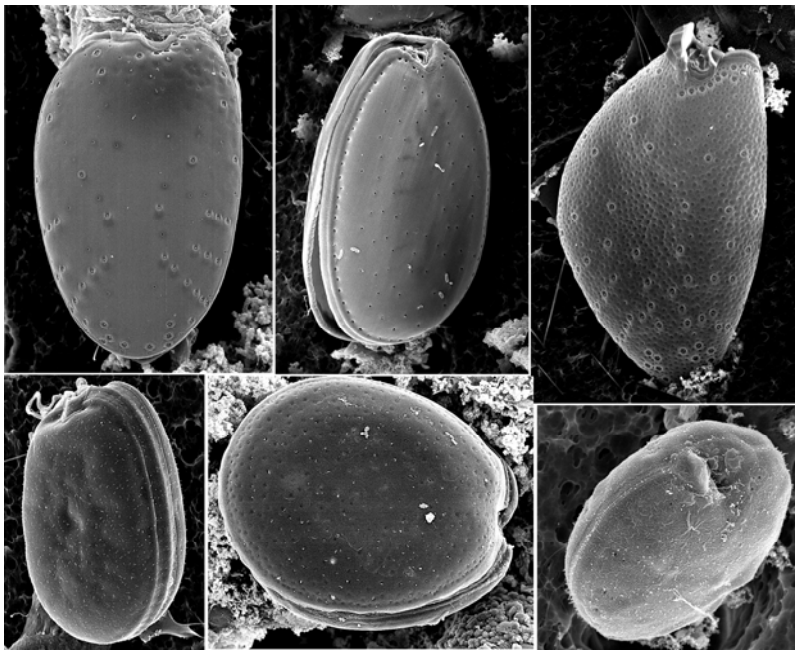
Universidade de Aveiro Departamento de Química
2005

Mariana Fonseca Hinzmann

Estudo de dinoflagelados bênticos da Ria de Aveiro (NW Portugal)

Study on benthic dinoflagellates from the Aveiro Lagoon (NW Portugal)



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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Métodos Biomoleculares Avançados, realizada sob a orientação científica de António José de Brito Fonseca Mendes Calado, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro. Dissertação realizada com o apoio da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

o júri

Presidente	Doutor Armando Jorge Domingues Silvestre, Professor Associado da Universidade de Aveiro.
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I dedicate this thesis to José Violante and thank him for all his patience and help, especially in the maintenance of my dinoflagellate cultures. If I manage to end this, to you I owe it, without your support and help it would be impossible.

Thank you all very much!

resumo

A Ria de Aveiro apresenta ao longo das margens pequenos charcos com características muito peculiares, que oferecem condições para a proliferação de um grande número de algas, especialmente algas bênticas pertencentes a vários grupos, com a dominância de diatomáceas, dinoflagelados e macroalgas verdes.

Verificou-se que a comunidade de dinoflagelados é dominada essencialmente por espécies pertencentes ao género *Prorocentrum* (*P. lima*, *P. micans*, *P. cassubicum*, *P. rhathymum* e duas prováveis novas espécies de *Prorocentrum*), mas também por outras espécies como *Coolia monotis*, *Amphidinium massartii*, *Bysmatrum subsalsum*, *Scrippsiella* cf. *trochoidea*, *Kryptoperidinium foliaceum*, *Oblea rotunda*, *Akashiwo sanguinea* e, embora menos frequentes, espécies dos géneros *Protoperidinium* e *Peridinium*.
Na costa portuguesa a maioria destas espécies já tinham sido anteriormente registadas, excepto *A. massartii*, *O. rotunda*, *B. subsalsum*, *P. cassubicum* e *P. rhathymum*, mas para a Ria de Aveiro apenas *S. trochoidea*, *Peridinium quinquecorne*, *P. lima*, *P. minimum* e *P. micans* já tinham sido referenciados (Moita & Vilarinho 1999).

O teste de *Artemia* demonstrou a toxicidade de apenas três espécies: *Prorocentrum lima*, *P. cassubicum* e *Coolia monotis*. As culturas de *P. lima* foram de longe as mais tóxicas para a larva de *Artemia salina*. A ocorrência de espécies tóxicas na Ria deve ser tida em conta uma vez que nesta região há produção de moluscos, que podem acumular estas toxinas afectando espécies em níveis tróficos superiores, incluindo o Homem.

Os charcos estudados foram bastante semelhantes quanto à variação dos parâmetros ambientais e comunidades biológicas. Apesar das variações climatéricas provocarem alterações acentuadas na salinidade e temperatura da água, há um leque de espécies que está sempre presente, enquanto outras só ocorrem em determinadas alturas, podendo atingir elevadas densidades.

Neste estudo da comunidade bêntica de dinoflagelados da Ria de Aveiro pretendeu-se incluir a correcta identificação das espécies, usando técnicas avançadas de microscopia, o estabelecimento de culturas em laboratório e a realização de ensaios de toxicologia (com *Artemia* e com *Hydrobia*) com o objectivo de descobrir o papel que estas espécies desempenham na cadeia alimentar. O conhecimento adquirido através do estudo de organismos tóxicos é importante para aumentar o conhecimento de quais as espécies, quando e como podem surgir nestas áreas como um surto nefasto. Outro aspecto interessante acerca dos dinoflagelados reside na sua filogenia; a análise detalhada de sequências genéticas como SSU-rDNA e LSU-rDNA vêm clarificar estas relações, podendo ainda ajudar em eventuais identificações ambíguas.

abstract

The Ria de Aveiro contain numerous ponds and sheltered areas, which, offer the appropriate conditions for the proliferation of a very diverse algal community, especially benthic algae, in which diatoms, dinoflagellates and green macroalgae predominate.

Some of the benthic dinoflagellates are known to produce toxic substances, the epibenthic dinoflagellate community is therefore the focus of this work. The dominant species of dinoflagellates in the ponds were from the genus *Prorocentrum* (*P. lima*, *P. micans*, *P. cassubicum*, *P. rhathymum* and probably two new species), but also other frequent species like *Coolia monotis*, *Amphidinium massartii*, *Bysmatrum subsalsum*, *Scrippsiella* cf. *trochoidea*, *Kryptoperidinium foliaceum*, *Oblea rotunda*, *Akashiwo sanguinea* and some species of the genera *Protoperidinium* and *Peridinium* were present.

Most of this species had been previously referred for Portuguese waters, except *A. massartii*, *O. rotunda*, *B. subsalsum*, *P. cassubicum* and *P. rhathymum*, although most of them had not been reported from the Ria de Aveiro (only *S. trochoidea*, *Peridinium quinquecorne*, *P. lima*, *P. minimum* and *P. micans* had already been cited from this area) (Moita & Vilarinho 1999).

Prorocentrum lima, *P. cassubicum* and *Coolia monotis* were found to be toxic to the brine shrimp, *Artemia salina*, but with different intensities, being *P. lima* the most toxic and *P. cassubicum* the less toxic. The occurrence of toxic species should be taken in consideration, since they can affect species in higher trophic levels, including humans that feed on organisms that accumulate the toxins, like molluscs. Ria de Aveiro is one of the major shellfish productions areas.

In relation to the environmental changes, most of the benthic dinoflagellates were found to be very tolerant to these modifications, although some species only appeared in some periods of the year, sometimes in large numbers.

The analysis of the benthic dinoflagellates from Ria de Aveiro includes the identification of the organisms, using light and electron microscopy (high resolution scanning and transmission electron microscopy), the establishment of laboratory cultures and the study of the toxicity of the species by bioassays (using *Artemia* and *Hydrobia*), with the aim of better understanding the role of these organisms in marine food webs.

This study also included a genetic approach, based on the sequence of the LSU-rDNA of the cultured species, for future incorporation in a phylogenetic tree, but the only sequences obtained were from *A. massartii* and *P. micans*.

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List of abbreviations

ASP – Amnesic Shellfish Poisoning

AZP – Azaspiracid Poisoning

CFP – Ciguatera Fish Poisoning

CTP – Cyanobacterial Toxin Poisoning

CTX – Ciguatoxin

DSP – Diarrhetic Shellfish Poisoning

DTX – Dinophysistoxins

HAB – Harmful Algal Bloom

HPLC – High Performance Liquid Chromatography

LSU-rDNA **–** Large Subunit of the ribossomal DNA

SSU-rDNA – Small Subunit of the ribossomal DNA

MNR – Magnetic nuclear resonance

MS – Mass Spectrometry

NSP – Neurotoxic Shellfish Poisoning

OA – Okadaic Acid

PP1 & PP2A – Protein Phosphatase type 1 & 2A

PSP – Paralytic Shellfish Poisoning

SEM – Scanning Electron Microscope

STX – Saxitoxin

TEM – Transmission Electron Microscope

YTX – Yesotoxins

General Introduction

The estuaries are one of most productive habitats on earth; they contain a diversity of species from marine to fresh water, providing conditions for the proliferation of characteristic species.

A variety of phytoplankton species can be found in this habitat, their importance is huge since they are primary producers, representing the first trophic level. Species from primitive blue-green algae (cyanobacteria), which are among the first photosynthetic organisms on the planet, to diatoms, dinoflagellates and green flagellates, can be found (Hallegraeff 2002). Phytoplankton is food for species from higher trophic levels like grazers or filter feeders, in which are included shellfish, crustacean's larvae and finfish (Daranas *et al.* 2001).

The occurrence of certain species or the development of algal blooms can have a serious and negative impact, causing severe economic losses to aquaculture, fisheries and tourism, affecting the environment and human health. Two mechanisms for this negative impact are of particular interest for the present study. One is the production of toxins that may accumulate through the food chain, affecting organisms at higher trophic level. Another mechanism is the drastic drop in oxygen level that happens during the decay of large blooms, which kills or inhibits the growth of other organisms (Hallegraef 1995).

Algal blooms (HAB) are a natural phenomenon, however it seems that in the last decades these episodes in general have been increasing and expanding geographically, affecting local ecosystems, public health and the economies of countries (Hallegraef 1995).

The dinoflagellates are one of the phytoplankton groups that have toxic species or species that form harmful blooms. Dinoflagellates are mostly unicellular algae, with two dimorphic flagella, that may be found in all aquatic environments, with the greatest diversity in the marine environment. Photosynthetic, heterotrophic and mixotrophic species of dinoflagellates occur, rendering the group interesting for both botanists and zoologists. They form an important part of the aquatic phytoplankton, but there are also many benthic species (Hoek 1995).

According to the symptoms observed in human intoxications by the secondary metabolites produced by dinoflagellates, it is possible to consider six types of illnesses caused by groups of marine toxins: Paralytic Shellfish Poisoning (PSP), Ciguatera Fish

Poisoning (CFP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning (NSP) and Azaspiracid Poisoning (AZP) (Daranas *et al.* 2001).

In estuaries, some dinoflagellate species find good conditions to grow and proliferate, taking advantage of stratified stable conditions, higher temperatures and high organic input from land run-off after heavy rain (Hallegraeff 2002).

The present work took place in Ria de Aveiro, a bar built estuary, in the NW cost of Portugal, is an important area at the economic level, since it is a place of salt, fish and shellfish production.

The estuary Ria de Aveiro exhibit a diverse community of epibenthic dinoflagellates, specially in particular areas designated by ponds, which represent old saltpans, their extensions can vary (from 100 to 1000 m^2), the water level is small at the margins and macroalgae find good conditions to develop in these areas. Some of the species found in these ponds are still insufficiently studied and many have the potential to produce several kinds of toxins. Therefore, it is essential to obtain information on these species, studying their structure, growth pattern, taxonomy, toxicity and role in the marine food webs. The ecology of these ponds has been little studied.

The knowledge about the evolution of dinoflagellates is not fully understood, the recent techniques of molecular biology seem to be the answer to this problem. The study of the SSU-rDNA and LSU-rDNA sequence from species in culture, together with all the other species of dinoflagellates already sequenced, can help to comprehend the relation between all of them and determinate which are the more primitive ones, which give rise to the others. For this kind of study the fossil record can also be very helpful.

Aim of study

Estuary ecosystems are dynamic and constitute one of the major contributors for primary production. They are the preferential habitat of thousands of species, especially algal species. In this ecosystem smaller communities can be established due to specific environmental characteristics, such as can be found in the benthic habitat.

The aims of this project are mainly the characterization of dinoflagellate species that choose these particular conditions (ponds with low depth in the margins and large masses of macroscopical algae), a benthic or epibenthic habit, and see how their abundances are related. Also, see how these species vary in relation with other surrounding species and environmental changes (season, temperature and salinity). Evaluate the impact that some of these benthic dinoflagellates can have in this ecosystem, having in consideration that some of them are toxic species, identified through the realization of the *Artemia* bioassay, and can be accumulated by gastropods like *Hydrobia ulvae*.

The identification of species is based on morphologic features observed using different microscopic techniques (light microscopy, epiflourescence microscopy, scanning electron microscopy and in some cases transmission electron microscopy), except for *A. massartii* which correct identification was only validated trough analyse of its LSU-rDNA sequence when compared with other *Amphidinium* sequences.

The study of the LSU-rDNA sequence from the cultured dinoflagellates was also an aim of this work, with the finality of adding this information to a phylogenetic tree, to better understand the relations between planktonic and benthic *Prorocentrum* species. Only for two species, *Prorocentrum micans* and *Amphidinium massartii*, this sequence was established; all the other species tested (*P. lima*, *P. cassubicum*, *P. rhathymum*, *Prorocentrum* sp.1, *Prorocentrum* sp. 2, *Coolia monotis* and *Bysmatrum subsalsum*) had no success. The main problems that contribute for this result were the mucilage, a substance produced by most of the benthic dinoflagellates that interferes with the PCR products and does not allowed the amplification process; and in some cases the insufficient number of cells (*B. subsalsum*), since some species in laboratory hardly reach dense cultures.

Thesis outline

To better understand the different methodologies used in this work this thesis is organized in four chapters: Chapter I - The ecology of sampling sites, Chapter II - Characterization of the dominant species of dinoflagellates, Chapter III - Toxicological study of the established cultures of dinoflagellates and Chapter IV – Preliminary phylogenetic study of selected species.

In each chapter a small introduction, description of the procedure, results and discussion is made. The references are organized in one single chapter at the end of the thesis. In the end of chapter four there is a brief general conclusion and some considerations on future work.

Chapter I

The ecology of the sampling sites

Introduction

The estuary

An estuary is an inlet of the sea reaching into a river valley as far as the upper limit of the tidal rise. Usually it can be divided into three sectors: a marine or lower estuary, in free connection with the open sea; a middle estuary where the salted water is mixed with the fresh water; and an upper or fluvial estuary, characterized by having essentially fresh water but still suffering the action of the tides (Day *et al.* 1989).

Ria de Aveiro is a complex extension of the Vouga River, ca. 60 km south of the city of Porto. It is the major lagoon on the coast of Portugal with more than 60 km of length and a maximum width of 20 km, depth usually smaller than 2 m, with only one small opening to the sea, a canal 300 m wide. It has been changing during the centuries due to human activity taking advantage of such a rich ecosystem, producing salt, using seaweed as manure, harvesting fish and shellfish, and conducting recreational activities.

Ecology of dinoflagellates

Although most of the dinoflagellates are planktonic, some species are benthic, living in the upper layers of marine sands, above macroalgae or in symbiosis in the tissue of some invertebrates (zooxanthellae) where they perform an important function (Hoek *et al.* 1995).

In favourable conditions, cells of some species can proliferate rapidly and produce dense blooms (Smayda 1997), as a result, the surface water can suffer discoloration. Some species produce poisonous blooms that can cause mass mortality to different types of organisms. Other blooms can light up the sea due to the bioluminescence of some dinoflagellate species (Hoek *et al.* 1995).

A property of some dinoflagellates is the capacity to produce cysts, which are resting stages produced during the sexual phase of the life cycle, which settle in the sediments where they remain until germination conditions become again suitable. Some cysts have very resistant walls and may fossilize, playing a very important role in the palaeontologics studies.

Toxic episodes in the "Ria de Aveiro"

The Ria de Aveiro has already a history of toxic episodes in shellfish, due to exposure to DSP (diarrhetic shellfish poisoning) toxins, being the blue mussel the best indicator species of this kind of intoxication, since it is the organism that accumulates the highest levels of this toxins (Vale & Sampayo 1999[a]; 2002[b]; 2003).

According to Vale & Sampayo 2003 it is in the driest and hottest months of the year that the occurrence of toxic blooms is higher, since the river influence is lower, the increase in the salinity give rise to a increase in the stability of the water column inside the lagoon, that turn the conditions favourable for the proliferation of toxic dinoflagellates. The rainy season is on the other hand the best time to avoid the proliferation of harmful algae.

Toxic blooms usually have a short lifetime, no more than a week in most of cases, but the impact that they can cause can be drastic if monitorization is not regularly made. The dominant dinoflagellate species that cause toxic blooms in the main canals of Ria de Aveiro (S. Jacinto and Costa Nova) are species from the genus *Dinophysis*, which contaminate most of the shellfish consumed by humans (clams, crabs and others) (Vale & Sampayo 2002[b]). These species are planktonic, occurring only where the water level is high. On the other hand, what happens in the areas where the depth is usually lower than 0,50 m is much less known.

The "Ria de Aveiro" offers a combination of different habitats. Some areas are more similar to freshwater habitats; others are more akin to the marine habitat. Even a particular area can change dramatically over a short period, due to changes in climatic conditions. The place where this influence is maximum is where the amount of water is smaller, that is, in small ponds. These ponds are appropriate for the study of benthic communities, and their relation to environmental changes.

Material and Methods

Study Site

Aveiro is a city located near the Atlantic Ocean, by a coastal lagoon called "Ria de Aveiro"(Fig. 1.1). The Aveiro estuary is a costal lagoon with a recent origin that has been developing fast under human influence. Table 1 shows its geographic parameters and the localization is shown in Fig. 1.1.

The Ria de Aveiro has along its margins a large area of natural and man-made puddles, ponds and saltpans (many of them no longer in use) (Fig. 1.2). A rich variety of organisms can be found in these highly variable and often sheltered habitats. Extensive macroscopical masses of green algae, mostly of the genera *Cladophora*, *Enteromorpha* and *Ulva* support a number of partly benthic, partly free-swimming photosynthetic and heterotrophic protists. Some of these areas develop important dinoflagellate communities, sometimes with large numbers of cells, which include *Prorocentrum lima* and several other potentially toxic species.

The sampling sites choosen for this study were costal ponds close to saltpans located near the Aveiro city. During a preliminary survey twelve sites were sampled, these sites were located in the area limited by “Canal de Navegação”, “Canal das Pirâmides”, the road IP5 and the red line marked in Fig. 1.2.

Two sites were studied more thoroughly, site 1 and site 2 (see Fig. 1.2 and 1.3), they are very close to each other, but they vary in terms of water supply and depth.

Site 1 is a pond where the depth varies in its extension, being lower in the margins (0,5 m) and higher in the centre (1,5 m to 2,0 m). This pond is not directly connected to the main canals, receiving water from adjacent ponds through conduits, in such a way that the influence of the tide in it is usually the opposite to the tides the sea.

Site 2 is a pond of low depth (0,5 m or less) in all its extension, is located very close to active saltpans. In some periods, the control of the water level was controlled by man.

Sampling methods and handling of samples

Planktonic and epibenthic communities from twelve sites in "Ria de Aveiro" were sampled irregularly for more than 2 years (2001-2004). Some of these sites had to be discarted during this period, because they became in use as saltpans. The criteria used to choose these sites was that they should have large masses of macroscopical green algae, low depth (max. 1 meter), suffer only small variation according with the tides and show some biodiversity. So in the end, only two sites (Site 1 and Site 2) were exhaustively studied (Fig. 1.2). At site 1, samples were collected from October 2001 until August 2004; at site 2, samples were collected from May 2003 until August 2004.

Qualitative phytoplankton samples were collected with 25 µm plankton net; aliquots of each sample were fixed in 4-6% Formalin in seawater and a live duplicate was kept for immediate examination under a light microscope. For a better evaluation of benthic species filamentous microalgae and macroalgal specimens were handpicked and processed in the same way as the plankton samples.

Table 1.1: Summary of the main geographic characteristics of "Ria de Aveiro" ("Ministério do Plano e da Admistração do território 1988"). *measured in the maximum limit of the level of water

Latitude	40 30' - 40 52' N
Longitude	8 35' – 8 47' W
Total area *	88 km^2
Submerse area	43 km^2
Intertidal area	20 km^2
Average volume	70.000.000 m^3
Average depth	1,5 m

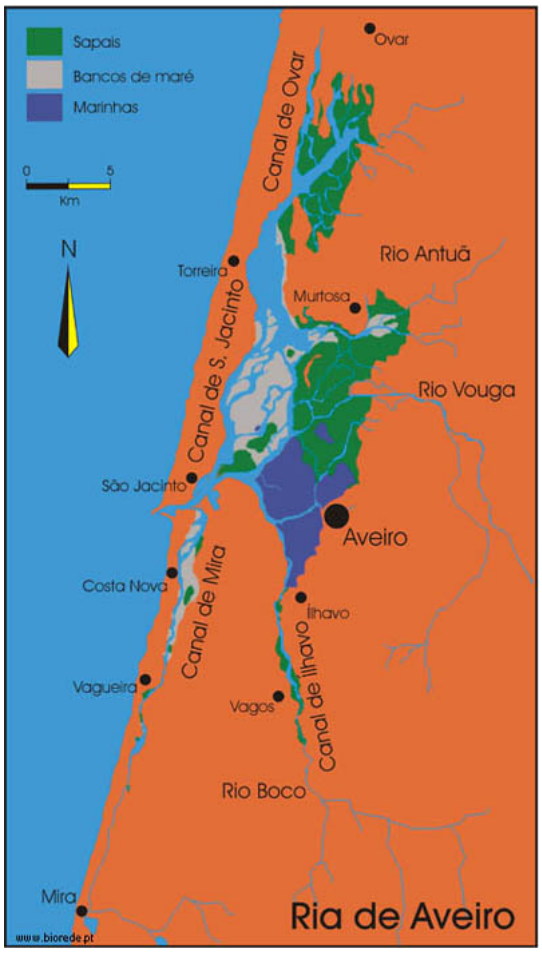


Fig. 1.1: Map showing the localization of the Aveiro estuary (Ria de Aveiro).

Fig. 1.2: Location of the sampling area. Site 1 and 2 are indicated

Temperature, pH and salinity were measured at each site during sampling. Quantitative samples (1 litre of seawater fixed with Lugol's solution) were taken in November 2003, February 2004, March 2004 and July of 2004.

Samples were studied with a light microscope connected to a video recorder. Subsamples were critical point dried and observed with a scanning electron microscope (Jeol JSM 5400). Specimens were also photographed with a traditional light microscope to register the variety of species on the samples as well as to document the morphology of the uncultured species.

Fig. 1.3: Pictures from the sampling sites from Aveiro Lagoon. a) site 1, b) site 2, collecting a plankton sample.

Results

Two sampling locations were selected for showing particular environmental conditions (for not changing drastically during the sample period, for having low depth and for exhibiting high masses of macroscopical algae). The results obtained in other sampling sites are partly redundant and are not presented here. These sites were sampled more irregularly and did not show to have the same level of biodiversity as the one observed in the selected sites (site 1 and 2) for this work.

Localities 1 and 2 are very close to each other, located on opposite's sites of one road. However, different communities and values of environmental parameters were often found during the same sampling occasion. Although these localities are under the influence of the tide, this can be considerably indirect, especially in site 1, in which the water level was often rising, while at the same time it was ebb tide in the nearby canals.

Environmental data

Salinity

Of the few measured parameters, salinity seems to be the most influential over the biodiversity in the ponds. The highest diversity was found when the salinity approached that of seawater, between 25 and 35 .

During one year it was possible to see how the values of salinity vary, according with the respective season, and this was parallel in the two study sites, but more marked in site 1. The salinity was lower in the winter, began to increase in the spring, and reached the maximum values around the end of summer and beginning of autumn, decreasing again in late autumn (see Fig. 1.4 a-b and Appendix 5 and 6).

In site 1 these variations of values were more intense, in the winter the salinity could be as low as 5 , whereas in site 2 it was never lower than 20 . Nevertheless, the maximum values of salinity were obtained in site 2, where it reached values in the order of 60 (being 50 for a long period. In site 1 the maximum salinity was 50 and only occurred once (see Fig. 1.4).

Temperature

The variation of temperature found for the two study sites was parallel to the variation of salinity, as would be expected since both are under climatic influence. In the winter, the atmospheric temperature is lower, so the water temperature is also lower, and during this period the rains are constant so the seawater is diluted into lower values of salinity.

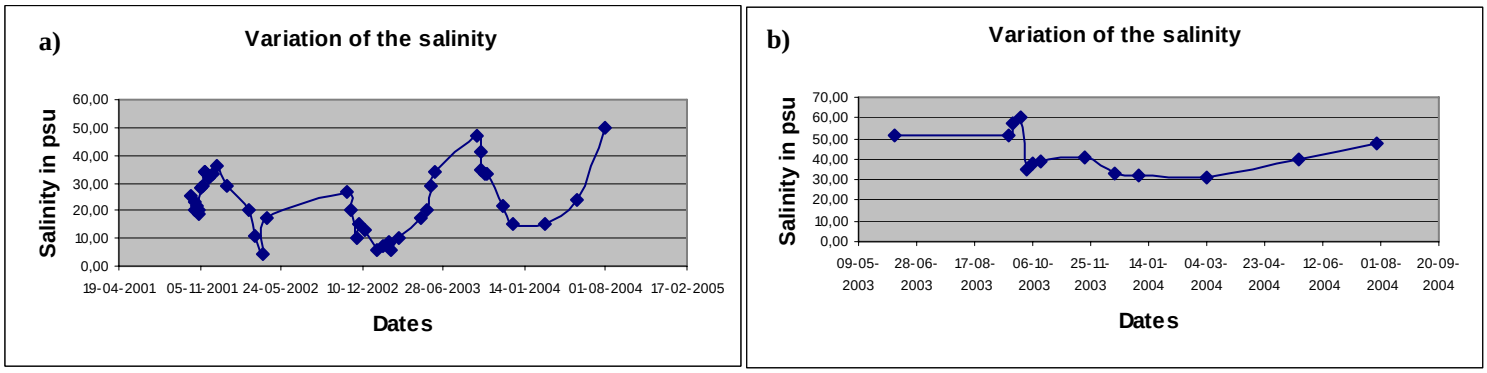


Fig.1.4: Variation of the salinity in the two sites studied: a) site 1 and b) site 2, through the period of sampling.

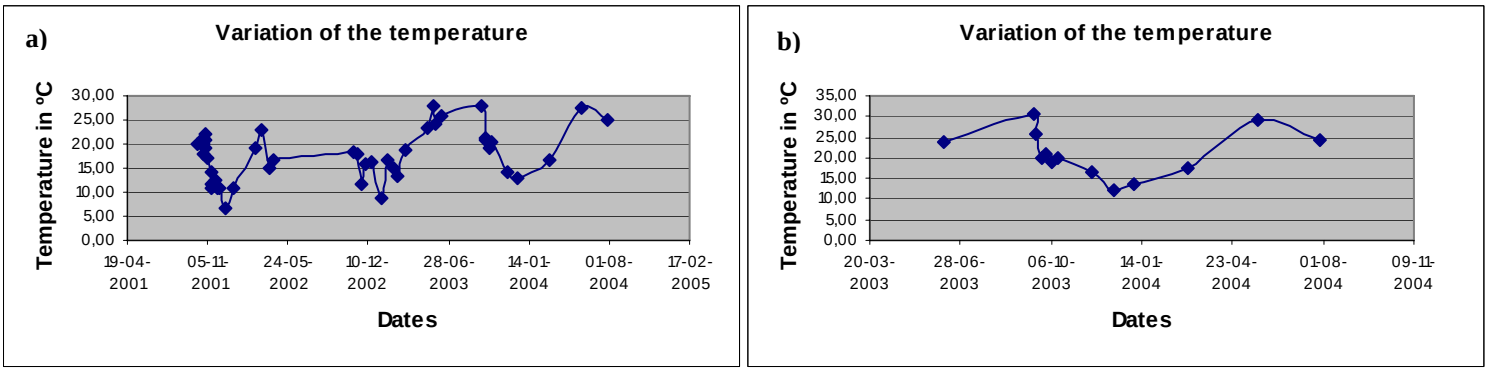


Fig.1.5: Variation of the temperature in the two studied sites: a) site 1 and b) site 2, through the period of sampling.

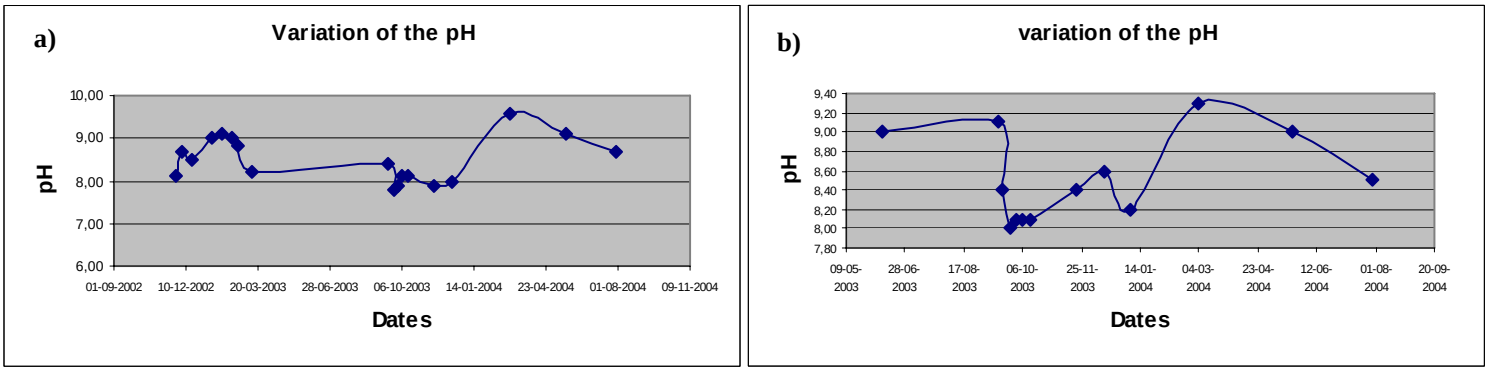


Fig.1.6: Variation of the value of pH in the two sites studied: a) site 1 and b) site 2, through the period sampling.

In the winter the temperature reached values low as 5ºC, but usually it was around 10ºC. In spring, the values increased to around 18ºC. The increase continued in summer, reaching values in the maximum of 30ºC. Finally in the autumn these values of water temperature began to decrease (see Fig. 1.5 a-b and Appendix 5 and 6).

pH

The pH seemed to be the parameter that less influences the biologic community in the ponds, since it only varied between 7,8 and 9,8 (see Fig. 1.6 a-b and Appendixes 5 and 6). The variations of these values can not be associated to changes in the wheather conditions.

How pH values influences the communities of organisms from the ponds was not understood. Usually elevations on pH are associated with an increase of photosynthetic rates from the primary producers.

The waters that are released to these ponds can also be responsible for the changes in the pH values, perhaps in some occasions there are residual water entering in the ponds, coming from freshwater effluents, but there is no way of evaluate the effects caused in the biodiversity established.

Biological data

The two ponds studied are quite similar in term of microalgal groups that are living there; the small flagellates are dominant in terms of numbers, and then come the dinoflagellates and the diatoms. However, detailed species composition was different in the two localities see Fig. 1.7, 1.8 and Table 1.2.

In site 1, in the samples of February and March similar populations were found, dominated especially by diatoms and small flagellates, at a time when salinities values were low. In July occurred an outbreak of a species of Euglenophyta, *Eutreptiella* cf. *gymnastica* (almost 13000000 cells/L), together with small flagellates, especially from the genus *Pyramimonas* (more than 6000000 cells/L), and dinoflagellates, with high number of cells of *Oxyrrhis marina* (more than 1000000 cells/L), *Bysmatrum subsalsum* and *Prorocentrum micans*, see Fig. 1.7 and Table 1.2. In the other samples, the abundances of the dinoflagellates were quite low.

Site 2 always showed higher variety of species, even in the winter samples. The dominant group were also the small flagellates (which included species from the genera *Cryptomonas* and *Pyramimonas* and other small green flagellates). The second most abundant group were the dinoflagellates, of which *Prorocentrum micans, Prorocentrum cassubicum* and *Oxyrrhis marina* occurred in the highest numbers, and, on one occasion (November 2003) *Heterocapsa niei* (with more than 180000 cells/L), see Fig. 1.8 and Table 1.2.

In site 2 the diatoms were in high numbers in the samples of November and February, but the abundances of dinoflagellates were of the same order, in the other samples this latter group was always with higher abundances. In March the most abundant dinoflagellates species were *Oxyrrhis marina* and an athecate dinoflagellate not identified (with more than 600000 cells/L of each), but in July *Oxyrrhis marina* reached the 570000 cell/L, being the most abundant taxa of the sample.

Site 2 showed usually higher abundances in all identified taxa, and was the only to register significant numbers of Cyanophyceae species (*Chroococcus* sp., *Oscillatoria* sp., *Lyngbya* sp. and *Spirogyra* sp.) during the samples of November and July.

The diversity of dinoflagellate species found during all the samples was much higher than the one found in these quantitative samples, see Appendixes 3, 7 and 8. Benthic, planktonic and tycoplanktonic dinoflagellates were recorded (see Appendix 3). Benthic dinoflagellates included 4 species from 2 genera: *Coolia monotis, Prorocentrum lima, P. cassubuicum* and *Prorocentrum* sp. 1; 7 tycoplanktonic species from 5 genera: *Amphidinium carterae, A. massartii, Kryptoperidinium foliaceum, Heterocapsa niei, Bysmatrum subsalsum, Prorocentrum rhathymum* and *Prorocentrum* sp. 2; and 11 planktonic species from 11 genera: *Akashiwo sanguinea, Gymnodinium catenatum, Gyrodinium* sp. 1, *Pheopolykrikos* sp. 1, *Protoperidinium minutum, Peridinium quinquecorne, Oblea rotunda, Scrippsiella trochoidea, Oxyrrhis marina, Prorocentrum minimum* and *P. micans.*

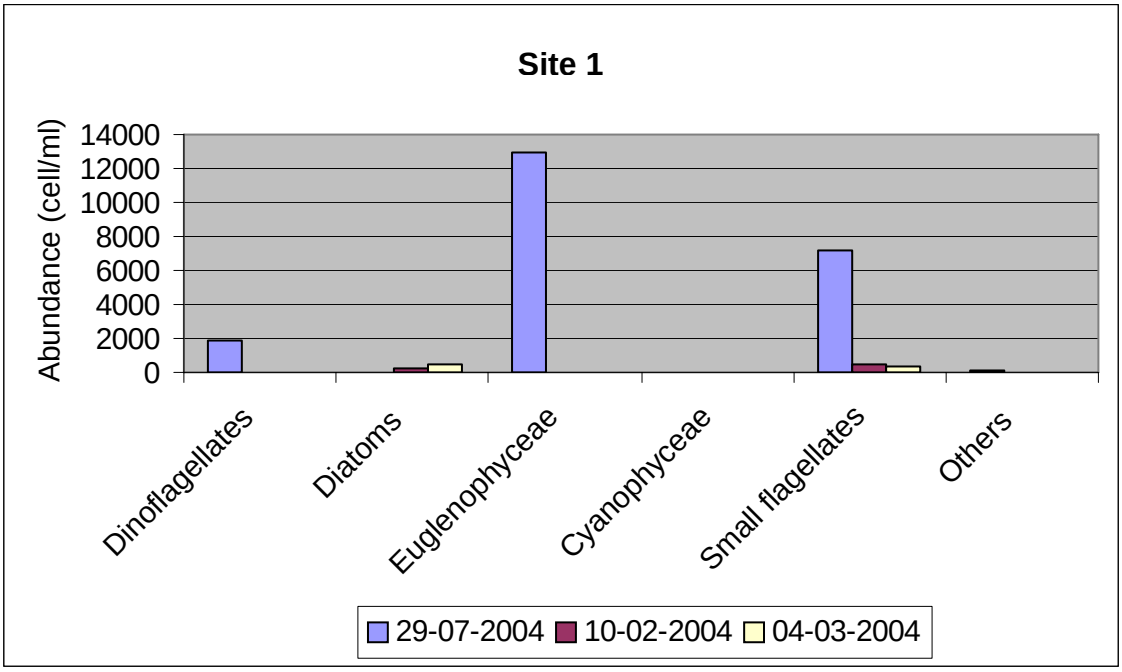


Fig. 1.7: Abundance of the major groups of organisms found in the samples collected from Site 1 at different moments of the year.

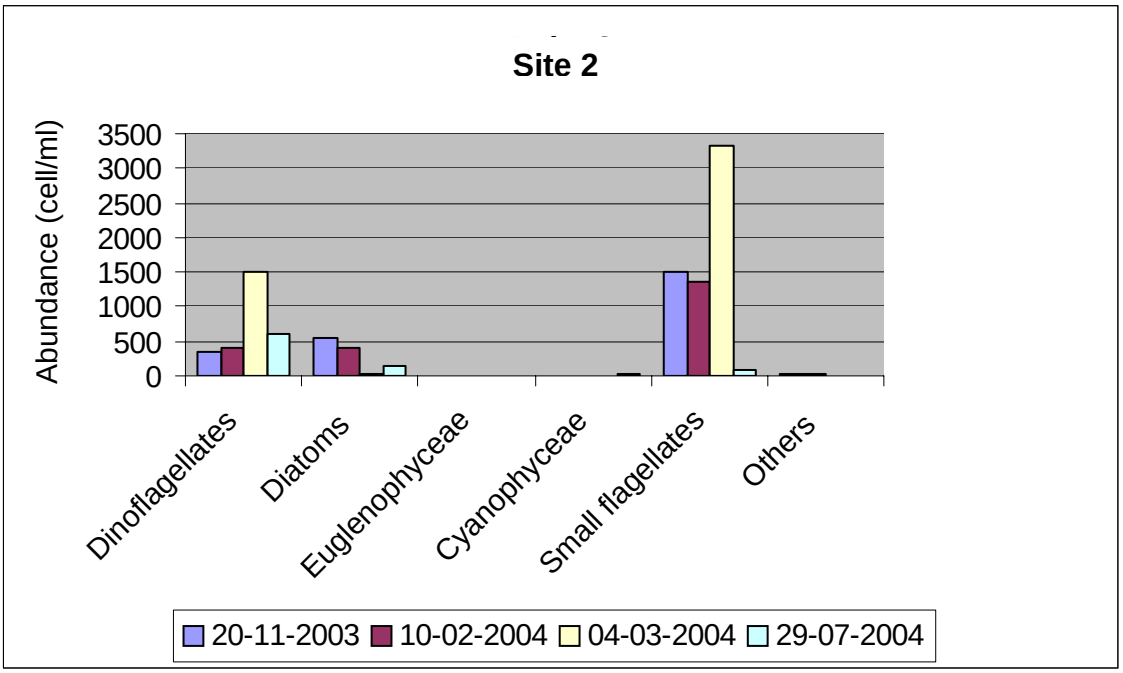


Fig. 1.8: Abundance of the major groups of organisms found in the samples collected from Site 2 at different moments of the year.

Table 1.2: Quantitative list of taxa found in the two sampling sites. Concentrations are given in cells/L.

Date	**20-11-2003**	**10-02-2004**		**04-03-2004**		**29-07-2004**	
Sites	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Taxa							
Dinoflagellates	361360	4643	366293	3467	1494322	1938202	610211
Prorocentrum lima	2346	0	240	0	4623	0	0
Prorocentrum sp.1	7039	0	4693	0	3467	0	5779
Prorocentrum cassubicum	14079	1156	86820	0	0	0	0
Prorocentrum sp. 2	16425	0	0	0	3467	0	1156
Prorocentrum micans	98553	0	2346	0	40450	539693	0
Amphidinium sp.	0	0	4693	0	36982	0	0
Oxyrrhis marina	35197	3467	53969	2311	669151	1173246	572072
Athecate dinoflagellate	0	0	0	1156	708445	0	0
Thecate dinoflagellate	0	20	213531	0	5779	0	10401
Gymnodinium sp.	0	0	0	0	21958	61009	4623
Bysmatrum subsalsum	0	0	0	0	0	164254	10401
Coolia monotis	0	0	0	0	0	0	5779
Heterocapsa niei	187719	0	0	0	0	0	0
Diatoms	542039	240466	410936	459969	34671	0	143327
Pennate diatoms	258114	41605	215877	65875	28893	0	128283
Centric diatoms	272193	197625	122018	307417	1156	0	0
Cylindrotheca closterium	2346	1156	65702	0	0	0	2311
Entomoneis sp.	0	20	7039	0	0	0	20
Pleurosigma angulatum	2346	40	300	8090	0	0	3467
Navicula spp.	0	20	0	0	0	0	3467
Cymbella spp.	0	0	0	11557	2311	0	0
Diploneis sp.	0	0	0	12713	0	0	0
Achnanthes coarctata	7039	0	0	40450	2311	0	3467
Eunotia sp.	0	0	0	8090	0	0	0
Surirella sp.	0	0	0	2311	0	0	0
Cocconeis sp.	0	0	0	3467	0	0	0
Striatella unipunctata	0	0	0	0	0	0	2311
Biddulphia sp.	0	0	0		0	0	19647
Euglenophyceae	0	0	0	0	0	12952632	3467
Eutreptiella cf. *gymnastica*	0	0	0	0	0	12952632	3467
Cyanophyceae	9386	0	0	0	0	0	4643
Chroococcus sp.	2346	0	0		0	0	3467
Oscillatoria sp.	2346	0	0		0	0	20
Lyngbya sp.	4693	0	0		0	0	0
Spirolina sp.	0	0	0		0	0	1156
Small flagellates	1511140	417208	1370351	357112	3322643	7161491	77432
Cryptophyceae	72741	23114	260461	21958	132906	539693	77432
Prasinophyceae	1438399	394094	1109890	335154	3189737	6621798	0
Others	35197	3587	32911	8100	12713	103246	11557
Ciliates	25811	3467	21118	1156	10401	75088	6934
Nematoda	0	10	2346	0	1156	0	1156
Arthropoda	9386	110	9386	6934	1156	28158	3467
Rotifera	0	0	60	10	0	0	0

Discussion

The phytoplankton diversity in the ponds from Ria de Aveiro

The ponds studied although being under the same environmental conditions show some heterogeneity in terms of the biodiversity found there, especially in terms of abundances.

