



# Response of the phytoplankton community to the cessation of wastewater discharges in the urdaibai estuary (SE bay of biscay) based on PIGMENTUM analysis of HPLC pigments

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## ARTICLE INFO

### Keywords:

Estuaries  
eutrophication  
nutrient enrichment  
Pigment analysis  
PIGMENTUM  
Pollution monitoring  
Wastewater  
sewerage works

## ABSTRACT

Phytoplankton responds rapidly to nutrient availability variations, becoming a useful indicator for eutrophication and/or management actions to reduce it. The present study evaluated the medium-term response of the phytoplankton community of a temperate estuary (Urdaibai estuary) to the cessation of discharges from a wastewater treatment-plant (WWTP), comparing the physicochemical conditions and the phytoplankton community before (2020) and after (2022) the sewerage works. The cessation led to a decrease of ammonium and phosphate, causing decreases of phytoplankton biomass in the outer and middle estuary and increases in the surroundings of the WWTP. Community composition also changed, recording an increase of prasinoxanthin-containing algae's contribution to total biomass, and a composition shift in the inner estuary, from mainly flagellates (alloxanthin-containing and chlorophyll *b*-containing algae) to the increase of diatoms, which could be prompted by the change of nutrient-ratios and the nitrogen source, and might indicate the recuperation of the system.

## 1. Introduction

Cultural eutrophication, i.e., water over-enrichment by nutrients (especially nitrogen and phosphorus) to produce an undesirable disturbance to the water balance of organisms and the water quality (European Commission, 1991; OSPAR, 2003), has become the primary and most widespread anthropogenic threat to the health of freshwater and coastal ecosystems (Field and Barros, 2014; Malone and Newton, 2020; Smith and Schindler, 2009). Although this excessive nutrient enrichment is mainly caused by anthropogenic nutrient inputs from human activities (e.g., wastewater, synthetic fertilizers, and aquaculture) (Malone and Newton, 2020), it is also driven and accelerated by climate change (Paerl, 2006; Sinha et al., 2017). Eutrophication is considered the main threat to the integrity of estuaries worldwide (e.g., Adams et al., 2020; Dutto et al., 2012; Niu et al., 2020), causing degradation of the systems via the enhancement of primary production in response to nutrient loadings. This results in the loss of biodiversity (mostly submerged aquatic vegetation), increased frequency and extent of harmful algal blooms (HABs) (Glibert et al., 2018; Paerl, 2018),

coastal acidification (Cai et al., 2011; Liang et al., 2021), turbidity (Cloern, 2001), hypoxic conditions due to microbial oxygen consumption (Li et al., 2020), imbalanced food webs, and altered biogeochemical cycling (Ferreira et al., 2011; Lemley and Adams, 2019).

Its basal position in the food chain makes phytoplankton the link between inorganic nutrients and the rest of the trophic levels (Seoane et al., 2011), and it is known to be the first autotrophic community to respond to variations in nutrient availability (Paerl et al., 2003). Unbalanced loadings of nitrogen (N) and phosphorus (P) can lead to changes in the N:P:Si nutrient ratio, creating Si-, P- or N-limitation and conditioning phytoplankton community composition (Lie et al., 2011; Wu et al., 2017a), which can have profound ecological consequences (Van Meerssche and Pinckney, 2019). Generally, eutrophication tends to favor the dominance of small, fast-growing phytoplankton organisms ("r"-selected species) (Bužančić et al., 2016; González and Roldán, 2019). Additionally, the forms of N, the primary cause of eutrophication in most coastal ecosystems (e.g., Paerl, 2018), can also result in alterations the community composition. Diatoms appear to be nitrate (NO<sub>3</sub><sup>-</sup>) opportunists, becoming the dominant protists in NO<sub>3</sub><sup>-</sup> rich waters, while

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<https://doi.org/10.1016/j.marenvres.2024.106668>

Received 1 February 2024; Received in revised form 4 July 2024; Accepted 2 August 2024

Available online 3 August 2024

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systems that are more enriched with chemically reduced N forms (ammonium) often result in communities dominated by mixotrophic dinoflagellates or (pico)cyanobacteria (Glibert et al., 2016). Consequently, phytoplankton is a useful indicator for the assessment of the ecological status of surface waters like estuaries (e.g., within the European Water Framework or Marine Strategy Directives; WFD or MSFD) (Seoane et al., 2011) and/or to assess the effectiveness of management strategies to mitigate adverse changes like nutrient enrichment (Eccles et al., 2020; Li et al., 2018).

Human sewage is the most prevalent urban source of nutrient pressure, with an estimated release of  $\sim 9 \times 10^9$  kg N yr<sup>-1</sup> into the environment in 2018 (Malone and Newton, 2020). However, growing evidence suggests there is a global trend towards reversing eutrophication (Ibáñez and Peñuelas, 2019). In developed countries, N and P inputs to coastal areas through riverine discharge are being reduced (termed reoligotrophication) with enforced water quality regulations and technological advances in wastewater treatment facilities (e.g., Groll, 2017). However, understanding of the effects of eutrophication and reoligotrophication mainly comes from studies of shallow lakes (e.g., Sand-Jensen et al., 2017; Tong et al., 2017). Changes in rivers and estuaries are less well understood (Ibáñez and Peñuelas, 2019), but several studies have reported an abrupt shift from phytoplankton to macrophytes as dominant primary producers (from green to clear waters) in response to reoligotrophication in rivers and estuaries (Ibáñez et al., 2012; Riemann et al., 2016).

In this context, the Urdaibai estuary received direct discharges of wastewaters coming from Gernikas' wastewater treatment plant (WWTP) from 1972 to 2021. The WWTP had primary and secondary treatment but did not allow efficient removal of N and P, discharging large amounts of inorganic nutrients, together with organic wastes and faecal bacteria (Franco et al., 2004; Revilla et al., 2000). As a result, the direct discharges to the innermost area of the Urdaibai estuary have represented the main source of pollution and water quality degradation of the system, making the inner estuary susceptible to eutrophication (Revilla et al., 2017). Indeed, some areas of the system did not fulfil the environmental objectives set by the WFD (Borja et al., 2021), with very high ammonium and phosphate concentrations that led to high phytoplankton biomass (eutrophication). However, sewerage system renovations were implemented in the area lately to address this issue (Revilla et al., 2017), which resulted in the diversion of the effluent from Gernikas' WWTP outside the estuary in July 2021, after five decades of discharges.

Several studies have addressed the physicochemical properties (e.g., Iriarte et al., 2010, 2015), human impacts (Castillo-Eguskizta et al., 2017; Iriarte et al., 2016), and phytoplankton community (e.g., Ansoategui et al., 2003; Bilbao et al., 2023; Franco, 1994; Revilla et al., 2000; Trigueros et al., 2000) of the Urdaibai estuary before the sewerage works. However, there is little knowledge on the effect of cessation of wastewater discharges to the estuary. Bilbao et al. (2022) evaluated the immediate effect of sewerage improvement on the water quality throughout the Urdaibai estuary based on nutrients and phytoplankton, confirming that the cessation led to an abrupt decrease of ammonium and phosphate concentrations and changes in the phytoplankton community composition of inner Urdaibai estuary. Nevertheless, the sampling-period of this study was short, covering just one summer month, and the real effect of the sanitation works in the Urdaibai estuary is still unknown.

The aim of this study was to evaluate the medium-term response of the phytoplankton community of the Urdaibai estuary to the cessation of the wastewater discharges by comparing it before (in 2020) and after (in 2022) the sewerage works. The phytoplankton biomass and community composition changes were analysed, focusing on variations in the dominance of groups and algal blooms (abundance and frequency), together with the relationship between local environmental changes and the phytoplankton variations detected. The research hypothesis was that the decrease of N and P concentrations would lead to a decrease of

phytoplankton biomass and blooms as well as a change in the community composition throughout the entire estuary.

## 2. Material and methods

### 2.1. Study area

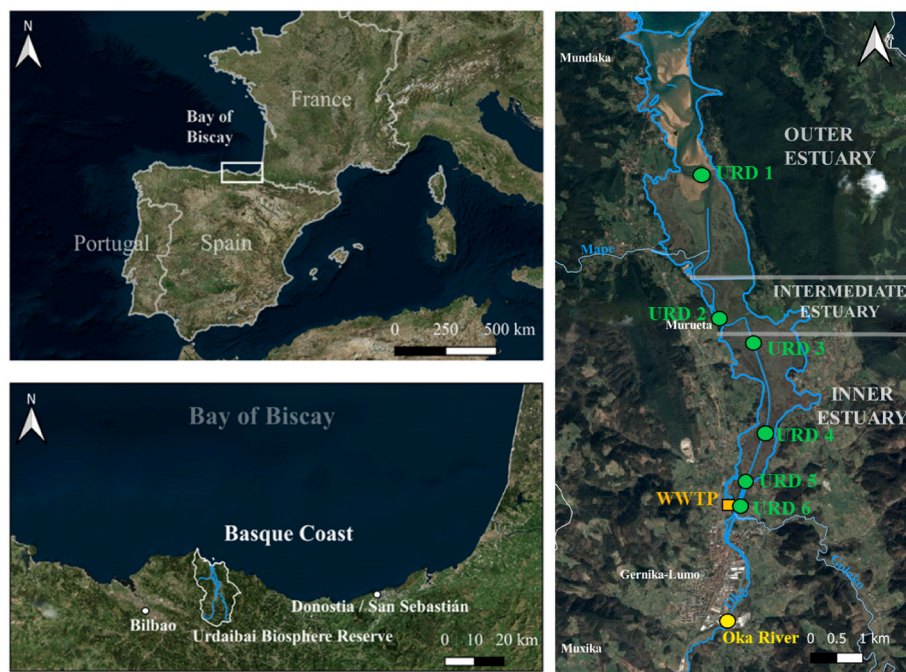
The present study was carried out at Urdaibai estuary, situated on the Basque coast (43° 22' N, 2° 43' W), in northern Spain, and draining into the southeaster Bay of Biscay (Fig. 1). The region experiences a temperate-oceanic climate, characterized by mild winters and warm summers, and it is influenced by both the Gulf Stream and the atmospheric westerlies in the middle and upper troposphere (Usabiaga et al., 2004).

Urdaibai estuary is a short (13.7 km) and shallow (average depth of 2.59 m) meso-macrotidal system formed by the tidal part of the Oka River (Barroeta et al., 2023). The estuary covers 1.89 km<sup>2</sup> (Villate et al., 1989), with a relatively small freshwater contribution (mean flow of 3.6 m<sup>3</sup> s<sup>-1</sup>) compared to the total volume of the estuary ( $3.29 \times 10^6$  m<sup>3</sup>) (Valencia et al., 2004). As a result, tidal cycles exert a significant influence, leading to a predominantly marine-dominated estuary during high tide. The water residence time and the stratification vary within the system (Villate et al., 2017). Longer residence times (between 3 and 86 days) and partially stratified conditions prevail in the middle and inner areas, while the outer zone features residence times shorter than 1 day and a well-mixed water column, due to the substantial tidal flushing (Barroeta et al., 2020; Franco, 1994). However, the system's volume and flushing rates can undergo noticeable variations, due to its geomorphology and, mostly, the torrential nature of the Oka River, with intermittent large flows disrupting the typical pattern of the estuary (Madariaga et al., 1994). Based on morphology, the estuary is divided into three areas (Villate et al., 2017) (Fig. 1): the outer estuary, with marine character, sandy beaches and intertidal flats; the intermediate estuary, comprising a central channel flanked by salt marshes and a complex system of secondary channels; and the inner estuary, a 4-km-long and 15-m-wide artificial channel bordered by reed beds that reaches Gernika. This spatio-temporal variability has direct effects on the physicochemical and biological characteristics of the Urdaibai estuary, leading to short-term changes in the environmental conditions and phytoplankton communities of the system (Madariaga et al., 1994).

### 2.2. Sampling and data acquisition

To determine the effect of the diversion of wastewaters outside the Urdaibai estuary, which was conducted in July 2021, samples were collected in 2020 (pre-sewerage works) and 2022 (post-sewerage works), from March to October, fortnightly. Six permanent sampling stations throughout the estuary's longitudinal axis (Fig. 1) were studied, strategically placed to cover the entire salinity gradient: one in the outer estuary (URD1), one in the middle estuary (URD2), and four in the inner estuary (URD3, URD4, URD5, and URD6). URD6 was situated in Gernikas' WWTP discharge area. An additional station in the Oka River was set up to monitor its physicochemical properties before arriving to the WWTP area. Due to COVID-19 restrictions, no sampling occurred in the first half of April 2020 and thus, to ensure data comparability between 2020 and 2022, the first sampling of April 2022 was excluded. Consequently, 182 samples were collected, corresponding to 26 sampling days (13 each in 2020 and 2022) across the seven sampling stations.

Estuarine water samples were collected during high tide, 0.75 m below the surface and using a 2.5 L plastic Niskin bottle, in an hour-long boat transect from URD1 to URD6. Throughout all sampling days, tidal heights ranged between 3.5 and 4 m, which is considered negligible for a meso-macrotidal estuary, aiming to mitigate its potential as a confounding factor in the physicochemical conditions of the estuary. Additionally, this study exclusively considered subsurface samples to avoid potential impacts from sediment resuspension processes and the



**Fig. 1.** Study area and sampling stations. On the left, the location of the Urdaibai estuary and the Biosphere Reserve in the Bay of Biscay (upper panel) and the Basque coast (lower panel). On the right, the Urdaibai estuary and the sampling stations (URD1-URD6 and Oka River). The discharge point of the wastewater treatment plant (WWTP) is also indicated.

freshwater of the surface layer (Madariaga and Orive, 1989). The collected water was analysed for inorganic nutrients, phytoplankton biomass, and community composition. Additionally, *in situ* measurements of physicochemical parameters were performed at each station, including salinity, temperature, conductivity, pH, dissolved oxygen (DO), oxygen saturation, and turbidity, employing the multi-parameter water quality meter EXO2 (YSI). Water column depth was recorded with the GPS sounder of the boat, while water transparency was estimated using a Secchi disc. At the Oka River station, due to its shallow depth, water for inorganic nutrient analysis was directly collected from the surface, together with the *in situ* measurements of the aforementioned physicochemical parameters.

The dissolved inorganic nutrients subjected to analysis were ammonium, nitrite, nitrate (derived from total oxidized nitrogen), orthophosphate, and silicate. Their determination was conducted at the Chemical Laboratory of the Marine Research Unit within AZTI (Pasaia, Gipuzkoa), using VIS/UV colorimetry in an automated 5-channel analyser with segmented flow. The determination of individual concentrations for these dissolved inorganic nutrients followed classical and widely accepted colorimetric reactions, applicable to both inland and marine waters (GO-SHIP manual by Hydes et al., 2010). The quantification limit for ammonium, nitrate, and silicate was  $1.6 \mu\text{mol L}^{-1}$ ,  $0.4 \mu\text{mol L}^{-1}$  for nitrite, and  $0.16 \mu\text{mol L}^{-1}$  for phosphate. When nutrient concentrations were recorded below the quantification limit, hypothetical values were applied for data analysis and graphical representation, assuming that the concentrations were 50% of the limit. Additionally, nutrient ratios of the Urdaibai estuarine waters were determined, comparing them with the balanced nutrient composition (DIN:Si:P ratio of 16:16:1) described by Redfield (1958) and observing their variability over time and space. For this, dissolved inorganic Nitrogen (DIN) was calculated by summing the concentrations of ammonium, nitrite, and nitrate, orthophosphate was considered dissolved inorganic phosphate (P), and silicate was assumed to be dissolved silicate (Si) (e.g., Sun et al., 2022).

