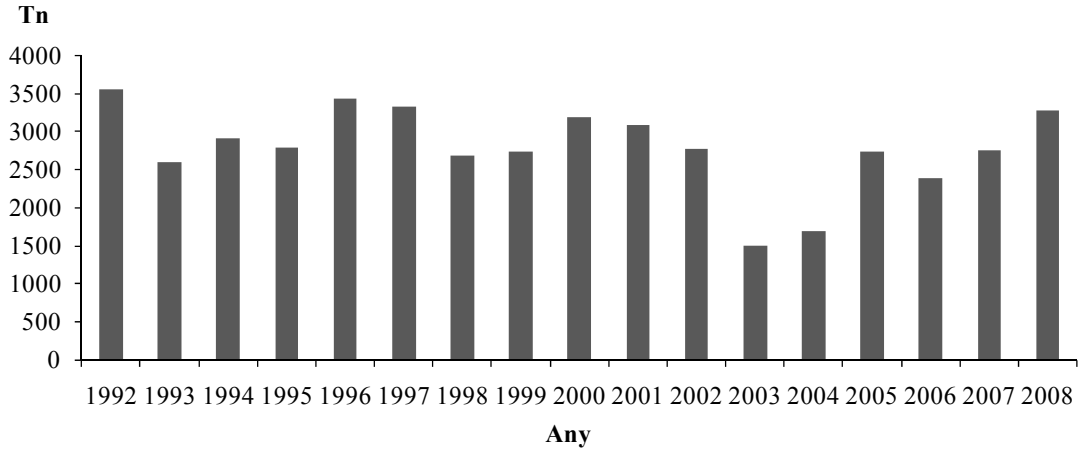

Fig. 7: Producció de musclo al Delta de l'Ebre (DAR, 2009).

De totes les regions productores de musclo del Mediterrani espanyol, la major producció s'obté a les badies del Delta de l'Ebre (Ramón et al., 2005), amb unes 3000 Tn anuals (Fig. 9). Tot i que la producció és força inferior a la de Galícia i la mundial en general, representa un ingrés local molt important.

2. OBJECTIUS

L'objectiu de la present tesi fou l'estudi del comportament alimentari dels musclos en termes de selecció i components fisiològics del balanç absortiu, i esbrinar si la ingestió d'algues tòxiques modula la seva resposta immunitària i els causa canvis patològics.

Per adreçar aquesta problemàtica es van usar dues espècies de musclos, *Mytilus galloprovincialis* i *M. edulis*. Es van emprar dos tipus de sistemes experimentals; els experiments d'alimentació es van dur a terme *in situ* sobre una instal·lació de musclos en condicions naturals, i es van exposar musclos a algues tòxiques en aquaris sota condicions controlades de laboratori.

Es van formular les següents hipòtesi:

Hipòtesi I: El comportament alimentari dels musclos a la Badia d'Alfacs (N.O. Mar Mediterrània) depèn de la quantitat i qualitat del sèston, que pot variar en el temps.

Hipòtesi II: Els musclos de la Badia d'Alfacs (N.O. Mar Mediterrània) seleccionen preferentment determinades partícules en suspensió però no d'altres.

Hipòtesi III: Les proliferacions d'algues tòxiques (HAB) modulen el sistema immunitari dels musclos i els causen canvis patològics.

Aquesta tesi es basa en sis capítols, cadascun referenciat amb la corresponent numerologia romana.

Les hipòtesi es van contrastar mitjançant el següent procediment:

1. Determinació dels paràmetres fisiològics més importants relacionats amb el comportament alimentari del musclo a la Badia d'Alfacs mitjançant el mètode de la biodeposició (Capítol I).

2. Estudi de la composició del fitoplàncton, determinació dels taxons dominants, i detecció de proliferacions de fitoplàncton durant quatre períodes en un cicle de cultiu (d'octubre a juliol) a la Badia d'Alfacs, on s'hi desenvolupa aqüicultura de musclo (Capítols II i III).

3. Estudi de la selecció i ingestió de partícules en musclos, incloent algunes espècies tòxiques, durant quatre períodes en un cicle de cultiu (d'octubre a juliol) a la badia del Alfacs (Capítols II i III).

4. Estudi dels efectes immunològics i histològics de 3 algues tòxiques: *Karlodinium veneficum*, *Prorocentrum minimum* i *Alexandrium fundyense*, en musclos mitjançant condicions experimentals de laboratori (Capítols IV, V i VI).

5. Anàlisi de la possible recuperació dels musclos després d'una exposició experimental a l'alga tòxica *Alexandrium fundyense* (Capítol VI).

3. RESULTATS

Capítol I. Comportament alimentari del musclo Myt*ilus galloprovincialis* (L.) a la Badia dels Alfacs (N.O. Mar Mediterrània), un estudi de camp

El comportament alimentari del musclo *Mytilus galloprovincialis* es va estudiar en condicions de camp sobre una plataforma de cultiu de musclo a la badia dels Alfacs, N.O. Mar Mediterrània. Es van realitzar quatre experiments de filtració de dues hores de durada cadascun per període de mostreig (novembre 2006, febrer, abril i juliol 2007) mitjançant dos aparells amb sistema de flux obert. Es van calcular la matèria particulada total (TPM), matèria particulada orgànica (POM) i matèria particulada inorgànica (PIM) de l'aigua de la badia, i femtes i pseudofemtes dels musclos, per obtenir diversos paràmetres fisiològics relacionats amb