The main group always present in the ponds were the small green flagellates, found in very high quantities. Then depending on the season other groups could be the second more abundant. From spring to autumn, the dinoflagellates were quite abundant, at these periods, the values of salinity were high, higher than 30 , and the water temperature mild, adequate conditions for the proliferation of these marine dinoflagellates, which reached high densities in some samples (*O. marina*, *P. micans, P. cassubicum, B. subsalsum*, *H. niei*, *C. monotis* and *A. massartii*). From autumn to the beginning of spring, when the salinity was lower, around 15 and the water colder, around 10ºC, the diatoms were found in high abundances. In the summer of 2004, in site 1 occurred an outbreak of an Euglenophyta species *Eutreptiella* cf. *gymnastica*.

The diversity of dinoflagellates in ponds

Dinoflagellate taxa found in the studied ponds from Ria de Aveiro include benthic, planktonic and tycoplanktonic (see Appendix 3).

Prorocentrum species were the dominant species in the ponds studied, specially the planktonic *P. micans*, and the benthic species *P. lima* and *P. cassubicum*, present in most of the year, occurring in some periods in huge amounts. This situation must be taken in consideration since these two benthic species are toxic. Nevertheless, species as *C. monotis*, *A. massartii* and *B. subsalsum* can occur with elevated abundances what should be taken in consideration, especially in the case of *C. monotis* that is also a toxic species.

Most of these species were already known for Portuguese waters but only few for the Ria de Aveiro (*P. quinquecorne*, *S.* cf. *trochoidea, P. minimum, P. lima*, *P. micans* and *O. marina*). Species like *Amphidinium massartii, Oblea rotunda*, *Bysmatrum subsalsum*, *Prorocentrum cassubicum* and *P. rhathymum* were never before reported for Portuguese waters (see Appendix 4) (Moita & Vilarinho 1999).

The benthic community of dinoflagellates

The community found in the ponds was not exclusively represented by benthic species. Many species were planktonic and other show a benthic and planktonic behaviour (tycoplanktonic). However, although there is no physical barrier between both types of organisms the benthic species tended to be the dominant group.

The dinoflagellates that attach themselves to a large variety of substrates (as sand, sea glass blades, macroalgae, dead corals, etc) sometimes also swimming freely, constitutes the epibenthic community. Benthic species can form a mucilaginous matrix (MacKenzie *et al.* 2002), swim freely within the water column, entangle in detritus aggregates, and glide within the open spaces between sediments. The population density that they can reach varies daily and it depends on the sites (Faust 1996).

In the work by Faust (1995) eleven dinoflagellates species were found that could be considered as being benthic, from this group *Prorocentrum hoffmannianum*, *P. lima*, *Gambierdiscus toxicus*, *Ostreopsis lenticularis* and *O. ovata* are epiphytic; *P. mexicanum*, *P. rueztlerianum*, *P. foraminosum*, *P. maculosum*, *P. hoffmannianum*, *P. emarginatum*, *Coolia monotis*, *Amphidinium* sp. and *P. foramen* are benthic-detrital; *P. elegans* and *P. caribbeaum* are bloom forming; and *P. sabulosum*, *P. sculptile*, *P. arenarium*, *P. lima*, *P. hoffmannianum* and *Amphidinium* sp. are sand-dwelling. These benthic species have been responsible for bloom outbreaks in some periods, especially in summer, and implicated in causing humans diseases, caused by toxins accumulated through the marine food web organisms (Faust 1996; Morton & Faust 1997).

The ecology of toxic species is complex and is not restricted to benthic or epiphytic habit, toxicity on dinoflagellates may depend of many factors like: clonal variations, life cycle stages and habitat preferences (Faust 1995).

Within the benthic species found in the Ria de Aveiro it was possible to distinguish (1) epiphytic species (associated with macroalgae): *Prorocentrum lima, P. cassubicum* and *Prorocentrum* sp. 1; (2) benthic detrital: *P. rhathymum*, *P. micans*, *Coolia monotis* and *Amphidinium massartii*; and (3) bloom forming: *P. lima*, *P. cassubicum*, *Prorocentrum* sp. 2; and *A. massartii*, all of then can still be found in the water column swimming freely.

The heterotrophic community of dinoflagellates

Dinoflagellates may combine autotrophic and heterotrophic features, consequently its species have been studied and described by both phycologists and protozoologists. Dinoflagellates include many species that are partly or entirely heterotrophic, about half of the known dinoflagellates are presumed to be heterotrophic or mixotrophic, found on 9 of the 13 orders recognized by Larsen & Sournia (1991). This is a very diverse group, with sizes ranging from a few micrometers to a couple of millimetres size, with very simple to rather elaborate morphologies. The heterotrophic species are not usually responsible for red tides, they are encountered in small numbers in plankton samples, but they may be important in controlling outbreaks of other dinoflagellates species (Larsen & Sournia 1991).

Heterotrophic dinoflagellates developed mechanisms that allow them to efficiently ingest other organisms (Calado & Moestrup 1997). They find food in the autotrophic organisms mostly. They can be distinguished from the others because they lack photosynthetic apparatus, which gives them a translucent aspect. Some species posses a photosynthetic apparatus, but it seems that it is not sufficient to respond to all their thophic necessities, which they complement with phagotrophic feeding.

The heterotrophic dinoflagellates species found in the present studies were: *Oxyrrhis marina*, *Oblea rotunda*, *Protoperidinium minutum*, a colourless *Gyrodinium* and *Polykrikos* sp..

The studied ponds have periods of high level of biodiversity or by contrast periods when a single species dominates the assemblage. It was in the latter situation the heterotrophic dinoflagellates were more abundant. For instance from the quantitative data was possible to see that the highest concentrations of the heterotrophic *Oxyrrhis marina* co-occurred with the high numbers of a small flagellate, which usually was a Prasinophyceae species.

Chapter II

Characterization of the dominant species of dinoflagellates

Introduction

The dinoflagellates

The Dinophyceae is a unique algal group of protists, with very peculiar characteristics that makes them an excellent subject for the scientists that study algae and for the ones that study invertebrates, since it is a controversial group with affinities with both phyla.

Main morphological and ecological characteristics

The principal characteristics of the Dinophyceae are that they are eukaryotic unicells, with two dimorphic flagella; the main pigments are chlorophylls a and c2. The nucleus is peculiar, since the chromosomes are continually condensed, they lack histones, and it is usually designate by dinokarion. Some species are involved by an armour called theca; this theca is organized in small pieces called plates, the organization of this plates form a unique pattern specific for each species (Steidinger & Tangen 1996).

The reproduction is done usually by binary fission, but sexual reproduction can also occur when the environmental conditions are not the ideal ones. Their nutrition can be autotrophic, auxotrophic or heterotrophic and possess food reserves in the form starch and oil. The habitat varies from marine, where 90% of the dinoflagellates live, to freshwater, pelagic, benthic and symbiotic (Steidinger & Tangen 1996).

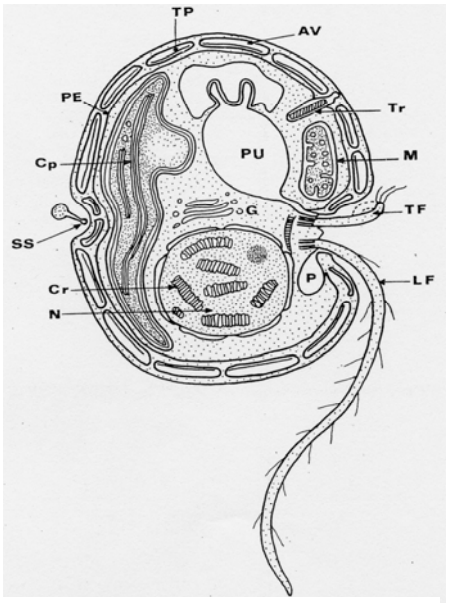


Fig. 2.1: Draw of a dinoflagellate cell in longitudinal view. AV- anphiesmal vesicle; Cp- chloroplast; Cr- chromosome; G- Golgi complex; LF- longitudinal flagellum; M- mitochondrion; N- nucleus; PE- pellicular layer; P, PU- pusule; SS- striated strand; TF- transverse flagellum; TP- thecal plate; Tr- trichocyst (adapted from Taylor 1980).

Terminology and morphology

The Dinophyceae include more than 2000 living and 2000 fossil species, belonging to about 130 genera, many have bizarre forms that makes them very interesting at the microscope (Graham & Wilcox 2000).

The dinoflagellates used to be differentiated in two general types of cells, based on the position of the flagella: (1) desmokont (fig. 2.2) – where the two dissimilar flagella emerge from the cell apex (the region where the flagella emerge is the apical region, and the opposite pole is the antapical region). And (2) dinokont cells (fig. 2.2): having dissimilar flagella that emerge from the median region of the cell that presents a groove - the cingulum, which divides the cell in two parts, the apical part – epicone or epitheca and the posterior part – hypocone or hypotheca. A smaller groove known as sulcus extends posteriorly from the cingulum to the hypotheca. At intersection between these two grooves is a pore from which the two dissimilar flagella emerge. The transversal flagellum lies in the cingulum and the longitudinal flagellum emerges from the sulcus, the region from where the flagella emerge is defined as being the ventral side of the cell (Graham & Wilcox 2000).

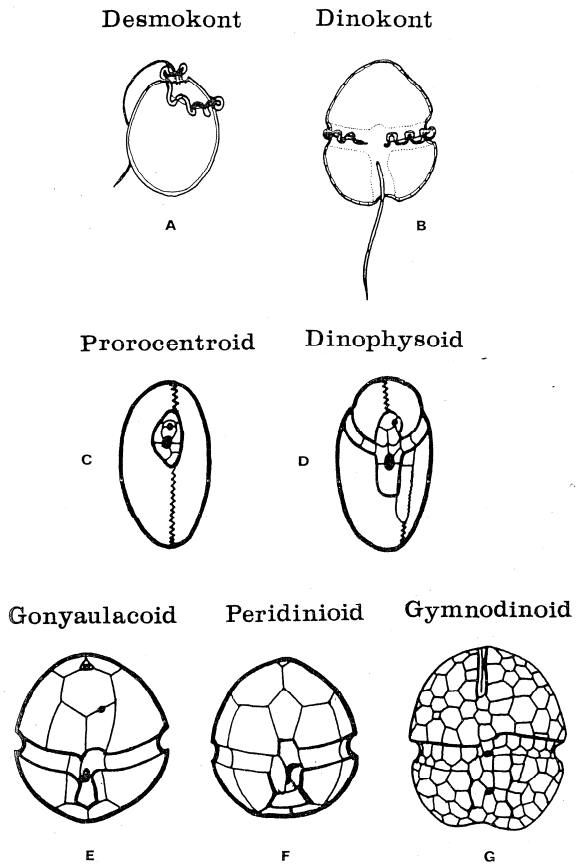


Fig. 2.2: Major types of organization of a dinoflagellate cell. A, B – basic flagellar arrangements; C-G – Basic thecal types (adapted from Taylor 1980).

In Taylor (1980), he proposed 5 major tabulation types of organizations of the cell: Prorocentroid, Dinophysoid, Gonyaulocoid, Peridinioid and Gymnodinoid (Taylor 1980).

The Prorocentroid cell organization is different from all the others since there is no epitheca or hypotheca. Species from this genus are characterized by the presence of two opposing plates or valves that are laterally compressed, connected by a well defined

intercalary band, with anteriorly inserted flagella. One valve, designated right valve, has a small apical depression in V-shape, where several small periflagellar plates are located, as well as the pore from which the flagella emerge. The other valve, designated left valve, is very flat and has no apical depression (Faust *et al.* 1999; Hansen *et al.* 2001).

Material and Methods

Establishment of cultures

Cultures of several species were cultivated by isolation of cells from samples from the field using the pipetting or washing method (Pringsheim 1946). In this method individual cells were picked up, using an inverted microscope (Wild Leitz Cmbh), washed at least three times in the medium and inoculated into 2 ml of F/2 medium (Guillard & Kelley 1984) (procedure to the preparation of medium in the Appendix 1) in stereo multi-well boxes (24 wells Starsted), one or two specimen for each well. These boxes were kept in culture chambers at 15, 18 and 20 °C with a photoperiod of 12:12 hours (L:D) (in Forma Scientific Diurnal Growth Chamber, Sanyo Growth Gabinet and Binder with light intensity of 584 lux, 1492 lux and 1030 lux respectively).

When unialgal culture begins were dense, aliquots were collected and transferred into sterile 50 ml culture bottles (Starsted 50 ml) containing F/2 medium. Time to time these bottles were replicate, so that the cultures could be kept in laboratory conditions for long periods.

Morphological study

Light microscopy

Aliquots from the field samples and cultures were observed and photographed in a light microscope, Zeiss Axioplan II imaging, equipped with 63X (n.a. 1,4) and 40X (n.a. 1,3) objectives with oil immersion and interferential contrast.

Light microscopy pictures from the cultures were also taken on an Olympus BX60 microscope with a 5X digital photo camera (3CCCD Sony Colour Video Camera). Pictures

that if possible could show dorsal and lateral views of the cell, the flagella, the pyrenoid and nucleus.

Two different methods were used to visualize the cells, Nomasrki to have a general view of the cell, to see the organelles and to determine the size of the cell (taking morphometric measurements – see Appendix 9). For tabulation study epiflourescence light microscopy was used using calcofluor white in a working stock solution of 10 mg/ml in distilled water. Calcofluor absorbs UV light radiation in the 340-400 nm wavelength and re-emits visible blue light, making possible to see the theca shape, the type of ornamentation, number and organization of plates (Fritz & Triemer 1985).

Scanning electron microscopy (SEM)

Procedure 1 (Portugal)

Samples from the field and species cultures were prepared for scanning electron microscope. Fixated with a final concentration of 2,5% of glutaraldehyde, filtered with a polyester filter with 5 μm diameter and washed several times with distilled water. The filter was then collected and put inside a porous capsule. Dehydration was done by introducing the capsule in a succession of increasing concentrations solutions of ethanol in water (30%, 50%, 75%, 90%, 96% and 100%) in intervals of 10 minutes. Following this procedure the capsule was allowed to rest into a new 100% ethanol solution until the critical point dryer is ready. The critical point was achieved in a Bal-Tec CPD 030-critical point dryer with carbon dioxide gas.

The filter was removed from the capsule and attached to a SEM stub.Then the sample was sputter-coated with palladium and gold. Samples were observed with a scanning electron microscope (Jeol JSM 5400 with photo-machine).

Procedure 2 (Denmark)

Fractions of the pure cultures species were fixed individually with 1% of osmium for 1 hour in a centrifuge tube. This mixture was then transfered to a syringe tube attached to a swineex system, whith a 8 μm Millipore filter. The sample allowed to pass through the filter, and was abundantly washed for one hour with distilled water. The dehydration

begins with a 30% concentration of ethanol; changes of ethanol were made every 15 minutes in increasing concentration of ethanol until 100% (30%, 50%, 70%, 96%, 99% commercial and 99% with molecular sieves). In the step of the ethanol 99% with the molecular sieves the swineex was introduced in a glass and field with this solution, it was kept there for 1 hour, with a solution change after 30 minutes.

In the end, the swineex was introduced into the critical point dryer with ethanol (Baltec CPD-030). As described above the filters were then inserted on metal stubs and coated with plattium/palladium in a high-resolution fine coater (Jeol JFC 2300 HR). The observations were made in an electron-scanning microscope (FE-SEM Jeol JSM-6335F) and digital pictures were taken (Pickett-Heaps 1998; Truby 1997).

Transmission electron microscopy (TEM) (Denmark)

The cells were fixed for 1 hour by adding 25 ml of 2% glutaraldehyde in 0,1 M cacodylate buffer with 0,3 M sucrose to 25 ml of dense pure culture at 4°C. The material was then centrifuged at 2300 rpm, at 20°C for 5 minutes. The pellet was washed in the same buffer 3 times for 20 minutes each, with decreasing concentration of sucrose (0,3 M, 0,15 M, 0,0 M).

The post fixation was done with osmium 1% in buffer for 2 hours at 4°C. Following a brief wash in buffer and in water, the cells were dehydrated in an ethanol series (15%, 30%, 50%, 70%, 96%, 99% commercial and 99% with molecular sieves) in intervals of 20 minutes and at 4°C until the 99% concentration, after which the tube was kept at room temperature. Following two changes in 1,2-propylene oxide for 5 minutes each, the pellets were embedded in a mixture of Spurr resin with 1,2-propylene oxide in a 1:1 proportion overnight. A new change of Spurr was made and let to rest for 3 hours, followed by the preparation of the blocks. The blocks were polymerised at 70°C for 8 hours.

For each species slight variations in the fixation step were tried, sometimes the fixator was diluted or different buffers, culture medium or seawater were used.

Results

Cultures established

Thirteen species were cultured and maintained during this study in F/2 medium. Attempts to grow heterotrophic species of *Gyrodinium* were not successful. The process of culturing these species was not always easy, especially due to problems with precipitation in the culture medium (solved by introducing the nutrients in the autoclaved seawater through a syringe with a filter attached) and with contamination by other species, like diatoms from the genus *Chaetoceros.*

Table 2.1. Cultures isolated and maintained during this study.

Species	Isolation Date	Isolation Place	Current Status
Prorocentrum lima	December 2001	Ria de Aveiro	Alive
Prorocentrum cassubicum	February 2002	Ria de Aveiro	Alive
Prorocentrum micans	March 2003	Ria de Aveiro	Alive
Prorocentrum rhathymum	September 2003	Ria de Aveiro	Alive
Prorocentrum sp.1	September 2003	Ria de Aveiro	Alive
Prorocentrum sp. 2	December 2003	Ria de Aveiro	Alive
Akashiwo sanguinea	June 2003	Ria de Aveiro	Alive
Kryptoperidinium foliaceum	June 2003	Ria de Aveiro	Alive
Amphidinium massartii	December 2003	Ria de Aveiro	Alive
Bysmatrum subsalsum	June 2003	Ria de Aveiro	Alive
Scrippsiella trochoidea	January 2004	Ria de Aveiro	Alive
Heterocapsa niei	June 2003	Ria de Aveiro	Alive
Coolia monotis	March 2003	Ria de Aveiro	Alive

Species descriptions - morphology

The synonymies used in the follow descriptions are a compilation of different sources: Steidinger & Tangen (1996); Dodge (1975); Dodge (1982); Hallegraeff (2002); Faust *et al.* (1999); Faust & Gulledge (2002).

Order Prorocentrales
Family Prorocentraceae

Genus *Prorocentrum* Ehrenberg

Description

The species from the genus *Prorocentrum* are characterized by having two laterally compressed valves, which gives to the cell a very thin aspect in side view. The area where both valves connect is the intercalary band, it can be easily distinguished and the thickness can vary slightly. The flagella come out through a pore (flagellar pore) in the apical area of the cell where a V-shaped depression is found, usually continuing down on the right valve; many little apical plates can be found associated in this area. Near the flagellar pore is usually another pore, called the auxiliary pore, but its function is not clear. The theca is usually ornamented and the kind of ornamentation is characteristic for each species.

All species are photosynthetic; they have two large peripheral chloroplasts. The nucleus is in a posterior position and is relatively large.

The main morphologic features, toxicity and habit of the different species of *Prorocentrum* species found in the ponds from the Ria de Aveiro are resumed in two tables in the end of this section, see Table 2.1 and Table 2.2.

Prorocentrum lima (Ehrenberg) Stein 1878
Basionym: *Cryptomonas lima* Ehrenberg1860
Synonyms: *Exuviaella marina* Cienkowski 1881
Exuviaella lima (Ehrenberg) Bütschli 1885
Exuviaella marina var. *lima* (Ehrenberg) Schiller 1933
Dinopyxis laevis Stein 1883
E. cincta Schiller 1918
E. ostenfeldii Schiller 1933
E caspica Kiselev 1940
Prorocentrum marinum Dodge & Bibby 1973 comb. invalid

Ehrenberg (1860); Ehrenberg (1873); Stein (1878); Schiller (1928); Schiller (1933); Dodge (1965); Dodge (1975); Fukuyo (1981); McLachlan *et al.* (1997); Faust (1991); Faust *et al.* (1999); Faust & Gulledge (2002).

Plate I and II

Description

Prorocentrum lima was the most predominant benthic dinoflagellate founf in the samples, present in all collecting sites at almost all the collecting times. It could be found in the samples swimming freely or attached to the thalli of green macroalgae like *Cladophora* sp., *Enteromorpha* sp. and *Ulva* sp.. In the summer, when the salinity of the ponds can be quite high, this species appears at higher densities.

The cultures of *P. lima* had the tendency to aggregate at the bottom of the culture vessels, specially when growing at temperatures lower than 20°C, the aggregates can be of thousands to millions of cells, visible to the naked eye, consistent with the benthic nature of this species. The feature that allows them to aggregate is the capacity to produce huge amounts of mucus. Fukuyo (1981) already observed this tendency of *P. lima*, having found it very often attached to algae, and in culture showing a tendency of adhering to the wall of the culture vessel and rarely swimming freely.

Cells of *P. lima* were ovate, medium size: 43 µm length, 25 µm width and a ratio l/w of about 1,6. In side view it is possible to see how narrow the cell is, since it is less than 20 µm thick. In the light microscope, the cells showed a yellow-brown colour, due to the two large chloroplasts and the big double pyrenoid in the centre of the cell. The nucleus is large and has a posterior position, in the anterior area two big vacuoles can be found. From the V-shape depression on the top of the right valve of the cell two dimorphic flagella come out.

With the scanning electron microscope it was possible to see that the theca had a smooth surface, in the right valve is the prominent V-shaped depression, where are inserted the flagellar and auxiliary pores, and several small apical plates. The surface of the valve is covered with randomly distributed round pores, numbering about 100; in the margin there are about 60 pores regularly distributed; all pores have smooth edges, and no pores were found in the centre of the theca, where a slight but wide depression is seen, with the same general shape of the pyrenoid.

Distribution

Prorocentrum lima was usually present in the ponds studied. It was more abundant in periods of higher salinity (higher than 20) and temperature (higher than 18ºC). This species seemed to prefer the late summer or beginning of autumn to form small blooms. In

winter when the salinity was low, only few cells were found and they were not in the best conditions.

Toxicity: Toxic species, produce okadaic acid, responsible for syndromes as ciguatera and DSP.

Remarks

Ehrenberg (1860) was the first to describe this organism and named it *Cryptomonas lima*, although his identification had a poor description of the species, some characteristics were already noted, but on his first drawing (1873) the cell appeared covered with spines, which are not found actually in the synonym species *P. lima* (has the theca covered by pores). Cienkowski (1881) described this species as being *Exuviaella marina* and he recognized its major features: an excavated plate in the right valve, pores in the valves, two dimorphic flagella, nucleus, chloroplasts and reserve organelles (McLanchlan *et al.* 1997). Although was Stein (1878) who first suggested the combination to *Prorocentrum lima*, only with the work of Dodge (1975) this name was established. In the same work Dodge put together all the *Prorocentrum* and *Exuviaella* species in the same genus *Prorocentrum*, assuming that these genus were too similar to be separated. This is now being discussed again, McLanchlan *et al.* (1997) proposed the separation of marine *Prorocentrum* species and the reinstatement of the genus *Exuviaella* for the benthic species.

Prorocentrum lima is known for including specimens with morphological variations like: different size range, shape and type of pores, especially when comparing specimens from temperate and tropical waters (Faust 1991; Morton & Tindall 1995; Bouaïcha *et al.* 2001).

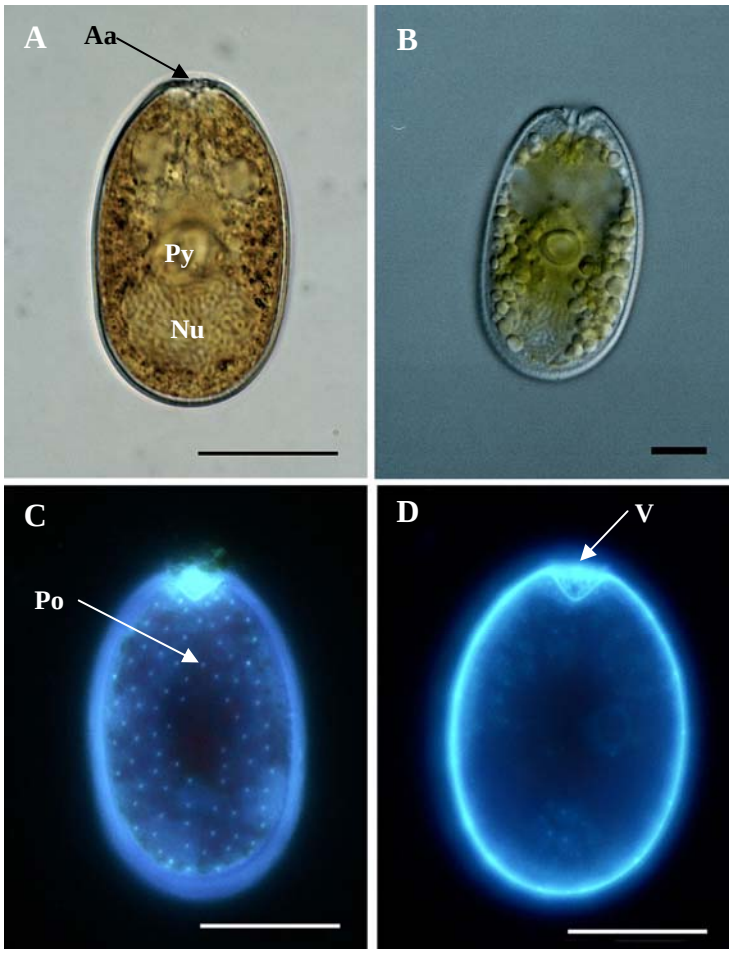


Plate I. *Prorocentrum lima*, cells in valve view. A and B light microscopy images; C and D epiflourescence images. Scale bars = 20 μm in A, C and D; 10 μm in B. Aa – apical aperture, Py – double pyrenoid, Nu – nucleus with condense chromosomes, Po – thecal pores and V – V-shape depression in the right valve.

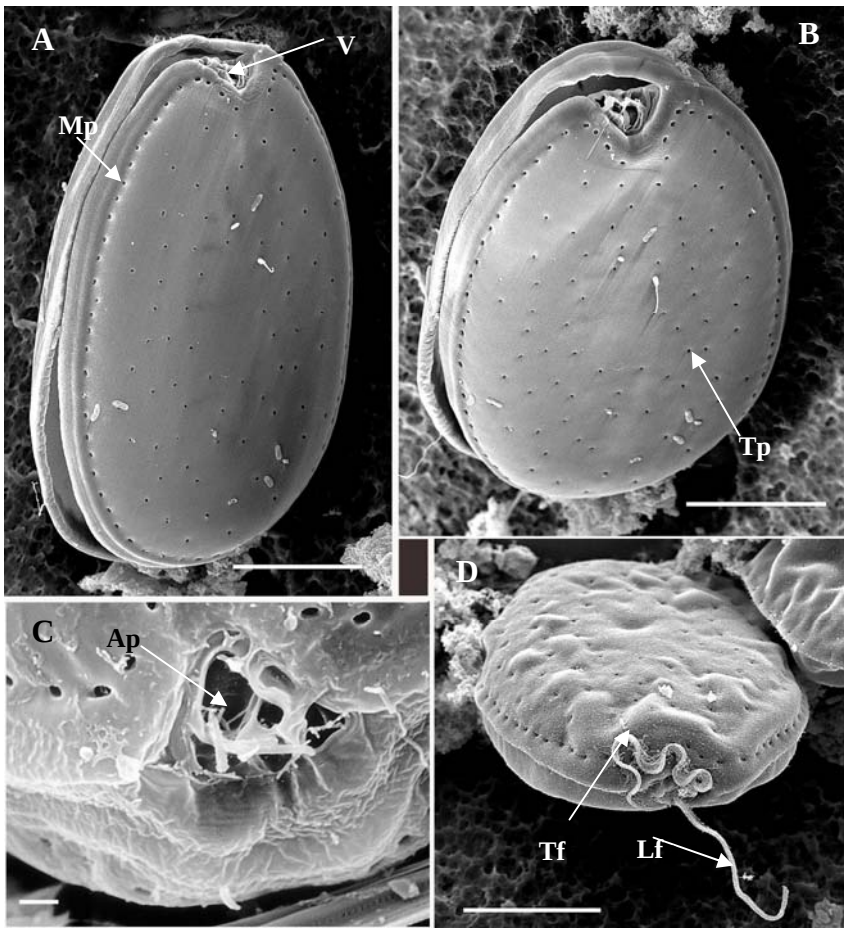


Plate II. *Prorocentrum lima*. A, lateral view; B valvar view; C and D apical views. Scale bars = 10 μm in A, B and D; 1 μm in C.V – V-shape depression in the right valve, Mp – marginal pore, Tp – thecal pore, Ap - apical pore, Tf – transversal flagellum and Lf – longitudinal flagellum.

Prorocentrum cassubicum (Woloszynska) Doge 1973

Syn: *Exuviaella cassubica* Woloszynska

Dodge (1965); Dodge (1973); Dodge (1975).

Plate III and IV

Description

Prorocentrum cassubicum is in many aspects similar to *P. lima*, the main difference is the size. It was also a constant species in the collected samples, where it appeared as an active swimmer, characteristic that it tend to loose after long periods in culture, where it choose a more benthic habit forming big and dense aggregates in the bottom of the culture flasks, where it produces mucus to maintain these aggregates.

Cells of *P. cassubicum* varied from an ovate to a more angulated shape in the edges, the size was small, with an average of 28 µm of length, 18 µm of width and a ratio of ca. 1,5. In similarity with *P. lima* the cells of *P. cassubicum* also have a big central double pyrenoid, two large peripheric chloroplasts and many bodies of starch accumulation spread by the cell, that give to the cell a yellow-green colour in light microscopy. The nucleus is large and has a posterior position.

In the scanning electron microscope, the theca has a smooth surface, with many small pores in aleatory distribution in the valve, but regularly distributed in the edge. In the centre of the theca no pores were found, instead was found a depression with the same shape of the pyrenoid. The anterior area of the right valve has a slight depression in V-shape, surrounding this depression is a robust apical curved collar, from which the two flagella emerge through the flagellar pore. The apical region is surrounded by several small apical plates and another pore, the auxiliary pore, but they are not easily distinguished. The intercalary band sometimes can be very thick and be formed by the association of several layers. This can be associated with division stages of the cell.

Distribution

Prorocentrum cassubicum was the most cosmopolitan species; found all the year, in spite of variations in the salinity and water temperature, which can be very low or very high in some periods. This species usually can be found in higher densities in the beginning of spring, when the salinity is around 25 and the temperature around 15ºC.

Toxicity: There are some reports of toxicity caused by this species, but its toxicity is not well known.

Remarks

Dodge (1975) described this species as being very similar to *Prorocentrum lima*, but in smaller size, differing slightly in shape and also in the lack of trichocysts. Recent studies confirmed that trichocysts are not present in *P. lima*, only mucocysts (Zhou & Fritz 1993).

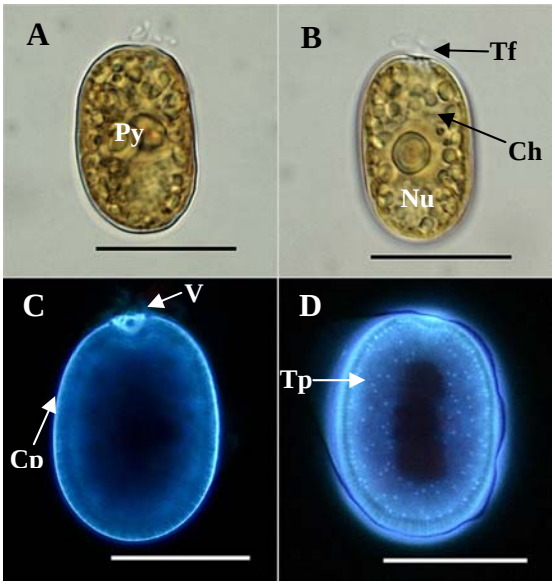


Plate III. *Prorocentrum cassubicum*. Scale bars= 20 μm. A and B light microscopy images; C and D epifluorescence. Py – double pyrenoid, Nu – nucleus, Ch – chloroplast, V – V-shape depression, Cp – cell periphery and Tp – thecal pore.

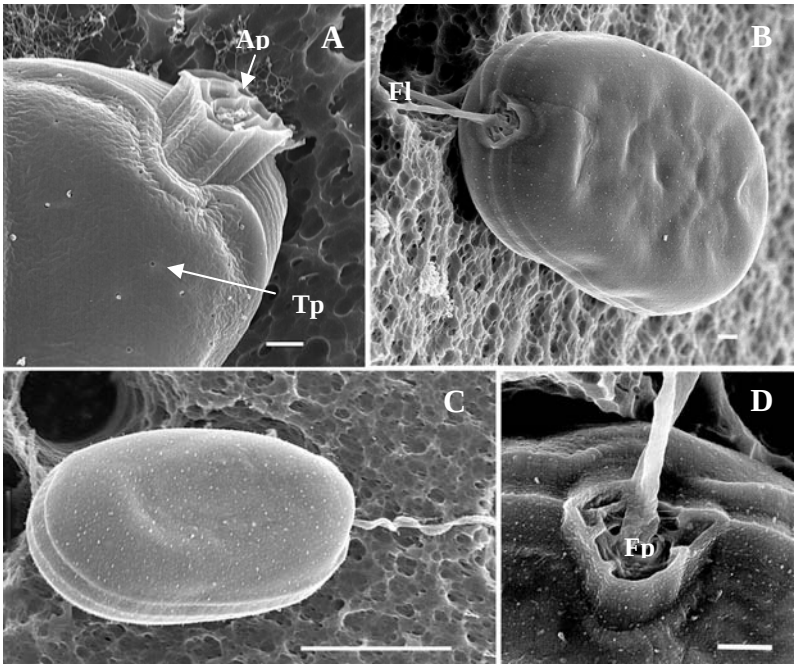


Plate IV. *Prorocentrum cassubicum*. Scale bars = 1 μm in A, B and D, 10 μm in C. A and D apical views; B right valve; C left valve. Ap – apical plates, Tp – thecal pores, Fl – flagella and Fp – flagellar pore.

Prorocentrum micans Ehrenberg 1834

Syn: *Prorocentrum schilleri* Böhm in Schiller 1933
P. lavantinoides Bursa 1959
P. pacificum Wood 1963

Stein (1878); Bursa (1959); Dodge (1965); Dodge (1982); Faust *et al.* (1999); Faust & Gulledge (2002).

Plate V, VI and VII

Description

Prorocentrum micans is a planktonic species, it is a very active swimmer and was found in almost all collecting sites, all the year, it shows high tolerance to different salinity values (10-50), but tend to occur in higher densities during the summer.

The shape of the cell is between a tear droop and heart shape, being more round in the anterior area and becomes thinner in the posterior area, the cell in side view is very thin. Cells are medium size, with 40 µm long and 30 µm wide, with a length/width ratio of 1,4, it appeared at the light microscope with a yellow-brown colour, due to the two large peripheric chloroplasts. In the anterior area besides the spine, two big vacuoles can be distinguished, and in the posterior end a large nucleus, where chromosomes were visible.

On the scanning electron microscope it was possible to see that the theca surface was very ornamented, due to numerous depressions and pores. Three different kinds of pores could be distinguished, very small pores in aggregates of 6 in the posterior end, simple pores distributed randomly in the valve, and composed pores distributed regularly in a radial form in the valve. The composed pores occurred with higher concentrations in the edges of the valves, and some of these pores appeared to be associated with trichocysts.

Prorocentrum micans like all the others *Prorocentrum* species has a theca composed by two valves. In the anterior part of the cell a large apical spine (up to 10 µm long) is projected, surrounding it are many small apical plates that continued to the right valve in a V-shape depression. In this depression two pores can also be found, the flagellar pore and the auxiliary pore.

The transmission electron microscope allows us to see that the cell of *P. micans* is full of trichocysts, but that it also has mucocysts. It has a very large posterior nucleus and two large peripheric chloroplasts. Using this technique was possible to estimate the number of apical plates, which is around six.

This species is not easy to maintain in culture, since after reaching the exponential growth stage the cells began to produce huge amounts of mucous. Rapidly all the nutrient are consumed and the culture enter in a stage of starvation, from which just few cells can survive, if the culture is not rapidly transfered into fresh medium it will die in a short period of time.

Distribution

Prorocentrum micans was a very common species in the ponds, especially in warmer periods, where it could be found in higher densities. This species shows preference for high salinities, higher than 25 and temperatures between 18 to 25 ºC.

Toxicity: Non-toxic, but *P. micans* is capable to form extensive blooms. There are some reports of causing shellfish kills, but the toxicity of this species is still not confirmed.

Remarks

Prorocentrum micans is a species with many shape variations, what can lead to misidentification, and be confused with other similar species like: *P. gracile, P. scutellum* and *P. caribbaeum* (Faust & Gulledge 2002).

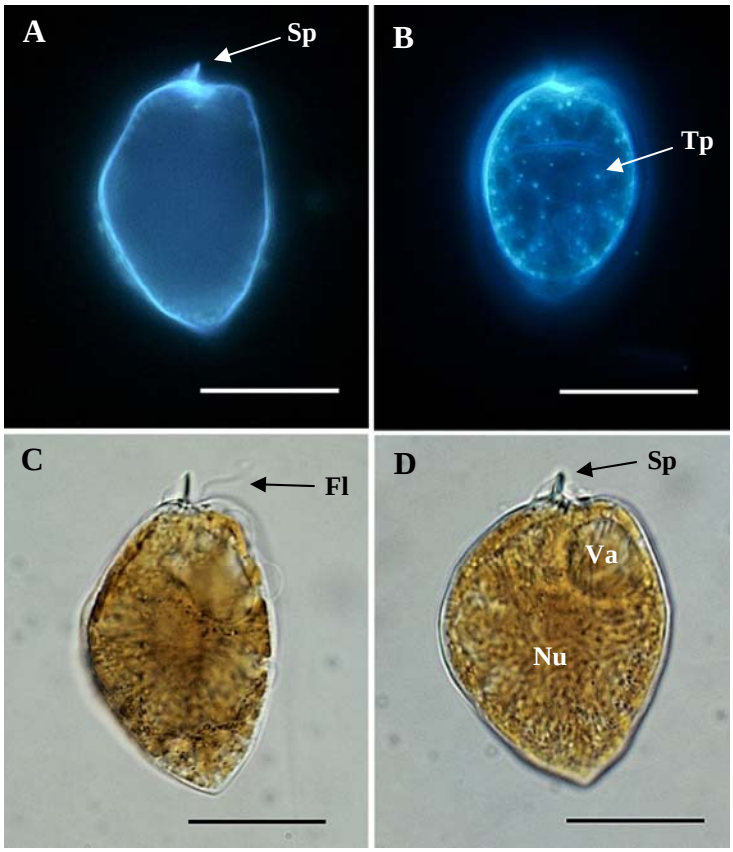


Plate V. *Prorocentrum micans*. Scale bars = 20 μm. C and D light microscopy images; A and B epifluorescence. Sp – apical spine, Nu – nucleus, Fl – flagella, Va - vacuole and Tp – thecal pore.

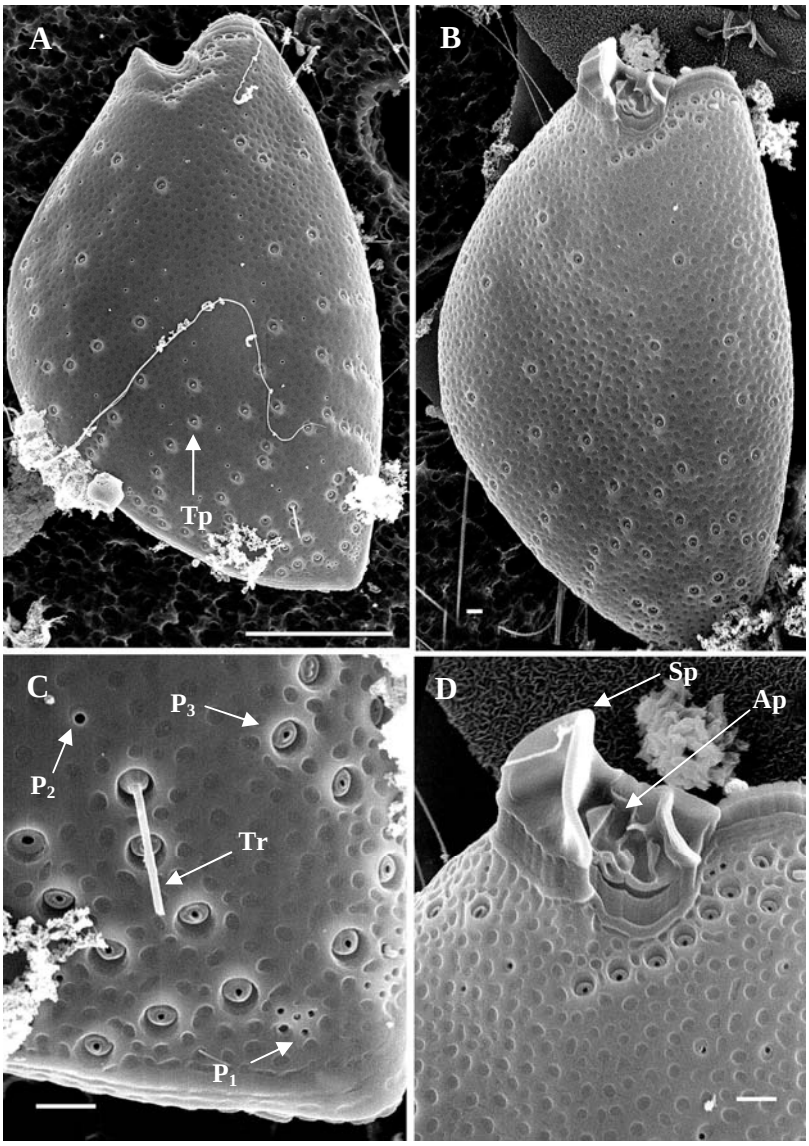


Plate VI. *Prorocentrum micans.* Scale bars = 10 μm in A; 1 μm in B, C and D. A is a right valve and B a left valve. C is the antapical area and D an apical view. Ap – apical plate, Sp – apical spine, Tr – trichocyst, Tp – thecal pores that can be of three types: P_1 – aggregate of small pores, P_2 – normal pores and P_3 – composed pores.

