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Immediate effect of sewerage improvement on the phytoplankton and physicochemical conditions in the Urdaibai estuary (southeastern Bay of Biscay)

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ABSTRACT

Anthropic nutrient enrichment has become a major environmental issue in estuaries around the world, being the effluents of wastewater treatment plants (WWTPs) one of the main causes. Phytoplankton is, together with nutrients, a useful indicator for the ecological status assessment of estuaries due to its basal position in the food chain. The present study aims to evaluate the immediate effect of the cessation of the wastewater discharges on the environmental conditions and phytoplankton community along the eutrophicated Urdaibai estuary (southeastern Bay of Biscay). Thus, a short-term comparison of the physicochemical conditions and the phytoplankton abundance and community composition (by pigment analysis and microscopy) was carried out before and after the sewerage works. Results confirmed that the cessation of wastewater discharges had an immediate effect in the estuary, mainly noticed in the inner part. The abrupt decrease of ammonium (from 72.3 to 18.2 μmol/L) and phosphate (from 3.5 to 2 μmol/L) concentrations led to the improvement of the water status, reaching values lower than the "good" status threshold along the whole estuary. The phytoplankton community composition also showed and immediate reaction in the surroundings of the WWTP, with the chlorophyll *b* becoming the dominant secondary pigment in the area after the diversion due to the immediate and rapid increase of *Eutreptiella* sp. However, since the sampling-period of this study did not exceed a month after the cessation, a longer study-period is necessary, including interannual studies, to test the real effect of the sanitation works in the Urdaibai estuary.

1. Introduction

Estuaries are partially enclosed coastal water bodies, which receive discharges from one or more rivers and are open to the sea, becoming a transition zone between riverine and marine environments (Zambrano-Monserrate and Ruano, 2021). These systems have always been attractive for humans due to the ecosystem services they provide, such as the supply of water and fish, protection against floods, receipt of residual waters, opportunity for transport, and cultural and recreational services (Beaumont et al., 2007). Consequently, large urban areas have developed near estuaries around the world (Boerema and Meire, 2017), and the resulting high population density and anthropic activities pose a strong pressure for these systems (Rodrigues et al., 2021).

Lately, anthropic nutrient enrichment has become a major environmental issue in estuaries around the world and, in many urbanized estuaries, wastewater treatment plants (WWTPs) generate point discharges of phosphorus and nitrogen (Silva et al., 2016). Therefore, although WWTPs are important structures for mitigating the impacts of human settlements (Cloern et al., 2016) by reducing pollutant concentration and avoiding the direct discharge of wastewater into rivers (Pascual-Benito et al., 2020), depending on the characteristics of the WWTPs' design, the effluents can cause very negative impacts.

High nutrient concentrations in estuaries often leads to eutrophication (Eccles et al., 2020) and to its negative biological effects, such as the increase of phytoplankton abundance and harmful algal blooms; since these organisms are known to increase their growth rates with the increase of essential nutrients (N, C, P, O, Fe, Si) concentrations (Wells et al., 2015; Vajravelu et al., 2018). This increase in production and the accumulation

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of organic matter can lead to hypoxic conditions, together with loss of biodiversity and changes in community structure of the systems (Scherner et al., 2013).

In this context, nutrient concentrations and phytoplankton are some of the most used indicators for the assessment of the ecological status of surface waters like estuaries (e.g. within the European Water Framework or Marine Strategy Directives) (Seoane et al., 2011). In the case of nutrients, its monitoring enables the control of the short-term direct effect that the discharges are having in the physico-chemical properties of the estuary, since WWTP effluents influence receiving waters not only in the immediate vicinity, but also at distances that extend beyond local discharge areas (Poikane et al., 2019). Meanwhile, phytoplankton, due to its basal position in the food chain, is considered the link between inorganic nutrients and the rest of the trophic levels (Seoane et al., 2011) and is known to be the first autotrophic community showing response to variations in nutrient availability (Paerl et al., 2003). Therefore, nutrient and phytoplankton monitoring are useful to assess the immediate impacts of anthropogenic changes and/or the effectiveness of management strategies to mitigate these adverse changes (Li et al., 2018; Eccles et al., 2020).

Phytoplankton monitoring has been traditionally carried out by optical microscopy identification and enumeration (e.g., Córdoba-Mena et al., 2020). However, this method is time-consuming (Wang et al., 2018) and requires extensive taxonomic knowledge (Naik et al., 2011), becoming highly dependent on personal skills (Muñiz et al., 2020). Lately, pigment analysis based on high-performance liquid chromatography (HPLC) is becoming an alternative for phytoplankton monitoring (e.g. Chai et al., 2016; Damar et al., 2020). This technique allows an easy and fast separation and identification of over 50 phytoplankton pigments (Zapata et al., 2000), based on a larger volume analyzed compared to microscopy (Agirbas et al., 2015) and, using them as biomarkers, enables the assessment of the phytoplankton community structure (by diagnostic pigments) and quantification of total biomass (using chlorophyll *a* as a proxy) (Chai et al., 2016; Nunes et al., 2018). Nevertheless, this technique has some limitations, like the presence of common pigments for some groups, limiting their discriminant capacity, and the fact that identification is only done at group level (Latasa, 2007; Nunes et al., 2018). Due to this, a combination of techniques (microscopy and pigment analysis) allows obtaining a more realistic image of the community (e.g., Dursun et al., 2020; Lee et al., 2020).

The Urdaibai estuary, also called Oka estuary, is located in the Basque coast (southeastern Bay of Biscay) and is part of the only Biosphere Reserve of the Basque Country, declared in 1984, due to its high naturalistic value and its interaction with socioeconomic activities (Castillo-Eguskitza et al., 2017). However, the estuary is under several significant anthropogenic pressures, making the balance between the economical, recreational and natural components difficult to reach (Monge-Ganuzas et al., 2017). Among these pressures, Gernika wastewater treatment plant (WWTP), located in the innermost area of the estuary, stands out. The WWTP has primary and secondary treatment, but does not allow the efficient removal of phosphorus and nitrogen, being these nutrients the main drivers of phytoplankton growth. As a result, since 1972, the direct discharges of wastewaters coming from Gernika WWTP to the estuary have represented the main source of pollution and environmental problems of the system. Consequently, some areas of the system do not fulfill the environmental objectives set by the WFD (Borja et al., 2021), with very high nutrient concentrations and eutrophication problems. During these years of wastewater discharges, studies on the physico-chemical properties and the human impact on the estuary have been performed by several authors (Iriarte et al., 2016; Castillo-Eguskitza et al., 2017), as well as studies of the phytoplankton community (e.g., Ansotegui et al., 2001; Trigueros and Orive, 2001). Nevertheless, sewerage works have been implemented in the area lately and, in the beginning of July 2021, the effluent of Gernika WWTP was diverted to the Lamiaran WWTP, which discharge the treated effluents in the coastal area in Bermeo. Therefore, nowadays, the nutrient input received during several decades from the WWTP of Gernika in the innermost part of the estuary has disappeared.

