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PHYTOPLANKTON DYNAMIC IN PERMANENT AND TEMPORARY WATERS OF EMPORDÀ SALT MARSHES (NE SPAIN)

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A Carlos, Pati y Omar, espero que
nada vuelva a robarme vuestro
tiempo.

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Resumen (*in Spanish*)

Se ha estudiado la dinámica del fitoplancton en las lagunas costeras de Aiguamolls de l'Empordà. Estas lagunas son un ejemplo de laguna mediterránea confinada. Son sistemas que se inundan esporádicamente durante los temporales marinos, que aun siendo más frecuentes en otoño y primavera, distan de seguir un patrón estacional. El resto del año las lagunas permanecen aisladas, lo que las lleva a un proceso de confinamiento, que se acentúa al final del verano. Esta falta de estacionalidad repercute directamente en la dinámica de la comunidad fitoplanctónica, de la que poco se conoce. En este estudio se abordan diferentes aspectos de la dinámica fitoplanctónica, que quedan recogidos en tres capítulos.

En el primer capítulo (IV) se estudia la evolución temporal, a lo largo de un ciclo anual, de los diferentes grupos funcionales del fitoplancton, mediante su composición pigmentaria. Los organismos del fitoplancton se agrupan en clases según su similitud en la composición de pigmentos. Así, la composición pigmentaria nos da información exclusiva de cada una de las clases fitoplanctónicas y nos permite estudiar su evolución frente a los diversos factores bióticos o abióticos del medio. Al mismo tiempo, también se han observado los cambios que los factores ambientales producen en la composición de tamaños del fitoplancton. Los resultados muestran que el fitoplancton, en estos ambientes, esta sujeto principalmente al control "bottom-up" y que la variabilidad hidrológica y la disponibilidad de nutrientes tienen una mayor influencia en la composición y distribución de tamaños del fitoplancton, que el zooplancton. Así, en estos sistemas donde la tasa de desnitrificación es muy elevada, la baja disponibilidad de nitrógeno inorgánico es el factor limitante de la producción estrictamente autótrofa. Se ha observado cómo la composición del fitoplancton cambia a lo largo de un gradiente relacionado con la ratio nitrógeno inorgánico: nitrógeno total, en función de las diferencias fisiológicas de cada organismo, en cuanto a su capacidad para obtener el nitrógeno del medio. En los momentos en que el confinamiento es más pronunciado, al final del verano, los nutrientes están mayoritariamente en forma orgánica y los dinoflagelados parecen sacar ventaja de su capacidad mixótrofa, compitiendo y depredando, al mismo tiempo, sobre el fitoplancton de menor tamaño.

En el segundo capítulo (V) se analizan las interacciones entre bacterio- y fitoplancton y los factores que favorecen su dominancia. Estas interacciones se estudian en dos lagunas temporales donde el efecto de la escorrentía durante las épocas de inundación es más importante. Se estudia, en especial, el efecto que los aportes de materia orgánica alóctona tienen sobre la comunidad microbiana. Para detectar los cambios a corto plazo del bacterio- y fitoplancton, se lleva a cabo un monitoreo intensivo a dos profundidades y en dos momentos del día, a la salida y a la puesta del sol. De los resultados se extrae que la concentración de materia orgánica disuelta es el factor ambiental más correlacionado con el crecimiento de la biomasa fitoplanctónica y que el papel de los nutrientes disueltos es secundario. Las diferencias observadas entre lagunas en cuanto a la dominancia del bacterio- o fitoplancton, están relacionadas indirectamente con la ratio cuenca de recepción: área de inundación, la cual explica la acumulación diferencial de materia orgánica y nutrientes entre las lagunas. Se dan muchas situaciones que van desde una relación positiva entre las biomasas del bacterio- y del fitoplancton, que sugiere una cierta relación sinérgica entre ellos, hasta una relación negativa, que probablemente es fruto de una intensa actividad fagotrófica por parte del fitoplancton.

Finalmente en el tercer capítulo (VI y VII), dada la proximidad entre las lagunas costeras y el mar, donde la ocurrencia de PANs (Proliferaciones de Algas Nocivas, equivalente en inglés, HABs) es cada vez más frecuente, se estudia el posible desarrollo de estas proliferaciones en los ambientes lagunares confinados. En primer lugar, se realiza un inventario general de las especies más abundantes de fitoplancton en Aiguamolls de l'Empordà (VI). Las muestras proceden de lagunas donde se alcanzan altas concentraciones de biomasa fitoplanctónica en los periodos estivales, y puntualmente de lagunas donde se han dado episodios de mortalidad de peces. A continuación, se llevan a cabo análisis extensivos de la toxicidad, mediante Microtox, y los resultados positivos se relacionan con la presencia de especies nocivas y con los factores ambientales que pueden favorecer su desarrollo. Se observa que la toxicidad se da en verano, coincidiendo con altas temperaturas y alta biomasa de bacterias y materia orgánica. Además la toxicidad se da solo en la fracción particulada y se descarta que sea debida a los metales pesados. Los episodios de toxicidad coinciden con elevadas biomasas de fitoplancton, aunque dado el carácter pulsátil de las

proliferaciones, resulta difícil establecer una relación directa entre ellos. Así, los resultados sugieren, que a pesar de que Microtox es un buen método para realizar monitoreos extensivos de toxicidad en sistemas acuáticos lagunares, la información que proporciona debe contrastarse con otras herramientas moleculares y taxonómicas.

En la segunda parte de este capítulo (VII), se hace un esfuerzo en la clasificación taxonómica de los dinoflagelados, que permite la comparación de la composición y dinámica de este grupo taxonómico entre las lagunas costeras y el agua del mar. Esta parte se centra principalmente en la comparación de las especies de dinoflagelados tóxicos y formadores de mareas rojas y la posibilidad de que se produzca intercambio de organismos entre los dos medios acuáticos. Los resultados permiten afirmar, que a pesar de la falta de un ciclo estacional concreto en las lagunas, las biomasas máximas de diatomeas y dinoflagelados se alcanzan de forma alternada, al igual que ocurre en el mar y como describe el modelo de la mandala de Margalef (Margalef, 1983). La mayoría de especies de dinoflagelados encontradas son responsables de desarrollo de altas biomasas o de la secreción de toxinas. Se encuentran pocas especies en común entre el mar y las lagunas. Los blooms algales en las lagunas, en su mayoría son producidos por especies que en las aguas marinas más cercanas no producen proliferaciones. Así la expansión de los PANs provocados por especies marinas parece poco probable en las lagunas, ya que la afinidad de cada una de las especies a los dos ecosistemas parece ser muy distinta. Sin embargo, existen especies productoras de PANs características de los ambientes lagunares, de manera que se hace necesaria una gestión individualizada de las marismas, que empiece por controlar la eutrofización progresiva de estas y en concreto las entradas de nitrógeno, ya que se trata de un factor determinante en el desarrollo de las PANs.

I. General introduction

General introduction

1. The phytoplankton in Mediterranean coastal marshes

Mediterranean coastal marshes are examples of fluctuating systems and are recognised for their ecological and economic value. Over the last decades several Mediterranean coastal marshes have disappeared because they have been dried up. Most of the rest of the marshes are overexploited and degraded, and few are protected. The hydrological regimes of coastal marshes have been subject to man-made changes which have lead to a progressive process of eutrophycation and to a loss in ecological value (Britton and Crivelli, 1993). Mediterranean coastal lagoons can be deltaic, connected with the sea, or confined, when only sporadic fresh and marine water exchanges occur.

The deltaic marshes and lagoons are influenced by freshwater. In most of these areas traditional rice crop land use has lead to the freshwater flooding period being artificially prolonged, and the phytoplankton composition and dynamics is related strongly with this altered hydrological regime (Comín, 1984). In the Delta de l'Ebre (Tarragona, Spain), human changes have resulted in a shift in the phytoplankton composition due to the change from a sea-water dominated phase to a fresh water-dominated phase (Comín and Valiela, 1993). In the Albufera of Valencia (Spain) the anthropic alteration of the hydrological cycle, due to the fresh water input from the rice crop, have lead to a larger number of phytoplankton assemblages than is normally expected for polymictic lakes (Romo and Miracle, 1995). In the Ter Vell, an eutrophic lagoon in Empordà wetlands (Girona, Spain), two main periods can be distinguished related to the temperature and the fresh water and sea water inflows, which control the differences in the phytoplankton community observed there (Quintana and Comín, 1989).

In a similar way, the coastal salt marshes and lagoons that have a continuous connection with the sea are expected to have a high marine water influence. Consequently, the phytoplankton composition is expected to be essentially marine (Britton and Crivelli, 1993). Nevertheless, human land use has altered the hydrological cycles and consequently, the phytoplankton composition has adapted to more eutrophic habitats. In the case of the Mar Menor (Murcia,

Spain), the low autotroph: heterotroph ratio was related with the importance of the microbial loop in this eutrophic lagoon (Gilabert, 2001). In the lagoon area of Comacchio (NE Adriatic, Italy), eutrophication was enhanced by the intensive fish farms in the late 1970s leading to a reduction in animal compartment of the food web, as a result of cyanobacterial blooms (Sorokin *et al.*, 1996). Likewise, Boutière *et al.* (1982) related the massive proliferation of *Nannochloris* sp. in l'étang de Salces-Leucate (Perpignan, France) during a dystrophic crisis with the mineralisation of the allochthonous anthropogenic supplies of organic matter.

In general, all the authors who worked on the cited Mediterranean lagoons, both sea or river influenced, all stated that anthropic changes have a big effect on the hydrological pattern, with regards to the increasing level of eutrophy of the lagoons. Furthermore, in the main cases the authors found that both factors, hydrological seasonal patterns and the increase in eutrophy, determined the phytoplankton composition.

Confined Mediterranean marshes and lagoons are ecosystems with an unusual hydrologic regime. These ecosystems do not have a continuous input of fresh or marine water and are less affected by human activities since they have less economical value. They are very affected by sudden sea water inputs, which even though they are more probable in autumn and spring are far from being seasonal (Quintana *et al.*, 1998a; Brucet *et al.*, 2005; Gascón *et al.*, in press). These ecosystems are isolated for most of the year, but the isolation becomes extreme during the summer months when the high evaporation rate causes the concentration of organic matter, nutrients and organisms.

The confined lagoons in the Empordà salt marshes are an example of these ecosystems where data about the phytoplankton community is available (Domínguez, 1987; Comín *et al.*, 1994). However, little is known about the phytoplankton dynamics in these marshes where the natural hydrological disturbances seem to affect the plankton community more than the anthropogenically induced seasonal changes.

Innovative and faster monitoring techniques, which, on the other hand, are well established in marine water research, are needed to allow us to obtain information about the community dynamics quickly in order to asses the preservation of the ecological value of these highly variable ecosystems. Some of

these techniques are: studying microbial loop and the microbial food web interactions using flow cytometry (Gasol *et al.*, 1991; Garcia *et al.*, 1994; Tarran *et al.*, 1999; Moreira-turcq *et al.*, 2001; DuRand *et al.*, 2001; Gasol *et al.*, 2004); using chromatography to assess the pigment composition of the phytoplankton community (Mackey *et al.*, 1996; Lemaire *et al.*, 2002; Lohrenz *et al.*, 2003; Ayadi *et al.*, 2004) and using the biomass-size spectra as a tool to evaluate the environmental factors affecting the size distribution of the phytoplankton community (Sprules and Goyke, 1994; Cottingham, 1999; Cavender-Bares *et al.*, 2001; Quintana *et al.*, 2002; Yamaguchi *et al.*, 2002; Rodríguez *et al.*, 2002). Thus, the traditional taxonomic approach is being supplied by modern methodologies (Macedo *et al.*, 2001), which probably will be also more appropriated in Mediterranean lagoons, where (Fathi *et al.*, 2001; Ayadi *et al.*, 2004)the hydrological variability is high and the response time of the phytoplankton is short.

Regarding the phytoplankton dynamics, the existing information on phytoplankton communities in marine and freshwater ecosystems needs to be adapted in order to support the research on confined Mediterranean lagoons. For example, it is known that phytoplankton succession can be determined by following Margalef's mandala (Margalef, 1983), Reynolds *et al.* (2002), and Smayda and Reynolds (2003), according to two main factors: turbulence and nutrient levels. Nevertheless, little is known about the factors determining phytoplankton temporal patterns in confined Mediterranean lagoons, where the hydrology is highly variable and the isolation process means that the phytoplankton community lives in extremely confined conditions.

2. The Empordà wetlands

Some studies have been performed in the Empordà wetlands, which have provided detailed information about the ecological functioning of these ecosystems, especially in reference to the hydrologic fluctuations, the eutrophy process and the structure of the high trophic levels of the planktonic community.

In the Empordà salt marshes, water intrusions into the lagoons are mainly due to sudden, large sea storms, but also to intense rains. These flood situations alternate with long periods of confinement without water intrusions, especially

during summer or winter anticyclones, when the lagoons progressively dry up. These special hydrological characteristics have been found to determine the structure and functioning of the aquatic community (Quintana *et al.*, 1998b; Trobajo *et al.*, 2002), the size structure of the community (Quintana *et al.*, 2002; Brucet, 2003; Gascón, 2003; Boix *et al.*, 2004) and the species composition (Gifre *et al.*, 2002; Trobajo *et al.*, 2004). Over the last decades tourism pressure has increased in this area, where traditionally agriculture was the main land use. Therefore, the eutrophy caused by the temporary (1995-98) regulation of the fresh water flow and its consequences (Quintana *et al.*, 1998a), and diffuse contamination by nutrients and pollutants (López-Flores *et al.*, 2003) are the main factors that have caused ecological loss in the Empordà wetlands.

The Empordà salt marshes are an example of a fluctuating Mediterranean environment, which brings up several questions about the phytoplankton dynamics. Even though there is no seasonal pattern, the Empordà wetland hydrology is determined by recurring phenomena, such as flooding due to sea storms and confinement due to lagoon isolation, which occur every year. The specific phytoplankton composition depends greatly on these factors. Some lagoons, both permanent and temporary, are completely confined during summer, when the extreme environmental conditions probably determine phytoplankton composition, dynamics and trophic strategies.

Allochthonous inputs are an important source of nutrients in the lagoons. Thus, differences in catchment areas between lagoons would be a significant factor for the microbial trophic web short-term dynamic. Moreover, the Empordà salt marshes show a lot of environmental variation in short periods of time and space, therefore intensive monitoring of lagoons will provide data about the short-term response of the microbial community.

3. The importance of harmful phytoplankton species

Studying potentially harmful algae has become very important over the last decades due to the economic and biological losses resulting from the proliferation of toxic algal blooms in coastal ecosystems. This global increase has also been detected in the Mediterranean where the bloom events are mainly associated with water bodies with restricted water exchange, such as harbours

(Vila *et al.*, 2001a), small bays and protected beaches (Garcés *et al.*, 1999), and coastal lagoons (Comín and Ferrer, 1978; Sarno *et al.*, 1993; Cabrini *et al.*, 1995; Giacobbe *et al.*, 2000). Harmful algal blooms (HABs) are either toxic or have other deteriorating effects, such as causing oxygen deficiency. In either case, they can harm marine ecosystems and resources such as tourism, fishing and aquaculture. The need to study HABs is especially urgent in aquatic ecosystems where endangered species are present or, in general, in any aquatic ecosystem subject to a restoration program or to any type of management. While research on marine HABs has been improved, nowadays little is known about HAB dynamics and composition in Mediterranean coastal salt marshes.

Salt marshes are vulnerable systems that are usually affected by anthropogenic activities. They are very interesting both in terms of nature and science since they are the habitat for a large number of species, several of which are endangered, e.g. *Aphanius iberus* (Quintana *et al.*, 2001). Thus, in order to assess the management of HABs in confined marshes, initial extensive monitoring of the water toxicity in the salt marsh basins and lagoons is required. Due to their hydrological characteristics, Mediterranean coastal lagoons can accumulate neritic phytoplankton, therefore increasing the phytoplankton density. Since confinement in coastal lagoons may concentrate phytoplankton, and sporadic connection with the marine coastal waters can occur, it would be expected that the same harmful species found in marine waters could be found in the lagoons.

4. Study approach

The phytoplankton dynamics will be investigated in Empordà salt marshes as an example of a confined salt marsh. This main objective is divided into three chapters:

In the first chapter, the evolution of the different phytoplankton functional groups during the annual cycle will be analysed by studying the pigment composition of the community. The pigment composition approach provides data about the phytoplankton classes that make up the community, and their relation with the environment. Changes in size composition of the community, which are associated with the environmental conditions and the organism dynamics (Rodríguez *et al.*, 2002; Brucet *et al.*, 2005) will be also analysed.

In the second chapter, phytoplankton and bacterioplankton biomass interactions and the environmental factors responsible for their dominance will be analysed. These interactions will be evaluated in temporary basins, which are very affected by runoff during the flooding periods. The influence that allochthonous nutrients have on the microbial community will also be examined. In order to detect short-term changes, the community will be studied intensively, with monitoring twice a day. Since bacterio- or phytoplankton dominance may be affected by other factors such as diel variations or vertical differences in nutrient composition and distribution, high frequency fluctuations due to these factors will also be taken into account. We will focus especially on the differences in the catchment area and the importance of the differential accumulation of allochthonous nutrients and organic carbon between basins.

Finally, in the third chapter, due to the close relationship between coastal lagoons and marine coastal waters where HABs are frequent, a general inventory of the most abundant phytoplanktonic species will be made, giving importance to those time periods when a large phytoplankton biomass accumulates and to the presence of potentially harmful species. Extensive toxicity tests will be performed in order to detect these conditions during which the ecosystems are more affected by phytoplankton toxins. Then a more focussed analysis will be carried out to define the cause of the toxicity. A taxonomic effort will be made here in order to compare the phytoplankton communities in coastal lagoons with those in marine coastal waters. This part of the study will be focussed on comparing potentially harmful dinoflagellates and the possibility of these species moving between the two ecosystems. The proliferation of dinoflagellates will be related with their specific environmental requirements.

5. Objectives

The main objective of this study is to analyse the dynamics of the phytoplankton in the Empordà salt marshes as an example of a confined Mediterranean salt marsh.

This main aim has been split into different specific objectives:

❖ To analyse the changes in the phytoplankton composition by studying the pigment groups (classes) in relation to environmental changes.

❖ To determine the effect of environment factors on phytoplankton size distribution by studying the phytoplankton biomass-size spectra.

❖ To determine the importance of the top-down and bottom-up controls in the microbial trophic web.

❖ To establish which factors or environmental parameters determine whether bacterioplankton or phytoplankton are dominant.

❖ To determine the importance of the allochthonous organic matter coming from the runoffs into the lagoons for the microbial community.

❖ To determine the degree of importance of harmful algae species in the Empordà salt marshes. To find out which taxa are present, their abundance and potential toxicity.

❖ To evaluate the viability of a toxicity test method (Microtox) to detect phytoplankton toxins in Mediterranean salt marshes.

❖ To compare the harmful dinoflagellate composition and dynamics of coastal lagoons and the external seawater. To establish the environmental factors that favour harmful dinoflagellates proliferating in both ecosystems, the marine and the lenitic.

❖ To determine if coastal lagoons act as concentrators of harmful marine dinoflagellates during confinement periods.

II. Study area

Study area

The Empordà salt marshes (NE Spain) are a set of shallow lenitic waters without tidal influence (Fig. 1). They lie on a plain formed by sedimentary materials from three rivers, the Muga, Fluvià and Ter, with highly variable and irregular flow rates (Julià *et al.*, 1994). The studied zones are characterised by occasional influxes of seawater. Water intrusions into the lagoons are mainly due to sudden, large sea storms and also to intense precipitation (autumn to spring). During the dry periods (mainly in winter and summer), the water level gradually decreases, mainly due to evaporation and infiltration, and conductivity increases. Water levels are usually very low, especially during summer when a large number of basins dry out completely. After sea storms or rainfall, the marshes become confined (no water supply) for a long time and tend to dry up. During the confinement period the lagoon water level gradually decreases and conductivity increases.

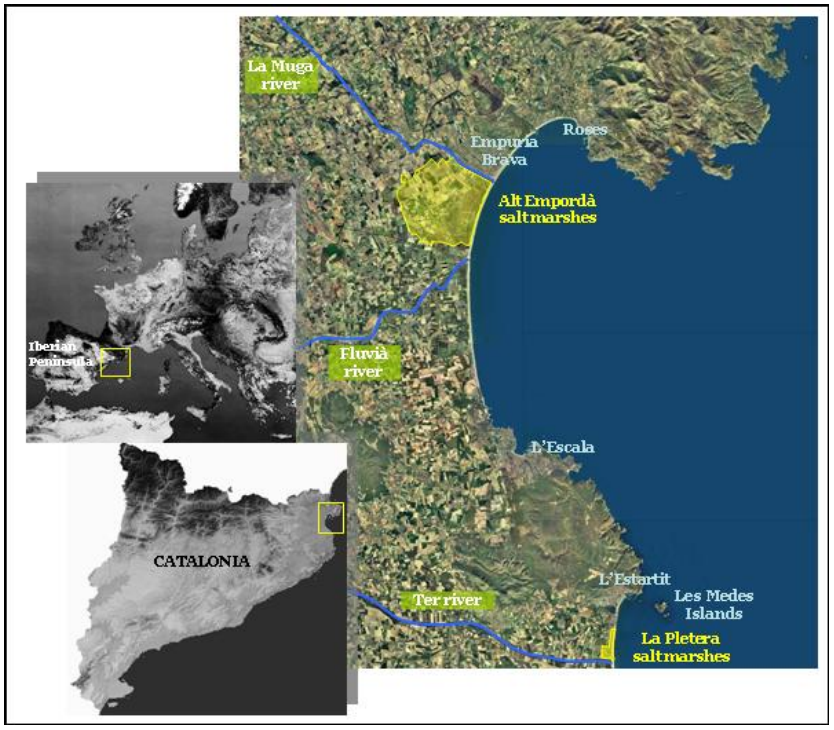

Fig. 1. Location of the salt marshes studied. Alt Empordà and La Pletera salt marshes.

Two salt marshes were included in this study: the Alt Empordà salt marshes in the Empordà Wetlands Natural Park and La Pletera salt marshes in the Baix Empordà Wetlands (Fig. 1). Permanent and temporary coastal lagoons from the two salt marshes were studied. Table 1 shows some physical and chemical characteristics of an example of the lagoons studied in each system. Some data about the vegetal community of the lagoons catchment area is also available.

Lagoon		***Fra Ramon* (o2)**	***Estany d'en Túries* (Turies)**
Salt marshes		La Pletera	Alt Empordà
Water level (cm above sea level)		101 (0-135)	64 (0-100)
Minimum depth (cm)		-175	0
Conductivity ($mS \cdot cm^{-1}$)		49 (8.8- 149)	18 (0.7- 51)
DIN (µM)		9.6 (0-186)	10.4 (0-148)
SRP (µM)		4.8 (0- 92)	1.2 (0- 10)
Water permanence		Permanent	Temporary*
Vegetal communities	hydrophytic	*Chaetomorpho-Ruppietum*	*Chaetomorpho-Ruppietum*
	halophytic	*Puccinellio-Arthrocnemetum fruticosi typicum*	*Puccinellio-Arthrocnemetum fruticosi typicum*
Main heleoplankton species		*Calanipeda aquae-dulcis, Eurytemora velox, Diacyclops bicuspidatus odessanus, Halicyclops rotundipes, Mesochra rapiens*	*Calanipeda aquae-dulcis, Eurytemora velox, Diacyclops bicuspidatus odessanus,Cyprideis torosa, Loxoconcha elliptica*

Table 1. Mean and range of variation (in brackets) of some physical and chemical parameters of the two lagoons, permanent and temporary, representative of La Pletera and the Alt Empordà salt marshes (NE Spain) respectively. Data from Trobajo *et al.* (2002). *In this study the term temporary include both, temporary and semi-permanent lagoons.

The permanent confined lagoons (La Pletera salt marshes)

La Pletera is a brackish water marsh situated between the urban nucleus of l'Estartit (Torroella de Mongrí, Girona) and the outfall of the Ter River (Fig. 2). This zone has a typical Mediterranean hydrologic regime (Fig. 3), with few human alterations and is very affected by the sea. Within a Life Restoration project (Quintana *et al.*, 2001), new shallow basins were constructed in this salt marsh in 2002, in order to preserve the population of the Iberian toothcarp (*Aphanius iberus*), an endemic and endangered fish from the Iberian Peninsula. The new lagoons and the old ones provided proximal waterbodies with a similar hydrology but with different trophic conditions. They include hypertrophic

conditions with occasional proliferations of phototrophic bacteria (Fig. 4), but also the oligotrophic conditions of the recently created lagoons.

Fig. 2. General view of La Pletera salt marshes (Photo by Bon Vent de l'Empordà).

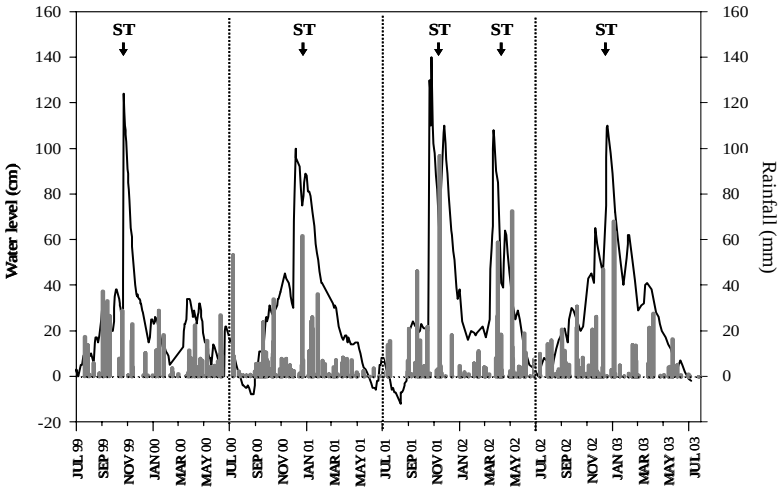

Fig. 3. Variation in the water level (cm above the average sea level) in O2 lagoon during four hydrological cycles (from 1999 to 2003, each cycle is separated by dotted lines). Bars represent rainfall (in mm). ST indicates sea storm events. The zero water level corresponds to the average sea level over the last 15 years (Badosa *et al.* submitted).

Fig. 4. O1 lagoon (Bassa del Pi), during a proliferation of *Chromatium* (summer 2003).

Fig. 5. Detail of the aerial view of O2 lagoon (Fra Ramon; Photo by Bon Vent de l'Empordà).

Fig. 6. Detail of the aerial view of the three newly created lagoons N3, N2, N1 from La Pletera salt marshes (Photo by Photo by Bon Vent de l'Empordà).