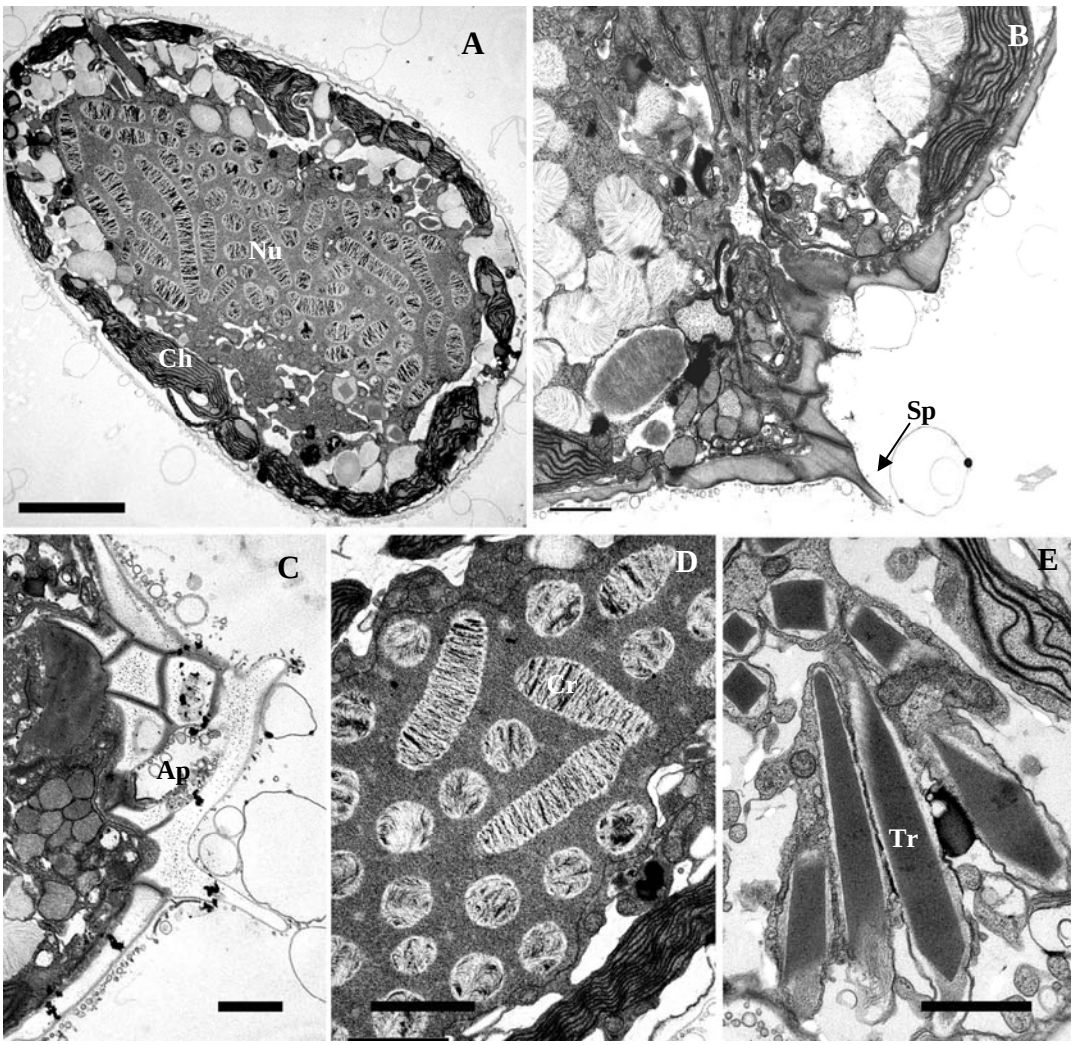


Plate VII. *Prorocentrum micans.* Scale bars = 5μm in A and B and 1μm in C, D and E. In A is a lateral section of a cell of *P. micans* where the large nucleus is visible (Nu) and the chloroplasts (Ch). B and C show in detail the apical area of the cell, formed by 7 apical plates (Ap) being the biggest one the apical spine (Sp). In D are the large condense chromosomes (Cr). E shows the trichocysts (Tr).

Prorocentrum rhathymum Loeblich III 1979

Loeblich et al. (1979); Fukuyo (1981); Cortés-Altamirano & Sierra-Beltrán (2003).

Plate VIII and IX

Description

Prorocentrum rhathymum was only found in a specific group of collected samples during the late summer of 2003 and summer of 2004, where it appeared in very high densities. It can be classified as being a planktonic species, since it is a very active swimmer, but also as a benthic, since in culture it produce big amounts of mucus, even if the cells do not tend to aggregate, as observed with other benthic species like *Prorocentrum lima*.

The cell had an ovoid shape in valve view, and ellipsoid in side view, the intercalary band was very thick. The cell is of medium size with 34 μm length, 24 μm width and a ratio l/w of 1,5. At the light microscope a very small spine (2-3 μm long) in the apical area could be distinguished, it was also visible the two large chloroplasts at the periphery that give to the cell a yellow-brown colour, and in the anterior area one or two big vacuoles could be observed, the large nucleus occupied a posterior position.

Under the scanning electron microscope and epiflourescence light microscope (using calcofluor), a regular pattern of pores in the valves was easily distinguished. However, in scanning we could see that these pores were composed pores, inside each is a small protuberance with a little pore on the top. The valves were also ornamented with other kinds of pores, very small pores in groups of 5 or 6 in the posterior end of the valves, and simple pores distributed more or less aleatory in the valve. These types of pores were also found in the theca of *P. micans*. The composed pores seemed to be associated with trichocysts. The rest of the theca surface is smooth. In lateral view it is possible to see the thick intercalary band, composed by several crossover layers.

Distribution

Prorocentrum rhathymum occurred only twice but in very huge amounts, it was in the beginning of autumn of 2003 and summer of 2004, when the salinity was higher than 40 and the water temperate around 25 ºC.

Toxicity: *Prorocentrum rhathymum* is a toxic benthic species associated with cases of ciguatera.

Remarks

Prorocentrum rhathymum was for a long time considered as a synonym of *Prorocentrum mexicanum*, but the work of Crtés-Altamirano & Beltran (2003) described the features that allow the distinction of these two species (see Fig. 2.3). The main differences are that *P. rhathymum* does not have pores located in depressions, the spine is simple and only one, in the periflagellar area, there are trichocysts pores but only in the right valve and the intercalary band is more think in this species that in *P. mexicanum* (Crtés-Altamirano & Beltran 2003).

Loeblich *et al.* (1979) noted that the living habitat of this species was embedding in mucilage and not moving actively. However, this non motile stage was not found in wild or cultured specimens. In culture *P. rhathymum* after reaching exponential growth tend to produce a kind of mucus, similar with the one produced by *P. micans*, but it is always an active swimmer, this observation was also registed by Fukuyo (1981).

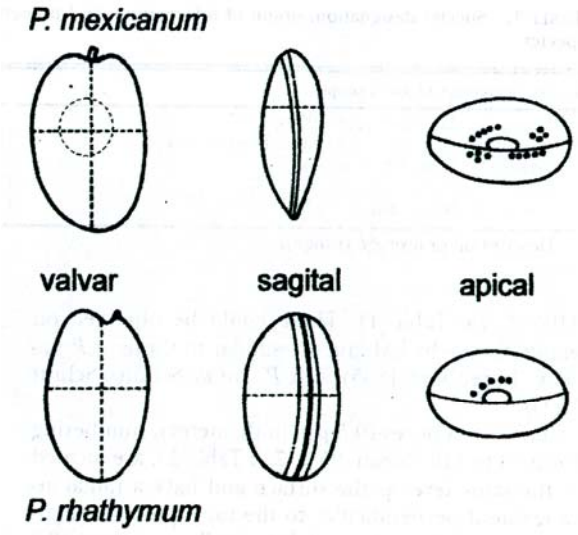


Fig.2.3. Diagram illustrating the main differences between *Prorocentrum rhathymym* and *Prorocentrum mexicanum*, adapted from Cortés-Altamirano and Sierra-Beltrán (2003).

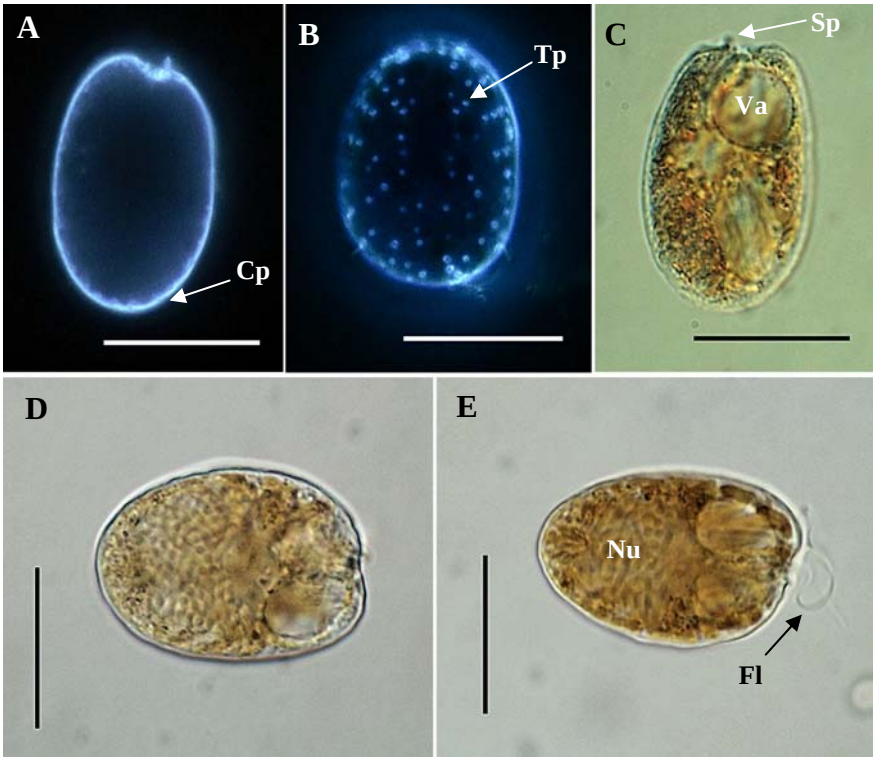


Plate VIII. *Prorocentrum rhathymum* cells in valvar view. Scale bars = 20 μm. A, B epifluorescence; C, D and E light microscopy images. Cp – cell periphery, Tp – thecal pore, Sp – spine, Va – vacuole, Nu – nucleus and Fl – flagella.

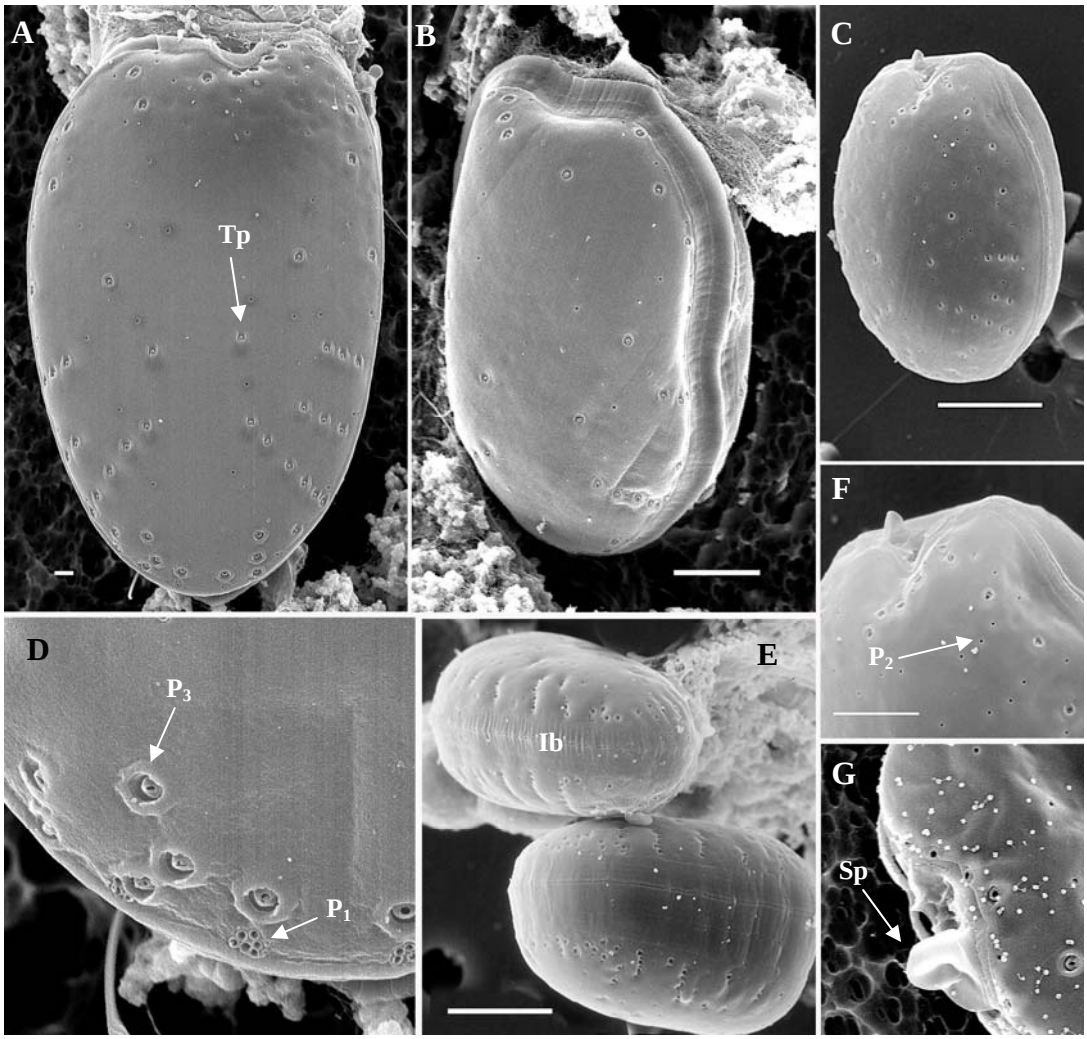


Plate IX. *Prorocentrum rhathymum*. Scale bars = 1 μm in A: 10 μm in B, C, E and F. A and B left valve. C - right valve. D - detail of pore types. E - two cells in antapical view. F and G detail of the apical area. Sp – apical spine, Ib – intercalary band, Tp – thecal pores that can be of three types: P_1 – aggregate of small pores, P_2 – normal pores and P_3 – composed pores.

Prorocentrum minimum (Pavillard) Schiller 1933

Syn*: Exuviaella minima* Pavillard 1916
Prorocentrum triangulatum Martin 1929
Exuviaella marie-lebouriae Parke & Ballantine 1957
Prorocentrum cordiformis Bursa 1959
Prorocentrum mariae-lebouriae (Parke & Ballantine) Loeblich 1970

Faust (1974); Faust & Gulledge (2002).

Description

Prorocentrum minimum is a small armoured dinoflagellate, easily distinguished from all the other in the scanning electron microscope, since it has the particularity of having the theca surface covered by fine spines and has a little apical spine.

Distribution

This species was only observed once, is a planktonic species, so its recorder was punctual.

Toxicity: *Prorocentrum minimum* is a toxic species, it produce venerupin (hepatotoxin), which has caused shellfish poisoning resulting in gastrointestinal illness in humans and a number of deaths.

Remarks

Prorocentrum minimum can be confused with *P. balticum*, although the size and shape are slightly different (Faust & Gulledge 2002) and with *P. cordatum* (Ostenfeld 1901) Dodge 1975.

***Prorocentrum* sp. 1**

Plate X, XI, XII and XIII

Description

Prorocentrum sp. 1 was a species found in the ponds especially in the summer, when the salinity was high. It is a predominantly benthic species, since it was found several times attached to the filaments of the green macro-algae *Cladophora* sp. through a mucus extension that involves all the cell. From the apical area, a prominent neck of mucus is

projected which allow the attachment of the cell to the tallus of the macro-algae. In live samples this species was also found swimming freely, but only in rare occasions and it is not a very vigorously swimmer.

In culture, this species only grew in F/2 medium with high salinity (higher than 30) and showed preference for high temperatures (20ºC). The cells grow attached to each other forming large condense groups, visible to the naked eye. When an aggregate becomes too dense, one or two cells release themselves from this group and swim freely to an open space in the bottom of the culture vessel, there they settle down and establish a new group (like a colony). Most of the time, cells of *Prorocentrum* sp.1 are in a non-motile stage, with all the mucilage surrounding them to form this aggregate, in a stage where the flagella are not needed.

Prorocentrum sp. 1 is very similar to *P. lima*, they even occur together, the obvious differences are the size and shape, what may indicate that they are not the same species and maybe we are dealing with a new species.

The size of the cell can be classified as being medium to large, with ca 48 µm length, 37 µm width and a ratio of 1,2, gives the cell a quite round shape, being broader in the posterior area. The cell at light microscope appeared with a yellow-brown colour, due to the two large chloroplasts, that occupied the entire periphery of the cell and the big double pyrenoid that lies at the centre. The nucleus is huge and has a posterior position, easily the condense chromosomes can be noted. At side view, the cells are quite thin, less than 20 µm thick, even with all the mucilage that surrounds them.

In the scanning electron microscope was possible to see that the surface of the theca is smooth, although there were many small pores (more then 100 with 0,5 µm diameter) inserted in slight depressions. The pattern of distribution of the pores is aleatory in the valves, but regularly in the margin of the valves, no pores were found in the centre of the valves, where an extensive depression is located (with the same shape of the pyrenoid). The neck of mucilage was also visible after a scanning fixation, showing the high consistence of the material produced by these cells. At the right valve, in the apical area, a U-shape depression was observed and inside of it are the apical pores (flagellar and auxiliary pore) and several small apical plates were observed. The left valve also showed a depression in the apical area, but of smaller dimensions. The intercalary band is thin and the area where both valves connect is even thinner than the rest and more prominent.

Distribution

This species was found very often in one of the studied ponds, the site 2, especially due to its preference for higher salinities (higher than 35). *Prorocentrum* sp. 1 was more abundant in warmer periods, like in the end of spring, summer and beginning of autumn.

Toxicity: Non-toxic.

Remarks

Prorocentrum sp. 1 in an initial stage of work was wrongly identified as *P. arenarium* (Faust 1994), but further observations using more advanced techniques of microscopy revealed some morphologic features between this two species, manly the type of pores and the size. *Prorocentrum* sp. 1 is a non-toxic species (at least to *Artemia* larvae) and *P. arenarium* is capable of producing DSP toxins.

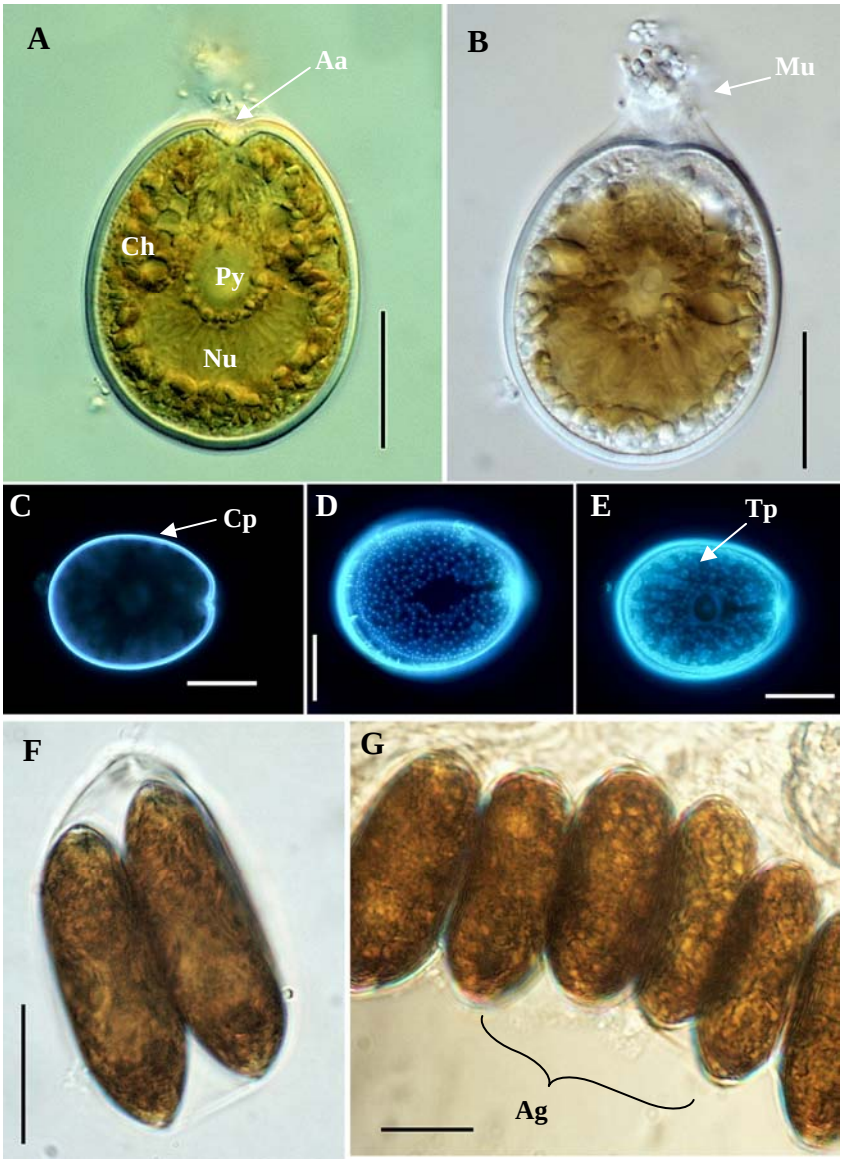


Plate X. *Prorocentrum* sp. 1. Scale bars = 20 μm. A, B, F and G light microscopy images. C, D and E epifluorescence. A and B cells in valvar view. F and G cells involved by mucilage. Aa – apical aperture, Ch – chloroplast, Py – double pyrenoid, Nu – nucleus, Mu – mucus, Cp – cell periphery, Tp – thecal pore and Ag – aggregate of cells.

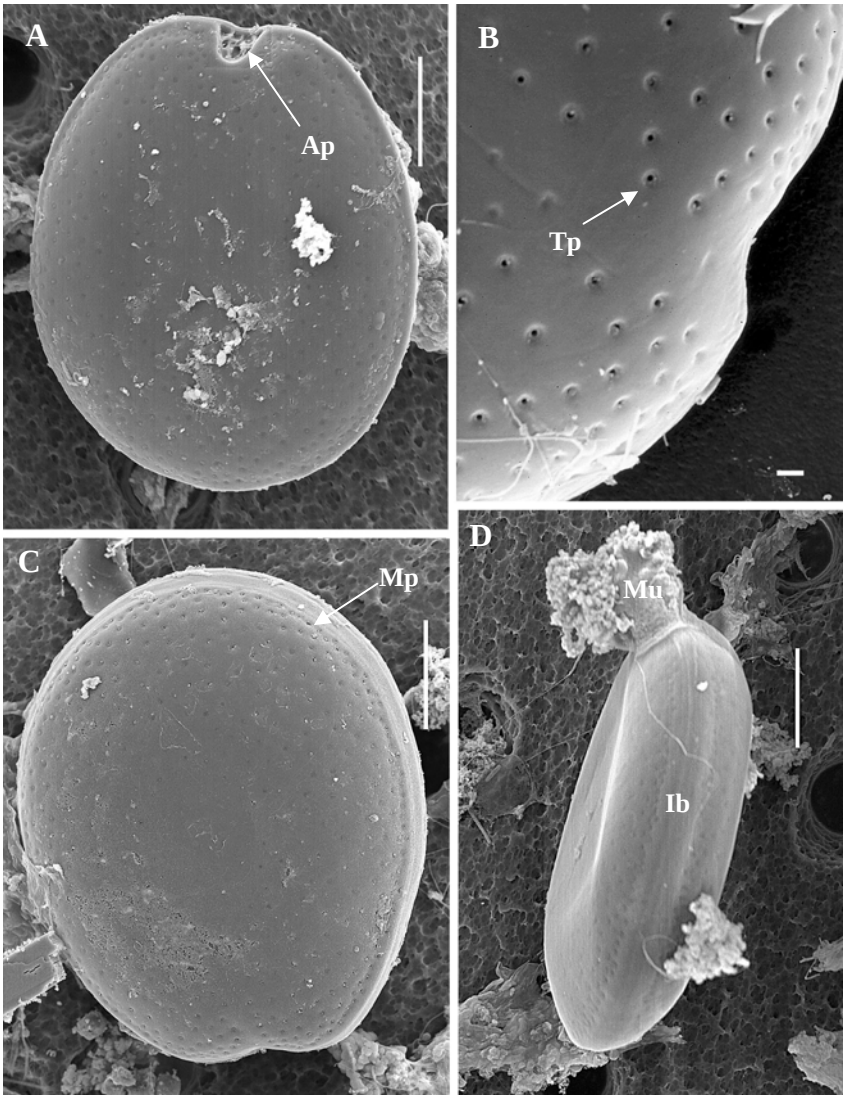


Plate XI. *Prorocentrum* sp. 1. Scale bars = 10 μm in A, C and D; 1 μm in B. A, and C cells in valvar view, right and left valves respectively. B detail of the pore pattern. D lateral view of the cell. Ap – apical plates, Tp – thecal pore, Mp – marginal pore, Mu – mucus and Ib – intercalary band.

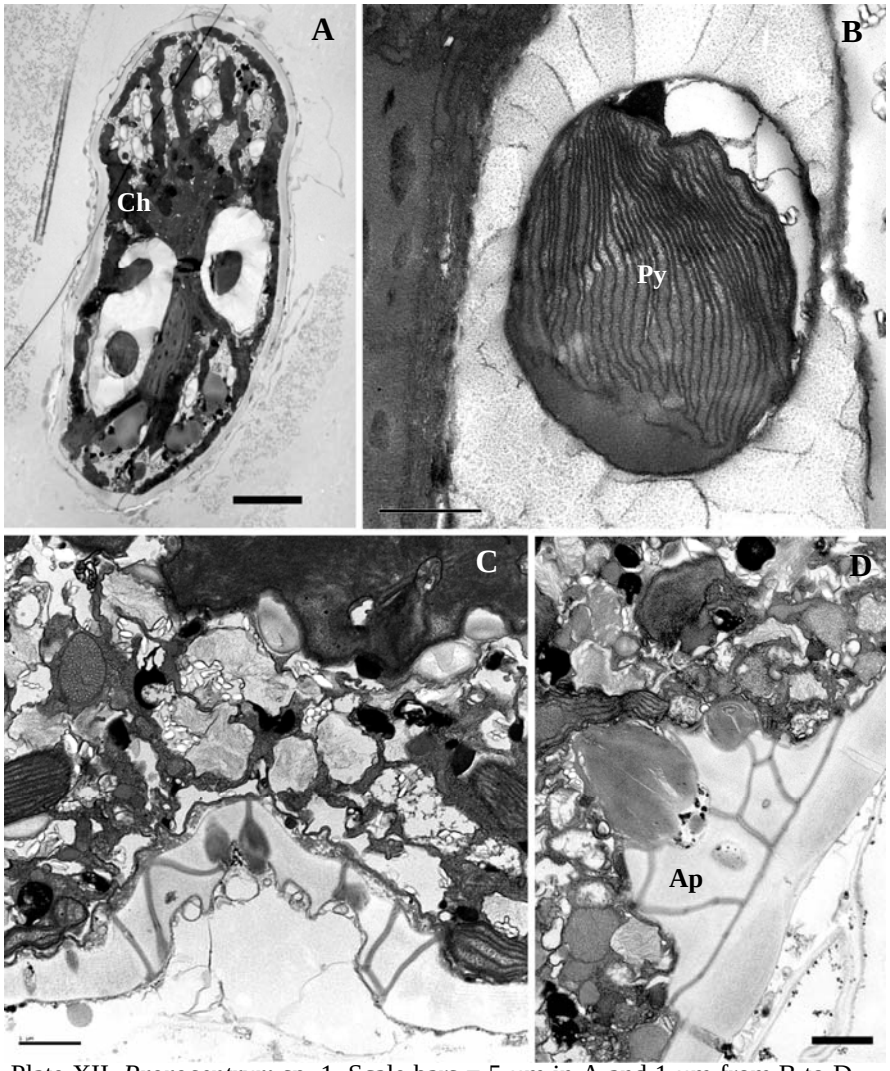


Plate XII. *Prorocentrum* sp. 1. Scale bars = 5 μm in A and 1 μm from B to D. In A is a longitudinal section of the cell, where is possible to see the chloroplasts (Ch), in B is the pyrenoid (Py), C and D ilustrate the apical region where is possible to detinguish 6 in C and 7 in D apical plates (Ap).

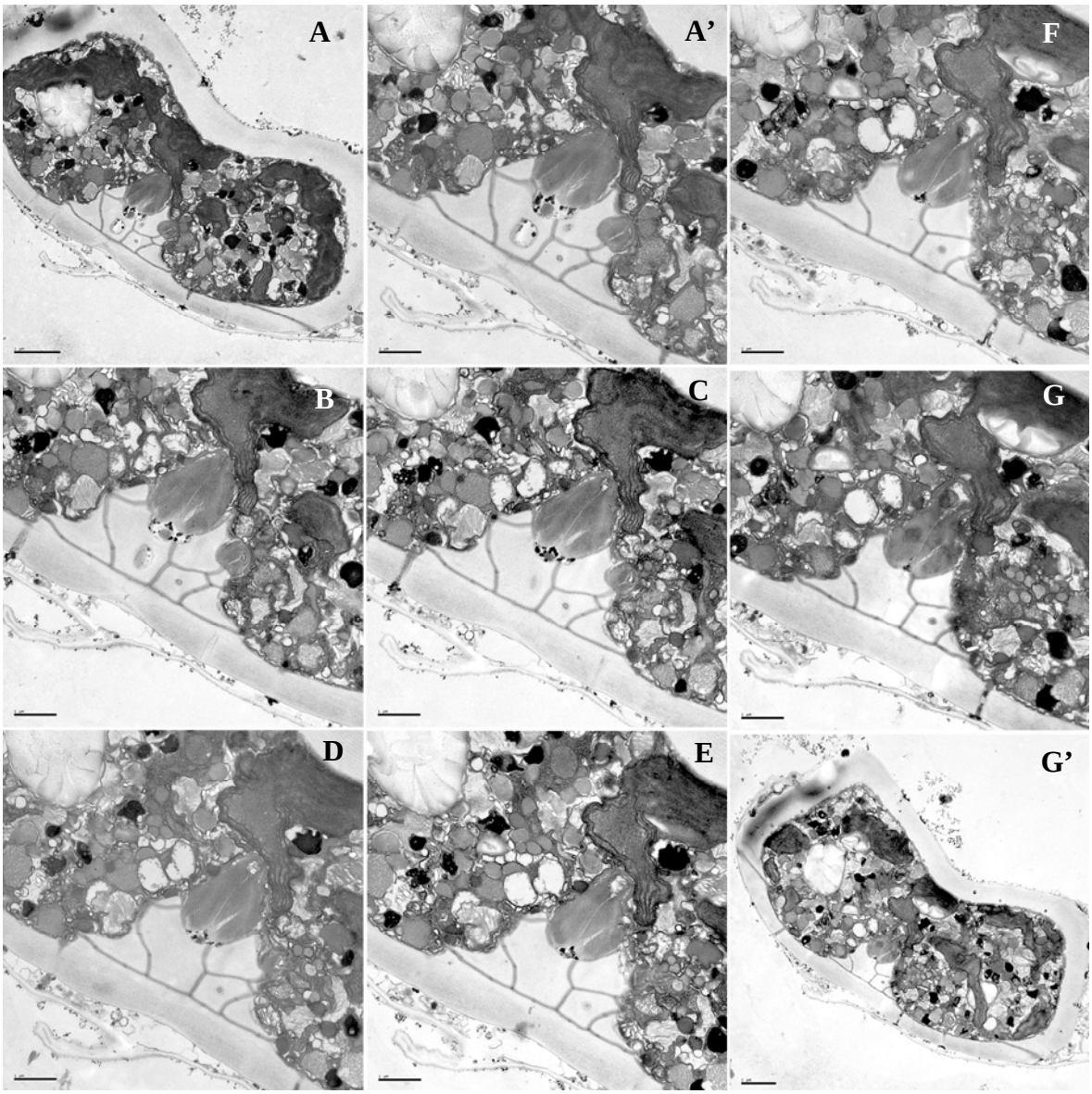


Plate XIII. *Prorocentrum* sp. 1. Scale bars = 2 μm in A and G'; 1μm in all the others TEM images. Serial tranversal sections of a single cell, where is possible to observe the number of apical plates, the maximum number count was 7 apical plates.

Table 2.1: Some morphological characteristics from the *Prorocentrum* species found in this study, and measured from clonal cultures, in comparison with the description from the literature.

				Valves		Apical periflagellar area		
Species and clone number	Length (µm)	Width (µm)	Ratio	Shape	Ornamentation	Pore pattern	Shape in apical view	Ornamentation
Prorocentrum micans	35-70	20-50		Pyriform to heart shaped, pointed posteriorly	Shallow depressions	Pores in radial lines, pores scattered on valves	Ovoid to oblong	Long spine
#439	34-45	24-33	1,4					
Prorocentrum lima	31-47	22-40		Ovate	None (smooth valves)	Pores scattered on valves, marginal pores	Broad, V-shaped	Protruding, curved apical collar
#241	37-51	21-32	1,6					
Prorocentrum cassubicum	22-25	16		Ovate, hexagonal	None	Very small pores	Broad slightly	Apical collar curved
#377	26-30	16-19	1,5					
Prorocentrum rhathymum	38-40	22-25		Ovate	Shallow depressions	Pores in radial lines, pores scattered on valves	Ovoid	Small spine
#328	29-37	18-28	1,5					
Prorocentrum sp.2 #326	15-20	11-16	1,2	Elliptic to square	None	Marginal pores	Broad, with a depression	Small spine
Prorocentrum sp.1 #390	41-54	31-43	1,2	Elliptic	None	Pores scattered on valves, marginal pores, none in the centre	Broad, V-shaped	Protruding, curved apical collar

Table 2.2: Other morphological characteristics, toxicity and habitat preferences from the *Prorocentrum* species found in this study. The empty spaces refer to organelles that were not seen. The toxicity refers to the results obtained with the *Artemia* bioaasay using clonal cultures.

	Organelles						Toxicity	Ecology
Species	Chloroplasts	Pyrenoid	Trichocysts	Mucocysts	Nucleus	Vacuoles		
Prorocentrum micans	2 yellow-brown peripherally	absent	several	some	kidney shape	2 anterior posterior	non-toxic form blooms	planktonic
Prorocentrum lima	2 yellow-brown	2 large central crossover	absent	several	large posterior	2 anterior	toxic (OA)	benthic and epiphytic
Prorocentrum cassubicum	2 yellow-green	2 large central crossover	absent	several	posterior		toxic	benthic and epiphytic
Prorocentrum rhathymum	2 yellow brown	absent	several	some	posterior	2 anterior	non-toxic	epibenthic
Prorocentrum sp.2	2 yellow-brown	2 central	several	absent	posterior	2 anterior	non-toxic	epibenthic
Prorocentrum sp. 1	2 yellow-brown	2 large central crossover	absent	many	posterior		non-toxic	benthic, embedding in mucilage stage

***Prorocentrum* sp. 2**

Plate XIV, XV and XVI

Description

Prorocentrum sp. 2 was a planktonic species that occurred very often in the samples, and found many times in very high concentrations. It is an active swimmer even in culture but its activity tends to decrease with the age of the culture; it can proliferate quickly when compared with other species of *Prorocentrum*.

Prorocentrum sp. 2 was the smallest *Prorocentrum* found in the collecting sites and put into culture. This species can easily be confused with *Prorocentrum minimum*, only electron microscopy proved that they are not the same species, because of the lack of the small spines on the theca surface that characterized the *P. minimum* cell, so maybe we are in the presence of a non-describe species of *Prorocentrum*.

Prorocentrum sp. 2 has a small size; 17 µm length, 13 µm width and a ratio of 1,2. The cell has a shape between elliptic and scare. At light microscope, it appeared with a yellow-brown colour, due to the chloroplasts. The nucleus has a posterior position and two anterior vacuoles can be seen sometimes at the anterior region of the cell. Epiflourescence showed that the theca is almost absent of pores, since these were only observed in the periphery of the cell, in side view in the area where both valves connect, this was also visible in the scanning electron microscope.

In the scanning electron microscope more details of the cell were seen, like a small apical spine in the apical area, and that both valves showed to have a depression, being wider in the right valve. The apical area, where usually are the apical plates, the flagellar pore and the auxiliary pore were not observed, this area appeared to be hidden and only one large pore was observed.

The surface of the valves is not very smooth, it seems to be covered with something that gives a wrinkle aspect to the theca. Pores were found in the periphery of the valves (about 10) regularly distributed, and some seemed to be associated with trichocysts.

The intercalary band is well defined and sometimes quite thick, what can be associated with division stages of the cell.

At transmission electron microscope, other features could be observed: a double pyrenoid associated with the two peripheric chloroplasts, and a little spine that makes part of a group of small apical plates (around 5). Inside the cell several trichocysts were found, but not mucocysts.

Distribution

This species appeared quite often in the ponds, being more abundant from spring to autumn. This species seems to prefer a range of salinity and temperature around 15 - 35 and 15-20 ºC respectively. In March of 2003 occurred a small bloom of this species.

Toxicity: Non-toxic, but this species has a fast growth rate, compared with the other *Prorocentrum* species.

Remarks

Initially this species was confused with *P. minimum*, since at light microscope, they were very similar, but observations in SEM and TEM eliminated this hypothesis. *P. minimum* is known for having all the theca covered with fine spines, this kind of ornamentation was not found in this species. However, looking at all the small *Prorocentrum* species that used to belong to the genus *Exuviaella, E. apora* (Schiller 1918) and *E. pusilla* (Schiller 1928), similar aspects were found like shape and the almost inexistent pores, but also other completely different aspects like the size range. In the literature was not found any species that resemble completely with the one described as *Prorocentrum* sp. 2.

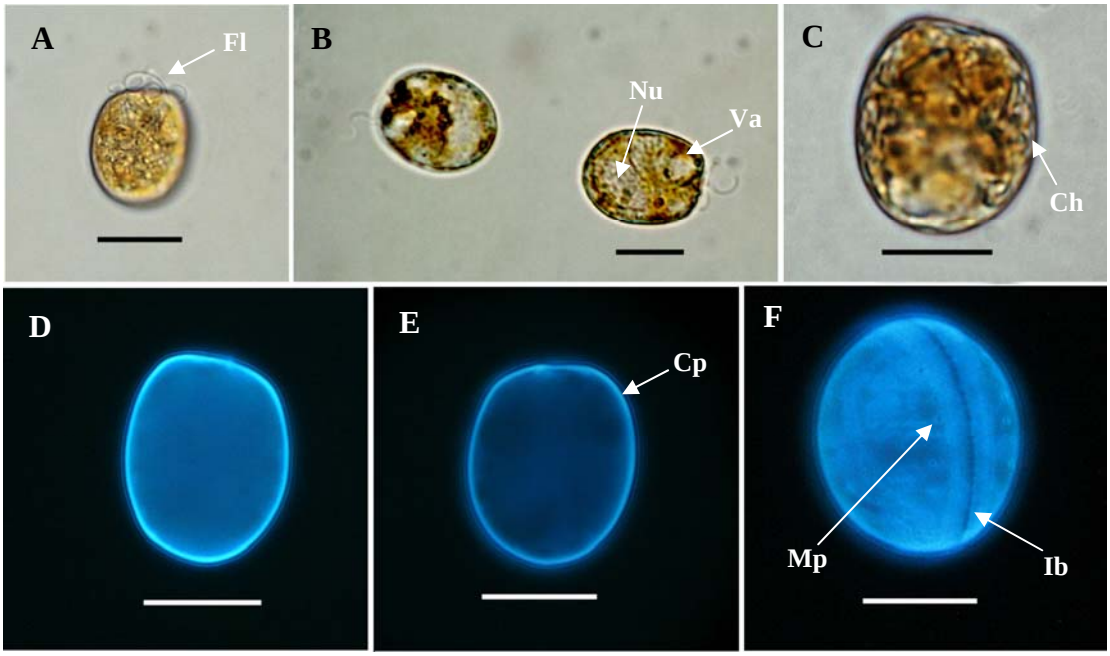


Plate XIV. *Prorocentrum* sp cells in valvar view (A to E) and in side view (F) 2. Scale bars = 10 µm. A-C light microscopy images. D-F epifluorescence. Fl – flagella, Nu – nucleus, Va – vacuole, Ch – chloroplast, Cp – cell periphery, Mp – marginal pore and Ib – intercalary band.