Meteorological data (i.e., air temperature, hours of sunshine, radiation, and monthly-accumulated rainfall) was provided by the Basque Agency of Meteorology (Euskalmet) and the Spanish State

Meteorological Agency (AEMET). Temperature and accumulated rainfall were available from two nearby meteorological stations (Euskalmet), Muxika ( $43^{\circ}17' \text{ N}$ ,  $2^{\circ}41' \text{ W}$ , 16 m height), and Arteaga ( $43^{\circ}20' \text{ N}$ ,  $2^{\circ}39' \text{ W}$ , 19 m height), and the average values from both stations were considered. Insolation hours and radiation (AEMET) data were available from Bilbao Airport (20 km from the estuary). Regarding the hydrological data of the Oka River, Euskalmet provided daily flow values corresponding to Muxikas' gauging station. Additionally, using the raw Oka River flow data, the river Flow Index was calculated following Shortreed and Stockner (1983).

### 2.2.1. Phytoplankton biomass and community composition

The studies of phytoplankton biomass and community composition were carried out mainly based on high performance liquid chromatography (HPLC) pigment analysis. Additionally, microscopy analysis was performed as a complementary technique, to identify and enumerate the dominant taxa of the community and phytoplankton blooms.

Water samples for pigment analysis, stored in opaque plastic bottles, were filtered (0.4–4 L) on the same sampling day, in dark conditions and with a gentle vacuum ( $<150 \text{ mm Hg}$ ) onto Whatman GF/F glass-fibre filters (47 mm diameter, Whatman International Ltd.). The filters were instantly frozen in liquid nitrogen and stored at  $-80^{\circ} \text{ C}$  until extraction. Extraction was done under low light, with 5 ml of 90% acetone, employing a glass rod for grinding, and the extracts underwent filtration through syringe filters (Millex,  $0.22 \mu\text{m}$  pore size) to eliminate cell and filter debris. The pigment analysis was conducted through HPLC, following the procedure described by Zapata et al. (2000), with the modification in solvent A outlined in Seoane et al. (2009). This method enabled the identification and quantification of more than 50 different phytoplankton pigments based on their absorbance spectra and retention times.

Chlorophyll *a* (Chl *a*) data was used to determine the spatio-temporal dynamics of the phytoplankton biomass throughout the Urdaibai estuary and to define the trophic status of estuarine waters (e.g., Bricker et al., 2003). Following Hagy et al. (2022), hypertrophic waters were defined as those with Chl *a* concentrations above  $20 \mu\text{g L}^{-1}$ , since this Chl *a* concentration is considered "high" in most estuaries and adverse



effects have been noted at various levels below it. Taking this into account, the frequency and magnitude of hypertrophic Chl *a* concentrations in the Urdaibai estuary was determined and compared between 2020 and 2022.

For the pigment-based description of the phytoplankton community composition, the chemotaxonomic tool *PIGMENTUM* (Bilbao and Seoane, 2024) was applied. This tool determines the contributions of the different phytoplankton pigment-groups to the total phytoplankton biomass (Chl *a*), using pigments as biomarkers, based on a set of simultaneous equations where the lack of exclusiveness of some of the diagnostic pigments (DPs) is considered. Thus, the raw pigment data obtained by HPLC is transformed in contribution percentages of different pigment-groups (Table 1) to total phytoplankton biomass. The DPs used by *PIGMENTUM* that were present in the HPLC pigment dataset of the present study were peridinin (Peri), fucoxanthin (Fuco), 19'-butanoyloxyfucoxanthin (But), 19'-hexanoyloxyfucoxanthin (Hex), alloxanthin (Allo), chlorophyll *b* (Chl *b*), zeaxanthin (Zea), and prasinoxanthin (Pras). The DP:Chl *a* ratios applied for the characterisation of the community composition were the original ones proposed in Bilbao and Seoane (2024).

As complementary information for the *PIGMENTUM* results, microscopy analysis was performed to identify and quantify the main taxa of the dominant pigment groups in each sample. Water samples for microscopy were immediately fixed with acidic Lugol's solution (0.4% v/v) after collection and stored in 125 ml topaz borosilicate bottles under dark and cool (4 °C) conditions until the analysis. The taxonomic identification and quantification of the dominant taxa relied on the Utermöhl sedimentation method (Edler and Elbrächter, 2010) and were conducted under a Nikon diaphot TMD inverted microscope for 10- or 50-ml subsamples. Transects at different magnifications (100 ×, 200 ×, or 400 ×) were performed based on the abundance and size of the dominant taxa. The identification of most of these organisms was done up to the genus or even species level, and the nomenclature was standardized following AlgaeBase (Guiry and Guiry, 2018).

Additionally, microscopy was used to determine phytoplankton blooms (frequency and intensity). Following the criteria from Revilla et al. (2009) in the Basque coast, the selected threshold to consider blooms was that of small phytoplankton taxa:  $7.5 \times 10^5$  cells L<sup>-1</sup>. Thus, in the present study, this bloom threshold was only considered for the outer and middle Urdaibai estuary (URD1 and URD2), as these were the stations with highest marine influence.

**Table 1**

Pigment-groups defined by *PIGMENTUM* that were present in the Urdaibai estuary and the description of the main phytoplankton taxa of each of these groups (modified from Bilbao and Seoane, 2024). In bold, the taxonomic group from which the DP:Chl *a* ratio was taken.

<i>PIGMENTUM</i> pigment-groups	Main taxa
Peridinin containing algae (PeCA)	Peri containing <b>dinoflagellates</b>
19'-hexanoyloxyfucoxanthin containing algae (HCA)	Hex containing <b>haptophytes</b> and dinoflagellates with Fuco-derivates
Alloxanthin containing algae (ACA)	<b>Cryptophytes</b> and <i>Dinophysis</i> spp.
Prasinoxanthin containing algae (PrCA)	Pras containing <b>prasinophytes</b>
Fucoxanthin containing algae (FCA)	<b>Diatoms</b> , crysophytes, other minor ochrophyte groups, Fuco-containing dinoflagellates, and haptophytes without Fuco-derivates
19'-butanoyloxyfucoxanthin containing algae (BCA)	<b>Pelagophyceae</b> , But-containing haptophytes, and dinoflagellates
Chlorophyll <i>b</i> containing algae (CbCA)	<b>Chlorophytes</b> , Pras non-containing <b>prasinophytes</b> , euglenophytes, and <i>Leptodinium</i> spp.
Zeaxanthin containing algae (ZCA)	<b>Cyanobacteria</b> excluding <i>Prochlorococcus marinus</i>

### 2.3. Data analysis

The main statistical parameters (median, range, and/or arithmetic mean) were calculated for environmental and phytoplankton data, and the graphical representations of their spatio-temporal variability for each sampling point was performed when remarkable dynamics were noticed. All this was performed with Windows Excel 2016.

Hydrometeorological, physicochemical, and biotic variables were compared among sampling stations and/or between years (2020 and 2022) to seek significant differences. Due to the rejection of normality and homoscedasticity assumptions in environmental data (Legendre and Legendre, 1979), non-parametric univariate and multivariate statistical tests were carried out in the present study using PAST 4.05 (Hammer et al., 2001) to test these differences.

The univariate Mann-Whitney *U* test was used to determine the significant differences (Monte Carlo permutation test, alpha: 0.05) in the median values of the studied variables (hydrometeorological, physicochemical, and biotic) before (in 2020) and after (in 2022) the diversion of the wastewaters outside the estuary. This test was performed independently for each variable at each sampling station. Additionally, multivariate analyses were performed to obtain a general overview of the spatial and inert-annual variability of the physicochemical conditions and phytoplankton community composition of Urdaibai estuary. Permutational multivariate analysis of variance (PERMANOVA) was conducted to test significant differences (alpha: 0.05) between groups based on the Bray-Curtis distance matrix (Anderson, 2001). A two-way PERMANOVA was used to account for the spatial (sampling stations) and inter-annual (2020 and 2022) variability, as well as for the interaction of these two factors, of the environmental conditions and phytoplankton community composition throughout the Urdaibai estuary. Thus, the PERMANOVA analysis was conducted independently for the physicochemical variables dataset (including salinity, temperature, pH, DO, turbidity, ammonium, phosphate, nitrate, and silicate) and the phytoplankton community composition (including all the pigment-group contributions to total Chl *a*). The analyses were performed for the whole study period (March–October), as well as for the different seasons (spring and summer) individually. To complement this, non-metric multidimensional scaling (nMDS) in 2D was carried out, with an ordination based on the Bray-Curtis similarity index, for the graphical representation of the interrelationships among samples according to their similarity/dissimilarities. The nMDS was also performed independently for each dataset (physicochemical conditions and community composition) and for each season.

Finally, the relationship between the physicochemical conditions and the phytoplankton community of the Urdaibai estuary was explored using the Spearman correlation (also in PAST). The correlations were examined independently for each year and the Bonferroni correction was applied to the correlation analysis. Results were considered significant when they showed a *p* value lower than 0.05.

## 3. Results

### 3.1. Hydrometeorological conditions

The meteorological and hydrological parameters analysed in the Urdaibai estuary showed similar conditions between March and October of 2020 and 2022 (Annexes, Table A1 and Table A2).

The median air temperature during the study period (March–October) was 17.9 °C in 2020 and 18.3 °C in 2022, being August the hottest month both years. The global monthly solar radiation (55,494 kJ m<sup>-2</sup> in 2020 and 55,252 kJ m<sup>-2</sup> in 2022) and the median daily insolation hours (5.5 h day<sup>-1</sup> and 5.9 h day<sup>-1</sup> in 2020 and 2022, respectively) during the study period were almost the same in both years. However, the maximum values were recorded in different months, being May in 2020 and July in 2022 the months with highest solar exposition. As for precipitation, median monthly-accumulated rainfall between March and

October was 62 L m<sup>-2</sup> in 2020 and 58 L m<sup>-2</sup> in 2022. In both cases, the maxima were recorded in spring, in March (164 L m<sup>-2</sup>) in 2020 and in April (128 L m<sup>-2</sup>) in 2022. Related to this, the median Oka River flow during the study period was 0.22 m<sup>3</sup> s<sup>-1</sup> in 2020 and 0.14 m<sup>3</sup> s<sup>-1</sup> in 2022, and the highest median flow values were recorded in spring, in March in 2020 and in April in 2022. As for the Flow Index (Annexes, Table A2), values were much higher in spring (0.77 m<sup>3</sup> s<sup>-1</sup> and 0.73 m<sup>3</sup> s<sup>-1</sup> in 2020 and 2022, respectively) than in summer (0.26 m<sup>3</sup> s<sup>-1</sup> and 0.19 m<sup>3</sup> s<sup>-1</sup> in 2020 and 2022, respectively), also recording the maxima in March in 2020 and in April in 2022. In addition, riverine water temperature and conductivity were similar in both years.

The multivariate two-way (seasonal and inter-annual) PERMANOVA performed (Annexes, Table A6) with the meteorological and hydrological data revealed that there were no significant differences in the hydrometeorological conditions of the study area between 2020 and 2022. However, the test determined significant seasonal changes (p = 0.0001) in the hydrological conditions of the Oka River. Additionally, when comparing every variable independently with the Mann–Whitney U test (Annexes, Table A7), none of the meteorological nor hydrological parameters analysed showed significant differences between 2020 and 2022, confirming that the environmental conditions were similar in both years.

### 3.2. Physicochemical conditions of the urdaibai estuary

The physicochemical parameters measured in the Urdaibai estuary during 2020 and 2022 (Table 2; Annexes, Table A3) showed different spatio-temporal patterns throughout the estuary and were affected by the cessation of wastewater discharges in diverse ways.

Estuarine water salinity and temperature did not vary significantly between 2020 and 2022 and showed similar spatio-temporal patterns (Table 2). There was a marked longitudinal salinity gradient that

decreased towards the inner estuary, categorizing stations in the following classes both years: URD1 was euhaline, URD2 and URD3 were polihaline, and URD 4, URD5, and URD6 were mesohaline. Water temperature between March and October registered a median value of 21.3 °C in 2020 and 21 °C in 2022, increasing from spring towards the summer season and registering the highest values in June (25 °C) in 2020 and July (24.7 °C) in 2022.

The pH, which showed a spatial gradient throughout the estuary with a slight decrease towards the inner area in both 2020 and 2022, registered a significant (p < 0.01) decrease after the sewerage works according to the Mann–Whitney U test (Table 2, Fig. 2). Regarding DO, no clear spatial pattern was defined throughout the estuary and the differences between 2020 and 2022 were not significant. Estuarine turbidity showed a marked increasing gradient towards the inner Urdaibai estuary, both in 2020 and 2022, and the small changes detected between the two years were not considered significant (Annexes, Table A8). Nevertheless, it should be highlighted that the turbidity values in 2022 were always below 13 NTU, while several high turbidity peaks were recorded in 2020, such as in April in URD4 (31 NTU), or in August in URD5 and URD6 (16 NTU and 23 NTU).

Inorganic nutrient concentration showed a marked longitudinal gradient throughout the Urdaibai estuary in 2020 and 2022, all increasing towards the inner area. However, the Oka River nutrient concentration revealed that the estuarine ammonium and phosphate were from anthropogenic origins, since concentrations were much higher in the surroundings of the WWTP (URD6) than in the Oka River, and estuarine nitrate and silicate had riverine origins, with lower concentrations in URD6 than in the Oka River (Table 2). Ammonium and phosphate concentrations showed significant decreases from 2020 to 2022 according to the Mann–Whitney U test (Table 2 and Fig. 2). The decrease in ammonium was the most noticeable, (p < 0.01), recording median concentrations three times lower from URD1 to URD3, seven

**Table 2**

Median values (and range) of the physicochemical variables analysed in the Urdaibai estuary for each sampling station in 2020 and 2022. The significance of the change between 2020 and 2022 for each sampling station according to the Mann–Whitney U test is indicated as: \* for p < 0.05 and \*\* for p < 0.01.

