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Phytoplankton community composition in relation to environmental variability in the Urdaibai estuary (SE Bay of Biscay): Microscopy and eDNA metabarcoding

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ABSTRACT

Phytoplankton monitoring is essential for the global understanding of aquatic ecosystems. The present research studies the phytoplankton community of the Urdaibai estuary, combining microscopy and eDNA metabarcoding for the first time in the area. The main aims were to describe the phytoplankton community composition in relation to the environmental conditions of the estuary, and to compare the two methods used. Diatoms *Minutocellus polymorphus* and *Chaetoceros tenuissimus* dominated the outer estuary, being replaced by *Teleaulax acuta* (cryptophyte), *Kryptoperidinium foliaceum* (dinoflagellate) and *Cyclotella* spp. (diatom) towards the inner area. This change was mainly prompted by salinity and nutrients. Metabarcoding revealed the presence of 223 species that were not observed by microscopy in previous studies in the estuary. However, several characteristic species (e.g., *K. foliaceum*) were only detected with microscopy. Additionally, microscopy covered the limitations of eDNA metabarcoding concerning quantification. Thus, to give a full insight, a combination of techniques is recommended.

1. Introduction

Estuaries are known as dynamic ecosystems where major changes occur along numerous environmental gradients (e.g. salinity, temperature, nutrients and turbulence) associated with the mixing of freshwater and seawater during tidal cycles (Cloern et al., 2017; Muylaert et al., 2000). The biological communities inhabiting these systems are subject to high spatial and temporal contrasts: spatial variations depending on the tidal and river influence; and very high temporal variability at different scales, from daily (mainly due to tidal fluctuations) to seasonal (fluctuations in river discharge and meteorology) (McLusky and Elliott, 2004). As a result, estuaries are considered unique environments that support high biodiversity.

In these ecosystems, the structure and biomass of phytoplankton communities vary continuously, mainly because of their adaptation to the environmental gradients caused by the tidal water circulation and the influence of the river (Jouenne et al., 2007; Lee et al., 2020). More precisely, phytoplankton communities in estuaries respond to environmental changes related to runoffs, water surface temperature, salinity, light availability and resuspension induced by waves and winds (e.g. Vajravelu et al., 2018; Wells et al., 2015). Freshwater inflows are also known to be a key determinant of phytoplankton abundance and community structure in estuaries, due to their influence on the water retention time and degree of stratification (e.g. Lemley et al., 2018; Nunes et al., 2018). Additionally, the inputs of inorganic nutrients (nitrogen:N, carbon:C, phosphorus:P, oxygen:O, iron:Fe, silicon:Si) on estuarine surface waters stimulate phytoplankton growth and modulate community composition, increasing the growth rates of certain taxa and leading to harmful algal blooms that can affect ecosystems negatively (Anderson et al., 2002; McCabe et al., 2016; Pinckney et al., 2001;

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Vajravelu et al., 2018; Wells et al., 2015). Phytoplankton is known to be the first autotrophic community showing response to nutrient availability variations (Paerl et al., 2003) and, due to its basal position in the food chain, it is considered the link between inorganic nutrients and the rest of the trophic levels (Seoane et al., 2012). This makes phytoplankton one of the most used biological indicators of water quality and ecosystem health, especially for monitoring eutrophication (e.g. Kitsiou and Karydis, 2011; Raveh et al., 2019) as applied in international directives (Seoane et al., 2012).

Light microscopy has been traditionally the most used technique to assess phytoplankton biomass and diversity (e.g. Agirbas et al., 2015; Aktan et al., 2005; Lee et al., 2020). The main strength of this method is that it enables both phytoplankton identification and enumeration at the same time, providing cell counts, size measurement and taxonomic information to genus or species level (Edler and Elbrächter, 2010). However, it is highly dependent on individual researcher's skills (Muñiz et al., 2020), since it requires extensive taxonomic knowledge (Naik et al., 2011) and it is time-consuming (Wang et al., 2018). In addition, fragile and small cells (i.e., picophytoplankton cells) are difficult or impossible to identify, usually leading to omissions or errors in taxonomic identifications (Agirbas et al., 2015; Jeffrey, 1997). Many studies (e.g. Huo et al., 2020; Penna et al., 2017) assume that morphological analyses alone cannot provide a complete description of the huge phytoplankton diversity. The use of molecular tools, like DNA metabarcoding, can be an alternative to overcome the limitations associated with microscopy-based diversity monitoring, especially after the development of high-throughput sequencing (HTS) technologies (Rimet et al., 2021; Trebitz et al., 2017).

Metabarcoding enables the simultaneous identification of taxa from environmental samples based on their DNA by sequencing specific marker genes (barcodes) (Keck et al., 2017; Zimmermann et al., 2015). This method generates large amounts of biodiversity information, as it is able to identify species at any life stage, as well as cryptic species and those overlooked by traditional methods (Comtet et al., 2015). The data sets obtained from this approach are more complete, quickly available, cost-effective and are less dependent on taxonomic expertise (Penna et al., 2017; Trebitz et al., 2017). Consequently, metabarcoding has been applied successfully in phytoplankton research (e.g. Chen et al., 2019b; De Luca et al., 2021; Liu et al., 2020; Mortagua et al., 2019; Muhammad et al., 2021; Pérez-Burillo et al., 2022) and in many global marine environment projects, including the Tara Oceans Expedition (Malviya et al., 2016) and the Ocean Sampling Day (Kopf et al., 2015). However, it is known that the copy number variation (CNV) could affect the abundance estimates when using metabarcoding (Kembel et al., 2012), which explains the lack of correlation between this approach and microscopy in some cases (e.g. Stoeck et al., 2014). The low molecular taxonomic resolution of the available reference barcode database also influences the correlation between this method and traditional microscopy (e.g. Rimet et al., 2021). In addition, metabarcoding results are dependent on choice of primer pair used for the amplification (e.g. Alberdi et al., 2018; Piñol et al., 2019).

The Urdaibai estuary is located on the Basque coast, North of Spain, and drains into the southeastern Bay of Biscay. The estuary is formed by the tidal part of the Oka River and constitutes the central area of the only Biosphere Reserve of the Basque Country, declared by UNESCO in 1984 because of its high naturalistic and cultural value (Castillo-Eguskitza et al., 2017). This estuary represents one of the main tidal marshes along the coast of northern Iberia and hosts an especially relevant richness of water birds (Arizaga et al., 2014), being added to the list of Ramsar Wetlands in 1993 and the network of the European Union Natura 2000; Castillo-Eguskitza et al. (2017). Previous studies in the area suggested that biodiversity and ecosystem services provided by the estuary are positively correlated (Onaindia et al., 2013), which in turn, results in economic benefits, since (eco)tourism and recreation are the main economic motor and attraction in the region (Castillo-Eguskitza et al., 2017). Nevertheless, the estuary has been receiving direct discharges from the Gernika wastewater treatment plant (WWTP) since 1972 in the inner area, as this is the principal source of pollution of the estuary. Consequently, the inner area of the system does not fulfil the EU Water Framework Directive (WFD; Directive, 2000/60/EC) environmental objectives (e.g. Borja et al., 2021), presenting very high nutrient and faecal bacteria concentrations and eutrophication problems. Sewerage system renovations are being implemented, with the aim of diverting the effluent of Gernika WWTP outside the estuary, using a water collector to connect the effluent to Lamiaran WWTP, which will discharge the treated effluents in to coastal area of Bermeo.

Due to its high naturalistic value, the Urdaibai estuary is a widely studied ecosystem and the phytoplankton community of the estuary has been the subject of numerous studies since the late 1980s. Several of these were centred on primary production, respiration and photosynthetic characteristics of phytoplankton (Iriarte et al., 1997; Madariaga and Orive, 1989; Madariaga et al., 1989; Revilla et al., 2000), while others described the communities and their dynamics in different time scales, based on microscopy identification and enumeration (Madariaga, 1995; Orive et al., 1998; 1998; Trigueros and Orive, 2000, 2001; Trigueros et al., 2000a, 2000b) or pigment analysis (Ansotegui et al., 2001, 2003). The latest intensive studies on the phytoplankton community of the estuary were performed in the late 90s, and for the last 20 years, the study of the phytoplankton community of the Urdaibai estuary has been reduced to quarterly samplings carried out to comply with the requirements of the WFD. Additionally, to the best of our knowledge, no previous studies targeting phytoplankton have been carried out in the Urdaibai estuary based on molecular techniques, and therefore, there is limited phytoplankton identification at the species level, especially for pico- and nano-phytoplankton.

The present study has two main aims: (1) the description of the phytoplankton community composition, by combining microscopy and V4 18S rDNA metabarcoding, in relation to the environmental conditions of the Urdaibai estuary with fortnightly samplings for an entire year, and (2) the comparison of the two methods used for the phytoplankton community characterisation. Regarding the former, strong correlations between salinity and/or nutrients and the dominant phytoplankton taxa are expected, due to the marine influence and the presence of the WWTP in the estuary. For the latter, differences in taxa richness and abundance estimations are presumed, due to the limitations of both techniques.

2. Materials and methods

2.1. Study area

This study was performed at the Urdaibai estuary (also known as Gernika estuary, Mundaka estuary or Oka estuary), a meso-macrotidal system located in the southeastern Bay of Biscay (43°22′N, 2°43′W) (Fig. 1). The climate of the area is temperate-oceanic, with moderate winters and warm summers, and it is under the influence of the Gulf Stream and the atmospheric westerlies in the middle and upper troposphere (Usabiaga et al., 2004).

The Urdaibai estuary is short (12.5 km long) and shallow, and it is formed by the tidal part of the Oka River (Villate et al., 2017). The total estuarine area (1.89 km^2) is big when compared to its drainage basin (140 km^2), and therefore, the contribution of freshwater (mean flow of 0.59 m^3/s) is small compared to the total volume of the estuary (3293100 m^3) (Villate et al., 1989, 2008). In addition, river discharge is usually low in relation to the tidal prism (Villate et al., 2017). Consequently, tidal cycles have a great impact and most of the estuary is marine dominated at high tide (Valdes-Weaver et al., 2006). The water residence time is short (days), and the stratification varies within the system, with partially stratified conditions in the middle and inner area and a well-mixed water column in the outer zone, due to the high tidal flushing (Barroeta et al., 2020; Villate et al., 2017). However, this estuarine system can vary considerably in its volume and flushing rates,

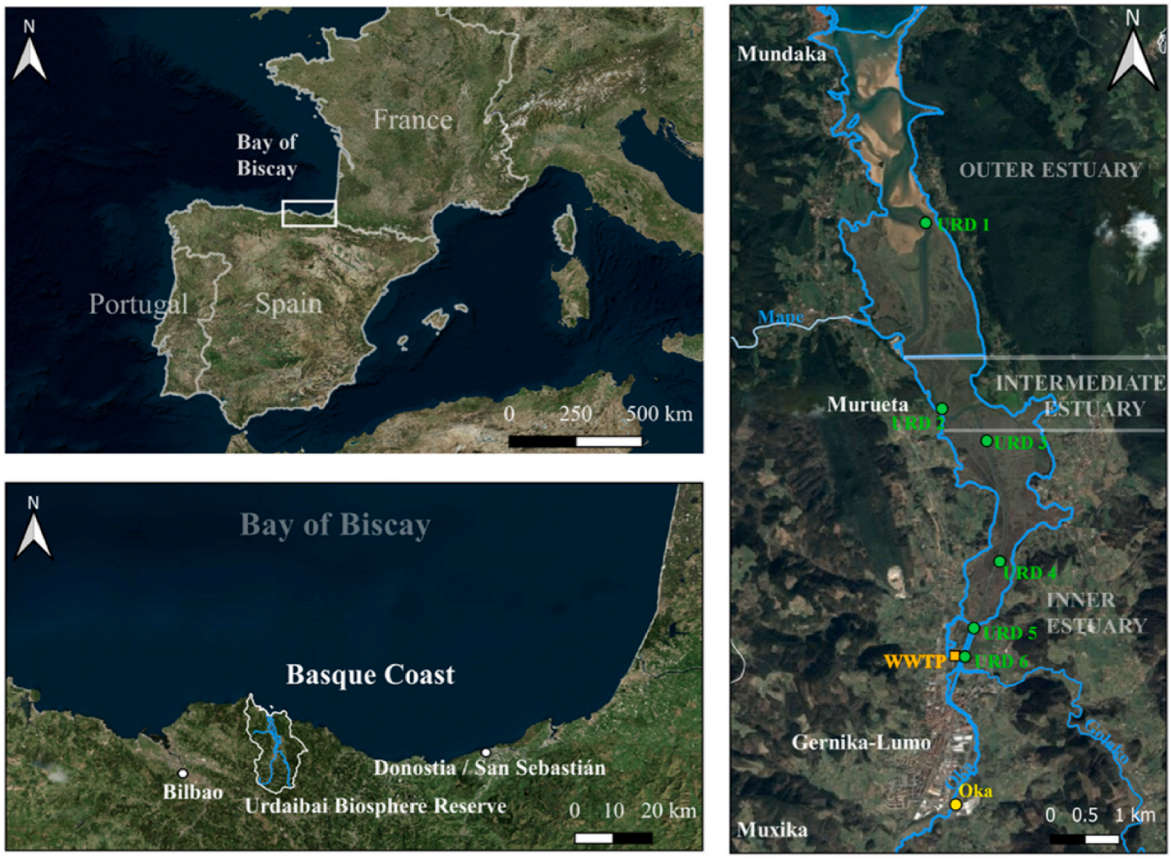

Fig. 1. Study area and sampling stations. On the left, the location of the Urdaibai estuary and the Urdaibai Biosphere Reserve in the Bay of Biscay and the Basque Coast is shown. On the right, the Urdaibai estuary is divided into the outer estuary (sampling station URD1), intermediate estuary (station URD2) and inner estuary (stations URD3, URD4, URD5 and URD6). The discharge point of the wastewater treatment plant (WWTP) and the sampling station at Oka River (Oka) are also located in the inner estuary.

owing to its geomorphology and, mainly, due to the torrential nature of the Oka River, since large flows that change the typical pattern appear occasionally (Madariaga et al., 1994). This has a direct effect on the physicochemical and biological properties of the estuary, causing changes in the phytoplankton communities on a short time scale (Madariaga et al., 1994; Uriarte, 2001) as well as the proliferation of particulate matter with a high proportion of inorganic materials (Ruiz et al., 1998).

Based on morphology, the estuary is divided in three main areas (Fig. 1) (Villate et al., 1989). The outer estuary is a zone with sandy beaches and extensive intertidal flats, which extends from the outer limit of the system (Mundaka) to Murueta. The middle area (i.e., intermediate estuary), from Murueta to the start of the artificial channel, consists of a central channel bordered by shalt-marshes and a complex system of interlaced secondary channels. The inner estuary is formed by a 4 km long and 15 m wide artificial channel built in the beginning of the 20th century that reaches the town of Gernika and is bordered by reed beds.

2.2. Sampling and data acquisition

Sampling was conducted every 15 days from September 2019 to September 2020, at six permanent sampling stations located within the longitudinal axis of the estuary (Fig. 1) to cover the entire salinity gradient: one in the outer part (URD1), one in the middle area (URD2) and four in the inner estuary (URD3, URD4, URD5 and URD6). The last sampling station (URD6) was located in the WWTP of Gernika and is near the inner limit of seawater penetration. An additional sampling station was set up in the Oka River to monitor the physicochemical properties of the river. Therefore, a total of 161 samples were collected, corresponding to 23 sampling days for each of the 7 sampling stations.

Estuarine water samples were always collected at high tide with a 2.5 L plastic Niskin bottle, 0.75 m below the surface in an hour long transect from URD1 to URD6. Only subsurface samples were considered in this study because bottom samples could have been affected by sediment resuspension processes (Madariaga and Orive, 1989). The collected water was used for the analysis of inorganic nutrients, phytoplankton abundance and composition and the determination of photosynthetic pigments. At each station, salinity, temperature, conductivity, pH, dissolved oxygen, oxygen saturation and turbidity were measured *in situ*, using the multi-parameter water quality metre EXO2 (YSI). Water transparency was estimated using a Secchi disc, and water column depth was registered with the GPS sounder of the boat. In the Oka River station, the water was collected from the surface directly, due to the very low depth present at this station, for the analysis of inorganic nutrients, together with the *in situ* measurement of the above-mentioned physicochemical parameters using the EXO2 (YSI).

The dissolved inorganic nutrients analysed were ammonium, nitrite, nitrate (calculated from the total oxidised nitrogen), orthophosphate and silicate. The analyses were carried out using VIS/UV colorimetry in an automatic 5-channel analyser with segmented flow at the Chemical Laboratory of the Marine Research Unit of the AZTI Foundation in Pasaia (Gipuzkoa). The individual determinations of these dissolved inorganic nutrients were based on methods that apply classical and widely used colorimetric reactions, both for inland and marine waters (GO-SHIP manual by Hydes et al., 2010). The quantification limit for ammonium, nitrate and silicate was 1.6 μmol/L, 0.4 μmol/L for nitrite and 0.16 μmol/L for phosphate. For measurements below the quantification limit, a concentration equal to 50% of the limit was assumed for

the estimation of average values.

The data on daily-accumulated rainfall, temperature and hours of sunshine were provided by the Basque Agency of Meteorology (Euskalmet). For accumulated rainfall and temperature, data were available from two nearby meteorological stations: Muxika (43°17′N, 2°41′W, 16 m height) and Arteaga (43°20′N, 2°39′W, 19 m height). Therefore, the average values from both stations were taken. As for the number of hours of sunshine, data were available from Bilbao Airport (20 km from the estuary). The Provincial Council of Bizkaia provided Oka River flow data, corresponding to the Muxikas' gauging station.

