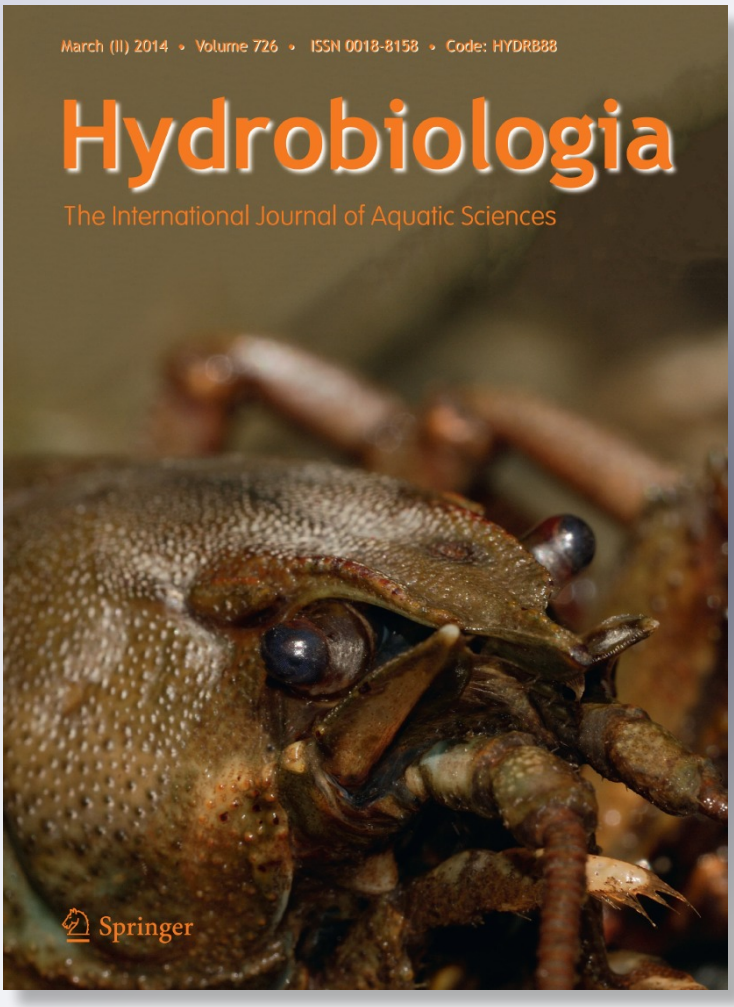
Complex patterns in phytoplankton and microeukaryote diversity along the estuarine continuum

Pauline Bazin, Fabien Jouenne, Anne-Flore Deton-Cabanillas, Ángel Pérez-Ruzafa & Benoît Véron

Hydrobiologia
The International Journal of Aquatic Sciences

ISSN 0018-8158
Volume 726
Number 1

Hydrobiologia (2014) 726:155-178
DOI 10.1007/s10750-013-1761-9



Springer

Hydrobiologia (2014) 726:155–178
DOI 10.1007/s10750-013-1761-9

PRIMARY RESEARCH PAPER

Complex patterns in phytoplankton and microeukaryote diversity along the estuarine continuum

Pauline Bazin · Fabien Jouenne · Anne-Flore Deton-Cabanillas · Ángel Pérez-Ruzafa · Benoît Véron

Received: 4 June 2013 / Revised: 16 November 2013 / Accepted: 18 November 2013 / Published online: 4 December 2013

Abstract Estuaries and coastal lagoons are included within the transitional waters category, according to the Water Framework Directive. However, criteria for their differentiation and characterisation are still under discussion and require more research. In particular, detailed observations of biodiversity in more complex transitional and coastal waters are lacking. Microscopic and molecular analyses were therefore used to investigate phytoplankton diversity and spatial community structure, in early spring, along the freshwater-to-marine continuum of the Segura River (Spain), an intensively regulated semiarid basin discharging into the Mediterranean Sea. In addition to the salinity gradient as the major factor determining taxa distribution, influence of multiple anthropogenic and climatic impacting factors (drought, confined waters, irrigation canal) leads to a significant spatial heterogeneity of the aquatic habitat types associated with variations in community composition. Several shifts within the phytoplankton distribution pattern along the continuum are revealed using multivariate analyses. An impressive bloom of the cryptophyte *Plagioselmis prolonga* occurred in the mixing zone, associated with a typical euryhaline community indicative of eutrophication. The 18S rDNA diversity revealed a microeukaryotic richness including several little-known groups, heterotrophic representatives, and potential parasites. By combining morphological and molecular approaches we revealed the presence of a 'hidden' diversity often neglected in traditional surveys.

Keywords Phytoplankton · Microeukaryotes · Diversity · Microscopy · 18S rDNA clone libraries · Estuarine gradient · Semiarid area

Electronic supplementary material The online version of this article (doi:10.1007/s10750-013-1761-9) contains supplementary material, which is available to authorized users.

Handling editor: Judit Padisak

P. Bazin (✉) · F. Jouenne · A.-F. Deton-Cabanillas · B. Véron
Normandie Université, Caen, France
e-mail: pauline.bazin@unicaen.fr

P. Bazin · A.-F. Deton-Cabanillas · B. Véron
BioMEA, SFR ICORE, UNICAEN, 14032 Caen, France

P. Bazin · A.-F. Deton-Cabanillas · B. Véron
FRE 3484, CNRS, 14032 Caen, France

F. Jouenne
UNICAEN, Algobank-Caen, 14032 Caen, France

Á. Pérez-Ruzafa
Departamento de Ecología e Hidrología, Universidad de Murcia, Campus de Espinardo, 30 100 Murcia, Spain

Introduction

Transitional and coastal water bodies such as estuaries and coastal lagoons represent complex environments linking freshwater and marine systems. Due to the

mixing of both distinct waters, they are characterised by pronounced gradients of physical, chemical components (McLusky, 1993). These factors strongly influence the communities and lead to changes in composition, structure and diversity of phytoplankton and other microbial eukaryotes along the resulting continuum. Inputs of nutrient-rich waters from the tributary rivers provide a high potential for proliferation of microorganisms that have developed tolerance to intermediate and fluctuating salinities.

The increasing human densities along with agricultural and industrial development have promoted the nutrient enrichment of the water bodies, and therefore contribute to eutrophication of many ecosystems (Smith et al., 1999). Given that more than half of the world's population currently lives within 60 km of the shoreline, this phenomenon is even more likely to affect the coastal areas. Phytoplankton, as major primary producer, is the first compartment impacted by the anthropogenic induced forcing factors, and because of its rapid responses to fluctuations of environmental conditions, its composition is then considered as a natural indicator of water quality (Livingston, 2001).

In order to avoid an increasing risk of biodiversity loss, the interest for documenting taxonomic richness in aquatic environments has increased in the last decades. As regards the aquatic transition systems, most of the phytoplankton surveys have been conducted in the well-known coastal lagoons and largest estuaries in the world. These investigations are generally based on microscopy with focus on the large phytoplankton fractions (Muylaert et al., 2009), or are restricted to a specific part of the transition zone (Lionard et al., 2008). Due to their position between the fields of interest of freshwater and marine scientists, water mixing areas have received moderate attention in ecological studies, and only few works emphasized the changes in phytoplankton diversity along a whole freshwater-marine gradient (Trigueros & Orive, 2000; Muylaert et al., 2009). The other members of the protistan community, i.e. heterotrophic protists, have been even less investigated in transitional waters (Muylaert et al., 2000; Lesen et al., 2010) in spite of their crucial role in aquatic ecosystems as important consumers of bacteria and small protists in the 'microbial loop' (Azam et al., 1983).

In recent years, the rise of molecular microbial ecology has revealed a high diversity of eukaryotic lineages, particularly the heterotrophic and/or small size protists (<3–5 μm), which usually escape detection with traditional microscopy and are difficult to isolate (Vaulot et al., 2008). These environmental surveys contributed to our current understanding of microbial food web structure in a wide variety of aquatic systems (e.g. Díez et al., 2001; Behnke et al., 2010). So far, very few diversity surveys using molecular techniques have been conducted on phytoplankton and other protists inhabiting rivers or marine–freshwater transition zones, except for recent studies restricted to one point following temporal dynamic (Vigil et al., 2009; Herfort et al., 2011).

The Mediterranean area is a hotspot for biodiversity of terrestrial (Myers et al., 2000) and marine species (Coll et al., 2010). The south-east of the Iberian Peninsula (Murcia and Alicante provinces) is of particular interest because of its semiarid climate (Peel et al., 2007) unique in Western Europe, and the richness of its associated endemic biota (Médail & Quezel, 1996). Nevertheless, the recent increase of anthropogenic perturbations and climate change could affect a great number of taxonomic groups inhabiting this region (Coll et al., 2010). Only a few investigations on freshwater phytoplankton/phytobenthos were performed in some streams of this area (Aboal et al., 2005; Ros et al., 2009). And although sporadic studies described the taxonomic structure of the marine phytoplankton in western Mediterranean Sea (e.g. Alborán sea: Delgado, 1990, Mercado et al., 2005; Balearic Sea: Estrada et al., 1999), few reports on the coastal waters of Alicante, Murcia (Gomis et al., 1987, Gras et al., 1991, Bouza & Aboal, 2008), and its coastal lagoon Mar Menor (Ros & Miracle, 1984; Gilabert, 2001) have been published.

Detailed observations of phytoplankton diversity in transitional and coastal water bodies in such semiarid and impacted environments are still lacking, which seriously limits our knowledge about ecological functioning of these complex systems. We investigated here the spatial distribution and the diversity of the phytoplankton community along a whole freshwater-to-marine continuum of the lower Segura River (province of Alicante). Data presented were obtained from a survey carried out during the late winter-early spring period, along a longitudinal gradient and in the water column of the coastal waters. This spatial investigation involved detailed taxonomic identifications of the nano-, microphytoplankton (>2 μm) and

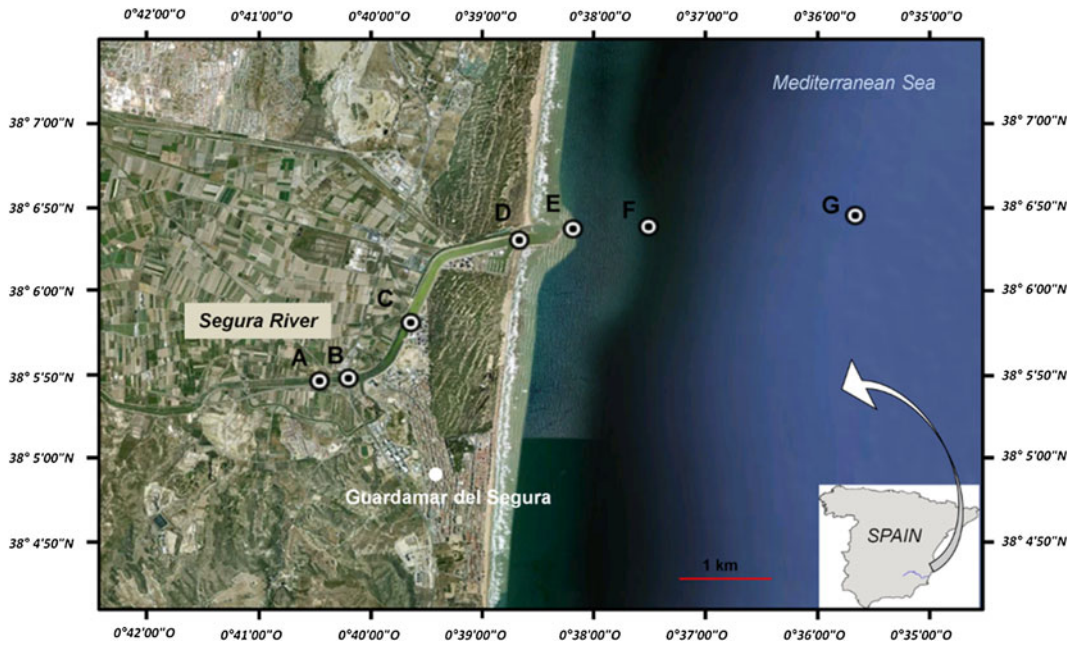


Fig. 1 Map of the Segura River—coastal zone continuum showing the seven sampling stations (*A*–*G*). (South-East Spain, coast of Alicante province)

in a lesser extent the heterotrophic nanoflagellates, by microscopy. We analyzed to what degree the distributional pattern was related to environmental variables. Genetic diversity of eukaryotes was for the first time explored in this site, through the construction of 18S rRNA gene clone libraries for three selected samples along the surface salinity gradient. This provided a first insight into the microbial eukaryotic diversity that can occur in such aquatic transition systems.

Materials and methods

Study area

The Segura River (Fig. 1) covers a surface area of about 18,870 km^2. It flows through the driest region of the Iberian Peninsula from the north-west to the south-east over a distance of 325 km. Within the area of its terminal part, mean annual temperature is around 18°C and the mean annual rainfall below 300 mm. The seasons are characterised by a long warm and dry summer and a temperate winter, both interrupted by spring and autumn rains. Intensive drought events occur during the summer season due to low rainfall and high temperatures (Confederación hidrográfica del Segura, 2007).

The Segura River is an intensively regulated basin, where many complex systems of irrigation ditches and reservoirs have been developed in order to prevent the great floods, but also to assure periodically irrigation of the fertile plain. The human activities modified the natural flow regimes of the river leading to important reduction in the magnitude of flows with droughts becoming more frequent and long-lasting (Vidal-Abarca Guitérrez et al., 2002). In its terminal part in the province of Alicante, a succession of cultivated areas and tourism infrastructures borders the Segura River (Fig. 1) that progressively broadens and deepens towards its mouth. The microtidal coastline (tidal range <1 m) and the coastal waters nearby the river mouth form a buffer zone between the inputs of nutrient-rich continental waters and the hypersaline and oligotrophic waters from the Mediterranean Sea.