l'alimentació mitjançant el mètode de la biodeposició (Iglesias et al., 1998), com per exemple: taxa d'aclariment (CR), taxa d'ingestió orgànica (OIR), taxa d'absorció, i eficiència d'absorció (AE). Els aparells de filtració es van validar abans de l'anàlisi de les dades obtingudes. Els resultats van mostrar que les variacions a curt terme (hores) de la quantitat i qualitat de l'aliment són similars a les variacions a llarg terme (mesos); tot i això, el contingut orgànic de l'aigua de la badia fou elevat al llarg de tot l'estudi obtenint uns valors de qualitat de l'aliment *f* (POM/TPM) de 0.51 a 0.72, comparables a altres àrees de gran producció de musclo, com les Ríes Gallegues. Els paràmetres fisiològics que caracteritzen l'adquisició i absorció d'aliment van presentar importants variacions a curt terme. Malgrat això, es van obtenir elevades CR i AE durant tot l'estudi. Com a resposta davant de la variabilitat ambiental de la badia d'Alfacs, quan les concentracions de seston eren altes, els musclos van reduir les seves taxes d'aclariment, en lloc d'incrementar la producció de pseudofemtes. L'eficiència d'absorció es va relacionar positivament amb el contingut orgànic de les partícules ingerides (i), el qual ja s'ha demostrat en la literatura que pot ser el determinant més important de l'eficiència d'absorció. No vam poder establir un patró estacional però es va observar una clara tendència a minvar CR, OIR i AR durant els mostrejos de juliol 2007. Aquesta restricció alimentària sembla explicar-se per l'elevada temperatura que va assolir l'aigua de la badia, fins 25 ºC.

Els nostres resultats mostren que la badia dels Alfacs reuneix unes condicions tròfiques estables amb una bona qualitat del seston pel cultiu de bivalves. Els paràmetres fisiològics mesurats en musclo van ser elevats excepte el mes de juliol, quan es van veure negativament influenciats per l'elevada temperatura de l'aigua. Aquests tipus d'estudis, amb dades de camp, són molt útils per gestionar zones amb aqüicultura de bivalves i ajuden a realitzar estudis de capacitat de càrrega dels ecosistemes.

Capítol II. Selecció pre-ingestiva del musclos, *Mytilus galloprovincialis* (L.), al alimentar-se de poblacions naturals de fitoplàncton en un estuari del N.O. de la Mar Mediterrània

La selecció pre-ingestiva dels musclos (*Mytilus galloprovincialis*) es va investigar en la Badia d'Alfacs, un estuari del N.O. de la Mediterrània. Per això, es van dur a terme experiments sobre una musclera durant quatre períodes al llarg d'un any (4 experiments per període). Per evaluar la capacitat de selecció pre-ingestiva dels musclos envers la població natural de fitoplàncton es van identificar i quantificar les espècies de fitoplàncton de l'aigua de la badia i de les pseudofemtes dels musclos analitzats; també es van identificar i quantificar les espècies de fitoplàncton en els continguts estomacals i les femtes dels musclos com a informació complementària per l'estudi.

Es van identificar quaranta-set espècies de fitoplàncton a l'aigua de la badia, i, a més, altres espècies no identificades es van categoritzar en 6 grups: (1) dinoflagel·lats petits (<20μm); (2) dinoflagel·lats grans (>20 μm); (3) cists de dinoflagel·lats; (4) diatomees bentòniques petites (<30μm); (5) diatomees bentòniques grans (>30 μm) i (6) diatomees cèntriques. Totes les espècies i grups es van reagrupar per analitzar la seva importància relativa en la selecció pre-ingestiva dels musclos de la següent manera: dinoflagel·lats, diatomees i "altres", que va incloure 4 espècies marines (*Cryptomonas* spp., *Dictyocha fibula*, *D. octonaria*, i *Hermesinum adriaticum*) i l'espècie d'aigua dolça *Scenedesmus* spp. Les ditomees planctòniques varen ser dominants a l'aigua de la badia durant tots els períodes estudiats. Malgrat això, els musclos van seleccionar i ingerir majoritàriament dinoflagel·lats. La preferència de selecció envers algunes espècies va variar segons la seva concentració en l'aigua, com es va observar per la diatomea *Cyclotella meneghiniana*. En aquest cas en particular, l'espècie es va seleccionar positivament durant el febrer però es va rebutjar durant juliol, quan va assolir concentracions en l'aigua de 10^5 cel l^{-1}.

Aquest estudi mostra les preferències alimentàries de *M. galloprovincialis* envers determinades espècies de fitoplàncton, majoritàriament dinoflagel·lades, malgrat la potencial toxicitat d'algunes de les espècies. A més, mostra que la selecció pre-ingestiva dels musclos a la Badia d'Alfacs és molt important per incrementar la qualitat de la seva dieta. Aquest estudi contribueix a entendre l'important paper dels musclos en els ecosistemes degut al seu potencial de canviar l'estructura de les comunitats de fitoplàncton com a conseqüència del seu comporatment alimentari.

Capítol III. Primera evidència de la ingestió de fibra de vidre per un invertebrat marí (*Mytilus galloprovincialis* L.) en un estuari del NO. Mediterrani

La badia d'Alfacs és un estuari del NO del Mar Mediterrani on s'hi desenvolupa aqüicultura de musclo (*Mytilus galloprovincialis*). Durant estudis realitzats en aquesta àrea es van detectar partícules de fibra de vidre de longitud i amplada mitjana 280.09 ± 301.69μm i 7.62 ± 1.27μm respectivament. Tot i observar una gran variabilitat en la longitud de les fibres de vidre, les fibres curtes (<200 μm) van ser les més abundants amb un valor modal de 65.83μm. La seva presència en l'aigua va ocórrer de forma natural durant les quatre èpoques de l'estudi (Novembre 2006, Febrer, Abril i Juliol 2007) i la seva abundància respecte el total de partícules de fitoplàncton va ser en tots els casos inferior al 1%. En una investigació duta a terme sobre la conducta alimentària dels musclos de la zona es va observar la presència de fibra de vidre en totes les mostres de musclo (contingut estomacal, femtes i pseudofemtes). Malgrat esperar rebuig de les fibres durant tot l'estudi, aquest només fou evident durant el segon període analitzat, Febrer 2007, on l'abundància de fibres de vidre en pseudofemtes fou molt superior a

la resta de mesos i tipus de mostres. Segons ens consta, aquest és el primer informe de la ingestió de fibra de vidre per un organisme marí. La nostra innovadora troballa evidencia la necessitat d'investigar la ingestió de fibra de vidre per organismes marins a diferents nivells de la xarxa tròfica i possibles implicacions per la salut humana i dels propis organismes. A més, proposem l'ús dels musclos com a organismes bioindicadors per detectar contaminació per fibra de vidre en els ecosistemes marins.

