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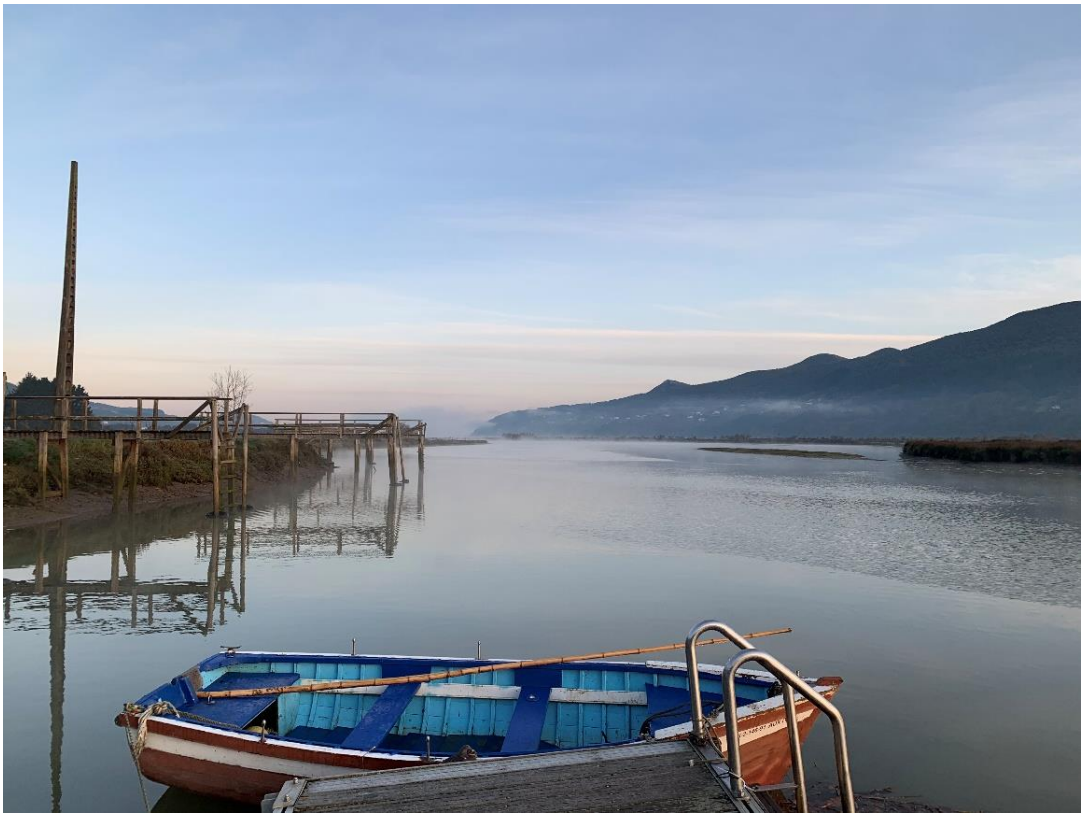

Jone Bilbao Antolin

2024

Characterisation of the phytoplankton community of the Urdaibai estuary: influence of anthropogenic nutrient loadings and analysis of monitoring techniques

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PhD Thesis

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Urdabai estuarioko fitoplankton-komunitatearen karakterizazioa: mantenugaien karga antropogenikoen eragina eta jarraipen tekniken analisia

Jone Bilbao Antolin

2024

Tesi Doktorala

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“The sun does not forget a village just because it is small”

African Proverb

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CONTENTS / EDUKIAK

LABURPENA

Fitoplanktona aztertzea ezinbestekoa da ekosistema urtarren funtzionamendu globala ulertzeko. Estuarioetan, fitoplankton-komunitateek denboran eta espazioan zeharreko aldakortasun handia jasaten dute, ingurumen-gradiente anitzen eraginpean baitaude. Gainera, mantenugaien gehiegizko aberaste antropogenikoa ingurumen-arazo garrantzitsu bihurtu da estuarioetan, bertako fitoplankton biomasari eta komunitatearen osaerari ere eraginez. Mantenugaiak eta fitoplanktona, estuarioen egoera ekologikoaren ebaluaziorako adierazle eraginkor eta oso erabiliak dira, eta presio antropogenikoen eraginen edo/eta kudeaketa-ekintzen eraginkortasuna ebaluatzeko ere erabili daitezke. Tradizionalki, fitoplanktonaren jarraipena egiteko mikroskopia erabili izan da, baina, azkenaldian, pigmentuen analisia edo DNA metabarcoding-a bezalako teknikak gero eta erabiliagoak dira.

Urdaibai estuarioak (Bizkaiko golkoko hego-ekialdea), Euskal Autonomia Erkidegoko (EAEko) Biosfera Erreserba bakarraren parte denak, Gernikako hondakin-uren araztegiko isurketak jaso izan ditu 1972-tik. Araztegiak ez zuen hondakin-uren nitrogenoaren eta fosforoaren desagertze eraginkorra ahalbidetzen, eta, ondorioz, estuarioko eremu batzuek ez zituzten Europako Uraren Esparru Zuzentarauak (UEZ-ak) ezarritako ingurumen-helburuak betetzen. Honi irtenbidea jartzeko helburuz, saneamendu lanak egin ziren eskualdean eta, 2021ean, araztegiko isurketak estuariotik kanpo bideratzea lortu zen. Honenbestez, 50 urtetan zehar Urdaibai estuarioak araztegitik jaso dituen hondakin-uren isurketak eten egin dira. Estuarioko fitoplanktonari dagokionez, oso ikertua izan den arren, azken jarraipen intentsiboak 90eko hamarkadaren amaieran egin ziren.

Testuinguru honetan, tesi hau, jarraipen-teknika desberdinak erabiliz Urdaibai estuarioko fitoplankton-komunitatearen deskribapen osoagoa eta eguneratuagoa lortzeko helburuz burutu da, araztegiko hondakin-uren isurketak eteteak estuarioan izan dezakeen eragina zehazteko eta teknika ezberdinen erabilgarritasuna ebaluatzeko. Ikerketak, araztegiko hondakin-uren isurketak egon bitarteko (2019-2020) eta saneamendu lanen ondorengo (2021 eta 2022) fitoplankton-jarraipenen datuak biltzen ditu, non estuarioaren baldintza fisiko-kimikoak zein fitoplankton-komunitatea aztertu diren. Fitoplanktonaren azterketarako erabilitako teknikak mikroskopia, pigmentuen analisia eta DNA metabarcoding-a izan dira.

1. Kapituluan, hondakin-uren isurketak egon bitarteko (2019-2020) estuarioko fitoplankton-komunitatearen deskribapena egiten da, eremuan lehen aldiz mikroskopia eta eDNA metabarcoding-a konbinatuz, baita bi jarraipen teknika hauen konparaketa ere. *2. Kapituluan,* hondakin-uren isurketen eteteak estuarioko ingurumen-baldintzetan eta fitoplankton-komunitatean (pigmentuen analisiaren eta mikroskopiaren bidez) izandako berehalako eragina aztertzen da, denbora-eskala laburrean (2021eko uztailean zehar). *3. Kapituluan,* pigmentuen analisian oinarritutako fitoplanktonaren jarraipenerako tresna kimiotaxonomiko berri bat (*PIGMENTUM*) proposatzen da, ikerketa honetan zehar aurkitutako beharrizanak asetzeko sortu dena. Azkenik, *4. Kapituluan*, saneamendu lanek epe ertainera (urte batera) estuarioko fitoplankton-komunitatean izan duten eragina deskribatzen da, komunitatea saneamendu lanen aurretik (2020an) eta ondoren (2022an) alderatuz, HPLC pigmentuen *PIGMENTUM* analisia lehen aldiz aplikatuz.

Kapitulu ezberdinetan lortutako emaitzak ikuspegi bateratzaile batetik aztertzen dira *Eztabaida Orokorra* atalean. Oro har, lan honek baieztatu du, 2019-2020 bitartean, Urdaibai estuarioko fitoplankton-komunitateak esperotako denboran eta espazioan zeharreko aldakortasuna erakutsi zuela, 90eko hamarkadako ikerketekin antzekotasunak erakutsiz. Fitoplankton biomasa estuarioaren barneko eremura hurbildu ahala handitzen zen eta baliorik altuenak uda-udaberrian aurkitu ziren. Komunitatearen osaerari dagokionez, kanpoko eta erdiko eremuan diatomeoak ziren nagusi eta, barneko eremuan, gehienbat flagelatuak (batez ere kriptofitoak) gailendu ziren. Aldaketa hauek, gehienbat, gazitasun eta mantenugaien gradienteek eragin zituzten. Gernikako araztegiko isurketen desbideratzeak berehalako eragina izan zuen estuarioan, batez ere barne-eremuan nabaritu zena. Amonio eta fosfato kontzentrazioen beherakada nabarmena egon zen eta fitoplankton-komunitatearen osaerak aldaketak jasan zituen, izan ere, *Eutreptiella* sp.-aren hazkunde azkarraren ondorioz, *b* klorofilak aloxantina (kriptofitoen pigmentu diagnostikoa) ordezkatu zuen eremuko pigmentu diagnostiko nagusi gisa. 2022an, saneamendu lanak amaitu eta urte batera, amonioaren eta fosfatoaren beherakada nabaria izan zen estuario osoan zehar eta fitoplankton-komunitateak jasandako aldaketak nabarmenagoak izan ziren. Estuarioko kanpoko eta erdiko eremuan biomasa gutxitu egin zen, mantenugaien kontzentrazioen beherakadaren ondorioz, eta araztegiaren inguruan, ordea, biomasa handitu egin zen, ziurrenik araztegitik zetozen gehiegizko amonio kargek fitoplanktonaren hazkuntza mugatzen zutelako. Komunitatearen osaerari dagokionez, prasinoxantinadun prasinofitoek biomasa totalari egindako ekarpena handitu egin zen, batez ere estuarioaren erdiko eremuan. Gainera, estuarioaren barruko eremuan, flagelatuen nagusitasuna murriztu eta diatomeoek

biomasa totalari egindako ekarpena handitu egin zen, mantenugaien konposizio eta ratio aldaketei erantzunez, hau estuarioaren berreskurapenaren seinale izan zitekeelarik. Fitoplankton-komunitatearen karakterizazioa eta saneamendu lanekiko izandako erantzun ezberdinen ebaluazioa, mikroskopiaren (identifikazio taxonomikoa eta kuantifikazioa egiteko aukera emanez), eDNA metabarcoding-aren (bereizmen handiko identifikazio taxonomikoa ahalbidetuz) eta pigmentuen analisiaren (komunitatearen deskribapen kualitatibo eta kuantitatibo azkarra eskainiz) erabilera bateratuari esker lortu da. Gainera, tesi honek Urdaibai estuarioko espezie-aberastasunari buruzko informazio berritzailea eskaintzen du, eremuan eDNA metabarcoding-a jarraipen-tresna gisa lehen aldiz aplikatzeari esker, eta fitoplankton-jarraipenerako tresna berri bat (*PIGMENTUM*) proposatu eta aplikatzen du, eraginkorra dela frogatuz.

Honenbestez, ikerketa hau baliagarria izan daiteke etorkizuneko disziplinarteko azterlanetarako oinarri gisa, Urdaibai estuarioko baldintza fisiko-kimikoen eta, batez ere, fitoplankton biomasaren eta komunitatearen osaeraren datu eguneratuak eskaintzen baitira. Horrez gain, fitoplanktonaren jarraipen-tekniken erabilgarritasunari buruzko argibideak ematen dira, etorkizuneko uraren kalitatearen kudeaketan eta/edo epe luzerako jarraipen ekologikoko programetan aplikatzeko metodologia posible bat iradokiz, non bereizmen taxonomiko handia derrigorrezkoa ez den.

SUMMARY

Phytoplankton is an essential element for the global understanding of aquatic ecosystems. In estuaries, phytoplankton communities are subject to high spatial and temporal contrasts along numerous environmental gradients. Additionally, anthropic nutrient enrichment has become a major environmental issue in estuaries, also affecting estuarine phytoplankton biomass and community composition. Both nutrient concentrations and phytoplankton are accurate indicators for the assessment of the ecological status of estuaries, as well as for the assessment of the impacts of anthropogenic pressures and/or the effectiveness of management actions. Traditionally, microscopy has been used for phytoplankton monitoring, but several alternatives are arising lately, such as pigment analysis or DNA metabarcoding.

Urdaibai estuary (SE Bay of Biscay), part of the only Biosphere Reserve of the Basque Country, has been receiving direct discharges from the Gernika wastewater treatment plant (WWTP) since 1972. The WWTP did not allow the efficient removal of N and P, and consequently, some areas of the system did not fulfil the environmental objectives set by the European Water Framework Directive (WFD). However, sewerage works were implemented in the area, diverting the effluent of the WWTP outside the estuary in 2021. Therefore, the wastewater discharges that the Urdaibai estuary received during five decades have disappeared. The latest intensive phytoplankton monitoring in the area, however, were in the late 90s.

In this context, this thesis aims to obtain a more complete and updated description of the phytoplankton community of the Urdaibai estuary, using different monitoring techniques, to determine the potential effect of the cessation of wastewater discharges and evaluate the usefulness of the different techniques. The study integrates data from phytoplankton monitorings conducted during the wastewater discharges (2019-2020) and after sewerage works (2021 and 2022), where both environmental variables and the phytoplankton community were analysed, using three different techniques for the latter, i.e., microscopy, pigment analysis and DNA metabarcoding.

Chapter 1 provides the description of the phytoplankton community of the Urdaibai estuary during the WWTP discharges (2019-2020), combining microscopy and eDNA metabarcoding for the first time in the area, as well as the comparison of the two methods. *Chapter 2* covers the immediate effect of the cessation of wastewater discharges on the

environmental conditions and phytoplankton community (by pigment analysis and microscopy) of the estuary in a short time scale (during July 2021). *Chapter 3* proposes a new chemotaxonomic tool (*PIGMENTUM*) for phytoplankton monitoring based on pigment analysis, conceived to fulfil the necessities encountered during the present study. Finally, *Chapter 4* describes the medium term effect of the cessation of wastewater discharges on the phytoplankton community of the estuary by comparing it before (in 2020) and after (in 2022) the sewerage works, applying *PIGMENTUM* analysis of HPLC pigments.

The results obtained in the different Chapters are analysed from an integrative point of view in the *General discussion* section. Overall, the present work confirmed that the phytoplankton community of the Urdaibai estuary showed the expected spatio-temporal variability during 2019-2020, with similarities to the studies from the late 90´s. The biomass values increased towards the inner estuary and the highest values were recorded during spring-summer. As for the community composition, the outer and middle estuary were dominated by diatoms and, towards the inner estuary, flagellates (especially cryptophytes) became more dominant. These changes were mainly prompted by salinity and nutrients. The sewerage works had an immediate effect in the estuary, mainly noticed in the inner area, with significant decreases of ammonium and phosphate concentrations and changes in the phytoplankton community composition, since, due to the rapid increase of *Eutreptiella* sp., chlorophyll *b* replaced alloxanthin as the dominant diagnostic pigment in the inner estuary. In 2022, the decrease in ammonium and phosphate was noticeable throughout the entire estuary and more significant changes were recorded in the phytoplankton community. Biomass decreased in the outer and middle Urdaibai estuary, due to the lower nutrient concentrations, and increased in the surrounding of the WWTP, probably because the excessive ammonium loadings used to suppress phytoplankton growth in the area. Additionally, the contribution of prasinoxanthin-containing prasinophytes increased, mostly in the middle estuary, and there was a community composition shift in the inner estuary from flagellates to diatoms as a response to changes in nutrient composition and ratios, which might indicate the recovery of the area. The characterisation of the phytoplankton community and the determination of the different responses was enabled by the combination of microscopy (providing taxonomic identification together with quantification), eDNA metabarcoding (with high resolution for taxonomic identification) and pigment analysis (rapid qualitative and quantitative description of the community). Moreover, this thesis provides novel information on the species richness of the Urdaibai estuary, due to the application of eDNA metabarcoding as a monitoring tool for the

first time in the area, and proposes and applies a new tool for phytoplankton monitoring (*PIGMENTUM*), which indeed was proven efficient.

In conclusion, the present study might be useful as a baseline for future interdisciplinary studies in the area that require updated data on the phytoplankton biomass and community composition of the Urdaibai estuary. Additionally, it provides insights in the usefulness of different phytoplankton monitoring techniques, suggesting a possible methodology for future water quality management and/or long-term ecological monitoring programmes, where high taxonomic resolution is not compulsory.

I. SARRERA

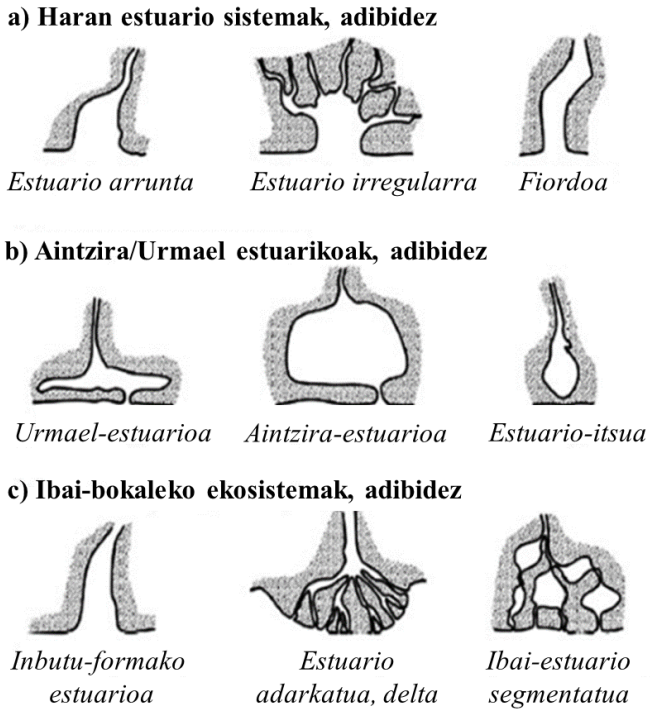
1. Estuarioak

1.1. Definizio eta sailkapena

Estuarioa definizio anitzeko hitza da, zaila baita definizio bakar batek sistema aldakor hauen ezaugarri guztiak biltzea. Urte askotan zehar Pritchard-ena (1967) izan da onartu eta erabilienetakoa, zeinak estuarioa partzialki mugatutako kostaldeko ur-masa bezala definitu zuen, itsaso zabalarekin lotura askea duena, non itsasoko ura lurretik drainatutako ur gezarekin nabarmen diluitzen den. Hala ere, asko izan dira definizio hau moldatu eta osatzeko ahaleginak egin dituztenak, besteak beste, Day (1981) edo Potter *et al.* (2010). Wolanski eta Elliott-ek (2015), definizio ezberdinak konbinatuz, hurrengo definizio eguneratu eta osoa proposatzen dute:

"Estuarioa partzialki mugatutako ur-masa da, mareen edo gatz-sarreren mugaraino itsasoarekin konektatua dagoena eta ur-gezaren isurketak jasotzen dituena, kontuan hartuz ur-gezaren sarrera ez dela iraunkorra izan behar (gerta daiteke urteko zenbait momentutan bakarrik egotea), itsasoarekiko konexioa aldizka itxita egon daitekeela (adibidez, hareazko barra baten bidez) eta mareen eragina arbuiagarria izan daitekeela."

Definizio honek, estuario klasikoez gain, ibai haranak, itsasadarrak, fiordoak, deltak, senadiak eta aintzira-estuarioak biltzen ditu, baita Itsaso Baltikoa bezalako eremu gazikaren eta ur-geza urria den eskualde lehorretako kostaldeko uren ezaugarriak ere biltzen ditu.



1. irudia. *Hiru estuario mota nagusien adibideak: (a) haran-estuarioak, (b) aintzira/urmael-estuarioak, (c) ibai-bokaleko sistemak; Wolanski eta Elliott-en (2015) irudi moldatua.*

Estuarioen sailkapen geomorfologikoa (*1. irudia*) alde batera utziz, eta estuario arruntak adibide moduan hartuz, asko dira ezaugarri fisiko, kimiko eta biologikoen arabera sailkatuak izan daitezkeen estuario motak. Pritchard-ek (1952) oreka-hidrikoaren araberako sailkapena proposatu zuen: (1) "positiboak", non ibai, lurpeko ur eta prezipitazioetatik eratorritako ur gezaren sarrera, lurruntzea baino handiagoa den; (2) "neutroak", ur gezaren sarrera eta lurrunketaren arteko oreka dutenak; eta (3) "negatiboak" edo "alderantzizkoak", non lurrunketak ur gezaren sarrera gainditzen duen. Gune epeletako ohikoenak estuario "positiboak" dira, ondorioz, hondoan itsasotik sartzen den ur gazia aurkitzen da, erdialdean nahasketa bertikal graduala eta azalean ibaitik jaisten den ura. Hala ere, eta baliagarria bada ere, estuarioak sailkatzeko oreka-hidrikoa bakarrik erabiltzea ez da ohikoa.

Beranduago, Pritchard-ek (1967) ur-zirkulazioan oinarritutako estuarioen sailkapen bat garatu zuen, mota hauek bereiziz: (1) oso estratifikatuak diren estuarioak, (2) estuario partzialki mistoak, neurrizko geruzapenarekin (3) alboko gazitasun gradientea duten eta bertikalki homogeneoak diren estuarioak, eta (4) guneka homogeneoak diren eta

longitudinalki gazitasun gradientea duten estuarioak. Honetatik abiatuta, Dyer-ek (1972) estuarioak sailkatzeko antzeko sistema bat proposatu zuen, gehiago erabiltzen dena, hiru nahaste erregimen identifikatuz: estratifikatua, partzialki mistoa edo homogeneoa.

Hayes-ek (1975) marea bitarteetan oinarritutako estuarioen sailkapen sistema proposatu zuen. Honen arabera, mikromareak (<2 m-ko marea bitartea), mesomareak (2-4 m) eta makromareak (>4 m) bereizten dira. Marea bitarte ezberdin hauek marea-korronteen aldaketa handiak eragiten dituzte, hiru estuario moten sedimentuen ezaugarrietan eragina nabarmena izanik. Hala ere, lehen esan bezala, badira aintzira/urmael-estuarioak eta denbora batez itxitako estuarioak, marea-prisma gutxi edo bat ere ez dutenak, baina oraindik ere estuario-ekosistema funtzionalak direnak.

Bestalde, orokorrean, edozein motatako estuarioetan, itsasoko uraren eta lurretik drainatutako ur gezaren nahastearen ondorioz, gazitasun ezberdineko ur-masak sortzen dira, gazitasun guneak bezala ezagutzen direnak. Venezia sistemak (1959), estuarioak sei gune ezberdinetan bereizten ditu uraren gazitasunaren arabera: ur gezakoa edo limnetikoa (gazitasuna <0,5), oligohalinoa (0,5-5), mesohalinoa (5-18), polihalinoa (18-30), euhalinoa (30-40) eta hiperhalinoa (>40). Zona hauek talde biotiko ezberdinen sailkapenarekin eta hauen estuarioan zeharreko banaketa ereduekin lotu daitezke. Ipar Hemisferioko estuario gehienetan gazitasun-guneek Veneziako sistema jarraitzen dute, sorburutik ahoraino, baina Hego Hemisferioko sistemetan, ordea, askotan ez da hori betetzen.

Definizio eta sailkapen mota anitzek ekar ditzaketen arazo edo zailtasunak ekiditeko helburuz, Europar Batasunak, Uraren Esparru Zuzentarauaren (UEZ; 2000/60/EE) bitartez, "trantsizio-urak" hitza sortu zuen estuario guztiak biltzeko, “ibai-ahoen inguruko gainazaleko ur-masak, hein batean gazi-izaera dutenak kostaldeko uretatik gertu egotearen ondorioz, non ur gezako emariek eragin nabarmena duten" gisa definituz. Beraz, ibaien muturrak edo itsasoko sarrerak bezala ikusi beharrean, gaur egun, estuarioak ekosistema gisa aintzat hartzen dira berez (adibidez, Elliott eta Whitfield, 2011).

1.2. Estuarioen garrantzia

Estuarioak sistema dinamikoak dira, ibai-fluxua, marea maila eta estuarioetako sedimentuen banaketa etengabe aldatzen ari baita, eta horrek ekosistema emankorrak bihurtzen ditu. Dinamismoarekin batera, estuarioen emankortasuna azaltzen duten beste hainbat faktore

daude (Day et al., 2012): mantenugaien sarrera ugariak, mantenugai hauen berriztatze azkarra eta kontserbazio altua, kanpo energia altua (nagusiki mareek eragindakoa), materia organikoaren sarrera ugariak eta ekoizle primarioen komunitate anitzen presentzia (makrofitoak, mikrofitobentosa eta fitoplanktona nagusiki). Dinamismo eta emankortasun honek estuarioak sistema garrantzitsuak bihurtzen ditu aspektu ekologikorako eta giza bizitzarako (McLusky eta Elliott, 2004).

Ekologia globalaren ikuspuntutik, estuarioek funtsezko rolak betetzen dituzte (Thrush et al., 2013). Alde batetik, biodibertsitatearen kontserbaziorako garrantzi handiko eremuak dira, izan ere, sistema hauek eskaintzen dituzten baldintza fisiko-kimiko anitzen ondorioz eta emankortasun altuari esker, fauna eta flora komunitate ezberdin askoren habitata dira. Gainera, hainbat espezieren babesleku eremuak dira migrazio- zein errute-garaian. Bestalde, estuarioak karbonoaren finkapenerako eremu eraginkorrak dira, atmosferako karbono kantitate handiak gordetzen baitituzte sedimentu eta landaredian, ezinbestekoa dena karbonoaren zikloaren erregulaziorako eta klima-aldaketa arintzeko. Horrez gain, estuarioek kutsatzaileen iragazketan eta mantenugaien zikloan funtsezko eginkizuna dute, uren kalitatearen erregularizazioan nabarmen lagunduz, elementuen sedimentazio, adsortzio eta flokulazio edo biodegradazio bidez.

Estuarioetako ekosistemek gizakiei eskaintzen dizkieten onurak ere anitzak dira eta zerbitzu-ekosistemiko izena jasotzen dute (Barbier et al., 2011). Zerbitzu-ekosistemikoak hornikuntzakoak (materialak) edo kulturalak (ez-materialak) izan daitezke. Hornikuntza zerbitzuen artean, janari (arrainak eta itsaskiak), ura, eraikuntzarako agregatu eta baliabide genetikoen erauzketa daude. Zerbitzu kulturalek, ordea, aisialdiari, gozamen estetikoari, esperientzia espiritualei eta onura fisiko eta psikologikoei egiten diete erreferentzia. Gainera, estuarioek hondamendi naturalen (uholdeak edo ekaitzak adibidez) aurkako babesa eskaintzen diete bertako espezieei eta inguruan bizi diren biztanleei. Nabigazio bide eta hondakinen deskarga eremu bezala ere erabiliak dira, eta ekonomia sustatzen dute, turismoaren bidez eta horren inguruan sortzen diren enpleguen bidez.

1.3. Estuarioak eta gizadia

Estuarioek zerbitzu-ekosistemiko baliotsuak eskaintzen dituzte, hori dela eta, hiri-, nekazaritza- eta industria-jarduerak azkar hedatu dira haien inguruan. Gaur egun, estuarioek lurraren azaleraren zati txikia (% 5,2) hartzen badute ere, giza-karga neurrigabea jasaten dute,

munduko biztanleriaren % 60 inguru estuarioetan eta kostaldean bizi baita (Wolanski eta Elliott, 2015). New York, Shangai, Londres, Rio de Janeiro, Buenos Aires edo San Frantzisko bezalako hiri garrantzitsu asko, adibidez, estuarioen inguruan eraikiak izan dira. Honen ondorioz, estuarioak presio askoren menpe daude, zuzenean edo zeharka, eta presio horien eragin metatua larria da (Elliott eta Whitfield, 2011).

Estuarioen degradazio arrazoiak asko dira, baina, funtsean, bi iturritatik datoz (McLusky eta Elliott, 2004): estuarioetara sartzen diren materialak eta estuarioetatik erauzten diren materialak. Estuarioetara sartzen diren materialen artean, kutsatzaileak, sedimentuak, energia (zentral elektrikoetatik isuritako ur beroa, adibidez) eta azpiegiturak (zubiak edo presak, esaterako) daude. Estuarioetatik kendutako materialek, ordea, gatza, ura, arrain edo itsaskiak, sedimentuak eta eremu fisikoari egiten diote erreferentzia. Gainera, estuarioen degradazioa eragin dezaketen jatorri zabalagoko arazoak ere badaude, adibidez, klima aldaketa. Honek guztiak eragin negatibo larriak ditu, bai estuarioetan baita bertan edo inguruan bizi diren bizidunengan.

Kostaldeko eremuen garapenak eta lurren erabilerak habitaten galera edo alterazioa dakar, estuarioetako habitat-kritikoak murriztuz (Mitsch eta Gosselink, 2015). Hala ere, ondorio zehatzagoak ere ezberdindu daitezke (Wolanski eta Elliott, 2015). Urbanizazioa era neurrigabean handitzeak, ibai-kanaletan aldaketak geomorfologikoak egotea eta euri-jasa handiko uneetan ur-fluxu azkarrago eta handiagoak sortzea eragiten du. Gainera, baso-soiltzeak, gehiegizko abeltzaintza eta bestelako nekazaritza-praktika txarrek, lurzoruaren higadura, eta ondoriozko ibaietako sedimentuen karga areagotzea eragiten dute. Sedimentazio honen erruz estuario ertzetako kalitate ekologikoa gutxitu eta uretako uhertasuna handitzen da, fotosintesirako eskuragarri dagoen argia gutxituz. Sedimentu hauen erauzketarako teknikak (dragak, esaterako), halaber, kaltegarriak dira sistema hauetan bizi diren organismo eta uraren kalitatearentzat. Agente kutsatzaileen isurketari dagokionez, hiriguneetako, industria-jardueretako eta nekazaritzako isuriek kutsatzaileak sartzen dituzte estuarioetan (kutsadura mikrobianoa, metal astunak, mantenugaiak eta bestelako kutsatzaile organikoak barne), uraren kalitatea okerragotuz eta bertako organismoak kaltetuz (Lotze et al., 2006). Kutsatzaileen isurketaren bi ondorio argi biodibertsitatearen galera eta eutrofizazioa dira, azken hau aurrerago garatuko delarik.

Laburbilduz, estuarioen inguruko urbanizazio eta industrializazioak, sedimentuen, mantenugaien eta bestelako kutsatzaileen areagotzea sustatzen du, eta honek estuarioko

uraren kalitatean eragin negatiboa dauka, baita bertan bizi diren organismoengan ere. Honek, aldi berean, eragin negatibo zuzena dauka estuarioen inguruan bizi diren gizakiengan, bizi kalitatean, baita aisialdirako erabilerak mugatuz edo galera ekonomikoak eraginez.

2. Fitoplanktona

2.1. Ezaugarri orokorrak

Reynolds-ek (2006) fitoplanktona, grekeratik *phyto* (landare) eta *planktos* (noraezeko), honela definitzen du:

"Mikroorganismo fotoautotrofoen multzo heterogeneoa, gehienetan zelulabakarrak direnak eta ur zutabean (itsasoak, aintzirak, urmaelak eta ibaiak) esekiduran bizitzeko egokituta daudenak, non sare trofiko pelagikoentzat eskuragarri dagoen karbono organiko gehiena ekoizten duten".

Honenbestez, fitoplanktona ekosistema urtarretan kate trofikoaren oinarri gisa jarduten duen mikroorganismo zelulabakar fotosintetikoen multzoa da. Ondorioz, fitoplankton-komunitateen tamaina-klaseen egiturak eta osaera taxonomikoak, kate-trofikoaren goragoko mailetan aurkituko ditugun organismo motak baldintzatzen ditu. Gainera, fitoplanktonak $50x10^{15}$ g C inguruko finkapen fotosintetikoa burutzen du urteko, Lurreko ekoizpen primario garbiaren erdia dena (Marañón et al., 2009). Ekoizle primario eta kate trofikoaren oinarri gisa duen garrantziari, sistema klimatiko globalean duen rola (Vaulot, 2001) eta hainbat elementuren (karbono, nitrogeno, oxigeno, sufre eta fosforoa) ziklo biogeokimikoetan duen partehartzea gehitzen zaizkio (Falkowski, 1994). Beraz, fitoplanktonak ezinbesteko eginkizuna betetzen du ekosistemen egitura eta funtzionamendu osasuntsua mantentzeko.

Fitoplanktona osatzen duten organismoen artean, tamaina, morfologia, exoeskeleto mota, bizi-estrategia eta osaera pigmentario aniztasun handia aurkitu daiteke (Clementson et al., 2021). Zelula-tamaina tartea oso zabala da, organismoak pikofitoplankton (0,2-2 μm), nanofitoplankton (2-20 μm) eta mikrofitoplanktonean (20-200 μm) bereiziz. Zelula-tamainak, halaber, fitoplanktonaren fisiologia eta ekologiaren alderdi askotan eragiten du, antolakuntza maila ezberdinetan, hala nola gizabanakoetan, populazioetan eta komunitateetan. Morfologiari dagokionez, organismoek askotariko ezaugarriak izan ditzakete, hala nola, flageloak, areola, arantzak, harizpi organikoak, silizezko exoeskeletoa

(frustulua), tekak edo kaltzio karbonatozko ezkatak. Bizi-estrategiak ere askotarikoak dira, zelula bakartiak (gehienetan), kateak eratzen dituztenak, koloniak, endosinbionteak (koralak) edo kisteak aurkituz.

Hala ere, nahiz eta fitoplanktonak aldakortasun handia bildu, badaude organismo fitoplanktonikoek partekatzen dituzten hainbat ezaugarri (Reynolds, 2006). Zelula-tamaina hainbat magnitude-orden aldatu daitekeen arren, espezie guztiek azalera-bolumen erlazioaren kontserbaziorako joera erakusten dute, organismo handien kasuan forma esferikotik urrunduz eta bestelako forma hartuz lortzen dena. Honek gasen, mantenugaien eta beste solutuen trukean laguntzen du eta organismoen esekidura-gaitasuna handitzea ahalbidetzen du. Era berean, protoplastoaren osaera elementala antzekoa da fitoplankton talde guztien artean. Zelula hormaren pisu lehorraren (errautsa kenduta) % 50 inguru karbonoa da, nitrogenoa % 8-9 inguru eta fosforoa % 1-1,5. Hidrogeno, oxigeno, silize, sufre eta burdina ere agertzen dira, ehuneko baxuetan, eta, aztarna-ehunekotan, beste 12 elementu (Ca, Mg, Na, Cl, K, Mn, Mo, Cu, Co, Zn, B, Va) aurkitu ditzakegu fitoplankton zeluletan. Bestetik, zelulen Chl *a* edukia hazkuntza-baldintzen arabera oso aldakorra den arren, zelularen errautsik-gabeko masa lehorraren % 1-a inguru izaten da, batez beste, eta karbono elementalaren % 2 inguru. Karbono:klorofila erlazio ohikoena 50:1-ekoa da, baina gerta daiteke 70:1 inguruko (argi asko dagoenean) edo 30:1 edo baxuagoak (etengabe argi gutxi dagoenean) diren erlazioak aurkitzea, aztergai den fitoplankton taldea kontuan hartu gabe.

2.2. Fitoplankton talde nagusiak

Reynolds-ek (2006) espezieak 14 filotan banatu zituen, bakterio zein eukariotoen domeinuetatik datozenak. Talde bakoitzak ezaugarri morfologiko zein osaera zelular bereizgarriak ditu, haien ekologian eta dinamikan ondorio zuzenak izango dituztenak (Reynolds, 2006; Paerl eta Justic, 2013).

Diatomeoak (Bacillariophyta) flagelorik ez duten alga zelulabakar hori-marroiak dira, itsastar zein ur gezakoak, banaka zein kateak eratuz bizi daitezkeenak (Round et al., 1990). Fitoplankton talde anitzena, zabalduena eta emankorrena da eta elikadura katean oinarrizko rola betetzen du ekosistema urtar guztietan, baina bereziki itsasoan. Diatomeoen zelula tamaina 2 eta 100 µm bitartekoa da eta zelula-horma pektinazeoa dute, bitan zatitua eta silize kriptokristalinoz inguratua, “frustulu” izena jasotzen duena. Diatomeoen baitan, frustuluaren formaren arabera, bi talde handi bereizten dira: zentrikoak (Coscinodiscophyceae eta

Mediophyceae) eta pennatuak (Fragilariophyceae eta Bacillariophyceae). Azken hauek, pennatuak, organismo bentonikoak izaten dira oro har, baina sarritan hondotik ur zutabera pasatzen dira, birsuspentzio bidez, plankton-komunitateetan garrantzitsuak bihurtuz. Gainera, nahiz eta flagelorik ez izan, diatomeoek mugitzeko bi estrategia ezberdin garatu dituzte: herrestatzea edo flotatzea. Herrestatzeari dagokionez, zenbait diatomeo (pennatuak, oro har) rafe izeneko egitura batetik ateratzen den eskrezio muzilaginosoaz baliatzen dira mugitzeko. Beste batzuek, ur zutabean flotatzeko gaitasuna handitzeko egitura ezberdinak garatu dituzte, hala nola arantzak edo zurdak izan edo/eta kateak eratzea. Horrez gain, diatomeoen osaera pigmentarioari dagokionez, plastido diskoide ugari dituzte, *a* klorofila (Chl *a*), c_1 klorofila (Chl c_1) eta c_2 klorofila (Chl c_2) dituztenak, bere pigmentu osagarri nagusia den fukoxantinarekin (Fuco) batera.

Dinoflagelatuak (Dinophyta), orientazio eta luzera ezberdineko bi flagelo dituzten mikroalgak dira, orokorrean zelulabakarrak (Hasle et al., 1996). Morfologikoki eta funtzionalki askotarikoak dira, oso eboluzionatuak, gehienbat itsastarrak, baina ur gezatan ere aurkitu daitezke. Dinoflagelatuen zelula tamaina 10 eta 1000 μm bitartekoa da eta zelula-horma sendoak dituzte. Espezie askotan, zelula horma "teka" izeneko zelulozazko plaka poligonalekin indartua egoten da. Gainera, dinoflagelatuak plastido konplexuz osatuta daude, non Chl *a*, Chl c_1 eta Chl c_2-z gain, pigmentu osagarri nagusia den peridinina (Peri) aurkitzen den. Hala ere, aipatzekoa da dinoflagelatu batzuek ez dutela Peri-rik eta, bere ordez, beste fitoplankton talde batzuen pigmentu osagarriak dituztela, hala nola, Fuco (adibidez, *Kryptoperidinium foliaceum*).

Alga berdeei dagokienez, oso talde anitza da, ur geza zein gazian aurkitu daitezke eta haien ezaugarri bereizgarri nagusia Chl *a*-z gain *b* klorofila (Chl *b*) izatea da, pigmentazio berdea ematen diena (Leliaert et al., 2012). Alga berdeen taldea klorofitaz osatua dago nagusiki, baina prasinofitak edo euglenofitak ere biltzen ditu. Klorofitak mikroalga zelulabakar, kolonial, harizpi formakoak, sifonazeoak edo taloideak izan daitezke, flagelodunak edo flagelo gabeak. Honenbestez, tamaina eta forma aldakortasun handia biltzen dute, pikoplanktonaren parte diren zelula kokoide txikietatik (2-5 μm), zelula oboide edo disko-formako zelula (desmidoak) handiagoetara, pilatutako zelula-talde txiki gisa egon daitezkeenak (adibidez, *Scenedesmus*). Prasinofitak, oro har, mugitzeko gai diren organismo zelulabakarrak dira, alboz edo apikalki kokatutako 1 eta 16 flagelo artean izan ditzaketenak, eta zelula horma ezkata finez estalita dutenak. Euglenofitak, ordea, zelulabakarrak eta biflagelatuak dira, plastido irregular ugaridunak.

Bestelako flagelatuen artean, haptofitak eta kriptofitak dira nagusi, nahiz eta beste talde garrantzitsu batzuk ere aurkitu ditzakegun, hala nola, rafidofitak (Raphidophyta) edo krisofitak (Chrysophyta). Haptofitak, 2 eta 20 μm bitarteko mikroalga hori-marroi gehienbat zelulabakarrak dira, bi flagelo eta haptonema bat dutenak (Green eta Leadbeater; 1994). Ur gezako espezieak egon arren, oro har organismo itsastarrak dira eta kostaldeko uretan elikagai-iturri garrantzitsua dira. Haptofitek bizi-ziklo konplexua izan dezakete, morfologia mugikorrak eta estatikoak tartekatuz, izan ere, fase ameboide, kokoide edo palmeloidea izan dezakete. Espezie askok, zelula-horma kaltzifikatutako ezkataz estalita dute, eta batzuek arantzak ere badituzte, askotariko morfologia eta egitura konplexuak sortuz. Eduki pigmentarioari dagokionez, Chl *a*, Chl c_1 eta Chl c_2-az gain, fukoxantina edo/eta 19'-hexanoiloxifukoxantina (Hex) pigmentu osagarri karotenoideak aurkitzen dira haien baitan.

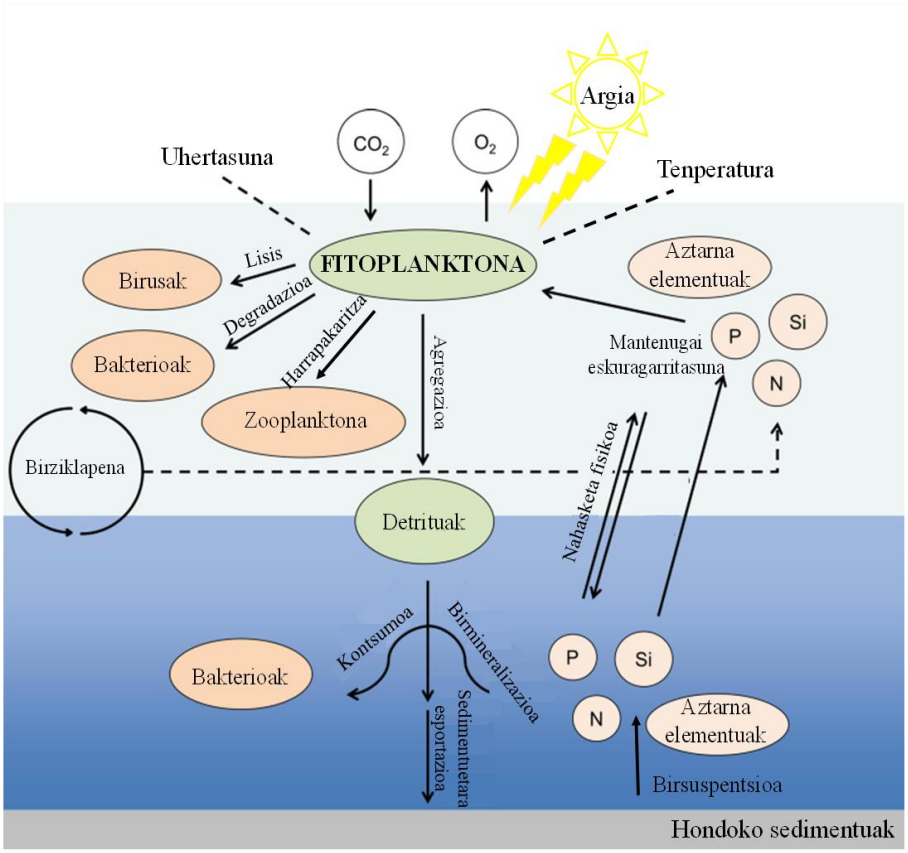
Bestalde, kriptofitak, geruza proteiko batez (periplasto) inguraturiko zelula eta bi flagelo ezberdin dituzten organismo itsastarrak zein ur gezakoak dira (Lee, 2008). Haien zelula tamaina, orokorrean, 5 eta 30 μm bitartekoa da (espezie handiagoak dauden arren) eta gehienbat organismo zelulabakar oboideak dira. Morfologikoki asimetrikoak direla esaten da, bi flagelo ezberdinak zelularen amaieran kokatuak baitituzte, modu bereizi eta eraginkorrean bultzatuz. Gainera, kriptofita gehienek aho moduan jarduten duen zirrikitu bat ere badute, harrapakin txikiak harrapatzeko balio duena, bakterioak adibidez. Honek malgutasun metaboliko handia ematen die, zelulek modu fotosintetiko autotrofo eta heterotrofoetan funtziona dezaten ahalbidetuz. Bestalde, plastido handi bakar bat edo bi dituzte, Chl *a* eta Chl c_2-z osatuak, eta pigmentu osagarri nagusia aloxantina (Allo) da, fikobiliproteinekin batera algei kolore marroi-gorrixka (normalean) edo berdexka emanez.

Zianobakterioak ere aipatzea ezinbestekoa da, bakterioen domeinuko organismo zelulabakar edo kolonial urdin-berdexkak (Whitton eta Potts, 2007). Honenbestez, bakterioen antzeko ezaugarri zelularrak dituzten (ondo definituriko nukleorik ez eta mintzez inguraturiko organulurik ez) fitoplankton prokariotoak dira. Itsaso zein ur gezako eremuetan aurkitu daitezke eta fotosintesi oxigenikoa burutzeko gai diren prokariota bakarrak dira. Zianobakterioak esfera forman (bakarka edo zelula-multzoetan), harizpi ez heterozisto moduan (bakarti edo multzoak) edo harizpi heterozisto taldeetan aurkitu daitezke. Hauen lehen-mailako pigmentu fotosintetikoa Chl *a* da, fikobilina (fikozianina eta fikoeritrina) osagarriekin batera, eta pigmentu osagarri nagusia zeaxantina (Zea) da.

Fitoplanktonaren baitan aurkitzen den aldakortasun handi honen ondorioz, taxonomiak etengabeko berrikuspena pairatzen du eta zaila da identifikatutako espezieen kopuru zehatza ezagutzea. Gutxi gorabehera 25000 espezie deskribatuak izan direla uste da, espezie eubakterio eta eukariotoak barne, ur gazi zein gezakoak (Marañón et al., 2009). Hala ere, asko dira oraindik deskribatu gabeko organismoak, izan ere, diatomeoak bakarrik kontutan hartuz, 10mila eta 100mila espezie bitartean existitzen direla esaten da (Clementson et al., 2021).

2.3. Fitoplanktonaren aldakortasuna kontrolatzen duten faktoreak

Fitoplanktonak, hazkuntza-tasa altua duenez, azkar erantzuten du baldintza fisiko (argia, tenperatura edo uhertasun), kimiko (mantenugaien edo kutsatzaileen kontzentrazioak) eta biotikoen (bazka) aldaketen aurrean (Paerl eta Justic, 2013) (*2. irudia*). Fitoplankton-komunitateek pairatzen dituzten aldaketa hauek, aldi berean, ekosistemen funtzionamenduan aldaketak dakartza, material-fluxu, oxigeno-balantze edo elikadura-sarean adibidez, baita goi-mailako landare eta animalietan ere.



2. irudia. *Fitoplanktonaren aldakortasuna kontrolatzen duten faktoreen irudi eskematikoa (Heinrichs et al. 2020-tik moldatua).*

Argiak funtsezko garrantzia du fitoplanktonaren biomasa, banaketa eta komunitateen osaeraren kontrolean. Organismo gehienak fototrofoak izanda, hauen hazkuntza-tasa argiaren kalitatearen eta eskuragarritasunaren menpe dago (Cloern, 1999). Hori dela eta, bai irradiantzia baita egunean zehar eskuragarri dagoen argi-kopurua ere ekoizpen primarioaren oso iragarle eraginkorrak dira. Fitoplanktonak, argi ikuskorraren espektroaren 400-700 nm-ko zatia erabiltzen du, *erradiazio fotosintetikoki aktiboa* edo *PAR* (Photosynthetic Active Radiation) deitua. Argiaren eskuragarritasuna, bestetik, uretan dagoen argia gutxitzen duten substantzien kontzentrazioaren (adibidez, materia organikoak), uhertasunaren, kolorearen eta organismoen fotopigmentuen araberakoa da. Era berean, uraren nahasketa-bertikaleko tasak eta fitoplanktonaren migrazio bertikalerako gaitasunak eragin zuzena dute argiaren eskuragarritasunean eta, ondorioz, fitoplanktonaren biomasa, banaketa eta komunitateen osaeran (Mallin eta Paerl, 1992).

Fitoplanktonak, hazteko, argiaz gain, hainbat mantenugai inorganiko eta organiko behar ditu, besteak beste, karbonoa (C), nitrogenoa (N), fosforoa (P), silizioa (Si), metalak (nagusiki burdina) eta oligoelementuak, baita B_{12} bezalako bitaminak ere (Cloern, 1999). Makromantenugaien (C, N, P eta Si) artean, N eta P dira garrantzitsuenak, hauek baitira, normalean, eskariarekiko eskaintzarik murritzena dutenak. Ondorioz, sarritan, N eta P-ren eskuragarritasunak fitoplanktonaren hazkuntza eta, beraz, lehen mailako ekoizpena, kontrolatzen edo mugatzen ditu (Ryther eta Dunstan; 1971). Redfield-ek eta Danielek (1935) itsasoko uraren eta fitoplankton itsastarraren oinarrizko konposizioan koherentzia nabarmena aurkitu zuten, “Redfield ratio” izena jarriz, non C:N:P elementuen pisu atomikoen erlazioa 106:16:1 den. Hala ere, ikusi da erlazio hori ez dela denbora eta espazioan konstante mantentzen, izan ere, giza zein jatorri naturaleko aldizkako mantenugaien sarrerek aldaketak eragin ditzakete. Gainera, fitoplankton talde, genero edo/eta espezie ezberdinek mantenugai hauek proportzio ezberdinetan behar dituztenez, elementu hauen eta beste batzuen hornikuntza-ratioek (adibidez N/Si edo N/Fe) fitoplankton talde ezberdinen hazkuntza eta haien arteko lehiakortasuna baldintzatzen dute.

Tenperaturak ere eragin zuzena dauka fitoplanktonaren biomasa eta komunitateen osaeran (Eppley, 1972). Fitoplanktonaren fotosintesi, karbono eta mantenugaien asimilazio-prozesuak, arnasketa, metabolismoa eta hazkuntza-zelularra tenperaturarekiko sentikorrak diren entzimek bideratzen dituzte (Goldman, 1979). Honenbestez, urtarokotasunak, uraren tenperatura eta argi kantitatea baldintzatzen duenez, paper garrantzitsua betetzen du ekoizpen primarioan eta komunitateen osaeraren kontrolean (Valdes-Weaver et al., 2006).

Bestetik, mikrozooplanktonak, makrozooplanktonak eta bibalbioak bezalako ornogabe bentoniko bazkatzaileek (*grazer*), fitoplankton-komunitatearen biomasaren eta osaeraren "goitik behera"-ko kontrola burutzen dute (Sterner, 1989). Gainera, elkarrekintza mikrobianoek ere, esaterako bakterio-fitoplankton asoziazio eta birusen efektua, fitoplankton-komunitatearen osaeran eta jardueran eragiten dute (Paerl eta Pinckney, 1996). Hala ere, organismo hauek burutzen duten predazioaren eragina aldakorra da, hain zuzen ere, urtaroen, taxonen, ur zutabearen nahasketa bertikalaren, ur gezaren sarreren eta egonaldi-denboraren araberakoa. Edonola ere, elkarreraginean dauden kontrol anitz hauek fitoplankton talde funtzional eta espezieen aniztasuna bermatzen dute hidrologikoki eta biogeokimikoki aldakorrak diren ekosistema urtarretan.

Hau guztia kontutan hartuz, ekosistema urtarrek jasaten dituzten aldakortasun tenporalek (urtaroak) zein espazialek eragin zuzena dute fitoplankton-komunitateen biomasan eta osaeran, uretako argi- eta mantenugaien- eskuragarritasuna, tenperatura, bazka eta bestelako faktore asko baldintzatzen baitituzte. Hala ere, fitoplankton talde edo/eta espezie guztiek ez dute berdin erantzuten argi, tenperatura eta mantenugaien gradienteen aldaketen aurrean; hau, hein handi batean, organismo bakoitzak hazkunde-optimoa lortzeko dituen mantenugai-eskakizunen, eskakizun-energetikoen eta bestelako eskakizunen (adibidez, gazitasuna, metal astunen beharra, pH-a edo karbono inorganikoa disolbatua) araberakoa baita.

2.4. Alga loraketa kaltegarriak (HAB)

Ekosistema urtarren biomasa fitoplanktonikoaren hazkunde azkar eta hautemangarriari loraketa edo *bloom*-a deritzo (Hallegraeff, 1993; Paerl eta Justic, 2013) (*3. irudia*). Orokorrean, fitoplanktonak hazkuntza-tasa azkarra izan arren, ekosistema urtarren elikadura katearen oinarria denez eta bere ugaritasuna eta konposizioa hainbat faktore fisiko, kimiko eta biotikoren menpe dagoenez, fitoplanktonaren biomasa totala baxua izan ohi da, galera-faktoreek berezko hazkuntza-tasa azkarrak orekatzen dituztelako. Hala ere, batzuetan, espezie baten edo gutxi batzuen hazkuntzak galerak gainditu ditzake, fitoplankton loraketak eraginez. Loraketak fitoplanktonaren hazkuntzarako onuragarriak diren faktoreen konbinazioarengatik gertatzen dira, prozesu hidrografiko edo meteorologikoen ondorioz haien kontzentrazioa handitu eta birusak, sedimentazioa eta bazkatzea bezalako faktoreen ondoriozko galerak murrizteagatik (Granéli eta Turner, 2006). Ingurumen-aldagai hauek guztiak eskala tenporal ezberdinetan agertzen dira, eta beraz, loraketak, pasarte puntualak edo urtaroko fenomenoak

izan daitezke, egunak edo asteak iraun ditzaketenak, non loraketa eragin duen espeziea komunitateko elementu nagusia bihurtuko den (Cloern, 1996).

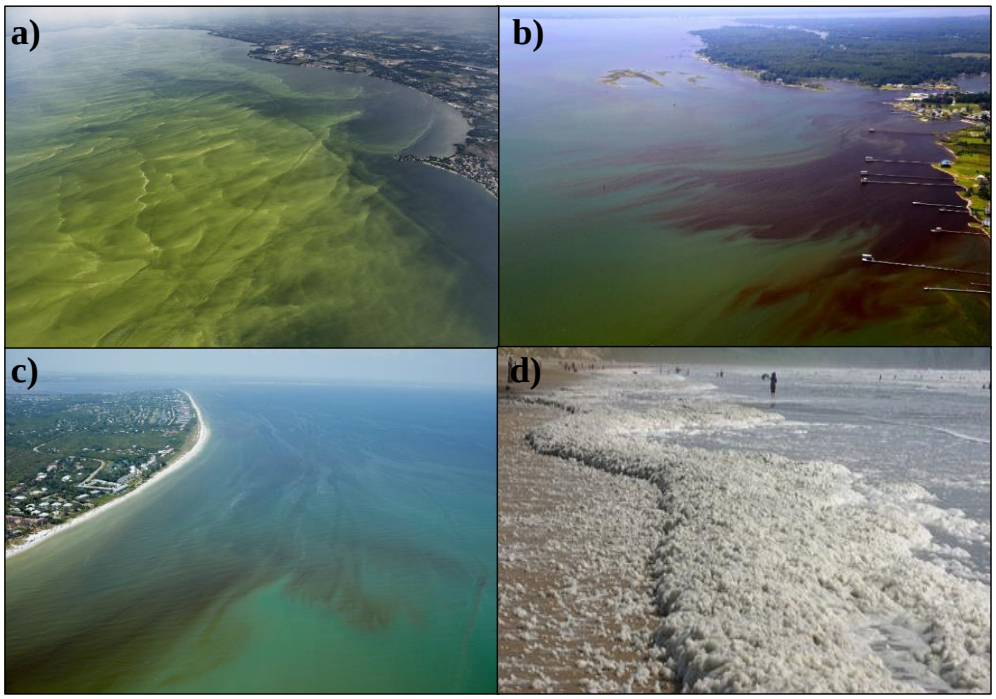
Fitoplankton loraketa batzuek oxigenoaren murrizketa, elikadura-kateen alterazioa, ingurumen-osasun arriskua eta ingurune urtarren kalitatearen murriztea edo habitaten degradazioa eragin dezakete, eta "alga loraketa kaltegarriak" edo HAB (Harmful Algal Bloom) izena jasotzen dute (Granéli eta Turner, 2006). Ezagutzen diren fitoplankton espezie guztien artean, 300 espezie inguruk ekosistema urtarrentzako era batera edo bestera kaltegarriak diren HAB-ak eratzeko gai dira, eta hauetatik 80 espezie inguru baino ez dira toxinak ekoizteko gai (Shumway et al., 2018).

Biomasa handiak eratzeagatik kaltegarriak diren organismoek eragindako arazo nagusiak oxigeno eskasia, uraren kalitatearen galera eta beste organismoengan kalte fisikoak eragitea dira (Granéli eta Turner, 2006). Loraketen ostean organismoak hil egiten direnean, zelulak hondoratu eta bertan bakterioek deskonposatu egiten dituzte, oxigeno kontsumo tasa handituz eta hondoko uretan oxigeno eskasia eraginez (hipoxia edo anoxia) (Diaz eta Rosenberg, 2008). Gainera, loraketa batzuek uraren usain edo zapore txarra eragite dute, ur-horniduraren, aisialdiaren edo akuikulturaren ikuspuntutik kaltegarria izan daitekeena. Beste batzuetan, loraketen arazoa algak inguratzen dituen muzilagoa da, izan ere, kontzentrazio altuetan daudenean arrainen zakatzak estali ditzakete edo/eta disolbaturiko toxinekiko iragazkorrak bihurtzen dituzte (Paerl eta Justic, 2013). Eremu geografiko gehienetan antzeman dira fitoplankton hazkunde masiboak, ia alga talde guztiek eragindakoak, nahiz eta flagelodunak izan loraketa gehienen protagonistak (Lassus et al., 2015). Loraketa hauen adierazpen ohikoena uraren kolorazioa da eta, urak kolore ezberdinak hartzen dituen arren (berdea, marroia, gorria, etab.), ohikoa da "marea gorria" izenaz deitzea.

Bestetik, fitoplankton espezie batzuek goi-faunarentzat toxikoak izan daitezkeen metabolito sekundarioak (toxinak) sortzen dituzte (Pinto et al., 2023). Fitoplankton toxina hauek elikadura-katean sartu eta bertan metatu edo/eta maila trofiko altuagotara garraiatu daitezke, itsaskiak kutsatu eta gizakien kontsumorako desegokiak bihurtuz, edo zuzenean goi-mailako kontsumitzaileak pozoituz, arrainak, hegazti itsastarrak, ugaztun itsastarrak eta gizakiak barne (Shumway et al., 2018). Espezie toxiko hauen agerpenak galera ekonomikoak eragiten ditu askotan, arrantzan, turismoan, uren kalitatearen kontrolen beharragatik eta osasun-zaintzako gastuengatik. Diatomeoen barruan *Pseudonitzschia* genero toxikoa dago, eta

dinoflagelatuen artean genero toxiko asko aurki daitezke, adibidez, *Alexandrium*, *Karlodinium*, *Karenia*, *Prorocentrum* eta *Dinophysis* (Lassus et al., 2015).

Smaydaren (1997) arabera, *bloom*ak definitzeko erabilitako irizpideak ezberdinak izan behar dira loraketaren osaera zehatzaren arabera: espezie ez-toxikoen kasuan, ugaritasuna irizpide gisa erabiltzen da eta, espezie toxikoentzat, nahikoa da presentzia hutsa edo toxina kontzentrazio jakin bat izatea. Esaterako, Swan eta Davidsonek (2012) ezarritako toxikotasun mugen arabera, *Dinophysis* generoaren *bloom* bat izateko nahikoa da litro batean 100 indibiduo egotea.