2.2.1. Microscopy

Water samples used for the study of the phytoplankton community by microscopy were fixed with acidic Lugol's solution (0.4% v/v) right after collection and stored until analysis in dark and cool (4 °C) conditions, in 125 ml topaz borosilicate bottles. Taxonomic identification and cell counts were performed following the Utermöhl sedimentation method (Edler and Elbrächter, 2010) under a Nikon diaphot TMD inverted microscope, for subsamples of 10 or 50 ml depending on the density. Transects at different magnifications (100 × , 200 × or 400 ×) were carried out depending on the taxa's abundance and size. In order to count the larger and less abundant taxa, the whole chamber was analysed at low magnifications (200 ×). Most of the taxa were identified to the genus level, and the nomenclature of the identified taxa was standardised according to AlgaeBase (Guiry and Guiry, 2018).

2.2.2. Phytoplankton biomass

Taking into account the limitations that the different techniques present to express the phytoplankton biomass, two different approaches were applied for its estimation: carbon content (which is based on the microscopy cell counts) and the chlorophyll *a* (chl *a*) concentration.

For the calculation of carbon content, first, the biovolume was determined by assigning each taxon a mean equivalent spherical diameter (ESD), based on Olenina et al. (2006), which takes into account cell shape and size. Afterwards, the biomass was calculated using the equation as reported for marine phytoplankton by Montagnes et al. (1994): Carbon content = $0.109 \times \text{Volume}^{0.991}$, where carbon content is expressed in pg C/cell and volume in μm^3.

Measurement of chl *a* concentration in water samples is commonly used (e.g. the WFD) as a direct proxy for total phytoplankton biomass. For its determination, samples collected with the Niskin bottle and stored in opaque plastic bottles were filtered (0.4–4 L) with gentle vacuum (<150 mm Hg) onto Whatman GF/F glass-fibre filters (47 mm diameter, Whatman International Ltd.) in dark conditions. Filters were immediately frozen in liquid nitrogen and stored at −80 °C until pigment extraction, which was always less than 15 days after filtration. Chl *a* was extracted, under low light, with 5 ml of 90% acetone, using a glass rod for grinding, and the extracts were then filtered through syringe filters (Millex, 0.22 μm pore size) to remove cell and filter debris. The analysis was carried out by high performance liquid chromatography (HPLC), following the method described by Zapata et al. (2000), with a modification in solvent A and the equipment explained in Seoane et al. (2009b).

2.2.3. eDNA metabarcoding

Water samples for eDNA analysis were pre-filtered *in situ* through a 200 μm mesh (Millipore Nylon Nets) and subsequently stored in opaque plastic bottles. Once in the laboratory, samples were filtered (0.2–3.5 L) through a 0.8 μm MCE-membrane filter (MF-Millipore) with gentle vacuum (<150 mm Hg). The filter was then kept in PowerWater DNA Bead Tubes (QIAGEN) and frozen at −80 °C, until further use for metabarcoding.

DNA extraction, amplification and sequencing of the 18S rRNA V4 region was carried out at the Sequencing and Genotyping Unit of Genomics Facilities from the University of the Basque Country (SGIker – UPV/EHU). The DNA was extracted from the filters with the DNeasy Power Water Kit (QIAGEN), following the kits' specific protocol. In order to account for contaminations, an extraction negative control (EXT-C) was included, obtaining a quantification value below the detection threshold (<2 ng/μL). As indicative data, the mechanical lysis for the extraction was carried out on a flat-bottom vortex, at 3000 rpm for 5 min, and the volume of the elution buffer was decreased to 60 μL. The primers used for amplification of V4 18S rRNA were TAReuk454FWD1 and TAReukREV3 (Stoeck et al., 2010), modified by Piredda et al. (2017) (5′-CCAGCASCYGCGGTAATTCC-3′ and 5′-ACTTTCGTTCTTGATYRATGA-3′), to which the necessary tails for later insertion of the adapters and indices compatible with the Illumina platform were added (5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC-3′ and 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA-3′). Amplification was carried out following the protocol described by Piredda et al. (2017) but with certain modifications. More specifically, KAPA HiFi Hotstart ReadyMix (Roche Kapa Biosystems) was used, and the activation time and temperature of the thermocycler programme were adapted: an initial 4 min at 95 °C, followed by 10 cycles at 95 °C for 10s, 44 °C for 30s, 72 °C for 15s; a second step by 15 cycles at 95 °C for 10s, 62 °C for 30s, 72 °C for 15s; and a final cycle of 7 min at 72 °C. 2 μL of DNA at stock concentration were added between 15 and 80 ng/μL by nanodrop. 1 μl of each primer (10 μM) was added to the total reaction volume (25 μL). Both extraction (EXT-C) and amplification (NTC) negative controls were included. After electrophoresis to assess PCR product yield, purification was carried out by beads (CleaNNA NGS kit), followed by the subsequent indexing reaction with the Illumina Nextera XT v2 set A and set B kit (following the standard protocol of the commercial house). Indexed products were checked by electrophoresis, and purified with beads was carried out in pool. Final library quantification was carried out by Qubit. The sequencing was performed on an Illumina MiSeq instrument with a 300-bp paired-end protocol (MiSeq v3 chemistry). The loading library concentration was 6 pM, and 10% PhiX was used as an internal control. Obtained quality control values for the sequencing run were > Q30 = 76,53% and %PF: 91.65. After post-filtering, a total of 2 × 17,753,716 reads assigned to marker 18S rRNA V4 were obtained. The average coverage of the samples was 120.000. Less than 1100 and 500 reads were assigned for both extraction and amplification negative controls, respectively. All raw sequence files have been submitted to the European Nucleotide Archive (ENA) (Cummins et al., 2022) with the study accession number PRJEB48801 (available at http://www.ebi.ac.uk/ena/data/view/PRJEB48801).

As for the bioinformatics, the sequence processing was performed using DADA2 (Callahan et al., 2016). The analysis was carried out through Zorba, the High Performance Computing (HPC) system of IMBBC (Institute of Marine Biology, Biotechnology and Aquaculture) (Zafeiropoulos et al., 2021). Within the pipeline, reads were quality filtered and trimmed by the *filterAndTrim* function with the following parameters: maxN = 0, maxEE = c(5,5), truncQ = 2, rm.phix = TRUE, minLen = 100, compress = TRUE, multithread = TRUE. Error rate calculation and dereplication were performed at default settings set at DADA2 tutorial. Amplicon sequence variant (ASV) inference was completed by a distance of 1 nucleotide (maxMismatch = 1). Merging was completed with a minimum overlap of 20 bp, and chimera removal was completed with the *removeBimeraDenovo* function under the "consensus" method. Details on the sequence processing of the 18S rRNA data are available in Supplementary Material I, Table 1. The reads were subsequently classified against the Protist Ribosomal Reference Database (PR2 4.14, https://pr2-database.org), using the *assignTaxonomy* function, which contains fewer sequences (~180,000) than Silva, but these sequences are periodically re-annotated by experts (Egge et al., 2021; Guillou et al., 2013).

The preparation of the ASV-table was done in R v.3.6.0. (R core Team, 2021) and Excel 2007. Out of the 138 samples processed, 8 were removed due to problems during amplification and scarce reads: URD3 and 4 from 20/11/2019; URD1, 2, 3, 4 and 5 from the 04/12/2019; and

Table 1
Spearman correlation analysis results for microscopy cell counts and eDNA metabarcoding reads of the taxa selected for the CCA, showing rho (ρ) and p values.

Microscopy	eDNA metabarcoding	Correlation analysis
Tetraselmis spp. – *Tetra(Mi)*	*Tetraselmis* spp. – *Tetra(Me)*	ρ 0.6; $p < 0.01$
	Oltmannsiellopsis viridis – *O.viri (Me)*	ρ 0.65; $p < 0.01$
Centric diatoms <10 μm – *Cen<10(Mi)*	*Cyclotella atomus* – *C.ato (Me)*	ρ 0.55; $p < 0.01$
	Cyclotella spp. – *Cycl (Me)*	ρ 0.58; $p < 0.01$
Centric diatoms >10 μm – *Cen>10(Mi)*	*Conticribra guillardii* – *C.gui (Me)*	ρ 0.66; $p < 0.01$
Chaetoceros tenuissimus – *C. ten(Mi)*	*Chaetoceros tenuissimus* – *C.ten (Me)*	ρ 0.79; $p < 0.01$
Minutecellus polymorphus – *M.pol(Mi)*	*Minutecellus polymorphus* – *M.pol (Me)*	ρ 0.85; $p < 0.01$
Blixaea quinquecornis – *B. quin(Mi)*	*Blixaea quinquecornis* – *B.quin (Me)*	ρ 0.73; $p < 0.01$
Plagioselmis sp. – *Plagio(Mi)*	*Hemiselmis cryptochromatica* – *H. crypto(Me)*	ρ 0.78; $p < 0.01$
Teleaulax acuta – *T.acu(Mi)*	*Teleaulax acuta* – *T.acu(Me)*	ρ 0.76; $p < 0.01$
Urgorri complanatus – *U. comp(Mi)*	*Urgorri complanatus* – *U.comp (Me)*	ρ 0.75; $p < 0.01$
Prymnesiales – *Prym(Mi)*	*Chrysochromulina* sp. – *Chryso (Me)*	ρ 0.76; $p < 0.01$
	Prymnesium spp. – *Prymne(Me)*	ρ 0.72; $p < 0.01$

URD2 from the 20/03/2020.

A total of 36,562 ASVs were defined by eDNA metabarcoding; however, since this study is focused on phytoplankton, all reads assigned to other organisms were excluded from the processed ASV tables, selecting a total of 14,672 ASVs. ASVs that were found only in the control samples were removed from the subsequent analysis; in addition, for ASVs that were found in the control samples in higher abundances than those in the actual samples, their abundances in the control samples were subtracted from the abundances in the actual samples. Among the selected ASVs, 615 unique phytoplankton taxa were identified, but only those that reached taxonomic assignment to at least the genus level were considered in this study. Moreover, we filtered ASVs for those that made up at least 0.01% of the total reads of at least one of the 130 samples and also excluded the taxa appearing in less than 1% of the samples processed.

2.3. Data analysis

Regarding environmental conditions, the Spearman correlation was performed to test for environmental variable correlation in the Urdaibai estuary. The Bonferroni correction was applied to the correlation analysis, and the results were considered significant when they showed a rho (ρ) value higher than |0.7| and a p value lower than 0.05 (Córdoba-Mena et al., 2020). In addition, the principal component analysis (PCA) was applied using 10 physicochemical variables (salinity, temperature, pH, dissolved oxygen, oxygen saturation, turbidity, ammonium, nitrate, phosphate and silicate) to elucidate the main environmental drivers shaping the abiotic environment of the Urdaibai estuary and the site-specific differences. Conductivity, Secchi disc depth and nitrite concentration were not considered in the PCA to avoid redundancy with salinity, turbidity and ammonium, respectively. The correlation-matrix was applied in the PCA, which implies normalising all variables using division by their standard deviations, since the variables were measured in different units (Hammer and Harper, 2006).

To analyse the phytoplankton community composition, non-metric multivariate analyses were performed to visualise similarity/dissimilarity among samples and determine the spatio-temporal dynamics within the Urdaibai estuary. Non-metric multidimensional scaling (nMDS) in 2D was performed, creating an ordination based on the Bray-Curtis similarity index, for the graphical representation of the interrelationships among samples according to the phytoplankton community composition. Independent nMDS were performed for the data sets obtained by microscopy (cell abundances) and metabarcoding (standardised read counts). To complement this, permutational multivariate analysis of variance (PERMANOVA), a non-parametric multivariate statistical permutation test, was performed to test significant differences between groups based on the Bray-Curtis distance matrix (Anderson, 2001). PERMANOVA was tested as a "two-way PERMANOVA" to account for the spatial (sampling stations) and seasonal variability of the community, as well as for the interaction of these two factors. Later, a "one-way PERMANOVA" was performed, with the Bonferroni corrected p values, for each of the factors (spatial and seasonal) for the pairwise comparison of groups along the gradients. This decision was based on the conclusions of Anderson and Walsh (2013), which conducted a simulation-based comparison of PERMANOVA and ANOSIM and found that PERMANOVA is more robust in general for ecological data. Finally, a similarity percentages analysis (SIMPER) was performed to determine which phytoplankton taxa contributed the most to the observed dissimilarity between samples, for both spatial and temporal variability. As for the nMDS, the PERMANOVA and SIMPER analysis were also conducted independently for microscopy and metabarcoding analysis.

The relationship between the phytoplankton community and the environmental data was explored using a canonical correspondence analysis (CCA). A single CCA was performed, with both microscopy (with cell abundance) and DNA metabarcoding (read abundances) data, after normalising the number of reads with sample volume. The environmental variables chosen for the CCA were the same as in the PCA. The phytoplankton taxa selection was based on choosing the most relevant (abundant and/or frequent) taxa of the estuary that were detected by both microscopy and metabarcoding. Thus, results from the SIMPER analysis were taken into account, since this determined the main taxa contributing for the variation in the community composition within the estuary. The taxa selection was done firstly by choosing common taxa in the top 15 organisms of the SIMPER results for microscopy and metabarcoding (Supplementary Material I, Table 10). For the remaining taxa, that did not have a match (e.g. unidentified centric diatoms), the Spearman correlation analysis was performed between these and the organisms from the metabarcoding analysis that morphologically could correspond to the same taxa. Matching was only accepted when the correlation was significant and the organisms from both techniques followed the same morphological and ecological characteristics. The selected taxa and the Spearman correlation analysis results for each matching are shown in Table 1.

Finally, the Spearman correlation was tested in order to correlate the chosen phytoplankton taxa and phytoplankton biomass with the environmental conditions of the Urdaibai estuary.

All the data analyses was performed with Past 4.05 (Paleontological Statistics), a software for scientific data analysis (Hammer et al., 2001).

3. Results

3.1. Environmental conditions

The physicochemical variables showed the expected spatial patterns and seasonal trends in a temperate estuary during the study period (Fig. 2; Supplementary Material I, Table 2). Salinity and temperature increased towards the outer part of the estuary and summer season. Oxygen concentration showed both the minimum (1.35 mg/L) and maximum (11.25 mg/L) values at the innermost station (URD6) and, overall the highest mean oxygen concentrations of the estuary were recorded in winter (8.79 mg/L) and the lowest in summer (6.27 mg/L). Both pH and turbidity increased towards the inner part of the estuary, and regarding seasonality, the former presented the highest mean values in summer (8.7) and the latter in autumn (7.1 NTU).

Inorganic nutrient concentrations showed a marked spatial gradient

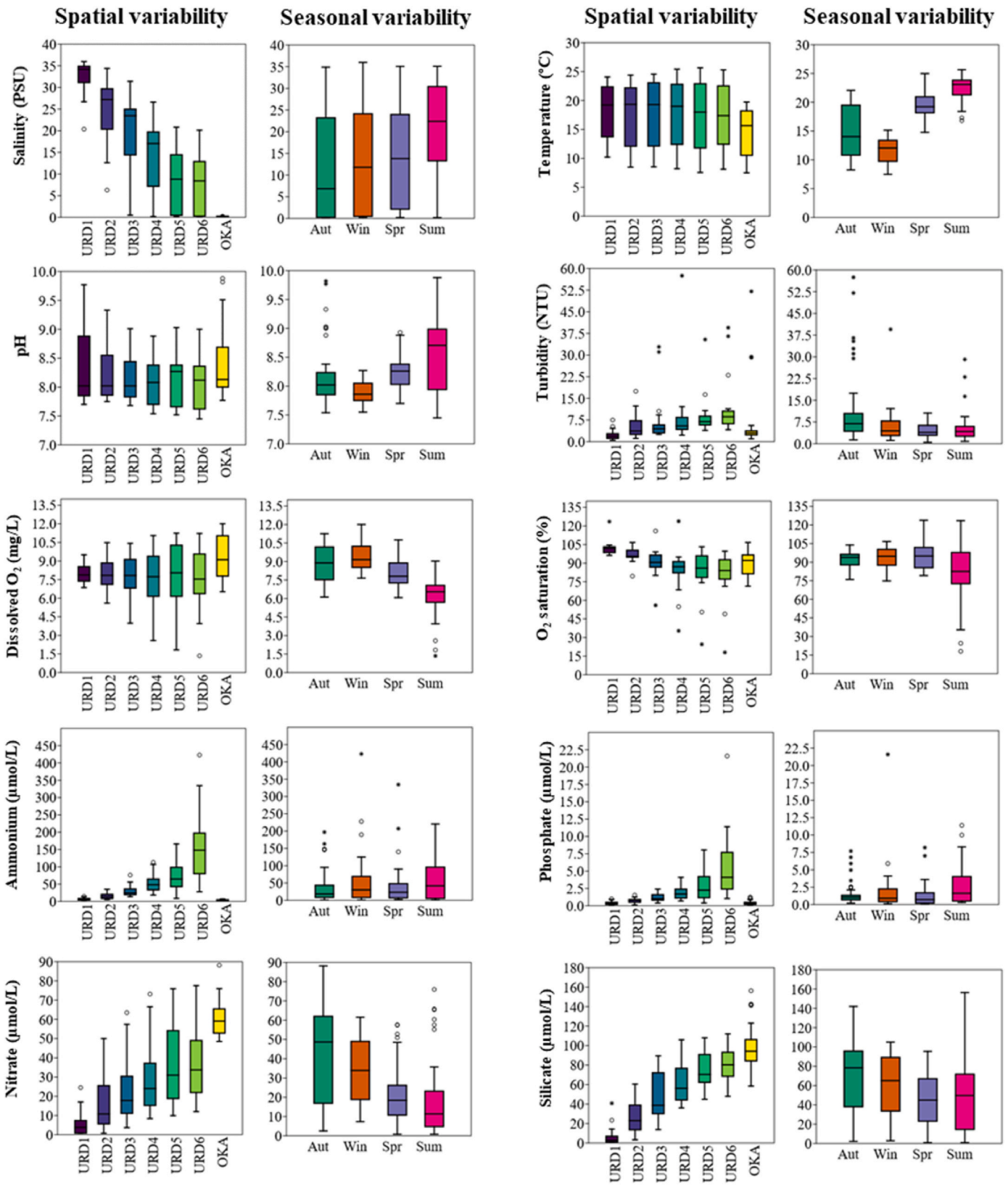

Fig. 2. Spatial and seasonal variability of the main hydrographic and physicochemical conditions in the Urdaibai estuary. For nutrients with values below the quantification limit, the value of half of the limit was considered. The box represents the Interquartile Range (IQR), data between Q1 (25th percentile) and Q3 (75th percentile); the line inside the box is the median. The lower whisker shows the Q1-1.5*IQR, and the upper Q3+1.5*IQR. Circles and asterisks represent outliers. Aut: autumn; Win: winter; Spr: spring; Sum: summer.