Capítol IV. Efectes en el musclo *Mytilus edulis* a l'alimentar-se de *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine)

Karlodinium veneficum (PLY # 103) és un dinoflagel·lat tòxic que produeix una poderosa toxina letal en diversos organismes. Es van investigar els efectes d'aquesta alga tòxica sobre el sistema immune del musclo *Mytilus edulis*, alhora que es va fer un estudi histopatològic de la possible afectació de l'alga en els teixits del musclo. Per això, els musclos es van recollir a Whitsand Bay, Cornwall (UK) i es van alimentar musclos amb *K. veneficum* (PLY # 103) durant 6 dies, recollint mostres d'hemolimfa i teixits el dia previ a l'exposició, i durant els dies 3 i 6. Les proves immunològiques van incloure recompte total i diferencial d'hemòcits, fagocitosi, i alliberament extracel·lular de radicals lliures derivats de l'oxigen. També es va estudiar en preparacions histològiques la glàndula digestiva i el mantell. La quantitat de toxina de *K. veneficum* (PLY # 103) es va mesurar mitjançant espectrometria de masses.

Tots els musclos van produir femtes però no pseudofemtes, indicant que l'alga no es va rebutjar prèviament a la seva ingestió. Es van detectar i aïllar dues toxines de *K. veneficum* (PLY # 103), KvTx1 (11.6 ± 5.4 ng/ml) i KvTx2 (47.7 ± 4.2 ng/ml). Aquesta soca original de *K. veneficum* aïllada per Mary Parke, malgrat portar en cultiu més de 50 anys, va causar efectes tòxics en els musclos ja que els exposats a l'alga tòxica van patir un increment significatiu de fagocitosi i alliberament de radicals lliures després de 6 dies d'exposició. En quant al recompte d'hemòcits, no es van trobar diferències significatives en el nombre total d'hemòcits, però sí en el recompte diferencial, després de 3 dies d'exposició a l'alga tòxica. Aquesta diferència pot ser deguda a una estimulació del sistema immune per part de les toxines provocant una migració dels hemòcits eosinofílics de la circulació cap als teixits afectats. Les glàndules digestives van mostrar ingestió de l'alga i infiltració d'hemòcits després de 3 dies d'exposició, mentre que el mantell no va mostrar diferències. Així doncs, les karlotoxines van afectar els teixits i cèl·lules dels musclos amb que primer van contactar, o sigui, els hemòcits i la glàndula digestiva. Es recomana estudiar si una concentració de *K. veneficum* més elevada o una exposició més prolongada podria provocar efectes en altres teixits

Capítol V. Patologia i resposta immunitària del musclo (*Mytilus edulis* L.) després de l'exposició al dinoflagel·lat tòxic *Prorocentrum mínimum*

El dinoflagel·lat tòxic *Prorocentrum minimum* causa efectes nocius en diverses espècies de bivalves quan l'ingereixen; aquests efectes també varien segons l'espècie i el període vital dels organismes. Els possibles efectes d'aquest dinoflagel·lat en musclos no estaven descrits; per això, vam realitzar experiments d'exposició de *P. minimum* a musclos adults *Mytilus edulis*. Els musclos es van recollir de la platja de Westcott, al nord de Long Island Sound (CT, USA), el juny del 2007. A continuació, es van netejar i posar en aquaris experimentals, on després d'un període d'aclimatació, es van exposar a cultius de l'alga tòxica *P. minimum* i no tòxica *Rhodomonas* sp. en continu. Es van recollir mostres d'hemolimfa i teixits en el dia 0 (abans de l'exposició a les algues), i després de 3, 6 i 9 dies d'exposició contínua. L'hemolimfa es va analitzar mitjançant citometria de flux per estudiar diversos paràmetres funcionals i immunològics dels hemòcits. Els teixits es van processar per fer el seu estudi histopatològic.

Es van trobar cèl·lules d'algues en els continguts estomacals, femtes i pseudofemtes dels musclos, indicant ingestió de *P. minimum* i *Rhodomonas* sp. Els musclos van respondre a *P. minimum* amb diapedesi dels hemòcits en l'intestí, presumiblement per isolar les cèl·lules de l'alga tòxica i minimitzar possibles danys a altres teixits. Aquest resposta immunitària es va mantenir durant els 9 dies d'exposició, i els hemòcits circulants van mantenir les seves propietats hematològiques i funcionals. De fet, les femtes dels musclos alimentats amb *P. minimum* van presentar hemòcits agregats al voltant de les cèl·lules d'alga tòxica. A més, en l'intestí dels musclos exposats a *P. minimum* van proliferar bacteris. Així doncs, els hemòcits en l'intestí estaven, o bé aclaparats pel gran nombre de bacteris, o més probablement, ocupats encapsulant cèl·lules de *P. minimum*, incapaços de mantenir el nombre de bacteris sota control. Finalment, en arribar els hemòcits a la llum de l'intestí van patir apoptosi i degradació bacteriana.

La relació filogenètica descrita entre *Perkinsus marinus* (paràsit de bivalves) i determinats dinoflagel·lats com *P. minimum*, suggereix la presencia de receptors de membrana comuns que permetrien als hemòcits reconèixer les partícules com a no pròpies. Malgrat això, la resposta immune varia ja que les cèl·lules de l'alga tòxica són molt majors que els hemòcits i, en lloc de ser fagocitades, són encapsulades. Els resultats d'aquest experiment mostren la importància d'usar diversos tipus d'anàlisi (immunològic i histopatològic) per assolir una major comprensió dels efectes de les algues tòxiques en els bivalves. En el nostre cas concret, es demostra que *M. edulis* es veu afectat per la ingestió de l'alga tòxica *P. minimum* i que les respostes específiques observades són diferents de les descrites per altres espècies de bivalves. Aquesta troballa destaca la necessitat d'estudiar els efectes de diversos HABs en diferents

espècies de bivalves, en lloc d'inferir que el resultat d'una espècie sigui la mateixa resposta en tots els bivalves.