The present study aims to evaluate the immediate effect of the cessation of the wastewater discharges from Gernika WWTP on the water quality status and phytoplankton community along the Urdaibai estuary by comparing, in a short time period (weeks), the environmental conditions and the phytoplankton abundance and community composition before and after the diversion. The research hypothesis is that there will be an immediate decrease of nutrient concentrations and that the phytoplankton community will change as a response to this change in nutrients.

2. Material and methods

2.1. Study area

This study was carried out at the Urdaibai estuary, a meso-macrotidal system located in the Basque coast (North of Spain) that drains into the southeastern Bay of Biscay (43°22′N, 2°41′W). The climate of the area is temperate-oceanic, with warm summers and moderate winters, and it is influenced by the Gulf Stream and the atmospheric westerlies in the middle and upper troposphere (Usabiaga et al., 2004). The estuary is formed by the tidal part of the Oka river and it is a short (12.5 km long) and shallow system (Villate et al., 2017). The contribution of freshwater to the system is small compared to the total volume of the estuary and the river discharge is usually low in relation to the tidal prism (Villate et al., 2017). As a result, most of the estuary is marine-dominated at high tide and tidal cycles have a great impact on the system (Valencia et al., 2004). The water stratification varies through the estuary, with a well-mixed water column in the outer area and stratified conditions in the middle and inner zones (Barroeta et al., 2020). Nevertheless, due to the geomorphology of the system and the torrential nature of the Oka river, showing occasional large flows that change the typical pattern, the volume and flushing rates of the estuary are highly variable (Madariaga, 1995). This has a direct influence on the physico-chemical and biological properties of the estuary, promoting changes in the phytoplankton community composition and abundance on a short time scale (Madariaga, 1995) as well as the proliferation of particulate matter with high inorganic matter proportions (Ruiz et al., 1998).

Based on morphology, three main areas can be identified in the estuary (Fig. 1). The outer estuary, which extends from the outer limit of the system (Mundaka) to Murueta, is a zone with extensive intertidal flats and sandy beaches. The middle area, from Murueta to the start of the artificial channel, is characterized by a significant narrowing when comparing to the lower estuary and consist of a central channel bordered by shalt-marshes and a complex system of interlaced secondary channels. Finally, the inner estuary is formed by a 4 km long and 15 m wide artificial channel that reaches the town of Gernika and is bordered by reed beds.

2.2. Sampling and data acquisition

Six samplings were conducted in 2021 in order to compare the situation before (2 sampling campaigns) and after (4 sampling campaigns) the diversion of Gernika WWTP effluent outside the Urdaibai estuary. The first sampling day was June 30th and

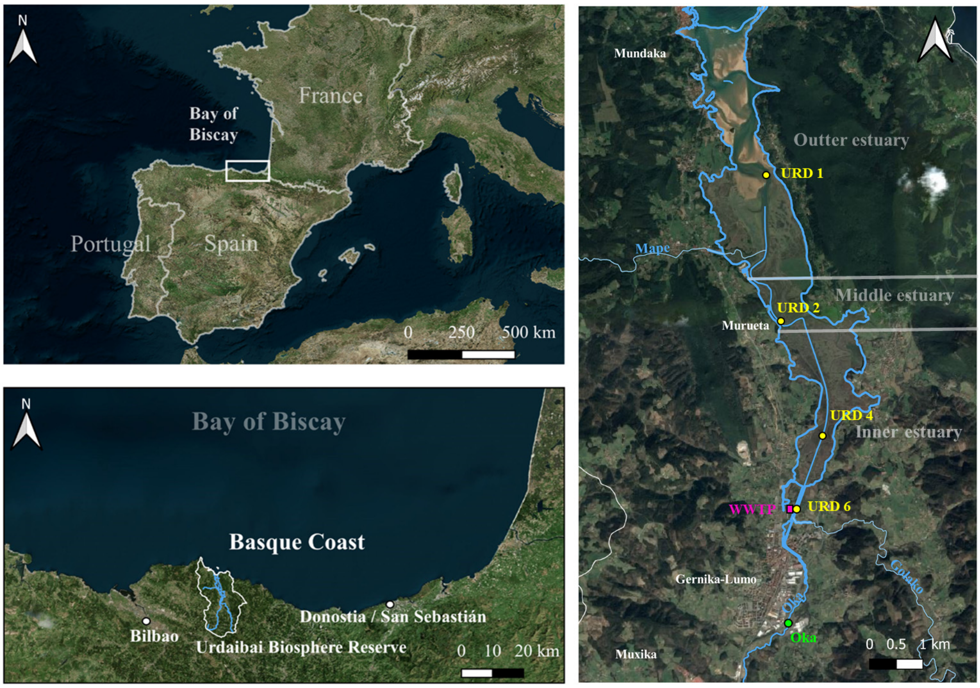

Fig. 1. Study area and sampling stations. On the left, location of the Urdaibai estuary and the Urdaibai Biosphere Reserve in the Bay of Biscay and the Basque Coast. On the right, the Urdaibai estuary divided in the outer estuary (sampling station URD1), middle estuary (station URD2) and inner estuary (stations URD4 and URD6). The discharge point of the Wastewater Treatment Plant (WWTP) and the sampling station at Oka River (Oka) are also located on the map.

the second on July 6th, the day that the wastewater discharges stopped definitively. From June 30th until the 6th of July intermittent cuts of the effluent were done as part of the final works. After this, 4 more sampling were done within a month period, with time distances of 48 h, 1 week, 2 weeks and 1 month since the diversion. Samplings were conducted always at high tide.

Samples were collected at four permanent sampling sites along the longitudinal axis of the estuary (Fig. 1) in order to cover the entire salinity gradient: one in the outer estuary (URD1), one in the middle estuary (URD2) and 2 in the inner estuary (URD4, and URD6). The last sampling point (URD6) was located in front of the WWTP of Gernika, where the discharges were happening. In addition, one more sample was collected in the Oka River, with the aim of monitoring the physicochemical properties of the river right before receiving the discharges of the WWTP. According to the average surface salinities, URD1 is considered euhaline, URD2 polyhaline and URD4 and URD6 mesohaline (unpublished data).