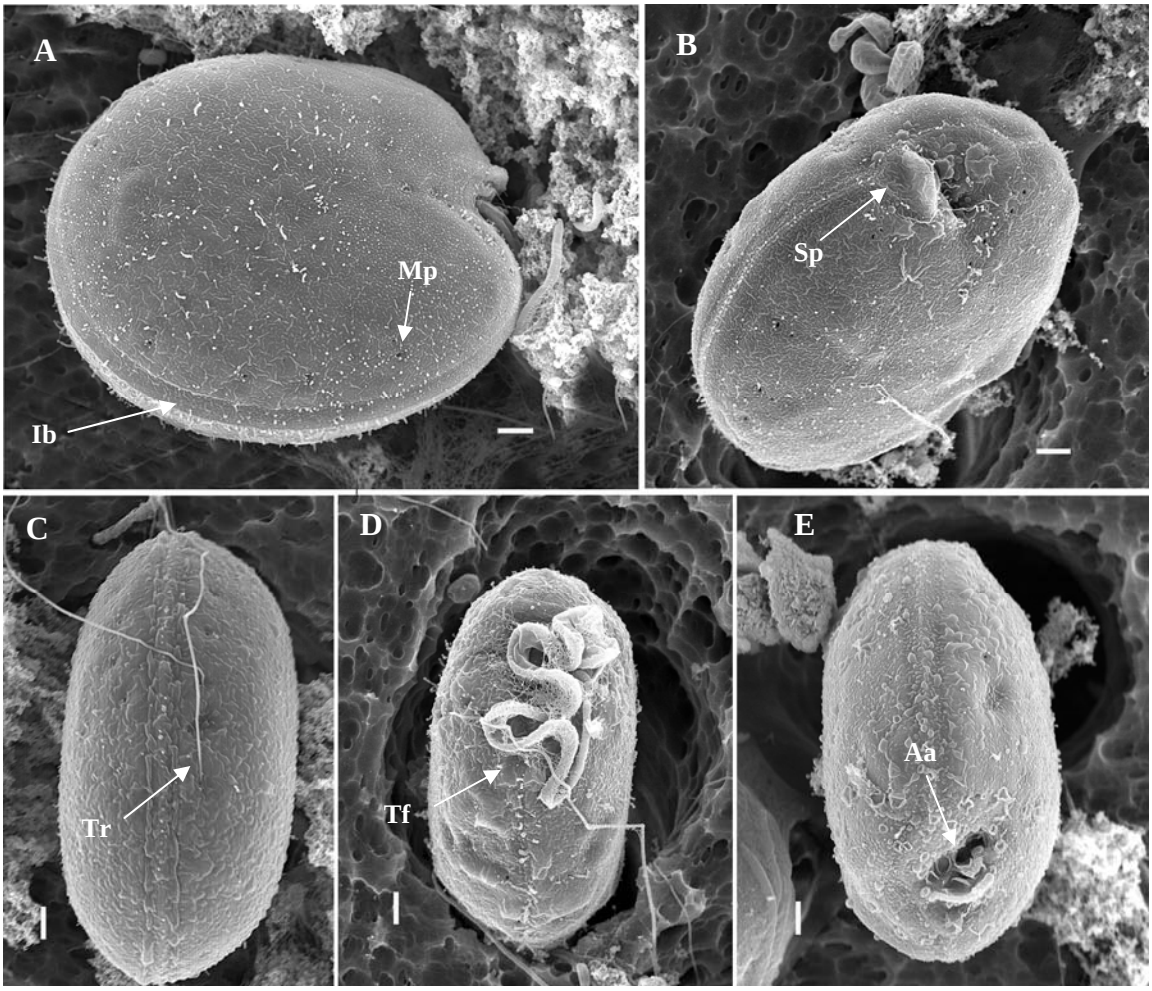


Plate XV. *Prorocentrum* sp.2. Scale bars = 1 μm. A valvar view. B and D apical views. C and E lateral views. Ib – intercalary band, Mp – marginal pore, Sp – apical spine, Tr – trichocyst, Tf – transversal flagellum and Aa – apical aperture.

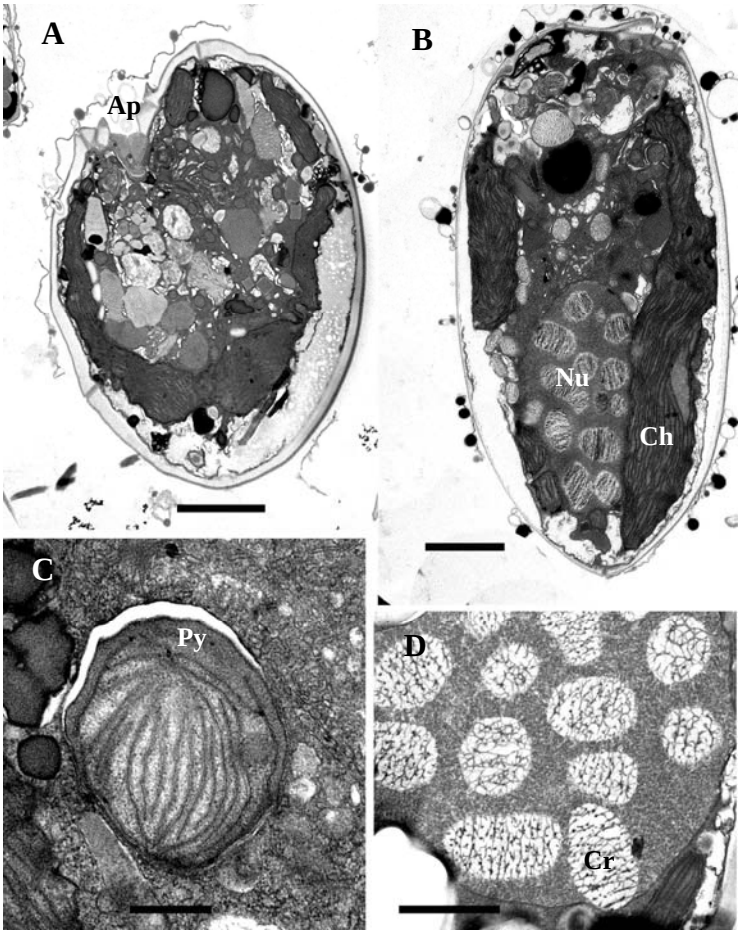


Plate XVI. *Prorocentrum* sp.2. Scale bars = 2 μm in A and B, 0,5 μm in C and 1 μm in D. A - lateral section and B a longitudinal section of a cell of *Prorocentrum* sp. 2, C is the pyrenoid (Py) associated with the chloroplast (Ch) and D show the condense chromosomes (Cr). Ap- apical plates, Nu – nucleus.

Order Peridiniales
Family Calciodinellaceae

Genus *Scrippsiella*

Scrippsiella* cf. *trochoidea (Stein) Loeblich III 1976

Syn: *Glenodinium trochoideum* Stein 1883
Glenodinium acuminatum Jorgensen 1899
Peridinium trochoideum Lemmermann 1910
Scrippsiella faeroense Dickensheeets & Cox 1971

Dodge (1982); Steidinger & Tangen (1996); Janofske (2000); Hallegraeff (2002).

Plate XVII and XVIII

Description

Scrippsiella cf. *trochoidea* is a small periforme armoured dinoflagellate, with conical epitheca and rounded and larger hypotheca, which gives to the cell a tear droop shape. The cell size is 20-30 μm long, 15-25 μm wide and a ratio length/width of 1,2. The cingulum is wide and displaced about half the girdle width. The nucleus is very large and located in the centre of the cell, surrounding are several small rounded yellow-brown chloroplasts.

In the scanning electron microscope was possible to see the plate formula: po, x, 4', 3a, 7'', 5''', 2''''. The plates are covered with scattered pores, which in the pre and post-cingular plates are aligned on the edge along the cingulum. The apical pore is circular and rises from an apical horn. The sulcus is ventral and extends into the hypotheca.

This species in culture takes long time to establish, but after that, it can proliferate quickly.

Distribution

Scrippsiella cf. *trochoidea* only appeared twice during the samples, in consecutive moments, during the autumn of 2003. It is a more planktonic than benthic species, this occurrence was probably due to a higher influence of the sea in the estuary. It seems that this species prefers high salinities, higher than 35 .

Toxicity: Non toxic.

Remarks

Scrippsiella cf. *trochoidea* is known to form spherical calcareous cysts, covered with numerous calcareous spines (Janofskel 2000; Lewis 1991).

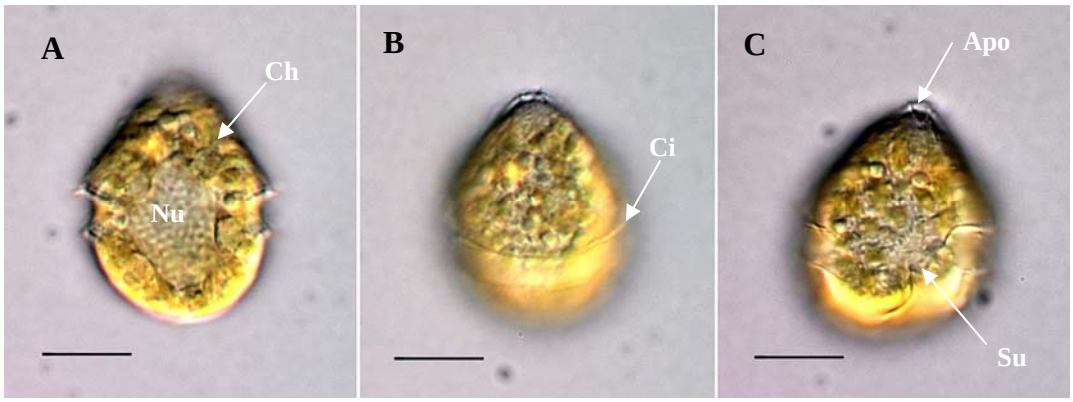


Plate XVII. *Scrippsiella trochoidea* in light microscopy. Scale bars = 10 μm. Nu – nucleus, Ch – chloroplast, Ci – cingulum, Apo – Apical pore and Su – sulcus.

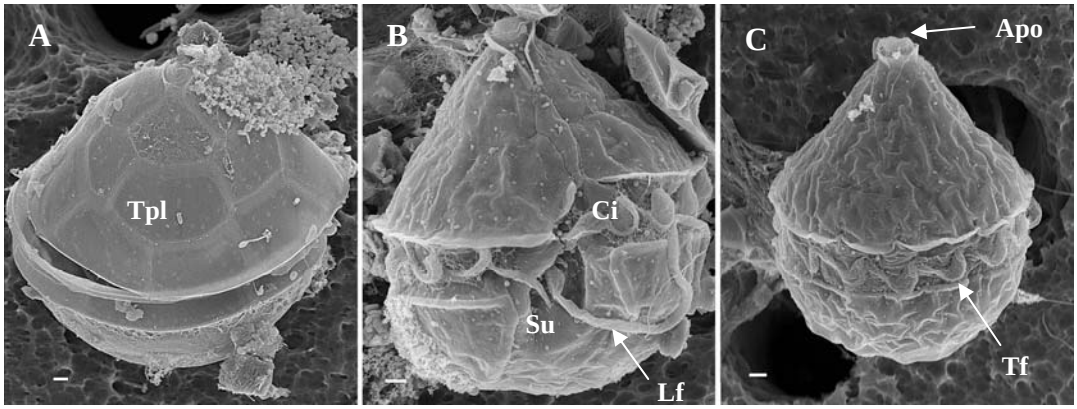


Plate XVIII. *Scrippsiella trochoidea*. Scale bars = 1 μm. A and C dorsal views. B frontal view. Tpl – thecal plates, Ci – cingulum, Su – sulcus, Lf – longitudinal flagellum, Tf- transversal flagellum and Apo – apical pore.

Genus *Bysmatrum*

Bysmatrum subsalsum Faust & Steidinger 1998

Syn: *Scrippsiella subsalsa* Steidinger & Balech 1977

Peridinium subsalsum Ostenfeld 1908

Steindinger & Balech (1977); Steidinger & Tangen (1996); Faust & Steidinger (1998).

Plate XIX

Description

Bysmatrum subsalsum is an armoured dinoflagellate of medium size 30-40 μm long and 30-50 μm wide. The cell is compressed dorsoventrally, the epitheca is conical and has a large pore in the apex. The hypotheca has a trapezoidal shape and is slight lobed in the antapical region.

The epitheca and hypotheca have almost the same length, in the scanning electron microscope was possible to determinate the plate formula: po, x, 4', 3a, 7'', 6c, 5''', op, 2'''' and 4s. The plates are connected through large growing strips. The theca has a wrinkled pattern of ornamentation. The cingulum is slightly displaced and well excavated. From the end of the sulcus besides the flagellum, a kind of wing is projected.

In the light microscope was possible to see numerous yellow-brown chloroplasts, radially arranged. The nucleus is in the central region, and a large pusula was visible.

Distribution

Bysmatrum subsalsum was a quite common species; present all the year in this benthic habitat, occurring sometimes as a bloom, especially in the end of spring and autumn, when the salinity was high (40-60) and the water was warmer (higher than 15ºC).

Toxicity: Non-toxic, but harmful if it proliferate to dense blooms.

Remarks

This benthic dinoflagellate had to be inserted in a new genus, *Bysmatrum*, together with two more benthic species, *B. arenicola* and *B. caponii*, Fuast & Steidinger (1998) distinguished them from the other species belonging to the genus *Scrippsiella*, that are

planktonic. The species from the genus *Bysmatrum* share exclusively a number of morphologic characteristics: apical plate 1' is wide, asymmetric, pentagonal and ends at the anterior margin of the cingulum; intercalary plates 2a and 3a separated by apical plates 3' and 4''; the apical pore complex is a chamber with a large P_o plate, elongated X plate, six cingular plates and four sulcal plates are present; and the thecal surface is reticulate.

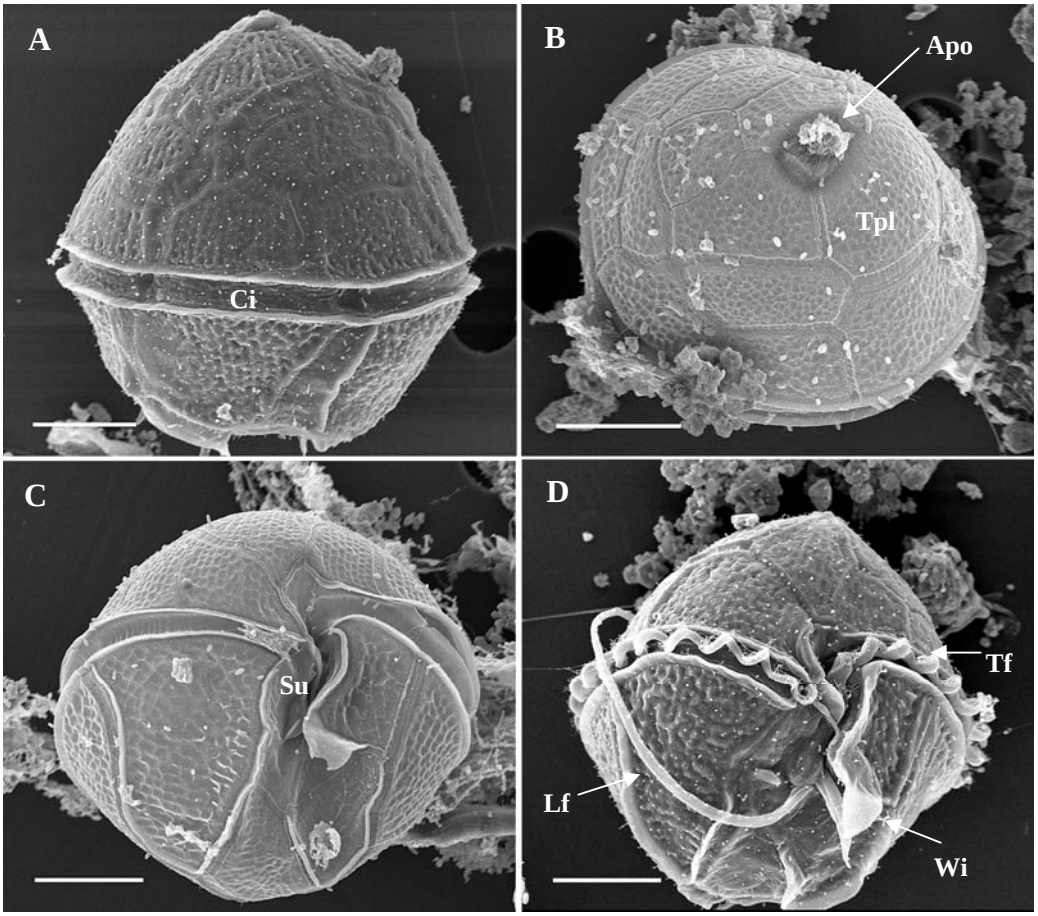


Plate XIX. *Bysmatrum subsalsum*. Scale bars = 10 μm. A dorsal view. B apical view. C and D frontal views. Ci – cingulum, Apo – Apical pore, Tpl – Thecal plates, Su – sulcus, Lf – longitudinal flagellum, Tf – transversal flagellum and Wi – wing.

Family Peridiniaceae

Genus *Heterocapsa*

Heterocapsa niei (Loeblich) Morril & Loeblich III 1981
Syn: *Cachonina niei* Loeblich 1968

Dodge (1982); Steidinger & Tangen (1996).

Plate XX

Description

Heterocapsa niei is a small spindle-shaped armoured dinoflagellate, being narrower in the cingulum region, the epitheca and hypotheca have similar shape and size. The cell size found was between 20-23 μm long, 14-16 μm width and a ratio length/width of 1,4.

At light microscope was possible to see the central nucleus and numerous yellow-brown chloroplasts surrounding a large pyrenoid.

At the scanning electron microscope was not possible to see the plate formula, due to the numerous scales that the cell has covering the theca.

Distribution

Heterocapsa niei is a planktonic species, found only a few times during the autumn and winter, but when the salinity was still higher than 30 . In November of 2003 occurred a small bloom of this species in one of the ponds (site 2), this lasted only few days.

Toxicity: Non-toxic but is a bloom forming species.

Remarks

Heterocapsa niei is very similar with *H. triquetra*, but distinguished because this latter species has a more conical hypotheca.

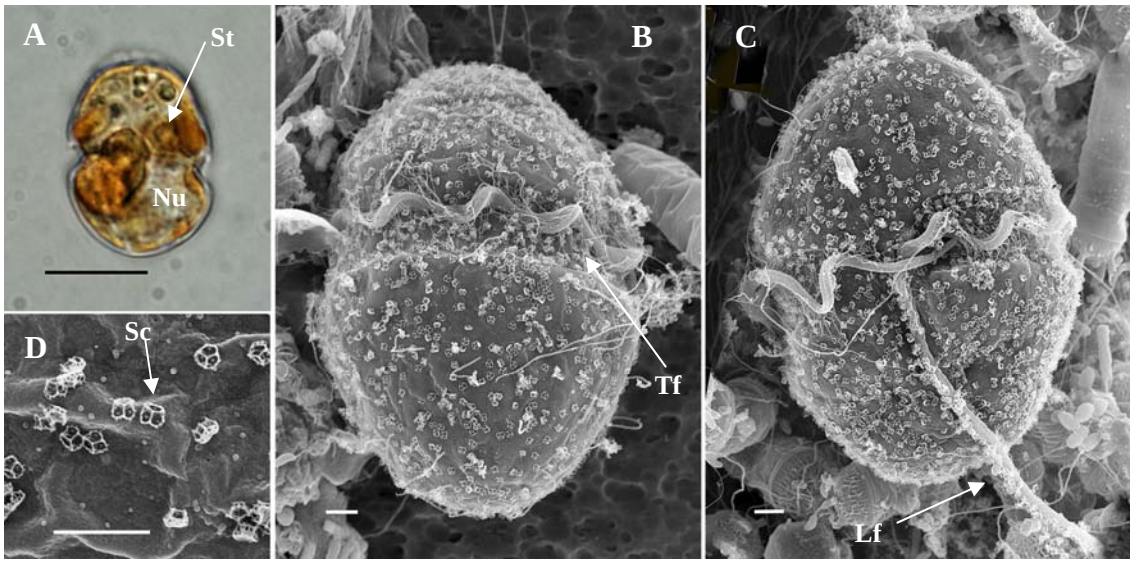


Plate XX. *Heterocapsa niei*. Scale bars = 10 μm in A; 1μm in B-D. A in light microscopy. B dorsal view. C frontal view. D scales in detail. Nu – nucleus, St – stigma, Tf- transversal flagellum, Lf – longitudinal flagellum and Sc – scale.

Genus *Kryptoperidinium*

Kryptoperidinium foliaceum Lindemann 1924
Syn: *Peridinium foliaceum* **(**Lindemann) Biecheler 1952
Glenodinium foliaceum (Lindemann) Dodge 1982

Dodge (1982); Trigueros *et al.* 2000; Hallegraeff (2002).

Plate XXI

Description

Kryptoperidinium foliaceum is an armoured dinoflagellate, with a very flat shape (markedly compressed dorsoventrally) and it is a very active swimmer. It can form cysts easily in culture when the medium conditions drop. The cell size can vary drastically, length between 15 to 35 μm and width from 10 to 30 μm.

The main characteristic of this species visible at light microscopy was the red stigma near the flagellar pore in the hypotheca, the numerous small yellow-brown chloroplasts and the nucleus with a central position.

At the scanning electron microscope was possible to see that the theca is thin, but the plates were not visible. The two flagella were preserved, and in the sulcus where the transversal flagellum emerges was also possible to see a small peduncle projected.

Distribution

Kryptoperidinium foliaceum was a species present very often in the ponds, occurring in some periods with high abundance, especially in the end of the autumn and beginning of winter, when the salinity was lower than 30 and the water temperature lower than 20ºC.

Toxicity: Non-toxic, but is a bloom-forming species.

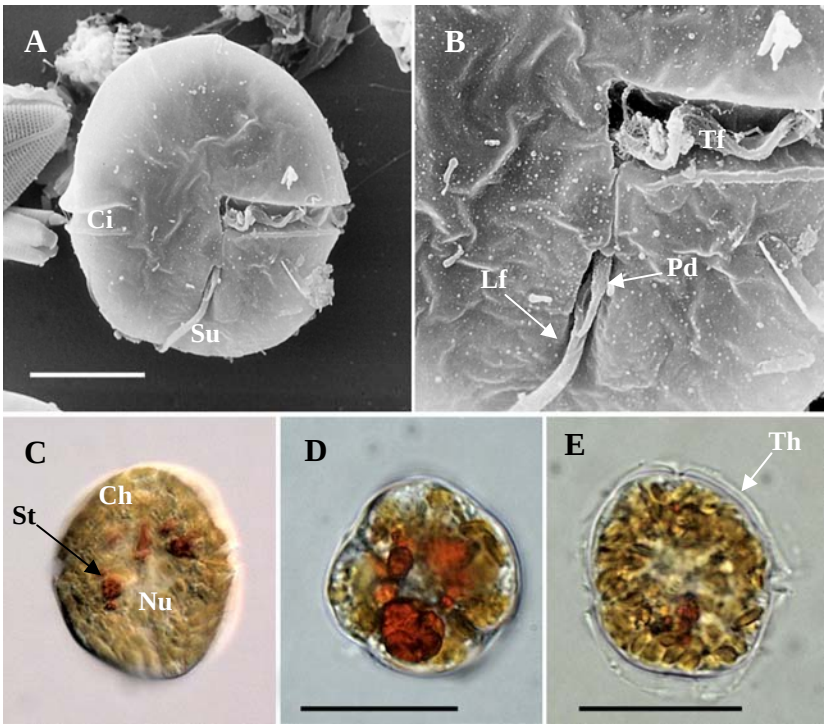


Plate XXI. *Kryptoperidinium foliaceum*. Scale bars = 10 µm in A. 20 µm in C-E. A frontal view of a cell with the sulcal region in detail in B. C frontal view. D early stage of encystment. E dorsal view. Ci – cingulum, Su – sulcus, Tf – transversal flagellum, Lf – longitudinal flagellum, Pd – peduncle, St – stigma, Ch – chloroplast, Nu – nucleus and Th – theca.

Genus *Peridinium*

Peridinium quinquecorne Abé 1927
Syn: *Protoperidinium quinquecorne* Balech 1974

Dodge (1982), Trigueros *et al.* 2000.

Plate XXII

Description

Peridinium quinquecorne cell has a typical *Peridinium* shape, slightly compressed dorsoventrally, with the particularity of having four small antapical horns in the hypotheca, which in lateral view can be mistaken for only two, and one apical horn in the epitheca.

The cell size is between 20-30 μm long and 20-30 μm broad, with ratio of 1,1, the epitheca has a more conical shape and the hypotheca is more rounded. The plate formula is: po, x, 3', 2a, 7'', 5c, 4s, 5''' and 2''''.

In lateral view is possible to see that the cingulum is more orientated to the left and the sulcus extends until the antapex.

This species has a red stigma near the sulcus, a central nucleus and several yellow-brow chloroplasts visible in light microscopy.

Distribution

Peridinium quinquecorne is a planktonic species, was only registed once in very high quantities, in a time when the influence of the sea in the estuary was higher.

Toxicity: Non-toxic.

Remarks

This species is considered a bloom forming species, producing discolouration of the waters.

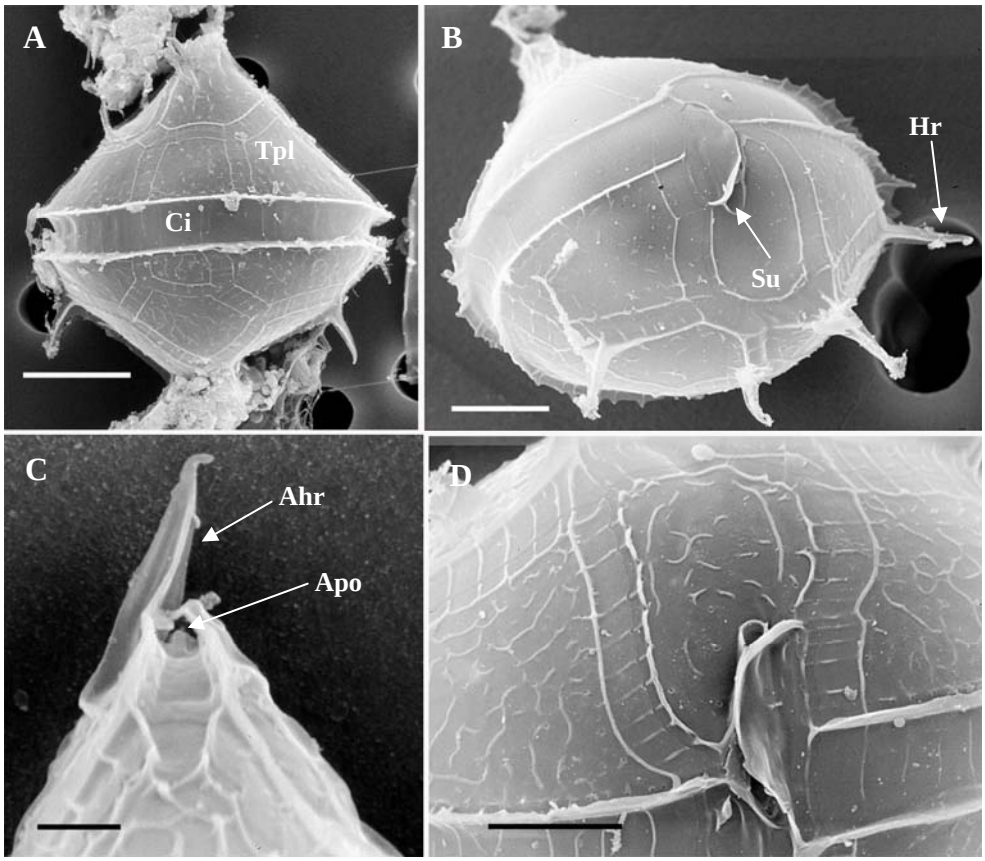


Plate XXII. *Peridinium quinquecorne*. Scale bars = 10 μm in A; 5μm in B and D; 1 μm in C. A dorsal view. B antapical view. C apicar horn (Ahr). D detail of the sulcal area. Ci – cingulum, Tpl – thecal plate, Su – sulcus, Hr – horne and Apo – apical pore.

Family Protoperidiniaceae

Genus *Protoperidinium*

Protoperidinium minutum (Kofoid) Loeblich III 1970
Syn: *Peridinium minutum* Kofoid 1907
Peridinium monospinum Paulsen 1907

Dodge (1982); Steidinger & Tangen (1996);

Plate XXIII

Description

Protoperidinium minutum is medium size, incolour and an armoured dinoflagellate, with a globular shape and an apical horn. The cell size is variable between 20-40 μm long and 20-55 μm wide. The hypotheca is slightly smaller than the epitheca.

The sulcus is posteriorly expanded and has a prominent short left sulcal list. The surface of theca is filed with short spines and randomly fine pores.

At the light microscope this species appeared completely colourless.

Distribution

Protoperidinium minutum occurred in the beginning of autumn, when the temperatures were mild and the salinity was still high, with values higher than 10ºC and 30 respectively. However, this species is more planktonic than benthic being more usual in areas that are in constant contact with the sea.

Toxicity: Non-toxic.

Remarks

Protoperidinium minutum is a cyst forming species, recorded by Labour (1925) in which the cysts were liberated by the theca opening at the girdle (Dodge 1982).

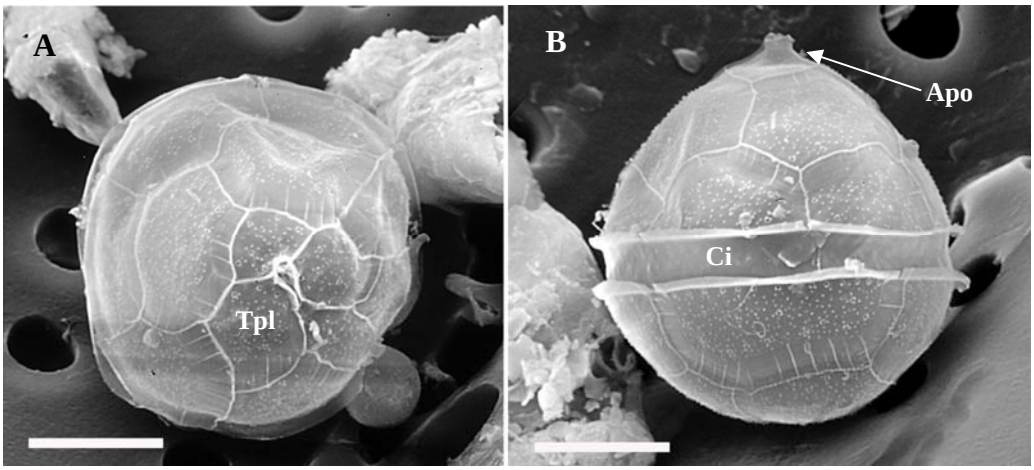


Plate XXIII – *Protoperidinium minutum*. Scale bars = 10 μm. A is a apical view of the cell and B is a dorsal view. Tpl – thecal plate, Ci – cingulum and Apo – apical pore.

Family Kolkwitziellaceae

Genus *Oblea*

Oblea rotunda (Lebour) Balech1964
Sournia 1973
Syn: *Peridiniopsis rotunda* Lebour 1922
Glenodinium rotundum Schiller 1937
Diplopsalis rotunda (Lebour) Wood 1968
D. rotundata Steidinger & Williams 1970

Dodge (1982); Thomsen (1992); Steidinger & Tangen (1996); Chomérat *et al.* (2004)

Plate XXIV

Description

Oblea rotunda is a small armoured dinoflagellate, the epitheca and hypotheca are rounded and with similar size. In the epitheca is possible to distinguish a small apical prominence, where lies an apical pore. In the hypotheca the sulcus is bordered to the left and ends with a wing.

The cell size varies between 20 to 30 μm of diameter. At the light microscope it was possible to see that the cell did not have chloroplasts, but it had a big pusula in the hypotheca. The nucleus lies in the centre of the cell. Using epiflourescence microscope was possible to determinate the plate formula: 3', 1a, 6'', 3c, 5''' and 2''''.

Distribution

Oblea rotunda was present quite often in the ponds, especially in autumn and winter, when it occurred in higher densities. It seems to have no preferences in terms of salinity or water temperature, since it was found in salinities from 13 to 36 and temperatures from 6 to 24°C. Usually appeared in times when many diatoms were present.

Toxicity: Non-toxic but is a bloom-forming species.

Remarks

Recently this species was found in more eutrophic conditions, where it occurred in high densities, especially after a diatom bloom, usually this species is found in open sea regions, but it seems that now its preferences are expanding (Chomérat *et al.* 2004). The *O.*

rotunda found in the ponds occurred when diatoms were quite abundant, probably because it feeds on them.

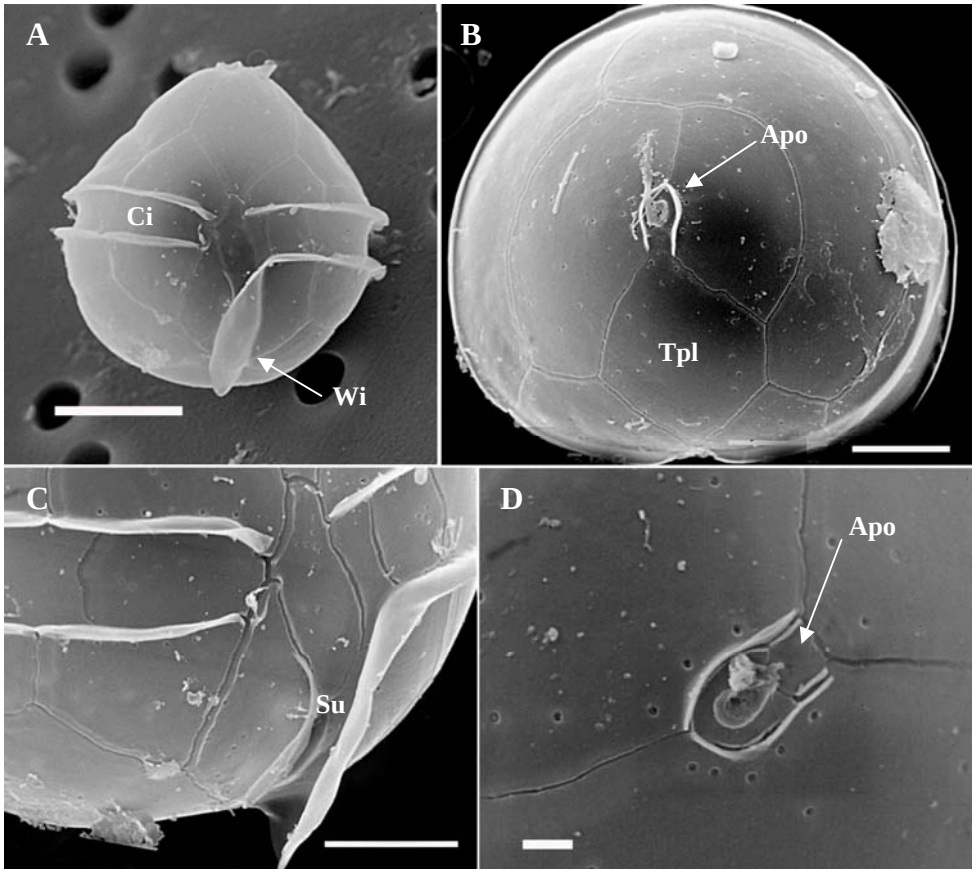


Plate XXIV. *Oblea rotunda*. Scale bars = 10μm in A; 5 μm in B and C; 1 μm in D. A is a frontal view. B is an apical view. C shows the sulcal region. In D is the apical pore in detail. Ci –cingulum, Wi – wing, Apo – apical pore, Tpl – thecal plate and Su – sulcus.

Order Gonyaulacales

Family Ostreopsidaceae

Genus *Coolia*

Coolia monotis Meunier 1919
Syn: O*streopsis monotis* (Meunier) Lindemann 1928
Glenodinium monotis (Meunier)Biecheler 1952

Fukuyo (1981); Dodge (1982); Faust (1992); Steidinger & Tangen (1996); Gert *et al.* (2001); Hallegraeff (2002); Faust & Gulledge (2002).

Plate XXV

Description

Coolia monotis is a benthic armoured dinoflagellate, the cell is compressed anterior-posteriorly in an oblique axis. The cell size range can vary from 28 to 49 μm length and 26 to 48 μm diameter, with a ratio of 1,1.

In light microscope the cell appeared with an elliptic shape, the epitheca has the same shape of hypotheca, but is slightly smaller. Inside the cell are numerous yellow-brown chloroplasts radially distributed; in the centre of the hypotheca is the nucleus, and near the sulcus is visible a big pusula.

In the scanning electron microscope was possible to see the characteristic plate distribution of this species, as well as the pore pattern that ornament the theca, they are regularly distributed and each pore as inside 5 to 7 smaller pores connected. The cingulum is descending in frontal view. The apex is also displaced, dorsally to the left.

In culture when the cells of *C. monotis* are in proliferation, they tend to swim together involved by common mucilage, produced by them. This mucilage is initially brown but with the age of the culture tend to turn to white and be denser.

Distribution

Coolia monotis is a benthic species quite frequent in the ponds, especially in spring and summer. This species seems to prefer warmer waters and relatively high salinities from 30 to 52, in this conditions it can occur in elevate densities. In the winter can also occur, if the salinity does not drop too much.

Toxicity: Toxic species, *C. monotis* produces yessotoxin analogues.

Remarks

This species shows similarity with the genus *Ostreopsis*, since *C. monotis* as the same fundamental epitheca plate arrangement of *O. siamensis*, and was once allocated in *Ostreopsis* genus by Schiller. Due to differences in the plate configuration in the

hypotheca, the size cell and the position of the apical plate, it was adequate to collocate this species in the original genus *Coolia* (Fukuyo 1981).

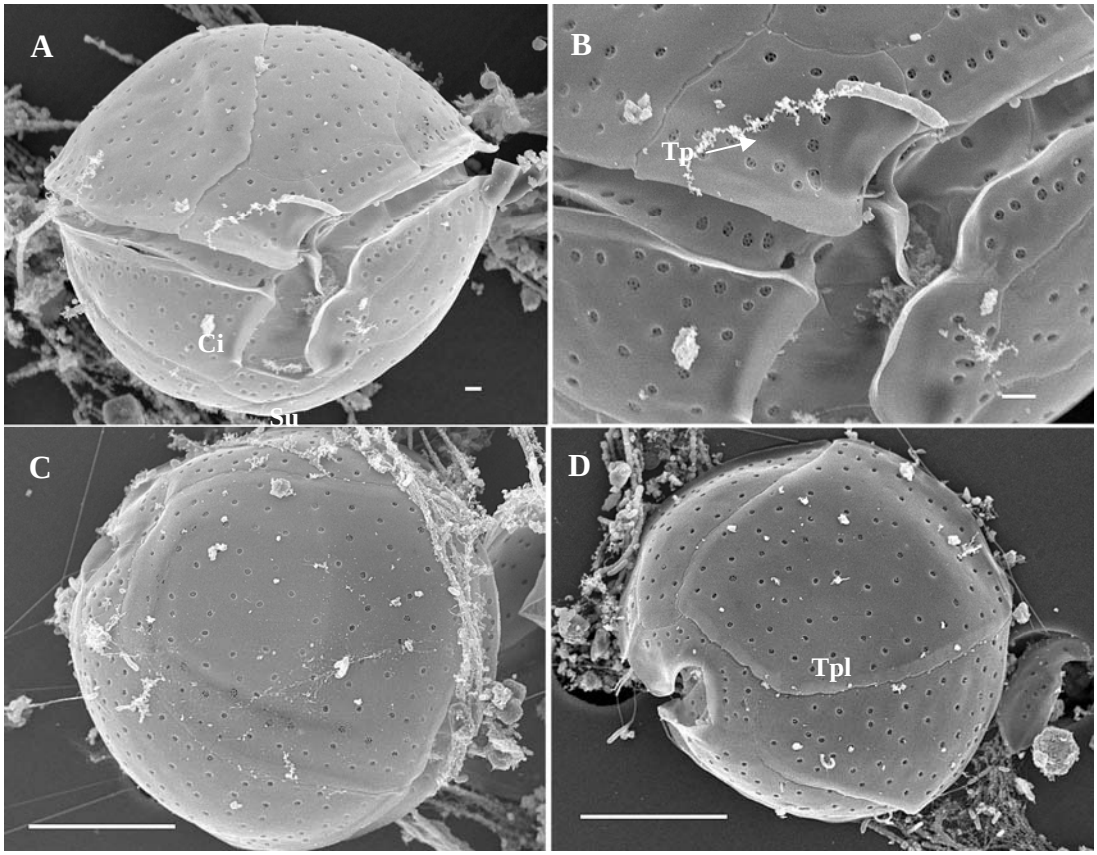


Plate XXV. *Coolia* monotis. Scale bars = 1 μm in A and B; 10 μm in C and D. A cell in frontal view, with the sulcal area in detail in B. C antapical view. D apical view. Ci – cingulum, Su – sulcus, Tp – thecal pore and Tpl – thecal plate.

Order Gymnodiniales

Family Gymnodiniaceae

Genus *Amphidinium*

Amphidinium massartii Biecheler 1952
Syn: *Amphidinium höfleri* Schiller & Diskus 1955

Hulbert (1957); Dodge (1982); Murray *et al.* (2004); Jørgensen *et al.* (2004).

Plate XXVI and XXVII

Description

Amphidinium massartii is a small unarmoured dinoflagellate; the cell has an oval shape in ventral view and is dorsoventrally flatted. Cell size range can vary from 16-26 μm long and from 11-22 μm wide.

The epicone is very small and emerge from the hypocone as an appendix pointing toward to the left. The cingulum is displaced and begins at 1/3 of the cell length from the apex, descending until the median region.

At light microscope was possible to see that the round nucleus is in the posterior part of the hypocone. In the middle of the cell lies a large pyrenoid, surrounding are several yellow-brown chloroplast disposed in radiating pattern.