		Salinity (PSU)	Temperature (°C)	pH	Dissolved oxygen (mg L <sup>-1</sup> )	Turbidity (NTU)	Ammonium (µmol L <sup>-1</sup> )	Phosphate (µmol L <sup>-1</sup> )	Nitrate (µmol L <sup>-1</sup> )	Silicate (µmol L <sup>-1</sup> )
URD1	2020	33.6 (28.07–35.05)	22.38 (15.15–24.59)	<b>8.88**</b> (7.85–9.77)	7.37 (6.71–8.69)	1.30 (0.48–4.63)	<b>4.65**</b> (3.5–7.85)	<b>0.35**</b> (0.08–0.65)	0.80 (0.8–10.3)	1.75 (0.8–14.3)
	2022	34.3 (25.47–35.79)	20.33 (12.62–23.05)	<b>7.94**</b> (7.57–8.06)	7.39 (6.94–9.45)	1.25 (0.61–2.74)	<b>2.15**</b> (0.8–3.4)	<b>0.20**</b> (0.08–0.4)	0.80 (0.8–5.6)	1.60 (5.95–38.55)
URD2	2020	28.4 (14.8–34.39)	21.39 (14.84–24.84)	<b>8.55**</b> (7.85–9.33)	7.11 (6.79–9.15)	3.34 (1.15–7.71)	<b>13.10**</b> (5.95–26.3)	<b>0.50*</b> (0.08–0.95)	7.50 (0.8–37.25)	15.50 (3.25–58.1)
	2022	29.6 (16.47–35.32)	20.85 (13.29–23.9)	<b>7.93**</b> (7.85–8.02)	6.99 (6.22–8.82)	2.91 (1.73–8.41)	<b>4.35**</b> (1.6–8.25)	<b>0.45*</b> (0.15–0.75)	4.20 (0.8–26.8)	14.90 (5.95–38.55)
URD3	2020	24 (4.9–31.42)	21.54 (14.46–25.06)	<b>8.44**</b> (7.83–9.01)	7.04 (6.04–9.76)	3.63 (2.54–9.27)	<b>24.05**</b> (13.8–56.1)	<b>0.95**</b> (0.35–1.75)	14.00 (9.85–5.6)	35.55 (13.75–84.1)
	2022	25.5 (8.82–34.2)	21.07 (13.55–24.62)	<b>7.88**</b> (7.74–8.05)	6.70 (5.97–9.29)	3.59 (1.81–8.38)	<b>7.00**</b> (0.8–14.75)	<b>0.60**</b> (0.25–0.8)	9.90 (2.35–26.8)	32.80 (12.75–64.85)
URD4	2020	18.6 (1.24–26.6)	21.21 (14– 61–25.66)	<b>8.38**</b> (7.7–8.88)	7.02 (5.21–10.75)	4.58 (2.26–31.03)	<b>57.45**</b> (21.5–112.9)	<b>1.65**</b> (0.7–4.05)	16.60 (8.4–66.55)	51.75 (36–96.5)
	2022	20.8 (2.26–31.64)	21.34 (13.64–25.1)	<b>7.79**</b> (7.61–8.13)	6.34 (5.52–9.87)	4.57 (3.39–9.25)	<b>8.65**</b> (1.6–20.75)	<b>0.70**</b> (0.3–1.2)	14.95 (3.4–31.55)	48.20 (19.15–66.2)
URD5	2020	13.5 (0.21–20.72)	20.96 (14.57–25.66)	<b>8.37**</b> (8.03–9.03)	7.13 (5.61–10.28)	6.22 (3.91–16.33)	<b>64.10**</b> (30.3–166.2)	<b>2.25*</b> (0.35–8.05)	21.40 (9.85–65.55)	64.45 (44.85–104.55)
	2022	15.8 (0.33–26.71)	20.98 (12.99–24.96)	<b>7.79**</b> (7.62–8.45)	7.00 (4.43–12.03)	8.33 (5.46–12.79)	<b>9.10**</b> (0.8–24)	<b>1.15*</b> (0.6–2.9)	21.85 (7.1–38.4)	64.50 (54.9–95.55)
URD6	2020	10 (0.21–18.85)	20.51 (14.42–25.3)	<b>8.38**</b> (7.73–9)	7.13 (5.72–10.16)	7.92 (5.62–23.03)	<b>153.35**</b> (65.65–334.45)	<b>5.80**</b> (2.4–11.4)	22.45 (12–77.55)	76.15 (47.9–89.25)
	2022	13.7 (0.24–25.69)	20.92 (13.1–25.15)	<b>7.74**</b> (7.6–8.37)	6.59 (5.21–10.24)	8.48 (5.28–11.36)	<b>10.70**</b> (0.8–24.2)	<b>1.30**</b> (0.5–2.15)	21.00 (9.35–49.3)	64.85 (45.15–91.8)
OKA	2020	0.22 (0.19–0.24)	17.3 (12.7–19.75)	<b>8.43**</b> (7.77–9.88)	8.38 (6.52–11.04)	3.01 (1–29.1)	4.35 (0.8–6.8)	<b>0.4*</b> (0.08–0.95)	57.55 (48.45–88.2)	92.4 (58.5–156.3)
	2022	0.22 (0.21–0.28)	17.71 (12.442–21.4)	<b>7.93**</b> (7.76–8.26)	7.45 (6.37–10.35)	2.14 (1.07–4.33)	3.3 (0.8–5.6)	<b>0.75*</b> (0.35–1.15)	57.35 (36.4–79.7)	158.5 (35.9–188.35)

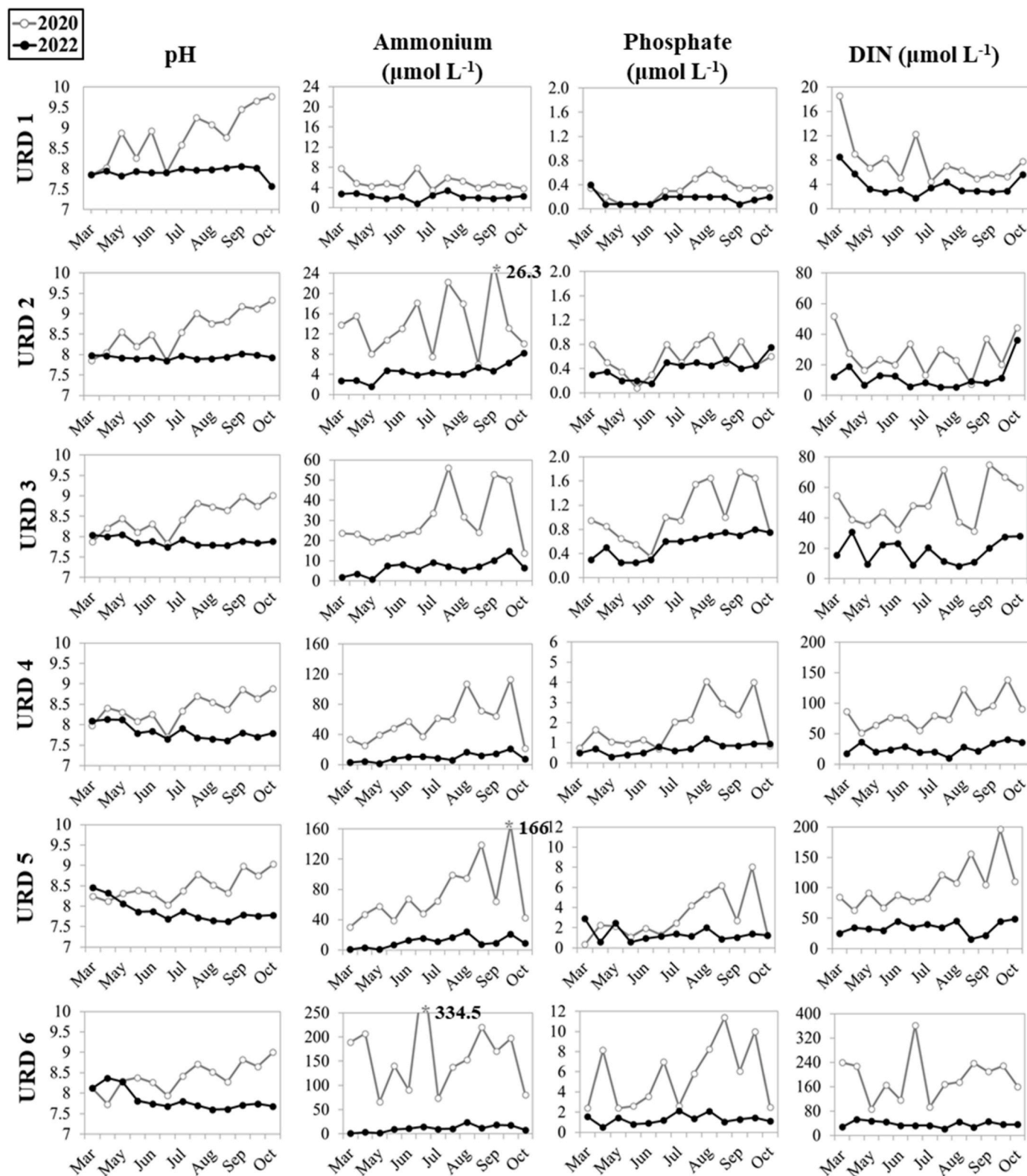


Fig. 2. Spatial and temporal variability of the physicochemical variables showing significant differences throughout the Urdaibai estuary between 2020 and 2022.

times lower in URD4 and URD5, and fourteen times lower in URD6. In the case of phosphate, although the decrease was significant throughout the entire estuary (at least  $p < 0.05$ ), the impact was more pronounced in the inner area (URD4–URD6), registering two times lower concentrations in URD4 and URD5 ( $p < 0.01$ ) and four times lower in URD6 ( $p < 0.01$ ). In addition, in the surroundings of the WWTP (URD6), the

maximum concentrations of ammonium ( $24.2 \mu\text{mol L}^{-1}$ ) and phosphate ( $2.5 \mu\text{mol L}^{-1}$ ) in 2022 were lower or similar to the minimum in 2020 ( $65.6 \mu\text{mol L}^{-1}$  and  $2.4 \mu\text{mol L}^{-1}$  of ammonium and phosphate, respectively). In contrast, nitrate and silicate did not show significant changes between 2020 and 2022 according to the Mann–Whitney  $U$  test in any sampling station (Table 2; Annexes, Table A9).



These changes in nutrient concentrations registered between 2020 and 2022 had several consequences. On the one hand, the composition of DIN varied between years (Annexes, Table A10), being mainly contributed by ammonium throughout the estuary in 2020 (72%) and by nitrate (48.5%) in 2022 (although ammonium was still 44.9% of the DIN). This change was more noticeable in the innermost estuary (URD6), where the contribution of ammonium to DIN changed from 82% in 2020 to 33% in 2022 (nitrate from 15% to 61%). Thus, the ammonium decrease (being the main contributor to DIN in 2020) explains the significant decrease ( $p < 0.01$ ) registered in DIN concentrations from 2020 to 2022, according to the Mann-Whitney  $U$  test (Fig. 2; Annexes, Table A8). On the other hand, changes in nutrient concentrations led to variations in the nutrient ratios (DIN:P, DIN:Si and Si:P) of Urdaibai

estuary (Fig. 3; Annexes, Table A11). DIN:Si ratio was the most altered by the diversion of the wastewaters, registering a significant decrease ( $p < 0.01$ ) from URD2 to URD6 in 2022 according to the Mann-Whitney  $U$  test. Additionally, there were several other significant ratio changes throughout the estuary, such as the DIN:P decrease in URD3, and the Si:P increase from URD2 to URD6, being especially notable in URD6.

As a general overview, and taking all the studied physicochemical variables into account (Table 2), the two-way PERMANOVA analysis determined that there was a significant ( $p = 0.0001$ ) spatial (between sampling stations) and inter-annual (between 2020 and 2022) variability of the physicochemical conditions in the Urdaibai estuary in both spring and summer (Annexes, Table A8). Additionally, the interaction of both factors (spatial and inter-annual) also explained the

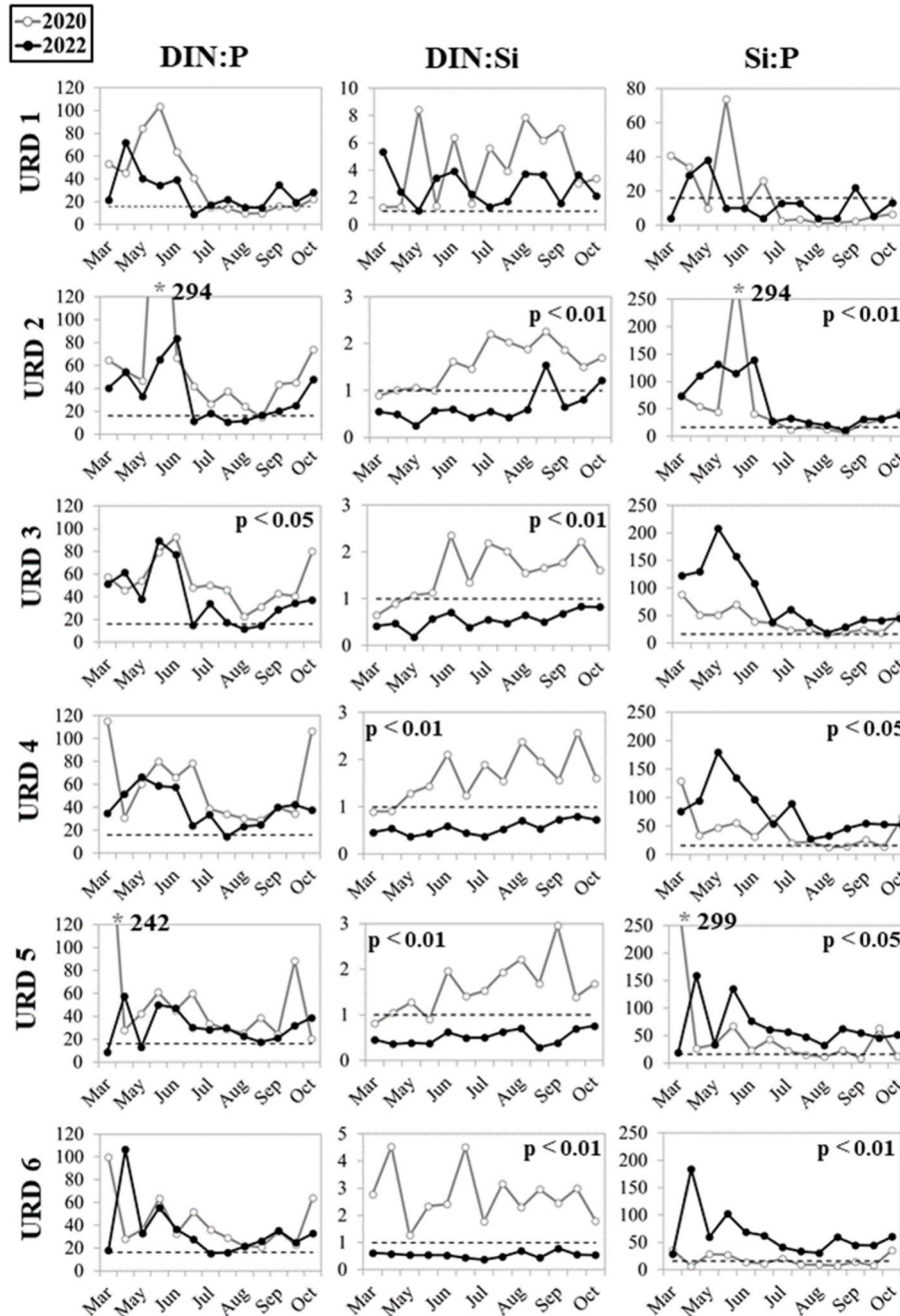


Fig. 3. Spatial and temporal variability of the nutrient molar ratios showing significant differences throughout the Urdaibai estuary between 2020 and 2022. Horizontal dashed lines indicate the Redfield ratio (Redfield, 1958). The significance of the change between 2020 and 2022 for each sampling station according to the Mann-Whitney  $U$  test is indicated in each graph:  $p < 0.05$  and  $p < 0.01$ .

physicochemical condition varied significantly in both seasons. Thus, and although most parameters did not register individual significant changes in each sampling station according to the Mann Whitney *U* test, the estuarine physicochemical conditions were considered different in 2020 and 2022, mainly due to the noticeable changes in nutrient concentrations. The non-metric multidimensional scaling (nMDS), containing the variables in Table 2, illustrates the variation in the physicochemical conditions throughout the sampling stations and between years, for both spring and summer seasons independently (Fig. 4). As can be seen, the physicochemical conditions in the surroundings of the WWTP (URD 5 and 6) after the wastewater diversion outside the estuary (2022) are similar to those in URD3 before diversion (2020).

### 3.3. Phytoplankton biomass and blooms

In both 2020 and 2022, a general increase of biomass was observed towards the inner estuary (Table 3; Annexes, Table A4). The lowest median biomass values were recorded in URD1, however, the biomass maxima area varied between years. In 2020, the highest median biomass was registered in URD5 and the Chl *a* maxima (54.61  $\mu\text{g L}^{-1}$ ) in URD4. In contrast, in 2022 the longitudinal biomass gradient was more marked, reaching the highest median values in URD6 and decreasing gradually until URD1. The Chl *a* maxima of 2022 was registered in URD 5 (251  $\mu\text{g L}^{-1}$ ), but was also noticeable in URD6 (151  $\mu\text{g L}^{-1}$ ).

Regarding the inter-annual variability of Chl *a*, the outer and middle estuary recorded significantly ( $p < 0.05$ ) lower biomass values in 2022 than in 2020, this decrease being especially notable in URD1 in spring and in URD2 in summer (Table 3). The inner estuary, on the contrary, did not register significant changes in Chl *a* concentration according to Mann–Whitney *U* test. Nevertheless, in the surroundings of the WWTP (URD6), although it was not considered significant, there was a notably higher median Chl *a* concentration (two times higher) in 2022 (16.8  $\mu\text{g L}^{-1}$ ) than in 2020 (7.57  $\mu\text{g L}^{-1}$ ) in 2020, which should be taken into account.