Sampling strategy

Seven sampling sites (stations A–G, Fig. 1) were established covering the salinity gradient along the Segura River-mouth-coastal zone continuum and defined a 6.8-km long transect. The cruise was

conducted during high tide at the beginning of the wet spring period, on 2nd–3rd March 2011, ensuring the continuous flow of the river. Stations A to D were situated in the Segura River. Station A corresponded to the lower reach of the river, B and C to the brackish mixing zone, and D was located at the river mouth nearby the Guardamar port. The three other stations were situated in the coastal part of the transect, from E located near the river mouth and behind the seawall, to G as the farthest station from the influence of the river (~3 km from the river mouth). For all the stations, surface water was sampled at 0–0.5 m. From stations D to G, where depth was not too shallow, water samples were also collected at 0.5–1 m above the bottom with a 5 L Niskin bottle. Additional samples were collected at mid-depth for the deepest stations F and G. Each sample is thus designated by the subscript: s (surface), ½ (mid-depth) or b (bottom), following the corresponding station name: A–G (e.g. Bb refers to the bottom water sample of station B).

Physical and chemical measurements

Vertical profiles of temperature, salinity and turbidity were recorded in situ using a Hydrolab multi-parameter probe (Hydrolab Data sonde 5 Options, USA). Vertical profiles of irradiance (μmole photons m^{-2} s^{-1}) were obtained by measuring PAR levels (Photosynthetically Active Radiation) with an underwater quantum sensor (LICOR LI-1400, Lincoln, NB, USA). Inorganic nutrient analysis was performed with a *Bran + Luebbe* Autoanalyzer AA3 according to Aminot & Kérouel (2007). Suspended particulate matter was determined by standard weights measurements after filtering water through pre-combusted Whatman GF/F glass-fibre filters and drying at 70°C. Total particulate organic matter (POM) and particulate inorganic matter (PIM) contents were determined from dry weights before and after combustion (Aminot & Chaussepied, 1983).

Phytoplankton analysis

For phytoplankton biomass measurements, water subsamples were filtered over GF/F filters. After extraction in 90% acetone overnight, chlorophyll *a* concentrations were estimated by fluorimetry (Trilogy 7200-000, Turner Designs, Sunnyvale, CA, USA) according to Welschmeyer (1994).

First observations with light microscopy on living material were carried out to establish a preliminary floristic list. For further identification and counting, subsamples (200 ml) were rapidly fixed after collection with glutaraldehyde (final concentration 1%) and stored in darkness at 4°C until analysis. Phytoplankton was identified using appropriate keys for marine and freshwater environments (Bourrelly, 1981; Tomas, 1997; John et al., 2002) and by referring to literature from similar or surrounding studied areas (*vide super*). Further examination was made by scanning and transmission electron microscopy (SEM, TEM) using a JEOL JSM-6400 and a JEOL/JEM-1011. Cells were enumerated using the Utermöhl settling method (Edler & Elbrächter, 2010) with a Leica DMI3000B inverted microscope. Because phytoplankton density can vary considerably along the estuarine gradient, volume of samples and settling time were adjusted to ensure a complete sedimentation of the organisms (1:2–1:20 dilutions for samples from the mixing zone). The entire chamber bottom was examined at 200×–400× to complete the list of species. Taxa were quantified at 400× in randomly selected microscopic fields. A minimum of 30 microscopic fields and 500 individual units were counted, leading to a standard deviation of <10% (Lund et al., 1958).

Clone libraries and phylogenetic analyses

Three surface samples (As, Bs and Gs) representative of the lower river reach, the mixing zone, and the coast were selected for clone library analysis. 500 ml to 2 l of collected water was filtered on GF/F fibreglass filters (47 mm diameter) with no initial prefiltration and stored at −80°C until analysis. Total DNA was extracted using the Invisorb Spin Plant mini Kit (Invitek, Berlin, Germany) with modification of the first steps of the manufacturer's protocol. Briefly, each filter was aseptically cut into pieces and divided into two 2-ml cryotubes filled with 600 μl of lysis buffer. Cells disruption was achieved by thermal shock, i.e. three freeze–thaw cycles by plunging the tubes into liquid N_2 (1 min) and into water bath at 65°C (2–4 min). Then 30 μl of proteinase K was added to the lysate which was incubated for 1 h at 65°C to complete lysis. The remaining procedures were conducted according to manufacturer's instructions. Quantification and purity of genomic DNA was assessed by measuring UV absorbance with a

NanoDrop 2000 Spectrophotometer (Wilmington, DE, USA).

Eukaryotic 18S rRNA genes (≈1,800 bp) were amplified using the eukaryotic-specific primer set: EukA/EukB (Medlin et al., 1988). The PCR mixtures (50 µl) contained about 10 ng of environmental DNA as template, and final concentrations of 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of each primer, 1.25 units of Taq DNA polymerase (Promega) and 1× of the PCR buffer solution supplied with the enzyme. Reactions were performed at two different annealing temperatures: 55 and 50°C, and pooled. The conditions were as follows: an initial hot-start at 95°C for 10 min, followed by 30 cycles (denaturing at 95°C for 1 min, annealing during 1.5 min at 50/55°C, extension at 72°C for 2 min), and a final extension at 72°C for 10 min. For all samples, several replicates of PCR products were pooled and cleaned with the Wizard PCR clean-up system kit (Promega). Clone libraries were constructed using the pCR2.1 TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The presence of the 18S rDNA inserts in the putative positive colonies was confirmed by re-amplifying 1.5 µl of transformed cells using the flanking vector M13 primers. Positive clones were sent to Macrogen (Europe) for plasmid purification followed by partial sequencing with ABI 3730XL system (Applied Biosystems, Foster City, CA, USA). The internal standard primer 895R (5′-AAATCCAAGAATTTCACCTC-3′) that covers conserved and rapidly evolving regions was selected for sequencing and resulted in reads of about 700–900 base pairs.

All the sequences were manually checked, trimmed and edited using the SeqAssem software (Hepperle, 2004), and then compared to those available in public databases (GenBank) using the NCBI BLAST web application (Altschul et al., 1990). Potential chimeras were detected with the online software Bellerophon (Huber et al., 2004) and with KeyDNAtools (Viprey et al., 2008). After removal of low-quality sequences, metazoan sequences, and putative chimeras, the remaining sequences were aligned using MAFFT 6.9 software (Katoh et al., 2005). The resulting alignment was checked and corrected manually. Based on this alignment, the sequences were clustered into distinct operational taxonomic units (OTUs) with MOTHUR 1.13 (Schloss et al., 2009) using a similarity threshold of 98% that roughly corresponds to the genus/species level (Romari & Vaulot, 2004). Phylogenetic trees including additional selected sequences from both GenBank and ARB databases were reconstructed using the maximum likelihood method (ML) with MEGA version 5. Bootstrap support values (BP) were calculated from 100 replicates.

Sequences reported in this paper have been deposited in the GenBank database under accession numbers KC911732–KC911802.

Diversity indices and statistical analyses

For the morphological approach, richness/diversity indices were calculated from cell counts and floristic lists using PAST software (Hammer et al., 2001). Hill's diversity numbers of the order 0, 1 and 2 (Hill, 1973) were calculated for each sample. N0 is equal to the total number of species in the sample (=species richness). In our case, all the taxa identified were considered, even though they couldn't be identified at the species level. It is then more appropriate to speak about 'Taxa' richness. N1 is the exponential of the Shannon–Wiener diversity index (e^H) and gives an indication about equitability. N2 is the reciprocal of Simpson's dominance index (1/D). Taxa richness and Shannon index for the molecular data were estimated with MOTHUR, based on OTUs defined at 98% sequence similarity level.

Multivariate statistical analyses in PAST were applied to environmental and phytoplankton data to identify spatial trends or discrete groups within the river-coastal transect. Principal component analysis (PCA) was performed to group samples according to the environmental variables (standardised and $\log(x + 1)$ transformed). Correspondence analysis (CA) was applied to phytoplankton abundance (square-root transformed). Taxa considered for the CA analysis contributed >1% of the total abundance in at least one sample or were found in at least five samples along the transect, corresponding to a data set of 49 taxa. A cluster analysis was also performed but on the original data set (94 taxa) using the Bray–Curtis similarity index, in order to identify the different groups of samples. Correlations between all variables (environmental and phytoplankton data) were investigated and their significance calculated through the Spearman's rank correlation coefficient (non-parametric).

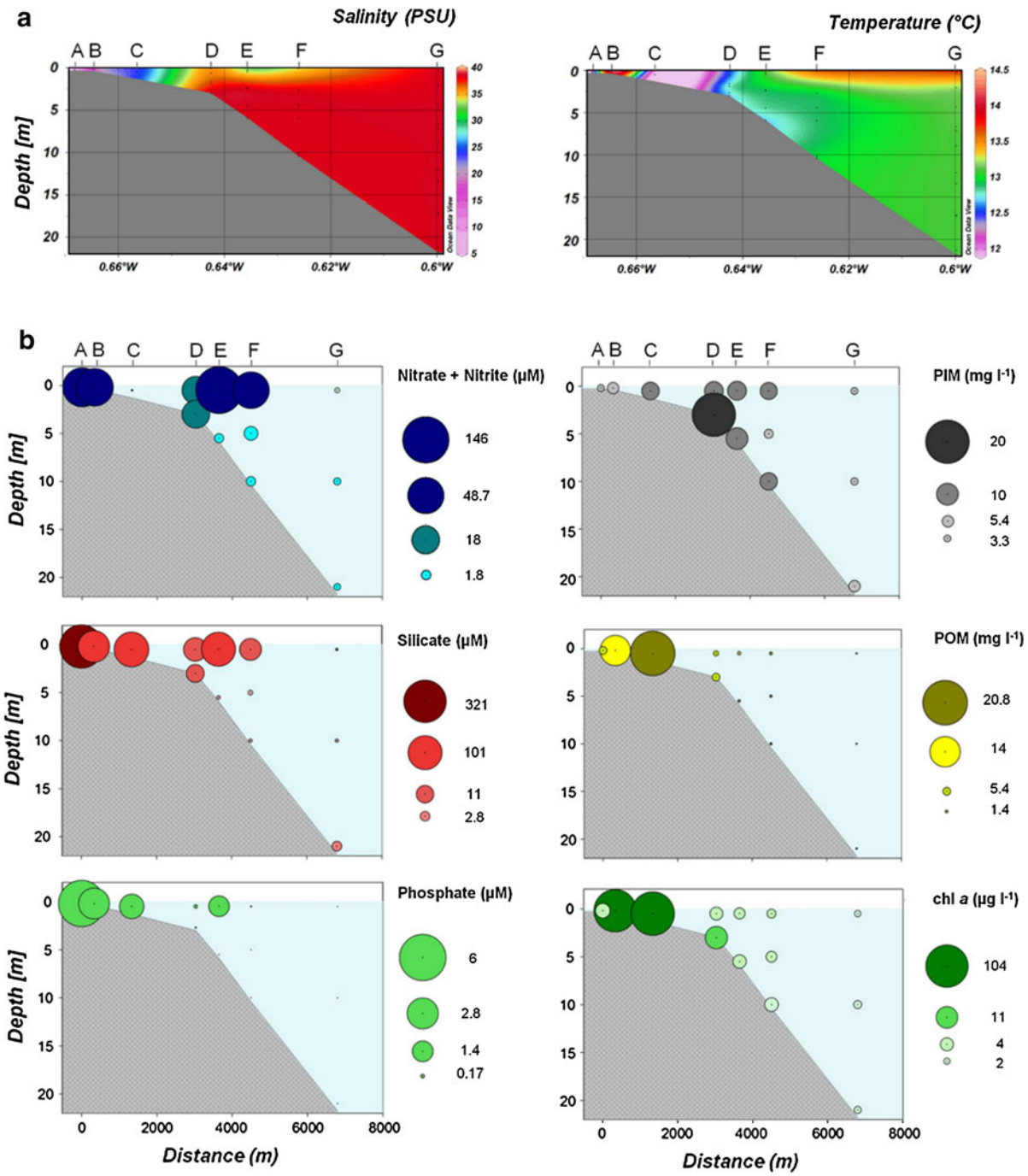


Fig. 2 Main physico-chemical and biological variables along the continuum from the Segura River to the adjacent coastal waters ($A \rightarrow G$). **a** Vertical profiles of salinity and temperature recorded in the water column with the multi-parameter probe. **b** Profiles of the other variables (nutrient concentrations, chl *a*, organic and inorganic particulate matter POM, PIM) analyzed in the samples collected in the water column (A logarithmic scale was used for the representation of nutrients and chl *a* measurements)

Results

Environmental parameters

Spatial variations of main environmental variables along the Segura River—coastal zone continuum at the time of the sampling are shown in Fig. 2. Salinity values varied between 4.5 psu (oligohaline) at the station A to 40 psu (hyperhaline) at G (Fig. 2a). The Segura river watershed is a naturally saline system (≥3 psu), which explains the high salinity observed in the lower river reach during our study. Station D just located at the river mouth was already hyperhaline (36 psu at surface) and the brackish stations B–C located 2–3 km upstream from the mouth were polyhaline (19–23 psu), overall highlighting the very low discharge of the river together with the seawater intrusion leading to the phenomenon of salinisation, also associated with the semiarid climate (evaporation). In the coastal part of the transect, water column was rather homogenous (38–40 psu). Nevertheless, a slight stratification was observed in the surface layer of E (33 psu) extending up to F (34 psu) because this zone receives inputs from riverine adjacent waters (discharge of an irrigation channel).