In the scanning electron microscope was possible to determinate the exact position from where the flagella emerge, there is a narrow ventral ridge that runs between the two sites of flagellar insertion.

This species was only confirmed as being *A. massartii* after comparing its LSU-rDNA sequence with other *Amphidinium* sequenced (Jørgensen *et al.* 2004), which revealed to be identical with the sequence of the strain CCMP 1821 of *A. massartii* with the accession number in the GenBank AY 455670.

Distribution

Amphidinium massartii was found in the ponds from the end of spring until the autumn, occurring sometimes as dominant species, being at high densities, it showed preference for high salinities and moderate water temperature (20 – 25 ºC).

Toxicity: Non toxic, but tend to form dense blooms.

Remarks

Amphidinium massartii showed similarity with other species from the genus *Amphidinium*, what can make the identification just based in morphologic characters a very difficult process, causing considerable taxonomic problems (Murray *et al.* 2004). *A. massartii* as a synonym of *A. hofleri* can create even more confusion since it is also a synonym of *A. operculatum* in the literature (Dodge 1982).

Amphidinium massartii is very similar with *A. carterae*, in terms of size and shape, the main difference is that *A. carterae* has a single reticulate chloroplast, that give to these cells a yellow-brown colour in opposition to the yellow-green colour of the cells of *A. massartii*. Only molecular data seems to be able to clarify completely this situation (Jørgensen *et al*. 2004 ; Murray *et al*. 2004).

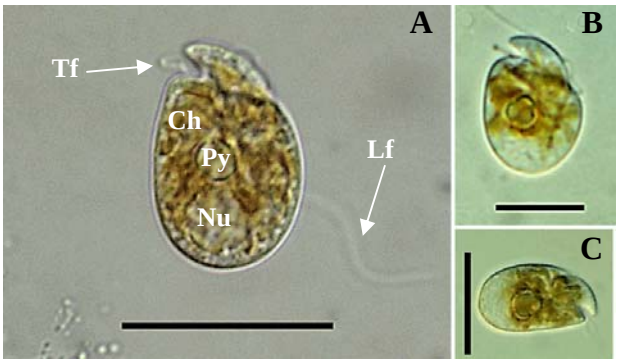


Plate XXVI. *Amphidinium massartii*. Scale bars = 20 µm. Cells in frontal view. Py – pyrenoid, Nu – nucleus, Ch – chloroplast, Tf – transversal flagellum and Lf – longitudinal flagellum.

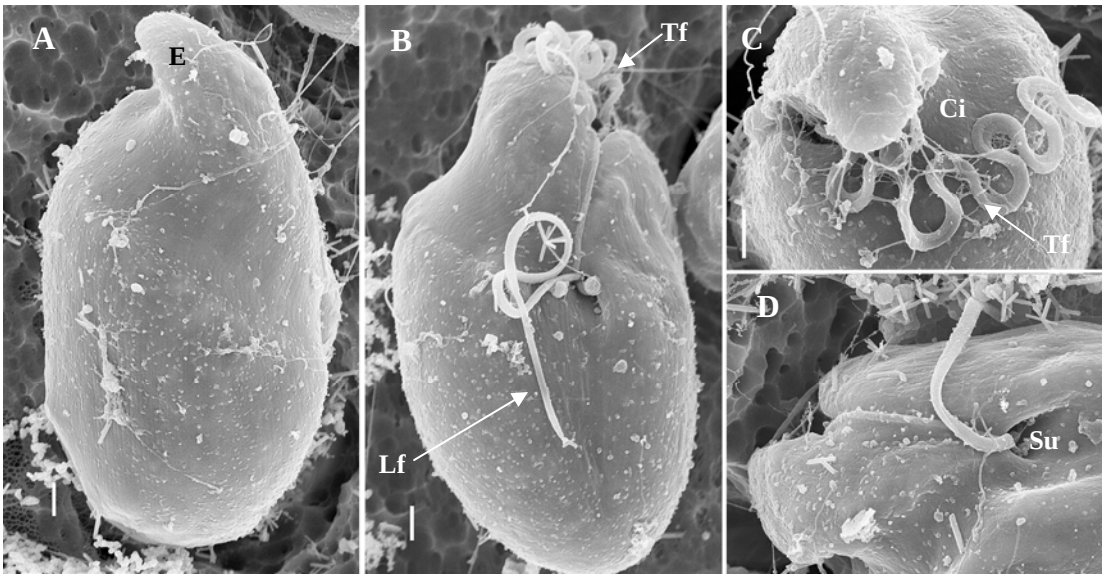


Plate XXVII. *Amphidinium massartii*. Scale bars = 1 µm. A dorsal view. B frontal view. C apical view. D sulcal view. E – epitheca, Tf – transversal flagellum, Lf – longitudinal flagellum, Ci - cingulum and Su – sulcus.

Genus *Akashiwo*

Akashiwo sanguinea G. Hansen & Moestrup 2000
Syn: *Gymnodinium sanguineum* Hirasaka 1922
Gymnodinium splendens Lebour 1925
G. nelsonii Martin 1929

Steidinger & Tangen (1996); Daugbjerg *et al.* (2000); Hallegraeff (2002); Faust & Gulledge (2002).

Plate XXVIII

Description

Akashiwo sanguinea is a large unarmoured dinoflagellate, the cell size range varies from 47 to 57 μm long, 40 to 50 μm wide and a ratio of 1,2: the cell has a pentagonal shape being more wide in the cingulum region. The epitheca tends to a conical shape and the hypotheca is bilobed in the posterior end, but in term of size, they are almost equal.

At the light microscope, the cell showed a red-brown intense colour, due to the numerous chloroplasts in radial distribution, the nucleus is also large and occupies the central region of the cell.

At the scanning electron microscope was possible to see that the cingulum is displaced in one width of the cingulum, and the sulcus does not reach the epitheca but enters deeply in the hypotheca. In the epitheca is possible to notice an apical groove, is a large clockwise spiral, only visible in apical view of the cell.

Akashiwo sanguinea is a naked dinoflagellate, during the fixation procedure numerous trichocysts were shoot and were visible in SEM, giving to the cell a tangled aspect.

In culture, this species does not reaches dense numbers of cells, and with the age of the culture the number of motile cells tend to decrease, with the increase on the number of cells in resting stages (cysts).

Distribution

This species was a constant in the ponds studied, especially from the summer to the winter, when the salinity was around 30 , in concern to the water temperature this species seemed to have no preference.

Toxicity: Non-toxic, but was already implicated in cases of oyster losses, probably due to physical clogging of the gills.

Remarks

The colour, size and shape of this species can vary during the growth or conditions of the cell. The cysts are very large and formed by a mucoid halo (Steidinger & Tangen 1996).

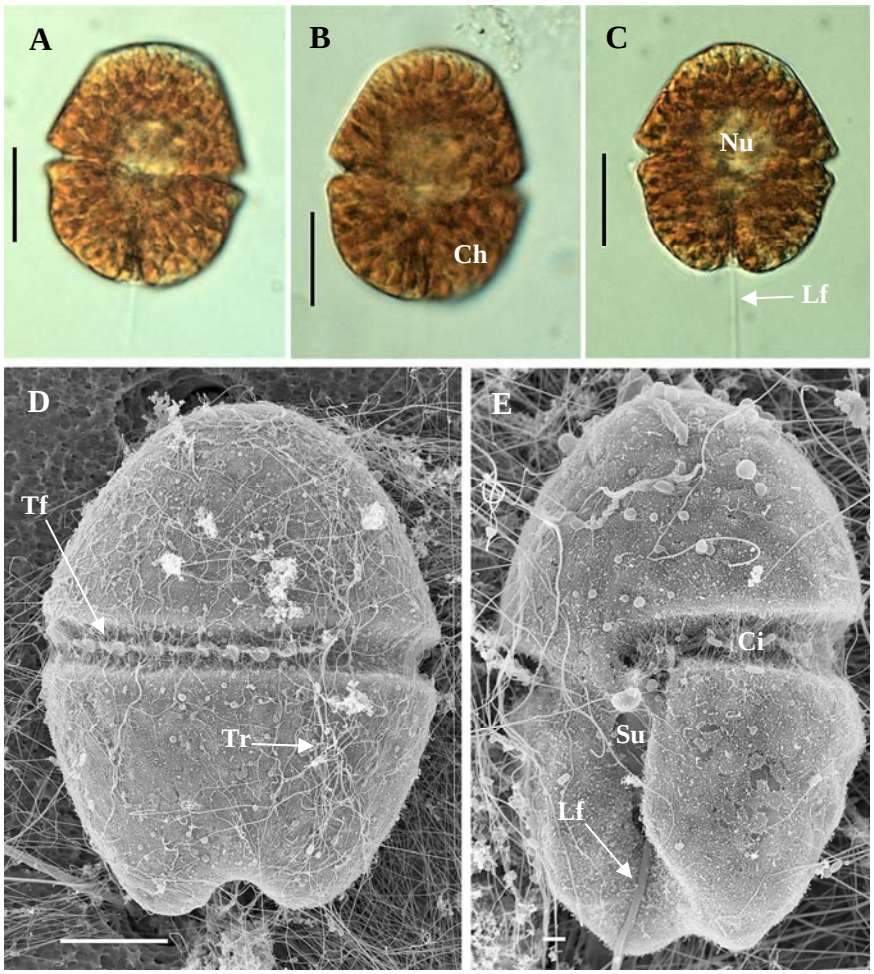


Plate XXVIII. *Akashiwo sanguinea*. Scale bars = 20 μm in A to C; 10 μm in D and 1 μm in E. A - D dorsal views of the cell. E is a frontal view, Ch – chloroplast, Nu – nucleus, Lf – longitudinal flagellum, Tf – transversal flagellum, Tr – trichocysts, Su – sulcus and Ci – cingulum.

Genus *Gyrodinium.*

Gyrodinium sp. 1

Steidinger & Tangen (1996)

Plate XXIX

Description

Gyrodinium sp. 1 is a medium size unarmoured naked dinoflagellate. The cell size was around 30 μm long and 20 μm wide, and is slightly dorsoventrally compressed, the cell has an ovoid to biconical shape. The cingulum is displaced more than one-fifth of body length in a descending left spiral, the sulcus invades the epicone. At the light microscope was not possible to see the apical groove. The cells are colourless since there are no chloroplasts, some colour inside the cell is due to the presence of some food vacuoles. In the cell it is also possible to see a kind of striations that comes from the apical apex to the antapical apex. The nucleus has a central position in the cell.

Distribution

Gyrodinium sp. 1 was only found once in the beginning of summer of 2004, but in high densities, when the salinity was very high (45).

Remarks

The identification to the species level was not possible, because not all morphologic features important to the identification were observed.

This genus is similar to the genus *Gymnodinium*, the main difference is in the displacement of the cingulum that in the genus *Gyrodinium* is higher, is more than one-fifth of the body length. This species has the particularity of not having any chloroplasts, so it is a heterotrophic species, in some cells was possible to distinguish clearly the food vacuoles, this genus is known for including phagotrophic species (Steidnger & Tangen 1996).

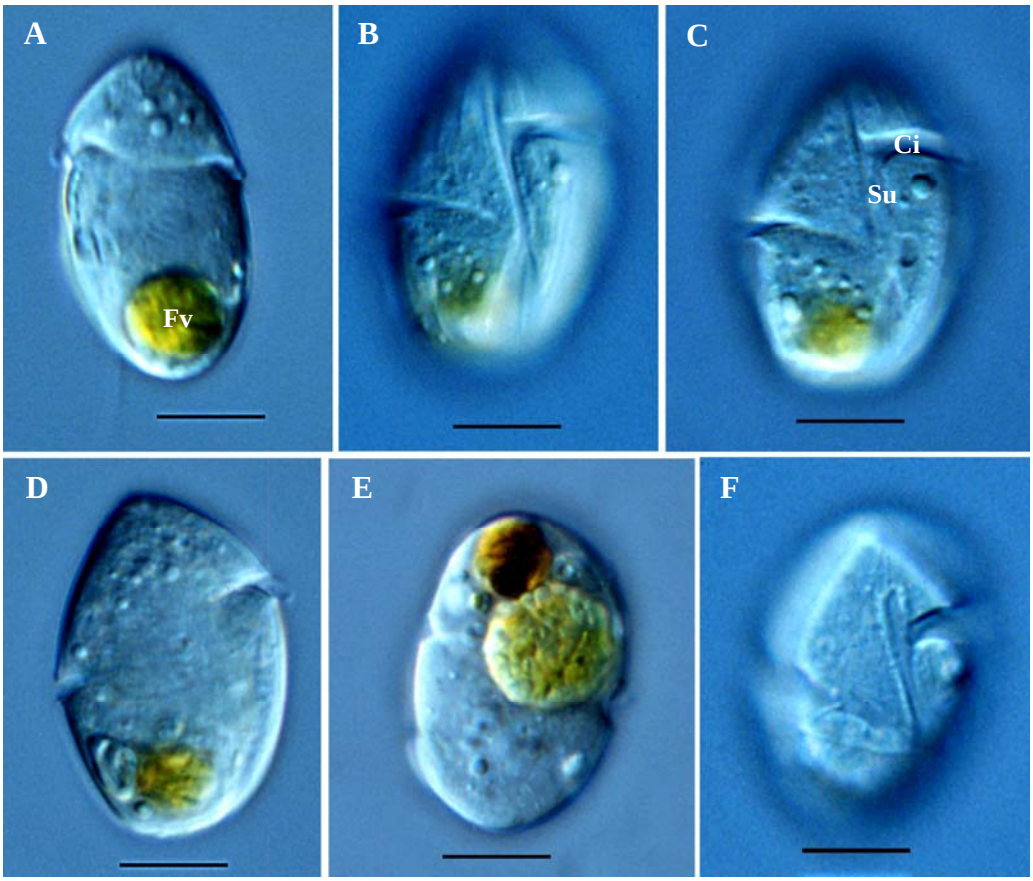


Plate XXIX. *Gyrodinium* sp. 1. Scale bars = 10 µm. Different cells of a colourless *Gyrodinium* with food inside (A to E), the F cell is completely colourless. Fv – food vacuole, Ci – cingulum and Su – sulcus.

Family Polykrikaceae

Genus *Pheopolykrikos*

***Pheopolykrikos* sp. 1**

Steidinger & Tangen (1996)

Plate XXX

Description

Pheopolykrikos sp. 1 is an unarmoured dinoflagellate, with small to medium size and has a twisted shape. The apexes are lobed, the epicone and hypocone have a rounded shape. The cingulum is displaced two times the width and about one-third the body length, the cell is not compressed dorsoventrally. The nucleus is in the centre of the cell, and

surrounding are several small rounded chloroplasts that give to the cell a yellow-green colour.

Distribution

Pheopolykrikos sp. 1 was only found once in the beginning of autumn of 2003, but with many cells, when the salinity was still very high (40).

Remarks

Pheopolykrikos sp. 1 species was not identified to the species level since not all important features were possible to be observed, this species was only recorded once and did not survive in culture. This organism must be a *Pheopolykriko*s species since it has visible chloroplasts, feature not observed in the species from the genus *Polykrikos* (Steidnger & Tangen 1996).

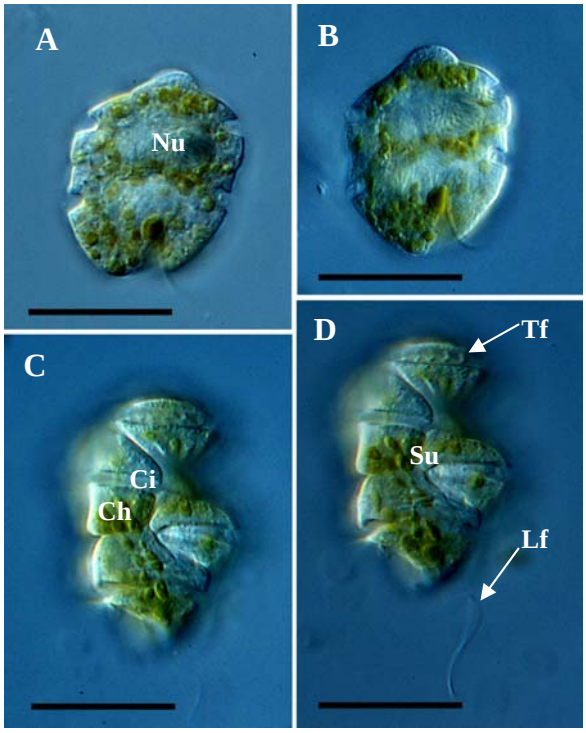


Plate XXX. *Pheopolykrikos* sp. 1. Scale bars = 20µm. A and B cells in a division stage, two nucleus (Nu) are visible. C and D motile cells in frontal view with visible chloroplast (Ch), D also shows both flagella: longitudinal flagella (Lf) and transversal flagella (Tf). Ci – cingulum and Su – sulcus.

Uncertain taxa

Genus *Oxyrrhis*

Oxyrrhis marina Dujardin 1841
Syn: *Oxyrrhis tentaculifera* Conrad 1939
O. maritima Van Mell 1969

Dodge (1982); Steidinger & Tangen (1996).

Plate XXXI

Description

The cell of *O. marina* is unarmoured, has an ovoid shape and is slightly laterally compressed, has no cingulum or sulcus. From the posterior part of the cell a kind of tentacular lobe is projected, inside is a depression from which two dissimilar flagella emerge.

At the light microscope was also possible to see that the nucleus lies at the anterior end of the cell. The cell is colourless due to the inexistence of chloroplasts, but frequently is possible to see some food vacuoles in the cytoplasm, that give to the cell some colour.

The size range of the cell can vary from 10 to 35 μm long and 10 to 30 μm wide.

Distribution

Oxyrrhis marina was a very common and abundant species in these ponds, being more abundant from spring to autumn. This species seemed to prefer high salinities from 30 to 60 , but has no ideal range of water temperature to occur, since it was found in temperatures that vary from 6 to 30°C.

Toxicity: Non-toxic but is a bloom-forming species (Begun *et al.* 2004).

Remarks

Oxyrrhis marina is a heterotrophic organism, has been found consuming other dinoflagellates, green algae, diatoms and yeast, but can survive as well in a defined medium (Dodge 1982).

The question of whether *Oxyrrhis marina* is a dinoflagellate remains, since it has no morphology comparable with the other groups of dinoflagellates, the microtubular

cytoskeleton, the flagella and nuclear structure are also different, this species seems to be between the dinoflagellates and other eukaryotes (Steidinger & Tangen 1996).

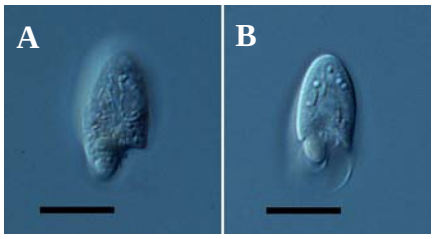


Plate XXXI. *Oxyrrhis marina*. Scale bars = 10 μm. Cells in frontal view.

Discussion

The correct identification of dinoflagellates is not an easy task. Early descriptions of dinoflagellates, produced with limited means of observation, are often lacking in detail and therefore ambiguous; some however are remarkably detailed and are still useful today.

Nowadays, less attention is given in general to species identification, originating incorrect identifications and attributions of some properties to species that do not posses them.

In the dinoflagellate group, the most studied species are the ones capable to produce toxins or blooms, because of their direct impact on economies and this generated considerable knowledge about them, which unfortunately does not extend to all dinoflagellate species.

This discussion is focused on the *Prorocentrum species* and on *Amphidinium massartii*, since these where subjected to more detailed studies, that include besides the morphological study, a toxicity study through *Artemia* bioassay and in some cases to a genetic study in which the LSU-rDNA was sequenced (*P. micans* and *A. massartii*).

The different species of *Prorocentrum* identified in the sheltered habitats of the Ria de Aveiro are evidenced in this discussion, because it includes toxic and bloom forming species. This genus has also a taxonomic interest, it includes species that should be transferred to another genus, the genus *Exuviaella*, face to recent data from morphologic, biochemical and genetic studies.

The Genus *Prorocentrum*

The genus *Prorocentrum* includes more than 40 species and this number is constantly increasing (Faust 1990; Faust 1993[a]; Faust 1993[b]; Faust 1994; Faust 1997; Morton & Tindall 1995; Puigserver & Zingone 2002; Morton *et al.* 2002; Ten-Hage *et al.* 2000). The cell is characterized by having two laterally compressed valves, dimorphic flagella inserted anteriorly and an ovate to rounded shape (see Fig 2.3).

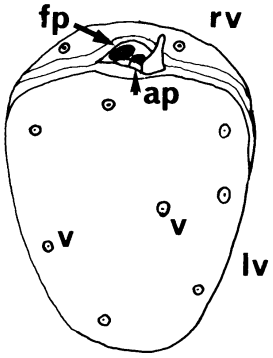


Fig. 2.3. Schematic diagram of *Prorocentrum* cell. ap- auxiliary pore; fp- flagellar pore; lv- left valve; rv- right valve; v- valve pore. Adapted from Loeblich *et al.* (1979).

The left valve is flat and the right has at the anterior end a V-shape depression, where the apical plates are inserted, as well as the flagellar pore from which the flagella emerge (see previous explanation in the introduction of this chapter). To distinguish the species it is sometimes necessary to examine these apical plates and to look for the presence of an apical spine. Valve morphology is also important, details about the surface of valves, ornamentation of thecal plates and the organization of the periflagellar area and intercalary band (see Fig. 2.5) (Dodge 1982; Faust *et al.* 1999). This genus is not known to produce characteristic cysts.

Genus *Prorocentrum versus* Genus *Exuviaella*

Dodge & Bibby (1973) analysed the fine structure of species from the genera *Prorocentrum* and *Exuviaella*, showed that there is no basic distinction between these two genera and proposed that the genus *Exuviaella* should be abandoned and its species incorporated into *Prorocentrum*. The only significant distinction was the presence or absence of an apical spine. However, this was contradicted by the inconsistent presence of other features: type of pyrenoid, ornamentation, structure of theca and apical plates, presence or absence of an apical spine and fibrilar bodies.

Dodge (1975) made a revision of the taxonomy within the genus *Prorocentrum* reducing 64 species to 21, by considering some as synonymous and others as aberrant forms. He concluded that characterization based only on shape and cell size was ambiguous, and to a correct identification a detail study on pore ornamentation, number and arrangement of apical plates, the type of pyrenoid, presence or absence of trichocysts and shape and size of the nucleus should be taken in consideration. So based on these features five distinct groups were established in the genus *Prorocentrum*:

a) No obvious pores, no anterior spines or ornamentation, no trichocysts (*P. aporum* and *P. cassubicum*);
b) No anterior spine or ornamentation, trichocyst pores present (*P. lima*, *P. ovum*, *P. dactylus* and *P. nanum*);
c) Small anterior spines and sometimes surface depressions or ornamentation present, trichocyst pores present (*P. compressum*, *P. magnum*, *P. rotundatum* and *P. triestinum*);

d) Large anterior spine present, posterior end pointed, surface of plates covered with depressions (*P. rostratum*, *P. micans*, *P. schilleri*, *P. arcuatum*, *P. gracile* and *P. scutellum*);

e) Spiny thecal plates present, trichocyst pores and anterior spine also occur (*P. dentatum*, *P. vaginulum*, *P. minimum*, *P. cordatum* and *P. balticum*).

In terms of phylogeny, *P. aporum* was considered by Dodge (1975) as being the most primitive *Prorocentrum* species. This organism is rounded and seems to lack pores, spines or any kind or ornamentation. The next most primitive species was *P. cassubicum*. Based on the complexity of the species Dodge also created a diagram showing the possible phylogenetic relationships of the 21 species recognized (see Fig. 2.4).

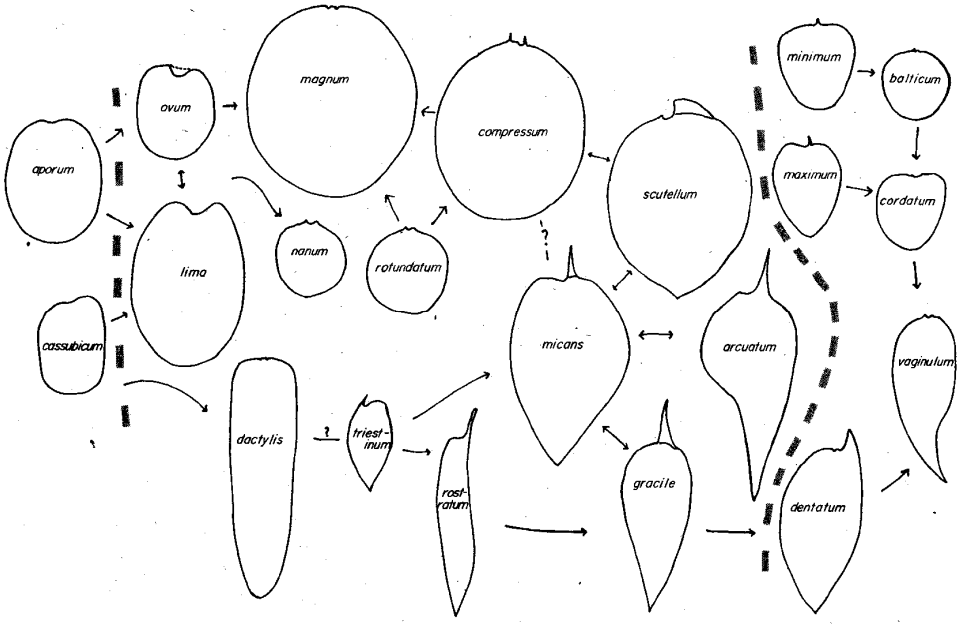


Fig. 2.4. Schematic diagram showing the phylogenetic relationships between *Prorocentrum* species, proposed by Dodge (1975).

Nowadays more information about the species of *Prorocentrum* in terms of ecology, morphology and genetics is available, and some evidence seems to favour the reinstatement of the genus *Exuviaella*. The genus *Exuviaella* would include marine prorocentroids, with a primary benthic life habit, which have no apical spine or tooth, no thecal spines, and thecal pores associated with mucocysts and capacity of producing secondary metabolites (DSP-type toxins). Based on these characteristics, species known as *P. lima* Ehrenberg, *P. maculosum* Faust and *P. hofmanianum* Faust, for instance should be

transferred to the genus *Exuviaella*. Although there is some molecular data that support the separation of the two genera, the issue remains controversial (McLachlam *et al.* 1997).

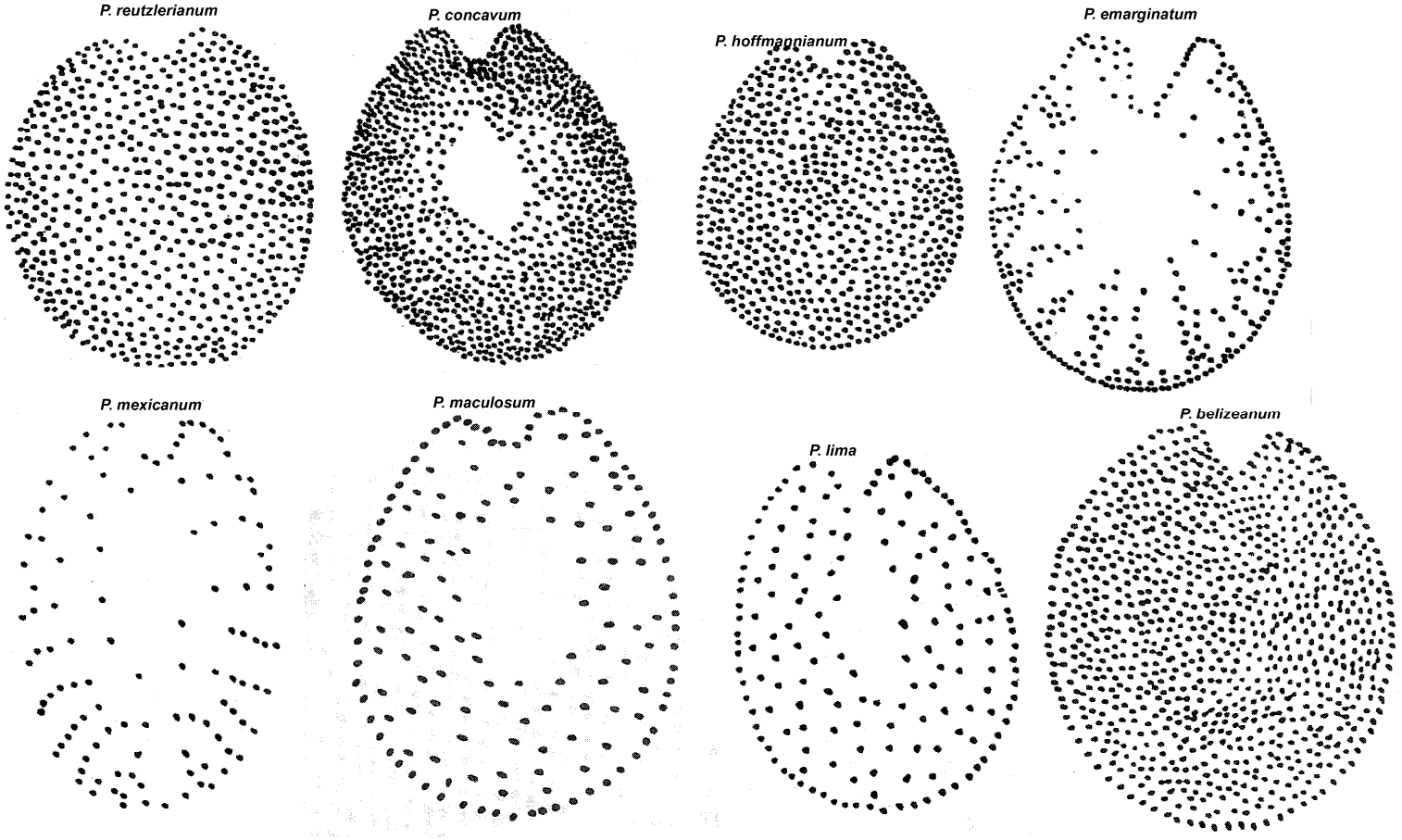


Fig. 2.5. Diagram showing diffrent types of pores organization in the eight benthic *Prorocentrum* species, adapted from Faust *et al.* (1999). The pore organization and number are good criteria to identify a *Prorocentrum* species.

Some of the features chosen by Dodge (1975) to subdivide the genus *Prorocentrum* in five sub-groups are no more valid, like trichocysts pores in *P. lima* and that each flagellum emerge from its own apical pore, when they really emerge by the same.

Zhou & Fritz (1993) showed that the benthic prorocentroids *P. lima* and *P. maculosum* have both flagella emerging from a single pore in the apical plates and that these species have no trichocysts associated with the pores, but instead have mucocysts. The mucocysts are responsible for mucus production, contributing to the attachment of cells and substrates (macro-algae, rocks or culture bottles), confirming the preference for a benthic habitat (Fukuyo 1981). Species with real trichocysts, like *P. micans*, tend to be much more active and do not tend to have a benthic life habit, they are essentially planktonic and usually not associated with the production of DSP- type of toxins (McLachlan *et al.* 1997).

Confusion also existed about the type species of *Prorocentrum* and *Exuviaella*, *P. micans* and *E. marina* respectively (assumed as a synonym of *P. lima*) and their original descriptions. While the first drawing and description of *P. micans* by Ehrenberg (1834) was very clear and unambiguous, the description of *P. lima*, named by Ehrenberg (1860) as *Cryptomonas lima* included some confusing features. In his description of the species he refered that the theca was covered with spines, a feature shown in the drawing published in 1873, he identified also an apical notch, a single flagellum and fluorescence activity in *Cryptomonas lima* (Ehrenberg 1860; Ehrenberg 1873; McLachlan *et al.* 1997).

Recently several specimens of *Cryptomonas lima* were found, in preparation from the same period and locality of the *C. lima* identified by Ehrenberg, which were very similar with his drawings and published figures (Ehrenberg 1873), except for one detail, the spines that he referred seem to correspond to large pores on the surface of the theca, pores much larger than the ones found usually in *P. lima* cells, so maybe we are in the presence of two different species (Hoppenrath *et al.* 2004).

Cienkowski (1881) in McLachlan *et al.* (1997), made a much more detailed description of type species of *Exuviaella*, *Exuviaella marina* that has many similarities with the species now known as *P. lima*. He described this species as having a cell with flat shape, two long flagella and two pyrenoids. The name *Exuviaella* comes from the capacity that this species shows of shedding the theca and growing a new one underneath, the old theca is then splitting in two longitudinal valves. Bütschli (1885) made the combination

Prorocentrum lima with *E. marina* as a synonym, illustrating this species and completing the description (McLachlan *et al.* 1997; Faust *et al.*1999).

Recent evidences suggest morphological and physiological differences exist between planktonic and benthic species: pores related to the production of trichocysts in actively motile cells (in planktonic forms) or to the production of mucocysts (in benthic forms) (Fukuyo 1981; Zhou & Fritz 1993; McLachlan *et al.* 1997).

Zardoya *et al.* (1995) showed strong genetic differences between benthic and planktonic species. Phylogenetic studies based on ribossomal SSU-rRNA showed that the genus *Prorocentrum* subdivides into three major sub-groups, with *P. lima* and P. *micans* falling into distinct groups, suggesting that *P. lima* should be included in a separate genus. However, some species that were originally described as belonging to the genus *Exuviaella*, merged in this phylogenetic study with the rest of the *Prorocentrum* species (Zardoya *et al.* 1995).

Grzebyk *et al.* (1998) found similar results, they encountered two separate groups in nine species of *Prorocentrum*, one formed by benthic species (*P. concavum*, *P. emarginatum*, *P. maculosum*, *P. lima* and *P. arenarium*) and the other by planktonic species (*P. panamensis*, *P. minimum*, *P. mexicanum* and *P. micans*) when analysing the 18S-rRNA sequence.

The genus *Prorocentrum* needs a clarification of what species it should include based on modern cytological, biochemical and genetic methods.

Of the species of *Prorocentrum* found in Ria de Aveiro, based on their main features three distinctive groups can be distinguished. *Prorocentrum lima*, *Prorocentrum cassubicum* and *Prorocentrum* sp.1 display *Exuviaella* characteristics: benthic habit, presence of mucocysts, absence of tricocysts, double pyrenoid and simple pores covering the theca. *Prorocentrum micans*, *P. rhathymum* and *P. minimum* fall into the main *Prorocentrum* features: planktonic life habit, presence of trichocysts and mucocysts, apical spine, theca more ornamented with different kinds of pores or covered with little spines, and pyrenoid absent. The species here refereed to as *Prorocentrum* sp. 2 seems to have characteristics of both groups, it shows both a benthic and a planktonic habit, has a little apical spine, the theca has almost no ornamentation (only few marginal pores), there is a double pyrenoid associated with the chloroplasts and trichocysts are present. It probably represents a new species.

Monitoring for red tides caused by potentially toxic *Prorocentrum* species appears important and should be in focus on areas with aquaculture importance within regions, such as shellfish farms, shrimp farms and coastal lagoons (Hernández-Bacerril *et al.* 2000).

The new species of *Prorocentrum*

Prorocentrum sp. 1 and *Prorocentrum* sp. 2 were two species found in the ponds that exhibit features that make them impossible to identify as any other *Prorocentrum* species already known, although some similarities with other species can be noted.

These two species present the main *Prorocentrum* features, two flat valves, two dimorphic flagella inserted in the apical area, a elliptic to ovate shape, right valve with a V-shape depression and the apical area formed by apical plates and two pores, the flagellar pore and the auxiliary pore.

Prorocentrum sp. 1 is a species similar to *P. lima* but bigger, it is rounded and showed no capacity of producing toxins. This species has similarities with *P. arenarium* (Faust 1994) in terms of shape and size, but the pore shape is different. *P. arenarium* is known for having kidney shape pores, the ones found in *Prorocentrum* sp. 1 are round and inserted in slight depressions. The capacity of *Prorocentrum* sp. 1 to produce large amounts of mucus that envelops the cells and aggregates of cells is also an unusual property, consistent with the benthic life habit of this species.

Prorocentrum sp. 2 is a small prorocentroid, can be confused with *P. minimum*, but a detailed examination shows differences. It has no spines covering the theca, has a double pyrenoid, tends to aggregate in culture (compatible with a benthic nature), is non toxic to *Artemia* and the apical area has a depression in both valves, although more pronounced in the right valve.

The Genus *Amphidinium*

Amphidinium cells are athecate, recognised for having a small asymmetric epicone with a crescent shape in ventral view, which size does not exceed one third of body length, being dorsoventrally compressed giving to the cell an oval shape in ventral view. The majority of species are sand-dwelling benthic dinoflagellates, with a worldwide distribution, some species can occur in so high densities that they can cause discolouration of the sand (Hulbert 1957; Jørgensen *et al*. 2004).

The genus *Amphidinium* includes some very similar but also very different species, which makes it difficult to establish a easy way to characterize this group, it includes marine and freshwater species that can posses all the different kinds of nutrition and even pelagic and benthic forms. So is believed that this is not a homogeneous group, but it includes species that don't share a common phylogeny, only some morphologic criteria, to resolve this ambiguity recent studies based on molecular phylogenetic analysis have started (Jørgensen *et al*. 2004; Murray *et al*. 2004).

From these studies based in the analysis on nuclear encoded partial large subunit (LSU) rDNA showed that the species that have a minute left deflected epicone form a monophyletic group, this was also proved using a cladistic analysis, that include the type species, *A. operculatum*, all the other species that have a different epicone were found to be unrelated. This monophyletic group assembles the true characteristics of the genus *Amphidinium*, includes at least *A. carterae*, *A. massartii*, *A. gibossum*, *A. trulla*, *A. operculatum*, *A. steinii*, *A. mootonorum*, *A. herdmanii* and *A. incoloratum*; all the other species need a further detail study that allow them to be included in this or other known genus or even in a new one (Jørgensen *et al*. 2004).

Chapter III

Toxicological study of the established cultures of dinoflagellates

Introduction

Toxicity

An essentially different phenomenon associated to certain species (especially dinoflagellates) is the production of potent toxins that can find their way through fish or shellfish to humans. In this case, even low densities of the toxic algae in the water column may be sufficient to cause such illnesses in humans as paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), azaspiracid poisoning (AZP) and ciguatera fish poisoning (CFP) (Hallegraeff 2002). In Table 1 is a resume of which species can cause these illnesses and how they affect the humans.

The major biotoxins responsible for DSP are okadaic acid (OA); its structural analogues called dinophysistoxins (DTX-1,2); the neutral polyether-lactones of the pectenotoxin group PTX-1,2,3,6; yessotoxin (YTX) and its analogue 45-hydroxy-yessotoxin (45-OH YTX) (Pavela-Vrancic *et al.* 2002). These toxins are produced by several species of marine dinoflagellates, mostly belonging to the genera *Dinophysis* and *Prorocentrum*. These lipophilic phycotoxins are accumulated in the digestive organs of mussels and may cause DSP in humans, an intoxication characterized by severe gastro-intestinal disturbances (Svensson & Förlin 2004).

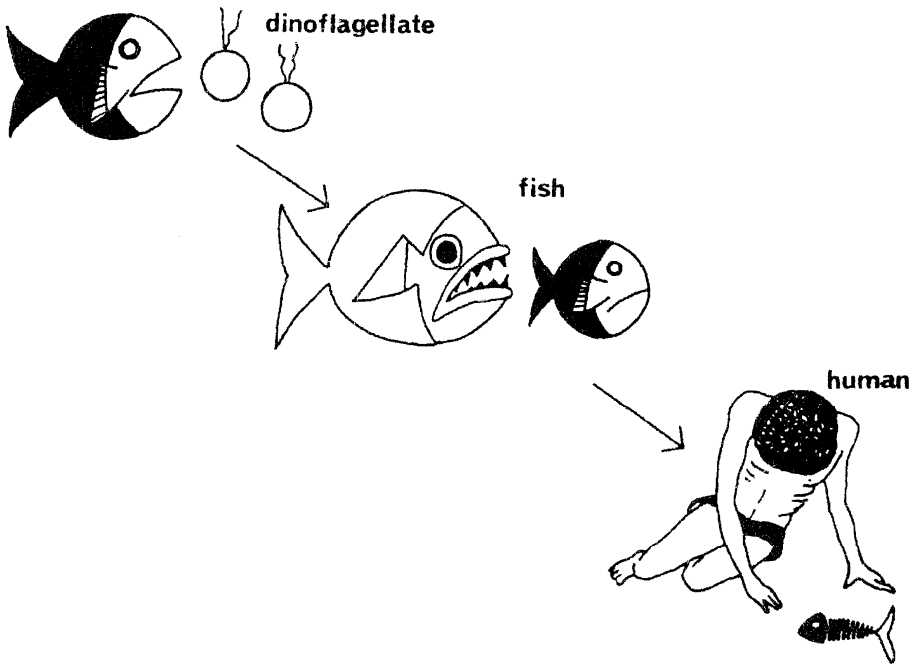


Fig.3.1. Schematic diagram illustrating the way of action of the CFP toxins, adapted from Hallegraeff (2002).

Okadaic acid and DTX-1 have considered as the principal causative agents of DSP outbreaks. They exert their toxic effects by inhibiting three of the four major classes of serine and threonine protein phosphatases (PP): PP1, PP2A, and PP2B. The sensitivity of these phosphatases to inhibition by okadaic acid varies depending on the class of phosphatase, the PP2A is inhibited by lower concentrations than PP1 and PP1 is inhibited by lower concentrations than PP2B (Cohen 1991). The relative toxicity of each of the okadaic acid analogues appears to be related to their affinity for PP (Holmes *et al.* 2001). Serine/threonine protein phosphatases are involved in many diverse cellular processes in eukaryotic organisms, including ion transport, signal transduction, cell cycle regulation and protein synthesis (Sugg & VanDolah 1999).