3. irudia. *Fitoplankton loraketak ekosistema urtar ezberdinetan. a) Udako zianobakterio loraketa Erie Lakuan (Zachary Haslick, National Oceanic and Atmospheric Administration); b) Alexandrium monilatum loraketa Chesapeake badian (W. Vogelbein, VIMS); c) Karenia brevis loraketa Floridako hego-mendebaldeko kostaldean (Ben Deep, National Geographic); d) Phaeocystis pouchetii loraketa, eta honek sortutako aparra, Gower Penintsulan, Galesen (David John, Algaebase).*

3. Fitoplanktona estuarioetan

Fitoplanktona estuarioetako oinarrizko elementua da, izan ere, sistema hauetan, lehen mailako ekoizle guztien artean fitoplanktona izan ohi da materia organiko autoktonoaren iturri nagusia (Cloern et al., 2014). Estuarioen dinamismoaren ondorioz, bertan bizi diren fitoplankton-komunitateek denboran eta espazioan zeharreko kontraste handiak jasaten dituzte: mareen eta ur gezaren eraginaren araberako aldaketa espazialak; eta aldakortasun

tenporal handia eskala ezberdinetan, bai mareek eragindako egunean zeharreko aldakortasuna baita urtaroko aldakortasuna (ur-gezaren sarrera eta meteorologia baldintzatuz) (McLusky eta Elliott, 2004). Honenbestez, estuarioetako fitoplankton-komunitateen biomasa eta egitura etengabe aldatzen dira, gehienbat mareen uren zirkulazioak eta ibaiaren eraginak baldintzatutako ingurumen-gradienteetara moldatuz (Jouenne et al., 2007).

Zehazki, estuarioetako fitoplankton-komunitateek, gazitasunari, jatorri ezberdinetako isurketei, uraren tenperaturari eta argiaren eta mantenugaien eskuragarritasunari loturiko ingurumen-aldaketei erantzuten diete (Paerl eta Justic, 2013). Aldagai biologikoek ere eragin zuzena dute, hala nola, bazkatzeak eta birus eta bakterioen erasoek. Beste eragile bat prozesu hidrodinamikoak dira, izan ere, mareek eragindako korronteek, haizeak edo dentsitate gradienteek ur masa ezberdinak horizontalki, bertikalean eta lateralki garraiatzen dituzte eta horiekin batera fitoplankton-komunitateak (Lucas et al., 1999). Gainera, ur gezaren sarrerak ere estuarioetako fitoplankton-komunitateen bilakaeran eragin zuzena dauka, estuarioetako uraren egonaldi-denboran eta estratifikazio mailan duen eraginagatik (Lancelot eta Muylaert, 2011). Hau guztiari, eremu biografikoak edo eskualdeak dituen mugak fitoplankton-komunitateetan duen eragina gehitu behar zaio. Honenbestez, estuarioko fitoplanktonak baldintza fisikoen (gazitasuna edo ur zutabearen nahasketa maila, adibidez) edo/eta faktore-mugatzaileen (argi edo mantenugai eskuragarritasuna) aldaketa azkarrei aurre egin behar die, eta aldaketa horiek biomasaren eta espezieen osaeran eragin dezakete.

3.1. Fitoplankton biomasaren aldakortasuna

Orokorrean, estuarioetako fitoplankton biomasaren banaketa espazio-tenporala arautzen duen faktore nagusia ur gezaren sarrerak (isurketak) dira. Ur gezaren sarrerak eragin positiboa izan dezake estuarioetako fitoplankton biomasaren aldakortasunean, nutriente-iturri nagusi gisa eta ur-zutabearen geruzapena sortuz, horrela, fitoplanktonaren loraketei hasiera emanez, baina eragin negatiboak ere izan ditzake, uhertasuna areagotuz, fitoplankton populazioak estuariotik ateraz (hustuz) edo estres osmotikoa eraginez (McLusky eta Elliott, 2004). Gainera, ur gezaren isurketak estuarioko uren emari-abiadurak eta ondoriozko egonaldi-denbora ere baldintzatzen ditu (Paerl eta Justic, 2013).

Estuarioetako mantenugaien banaketa, oro har, sarrera puntual eta ez-egonkorren bitartez gertatzen da. Estuarioetan fitoplanktonaren hazkuntza mugatzen duen mantenugaia N dela ikusi bada ere (esaterako, Ryther eta Dunstan, 1971; Nixon, 1995), N eta P-ren aldibereko mugatzea aurkitzea ere ohikoa da (Malone et al., 1996), batez ere estuarioen gazitasun baxuko

barrualdeko eremuetan (Fisher et al., 1988; Paerl et al., 1995). Fitoplankton biomasaren hazkundea baldintzatzen duten N eta P-ren aldibereko mugatzea edo P mugaketa esklusiboa ur gezaren sarrerak altuen diren garaietan ematen da normalean, ur geza hauek N-an aberatsak izaten baitira (Fisher et al., 1988; Sylvan et al., 2006). Baldintza hauetan, N:P (16:1)-ren "Redfield ratio" molarra asko gainditu daiteke, batzuetan 200:1era iritsiz, P-ren faltaren ondoriozko fitoplankton hazkundearen mugaketa handia eraginez.

Gainera, estuarioetan fitoplanktonaren hazkuntza mugatzen duen beste faktore bat uraren uhertasuna da. Estuarioko uren uhertasun maila, jatorri naturalekoa denean, hainbat faktoreren araberakoa izaten da, hala nola, itsaso zein ibaiko urek dakarten partikula kantitatea, estuarioko zirkulazioa eta partikulen sedimentazio-abiadura (McLusky eta Elliott, 2004). Orokorrean, "uhertasun maximoa" estuarioaren erdialdeko edo barrualdeko eremuetan ematen da, ur geza eta gaziaren arteko nahasketa eremutik hurbil, bertan ematen diren prozesu fisiko-kimikoen eraginez. Hala ere, maximoa honen kokalekua eta tamaina aldakorra da, ur gezaren sarreraren (ibaiaren emariaren) indarraren arabera, emari txikiarekin estuarioaren barrualdean kokatuz eta ibai emari handiarekin kanporago mugituz. Gainera, giza jatorria duten materia partikulatuaren sarrera puntualak ere gerta daitezke estuarioetan, inguruko lurren erabileraren ondoriozkoak direnak, uraren uhertasun maila handitzen dutenak. Edonola ere, baldintza naturalek edo gizakiak eragindako uhertasunak estuarioetako ur-zutabean dagoen argi-eskuragarritasuna mugatzen du, horrela fitoplanktonaren hazkuntza mugatuz.

Bestetik, estuarioetako hustuketa-tasak eta egonaldi-denborak fitoplanktonaren hazkuntzarako elementu garrantzitsuak dira, biomasa fitoplanktoniko maximoa zenbatekoa den eta non garatu daitekeen baldintzatzen baitute (Lancelot eta Muylaert, 2011). Ibai fluxu handiko momentuetan, emari handiak egonaldi-denbora laburrak eta hustuketa-tasa altuak sortuko ditu estuarioan zehar, eta honen ondorioz estuarioko fitoplanktona itsasora ateratzen da haien hazkuntza-tasak loraketak garatzea ahalbidetu baino lehen. Honek, fitoplanktonaren hazkuntza oztopatu eta estuarioan biomasa fitoplanktoniko baxuak izatea eragiten du, fitoplanktonaren hazkuntza eta biomasa maximoak estuarioko eremu zabal eta egonaldi-denborarik luzeagoetara mugatuz.

Aipatutako faktoreen heterogeneotasun handiak estuarioetako biomasa maximoko eremuak aldakorrak izatea eragiten du. Estuario gehienetan, biomasa maximoak barrualdean ematen dira, ekarpen flubialek eragindako mantenugaien eskuragarritasun altuagoagatik, itsasoko ura, orokorrean, oligotrofikoagoa baita (Paerl eta Justic, 2013). Honek azaltzen du biomasa

maximoak gazitasun baxuko eremuekin (adibidez, Kromkamp et al., 1995) edo uhertasun altuko eremuekin (adibidez, Fichez et al., 1992) erlazionatzea. Hala ere, gerta daiteke biomasa maximoak estuarioaren eremu itsastarrean edo tarteko eremuan aurkitzea, bestelako mantenugaien sarrera puntualen kokapenagatik edo barrualdeko eremuen gehiegizko uhertasunak fitoplanktonaren hazkuntza mugatzen duelako (adibidez, Muylaert eta Raine, 1999). Azken kasu hauetan, mantenugaien murrizketa uraren gardentasunaren areagotzearekin konpentsatzen da.

Era berean, urtarokotasunak ere eragin zuzena dauka estuarioetako fitoplankton biomasaren aldakortasunean. Urtaroko biomasa aldakortasuna, hein handi batean, ibai-arroaren baldintzen araberakoa da, hau da, ur gezaren sarreren araberakoa, lurretik estuarioetara egiten den mantenugai eta sedimentuen sarrera arautzen baitute (McLusky eta Elliott, 2004). Sarrera hauek fitoplanktonaren hazkuntza susper dezakete sisteman mantenugai-eskuragarritasuna mugatua denean edo argi-baldintzak hobetzen direnean, edo hazkuntza mugatu dezakete, sedimentuez betetako uren sarrerak argiaren eskuragarritasuna gutxituz edo populazioak estuariotik ateraz. Orokorrean, fitoplankton biomasa maximoak udaberrian izaten dira, argia edo/eta mantenugaien eskuragarritasuna altuak direnean, udaberriko loraketak ahalbidetuz. Udan mantenugaien eskuragarritasunak altua izaten jarraitzen badu, sarrera berrien edo/eta birziklapenaren ondorioz, loraketek iraun dezakete (Paerl eta Justic, 2013). Urtaro hauek, gainera, tenperatura maximoen garaiak ere badira, fitoplanktonaren hazkunde-tasa altuak ahalbidetuz. Argitu behar da, urtarokotasun honek estuarioaren barruko eta erdialdeko eremuetan duela eragina batez ere, izan ere, eremu itsastarreko baldintzak egonkorragoak dira urtean zehar.

3.2. Fitoplankton-komunitateen osaeraren aldakortasuna

Estuarioetako fitoplankton-komunitateak osatzen dituzten talde eukarioto nagusiak diatomeoak, dinoflagelatuak, alga berdeak, kriptofitak eta haptofitak dira (Paerl eta Justic, 2013). Talde hauetako bakoitzak zeregin garrantzitsua betetzen du estuarioetako lehen-mailako ekoizpenean, elikadura sarearen dinamikan eta ziklo biogeokimikoetan. Era berean, modu desberdinean erantzuten diete mantenugaien aberasteari, presio hidrologikoari (ur gezaren isurketak eta gazitasun erregimenak), argi eskuragarritasunari (irradiazio-gradienteak eta uhertasuna) eta bazka-presioari. Hori dela eta, biomasarekin gertatzen den bezala, estuarioetako fitoplankton-komunitateen osaerak denboran eta espazioan zeharreko aldakortasuna jasaten du, izan ere, talde fitoplanktoniko bakoitzak mantenugai, argi edo bestelako baldintza fisiko-kimiko (ur-zutabearen egonkortasuna, gazitasuna, aztarna-metalen

presentzia, pH-a edo disolbaturiko karbono inorganikoa) ezberdinetan lortzen du hazkuntza-tasa optimoa. Gainera, talde edo espezie hauen bazkatze-tasa ezberdina izan daiteke, "goitik-behera"-ko kontrol selektiboa aurkituz.

Diatomeoak adibidez, estuarioetako talderik ugari, hedatu eta emankorrenetakoak dira, eta zeregin nagusia betetzen dute elikadura-sare planktonikoetan (Hasle et al., 1996). Talde honen presentzia eta hazkuntza mantenugaien eskuragarritasunarekin oso lotuta dago, izan ere baldintza zehatzak eskatzen ditu (Paerl eta Justic, 2013). Mantenugai-kontzentrazio moderatua edo altua duten urak nahiago izaten dituzte, organismo oportunistak (r estrategak) kontsideratuz, beraz, udaberrian eta udan loratzen dira gehienbat. Gainera, tenperatura nahiko baxuetan hazkuntza-tasa bizkorrak izateko gai dira eta, beraz, udaberriaren hasierako ur gezaren sarrerak aprobetxatuz, ohikoa da diatomeoek udaberri hasieran fitoplankton-komunitateak menderatzea (Harding et al., 2002; Valdes-Weaver et al., 2006; Harding eta Miller, 2009). Hala ere, haien zelula-horma eratzeko silizioaren beharra dutenez, diatomeoen hazkuntza Si-aren eskuragarritasunagatik kontrolatua edo mugatua dago. Estuarioko uretan disolbatutako Si-a estuarioa eratzen duen ibai-arroaren lurzoruaren meteorizazioaren produktua da, beraz, lurzoru hauek silizikoak ez badira edo edukia urria bada, Si-aren hornidura, eta beraz diatomeoen hazkuntza, mugatua izan daiteke. Si-aren mugaketa bereziki nabarmena egiten da estuarioak jatorri antropikoko mantenugaien sarrerak jasotzen dituenean, izan ere, N edo/eta P-ren kontzentrazioen igoerak eragiten dituzte baina ez Si-arenak, fitoplankton-komunitatearen osaeran aldaketak eraginez (Officer eta Ryther, 1980; Turner et al., 1998).

Beste talde batzuek, dinoflagelatuek adibidez, bizi-estrategia ezberdinak garatu dituzte estuarioen baldintzetara egokitzeko, organismo espezialisten (k estrategak) eredua jarraituz (Hasle et al., 1996). Orokorrean, uda garaian aurkitzen da dinoflagelatuen presentzia handiena estuarioetako fitoplankton-komunitateetan, mantenugaien kontzentrazioa jaisten hasten denean eta ur-zutabearen egonkortasuna handitzen denean, lehiakide hobeak baitira mantenugaien eskuragarritasuna baxua den inguruneetan eta mugimendu gutxiko uretan (Paerl eta Justic, 2013). Honen arrazoietako bat zenbait dinoflagelatu heterotrofo fakultatiboak izatea da, bakterio eta beste organismo fitoplanktoniko txikiago batzuk irentsiz edo/eta disolbatutako karbono organikoa kontsumituz hazteko aukera baitute. Honi esker, eta haien hazkuntza-tasa beste talde batzuena (diatomeoak, kriptofitak edo klorofitak) baino geldoagoa izan arren, dinoflagelatuek loraketa handiak sortu ditzakete estuarioetan, batzuetan kaltegarriak izan daitezkeenak. Gainera, ohikoa da negu amaieran ere estuarioetan zenbait

dinoflagelatuen (adibidez, *Heterocapsa* spp.) bat-bateko loraketak aurkitzea, argi-eskuragarritasunaren gorakadak (intentsitatea eta ordu kopurua), mantenugaietan aberatsak diren ur gezaren sarrerak eta bazka-presio ezak bultzatuta (Paerl et al., 1998; Litaker et al., 2002).

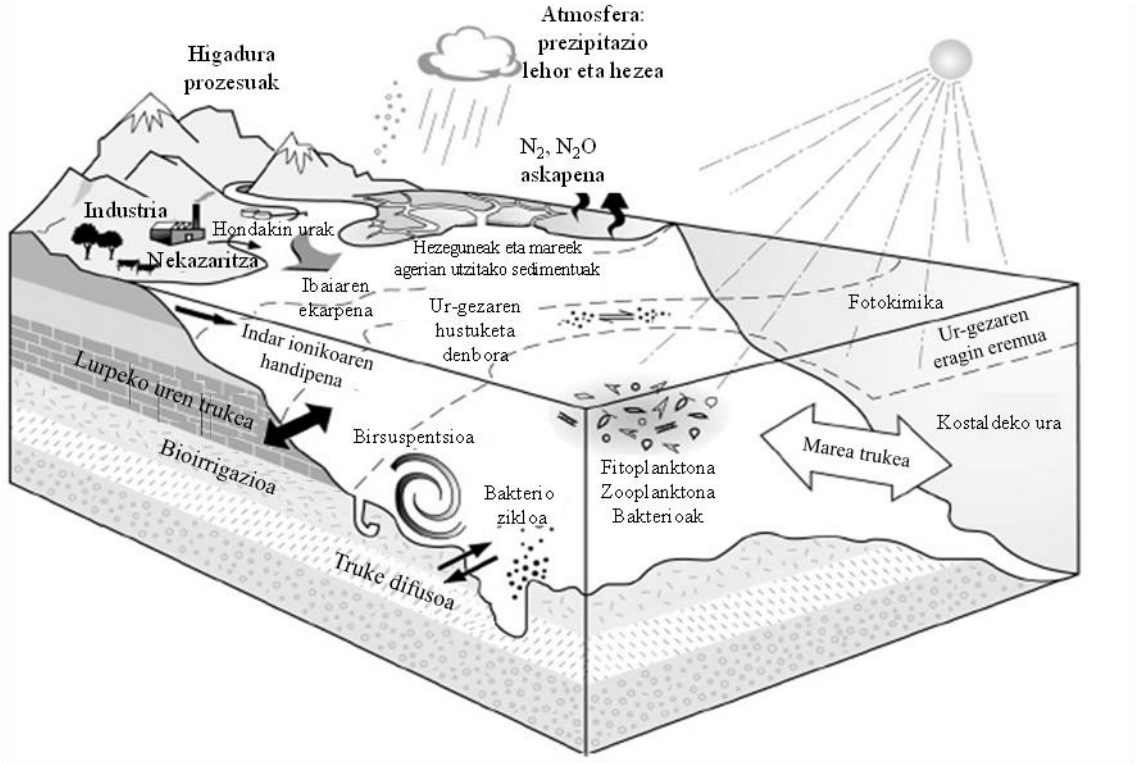
Badaude mareen uren zirkulazioak edo/eta ibaien eraginak baldintzatutako aldakortasuna erakusten duten taldeak ere, hazteko eta bizitzeko gazitasun lehentasun zehatzak dituztelako. Haptofitak adibidez, gehienbat banaketa itsastarra dutenak (Eikrem et al., 2016), estuarioaren kanpoaldeko eremuetara mugatzen dira, ur itsastar eta gazikaretara, eta barrurantz joan ahala fitoplankton-komunitatearen osaeran duen garrantzia galduz doa (Seoane et al., 2005). Alga berdeei dagokienez, klorofitak ohikoak dira estuarioen gazitasun baxuko barrualdeko eremuetan, batzuetan komunitateen talde nagusia bihurtuz (Tomas, 1997). Klorofitek hazkuntza-tasa azkarra dute eta ondo hazten dira indar handiko eta egonaldi-denbora baxuko gazitasun baxuko uretan (Paerl eta Justic, 2013). Normalean ur gezaren sarreratik hurbil dauden eremuek aipatutako baldintzak betetzen dituzte, eta mantenugaien eskuragarritasun altua izan ohi dute, alga berdeak bezalako hazkuntza azkarreko espezieen loraketak lagunduz.

Kriptofitei dagokienez, haien hazkuntza baldintza optimoak mantenugaietan aberatsak diren ur gazikarak dira. Ondorioz, normalean, kriptofiten loraketak gazitasun-gradiente handiko eremuetan ematen dira, estuarioen erdialde edo barrualdean, eremu meso- edo oligohalinoetan (Seoane et al., 2005; Vilicic et al., 2008). Loraketa hauek, askotan, diatomeoen loraketen ondoren ematen dira, izan ere, krirptofitek mugitzeko duten gaitasunari esker, gai dira ur zutabearen goiko partean mantentzeko, eskuragarri dagoen argia aprobetxatuz, udaberriko diatomeo loraketak amaitu eta sedimentuz beteriko ur gezaren sarrerek uraren gardentasuna gutxitu dutenean. Gainera, kriptofitak nahiko eraginkorrak dira mantenugaiak bahitzen mantenugaien eskuragarritasuna murritza denean, beraz ez dute arazorik diatomeo loraketen ondorengo baldintzetan hazteko, eta zenbait espeziek jarrera mixotrofoa ere erakusten dute. Honenbestez, kriptofitak lehiakide onak direla esan daiteke, askotan estuarioen erdiko edo barruko eremuetan fitoplankton-komunitateetan talde nagusia bihurtuz.

Talde nagusi hauek estuarioan zehar erakusten duten aldakortasunaz gain, aipatzekoa da, udako hilabeteetan, estuarioan dagoen ur gezaren sarrera txikia denean, egonaldi denbora altua denean, mantenugaiak agortzen hasten direnean eta tenperaturak eta ur-zutabearen geruzapena altuak direnean, hazkuntza motela eta mantenugaien erabilera eraginkorra duten

zenbait taxon ugaritzen direla komunitateetan, hala nola, organismo pikoplanktonikoak eta N2 finkatzaileak diren zianobakterio espezieak (Paerl eta Justic, 2013).

Estuarioetan dagoen fitoplanktonaren gaineko bazka-presioak eta "goitik-beherako" kontrolak aldakortasun espaziala (estuarioko gazitasun-gradientean zehar) eta tenporala (urtaroen araberakoa) erakusten du, estuarioko baldintza fisiko-kimikoekin (mantenugai eskuragarritasuna edo tenperatura) elkar-eraginean. Honek, fitoplankton-komunitatearen osaeraren aldakortasunean eragiten duten gainerako baldintzekin batera, hidrologikoki eta biogeokimikoki aldakorrak diren estuarioetan fitoplankton espezieen eta talde funtzionalen aniztasuna bermatzen du.



4. irudia. *Estuarioko prozesuen eskema (Statham, 2012-etik moldatua).*

4. Eutrofizazioa eta fitoplanktonaren rola uraren kalitatearen adierazle gisa

Eutrofizazio hitzak ur masek jasaten duten mantenugaien gehiegizko aberasteari eta horrek eragindako ondorio biologiko kaltegarriei egiten die erreferentzia (Sinha et al., 2017). Mantenugaien gehiegizko karga hauek 1950etik aurrera handitzen hasi ziren eta, gaur egun, eutrofizazioa arazo globala bihurtu da (Van Beusekom, 2018). Europan, konkretuki, eutrofizazioa trantsizio eta kostaldeko urek jasaten duten presio nagusienetako da (CIS, 2005).

Populazio-hazkundeak eta, hau asetzeko, elikagai-ekoizpenaren areagotzeak (nekazaritza, abeltzaintza eta akuikultura) landa-eskualdeetan zein hiriguneetan azaleko-isurketen eta hondakin-uren isurketen bidez uretako mantenugaien kontzentrazioak areagotzea eragin du (Doney et al., 2010; Glibert eta Burkholder, 2018). Estuarioek jasotzen dituzten jatorri antropikoko N eta P-ren sarrerak iturri desberdinetakoak dira eta horien proportzioak geografikoki eta demografikoki aldatzen dira (Paerl eta Justic, 2013). Nekazaritza nagusi den landa-eskualdeetan, N eta P sarreren % 50 eta % 75 bitartean iturri difuso eta "ez-puntualetakoa" da, hala nola, gainazaleko isurketek, euriteek eta lurpeko urek lixibiazio bidez eragindakoak (Paerl, 1997). Kasu hauetan, N eta P kargak nekazaritzan erabiltzen diren ongarrietatik datoz gehienbat (Howarth, 2008). Hiriguneetan, ordea, N eta P kargak iturri puntualetakoak izaten dira nagusiki (% 50etik gora), hondakin-uren isurketek, isurketa industrialek eta udal-isuriek eragindakoak (Castro et al., 2003). Gainera, ekosistema urtarretatik hurbil kokatzen diren abeltzaintza intentsiboko eremuak eta akuikultura eremuak ere mantenugai-iturri garrantzitsuak bihurtu dira (Glibert eta Burkholder, 2018). Abeltzaintza intentsiboko eremuetan, abeltzaintza estentsiboarekin alderatuz, animalia kopuru handia dago lur-eremu unitateko, eta beraz hondakin kopuru handia ere bai, inguruko ekosistema urtarretan mantenugaien aberastearekin eta bestelako kutsatzaileekin (mikroorganismo patogenoak edo solido esekiak) lotutako arazoak eraginez (Burkholder et al., 2007). Akuikulturari dagokionez, organismoen hazkuntzarako isuritako mantenugaien eta organismoek ekoitzitako iraitz-materialen bidez mantenugaien gehiegizko aberastea gerta daiteke (Burridge et al., 2010; Glibert eta Burkholder, 2018).

Estuarioek, beraz, ibaiek dakarten mantenugaien sarreraz gain (iturri naturala), inguruko nekazaritza lurretatik edo hiriguneetatik datozen mantenugai sarrerak jasotzen dituzte (iturri antropikoa). Gainera, sistema hauetan ematen diren prozesu geokimiko eta biologikoei esker, estuarioek "iragazki" moduan jarduten dute eta sartzen diren mantenugaien parte handi bat mantentzea lortzen dute, mantenugaien erretentzio eta erregenerazio gaitasun altua izanik (Cloern, 2001). Mantenugaien iturria edozein dela ere, ekosistema urtarretan elikadura-kate produktibo eta osasuntsu bati eusteko beharrezkoa den mantenugaien aberaste "onuragarriaren" eta, ekoizpen primarioa asko bizkortu eta materia organikoaren gehiegizko ekoizpena (hau da, eutrofizazioa) sustatzen duen mantenugaien aberaste "kaltegarriaren" artean dagoen aldea, oso txikia da. Ur gezako sistemetan kezka nagusia P den bitartean, estuarioetan eta kostaldeko uretan gehiegizko N da eutrofizazioaren eraile nagusia (Howarth eta Marino, 2006).

Eutrofizazioak estuarioak bezalako ur masetan eragiten dituen ondorio biologikoen artean fitoplankton loraketen ugaritzea, itsas-larreen murrizketa, disolbaturiko oxigenoaren murrizketa eta arrainen heriotza daude (Paerl, 1988, 2004). Fitoplanktonaren loraketen ondoren, bat-bateko tenperatura aldaketek, argiaren eskuragarritasun murrizketak edo/eta mantenugaien agortzeak loraketak desagertzea eragin dezakete, honek dakarren materia organikoaren pilaketarekin (Paerl, 1988). Materia organiko hau hondoan pilatu eta, bertan, bakterioek deskonposatu egiten dute, oxigeno kontsumo-tasa handituz. Hondoko ur hauek ez badira gainazaleko ur oxigenatuekin nahasten, oxigeno eskasia sortzen da (hipoxia edo anoxia), estuarioen ekosistema bentiko eta pelagikoetan ondorio kaltegarriak garatuz, hala nola, arrainen eta itsaskien heriotza (Paerl eta Justic, 2013).

Eutrofizazioak fitoplanktonean duen eragina ez da loraketen ugaritasunera bakarrik mugatzen. Mantenugaien kontzentrazio eta N:P:Si ratioen aldaketek fitoplankton-komunitateen osaeran eragin zuzena dute, fitoplankton talde ezberdinen ugaritasun erlatiboa eraldatu egiten baitu, eta horrek ondorio ekologiko sakonak izan ditzake (Meerssche eta Pinckney, 2019). Esaterako, jatorri antropikoko mantenugaien sarrerek N eta P kontzentrazioa handitu baina Si-arena berdin mantentzea eragiten dute, azken hau mugatzailea bihurtuz, eta honek diatomeoen hazkuntza mugatzen du. Gainera, fitoplankton talde bakoitzak N forma desberdinen aprobetxamendu-tasa espezifikoak ditu, eta honek haien hazkunde-tasan eta komunitatearen osaeran aldaketak eragin ditzake (Glibert et al., 2016). Beste batzuetan, gehiegizko mantenugaien-aberasteak HABak eratzen dituzten espezieen loraketak sustatu ditzake (Glibert eta Burkholder, 2018), izan ere, hauek, mantenugai-baldintza desorekatuetan toxina eta alelokimiko gehiago ekoizteko joera dute, beste fitoplankton espezie batzuen aurka lehiatzeko eta hauek gainditzeko abantaila emanez (Granéli eta Turner, 2006; Van Meerssche et al., 2018).

Testuinguru honetan, uretako mantenugaien kontzentrazioa eta fitoplanktona estuarioak bezalako ekosistema urtarren egoera ekologikoaren, eta bereziki eutrofizazioaren, ebaluaziorako adierazle erabilienetakoak dira, Europako Uraren Esparru Zuzentarauaren (UEZ; 2000/60/EE) edo Itsas Estrategiaren Zuzentarauaren (IEZ; 2008/56/EE) parte izanez (Seoane et al., 2011). Mantenugaien jarraipenaren bidez, sistemara egiten diren isurketa-antropikoek ur-masaren baldintza fisiko-kimikoetan dituzten efektu zuzenak kontrolatu daitezke, izan ere, isurketen efektuak, inguruko uretan ez ezik, isurketa eremuetatik haratago dauden uretara ere hedatzen dira (Poikane et al., 2019). Estuarioko mantenugaien

kontzentrazioen dinamikak ezagutzea beraz, sistemaren eutrofizazio arriskua kontrolatzeko erraminta bihurtu daiteke. Fitoplanktonari dagokionez, mantenugai-eskuragarritasun aldaketei erantzuna ematen dien lehen komunitate autotrofoa da (Paerl et al., 2003) eta, ekosistema urtarren elikadura-katean duen oinarrizko posizioa dela eta, mantenugai inorganikoen eta gainerako maila trofikoen arteko loturatzat hartzen da (Seoane et al., 2011). Ondorioz, aurretik azaldu bezala, estuarioetan ematen diren mantenugai inorganikoen aberasteak fitoplanktonaren hazkuntza sustatzen du eta komunitatearen osaera eraldatzen du, adierazle bikaina bihurtuz.

Europako Uraren Esparru Zuzentarauak uren egoera ekologia zehazteko adierazle gisa erabiltzen du fitoplanktona, ekosistema urtar kontinental, itsastar zein trantsiziokoetan. Bertan, fitoplanktonaren honako alderdi hauek hartzen ditu kontuan ur-masa mota desberdinak sailkatu eta zehazten direnean: fitoplankton-komunitatearen osaera taxonomikoa eta ugaritasuna, ohiko baldintzekin alderatzean dagoen aldakortasun maila kontuan hartuz; fitoplanktonaren batez besteko ugaritasuna, komunitate-tipoaren baldintza fisiko eta kimikoekin alderatuz, eta biomasaren batez besteko kontzentrazioen aldaketa hauek bertan dauden organismoen orekan eta uren eta sedimentuen kalitate fisiko-kimikoetan izan dezaketen eragina; eta loraketen maiztasun eta intentsitatea, eta hauen koherentzia komunitate tipoaren baldintza fisiko eta kimiko espezifikoekin, udako hilabeteetan gerta daitezkeen loraketa iraunkorretan interes berezia erakutsiz. Gainera, Itsas Estrategiaren Esparru Zuzentarauak (IEEZ), lehen aldiz 2008an ezarri (2008/56/EC) eta 2017an eguneratu (2017/848/EU) zenak, "egoera ona" lortu beharreko 11 ingurumen deskriptoreez osatutako zerrenda bat ezartzen du eta fitoplanktona 1, 2, 3 eta 5 deskriptoreen parte da.

Estuarioetako fitoplanktonaren ekoizpena eta konposizioa kontrolatzen duten mantenugaien sarrerak identifikatu, karakterizatu eta kudeatzea zaila den arren, ikerketak eta jarraipenek ezinbesteko informazioa eskaintzen dute epe-ertain eta luzean eutrofizazioa kudeatzeko estrategiak ezarri eta uren kalitatea berreskuratu eta babesteko. Fitoplanktonari dagokionez, ekosistema urtarren elikadura-kateari eusteko ezinbestekoa den fitoplankton ekoizpena lortzea da helburua, gehiegizko loraketarik gabe, eta taxoi kaltegarrien agerpena eta loraketak saihestu edo, gutxienez, kontrolatzea. Gainera, mantenugaien eta fitoplanktonaren jarraipena egitea ekosistema urtarrek jasaten dituzten aldaketa antropikoen berehalako inpaktuak neurtzeko baliagarriak izateaz gain, aldaketa kaltegarri horiek arintzeko kudeaketa-estrategien eraginkortasuna ebaluatzeko ere balio du (adibidez, Eccles et al., 2020).

5. Fitoplanktonaren jarraipenerako teknikak

Aurreko ataletan azaldu bezala, fitoplanktonaren jarraipena ezinbestekoa da ekosistema urtarren funtzionamendua ulertu edo/eta uraren kalitatea ebaluatzeko. Honenbestez, asko dira helburu ekologikoekin (Mao et al., 2020; Diana et al., 2021; Dedman et al., 2022; Chen et al., 2023) edo uren kalitatearen ebaluaziorako (Cupertino et al., 2019; Amorim eta Nascimento, 2021; Kanboj et al, 2022; Chandel et al., 2023) ur gezetan, estuarioetan edo itsasoan egin diren fitoplanktonaren jarraipenak.

Fitoplanktonaren jarraipena egiterako orduan kontuan hartu beharreko faktore garrantzitsu bat fitoplankton-komunitateen dinamismoa da, izan ere, sakabanatze espazial (mikrometroetatik metroetara) eta denbora-aldakortasun (minutuetatik egunetara bitarteko eskaletan) handia erakusten dute (Paerl eta Justic, 2013). Honek, ekosistemen berezko aldakortasun espazio-tenporal altuekin batera, fitoplanktonaren jarraipenerako teknikek lagin kopuru handiak azkar eta modu eraginkorrean, kostu minimoarekin, prozesatzeko beharra azaltzen du.

Asko dira gaur egun fitoplankton-komunitateen biomasa eta osaera ezagutzeko erabiltzen diren teknikak, hala nola (Kramer et al., 2023): mikroskopia (Karlson et al., 2010), errendimendu handiko kromatografia likido (HPLC) bidezko pigmentuen analisia (Mackey et al., 1996), teknika molekularrak (Jerney al., 2023), fluxu-zitometria (Sosik et al., 2010), *in situ* edo teledetekzio tekniketan oinarritutako estimazio optikoko metodoak (Uitz et al., 2015) edo zelula-eskaner kuantitatiboak (adibidez, Imaging FlowCytobot-arekin; Olson eta Sosik, 2007). Teknika hauetako bakoitzaren bereizmen-taxonomikoa ezberdina da eta, beraz, emaitzen zehaztasun maila ere ezberdina da. Honenbestez, teknikaren aukeraketa jarraipen-programaren xedearen araberakoa izango da. Hala ere, eskura dauden tresna guztien artean, ez dago indibidualki ekosistema bateko fitoplanktonaren benetako aniztasun guztia antzemateko eta zenbatzeko gai den teknikarik (Eriksen, 2021). Hori dela eta, posible izatekotan, fitoplankton-komunitateen jarraipenerako tekniken konbinazioa erabiltzea gomendatzen da, emaitza fidagarriagoak eta osatuagoak lortzeko (Huo et al., 2020; Pérez-Burillo et al., 2022).

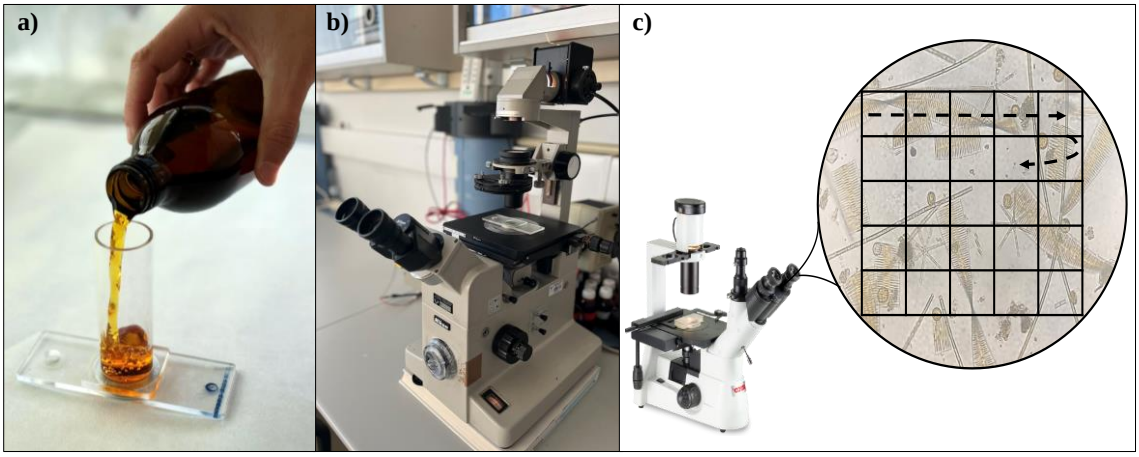
Jarraian, fitoplankton ugaritasuna eta osaera aztertzeko gehien erabiltzen diren hiru metodoak garatuko dira: teknika mikroskopikoetan oinarritutakoak, pigmentuak erabiltzen dituztenak eta teknika molekularrak aplikatzen dituztenak.

5.1. Mikroskopia

XVII. mendean Anton van Leeuwenhoek mikroskopioa asmatzeak fitoplanktonaren behaketa zehatzagoa ahalbidetu zuen, Christian Gottfied Ehrenberg eta Ernst Heinrich Philipp August Haeckel mikroalgen behaketan aitzindari bihurtuz (Karlson et al., 2010). Ordutik, mikroskopio optikoan oinarritutako fitoplanktonaren analisirako hainbat teknika garatu dira eta, mende bat baino gehiagoan zehar, mikroskopia optikoa fitoplanktonaren azterketarako oinarrizko tresna izan da, gaur egun ere espezieen identifikaziorako metodo baliagarria eta erabilia izaten jarraitzen duelarik.

Metodo mikroskopikoetan, fitoplanktonaren identifikazioa espezieen morfologian eta ikusgai dauden bestelako irizpideetan oinarritzen da. Hori dela eta, ezinbestekoa da taxonomistek fitoplanktonaren identifikazioan trebetasun eta esperientzia maila handia izatea. Gainera, beharrezkoa da fitoplanktona identifikatzeko oinarrizko literatura erabiltzea, hala nola, Horner (2002), Tomas (1997) eta Throndsen et al.-ena (2003, 2007). Mikroskopioaren kalitatea ere funtsezkoa da espezieen identifikaziorako (Karlson et al., 2010).

Gaur egun, fitoplanktonaren azterketarako gehien erabiltzen den teknika mikroskopikoa Utermöhl metodoa da (Utermöhl, 1931, 1958). Teknika honek, fitoplanktonaren identifikazioaz gain, zelulen zenbaketa ere ahalbidetzen du, hau bere abantaila nagusia delarik. Gainera, honen bidez, zelula bakoitzaren tamaina, forma, biobolumena (Olenina et al., 2006) eta atseden fasea zehazteko aukera dago. Utermöhl metodoan (*5. irudia*), laginen analisirako, oro har Lugolez fixatuta dagoen ur laginaren alikuota bat (50 ml edo 10 ml, normalean) sedimentatzen da zenbaketa-kameraren gainean eta, bolumenaren araberako sedimentazio-denbora bat itxaron ondoren, grabitatearen ondorioz zelulak kameraren beheko partean geratu eta alderantzizko mikroskopioa erabiliz zelulak identifikatu eta zenbatu egiten dira (Edler eta Elbrächter, 2010) (*5. irudiak*). Utermöhl metodoa sedimentazioaren ostean zelulek zenbaketa-kameran poisson banaketa dutenaren ustean oinarritzen da, beraz, fitoplanktonaren zenbaketa eta identifikazioa kamera osoan zehar edo trantsektuka egin daiteke, azalera konkretu bat bakarrik aztertuz. Teknika honen bidez lortzen diren emaitzak zelula/bolumen unitateetan ematen dira.



5. irudia. *Fitoplanktonaren identifikazio eta zenbaketarako Utermöhl metodoaren materiala eta irudikapena: laginaren sedimentazioa (a), alderantzizko-mikroskopioa eta zenbaketa kameraren kokapena (b) eta laginaren analisia (c).*

Gaur egun Utermöhl metodoa fitoplankton-komunitateen jarraipenean asko erabiltzen den arren (adibidez, Barth et al., 2020; Chin et al., 2022; Abirhire et al., 2023), hainbat muga eta desabantaila ditu. Alde batetik, mikroskopio optiko bidezko fitoplanktonaren identifikazioa oso konplexua da, izan ere, espezieen arteko antzekotasun ugari daude eta ezaugarri diagnostikoak mugatuak dira askotan, espezie kriptikoak agertzen direnean bereziki. Beraz, mikroskopiak ezagutza taxonomiko zabala eskatzen du (Naik et al., 2011) eta, ondorioz, emaitzak gaitasun pertsonalen araberakoak dira (Muñiz et al., 2020). Gainera, laginen analisirako denbora dezente behar da (Edler eta Elbrächter, 2010), 2 eta 10 h bitartean laginaren eta analisiaren sakontasunaren arabera, eta sedimentazio denborak lagina berehala aztertzeko aukera eragozten du. Bestetik, Utermöhl metodoak ez du pikoplankton autotrofoa aztertzeko aukerarik ematen (Edler eta Elbrächter, 2010), ezta beste organismo txiki batzuk ere, tamaina txikiegia edo estruktura hauskorrak dituztelako eta beraz ezin direlako mikroskopio optikoarekin identifikatu ezta zenbatu. Honen ondorioz, askotan hainbat espezie edo genero baztertuak izan dira fitoplankton-komunitateen azterketetan, haien omisio edo identifikazio okerren bidez (Jeffrey et al., 1997; Agirbas et al., 2015). Horrez gain, emaitzen zehaztasuna onargarria izan dadin, gutxienez 500 zelula zenbatu beharko lirateke lagin bakoitzeko (Edler, 1979), honek daraman denbora eta ahaleginarekin, batzuetan (biomasa gutxi edo materia organiko gehiegi dagoenean, adibidez) ezinezkoa dena. Halaber, mikroskopioaren bidezko zenbaketarekin, ezin da populazioen egoera fisiologikoa zehaztu, ezta talde bakoitzak Chl *a* totalari egiten dion ekarpena ezagutu ere.

Utermöhl metodoaz gain, mikroskopiaren beste aldaera batzuk ere oso erabiliak dira fitoplanktonaren azterketan. Batetik, 1950. urtetik aurrera, identifikazio taxonomiko zehatzago bat egiteko helburuz, mikroskopia elektronikoaren erabilera ugaritzen hasi zen, bai ekorketa-mikroskopia baita transmisiokoa ere (Karlson et al., 2010). Teknika hauek, fitoplankton zelulen egitura xehetasun handiagoz ikusteko aukera ematen zuten, hala nola, horma-zelularra, gorputzeko ezkatak edo flageloaren oinarriaren egitura, baina ez dira zenbaketarako erabiltzen (Vaulot, 2001). Ekorketa-mikroskopia elektronikoa (EME), konkretuki, oso erabilia da diatomeoa espezie desberdinak identifikatzeko (adibidez, Li et al., 2019), diatomeoen frustuluek handipen handietan bakarrik ikusi daitezkeen espezie-espezifikoak diren mikro- eta nano-patroiak dituztelako (Soleimani et al., 2021). Bestetik, fluoreszentzian oinarritutako mikroskopia ere aplikatzen da fitoplanktonaren azterketan, izan ere, fitoplanktonaren pigmentu fotosintetikoek eta bestelako pigmentuek sortutako fluoreszentzia espezieen identifikazio eta zenbaketarako laguntza gisa erabil daiteke (Karlson et al., 2010). Hori dela eta, fluoreszentzia mikroskopia oso erabilia da pikoplankton espezieen ikerketarako, adibidez, *Synechoccocus* generoko zianobakterioa eta proklorofizeentzako (Di Tullio et al., 2003). Fluoreszentzia hau, gainera, bi modutara aplikatu daiteke (Karlson et al., 2010): naturala edo tindaketen bidezkoa. Fluoreszentzia naturalari dagokionez, organismo autotrofoen fluoreszentzia-gaitasun propioa baliatzean datza eta lagin biziekin edo formaldeido/glutaraldeidoz fixatutako laginekin egiten da, Lugolak ez du balio. Honek, zenbaketaz gain, organismo heterotrofoak eta autotrofoak bereizteko ere balio du. Bestetik, zelulen tindaketan oinarritutako floreszentzia teknikak ere badaude, hala nola kalkofluorra (Fritz eta Triemer 1985) edo DAPI (Porter eta Feig, 1980), non tindagai fluoreszentea (fluorokromoak) organismoen egituretan itsatsi eta haien identifikazioan laguntzen duen. Zehazki, kalkofluorra, dinoflagelatuen teken zelulosa tindatzeko erabiltzen da (Fritz eta Triemer 1985) eta DAPI-k azido nukleikoa tindatzeko balio du, zelulen DNA edo nukleoa tindatuz zelula guztien ikuspegi orokorra ahalbidetuz (Porter eta Feig, 1980). Hala ere, aipatutako teknika mikroskopiko hauek, normalean, ez dira fitoplanktonaren jarraipena egiteko erabiltzen, baizik eta teknika osagarri gisa, komunitate osoaren analisian zentratu beharrean organismo edo talde konkretuagoen azterketa sakonagoan laguntzen baitute.

5.2. HPLC bidezko fitoplankton pigmentuen analisia

Fitoplanktonaren jarraipenaren xedearen arabera, gerta daiteke espezie-mailako identifikazioa eta zenbaketa beharrezkoak ez izatea. Eskala handian fitoplanktonak ziklo biogeokimikoetan edo dinamika-trofikoetan duen inpaktua aztertzeko, adibidez, nahikoa da

fitoplanktonaren jarraipen teknikek maila taxonomiko altuetan (hau da, klase edo taldeak) identifikazio eta zenbaketa eraginkorrak izatea. Hori dela eta, pigmentu fotosintetikoen analisia funtsezko informazio iturri bilakatu da fitoplanktonaren azterketa ekologiko eta fisiologikoak egiteko. Azterketa pigmentarioek dituzten aplikazio zehatzen artean, fitoplanktonaren populazio aldaketak ikertzea (adibidez, aldaketa klimatikoarekin lotutakoak), sateliteetatik eratorritako alga-biomasa kalkuluen egiaztapenak egitea, uretako argi-aldaketen erantzun fotosintetikoak aztertzea edo ekoizle primarioen transferentzia trofikoa ezagutzea daude (Garrido eta Roy, 2015).

Fitoplanktonaren pigmentu fotosintetikoak hiru familia kimikotan banatzen dira: fikobiliproteinak, karotenoideak eta klorofilak (Wright eta Jeffrey, 2006). Gehienetan, "fitoplankton pigmentuen analisia"-ren bidezko fitoplankton-komunitateen azterketa aipatzen denean, klorofilen eta karotenoideen azterketa bateratuari egiten zaio erreferentzia, izan ere, bi konposatu hauek alga fotosintetiko guztietan agertzen dira, disolbatzaile organikoetan erraz erauzten dira eta sentikortasun handiz hauteman daitezke eremu ikuskorrean (Garrido eta Roy, 2015). Klorofila eta karotenoide hauek fitoplankton taxoi ezberdinetan agertzen dira espezifikotasun maila aldakorrarekin (Roy et al., 2011): batzuk fitoplankton talde ezberdinetan agertzen dira, izaera orokorragoa erakutsiz; eta beste batzuk fitoplankton talde bakarrera edo gutxi batzuetara mugatuta daude, "pigmentu diagnostiko" (PD) bezala jokatuz. Hori dela eta, klorofila eta karotenoideak markatzaile kimiotaxonomiko anbiguoak direla esan daiteke. Fitoplankton talde ezberdinen eredu-pigmentarioak *1. taulan* zehazten dira, pigmentu ezberdinek klase fitoplanktoniko nagusietan duten garrantzia irudikatuz.

60ko hamarkadatik, espektrofotometria edo fluorimetria bezalako teknikak ohikoak izan dira fitoplanktonaren azterketa pigmentarioan (Richards eta Thompson, 1952; Parsons eta Strickland 1963; Yentsch eta Menzel, 1963), baina teknika hauek muga anitz dituzte. Mugen artean, azpimarratzekoak dira, espektrofotometrian, argitaratutako ekuazio normalizatu eta xurgapen koefiziente anitzen artean aukeratzeko beharra eta, fluorimetrian, klorofila taldeen bereizmen txikia eta karotenoideak detektatzeko ezintasuna. Honen ondorioz, alternatiba bezala, fluoreszentziaren zuzeneko neurketa eta kromatografia likidoa bezalako tekniken erabilera asko areagotu da.

1. taula. *Fitoplankton klase nagusien pigmentu ugari eta taxonomikoki adierazgarrienen banaketa (Jeffrey eta Vesk, 1997-etik moldatua). Chl a: a klorofila; Chl b: b klorofila; Chl c_1: c_1 klorofila; Chl c_2: c_2 klorofila; Chl c_3: c_3 klorofila; Chl c_2-MGDG: c_2-MGDG klorofila; MgDVP: Mg-2,4-dibinil feoporfirin a5 monometil ester; DV Chl a: Dibinil Chl a; DV Chl b: Dibinil Chl b; βε–Car: α karotenoa; ββ–Car: β karotenoa; βγ–Car: γ karotenoa; εε–Car: ε karotenoa.*

Kodea: ●= pigmentu nagusia (>% 10); •= pigmentu minoritarioa (% 1-10); ⋅ = aztarna pigmentua (<% 1)

	Zianobakterioak I	**Zianobakterioak II**	**Errodofitoak**	**Kriptofizeoak**	**Klorofizeoak**	**Prasinofizeoak**	**Euglenofizeoak**	**Eustigmatofizeoak**	**Diatomeoak**	**Dinoflagelatuak**	**Primnesiofizeoak**	**Krisofizeoak**	**Rafidofizeoak**
I. Klorofilak													
Chl *a*	●		●	●	●	●	●		●	●	●	●	●
Chl *b*					●	●	●	●					
Chl c_1									●		●		●
Chl c_2				●					●	●	●	●	●
Chl c_3											●	●	
Chl c_2-MGDG											⋅		
MgDVP		⋅				●							
DV Chl *a*		●											
DV Chl *b*		●											
II. Karotenoak													
βε–Car				⋅						⋅			
ββ–Car	⋅	⋅			⋅	⋅	⋅		⋅	⋅	⋅	⋅	●
βγ–Car					⋅			●				⋅	
εε–Car				⋅								⋅	
Xantofilak													
Aloxantina				●									
Anteraxantina					⋅	⋅	⋅						
Biolaxantina					●	●		●					
19'-butanoiloxifukoxantina											●	●	
Diadinoxantina							●		●	●	●	●	●
Diatoxantina							⋅		⋅	⋅	⋅	⋅	⋅
Dinoxantina										●			
Fukoxantina									●		●	●	●
19'-hexanoiloxifukoxantina											●		
Krokoxantina				⋅									
Luteina					●	⋅							
Monadoxantina				⋅									
Neoxantina					●	●	⋅						
Peridinina										●			
Prasinoxantina						●							
Zeaxantina	●	●	●		⋅			●					

Gaur egun, bereizmen altuko kromatografia likidoa (HPLC) da fitoplanktonaren klorofila eta karotenoideen identifikazio eta kuantifikazio zehatza egiteko aukerazko teknika (Garrido eta Roy, 2015). Hala ere, hain erabilia den teknika honek, urteetan zehar, hainbat hobekuntza jasan ditu, fitoplankton-komunitatearen azterketa eraginkorra burutu ahal izateko (Garrido et al., 2011; Roy eta Garrido 2013). Metodo klasikoek fase alderantzikatuko C18 zutabea eta metanol (Gieskes eta Kraay, 1983) edo azetonitriloa (Wright eta Shearer, 1984) bezalako disolbatzaileak erabiltzen zituzten. Mantoura eta Llewellynek (1983) eta Zapata et al.-ek (1987) klorofila polarrenen erretentziorako hobekuntzak egin zituzten eta, beranduago, aurreko metodoek zituzten abantailak konbinatuz, Wright et al.-ek (1991) nazioarteko ozeanografia programetan protokolo gisa erabili zen metodo bat garatu zuten, ozeanoen ikerketarako batzorde zientifikoak (SCOR) gomendatzen zuena (Wright eta Jeffrey, 1997). Hala ere, metodo honek ez zuen Chl *c*-en banaketa behar bezala egiten, ezta Chl *a* eta Chl *b*-ren mono-dibinil bikoteena ere. Honen harira, Rodriguez et al.-ek (1998) C8 zutabean oinarritutako metodo bat aurkeztu zuten, aurretik aipatutako arazoak ebazten zituena kultibo monoalgaletan, baina ur lagin arruntekin ordea ez. Zapata et al.-ek (2000), fase mugikorrari piridina gehituz, bereizmen eta errepikakortasun handiko metodoa aurkeztu zuten, gutxienez 20 klorofila eta 34 karotenoide bereizteko gai zena. Gaur egun, HPLC bidezko fitoplankton pigmentuen analisian gehien erabiltzen diren metodoek (Zapata et al., 2000; Van Heukelem eta Thomas, 2001) C8 zutabean oinarritzen dira, Chl c_1/c_2 eta Chl *a*/DVChl *a* bikoteen bereizmen ona lortuz, baina ez dira Chl *b*/DVChl *b* bikotearen banaketa egokia egiteko gai (Wright eta Jeffrey, 2006). Hori dela eta, azken urteetan zutabe ezberdinen erabilera proposatzen duten metodo alternatibo batzuk garatuz joan dira, hala nola, Jayaraman et al.-ek (2011) (C16-Aminda zutabea) edo Sanz et al.-ek (2015) (Silize-pentafluorofeniloctadecil zutabea) proposatutakoa.

Edonola ere, gaur egun, HPLC-ak fitoplankton talde ezberdinek dituzten 50 pigmentu baino gehiago identifikatzeko eta kuantifikatzeko aukera ematen du (adibidez, Zapata et al., 2000). Teknika honek, Chl *a*-ren kuantifikazioa zehaztasun handiz egiteko aukera ematen du, fitoplankton talde guztietan dagoen pigmentua eta, ondorioz, fitoplanktonaren biomasa totalaren estimaziorako erabiltzen dena (Jeffrey et al., 1997). Gainera, fitoplankton-komunitatearen osaera zehazteko balio duten pigmentu diagnostiko anitzen identifikazio eta kuantifikazioa ahalbidetzen du (Barlow et al., 2008). Honenbestez, HPLC bidezko pigmentuen analisia, mikroskopiaren alternatiba baliagarria izan daiteke, fitoplanktonaren biomasa totala eta komunitatearen konposizioa zehazteko helburuak betez, aztertutako bolumen handiago batean oinarrituta (Agirbas et al., 2015). Gainera, mikroskopiak ez bezala,

pigmentuen analisiak, piko- eta nano-fitoplanktonaren kuantifikazioa ahalbidetzen du, populazioen egoera fisiologikoa balioztatzeko aukera ematen du, analisia azkarra da eta teknikaren errepikakortasuna eta objetibotasuna altua da (Wright eta Jeffrey, 2006; Barlow et al., 2008; Roy eta Garrido, 2013). Ondorioz, pigmentuen analisia asko erabiltzen da fitoplanktonaren jarraipenerako (Damar et al., 2020; Miranda-Alvarez et al., 2020; Mudakikwa et al., 2021; Cereja et al., 2022; Lee et al., 2022). Hala ere, metodo honek muga garrantzitsu bati aurre egin behar dio fitoplankton-komunitatearen osaera aztertzeko erabiltzen denean: fitoplankton talde batzuek PD esklusiborik ez izateak eta zenbait PD hainbat taldetan agertzeak hauen bereizketa-ahalmena murrizten du (Latasa, 2007; Nunes et al., 2018).

Muga honi aurre egiteko asmoz, pigmentuen datu multzoen interpretazio taxonomikorako (kimiotaxonomia) tresna matematiko desberdinak garatu dira urteetan zehar (Letelier et al., 1993; Mackey et al., 1996; Uitz et al., 2006; Latasa, 2007; Van den Meersche, et al. 2008; Kramer eta Siegel, 2019; Hayward et al., 2023). Tresna hauek, PDak eta fitoplankton taldeak erlazionatzeko eta talde horietako bakoitzak fitoplanktonaren biomasa totalean (Chl *a*) duen ekarpena zehazteko, PD:Chl *a* ratioen erabileran oinarritzen dira (Higgins et al., 2011). Gieskes eta Kraay-k (1983) erregresio lineal anitz proposatu zituzten PD espezifikoen eta Chl *a*-ren arteko erlazioa zehazteko, baina ez zuten kontuan hartu zenbait PD hainbat talderen artean partekatzen direla. Partekatutako PD-en arazoari aurre egiteko, hainbat autorek (adibidez, Letelier et al., 1993; Vidussi et al., 2001) alderantzizko aldibereko ekuazioak (ISE) erabiltzea proposatu zuten, DPen kontzentrazioen kenketak eginez, fitoplankton talde bakoitzak Chl *a* totalari egindako ekarpena kalkulatuz. ISE metodoaren antzeko oinarriarekin, Mackey et al. (1996) CHEMTAX softwarea garatu zuten, erabilera errazagoa zuena (Higgins et al., 2011). Kasu honetan, ekuazioak eraiki beharrean, erabiltzaileak pigmentu:Chl *a* ratio matrize bat eraikitzen du espero den fitoplankton talde guztiak biltzen dituena. Horren ondoren, Bayesian Compositional Estimator (BCE) (Van den Meersche et al., 2008) eta Phytoclass (Hayward et al., 2023) bezalako metodo kimiotaxonomiko alternatiboak proposatu dira pigmentuen bidez fitoplankton-komunitateen osaera deskribatzeko. Gainera, pigmentuen interpretazio ez-taxonomikoak ere proposatu dira, Vidussi et al-ek (2001) eta, geroago, Uitz et al.-ek (2006) deskribatutakoak esaterako, fitoplankton-komunitatearen tamaina-klaseak zehaztea helburu dutenak.

Pigmentuen datuetatik abiatuta fitoplankton-komunitateen osaera eta talde bakoitzak fitoplanktonaren biomasa totalari egiten dion ekarpena kalkulatzeko aipatutako tresnak oso

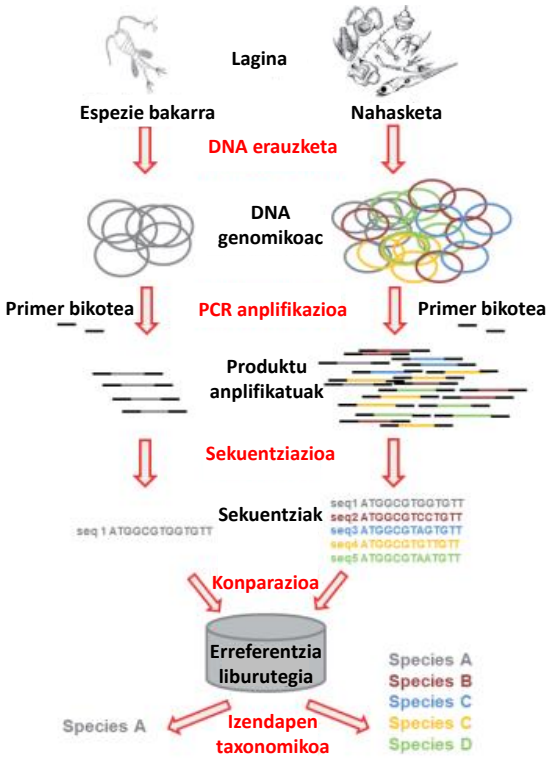
erabiliak dira fitoplanktonaren jarraipenetan (adibidez, Uitz et al., 2009; Chase et al., 2020; Flander-Putrle et al., 2021), batez ere CHEMTAX-a (adibidez, Miranda-Alvarez et al., 2020; Wang et al., 2021; Hyun et al., 2022). Hala ere, kontuan hartu beharreko eragozpen komun batzuk partekatzen dituzte, hala nola, PD bat taxoi fitoplanktoniko batekin zuzenean lotzeak dakarren gehiegizko sinplifikazioa (PD batzuk hainbat taldek elkar banatzen dituztelako) eta tresnak modu egokian aplikatzeko komunitatearen osaeraren aurretiazko ezagutzaren beharra.