Capítol VI. Exposició experimental de musclo (*Mytilus edulis*, L.) al dinoflagel·lat tòxic *Alexandrium fundyense*: histopatologia, resposta immunitària, i recuperació

Es van exposar musclos *Mytilus edulis* als cultius del dinoflagel·lat tòxic *Alexandrium fundyense* i a l'alga no tòxica *Rhodomonas* sp. Es van avaluar els efectes de l'alga tòxica en el sistema immunitari dels musclos,i a continuació, es va estudiar si havia recuperació per part dels musclos després d'interrompre l'exposició a *A. fundyense* i alimentar-se d'alga no tòxica. Per això, els musclos es van alimentar durant 9 dies de les diferents algues i, a continuació, es van alimentar de *Rhodomonas* sp. durant 6 dies més. Es van prendre mostres d'hemolimfa i teixits abans, durant l'exposició a les algues, i en el període de recuperació. L'hemolimfa es va analitzar mitjançant citometria de flux per estudiar diversos paràmetres funcionals i immunològics dels hemòcits. Els teixits es van processar per fer un estudi histopatològic.

Els musclos van filtrar i ingerir els dos cultius de microalgues, produint femtes que contenien algues degradades, parcialment degradades i cèl·lules intactes. La toxicitat de *A. fundyense* es va determinar mitjançant HPLC obtenint una toxicitat total estimada de 0.43 pg STXeq per cèl·lula. Malgrat el baix nivell de toxicitat, els musclos exposats a *A. fundyense* es van veure afectats per la seva ingestió, resumit en la figura 6. Van presentar resposta inflamatòria que va consistir en degranulació i diapedesi dels hemòcits en el canal alimentari; a mesura que l'exposició va continuar, es va observar migració dels hemòcits en el teixit connectiu entre els fol·licles gonadals. També es va evidenciar peroxidació lipídica, similar al patró de detoxificació descrit per diversos xenobiòtics, formant-se grànuls insolubles de lipofucsina (ceroidosi), que els hemòcits portaven al canal alimentari, eliminant així toxines del dinoflagel·lat en les femtes. A mesura que el nombre d'hemòcits circulants en musclos exposats a *A. fundyense* van minvar, els musclos van esdevenir immuno-compromesos, seguint un conjunt de canvis patològics com augmentar la prevalença de ceroidosi i trematodes després de 9 dies d'exposició i l'aparició de cèl·lules secretores de mucus en brànquies. A més, el nombre total de canvis patològics va incrementar des de l'inici de l'exposició fins el darrer dia (dia 9).

Després de 6 dies d'exposició, els musclos d'un dels tres tancs exposats a l'alga tòxica van fer posta massiva. Aquests musclos van presentar uns efectes molt més severs de l'alga tòxica que els musclos que no van fer posta també exposats a *A. fundyense*.

No es van observar diferències significatives entre els dos tractaments durant el període de recuperació, indicant un ràpid procés homeostàtic en els teixits i hemòcits circulants. Els musclos amb prèvia exposició a l'alga tòxica, però, no es van poder recuperar completament dirigint els hemòcits a recuperar primordialment la glàndula digestiva però no les gònades.

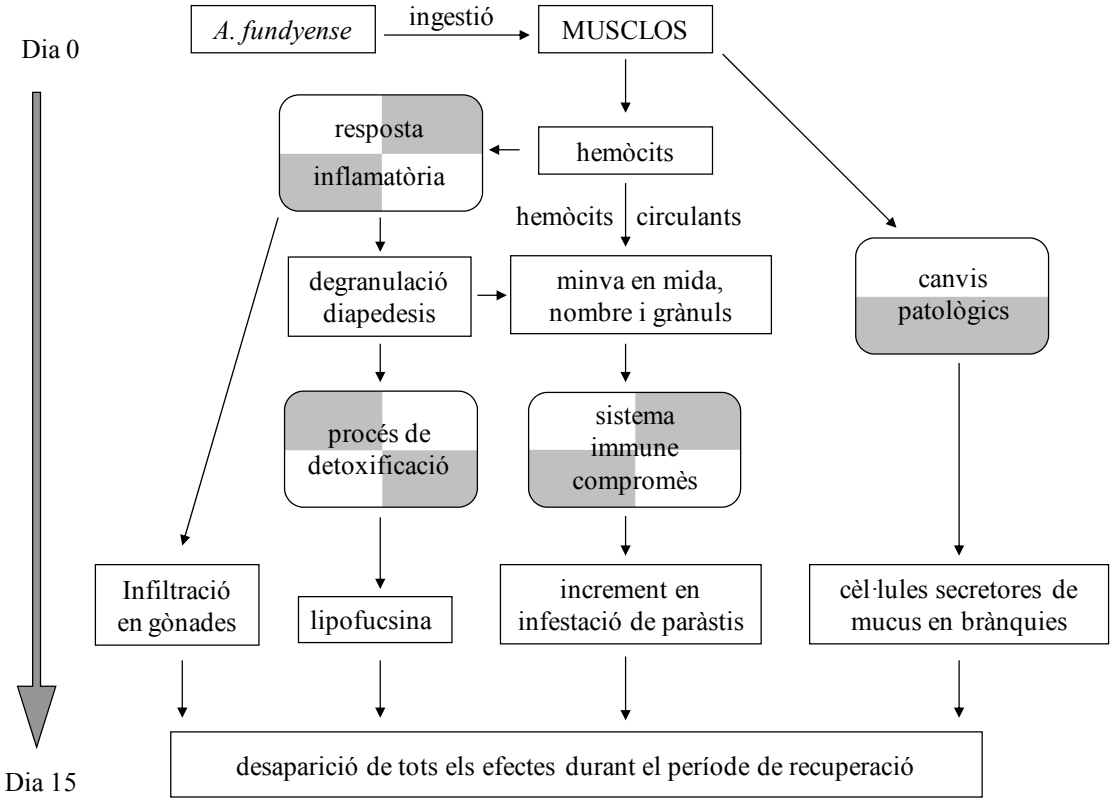

Fig. 8. Diagrama sumari de la resposta del musclo *M. edulis* a la ingestió d'*A. fundyense*.