Table 2
The number of phytoplankton taxa identified, at least to the genus level, by microscopy, eDNA metabarcoding or both techniques within the Urdaibai estuary. "Others" category contains: pelagophytes, bolidophytes, raphidophytes, synurophytes, olisthodiscophytes and rhodophyta.

	Diatoms	Dinoflagellates	Green algae	Cryptophytes	Haptophytes	Chrysophyceans	Dictyochophyceans	Others	Total
Microscopy	32	28	11	1	5	0	1	0	78
eDNA metabarcoding	124	83	55	20	19	16	14	18	349
Both techniques	35	14	2	5	1	0	1	0	58

along the longitudinal axis of the estuary, with increasing concentrations towards the inner area (Fig. 2; Supplementary Material I, Table 2). However, the source of the different nutrients varied. The station located in the Oka River showed very low median concentrations of ammonium (3.4 μg/L) and phosphate (0.35 μg/L), which increased highly at the URD6 station (143 μg/L and 4.1 μg/L, respectively) located close to the WWTP. On the contrary, the silicate and nitrate median concentrations in the Oka River (94.3 μg/L and 59.1 μg/L, respectively) were higher than the maxima values observed at URD6 (80.3 μg/L and 33.7 μg/L, respectively). Nutrient concentrations varied, ammonium (2.9–423 μg/L), phosphate (0.2–5.58 μg/L), nitrate (0.8–77.55 μg/L) and silicate (0.8–112 μg/L), and decreased towards the outer estuary (Fig. 2). As for their seasonal variability, ammonium and phosphate reached their highest median concentrations in summer (58 μg/L and 1.95 μg/L, respectively), while the lowest were found in autumn. Conversely, nitrate and silicate followed an inverse temporal pattern, reaching their highest median concentrations in autumn (36 μg/L and 64.7 μg/L, respectively), and their lowest in summer (11 μg/L and 42 μg/L, respectively).

In addition, several of the analysed environmental variables showed strong correlations. As expected, due to the dependence of these variables, Secchi disc depth and turbidity were negatively correlated (ρ −0.89; $p < 0.01$); salinity and conductivity showed a strong positive correlation (ρ 0.98; $p < 0.01$); and nitrite and ammonium were also positively correlated (ρ 0.77; $p < 0.01$). Salinity was negatively correlated with turbidity (ρ −0.75; $p < 0.01$), nitrate (ρ −0.91; $p < 0.01$) and silicate (ρ −0.92; $p < 0.01$). Temperature and dissolved oxygen were also negatively correlated (ρ −0.88; $p < 0.01$), and ammonium and phosphate showed positive correlation (ρ 0.93; $p < 0.01$).

The application of PCA (Fig. 3) to 10 physicochemical parameters indicated that 68.5% of the variability contained in the data set was explained by only two PCs (principal components). The loading values (correlations) of the first two PCs are displayed in Supplementary Material I (Table 4). PC1 was interpreted as the spatial variation of the environmental conditions along the longitudinal axis of estuary, as it was highly correlated with salinity, silicate and nitrate, with large factor loadings (>|0.91|). PC2 was interpreted as the trophic status variation of the samples, since it is mainly correlated with dissolved oxygen, oxygen saturation, ammonium and phosphate (with loadings above |0.78|). Among the physicochemical parameters analysed, salinity was the main environmental driver shaping the spatial variability of the abiotic environment along the Urdaibai estuary, with a correlation of −0.926. This indicates that the environmental conditions of the estuary are mostly dependent on the tidal effect and/or the Oka River flow. This graphical representation confirmed the description of environmental parameters explained above and the strong spatial variability present in the estuary. It also reflected the different variability of environmental conditions during the year at the different stations, as the stability was higher towards the outer estuary since the range of values was the narrowest for most of the analysed parameters.

Regarding the meteorological conditions, autumn-winter was the period with the highest precipitation, with a monthly maximum in November 2019 (453 mm), and a minimum in July 2020 (22 mm). River flow values ranged between 0.001 and 7.4 m^3/s during sampling weeks, with the highest values found in autumn-winter (median of 0.56 m^3/s) and the lowest in summer-spring (0.15 m^3/s). The insolation (hours of sunshine) was the highest in spring and summer (Supplementary Material I, Table 3).

3.2. Phytoplankton biomass estimations

Total phytoplankton biomass, estimated by carbon content and chl *a* concentration, showed spatio-temporal patterns in the Urdaibai estuary (Fig. 4).

Total carbon content of the phytoplankton community ranged between 1 and 916 μg C/L within the estuary during the study period. URD1 registered the lowest annual median carbon content of the estuary (38.9 μgC/L), and the median maxima were recorded in URD 5 and URD6, with concentrations of 112 μgC/L and 94 μgC/L, respectively. However, there was not an increasing gradient from outer to inner

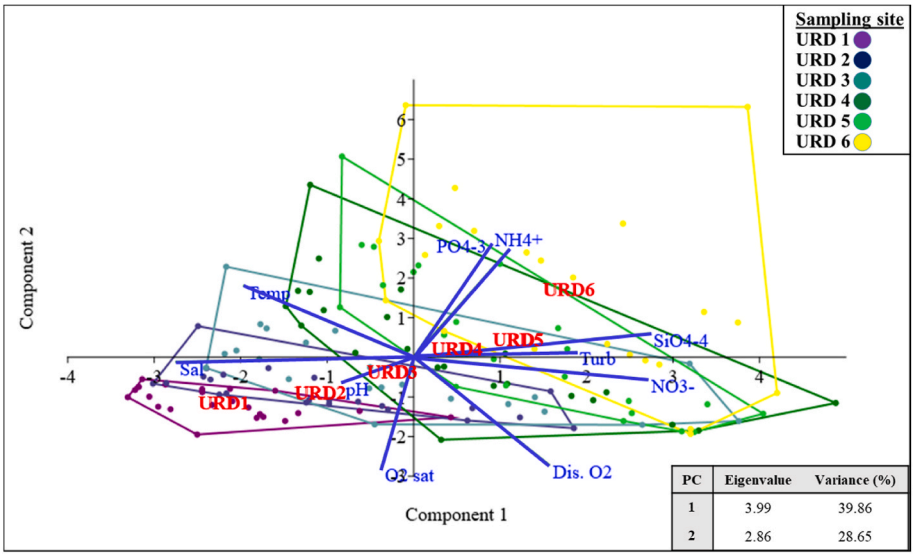

Fig. 3. PCA plot of the main hydrographic and physicochemical conditions in the Urdaibai estuary. A correlation matrix was used, and the Bootstrap N was 999. Samples are coloured based on the sampling stations and grouped by convex hulls, which include the name of the group (station names). Environmental factors are shown as gradients with blue lines. Eigenvalues and variance percentages for PC1 and PC2 and the legend are located on the right side of the figure. Sal: salinity; Temp: temperature; O2 sat: oxygen saturation; Dis.O2: dissolved oxygen; Turb: turbidity; NH_4^+: ammonium; PO_4^{-3}: phosphate; NO^{3-}: nitrate; SiO_4^{-4}: silicate.

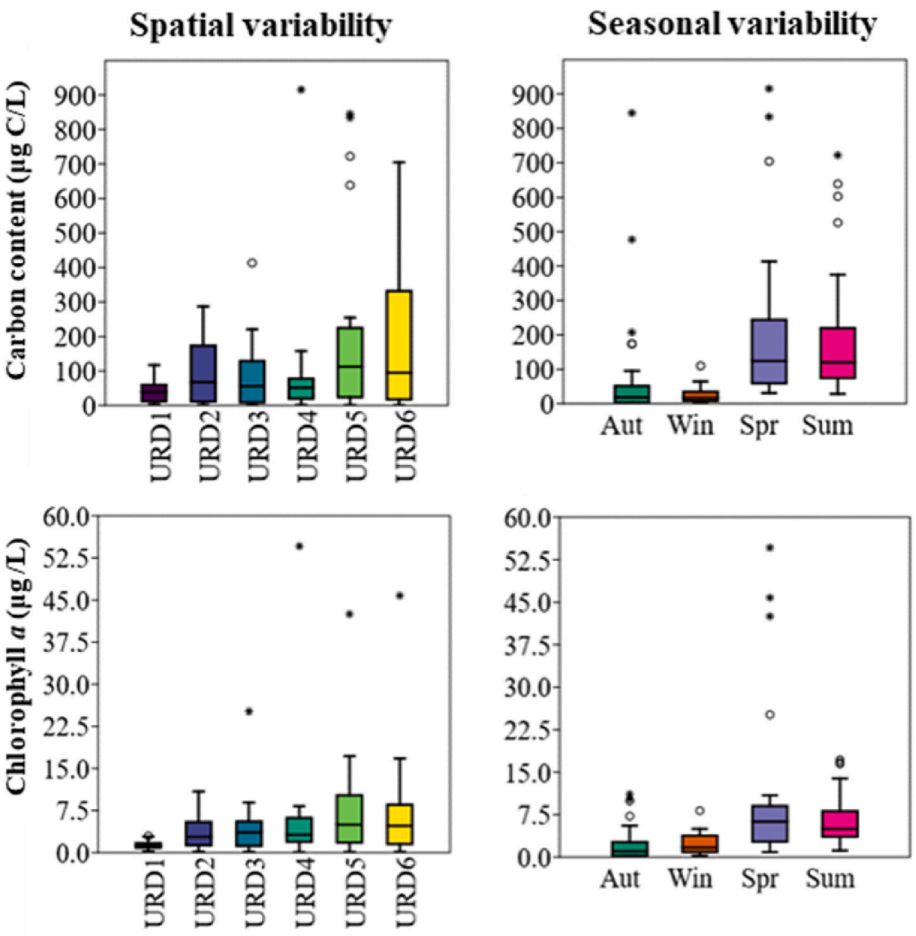

Fig. 4. Spatial and seasonal variability of total phytoplankton biomass (up, carbon content; down, chl *a*) within the Urdaibai estuary during the study period. The box represents the Interquartile Range (IQR), data between Q1 (25th percentile) and Q3 (75th percentile); the line inside the box is the median. The lower whisker shows the Q1-1.5*IQR, and the upper Q3+1.5*IQR. Circles and asterisks represent outliers. Aut: autumn; Win: winter; Spr: spring; Sum: summer.

estuary, since the median carbon content was higher in URD2 (67.3 µgC/L) than in URD3 and URD4 (approximately 55 µgC/L). As for the temporal patterns, the lowest concentrations were found in winter, with a median value of 16.4 µg C/L in the estuary, and the highest in spring (124 µgC/L) and summer (120 µgC/L).

Chl *a* ranged between 0.09 and 54.61 µg/L throughout the year within the estuary and showed an increasing gradient towards the inner estuary. The outer estuary (URD1) registered chl *a* values between 0.21 and 2.97 µg/L and had the lowest annual median chl *a* concentration (1.18 µg/L). In the middle estuary (URD 2), the chl *a* concentration ranged between 0.17 and 10.89 µg/L, with a median annual concentration of 2.8 µg/L. In URD3 and URD4, chl *a* median concentration was slightly higher than in the middle estuary (3.5 µg/L approximately), and the chl *a* concentration range was much wider than in the outer and middle estuary, with values between 0.09 µg/L and 54.61 µg/L. URD4 registered the chl *a* maximum of the year in the estuary. At URD5 and URD6, the chl *a* concentration was the highest of the entire estuary on average, with a median annual concentration of approximately 5 µg/L. As for the temporal patterns, the studied year was divided into two main periods when it comes to chl *a*: a low concentration period during autumn and winter, with median concentrations of 1.04–1.67 µg/L; and a high concentration period during spring and summer, with median concentrations of 5–6.26 µg/L. The highest chl *a* concentrations registered during the year were in spring (54.61 µg/L in April 2020).

3.3. Phytoplankton community composition

The phytoplankton community composition of the Urdaibai estuary was surveyed with both microscopy and eDNA metabarcoding, showing some common and unique aspects for each of the techniques.

The phytoplankton community of the Urdaibai estuary included 136 taxa identified with microscopy, and 407 taxa identified with eDNA metabarcoding (Supplementary Material I, Table 5), of which 58 taxa were shared by both approaches. A total of 349 species or genera were unique to eDNA metabarcoding (Table 2; Supplementary Material I, Table 5), including green algae, cryptophytes and haptophytes that presented three to four times more diversity than with microscopy. Other minor groups, such as chrysophyceans or dictyochophyceans were also identified by eDNA metabarcoding, while were mostly absent from microscopy. A total of 78 taxa were identified with microscopy only (Table 2; Supplementary Material I, Table 5). Despite the difference in the taxa richness obtained by each approach, diatoms were the most diverse group, containing almost half of the taxa found, followed by dinoflagellates and green algae.

The outer estuary registered 127 and 363 different phytoplankton taxa with microscopy and metabarcoding, respectively. Diatoms were the dominant phytoplankton group, with a 44% and 40% of average contribution to the total abundance during the study year according to microscopy cell counts and metabarcoding reads, respectively (Fig. 5). Among them, microscopy identified *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C.Lewin, *Minutocellus polymorphus* (Hargraves & Guillard) Hasle, Stosch, & Syvertsen, *Chaetoceros* spp. Ehrenberg,

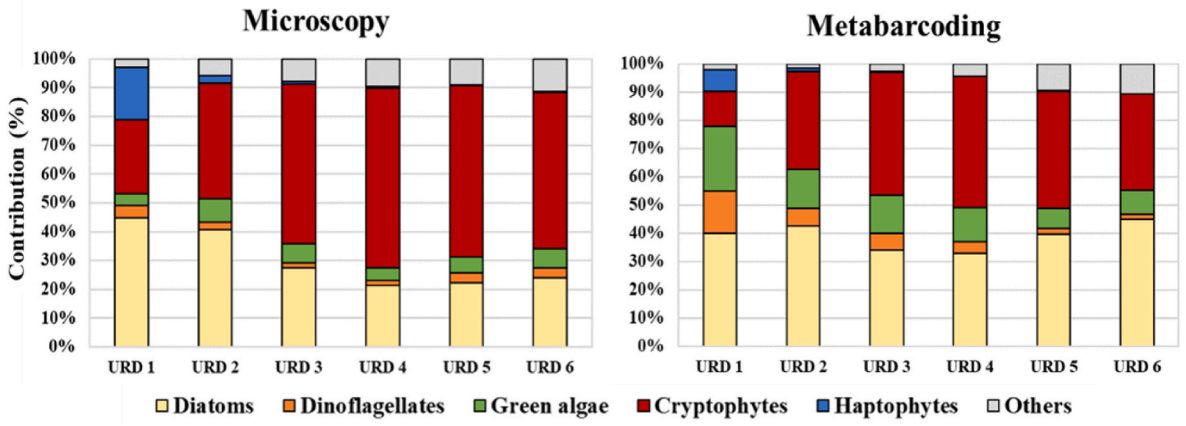

Fig. 5. Average relative contribution percentages of the main phytoplankton groups to the total community composition of each sampling station within the Urdaibai estuary, by microscopy cell counts (left) and DNA metabarcoding reads (right), at each sampling station.

Leptocylidrus danicus Cleve and *Pseudo-nitzschia* spp. H.Peragallo as the most frequent taxa, together with unidentified centric and unidentified pennate diatoms, that were recorded in more than 60% of the samples. For metabarcoding, *Chaetoceros tenuissimus* Meunier, *Minutocellus polymorphus*, *Minidiscus variabilis* Kaczmarska and *Mediolabrus comicus* (H. Takano) Yang Li occurred in all samples analysed and were the dominant taxa in the outer estuary. *Pseudo-nitzschia* spp. (*P. seriata* (Cleve) H. Peragallo especially), *Cylindrotheca closterium* and *Leptocylindrus convexus* D.Nanjappa & A.Zingone were also frequent diatoms of URD1, which were more recurrent than in the remaining sampling stations. In addition, microscopy revealed that *M. polymorphus* was the species that recorded the highest cell abundance in URD1, with a maximum of 9.1×10^6 cells/L in summer (Supplementary Material II). In contrast to the other sampling stations, haptophytes had a significant importance in the phytoplankton community of URD1, contributing 18% and 8% to the total community composition according to microscopy and metabarcoding, respectively. While microscopy was only able to detect a few taxa (Prymnesiales and *Emiliania huxleyi* (Lohmann) W.W.Hay & H. Mohler being the most frequent), genetic method identified *Chrysochromulina* spp. Lackey (especially *C. scutellum* Eikrem & Moestrup and *C. rotalis* Eikrem & Throndsen), *Prymnesium* spp. Massart, *Phaeocystis* spp. Lagerheim (mainly *P. cordata* A.Zingone & M.J.Chrétiennot-Dinet, but also *P. globose Scherffel*), *Gephyrocapsa oceanica* Kamptner, *Haptolina* sp. Edvardsen & Eikrem and *Dicrateria rotunda* (N.Reynolds) El M.Bendif & I.Probert as the most recurring taxa. URD1 was the station with the highest contribution of dinoflagellates to the total community composition (4% and 15% for microscopy and metabarcoding, respectively), which was especially important in spring and summer, with presence of *Warnowia* sp. Lindemann, *Heterocapsa* spp. F. Stein (especially *H. pygmaea* Lobelich III, R.J.Schmidt & Sherley *and H. rotundata* (Lohmann) Gert Hansen), *Gyrodinium* spp. Kofoid & Swezy (*G. fusiforme* Kofoid & Swezy, *G.dominans* Hulburt and *G.spirale* (Bergh) Kofoid & Swezy), *Tripos furca* (Ehrenberg) F.Gómez and *Torodinium robustum* Kofoid & Swezy in all samples analysed by metabarcoding. Chlorophytes were largely overlooked by microscopy at the outer station. However, through metabarcoding analysis, chlorophytes were identified as contributing to 24% of the community, being primarily represented by *Ostreococcus lucimarinus* Palenick et al., *Bathycoccus prasinos*, *Micromonas* spp. Manton & Parke (mainly *M. bravo* N.Simon, Foulon & B. Marin and *M. commoda* Baren, Bachy & Worden) and *Pyramimonas australis* Andreoli & Moro, that were the most frequent (100% of the samples) taxa.