The other physical and chemical variables showed great differences along the continuum, contrasting with the typical gradual change from the river to the sea as expected in estuarine systems. Vertical differences were observed within the water column, although less pronounced seaward.

Water temperatures ranged from 11.6 to 14.2°C with intermediate values for coastal waters (13–13.5°C). The high temperature found at B (14.2°C) sharply contrasting with the surrounding stations A (11.9°C) and C (11.6°C) was explained by the local topography, which defined a small shallow basin with stagnant conditions resulting in warmer waters. Nutrient concentrations except nitrate were highest at the uppermost station A (>300 μM for silicate, >6 μM for phosphate) and decreased towards the river mouth (Fig. 2b). The input of nutrient-rich water at the surface of E resulted in a sharp increase in the concentration of all nutrients, and particularly nitrate/nitrite (NOx), which reached a maximum concentration of 146 μM, that is more than twice as much as in the upper river station A (63 μM). Then, nutrient levels decreased drastically seawards with lowest values at the surface of G.

Chlorophyll *a* concentrations (chl *a*) used as proxy for phytoplankton biomass, and particulate organic matter (POM), showed the same spatial pattern with significant variations along the continuum. The highest chl *a* and POM concentrations were observed at the polyhaline stations B and C with values around 100 μg l^{-1} for chl *a* and 15–20 mg l^{-1} for POM, indicating a massive phytoplankton bloom. Then, concentrations showed a decreasing seaward gradient with values always lower at the surface than at the bottom. The lowest values were recorded at the surface of G with 2 μg l^{-1} for chl *a* and 0.7 mg l^{-1} for POM.

Particulate inorganic matter (PIM) showed an increasing pattern from the uppermost station A (3.3 mg l^{-1}) to the river mouth in the station D, where the highest level of PIM was reached at the bottom (20 mg l^{-1}). PIM levels were still high in the vicinity of river mouth (E = 9–10 mg l^{-1}) before decreasing seaward (G = 3.3–5 mg l^{-1}).

The PCA analysis provided a good description of the environmental structure with the first three components (PC) together explaining 83% of total variance (Fig. 3). The first axis (PC1) illustrates the natural estuarine gradient, with $Si(OH)_4$ and PO_4 as main positive contributors, while salinity loads negatively. A group of typical marine samples is on the left part of the diagram, characterised by high salinity and low nutrient concentrations. It includes the bottom coastal waters (samples Eb, Fb and Gb), and all the samples from the station G (Gs, $G_{1/2}$, Gb), indicating the well-mixing of the water column at this location. The other samples are spread out on the diagram, reflecting the great spatial variations of the environmental conditions along the continuum, which are added to the natural salinity gradient. Supporting this interpretation, PC2 is positively linked with the variables PIM, chl *a* and POM, and negatively with the temperature (T °C). This component is mainly influenced by the particular characteristics of Cs and Db. Both were characterised by a high turbidity due to high concentration of suspended mineral matters (PIM) for Db at the river mouth, and mainly due to the occurrence of the phytoplankton bloom for Cs (chl *a*, POM and total phytoplankton abundance are strongly correlated, Spearman's coefficient: $r_s = 0.92$, $P < 0.05$). PC3, still significant, is mainly associated with the strong variability of NO_3 levels, especially its depletion at Cs (Fig. 2b) due to consumption by the massive phytoplankton bloom, in contrast to the sharp increase at Es due to the nutrient-rich water inputs at this location.

These water inputs from an irrigation channel close to the river mouth explained the position of Es (and in a lesser extent Fs) in the right part of the diagram, nearby the river station A.

Phytoplankton composition and abundance

A total of 202 taxa were observed along the whole transect (Floristic list in Table S1—Supplementary material). Diatoms (Bacillariophyceae) were the richest algal group with 98 taxa. Other algal groups were Dinoflagellata (40), Chlorophyta (22), Haptophyta (14), Cryptophyta (6), Euglenozoa (4), Chrysophyceae (4), Dictyochophyceae (4) and Xanthophyceae (2).

The highest number of diatom species was found in the vicinity of the river mouth (D–E) with 57–67 different taxa. Diatom richness decreased towards the coastal waters (F–G) but also upriver (A–B–C) where it was lowest (8–14 species). The highest species richness among dinoflagellates was found in the bottom waters of E and F (22–24 species) with a high number of *Protoperidinium* species and various unarmoured species. This richness significantly decreased towards the farthest coastal station (G), where only 3–7 species were observed. The third richest group of taxa was the Chlorophyta, especially found in samples As and Es (12 species in both samples).

Fig. 4 Spatial distribution of the main phytoplankton groups (cell ml^{-1}) along the Segura River-coastal zone continuum, in the surface (*left*) and near-bottom (*right*) layers of the water column. The different groups, from top to bottom, are presented in descending order of abundance (Note the differences in scale on the *y* axis)

Total cell abundance along the estuarine continuum (Table S2) was highest in the brackish stations B and C reaching bloom concentrations (maximum 181 $\times$ 10^3 cells ml^{-1}), then it decreased seaward with the lowest phytoplankton abundance found at Gs (2 $\times$ 10^3 cells ml^{-1}). In addition, vertical variations were observed in the column water of all euhaline and hyperhaline stations (D–G) with total cell densities always increasing with depth.

Cell abundances along the whole transect (Fig. 4) showed clearly different patterns of distribution depending on the taxonomic groups. In the uppermost oligohaline zone (As), the small thalassiosiroid diatoms (±5 µm), almost exclusively composed of *Thalassiosira pseudonana*, were largely the main contributor of the phytoplankton riverine community (90%) reaching here their maximum abundance

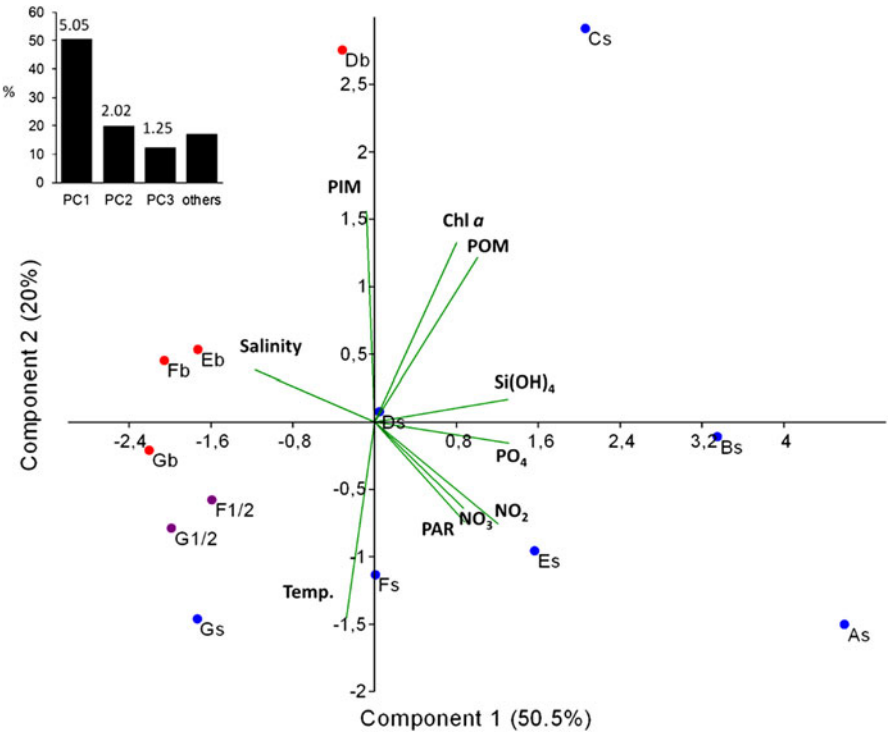
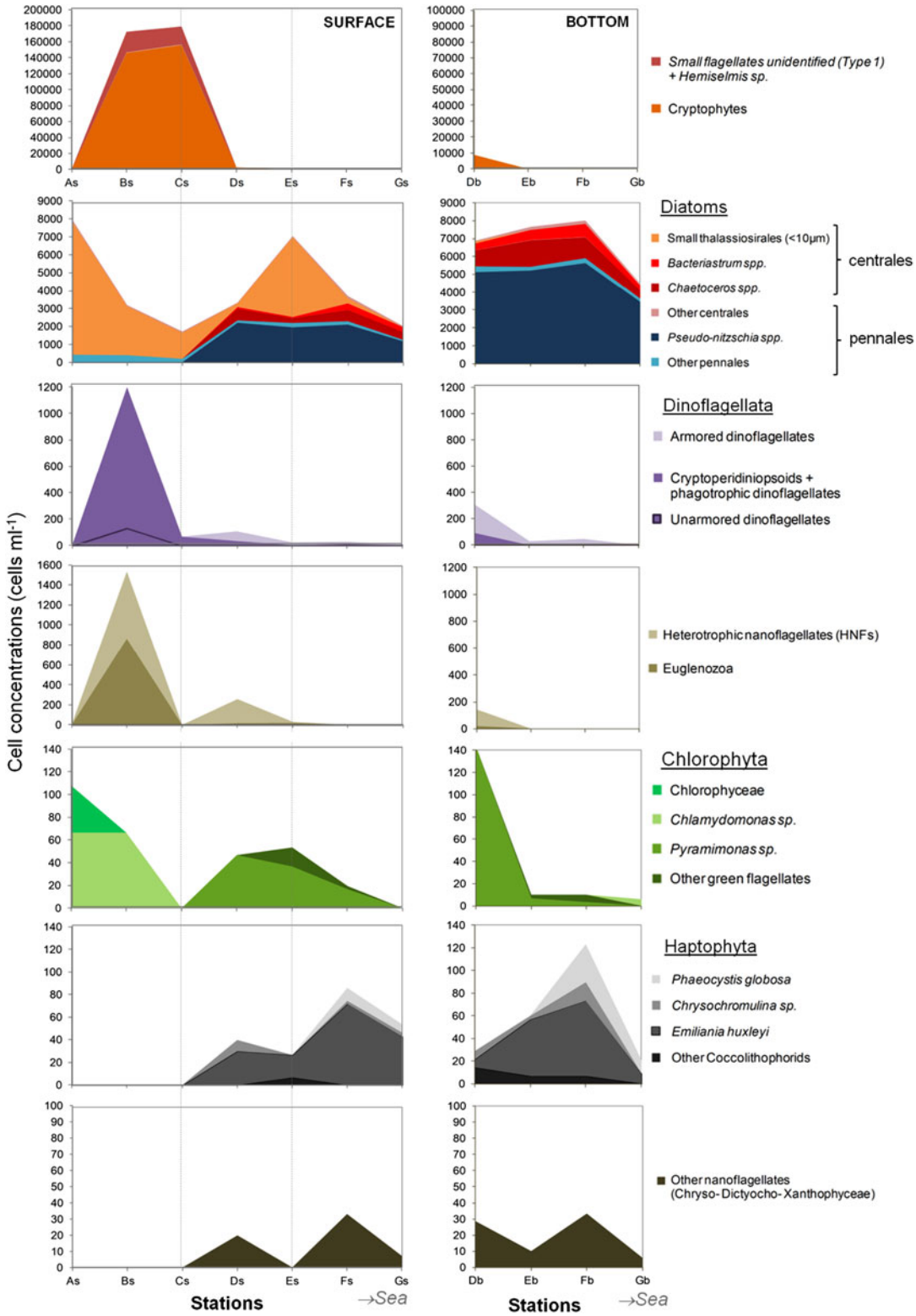


Fig. 3 Principal component analysis (PCA) of environmental data. The ordination diagram shows position of the samples (*dots*) and major variables (*green lines*) in the space defined by the first two PCA axes. Proportion of variance (%) explained by each PC and eigenvalues of the correlation matrix are indicated on the histogram (*top left*). *Blue dots* correspond to surface samples (As → Gs), *purple dots* to mid-depth samples ($F_{1/2}$ and $G_{1/2}$), and *red dots* to samples of the near-bottom water layer (Db → Gb). *PIM* particulate inorganic matter, *POM* particulate organic matter, *Temp*. temperature, *PAR* photosynthetically active radiation



(7.4×10^3 cells ml^{-1}). The Cryptophyta, including *Cryptomonas* spp. and *Plagioselmis*-like taxa, was the second most abundant group in As, although it only contributed to 2.3% of total species (Table S2). The Chlorophyta represented 1.3% of total riverine phytoplankton and included especially *Chlamydomonas* species (Fig. 4, Table S2).

Polyhaline stations B and C were characterised by a phytoplankton bloom with Cryptophyta as dominant group (Fig. 4), mainly composed of *Plagioselmis prolonga* accounting for 86.5% of the total community in Cs (Table S2), with 156×10^3 cells ml^{-1}. Small cryptophytes such as *Hemiselmis* sp. and small unidentified flagellates (3–5 μm) were associated with this bloom, showing a similar pattern but with lower number of cells. Bs differed from Cs by the presence of other taxa in relatively high concentration associated with the *P. prolonga* bloom. Among them, the heterotrophic/phagotrophic dinoflagellates 'Cryptoperidiniopsoids' or *Pfiesteria*-like showed an increase in cell concentration restricted to Bs and reaching 1.1×10^3 cells ml^{-1} (Fig. 4). Similarly, a peak of abundance was observed for the euglenophytes (*Euglena/Eutreptiella* sp., 860 cells ml^{-1}) and the heterotrophic nanoflagellates (HNFs, 667 cells ml^{-1}) with *Katablepharis* sp. (Cryptophyta *incertae sedis*) as major representative (Fig. 4). Cell concentration of these three groups decreased in Cs. Along with this, abundance of chlorophytes and small thalassiosiroid diatoms decreased considerably from As to Cs, so that the bloom of *P. prolonga* in Cs was almost monospecific.