4. DISCUSSIÓ

4.1 El paper ecològic dels musclos

La importància ecològica dels musclos en els ecosistemes marins es resumeix en la figura 7. Els musclos s'alimenten de matèria particulada suspesa en l'aigua, majorment fitoplàncton. Com a conseqüència de la selecció i ingestió de determinades espècies de microalgues i el rebuig d'altres en les pseudofemtes, els musclos poden provocar canvis en la comunitat de fitoplàncton. Entre les espècies de microalgues que es troben de forma natural en els ecosistemes, algunes poden ser tòxiques. Malgrat això, els musclos poden ingerir-les i acumular les seves toxines, transferint-les a nivells tròfics superiors (Shumway, 1990; Landsberg, 2002) o bé dipositant-les al bentos mitjançant biodipòsits a través dels cists de resistència que poden formar algunes espècies de microalgues (Harper et al., 2002; Hégaret et al., 2008).

Els musclos participen en el cicle de nutrients dels ecosistemes ja que poden assimilar un gran nombre de compostos de la columna d'aigua, com calci i nitrogen, i transferir-los a diversos nivells tròfics, tant bentònics com pelàgics (Fig. 10). Els biodipòsits es poden acumular en el sediment però també resuspendre, alliberant nutrients de nou a la columna

d'aigua i afavorint la regeneració de la comunitat de fitoplàncton (Asmus i Asmus, 1991; Prins et al, 1998; Smaal et al, 2001; Newell, 2004).

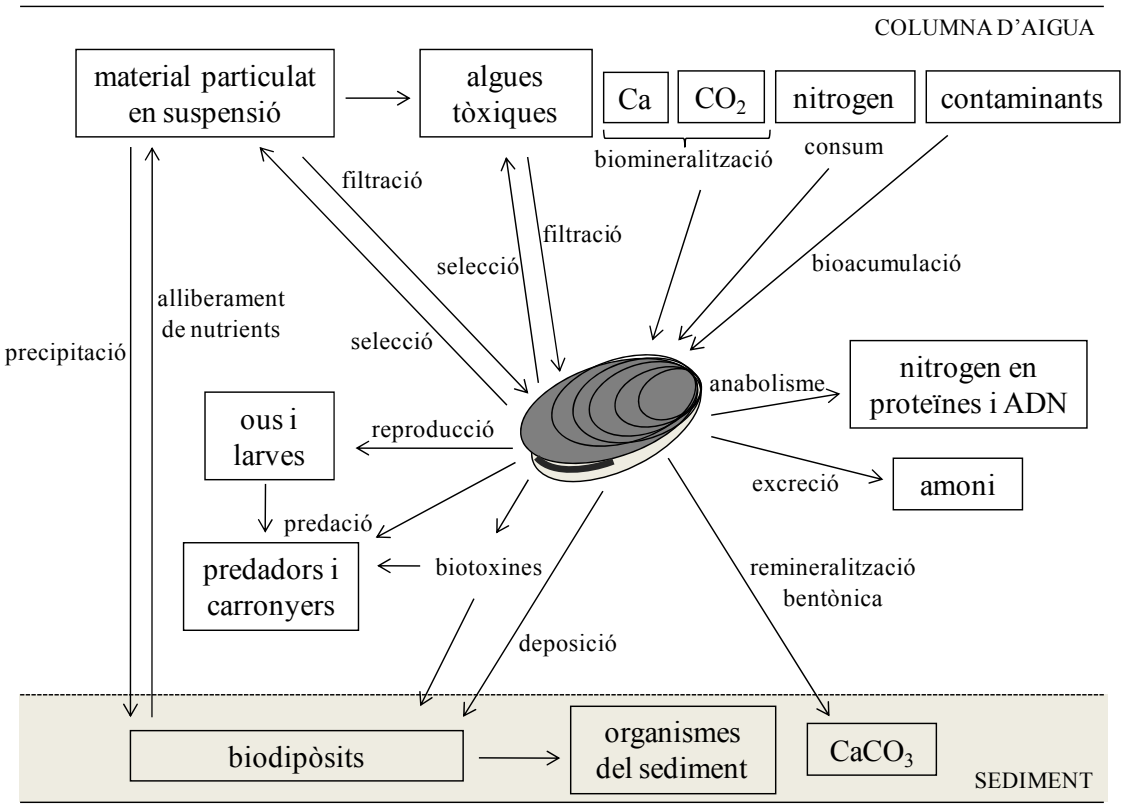

Fig. 9: Resum del paper ecològic que els musclos desenvolupen en els ecosistemes.

Com a conseqüència de l'important paper ecològic i econòmic dels bivalves, i malgrat la recerca ja feta en aquestes espècies, encara manca investigació en les interaccions bivalves-fitoplàncton *in situ* en els ecosistemes així com en els efectes de les proliferacions de fitoplàncton tòxic, cada vegada més esteses i freqüents.