In the middle estuary (URD2), the number of identified taxa decreased to 101 by microscopy and 349 by metabarcoding. Similarly to URD1, according to both techniques, diatoms were still the dominant group of the community in terms of average contribution (41–43%), but the contribution of cryptophytes increased, up to 35–40%, becoming co-dominant in this area. Among diatoms, *M. polymorphus* and *C. tenuissimus* were the dominant taxa in URD2, according to both techniques, together with the continuous presence of *Tryblionella apiculata* W.Gregory and *Thalassiosira profunda* (Hendey) Hasle, which were identified by metabarcoding. *M. polymorphus* registered higher abundances in this area than at URD1, with a maximum of 1.5×10^7 cells/L, and showed a clear preference for the summer season. However, *C. tenuissimus* was the dominant diatom species at URD2, reaching an abundance maximum of 2×10^7 cells/L. As for cryptophytes, according to the microscopy analysis, *Plagioselmis* spp. Butcher ex G.Novarino, I.A. N.Lucas, & S.Morrall and *Teleaulax* spp. D.R.A. Hill (especially *T. gracilis* Laza-Martínez) were present in all the samples analysed, with median cell abundances of 5.5×10^5 cells/L and 1×10^5 cells/L, respectively. As for metabarcoding, *T. acuta* (Butcher) D.R.A.Hill and *Hemiselmis cryptochromatica* C.E.Lane & J.M.Archibald were the dominant cryptophytes in the area. Among dinoflagellates, both techniques highlighted *Heterocapsa* spp. (mostly *H. pygmea*, according to the molecular analysis) and *B. quinquecornis* (T.H.Abé) Gottschling as the most abundant and frequent.

In the first section of the channeled area (URD3 and URD4), the number of taxa identified was 85 and 342 by microscopy and metabarcoding, respectively. Cryptophytes were the dominant group of the community, with a median contribution of around 60% according to microscopy and 45% according to metabarcoding. Microscopy described *Plagioselmis* spp., identified in every sample, as the taxa that recorded the highest median abundances in this area, between 7.6×10^6 cells/L (URD3) and 9.6×10^6 cells/L (URD4), together with the frequent presence of *T. gracilis* (91% of the samples) and *T. acuta* (80%). According to the metabarcoding analysis, *T. acuta*, *H. cryptochromatica* and *Urgorri complanatus* Laza-Martínez were the dominant cryptophytes in this area. In addition, both techniques determined that diatoms were the second most contributing group (25–33%), with *M. polymorphus, C. tenuissimus* and *T. apiculata* being the most representative taxa. Metabarcoding revealed that URD4 was the station with the highest chlorophyte diversity (49 taxa) within the entire estuary, among which *Picochlorum* spp. W.J Henley et al., *Oltmannsiellopsis viridis* (P.E.Hargraves & R.L.Steele) M.Chihara & Inouye, *Nannochloris* sp. Naumann, *Chlamydomonas kuwadae* Gerloff and *Ostreococcus* spp. C.Courties & M.-J.Chrétiennot-Dinet (*O. mediterraneus* C.Courties & M.-J.Chrétiennot-Dinet and *O. tauri* C.Courties & M.-J.Chrétiennot-Dinet) were dominant. According to microscopy, which did not identify any of the previously mentioned chlorophytes, this area recorded a higher presence of *Eutreptiella* spp. A.M.Cunha and *Tetraselmis* spp. F.Stein than URD2.

At the innermost area of the estuary (URD 5 and URD 6), the number of identified taxa by microscopy was 58, while DNA metabarcoding

identified 264 taxa in the area. The phytoplankton community description given by both techniques differed substantially in the surroundings of the WWTP (Fig. 5). According to microscopy, cryptophytes were still the dominant group in terms of abundance (57% of the total cell abundance). Specifically, *U. complanatus* was more prevalent in the inner area of the estuary compared to the other sampling stations, as well as this taxon was present in the majority of the analysed water samples, reaching a maximum abundance of 7.7×10^6 cells/L at URD5. Microscopy defined diatoms as the second most contributing group (23%), with the unidentified small (less than 10 μm) centric diatoms being the dominant taxon, with presence in every sample analysed. In this area, additionally, the dinoflagellate *K. foliaceum* (F.Stein) Lindemann was one of the most representative taxa, being present in 90% of the samples, with a median cell abundance of approximately 4.5×10^4 cells/L, and showing a clear preference for the innermost estuary. Metabarcoding, however, described a shared dominance in terms of contribution between diatoms (around 43%) and cryptophytes (around 38%), and *K. foliaceum* was not detected. In addition, molecular techniques revealed that the small centric *Cyclotella* spp. (Kützing) Brébisson was the dominant diatom, together with important presences of *Nitzschia draveillensis* Coste & Ricard, *Navicula phyllepta* Kützing, *Navicula gregaria* Donkin and *Suriella angusta* Kützing. According to metabarcoding, this was the area with the highest cryptophyte diversity (25 taxa), and the most important taxa were the same as at URD3 and URD4, but with an increase frequency of occurrence and abundance of *U. complanatus,* as revealed by microscopy.

The spatial and temporal variability of the community composition described in the Urdaibai estuary with both microscopy and DNA metabarcoding have been verified by several non-parametric multivariate analyses. The two-way (spatial and seasonal) PERMANOVA analysis performed (Supplementary Material I, Table 8) with the microscopy and metabarcoding data revealed that phytoplankton community composition variability was explained by both the spatial gradient (different sampling stations) within the Urdaibai estuary and seasonal changes observed during the study period ($p = 0.0001$). In addition, when performing PERMANOVA with metabarcoding data, the interaction of both factors (spatial and seasonal) was also significant ($p = 0.0007$). The pairwise PERMANOVA (Supplementary Material I, Table 9), both for microscopy and metabarcoding, revealed significant differences between every season ($p < 0.01$). Regarding sampling stations, URD1 was significantly different from all stations except URD2, and URD2 was also different from URD5 and URD6 ($p < 0.05$). Non-metric multidimensional scaling (nMDS) illustrated the variation in the phytoplankton community composition along the sampling stations of the Urdaibai estuary and between seasons (Fig. 6). SIMPER analysis revealed that the top 15 key phytoplankton taxa that contributed the most to the dissimilarities observed between sampling stations and between seasons were common in each approach (Supplementary Material I, Table 10). Among these top 15 taxa, a few similar dominant species were identified by both microscopy and metabarcoding method: *C. tenuissimus, M. polymorphus, T. acuta* and *U. complanatus*. Apart from these shared species, metabarcoding considered the following taxa responsible for spatio-temporal differences in the community: *Cyclotella* spp., *H. cryptochromatica, N. draveillensis, O. tauri, Cladophora striolata, C. guillardii, N. phyllepta, S. angusta, O. lucimarinus, O. mediterraneus* and *Suriella* sp. As for microscopy, the organisms completing the top 15 taxa responsible for the dissimilarities were *Plagioselmis* sp., *T. gracilis, K. foliaceum, Tetraselmis* spp., Prymnesiales, *Heterocapsa* spp., *Eutreptiella* spp., *Chaetoceros* spp. and unidentified small centric and pennate diatoms.

3.4. Relationship between phytoplankton community and environmental variables

The CCA (Fig. 7) showed that the 10 physicochemical variables selected explained 80.8% of the phytoplankton abundance variability ($p < 0.001$) (Supplementary Material I, Table 11). The first axis

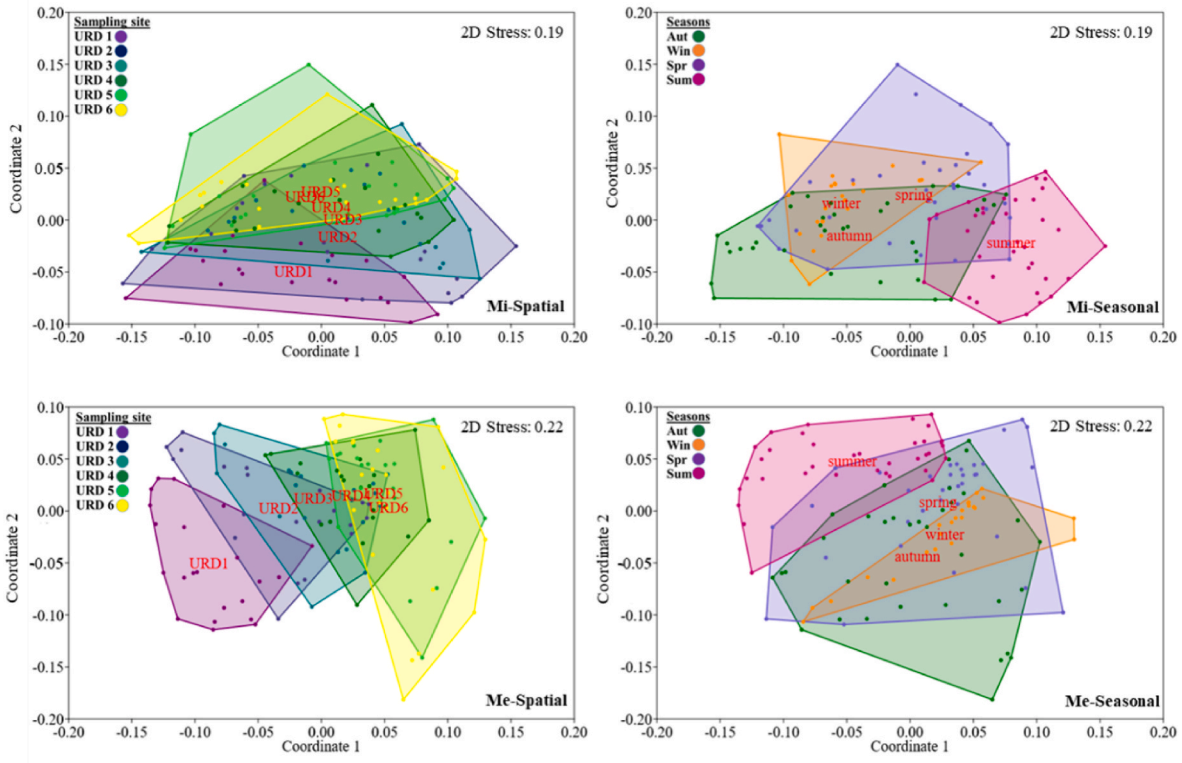

Fig. 6. Non-metric multidimensional scaling (nMDS) of phytoplankton cell abundance (microscopy; Mi) and relative read abundance (metabarcoding; Me) data using Bray-Curtis distances. Data are shown separately for microscopy (up) and metabarcoding (down). Different symbol colours represent the different sampling stations (left) or seasons (right).

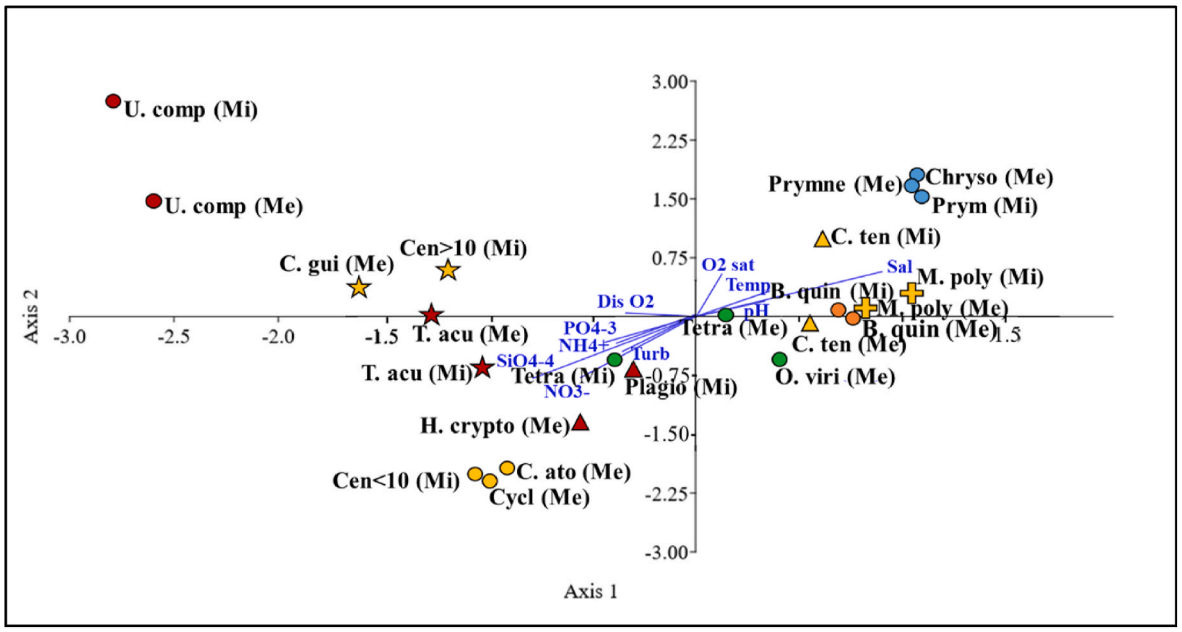

Fig. 7. Canonical Correspondence Analysis (CCA) plot showing the correspondence (influence) of the main environmental factors (blue lines) with the standardised abundance of the most relevant phytoplankton taxa of the Urdaibai estuary for the microscopy (*Mi*) and eDNA metabarcoding (*Me*) analysis. The analysis was significant overall at $p < 0.001$ (permutation test for CCA). Sal: salinity; Temp: temperature; Turb: turbidity; NH_4^+: ammonium; PO_4^{-3}: phosphate; NO_3^-: nitrate; SiO_4^{-4}: silicate. Phytoplankton taxa abbreviations are detailed in Table 1. The colours of the symbols indicate different phytoplankton groups: diatoms in yellow, dinoflagellates in orange, green algae in green, cryptophytes in red and haptophytes in blue. Symbols with the same shape and colour indicate correlated (matching) taxa from both techniques.

represented the spatial variation of the environmental conditions (mainly influenced by salinity and nutrient concentration) and was the axis that explained most of the abundance variability of phytoplankton (53.5%). The Spearman correlation analysis confirmed the relationships between the chosen phytoplankton taxa and the environmental variables visible in the CCA (Supplementary Material I, Tables 12, 13).

The taxa that seemed to be more abundant as salinity increased were the haptophytes; diatoms, *M. polymorphus* and *C. tenuissimus;* and the dinoflagellate, *B. quinquecornis*. There was a significant positive correlation between salinity and Prymnesiales (ρ 0.78; $p < 0.01$), *M. polymorphus* (ρ 0.57; $p < 0.01$), *C. tenuissimus* (ρ 0.45; $p < 0.01$) and *B. quinquecornis* (ρ 0.58; $p < 0.01$) based on microscopy cell counts. These significant correlations with salinity were also detected for the metabarcoding data for *Chrysochromulina* sp. (ρ 0.82; $p < 0.01$), *Prymnesium* spp. (ρ 0.73; $p < 0.01$), *M. polymorphus* (ρ 0.7; $p < 0.01$), *C. tenuissimus* (ρ 0.58; $p < 0.01$) and *B. quinquecornis* (ρ 0.4; $p < 0.01$). The CCA also showed that cryptophytes (e.g., *U.complanatus*, and *T. acuta*) and centric diatoms (e.g., *C. guillardii* or *Cyclotella* spp.) increased their abundances as nutrient concentration increased, which is in accordance with the community composition description previously mentioned. Spearman correlation analysis confirmed this relationship for metabarcoding data, registering positive correlations between *Cyclotella* spp. and ammonium (ρ 0.63; $p < 0.01$), nitrate (ρ 0.51; $p < 0.01$), phosphate (ρ 0.66; $p < 0.01$) and silicate (ρ 0.62; $p < 0.01$); between *U.complanatus*, and ammonium (ρ 0.53; $p < 0.01$) and phosphate (ρ 0.48; $p < 0.01$); and *T. acuta* and ammonium (ρ 0.43; $p < 0.01$). Several strong correlations were also detected with temperature for both microscopy and metabarcoding. Microscopy cell counts revealed strong positive correlations between temperature and *M. polymorphus* (ρ 0.76; $p < 0.01$), *B. quinquecornis* (ρ 0.72; $p < 0.01$), *Plagioselmis* spp. (ρ 0.73; $p < 0.01$) and *Tetraselmis* spp. (ρ 0.7; $p < 0.01$). These correlations were also significant for the metabarcoding data, but were not so strong. However, relative abundances, resulting from the metabarcoding analysis, determined a strong correlation between temperature and *C. tenuissimus* (ρ 0.76; $p < 0.01$) that was not so marked (ρ 0.6; $p < 0.01$) for microscopy data. As for total phytoplankton biomass, significant positive correlations were detected between chl *a* and temperature (ρ 0.59; $p < 0.01$), ammonium (ρ 0.42; $p < 0.01$) and phosphate (ρ 0.39; $p < 0.01$), although they were not considered strong.

