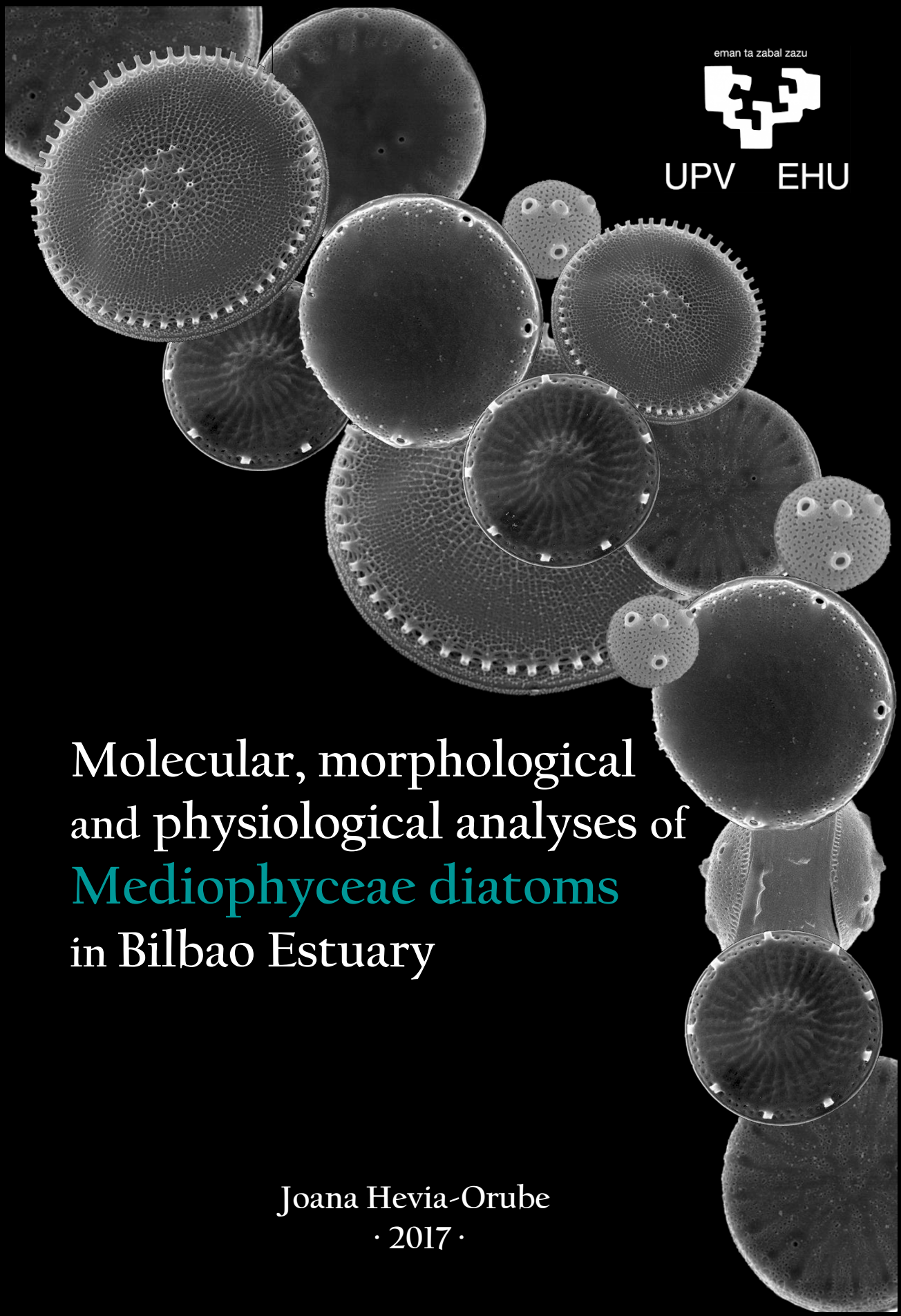

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UPV EHU

Molecular, morphological and physiological analyses of Mediophyceae diatoms in Bilbao Estuary

Joana Hevia-Orube

· 2017 ·

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RESUMEN

La evaluación medioambiental de estuarios, ríos o lagos se puede realizar basada en las comunidades de fitoplancton. En el estuario de Bilbao se lleva a cabo un monitoreo rutinario desde el año 2000, con el objetivo de evaluar la calidad del agua. Para realizar esta evaluación, se tienen en cuenta los grupos generales de fitoplancton en términos taxonómicos, creando subgrupos en función del tamaño celular y registrando su abundancia. De acuerdo con este procedimiento, las diatomeas céntricas se clasificaban en tres grupos: <5µm; 5-15 µm; >15 µm (desde el año 2000 al 2009) y 3-10µm; 10-15 µm; > 15 µm (desde 2010 a la actualidad). Ésta evaluación se hacía usando microscopía óptica, la cual no es resolutiva a nivel de especie. Sin embargo, la identificación de estas especies es importante porque se han registrado floraciones de diatomeas céntricas al final de la primavera y el verano durante años, siendo éstas las especies dominantes del estuario. El objetivo de este estudio es demostrar que los grupos de diatomeas establecidos en clases de tamaños y analizados con microscopía óptica, en realidad son grupos heterogéneos que contienen una gran diversidad específica. Para ello se lleva a cabo una exhaustiva identificación de las diferentes cepas de especies de la clase Mediophyceae a nivel morfológico y molecular, que revelarán una gran diversidad, incluyendo la presencia de especies cripticas o complejos de especies (como el caso del género *Skeletonema* o el complejo de especies de *Cyclotella meneghiniana*). Estas especies, fueron sometidas, a los análisis anteriormente nombrados y a análisis fisiológicos complementarios para analizar su potencial como especies formadoras de floraciones en el área de estudio.

Capítulo 1- Es un capítulo introductorio dónde se habla de las aplicaciones de las diatomeas, su clasificación taxonómica y cómo ésta ha ido evolucionando. Aquí

también se recogen la situación de los distintos análisis a utilizar, tanto morfológicos como moleculares, su utilidad, sus limitaciones y su aplicación. Posteriormente estos análisis serán utilizados y explicados en más detalle en los siguientes capítulos. Además, se explica la importancia del estudio de la ecofisiología en especies de estuario, junto con los métodos usados, que se desarrollarán en detalle en los **capítulos 3** y **4**. Respecto a la técnica de la metabarcoding, ésta se introducirá en aspectos generales en este capítulo y será desarrollada en detalle en el **capítulo 6**. Los objetivos y la hipótesis están recogidos al final de este **capítulo 1**.

Capítulo 2- Para entender mejor la composición de especies de las floraciones de diatomeas de la clase Mediophyceae en el estuario de Bilbao, se realizaron varios análisis. En primer lugar se llevaron a cabo análisis morfológicos mediante microscopía electrónica de barrido, tanto de muestras de campo, como de cultivos de cepas aisladas de ésas muestras. Además, se realizaron análisis moleculares de esas cepas, basados en la región ITS (ITS1+5.8S+ITS2) y 28S rARN. Como resultado, se identificaron 7 especies de diatomeas céntricas solitarias, las cuales pertenecen a 4 géneros diferentes: *Conticribra weissflogii*, *Cyclotella atomus* var. *atomus*, *Cyclotella cryptica*, *Cyclotella marina*, *Cyclotella meneghiniana*, *Discostella pseudostelligera* y *Thalassiosira pseudonana*. De éstas, las especies dominantes durante las floraciones fueron *C. meneghiniana* y *Co. weissflogii* en la parte interior del estuario y *D. pseudostelligera* y *T. pseudonana* en la parte media. Se observó que los rasgos morfológicos usados para diferenciar entre pares de especies similares (*C. meneghiniana*/ *C. cryptica* o *D. pseudostelligera*/*D. woltereckii*) variaban con las condiciones medioambientales, denotando una gran plasticidad fenotípica que puede dificultar una identificación precisa de especies cuándo se usa sólo un enfoque morfológico.

Capítulo 3- Para conocer más sobre la composición de especies identificadas en el **capítulo 2**, se estudiaron también las especies de diatomeas céntricas que formaban cadenas. Estas especies pertenecen al género *Skeletonema,* el cual es frecuente en el estuario de Bilbao, dónde está presente a lo largo de todo el gradiente de salinidad. Por medio de análisis morfológicos y moleculares, seis cepas, pertenecientes a 3 especies fueron identificadas: *S. costatum, S dohrnii* y *S.menzelii.* Se analizó mediante un fluorímetro PAM la respuesta a la salinidad (5, 10, 15, 20, 25 30 y 35 psu) para saber si pueden crecer activamente en las diferentes masas de agua o si son desplazadas por la marea, para ello se analizaron las tasas de crecimiento (GR), la eficiencia cuántica máxima del fotosistema II (F_v/F_m) y la tasa relativa de transporte de electrones (rETR). Los resultados desvelaron que las tres especies pueden considerarse eurihalinas, creciendo con un buen estado fisiológico entre 5 y 35 psu. Las tasas de crecimiento máximo fueron de 1.17 d^{-1} para *S. costatum* a 15 psu; 1.61 d^{-1} para *S. dohrnii* a 25 psu y 1.7 d^{-1} para *S. menzelii* a 20 psu.

Capítulo 4- Las especies identificadas como crípticas en el **capítulo 2**, *Cyclotella cryptica* y *C. meneghiniana*, fueron sometidas a un análisis exhaustivo en éste capítulo. Se realizaron análisis morfológicos, moleculares y fisiológicos para distinguir entre estas morfoespecies del complejo de especies de *C. meneghiniana*, las cuales podrían presentar, según estudios anteriores, plasticidad morfológica bajo determinadas condiciones medioambientales, particularmente con cambios en salinidad. Las características morfológicas fueron medidas en especímenes de las dos especies creciendo en cultivos a 5 salinidades diferentes, de 0.5 a 30 psu, las cuales son típicas del ambiente estuarino. Además se identificaron molecularmente, mediante la región ITS, incluyendo el análisis de la estructura secundaria de ITS2. El estado fisiológico de

las cepas creciendo a distintas salinidades (7 salinidades, desde 0.5 a 35 psu) fueron analizadas mediante la fluorímetro PAM, el cual proporcionó datos como la biomasa o el estrés por salinidad al que estaban sometidas las cepas. Los resultados de los análisis moleculares revelaron la ausencia de solapamiento en las características morfológicas de ambas especies a las salinidades testadas, aunque *C. meneghiniana* presentó una marcada plasticidad morfológica con las salinidad. Ambas especies estaban claramente diferenciadas por su estructura secundaria de ITS2, las cuales presentan CBC y HCBC's entre ellas, lo que indica una incompatibilidad reproductiva. Ambas especies crecieron en buen estado fisiológico en todas las salinidades testadas.

Capítulo 5- La fracción de tamaño del picoplancton (0.2-2nm), a pesar de ser un componente abundante de la comunidad de fitoplancton, es generalmente ignorada o incorrectamente identificada, ya que sus individuos pasan desapercibidos en microscopía óptica o incluso electrónica. Además, las especies de algunos géneros presentan características similares, como ocurre con el género *Minidiscus*. Éste puede llegar a confundirse incluso con especies de otros género como *Skeletonema menzelii*. Su pequeño tamaño y la presencia de especies pseudocrípticas hacen de Minidiscus uno de los géneros menos conocidos y estudiados de diatomeas céntricas, a pesar de estar ampliamente distribuido geográficamente. Por esta razón se le dedica a éste género un capítulo, además de por ser un género muy presente en el estuario de Bilbao y para clarificar algunas incongruencias detectadas en los análisis filogenéticos realizados para el **capítulo 3**, en el que se detectó una relación filogenética con *Skeletonema* inesperada. Muy pocos estudios sobre *Minidiscus* están basados en análisis de cultivos, lo que explica la falta de datos moleculares o morfológicos o la ausencia de estudios sobre su ciclo vital. En este trabajo, se hizo un estudio detallado sobre *Minidiscus comicus*, basado en cultivos y en muestras de campo del estuario de Bilbao. Los resultados

demostraron una variación morfológica más amplia de la descrita anteriormente, con especímenes pertenecientes a dos morfologías diferentes. Para comprobar si estas características morfológicas correspondían con diferencias moleculares, se analizaron dos marcadores: la región ITS y el 28S del rDNA, los cuales no revelaron correspondencia con la variabilidad morfológica.

Capítulo 6- La técnica de metabarcoding fue testada y los resultados fueron comparados con los obtenidos previamente con métodos tradicionales, descritos en anteriores capítulos. La técnica de Illumina Hi-seq, basada en la región variable V4 del gen 18S del rDNA, fue usada en muestras de campo. Se identificaron así 20 géneros de la clase Mediophyceae en el estuario de Bilbao. Presentando una gran similitud entre los resultados de metabarcoding y taxonomía basada en imágenes de SEM, los resultados demuestran la eficiencia de la técnica de metabarcoding en la identificación de Mediophyceae. El principal problema que presenta es la inexactitud y la falta de datos en las bases de datos existentes. Las secuencias disponibles en los bancos de genes no son fiables en la identidad de las especies, ya que estas no están siempre correctamente identificadas o están identificadas en un nivel taxonómico por encima de especie (en caso de *Thalassiosira, Minidiscus* o *Skeletonema*). Debería mejorarse la técnica con una combinación de métodos, incluyendo análisis de barcoding de cultivos y microscopía electrónica para completar los datos de cepas de diatomeas, con el objetivo de completar los vacíos de información que existen actualmente en las bases de datos.

SUMMARY

The environmental assessment of the estuaries, rivers or lakes can be made based on features of the phytoplankton community. In the Bilbao estuary, a routine monitoring program has been performed since 2000 to supervise the evolution of the water quality. The phytoplankton community assessment has been done taking into account the general groups, in terms of taxonomy, and the abundance and size of the cells. According to this procedure, centric diatoms were classified into 3 groups: <5µm; 5-15 µm; >15 µm (from 2000 to 2009) and 3-10µm; 10-15 µm and > 15 µm (from 2010 to nowadays). The classification was made using light microscopy, which is not resolute at species level. The identification of these species is important due to that blooms of centric diatoms have been reported in latter spring and summer for years, being the dominant species in the study area. The aim of this study was to demonstrate that a heterogeneous group of centric diatoms underlies the discrete size-classes counted with the light microscope. A detailed identification of the different strains based on morphological, molecular and physiological analyses will discover a higher specific diversity, with particular species within the class Mediophiceae differentiated by their blooming potential in the study area.

Chapter 1- This chapter introduces the diatoms: their applications, their taxonomical classification and how it has evolved. The state of the art of the morphological and molecular analyses and their limitations are also discussed here and referred to in **Chapters 2, 3, 4** and **5**. Besides, the importance of the study of the ecophysiology in estuarine species is explained, together with the methods used, which are developed in **Chapters 3** and **4**. The method of metabarcoding is introduced here

and developed in **Chapter 6**. The objectives and the hypothesis are listed at the end of this introductive **Chapter 1**.

Chapter 2- To gain insight into the specific composition of diatom blooms in the Bilbao estuary, net samples and cultures of estuarine isolates were analysed under the scanning electron microscope (SEM) and by molecular analyses of the Internal Transcribed Spacers 1 and 2 plus the coding region 5.8S (ITS region) and the 28S rRNA gene. Seven species of solitary centric diatoms belonging to four genera were found in the estuary including: *Conticribra weissflogii, Cyclotella atomus* var. *atomus, Cyclotella cryptica, Cyclotella marina, Cyclotella meneghiniana, Discostella pseudostelligera* and *Thalassiosira pseudonana.* Dominant species during blooms were *C. meneghiniana* and *Co. weissflogii* in the upper estuary and *D. pseudostelligera* and *T. pseudonana* in the middle estuary. The morphological traits used to differentiate between species pairs of similar morphology (*C. meneghiniana*/ *C. cryptica* or *D. pseudostelligera*/*D. woltereckii*) were observed to vary with environmental conditions, denoting a great deal of phenotypic plasticity which would hinder accurate identification of the species when using morphological approaches alone.

Chapter 3- To complete the knowledge on the species composition identified in **Chapter 2**, the species of centric diatoms that forms chains were also studied in this chapter. These species belongs to the genus *Skeletonema,* which is frequently found in Bilbao estuary where it is broadly distributed along the salinity gradient. By means of morphological and molecular analyses, six strains belonging to three species, *S.*

costatum, S dornhii and *S.menzelii,* have been identified. To know if they can actively grow in the different water masses or whether they are simply displaced by the tide, their response to salinity (5, 10, 15, 20, 25, 30 and 35 psu) was measured with a PAM fluorometer providing the specific growth rates, the maximal quantum efficiency of the Photosystem II (F_v/F_m) and the relative Electron Transport Rate (rETR). All three species can be considered as marine euryhaline, growing with good physiological state between 5-35 psu. The maximum growth rates were 1.17 d^{-1} for *S. costatum* (strain Bc18EHU) at 15 psu; 1.61 d^{-1} for *S. dohrnii* (strain Bc21EHU) at 25 psu and 1.7 d^{-1} for *S. menzelii* (strain Bc30EHU) at 20 psu.

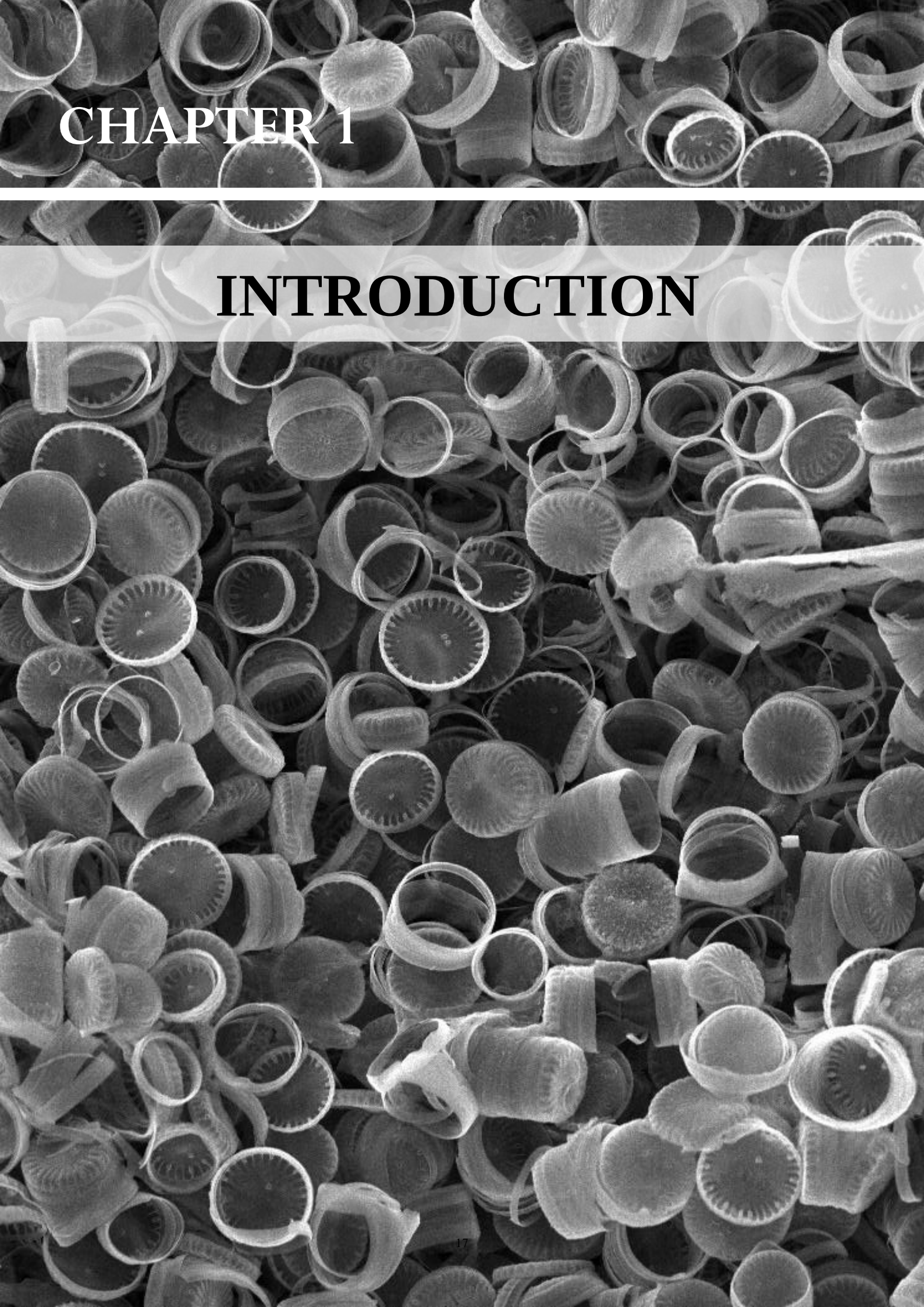
Chapter 4- An exhaustive study was done to gain insight into the cryptic species identified in **Chapter 2**, *Cyclotella cryptica* and *C. meneghiniana.* Morphological, molecular and physiological approaches have been used to distinguish between these diatoms morphospecies, from the *C. meneghiniana* complex, which might show morphological plasticity under determined environmental conditions, particularly with changing salinity. Morphological traits have been measured for specimens of both species growing in culture at 5 selected salinities, from 0.5 to 30, which are typical of the estuarine gradient. Molecular identity has been assessed by means of the Internal Transcribed Spacer (ITS1and ITS2) and 5.8 regions of the rDNA, including the analysis of the secondary structure of the ITS2. The physiological status of the strains when growing at different salinities (7 salinities, from 0.5 to 35) was tested with a PAM fluorometer which provided photosynthetic parameters used as proxy of algae biomass (F_0) and of saline stress (F_v/F_m). Results of the morphological analyses revealed the absence of overlapping features between both species at all the salinities tested,

although *C. meneghiniana* showed marked morphological plasticity with salinity. Both species are clearly differentiated by the secondary structure of the ITS2, which present one CBC and several HCBCs between them. Both species are euryhaline growing with good physiological status at all the salinities tested.

Chapter 5- The picophytoplankton (0.2-2nm) size fraction, despite being an abundant component of the phytoplankton community, has not been generally identified to species level due to that their individuals are inconspicuous under the light microscope, even under the scanning electron microscopy. In addition, species within some genera show similar features, as occurs with species of the diatom genus *Minidiscus*. Both the small size and the presence of pseudocryptic species make of *Minidiscus* one of the less known genus of centric diatoms despite it is widely distributed in different marine areas. For this reason a special chapter was dedicated to this genera, which was present in the Bilbao estuary, also some unexpected phylogenetic relationships with *Skeletonema* species were detected during the analyses of **Chapter 3**. Only a few studies on *Minidiscus* are based on cultured species, what explain the scarcity of data on molecular or morphological analyses or life cycles studies. In this work, a detailed study of *Minidiscus comicus* based on cultures and field samples is performed to strains isolated from the Bilbao estuary. The results show a wider morphological variation than that previously described, with specimens belonging to two different morphologies. In order to check if these morphological characteristics correspond with molecular differences, two markers were analyzed: ITS and 28S regions of the rDNA, which did not reveal correspondence with the morphological variability.

Chapter 6- A metabarcoding technique was tested to compare the previous results performed with traditional methods (scanning electron microscopy and barcoding of cultured samples). The Illumina Hi-seq technique, based on the V4 of the 18S rDNA barcode, on field samples were used. The class Mediophyceae was formed by at least 20 genera in the estuary area. By exhibiting strong similarity between the results of metabarcoding and taxonomy based on SEM micrographics, the results demonstrated the efficiency of metabarcoding techniques in analyzing Mediophyceae. The main problem was the incompleteness and inaccuracy of the databases. It appears that the sequences available in the banks of genes are not reliable as species identity is not always correct or they are identified at taxonomic levels higher than that of species (the case of *Thalassiosira, Minidiscus* or *Skeletonema*). It is of great concern to improve metabarcoding with a combination of methods including barcoding of cultured cells and SEM taxonomy to complete data for diatom strains, with the aim to filling the gaps in diatoms inventories.

Chapter 7- In this chapter, the main conclusions of the previous chapters are presented. They are listed together and briefly expounded before the thesis, which conclude this manuscript.



CHAPTER 1

INTRODUCTION

Phytoplankton are defined as planktonic microalgae drifting or moving by flagella in the illuminated part of the water column of almost all the water bodies. Most of them are photosynthetic organisms although some algal classes have mixotrophic or even particular cases of heterotropic representatives. They are extremely diverse both in morphology and physiology and due to their high abundance are significant contributors to major global processes. These include oxygen production, carbon fixation and sinking and nutrient recycling, sustaining the life of most other aquatic organisms. In the oceans, diatoms, dinoflagellates, haptophytes and small prasinophyceans are the most diverse eukaryotic phytoplankton taxa, some of which can form blooms (Not et al. 2012).

Diatoms are perhaps the most widespread group of phyoplanktonic organisms on Earth. They are abundant in all aquatic ecosystems, which cover about 70% of the earth surface, and even occur in terrestrial environments, including soils, moist surfaces of rocks and some plants (Round et al. 1990). There are over 250 genera of living diatoms belonging to over 100.000 different species (Van den Hoek et al. 1995), classified by their frustules morphology (Round et al. 1990). They form the base of short, energy-efficient food webs that support large-scale coastal fisheries. Photosynthesis by diatoms generates as much as 40% of the organic carbon produced each year in the sea and the role in global carbon cycling is comparable to that of all terrestrial rain forest together (Armbrust et al. 2004).

APLICATIONS

Diatoms are extremely abundant and ecologically diverse and their short lifespan and fast migration rates enable them to respond quickly to environmental changes. For these reasons, they are very good bioindicators used as sensors of environmental changes including biogeochemical silica depletion, surface water acidity, eutrophication, paleo-climatology and sea level changes. They are among the most used indicators of fresh water quality and water ecological status (Guzkowska & Gasse 1990, Kelly et al. 2008). Recently, they have shown to be very useful in ecotoxicological assays (Araújo et al. 2010). They are also used for oil and gas exploration, forensic analysis, or as markers of atmospheric transport (Stoermer & Smol 1999, Cermeño et al. 2005). The pharmaceutical industry makes use of the anticarcinogenic capacity of some of their pigments and other organic compounds (Peng et al. 2011). Actually, there are industries focussing their activity on the many uses of the marine phytoplankton with diatoms as one of the main components (e.g. *fitoplanctonmarino.com*). In addition, to all these applications, their special morphology makes diatoms very interesting in the field of nanotechnology, which designs and produces specific frustule morphologies (Parkinson & Gordon 1999, Parkinson et al. 1999, Drum & Gordon 2003, Gordon et al. 2009). Besides, advances in molecular genomics are facilitating the use of diatom-specific genes or pathways for biotechnology (Lopez et al. 2005).

Diatoms are a wide group with a great future in science due to their many applications. However, whereas some species are well known, there is a lack of information on many others. To know all the potential uses of diatoms, the first step is to identify them accurately.

DIATOMS CLASSIFICATION

Heterokont algae are a monophyletic group that includes all photosynthetic organisms with tripartite tubular hairs on the mature flagellum, as well as some nonphotosynthetic relatives and some that have secondarily reduced or lost tripartite hairs (Andersen 2004). Diatoms are heterokont algae, and their placement in this kingdom is confirmed by their molecular signal and by the tripartite hairs on the uniflagellate flagellum of the sperm of the centric diatoms. They have lost all traces of the smooth posterior flagellum. In diatoms, the flagellar apparatus is reduced or absent, with only the centric diatom sperm bearing a flagellum.

It has been long assumed that diatoms were split in two groups: centric (Class *Coscinodiscophyceae*) and pennates (raphids, Class *Bacillariophyceae;* araphids, class *Fragilariophyceae*), which could be distinguished by their valves symmetry, mode of sexual reproduction and plastid number and structure (**Fig.1**):

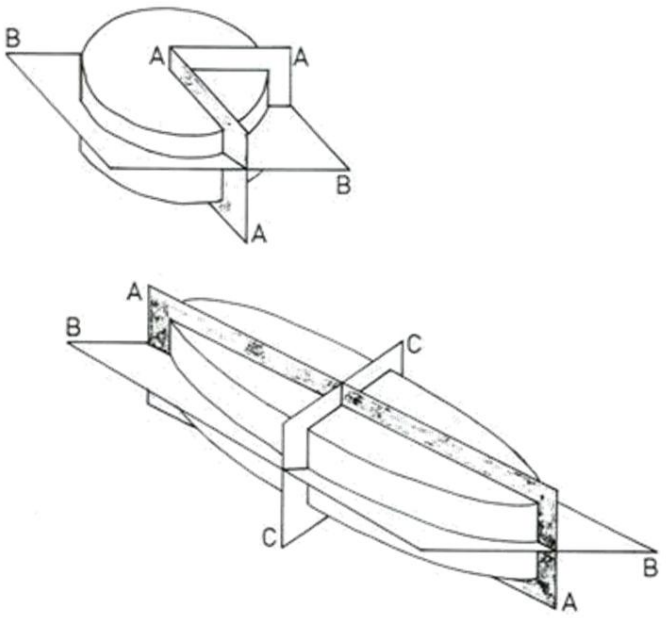


Fig.1: Morphological representation of centric and pennate diatoms. (Round et al. 1990)

·**Centric diatoms:** oogamous, with radial symmetry on valves from a central region or ring and numerous discoid plastids.

·**Pennate diatoms:** isogamous, with bilateral symmetry of the valves, i.e. elongated bilateral valves with striae oriented perpendicular to the long axis, and generally fewer, plate-like plastids.

This classification is descriptive and has not taxonomic value, except for the pennates. Medlin and Kaczmarska (2004) revised the systematic of diatoms based on more than 20 years of consistent recovery of two major clades of diatoms that did not correspond to the previous traditional concept of centrics and pennates and, as a result, they established the following three classes of diatoms (**Fig.2**):

·**Coscinodiscophyceae**: centric diatoms with radial symmetry of valve shape and structure.

·**Mediophyceae**: bipolar and multipolar centrics + radial Thalassiosirales (Order Thalassiosirales).

·**Bacillariophyceae**: pennates

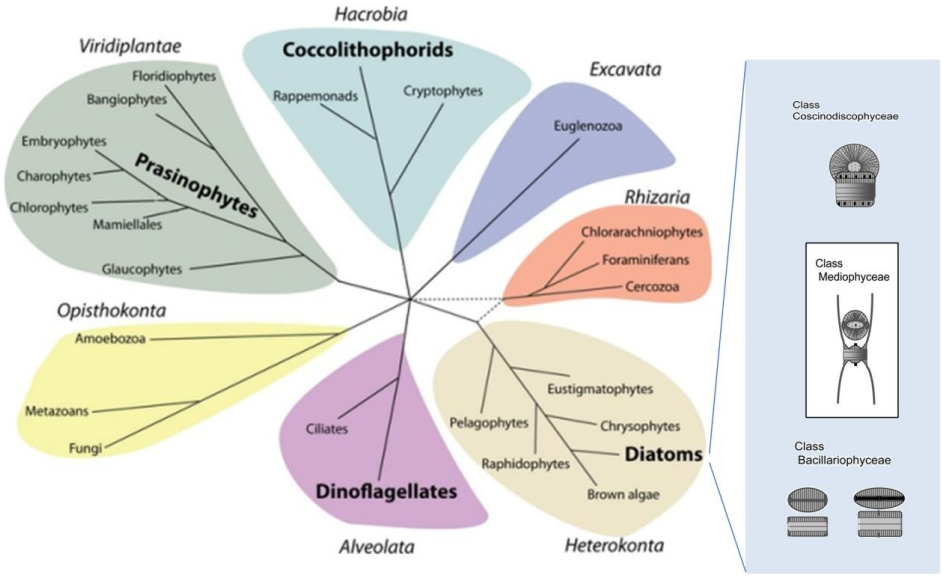


Fig.2: Modified from Collins et al. 2013. Phylogenetic diversity of eukaryotes. With the representation of the three classes that conforms the diatoms.

TAXONOMIC IDENTIFICATION ANALYSES: MORPHOLOGICAL AND MOLECULAR

Typically each cell possesses an extracellular shell, called frustule, which contains, among other compounds, amorphous silica (SiO_2). These frustules, formed by two valves, are elaborately ornamented and sculptured with species-specific patterns. Vegetative growth carries out with a progressive separation of the two valves, culminating in periodical sexual reproduction to restore the specific size reduced after a period of asexual reproduction (**Fig.3**).

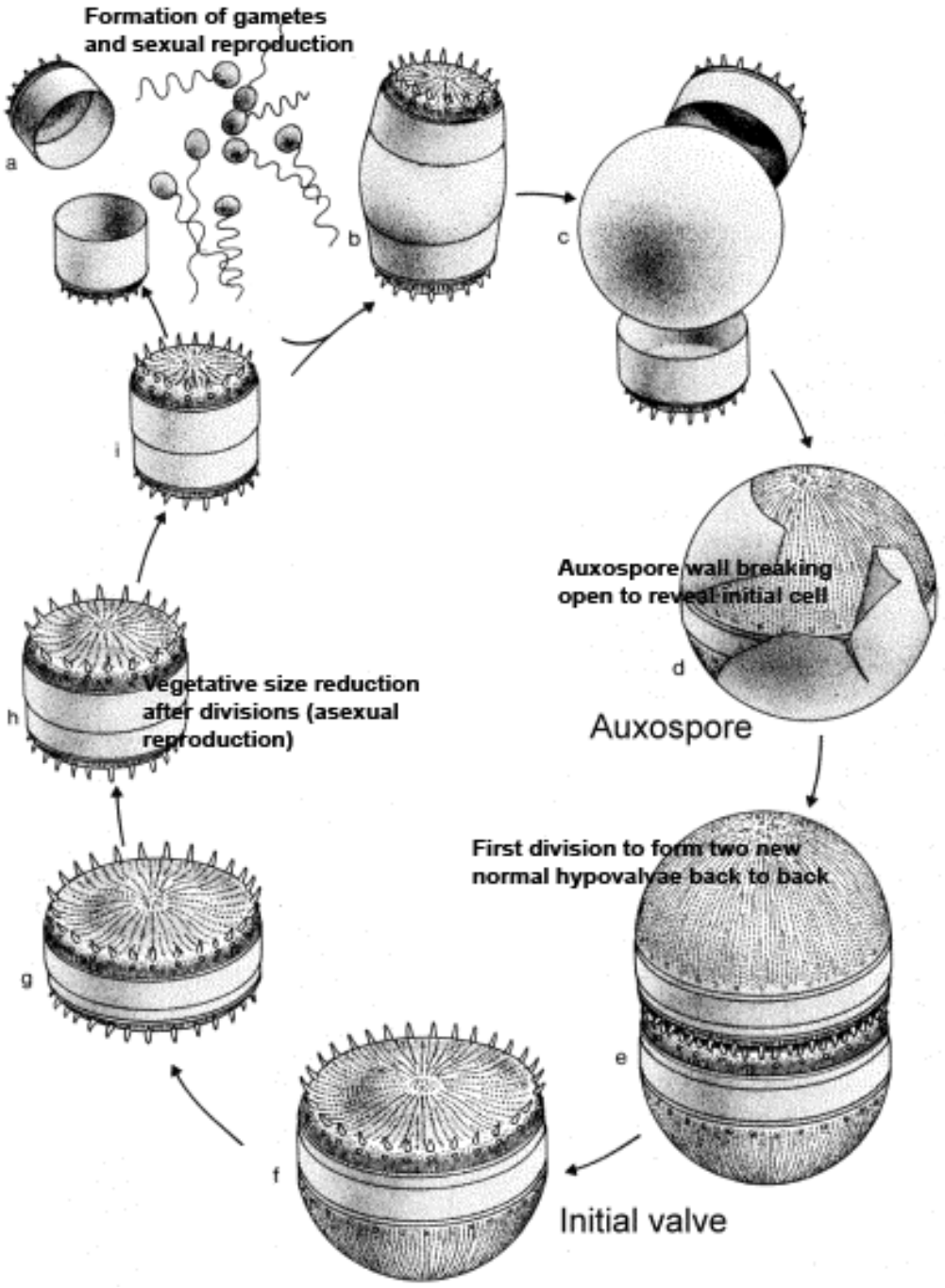


Fig.3: Standart life cycle of a diatom, modified from (Round et al. 1990).

Almost all species of diatoms could be identified by a detailed morphological analysis of the frustule, for which the protoplasm has to be removed. To identify to species level, generally the ultrastructure of the frustule has to be analysed by electron microscopy. Morphological identification has traditionally been done, although this procedure creates certain 'uncertainty' due to the following:

·Depending on the species of diatom and the growth conditions, frustules can display a wide range of different morphologies. Even the same cell, can be heterovalvate, which means that the two valves from the same cell can be different in morphology.

·The presence of cryptic (identical morphology) or pseudocryptic species (differentiated by a few not well marked morphological traits), which have different genotype and can thus considered different species with even different ecophysiology.

·The concept of 'species', which has been a widely debated theme in biology and still not clear nowadays, makes the taxonomy in diatoms messy. The evidence from morphology, genetic data, mating system, physiology, ecology and crossing behavior suggests that species boundaries in diatoms have traditionally been drawn too broadly. The species concept in diatoms was addressed by Mann (1999) discussing how this problem has been evolved during time.

As discussed by Round et al. (1990), various diatoms were described in the latter half of the 18th century, since then several species were changed from one taxon to another based on the use of more advanced morphological or genetics tools. Despite some recent studies of taxonomy have been carried out taking into account only morphological features, it is nowadays assumed that molecular analyses have to be performed for an accurate identification to species level.

In 2004, Armbrust et al. published the first diatom genome from a marine strain of *Thalassiosira pseudonana*, which was a breakthrough to understand the ecology and evolution of diatoms. The genome revealed unexpected metabolic pathways and uncovered the nature of nuclear genomes, which contain a mix of genetic material from the stramenopile host cell, diverse bacterial donors and a succession of green and red

algal endosymbionts (Alverson et al. 2011). Indeed, it revealed a 'mosaic' of features, with genes derived from plant, animal and bacterial lineages (Lopez et al. 2005).

ECO-PHYSIOLOGY OF ESTUARINE SPECIES

Small meso-macrotidal estuaries receiving fast-flowing rivers can experience drastic changes in salinity at different time scales, from hourly to seasonal. Bloom-forming species within these areas have to be salinity tolerant to a certain extent, at least those blooming in the inner and middle reaches of these estuaries, which are the most unstable with respect to salinity. Before the use of molecular methods to support the identification of microalgae, species of several genera, now known to contain morphologically cryptic or pseudo-cryptic species, were identified as single species with broad salinity tolerances.