At each sampling site, several physico-chemial parameters (salinity, temperature, conductivity, pH, dissolved oxygen, oxygen saturation and turbidity) were measured *in situ* using the multi-parameter water quality meter EXO2 (YSI). In addition, the water column depth was registered with the Plastimo Echotech II sounder and the Secchi disk depth was measured as an estimation of water transparency. As for the water sample collection, a 2.5 L plastic Niskin bottle was used to collect water from 0.75 m below the surface. The collected water was used for the analysis of inorganic nutrients and phytoplankton abundance and composition.

The dissolved inorganic nutrients analyzed were ammonium, nitrite, nitrate (calculated from the total oxidized nitrogen), orthophosphate and silicate. The nutrient analysis was carried out at the Chemical Laboratory of the Marine Research Unit of the AZTI Foundation in Pasaia (Gipuzkoa). The technique used for the determination was UV/VIS colorimetry, with an automatic 5-channel analyzer with segmented flow (BRAN-LUEBBE, AXFLOW). The individual determinations of each of the nutrients were based on methods that apply classical and widely used colorimetric reactions (GO-SHIP manual by Hydes et al., 2010). The quantification limit for ammonium, nitrate and silicate was 1.6 μmol/L, for nitrate 0.4 μmol/L and for phosphate 0.16 μmol/L. When concentration values were below the quantification limit, a concentration equal to 50% of the limit was assumed for statistical analysis and graphical representations.

The study of the phytoplankton community was carried out based on HPLC pigment analysis, together with the observation of the sample by optical microscopy for the identification of the dominant taxa.

Water samples for pigment analysis were stored in opaque plastic bottles and filtered (0.4–4 L) on the same sampling day. Filtration was carried out in dark conditions, with gentle vacuum ($<$150 mm Hg), onto Whatman GF/F glass-fiber filters (47 mm diameter, Whatman International Ltd.). Immediately after filtration, filters were frozen in liquid nitrogen and stored until extraction (never more than a month after filtration) at −80 °C. Pigment extraction and analysis was carried following Zapata et al. (2000), with the equipment described in Seoane et al. (2009). Extraction was done under low light, in screw-capped tubes with 5 ml of 90% acetone, and the extracts were filtered through syringe filters (Millex, 0.22 μm pore size) to remove cell and filter debris. The pigment analysis was performed with HPLC, identifying pigments depending on their absorbance spectra and retention times (Jeffrey et al., 1997; Zapata et al., 2000). HPLC calibration was performed using chlorophyll (*a*, *b*, and *c*2) and carotenoids (fucoxanthin, 19′hexanoiloxyfucoxanthin (HFU), lutein, zeaxanthin, peridinin, violaxanthin, alloxanthin and β carotene) standards obtained commercially from DHI. For the quantification of pigments that were not calibrated with commercial standards, the molar extinction coefficients obtained from Jeffrey et al. (1997) were applied.

Water samples for the identification of the dominant phytoplankton taxa by microscopy were fixed with acidic Lugol's solution (0.4% v/v) right after collection, and stored in 125 ml topaz borosilicate bottles in dark and cool (4 °C) conditions until analysis. Taxonomic identification was based on the Utermöhl

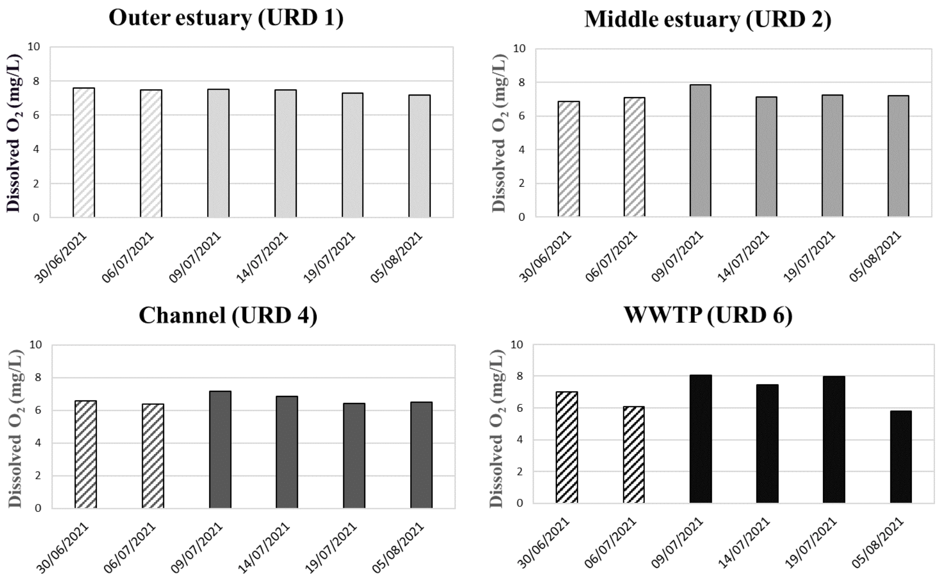

Fig. 2. Dissolved oxygen concentration in the different sampling stations along the Urdaibai estuary. Barred columns represent samplings with wastewater discharges to the estuary; filled columns correspond to samplings after the diversion of the wastewaters outside the estuary.

sedimentation method (Edler and Elbrächter, 2010) and was performed under a Nikon diaphot TMD inverted microscope, for subsamples of 10 or 50 ml, depending on the density of the samples. The whole chamber was analyzed at low magnifications (100× and 200×) to identify larger cells and, for smaller cells, transects at 400× magnification were carried out. Generally, the most dominant taxa were identified to genus or even species level, standardizing nomenclature according to AlgaeBase (Guiry and Guiry, 2018).

2.3. Data analysis

The main statistical parameters (range, median, and/or arithmetic mean) were calculated and graphical representations for physicochemical variables, phytoplankton total biomass and community composition for each sampling point were performed; all this was performed with Windows Excel 2016.