In addition, the CCA enabled the comparison of the results obtained by both microscopy and metabarcoding. Overall, the CCA revealed that the relationship of the selected phytoplankton taxa distribution with the environmental conditions along the Urdaibai estuary was similar with both approaches. However, some taxa showed different patterns with the different approaches, for example *Tetraselmis* sp. showed greater preference for salinity with metabarcoding. This might be caused by the misidentification or omission of this taxon in microscopy samples.

4. Discussion

4.1. Phytoplankton community composition and dynamics

Defining the drivers that promote the changes in the phytoplankton community is essential for the understanding of its dynamics and foreseeing the possible impacts in the whole ecosystem. The phytoplankton biomass and community composition showed substantial changes within the Urdaibai estuary, which are promoted by the strong longitudinal gradients of salinity, seasonal changes and inorganic nutrient concentrations caused by the effect of the tidal incursion and the wastewater discharges coming from Gernikas' WWTP.

Regarding the variability of the phytoplankton biomass, the seasonal biomass cycle detected by both the total carbon content and chl *a* in the Urdaibai estuary was typical of temperate estuaries in the Bay of Biscay (Seoane et al., 2005; Trigueros and Orive, 2001; Varela, 1996), and also in other temperate areas (i.e. Changjiang River Estuary in China, the fjords of Kategatt or Atlantic Iberian Peninsula) (Cartensen et al., 2007; Domingues et al., 2007; Gameiro et al., 2007; Wang et al., 2019). This seasonal cycle comprehends a low biomass period during autumn and winter, and a high biomass period during spring and summer. In temperate regions, higher water temperatures usually indicate higher solar radiation, and light availability is one of the main factors influencing phytoplankton growth (Morison et al., 2020), which may explain

the phytoplankton biomass-temperature relationship observed in the present study. In addition, the high biomass values coincide with periods of low to moderate river flow (in spring and summer), while the lowest biomass values were registered with seasonal periods of high river flow, in agreement with previous works in the estuary (Ansotegui et al., 2001; Madariaga and Orive, 1989; Madariaga et al., 1989, 1994), and also observed in other studies (O'Boyle and Silke, 2010; Snow et al., 2000), where high flow rates could lead to the dilution of the chl *a* (Du et al., 2017). Considering the torrential nature of the Oka River, when large flows occur after punctual precipitations, the flushing rate increases and the estuary is almost flushed. The seasonal biomass varied significantly in the middle and inner estuary, while concentrations in the outer estuary remained fairly stable during the study period.

The spatial pattern of biomass showed the lowest concentrations in the outer estuary, while the maxima were recorded in the middle and inner area. This pattern coincided with that observed in late 90's in the studies performed along the Urdaibai estuary by Ansotegui et al. (2001). Ansotegui et al. (2001) recorded values higher than 100 μg/L of chl *a* in the inner part, corresponding to the stations URD4 to URD6 of our study, and values below 6 μg/L of chl *a* in the outer estuary, corresponding to URD1. In the present study, the maximum values registered were not so high, but the pattern was the same. This trend is repeated in many estuaries around the world (Brito et al., 2015; Santos et al., 2022; Seoane et al., 2005; Shi et al., 2019). For example, MCGarrigle et al. (2001) reported on the distribution of chl *a* in 25 estuarine and coastal areas around Ireland in 1998–2000 and the range of concentrations found in the inner part of the estuaries were approximately 10 times higher than those obtained in the outer and coastal adjacent areas. This distribution of chl *a* is explained by the factors governing phytoplankton growth (e.g., nutrients, light, and grazing). Wang et al. (2019) reported that, in the inner areas of the estuaries, phytoplankton growth is mostly limited by light availability, while in the outer areas the limiting factor is mostly nutrient availability. In the outer area of the Urdaibai estuary, nutrients were usually found in low concentrations, in accordance with several authors that have stated that during stratification periods, in shelf waters off the Basque coast, the residual concentrations in the water layer above the thermocline are comparable to those found in oligotrophic areas (Muñiz et al., 2019). Therefore, this nutrient limitation could explain the lower phytoplankton biomass recorded in the outer area. However, high nutrient concentrations (like N, P and Si) were found in the inner estuary, which might have enhanced the phytoplankton growth in the area. This finding aligns with previous studies (e.g., McCabe et al., 2016; Vajravelu et al., 2018) and is supported by the positive correlations observed between chl *a* and ammonium as well as phosphate in this study. Additionally, although turbidity values were much higher in the inner estuary, reducing light availability, it seems that it is not a limiting factor for phytoplankton growth, probably due to the high heterogeneity of environmental conditions found in this area (clearly represented by the PCA) due to the Oka River inflow. The spatial pattern of increasing biomass with increasing nutrients (and decreasing salinity) was also registered in previous studies of the Urdaibai estuary (e.g. Orive et al., 1998). Indeed, when observing the nutrient concentrations, and referring to the accepted standard molar ratios of nutrients for phytoplankton growth, N:Si:P (16:16:1), the phosphorous was usually the limiting nutrient for phytoplankton growth along the estuary, due to the high concentrations of Si, coming from the Oka River, and N, coming both from the river and mostly from the WWTP. This explains the important presence of diatoms along the entire Urdaibai estuary, since there is no Si limitation, but their density varies because it is directly related to the P concentration, which in this case is a limiting factor, leading to lower cell abundances in the outer estuary.

As for the community composition, although the sampling frequency and stations do not exactly coincide with the studies performed in the system before, the phytoplankton community of the Urdaibai estuary described in the present study was similar to results reported in the late 90's (Madariaga et al., 1989, 1994; Orive et al., 1998; 1998; Trigueros and Orive, 2001). In the outer estuary, the community was mainly dominated by diatoms, with the recurrent presence of dinoflagellates in this area, while the dominance of cryptophytes, green algae, centric diatoms and *K. foliaceum* was restricted to the inner part. In addition, the trends observed of taxa richness decreasing towards the inner estuary were also similar to the studies performed in late 90's (e.g., Trigueros and Orive, 2001). This higher taxonomic richness in the highest salinity zone has also been described in other estuaries by several authors (e.g. Bharathi et al., 2022; Paczkowska et al., 2019; Saifullah et al., 2019). It may be a result of the salinity range that populations can tolerate, since, in estuaries, salinity is known to be a fundamental modulator of the abundance dynamics of the species (e.g. de Affe et al., 2018; Córdoba-Mena et al., 2020). Additionally, the trend of decreasing richness towards the inner estuary may also reflect the effect of the eutrophic conditions of this area. Biodiversity measures (e.g., species richness, Shannon–Wiener diversity, and Pielou's equitability indexes), together with the abundance of some specific taxonomic groups of microalgae, have been suggested as indicators of nutrient enrichment (Machado et al., 2023). Several studies found a reduction in diversity and species richness as a consequence of nutrient enrichment (e.g., Baho et al., 2017; Machado et al., 2023; Soares et al., 2013), mainly caused by the dominance of a few species and the decrease in the presence and abundance of rare species or functional groups (Ansari et al., 2011).

Diatoms dominated the outer and middle estuarine waters. This pattern was also observed in other areas around the globe, such as Ireland (Oboyle and Silke, 2010), Rio de la Plata (Argentina) (Gómez et al., 2004) and Sado Estuary (Portugal) (Santos et al., 2022). Among the diatom taxa found in these areas, *M. polymorphus* and *C. tenuissimus* both were the most important bloom-forming diatoms at URD1 and URD2. *M. polymorphus* had not been reported previously in any scientific research paper of the Urdaibai estuary, but was described in several technical reports done for the administration (URA, Basque Water Agency). This marine, planktonic and cosmopolitan species usually occurs in estuaries and open ocean (Walsh et al., 1988), which explains the significant correlation (ρ 0.7; $p < 0.01$) established in the present study with salinity. In addition, *M. polymorphus* has shown a clear preference for the summer season, which was confirmed by the positive correlation with temperature, in accordance with previous studies that reported summer blooms of this species in the Fusaro Lagoon (Mediterranean Sea) or the Bohai Sea (China) (Chen et al., 2019a; Sarno et al., 1993). As for *C. tenuissimus*, also identified by both techniques, it is a small cosmopolitan species that thrives in coastal waters around the world between tropical and temperate waters (De Luca et al., 2019; Grzebyk et al., 2022), being frequently found in the Mediterranean Sea, Atlantic Ocean and Japanese coastal waters among others (Hongo et al., 2021). This species is characterised by having a high growth rate (at least three divisions per day) and causes blooms mostly in the spring and autumn (Hongo et al., 2021). This marine character and rapid growth agree with its higher presence in the poly-euhaline zone of the Urdaibai estuary (positive correlation with salinity) and recording high cell abundances in late spring-summer (positive correlation with temperature), like the bloom of 20 million cells/L of June 2020 in URD2 (Supplementary Material II). Comparing with previous studies, it is remarkable the low densities of *Asterionellopsis glacialis* found in the present research, which was one of the most dominant diatom species in the studies performed in late 90's, being sometimes more than 90% of the community (Ansotegui et al., 2003).

The outer area also recorded the highest dinoflagellate and haptophyte diversity along the estuary, which is mainly explained by the marine nature of most of the species of these two groups. This is in accordance with previous studies in the Urdaibai estuary and in the nearby estuary of Bilbao (Trigueros and Orive, 2000; Trigueros and Orive 2001; Seoane et al., 2005; Seoane et al., 2009a). Additionally, the mixotrophy explains the high presence of these groups in the outer estuary, providing them with the ability to grow in low nutrient regions (Unrein et al., 2014; Zhang et al., 2013). However, in the case of

dinoflagellates, their contribution to the total phytoplankton abundance in the inner estuary is similar to the outer estuary, even if the species richness is much lower. This is explained by the large cell abundances that some of these dinoflagellate species (e.g., *K. foliaceum*) record in the inner estuary.

From the middle estuary towards the inner Urdaibai estuary, cryptophytes started to increase in abundance, becoming the dominant group in the phytoplankton community in the channelled section of the estuary, following the pattern of other eutrophicated estuaries such as Chesapeake Bay (Adolf et al., 2006), Neuse River Estuary (Valdes--Weaver et al., 2006) or Galveston Bay in Texas (Paerl et al., 2003). This trend of increasing abundance in this middle and inner section of the estuary was also recorded in previous studies in Urdaibai estuary (Ansotegui et al., 2001; Madariaga, 1995; Orive et al., 1998). However, in the present study, their dominance is more evident compared to the late 90s', obtaining higher cryptophyte abundances than in previous studies. The increase of cryptophytes dominance has been recently observed in other temperate estuaries as well, such as Tagus (Brito et al., 2015) and Sado (Santos et al., 2022) estuaries. The studies of both Brito et al. (2015) and Santos et al. (2022) compared the phytoplankton communities in the estuaries with the community observed historically and both agreed with the trend of the increasing dominance of cryptophytes. The dominance of cryptophytes over other groups in the middle and inner Urdaibai estuary may have been triggered by higher turbidity and organic matter concentration found in these areas of the estuary, since several authors (e.g. Adolf et al., 2008; Bergmann, 2004) have described the capability of crytophytes for growing in such conditions. Their success in this niche, over other groups like diatoms, is explained by their ability to survive under restricted light conditions, since cryptophytes adapt the absorption spectrum of their phycobiliprotein antennas by replacing a bilin pigment with another one, capable of more efficiently harvesting the available wavelengths (Collini, 2022). Being small flagellates, their motility could also be advantageous in these light restricted zones. In addition, they show mixotrophic character, which allows them to utilise dissolved organic carbon for growth (Johnson et al., 2013). Another reason for the substitution of dominant group from the outer area (diatoms) to this middle and inner area (cryptophytes) could be the different nutritional preferences of the dominant groups. While diatoms, along with the silicate, preferably make use of nitrate, cryptophytes have a higher advantage in ammonium enriched waters (Horner Rosser and Thompson, 2001). This is in agreement with the positive correlations recorded between ammonium and *U.complanatus* and *T. acuta* in the present study. Among the cryptophytes found in the estuary, some showed a preference for higher salinity areas like *T. amphioxeia* and *T. gracilis*, and others were more restricted to the inner estuary, like *H. cryptochromatica*, *T. acuta* and, especially, *U. complanatus*. *T. amphioxeia,* which was not identified by microscopy, is well known from brackish waters in Europe (Gran-Stadniczeňko et al., 2019; Throndsen et al., 2007), whereas *T. gracilis,* detected by both approaches, was described in 2012 from the Atlantic coast of Spain for the first time (Laza-Martínez, 2012). *U. complanatus*, considered one of the most characteristic species of the inner Urdaibai estuary, is a common bloom-forming euryhaline cryptophyte in the estuaries of SE Bay of Biscay (Seoane et al., 2012). Recently, booms of *U. complanatus* have also been registered in Japanese estuaries, in brackish waters in Ehime, Hiroshima and Kochi prefectures (Mizobuchi et al., 2021).

In the surroundings of the WWTP, the dinoflagellate *K. foliaceum,* only identified by microscopy, became very abundant and was present in most of the samples, being among the taxa that most explained the spatio-temporal variability of the community according to SIMPER. This dinoflagellate has been previously reported in Urdaibai as a characteristic species of the inner zone (e.g. Ansotegui et al., 2001; Trigueros et al., 2000b). It has also been observed in the meso-polyhaline region of other small shallow estuaries of the Basque coast (Orive et al., 1998) and other temperate estuaries like the Guadiana estuary (Domingues et al., 2011), Maruca estuary (Seoane et al., 2012), Deel estuary (Jenkinson, 1985), Lough Atalia estuary (Pybus et al., 1984) or Christchurch Harbour estuary (Charoenvattanaporn, 2016). Jenkinson (1985) suggested that the confinement of this species within estuaries might result from the ability of *K.foliaceum* to vertically migrate and interact with water movements. Additionally, Domingues et al. (2011) found that *K. foliaceum* showed higher growth rates in response to N additions in the absence of Si, which is typical of anthropogenic nutrient inputs typically originating from the WWTP, since they are typically high in N and P, but no Si (mainly coming from the chemical weathering). Therefore, *K. foliaceum* proliferated in the inner Urdaibai estuary, especially in the surroundings of the wastewater discharges, due to favourable growth conditions i.e., low salinity and increased nutrient availability derived from the WWTP, and the possibility of migrating in the water column, avoiding the usual high turbidity of the area.

Diatoms, both pennated and centric, were also an important part of the phytoplankton community of the inner Urdaibai estuary. Species such as the epipelic pennates *Navicula phyllepta* and *Navicula gregaria,* found in Urdaibai, have been recorded as the dominant species of the benthic substrate from intertidal mudflats (Admiraal et al., 1984; Haubois et al., 2005) and from oligo and mesohaline areas of estuaries (Underwood et al., 1998). The ability of these small-fast growing diatoms to move in the sediment allows them to migrate to the surface for receiving higher light intensity, which at the same time increases the possibility of their resuspension to the water column. The resuspension rates of these benthic diatoms are greater in shallow waters of well mixed estuaries (Baillie and Welsh, 1980), such as the Urdaibai estuary, due to the higher influence of wind action, tidal currents and convective currents on theses ecosystems in comparison to deeper estuaries with marked halocline (Anderson, 1973). This explains the high abundance in which both *N. phyllepta* and *N. gregaria* were found in the waters of the inner Urdaibai estuary. Regarding the centric diatoms, the genus *Cyclotella* was the main representative of the inner Urdaibai estuary. *Cyclotella* is a common genus from the inner areas of temperate estuaries, due to its oligo or mesohaline character. This genus has been previously recorded in high abundances in several estuaries, such as the Schelde estuary (Muylaert et al., 2000), Bahía Blanca estuary (Popovich and Marcovecchio, 2008) and the nearby Bilbao estuary (Seoane et al., 2005).

4.2. Comparison of the community characterization methods

Both microscopy and eDNA metabarcoding revealed similar trends in phytoplankton community composition along the Urdaibai estuary, especially on the dominance of phytoplankton groups, spatio-temporal variations of the community composition and relationship between relevant taxa and environmental conditions. However, most of these analyses were done with the aim of obtaining a general image of the community composition, which was based on the whole data set (nMDS and PERMANOVA) or centred in high taxonomic levels, like phytoplankton groups. The present study revealed that the lower the taxonomic level, the higher the inconsistency between microscopy and metabarcoding when describing the community (e.g. taxa richness or SIMPER analysis). Several studies, focused on the comparison of morphological and molecular methodologies for freshwater (MacKeigan et al., 2022), estuaries (Abad et al., 2016; Nunes et al., 2018) and marine waters (Gran-Stadniczeňko et al., 2019; Santi et al., 2021; Wang et al., 2022), have enumerated possible reasons to explain the discrepancies between the two approaches, uncovering the strengths and weaknesses of each approach, that were also detected in the present study.