There are three hypotheses of mechanisms of self-protection used by *Prorocentrum lima* a okadaic acid producer:

1 - They can be protected from such a powerful phosphatase inhibitor because they confined the toxic molecules away from their cytoplasm phosphatases, compartmentalized the toxins in plastids, vacuoles and lysossomes (as the site of toxin synthesis or storage) which were free of such phosphatases or they were unaffected by okadaic acid (Zhou & Fritz 1994[a;b]);

2 - Barbier *et al*. (1999) and Sugg & VanDolah (1999) proved that these protein phosphatases are also present in *P. lima* cells. In the cell the main toxic products found are DTX-4 and DTX-5, are large, water-soluble, sulphated diol-ester derivates, less toxic than the other OA analogues, considered to be inactive forms of OA storage in the cell (Holmes *et al*. 2001). The toxicity of these compounds result from their release into the extracelular environment and subsequent hydrolysis into active forms of OA, forms possessing antiphosphatase protein activities, resulting in an increase of protein phosphorilation. This event is concomitant with the onset of mitosis, leading to other property of OA that is its capacity to be a potent tumour promoter (Zhou & Fritz 1994[a]; Barbier *et al.* 1999);

3 – The last hypothesis is that a structural modification of the target of the toxin occurs (algal protein phosphatases) (Barbier *et al.* 1999).

The second hypothesis is the considered more valid, DTX-4 type compounds have been detected in benthic *Prorocentrum* species that produce DSP toxins, but it has not been found in bivalves or pelagic plankton, instead acyl derivates of DTX-3 have been reported in the digestive glands of contaminated bivalves (Vale & Sampayo 1999; Moroño *et al.*

2003); it seems to be originated from the transformation of OA and analogues in the bivalves. Once the toxin is ingested they can be accumulated or transformed (Moroño *et al.* 2003).

The first's official reports of DSP episodes occurred in the beginning of the eighties in Japanese waters. The toxin producers were mainly benthic dinoflagellates, from which *Prorocentrum lima* was identified as an okadaic acid and DTX-1 producer, through chromatographic analysis to the hepatopancreas of *Mytilus edulis* and mouse bioassay (Yasumoto *et al.* 1980; Murata *et al.* 1982; Yasumoto *et al.* 1987).

Ciguatera (CFP) is another pathology associated with fish poisoning, occurring usually in tropical waters where the conditions for the proliferation of epibenthic dinoflagellates are maximum (Yasumoto *et al.* 1987). The main species responsible for this kind of intoxication is *Gambierdiscus toxicus*, but others like *Coolia monotis* and species from the genus *Ostreopsis* are also implicated in these cases (Vila *et al*. 2001; Hansen *et al.* 2001). The benthic *Prorocentrum lima* from tropical waters, is also associated with this type of intoxication, and okadaic acid was already identified as being one of the toxins involved in ciguatera (Murakami *et al.* 1982; Dickey *et al.* 1990; Morton *et al.* 1998).

The toxins involved in CFP are ciguatoxins, a family of heat-stable and lipid-soluble cyclic polyethers toxins that includes more than 20 types, which can vary with the geographical locations or in terms of the fish species (see Fig. 3.1). These toxins act upon sodium/calcium channels (more details in Table 3.1) (Vila *et al*. 2001; Hansen *et al.* 2001).

The aim of dinoflagellates producing toxins is not clear, since if it is for defence why is it not excreted into the external environment. Since the toxin is only active with the death of the dinoflagellate cell, at the individual point of view it is not an advantage, but maybe at the population level it is. Recently some studies are focus on proving the capacity of these toxins to have an allelopathic functions, which is to inhibit the growth of competitive microalgal species, thereby conferring a competitive advantage to the toxin producer (Holmes & Teo 2002; Sugg & VanDolah 1999).

On a global scale, close to 2000 cases of human poisoning through fish or shellfish consumption are reported each year and if not controlled, the economic damage through reduced local consumption and reduced export seafood products can be considerable (Hallegraeff 2002; Svesson & Förlin 2004).

The number and intensity of algal blooms seems to be on the rise and their geographic extension seems to be spreading. Four explanations for this apparent global increase have been suggested (Hallegraeff 2002):

(1) Increased scientific awareness of toxic species;
(2) Increased utilisation of coastal waters for aquaculture;
(3) Stimulation of plankton blooms by coastal eutrophization and/or unusual climatic conditions;
(4) Transport of dinoflagellates or their resistant benthic resting cysts either in ships' ballast water or associated with movement of shellfish stocks from one area to another (Hallegraeff 2002).

Finfish in intensive aquaculture systems are much more vulnerable to environmental perturbations and noxious algal blooms than wild stocks, since the latter have the freedom to swim away from problematic areas (Hallegraeff 2002).

Nowhere is the need for correct identification of plankton organisms more critical than in the study of the toxic species. As algal blooms are often composed of a single species, identifying correctly that species becomes crucial not only to understand the bloom event but also when deciding on possible measures for its control (Hallegraeff 2002).

Monitoring algal phytoplankton can give advanced warning of algal bloom problems. If it is possible to known which problematic species are involved, contingency plans need to be put in place to allow towing cages for rearing finfish away from bloom affected areas. Many of these toxic dinoflagellates produce resistant benthic resting stages (cysts) that survive to unfavourable conditions, waiting in the sediment until the environmental conditions are once the appropriate for proliferation (Hallegraeff 2002).

Table 3.1. Intoxication Syndromes Caused by Phycotoxins consumed in seafood (Hallegraef 1995; Hallegraef 2002).

Disease	PSP	NSP	ASP	DSP	Ciguatera	Puffer Fish
Causative Organism	Pelagic Dinoflagellate	Pelagic Dinoflagellate	Pelagic Diatom	Pelagic or Benthic Dinoflagellate	Epibenthic Dinoflagellate	Bacteria?
Most often Species	*Alexandrium catenella*	*Karenia brevis*	*Pseudo-nitzshia pungens*	*Dinophysis acuminata, Prorocentrum lima*	*Gambierdiscus toxicus, Coolia monotis*	
Major Transvector	Shellfish	Shellfish	Shellfish	Shellfish	Fish	Fish
Geographic Distribution	Temperate to Tropical World-wide	Gulf Mexico, Japan, New Zealand	Canada, NW USA	Temperate World-wide	Sub-Tropical to Tropical World-wide	Japan, World-wide
Major Toxin	Saxitoxin	Bretoxin	Domoic acid	Okadaic acid	Ciguatoxin, Scaritoxin, Maitotoxin	Tetrodotoxin
Neuro-Mechanism	Na^+ Channel Blocker	Na^+ Channel Activator	Glutamate Receptor Agonist	Phosphorylase Phosphatase Inhibitor	Na^+, Ca^{2+} Channel Activators	Na^+ Channel Blocker
Incubation Time	5-30 min	30 min to 3hr	Hours	Hours	Hours	5-30 min
Duration	Days	2 Days	Years	Days	Years	Days
Acute Symptoms	Nausea, vomiting, diarrhea, **paraesthesias, respiratory despression**	Nausea, vomiting, diarrhea, **bronchoconstriction, reversal of temperature sensation**, paraesthesias	Nausea, vomiting, diarrhea, **amnesia**, paraesthesias, respiratory despression	**Diarrhea**, nausea, vomiting	Nausea, vomiting, diarrhea, **reversal of temperature sensation,** paraesthesias	Nausea, vomiting, diarrhea, **paraesthesias, respiratory despression, decrease blood pression**
Chronic Symptoms	None	None	Amnesia	None	Paraesthesias	None
Fatality Rate	1-14%	0%	3%	0%	<1%	60%
Diagnosis	Clinical, mouse bioassay of food, HPLC	Clinical, mouse bioassay of food, ELISA	Clinical, mouse bioassay of food, ELISA	Clinical, mouse bioassay, ELISA, HPLC	Clinical, mouse bioassay, immunoassay	Clinical, mouse bioassay, fluorescence
Therapy	Supportative (respiratory)	Supportative	Supportative	Supportative	Mannitol, TCA, supportative	Supportative (respiratory)
Prevention	Red tide and seafood surveillance, report cases	Red tide and seafood surveillance, report cases	Seafood surveillance, report cases	Seafood surveillance, some red tide, report cases	Seafood surveillance, report cases	Regulated food preparation, report cases

Material and Methods

Toxicological study - Bioassays

Artemia test (Denmark)

Artemia cyst hatching is initiated 48 hours before the start of the toxicity test by placing the cysts in a Petri dish, covering them with the same medium of the cultures and exposing them to a light source for about one hour. The Petri dish is then covered with aluminium foil and put in a chamber, at the same temperature as the cultures that are going to be tested. After 24 hours, the cysts are transferred to a new Petri dish with fresh medium and put again in the chamber, completely covered for more 24 hours.

The bioassay is conducted in a disposable multiwell test plate, with 24 (6x4) test wells, following the standard procedure from the ARTOXKIT M™ (Demaret *et al.* 1993; Ismael *et al.* 1999; Caldwell *et al*. 2003).

The multiwell box is filled distributing the test culture in different concentrations in the wells, each column with a different one. The first column is for the control, three replicates are made for each condition. The control column (column 1) is filled with 1ml of the medium. To the other columns a dilution of the culture is added progressing from low to high concentration in columns 2 to 6.

Under a dissection microscope, 10 larvae are transferred to each well, using the fourth line of wells to help isolate and count the larvae. During this operation, the quantity of medium that is transferred should be minimized, and the condition of the larvae preserved. The test plate is covered with aluminium foil and transferred to the chamber. After 24, 48 and 72 hours the number of dead larvae in each well is registered. A species is considered toxic if it causes the death of more than 50% of the larvae. The test is valid as long as the mortality in the control wells is less than 10%.

Test using *Hydrobia ulvae* (Portugal)

To perform this test is necessary to begin the preparation at least one month in advance.

To present the test species (*Prorocentrum lima* in this case) in a way that *Hydrobia ulvae* is capable of feeding on it, cultures must be prepared and grow into an exponential phase, inside multi-well boxes in which the test will be preformed.

The culture in exponential phase is divided into several multi-well boxes, three times more than the number of days of the test to provide three replicates; each well is filled with 1ml of medium. Twenty day assays were preformed, so 60 wells containing *P. lima* growing were prepared. These boxes were kept for one month in the culture chamber at 20ºC, a temperature that allows fast growth of this species.

Fig.3.2 – *Hydrobia ulvae* specimens.

One week before beginning the assay, several specimens from *Hydrobia ulvae* (see Fig. 3.2) were collected from the field. Typically, found on muddy sand, in estuaries and salt marshes, sometimes in lagoons and other areas of more reduced salinity and frequently associated with sea grass beds.

They were washed with autoclaved seawater and then put into multi well boxes, one per well with medium, they are changed every day for 6 days to a new well until the beginning of the assay, so that all food particles that they had are eliminated as well as to remove eventual contaminants.

The assay begins by putting one specimen of *Hydrobia* in a well where *P. lima* is growing, forming yellow-brown agglomerates visible to the naked eye. This was done three times so that there were three replicates. The boxes were kept at a controlled temperature, the same that was in the field when the specimens of *H. ulvae* were collected, around 15ºC. Every day the specimens were moved to a new well with *P. lima*, so that the food was not limiting and the conditions in the well do not deteriorate. This procedure was repeated for 20 days.

The control to this assay was done in the same way than the test, except that there were no organisms growing in the control wells (it is a starvation control). There were three replicates of the control.

Every day the specimens were observed through a stereo microscope, to see if they were feeding on the tested species, if they were losing mobility or if they were still alive.

At the end of the test the specimens that were feeding on *P. lima* where kept in a nitrogen chamber for future intestinal analysis. This was not possible during the present study.

Results

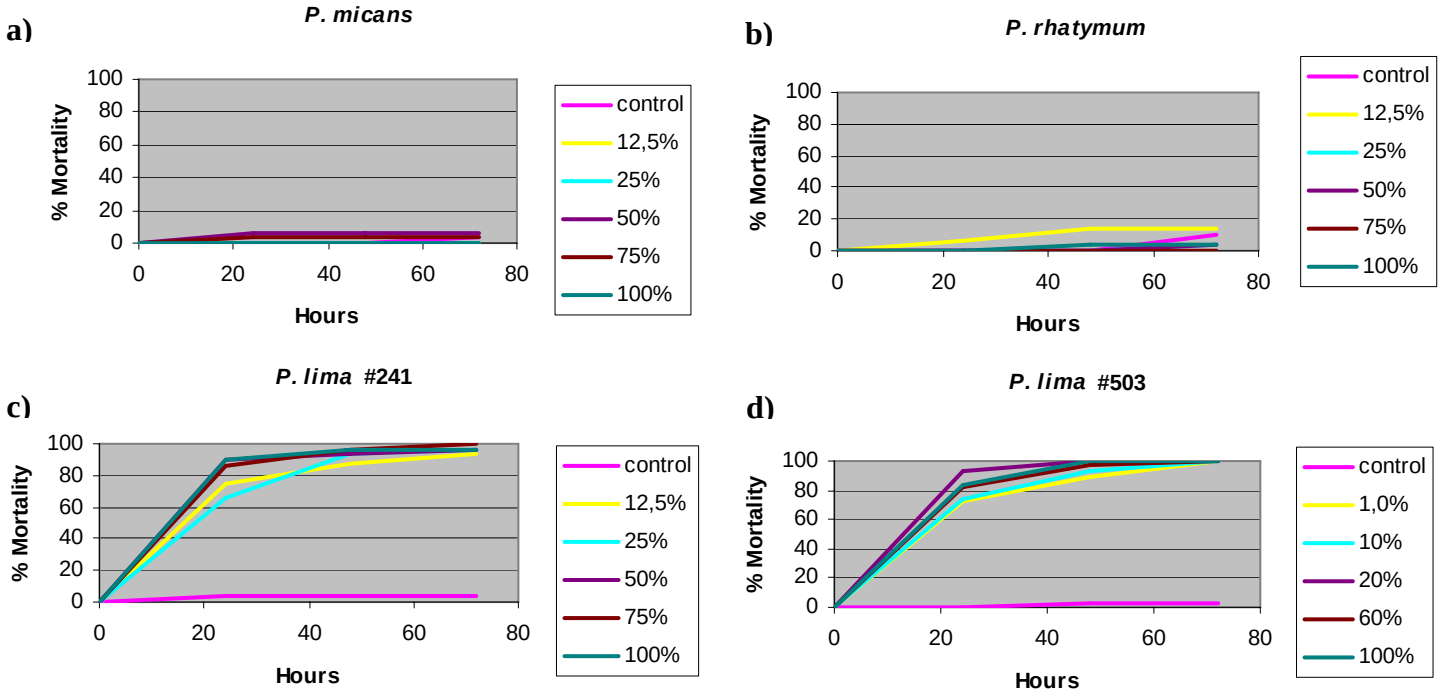
Artemia test

The *Artemia* test was conducted with eight different species of dinoflagellates, as is shown in Table 3.2, using the densest cultures that could be prepared for each species and dilutions of these. The results are illustrated by the graphs in Fig. 3.3 a-i.

The number of dead larvae is obtained by counting them after 24, 48 and 72 hours of exposure to the respective species of dinoflagellates.

Table 3.2. Cultures tested for toxicity using the *Artemia* bioassay.

Species	Culture number	Concentration cells/ml	Toxicity
Prorocentrum lima	#241	7290	Very toxic after 24 hours of exposure
Prorocentrum lima	#503	4290	
Prorocentrum cassubicum	#274	17468	Toxic after 72 hours of exposure
Prorocentrum micans	#498	5510	Non-toxic
Prorocentrum rhathymum	#495	7810	Non-toxic
Prorocentrum sp.1	#390	2195	Non-toxic
Prorocentrum sp. 2	#326	7210	Non-toxic
Amphidinium massartii	#455	4770	Non-toxic
Coolia monotis	# 563	2460	Toxic after 48 hours of exposure



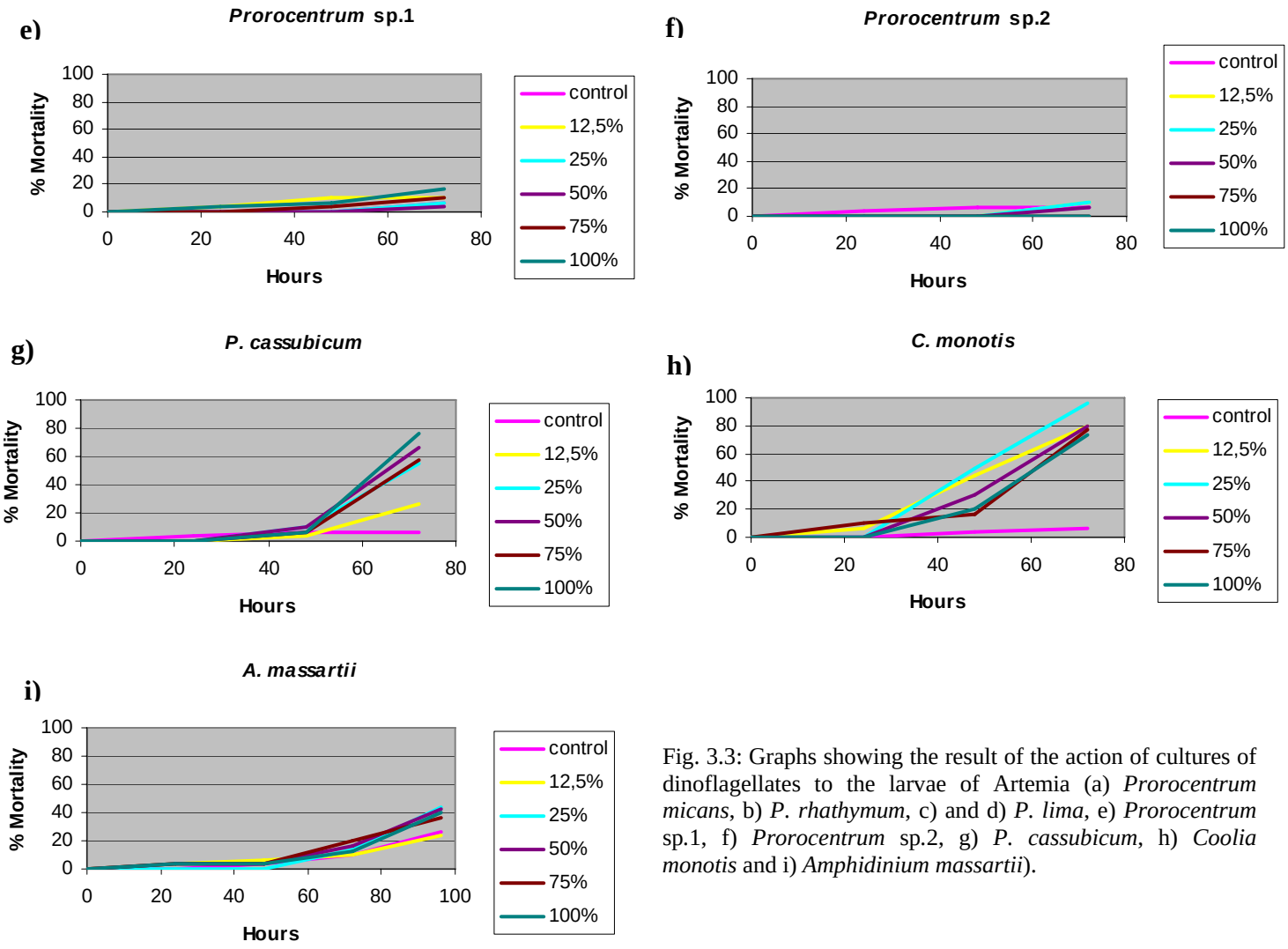


Fig. 3.3: Graphs showing the result of the action of cultures of dinoflagellates to the larvae of Artemia (a) *Prorocentrum micans*, b) *P. rhathymum*, c) and d) *P. lima*, e) *Prorocentrum* sp.1, f) *Prorocentrum* sp.2, g) *P. cassubicum*, h) *Coolia monotis* and i) *Amphidinium massartii*).

The Fig. 3.3 shows that only three species out of the eight species tested were toxic to the larvae of *Artemia salina*: *Prorocentrum lima* (Fig. 3.3 c and d), *Prorocentrum cassubicum* (Fig. 3.3g) and *Coolia monotis* (Fig. 3.3h). They caused the death of more than 50% of the larvae while the control was still valid (i.e. death percentage less than 10%).

All the other species tested, *Prorocentrum micans*, *P. rhathymum*, *Prorocentrum* sp. 1, *Prorocentrum* sp. 2 and *Amphidinium massartii*, were found to be harmless to the larvae, perhaps serving as food for them, since in these cases the rate of mortality was lower than the one registered in the control.

Amphidinium massartii (Fig. 3.3.i) seemed to cause an increase in the death rate after 92 hours, but at this time the test was no longer valid, since the mortality in the control was already higher than 10%, so its toxicity could not be proved.

Of the three toxic species found, it is possible to see that the toxicity of the culture was not the same, some causing killings sooner than others. *Prorocentrum lima* cultures killed 90% of the larvae in 24 hours. *Prorocentrum cassubicum* was the least toxic, only after 72 hours the mortality was higher than 50%. *Coolia monotis* was a middle case, causing the death of more than 70% of larvae after 48 hours of assay.

In this test different concentrations of species culture were used, they did not caused different rates of mortality. In the case of *P. lima* concentrations low as 1% (less than 100 cells) were used and even so after 24 hours the mortality was almost the same as in the 100% concentration of the culture, causing a mortality of 90%. It seems that the culture was already with such a high concentration of toxin that the different dilutions made were not enough to register different results, the relation concentration of toxin/mortality of larvae could not be established. These results were similar to the other cultures tested.

Test using *Hydrobia ulvae*

The snail mud *Hydrobia ulvae* is a small spiralling shell with six whorls, up to 6 mm high but more typically around 4 mm. The shell is brown to yellow in colour. The body of the snail is a clear grey frequently with various pigment spots. This species can occur in very high quantities.

Hydrobia ulvae is a very resistant organism, capable of surviving for long periods without eating, making of this species an excellent test species, since some of the specimens are going to spend almost one month without eating.

An important step of this test is the acclimatization, consisting in the diary changes of the specimens to new wells with seawater at controlled temperature (15ºC chamber), with the aims of cleaning the shells, eliminate attached contaminates and remove intestinal contents. Only after this step it is possible to study if this organism is capable of graze on dense cultures of *Prorocentrum lima*.

This test did not reveal if *P. lima* is toxic to *Hydrobia ulvae*, but showed that this species in laboratory can feed cells of *P. lima*, since marks of feeding were visible in the button of the wells, and when observing under a stereo microscope was possible to see the

foot of the snail collecting cells of *P. lima* to eat. In the field this two species co-habit, so maybe this phenomena also occur there.

Both control and tested specimens were alive in the end of the 20 days assay. The specimens put together with *P. lima* in the beginning of the test (the first 8 days) were very active and eating constantly, confirmed by the large number of faecal pellets produced. From the 9th day until the end of the assay their activity began to decay, the observations of organisms feeding on *P. lima* also become rare. In the end of the assay, the snails only moved if stimulated. The faecal pellets also began to be rarer through the test in both tested situations. The *Hydrobia ulvae* from the control were not as active as the first week of the ones that had *P. lima* as food, but their behaviour did not change through the assay, after the 20 days they were still alive and moving.

Discussion

Although positive results of the *Artemia* test demonstrate the existence of toxic properties in an organism, negative results are not enough to prove an organism incapability to produce toxins. Other methods need to be applied to detect the production of toxins to which *Artemia* larvae are not sensitive. Commonly used methods include other types of bioassays (mouse bioassay) (Cembella *et al.* 1995; Pavela-Vrancic *et al.* 2002), immunoassays (enzyme-binding-assays, neuroreceptor binding assays and cytotoxicity assays) (Cembella *et al.* 1995; Cembella *et al*. 1999; Sugg & VanDolah 1999; Barbier *et al.* 1999) and analytical methods for detection of particular types of toxins (LC-MS, HPLC, HPLC-MS, LC-SRM MS, CID MS-MS and RMN) (Pavela-Vrancic *et al.* 2002; Quilliam & Wright 1995; Moroño *et al.* 2003; Sugg & VanDolah 1999; Izumikawa *et al*. 2000; Vale & Sampayo 1999[b]; Vale & Sampayo 2002[a]).

On the other hand, a species can be harmful without being toxic. Excessive growth can be catastrophic. When a species reaches a bloom state few organisms can grow and the decay of a bloom can bring oxygen depletion, leading to massive deaths of aerobic organisms.

Eight species of benthic or planktonic dinoflagellates isolated from ponds from Ria de Aveiro were isolated into unialgal cultures: *Prorocentrum lima, P. micans, P. cassubicum, P. rhathymum, Prorocentrum* sp. 1, *Prorocentrum* sp. 2, *Coolia monotis* and *Amphidinium massartii*. Each culture was tested by *Artemia* bioassay, three confirmed to be toxic to the *Artemia salina* larvae. The three species that showed toxicity were *Prorocentrum lima, P. cassubicum* and *Coolia monotis*, three benthic dinoflagellates, supporting the idea that the benthic dinoflagellates communities are a rich source of biologically active compounds, potent toxins are at much higher incidence in epiphytic species than in planktonic species (Nakajima *et al.* 1981; Tanaka *et al.* 1998). Although several planktonic species of *Prorocentrum* (*P. micans, P. rhathymum* and *P. minimum*) may form extensive blooms (red tides), there are few reports of them being harmful to the flora and fauna (Faust *et al.* 1999; Faust & Gulledge 2002).

Among the benthic species, there are several toxin producers of which *Prorocentrum lima* is the best known toxic species. *Prorocentrum lima* is capable of producing several kinds of toxins, they enter in the chain food as DSP (diarrhetic shellfish poisons) toxin type, due to the accumulation of okadaic acid and DTX toxins in shellfish in temperate

waters and as ciguatoxins in tropical waters through ingestion of tropical fishes causing CFP (Murakami *et al.* 1982; Dickey *et al.* 1990; Morton *et al.* 1998; Vila *et al.* 2001; Lawrence & Cembella 1999). Another path of toxicity is due to nitrogenous macrocyclic lactone toxin (Faust *et al.* 1999; Lu et al. 2001).

Prorocentrum cassubicum toxicity is not well known, few reports exist for this species, it seems that it produces a fast acting toxin (FAT) (Faust 1995), a kind of secondary metabolite that maybe responsible for the death of the *Artemia* larvae.

Coolia monotis is a known toxic dinoflagellate that produces cooliatoxin, a neurotoxin analogue to yessotoxin, known to be toxic to *Artemia* and *Haliotis* (Hallegraeff 2002; Faust & Gulledge 2002). This species also produces cytotoxins, which main constituent is a diacylgalactosylcerolipid a ceroamida, this component is a common metabolite of marine algae, one of the major active components (Tanaka *et al.* 1998).

Hydrobia ulvae does not form a known unique food source for any other species but it does form a dietary component of the opisthobranch mollusc *Retusa obtusa*, crabs and some seabirds. Its food source can very in the field, but usually assimilates sedimentary microorganisms, from which bacteria, diatoms and cyanobacteria have already been reported. Non-living material seems to be inadequate as food, contributing little to the nutrition of this species. Coprophagy is another possible source of food, associated with the production and ingestion of faecal pellets, avoids competition for algal recourses and provides an alternative food, especially when food is not abundant (López-Figueroa & Niell 1987).

The test using *Hydrobia ulvae* did not reveal if *P. lima* is toxic to this species, but revealed that this species in laboratory can feed on cells of *P. lima*, maybe in the field the same situation is occurring. The ponds where *P. lima* was collected are full of gastropods like *Hydrobia ulvae* and other shellfish. Since no death was registed is possible that the okadaic acid produced by *P. lima* is being accumulated.

The problem was that no tests were made to the intestinal contents of *H. ulvae* to prove that *P. lima* was being consumed and its toxin accumulated, using chromatographic methods (HPLC). These methods were already used with other organisms, like the blue mussels *Mytilus edulis* that showed accumulations of okadaic acid produced by *Prorocentrum lima* (Pillet *et al.* 1995; Svensson & Förlin 2004) and *Mytilus*

galloprovincialis that showed accumulation and transformation of DSP toxins produced by *Dinophysis acuminata* (Moroño *et al.* 2003).

Pillet *et al.* (1995) made a study where was demonstrated the accumulation of okadaic acid and DTX1 in blue mussels (*Mytilus edulis*), that experimentally fed on a strain of *Prorocentrum lima* that produces DSP toxins. During the experiment they registered that the mussels avoid to eat the *P. lima* cells, reducing the filtration rate in the presence of high concentrations of cells of this species, surviving using reserves in the tissues, but starvation could finally lead the mussels to use *P. lima* cells as food. During the 20 days assay no mortality was registered, proving the capacity of *Mytilus edulis* to feed on *P. lima* cells and accumulate its toxins (Pillet *et al.* 1995; Pillet & Houvenaghel 1995). Pillet & Houvenaghel (1995) refereed that mussels from areas unaffected by toxic events are more sensitive to intoxication than mussels from areas where blooms are recurrent, so mussels can suffer directly from high levels of toxins, cytotoxic effect on ctenidia cells were observed, or indirectly when the mussels slow down their feeding rate, what leads to a reduce growth rate.

The bivalves are filter-feeders, so they can also accumulate DSP toxins not only by ingesting cells that produce toxins, but also from the filtered seawater, since toxins from *Prorocentrum lima* cells can be found in the extracellualr medium at low but significant amounts (0,1 to 1 %) (Traubenberg & Morlaix 1995).

In the Ria de Aveiro, human intoxications due to ingestion of green crabs (*Carcinus maenas*) and razor clams (*Solen marginatus*) were reported in 2001, the analysis of these organisms confirmed the presence of okadaic acid (Vale & Sampayo 2002).

Proving that *H. ulvae* accumulates okadaic acid and other toxins produced by *P. lima* will lead to the discovery of a new path of entry of these toxins in the food chain. *Hydrobia ulvae* makes part of a group of organisms, the gastropods, which are food to many other organisms: crabs, fishes, seabirds and others. The consequence of these organisms feeding on contaminated gastropods is that they will be in contact with much higher concentrations of these toxins, millions times higher that the one possible to found in a *P. lima* cell. The toxins are moved through the food chain from the toxic phytoplankton to herbivorous consumers (molluscs, crustaceans or phytophagous fish) and then on to carnivorous fish, piscivorous and scavenging birds, and mammals (Shumway *et al.* 2003). The ultimate consumers are the humans, which will suffer more drastically from this toxin since they are

not immune to them and the toxin is at this point in much higher concentrations, it has been accumulated through the food chain.

Chapter IV

Preliminary phylogenetic study of selected species

Introduction

Evolution of Dinoflagellates

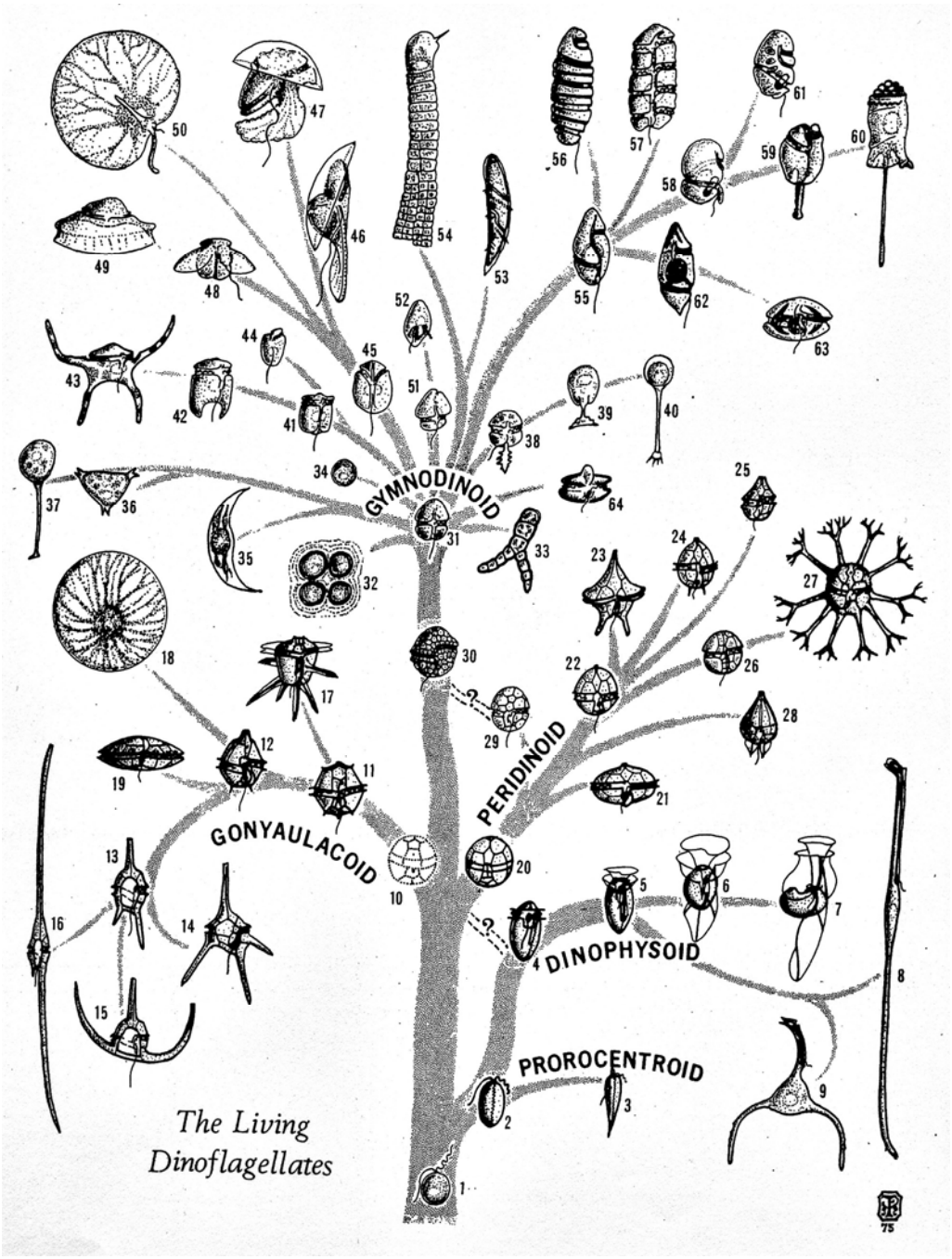


Fig. 4.1. Schematic representation of the origin of the different orders of dinoflagellates, proposed by Taylor (1980).

The origin of dinoflagellates has been the subject of many studies. Nowadays is accepted that dinoflagellates, euglenoids and cryptomonads were originated from a heterotrophic ancestor, this hypothesis is supported by the occurrence of modern heterotrophic dinoflagellates (Graham & Wilcox 2000). However, some scientists have suggested that the dinoflagellates acquired peridinium type plastids in a very early stage of evolution, and that the modern dinoflagellates that now lack them have lost an ancestral autotrophic capacity (Hoek *et al.* 1995). Another phylogenetic hypothesis is associated with the origin of the dinoflagellate theca, the armoured dinoflagellates are believed to be more primitive forms than the unarmored, yet this is still very controversial, since there are dinoflagellates with few plates (*Oxyrrhis* and *Noctiluca*) that can not be consider very evoluted forms (Graham & Wilcox 2000). The Dinophyceae includes a high variety of species some with the most bizarre forms, see Fig. 4.1.

Evolution of the classification of Dinoflagellates

The majority of genera of dinoflagellates were described during the end of the 19th century and early 20th century, when the light microscope was the only instrument available. These descriptions were only based on the morphological features that could be seen, with the result that in some cases poor drawing and ambiguous descriptions were produced, due to the small size of many species (Daugbjerg *et al.* 2000).

The natural variability of some species was often not taken into consideration, originating different names for the same species. Another conflict comes from cyst identification, since the morphology of the motile stage of a species and its cyst vary drastically, causing most of these species to have two names, since motile cells and cysts were named independently by phycologists and palaeontologists respectively (Ellegaard *et al.* 2003).

Since then several phycologists tried to improve the classification of dinoflagellates, using advanced microscopic techniques. Electron microscopy (scanning and transmission electron microscopy) brought an enormous contribution to our knowledge of dinoflagellates (Dodge 1965; Loeblich 1976; Roberts *et al.* 1995; Heimann *et al.* 1995; Calado *et al.* 1999; Hansen *et al.* 2000[a;b]).

Molecular data

The advances in the field of genetics make it possible to combine morphology and genetic information that is unique for each species. This allows a better comparison between species, establishing relationships and studying the phylogeny of the group, an issue that has long been controversial.

Studies have already been done in this area using the gene of the SSU-rRNA as a consensus sequence, but the phylogenetic resolution obtained is often insufficient. Some of these studies have created phylogenies that go against the ones made before based on morphologic criteria (Fig. 4.2), so the question of the evolution of dinoflagellates is still unsolved (Grzebyk *et al.* 1998).

The nucleus encoded sequence of the LSU-rDNA is longer and comprises more variable areas than the SSU-rDNA providing a stronger phylogenetic signal. The study using the LSU-rDNA sequence is based on the comparison of four regions, from D1 to D4, which include more than 1200 base pairs, so the probability of obtaining better results using this sequence is higher.

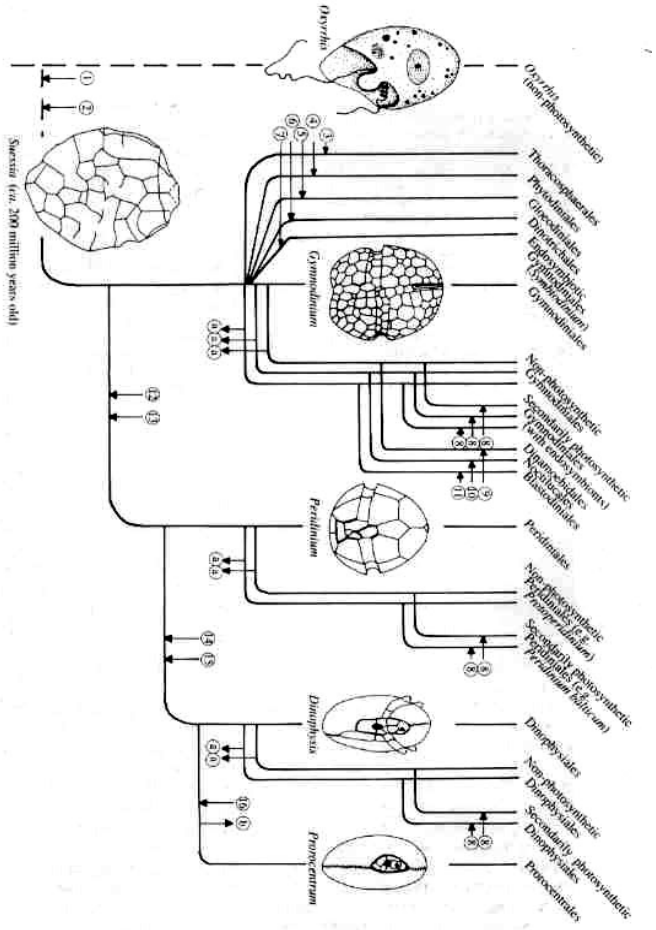


Fig.4.2 - Phylogenetic tree for some genus of dinoflagellates, based in morphologic criteria (Hoek *et al.* 1995).

Material and Methods

Genetic study

Dense cultures of the species were used. To proceed with genetic analysis cells were concentrated into a pellet, centrifuging 10 ml of culture for 10 minutes at 2500 rpm, at the same temperature as the growing chamber (20ºC). If the pellet is not very big, this procedure is repeated with more 10 ml of culture and in the end, the pellets are put together with just a small amount of medium and centrifuged again in the same conditions. In the end, this pellet is kept in a microtube and stored in the freezer at –20ºC until further utilization.

Some dinoflagellate species do not develop into dense cultures, and in these cases the final pellet obtained is not big enough to allow a procedure of DNA extraction, in which a large fraction of the DNA is lost.

DNA-Extraction

Only when the amount of cells is very large a DNA extraction procedure can and should be applied, to obtain DNA in is more pure form, using the CTAB method and precipitated with ethanol (Daugbjerg *et al.* 1994).

DNA amplification (PCR)

To amplify a fragment with approx. 1400bp of the LSU rDNA, the polymerase chain reaction (PCR) is the best strategy.

We begin be centrifuging the pellets of the cultures again in an ultracentrifuge at 4000 rpm for 10 minutes at 20ºC, so a dense pellet is formed and most of the medium may be removed.

The master mix solution for the PCR reaction contained in a microtube the following reagents:

- 5 μl Taq buffer (vortex before using because of the $MgCl_2$ that is hard to dissolve completely)
- 20 μl of the dNTPs

- 5 µl Primer D1R
- 5 µl Primer 1483R
- 5 µl TMA (helps the DNA chain to become more linear)
- 9 µl dH_2O

Table 4.1. List of oligonucleotide primer sequences used to amplify and determine the LSU rDNA (domains D1-D3) in dinoflagellates (Hansen *et al.* 2000[a]).

Primer name	Annealing Position	Primer sequence (5'-3')	Reference
D1R	24-31	ACCCGCTGAATTTAAGCATA	Scholin *et al.* (1994)
28-1483R		GCTACTACCACCAAGATCTGC	Daugbjerg *et al.* (2000)
D3A	708-727	GACCCGTCTTGAAACACGGA	Nunn *et al.* (1996)
D2C	733-714	CCTTGGTCCGTGTTTCAAGA	Scholin *et al.* (1994)
D3B	1011-992	TCGGAGGGAACCAGCTACTA	Nunn *et al.* (1996)

A little more of each reagent should be added (plus 1 µl) so that in the end there is enough reaction solution for all the samples. For more details, see Table 1 in the Appendix 2.

A negative control is added to guarantee the state of all reagents used, and to be sure that there are no contaminants.

The master mix is divided for all the PCR tube, without the Taq polymerase, then 2 µl or 1 µl of each pellet is added to the correspondent tubes, depending of the density of the pellet, and the tubes are spun down to mix all components.