5.3. Teknika molekularrak – DNA metabarcoding-a

70eko hamarkadatik aurrera, errendimendu altuko sekuentziazio (High-Throughput Sequencing, HTS; edo Next-Generation Sequencing, NGS) teknologien garapenari esker, metodo molekularrak fitoplankton taxoiak identifikatzeko aukerazko teknika bihurtzen ari dira dibertsitatearen ezagutza helburu duten jarraipen-lanetan (Trebitz et al., 2017; Rimet et al., 2021). Teknika molekular anitz daude, nukleotido sekuentziak aztertuz, komunitate biologikoen karakterizazio taxonomiko eta metabolikoa egiteko, espezie toxikoak edo inbaditzaileak azkar detektatzeko, populazioen arteko konektibitatea zehaztu edo gizabanakoei populazioak esleitzeko gai direnak (Bourlat et al., 2013). Teknika genomiko hauen artean barcoding-a, metabarcoding-a, metagenomika, metatranskriptomika, qPCR eta SNP genotyping-a daude, baina itsasoan zein trantzisio uretan fitoplanktonaren dibertsitatea eta banaketaren jarraipena egiteko teknikarik erabiliena eDNA metabarcoding-a da (Jerney et al., 2023).

Alde batetik, ingurumen DNA (eDNA) biodibertsitatearen karakterizaziorako tresna indartsua da, izan ere, komunitateen detekzio sentikorra eta erresoluzio altukoa eskaintzen du eta eraginkorra da gutxi aztertutako edo arraroak diren espezieak aztertzeko (Bunholi et al., 2023). Bestetik, "barcoding"-a, DNA zati estandarizatu labur baten (barcode) sekuentziazioaren bidez ale bati taxoi bat esleitzeko metodoa da (Bucklin et al., 2011). *Barcode*-a, espezie bakoitzarentzat esklusiboa da, beraz, espeziaren identifikazio eta diskriminaziorako balio du. Metodo hau, espezie bakar batentzat izan beharrean, komunitate oso bat barne hartzen duen laginetan aplikatzen denean, "DNA metabarcoding" izenez ezagutzen da (Bourlat et al., 2013) (*6. irudia*). Beraz, eDNA metabarcoding-a, DNA eskualde laburren sekuentziazioan oinarritutako lagin naturalen komunitate osoaren aldibereko analisi molekularra da (Jerney et al., 2023). Teknika honetan, *6. irudian* ikusten denez (Corell eta Rodriguez-Ezpeleta, 2014), *barcode*-aren albo bietan kokatzen diren *primer*-ak (DNAa

sekuentzia laburrak) erabiltzen dira DNA genomikotik abiatutako *barcode*-aren anplifikaziorako, PCR (Polymerase Chain Reaction) bidez egiten dena (Green eta Sambrook, 2012). Metabarcoding-aren kasuan, DNA lagin osotik erauzten denez, PCRak ez du produktu bakarra anplifikatzen, lagineko espezie ezberdinei dagozkien produktuen nahasketa baizik. Metabarcoding-etik lortutako produktuen sekuentziaziorako, errendimendu handiko sekuentziazio teknologiak (edo NGS) erabiltzen dira (hala nola, Illumina edo 454) produktu nahasketen sekuentziazio zuzena ahalbidetzen baitute (Nowrousian, 2010). Amaitzeko, metabarcoding-a (baita barcoding-a ere) aurretik eraikitako erreferentziazko datu-baseetan oinarritzen da sekuentzien eta espezieen arteko loturak zehazteko (Bourlat et al., 2013).



6. irudia. *Barcoding (ezker) eta metabarcoding (eskuin) tekniken urratsen irudikapen eskematikoa. Corell eta Rodriguez-Ezpeleta-tik (2014) moldatua.*

Mikroskopia bezalako metodo tradizionalekin konparatuz, metabarcoding-a komunitateen biodibertsitatearen azterketarako metodo eraginkorragoa da, izan ere, edozein bizitza-fasetan dauden espezieak, espezie kriptikoak eta hauskorregiak edo txikiegiak izateagatik baztertuak izan diren espezieen identifikazioa ahalbidetzen du (Comtet et al., 2015; Jerney et al., 2023). Gainera, datu-multzoak osoagoak dira, bizkorrago eskuratu daitezke eta ez daude trebetasun taxonomikoaren menpe (Penna et al., 2017; Trebitz et al., 2017). Era berean, barcoding-arekin konparatuz, prozesu neketsua dena espezie bakoitza bakarka prozesatu behar delako (Stein et al., 2014), metabarcoding-ak lagin osoak aztertzea ahalbidetzen du, organismoak isolatu beharrik gabe. Horrek, lagin kopuru handiagoak denbora tarte txikiagoan aztertzeko aukera

ematen du, eta ondorioz, jarraipenen kostuak murriztu eta eskala handiagoko jarraipenak egitea ahalbidetzen du (Jerney et al., 2023). Hau guztia kontuan hartuz, eta azken urteotako errendimendu altuko sekuentziazio teknologien garapenari esker, fitoplankton-komunitateen jarraipena egiteko metabarcoding-a erabiltzen duten ikerketa asko daude gaur egun (adibidez, Chen et al., 2019a; Liu et al., 2020; De Luca et al., 2021; Pérez-Burillo et al., 2022; Zhang et al., 2023), mikroskopia bezalako metodo tradizionaletan oinarritutako komunitateen azterketetan aurkitutako mugak gaindituz (adibidez, Gran-Stadniczeñko et al., 2019). Gainera, munduan zehar egiten diren itsas-ingurumeneko proiektu askotan aplikatzen da metabarcoding-a fitoplanktona aztertzeko, hala nola, Tara Oceans Expedition (Malviya et al., 2016) eta Ocean Sampling Day (Kopf et al., 2015). Honek, metabarcoding-a fitoplankton-komunitateen dibertsitate espezifikoaren informazioa eskaintzeko eta fitoplanktonaren jarraipen-programetan erabiltzeko teknika aproposa dela frogatzen du.

Hala ere, metabarcoding teknikaren erabilera eraginkorra izan dadin eta emaitza fidagarriak lortu ahal izateko, hainbat premisa esperimental eta analitiko bete behar dira: "barcode"-aren aukeraketa egokia, datu-baseen osotasuna eta protokolo estandarizatuen erabilera. Lehenik eta behin, aztergai diren organismo guztietan dagoen DNA zati ("barcode") bat aukeratu behar da, espezieen arteko bereizketa ahalbidetzeko nahikoa sekuentzia-aldakortasuna duena (Hebert et al., 2003) eta, PCR bidezko anplifikazioan parte hartuko duten *primer* unibertsalen diseinua errazteko, alboetan kontserbatutako eskualdeak dituena (Leray et al., 2015). Gainera, "barcode" eta taxonomiaren arteko lotura egin ahal izateko datu-base osatuak eta zehatzak egon behar dira (Zepeda-Mendoza et al., 2015), izendapen taxonomikoa datu-basea bezain zehatza eta fidagarria baita. Azkenik, laginak prozesatzeko, baita datuak aztertzeko, protokolo estandarizatuak erabili behar dira, aztergai den komunitatearen karakterizazio fidagarria bermatu eta emaitza erreproduzigarriak eta konparagarriak sortzea ahalbidetuz (Creer et al., 2016).

Gaur egun, ingurumen laginetan metabarcoding bidez mikroorganismo itsastar eukariotoen aniztasuna eta komunitate-egiturak zehaztasunez aztertzeko markagailurik erabiliena 18S rRNA genea da (Martin et al., 2022). Gene honetan, fitoplankton biodibertsitatea aztertzeko balio duten oso aldagarriak diren hainbat eskualde daudela ikusi da (V1-3, V4 edo V9, adibidez), eta hauen hautaketak eztabaidagai izaten jarraitzen duen arren, eskualde erabilienak V4 (450 base-pare [bp] inguru) eta V9 (150 bp inguru) dira (Dos Santos et al., 2022). Hasieran, Illumina teknologiak sekuentzia-tamainan mugatua zuenez, V9-aren erabilera nagusitzen zen (Amaral-Zettler et al., 2009), baina Illumina MiSeq-aren garapenak

V4 bezalako eskualde luzeagoen sekuentziazioa ahalbidetu zuen (van Dijk et al., 2014). Ordutik, hainbat ikerketek V4 eta V9-ak eukarioto urtarren dibertsitatea aztertzeko duten eraginkortasuna alderatu dute (Traging et al., 2018; Zhang et al., 2019), eskualdearen aukeraketa aztertu nahi den maila taxonomiko edo organismo taldearen araberakoa izan behar dela ondorioztatuz (Tragin et al., 2018). Hala ere, orokorrean, fitoplankton-komunitatearen osaera aztertzeko, 18S rRNA V4 eskualdea da gehien erabiltzen dena (adibidez, Piredda et al., 2017; Gran-Stadniczeñko et al., 2019; Liu et al., 2020; Wang et al., 2022), luzeagoa eta aldakorragoa delako, eta bere datu-baseak osatuagoak direlako (Dos Santos et al., 2022). Aipatu behar da, berriki, V7 eskualdea erabiltzen duen ikerketa bat argitaratu dela (Huo et al., 2020) eta 18S ez den beste markatzaile batzuk ere erabiliak direla fitoplankton-komunitateen ikerketetan, adibidez ITS (Zhang et al., 2019), 16S (Eiler et al., 2013; Esenkulova et al., 2019) eta 23S (Yoon et al., 2016).

Bestalde, *primer*-en hautaketa ere funtsezkoa da fitoplankton-komunitateen identifikazio taxonomiko zehatza lortu ahal izateko (Vaulot et al., 2021), baita tratamendu bioinformatikoa ere (Pauvert et al., 2019). Metabarcoding-en bidezko espezieen hautematea PCR *primer*-en aukeraketaren araberakoa da, hauek taxoi guztiak anplifikatzeko nahikoa generiko eta aldi berean espezieak bereizteko adina espezifikoa izan behar dutelarik (Jerney et al., 2023). Fitoplanktonaren kasuan, bi baldintza hauek betetzen dituen *primer* bakarra aukeratzea zaila da, izan ere, fitoplanktonak elkarrengandik urrun dauden talde taxonomiko ugari biltzen ditu. Fitoplankton eukariotoaren dibertsitatea aztertzeko *primer* unibertsalen erabilera oso hedatua dago (adibidez, Stoeck et al., 2010; Hadziavdic et al., 2014; Tanabe et al., 2016), eta aukera onenaren inguruko adostasunik egon ez arren, 18S rRNA V4 markagailuarekin lan egiterako orduan gomendagarrienak Balzano et al. (2015)-ena (Latz et al., 2022) edo Stoeck et al. (2010)-ena dira (Vaulot et al., 2022). Hala ere, ohikoa da aniztasun handiagoa detektatzeko helburuz markatzaile eta *primer* bikote anitzak erabiltzea (Smith et al., 2017; Alberdi et al., 2018; Zhang et al., 2018; Choi eta Park, 2020). Metabarcoding-aren tratamendu bioinformatikoari dagokionez, kanalizazio konputazionalen (*computational pipeline*-en) bidez burutzen da eta hurrengo pausuak biltzen ditu: luzera eta kalitatean oinarritutako sekuentzia gordinen iragazketa, sekuentziak Unitate Taxonomiko Operatiboetan (*Operational Taxonomic Unit*; OTU) edo Anplikon Sekuentziaren Aldaeretan (*Amplicon Sequence Variants*; ASV) taldekatzeko algoritmo eta baldintzak ezartzea eta datu-baseetan oinarritutako identifikazio-taxonomikoen esleipena (Tragin et al., 2018; Mortagua et al., 2019). Kanalizazio softwar-aren artean erabilienetakoa DADA2 (Callahan et al., 2016) da, R softwar-aren bitartez edo QIIME2 (Boylen et al., 2019) softwar-aren parte bezala, baina

Mothur (Schloss et al., 2009) edo PEMA (Zafeiropoulos et al., 2020) bezalako "pipeline"-ak ere erabiliak dira. Datu baseei dagokienez, NCBI GenBank (Benson et al., 2012) da informazio gehien biltzen duena, baina badaude fitoplanktonarentzat zehatzagoak diren eta adituek osatuak diren datu base eraginkorragoak, hala nola, SILVA (Quast et al., 2013), PR2 (Guillou et al., 2013) eta GTDB (Parks et al., 2022). Gainera, datu molekularrak taxon-inbentario ekologikoetan eraldatzea ahalbidetzen duten software hauek eta izendapen taxonomikoa baldintzatzen duten datu-baseak etengabe hobetzen ari dira (Brandt et al., 2021; Jenreyet al., 2023).

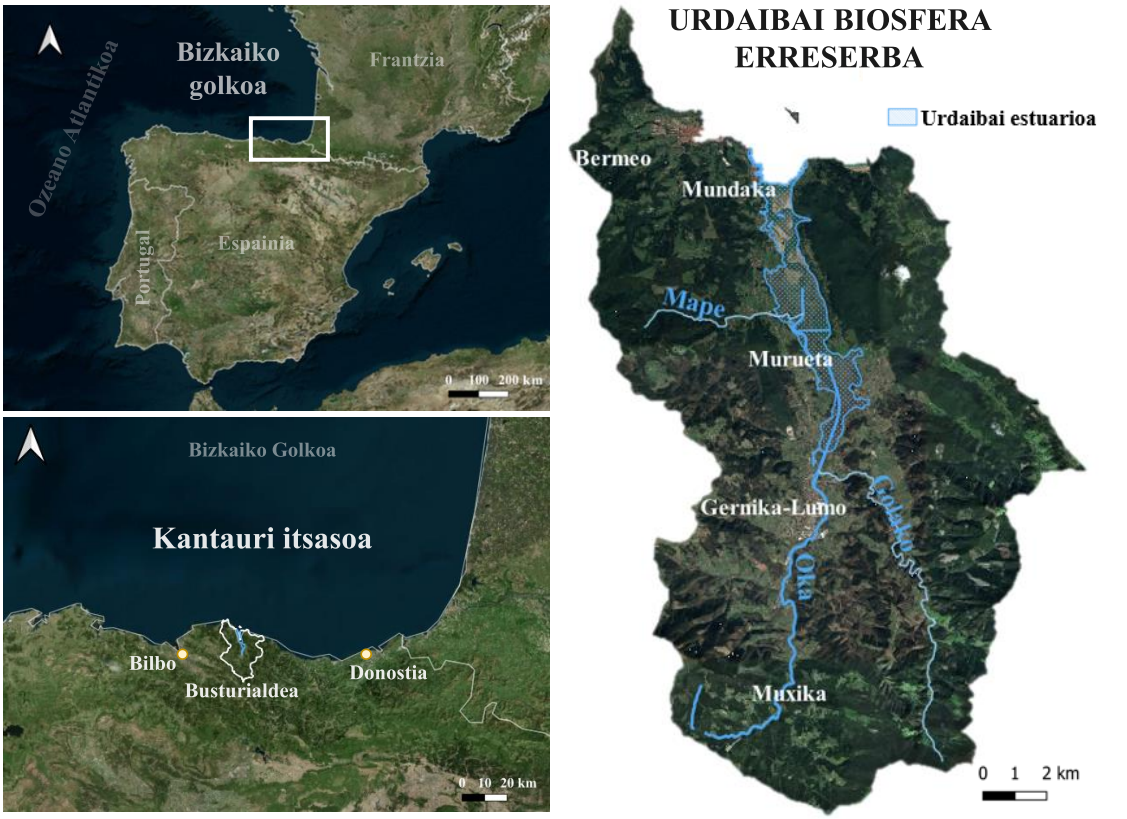
Hala ere, metabarcoding-a fitoplankton-komunitateen biodibertsitatea aztertzeko gero eta aukera hobea bihurtzen ari den arren, hainbat muga ditu. Alde batetik, emaitzen eraginkortasuna eta zehaztasun-maila, hein handi batean, oinarrizko hautaketa metodologikoen (gene markagailua, *primer* bikotea edo bioinformatika erabakiak) menpe dago (Santoferrara, 2019). Gainera, metabarkoding-aren lan-fluxuan zehar gerta daitezkeen alborakuntza teknikoek ere eragin negatiboa izan dezakete emaitzetan (Taberlet et al., 2012; Martin et al., 2022), hala nola, kutsadura edo/eta laginen kontserbazioan (Mäki et al., 2017), DNA erauzketan (Van der Loos eta Nijland, 2017) eta anplifikazioan (Latz et al., 2022) gerta daitezkeen aldakortasunak. Emaitzen aldakortasun eta arazo tekniko hauetako asko, ordea, protokolo estandarizatuak jarraituz ekidin daitezke (Bunholi et al., 2023), izan ere, eDNA metabarcoding-a burutzeko gida eta protokolo batzuk eskuragarri daude jada, hala nola, Elersek et al.-ek (2021) ur gezako fitoplanktona aztertzeko egindakoa edo Jerney et al.-ek (2023) fitoplankton itsastarrarentzako egindakoa. Bestetik, fitoplankton espezie asko mikroskopiaren bidez identifikatzeko edo/eta isolatzeko zailak dira, eta, beraz, ez dago hauen erreferentzia molekularrik eskuragarri eta zaila da datu-base osatuak sortzea. Hori dela eta, datu baseen osotasun falta de teknika honen mugarik handienetako bat (Rimet et al., 2021). Azkenik, DNA metabarcoding-aren muga nabarmenena eta fitoplanktonaren jarraipen metodo eraginkorra izatea ekiditen duena kuantifikazio gaitasun eza da. 18S rRNA-ren multikopia izaerak eragin zuzena dauka metabarcoding bidez identifikatutako taxoien ugaritasun emaitzetan, izan ere, fitoplankton espezie, genero eta talde bakoitzaren gene kopia kopurua aldakorra da (Santi et al., 2021; Martin et al., 2022). Honen ondorioz, metabarcoding-ren bidez taxoien ugaritasun erlatiboak kalkulatu daitezke, baina ez dago taxoien ugaritasun absolutua zehazteko aukerarik (Jenrey et al., 2023), metodo erdi-kuantitatiboa izatera mugatuz (Albaina et al., 2016). Hala ere, hainbat ikertzailek (Martin et al., 2022; Piwosz et al., 2020) defendatzen dute eDNA metabarcoding bidez zehazten diren

komunitate ugaritasun erlatiboak erabilgarriak eta fidagarriak direla interpretazio ekologikoen testuinguruan.

II. IKERKETA EREMUA

Urdaibai estuarioa, Oka, Mundaka edo Gernikako estuarioa bezala ere izendatua izan dena, Oka ibaiaren bokalean eratutako eremu naturala da, Urdaibai Biosfera Erreserbaren ardatz zentrala. Euskal Autonomia Erkidegoko (EAE-ko) mendebaldeko kostaldean kokatua dago, Bizkaian, Busturialdeko eskualdean hain zuzen ere (*7. irudia*). Honenbestez, estuarioa Kantauri itsasoan itsasoratzen da, Bizkaiko golkoan, Matxitxako eta Ogoño lurmuturren artean konkretuki (43º 22´ I, 2º 40´ M).

Kantauri itsasoko kostalde osoa bezala, Urdaibaiko eskualdea klima ozeanikoaren menpe dago, honen ezaugarri nagusiak aldaketa termiko txikiak, hezetasun erlatibo altua, urtean zeharreko euri-jasen ugaritasuna eta hauen banaketa homogeneoa eta izozteen urritasuna dira. Honenbestez, eremu honetan, prezipitazioak ugariak izaten dira (1000-1200 mm, urtean 180-200 egun), maximoak udaberri/udazkenean eta minimoak udan izanik, eta eguzki-erradiazioaren balioak nahiko baxuak (urteko batez bestekoa 3,8 kWh m^{-2} baino txikiagoa). Tenperaturari dagokionez, Mexikoko Golkotik iristen den ur korronte beroa (Gulf Stream) aipatu behar da, kostalde honetako urak nabarmen epeltzen baititu, baita troposferaren erdialde eta goiko aldeko mendebaldeko haize (Westerlies) atmosferikoek duten eragina ere (Usabiaga et al., 2004).



7. irudia. *Ikerketa eremuaren kokapena. Ezkerraldean, Busturialdea eskualdearen eta Urdaibai estuarioaren kokapena, Bizkaiko golkoaren (goian) eta Kantauriar kostaldearen (behean) baitan. Eskuinaldean, estuarioaren kokapena Urdaibai Biosfera Erreserbaren barruan.*

Estuariora urak isurtzen dituen ibai nagusia Oka da. Ibai honek Zugaztietan du iturburua, Oiz, Gorroño eta Bizkargai mendietatik datozen errekastoak elkartzen diren lekuan. Kantauriar isurialdeko gainerako ibaiak bezala, Oka ibaia, laburra, malda handikoa eta izaera torrentzialekoa da, eta 3,6 m^3 s^{-1}-ko batez besteko emaria dauka (Monge-Ganuzas, 2019; Barroeta et al., 2023), urtaroaren arabera nahiko aldakorra dena, udako lehorte garaietan 0 m^3 s^{-1}-koa izatetik neguko garai euritsuenetan 17 m^3 s^{-1}-koa izatera arte (Madariaga eta Orive, 1995). Guztira, Okaren ibai-arroak 183,21 km^2 inguruko azalera drainatzen du (Barroeta et al., 2023). Gainera, Oka-z gain, badira estuariora urak zuzenean isurtzen dituzten beste hainbat ibai ere (*7. irudia*), hala nola, Golako ibaia (eskuineko isurialdean, Gernika parean), Mape ibaia (ezkerreko isurialdean, Forua parean), eta beste zenbait errekasto (adibidez, Amunategi, Busturian). Hauen artean, Golako ibaia da, Oka ibaiaren atzetik, estuarioari ekarpen handiena egiten diona. Horrez gain, Urdaibaiko lur azpiko baliabide hidrikoak ere aipatzekoak dira (Santa Eufemia-Ereñozar unitate karstikoa eta Gernikako unitate detritikoa), gainazaletik doazen urekin batera sistema hidrodinamiko bakarra eratzen baitute, zeinetan batzuen eta besteen fluxuak elkar eragiten duten eta elkarren menpekoak diren.

2. taula. *Urdaibai estuarioko ezaugarri nagusiak (Villate et al., 1989[a]; Valencia et al., 2004[b]; Monge-Ganuzas et al., 2019[c]; Barroeta et al., 2023[d]).*

Parametro hidromorfometrikoak	
Estuarioaren luzera (km)[d]	13,7
Bokalaren sekzioa (m^2)[a]	1.286
Estuarioaren batez besteko sakonera (m)[a,d]	2,59
Estuarioaren sakonera maximoa (m)[b]	10
Urteko batez besteko ibaiaren emaria ($m^3\ s^{-1}$)[b,c,d]	3,6
Arroaren azalera (km^2)[d]	183,21
Estuarioaren azalera (km^2)[a,d]	1.89
Estuarioaren batez besteko bolumena (m^3)[b]	$3{,}29 \times 10^6$
Estuarioaren batez besteko marea prisma (m^3)[a,d]	$4{,}86 \times 10^6$
Estuarioaren bolumena/Ibaiaren emaria (egunak)[b]	10,6
Prisma mareala/Bolumena[d]	1,47

Estuarioaren berezko ezaugarri fisikoei eta geomorfologikoei dagokienez (*2. taula*), estuarioaren goiko mugatik (Gernikan) kanpoko mugaraino (Mundakan) estuarioak 13,7 km inguruko luzera dauka, zabalera maximoa 1 km-koa da eta, guztira, 1,89 km^2-ko azalera hartzen du (Barroeta et al., 2023). Sakonera txikiko estuarioa da, batez besteko sakonera 2,6 m ingurukoa izanik, baina hau estuarioaren kanpoaldean asko handitzen da, Astilleros Murueta S.A. ontziolak itsasontziak atera ahal izateko egin diren dragatze lanen ondorioz. Geomorfologia kontuan hartuz, Pritchard-ek (1952) proposatutako sailkapenaren arabera, Urdaibaiko estuarioa urperatutako ibai-harana da eta hiru zati ezberdinetan banatua izan da (Villate et al., 1989) (*8. irudia*): kanpoaldeko eremua edo itsastarra, Mundakatik Kanala hondartzaraino (3,5 km-ra), zabalera handieneko eremua da eta itsasbeheran agerian geratzen diren harea eta limozko sedimentuez osatutako mareartеko eremuak dira nagusi; erdiko eremuan, Muruetatik ubide-artifizialeraino, estuarioaren zabaleraren murrizketa nabarmena dago, sedimentuak buztinez, hondarrez eta legarrez osatuta daude (proportzio aldakorretan) eta kanal nagusiaren inguruan kanal bihurgunetsuen sare konplexua eta padura garatuagoa agertzen da; eta barruko eremua, Gernika herriraino heltzen den 15 m inguruko zabalera eta 4 km-ko luzera duen ubide artifiziala, eremu erabat eraldatua da, limoz eta lokatzez osatutako sedimentuak ditu eta espezie inbaditzaile zuhaixkarez (*Baccaris halimifolia)* inguratuta dago. Geologiari erreparatuz, estuarioa txangatik hautsitako tolestura antiklinal batean zehar luzatzen den ibai-harana da, eta Gernikako antiklinalaren bi aldeetan azaleratutako kareharrizko materialek estuarioaren inguruan dauden mendilerroak osatu dituzte. Oka ibaiaren arroa material sedimentarioek (hareharriak, tupak…) osatzen dute batez ere, nahiz eta basaltozko azaleramenduak aurki daitezkeen (Irabien eta Velasco, 1999).

***8. irudia**. Urdaibai estuarioaren ikuspegi orokorra (goian, ezkerrean), estuarioaren kanpoaldeko eremua (goian, eskuinean), estuarioaren erdiko eremua (behean, ezkerrean) eta barrualdeko eremu kanalizatua (behean, eskuinean).*

Urdaibaiko estuarioaren ezaugarri hidrografikoei dagokienez, estuarioa sistema meso-makromareala da, izan ere, marea bitartea metro bat baino gutxiago izatetik (marea hilenetan) 4,5 m izatera (marea bizietan) hel daiteke (Villate et al., 1989). Mareen erregimena egun-erdikoa da, euskal kostalde osoan bezala, egunean bi itsasgora eta bi itsasbehera inguru daudelarik, eta 15 egunetik behin marea hil-bizi zikloa gertatzen da. Estuario laburra denez, itsasoarekin ondo komunikatua dagoenez (ez dago oztoporik) eta hondoan frikzio ezberdintasunik ez dagoenez (Eusko Jaurlaritza, 1985), marea uhin egonkor bat eratzen da, hau da, ur mailaren igoera eta jaitsiera aldi berean gertatzen da estuarioaren puntu guztietan (Ketchum, 1983). Hala ere, estuarioak desfase txikiak aurkezten ditu itsasbehera gertatzen den denborak aztertzerakoan, estuarioaren geomorfologiak eragindako hustutzea atzeratzen duen inbutu-efektuagatik. Estuarioaren batez besteko bolumena 3,3 x 10^6 m^3–koa da eta urak denbora gutxi irauten du sisteman (10 egun inguru estuarioaren bolumena berriztatzeko), batez besteko 4,8 x 10^6 m^3-ko marea-prismarekin (Valencia et al., 2004). Geruzapenari dagokionez, orokorrean, estratifikazioa estuarioaren barruko eremuan ematen da bakarrik, kanal artifizialean. Estuarioaren kanpoaldeko eremuak fluxu-mareal handiak eta ondo nahastutako ur-zutabea izatea du ezaugarri, erdialdeko eta barrualdeko eremuetan ordea, gazitasun-gradiente horizontal nabarmenak eta partzialki estratifikatutako ur zutabea aurkitu daitezke (Villate et al., 2017). Gainera, estuarioaren kanpoko aldean uraren iraunkortasun-

denbora egun 1 baino laburragoa da, baina, erdiko eta barruko eremuek ibaiaren ur-emariaren eragin handiagoa jasaten dutenez, bertako ur masek 3 eta 83 egun tartean iraun dezake, deskarga flubialen arabera aldatzen dena (Franco, 1994; Sarobe, 2009).

Estuarioak duen garrantziari dagokionez, Urdaibaiko estuarioa EAE-ko Biosfera Erreserba bakarraren (Urdaibaiko Biosfera Erreserba) ardatz zentrala da, UNESCO-k 1984an "Man and Biosphere" programaren barruan izendatua, eremuaren balio paisajistiko, faunistiko, floristiko, sozial eta kulturalari esker (Castillo-Eguskitza et al., 2017). Gainera, estuarioa, EAEko eta Iberiar Penintsulako iparraldeko hezegune garrantzitsuenetakoa da, bere hedapen eta kontserbazio mailaren ondorioz, baita hegazti-migratzaileen dibertsitate handiko eremua izateagatik ere, hauen atseden eta negu-pasa gunea delarik (Arizaga et al., 2014), bertan lertxun hauskara (*Ardea cinerea*), ubarroia (*Phalacrocorax* sp.), txenada (*Sternidae* sp.) eta mokozabala (*Platalea leucorodia*) bezalako espezieak aurkituz. Hori dela eta, Biosfera Erreserba izateaz gain, 1993an Ramsar Hitzarmeneko nazioarteko garrantzia duten hezeguneen zerrendan sartu zen eta Europar Batasuneko Natura 2000 Sarearen parte ere bada, Hegaztientzako Babes Bereziko Eremu (HBBE) bezala (Castillo-Eguskitza et al., 2017). Hainbat ikerketek iradoki dute estuarioak eskaintzen duen balio ekologikoa eta bere zerbitzu-ekosistemikoak positiboki erlazionatuta daudela (Onaindia et al., 2013), eta horrek onura ekonomikoak eragiten ditu, (eko)turismoa eta aisialdia baitira eskualde honetako ekarpen ekonomiko nagusia (Castillo-Eguskitza et al., 2017). Gainera, biztanleria elikagaiez hornitzeko zerbitzua ere eskaintzen du estuarioak, itsaski-bilketa eta arrantza-jarduerak egiten baitira (Onaindia et al., 2011). Horrez gain, bere balio naturalistiko handia dela eta, Urdaibaiko estuarioa oso ekosistema ikertua da eta, beraz, zientzia/dibulgazioari loturiko zerbitzu garrantzitsua ere ematen du estuarioak eskala globalean (Onaindia et al., 2011). Estuarioko fitoplankton-komunitateen inguruan ere ikerketa ugari egin dira, 1980ko hamarkadaren amaieratik, fitoplanktonaren ekoizpen primarioan (Iriarte et al., 1997), ezaugarri fotosintetikoetan (Revilla et al., 2000), komunitateen dinamikan (adibidez, Madariaga, 1995; Orive et al., 1998; Trigueros et al., 2000) edo pigmentuen analisian (Ansotegui et al., 2003) oinarrituak. Hala ere, azken 20 urteotan, Urdaibai estuarioko fitoplanktona UEZ-ak ezarritako laginketen bidez (urtean 4 laginketa, estuarioko 2 puntuan) bakarrik ikertu da, komunitatearen azken ikerketa intentsiboak 90eko hamarkadako amaieran egin zirelarik.

Biosfera Erreserban giza jardueren eta natura-ingurunearen arteko oreka mantentzen saiatzen den arren, Urdaibaiko estuarioak, hainbat inpaktu eta presio jasaten ditu. 45.000 biztanle

inguru bizi dira Biosfera Erreserbaren baita, honen % 80a Gernika edo Bermeo bezalako hiriguneetan biltzen delarik, eta, gainerakoak, eskualdean zehar banatutako hainbat landa-eremuko edo kostaldeko giza-kokaleku txikiagoetan. Eskualdeko jarduera sozioekonomiko nagusia zerbitzuetan oinarritzen den arren, nekazaritza, abeltzaintza, arrantza, eta industria jarduerak (metalurgia, ontziolak, etab.) ere aurkitu daitezke estuarioaren inguruan. Giza-karga altu eta lurren erabilera honek estuarioak hainbat presio antropogenikoren menpe egotea eragin du (Monge-Ganuzas et al., 2017), hauen artean garrantzitsuena Gernikako Hondakin Uren Araztegiak eragindako kutsadura izanik.

9. irudia. *Gernikako Hondakin Uren Araztegiaren kokapena Urdaibai estuarioko barrualdean (ezkerraldea) eta isurketen iturburua (eskuinaldean).*

Gernikako araztegia estuarioaren barruko eremuan kokatuta dago eta, 1972tik, bertan tratatutako urak estuarioaren barnealdeko eremura isuri dira (*9. irudia*). Arazoa da, araztegiak lehen eta bigarren (2001-etik aurrera) mailako tratamenduak burutu arren, hondakin organiko, bakterio eta mantenugai ez-organiko kopuru handiak isuri dituela urte askoan zehar, estuarioko uren kalitatearen degradazioa eraginez (Revilla et al., 2000; Franco et al., 2004). Araztegiak ez duenez nitrogeno eta fosforoaren desagertze eraginkorra bermatzen, Urdaibai estuarioa da, normalean, EAE-n UEZ-aren jarraipenetan ikertzen diren estuarioen artean (12) mantenugai kontzentrazio altuena duena (adibidez, Borja et al. 2021), estuarioaren barrualdea eutrofizazioarekiko sentikorra bihurtuz (Revilla et al., 2017). Gainera, isurketa hauen ondorioz, Urdaibai estuarioaren barruko eremuak ez ditu UEZ-aren ingurumen-helburuak betetzen, gutxienez 2008-tik 2020-ra arte, egoera ekologiko “urria” edo “txarra” erregistratuz (adibidez, Borja et al., 2021). Honen arrazoi nagusia amonioa edo fosfatoa bezalako adierazle fisiko-kimikoek sistematikoki helburuak ez betetzea da, kontzentrazio altuegiak izateagatik, izan ere, honek fitoplanktona bezalako adierazle biologikoek ere kontzentrazio altuegiak (eutrofizazioa) izatea, eta beraz, helburuak ez betetzea, eragiten du. Horrez gain, araztegiaren

isurketek estuarioaren barruko uretan bakterio fekalen ugaritasuna areagotu dute. Uraren kalitatearen degradazio honek ondorio zuzenak izan ditu eremu honetako giza-baliabideen erabilgarritasunean, adibidez itsaski-bilketa debekatuz edo bainua "ez-gomendagarria" izendatuz.

Honi guztiari erantzuna emateko, 2004an, Urdaibaiko Saneamenduaren Plan Orokorra abian jarri zen, arroan sortutako industria eta hiri hondakin-uren bilketa eta tratamendua barne hartzen dituena, Urdaibaiko uraren egoera ekologikoa babestu eta saneamendu eraginkorra lortzeko helburuarekin. Ideia nagusia estuarioaren bi alboetako hondakin-urak bildu eta Bermeoko Lamiaran araztegira eramatea zen, handik itsaspeko hustubide baten bidez itsaso zabalera isurtzeko. 2007-2014 urteen bitartean, Gernikatik Bermeora doan kolektore nagusiaren tarte ezberdinen lanak egin ziren, 2014ean, Lamiaran araztegia martxan jarri eta Mundaka eta Bermeoko hondakin-urak bertara lotu ziren eta, 2018an, Sukarrieta eta Busturiakoak (Revilla et al., 2017). Azkenik, 2021eko uztailean, Gernikako kolektorea martxan jarri eta, ordutik, Lamiaranera bideratzen dira hondakin-urak, estuarioaren barrualdera egiten ziren isurketekin amaituz.

III. HIPOTESIA ETA HELBURUAK

Tesi hau Urdaibai estuarioko fitoplankton-komunitatea hobeto ezagutzeko asmoz burutu da, estuarioak, Euskal Herriko Biosfera Erreserba bakarreko parte izanik, duen balio naturalistiko handiagatik, 90eko hamarkadatik egon den ikerketa faltagatik eta, batez ere, inguruan egin diren saneamendu lanek izan dezaketen efektuagatik.

Hipotesia

Urdaibai estuarioko fitoplankton-komunitateak (i) gazitasun eta mantenugai gradienteek eragindako denboran eta espazioan zeharreko aldakortasuna aurkezten du, bai biomasan baita osaketan ere, eta (ii) aldaketak jasango ditu Gernikako araztegiko hondakin-urak estuariotik kanpo desbideratzearen ondorioz; hauek (iii) jarraipen-tekniken konbinazioari esker hobeto ulertuko direlarik.

Helburuak

Hipotesia egiaztatzeko, Tesiak honako helburu orokor hau jorratzen du:

"Urdaibai estuarioko fitoplankton-komunitatea deskribatzea, teknika desberdinak erabiliz, (i) honen denboran eta espazioan zeharreko aldakortasuna ezagutu eta hau kontrolatzen duten faktoreak zehazteko (ekologikoa), (ii) saneamendu lanek estuarioan duten eragina aztertzeko (aplikatua), eta (iii) jarraipen-tekniken erabilgarritasuna ebaluatzeko (metodologikoa)."

Helburu nagusi hau, Tesiaren kapitulu ezberdinetan jorratzen diren eta jarraian azaltzen diren helburu zehatzetan banatu da:

- Urdaibai estuarioko fitoplankton-komunitatearen karakterizazioa, Gernikako araztegiko hondakin-uren isurketak egon bitartean eta saneamendu lanen ondoren. (*1. Kapitulua* eta *4. Kapitulua*)
- Urdaibai estuarioko baldintza fisiko-kimikoen eta fitoplankton-komunitatearen arteko erlazioa zehaztea, biomasari eta komunitatearen osaerari dagokionez. (*1. Kapitulua* eta *4. Kapitulua*)
- Fitoplanktonaren jarraipenerako mikroskopia eta eDNA metabarcoding tekniken konparaketa. (*1. Kapitulua*)
- Gernikako araztegiaren hondakin-uren isurketen desbideratzeak Urdaibai estuarioko uraren kalitatean eta fitoplankton-komunitatean duen berehalako eragina ebaluatzea. (2. Kapitulua)
- Saneamendu lanek, epe-ertainean, Urdaibai estuarioan izandako eragina ebaluatzea, fitoplankton biomasan eta komunitatearen osaeran izandako aldaketak aztertuz eta aldaketa hauen eta eremuko aldaketa fisiko-kimikoen arteko erlazioa zehaztuz (*4. Kapitulua*).
- Pigmentuen analisiaren bidez fitoplankton-komunitatearen deskribapen kualitatibo eta kuantitatiboaren hurbilketa lortzea. (*3. Kapitulua*)
- HPLC bidezko pigmentuen *PIGMENTUM* analisiak fitoplankton-komunitatea eta haren denboran eta espazioan zeharreko aldakortasuna deskribatzeko duen eraginkortasuna frogatzea. (*4. Kapitulua*)

III. HYPOTHESIS AND OBJECTIVES

This thesis aims to obtain a better understanding of the phytoplankton community of the Urdaibai estuary, part of the only Biosphere Reserve of the Basque Country, considering the significant naturalistic value of the system, the lack of research since the late 90s and, above all, the potential effect of the sewerage improvement works carried out in the area.

Hypothesis

The phytoplankton community of the Urdaibai estuary (i) shows spatio-temporal variability in both biomass and composition prompted by salinity and nutrients and (ii) will undergo changes in response to the diversion of the wastewater discharges of the Gernika WWTP outside the estuary, which (iii) will be better understood due to the combined application of different monitoring techniques.

Objectives

In order to test the hypothesis, the Thesis addresses the following general objective:

"The general objective is to describe the phytoplankton community of Urdaibai estuary using different techniques, (i) to determine its spatio-temporal dynamics and the factors controlling them (ecological), (ii) to evaluate the effect of the sewerage works on the estuary (applied), and (iii) to provide insight into the usefulness of different monitoring techniques (methodological)."

This general objective has been subdivided in a series of operational objectives that are shown below and are addressed in the different Chapters of the Thesis:

- Characterization of the phytoplankton community in the Urdaibai estuary, during the wastewater discharges from Gernika WWTP and after the sewerage works. (*Chapter 1 and Chapter 4*)
- Determination of the relationship between the physicochemical conditions of the Urdaibai estuary and the phytoplankton community, in terms of biomass and community composition. (*Chapter 1 and Chapter 4*)
- Comparing microscopy and eDNA metabarcoding for phytoplankton monitoring. (*Chapter 1*)
- Assessment of the immediate effect of the cessation of wastewater discharges from Gernika WWTP on the water quality and the phytoplankton community in the Urdaibai estuary. (*Chapter 2*)
- Evaluation of the medium-term effect of the cessation of the wastewater discharges on the phytoplankton community of the Urdaibai estuary, analysing the biomass and community composition changes as well as the relationship between these changes and the local environmental alterations. (*Chapter 4*)
- Obtaining an approximation for the qualitative and quantitative description of the phytoplankton community through pigment analysis. (*Chapter 3*)
- Proving the efficiency of *PIGMENTUM* analysis of HPLC pigments to describe the phytoplankton community and its spatio-temporal variability. (*Chapter 4*)

Chapter 1

Phytoplankton community composition in relation to environmental variability in the Urdaibai estuary: Microscopy and eDNA metabarcoding

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Abstract

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

1. Introduction

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

In these ecosystems, the structure and biomass of phytoplankton communities vary continuously, mainly because of their adaptation to the environmental gradients caused by the tidal water circulation and the influence of the river (Jouenne et al., 2007; Lee et al., 2020). More precisely, phytoplankton communities in estuaries respond to environmental changes related to runoffs, water surface temperature, salinity, light availability and resuspension induced by waves and winds (e.g. Wells et al., 2015; Vajravelu et al., 2018). Freshwater inflows are also known to be a key determinant of phytoplankton abundance and community structure in estuaries, due to their influence on the water retention time and degree of stratification (e.g. Lemley et al., 2018; Nunes et al., 2018). Additionally, the inputs of inorganic nutrients (nitrogen:N, carbon:C, phosphorus:P, oxygen:O, iron:Fe, silicon:Si) on estuarine surface waters stimulate phytoplankton growth and modulate community composition, increasing the growth rates of certain taxa and leading to harmful algal blooms that can affect ecosystems negatively (Pincknet et al., 2001; Anderson et al., 2002; McCabe et al., 2016; Wells et al., 2015; Vajravelu et al., 2018). Phytoplankton is known to be the first autotrophic community showing response to nutrient availability variations (Paerl et al., 2003) and, due to its basal position in the food chain, it is considered the link between inorganic nutrients and the rest of the trophic levels (Seoane et al., 2011).

Light microscopy has been traditionally the most used technique to assess phytoplankton biomass and diversity (e.g. Aktan et al., 2005; Agirbas et al., 2015; Lee et al., 2020). The main strength of this method is that it enables both phytoplankton identification and enumeration at the same time, providing cell counts, size measurement and taxonomic information to genus or species level (Edler and Elbrächter, 2010). However, it is highly dependent on individual researcher's skills (Muñiz et al., 2020), since it requires extensive taxonomic knowledge (Naik et al., 2011) and it is time-consuming (Wang et al., 2018). In

addition, fragile and small cells (i.e., picophytoplankton cells) are difficult or impossible to identify, usually leading to omissions or errors in taxonomic identifications (Jeffrey et al., 1997; Agirbas et al., 2015). Many studies (e.g. Penna et al., 2017; Huo et al., 2020) assume that morphological analyses alone cannot provide a complete description of the huge phytoplankton diversity. The use of molecular tools, like DNA metabarcoding, can be an alternative to overcome the limitations associated with microscopy-based diversity monitoring, especially after the development of high-throughput sequencing (HTS) technologies (Trebitz et al., 2017; Rimet et al., 2021).

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

The Urdaibai estuary (southeastern Bay of Biscay) constitutes the central area of the only Biosphere Reserve of the Basque Country, declared by UNESCO in 1984 because of its high naturalistic and cultural value (Castillo-Eguskitza et al., 2017). This estuary represents one of the main tidal marshes along the coast of northern Iberia and hosts an especially relevant richness of water birds (Arizaga et al. 2014), being added to the list of Ramsar Wetlands in 1993 and the network of the European Union Natura 2000 (Castillo-Eguskitza et al., 2017). Previous studies in the area suggested that biodiversity and ecosystem services provided by the estuary are positively correlated (Onaindia et al., 2013), which in turn, results in economic benefits, since (eco)tourism and recreation are the main economic motor and attraction in the

region (Castillo-Eguskitza et al., 2017). Nevertheless, the estuary has been receiving direct discharges from the Gernika wastewater treatment plant (WWTP) since 1972 in the inner area, as this is the principal source of pollution of the estuary.

Due to its high naturalistic value, the Urdaibai estuary is a widely studied ecosystem and the phytoplankton community of the estuary has been the subject of numerous studies since the late 1980s. Several of these were centred on primary production, respiration and photosynthetic characteristics of phytoplankton (Madariaga and Orive, 1989; Madariaga et al., 1989; Iriarte et al., 1997; Revilla et al., 2000), while others described the communities and their dynamics in different time scales, based on microscopy identification and enumeration (Madariaga, 1995; Orive et al., 1995, 1998; Trigueros and Orive, 2000, 2001; Trigueros et al., 2000a, 2000b) or pigment analysis (Ansotegui et al., 2001, 2003).

The latest intensive studies on the phytoplankton community of the estuary were performed in the late 90s, and for the last 20 years, the study of the phytoplankton community of the Urdaibai estuary has been reduced to quarterly samplings carried out to comply with the requirements of the EU Water Framework Directive (WFD; Directive, 2000/60/EC). Knowing that sewerage system renovations were planned in the area in the near future, to divert the effluent of Gernika WWTP outside the estuary, an updated description of the phytoplankton community was needed before it, in order to evaluate the future effect of these works on the biomass and community composition. Additionally, to the best of our knowledge, no previous studies targeting phytoplankton have been carried out in the Urdaibai estuary based on molecular techniques, and therefore, there is limited phytoplankton identification at the species level, especially for pico- and nano-phytoplankton.

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

2. Materials and methods

2.1 Study area

This study was performed at the Urdaibai estuary (also known as Gernika estuary, Mundaka estuary or Oka estuary), a meso-macrotidal system located in the southeastern Bay of Biscay (43°22'N, 2°43'W) (*Figure 1.1*). The climate of the area is temperate-oceanic, with moderate winters and warm summers, and it is under the influence of the Gulf Stream and the atmospheric westerlies in the middle and upper troposphere (Usabiaga et al., 2004).

The Urdaibai estuary is short (13.7 km long) and shallow, and it is formed by the tidal part of the Oka River (Barroeta et al., 2023). The total estuarine area (1.89 km^2) is big when compared to its drainage basin (183 km^2), and therefore, the contribution of freshwater (mean flow of 3.6 $m^3\ s^{-1}$) is small compared to the total volume of the estuary (3293100 m^3) (Valencia et al., 2004; Barroeta et al., 2023). In addition, river discharge is usually low in relation to the tidal prism (Villate et al., 2017). Consequently, tidal cycles have a great impact and most of the estuary is marine dominated at high tide (Valencia et al., 2004). The water residence time is short (days), and the stratification varies within the system, with partially stratified conditions in the middle and inner area and a well-mixed water column in the outer zone, due to the high tidal flushing (Villate et al., 2017; Barroeta et al., 2020). However, this estuarine system can vary considerably in its volume and flushing rates, owing to its geomorphology and, mainly, due to the torrential nature of the Oka River, since large flows that change the typical pattern appear occasionally (Madariaga et al., 1994). This has a direct effect on the physicochemical and biological properties of the estuary, causing changes in the phytoplankton communities on a short time scale (Madariaga et al., 1994; Uriarte, 2001) as well as the proliferation of particulate matter with a high proportion of inorganic materials (Ruiz, 1998).

Based on morphology, the estuary is divided in three main areas (*Figure 1.1*) (Villate et al., 1989). The outer estuary is a zone with sandy beaches and extensive intertidal flats, which extends from the outer limit of the system (Mundaka) to Murueta. The middle area (i.e., intermediate estuary), from Murueta to the start of the artificial channel, consists of a central channel bordered by shalt-marshes and a complex system of interlaced secondary channels. The inner estuary is formed by a 4 km long and 15 m wide artificial channel built in the beginning of the 20th century that reaches the town of Gernika and is bordered by reed beds.

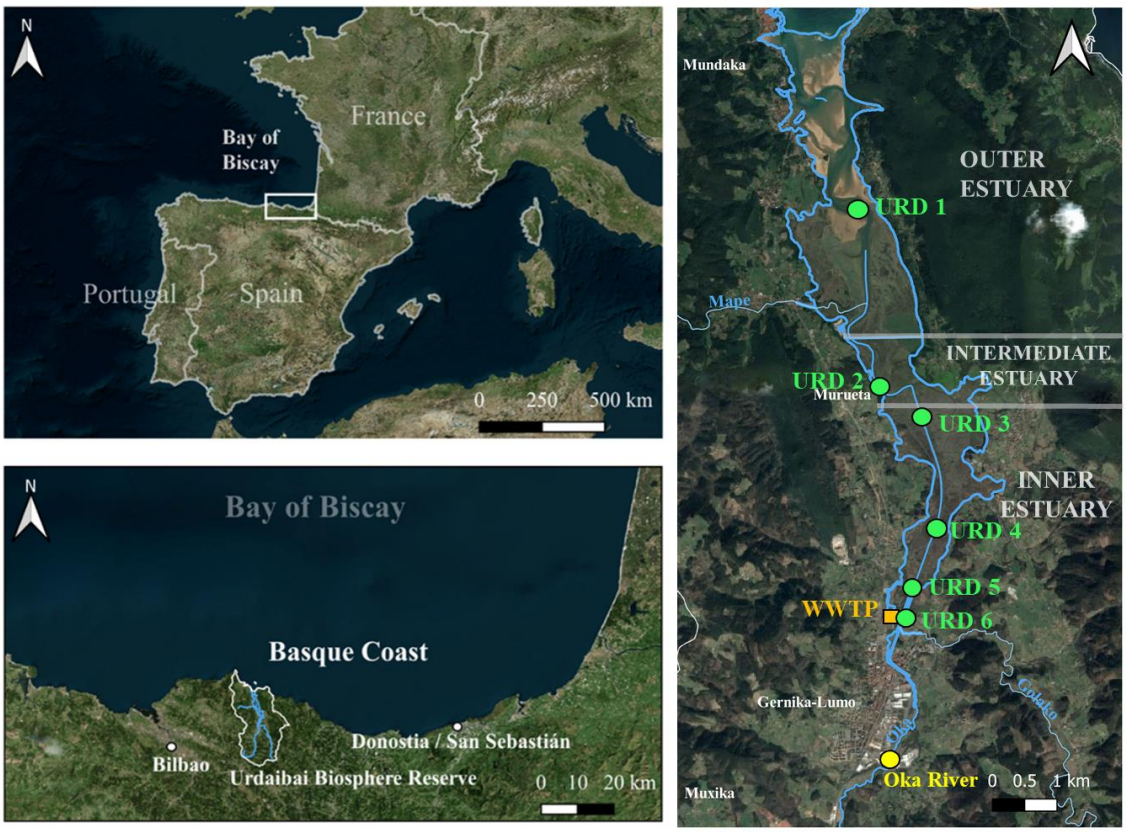

Figure 1.1. *Study area and sampling stations. On the left, the location of the Urdaibai estuary and the Urdaibai Biosphere Reserve in the Bay of Biscay and the Basque coast is shown. On the right, the Urdaibai estuary is divided into the outer estuary (sampling station URD1), intermediate estuary (station URD2) and inner estuary (stations URD3, URD4, URD5 and URD6). The discharge point of the wastewater treatment plant (WWTP) and the sampling station at Oka River (Oka) are also located in the inner estuary.*

2.2 Sampling and data acquisition

Sampling was conducted every 15 days from September 2019 to September 2020, at six permanent sampling stations located within the longitudinal axis of the estuary (*Figure 1.1*) to cover the entire salinity gradient: one in the outer part (URD1), one in the middle area (URD2) and four in the inner estuary (URD3, URD4, URD5 and URD6). The last sampling station (URD6) was located in the WWTP of Gernika and is near the inner limit of seawater penetration. An additional sampling station was set up in the Oka River to monitor the physicochemical properties of the river. Therefore, a total of 161 samples were collected, corresponding to 23 sampling days for each of the 7 sampling stations. Only two samplings were cancelled along the year: the first of November (due to prolonged adverse climatic conditions) and the first of April (due to COVID-19 prohibitions).

Estuarine water samples were always collected at high tide with a 2.5 L plastic Niskin bottle, 0.75 m below the surface in an hour long transect from URD1 to URD6. Only subsurface

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

The dissolved inorganic nutrients analysed were ammonium, nitrite, nitrate (calculated from the total oxidised nitrogen), orthophosphate and silicate. The analyses were carried out using VIS/UV colorimetry in an automatic 5-channel analyser with segmented flow at the Chemical Laboratory of the Marine Research Unit of the AZTI Foundation in Pasaia (Gipuzkoa). The individual determinations of these dissolved inorganic nutrients were based on methods that apply classical and widely used colorimetric reactions, both for inland and marine waters (GO-SHIP manual by Hydes et al., 2010). The quantification limit for ammonium, nitrate and silicate was 1.6 μmol L^{-1}, 0.4 μmol L^{-1} for nitrite and 0.16 μmol L^{-1} for phosphate. For measurements below the quantification limit, a concentration equal to 50% of the limit was assumed for the estimation of median values.

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

2.2.1 Microscopy

Water samples used for the study of the phytoplankton community by microscopy were fixed with acidic Lugol's solution (0.4% v/v) right after collection and stored until analysis in dark

and cool (4 ºC) conditions, in 125 ml topaz borosilicate bottles. Taxonomic identification and cell counts were performed following the Utermöhl sedimentation method (Edler and Elbrächter, 2010) under a Nikon diaphot TMD inverted microscope, for subsamples of 10 or 50 ml depending on the density. Transects at different magnifications (100×, 200× or 400×) were carried out depending on the taxa's abundance and size. In order to count the larger and less abundant taxa, the whole chamber was analysed at low magnifications (200×). Most of the taxa were identified to the genus level, and the nomenclature of the identified taxa was standardised according to AlgaeBase (Guiry and Guiry, 2018).

2.2.2 Phytoplankton biomass

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

For the calculation of carbon content, first, the biovolume was determined by assigning each taxon a mean equivalent spherical diameter (ESD), based on Olenina et al. (2006), which takes into account cell shape and size. Afterwards, the biomass was calculated using the equation as reported for marine phytoplankton by Montagnes et al. (1994): Carbon content = $0.109 \times \text{Volume}^{0.991}$, where carbon content is expressed in pg C $cell^{-1}$ and volume in μm^3.

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

2.2.3 eDNA Metabarcoding

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

DNA extraction, amplification and sequencing of the 18S rRNA V4 region was carried out at the Sequencing and Genotyping Unit of Genomics Facilities from the University of the Basque Country (SGIker – UPV/EHU). The DNA was extracted from the filters with the DNeasy Power Water Kit (QIAGEN), following the kits´ specific protocol. In order to account for contaminations, an extraction negative control (EXT-C) was included, obtaining a quantification value below the detection threshold (< 2 ng μL^{-1}). As indicative data, the mechanical lysis for the extraction was carried out on a flat-bottom vortex, at 3000 rpm for 5 minutes, and the volume of the elution buffer was decreased to 60 µL. The primers used for amplification of V4 18S rRNA were TAReuk454FWD1 and TAReukREV3 (Stoeck et al., 2010), modified by Piredda et al. (2017) (*5'-CCAGCASCYGCGGTAATTCC-3'* and *5'-ACTTTCGTTCTTGATYRATGA-3'*), to which the necessary tails for later insertion of the adapters and indices compatible with the Illumina platform were added (*5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC-3'* and *5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA-3'*).

Amplification was carried out following the protocol described by Piredda et al. (2017) but with certain modifications. More specifically, KAPA HiFi Hotstart ReadyMix (Roche Kapa Biosystems) was used, and the activation time and temperature of the thermocycler programme were adapted: an initial 4 min at 95 °C, followed by 10 cycles at 95 °C for 10s, 44 °C for 30s, 72 °C for 15s; a second step by 15 cycles at 95 °C for 10s, 62 °C for 30s, 72 °C for 15s; and a final cycle of 7 min at 72 °C. 2 µL of DNA at stock concentration were added between 15 and 80 ng μL^{-1} by nanodrop. 1 µL of each primer (10 µM) was added to the total reaction volume (25 µL). Both extraction (EXT-C) and amplification (NTC) negative controls were included. After electrophoresis to assess PCR product yield, purification was carried out by beads (CleaNNA NGS kit), followed by the subsequent indexing reaction with the Illumina Nextera XT v2 set A and set B kit (following the standard protocol of the commercial house). Indexed products were checked by electrophoresis, and purified with beads was carried out in pool. Final library quantification was carried out by Qubit. The

sequencing was performed on an Illumina MiSeq instrument with a 300-bp paired-end protocol (MiSeq v3 chemistry). The loading library concentration was 6 pM, and 10% PhiX was used as an internal control. Obtained quality control values for the sequencing run were > Q30 = 76,53% and %PF: 91.65. After post-filtering, 2 x 17,753,716 reads assigned to marker 18S rRNA V4 were obtained. The average coverage of the samples was 120,000. Less than 1,100 and 500 reads were assigned for both extraction and amplification negative controls, respectively. All raw sequence files have been submitted to the European Nucleotide Archive (ENA) (Cummins et al., 2022) with the study accession number PRJEB48801 (available at http://www.ebi.ac.uk/ena/data/view/PRJEB48801).

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

The preparation of the ASV-table was done in R v.3.6.0. (R core Team, 2021) and Excel 2007. Out of the 138 samples processed, 8 were removed due to problems during amplification and scarce reads: URD3 and 4 from 20/11/2019; URD1, 2, 3, 4 and 5 from the 04/12/2019; and URD2 from the 20/03/2020.

A total of 36,562 ASVs were defined by eDNA metabarcoding; however, since this study is focused on phytoplankton, all reads assigned to other organisms were excluded from the processed ASV tables, selecting a total of 14,672 ASVs. ASVs that were found only in the control samples were removed from the subsequent analysis; in addition, for ASVs that were found in the control samples in higher abundances than those in the actual samples, their

abundances in the control samples were subtracted from the abundances in the actual samples. Among the selected ASVs, 615 unique phytoplankton taxa were identified, but only those that reached taxonomic assignment to at least the genus level were considered in this study. Moreover, we filtered ASVs for those that made up at least 0.01 % of the total reads of at least one of the 130 samples and also excluded the taxa appearing in less than 1% of the samples processed.

2.3 Data analysis

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

To analyse the phytoplankton community composition, non-metric multivariate analyses were performed to visualise similarity/dissimilarity among samples and determine the spatio-temporal dynamics within the Urdaibai estuary. Non-metric multidimensional scaling (nMDS) in 2D was performed, creating an ordination based on the Bray-Curtis similarity index, for the graphical representation of the interrelationships among samples according to the phytoplankton community composition. Independent nMDS were performed for the data sets obtained by microscopy (cell abundances) and metabarcoding (standardised read counts). To complement this, permutational multivariate analysis of variance (PERMANOVA), a non-parametric multivariate statistical permutation test, was performed to test significant differences between groups based on the Bray-Curtis distance matrix (Anderson, 2001). PERMANOVA was tested as a “two-way PERMANOVA” to account for the spatial (sampling stations) and seasonal variability of the community, as well as for the interaction

of these two factors. Later, a "one-way PERMANOVA" was performed, with the Bonferroni corrected p values, for each of the factors (spatial and seasonal) for the pairwise comparison of groups along the gradients. This decision was based on the conclusions of Anderson and Walsh (2013), which conducted a simulation-based comparison of PERMANOVA and ANOSIM and found that PERMANOVA is more robust in general for ecological data. Finally, a similarity percentages analysis (SIMPER) was performed to determine which phytoplankton taxa contributed the most to the observed dissimilarity between samples, for both spatial and temporal variability. As for the nMDS, the PERMANOVA and SIMPER analysis were also conducted independently for microscopy and metabarcoding analysis.