The temporary confined lagoons (Alt Empordà salt marshes)

The Alt Empordà salt marshes are situated within the integral reserve of the Empordà wetlands Natural Park (Fig. 7). The hydrology of this area depends mainly on sudden and irregular intrusions during sea storms and intense rainfall (Fig. 8). There is subterranean circulation of both fresh and salt water due to the abundance of sand deposits in the surface aquifer (Bach, 1990; Quintana, 2002). The salt marsh area is mainly occupied by temporary and semi-permanent basins, permanent lagoons (e.g. La Rogera) are scarce.

Fig. 7. Aerial view of l' Estany d'en Tùries lagoons group in Alt Empordà salt marshes (Photo by E. Marqués).

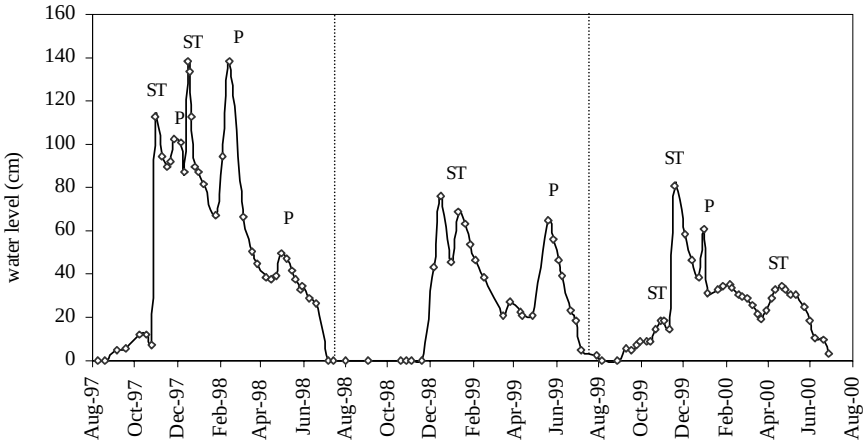

Fig. 8. Variation in the water level (cm above or below the average sea level) in Turies lagoon during two hydrological cycles (from 1997 to 2000, separated by dotted lines). ST indicates sea storm events and P indicates rainfalls > 50 mm. The zero water level corresponds to the average sea level over the last years. Modified from Gascón (2003).

Fig. 9. Aerial view of Turies lagoon in Alt Empordà salt marshes (Photo by E. Marqués).

Fig. 10. View of Turies lagoon in the Alt Empordà salt marshes.

Fig. 11. View of Litoral lagoon in Alt Empordà salt marshes (Photo by X. Quintana).

More details about specific characteristics of the basins and the sampling design can be found in each chapter.

III. Material and methods

Material and methods

1. Sampling

Samples were taken between June 2001 and December 2003, at a central point of each lagoon at a depth of 15-30 cm. For marine waters, surface samples were collected at a fixed point (water depth 1m) monthly during 2003. More details about sampling frequency are shown in each chapter. Sampling always included *in situ* measurements of temperature, electrical conductivity (EC25), pH and water level. Water samples for soluble inorganic nutrients analysis were filtered and stored on ice during transport to the laboratory and directly frozen upon arrival. Unfiltered samples were either frozen for later analysis of total nitrogen (TN) and total phosphorus (TP) or refrigerated for total organic carbon analysis (TOC). Concentrations of TN, TP, nitrate, nitrite, ammonia and soluble reactive phosphate (SRP) were analysed following Grasshoff *et al.* (1983). TOC and DOC (Dissolved organic carbon) were analysed using a TOC 500 SHIMADZU analyser. Gilvin (g_{440}) was analysed according with Kirk (1994) and corresponds to the absorbance at 440 nm of the filtered water sample. Gilvin are those soluble yellow substances, whatever their chemical structure, which occur in natural waters at concentrations sufficient to contribute significantly to the attenuation of the photosynthetically active radiation (PAR).

2. Phytoplankton pigments

For pigments samples, 500 ml of water was filtered through a Whatman GF/C filter that was frozen for later analysis. Frozen filters from samples were extracted in a Pyrex tube with 5 ml 95% methanol. Filters were extracted by sonication and preserved for 12 h at -20°C to prolong the extraction time. Subsequently, the extract was centrifuged and 0.5 ml of the supernatant was mixed with 0.2 ml of double distilled water to avoid the shape distortion of earlier eluting peaks (Zapata and Garrido, 1991). Extracts were analysed for pigment content using a high-performance liquid chromatographic (HPLC) method based on a reversed-phase C_8 column and pyridine-containing mobile phases (Zapata *et al.*, 2000). A Waters HPLC with a 996 Photodiode Array Detector was used. Chlorophylls and carotenoids were detected by diode array spectroscopy (350 to 700 nm). Every pigment peak was integrated in their

maxima emission wavelength in order to detect those pigments with lower concentrations. Pigment standards provided by DHI Water & Environment allowed the calculation of pigment concentration (Table 2). Pigments nomenclature and abbreviations suggested by SCOR WG 78 (Jeffrey *et al.*, 1996) were used.

Pigment	*Abbreviation*	*Regression equation*	R^2	*SD*
Chlorophyll *a*	Chl a	Area= 0,716x + 0,146	0,998	± 0,015
Divinyl Chlorophyll *a*	DVchl a	Area= 3,249x + 0,092	0,998	±0,020
Chlorophyll *b*	Chl b	Area= 1,564x + 0,085	0,999	± 0,066
Alloxanthin	Allo	Area=13,187x +0,181	0,992	± 0,135
Zeaxanthin	Zea	Area= 7,479x +0,049	0,997	± 0,080
Lutein	Lut	Area= 12,39x + 3,644	0,939	± 2,331
19' hexabutfucoxantin	Hex fuco	Area= 6,393x + 0,355	0,999	± 0,082
Fucoxantin	Fuco	Area= 5,646x + 0,322	0,996	± 0,164
Peridinin	Perid	Area= 5,899x +0,308	0,999	± 0,038

Table 2. Regression equations, Pearson's coefficients and standard deviation between the integrated area at the maximum wavelength and the concentration of every analysed pigment.

3. Chemotaxonomic determination of phytoplankton classes

The relative contribution of each phytoplankton taxonomic group to chlorophyll *a* was analysed using the programme Chemtax (Mackey *et al.*, 1996) and the pigment ratio matrix (Table 3) from Lohrenz *et al.* (2003). Discrimination of phytoplankton classes for the Chemtax analysis was based on inverted microscopy counts. Prasinophytes were treated within the chlorophytes, because prasinoxanthin was not detected.

	Perid	*Fuco*	*Hexfuco*	*Allox*	*Lut*	*Zea*	*Chl b*	*Chl a*
Dinoflagellates	0.80	0	0	0	0	0	0	1
Cryptophytes	0	0	0	0.23	0	0	0	1
Haptophytes	0	0.05	1.20	0	0	0	0	1
Chrysophytes	0	0.20	0.01	0	0	0	0	1
Chlorophytes	0	0	0	0	0.20	0.05	0.26	1
Cyanobacteria	0	0	0	0	0	1.20	0	1
Diatoms	0	0.75	0	0	0	0	0	1

Table 3. Initial pigment ratio used for CHEMTAX program from Lohrenz *et al.* (2003)

4. Microphytoplankton counting

Inverted microscopy: aliquots (150 ml) were fixed with lugol (1% final concentration) for quantifying phytoplankton. The general procedure for identifying and quantifying phytoplankton cells involved sedimentation of a subsample in a 50 ml settling chamber for 24 hours and then counting cells in a certain area (Throndsen, 1995) using an inverted microscope. The following taxonomic works Desikachary (1959), Dodge (1985), Sournia (1986), Chrétiennot-Dinet (1990), Hasle *et al.* (1997), Whitton and Potts (2000), Faust and Gulledge (2002), and Hallegraeff *et al.* (2003) were used to determine the taxa.

Epifluorescence microscopy: observations by means of the stain of thecate dinoflagellates with calcofluor (Hallegraeff *et al.*, 2003) were carried out in order to identify the *Alexandrium* species.

Scanning electron microscopy (SEM): the identification of some athecated dinoflagellates, cyanobacteria and other flagellates was supported by SEM. Samples were centrifuged, dehydrated and gold coated.

5. Phytoplankton and bacterioplankton counting.

Phytoplankton (pico- and nano-) and bacterioplankton abundance and biovolume were performed with a flow cytometer. Samples were filtered through 50 µm mesh, fixed with 1% parafomaldehyde and 0.05% glutaraldehyde (final concentration) and deep frozen in liquid nitrogen and stored frozen at -20 ºC. The cytometer was a FACScalibur of Becton & Dickinson with laser emitting at 488 nm. All information about light dispersion (Forward Scatter -FSC– and Side Scatter –SSC) as well as fluorescence emitted (Green Fluorescence –FL1–, Orange Fluorescence –FL2– and Red Fluorescence –FL3) was collected. Data were acquired in list mode, processed with WinMDI and analysed statistically with the SPSS program.

Picophytoplankton (PP) and Nanophytoplankton (NP) biomass. After the samples were thawed, a 10^5 ml $^{-1}$ solution of yellow green 2 µm Molecular Probes latex beads was added as an internal standard (Yentsch and Phinney, 1984; Olson *et al.*, 1985) and all parameters were normalized to them. When the sample acquisition rate was higher than 500 event s^{-1}, the sample was diluted

until it reached more or less the acquisition limit. Phytoplankton was detected by their signature in a plot of FSC *vs*. FL3.

In order to better discriminate between the populations, two methodological size divisions were done: PP for sizes between 0.5 – 3 µm and NP for sizes up to 3 µm ESD (Equivalent Spherical Diameter). The transformation of the FSC signals of the flow cytometer to cell volume was carried out through a calibration curve as described elsewhere (Olson *et al.*, 1989; Chisholm, 1992; Garcia *et al.*, 1994; Rodriguez *et al.*, 2002; Rodríguez *et al.*, 2002). A group of phytoplankton cultures were run on the flow cytometer and the average FSC value of the phytoplankton population, as normalized to that of the beads, was correlated with the population mean volume, obtained by inverted microscopy (Table 4).

ESD	Regression equations	r^2	*N*
PP (0- 3 µm)	Log (cell FSC/beadsFSC) = 0.78· (log Biovolume) - 0.31	0.97 (p<0.001)	6
NP (3- 20 µm)	Log (cell FSC/beadsFSC) = 0.69· (log Biovolume) - 0.27	0.98 (p<0.001)	9

Table 4. Relationship between Forward Scatter Channel (FSC) and cell volume for phytoplankton cultures. All the biovolume measures are given in $\mu m^{3} \cdot ml^{-1}$.

Bacterial biomass. After the samples were thawed, 500 µl were stained with 2 µl of Syto 13 at 500 µM (diluted in DMSO). When the sample acquisition rate was higher than 1500 event s^{-1}, the sample was diluted until the acquisition limit was reached. Usually, a 10^{6} ml^{-1} solution of yellow green 1 µm Polysciences latex beads was added as an internal standard and all parameters were normalized to them. During the entire process, samples were protected from light. Bacteria were detected by their signature in a plot of SSC *vs*. FL1. The bacterial biomass estimations were performed following Gasol and Del Giorgio (2000).

6. Biomass-size distributions

Phytoplankton biomass-size distributions were analysed by means of the non linear Pareto II probability density function, which has been described as adequately fitting most aquatic organism distributions (Vidondo *et al.*, 1997), especially when all organisms were considered belong to a single functional group (Boix, 2000; Brucet *et al.*, 2005).

This function is given by the following equation:

$$p_i(s) = c(K + D)^c (s + D)^{-(c+1)}$$

According to these authors, the Pareto type II model has a cumulative distribution of probability defined as:

$$prob(s \geq S) = (K + D)^c (s + D)^{-c}$$

where (s) is the size of an individual taken at random and S is a threshold size expressed as a function of s. Estimators of c, K and D can be obtained using this equation by means of an iterative nonlinear regression. In practice, the term $prob(s \geq S)$ is calculated for each individual as the fraction of all individuals larger than or equal to itself ($N_{s \geq S} / N_t$).

The size of each organism (s) was standardised by dividing it by the minimum size of each sample (s_{min}) following Winiwarter and Cempel (1992):

$$s' = s / s_{min}$$

7. Zooplankton counting

For zooplankton samples, 5 L of water were filtered through a 50 μm mesh and preserved in situ in 4% formalin. Measurements of zooplankton were conducted using an inverted microscope. Biomass dry weight estimations were obtained, for most invertebrate species, from the allometric correlation between weight and body length (Dumont *et al.*, 1975; Smock, 1980; Malley *et al.*, 1989). For rotifers and ciliates biomass was calculated by converting volume into dry weight (Ruttner-Kolisko, 1977; Malley *et al.*, 1989; Schönborn, 1992).

8. Microtox® toxicity assays.

Frozen filters from natural samples were extracted using a Pyrex tube with 5 ml of Milli Q water (Millipore). The tub was placed in an ultrasonic bath with ice and water and then conserved for 24 hours in the refrigerator (4ºC to prolong the extraction time). Extracts were then centrifuged at 4000 r.p.m. for 10 minutes and the supernatant was immediately analysed. We also analysed the soluble fraction of the samples for which the Microtox® results were positive for that particulate fraction. The Microtox® test was carried out according to the trademark directives. Microtox® values are given to the line calculated based on

a 5-point curve for each experiment (Volterra *et al.*, 1992). $EC50_5$ is the Effective Concentration (corresponding to 50% of the extinction coefficient) after exposing the sample to the bacteria *Vibrio fischeri* for five minutes. High values of Effective Concentration indicate low toxicity, while low values indicate high toxicity. Toxicity Units (TU_5) have been calculated as $TU = EC50^{-1}$ for 5 minutes exposure.

9. Heavy metals analyses

For metals samples, 500 ml of water was filtered through a Whatman GF/C filter that was frozen for later analysis. Frozen filters from samples were digested for 6 hours by a mixture of 4 ml HNO_3 (1:1) + 10 ml HCl (1:4) and filtered before analysis. Flame Atomic Absorption Spectrometry was employed to determine the metal concentration.

10. Microcystin analyses

For microcystin analysis, between 50 and 500 ml (until filter saturation) of lagoon water was filtered through glass fibre filters (Whatman GF/C) using a vacuum pump. The filters were packed in aluminium foil and stored frozen (<20°C). Filtered samples were extracted by adding 9 ml of 70% (v/v) aqueous methanol, followed by ultrasonication. Filter material and phytoplankton debris were removed by centrifugation. The supernatant was evaporated to dryness at 50°C. The residuals including the toxins were redissolved in 1 ml 70% v/v) methanol. This solution was used to analyse microcystin using high performance liquid chromatography. The analyses were carry out by the Biology Department of the Universidad Autónoma de Madrid. The method described by Lawton *et al.* (1995) was followed, but the TFA (trifluoroacetic acid) volume was modified from 0,1% to 0,05%, in order to obtain a better baseline response without modifying the method's separation capacity. The stationary phase used was a Purospher RP-18e (5 µm) 250 mm×46 mm column. The calculation standards were microcystin-LR, microcystin-RR, and microcystin-YR.

11. Statistical analyses

Canonical Correspondence Analysis (CCA)

In order to quantify the influence of the distinct environmental variables on phytoplankton, different CCA were performed. All canonical axes were used to

evaluate the significant variables under analysis by means of a Monte Carlo test (1000 permutations). CCA test were performed using version 4.5 of CANOCO (ter Braak and Šmilauer, 2002). Phytoplankton classes and species abundance matrix was square-root transformed, since logarithmic transformation is not suitable in data matrices with abundance of zeroes, and square root transformation also allows the stabilisation of the variance on count data (ter Braak and Šmilauer, 2002). Environmental variables were log transformed.

Clustering analysis

Hierarchical cluster analysis was used to establish similarity between dinoflagellate species. The data used in this case were the coordinates obtained for each species with the CCA using the abundances of species. Euclidian distance was used because it underlines differences between high values. The cluster method chosen was the average linkage.

Analysis of variance and covariance

To analyze the contribution of different sources of variation to bacterioplankton and phytoplankton biomass, a multivariate analysis of covariance (MANCOVA) was performed using both biomasses as variables. The covariables were temperature, nitrate, nitrite, ammonia, SRP, DOC and g440, while the factors were basin, period, depth, and hour. Starting from the results of the MANCOVA, two univariate analysis of the covariance (ANCOVA) were also performed. They related each ecological group with the variables and factors significant in the general MANCOVA model. Likewise, with the aim of analyse the possible interactions among phyto- and bacterioplankton, biomass of each group was introduced as covariable in the ANCOVA of the other group. Partial eta squared was used as a measure of effect size of factors and covariables. The level of statistical significance used was $p<0.05$.

All the analyses of variance and correlations were carried out using SPSS® for Windows 12.0. (SPSS, Chicago, Illinois).

IV. Phytoplankton composition and size distribution in the confined salt marshes of La Pletera

1. Introduction

Nutrient availability has been described as one of the main factors controlling phytoplankton community composition and biomass in shallow waters and marshes (e.g. Ortega-Mayagoitia *et al.*, 2003). On the other hand, most authors attribute phytoplankton community composition to top-down control by zooplankters (e.g. Gasiunaite and Olenina, 1998; Franks, 2001; Muylaert *et al.*, 2003). Moreover, according to Capblancq (1990) the factors determining phytoplankton composition, such as nutrient uptake or grazing, are strongly size-dependent and basically similar in lakes and in oceans.

Most Mediterranean coastal lagoons have negligible tidal inputs (Britton and Crivelli, 1993), but they are suddenly flooded during sea storms, after which mostly become confined. Water level fluctuations are characteristic of these ecosystems and they cause extreme variation in the physico-chemical parameters and species composition, both spatially and temporally (Perez-Ruzafa and Diego, 1993; Guelorget *et al.*, 1994; Quintana *et al.*, 1998a; Serrano *et al.*, 1999; Golterman, 1999). Connections between coastal lagoons and the sea, which occur during sea storms, allow exchange of nutrients and organisms. During confinement, a decrease in the water level causes the concentration of nutrients, which would favour phytoplankton growth and the concentration of zooplankters, which in turn limits phytoplankton development. Thus, phytoplankton growth may be controlled by both, nutrient supply and zooplankton grazing. Further, it has not been established whether bottom-up or top-down control dominates when nutrient and zooplankton are concentrated simultaneously during confinement.

The aim of this study was to analyse which factors determine the phytoplankton composition and size distribution in a confined Mediterranean salt marsh, and to establish if phytoplankton was mainly controlled by nutrient concentrations or by zooplankton biomass.

2. Study site and sampling

This study was carried out in La Pletera salt marshes (see Study Site). The creation of the new lagoons in this zone and the existence of the old ones provided proximal waterbodies with similar hydrology, but with different trophic conditions. Two “old” and three “new” waterbodies were chosen for this

study (Fig. 12). Samples were taken monthly between June 2002 and December 2003, at a central point of each basin at a depth of 15-30 cm.

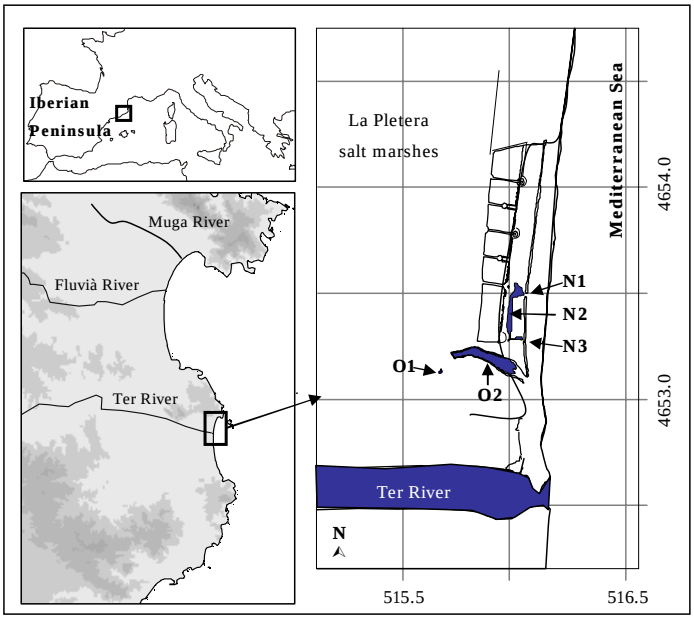

Fig. 12. Collection sites in La Pletera salt marshes. Grid indicates UTM coordinates (1000 m).

3. Results

Physical and chemical characteristics.

Floods mainly occurred during the winter months, reaching their highest water level after sea storms (December and January), and decreasing progressively the rest of the year until summer when confinement was the maximum (Fig. 13). Conductivity minimums coincided with the sea water input, after sea storms, and rainfalls. After these flooding events, conductivity increased progressively reaching its highest value at the end of summer, when evaporation rate was the highest. Similarly, the hydrological variations had a direct effect on nutrient concentrations. TP concentrations were the highest at the end of summer coinciding with high confinement and low water levels. Temporal variation of

TOC and TN (not represented) was similar to TP. Nitrate showed a concentration increase just after the main water inputs. Subsequently, nitrate diminished to an undetectable concentration the remainder of the year. In contrast, ammonia increased immediately after the nitrate peak, but also in summer when total nutrients were the highest. All the lagoons studied follow similar temporal patterns in nutrient composition, therefore, only one lagoon was used as a representive example in Fig. 13.

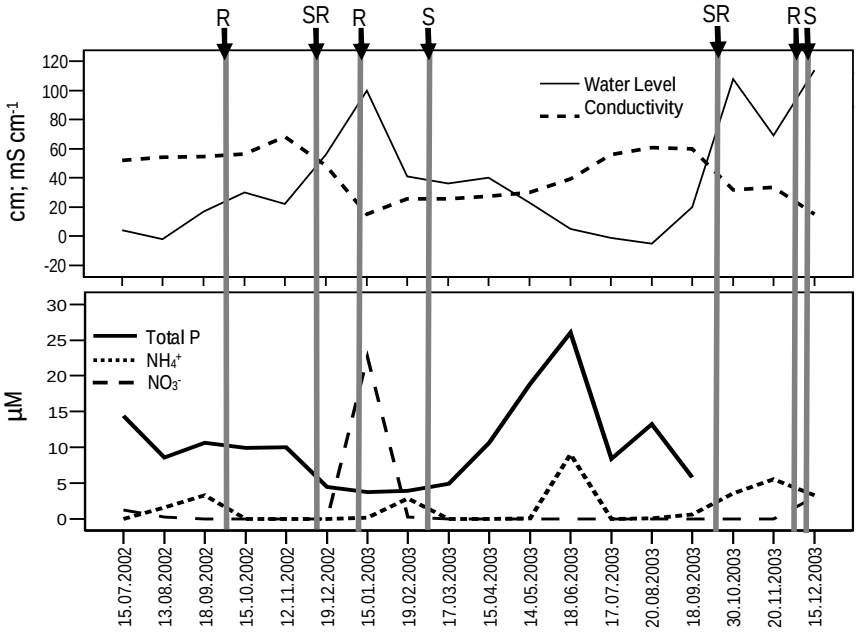

Fig. 13. Water level and nutrient fluctuations in O2 lagoon (2002-2003). Arrows indicate precipitation of more than 40 mm in 24h (R) and sea storms (S).

Old lagoons had significantly higher values of conductivity, TOC, TN, TP and SRP (Table 5). On the other hand, no significant differences were found in inorganic nitrogen concentrations. Oxygen values over 100% saturation were occasionaly found, more frequently in the old lagoons, where nutrients concentrations were higher.The occurrence of nocturnal anoxia was not verified, since samples were always taken at midday.

	Sig.	*N1*		*N2*		*N3*		*O1*		*O2*	
Number of cases		17		14		17		18		18	
Level (cm a.s.l.)	n.s.	**30.5**	42.11	**43.5**	43.99	**39**	40.97	**38**	39.65	**26.5**	37.84
Temperature (ºC)	n.s.	**18.7**	7.8	**15.2**	8.5	**16.6**	8.1	**20.0**	8.4	**19.0**	8.1
Conductivity ($mS \cdot cm^{-1}$)	**	**31.80**	11.18	**43.50**	14.69	**25.50**	6.40	**63.10**	35.44	**43.75**	16.73
pH	n.s.	**8.07**	0.63	**8.08**	0.50	**8.04**	0.51	**7.90**	0.51	**7.93**	0.49
% Oxygen	n.s.	**90**	28	**88**	21	**92**	26	**99**	83	**88**	43
Alkalinity	**	**4.60**	1.05	**5.07**	1.17	**5.01**	0.92	**7.71**	2.25	**5.36**	1.32
TOC (ppm)	**	**12.49**	15.88	**13.48**	15.79	**11.41**	6.36	**87.69**	103.99	**27.94**	15.42
TN (µM)	**	**96.13**	70.59	**120.10**	62.78	**76.04**	62.75	**408.01**	199.68	**246.38**	87.62
TP (µM)	**	**2.02**	3.45	**2.48**	3.41	**1.32**	1.60	**14.86**	29.11	**9.92**	6.10
NH_4^+ (µM)	n.s.	**<0.69**	9.69	**2.04**	5.28	**1.34**	7.98	**<0.69**	6.27	**<0.69**	2.27
NO_2^- (µM)	n.s.	**<0.04**	0.18	**<0.04**	0.22	**<0.04**	0.23	**0.07**	2.47	**<0.04**	0.42
NO_3^- (µM)	n.s.	**<0.44**	11.40	**<0.44**	10.55	**<0.44**	7.15	**<0.44**	35.30	**<0.44**	5.21
DIN (µM)	n.s.	**3.82**	14.37	**3.37**	10.83	**3.54**	10.00	**1.70**	43.07	**2.11**	5.48
SRP (µM)	**	**0.14**	1.27	**0.65**	1.14	**0.11**	0.86	**2.59**	1.80	**0.35**	0.53

Table 5. Median (in bold) and SD values of several physical and chemical variables in the studied lagoons (**) significant differences ($p<0.01$) between new and old lagoons.

Relationships between zooplankton, nutrients and phytoplankton community composition.

No significant correlations were found between the absolute amount of each phytoplankton class and the total zooplankton biomass (Table 6). The relative contribution of phytoplankton classes to chlorophyll *a* (relative amount) and total chlorophyll *a* did not correlate with total zooplankton biomass. On the other hand, significant correlations were found between TP and total and relative amount of chlorophyll *a* due to some phytoplankton classes. The relationship between TP and the absolute amount of phytoplankton classes was positive overall, except for diatoms and haptophytes, which did not have a significant correlation. However, the relative amount of chlorophyll *a* of diatoms and haptophytes had a negative correlation with TP. Similar, but lower correlations were found with TN and TOC concentrations (not included in Table 6).