Diatoms are a dominant component of the phytoplankton assemblages in estuaries (Marshall et al. 2006, Bazin et al. 2014). Although these microalgae are generally associated with well mixed waters, which allow them to obtain enough nutrients, nanoplanktonic centric diatoms are also frequent and abundant in stratified and partially mixed estuaries. There they can remain in the illuminated layers and be more abundant than in bottom waters (Bazin et al. 2014); organic secretions of the marginal strutted processes (fultoportulae) are thought to contribute to their buoyancy (Stoermer & Julius 2003). The elevated biomass generated by these diatoms (and their associated bacteria and fungi) during maintained periods of growth can be harmful for estuarine invertebrates and fishes, causing oxygen depletion and clogging of filtering structures with their mucilaginous excretions (Fryxell & Hasle, 2003). As pointed out

by these authors, any diatom frequently occurring in bloom proportions may be regarded as potentially harmful. The blooming of centric diatoms in the upper and middle segments of estuaries is well documented (Muylaert & Sabbe 1996, Orive et al. 1998, Trigueros & Orive 2000, 2001, Marshall et al. 2006, Lionard et al. 2008, Bazin et al. 2014). The assemblages are formed by freshwater euryhaline algae, which can also grow at relatively high rates in the brackish waters, together with brackish water species and marine euryhaline representatives. In nutrient-replete estuaries, these blooms are generally associated with spring or summer conditions of higher temperature and irradiance and longer water residence time. The enhanced synthesis of amino acids for osmoregulation requires nutrient rich waters (Schobert 1974), which explains to a large extent why eutrophic conditions appear to favour these blooms (Weckstrom & Juggins 2006). Although their distribution along the salinity gradient can be a consequence of the tidal flushing (Trigueros & Orive 2000), several of these taxa are euryhaline and as such able to grow in the estuary under different salinity ranges, as has been observed for particular species (see Orive et al. 2004, Prasad and Nienow 2006, Roubeix and Lancelot 2008). Thus, it is of paramount importance to know the response of particular species to salinity and irradiance as a means of estimating their bloom potential along the salinity gradient.

Photosynthetic parameters, as derived from the *in vivo* chlorophyll fluorescence associated with photosystem II (PSII), can be used to estimate the response of microalgae to environmental parameters (Maxwell & Johnson 2000, Baker 2008). When using pulse amplitude modulation (PAM) fluorometry, the derived parameter F_0 can be used as a proxy of algal biomass (Dijkman and Kromkamp 2006, Kromkamp et al. 2008 and references herein), whereas (Fm $-F$0)/Fm (Fv/Fm), the maximum quantum efficiency of PSII photochemistry, can be used as a measure of stress (Dijkman &

Kromkamp 2006, Kromkamp et al. 2008, Baker 2008, Suggett et al. 2012, Betancor et al. 2015). In addition, rapid light curves (RLCs) can be made under controlled conditions to estimate the relative electron transport rate (rETR) of PSII (Ralph & Gademann 2005). Parameters derived from these curves, namely light-limited initial slope (α), saturation light intensity (Ek) and maximal relative ETR (rETRmax), are good indicators of the light regime and photoacclimatory behaviour of the cells (Hennige et al. 2008, Suggett et al. 2012), and can provide a good estimate of the photosynthetic rate (Kromkamp et al. 2008, Napoléon & Claquin 2012, Figueroa et al. 2013).

METABARCODING

A new concept of sampling and community identification have been developed recently consisting in the molecular analysis of a community from natural samples based on their DNA sequences. This molecular procedure, called metabarcoding (**Fig.4**), has proven to be very useful for study phytoplankton specific diversity (Logares et al. 2014), including diatoms (Nanjappa et al. 2014), and for the use of these microalgae in monitoring programs (Corell and Rodriguez-Ezpeleta 2014; Amorim et al. 2015). An ideal DNA barcode is one with conserved flanking fragments to facilitate the design of universal primers; with a sequence length obtainable in a single amplification, and; with the power to identify organism at the species level (Moniz & Kaczmarska 2010). Taking into account these conditions, recent advances in DNA sequencing have revolutionized the field of genomics, making possible to generate large amounts of sequence data very rapidly and at a substantially lower cost (Kircher & Kelso 2010). Although this new method allows detecting organisms that are usually undetectable by traditional method due to their small size or their cryptic nature (Bazin

et al. 2014), they do not resolve at species level in most of the groups. The deep knowledge on Mediophiceae community in a study area allows comparing these new methods with traditional method of identification.

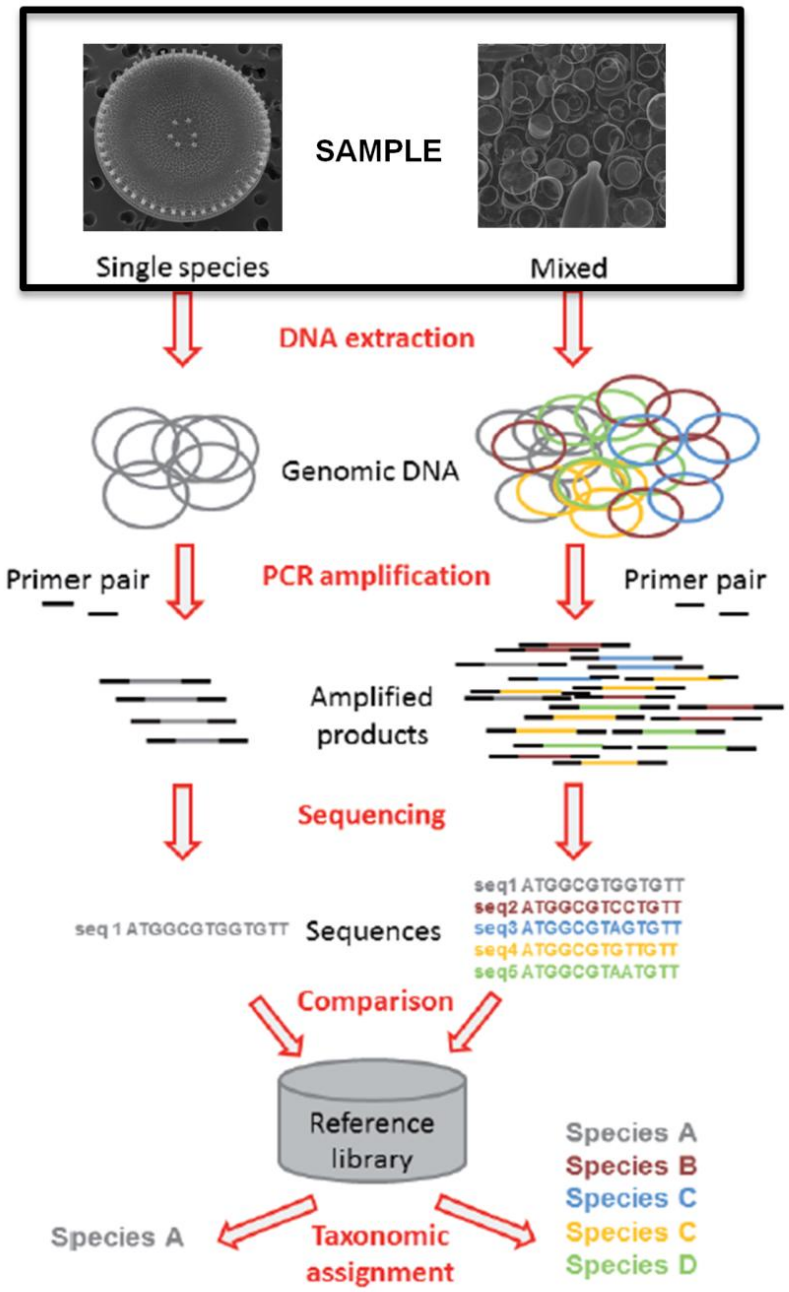


Fig.4: Schematic representation of the processes of DNA barcoding (left) and metabarcoding (right). Coloured circles represents extracted genomic DNA, wich is composed of multiple copies of the genomes of the species composing the sample (metabarcoding). Amplified products are identical in barcoding, whereas a mixture of amplified products from the diferent genomes is obtained in metabarcoding. Once the amplified products have been sequenced, taxonomic assigments is preformed based on comparison of the obtained sequences with a reference database. Modified from (Corell & Rodriguez-Ezpeleta 2014).

BACKGROUND OF THE STUDY AREA

In the Bilbao estuary, other phytoplankton groups have been studied during the last decades (Orive et al., 2013, Seoane et al. 2005, 2009, Laza-Martinez et al. 2007, Alonso-González et al. 2014), but a detailed morphological and molecular study on Mediophiceae is still lacking despite they have been observed to be a dominant group in monitoring studies of the estuary (Orive et al. 2004). In the Bilbao estuary, a routine monitoring program is being performed since 2000 to supervise the evolution of the water quality after the implementation of a sewerage scheme (Garcia- Barcina et al. 2012). In this context, the phytoplankton community assessment is done taking into account the abundance and size of the cells at the level of genus or higher taxonomic level, which are the taxonomic categories accurately identified by the Utermöhl method. Only a few specimens can be identified to species level. According to this procedure, centric diatoms are classified into 3 size classes: <5µm, 5-15 µm; >15 µm (from 2000 to 2009) and 3-10µm; 10-15 µm and > 15 µm (from 2010 to nowadays), without any taxonomic information.

It is well known that phytoplankton blooms can cause environmental and human health problems, associated in cases with great economic losses. In the Bilbao estuary, during blooms, centric diatoms have been observed in latter spring and summer for most years. In some cases, as in July 2003 or July 2007, some fishes were found death in the inner part of the estuary coinciding with high concentration of centric diatoms, which are known to be harmful by causing anoxia when growing in bloom proportions.

OBJECTIVES

· To identify the species of the class Mediophyceae present along the Bilbao estuary by using both ultrastructural and molecular analyses.

· To analyze the physiological response to salinity of congeners species including cryptic species or complex of species by means of chlorophyll *a* fluorescence.

· To estimate the blooming potential of Mediophyceae species along the salinity gradient, to know which species could be able to grow actively at the different estuarine salinities or be passively transported by the tide.

· To compare the metabarcoding procedure with illumine sequencing with traditional taxonomy (morphological and barcoding analyses) for discriminate at species level in Mediophyceae.

HYPOTHESIS

We presume that a heterogeneous group of centric diatoms underlies the discrete size-classes counted with the light microscope. A detailed identification of the different strains based on morphological, molecular and physiological analyses will allow us to discover a higher specific diversity and will confirm the importance of class Mediophyceae as blooming species within the phytoplankton community of the Bilbao estuary.

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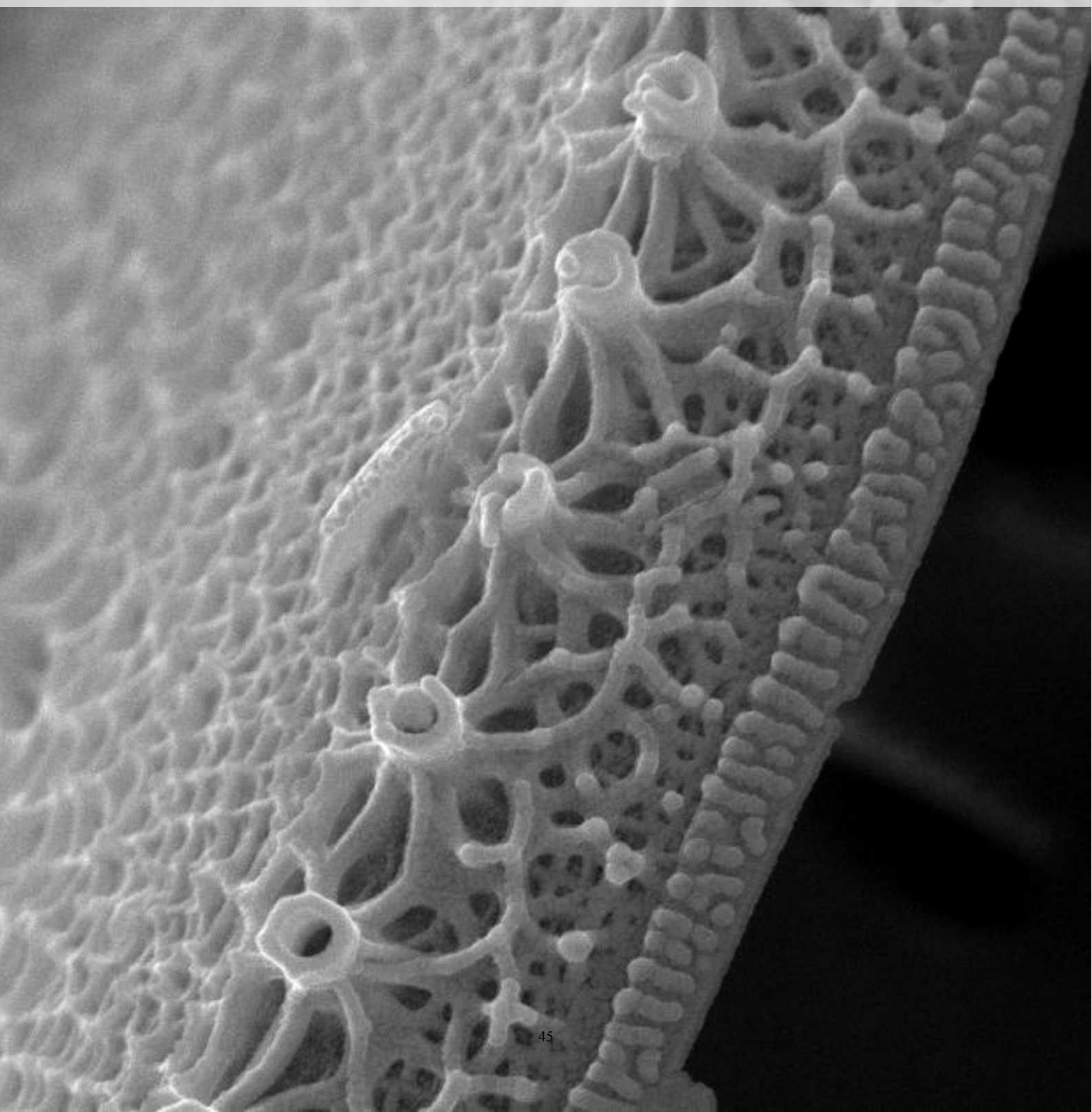
CHAPTER 2

Molecular and morphological analyses of solitary forms of brackish Thalassiosiroid diatoms (Coscinodiscophyceae), with emphasis on their phenotypic plasticity



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Name of the conference: ASLO - 2015 Aquatic Sciences Meeting
City: Granada, Andalusia, Spain
Date: 22/02/2015 - End date: 27/02/2015
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Authors: Joana Hevia-Orube; Helena David; Aitor Laza-Martínez; Irati Miguel; Sergio Seoane; Emma Orive.

Title: Bloom-forming diatoms of estuarine waters as potential fuel source
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Organising institution: University of Copenhagen
Authors: Joana Hevia-Orube; Helena David; Aitor Laza-Martínez; Irati Miguel; Sergio Seoane; Emma Orive.

INTRODUCTION

Estuaries form a freshwater-to-marine continuum where salinity, inorganic nutrients and water transparency experience marked spatial and temporal changes, particularly in shallow meso- or macrotidal estuaries. Consequently, phytoplankton populations are a mixture of freshwater, brackish water or marine species which changes along the longitudinal axis, depending on the tidal state and the flushing rate of the water. Diatoms are a dominant component of the phytoplankton assemblages in estuaries (Marshall *et al*., 2006, Bazin *et al.*, 2014). Although these microalgae are generally associated with well-mixed waters, which allow them to obtain enough nutrients, nanoplanktonic centric diatoms are also frequent and abundant in stratified and partially mixed estuaries. There they can remain in the illuminated layers and be more abundant than in bottom waters (Bazin *et al*., 2014); organic secretions of the marginal strutted processes (fultoportulae) are thought to contribute to their buoyancy (Stoermer & Julius, 2003). The elevated biomass generated by these diatoms (and their associated bacteria and fungi) during maintained periods of growth can be harmful for estuarine invertebrates and fishes, causing oxygen depletion and clogging of filtering structures with their mucilaginous excretions (Fryxell & Hasle, 2003). As pointed out by these authors, any diatom frequently occurring in bloom proportions may be regarded as potentially harmful and the blooming of centric diatoms in the upper and middle segments of estuaries is well documented (Muylaert & Sabbe, 1996; Orive *et al*., 1998; Muylaert *et al*., 2000; Orive *et al*., 1998; Trigueros *et al*., 2000; Trigueros & Orive, 2001; Marshall *et al*., 2006; Linard *et al*., 2008; Mitrovic *et al*., 2008; Bazin *et al*., 2014). The assemblages are formed by freshwater euryhaline algae, which can also grow at relatively high rates in the brackish waters, together with brackish water species and marine euryhaline representatives. In nutrient-replete estuaries, these blooms are

generally associated with spring or summer conditions of higher temperature and irradiance and longer water residence time. The enhanced synthesis of amino acids for osmoregulation requires nutrient rich waters (Schobert, 1974), which explains to a large extent why eutrophic conditions appear to favour these blooms (Weckström & Juggins, 2005). Although their distribution along the salinity gradient can be a consequence of the tidal flushing (Trigueros & Orive, 2000), several of these taxa are euryhaline and as such able to grow in the estuary under different salinity ranges, as has been observed for particular species (see Prasad *et al.*, 1990; Orive *et al*., 2004; Prasad & Nienow, 2006; Roubeix & Lancelot, 2008).

During 15 years of phytoplankton monitoring in the Bilbao estuary (also known as Nervion River estuary), blooms of centric diatoms have been seen, mainly in summer but also in spring, to extend from the river into the estuary to a variable extent (Orive *et al*., 2004, Seoane *et al*., 2005). Taking into account the difficulty in identifying these diatoms with a light microscope (LM), during monitoring, only the abundance of cells (solitary centric diatoms) of different size classes was recorded. To gain insight into the taxonomic composition of the centric diatoms present in the blooms, ultrastructural (SEM) analyses were applied to fixed samples from the blooms and cultured cells isolated from the estuary. Molecular analyses (28S and ITS regions) of these cultures were also performed. Over the last years, the taxonomy of solitary forms of thalassiosiroid diatoms, which include fultoportulate diatoms constituting the order Thalassiosirales Glezer and Makarova 1986, has been revised and new genera and species have been described (Hånkansson, 2002; Houk & Klee, 2004; Stachura-Suchoples & Williams, 2009; Aké-Castillo *et al*., 2012). The taxonomy of the group is confusing due to the existence of cryptic or pseudo-cryptic species forming complexes, which are difficult to differentiate at the species level even under the EM (Bezteri *et al*.,

2005, 2007). Conversely, different morphotypes can be found within a single species due to the existence of phenotypic plasticity in response to a changing environment (Cherepanova *et al.,* 2010; Kociolek & Stoermer, 2010, Shirokawa *et al.*, 2012),without molecular analyses, many of these ecomorphotypes could be described as different species.

The aim of this study was to gain insight into the specific composition of the bloom forming solitary centric diatoms, to ascertain if the blooms were monospecific or not and if they are formed by recurrent species. We also discuss the difficulty in identifying thalassiosiroid diatoms, due to the existence of pseudo-cryptic species and phenotypic plasticity.

MATERIALS AND METHODS

Study area

The study area was the Bilbao estuary (43º20'N, 2º70'W, Basque Country, Northern Spain), a meso-macro tidal estuary with a semidiurnal tidal regime. The estuary is relatively shallow for most its length (2–9 m depth), except at the mouth, which consists of a semi-confined harbour 3.8 km wide and 14–30 m deep. More details on the geomorphology and hydrology are given in Borja *et al.* (2004).

Sampling and cultures

For phytoplankton analyses, bottle samples were taken near monthly from March to September 2009 and from June to September 2010 at eight stations (1–8) located along the salinity gradient of the estuary (**Fig.1**). All the samples were regularly

analysed for cell identification and counting as part of a programme to monitor changes in water quality after installation of a wastewater treatment system. After being enumerated by the Utermöhl method for quantification (**Supplementary Table S1**), fixed samples containing small centric diatoms in bloom proportions (more than1 million cells per litre in this case) were processed, as described below, for more detailed morphological analysis. In addition, live samples were taken in 2004, 2011 and 2012 from bloom locations to isolate and culture centric diatoms for morphological and molecular analyses (**Supplementary Table S2**). Unialgal cultures were grown at 17–20ºC, under a 14:10 h light:dark cycle, at 100 µmol photons $m^{-2}s^{-1}$ in f/2 medium (Guillard & Ryther, 1962).

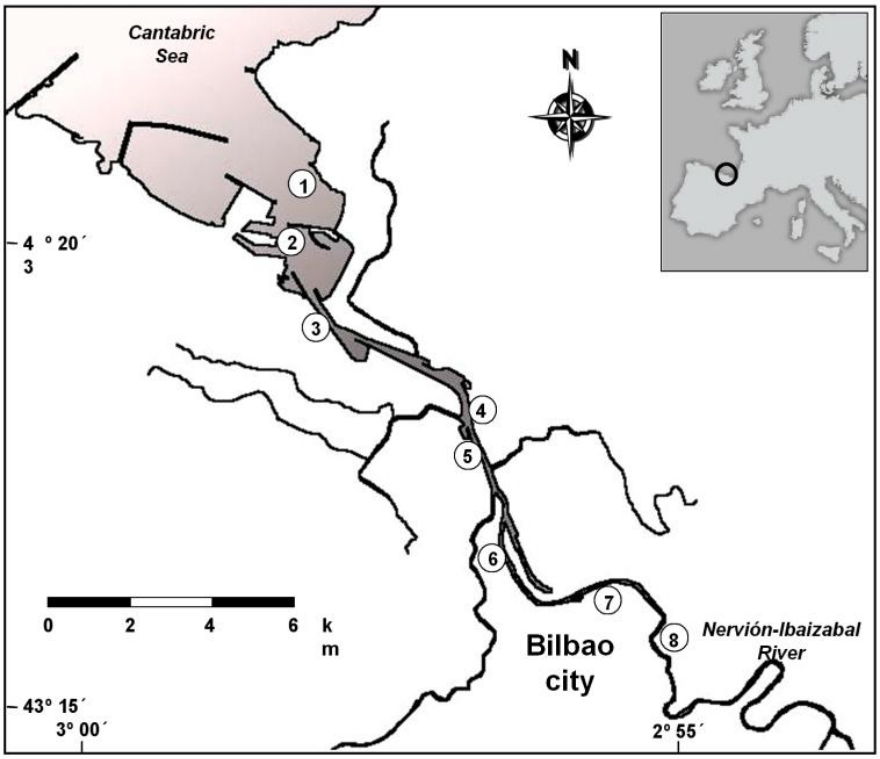


Fig. 1. Study area and sampling sites for the permanent monitoring.

Microscopy

Glutaraldehyde (0.1% final concentration) preserved samples were identified and counted according to the Utermöhl method as in Hasle (1978) under a Nikon Diaphot TMD inverted microscope with phase contrast. For ultrastructural analyses, both natural samples and cultured cells were rinsed and centrifuged to eliminate dissolved salts, after which the samples were boiled in acids (HNO_3 and H_2SO_4) to eliminate the organic matter. Samples for electron microscopy were filtered (Millipore TMTP, 1.2 µm pore size), rinsed with distilled water a few times before dehydrating with ethanol and finally rinsed with Hexamethyldisilazane (HMDS 98º) for 2 min. Filters were stub mounted (Agar Scientific Lt.), coated with 10 nm of chromium and observed with a Hitachi S-4800 SEM. In this study, both field samples and cultured cells were analysed for morphology. SEM images correspond usually to field samples and when this was not possible, micrographs of cultured strains were used and referred to in the corresponding figure legends.

The taxonomic description has been performed following the *terminology for the siliceous components of diatom cell* by Ross *et al.* (1979). Main morphological traits used to identify species were size and frustule ornamentation, including the following structures: stria, interstria (which when thick can be equivalent to costa), areolae, siliceous granules, spines, fultoportulae and rimoportulae. We do not describe girdle bands as during the process of cleaning cells, the girdle bands separate from the valves and cannot be related to a particular species in multispecific samples. The valve features were measured according to Genkal (1977) as in Genkal & Kiss (1993). The mathematical formulas used to measure valve dimensions and structures were: τ=

$10n/\pi D$; where τ= number of striae in 10µm; n= number of striae on circumference of valvae face; D= diameter of the valve.

DNA extraction, amplification and sequencing

For molecular analyses, unialgal cultures were centrifuged and the pellets frozen in ethanol until analysis. DNA was extracted with the commercial DNA extraction kit DNeasy Plant Mini (Qiagen, Hilden, Germany). The 28S and ITS rDNA regions were amplified with D1R-D2C (Scholin *et al*., 1993) and ITS1F-ITS1R (Pin *et al*., 2001) primer pairs, respectively. A mixture of 20 µl of ultrapure water, 5 µl of DNA extraction solution, 2.5 µl of each primer and 20 µl of a Bioline BioMix (Bioline GmbH, Luckenwalde, Germany) was transferred to the BIOER TC-24/H(b) thermo-cycler (BIOER Technology Co., Hangzhou, China). PCR amplification was achieved with the following program: one cycle at 95ºC for 2 min, 50ºC for 30 s and 72ºC for 45 s; 35 cycles at 94ºC for 30 s, 50ºC for 90 s and 72ºC for 30 s; and a final elongation step of 72ºC for 10 min. Amplification products were purified with the MultiScreen HTS PCR 96 kit (Millipore). Sequencing was carried out by using an ABI PRISM™ BIGDYE v3.1 Terminator Sequencing Reaction and an automatic sequencer ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were read and corrected using BioEdit 7.1.3 (Hall 1999).

Alignment and phylogenetic analyses

Phylogenetic relationships were inferred from 28 sequences (5 from this study plus 23 from GenBank) for the 28S rDNA and 16 (5 from this study plus 11 from GenBank) for the ITS rDNA. *Aulacoseira granulata* was used as outgroup for the 28S rDNA tree

and *Pseudo-nitzschia pungens* for the ITS rDNA tree. Sequences alignment was achieved using MUSCLE 3.7 alignment method (Edgar, 2004) and manually improved.

Phylogenetic analyses were inferred using Bayesian methods, with MrBayes v.3.2 software (Huelsenbeck & Ronquist, 2001), and Maximum Likelihood (ML) and Neighbour Joining (NJ) methods by using MEGA6 software (Tamura *et al*., 2011). For Bayesian method, GTR + 6 number of substitution types, 4by4 substitution model and invariable + gamma rates variation across sites were considered. Monte Carlo Markov chains (MCMC) (Yang & Rannala, 1997) were run from random trees for a total of 10,000 generations. One of every 10 trees was sampled. After excluding the first 250 sampled trees categorized as the "burn-in period", a consensus tree was visualized using MEGA 6 software (Tamura *et al*., 2011). For NJ and ML analyses, bootstrap confidence values were calculated from 5000 replications. Prior to phylogenetic analyses, the best DNA substitution model was carried out and a Bayesian information criterion (BIC) was selected to describe the best nucleotide substitution pattern.

For 28S, NJ algorithm and ML searches were conducted using the chosen model TN93+G (Tamura & Nei, 1993). For ML, a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.28) and transition/transversion rate (R = 2.44) and rates of base substitutions: r(AT) = 0.04; r(AC) = 0.03; r(AG) = 0.13; r(TA) = 0.03; r(TC) = 0.22; r(TG) = 0.04; r(CA) = 0.03; r(CT) = 0.28; r (CG)=0.04; r(GA) = 0.1; r(GT) = 0.04; r(GC)=0.03]. For ITS region, NJ algorithm and ML searches were conducted using the chosen model K2-P (Kimura, 1980) with a discrete Gamma distribution (+G, parameter = 0.53), transition/transversion rate (R = 1.87) and rates of base substitutions: r(AT) = 0.04; r(AC) = 0.04; r(AG) = 0.16; r(TA) = 0.04; r(TC) = 0.16; r(TG) = 0.04; r(CA) = 0.04; r(CT) = 0.16; r (CG)=0.,04; r(GA) = 0.15; r(GT) = 0.04; r(GC)=0.04].

RESULTS

Environmental conditions and phytoplankton biomass

The general features of the physical environment from January 2009 to September 2010 are depicted in **Fig. 2**. Temperature, salinity and dissolved oxygen experienced marked seasonal changes along the estuarine continuum. In summer, temperature increased over 20ºC along the entire estuary and salinity intrusion allowed the uppermost estuary (stations 6, 7 and 8) to reach meso-polyhaline conditions. During this period, oxygen concentration dropped to values as low as 60% saturation in this upper part. Maxima of chlorophyll *a* (**Fig.2**) and centric diatoms (**Supplementary Table S1**.) occurred in summer, particularly in the upper and middle estuary.

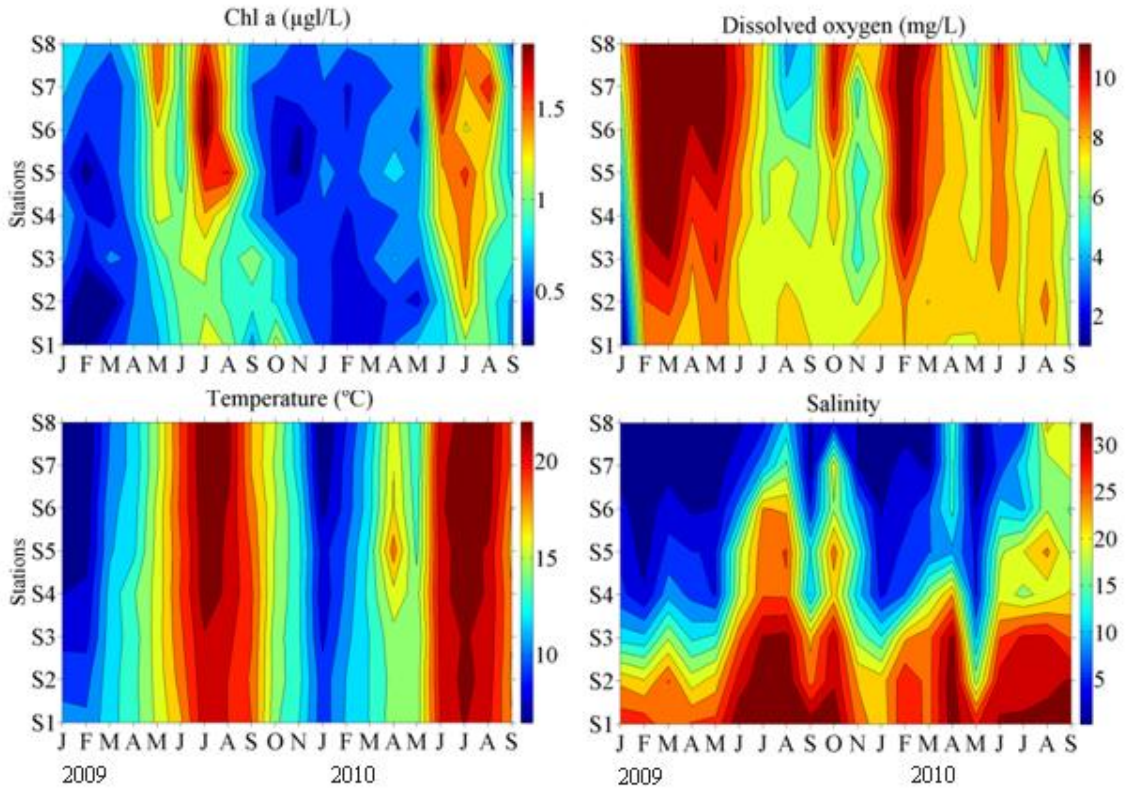


Fig.2: Contour plots representing environmental conditions and total phytoplankton biomass along the estuary from January 2009 to September 2010.

Average values of Chl *a* oscillated from a minimum of 2.44 µg l^{-1} in February 2009 and 3.86 µg l^{-1} in January 2010, to a maximum of 34.19 µg l^{-} in July 2009 and 22.65 µg l^{-1} in June 2010. Values as high as 85.60 µg l^{-1} were recorded in the inner stations during summer. These pigment values coincided with concentrations of diatom cells above 9×10^{6} cells l^{-1} in 2009 and more than 34×10^{6} cells l^{-1} in 2010.

Morphology

The following species of thalassiosiroid diatoms were found in the estuary during blooms of centric diatoms: *Cyclotella atomus* var. *atomus, Cyclotella marina, Cyclotella meneghiniana, Cyclotella cryptica, Conticribra weissflogii*, *Discostella pseudostelligera* and *Thalassiosira pseudonana.* On account of the marked morphological variability showed by centric diatoms, particularly in estuaries with very changeable conditions, a detailed description of the valve ultrastructure is given.

***Conticribra weissflogii* (Grunow) Stachura-Suchoples & Williams (Figs 3-8)**

Stachura-Suchoples & Williams, 2009, *European Journal of Phycology*: **44**, p. 477-486.

Basionym: *Micropodiscus weissflogii* Grunow in Van Haurck, 1885, *Synopsis des Diatomées de Belgique*: 210.

Synonyms: *Eupodiscus weissflogii* Grunow in Van Heurck, 1882-1885, *Types du Synopsis des Diatomées de Belgique*: **3** (No.11), 100 (No.416), *nom.invalid*.

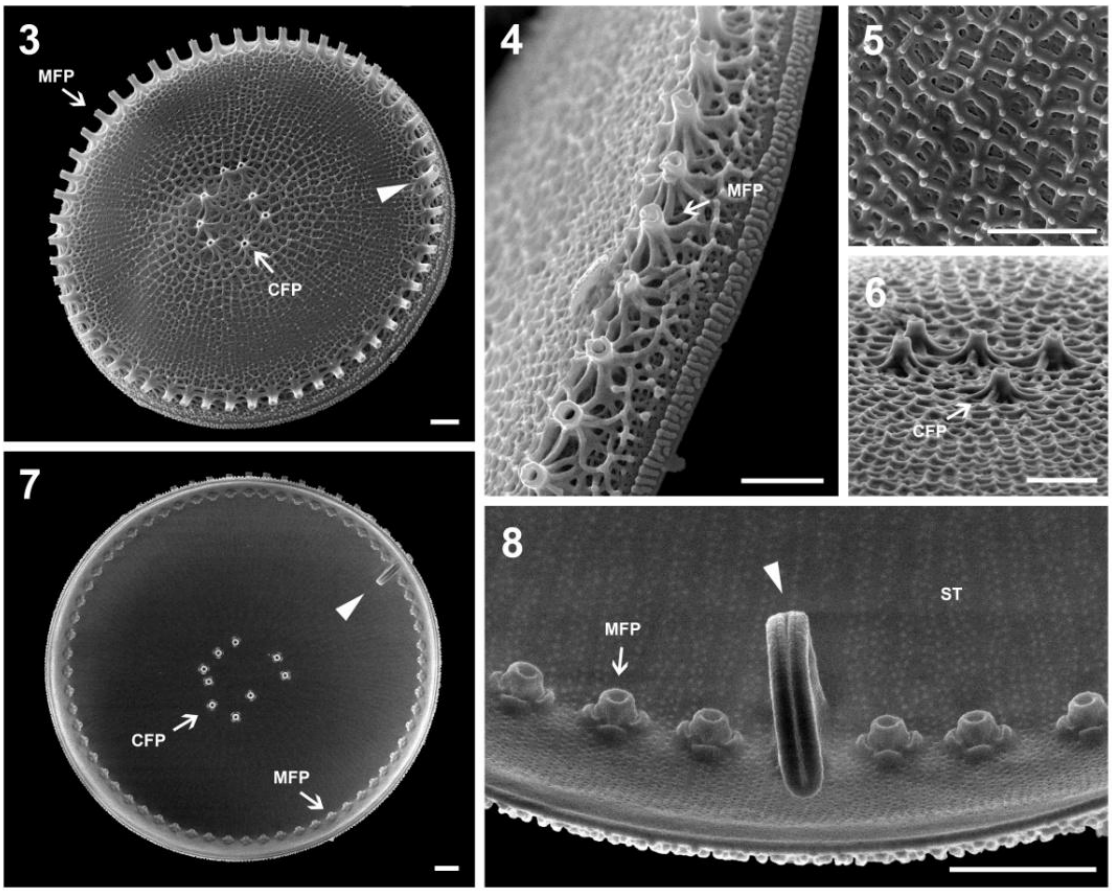
Micropodiscus weissflogii Grunow in Van Heurck, 1882-1885, *Types du Synopsis des Diatomées de Belgique*: **3** (No. 11), 100 (No. 416), *nom. invalid*.

Eupodiscus weissflogii (Grunow in Van Haurck) De Toni, 1894, *Sylloge algarum ómnium hucusque cognitarum*. Vol. II. Bacillarieae; section III. Cryptoraphideae. Typis Seminarrii: 1087.

Thalassiosira weissflogii (Grunow) G. Fryxell & Hasle, 1977, Nova Hedwigia, Beiheft,: **54**, p. 68.

Thalassiosira fluviatilis Hustedt, 1926, *Ber. Deutsch. Bot. Ges*.: **43**, p. 565.

Valve circular, with flat surface and 14.2–17 µm in diameter. A net of silicified structures and siliceous granules cover the external valve in most cases (**Figs 3–6**). In external view areolae are not conspicuous, whereas in internal view rows of continuous cribra obscuring the areola pattern can be observed (**Figs 7–8: ST**). The fultoportulae are distributed into two rings, one central and the other marginal, with 5 to 12 fultoportulae in the central and 7.7–13.7 fultoportulae in 10 µm, interrupted by an evident rimoportula, in the external. In valve external view, the fultoportulae present elongated tubes ornamented with ribs (**Figs 3, 4, 6**). In valve internal view, marginal and central fultoportulae show four satellite pores surrounding the tube (**Figs 7–8**). In external view, the rimoportula has an elongated tube located slightly in front of the marginal fultoportula ring (**Fig. 3: arrowhead**). In internal view, the rimoportula is larger than the fultoportulae, appearing as a pedunculated slit radially oriented. A net of silicified structures and siliceous granules cover the external valve in most cases (**Fig. 5**). These structures appear like a buttress in fultoportulae and rimoportula tubes. In oblique view, the valve is rimmed by a ring of siliceous granules (**Fig. 4**).