The relationship between the phytoplankton community and the environmental data was explored using a canonical correspondence analysis (CCA). A single CCA was performed, with both microscopy (with cell abundance) and DNA metabarcoding (read abundances) data, after normalising the number of reads with sample volume. The environmental variables chosen for the CCA were the same as in the PCA. The phytoplankton taxa selection was based on choosing the most relevant (abundant and/or frequent) taxa of the estuary that were detected by both microscopy and metabarcoding. Thus, results from the SIMPER analysis were taken into account, since this determined the main taxa contributing for the variation in the community composition within the estuary. The taxa selection was done firstly by choosing common taxa in the top 15 organisms of the SIMPER results for microscopy and metabarcoding (*Annexes, Table A1.10*). For the remaining taxa, that did not have a match (e.g. unidentified centric diatoms), the Spearman correlation analysis was performed between these and the organisms from the metabarcoding analysis that morphologically could correspond to the same taxa. Matching was only accepted when the correlation was significant and the organisms from both techniques followed the same morphological and ecological characteristics. The selected taxa and the Spearman correlation analysis results for each matching are shown in *Table 1.1*. Finally, the Spearman correlation was tested in order to correlate the chosen phytoplankton taxa and phytoplankton biomass with the environmental conditions of the Urdaibai estuary.

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

Table 1.1. *Spearman correlation analysis results for microscopy cell counts and eDNA metabarcoding reads of the taxa selected for the CCA, showing rho (ρ) and p values.*

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

3. Results

3.1 Environmental conditions

The physicochemical variables showed the expected spatial patterns and seasonal trends in a temperate estuary during the study period (*Figure 1.2*; *Annexes, Table A1.1*). Salinity and temperature increased towards the outer part of the estuary and summer season. Oxygen concentration showed both the minimum (1.35 mg L^{-1}) and maximum (11.25 mg L^{-1}) values at the innermost station (URD6) and, overall the highest median oxygen concentrations of the estuary were recorded in winter (8.79 mg L^{-1}) and the lowest in summer (6.27 mg L^{-1}). Both pH and turbidity increased towards the inner estuary, and regarding seasonality, the former presented the highest median values in summer (8.7) and the latter in autumn (7.1 NTU).

Inorganic nutrient concentrations showed a marked spatial gradient along the longitudinal axis of the estuary, with increasing concentrations towards the inner area (*Figure 1.2*). However, the source of the different nutrients varied. The station located in the Oka River showed very low median concentrations of ammonium (3.4 µmol L^{-1}) and phosphate (0.35 µmol L^{-1}), being much higher at URD6 (143 µmol L^{-1} and 4.1 µmol L^{-1}, respectively), located close to the WWTP. On the contrary, the silicate and nitrate median concentrations in the Oka River (94.3 µmol L^{-1} and 59.1 µmol L^{-1}, respectively) were higher than the maxima values observed at URD6 (80.3 µmol L^{-1} and 33.7 µmol L^{-1}, respectively). Nutrient concentrations varied, ammonium (2.9–423 µmol L^{-1}), phosphate (0.2–5.58 µmol L^{-1}), nitrate (0.8–77.55

µmol L^{-1}) and silicate (0.8–112 µmol L^{-1}), and decreased towards the outer estuary (*Figure 1.2*). As for their seasonal variability, ammonium and phosphate reached their highest median concentrations in summer (58 µmol L^{-1} and 1.95 µmol L^{-1}, respectively), while the lowest were found in autumn. Conversely, nitrate and silicate followed an inverse temporal pattern, reaching their highest median concentrations in autumn (36 µmol L^{-1} and 64.7 µmol L^{-1}, respectively), and their lowest in summer (11 µmol L^{-1} and 42 µmol L^{-1}, respectively).

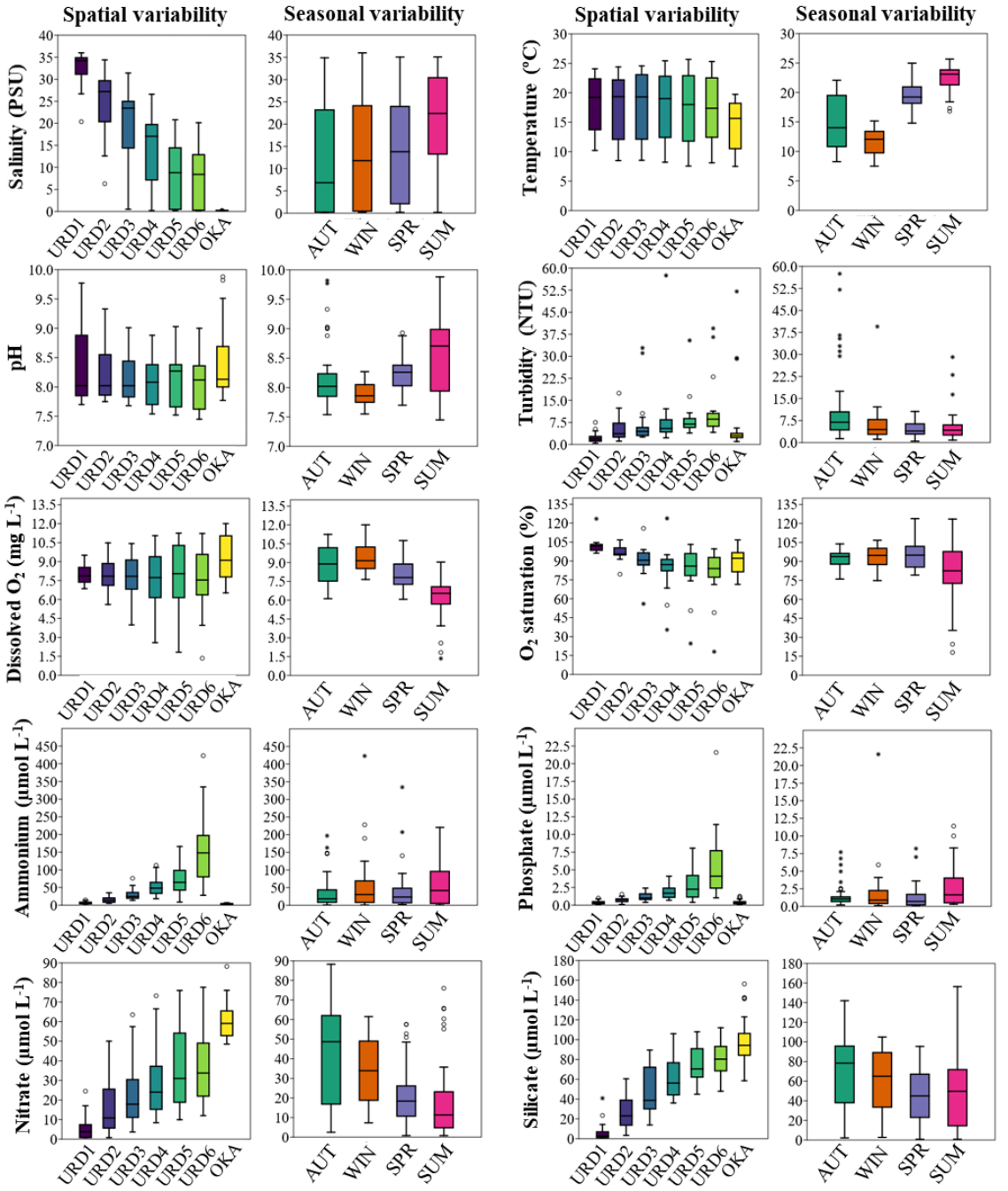

Figure 1.2. *Spatial and seasonal variability of the main physicochemical parameters in the Urdaibai estuary. For nutrient concentrations below the quantification limit, the value of half of the limit was considered. The box represents the Interquartile Range (IQR), data between Q1 (25th percentile) and Q3 (75th percentile); the line inside the box is the median. The lower whisker is the Q1-1.5*IQR, and the upper Q3+1.5*IQR. Circles and asterisks show outliers. Aut: autumn; Win: winter; Spr: spring; Sum: summer.*

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

The application of PCA (*Figure 1.3*) to 10 physicochemical parameters indicated that 68.5% of the variability contained in the data set was explained by only two PCs (principal components). The loading values (correlations) of the first two PCs are displayed in *Annexes, Table A1.4*. PC1 was interpreted as the spatial variation of the environmental conditions along the longitudinal axis of estuary, as it was highly correlated with salinity, silicate and nitrate, with large factor loadings (> |0.91|). PC2 was interpreted as the trophic status variation of the samples, since it is mainly correlated with dissolved oxygen, oxygen saturation, ammonium and phosphate (with loadings above |0.78|). Among the physicochemical parameters analysed, salinity was the main environmental driver shaping the spatial variability of the abiotic environment along the Urdaibai estuary, with a correlation of -0.926. This indicates that the environmental conditions of the estuary are mostly dependent on the tidal effect and/or the Oka River flow. This graphical representation confirmed the description of environmental parameters explained above and the strong spatial variability present in the estuary. It also reflected the different variability of environmental conditions during the year at the different stations, as the stability was higher towards the outer estuary since the range of values was the narrowest for most of the analysed parameters.

Regarding the hydrometeorological conditions (*Annexes, Table A1.2* and *Table A1.3*), autumn-winter was the period with the highest precipitation, with a monthly maximum in November 2019 (453 mm), and a minimum in July 2020 (22 mm). River flow values ranged between 0.001 and 7.4 $m^3\ s^{-1}$ during sampling weeks, with the highest values found in autumn-winter (median of 0.56 $m^3\ s^{-1}$) and the lowest in summer-spring (0.15 $m^3\ s^{-1}$). The insolation (hours of sunshine) was the highest in spring and summer.

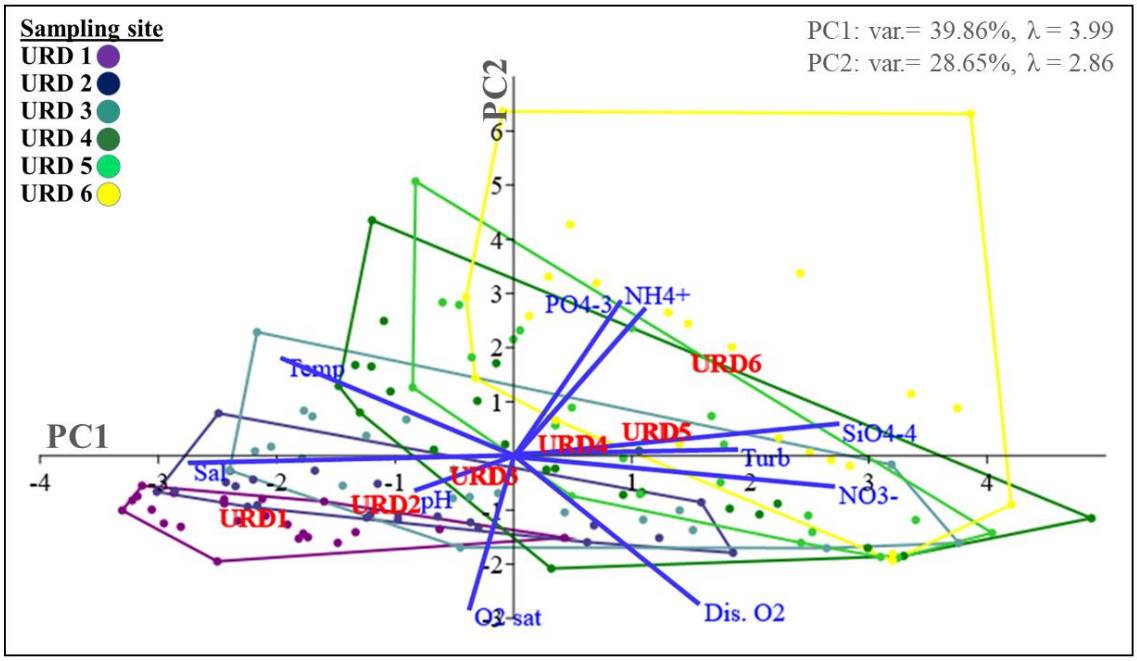

Figure 1.3. *PCA plot of the main hydrographic and physicochemical conditions in the Urdaibai estuary. A correlation matrix was used, and the Bootstrap N was 999. Samples are coloured based on the sampling stations and grouped by convex hulls, which include the name of the group (station names). Environmental factors are shown as gradients with blue lines. Eigenvalues and variance percentages for PC1 and PC2 and the legend are located on the right side of the figure. Var: variance; λ: eigenvalue; Sal: salinity; Temp: temperature; O2 sat: oxygen saturation; Dis.O2: dissolved oxygen; Turb: turbidity;* NH_4^+*: ammonium;* PO_4^{-3}*: phosphate;* NO^{3-}*: nitrate;* SiO_4^{-4}*: silicate.*

3.2 Phytoplankton biomass estimations

Total phytoplankton biomass, estimated by carbon content and Chl *a* concentration, showed spatio-temporal patterns in the Urdaibai estuary (*Figure 1.4*).

Total carbon content of the phytoplankton community ranged between 1 and 916 µg C L^{-1} within the estuary during the study period. URD1 registered the lowest annual median carbon content of the estuary (38.9 µg C L^{-1}), and the median maxima were recorded in URD 5 and URD6, with concentrations of 112 µg C L^{-1} and 94 µg C L^{-1}, respectively. However, there was not an increasing gradient from outer to inner estuary, since the median carbon content was higher in URD2 (67.3 µg C L^{-1}) than in URD3 and URD4 (approximately 55 µg C L^{-1}). As for the temporal patterns, the lowest concentrations were found in winter, with a median value of 16.4 µg C L^{-1} in the estuary, and the highest in spring (124 µg C L^{-1}) and summer (120 µg C L^{-1}).

Chl *a* ranged between 0.09 and 54.61 µg L^{-1} throughout the year within the estuary and showed an increasing gradient towards the inner estuary. The outer estuary (URD1) registered

Chl *a* values between 0.21 and 2.97 µg L^{-1} and had the lowest annual median Chl *a* concentration (1.18 µg L^{-1}). In the middle estuary (URD2), the Chl *a* concentration ranged between 0.17 and 10.89 µg L^{-1}, with a median annual concentration of 2.8 µg L^{-1}. In URD3 and URD4, Chl *a* median concentration was slightly higher than in the middle estuary (3.5 µg L^{-1} approximately), and the Chl *a* concentration range was much wider than in the outer and middle estuary, with values between 0.09 µg L^{-1} and 54.61 µg L^{-1}. URD4 registered the Chl *a* maximum of the year in the estuary. At URD5 and URD6, the Chl *a* concentration was the highest of the entire estuary on average, with a median annual concentration of approximately 5 µg L^{-1}. As for the temporal patterns, the studied year was divided into two main periods when it comes to Chl *a*: a low concentration period during autumn and winter, with median concentrations of 1.04–1.67 µg L^{-1}; and a high concentration period during spring and summer, with median concentrations of 5–6.26 µg L^{-1}. The highest Chl *a* concentrations registered during the year were in spring (54.61 µg L^{-1} in April 2020).

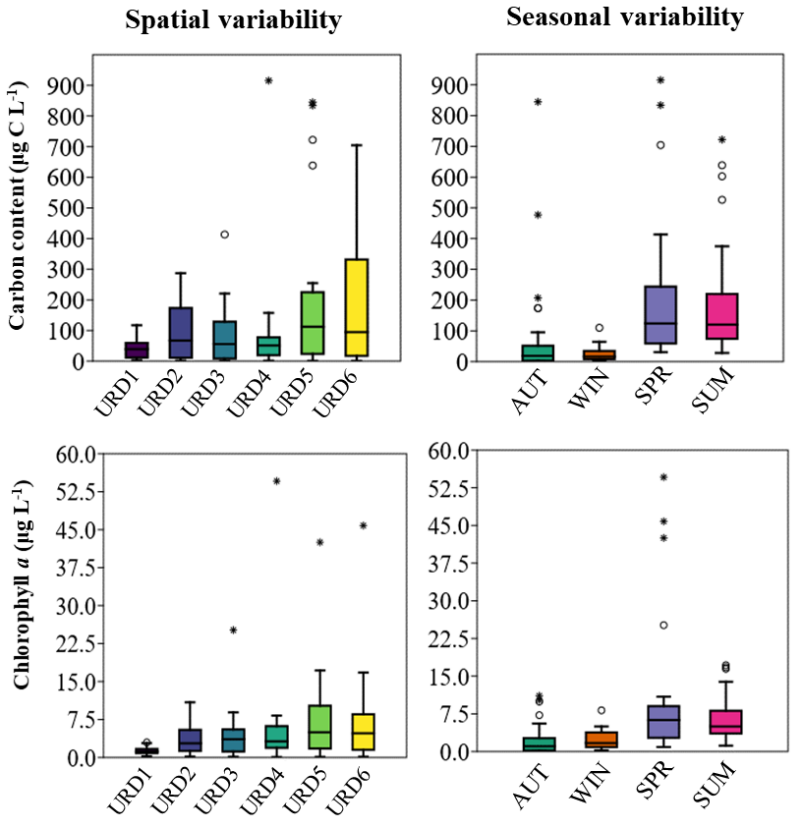

Figure 1.4. *Spatial and seasonal variability of total phytoplankton biomass (up, carbon content; down, Chl a) within the Urdaibai estuary during the study period. The box represents the Interquartile Range (IQR), data between Q1 (25th percentile) and Q3 (75th percentile); the line inside the box is the median. The lower whisker shows the Q1-1.5*IQR, and the upper Q3+1.5*IQR. Circles and asterisks represent outliers. Aut: autumn; Win: winter; Spr: spring; Sum: summer.*

3.3 Phytoplankton community composition

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

The phytoplankton community of the Urdaibai estuary included 136 taxa identified with microscopy, and 407 taxa identified with eDNA metabarcoding (*Annexes, Table A1.5*), of which 58 taxa were shared by both approaches. A total of 349 species or genera were unique to eDNA metabarcoding (*Table 1.2*), including green algae, cryptophytes and haptophytes that presented three to four times more diversity than with microscopy. Other minor groups, such as chrysophyceans or dictyochophyceans were also identified by eDNA metabarcoding, while were mostly absent from microscopy. A total of 78 taxa were identified with microscopy only (*Table 1.2*). Despite the difference in the taxa richness obtained by each approach, diatoms were the most diverse group, containing almost half of the taxa found, followed by dinoflagellates and green algae.

Table 1.2. *The number of phytoplankton taxa identified (taxa richness), at least to the genus level, by microscopy, eDNA metabarcoding or both techniques within the Urdaibai estuary. "Others" category contains: pelagophyceans, bolidophyceans, raphidophyceans, synurophyceans, olisthodiscophyceans and rhodophytes.*

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

The outer estuary registered 127 and 363 different phytoplankton taxa with microscopy and metabarcoding, respectively. Diatoms were the dominant phytoplankton group, with a 44% and 40% of median contribution to the total abundance during the study year according to microscopy cell counts and metabarcoding reads, respectively (*Figure 1.5*). Among them, microscopy identified *Cylindrotheca closterium*, *Minutocellus polymorphus*, *Chaetoceros* spp., *Leptocylidrus danicus* and *Pseudo-nitzschia* spp. as the most frequent taxa, together with unidentified centric and unidentified pennate diatoms, that were recorded in more than 60% of the samples. For metabarcoding, *Chaetoceros tenuissimus, M. polymorphus*,

Minidiscus variabilis and *Mediolabrus comicus* occurred in all samples analysed and were the dominant taxa in the outer estuary. *Pseudo-nitzschia* spp. (*P. seriata* especially), *C. costerium* and *Leptocylindrus convexus* were also frequent diatoms of URD1, and were more recurrent than in the remaining sampling stations. In addition, microscopy revealed that *M. polymorphus* was the species that recorded the highest cell abundance in URD1, with a maximum of $9.1x10^6$ cells L^{-1} in summer. In contrast to the other sampling stations, haptophytes had a significant importance in the phytoplankton community of URD1, contributing 18% and 8% to the total community composition according to microscopy and metabarcoding, respectively. While microscopy was only able to detect a few taxa (Prymnesiales and *Gephyrocapsa huxleyi* being the most frequent), genetic method identified *Chrysochromulina* spp. (especially *C. scutellum* and *C. rotalis*), *Prymnesium* spp., *Phaeocystis* spp. (mainly *P. cordata*, but also *P. globosa*), *Gephyrocapsa oceanica, Haptolina* sp. and *Dicrateria rotunda* as the most recurring taxa. URD1 was the station with the highest contribution of dinoflagellates to the total community composition (4% and 15% for microscopy and metabarcoding, respectively), which was especially important in spring and summer, with presence of *Warnowia* sp., *Heterocapsa* spp. (especially *H. pygmaea and H. rotundata*), *Gyrodinium* spp. (*G. fusiforme*, *G. dominans* and *G. spirale*), *Tripos furca* and *Torodinium robustum* in all samples analysed by metabarcoding. Green algae were largely overlooked by microscopy at the outer station. However, through metabarcoding analysis, green algae were identified as contributing to 24% of the community, being primarily represented by *Ostreococcus lucimarinus, Bathycoccus prasinos*, *Micormonas bravo*, *Micormonas commoda* and *Pyramimonas australis*, which were the most frequent (100% of the samples) taxa.

In the middle estuary (URD2), the number of identified taxa decreased to 101 by microscopy and 349 by metabarcoding. Similarly to URD1, according to both techniques, diatoms were still the dominant group of the community in terms of median contribution (41–43%), but the contribution of cryptophytes increased, up to 35–40%, becoming co-dominant in this area. Among diatoms, *M. polymorphus* and *C. tenuissimus* were the dominant taxa in URD2, according to both techniques, together with the continuous presence of *Tryblionella apiculata* and *Thalassiosira profunda*, which were identified by metabarcoding. *M. polymorphus* registered higher abundances in this area than at URD1, with a maximum of $1.5x10^7$ cells L^{-1}, and showed a clear preference for the summer season. However, *C. tenuissimus* was the dominant diatom species at URD2, reaching an abundance maximum of $2x10^7$ cells L^{-1}. As for cryptophytes, according to the microscopy analysis, *Plagioselmis* spp. and *Teleaulax* spp.

(especially *T. gracilis*) were present in all the samples analysed, with median cell abundances of $5.5x10^5$ cells L^{-1} and $1x10^5$ cells L^{-1}, respectively. As for metabarcoding, *T. acuta* and *Hemiselmis cryptochromatica* were the dominant cryptophytes in the area. Among dinoflagellates, both techniques highlighted *Heterocapsa* spp. (mostly *H. pygmea*, according to the molecular analysis) and *B. quinquecornis* as the most abundant and frequent.

In the first section of the channelled area (URD3 and URD4), the number of taxa identified was 85 and 342 by microscopy and metabarcoding, respectively. Cryptophytes were the dominant group of the community, with a median contribution of around 60% according to microscopy and 45% according to metabarcoding. Microscopy described *Plagioselmis* spp., identified in every sample, as the taxa that recorded the highest median abundances in this area, between $7.6x10^6$ cells L^{-1} (URD3) and $9.6x10^6$ cells L^{-1} (URD4), together with the frequent presence of *T. gracilis* (91% of the samples) and *T. acuta* (80%). According to the metabarcoding analysis, *T. acuta*, *H. cryptochromatica* and *Urgorri complanatus* were the dominant cryptophytes in this area. In addition, both techniques determined that diatoms were the second most contributing group (25–33%), with *M. polymorphus, C. tenuissimus* and *T. apiculata* being the most representative taxa. Metabarcoding revealed that URD4 was the station with the highest green algae diversity (49 taxa) within the entire estuary, among which *Ostreococcus* spp. (mainly *O. mediterraneus* and *O. tauri*), *Picochlorum* spp., *Nannochloris* sp., *Micromonas* spp. (mostly *M. bravo* and *M. commoda*), *Oltmannsiellopsis viridis* and *Pyramimonas* spp. were dominant. According to microscopy, which did not identify most of the previously mentioned green algae, this area recorded a higher presence of *Eutreptiella* spp. and *Tetraselmis* spp. than URD2.

At the innermost area of the estuary (URD 5 and URD 6), the number of identified taxa by microscopy was 58, while DNA metabarcoding identified 264 taxa in the area. The phytoplankton community description given by both techniques differed substantially in the surroundings of the WWTP (*Figure 1.5*). According to microscopy, cryptophytes were still the dominant group in terms of abundance (57% of the total cell abundance). Specifically, *U. complanatus* was more prevalent in the inner area of the estuary compared to the other sampling stations, as well as this taxon was present in the majority of the analysed water samples, reaching a maximum abundance of $7.7x10^6$ cells L^{-1} at URD5. Microscopy defined diatoms as the second most contributing group (23%), with the unidentified small (less than 10 μm) centric diatoms being the dominant taxon, with presence in every sample analysed. In this area, additionally, the dinoflagellate *K. foliaceum* was one of the most representative

taxa, being present in 90% of the samples, with a median cell abundance of approximately $4.5x10^4$ cells L^{-1}, and showing a clear preference for the innermost estuary. Metabarcoding, however, described a shared dominance in terms of contribution between diatoms (around 43%) and cryptophytes (around 38%), and *K. foliaceum* was not detected. In addition, molecular techniques revealed that the small centric *Cyclotella* spp. were the dominant diatom, together with important presences of *Nitzschia draveillensis*, *Navicula phyllepta*, *Navicula gregaria* and *Suriella angusta*. According to metabarcoding, this was the area with the highest cryptophyte diversity (25 taxa), and the most important taxa were the same as at URD3 and URD4, but with an increase frequency of occurrence and abundance of *U. complanatus*, as revealed by microscopy.

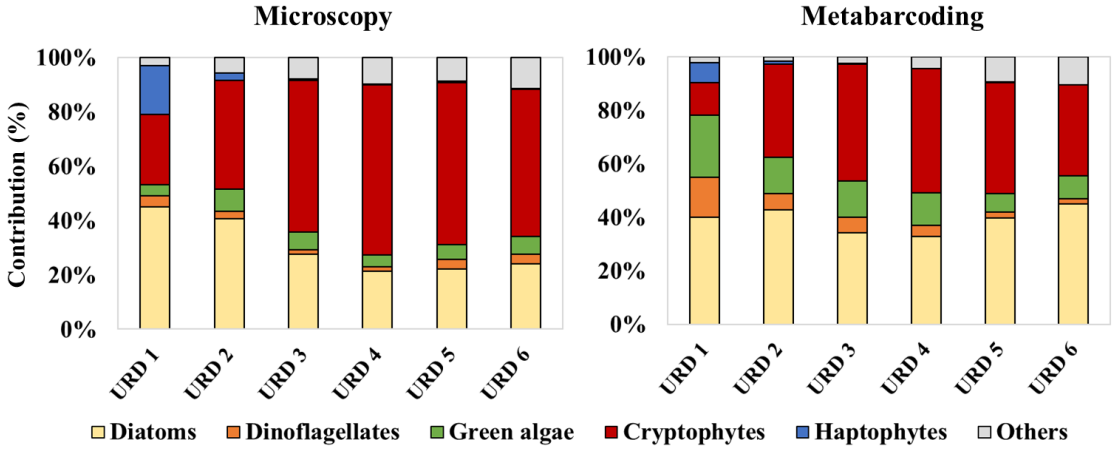

Figure 1.5. *Median relative contribution percentages of the main phytoplankton groups to the total community composition of each sampling station within the Urdaibai estuary, by microscopy cell counts (left) and DNA metabarcoding reads (right), at each sampling station.*

The spatial and temporal variability of the community composition described in the Urdaibai estuary with both microscopy and DNA metabarcoding have been verified by several non-parametric multivariate analyses. The two-way (spatial and seasonal) PERMANOVA analysis performed (*Annexes, Table A1.8*) with the microscopy and metabarcoding data revealed that phytoplankton community composition variability was explained by both the spatial gradient (different sampling stations) within the Urdaibai estuary and seasonal changes observed during the study period (p = 0.0001). In addition, when performing PERMANOVA with metabarcoding data, the interaction of both factors (spatial and seasonal) was also significant (p = 0.0007). The pair-wise PERMANOVA (*Annexes, Table A1.9*), both for microscopy and metabarcoding, revealed significant differences between every season ($p < 0.01$). Regarding sampling stations, URD1 was significantly different from all stations except URD2, and URD2 was also different from URD5 and URD6 ($p < 0.05$). Non-metric

multidimensional scaling (nMDS) illustrated the variation in the phytoplankton community composition along the sampling stations of the Urdaibai estuary and between seasons (*Figure 1.6*). SIMPER analysis revealed that the top 15 key phytoplankton taxa that contributed the most to the dissimilarities observed between sampling stations and between seasons were common in each approach (*Annexes, Table A1.10*). Among these top 15 taxa, a few similar dominant species were identified by both microscopy and metabarcoding method: *C. tenuissimus, M. polymorphus, T. acuta* and *U. complanatus*. Apart from these shared species, metabarcoding considered the following taxa responsible for spatio-temporal differences in the community: *Cyclotella* spp., *H. cryptochromatica, N. draveillensis, O. tauri, C. guillardii, N. phyllepta, S. angusta, O. lucimarinus, O. mediterraneus* and *Suriella* sp. As for microscopy, the organisms completing the top 15 taxa responsible for the dissimilarities were *Plagioselmis* sp., *T. gracilis, K. foliaceum, Tetraselmis* spp., Prymnesiales, *Heterocapsa* spp., *Eutreptiella* spp., *Chaetoceros* spp. and unidentified small centric and pennate diatoms.

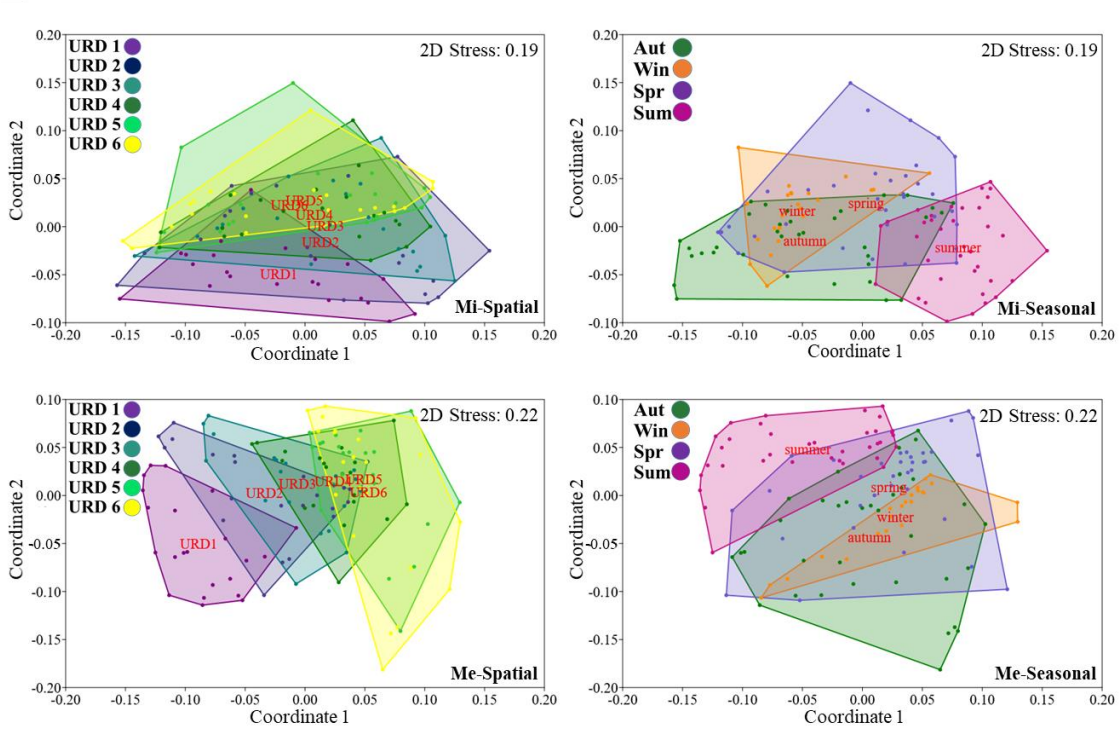

Figure 1.6. *Non-metric multidimensional scaling (nMDS) of phytoplankton cell abundance (microscopy; Mi) and relative read abundance (metabarcoding; Me) data using Bray-Curtis distances. Data are shown separately for microscopy (up) and metabarcoding (down). Different symbol colours represent the different sampling stations (left) or seasons (right).*

3.4 Relationship between phytoplankton community and environmental variables

The CCA (*Figure 1.7*) showed that the 10 physicochemical variables selected explained 80.8% of the phytoplankton abundance variability ($p < 0.001$). The first axis represented the

spatial variation of the environmental conditions (mainly influenced by salinity and nutrient concentration) and was the axis that explained most of the abundance variability of phytoplankton (53.5%). The Spearman correlation analysis confirmed the relationships between the chosen phytoplankton taxa and the environmental variables visible in the CCA (*Annexes, Table A1.11* and *Table A1.12*).

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

In addition, the CCA enabled the comparison of the results obtained by both microscopy and metabarcoding. Overall, the CCA revealed that the relationship of the selected phytoplankton taxa distribution with the environmental conditions along the Urdaibai estuary was similar

with both approaches. However, some taxa showed different patterns with the different approaches, for example *Tetraselmis* sp. showed greater preference for salinity with metabarcoding. This might be caused by the misidentification or omission of this taxon in microscopy samples.

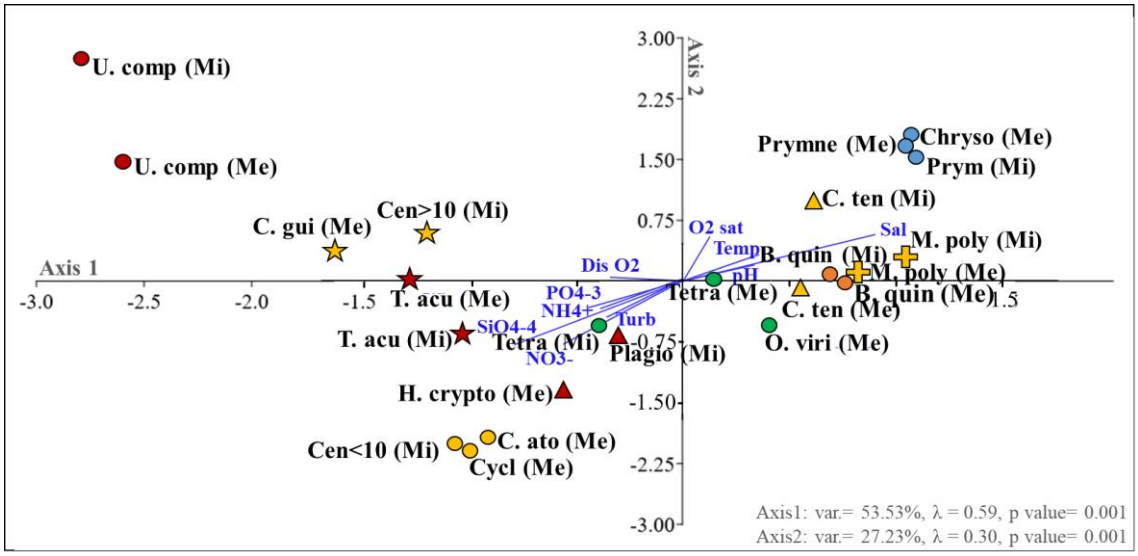

Figure 1.7. *Canonical Correspondence Analysis (CCA) plot showing the correspondence (influence) of the main environmental factors (blue lines) with the standardised abundance of the most relevant phytoplankton taxa of the Urdaibai estuary for the microscopy (Mi) and eDNA metabarcoding (Me) analysis. The analysis was significant overall at p < 0.001 (permutation test for CCA). Sal: salinity; Temp: temperature; Turb: turbidity; NH_4^+: ammonium; PO_4^{-3}: phosphate; NO_3^-: nitrate; SiO_4^{-4}: silicate; Var: variance; λ: eigenvalue. Phytoplankton taxa abbreviations are detailed in Table 1.1. The colours of the symbols indicate different phytoplankton groups: diatoms in yellow, dinoflagellates in orange, green algae in green, cryptophytes in red and haptophytes in blue. Symbols with the same shape and colour indicate correlated (matching) taxa from both techniques.*

4. Discussion

4.1. Phytoplankton community composition and dynamics

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

Regarding the variability of the phytoplankton biomass, the seasonal biomass cycle detected by both the total carbon content and Chl *a* in the Urdaibai estuary was typical of temperate estuaries in the Bay of Biscay (Varela, 1996; Trigueros and Orive, 2001; Seoane et al., 2005), and also in other temperate areas (i.e. Changjiang River Estuary in China, the fjords of Kategatt or Atlantic Iberian Peninsula) (Cartensen et al., 2007; Domingues et al., 2007; Gameiro et al., 2007; Wang et al., 2019). This seasonal cycle comprehends a low biomass period during autumn and winter, and a high biomass period during spring and summer. In temperate regions, higher water temperatures usually indicate higher solar radiation, and light availability is one of the main factors influencing phytoplankton growth (Morison et al., 2020), which may explain the phytoplankton biomass-temperature relationship observed in the present study. In addition, the high biomass values coincide with periods of low to moderate river flow (in spring and summer), while the lowest biomass values were registered with seasonal periods of high river flow, in agreement with previous works in the estuary (Madariaga and Orive, 1989; Madariaga et al., 1989, 1994; Ansotegui, 2001), and also observed in other studies (O'Boyle and Silke, 2010; Snow et al., 2000), where high flow rates could lead to the dilution of the Chl *a* (Du et al., 2017). Considering the torrential nature of the Oka River, when large flows occur after punctual precipitations, the flushing rate increases and the estuary is almost flushed. The seasonal biomass variability was more significant in the middle and inner estuary, while concentrations in the outer estuary remained fairly stable during the study period.

The spatial pattern of biomass showed the lowest concentrations in the outer estuary, while the maxima were recorded in the middle and inner area. This pattern coincided with that observed in late 90's in the studies performed along the Urdaibai estuary (e.g. Orive et al., 1998; Ansotegui et al., 2001). Ansotegui et al. (2001) recorded values higher than 100 µg L^{-1} of Chl *a* in the inner part, corresponding to the stations URD4 to URD6 of our study, and values below 6 µg L^{-1} of Chl *a* in the outer estuary, corresponding to URD1. In the present study, the maximum values registered were not so high, but the pattern was the same. This trend is repeated in many estuaries around the world (Brito et al., 2015; Santos et al., 2022; Seoane et al., 2005; Shi et al., 2019). For example, McGarrigle et al. (2001) reported on the distribution of Chl *a* in 25 estuarine and coastal areas around Ireland in 1998-2000 and the range of concentrations found in the inner part of the estuaries were approximately 10 times higher than those obtained in the outer and coastal adjacent areas. This distribution of Chl *a* is explained by the factors governing phytoplankton growth (e.g., nutrients, light, and grazing). Wang et al. (2019) reported that, in the inner areas of the estuaries, phytoplankton

growth is mostly limited by light availability, while in the outer areas the limiting factor is mostly nutrient availability. In the outer area of the Urdaibai estuary, nutrients were usually found in low concentrations, in accordance with several authors that have stated that during stratification periods, in shelf waters off the Basque coast, the residual concentrations in the water layer above the thermocline are comparable to those found in oligotrophic areas (Muñiz et al., 2019). Therefore, this nutrient limitation could explain the lower phytoplankton biomass recorded in the outer area. However, high nutrient concentrations (like N, P and Si) were found in the inner estuary, which might have enhanced the phytoplankton growth in the area. This finding aligns with previous studies (e.g., McCabe et al., 2016; Vajravelu et al., 2018) and is supported by the positive correlations observed between Chl *a* and ammonium as well as phosphate in this study. Additionally, although turbidity values were much higher in the inner estuary, reducing light availability, it seems that it is not a limiting factor for phytoplankton growth, probably due to the high heterogeneity of environmental conditions found in this area (clearly represented by the PCA) due to the Oka River inflow.

As for the community composition, although the sampling frequency and stations do not exactly coincide with the studies performed in the system before, the phytoplankton community of the Urdaibai estuary described in the present study was similar to results reported in the late 90's (Madariaga et al., 1989, 1994; Orive et al., 1995, 1998; Trigueros and Orive, 2001). In the outer estuary, the community was mainly dominated by diatoms, with the recurrent presence of dinoflagellates in this area, while the dominance of cryptophytes, green algae, centric diatoms and *K. foliaceum* was restricted to the inner part. In addition, the trends observed of taxa richness decreasing towards the inner estuary were also similar to the studies performed in late 90's (e.g., Trigueros and Orive, 2001). This higher taxonomic richness in the highest salinity zone has also been described in other estuaries by several authors (e.g. Paczkowska et al., 2019; Saifullah et al., 2019; Bharathi et al., 2022). It may be a result of the salinity range that populations can tolerate, since, in estuaries, salinity is known to be a fundamental modulator of the abundance dynamics of the species (e.g. de Affe et al. 2018; Córdoba-Mena et al., 2020). Additionally, the trend of decreasing richness towards the inner estuary (*Annexes, Figure A1.1*) may also reflect the effect of the eutrophic conditions of this area. Biodiversity measures (e.g., species richness, Shannon–Wiener diversity, and Pielou's equitability indexes), together with the abundance of some specific taxonomic groups of microalgae, have been suggested as indicators of nutrient enrichment (Machado et al., 2023). Several studies found a reduction in diversity and species richness as a consequence of nutrient enrichment (e.g., Soares et al., 2013; Baho et al., 2017; Machado

et al., 2023), mainly caused by the dominance of a few species and the decrease in the presence and abundance of rare species or functional groups (Ansari et al., 2011).

Diatoms dominated the outer and middle estuarine waters. This pattern was also observed in other areas around the globe, such as Ireland (Oboyle and Silke, 2010), Rio de la Plata (Argentina) (Gómez et al., 2004) and Sado Estuary (Portugal) (Santos et al., 2022). Among the diatom taxa found in these areas, *M. polymorphus* and *C. tenuissimus* both were the most important bloom-forming diatoms at URD1 and URD2. *M. polymorphus* had not been reported previously in any scientific research paper of the Urdaibai estuary, but was described in several technical reports done for the administration (URA, Basque Water Agency). This marine, planktonic and cosmopolitan species usually occurs in estuaries and open ocean (Walsh et al., 1988), which explains the significant correlation (ρ 0.7; $p < 0.01$) established in the present study with salinity. In addition, *M. polymorphus* has shown a clear preference for the summer season, which was confirmed by the positive correlation with temperature, in accordance with previous studies that reported summer blooms of this species in the Fusaro Lagoon (Mediterranean Sea) or the Bohai Sea (China) (Sarno et al., 1993; Chen et al., 2019a). As for *C. tenuissimus,* also identified by both techniques, it is a small cosmopolitan species that thrives in coastal waters around the world between tropical and temperate waters (De Luca et al., 2019; Grzebyk et al., 2022), being frequently found in the Mediterranean Sea, Atlantic Ocean and Japanese coastal waters among others (Hongo et al., 2021). This species is characterised by having a high growth rate (at least three divisions per day) and causes blooms mostly in the spring and autumn (Hongo et al., 2021). This marine character and rapid growth agree with its higher presence in the poly-euhaline zone of the Urdaibai estuary (positive correlation with salinity) and recording high cell abundances in late spring-summer (positive correlation with temperature), like the bloom of 20 million cells L^{-1} of June 2020 in URD2. Comparing with previous studies, it is remarkable the low densities of *Asterionellopsis glacialis* found in the present research, which was one of the most dominant diatom species in the studies performed in late 90's, being sometimes more than 90% of the community (Ansotegui et al., 2003).

The outer area also recorded the highest dinoflagellate and haptophyte diversity along the estuary, which is mainly explained by the marine nature of most of the species of these two groups. This is in accordance with previous studies in the Urdaibai estuary and in the nearby estuary of Bilbao (Trigueros et al., 2000b; Trigueros and Orive, 2001; Seoane et al., 2005; Seoane et al., 2009a). Additionally, the mixotrophy explains the high presence of these groups

in the outer estuary, providing them with the ability to grow in low nutrient regions (Zhang et al., 2013; Unrein et al., 2014). However, in the case of dinoflagellates, their contribution to the total phytoplankton abundance in the inner estuary is similar to the outer estuary, even if the species richness is much lower. This is explained by the large cell abundances that some of these dinoflagellate species (e.g., *K. foliaceum*) record in the inner estuary.

From the middle estuary towards the inner Urdaibai estuary, cryptophytes started to increase in abundance, becoming the dominant group in the phytoplankton community in the channelled section of the estuary, following the pattern of other eutrophicated estuaries such as Chesapeake Bay (Adolf et al., 2006), Neuse River Estuary (Valdes-Weaver et al., 2006) or Galveston Bay in Texas (Paerl et al., 2003). This trend of increasing abundance in this middle and inner section of the estuary was also recorded in previous studies in Urdaibai estuary (Madariaga, 1995; Orive et al., 1998; Ansotegui, 2001). However, in the present study, their dominance is more evident compared to the late 90s', obtaining higher cryptophyte abundances than in previous studies. The increase of cryptophytes' dominance has been recently observed in other temperate estuaries as well, such as Tagus (Brito et al., 2015) and Sado (Santos et al., 2022) estuaries. The studies of both Brito et al. (2015) and Santos et al. (2022) compared the phytoplankton communities in the estuaries with the community observed historically and both agreed with the trend of the increasing dominance of cryptophytes. The dominance of cryptophytes over other groups in the middle and inner Urdaibai estuary may have been triggered by higher turbidity and organic matter concentration found in these areas of the estuary, since several authors (e.g. Bergmann, 2004; Adolf et al., 2008) have described the capability of crytophytes for growing in such conditions. Their success in this niche, over other groups like diatoms, is explained by their ability to survive under restricted light conditions, since cryptophytes adapt the absorption spectrum of their phycobiliprotein antennas by replacing a bilin pigment with another one, capable of more efficiently harvesting the available wavelengths (Collini, 2022). Being small flagellates, their motility could also be advantageous in these light restricted zones. In addition, they show mixotrophic character, which allows them to utilise dissolved organic carbon for growth (Johnson et al., 2013). Another reason for the substitution of dominant group from the outer area (diatoms) to this middle and inner area (cryptophytes) could be the different nutritional preferences of the dominant groups. While diatoms, along with the silicate, preferably make use of nitrate, cryptophytes have a higher advantage in ammonium-enriched waters (Horner Rosser and Thompson, 2001). This is in agreement with the positive correlations recorded between ammonium and *U.complanatus* and *T. acuta* in the present

study. Among the cryptophytes found in the estuary, some showed a preference for higher salinity areas like *T. amphioxeia* and *T. gracilis*, and others were more restricted to the inner estuary, like *H. cryptochromatica*, *T. acuta* and, especially, *U. complanatus*. *T. amphioxeia,* which was not identified by microscopy, is well known from brackish waters in Europe (Throndsen et al. 2007; Gran-Stadniczeñko et al., 2019), whereas *T. gracilis*, detected by both approaches, was described in 2012 from the Atlantic coast of Spain for the first time (Laza-Martínez et al., 2012). *U. complanatus*, considered one of the most characteristic species of the inner Urdaibai estuary, is a common bloom-forming euryhaline cryptophyte in the estuaries of SE Bay of Biscay (Seoane et al., 2012). Recently, blooms of *U. complanatus* have also been registered in Japanese estuaries, in brackish waters in Ehime, Hiroshima and Kochi prefectures (Mizobuchi et al., 2021).

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

Diatoms, both pennated and centric, were also an important part of the phytoplankton community of the inner Urdaibai estuary. Species such as the epipelic pennates *Navicula phyllepta* and *Navicula gregaria,* found in Urdaibai, have been recorded as the dominant

species of the benthic substrate from intertidal mudflats (Adlmiraal et al., 1984; Haubois et al., 2005) and from oligo and mesohaline areas of estuaries (Underwood et al., 1998). The ability of these small fast growing diatoms to move in the sediment allows them to migrate to the surface for receiving higher light intensity, which at the same time increases the possibility of their resuspension to the water column. The resuspension rates of these benthic diatoms are greater in shallow waters of well mixed estuaries (Baillie and Welsch, 1980), such as the Urdaibai estuary, due to the higher influence of wind action, tidal currents and convective currents on theses ecosystems in comparison to deeper estuaries with marked halocline (Anderson, 1973). This explains the high abundance in which both *N. phyllepta* and *N. gregaria* were found in the waters of the inner Urdaibai estuary. Regarding the centric diatoms, the genus *Cyclotella* was the main representative of the inner Urdaibai estuary. *Cyclotella* is a common genus from the inner areas of temperate estuaries, due to its oligo or mesohaline character. This genus has been previously recorded in high abundances in several estuaries, such as the Schelde estuary (Muylaert et al., 2009), Bahía Blanca estuary (Popovich and Marcovecchio, 2008) and the nearby Bilbao estuary (Seoane et al., 2005).

4.2. Comparison of the community characterization methods

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

Differences in the identification capacity of molecular and morphological data sets could be one of the main causes of dissimilarity between the two methods (Zimmermann et al., 2015;

Kim et al., 2019; Santi et al., 2021). In the present study, DNA metabarcoding showed pronounced differences with microscopy when comparing the number of species/genera identified, the number of identified taxa (to, at least, the genus level) being 3 times higher in eDNA metabarcoding. Overall, metabarcoding revealed the presence of 349 phytoplankton taxa that were not identified by microscopy, among which 276 taxa were not previously recorded for the Urdaibai estuary (*Annexes, Table A1.5* and *Table A1.6*). Among these, 223 reached the species level, registering 77 new diatom species, 50 dinoflagellates, 40 green algae (mostly chlorophytes), 15 cryptophytes, 13 haptophytes, 7 dictyochophyceans, 7 chrysophyceans, 4 bolidophyceans, 4 pelagophyceans, 3 rhodophyceans, 2 synurophyceans and 1 raphidophycean for the first time in the Urdaibai estuary. The reason for the omission or misidentification of the phytoplankton taxa by microscopy varies depending on characteristics of the phytoplankton groups. In the case of green algae and haptophytes, their small cell size and fragility may be the main reason for their misidentification (Agirbas et al., 2015; Lee et al., 2020), since pico- and nanoplanktonic organisms require electron microscopy or molecular methods for identification (Gran-Stadniczeñko et al., 2019). As an example, 6 haptophyte taxa were identified by microscopy (3 species), while 19 taxa (14 species) were identified by molecular techniques, some of them being especially recurrent in the outer estuary, such as *Chrysochromulina scutellum* and *Chrysochromulina rotalis*. Many of them were previously identified in the nearby Nervion estuary (Seoane et al., 2009a) from natural samples and uni-algal cultures using light and mainly electron microscopy; however, this procedure is unfeasible for regular phytoplankton monitoring. The difference was even bigger for green algae, since the number of taxa identified increased from 12 to 61, as represented by *Picochlorum* spp., *Ostreococcus* spp. (*O. mediterraneus*, *O. lucimarinus* and *O. tauri*) and *Micromonas* spp. (mainly *M. bravo* and *M. commoda*), some of the most frequent taxa of the estuary according to metabarcoding, and were reported for the first time in the Urdaibai estuary in this study. This limitation was previously described in nearby estuaries (Abad et al., 2016) and marine areas (e.g. Gran-Stadniczeñko et al., 2019). Diatom misidentification, which was notable in the present study, can be due to the presence of cryptic species that cannot be identified. Additionally, the lack of resolution of the microscopy method used to identify diagnostic morphological characters may have resulted in overly coarse or erroneous taxonomic identification optically (Kim et al., 2019; Santi et al., 2021). In the present study, eDNA metabarcoding helped improving the resolution in the "unidentified centric" and "unidentified pennate" diatoms groups. Among the unidentified centric diatoms, we could determine the presence of three different *Cyclotella* species (*C. atomus*, *C. choctawhatcheeana* and *C. striata*), 2 *Minidiscus* (*M. spinulatus* and *M.*

variabilis), 12 *Thalassiosira* species, *Mediolabrus comicus* and *Stephanocyclus meneghinianus,* among others, by molecular techniques. As for unidentified pennate diatoms, 11 *Nitzschia* species, 10 *Navicula* species and 2 *Haslea* (*H. nipkowii* and *H. pseudostrearia*) were identified by eDNA metabarcoding. Scanning electron microscopy (SEM) is commonly used (e.g. Li et al., 2019) to allow a more detailed view at a higher magnification of many of the previously mentioned diatom species, since the diatom frustule, composed of two valves and a number of overlapping girdle bands, possesses a species-specific morphology of micro- and nanopatterns (Soleimani et al., 2021). In order to observe these patterns, in addition to the higher magnification provided by the SEM in comparison to the inverted microscope, diatom cells must overcome a digestion process to eliminate their intra-celular organic material, which is not part of the Utermöhl method followed in most of the phytoplankton monitoring programmes and the present study. In addition, as mentioned in several similar studies (e.g. Gran-Stadniczeñko et al., 2019), an important aspect in this comparison is that eDNA metabarcoding results are based on a bigger sample volume analysed (0.2–3.5 L) in comparison to microscopy (50 ml), which has also helped to reach a higher taxonomic richness. This proficiency of metabarcoding over phytoplankton for identifying phytoplankton taxa makes it a more accurate tool for monitoring rare and endangered species or detecting invasive species (Keck et al., 2022).

Nevertheless, among the 615 phytoplankton taxa identified in the Urdaibai estuary, only 407 were identified to the genus or species level, which leaves a high number of sequences with an incomplete taxonomic assignment. There are several causes behind these incomplete identifications (Santoferrara et al., 2019): insufficient marker resolution (e.g. not variable and/or long enough), unsuitable method or parameters (e.g. BLAST assignments based on suboptimal sequence similarity/coverage) or incomplete or inaccurate databases. Although the 18S rRNA gene is the most widely used marker for group and species detection within marine eukaryotic microorganisms (Martin et al., 2022), in some taxonomic groups (e.g. diatoms and haptophytes) the V4 18S rRNA gene, the one applied in the present study and many others (e.g. Piredda et al., 2017; Wang et al., 2022), shows identical sequences for different species, leading to incomplete identifications (Gran-Stadniczeñko et al., 2019). Not only the marker gene used, but also the primer choice can be crucial for an effective taxonomic assignment, since its amplification and binding affinity are critical factors to bring about taxonomic biases in eDNA metabarcoding identification (Kim et al., 2019; Van der Loos and Nijland, 2021). However, primer efficiency is highly species-specific, which would prevent straightforward assessments of species abundance (Elbrecht and Leese, 2015) and

might imply group-specific primer choice to obtain a higher rate of taxonomic assignment. Indeed, it is believed that a multimarker approach, using several primer sets, will result in a more reliable estimation of species richness (Alberdi et al., 2018). As for the bioinformatics decisions, its flexibility makes it an important source of variability of the final results obtained, and therefore, data sharing is key to enhancing result reproducibility and information discoverability, in order to develop community standards for bioinformatics methods (Santoferrara, 2019). In addition, many phytoplankton taxa are difficult to cultivate and/or identify through microscopy, and therefore, no molecular references are available, resulting in poor taxonomic coverage and data quality of reference libraries, which might also explain a high number of unclassified taxa obtained in the study (Gran-Stadniczeñko et al., 2019). Thus, species identification varies with accuracy and coverage of reference databases (Kim et al., 2019), which is considered one of the main drawbacks of the DNA metabarcoding approach (e.g. Weigand et al., 2019; Rimet et al., 2021).

This incomplete identification of ca. 200 unique phytoplankton taxa detected by metabarcoding leads to some "false negatives" when comparing it with microscopic identification. Among the species identified by microscopy that were not part of the metabarcoding results (*Annexes, Table A1.5* and *Table A1.7*), the dinoflagellate *K. foliaceum* must be highlighted. *K. foliaceum* was present in almost all the samples of the inner Urdaibai estuary and has been recorded in previous studies in the areas as well (Trigueros et al., 2001); however, molecular techniques did not identify it, with Kryptoperidiniaceae being the lowest identified taxonomic level. In addition, *K. foliaceum* is included in the database used in the present study (PR2 4.13.), and the sequence corresponds to the same region of 18S rRNA of the target of the primer used in the present study. Thus, this "false negative" may be caused by technical biases introduced throughout the DNA metabarcoding workflow (Martin et al., 2022): sample preservation (Mäki et al., 2017), DNA extraction (Van der Loos and Nijland, 2021) and/or PCR (Latz et al., 2022). The same may have happened with *Prorocentrum micans* and *Gephyrocapsa huxleyi*, which are both available in the PR2 database, but were not detected in our samples by molecular techniques, even if they were observed by microscopy. Several options for mitigating these false negatives are the optimisation of nucleic acid extraction and storage, improving primers and sequence processing and the adequate biological and technical replication (Santoferrara, 2019). Moreover, and in accordance with what Gran-Stadniczeñko et al. (2019) described, the molecular techniques almost overlooked the Euglenophyceae of the Urdaibai estuary, which are relevant and frequent green algae according to microscopy, but register much lower relative abundances

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

Additionally, besides the inherent technical biases, the major source of bias causing discrepancies between both approaches is the 18S rRNA gene copy number variation within species, genera and plankton groups (Santi et al., 2021; Martin et al., 2022). This variation of gene copy numbers can be substantial (ranging from tens to thousands), affecting the proportion of reads found for each species present in complex environmental assemblages and leading to misinterpretation of relative abundances when comparing to microscopic counts (Santi et al. 2021). In the present study, even if the relative abundance results for both techniques were quite similar in some cases (e.g. defining the dominant phytoplankton group), in most of the cases, these results differed (*Figure 1.5*). As an example, the median relative abundance of dinoflagellates and green algae within the Urdaibai estuary was two times higher according to metabarcoding when compared to microscopy. This is similar to the findings of Piredda et al. (2017) and Santi et al. (2021), which registered higher percentages of dinoflagellate contribution produced by metabarcoding. The main reasons for this frequent difference might be that, compared to taxa with similar cell size, dinoflagellates have large genomes and putatively high rRNA gene copy number, thus, an overrepresentation may be expected by molecular techniques (Martin et al., 2022). However, another reason for the discrepancies between techniques may be that the broadly used fixatives (e.g. Lugol) cannot preserve the morphology of unarmoured dinoflagellates and might lead to misidentification or omission by microscopy (Santi et al., 2021), decreasing their contribution to the total abundance. Therefore, although several authors (Piwosz et al., 2020; Martin et al., 2022) agree that community relative abundances determined by eDNA metabarcoding are useful and reliable in the context of ecological interpretations (like in the present study), microscopy cell counts are still essential when studying bloom-forming taxa and/or toxic species in the community.

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

two methods provides a much more complete image of the phytoplankton abundance and community composition and its spatio-temporal variability of the Urdaibai estuary.