The Mann–Whitney U test was used to determine whether there were significant differences (alpha: 0.05) in the median values of the studied parameters before and after the diversion of the wastewaters outside the estuary. It is a non-parametric test for independent samples and does not assume normal distribution. In order to make the results more reliable, additional data was used for this test, adding two samplings from the 17th and 29th of July 2020 (Bilbao et al. unpublished data; Supplementary Material 1, Table 3) to the "before" situation. This data was chosen because they represented the same time-period to the ones appearing in the "after section" (July 2021) and no significant alterations were made in the estuary, except for the diversion of the wastewaters, so they should be comparable. The test was performed independently for each sampling station. The statistical analysis was carried out using PAST 4.05 (Paleontological STatistics), a software package for data analysis (Hammer et al., 2001).

3. Results

Besides salinity and temperature, which are considered essential oceanographic variables to characterize the water masses in estuaries, in this section the parameters more likely to be affected by the cessation of the discharges from the WWTP into the estuary will be described and represented graphically. The rest of the parameters analyzed can be found in the Supplementary Material 1 (Table 1 and 2).

In addition, the results from the 6 sampling campaigns carried out are divided into two groups: samplings before the wastewater diversion (samplings from the 30/06/2021 and 06/07/2021) and samplings after the diversion (sampling from the 09/07/2021, 14/07/2021, 19/07/2021 and 05/08/2021).

3.1. Environmental conditions

Salinity showed a clear zonation in the Urdaibai estuary, with a decreasing gradient from outer to inner estuary. The highest (average of 34.1) and most stable values (ranging 33.8–34.3) were observed in the outer station (URD1). Middle estuary (URD2) showed an average salinity of 28.3 during the study period, ranging from 26.5 to 30. The inner estuary showed average salinity values of 18.5 and 11.5 in URD4 and URD6, respectively. In addition, it was the most variable area when it comes to salinity, with values ranging from 16.6 to 20.9 in URD4 and from 9.3 to 14.1 in URD6.

The average temperature recorded in the estuary was 21.6 °C, with values ranging from 18.3 to 25.3 °C during the study period. Besides, the average temperature increased from the beginning of the study (18.5 °C on June 30th) to the end (24 °C on August 5th). As for the Oka river, temperature was lower than in the estuary (ranging from 16.8 to 19.3 °C), with an average value of 18.1 °C.

Dissolved oxygen concentration was one of the environmental variables on which an immediate change was expected after the diversion of the wastewater discharges (Fig. 2). In the outer estuary (URD1), oxygen concentration was stable, with values between 7.2 and 7.6 mg/L. However, values decreased and became more variable towards the inner estuary, with an average value of 7.2 mg/L (6.9–7.9 mg/L) in middle estuary (URD2), 6.7 mg/L (6.4–7.2 mg/L) in the channel (URD4) and 7.1 mg/L (5.8–8.1 mg/L) in the surroundings of the WWTP (URD6). As for the changes resulting from the sanitation works, middle and inner estuary recorded higher oxygen concentration values after the cessation of the wastewater discharges. This was especially remarkable in the middle estuary, where the Mann–Whitney test determined that the increase of dissolved oxygen concentration after the cessation of wastewater discharges was significant (p value of 0.03).

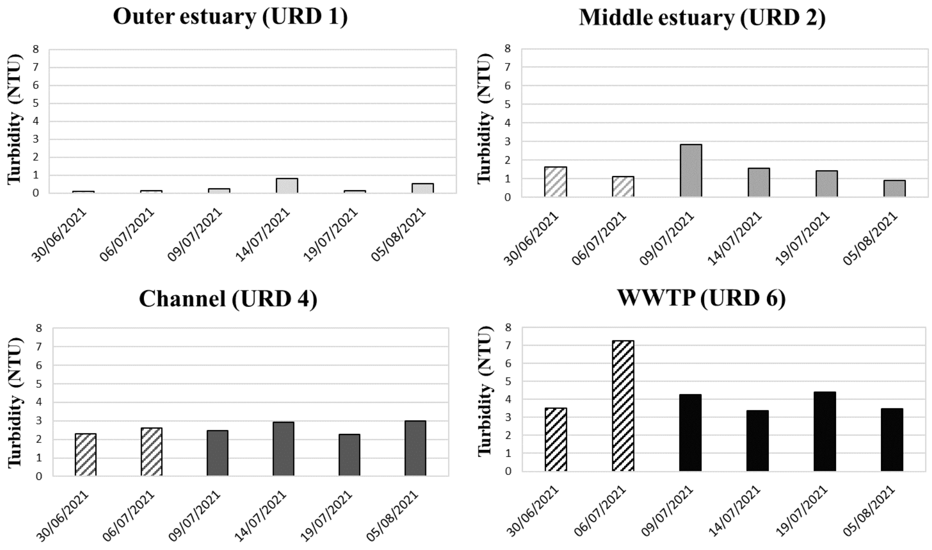

Fig. 3. Turbidity values in the different sampling stations along the Urdaibai estuary. Barred columns represent samplings with wastewater discharges to the estuary; filled columns correspond to samplings after the diversion of the wastewaters outside the estuary.

Turbidity, like salinity and oxygen, also showed the clear zonation of the Urdaibai estuary, with an increasing turbidity gradient towards the inner estuary (Fig. 3). Average turbidity in URD1 during the study period was 0.3 NTU and increased to an average of 4.4 NTU in the surroundings of the WWTP. Regarding the immediate change of turbidity after the sanitation works, only the surroundings of the WWTP showed a rapid decrease after the cessation of the wastewater discharges, with a change in average turbidity from 5.4 NTU (before) to 3.9 NTU (after) and values always lower than 4.5 NTU after the diversion. However, according to the Mann–Whitney test, these changes were no significant. The median turbidity in the Oka river was 1.6 NTU.

3.2. Inorganic nutrient concentrations

Inorganic nutrient concentration showed the expected marked gradient along the estuary, with much higher concentrations in the innermost area. In addition, some of them showed an immediate decrease after the diversion of the wastewaters outside the estuary, being this effect mostly noticed in the surroundings of the WWTP.

Ammonium was a clear example of the patterns mentioned above (Fig. 4). The average ammonium concentration in the outer estuary (URD1) during the study was 2.3 μmol/L and increased to an average concentration of 36.2 μmol/L in the surroundings of the WWTP, where it reached a maximum of 89.6 μmol/L. The average ammonium concentration in the Oka river was 3.3 μmol/L, much lower than in the surroundings of the WWTP, reflecting the main anthropogenic source of this nutrient in this system. As for the immediate change caused by the cessation of the wastewater discharges to the estuary, a rapid and significant (p values of 0.03) decrease was clearly seen in middle and inner estuary. In the middle estuary (URD2), average ammonium concentrations decreased from 14.8 μmol/L (before) to 4.3 μmol/L (after); in the channel (URD4), the change was from 47 μmol/L to 16.8 μmol/L; and, in the surroundings of the WWTP (URD6), from 72.3 μmol/L to 18.2 μmol/L.