Absolute amount of chlorophyll a due to pigment group ($\mu g \cdot l^{-1}$)		
Phytoplankton class	**Total phosphorus**	**Zooplankton Biomass**
Chrysophytes	0.712**	n.s.
Cyanophytes	0.553**	n.s.
Dinoflagellates	0.514**	n.s.
Cryptophytes	0.505**	n.s.
Chlorophytes	0.360*	n.s.
Diatoms	n.s	n.s.
Haptophytes	n.s.	n.s.
Total Chlorophyll *a*	0.487**	n.s.
Relative pigment group contribution to chlorophyll a		
Phytoplankton class	**Total phosphorus**	**Zooplankton Biomass**
Chrysophytes	0.446**	n.s.
Cyanophytes	n.s.	n.s.
Dinoflagellates	n.s.	n.s.
Cryptophytes	n.s.	n.s.
Chlorophytes	n.s.	n.s.
Diatoms	-0.337**	n.s.
Haptophytes	-0.333**	n.s.

Table 6. Pearson correlation coefficients between TP concentration, zooplankton biomass and pigment groups (phytoplankton classes) absolute and relative contribution to chlorophyll a. All data are logarithm transformed. (**) significant correlations ($p<0.01$) and (*) significant correlations ($p<0.05$).

Relationships between the phytoplankton community composition and the environmental factors were analysed by means of a Canonical Correspondence Analysis (CCA) (Fig. 14). The first two dimensions of the CCA accounted for 70% of the total variance of phytoplankton classes and environmental data. The first axis accounted for 40% of the total variance and the second axis for 30% of the total variance. The first axis mainly separated samples of the old lagoons with positive coordinates from samples of the new lagoons with negative coordinates. The second axis distinguished samples of the confinement periods with positive coordinates from samples of the flooding periods with negative coordinates. Temperature and conductivity were high during confinement, and covariated

with total nutrients, while water level increased during flooding periods, coinciding with the inorganic nutrients input.

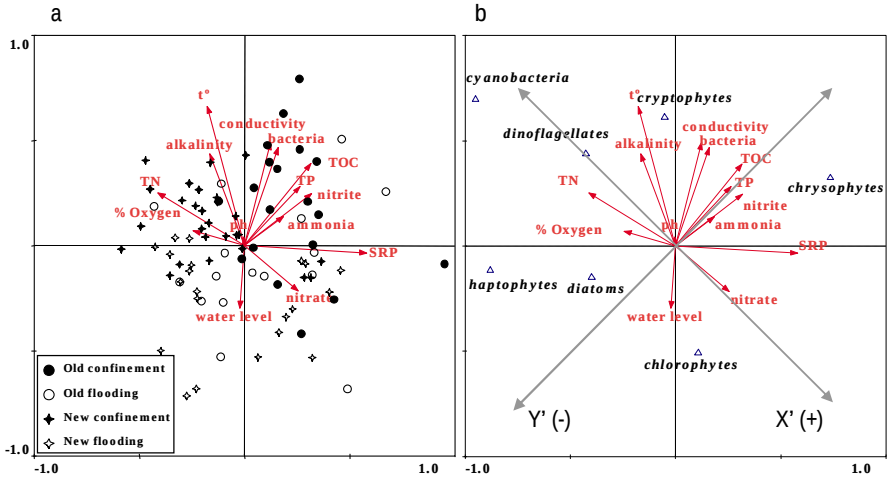

Fig. 14. a) Distribution in the two first CCA axes of samples identified by means of periods and lagoon type; b) Distribution of the phytoplankton classes and environmental parameters. Arrows indicate the diagonal gradients: X', related to the ratio of nitrate:TN; Y', related to the nutrient load.

A 45º rotation of the CCA axes provided a more general interpretation of the CCA results. After this rotation, significant correlations were found between the new axes, X' and Y' (see Fig. 14b) and some environmental variables. X' axis correlated positively with the nitrate:TN ratio (r=0.463, p<0.001, N=68). Y' axis correlated positively with TP (r=0.670, p<0.001, N=68) and TOC (r=0.471, p<0.001, N=83). Therefore, these diagonal axes showed the existence of two different environmental gradients, the first one related to the nutrient availability (X'), especially to the dominance of the inorganic or organic forms of nitrogen, and the second related to the total nutrient load (Y'). Low nitrate:NT ratios were mainly found in new lagoons under confinement conditions (negative X' values), while total nutrient loads were especially high in old lagoons during confinement (positive Y' values).

Phytoplankton classes were sorted along the diagonal axis, X'. Cyanobacterial phytoplankton was positioned in the most negative X' values. Dinoflagellates, cryptophytes and haptophytes were also dominant in negative X' values. On the

other hand, chrysophytes and chlorophytes were abundant in samples with positive X' values, while diatoms were located in X' values close to zero. Following the second diagonal *Y'*, cryptophytes and chrysophytes were abundant in Y' positive values, when TP and TOC concentrations where high, while haptophytes, diatoms and chlorophytes dominated in samples with negative Y' values with low concentrations of total nutrients. Cyanobacteria and dinoflagellates were located in Y' values close to zero.

Phytoplankton size distributions

Analysis of the biomass-size spectra showed two different types of phytoplankton size distributions, which differed by discontinuous or continuous intermediate sizes (Fig. 15). Size distributions were discontinuous, since there was a lack of size classes between 2.5 - 4 µm ESD (Equivalent Spherical Diameter) corresponding to $\log_2$ biovolumes 6 to 8. Thus, this kind of distribution separated two groups of phytoplankton, PP (smaller than 2.5 µm ESD) and NP (between 2.5 and 20 µm ESD). In most of the discontinuous distributions, PP was the most abundant (e.g. Fig. 15b), but distributions where NP dominated were also found (e.g. Fig. 15a). In continuous size distributions, intermediate sizes between PP and NP were present (e.g. Fig. 15c and d). In the new lagoons and during flooding periods, distributions were generally continuous, while in the old lagoons during confinement, when the nutrient loading gradient was high, all the distributions were discontinuous. The fit to the Pareto II model (r^2) allowed us to quantify lacks in the size distributions, since the higher a lack of intermediate sizes, the lower is r^2 (Fig. 15). Additionally, the lowest values of r^2 were found when there is a lack at intermediate sizes and the abundance of PP sizes is lower than the NP sizes (e.g. Fig. 15a). Thus, continuous distributions had high r^2 values, while discontinuous distributions had low r^2 values.

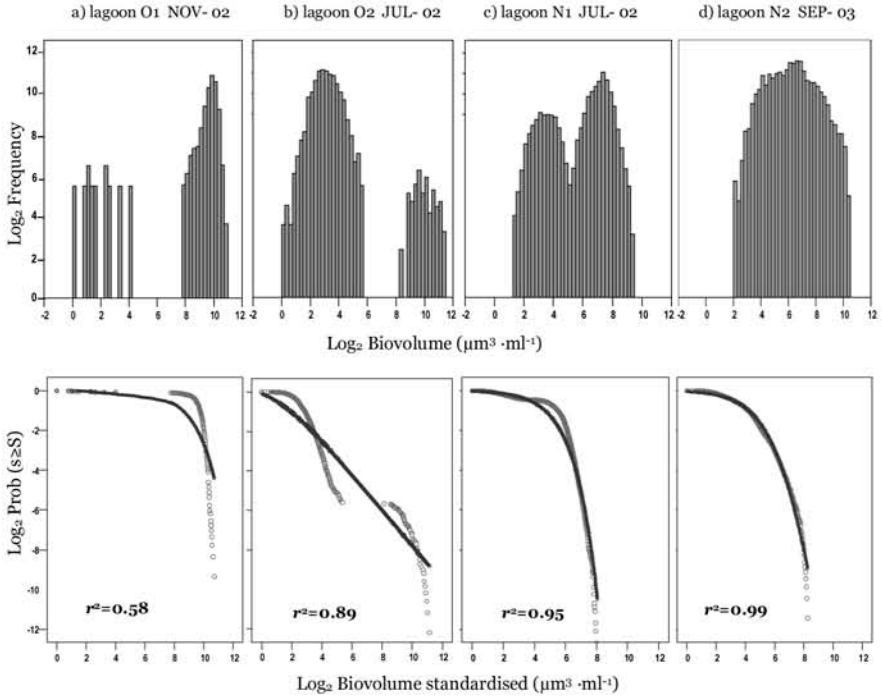

Fig. 15. Four examples of the phytoplankton biomass-size distributions (above) and their underlying Pareto II spectra (below). A gradient is shown, with increasing r^2, from a discontinuous distribution where PP abundance is extremely low (left) to continuous distribution with maximal abundances in PP size classes (right). The size of each organism (s) was standardised by dividing it by the minimum size of each sample (s_{min}) following Winiwarter and Cempel (1992): $s' = s/s_{min}$

In order to establish the possible effects of zooplankton on the phytoplankton size distribution, zooplankton biomass was correlated with the biomass of the two different phytoplankton size classes, PP and NP (Table 7). Zooplankton was also divided in two groups: (1) the small zooplankton (<200 µm length), which includes copepod naupli, rotifer and ciliate, and (2) the big zooplankton (>200 µm length), which includes copepodites, adult copepods and other invertebrates. Additionally, zooplankton biomass was correlated with the mode of the phytoplankton distribution, as indicative of the most abundant phytoplankton size. Most of these correlations were not significant and when significant they were weak and even positive (Table 7). Only a negative correlation was found between the total zooplankton biomass and the NP mode

in the old lagoons. These results indicated that the effect of zooplankton on phytoplankton size distributions was nearly negligible.

		Zooplankton biomass								
		Total zooplankton			Small zooplankton			Big zooplankton		
	Lagoons	Overall	Old	New	Overall	Old	New	Overall	Old	New
Phytoplankton biovolume	Total phytoplankton	0.338**	n.s.	0.396*	0.324*	n.s.	0.392*	n.s.	n.s.	n.s.
	PP	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	NP	0.329*	n.s.	0.473**	0.338**	n.s.	0.520**	n.s.	n.s.	n.s.
Phytoplankton size mode	Total phytoplankton	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	PP	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	NP	n.s.	-0.523**	n.s.	n.s.	n.s.	n.s.	0.310*	n.s.	0.505**

Table 7. Pearson correlation coefficients between phytoplankton and zooplankton sizes and biomass for all and every kind of lagoons (**) significant correlations ($p<0.01$) and (*) significant correlations ($p<0.05$).

The fit to the Pareto II model of the phytoplankton size distributions was also related with the zooplankton biomass, and no significant correlations were found for the whole of the lagoons or for the old lagoons (Table 8). However, in the new lagoons, the fit to the Pareto II model correlated negatively with the total zooplankton biomass and especially with the big zooplankton biomass, indicating that the discontinuous phytoplankton size distributions in the new lagoons were more frequent when zooplankton biomass increased. Correlations of the fit to the Pareto II model with nutrient concentrations were always weak or not significant. Some correlation was found with some nutrient ratios, such as DIN:TN and DIN:TOC, showing that the higher the relative abundance of inorganic nitrogen, the smaller was the discontinuity in the phytoplankton size distribution.

The fit to the Pareto II model was strongly correlated with the density of dinoflagellates (relative and absolute amount) in the more nutrient rich old lagoons. However, some samples of these old lagoons, where the dinoflagellates class was not detected, also showed low fit to the Pareto II model. In these

samples, the fit to the Pareto II model showed a negative correlation with the heterotrophic dinoflagellates biomass (examined by microscopy). No significant correlations were found with the biomass of other phytoplankton groups.

	Fit to the Pareto II model		
	Overall	Old Lagoons	New Lagoons
Zooplankton biomass	n.s.	n.s.	-0.347*
Small zooplankton biomass	n.s.	n.s.	n.s.
Big zooplankton biomass	n.s.	n.s.	-0.485**
DIN / TN	0.388**	n.s.	0.481**
DIN / TOC	n.s.	0.439**	n.s.
Dinoflagellates (relative amount to Chl a)	-0.341**	-0.716***	n.s.
Dinoflagellates (absolute amount of Chl a)	-0.490**	-0.578**	n.s.
Heterotrophic dinoflagellates (microscope)	-0.587**	-0.634**	n.s.

Table 8. Pearson correlation coefficients between nutrients and organisms with the fit to the Pareto II model (***) significant correlations ($p<0.001$), (**) significant correlations ($p<0.01$) and (*) significant correlations ($p<0.05$).

4. Discussion

Bottom-up or top-down control of phytoplankton

In the confined Mediterranean salt marshes studied, zooplankton predation pressure did not control either phytoplankton community composition, or their size distribution. Relationships were not observed with the biomass of the zooplankton either when analysing data of the phytoplankton separated by groups of sizes, or integrating them into a single spectrum. Only a few significant correlations were found between zooplankton and NP biomass, which rejected any top-down control on phytoplankton. Nutrients seem to be the main factor affecting phytoplankton variability as regarding the community composition with respect to their variations of size distribution. Thus, it seems that phytoplankton variability was mainly determined by the original trophic status of the basin, their internal loading of nutrients, and the external supply of nutrients.

Generally, the plankton community has been described as controlled by higher trophic levels. Predator presence controlled directionality in shallow lakes (Moss *et al.*, 1994; Pérez-Ruzafa *et al.*, 2002; Muylaert *et al.*, 2003), deep lakes (Cruz-Pizarro and Carrillo, 1991; Vanni *et al.*, 1997; Sommer *et al.*, 2001) and ocean ecosystems (Franks, 2001; Granéli and Turner, 2002). On the other hand, in shallow lakes with a high confinement level, nutrient control has been described as the main process controlling phytoplankton growth (Fathi *et al.*, 2001; Ortega-Mayagoitia *et al.*, 2003). Thus, the confined shallow lakes and lagoons seem to be systems susceptible to nutrient control. Probably confinement would subject a limitation on primary production, which is caused by a reduction of inorganic nutrient availability, as indicated by the low inorganic:organic nutrient ratio. Thus, our data showed that the phytoplankton of the Mediterranean salt marshes was mainly subjected to bottom up control.

Factors affecting phytoplankton composition

The low ratio of inorganic:organic nutrients is characteristic of Mediterranean salt marshes, where inorganic nitrogen is especially scarce due to denitrification (Comín and Valiela, 1993; Quintana *et al.*, 1998a; Golterman, 2000) and it becomes a limiting factor for autotrophic production. In this context, physiological differences in nitrogen uptake among the different phytoplankton groups may explain their ordination along the X' dimension, related to the ratio of nitrate:TN. Cyanobacteria showed a minor dependence on nitrate due to the capacity of most species to fix atmospheric nitrogen (Quesada *et al.*, 1998; Stal, 2000). Cryptophytes, dinoflagellates, haptophytes and chrysophytes, which have many mixotrophic species, may feed on bacteria or on other small eukaryotes to compensate for nutrient scarcity (Isaksson, 1998; Jones, 2000). Recently, some investigators (Cerón García *et al.*, 2000; Bavestrello *et al.*, 2000; Wen and Chen, 2003) have observed the capacity of some diatoms species to incorporate organic molecules. Thus, even diatoms could be successful when the ratio of nitrate:TN is low. The presence of chlorophytes, a group where species are rarely described as mixotrophic (Isaksson, 1998), is clearly related to a high nitrate:TN ratio.

The ordination of the different phytoplankton groups along the Y' gradient, was related to the organic matter and total nutrient loadings. Chrysophytes and

cryptophytes dominated the community when the water has high organic matter and total nutrient loadings, while diatoms, chlorophytes and haptophytes dominated when total nutrient loadings were low. Dinoflagellates and cyanobacteria would be more affected by the nitrate:TN ratio than by the total nutrient loading.

The trophic role of dinoflagellates

Dinoflagellates appeared to be determinants in phytoplankton biomass-size distributions of the lagoons studied and their abundance affected the lack of some NP intermediate sizes in the biomass spectra, especially in old lagoons. Two hypotheses could explain this lack of some intermediate phytoplankton sizes. One, dinoflagellates outcompete NP minor sizes (2.5 - 4 µm ESD), and two, direct grazing by mixotrophic or heterotrophic dinoflagellates affects NP sizes.

Regarding the nutrient composition, Malone (1980) and Harrison and Wood (1988) found a competitive advantage of microphytoplankton (>20 µm, where dinoflagellates are included), over other smaller algae when nitrate was abundant. Alternately, smaller sizes (<20 µm) dominated in nitrate poor waters when ammonia was most abundant. This disagrees with our results, since dinoflagellates mainly proliferated when the ratio of nitrate:TN was especially low. On the other hand, most of the dinoflagellate taxa have been described as mixotrophic organisms, which feed mainly on other smaller algae (Li *et al.*, 1996; Stoecker *et al.*, 1997; Isaksson, 1998; Stoecker, 1999; Jones, 2000; Li *et al.*, 2001; Ismael, 2003). Mixotrophic activity is favoured by inorganic nutrient depletion and by a low ratio of inorganic:organic nutrients (Granéli *et al.*, 1999; Jones, 2000). Although mixotrophic activity has not been measured in this study, it could explain the depletion of small NP sizes under a low inorganic:organic nutrient ratio. This ratio was especially low in old lagoons, where a strong relationship was found between discontinuous distributions and dinoflagellate biomass.

The biomass-size distribution within a functional group (i.e. phytoplankton, zooplankton or fish) shows a non linear continuous distribution (Dickie *et al.*, 1987; Sprules and Stockwell, 1994), as a consequence of competitive interactions. Thus, it is well modelled by the Pareto type II distribution

(Vidondo *et al.*, 1997). Furthermore, the presence of discontinuity in the size distribution, within a functional group and under stable conditions, has been related to intense predation within the same functional group (Sprules and Goyke, 1994). Stepped or discontinuous shapes not related to active predation, rather to population dynamics, have also been described, but they are not stable (Boix, 2000; Brucet *et al.*, 2005). Thus, phytoplankton size distribution patterns also suggest an important predation strategy by dinoflagellates.

In conclusion, our results suggest that in these confined environments, where nutrients are mainly in an organic form, dinoflagellates take advantage of their mixotrophy by the strategy of "eating their competitor" (Thingstad *et al.*, 1996), competing with and grazing on smaller phytoplankton at the same time.

V. Environmental factors affecting bacterioplankton or phytoplankton dominance and relationships in the Alt Empordà salt marshes

1. Introduction

Salt marshes are described as a sink for all kinds of particulate matter of which a considerable part is organic (Boorman *et al.*, 1994). Surface runoff is one of the most important sources of water in a wetland's hydrology budget (Wetzel, 1992; Mitsch and Gosselink, 1993). There are some controversies about the environmental factors determining the dominance of bacterioplankton or phytoplankton biomass in wetlands. According to Garnier and Benest (1990) and Revilla *et al.* (2000), variations of phytoplankton biomass are controlled mainly by inorganic nutrient input while variations of bacterial biomass are further influenced by allochthonous inputs of organic carbon. On the other hand, other authors (Fuhrman, 1992; Cotner and Biddanda, 2002) affirmed that bacteria and phytoplankton compete for the uptake of inorganic nutrients. Thus, at higher concentrations of nutrients, such as during pulses, the phytoplankton does proportionally better. In wetlands, inorganic nutrients and organic matter are mainly allochthonous (Wetzel, 1992), as a consequence the basin catchment area could play a main role in phytoplankton and bacterioplankton dynamics.

Mediterranean salt marshes are flooded, by sudden sea storms and/or rainfalls, after which marshes become mostly confined (Quintana *et al.*, 1998a). The confinement process involves the concentration of nutrients and organic matter of the whole catchment's area in a minor flooded area, the basin. Thus, the supply of allochthonous organic matter and nutrients into a basin during sea storms and rainfalls is the main source of nutrients for the organisms within. After that, only recycling can provide resource availability (Charpy and Charpy-Roubaud, 1990). Therefore, allochthonous nutrient supplies depend on the characteristics of the catchment's area and in the case of marshes, on the emergent herbaceous vegetation adapted to saturated soil condition (Mitsch and Gosselink, 1993). As a result, a higher vegetation cover of the catchment's area, will lead to a major organic matter loading associated to water inputs into the basin (Garnier and Benest, 1990).

The aim was to establish which factors or environmental parameters determinate the dominance of bacterioplankton or phytoplankton biomass in a Mediterranean salt marsh. In order to achieve this objective four environmental

factors have been taken into account. 1) size of the basins and the catchment's area; 2) different hydrological situations along the hydroperiod; 3) high frequency fluctuations due to day-night variations; and 4) differences in depth along the water column.

2. Study site

Two waterbodies (Turies and Litoral) located in the Alt Empordà salt marshes (see Study Site) were chosen for the study of phytoplankton and bacterioplankton biomass (Fig. 16). Both, are proximal to each other and are flooded with water from the same origin.

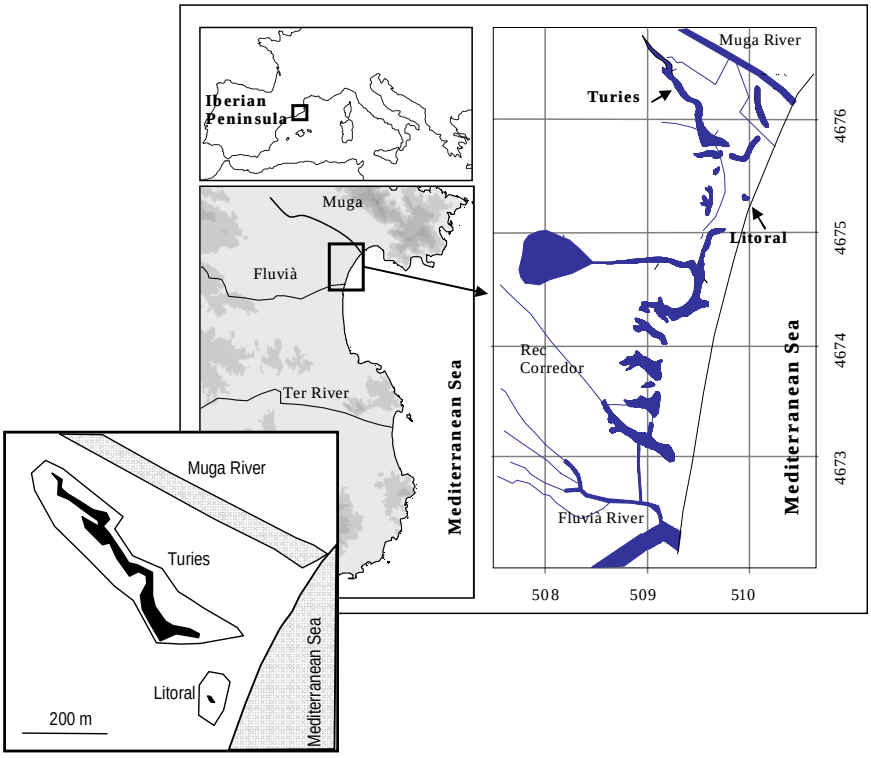

Fig. 16. a) Sampling sites in the Empordà marsh ecosystem. Grid indicates UTM coordinates (1000 m). b) Scheme of the flooded and catchment areas of Turies and Litoral lagoons.

Litoral is a little basin receiving inputs from a large hydrological catchment's area, whilst Turies has a higher flooded surface and, proportionally a minor external influence (Table 9). Turies is semi-permanent, that is, it remains flooded all the year but it dries out some years. Litoral is temporary and dries out every summer. However the average time of flooding per year is similar in both basins (Table 9). The hydrology of this area depends mainly on sudden and irregular intrusions during sea storms, intense rainfalls or entry of fresh water from rivers. Subterranean circulation of both, fresh and salt waters is active due to the abundance of sand deposits in the surface aquifer (Bach, 1990; Quintana, 2002). In spite of having similar water depth, the higher CF ratio (catchment:flooded area ratio) presupposes a proportionally greater organic matter input into Litoral.

	Litoral	*Turies*
Mean month flooded per year	9.3	10
Maximum depth (cm)	140	139
Mean depth (cm)	36	37
Flooded area (m^2)	983	132108
Catchment's area (m^2)	18750	277000
CF ratio	19	2.1

Table 9. Some morphometric characteristics of the basins. CF ratio is the relationship between the Catchment's and the Flooded area.

3. Sampling design

Previous studies in these marshes showed the lack of seasonal pattern in nutrient dynamics and species composition (Quintana *et al.*, 1998a; Quintana *et al.*, 1998b; Brucet *et al.*, 2005). Contrarily, they mainly depend on hydrology, which is highly variable along the year. Thus, bacterio- and phytoplankton dynamics may remain constant during a long period, but may change significantly in specific hydrological situations. For this reason, sampling frequency was not regular during the year. On the other hand, several sampling periods were selected coinciding with some characteristic hydrological situations that took place during the study period. These situations are similar to that found by Quintana *et al.* (1998b) to be a determinant for zooplankton

composition and community structure. Thus, basins were intensively sampled within each sampling period. The sampling periods selected were:

The sea storm period. The first period was immediately after an intense winter sea storm which occurred on December 26th 2000. Fresh and marine water remained separated in two different layers just after the storm until December 28th. Samples were taken daily during this period.

The column mix period. The second period started just three days after the sea storm (December 30th 2000- January 11th 2001), when the increase of wind speed provoked the column homogenization and the marine and fresh water layers became mixed. Sampling dates were 30th December and 2, 4, 7, 9, 11 of January.

The winter calm period. The third period was 3 months after the first and second periods (20th- 23rd March 2001), during an anticyclone when the basins remained confined and did not have any kind of water inputs. Samples were taken daily during this period.

The summer desiccation. The fourth period was characteristic of maximum confinement, during early summer, between June 20th and July 7th 2001, when basins were close to desiccation. Only evaporation occurred and no water inputs affected the basins during this period. Sampling dates were 20, 22, 26, 28 of June and 1, 4 of July.

To take into account the day-night variability, samples were taken twice on each sampling day, one approximately at 6 h, immediately before sunrise (night sample), and another one at 18 h immediately before sunset (day sample). Two different depths were sampled, 10 cm below the water surface (surface sample) and 10 cm above the sediment (bottom sample). During the summer desiccation period only one layer was sampled because the water column was not deep enough (average depth of 15 cm along this period). Two replicas of each sample were taken.

4. Results

Differences among basins in physico-chemical composition.

All nutrients analyzed, the g_{440}, the $\%O_2$ and the pH showed significant differences between basins, being all, except nitrate, higher in Litoral, the small

basin with the highest CF ratio (Table 10). On the other hand, there were no significant differences of temperature and conductivity between the basins.