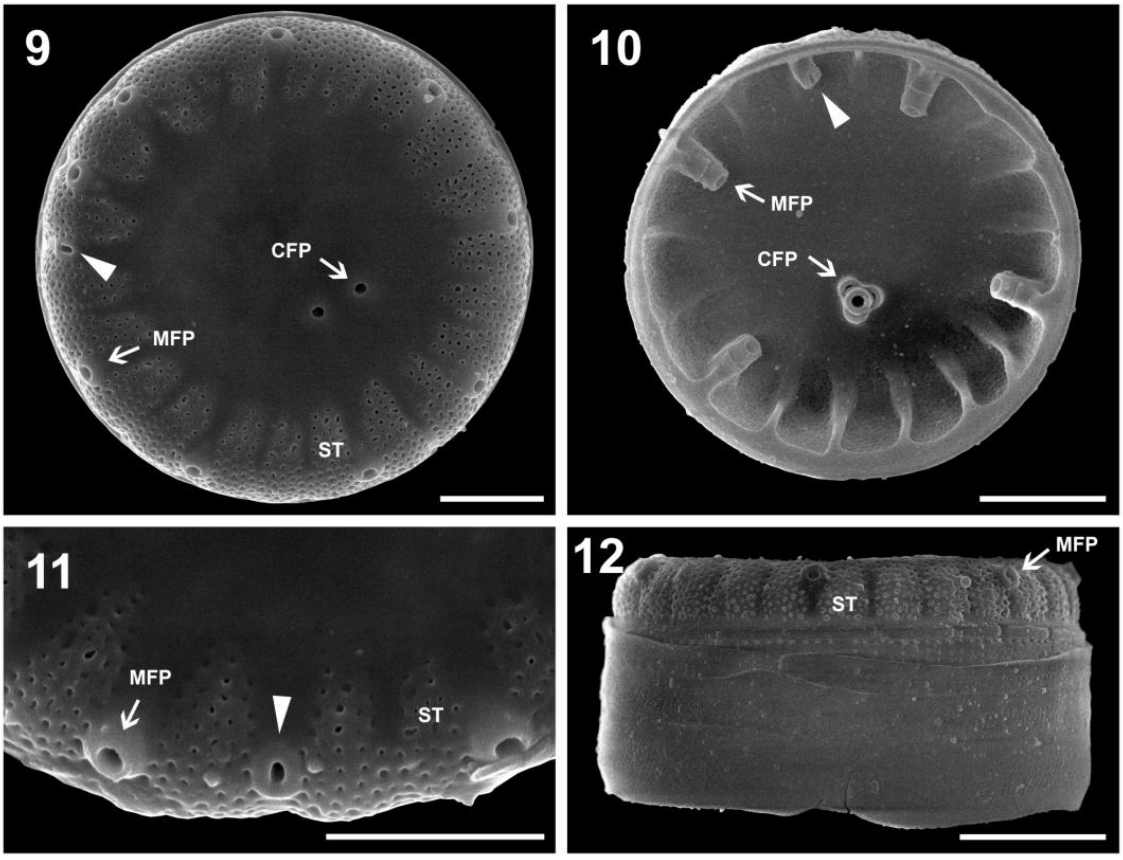
Figs 3-8. *Conticribra weissflogii*. SEM micrographs. Field samples. **Fig. 3**. External valve view. **Fig. 4.** Valve mantle view and mantle ends on a rimmed ring. Fultoportulae marginal ring (MFP) at the edge between the mantle and the valve. **Fig. 5**. Detail of the silicified net and siliceous granules. **Fig. 6**. Detail of the buttressed central fultoportulae. **Fig. 7**. Valve internal view. **Fig. 8**. Details of the marginal structures in the internal valve view, the marginal fultoportula with four satellite pores and the ring interrupted by a large pedunculated rimoportula (arrowhead). Scale bars: 1µm. MFP: marginal fultoportulae; CFP: central fultoportulae; ST: striae.

Cyclotella atomus Hustedt var. *atomus.* (Figs 9-12)

Hustedt, 1937, *Archiv f. Hydrobiologie*: **15** (suppl.), p.143 t.9, **figs 1–4**.

Valve with a flat surface and 3.5–6.2 µm in diameter. The central area is smooth, without ornamentation and with one, rarely two, central fultoportulae (**Figs 9–10: CFP**). In contrast, the marginal area is striated (**Figs 9, 11, 12: ST**), presenting 13.5–16 striae in 10µm, with 6 rows of pores near the mantle that become 4 rows towards the center. This characteristic is not very clear in some cells, where the areolae are irregular and do not follow a consistent pattern. In internal view, marginal alveolar chambers are well defined on the mantle, becoming diffuse towards the center. There is one, rarely two

central fultoportulae, externally open as a pore and without any ornamentation. There is a ring of marginal fultoportulae (**Figs 9–10: MFP**), 4–9 in each valve, located in the valve face-mantle junction, one on every 2nd–4th (rarely 6th) costa (**Table 1**). Marginal fultoportulae open to the exterior through a rimmed pore without ornamentation (**Fig. 11**). Marginal fultoportulae have two satellite pores radially oriented, whereas the central fultoportula has three pores. A single rimoportula (**Figs 9–11: arrowhead**) is present on a costa (interstria) between two fultoportulae. The rimoportula opens to the valve exterior through a slit. In internal view, the rimoportula is smaller than the fultoportulae and slightly oblique, inserted between two marginal fultoportulae (**Fig. 10: arrowhead**).



Figs 9-12. *Cyclotella atomus* var. *atomus*. SEM micrographs. Field samples (except when noted). **Fig. 9.** External valve view. **Fig. 10.** Internal valve view (strain Bc5EHU). **Fig. 11.** Detail of rimoportula (arrowhead) and fultoportulae. **Fig. 12.** Girdle view (strain Bc5EHU). Scale bars: 1 µm. MFP: marginal fultoportulae; CFP: central fultoportulae; ST: striae.

***Cyclotella cryptica* Reimann, Lewin & Guillard (Figs 13–16)**

Reimann, Lewin & Guillard, 1963, *Phycologia*: **3**, p. 82, **figs 4–11**.

The valve is circular, 5.2–9.4 µm in diameter and lacking a clear border between the central and marginal areas, which are quite continuous (**Fig.13–14**). It presents radiating raised striae and depressed interstriae. In internal view, alveolar chambers can be observed (**Fig.15**). The marginal striated area, with 7–9 striae in 10 µm, continues towards the center of the valve. Each stria is composed of 6 rows of pores that decrease in number towards the center. In most cases, the areolae, which are irregular in size and shape, do not follow a constant pattern lacking clear rows (**Figs 13, 16: ST**). There is a marginal ring of fultoportulae at the edge between the valve face and the mantle and 1–3 central fultoportulae. Marginal fultoportulae are present on every interstria, with each small tube surrounded externally by a thick raised silica wall (**Fig. 16**) and internally by three satellite pores (**Fig. 15**). In internal view, the rimoportula appears as a short stalk, oriented radially or slightly oblique, that occupies the place of one fultoportula (**Fig. 15**).

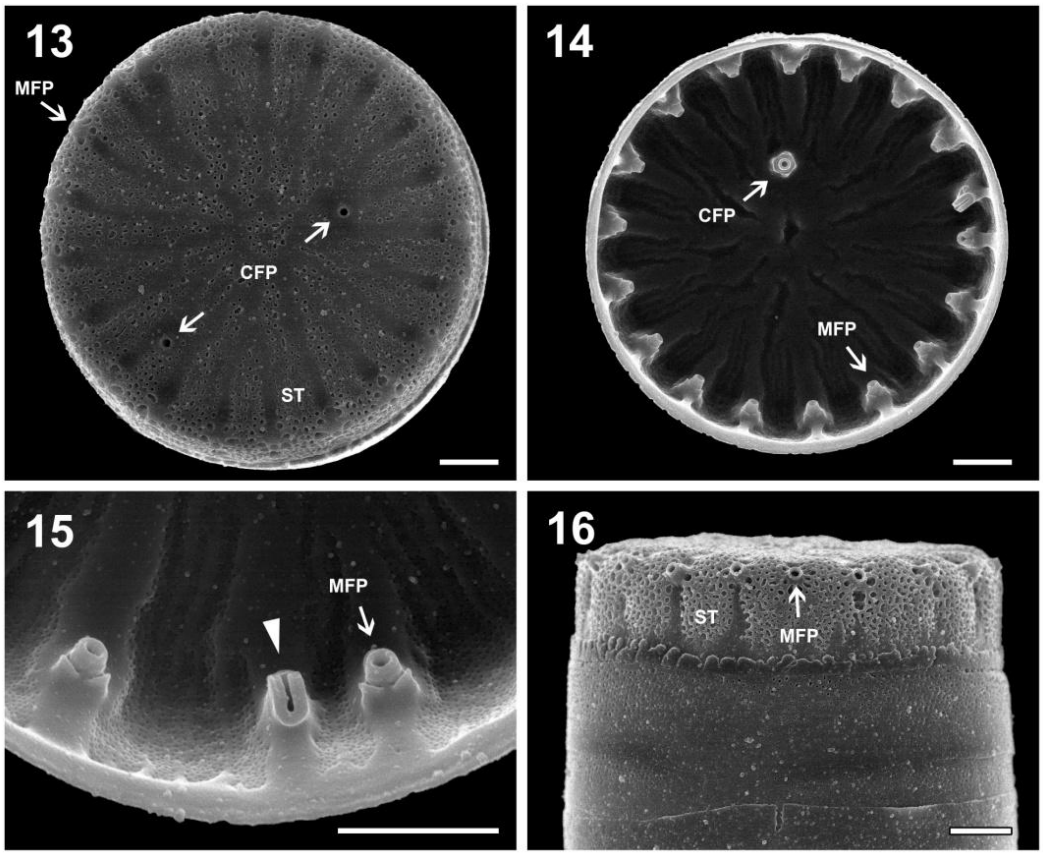
***Cyclotella marina* (Tanimura, Nagumo & Kato) Aké-Castillo (Figs 17–20)**

Aké- Castillo *et al*., 2012, *Nova Hedwigia, Beiheft*: **141**, p. 263-274, **figs 2–9**.

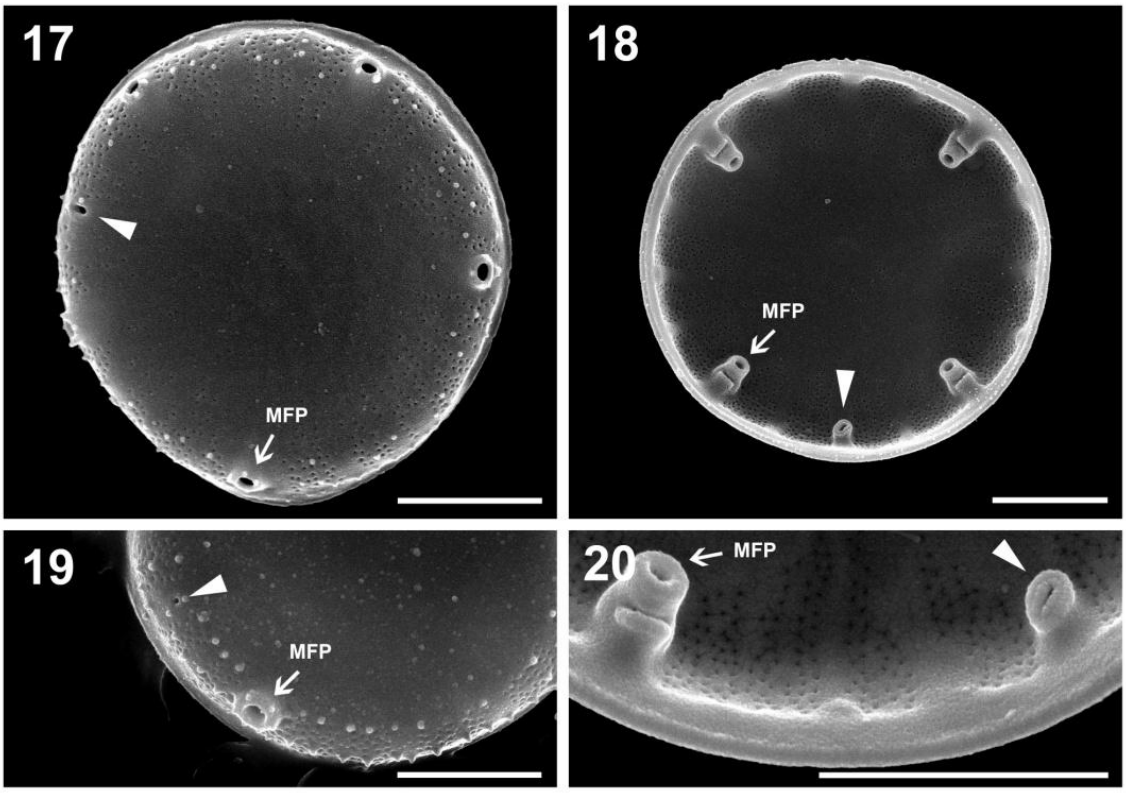
Basionym: *Cyclotella atomus* var. *marina* Tanimura, Nagumo & Kato, 2004: *Bull. Natn. Sci. Mus., Tokyo, Ser*.: **30**, p. 6-7, **figs 3–15**.

Small cells, 2.7–4µm in diameter. Striae are present in the valve margin, even though they are not well defined in external valve view (**Fig. 17**). In most specimens, they are only visible in the very ends of the valve margin and mantle. In internal view, the costae are not very clear and are less marked than in other species of the genus (**Fig. 18**). The central part of the valve is not ornamented, appearing as a smooth surface. It

lacks a central fultoportula, whereas the marginal fultoportulae are located on the valve face and mantle junction, or above in the mantle. In each valve, there are 3–4 fultoportulae situated every 4th or 5th interestria (costa). The marginal fultoportulae develop externally into very short tubes, sometimes looking like rimmed pores. The principal characteristic of these fultoportulae are the siliceous granules or small spinules present around the pores (**Fig. 19**). In internal view, each marginal fultoportula is surrounded by two satellite pores, which are radially oriented (**Figs 18, 20**). The rimoportula is located on one costa, externally appearing as a simple split or small pore without tubes or any ornamentation (**Figs 17, 19: arrowheads**), whereas internally it is obliquely oriented (**Fig. 20: arrowhead**). The marginal part of the valve contains siliceous granules or small spinules, which extend to the mantle (**Figs 17, 19**).



Figs 13-16. *Cyclotella cryptica*. SEM micrographs. Strain Bc1EHU. **Fig. 13.** External valve view. **Fig. 14.** Internal valve view. **Fig. 15.** Detail of rimoportula (arrowhead) and fultoportulae in internal view. **Fig. 16**. Girdle view. Scale bars: 1 µm. MFP: marginal fultoportulae; CFP: central fultoportulae; ST: striae.



Figs 17-20. *Cyclotella marina.* SEM micrographs. Field samples. **Fig. 17.** External valve view. **Fig. 18.** Internal valve view. **Fig. 19.** Detail of marginal fultoportula and rimoportula (arrowhead) in external view. **Fig. 20.** Detail of marginal fultoportula and rimoportula in internal view. Scale bars: 1 µm. MFP: marginal fultoportulae.

Cyclotella meneghiniana Kützing (Figs 21–30)

Kützing, 1844, *Die Kieselschaligen Bacillarien oder Diatomeen*: p. 50, pl. 30, **fig. 68**.

Valve circular, 6.1–19.6 µm in diameter. The valve margin has conspicuous striae (**Fig. 21**), each one formed by eight rows of poroid areolae (**Fig. 22**), which continue on the mantle (**Fig. 23**). The marginal striae, 6.3–13 per 10 µm, are radiating, raised and are alternating with depressed interestriae (**Fig. 24**). In internal view, marginal alveolar chambers are very evident and clearly differentiated from the center of the valve (**Fig. 25**). Some individuals present spines associated with interestriae at the edge, between the valve face and the mantle (**Figs 23, 24**). Spines are located in groups of 3 or 4 and beneath the larger ones there are the fultoportulae openings (**Fig. 26**).

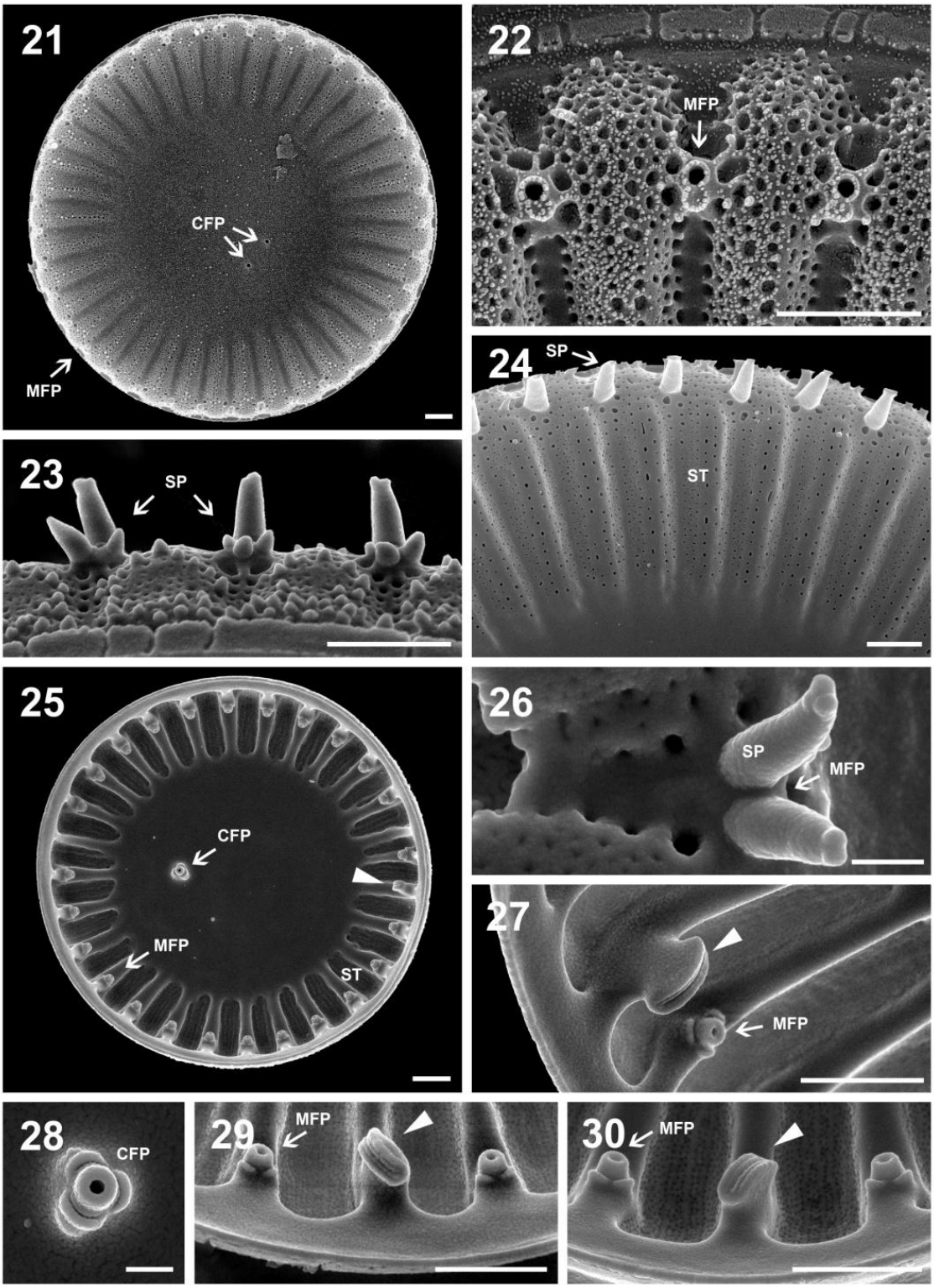
Some individuals lack spines, presenting an unornamented simple pore per fultoportula at the mantle edge (**Fig. 22**). The fultoportulae are positioned on nearly every single costa. In internal view, the fultoportulae are surrounded by 3 satellite pores (**Figs 27–30**).The rimoportula, whose opening has a short slit shape, occupies the place of one fultoportula on one costa, opposite to the central fultoportula. The rimoportula internal opening is a large, elongated labium, oriented radially or slightly oblique (**Figs 29, 30**).

***Discostella pseudostelligera* (Hustedt) Houk & Klee (Figs 31–36)**

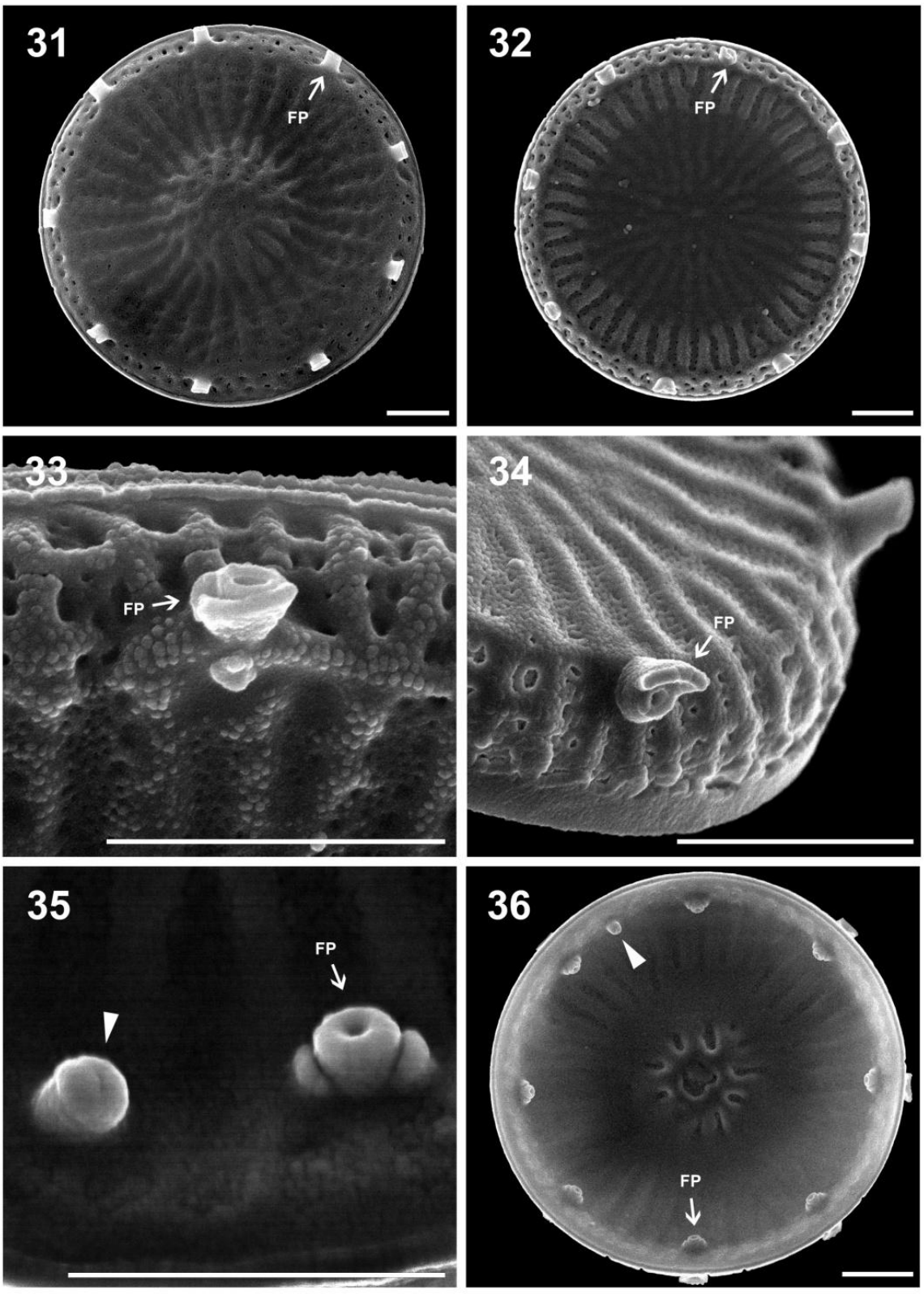
Houk & Klee, 2004, *Diatom Research*, **19**, p. 223, **figs 109–110**.

Basionym: Cyclotella pseudostelligera Hustedt (1939). *Abhhandlungen des Naturwissen-schaftlichen Vereins Bremen:* **31**, p.591, **figs 1, 2**.

Cells cylindrical, 4–6.4 µm in diameter. The variability in valve pattern is very large. he centre of the valve has a distinct star-shaped structure, although this can vary from one morphotype presenting an unornamented central zone, to another presenting a complete stellate arrangement of striae in the central area. In our specimens, both types and transitional forms between them were found (**Figs 31–32**). The striate marginal area has 18.8–25.8 striae in 10µm, separated by elevated interestriae (**Figs 33–34**). These can branch into two on the valve margin. Each stria is usually outlined by two rows of larger areolae, at the edge between the stria and interestria, with smaller areolae between them. In internal valve view (**Figs 35–36**) the striae are sunk in grooves. The central area is concave, convex or flat, with a stellate arrangement of striae or smooth central area. Marginal fultoportulae, in a ring, 3.9–5.5 in 10 µm, 7–9 per valve; located on every third to seventh stria. The external development of the fultoportulae into tubes is very characteristic, being tangentially sectioned at the opening (**Figs 33–34: MFP**).



Figs 21-30. *Cyclotella meneghiniana*. SEM micrographs. Field samples. **Fig. 21.** External valve view. **Fig. 22.** Details of triae and fultoportulae. **Fig. 23**. Spines and spinules in the valve mantle. **Fig. 24.** Detail of striae and spines in valve margin. **Fig. 25**. Internal valve view. **Fig. 26.** Detail of fultoportula surrounded by spines. **Fig. 27**. Internal view of valve margin and mantle, detailed alveolar chamber, rimoportula (arrowhead) and fultoportulae in costa. **Fig. 28**. Internal view of central fultoportula with 3 satellite pores. **Figs 29-30.** Internal valve view of fultoportulae and rimoportula. Scale bars: Figs 26, 28: 250 nm; Figs 21-25, 27, 29-30: 1 µm. MFP: marginal fultoportulae; CFP: central fultoportulae; ST: striae; SP: spine.



Figs 31-36.*Discostella pseudostelligera*. SEM micrographs. Field samples. **Fig. 3**1. External valve view, central star-shaped structure valve. **Fig. 32**. External valve view, unornamented central zone valve. **Fig. 33**. Detailed fultoportulae external tubes. **Fig. 34**. Fultoportula in the mantle, with the characteristic oblique section. **Fig. 35**. Detail of the rimoportula (arrowhead) and fultoportula in internal view. **Fig. 36**. Internal valve view. Scale bars: 1 µm. MFP: marginal fultoportulae.

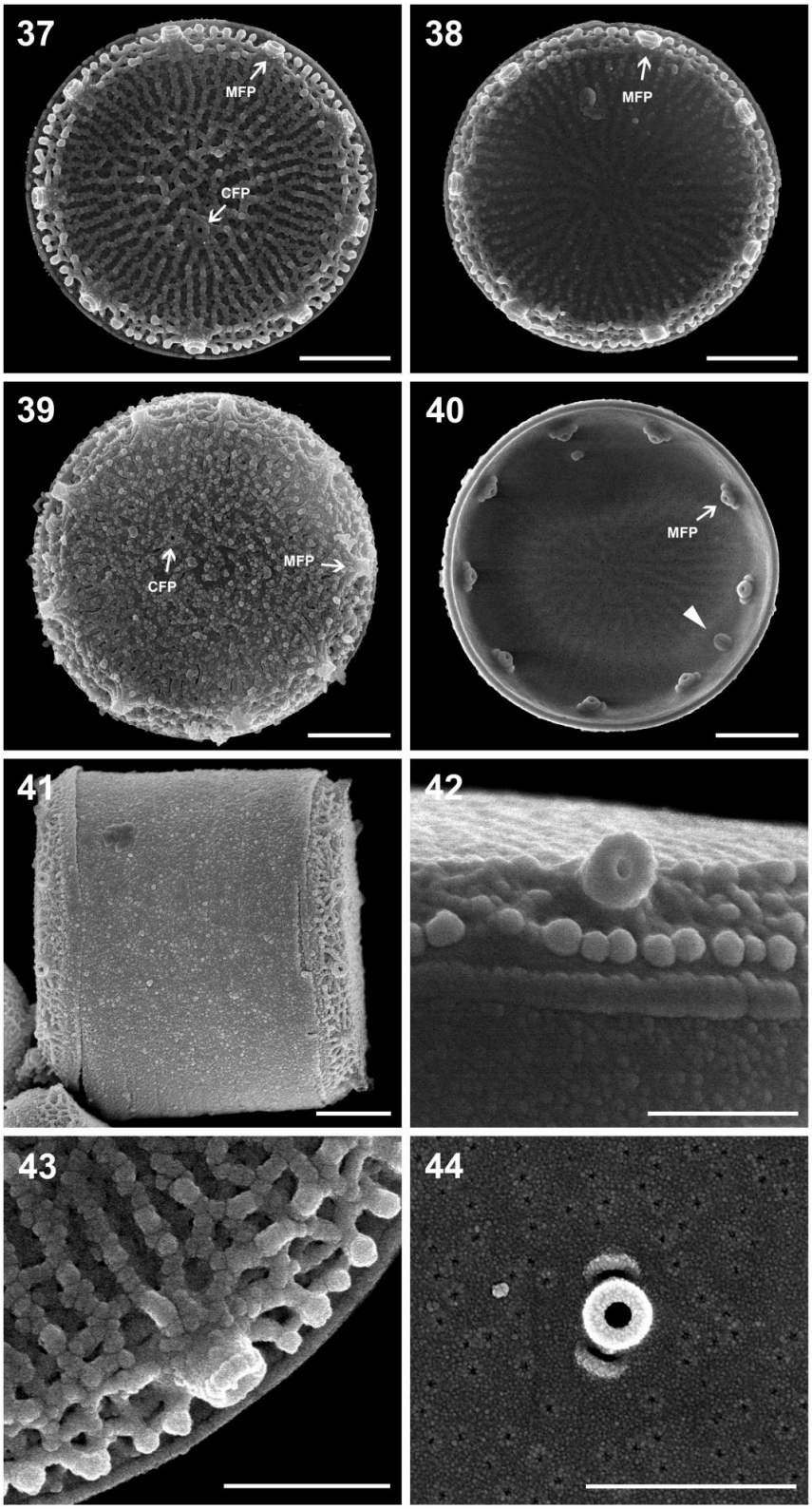
The fultoportulae have two satellite pores tangentially oriented (circumferentially). This species lacks central fultoportulae. The rimoportula is located on the mantle as a sessile labium radially oriented. The mantle ends in a conspicuous silica rim.

***Thalassiosira pseudonana* Hasle & Heimdal (Figs 37–44)**

Hasle & Heimdal, 1970, *Nova Hedwigia, Beiheft*: **31**, p. 565, **figs 27–38**.

Synonym: *Cyclotella nana*, Hustedt, 1957, *Abh. naturw. Ver. Bremen*: **38**, p. 212, **figs 1–2**.

Small cells, 2.1–4.5 (7.1) µm in diameter. The valve morphology is very variable. One of the most evident variations in this study is the degree of silicification, with highly silicified valves (**Fig. 37**) being less abundant than those less silicified (**Fig. 38**). The striation is radial but it is not always conspicuous due to the high silica content of the cell, which obscures the areolae (**Fig. 39**). Moreover their size is very small which hampers measurement of the areolation. On the valve face, there is 0–1 central fultoportula with external openings as a rimmed hole. However, valves with one central fultoportula were rare in this study. Additionally there is one marginal ring of fultoportulae, with 6–12 fultoportulae in 10 µm (7–12 per valve). External openings of the marginal fultoportulae are short rimmed tubes (**Figs 41–43**). In some cases they are obliquely sectioned at the opening (**Fig. 42**). In internal view, the central fultoportula is surrounded by two satellite pores (**Fig. 44**) and the marginal ones by three satellite pores. The rimoportula, similar in size to the fultoportula, is midway between two fultoportulae. It is a rimmed pore in the valve face, whereas the fultoportulae are in the mantle. The pervalvar axis is shorter than or as long as the valve diameter (**Fig. 41**).



Figs 37-44. *Thalassiosira pseudonana*. SEM micrographs. Field samples (except when noted). **Fig. 37**. External view of a highly silicified valve. Siliceous ridges and warts hide areolae. **Fig. 38**. External view of a valve lower silicified than those in Fig 37 and 39. **Fig. 39**. External view of a very silicified valve (Strain Bc12EHU). **Fig. 40**. Internal valve view. **Fig. 41**. Girdle view (Strain Bc3EHU). **Fig. 42**. Detail of fultoportula external tube, obliquely sectioned. **Fig. 43**. Detailed rimmed fultoportula tube. **Fig. 44**. Internal view of central fultoportula with two satellite pores. Areolation is more evident in the internal valve view. Scale bars: Figs 37-41: 1 µm; Figs 42-44: 500 nm. MFP: marginal fultoportulae; CFP: central fultoportulae.

Phylogeny

Based on a previous morphological description of the field samples and cultured cells, a selection of sequences from GenBank was made for the phylogenetic analyses to include species that could be considered cryptic or semicryptic with respect to our identified morphospecies. We used 18 strains isolated from the estuary which constituted 5 different genotypes; only the latter were used to build the tree. The other strains, which were identical to them, are indicated in the tree. We were unable to isolate strains from *Discostella pseudostelligera* and *Cyclotella marina* whose identification in this study was based solely on ultrastructural details. Four main groups were obtained from the 28S rDNA phylogenetic tree with *Aulacoseira granulata* as outgroup (**Fig. 45**). The first (group 1) included species of the genus *Cyclotella* along with *Thalassiosira pseudonana* (seven strains from this study plus one strain from GenBank) as a sister taxon. In this group, composed of strains of the *Cyclotella* genus: *C. cryptica* appeared in a group with *C. distinguenda* and *C. atomus* grouped with *C. choctawhatcheeana* and *C. meneghiniana* in another group not well delimited with other strains of this species. The second group (group 2) was formed by strains of *Conticribra weissflogii*, including the local strains. This group was highly supported only in NJ analysis, although the local strains were strongly supported with a sequence HM991687 of *Co. weissflogii* from GenBank. The third group (group 3) included a strain of *Skeletonema potamos* from Gen Bank and *Skeletonema* sp. from the Bilbao estuary. The last group (group 4) contained strains from *Stephanodiscus*, *Cyclostephanos* and *Discostella* from GenBank. None of the strains from this study belonged to this group, which contained two subgroups: one formed by *Stephanodiscus agassizensis* and *Cyclostephanos tholiformis*, highly supported only by the NJ analysis, but not very

robustly in Bayesian and MJ analyses, and a second one formed by *Discostella* species, including *D. pseudostelligera* and *D. woltereckii*.

Two groups were clearly delineated in the ITS tree (**Fig. 46**), one of them containing species of *Cyclotella* and the other species of *Thalassiosira* plus *Conticribra* (as *Thalassiosira* in GenBank) and *Skeletonema costatum.* The local strains of *C. cryptica*, *C. meneghiniana* and *C. atomus* appeared well differentiated in different subgroups of group 1 as well as *Conticribra weisfloggii* and *Thalassiosira pseudonana* in group 2.

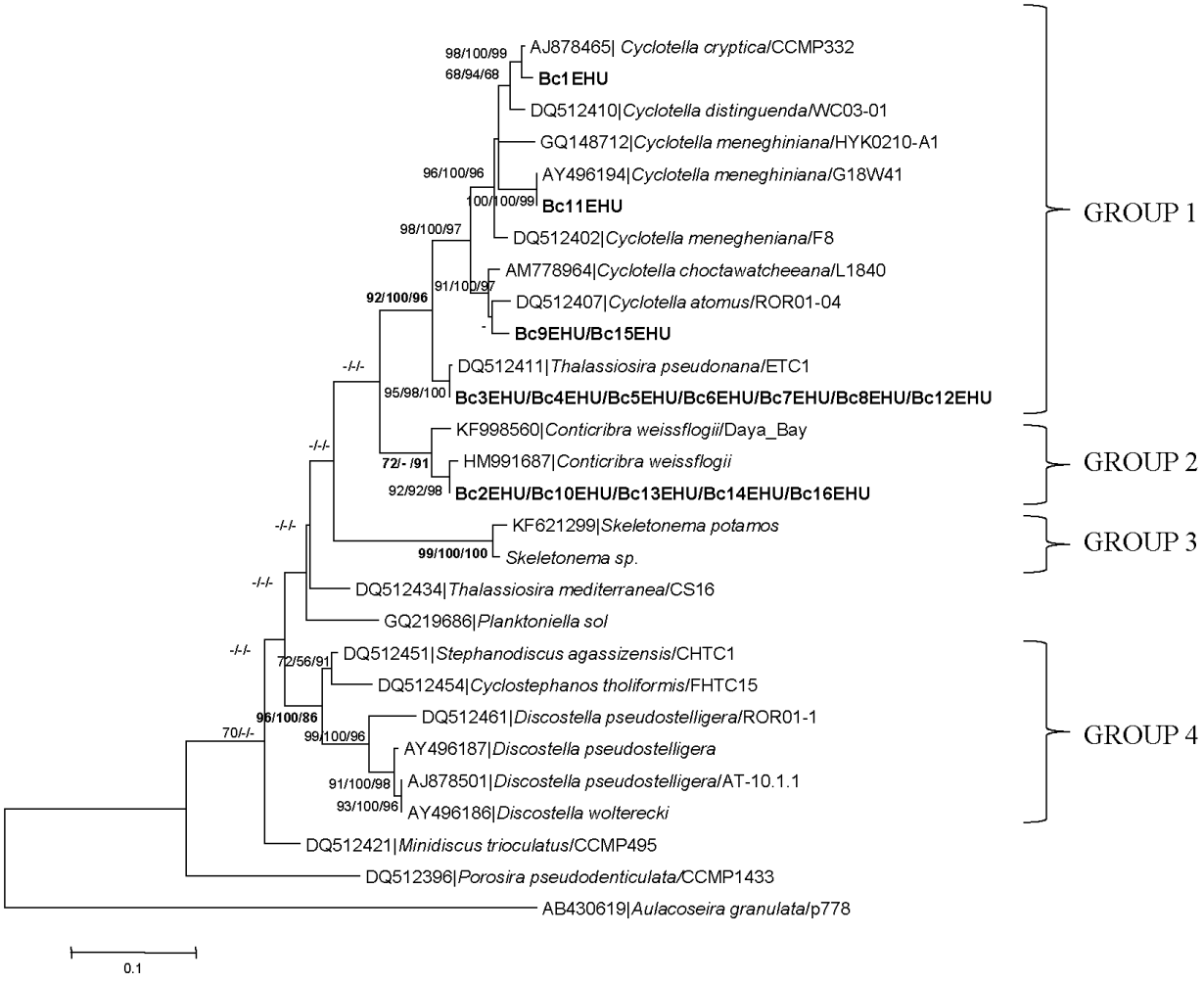


Fig. 45. Maximum Likelihood tree based on the 28S rDNA sequences of the local strains (in bold) plus other relatives from GenBank. Numbers on the nodes represent ML (before slash), Bayesian (between slashes), NJ (after slash) support values. *Aulacoseira granulata* was used as outgroup.