Regarding high biomass situations, several differences were detected between 2020 and 2022 (Table 4). Chl *a* values above 20  $\mu\text{g L}^{-1}$  indicating hypertrophic status were registered several times in the Urdaibai estuary, always in the inner area (from URD3 to URD6) and more

**Table 3**

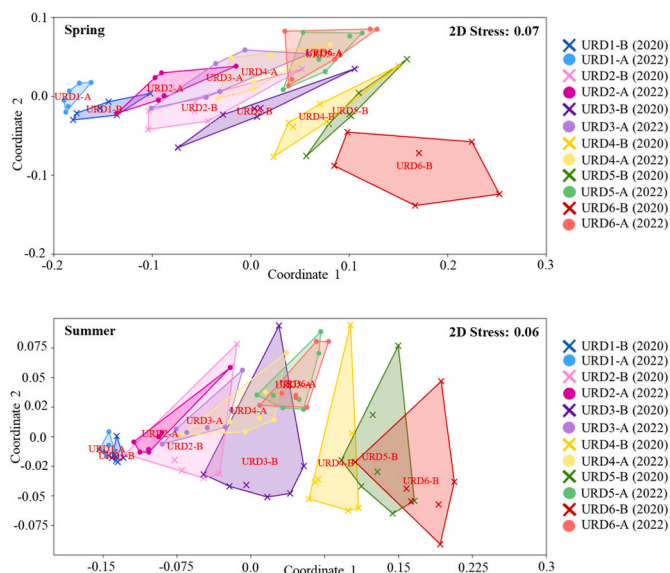
Median values (and range) of the Chl *a* in each sampling station in 2020 and 2022. The significance of the change between 2020 and 2022 according to the Mann–Whitney *U* test is indicated as: \* for  $p < 0.05$  and \*\* for  $p < 0.01$ .

		March–October ( $\mu\text{g L}^{-1}$ )	Spring ( $\mu\text{g L}^{-1}$ )	Summer ( $\mu\text{g L}^{-1}$ )
URD1	2020	1.61 (0.88–2.97)*	1.48 (0.88–2.04)*	1.87 (1.14–2.97)
	2022	1.04 (0.64–2.14)*	0.80 (0.64–1.55)*	1.22 (0.83–2.14)
URD2	2020	5.08 (1.29–10.89)*	4.21 (1.29–10.89)	5.08 (3.88–10.76)*
	2022	3.04 (0.67–6.96)*	3.15 (0.67–5.4)	3.04 (1.88–6.96)*
URD3	2020	4.89 (0.44–25.15)	6.22 (0.44–25.15)	3.93 (3.59–8.91)
	2022	4.15 (0.77–9.61)	5.33 (0.77–9.61)	4.14 (1.93–4.38)
URD4	2020	4.72 (0.49–54.61)	7.26 (0.49–54.61)	4.55 (2.01–8.35)
	2022	4.77 (1.73–41.12)	5.75 (2.65–41.12)	4.04 (1.73–7)
URD5	2020	9.94 (0.22–42.51)	9.40 (0.22–42.51)	9.94 (4.41–17.18)
	2022	10.06 (0.74–251.51)	9.80 (0.74–251.51)	10.06 (3.3–31.36)
URD6	2020	7.57 (0.5–45.82)	5.06 (0.5–45.82)	7.67 (7.08–16.76)
	2022	16.80 (0.9–151.57)	15.45 (0.9–151.57)	17.68 (3.73–41.83)

**Table 4**

Hypertrophic status of the water masses (Chl *a* > 20  $\mu\text{g L}^{-1}$ ) throughout the Urdaibai estuary in 2020 and 2022.

Year	Date	Sampling point	Chl <i>a</i> ( $\mu\text{g L}^{-1}$ )	Dominant taxa	Cell abundance (cells $\text{L}^{-1}$ )
2020	17th April	URD3	25.2	<i>Teleaulax</i> spp.	$5.95 \times 10^6$
		URD4	54.6	<i>Urgorri complanatus</i>	$7.61 \times 10^6$
		URD5	42.5	<i>Urgorri complanatus</i>	$7.73 \times 10^6$
		URD6	45.8	<i>Urgorri complanatus</i>	$6.35 \times 10^6$
2022	23rd March	URD4	41.1	<i>Teleaulax gracilis</i>	$3.07 \times 10^7$
		URD5	161.3	<i>Kryptoperidinium foliaceum</i>	$1.08 \times 10^7$
	6th May	URD5	251.5	<i>Urgorri complanatus</i>	$5.39 \times 10^7$
		URD6	151.6	<i>Urgorri complanatus</i>	$3.74 \times 10^7$
	6th June	URD5	23.2	<i>Tetraselmis</i> sp.	$3.40 \times 10^5$
		URD6	41.8	<i>Eutreptiella</i> sp.	$9.35 \times 10^4$
20th July	URD6		33.6	<i>Apedinella radians</i>	$1.69 \times 10^7$
				Centric diatoms 10–20 $\mu\text{m}$	$4.06 \times 10^6$
21st September	URD5	31.4	<i>Teleaulax acuta</i>	$9.01 \times 10^6$	
	URD6	26.2	<i>Teleaulax acuta</i>	$7.29 \times 10^6$	



**Fig. 4.** Non-metric multidimensional scaling (nMDS) of physicochemical conditions in the Urdaibai estuary using Bray–Curtis distances. Data are shown separately for spring (upper) and summer (lower) seasons, and contain 9 physicochemical variables (i.e., salinity, temperature, pH, DO, turbidity, ammonium, phosphate, nitrate, and silicate). B: before wastewater diversion (2020); A: after wastewater diversion (2022).

frequently in 2022 than in 2020 (Table 4). During 2020, there was just one occasion (April) in which hypertrophic conditions were registered, from URD3 to URD6, reaching Chl *a* concentrations up to 54.6  $\mu\text{g L}^{-1}$



L<sup>-1</sup> due to blooms of cryptophytes like *Urgorri complanatus* or *Tealeulax* spp. However, in 2022, Chl a concentration above 20 µg L<sup>-1</sup> were found in 38% of the samplings (5 out of 13), always registering this hypertrophic status in the inner estuarine area (mostly URD5 and URD6). In

2022, the main cause of the high biomass was the combined blooms of cryptophytes (mainly *U. complanatus*) and/or the dinoflagellate *Kryptoperidinium foliaceum*, reaching a Chl a maximum of 251.5 µg L<sup>-1</sup>. Thus, when comparing the years, 2022 showed hypertrophic situations more

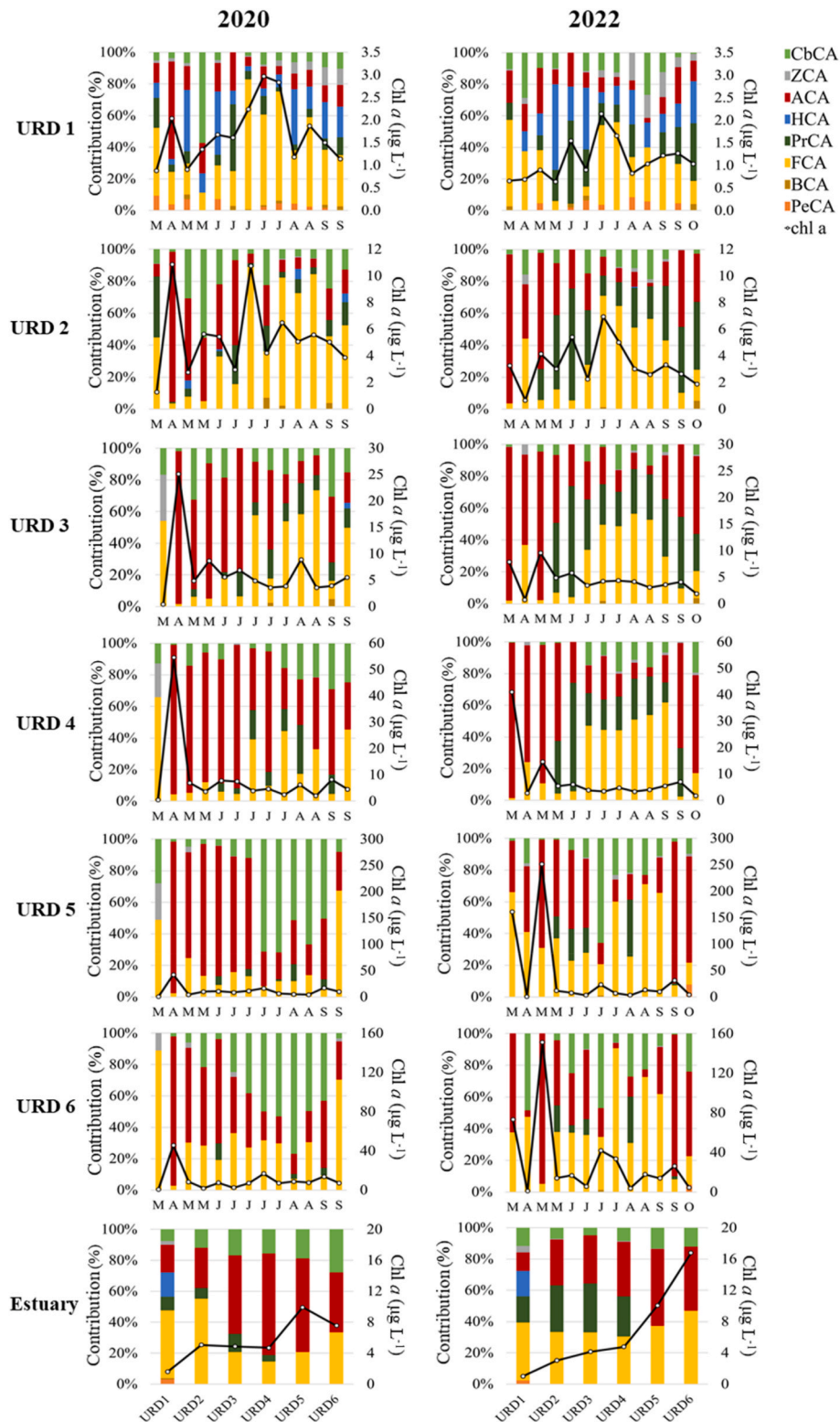


Fig. 5. Spatial and temporal variability of the contribution percentage of the different groups of the phytoplankton community to the total Chl a throughout the Urdaibai estuary in 2020 and 2022, by sampling stations and the annual median contribution to the whole estuary. PeCA: Peridinin Containing Algae; BCA: 19'-butanoyloxyfucoxanthin Containing Algae; FCA: Fucoxanthin Containing Algae; PrCA: Prasinocanthin Containing Algae; HCA: 19'-hexanoyloxyfucoxanthin Containing Algae; ACA: Alloxanthin Containing Algae; ZCA: Zeaxanthin Containing Algae; CbCA: Chl b Containing Algae.

frequently and the biomass maxima recorded were also higher.

Additionally, and although the Chl *a* values recorded were not as high as for indicating hypertrophic status, several additional remarkable phytoplankton blooms (taxa above  $7.5 \times 10^5$  cells L<sup>-1</sup>) were recorded in the outer and middle Urdaibai estuary in 2020 and 2022 (Annexes, Table A5). Phytoplankton blooms in URD1 and/or URD2 were detected in almost every sampling in 2020 and 2022, however, the number of bloom-forming organisms per sampling was much higher in 2020, since bloom thresholds were exceeded 40 times in 2020 (67.5% in URD2) and 21 times in 2022 (76% in URD2). Diatoms *Minutocellus polymorphus* and *Chaetoceros tenuissimus* and the cryptophyte *Plagioselmis* spp. were the main bloom-forming taxa in the outer and middle estuary, being responsible for 82% of the blooms in 2020 and 86% in 2022. *Minutocellus polymorphus* registered blooms in every sampling from July to September in both years and recorded maximum abundances of  $1.47 \times 10^7$  cells L<sup>-1</sup> and  $5.37 \times 10^6$  cells L<sup>-1</sup> in 2020 and 2022, respectively. *Chaetoceros tenuissimus* blooms were registered between June and August in 2020 and July and September in 2022, with maximum abundances of  $2.05 \times 10^7$  cells L<sup>-1</sup> and  $1.58 \times 10^7$  cells L<sup>-1</sup> in 2020 and 2022, respectively. Regarding *Plagioselmis* spp., 12 blooms were registered during 2020, appearing in high abundances in almost every sampling, however, in 2022, these blooms decreased (4 were registered) and were limited to May and June. Cell abundances of  $7.5 \times 10^5$  cells L<sup>-1</sup> were frequently exceeded in the inner Urdaibai estuary (URD3–URD6), but this bloom threshold is only acceptable for coastal waters, and therefore they were not taken into account in the present study.

### 3.4. Phytoplankton community composition

The community composition study revealed that there were several similarities and dissimilarities between the phytoplankton communities of the Urdaibai estuary in 2020 and 2022 (Fig. 5 and Table 5).

In 2020, FCA dominated the phytoplankton community of the outer and middle estuary, with median contributions of 34% and 42% to the total biomass in URD1 and URD2, respectively. Indeed, the dominance of this group was more noticeable in summer, with median contributions of 56% in URD1 and 73% in URD2. This FCA group in the outer and middle estuary was mostly formed by diatoms, especially by taxa such as *Chaetoceros* spp., *Minutocellus polymorphus*, *Cylindrotheca costerium*, *Leptocylindrus* spp., and *Pseudo-nitzschia* spp. Additionally, the presence of groups like PeCA and HCA was limited to the outer estuary, which is confirmed by their positive correlation with salinity (PeCA  $\rho$  0.5,  $p < 0.01$ ; HCA  $\rho$  0.65,  $p < 0.01$ ) (Annexes, Table A15). URD3 was a transitional zone, with shared dominance of FCA and ACA, the first

dominating in summer (54%) and the latter in spring (68%). Moving to the inner estuary, the median ACA contribution to total biomass increased, reaching the 54% in URD4, where it dominated mostly in spring (81%), but also in summer (40%). This ACA group was mainly formed by cryptophytes like *Plagioselmis* spp., *Hemiselmis* spp., *Teleaulax* (*T. gracilis* and *T. acuta*), and *Urgorri complanatus*. The innermost area of the estuary (URD5 and URD6) registered the highest temporal variabilities of the community composition. In URD5, ACA was the most contributing group to the total biomass in spring (median value of 78%), while CbCA dominated (51%) in summer, containing green algae like *Tetraselmis* spp. or *Eutreptiella* spp., among others. As for URD6, ACA also dominated in spring (55%) and CbCA in summer (50%), but the contribution of the FCA group remained around 30% throughout the entire study period, being higher than in URD5. On the contrary to the FCA group of the outer estuary, in the inner area one of the main contributing taxa was the dinoflagellate *K. foliaceum*, together with small centric diatoms like the genus *Cyclotella*.