Phosphate concentrations showed similar patterns to ammonium, but immediate changes were not so drastic (Fig. 5). Phosphate appeared in much lower concentrations than ammonium, with an average concentration of 0.1 μmol/L in the outer estuary, increasing to an average of 2.5 μmol/L in the surroundings of the WWTP (reaching a maximum of 3.8 μmol/L). In addition, as it happens with ammonium, the average concentration of this nutrient in the Oka river (0.7 μmol/L) is lower than in the surroundings of the WWTP. The immediate effect of the diversion of the wastewater discharges was mostly noticed in the inner estuary, with decreases from 1.9 μmol/L (before) to 1.2 μmol/L (after) in the channel area (URD4), and from 3.5 μmol/L to 2 μmol/L in the surroundings of the WWTP (URD6). The Mann–Whitney test determined that this decrease of phosphate concentration after the cessation of wastewater discharges was significant (p values of 0.03) in URD4 and URD6.

Nitrate and silicate had a different behavior in the estuary than ammonium and phosphate (Supplementary Material 1). As for nitrate, it also increased towards the inner estuary, with concentration values below quantification limit in the outer estuary and an average concentration of 20 μmol/L in the innermost station (URD6). However, on the contrary to the previously described nutrients, the average concentration of nitrate was higher in the Oka river (around 45 μmol/L) than in the surroundings of the WWTP, reflecting its main riverine origin. There was a general decrease of nitrate concentration after the diversion in the middle and inner estuary. The average concentration of nitrate decreased from 8.1 μmol/L (before) to 2.7 μmol/L (after) in middle estuary (URD2), and from 23.4 μmol/L to 18.5 μmol/L in the innermost station (URD6). Silicate showed a similar distribution to nitrate along the estuary, but appeared in higher concentrations. Average silicate concentrations increased from outer estuary (1.3 μmol/L) to the innermost estuary (76.6 μmol/L), but the highest average value was recorded in the Oka river (94.2 μmol/L), reflecting, as for nitrate, its terrestrial origin. As for the effect of the wastewater diversion, no clear immediate changes were detected in silicate concentration. According to the Mann–Whitney test, there were no significant changes in nitrate and silicate concentrations after the diversion, as expected due to their riverine origin.

3.3. Phytoplankton abundance and community composition

Total phytoplankton biomass was estimated with chlorophyll *a* concentration (Fig. 6). A general increase of biomass was observed towards the inner estuary, since average chl *a* values were 1.9 μg/L, 7.2 μg/L, 9.3 μg/L and 26.6 μg/L in URD1, URD2, URD4 and URD6 respectively. In addition, the chl *a* maximum (55.9 μg/L) recorded during the study was also found in the surroundings of the WWTP. Regarding changes in phytoplankton

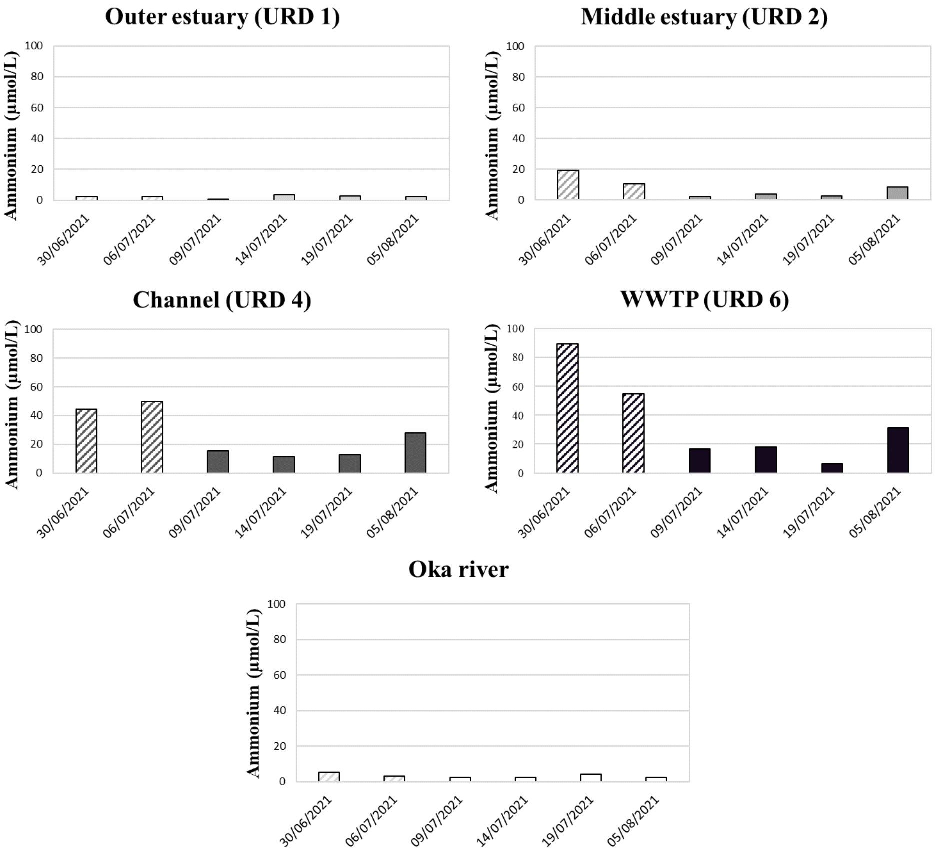

Fig. 4. Ammonium concentration in the different sampling stations. Barred columns represent samplings with wastewater discharges to the estuary; filled columns correspond to samplings after the diversion of the wastewaters outside the estuary.

biomass after the sanitation works in the estuary, large biomass changes were detected but they did not follow a specific pattern in relation to the wastewater diversion, with very high spatial (stations) and temporal (campaigns) variability.

In relation to the community composition, different phytoplankton communities were found along the longitudinal axis of the estuary, and immediate changes were detected in several areas after the diversion of the wastewaters (Fig. 7).

In the outer estuary, during the wastewater discharges from the WWTP, the dominant pigment was fucoxanthin, followed by chlorophyll *b* (chl *b*) and 19′hexanoyloxyfucoxanthin (HFU). The diatoms *Minutocellus polymorphus* and *Chaetoceros salsugineus* were the dominant taxa in the community, together with the important presence of the chlorophyte *Tetraselmis* spp., and the haptophytes *Emiliania huxleyi* and Prymnesiales. This community composition did not show big immediate changes after the diversion of the wastewaters outside the estuary. The only differences detected were a slight increase in the importance of the chl *b*, the higher presence of *Eutreptiella* sp. and the appearance of *Chamydomonas* sp. and *Pyramimonas* sp.