Composition of the phytoplankton community changed towards the Segura River mouth, by the apparition of many marine species (Table S1) leading to higher diversity together with changes in abundance of the main taxonomic groups. In the station D, the diversity and abundance of diatoms increased (Fig. 4) with the pennates more abundant than the centrics, and especially *Pseudo-nitzschia* spp. accounting for 66–75% (Ds–Db) of the diatom community. The increase in total diatom abundance was more remarkable at the bottom of the water column, since cell concentrations doubled with depth (from 3.3 to 6.9×10^3 cells ml^{-1} at Ds and Db, respectively). The same pattern was observed for the Chlorophyta and Dinoflagellata (Fig. 4). The genus *Pyramimonas* sp. was the exclusive representative of Chlorophyta, reaching 140 cells ml^{-1} in Db, and armoured taxa were most abundant among the dinoflagellates, dominated by *Heterocapsa rotundata* (207 cells ml^{-1} in Db) and rich in *Protoperidinium* diversity. Proportion of mixotrophic and heterotrophic taxa was more important at this location than the others (Table S2), reaching 5.8% of the total community at the surface (Ds). Some prymnesiophytes, mainly *Emiliania huxleyi*, and other nanoflagellates (e.g. *Apedinella spinifera,* Dictyochophyceae) appeared at this station (Fig. 4). However, the cryptophyte *P. prolonga* remained the most abundant representative of the community with contributions of 39 and 54% at Ds and Db, respectively (Table S2).

Disturbance in the natural change in the species assemblages occurred at station E. Inputs from riverine adjacent waters (irrigation channel) resulted in a local change in the phytoplankton composition, such as an 'artefact' in the natural estuarine gradient, and were responsible for significant differences between surface and bottom of the water column (Figs. 2, 4). Allochthonous riverine taxa were identified at the surface (Es), including some Chlorophyceae (Table S1), but the most noticeable were the small thalassiosiroids (Fig. 4), composed of *T. pseudonana* and *Cyclotella meneghiniana*, dominating the whole surface community with a contribution of 66% (Table S2).

The phytoplankton assemblages found in the bottom layer (Eb) and the remaining coastal samples (stations F and G) were characteristic of marine communities. Diatoms dominated with *Pseudo-nitzschia* spp. as the most abundant taxon (reaching 75% of total phytoplankton abundance in Gb), and cell numbers of dinoflagellates and chlorophytes decreased towards the open sea (Fig. 4). The Haptophyta were the second most important group in this part of the continuum, and cell abundance was highest at F reaching 123 cells ml^{-1} in the bottom waters (Fb). This group was dominated by the coccolithophorid *Emiliania huxleyi* among the 14 other taxa identified (Table S1). Cell concentrations of all taxonomic groups decreased towards the farthest coastal station G.

Diversity indices along the estuarine continuum

The taxa richness N0′ obtained from the floristic list, thus including the 'rare' species, and N0 determined during the cell count display the same pattern (Fig. 5a): the number of taxa varied significantly

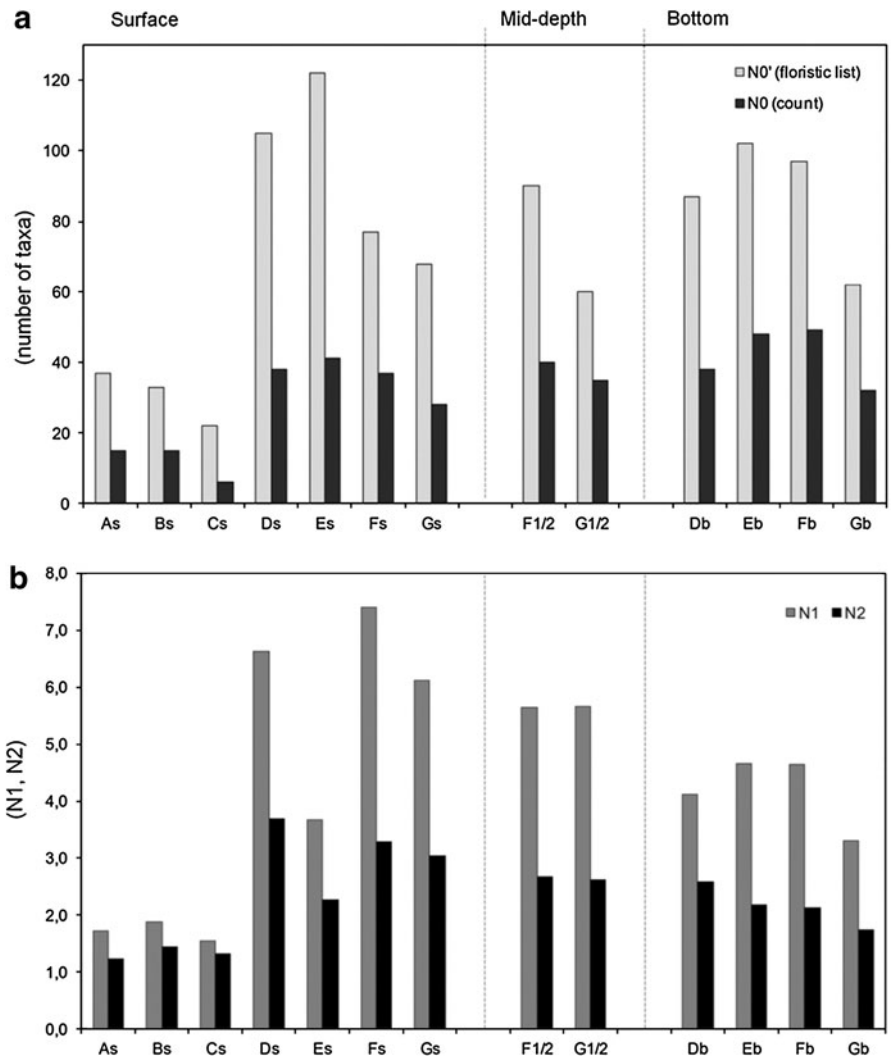


Fig. 5 Hill's diversity numbers N0, N1 and N2 for the sampling stations (A → G) along the estuarine continuum. As → Gs corresponding to surface samples, $F_{1/2}$ and $G_{1/2}$ to mid-depth samples, and Db → Gb to samples from the bottom. **a** Total number of taxa (=taxa richness). With N0′ as the taxa richness obtained from the floristic list established after detailed observations of each sample, whereas N0 is the taxa richness revealed during the process of cell count (randomly-selected fields). **b** N1 as the exponential of the Shannon–Wiener diversity index and N2 as the reciprocal of Simpson's dominance index

along the whole transition zone, but more moderately within the water column in the coastal zone, and the lowest richness was found in the upstream section including the riverine and brackish stations A–B–C. The minimum was found in Cs with 22 taxa identified (N0′), of which only 6 appeared in the cell count (N0). The highest taxa richness were found in the hyper-euhaline stations D-E, and more particularly at the surface with a maximum of 122 taxa observed in Es (N0′). Then, values of taxa richness tended to decrease progressively towards the open sea (F–G).

The comparison of N0′ and N0 (N0′–N0) highlights the significant presence of 'rare' species in the whole area, which contributed considerably to the phyto-plankton composition in the samples: between 42 and 73% of total species. Their maximum proportion was found in the middle part of the transect at the surface of C–D–E, then it decreased seawards. This indicates the presence in the samples of a minority of species with high abundance that dominated the community. In total, only 5 taxa among the 202 identified, but grouped at the genus level, contributed >10% of total species.

N1 and N2 displayed the same pattern (Fig. 5b) and were similar to N0 and N0′: overall diversity was lowest in the riverine community (A) and in the zone of the cryptophyte bloom (B–C), whereas it was highest in the marine samples and particularly in the surface waters with a maximum in Fs. This implies a more uniform distribution of individuals among the species together with higher richness in the marine community. However, a clear decrease in the diversity

indices restricted to Es highlights the effects of the disturbance in the community composition due to the allochthonous inputs at the surface. This indicates that the high taxa richness observed in this location (Fig. 5a) is mainly attributed to the presence of a large number of allochthonous and 'rare' taxa, while only a few species (thalassiosiroids) locally dominated the community. Except for this disturbance, overall diversity in the marine part of the transect tends to decrease progressively towards the open sea, and with depth.

Multivariate analysis of phytoplankton community

The general CA ordination in Fig. 6a shows the samples from As to Es widely spread out on the diagram, indicating that the taxonomic composition changed considerably within the upstream part of the transect including the lower river reach/mixing zone/ and river mouth. This heterogeneous distribution of samples on the diagram indicates the lack of a typical gradual change in phytoplankton composition along the estuarine gradient, although a salinity gradient can be depicted from the upper-right corner to the down-left corner. Sample Es, although euhaline (33 psu), is plotted close to As (4.5 psu) due to similarities in species composition after receiving riverine inputs (predominance of the small thalassiosiroid diatoms). Bs and Cs of the mixing zone are clustered in the right bottom portion of the biplot, both presenting almost the same community (Bray–Curtis similarity = 90%) dominated by the bloom-forming species *P. prolonga.* This taxon was positively correlated with chl *a*, POM, PO4 and Si (Spearman's coefficient: $r_s = [0.73 - 0.91]$, $P < 0.01$), and negatively with salinity ($r_s = -0.77$, $P < 0.01$). Associated taxa such as the cryptoperidiniopsoids and the HNFs tend to extend their distribution in more intermediate conditions towards the station D (cluster Ds–Db, Bray–Curtis similarity = 72%), which constitutes the zone of the approximate optimum for *H. rotundata*. This taxon was positively correlated with particulate inorganic matters ($r_s = 0.67$, $P < 0.05$) that characterised this zone at the river mouth.

Samples from the coastal part (except for Es) are grouped in the same cluster (Bray–Curtis similarity = 70%). However, the CA ordination focusing on this marine group (Fig. 6b) reveals some differences in the taxa composition. Fs is plotted separately because of the river influence. In contrast, samples of the less riverine-influenced station G (Gs, $G_{1/2}$, Gb) are plotted separately from the others, with *Phaeocystis globosa*, *Chaetoceros curvisetus* and *Thalassionema* spp. as most typical taxa, all highly correlated positively with salinity ($r_s = [0.71 - 0.74]$, $P < 0.01$), and negatively with nutrients ($r_s = -[0.71 - 0.84]$, $P < 0.01$), POM ($r_s = -[0.80 - 0.82]$, $P < 0.01$) and chl *a* ($r_s = -[0.5 - 0.65]$, $P < 0.05$). Samples from the near-bottom coastal waters (Eb, $F_{1/2}$, Fb) are grouped together, with *Chaetoceros affinis* as typical taxa among others. A number of taxa almost exclusively restricted to these samples tend to stretch out the sample plots towards the bottom portion of the biplot. Most of them are rare species (<1%), such as *Protoperidinium* spp. and *Chaetoceros didymus,* negatively correlated with the light availability in the water column ($r_s = -0.75$, $P < 0.05$). A group of several abundant species (surrounded by dotted line) forms an intermediate community with a wide coastal distribution, including *Pseudo-nitzschia* spp., *Bacteriastrum* spp., several *Chaetoceros* species and *Emiliania huxleyi*.

Clone library composition

The genetic diversity of planktonic microeukaryotes was investigated through the construction of clone libraries of 18S rRNA genes from three contrasting surface samples: the A0Esp clone library for the oligohaline river station A, B0Esp for the brackish mixing zone where the cryptophyte bloom occurred (station B), and G0Esp for the marine station G. A total of 405 clones were sequenced, of which 315 partial sequences ($\sim$825 pb $\pm$ 58) could be used for the taxonomy-based clustering after excluding low-quality results, putative chimeras, and metazoan sequences. Clustering of sequences based on a 98% similarity level revealed a total of 71 different operational taxonomic units (OTUs), with 30 OTUs found in A0Esp, 27 in B0Esp, and 21 in G0Esp (Table 1, Table S3). Only seven OTUs were shared between the two libraries A0Esp and B0Esp, but none was shared between G0Esp and the other two. The total richness highlighted in the marine G0Esp library (=21 OTUs) was lower than in A0Esp and B0Esp, in spite of a higher number of clones analysed (Table 1).