	Litoral		*Turies*		*P-values*
Number of cases	98		98		
Temperature (°C)	**15.0**	5.7-65.7	**14.4**	6.4-28.4	n.s.
Conductivity (mS cm^{-1})	**43.97**	7.99-81.00	**45.60**	28.00-65.30	n.s.
pH	**7.51**	3.00-9.40	**7.87**	6.13-10.30	0.0038
% O_2	**98**	2-554	**84**	29-220	<0.0001
NH_4^+ (µM)	**4.60**	<1.15-41.15	**1.45**	<1.15-5.29	<0.0001
NO_2^- (µM)	**1.98**	0.27-10.02	**0.52**	<0.19-2.30	<0.0001
NO_3^- (µM)	**<0.45**	<0.45-2.57	**0.89**	<0.45-4.78	0.0004
DIN (µM)	**7.13**	<1.88-50.77	**2.86**	<1.88-7.65	<0.0001
SRP (µM)	**5.32**	1.27-26.50	**1.69**	0.45-6.19	<0.0001
TOC (ppm)	**29.95**	7.08-112.33	**17.71**	9.23-380.50	<0.0001
DOC (ppm)	**27.17**	6.73-70.90	**18.85**	9.19-536.10	<0.0001
g_{440} (m^{-1})	**6.06**	1.17-13.98	**2.62**	0.93- 4.66	<0.0001
DOC: DIN mol	**533**	52-1201	**474**	188-739	n.s.
DOC: SRP mol	**529**	179-1129	**891**	300-2210	<0.0001

Table 10. Median (in bold) variation range and P-values of ANOVA (p<0.05) of several physical and chemical variables in the studied basins.

TOC and DOC had, always, similar values, suggesting that the main part of total organic matter was in dissolved form. The DOC:DIN ratio was similar between the two basins, but the ratio DOC:SRP was significantly higher in Turies.

The evolution along time of some environmental parameters is represented in Fig. 17. Nitrate showed only one peak during the sea storm period, coinciding with allochthonous inputs. After that, nitrate concentrations of the two basins remained close to zero the rest of the sampling periods. SRP concentration was high during the sea storm period in Litoral but reached its maximum during the summer desiccation period in both. SRP values were low in both basins during the winter calm period and the column mix period. DOC concentration was lower during sea storm period and started to increase during winter calm period, reaching its maximum during summer desiccation period, when the confinement of the basins was the highest.

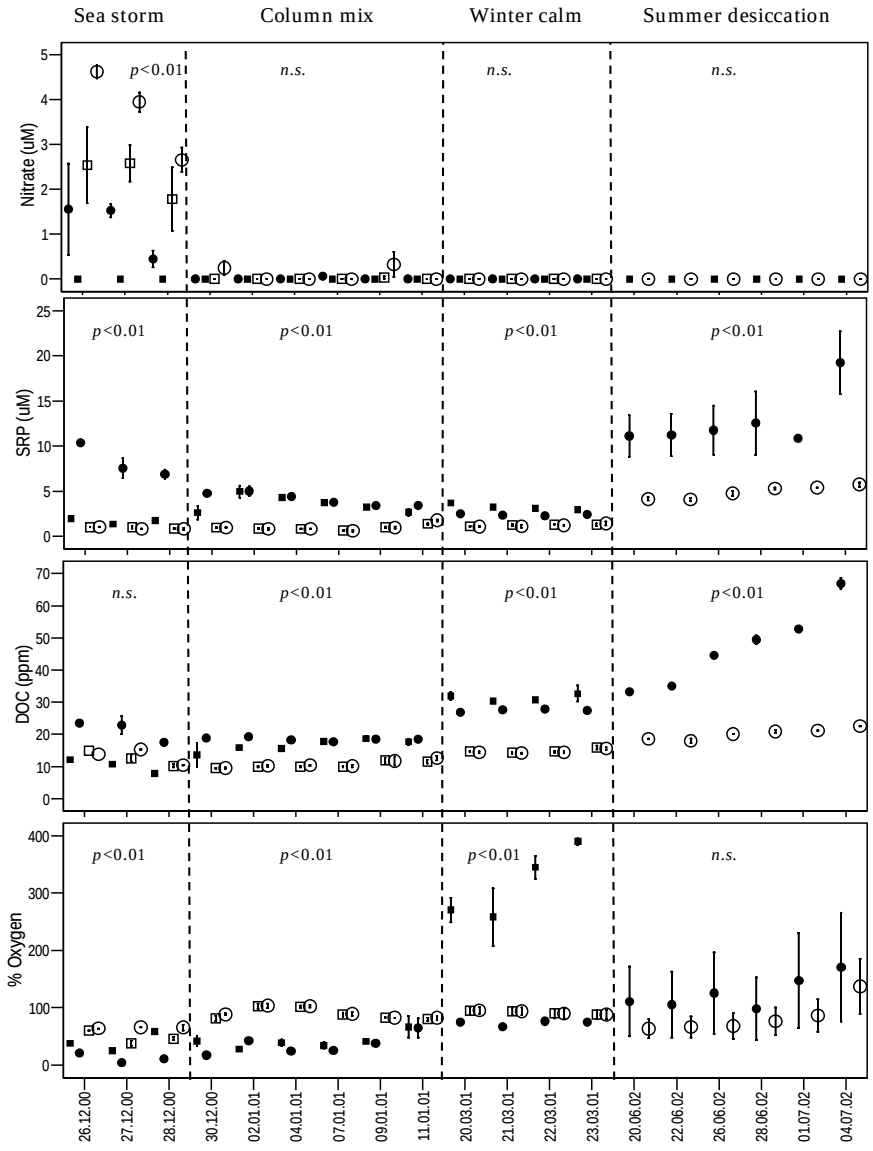

Fig. 17. Error bars showing the evolution of nitrate, SRP, DOC and % oxygen during the four periods. o represents the top layer and □ represents the bottom layer. Empty symbols belong to Turies and the full ones to Litoral basin. Significance test (ANOVA) data, for differences of the variables between basins in each period, are shown ($p<0.05$).

Nitrate concentration was significantly higher in Litoral than in Turies during the sea storm period (Fig. 17). During the other periods there was no significant differences of nitrate concentration between basins. SRP concentration was significantly higher in Litoral during the four periods. Similarly, DOC concentration was significantly higher in Litoral during all the sampling periods, except during the sea storm period. The $\%O_2$ was significantly higher in Turies during sea storm and column mix periods and in Litoral during winter calm period. During summer desiccation period, no significant differences were found between the $\%O_2$ of the two lagoons.

Regarding depth, significant differences were found in three periods in Litoral basin (Table 11). During the sea storm period, the surface layer had lower conductivity and $\%O_2$ and higher nitrate, SRP and DOC than the bottom layer.

LITORAL									
	Sea storm period			Column mix period			Winter calm period		
	top	bottom	*p*	top	bottom	*p*	top	bottom	*p*
NO_3^- (µM)	<0.45	1.14	<0.01	<0.45	<0.45	n.s.	<0.45	<0.45	n.s.
SRP (µM)	7.85	1.67	<0.01	4.14	3.59	0.04	2.38	3.24	<0.01
DOC (ppm)	20.86	9.42	<0.01	18.55	16.31	0.02	27.43	31.39	<0.01
% O_2	10	41	<0.01	35	41	n.s.	73	315	<0.01
Conductivity ($mS \cdot cm^{-1}$)	35.40	50.60	<0.01	43.92	47.02	<0.01	24.23	27.08	<0.01
TURIES									
	Sea storm period			Column mix period			Winter calm period		
	top	bottom	*p*	top	bottom	*p*	top	bottom	*p*
NO_3^- (µM)	3.57	2.53	n.s.	0.49	<0.45	n.s.	<0.45	<0.45	n.s.
SRP (µM)	0.88	0.87	n.s.	1.00	0.95	n.s.	1.22	1.25	n.s.
DOC (ppm)	12.61	12.13	n.s.	10.68	10.42	n.s.	14.64	14.87	n.s.
% O_2	65.40	47.84	<0.01	91.46	89.53	n.s.	91.93	91.54	n.s.
Conductivity ($mS \cdot cm^{-1}$)	47.52	50.40	<0.01	51.85	54.15	n.s.	30.44	30.14	n.s.

Table 11. Mean and *p*-values of ANOVA ($p<0.05$) of some environmental parameters for the top and bottom layers of Litoral and Turies basins during the three first studied periods.

Likewise, during column mix period SRP and DOC concentrations were also significantly higher in the surface layer. During the winter calm period SRP and DOC concentrations, and $\%O_2$ were higher in the bottom layer of the basin. On

the other hand, in Turies the $\%O_2$ was significantly higher and the conductivity was significantly lower in the top layer, but only during the sea storm period. No other differences between layers were detected in Turies basin. DOC differences among layers in Litoral were higher than differences among both basins.

Regarding the day-night samples, no significant differences were found for any of the variables analysed and for any period, with the only exception of the summer desiccation period in Litoral (Table 12). There, O_2 oversaturation took place during the day, but O_2 values close to anoxia and higher SRP concentrations were found at night. These SRP differences were greater than that found between different periods and basins.

Summer desiccation period in Litoral	*day*		*night*		*p*
% O_2	247	193-335	5	2-6	<0.001
NO_3^- (µM)	<0.45	<0.45-<0.45	<0.45	<0.45-<0.45	n.s.
SRP (µM)	9.09	7.06-13.30	18.32	15.09-25.19	0.001
DOC (ppm)	48.03	33.72-69.32	45.96	32.82-64.59	n.s.
Phytoplankton biomass ($x10^6 \cdot \mu m^3 \cdot ml^{-1}$)	13.8	5.4-34.5	14.7	6.6 -33.7	n.s.
Bacterioplankton biomass ($x10^4 \cdot \mu m^3 \cdot ml^{-1}$)	18.5	7.7-42.3	17.9	11.5-28.2	n.s.

Table 12. Mean (in bold) variation range and *p*-values of ANOVA (last column, $p<0.05$) of some environmental parameters and the phytoplankton and bacterioplankton biomass between day-night for Litoral basin during summer desiccation period.

Phytoplankton and bacterioplankton biomass.

Variations of phytoplankton and bacterioplankton biomass among the four periods are shown in Fig. 18. Generally, phytoplankton biomass increased from the sea storm period to the summer desiccation period. On the other hand, bacterioplankton biomass was the highest during these two periods. Phytoplankton biomass was higher in Turies than in Litoral during the sea storm and column mix periods, and lower during the winter calm and summer desiccation periods (Fig. 18). During the sea storm period, bacterioplankton biomass was significantly higher in Litoral, where organic matter was also the highest, while during the winter calm period bacterioplankton was higher in Turies than in Litoral. The two other periods did not show significant differences regarding bacterioplankton biomass. Neither bacterioplankton nor

phytoplankton biomass showed significant differences in response to day-night or depth.

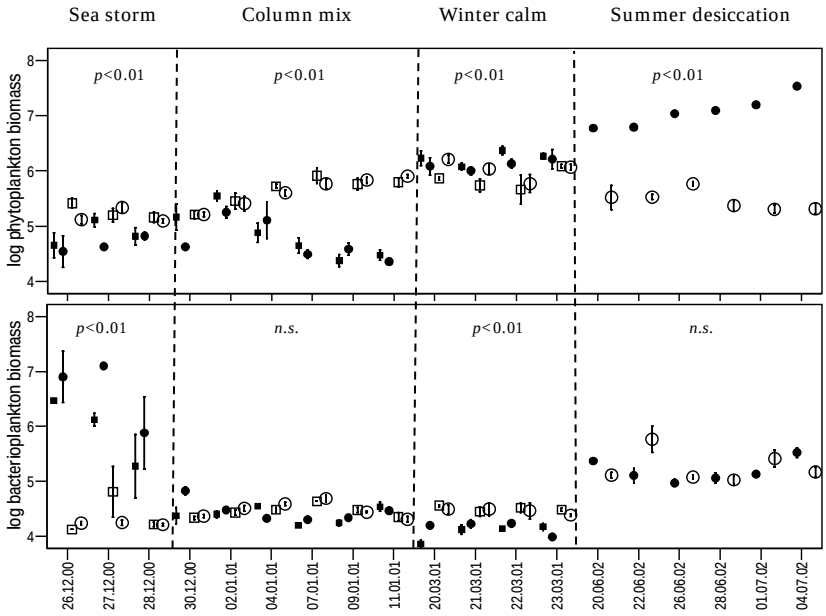

Fig. 18. Error bars showing the evolution of bacteria and phytoplankton during the four studied periods. O represents the top layer and □ represents the bottom layer. Empty symbols belong to Turies and the full ones to Litoral basin. Significance test (ANOVA) data, for differences of phytoplankton and bacterioplankton biomass between basins in each period, are shown ($p<0.05$).

Results from the MANCOVA (Table 13) showed that the interactions of the factor period with the covariable DOC were the only significant correlation to bacterioplankton biomass. Regarding phytoplankton, the interactions of basin x period and basin x DOC were significant for the model. The rest of the factors, covariables and their combinations were not significant.

BACTERIOPLANKTON				
Source of variation	***df***	***F***	***p***	**Partial ETA squared**
Period x DOC	3, 57	4.292	0.008	0.172
PHYTOPLANKTON				
Source of variation	***df***	***F***	***p***	**Partial ETA squared**
Period x Basin	3, 57	3.983	0.012	0.162
Basin x DOC	1, 57	7.509	0.008	0.108

Table 13. Results of multivariate analysis of covariance (MANCOVA) of bacterioplankton and phytoplankton against environmental variables (see Methods). Only significant differences with variables were shown ($p<0.05$).

Results from the ANCOVA (Table 14) reinforced those of the preceding multivariate analysis for phytoplankton, confirming its relation with DOC. On the other hand, bacterial biomass showed a significant relationship with phytoplankton biomass and mainly with the interaction of the factor period and the phytoplankton biomass.

BACTERIOPLANKTON				
Source of variation	***df***	***F***	***p***	**Partial *eta* squared**
Period x Basin	3, 17	8.300	0.000	0.193
Period x Phytoplankton biomass	3, 17	3.835	0.012	0.100
Phytoplankton biomass	1, 17	6.384	0.013	0.058
PHYTOPLANKTON				
Source of variation	***df***	***F***	***p***	**Partial *eta* squared**
Period x Basin	3, 17	7.813	0.000	0.184
DOC	1, 17	5.192	0.025	0.048

Table 14. Results of univariate analysis of covariance (ANCOVA) of bacterioplankton and phytoplankton against period, basin and DOC, and phytoplankton and bacterioplankton biomass respectively (see Methods). Only significant differences with variables were shown ($p<0.05$).

A positive relationship was found between phytoplankton biomass and DOC (Fig. 19) and this relationship increased when samples from the top layer during stratification events were excluded from the analysis. Although data of fresh water inputs was not available, these inputs often increased after sea storms,

coinciding with fresh water runoff. The relationship between bacterioplankton biomass and DOC was highly variable among periods and was lower than that found with phytoplankton (Fig. 19). Significant differences between periods obtained with the MANCOVA (Table 13), could be also observed (Fig. 20).

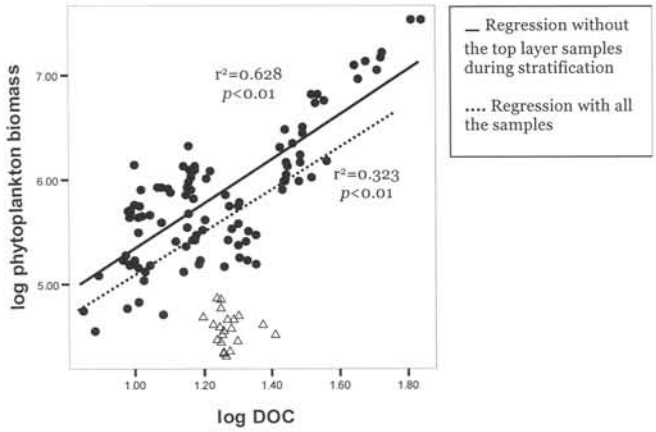

Fig. 19. Regression between phytoplankton biomass and DOC during the four periods in Litoral basin. Both variables are in logarithmic values. + correspond to the samples of the top layer during stratification and # to the rest of the samples.

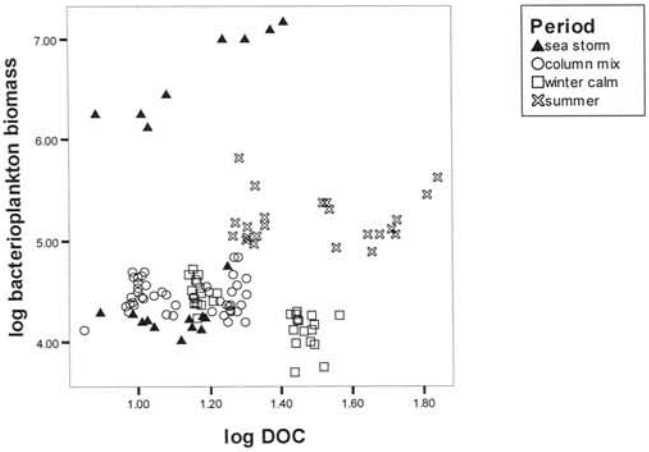

Fig. 20. Regression between bacterioplankton biomass and DOC during the four periods in Litoral basin. Both variables are in logarithmic values.

When the relationship between bacterioplankton and phytoplankton was represented, large differences were found among periods and basins (Fig. 21). In Litoral, phytoplankton dominance was documented during stability periods (winter calm and summer desiccation periods). On the other hand, dominance of bacterioplankton (sea storm period) or similar values of both biomasses (column mix period) was found during disturbance periods, when the input of allochthonous organic matter was the highest. Phytoplankton biomass always dominated Turies, except during summer desiccation period when similar dominances were found.

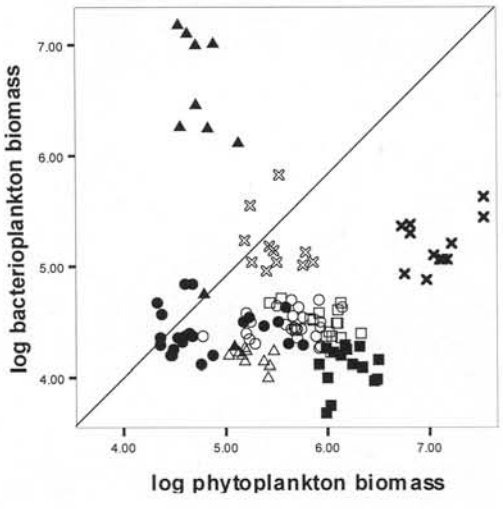

Fig. 21. Regression showing the relationships between phytoplankton and bacterioplankton biomass during the four periods. Both variables are in logarithmic values. Symbols represent periods and basins, full symbols belong to Litoral and empty symbols to Turies; and the shape corresponds to the different periods (↖ Sea storm, ↓ Column mix, ɜ Winter calm and ⊗ Summer desiccation).

The regressions between bacterio- and phytoplankton biomass showed variable and even opposite correlation among the different periods (Table 15). Correlation was negative during the winter calm period in Turies and during the sea storm period in both basins, but positive during the summer desiccation period in Litoral. No correlation was significant during the other periods.

	Litoral basin			*Turies basin*		
Period	***b***	***r***	***p***	***b***	***r***	***p***
Sea storm period	-1.472	0.379	0.100	-0.215	0.401	0.080
Column mix period	-	-	n.s.	-	-	n.s.
Winter calm period	-	-	n.s.	-0.249	0.476	0.006
Summer desiccation period	0.332	0.362	0.082	-	-	n.s.

Table 15. Slopes, correlation coefficients and significances ($p<0.1$) of the regressions between phytoplankton and bacterioplankton biomass, for each period, in Litoral and Turies basins.

5. Discussion

Nutrient cycling in Mediterranean marshes.

Results showed remarkable differences in nutrient and organic carbon accumulation among basins. Differences in catchment area, specifically in CF ratio appeared to be the more plausible explanation of this fact, since during confinement basins essentially accumulate the allochthonous nutrient and organic matter supplies that previously entered by runoff. Differences among basins are appreciable not only in average contents, but also in its variability during the year. Temporal variations in nutrient and organic matter accumulations may be related to variations in the hydrological pattern. Sudden flooding during sea storms and rainfalls causes an increase in the input of organic matter and inorganic nutrients; after that, the progressive isolation of basins, undergoes an intense confinement, during which the accumulation of the allochthonous organic matter and the depletion of inorganic nutrients occurs (Quintana *et al.*, 1998a; Quintana and Moreno-Amich, 2002). Water permanency or substrate granulometry has been described as a determinate of the mainly sediment-nutrient contents in these marshes and have less effect in the water nutrient composition (Gascón, 2003). Likewise, other factors not analysed here, such as vegetation biomass and productivity in the catchment area or interactions between the pelagic and the littoral zones, must also affect nutrient accumulation in these marshes (Mitsch and Gosselink, 1993). According to Wetzel (1992) most of the DOC of the lacustrine ecosystem is derived from photosynthesis of higher plants and microflora associated with detritus.

The role of DOC

DOC favours the bacterioplankton biomass abundance, but also is the main variable accounting significantly for phytoplankton biomass. The relationship between bacterioplankton biomass and DOC is well known in aquatic ecosystems (Wetzel, 1992; Mayer *et al.*, 1997; Kopylov *et al.*, 2002). However, this relationship is not so clearly established for phytoplankton, since phytoplankton biomass is habitually related to inorganic nutrient availability (Capblancq, 1990; Comín and Valiela, 1993; Vrede *et al.*, 1999). The significant correlation between DOC and phytoplankton biomass could be related to a high mixotrophic activity by phytoplankters. Although this fact is still not fully documented, most phytoplankton groups found in these basins and in nearby similar ecosystems are potentially mixotrophic (Quintana and Moreno-Amich, 2002). The significant effect of phytoplankton on bacterioplankton, but not *vice versa*, as found in the ANCOVA tests also agrees with a high phagotrophic activity in phytoplankton. Mixotrophy have also been described to be important in water bodies with a high degree of allochthonous organic matter supply (Isaksson, 1998; Jones *et al.*, 1998; Isaksson *et al.*, 1999; Jones, 2000; Hitchman and Jones, 2000). It is also known that a high ratio of organic:inorganic nutrients and the light limitation due to the presence of soluble organic matter affects the success of mixotrophic species (Isaksson, 1998; Granéli *et al.*, 1999; Jones, 2000), and both values are high in these marshes. The strong relation between DOC and phytoplankton biomass became weak during the stratification, when inputs of organic matter into the surface layer were the highest. Two hypotheses would explain this fact. Firstly, the high organic matter input rate during a hydrological disturbance probably overcame the phytoplankton heterotrophic organic matter assimilation capacity. Secondly, most of the organic matter inputs in these conditions should be recalcitrant, coming from the degradation of the surrounding vegetation, such as humic acids or tannins, which according to Wetzel (1992) may slow down the bacterioplankton and phytoplankton heterotrophic growth (although they can be an important DOC source for these organisms in wetlands).

Phytoplankton-bacterioplankton interactions

Basins showed marked differences in bacterio- and phytoplankton dominance. Whilst Turies was mainly dominated by phytoplankton, this was highly irregular

in Litoral, in the same way, the nutrient and organic matter contents, were also highly variable. Although diel oscillations or differences in depth caused changes in nutrient composition, they did not have a substantial effect on phyto- and bacterioplankton biomass variations. As in the case of nutrients, differences in biomass dominance among basins may also be related to differences in CF ratio. Higher inputs of allochthonous resources would cause greater variability in phytoplankton:bacterioplankton ratios. A higher CF ratio would allow a higher immediate development of bacterioplankton biomass after flooding periods, but it also would provide the nutrient sources for posterior autotrophic production along the confinement periods. On the other hand, a minor CF ratio would suggest less input loading of organic matter and nutrients, and these minor external exchanges would result in a more "buffered" community where changes are slower and less intense. Thus, the more input of allochthonous supplies, the higher is the variability of the microbial biomass dominance.

Relationships between phytoplankton and bacterioplankton were positive, negative or not significant, depending on the basin and on the period of the year. Strong phagotrophy by mixotrophic phytoplankton could explain negative relationships between both trophic groups, since bacterial ingestion rates by phytoplankton may be especially high (Hitchman and Jones, 2000; Burkert *et al.*, 2001). Competence in inorganic nutrients uptake may also explain this negative relationship (Fuhrman, 1992). However, the high correlation between DOC and phytoplankton and the lack of significance of inorganic nutrients, in the general MANCOVA and in the ANCOVA test, reduce the reliability of this hypothesis. On the other hand, a positive relationship among phyto- and bacterioplankton suggested that, in some situations, phytoplankton growth is mainly autotrophic, favouring synergy between autotrophic phytoplankton growth and heterotrophic bacterioplankton growth; each profiting each other (Capblancq, 1990). Many intermediate situations may occur between a predominance of autotrophic growth and a strong phagotrophy by phytoplankton leading to a relationship that is not significant between phytoplankton and bacterioplankton biomass.

VI. Harmful Algae: Is Microtox® toxicity related with potentially harmful algae proliferation in Empordà salt marshes?

1. Introduction

Mediterranean salt marshes are ecosystems that are highly influenced by sea exchanges and freshwater inputs from runoffs (Britton and Crivelli, 1993). The composition of the phytoplankton community of these areas is determined to a large extent by the hydrological variability (chapters IV & V). During disturbance periods the lagoons are flooded mainly by seawater. Then, the salt marsh basins become confined (which is most extreme during summer) leading to high concentrations of organic matter, nutrients and organisms in the water (Quintana *et al.*, 1998a). The proliferation of harmful phytoplankton in nearby Mediterranean beaches and harbours has been reported by several authors (Garcés *et al.*, 2000; Vila *et al.*, 2001a). Therefore, we can expect that harmful marine organisms, which are introduced into the lagoons during the disturbance periods, will proliferate while the lagoon is confined.

Previous studies have identified potentially harmful phytoplankton taxa in the confined lagoons of the Empordà salt marshes (Domínguez, 1987; Quintana and Moreno-Amich, 2002). Some authors have also reported HAB (Harmful Algae Blooms) events in other Mediterranean coastal lagoons e.g. (Comín and Ferrer, 1978; Sarno *et al.*, 1993). However, little is known about potentially harmful algae developing a high biomass and toxicity in confined Mediterranean salt marshes, in which the species composition is highly variable both in terms of space and time. In fact, this variability makes it difficult to monitor the toxicity in these ecosystems, since a method which allows extensive and non-time consuming measures is needed.