Regarding 2022, in the outer estuary the dominant group of the phytoplankton community in terms of biomass was FCA (34%), followed by PrCA and HCA, with a median contribution of 15% each. Positive significant correlations were also recorded between salinity and PrCA ( $\rho$  0.52,  $p < 0.01$ ) and HCA ( $\rho$  0.56,  $p < 0.01$ ). Like in 2020, the taxa belonging to FCA in the outer and middle estuary were mostly diatoms, such as *Chaetoceros* spp., *M. polymorphus*, *Guinardia delicatula*, *C. costerium*, *Proboscia alata*, and *Pseudo-nitzschia* spp. PrCA mostly corresponded to small unidentified prasinophytes, and HCA to haptophyte taxa like Prymnesiales (e.g., *Chrysochromulina* spp.) or coccolithophorids (e.g., *Gephyrocapsa* spp.). From URD2 to URD4, there was a shared dominance of FCA (24–30%), PrCA (20–30%), and ACA (25–28%), FCA contributing more to total biomass in summer, ACA in spring, and PrCA's contributions remaining similar from May to October. This seasonality was confirmed by the positive correlation between FCA and temperature ( $\rho$  0.44,  $p < 0.01$ ) and the negative between ACA and temperature ( $\rho$  -0.52,  $p < 0.01$ ). The taxa forming the ACA were cryptophytes like *Plagioselmis* spp., *Teleaulax* spp., and *Urgorri complanatus*. In the innermost area of the estuary (URD5 and URD6), FCA and ACA were also the groups with the highest median contribution to the phytoplankton biomass, with a contribution of 31% FCA and 41% ACA in URD5 and 37% FCA and 33% ACA in URD6. Additionally, in the inner area, CbCA replaced the PrCA group that appeared in the middle estuary, with a median contribution around 10% in both URD5 and URD6. The taxa of the FCA in the inner estuary were also different from the outer estuary, being mostly represented by the dinoflagellate *K. foliaceum* and the diatom genera *Thalassiosira*, *Skeletonema*, and

**Table 5**

Median values (and range) of the contribution percentage of the different groups of the phytoplankton community to the total Chl *a* in each sampling station in 2020 and 2022. The significance of the change between 2020 and 2022 according to the Mann-Whitney *U* test is indicated as: \* for  $p < 0.05$  and \*\* for  $p < 0.01$ . PeCA: Peridinin Containing Algae; BCA: 19'-butanoyloxyfucoxanthin Containing Algae; FCA: Fucoxanthin Containing Algae; PrCA: Prasinocanthin Containing Algae; HCA: 19'-hexanoyloxyfucoxanthin Containing Algae; ACA: Alloxanthin Containing Algae; ZCA: Zeaxanthin Containing Algae; CbCA: Chl *b* Containing Algae.

		Contribution to Chl <i>a</i> (%)							
		PeCA	BCA	FCA	PrCA	HCA	ACA	ZCA	CbCA
URD1	2020	2.4 (0–9.3)	0.6 (0–2.9)	33.7 (11.2–82.2)	6.8* (0–42)	12.2 (2.9–39.8)	13.7 (5.3–61.6)	1.9 (0–11.5)	5.8 (0–57.2)
	2022	2.1 (0–8.4)	0 (0–4.1)	33.6 (0–55.9)	15.1* (0–52.7)	14.8 (0–54.4)	10.6 (2.9–28.7)	3.9 (0–17)	10.4 (0–28.6)
URD2	2020	0	0 (0–7.1)	41.9 (3.5–88.4)	5.1* (0–38.1)	0 (0–6.5)	19.7 (5.5–93.9)	0 (0–0.9)	9 (1.6–55.4)
	2022	0	0 (0–5.3)	27.9 (3.5–68.8)	25* (0–70)	0 (0–0.8)	24.5 (2.2–93.4)	0.3 (0–6.2)	5.9 (0–18.7)
URD3	2020	0	0 (0–4.7)	17 (1.7–73.4)	9.7** (0–19.5)	0 (0–3.6)	41.3 (0–96.4)	0	13.8* (0–32.6)
	2022	0	0 (0–3.5)	29.6 (2–56)	28.1** (0–69.6)	0	27.4 (5.5–96.4)	0 (0–6.4)	4.4* (0–15.7)
URD4	2020	0	0	12.1 (4.4–65.8)	3.4* (0–31)	0	54.1 (0–94.5)	0 (0–21.4)	12.7 (0–29)
	2022	0	0	24.3 (1.3–61.7)	20.5* (0–68.6)	0	27.7 (5.7–98.3)	0.4 (0–2.2)	6.7 (0–19.3)
URD5	2020	0	0	13.2** (2.3–67.5)	0 (0–10.6)	0	52.6 (0–96.1)	0 (0–23.2)	11.9 (1.6–71.7)
	2022	0 (0–7.9)	0 (0–0.9)	31.1** (7.4–71.1)	0 (0–35.8)	0	41.3 (5.8–89.4)	0.1 (0–2.8)	11.2 (0.9–65.8)
URD6	2020	0	0	29.9 (2.9–89)	0 (0–10.7)	0	35.2 (0–95)	0 (0–11)	24.8 (0–76.9)
	2022	0 (0–4.7)	0 (0–1.4)	37.4 (5.2–90.8)	0 (0–29)	0	32.7 (3.5–94.8)	0 (0–0.7)	9.7 (0–48.3)

### Cyclotella.

The two-way (spatial and inter-annual) PERMANOVA analysis (Annexes, Table A12) revealed that the community composition variability was significantly explained ( $p = 0.0001$ ) by the spatial gradient (different sampling stations) within the Urdaibai estuary in spring and summer, confirming what Fig. 5 shows. However, the inter-annual variability could only explain the community composition variability of Urdaibai estuary in summer ( $p = 0.003$ ). In addition, the interaction of both factors did not explain the community composition variability significantly. Therefore, the non-metric multidimensional scaling (nMDS), containing the contributions of the different pigment-groups, illustrated the variation in the community composition among the sampling stations but was not useful for showing differences between years (Annexes, Fig. A1).

Nevertheless, several significant changes were detected when comparing the contribution of each pigment group individually, in each sampling station, with the Mann-Whitney  $U$  test (Table 5; Annexes, Table A13 and Table A14). One of the most noticeable difference was the increase in the contribution of the PrCA group to the total phytoplankton biomass in 2022, from URD1 to URD4, which was significant according to the Mann-Whitney  $U$  test. In 2022, the median PrCA contribution was two times higher in URD1, five times in URD2, three times in URD3, and six times higher in URD4. Additionally, FCA showed higher median values in 2022 from URD3 to URD6 and, especially, in URD 5 (from 1.3% to 31%,  $p < 0.01$ ). On the contrary, ACA and CbCA became less dominant in 2022, although the changes were not considered significant according to the Mann-Whitney  $U$  test. The median contribution of the ACA to Chl  $a$  in 2022 was two times lower in URD3 and URD4. As for the CbCA, noticeable decreases were registered in their median contributions of in URD3 (from 13.8% to 4.4%,  $p < 0.05$ ), but also in URD4 (from 12.7% to 6.7%) and URD 6 (from 24.8% to 9.7%).

## 4. Discussion

In some estuaries, like the Urdaibai estuary, the natural cyclic variations that are to some extent predictable can be altered by factors such as human activities (i.e., wastewater discharges to the estuary). Unravelling the combined effects of natural variability and human-induced pressures and/or the management actions on the physicochemical conditions and biological communities of this kind of estuary poses a challenging task (e.g., Elliott and Quintino, 2007). In the present study, to address this, different environmental parameters of the Urdaibai estuary were analysed, related to natural cycles (e.g., salinity or temperature) and wastewater discharges (e.g., turbidity or nutrient concentrations), to determine the mid-term effect of direct management action (cessation of wastewater discharges) on the physicochemical conditions, phytoplankton biomass and community composition of the system.

Studies focusing on coastal marine systems of Western Europe have revealed the high sensitivity of these systems to climate variability, suggesting that climate factors could significantly shape the changes in the biological and ecological conditions of estuaries, potentially acting in synergism with local human-induced pressures (Goberville et al., 2010). The comparison of several hydrometeorological variables between 2020 (during wastewater discharges) and 2022 (after sewerage works) revealed that no significant differences were detected in these conditions in the Urdaibai estuary between years. Both years showed a marked seasonality, with an increase in temperature (air and estuarine water) from spring to summer, as described by Valencia et al. (2004) for Basque coastal waters, and higher freshwater discharges to the estuary in spring, as expected for the Cantabrian coast (Prego et al., 2008; Revilla et al., 2009). Thus, the lack of differences in the climatic conditions could confirm that the significant differences found in the Urdaibai estuary between 2020 and 2022 for both physicochemical conditions and the phytoplankton community might be caused by differences in human impacts, mainly the cessation of wastewater

discharges to the estuary.

### 4.1. Physicochemical conditions

The physicochemical parameters that showed significant changes between 2020 and 2022 in the Urdaibai estuary were pH and the nutrient concentrations of anthropogenic origin (ammonium and phosphate). Regarding pH, a decrease was registered in the pH values of the Urdaibai estuary in 2022. The pH of the WWTPs effluents, overall, is usually in the range of 7–8 (Odjadjare and Okoh, 2010). Thus, it is hard to explain the acidification of the estuary with the cessation of wastewater from the WWTP of Gernika, since the pH of the WWTP effluent should not have been high enough to cause of the alkalisation of the system during the discharges. As previously described by Franco (1994), the pH of the Urdaibai estuary depends largely on the pH of the river, the pH of the sea, and the degree of mixing between both systems. Therefore, the pH decrease registered in URD1 and the Oka River might be the main causes of the acidification of the system. This was also recently described in Chesapeake Bay and the Neuse River Estuary (Hall et al., 2023). Nevertheless, estuarine systems are commonly perceived as being well buffered, so pH is frequently assumed to be of little significance (Ringwood and Keppler, 2002).

In contrast, the marked decrease of ammonium and phosphate concentrations registered in the Urdaibai estuary in 2022 was a direct result of the sewerage works in the area. Several studies (e.g., Iriarte et al., 2015; Revilla et al., 2000) have previously reported that the ammonium and phosphate over-enrichment in Urdaibai estuarine waters was caused by the discharges coming from the WWTP of Gernika, due to the low efficiency of the WWTP (Franco et al., 2004). This high ammonium and phosphate concentrations have led to “deficient” or “bad” ecological statuses of the inner estuary from at least 2008 and until 2020 (Borja et al., 2021). In 2021, immediately after (within one month) the diversion of the WWTP effluent outside the estuary, a significant decrease of ammonium and phosphate was seen (Bilbao et al., 2022), and the present study confirmed it was not something punctual or temporary. The most noticeable ammonium and phosphate decreases were recorded in the inner estuary, in the surroundings of the WWTP, where the ammonium and phosphate concentrations were 14 times and 4 times lower in 2022, respectively. Many studies (e.g., Ho et al., 2008; Liu et al., 2022; Mallin et al., 2005) showed positive results similar to those in Urdaibai estuary after upgrading the local WWTP or sewerage system, with significant reductions of ammonium and phosphate concentrations. However, and in accordance with what it was expected, these concentration changes only affected the nutrients of anthropogenic origin, while nitrate and silicate, nutrients from diffuse origins from land drainage and rock weathering (Turner et al., 2003; Valencia and Franco, 2004), did not show significant differences in their concentrations between 2020 and 2022 throughout the Urdaibai estuary.

The disparity in the nutrient loading changes between 2020 and 2022 caused by the cessation of the wastewater discharges (DIN and P changed, but Si did not) has led to changes in estuarine DIN:Si:P nutrient ratios. If the atomic DIN:Si:P ratio of 16:16:1 (Redfield, 1958) is taken as a criterion for balanced nutrient composition (e.g., Lane et al., 2004), the ratios revealed an excess of DIN throughout the entire Urdaibai estuary in 2020 and 2022, although sewerage works softened this imbalance. This has led to Si and P being potential limiting nutrients in the estuary (Justic et al., 1995; Redfield, 1958). In both years (2020 and 2022) the outer Urdaibai estuary was Si-deficient and, from URD2 to UR5, P-deficient, in agreement with previous descriptions by Madariaga et al. (1994). The change was found in the surroundings of the WWTP (URD6), where waters were Si-deficient in 2020 but became P-deficient after the sewerage works, due to the marked decreases of ammonium and phosphate. Changes in nutrient ratios and limitations caused by anthropogenic activities have become common in many other coastal areas (Guo et al., 2020; Sun et al., 2022), also registering deficiencies of P and Si associated with large amounts of N loadings (e.g., Wang et al.,



2012; Yuan et al., 2018). These differences in nutrient molar ratios and limiting factors are commonly associated with biological responses, especially from phytoplankton (Lie et al., 2011; Wu et al., 2017a).

#### 4.2. Phytoplankton biomass and community composition

Focusing on phytoplankton, both biomass and community composition changes were recorded between 2020 and 2022 in the Urdaibai estuary. Previous works have described the noticeable spatio-temporal variability of the phytoplankton biomass (Chl *a*) and/or the community composition in relation with the salinity, temperature, and nutrient gradients of the estuary, mostly prompted by the effect of the river, tidal incursion, and wastewater discharges coming from the WWTP (e.g., Ansotegui et al., 2003; Madariaga, 1995; Orive et al., 1998; Trigueros and Orive, 2001). In the present study, among all the analysed variables that have direct effects on phytoplankton abundance and composition (i. e., temperature, salinity, turbidity, and hydrometeorology), nutrient concentration was the only one showing significant differences between 2020 and 2022 in the estuary. Thus, it was assumed that the inter-annual changes observed in the present study in the phytoplankton community of the Urdaibai estuary might be explained by the significant changes in nutrient concentration, its composition, and its ratios resulting from the sewerage works. However, it is known that phytoplankton population dynamics are the result of imbalances between reproduction and losses, the latter including grazing, sinking, and natural mortality (Brussaard, 2004). Therefore, there are several biotic factors that were not considered but could have also influenced the changes in the phytoplankton community of the Urdaibai estuary, such as viruses (Brussaard, 2004), bacteria (Seymour et al., 2017), and grazers (Lürling, 2021).

Regarding phytoplankton biomass, the spatio-temporal patterns observed in 2020 and 2022 coincided with that observed in late 90's in the studies performed along the Urdaibai estuary (e.g., Ansotegui et al., 2001), however, several differences were detected after sewerage works. In the outer and middle Urdaibai estuary, the Chl *a* concentration was significantly lower after sewerage works, probably due to the decrease in DIN and P, in agreement with several studies that reported a positive relationship between the reduction of nutrient inputs and the decrease of phytoplankton biomass (e.g., Beardall et al., 2001; Wetz et al., 2011). Nutrient limitation in the outer and middle estuary might be explained by the oligotrophic character of the Basque coast (Muñiz et al., 2018) and, mainly, the scarce amount of nutrients reaching these areas from inner estuary, due to the cessation of high nutrient loadings and to the dilution effect caused by tidal incursion (Villate et al., 2017). Slight biomass decreases were preliminarily detected in URD1 and URD2 immediately after the cessation of wastewater discharges (Bilbao et al., 2022), but the present study confirmed the change is notable and significant. Additionally, in the outer and middle estuary, sewerage works led to a decrease in the number of phytoplankton blooms (cell abundances higher than  $7.5 \times 10^5$  cells  $L^{-1}$ ; Revilla et al., 2009). This might also be explained by nutrient limitation in this area, promoting competition for the limited resources and leading to an alternation of the blooming taxa.