Various microeukaryotic OTUs in a broad range of taxonomic lineages were identified, including: 22

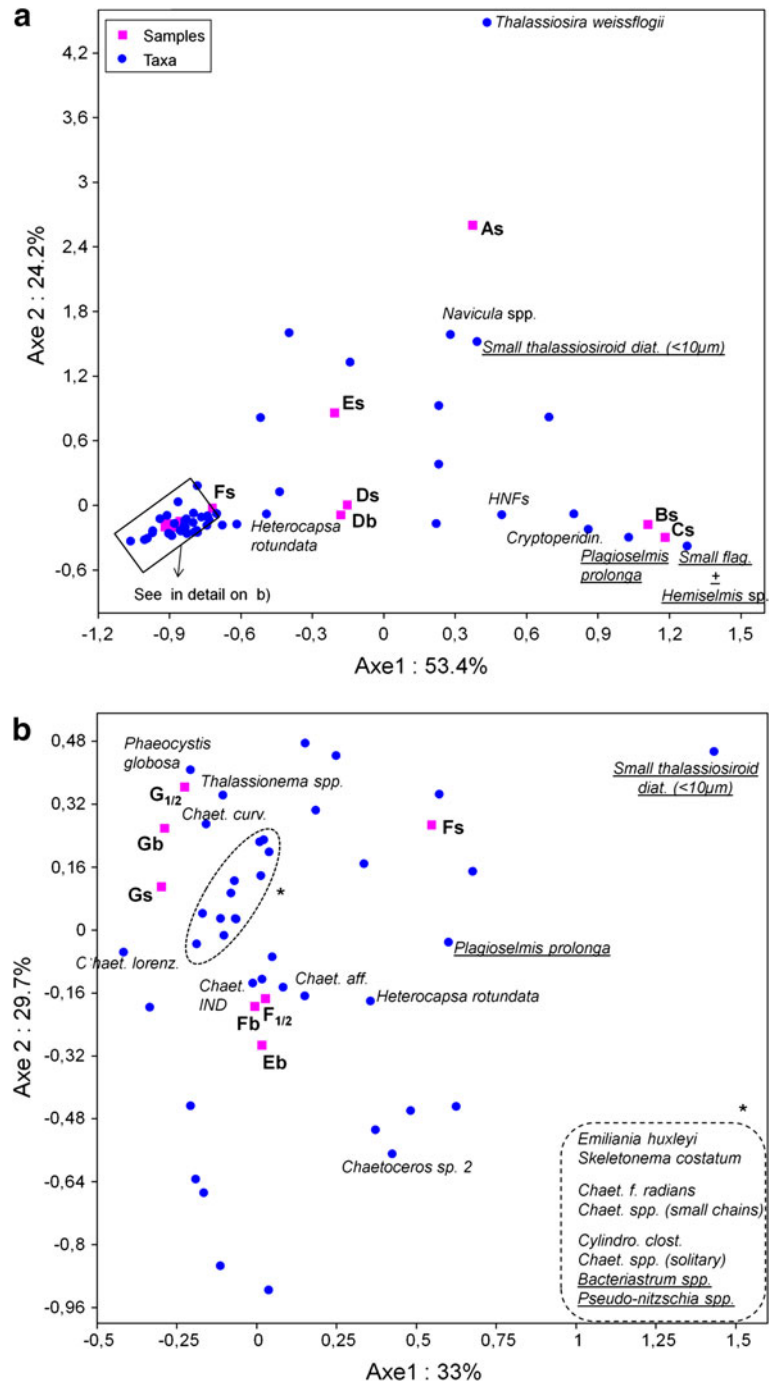


Fig. 6 Correspondence Analysis (CA) of samples (*squares*) collected along the estuarine continuum, calculated from taxa abundance. The data set included 49 taxa (*circles*), which contributed >1% of total abundance (in at least one sample) or present in at least five samples along the transect. Only taxa with contribution >1% are labelled and the dominant species (i.e. with a contribution >10% in at least one sample) are *underlined*. **a** CA including all the samples along the transect (13 samples), and **b** a detailed CA of the samples from the marine section (7 samples). Sample designation: station name (A–G) followed by the subscripts s (=surface), ½ (=mid-depth) or b (=bottom). (Abbreviations are Cryptoperidin. = Cryptoperidiniopsoids, Small flag. = small flagellates unidentified (Type 1), HNFs = heterotrophic nanoflagellates, Chaet. Curv. = *Chaetoceros curvisetus*, Chaet. lorenz. = *Chaetoceros lorenzianus/decipiens*, Chaet. IND = *Chaetoceros* indeterminate, Chaet. aff. = *Chaetoceros affinis*, Chaet. f. radians = *Chaetoceros socialis f. radians*, Cylindro. clost. = *Cylindrotheca closterium*)

Table 1 Distribution of OTUs and clones among the major phylogenetic groups identified in the three eukaryotic 18S rDNA libraries: A0Esp (river), B0Esp (mixing zone) and G0Esp (coast)

Major eukaryotic lineages		Nb of OTUs (clones) in			Nb of shared OTUs (libraries)
First rank	Second rank (~phyla/classes)	A0Esp	B0Esp	G0Esp	
Fungi	Ascomycota	2 (2)		1 (1)	
	Basidiomycota	1 (1)		1 (1)	
	Chytridiomycota	1 (1)			
	Environmental clade LKM11	14 (70)	7 (9)		6 (A0Esp-B0Esp)
	Zygomycota	1 (1)			
Cercozoa	Cryomonadida clade			1 (1)	
Euglenozoa			5 (14)		
Chlorophyta	Chlorophyceae	3 (6)	2 (2)	1 (1)	1 (A0Esp-B0Esp)
	Trebouxiophyceae	1 (1)			
	Prasinophyceae			1 (2)	
	Prasinophyceae (clade VII a)			1 (1)	
Stramenopiles	Xanthophyceae	1 (1)			
	Chrysophyceae	1 (2)			
	Oomycota		1 (2)		
	Labyrinthulomycota		1 (1)	2 (5)	
Cryptophyta			1 (5)		
Haptophyta				2 (2)	
Alveolates	'Core' dinoflagellates			4 (104)	
	Syndiniales gp I, Dinoflagellates			3 (7)	
	Syndiniales gp II, Dinoflagellates		1 (1)	1 (1)	
	Ciliophora	5 (7)	5 (11)	1 (1)	
	Apicomplexa (eugregarines)			2 (3)	
Unclear affiliations	Apicomplexa (putative)		3 (47)		
	Group LG-B* (Stramenopiles)		1 (1)		
Total		30 (92)	27 (93)	21 (130)	

'Major eukaryotic lineages' (first and second ranks) refer to the classification and nomenclature of eukaryotes according to Adl et al. (2005). '(A0Esp/B0Esp)' refers to the two clone libraries sharing OTUs. * (defined in Richards et al., 2005)

Fungi and 22 Alveolata as the two most diverse groups represented, and 8 Chlorophyta, 6 stramenopiles, 5 Euglenozoa, 2 Haptophyta, 1 Cryptophyta and 1 Cercozoa (Table S3). Sequences belonging to dinoflagellates and Fungi dominated the total genetic library obtained in this area (Table 1), accounting for 36 and 27% of the total clones, respectively.

At a high taxonomic level, differences in the community composition were observed between the three sampled areas (Table 1). Four of the eight major taxonomic groups (first rank taxa) were detected in the river (A0Esp) with a high OTU richness among Fungi, while six taxonomic groups were found in the two other clone libraries. In the coastal library G0Esp, Dinoflagellata showed clearly a higher richness than the other groups, whereas the OTUs detected in B0Esp were more evenly distributed among the different major groups.

Phylogenetic analyses of the main OTUs detected

Overall, most of the OTUs retrieved in this study belonged to heterotrophic or mixotrophic groups representing, with 55 OTUs, 82% of the total diversity, and particularly in the B0Esp library, where they reached 87%.

Among these groups, the Fungi exhibited highest diversity (22 OTUs, Table 1, Table S3), especially in A0Esp, where they dominated the riverine community (63% of the OTU richness). Most of the OTUs (16 out

of the 22) were affiliated with sequences of the group LKM11 recently named Rozellida (Lara et al., 2009) or Cryptomycota (Jones et al., 2011) and described as a novel intermediate form of Fungi composed of small eukaryotes (3–5 μm) flagellated or not. This group was detected in the river (A0Esp) and in the zone of the bloom (B0Esp), with 6 OTUs shared between both libraries. According to the phylogenetic tree based on the alignment of partial sequences (Fig S1.a), the LKM11-affiliated OTUs were robustly nested with the other LKM-related clones from the public databases. Among them, A0Esp_2_24 branched with *Rozella allomycis*, which is the unique cultivated genus associated with the LKM11 clade and known to be parasitic (Held, 1981).

Alveolata was, equally to Fungi, the most diverse group (22 OTUs). It was primarily composed of heterotrophic representatives of the Ciliophora, Dinoflagellata, and in a lesser extent the Apicomplexa (Table 1, Table S3). No dinoflagellate OTU was found in A0Esp and only one in B0Esp, while it was the most diverse phylum retrieved in the coastal sample (8 OTUs in G0ESp). Four OTUs from G0Esp were affiliated with the classical dinoflagellates, such as *Protoceratium reticulatum* (G0Esp_1_20, 100% similarity) and *Gyrodinium fusiforme* (G0Esp_7_27) (Table S3 and phylogenetic tree in Fig. S1.b). Interestingly, the predominant coastal OTU (G0Esp_50C_15, containing 61% of the clones) was close to the clone NPK2_155 (99% similarity) retrieved from an Arctic fjord (Luo et al., 2009), and together with clones from the Sargasso Sea (SCM38C55) and from the East Sea (E130908_30, Lee et al., 2012), they formed a robust clade (100% BP) with no affiliation to any characterised species. The remaining dinoflagellate OTUs (4 from the coast and 1 from the mixing zone) belonged to the *Syndiniales* groups I and II (formerly 'novel marine alveolate groups', MALV), in which all the known species are parasites or parasitoids (Guillou et al., 2008).

Ciliates were detected as an important contributor to the eukaryotic diversity in A0Esp and B0Esp (17 and 19% respectively), and 2 OTUs from the coastal zone were in the radiation of the Apicomplexa, robustly allied with the parasitic 'Crustacean' eugregarine clade (Rueckert et al., 2011) (Table 1).

Stramenopiles did not account for a significant proportion of clones in genetic libraries (3.5% of the total clones falling in 6 OTUs) and no diatom sequences were detected in samples (Table 1, Table S3, and phylogenetic tree in Fig. S1.c). Only one OTU (A0Esp_1_4) was affiliated with a group of photosynthetic stramenopiles (Xanthophyceae), while the other stramenopile OTUs were affiliated with heterotrophic taxa. Among them, A0Esp_3_20 from the river fell into the class Chrysophyceae, with a 99.4% homology with a colourless 'Spumella'-like strain GOT220 (Boenigk et al., 2007). Three OTUs belonged to the widely osmoheterotrophic marine group of Labyrinthulomycota, including 2 coastal OTUs close to *Aplanochytrium* strains known to be parasites (97.7–100% similarity), and an OTU from the mixing zone (B0Esp_1-2_15) closely affiliated with an environmental clone BBW042908_16 (100% BP) from the marine novel group 'uTh1', which is not related to any cultivated species (Collado-Mercado et al., 2010).

The main representatives of the primary producers are the Chlorophyta with 8 OTUs (Table 1, Table S3). Chlorophyte-affiliated sequences were found in all libraries and corresponded either to small-sized coccoid species such as *Mychonastes homosphaera* (Chlorophycea) and *Choricystis minor* (Trebouxiophyceae) found in A0Esp or B0Esp, or to flagellated taxa such as the two different *Chlamydomonas* species found in A0Esp and G0Esp (Table S3). The class Prasinophyceae was detected only in the coastal sample with two OTUs, whose closest matches were environmental sequences rather than described species. G0Esp_4_29 was nonetheless related to the genus *Tetraselmis* (98% similarity), and G0Esp_4_17 clustered with sequences of the picoplanktonic prasinophyte clade VII (lineage A), which contains clones retrieved from coastal/oceanic ecosystems and cultured strains of undescribed coccoid species (Guillou et al., 2004).

All the remaining OTUs and lineages were restricted to one of the three libraries (Table S3). Among them, two other autotrophic lineages were detected: the Haptophyta, with 2 OTUs only found in G0Esp and closely related to *Gephyrocapas oceanica*/*Emiliania huxleyi* (99.5% similarity) or *Chrysochromulina strobilis* (99.5%), and the Cryptophyta, with one OTU from the mixing zone. Surprisingly, the closest related known species of the cryptophyte OTU was *Teleaulax acuta* (100% similarity), while we identified *P. prolonga* by microscopy (LM and SEM) as the main contributor of the bloom. As regards this mixing zone, 5 OTUs belonged to the mixotrophic

lineage Euglenozoa. Four of these OTUs were related to the species *Euglena viridis* but with very variable homology percentages (85–97% similarity). Those showing the lowest similarity values (B0Esp_1_4, B0Esp_3_29) may correspond to divergent fast-evolving strains of *E. viridis*.

Four OTUs, all recovered from the mixing zone, were composed of divergent and long-branch sequences that could not be clearly assigned to major eukaryotic groups ('unclear affiliations' Table 1 and S3). Among them, two predominant OTUs (B0Esp_3_3 and B0Esp_2-2_59) had a very low homology with the sequences from the public database (72.6–80% similarity). However, it appears in analysis that they were associated with alveolate sequences, as KRL01E13 from the hypertrophic Lake Karla (Greece, Oikonomou et al., 2012) and the parasitic genus *Cryptosporidium* (Apicomplexa).

Discussion

Our first investigation along the Segura River-coastal zone continuum was carried out in late winter-early wet spring. Only low rainfall was observed in February (monthly average = 2,245 mm) but 0.2 mm precipitation was recorded the day before sampling (Agencia Estatal de Meteorología del Gobierno de España). Unusually high temperatures were recorded during the preceding week, reaching up to 26°C. Under these weather conditions, phytoplankton attained impressive bloom proportions in the brackish mixing zone. As regularly reported in other estuarine ecosystems (Seoane et al., 2005), such bloom events could be recurrent in the terminal part of the Segura River, especially as the regional semiarid climate involves long periods of stable weather with low river discharges (low turbulence) and the human activities are a major source of continuous nutrient enrichment of waters.