Middle estuary showed almost the same community composition as the outer estuary and there were not rapid significant changes detected in this area after the sanitation works. The community was dominated by fucoxanthin during the whole study period and Chl *b* was the second most abundant secondary pigment, increasing its importance after the stop of the wastewater discharges. In addition, alloxanthin gained importance in this area, replacing HFU. The dominant taxa of the community was *Chaetoceros salsugineus* and *Tetraselmis* spp. became more important in the area after the sanitation works. Besides, the presence of cryptophytes like *Plagioselmis* spp. and *Teleaulax* spp. was higher than in URD1.

The inner estuary, on the contrary to the outer and middle areas, showed a rapid and marked change after the diversion of the wastewaters in the phytoplankton community of both the channel area and the surroundings of the WWTP. On the one hand, in the channel area, during the wastewater discharges, alloxanthin was the most abundant secondary pigment, but, immediately after the diversion, the importance of alloxanthin decreased and chl *b* increased. This change was also reflected in the dominant taxa of the community. Before the diversion, there was an important presence of not only *Plagioselmis* spp. and *Teleaulax* spp., but also *Urgorri complanatus.* However, after the sanitation works, the presence of green algae like *Tetraselmis* spp. and *Eutreptiella* sp. increased, and *Urgorri complanatus* disappeared.

As for the surroundings of the WWTP, during the wastewater discharges there was a shared dominance between fucoxanthin and alloxanthin. Nevertheless, with the stop of the discharges, there was an abrupt increase of chl *b,* becoming the dominant secondary pigment in the area. This change of dominances was also seen in the taxonomic composition of the community, since the dinoflagellate *Kryptoperidinium foliaceum* and cryptophytes (especially *Teleaulax* spp. and *Urgorri complanatus*) were playing an important role in the phytoplankton community in this area until the cessation of the discharges, but there was an immediate and rapid growth of *Eutreptiella* sp., becoming the dominant taxon after the diversion.

4. Discussion

In marine systems, disentangling the combined effects of natural variability and anthropogenic pressures and/or the management actions is a difficult task (e.g., Elliott and Quintino, 2007).

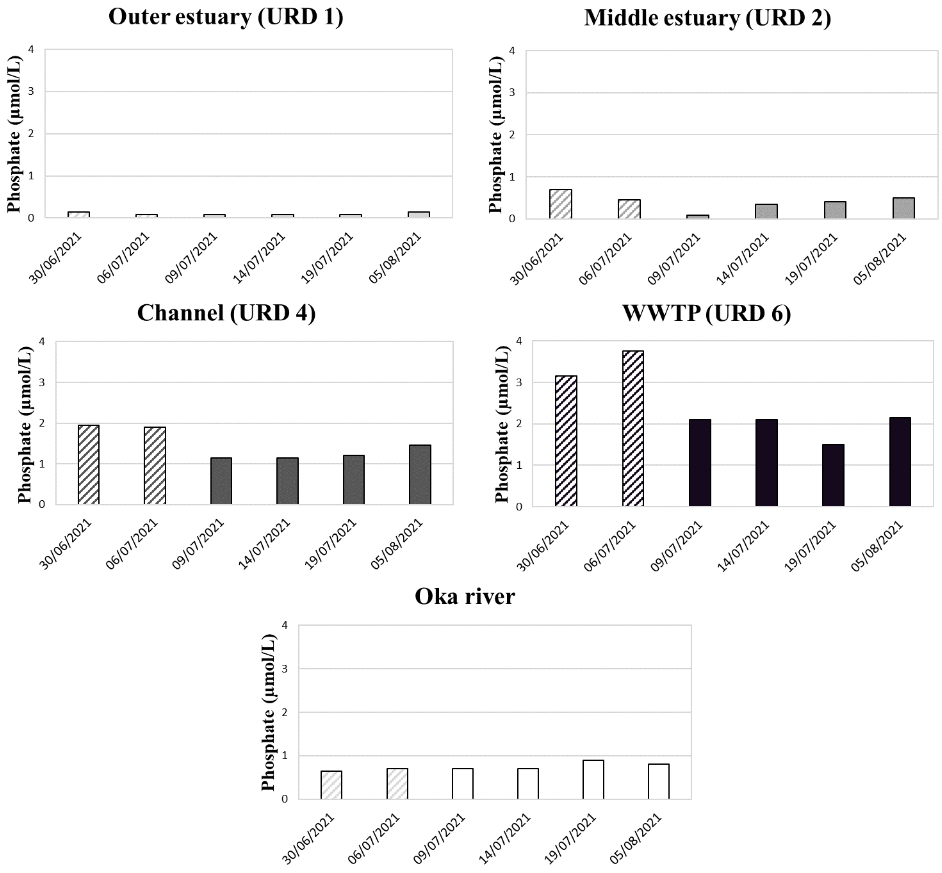

Fig. 5. Phosphate concentration in the different sampling stations. Barred columns represent samplings with wastewater discharges to the estuary; filled columns correspond to samplings after the diversion of the wastewaters outside the estuary.

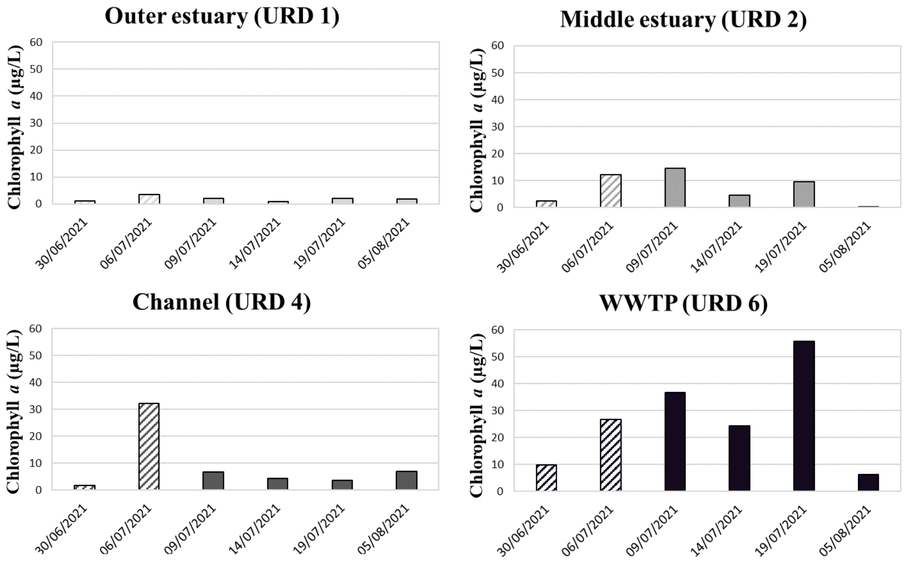

Fig. 6. Chlorophyll *a* concentration in the different sampling stations. Barred columns represent samplings with wastewater discharges to the estuary; filled columns correspond to samplings after the diversion of the wastewaters outside the estuary.