5. Conclusion

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

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

2. Kapitulua

Gernikako araztegiko isurketen desbideratzeak Urdaibai estuarioko fitoplanktonean eta baldintza fisiko-kimikoetan izandako berehalako eragina

(*Chapter 2: Immediate effect of sewerage improvement on the phytoplankton and physicochemical conditions in the Urdaibai estuary*)

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Laburpena

Mantenugaien aberaste antropikoa ingurumen-arazo nagusia bihurtu da mundu osoko estuarioetan, hondakin-uren araztegien (HUA) isurketak honen erantzule nagusietako bat izanik. Fitoplanktona, mantenugaien kontzentrazioarekin batera, estuarioen egoera ekologikoaren ebaluaziorako adierazle erabilienetako da, kate-trofikoan duen oinarrizko posizioaren ondorioz. Ikerketa honek, Gernikako HUAren isurketak Urdaibai estuariotik kanpo desbideratzeak sistemaren ingurumen baldintzetan eta fitoplankton komunitatean izandako berehalako eragina ebaluatzea zuen helburu. Horretarako, saneamendu lanak burutu baino lehen eta lanak amaitu osteko baldintza fisiko-kimikoen eta fitoplankton biomasa eta komunitatearen osaeraren (pigmentuen analisi eta mikroskopia bidez) konparaketa egin zen, epe laburrean. Emaitzek, saneamendu lanek estuarioan berehalako eragina izan zutela baieztatu zuten, bereziki estuarioaren barrualdeko eremuan nabaritu zena. Amonio (72,3 μmol L^{-1}-tik 18,2 μmol L^{-1}-ra) eta fosfato (3,5 μmol L^{-1}-tik 2 μmol L^{-1}-ra) kontzentrazioek bat-bateko jaitsierak pairatu zituzten, uraren kalitatea hobetzea eragin zuena, estuario osoan zehar egoera “ona” lortuz, gutxienez. Fitoplankton-komunitatearen osaerak ere berehalako erantzuna erakutsi zuen araztegiaren inguruan, saneamendu lanen ondoren *b* klorofila (Chl *b*) pigmentu diagnostiko ugariena bihurtu baitzen, *Eutreptiella* sp.-ren hazkuntza azkarraren ondorioz. Hala ere, ikerketa honen iraupena hilabete bakarrekoa izan da eta, beraz, denbora gehiago behar da Urdaibai estuarioan egindako saneamendu-lanek sisteman duten benetako eragina frogatzeko.

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. 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^{-1}) and phosphate (from 3.5 to 2 μmol L^{-1}) 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* (Chl *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. Sarrera

Estuarioak, ibai batetik edo gehiagotik isurketak jasotzen dituzten eta, batzuetan partzialki itxita egon daitezkeen arren, itsasora konektatuta dauden kostaldeko ur-masak dira, ibai- eta itsas-ekosistemen arteko trantsizio guneak alegia (Zambrano-Monserrate eta Ruano, 2021). Sistema hauek betidanik izan dira erakargarriak gizakiontzat, eskaintzen dituzten zerbitzu-ekosistemikoengatik, hala nola, ur eta elikagaien hornidura, uholdeen aurkako babesa, hondakin-uren jasotzea, garraiorako aukera edo/eta kultura eta aisialdi zerbitzuak (Beaumont et al., 2007). Ondorioz, estuarioen inguruan hiri-eremu handiak garatu dira mundu osoan zehar (Boerema eta Meire, 2017) eta horrek eragindako biztanleria-dentsitate handiek eta jarduera antropikoek presio handia eragiten dute sistema hauetan (Rodrigues et al., 2021).

Azkenaldian, mantenugaien aberaste antropikoa ingurumen-arazo nagusia bihurtu da mundu osoko estuarioetan eta, estuario hauetako askotan, hondakin-uren araztegiak (HUA) dira fosforo (P) eta nitrogeno (N) isurketa puntualen erantzule nagusiak (Silva et al., 2016). Honenbestez, askotan HUAk giza kokalekuen inpaktuak arintzeko egitura garrantzitsuak diren arren (Cloern et al., 2016), kutsatzaileen kontzentrazioa murriztuz eta hondakin-urak zuzenean ibaietara isurtzea saihestuz (Pascual-Benito et al., 2020), kutsadura iturri bihurtu daitezke tratamenduen eraginkortasun faltaren ondorioz.

Estuarioetan, mantenugai kontzentrazio handiek eutrofizazioa eragin dezakete (Eccles et al., 2020), honek dakartzan ondorio biologiko negatiboekin, hala nola, fitoplankton biomasaren hazkundea eta alga loraldi kaltegarriak ugaritzea; izan ere, organismo hauen hazkuntza-tasa oinarrizko mantenugaien (N, C, P, O, Fe, Si) kontzentrazioen hazkuntzarekin handitzen da (Wells et al., 2015; Vajravelu et al., 2018). Ekoizpenaren gorakada honek eta materia organikoaren metaketak baldintza hipoxikoak eragin ditzakete, biodibertsitatearen galera eta komunitatearen egitura-aldaketarekin batera (Scherner et al., 2013).

Testuinguru honetan, mantenugaien kontzentrazioa eta fitoplanktona dira estuarioak bezalako azaleko uren egoera ekologikoaren ebaluaziorako adierazlerik erabilienetakoak (adibidez, Europako Uraren Esparru edo Itsas Estrategiaren Esparru Zuzentarauetan; UEZ eta IEEZ) (Seoane et al., 2011). Mantenugaien kasuan, hauen jarraipenak isurketek estuarioaren ezaugarri fisiko-kimikoetan dituzten ondorio zuzenen kontrola ahalbidetzen du, ez bakarrik isurketak jasotzen dituzten eremu puntualetan, baizik eta isurketa eremuetatik haratago ere, orokorrean sistema osoan zehar nabaritzen baitira ondorioak (Poikane et al., 2019).

Fitoplanktona, bestalde, kate-trofikoan duen oinarrizko posizioa dela eta, mantenugai ez-organikoen eta gainerako maila trofikoen arteko loturatzat hartzen da (Seoane et al., 2011) eta mantenugaien eskuragarritasun aldaketei erantzuten dien lehen komunitate autotrofoa dela uste da (Paerl et al., 2003). Hori dela eta, mantenugaien eta fitoplanktonaren jarraipena egitea lagungarria da aldaketa antropikoen berehalako eraginak hauteman eta/edo aldaketa kaltegarri horiek arintzeko kudeaketa-estrategien eraginkortasuna ebaluatzeko (Li et al., 2018; Eccles et al., 2020).

Fitoplanktonaren jarraipena, tradizionalki, mikroskopia optiko bidezko identifikazio eta zenbaketaren bidez egin da (adibidez, Córdoba-Mena et al., 2020). Hala ere, metodo honek denbora asko (Wang et al., 2018) eta ezagutza taxonomiko zabala (Naik et al., 2011) eskatzen ditu, emaitzek gaitasun pertsonalen menpekotasun handia izatea eraginez (Muñiz et al., 2020). Bereizmen altuko kromatografia likido (High Performance Liquid Chromatography; HPLC) bidezko pigmentuen analisia fitoplanktonaren jarraipena egiteko alternatiba bilakatzen ari da (adibidez, Chai et al., 2016; Damar et al., 2020). Teknika honek, 50 pigmentu fitoplanktoniko baino gehiago modu erraz eta azkarrean identifikatu eta kuantifikatzeko aukera ematen du (Zapata et al., 2000), mikroskopian baino bolumen handiagoa aztertuz (Agirbas et al., 2015). Pigmentuak biomarkatzaile gisa erabiliz, fitoplankton-komunitatearen osaera zehaztu (pigmentu diagnostikoekin) eta biomasa totalaren kuantifikazioa (*a* klorofila erabiliz) egin daiteke (Chai et al., 2016; Nunes et al., 2018). Hala ere, teknika honek zenbait muga ere baditu, adibidez, hainbat fitoplankton taldek pigmentu diagnostikoak partekatzea, honek hauen bereizmen-ahalmena mugatzen baitu, eta identifikazioa talde mailaraino baino ez dela heltzen (Latasa, 2007; Nunes et al., 2018). Ondorioz, tekniken konbinazioa (mikroskopia eta pigmentuen analisia) da komunitatearen irudi osatuena lortzeko aukerarik erabiliena (adibidez, Dursun et al., 2020; Lee et al., 2020).

Urdaibai estuarioak, Euskal Autonomia Erkideko (EAEko) Biosfera Erreserba bakarraren parte izan arren, presio antropogeniko nabarmena jasaten du, alderdi natural, ekonomiko eta kulturalaren arteko oreka lortzea zaila izanik (Monge-Ganuzas et al., 2017). Presio eragileen artean, estuarioaren barruko eremuan kokatua dagoen Gernikako HUA nabarmentzen da. Araztegiak lehen eta bigarren mailako tratamenduak burutzen zituen, baina ez zen P eta N eraginkortasunez ezabatzeko gai, mantenugai horiek eutrofizazioaren eragile nagusiak izanik. Ondorioz, 1972az geroztik, Gernikako HUAtik estuariora egin diren isurketa zuzenak sistemaren kutsadura- eta ingurumen-arazoen iturri nagusia izan dira eta estuarioaren barruko

eremuak ez ditu UEZ-ak ezarritako ingurumen-helburuak bete azken urteotan (Borja et al., 2021), mantenugai eta fitoplanktonaren gehiegizko ugaritasunagatik.

Estuarioak Gernikako araztegiaren isurketak jaso bitartean, hainbat izan dira sistemaren ezaugarri fisiko-kimikoen (adibidez, Iriarte et al., 2016) edo giza inpaktuen (Castillo-Eguskitza et al., 2017) inguruan egin diren ikerketak, baita fitoplanktonaren ingurukoak ere (adibidez, Ansotegui et al., 2001; Trigueros eta Orive, 2001). Hala ere, azken urteotan saneamendu-lanak egin dira eremuan eta, 2021 uztailaren hasieran, Gernikako araztegiaren isurketak estuariotik kanpo desbideratzea lortu zen. Gaur egun, isurketak, kolektore baten bidez, Lamiarango HUAra (Bermeora) bideratzen dira eta, handik, araztutako efluenteak kostaldera isurtzen dira. Honenbestez, 2021an, 50 urtetan zehar Urdaibai estuarioko barnealdera isuritako Gernikako araztegiaren mantenugai-karga antropikoak eten egin ziren.

Ikerketa honek Gernikako HUAren isurketen eteteak Urdaibai estuarioaren ur-kalitatean eta fitoplankton-komunitatean duen berehalako eragina ebaluatzea du helburu, epe laburrean (asteak), estuarioko baldintza fisiko-kimikoak eta fitoplankton biomasa eta osaera desbideratzea baino lehen eta ostean alderatuz. Ikerketaren hipotesia mantenugai kontzentrazioaren berehalako jaitsiera egongo dela da, eta honek estuarioko fitoplankton-komunitatean aldaketak eragingo dituela.

2. Material eta metodoak

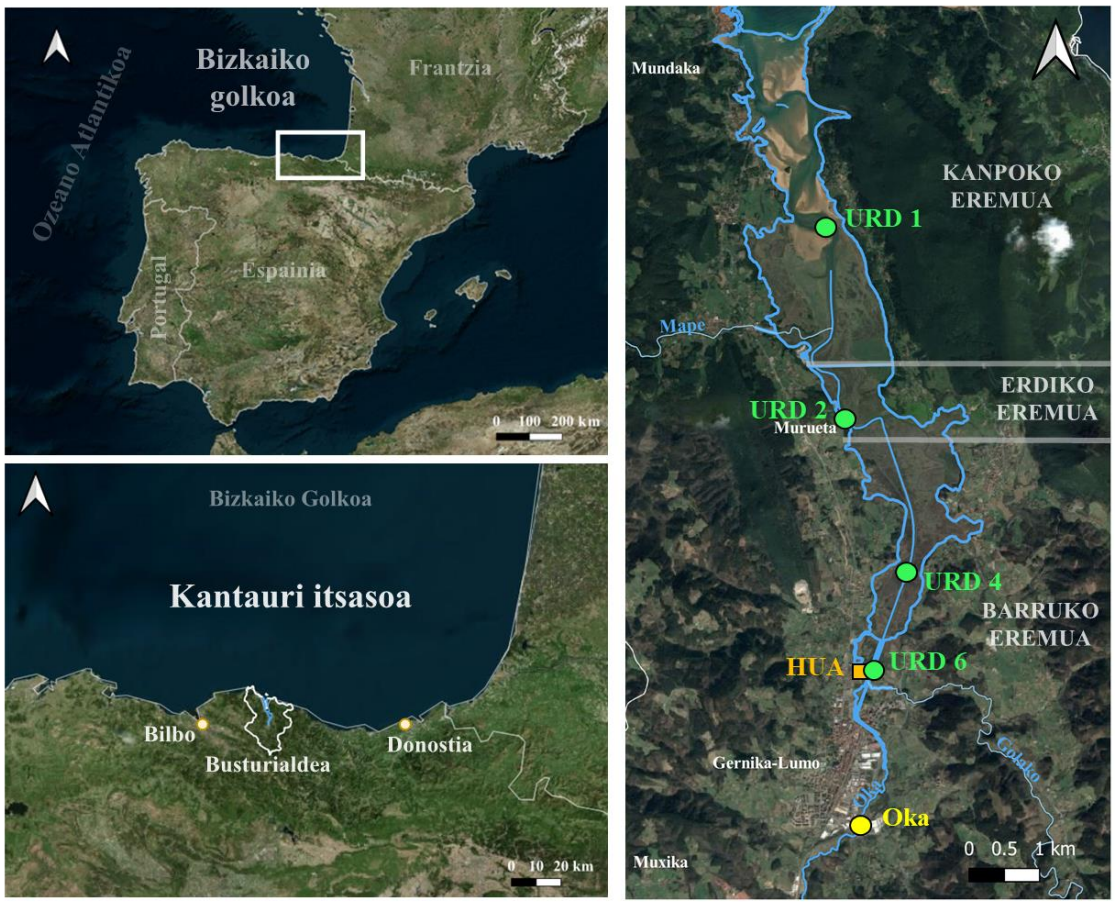
2.1. Ikerketa eremua

Urdaibaiko estuarioa Bizkaiko Golkoko hego-ekialdean kokatutako (43°22'N, 2°41'W) sistema meso-makromareala da (*2.1. irudia*). Eremuko klima epel-ozeanikoa da, uda epel eta negu leunekin, eta Golkoko Korrontearen eta trosposferaren erdialde eta goialdeko mendebaldeko haize atmosferikoen eraginpean dago (Usabiaga et al., 2004). Estuarioak Oka ibaiaren itsasoratze eremua hartzen du, sistema laburra (13,7 km) eta sakonera txikikoa (Barroeta et al., 2023) izanik. Ur gezak sistemari egiten dion ekarpena txikia da estuarioaren guztizko bolumenarekin alderatuta eta ibaiaren isurketa txikia izan ohi da marea-prismarekin alderatuta (Villate et al., 2017). Ondorioz, itsasgoran zehar, estuarioaren gehiengoa itsasoaren eraginaren menpe dago eta marea zikloek eragin handia dute sisteman (Valencia et al., 2004). Ur zutabearen estratifikazioa estuarioan zehar aldatuz doa, kanpoaldean ur-zutabea guztiz nahastuta egotetik, erdiko eta barruko eremuan geruzatuta egotera pasatuz (Barroeta et al., 2020). Hala ere, estuarioaren geomorfologia eta Oka ibaiaren izaera torrentziala dela eta,

estuarioaren bolumena eta hustuketa tasak oso aldakorrak dira (Madariaga et al., 1995). Honek eragin zuzena du estuarioaren ezaugarri fisiko-kimiko eta biologikoetan, denbora eskala laburrean fitoplankton-komunitatearen osaera eta ugaritasunean aldaketak sustatuz (Madariaga et al., 1995).

Morfologian oinarrituta, estuarioan hiru gune nagusi identifikatu daitezke (*2.1. irudia*) (Villate et al., 1989). Kanpoko eremua, sistemaren kanpo-mugatik (Mundaka) Muruetaraino hedatzen dena, marearteko lautada zabalak eta hondartza hareatsuak dituen eremua da. Erdialdeko eremua, Muruetan hasi eta kanal artifizialaren hasieraraino doana, nabarmen estutzen da kanpoaldeko eremuarekin alderatuz eta kanal bihurgunetsuen sare konplexu batez eta padura garatuz inguraturiko kanal zentral batek osatzen du. Barrualdeko eremua, 4 km-ko luzera eta 15 m-ko zabalera duen ubide artifizial bat da, Gernikako herrira iristen dena eta lezkadiz inguratuta dagoena.

2.2. Laginketa eta datuen bilketa



2.1. irudia. *Ikerketa eremua eta laginketa puntuak. Ezkerrean, Urdaibai estuarioaren kokapena, Bizkaiko Golkoan (goian) eta Euskal kostaldean (behean). Eskuinean, laginketa puntuen kokapena estuarioan zehar eta Gernikako HUA.*

Urdaibai estuarioko egoera Gernikako HUAaren isurketak gelditu aurretik eta ostean konparatu ahal izateko, sei laginketa burutu ziren 2021an, bi isurketak egon bitartean, eta lau desbideratzearen ondoren. Lehenengo laginketa eguna ekainaren 30a izan zen eta, bigarrena, uztailaren 6a, hondakin-uren isurketak behin-betiko gelditu ziren eguna. Ekainaren 30etik uztailaren 6ra bitartean isurketen tartekako mozketak egon ziren, hobekuntza lanen azken frogen ondorioz. Horren ostean, hilabete baten buruan, beste 4 laginketa egin ziren: desbideratzetik 48 h-ra, aste batera, bi astera eta hilabete batera. Laginketak itsasgoran burutu ziren beti.

Laginak estuarioan zeharreko lau laginketa-puntu egonkorretan bildu ziren (*2.1. irudia*), estuarioko gazitasun-gradiente osoa hartzen zutenak: bat kanpoaldeko eremuan (URD1), bat erdiko eremuan (URD2) eta bi barneko eremuan (URD4 eta URD6). Azken laginketa-puntua (URD6) Gernikako HUAren aurrean kokatua zegoen, efluentea estuariora isurtzen zen lekuan. Horrez gain, Oka ibaian beste laginketa puntu bat ezarri zen, HUAren isurketak jaso baino lehen ibaiaren propietate fisiko-kimikoak kontrolatzeko helburuarekin. Uraren gazitasunaren arabera, URD1 eremu euhalinoa da, URD2 polihalinoa eta URD4 eta URD6 mesohalinotzat hartzen dira (*1. Kapituluko* datuen arabera).

Laginketa puntu bakoitzean, hainbat parametro fisiko-kimiko (gazitasuna, tenperatura, konduktibitatea, pH-a, disolbaturiko oxigenoa, oxigeno saturazioa eta uhertasuna) neurtu ziren *in situ* EXO2 (YSI) zunda multiparametrikoa erabiliz. Horrez gain, Plastimo Echotech II zundarekin, ur-zutabearen sakonera erregistratu zen eta Secchi diskoaren sakonera neurtu zen uraren gardentasunaren estimazio gisa. Ur laginen bilketari dagokionez, 2,5 L-ko plastikozko Niskin botila bat erabili zen 0,75 m-ko sakonerara ura biltzeko. Bildutako ura mantenugai ez-organikoen analisirako eta fitoplanktonaren biomasa eta komunitatearen osaera aztertzeko erabili zen.

Aztertutako mantenugai ez-organiko disolbatuak amonioa, nitritoa, nitratoa (nitrogeno oxidatu osotik kalkulatua), ortofosfatoa eta silikatoa izan ziren. Analisia, Pasaiako (Gipuzkoa) AZTIn, Itsas Ikerketa Unitateko Kimika Laborategian, egin zen. Erabilitako teknika UV/VIS kolorimetria izan zen, fluxu segmentatua duen 5 kanaleko analizatzaile automatiko batekin (BRAN-LUEBBE, AXFLOW). Mantenugai bakoitzaren analisi indibidualak klasikoak eta oso erabiliak diren erreakzio kolorimetrikoak aplikatzen dituzten metodoen bidez burutu ziren (GO-SHIP eskuliburua, Hydes et al., 2010). Amonio, nitrato eta silikatoaren kuantifikazio-muga 1,6 μmol L^{-1} zen, nitritoarena 0,4 μmol L^{-1} eta fosfatoarena

0,16 µmol L^{-1}. Kontzentrazio balioak kuantifikazio-mugaren azpitik zeudenean, analisi estatistikoak eta irudikapen grafikoak egiteko, mugaren % 50eko kontzentrazio balioa ezarri zitzaien.

Fitoplankton-komunitatearen azterketa HPLC bidezko pigmentuen analisian oinarritu zen nagusiki. Hala ere, emaitza osoagoak lortzeko asmoz, mikroskopia optiko bidez laginak behatuz taxon nagusien identifikazioa ere burutu zen. Pigmentuak aztertzeko ur laginak plastikozko botila opakuetan biltzen ziren eta laginketa egunean bertan iragazten (0,4-4 L) ziren. Iragazketa ilunpetan egin zen, presio leunarekin (<150 mm Hg), Whatman GF/F beira-zuntzezko iragazkiak (47 mm-ko diametroa, Whatman International Ltd.) erabiliz. Iragazi eta berehala, iragazkiak nitrogeno likidoan izoztu eta, erauzketa egin arte, -80 ºC-tan gordetzen ziren. Pigmentuen erauzketa ere ilunpetan burutu zen, saiodi laburretan, % 90 azetona 5 ml-rekin, beirazko barra batez filtroa puskatuz. Jarraian, erauzketan lortutakoa xiringa-iragazkien (Millex, 0,22 µm-ko poro-tamaina) bidez iragazi zen, iragazki-hondakinak kentzeko. Pigmentuen analisia HPLC bidez egin zen, Zapata et al.-ek (2000) deskribatutako metodoa jarraituz eta Seoane et al.-en (2009b) ekipoekin, pigmentuak xurgapen espektroen eta atxikipen denboren arabera identifikatuz (Jeffrey, 1997; Zapata et al., 2000). HPLC-aren kalibrazioa DHI-ren klorofila (*a*, *b* eta c_2) eta karotenoide (fukoxantina, 19'-hexanoiloxifucoxantina, luteina, zeaxantina, peridinina, biolaxantina, aloxantina eta β-karoteno) estandarrak erabiliz egin zen. Estandar komertzialekin kalibratu ez ziren pigmentuen kuantifikaziorako, Jeffrey-k (1997) lortutako desagertze-koefiziente molarrak aplikatu ziren.

Mikroskopia bidezko identifikaziorako erabilitako ur-laginak 125 ml-ko topazio borosilikatoko botiletan gorde ziren. Laginak bildu eta berehala, Lugol disoluzio azidoarekin (% 0,4 v/v) fixatu eta hotzean (4 °C) eta ilunpetan gorde ziren, aztertuak izan arte. Fitoplankton taxon nagusien identifikazio taxonomikoa Utermöhl sedimentazio metodoan oinarritu zen (Edler eta Elbrächter, 2010) eta Nikon diaphot TMD alderantzizko mikroskopioarekin egin zen, laginen dentsitatearen arabera 10 edo 50 ml-ko azpi-laginak aztertuz. Zelula handiak identifikatzeko plaka osoa handipen baxuetan (100× eta 200×) aztertu zen eta, zelula txikiagoentzako, 400× handipenarekin transektuak egin ziren. Orokorrean, taxon nagusiak genero edo espezie mailan identifikatu ziren, hauen nomenklaturaren estandarizaziorako AlgaeBase erabiliz (Guiry eta Guiry, 2018).

2.3. Datuen analisia

Laginketa-puntu bakoitzerako parametro estatistiko nagusiak (tartea, mediana eta/edo batezbesteko aritmetikoa) kalkulatu eta aldagai fisiko-kimikoen, fitoplankton biomasa totalaren eta komunitatearen osaeraren irudikapen grafikoak egin ziren, Windows Excel 2016 bidez.

Mann-Whitney U proba erabili zen, lagin independenteentzako proba ez-parametrikoa, desbideratze lanen aurretik eta lanen ostean aztertutako parametroen mediana-balioen artean desberdintasun adierazgarriak (alfa: 0,05) zeuden zehazteko. Emaitzak fidagarriagoak izan zitezen, datu osagarriak erabili ziren proba honetan, desbideratzearen "aurreko" egoerari 2020ko uztailaren 17ko eta 29ko bi laginketa gehituz (*1. Kapituluko* datuak). Gehitutako laginketa hauen aukeraketa datetan oinarritu zen. 2020ko uztaileko datuak gehitzea erabaki zen desbideratzearen "ondoren" egindako laginketak 2021ko uztailean burutu zirelako (urteko sasoi berdinean) eta, bi urte hauen artean, estuarioan egondako aldaketa nabarmen bakarra hondakin-uren desbideratzea izan zelako. Proba estatistikoa laginketa puntu bakoitzean burutu zen, independenteki, PAST 4.05 (Paleontological STatistics) erabiliz (Hammer et al., 2001).

3. Emaitzak

Gazitasunaz eta tenperaturaz gain, estuarioetako ur-masak deskribatzeko ezinbesteko aldagai ozeanografikoak direnak, atal honetan HUAren isurketen gelditzearen ondorioz aldaketak pairatu ditzaketen aldagaiak deskribatu eta irudikatuko dira. Aztertutako gainerako parametroak material osagarrian aurki daitezke (*Annexes, A2.1. taula* eta *A2.2. taula*).

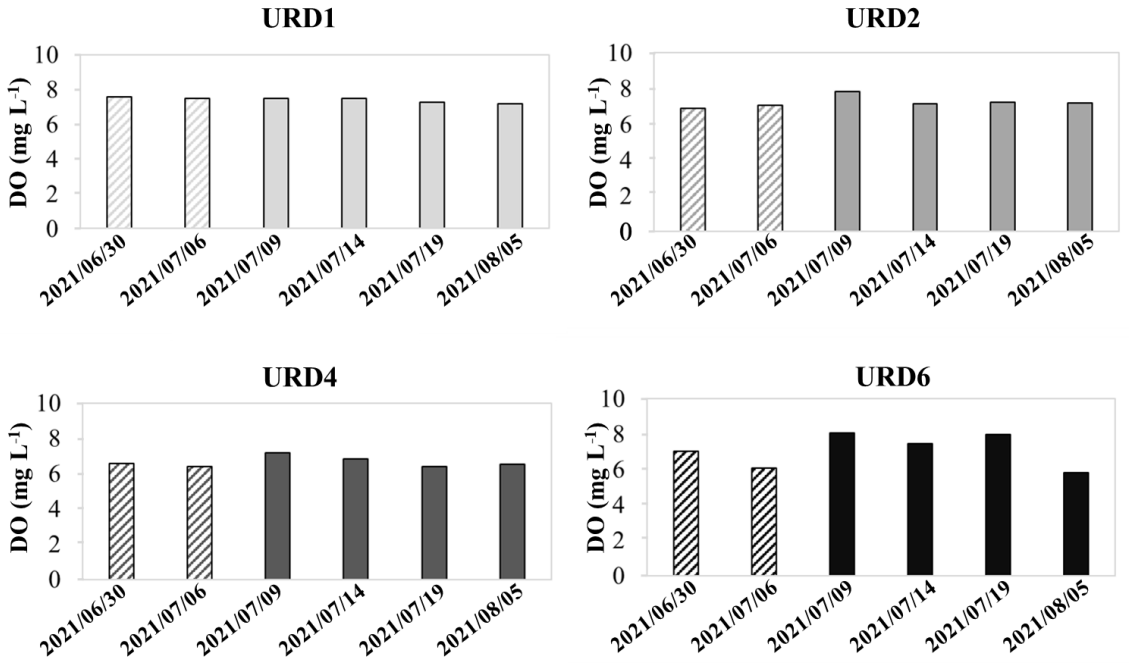
Egindako sei laginketen emaitzak bi taldetan banatzen dira: hondakin-uren desbideratzearen aurreko laginketak (2021/06/30 eta 2021/06/07) eta desbideratze osteko laginketak (2021/07/09, 2021/07/14, 2021/07/19 eta 2021/08/05).

3.1. Ingurumen baldintzak

Gazitasunak zonifikazio argia erakutsi zuen Urdaibaiko estuarioan zehar, estuarioaren kanpoko eremutik barrurantz joan ahala gutxituz. Baliorik altuenak (34,1eko batez bestekoa) eta egonkorrenak (33,8–34,3 bitartekoak) kanpoko eremuan (URD1) ikusi ziren. Erdiko eremuan (URD2) batez besteko gazitasuna 28,3koa izan zen azterketa-aldian, 26,5–30

bitartekoa. Estuarioaren eremu kanalizatuan batez besteko gazitasun-balioak 18,5 eta 11,5 izan ziren URD4n eta URD6an, hurrenez hurren. Gainera, gazitasunari dagokionez, hau izan zen eremurik aldakorrena, 16,6–20,9 bitarteko balioekin URD4n eta 9,3–14,1 bitarteko balioekin URD6n.

Estuarioan, ikerketan zehar, batez besteko tenperatura 21,6 ºC-koa izan zen, 18,3–25,3 ºC bitarteko balioekin. Gainera, batez besteko tenperatura handituz joan zen ikerketaren hasieratik (18,5 ºC ekainaren 30ean) amaierara arte (24 ºC abuztuaren 5ean). Oka ibaiaren tenperaturari dagokionez, estuarioan aurkitutakoa baino baxuagoa izan zen (16,8–19,3 ºC artean), 18,1 ºC-ko batez besteko balioarekin.

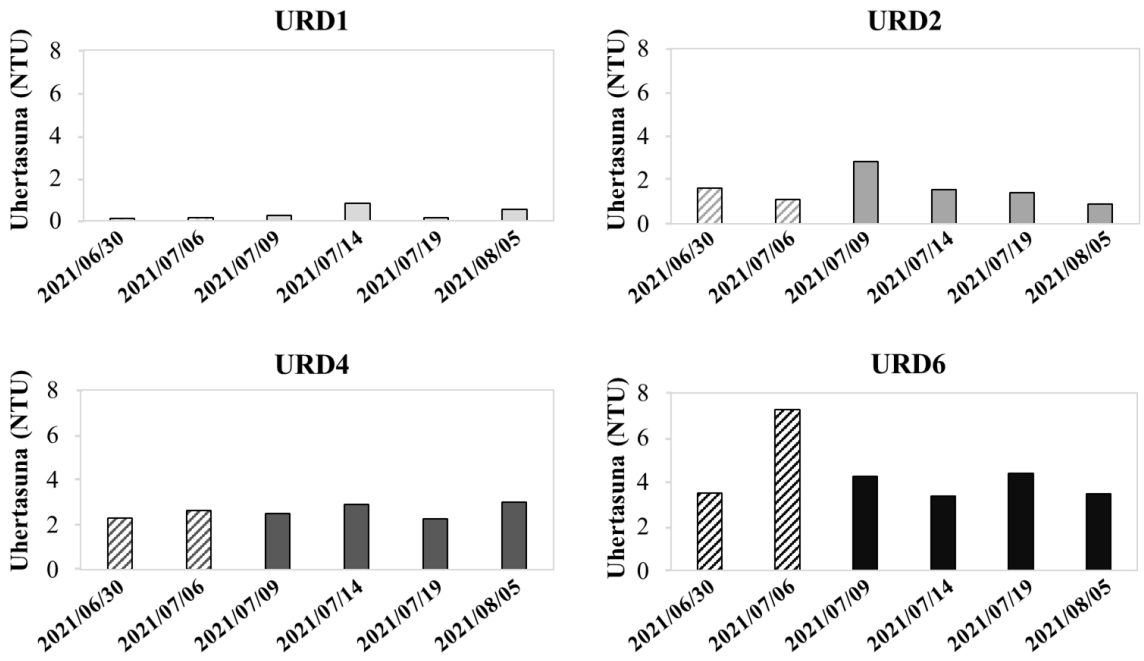


2.2. irudia. *Disolbaturiko oxigeno kontzentrazioa (DO) Urdaibai estuarioko laginketa puntu desberdinetan. Marradun zutabeek saneamendu lanen aurreko laginketak irudikatzen dituzte eta betegarri homogeneodun zutabeek desbideratzearen osteko laginketak.*

Hondakin-uren desbideratzearen ondorioz berehalako aldaketak pairatu zitzakeen aldagaietako bat disolbaturiko oxigeno (DO) kontzentrazioa da (*2.2. irudia*). Kanpoko estuarioan (URD1), DO kontzentrazioa egonkorra izan zen ikerketaren denbora-tartean zehar, 7,2–7,6 mg L^{-1} arteko balioekin. Hala ere, balioak aldakorrago bihurtuz joan ziren barne-estuariorantz hurbildu ahala: URD2n batez besteko balioa 7,2 mg L^{-1} (6,9-7,9 mg L^{-1}) izan zen, URD4n 6,7 mg L^{-1} (6,4-7,2 mg L^{-1}) eta URD6n 7,1 mg L^{-1} (5,8-8,1 mg L^{-1}). Saneamendu-lanen ondoriozko aldaketei dagokienez, estuarioko erdiko eta barneko eremuek DO balio arinki altuagoak erregistratu zituzten hondakin-uren isurketak gelditu ondoren. Hau bereziki nabarmena izan zen estuarioaren erdiko eremuan, Mann-Whitney-ren probak hondakin-uren

isurketak eten osteko DO kontzentrazio-igoera adierazgarria zela zehaztu baitzuen (p balioa 0,03).

Uhertasunak, gazitasunak bezala, zonazio argia erakutsi zuen Urdaibaiko estuarioan, uhertasun-gradientea estuarioaren barrualderantz handitzen zelarik (*2.3. irudia*). URD1en batez besteko uhertasuna 0,3 NTU-koa izan zen ikerketan zehar, eta batez bestekoa 4,4 NTU-ra igo zen HUAren inguruan (URD6). Saneamendu-lanen ondoriozko uhertasunaren berehalako aldaketari dagokionez, HUAren inguruan bakarrik nabaritu zen. URD6n uhertasunaren beherakada azkarra egon zen hondakin-uren isurketak eten ondoren, batez besteko uhertasuna 5,4 NTU izatetik (aurretik) 3,9 NTU izatera (ondoren) pasatuz. Gainera, desbideratzearen ondoren, uhertasun balioak 4,5 NTU baino txikiagoak izan ziren. Hala ere, Mann-Whitney probaren arabera, aldaketa horiek ez ziren adierazgarriak izan. Oka ibaian uhertasun mediana 1,6 NTU izan zen ikerketan zehar.

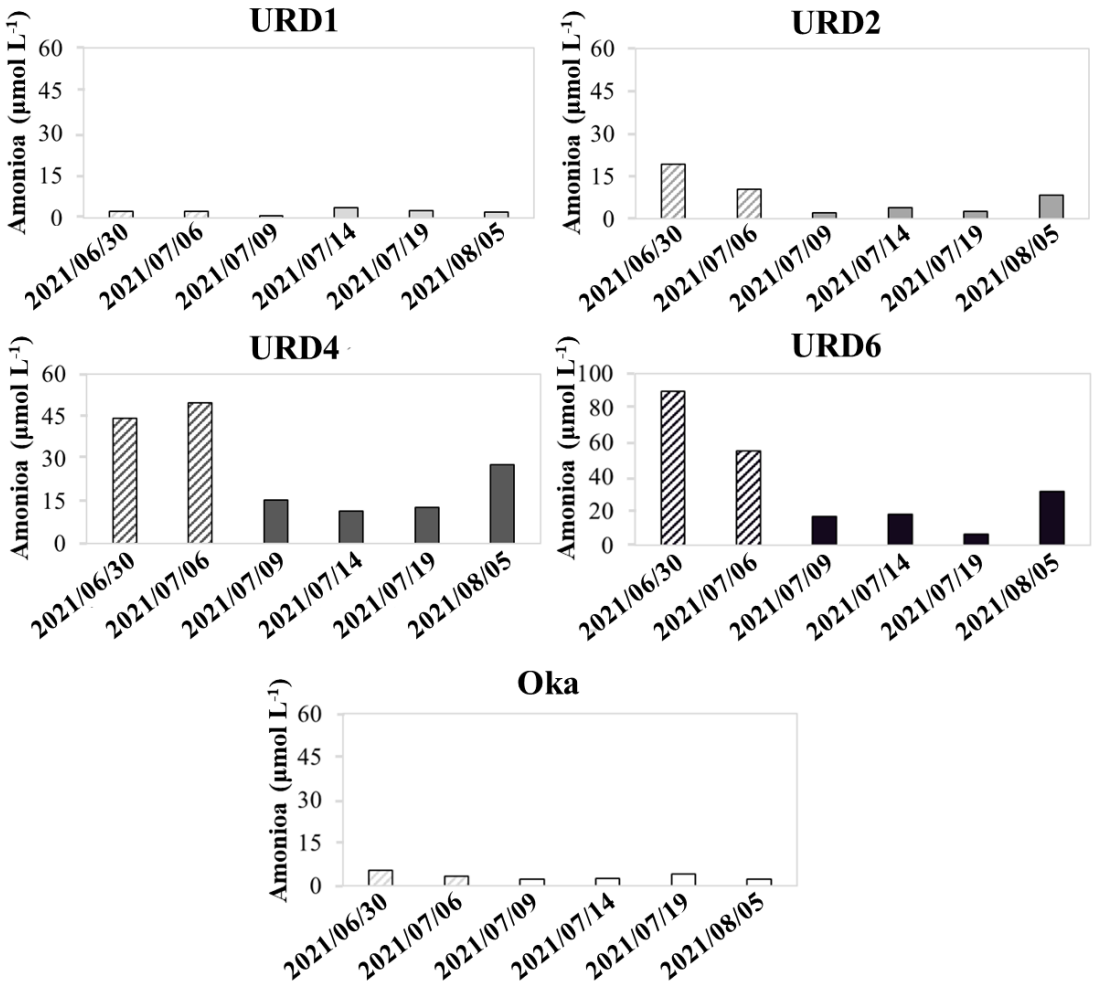


2.3. irudia. *Uhertasun balioak Urdaibai estuarioko laginketa puntu desberdinetan. Marradun zutabeek saneamendu lanen aurreko laginketak irudikatzen dituzte eta betegarri homogeneodun zutabeek desbideratzearen osteko laginketak.*

3.2. Mantenugai ez-organikoen kontzentrazioa

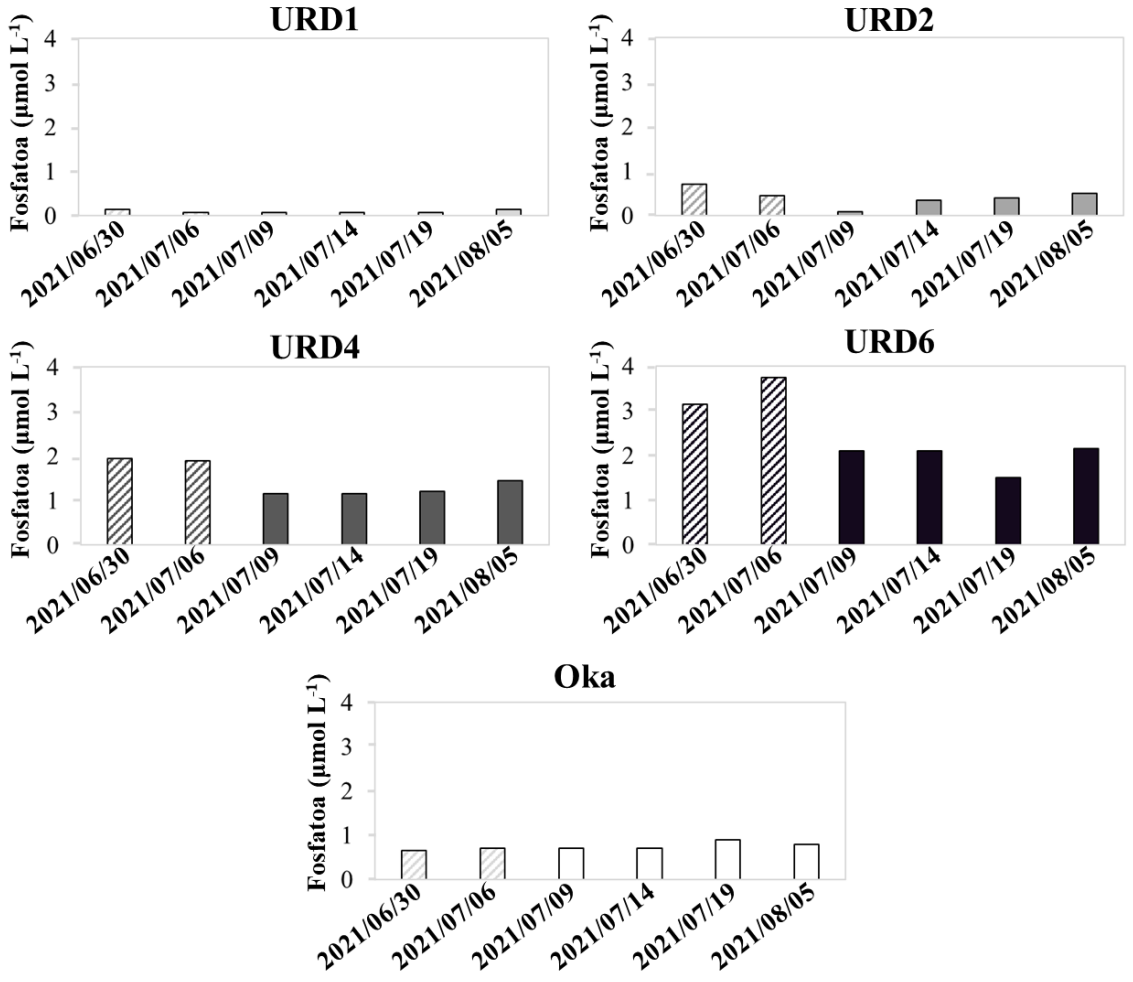
Estuarioko mantenugai ez-organikoen kontzentrazioak esperotako gradiente espaziala erakutsi zuen, estuarioaren barneko eremuan kontzentrazio altuagoak aurkituz. Gainera, aztertutako mantenugai horietako batzuek, berehalako beherakada jasan zuten hondakin-urak estuariotik kanpo desbideratu ostean, eragina HUAren inguruan bereziki nabarmena izanik.

Amonioa izan zen hondakin-urak desbideratzearen eragina gehien pairatu zuen aldagaia (*2.4. irudia*). Orokorrean, ikerketan zehar, estuarioaren kanpoko eremuan (URD1) batez besteko amonio kontzentrazioa 2,3 µmol L^{-1}-koa izan zen, hau estuarioren barrualdera hurbildu ahala handituz joan zelarik, HUAren inguruan (URD6) 36,2 µmol L^{-1}-ko batez besteko kontzentraziora helduz (maximoa 89,6 µmol L^{-1}). Oka ibaian, ordea, batez besteko amonio kontzentrazioa 3,3 µmol L^{-1} zen. Honek argi utzi zuen estuarioko amonioak jatorri antropikoa zuela, iturri nagusia HUA zelarik. Ondorioz, HUAren hondakin-urak estuariotik kanpo desbideratzerakoan, amonio kontzentrazioak beherakada azkarra eta adierazgarria (p balioa 0,03) jasan zuen estuarioaren erdiko eta barruko eremuetan. URD2n, batez besteko amonio kontzentrazioa 14,8 µmol L^{-1} (aurretik) izatetik 4,3 µmol L^{-1} (ondoren) izatera pasa zen. Kanalean (URD4), batez besteko kontzentrazioaren jaitsiera 47 µmol L^{-1}-tik 16,8 µmol L^{-1}-ra izan zen eta, araztegiaren inguruan (URD6), 72,3 µmol L^{-1}-tik 18,2 µmol L^{-1}-ra.



2.4. irudia. *Amonio kontzentrazioak Urdaibai estuarioko laginketa puntu desberdinetan eta Oka ibaian. Marradun zutabeek saneamendu lanen aurreko laginketak irudikatzen dituzte eta betegarri homogeneodun zutabeek desbideratzearen osteko laginketak.*

Fosfato kontzentrazioak amonioaren antzeko jokaera erakutsi zuen, baina berehalako aldaketak ez ziren hain esanguratsuak izan (*2.5. irudia*). Estuarioko fosfato kontzentrazioa amonioarena baino askoz baxuagoa zen, URD1n 0,1 µmol L^{-1}-ko batez besteko balioak erregistratuz eta URD6n 2,5 µmol L^{-1} (gehienez 3,8 µmol L^{-1}-ra iritsiz). Gainera, amonioarekin gertatzen zen bezala, Oka ibaiaren batez besteko fosfato kontzentrazioa (0,7 µmol L^{-1}) araztegiaren ingurukoa baino askoz txikiagoa zen, bere jatorri antorpikoa agerian utziz. Hondakin-uren isurketak estuariotik kanpo desbideratzearen berehalako eragina barneko eremuan nabaritu zen nagusiki, kontzentrazioak URD4n 1,9 µmol L^{-1} (aurretik) izatetik 1,2 µmol L^{-1} (ondoren) izatera jaitsiz eta URD6n 3,5 µmol L^{-1}-tik 2 µmol L^{-1}-ra jaitsiz. Mann-Whitney-ren probaren arabera, URD4n eta URD6n ikusitako fosfato kontzentrazio jaitsiera hau esanguratsua (p balioa 0,03) izan zen.

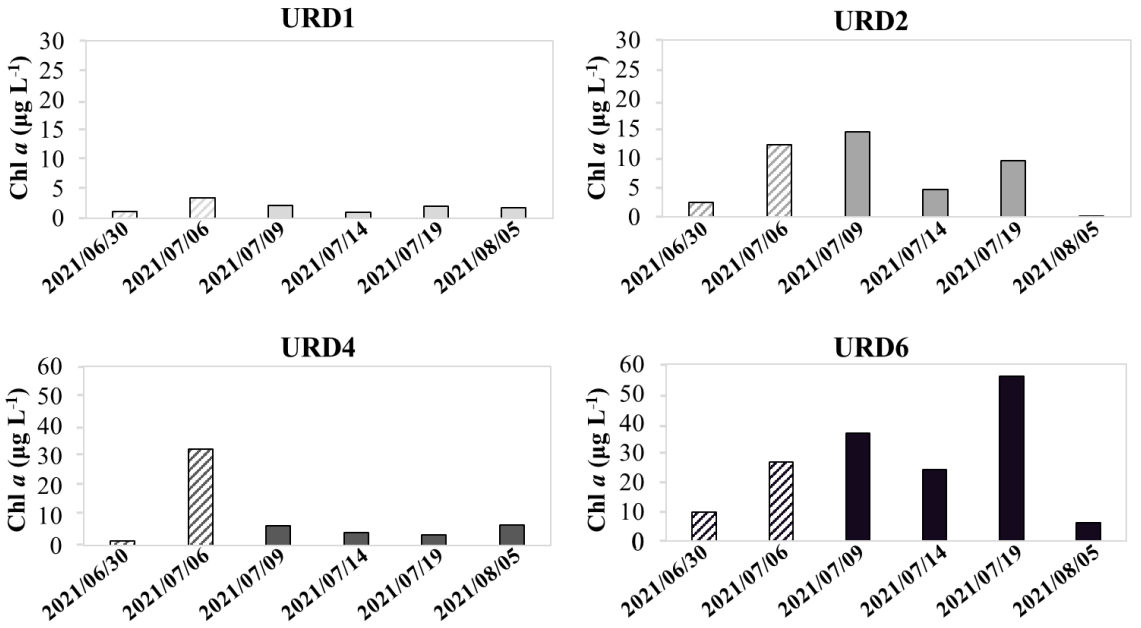


2.5. irudia. *Fosfato kontzentrazioak Urdaibai estuarioko laginketa puntu desberdinetan eta Oka ibaian. Marradun zutabeek saneamendu lanen aurreko laginketak irudikatzen dituzte eta betegarri homogeneodun zutabeek desbideratzearen osteko laginketak.*

Nitratoak eta silikatoak ordea, portaera desberdina erakutsi zuten estuarioan zehar (material osagarriak; *Annexes, A2.2. taula*). Hauen kontzentrazioak handituz joan ziren estuarioaren barrualderantz hurbildu ahala, amonio eta fosfatoarekin gertatzen zen bezala, URD1n kuantifikazio-muga azpiko nitrato kontzentrazioak eta batez besteko 1,3 µmol L^{-1} silikato kontzentrazioak aurkituz eta URD6n, ordea, batez besteko 20 µmol L^{-1} nitrato eta 76,6 µmol L^{-1} silikato kontzentrazioekin. Hala ere, bai nitrato baita silikatoaren kasuan ere, Oka ibaian kontzentrazio altuagoak aurkitu ziren, batez besteko 45 µmol L^{-1} nitrato eta 94,2 µmol L^{-1} silikato kontzentrazioak hain zuzen ere, hauen jatorri naturala islatuz. Ondorioz, hondakin-uren isurketak estuariotik kanpo desbideratzeak ez zuen ondorio argirik izan mantenugai hauen kontzentrazioetan. Nitratoaren kasuan, kontzentrazioek behera egin zuten orokorrean, URD2n 8,1 µmol L^{-1} (aurretik) izatetik 2,7 µmol L^{-1} (ondoren) izatera pasa zen eta URD6n 23,4 µmol L^{-1}-tik 18,5 µmol L^{-1}-ra jaitsi zen. Silikatoari dagokionez, ez zen berehalako aldaketarik hauteman. Are gehiago, Mann-Whitney probaren arabera, desbideratzearen ondoren ez zen aldaketa esanguratsurik egon estuarioko ez nitrato ezta silikato kontzentrazioetan ere.

3.3. Fitoplankton biomasa eta komunitatearen osaera

Biomasa fitoplanktoniko totala *a* klorofila (Chl *a*) kontzentrazioarekin zenbatetsi da (*2.6. irudia*). Ikerketan zehar, biomasak zonazio argia erakutsi zuen Urdaibaiko estuarioan, balioak barruko eremura hurbildu ahala handituz, honako batez besteko Chl *a* balioekin: URD1 1,9 µg L^{-1}, URD2 7,2 µg L^{-1}, URD4 9,3 µg L^{-1} eta URD6 26,6 µg L^{-1}. Gainera, ikerketan zehar estuarioan erregistratutako Chl *a* maximoa (55,9 µg L^{-1}) ere URD6n aurkitu zen. Saneamendu lanen ondoren ikusitako biomasa fitoplanktonikoaren bilakaerari dagokionez, aldaketa nabarmenak antzeman ziren, aldakortasun espaziala (laginketa puntuak) eta denborazkoa (laginketa egunak) erakutsiz, baina ez zuten eredu zehatzik jarraitu hondakin-uren desbideratzearekin erlazionatu ahal izateko.

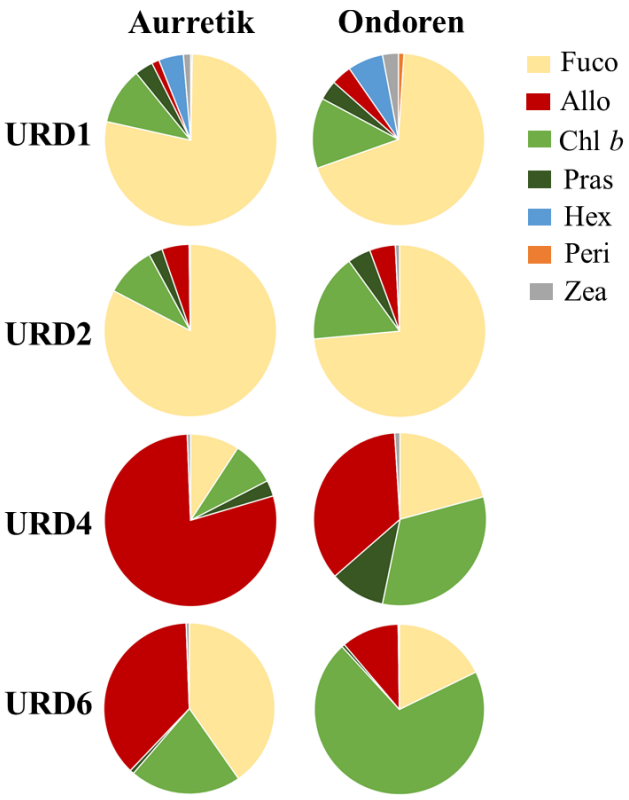


2.6. irudia. *Fitoplankton biomasa totala (Chl a kontzentrazioa) Urdaibai estuarioko laginketa puntu desberdinetan. Marradun zutabeek saneamendu lanen aurreko laginketak irudikatzen dituzte eta betegarri homogeneodun zutabeek desbideratzearen osteko laginketak.*

Komunitatearen osaerari dagokionez, aitzitik, estuarioaren luzetarako ardatzean fitoplankton-komunitate desberdinak aurkitu ziren eta, zenbait eremutan, berehalako aldaketak hauteman ziren hondakin-urak estuariotik kanpo desbideratu ostean (*2.7. irudia*). Kanpoko eremuan (URD1), komunitatearen osaerak ez zuen berehalako aldaketa nabarmenik jasan hondakin-urak estuariotik kanpo bideratu ostean. Eremu honetan, pigmentu diagnostiko nagusia fukoxantina (Fuco) zen, *b* klorofila (Chl *b*) eta 19'-hexanoiloxifukoxantina (Hex) atzetik zituelarik. *Minutocellus polymorphus* eta *Chaetoceros tenuissimus* diatomeoak (fukoxantina-dunak) izan ziren eremu horretako taxon nagusiak, *Tetraselmis* spp. klorofitaren (Chl *b*-duna) eta *Gephyrocapsa huxleyi* eta Prymnesiales bezalako haptofitoen agerpen garrantzitsuarekin batera. Kanpoko estuarioko komunitatean antzemandako desberdintasun bakarrak Chl *b*-aren ugaritasunaren handipen arina izan zen, *Eutreptiella* sp. -en presentzia handiagoagatik eta *Chamydomonas* sp. eta *Pyramimonas* sp.-ren agerpenaren ondorioz.

Erdialdeko eremuko fitoplankton-komunitatearen osaera kanpoko eremuaren antzekoa izan zen ikerketan zehar, eta honek ere ez zuen aldaketa adierazgarririk pairatu hondakin-uren desbideratzearen ondorioz. Fukoxantina izan zen eremu honetako pigmentu diagnostiko ugariena eta Chl *b* bigarrena, hondakin-uren isurketak gelditu ondoren bere garrantzia areagotuz. Gainera, kanpoaldeko eremuan ez bezala, aloxantina ugaritzen hasi zen, 19'-hexanoiloxifucoxantina (Hex) ordezkatuz. Komunitatearen taxon gainartzailea *C.*

tenuissimus diatomeoa izan zen, nahiz eta saneamendu lanen ostean *Tetraselmis* spp.-ren garrantzia handitu. Gainera, *Plagioselmis* spp. eta *Teleaulax* spp. bezalako kriptofitoen (aloxantina-dunak) agerpena URD1ean baino handiagoa zen, eta haptofitoena txikiagoa.



2.7. irudia. *Pigmentu diagnostikoen proportzioak (batez besteko balioak) Urdaibai estuarioko laginketa puntu ezberdinetan HUAren isurketak desbideratu aurretik (ezkerreko zutabea) eta desbideratu ondoren (eskuineko zutabea).Fuko: fukoxantina; Allo:aloxantina, Chl b: b klorofila; Pras: prasinoxantina; Hex: 19'-hexanoiloxifucoxantina; Peri: peridinina; Zea: zeaxantina.*

Barrualdeko eremuan (URD4 eta URD6), kanpoaldeko eta erdiko eremuetan ez bezala, fitoplankton-komunitatearen osaeraren aldaketa azkar eta adierazgarria ikusi zen hondakin-urak estuariotik kanpo bideratu ostean. Alde batetik, URD4n, saneamendu lanak burutu baino lehen, aloxantina zen pigmentu diagnostiko ugariena, baina, lanak burutu eta berehala, aloxantinaren garrantzia gutxitu eta Chl *b* kontzentrazioa handitu egin zen. Aldaketa hori eremuko taxon nagusietan ere islatu zen. Desbideratzea baino lehen, *Plagioselmis* spp., *Teleaulax* spp. eta *Urgorri complanatus* kriptofitoak ziren nagusi URD4n. Saneamendu lanen ostean, ordea, *Tetraselmis* spp. eta *Eutreptiella* sp. bezalako alga berdeen presentzia handitu eta *Urgorri complanatus* desagertu egin zen.

Araztegiaren inguruan, URD6n, araztegiko urak estuariora isuri bitartean, fukoxantina eta aloxantinaren arteko nagusitasun partekatua zegoen. Isurketak eten zirenean, aldiz, Chl *b*

kontzentrazioak igoera bortitza pairatu zuen, eremuko pigmentu diagnostiko nagusia bihurtuz. Nagusitasun-aldaketa hori komunitatearen osaera taxonomikoan ere ikusi zen. Saneamendu lanak burutu baino lehen, *Kryptoperidinium foliaceum* dinoflagelatuak (fukoxantina-duna) eta kriptofitoak (batez ere *Teleaulax* spp. eta *Urgorri complanatus*) ziren URD6ko fitoplankton-komunitatean garrantzi handiena zuten organismoak. Hala ere, hondakin-uren isurketak eten eta berehala, *Eutreptiella* sp.-ren hazkuntza azkarra ikusi zen, URD6ko taxon gainartzailea bihurtuz.

4. Eztabaida

Kostaldeko ekosistemetan, aldakortasun naturala eta presio antropogenikoen edo/eta kudeaketa-ekintzen ondoriozko aldakortasuna bereiztea lan zaila da (adibidez, Elliott eta Quintino, 2007). Ikerketa honetan, estuarioan eragin zuzena daukan kudeaketa-ekintza batek (Gernikako HUAko hondakin-urak estuariotik kanpo desbideratzea) sistemaren aldagai fisiko-kimikoetan eta fitoplankton-komunitatean dituen berehalako ondorioak aztertu dira, hainbat aldaketa antzemanez.

Orokorrean, estuarioetako uhertasuna hainbat faktoreren menpe dago, hala nola, ibaiaren ekarpena/emaria, marea-efektua eta, sistema askotan, Urdaibai estuarioan esaterako, hondakin-uren isurketak (Toublanc et al., 2016; Eccles et al., 2020). Uhertasunaren aldakortasuna faktore anitzen menpe egoteak, zaildu egiten du Gernikako araztegiak estuarioko uraren uhertasunaren jaitsieran izan zuen eragina zehaztea. Ikerketa honetan, Oka ibaiaren uhertasuna ere aztertu zenez, jakina da ibaiaren ekarpenak ez zuela eraginik izan saneamendu lanen ondoren estuarioan ikusitako uhertasun aldaketekin. Gainera, marea-efektua ere ezin da kontuan hartu, laginketa guztiak marea-momentu berdinean (itsasgora baino 1h lehenago) egin baitziren, eta antzeko marea-maila zegoen guztietan. Ondorioz, pentsa daiteke saneamendu lanak izan zirela URD6an ikusitako uhertasunaren arintzearen arrazoi nagusia, izan ere, araztegietako efluenteek partikula-kontzentrazio altuak izaten dituzte sarri, eta horrek uraren uhertasuna areagotzen du ur-hartzaileetan (Carey eta Miggliaccio, 2009). Oxigenoari dagokionez, oxigeno-eskaera biologikoaren murrizketak (materia organikoaren sarreraden murrizketaren ondorioz) uretako oxigeno kontzentrazioa areagotu beharko luke (García-Barcina et al., 2016), eta hau Urdaibai estuarioaren erdiko eremuan desbideratzearen ondoren gertatutako DO-aren gorakada adierazgarriaren bidez baieztatu da.