The Microtox® test have been described as a successful, quick method for detecting cyanobacterial toxicity (Lawton *et al.*, 1990; Volterra *et al.*, 1992; Bruno *et al.*, 1994; Campbell *et al.*, 1994), dinoflagellates toxicity (Bruno *et al.*, 1990; Giacobbe and Yang, 1999; Derby *et al.*, 2003), and diatoms toxicity (Gallina, 2002). Thus it could be a feasible method for taking an initial measurement of toxicity in the Empordà salt marshes, where the different types of waterbodies (Trobajo *et al.*, 2002; Gascón *et al.*, in press) and the high variability of the phytoplankton community in the different environmental conditions (Gascón, 2003; Chapter IV & V) make it necessary to use an extensive toxin detection method.

The aims of this study were 1) to determine the efficiency of Microtox® for detecting phytoplanktonic toxins in the water of several confined Mediterranean salt marsh basins and lagoons; 2) to analyse the phytoplankton taxa composition during the periods of high biomass development, during fish kill episodes and in the periods when Microtox® toxicity was detected; and 3) to discuss the possible relationship between Microtox® toxicity and the proliferation of potentially harmful phytoplankton species.

2. Study site and sampling

The study was carried out in the whole Empordà wetlands (Fig. 22; see Study Site). Five lagoons were sampled within La Pletera salt marshes, two old, O1 and O2, which are more eutrophic, and three new, N1, N2 and N3, which are less eutrophic (chapter IV). In the Alt Empordà salt marshes, four lagoons were sampled, Litoral and Rogera, which were more eutrophic, and Turies and Connectada which are less eutrophic (Gascón, 2003; Trobajo, 2003; chapter V).

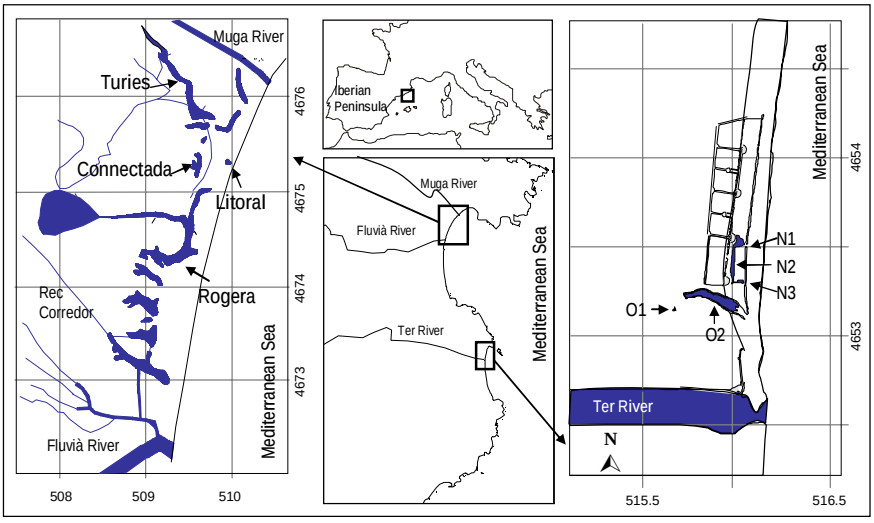

Fig. 22. Collection sites in the Alt Empordà and La Pletera salt marshes. Grid indicates UTM coordinates (1000 m).

Samples were taken during two monitoring campaigns (chapters IV & V) between December 2000 and December 2003, at a central point of each lagoon

at a depth of 15-30 cm. Some additional samples were also taken, coinciding with fish kill episodes (e.g. Rogera lagoon in August 2001).

3. Results

The phytoplankton species identified are shown in Table 16 and in the Appendix. The potentially harmful species belonged mainly to cyanobacteria and dinoflagellate classes and were found in both salt marshes, however, they were not detected in all the lagoons. Diatom species from the genus *Chaetoceros* were also detected forming a bloom in the N3 lagoon during spring 2003, however, no fish kills were related to this genus. The harmful cyanobacteria genera identified were *Anabaena* with low densities in different lagoons in both salt marshes, and *Aphanocapsa*, whose blooms joined with *Dactylococcopsis* cf. *raphidiodes* during a fish kill in Rogera in August 2001. Haptophytes from the genus *Prymnesium* were also found in high densities in O1 and with a lower abundance in the new lagoons (N1, N2, N3). *Alexandrium pseudogonyaulax* and *Prorocentrum minimum* were identified in the new lagoons of La Pletera salt marshes, the last developing high biomass in the three lagoons. The *Protoperidinium*-like was found in O2 every summer, between the years 2001 and 2003. At least two different species from the genus *Gymnodinium* were identified in most of the lagoons, however only the species *G. instriatum* was identified to species level.

	Species	O1	O2	N1	N2	N3	Litoral	Turies	Rogera	Connectada
Diatoms	*Amphora* spp.	+			+		b	+		+
	Chaetoceros sp.			+		B				
	Cocconeis placentula		++	+	++	b	++	+		
	Cyclotella spp.	+	++	+	+	+			++	
	Entonomeis spp.	+		+		+	++	+		
	Navicula spp.	++	++	+	++	+	+	++	++	+
	Nitzchia closterium	++	++	B	++	++	b	b		
	Nitzchia sp.	+	+	+	+	+	+	+		
Chlorophytes	*Nannochloris* sp.	B	B							
Chrysophytes	*Ochromonas* spp.		++			b	b	b		
Cryptophytes	*Chroomonas* sp.	b	b	++	+	+	b			
	Hemiselmis sp.						++	+		
Cyanobacteria	***Anabaena* spp.**	+	+					+		
	***Aphanocapsa* sp.**								B	
	Dactylococcopsis cf. *raphidioides*								B	
	Merismopedia spp.			+	++	++		+		
	***Synechococcus* sp.**					+		+		
Dinoflagellates	***Alexandrium pseudogonyaulax***			++	+	++				
	Protoperidinium-like*		b	+		+				
	***Amphidinium* sp.**		B			+		b		
	Glenodinium foliaceum		B	+	++		b	++		b
	***Gymnodinium* spp.**	B	B	b	b	+	++	++		
	Gymnodinium instriatum		++							
	Gyrodinium sp.				++					
	***Heterocapsa* spp.**	+		b	+					
	Oxyrrhis marina	b	B	b	b	+	B	+		
	Prorocentrum micans	+	+					+		
	Prorocentrum minimum		+	B	B	B				
	Scripsella spp.	+	++	+	b	+		++		
Euglenophytes	*Euglena* cf. *proxima*		+	+						
Prasinophytes	*Pyramimonas* spp.	b		B	b	b	B	b		
	Tetraselmys cf. *gracilis*	b			++			++		
Haptophytes	***Chrysochromulina* sp.**						+			
	Pavlova cf. *lutheri*							+		++
	***Prymnesium* sp.**	B		+	+	+				

Table 16. List of the phytoplankton taxa in the different lagoons studied. Potentially harmful species are in bold (Hallegraeff *et al.*, 2003). The symbol + corresponds to mean values $<10^5$ cells l^{-1} and ++ to mean values between 10^5 and 10^6. Blooms are identified with b and B, if the number of cells per litre is between 10^6 and 10^7, and $>10^7$ respectively. *The thecal plates arrange showed by these individuals was similar to that of *Protoperidinium*, however its taxonomic identification is currently being studied.

The Microtox® test obtained positive values for the particulate fraction in samples from the two salt marshes (Table 17), however, not all the lagoons had positive results. The results for the soluble fraction were negative, indicating that positive toxicity results are only related to the particulate fraction.

				Biomass (µg·ml^{-1})				
lagoon	**date**	**EC50$_5$**	**TU$_5$**	*Gymnodinium* spp.	*P. minimum*	*Protoperidinium* -like	*Prymnesium* sp.	*Aphanocapsa* sp.
O2	17.07.03	2.74	36.54			0.01		
O1	17.07.03	6.71	14.91				0.16	
O2	18.09.02	7.85	12.75	172.65				
O1	18.09.02	10.48	9.54	4.05				
O1	20.08.03	15.58	6.42				0.15	
O2	18.06.03	17.64	5.67			0.18		
O2	18.08.01	24.24	4.10			9.35		
N1	14.05.03	36.30	2.75	0.79	0.12			
O2	15.10.02	40.39	2.48	45.56				
Litoral	01.07.02	50.23	1.99	24.24				
O2	15.07.02	51.29	1.90			17.38		
O2	13.08.02	52.06	1.92	5.69				
Rogera	18.08.01	52.59	1.90					33.45

Table 17. List of samples with TU_5>1 ($EC50_5$<100 ml). $EC50_5$ is the Effective Concentration expressed as ml of a sample and TU_5 corresponds to $EC50_5^{-1}$. The biomass of each potentially harmful species is also shown.

Samples from the less eutrophic lagoons (N2, N3, Turies and Connectada), never had values of TU_5>1 ($EC50_5$<100 ml). In one case toxicity appeared in later spring (May 2003 in lagoon N1), but generally high TU_5 coincided with later or mid summer.

Heavy metal concentrations in the lagoons were compared with the corresponding $EC50_5$ found in the literature (Ronco, 1992; Villaescusa *et al.*, 1996; Villaescusa *et al.*, 1997; Villaescusa *et al.*, 1998), in order to measure their contribution to sample toxicity (Table 18). Generally, concentrations did not

reach the method's detection limit. Only chrome concentrations were detectable in some lagoons, however they never reached the $EC50_5$ values established in studies with Microtox®.

Lagoon	*Source*	*Cadmium* ($mg \cdot l^{-1}$)	*Chrome* ($mg \cdot l^{-1}$)	*Copper* ($mg \cdot l^{-1}$)	*Nickel* ($mg \cdot l^{-1}$)	*Lead* ($mg \cdot l^{-1}$)
O1	this study	<0.02	<0.03	0.03	<0.02	<0.07
O2	this study	<0.02	<0.03	<0.02	<0.02	<0.07
N1	this study	<0.02	<0.03	<0.02	<0.02	<0.07
N2	this study	<0.02	<0.03	<0.02	<0.02	<0.07
N3	this study	<0.02	0.034	<0.02	<0.02	<0.07
Litoral	(Salvadó *et al.*, 2001)	<0.02	0.149	<0.02	<0.02	<0.07
Connectada	(Salvadó *et al.*, 2001)	<0.02	0.130	<0.02	<0.02	<0.07
Rogera	(Salvadó *et al.*, 2001)	<0.02	0.089	<0.02	<0.02	<0.07
Turies	(Salvadó *et al.*, 2001)	<0.02	0.148	<0.02	<0.02	<0.07
$EC50_5$ ($mg \cdot l^{-1}$)		7.82(1)	4.30(2)	8.00(3)	10.00(4)	0.36(4)

Table 18. Heavy metal concentrations in the studied lagoons. $EC50_5$ values calculated by means the Microtox® test, extracted from (1) Villaescusa *et al.*, 1996; (2) Villaescusa *et al.*, 1997; (3) Ronco, 1992; (4) Villaescusa *et al.*, 1998.

Often high TU_5 coincided with a high biomass of cyanobacteria, dinoflagellates or haptophytes. However, proliferations of some potentially harmful species were also identified in lagoons where TU_5 was close to zero (Table 19). In these samples, *P. minimum* was the main species found, but *A. pseudogonyaulax* and *Gymnodinium* spp. were also found.

		Biomass (µg·ml^{-1})		
Lagoon	**Date**	*Gymnodinium* spp.	*P. minimum*	*A. pseudogonyaulax*
N1	15.10.02			
	12.11.02		0.05	0.42
	19.02.03	0.76	0.10	
	17.03.03		3.76	
	15.04.03		4.48	
	18.06.03	27.98		
N2	19.02.03	0.83	0.42	
	17.03.03		2.45	
	15.04.03		8.96	
	18.06.03	18.46		
N3	18.09.02		0.02	0.12
	19.02.03		0.52	
	17.03.03		24.23	
	15.04.03		4.48	

Table 19. List of samples with high biomass of potentially harmful species and with $TU_5<1$ ($EC50_5>100$ ml). $EC50_5$ is the Effective Concentration expressed as ml of a sample and TU_5 corresponds to $EC50_5^{-1}$. The biomass of each potentially harmful species is also shown.

TU_5 was positively correlated with the biomass of bacterioplankton and phytoplankton (pico- and nano-), chlorophyll *a*, TOC concentration, conductivity and temperature (Table 20), while the water level was negatively correlated with TU_5. No significant correlations were found between TU_5 and the biomass of each harmful species, or with the total biomass of harmful species.

	TU$_5$		
	r^2	*p*	*N*
Bacterioplankton biomass	0.521	<0.0001	46
Conductivity	0.460	0.001	45
Chl a	0.454	0.005	37
TOC	0.441	0.002	45
Pico- and nanophytoplankton biomass	0.392	0.007	46
Water level	-0.401	0.019	34
Temperature	0.310	0.038	45

Table 20. Pearson's coefficients obtained from the correlation between TU_5 and several environmental parameters. Only significant results are shown ($p<0.05$).

Microcystin concentrations were under the method detection limit in all the samples in which a large cyanobacteria biomass was found and Microtox® toxicity was positive.

4. Discussion

In the Empordà salt marshes Microtox® toxicity occurs during summer coinciding with high temperatures, high bacterial biomass and high organic matter concentration, as well as high densities of chlorophyll *a* and high pico- and nanophytoplankton biomasses. The toxicity was not due to substances arriving in the water, since the Microtox® test was negative for the dissolved fraction. Moreover, toxicity was not caused by heavy metals in particulate matter, since concentrations were under the $EC50_5$ for the metals analysed. Previous studies have ruled out the presence of harmful concentrations of pesticides in the Empordà salt marshes (Salvadó *et al.*, submitted; López-Flores *et al.*, 2003). Anoxia is one of the most likely causes of fish and zooplankton mortality in this ecosystem because during summer high phytoplankton biomasses are reached and there are nocturnal anoxias. However, the lack of oxygen in the water has no consequences for Microtox® toxicity, thus positive values could not be related with it. The pulse rhythm of the phytoplankton proliferation and the spatial variability of the species composition did not allow us to correlate the harmful species biomass to Microtox® toxicity. Despite this, it was found a coincidence on time between HABs and Microtox® toxicity.

Previous studies have found a relationship between Microtox® toxicity and harmful algae proliferation (Bruno *et al.*, 1990; Bruno *et al.*, 1994; Gallina, 2002; Derby *et al.*, 2003). However, in the present study, some high densities developed by some harmful species could not be related to Microtox® toxicity (e.g. *P. minimum* proliferation). These negative Microtox® test results could be because there are no toxic substances or the substances are not toxic for *Vibrio fischeri*. *P. minimum* have often been related to fish mortality and the production of venerupin, a hepatotoxin (Tangen, 1983; Faust and Gulledge, 2002). This species has also been shown to contain a water soluble neurotoxic component which can kill mice (Heil *et al.*, 2005). However, its toxicity is known to be conditioned by the species clone and the toxic effects may be elicited under certain growth conditions (Glibert and Sellner, 2005).

These contradictions bring into doubt the efficiency of the Microtox® method in detecting harmful algae toxins. Moreover, some authors criticise immersion exposure methods, like Microtox®, since they suppose an indiscriminate whole organism exposure, while in natural conditions exposure to toxins is mainly through the grazer's digestive system (Caldwell *et al.*, 2004). In addition, Microtox® toxicity detection is limited because not all the phytoplankton toxins affect the viability of the tested bacteria in the same way. Other methods such as high performance liquid chromatography (HPLC), lipid emulsions and liposome or microparticulate zooplankter diets (Caldwell *et al.*, 2004) are more rigorous, but are only recommended if taxonomic knowledge of the community composition is available and the chemical structure of the toxin is well known, since the analysis is extremely focussed on a particular toxin or organism. Even with a perfect knowledge of the taxonomic composition, differences between species strains could lead to confused results. Moreover, these methods have the disadvantage of being more expensive, slow and generally not available for non-qualified technicians. As an example, the harmful species *A. taylori* in Marina di Melilli (Sicily, Italy) was related with positive Microtox® results, however, no PSP toxins were detected using HPLC (Giacobbe and Yang, 1999). In the present study, the positive Microtox® test results when there was a high cyanobacteria biomass did not coincide with the presence of microcystin in the lagoons.

Thus, it could be concluded that there are substances in the particulate matter of several lagoons in the Empordà salt marshes that produce toxicity detected by the Microtox® test. This Microtox® toxicity coincides with periods when potentially harmful cyanobacteria dinoflagellates and haptophytes have a high biomass. In addition, in some lagoons HABs and Microtox® toxicity co-occur with fish kills. The results suggest that potentially harmful phytoplankton cannot be ruled out as a source Microtox® toxicity and also as a source of other organism kills or sub-acute effects.

VII. Harmful Algae: Comparative composition and dynamics of harmful dinoflagellates in La Pletera salt marshes and nearby external marine waters.

1. Introduction

Harmful microalgae blooms (HAB) comprise a widely extended problem, and recent studies indicate an increasing tendency of these events worldwide (Smayda, 1997). These increments are, in most cases, related to anthropogenic activities. This includes eutrophication (Anderson *et al.*, 2002), and increases of confined areas associated with construction of jetties, leisure harbours etc. (Garcés *et al.*, 2000). It should, however, be noted that the increasing tendency is partially a consequence of the enhanced monitoring programmes. The increase of HAB events also applies to the Mediterranean Sea, where they are mainly associated with locations characterised by a restricted water exchange, such as harbours (Vila *et al.*, 2001a), small bays and protected beaches (Garcés et al. 1999), and coastal lagoons (Sarno *et al.*, 1993; Cabrini *et al.*, 1995; Giacobbe *et al.*, 2000).

Especially interesting are the Mediterranean confined lagoons, which remain isolated during most the year and concentrate phytoplankton to a very high biomass. These coastal lagoons have negligible tidal inputs (Britton and Crivelli, 1993), but they are occasionally flooded during sea storms, after which they usually recover their confined character without marine exchange (Picot *et al.*, 1990; Quintana *et al.*, 1998a; Pérez-Ruzafa *et al.*, 2002). During periods of confinement, water levels tend to decrease, subsequently enhancing conductivity, nutrient levels and phytoplankton concentration. Mediterranean coastal lagoons may, therefore, act as an accumulator of neritic phytoplankton. Sporadic connections of lagoons with external waters will lead to posterior concentration of a similar phytoplankton population found in the surrounding coastal regions (including species related to HAB). However, when the confined character of lagoons is more pronounced the deviating characteristics of their water bodies may lead to significant modifications of the phytoplankton composition (Chapters IV & V). Detailed analyses and comparisons of ecosystems are needed to understand why only specific lagoons are susceptible to development of HAB events.

Stability and nutrients levels are known to determine the phytoplankton succession in temperate coastal waters (Margalef, 1983; Reynolds *et al.*, 2002; Smayda and Reynolds, 2003). Nevertheless, little is known about the factors

influencing phytoplankton temporal patterns in confined coastal lagoons (Quintana and Moreno-Amich, 2002; chapter IV & V). In the present study the taxonomic structure of phytoplankton populations in two Mediterranean coastal lagoons are compared with those of the nearby external waters. Main aims were to determine if coastal lagoons actually act as concentrators of marine toxic dinoflagellates during confinement periods, and to establish the common environmental factors that favour growth of specific harmful species in the two ecosystems considered.

2. Study site and sampling

Two Mediterranean coastal lagoons located along La Pletera salt marshes (Fig. 23) were compared with the nearby external marine ecosystems. The present study included one natural- (O2: Fra Ramon 42°01'48"N 3°11'31"E) and one artificial lagoon (N3: Pletera Nova 42°01'51"N 3°11'40"E).

Regarding reference points for the external waters, two sampling stations were selected along the beaches of La Fosca (41°51'42"N 3°09'02"E) and l'Estartit (42°01'51" 3°11'45"). Both stations are located in areas where human population is highly intensified by seasonal tourism. The first one is an approximately rectangular protected pocket beach opening towards the SE, free from direct river influences, and dimensions of 525 x 300 m. The second one is an open long-shaped 3000 x 150 m beach orientated to the E, and suffering direct influences of the Ter river outlet. The routine sampling point of this specific beach was located at a distance of approximately 50 m from the two studied lagoons and 500 m N from the river outlet. Both beaches were intensively studied during the summer months, when they are known to be affected by a dense dinoflagellate bloom that last almost 3 months. These blooms are dominated by *Alexandrium taylori*, whereas *Gymnodinium* sp. is the accompanying dinoflagellate species (Garcés *et al.*, 1999).

Between June 2002 and December 2003 samples were collected on a monthly base, at a central point of each lagoon (depth of 15-30 cm). During 2003 surface samples were collected, on a monthly base, at fixed stations (water depth 1 m) along the marine beaches.

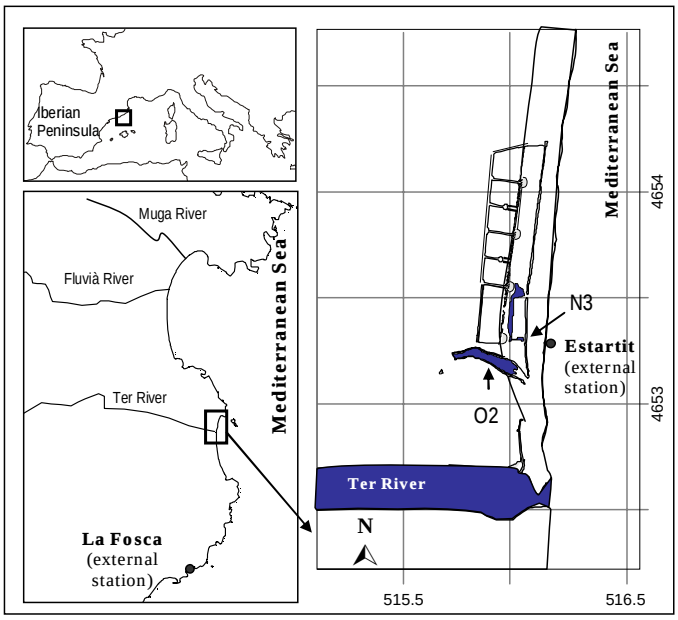

Fig. 23. Geographic location of the sampling sites in La Pletera saltmash and nearby external Mediterranean water. Grid indicates UTM coordinates (1000 m).

3. Results

Confinement conditions.

Confinement of coastal lagoons, in the present study, was defined as an isolation period without marine water exchange and characterised by low water levels and high conductivity conditions (Fig. 24). The shaded area in the figure marked the most pronounced period of confinement, characterised by high values of SRP (Soluble Reactive Phosphorus) and bacteria biomass. During June, the first month of this period, the concentration of DIN (Dissolved Inorganic Nitrogen) was high, and mainly present in the regenerated form ammonia. After that concentrations of DIN dropped close to zero during the rest of the confinement period. Contrarily, during the winter season concentrations of DIN were mainly found in the form of nitrate.

During this period the external waters (coastal stations) are characterized by high DIN and SRP concentrations, high concentration of bacteria, and high conductivity.

The microplankton community and harmful dinoflagellate species.

The abundance of dinoflagellates was generally higher in the lagoons than in the coastal waters (Fig. 25). The maximum abundances of dinoflagellate (cell numbers) detected at the four stations sampled did not coincide in time. The two external water stations showed a maximum during summer. At l'Estartit detection of the maximum occurred in June, after which the dinoflagellates showed a marked decrease, whereas the La Fosca maximum occurred in August. The O2 lagoon, on the other hand showed a pronounced increase in dinoflagellate cell numbers after the winter, coinciding with the firsts moments without water exchange, although the absolute maximum was not attained until September. At N3 dinoflagellate abundance was high during summer, but this location showed maximum values during spring. Although maximum values did not coincide, both lagoons initiated a relative pronounced increase of dinoflagellates in June, when the nearest external water station (l'Estartit) already showed maximum values. Periods of maximum dinoflagellate cell numbers were generally altered with periods dominated by diatoms, especially in coastal stations.

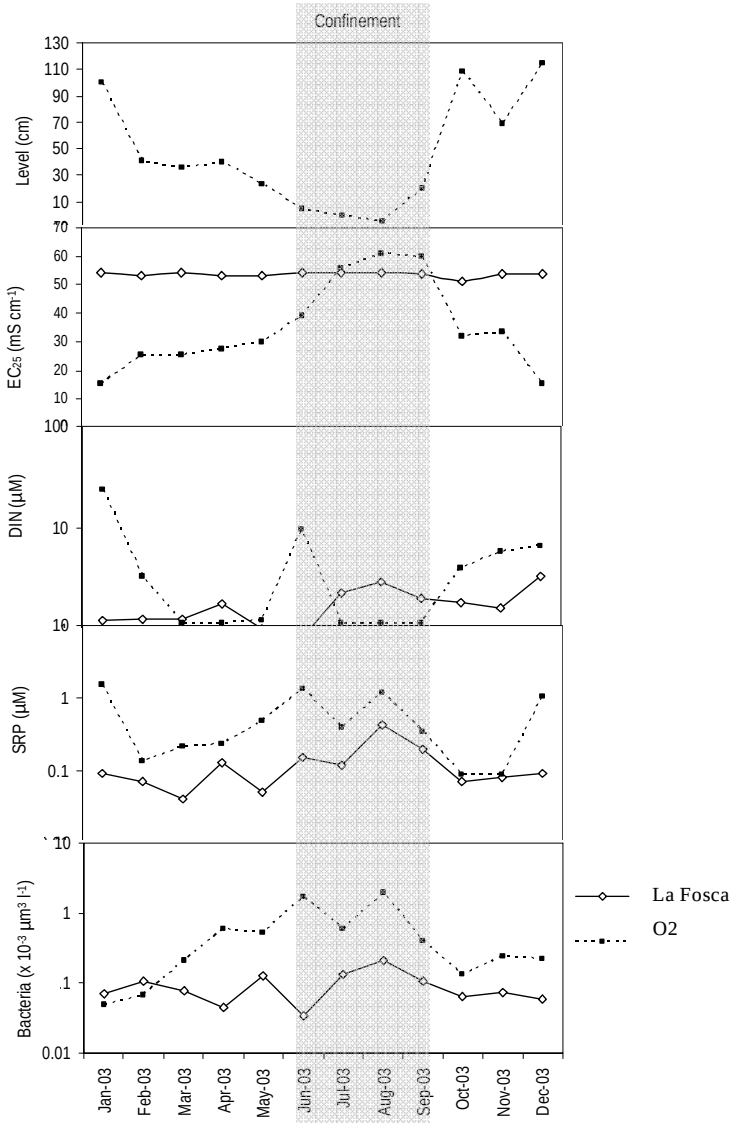

Fig. 24. Evolution of water level (lagoon values with respect to sea levels outside), conductivity, inorganic nutrients and bacteria along the studied period. Represented data correspond to results obtained in one coastal lagoon (O2) and at one coastal sampling point (La Fosca). The months where the lagoons were most affected by confinement were represented in grey.