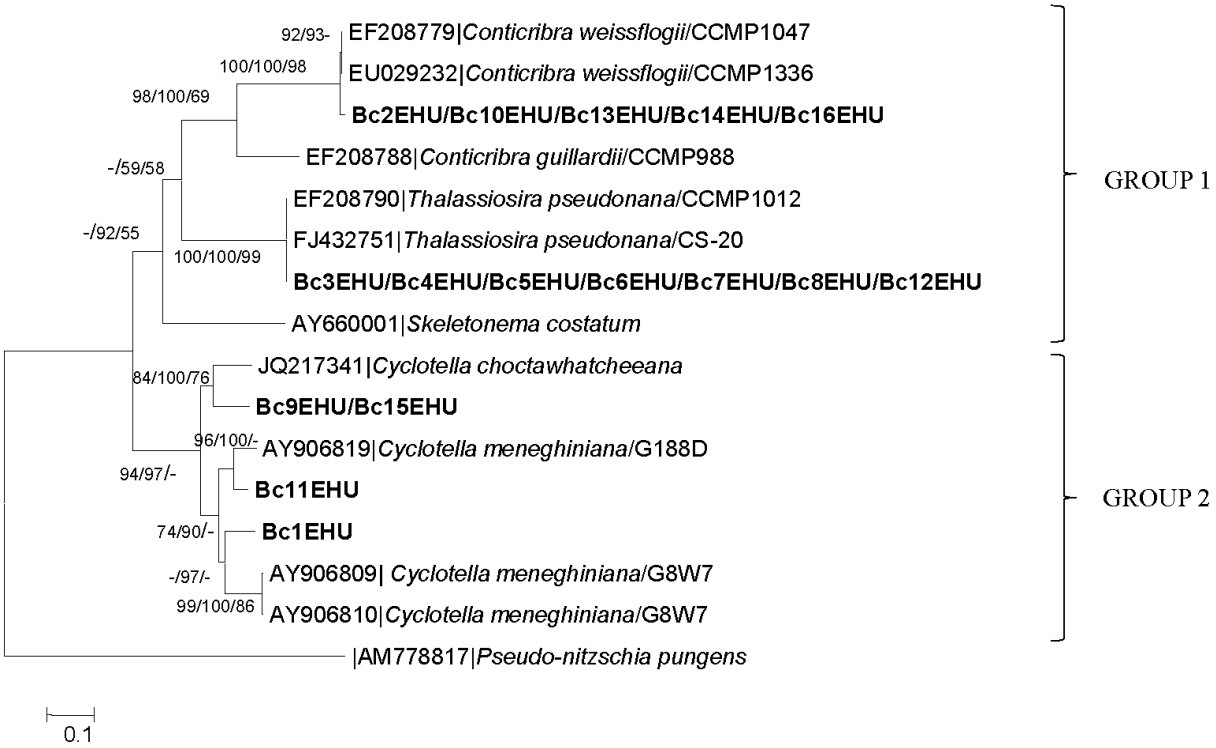


Fig. 46. Maximum Likelihood tree based on the ITS and 5.8 rDNA sequences of the local strains (in bold) plus other relatives from GenBank. Numbers on the nodes represent ML (before slash), Bayesian (between slashes), NJ (after slash) support values. *Pseudo- nitzschia pungens* was used as outgroup.

DISCUSSION

Morphological traits of the species identified from the Bilbao estuary agree quite well with those reported in the literature, except for the valve size of the different specimens which differed from those reported by other authors (**Table** 1). This can be due to the limited size range covered in each study, but also to the existence of phenotypic plasticity. The morphological differences between some species are very subtle -some morphological traits are shared by different species and in some cases, there is not a clear limit between two species' morphology - this is the case in the species-pairs: *Discostella pseudostelligera*/*Discostella woltereckii; Discostella*

pseudostelligera/Thalassiosira pseudonana (only in external valve view and in some exceptional cases); *Cyclotella atomus* var. *atomus*/*Cyclotella marina*; and *Cyclotella meneghiniana*/*Cyclotella cryptica*.

Discostella pseudostelligera is one of the identified species with varying valve morphology, which hampers an accurate identification. The valve oscillates between an unornamented central zone and a complete stellate arrangement of striae. The former feature is characteristic of *D. woltereckii* and the latter of *D. pseudostelligera*. However, although they have different typical valve morphologies, there is a continuum of forms between them. Houk *et al*. (2010) differentiated the morphotypes based on the stellate pattern of the central valve. The central area of the stellate pattern typical of *D. pseudostelligera* is broad and the marginal striae are of equal length, whereas the central area of the dichotomous pattern typical of *D. woltereckii* is smaller and the marginal striae dichotomous. Specimens from this study presented a range of characteristics very similar to both morphotypes. Although those with a stellate arrangement of striae were more frequent, some intermediate morphologies were closer to the description of the dichotomous pattern, although never matching it completely. This continuous transition between both morphotypes is present in both species, which only differ in their ecology (Houk *et al*., 2010). Other authors consider *D. woltereckii* as a form of *D. pseudostelligera* (Haworth & Hurley, 1986; Guerrero *et al.*, 2006). Furthermore, it was suggested that the valve pattern changes with the availability of nutrients in the water and that the *D. wolterecki* type undergoes a morphological change in nutrient-poor water to resemble the *D. pseudostelligera* type (Tanaka, 2007). Due to the close proximity of both species in the molecular analyses, it cannot be ruled out that the morphotypes are conspecific. The absence of cultures of the local strains of *D. pseudostelligera* did not allow us to check this hypothesis.

At low magnifications, *Discostella pseudostelligera* can be misidentified as *Thalassiosira pseudonana*, which is similar in size and morphology, as reported by Scheffler *et al*. (2003). Both species can appear together, creating the false impression that the sample contains a monospecific bloom. For a reliable identification of these species it is necessary to observe the valves at high resolution or look at the internal view of the valve. When observing natural samples at low magnifications, some characteristics can be ambiguous due to the large variability in valve pattern in both species, mainly in external valve view. When *T. pseudonana* presents high silicification, the identification can be accurate as it has siliceous granules and ornamentation in the valve mantle, both features which are lacking in *D. pseudostelligera*. However, when *T. pseudonana* presents low silicification, the central area of both species can appear as a smooth area without ornamentation, making correct identification more difficult. In this study, several features were taken into account to distinguish between the pair, but the most indicative was that *D. pseudostelligera* usually presents fultoportulae tangentially sectioned at the opening, with a kind of visor in the upper part (**Fig. 34**), whereas *T. pseudonana* fultoportulae are rimmed tubes (**Figs 42–43**). Also, the central fultoportula is only characteristic of *T. pseudonana*.

Thalassiosira pseudonana was first described as *Cyclotella nana* Hustedt, Hasle & Heimdal and then transferred to the genus *Thalassiosira*. Since the name *T. nana* already existed, *C. nana* was renamed as *T. pseudonana* Hasle & Heimdal to avoid homonymy. The synonym *C. pseudonana* Chang (Chang & Steinberg, 1989) was then created and later Chang & Chang-Scheider (2008) transferred *C. nana* to the genus *Discostella*. Alverson *et al*. (2011) demonstrated with molecular analysis that *T. pseudonana* has a freshwater *Cyclotella* antecessor and suggested that *T. pseudonana* is more appropriately classified by its original name, *Cyclotella nana*. Our phylogenetic

analyses of several thalassiosiroid strains using 28S rDNA as a marker, clustered *T. pseudonana* as sister taxon of a group containing *Cyclotella* species, as in Alverson *et al.* (2011) and Lee *et al.* (2013), suggesting that *T. pseudonana* is a close relative of *Cyclotella*. However, the ITS tree built with several thalassiosiroid strains separated *T. pseudonana* and *Cyclotella* species into two different clades. The first included several strains of *Thalassiosira* and *Conticribra* and the second different species of *Cyclotella*, in agreement with Tuji *et al.* (2012), who used 18S rDNA as marker. Taking into account that the ITS region is considered a better discriminating marker than the LSU to delineate species in diatoms (Amato *et al*., 2007; Von Dassow *et al*., 2008; Orive *et al*., 2013), the ITS region tree confirms the identification of the local strains and the exclusion of *Thalassiosira pseudonana* from the genus *Cyclotella*. Moreover, some morphological features of *T. pseudonana* are ambiguous as representatives of the genus *Thalassiosira* have fultoportulae with four satellite pores, whereas *T. pseudonana* presents two satellite pores in the central fultoportula and three in the marginal ones, which is characteristic of *Cyclotella*. However, *T. pseudonana* does not have striae (costae), which is one of the principal characteristics of *Cyclotella*. Currently, the classification of this species remains controversial.

Cyclotella marina and *Cyclotella atomus* var. *atomus* are also taxonomically problematic. Tanimura *et al*. (2004) described *C. atomus* var. *marina* as a variety of *C. atomus* Hustedt (1938). Previously, Genkal & Kiss (1993) described the two varieties *C. atomus* var. *atomus* and *C. atomus* var. *gracilis*. The principal differences to other varieties were the absence of central fultoportula and the poor development of the costae. Finally, Aké-Castillo *et al.* (2012) gave *C. atomus* var. *marina* species status as *C. marina* (Tanimura, Nagumo & Kato) Aké-Castillo, Okolodkov & Ector. Only four records of *C. marina* have been reported: from Tokyo Bay (Tanimura *et al*., 2004),

Korea (Chung *et al*., 2010), Brazil (Cavalcante *et al.*, 2013) and the Gulf of México (Aké-Castillo *et al*., 2012). This study constitutes the first record in the East Atlantic. *C. marina* and *C. atomus* var. *atomus* are very similar and only one of them, *C. atomus*, could be isolated to perform molecular analyses for this study. Morphological characteristics were used to distinguish *C. marina* from *C. atomus*: the absence of central fultoportula in the valve and the presence of granules/spinules around the fultoportula in the former, in addition, *C. atomus* var. *atomus* is larger. Taxonomically useful characteristics based on valvocopulae were not visible in the specimens of this study.

Cyclotella marina is a bloom forming diatom in the southeastern Gulf of Mexico (Aké- Castillo *et al*., 2012). Even though this species was found in very low concentrations generally, it reached high concentrations in the middle estuary (station 6) in August 2009. Although *T. pseudonana* was the dominant species, taking into account that the quantity of cells smaller than 5µm containing both species was higher than 3×10^6 cells l^{-1}, the presence of *C. marina* was also noticeable. *C. marina* is known to have a preference for marine salinities, Tanimura *et al.* (2004) suggested that it grows profusely in waters with salinity of around 30 and this was confirmed by Aké-Castillo *et al*. (2012). In this study, station 6 had a salinity of 24 when *C. marina* was present in the samples. Observations in Aké-Castillo *et al.* (2012) support the view that *C. marina* is restricted to neritic shallow waters enriched with nutrients from freshwater discharges and with high detritus content. They propose that it may be attached to submerged macrophytes until environmental conditions favour a rapid growth to form blooms in the water column. However, we could not test this affirmation as in the Bilbao estuary as macrophytes are only present in the outer part.

The 28S rDNA phylogenetic tree clustered our strain of *C. atomus* together with other strains of this species from GenBank and with C. *choctawhatcheeana* as sister taxon of the group. *Cyclotella atomus* lacked ITS sequences in GenBank and our strain appeared together with *C. choctawhatcheeana* in a subgroup of the clade containing other *Cyclotella* species. Unfortunately, there is no morphological information about the *C. choctawhatcheeana* strain which was included in Bruder & Medlin (2007) as outgroup of a tree formed by species of other genera. Based on morphology, our cultures matched the description of *C. atomus* (as explained in morphological description) and the 28S rDNA phylogenetic tree allowed us to conclude that the strains Bc9EHU and Bc15EHU belong to this species.

Its large size and characteristic features make *Conticribra weissflogii* very easy to identify with EM, although its adscription to a particular genus has been the subject of much debate. It was described as *Micropodiscus weissflogii* Grunow in 1885, then changed to *Eupodiscus weissflogii* (Grunow) De Toni, after which Fryxell & Hasle (1977) changed the genus to *Thalassiosira weissflogii* (Grunow) Fryxell & Hasle. Thereafter, the new genus *Conticribra* was described by Stachura-Suchoples & Williams (2009) based on the morphology of the cribra and several species were transferred from *Thalassiosira* to *Conticribra*, including *Co. weissflogii*. Lee *et al.* (2013) later concluded that *Conticribra* was a later synonym of *Spicaticribra* as they described a similar internal cribra structure, so following this concept they referred the species as *Spicaticribra weissflogii*. However, in our study, using the original description of both genera (*Spicaticribra* and *Conticribra)*, some differences were found from the original description of the *Spicaticribra* genus: Johansen *et al.* (2008) described the absence of central fultoportulae on the valve face and absence of external extensions of marginal strutted processes. This is not the case of *Co. weissflogii*, which

matches with the original description of *Conticribra* genus (Stachura-Suchoples & Williams, 2009). The lack of molecular information for the type species of the genus *Spicaticribra* prevents a molecular comparison of genera and species.

Cyclotella *meneghiniana* and *Cyclotella cryptica* were problematic in this study as they showed morphological similarities, with transitional morphologies among them. Houk *et al*. (2010) suggested that these two species could be conspecific. However, Beszteri *et al*. (2007) concluded that *C. meneghiniana* does not constitute a genetically homogeneous species, whereas strains identified as *C. cryptica* were genetically homogeneous and differed from those of *C. meneghiniana*. The authors observed that although their rDNA and psaA variants of *C. cryptica* were not more different from other *C. meneghiniana* sequences than *C. meneghiniana* was from itself, *C. cryptica* formed a distinct group within the *C. meneghiniana* complex, denoting either that *C. meneghiniana* is a taxon that includes more than one species, or that *C. cryptica* is a variety of *C. meneghiniana*. In this study, the subgroup of the genus *Cyclotella* was divided into two clusters in the 28S tree, one of them formed by a mixture of species including *C: meneghiniana, C. cryptica* and *C. distinguenda*. All these species belong to the *C. meneghiniana* species complex in the sense of Beszteri *et al*. (2007). This analysis confirmed the identity of the strain Bc1EHU as *C. cryptica*, which appears in the tree grouped with a strain of the same species from Martha's Vineyard, USA. The sequence of the strain Bc11EHU is identical to that of a *C. meneghiniana* strain from the Geeste estuary, Bremerhaven (Germany). A *C. distinguenda* sequence from GenBank appeared within the group of the *C. meneghiniana* complex, but this relationship was supported only by the Bayesian analysis. The same strain of *C. distinguenda* was studied by Alverson *et al*. (2007) appearing, in this case, in the *Cyclotella* group but outside the *C. meneghiniana* complex.

The morphological features of *C. meneghiniana* from the literature matched those recorded in field samples from this study. This species has a very wide size range and its characteristic features are very variable. The only difference with respect to the features reported in the literature concerns the presence/absence of spines. Although Cavalcante *et al*. (2013) described the species as having external openings always associated with elongated spines, it was not the case in this study in which some of the valves lack spines (**Fig. 22**). Molecular analyses confirmed that, in strains isolated from the estuary, both species were present: *C. meneghiniana* (strain Bc11EHU) and *C. cryptica* (strain Bc1EHU). *Cyclotella cryptica* was not observed in field samples, although it could be isolated from the estuary. Cultured specimens of *C. cryptica* matched well with previous descriptions, so one possible explanation for this apparent absence from field samples could be that it was overlooked due to its morphological variability. Schultz (1971) showed that *C. cryptica* displayed extreme morphological plasticity, with features characteristic of *C. meneghiniana* when growing at salinities less than 1.4, whereas at salinities higher than 4.3 it presented morphological features typical of *C. cryptica*. In our study, Bc1EHU was grown in culture at a salinity of 30, which could account for it displaying morphology typical of *C. cryptica*. Most of the individuals of the *C. meneghiniana* type studied came from a field sample of the Nervión River, which could explain why specimens of the *C. cryptica* type morphology were not observed in these samples. If present in the samples, *Cyclotella cryptica* could be misidentified even under the EM, as using EM alone does not distinguish between these two species with overlapping morphologies.

In this study, some species showed high morphological variability not only between stations, but also within the same sample. Morphology of cultured cells can differ from field specimens, as seen in *Thalassiosira pseudonana* in **Figs 37–38** from field samples

and in **Fig. 39** from culture. Culture conditions, such as lack of hydrodynamism, flask type, specific salinity, temperature and light regimes might be the cause of this morphological change. In this study both freshwater and more marine species were cultured under the same conditions, which could be far from their salinity optimum. Although different morphologies within the same species can be explained by environmental factors (Paasche, 1975; García *et al*., 2012; Adenan *et al*., 2013), they can also be a result of biological factors such as size-selective grazing (Muylaert *et al*., 2000) and also possible intraspecific genetic differences. The large amount of phenotypic plasticity observed in this study could be a consequence of the varying environmental conditions experienced by the species in estuarine waters, which makes them very suitable environments to study the coupling between morphology and physiology in response to the changes in selected environmental variables such as temperature and salinity.

Bloom forming species in the Bilbao estuary are *Conticribra weissflogii*, *Cyclotella meneghiniana*, *Discostella pseudostelligera* and *Thalassiosira pseudonana* whose abundances are listed as supplementary information (**Supplementary Table S1**). These species can form nearly monospecific blooms or blooms of a few species, and although a clear pattern was not detected in this study, *Co. weissflogii* and *C. meneghiniana* appear as bloom formers in the inner part of the estuary and *D. pseudostelligera* and *T. pseudonana* as the species more abundant in the blooms of the middle estuary.

Supplementary material

Table S1. Supplementary material. Comparison between the total number of cells of solitary centric diatoms by size classes as assessed by the Utermöhl method and the dominant species in the sample as viewed under the SEM.

Date	Stations	Dominant species [a]	Number of cells
20/7/2009	S3	*Thalassiosira pseudonana* *Discostella pseudostelligera*	<5µm: 849.600
	S5	*Thalassiosira pseudonana* *Discostella pseudostelligera*	<5µm: 2.973.600
	S6	*Thalassiosira pseudonana* *Discostella pseudostelligera*	<5µm: 3.186.000
	S8	*Conticribra weissflogii* *Thalassiosira pseudonana* *Discostella pseudostelligera*	<5µm: 8.665.920; 5-15µm: 934.560; >15µm: 169.920
27/7/2009	S6	*Thalassiosira pseudonana*	No data
25/8/2009	S4	*Thalassiosira pseudonana* *Discostella pseudostelligera*	<5µm: 1.571.760
	S6	*Thalassiosira pseudonana*	<5µm: 3.016.080; 5-15µm: 42.480
13/7/2010	S6	*Conticribra weissflogii*	3-10µm: 297.360; 10-15µm: 3.186.000
	S7	*Conticribra weissflogii*	3-10µm: 53.100; 10-15µm: 12.510.360
28/7/2010	S4	*Thalassiosira pseudonana*	3-10µm: 11.894.400; 10- 15µm: 106.200
	S5	*Thalassiosira pseudonana*	3-10µm: 28.780.200; 10- 15µm: 212.400
	S6	*Thalassiosira pseudonana* *Discostella pseudostelligera*	3-10µm: 34.196.400; 10- 15µm: 318.600
	S7	*Thalassiosira pseudonana*	3-10µm:

		Discostella pseudostelligera	24.638.400: 10-15µm: 584.100
	S8	*Conticribra weissflogii* *Cyclotella meneghiniana* *Thalassiosira pseudonana* *Discostella pseudostelligera*	3-10µm: 26.337.600; 10-15µm: 1.380.600
17/9/2010	River	*Cyclotella meneghiniana*	3-10µm: 392.940; 10-15µm: 175.230; >15µm:79.650

[a] Species constituting more than 90% of the bloom.

Table S2. Supplementary material. List of cultures

Code	Station	Date	Species
Bc1EHU	Bilbao, E2	Sept. 2004	*Cyclotella cryptica*
Bc2EHU	Bilbao	Sept. 2004	*Conticribra weissflogii*
Bc3EHU	Bilbao, E5 (2m)	07/07/2011	*Thalassiosira pseudonana*
Bc4EHU	Bilbao, E5	31/05/2012	*Thalassiosira pseudonana*
Bc5EHU	Bilbao, E5	31/05/2012	*Thalassiosira pseudonana*
Bc6EHU	Bilbao, E5	31/05/2012	*Thalassiosira pseudonana*
Bc7EHU	Bilbao, E5	31/05/2012	*Thalassiosira pseudonana*
Bc8EHU	Bilbao, E5	31/05/2012	*Thalassiosira pseudonana*
Bc9EHU	Bilbao, E5	14/06/2012	*Cyclotella atomus* var. *Atomus*
Bc10EHU	Bilbao, E5	14/06/2012	*Conticribra weissflogii*
Bc11EHU	Bilbao, E5	14/06/2012	*Cyclotella meneghiniana*
Bc12EHU	Bilbao, E5	14/06/2012	*Thalassiosira pseudonana*
Bc13EHU	Bilbao, E5	14/06/2012	*Conticribra weissflogii*
Bc14EHU	Bilbao, E5	26/06/2012	*Conticribra weissflogii*
Bc15EHU	Bilbao, E5	26/06/2012	*Cyclotella atomus* var. *Atomus*
Bc16EHU	Bilbao, E5	26/06/2012	*Conticribra weissflogii*

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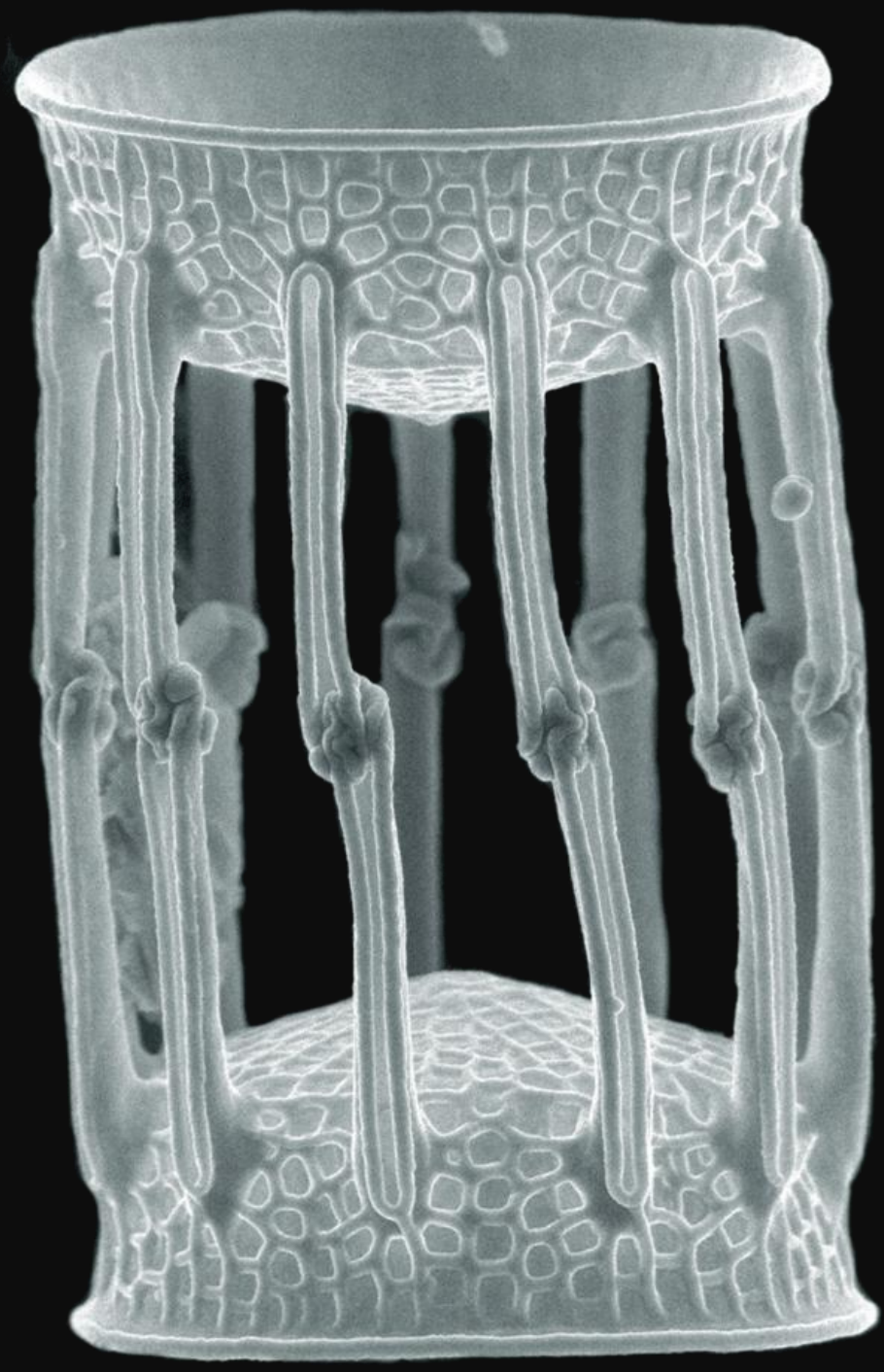
CHAPTER 3

Skeletonema species in a temperate estuary: a morphological, molecular and physiological approach



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INTRODUCTION

Small meso-macrotidal estuaries receiving fast-flowing rivers can experience drastic changes in salinity at different time scales, from hourly to seasonal. Bloom forming species within these areas have to be salinity tolerant to a certain extent, at least those blooming in the inner and middle reaches of these estuaries, which are the most instable with respect to salinity. Before the use of molecular methods as a tool to support the identification of microalgae, species of several genera, now known to contain morphologically cryptic or pseudo-cryptic species, were identified as single species with a broad salinity tolerance. Such is the case of the genus *Skeletonema*, whose most widespread and well known species, *S. costatum* (Greville) Cleve, could have been misidentified as other congener species recently separated from it. *Skeletonema costatum* was described by Greville (Greville 1866) as *Melosira costata* Greville and transferred to the genus *Skeletonema* by Cleve (Cleve 1873). A large variability in shape and size was observed by Hasle (Hasle 1973a) in *Skeletonema costatum* and genetic variability between bloom forming strains of *Skeletonema costatum* s.l. was found by Gallagher (Gallagher 1980) in Narragansett Bay. This author also observed a great deal of physiological differences in terms of growth rates and photosynthetic response among groups of clones from that estuary (Gallagher 1982). In agreement with these observations, later studies using both morphological and molecular analyses demonstrated that *S. costatum* (*sensu lato*, s. l.) actually comprises a complex of different species, which have been subsequently described as *S. pseudocostatum* Medlin (Medlin 1991); *S. grevillei* Sarno & Zingone (Zingone et al. 2005); and *S. dohrnii* Sarno & Kooistra, *S. grethae* Zingone & Sarno, *S. japonicum* Zingone & Sarno, *S. marinoi* Sarno & Zingone and *S. ardens* Sarno & Zingone (Sarno et al. 2005, 2007) . The original species *S. costatum* s. l. has thus been split into several

species, most of them widespread, although each with a preference for a particular latitude (Kooistra et al. 2008). Currently, there are 13 accepted species within the genus *Skeletonema* and several studies have shown larger species richness within particular areas than previously thought (Bergesch et al. 2009, Naik et al. 2010, Sar et al. 2010, Yamada et al. 2010, 2013, Gu et al. 2012). For some time, one of the most representative species in reports on phytoplankton assemblages was *Skeletonema costatum* s.l. It was recognized as the most abundant and cosmopolitan species within the genus, which was found worldwide except in Antarctic waters (Kooistra et al. 2008, Degerlund & Eilertsen 2010). It has been cited recurrently as a bloom-forming species in coastal waters, although recent studies on phytoplankton assemblages leave the identification of this complex at the genus level when observed in the light microscope (Borkman & Smayda 2009, Hunt et al. 2010, Verity & Borkman 2010, Gettings et al. 2014).

In order to know the response of a particular species to environmental parameters it is critical to assure its taxonomic identity given that the physiological features can differ among phylogenetically close congener species or even within species. Photosynthetic parameters as derived from the *in vivo* chlorophyll fluorescence associated to Photosystem II (PSII) can be used to estimate the response of microalgae to environmental parameters (Maxwell & Johnson 2000, Baker 2008). When using Pulse Amplitude Modulation (PAM) fluorometry, the derived parameter F_0 can be used as a proxy of algae biomass (Dijkman & Kromkamp 2006, Kromkamp et al. 2008 and references herein), whereas $(F_m - F_0)/F_m$ (F_v/F_m), the maximum quantum efficiency of PSII photochemistry, can be used as a sensor of stress (Dijkman & Kromkamp 2006, Kromkamp et al. 2008, Baker 2008, Suggett et al. 2012, Betancor et al. 2015). In addition, rapid light curves (RLC) can be made at fixed conditions to estimate the

relative electron transport rate (rETR) of the PSII (Ralph & Gademann 2005). Parameters derived from these curves, viz. light limited initial slope (α), saturation light intensity (E_k) and maximal relative ETR ($rETR_{max}$) are good indicators of the light regime and photoacclimatory behavior of the cells (Hennige et al. 2008, Suggett et al. 2012) and can provide a good estimate of the photosynthetic rate (Kromkamp et al. 2008, Napoléon & Claquin 2012, Figueroa et al. 2013).

As in other temperate coastal areas, *Skeletonema* species appear frequently in bloom proportions in Bilbao estuary (Seoane et al. 2005, Laza-Martinez et al. 2007). However, only *S. costatum* s. l. was named in previous studies. Taking into account the high variability in salinity and other environmental parameters, it was thought that the putative species *Skeletonema costatum* could include other species of the complex. The aim of this study was to verify the identity of the strains of the genus *Skeletonema* present in Bilbao estuary, and their response to salinity and irradiance as a means to estimate their blooming potential along the salinity gradient. The identification of the isolated strains has been carried out by means of ultrastructural and molecular analyses, whereas the physiological response has been evaluated by measuring the chlorophyll *a* fluorescence with a Water-PAM fluorometer. The molecular markers have been selected taking into account the availability of sequences of *Skeletonema* species in GenBank as well as their capacity to discriminate at the species level. With this study we want to test the hypothesis that different *Skeletonema* species are present in the Bilbao estuary, and that these species are able to grow actively in different salinity environments rather than being merely transported passively by the tide. We also want to establish the potential salinity niche which the different species can occupy.

METHODS

Study area

The study area was the Bilbao estuary (43º20'N, 2º70'W, Basque Country, Northern Spain). This system is forced by a semidiurnal tidal regime, which oscillates from 4.6 m during spring tides to 1.2 m during neap tides (Uriarte et al. 2014). The estuary is relatively shallow for most of its length (2-9 m depth), except at the mouth, which forms a semi-confined harbor 3, 8 km in width and 14-30 m in depth. The inner estuary is channelled and highly stratified for most of its length. Secchi Disc values lower than 4 m are frequent in most of the estuary, except in its outermost part where values up to 12 m are registered during periods of low river discharge (Valencia & Franco 2004). The salinity regime varies inter-annually and seasonally (for more details, Hevia-Orube et al. 2016).

Sampling, isolation and culture

For phytoplankton analyses, bottle samples were taken monthly at eight permanent stations (1–8) located along the salinity gradient of the estuary (**Figure 1**), which is seasonally variable (Hevia-Orube et al., 2016). Fixed samples were regularly analyzed for identification and counting (Utermöhl method) as part of a monitoring program which started in 2000. In addition, live samples were regularly examined in the light microscope and cultures of *Skeletonema* strains were established by isolating a single cells or small chains by micropipetting. Details on the origin of the strains are given in **Table 1**. Unialgal cultures were maintained in f/2 medium (Guillard & Ryther 1962) at 30 psu of salinity, 17-20ºC, and100 µmol photons $m^{-2}\cdot s^{-1}$ with a photoperiod of 14-10 h light-dark cycle.

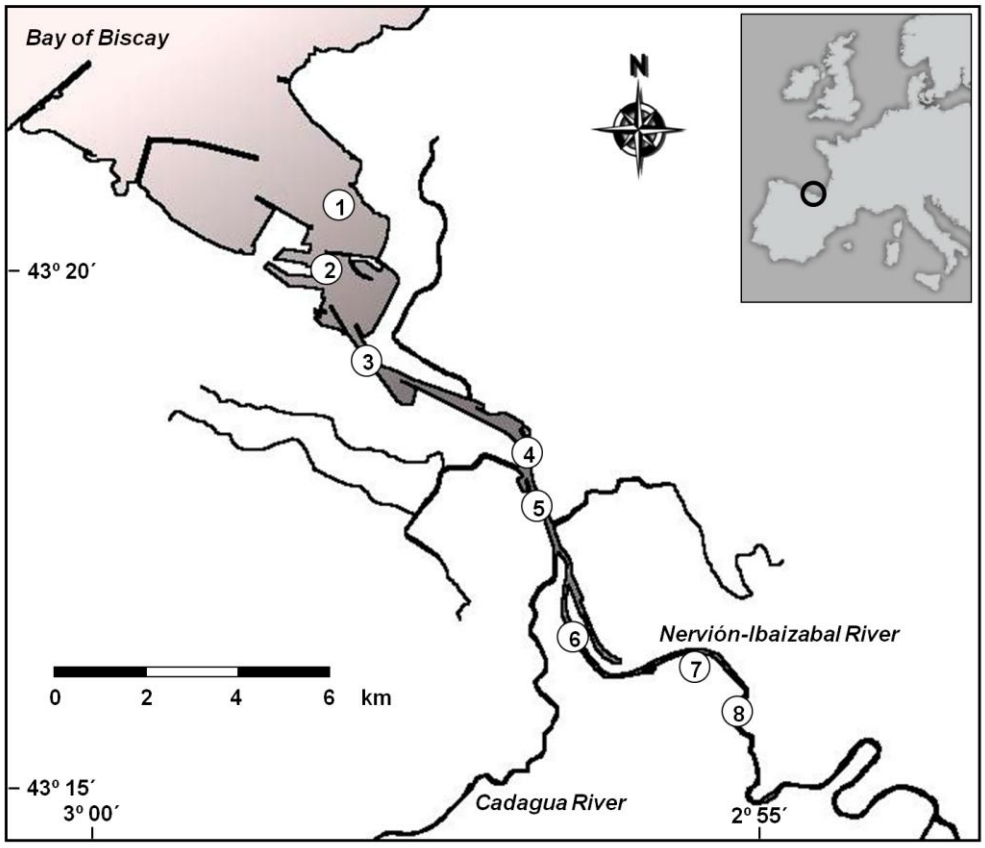


Figure 1. Study area with sampling sites 1-8 from the routine phytoplankton monitoring.

Table 1. Data from isolated strains. Salinity values refer to the salinity range measured at each station at the time of sampling.

Strain	Species	GenBank acc.	Date isolation	Station	Field salinity (psu)
Bc17EHU	*S.costatum*	KT223364	26 June 2012	5	15-20
Bc18EHU	*S.costatum*	KT223365	26 June 2012	5	15-20
Bc20EHU	*S. dohrnii*	KT223366	17 June 2014	5	15-20
Bc21EHU	*S. dohrnii*	KT223367	17 June 2014	5	15-20
Bc24EHU	*S.costatum*	KT223368	17 June 2014	5	15-20
Bc30EHU	*S. menzelii*	KT223369	1 September 2014	2	30

Microscopy

For ultrastructural analysis, both natural community samples (in which *Skeletonema* was detected by LM) and cultured cells were centrifuged and rinsed to eliminate dissolved salts, after which the samples were boiled in acids (HNO_3 and H_2SO_4) to eliminate the organic matter. Samples for electron microscopy were filtered (Millipore TMTP, 1,2 µm pore size), rinsed with distilled water for a few times and then dehydrated with a graded ethanol series and finally desiccated with hexamethyldisilazane (HMDS 98º) for 2 minutes (adapted from Hasle & Fryxell 1970). Filters were mounted on a stub (Agar Scientific Lt.), coated with a 10 nm chromium layer and observed with a Hitachi S-4800 SEM. For TEM observations, drops of organic matter cleaned samples were placed over a cooper mesh.

DNA extraction, amplification and sequencing

For molecular analyses, unialgal cultures were centrifuged and the pellets kept frozen in ethanol until analysis. DNA was extracted with the commercial DNA extraction kit DNeasy Plant Mini (Qiagen, Hilden, Germany). The LSU region was amplified by using D1R-D2C primers (Scholin & Anderson 1993) . A mixture of 20 µL of ultrapure water, 5 µL of DNA extraction solution, 2.5 µL of each primer and 20 µL of Bioline BioMix (Bioline GmbH, Luckenwalde, Germany) were transferred to the BIOER TC-24/H(b) thermo-cycler (BIOER Technology Co., Hangzhou, China) for PCR amplification with the following program: one cycle at 95ºC for 2 minutes, 50ºC for 30 seconds and 72ºCfor 45 seconds; 35 cycles at 94ºC for 30 seconds, 50ºC for 90 seconds and 72ºC for 30 seconds; and a final elongation step of 72ºC for 10 minutes. Amplification products were purified with the MultiScreen HTS PCR 96 kit (Millipore).

Sequencing was carried out by using an ABI PRISM™ BIGDYE v3.1 Terminator Sequencing Reaction and an automatic sequencer ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were read and corrected using BioEdit 7.1.3(Hall 1999).

Sequence alignment and phylogenetic analyses

The final LSU dataset contained 35 nucleotide sequences of *Skeletonema*: 6 from our study area and 29 from GenBank. A sequence of *Thalassiosira pseudonana* (DQ512411) was used as outgroup to root the tree. Sequence alignment was achieved using ClustalW2 software (Larkin et al. 2007) and improved manually. Phylogenetic analyses were inferred using Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbour Joining (NJ) methods, all conducted in MEGA6 (Tamura et al. 2013). In all cases (NJ, MP and ML), bootstrap confidence values were calculated from 1000 replications. Prior to ML analyses, a test to find the best fitting model of DNA substitution was performed (with the Akaike information criterion). ML analyses were performed with the Kimura 2 – parameter model (Kimura 1980), and a discrete Gamma distribution was used to identify evolutionary rate differences among sites (5 categories (+G, parameter = 0.51)), transition/transversion rate (R = 1.37), with substitution rates for transitions and transversion 0.144 and 0.053 respectively. The tree with the highest log likelihood is shown (-1473, 6507). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar 2000) with a search level of 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). NJ was computed with the Tamura-Nei method (Tamura & Nei 1993). All

positions containing gaps and missing data were eliminated resulting in a total of 467 positions in the final dataset.

Physiological experiments

To compare their physiological response to salinities, strains Bc18EHU (*S. costatum*), Bc21EHU (*S. dohrnii*) and Bc30EHU (*S. menzelii*) were cultured at 7 salinities (5, 10, 15, 20, 25, 30, 35) at a fixed irradiance of 120 µmol photons $m^{-2} \cdot s^{-1}$ in seawater enriched with f/2 (Guillard & Ryther 1962). The in vivo chlorophyll *a* fluorescence was measured with a Water-PAM fluorometer (Heinz Walz, Effeltrich, Germany) to estimate the photochemical efficiency and photosynthetic features of PSII. From the original culture (salinity 30 psu), 2 mL of inocula were progressively transferred to 10 ml of culture medium of other salinities at a rate of 5 units of salinity every 2 days until 5 psu. After this pre-acclimation to different salinities, the experiments were carried out within the salinity range at which the strains were able to grow. The acclimation period started with an inoculum of about $4 \cdot 10^4$ cells in 40 mL (10^3 cell/mL) of new medium, in a plastic culture flask Nuclon ™. During the acclimation period, the fluorescence parameters F_0 and F_v/F_m (**Table 2**) were measured every 24h.