4.2 Validació dels mètodes

L'objectiu de la present tesi, "l'estudi del comportament alimentari dels musclos en termes de selecció i components fisiològics del balanç absortiu, i esbrinar si la ingestió d'algues tòxiques modula la seva resposta immunitària i els causa canvis patològics", es va adreçar mitjançant dos tipus de sistemes experimentals i dues espècies del gènere *Mytilus*. Els experiments d'alimentació es van dur a terme *in situ* sobre una musclera en condicions naturals (Capítols I, II i III), i es van exposar musclos a algues tòxiques en aquaris sota condicions controlades de laboratori (Capítols IV, V i VI). Les espècies de musclo estudiades representen espècies model de cada àrea d'estudi (Gosling, 2003); així, *Mytilus galloprovincialis* es va usar

pels experiments de la Badia d'Alfacs (Mar Mediterrània) i *M. edulis* es van usar pels experiments realitzats en dos estuaris Atlàntics; Plymouth Sound (UK) a la costa Est de l'Oceà Atlàntic (Capítol IV), i Long Island Sound (USA) a la costa Oest de l'Atlàntic (Capítols V i VI). Quan l'objectiu de l'estudi, com en els Capítols I, II i III de la present tesi, era l'aproximació de valors reals del comportament alimentari dels musclos en el medi natural, es recomanen dissenys experimentals usant el sèston natural de l'aigua (Velasco and Navarro, 2005). Però si l'objectiu d'estudi era testar els efectes d'una única variable en els musclos, com els canvis immunològics i histopatològics causats per la ingestió d'alga tòxica, es recomanen condicions controlades de laboratori davant la impossibilitat de controlar tots els paràmetres ambientals que ocorren a camp (Walne, 1972; Bernard, 1983; Denis et al., 1999).

4. 2. 1 Els experiments d'alimentació *in situ*

Les característiques del sèston de la Badia d'Alfacs es van avaluar en quantitat i composició de les espècies de fitoplàncton per relacionar-ho amb el comportament alimentós dels musclos (Capítols I i II). La Badia va tenir una elevada qualitat de l'aigua durant tot el període d'estudi (Capítol I). La Taula 3 mostra les característiques del sèston de diverses localitats amb aqüicultura de musclos a Espanya, França, Holanda, i Austràlia. El valor màxim de TPM es va registrar a Brisbane Water (Austràlia) però la major fracció era inorgànica. Thau Lagoon (França) va presentar els valors màxims de qualitat d'aigua però no fou constant al llarg de l'estudi ja que també s'hi van registrar valors molt baixos degut a fenòmens de resuspensió de sediment.

Les característiques del sèston són un component clau per l'estudi de la fisiologia d'alimentació dels bivalves. A la Badia d'Alfacs, on les variacions a curt termini (dies) de la quantitat de sèston eren importants, els musclos van regular la seva capacitat de filtració com a resposta a les variacions del medi (Capítol I), com va trobar també Babarro et al. (2000) a les Ries Gallegues però al contrari del què va trobar Urrutia et al. (1996) a Marennes-Oléron per l'espècie *Cerastoderma edule*.

La pressió que exerceixen els bivalves sobre les partícules suspeses en el medi és variable. L'objectiu de la selecció d'unes determinades partícules però no d'altres és millorar la qualitat del material ingerit. El Capítol II presenta l'estudi de selecció pre-ingestiva del musclo sobre la comunitat de fitoplàncton a la Badia d'Alfacs. En acord amb previs estudis realitzats a d'altres localitats, la selecció pre-ingestiva varia segons la composició i abundància relativa de les espècies de fitoplàncton suspeses en el sèston (Kiørboe i Møhlenberg 1981; Bougrier et al., 1997). Tot i això, en termes generals, els resultats del Capítol II van mostrar una selecció preferencial de dinoflagel·lats, en acord a previs estudis (Shumway et al, 1985; Sidari et al., 1998), malgrat la dominància de diatomees a l'aigua de la badia.

Localitat	TPM (mg l^{-1})	POM (mg l^{-1})	PIM (mg l^{-1})	*f*	Bibliografia
Badia d'Alfacs (España)	2.30 - 1.02	1.36 – 0.67	1.11 – 0.35	0.72 – 0.51	Present tesi (Capítol I)
Ría Arousa (España)	2.56 - 0.49	1.00 – 0.28	1.56 – 0.20	0.60 – 0.29	Babarro et al. (2003)
Ría Arousa (España)	2.71 – 0.74	1.08 – 0.35		0.54 – 0.35	Navarro et al. (1991)
Thau Lagoon (França)		1.7 – 0.1		0.88 – 0.12	Gangnery et al. (2004)
Marennes-Oléron (França)				0.28 – 0.08	Hawkins et al. (1996)
Marennes-Oléron (França)				0.33 – 0.03	Grant i Bacher (1998)
Oosterschelde (Holanda)				0.20 – 0.15	Scholten i Smaal (1998)
Brisbane Water (Austràlia)	6.95 – 3.54	1.87 – 0.97	5.08 – 2.57		Paterson et al. (2003)

Taula 3: Característiques del sèston de diferents àrees amb aqüicultura de bivalves, s'indica TPM: material total particulat; POM: material orgànic particulat; PIM: material inorgànic particulat; *f*: fracció orgànica del sèston expressada com POM/TPM, per cada localitat. Els valors corresponen a màxims i mínims descrits.

Com a complement del mateix capítol, la taula 4 mostra la llista dels taxons de fitoplàncton identificats durant els experiments realitzats a la Badia d'Alfacs. A més de fitoplàncton, es van identificar altres partícules en l'aigua i les diverses mostres de musclos estudiades, no descrits en el Capítol II, degut a les seves baixes abundàncies, ex., cocolitoforals, nanoflagel·lats i espores de fongs. També es van identificar diverses espècies de zooplàncton.

Com es resumeix en la figura 10, els contaminants també es troben suspesos en la columna d'aigua. La fibra de vidre trobada a l'aigua de la Badia d'Alfacs (Capítol III) es pot considerar un contaminant marí ja que aquest material és nociu quan es presenta en forma de pols a l'aire (IARC, 2002). Les fibres de vidre també es van identificar en els continguts estomacals dels musclos evidenciant la transferència d'aquestes partícules a la cadena alimentària marina. Es recomana estudiar a fons aquesta temàtica ja que alguns estudis han demostrat que la ingestió de fibres de vidres por produir greus malalties en mamífers com càncer d'estómac o peritonitis (Maresca et al., 1984; Hardie et al., 1994; Kærheim et al., 2005).