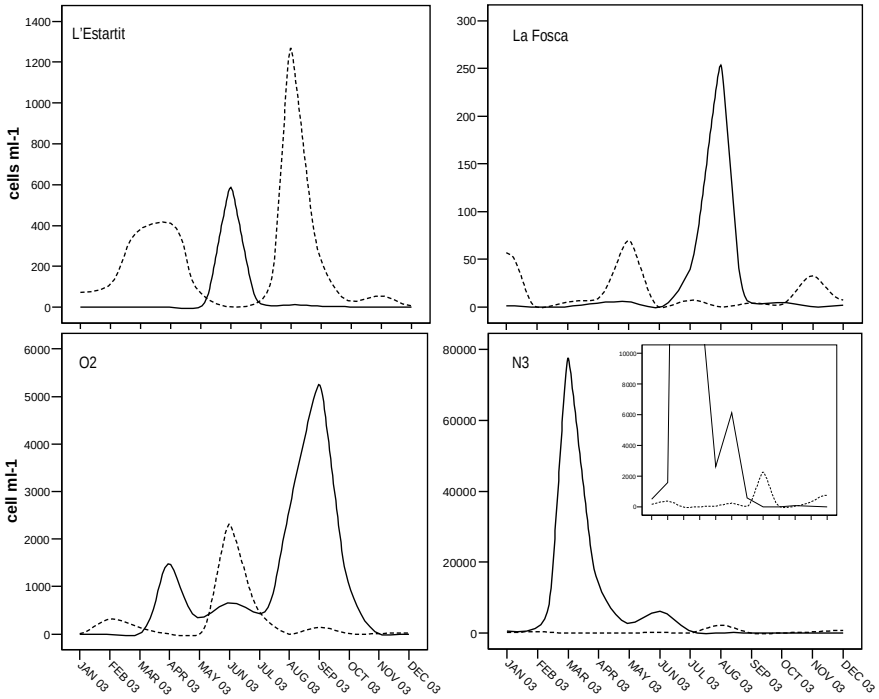

Fig. 25. Evolution of dinoflagellates (continuous) and diatoms (discontinuous) along the sampled period in the two coastal water points and the two lagoons. Note that scale bars are different.

The different dinoflagellate species detected showed different periods of permanence in the ecosystems studied. At O2, *Oxyrrhis marina* and *Glenodinium foliaceum* were present in more than 40% of the samples collected (Table 21), whereas N3 was dominated by *Prorocentrum minimum* and *Alexandrium pseudogonyaulax*, 66% and 28% of the time, respectively. At l'Estartit, the three main taxa *Alexandrium taylori*, *Prorocentrum minimum* and *Gymnodinium* spp. were present in approximately 30% of the samples collected. At La Fosca, *A. taylori*, *Gymnodinium* spp. and *Ostreopsis* cf. *siamensis* were present in 20% of the samples only.

					O2			N3			Estartit			La Fosca		
Species	Trophic Strategy[b]	Habitat[b]	Harmful[b]	HB[b]	%Np (N=18)	BI	Max (cell ml^{-1})	%Np (N=18)	BI	Max (cell ml^{-1})	%Np (N=12)	BI	Max (cell ml^{-1})	%Np (N=12)	BI	Max (cell ml^{-1})
Alexandrium pseudogonyaulax	auto	coastal & brackish	GA	yes				28	31	473						
Protoperidinium-like [a]	hetero	cosmopolitan	Positive Microtox	yes	28	55	4688	6	10	57						
Alexandrium minutum	auto	coastal	PSP	yes							8	21	10			
Alexandrium taylori	auto	coastal	PSP[e]	yes							25	6	36	17	53	26
Amphidinium sp. [a]	all	benthic, brackish	Amphidinilides	yes		98	27569	6	1	5						
Glenodinium foliaceum	mixo	Brackish	no	yes	44	77	9529									
Gymnodinium spp.[a]	Auto/mixo/ hetero	cosmopolitan	PSP, NSP, Ichthyotoxins	yes	22	56	10417	17	47	286	33	92	542	17	93	239
Ostreopsis cf. siamensis	auto	benthic, coastal	Palytoxin	yes[c]										17	4	1
Oxyrrhis marina	hetero	brackish	no	yes	67	100	18703	6	4	24						
Prorocentrum minimum	mixo	cosmopolitan	DSP	yes	11	6	95	66	100	77606	8	0.1	1			
Prorocentrum micans	mixo	cosmopolitan	no[d]	yes	6	0.5	29				25	5	1	8	13	1

Table 21. List of the species found at each sampled point. Trophic strategy: trophic strategy of each taxa (auto: autotrophy, mixo: mixotrophy and hetero: heterotrophy); Habitat: Most common habitat where the species is found (coastal, brackish or cosmopolitan); Harmful: the toxins and syndromes related with intoxications by harmful species (DSP: diarrhetic shellfish poisoning, PSP: paralytic shellfish poisoning and NSP: neurotoxic shellfish poisoning, GA: Goniodomin A, Positive Microtox: in this study); HB: High biomass developing species; %Np: percentage of occurrence of the species; BI: maximum percentage of the species regarding to the total microplankton community; Max: maximum cell concentration (cell·ml^{-1}). [a] For species non identified, trophic strategy, habitat, harmful, and HB were the characteristics of the genera. [b]Data extracted from Faust and Gulledge (2002) and, Hallegraeff *et al.* (2003). [c] *Ostreopsis* cf. *siamensis* has been found with densities of 300 cell ml^{-1} in Llavaneras (Spain) in 1998 (Garcés, personal communication). [d] There are only a few reports of *P. micans* having caused problems: shellfish kills in Portugal (Pinto and Silva, 1956) and South Africa (Horstman, 1981). [e] *A. taylori* causes discoloration of water, but also the production of low concentrations of PSP toxins have been related with this species (Emura *et al.*, 2004).

Detection of harmful dinoflagellate species.

Most dinoflagellate species detected in the present study have been described in literature as harmful or able to occur at high biomass. The species *Amphidinium* sp., *Gymnodinium* spp., *P. minimum*, *G. foliaceum* and *O. marina* formed mono-specific blooms (episodes during which their contribution to total microplankton cell numbers was close to 100%). Some potentionally harmful species such as *A. taylori* and *A. pseudogonyaulax*, were also found at bloom concentrations, but their relative abundance never exceeded 60% of total microplankton cell numbers (see BI in Table 21).

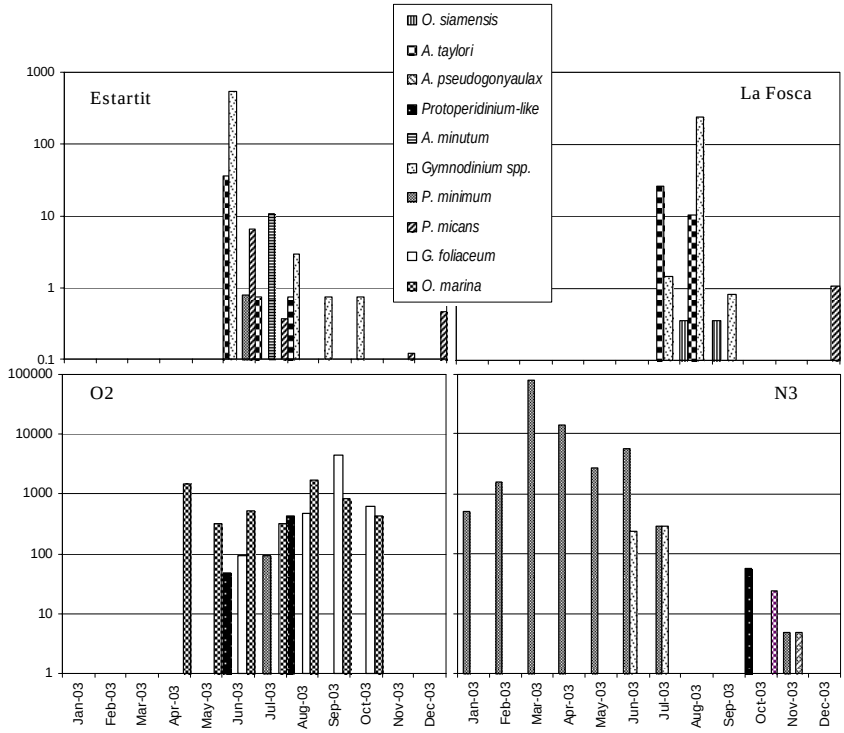

Fig. 26. Evolution of harmful dinoflagellate species along the sampled period. Two coastal water and two coastal lagoon ecosystems were represented.

Most of the species found in external coastal water were common for both external stations sampled (Table 21 & Fig. 26). However, the specific composition strongly differed among the lagoons, and also between lagoons and

coastal waters. Only *Gymnodinium* spp. were common for the four sites sampled, and were mainly detected during summer months. At least two morphotypes of the genus *Gymnodinium* were detected by means of Scanning Electronic Microscope and one of them was common in the four sites. Since they were not distinguishable under inverted microscope, all the morphotypes were clustered in a single taxa (*Gymnodinium* spp.). *P. minimum* was detected at most locations, except for La Fosca. Distribution of the other species was restricted to lagoons or coastal waters only. At N3 *P. minimum* dominated in most of the samples collected along the year. At O2, *P. minimum* and *Protoperidinium*-like were only present during the summer months. *A. taylori* and *Gymnodinium* spp. were the main species in coastal waters, and also appeared during summer only.

Whereas N3 showed potentially harmful dinoflagellates almost the whole year round, except for the summer period (August), the coastal stations showed an opposite pattern, were the presence of dinoflagellates was restricted to summer.

Environmental factors associated with bloom forming and harmful species.

The effect of environmental conditions on pre-establish general microplankton groups was analysed by means of Canonical Correspondence Analysis (CCA). The groups considered were: 1) diatoms, 2) dinoflagellates (separated in HD; harmful dinoflagellates and NHD; non-harmful dinoflagellates), 3) small flagellates and 4) ciliates (Fig. 27). HD and NHD were managed as two different groups to find a possible common specific response within each of them. The first two dimensions of the CCA accounted for 79.8% of the total variance of phytoplankton classes and environmental data. The first axis accounted for 46.4% and the second for 33.4% of the total variance, respectively. All canonical axes were significant ($p<0.01$, Montecarlo test). The first axis mainly separated samples with positive coordinates (where diatoms and ciliates were abundant, and with more ammonia and less organic matter content) from samples with flagellates (with high conductivity, bacterial biomass and TOC concentration in negative coordinates). Following the second axis, flagellates and diatoms were abundant in positive coordinates, when the concentration of the most regenerated forms of nitrogen were higher. Ciliates dominated in samples with negative coordinates, coinciding with samples from the confinement period.

NHD were situated in the positive values of the second axis and were abundant in samples with negative first axis values, coinciding with higher concentrations of TOC. HD, on the other hand, were positioned in the positive values of the first axis and negative values of the second axis, where nitrogen was mainly found in its most reduced form, ammonia (Fig. 27).

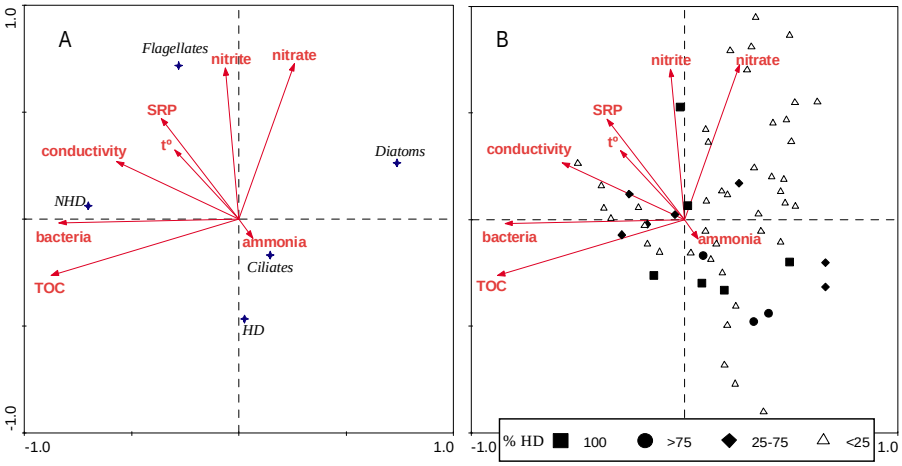

Fig. 27. A) Representation of microplankton functional groups in the CCA axes space (1, 2). Harmful (HD) and non-harmful dinoflagellates (NHD) as well as diatoms, ciliates and other flagellates, were considered separately. B) Representation of samples in the CCA axes space (1, 2). Samples have been classified following the percentage of HD respect of the rest of the microplankton community abundance.

The relative abundance of HD (% of total microplankton) was plotted in Fig. 27b to distinguish the location of samples with a high HD content in the CCA. Several HD-dominated samples coincided with confinement conditions (Fig. 27b). However, this pattern did not apply for some samples dominated by HD.

The apparently different responses of HD populations to environmental conditions induced the need for an additional analysis in which every dinoflagellate species was considered individually. This second CCA was performed to relate the proliferation of each dinoflagellate species with the specific environmental variables (Fig. 28a & b). The first two dimensions of the second CCA accounted for 67.7% of the total variance of phytoplankton classes and environmental data observed. The first and second axis accounted for

36.7% and 31.0% of the total variance, respectively. All canonical axes were significant ($p<0.01$, Montecarlo test).

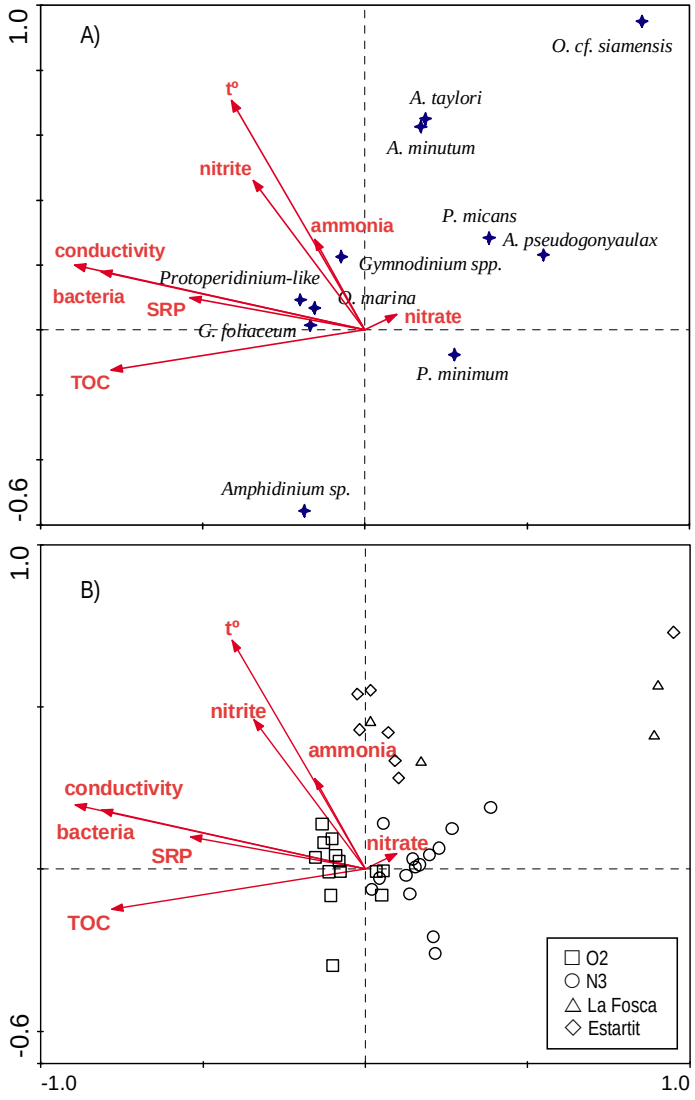

Fig. 28. A) Distribution in the two first CCA axes of harmful dinoflagellate species. B) Distribution in the two first CCA axes of samples. Samples have been classified by their origin.

The first axis separated samples with nitrate and positive coordinates from samples with high TOC and bacteria concentrations with negative coordinates. The second axis mainly separated samples with high temperature and high concentrations of regenerated nitrogen with positive coordinates from samples with higher concentrations of TOC, and negative coordinates. Following the first axis, *Ostreopsis* cf. *siamensis*, *A. pseudogonyaulax*, *P. micans*, *A. taylori*, *A. minutum* and *P. minimum* were abundant in samples dominated by high nitrate concentrations while *Protoperidinium*-like, *Gymnodinium* spp., *O. marina*, *G. foliaceum* and *Amphidinium* sp. dominated in samples with high TOC and bacteria concentration (Fig. 28a). *P. minimum*, *Protoperidinium*-like, *O. marina* and *G. foliaceum* were positioned at medium values of the second axis. *Amphidinium* sp. was abundant in samples with a high TOC concentration. The rest of dinoflagellate species coincided with high temperatures and high concentrations of all inorganic nitrogen forms. The Fig. 28b showed the distribution of samples according their origin. Samples from lagoons were situated mainly in mid and negative values of the second axis and samples from external waters were situated in positive values of this axis.

One hierarchical cluster analysis was performed with the first two axes of the CCA. The genera *Alexandrium* and *Prorocentrum* grouped together, also, the species *Gymnodinium* spp., *O. marina*, *G. foliaceum* and *Protoperidinium*-like cluster in a group, whereas the two other taxa were separated in the cluster. Within *Alexandrium* the species *A. taylori* and *A. minimum* defined a subgroup, which was well separated from *A. pseudogonyaulax*, *P. minimum and P. micans.*

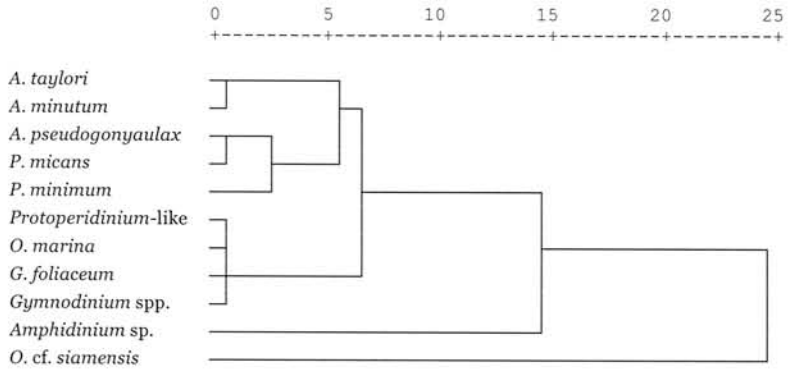

Fig. 29. Cluster analysis of harmful dinoflagellate species organised by coordinates of the two first dimensions of the CCA analysis. Euclidian distances and average linkage were used.

4. Discussion

The microplankton community dynamics.

Along the sampled beaches of the Mediterranean, the phytoplankton community described a seasonal pattern that was not observed in the lagoons. In the lagoons the phytoplankton community appeared mainly influenced by the variability of the hydrological regime. Coinciding with previous observations (Comín and Valiela, 1993; Gilabert, 2001; Quintana and Moreno-Amich, 2002), the characteristic hydrological conditions of Mediterranean coastal lagoons strongly determined dynamics of its phytoplankton community. The observed alternation between the dominance of diatoms or dinoflagellates coincided with that described in the Margalef's mandala (Margalef, 1983), and occurred in external waters as well as the coastal lagoons. These results confirmed previous alternations between the two functional groups detected at La Fosca (Vila and Masó, 2005; Garcés, unpublished results). The patterns of alternation were less stable in coastal lagoons than in the external waters, where the alternation follows a seasonal cycle. This was probably due to the proximity of benthos (Gascón, 2003) or the influence of allochthonous organic matter inputs (chapter V) in the lagoons.

In spite of the minor distances between the beaches and the coastal lagoons, the specific composition of the dinoflagellate populations appeared quite different during the studied period. Among the HD, one of the *Gymnodinium* morphotypes was common for all four locations sampled. However, since *Gymnodinium* species were not differentiable, it cannot be affirmed that the *Gymnodinium* individuals found in external and in coastal lagoon waters belong to the same species. Further genetic analysis will needed in order to discern among the species of this genus. The genus *Alexandrium* was also common, but the species composition of this taxonomic group differed, however, between coastal waters and lagoons. The usual brackish species *A. pseudogonyaulax* (Hasle *et al.*, 1997) was indeed found in the lagoons, while *A. minutum* and *A. taylori* only appeared in coastal waters, were blooms of these organisms are usually detected. Detection of the benthic species *Amphidinium* sp. and O. cf. *siamensis* in the brackish waters of the lagoons and in coastal waters, respectively, confirmed previous observations of Hasle *et al.* (1997) about these species. The species composition of the dinoflagellates detected in the present study, and formation of their corresponding blooms, appeared indeed highly variable in time and space, and they could not be classified in a single category. These results corroborated previous conclusions of Smayda and Reynolds (2003), and Vila and Masó (2005).

Harmful dinoflagellates dynamics.

Most of the dinoflagellates species detected in the samples collected from the Mediterranean coastal waters and lagoons are known as harmful organisms. High concentrations of ammonia seemed to stimulate formation of HD blooms, but the dominance of this functional group did, however, not always coincide with this specific environmental condition. Blooms of *P. minimum* for example did in fact occurred during periods with a high nitrate status, rather than its regenerated form. Differences in habitat preferences among individual HD species would explain formation of toxic blooms under a wide range of different environmental conditions.

The species from the genus *Alexandrium*, together with *Prorocentrum* (from the habitat types IV and II described by Smayda and Reynolds (2001), respectively, shared a similar response to the environmental variables

considered. These organisms always appeared when water temperature and nitrogen status were high. Within the genus *Alexandrium*, growth of *A. pseudogonyaulax*, *A. taylori* and *A. minutum* seemed to be favoured by the presence of different inorganic forms of nitrogen. The *Gesnerium* group species, *A. pseudogonyaulax* and *A. taylori*, behaved similar. Their proliferations responded to high concentrations of nitrogen (to nitrate *A. pseudogonyaulax* and to more regenerated nitrogen *A. taylori*), although they were found at different niches (lagoons and coastal water, respectively). They also shared other common characteristics, including the production of temporary cysts as part of the vegetative cell division. Formation of these cysts is an advantage as it allows the population to flourish in a later stage from previously sedimentated material (Garcés et al. 2002). In contrast, it is known that *A. minutum* environmental requirements differ from that of *A. pseudogonyaulax* and *A. taylori* (Vila *et al.*, 2005). Among the *Prorocentrum* genus, *P. minimum* is a good example of Type II (Smayda and Reynolds, 2001), and comprises an important bloom-forming species in coastal lagoons. In spite of its apparently mixotrophic behaviour (Stoecker *et al.*, 1997), the present study clearly showed that *P. minimum* was favoured by high nitrate concentrations. This observation confirmed results obtained by other authors (Witek and Plinski, 2000; Macedo *et al.*, 2001). *Protoperidinium*-like and *Gymnodinium* spp., joint with *G. foliaceum* and the heterotrophic *O. marina*, were strongly related with high bacteria biomass and TOC concentration. *O.* cf. *siamensis* and *Amphidinium* sp., the two benthic species, responded to opposed environmental conditions and were present in different habitats. *Amphidinium* sp. appeared after the confinement period in the lagoons, probably related with a process of sediment resuspension, whereas *O.* cf. *siamensis* was found in external waters during summer, when the temperatures were higher, since this species has been found often in tropical marine habitats (Faust and Gulledge, 2002).

The HD species performed well in both marine coastal waters and lagoons, but the specific species-dependent affinity to each of these environments determined which organism bloomed. Since nutrient enrichments were shown to stimulate formation of some HABs (Anderson, 2002), as well as their duration (Riegman and Kuipers, 1994), closed bays, estuarine waters and coastal lagoons are currently highly vulnerable ecosystems, especially during

VIII. General discussion

General discussion

1. The phytoplankton in coastal marshes

In the Empordà coastal lagoons the phytoplankton dynamics are mainly related to the hydrological variability. The hydrological variability, which determines the nutrient availability, plays the most important role in the community composition and structure, which has also been found to be true for zooplankton (Quintana *et al.*, 1998b; Gifre *et al.*, 2002; Brucet, 2003), periphyton (Trobajo, 2003) and zoobenthos (Gascón, 2003) in these ecosystems. Top-down control has little effect on phytoplankton biomass and its community composition.

In the Empordà salt marshes, the maximum density of phytoplankton occurs in summer and generally dinoflagellates and cyanobacteria dominance coincides with maximum confinement and when inorganic nitrogen is most scarce. During hydrologic instability haptophytes and diatoms dominate, but with a considerably lower biomass than the biomass that dinoflagellates reach during summer. Unlike what has been observed in marine coastal waters, the phytoplankton community in the lagoons does not follow a seasonal pattern, since the hydrological cycle is not seasonal. Even though these salt marshes have high hydrological variability, alternation between dinoflagellates and diatoms took place in the lagoons according to the mandala model (Margalef, 1983). Due to the lack of seasonality, the time when diatoms and dinoflagellates proliferated did not coincide with that predicted by the model, however, diatom proliferations coincided with large concentrations of inorganic nutrients, while dinoflagellate blooms coincided with the most confined period, when the organic matter concentration was high and turbulence was very low.

Confinement and flooding periods are the most extreme situations of the hydroperiod. Lagoon flooding is mainly caused by natural pulse disturbances (Zedler *et al.*, 1979; Brey, 1991; Quintana *et al.*, 1998b; Quintana and Moreno-Amich, 2002; Bertrand *et al.*, 2004). During sea storms or intense rainfall, the confined lagoons have high inputs of energy and nutrients that change the water and the community composition. During this period bacterioplankton dominates the microbial community, since their growth is favoured by the

inputs of allochthonous organic matter into the lagoons coming from runoffs. The phytoplankton mainly develops when the system recovers hydrological stability. After sea storms, the phytoplankton community starts to develop slowly, and this development becomes quicker as confinement increases. Generally the confinement period is when there is most microbial biomass accumulation, although extreme confinement conditions may lead to nutrient depletion, and consequently to limited production (Trobajo *et al.*, 2002). Data suggest that the dominance of phytoplankton or bacterioplankton, and the phytoplankton composition depend on the inorganic to organic nutrient ratio. It seems that the confinement periods favour more phytoplankton organisms, while pulse disturbances lead to the periodical dominance of bacterioplankton.

In unconfined Mediterranean lagoons the community composition is influenced greatly by the annual seasonal pattern, which is often anthropically determined due to a controlled flushing cycle (Comín and Valiela, 1993; Romo and Miracle, 1995; Gilabert, 2001). The higher phytoplankton biomasses are reached in these lagoons during the spring and they are related to external nutrient inputs. Blooms are usually of diatoms, chlorophytes and cyanobacteria during the inflow periods (Comín and Valiela, 1993; Quesada *et al.*, 1998; Gilabert, 2001; Villena and Romo, 2003).