To calculate F_0, a modulated light source was applied to cells adapted to dark for 10 min. The minimum or "back ground" fluorescence yield when all PSII reaction centers are open and ready to receive photons is thus determined. A strong correlation was observed between F_0 and the number of cells (data not shown here) supporting its use as a proxy of microalgal biomass. To calculate F_m, a high intensity (> 6000 µmol

photons m^{-2} s^{-1}) short (0.8 s) saturating pulse was applied to close all PSII reaction centers and to reduce the pool of electron acceptors. With both data, the maximum quantum efficiency of photosystem II (PSII) was calculated as $(F_m–F_0)/F_m= F_v/F_m$, where F_v is the variable fluorescence. When four consecutive measurements indicated that the culture was in exponential growth phase, the experiment was initiated by inoculating $4{\cdot}10^4$ cells in new flasks with 40 mL of medium. Three replicates for each salinity and strain were used.

Table 2. Chlorophyll fluorescence parameters

Parameter	Definition	Units	Measurement conditions
A	Initial slope of the Rapid Light Curve	µmol electrons m^{-2} s^{-1}/ µmol photons m^{-2} s^{-1}	
E_k	Light saturation coefficient	µmol photons m^{-2} s^{-1}	
$rETR_{max}$	Maximum relative electron transport rate	µmol electrons m^{-2} s^{-1}	
F_0	Minimum fluorescence yield	Dimensionless	Dark adapted
F_m	Maximum fluorescence yield	Dimensionless	Dark adapted
F_v	Variable fluorescence yield (F_m-F_0)	Dimensionless	Dark adapted
F_v/F_m	Maximum photochemical efficiency of PSII (Maximum quantum yield)	Dimensionless	Dark adapted

Samples were taken every 24h for F_0, F_m and F_v/F_m measurements. Growth rates were estimated using daily F_0 values of the exponential growth phase plotted vs. time. Growth was considered in exponential phase when, after transforming the data to natural logarithm (ln), the following criteria (adapted from Maier Brown et al. 2006)

were reached: (1) the F_0 increased; (2) at least four data points matched with a straight line; and (3) the correlation coefficient (r^2) for the slope was more than 0.95. To calculate the specific growth rates (d^{-1}), the following formula was used: $\mu = (\ln C_2 - \ln C_1)/t_2 - t_1$, where C_1 and C_2 are F_0 values in the exponential phase at times t_1 and t_2 respectively. In order to evaluate the response to light intensity, one RLC was performed for each strain by measuring the rETR in function of increasing actinic light (10, 200, 250, 300, 500, 600, 1000, 1500 and 1800 µmol photons m^{-2} s^{-1}). As discussed by Ralph & Gademann (2005), rapid light curves can be used to assess the photosynthetic response of plants to a wide range of light intensities. All RLC were established at a salinity of 25 psu. The analysis was performed by setting a PAM program which was applied to 2 mL of culture, avoiding manual manipulation during the RLC process. The rETR through PSII was calculated for each level of actinic light as rETR=(F'm-F/F'm)·PAR·0.5, where F is the minimal fluorescence at steady-state for each actinic light intensity and F'm the maximal fluorescence for each actinic light intensity after a short pulse (10 seconds) of saturating light (2000 µmol photons m^{-2} s^{-1}) to light-adapted cells. The multiplication factor 0.5 means that 50% of the absorbed quanta are distributed to the PSI and PSII reaction centers respectively (Cosgrove & Borowitzka 2006). RLC data were fitted to the model of Jassby & Platt (1976) to obtain the following derived parameters: light-limited ETR (αETR); saturation light intensity (E_k, µmol photons · m^{-2}· s^{-1}); and maximal electron transport rate (ETR_{max}, µmol electrons m^{-2} s^{-1}) (**Table 2**). This information can be used to compare the photosynthetic efficiency of PSII of the different strains acclimated to the same conditions of nutrients, salinity and light.

Statistical analyses

Statistical analyses were performed with R software. For each species, the differences of the daily measures of F_v/F_m during the exponential growth phase at the different salinity conditions were examined using a Kruskal-Wallis test. In the case of differences, a Post Hoc Nemenyi test (Pohlert 2014) was performed.

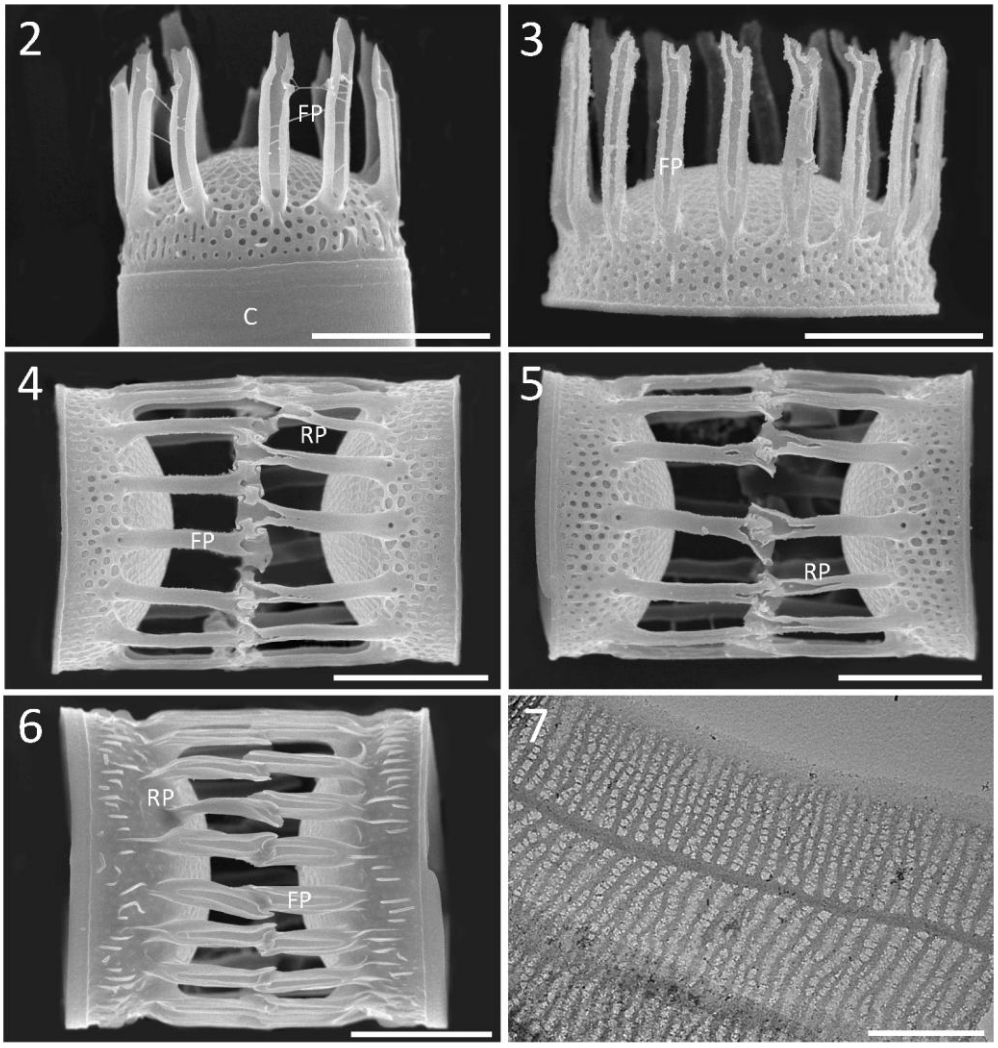
RESULTS

Morphological and molecular identification

Three species of the genus *Skeletonema* were identified: *Skeletonema costatum* (Greville) Cleve, *Skeletonema dohrnii* Sarno and Kooistra and *Skeletonema menzelii* Guillard, Carpenter and Reimann. The identification of the tree species based on morphological characteristics was supported by the molecular analysis.

Skeletonema costatum forms long chains, with a valve diameter of 3.8-7.9 µm (mean 5.6, n=60). The valves are convex and present a high mantle perpendicular to the valve face. Externally, the terminal cell fultoportulae are split tubes, with a distal part ending in a spine or claw-like form (**Figures 2-3**). The fultoportulae of intercalary cells are flattened closed tubes, with a suture throughout and a pore at the base (**Figures 4-5**). In old cultures the suture line is open (**Figure 6**). Opposite intercalary valves are displaced, and each intercalary fultoportula process (IFPP) links with two IFPPs of the adjacent valve (1:2 junction) (**Figure 4**). In some cases, the sibling valves are aligned and in this case the IFPP connects with one IFPP of the adjacent valve (1:1 junction) (**Figures 5-6**). The links between IFPP are knot- or knuckle-like junctions. The external rimoportula tube is long, situated slightly inside the ring of fultoportulae, and occupies

the place of one fultoportula. In intercalary valves, the rimoportula is connected with two IFPP of the sibling valve (**Figures 4-6**; RP). The copulae (**Figure 2**; C) have a main longitudinal ridge with perpendicular branching ribs separated by rows of irregular pores (**Figure 7**).



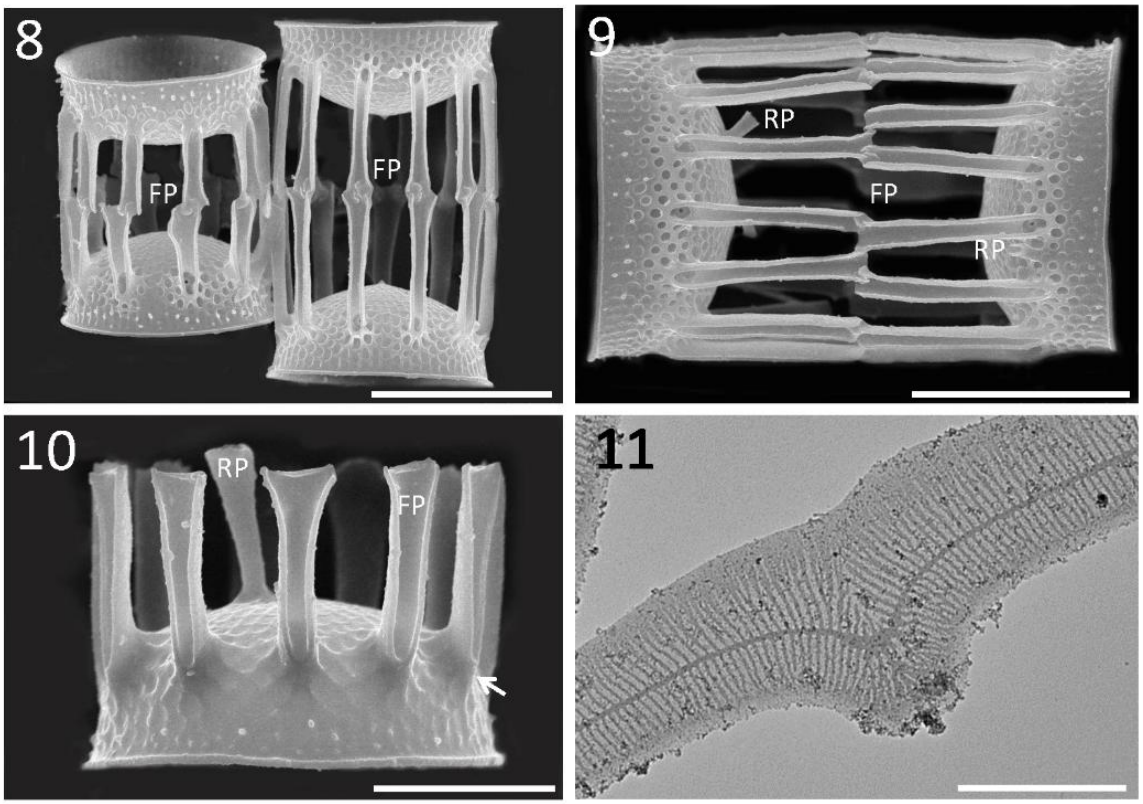
Figures 2-7. Micrographs of *Skeletonema costatum*. **Figure 2**. SEM. Strain Bc18EHU. Scale bar 3µm. Terminal valve of a colony in girdle view, note copula (C) with cingular bands. **Figure 3**. SEM. Strain Bc24EHU. Scale bar 3µm. Terminal valve of a colony, with the claw-like marginal end of the fultoportulae (FP). **Figure 4**. SEM. Strain Bc17EHU. Scale bar 3µm. Intercalary valves with 1:2 junction, the rimoportula (RP) replaces a fultoportula (FP). **Figure 5**. SEM. Strain Bc17EHU. Scale bar 3 µm. Intercalary valves with 1:1 junction. **Figure 6**. SEM. Strain Bc24EHU. Scale bar 3µm. Intercalary valves with fultoportulae (FP) open throughout, characteristic of cultured strains. **Figure 7**. TEM. Strain Bc24EHU. Scale bar 500nm. Detail of the cingular band, with the characteristic pattern of uniseriate rows of pores.

Cells of *Skeletonema dohrnii* form long chains, with a valve diameter of 4.7- 9.8 µm (mean 5.6, n=30).The valves are convex and present a high mantle perpendicular to the valve face (**Figures 8-10**). Terminal cells have FPPs open throughout, including the base (**Figure 10**; arrow), with flattened distal ends. Intercalary valves have narrower FPPs linked with a 1:1 (**Figure 8**) or 1:2 (**Figure 9**) junction. The IFPPs are split tubes. The rimoportula is located close to the valve face margin in intercalary valves and, and in a subcentral position in terminal valves. The terminal rimoportula process (TRPP) is a long tube, with the distal part cup shaped (**Figure 10**; RP). The intercalary rimoportula process (IRPP) is a short tube (**Figure 9**; RP). The copulae have a longitudinal ridge with transverse branching ribs that are interspaced by hyaline areas (**Figure 11**).

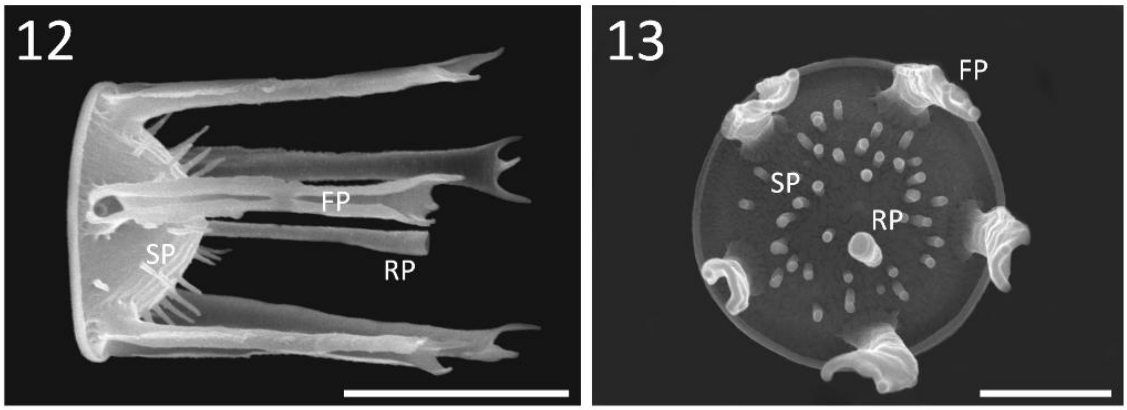
Skeletonema menzelii appears as solitary cells (in pairs when they are dividing), with a valve diameter of 2.3-4.2 µm (mean 2.7, n=20). The valves are weakly silicified and convex (**Figure 12**). The mantle is shallow, ringed and inconspicuous. The valve face has radial, delicate and barely visible ribs. There are large spines on the valve (**Figures 12-13**; SP). The fultoportulae are long and open throughout, the distal part ends in 2-3 spines (**Figure 12**; FP). The rimoportula, located between the central area and the fultoportulae ring, is a closed tube with a bell-shaped end, similar in size to the external fultoportula (**Figures 12-13**; RP).

Three of the strains corresponded morphologically to *S. costatum* s.s. (Bc17EHU; Bc18EHU; Bc24EHU). In the molecular analysis, they are grouped with four other strains identified as *S. costatum* from Japan, Uruguay and USA, with high bootstrap values (**Figure 14**). Two strains in this study were morphologically recognized as *S. dohrnii* (Bc20EHU; Bc21EHU), appearing in the phylogenetic tree together with strain AB630038 of *S. dohrnii* from Japan albeit with low bootstrap values. These three strains are included in a highly supported group, in which other *S.*

dohrnii and strains of *S. marinoi* are clustered. One strain was identified as *S.menzelii* (Bc30EHU). In the molecular analysis it was grouped with strain AJ633528 from Massachusetts (USA) with high bootstrap value.



Figures 8-11. Micrographs of *Skeletonema dohrnii*. Strain Bc21EHU. **Figure 8**. SEM. Scale bar 4µm. Intercalary valves with fultoportulae (FP) open throughout. **Figure 9**. SEM. Scale bar 4µm. Intercalary valves with fultoportulae junction 1:2. Note the short tubes of the rimoportula (RP). **Figure 10**. SEM. Scale bar 2µm. Terminal valve of the colony, with flattened open tubes of the fultoportulae (FP) and the long tubular rimoportula (RP). **Figure 11**. TEM. Scale bar 1µm. Detail of the cingular band, with ribs delimiting hyaline interspaces.



Figures 12-13. Micrographs of *Skeletonema menzelii*. Strain Bc30EHU. **Figure 12**. SEM. Scale bar 2µm. Valve with the long tubes of the fultoportula (FP) open throughout and with the close tube of the rimoportula (RP). **Figure 13**. SEM. Scale bar 1µm. Valve view, fultoportulae (FP) ring with a subcentral rimoportula (RP). Note scattered spines (SP) in the valve face.

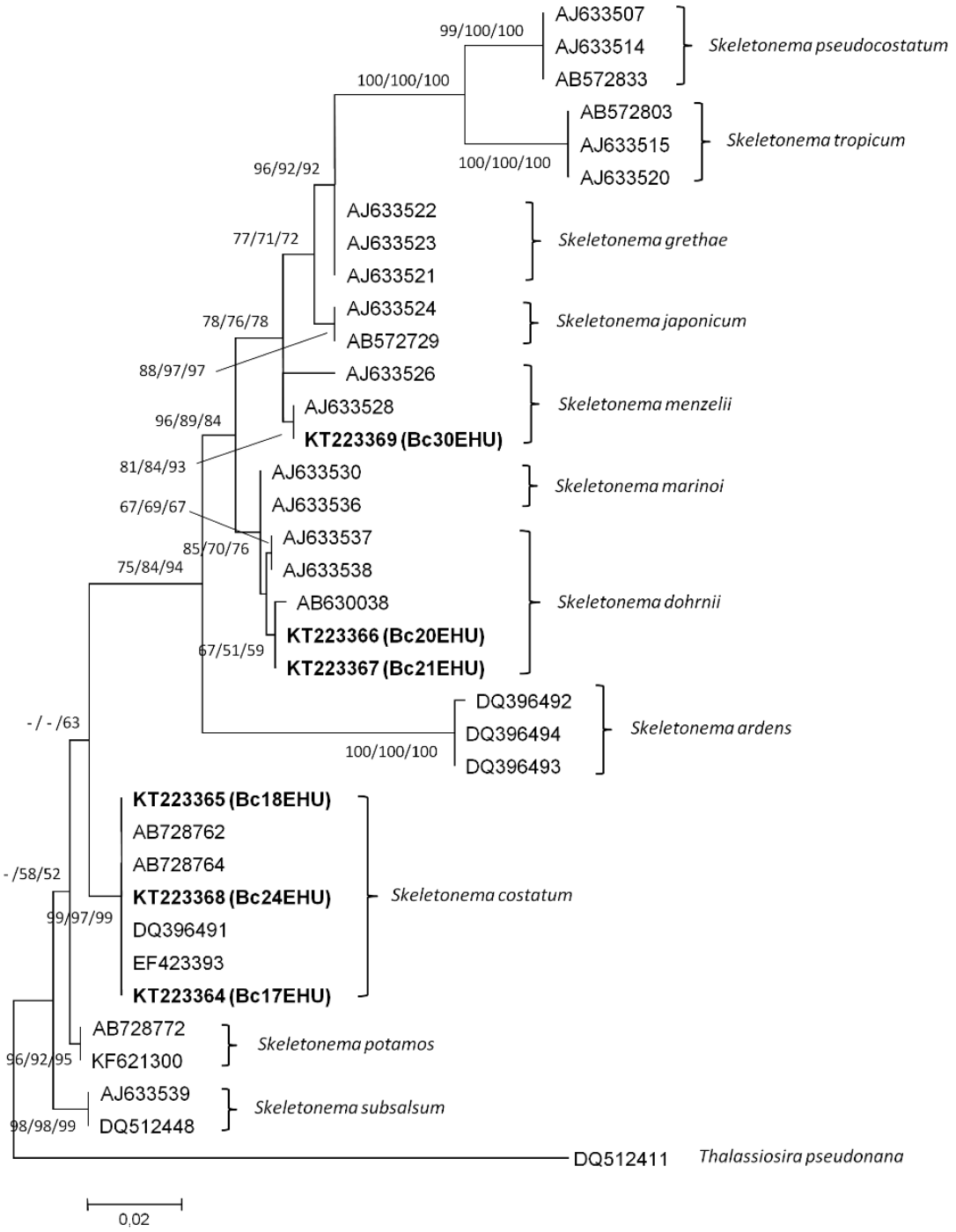


Figure 14. Maximum Likelihood tree based on the 28S rDNA sequences of the local strains plus other relatives from GenBank. Numbers on the nodes represent ML (before slash), MP (between slashes) and NJ (after slash)

Physiological response to varying salinity

Growth rates

The average growth rates (n=3) from each species at different salinities is shown in **Figure 15**. *Skeletonema costatum* can grow along a broad salinity range, between 5 and 35 psu (**Figure 15**). Within this range, the lowest values (0.10 d^{-1}) were obtained at 5 psu and the highest (1.16 d^{-1}) at 15 psu. Nevertheless, growth rate values between 15 and 35 psu were higher than 1 division per day. The minimum growth rate (0.13 d^{-1}) for *S. dohrnii* was registered at 5 psu and the maximum (1.61 d^{-1}) at 25 psu, with values between 15 and 35 psu also high (about 2 divisions per day). The minimum growth rate for *Skeletonema menzelii* was registered at 5 psu (0.70 d^{-1}) and the maximum at 20 psu (1.7 d^{-1}). Growth rate response to salinity followed the same pattern in the three species, being maximal in the range 15-35 psu. *S. costatum* overall had the lowest growth rate.

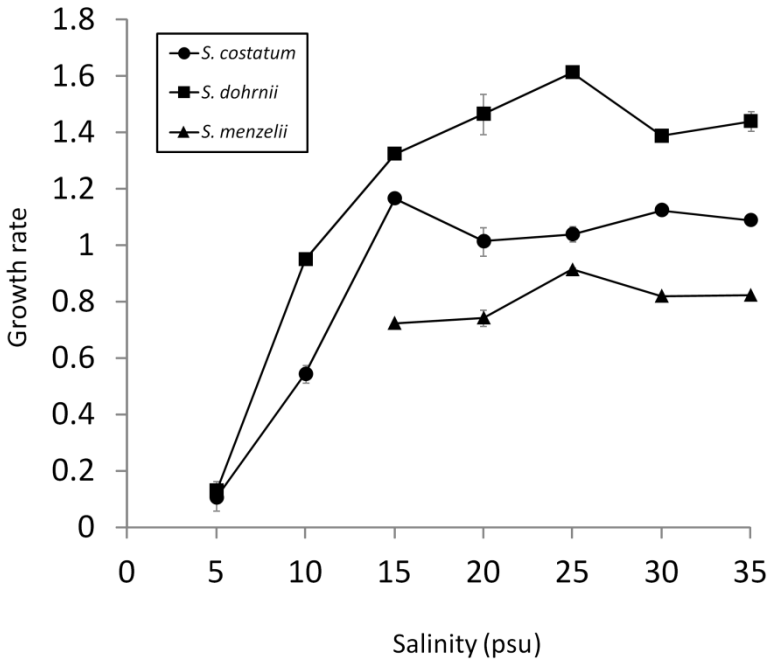


Figure 15. Average growth rates between replicates during the exponential growth phase of *S. costatum* (strain Bc18EHU), *S. dohrnii* (strain Bc21EHU) and *S. menzelii* (strain Bc30EHU) at different salinities. Error bars indicate ± standard deviation (n=3).

Photochemical efficiency (F_v/F_m) and relative electron transport rates (rETR)

S.costatum and *S.dohrnii*, showed significant differences in photochemical efficiency with salinity (**Figure 16**), with *p*-values <0.001 and <0.01, respectively, while *S. menzelii* did not (*p*-value >0.1). The minimum F_v/F_m values for *S. costatum* occurred at salinities of 5 psu (mean 0.16) and 10 psu (mean 0.49) whereas between 15 and 35 they were higher than 0.65. *Skeletonema dohrnii* also showed the lowest minimum photochemical efficiency value at 5 psu (mean 0.58), but on average no differences were observed between the different salinities.

Rapid light curves were performed for cultures growing at a salinity of 25 psu, which was within the optimal salinity ranges for the three species (**Table 3**). Although the derived parameters (α, rETRm and E_k) were highest for *S. menzelii*, all the three species appeared to be adapted to high light intensities.

Table 3. Parameters of the rapid light curves: α (µmol electrons m^{-2} s^{-1}/ µmol photons m^{-2} s^{-1}), rETRm (µmol electrons m^{-2} s^{-1}), E_k (µmol photons m^{-2} s^{-1})

Species	Salinity	A	rETRm	E_k
S. costatum	25	0.20	141.81	697.35
S. dohrnii	25	0.21	163.98	792.24
S. menzelii	25	0.39	163.8	1000

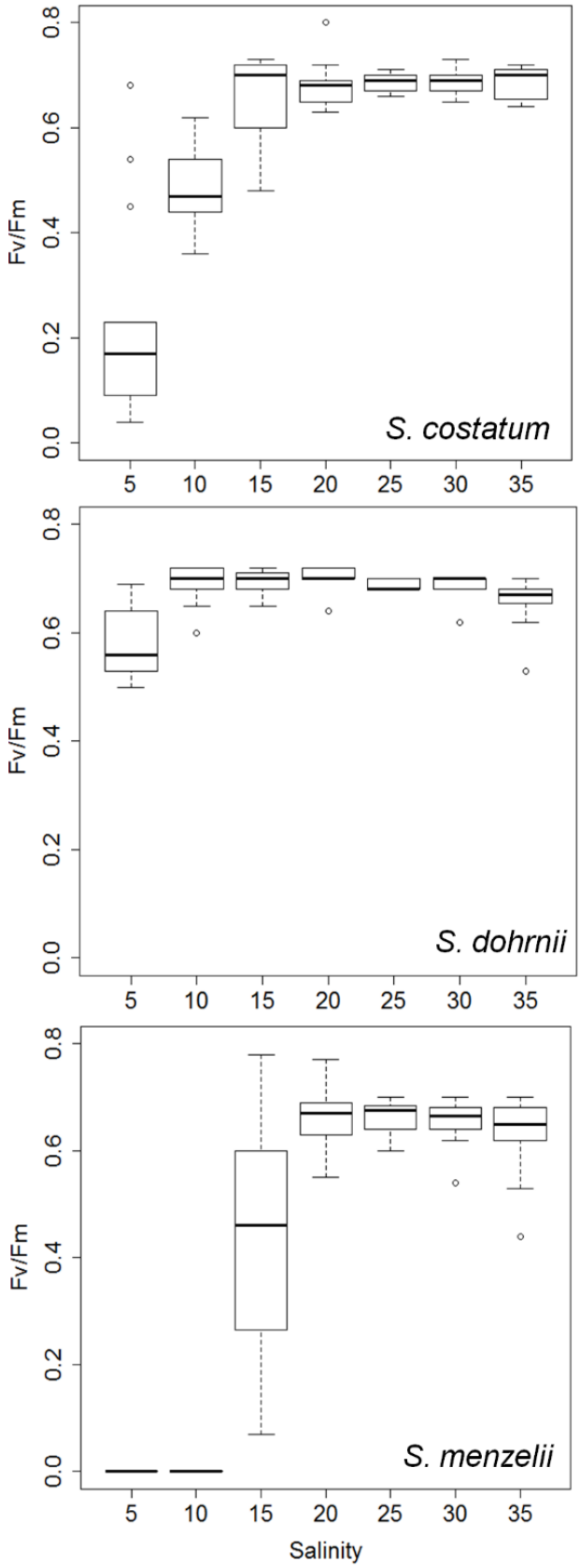


Figure 16. Average F_v/F_m daily values during the exponential growth phase of *S. costatum* (strain Bc18EHU), *S. dohrnii* (strain Bc21EHU) and *S. menzelii* (strain Bc30EHU) at different salinities.

DISCUSSION

Species identity

Three species of the genus *Skeletonema* were isolated from Bilbao estuary: *S. costatum* (Bc17EHU, Bc18EHU and Bc24EHU), *S. dohrnii* (Bc20EHU and Bc21EHU) and *S. menzelii* (Bc30EHU). Both morphological and molecular analyses matched with the data reported in the literature for these species (Guillard et al. 1974, Sarno et al. 2005, Zingone et al. 2005), although the cells of our strain of *S. menzelii* are smaller than those reported in Sarno et al. (Sarno et al. 2005). There is some controversy on species delimitation of *S. dohrnii* due to its similarity to *S. marinoi*. Sarno et al. (Sarno et al. 2005) grouped *S. dohrnii* and *S. marinoi* into two different clades although the support values for the *S. dohrnii* clade were weak in both SSU and LSU rDNA-based phylogenies. Despite the morphological similarity between both species, some differences were observed in the ultrastructure of the cingular bands: whereas *S. dohrnii* presented hyaline areas between the ribs of the bands, *S. marinoi* ribs were characterized by rows of pores. However, in later studies, Ellegaard et al. (2008) and Kooistra et al. (2008) found both types of cingular bands in clones of *S. marinoi*. Besides, Yamada et al. (2010) reported that some specimens of cultured *S. dohrnii* showed typical *S. marinoi* cingular bands. Furthermore, in the same study, these authors identified a strain as *S. dohrnii* using LSU rDNA, but as *S. marinoi* using the SSU rDNA. It was not the aim of the present study to address the challenge of the species delimitation between these two nominal species, and strains Bc20EHU and Bc21EHU were ascribed to *S. dohrnii* following the identification of their closest sequences from the databases in the LSU rDNA analysis. As in previous studies (Ellegaard et al. 2008, Yamada et al. 2010), our LSU rDNA phylogeny did not recover reciprocally monophyletic well-supported clades for the two putative species. With respect to

cingulum structure, both strains corresponded with the original morphological description of *S. dohrnii* (hyaline areas between the ribs of the cingular bands).

The use of molecular methods in conjunction with morphological analyses allowed us to identify three different species of the genus *Skeletonema* in Bilbao estuary. However, although these species have been shown as bloom-forming, it cannot be excluded that even more *Skeletonema* species are present.

Physiological response to salinity and light intensity

The three *Skeletonema* species found in Bilbao estuary can be considered as marine euryhaline and, as such, brackish as well as marine waters of estuaries and other coastal areas appear to be suitable environments for the active growth of these species (i.e. they are not being merely transported by the tide along the salinity gradients). The euryhaline character of several species within the genus has been pointed out by Erga et al. (2015) and Balzano et al. (2011). Euryhalinity seems to be a common feature in many coastal microalgae, which in some cases has been attributed to a possible freshwater origin (Alverson et al. 2011). For those of marine origin, euryhalinity can constitute a selective advantage to colonize the brackish waters of estuaries and coasts where more nutrients are available than offshore. However, the fact that these species can survive at a broad range of salinities does not mean that they are in good physiological state across the whole range. This is the case for *S. costatum*, which, although surviving for a few days at 5 psu, never reached high biomass, showed very low growth rate (0.10 d^{-1}) and a F_v/F_m value (0.16) indicative of physiological stress. The maximum F_v/F_m for *S. costatum* was 0.69 at 25 psu. This value can be considered high taking into account that the theoretical maximum for this parameter is 0.83 in

green plants (Ritchie 2006) and oscillates more frequently between 0.6 and 0.7 in microalgae, both in communities and single cells cultures (Dijkman & Kromkamp 2006). Values lower than 0.4 can be considered as indicative of stress (Dijkman & Kromkamp 2006). Higher variability between replicates and lower values of the photochemical efficiency were also observed at 10 psu and, although the values at 15 psu were high, they also displayed more variation than at the highest salinities (20-35 psu), suggesting that the latter represents the optimum salinity range for this strain of *S costatum*. The optimum range deduced from growth rates extends to 15-35 psu, in contrast with the observations of Balzano et al. (2011), whose (estuarine) strains showed optima extending below 10 psu. The growth rate of *S. dohrnii* at 5 psu was also very low (0.13 $d^{-1,}$ more than 10 times lower than its optimum at 25 psu), but its average F_v/F_m was 0.56, which is lower than the optimum (0.70) but can still be considered as indicative of a good quantum efficiency. The response to salinity of this strain was more similar to strain CCMP781 (*S. marinoi*, Balzano et al. (2011) In our *S. dohrnii* strain (Bc21EHU), the growth rates at 5 and 10 psu are low, although the strain maintained a good physiological status. . As pointed out by Dijkman & Kromkamp (2006), salinity induced stress could affect the growth rate before F_v/F_m is affected. *Skeletonema dohrnii* and *S. costatum* cultured at low salinity had similar growth rates but very different physiological status, suggesting that the strain of *S. dohrnii* could be more successful at low salinity. *S. menzelii* showed good physiological status at all the salinities tested, although the growth rates were lower at the lowest salinities. Regardless of the maximum growth rate achieved by each strain, they all showed the same maximum F_v/F_m value (~0.7) which can be observed at all salinities where the species grow without stress, even at sub-maximal growth rates. The high values of E_k and rETR presented by the three species are indicatives of photoacclimation to high

light intensities, although this fact does not preclude that these species can live under lower light intensities by changing the pigment concentrations or the number and size of the reaction centres as observed by Falkowski & Owens (1980) in *Skeletonema costatum* s.l. and Hennige et al. (2008) in symbionts of corals. Thus, *Skeletonema costatum* s.l. has been found to be the only group of species surviving, although in low concentrations, at the low irradiances of the northern high latitude winter (Eilertsen & Degerlund 2010). *Skeletonema menzelii* has the highest growth rates under the experimental conditions of temperature, irradiance and salinity used in this study, which correspond to conditions frequently found in the field during the warm season (Orive et al., 2004). *S. menzelii* also had good physiological status at 5 psu suggesting it can survive along the whole salinity gradient, although it has been pointed out as a marine species (Gu et al. 2012).

Within species, differences in salinity tolerance have been observed in coastal waters, where a shift in the composition of the populations with changing salinity can be observed. Balzano et al. (2011) observed noticeable differences in the growth rates and salinity range tolerance among different strains of *Skeletonema costatum, S. ardens* and *S. marinoi* from the same or different geographic area, which the authors attribute to possible physiological and genetic differences. The authors also pointed out that the strains that were isolated from these changing environments presented broader salinity ranges, even comparing with strains of the same species, suggesting that they were adapted to varying conditions. Godhe et al. (2016) reported that blooms of *S. marinoi* in the Baltic Sea are composed of significantly different populations separated by distance and by the South-North salinity gradient. In a related study, Sjöqvist et al. (2015) , on the basis of genetic and experimental data, observed potential local salinity adaptation in populations of *S. marinoi*.

The response to salinity and light of the three tested strains of *Skeletonema* isolated from the Bilbao estuary reveals that they could occupy similar niches concerning these variables. However, this assumption does not mean that these results could match the species distribution in the area.

This study confirms that *S. costatum*, *S. dohrnii* and *S. menzelii* are euryhaline species which can potentially grow actively along most of the salinity range of the Bilbao estuary, where the genus *Skeletonema* is frequently found as dominant taxa during phytoplankton blooms (Hevia-Orube, unpublished data).

The physiology of *S. dohrnii* and *S. costatum* s.s. was poorly known due to their relatively recent descriptions and, although a single strain is not representative of the whole within species variability, this study contributes to the knowledge of their environmental preferences, with data from estuarine strains of these species.

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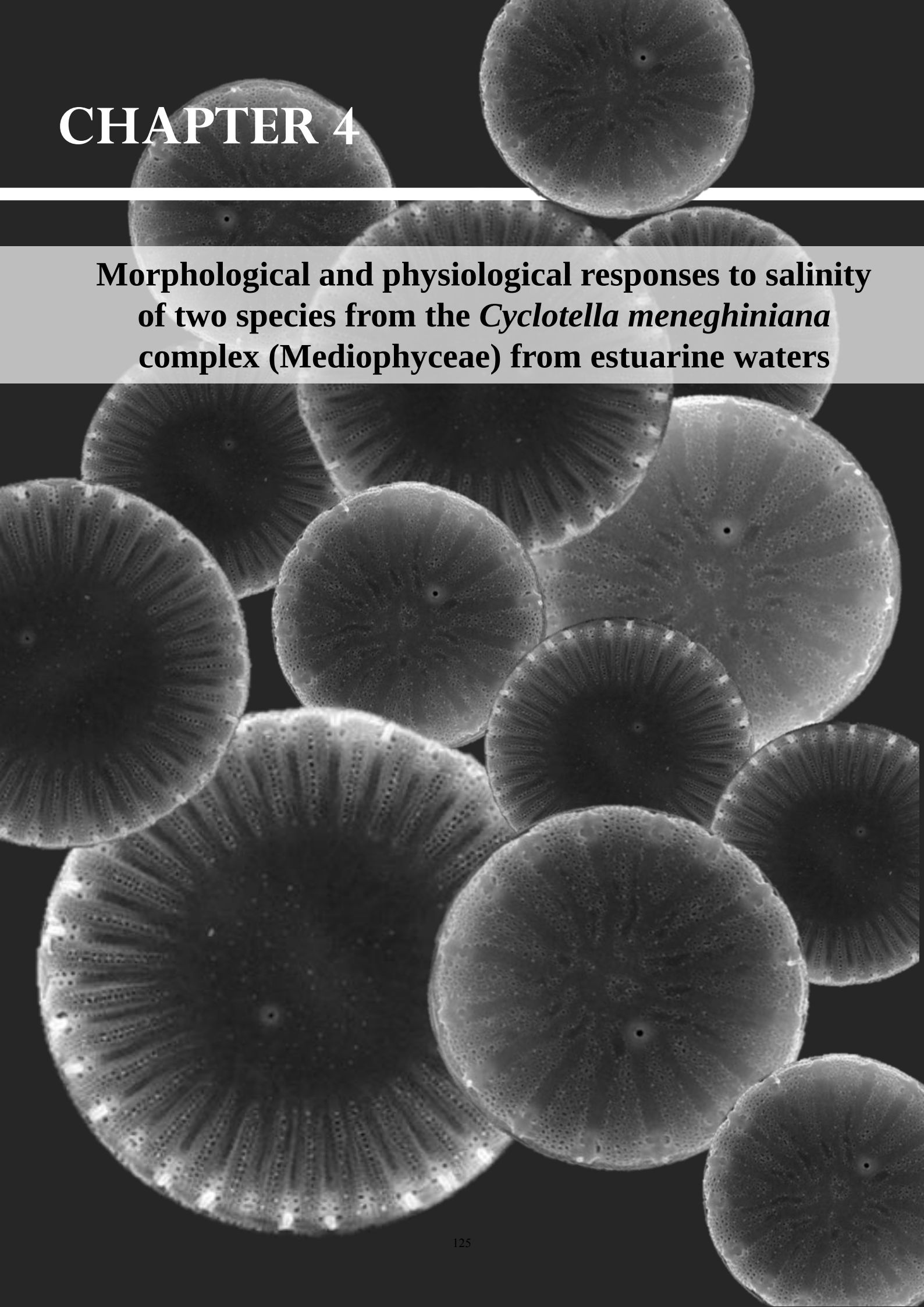
CHAPTER 4

Morphological and physiological responses to salinity of two species from the *Cyclotella meneghiniana* complex (Mediophyceae) from estuarine waters



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INTRODUCTION

The diatom *Cyclotella meneghiniana* is widely distributed worldwide in both freshwater and coastal environments and, consequently, it has been the subject of much research work dealing with its taxonomy as well as physiology. Its delimitation from related *Cyclotella* species is often difficult due to the existence of marked morphological plasticity as well as within species genetic diversity (Beszteri et al. 2005a, 2005b, 2007). The species *C. scaldensis* and *C. cryptica*, which together with the different lineages of the *C. meneghiniana* morphospecies, and *C. quillensis* conform the *Cyclotella meneghiniana* complex, show overlapping values of structures of taxonomic value (Beszteri et al., 2005a; Houk et al., 2010; Muylaert & Sabbe, 1996; Schultz & Trainor, 1968). The observed plasticity within this complex can be the result of phenotypic adaptation to different environmental conditions (Ghalambor et al. 2007), with salinity as one of the factors that could trigger this morphological plasticity, especially when dealing with estuarine environments. Several authors have reported the changes in morphological features of taxonomic value in relation with salinity in both *C. cryptica* and *C. meneghiniana* (Håkansson & Chepurnov, 1999; Reimann et al., 1963; Schultz, 1971; Shirokawa et al., 2012; Tuchman et al., 1984).