To begin, a hot start was made, 10 minutes at 95ºC in a PCR machine, to help disrupt the cells and allow the DNA to come out.

Only after this procedure the taq polymerase was added to the tubes kept on ice. The tubes were again spun down and the PCR was performed with the following sequence:

- 94ºC 10' 1 cycle
- 94ºC 1'
- 52ºC 1'
- 72ºC 2'

} 35 cycles

- 72ºC 6' 1 cycle

When the PCR is finished, the tubes are collected and put in the freezer until confirmation in a DNA electrophoresis gel.

Electrophoreses

An electrophoresis gel was prepared, 2% Nusieve agarose with Ethidium bromide keep at 64ºC. For a quicker polymerisation, the gel is put in the refrigerator for 10 minutes. The PCR tubes are spun down before opening. The samples to load are prepared by mixing in a piece of parafilm 2 μl of loading buffer with 5 μl of the PCR product, by making up and down movements with the pipette. Then the sample is loaded to the gel, after quickly cleaning the loaded tip in the TBE buffer that is in the electrophoresis machine. This procedure is repeated for all samples and the leftovers are kept in the refrigerator. Before loading the control sample a molecular weight marker is loaded so that we can estimate the size of the DNA fragments amplified.

The electrophoresis is run at 150 volts for 20 minutes, using TBE buffer as the surrounding medium.

At the end, the gel is cheeked using UV light (254 nm) and a photograph of the results is taken.

Nested PCR

Each band found in the DNA gel is used in a new PRC reaction using intermediary primers from the ones used in the beginning to further increase the amount of amplified DNA.

The bands are cut with a clean blade and put in a microtube with 300 μl of distilled water, heated at 75ºC for 30 minutes to melt the agarose. This procedure is repeated for all positive results.

A new master mix solution is prepared by adding for each sample following amounts of each reagent, they have to be prepared in duplicate because two intermediary primers from the ones used in the beginning are going to be used:

- 5 μl Taq buffer (vortex before using because of the $MgCl_2$ that is hard to dissolve completely)
- 20 μl of the dNTPs
- 5 μl of each primer (combining DIF and D3B; D3A and 1483R)
- 0.2 μl Taq polymerase
- 5 μl TMA (helps the DNA chain to become more linear)
- 9 μl dH_2O

A negative control is prepared for each combination of primers. See Table 2 in the Appendix 2.

The addition of the DNA template is done in a laminar flux chamber (to avoid contamination), and the samples are first spun down.

The PCR machine is programmed as follows:

- 94ºC 10' 1 cycle
- 94ºC 1'
- 52ºC 1'
- 72ºC 2'

} 20 cycles (enough because of the large amount of DNA template)

- 72ºC 6' 1 cycle

At the end, the PCR products are collected and put in the freezer until confirmation in an electrophoresis gel.

Purification and Sequencing of the DNA

Purification of the PCR products was made using the QIAquick PCR purification Kit (Qiagen).

To sequence our DNA template with the Dye Terminator Cycle, its concentration has to be higher than 20.0 ng/μl.

Discussion

This study due to laboratory problems was not completed, in a way of obtaining relevant results.

The benthic dinoflagellates are known for producing mucus, this mucus interfere with the processes of DNA amplification, making the PCR amplification very difficult, only with the assistance of a special Kit this mucilage can be neutralized. During the realization of this work was not possible to have access to this material. Only for two species (*Prorocentrum micans* and *Amphidinium massartii*) was possible to obtain the LSU-rDNA sequence.

Conclusion

The occurrence of HAB (harmful algal bloom) events tends to increase in the future. The climatic changes (global warming), pollution, eutrophication and massive aquaculture productions are some of the factors that will lead to the proliferation of harmful algae.

Governments and people in general are not aware of this kind of problems, some regarding harmful algae as a myth or only occurring in overpopulated countries. Monitorization programs are important and it is urgent that they are applied in coastal areas where shellfish or fish productions exist, but before that it is essential to inform the population in general about this problems, and how the so called "inferior species" can influence higher species through the food chain and reach in some cases man as final consumer.

Ria de Aveiro has already suffered the consequences of proliferations of HAB species, species of the genus *Dinophysis* responsible for DSP intoxications (Vale & Sampayo 2000) and *Gymnodinium catenatum*, a PSP toxin producer. The confirmations of the occurrence in this area of more toxic species like *Prorocentrum lima*, *P. cassubicum* and *Coolia monotis*, species capable of causing ciguatera and DSP, expose other potential causes for toxic episodes in this coastal lagoon. It is important to know which species usually inhabit these areas, how they vary through the seasons and in which conditions they proliferate better, being capable of forming a HAB event. However, this kind of information is only possible to obtain through exhaustive monitorization programs, that imply long time research and resources which are often lacking.

This survey allows us to understand a little better the benthic algal communities that share these ecosystems, the sheltered ponds. A list of dinoflagellate species was produced and the main periods of their occurrence identified, as well as which other species they may associate with in these environmental conditions.

The community of dinoflagellates found in these sheltered ponds from Ria de Aveiro is well established. It is dominated by benthic species: *Prorocentrum lima*, *P. cassubicum*, *Prorocentrum* sp. 1, *Prorocentrum* sp. 2, *Bysmatrum subsalsum* and C*oolia monotis*; but also includes more tycoplanktonic species: *Prorocentrum micans*, *P. rhathymum*, *Amphidinium massartii*, *Akashiwo sanguinea*, *Kryptoperidinium foliaceum*, *Scrippsiella* cf. *trochoidea* and *Heterocapsa niei*. Occasionally heterotrophic dinoflagellates (*Oxyrrhis*

marina, *Oblea rotunda* and *Protoperidinium minutum*) also proliferate, especially after diatoms or others species blooms.

Toxic strains of *P. lima*, *P. cassubicum* and *C. monotis* were identified through *Artemia* bioassay. *Prorocentrum lima* was also identified in the laboratory as being food for *Hydrobia ulvae*, what can indicate a new path of accumulation and transport of DSP toxins through the food chain. Gastropods are food to other higher organisms: crabs, fishes and seabirds, reaching the humans as ultimate consumers. This last one is exposed too much higher concentrations of toxins, which can cause severe disorders. Mass death can also occur in the other organisms that form the chain of the path of these toxins, since in each level more toxins are accumulated, reaching levels that can be intolerant to any organisms. The grazers organisms, like *H. ulvae*, that share the same habitats of these benthic species are not included in the monitorization programs for identifications of DSP toxins, that usually only includes filter-feeders, and with this preliminary test they may represent a new path of toxin accumulation.

Future perspectives:

This work is only a preliminary survey of the variety of species that share this sheltered ecosystem during the environmental changes caused by the different seasons of the year. The ponds are more favourable to the development of benthic species, since they are of low depth and usually covered by a layer of macroalgae at the surface. The benthic dinoflagellates are known for being a potential source of species capable of forming blooms or of being toxic.

To fully understand these environments more deep studies should be conducted. More sampling sites should be established, sampling more times and in different tides, the growth pattern of the toxic or bloom forming species studied and toxicity tested by different methods, using bioassays but also advanced chromatographic methods, like HPLC and capillary electrophoresis in connection with mass spectrometry, to identify the toxins present.

Only with detailed studies the interactions and occurrence of these benthic species can be fully understood and in some cases predicted, avoiding situations as contaminations to humans. Nevertheless, the information about harmful algae and their way of action must reach populations in general and fishermen in particular.

References:

Barbier M., Amzil Z., Mondeguer F., Bhaud Y., Soyer-Gobillard M. & Lassus P. 1999. Okadaic acid and PP2A cellular immunolocalization in *Prorocentrum lima* (Dinophyceae). *Phycologia* 38 (1): 41-46.

Begun A., Orlova T. & Selina M. 2004. A “Bloom” in the water of Amursky Bay (Sea of Japan) caused by the dinoflagellate *Oxyrrhis marina* Dujardin, 1841. *Russian Journal of Marine Biology* 30 (1): 51-55.

Bouaïcha N., Chézeau A., Turquet J., Quod J. P.& Puiseux-Dao S. 2001. Morphological and toxicological variability of *Prorocentrum lima* clones isolated from four locations in south-west Indian Ocean. *Toxicon* 39: 1195-1202.

Bursa A. 1959. The genus *Prorocentrum* Ehrenberg. Morphodynamics, protoplasmatic structures, and taxonomy. *Canadian Journal of Botany* 37 (1): 1-31.

Calado A. & Moestrup Ø. 1997. Feeding in *Peridiniopsis berolinensis* (Dinophyceae): new observations on tube feeding by an omnivorous, heterotrophic dinoflagellates. *Phycologia* 36: 47-50.

Calado A., Hansen G. & Moestrup Ø. 1999. Architecture of the flagellar apparatus and related structures in the type species of *Peridinium, P. cinctum* (Dinophyceae). *European Journal of Phycology* 34: 179-191.

Caldwell G., Bentley M. & Olive P. 2003. The use of a brine shrimp (*Artemia salina*) bioassay to assess the toxicity of diatom extracts and short chain aldehydes. *Toxicon* 42: 301-306.

Cembella A., Milenkovic, L., Doucette G. & Fernandez M. 1995. In: *In vitro* Biochemical Methods and Mammalian Bioassays for Phycotoxins. In: Manual on Harmful Marine Microalgae (Ed. By Hallegraeff G., Anderson D. & Cembella A.). UNESCO. France. pp. 177-224.

Chomérat N., Couté A., Fayolle S., Mascarell G. & Cazaubon A. 2004. Morphology and ecology of *Oblea rotunda* (Diplopsalidaceae, Dinophyceae) from a new habitat: a brackish and hypertrophic ecosystem, the Étang de Bolmon (South of France). *European Journal of Phycology* 39: 317-326.

Cohen P. 1991. Classification of protein-serine/threonine phosphatases: identification and quantification in cell extracts. *Methods Enzymol*. 201: 389-398.

Cortés-Altamirano R. & Sierra- Beltrán A. 2003. Morphology and taxonomy of *Prorocentrum mexicanum* and reinstatement of *Prorocentrum rhathymum* (Dinophyceae). *Journal of Phycology* 39: 221-225.

Daranas A., Norte M. & Fernández J. 2001. Toxic marine microalgae. *Toxicon* 39: 1101-1132.

Daugbjerg N., Hansen G., Larsen J. & Moestrup Ø. 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the three new genera of unarmoured dinoflagellates. *Phycologia* 39 (4): 302-317.

Daugbjerg N., Moestrup Ø. & Arctander P. 1994. Phylogeny of the genus *Pyramimonas* (Prasinophyceae, Chlorophyta) inferred from the rbcL gene. *Journal of Phycology* 30: 991-999.

Day J. Jr, Hall C., Kemp W. & Yañez-Anancibia A. 1989.Estuary Ecology. pp 45-78. John Wiley & Sons. New York USA.

Demaret A., Sohet K. & Houvenaghel G. 1993. Effects of toxic dinoflagellates on the feeding and mortality of *Artemia franciscana* larvae. *Harmful Marine Algal Blooms* 427-432.

Dickey R., Bobzin S., Faulkner D., Bencsath F. & Andrzejewski D. 1990. Identification of okadaic acid from a Caribbean dinoflagellate, *Prorocentrum concavum*. *Toxicon* 28 (4): 371-377.

Dodge J. & Bibby B. 1973. The Prorocentrales (Dinophyceae) I. A comparative account of fine structure in the genera *Prorocentrum* and *Exuviaella*. *Botanical Journal of the Linnean Society* 67: 175-187.

Dodge J. 1965. Thecal fine-structure in the dinoflagellate genera *Prorocentrum* and *Exuviaella*. *Journal of Marine Biology* 45: 607-614.

Dodge J. 1975. The Prorocentrales (Dinophyceae) II. Revision of the taxonomy within the genus *Prorocentrum*. *Botanical Journal of the Linnean Society* 71 (2): 103-125.

Dodge J. 1982. Marine dinoflagellates of the British Isles. Her Majesty's Stationery Office, London. 303 pp.

Ehrenberg C. 1860. Über das Leuchten und über neue mikroskopische Leuchttiere des Mittelmeeres. Monatsber. Königl. Preuss. Akad. Wissensch. Berlin. 1859: 727-738.

Ehrenberg C. 1873. Die das Funkeln und Ausblitzen des Mittelmeeres bewirkenden unsichtbar kleinen Lebensformen. Gesellsch. Naturforsch. Freunde Berlin, Festschr. Feier Hundertjährigen bestehens, 1-4.

Faust M. & Steidinger K. 1998. *Bysmatrum* gen. nov. (Dinophyceae) and three new combinations for benthic scrippsielloid species. *Phycologia* 37 (1): 47-52.

Faust M. 1974. Micromorphology of a small dinoflagellate *Prorocentrum mariae-lebouriae* (Parke & Ballatine) comb. nov.. *Journal of Phycology* 10: 315-322.

Faust M. 1990. Morphologic details of six benthic species of *Prorocentrum* (Pyrrophyta) from a mangrove island, Twin Cays, Belize, including two new species. *Journal of Phycology* 26: 548-558.

Faust M. 1991. Morphology of ciguatera-causing *Prorocentrum lima* (Pyrrophyta) from widely differing sites. *Journal of Phycology* 27: 642-648.

Faust M. 1992. Observations on the morphology and sexual reproduction of *Coolia monotis* (Dinophyceae). *Journal of Phycology* 28: 94-104.

Faust M. 1993[a]. *Prorocentrum belizeanum*, *Prorocentrum elegans*, and *Prorocentrum caribbaeum*, three new benthic species (Dinophyceae) from a mangrove island, Twin Cays, Belize. *Journal of Phycology* 29: 100-107.

Faust M. 1993[b]. Three new benthic species of *Prorocentrum* (Dinophyceae) from Twin Cays, Belize: *P. maculosum* sp. nov., *P. foraminosum* sp. nov. and *P. formosum* sp.nov. *Phycologia* 32 (6): 410-418.

Faust M. 1994. Three new benthic species of *Prorocentrum* (Dinophyceae) from Carrie Bow Cay, Belize: *P. sabulosum* sp. nov., *P. sculptile* sp. nov., and *P. arenarium* sp. nov. *Journal of Phycology* 30: 755-763.

Faust M. 1995. Benthic, toxic dinoflagellates: an overview. In: Harmful Marine Algal Blooms (Edited by Lassus P., Arzul G., Erard P. & Marcaillou C.) .pp. 847-854.

Faust M. 1996. Dinoflagellates in a mangrove ecosystem, Twin Cays, Belize. *Nova Hedwigia* 112: 447-460.

Faust M. 1997. Three new benthic species of *Prorocentrum* (Dinophyceae) from Belize: *P. norrisianum* sp. nov., *P. tropicalis* sp. nov., and *P. reticulatum* sp. nov. *Journal of Phycology* 33: 851-858.

Faust M. & Gulledge A. 2002. Identifying harmful marine dinoflagellates – Vol. 42:1-144. Smithsonian Institution. Washington DC. 144 pp.

Faust M., Larsen J. & Moestrup Ø. 1999. Potentially toxic phytoplankton – 3 Genus *Prorocentrum* (Dinophyceae). *ICES Identification Leaflets for Plankton* 184: 1-24.

Fernández J., Suárez-Gómez B., Souto M. & Norte M. 2003. Identification of new okadaic acid derivates from laboratory cultures of *Prorocentrum lima*. *Journal of Natural Products* 66: 1294-1296.

Fritz L. & Triemer E. 1985. A rapid simple technique utilizing calcofluor white M2R for the visualization of dinoflagellate thecal plates. *Journal of Phycology* 21: 662-664.

Fukuyo Y. 1981. Taxonomical Study on Benthic Dinoflagellates Collected in Coral Reefs. Bulletin of the Japanese Society of Scientific Fisheries 47(8): 967-978.

Graham L. E., Wilcox L. W. 2000. Algae. Prentice Hall. London. pp 198-231.

Grzebyk D., Sako Y. & Berland B. 1998. Phylogenetic analysis of nine species of *Prorocentrum* (Dinophyceae) inferred from 18S ribosomal DNA sequences, morphological comparisons, and description of *Prorocentrum panamensis*, sp. nov.. *Journal of Phycology* 34: 1055-1068.

Guillard R. & Keller M. 1984. Culturing dinoflagellates. In Dinoflagellates (Ed. by Spector D.). Academic press. Orlando. pp 391-442.

Hallegraeff G. 1995. Harmful Algal Blooms: A global overview. In: Manual on Harmful Marine Microalgae (Ed. By Hallegraeff G., Anderson D. & Cembella A.). UNESCO. France. pp. 1-18.

Hallegraeff G. M. 2002: Aquaculturists'Guide to Harmful Australian Microalgae, 2nd edition, The Print Centre, Australia. 136 pp.

Hansen G., Daugbjerg N. & Henriksen, P. 2000[a]. Comparative study of *Gymnodiniun mikimotoi* and *Gymnodiniun aurelum*, comb. nov. (= *Gyrodinium aurelum*) based on morphology, pigment composition, and molecular data. *Journal of. Phycology*. 36: 394-410.

Hansen G., Moestrup Ø. & Roberts K. 2000[b]. Light and electron microscopical observations on the type species of *Gymnodinium*, *G. fuscum* (Dinophyyceae). *Phycologia* 39 (5): 365-376.

Hansen G., Turquet J., Quod J., Ten-Hage L., Lugomela C., Kyewalyanga M., Hurbungs M., Wawiye P., Ogongo B., Tunje S. & Rakotoarinjanahary H. 2001. Potentially Harmful Microalgae of the western Indian Ocean – A guide based on a preliminary survey. IOC Manuals and Guides No.41. Intergovernmental Oceanographic Commission of UNESCO. France. 105 pp.

Heimann K, Roberts K. & Wetherbee R. 1995. The flagellar apparatus transformation and development in *Prorocentrum micans* and *P. minimum* (Dinophyceae). *Phycologia* 34 (4): 323-335.

Hernández-Becerril D., Altamirano R. & Alonso R. 2000. The dinoflagellate genus *Prorocentrum* along the coasts of the Mexican Pacific. *Hydrobiologia* 418: 111-121.

Hoek C., Mann D. & Jahns H. 1995. Algae – An introduction to phycology. Cambridge Press. Cambridge. pp 244-285.

Holmes M. & Teo S. 2002. Toxic marine dinoflagellates in Singapore waters that cause seafood poisonings. *Clinical and Experimental Pharmacology and Physiology* 29: 829-836.

Holmes M. J., Lee F. C., Teo S. L. & Khoo H. W. 2001. Production of 7-Deoxy-Okadaic acid by new Caledonian strain of *Prorocentrum lima. J. Phycology* 37: 280-288.

Hoppenrath M., Jahn R., Elbrächter M. & Kusber W. 2004. Original material of Ehrenberg's "*Cryptomonas lima*" rediscovered: The identity of *Prorocentrum lima* reinvestigated. In: Conference on Harmful Algae Cape Town - Program and Abstracts. South Africa. pp. 142.

Hulburt E. 1957. The taxonomy of unarmoured Dinophyceae of shallow embayments on Cape Cod, Massachusetts. *The Biological Bulletin* 112 (2): 196- 219.

Ismael A., Halim Y. & Khalil A-G. 1999. Optimum growth conditions for *Amphidinium carterae* Hulburt from eutrophic waters in Alexandria (Egypt) and its toxicity to the brine shrimp *Artemia salina*. *Grana* 38: 179-185.

Izumikawa M., Murata M., Tachibana K., Fujita T. & Naoki H. 2000. ^{18}O-Labelling pattern of okadaic acid from $H_2{}^{18}O$ in dinoflagellate *Prorocentrum lima* elucidated by tandem mass spectrometry. *European Journal Biochemistry* 267: 5179-5183.

Janofske D. 2000. *Scrippsiella* cf. *trochoidea* and *Scrippsiella regalis*, nov. com. (Peridiniales, Dinophyceae): a comparison. *Journal of Phycology* 36: 178-189.

Jørgensen M., Murray S. & Daugbjerg N. 2004. *Amphidinium* revisited. I. Redefinition of *Amphidinium* (Dinophyceae) based on cladistic and molecular phylogenetic analyses. *Journal of Phycology* 40: 351-365.

Larsen J. & Sournia A. 1991. The diversity of heterotrophic dinoflagellates. In: The Biology of Free-living Heterotrophic Flagellates (Ed. by Patterson D. & Larsen J.). Clarendon Press. Oxford. pp 313-332.

Lawrence J. & Cembella A. 1999. An immunolabeling technique for the detection of diarrhetic shellfish toxins in individual dinoflagellate cells. *Phycologia* 38 (1): 60-65.

Lewis J. 1991. Cyst-theca relationships in *Scrippsiella* (Dinophyceae) and related Orthoperidinioid genera. *Botanica Marina* 34: 91-106.

Loeblich III A. R. 1976. Dinoflagellate Evolution: Speculation and Evidence. *Journal of Protozoology*. 23(1): 13-28.

Loeblich III A., Sherley J. & Schmidt R. 1979. The correct position of flagellar insertion in *Prorocentrum* and description of *Prorocentrum rhathymum* sp. nov. (Pyrrhophyta). *Journal of Plankton Research* 1 (2): 113-120.

López-Figueroa F. & Niell F. 1987. Feeding behaviour of *Hydrobia ulvae* (Pennant) in microcosmos. *Journal of Experimental Marine Biology and Ecology* 114: 153-167.

Lu C., Lee G., Huang R. & Chou H. 2001. Spiro-prorocentrimine, a novel macrocyclic lactone from a benthic *Prorocentrum* sp. of Taiwan. *Tetrahedron Letters* 42: 1713-1716.

Mackenzie L., Sims I., Beuzenberg V. & Gillespie P. 2002. Mass accumulation of mucilage caused by dinoflagellate polysaccharide exudates in Tasman Bay, New Zealand. *Harmful Algae* 1: 69-83.

McLachlan J. L., Boalch G. T. & Jahn R. 1997. Reinstatement of the genus *Exuviaella* (Dinophyceae, Prorocentraphycidae) and an assessment of *Prorocentrum lima*. *Phycologia*. 36(1): 38-46.

Ministério do Plano e da Administração do Território Serviço Nacional de Parques.1988. Estudo sobre zonas húmidas costeiras em Portugal. pp. 1-15. MPAT SNPRCN. Lisboa Portugal.

Moita M. & Vilarinho M. 1999. Checklist of phytoplankton species of Portugal: 70 years (1929-1998) of studies. *Portugaliae Acta Biol., Sér. B, Sist.* 18: 5-50.

Moroño A., Arévalo F., Fernández M., Maneiro J., Pazos Y., Salgado C. & Blanco J. 2003. Accumulation and transformation of DSP toxins in mussels *Mytilus galloprovincialis* during a toxic episode caused by *Dinophysis acuminata*. *Aquatic Toxicology* 62: 269-280.

Morton S. & Faust M. 1997. Survey of toxic epiphytic dinoflagellates from the Belizean barrier reef ecosystem. *Bulletin of Marine Science* 61 (3): 899-906.

Morton S. & Tindall D. 1995. Morphological and biochemical variability of the toxic dinoflagellate *Prorocentrum lima* isolated from three locations at Heron Island, Australia. *Journal of Phycology* 31: 914-921.

Morton S., Faust M., Fairey E. & Moeller P. 2002. Morphology and toxicology of *Prorocentrum arabianum* sp. nov., (Dinophyceae) a toxic planktonic dinoflagellate from the Gulf of Oman, Arabian Sea. *Harmful Algae* 1: 393-400.

Morton S., Moeller P., Young K. & Lanoue B. 1998. Okadaic acid production from the marine dinoflagellate *Prorocentrum belizeanum* Faust isolated from the Belizean coral reef ecosystem. *Toxicon* 36 (1): 201-206.

Murakami Y., Oshima Y. & Yasumoto T. 1982. Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima*. *Bulletin of the Japanese Society of Scientific Fisheries* 48 (1): 69-72.

Murata M., Shimatani M., Sugitani H., Oshima Y. & Yasumoto T. 1982. Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. *Bulletin of the Japanese Society of Scientific Fisheries* 48 (4): 549-552.

Murray S., Jørgensen M., Daugbjerg N. & Rhodes L. 2004. *Amphidinium* revisited. II. Resolving species boundaries in the *Amphidinium operculatum* species complex (Dinophyceae), including descriptions of *Amphidinium trulla* sp. nov. and *Amphidinium gibbosum* comb. nov.. *Journal of Phycology* 40: 366-382.

Nakajima I., Oshima Y. & Yasumoto T. 1981. Toxicity of benthic dinoflagellates in Okinawa. *Bulletin of the Japanese Society of Scientific Fisheries* 47(8): 1029-1033.

Pavela-Vrancic M., Mestrovic V., Marasovic I., Gillman M., Furey A. & James K. 2002. DSP toxin profile in the coastal waters of the central Adriactic Sea. *Toxicon* 40: 1601-1607.

Pickett-Heaps J. 1998. A rapid, highly efficient method for collecting, fixing, and embedding planktonic and other small cells for electron microscopy. *Journal of Phycology* 34: 1088-1089.

Pillet S. & Houvenaghel G. 1995. Influence of experimental toxification by DSP producing microalgae, *Prorocentrum lima*, on clearance rate in blue mussels *Mytilus edulis*. In: Harmful Marine Algal Blooms (Edited by Lassus P., Arzul G., Erard P. & Marcaillou C.). pp 481-486.

Pillet S., Pereira A., Braekman J-C. & Houvenaghel G. 1995. Patterns in long term accumulation of okadaic acid and DTX1 in blue mussels, *Mytilus edulis*, experimentally fed with the DSP- containing alga *Prorocentrum lima*. In: Harmful Marine Algal Blooms (Edited by Lassus P., Arzul G., Erard P. & Marcaillou C.). pp 487-492.

Pringsheim E. G. 1946. Pure Cultures Of Algae. Cambridge University Press, London. 119 pp.

Puigserver M. & Zingone A. 2002. *Prorocentrum nux* sp. nov. (Dinophyceae), a small planktonic dinoflagellate from the Mediterranean Sea, and discussion of *P. nanum* and *P. pusillum*. *Phycologia* 41 (1): 29-38.

Quilliam M. & Wright J. 1995. Methods for Diarrhetic Shellfish Poisons. In: Manual on Harmful Marine Microalgae (Ed. By Hallegraeff G., Anderson D. & Cembella A.). UNESCO. France. pp. 95-104.

Roberts K., Heimann K. & Wetherbee R. 1995. The flagellar apparatus and canal structure in *Prorocentrum micans* (Dinophyceae). *Phycologia* 34 (4): 313-322.

Schiller J. 1918. Über neue *Prorocentrum*- und *Exuviella*-Arten aus der Adria. *Archiv für Protistenkunde* 38 (2): 250-262.

Schiller J. 1928. Die planktischen vegetationen des adriatischen Meeres. *Archiv für Protistenkunde* 61: 45-90.

Scholin C. A., Herzog M., Sogin M. & Anderson D. M. 1994. Identification of group- and strain- specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. *Journal of. Phycology*. 30: 999-1011.

Shumway S., Allen S. & Boersma P. 2003. Marine birds and harmful algal blooms: sporadic victims or under-reported events?. *Harmful Algae* 2: 1-17.

Smayda T. 1997. What is a bloom? A commentary. *Limnology and Oceanography* 42 (5,part 2): 1132-1136.

Steidinger K. & Balech E. 1977. *Scrippsiella subsalsa* (Ostenfeld) comb. nov. (Dinophyceae) with a discussion on *Scrippsiella*. *Phycologia* 16 (1): 69-73.

Steidinger K. A. & Tanger K. 1996. Dinoflagellates. In: Identifying Marine Diatoms and Dinoflagellates (Ed. by Tomas C.). Academic Press. San Diego. pp 387-598.

Stein F. 1883. Der organismus der flagellaten nach eigenen forschungen in systematischer reihenfolge bearbeitet, 3 (2): 1-30. Leipzig.

Sugg L. & VanDolah F. 1999. No evidence for an allelopathic role of akadaic acid among ciguatera-associated dinoflagellates. *Journal of Phycology* 35: 93-103.

Svensson S. & Förlin L. 2004. Analysis of the importance of lipid breakdown for elimination of okadaic acid (diarrhetic shellfish toxin) in mussels, *Mytilus edulis*: results from a field study and a laboratory experiment. *Aquatic Toxicology* 66: 405-418.

Tanaka I., Matsuoka S., Murata M. & Tachibana K. 1998. A new ceramid with a novel branched-chain fatty acid isolated from the epiphytic dinoflagellate *Coolia monotis*. *Journal of Natural Products* 61: 685-688.

Taylor F. 1980. On dinoflagellate evolution. *BioSystems* 13: 65-108.

Ten-Hage L., Tourquet J., Quod J., Puiseux-Dao & Coute A. 2000. *Prorocentrum borbonicum* sp. nov. (Dinophyceae), a new toxic benthic dinoflagellate from the southwestern Indian Ocean. *Phycologia* 39 (4): 296-301.

Thomsen H.1992. Havforskning fra Miljøstyrelsen-Nr.11-Plankton I de indre danske farvande. Miljøministeriet Miljøstyrelsen. Denmark. 331pp.

Traubenberg C. & Morlaix M. 1995. Evidence of okadaic acid release into extracelular medium in cultures of *Prorocentrum lima* (Ehrenberg) Dodge. In: Harmful Marine Algal Blooms (Edited by Lassus P., Arzul G., Erard P. & Marcaillou C.). pp 493-498.

Trigueros J., Ansotegui A. & Orive E. 2000. Remarks on morphology and ecology of recurrent dinoflagellate species in the Estuary of Urdaibai (Northern Spain). *Botanica Marina* 43: 93-103.

Truby E. 1997. Preparation of single-celled marine dinoflagellates for electron microscopy. *Microscopy Research and Technique* 36: 337-340.

Vale P. & Sampayo M. 1999[a]. Esters of okadaic acid and dinophysistoxin-2 in Portuguese bivalves related to human poisonings. *Toxicon* 37: 1109-1121.

Vale P. & Sampayo M. 1999[b]. Comparison between HPLC and a commercial immunoassay kit for detection of okadaic acid and esters in Portuguese bivalves. *Toxicon* 37: 1565-1577.

Vale P. & Sampayo M. 2002[a]. Evaluation of extraction methods for analysis of domoic acid in naturally contaminated shellfish from Portugal. *Harmful Algae* 1: 127-135.

Vale P. & Sampayo M. 2002[b]. First confirmation of human diarrhoeic poisonings by okadaic acid esters after ingestion of razor clams (*Solen marginatus*) and green crabs (*Carcinus maenas*) in Ria de Aveiro, Portugal and detection of okadaic acid esters in phytoplankton. *Toxicon* 40: 989-996.

Vale P. & Sampayo M. A. 2003. Seasonality of diarrhetic shellfish poisoning at a coastal lagoon in Portugal: rainfall patterns and folk wisdom. *Toxicon* 41: 187–197.

Vila M., Garcés E. & Masó M. 2001. Potentially toxic epiphytic dinoflagellate assemblages on macroalgae in the NW Mediterranean. *Aquatic Microbial Ecology* 26: 51-60.

Yasumoto T., Oshima Y., Murakami Y., Nakajima I., Bagnis R. & Fukuyo Y. 1980. Toxicity of benthic dinoflagellates found in coral reef. *Bulletin of the Japanese Society of Scientific Fisheries* 46 (3): 327-331.

Yasumoto T., Seino N., Murakami Y. & Murata M. 1987. Toxins produced by benthic dinoflagellates. *Biological Bulletin* 172: 128-131.

Zardoya R., Costas E., López-Roda V., Garrido-Pertierra A. & Bautista J. 1995. Revised dinoflagellates phylogeny inferred from molecular analysis of large-subunit ribosomal RNA gene sequences. *Journal of Molecular Evolution* 41:637-645.

Zhou J. & Fritz L. 1993. Ultrastructure of two toxic marine dinoflagellates, *Prorocentrum lima* and *Prorocentrum maculosum*. *Phycologia* 32: 444-450.

Zhou J. & Fritz L. 1994[a]. Okadaic acid antibody localizes to chloroplast in the DSP-toxin-producing dinoflagellates *Prorocentrum lima* and *Prorocentrum maculosum*. *Phycologia* 33: 455-461.

Zhou J. & Fritz L. 1994[b]. The PAS/ Accumulation bodies in *Prorocentrum lima* and *Prorocentrum maculosum* (Dinophyceae) are dinoflagellate lysossomes. *Journal of Phycology* 30: 39-44.

Appendixes

Appendix 1: Recipe to make f/2 medium.

f/2 Medium and Derivatives
(Guillard & Ryther 1962, Guillard 1975)

Below are recipes for f/2 medium, its derivatives (e.g, f/2 agar, f/2-Si, f/2 + Se, f/4, f/50) and related media (e.g., Black Sea). F/2 is listed first, followed by derivatives of f/2.

f/2 Medium

(Guillard & Ryther 1962, Guillard 1975)

To 950 mL filtered and autoclaved seawater add:

Quantity	Compound	Stock Solution	Molar Concentration in Final Medium
1 mL	$NaNO_3$	75 g/L dH_2O	8.83×10^{-4} M
1 mL	$NaH_2PO_4 \cdot H_2O$	5 g/L dH_2O	3.63×10^{-5} M
1 mL *	$Na_2SiO_3 \cdot 9H_2O$*	30 g/L dH_2O*	1.07×10^{-4} M*
1 mL	f/2 trace metal solution	(see recipe below)	-
0.5 mL	f/2 vitamin solution	(see recipe below)	-

Make final volume up to 1 L with filtered seawater .
***Note:** Autoclaved f/2 medium produces extensive silica precipitate. We do not add silicate when it is not required by the alga (see f/2-Si medium below).

f/2 Trace Metal Solution
(Guillard & Ryther 1962, Guillard 1975)

To 950 mL dH_2O add:

Quantity	Compound	Stock Solution	Molar Concentration in Final Medium
3.15 g	$FeCl_3 \cdot 6H_2O$	-	1×10^{-5} M
4.36 g	$Na_2EDTA \cdot 2H_2O$	-	1×10^{-5} M
1 mL	$CuSO_4 \cdot 5H_2O$	9.8 g/L dH_2O	4×10^{-8} M
1 mL	$Na_2MoO_4 \cdot 2H_2O$	6.3 g/L dH_2O	3×10^{-8} M
1 mL	$ZnSO_4 \cdot 7H_2O$	22.0 g/L dH_2O	8×10^{-8} M
1 mL	$CoCl_2 \cdot 6H_2O$	10.0 g/L dH_2O	5×10^{-8} M
1 mL	$MnCl_2 \cdot 4H_2O$	180.0 g/L dH_2O	9×10^{-7} M

Make final volume up to 1 L with dH_2O. Autoclave.

f/2 Vitamin Solution
(Guillard & Ryther 1962, Guillard 1975)

To 950 mL dH_2O add:

Quantity	Compound	Stock Solution	Molar Concentration in Final Medium
1 mL	Vitamin B_{12} (cyanocobalamin)	1.0 g/L dH_2O	1×10^{-10} M
10 mL	Biotin	0.1 g/L dH_2O	2×10^{-9} M
200 mg	Thiamine · HCl	-	3×10^{-7} M

Make final volume up to 1 L with dH_2O. Autoclave and store in refrigerator. **Note**: Vitamin B_{12} and biotin are obtained in a crystalline form. When preparing the vitamin B_{12} stock solution, allow for approximately 11% water of crystallization (for each 1 mg of Vitamin $B_{12,}$ add 0.89 mL dH_2O). When preparing the biotin stock solution, allow for approximately 4% water of crystallization (for each 1 mg of biotin, add 9.6 mL dH_2O).

f/2 Derivatives

Black Sea Medium: For brackish water organisms (16 , half-strength nutrients). Combine 500 mL f/2 medium and 500 mL dH_2O. Autoclave.

f/2 agar: Prepare 1 litre of f/2 medium and dissolve 9g Bacto-agar (heat and mix). For test tubes, dispense dissolved agar medium into tubes, autoclave, and then cool with tubes slanted at an angle. For Petri plates, autoclave in a flask, cool almost to the gelling point, and then aseptically dispense into sterile Petri plates. **Note:** Agar can be added to other media (e.g., f/50 agar), and agar concentration can be varied to produce softer or firmer substrates.

f/2-Si: Prepare as for f/2 medium but omit $Na_2SiO_3 \cdot 9H_2O$. This is preferred over f/2 medium for organisms with no silica requirement because less precipitation forms.

f/2 + Se: Extra silicon and selenium are beneficial to several diatom strains. Prepare 1 L of f/2 medium but use 2 mL of silicate stock, then add 1.0 mL of selenium stock solution (1.29 mg H_2SeO_3 /L distilled H_2O). Autoclave.

f/2 (11): For brackish water organisms. Mix 650 mL distilled H_2O and 350 mL filtered seawater. Add f/2 medium nutrients and autoclave.

f/2-Si (24): Mix 750 mL distilled H_2O and 250 mL filtered seawater. Prepare as for f/2 medium but omit $Na_2SiO_3 \cdot 9H_2O$.

f/4: Add 500 mL f/2 medium to 500 mL filtered seawater, then autoclave.

f/4-Si: Autoclave 1 L of filtered seawater. When cool, aseptically add f/2-Si nutrients at half concentration (i.e., 0.5 mL).

f/20-Si: Autoclave 1 L of filtered seawater. When cool, aseptically add f/2-Si nutrients at one tenth concentration (i.e., 100 µL).

f/50-Si: This is more than a 1/25 dilution of f/2-Si medium. We autoclave 1 L of seawater in a Teflon-lined bottle. Wait for the autoclaved seawater to cool to room temperature (important). Aseptically add 40 µL of sterile f/2 nutrients (20 µL of vitamins).

f/50-Si + CCMP1320 as food: Prepare f/50 and aseptically add 50 µL of healthy, moderately dense culture of CCMP1320.

f/2m: To 1L f/2 medium add 1 g methylamine · HCl, mix until dissolved and autoclave. This medium is used to test for contamination by methylaminotrophic bacteria.

f/2p: To 1 L f/2 medium, add 1 g Bacto-peptone, mix until dissolves and autoclave. This medium is used to test for contamination by non- methylaminotrophic bacteria and fungi.

f/2pm: To 1L f/2 medium add 1 g Bacto-peptone and 1 g methylamine · HCl, mix until dissolved and autoclave. This general medium is used to test for contamination by bacteria and fungi.

f/2 + NPM: Add f/2 nutrients to 900 mL of seawater and autoclave. After cooling, aseptically add 100 mL of the following organic stock solution. Dispense aseptically into test tubes.

Organics Stock Solution

(modified from Guillard 1960)

To 900 mL dH_2O add:

Quantity	Compound
1 g	sodium acetate
6 g	glucose
3 g	(di-) sodium succinate · $6H_2O$
4 g	neopeptone
1 g	Bacto-tryptone
100 mg	yeast extract

Bring up to 1 L with dH_2O. Dispense in small aliquots and autoclave.

References

Guillard, R.R.L. 1960. A mutant of *Chlamydomonas moewusii* lacking contractile vacuoles. **J. Protozool. 7:** 262-268.

Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates. pp 26-60. *In* Smith, W.L. & Chanle,y M.H. (eds.) Culture of Marine Invertebrate Animals. Plenum Press, New York, USA.

Guillard, R.R.L. & Ryther, J.H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. **Can. J. Microbiol. 8:** 229-239.

Appendix 2: Genetic experiences to amplify the LSU-rDNA sequence.

Table 1 – PCR experiment MH1

Tube	**10xTaq buffer**	**dNTPs**	**Primer1 DIF**	**Primer2 1483R**	**Template(species)**	**Taq**	**TMA**	**dH_2O**	**Total reaction volume**
1	5µl	20µl	5µl	5µl	Prorocentrum sp. 2 482 2µl	0.2µl	5µl	9µl	50µl
2	5µl	20µl	5µl	5µl	B. subsalsum 405 2µl	0.2µl	5µl	9µl	50µl
3	5µl	20µl	5µl	5µl	Heterocapsa 408 1µl	0.2µl	5µl	9µl	50µl
4	5µl	20µl	5µl	5µl	P.micans 501 1µl	0.2µl	5µl	9µl	50µl
5	5µl	20µl	5µl	5µl	Amphidinium 458 2µl	0.2µl	5µl	9µl	50µl
6	5µl	20µl	5µl	5µl	P. rhathymum 515 1µl	0.2µl	5µl	9µl	50µl
7	5µl	20µl	5µl	5µl	Control -	0.2µl	5µl	9µl	50µl
Total	35µl	140µl	35µl	35µl		1.4µl	35µl	63µl	345µl

Table 2 – PCR experiment MH2

Tube	**10xTaq buffer**	**dNTPs**	**Primer1 DIF**	**Primer2 D3B**	**Template (species)**	**Taq**	**TMA**	**dH_2O**	**Total reaction volume**
1	5µl	20µl	5µl	5µl	B. subsalsum 405 2µl	0.2µl	5µl	9µl	50µl
2	5µl	20µl	5µl	5µl	P.micans 501 2µl	0.2µl	5µl	9µl	50µl
3	5µl	20µl	5µl	5µl	Amphidinium 458 2µl	0.2µl	5µl	9µl	50µl
4	5µl	20µl	5µl	5µl	Control	0.2µl	5µl	9µl	50µl
Total	20 µl	80µl	20µl	20µl		0.8µl	20µl	36µl	200µl
Tube	**10xTaq buffer**	**dNTPs**	**Primer1 D3A**	**Primer2 1483R**	**Template (species)**	**Taq**	**TMA**	**dH_2O**	**Total reaction volume**
5	5µl	20µl	5µl	5µl	B. subsalsum 405 2µl	0.2µl	5µl	9µl	50µl
6	5µl	20µl	5µl	5µl	P.micans 501 2µl	0.2µl	5µl	9µl	50µl
7	5µl	20µl	5µl	5µl	Amphidinium 458 2µl	0.2µl	5µl	9µl	50µl
8	5µl	20µl	5µl	5µl	Control	0.2µl	5µl	9µl	50µl
Total	20 µl	80µl	20µl	20µl		0.8µl	20µl	36µl	200µl

Appendix 3: List of coastal dinoflagellates species found in this study.