2. The importance of mixotrophy

The phytoplankton biomass is strongly related to the DOC concentration. In contrast, inorganic nutrients are weakly related to the increase in the phytoplankton biomass. In fact, the highest phytoplankton biomass is reached during confinement, when the inorganic to organic nutrient ratio is at its lowest. Out of all the phytoplankton, dinoflagellates are the class that is most favoured by confinement and the low inorganic to organic nutrient ratio. It is known that mixotrophic activity is favoured by inorganic nutrient depletion and by a low inorganic:organic nutrient ratio (Granéli *et al.*, 1999; Jones, 2000). Although it has not been tested, our data strongly suggest that some dinoflagellates are mixotrophs during the confinement periods. In fact, the importance of mixotrophic phytoplankton in wetlands has been well documented before (Comín and Ferrer, 1978; Stickney *et al.*, 2000; Macedo *et al.*, 2001). Within the mixotrophic phytoplankton from the Empordà salt marshes, phagotrophy has

been reported most in dinoflagellates (Li *et al.*, 1996; Stoecker *et al.*, 1997; Isaksson, 1998; Stoecker, 1999; Jones, 2000; Li *et al.*, 2001; Ismael, 2003). The analysis of the community's specific composition showed that the dinoflagellate species responsible for the highest biomass proliferation are those described as mixotrophic. The mixotrophic activity of dinoflagellates could affect the phytoplankton size distribution. It has been observed in phytoplankton size distribution patterns that small nanophytoplankton sizes decrease and disappear when there is a low inorganic:organic nutrient ratio, which suggests strong predation by dinoflagellates.

Although dinoflagellate biomass is significant in this ecosystem, other organisms found and described as potentially phagotrophs such as some haptophytes (Jones, 2000) and cryptophytes (Urabe *et al.*, 2000; Marshall and Laybourn-Parry, 2002), have to be taken into account when the microbial food web is analysed. In fact, mixotrophic nanoflagellates are known to prey on bacteria and also on smaller phytoplankton (Granéli *et al.*, 1999; Marshall and Laybourn-Parry, 2002; Stibor and Sommer, 2003). However, the methodology to detect phagotrophy becomes more complex when the algae are small. Thus, future studies will be needed to measure the degree of importance of this niche in the whole food chain.

3. Toxicity repercussions

Evidence regarding the presence of harmful phytoplankton in Mediterranean lagoons (Sarno *et al.*, 1993; Cabrini *et al.*, 1995; Giacobbe *et al.*, 2000) and coastal waters (Garcés *et al.*, 2000; Vila *et al.*, 2001b; Basterretxea *et al.*, 2004) have been reported before. In the Empordà salt marshes, the potentially harmful phytoplankton species found were haptophytes and cyanobacteria and dinoflagellates. High biomasses of cyanobacteria of different genera were found in the lagoons, and positive results of the Microtox® test were related to them. However, the microcystin concentration was as a whole undetectable, which suggests that toxicity, during high cyanobacterial biomass proliferations, would be caused by another cyanobacterial toxin. Haptophytes from the genus *Prymnesium* were also related with episodes of Microtox® toxicity. Interestingly, most of the dinoflagellate species found in this study have been described as harmful and large biomass developers.

The specific composition of harmful dinoflagellates is different in marine waters compared to coastal lagoons. Only the mixotrophic and bloom-forming species *P. minimum* (Stoecker *et al.*, 1997) and *Gymnodinium spp.* (Uchida *et al.*, 1997) were present in the two ecosystems analysed. The rest of the harmful species were only in the lagoons or marine coastal waters. Differences in habitat preferences between harmful dinoflagellate species, would explain their presence in a wide range of environmental conditions (Smayda and Reynolds, 2003). It seems difficult to predict the episodes of harmful dinoflagellate proliferation or the bloom dynamics in confined lagoons, since they do not occur seasonally, and the species composition is different from that of the nearby marine ecosystems, for which more data on bloom dynamics are available.

Generic methods to detect toxicity, like Microtox, allow us to monitor the toxicity to which the organisms inhabiting the aquatic ecosystems are exposed. However, given that there are many potentially toxic substances present in natural ecosystems, such as heavy metals and pesticides (López-Flores *et al.*, 2003), when using this method it is difficult to relate the positive results to the harmful phytoplankton. The specific toxin measurements obtained using chromatography, are also often not a definitive solution, since there are a large amount of different phytoplankton toxins and most of them are still unknown. This kind of analysis is only appropriate when the species, or at least the genera of the algae is known, and even if the species is known, acceptable results are not guaranteed. Thus, the Microtox® test can supply some data about the toxicity level in highly variable salt marsh lagoons and temporary basins, which although incomplete, could assist in conserving the ecological value of Mediterranean coastal marshes.

It is known that within the same species, some strains could be toxin-producers while others are not, depending on their habitat characteristics (Granéli *et al.*, 1999; Hamasaki *et al.*, 2001; Hallegraeff *et al.*, 2003). Likewise, mixotrophy in many harmful, bloom-forming algae may contribute to their ability to form mono-specific blooms and may be related, in some cases, to their harmful effects on other organisms (e.g. Leeper and Porter, 1995). The effect that harmful phytoplankton could have on the trophic chain is not limited to lethal consequences. In fact, some authors have described toxicity effects on the

immediately higher trophic levels that may lead to subaccute symptoms, such as a reduced copepod male fertilization capacity (Ianora *et al.*, 1999), and the production of antiproliferative compounds by non-neurotoxic dinoflagellate strains that can reduce copepod egg production and hatching success (Ianora *et al.*, 2004). The important role that dinoflagellates play in lagoons is due to their capacity to outcompete the other phytoplankton and also the zooplankton organisms. The mixotrophic capabilities as well as the ability to produce toxins of some dinoflagellates present in coastal lagoons could explain why top-down control in these ecosystems is not very significant.

Therefore, it seems that assessing the management of coastal lagoons, such as the conservation of the endangered species *Aphanius iberus* in the Empordà salt marshes, where the proliferation of harmful algae is usual, depends mainly on controlling the specific development of the most dangerous phytoplanktonic species, as well as monitoring the factors controlling their bloom, such as the confinement of water and the nutrient composition. In addition, the decrease in allochthonous organic or inorganic nutrient inputs into the wetland ecosystem would reduce the stimulation of some harmful algae blooms (Anderson, 2002) or at least their duration (Riegman et al., 1994).

IX. Conclusions

Conclusions

❖ Phytoplankton in Mediterranean salt marshes are subjected to bottom up control more than top-down control. Hydrological disturbances and nutrient availability control the structure and composition of the phytoplankton community.

❖ The low inorganic:organic nutrient ratio is characteristic of Mediterranean salt marshes, in which inorganic nitrogen is especially scarce due to denitrification. This ratio becomes a limiting factor for autotrophic production. The different phytoplankton classes were ordered along a gradient related to the nitrate to total nitrogen ratio according to their physiological differences in relation to nitrogen uptake.

❖ In confined lagoons, nutrients are mainly in organic form and dinoflagellates take advantage of the mixotrophy by "eating their competitor", as well as competing with and grazing on smaller phytoplankton.

❖ DOC is the main variable that accounts for phytoplankton biomass. This significant correlation between DOC and phytoplankton biomass could be related to high mixotrophic activity by phytoplankters. Although this fact is still not fully documented, most phytoplankton groups found in these basins and in nearby similar ecosystems are potentially mixotrophic.

❖ Differences in catchment areas, specifically in the CF ratio, explain the differences in nutrient and organic carbon accumulation found in the different basins. During confinement, basins accumulate the allochthonous nutrient and organic matter that entered the lagoon through runoffs. The magnitude of this accumulation is related to the CF ratio.

❖ Many intermediate situations may occur between the predominance of autotrophic growth and the phytoplankton becoming phagotrophic. This leads to a relationship between phytoplankton and the bacterioplankton biomass that is not significant.

❖ Toxicity takes place during summer coinciding with high temperatures, high bacterial biomasses and high organic matter concentrations. The toxicity

was related with the particulate fraction, and was not due to heavy metals, since their concentrations in the particulate matter was always under the $EC50_5$ for the metals analysed.

❖ Toxicity also coincided with high densities of chlorophyll *a* and high pico- and nanophytoplankton biomasses. In most cases, high TU_5 coincided with the presence of harmful algae, however the pulsated proliferation of the algae did not allow us to establish a positive correlation between the harmful species biomass and the Microtox® results.

❖ Microtox® is a useful tool for detecting toxins in water, since it is quick and allows cheap and easy overall monitoring in systems like the Empordà salt marshes, where the phytoplankton composition varies greatly both in terms of time and space. Nevertheless, the Microtox® results should be assessed by other analytical and taxonomic tools.

❖ Generally in external Mediterranean waters the phytoplankton community has a seasonal pattern. This was not observed in the lagoons, in which the phytoplankton community appeared to be mainly influenced by the variability of the hydrological regime. Despite this, the alternation between the dominance of diatoms and dinoflagellates occurring in external waters and coastal lagoons corresponds to that described by Margalef's mandala (Margalef, 1983).

❖ Most of the dinoflagellate species found in Mediterranean marine coastal waters and lagoons have been previously described as harmful. These harmful dinoflagellate species perform well in both marine coastal water and lagoons, but the specific species-dependent affinity to each of these environments determines which organism blooms in each environment.

❖ Expansion of HAB to inland waters due to coastal marine water inputs is not likely, due to the low affinity of the species with such brackish ecosystems. The level of confinement and the oxidised state of available nitrogen are the main factors that determine the success and possible bloom formation of dinoflagellate species introduced into a new salt marsh ecosystem.

X. References

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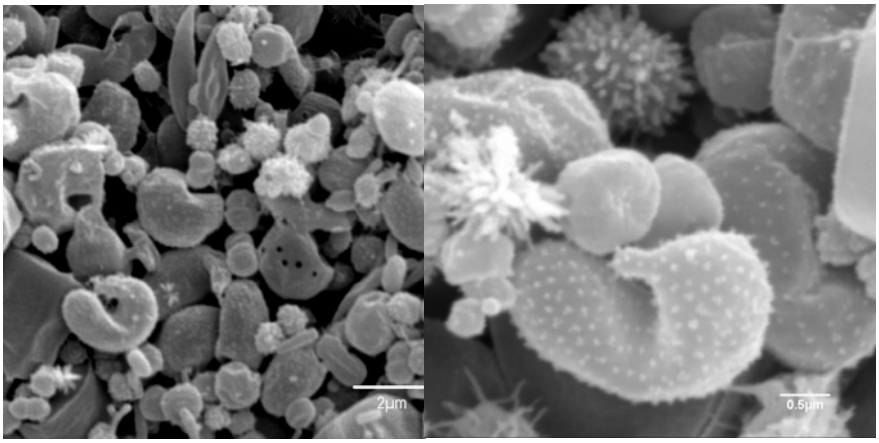
Appendix

Plates of some of the most abundant phytoplankton species found in the Empordà salt marshes.

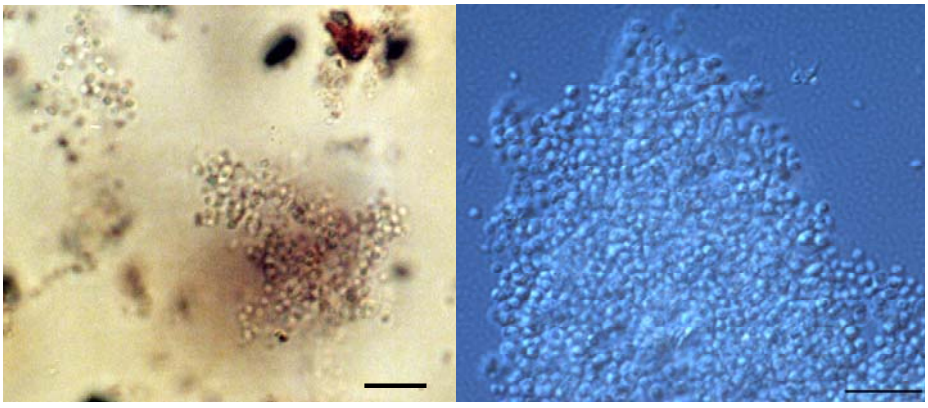
In all the pictures the scale is 10 µm, except in those cases in which it is indicated.

1. *Cyanophytes*

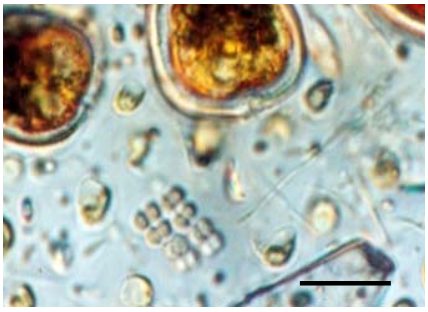
Dactylococcopsis cf. *raphidioides* (Rogera, August 2001)



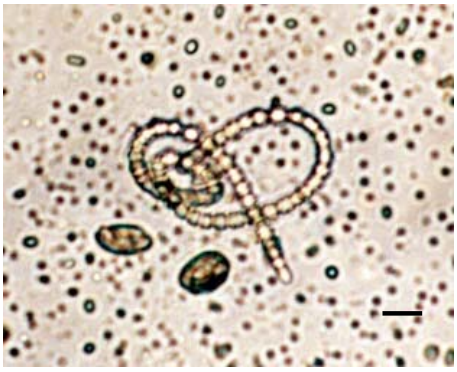
Aphanocapsa sp. (Rogera, August 2001)



Merismopedia spp. (N1, April 2003)

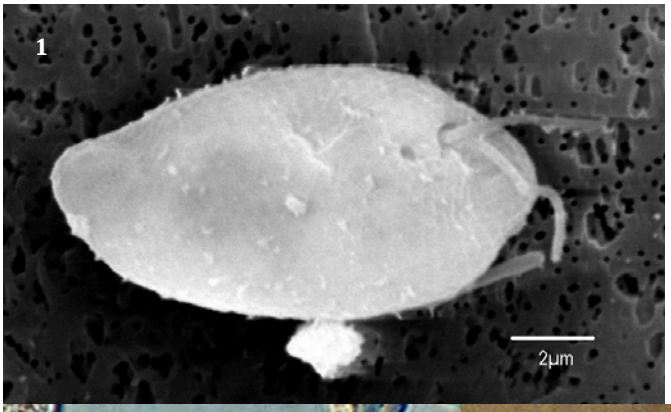


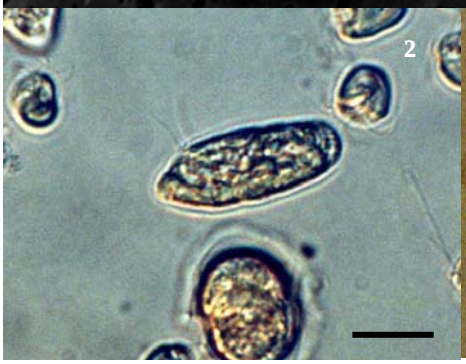
Anabaena sp. (Turies, March 2001)

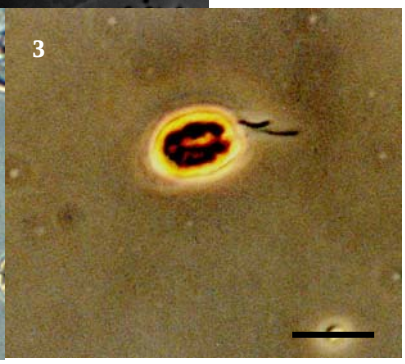


2. Cryptophytes

Chroomonas spp. (1, 2: Litoral, July 2002; 3: O2, July 2003)



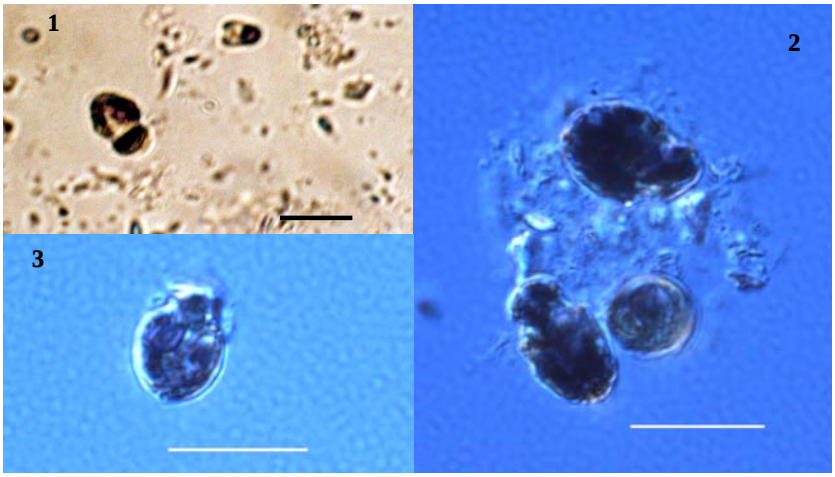




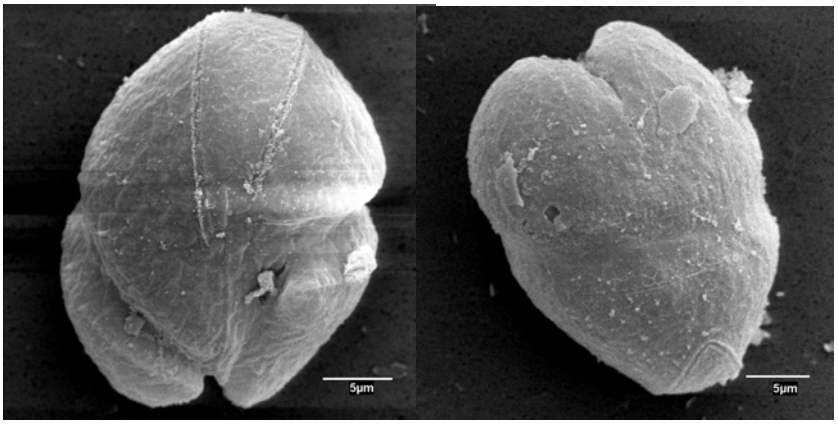
3. Dinoflagellates

3.1. Gymnodiniales

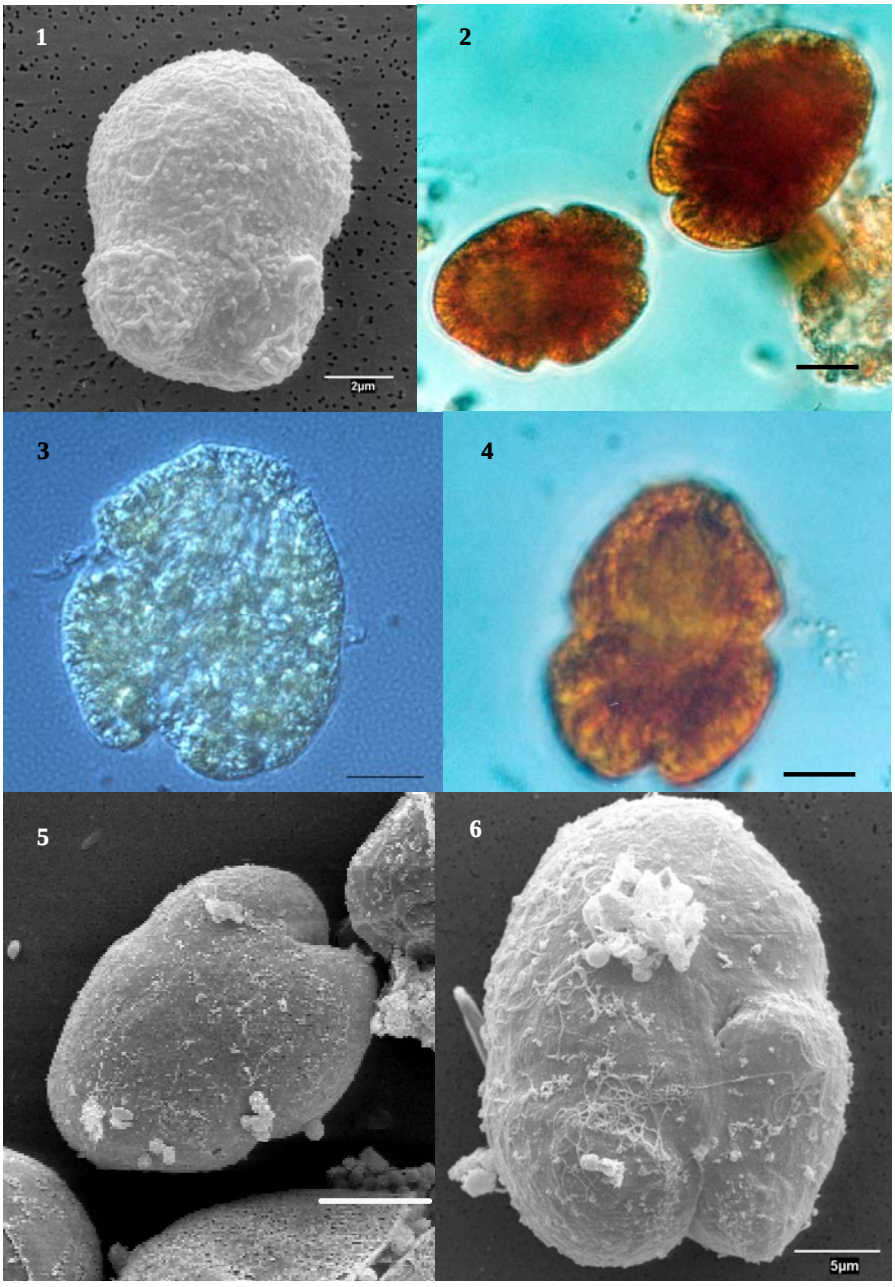
Amphidinium spp. (1: Turies, December 2000; 2, 3: O2, December 2002)



Gymnodinium instriatum (O2, September 2002)

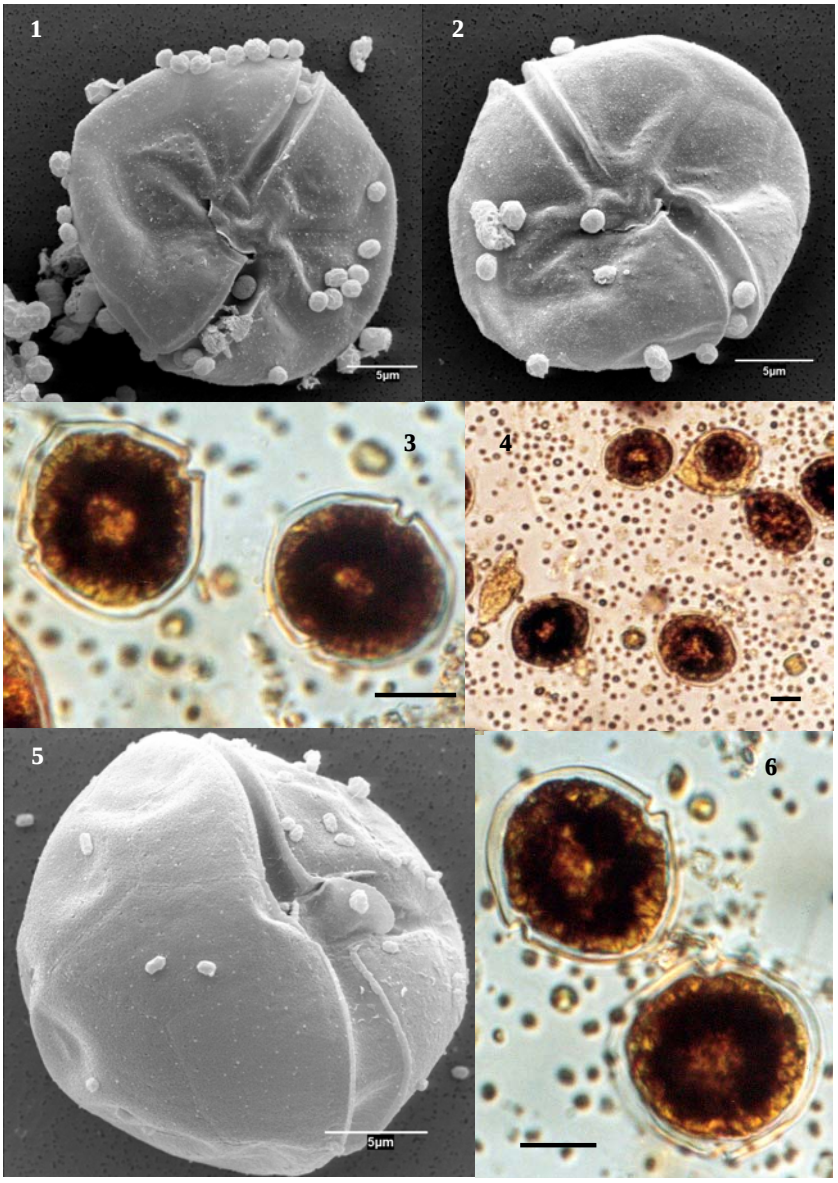


Gymnodinium spp. (1: N1, September 2002; 3: O2, September 2002; 2, 4: N1, June 2003; 5: O2, December 2002; 6: O2, September 2002)