In field samples from the Bilbao estuary viewed under the SEM, *C. meneghiniana* was observed to bloom in the inner part of the estuary during the warm season, being one of the most abundant centric diatom in the area (Hevia-Orube et al. 2016). *Cyclotella cryptica* was not visualized under the SEM in any of these fixed samples, but it could be isolated alive from the same water samples. In this previous study, strains of both species could be isolated from the estuary and characterized by ultraestructural features and molecular analyses. However, in that occasion, only specimens growing at a fixed salinity (30) were analyzed despite this parameter might

be responsible for the morphological plasticity causing the taxonomic confusion between the two species. Some authors described *C. meneghiniana* as a freshwater species (Houk et al. 2010), while others reported this species as euryhaline or brackish water species (Liu & Hellebust 1976, Tuchman et al. 1984, Håkansson 2002). Its presence in great proportions in the inner part of the Bilbao estuary (from 0 to 22 salinity) prompted the question whether *C. meneghiniana* is transported by river flow, being here out of its salinity tolerance, or it is a euryhaline species able to grow actively in the estuary at different salinities. In the case of complexes with morphologically cryptic species, it is a matter of discussion whether the different species occurring simpatrically can be distributed occupying each one a different niche along gradients of environmental variables, as those occurring in estuaries. Once the taxonomic identity of a strain is clarified, its response to key environmental variables has to be tested in order to know its ability to grow without stress in a particular habitat. To know the physiological status of both strains at different salinities, photosynthetic parameters as derived from the *in vivo* chlorophyll fluorescence associated to Photosystem II (PSII) were estimated by means of Pulse Amplitude Modulation (PAM) fluorometry (Maxwell & Johnson 2000, Baker 2008). The derived parameter F_0 can be considered as proxy of algae biomass (Dijkman & Kromkamp, 2006; Kromkamp et al., 2008), whereas the maximum quantum efficiency of PSII photochemistry (F_v/F_m) can be used as a sensor of stress (Baker, 2008; Betancor et al., 2015; Dijkman & Kromkamp, 2006; Kromkamp et al., 2008; Suggett et al., 2012).

The characterization of the two strains before undertaking the physiological experiments has been accounted for by electronic microscopy and molecular methods. In a previous study (Hevia-Orube et al. 2016), the molecular phylogeny of both strains based on the 28S rDNA and the Internal Transcribed Spacer (ITS1and ITS2) and 5.8

regions of the rDNA markers was addressed in a wider taxonomic context. In this study, the strains are analyzed more in detail within the frame of the *C. meneghiniana* complex. Moreover, the approach of the analysis of the ITS2 secondary structure as a proxy to address sexual incompatibility among lineages is tackled for the first time for this complex. The advantage of the secondary structure of the ITS2 to define species boundaries in microalgae including diatoms has been discussed in Orive et al. (2013). The folding on itself of the ITS2 forms a structure of four main helixes, which is characteristic of most eukaryotes. In this structure, helixes II and III are less variable than helixes I and IV, being suitable for comparing homologous positions between different strains. The presence of compensatory base changes (CBCs) or hemi compensatory base changes (HCBCs) among the ITS2 secondary structures of related species can be used as a criterion for species discrimination (Coleman 2000, 2007, Müller et al. 2007, Caisová et al. 2011).

In this study, the morphology of two species under different salinities was analyzed to deal with the controversy concerning the possible morphological overlapping between putative morphospecies (*C. cryptica* and *C. meneghiniana*) within the otherwise cryptic complex. Moreover, the physiological response to salinity has been monitored to gain insight into their preference for particular saline environments and define their degree of eurihalinity. The aim of this study was also to contribute to the delimitation of species within this complex addressing the sexual incompatibility among lineages by means of the analysis of the secondary structure of the ITS2.

METHODS

Strains

The tested strains, Bc1EHU (*C. cryptica*) and Bc11EHU (*C. meneghiniana*), were isolated from the Bilbao estuary (Basque Country, Northern Spain) and identified in a previous study (Hevia-Orube et al. 2016). They were maintained until analyses in f/2 medium (Guillard & Ryther, 1962) in the following conditions: 30 salinity, 17-20ºC and a 14:10 h light-dark cycle illuminated with 100 µmol photons· m^{-2}· s^{-1}.

Physiological experiments

To compare the physiological response to varying salinity, strains BC1EHU of *C. cryptica* and Bc11EHU of *C. meneghiniana* were cultured in f/2 medium (Guillard & Ryther 1962) at 8 salinities (0.5, 5, 10, 15, 20, 25, 30, 35) prepared with seawater and at a fixed irradiance of 120 µmol photons m^{-2}· s^{-1}. The in vivo chlorophyll *a* fluorescence was measured with a Water-PAM fluorometer (Heinz Walz, Effeltrich, Germany) allowing estimating the specific growth rate and the photochemical efficiency of photosystem II (PSII). From the original culture (salinity 30), 2 ml of inoculum were progressively transferred to the other salinities at a rate of 5 units of salinity every 2 days until a salinity of 0.5, maintaining the cultures during one week in these new conditions. After this pre-acclimation to different salinities, the acclimation and experiment were carried out. The acclimation period started with an inoculum of about $4 \cdot 10^4$ cells in 40 ml (10^3 cell/ml) of new medium, in a plastic culture flask Nuclon ™. During the acclimation period, the fluorescence parameters F_0 and F_v/F_m were measured every 24h. To calculate F_0, used as proxy of microalgae biomass, a modulated light source was applied to cells adapted to dark for 10 min. The minimum or “back ground”

fluorescence yield when all PSII reaction centers are open and ready to receive photons are thus determined. To calculate F_m, a high saturating pulse was applied to close all PSII reaction centers and to reduce the pool of electron acceptors. With both data, the maximum quantum efficiency of photosystem II (PSII) was calculated as $(F_m–F_0)/F_m= F_v/F_m$, where F_v is the variable fluorescence. When four consecutive measures indicated that the culture was in exponential growth phase, the experiment started by inoculating $4·10^4$ cells in new flasks with 40 ml of medium. Four replicates for each salinity and strain were used, one of them for morphological analysis only. Samples were taken every 24h for F_0, F_m and F_v/F_m measures. Growth rates (GR) were estimated from daily F_0 values of the exponential growth phase plotted vs. time. Growth was considered in exponential phase when, after transforming the data to natural logarithm (ln), the following criteria (adapted from Maier et al. 2006) were reached: (1) the F_0 increased; (2) at least four data point matched with a straight line; and (3) the correlation coefficient (r^2) for the slop was more than 0.95. To calculate the specific growth rates (d^{-1}), the following formula was used: $\mu= (\ln C_2-\ln C_1)/t_2-t_1$, where C_1 and C_2 are F_0 values in the exponential phase at times t_1 and t_2 respectively.

Microscopy

Cultures of each strain corresponding to the salinities of 0.5, 5, 10, 20 and 30 were cleaned the fourth day of the exponential phase for SEM analysis. After eliminating the dissolved salts, the samples were boiled in acids (HNO_3 and H_2SO_4) to eliminate the organic matter. Samples for electron microscopy were filtered (Millipore TMTP, 1.2 µm pore size), rinsed with distilled water for a few times before dehydrating with ethanol and finally rinsed with hexamethyldisilazane (HMDS 98º) for 2 minutes.

Filters were mounted on a stub (Agar Scientific Lt.), coated with a 10 nm chromium layer and observed with a Hitachi S-4800 SEM. From each salinity, at least 20 cells were analysed for valve size, number of striae (NSTR), length of striae (LSTR), number of marginal fultoportulae (MFP) and number of central fultoportulae (CFP). In addition, the cells were identified as *C. cryptica* or *C. meneghiniana* based on the original descriptions (Reimann et al. 1963, Håkansson 2002)

Statistical analyses

Statistical analyses were applied to the data to test: 1) whether the distribution of valve size, number of central fultoportulae, number of marginal fultoportulae, number of striae, and length of striae varied with salinity; 2) If this differences were related to valve size in the case of significant differences; and 3) whether there was an overlapping in the morphological traits of both strains at any salinity. The one-way ANOVA and Kruskall-Wallis test were used in looking for differences between the two strains for which it was taking into account the normality/no normality of each variable. Then, in the case of differences, a HSD Tukey multiple comparison post-test was used for normal variables. One-way ANOVA was used to test differences in size between salinities for each strain independently and a two-ways ANOVA (normality) or the Poisson regression generalized linear model (no normality) were use in order to test independent influence, no influence or interaction between both factors: salinity and valve size. Then, a Tukey test (normality) and F- Fisher test (no normality) were used to analyze difference between size and/or salinity categories. Statistical analyses have been performed with SPSS software.

DNA extraction, amplification and sequencing

For molecular analyses, unialgal cultures were centrifuged and the pellets frozen in ethanol until analysis. DNA was extracted with the commercial DNA extraction kit DNeasy Plant Mini (Qiagen, Hilden, Germany). The ITS rDNA region was amplified with ITS1F-ITS1R (Pin et al., 2001) primer pair. A mixture of 20 µl of ultrapure water, 5 µl of DNA extraction solution, 2.5 µl of each primer and 20 µl of a Bioline BioMix (Bioline GmbH, Luckenwalde, Germany) was transferred to the BIOER TC-24/H(b) thermo-cycler (BIOER Technology Co., Hangzhou, China). PCR amplification was achieved with the following program: one cycle at 95ºC for 2 min, 50ºC for 30 s and 72ºC for 45 s; 35 cycles at 94ºC for 30 s, 50ºC for 90 s and 72ºC for 30 s; and a final elongation step of 72ºC for 10 min. Amplification products were purified with the MultiScreen HTS PCR 96 kit (Millipore). Sequencing was carried out by using an ABI PRISM™ BIGDYE v3.1 Terminator Sequencing Reaction and an automatic sequencer ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were read and corrected using BioEdit 7.1.3 (Hall 1999).

Phylogenetic analyses

A total of 12 sequences, representative of five lineages of the *C. meneghiniana* complex were used for the phylogenetic analyses. Two of these came from the local strains, one belonging to the *C. cryptica* morphospecies and another to the *C. meneghiniana* morphospecies. All the sequences of *C. meneghiniana*, except the one from the local strain Bc1EHU, came from strains from Bremerhaven, in the North Atlantic and named according to the ribotypes of Beszteri et al. (2007); the strain CCAP 1070/2 of *C. cryptica* was from Martha´s Vineyard, the type locality of the species.

Sequences alignment was achieved using MUSCLE 3.7 alignment method (Edgar, 2004) and manually improved. All positions containing gaps and missing data were eliminated. There were a total of 300 positions in the final dataset. Phylogenetic analyses were inferred using Maximum likelihood (ML) method with MEGA6 software (Tamura et al. 2013). For this analysis, bootstrap confidence values were calculated from 10000 replications. Prior to phylogenetic analyses, the best DNA substitution model was carried out and a Bayesian information criterion (BIC) was selected to describe the best nucleotide substitution pattern. ML searches were conducted using the chosen model K2-P (Kimura, 1980) with a discrete Gamma distribution (+G, parameter = 0. 90), transition/transversion rate (R = 1.96) and rates of base substitutions: r(AT) = 0.04; r(AC) = 0.04; r(AG) = 0.16; r(TA) = 0.04; r(TC) = 0.16; r(TG) = 0.04; r (CA) = 0.04; r(CT) = 0.16; r (CG)=0.04; r(GA) = 0.16; r (GT) = 0.04; r(GC)=0.04.

ITS2 secondary structure

The secondary structure of the ITS2 has been delineated for selected strains (two of *C. cryptica* and seven of *C. meneghiniana*) in looking for CBC clades in the sense of Coleman (2000). The presence of CBCs in the most conserved parts of the helixes conforming the secondary structure has been related by this author with mating incompatibility among the compared strains. The comparisons have been made between the two local strains and 7 strains from GenBank used in the ITS phylogenetic tree.

The ITS2 secondary structure was predicted by homology modelling (Wolf et al. 2005a) for the selected *C. cryptica* and *C. meneghiniana* sequences. The ITS2 secondary structure was based on *C. meneghiniana* (strain G17W3, accession number: AY906805) available from ITS2-Database version v.5 (Ankenbrand et al. 2015).

Structures were visualized with VARNA (Darty et al. 2009) and then alignment was performed with both 4SALE (Seibel et al. 2006) and ITS2-Database version v.5 (Ankenbrand et al. 2015). Finally, the CBCs and HCBCs were identified using the software CBCA analyzer (Wolf et al. 2005b).

RESULTS

Physiological response to salinity

Strains Bc1EHU of *C. cryptica* and Bc11EHU of *C. meneghiniana* survived at the whole range of salinities tested (from 0.5 to 35) with relatively high growth rates and good physiological status. The response to salinity in terms of growth rates was similar for both strains, with slightly higher growth rates at intermediate salinities (**Fig.1**).

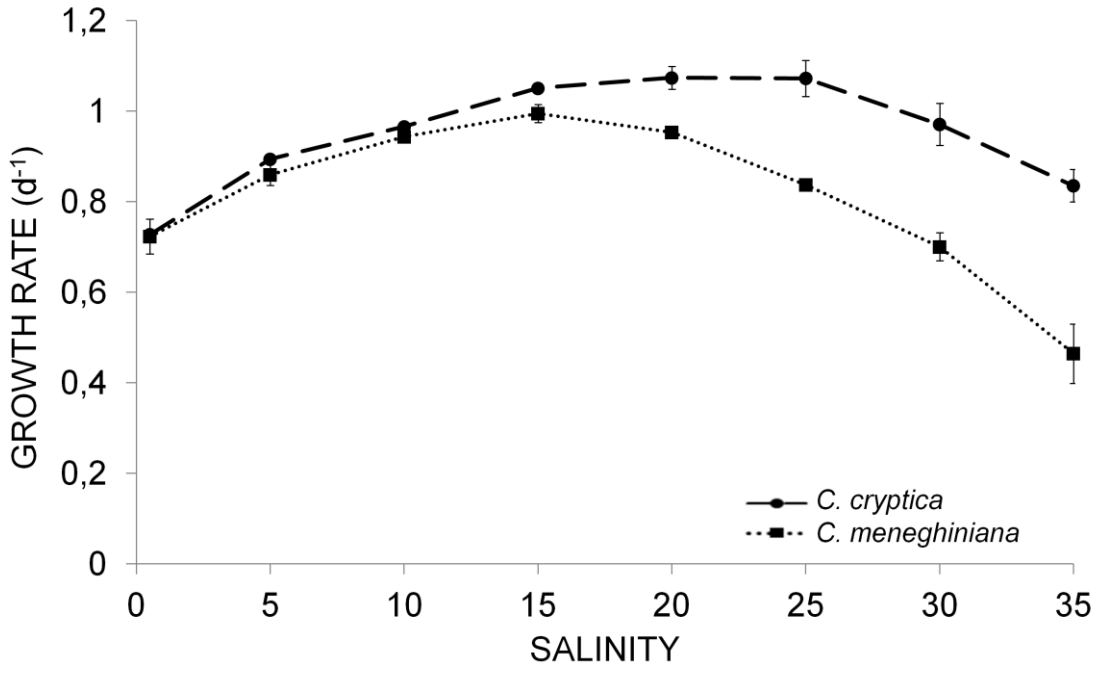


Fig. 1 - GR average of culture replicates (n=3) at different salinities.

Cyclotella cryptica showed low variability among salinities, with the maximum growth rate (1.07 d^{-1}) at 20 and 25, and the minimum (0.73 d^{-1}) at 0.5. *Cyclotella meneghiniana* showed marked differences in growth rate with salinity with a maximum of 0.99 d^{-1} at 15 of salinity and a minimum of 0.46 d^{-1} at 35. *Cyclotella meneghiniana* showed lower growth rates than *C. cryptica* at all salinities, although the values were similar at the lowest salinities.

The maximum quantum efficiency of the PSII (F_v/F_m) was relatively high in both strains (**Fig. 2**), with values varying between 0.61 and 0.66 in *C. cryptica* and between 0.50 and 0.71 in *C. meneghiniana*. In agreement with the decreases in growth rates, *C. meneghiniana* experienced a sharp decreases in F_v/F_m at 35 of salinity The maximum quantum efficiency of *C. meneghiniana* was higher than that of *C. cryptica* at all the salinities tested except at 35.

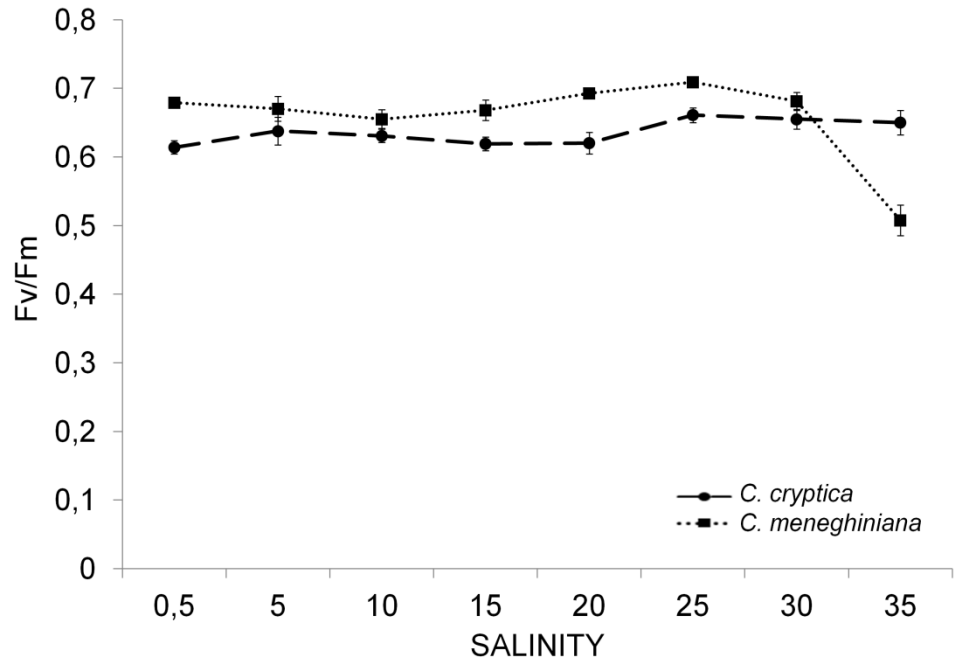


Fig. 2 - Average values of photosynthetic parameter F_v/F_m during the exponential phase days at different salinities.

Morphological plasticity with salinity

The following variables were measured and analyzed statistically: number and length of striae and number of marginal and central fultoportulae, all of them related to salinity and valve size. These analyses were performed for strains of *C. cryptica* and *C. meneghiniana* independently, which were then compared.

The average valve size was similar for each strain at the different salinities (**Fig. 3**), with values markedly higher for *C. meneghiniana* at all the salinities tested. The valve size ranged from 5.49-10.03µm in *C. cryptica* and from 13.54-18.82 µm in *C. meneghiniana*. The average number of central fultoportulae increased with salinity in both species, from one at the lowest salinities to two at the highest (**Fig. 4**). The dispersion of the data was very high, particularly in *Cyclotella cryptica*, in which some individuals presented 5 central fultoportula at a salinity of 30. In *C. meneghiniana*, the central fultoportulae were also related to salinity, with always 1 central fultoportula at 0.5, whereas at higher salinities individuals with 2 and 3 central fultoportulae were frequents. *Cyclotella cryptica* did not present differences between salinities in the other characteristics measured, unlike *C. meneghiniana*, which showed significant differences in the number of marginal fultoportulae with valve size and salinity (**Fig.5**), and in the number of striae with valvae size (**Fig. 6**) and the length of striae with salinity (**Fig. 7**). The number of striae oscillated from 14-26 per valve in *C. cryptica* and from 29-50 in *C. meneghiniana*. Despite the morphological variations experienced by both species, they fitted the corresponding original descriptions along the whole salinity gradient (**Figs 8-11**).

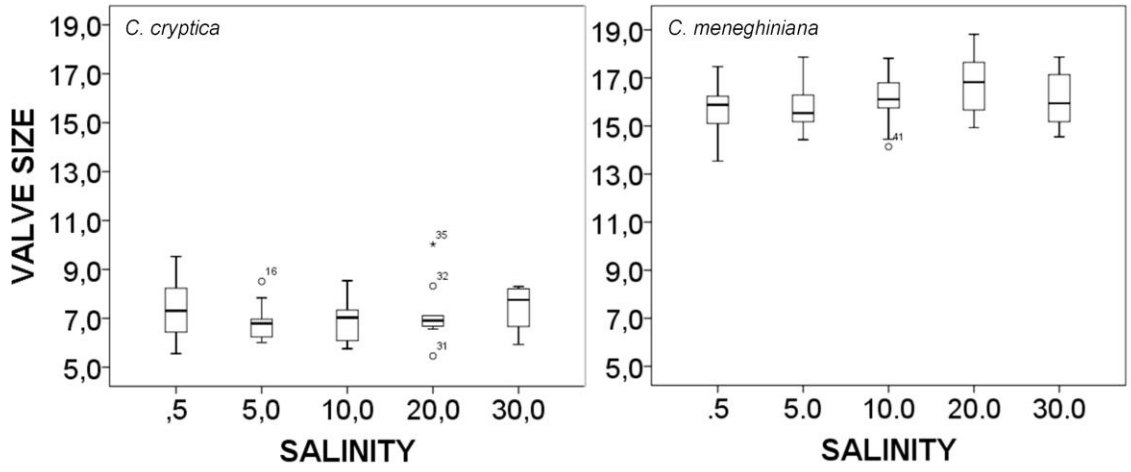


Fig. 3 - Valve cell size in function of salinity.

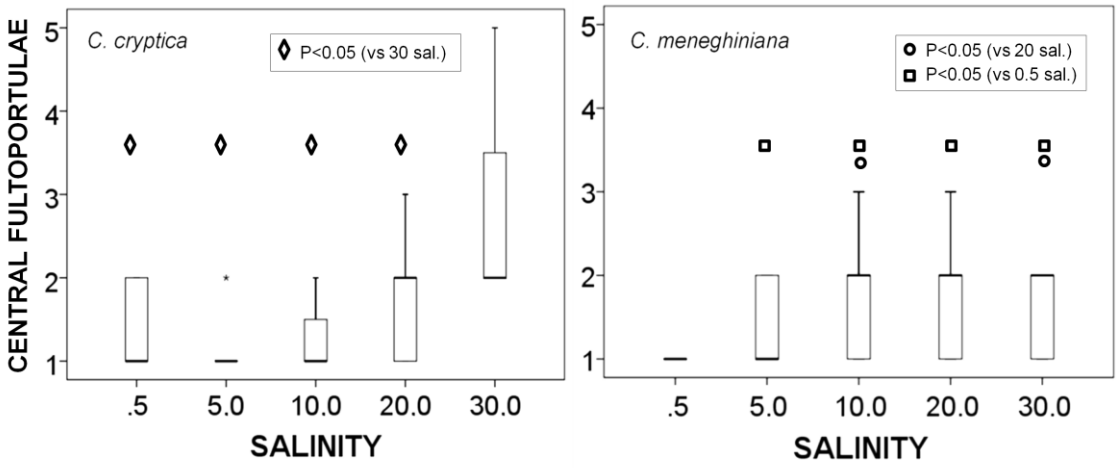


Fig. 4 - Relationships between salinity and central fultoportulae number (CFP).

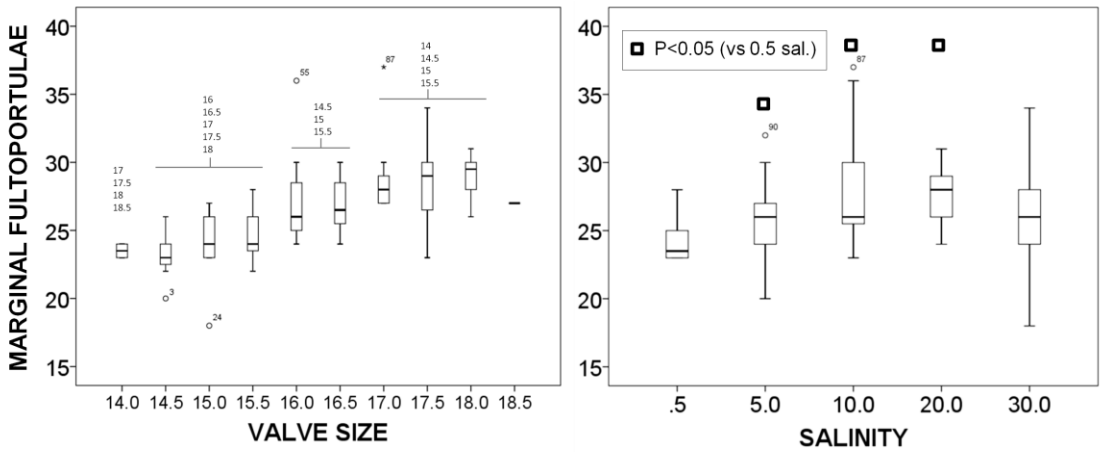


Fig. 5 - Marginal fultoportulae number (MFP) in function of valve size and salinity, in *Cyclotella meneghiniana*.

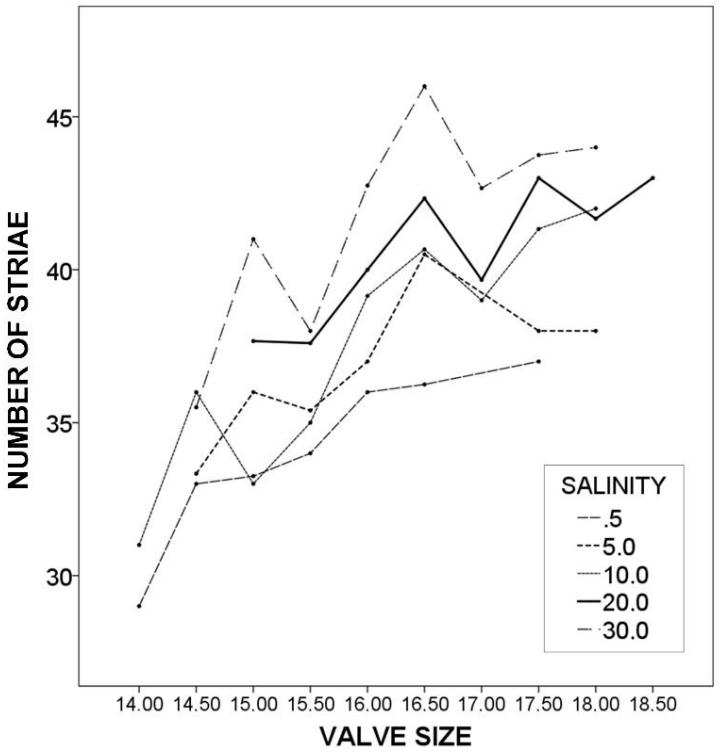


Fig. 6 - Correlation of number of striae (NSTR) with valve size and salinity in *Cyclotella meneghiniana.*

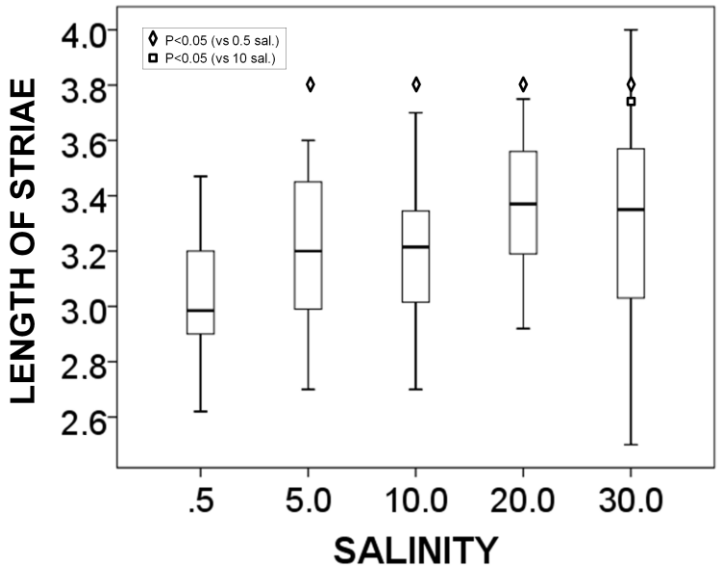
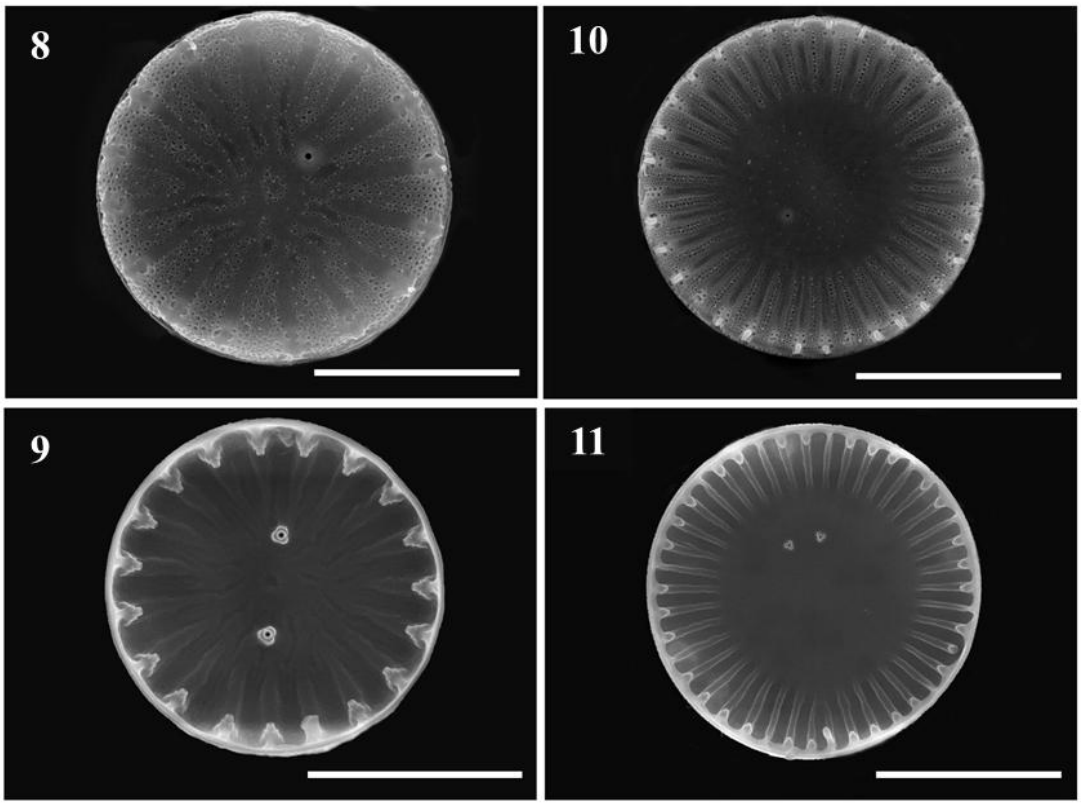


Fig. 7 - Relationships between the length of the striae (LSTR) and salinity in *Cyclotella meneghiniana*.



Figs. 8-11 - Electron micrographs of valves of *Cyclotella cryptica* in external (8) and internal valve view (9) and *C. meneghiniana* in external (10) and internal valve view (11). Scale bars: 10 µm.

ITS Phylogeny and ITS2 secondary structure

The ITS tree showed five well supported groups (**Fig. 12**), one of them (Cry+C) with higher internal variability. This group included a subgroup with strains G183K and G188D from Bremerhaven and named *C. meneghiniana,* and other two strains identified as *C. cryptica*, including the local strain Bc1EHU and the strain CCAP 1070/2 isolated from Martha´s Vineyard, the type locality of the species. The other four groups contained strains identified as *C. meneghiniana* (groups B, A and D) and *Cyclotella* cf. *scaldensis* (group Amb). The other local strain Bc11EHU clustered in group D of *C. meneghiniana* complex.

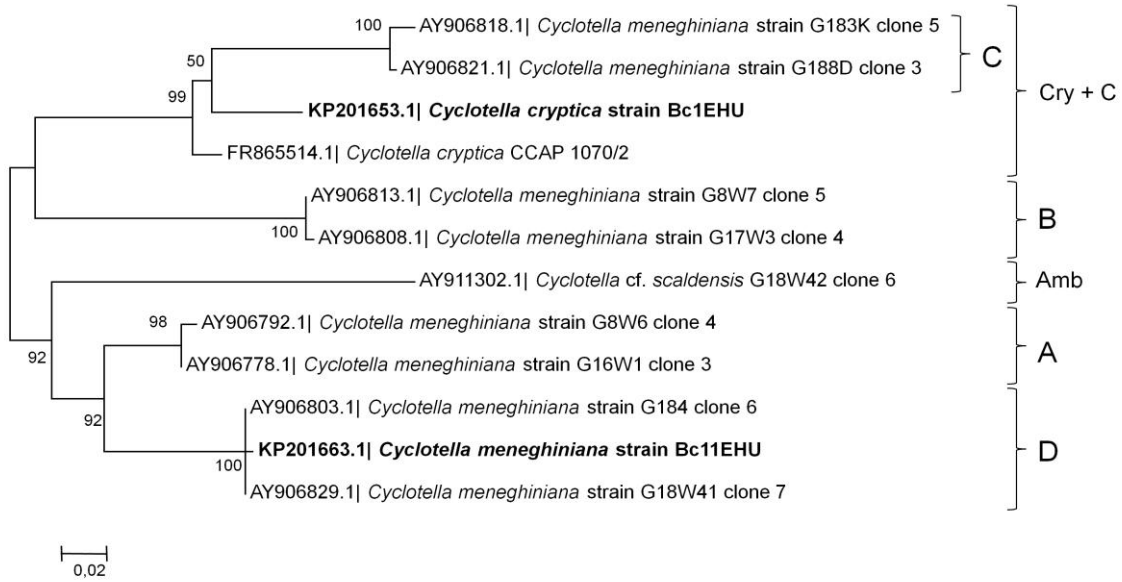


Fig. 12 - Maximum likelihood tree based on the ITS and 5.8 rDNA sequences of the local strains (in bold) plus strains selected from different ribotypes of the *C. meneghiniana* complex delineated by Bezteri et al. (2005b) and a strain of *C. cryptica*. Numbers on the nodes represent ML support values.

A common pattern of the ITS2 secondary structure was delineated for all the 9 strains analyzed and selected among those of the different subgroups of the ITS tree. Four main helixes (I-IV) and a pyrimidine- pyrimidine bulge in helix II were recovered and the corresponding CBCs represented (**Fig. 13**). The total of CBCs and HCBCs among the different strains are depicted in **Table 1**. The different groups based on the ITS tree (Cry+C, B, A and D) contained strains that did not present CBCs within them, but they showed at least 1CBC in helixes II and III with respect to strains of the other groups (**Fig. 13**). The number of HCBCs (**Table 1**) was also larger between strains from different groups (from 7 to 13) than within strains of the same group (from 0 to 6). The local strains Bc1EHU (*C. cryptica*) and Bc11EHU (*C. meneghiniana*) showed one CBC (G:C →A:T) between them, positioned in helix II, in the bp 58- 77 near the base of the helix. In addition, 8 HCBCs were identified, one of them in helix I, another in helix IV, and the rest along helix III (not shown).