Differences in the identification capacity of molecular and morphological data sets could be one of the main causes of dissimilarity between the two methods (Kim et al., 2019; Santi et al., 2021; Zimmermann et al., 2015). In the present study, DNA metabarcoding showed pronounced differences with microscopy when comparing the number of species/genera identified, the number of identified taxa (to, at least, the genus level) being 3 times higher in eDNA metabarcoding. Overall,

metabarcoding revealed the presence of 349 phytoplankton taxa that were not identified by microscopy, among which 276 taxa were not previously recorded for the Urdaibai estuary (Supplementary Material I, Tables 5 and 7). Among these, 223 reached the species level, registering 77 new diatom species, 50 dinoflagellates, 40 green algae (mostly chlorophytes), 15 cryphytophytes, 13 haptophytes, 7 dictyochophyceans, 7 chrysophyceans, 4 bolidophyceans, 4 pelagophyceans, 3 rhodophyceans, 2 synurophyceans and 1 raphidophycean for the first time in the Urdaibai estuary. The reason for the omission or misidentification of the phytoplankton taxa by microscopy varies depending on characteristics of the phytoplankton groups. In the case of chlorophytes and haptophytes, their small cell size and fragility may be the main reason for their misidentification (Agirbas et al., 2015; Lee et al., 2020), since pico- and nanoplanktonic organisms require electron microscopy or molecular methods for identification (Gran-Stadniczeñko et al., 2019). As an example, 6 haptophyte taxa were identified by microscopy (3 species), while 19 taxa (14 species) were identified by molecular techniques, some of them being especially recurrent in the outer estuary, such as *Chrysochromulina scutellum* and *Chrysochromulina rotalis*. Many of them were previously identified in the nearby Nervion estuary (Seoane et al., 2009a) from natural samples and uni-algal cultures using light and mainly electron microscopy; however, this procedure is unfeasible for regular phytoplankton monitoring. The difference was even bigger for chlorophytes, since the number of taxa identified increased from 12 to 61, as represented by *Picochlorum* spp., *Ostreococcus* spp. (*O. mediterraneus*, *O. lucimarinus* and *O. tauri*) and *Micromonas* spp. (mainly *M. bravo* and *M. commoda*), some of the most frequent taxa of the estuary according to metabarcoding, and were reported for the first time in the Urdaibai estuary in this study. This limitation was previously described in nearby estuaries (Abad et al., 2016) and marine areas (e.g. Gran-Stadniczeñko et al., 2019). Diatom misidentification, which was notable in the present study, can be due to the presence of cryptic species that cannot be identified. Additionally, the lack of resolution of the microscopy method used to identify diagnostic morphological characters may have resulted in overly coarse or erroneous taxonomic identification optically (Kim et al., 2019; Santi et al., 2021). In the present study, eDNA metabarcoding helped improving the resolution in the "unidentified centric" and "unidentified pennate" diatoms groups. Among the unidentified centric diatoms, we could determine the presence of three different *Cyclotella* species (*C. atomus* (Hustedt) *C. choctawhatcheeana* Prasad and *C. striata* (Kützing) Grunow), 2 *Minidiscus* Hasle (*M. spinulatus* (H.Takano) J.S.Park & J.H.Lee and *M. variabilis*), 12 *Thalassiosira* Cleve species, *Mediolabrus comicus* and *Stephanocyclus meneghinianus* (Kützing) Kulikovskiy, Genkal & Kociolek among others by molecular techniques. As for unidentified pennate diatoms, 11 *Nitzschia* species, 10 *Navicula* Bory species and 2 *Haslea* Simonsen (*H. nipkowii* (Meister) M.Poulin & G.Massé and *H. pseudostrearia* Massé, Rincé & E.J.Cox) were identified by eDNA metabarcoding. Scanning electron microscopy (SEM) is commonly used (e.g. Li et al., 2019) to allow a more detailed view at a higher magnification of many of the previously mentioned diatom species, since the diatom frustule, composed of two valves and a number of overlapping girdle bands, possesses a species-specific morphology of micro- and nanopatterns (Soleimani et al., 2021). In order to observe these patterns, in addition to the higher magnification provided by the SEM in comparison to the inverted microscope, diatom cells must overcome a digestion process to eliminate their intra-celular organic material, which is not part of the Utermöhl method followed in most of the phytoplankton monitoring programmes and the present study. In addition, as mentioned in several similar studies (e.g. Gran-Stadniczeñko et al., 2019), an important aspect in this comparison is that eDNA metabarcoding results are based on a bigger sample volume analysed (0.2–3.5 L) in comparison to microscopy (50 ml), which has also helped to reach a higher taxonomic richness. This proficiency of metabarcoding over phytoplankton for identifying phytoplankton taxa makes it a more accurate tool for monitoring rare and endangered species or detecting invasive species (Keck et al., 2017).

Nevertheless, among the 615 phytoplankton taxa identified in the Urdaibai estuary, only 407 were identified to the genus or species level, which leaves a high number of sequences with an incomplete taxonomic assignment. There are several causes behind these incomplete identifications (Santoferrara, 2019): insufficient marker resolution (e.g. not variable and/or long enough), unsuitable method or parameters (e.g. BLAST assignments based on suboptimal sequence similarity/coverage) or incomplete or inaccurate databases. Although the 18S rRNA gene is the most widely used marker for group and species detection within marine eukaryotic microorganisms (Martin et al., 2022), in some taxonomic groups (e.g. diatoms and haptophytes) the V4 18S rRNA gene, the one applied in the present study and many others (e.g. Piredda et al., 2017; Wang et al., 2022), shows identical sequences for different species, leading to incomplete identifications (Gran-Stadniczeñko et al., 2019). Not only the marker gene used, but also the primer choice can be crucial for an effective taxonomic assignment, since its amplification and binding affinity are critical factors to bring about taxonomic biases in eDNA metabarcoding identification (Kim et al., 2019; Van der Loos and Nijland, 2021). However, primer efficiency is highly species-specific, which would prevent straightforward assessments of species abundance (Elbrecht and Leese, 2015) and might imply group-specific primer choice to obtain a higher rate of taxonomic assignment. Indeed, it is believed that a multimarker approach, using several primer sets, will result in a more reliable estimation of species richness (Alberdi et al., 2018). As for the bioinformatics decisions, its flexibility makes it an important source of variability of the final results obtained, and therefore, data sharing is key to enhancing result reproducibility and information discoverability, in order to develop community standards for bioinformatics methods (Santoferrara, 2019). In addition, many phytoplankton taxa are difficult to cultivate and/or identify through microscopy, and therefore, no molecular references are available, resulting in poor taxonomic coverage and data quality of reference libraries, which might also explain a high number of unclassified taxa obtained in the study (Gran-Stadniczeñko et al., 2019). Thus, species identification varies with accuracy and coverage of reference databases (Kim et al., 2019), which is considered one of the main drawbacks of the DNA metabarcoding approach (e.g. Rimet et al., 2021; Weigand et al., 2019).

This incomplete identification of ca. 200 unique phytoplankton taxa detected by metabarcoding leads to some "false negatives" when comparing it with microscopic identification. Among the species identified by microscopy that were not part of the metabarcoding results (Supplementary Material I, Table 6), the dinoflagellate *K. foliaceum* must be highlighted. *K. foliaceum* was present in almost all the samples of the inner Urdaibai estuary and has been recorded in previous studies in the areas as well (et al., 2001); however, molecular techniques did not identify it, with Kryptoperidiniaceae being the lowest identified taxonomic level. In addition, *K. foliaceum* is included in the database used in the present study (PR2 4.13.), and the sequence corresponds to the same region of 18S rRNA of the target of the primer used in the present study. Thus, this "false negative" may be caused by technical biases introduced throughout the DNA metabarcoding workflow (Martin et al., 2022): sample preservation (Mäki et al., 2017), DNA extraction (Van der Loos and Nijland, 2021) and/or PCR (Latz et al., 2022). The same may have happened with *Prorocentrum micans* Ehrenberg and *Emiliania huxleyi*, which are both available in the PR2 database, but were not detected in our samples by molecular techniques, even if they were observed by microscopy. Several options for mitigating these false negatives are the optimisation of nucleic acid extraction and storage, improving primers and sequence processing and the adequate biological and technical replication (Santoferrara, 2019). Moreover, and in accordance with what Gran-Stadniczeñko et al. (2019) described, the molecular techniques almost overlooked the Euglenophyceae of the Urdaibai estuary, which are relevant and frequent green algae according to microscopy, but register much lower relative abundances and presence for

metabarcoding. However, this can be partly explained by the V4 18S rRNA gene PCR primers used that seem to be poor in amplifying members of Euglenophyta compared to amplification using chloroplast gene targeting primers (Amaral-Zettler et al., 2011).

Additionally, besides the inherent technical biases, the major source of bias causing discrepancies between both approaches is the 18S rRNA gene copy number variation within species, genera and plankton groups (Martin et al., 2022; Santi et al., 2021). This variation of gene copy numbers can be substantial (ranging from tens to thousands), affecting the proportion of reads found for each species present in complex environmental assemblages and leading to misinterpretation of relative abundances when comparing to microscopic counts (Santi et al., 2021). In the present study, even if the relative abundance results for both techniques were quite similar in some cases (e.g. defining the dominant phytoplankton group), in most of the cases, these results differed (Fig. 5). As an example, the average relative abundance of dinoflagellates and green algae within the Urdaibai estuary was two times higher according to metabarcoding when compared to microscopy. This is similar to the findings of Piredda et al. (2017) and Santi et al. (2021), which registered higher percentages of dinoflagellate contribution produced by metabarcoding. The main reasons for this frequent difference might be that, compared to taxa with similar cell size, dinoflagellates have large genomes and putatively high rRNA gene copy number, thus, an overrepresentation may be expected by molecular techniques (Martin et al., 2022). However, another reason for the discrepancies between techniques may be that the broadly used fixatives (e.g. Lugol) cannot preserve the morphology of unarmored dinoflagellates and might lead to misidentification or omission by microscopy (Santi et al., 2021), decreasing their contribution to the total abundance. Therefore, although several authors (Martin et al., 2022; Piwosz et al., 2020) agree that community relative abundances determined by eDNA metabarcoding are useful and reliable in the context of ecological interpretations (like in the present study), microscopy cell counts are still essential when studying bloom-forming taxa and/or toxic species in the community.

Thus, the present study underlines that, while metabarcoding is a more accurate approach for the assessment of the phytoplankton taxonomic richness of an aquatic ecosystem like the Urdaibai estuary, it may under- or overestimate the abundance of the identified taxa, making the combination with microscopy necessary for obtaining reliable quantitative results. Therefore, each approach answers different ecological questions, and the combined use of the two methods provides a much more complete image of the phytoplankton abundance and community composition and its spatio-temporal variability of the Urdaibai estuary.

5. Conclusion

This present study investigated the phytoplankton abundance and community composition of the Urdaibai estuary at a fine-scale resolution during a 12-month period. Results determined that chl *a* increased towards the inner estuary and spring/summer seasons, showing a significant positive relationship with nutrients and temperature. As for community composition, diatoms like *M. polymorphus* and *C. tenussimus* dominated the outer and middle area of the estuary and were replaced by cryptophytes, like *T. acuta* and *U. complanatus*, and the dinoflagellate *K. foliaceum* and diatoms *Cyclotella* spp. towards the inner area. These changes in dominant taxa were promoted mainly by the strong longitudinal gradients of salinity and inorganic nutrient concentrations of the Urdaibai estuary, but other factors such as light availability and mixotrophy may also explain the taxa distribution.

Both microscopy and eDNA metabarcoding revealed similar patterns within the Urdaibai estuary regarding dominance of phytoplankton groups, spatial and temporal differences of the community composition and influence of the main environmental factors with the most relevant taxa. Nevertheless, both approaches were also complementary, since metabarcoding was able to overcome the lack of taxonomic resolution of microscopy, especially for picoplankton, and revealed the presence of 223 species that had not been previously recorded in the Urdaibai estuary, providing new information on the species richness of this protected area. On the other hand, the microscopic analysis of the community was useful to cover the gaps that still exist in eDNA metabarcoding, since cell counts are essential when studying bloom-forming taxa and/or toxic species in the community. Thus, considering the different characteristics of microscopy and DNA metabarcoding, this work emphasizes that the choice of the approach should be based on the objective of the research, and if possible, a combination of techniques is recommended to obtain more reliable and accurate results of the phytoplankton community.

CRediT author statement

Jone Bilbao: Conceptualization; Methodology; Data curation; Investigation; Visualization; Writing - original draft. Christina Pavloudi: Formal analysis; Data curation; Resources; Writing- Reviewing and Editing. Esther Blanco-Rayón: Investigation, Writing- Reviewing and Editing. Javier Franco: Conceptualization; Methodology; Writing-Reviewing and Editing. Sergio Seoane: Conceptualization; Methodology; Funding acquisition; Supervision; Validation; Visualization; Writing- Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

Abad, D., Albaina, A., Aguirre, M., Laza-Martínez, A., Uriarte, I., Iriarte, A., et al., 2016. Is metabarcoding suitable for estuarine plankton monitoring? A comparative study with microscopy. Mar. Biol. 163 (7), 1–13. https://doi.org/10.1007/s00227-016-2920-0.

Admiraal, W., Peletier, H., Brouwer, T., 1984. The seasonal succesion patterns of diatoms species on an intertidal mudflat: an experimental analysis. Oikos 42, 30–40. https://doi.org/10.2307/3544606.

Adolf, J.E., Bachvaroff, T., Place, A.R., 2008. Can cryptophyte abundance trigger toxic *Karlodinium veneficum* blooms in eutrophic estuaries? Harmful Algae 8 (1), 119–128. https://doi.org/10.1016/j.hal.2008.08.003.

Adolf, J.E., Yeager, C.L., Miller, W.D., Mallonee, M.E., Harding Jr., L.W., 2006. Environmental forcing of phytoplankton floral composition, biomass, and primary productivity in Chesapeake Bay, USA. Estuar. Coast Shelf Sci. 67 (1–2), 108–122. https://doi.org/10.1016/j.ecss.2005.11.030.

Agirbas, E., Feyzioglu, A.M., Kopuz, U., Llewellyn, C.A., 2015. Phytoplankton community composition in the south- eastern Black Sea determined with pigments measured by HPLC-CHEMTAX analyses and microscopy cell counts. J. Mar. Biolog. Assoc. 95 (1), 35–52. https://doi.org/10.1017/S0025315414001040.

Aktan, Y., Tüfekçi, V., Tüfekçi, H., Aykulu, G., 2005. Distribution patterns, biomass estimates and diversity of phytoplankton in İzmit Bay (Turkey). Estuar. Coast Shelf Sci. 64, 372–384.

Alberdi, A., Aizpurua, O., Gilbert, M.T.P., Bohmann, K., 2018. Scrutinizing key steps for reliable metabarcoding of environmental samples. Methods Ecol. Evol. 9 (1), 134–147. https://doi.org/10.1111/2041-210X.12849.

Amaral-Zettler, L.A., Zettler, E.R., Theroux, S.M., Palacios, C., Aguilera, A., Amils, R., 2011. Microbial community structure across the tree of life in the extreme Rio Tinto. ISME J. 5 (1), 42–50.

Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuar. Coast 25 (4), 704–726. https://doi.org/10.1007/BF02804901.

Anderson, F.E., 1973. Observations of some sedimentary processes acting on a tidal flat. Mar. Geol. 14, 101–116.

Anderson, M.J., Walsh, D.C., 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? Ecol. Monogr. 83 (4), 557–574. https://doi.org/10.1890/12-2010.1.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32–46.

Ansari, A.A., Gill, S.S., Khan, F.A., 2011. Eutrophication: threat to aquatic ecosystems. In: Ansari, A.A., Gill, S.S., Lanza, G., Rast, W. (Eds.), Eutrophication: Causes, Consequences and Control. Springer, Dordrecht, pp. 143–170.

Ansotegui, A., Sarobe, A., Trigueros, J.M., Urrutxurtu, I., Orive, E., 2003. Size distribution of algal pigments and phytoplankton assemblages in a coastal—estuarine environment: contribution of small eukaryotic algae. J. Plankton Res. 25 (4), 341–355. https://doi.org/10.1093/plankt/25.4.341.

Ansotegui, A., Trigueros, J.M., Orive, E., 2001. The use of pigment signatures to assess phytoplankton assemblage structure in estuarine waters. Estuar. Coast Shelf Sci. 52 (6), 689–703. https://doi.org/10.1006/ecss.2001.0785.

Arizaga, J., Cepeda, X., Maguregi, J., Unamuno, E., Ajuriagogeaskoa, A., Borregón, L., Azkona, A., Unamuno, J.M., 2014. The influence of the creation of a laggon on waterbird diversity in Urdaibai, Spain. Waterbirds 37 (1), 111–118.

Baho, D.L., Drakare, S., Johnson, R.K., Allen, C.R., Angeler, D.G., 2017. Is the impact of eutrophication on phytoplankton diversity dependent on lake volume/ecosystem size? J. Limnol. 76, 199–210. https://doi.org/10.4081/jlimnol.2016.1562.

Baillie, P.W., Welsh, B.L., 1980. The effect of tidal resuspension on the distribution of intertidal epipelic algae in an estuary. Estuar. Coast Shelf Sci. 10, 753–765.

Barroeta, Z., Villate, F., Uriarte, I., Iriarte, A., 2020. Differences in the colonization success and impact of non-indigenous and other expanding copepod species on the zooplankton of two contrasting estuaries of the Bay of Biscay. Biol. Invasions 22 (11), 3239–3267. https://doi.org/10.1007/s10530-020-02320-7.

Bergmann, T.I., 2004. The Physiological Ecology and Natural Distribution of Cryptomonad Algae in Coastal Aquatic Ecosystems. Ph.D. Dissertation. Graduate Program in Oceanography. Rutgers University, New Brunswick, NJ, USA, p. 144.

Bharathi, M.D., Venkataramana, V., Sarma, V.V., 2022. Phytoplankton community structure is governed by salinity gradient and nutrient composition in the tropical estuarine system. Continent. Shelf Res. 234, 104643 https://doi.org/10.1016/j.csr.2021.104643.

Brito, A.C., Moita, T., Gameiro, C., Silva, T., Anselmo, T., Brotas, V., 2015. Changes in phytoplankton composition in a temperate estuarine system (1960 to 2010). Estuar. Coast 38, 1678–1691.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Cartensen, J., Henriksen, P., Heiskanen, A.-S., 2007. Summer algal blooms in shallow estuaries: definition, mechanisms, and link to eutrophication. Limnol. Oceanogr. 52 (1), 370–384. https://doi.org/10.4319/lo.2007.52.1.0370.