On the contrary, in the surroundings of the WWTP of Gernika (URD6), phytoplankton biomass increased in 2022, which means that sewerage improvements did not cause nutrient limitation and phytoplankton growth persisted throughout the year. Indeed, Chl *a* concentrations above  $20 \mu g L^{-1}$  that indicate hypertrophic conditions in the inner estuary (Hagy et al., 2022) were more frequently recorded after the sewerage works (Table 4), reaching a Chl *a* maximum of  $251.5 \mu g L^{-1}$ , the highest ever reported in previous works in the area. This biomass increase could respond to the excessive ammonium loads received from the WWTP before the sewerage works, which might have caused phytoplankton growth suppression in the area during the discharges. Highly elevated ammonium concentrations (exceeding several tens to hundreds of  $\mu mol L^{-1}$ ) can lead to phytoplankton growth suppression rather than enhancement (Glibert et al., 2016). Thus, in 2020,

when the median ammonium concentration in URD6 was  $153 \mu mol L^{-1}$  (with a range of  $65\text{--}334 \mu mol L^{-1}$ ), the concentration might have been excessive for phytoplankton growth, while the decrease to  $10 \mu mol L^{-1}$  (reaching a maximum of  $24 \mu mol L^{-1}$ ) after the sewerage works enabled adequate growth, leading to the biomass increase. Several studies previously observed phytoplankton growth suppression with increasing ammonium concentrations (above  $10 \mu mol L^{-1}$ ), such as Yoshiyama and Sharp (2006) in the Delaware Bay and Dugdale et al. (2007) in the San Francisco Bay Delta. Additionally, sewerage works might have reduced the input of particulate matter in URD6, as happened in other estuaries (Carey and Migliaccio, 2009; Eccles et al., 2020; Toubanc et al., 2016). Therefore, the higher biomass values registered in 2022 might have also responded to the higher light availability due to the decreased turbidity in the area (Karlsson et al., 2009; Yamamichi et al., 2018). Indeed, the lack of significant differences registered in the water turbidity values between 2020 and 2022 might be explained by the increase of phytoplankton biomass, masking the reduction of the turbidity achieved with the sewerage works.

Regarding phytoplankton community composition, although the sampling stations and frequency and do not exactly coincide with the studies performed in the estuary before, the phytoplankton community of the Urdaibai estuary in 2020 was similar to that described in the late 90's (Madariaga et al., 1994; Orive et al., 1998; Trigueros and Orive, 2001). However, several differences were registered in 2022 related to variations in nutrient loadings. The most noticeable changes were the increase in the contribution of PrCA to total phytoplankton biomass from URD1 to URD4 and higher presence of FCA in the inner estuary, leading to the decrease of ACA and CbCA.

Concerning Pras containing prasinophytes (PrCA), which comprise the Prasinophyceae orders Mamiellales, Pseudosourfieldiales (Pycnococcales), and Prasinococcales (Latasa et al., 2004), the most commonly and abundantly reported in the estuary in 2020 were *Bathycoccus prasinos*, *Micromonas* spp. (*M. bravo*, *M. commoda* and *M. pusila*), and *Ostreococcus* spp. (*O. lucimarinus*, *O. mediterraneus*, and *O. tauri*) (Bilbao et al., 2023). Some of these were previously reported in the Urdaibai estuary and other nearby systems like the Nervion Estuary (Ansotegui et al., 2003; Laza-Martinez et al., 2007; Seoane et al., 2005) and most of them belong to the picoplanktonic fraction (cells  $<3 \mu m$  in diameter) (Guillou et al., 2004), being hard or impossible to identify and quantify them by microscopy (e.g., Ansotegui et al., 2003). In the present study, pigment analysis determined a significant increase of PrCAs' contribution to the total biomass of the Urdaibai estuary after the sewerage works, being two times higher in URD1, five times in URD2, three times in URD3 and six times in URD4, although it was not possible to identify the organisms responsible for it. The absence of PrCA in URD5 and URD6 is explained by the marine character of these organisms, being typical in coastal waters (Collado-Fabbri et al., 2011), open ocean (Treich et al., 2012; Vaulot et al., 2012) and the outermost estuarine areas (Lemaire et al., 2002), becoming one of the major contributors to marine picophytoplankton (Lohrenz et al., 2003; Wu et al., 2017b). Their increase from URD1 to URD4 after sewerage works might be explained by their high surface:volume ratio, efficient growth rates, and enhanced nutrient uptake rates (Paerl et al., 2003), PrCA being more competitive than other phytoplankton functional groups when nutrients are not so abundant (Glibert et al., 2010). An increase of PrCA after the sewerage works (and reduction of anthropogenic inputs of N and P) was also described by Leruste et al. (2016) in Mediterranean lagoons, where prasinophytes in the 3–6  $\mu m$  size range replaced small diatoms. Nevertheless, this significant change registered in Urdaibai should be further studied, to determine if PrCA will continue having an important contribution to the total phytoplankton biomass of the estuary and which are the taxa responsible for this, or if it was just a mid-term effect of the sewerage works.

Regarding the community composition shift in the inner estuary, the increase of FCA over the decrease of ACA (cryptophytes) and CbCA (green algae except for PrCA) might be interpreted as recovery of the

system after years of anthropogenic nutrient enrichment by the WWTP. The community composition of the inner Urdaibai estuary during the WWTP discharges (2020) was the expected for human impacted estuaries, as described in the late 90s (e.g., Ansotegui et al., 2003; Orive et al., 1998; Trigueros and Orive, 2001), with a high contribution of flagellates to the total biomass, due to the dominance of ACA and the high presence of CbCA and *K. foliaceum* (contributing to FCA). Smayda (1990) suggested that the elevation of inorganic N:Si ratios in coastal areas might affect species dominance within the phytoplankton assemblage, prompting the growth of flagellates over diatoms (Roberts et al., 2003; Sommer, 1994). Thus, from a nutrient composition perspective, in the Urdaibai estuary, before sewerage works, non-siliceous phytoplankton (e.g., ACA, CbCA and *K. foliaceum*) probably used the excess of N and P coming from the WWTP, while the Si limited diatoms could not use it, and became dominant in the inner area (Jiang et al., 2014; Tréguer and De La Rocha, 2013). Indeed, similar shifts in phytoplankton assemblage composition have been observed in many estuarine ecosystems related to anthropogenic N and P enrichment (Dutto et al., 2012; Glibert et al., 2011; Jiang et al., 2014; Liu et al., 2022). Cryptophytes (ACA) are known to dominate the inner area of eutrophicated estuaries other than Urdaibai (Adolf et al., 2006; Santos et al., 2022; Valdes-Weaver et al., 2006), due to their ability to adapt to turbid environments (Collini, 2022; Weng et al., 2009), their high growth rate (Paerl et al., 2003), their advantage in ammonium-enriched waters (Horner and Thompson, 2001), and their mixotrophic character (Johnson et al., 2013). As for the green algae (CbCA) present in the inner Urdaibai estuary before sewerage works, the main taxa were euglenoids like *Eutreptiella* spp. and chlorophytes, such as *Tetraselmis* spp. and several unidentified picoplanktonic coccoids. Euglenoids are known to thrive in shallow, nutrient rich, and low light waters (Poniewozik and Jurán, 2018), where they coexist with other flagellates like cryptophytes (Jeong et al., 2021), sharing features such as fast reproduction and mixotrophy, which explains their presence in the inner Urdaibai estuary during the wastewater discharges. As for small (<5 µm) chlorophytes, being *Picochlorum* spp. and *Nannochloris* sp. the most representatives in the area (Bilbao et al., 2023), they have particularly high affinity for ammonium uptake compared to diatoms and dinophytes (Leruste et al., 2016; Litchman et al., 2007), which might explain the frequent presence of these non-siliceous algae.

However, one year after the cessation of wastewater discharges, there was an overall decrease of the contribution to total biomass of ACA (e.g., from 54% to 27% in URD4) and CbCA (e.g., from 25% to 5% in URD6) in the inner estuary, being replaced by a higher presence FCA (Fig. 5). The FCA group of the inner Urdaibai estuary is mostly comprised of small diatoms and the Fuco-containing dinoflagellate *K. foliaceum*. However, the higher contribution of FCA to the total phytoplankton biomass during 2022 (from URD3 to URD6), being two times higher in URD4 and three times higher in URD5, was mostly caused by the higher diatom abundance, with the increase of taxa like *Cyclotella*, *M. polymorphus*, *Skeletonema*, and *Thalassiosira*. In general, diatoms show high maximum nutrient uptake rates and high growth rates, being favoured under high or fluctuating nutrients (Leruste et al., 2016; Litchman et al., 2007), which explains their presence in 2020 and 2022. The lower diatom presence in 2020 might be associated with the elevated levels of ammonium coming from the WWTP rather than with nutrient limitation or stoichiometry (varying nutrient ratios) (Glibert et al., 2011). Many organisms prefer ammonium as an N source because it is already in reduced form, being easily assimilated (e.g., McCarthy et al., 1977), however, some diatoms physiologically prefer, and sometimes require, nitrate over ammonium (Lomas and Glibert, 1999). Additionally, this is accentuated when cells are in an energy-imbalance state, such as in situations involving fluctuating light conditions like in the inner Urdaibai estuary, when nitrate serves not only as a nutrient, but also contributes to maintaining the cellular energy balance (Lomas and Glibert, 1999). Thus, the increase of the FCA contribution to total phytoplankton biomass in the inner estuary in 2022 could be explained

by the lower ammonium concentrations and higher availability of nitrate for diatom growth resulting from sewerage works in the Urdaibai estuary. Indeed, declines in diatoms were significantly correlated with the increase in ammonium concentration in the San Francisco Estuary (Glibert et al., 2011), and several studies have confirmed the favoured growth of diatoms under nitrate enrichment (Domingues et al., 2011; Glibert et al., 2014). Apart from this, Si was no longer the limiting nutrient in URD6 in 2022, since after sewerage works the innermost estuary was P-deficient. Consequently, the growth of non-siliceous phytoplankton groups (ACA and CbCA, or *K. foliaceum*) was not favoured over diatoms anymore. Taking all this into account, the higher contribution of FCA to total phytoplankton biomass in 2022 might be considered a recovery sign of the Urdaibai estuary.

Thus, overall, the present study reveals mid-term changes in the nutrient concentrations of the Urdaibai estuary following sewerage works, leading to noticeable shifts in phytoplankton biomass and community composition. However, other factors, such as biotic changes (Brussaard, 2004; Lürling, 2021; Seymour et al., 2017) or inherent annual variability, could also account for the variations registered in the phytoplankton community. Additionally, it is important to acknowledge that years of excessive external nutrient loadings might result in the storage of nutrients in benthic sediments, which may be slowly released even after external loading disappear (e.g., Corbett, 2010). The resilience of the estuary will also determine the medium- and long-term effects of the sewerage works on the system, especially the recovery capacity (Wainger et al., 2017). Therefore, these results should be interpreted with caution, as they represent preliminary findings based on data from the first year after the sewerage works. Future studies including a more comprehensive temporal dataset will help to better understand the underlying causes of the observed changes and to define the system's long-term response to sewerage works.

Nevertheless, the insights presented in this study provide preliminary findings on the effect of restoration works on the Urdaibai estuary, contributing to the understanding of interactions between anthropogenic activities, estuarine ecosystems and phytoplankton. These findings may be applicable to other temperate estuaries and will serve as a basis for future phytoplankton and interdisciplinary studies in the Urdaibai estuary, as well as for developing future temporal datasets. Moreover, in the present study, pigment analysis through *PIGMENTUM*, novel in its application, was found to be very useful for determining changes on the phytoplankton community composition, including groups containing small and fragile cells underestimated by other procedures (Jeffrey et al., 1997). This pioneering application demonstrates the efficacy and utility of *PIGMENTUM* and establishes a foundation for its future application in phytoplankton research.

## 5. Conclusion

The cessation of wastewater discharges coming from the Gernika WWTP to the Urdaibai estuary had significant medium-term effects on the system. Ammonium and phosphate concentration decreased significantly, leading to changes in nutrient ratios and composition, which had a direct effect on phytoplankton biomass and community composition. In the outer and middle estuary, biomass decreased significantly due to lower nutrient concentrations, while in the surroundings of the WWTP, it increased after the sewerage works, probably because the excessive ammonium loadings coming from the WWTP discharges were causing phytoplankton growth suppression in the area. As for community composition, two significant changes were recorded after the sewerage works: PrCA contribution to total phytoplankton biomass increased from URD1 to URD4 and, in the inner Urdaibai estuary (URD3–URD6), there was a shift from mainly flagellates (ACA and CbCA) to FCA, due to the increase of diatoms, that might indicate the recovery of the area. These changes might have potential ecological implications in the near future, since phytoplankton community composition changes may have effects throughout the trophic chain and

therefore, they should be further studied. Additionally, *PIGMENTUM* was proven a useful tool for studying community composition and its variability, recording contribution changes of groups like PrCA, contain picoplanktonic organisms, that would have been ignored with microscopy.

### CRedit authorship contribution statement

**Jone Bilbao:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sergio Seoane:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

### 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.

### Acknowledgements

This study was partially supported by project IT1471-22 funded by the Department of Education of the Basque Government and project TR41828 funded by “Agencia Vasca del Agua (URA)”. J. Bilbao was funded by a grant from the University of the Basque Country (UPV/EHU – PIF 18/306).

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2024.106668>.