Dinoflagel·lats	Diatomees	Altres
	Species	
Alexandrium minutum	*Asteromphalus heptactis*	*Cryptomonas* spp.
Alexandrium spp.	*Bacillaria paxillifer*	*Dictyocha fibula*
Ceratium furca	*Bacteriastrum delicatulum*	*Dictyocha octonaria*
Dinophysis caudata	*Bellerochea malleus*	*Hermesinum adriaticum*
Dinophysis rotundata	*Corataulina pelagica*	
Dinophysis sacculus	*Chaetoceros* spp.	
Gonyaulax verior	*Coscinodiscus* spp.	
Gonyalaux spp.	*Cyclotella meneghiniana*	
Gymnodinium spp.	*Cylindrotheca closterium*	
Heterocapsa niei	*Grammatophora marina*	
Peridinium quinquecorne	*Guinardia flaccida*	
Prorocentrum micans	*Guinardia striata*	
Prorocentrum minimum	*Leptocylindrus minimus*	
Prorocentrum triestinum	*Lioloma pacificum*	
Protoceratium reticulatum	*Paralia sulcata*	
Protoperidinium diabolus	*Pleurosigma* spp.	
Protoperidinium spp.	*Proboscia alata*	
Pyrophacus horologium	*Pseudo-nitzschia* spp.	
Scrippsiella trochoidea	*Rhizosolenia* spp.	
Scrippsiella spp.	*Striatella unipunctata*	
	Synedra spp.	
	Thalassionema nitzschioides	
	Thalassiosira spp.	

Taula 4: Espècies de fitoplàncton trobades en l'aigua de la Badia d'Alfacs durant novembre 2006 i febrer, abril, i juliol 2007 (Comunicació personal de M. Delgado, ICM-CSIC, 2009).

4. 2. 2 Els experiments d'exposició a algues tòxiques

Els musclos s'ha descrit com a organismes molt resistents a l'efecte de les biotoxines, especulant que eren simplement vectors de transferència de toxines a elevats nivells tròfics (Twarog et al., 1972; Bricelj et al., 1990; Marsden i Shumway, 1991). Els estudis dels efectes histopatològics en bivalves després de la ingestió d'algues tòxiques són escassos però demostren canvis patològics en la glàndula digestiva (Wikfors i Smolowitz, 1993, 1995). Per això, els Capítols IV, V I VI d'aquesta tesi investiguen els efectes de tres espècies potencialment tòxiques: *Karlodinium veneficum*, *Prorocentrum minimum,* i *Alexandrium fundyense*, en musclos sota condicions controlades de laboratori.

Com a resposta comú a les tres espècies estudiades (Capítols IV, V i VI), les biotoxines van afectar primer els hemòcits i la glàndula digestiva dels musclos. Les biotoxines s'acumulen en la glàndula digestiva dels bivalves (Yasumoto et al., 1978, Murata et al., 1982; Bricelj et al., 1990) així que l'afectació d'aquest òrgan era esperable. Les glàndules digestives dels musclos van presentar infiltració d'hemòcits entre o dintre els túbuls digestius. Els musclos exposats a *K. veneficum* (Capítol IV), no van presentar infiltració hemocitària en el teixit connectiu d'entre els fol·licles gonadals, possiblement degut a la baixa concentració de cèl·lules usada o un baix temps d'exposició.

	Karlodinium veneficum (Capítol IV)	*Prorocentrum minimum* (Capítol V)	*Alexandrium fundyense* (Capítol VI)
Hemòcits			
Complexitat interna	no estudiat	minvat	minvat
Mida	no estudiat	mantingut	minvat
Nombre total	mantingut	mantingut	minvat
Assajos immunològics			
Espècies reactives d'oxigen	augmentat	mantingut	mantingut
Fagocitosi	augmentat	mantingut	mantingut
Histopatologia			
Teixit del mantell	mantingut	hemòcits en teixit connectiu entre fol·licles gonadals	hemòcits en teixit connectiu entre fol·licles gonadals
Glàndula digestiva	hemòcits infiltrats entre els túbuls digestius	- diapedesi - bacteris en estómac i intestí	diapedesi

Taula 5: principals canvis immunològics i histopatològics dels musclos després de la seva exposició a les algues potencialment tòxiques: *Karlodinium veneficum*, *Prorocentrum minimum* i *Alexandrium fundyense*.

Les espècies del gènere *Alexandrium* poden provocar una paràlisi del múscul abductor en diverses espècies de bivalves (Connell et al., 2007; Hégaret et al., 2007b). En el Capítol VI, els musclos que van presentar paràlisi en el múscul van ser aquells que havien fet posta prèviament, suggerint que l'estat fisiològic dels organismes és molt important pel grau d'afectació de la lesió i possible recuperació. Si la concentració de toxines fos més elevada, aquest efecte de paràlisi s'hagués observat en tots els musclos.

La resposta global i els efectes immunològics i histopatològics de les biotoxines en els musclos va variar per les tres espècies de fitoplàncton estudiades. Els hemòcits dels musclos exposats a *K. veneficum* van incrementar la producció d'espècies reactives d'oxigen i la capacitat fagocítica, mentre que els hemòcits dels musclos exposats a les altres dues espècies no van presentar canvis significatius amb els controls. Hégaret i Wikfors (2005) van observar un augment en la capacitat fagocítica d'ostres (*Crassostrea virginica*) exposades a *Prorocentrum minimum*, com el que es va trobar per *K. veneficum* en musclos en el Capítol IV, però al contrari de l'observat en el Capítol V per *P. minimum*. Al ingerir *P. minimum* la resposta dels musclos va ser similar a l'observat en cloïsses infectades per *Perkinsu* (Capítol V), mentre que la ingestió de *A. fundyense* va induir el procés bioquímic de peroxidació lipídica com a via de detoxificació (Capítol VI). En resum, els resultats dels Capítols IV, V i VI i la seva comparació amb previs estudis verifiquen la hipòtesi que les respostes dels bivalves davant la ingestió d'algues potencialment tòxiques és específica per cada espècie (Shumway et al., 2003; Hégaret et al., 2007a).