Castillo-Eguskitza, N., Rescia, A.J., Onaindia, M., 2017. Urdaibai biosphere reserve (Biscay, Spain): conservation against development? Sci. Total Environ. 592, 124–133. https://doi.org/10.1016/j.scitotenv.2017.03.076.

Charoenvattanaporn, J., 2016. Processes Influencing Phytoplankton Growth and Primary Production in Shallow Temperate Estuary Christchurch Harbour, United Kingdom. Ph.D. Thesis. Univ. of, Southampton, England, United Kingdom.

Chen, Y., Gao, Y., Chen, C., Li, X., Liang, J., Sun, L., Wang, Y., 2019a. Interactions between the brown tide-causing microalga *Aureococcus anophagefferens* and the small diatom *Minutocellus polymorphus* under laboratory culture. J. Appl. Phycol. 31 (3), 1793–1803. https://doi.org/10.1007/s10811-018-1704-y.

Chen, Z.F., Zhang, Q.C., Kong, F.Z., Liu, Y., Zhao, Y., Zhou, Z.X., et al., 2019b. Resolving phytoplankton taxa based on high-throughput sequencing during brown tides in the Bohai Sea, China. Harmful Algae 84, 127–138. https://doi.org/10.1016/j.hal.2019.03.011.

Cloern, J.E., Jassby, A.D., Schraga, T.S., Nejad, E., Martin, C., 2017. Ecosystem variability along the estuarine salinity gradient: examples from long-term study of San Francisco Bay. Limnol. Oceanogr. 62, 272–291. https://doi.org/10.1002/lno.10537.

Collini, E., 2022. Light-harvesting: the never-ending lesson of nature. ACS Cent. Sci. 8 (3), 306–308. https://doi.org/10.1021/acscentsci.2c00137.

Comtet, T., Sandionigi, A., Viard, F., Casiraghi, M., 2015. DNA (meta)barcoding of biological invasions: a powerful tool to elucidate invasion processes and help managing aliens. Biol. Invasions 17, 905e922. https://doi.org/10.1007/s10530-015-0854-y.

Córdoba-Mena, N., Florez-Leiva, L., Atehortúa, L., Obando, E., 2020. Changes in phytoplankton communities in a tropical estuary in the Colombian caribbean Sea. Estuar. Coast 43, 2106–2127. https://doi.org/10.1007/s12237-020-00750-z.

Cummins, C., Ahamed, A., Aslam, R., Burgin, J., Devraj, R., Edbali, O., Gupta, D., Harrison, P.W., Haseeb, M., Holt, S., Ibrahim, T., Ivanov, E., Jayathilaka, S., Kadhirvelu, V., Kay, S., Kumar, M., Lathi, A., Leinonen, R., Madeira, F., Madhusoodanan, N., Mansurova, M., O'Cathail, C., Pearce, M., Pesant, S., Rahman, N., Rajan, J., Rinck, G., Selvakumar, S., Sokolov, A., Suman, S., Thorne, R., Totoo, P., Vijayaraja, S., Waheed, Z., Zyoud, A., Lopez, R., Burdett, T., Cochrane, G., 2022. The European nucleotide archive in 2021. Nucleic Acids Res. 50 (D1), D106–D110.

De Luca, D., Kooistra, W.H.C.F., Sarno, D., Gaonkar, C.C., Piredda, R., 2019. Global distribution and diversity of *Chaetoceros* (Bacillariophyta, Mediophyceae): 18 L. ARIN ET AL.integration of classical and novel strategies. PeerJ. https://doi.org/10.7717/peerj.7410.

De Luca, D., Piredda, R., Sarno, D., Kooistra, W.H.C.F., 2021. Resolving cryptic species complexes in marine protists: phylogenetic haplotype networks meet global DNA metabarcoding datasets. ISME J. https://doi.org/10.1038/s41396-021-00895-0.

Domingues, R.B., Barbosa, A.B., Sommer, U., Galvão, H.M., 2011. Ammonium, nitrate and phytoplankton interactions in a freshwater tidal estuarine zone: potential effects of cultural eutrophication. Aquat. Sci. 73 (3), 331–343. https://doi.org/10.1007/s00027-011-0180-0.

Domingues, R.B., Sobrino, C., Galvao, H., 2007. Impact of reservoir filling on phytoplankton succession and cyanobacteria blooms in a temperate estuary. Estuar. Coast Shelf Sci. 74, 31–43. https://doi.org/10.1016/j.ecss.2007.03.021.

Du, C., Li, Y., Wang, Q., Liu, G., Zheng, Z., Mu, M., Li, Y., 2017. Tempo-Spatial dynamics of water quality and its response to river flow in estuary of Taihu Lake based on GOCI imagery. Environ. Sci. Pollut. Res. 24 https://doi.org/10.1007/s11356-017-0305-7, 29079-28101.

Edler, L., Elbrächter, M., 2010. Chapter 2: the Utermöhl method for quantitative phytoplankton analysis. In: Karlson, B., Cusak, C., Bresnan, E. (Eds.), Microscopic and Molecular Methods for Quantitative Phytoplankton Analysis, 55. UNESCO, Paris, pp. 13–20.

Egge, E., Elferink, S., Vaulot, D., John, U., Bratbak, G., Larsen, A., Edvardsen, B., 2021. An 18S V4 rDNA Metabarcoding Dataset of Protist Diversity in the Atlantic Inflow to the Arctic Ocean, through the Year and Down to 1000 M Depth. https://doi.org/10.5194/essd-2021-133 bioRxiv.

Elbrecht, V., Leese, F., 2015. Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass—sequence relationships with an innovative metabarcoding protocol. PLoS One 10 (7), e0130324. https://doi.org/10.1371/journal.pone.0130324.

Gameiro, C., Cartaxana, P., Brotas, V., 2007. Environmental drivers of phytoplankton distribution and composition in Tagus Estuary, Portugal. Estuar. Coast Shelf Sci. 75, 21–34.

Gran-Stadniczeñko, S., Egge, E., Hostyeva, V., Logares, R., Eikrem, W., Edvardsen, B., 2019. Protist diversity and seasonal dynamics in Skagerrak plankton communities as revealed by metabarcoding and microscopy. J. Eukaryot. Microbiol. 66 (3), 494–513. https://doi.org/10.1111/jeu.12700.

Grzebyk, D., Pasqualini, V., Garrido, M., Quilichini, Y., Pereto, C., Cecchi, P., 2022. Insight into the morphology and genetic diversity of the *Chaetoceros tenuissimus* (Bacillariophyta) species complex. Eur. J. Phycol. 1–19 https://doi.org/10.1080/09670262.2022.2029949.

Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, et al., 2013. The protist ribosomal reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. Nucleic Acids Res. 41, D597–D604. https://doi.org/10.1093/nar/gks1160.

Guiry, M.D., Guiry, G.M., 2018. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org.

Hammer, Ø., Harper, D.A.T., 2006. Paleontological Data Analysis. Blackwell.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological Statistics software package for education and data analysis. Palaeontol. Electron. 4 (1), 9.

Haubois, A.G., Sylvestre, F., Guarini, J.-M., Richard, P., Blanchard, G.F., 2005. Spatio-temporal structure of the epipelic diatom assemblage from an intertidal mudflat in Marennes-Olerón Bay, France. Estuar. Coast Shelf Sci. 64, 385–394.

Hongo, Y., Kimura, K., Takaki, Y., Yoshida, Y., Baba, S., Kobayashi, G., et al., 2021. The genome of the diatom *Chaetoceros tenuissimus* carries an ancient integrated fragment of an extant virus. Sci. Rep. 11 (1), 22877 https://doi.org/10.1038/s41598-021-00565-3.

Horner Rosser, S.M.J., Thompson, P.A., 2001. Phytoplankton of the Swan-Canning Estuary: a comparison of nitrogen uptake by different bloom assemblages. Hydrol. Process. 15, 2579–2594. https://doi.org/10.1002/hyp.288.

Huo, S., Li, X., Xi, B., Zhang, H., Ma, C., He, Z., 2020. Combining morphological and metabarcoding approaches reveals the freshwater eukaryotic phytoplankton community. Environ. Sci. Eur. 32, 1–14. https://doi.org/10.1186/s12302-020-00321-w.

Hydes, D.J., Aoyama, M., Aminot, A., Bakker, K., Becker, S., Coverly, S., Zhang, J.Z., 2010. Recommendations for the Determination of Nutrients in Seawater to High Levels of Precision and Inter-comparability Using Continuous Flow Analysers. GO-SHIP (Unesco/IOC), 1–87.

Iriarte, A., de Madariaga, I., Diez-Garagarza, F., Revilla, M., Orive, E., 1997. Primary plankton production, respiration and nitrification in a shallow temperate estuary during summer. J. Exp. Mar. Biol. 208 (1–2), 127–151.

Jeffrey, S.W., 1997. Application of pigment methods to oceanography. In: Jeffrey, S.W., Mantoura, R.F.C., Wright, S.W. (Eds.), Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods. UNESCO Monogr. Oceanogr. Methodol. 10. UNESCO Publishing, Paris, pp. 127–165.

Jenkinson, I.R., 1985. The microplankton biomass and diversity of the upper Shannon Estuary (Ireland) and two tributary estuaries. Br. Phycol. J. 20, 187.
Johnson, M.D., Stoecker, D.K., Marshall, H.G., 2013. Seasonal dynamics of *Mesodinium rubrum* in Chesapeake bay. J. Plankton Res. 35 (4), 877–893. https://doi.org/10.1093/plankt/fbt028.
Jouenne, F., Lefebvre, S., Véron, B., Lagadeuc, Y., 2007. Phytoplankton community structure and primary production in small intertidal estuarine-bay ecosystem (eastern English Channel, France). Mar. Biol. 151, 805–825. https://doi.org/10.1007/s00227-006-0440-z.
Keck, F., Vasselon, V., Tapolczai, K., Rimet, F., Bouchez, A., 2017. Freshwater biomonitoring in the information age. Front. Ecol. Environ. 15, 266–274. https://doi.org/10.1002/fee.1490.
Kembel, S.W., Wu, M., Eisen, J.A., Green, J.L., 2012. Incorporating 16S gene copy number information improves estimates of microbial diversity and abundance. PLoS Comput. Biol. 8 (10), e1002743 https://doi.org/10.1371/journal.pcbi.1002743.
Kim, D.K., Park, K., Jo, H., Kwak, I.S., 2019. Comparison of water sampling between environmental DNA metabarcoding and conventional microscopic identification: a case study in Gwangyang Bay, South Korea. Appl. Sci. 9 (16), 3272. https://doi.org/10.3390/app9163272.
Kitsiou, D., Karydis, M., 2011. Coastal marine eutrophication assessment: a review on data analysis. Environ. Int. 37, 778–801. https://doi.org/10.1016/j.envint.2011.02.004.
Kopf, A., Bicak, M., Kottmann, R., et al., 2015. The ocean sampling day consortium. GigaScience 4, 27. https://doi.org/10.1186/s13742-015-0066-5.
Latz, M.A., Grujcic, V., Brugel, S., Lycken, J., John, U., Karlson, B., et al., 2022. Short-and long-read metabarcoding of the eukaryotic rRNA operon: evaluation of primers and comparison to shotgun metagenomics sequencing. Mol. Ecol. Resour. 22 (6), 2304–2318. https://doi.org/10.1111/1755-0998.13623.
Laza-Martínez, A., 2012. *Urgorri complanatus* gen. Et sp. Nov. (cryptophyceae), a red-tide-forming species in brackish waters. J. Phycol. 48 (2), 423–435. https://doi.org/10.1111/j.1529-8817.2012.01130.x.
Lee, M., Won, N.I., Baek, S.H., 2020. Comparison of HPLC pigment analysis and microscopy in phytoplankton assessment in the seomjin River Estuary, korea. Sustainability 12 (4), 1675. https://doi.org/10.3390/su12041675.
Lemley, D.A., Adams, J.B., Strydom, N.A., 2018. Triggers of phytoplankton bloom dynamics in permanently eutrophic waters of a South African estuary. Afr. J. Aquat. Sci. 43 (3), 229–240. https://doi.org/10.2989/16085914.2018.1478794.
Li, Z., Liu, X., Yu, Y., Huang, H., Li, X., Ji, Q., et al., 2019. Barcoding for diatoms in the Yangtze River from the morphological observation and 18S rDNA polymorphic analysis. Forensic Sci. Int. 297, 81–89. https://doi.org/10.1016/j.forsciint.2019.01.028.
Liu, S., Gibson, K., Cui, Z., Chen, Y., Sun, X., Chen, N., 2020. Metabarcoding analysis of harmful algal species in Jiaozhou Bay. Harmful Algae 92, 101772. https://doi.org/10.1016/j.hal.2020.101772.
Machado, K.B., Bini, L.M., Melo, A.S., Andrade, A.T.D., Almeida, M.F.D., Carvalho, P., Nabout, J.C., 2023. Functional and taxonomic diversities are better early indicators of eutrophication than composition of freshwater phytoplankton. Hydrobiologia 850 (6), 1393–1411. https://doi.org/10.1007/s10750-022-04954-1.
MacKeigan, P.W., Garner, R.E., Monchamp, M.È., Walsh, D.A., Onana, V.E., et al., 2022. Comparing microscopy and DNA metabarcoding techniques for identifying cyanobacteria assemblages across hundreds of lakes. Harmful Algae 113, 102187. https://doi.org/10.1016/j.hal.2022.102187.
Madariaga, I., 1995. Photosynthetic characteristics of phytoplankton during the development of a summer bloom in the Urdaibai Estuary, Bay of Biscay. Estuar. Coast Shelf Sci. 40 (5), 559–575.
Madariaga, I., Garagarza, F.D., Revilla, M., 1994. Caracterización hidrográfica del estuario de Urdaibai durante el desarrollo de una floración fitoplanctónica estival. Kobie. Ciencias naturales 22, 13–22.
Madariaga, I., Orive, E., 1989. Spatio-temporal variations of size-fractionated primary production in the Gernika estuary. J. Exp. Mar. Biol. Ecol. 127 (3), 273–288. https://doi.org/10.1016/0022-0981(89)90079-8.
Madariaga, I., Orive, E., Boalch, G.T., 1989. Primary production in the Gernika Estuary during a summer bloom of the dinoflagellate *Peridinium quinquecorne Abé*. Bot. Mar. 32 (2), 159–165. https://doi.org/10.1515/botm.1989.32.2.159.
Mäki, A., Salmi, P., Mikkonen, A., Kremp, A., Tiirola, M., 2017. Sample preservation, DNA or RNA extraction and data analysis for high-throughput phytoplankton community sequencing. Front. Microbiol. 8 https://doi.org/10.3389/fmicb.2017.01848.
Malviya, S., Scalco, E., Audic, S., Vincent, F., Veluchamy, A., Poulain, J., Wincker, J., Ludicone, D., De Vargas, C., Bittner, L., Zingone, A., Bowler, C., 2016. Insights into global diatom distribution and diversity in the world's ocean. Proc. Natl. Acad. Sci. USA 113, 1516–1525. https://doi.org/10.1073/pnas.1509523113.
Martin, J.L., Santi, I., Pitta, P., John, U., Gypens, N., 2022. Towards quantitative metabarcoding of eukaryotic plankton: an approach to improve 18S rRNA gene copy number bias. Metabarcoding Metagenom 6, e85794. https://doi.org/10.3897/mbmg.6.85794.
McCabe, R.M., Hickey, B.M., Kudela, R.M., Lefebvre, K.A., Adams, N.G., Bill, B.D., Trainer, V.L., 2016. An unprecedented coastwide toxic algal bloom linked to anomalous ocean conditions. Geophys. Res. Lett. 43 (19), 366–376. https://doi.org/10.1002/2016GL070023.
McGarrigle, M., Bowman, J.J., Clabby, K.J., Cunninghan, P., MacCarthaig, M., Keegan, M., Cantrell, B., Lehane, M., Clenaghan, C., Toner, P.F., 2001. Water Quality in Ireland, 1998-2000. EPA, Wexford.
McLusky, D.S., Elliott, M., 2004. The Estuarine Ecosystem: Ecology, Threats and Management. OUP, Oxford.
Mizobuchi, A., Tsuchioka, H., Handa, S., 2021. Cryptophyte *Urgorri complanatus* causes red tides in brackish waters in Japan. Jpn. J. Phycol. 69, 10–13. https://doi.org/10.1111/j.1529-8817.2012.01130.x.
Montagnes, D.J.S., Berges, J.A., Harrison, P.J., Taylor, F.J.R.L., 1994. Estimating carbon, nitrogen, protein, and chlorophyll *a* from volume in marine phytoplankton. Limnol. Oceanogr. 39 (5), 1044–1060. https://doi.org/10.4319/lo.1994.39.5.1044.
Morison, F., Franzè, G., Harvey, E., Menden-Deuer, S., 2020. Light fluctuations are key in modulating plankton trophic dynamics and their impact on primary production. L&O Letters 5 (5), 346–353. https://doi.org/10.1002/lol2.10156.
Mortagua, A., Vasselon, V., Oliveira, R., Elias, C., Chardon, C., Bouchez, A., Almeida, S. F., 2019. Applicability of DNA metabarcoding approach in the bioassessment of Portuguese rivers using diatoms. Ecol. Indicat. 106, 105470 https://doi.org/10.1016/j.ecolind.2019.105470.
Muhammad, B.L., Kim, T., Ki, J.S., 2021. 18S rRNA analysis reveals high diversity of phytoplankton with emphasis on a naked Dinoflagellate Gymnodinium sp. at the han river (Korea). Diversity 13 (2), 73. https://doi.org/10.3390/d13020073.
Muñiz, O., Revilla, M., Rodríguez, J.G., Laza-Martínez, A., Fontán, A., 2019. Annual cycle of phytoplankton community through the water column: study applied to the implementation of bivalve offshore aquaculture in the southeastern Bay of Biscay. Oceanologia 61 (1), 114–130. https://doi.org/10.1016/j.oceano.2018.08.001.
Muñiz, O., Rodríguez, J.G., Revilla, M., Laza-Martínez, A., Seoane, S., Franco, J., 2020. Inhomogeneity detection in phytoplankton time series using multivariate analyses. Oceanologia 62 (3), 243–254. https://doi.org/10.1016/j.oceano.2020.01.004.
Muylaert, K., Sabbe, K., Vyverman, W., 2000. Spatial and temporal dynamics of phytoplankton communities in a freshwater tidal estuary (Schelde, Belgium). Estuar. Coast Shelf Sci. 50, 673–687. https://doi.org/10.1006/ecss.2000.0590.
Naik, R.K., Anil, A.C., Narale, D.D., Chitari, R.R., Kulkarni, V.V., 2011. Primary description of surface water phytoplankton pigment patterns in the Bay of Bengal. J. Sea Res. 65, 435–441. https://doi.org/10.1016/j.seares.2011.03.007.
Nunes, S., Latasa, M., Gasol, J.M., Estrada, M., 2018. Seasonal and interannual variability of phytoplankton community structure in a Mediterranean coastal site. Mar. Ecol. Prog. Ser. 592, 57–75.
Olenina, I., Hajdu, S., Edler, L., Andersson, A., Wasmund, N., Busch, S., Göbel, J., Gromisz, S., Huseby, S., Huttunen, M., Jaanus, A., Kokkonen, P., Ledaine, I., Niemkiewicz, E., 2006. Biovolumes and Size-Classes of Phytoplankton in the Baltic Sea, 106. Helsinki Commission, 144. Baltic Marine Environ. Prot. Comm.
Orive, E., Iriarte, A., Madariaga, I., Revilla, M., 1998. Phytoplankton blooms in the Urdaibai estuary during summer: physico-chemical conditions and taxa involved. Oceanol. Acta 21, 293–305.
Paczkowska, J., Rowe, O.F., Figueroa, D., Andersson, A., 2019. Drivers of phytoplankton production and community structure in nutrient-poor estuaries receiving terrestrial organic inflow. Mar. Environ. Res. 151, 104778 https://doi.org/10.1016/j.marenvres.2019.104778.
Paerl, H.W., Valdes, L.M., Pinckney, J.L., Piehler, M.F., Dyble, J., Moisander, P.H., 2003. Phytoplankton photopigments as indicators of estuarine and coastal eutrophication. Bioscience 53 (10), 953–964.
Penna, A., Casabianca, S., Guerra, A.F., Vernesi, C., Scardi, M., 2017. Analysis of phytoplankton assemblage structure in the Mediterranean Sea based on high-throughput sequencing of partial 18S rRNA sequences. Mar. Genomics 36, 49–55. https://doi.org/10.1016/j.margen.2017.06.001.
Pérez-Burillo, J., Valoti, G., Witkowski, A., Prado, P., Mann, D.G., Trobajo, R., 2022. Assessment of marine benthic diatom communities: insights from a combined morphological–metabarcoding approach in Mediterranean shallow coastal waters. Mar. Pollut. Bull. 174, 113183 https://doi.org/10.1016/j.marpolbul.2021.113183.
Pinckney, J.L., Paerl, H.W., Tester, P., Richardson, T.L., 2001. The role of nutrient loading and eutrophication in estuarine ecology. Environ. Health Perspect. 109, 699–706. https://doi.org/10.1289/ehp.01109s5699.
Piñol, J., Senar, M.A., Symondson, W.O., 2019. The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. Mol. Ecol. 28 (2), 407–419. https://doi.org/10.1111/mec.14776.
Piredda, R., Tomasino, M.P., D'erchia, A.M., Manzari, C., Pesole, G., Montresor, M., Zingone, A., 2017. Diversity and temporal patterns of planktonic protist assemblages at a Mediterranean Long Term Ecological Research site. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 93 (1) https://doi.org/10.1093/femsec/fiw200.
Piwosz, K., Shabarova, T., Pernthaler, J., Posch, T., Šimek, K., Porcal, P., Salcher, M.M., 2020. Bacterial and eukaryotic small-subunit amplicon data do not provide a quantitative picture of microbial communities, but they are reliable in the context of ecological interpretations. mSphere 5 (2), e00052-20. https://doi.org/10.1128/mSphere.00052-20.
Popovich, C.A., Marcovecchio, J.E., 2008. Spatial and temporal variability of phytoplankton and environmental factors in a temperate estuary of South America (Atlantic coast, Argentina). Continent. Shelf Res. 28, 236–244.
Pybus, C., McEvoy, S., McGrath, D., 1984. Red water caused by *Genodinium foliaceum* (dinophyta) in Lough Atalia, Co galway. Ir. Nat. J. 21 (5), 226–228.
Raveh, O., Angel, D.L., Astrahan, P., Belkin, N., Bar-Zeev, E., Rahava, E., 2019. Phytoplankton response to N-rich well amelioration brines: a mesocosm study from the southeastern Mediterranean Sea. Mar. Pollut. Bull. 146, 355–365. https://doi.org/10.1016/j.marpolbul.2019.06.067.
Revilla, M., Iriarte, A., Madariaga, I., Orive, E., 2000. Bacterial and phytoplankton dynamics along a trophic gradient in a shallow temperate estuary. Estuar. Coast Shelf Sci. 50, 297–313.
Rimet, F., Aylagas, E., Borja, A., Bouchez, A., Canino, A., Chauvin, C., Ekrem, T., 2021. Metadata standards and practical guidelines for specimen and DNA curation when building barcode reference libraries for aquatic life. Metabarcoding Metagenom 5. https://doi.org/10.3897/mbmg.5.58056.