### References

- Adams, J.B., Taljaard, S., Van Niekerk, L., Lemley, D.A., 2020. Nutrient enrichment as a threat to the ecological resilience and health of South African microtidal estuaries. *Afr. J. Aquat. Sci.* 45 (1–2), 23–40. <https://doi.org/10.2989/16085914.2019.1677212>.
- 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>.
- Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26, 32–46.
- 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>.
- Barroeta, Z., Uriarte, I., Iriarte, A., Villate, F., 2023. Intraregional variability of exotic and native zooplankton in Basque coast estuaries (inner Bay of Biscay): effect of secondary dispersion, estuary features and regional environmental gradients. *Hydrobiologia* 1–21. <https://doi.org/10.1007/s10750-023-05363-8>.
- 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>.
- Beardall, J., Young, E., Roberts, S., 2001. Approaches for determining phytoplankton nutrient limitation. *Aquat. Sci.* 63, 44–69.
- Bilbao, J., Seoane, S., 2024. *PIGMENTUM: an easy pigment-based tool for monitoring phytoplankton community composition*. *Mar. Ecol. Prog. Ser.* (in press).
- Bilbao, J., Larreta, J., Franco, J., Seoane, S., 2022. Immediate effect of sewerage improvement on the phytoplankton and physicochemical conditions in the Urdaibai estuary (southeastern Bay of Biscay). *Reg. Stud. Mar. Sci.* 56, 102707. <https://doi.org/10.1016/j.rsm.2022.102707>.
- Bilbao, J., Pavlou, C., Blanco-Rayón, E., Franco, J., Madariaga, I., Seoane, S., 2023. Phytoplankton community composition in relation to environmental variability in the Urdaibai estuary (SE Bay of Biscay): microscopy and eDNA metabarcoding. *Mar. Env. Res.* 191, 106175. <https://doi.org/10.1016/j.marenvres.2023.106175>.
- Borja, A., Bald, J., Uyarra, M.C., Franco, J., Larreta, J., et al., 2021. *Red de Seguimiento Del Estado Ecológico de Las Aguas de Transición y Costeras de la Comunidad Autónoma Del País Vasco. Informe de Resultados. Campaña 2020. Technical report, Informe de AZTI para la Agencia Vasca del Agua*, p. 385.
- Bricker, S.B., Ferreira, J.G., Simas, T., 2003. An integrated methodology for assessment of estuarine trophic status. *Ecol. Modell.* 169 (1), 39–60. [https://doi.org/10.1016/S0304-3800\(03\)00199-6](https://doi.org/10.1016/S0304-3800(03)00199-6).
- Brussaard, C.P., 2004. Viral control of phytoplankton populations - a review. *J. Eukaryot. Microbiol.* 51 (2), 125–138. <https://doi.org/10.1111/j.1550-7408.2004.tb00537.x>.
- Buzanić, M., Gladan, Ž.N., Marasović, I., Kušpilić, G., Grbec, B., 2016. Eutrophication influence on phytoplankton community composition in three bays on the eastern Adriatic coast. *Oceanologia* 58 (4), 302–316. <https://doi.org/10.1016/j.oceano.2016.05.003>.
- Cai, W.J., Hu, X., Huang, W.J., Murrell, M.C., Lehrter, J.C., Lohrenz, S.E., Chou, W.C., Zhai, W., Hollibaugh, J.T., Wang, Y., 2011. Acidification of subsurface coastal waters enhanced by eutrophication. *Nat. Geosci.* 4, 766–770.
- Carey, R.O., Migliaccio, K.W., 2009. Contribution of wastewater treatment plant effluents to nutrient dynamics in aquatic systems: a review. *Environ. Manage.* 44, 205–217. <https://doi.org/10.1007/s00267-009-9309-5>.
- Castillo-Eguskiza, 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>.
- Cloern, J.E., 2001. Our evolving conceptual model of the coastal eutrophication problem. *Mar. Ecol. Prog. Ser.* 210, 223–253. <https://doi.org/10.3354/meps210223>.
- Collado-Fabris, S., Vulot, D., Ulloa, O., 2011. Structure and seasonal dynamics of the eukaryotic picophytoplankton community in a wind-driven coastal upwelling ecosystem. *Limnol. Oceanogr.* 5, 2334–2346. <https://doi.org/10.4319/lo.2011.56.6.2334>.
- 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>.
- Corbett, D.R., 2010. Resuspension and estuarine nutrient cycling: insights from the Neuse River Estuary. *Biogeosciences* 7, 3289–3300. <https://doi.org/10.5194/bg-7-3289-2010>.
- Domingues, R.B., Barbosa, A.B., Sommer, U., Galvao, H.M., 2011. Ammonium, nitrate and phytoplankton interactions in a freshwater tidal estuarine zone: potential effects of cultural eutrophication. *Aquat. Sci.* 73, 331–343. <https://doi.org/10.1007/s00027-011-0180-0>.
- Dugdale, R.C., Wilkerson, F.P., Hogue, V.E., Marchi, A., 2007. The role of ammonium and nitrate in spring Bloom development in San Francisco Bay. *Estuar. Coast Shelf Sci.* 73, 17–29. <https://doi.org/10.1016/j.ecss.2006.12.008>.
- Dutto, M.S., López Abbate, M.C., Biancalana, F., Berasategui, A.A., Hoffmeyer, M.S., 2012. The impact of sewage on environmental quality and the mesozooplankton community in a highly eutrophic estuary in Argentina. *ICES J. Mar. Sci.* 69 (3), 399–409. <https://doi.org/10.1093/icesjms/fsr204>.
- Eccles, R., Zhang, H., Hamilton, D., Maxwell, P., 2020. Trends in water quality in a subtropical Australian river—estuary system: responses to damming, climate variability and wastewater discharges. *J. Environ. Manag.* 269, 110796. <https://doi.org/10.1016/j.jenvman.2020.110796>.
- Edler, L., Elbrächter, M., 2010. In: Karlson, B., Cusack, C., Bresnan, E. (Eds.), *Microscopic and Molecular Methods for Quantitative Phytoplankton Analysis*, 55. UNESCO, Paris, pp. 13–20.
- Elliott, M., Quintino, V., 2007. The estuarine quality paradox, environmental homeostasis and the difficulty of detecting anthropogenic stress in naturally stressed areas. *Mar. Pollut. Bull.* 54, 640–645. <https://doi.org/10.1016/j.marpollbul.2007.02.003>.
- European Commission, 1991. Council Directive 91/271/EEC of 21 May 1991 Concerning Urban Waste Water Treatment. O.J. L135, 30.5. European Commission, Brussels.
- Ferreira, J.G., Andersen, J.H., Borja, A., Bricker, S.B., Camp, J., Silva, M.C., et al., 2011. Overview of eutrophication indicators to assess environmental status within the European Marine Strategy Framework Directive. *Estuar. Coast Shelf Sci.* 93, 117–131. <https://doi.org/10.1016/j.ecss.2011.03.014>.
- Field, C.B., Barros, V.R. (Eds.), 2014. *Climate Change 2014—Impacts, Adaptation and Vulnerability: Regional Aspects*. Cambridge University Press.
- Franco, J., 1994. *Variabilidad espacio-temporal de la biomasa y produccion de fitoplancton en el estuario de Urdaibai*. University of the Basque Country, Universidad del País Vasco – Euskal Herriko Unibertsitatea (UPV/EHU), Leioa, Spain. PhD thesis.
- Franco, J., Borja, A., Valencia, V., 2004. Overall assessment—human impacts and quality status. *Oceanography and Marine Environment of the Basque Country*. Elsevier Oceanogr. Ser. 70, 581–597.
- Glibert, P.M., Fullerton, D., Burkholder, J.M., Cornwell, J.C., Kana, T.M., 2011. Ecological stoichiometry, biogeochemical cycling, invasive species, and aquatic food webs: San Francisco Estuary and comparative systems. *Rev. Fish. Aquac.* 19 (4), 358–417. <https://doi.org/10.1080/10641262.2011.611916>.
- Glibert, P.M., Allen, J.I., Bouwman, L., Brown, C., Flynn, K.J., Lewitus, A., Madden, C., 2010. Modeling of HABs and eutrophication: status, advances, challenges. *J. Mar. Systems* 83, 262–275. <https://doi.org/10.1016/j.jmarsys.2010.05.004>.
- Glibert, P.M., Wilkerson, F.P., Dugdale, R.C., Parker, A.E., Alexander, J., Blaser, S., Murasko, S., 2014. Microbial communities from San Francisco Bay Delta respond differently to oxidized and reduced nitrogen substrates—even under conditions that would otherwise suggest nitrogen sufficiency. *Front. Mar. Sci.* 1, 17. <https://doi.org/10.3389/fmars.2014.00017>.
- Glibert, P.M., Wilkerson, F.P., Dugdale, R.C., Raven, J.A., Dupont, C.L., Leavitt, P.R., Kana, T.M., 2016. Pluses and minuses of ammonium and nitrate uptake and



- assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. *Limnol. Oceanogr.* 61 (1), 165–197. <https://doi.org/10.1002/lno.10203>.
- Glibert, P.M., Al-Azri, A., Icarus Allen, J., Bouwman, A.F., Beusen, A.H., Burford, M.A., Harrison, P.J., Zhou, M., 2018. Key questions and recent research advances on harmful algal blooms in relation to nutrients and eutrophication. *Global Ecol. Oceanogr.* 229–259. [https://doi.org/10.1007/978-3-319-70069-4\\_12](https://doi.org/10.1007/978-3-319-70069-4_12).
- Goberville, E., Beaugrand, G., Sautour, B., Tréguer, P., SOMLIT Team, 2010. Climate-driven changes in coastal marine systems of western Europe. *Mar. Ecol. Prog. Ser.* 408, 129–148. <https://doi.org/10.3354/meps08564>.
- González, E.J., Roldán, G., 2019. Eutrophication and phytoplankton: some generalities from lakes and reservoirs of the Americas. In: Vítová, M. (Ed.), *Microalgae - From Physiology to Application*. IntechOpen, pp. 27–47.
- Groll, M., 2017. The passive river restoration approach as an efficient tool to improve the hydromorphological diversity of rivers – case study from two river restoration projects in the German lower mountain range. *Geomorphology* 293, 69–83. <https://doi.org/10.1016/j.geomorph.2017.05.004>.
- Guillou, L., Eikrem, W., Chrétiennot-Dinet, M.J., 2004. Diversity of picoplanktonic Prasinophytes assessed by direct nuclear SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. *Protist* 155, 193–214. <https://doi.org/10.1078/143446104774199592>.
- Guiry, M.D., Guiry, G.M., 2018. *AlgaeBase*. World-wide Electronic Publication. National University of Ireland, Galway. <http://www.algaebase.org>.
- Guo, C.C., Zhang, G.C., Sun, J., Leng, X.Y., Xu, W.Z., Wu, C., Li, X.Q., Pujari, L., 2020. Seasonal responses of nutrient to hydrology and biology in the southern Yellow Sea. *Cont. Shelf Res.* 206, 104207. <https://doi.org/10.1016/j.csr.2020.104207>.
- Hagy, J.D. 3rd, Kreakie, B.J., Pelletier, M.C., Nojavan, F., Kiddon, J.A., Oczkowski, A.J., 2022. Quantifying coastal ecosystem trophic state at a macroscale using a Bayesian analytical framework. *Ecol. Indic.* 1 (142), 1–12. <https://doi.org/10.1016/j.ecolind.2022.109267>.
- Hall, N., Testa, J., Li, M., Paerl, H., 2023. Assessing drivers of estuarine pH: a comparative analysis of the continental USA's two largest estuaries. *Limnol. Oceanogr.* 68 (10), 2227–2244. <https://doi.org/10.1002/lno.12418>.
- Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. *PAST: paleontological statistics software package for education and data analysis*. *Palaentol. Electron.* 4, 9.
- Ho, A.Y., Xu, J., Yin, K., Yuan, X., He, L., Jiang, Y., Harrison, P.J., 2008. Seasonal and spatial dynamics of nutrients and phytoplankton biomass in Victoria Harbour and its vicinity before and after sewage abatement. *Mar. Pollut. Bull.* 57 (6–12), 313–324. <https://doi.org/10.1016/j.marpolbul.2008.04.035>.
- Horner, S.M.J., Thompson, P.A., 2001. Phytoplankton of the Swan-Canning Estuary: a comparison of nitrogen uptake by different bloom assemblages. *Hydrobiol. Process.* 15, 2579–2594. <https://doi.org/10.1002/hyp.288>.
- 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.
- Ibáñez, C., Alcaraz, C., Caiola, N., Rovira, A., Trobajo, R., Alonso, M., Prat, N., 2012. Regime shift from phytoplankton to macrophyte dominance in a large river: top-down versus bottom-up effects. *Sci. Total Environ.* 416, 314–322. <https://doi.org/10.1016/j.scitotenv.2011.11.059>.
- Ibáñez, C., Peñuelas, J., 2019. Changing nutrients, changing rivers. *Science* 365 (6454), 637–638. <https://doi.org/10.1126/science.aay2723>.
- Iriarte, A., Aravena, G., Villate, F., Uriarte, I., Ibáñez, B., Llope, M., Stenseth, N.C., 2010. Dissolved oxygen in contrasting estuaries of the Bay of Biscay: effects of temperature, river discharge and chlorophyll a. *Mar. Ecol. Prog. Ser.* 418, 57–71. <https://doi.org/10.3354/meps08812>.
- Iriarte, A., Villate, F., Uriarte, I., Arranz, S., 2016. Assessment of the climate and human impact on estuarine water environments in two estuaries of the Bay of Biscay. *Oceanol. Hydrobiol. Stud.* 45, 505–523. <https://doi.org/10.1515/ohs-2016-0043>.
- Iriarte, A., Villate, F., Uriarte, I., Alberdi, L., Intxausti, L., 2015. Dissolved oxygen in a temperate estuary: the influence of hydro-climatic factors and eutrophication at seasonal and inter-annual time scales. *Estuaries Coast* 38, 1000–1015. <https://doi.org/10.1007/s12237-014-9870-x>.
- Jeffrey, S.W., Mantoura, R.F., Bjørnland, T., 1997. Data for the identification of 47 key phytoplankton pigments. In: Jeffrey, S.W., Mantoura, R.F., Wright, S.W. (Eds.), *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*. In: *Monographs on Oceanographic Methodology*. UNESCO, Paris.
- Jeong, H.J., Du, Y.Y., Lee, K., Kang, H.C., Kim, J.S., 2021. Annual carbon retention of a marine-plankton community in the eutrophic Masan Bay, based on daily measurements. *Mar. Biol.* 168, 1–10. <https://doi.org/10.1007/s00227-021-03881-4>.
- Jiang, Z., Liu, J., Chen, J., Chen, Q., Yan, X., Xuan, J., Zeng, J., 2014. Responses of summer phytoplankton community to drastic environmental changes in the Changjiang (Yangtze River) estuary during the past 50 years. *Water Res.* 54, 1–11. <https://doi.org/10.1016/j.watres.2014.01.032>.
- 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>.
- Justic, D., Rabalais, N., Turner, R., 1995. Stoichiometric nutrient balance and origin of coastal eutrophication. *Mar. Pollut. Bull.* 30, 41–46. [https://doi.org/10.1016/0025-326X\(94\)00105-1](https://doi.org/10.1016/0025-326X(94)00105-1).
- Karlsson, J., Byström, P., Ask, J., Ask, P., Persson, L., 2009. Light limitation of nutrient-poor lake ecosystems. *Nature* 460, 506–509. <https://doi.org/10.1038/nature08179>.
- Lane, R.R., Day, J.W., Justic, D., Reyes, E., Marx, B., Day, J.N., Hyfield, E., 2004. Changes in stoichiometric Si, N and P ratios of Mississippi River water diverted through coastal wetlands to the Gulf of Mexico. *Estuar. Coast Shelf Sci.* 60 (1), 1–10. <https://doi.org/10.1016/j.ecss.2003.11.015>.
- Latasa, M., Scharek, R., Gall, F.L., Guillou, L., 2004. Pigment suites and taxonomic groups in Prasinophyceae 1. *J. Phycol.* 40 (6), 1149–1155. <https://doi.org/10.1111/j.1529-8817.2004.03136.x>.
- Laza-Martínez, A., Seoane, S., Zapata, M., Orive, E., 2007. Phytoplankton pigment patterns in a temperate estuary: from unialgal cultures to natural assemblages. *J. Plankton Res.* 29 (11), 913–929. <https://doi.org/10.1093/plankt/fbm069>.
- Legendre, L., Legendre, P., 1979. *Ecologie Numérique. 1. Le Traitement des Données Ecologiques*. Masson and Cie, Paris, p. 871.
- Lemaire, E., Abril, G., De Wit, R., Etcheber, H., 2002. Distribution of phytoplankton pigments in nine European estuaries and implications for an estuarine typology. *Biogeochemistry* 59, 5–23.
- Lemley, D.A., Adams, J.B., 2019. Eutrophication. In: Faith, B. (Ed.), *Encyclopedia of Ecology*, second ed. Elsevier Science and Technology, Oxford, UK.
- Leruste, A., Malet, N., Munaron, D., Derolez, V., Hatey, E., Collos, Y., Bec, B., 2016. First steps of ecological restoration in Mediterranean lagoons: shifts in phytoplankton communities. *Estuar. Coast Shelf Sci.* 180, 190–203. <https://doi.org/10.1016/j.ecss.2016.06.029>.
- Li, D., Lu, X.X., Yang, X., Chen, L., Lin, L., 2018. Sediment load responses to climate variation and cascade reservoirs in the Yangtze River: a case study of the Jinsha River. *Geomorphology* 322, 41–52. <https://doi.org/10.1016/j.geomorph.2018.08.038>.
- Li, X.Q., Lu, C.Q., Zhang, Y.F., Zhao, H.D., Wang, J.Y., Liu, H.B., Yin, K.D., 2020. Low dissolved oxygen in the Pearl River estuary in summer: long-term spatio-temporal patterns, trends, and regulating factors. *Mar. Pollut. Bull.* 151, 110814. <https://doi.org/10.1016/j.marpolbul.2019.110814>.
- Liang, B., Xiu, P., Hu, J., Li, S., 2021. Seasonal and spatial controls on the eutrophication-induced acidification in the Pearl River Estuary. *J. Geophys. Res.: Oceans*, e2020JC017107. <https://doi.org/10.1029/2020JC017107>.
- Lie, A.A.Y., Wong, C.K., Lam, J.Y.C., Liu, J.H., Yung, Y.K., 2011. Changes in the nutrient ratios and phytoplankton community after declines in nutrient concentrations in a semi-enclosed bay in Hong Kong. *Mar. Environ. Res.* 71, 178–188. <https://doi.org/10.1016/j.marenvres.2011.01.001>.
- Litchman, E., Klausmeier, C.A., Schofield, O.M., Falkowski, P.G., 2007. The role of functional traits and trade-offs in structuring phytoplankton communities: scaling from cellular to ecosystem level. *Ecol. Lett.* 10, 1170e1181. <https://doi.org/10.1111/j.1461-0248.2007.01117.x>.
- Liu, J., Yao, Q., Mi, T., Wei, Q., Chen, H., Yu, Z., 2022. Change of the long-term nitrogen and phosphorus in the Changjiang (Yangtze) river estuary. *Front. Mar. Sci.* 9, 885311. <https://doi.org/10.3389/fmars.2022.885311>.
- Lohrenz, S.E., Carrolla, C.L., Weidemann, A.D., Tuel, M., 2003. Variations in phytoplankton pigments, size structure and community composition related to wind forcing and water mass properties on the North Carolina inner shelf. *Cont. Shelf Res.* 23, 1447–1464. [https://doi.org/10.1016/S0278-4343\(03\)00131-6](https://doi.org/10.1016/S0278-4343(03)00131-6).
- Lomas, M.W., Glibert, P.M., 1999. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnol. Oceanogr.* 44, 556–572.
- Lürling, M., 2021. Grazing resistance in phytoplankton. *Hydrobiologia* 848 (1), 237–249. <https://doi.org/10.1007/s10750-020-04370-3>.
- 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. <https://doi.org/10.1006/ecss.1995.0038>.
- 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](https://doi.org/10.1016/0022-0981(89)90079-8).
- 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.
- Mallin, M.A., McIver, M.R., Wells, H.A., Parsons, D.C., Johnson, V.L., 2005. Reversal of eutrophication following sewage treatment upgrades in the New River Estuary, North Carolina. *Estuaries* 28, 750–760.
- Malone, T.C., Newton, A., 2020. The globalization of cultural eutrophication in the coastal ocean: causes and consequences. *Front. Mar. Sci.* 7, 670. <https://doi.org/10.3389/fmars.2020.00670>.
- McCarthy, J.J., Taylor, W.R., Taft, J.L., 1977. Nitrogenous nutrition of the plankton in the Chesapeake Bay. 1. Nutrient availability and phytoplankton preferences. *Limnol. Oceanogr.* 22, 996–1011.
- Muñiz, O., Rodríguez, J.G., Revilla, M., Laza-Martínez, A., Seoane, S., Franco, J., 2018. Seasonal variations of phytoplankton community in relation to environmental factors in an oligotrophic area of the European Atlantic coast (southeastern Bay of Biscay). *Reg. Stud. Mar. Sci.* 17, 59–72. <https://doi.org/10.1016/j.rmsa.2017.11.011>.
- Niu, L., van Gelder, P., Luo, X., Cai, H., Zhang, T., Yang, Q., 2020. Implications of nutrient enrichment and related environmental impacts in the Pearl River Estuary, China: characterizing the seasonal influence of riverine input. *Water* 12 (11), 3245. <https://doi.org/10.3390/w12113245>.
- Odjadjare, E.E., Okoh, A.I., 2010. Physicochemical quality of an urban municipal wastewater effluent and its impact on the receiving environment. *Environ. Monit. Assess.* 170, 383–394. <https://doi.org/10.1007/s10661-009-1240-y>.
- Orive, E., Iriarte, A., De Madariaga, I., Revilla, M., 1998. Phytoplankton blooms in the Urdaibai estuary during summer: physico-chemical conditions and taxa involved. *Oceanol. Acta* 21 (2), 293–305.
- OSPAR, 2003. Strategies of the OSPAR commission for the protection of the marine environment of the north-east Atlantic (reference number: 2003e21). In: *OSPAR Convention for the Protection of the Marine Environment of the Northeast Atlantic: Ministerial Meeting of the OSPAR Commission, Bremen, 25 June 2003, Annex 31*. OSPAR Commission, London, United Kingdom (Ref. B-4.2).