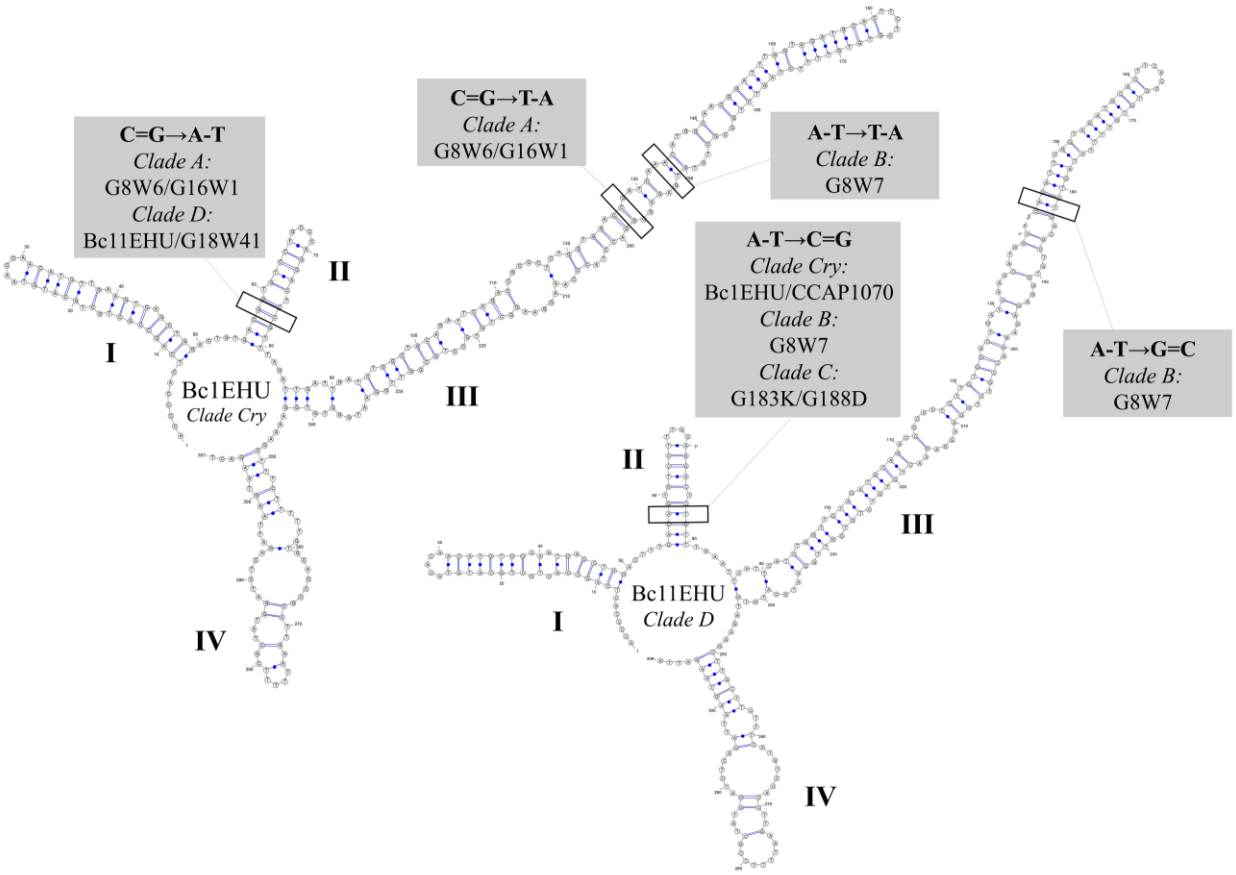


Fig. 13 - Comparison of the ITS2 secondary structures of the local strains of *Cyclotella cryptica* (Bc1EHU) and *C. meneghiniana* (Bc11EHU). The presence of CBCs is highlighted with squares.

Table 1. Number of CBCs (in parenthesis) plus HCBCs among selected strains.

	Bc1EHU[a]	CCAP 1070/2[a]	G183[b]	G188[b]	G8W6[b]	G16W1[b]	G8W7[b]	G18W41[b]	BC11EHU[b]
Bc1EHU[a]	-	3 (0)	6 (0)	6 (0)	13 (2)	12 (2)	11 (1)	9 (1)	9 (1)
CCAP 1070/2[a]	3 (0)	-	4 (0)	4 (0)	11 (2)	10 (2)	7 (1)	8 (1)	8 (1)
G183[b]	6 (0)	4 (0)	-	2 (0)	14 (2)	14 (2)	10 (1)	11 (1)	11 (1)
G188[b]	6 (0)	4 (0)	2 (0)	-	15 (2)	15 (2)	10 (1)	11 (1)	11 (1)
G8W6[b]	13 (2)	11 (2)	14 (2)	15 (2)	-	1 (0)	15 (3)	8 (1)	8 (1)
G16W1[b]	12(2)	10 (2)	14 (2)	15 (2)	1 (0)	-	14 (3)	7 (1)	7 (1)
G8W7[b]	11 (1)	7 (1)	10 (1)	10 (1)	15 (3)	14 (3)	-	11 (2)	11 (2)
G18W41[b]	9 (1)	8 (1)	11 (1)	11 (1)	8 (1)	7 (1)	11 (2)	-	0 (0)
BC11EHU[b]	9 (1)	8 (1)	11 (1)	11 (1)	8 (1)	7 (1)	11 (2)	0 (0)	-

[a]*C. cryptica*; [b]*C. meneghiniana*

DISCUSSION

Physiological response to varying salinity

The photosynthetic features of the microalgae and their growth rates can be accurately estimated with PAM fluorescence, which can also provide information on their physiological state. In highly mobile systems such as estuaries, which experience drastic changes in salinity at short spatial and temporal scales, it is of paramount importance to know whether a species is able to grow actively in a particular place along the salinity gradient or if its presence in the place is the result of the transport by the tide, experiencing there a certain degree of stress. Strains of *C. cryptica* (Bc1EHU) and *C. meneghiniana* group D (Bc11EHU) isolated from the Bilbao estuary, a short and shallow meso-macrotidal system, were tested for this purpose after being identified by means of morphological and molecular methods and the results show that both are able to survive within the salinity range of 0.5 to 35 with good physiological status. Only *C. cryptica* at the highest salinity show a value of the maximum quantum efficiency of PSII (F_v/F_m) relatively low (0.5), which although not within the range of 0.6-0.7, considered by Dijkman & Kromkamp (2006) as indicative of good physiological status for healthy microalgae, cannot be considered as indicative of stress, which these authors place below 0.4. The growth rates of both strains were more variable than the F_v/F_m values what can be due to that they grow at lower rates out of its salinity optima but maintaining a good physiological status. Both strains behave thus as euryhaline species with lower response in terms of growth rates at extreme salinities, as revealed also by other authors (Liu & Hellebust, 1976; Roubeix & Lancelot, 2008). Despite the physiological response to high salinity is slightly different in both strains, they can be characterized as brackish water species. They could occupy the whole salinity range tested, and the tolerance to salinity could not be a distinctive feature between *C.*

cryptica and the strain of *C. meneghiniana*, except for the highest salinity, more favorable for the former.

Morphological identity and plasticity

Both strains, Bc1EHU and Bc11EHU maintained the diagnostic characteristics of the original descriptions of *Cyclotella cryptica* and *C. meneghiniana,* respectively, and, although they showed morphological changes with salinity, these differences did not mask the identity of the cells, which maintained at all the salinities tested their main differential features. These concerned the border between the central area and the radially striated margin, poorly defined in *C. cryptica*, and marked in *C. meneghiniana*, and the internal alveolar chambers, which are diffuses in *C. cryptica* (**Figs 8-11**). In this study, all specimens of *C. cryptica* matched with the original description by Reimann et al. (1963), although the formation of auxospores and cell sizes higher than 10.03 µm were not observed. The authors suggested that a change in salinity triggered the formation of auxospores (also reported by Chepurnov et al., 2012), which resulted in larger cells, with the striae occupying half the radius of the valve, as occurs in *C. meneghiniana*, whereas in the smaller ones the central area may disappear altogether. The latter is the kind of morphology that was found in the present study, where changes in salinity did not trigger auxospores formation. In a later study, Schultz (1971) concluded that within the salinity range of 4.3-1.4, *C. cryptica* presented the pattern of *C. meneghiniana*. These results have not been observed in this study, where the only morphological change that *C. cryptica* experienced with salinity was the increase in the number of central fultoportulae with increasing salinity.

Cells from strain Bc11EHU were morphologically identified as *C. meneghiniana* Kützing as in Håkansson (2002), although marked morphological changes in central fultoportulae, marginal fultoportulae, number of striae and length of striae were observed with salinity. The central and marginal fultoportulae increased from 0.5 to 20 of salinity, decreasing slightly at 30, coinciding with the growth rate pattern. Nevertheless, the length and number of striae increased with salinity. Previous studies also observed that *C. meneghiniana* had a unique central fultoportula in freshwater, and several at increasing salinity (Håkansson & Chepurnov, 1999; Shirokawa et al., 2012) . These differences were not linked with valve size (Desikachary & Rao, 1973). In Shirokawa et al. (2012) the number of striae also increased with salinity in one strain of *C. meneghiniana*. Furthermore, (Håkansson & Chepurnov, 1999) detected differences in the length of the striae, although they were 3.5µm long generally. The number of marginal fultoportulae and number of striae are two variables closely related, as almost each stria is occupied by one marginal fultoportula. They were size dependent with their number decreasing with cell size due to the minimum distance they have to maintain between adjacent processes (Cox 2014). The fultoportulae produce a chitin thread that connects the cells in chains, hence they have a colony linking function (Kaczmarska et al. 2005, Hernández-Becerril et al. 2009). Furthermore, the fibers decrease the sinking rate of the cells by increasing their form resistance (Walsby & Xypolyta, 1977). In addition, there is a possibility that the formation of a local environment around cells by aggregation could reduce salinity stress (Silsbe & Kromkamp, 2012). These hypotheses cannot explain the results obtained, as the higher number of central and marginal fultoportulae appeared at intermediate salinity, which are the optimum for these strains. The diversity of results obtained by different authors in previous studies (Håkansson & Chepurnov, 1999; Reimann et al., 1963; Schultz,

1971), suggest the existence of factors other than salinity in triggering the morphological changes. In previous studies, different media were used to test differences in salinity, thus introducing new variables as could be nutrient concentration. Besides, the large variability of results observed in published studies could be the result of the analyses of different species morphologically cryptic within the *C. meneghiniana* complex and, consequently, with possible different response to particular environmental variables.

ITS phylogeny and ITS2 secondary structure

Among molecular markers, the ITS region, and particularly the ITS2, has been widely used to distinguish among species in microalgae and particularly in diatoms (Caisová et al., 2011; Orive et al., 2013). Therefore, it has been used to know if *C. cryptica* and *C. meneghiniana* are not only different morphospecies, but also different genotypes potentially unable to reproduce giving a fertile progeny. For this to account, an ITS phylogenetic tree has been delineated and the ITS2 secondary structure developed. Based on previous studies (Coleman 2000, 2007, 2009, Müller et al. 2007, Caisová et al. 2011, 2013), the existence between two strains of a CBC of the secondary structure of the ITS2 , can be interpreted as proxy of mating incompatibility and as such can be used to define different biological species. A group of organisms lacking CBCs in a conserved region form a CBC-clade sensu Coleman (2000, 2003, 2009).This author defended that a CBC-clade predicts meaningful intercrossing ability. However, as stated by Caisová et al. (2011), the absence of CBCs in a group does not mean that they could reproduce successfully forming a Z clade, which is that one formed by organisms capable to produce compatible gametes and form viable zygotes (biological species

concept). One CBC clade contains at least one Z clade, and different Z clades can be grouped in the same CBC clade. Caisová et al. (2011) studied this concept in Ulvales, concluding that genes which control gametes compatibility and genes involved in structural differentiation evolve much faster than most CBCs. However, there is a discussion about the location of the CBCs within the ITS2 secondary structure. While Müller et al. (2007) defended that the existence of CBCs separate species in the 93% of studied cases, regardless of where they were located, Coleman (2009) defined the helix III as the conserved region of the structure and therefore, concluded that only CBCs situated in this region delimited biological species. Despite this author had previously defended the helix II, helix III and adjacent regions as conserved regions (Coleman 2007), the latter conclusion was reached also by Caisová et al. (2011). According to this, we presume that strains from the different tested lineages (C, Cry, B, A and D) belongs to different CBC clades. These lineages were formed by at least one CBC clade, as there were one or more CBCs in a conserved region of the ITS2 secondary structure between stains of different lineages, denoting the existence of different species. Therefore, the *C. meneghiniana* complex is formed by different cryptic species which are pending of formal taxonomic description. In this study, the results of the ITS2 secondary structure reveals that the local strain of *C. meneghiniana* shares the same ITS structure as one strain of group D of Beszteri et al. (2005b, 2007), differing from the other lineages which might constitute different biological species. Beszteri et al. (2005b, 2007), based on the phylogenies of the regions D1/D2 of the 28S rDNA and the ITS and 18S rDNA and a segment of the plastid encode psaA gene, concluded that the morphospecies *C. meneghiniana* included several cryptic species. Furthermore, Chepurnov et al. (2012) reported the existence of allogamous reproduction in *C. meneghiniana* supporting thus that *C. meneghiniana* is a complex of cryptic sexual

species. The local strain of *C. cryptica* (Bc1EHU) belongs to a heterogeneous group (Cry+C) formed by strains identified as *C. cryptica* and a homogeneous group of *C. meneghiniana* (group C). This lineage forms a CBC clade, the local strain of *C. cryptica* differed in only three HCBCs from the strain CCAP 1070/2 of Martha´s Vineyard, the type locality of *C. cryptica* and in six HCBCs with strains of group C of *C. meneghiniana*. Inside this complex, Bc1EHU (*C. cryptica*) belonged to group Cry, and Bc11EHU (C. *meneghiniana*) is part of the group D. Both strains presented one CBC in the helix II and several HCBCs in conserved parts of the ITS2 secondary structure between them and as such we can conclude that they are effectively two different species, unable to reproduce giving a fertile progeny. Recent studies (Caisová et al. 2013) discussed the concepts related to the secondary structure, the way to obtain it, the reliability of the structure and the quality of the conclusions that could be reached with this method, affirming that this method is not useful to discriminate species by their own in the chlorophyta group they studied, remarking the necessity of studying also the phylogeny based on other molecular markers. Beszteri et al. (2005b, 2007), based on the phylogenies of the regions D1/D2 of the 28S rDNA and the ITS and 18S rDNA and a segment of the plastid encode psaA gene, concluded that the morphospecies *C. meneghiniana* included several cryptic species. Furthermore, Chepurnov et al. (2012) reported the existence of allogamous reproduction in *C. meneghiniana* supporting thus that *C. meneghiniana* is a complex of cryptic sexual species.

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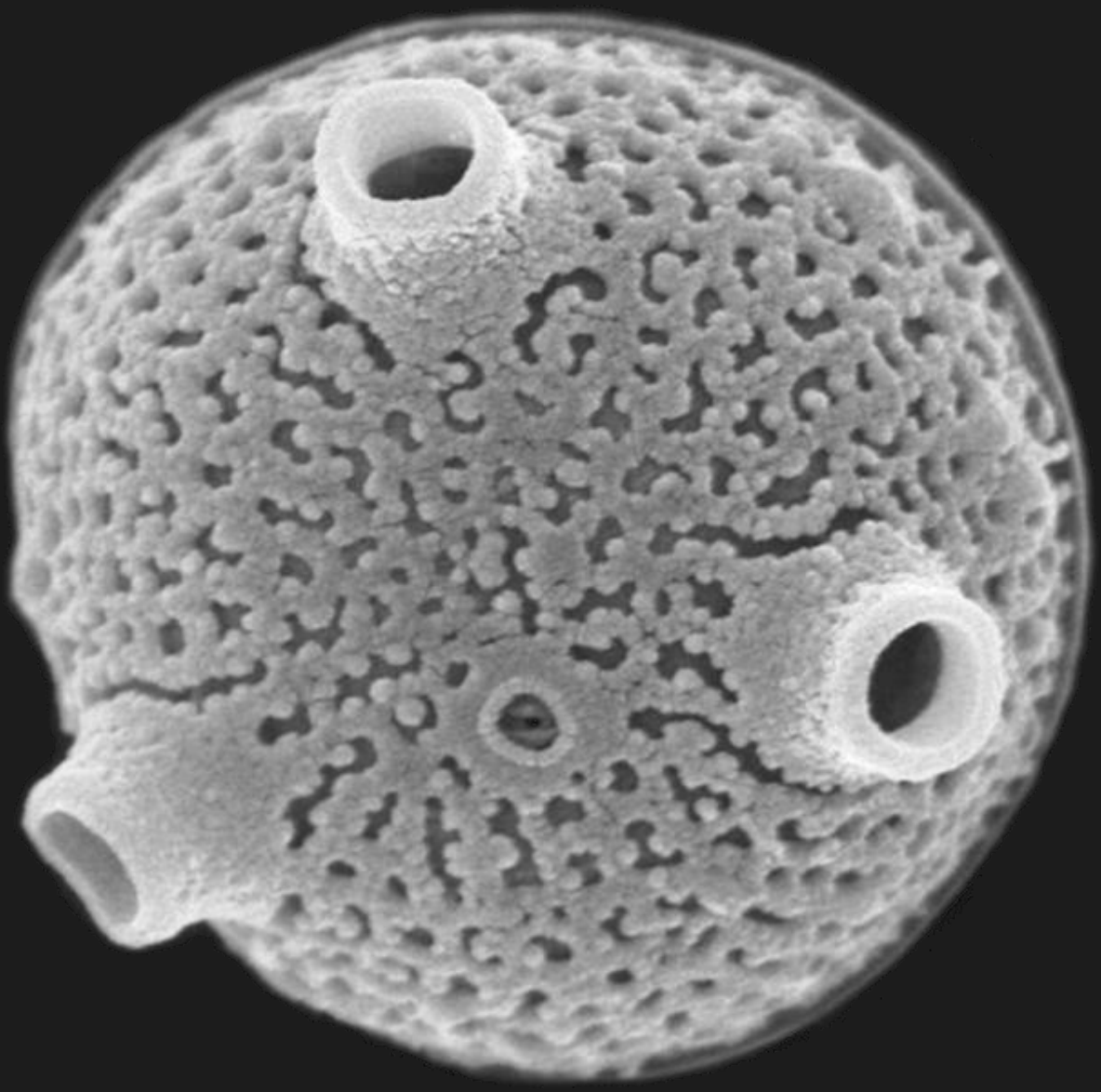
CHAPTER 5

A comprehensive molecular and morphological analyes of *Minidiscus comicus* (Mediophyceae) reveals two distinctive morphologies within the species



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INTRODUCTION

Minidiscus Hasle (1973a) is a cosmopolitan genus whose species can reach noticeable concentrations in the plankton of different geographic areas as revealed by analyzing natural samples under the SEM (Kang et al. 2003, Aizawa et al. 2005, Buck et al. 2008, Garibotti et al. 2011, Zingone et al. 2011, Lee et al. 2012, Hernández-Becerril 2014, Daniels et al. 2015). Species of *Minidiscus* have been revealed as planktonic by analyzing sediment traps (Kang et al. 2003) and bottle samples (Jewson et al. 2016), but have also been found in high concentrations in mud samples (Kaczmarska et al. 2009, Manoylov & Dominy 2013), what is not necessarily controversial due to the possibility of appearance of high concentrations of cells in the sediment after blooming in the plankton. As observed by Buck et al. (2008), in upwelling waters of California, *Minidiscus trioculatus* cells spent a part of their life cycle in the sediment as intact cells in an active or dormant state, from which they can be resuspended in the water column to form blooms.

The identification of the genus *Minidiscus* by Hasle (1973) was based on the following diagnostic criteria: valves without a marginal ring of fultoportulae; varying number of strutted process (fultoportulae) in the valve plane distant from the margin; and a labiate process, also distant from the margin. With these criteria, Hasle (1973) renamed the species *Coscinodiscus trioculatus* reported by Taylor (1967) to *Minidiscus trioculatus* (Taylor) Hasle. Since then, seven new species have been described: *M. comicus* (Takano 1981), *M. chilensis* (Rivera & Koch 1984), *M. subtilis* (Gao & Cheng 1992), *M. ocellatus* and *M. spinulosus* (Gao et al. 1992), *M. decoratus* (Quiroga & Chrétiennot-Dinet 2004), and *M. variabilis* (Kaczmarska et al. 2009).

The small size of the *Minidiscus* cells (1.5-7.5µm) difficults their identification

and isolation, making that most of the research works on the genus, including type materials, are based on images of field samples. Consequently, some species of *Minidiscus* have been misidentified, as pointed out by Quiroga & Chrétiennot-Dinet (2004). In *Minidiscus*, as accounts for most if not all microalgae, an accurate identification to species level requires the concourse of molecular methods in addition to the morphological description. Even so, it must be taken into account the existence in the gene banks of sequences corresponding to morphologically misidentified species. Concerning this, in Gen Bank there are 42 sequences corresponding to organisms named as *Minidiscus*, but 66% of them can be considered misidentified or whith incorrect spelling of the specific name. Despite some species have been reidentify in recent works, they still appear with the erroneous name in the data base. This kind of mistakes difficults a correct identification of species using DNA sequences as complementary information, but even more for studies that are only based on molecular analyses. This is also a trouble for the aplication of metabarcoding methods to the identification of the whole phyloplankton assemblage in natural samples, for whose purpose it is crucial the existence of an accuratelly identified dataset.

In a previous study on small bloom forming Mediophyceae diatoms from the Bilbao estuary (Hevia-Orube et al. 2016), individuals of *Minidiscus* were identified under the SEM, appearing generally in low concentrations and consequently were not reported therein. Recently, individuals of this genus have been isolated from the Bilbao estuary and cultured to identify them by means of both morphological and molecular analyses. Taken into account that only a few studies on the genus *Minidiscus* have been accomplished jointly by morphological and molecular methods, the aim of this work was to gain insight into the identity of *Minidiscus* species as assessed by SEM and by ITS1, 5.8S and ITS2 (ITS) and 28S as DNA molecular markers. Cultures of local strains

and natural samples of the Bilbao estuary were used for morphological purpose. In this study we present two morphotypes of *Minidiscus comicus* found in natural samples as well as in cultures.

MATERIAL AND METHODS

Study area

The Bilbao estuary (43º20'N, 2º70'W, Basque Country, Northern Spain) is meso-macro tidal, with a semidiurnal tidal regime. The estuary is relatively shallow (2-9 m depth), except at the mouth, which consists of a semi-confined harbor 3,8 km in width and 14-30 m in depth. More details are given in Borja et al. (2004).

Sampling and cultures

Monthly phytoplankton samples were taken at eight permanent stations located along the longitudinal axis of the estuary (Hevia-Orube et al. 2016). *Minidiscus* cells were isolated from live samples by pippeting and unialgal cultures were established in f /2 medium (Guillard & Ryther 1962) and maintained in stock at 30 of salinity, 17-20ºC and a 12:12 h light-dark cycle illuminated with 100 µmol· m $^{-2}$· s^{-1} . Seven strains: Bc40EHU, Bc49EHU, Bc50EHU, Bc51EHU, Bc52EHU, Bc53EHU and Bc54EHU were identified as *Minidiscus* and analyzed in this work.

Morphological analysis

The ultrastructural analysis was base in SEM micrographs (Hitachi S-4800). The cleaning, following Von Stoch's procedure, and samples preparation for SEM was

explained in detail in Hevia-Orube et al. (2015). From each culture, at least 20 images of cells were taken and some features were measured (with image J software) or counted. Theriot & Serieyssol (1994) were followed for morphological characters and features description. Data analysis was performed with SPSS software.

Molecular analysis

DNA extraction, amplification and sequencing

For molecular analyses, cultures were centrifuged, and the DNA was extracted with the commercial DNA extraction kit DNeasy Plant Mini (Qiagen, Hilden, Germany). D1R-D2C and ITS1-ITS4 pairs of primers were used to amplify 28S (Scholin et al. 1993) and ITS rDNA regions (White et al.1990), respectively. A mixture of 20 µL of ultrapure water, 5 µL of DNA extraction solution, 2.5 µL of each primer and 20 µL of Bioline BioMix (Bioline GmbH, Luckenwalde, Germany) was transferred to the BIOER TC-24/H(b) thermo-cycler (BIOER Technology Co., Hangzhou, China) for PCR amplification with the following program: one cycle at 95ºC for 2 minutes, 50ºC for 30 seconds and 72ºCfor 45 seconds; 35 cycles at 94ºC for 30 seconds, 50ºC for 90 seconds and 72ºC for 30 seconds; and a final elongation step of 72ºC for 10 minutes. Amplification products were purified with the MultiScreen HTS PCR 96 kit (Millipore). Sequencing was carried out by using an ABI PRISM™ BIGDYE v3.1 Terminator Sequencing Reaction and an automatic sequencer ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were read and corrected using BioEdit 7.1.3 (Hall 1999).

Sequence alignment and phylogenetic analyses

Three strains of *Minidiscus* (Bc49EHU, Bc50EHU, and Bc53EHU) from this study were used to build the phylogenetic tree. In addition, 12 strains from Gen Bank were included, four of them among those available from *Minidiscus* and 8 from *Skeletonema*. Another two strains of *Minidiscus variabilis* from our culture collection were used to complete the phylogenetic tree. A strain of *Cyclotella atomus* was used as outgroup.

Sequence alignment was achieved using ClustalW2 software (Larkin et al. 2007) and improved manually. Phylogenetic analyses were inferred using Maximum Likelihood (ML) and Neighbour Joining (NJ) methods, all conducted in MEGA6 (Tamura et al. 2013). In all cases (NJ, MP and ML), bootstrap confidence values were calculated from 1000 replications. Prior to ML analyses, a test to find the best fitting model of DNA substitution was performed (with the Akaike information criterion). ML analyses were performed with the Kimura 2 – parameter model (Kimura 1980), and a discrete Gamma distribution was used to identify evolutionary rate differences among sites (5 categories (+G, parameter = 0.51)), transition/transversion rate (R = 1.37), with substitution rates for transitions and transversion 0.144 and 0.053, respectively.

RESULTS

Morphological analysis

Based on morphological analyses, the seven strains of *Minidiscus* corresponded to *Minidiscus comicus*. The morphological characteristics matched with bibliography data (**Table 1**).

Table 1. Morphological features of *Minidiscus* species comparing this study with other published studies.

Species	Ø(µm)	Areolae (in 10 µm)	FP	RP	Valvae face	From culture/ field	Identified as	Sour.
M. trioculatus* var. *trioculatus	3.3 2.6-4.2	54 44-65	2.03 2-3	1	Tangential- linear areolation	Culture CCMP496	*M. trioculatus var. trioculatus*	1
M. trioculatus* var. *monoculatus	3.0 2.4-3.7	59 48-68	1.02 1 (2)	1	Tangential-linear areolation	Culture MiniNova2. From Bay of Fundy (Atlantic coast North America)	*M. trioculatus var. monoculatus*	1
M. trioculatus	2.0-6.8	40-50	3-5 (2s.p.)	1	13-30 rows of areolae, circular an protuberant pattern	Not indicated	*M. trioculatus*	2
M. trioculatus	1.5-3.3	60 (40-70)	2-3 (2s.p.)	1	Linear or slightly excentric. Hexagonal areolae	Field samples: Mexican Pacific	*M. trioculatus*	3
M. chilensis	4-5.5	11-12 perforations	2	1	Radial areolation, diminishing towards center	Field samples: Argentina (South Atlantic)	*M. chilensis**	4
M. chilensis	3.1-5.1	90-100	2 (2s.p.)	1	Marginal ring of short radial rows of areolae	Field samples: Bay of Fundy (Atlantic coast North America)	*M. chilensis*	1
M. chilensis	3.0-7.5	90	-	-	Radial rows of areolae	Not indicated	*M. chilensis*	2
M. decoratus	2.5-2.9	-	2-3 (2 s.p.)	1	Smooth except one or more rows of areolae forming a hexagonal pattern	Field samples: English Channel (North Sea-Atlantic)	*M. decoratus (including the type)*	2
M. comicus	2-3.5	50 (60-80)	3 (3 s.p.)	1	Radial areolation. Polygonal areolae	Field samples: Mexican Pacific	*M. comicus*	3
M. comicus	2.1-3.2	60-80	3	1	Net of loculate and/or pseudoloculate areolae	Field samples: Bay of Fundy (Atlantic coast North America)	*M. comicus*	1
M. comicus	2.58 1.9-6		3 (3 s.p.)	1(0)		Field samples: Bilbao Estuary	*M. comicus*	7
M. comicus	4.36 2.2-6.7		3 (9) (3 s.p.)	1		Cultures: Bc49EHU, Bc50EHU, Bc52EHU, Bc53EHU	*M. comicus*	7

M. comicus	1.9-7	50-100	3-7 (3 s.p.)	1	Radial areolation. Polygonal areolae. Foramina external, cribra internal	Field samples: Tokyo Bay	*M. comicus (including the type)*	5
M. comicus	1.9-7.0	-	-	-	Areolae non linear	Not indicated	*M. comicus*	2
M. spinulosus	3.0-5.0	-	-	-	Radial rows of areolae	Not indicated	*M. spinulosus*	2
M. subtilis	3.5-5.5	85	-	-	Radial rows of areolae	Not indicated	*M. subtilis*	2
M. variabilis* var. *variabilis	3.9 3.2-5.5	61 (54-72)	2.75 (2-4)	1	Radial rows of areolae	Culture CCMP 495 Gulf of Maine, 1976	*M. trioculatus*	1
M. variabilis* f. *inornata	2.8 2.0-3.7	65 (57-75)	2.73 (2-4)	1	Radial rows of areolae (if detectable)	Culture MiniIKE Gulf of St Lawrence Winter, 2006	*M. variabilis f. inornata*	1
M. ocellatus	6	30-40	-	-	Radial or spiral areolation. Polygonal areolae	Not indicated	*M. ocellatus*	6

Ø: Diameter; S.p.: satellite pores; FP: fultoportulae; RP: rimoportulae; Sour.: Source; 1. Kaczmarska et al. 2009; 2. Quiroga & Chrétiennot-Dinet 2004; 3. Aké-Castillo et al. 2001; 4. Ferrario 1988; 5. Takano 1981; 6. Gao et al. 1992; 7. This study.

The cells appeared as solitary forms or in pairs or aggregates. Valvae were circular, with 1.87-6.69 µm in diameter. There were marked differences in size between cultures and field samples, with average values of 4.36 µm and 2.58 µm, respectively. The mantle was not distinguishable from the valve face. In the valve central area there was a rimoportula, ornamented with a small tube or a ringed hole. There were three satellite pores at the base of each fultoportulae in internal view. Two different morphologies were found in cultures: convex (**Fig. 1**) and flat (**Fig. 2**). The first, with convex valvae, radial areolation, irregular areolas, three fultoportulae equipped with external tubes and a ring at the edge of the mantle, appeared more frequently in smaller cells. The second, which presented a flatter valva, 3-5 fultoportulae surrounded by rims, and ribs at the edge of the mantle, was more frequent in larger cells. There was not a

noticeable difference between the maximum and minimum size between the two morphologies, but the average size of the valva of the convex specimens was 2.32 µm whereas that of the flat specimens was 5.14 µm. Each culture seemed to have a vast majority of cells of one or other morphology, although cells with both morphologies and intermediate morphologies could be found in the same culture.

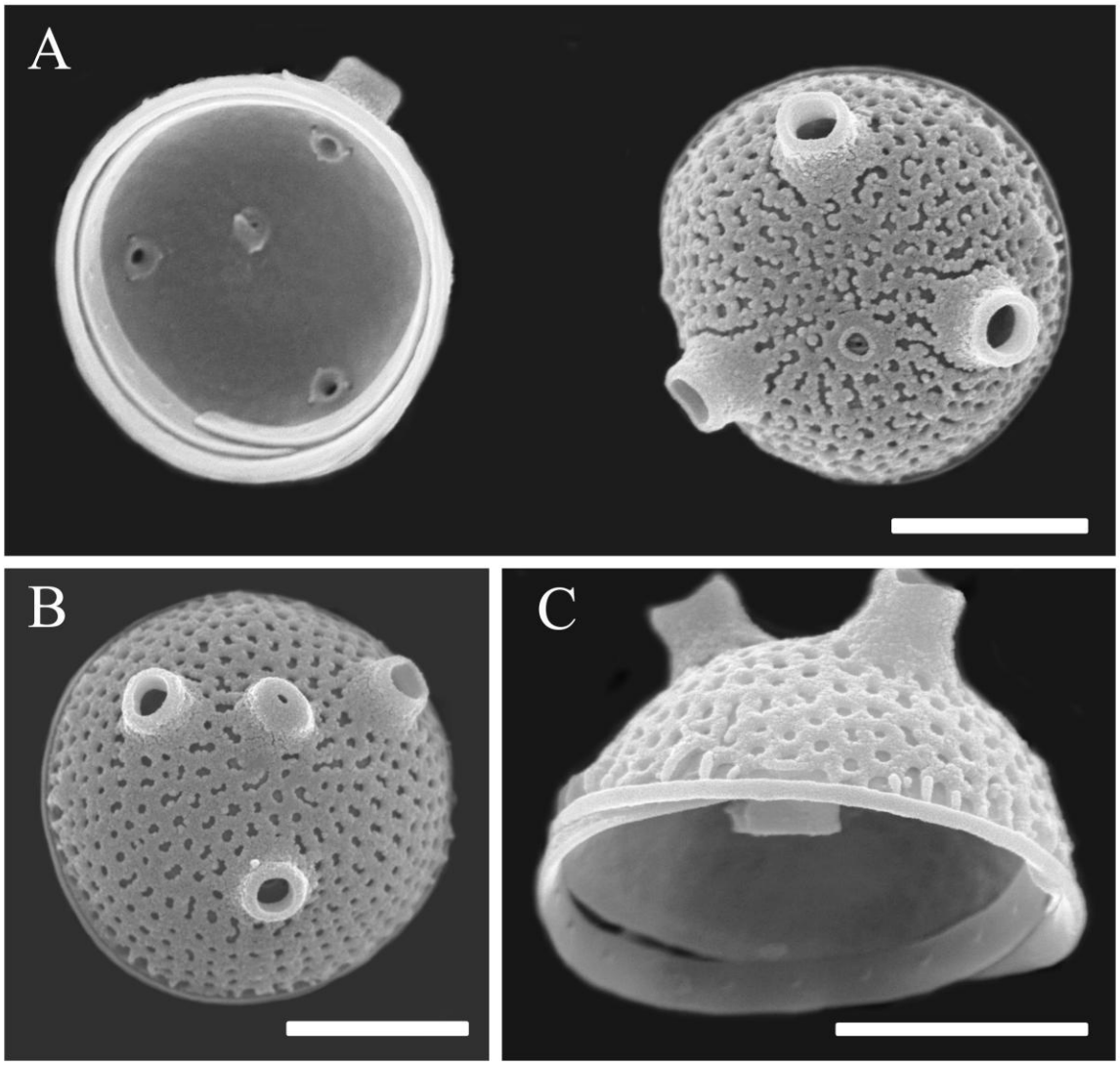

Fig. 1: A-C. Electron micrographs of valves of *Minidiscus comicus,* Morphology Convex, in internal (A), external valve view (B) and girdle view (C). Scale bar: 1µm.

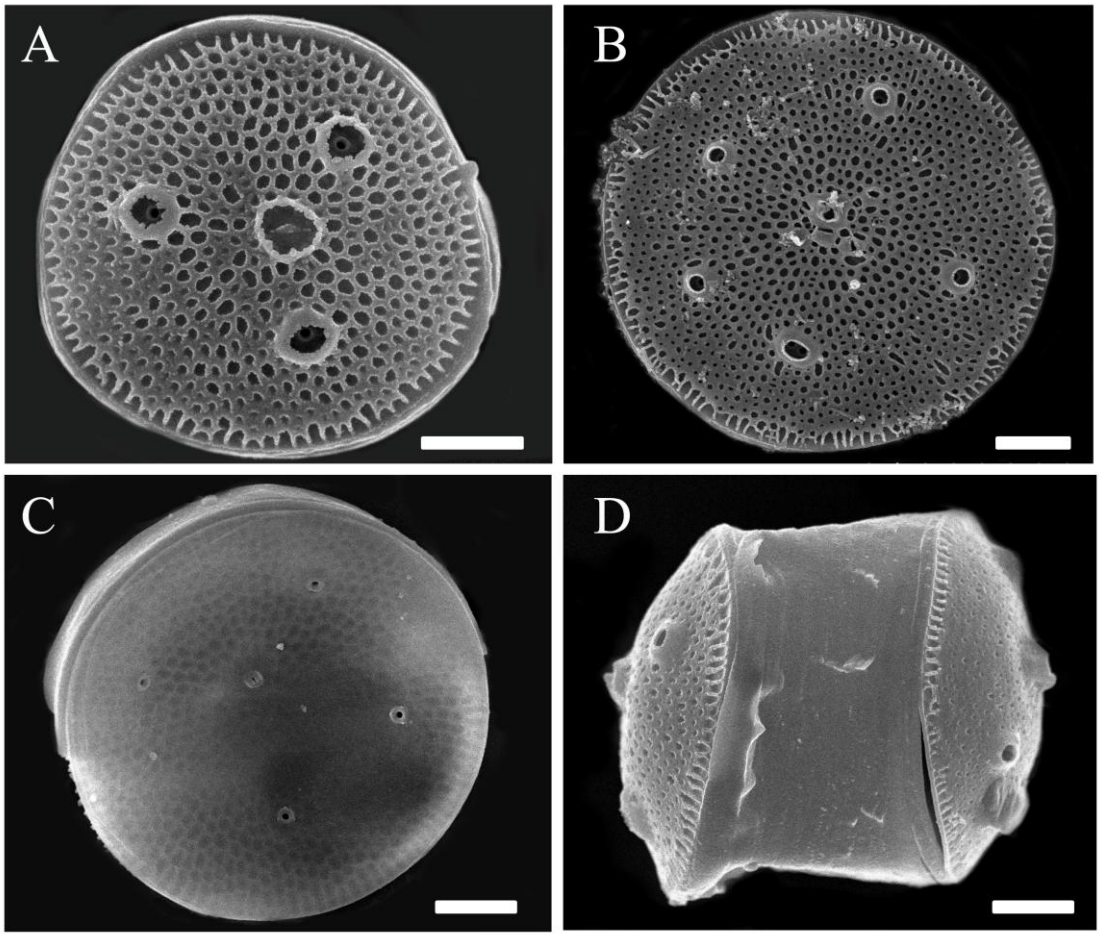

Fig. 2: A-D. Electron micrographs of valves of *Minidiscus comicus,* Morphology Flat, in external (A-B), internal valve view (C) and girdle view (D). Scale bar: 1µm

Molecular analyses and phylogeny

The resulting sequences for both markers, ITS rDNA and 28S rDNA regions, are detailed in **Table 2**. Only the ITS of the strain Bc50EHU could be used due to the presence of pseudogenes in the other analysed strains. On the other hand, there were not sequences of this marker for this species in GenBank as to build a phylogenetic tree. The seven nucleotide sequences analysed in this study were identical (identity=1) for the 28S marker, except Bc49EHU, which showed an identity of 0.9 with the other six sequences. For this reason, the strain Bc49EHU and other two sequences (Bc50EHU and Bc53EHU), which corresponded with the largest sequences obtained, were used to build the phylogenetic tree.

Table 2. ITS and 28S rDNA sequences.