Ruiz, A., Franco, J., Villate, F., 1998. Microzooplankton grazing in the Estuary of Mundaka, Spain, and its impact on phytoplankton distribution along the salinity gradient. Aquat. Microb. Ecol. 14 (3), 281–288. https://doi.org/10.3354/ame014281.

Saifullah, A.S.M., Kamal, A.H.M., Idris, M.H., Rajaee, A.H., 2019. Community composition and diversity of phytoplankton in relation to environmental variables and seasonality in a tropical mangrove estuary. Reg. Stud. Mar. Sci. 32, 100826 https://doi.org/10.1016/j.rsma.2019.100826.

Santi, I., Kasapidis, P., Karakassis, I., Pitta, P., 2021. A comparison of DNA metabarcoding and microscopy methodologies for the study of aquatic microbial eukaryotes. Diversity 13 (5), 180. https://doi.org/10.3390/d13050180.

Santoferrara, L.F., 2019. Current practice in plankton metabarcoding: optimization and error management. J. Plankton Res. 41 (5), 571–582. https://doi.org/10.1093/plankt/fbz041.

Santos, M., Amorim, A., Brotas, V., Cruz, J.P.C., Palma, C., Borges, C., Favareto, L.R., Veloso, V., Damaso-Rodrigues, M.L., Chainho, P., Felix, P.M., Brito, A.C., 2022. Sci. Rep. 12 (1), 16423.

Sarno, D., Zingone, A., Saggiomo, V., Carrada, G.C., 1993. Phytoplankton biomass and species composition in a Mediterranean coastal lagoon. Hydrobiologia 271 (1), 27–40. https://doi.org/10.1007/BF00005692.

Seoane, S., Eikrem, W., Arlucea, J., Orive, E., 2009a. Haptophytes of the nervión river estuaries (northern Spain). Bot. Mar. 52, 47–59. https://doi.org/10.1515/bot.2009.027.

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

Seoane, S., Puente, A., Guinda, X., Juanes, J.A., 2012. Bloom forming and toxic phytoplankton in transitional and coastal waters of Cantabria region coast (Southeastern Bay of Biscay, Spain). Mar. Pollut. Bull. 64 (12), 2860–2866. https://doi.org/10.1016/j.marpolbul.2012.08.023.

Seoane, S., Zapata, M., Orive, E., 2009b. Growth rates and pigment patterns of haptophytes isolated from estuarine waters. J. Sea Res. 62 (4), 286–294. https://doi.org/10.1016/j.seares.2009.07.008.

Shi, T., Li, Z., Zhao, Y., 2019. Spatial variation in water column structure, nutrients, chlorophyll and zooplankton in an estuarine transect of Southampton water. IOP Conf. Ser. Earth Environ. Sci. 281, 012008.

Snow, G.C., Adams, J.B., Bate, G.C., 2000. Effect of river flow on estuarine microalgal biomass and distribution. Estuar. Coast Shelf Sci. 51 (2), 255–266. https://doi.org/10.1006/ecss.2000.0638.

Soares, E.M., Figueredo, C.C., Gücker, B., Boëchat, I.G., 2013. Effects of growth condition on succession patterns in tropical phytoplankton assemblages subjected to experimental eutrophication. J. Plankton Res. 35 (5), 1141–1153. https://doi.org/10.1093/plankt/fbt061.

Soleimani, M., van Breemen, L.C., Maddala, S.P., Joosten, R.R., Wu, H., et al., 2021. In situ manipulation and micromechanical characterization of diatom frustule constituents using focused ion beam scanning electron microscopy. Small Methods 5 (12), 2100638. https://doi.org/10.1002/smtd.202100638.

Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M.D.M., Breiner, H.W., Richards, T.A., 2010. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Mol. Ecol. 19, 21–31. https://doi.org/10.1111/j.1365-294X.2009.04480.x.

Stoeck, T., Breiner, H.W., Filker, S., Ostermaier, V., Kammerlander, B., Sonntag, B., 2014. A morphogenetic survey on ciliate plankton from a mountain lake pinpoints the necessity of lineage-specific barcode markers in microbial ecology. Environ. Microbiol. 1, 430–444. https://doi.org/10.1111/1462-2920.12194.

Throndsen, J., Hasle, G.R., Tangen, K., 2007. Phytoplankton of Norwegian Coastal Waters. Almater. Oslo, p. 343.

Trebitz, A.S., Hoffman, J.C., Darling, J.A., Pilgrim, E.M., Kelly, J.R., Brown, E.A., Chadderton, W.L., Egan, S.P., Grey, E.K., Hashsham, S.A., Klymus, K.E., Mahon, A.R., Ram, J.L., Schultz, M.T., Stepien, C.A., Schardt, J.C., 2017. Early detection monitoring for aquatic non-indigenous species: optimizing surveillance, incorporating advanced technologies, and identifying research needs. J. Environ. Manag. 202, 299–310. https://doi.org/10.1016/j.jenvman.2017.07.045.

Trigueros, J.M., Orive, E., 2001. Seasonal variations of diatoms and dinoflagellates in a shallow, temperate estuary, with emphasis on neritic assemblages. Hydrobiologia 444, 119–133. https://doi.org/10.1023/A:1017563031810.

Trigueros, J.M., Ansotegui, A., Orive, E., 2000a. Morphology and distribution of two brackish diatoms (Bacillariophyceae): *Cyclotella atomus Hustedt* and *Thalassiosira guillardii Hasle* in the estuary of Urdaibai (northern Spain). Nova Hedwigia 70, 431–450. https://doi.org/10.1127/nova.hedwigia/70/2000/431.

Trigueros, J.M., Ansotegui, A., Orive, E., 2000b. Remarks on morphology and ecology of recurrent dinoflagellate species in the estuary of Urdaibai (northern Spain). Bot. Mar. 43, 93–103. https://doi.org/10.1515/BOT.2000.009.

Trigueros, J.M., Orive, E., 2000. Tidally driven distribution of phytoplankton blooms in a shallow, macrotidal estuary. J. Plankton Res. 22 (5), 969–986.

Underwood, G.J.C., Phillips, J., Saunders, K., 1998. Distribution of estuarine benthic diatom species along salinity and nutrient gradients. Eur. J. Phycol. 33, 173–183. https://doi.org/10.1080/09670269810001736673.

Uriarte, I., 2001. Evaluación de la respuesta del zooplancton al estrés ambiental en un sistema con elevado grado de alteración antrópica (estuario de Bilbao) en relación a un sistema poco alterado (estuario de Urdaibai). PhD Thesis. Univ. of the Basque Country, Basque Country, Spain, p. 118.

Vajravelu, M., Martin, Y., Ayyappan, S., 2018. Seasonal influence of physicochemical parameters on phytoplankton diversity, community structure and abundance at Parangipettai coastal waters Bay of Bengal, South East Coast of India. Oceanologia 60 (2), 114–127. https://doi.org/10.1016/j.oceano.2017.08.003.

Valdes-Weaver, L.M., Piehler, M.F., Pinckney, J.L., Howe, K.E., Rossignol, K., Paerl, H. W., 2006. Long-term temporal and spatial trends in phytoplankton biomass and class-level composition in the hydrologically variable Neuse-Pimlico Estuarine Continuum, North Carolina, USA. Limnol. Oceanogr. 51 (3), 1410–1420. https://doi.org/10.4319/lo.2006.51.3.1410.

Van der Loos, L.M., Nijland, R., 2021. Biases in bulk: DNA metabarcoding of marine communities and the methodology involved. Mol. Ecol. 30 (13), 3270–3288. https://doi.org/10.1111/mec.15592.

Varela, M., 1996. Phytoplankton ecology in the bay of Biscay. Sci. Mar. 60 (2), 45–53.

Villate, F., Aravena, G., Iriarte, A., Uriarte, I., 2008. Axial variability in the relationship of chlorophyll *a* with climatic factors and the North Atlantic Oscillation in a Basque coast estuary, Bay of Biscay (1997–2006). J. Plankton Res. 30 (9), 1041–1049. https://doi.org/10.1093/plankt/fbn056.

Villate, F., Franco, J., Ruiz, A., Orive, E., 1989. Caracterización geomorfológica e hidrológica de cinco sistemas estuáricos del País Vasco. Kobie 18, 157–170.

Villate, F., Iriarte, A., Uriarte, I., Sanchez, I., 2017. Seasonal and interannual variability of mesozooplankton in two contrasting estuaries of the Bay of Biscay: relationship to environmental factors. J. Sea Res. 130, 189–203. https://doi.org/10.1016/j.seares.2017.05.002.

Walsh, G.E., McLaughlin, L.L., Yoder, M.J., Moody, P.H., Lores, E.M., Forester, J., Wessinger-Duvall, P.B., 1988. *Minutocellus polymorphus*: a new marine diatom for use in algal toxicity tests. Environ. Toxicol. Chem. 7 (11), 925–929.

Wang, L., Ou, L., Huang, K., Chai, C., Wang, Z., et al., 2018. Determination of the spatial and temporal variability of phytoplankton community structure in Daya Bay via HPLC-CHEMTAX pigment analysis. J. Oceanol. Limnol. 36 (3), 750–760. https://doi.org/10.1016/j.hal.2022.102297.

Wang, Y., Wu, H., Lin, J., Zhu, J., Zhang, W., Li, C., 2019. Phytoplankton blooms off a high turbidity estuary: a case study in the Changjiang River Estuary. J. Geophys. Res.: Oceans 124, 8036–8059. https://doi.org/10.1029/2019JC015343.

Wang, Z., Liu, L., Tang, Y., Li, A., Liu, C., Xie, C., et al., 2022. Phytoplankton community and HAB species in the South China Sea detected by morphological and metabarcoding approaches. Harmful Algae 118, 102297. https://doi.org/10.1016/j.hal.2022.102297.

Weigand, H., Beermann, A.J., Čiampor, F., Costa, F.O., Csabai, Z., Duarte, S., Geiger, M. F., Grabowski, M., Rimet, F., Rulik, B., et al., 2019. DNA barcode reference libraries for the monitoring of aquatic biota in Europe: gap-analysis and recommendations for future work. Sci. Total Environ. 678, 499–524.

Wells, M.L., Trainer, V.L., Smayda, T.J., Karlson, B.S.O., Trick, C.G., Kudela, R.M., Ishikawa, A., Bernard, S., Wulff, A., Anderson, D.M., Cochlan, W.P., 2015. Harmful algal blooms and climate change: learning from the past and present to forecast the future. Harmful Algae 49, 68–93. https://doi.org/10.1016/j.hal.2015.07.009.

Zafeiropoulos, H., Gioti, A., Ninidakis, S., Potirakis, A., Paragkamian, S., Angelova, N., Antoniou, A., Danis, T., Kaitetzidou, E., Kasapidis, P., Kristoffersen, J.B., Papadogiannis, V., Pavloudi, C., Ha, Q.V., Lagnel, J., Pattakos, N., Perantinos, G., Sidirokastritis, D., Vavilis, P., Kotoulas, G., Manousaki, T., Sarropoulou, E., Tsigenopoulos, C.S., Arvanitidis, C., Magoulas, A., Pafilis, E., 2021. 0s and 1s in marine molecular research: a regional HPC perspective. GigaScience 10 (8), giab053.

Zapata, M., Rodríguez, F., Garrido, J.L., 2000. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. Mar. Ecol. Prog. Ser. 195, 29–45. https://doi.org/10.3354/meps195029.

Zhang, Q.-C., Song, J.-J., Yu, R.-C., Yan, T., Wang, Y.-F., Kong, F.-Z., Zhou, M.-J., 2013. Roles of mixotrophy in blooms of different dinoflagellates: implications from the growth experiment. Harmful Algae 30, 10–26. https://doi.org/10.1016/j.hal.2013.08.003.

Zimmermann, J., Glöockner, G., Jahn, R., Enke, N., Gemeinholzer, B., 2015. Metabarcoding vs. morphological identification to assess diatom diversity in environmental studies. Mol. Ecol. Resour. 15, 526–542.