Although we studied this area at a single date, our taxonomic inventory along the estuarine gradient revealed a relatively high total richness (202 taxa from 13 samples), when compared with extensive temporal surveys carried out in other Mediterranean estuaries (e.g. Brogueira et al., 2007; Pérez et al., 2009 along the Tagus estuary and the Ebro River estuary, respectively). Moreover, by using two contrasting but complementing methods, we enhanced our assessment of the global diversity. The 18S rDNA clone libraries revealed an unexpectedly high diversity among the heterotrophic groups that usually escape detection with traditional microscopy, including many organisms still poorly known. As previously found in other similar environmental surveys (Savin et al., 2004; Luo et al., 2011), the microscopic analyses were more efficient in revealing the high diversity among the phototrophic organisms than the molecular method using universal primers did. The non-recovery of some phytoplankton taxa/groups (e.g. the diatoms) in the clone libraries is most probably due to the strong competition for the primers between the co-occurring sequences in the sample, increased by the presence of taxa containing high copy number of 18S rRNA genes (Zhu et al., 2005), as the Alveoloates. In addition, as commonly found our clone sampling effort is not sufficient to reach saturation and inevitably missed a part of the protist diversity.

As often reported in estuaries (Lancelot & Muylaert 2011), our results suggested that salinity was a major factor influencing the distribution of phytoplankton taxa in the Segura River transitional waters, according to the salinity tolerance of species. However, salinity alone did not explain the considerable changes in the biodiversity pattern along the continuum. The combined effects of multiple factors associated with the climatic and meteorological conditions as well as the anthropogenic pressures (e.g. the low river-flow, the nutrient-rich water discharges) are also involved in the great spatial variations in the phytoplankton composition, primarily in the continental part of the transect. The presence of man-made coastal infrastructures such as the seawall at the river mouth, the proximity of the harbour, and the adjacent outlet of a complex system of channels for irrigation and drainage of cultivated area, constitute an additional physical source of disturbance in the natural estuarine gradient impacting the distributional pattern of species.

Thus, based on the environmental and biological characteristics, we defined four major spatial shifts in the assemblage of the phytoplanktonic taxa, delimiting the following main communities along the continuum: (1) an upper-riverine community of euryhaline species due to the salinisation of the river, (2) a cryptophyte bloom and its associated community in the mixing zone, (3) an intermediate marine community accumulated in the sheltered waters of the river mouth, (4) a surface-localised community disturbed by inputs of allochthonous species also near the river mouth, and (5) a coastal community:

The euryhaline riverine community

Despite the nutrient-replete conditions in the upper oligohaline zone (As), the taxonomic richness and diversity indices were rather moderate when compared with the coastal communities. This early-spring riverine community was mainly composed of diatoms and chlorophytes species, but the contribution of the latter to the total phytoplanktonic abundance was low (1.3% of total). Chlorophyta richness was mainly composed of dispersed and isolated cells easily identified by microscopy in spite of their low abundance (e.g. *Scenedesmus* spp., *Monoraphidium* spp.). However, the clone library approach also allowed the detection of some coccoid chlorophytes that escaped our observations, such as *Mychonastes homosphaera* and *Choricystis minor*.

The small thalassiosiroid diatoms were largely predominant (90%) and almost exclusively composed of nano-sized taxa of *Thalassiosira pseudonana*. This species recorded with high density in the oligohaline waters (4.5 psu) was also dominant in euhaline waters at the river mouth (33–34 psu), originating from allochthonous inputs. This confirmed the considerable tolerance of this euryhaline species to a wide range of salinity (Hasle, 1978).

Most of the other taxa in the river, even in low densities, have also been previously reported in both marine and freshwater environments. Among them are the diatom *Thalassiosira weissflogii,* known from marine waters and inland rivers (Sorhannus et al., 2010), the halotolerant freshwater green alga *Chlamydomonas reinhardtii* (León & Galván, 1994), *Nitzschia constricta* (Pérez et al., 2009), and several naviculaceae typical of brackish and eutrophic waters (e.g. *Navicula gregaria*). The specific salinity-driven composition of taxa forming the riverine community is most likely due to the high salinity level inherent to the Segura river, which is naturally oligohaline (>3 psu, at least up to 10 km upstream of Station A). River salinisation is a growing worldwide phenomenon (Cañedo-Argüelles et al., 2013) that also affects the Segura River, and will probably be amplified by climate change (Observatorio de la Sostenibilidad en Espagna, 2008). Many different causes can be involved and are more recently accentuated by anthropogenic activities, posing a risk of causing biodiversity losses in the freshwater ecosystem (Blinn & Bailey, 2001). The arid climate and the intensive irrigation of crops consuming large quantities of water are two of the main factors increasing salinisation (Cañedo-Argüelles et al., 2013), and both are characteristic of the lower Segura River basin.

The genetic diversity revealed within the oligohaline river community was comparable to the clone libraries previously obtained from rivers and soil ecosystems, with among others the Fungi as a major contributor of the detected diversity (Berney et al., 2004, Lefèvre et al., 2007). Our observation was even more impressive since fungal OTUs contributed to 63% of the total richness, with the large majority belonging to the deep-branching fungal clade LKM11, characterised as a group of small eukaryotes with different life cycle stages, including one as attached non-flagellate cells (possibly parasitic or saprotrophic) frequently found on diatoms, and another as uniflagellate spores (Jones et al., 2011). Our personal observations of similar small heterotrophic flagellates (HFs) and other small eukaryotes associated with the thalassiosiroid diatoms dominating in the river could correspond to this description. This highly diverse group might represent a major component of the microbial food web in the lower part of the Segura River, and probably has ecological implications. Unfortunately, it remains a largely unexplored group, whose potential roles on the phytoplankton communities should be further investigated.

Bloom of cryptophytes in the mixing zone

The meteorological conditions associated with the local configuration of the Segura River like a retention pool and the high stability of the nutrient-enriched water column, led to a bloom largely dominated by cryptophytes that we identified as *Plagioselmis prolonga,* forming an impressive red tide in the brackish mixing zone. A cryptophyte OTU detected by our molecular approach was identical to that of *Teleaulax acuta* (100% similarity) but not with total coverage of sequences (97%). The second closest organism was *Geminigera cryophila* (DQ452091) with 99% similarity, indicating that only few differences in the sequences (1%) lead to another genus. The phylogenetic relationships between the members of the *Teleaulax*-like lineage are still unclear (Shalchian-Tabrizi et al., 2008) and include short basal and internal branches, suggesting slow evolutionary rates, and several misclassifications of the *Plagioselmis* are

suspected. This is why we cannot resolve the discrepancy between our morphological identification of *P. prolonga* and the genetic assignation to *Teleaulax acuta*. However, the bloom-forming cryptophyte in our study could in fact belong to a species complex within the *Teleaulax*-like group presenting a *Plagioselmis*-like morphology and cell size.

Only few events of water discolouration caused by cryptophytes have been reported (four according to Laza-Martínez, 2012). However, Cryptophyceae are in general abundant members of phytoplankton communities in various water transition zones along the Iberian coast (Sebastiá et al., 2012; Martínez-Guijarro et al., 2013). To our knowledge, the red tide we reported in the Segura River showed the highest cryptophytes abundances recorded so far ($>150 \times 10^3$ cells ml^{-1}), exceeding those of *Urgorri complanatus*, a new red-tide-forming species recurrent in the Spanish Nervion River estuary (a peak $>40 \times 10^3$ cells ml^{-1}, Laza-Martínez, 2012), and even the observations of *Hemiselmis virescens* in the Baltic Sea (125×10^3 cells ml^{-1}, Hill et al., 1992). *P. prolonga* is a marine euryhaline species that enters into the Segura River as the river flow is very low. These blooming species is then certainly retained inside the mixing zone at both the ebb and low tide due to the microtidal regime. The *Plagioselmis* bloom co-occurred with cryptoperidiniopsoid dinoflagellates (or *Pfiesteria*-like) which prey on it and are capable of retaining photosynthetically functional the chloroplasts of their prey as kleptoplasts (Lewitus et al., 1999). Such potentially toxic dinoflagellates (Glasgow et al., 1995) are known to have high grazing rates and they may control the population of their prey (Lin et al., 2004). At the time of sampling, the grazing pressure in the mixing zone was insufficient to prevent the massive *Plagioselmis* bloom. However, knowing that dinoflagellates can respond quickly to ephemeral blooms of cryptophytes, the occurrence of a consecutive *Pfiesteria*-like bloom in the waters of the Segura River is highly probable, and should be monitored in this area. Other taxa were also associated with *P. prolonga* and the *Pfiesteria*-like, together forming a typical euryhaline community indicative of highly eutrophic conditions. Among them are the small cryptophytes *Hemiselmis* sp., many heterotrophic nanoflagellates including the colourless *Katablepharis* feeding on the blooming cryptophytes (personal observations), and members of the Euglenophyta, a group considered as an indicator of pollution with organic matter (Stonik & Selina, 2001).

The B0Esp clone library was interesting because of the dominance of 3 OTUs with no homology to any known organism or even environmental clone from the public databases. We suppose these clones to belong to the fast-evolving lineages of the alveolates according to our blast search and preliminary reconstruction trees including the major eukaryotic groups (data not shown). Indeed, we cannot exclude the presence of unknown fast-evolving organisms, potentially parasites or parasitoids, occurring in this highly impacted and eutrophic brackish zone of the Segura River, especially as environmental molecular surveys in such transitional waters are still lacking and suggest that a significant part of the genetic microbial diversity has not yet been discovered.

Communities of the modified Segura River mouth

The inner part of the Segura River mouth (station D) can be considered as a restricted sheltered area due to the construction of the seawall and the harbour at its outlet. In this modified water body, environmental and biological characteristics were different from the offshore coastal waters and constituted an intermediate zone along the continuum, where the contribution of the euryaline taxa found in upstream mixing zone balanced with the contribution of typical marine species rich in diatoms and dinoflagellates. This hyperhaline zone (36–38 psu) showed relatively high total cell abundance and a high turbidity related to the increase of particulate inorganic matters (PIM), especially at the bottom. This phenomenon is probably due to the accumulation process including the transport of coastal phytoplankton and other particles in the inner river mouth, followed by their retention in this zone because of the restricted configuration and the probable slow renovation of water (as in Varela et al., 2001). Moreover, the general low flushing rates and the shallow waters can promote high algal production (Anderson et al., 2002). These conditions seemed to be favourable for the presence of the dinoflagellate *Heterocapsa rotundata*, which was typical of this confined area and occurred preferentially in the bottom layer.

The allochthonous freshwater inputs from an irrigation channel adjoining the Segura River and discharging close to its outlet causes the brutal change

in the phytoplankton community composition and in the physico-chemical parameters in the coastal surface waters (station E). This localised disturbance in the natural gradient of species was revealed by the decrease of the diversity indices N1, N2 reflecting a less evenly distribution of individuals among species. Artificial channels are commonly found on the Spanish Mediterranean coast (Sebastiá et al., 2012) due to the vast extent of the agricultural practice and the recurrent drought issues. Their water discharges are characterised by high nitrogen loads, as highlighted by the high concentration of $NO_3 + NO_2$ at the surface of the station E. The inputs lead to a local shift in the surface community and a high taxonomic richness, composed of a mixture of marine and freshwater species. However, most of the typical freshwater taxa in the surface waters are isolated cells transported with the water discharge and decreasing because of the osmotic stress. Due to their wide tolerance to salinity, the small thalassiosiroids were the only freshwater-originated taxa supporting the high salinity variation, and even, dominated the community.

Coastal community

The remaining coastal water samples off the Segura River presented the physico-chemical characteristics and phytoplankton communities typical of late winter-early spring: a period with no stratification, cool waters (13–14°C) compared to other seasons, and relatively high nutrient level associated with diatoms as dominant group (Margalef, 1978) and dinoflagellates in relatively low concentrations (4–80 cells ml^{-1}). These observations are similar to those of other studies carried out in the coastal NW Mediterranean waters at the same season (Varela et al., 2001; Vila & Masó, 2005). The spatial analysis of the phytoplankton distribution in the coastal waters highlighted the progressive change in the community composition both longitudinally and vertically, from a rather euryhaline community to a typical marine community with preferences for less nutrient-rich waters and higher salinities (e.g. *T. nitzschoides/frauenfeldii, C. curvisetus*). Through the use of the multivariate analysis, a group of intermediate coastal taxa with a wide distribution within the coastal waters was defined, including the diatom genera *Pseudo-nitzschia* and *Bacteriastrum,* and the coccolithophorid *Emiliania huxleyi*. *Pseudo-nitzschia* has been known to occur along the Spanish Mediterranean coast most of the year (Vila & Masó, 2005; Pérez et al., 2009) and with high cell abundance during winter-early spring (Quijano-scheggia et al., 2008). During our study, it was the dominant taxa in the coastal waters, and composed of at least three species: *P. fraudulenta*, *P. delicatissima* and *P. pseudo-delicatissima,* among which the latter two are known to be potentially toxic (Rhodes et al., 1997; Pan et al., 2001).

Interestingly, phytoplankton abundances and nutrient concentrations increased with depth, even though it tended to be less evident towards the open sea. This probably indicates that organisms reached deeper layer, where they can develop under higher nutrient load with sufficient light penetration due to the late-winter clear waters in the absence of stratification (Townsend et al., 1992). In addition, photoinhibition effect, which reduces growth and photosynthesis, is known to occur in the superficial water layers (Helbling et al., 2005), and may also contribute to the induction of vertical phytoplankton migration. Some of the taxa were typical of the bottom waters closer to the coast (E–F). Most of them were rare taxa (<1%) almost exclusively restricted to these waters such as *Chaetoceros didymus* and the dinoflagellates *Protoperidinium* spp. The Dinoflagellata were more diverse in this closer coastal zone especially in the bottom layer. Our investigation may coincide with the period of global cyst germination for most taxa, including the numerous *Protoperidinium* species, known to produce benthic resting cysts during their life style cycles (Head, 1996). The cysts germinate when environmental conditions are favourable for growth in the water column (Ishikawa & Taniguchi, 1996), as it seemed to be initiated in the coastal waters off the Segura River in late-winter–early-spring (e.g. longer days, light availability).