- Paerl, H.W., 2006. Assessing and managing nutrient-enhanced eutrophication in estuarine and coastal waters: interactive effects of human and climatic perturbations. *Ecol. Eng.* 26 (1), 40–54. <https://doi.org/10.1016/j.ecoleng.2005.09.006>.
- Paerl, H.W., 2018. Why does N-limitation persist in the world's marine waters? *Mar. Chem.* 206, 1–6. <https://doi.org/10.1016/j.marchem.2018.09.001>.
- Paerl, H.W., Valdes, L.M., Pinckney, J.L., Piehler, M.F., Dyble, J., et al., 2003. Phytoplankton photopigments as indicators of estuarine and coastal eutrophication. *Bioscience* 53, 953–964. [https://doi.org/10.1641/0006-3568\(2003\)053\[0953:PPAIOE\]2.0.CO;2](https://doi.org/10.1641/0006-3568(2003)053[0953:PPAIOE]2.0.CO;2).
- Poniewozik, M., Jurán, J., 2018. Extremely high diversity of euglenophytes in a small pond in eastern Poland. *Plant Ecol. Evol.* 151, 18–34. <https://doi.org/10.5091/plecevo.2018.1308>.
- Prego, R., Boi, P., Cabelo-García, A., 2008. The contribution of total suspended solids to the Bay of Biscay by Cantabrian Rivers (northern coast of the Iberian Peninsula). *J. Mar. Syst.* 72, 342–349. <https://doi.org/10.1016/j.jmarsys.2007.01.011>.
- Redfield, A.C., 1958. The biological control of chemical factors in the environment. *Am. Sci.* 46, 205e222.
- Revilla, M., Solaun, O., Menchaca, I., Zorita, I., Franco, J., Borja, A., Valencia, V., Bald, J., 2017. Estudio de la sensibilidad a la eutrofización de los estuarios del País Vasco. Elaborado por AZTI para URA, p. 95. Informe 2017.
- Revilla, M., Franco, J., Bald, J., Borja, A., Laza, A., Seoane, S., Valencia, V., 2009. Assessment of the phytoplankton ecological status in the Basque coast (northern Spain) according to the European Water Framework Directive. *J. Sea Res.* 61 (1–2), 60–67. <https://doi.org/10.1016/j.seares.2008.05.009>.
- 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. <https://doi.org/10.1006/ecss.1999.0576>.
- Riemann, B., Carstensen, J., Dahl, K., Fossing, H., Hansen, J.W., Jakobsen, H.H., Andersen, J.H., 2016. Recovery of Danish coastal ecosystems after reductions in nutrient loading: a holistic ecosystem approach. *Estuar. Coast* 39, 82–97. <https://doi.org/10.1007/s12237-015-9980-0>.
- Ringwood, A.H., Keppler, C.J., 2002. Water quality variation and clam growth: is pH really a non-issue in estuaries? *Estuaries* 25, 901–907.
- Roberts, E.C., Davidson, K., Gilpin, L.C., 2003. Response of temperate microplankton communities to N: Si ratio perturbation. *J. Plankton Res.* 25 (12), 1485–1495. <https://doi.org/10.1093/plankr/fgb109>.
- Sand-Jensen, K., Bruun, H.H., Baastrup-Spohr, L., 2017. Decade-long time delays in nutrient and plant species dynamics during eutrophication and re-oligotrophication of Lake Fure 1900–2015. *J. Ecol.* 105 (3), 690–700. <https://doi.org/10.1111/1365-2745.12715>.
- Santos, M., Amorim, A., Brotas, V., Cruz, J.P.C., Palma, C., Borges, C., Brito, A.C., 2022. Spatio-temporal dynamics of phytoplankton community in a well-mixed temperate estuary (Sado Estuary, Portugal). *Sci. Rep.* 12 (1), 16423 <https://doi.org/10.1038/s41598-022-20792-6>.
- Seoane, S., Garmendia, M., Revilla, M., Á, Borja, Franco, J., et al., 2011. Phytoplankton pigments and epifluorescence microscopy as tools for ecological status assessment in coastal and estuarine waters, within the Water Framework Directive. *Mar. Pollut. Bull.* 62 (7), 1484–1497. <https://doi.org/10.1016/j.marpolbul.2011.04.010>.
- Seoane, S., Laza, A., Urrutxurtu, I., Orive, E., 2005. Phytoplankton assemblages and their dominant pigments in the Nervion River estuary. *Hydrobiologia* 549, 1–13. <https://doi.org/10.1007/s10750-005-1736-6>.
- Seoane, S., Zapata, M., Orive, E., 2009. 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>.
- Seymour, J.R., Amin, S.A., Raina, J.B., Stocker, R., 2017. Zooming in on the phycosphere: the ecological interface for phytoplankton–bacteria relationships. *Nature Microbiol.* 2 (7), 1–12. <https://doi.org/10.1038/nmicrobiol.2017.65>.
- Shortreed, K.S., Stockner, J.G., 1983. Periphyton biomass and species composition in a coastal rainforest stream in British Columbia: effects of environmental changes caused by logging. *Can. J. Fish. Aquat. Sci.* 40 (11), 1887–1895. <https://doi.org/10.1139/F83-219>.
- Sinha, E., Michalak, A.M., Balaji, V., 2017. Eutrophication will increase during the 21st century as a result of precipitation changes. *Science* 357 (6349), 405–408. <https://doi.org/10.1126/science.aan240>.
- Smayda, T.J., 1990. Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In: Granéli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), *Toxic Marine Phytoplankton*. Elsevier, New York, pp. 29–40.
- Smith, V.H., Schindler, D.W., 2009. Eutrophication science: where do we go from here? *Trends Ecol. Evol.* 24, 201–207. <https://doi.org/10.1016/j.tree.2008.11.009>.
- Sommer, U., 1994. Are marine diatoms favoured by high Si: N ratios? *Mar. Ecol. Prog. Ser.* 115, 309–315.
- Sun, X., Dong, Z., Zhang, W., Sun, X., Hou, C., Liu, Y., Chen, L., 2022. Seasonal and spatial variations in nutrients under the influence of natural and anthropogenic factors in coastal waters of the northern Yellow Sea, China. *Mar. Pollut. Bull.* 175, 113171 <https://doi.org/10.1016/j.marpolbul.2021.113171>.
- Tong, Y., Zhang, W., Wang, X., Couture, R.M., Larsen, T., Zhao, Y., Lin, Y., 2017. Decline in Chinese lake phosphorus concentration accompanied by shift in sources since 2006. *Nat. Geosci.* 10 (7), 507–511. <https://doi.org/10.1038/ngeo2967>.
- Toublanc, F., Brenon, I., Coulombier, T., 2016. Formation and structure of the turbidity maximum in the macrotidal Charente estuary (France): influence of fluvial and tidal forcing. *Estuar. Coast Shelf Sci.* 169, 1–14. <https://doi.org/10.1016/j.ecss.2015.11.019>.
- Tréguer, P.J., De La Rocha, C.L., 2013. The world ocean silica cycle. *Annu. Rev. Mar. Sci.* 5, 477e501. <https://doi.org/10.1146/annurev-marine-121211-172346>.
- Treusch, A.H., Demir-Hilton, E., Vergin, K.L., Worden, A.Z., Carlson, C., Donatz, M.G., 2012. Phytoplankton distribution patterns in the northwestern Sargasso Sea revealed by small subunit rRNA genes from plastids. *ISME J.* 6, 481–492. <https://doi.org/10.1038/ismej.2011.117>.
- 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.
- Trigueros, J.M., Ansoategui, A., Orive, E., 2000. 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>.
- Turner, R.E., Rabalais, N.N., Justic, D., Dortch, Q., 2003. Global patterns of dissolved N, P and Si in large rivers. *Biogeochemistry* 64, 297–317.
- Usabiaga, J., Sáenz, J., Valencia, V., Borja, A., 2004. Climate and meteorology: variability and its influence on the Ocean. In: Borja, A., Collins, M. (Eds.), *Oceanography and Marine Environment of the Basque Country*. Elsevier Oceanography Series, Elsevier, Amsterdam, pp. 133–157.
- Valdes-Weaver, L.M., Piehler, M.F., Pinckney, J.L., Howe, K.E., Rossignol, K., 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, 1410–1420. <https://doi.org/10.4319/lo.2006.51.3.1410>.
- Valencia, V., Franco, J., 2004. Main characteristics of the water masses. In: Borja, A., Collins, M. (Eds.), *Oceanography and Marine Environment of the Basque Country*, vol. 70. Elsevier Oceanography Series, Amsterdam.
- Valencia, V., Franco, J., Borja, A., Fontán, A., 2004. Hydrography of the southeastern bay of Biscay. In: Borja, Collins (Eds.), *Oceanography and Marine Environment of the Basque Country*, vol. 70. Elsevier Oceanography Series, Elsevier, Amsterdam, pp. 159–194.
- Van Meerssche, E., Pinckney, J.L., 2019. Nutrient loading impacts on estuarine phytoplankton size and community composition: community-based indicators of eutrophication. *Estuar. Coast* 42 (2), 504–512. <https://doi.org/10.1007/s12237-018-0470-z>.
- Vaulot, D., Lepère, C., Toulza, E., de la Iglesia, R., Poulain, J., Gaboyer, F., 2012. Metagenomes of the picoalga *Bathycoccus* from the Chile coastal upwelling. *PLoS One* 7, e39648. <https://doi.org/10.1371/journal.pone.0039648>.
- 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>.
- Wainger, L.A., Secor, D.H., Gurbisz, C., Kemp, W.M., Glibert, P.M., Houde, E.D., Barber, M.C., 2017. Resilience indicators support valuation of estuarine ecosystem restoration under climate change. *Ecosyst. Health Sustain* 3 (4), e01268. <https://doi.org/10.1002/ehs2.1268>.
- Wang, Y.J., Liu, D.Y., Dong, Z.J., Di, B.P., Shen, X.H., 2012. Temporal and spatial distributions of nutrients under the influence of human activities in Sishili Bay, northern Yellow Sea of China. *Mar. Pollut. Bull.* 64, 2708–2719. <https://doi.org/10.1016/j.marpolbul.2012.09.024>.
- Weng, H.-X., Qin, Y.-C., Sun, X.-W., Chen, X.-H., Chen, J.-F., 2009. Effects of light intensity on the growth of *Cryptomonas* sp (Cryptophyceae). *Environ. Geol.* 57, 9e15. <https://doi.org/10.1007/s00254-008-1277-1>.
- Wetz, M.S., Hutchinson, E.A., Lunetta, R.S., Paerl, H.W., Taylor, J.C., 2011. Severe droughts reduce estuarine primary productivity with cascading effects on higher trophic levels. *Limnol. Oceanogr.* 56, 627–638. <https://doi.org/10.4319/lo.2011.56.2.0627>.
- Wu, M., Wang, Y., Wang, Y., Yin, J., Dong, J., Jiang, Z., Sun, F., 2017a. Scenarios of nutrient alterations and responses of phytoplankton in a changing Daya Bay, South China Sea. *J. Mar. Syst.* 165, 1–12. <https://doi.org/10.1016/j.jmarsys.2016.09.004>.
- Wu, W., Wang, L., Liao, Y., Xu, S., Huang, B., 2017b. Spatial and seasonal distributions of photosynthetic picocaryotes along an estuary to basin transect in the northern South China Sea. *J. Plankton Res.* 39 (3), 423–435.
- Yamamichi, M., Kazama, T., Tokita, K., Katano, I., Doi, H., 2018. A shady phytoplankton paradox: when phytoplankton increases under low light. *Proc. Royal Soc. B* 285, 20181067. <https://doi.org/10.1098/rspb.2018.1067>.
- Yoshiyama, K., Sharp, J.H., 2006. Phytoplankton response to nutrient enrichment in an urbanized estuary: apparent inhibition of primary production by overeutrophication. *Limnol. Oceanogr.* 51, 424–434. [https://doi.org/10.4319/lo.2006.51.1\\_part\\_2.0424](https://doi.org/10.4319/lo.2006.51.1_part_2.0424).
- Yuan, H.M., Song, J.M., Xing, J.W., Li, X.G., Li, N., Duan, L.Q., Qu, B.X., Wang, Q.D., 2018. Spatial and seasonal variations, partitioning and fluxes of dissolved and particulate nutrients in Jiaozhou Bay. *Cont. Shelf Res.* 171, 140–149. <https://doi.org/10.1016/j.csr.2018.11.004>.
- 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>.