Mantenugai ez-organikoen kontzentrazioari dagokionez, jakina da araztegien isurketek ur hartzaileak degradatzen dituztela N eta P kontzentrazioak handituz, isurketa puntutik haratago dauden ur-masetan ere eraginez (Carey eta Miggliaccio, 2009). Ikerketa honek, aurretik egindako beste ikerketa batzuek bezala (adibidez, Revilla et al., 2000), Urdaibai estuarioko amonio eta fosfato iturri nagusia Gernikako HUAren isurketak zirela baieztatzeko aukera eman du. Nitratoa eta silikatoa balio maximoak, aitzitik, ibaian lortu ziren, hauen jatorri naturala frogatuz. Estuarioko nitratoak inguruko lurrazaleko uren drainatzean du bere jatorria eta silikatoak, ordea, arroken meteorizazioan (Turner et al., 2003; Valencia eta Franco, 2004). Honenbestez, saneamendu lanek, jatorri antropikoko mantenugai ez-organikoetan, amonio eta fosfatoan hain zuzen ere, izan zuten eragina, estuarioaren erdialde eta barrualdean hauen kontzentrazioen murrizketa % 50 baino gehiagokoa izan zelarik. Horrez gain, estuarioaren erdiko eta barrualdeko eremuetan erregistratutako nitrato kontzentrazio jaitsieraren zati bat amonioaren jaitsierarekin erlazionatuta egon liteke, izan ere, sarritan estuarioetan nitratoa amonioaren oxidazioaren ondorioz (nitrifikazioa) sortzen da (Dai et al., 2008; Damashek et al., 2016).

Euskal Autonomia Erkidegoko (EAEko) kostaldeko eta trantsiziozko eremuen uraren kalitatearen eta egoera ekologikoaren ebaluazioa 817/2015 Errege Dekretuan ezarritako irizpide eta muga balioen araberakoa da, Kantauri Ekialdeko Demarkazio Hidrografikorako balioak kontutan hartuz. EAEko trantsizio- eta kostaldeko uren jarraipen-sarean lortutako emaitzek erakutsi zuten Urdaibai estuarioko barrualdeko eremuak ez dituela ezarritako ingurumen-helburuak bete, gutxienez 2008az geroztik eta 2020ra arte (adibidez, Borja et al., 2021), egoera ekologiko “eskasa” edo “txarra” erregistratuz. Hau, amonioa eta fosfatoa bezalako adierazle fisiko-kimikoek kontzentrazio muga-balioak sistematikoki gainditzeagatik gertatu da, eta honek, aldi berean fitoplankton biomasaren (adierazle biologikoa) gehiegizko balioak sustatu eta honek ere helburuak ez betetzeagatik (Borja et al., 2021). Hala ere, baliteke burututako saneamendu-lanek Urdaibai estuarioko adierazle fisiko-kimikoen egoera hobetu eta, ondorioz, uraren kalitatea hobetzea.

Ikerketa honetan ikusi da, 817/2015 Errege Dekretuan EAErako ezarritako muga balioak (material osagarrietan eskuragarri; *Annexes, A4.16. taula*) kontutan hartuz, saneamendu lanak baino lehen Urdaibai estuarioko erdialdeko eta barneko eremuan amonio- eta fosfato-kontzentrazioek egoera “moderatu”-tik (hau da, uraren kalitatearen helburu minimoa) gorako balioak zituztela. Araztegiko isurketak desbideratu ondoren, ordea, mantenugai hauen kontzentrazio jaitsierari esker, estuario osoan zehar, gutxienez, egoera “ona” izatea lortu zen,

zenbait kasutan “oso ona” ere lortuz. Gainera, saneamendu lanen ostean, oxigeno saturazioaren (egoera ekologikoa ebaluatzeko beste parametro bat, datuak material osagarrietan eskuragarri; *Annexes, A2.1.taula*) gorakada ere eman zen, estuario osoan egoera "oso ona"- ren muga-balioetatik gorako emaitzak lortuz, nahiz eta saneamendu lanen aurretik zonalde batzuetan egoera “ona”-ra baino ez zen heltzen. Hortaz, hondakin-uren isurketak amaitu ondoren, adierazle fisiko-kimikoek Urdaibaiko estuarioko laginketa puntu guztien uraren egoera, gutxienez, “ona” zela adierazi zuten.

Fitoplanktonari dagokionez, ikerketa honen helburuetako bat biomasa fitoplanktonikoak hondakin-uren isurketen eteteari berehalako erantzunik emango ote zion zehaztea zen. Orokorrean, estuarioetako fitoplankton biomasak denboran eta espazioan zeharreko aldakortasuna erakusten du, prozesu fisikoen (gazitasuna eta tenperatura baldintzatuz) eta, batez ere, argi eta mantenugaien eskuragarritasunaren araberakoa dena (Masotti et al., 2018). Saneamendu lanen ondorioz estuarioko biomasak erantzun adierazgarririk erakutsi ez arren, badira aipatu beharreko zenbait aldaketa arin. Urdaibai estuarioko erdialdeko eremuan, Chl *a* balioek jaitsiera leuna erregistratu zuten saneamendu lanen ondoren. Hau, ziurrenik, mantenugaien kontzentrazio jaitsierari erantzunez gertatu zen, izan ere, hainbat ikerketen arabera, mantenugai-sarrerak murriztearen eta fitoplanktonaren biomasaren gutxitzearen artean erlazio positiboa dago (adibidez, Wetz et al., 2011). Araztegiaren inguruko eremuan (URD6), ordea, biomasak gora egin zuen. Hau, saneamendu lanen ondoren eremu honetan lortutako argi eskuragarritasun altuagoarekin (uhertasun baxuagoa) azaldu daiteke (Karlsson et al., 2009; Yamamichi et al., 2018).

Estuarioak bezalako ingurune aldakorretan, fitoplankton-komunitatearen osaera ere moldatuz joaten da taxon ezberdinen lehiakortasun-gaitasunen aldaketen ondorioz (Cardinale et al., 2011). Urdaibai estuarioko kanpoko eta erdiko eremuetako baldintza fisiko-kimikoek ez zuten aldaketa nabarmenik jaso saneamendu lanen ondorioz, ziurrenik HUAtik urrun egoteagatik, beraz, fitoplankton-komunitateak ere ez zuen aldaketa adierazgarririk pairatu. Hala ere, estuarioaren barruko eremu kanalizatuan fitoplankton-komunitatearen osaeran aldaketa azkarrak eta bortitzak eman ziren, ziurrenik eremu horretan mantenugaien kontzentrazioan eta argiaren eskuragarritasunean egondako aldaketek eraginda.

Urdaibai estuarioko barrualdeko eremuan, URD4n konkretuki, saneamendu lanak burutu baino lehen, pigmentu diagnostiko ugariena aloxantina zen, kriptofitoak baitziren eremu honetako fitoplankton talderik ugariena, bereziki *Teleaulax* spp. eta *U. complanatus*. Ohikoa

da presio antropogeniko handia jasaten duten sistemetan kriptofitoak aurkitzea, argi eskuragarritasun gutxiko eremuetan bizitzeko duten tolerantzia eta kutsadura iturburuetan ugaritasun handietan hazteko duten erraztasunagatik (adibidez, Paches et al., 2019). Kriptofitoek materia organikotan aberatsak diren ur uherretan hazteko duten gaitasunak (Bergmann, 2004; Adolf et al., 2008), Urdaibai estuarioaz gain beste hainbat estuario eutrofikoetan duten nagusitasuna azaltzen du, hala nola Chesapeake, Neuse edo Galvestonen (Paerl et al., 2003; Adolf, 2008; Valdes-Weaver et al., 2006).

Araztegiaren inguruko eremuari dagokionez (URD6), saneamendu lanen aurretik, fitoplankton-komunitatea kriptofitoek eta fukoxantina-dun *K. foliaceum* dinoflagelatuak osatzen zuten gehienbat. Urdaibai estuarioan aurretik egindako hainbat ikerketek azpimarratu dute *K. foliaceum*-ek estuarioaren barruko eremuan duen garrantzia (adibidez, Ansotegui et al., 2001; Trigueros eta Orive, 2001). Gainera, inguruko beste estuario batzuetan ere aurkitu da, hala nola, Nerbioi ibaiaren estuarioan (Seoane et al., 2005) edo Espainiako hegoaldeko Guadiana estuarioan (Domingues et al., 2011). Espezie honek hondakin-uren isurketa eremutik gertu duen garrantzia bere mantenugai-lehentasunekin azaldu daiteke, izan ere, jakina da N aberasteari positiboki erantzuten diola, batez ere Si-ren aberasterik ez dagoenean (Domingues et al., 2011). Honek, mantenugaien aberaste antropikoaren adierazle onak bihurtzen ditu, izan ere, N aberastearekin batera Si aberastea badator, gerta liteke mantenugai ekarpena ibaiek egitea, baina, aldiz, N aberastea bakarrik badator, edo P-rekin batera, posibleena da jatorria isurketa antropikoak izatea, Urdaibain HUArekin gertatzen den bezala.

Gernikako HUAren isurketak estuariotik kanpo desbideratzeak berehalako aldaketa eragin zuen estuarioaren barrualdeko fitoplankton-komunitatearen osaeran. Saneamendu lanen ostean, aloxantina eta fukoxantina kontzentrazioak jaitsi eta Chl *b* kontzentrazioa nabarmen hazi zen. Hau, maila taxonomikoan, kriptofitoen agerpenaren murrizketan eta *K. foliaceum*-en ia desagertzean ikusi zen, *Eutreptiella* generoaren hazkuntza bortitzarekin batera, azken honek barrualdeko eremuan gainjartzea lortu zuelarik. *Eutreptiella* euglenoide fotosintetiko flagelatuen genero bat da, kostaldeko eta ur gazikaretako zenbait sistemetan nahiko ugaria dena (Henriksen et al., 2002). Euglenoideek sakonera gutxiko ur-masa txikiak gustuko dituzte, mantenugaietan aberatsak direnak eta argi-baldintza eskasak dituztenak (Poniewozik eta Juráň, 2018). Hau kontutan hartuz, Urdaibai estuarioko barrualdeko eremua, mantenugaietan aberatsa dena eta sakonera gutxi duena, leku aproposa da *Eutreptiella* sp.-ren hazkuntzarako. Hainbat ikerketek deskribatu dituzte *Eutreptiella* loraketak (*bloom*-ak) mantenugaietan aberatsak ziren kostaldeko zein trantsizioko uretan, komunitate hauetan

taxon gainartzailea bihurtuz (adibidez, Olli et al., 1996; Stonik et al., 2007), Urdaibai estuarioko barrualdean gertatu bezala. Gainera, normalean, euglenofitoak kriptofito eta dinoflagelatuak bezalako bestelako flagelatuekin batera bizi ohi dira (adibidez, Jeong et al., 2021), baldintza zailetan bizirauteko ezaugarri berdinak partekatzen baitituzte, hala nola, ugalketa azkarra, mixotrofia eta argi urritasunarekiko tolerantzia (Plachno et al., 2015). Horrez gain, jakina da euglenoideak oso sentikorrak direla ingurumen-baldintzen aldaketekiko, eta populazioak oso azkar hazi edo desager daitezkeela (Heckman et al. 1996), beraz, ziurrenik, araztegiaren inguruan gertatutako aldaketek *Eutreptiella* spp.-ren hazkundea sustatu zuen.

Honenbestez, Urdaibai estuarioan burututako saneamendu lanen ondoriozko mantenugaien kontzentrazioen eta uhertasunaren gutxitzea ez zen nahikoa izan ur garbiko fitoplankton-komunitate tipikoak agertzeko, baina abantaila lehiakorra eman zion *Eutreptiella* generoari, kriptofitoak eta *K. foliaceum* ordezkatu eta komunitateko taxon nagusia bihurtzeko. Datozen hilabete eta urteetako jarraipen programek frogatuko dute estuarioan egindako saneamendu lanek eutrofizatu gabeko ur-sistemetan tipikoak diren komunitateak lortzeko bidea zabaldu duten edo, aitzitik, ikusitako aldaketa ur-baldintzen aldaketa bortitzaren ondoriozko erantzun puntuala izan den. Gainera, Urdaibai estuarioaren erresilientzia gaitasunak rol garrantzitsua izango du sistemaren etorkizuneko fitoplankton-komunitatearen osaeran.

5. Ondorioak

Ikerketa honetatik ondorioztatu daiteke Gernikako HUAren isurketak Urdaibaiko estuariotik kanpo desbideratzeak, eta, ondorioz, estuarioaren barruko eremuko hondakin-uren isurketekin amaitzeak, berehalako eragina izan zuela sisteman, batez ere barrualdeko eremuan. Aldaketarik bortitzenak, baldintza fisiko-kimikoen artean, estuarioaren erdiko eta barruko eremuan ikusitako amonio eta fosfato kontzentrazioen beherakadak adierazgarriak izan ziren, estuario osoan zehar, gutxienez, egoera “ona” lortzea ahalbidetu zutelarik. Gainera, estuarioaren barruko eremuan, fitoplankton-komunitatearen osaera ere aldatu zen saneamendu lanen ondoren, fitoplanktonak baldintza fisiko-kimikoen aldaketen aurrean azkar erantzun dezakeela frogatuz. Nolanahi ere, ikerketa honen iraupena laburregia da, desbideratzea gauzatu eta hilabete baten buruan gertatutako aldaketak bakarrik aztertzen baititu, beraz, ondorioak, “atariko efektu” bezala hartu beharko lirateke. Ikerketa-denbora

luzeagoa behar da Urdaibai estuarioko saneamendu lanek sisteman izango duten benetako eragina zehaztu eta sistemaren erresilientzia gaitasuna frogatzeko.

Chapter 3

PIGMENTUM: An easy pigment-based tool for monitoring phytoplankton community composition

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Abstract

Different mathematical tools have been developed to relate pigments and phytoplankton groups and determine the contribution of each of these groups to the total phytoplankton biomass. However, most of them present several drawbacks, such as the need of a minimum sample number or previous knowledge on the community composition. The present study proposes a new chemotaxonomic tool, *PIGMENTUM*, based on simultaneous equations that take into account the lack of exclusiveness of diagnostic pigments and defends the use of "pigment-groups" to define the community composition over "taxonomic-groups". Additionally, *PIGMENTUM* does not require having prior knowledge on the community composition of the study area, generic pigment ratios are applied, samples are treated independently and it allows individualised ratio correction if necessary. The accuracy of *PIGMENTUM* was tested with a data series of 330 samples from Urdaibai estuary containing different trophic statuses (0.1–251 μg L^{-1} of chlorophyll *a* [Chl *a*]) and water masses (salinities between 0 and 36 PSU). The median similarity between the calculated and the real Chl *a* was 82%, and 98% of the samples obtained reliable results, the tool being especially accurate for eutrophic and hypereutrophic waters. *PIGMENTUM* was thus proven efficient for phytoplankton monitoring in a wide variety of aquatic systems and environmental conditions, allowing following up the variability of the community over time and space and/or determining the groups causing eutrophication or isolated blooms, without the need of using additional techniques.

1. Introduction

Phytoplankton monitoring is essential for understanding the functioning of aquatic ecosystems and for water quality assessment. Its basal position in the food chain and being the main primary producer of aquatic ecosystems make phytoplankton a key biological component (Cordoba-Mena et al., 2020; Damar et al., 2020). Its suitability as a bioindicator is mostly due to the rapid response of phytoplankton to alterations in the environmental conditions of systems, showing changes in community composition and biomass (Paerl et al., 2003). Thus, phytoplankton monitoring for ecological purposes (Scharfe and Wiltshire, 2019; Mao et al., 2020; Diana et al., 2021; Dedman et al., 2022; Chen et al., 2023) or water quality assessments (Boyer et al., 2009; Katsiapi et al., 2016; Cupertino et al., 2019; Amorim and Nascimento, 2021; Zhang et al., 2021) have been extensively performed in freshwater, estuarine and marine ecosystems.

For many years, light microscopy has been the most widely used method to assess phytoplankton biomass and biodiversity (Córdoba-Mena et al., 2020; Lee et al., 2020), providing both qualitative and quantitative data at the genus or even species level (Edler and Elbrächter, 2010). However, this technique is time-consuming (Wang et al., 2018), is limited to small water volumes (usually up to 50 ml) (Gran-Stadniczeñko et al., 2019) and fragile and small cells (i.e. pico- and nanophytoplankton cells) are difficult to identify, leading to omissions or errors in taxonomic identifications (Agirbas et al., 2015). Furthermore, microscopic identification requires extensive taxonomic knowledge (Naik et al., 2011), and therefore, results are highly dependent on personal skills (Muñiz et al., 2020). Consequently, alternative techniques like pigment analysis are arising, trying to overcome the limitations that microscopy entails.

High-performance liquid chromatography (HPLC) enables the easy and fast identification and quantification of over 50 phytoplankton pigments (e.g., Zapata et al., 2000). Among the obtained pigments, chlorophyll *a* (Chl *a*), present in every phytoplankton group, is used as a proxy for total algal biomass estimation (Jeffrey et al., 1997), and several others are considered diagnostic pigments (DPs) of specific phytoplankton groups to determine community composition (Barlow et al., 2008; Chai et al., 2016). In this context, pigment analysis might be a useful alternative to microscopy, meeting the objectives of determining both phytoplankton total biomass and community composition, based on a larger volume analysed (Agirbas et al., 2015) and enabling the quantification of pico- and nano-sized

phytoplankton (Wright and Jeffrey 2006; Barlow et al., 2008). Consequently, pigment analysis is widely used in phytoplankton monitoring (Damar et al., 2020; Miranda-Alvarez et al., 2021; Mudakikwa et al., 2021; Cereja et al., 2022; Lee et al., 2022). Nevertheless, this method has to deal with a limitation when used for the study of phytoplankton community composition: the lack of exclusive DPs for some phytoplankton groups, and the presence of common pigments in others, reduces the discriminant capacity of the DPs (Latasa, 2007; Nunes et al., 2018).

Different mathematical tools for taxonomic interpretation of pigment datasets have been developed over the years (e.g., Letelier et al., 1993; Mackey et al., 1996; Uitz et al., 2006; Latasa, 2007; Van den Meersche et al., 2008; Higgins et al., 2011; Kramer and Siegel, 2019; Hayward et al., 2023). These tools are based on the use of DP:Chl *a* ratios, mainly based on literature, to relate DPs and phytoplankton groups, and determine the contribution of each of these groups to the total phytoplankton biomass (Chl *a*) (Higgins et al., 2011). Gieskes and Kraay (1983) employed multiple linear regressions to determine the relationship between single DPs and Chl *a*, which is statistically sound, but cannot account for the DP that are shared between several taxa.

To tackle the problem of shared pigments, several authors (e.g., Letelier et al., 1993; Vidussi et al., 2001) proposed the use of inverse simultaneous equations (ISE), based on calculating the contribution of each phytoplankton group to total Chl *a* from the concentrations of DPs, while subtracting contributions to those DPs by other groups. With a similar base as the ISE method, Mackey et al. (1996) developed the CHEMTAX software. In this case, rather than building equations, the user constructs a pigment:Chl *a* ratio matrix for every phytoplankton group expected, in order to calculate the total Chl *a* content of each sample. The CHEMTAX algorithm then iteratively adjusts the ratio matrix so that the differences between the calculated and observed Chl *a* are minimised. The main advantage of CHEMTAX over ISE is the ease with which changes can be introduced in the taxonomic composition of the populations by simply subtracting or adding rows in the matrix (Higgins et al., 2011). Additionally, as an alternative to CHEMTAX, a Bayesian Compositional Estimator (BCE) was developed to determine probability intervals and point estimates for the phytoplankton classes' biomass (Van den Meersche et al., 2008), but this tool required either prior knowledge or extensive predictive simulation and, therefore, is not commonly used. Hayward et al. (2023) also presented an alternative chemotaxonomic method (*phytoclass*) that utilises simulated annealing with a steepest descent algorithm to derive class abundances and

pigment:Chl *a* ratios, which displays a higher accuracy than two common configurations of the CHEMTAX method. Moreover, non-taxonomic interpretation of pigment datasets have also been proposed, such as the one described by Vidussi et al. (2001) and later modified by Uitz et al. (2006) for determining the size classes of the phytoplankton community.

Previously mentioned tools for determining the quantitative composition of phytoplankton communities from pigment data have been widely employed for phytoplankton monitoring (e.g. Uitz et al., 2009; Chase et al., 2020; Flander-Putrle et al., 2021), especially the CHEMTAX software (e.g., Miranda-Alvarez et al., 2020; Wang et al., 2021; Hyun et al., 2022). However, they share some common drawbacks that should be taken into account in order to interpret the results accurately. One of the main weaknesses of these tools is the oversimplification of directly linking a "taxonomic-group" and a DP when trying to determine the community composition, since it is well known that not all the DPs are specific for one phytoplankton group and not all members of a taxonomic-group contain the same DP (Latasa, 2007). Clear examples of this situation are *Kryptoperidinium foliaceum,* a fucoxanthin (DP for diatoms) containing dinoflagellate with no peridinin (DP for dinoflagellates) (Higgins et al., 2011), or *Isochrysis,* a fucoxanthin containing haptophyte genus with no 19'-hexanoyloxyfucoxanthin (DP for haptophytes) (Seoane et al., 2005). These two taxa could be dominant in several ecosystems and the use of this assignation would result in a misinterpretation. In addition, this simplification requires a previous knowledge of the community for each sample before applying any of the tools, in order to make a correct ratio and taxonomic-group choice, since the ratios will remain constant across all samples of the dataset and will determine the contribution of a certain taxonomic group. Besides, this lack of variability of the ratios is rarely true given the effects of light, nutrients and other factors on pigment ratios and could only be solved by subdividing the dataset. Furthermore, these ratios are varied automatically in tools like CHEMTAX and could result in unrealistic final ratios according to algae physiology and ecology (Higgins et al., 2011).

The present study proposes a new chemotaxonomic tool (*PIGMENTUM*), based on simultaneous equations that take into account the lack of exclusiveness of diagnostic pigments, giving the possibility of ratio correction if necessary, without the need of observing the sample prior to analysis. The main aims are to leave behind the paradigm of using HPLC pigment analysis to define the phytoplankton community composition through "taxonomic-groups", by proposing the definitive use of "pigment-groups" and leverage all the advantages of *PIGMENTUM* for being used in phytoplankton monitoring.

2. Materials and Methods

2.1. Description of PIGMENTUM

The aim of this method is to determine the contribution of the different phytoplankton pigment-groups to the total phytoplankton biomass (Chl *a*) in water samples, using pigments as biomarkers. In order to fulfil this objective, different equations were built (one for each pigment-group) based on the selected diagnostic pigments, the contributions of the DPs to total Chl *a* (ratios) and the lack of exclusiveness of several DPs.

Pigment data obtained by HPLC is generally large (i.e. 50 different pigments are differentiated), and consequently, some pigments may be redundant or covary with others, making data visualisation and interpretation difficult. Therefore, in order to extract the most relevant information of the dataset, pigment data reduction is required. The present method selects the following DPs to build the equations (*Table 3.1*): peridinin (Peri), fucoxanthin (Fuco), 19'-butanoyloxyfucoxanthin (But), 19'-hexanoyloxyfucoxanthin (Hex), alloxanthin (Allo), chlorophyll *b* (Chl *b*), zeaxanthin (Zea), prasinoxanthin (Pras), Divinyl chlorophyll *a* (DVChl *a*) and vaucheriaxanthin (Vauch).

Table 3.1. *Table containing the pigment-groups defined by PIGMENTUM, the main DP ratio of each of the groups, the calculated K value used in the equations and the description of the main phytoplankton taxa of each of the groups. In bold, the taxonomic group from which the ratio was taken.*

***PIGMENTUM* pigment-groups**	**DP ratios**	**K (1/ratio)**	**Main taxa**
Peridinin containing algae (PeCA)	Peri:Chl *a* = 0.56	K_{Peri} = 1.79	Peri-containing **dinoflagellates**
19'-hexanoyloxyfucoxanthin containing algae (HCA)	Hex:Chl *a* = 0.47	K_{Hex}= 2.13	Hex-containing **haptophytes** and dinoflagellates with Fuco-derivates
Alloxanthin containing algae (ACA)	Allo:Chl *a* = 0.38	K_{Allo}= 2.63	**Cryptophytes** and *Dinophysis* spp.
Prasinoxanthin containing algae (PrCA)	Pras:Chl *a* = 0.25	K_{Pras}= 4.00	Pras-containing **prasinophytes**
Vaucheriaxanthin containing algae (VCA)	Vauch:Chl *a* = 0.049	K_{Vauch}= 20.41	**Eustigmatophytes**
Fucoxanthin containing algae (FCA)	Fuco:Chl *a* = 0.805	K_{Fuco}= 1.24	**Diatoms**, crysophytes, other minor ochrophyte groups, Fuco-containing dinoflagellates and haptophytes without Fuco-derivates
19'-butanoyloxyfucoxanthin containing algae (BCA)	But:Chl *a* = 0.658	K_{But}= 1.52	**Pelagophyceae**, But-containing haptophytes and dinoflagellates
Chlorophyll *b* containing algae (CbCA)	Chl *b*:Chl *a* = 0.525	K_{chlb}= 1.90	**Chlorophytes**, Pras non-containing prasinophytes, euglenophytes and *Lepidodinium* spp.
Zeaxanthin containing algae (ZCA)	Zea:Chl *a* = 0.64	K_{Zea}= 1.56	**Cyanobacteria** excluding *Prochlorococcus marinus*.
Divinyl chlorophyll *a* containing algae (DCA)	-	-	***Prochlorococcus marinus***

Assuming that each of the chosen DPs contributes equally to Chl *a* is not strictly realistic, and thus, DP:Chl *a* ratios must be applied for the characterisation of the community composition. Moreover, these ratios vary with species and their biological state (e.g. Vidussi et al., 2001). The ratios suggested by *PIGMENTUM* for each DP are based on average values from cultures for low, medium and high light of the most representative taxonomic group for this pigment, taken from Higgins et al. (2011), with the exception of DVChl *a*, which was defined according to Claustre and Marty (1995).

Besides, *PIGMENTUM* addresses the problem of shared pigments when calculating the contribution of each pigment-group to total biomass, following the example of Letelier et al. (1993). The method proposes equations for each pigment-group, intending to leave in each of these groups those organisms that do not have any other DP more specific than the one chosen for the group. This leads to simple equations (equations 1 to 5) when the DP of the pigment-group is very specific, based on the multiplication between the concentrations of the DP of the pigment-group and the K value that corresponds in each case (see *Table 3.1*). The K value of each DP corresponds to the inverse value of the DP:Chl *a* ratio (1/ratio). On the contrary, when the DP of a pigment-group is not so specific and is also present in other organisms outside the group, the equation (equations 6 to 9) subtracts the concentration of the DP given by other organisms belonging to other groups with more specific pigments of that chosen, before multiplying the concentration by the K value. Additionally, a different strategy was followed to determine the contribution of the DCA to the total phytoplankton biomass (see eq. 10), resulting from Claustre and Marty (1995). The DCA group is mainly represented by *Prochlorococcus marinus*, choosing DVChl *a* as the DP for their determination, nevertheless, and on the contrary to the rest of the groups mentioned, they do not contribute to the Chl *a* concentration, they just provide DVChl *a* (Claustre and Marty, 1995), which explains the simplicity of eq 10.

These are the equations suggested by *PIGMENTUM*:

$$(1)\ [\text{Chl } a]_{\text{PeCA}} = K_{\text{Peri}} \cdot [\text{Peri}]$$

$$(2)\ [\text{Chl } a]_{\text{HCA}} = K_{\text{Hex}} \cdot [\text{Hex}]$$

$$(3)\ [\text{Chl } a]_{\text{ACA}} = K_{\text{Allo}} \cdot [\text{Allo}]$$

$$(4)\ [\text{Chl } a]_{\text{PrCA}} = K_{\text{Pras}} \cdot [\text{Pras}]$$

$$(5)\ [\text{Chl } a]_{\text{VCA}} = K_{\text{Vauch}} \cdot [\text{Vauch}]$$

$$(6)\ [\text{Chl } a]_{FCA} = K_{Fuco} \cdot ([Fuco] - 0.64 \cdot [Hex] - 1.18 \cdot [But])$$

$$(7)\ [\text{Chl } a]_{BCA} = K_{But} \cdot ([But] - 0.08 \cdot [Hex])$$

$$(8)\ [\text{Chl } a]_{CbCA} = K_{chlb} \cdot ([\text{Chl } b] - 2.92 \cdot [Pras])$$

$$(9)\ [\text{Chl } a]_{ZCA} = K_{Zea} \cdot ([Zea] - 0.1 \cdot [\text{Chl } b] - 0.321 \cdot [\text{DVChl } a])$$

$$(10)\ [\text{Chl } a]_{DCA} = [\text{DVChl } a]$$

In order to determine the concentrations of the DPs that need to be subtracted in each of the complex equations when DPs are not specific (eq. 6 to 9), the relation among the different pigments belonging to the different pigment groups are taken into account. As an example, in the case of the FCA pigment-group (eq. 6), the DP is Fuco, but this pigment is also present in high abundance in organisms belonging to HCA and BCA groups. Since both HCA and BCA have more specific DPs than Fuco (Hex and But, respectively), the amount of Fuco given by the organisms belonging to these two groups must be subtracted, before determining the contribution of FCA to total phytoplankton biomass. For determining the amount of Fuco that needs to be subtracted, the relation between their respective DP and Fuco must be calculated. Following the example, organisms belonging to HCA have, according to Higgins et al. (2011), a Hex:Chl *a* ratio (their DP) of 0.47 (see *Table 3.1*), and their Fuco:Chl *a* ratio (the pigment we want to extract) is 0.3, corresponding to the haptophytes types HAPTO-6, -7 and -8 (Higgins et al., 2011); therefore, the amount of Fuco corresponding to HCA that needs to be subtracted from the FCA is the relation among the two pigment ratios (0.3/0.47 = 0.64) multiplied by the concentration of their DP (Hex in this case) (see eq. 7). As for organisms belonging to BCA, the But:Chl *a* ratio is 0.658 (see *Table 3.1*), and their Fuco:Chl *a* is 0.779 (Higgins et al., 2011), being 1.18 multiplied by the But concentration of the sample the amount of Fuco belonging to BCA that needs to be subtracted from FCA (see eq. 6). This same procedure is followed for BCA, CbCA and ZCA pigment-groups, since But, Chl *b* and Zea are, respectively, pigments shared by more than their main groups, even if they do not act as the predominant DP. It should be noted that whenever any of these complex equations has a value lower than 0, it will be replaced by 0, since this would indicate that all the DP concentration of the chosen pigment-group is contributed by organisms belonging to other groups.

Once equations are executed for each water sample individually, the obtained result will give us an estimation of the Chl *a* concentration that each pigment-group present in the sample provides ($[\text{Chl } a]_i$; where "i" refers to any of the different pigment groups). Based on this, the contribution percentages of each group ($\%_i$) to the total phytoplankton biomass will be

calculated (eq. 11 and 12). First, the sum of the Chl *a* given by each pigment group is calculated ($[Chl\ a]_{SUMpg}$) (eq.11) and, this will be later used for the estimation of the contribution of each pigment group to the total biomass of the community ($\%_i$) (eq. 12), instead of using the real Chl *a* given by the HPLC.

$$(11)\ [Chl\ a]_{SUMpg} = [Chl\ a]_{PeCA}+[Chl\ a]_{FCA}+[Chl\ a]_{BTA}+[Chl\ a]_{HTA}+[Chl\ a]_{ACA}+[Chl\ a]_{CbCA}+[Chl\ a]_{ZCA}+[Chl\ a]_{PrCA}+[Chl\ a]_{VCA}+[Chl\ a]_{DCA}$$

$$(12)\ \%_i = ([Chl\ a]_i/[Chl\ a]_{SUMpg}) \cdot 100$$

Knowing that pigment ratio choice is one of the main constraints of chemotaxonomic tools like CHEMTAX (Latasa, 2007), *PIGMENTUM* gives the opportunity to correct it. It was assumed that, in the samples where any of the pigment-groups dominates the community by more than 75%, the direct field DP:Chl *a* ratio may be more accurate than the literature ratio for the calculation of the contribution of this group to the total phytoplankton biomass. Therefore, when this happens, the DP ratio of the dominant pigment-group taken from literature (*Table 3.1*) will be replaced by the field ratio for building the corresponding K equation (eq. 13). As an example, if the ACA pigment-group dominates the community by more than 75% after performing the equations (1 to 12), the K_{Allo} will be corrected following eq. 13, with field Allo and Chl *a* concentrations of this particular sample, and will be replaced in eq. 3 as the new K_{Allo}.

$$(13)\ K_{DPcorrected} = (1 / ([DP] / [Chl\ a]_{HPLC}))$$

After applying the required corrections, *PIGMENTUM* will deliver a new data output of the concentration of Chl *a* provided by each of the pigment-groups present in the samples. Once the final output is obtained, *PIGMENTUM* will give the possibility of estimating the accuracy of the final approximation, that will be evaluated by determining the similarity percentage (Sim%) between the calculated sum of the Chl *a* given by the pigment-groups ($[Chl\ a]_{SUMpg}$) and the real Chl *a* ($[Chl\ a]_{field}$) concentration, given by HPLC (eq. 14). It must be highlighted that, in order to account with the possible presence of the DCA group, which provides DVChl *a* instead of Chl *a* (Claustre and Marty, 1995), the $[Chl\ a]_{field}$ of each sample applied in eq. 14 is formed by the sum of $[Chl\ a]_{HPLC}$ and $[DVChl\ a]_{HPLC}$. The Sim% is obtained for each sample, allowing the evaluation of the adjustment accuracy in each of the samples. Considering this, the correction of changing the literature ratio by the field ratio will only be applied to the final result if the Sim% is improved with the new field ratio. On the contrary, if the Sim% is lower after the correction, literature ratios will again be used to return to the

initial Sim% values. This latter situation will happen whenever the literature ratio chosen for the dominant pigment-group, although inaccurate, helps to compensate for the possible variability in the ratio choice for the other pigment-groups, making the calculated Chl *a* more similar to the real Chl *a*.

$$(14)\ \mathrm{Sim\%} = ([\mathrm{Chl\ a}]_{\mathrm{field}} - |[\mathrm{Chl\ a}]_{\mathrm{field}} - [\mathrm{Chl\ a}]_{\mathrm{SUMpg}}|) \cdot 100/[\mathrm{Chl\ a}]_{\mathrm{field}}$$

$$\textit{where, } [\textit{Chl a}]_{\textit{field}} = [\textit{Chl a}]_{\textit{HPLC}} + [\textit{DVChl a}]_{\textit{HPLC}}$$

Once the final calculations are performed, by only applying the efficient corrections, the *PIGMENTUM* final output will be delivered. Results of the contribution of each pigment-group to the total phytoplankton biomass will be given in percentages and raw Chl *a* concentrations (μg L^{-1}) for each sample analysed. Additionally, the estimation-accuracy will be provided for each sample, defined by the Sim%.

2.2. Testing dataset: Urdaibai estuary (SE Bay of Biscay)

The development of *PIGMENTUM* required the assessment of the method by using real field datasets of HPLC-derived pigment concentrations, and thus, the tool was tested using data from the Urdaibai estuary (SE Bay of Biscay).

Urdaibai estuary is a short and shallow meso-macrotidal system, where tidal cycles have a great impact, being mostly marine dominated at high tide. However, this estuarine system is highly variable in volume and flushing rates, due to the torrential nature of the Oka River, since large flows that change the typical pattern appear occasionally (Madariaga et al., 1994). This has a direct effect on the physicochemical and biological properties of the estuary, causing changes in the phytoplankton communities on a short time scale (Madariaga et al., 1994). In addition to the temporal variability, Urdaibai estuary shows a marked spatial variability, mostly marked by the morphology of the system. Considering this, three differentiated areas can be identified in the estuary: an outer wide zone of marine character, a middle area characterised by a significant narrowing formed by a central channel bordered by salt-marshes and secondary channels, and an inner zone consisting of an artificial channel that reaches the main town in the area (Gernika).

The data series used to test the program is made up of 330 samples collected over three years (2019–2022) at 6 sampling stations (see map in *Chapter 1*) located throughout the entire longitudinal axis of the estuary (1 outer [URD1], 1 middle [URD2] and 4 inner estuary

[URD3–URD6]). Samples were collected quarterly from September 2019 to September 2020, and from March to October in 2021 and 2022, concluding with 55 sampling campaigns. This allowed us to test *PIGMENTUM* in a wide variety of scenarios due to the high spatial and temporal variability covered in the dataset. The samples contained waters with salinities between 0 and 36, temperatures from 8 to 26 ºC, turbidity ranging between 0.5 and 57.5 NTU and phytoplankton abundances between 0.1 and 251 μg L^{-1} of Chl *a*. Pigment data was obtained following the methodology detailed in *Chapter 2*. Water samples were collected using a Niskin bottle, stored in opaque plastic bottles and then filtered (0.4–4 L) with a gentle vacuum (<150 mm Hg) through Whatman GF/F glass-fibre filters (Whatman International Ltd.) in dark conditions. Pigment extraction and analysis by HPLC was performed following Zapata et al. (2000), with a modification in solvent A and the equipment described in Seoane et al. (2009b).

2.3. Data analysis

The main statistical parameters (range, median and/or arithmetic mean) were calculated for Sim% results obtained from *PIGMENTUM*, before and after correction, for the overall dataset, but also for the different trophic conditions and water masses. The trophic status was defined based on Chl *a* concentration intervals (OECD, 1982) and the different water masses based on salinity (Water Framework Directive, WFD).

Additionally, the Wilcoxon signed-rank test (Woolson, 2007), a non-parametric two-sample paired test, was performed to determine whether there were significant differences (alpha = 0.01) in the Sim% values of the corrected samples before and after correction. The Monte Carlo significance (Hope, 1968) value was considered, which is based on 99,999 random reassignments of values to columns within each pair (Hammer and Harper, 2006).

All statistical analyses were carried out using PAST 4.0 (Paleontological STatistics), a software package for data analysis (Hammer et al., 2001).

3. Results

PIGMENTUM, tested with pigment data from the Urdaibai estuary, seemed to be accurate for the phytoplankton community composition monitoring in a wide variety of environmental conditions (salinity, temperature, turbidity and trophic conditions).

Among the 330 samples analysed, 22% had a dominant pigment-group in the community that contributed more than 75% to the total phytoplankton biomass and, therefore, were exposed to ratio correction. The pigment ratio most frequently changed was Allo:Chl *a* (67%), followed by Fuco:Chl *a* (23%), Chl *b*:Chl *a* (8%) and Pras:Chl *a* (1%). Peri:Chl *a*, Zea:Chl *a*, Hex:Chl *a* and But:Chl *a* ratios were not modified in any of the samples, because their corresponding pigment-groups did not dominate the community. As for Vauch and DVChl *a*, they were not detected within the dataset.

Within the samples exposed to correction, 66% led to the improvement of the similarity between the sum of the Chl *a* given by the pigment-groups and the real Chl *a* concentration, while 34% registered lower Sim% after correction. Nevertheless, the Wilcoxon signed-rank test determined that the corrections, accounting improves and worsening, entailed a significant overall improvement ($p<0.001$) of Sim% in the samples applied. This might be explained by the fact that, among the corrected samples, the average improvement percentage of Sim% was 22%, registering a maximum improvement of 62%, way higher than the worsening average (8%). Besides, as previously mentioned, only the corrections that led to improvements were considered for the final calculation.

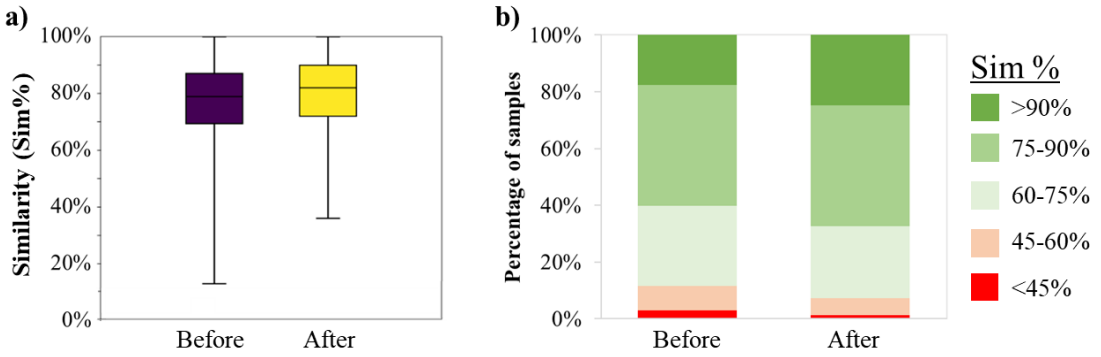

Figure 3.1. *a) Sim% before and after correction for the whole dataset. The box represents the Interquartile Range (IQR), data between Q1 (25th percentile) and Q3 (75th percentile); the line inside the box is the median, the lower whisker shows the Q1-1.5*IQR, the upper Q3+1.5*IQR and circles and asterisks represent outliers. b) The percentage of samples belonging to the different Sim% ranges (<45%, 45–60%, 60–75%, 75–90% and >90%) before and after applying the corrections.*

Once the pigment-ratio corrections were applied and the definitive results were obtained, Sim% values were recalculated in order to determine the accuracy of *PIGMENTUM* (*Figure 3.1*; *Annexes, Table A3.1*). The median Sim% of the whole dataset was 82%, with the 93% of the samples obtaining a Sim% above 60%, and the 67% of them a Sim% above 75% (*Figure 3.1.b*). In addition, only the 1% of the samples (3 out of 330 samples) showed a Sim% below 45%. These Sim% values were improved due to correction, since previously only the

89% of the samples reached a Sim% above 60%, and the 3% of them recorded Sim% values below 45%.

When analysing the accuracy of *PIGMENTUM* by the different trophic statuses of the water samples, taking Chl *a* concentration into account, results showed that Sim% increased when Chl *a* concentration was high (*Figure 3.2*; *Annexes, Table A3.1*). The hypereutrophic (Chl *a* above 25 µg L^{-1}) samples showed the highest median Sim% (90%), followed by eutrophic waters (8–25 µg L^{-1} Chl *a*) with 84% median Sim%. Mesotrophic (2.5–8 µg L^{-1} Chl *a*) and oligotrophic (1–2.5 µg L^{-1} Chl *a*) samples both showed a median Sim% of 82%, and ultraoligotrophic (Chl *a* <1 µg L^{-1}) recorded the lowest median Sim% of 75%. It must be outlined that every hypereutrophic sample obtained Sim% values above 65%, more than half of them having Sim% above 90%. The Sim% of most of the eutrophic samples (95%) was also higher than 60%, and 34% had Sim% values higher than 90%. As for ultraoligotrophic samples, even if the average Sim% was the lowest, 90% of the samples showed Sim% higher than 60%, and only 2% of them registered Sim% values lower than 45%.

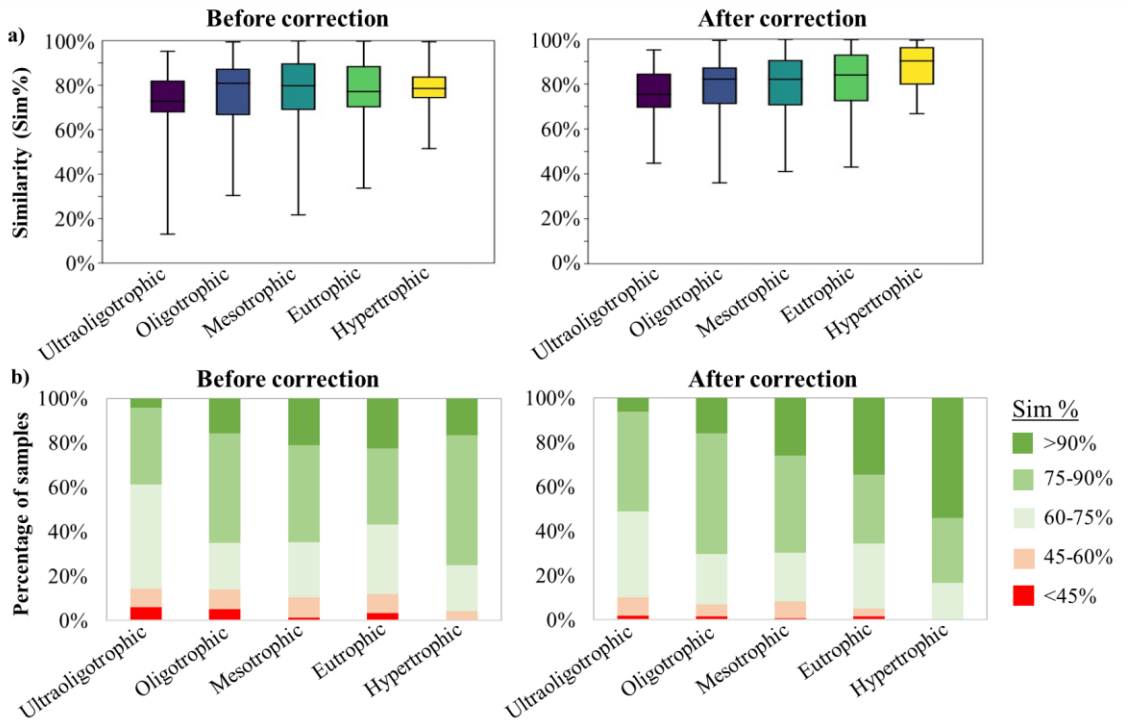

Figure 3.2. *a) Sim% before and after correction for each Chl a concentration category. The box represents the Interquartile Range (IQR), data between Q1 (25th percentile) and Q3 (75th percentile); the line inside the box is the median, the lower whisker shows the Q1-1.5*IQR, the upper Q3+1.5*IQR and circles and asterisks represent outliers. b) The percentage of samples belonging to the different Sim% ranges (<45%, 45–60%, 60–75%, 75–90% and >90%) before and after applying the corrections, taking into account the trophic statuses of the samples.*

Regarding the *PIGMENTUM* precision for different water masses, making the categorization based on salinity, there was not a clear pattern (*Figure 3.3*; *Annexes, Table A3.1*). Mesohaline waters (5–18 PSU) recorded the highest median Sim% (84%), followed by polihaline (18–30 PSU) and euhaline (higher than 30 PSU) waters, with 82% and 81% median Sim%, respectively. In addition, mesohaline samples did not register Sim% below 45%, most of them (95%) recording Sim% higher than 60%, and 37% higher than 90% of similarity. As for polihaline and euhaline samples, more than 90% recorded Sim% higher than 60%, and around 20% of the samples reached Sim% values higher than 90%. The lowest median Sim% was obtained in oligohaline water samples (0.5–5 PSU) (74%) and the second lowest in freshwater samples (76.5%). Nevertheless, half of the oligohaline water samples recorded Sim% values between 60 and 75%; among the rest of the samples, 45% recorded Sim% values above 75%, and only 6% were below 45% of similarity. Regarding the freshwater samples, half of them registered Sim% values between 75 and 90%, and there was no sample with Sim% lower than 45%. The lack of clear trends relating salinity and Sim% might be explained by the strong influence of Chl *a* concentration in the samples.

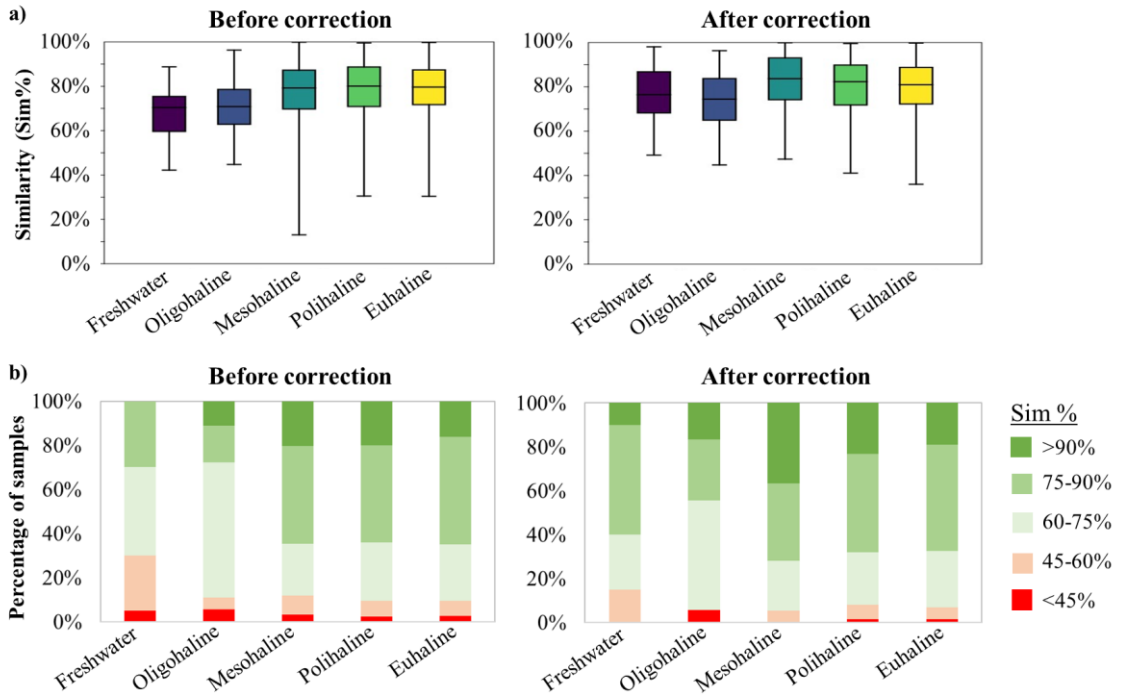

Figure 3.3. *a) Sim% before and after correction for each water-mass category according to salinity. The box represents the Interquartile Range (IQR), data between Q1 (25th percentile) and Q3 (75th percentile); the line inside the box is the median, the lower whisker shows the Q1-1.5*IQR, the upper Q3+1.5*IQR and circles and asterisks represent outliers. b) The percentage of samples belonging to the different Sim% ranges (<45%, 45–60%, 60–75%, 75–90% and >90%) before and after applying the corrections, taking into account the different salinity categories.*

Leaving the *PIGMENTUM* accuracy testing aside, and after confirming that the tool is capable of obtaining high similarity percentages between the sum of the Chl *a* given by the pigment-groups and the real Chl *a* concentration, a community composition estimation was delivered. For this, *PIGMENTUM* displayed a final contribution table, with every sample analysed, where the contribution of each pigment-group to total calculated Chl *a* was determined by means of contribution percentages and raw Chl *a* concentration provided by each pigment-group. As an example of the final results obtained, samples from 2019–2020 (138) were represented in *Figure 3.4*, divided by sampling station, to observe the community composition in the different areas of the Urdaibai estuary and the annual variability of these communities in each area. As can be observed, this enables a fast description of the community composition. *Figure 3.4* shows a higher pigment-group diversity at URD1 and the lowest at URD5 and URD6. In addition, it is easily detected that the dominant pigment-group changes along the estuary, with FCA dominating at URD1 and ACA dominating at URD3, URD4 and URD5, and that some groups have a clear station preference, like HCA with URD1. Temporal changes can also be seen, like the higher dominance of CbCA in the summer months at URD5 and URD6. Moreover, this gives the opportunity to clearly see that the bloom forming pigment-group on April 2020 was ACA.

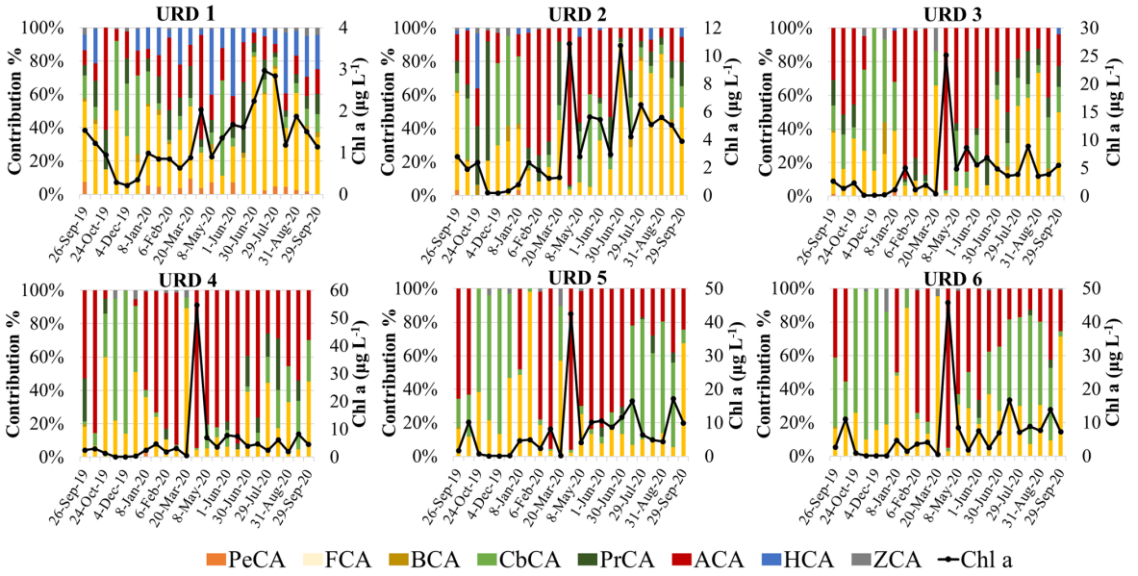

Figure 3.4. *Graphical result of PIGMENTUM for samples from 2019–2020 at the six different sampling stations of Urdaibai estuary, showing the spatial and temporal variability of the community composition and phytoplankton biomass. The contribution percentage of each of the pigment-groups is represented for each sampling campaign by areas and the black line represents the real Chl a concentration obtained by HPLC. PeCA: Peri Containing Algae; FCA: Fuco Containing Algae; BCA: But Containing Algae; CbCA: Chl b Containing Algae; PrCA: Pras Containing Algae; ACA; Allo Containing Algae; HCA: Hex Containing Algae.*

4. Discussion

Pigment analysis by HPLC has been widely used for phytoplankton monitoring in marine (e.g., Lee et al., 2022; Zhang et al., 2023a), estuarine (e.g., Cereja et al., 2022; Anil et al., 2023) and freshwater (e.g., Marguš et al., 2023; Tanttu et al., 2023) systems due to its capacity to obtain quick, reproducible and representative results. Knowing the existence of several approaches that use phytoplankton pigments to describe the community, *PIGMENTUM* was built to serve as a rapid and easy tool for obtaining a realistic insight of the phytoplankton community by both simplifying the calculations and advocating the assignation of DPs to "pigment-groups", instead of "taxonomic-groups".

The present research has proven that *PIGMENTUM* is an efficient tool for the description of the phytoplankton community composition and the contribution of the different groups to total phytoplankton biomass. When testing the tool with a dataset containing 330 samples within a wide range of Chl *a* concentrations and salinities, the median similarity between the Chl *a*, calculated with *PIGMENTUM*, and the real Chl *a* concentration, given by HPLC, was 82%. Approximately 98% of the samples fit accurately with real Chl *a*, only recording low similarities around 50% in 8 out of the 330 samples analysed, which indeed corresponded mostly to samples of low Chl *a* concentrations. Thus, the approximation made by the proposed equations of *PIGMENTUM* for subdividing the Chl *a* among the different pigment-groups was highly reliable. Additionally, this median similarity increased for eutrophic (84%) and, especially, for hypereutrophic (90%) samples, which makes *PIGMENTUM* an optimum monitoring tool for ecosystems with eutrophication problems. Moreover, the tool did not show specific weaknesses in any of the different salinity categories tested, from freshwater to marine samples, even if the phytoplankton taxa of those samples varied according to their salinity preferences. The non-dependence of the accuracy of results with the origin of the water mass or its trophic state achieved by *PIGMENTUM* proves it a versatile tool for a wide range of aquatic ecosystems.

The main advantage of pigment-based phytoplankton monitoring tools over other phytoplankton monitoring techniques (e.g. microscopy or eDNA metabarcoding), together with its rapidity and reproducibility, is the possibility of obtaining an accurate biomass estimation by determining Chl *a* in a reliable way. Chl *a* is the parameter used nowadays as the official measurement of phytoplankton biomass for water quality and eutrophication assessment in different International Policies (e.g. OSPAR, HELCOM) and European Directives (the WFD and the Marine Strategy Framework Directive) (Seoane et al., 2011).

Thus, its proper calculation and the use of secondary pigments to determine how this Chl *a* is subdivided among the phytoplankton community is more appropriate than the use of microscopic enumeration and its subsequent conversion to biovolume and/or carbon biomass, since Chl *a* determines primary production, while carbon biomass describes carbon fluxes (Higgins et al., 2011). This explains the difficulty of comparing pigment chemotaxonomy and microscopy results since the methods measure different parameters and have different units (Higgins et al., 2011). When trying to distribute the Chl *a* in percentages of taxonomic groups observed by microscopy, we are making a basic mistake that, together with the errors introduced by the low precision of microscopic identification and enumeration, and with those introduced in the conversion to biovolume and/or biomass, pose a problem if they are taken as "true values". This problem is especially remarkable in ecosystems dominated by pico- and nano-phytoplankton species, due to the underestimation or even omission of small and fragile cells that microscopy entails (Agirbas et al., 2015), while chemotaxonomic methods allow the detection of these pico- and nanophytoplankton fractions. Consequently, the effectiveness of *PIGMENTUM* or other chemotaxonomic tools should not rely on a comparison with microscopy results.

Most likely motivated by the tradition of using microscopy for the characterization of the phytoplankton community, most of the studies using chemotaxonomic tools (e.g. CHEMTAX) assign the DPs obtained in pigment analysis to phytoplankton taxonomic-groups (e.g., Jiang et al., 2022; Lee et al., 2022). However, these taxonomic-groups (e.g. diatoms or dinoflagellates) derived from chemotaxonomic tools or microscopy, cannot define unique functional groups within their classes either. Indeed, when Reynolds et al. (2002) studied the functional classification of the freshwater phytoplankton, they classified taxa belonging to "diatoms" and "cyanobacteria" in several of the different trait-separated functional groups. Thus, from a management perspective, determining the taxonomic groups of the phytoplankton community is not strictly necessary, since the taxonomic groups proposed usually correspond to high taxonomic levels and do not always refer to ecological characteristics. Based on this, the key novelty that *PIGMENTUM* offers is the definitive paradigm shift from assigning DPs to "taxonomic-groups" to "pigment-groups", which defends the fact that taxonomic-groups should be defined by techniques focused on the morphological description of the taxa, while pigment analysis should define pigment-groups. The change proposed aims to avoid the possible misinformation that the assignation of "taxonomic-groups" may entail due to the simplification made by the direct omission of certain groups. Several recent studies have fallen in this simplification, e.g. Jiang et al. (2022)

described the contribution of "haptophytes" or "diatoms" in the Bohai Sea and Lee et al. (2022) in the East/Japan Sea without taking into account the possible presence of Fuco-containig or Fuco-derivates containing dinoflagellates in these groups. In order to address this problem, each of the *PIGMENTUM* pigment-groups includes several taxonomic-groups (*Table 3.1*), driven by the lack of exclusiveness of DPs, and therefore, when referring to the contribution of certain pigment groups to the phytoplankton community, results are more realistic than when using taxonomic groups. Additionally, from a functional point of view, the use of pigment-groups over taxonomic-groups does not entail any disadvantage either because since several of the taxonomic-groups belong to different functional groups, their responses to the physicochemical variations may be very different, making data interpretation complicated (Reynolds et al., 1997).