A high richness in dinoflagellate OTUs also characterised the marine clone library, including the highly widespread Syndiniales and several 'core' dinoflagellates. Interestingly, the dominant marine OTU (G0Esp_50C_15) formed with three other environmental clones a new clade among the 'core' dinoflagellates that has never been highlighted before. No described species were closely related nor clustered with this clade, and the first BLAST matches (84–85% similarity) were a *Pfiesteria*-like isolate (PL021, Litaker et al., 2003), and *Blastodinium navicula* a parasite of Copepod's gut (Skovgaard et al., 2012). Further analyses and more sequences are needed to know more about this

clade, but according to the origin of the current environmental clones (East Sea, Arctic Fjord and Sargasso Sea) this may correspond to species with a widespread distribution in surface marine waters.

The Syndiniales are a parasitic order at the base of the Dinoflagellata known to have a wide range of potential hosts, and which commonly dominates clone libraries from coastal and oceanic waters (López-García et al., 2001; Massana & Pedrós-Alió, 2008). In the coastal waters off the Segura River, we found sequences belonging to the two major groups I and II (Guillou et al., 2008). Among them we can notice two OTUs probably parasitoids of other dinoflagellates, as are their closely related species of the genus *Amoebophrya* (Group II). The other Syndiniales OTUs included in the Group I were affiliated with environmental clones, so we do not have information about their potential hosts, except that *Duboscquella*, known to infect ciliates, is a member of a clade we retrieved. Several others OTUs belonged to various taxonomic groups also known to be (or to be potentially) parasite of a variety of invertebrates, such as eugregarine taxa (Apicomplexa) and the Labyrinthulomycota. Our results suggest therefore a potentially important role of parasitism in the coastal waters off the Segura River.

Conclusion

In the present study, thanks to a detailed microscopical approach, we provided an extensive spatial description of phytoplankton communities that can occur in the impacted waters along the continuum of the Segura River and its coast. By coupling this with a molecular survey in the three contrasting surface zones of the continuum, we extended our access to the genetic diversity within communities. Both approaches provided complementary rather than redundant information about the taxa developing in this area of major interest that had not been explored before. This site offers us an original environment under the influence of multiple climatic and anthropogenic impacting factors (e.g. droughts, salinisation, confined and sheltered areas, accumulation process, and inputs from irrigation channel) which are superimposed on the natural gradient of salinity. We consider the transition waters of the lower Segura River as an area with high risk of high-biomass bloom occurrence, which should be investigated more thoroughly in the future, and especially monitored for the possible development of harmful species. The several major shifts in the species assemblages promoted, at a global scale, a high taxa richness involving various trophic levels, life strategies, and adaptability. Our thorough taxonomic inventory using microscopy allowed to highlight a considerable richness of 'rare' taxa (<1%). Unfortunately, this richness has often been neglected in most environmental surveys, despite the fact that it represents an important reservoir of various taxa that are ready to take advantage when conditions are favourable and may have important ecological implications. The 18S rDNA survey was useful in revealing a significant diversity missed by the morphological approach, including a large proportion of the sequences belonging to groups of organisms still little known, such as several potential parasites, fast-evolving organisms or other heterotrophic microeukaryotes. This suggests that a significant part of the genetic microbial diversity remains to be discovered in such complex ecosystems.

Finally, our study highlights the importance of investigating such impacted estuaries in their entire continuum, given the spatial heterogeneity of environmental conditions leading to important changes in the diversity. By carrying out an in-depth analysis of the phytoplankton and microeukaryote diversity, we provided an overview of the 'hidden' diversity that can occur in such ecosystems yet considered as endangered water bodies. This demonstrates the need to further investigate the water transition zones in order to better estimate the extent of the whole protsitan diversity and to assess their ecological relevance in these environments.

Acknowledgments The authors wish to thank Dr. David Gras Olivares and Captain Carles (Instituto d'Ecología Litoral, Alicante, Spain) for logistical support during cruise and their dedicated efforts in the field. We thank Dr. Elodie Foulon for her valuable help in the field and in the laboratory during the sampling mission. We are very grateful to IFREMER Port-en-Bessin (particularly Olivier Pierre-Duplessix) for the loan of equipment and the nutrient measurements, and to Didier Goux (CMABio, UCBN) for his valuable assistance with SEM. For providing the meteorological data from at the Academia General del Aire (San Javier), we thank the Agencia Estatal de Meteorología del Gobierno de España. We would like to thank the two anonymous reviewers for their insightful and constructive comments.

References

Aboal, M., M. Á. Puig & A. D. Asencio, 2005. Production of microcystins in calcareous Mediterranean streams: the

Alharabe River, Segura River basin in south-east Spain. Journal of Applied Phycology 17: 231–243.

Adl, S. M., A. G. B. Simpson, M. A. Farmer, R. A. Andersen, O. R. Anderson, J. R. Barta, S. S. Bowser, G. Brugerolle, R. A. Fensome, S. Fredericq, T. Y. James, S. Karpov, P. Kugrens, J. Krug, C. E. Lane, L. A. Lewis, J. Lodge, D. H. Lynn, D. G. Mann, R. M. McCourt, L. Mendoza, O. Moestrup, S. E. Mozley-Standridge, T. A. Nerad, C. A. Shearer, A. V. Smirnov, F. W. Spiegel & M. F. J. R. Taylor, 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. The Journal of Eukaryotic Microbiology 52: 399–451.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers & D. J. Lipman, 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403–410.

Aminot, A. & M. Chaussepied, 1983. Manuel des analyses chimiques en milieu marin. Centre national pour l'exploitation des Océans, France: 395.

Aminot, A., & R. Kérouel, 2007. Dosage automatique des nutriments dans les eaux marines: méthodes en flux continu: 188 pp.

Anderson, D. M., P. M. Glibert & J. M. Burkholder, 2002. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 25: 704–726.

Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil & F. Thingstad, 1983. The ecological role of water-column microbes in the sea. Marine Ecology Progress Series 10: 257–263.

Behnke, A., K. J. Barger, J. Bunge & T. Stoeck, 2010. Spatio-temporal variations in protistan communities along an O2/H2S gradient in the anoxic Framvaren Fjord (Norway). FEMS Microbiology Ecology 72: 89–102.

Berney, C., J. Fahrni & J. Pawlowski, 2004. How many novel eukaryotic "kingdoms"? Pitfalls and limitations of environmental DNA surveys. BMC Biology 2: 1–13.

Blinn, D. W. & P. C. E. Bailey, 2001. Land-use influence on stream water quality and diatom communities in Victoria, Australia: a response to secondary salinization. Hydrobiologia 466: 231–244.

Boenigk, J., S. Jost, T. Stoeck & T. Garstecki, 2007. Differential thermal adaptation of clonal strains of a protist morphospecies originating from different climatic zones. Environmental Microbiology 9: 593–602.

Bourrelly, P., 1981. Les algues d'eau douce – Tome 1,2 et 3. Paris.

Bouza, N. & M. Aboal, 2008. Checklist of phytoplankton on the south coast of murcia (SE spain, SW Mediterranean sea). In Evangelista, V., L. Barsanti, A. M. Frassanito, V. Passarelli & P. Gualtieri (eds.), Algal Toxins: Nature, Occurence, Effect and Detection. Springer, Netherlands: 179–196.

Brogueira, M. J., M. D. R. Oliveira & G. Cabeçadas, 2007. Phytoplankton community structure defined by key environmental variables in Tagus estuary, Portugal. Marine Environmental Research 64: 616–628.

Cañedo-Argüelles, M., B. J. Kefford, C. Piscart, N. Prat, R. B. Schäfer & C.-J. Schulz, 2013. Salinisation of rivers: an urgent ecological issue. Environmental pollution 173: 157–167.

Coll, M., C. Piroddi, J. Steenbeek, K. Kaschner, F. Ben Rais Lasram, J. Aguzzi, E. Ballesteros, C. N. Bianchi, J. Corbera, T. Dailianis, R. Danovaro, M. Estrada, C. Froglia, B. S. Galil, J. M. Gasol, R. Gertwagen, J. Gil, F. Guilhaumon, K. Kesner-Reyes, M.-S. Kitsos, A. Koukouras, N. Lampadariou, E. Laxamana, C. M. López-Fé de la Cuadra, H. K. Lotze, D. Martin, D. Mouillot, D. Oro, S. Raicevich, J. Rius-Barile, J. I. Saiz-Salinas, C. San Vicente, S. Somot, J. Templado, X. Turon, D. Vafidis, R. Villanueva & E. Voultsiadou, 2010. The biodiversity of the Mediterranean Sea: estimates, patterns, and threats. PloS One 5: e11842.

Collado-Mercado, E., J. C. Radway & J. L. Collier, 2010. Novel uncultivated labyrinthulomycetes revealed by 18S rDNA sequences from seawater and sediment samples. Aquatic Microbial Ecology 58: 215–228.

Confederación hidrográfica del Segura, 2007. Estudio general sobre la Demarcación Hydrográfica del Segura: 359 pp.

Delgado, M., 1990. Phytoplankton distribution along the Spanish coast of the Alboran Sea. Scientia Marina 54: 169–178.

Díez, B., C. Pedrós-Alió & R. Massana, 2001. Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Applied and Environmental Microbiology 67: 2932–2941.

Edler, L. & M. Elbrächter, 2010. The Utermöhl method for quantitative phytoplankton analysis. In Karlson, B., C. Cusack & E. Bresnan (eds.), Microscopic and Molecular Methods for Quantitative Phytoplankton Analysis (IOC Manuals an Guide, N 55). Paris, UNESCO: 110.

Estrada, M., R. A. Varela, S. Jordi, C. Antoni & Arias Enric, 1999. Spatio-temporal variability of the winter phytoplankton distribution across the Catalan and North Balearic fronts (NW Mediterranean). Journal of Plankton Research 21: 1–20.

Gilabert, J., 2001. Seasonal plankton dynamics in a Mediterranean hypersaline coastal lagoon: the Mar Menor. Journal of Plankton Research 23: 207–218.

Glasgow, H. B., J. M. Burkholder, D. E. Schmechel, P. Tester & P. A. Rublee, 1995. Insidious effects of a toxic estuarine dinoflagellate of fish survival and human health. Journal Toxicolgy and Environmental Health 45: 501–522.

Gomis, C., M. J. Sanz, J. Alcober & J. Martínez, 1987. Phytoplankton and nutrients in the harbour of El Campello (Alicante). Limnetica 3: 273–280.

Gras, D., M. Planelles, C. Gomis, & J. Alcober, 1991. La comunidad fitoplanctonica de la reserva marina de Tabarca (Primavera-verano 1989). Ministerio de Agricultura, Pesca y Alimentación (Secretaría General Técnica). Madrid: 51–75.

Guillou, L., W. Eikrem, M.-J. Chrétiennot-Dinet, F. Le Gall, R. Massana, K. Romari, C. Pedrós-Alió & D. Vaulot, 2004. Diversity of picoplanktonic prasinophytes assessed by direct nuclear SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. Protist 155: 193–214.

Guillou, L., M. Viprey, A. Chambouvet, R. M. Welsh, A. R. Kirkham, R. Massana, D. J. Scanlan & A. Z. Worden, 2008. Widespread occurrence and genetic diversity of marine parasitoids belonging to Syndiniales (Alveolata). Environmental Microbiology 10: 3349–3365.

Hammer, Ø., D. A. T. Harper & P. D. Ryan, 2001. PAST: paleontological statistics software package for education and data analysis. Paleontologia Electronica 4: 1–9.

Hasle, G. R., 1978. Some freshwater and brackish water species of the diatom genus *Thalassiosira* Cleve. Phycologia 17: 263–292.

Head, M., 1996. Modern dinoflagellate cysts and their biological affnities In Jansonius, J., & D. C. McGregor (eds), Palynology: Principles and Applications. American Association of Stratigraphic Palynologists. 1197–1248.

Helbling, E. W., E. S. Barbieri, M. A. Marcoval, R. J. Gonçalves & V. E. Villafañe, 2005. Impact of solar ultraviolet radiation on marine phytoplankton of Patagonia, Argentina. Photochemistry and Photobiology 81: 807–818.

Held, A. A., 1981. *Rozella* and *Rozellopsis*: naked endoparasitic Fungi which dress-up as their hosts. Botanical Review 47: 451–515.

Hepperle, D., 2004. SeqAssem©. A sequence analysis tool, contig assembler and trace data visualization for molecular sequences. Win32-Version.

Herfort, L., T. D. Peterson, L. McCue & P. Zuber, 2011. Protist 18S rRNA gene sequence analysis reveals multiple sources of organic matter contributing to turbidity maxima of the Columbia River estuary. Marine Ecologie Progress Series 438: 19–31.

Hill, M. O., 1973. Diversity and evenness: a unifying notation and its consequences. Ecology 54: 427–432.

Hill, D. R. A., Ø. Moestrup, & N. Vørs, 1992. Rekylalger (Cryptophyceae) In Thomsen, H. A. (ed), Plankton I de indre danske farvande. Miljøministeriet Milijøstyrelsen, Copenhagen: 251–265.

Huber, T., G. Faulkner & P. Hugenholtz, 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20: 2317–2319.

Ishikawa, A. & A. Taniguchi, 1996. Contribution of benthic cysts to the population dynamics of *Scrippsiella* spp. (Dinophyceae) in Onagawa Bay. Marine Ecology Progress Series 140: 169–178.