In the present study, the immediate effects of a direct management action (wastewater diversion) on physico-chemical variables and phytoplankton communities were studied and some rapid changes have been observed.

Turbidity in estuaries depends on several factors, such as the riverine input, the tidal effect and, in many systems, like the Urdaibai estuary, by wastewater effluents (Toublanc et al., 2016; Eccles et al., 2020). This dependence from several factors makes difficult to isolate the effect that the WWTP has on water turbidity. Nevertheless, since the turbidity of the Oka river was also analyzed and it did not show any anomalies during this study, it can be confirmed that sanitation works might be the main reason for the slight decrease in turbidity registered in the surroundings of the WWTP (station URD6). WWTP effluents often contain high

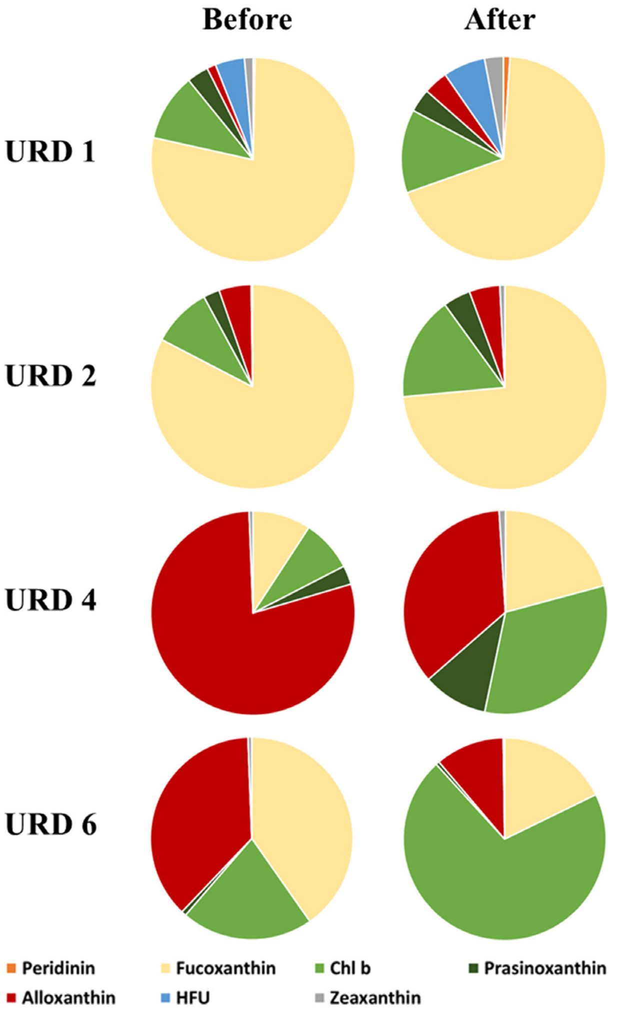

Fig. 7. Marker pigment proportions (average values) in the different sampling stations, before (left) and after (right) the diversion of the wastewaters outside the Urdaibai estuary.

concentrations of particulate matter and this increases water turbidity in the receiving waters (Carey and Migliaccio, 2009). Regarding the oxygen, the reduction of the biological oxygen demand should increase the oxygen concentration (García-Barcina et al., 2006), which seems to be confirmed by the significant increase of dissolved oxygen concentration registered in the middle Urdaibai estuary after the wastewater diversion.

As for the inorganic nutrient concentrations, the WWTP effluents have been degrading the receiving waters by increasing their nitrogen and phosphorus concentration, affecting water masses quite beyond local discharge areas (Carey and Migliaccio, 2009). The present study confirmed that the main source of ammonium and phosphate to the Urdaibai estuary was the WWTP of Gernika, as previously stated by several authors (e.g. Revilla et al., 2000). Nitrate and silicate, on the contrary, registered their maxima in the river, reflecting its diffuse origin from land drainage and rock weathering, respectively (Turner et al., 2003; Valencia and Franco, 2004). Consequently, the greatest immediate changes after the sanitation works in the Urdaibai estuary were recorded in the nutrients of anthropogenic origin, ammonium and phosphate, with notable reductions in the middle and inner estuary, of more than 50%. In addition, part of the decrease of nitrate concentration registered in middle and inner estuary might be related to the decrease of ammonium as well, since nitrate is often formed in estuaries by the oxidation of ammonium (nitrification) (Dai et al., 2008; Damashek et al., 2016).

Water quality and ecological status assessment of coastal and transitional waters of the Basque Country are determined by the criteria and thresholds established in the Royal Decree 817/2015 for the Eastern Cantabrian Hydrographic Demarcation. The results obtained in the monitoring network of the transitional and coastal waters of the Basque Country showed that the inner Urdaibai estuary has failed to meet the environmental objectives at least since 2008, registering "deficient" or "bad" ecological status until 2020 (e.g., Borja et al., 2021). This is caused, among others, by the non-compliance of the physicochemical indicators, like ammonium and phosphate, which have systematically failed to meet the objectives, consequently leading to the non-compliance of the biological indicators like phytoplankton (Borja et al., 2021). However, sanitation works might have improve the water quality in the Urdaibai estuary, as far as physicochemical indicators are concerned.

In the present study, based on the thresholds established in the Royal Decree 817/2015 for the Basque Country (Supplementary Material 2), before the cessation, ammonium and phosphate concentrations recorded values above the "moderate" status threshold (which is established as the minimum water quality objective) in the middle and inner estuary. However, the significant decrease registered after the cessation led to concentrations lower than those considered as "good" or even "very good" status threshold. In addition, the increase of oxygen saturation (a parameter used for evaluating the ecological status, data available in Supplementary Material 1) after the cessation led to reach values above the "very good" status threshold along the whole estuary, while the values during the discharges used to be between the "good" and "very good" status limits. Therefore, physicochemical indicators recorded values better than those taken as "good" status threshold, at least, in the whole estuary after the cessation of the wastewater discharges.