One of the main advantages that *PIGMENTUM* offers over other chemotaxomic tools is the possibility of providing an accurate description of the spatiotemporal dynamics of the phytoplankton community and determining the groups responsible for the recorded phytoplankton blooms, without the need for previous knowledge on the phytoplankton community of the area. This has a direct relation to the paradigm shift to "pigment-groups" and to the use of average values for ratios. When chemotaxonomic tools define taxonomic-groups (e.g. CHEMTAX), having some knowledge of the phytoplankton communities of the area sampled is essential to determine the major taxa and the DP:Chl *a* ratios chosen for each taxonomic-group (Higgins et al., 2011). This is because organisms with unusual pigment signatures may be present in the community, such as dinoflagellates lacking Peri but with Fuco-derivates (e.g., *Karlodinium*) (Bachvaroff et al., 2009) or different subtypes of haptophytes with different pigment content (Zapata et al., 2004), and may otherwise introduce errors in the chemotaxonomic analyses when generic taxonomic-groups are defined. Thus, most of the studies using chemotaxonomy for phytoplankton monitoring highlight the importance of having previous information on the community composition of the area, not only for choosing pigment ratios, but also for defining taxonomic-groups based on that (e.g., Cereja et al., 2022; Jiang et al., 2022; Lee et al., 2022; Anil et al., 2023). In order to account for this background information, the research region must have been studied previously, or a preliminary analysis of the samples may be done with other techniques (e.g. microscopy or metabarcoding). Conversely, the ratios applied by *PIGMENTUM* (*Table 3.1*) for the Urdaibai estuary samples are based on average values taken from Higgins et al. (2011), which have proven efficient in at least 98% of the approximations done, and this makes prior knowledge of the community unnecessary in most cases. Thus, *PIGMENTUM* provides a quick insight

of the community composition by itself, without the need for prior microscopy or genetic analysis, saving time and effort for the researcher. Complementary monitoring approaches nonetheless provide valuable taxonomic information that help in the result interpretation and may be enriching for a deeper study of the community composition, for example for determining bloom forming organisms, when the pigment-group that dominates the community contains toxic species (e.g., PeCA) or to study species diversity.

The equation system proposed by *PIGMENTUM* to determine "pigment-groups" is formed by 10 new equations that were built with the main aim of addressing the non-exclusiveness of several of the DPs. The problem of the shared DPs is tackled by subtracting to the "pigment-group" defined by non-exclusive DPs the corresponding part of a "pigment-group" with more specific DPs. This makes the pigment choice an important aspect of *PIGMENTUM*. Among the chosen pigments, some of them (i.e. Peri, Fuco, But, Hex, Allo, Chl *b*, Zea) are those commonly used in previous chemotaxonomic studies (e.g., Gieskes and Kraay; 1983; Vidussi et al., 2001; Flander-Putrle et al., 2021). Nevertheless, *PIGMENTUM* incorporates 3 other DPs, prasinoxanthin (Pras), Divinyl chlorophyll *a* (DVChl *a*) and vaucheriaxanthin (Vauch), which do not appear in high concentrations in water samples in some ecosystems, but are very specific marker pigments of certain phytoplankton taxa in others. Thus, the equations proposed for *PIGMENTUM* contain more DPs than most of the previous tools (e.g. Vidussi et al., 2001), since pigment analysis has undergone continuous development in the last years, and higher resolution HPLC measurements have revealed that minor concentrations of many pigments are present in major algal classes, leaving few previously classified DPs unambiguous (Higgins et al., 2011). Pras is fundamental for the determination of the Pras-containing Prasinophyceae, helping distinguish them from the rest of green algae (Yu et al., 2007). DVChl *a* is an essential marker pigment to distinguish *Prochlorococcus marinus*, a marine unicellular green cyanobacterium that belongs to the most abundant and photosynthetically productive genus of cyanobacteria in the oceans (Goericke and Repata, 1992; Barrera-Rojas et al., 2018). Vauch facilitates the differentiation of Eustigmatophytes, a small group of 17 genera from marine and freshwater origin, over other Ochrophytes (Li et al., 2012). Therefore, the 10 "pigment-groups" defined by *PIGMENTUM* cover the entire spectrum of the major phytoplankton groups present in any aquatic ecosystem, without excluding any taxonomic group.

Together with the effectiveness in a wide range of aquatic ecosystems and the non-dependence on previous knowledge of the studied community, *PIGMENTUM* offers the

advantage of analysing the samples independently. In contrast to other robust chemotaxonomy tools like CHEMTAX, *PIGMENTUM* quantifies the contribution of each group to the total community by treating each sample independently, allowing the introduction of DPs that rarely appear (e.g., Vauch) without influencing the rest of the samples in any way. Additionally, CHEMTAX requires a minimum of 10–20 samples from homogeneous environmental conditions (Mackey et al., 1997) for its adequate use, as it is a matrix factorisation programme. This necessity of analysing samples with homogeneous environmental conditions or community compositions for the adequate ratio and taxonomy-group election counters the need of having a minimum number of samples, since in most of the cases researchers are forced to divide their sample sets to fulfil this first requirement, reducing their number of samples. This is a common problem faced by CHEMTAX users in ecosystems like estuaries and lakes, where environmental and ecological conditions of the samples are very heterogeneous and the subdivision of sample sets is something almost assumed (e.g., Cereja et al., 2022). Indeed, Tanttu et al. (2023) revealed the difficulties in using CHEMTAX with studies focusing on differences in phytoplankton composition among a large number of lakes of different trophic statuses. Conversely, *PIGMENTUM* may be a powerful tool for heterogeneous ecosystems like estuaries, as well as for studies with a limited amount of samples.

Additionally, in order to obtain meaningful results in several of the commonly used chemotaxonomic tools, such as ISE (e.g., Letelier et al., 1993; Vidussi et al., 2001) and CHEMTAX, certain assumptions and constraints must be considered (Higgins et al., 2011): normalisation and ratio variation. Pigment data and pigment:Chl *a* ratios in CHEMTAX are normalised, so that the sum of the contributions of taxonomy-groups will always equal the real sample Chl *a*, but this means that the residual errors (calculated – real Chl *a*) are also relative, making it difficult to compare different scenarios or to compare the absolute fit between different datasets. As for ratio variability, both ISE and CHEMTAX employ a 'steepest descent' algorithm to optimise the pigment:Chl *a* ratios used with the aim of minimising the difference between real Chl *a* and calculated Chl *a* (Higgins et al., 2011). This variation is done automatically and, if unconstrained, may venture outside realistic physiological and ecological pigment values (Goericke and Montoya, 1998). Mackey et al. (1996) recommended bounds of 500% for CHEMTAX, which still may cause significant modifications in the final output. *PIGMENTUM*, however, does not assume the normalisation of the introduced dataset and does not allow the automatic variation of the introduced pigment

ratios, believing in the accuracy of the pigment ratios introduced, which are derived from years of research and taking several studies into account (Higgins et al., 2011).

The need for ratio variation seen in CHEMTAX or ISE for the accurate calculation of phytoplankton community composition is explained by the high pigment-composition variability of microalgae. The pigment content, and therefore DP:Chl *a* ratio, is strongly influenced by several environmental factors, such as irradiance, nutrient status and growth phase, showing qualitative variations not only between species, but also between strains of single species (Zapata et al., 2004; Laza-Martinez et al., 2007). Thus, the accurate choice of the pigment ratios suitable for a whole community is difficult, even when the species present in the community are known. In order to address this difficulty, *PIGMENTUM* offers the possibility of ratio correction whenever the composition of the community requires it. The tool was developed with the aim of allowing the initial ratio modification when one of the pigment-groups substantially dominates the community (e.g. blooms), replacing the initial ratio of the dominant group taken from literature with its field ratio. Therefore, unlike the previously mentioned ratio variability experimented by other tools, the ratio modification will never be unrealistic and will only affect the sample that requires it, without modifying the entire dataset. The present study proved that this correction led to the improvement of the similarity between the real Chl *a* and calculated Chl *a* in 2 out of the 3 samples in which the ratio was modified, increasing the similarity by an average of 20% in each sample.

Nevertheless, and although they were few in the present study, there were still samples that registered a low similarity percentage between the real Chl *a* and the calculated one. Only 8 out of the 330 samples tested obtained Sim% lower than 50%, with a minimum Sim% of 36%. The low similarity percentage indicates that the calculations made by *PIGMENTUM* for the subdivision of the Chl *a* among the different groups in these samples may not be fully adjusted to reality. While the residual errors in CHEMTAX are given as relative values, *PIGMENTUM* allows the determination of the reliability of the adjustment for each sample independently, facilitating the identification of the problematic samples. This allows the researcher to decide whether the samples with low SIM% are relevant in the context of the study or if, conversely, due to different reasons (e.g. the low Chl *a* of the sample or anomalies registered during sampling), the low efficiency could be justified or ignored, since this does not affect the rest of the samples. *PIGMENTUM* is mainly designed to be used with the initial ratios proposed in *Table 3.1*, only changing them to field ratios when the correction is required, but the tool allows the introduction of different initial ratios if the researcher finds

it necessary. This is suitable in the situations of low similarity percentages mentioned previously and/or when the community composition of our study area is known, changing the generic initial ratios proposed by ratios belonging to organisms present in the water, in order to see if it is possible to achieve a better adjustment. However, it should be noted that this, unlike in previous tools, is a possibility, not a requirement.

5. Conclusion

PIGMENTUM was proven to be a powerful tool for phytoplankton monitoring in a wide range of Chl *a* concentrations and salinities. It enables the accurate subdivision of Chl *a* in 10 different pigment-groups based on a pigment dataset obtained by HPLC, providing information on phytoplankton community composition that allows us to follow up on the variability of the community over time and space and/or determine the groups causing eutrophication or isolated blooms, without the need of using additional techniques. *PIGMENTUM* is thus very appropriate for water quality management and long-term ecological monitoring programmes, where high taxonomic resolution is not compulsory, allowing measurements of responses to short-term perturbations, seasonal changes or climate change. Additionally, *PIGMENTUM* addresses several drawbacks present in previous chemotaxonomic approaches such as ISE or CHEMTAX. In this new tool, the Chl *a* is assigned to "pigment-groups", instead of "taxonomic-groups", avoiding simplifications that may distort the real image of the community. Moreover, *PIGMENTUM* does not require prior knowledge of the community composition of the study area, samples are treated individually, and generic pigment ratios are applied. Furthermore, *PIGMENTUM* allows individualised pigment-ratio correction whenever there is a dominant group in the community, using its field ratio, which has shown to be efficient.

Chapter 4

Medium-term response of the phytoplankton community to the cessation of wastewater discharges in the Urdaibai estuary based on *PIGMENTUM* analysis of HPLC pigments

Sent as: **Bilbao, J.** and Seoane, S. 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. *Frontiers in Marine Science.*

Abstract

Cultural eutrophication has become the most widespread anthropogenic threat to freshwater and coastal ecosystems' health. Phytoplankton is known to have a rapid response 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), which released high nutrient concentrations for 50 years, by comparing the physicochemical conditions and the phytoplankton biomass and composition (*PIGMENTUM* and microscopy) before (2020) and after (2022) the sewerage works. The cessation led to a significant decrease of ammonium and phosphate concentrations throughout the estuary, causing decreases of phytoplankton biomass in the outer and, especially, middle estuary (from 5 to 3 μg L^{-1} of chlorophyll *a*) and increases in the surroundings of the WWTP (from 7.6 to 16.8 μg L^{-1}), since the excessive ammonium probably suppressed phytoplankton growth. Changes in nutrient loadings also altered the community composition, recording an increase of prasinoxanthin-containing algae's (PrCA) contribution to total biomass (e.g., from 5% to 25% in URD2), and a composition shift from mainly flagellates (alloxanthin-containing and chlorophyll *b*-containing algae; ACA and CbCA) to fucoxanthin-containing algae (FCA) (mostly diatoms) in the inner estuary (e.g., from 13% to 31% in URD5), which could have been prompted by the higher nitrate availability over ammonium, and might indicate recuperation. This response of the phytoplankton community might have ecological implications in the future by affecting higher trophic levels in the estuary.

1. Introduction

Nitrogen (N) and phosphorus (P) are essential nutrients for the growth and reproduction of phytoplankton (Ryther and Dunstan, 1971; Conley et al., 2009). Thus, their concentration and distribution directly affect the primary productivity, carbon cycling, and food chains of aquatic ecosystems by influencing the species composition, abundance, and distribution of phytoplankton communities (Anderson et al., 2002; Davidson et al., 2014; Ke et al., 2022). In the past few decades, there has been an increase in anthropogenic inputs of N and P to coastal areas via river discharge (Liu et al., 2022; Sun et al., 2022), which has led to water quality degradation related to eutrophication worldwide (Conley et al., 2009; Romero et al., 2013; Paerl et al., 2014).

Cultural eutrophication, i.e., water over-enrichment by nutrients (especially N and P) 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 (Smith and Schindler; 2009; Field and Barros, 2014; Malone and Newton, 2020). 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). Estuaries, being systems with high land-ocean interaction (Frigstad et al., 2020), are particularly vulnerable to nutrient enrichment because they are the final receiving waters of rivers in the upper reaches (Zhang et al., 2022). Thus, eutrophication is considered the main threat to the integrity of estuaries worldwide (e.g., Dutto et al., 2012; Adams et al., 2020; 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). Thus, the effect of eutrophication on phytoplankton is not limited to high biomass production

and driving HABs. Unbalanced loadings of N and 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). In addition, it is generally agreed that N is the primary cause of eutrophication in most coastal ecosystems (e.g., Paerl, 2018), and since each phytoplankton group has specific uptake rates of different forms of N, this can also result in alterations in their growth rates and community composition (Glibert et al., 2016). Among the structural changes caused, generally, eutrophication tends to favour the dominance of small, fast-growing phytoplankton organisms ("r"-selected species) (Bužančić et al., 2016; González and Roldán, 2019). As for the effect of the N source, diatoms appear to be nitrate (NO_3^-) opportunists, becoming the dominant protists in NO_3^- rich waters, while 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 (Li et al., 2018; Eccles et al., 2020).

Human sewage is the most prevalent urban source of nutrient pressure, with an estimated release of $\sim 9 \times 10^9$ kg N yr^{-1} into the environment in 2018 (Malone and Newton, 2020). This is because, globally, 80% of municipal wastewater is released into the environment untreated (World Water Assessment Programme [WWAP], 2017), although the percentage of treated sewage varies regionally: 90% in North America, 66% in Europe, 35% in Asia, 14% in Latin America and the Caribbean, and < 1% in Africa (Selman and Greengalgh, 2010). 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 (Revilla et al., 2000; Franco et al., 2004). 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. Currently, the effluent is redirected to the Lamiaran WWTP, which discharges the treated effluents into the coastal area of Bermeo, and therefore the wastewater discharges that Urdaibai estuary received for five decades have disappeared.

Several studies have addressed the physicochemical properties (e.g., Iriarte et al., 2010; 2015), human impacts (Iriarte et al., 2016; Castillo-Eguskitza et al., 2017), and phytoplankton community (e.g., Franco et al., 1994; Iriarte et al., 1997; Revilla et al., 2000; Trigueros et al., 2000a; Ansotegui et al., 2003) of the Urdaibai estuary before the sewerage works. However, there is little knowledge on the effect of cessation of wastewater discharges to the estuary. *Chapter 2* 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 did not show the full annual dynamic of the estuary after diversion. Thus, the real effect of the sanitation works and the consequent nutrient input reduction in the Urdaibai estuary is still unknown. Indeed, the resilience of the estuary will determine the medium- and long-term effects of the deviation on the system, especially the recovery capacity, which defines the rapidity with which a system returns to its prior state following a disturbance (Wainger et al., 2017).

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 (*Figure 4.1*). 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), 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^2 (Villate et al., 1989), with a relatively small freshwater contribution (mean flow of 3.6 $m^3 s^{-1}$) compared to the total volume of the estuary (3.29 x 10^6 m^3) (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 (Franco, 1994; Barroeta et al., 2020). 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) (*Figure 4.1*): 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).

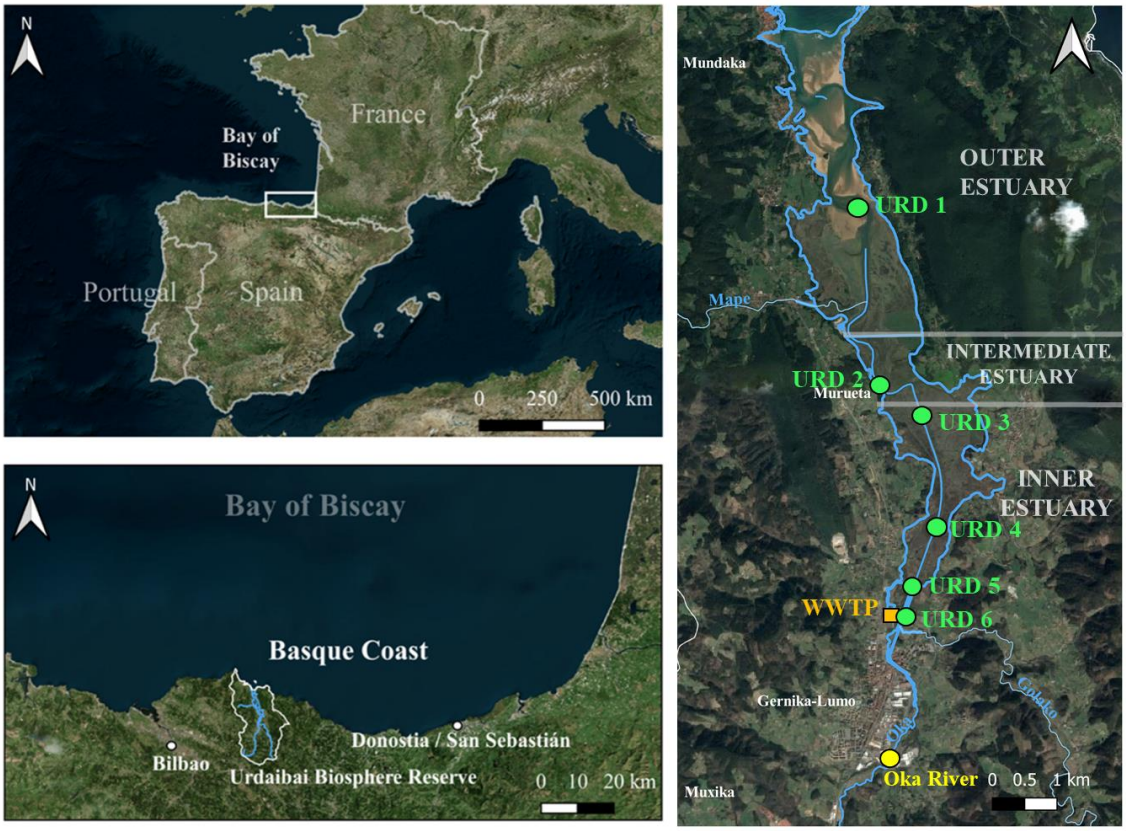

Figure 4.1. *Study area and sampling stations. On the left, the location of the Urdaibai estuary and the Biosphere Reserve in the Bay of Biscay (upper panel) and the Basque coast (lower panel). On the right, the Urdaibai estuary and the sampling stations (URD1-URD6 and Oka River). The discharge point of the wastewater treatment plant (WWTP) is also indicated.*

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, with a bi-weekly frequency. Six permanent sampling stations throughout the estuary's longitudinal axis (*Figure 4.1*) 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. This study exclusively considered subsurface samples to avoid potential impacts from sediment resuspension processes (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 μmol L^{-1}, 0.4 μmol L^{-1} for nitrite, and 0.16 μmol 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 (43º17’ N,

2º41' W, 16 m height), and Arteaga (43º20' N, 2º39' 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 based on high performance liquid chromatography (HPLC) pigment analysis, following the same methodology as in *Chapter 2*. Additionally, microscopy analysis was performed to identify and enumerate the dominant taxa, using it as complementary data for the pigment analysis, and the identification of 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 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 °C until extraction, ensuring that this occurred within a month after filtration. Pigments were extracted under low lighting conditions, using screw-capped tubes containing 5 ml of 90% acetone and employing a glass rod for grinding. Subsequently, the extracts underwent filtration through syringe filters (Millex, 0.22 μm 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. (2009b). 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*) concentration is commonly used as a direct proxy for total phytoplankton biomass in water samples in international directives like the WFD. Thus, in the present study, HPLC Chl *a* data was used to determine the spatio-temporal dynamics of the phytoplankton biomass throughout the Urdaibai estuary, including the definition of the maximum biomass areas and changes between 2020 and 2022. Additionally, Chl *a* values are also used to define the trophic status of estuarine waters (e.g., Bricker et al., 2003). In the present study, following Hagy et al. (2022), hypertrophic waters were defined as those with

Chl *a* concentrations above 20 μg 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* (developed in *Chapter 3*) 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 4.1*) 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 *Chapter 3*.

Table 4.1. *Pigment-groups defined by PIGMENTUM and the description of the main phytoplankton taxa of each of the groups (modified from Chapter 3). In bold, the taxonomic group from which the DP:Chl a ratio was taken.*

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

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) in the Urdaibai estuary during 2020 and 2022. Following the criteria from Revilla et al. (2009) in the Basque coast, the selected threshold to consider blooms was that of small phytoplankton taxa: 7.5×10^5 cells L^{-1}. 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. Additionally, toxic phytoplankton blooms were determined based on alert levels of cell concentrations. The threshold levels employed were the most restrictive ones among those previously used for Basque marine waters (Muñiz et al., 2017; Bilbao et al., 2020), which correspond to Swan and Davidson (2012), these being also commonly used in European monitoring programmes for HABs (ICES, 2015).

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 (2020) and after (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. Similar to the PERMANOVA analysis, 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 Urdaibay 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 A4.1* and *Table A4.2*).

The median air temperature during the study period (March–October) was 17.9 ºC in 2020 and 18.3 ºC in 2022, with springs at 16 ºC and 14.5 ºC and summers at 20.2 ºC and 20.9 ºC in 2020 and 2022, respectively. In both years, the hottest month was August, recording median temperatures of 20.8 ºC in 2020 and 21.4 ºC in 2022. The global monthly solar radiation (55,494 kJ m^{-2} in 2020 and 55,252 kJ m^{-2} in 2022) and the median daily insolation hours (5.5 h day^{-1} and 5.9 h day^{-1} in 2020 and 2022, respectively) during the study period were almost the same in both years. However, the maximum radiation values and insolation hours were recorded in different months, with May (64,034 kJ m^{-2} and 6.8 h day^{-1}) in 2020 and July (69,094 kJ m^{-2} and 9.2 h day^{-1}) in 2022 being the months with highest solar exposition. As for precipitation, median monthly-accumulated rainfall between March and October was 62 L m^{-2} in 2020 and 58 L m^{-2} in 2022. In both cases, the monthly-accumulated rainfall maxima were recorded in spring, in March (164 L m^{-2}) in 2020 and in April (128 L m^{-2}) in 2022.

Regarding the effect of the Oka River on the estuary, both the river flow and the Flow Index showed similar freshwater inputs into the Urdaibai estuary in 2020 and 2022. The median Oka River flow during the study period in 2020 was 0.22 m^3 s^{-1} and, in 2022, 0.14 m^3 s^{-1}. In accordance with the precipitation maxima, the highest median flow values were recorded in spring, in March (0.71 m^3 s^{-1}) in 2020 and in April (0.52 m^3 s^{-1}) in 2022. As for the Flow Index (*Annexes, Table A4.2*), values were much higher in spring (0.77 m^3 s^{-1} and 0.73 m^3 s^{-1} in 2020 and 2022, respectively) than in summer (0.26 m^3 s^{-1} and 0.19 m^3 s^{-1} in 2020 and 2022, respectively), also recording the maxima in March (4.7 m^3 s^{-1}) in 2020 and in April (4.3 m^3 s^{-1}) in 2022. In addition, riverine water temperature (median values of 17.3 ºC in 2020 and 17.7 ºC in 2022) and conductivity (388.7 µS cm^{-1} and 406.7 µS cm^{-1}) were similar in both years.

The multivariate two-way (seasonal and inter-annual) PERMANOVA performed (*Annexes, Table A4.5*) 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 A4.6*), 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 4.2*; *Annexes, Table A4.3*) showed different spatio-temporal patterns throughout the estuary and were affected by the cessation of wastewater discharges in diverse ways.

In accordance with the similarity of the hydrometeorological conditions between the studied years, estuarine water salinity and temperature did not vary significantly between 2020 and 2022 in any of the sampling stations neither and showed similar spatio-temporal patterns (*Table 4.2*). 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 ºC in 2020 and 21 ºC in 2022, increasing from spring towards the summer season and registering the highest values in June (25 ºC) in 2020 and July (24.7 ºC) in 2022.

Table 4.2. *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 $\rho < 0.05$ and ** for $\rho < 0.01$.*

		Salinity (PSU)	Temperature (ºC)	pH	Dissolved oxygen (mg L^{-1})	Turbidity (NTU)	Ammonium (µmol L^{-1})	Phosphate (µmol L^{-1})	Nitrate (µmol L^{-1})	Silicate (µmol L^{-1})
URD1	**2020**	33.6 (28.07–35.05)	22.38 (15.15–24.59)	**8.88**** (7.85–9.77)	7.37 (6.71–8.69)	1.30 (0.48–4.63)	**4.65**** (3.5–7.85)	**0.35**** (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)	**7.94**** (7.57–8.06)	7.39 (6.94–9.45)	1.25 (0.61–2.74)	**2.15**** (0.8–3.4)	**0.20**** (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)	**8.55**** (7.85–9.33)	7.11 (6.79–9.15)	3.34 (1.15–7.71)	**13.10**** (5.95–26.3)	**0.50*** (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)	**7.93**** (7.85–8.02)	6.99 (6.22–8.82)	2.91 (1.73–8.41)	**4.35**** (1.6–8.25)	**0.45*** (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)	**8.44**** (7.83–9.01)	7.04 (6.04–9.76)	3.63 (2.54–9.27)	**24.05**** (13.8–56.1)	**0.95**** (0.35–1.75)	14.00 (0.65–5.6)	35.55 (13.75–84.1)
	2022	25.5 (8.82–34.2)	21.07 (13.55–24.62)	**7.88**** (7.74–8.05)	6.70 (5.97–9.29)	3.59 (1.81–8.38)	**7.00**** (0.8–14.75)	**0.60**** (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)	**8.38**** (7.7–8.88)	7.02 (5.21–10.75)	4.58 (2.26–31.03)	**57.45**** (21.5–112.9)	**1.65**** (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)	**7.79**** (7.61–8.13)	6.34 (5.52–9.87)	4.57 (3.39–9.25)	**8.65**** (1.6–20.75)	**0.70**** (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)	**8.37**** (8.03–9.03)	7.13 (5.61–10.28)	6.22 (3.91–16.33)	**64.10**** (30.3–166.2)	**2.25*** (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)	**7.79**** (7.62–8.45)	7.00 (4.43–12.03)	8.33 (5.46–12.79)	**9.10**** (0.8–24)	**1.15*** (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)	**8.38**** (7.73–9)	7.13 (5.72–10.16)	7.92 (5.62–23.03)	**153.35**** (65.65–334.45)	**5.80**** (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)	**7.74**** (7.6–8.37)	6.59 (5.21–10.24)	8.48 (5.28–11.36)	**10.70**** (0.8–24.2)	**1.30**** (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)	**8.43**** (7.77–9.88)	8.38 (6.52–11.04)	3.01 (1–29.1)	4.35 (0.8–6.8)	**0.4*** (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)	**7.93**** (7.76–8.26)	7.45 (6.37–10.35)	2.14 (1.07–4.33)	3.3 (0.8–5.6)	**0.75*** (0.35–1.15)	57.35 (36.4–79.7)	158.5 (35.9–188.35)

The pH, which showed a spatial gradient throughout the estuary with a slight decrease towards the inner area in both 2020 and 2022, registered significant differences after the sewerage works (*Table 4.2*). The Mann–Whitney U test determined that the median pH values were significantly lower ($\rho < 0.01$) in every sampling station during 2022, with a mean decrease of 0.66, which was more notable in the summer season (*Figure 4.2*). In addition, this significant decrease in the pH values was also detected in the Oka River. Regarding DO, no clear spatial pattern was defined throughout the estuary and the differences between 2020 and 2022 were not significant. However, the median DO concentration was lower in 2022 in most of the estuary (URD2–URD6), decreasing, on average, 0.36 mg L^{-1} in each station and being especially noticeable in URD 4 (from 7.02 mg L^{-1} in 2020 to 6.34 mg L^{-1} in 2022, a 10% decline). Estuarine turbidity varied spatially throughout the Urdaibai estuary in 2020 and 2022, with a marked increasing gradient towards the inner area, recording median values ranging between 1.3 NTU (URD1) and 7.92 NTU (URD6) in 2020 and between 1.25 NTU (URD1) and 8.48 NTU (URD6) in 2022. However, as for DO, the small changes detected between 2020 and 2022 were not considered significant (*Annexes, Table A4.8*). 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 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 4.2*).

Ammonium and phosphate concentrations, the nutrients with anthropogenic origins, showed significant decreases from 2020 to 2022 in every sampling station throughout the Urdaibai estuary according to the Mann–Whitney U test (*Table 4.2* and *Figure 4.2*). The decrease in ammonium was the most noticeable, ($p < 0.01$), recording median concentrations three times lower from URD1 to URD3, seven times lower in URD4 and URD5, and fourteen times lower in URD6. In the case of phosphate concentration, 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 µmol L^{-1}) and phosphate (2.5 µmol L^{-1}) in 2022 were lower or similar to the minimum in 2020 (65.6 µmol L^{-1} and 2.4 µmol 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 4.2*; *Annexes, Table A4.8*). Both median nitrate and silicate concentrations were generally lower throughout the estuary in 2022, but these changes were not considered significant because the difference was slight and there were also several months with higher nitrate and/or silicate concentrations in 2022 than in 2020.

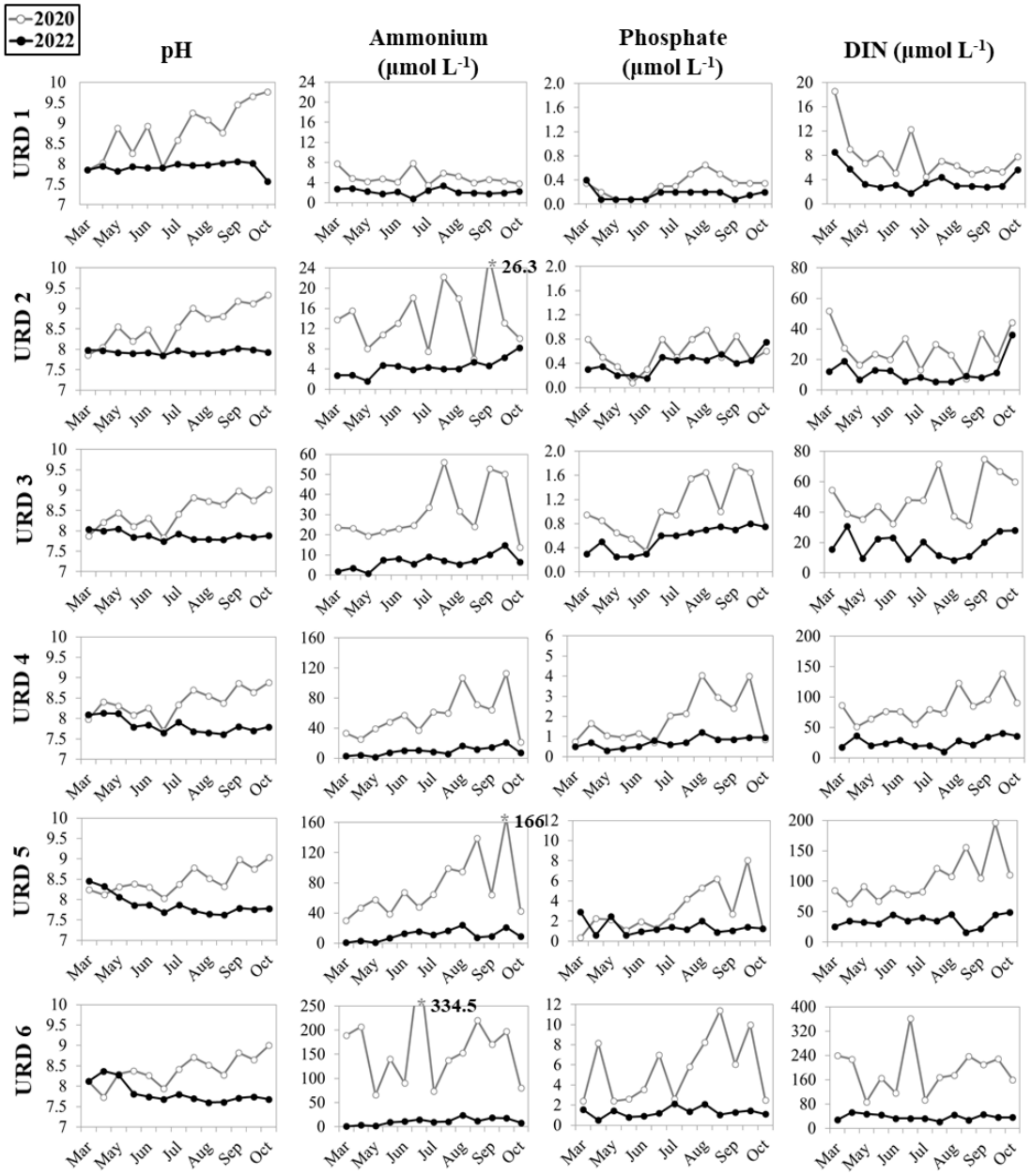

Figure 4.2. *Spatial and temporal variability of the physicochemical variables showing significant differences throughout the Urdaibai estuary between 2020 and 2022.*

These changes in nutrient concentrations registered between 2020 and 2022 led to variations in the nutrient ratios (DIN:P, DIN:Si and Si:P) of Urdaibai estuary (*Figure 4.3*). On the one hand, the composition of DIN varied between years (*Annexes, Table A4.9*), 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 not so marked in the outer estuary, where ammonium was 77.8% of the DIN in 2020 and the 66% in 2022 but was noticeable in the innermost estuary (URD6), were the contribution of ammonium to DIN changed from 82% in 2020 to 33% in 2022 (nitrate being 61%). Nitrite median contribution to DIN was below 7% in both years. 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, in every sampling station throughout the estuary, according to the Mann–Whitney U test (*Figure 4.2*; *Annexes, Table A4.8*).

Regarding the effect of this nutrient concentration change on the ratios (*Figure 4.3*; *Annexes, Table A4.10*), the 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, which was particularly marked in URD6 (from 2.4 to 0.5). 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. In the outer Urdaibai estuary, the DIN:P ratio remained stable (22:1), DIN:Si decreased from 4:1 (2020) to 2.5:1 (2022), and Si:P increased from 6:1 to 10:1. From URD2 to URD5, DIN:P ratios decreased from approximately 45:1 (2020) to 30:1 (2022) and DIN:Si from 1.6:1 (2020) to 1:2 (2022), and Si:P increased from 30:1 (2020) to 45:1 (2022). In the surroundings of the WWTP (URD6), DIN:P ratios remained stable with average values of 35:1 (2020) and 28:1 (2022), but DIN/Si decreased from 2.5:1 (2020) to 1:2 (2022) and Si:P increased from 13:1 (2020) to 60:1 (2022).

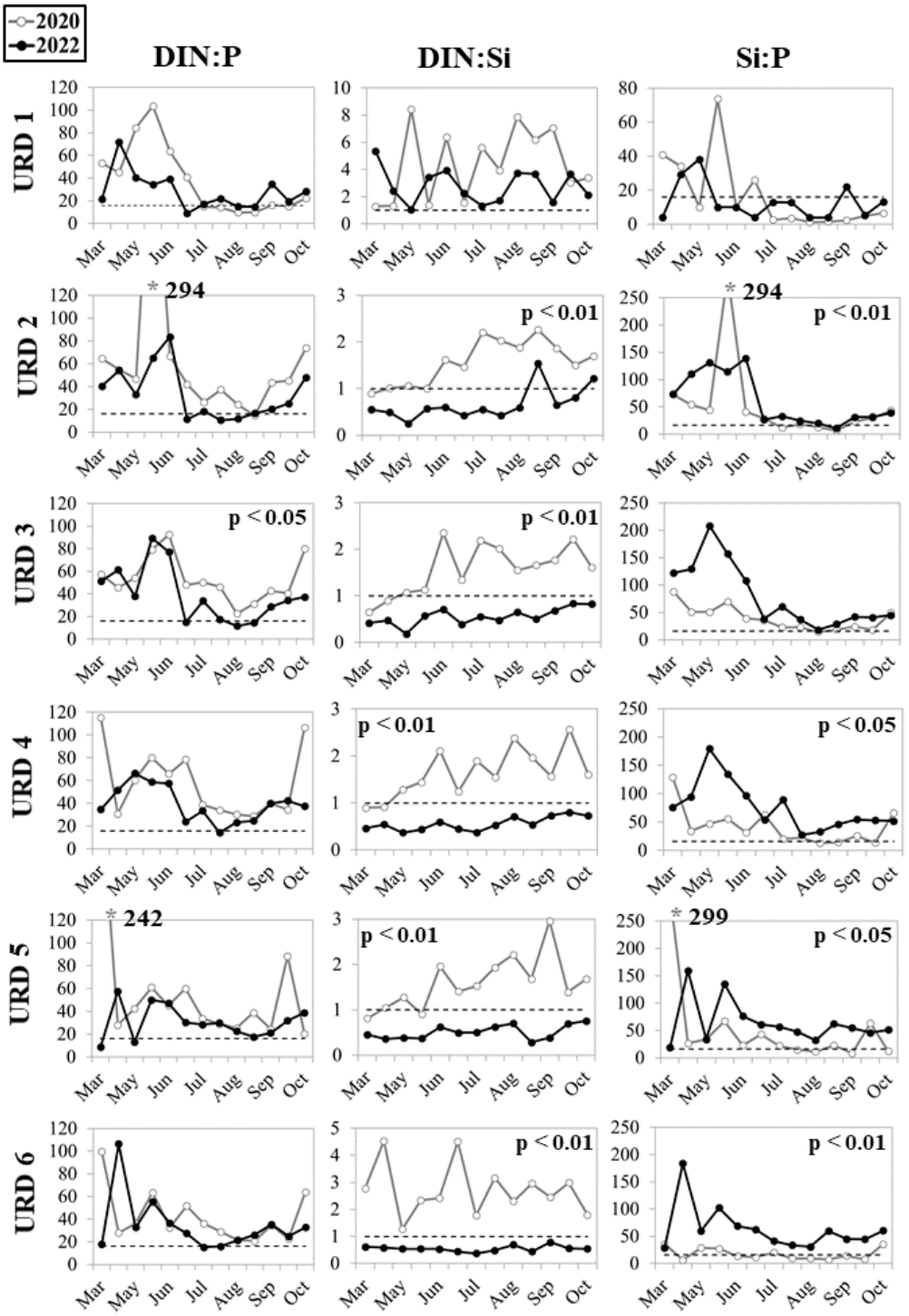

Figure 4.3. *Spatial and temporal variability of the nutrient molar ratios showing significant differences throughout the Urdaibai estuary between 2020 and 2022. Horizontal dashed lines indicate the Redfield ratio (Redfield, 1958). The significance of the change between 2020 and 2022 for each sampling station according to the Mann–Whitney U test is indicated in each graph: p < 0.05 and p < 0.01.*

As a general overview, and taking all the studied physicochemical variables into account (*Table 4.2*), 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 A4.7*). Additionally, the interaction of both factors (spatial and inter-annual) also explained the physicochemical condition varied significantly in both seasons. Thus, and although some parameters (e.g., salinity and temperature) 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 pH and nutrient concentrations. The non–metric multidimensional scaling (nMDS), containing the variables in *Table 4.2*, illustrates the variation in the physicochemical conditions throughout the sampling stations and between years, for both spring and summer seasons independently (*Figure 4.4*). 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).

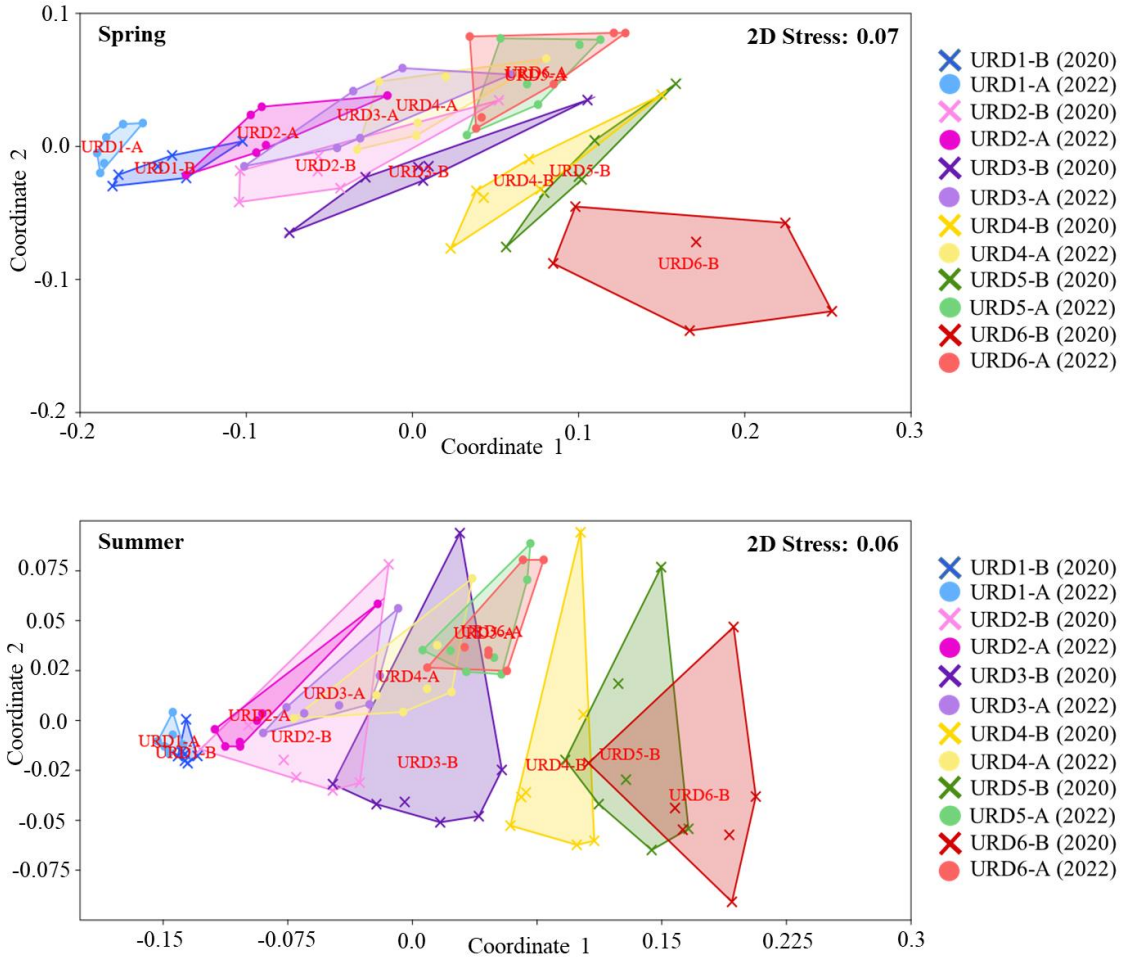

Figure 4.4. *Non–metric multidimensional scaling (nMDS) of physicochemical conditions in the Urdaibai estuary using Bray–Curtis distances. Data are shown separately for spring (upper) and summer (lower) seasons, and contain 9 physicochemical variables (i.e., salinity, temperature, pH, DO, turbidity, ammonium, phosphate, nitrate, and silicate). B: before wastewater diversion (2020); A: after wastewater diversion (2022).*

3.3. Phytoplankton biomass and blooms

Total phytoplankton biomass was estimated with Chl *a* concentration (*Table 4.3*; *Annexes, Table A4.4*). In both 2020 and 2022, a general increase of biomass was observed towards the inner estuary, recording the lowest median biomass values in URD1 (1.61 μg L^{-1} in 2020 and 1.04 μg L^{-1} in 2022). However, the biomass maxima area varied between years. In 2020, the highest median biomass (9.94 μg L^{-1}) was registered in URD5 and the Chl *a* maxima (54.61 μg L^{-1}) in URD4. In contrast, in 2022 the longitudinal biomass gradient was more marked, reaching the highest median values (16.8 μg L^{-1}) in URD6 and decreasing gradually until URD1. The Chl *a* maxima of 2022 was registered in URD 5 (251 μg L^{-1}), but was also noticeable in URD6 (151 μg L^{-1}).

Together with the aforementioned differences in the distribution of the phytoplankton biomass throughout the estuary, the Mann–Whitney U test confirmed several significant Chl *a* concentration changes between 2020 and 2022 (*Table 4.3*). The outer and middle estuary recorded significantly ($\rho < 0.05$) lower biomass values in 2022 than in 2020, this decrease being especially notable in URD1 in spring (from 1.48 μg L^{-1} in 2020 to 0.8 μg L^{-1} in 2022) and in URD2 in summer (from 5.08 μg L^{-1} in 2020 to 3.04 μg L^{-1} in 2022). 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 μg L^{-1}) than in 2020 (7.57 μg L^{-1}) in 2020, which should be taken into account.

Table 4.3. *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 $\rho < 0.05$ and ** for $\rho < 0.01$.*

		March–October (μg L^{-1})	Spring (μg L^{-1})	Summer (μg 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)

Regarding high biomass situations and phytoplankton blooms, several differences were detected between 2020 and 2022 (*Table 4.4* and *Table 4.5*). Chl *a* values above 20 μg L^{-1} indicating hypertrophic status of estuarine waters were registered several times in the Urdaibai estuary, always in the inner area (from URD3 to URD6) and more frequently in 2022 than in 2020 (*Table 4.4*). During 2020, there was just one occasion (April) in which hypereutrophic conditions were registered, from URD3 to URD6, reaching Chl *a* concentrations up to 54.6 μg L^{-1} due to blooms of cryptophytes like *Urgorri complanatus* or *Teleaulax* spp. However, in 2022, Chl *a* concentration above 20 μg L^{-1} were found in 38% of the samplings (5 out of 13), always registering this hypereutrophic status in the inner estuarine area (mostly URD5 and URD6). In March and May 2022, the main cause of the high biomass was the combined blooms of *U. complanatus* and/or the dinoflagellate *Kryptoperidinium foliaceum*, reaching a Chl *a* maximum of 251.5 μg L^{-1}, and with maximum cell abundances of 5.39 x 10^7 cells L^{-1} of *U. complanatus* and 1.08 x 10^7 cells L^{-1} of *K. foliaceum*. In June 2022, green algae (*Tetraselmis* sp. and *Eutreptiella* sp.) combined with *K. foliaceum* were the main causes of the hypereutrophication of the estuary and, in July, *Apedinella radians* (1.69 x 10^7 cells L^{-1}) and small centric diatoms (4 x 10^6 cells L^{-1}). In late summer, *Teleaulax acuta* was responsible of the high phytoplankton biomass in the inner estuary, with cell abundances around 9 x 10^6 cells L^{-1}. Thus, when comparing the years, 2022 showed more frequent hypereutrophication situations and the biomass maxima recorded were also higher.

Table 4.4. *Hypertrophic status of the water masses (Chl a > 20 μg L^{-1}) throughout the Urdaibai estuary in 2020 and 2022.*

Year	Date	Sampling point	Chl *a* (μg L^{-1})	Dominant taxa	Cell abundance (cells L^{-1})
2020	17th April	URD3	25.2	*Teleaulax* spp.	5.95 x 10^6
		URD4	54.6	*Urgorri complanatus*	7.61 x 10^6
		URD5	42.5	*Urgorri complanatus*	7.73 x 10^6
		URD6	45.8	*Urgorri complanatus*	6.35 x 10^6
2022	23rd March	URD4	41.1	*Teleaulax gracilis*	3.07 x 10^7
		URD5	161.3	*Kryptoperidinium foliaceum*	1.08 x 10^7
				Urgorri complanatus	8.75 x 10^6
		URD6	73.4	*Urgorri complanatus*	5.52 x 10^6
				Kryptoperidinium foliaceum	2.24 x 10^6
	6th May	URD5	251.5	*Urgorri complanatus*	5.39 x 10^7
		URD6	151.6	*Urgorri complanatus*	3.74 x 10^7
	6th June	URD5	23.2	*Tetraselmis* sp.	3.40 x 10^5
				Eutreptiella sp.	9.35 x 10^4
		URD6	41.8	*Eutreptiella* sp.	2.80 x 10^6
				Kryptoperidinium foliaceum	7.52 x 10^5
	20th July	URD6	33.6	*Apedinella radians*	1.69 x 10^7
				Centric diatoms 10–20 μm	4.06 x 10^6
	21st September	URD5	31.4	*Teleaulax acuta*	9.01 x 10^6
		URD6	26.2	*Teleaulax acuta*	7.29 x 10^6

Additionally, and although the Chl *a* values recorded were not as high as for indicating hypereutrophic status of the water masses, several additional remarkable phytoplankton blooms (taxa above 7.5 x 10^5 cells L^{-1}) were recorded in the outer and middle Urdaibai estuary in 2020 and 2022 (*Table 4.5*). 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 x 10^7 cells L^{-1} and 5.37 x 10^6 cells L^{-1} 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 x 10^7 cells L^{-1} and 1.58 x 10^7 cells L^{-1} 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. Apart from the mentioned taxa, other organisms such as *Pyramimonas* sp. and *Teleaulax gracile* also formed blooms in the outer and middle Urdaibai estuary in 2020 and 2022 but were not so common. Cell abundances of 7.5 x 10^5 cells L^{-1} 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.

Table 4.5. (Part 1) *Phytoplankton blooms (>7.5 x 10^5 cells L^{-1}) in the outer and middle Urdaibai estuary.*

Year	Date	Sampling point	Chl *a* (µg L^{-1})	Bloom forming taxa	Cell abundance (cells L^{-1})
2020	17th April	URD1	2.04	*Teleaulax gracilis*	8.28 x 10^5
		URD2	10.89	*Plagioselmis* spp.	2.25 x 10^6
				Teleaulax gracilis	5.95 x 10^6
	4th May	URD2	2.77	*Pyramimonas* sp.	1.36 x 10^6
	18th May	URD1	1.35	*Pyramimonas* sp.	1.08 x 10^6
		URD2	5.65	*Plagioselmis* spp.	8.84 x 10^5
				Pyramimonas sp.	2.65 x 10^6
	1st June	URD1	1.68	*Plagioselmis* spp.	9.13 x 10^5
				Minutocellus polymorphus	2.15 x 10^6
		URD2	5.45	*Plagioselmis* spp.	2.29 x 10^6
				Minutocellus polymorphus	6.03 x 10^6
	16th June	URD2	2.96	*Plagioselmis* spp.	1.15 x 10^6
	30th June	URD1	2.23	*Chaetoceros tenuissimus*	5.71 x 10^6
		URD2	10.75	*Chaetoceros tenuissimus*	2.05 x 10^7
				Plagioselmis spp.	2.63 x 10^6

Table 4.5. (Continued).

Year	Date	Sampling point	Chl *a* (µg L^{-1})	Bloom forming taxa	Cell abundance (cells L^{-1})
2020	17th July	URD1	2.97	*Plagioselmis* spp.	1.03 x 10^6
				Minutocellus polymorphus	5.42 x 10^6
		URD2	4.2	*Chaetoceros tenuissimus*	8.29 x 10^5
				Plagioselmis spp.	2.44 x 10^6
				Minutocellus polymorphus	3.4 x 10^6
				Tetraselmis sp.	8.07 x 10^5
	29th July	URD1	2.84	*Chaetoceros tenuissimus*	1.87 x 10^6
				Minutocellus polymorphus	9.14 x 10^6
		URD2	6.49	*Chaetoceros tenuissimus*	3.12 x 10^6
				Plagioselmis spp.	1.55 x 10^6
				Minutocellus polymorphus	1.37 x 10^7
	17th August	URD1	1.18	*Minutocellus polymorphus*	2.04 x 10^6
		URD2	5.08	*Chaetoceros tenuissimus*	8.92 x 10^5
				Plagioselmis spp.	1.43 x 10^6
				Minutocellus polymorphus	1.38 x 10^7
	31st August	URD1	1.87	*Minutocellus polymorphus*	6.01 x 10^6
		URD2	5.57	*Chaetoceros tenuissimus*	2.65 x 10^6
				Plagioselmis spp.	1.06 x 10^6
				Minutocellus polymorphus	1.29 x 10^7
	14th September	URD1	1.5	*Minutocellus polymorphus*	4.67 x 10^6
		URD2	5.04	*Plagioselmis* spp.	1.91 x 10^6
				Minutocellus polymorphus	1.47 x 10^7
				Pyramimonas sp.	1.28 x 10^6
	29th September	URD1	1.14	*Minutocellus polymorphus*	1.39 x 10^6
		URD2	3.88	*Minutocellus polymorphus*	9.6 x 10^5
2022	25th March	URD2	3.27	*Teleaulax gracilis*	3.34 x 10^6
	6th May	URD2	4.15	*Plagioselmis* spp.	3.11 x 10^7
	23rd May	URD2	3.02	*Plagioselmis* spp.	5.31 x 10^6
	6th June	URD2	5.4	*Plagioselmis* spp.	8.96 x 10^6
	20th June	URD2	2.25	*Plagioselmis* spp.	5.93 x 10^6
	6th July	URD1	2.14	*Chaetoceros tenuissimus*	3.36 x 10^6
		URD2	6.96	*Chaetoceros tenuissimus*	1.58 x 10^7
	20th July	URD2	5.02	*Chaetoceros tenuissimus*	5.93 x 10^6
				Minutocellus polymorphus	1.38 x 10^6
	3rd August	URD1	0.83	*Minutocellus polymorphus*	9.6 x 10^5
		URD2	3.04	*Chaetoceros tenuissimus*	3.99 x 10^6
				Minutocellus polymorphus	5.37 x 10^6
	18th August	URD1	1.04	*Minutocellus polymorphus*	1.05 x 10^6
		URD2	2.61	*Chaetoceros tenuissimus*	3.02 x 10^6
				Minutocellus polymorphus	3.63 x 10^6
	5th September	URD1	1.22	*Minutocellus polymorphus*	1.91 x 10^6
		URD2	3.33	*Chaetoceros tenuissimus*	1.74 x 10^6
				Minutocellus polymorphus	2.38 x 10^6
	21st September	URD1	1.27	*Minutocellus polymorphus*	8.84 x 10^5
		URD2	2.65	*Teleaulax gracilis*	1.89 x 10^6
	4th October	URD2	1.88	*Teleaulax gracilis*	3.89 x 10^6

Moreover, toxic phytoplankton blooms were recorded several times in 2020 and 2022, always in URD1 and/or URD2, registering abundances above the threshold values established by Swan and Davidson (2012) for both *Dinophysis* spp. and *Pseudonitzschia* spp. (*Annexes,*

Table A4.11). *Dinophysis* spp. abundances above the threshold (100 cells L^{-1}) were found four times in 2020 and once in 2022, and in both years, the maximum abundance was 1,200 cells L^{-1} and was recorded in May. *Pseudonitzschia* spp. abundance threshold (5 x 10^4 cells L^{-1}) was exceeded twice in 2020, with a maximum of 4.4 x 10^5 cells L^{-1} in April, and once in 2022 (1.2 x 10^5 cells L^{-1} in July).

3.4. Phytoplankton community composition

Phytoplankton community composition was described with diagnostic pigments, determining the contribution percentage of each pigment-group to the total phytoplankton biomass (Chl *a*), with additional information of the dominant taxa of these groups given by microscopy analysis. The community composition study revealed that there were several similarities and dissimilarities between the phytoplankton communities of the Urdaibai estuary in 2020 and 2022 (*Figure 4.5* and *Table 4.6*).

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. The FCA group in the outer and middle estuary was mostly formed by diatoms, especially by taxa such as *Chaetoceros*, *Minutocellus polymorphus*, *Cylindrotheca costerium*, *Leptocylindrus convexus*, and *Pseudonitzschia*. 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 A4.15*). 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 during the study period increased, registering 54% in URD4, where it dominated mostly in spring (81%), but also in summer (40%). This ACA group of the inner estuary was mainly formed by cryptophytes like *Plagioselmis*, *Hemiselmis*, *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 the surroundings of the estuary, 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, increasing from 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*.

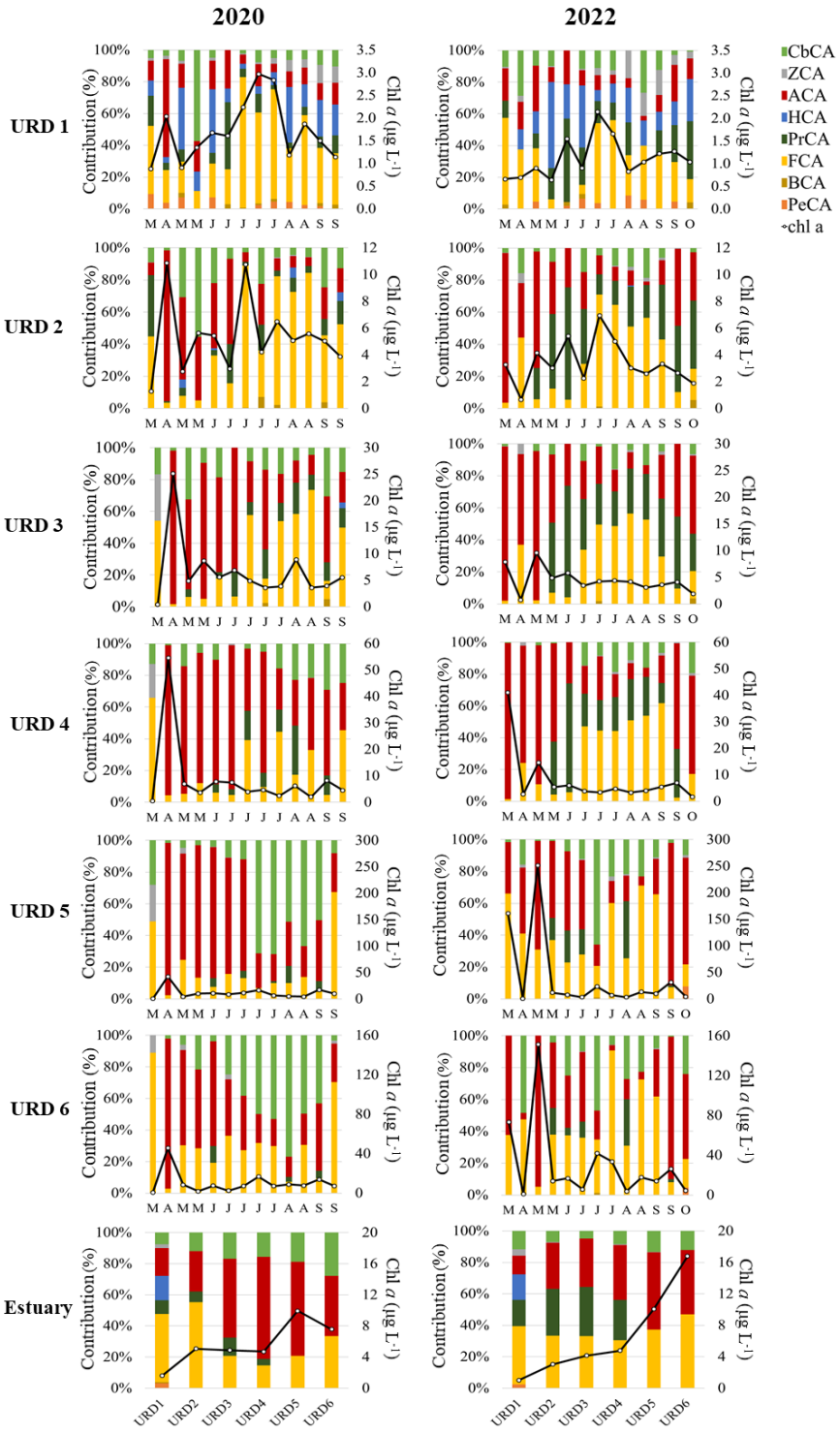

Figure 4.5. *Spatial and temporal variability of the contribution percentage of the different groups of the phytoplankton community to the total Chl a througout the Urdaibai estuary in 2020 and 2022, by sampling stations and the annual median contribution to the whole estuary. PeCA: Peridinin Containing Algae; BCA: 19'–butanoyloxyfucoxanthin Containing Algae; FCA: Fucoxanthin Containing Algae; PrCA: Prasinoxanthin Containing Algae; HCA: 19'–hexanoyloxyfucoxanthin Containing Algae; ACA: Alloxanthin Containing Algae; ZCA: Zeaxanthin Containing Algae; CbCA: Chl b Containing Algae.*

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 *Pseudonitzschia* spp. PrCA mostly corresponded to small unidentified prasinophytes, and HCA to haptophyte taxa like Prymnesiales (e.g., *Chrysochromulina*) or cocolithophorids (e.g., *Gephyrocapsa*). 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*. FCA and ACA were also the groups with the highest median contribution to the phytoplankton biomass of the innermost area of the estuary (URD5 and URD6), 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 *Cyclotella*. CbCA contained mainly chlorophytes and euglenophytes.