John, D. M., B. A. Whitton & A. J. Brook, 2002. The Freshwater Algal Flora of the British Isles: An Identification Guide to Freshwater and Terrestrial Algae. Cambridge University Press, Cambridge: 714.

Jones, M. D. M., I. Forn, C. Gadelha, M. J. Egan, D. Bass, R. Massana & T. A. Richards, 2011. Discovery of novel intermediate forms redefines the fungal tree of life. Nature 474: 200–203.

Katoh, K., K. Kuma, H. Toh & T. Miyata, 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic acids research 33: 511–518.

Lancelot, C. & K. Muylaert, 2011. Trends in estuarine phytoplankton ecology. In Wolanski, E., & D. McLusky (eds.), Treatise on Estuarine and Coastal Science. Elsevier: 5–16.

Lara, E., D. Moreira & P. López-García, 2009. The environmental clade LKM11 and *Rozella* form the deepest branching clade of fungi. Protist 161: 116–121.

Laza-Martínez, A., 2012. *Urgorri Complanatus* Gen. et Sp. Nov. (Cryptophyceae), a Red-tide-forming species in brackish waters. Journal of Phycology 48: 423–435.

Lee, S.-R., T. Rho, J. H. Oak, J. A. Lee, T. Lee & I. K. Chung, 2012. Metagenomic examination of diversity within eukaryotic plankton from the Ulleung Basin in the East Sea of Korea. Journal of Plant Biology 55: 310–315.

Lefèvre, E., C. Bardot, C. Noël, J.-F. Carrias, E. Viscogliosi, C. Amblard & T. Sime-Ngando, 2007. Unveiling fungal zooflagellates as members of freshwater picoeukaryotes: evidence from a molecular diversity study in a deep meromictic lake. Environmental Microbiology 9: 61–71.

León, R. & F. Galván, 1994. Halotolerance studies on Chlamydomonas reinhardtii: glycerol excretion by free and immobilized cells. Journal of Applied Phycology 6: 13–20.

Lesen, A., A. R. Juhl & O. R. Anderson, 2010. Heterotrophic microplankton in the lower Hudson River Estuary: potential importance of naked, planktonic amebas for bacterivory and carbon flux. Aquatic Microbial Ecology. 61: 45–56.

Lewitus, A. J., H. B. Glasgow & J. M. Burkholder, 1999. Kleptoplastidy in the toxic dinoflagellate *Pfiesteria piscicida* (Dinophyceae). Journal of Phycology 35: 303–312.

Lin, S., M. R. Mulholland, H. Zhang, T. N. Feinstein, F. J. Jochem & E. J. Carpenter, 2004. Intense grazing and prey-dependent growth of *Pfiesteria piscicida* (Dinophyceae). Journal of Phycology 40: 1062–1073.

Lionard, M., K. Muylaert, A. Hanoutti, T. Maris, M. Tackx & W. Vyverman, 2008. Inter-annual variability in phytoplankton summer blooms in the freshwater tidal reaches of the Schelde estuary (Belgium). Estuarine, Coastal and Shelf Science 79: 694–700.

Litaker, R. W., M. W. Vandersea, S. R. Kibler, K. S. Reece, N. A. Stokes, K. A. Steidinger, D. F. Millie, B. J. Bendis, R. J. Pigg & P. A. Tester, 2003. Identification of Pfiesteria piscida (Dinophyceae) and *Pfiesteria*-like organisms using internal transcribed spacer-specific PCR assays. Journal of Phycology 39: 754–761.

Livingston, R. J., 2001. Eutrophication processes in coastal systems: Origin and succession of plankton blooms and effects on secondary production in Gulf Coast Estuaries. Florida State University, 327 pp.

López-García, P., F. Rodríguez-Valera, C. Pedrós-Alió & D. Moreira, 2001. Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature 409: 603–607.

Lund, J. W. G., C. Kipling & E. D. Le Cren, 1958. The Inverted Microscope method of estimating algal numbers and the statistical basis of estimations by counting. Hydrobiologia 11: 143–170.

Luo, W., H. Li, M. Cai & J. He, 2009. Diversity of microbial eukaryotes in Kongsfjorden, Svalbard. Hydrobiologia 636: 233–248.

Luo, W., C. Bock, H. R. Li, J. Padisák & L. Krienitz, 2011. Molecular and microscopic diversity of planktonic eukaryotes in the oligotrophic Lake Stechlin (Germany). Hydrobiologia 661: 133–143.

Margalef, R., 1978. Life-forms of phytoplankton as survival alternatives in an unstable environment. Oceanologica Acta 1: 493–509.

Martínez-Guijarro, R., I. Romero, M. Pachés, C. M. Martí & J. Ferrer, 2013. Chlorophyll *a*, nutrients and phytoplanktonic community in a continental ecosystem highly influenced by marine waters. Journal of Experimental Marine Biology and Ecology. 442: 30–38.

Massana, R. & C. Pedrós-Alió, 2008. Unveiling new microbial eukaryotes in the surface ocean. Current Opinion in Microbiology 11: 213–218.

McLusky, D. S., 1993. Marine and estuarine gradients – an overview. Netherlands Journal of Aquatic Ecology 27: 489–493.

Médail, F. & P. Quezel, 1996. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean basin. Annals of the Missouri Botanical Garden 84: 112–127.

Medlin, L., H. J. Elwood, S. Stickel & M. L. Sogin, 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71: 491–499.

Mercado, J. M., T. Ramírez, D. Cortés, M. Sebastián & M. Vargas-yáñez, 2005. Seasonal and inter-annual variability of the phytoplankton communities in an upwelling area of the Alborán Sea (SW Mediterranean Sea). Scientia Marina 69: 451–465.

Muylaert, K., R. Van Mieghem, K. Sabbe, M. Tackx & W. Vyverman, 2000. Dynamics and trophic roles of heterotrophic protists in the plankton of a freshwater tidal estuary. Hydrobiologia 432: 25–36.

Muylaert, K., K. Sabbe & W. Vyverman, 2009. Changes in phytoplankton diversity and community composition along the salinity gradient of the Schelde estuary (Belgium/The Netherlands). Estuarine, Coastal and Shelf Science 82: 335–340.

Myers, N., R. A. Mittermeier, C. G. Mittermeier, G. a da Fonseca & J. Kent, 2000. Biodiversity hotspots for conservation priorities. Nature 403: 853–858.

Observatorio de la Sostenibilidad en Espagna, 2008. Water and Sustainability: Water Basin Functionality. Alcala de Henares, Spain: 203.

Oikonomou, A., M. Katsiapi, H. Karayanni, M. Moustaka-Gouni & K. A. Kormas, 2012. Plankton microorganisms coinciding with two consecutive mass fish kills in a newly reconstructed lake. TheScientificWorldJournal 2012: 1–14.

Pan, Y., M. L. Parsons, M. Busman, P. Moeller, Q. Dortch, C. L. Powell, G. A. Fryxell & G. J. Doucette, 2001. *Pseudo-nitzschia pseudodelicatissima* – a confirmed producer of domoic acid from the northern Gulf of Mexico. Marine Ecology Progress Series 220: 343–359.

Peel, M. C., B. L. Finlayson & T. A. Mcmahon, 2007. Updated world map of the Köppen-Geiger climate classification. Hydrology and Earth System Sciences Discussions 4: 439–473.

Pérez, M. C., N. L. Maidana & A. Comas, 2009. Phytoplankton composition of the Ebro River estuary, Spain. Acta Botanica Croatica 68: 11–27.

Quijano-scheggia, S., E. Garcés, N. Sampedro, K. van Lenning, K. Andree, J.-M. Fortuno & J. Camp, 2008. Identification and characterisation of the dominant *Pseudo-nitzschia* species (Bacillariophyceae) along the NE Spanish coast (Catalonia, NW Mediterranean). Scientia Marina 72: 343–359.

Rhodes, L. C., A. Scholin, I. Garthwaite, A. Haywood & A. Thomas, 1997. Domoic acid producing *Pseudo-nitzschia* species educed by whole cell DNA probe-based and immunochemical assays. In Reguera, B., J. Blanco, M. L. Fernández & T. Wyatt (eds.), Harmful algae. Proceedings of the VIII International Conference on Harmful Algae. Vigo, Spain: 274–277.

Richards, T. A., A. A. Vepritskiy, D. E. Gouliamova & S. A. Nierzwicki-Bauer, 2005. The molecular diversity of freshwater picoeukaryotes from an oligotrophic lake reveals diverse, distinctive and globally dispersed lineages. Environmental Microbiology 7: 1413–1425.

Romari, K. & D. Vaulot, 2004. Composition and temporal variability of picoeukaryote communities at a coastal site of the English Channel from 18S rDNA sequences. Limnology and Oceanography 49: 784–798.

Ros, M. & R. M. Miracle, 1984. Variación estacional del fitoplancton del Mar Menor y su relación con la de un punto próximo en el Mediterráneo. Limnética 1: 32–42.

Ros, M. D., J. P. Marín-Murcia & M. Aboal, 2009. Biodiversity of diatom assemblages in a Mediterranean semiarid stream: implications for conservation. Marine and Freshwater Research 60: 14–24.

Rueckert, S., T. G. Simdyanov, V. V. Aleoshin & B. S. Leander, 2011. Identification of a divergent environmental DNA sequence clade using the phylogeny of gregarine parasites (Apicomplexa) from crustacean hosts. PloS One 6: e18163.

Savin, M. C., J. L. Martin, M. LeGresley, M. Giewat & J. Rooney-Varga, 2004. Plankton diversity in the Bay of Fundy as measured by morphological and molecular methods. Microbial Ecology 48: 51–65.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn & C. F. Weber, 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75: 7537–7541.

Sebastiá, M.-T., M. Rodilla, J.-A. Sanchis, V. Altur, I. Gadea & S. Falco, 2012. Influence of nutrient inputs from a wetland dominated by agriculture on the phytoplankton community in a shallow harbour at the Spanish Mediterranean coast. Agriculture, Ecosystems & Environment 152: 10–20.

Seoane, S., A. Laza, I. Urrutxurtu & E. Orive, 2005. Phytoplankton assemblages and their dominant pigments in the Nervion River estuary. Hydrobiologia 549: 1–13.

Shalchian-Tabrizi, K., J. Bråte, R. Logares, D. Klaveness, C. Berney & K. S. Jakobsen, 2008. Diversification of unicellular eukaryotes: cryptomonad colonizations of marine and fresh waters inferred from revised 18S rRNA phylogeny. Environmental Microbiology 10: 2635–2644.

Skovgaard, A., S. A. Karpov & L. Guillou, 2012. The parasitic dinoflagellates *Blastodinium* spp. inhabiting the gut of marine, planktonic copepods: morphology, ecology, and unrecognized species diversity. Frontiers in Microbiology 3: 1–22.

Smith, V. H., G. D. Tilman & J. C. Nekola, 1999. Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environmental Pollution 100: 179–196.

Sorhannus, U., J. D. Ortiz, M. Wolf & M. G. Fox, 2010. Microevolution and speciation in *Thalassiosira weissflogii* (Bacillariophyta). Protist 161: 237–249.

Stonik, I. V. & M. S. Selina, 2001. Species composition and seasonal dynamics of density and biomass of Euglenoids in Peter the Great Bay, Sea of Japan. Russian Journal of Marine Biology 27: 174–176.

Tomas, C. R., 1997. Identifying Marine Phytoplankton. San Diego. 858 pp.

Townsend, D. W., M. D. Keller, M. E. Sieracki & S. G. Ackleson, 1992. Spring phytoplankton blooms in the absence of vertical water column stratification. Nature 360: 59–62.

Trigueros, J. M. & E. Orive, 2000. Tidally driven distribution of phytoplankton blooms in a shallow, macrotidal estuary. Journal of Plankton Research 22: 969–986.

Varela, M., R. Prego, M. J. Belzunce & F. M. Salas, 2001. Inshore–offshore differences in seasonal variations of phytoplankton assemblages: the case of a Galician Ria Alta (Ria de A Coruña) and its adjacent shelf (NW of Spain). Continental Shelf Research 21: 1815–1838.

Vaulot, D., W. Eikrem & M. Viprey, 2008. The diversity of small eukaryotic phytoplankton (≤3 μm) in marine ecosystems. FEMS Microbiology Reviews 32: 785–820.

Vidal-Abarca Guitérrez, M. R., M. L. Suárez Alonso, & R. Gómez Cerezo, 2002. Caudales y aportaciones en la cuenca del Río Segura: Son fiables los datos hidrológicos? III Congreso Ibérico de la Fundación Nueva Cultura del Agua: La Directiva del Agua: realides y futuros. Seville.

Vigil, P., P. Countway, J. Rose, D. Lonsdale, C. Gobler & D. Caron, 2009. Rapid shifts in dominant taxa among microbial eukaryotes in estuarine ecosystems. Aquatic Microbial Ecology 54: 83–100.

Vila, M. & M. Masó, 2005. Phytoplankton functional groups and harmful algal species in anthropogenically impacted waters of the NW Mediterranean Sea. Scientia Marina 69: 31–45.

Viprey, M., L. Guillou, M. Ferréol & D. Vaulot, 2008. Wide genetic diversity of picoplanktonic green algae (Chloroplastida) in the Mediterranean Sea uncovered by a phylum-biased PCR approach. Environmental Microbiology 10: 1804–1822.

Welschmeyer, N., 1994. Fluorometric analysis of chlorophyll chlorophyll *b* and pheopigments. Limnology and Oceanography 39: 1985–1992.

Zhu, F., R. Massana, F. Not, D. Marie & D. Vaulot, 2005. Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS Microbiology Ecology 52: 79–92.