In relation to phytoplankton, one of the objectives of this work was to see if phytoplankton abundance showed an immediate response to the cessation of the wastewater discharges. Phytoplankton biomass in estuaries is highly variable over temporal and spatial scales, mainly regulated by light availability, nutrient supply and physical processes (Masotti et al., 2018). In the middle Urdaibai estuary, a slight chl *a* decrease was recorded after the stop on the wastewater discharges, in accordance with several studies that reported positive relationship between the reduction of nutrient inputs and the decrease of phytoplankton biomass (e.g., Wetz et al., 2011). However, in the surroundings of the WWTP an increase in biomass was observed, which might seem contradictory to the decrease in nutrient concentration, but might respond to the higher light availability due to the decrease of turbidity in the area (Karlsson et al., 2009; Yamamichi et al., 2018).

In changing environments, phytoplankton community composition shifts due to the differences in the competitive ability of the different taxa (Cardinale et al., 2011). The outer and middle Urdaibai estuary did not record significant changes in the physicochemical conditions after the sanitation works, probably due to the distance from the WWTP, and the community composition remained unaltered. However, the inner estuary showed rapid and abrupt changes in the phytoplankton community composition that were probably triggered by the changes in nutrient concentration and light availability.

Before the diversion of the wastewater discharges outside the Urdaibai estuary, in the inner area, and especially in the channeled area (URD4), the most abundant secondary pigment was alloxanthin. This was explained by the dominance of cryptophytes in this area, especially *Teleaulax* spp. and *Urgorri complanatus*. The tolerance of cryptophytes to low light intensity and the ability to reach high abundances in point source discharge areas makes them the dominant group in many aquatic systems with high anthropogenic pressures (e.g., Paches et al., 2019). Indeed, this ability of growing in turbid waters with high organic matter concentrations (Bergmann, 2004; Adolf et al., 2008) explains its high presence in eutrophic estuaries others than Urdaibai, such as the Chesapeake, Neuse or Galveston (Paerl et al., 2003; Adolf et al., 2008; Valdes-Weaver et al., 2006). As for the surroundings of the WWTP, before the sanitation works, the phytoplankton community was composed by a shared dominance between cryptophytes and the fucoxanthin containing dinoflagellate *Kryptoperidinium foliaceum*. Previous studies also reported the importance of *K. foliaceum* in the innermost area of the Urdaibai estuary (e.g. Ansotegui et al., 2001; Trigueros and Orive, 2001), and other adjacent areas like the Nervion River estuary (Seoane et al., 2005) or the Guadiana estuary in the south of Spain (Domingues et al., 2011). In addition, *K. foliaceum* is known for responding to nitrogen enrichment in the absence of silica, which makes them good indicators of anthropogenic nutrient enrichment, since in this cases nitrogen and phosphorus concentration increase but silica does not (Domingues et al., 2011). This could explain their high presence during the wastewater discharges to the inner Urdaibai estuary.

The diversion of Gernika WWTP effluent outside the estuary provoked an immediate change in the phytoplankton community composition of the inner estuary, decreasing alloxanthin and fucoxanthin concentrations due to the lower presence of cryptophytes and the almost disappearance of *K. foliaceum*. The group that dominated the channeled area and the surroundings of the WWTP after the sanitation works was the euglenoids, due to a rapid and drastic growth of the genus *Eutreptiella,* leading to an increase of chl *b* concentration in the area.

Eutreptiella is a genus of photosinthetic euglenoid flagellates that are rather abundant in some coastal and brackish water areas (Henriksen et al., 2002). Euglenoids show a preference for small and shallow fertile water bodies, with poor light conditions (Poniewozik and Juráň, 2018). Therefore, the nutrient rich and shallow inner Urdaibai estuary might be the ideal site for *Eutreptiella spp*. growth. Several studies have previously reported *Eutreptiella spp*. blooms in nutrient-rich coastal and transitional waters, becoming the dominant taxa in these phytoplankton communities (e.g., Olli, 1996; Stonik, 2007), like it happened in the inner Urdaibai estuary. Besides, euglenophytes usually coexist with other flagellates belonging to cryptophytes and dinoflagellates (e.g., Jeong et al., 2021), which share the same features to survive in unfavorable conditions, such as fast reproduction, mixotrophy and tolerance to low light (Plachno et al., 2015). In addition, it is proved that Euglenoids are highly sensitive to changes in environmental conditions, and populations might grow or disappear very rapidly (Heckman et al., 1996), so the changes that occurred in the surroundings of the WWTP might have favored its rapid growth.

Therefore, the decrease of nutrient concentration and turbidity after the diversion was not enough for typical clean water species to appear, but it gave competitive advantage to the genus *Eutreptiella*, becoming the dominant of the community and replacing the cryptophytes and *K. foliaceum*. The monitoring of the changes in the next months and years will confirm if the observed change is the beginning of a change towards a community of less eutrophicated waters or only an instant change due to an abrupt modification in water conditions. Moreover, the resilience of the system will play an important role in the future phytoplankton community composition of the system.

5. Conclusion

From the present study it can be concluded that the diversion of the Gernika WWTP effluent, and the consequent cessation of wastewater discharges to the inner estuary had an immediate effect in some water properties in the Urdaibai estuary, mainly noticed in the innermost area. The biggest change, within the environmental conditions, was the abrupt significant decrease of ammonium and phosphate concentrations in middle and inner estuary, reaching concentrations lower than, at least, the "good" status threshold along the whole estuary. In addition, the community composition also changed after the diversion in the innermost estuary, showing how quick phytoplankton could respond to alterations in the environmental conditions. However, the sampling-period of this study did not exceed a month after the diversion, so the conclusions must be taken with caution. A longer period of time is necessary, including interannual studies, to test the real effect of the sanitation works in the Urdaibai estuary and the resilience of the system.

CRediT authorship contribution statement

Jone Bilbao: Conceptualization, Methodology, Data curation, Investigation, Visualization, Writing – original draft. **Joana Larreta:** Data curation, Writing – review & editing. **Javier Franco:** Conceptualization, Methodology, Writing – review & editing. **Sergio Seoane:** Conceptualization, Methodology, Funding acquisition, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.rsma.2022.102707.

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