Culture	Marker	bp sequence
Bc50EHU	ITS	TGCGGAAGGATCATTATCACAGTTTTATCCAACCTTTACTTCCCCGAGAAGAGGTGCCCGATGTTG CCTGCCGATACACTGTGCAGGGAGCGCCGGGTGCCAAACTTATATAAACCAAACCAGGAGCAAAG CGGGCCCCAGCACCTCGTGTGCTGGCCGGCTAGCCGAGCTCCGCCTCCATACTGAACTGAAACCTG TAACTGAATCTAAACTGCTTTTGGAGCCTCGTGCTCTTCTAGCTGTATAATCTATTACAACTTTTAG CAATGGATGTCTTGGGTCCCACAACGATGAAGAACGCAGCGAAATGCGATACGTAATGCGAATTG CAGAACTTCGTGAATCATCAAAATTTTGAACGCACATTGCGCTACTGAGTAATCTCAGTAGCATGC TTATTTGAGTGTCTCTAAACCCCACTCAGCCGCTGCTGCACTTGATTGTGCATGTGGCGTGCTGGA CTGTGATGGTGCTTGGCCCTGCTGCCGAGCTCCGTTCAAGTTCATTGGTTTGTTGTTGACGCCCTCG CTGGCTGAACGCAGATCGCAACTAGATTTGGTAGGTGCACTTTATGTGTATTTTGAATCTGGCTTG TGGCTTGTGTAGGCTTACTTGAGCGCCTTCCGCGAACTGGGCCTCCAGCATTACCTGCTGGCGGGT CCGGAATGAATGTACCTCATCTATGGATCTCAAATTAAGTAAGACTACCCGCTGAATTTAA
Bc50EHU Bc51EHU Bc52EHU Bc53EHU Bc54EHU	28S	TTTTTCCGCGTACTATTACGCGGAAAGATACAAGATTCCTGTACGGCGAGTGAAGCGGGAAGAGC TCACCATGTGAATCTCTATAACCAGCAATGGTATAGCGAATTGTGGTCTGGAGAAGTATTGTCGGC CGTGAATCCGGGCCAAGTCTCTTGGAAAAGGGCAGCTGAGAGGGTGAAACTCCCGTTCTTGCCTG GAATCATTGCGCTCTGGCACATGCTTTCTACGAGTCGAGTTGTTTGGGATTACAGCTCAAAGTATG TCGTAACTTCGATATAAAGCTAAATATTGGTGGGACACCGATAGTGTACAAGTACCGTGAGGGAA AGATGCAAAGAACTTTGAAAAGAGAGTTAAAGAGTACCTGAAATTGTTAAAAGGGAAGCGAAGG AAACCAGTGTTTGGCGAATCCATTCTTCCCAAGCTACTTGTAGTTTGGGCGCTGTGGTTAGCAATA GTCAGCATTGGCTTAATCTGGGTCAAACGTTTGGGTGGGTAGGCGGCTCTCGGAGTTAGTGCCCAT CTTTCATGCTCTGGATTGGGCTGAGGTCAGTCACTCTTGCTCGTGATGCTGCAAAACGGTT

Two main groups were obtained from the 28S rDNA phylogenetic tree with *Cyclotella atomus* as outgroup. The group A included strains of different genera in three subgroups: subgroup A1, which grouped different strains of *Skeletonema* (*Skeletonema* sp., *S. menzelii, S. pseudocostatum, S. japonicum* and *S. dohrnii*); subgroup A2, formed by a strain of *S. potamos*; and subgroup A3, which grouped strains from different species of *Minidiscus* (*M. spinulosus* and other clade which grouped the strains from *Minidiscus comicus*: the three strains from this study and other two strains from Gen Bank). The group B included three strains of *Minidiscus variabilis*, two from the culture collection of the UPV/EHU and isolated from New Zealand plus one from the Gulf of Maine (CCMP495).

DISCUSSION

Minidiscus comicus has been identified from samples taken at the Bilbao estuary after studying unialgal cultures of several strains of this species by means of morphological and molecular analyses. As can be observed in **Table 1**, only eight species of *Minidiscus* have been described to date and only a few of them have been cultured and the 28S rDNA marker sequenced. Two morphologies of *Minidiscus comicus* have been observed in our cultures: one convex and other flat. Both morphologies were described by Takano (1981), which associated the different morphology with the cell size. Meanwhile, although in most cases they are related, both morphologies can be found in cells of the same size. Kaczmarska et al. (2009) and Aké-Castillo et al. (2001) observed the same size range in the cells of *M. comicus* (2.1-3.2 µm and 2-3.5 µm, respectively) but, whereas Aké-Castillo et al. (2001) showed in **Fig 4** cells which matched with flat morphology, Kaczmarska et al. (2009) described in **Figs 3-6** cells strongly convex, despite the cells of **Fig. 6** shows a rather flat morphology.

Individuals corresponding to the two different morphologies showed the same ribotype when using the 28S rDNA as marker. The presence of cells from both morphologies in the same culture is an indicative that these morphological differences are not related with molecular differences, as the cultures were established from a single cell producing a unialgal (clonal) culture. It is well known that the ITS rDNA region is more variable and appropriate marker to discriminate at the species or even at the variety levels than 18S rDNA or 28S rDNA, providing a greater level of divergence when sequences are compared directly (uncorrected distances) (Kaczmarska et al. 2005). However, we were unsuccessful in obtaining most of the ITS sequences, due to the presence of pseudogenes, so we cannot corroborate this extreme. In absence of

molecular differences with the used marker, these morphological differences could be explained by differences in the life cycle stages of the cultures or the age classes of the cells, as was pointed out by (Jewson et al. 2016), which related these differences in morphology with the size restoration strategy. According to these authors, larger cells are flatter than the older smaller cells, which dome to maintain a major cellular volume to compensate for the diminution of cell size. Kaczmarska et al. (2005) observed differences in cultures of different phases, with solitary cells in the exponential growing phase and aggregates in stationary one of *M. trioculatus* cultures. Similar behaviour has been observed with *M. comicus* in which those authors also detected two ways of growing: solitary cells and 2-3 cells colonies. Kaczmarska et al. (2005) suggested that the morphological and genetic diversity within *Minidiscus* has not yet been fully explored, and that the taxonomic affiliation of these species requires critical re-evaluation and further sequencing of taxonomical informative genes in described species. In this study, the 28S rDNA marker was used to clarify the phylogeny of species within the genus but, unfortunately, there are few DNA sequences available in the data bases and few available cultures of species of *Minidiscus*. The results of the 28S phylogenetic tree revealed that *M. comicus* and *M. spinulosus* are genetically closer to *Skeletonema* species than to strains of *M. variabilis*. Kaczmarska et al. (2005) concluded that some species of the *Skeletonema costatum* complex as well as the morphologically distinct species *Fragilariopsis nana*, have fewer interspecific base pair differences than those recovered between two species of *Minidiscus*. These results could uncover that *Minidiscus variabilis* belongs to a different genus than *Minidiscus*. To prove the hypothesis that *Minidiscus* is composed by at least two different genera, other molecular markers and morphological traits need to be analysed in depth. Re-evaluation of the diagnosis of the genus seems necessary. Originally, the generic diagnosis

specifies the absence of a marginal ring of fultoportulae, which is irregularly scattered around the centre of the valve face. Also, each fultoportulae has a wide ring of silica at the base of the external tube, and there is a sub-central rimoportula (Kaczmarska et al. 2005). The species of the genus share these characteristics, which could belong to a common ancestor shared with *Skeletonema* species. Species of this genus might be lost these features during their evolution.

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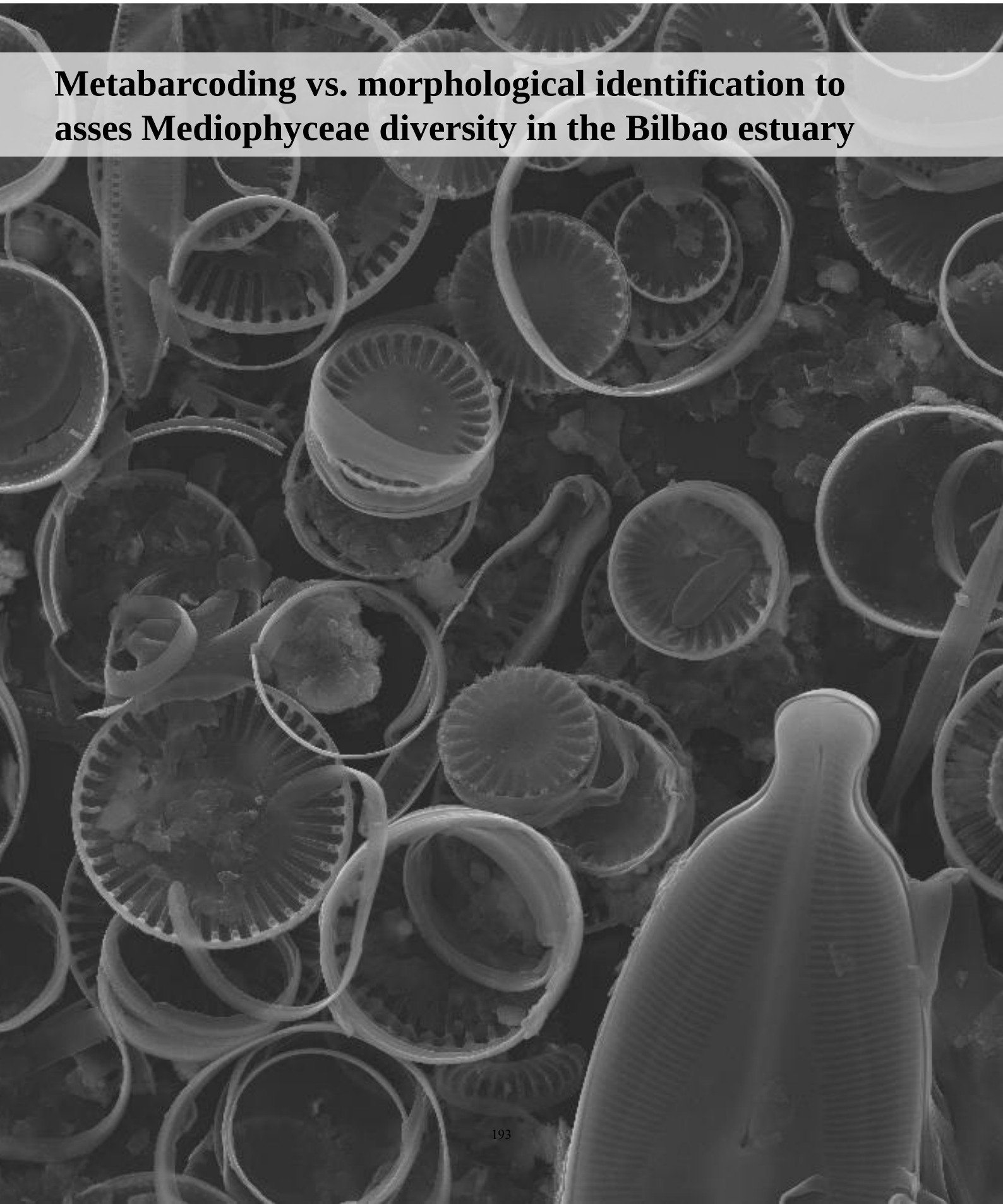
CHAPTER 6

Metabarcoding vs. morphological identification to asses Mediophyceae diversity in the Bilbao estuary



INTRODUCTION

Diatoms are highly sensitive to environmental conditions due to their short life cycle, which allows them to respond quickly to environmental changes in chemical, physical and biological features. This makes of the study of the whole diatom community present in a particular area, a good tool to detect environmental changes indicatives of different ecological conditions (Corell & Rodriguez-Ezpeleta 2014, Amorim Visco et al. 2015), Diatoms identification is time-consuming and requires a high level of taxonomic expertise and, due to that, molecular methods have been developed to overcame many of the difficulties encountered in the classical taxonomic identification based on morphological traits (Kermarrec et al. 2013). Barcoding of the rDNA has been used to identify diatoms to species levels, particularly in the case of cryptic species (Kaczmarska et al. 2005, 2009, Zingone et al. 2005, Sarno et al. 2007, Orive et al. 2013). This molecular analysis, based on Sanger-sequencing, requires a unialgal culture or a single cell analysis from field samples, which is time-consuming and expensive. Besides, only certain species can be cultured, what difficult the knowledge of the entire diversity of the selected taxonomic group. Recently, alternative sequencing strategies appeared, called "high-throughput sequencing" as they are 100-1000 times faster than Sanger-sequencing and cheaper (Kircher & Kelso 2010). The molecular analysis of the whole community of natural samples based on sequences of short rDNA regions (metabarcoding) has proven to be very useful for studying phytoplankton specific diversity (Logares et al. 2014), including diatoms (Nanjappa et al. 2014). The sequenced DNA regions should fulfil the following properties, following Moniz & Kaczmarska (2010): 1) they have to be conserved flanking fragments to facilitate the design of universal primers; 2) the sequence length should be obtainable in a single amplification; 3) they should be able to identify organisms at the species level.

In previous studies with diatoms, the selected target region was the V4 of the 18S rDNA, as this barcode was observed to separate 96.9% of all tested species of diatoms (Zimmermann et al. 2011, Luddington et al. 2012, Guo et al. 2015). Despite Moniz & Kaczmarska (2009, 2010) demonstrated that the 5.8S + ITS2 region of the rDNA was a better barcode in terms of feasibility in separating species as it had a 99.5% of success in the diatom species tested, this marker lacks of enough data sets. As to be able for metabarcoding. Thus, it is preferable to use a more universal marker, such as a variable regions of the 18S rDNA, with more complete data sets even though the success in identifying species is a bit lower.

In this study the Illumina Hi-seq technique, based on the V4 of the 18S rDNA barcode, has been used to know if *Mediophyceae* diatoms are the most abundant group in the Bilbao estuary as the use of barcoding presumed. In addition, details on the temporal and spatial differences in *Mediophyceae* species composition has been tested. To gain insight into the efficiency of the marker V4 18S to species identification, ultramicrographs of natural samples have been compared with the data provided with metabarcoding.

MATERIAL AND METHODS

Study area and sampling

The study was carried out in the Bilbao estuary (43º20'N, 2º70'W, Basque Country, Northern Spain). Three samples representing the outer, middle and inner segments of the estuary were taken in six different days along the period of October 2014-September 2015 (**Fig.1**).Ten litres of surface water were collected in hydrochloric acid-washed plastic carboys. Once in the lab, samples were prefiltered through a 45 µm

nylon mesh. The resulting sample was then filtered by pumping through a 3µm pore cellulose filter (Millipore, Billerica, MA) placed in a kitasato with a filter holder. Filtration time was kept to a minimum (30 min) to minimise RNA degradation. Two filters were obtained from each sample, which were removed from the filter holders and placed in a crystal tube and immediately frozen in liquid nitrogen and kept at -80 °C until RNA extraction.

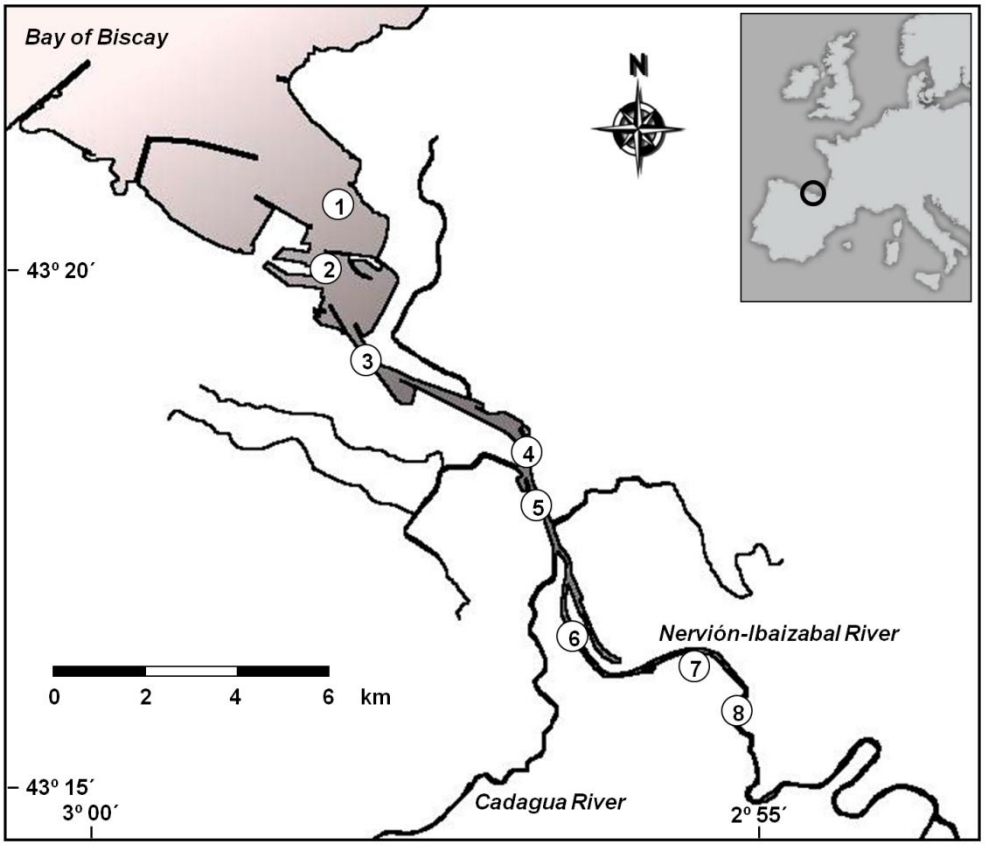


Figure 1. Study area with sampling sites 1-8. Inner estuary: 6-8, middle estuary: 4-5, Outer estuary: 1-3.

DNA extraction, PCR, and sequencing

Genomic DNA was isolated from filters with PowerWater® DNA Isolation Kit (MoBIO) and quantified with Qubit® dsDNA HS Assay Kit. Library construction was

performed following *Metagenomics sequencing library preparation guide* of Illumina, which includes one marker for 18 samples. The primers used for amplification of V4 18S were D512for / D978rev (Luddington et al. 2012), modified by adding the Illumina adapter sequences. Indexing of amplicons was performed in a second step using Nextera XT Index kit. Throughout the process, both extraction and amplification negative controls were added. The sequencing was performed on a MiSeq desktop sequencer (Illumina) using paired 300bp reads (MiSeq v3_600 cycles_2x300bp). To increase the genetic diversity, 30% of quality control genome (PhiX) was added to the final library. An output of 10 Gb was obtained, were 31% corresponds to PhiX.

Metabarcoding, OTU definition and taxonomic assignation

A total of 18 samples were analysed corresponding to 3 estuarine places corresponding to the outer, middle and inner segments and 6 different dates. Mothur v.1.36.1 was used to process the illumine reads. The reads (minimum size 220 bp) were quality checked by using a sliding 50 bp window. Only the windows that presented an average quality score over 20 passes the control and the sequences were trimmed before the quality of the sliding window dropped under the quality limit. The resulting sequences were made contigs and clustered into OTUS with QIIME. The method used was “open reference OTU”, which means that reads are clustered against a reference sequence collection and any reads which do not hit the reference sequence collection are subsequently clustered de novo. The threshold of identity chosen was 98%, and the sequences that did not overcome this threshold were assigned as “new reference OTU”. Each OTU was taxonomically assigned to a reference PR2 (Protist Ribosomal Reference) Data base. From this data the target groups (Main photosynthetic

phytoplankton, Bacillariophyta classes, Mediophyceae genera) were selected, analyzed and represented.

Scanning Electronic Microscopy

For ultrastructural analyses, aliquots of natural samples were rinsed and centrifuged to eliminate dissolved salts, after which the samples were boiled in acids (HNO_3 and H_2SO_4) to eliminate the organic matter. Samples for electron microscopy were filtered (Millipore TMTP, 1.2 μm pore size), rinsed with distilled water a few times before dehydrating with ethanol and finally rinsed with hexamethyldisilazane (HMDS 98º) for 2 min. Filters were stub mounted (Agar Scientific Ltd), coated with 10 nm of chromium and observed with a Hitachi S-4800 SEM. In this study, field samples were analysed for taxonomic identification.

RESULTS

OTU definition and taxonomic assignation

After the taxonomic assignation a total of 171767 OTUs and 4869221 reads were obtained. From them, the OTUs with less than 10 reads were eliminated. The main photosynthetic phytoplankton groups were selected, obtaining a total of 6971 OTUs and 20822654 reads. This data was rarefy, with the aim to standardized the number of reads by sample, in this case 115703 reads.

Taxa abundance by groups

The photosynthetic phytoplankton was classified by groups (Bacillariophyta, Cholorophyta, Chrysophyceae, Cryptophyta, Dictyochophyceae, Dinoflagellata, Haptophyta and Other Stramenophiles). Bacillariophyta was the most abundant group (**Fig. 2**), with 50.29% of the reads belonging to this group. The second most abundant group were the Chrysophyceae, with 25.5% of the reads. In decreasing order the abundance were the following groups: other stramenophiles (16.56%), Dictyochophyceae (3.03%), Chlorophyta (2.48%), Dinoflagellata (2.1%), Haptophyta (0.04%) and Cryptophyta (0.008%).

The most abundant group within the Bacillariophyta were the class Mediophyceae, which conformed the 93.28% of abundance of the group, followed by the Bacillariophyceae (4.84%) and Concinodiscophyceae (1.85%). The 0.03% of the reads where unassigned. The OTUS's number was also was much higher in the class Mediophyceae.

Species composition in the estuary

The number of reads of species of Mediophyceae presented in each estuarine area (outer, middle and inner) are represented in table 1. This table was obtained by adding the reads from the sequences that belonged to the same species, including sequences that were only identified at the genus level (i.e. *Skeletonema* sp. appeared several times and all them were represented together). As a result of this aggrupation, 88 different taxa were registered. From these, species with less than 100 reads after summing all the samples were considered not relevant in this study in comparison with the dominant taxa. Accordingly, the list of species was reduced to 53 taxa.

From the data of this table, the analysis of species by date and estuarine area was focused in dominant species (**Fig. 3**). For this propose, the 13 most abundant taxa, which corresponded with the sequences with more than 10,000 reads, were selected, and represented in the barplot.

The most abundant taxa in decreasing order are the following: *Cyclotella meneghiniana, Skeletonema* sp., *Cyclotella atomus, Skeletonema menzelii, Conticribra guillardii, Stephanodiscus* sp., *Chaetoceros* sp., *Chaetoceros muellerii, Thalassiosira pseudonana, Thalassiosira* sp., *Conticribra weissflogii, Cerataulina pelagica* and *Thalassiosira anguste lineata.*

Every taxon presented a temporal or spatial distributional pattern, with some of them presenting both temporal and spatial patterns (**Fig.3**). Species of the genus *Chaetoceros* were present in the outer estuary, where *C. muellerii* appeared during the warmer period (June and August). Nevertheless, *Conticribra guillardii* and *C. weissflogii* were present in the inner, middle and outer estuary, although the abundance of *C. guillardii* increased towards the outer estuary. This species was present during the whole year, blooming in March and April in outer estuary. *Cyclotella atomus* was other specie present in the whole estuary, and, although it did not follow a pattern of distribution along the estuary, was highly abundant in August and September. *Cyclotella meneghiniana* was present along the year, but its abundance could be negligible from October to April, increasing from June to September when it reached concentrations that made this species the dominant one in the inner estuary and also in the middle estuary in June. The abundance during summer decreased towards the outer estuary, where there was negligible. Among *Skeletonema* species, *S. menzelii* was present in August, September and October in the outer and middle estuary, whereas *Skeletonema* sp. was observed in June-August-September-October along the estuary.

Stephanodiscus sp. appeared in March and April, its abundance decreased from the inner to the outer estuary. Species of the genus *Thalassiosira* appeared from March to April or June, although they did not follow a pattern of distribution along the estuary.

Figure 2. Main phytoplankton groups, Bacillariophyta classes and Mediophyceae genera proportions in terms of reads and OTUs.

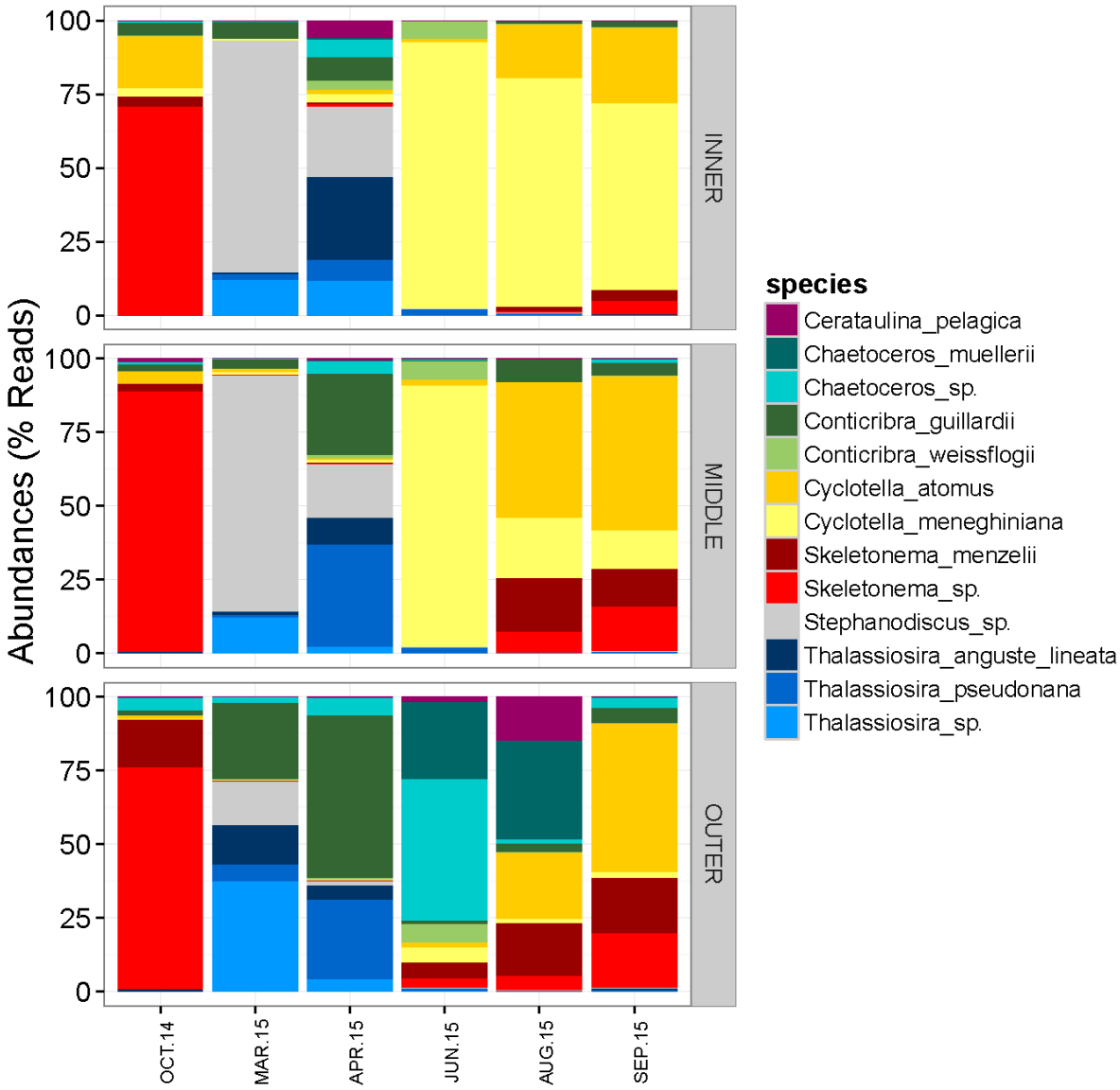


Figure 3. Barplot of the abundances (% of reads) of 13 most abundant Mediophyceae taxa in the three areas of the estuary.

Table 1. List of species and the number of reads (>50) present in each estuarine area.

SPECIES	INNER	MIDDLE	OUTER
Bacterosira sp.	145	<50	<50
Cerataulina pelagica	417	1161	11921
Cerataulina sp.	<50	146	463
Chaetoceros calcitrans	<50	56	207
Chaetoceros decipiens	<50	<50	663
Chaetoceros muellerii	117	289	38121
Chaetoceros pumilum	693	2260	5697
Chaetoceros sp.	887	3109	34914
Conticribra guillardii	7229	17978	35914
Conticribra weissflogii	5047	5068	3821
Cyclostephanos sp.	79	98	<50
Cyclotella atomus	40283	79419	63174
Cyclotella choctawhatcheeana	1287	1642	1211
Cyclotella cryptica	<50	55	<50
Cyclotella meneghiniana	158226	90505	5940
Cyclotella scaldensis	1860	1679	2077
Cyclotella sp.	360	967	1475
Cyclotella striata	456	292	167
Discostella pseudostelligera	166	121	<50
Discostella stelligera	<50	<50	98
Ditylum brightwellii	59	73	<50
Ditylum intricatum	903	2866	220
Ditylum sp.	<50	55	119
Eucampia sp.	<50	<50	857
Eunotogramma laevis	<50	<50	137
Lauderia annulata	<50	<50	4533
Minidiscus comicus	<50	82	239
Minidiscus trioculatus	740	1308	4090
Minutocellus polymorphus	67	653	666
Minutocellus sp.	408	2905	2771
Peridiniopsis penardii	299	266	79
Peridiniopsis sp.	998	559	94
Plagiogrammopsis sp.	<50	58	183
Plagiogrammopsis vanheurckii	<50	<50	183
Skeletonema costatum	<50	617	465
Skeletonema marinoi	<50	<50	932
Skeletonema menzelii	5495	26451	46486
Skeletonema sp.	55484	102305	86551
Stephanodiscus hantzschii	173	150	59
Stephanodiscus minutulus	82	82	<50
Stephanodiscus niagarae	58	<50	<50
Stephanodiscus sp.	25292	20683	4224
Thalassiosira allenii	<50	<50	74
Thalassiosira angustelineata	1759	2848	5996

Thalassiosira concaviuscula	85	100	642
Thalassiosira delicatula	<50	59	220
Thalassiosira gessneri	309	405	1055
Thalassiosira minima	65	69	140
Thalassiosira nordenskioeldii	864	769	3301
Thalassiosira oceánica	<50	90	609
Thalassiosira profunda	<50	<50	137
Thalassiosira pseudonana	3373	10038	12871
Thalassiosira sp.	4439	3094	10032
Unassigned	23	54	1396

Morphological data

The identity of the taxa was checked morphologically from ultramicrographs taken under the SEM and compared with the data obtained with metabarcoding. Only qualitative analyses were done, as some samples did not contain enough cells for reliable quantitative data. Besides, the final metabarcoding data from **Fig. 3** is a representation of relative abundance of taxa with more than 10,000 reads, which is not comparable with the total abundance presented in the field samples. Qualitative analyses effort was focussed in the taxa that was only identified at the genus level or taxa which creates disagreements in the identification by the different methods. Such was the case of *Minidiscus* and *Skeletonema* in samples from Oct.14. In metabarcoding, *Skeletonema* sp. appeared as the most abundant taxon, whereas under the SEM it was not detected, whereas several individuals of *Minidiscus comicus* were identified.

DISCUSSION

Relative abundance of Mediophiceae in the study area

Metabarcoding of natural samples confirmed the dominance of the class Mediophyceae in the Bilbao estuary, both in terms of abundance and number of OTUs,

what means a higher diversity comparing with other phytoplankton groups. The class Mediophyceae contained at least 20 genera in the estuary (**Fig. 2**).

Temporal and spatial distribution of species

The vast majority of species followed both a temporal and spatial pattern of distribution, appearing during a continuous period of the year preferably either in the inner or the outer estuary. Bazin et al. (2014) explained the spatial distribution of diatoms in an estuary based on the effect of salinity and tidal force. These authors grouped the Mediophyceae in "*Cyclotella*-like" species, locating them in the inner and middle estuary of the Baie des Veys (English Channel). However, the named *Cyclotella*-like species must be separated by genera or even by species, because species from the same genus can have different environmental preferences, do not appearing in the same area of the estuary. Thus, species such as *Cyclotella meneghiniana* were present in the inner estuary during summer and autumn, while species such as *Thalassiosira pseudonana* were present in the outer estuary in spring. The species composition of dominant Mediophyceae as revealed by metabarcoding matched with previous studies performed in the same estuary (Hevia-Orube et al. 2016). However, the metabarcoding approach provided greater insight into the community composition, covering even the identity of the low abundant species.

Comparisson between metabarcoding of V4 region (18S) and taxonomy by SEM.

By exhibiting strong similarity between the results of metabarcoding of region V4 from 18S and taxonomy based on SEM micrographics, the results demonstrated the

efficiency of metabarcoding techniques in analyzing Mediophyceae. Other studies on diatoms also reached this conclusion by sequencing the V4 region (18S(Zimmermann et al. 2015, Amorim et al. 2015) and other markers (Kermarrec et al. 2013). The majority of species were identified with both methods, although the species that were present in low abundance were very time consuming to find or even impossible by SEM microscopy. This was due to that the metabarcoding allows obtaining a higher number of retrieved sequences comparing with the few species counted by SEM, under which cells in low abundance were difficult to observe. In addition, identification by morphology is time-consuming and costly and requires a high level of taxonomic expertise (Mandelik et al. 2010). Special cases are that of morphologically cryptic species, which are impossible or highly difficult to identify by morphological characteristics, whereas they can be identified by molecular methods. This is the case of *Cyclotella cryptica* and *C. meneghiniana* (Beszteri et al. 2005, 2007). Besides, metabarcoding provides data on taxa relative abundances, while microscopy requires the identification cell by cell, making unable to estimate the abundance of the different taxa.

The main problem of metabarcoding was not the method by itself but the incompleteness and inaccuracy of databases. The sequences available in the banks of genes are not as reliable as expected due to that the species identification is not always correct or they are identified at taxonomic levels higher than that of species (Becker et al. 2011, Yoccoz 2012, Zimmermann et al. 2015). It is the case of OTUs that were identified at the genus level, such as *Thalassiosira* sp., which appear in the estuary in high abundance from March to April. This taxon was analyzed with detail under the SEM, which revealed that the group contained at least five different species: *T. Anguste-*

lineata, T. gravida, T. mediterranea, T. minima and *T. punctigera*. Each species of this group independently could not be dominant in the estuary, not appearing probably in the plot of dominant species whose threshold was established in 10000 reads.

The genus *Skeletonema* was not well resolved with the metabarcoding technique. Although the presence of the taxon identified as *S. menzelii* matched with both methods, the taxa identified as *Skeletonema* sp. in metabarcoding, grouped at least *S. dohrnii* or/and *S. marinoii* and *Minidiscus comicus* in microscopy Samples from October, August and September did not present individuals of *Skeletonema* others than *S. menzelii*, which appear well identified by metabarcoding. However these samples presented high number of cells of *Minidiscus comicus* whose relative abundance was much higher than the expected with data of reads of this taxon. This was very evident in the outer station in September, where the reads of *Skeletonema* sp. were 16574 and the reads of *Minidiscus comicus* 149. However, under the SEM, *Minidiscus comicus* was the most abundant taxon after *Cyclotella atomus* and there were not cells of *Skeletonema* different from *S. menzelii*.

Luddington et al. (2012) concluded that the V4 region could serve as an effective barcode for diatoms as it separated 96.9% of all tested species, but, nevertheless, they faced problems in the identification among species of *Skeletonema* and also between *Minidiscus variabilis* and *M. trioculatus*. Moreover, the genus *Minidiscus* presented a lack of molecular information in the data bases (Hevia-Orube et al., chapter 4 of this manuscript), where not all the species are represented with the marker 18S. The third group that presented troublesome in species separation by the V4 in Luddington et al. (2012) were those of the genus *Thalassiosira*, where three pairs of species were identical over the V4 region. Those groups of species are the same that presented problems in this study, due to that some revisions of the data bases need be

done, to discard that the bad identification of species was the cause of the bad results of the metabarcoding with these groups.

The water quality assessment using diatoms are based on indices which demand the identification at the species level (Zimmermann et al. 2015). Consequently, it is of great concern to improve metabarcoding with a combination of methods including barcoding of cultured cells and SEM taxonomy to complete data bases as pointed out by Amorim et al. (2015) for diatom strains, with the aim to filling the gaps in diatoms inventories. The studies done in previous chapters of this manuscript are necessary to complete the databases in order to gain insight into the species identification and to complete the databases of phytoplankton species.

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CHAPTER 7

General conclusions & Thesis

GENERAL CONCLUSIONS

In this study, several species of the class Mediophyceae were isolated and characterized morphologically and molecularly. Some of them were subjected to physiological experiments to know their response to different salinities. Besides, a metabarcoding study was done to identify the whole Mediophyceae community in the study area. Below, the main conclusions of the thesis can be found:

- Mediophyceae species identified by morphological and/or molecular analyses in the Bilbao estuary were: *Conticribra weissflogii*, *Cyclotella atomus* var. *atomus*, *Cyclotella cryptica*, *Cyclotella marina*, *Cyclotella meneghiniana*, *Discostella pseudostelligera*, *Minidiscus comicus*, *Skeletonema costatum*, *Skeletonema dohrnii*, *Skeletonema menzelii* and *Thalassiosira pseudonana.*

- From all these, bloom-forming species of solitary forms of Mediophyceae in the Bilbao estuary were *Conticribra weissflogii*, *Cyclotella meneghiniana*, *Discostella pseudostelligera* and *Thalassiosira pseudonana*. These species can form nearly monospecific blooms or blooms of a few species, and although a clear pattern was not detected in this study, *C. weissflogii* and *C. meneghiniana* appear as bloom formers in the inner part of the estuary whereas *D. pseudostelligera* and *T. pseudonana* are the most abundant species in the blooms of the middle estuary.

- The dominant chain-forming species in the estuary belonged to the genus *Skeletonema*, which is frequently found as dominant taxon during phytoplankton blooms. Three species were identified and physiologically analysed: *S. costatum*, *S. dohrnii* and *S. menzelii*. They can be characterized as euryhaline species, which can potentially grow actively along most of the salinity range of the Bilbao estuary.

- *Cyclotella Cryptica* and *Cyclotella meneghiniana* are considered by some authors as cryptic species, but, concerning the specimens from the Bilbao estuary, morphological analyses revealed the absence of overlapping features between both species at all the salinities tested, although *C. meneghiniana* showed marked morphological plasticity with salinity. Both species are clearly differentiated by the secondary structure of the ITS2, which revealed interbreeding incompatibility. Both species behave as euryhaline, growing with good physiological status along the whole salinity range of the Bilbao estuary, from fresh to marine waters.

- *Minidiscus comicus* showed a wider morphological variation than that previously described, with specimens belonging to two different morphologies, "convex" and "flat". This variation did not corresponded with differences in cell size, neither corresponded with molecular differences based on 28S region of the rDNA. The genetic diversity within *Minidiscus* has not yet been fully explored, and the taxonomic affiliation of these species requires critical re-evaluation and further sequencing of taxonomical informative genes.

- Mediophyceae is the most abundant group in the Bilbao estuary as assessed by metabarcoding. This approach allows us to gain insight into the taxonomic composition of the phytoplankton community better than microscopy does, uncovering even the identity of the species with lowest abundances. Mediophyceae was formed in the estuary by at least 20 genera. Some OTUs could not be identified at the species level due to that the differences in nucleotides base pairs were not well resolved in some taxa or that the databases were incompletes or incorrect.

THESIS

In the Bilbao estuary, Mediophyceae constitute the most abundant taxa. This class contains a great diversity of species belonging at least to 20 genera, underlying the discrete size-classes established to characterize the community by light microscopy. Within them, the six most abundant genera: *Conticribra, Cyclotella, Discostella, Minidiscus, Skeletonema* and *Thalassiosira* were studied in detail by means of molecular, morphological and/or physiological approaches. The species from these genera have been identified, and some of them have been established as potential blooming species in the study area.