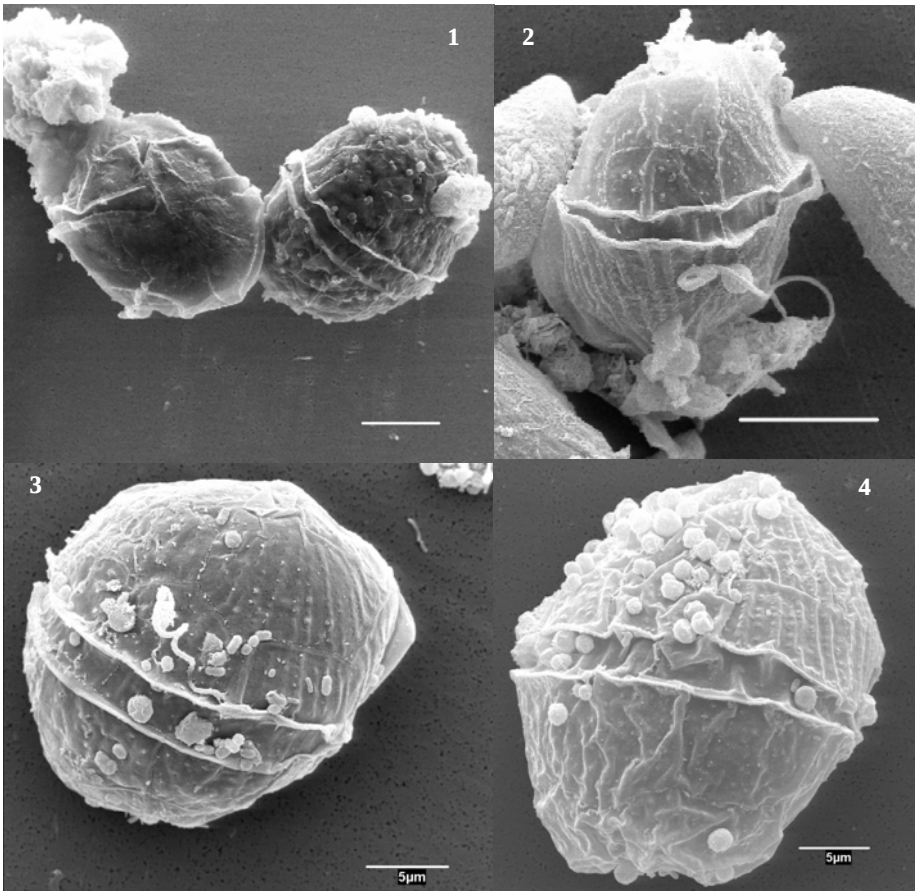


3.2. Peridiniales

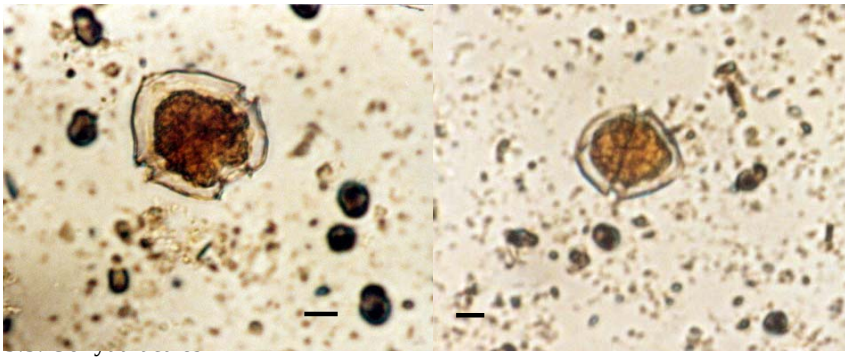
Glenodinium foliaceum (1-3, 6: O2, October 2003; 4: O1, November 2003; 5: O2, July 2002)



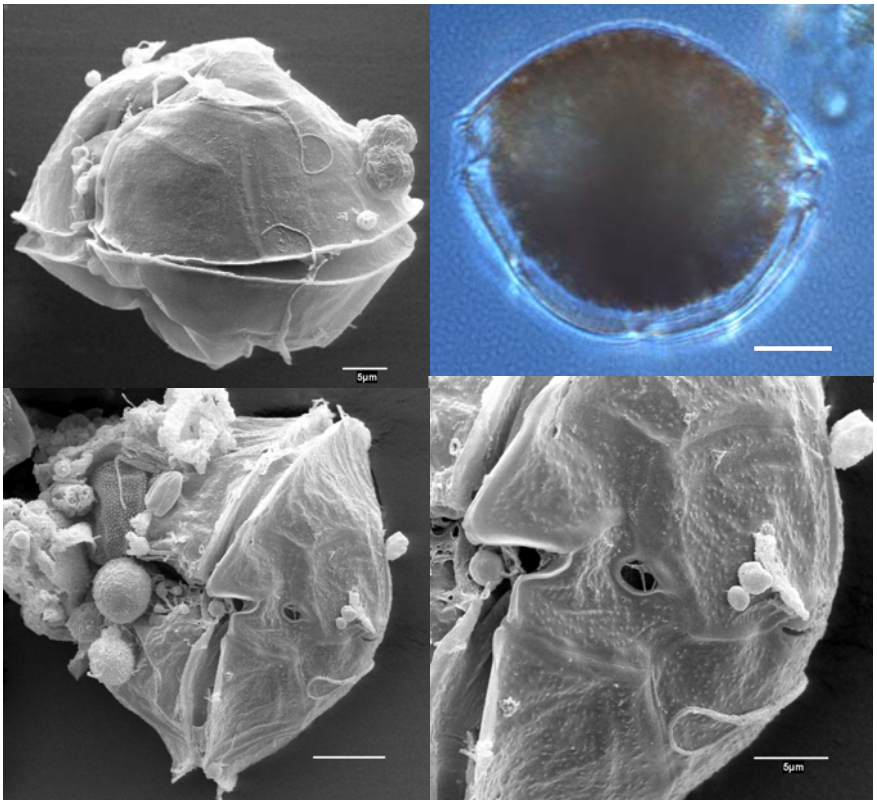
Heterocapsa spp (1: N1, September 2002; 2: O2, September 2002; 3: O2, December 2002; 4: O2, August 2001)



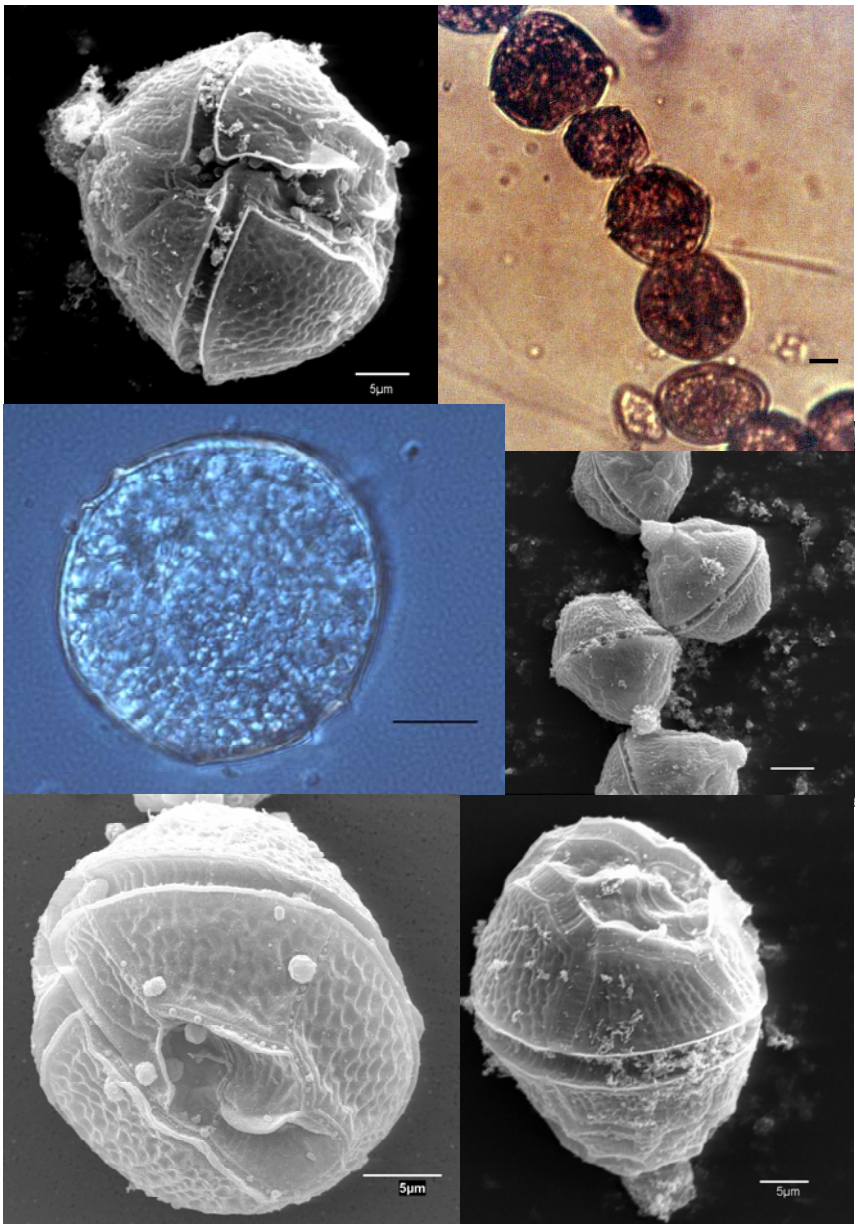
Scripsella spp. (Turies, December 2000)



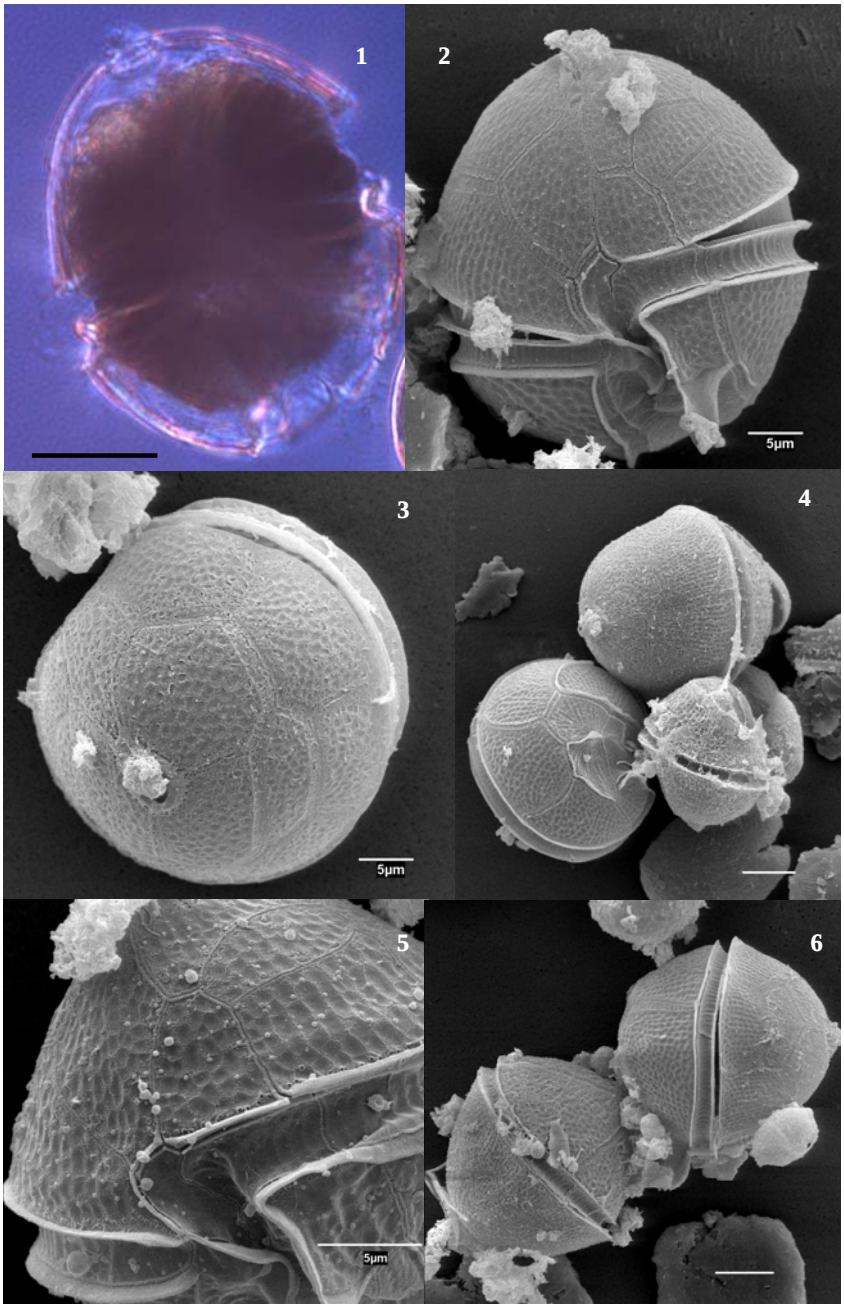
Alexandrium pseudogonyaulax (N3, September 2002)



Protoperidinium like. (O2, August 2001)

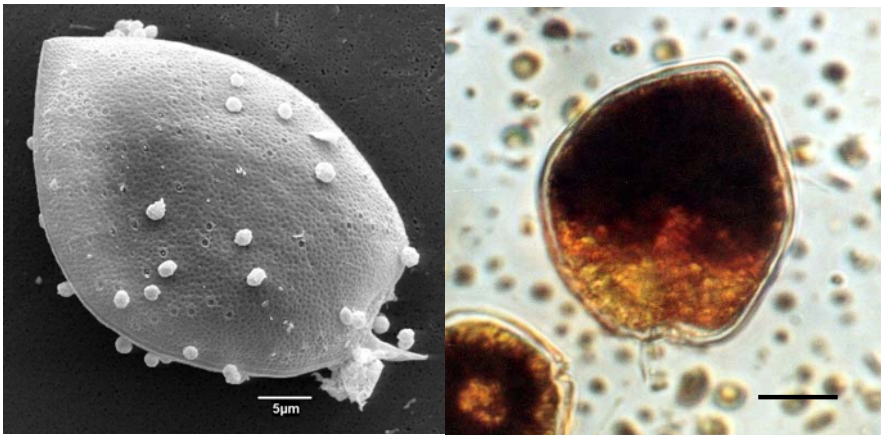


Protoperidinium like. (1: O2, August 2002; 2-6: O2, September 2002)

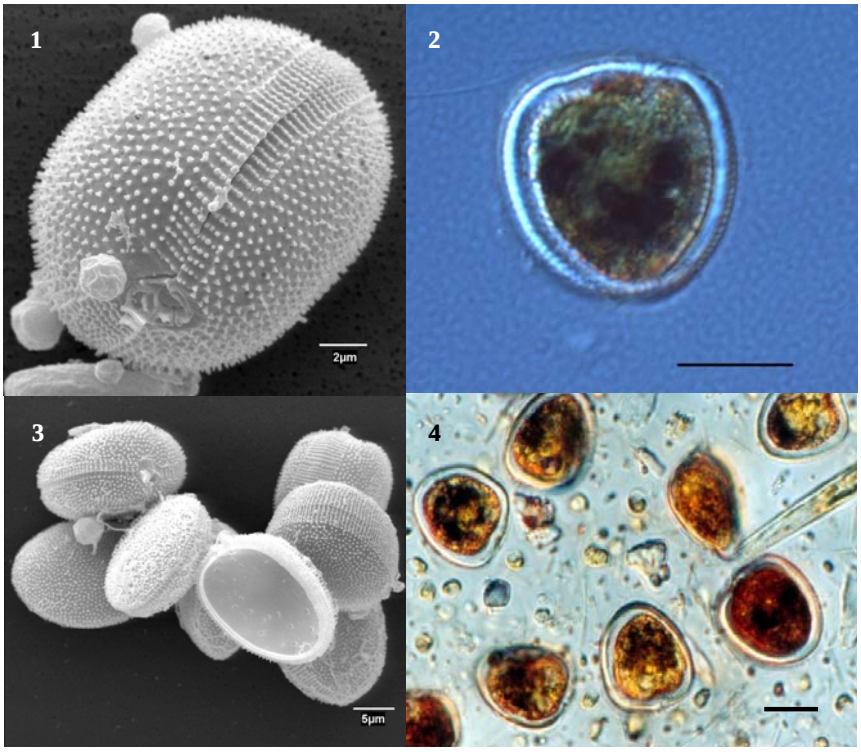


3.4. Prorocentrales

Prorocentrum micans (O2, October 2003)

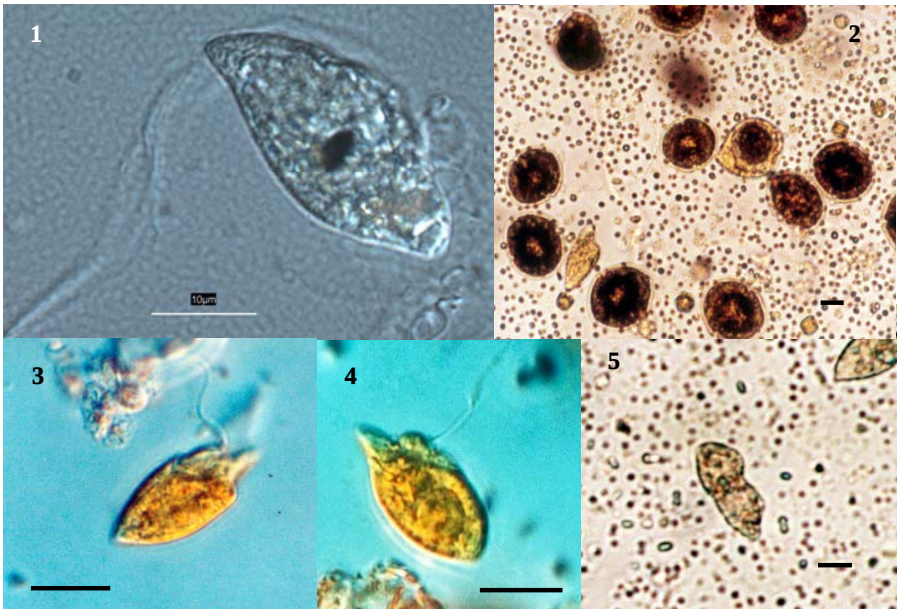


Prorocentrum minimum *(1: N1 September 2002; 2: N3 April 2003; 3: N1 July 2003; 4: N1 April 2003)*

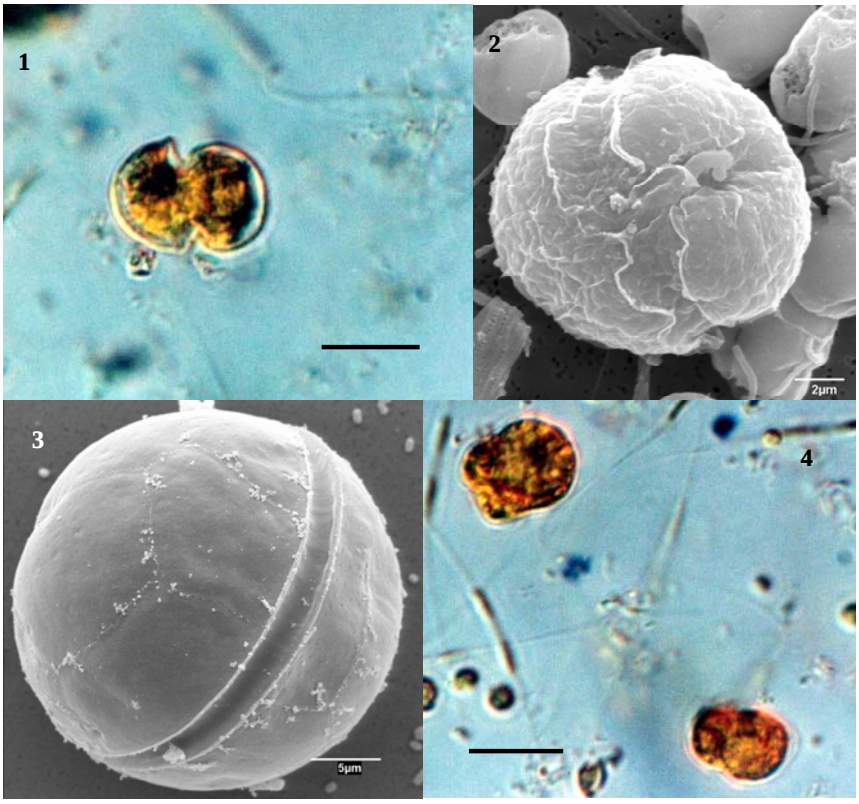


3.5. Uncertain taxa

Oxyrrhis marina (1: O2, September 2002; 2: O1, November 2003; 3, 4: N1, June 2003; 5: Turies March 2001)

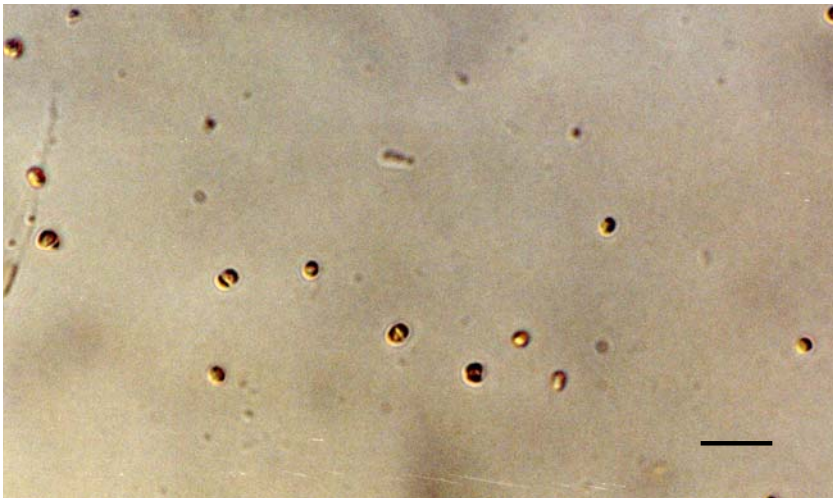


Non-identified dinoflagellates. (1, 4 : N1, October 2002; 2: Litoral July 2002; 3: O2, July 2002)



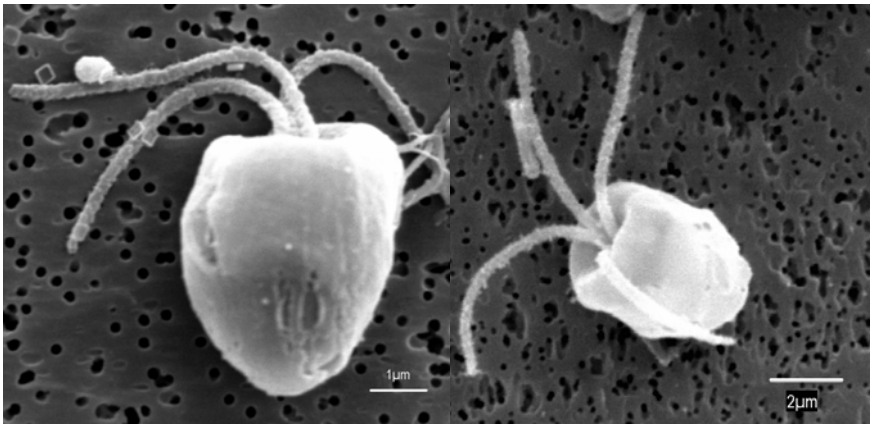
4. Chlorophytes

Nannochloris sp. (O2, June 2003)

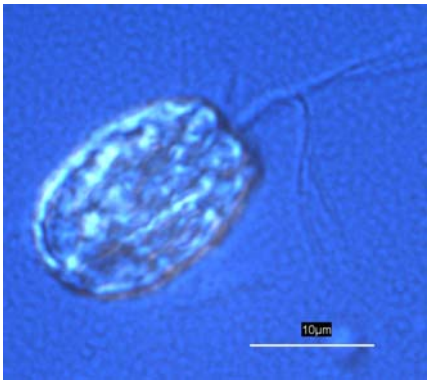


5. Prasinophytes

Pyramimonas spp. (Litoral, July 2002)

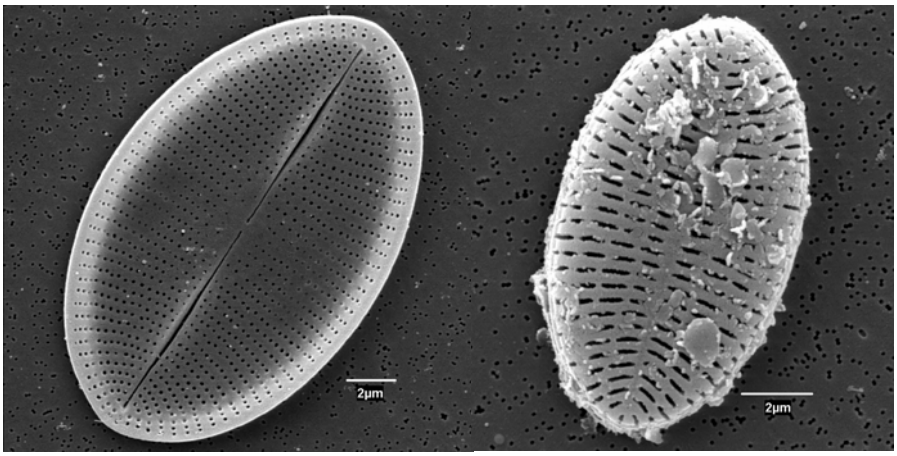


Tetraselmys cf. *gracilis* (O1, September 2002)

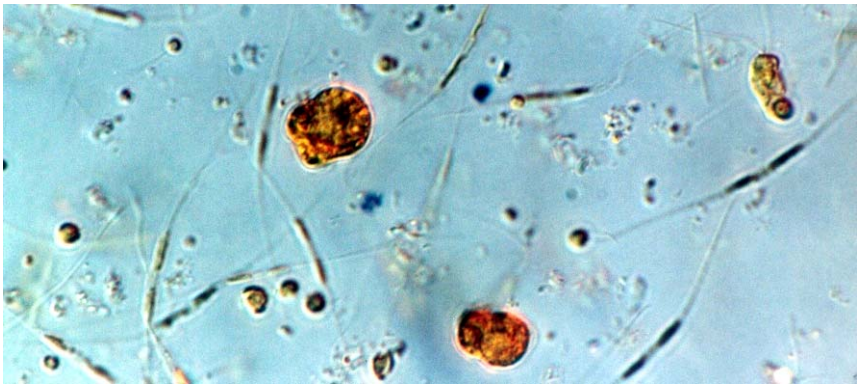


6. Diatoms

Cocconeis placentula (N1, July 2003)

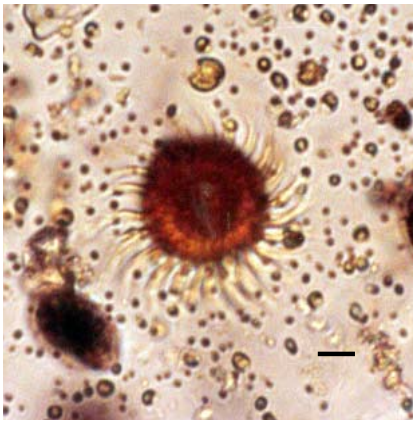


Nitzchia closterium. (N1, October 2002)

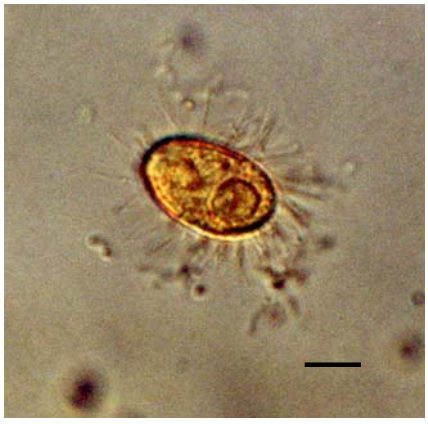


7. Ciliates

Strombidium sp. (O2, October 2003)



Cyclidium sp. (O2, July 2003)



Unknown ciliate (Litoral, June 2001)