The columns indicate the authority, whether if the species is planktonic and/or benthic and if it contains plastids.

Taxa	Authority and Date	Habitat	Planktonic or Benthic	Plastids Present
Gymnodiniaceae	**Lankester 1885**			
Akashiwo	**G. Hansen & Moestrup 2000**			
Akashiwo sanguinea	Hansen & Moestrup 2000	marine	planktonic	yes
Amphidinium	**Claparede & Lachmann 1859**			
Amphidinium carterae	Hulbert 1957	marine	p/b	yes
Amphidinium massartii	Biecheler 1952	marine	p/b	yes
Gymnodinium	**Stein 1878**			
Gymnodinium catenatum	Graham 1943	marine	planktonic	yes
Gyrodinium	**Kofoid & Swezy 1921**			
Gyrodinium sp. 1	**?**	marine	planktonic	no
Polykrikaceae	**Kofoid & Swezy 1921**			
Pheopolykrikos	**Chatton 1933**			
Pheopolykrikos sp. 1.	**?**	marine	planktonic	yes
Peridiniales	**Schütt 1896**			
Peridiniaceae	**Ehrenberg 1838**			
Peridinium	**Ehrenberg 1838**			
Kryptoperidinium foliaceum	Biecheler 1952	marine	p/b	yes
Peridinium quinquecorne	Abe 1927	marine	p/b	yes
Heterocapsa	**Stein 1883**			
Heterocapsa niei	Morril & Loeblich III 1981	marine	p/b	yes

Protoperidiniaceae	**F. J. R. Taylor 1987**			
Protoperidinium	**Bergh 1881**			
Protoperidinium minutum	Loeblich III 1970	marine	planktonic	no
Kolkwitziellaceae	**Lindemann 1928**			
Oblea	**Balech ex Loeblich Jr. & Loeblich III 1966**			
Oblea rotunda	Balech 1964	marine	planktonic	no
Calciodinellaceae	**F. J. R. Taylor 1987**			
Scrippsiella	**Balech ex Loeblich III 1965**			
Scrippsiella trochoidea	Loeblich III 1976	marine	planktonic	yes
Bysmatrum	**Faust & Steidinger 1998**			
Bysmatrum subsalsum	Faust & Steidinger 1998	marine	p/b	yes
Gonyaulacales	**Taylor 1980**			
Ostreopsidaceae	**Lindemann 1928**			
Coolia	**Meunier 1919**			
Coolia monotis	Meurier 1919	marine	benthic	yes
Prorocentrales	**Lemmermann 1910**			
Prorocentraceae	**Stein 1883**			
Prorocentrum	**Ehrenberg 1834**			
Prorocentrum cassubicum	Dodge 1973	marine	benthic	yes
Prorocentrum minimum	Pavillard 1916	marine	planktonic	yes
Prorocentrum lima	Ehrenberg 1859	marine	benthic	yes
Prorocentrum micans	Ehrenberg 1834	marine	planktonic	yes
Prorocentrum rhathymum	Loeblich 1976	marine	p/b	yes
Prorocentrum sp. 1	new species	marine	benthic	yes
Prorocentrum sp. 2	new species	marine	p/b	yes

Appendix 4: Comparison with previous records from the coast of Portugal (based on Moita e Vilarinho, 1999)

Legend: **X** – recorded by Moita & Vilarinho (1999); O – identified in the present work; and + indicates that they are recognise as being harmful. The species in yellow have in the present work their first occurrence.

HAB	Taxa	Synonyms in Portuguese references	Coast			Ria Formosa	Óbidos Lag.	Tejo Est.	Sado Est.	Ria Aveiro	S.Martinho Bay	Albufeiro Lag.	Mondego Est.	Minho Est.
			NW	SW	S									
	Dinophyceae		X	X	X	X	X	X	X	X	X	X	X	X
	Akashiwo		X	X	X	X	X	X	X	O	X	X	X	
+	*Akashiwo sanguinea*	*Gymnodinium sanguineum*, *G. splendens*	X	X	X	X	X	X	X	O				
	Amphidinium		X	X	X	X	X	X	X	O	X	X	X	
+	*Amphidinium carterae*					X	X	X	X	O	X	X	X	
	Amphidinium massartii									O				
	Gymnodinium		X	X	X	X	X	X	X	X	X	X	X	X
+	*Gymnodinium catenatum*		X	X	X	X	X	X	X	X	X	X	X	X
	Peridinium			X		X	X	X	X	X			X	
	Peridinium foliaceum	*Glenodinium foliaceun*				X	X	X	X	O				
	Peridinium quinquecorne	*Protoperidinium quinquecorne*		X		X	X			X			X	
	Heterocapsa		X	X	X		X			O				
	Heterocapsa niei	*Cachonina niei*	X	X	X		X			O				
	Protoperidinium	*Peridinium*	X	X	X		X	X	X	X	X			
	Protoperidinium minutum	*Peridinium monospinum*	X	X	X	X	X							
	Oblea									X				
	Oblea rotunda									O				
	Scrippsiella		X	X	X	X	X	X	X	X	X	X	X	X
	Scrippsiella trochoidea	*Peridinium trochoideum*	X	X	X	X	X	X	X	X	X	X	X	X
	Bysmatrum									O				
	Bysmatrum subsalsum									O				
	Coolia						X			X				
+	*Coolia monotis*			X						O				
	Prorocentrum		X	X	X	X	X	X	X	X	X	X	X	X
	Prorocentrum cassubicum									O				
+	*Prorocentrum minimum*	*Exuviella minima*	X	X	X		X			X	X	X	X	
+	*Prorocentrum lima*	*Exuviella marina*	X	X	X		X	X	X	X	X	X	X	
	Prorocentrum micans		X	X	X	X	X	X	X	X	X	X	X	X
	Prorocentrum rhathymum									O				
	Oxyrrhis			X		X	X	X	X	X	X	X	X	
	Oxyrrhis marina			X		X	X	X	X	X	X	X	X	

Appendix 5: Data from the field samples from site 1:

Site 1

Date	Hour	Tide	Temperature	Salinity ‰	pH	Species more abundant in the net	Species more abundant in the filaments
11-10-01				25		Prorocentrum lima +++	
23-10-01	10h	Full	18	23		Chaetoceros sp., spherical ciliate, Cryptophyceae, small pennate diatoms, Entomoneis, small colourless Gymnodinium, Gymnodinium, Gyrodinium, Microcystis, Oscillatoria, Oxyrrhis, Prorocentrum lima, Prorocentrum cassubicum, Pyramimonas, Bysmatrum subsalsum, small round Dino with theca, large Dino with colour, Euglena sp..	
23-10-01	17h	Low	21.3	20		Cryptophyceae, pennate diatoms, small round and thecate dino, large Dino with colour, Entomoneis, Euglena sp., Gyrodinium, Oxyrrhis, Prorocentrum lima, Prorocentrum cassubicum, Pyramimonas, Bysmatrum subsalsum, Rhopalodia, Kryptoperidinium foliaceum.	
26-10-01	13:20h	Full	20.1	22		Prorocentrum lima, Prorocentrum cassubicum, Euglena, Microcystis, Chaetoceros spp., small diatoms.	
29-10-01	14:35	Full	21	20		Prorocentrum lima, Euglena, Microcystis, Chaetoceros spp., small diatoms.	
31-10-01	9:30h	Low	19	19		Prorocentrum lima, Euglena sp., Microcystis, Chaetoceros spp., small diatoms.	
31-10-01	Afternoon	Full	22,1	20		Prorocentrum lima, Prorocentrum cassubicum, Euglena, Microcystis, Chaetoceros spp., small diatoms.	
6-11-01	15h	Low	17	28		Prorocentrum lima, Euglena sp., Microcystis, Chaetoceros spp., small diatoms.	

13-11-01	14:50h	Full	14.0	29			
16-11-01	10:30h	Low	10.7	34		Centric ciliate, large Cryptomonas, cf. Polykrikos, Cylindrotheca, pennate diatom, centric diatom, Dino cell with theca, small round Dino with theca, Entomoneis, Euglena sp. (+++), Gyrodinium, Microcystis, Oxyrrhis (++), Pleurosigma, Prorocentrum cassubicum, Prorocentrum lima, Prorocentrum micans, Pyramimonas, Rhopalodia, Striatella unipunctata, colourless zooplankton.	
16-11-01	17h	Full	11.5	34		Oscillatoria, colourless Dino, Bysmatrum subsalsum, ciliate, big Cryptomonas, cf. Polykrikos, Cylindrotheca, pennate diatom, centric diatom, Dino with theca, small round dino with theca, Entomoneis, Euglena sp. (+++), Gyrodinium, Microcystis, Oxyrrhis (++), Prorocentrum cassubicum, Prorocentrum lima, Pyramimonas, Rhopalodia, Striatella unipunctata, zooplankton.	
26-11-01	16h	Low?	12,4	32			
29-11-01	9:45h		11	32,5		Prorocentrum lima.	
06-12-01	14:25h	Low	11.0	33		Centric ciliate, oval ciliate, big Cryptomonas, centric and pennate diatoms, small round dino with theca, Entomoneis, Euglena sp. (++++), Gyrodinium, Microcystis, Oxyrrhis, Pleurosigma sp., Prorocentrum cassubicum, Prorocentrum lima, Pyramimonas, Rhopalodia, Striatella unipunctata, zooplankton, Bysmatrum subsalsum.	
18-12-01		Low	6.5	36		Centric ciliate, oval ciliate, big Cryptomonas, pennate diatom, Dino with theca, large Dino with colour, small round Dino with theca, Euglena sp. (++++), Gyrodinium, Microcystis, Oxyrrhis (++), Prorocentrum cassubicum, Prorocentrum lima, Prorocentrum micans, Protoperidinium, Pyramimonas, Striatella	P. lima, P.cassubicum, Euglena, small Chaetoceros (+++), round Protoperidinium (+++), Cryptophyceae, Striatella, Oxyrrhis.

						unipunctata, Bysmatrum subsalsum.	
09-01-02	14:30h		10.7	29		cf. Polykrikos, centric ciliate, ciliate with tail and a ring of fine hairs, oval ciliate, large Cryptomonas, Cylindrotheca, pennate and centric diatoms, Dino with theca, big Dino with intense colour, small round Dino with theca, Euglena sp.(++++), Gyrodinium, Microcystis, Oxyrrhis (++), Prorocentrum cassubicum, Prorocentrum lima, Prorocentrum micans, B. subsalsum +++, Kryptoperidinium foliaceum Protoperidinium (++), Pyramimonas, Striatella unipunctata, zooplankton.	Prorocentrum cassubicum, Prorocentrum lima, Gyrodinium sp., Euglena sp., Cryptophyceae, Cylindrotheca, round Dino with theca and food inside, colourless Katodinium sp.
06-03-02	15h	Low	19.3 ºC	20	---	Prorocentrum sp. 2 (+++), Prorocentrum micans, Cryptomonas.	Prorocentrum sp. 2 (+++), Prorocentrum micans, Cryptomonas.
20-03-02	15h	± Low	23 ºC	9 a 13	---		
08-04-02	10:15h	Full	14.8 ºC	3 a 5	---		
17-04-02	11:15h	Low	16.8 ºC	17	---		
04-11-02	11:45h	Full	18.2 ºC	27	---	Gymnodinium cf. catenatum	Cryptomonas (+++), P. cassubicum, Prorocentrum sp. 2, Amphidinium cf. carterae, Heterocapsa, Planozigote of a Dino with theca, chloroplasts and stigma, Entomoneis, Chaetoceros (+++), Euglena, many small flagellates with plastids.
11-11-02	11h	± Full	17.8 ºC	20	---		Amphidinium, P. cassubicum, Cryptomonas, P. lima, Diatoms, Dino with theca and stigma similar with a Gyrodinium cell, Ciliates.
25-11-02	11:30h	Full	11.7 ºC	10	8.1	P. micans, Prorocentrum sp. 2, Ciliate, Entomoneis,	Many diatoms – Melosira, Pinnularia, Entomoneis, Gyrosigma; P. cassubicum.

						diatoms spp., Oscillatoria.	
04-12-02	15:30h	Full	15.9 ºC	15	8.7	Prorocentrum sp. 2, Entomoneis.	Cladophora, P. cassubicum, many diatoms (Navicula, Achnanthes, Nitzschia), Oscillatoria, pink Cyanophyta.
18-12-02			16.3 ºC	13	8.5	Prorocentrum sp. 2 (++), P. lima (+), P. cassubicum, P. micans, Cyst of a Dino with theca, theca of a Gyrodinium, small round Dino with plastids, colourless Protoperidinium (++), many diatoms (Rhopalodia), Kryptoperidinium foliaceum, Oscillatoria, Chlorophyta.	P. cassubicum, diatoms, Oscillatoria, Prorocentrum sp. 2.
14-01-03	13:30h	Full	8.8 ºC	6	9	Centric diatoms +, Oscillatoria, Euglena, P. cassubicum, Gyrosigma, Criptophyceae, Epithemia.	P. cassubicum, Diatoms +++, Oscillatoria, Entomoneis, Centric diatoms, P. lima (-), Gyrosigma, Euglena, Amphora, Microcystis sp., Ciliate, Navicula.
28-01-03	14h	Low	16.6 ºC	7	9.1	Cyclotella, Diatoms spp., Navicula, cysts of a Dino with two stigmas, Eunotia, Melosira, Ciliate, Cyanophyta sp., Entomoneis, Oscillatoria, Gyrosigma.	P. cassubicum, Diatom sp., Navicula, Eunotia (+++), Spirulina, Cryptomonas, Entomoneis, Navicula, colourless Katodinium with stigma, Nitzschia, Cyclotella, diatoms spp., Oscillatoria, Euglena, Nitzschia sp.
12-02-03	12h	Full	14.8ºC	9	---	Colourless Dino with theca, P. cassubicum, Oscillatoria, Euglena.	P. cassubicum, Amphora, Oscillatoria, Amphidinium sp.
20-02-03	14:30h		13.5 ºC	6	8.8	Colourless Dino – Peridiniopsis sp. Oscillatoria, Enthomoneis, Coolia sp., colourless Euglenophyta that eats, Euglena, Cryptomonas, Rhopalodia, Cysts of Dinoflagellates with plastids and stigma, Kryptoperidinium foliaceum,	P. cassubicum, Euglenophyta, Cryptomonas - Cryptophyceae, Amphora, Rhapolodia, Cladophora, Heliozoa.

						Protoperidinium (+++), Prorocentrum cassubicum.	
12-03-03	11:50h	Full	18.6 ºC	10	8.2	Protoperidinium (+++), Kryptoperidinium foliaceum, Prorocentrum cassubicum, Oscillatoria, Beggiatoa, Tetraselmis, Petalomonas (colourless Euglenophyta), Euglena, Entomoneis spp..	Prorocentrum cassubicum (+++), Oscillatoria, Ochromonas, Amphora (++), Pleurosigma, Spirulina, Tetraselmis, Entomoneis.
05-05-03	14:30h	Low?	23.5	17	---	P. cassubicum, Cryptomonas (+++), Rotifers, Ciliate, Entomoneis, Pleurosigma, Oscillatoria, Thecate Dino; Achnathes, Euglena, P. lima, Criptomonas, Microcystis sp.	Cladophora, Lyngbia, small diatoms, Achnanthes cf. coarctata.
19-05-03	14:45h		28	20		Small arthropod, Rotifer and Ostracops. Gomphospheria; Crypto?; Entomoneis; Gyrosigma, Bysmatrum subsalsum, P. cassubicum, Amphidinium cf. carterae, P. lima.	Cladophora, small flagellates, Amphora, Rhopalodia, Gyrodinium, Gyrosigma, Cryptos, P. cassubicum.
27-05-03	11:30h		24	29		Small arthropod +++, flagellates, Cocconeis, Amphidinium.	Cladophora, flagellates, Amphidinium cf. carterae, Cocconeis +++, cysts and small arthropod.
09-06-03	11:20		26	34		P.lima ++, Entomoneis, B. subsalsum, Gyrosigma.	Cladophora, Lyngbia, Euglenophyta, Amphidinium cf. carterae, Cryptomonas, B. subsalsum.
16-09-03			27,8	47	8.4	Eutreptiella, Euglena sp.; Prorocentrum sp. 1, Chaetoceros, Gyrodinium, B. subsalsum, Oscillatoria, Entomoneis, Gyrosigma, Nematoda, Oblea rotunda, Striatella, K. foliaceum.	Gyrodinium. Rhopalodia, P. cassubicum, Oxyrrhis marina.
26-09-03			21.1	41	7.8	Rhopalodia, P. cassubicum, small flagellates ++, B. subsalsum, Cylindrotheca closterium, small	Prorocentrum sp. 1, Gyrodinium ++, Rhopalodia, P. cassubicum, Oxyrrhis marina, Amphidinium cf. carterae,

						arthropod, Gyrodinium+, Gyrosigma, Oscillatoria, P. lima.	small arthropod.
30-09-03			20.9	35	7.9	Dino (round with theca, plastids and stigma), colourless Dino with theca, Oblea rotunda, Gyrodinium, Oxyrrhis marina, B. subsalsum, Cylindrotheca closterium, P. lima, Prorocentrum sp. 1, ciliates+, Crypto.	Large Gyrodinium, Prorocentrum sp. 1, Cylindrotheca closterium, Navicula, Rhopalodia, P. lima, P. cassubicum, Oscillatoria, Lyngbia, Amphidinium cf. carterae.
06-10-03	13:45		19,2	33	8.1	Chaetoceros spp., Gyrodinium +, Oscillatoria spp, Striatella, Gyrosigma, Oxyrrhis marina, Prorocentrum sp. 2, Rhopalodia, Entomoneis, small flagellates, P. cassubicum, Eutreptiella, Cylindrotheca closterium, Spirulina.	P. cassubicum +, flagellates, Cymbela, Oscillatoria, Euglenophyta (Entosiphon), Spirulina, Clorophyta, Gyrodinium, Rhopalodia, Prorocentrum sp. 1, Oxyrrhis marina, Entomoneis, P. lima, Achnanthes coarctata, Lyngbia.
13-10-03			20,4	33	8.1	Chaetoceros spp +++, Euglena, Prorocentrum sp. 2, Protoperidinium without spines, small Protoperidinium with 2 spines, Gyrodinium, Eutreptia sp. +, large colourless Dino with theca.	P. cassubicum, Lyngbia, Chroococcus, Gyrosigma, Oscillatoria, Prorocentrum sp. 1, Gyrodinium, Rhopalodia, Nematoda, Euglena, small diatoms, P. lima, Cladophora, large colourless Dino without spines.
20-11-03			14,1	22	7.9	P. micans, Euglena, Oscillatoria, Lyngbia, Oblea rotunda, Gyrosigma, Prorocentrum sp. 2, Chroococcus, Nematoda, Cladophora, Rhopalodia.	
15-12-03			13	15	8.0	Gyrosigma, Small diatoms, Navicula, Cylindrotheca closterium, Entomoneis, Oscillatoria, Lyngbia, Euglena sp, small arthropod ++, Rhopalodia,	Cladophora, Achnanthes cf. coarctata ++, P. cassubicum, Gyrosigma, Nematoda, small arthropod, Rhopalodia, Amphidinium sp., Euglena, Cylindrotheca closterium
10-02-04						Small arthropod +,	Cladophora, Ulva sp., small diatoms.
04-03-04			16.5	15	9.6	Round Dino with theca and	Enteromorpha, Cladophora, Oxyrrhis

						plastids, large Surirella, Gyrosigma.	marina, Ulva sp..
22-05-04			27.6	24		B. subsalsum +++, small arthropod.	Cladophora, Ulva sp..
29-07-04			25	50	8.7	Eutreptiella gymnastica +++, P. micans + , Gyrosigma, Oxyrrhis marina, B. subsalsum, colourless Gyrodinium with food, small arthropod +, Entomoneis.	Enteromorpha, Ulva sp., Eutreptiella gymnastica +++, P. micans, B. subsalsum, Gyrosigma, round Dino with theca (B. subsalsum), Oxyrrhis marina, colourless striate Gyrodinium with food, Pyramimonas.

Appendix 6: Data from the field samples from site 2

Site 2

Date	Hour	Tide	Temperature	Salinity ‰	pH	Species more abundant in the net	Species more abundant in the filaments
27-05-03	11:30						Cladophora, Ulva sp, P. lima, Achnanthes coarctata, small arthropod, flagellates, many small diatoms, Gyrosigma, Amphidinium cf. carterae, Cocconeis, Oxyrrhis marina, B. subsalsum, C. monotis, Cryptos +, Prorocentrum sp. 1
09-06-03	11		24	52		Entomoneis +, B. subsalsum, Gyrosigma, Oxyrrhis marina, Glenodinium sp., Prorocentrum sp. 1, Cylindroteca closterium, Gyrodinium, Achnanthes coarctata, C. monotis, P. micans.	Glenodinium +, B. subsalsum ++, Oxyrrhis marina +, Katodinium, Amphidinium cf. carterae, P. cassubicum, Cladophora, Striatella.
16-09-03			30,5	52	9.1		Prorocentrum sp. 1, Oxyrrhis marina, P. cassubicum, Prorocentrum sp. 2, B. subsalsum, large Gyrodinum, Protoperidinium, Chroococcus, Nematoda, Gyrosigma, small diatoms, Microcystis +, Entomoneis
19-09-03				57		B. subsalsum ++, Prorocentrum sp. 1, Heterocapsa, Gyrosigma,	B. subsalsum ++, Prorocentrum sp. 2 (++), P. cassubicum, Cymbela, Chroococcus, Nitzschia, Euglena sp.,

						Microcystis, large Navicula.	Gyrosigma, Gyrodinium, Microcystis, Heterocapsa, Navicula, Prorocentrum sp. 1, small diatoms +++, Amphora, Nematodes, Cylindrotheca closterium.
26-09-03			19,7	60	8	Oxyrrhis marina ++, B. subsalsum +, Glenodinium, Euglena sp, Gyrosigma, Heterocapsa, Prorocentrum sp. 2, Chroococcus, Snowella (Cyanophyta), Entomoneis, Prorocentrum sp. 1, large Gyrodinium.	Prorocentrum sp. 2 +++, Prorocentrum sp. 1 +, Chroococcus, Rhopalodia, small diatoms ++, Entomoneis, Gyrosigma, Snowella, P. lima, Gyrodinium, B. subsalsum, P. cassubicum, Glenodinium, Oxyrrhis marina.
30-09-03			20,9	35	8.1	S. trochoidea, P. micans, B. subsalsum, Cochlodinium, cf. Gymnodinium catenatum, Prorocentrum sp. 1, Ceratium fusus, Protoperidinium, colourless Amphidinium, Euglena sp.	Cladophora, Prorocentrum sp. 1, P. cassubicum +, Gyrodinium, B. subsalsum +, Prorocentrum sp. 2, Cymbela, Euglena sp., Spirulina.
06-10-03	13:45		19	38	8.1	Skeletonema, Chaetoceros spp., B. subsalsum, Protoperidinium minutum +, P. micans +, Oscillatoria, Rhizosolenia, S. trochoidea, Prorocentrum sp. 2, Gymnodinium, Polykricos (pheopolykricos).	Cladophora, large Gyrodinium, Chroococcus, P. cassubicum, Pinularia, Tetraselmis (Clorophyta), Beggiatoa (sulphurous Cyanophyta), Cryptos, Prorocentrum sp. 2, Oscillatoria, Lingbya, small flagellates.
13-10-03			19,8	39	8,1	Prorocentrum sp. 1, small round Dino with plastids, Protoperidinium (two small spines) +, Scrippsiella cf. trochoidea +, P. minimum, Oscillatoria, Protoperidium minimum?, B. subsalsum,	Cladophora, Oxyrrhis marina, Prorocentrum sp. 2, Prorocentrum sp. 1, Oscillatoria spp, C. monotis, P. cassubicum, Entomoneis, P. micans,

						Gyrodinium, P. micans, Chroococcus, Chaetoceros, K. foliaceum, Oxyrrhis marina, Protoperidinium (without spines), P. cassubicum, P. lima, Euglenophyta, Amphidinium cf. carterae.	Chroococcus, Small diatoms, B. subsalsum, Gyrosigma, P. lima, Gyrodinium.
20-11-03			16.3	41	8.4	Heterocapsa ++, P. micans, cf. gyrodinium small, Chrysochromulina, large Gynodinium, Prorocentrum sp. 1, Entomoneis, Chroococcus, P. cf. minimum, P. cassubicum, Lingbya, P. lima, Euglena sp.	P. cassubicum, Heterocapsa, Prorocentrum sp. 1, Cryptomonas, Amphidinium cf. carterae, Lingbya, large Gyrodinium, Euglena sp., Chroococcus, P. micans, P. lima, Oxyrrhis marina, Entomoneis.
15-12-03			12,3	33	8.6	Prorocentrum sp. 1, Gyrosigma, P. cf. minimum ++, Oscillatoria, Lingbya, P. cassubicum, Gyrodinium, Entomoneis, large Amphidinium, Cryptos, P. lima, Amphidinium cf. carterae, C. monotis, Euglena sp., Cylindrotheca closterium, Eutreptiella, Chroococcus, Striatella unipunctata, small colourless Gyrodinium, P. micans.	P. lima, large Gyrodinium, many small flagellates, P. cassubicum, C. monotis, P. cf. minimum, Heterocapsa, Amphidinium cf. carterae, Chroococcus, Gyrosigma.
6-01-04			13.8	32	8.2	Oxyrrhis marina, Lingbya, Entomoneis, Cylindrotheca closterium, Gyrosigma (Pleurosigma angulatum) Gyrodinium large, Euglena sp., small arthropod, Prorocentrum sp. 1, P. micans.	Cladophora, P. lima, Amphidinium cf. carterae, P. cassubicum, Pleurosigma angulatum, Prorocentrum sp. 1, small arthropod, Euglena sp., Achnanthes coarctata, O. marina, Lingbya, large Gyrodinium, colourless Katodinium, Beggiatoa, small flagellates, Cylindrotheca closterium, P. lima.
10-02-04						P. micans +, Cylindrotheca closterium, P. cassubicum, small arthropod,	P. lima, P. micans, pennate diatom, S. trochoidea,

						Entomoneis, Pleurosigma angulatum, P. lima, Cymbela, S. trochoidea, Prorocentrum sp. 1.	Cyanophyta with 8 cells, Achnanthes coarctata.
04-03-04			17,4	31	9.3	P. micans +++, P. lima +, P. cf. minimum, Prorocentrum sp. 1 +, P. rhathymum, Entomoneis, Pleurosigma angulatum, Cymbela, C. monotis, small arthropod and nematoda.	Amphidinium cf. carterae, Oxyrrhis marina, P. lima, colourless Gymnodinium, Chrysochromulina +++, C. monotis, large Gymnodinium, many small diatoms, small arthropod, Striatella unipunctata.
22-05-04	13		29,2	40		B. subsalsum +++++, Pleurosigma angulatum, Fragilaria, Prorocentrum sp. 1, small arthropod.	Chroococcus, Nematoda, Pleurosigma angulatum, Lingbya, P. cassubicum, Oscillatoria, B. subsalsum+, small arthropod.
29-07-04			24.4	48	8.5	C. monotis, Striatella unipunctata, B. subsalsum, Pleurosigma angulatum, Prorocentrum sp. 1, large Gyrodinium, Entomoneis, Navicula, Oxyrrhis marina.	Ulva sp., Cladophora, Achnanthes coarctata, Navicula, Chroococcus, Oxyrrhis marina, Amphidinium, Pleurosigma angulata, Cryptos, Cylindrotheca closterium, Biddulphia, C. monotis, small arthropod.

Appendix 7: Species of dinoflagellates found in Site 1

Temperature ºC	18	21,3	20,1	21	19	17	14	10,7	12,4	11	11	6,5	10,7	19,3	23	14,8	16,8	18,2	17,8	11,7	15,9	16,3
Salinity (‰)	23	20	22	20	19	28	29	34	32	32,5	33	36	29	20	11	4	17	27	20	10	15	13
pH																				8,1	8,7	8,5
Species \ Date	11-10-01	23-10-01	26-10-01	29-10-01	31-10-01	06-11-01	13-11-01	16-11-01	26-11-01	29-11-01	06-12-01	18-12-01	09-01-02	06-03-02	20-03-02	08-04-02	17-04-02	04-11-02	11-11-02	25-11-02	04-12-02	18-12-02
Prorocentrum lima	++	++	+	+	+	+		+		+	+	+	+						+			+
Prorocentrum cassubicum		+	+		+	+		+			+	+	+					+	+	+	+	+
Prorocentrum micans						+		+			+	+	+	+						+		+
Prorocentrum sp. 2														+++				+		+	+	++
Prorocentrum minimum														+								
Prorocentrum rhathymum																						
Prorocentrum sp. 1																						
Oxyrrhis marina		+						+			+	++	+									
Bysmatrum subsalsum		+			+	+		+			+	+	+++									

Kryptoperidinium foliaceum		+			+	++						+	+								+
Protoperidinium minutum						+					+	+	+								
Coolia monotis																					
cf. *Polykrikos*								+													
Amphidinium massartii		+																+	+		
cf. *Glenodium*																					
cf. *Gymnodinium*		+										+									
Gymnodinium cf. *catenatum*																		+	+		
cf. *Gyrodinium*		+						+			+		+								+
Oblea rotunda												++	++								++
cf. *Katodinium*													+								
Akashiwo sanguineum																					
Scrippsiella trochoidea																					
Heterocapsa niei																		+			

Temperature ºC	8,8	16,6	14,8	13,5	18,6	23,5	28	24	26	27,8	21,2	20,9	19,2	20,4	14,1	13	?	16,5	27,6	24
Salinity (‰)	6	7	9	6	10	17	20	29	34	47	41	35	33	33	22	15	?	15	24	50
pH	9	9,1		8,8	8,2					8,4	7,8	7,9	8,1	8,1	7,9	8,0	?	9,6		8,7
Species \ Date	14-01-03	28-01-03	12-02-03	20-02-03	12-03-03	05-05-03	19-05-03	27-05-03	09-06-03	16-09-03	26-09-03	30-09-03	06-10-03	13-10-03	20-11-03	15-12-03	10-02-04	04-03-04	22-05-04	29-07-04
Prorocentrum lima	+					+			++		+	+	+	+						
Prorocentrum cassubicum	+	+	+	+	++	+	+			+	+	+	+	+		+				
Prorocentrum micans															+					++
Prorocentrum sp. 2													+	+	+					
Prorocentrum minimum															+					
Prorocentrum rhathymum																				
Prorocentrum sp. 1										+	+	+	+	+						
Oxyrrhis marina.										+	+	+	+					+		+
Bysmatrum subsalsum						+			+	+	+	+							+++	+

Kryptoperidinium foliaceum				+	+					+									
Protoperidinium minutum														+					
Coolia monotis				+															+
cf. *Polykrikos*																			
Amphidinium massartii						+		+	+		+	+				+			
cf. *Glenodium*			+																
cf. *Gymnodinium*																			
Gymnodinium cf. *catenatum*																			
cf. *Gyrodinium*							+						+	+					+
Oblea rotunda				++	++					+		+		+	+				
cf. *Katodinium*																			
Akashiwo sanguineum										+	+	+							
Scrippsiella trochoidea																	+		
Heterocapsa niei																			

Appendix 8: Species of dinoflagellates found in Site 2

Temperature ºC		24	30,5		19,7	20,9	19	19,8	16,3	12,3	13,8	?	17,4	29,2	24,4
Salinity (‰)		52	52	57	60	35	38	39	41	33	32	?	31	40	48
pH			9,1		8	8,1	8,1	8,1	8,4	8,6	8,2	?	9,3		8,5
Species \ Date	27-05-03	09-06-03	16-09-03	19-09-03	26-09-03	30-09-03	06-10-03	13-10-03	20-11-03	15-12-03	06-01-04	10-02-04	04-03-04	22-05-04	29-07-04
Prorocentrum lima	+	+			+			+	+	+	+	+	+		+
Prorocentrum cassubicum	+	+	+	+	+	+	+	+	+	+	+	+		+	
Prorocentrum micans		+				+	+	+	+	+	+	+	+++		
Prorocentrum sp. 2			+	++	+++	+	+	+	+	+			+		
Prorocentrum minimum															
Prorocentrum rhathymum													+		
Prorocentrum sp. 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxyrrhis marina.	+	+	+		++			+	+		+		+		+
Bysmatrum subsalsum	+	++	+	++	+	+	+							+++	+
Kryptoperidinium foliaceum								+							
Protoperidinium minutum						+	+	+							

Coolia monotis	+	+								+			+		+
cf. *Polykrikos*							+								
Amphidinium massartii	+	+				+		+	+	+	+		+		+
cf. *Glenodium*		+			+										
cf. *Gymnodinium*													+		
Gymnodinium cf. *catenatum*						+									
cf. *Gyrodinium*		+		+		+		+	+	+					+
Oblea rotunda			+												
cf. *Katodinium*		+									+				
Akashiwo sanguineum			+		+		+	+	+		+		+		
Scrippsiella trochoidea							+	+				+			
Heterocapsa niei				+	+				++	+					
Ceratium fusus						+									

Appendix 9: Morphometric measurements of some cells from different species in culture

Prorocentrum cassubicum

Nº	Length (μm)	Width (μm)	Ratio
1	28,1	19,0	1,48
2	28,0	19,0	1,47
3	27,0	18,2	1,48
4	27,0	17,4	1,55
5	26,8	17,3	1,55
6	27,4	17,9	1,53
7	27,1	17,9	1,51
8	29,3	19,0	1,54
9	28,8	18,5	1,56
10	28,5	18,3	1,56
11	28,8	18,9	1,52
12	27,1	18,8	1,44
13	28,6	18,3	1,56
14	27,3	17,8	1,53
15	28,1	18,6	1,51
16	28,3	18,6	1,52
17	26,7	18,5	1,45
18	28,0	17,9	1,57
19	26,7	17,0	1,57
20	28,9	19,2	1,50
21	27,1	16,6	1,63
22	26,1	16,2	1,62
23	26,9	17,3	1,56
24	26,3	15,9	1,66
25	27,8	16,4	1,70
26	26,8	18,6	1,44

Average	27,59	17,95	1,54
Deviation	0,876	0,951	0,063
Max	29,26	19,20	1,70
Min	26,13	15,88	1,44
Nº cells	26	26	26

Prorocentrum lima

Nº	Length (μm)	Width (μm)	Ratio
1	45,3		
2	42,3	20,9	2,03
3	50,9	24,7	2,06
4	48,7	31,7	1,54
5	45,2	29,7	1,52
6		25,5	
7	37,7	23,2	1,62
8	39,7	24,5	1,62
9	45,1	27,1	1,66
10	44,8	26,5	1,69
11	45,1	27,4	1,65
12	42,1	27,0	1,56
13	44,8	27,6	1,62
14	45,2	26,9	1,68
15	46,2	25,8	1,79
16	45,5	29,9	1,52
17	45,2	29,8	1,52
18	45,3	28,0	1,62
19	42,6	25,2	1,69
20	43,9	27,8	1,58
21	43,3	26,7	1,62
22	39,8	26,3	1,51
23	41,6	28,0	1,49
24	44,1	26,8	1,64
25	46,4	28,6	1,62
26	46,1	29,4	1,57
27	43,0	28,1	1,53
28	47,8	28,3	1,69
29	45,4	27,9	1,63
30	41,3	27,3	1,51
31	43,5	27,1	1,61
32	45,2	25,5	1,77

Average	44,29	27,07	1,64
Deviation	2,663	2,113	0,134
Max	50,91	31,71	2,06
Min	37,69	20,87	1,49
Nº cells	31	31	30

Prorocentrum sp. 2

Nº	Length (μm)	Width (μm)	Ratio
1	18,6	15,9	1,17
2	17,3	14,1	1,22
3	17,5	14,8	1,18
4	16,3	13,6	1,20
5	19,4	14,3	1,36
6	17,8	14,7	1,21
7	17,8	14,5	1,23
8	18,3	15,9	1,15
9	17,4	15,4	1,13
10	18,3	14,6	1,25
11	15,9	13,7	1,16
12	17,2	14,8	1,16
13	17,4	14,9	1,17
14	16,8	14,0	1,20
15	15,9	13,7	1,16
16	18,9	16,5	1,14
17	17,7	14,8	1,20
18	17,6	14,1	1,25
19	16,8	14,0	1,20
20	17,4	14,3	1,21
21	17,3	13,9	1,25
22	17,7	14,9	1,18
23	16,5	11,7	1,41
24	18,8	16,0	1,18
25	17,1	14,8	1,15
26	15,2	10,6	1,42
27	16,6	14,2	1,17
28	16,9	14,4	1,18
29	17,7	15,4	1,15
30	16,7	12,2	1,37
31	17,8	14,9	1,19
32	18,7	15,9	1,18
33	16,4	13,0	1,26
34	15,8	13,8	1,15
35	15,5	12,4	1,25
36	16,6	14,9	1,12

Average	17,26	14,32	1,21
Deviation	0,993	1,216	0,074
Max	19,44	16,50	1,424
Min	15,15	10,64	1,116
Nº cells	36	36	36

Prorocentrum sp. 1

Nº	Length (μm)	Width (μm)	Ratio
1	47,6		
2	45,0		
3	41,1		
4	41,2		
5	47,9	37,5	1,28
6	47,4	39,8	1,19
7	44,6	38,4	1,16
8	44,8	37,6	1,19
9	45,5	31,2	1,46
10	50,1	40,2	1,25
11	44,8	36,4	1,23
12	49,6	39,9	1,24
13	53,6	43,2	1,24
14	47,4	40,0	1,19
15	49,0	39,7	1,24

Average	46,65	38,53	1,24
Deviation	3,32	3,04	0,08
Max	53,55	43,21	1,460
Min	41,05	31,16	1,164
Nº cells	15	11	11

Prorocentrum rhathymum

Nº	Length (μm)	Width (μm)	Ratio
1	34,1	23,3	1,47
2	34,5	21,6	1,59
3	33,9	21,6	1,57
4	31,8	18,3	1,74
5	34,2	22,1	1,55
6	34,3	21,9	1,57
7	29,3	19,3	1,52
8	31,7	18,9	1,68
9	32,9	21,7	1,52
10	32,7	21,7	1,51
11	30,5	22,3	1,36
12	32,2	21,8	1,48
13	33,3	24,8	1,34
14	36,7	28,2	1,30
15	32,5	22,6	1,44
16	32,4	21,6	1,50
17	32,2	20,7	1,55
18	30,8	19,4	1,59
19	30,4	19,9	1,52
20	34,5	24,5	1,41

Average	32,74	21,81	1,510
Deviation	1,756	2,259	0,106
Max	36,67	28,19	1,738
Min	29,34	18,29	1,301
Nº cells	20	20	20

Prorocentrum micans

Nº	Length (μm)	Width (μm)	Ratio
1	38,8	27,0	1,44
2	39,3	29,7	1,32
3	42,4	28,5	1,49
4	43,6	32,9	1,33
5	42,9	31,0	1,39
6	43,3	28,6	1,52
7	44,5	29,3	1,52
8	45,1	29,3	1,54
9	34,3	24,4	1,41
10	34,1	24,6	1,39
11	40,4	30,3	1,33
12	43,2	33,1	1,31
13	41,8	30,3	1,38
14	41,6	30,0	1,39

Average	41,10	29,20	1,410
Deviation	3,435	2,567	0,079
Max	45,12	33,11	1,541
Min	34,08	24,39	1,306
Nº cells	14	14	14

Akashiwo sanguinea

Nº	Length (μm)	Width (μm)	Ratio
1	50,9	47,5	1,07
2	54,2	44,8	1,21
3	49,9	39,7	1,26
4	50,1	44,9	1,12
5	50,3	43,8	1,15
6	50,5	46,3	1,09
7	50,8	44,7	1,14
8	55,8	41,9	1,33
9	55,2	45,0	1,23
10	52,7	44,1	1,20
11	53,0	44,7	1,19
12	57,0	46,4	1,23
13	57,1	46,1	1,24

Average	52,9	44,6	1,188
Deviation	2,699	2,020	0,073
Max	57,08	47,47	1,332
Min	49,91	39,69	1,073
Nº cells	13	13	13

Coolia monotis

Nº	Length (μm)	Width (μm)	Ratio
1	49,4	45,2	1,09
2	48,5	47,6	1,02
3	31,3	31,4	1,00
4	33,6	28,9	1,16
5	45,5	41,6	1,09
6	45,1	39,4	1,15
7	42,3	37,1	1,14
8	37,9	31,9	1,19
9	36,6	35,9	1,02
10	31,1	27,8	1,12
11	30,5	26,4	1,15
12	35,2	34,3	1,03
13	28,5	26,4	1,08
14	28,5	26,7	1,07
15	32,9	28,1	1,17
16	33,5	29,1	1,15

Average	36,9	33,6	1,102
Deviation	7,083	6,887	0,062
Max	49,40	47,64	1,190
Min	28,50	26,42	0,997
Nº cells	16	16	16

Heterocapsa niei

Nº	Length (μm)	Width (μm)	Ratio
1	21,2	15,6	1,36
2	19,9	14,2	1,40
3	22,9	14,3	1,60

Average	21,3	14,7	1,454
Deviation	1,511	0,760	0,127
Max	22,91	15,57	1,599
Min	19,9	14,19	1,360
Nº cells	3	3	3

Amphidinium massartii

Nº	Length (μm)	Width (μm)	Ratio
1	21,1	16,3	1,30
2	20,3	13,2	1,53
3	20,0	15,2	1,31
4	24,8	21,3	1,17
5	25,9	22,5	1,15
6	22,6	18,4	1,23
7	22,3	16,9	1,33
8	16,1	11,6	1,39
9	16,5	12,5	1,33
10	17,0	12,8	1,33

Average	20,7	16,1	1,306
Deviation	3,405	3,767	0,110
Max	25,93	22,53	1,531
Min	16,06	11,55	1,151
Nº cells	10	10	10