4.3. Beneficis de la recerca

1. Els experiments d'alimentació duts a terme *in situ* sobre una instal·lació de musclos (Capítols I i II) beneficiaran a la comunitat científica i als productors de musclo al millorar el coneixement de les preferències alimentàries de fitoplàncton en musclos i de la fisiologia involucrada en el seu comportament alimentari al camp. El protocol d'usar aparells dissenyats per distribuir aigua de mar en aquaris individuals amb musclo, i llavors mesurar els processos fisiològics de l'alimentació segons el mètode de la biodeposició, es pot usar com a primer pas per mesurar la capacitat de càrrega dels ecosistemes. En detall, els experiments d'alimentació a la Badia d'Alfacs ajudaran al Govern Català a optimitzar la gestió dels cultius de la zona. Els aparells es poden ajustar a altres espècies de bivalves ajudant als productors a diversificar i incrementar la producció aqüícola.

2. Els estudis de canvis immunològics i patològics de musclos després de l'exposició a algues tòxiques augmenten el coneixement bàsic de les respostes d'un organisme que, prèviament, estava considerat com un vector inert de toxines cap a nivells tròfics superiors. Aquest tipus d'estudis, on una espècie clau s'usa com a organisme indicador, ajudarà als gerents dels recursos naturals a estimar els efectes de l'increment de proliferacions d'algues tòxiques (HAB) en els ecosistemes marins. A més, ajudaran a representants i reguladors de la industria musclaire a estimar l'impacte potencial de l'increment d'HAB en la producció de musclos.

3. L'estudi de recuperació després de l'exposició de musclo a *Alexandrium* pot beneficiar els organismes de salut pública a determinar la durada dels períodes de tancament després d'un esdeveniment d'HAB.

4. La innovadora troballa de la ingestió de fibra de vidre per musclos indica la necessitat d'investigar la presència d'aquest material arreu del món, la seva possible ingestió per part d'altres organismes marins, i la transferència a diversos nivells de la cadena alimentària marina. A més, caldria evaluar les possibles implicacions per la salut pública i la salut dels propis organismes. Com a mesura de precaució, en el cas dels bivalves comercials, advertim al Programa de Seguiment de Qualitat de l'Aigua d'estar alerta d'una nova partícula potencialment perillosa en àrees de cultiu de marisc.

5. CONCLUSIONS

Les hipòtesi formulades en la present tesi es van testar mitjançant experiments i es van obtenir les següents conclusions:

1. Hipòtesi I: El comportament alimentari dels musclos a la Badia d'Alfacs (N.O. Mar Mediterrània) depèn de la quantitat i qualitat del sèston, que pot variar en el temps.

Conclusions:

1.1 La Badia d'Alfacs té una bona qualitat de l'aigua per desenvolupar cultiu de bivalves, similar a altres zones productores conegudes arreu del món, malgrat que les variacions a curt termini (dies) en quantitat i qualitat del sèston són semblants a les variacions a llarg termini (mesos), una característica de la zona.

1.2 La capacitat de filtració i l'eficiència d'absorció dels musclos a la Badia d'Alfacs són elevades al llarg del cicle de cultiu i depenen de TPM i del contingut orgànic de les partícules ingerides, respectivament.

1.3 Els musclos a la Badia d'Alfacs van reduir la taxa d'aclariment davant d'elevades concentracions de sèston, en lloc de incrementar la producció de pseudofemtes.

2. Hipòtesi II: Els musclos de la Badia d'Alfacs (N.O. Mar Mediterrània) seleccionen preferentment determinades partícules en suspensió però no d'altres.

Conclusions:

2.1. Els musclos van seleccionar i ingerir cèl·lules de dinoflagel·lats, tot i que les diatomees van ser dominants en el ecosistema. El comportament alimentari dels musclos pot causar canvis en la comunitat de fitoplàncton mitjançant alimentació diferencial.

2.2 La selecció pre-ingestiva dels musclos envers fitoplàncton va variar d'acord amb la composició i l'abundància relativa de les diverses espècies de fitoplàncton en el sèston disponible.

2.3. Els musclos de la Badia d'Alfacs van ingerir fibra de vidre, un component tòxic antropogènic, i la van alliberar en els biodipòsits. A més, informem de la introducció de la fibra de vidre en les xarxes tròfiques marines.

3. Hipòtesi III: Les proliferacions d'algues tòxiques (HAB) modulen el sistema immunitari dels musclos i els causen canvis patològics.

Conclusions:

3.1. La ingestió de fitoplàncton tòxic causa primerament canvis patològics en els teixits que estan en contacte directe amb les cèl·lules, com són els hemòcits i la glàndula digestiva.

3.2. Els efectes de les esmentades espècies en musclo són espècies-específiques:

3.2.1. *Karlodinium veneficum*: els musclos van incrementar la capacitat fagocítica i la producció d'espècies reactives d'oxigen, van presentar infiltració d'hemòcits en la glàndula digestiva.

3.2.2. *Prorocentrum minimum*: els musclo van desenvolupar diapedesi, agregació i encapsulació d' hemòcits en el canal alimentari, i proliferació de bacteris en l'estómac i l'intestí;

3.2.3. *Alexandrium fundyense*: els musclos van desenvolupar una resposta inflamatòria amb degranulació, diapedesi, i infiltració d'hemòcits en les gònades, amb un subseqüent increment d'infestacions de paràsits oportunistes.

3.3. L'aproximació global incloent tècniques d'estudi d'immunologia i histopatologia són necessàries per detectar els efectes de les espècies de microalgues potencialment tòxiques en bivalves.

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