The spatial and inter-annual variability of the phytoplankton community composition described in the Urdaibai estuary with pigment groups was tested by a two-way (spatial and inter-annual) PERMANOVA analysis (*Annexes, Table A4.12*). The results 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. 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 (spatial and inter-annual) did not explain the community composition variability significantly in any season. The non-metric multidimensional scaling (nMDS), containing the contributions of the different pigment-groups, illustrates the variation in the community composition among the sampling stations and between years, for the spring and summer seasons independently (*Figure 4.6*). As can be seen, the community of URD1 is clearly

differentiated from the rest of the estuary (spatial variability), while the distances between the communities in 2020 (B) and 2022 (A) of the same sampling station (inter-annual variability) are higher in summer (e.g., distances between URD6 B and URD6 A).

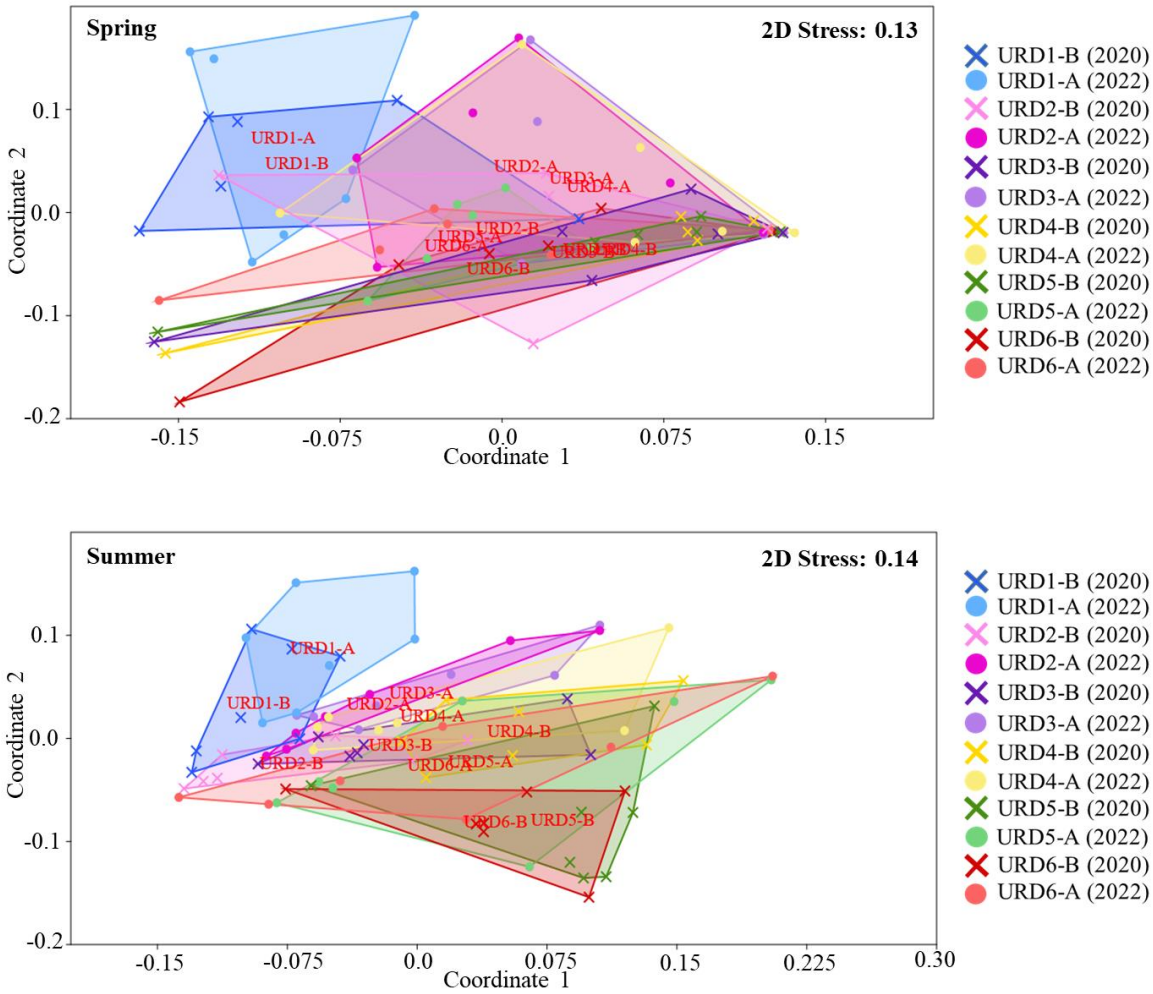

Figure 4.6. *Non-metric multidimensional scaling (nMDS) of phytoplankton community composition in the Urdaibai estuary using Bray–Curtis distances. Data are shown separately for spring (upper) and summer (lower) seasons. B: before wastewater diversion (2020); A: after wastewater diversion (2022).*

Although the overall differences in the phytoplankton community composition between 2020 and 2022 do not seem to be noticeable, 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 4.6*; *Annexes, Table A4.13 and Table A4.14*). 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 comparison to 2020, 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, the contribution of other groups to the total biomass also varied, such

as the FCA, which showed higher median values in 2022 in URD3, URD4, and, especially, in URD 5 (from 13% to 31%, $\rho < 0.01$). On the contrary, ACA and CbCA became less dominant in 2022, probably due to the increase of PrCA and FCA. 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 the median contributions of 2022 in URD3 (from 13.8% to 4.4%, $\rho < 0.05$), but also in URD4 and URD 6.

Table 4.6. *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 $\rho < 0.05$ and ** for $\rho < 0.01$. PeCA: Peridinin Containing Algae; BCA: 19'–butanoyloxyfucoxanthin Containing Algae; FCA: Fucoxanthin Containing Algae; PrCA: Prasinoxanthin Containing Algae; HCA: 19'–hexanoyloxyfucoxanthin Containing Algae; ACA: Alloxanthin Containing Algae; ZCA: Zeaxanthin Containing Algae; CbCA: Chl b Containing Algae*.

		Contribution to Chl *a* (%)							
		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 0–29.1)	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)

4. Discussion

Estuaries are dynamic ecosystems where significant spatio-temporal fluctuations occur across various environmental gradients, mostly linked to the mixing of seawater and freshwater

during tidal cycles (Cloern et al., 2017). However, in some estuaries, like the Urdaibai estuary, the 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.

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 previously described by Valencia et al. (2004) for Basque coastal waters, and higher freshwater discharges to the estuary occurred in spring compared to summer, as expected for the Cantabrian coast (Revilla et al., 2009; Prego et al., 2008). Studies focusing on coastal marine systems of western Europe, where the Urdaibai estuary is located, 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). Thus, the lack of differences in the climatic conditions might explain the lack of significant differences in the water temperature and salinity of the Urdaibai estuary in any of the sampling stations between 2020 and 2022, as these two physicochemical parameters show variability due to natural cycles. More importantly, this also confirms 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), since climatic conditions did not vary between years.

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). Estuarine systems are commonly perceived as being well

buffered, so pH is frequently assumed to be of little significance (Ringwood and Keppler, 2002). However, pH levels can decrease or increase in estuarine systems to levels that can adversely affect biological responses by being toxic to aquatic life and altering the solubility of chemical pollutants and essential elements in water systems (Saalidong et al., 2022). Changes in waters' pH is induced by the minerals dissolved in the water, atmospheric deposition of aerosols and dust, wastewater discharges of anthropogenic origin, and by the photosynthesis and respiration of the inhabiting organisms (EPA, 2006). In this context, pH values of the Urdaibai estuary were significantly lower in 2022 in every sampling station. Although the pH of the WWTPs effluents vary according to level of treatment and type of households and/or industries discharging into the system, overall, it 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 alkalinisation 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, ocean acidification (significantly lower pH registered in URD1) and the significant pH decrease found in 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). Due to this decrease in pH, every value registered in 2022 in Urdaibai estuary was included in the preferred pH conditions (6.5–8.5) of estuarine organisms (EPA, 2006); while in 2020, values above 9 were registered in the estuary during the summer, which might be problematic for the survival of many species (tolerance limit 6–9 pH units) (Chapman, 1996; EPA, 2006). Nevertheless, pH is a key variable to control the bioavailability and the toxicity of heavy metals bound to sediments in estuaries and acidification usually provokes an increase of the mobility of metals (Riba et al., 2003). Thus, this pH decrease should be controlled in future years because, if the pH levels continue decreasing, toxic metals in the estuarine sediment can be resuspended in the water column and have negative impacts on many aquatic species.

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., 1997; Revilla et al., 2000; Iriarte et al., 2015) 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). 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 (*Chapter 2*), 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. In fact, 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 nutrient enrichment caused by WWTP effluents and wastewater is also a threat to the ecological health of many estuaries around the world, (e.g., Dutto et al., 2012; Silva et al., 2013; Adams et al., 2020; Ke et al., 2022). However, many studies (e.g., Mallin et al., 2005; Ho et al., 2008; Liu et al., 2022) 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.

While P is the main concern for causing eutrophication in freshwater systems, estuaries and coastal waters are more threatened by excess N (Howarth and Marino, 2006) and, thus, the greater decrease of ammonium (and consequently DIN) registered in the Urdaibai estuary in comparison to phosphate should be beneficial for the ecosystem's health. However, 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), overall, 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 becoming potential limiting nutrients at the different estuarine sampling points (Redfield, 1958; Justic et al., 1995). The outer Urdaibai estuary was Si-deficient in 2020 and 2022, and from URD2 to UR5, the estuarine waters were P-deficient in both years, 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 in the area. Changes in nutrient ratios and limitations caused by anthropogenic activities similar to those in the Urdaibai estuary 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 generated from

agricultural activities, precipitation, and wastewater inputs (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, since nutrient ratios are known to strongly affect phytoplankton community structures (Lie et al., 2011; Wu et al., 2017a).

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., Madariaga, 1995; Iriarte et al., 1997; Orive et al., 1998; Trigueros and Orive, 2001; Ansotegui et al., 2003). 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).

Several remarkable differences were found in the phytoplankton biomass and its dynamics in the Urdaibai estuary after sewerage works, such as a biomass decrease in the outer and middle estuary, the biomass increase in the innermost area (URD6), and differences in the spatial distribution of the biomass throughout the estuary. In the outer and middle Urdaibai estuary, the Chl *a* concentration was significantly lower after the cessation of wastewater discharges, 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 the outer and middle area from inner estuary, due to the cessation of high nutrient loadings after the sewerage works and to the dilution effect caused by tidal incursion in the area (Villate et al., 2017). Slight biomass decreases were preliminarily detected in URD1 and URD2 immediately after the cessation of wastewater discharges (*Chapter 2*), 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 x 10^5 cells L^{-1}; Revilla et al., 2009). In 2020 and 2022, diatoms (*M. polymorphus* and *C. tenuissimus*) and cryptophytes (*Plagioselmis* spp.) were the main blooming taxa in URD1 and URD2, as described for most of the estuaries and several coastal sites in the SE Bay of Biscay (Seoane et al., 2012). However, in 2020, diatoms and cryptophytes were able to bloom together, exceeding bloom thresholds 40 times from March to October, while in 2022, it decreased to 21, because there was just one species blooming each time and if there were more (two), they corresponded to the same phytoplankton group (*Table 4.5*). This reduction in the number of simultaneous bloom-forming taxa in the outer and middle estuary 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. Moreover, the toxic phytoplankton blooms detected after sewerage works (2) were also less that during the WWTP discharges (6), which makes sense since eutrophication is known to increase the frequency and extent of HABs (Glibert et al., 2018; Paerl, 2018).

In the surroundings of the WWTP of Gernika (URD6), on the contrary, 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 μg L^{-1} that indicate hypertrophic conditions in the inner estuary (Hagy et al., 2022) were more frequently recorded after the sewerage works (*Table 4.4*), reaching a Chl *a* maximum of 251.5 μg L^{-1}, the highest ever reported in previous works in the area. The biomass increase recorded in URD6 in 2022 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 2020. It has been documented that highly elevated ammonium concentrations (exceeding several tens to hundreds of μmol 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 μmol L^{-1} (with a range of 65–334 μmol L^{-1}), the concentration might have been excessive for phytoplankton growth, while the decrease to 10 μmol L^{-1} (reaching a maximum of 24 μmol

L^{-1}) after the sewerage works enabled adequate growth, leading to the biomass increase. Indeed, several studies previously observed phytoplankton growth suppression with increasing ammonium concentrations (above 10 μmol L^{-1}), such as Yoshiyama and Sharp (2006) in the Delaware Bay and Dugdale et al. (2007) in the San Francisco Bay Delta. Additionally, stopping wastewater discharges to the estuary might have reduced, apart from ammonium, the input of particulate matter in URD6 (Carey and Migliaccio, 2009), as happened in other estuaries (Toublanc et al., 2016; Eccles et al., 2020). 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 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. Moreover, the variation of the maximum biomass area of the estuary, moving from URD5 in 2020 to URD6 in 2022 (*Figure 4.5*), was probably explained by the no longer phytoplankton growth suppression due to ammonium and the higher light availability registered in URD6 after the sewerage works.

Regarding phytoplankton community composition, several differences were registered between 2020 and 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 (e.g., from 5% to 25% in URD2) and higher presence of FCA in the inner estuary (e.g., from 13% to 31% in URD5), leading to the decrease of ACA and CbCA.

Among the Pras containing prasinophytes (PrCA taxa), which comprise the Prasinophyceae orders Mamiellales, Pseudoscourfieldiales (Pycnococcaceae), 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 *Ostreococcu*s spp. (*O. lucimarinus*, *O. mediterraneus*, and *O. tauri*) (*Chapter 1*), corresponding to Mamiellophyceae (Lopes dos Santos et al., 2017). Additionally, some of these were previously reported in the Urdaibai estuary and other nearby systems like the Nervion Estuary (Ansotegi et al., 2003; Seoane et al., 2005; Laza-Martinez et al., 2007). These, and most of the organisms belonging to PrCA, belong to the picoplanktonic fraction (cells < 3 μm in diameter) (Guillou et al., 2004) and, therefore, it is hard or impossible to identify and quantify them by microscopy due to their small size (e.g., Ansotegui et al., 2003). Nevertheless, in the present study, pigment analysis enabled the determination of 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. Research has shown that PrCA are typical in coastal waters (Not et al., 2004; Collado-Fabri et al., 2011) and open ocean (Treusch et al., 2012; Vaulot et al., 2012), being one of the major contributors to marine picophytoplankton (Lohrenz et al., 2003; Wu et al., 2017b). This explains their distribution in many estuarine systems, like the Pearl River Estuary (Chai et al., 2016), tropical estuaries in Indonesia (Damar et al., 2020), and several European estuaries (Lemaire et al., 2002), where the presence of PrCA (determined through Pras) is limited to the outermost estuarine areas and is generally recorded in low concentrations and frequencies. Due to their high surface:volume ratio, efficient growth rates, and enhanced nutrient uptake rates (Paerl et al., 2003), PrCA are more competitive than other phytoplankton functional groups when nutrients are not so abundant (Glibert et al., 2010), which might explain their increase from URD1 to URD4. 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 μm 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, with the increase of FCA (mainly diatoms) over the decrease of ACA (cryptophytes) and CbCA (green algae except for Pas containing prasinophytes), it 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, 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*. 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 (Sommer, 1994; Roberts et al., 2003). Indeed, a shift in phytoplankton assemblage composition has been observed in many estuarine ecosystems related to anthropogenic N and P enrichment, most of them reporting that diatoms were replaced by smaller phototrophs, such as cyanobacteria and various flagellates (Glibert et al., 2011; Dutto et al., 2012; Jiang et al., 2014; Liu et al., 2022). Thus, from a nutrient composition perspective, in Urdaibai estuary, during 2020, non-siliceous phytoplankton groups (e.g. ACA and CbCA) probably used the

excess of N and P coming from the WWTP, while the Si limited diatoms could not use it, and thus with increasing DIN:Si these non-siliceous algae gradually became more dominant and influenced the phytoplankton assemblage composition (Tréguer and De La Rocha, 2013; Jiang et al., 2014). In fact, cryptophytes (ACA) are known to dominate the inner area of many eutrophicated estuaries other than Urdaibai (Adolf et al., 2006; Valdes-Weaver et al., 2006; Santos et al., 2022), where their success over other groups like diatoms is explained, among others, by their ability to adapt to turbid environments by photoacclimation to low light intensities (Weng et al., 2009; Collini, 2022). Additionally, they respond rapidly to nutrient loads due to their high growth rate (Paerl et al., 2003), have higher advantage in ammonium-enriched waters (Horner and Thompson, 2001), and show a mixotrophic character, which allows them to utilise dissolved organic carbon for growth if necessary (Johnson et al., 2013). As for the green algae (CbCA) in the inner Urdaibai estuary in 2020, the main taxa were euglenoids like *Eutreptiella* spp. and chlorophytes, such as *Tetraselmis* spp. and several unidentified picoplanctonic coccoids (*Chapter 1*). Euglenoids are known to thrive in shallow, nutrient rich, and low light waters (Poniewozik and Juráň, 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 (*Chapter 1*), they have particularly high affinity for ammonium uptake compared to diatoms and dinophytes (Litchmann et al., 2007; Laruste et al., 2016), which was in high concentrations during the discharges and might explain the frequent presence of these non-siliceous algae. In *Chapter 2*, an immediate increase of CbCA was recorded after the sewerage works in 2021, due to the increase of *Eutreptiella* spp. However, the present study revealed that, in the mid-term, there has been an overall decrease of the contribution to total biomass of this group in the inner estuary (e.g. from 25% to 5% in URD6), together with the decrease of cryptophytes (e.g., from 54% to 27% in URD4), being replaced by a higher presence FCA (*Figure 4.5*).

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 (form 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*. The increase of diatom contribution to total phytoplankton biomass can be easily explained by the cessation of wastewater discharges to the Urdaibai estuary and the consequent change

in nutrient loadings. In general, diatoms show high maximum nutrient uptake rates and high growth rates, being favoured under high or fluctuating nutrients (Litchman et al., 2007; Laruste et al., 2016), which explains their presence in 2020 and 2022. However, 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., 1997), 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). In the present study, during WWTP discharges, ammonium was the main component of DIN throughout the entire estuary, but this changed after the sewerage works, when ammonium concentrations decreased and nitrate became the main component of DIN in the inner area (at least 50%, and 65% in URD5 and URD6). 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 (Dominges 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.

The mid-term changes recorded in nutrient concentrations and phytoplankton community after sewerage works in the Urdaibai estuary might have resulted in a change of the ecological status of the estuarine waters as well. In coastal and transitional waters of Basque Country, the water quality and ecological status assessment criteria are stablished by Royal Decree 817/2015, as part of the Eastern Cantabrian Hydrographic Demarcation. Previous studies showed that ammonium, phosphate, and phytoplankton (Chl *a*) have registered "deficient" or "bad" statuses in the inner estuary from at least 2008 and until 2020 (Borja et al., 2021). In the present study (*Annexes*, *Table A4.16* and *Table A4.17*), in accordance with it, ammonium

concentrations were below the "moderate" status from URD3 to URD6 in 2020, registering higher concentrations than the reference "very bad status" in the innermost station. Phosphate registered a "good" status throughout the estuary except in the innermost area, where concentrations were also higher than the reference "very bad status". In 2022, however, due to the significant differences registered in nutrient concentrations, "very good" status was achieved for ammonium and phosphate concentrations throughout the estuary. As for phytoplankton, in 2020 there were "good" or "moderate" statuses in the outer and middle estuary, but "deficient" in URD5 and URD6. In 2022, the decrease of biomass in the outer estuary led to "very good" and "good" statuses in URD1 and URD2, respectively, but the increase in the inner estuary caused "bad" statuses in URD5 and URD6. Therefore, an improvement was registered after the sewerage works in the inner Urdaibai estuarine water status for nutrient concentrations, confirming what *Chapter 2* anticipated, but not for phytoplankton.

These results are a clear example that although phytoplankton biomass (Chl *a*) is commonly employed (e.g., WFD or MSFD) as an indicator of eutrophication (Bricker et al., 1999) because it provides consistent insights on a stressed area, it should be monitored with phytoplankton assemblage changes (Niveditha et al., 2022). If the mid-term effect of the sewerage works in the Urdaibai estuary on phytoplankton had been evaluated just based on biomass, the results would have been negative due to the Chl *a* increase in the inner estuary, although this increase was the result of a community composition shift that might indicate recovery. The study of phytoplankton assemblages by microscopy might be too time-consuming (Wang et al., 2018), dependent on personal taxonomic skills, and difficult to intercalibrate (Muñiz et al., 2020) to be applied in international monitoring programs with intensive samplings together with Chl *a*. Nevertheless, pigment analysis was proven efficient for studying phytoplankton community composition (e.g., Chai et al., 2016; Damar et al., 2020). Indeed, in the present study, the use of diagnostic pigments through *PIGMENTUM* was found to be very useful for estimating the impacts of nutrient loading changes on the phytoplankton community composition of the Urdaibai estuary, including groups containing small and fragile cells underestimated by other procedures (Jeffrey et al.,1997) and based on a larger volume analysed (Agirbas et al., 2015). This provided insights into the potential ecological implications that this sewerage works could have, which would not have been possible with Chl *a* concentrations only, since alterations in phytoplankton community structure may have cascading effects on higher trophic levels (Baum and Worm, 2009). Thus, as stated by several previous authors (Ferreira et al., 2011; Van Meerssche and Pinckney,

2019), water quality criteria based on phytoplankton abundance should also include consideration of community composition.

5. Conclusion

Overall, the cessation of wastewater discharges coming from Gernikas' WWTP to the Urdaibai estuary had significant medium-term effects on the system. Ammonium and phosphate concentration decreased significantly, achieving "very good" statuses throughout the entire estuary (Royal Decree 817/2015) and leading to changes in nutrient ratios. These loading changes had a direct effect on phytoplankton biomass and community composition. In the outer and middle estuary, phytoplankton biomass decreased significantly due to lower nutrient concentrations, while in the surroundings of the WWTP, biomass increased after the sewerage works, probably because the excessive ammonium loadings coming from the WWTP discharges might have been causing phytoplankton growth suppression in the area. This biomass increase led to the deterioration of the waters in URD5 and URD6, recording "bad" ecological statuses according to phytoplankton (Royal Decree 817/2015). 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 (especially URD5–URD6), there was a shift from mainly flagellates (ACA and CbCA) to diatoms (FCA) 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. Thus, phytoplankton dynamics in the Urdaibai estuary should be further studied to determine whether the medium-term changes detected will remain over time or if, on the contrary, new alterations will appear in the future. 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.

V. EZTABAIDA OROKORRA

Ikerketa honek, Gernikako araztegiaren hondakin-urak Urdaibai estuariora isuri bitarteko fitoplankton-komunitatearen jarraipen intentsibo baten datuak (2019-2020) eta saneamendu lanen osteko datu eguneratuagoak (2021 eta 2022) biltzen ditu. Gainera, fitoplankton jarraipen hauetan, teknika ezberdinak aplikatu ziren komunitatearen biomasa eta osaera ezagutzeko (mikroskopia, pigmentuen analisia eta DNA metabarcoding-a, hain zuzen ere), fitoplankton-komunitatearen deskribapen osoagoa eskainiz eta teknika hauen erabilgarritasunaren konparazioa ahalbidetuz.

Jarraipen hauek izan ditzaketen mugak alde batera utziz (saneamendu lanak amaitu eta urtebete osteko datuak soilik daude eskuragarri eta ez da bestelako aldagai biotikoen inguruko daturik kontutan hartzen), ikerketa honek funtsezko informazioa lortzeko aukera eman du. Lortutako fitoplankton-komunitatearen inguruko datuek, honen aldakortasunaren eta estuarioaren baldintza fisiko-kimikoen arteko harremanen deskribapenak eta saneamendu lanek estuarioan izandako eraginaren inguruko informazioak, Urdaibai estuarioan aurretik egindako lanak osatu eta eguneratzen lagundu dute. Hori dela eta, ikerketa hau oso erabilgarria izan daiteke etorkizunean egingo diren ikerketen oinarri gisa, batez ere helburu ekologikoetara zein saneamendu lanen eragina aztertzera bideratutako fitoplanktonaren inguruko ikerketentzako, baina baita bestelako diziplinarteko ikerketentzako ere. Gainera, tesi honek, Urdaibai estuarioaren fitoplankton espezie-aberastasunaren inguruko informazio berria eskaintzen du, eremuan eDNA metabarcoding-a lehendabiziko aldiz erabiltzearen ondorioz, eta pigmentuen analisian oinarritutako fitoplanktonaren azterketarako tresna berri

bat (*PIGMENTUM*) proposatu eta aplikatzen du, tesian zehar aurkitutako beharrak asetzeko helburuz garatu zena baina eremu itsastar zein ur gezetan erabiltzeko balio duena.

1. Urdaibai estuarioko fitoplankton-komunitatea

Estuarioko fitoplankton-komunitateek, mareen zikloetan zehar gertatzen den itsasoko uraren eta ur gezaren nahastearekin lotutako denboran eta espazioan zeharreko aldakortasuna pairatzen dute (Cloern et al., 2017). Estuario batzuetan, Urdaibain adibidez, neurri batean aurreikus daitezkeen aldakuntza zikliko hauek, giza jardueren (estuariora hondakin-urak isurtzea, esaterako) ondorioz aldaketak jasan ditzakete. Orokorrean, Urdaibai estuarioko fitoplankton-komunitatean ikusitako aldakortasuna, mareen eraginak eta Gernikako araztegiko hondakin-uren isurketek eragindako estuarioan zeharreko gazitasun gradiente handiek eta mantenugai ez-organikoen eskuragarritasun-aldaketek azaltzen dute, urtarokotasunarekin batera (*1. Kapitulua*, *2. Kapitulua* eta *4. Kapitulua*).

Urdaibai estuarioko fitoplankton biomasak, espero zen urtarokotasuna eta aldakortasun espaziala erakutsi zuen 2019-2020 bitartean (adibidez, Ansotegui et al., 2001). Urtean zeharreko biomasaren aldakortasunari dagokionez, Bizkaiko Golkoko estuarioetan (Seoane et al., 2005) eta beste gune epeletan (Domingues et al., 2007) aurkitzen den ohiko zikloa jarraitu zuen, udazkenean eta neguan biomasa balio baxuak eta udaberrian eta udan balio altuak lortuz. Eskualde honetan, udaberrian eta udan ur-tenperatura altuagoak, eguzki-erradiazio (eta argi-eskuragarritasun) handiagoa eta ibai-emari txiki edo moderatuak egon ohi dira, estuarioetako fitoplanktonaren hazkundea erraztuz. Biomasa baliorik baxuenak, ordea, prezipitazio eta ibai emari handieneko sasoietan erregistratu ziren, baldintza hauek Chl *a*-ren diluzioa eragiten baitute (Du et al., 2017), bereziki, Oka ibaiaren izaera torrentzialaren ondorioz eurite puntualen ondoren estuarioa ia erabat hustu egiten dela kontuan izanda. 2019-2020 bitartean ikusitako biomasaren urtarokotasun hau hurrengo fitoplanktonaren jarraipenen plangintzarako kontuan hartu zen, azterketa-aldia martxotik urrira arte murriztuz, biomasa altuko sasoia hartzeko asmoz.

Estuarioko biomasaren aldakortasun espazialari dagokionez, 2019-2020an ikusitako ereduak 90eko hamarkadan deskribatutakoa bezalakoa izaten jarraitzen zuen (adibidez, Orive et al., 1998; Ansotegui et al., 2001), kanpoaldean balio baxuenak erregistratuz eta estuarioaren barrualdera hurbildu ahala handituz. Hau, estuario askoren ohiko Chl *a* banaketa-eredua da (McGarrigle et al., 2001; Santos et al., 2022), fitoplanktonaren hazkuntza arautzen duten

faktoreek (adibidez, mantenugaiak eta argi eskuragarritasunak edo bazkak) eraginda. Araztegiak hondakin-urak (eta mantenugaiak) isuri arren, mantenugai falta izan liteke Urdaibai estuarioko kanpoaldeko eremuan biomasa balio baxuak aurkitzearen arrazoi nagusia, mareek eragindako diluzio-efektuagatik eta Euskal Kostaldearen izaera oligotrofikoagatik (Muñiz et al., 2019). Estuarioaren barrualdeko mantenugaien kontzentrazio altuagoek (araztegitik zein Oka ibaitik etorritakoak), ordea, fitoplanktonaren hazkundea suspertu eta bertan aurkitutako biomasa balio altuagoak azaltzen ditu (McCabe et al., 2016; Vajravelu et al., 2018). Gainera, 2019-2020 artean ikusitakoaren arabera (*1. Kapitulua*), estuarioaren barrualdean aurkitutako uhertasun handiagoak ez zuen fitoplanktonaren hazkuntza oztopatzen.

Komunitatearen osaerari dagokionez, oro har, 2019-2020an deskribatutako Urdaibai estuarioko fitoplankton-komunitatea (*1. Kapitulua* eta *4. Kapitulua*) 90eko hamarkadan deskribatutakoaren antzekoa da (Madariaga et al., 1989, 1994; Orive et al., 1995, 1998; Trigueros eta Orive, 2001). Estuarioaren kanpoko eremua zen taxon-aberastasun handieneko eremua eta komunitatean diatomeoak ziren nagusi, dinoflagelatuen eta haptofitoen ohiko agerpenarekin, eta barrualderantz joan ahala flagelatuen nagusitasuna areagotzen zen, batez ere kriptofitoena, baita alga berdeena eta *Kryptoperidinium foliaceum*-ena ere. Gainera, ikerketa honek, estuarioko organismo nano- eta pikoplanktonikoen ugaritasuna eta dibertsitatea agerian utzi du, DNA metabarcoding-a jarraipen-teknika gisa aplikatzeari esker, aurretik Urdaibai estuarioan identifikatuak izan ez diren tamaina txikiko espezie askoren presentzia ezagutzera eman baitu (*1. Kapitulua*).

Saneamendu lanak burutu baino lehen (2019-2020), Urdaibai estuarioko kanpoko eta erdiko eremuan fukoxantinadun algak (FCA), batez ere diatomeoak, ziren nagusi, hau munduan zeharreko estuario askotan ohikoa izanik, hala nola, Rio de la Platan (Gómez et al., 2004) eta Sadoko estuarioan (Santos et al., 2022). *Minutocellus polymorphus* eta *Chaetoceros tenuissimus* izan ziren eremu honetan loraketak sortzen zituzten diatomeorik garrantzitsuenak (*4. Kapitulua*), Mediterraneo Itsasoko eta Ozeano Atlantikoko estuarioetan eta kostaldeko uretan egon ohi diren organismo kosmopolita txikiak (Walsh et al., 1988; De Luca et al., 2019). Gainera, teknika molekularrek laginketa guztietan agertzen ziren beste diatomeo batzuen presentzia agerian utzi zuten, hala nola *Minidiscus variabilis* eta *Mediolabrus comicus* URD1-en eta *Thalassiosira profunda* URD2-n, estuarioan lehen aldiz identifikatu zirenak (*1. Kapitulua*). Kanpoko eremuan, diatomeoez gain, dinoflagelatuen eta haptofitoen ugaritasuna ere nabarmena izan zen, estuarioko handiena izatez, eta PeCAk (peridininadun

algak) eta HCAk (19'-hexanoiloxifukoxantinadun algak) fitoplankton biomasa totalari egindako ekarpen balio handienak ere eremu honetan aurkitu ziren, *Warnowia* sp., *Heterocapsa* spp. (bereziki *H. pygmaea* eta *H. rotundata*) eta *Gyrodinium* spp. (*G. fusiforme, G. dominans* eta *G. spirale*) bezalako dinoflagelatuen eta *Chrysochromulina* spp. (batez ere *C. scutellum* eta *C. rotalis*), *Prymnesium* spp. and *Phaeocystis* spp. (*P. cordata* nagusiki) bezalako haptofitoen etengabeko agerpenarekin. Hau, bi talde hauek osatzen dituzten espezie gehienen izaera itsastarrak eta mixotrofikoak azaltze du, azken honek mantenugai gutxiko eskualdeetan hazteko gaitasuna eskaintzen dielarik (Unrein et al., 2014; Zhang et al., 2013). Estuarioaren erdialdeko eremuan, ordea, hondakin uren isurketak egon bitartean, diatomeoekin batera kriptofitoak ugariak ziren (aloxantinadun algak, ACA), *Plagioselmis* spp. eta *Teleaulax* spp. bezalako taxoiak garrantzitsu bihurtuz.

Estuarioaren erdiko eremutik barrualderantz hurbiltzerakoan, ACA zen 2019-2020an fitoplankton biomasa totalari ekarpen handiena egiten zion taldea, kriptofitoen ugaritasunaren gorakadagatik. Estuarioan aurkitutako kriptofitoen artean, batzuek gazitasun altuko eremuekiko lehentasuna erakutsi zuten, hala nola, *Teleaulax amphioxeia* eta *Teleaulax gracilis*, eta beste batzuen agerpena estuarioaren barrualdeko eremura mugatu zen, *Hemiselmis cryptochromatica*, *Teleaulax acuta* eta, batez ere, *Urgorri complanatus* bezalakoak. Jakina da kriptofitoak eutrofizatutako estuario askoren barne-eremuko talde nagusia direla (adibidez, Valdes-Weaver et al., 2006; Santos et al., 2022), non argi intentsitate baxuetan (ur uherretan) fotoaklimatatzeko duten gaitasunagatik (Collini, 2022), hazkunde-tasa altuak izateagatik (Paer et al., 2003), amonioz aberastutako uretan erraz hazteagatik (Horner eta Thompson, 2001) eta izaera mixotrofikoa izateagatik (Johnson et al., 2013) diatomeoak bezalako beste talde batzuk baino arrakasta handiagoz hazten diren. Kriptofitoek Urdaibai estuarioko barruko eremuan duten nagusitasun hau eremuan egindako aurreko ikerketetan jasota zegoen arren (Madariaga, 1995; Orive et al., 1998; Ansotegui, 2001), 2019-2020an talde honek erakutsitako nagusitasuna 90eko hamarkadan ikusitakoa baino nabarmenagoa izan zen, horrek estuarioaren eutrofizazio maila handitu dela adierazi dezakeelarik.

Kriptofitoez gain, saneamendu lanen aurretik, estuarioaren barrualdeko eremuan, CbCA taldeak (prasinoxantinarik gabeko alga berdeak) fitoplankton biomasa totalari egindako ekarpena ere aipatzekoa da, batez ere uda garaian eta URD5 eta URD6 eremuetan. Bertan, *Eutreptiella* spp. bezalako euglenoideak eta hainbat klorofita, *Tetraselmis* spp. edo *Picochlorum* spp. eta *Nannochloris* sp. bezalako organismo picoplanktoniko kokoideak

esaterako (*1. Kapitulua*), izan ziren Chl *b*-aren ekarpen handiena egiten zuten organismoak. Euglenoideak mantenugaietan aberatsak diren sakonera gutxiko eta argi gutxiko uretan hazteko gai dira (Poniewozik eta Juráň, 2018), duten ugalketa azkarragatik eta izaera mixotrofoagatik. Klorofito txikiei dagokienez (<5 μm), amonioa hartzeko afinitate bereziki handia dute (Litchmann et al., 2007; Laruste et al., 2016), eremu horretan hondakin uren isurketak egon bitartean oso ugaria zena. Gainera, araztegiaren inguruan, FCA zen biomasa totalari ekarpen handiena egiten zion hirugarren taldea, *Kryptoperidinium foliaceum* fukoxantinadun dinoflagelatuaren eta diatomeo txikien agerpenagatik. *K. foliaceum*-ek, Urdaibai estuarioaren barnealdeko espezie bereizgarrienetakoa (Ansotegui et al., 2001; Trigueros et al., 2000b), Si-aren sarrerarik gabeko N-aren aberasteari positiboki erantzuten dio (Domingues et al., 2011) eta, beraz, eremu honetan aurkitzen zen bere ugaritasun altua estuarioak jasaten zuen mantenugaien aberaste antropogenikoaren adierazlea da. Barrualdeko eremuan agertzen ziren diatomea txikiei dagokienez, *Navicula phyllepta* eta *Navicula gregaria* pennatu epipelikoak eta *Cyclotella* spp. bezalako diatomeo zentrikoak esaterako, FCAri egindako ekarpena mugatua zen saneamendu lanen aurretik (2019-2020).

Honenbestez, orokorrean, Urdaibai estuarioko kanpoaldeko eta erdialdeko fitoplankton-komunitateak ez zuen, itxuraz, Gernikako araztegiaren isurketen ondoriorik pairatzen, 2019-2020an estuario epeletan ohikoak diren biomasa eta osaera erakutsiz, ziurrenik isurketa-eremutik dagoen distantziagatik eta horren ondoriozko diluzio-efektuagatik. Aitzitik, estuarioaren barrualdeko komunitatearen osaera (kriptofitoak bezalako organismo flagelatu txikien nagusitasunarekin), presio antropogenikoen ondorioz eutrofizatuta dauden estuarioetan ohikoak diren komunitateen antzekoa zen (e.g., Smayda, 1990; Glibert et al., 2011). Kanpoaldean diatomeoak egotetik barrualdean flagelatuen nagusitasuna izatera (ACAen nagusitasuna, CbCA eta *K.foliaceum*-en presentzia altuarekin batera) pasatzearen arrazoi nagusienetako bat araztegiaren isurketek eragindako mantenugai ratioen aldaketak izan daitezke. Isurketek, DIN:Si eta DIN:P erlazioak handitzen zituzten eta, ziurrenik, N eta P aberasteak aprobetxatuz alga ez-silizikoak pixkanaka nagusitzen joan ziren (Tréguer eta De La Rocha, 2013; Jiang et al., 2014), diatomeoek ordea, ez zuten mantenugai aberaste hau erabiltzeko aukerarik, haien hazkuntza Si-agatik mugatuta egoteagatik, bereziki URD6n, non saneamendu lanen aurretik Si eskasi nabarmena zegoen (*4. Kapitulua*).

2021eko uztailean, 50 urtez Urdaibai estuarioaren barruko eremura hondakin-urak isuri ostean, Gernikako araztegiko efluentea estuariotik kanpo desbideratu zen, kolektore baten bidez Bermeoko Lamiaran araztegira eramanez. Ikerketa honen bidez, saneamendu lanek

Urdaibai estuarioko fitoplankton biomasan eta komunitatearen osaeran izandako berehalako (*2. Kapitulua*) eragina eta, batez ere, epe-ertaineko (*4. Kapitulua*) efektua ikusi da, gehienbat baldintza fisiko-kimikoen eta, batez ere, nutrienteen eskuragarritasun aldaketei erantzunez.

Araztegiko efluentea estuariotik kanpo desbideratu eta berehala (*2. Kapitulua*), amonio eta fosfato kontzentrazioak nabarmen gutxitu ziren (% 50 baino gehiago) estuarioaren erdiko eta barneko eremuan, jakina baitzen Gernikako araztegia zela estuarioko mantenugai hauen gehiegizko aberastearen erantzule nagusia (Iriarte et al., 1997; Revilla et al., 2000; Iriarte et al., 2015). Honi esker, Urdaibai estuarioko uraren kalitatea hobetu egin zen, adierazle fisiko-kimikoei dagokienez, 817/2015 Errege Dekretuan ezarritako irizpideen arabera. 2020ra arte estuarioaren barruko eremuak ingurumen-helburuak bete izan ez arren, adierazle fisiko-kimikoen (amonio eta fosfatoa) eta fitoplanktona bezalako adierazle biologikoen ondorioz egoera ekologiko "eskasa" edo "txarra" lortuz (Borja et al., 2021), saneamendu lanak amaitu eta hilabetera, amonio eta fosfato kontzentrazioek uraren egoera "ona" adierazi zuten estuario osoan zehar. Gainera, estuarioaren baldintza fisiko-kimikoetan beste aldaketa txiki batzuk ere egon ziren, hala nola, uhertasunaren jaitsiera eta oxigeno-saturazioa handitzea, baina ez ziren erabat esanguratsuak izan ikerketa-tarte honetan.

Ingurune aldakorretan, fitoplankton-komunitatearen osaera aldatu egiten da taxonen lehiakortasun-gaitasun ezberdinen ondorioz (Cardinale et al., 2011). Urdaibaiko estuarioaren barruko eremuko komunitatearen osaerak saneamendu lanekiko berehalako erantzuna erakutsi zuen. *Eutreptiella* spp.-ren hazkuntza bizkor eta nabarmenak Chl *b*-ren gorakada eragin zuen, barrualdeko eremuan pigmentu diagnostiko nagusia bihurtuz, eta, ondorioz, aloxantinak eta fukoxantinak garrantzia galdu zuten, kriptofitoen ugaritasuna murriztuz eta *K. foliaceum*-en ia erabateko desagerpenarekin. *Eutreptiella* generoak loraketak sortzen ditu elikagaietan aberatsak diren kostaldeko eta trantsizioko uretan (adibidez, Olli et al., 1996; Stonik et al., 2007) eta, normalean, kriptofito eta dinoflagelatuak bezalako bestelako flagelatuekin batera agertzen da (adibidez, Jeong et al., 2021). Honenbestez, komunitatearen berehalako aldaketa honek ez zuen estuarioko uraren kalitatearen hobekuntza adierazi. Euglenoideak ingurune-baldintzen aldaketekiko oso sentikorrak dira eta populazioak oso azkar hazten edo desagertzen dira (Heckman et al., 1996), beraz, litekeena da saneamendu lanen ondoren araztegiaren inguruan gertatutako aldaketa fisiko-kimikoek *Eutreptiella* spp.-ri kriptofitoekin edo/eta *K. foliaceum*-ekin lehiatzeko abantaila eman izatea, bere hazkundea azkartuz. Gainera, estuarioko biomasak ere berehalako aldaketa arinak jasan zituen, tarteko eremuan pixka bat gutxituz eta araztegiaren inguruan handituz, ziurrenik mantenugaien

sarrerak murrizteagatik (adibidez, Wetz et al., 2011), baina aldaketa horiek ez ziren guztiz esanguratsuak izan.

Hondakin-urak estuariotik kanpo desbideratu eta urtebetera, 2022an, aldaketa nabarmenagoak nabaritu ziren Urdaibai estuarioan (*4. Kapitulua*). Gainera, baldintza hidrometeorologikoek eta estuarioko gazitasunak eta tenperaturak ez zuten ezberdintasun adierazgarririk erakutsi 2020 eta 2022an aztertutako denbora tartean eta, beraz, aurkitutako aldaketak estuarioko saneamendu lanek eragindakoak izan zitezkeela ondorioztatu da.

Hondakin-uren desbideratzea egin eta berehala (2021) ikusi zenarekin bat eginez, 2022an, 2020arekin alderatuz, amonio eta fosfato kontzentrazioen beherakada nabarmena izan zen Urdaibai estuario osoan zehar. Kontzentrazioen beherakada honekin, 2022an estuarioko egoera ekologikoa hobetu zen, adierazle fisiko-kimikoei dagokienez, estuario osoan zehar egoera "oso ona" lortuz (817/2015 Errege Dekretua). Mantenugaien beherakada hau bereziki nabarmena izan zen amonioaren kasuan, DINaren (disolbatutako nitrogeno organikoa) beherakada ere eraginez. Estuario eta kostaldeko uren mehatxu handiena gehiegizko N-aren aberastea izanda (Howarth eta Marino, 2006), Urdaibain geratutako N-aren beherakada handiagoa onuragarria izan beharko litzateke sistemaren osasunerako. Bestetik, espero bezala, nitrato eta silikato kontzentrazioek, estuariora iturri naturaletatik heltzen direnak (Turner et al., 2003; Valencia eta Franco, 2004), ez zuten alde handirik erakutsi saneamendu lanen ondoren. Hala ere, mantenugaien jatorriaren araberako karga-aldaketen desberdintasun horrek estuarioko DIN:Si:P ratioetan aldaketak eragin zituen. 2022an, gehiegizko DIN kontzentrazioak zeuden oraindik Urdaibai estuario osoan zehar, nahiz eta saneamendu lanek desoreka hori leundu zuten. Ondorioz, estuarioaren kanpoko eremuan Si falta zegoen eta URD2tik URD5era P eskasiak mugatzen zuen fitoplanktonaren hazkuntza, 2020an bezala. Araztegiaren inguruan (URD6) ordea, hondakin-uren isurketak egon bitartean Si falta zegoen arren, saneamendu lanen ondoren P bihurtu zen fitoplanktonaren hazkuntzaren mugatzailea.

Mantenugai sarreren eta ratioen aldaketek fitoplankton-komunitateen aldakortasuna eragin ohi dute (Lie et al., 2011; Wu et al., 2017a), Urdaibai estuarioko fitoplankton-komunitatearekin gertatu den bezala. Saneamendu lanak amaitu eta urtebetera, 2021ean aurreikusitako aldaketa batzuk (*2. Kapitulua*) nabarmenagoak izan ziren, hala nola fitoplankton biomasa totalaren aldakuntza. Urdaibai estuarioko kanpoko eta erdiko eremuan, Chl *a* kontzentrazioa eta fitoplankton-loraketen ($7{,}5x10^5$ zelula L^{-1} baino gehiago; Revilla et al., 2009) kopurua nabarmen jaitsi zen saneamendu lanen ostean. Hau DIN eta P-ren

jaitsierarekin azaldu daiteke, izan ere, honek fitoplankton hazkundearen murrizketa eragin (adibidez, Beardall et al., 2001) eta, aldi berean, loraketak sortzeko gai diren taxonen arteko lehia sustatu zuen, mantenugai eskuragarritasuna mugatuagoa izateagatik, loraketen kopurua murriztuz. Araztegiaren inguruan ordea, *4. Kapituluko* emaitzetan ikusitakoaren arabera, pentsa daiteke hondakin-uren isurketak zeuden bitartean amonio sarrera altuegiek eremuko fitoplanktonaren hazkuntza oztopatzen zutela (Dugdale et al., 2007), biomasaren igoera adierazgarria izan baitzen saneamendu lanak burutu eta urtebetera. 2020an, saneamendu lanak baino lehen, URD6-ko amonio-kontzentrazioaren mediana 153 μmol L^{-1}-koa zen (65–334 μmol L^{-1} bitarteko balioak), fitoplanktonaren hazkuntza ekiditeko nahikoa (Glibert et al., 2016); 2022an ordea, 10 μmol L^{-1}-era jaitsi zen (24 μmol L^{-1} gehienez), fitoplanktonaren hazkuntza egokia ahalbidetuz. Are gehiago, baldintza hipertrofikoak adierazten dituzten Chl *a* kontzentrazioa altuak (Hagy et al., 2022) maiztasun handiagoz aurkitu ziren estuarioaren barruko eremuan saneamendu lanen ondoren (*4. Kapitulua*), 251,5 μg L^{-1}-ko Chl *a* maximora iritsiz. Saneamendu lanen ondoren araztegiaren inguruan fitoplanktonaren hazkuntza areagotzeko beste arrazoi bat, hondakin-uren isurketak eteteak eragindakoa materia-partikulatuaren sarreren murrizketa, eta ondoriozko argi-eskuragarritasun handiagoa, izan daiteke (Carey eta Migliaccio, 2009), nahiz eta 2020an ez zirudien uhertasunak fitoplanktonaren hazkuntza oztopatzen zuenik (lortutako Chl a balio altuengatik). 2020an eremuko uren uhertasunak jaitsiera esanguratsurik erakutsi ez izanak ez du esan nahi gertatu ez denik, izan ere, posibleena da fitoplankton biomasaren hazkuntzak uraren uhertasuna handitu eta aldaketa hau estali izana. Hala eta guztiz ere, eta araztegiaren inguruan ikusitako fitoplankton biomasaren igoerak emaitza positiboa zirudien arren (amonioak jada ez zuelako hazkundea oztopatzen), URD5 eta URD6an egoera ekologikoa okerragotzea eragin zuen, Chl *a*-rentzat egoera "txarra" lortuz (Errege Dekretua 817/2015).

Fitoplankton-komunitatearen osaerari dagokionez, hondakin-uren isurketak gelditu eta urtebetera, estuarioaren kanpoko eremuan (URD1) ez zen aldaketarik ikusi, araztegiaren isurketek eremu honetako fitoplanktonean zuten eragina txikia edo hutsa zela baieztatuz. Gainera, *4. Kapituluan* frogatu zen saneamendu lanak burutu eta berehala estuarioaren barrualdean egondako CbCA-ren bat-bateko igoera (*2. Kapitulua*) aldaketa puntuala izan zela, ez baitzen 2022ra arte luzatu. Hala ere, 2022an, Urdaibai estuarioko fitoplankton-komunitatean bi desberdintasun esanguratsu ikusi ziren, *2. Kapituluan* aurreikusi ez zirenak: prasinoxantinadun prasinofitoek (PrCA) biomasa totalari egiten dioten ekarpenaren handiagotzea (URD1-URD4) eta FCAen garrantzi handiagoa estuarioaren barruko eremuan (URD3-URD6).

Urdaibai estuarioan PrCAen gorakada nabarmena eman zen saneamendu lanen ostean, URD1etik URD4ra, estuarioaren erdiko eremuan, adibidez, biomasa totalari egiten zion ekarpena bost aldiz handiagoa izanik (Chl *a*-ren % 5 izatetik % 25 izatera). PrCAen parte diren organismo gehienak frakzio pikoplanktonikoari dagozkio (<3 μm-ko diametroa duten zelulak) (Guillou et al., 2004), haien mikroskopia bidezko identifikazio eta kuantifikazioa ezinezkoa izanik (adibidez, Ansotegui et al., 2003). Hala ere, DNA metabarcoding-ak, *Bathycoccus prasinos*, *Micromonas* spp. (*M. bravo*, *M. commoda* eta *M. pusila*) eta *Ostreococcus* spp. (*O. lucimarinus*, *O. mediterraneus* eta *O. tauri*) identifikatu zituen 2019-2020an Urdaibai estuarioko PrCA taxon garrantzitsuenak bezala (*1. Kapitulua*) eta, *PIGMENTUM* bidezko HPLC pigmentuen analisiak, 2022an PrCA taldeak pairatu zuen hazkunde nabarmena zehaztea ahalbidetu du (*4. Kapitulua*). Organismo hauek duten azalera:bolumen erlazio altuari, hazkunde-tasa eraginkorrari eta mantenugaiak bereganatzeko-tasa altuari (Paerl et al., 2003) esker, PrCA beste fitoplankton talde funtzional batzuk baino lehiakorragoa da mantenugaiak hain ugariak ez direnean (Glibert et al., 2010). Honek azaldu lezake Urdaibai estuarioan saneamendu lanen ondoren egondako gorakada, baita antzeko hobekuntza lanak jasan dituzten beste ekosistema batzuetan agertu izana ere (Leruste et al., 2016). Hala ere, estuarioetako PrCAen lehentasun ekologikoen eta aldakortasunari buruzko ezagutza mugatua da, izan ere, haien izaera nagusiki pikoplanktonikoa dela eta, mikroskopian oinarrituta jarraipen gehienetan alde batera uzten da, *Pyramimonas* bezalako Pras-ik ez duten prasinofito handiagoetan zentratuz. Beraz, Urdaibai estuarioaren PrCAen ugaritze hau gehiago aztertu beharko litzateke, estuarioko fitoplankton biomasa totalaren zati garrantzitsua izaten jarraituko duen jakiteko eta gorakada honen erantzuleak diren taxoiak zehazteko.

Saneamendu lanen ostean Urdaibai estuarioko barneko eremuan ikusitako FCAen igoera, batez ere URD5n eta URD6n, sistemaren berreskurapen seinale gisa interpretatu liteke. 2019-2020an, aurretik esan bezala, Urdaibai estuarioaren barnealdeko eremuan, flagelatuak batez ere ACA) ziren nagusi, eta FCA taldean, biomasari ekarpena nabarmena egiten ziona, *K. foliaceum* dinoflagelatua zen taxonik ugariena, diatomeoen ekarpena txikia izanik. Hala ere, saneamendu lanen ondoren ikusitako FCAaren igoera (ACA eta CbCAren ekarpenak gutxituz) diatomeoen ugaritasunaren gorakadaren ondoriozkoa izan zen (adibidez, *Cyclotella* eta *Thalassiosira*). Araztegiko isurketak zeuden bitartean diatomeoen presentzia baxuagoa izatea hondakin-uren amonio kontzentrazio altuekin lotu liteke (Glibert et al., 2011). Fisiologikoki, diatomeo askok N iturri gisa nitratoa nahiago dute amonioaren aldean, eta hau batzuetan beharra bihurtu daiteke, batez ere zelulak energia-desoreka egoeran daudenean

(argi-baldintza aldakorra duten egoeretan adibidez, Urdaibai estuarioaren barrualdean bezala), izan ere, nitratoak mantenugai gisa jardun ez ezik, energia-oreka zelularra mantentzen laguntzen baitu (Lomas eta Glibert, 1999). Beraz, diatomeoen ugaritasunaren areagotzea, amonio-kontzentrazio baxuagoekin eta nitratoaren eskuragarritasun handiagoarekin azal liteke (adibidez, Dominges et al., 2011; Glibert et al., 2014). Horrez gain, saneamendu lanen ondoren, Si jada ez zen URD6n mantenugai mugatzailea, eta, beraz, fitoplankton talde ez-silizikoen (ACA, CbcA edo *K. foliaceum*, adibidez) hazkuntzak ez zeukan abantailarik diatomeoen aurrean. Hori dela eta, estuarioaren barneko eremuan egondako fitoplankton-komunitatearen osaera aldaketa, FCAen (batez ere diatomeoen) ugaritzea eta flagelatuen (ACA eta CbCA) murrizketa, Urdaibai estuarioaren berreskurapen seinaletzat har liteke.

Ikerketa honetan zehar fitoplankton-komunitatean hautemandako aldaketa gehienak mantenugaien kontzentrazioaren edo/eta argiaren eskuragarritasun aldaketekin lotu diren arren, jakina da fitoplankton-populazioen dinamikak hazkuntzaren eta galeren arteko desoreken ondorio direla, galera hauen baitan fitoplanktonaren bazka, hondoratzea eta hilkortasun naturala biltzen direlarik (Brussaard, 2004). Hortaz, badira kontuan hartu ez diren baina Urdaibai estuarioko fitoplankton-komunitatean ikusitako aldaketetan ere eragina izan dezaketen edo aldaketen erantzuleak izan zitezkeen hainbat faktore biotiko, hala nola, birusak (Brussaard, 2004), bakterioak (Seymour et al., 2017) eta bazkatzaileak (Lürling, 2021). Hala ere, ikerketa honek Urdaibai estuarioko baldintza fisiko-kimikoen eta fitoplankton-komunitatearen deskribapen zehatza eskaintzen du, Gernikako araztegiko hondakin-uren isurketak eten aurretik (2019-2020), eta saneamendu lanek estuarioan izandako berehalako eta epe-ertaineko (urtebete geroago) eragina ebaluatzen du, gizakion inpaktu zuzenek edo/eta kudeaketa ekintzek estuario honetan eta antzekoetan izan ditzaketen ondorio ekologiko posibleak ezagutzeko baliogarria izan daitekeena.

2. Fitoplanktonaren jarraipen tekniken analisia

Fitoplanktona uraren kalitatearen adierazle garrantzitsua eta uretako ekosistemen funtzionamendu globala ulertzeko ezinbesteko elementua da, kate-trofikoaren oinarria izateagatik, espezieen bereizketa handiagatik, hazkuntza-tasa altuengatik eta ingurumen-baldintzei erantzuteko duten gaitasunagatik. Hori dela eta, fitoplanktonaren jarraipen asko egin dira helburu ekologikoekin (Diana et al., 2021; Dedman et al., 2022; Chen et al., 2023)

edo uraren kalitatearen ebaluaziorako (Amorim eta Nascimento, 2021; Zhang et al., 2021). Hala ere, jarraipen horietatik lortutako datu-mota eta kopurua erabilitako azterketa-teknikaren araberako da. Ikerketa honetan, Urdaibai estuarioko fitoplanktonaren biomasaren eta komunitatearen osaeraren analisirako hiru teknika ezberdin erabili dira: mikroskopia, eDNA metabarcodig-a eta HPLC bidezko pigmentuen analisia. Hiru metodo hauen konbinazioak estuarioko fitoplankton-komunitatearen ikuspegi integratua eta osoa lortzeko aukera eman du eta teknika bakoitzaren abantaila eta ahulgune nagusiak agerian utzi ditu.

Estuarioko fitoplankton-komunitatearen identifikazio taxonomikoa eta kuantifikazioa egiteko mikroskopia erabili zen, Utermöhl metodoan oinarrituz (Edler eta Elbrächter, 2010). Metodo honek eskaini duen abantaila nagusia identifikazioarekin batera taxonen zenbaketa egiteko aukera izan da, horrela, informazio taxonomikoaz gain, zelula-kontzentrazioen datuak eskuratu baitira, eta, beharrezkoa izatekotan, zelulen tamaina neurtzeko aukera ere eman du (biobolumenen kalkuluak egiteko adibidez). Zelula-ugaritasun datuak bereziki erabilgarriak dira komunitatean loraketak sortzen dituzten taxoiak eta/edo espezie toxikoak aztertzerakoan. Ikerketa honetan, mikroskopiak, Urdaibai estuarioan gertatutako loraketak identifikatzea eta kuantifikatzea ahalbidetu zuen, Revilla et al.-ek (2009) UEZan oinarrituz Euskal kostaldeko fitoplanktonaren egoera ekologikoaren ebaluazioa egiteko ezarritako irizpideak jarraituz (*4. Kapitulua*). Honek, aldi berean, saneamendu lanen ondoren estuarioaren kanpoko eta erdiko eremuetan gertatutako loraketa kopuruaren murrizketa antzematea ahalbidetu zuen, estuarioan egondako aldaketa nagusietako bat dena. Bestetik, ur zutabean fitoplankton toxikoaren ondoriozko “toxikotasun arrisku egoera” markatzen duten muga-balioak ere mikroskopia bidez lortutako zelula-ugaritasun datuetan oinarritzen dira (adibidez, Swan eta Davidson, 2012). Ikerketa honetan, mikroskopiak, estuarioaren kanpoko eta erdiko eremuan *Pseudo-nitzschia* spp.-k eta *Dinophysis* spp.-k toxikotasun muga-balioak hainbat aldiz gainditzen zituztela jakiteko aukera eman zuen, 2022an ere hauen kopurua murriztu zelarik (*4. Kapitulua*). Beste teknikekin identifikatu ez ziren estuarioko taxon garrantzitsu batzuen agerpenari buruzko informazioa ere eskaini zuen, hala nola *Gephyrocapsa huxleyi*, *Prorocentrum micans* eta, bereziki, *K. foliaceum* (*1. Kapitulua*). Gainera, mikroskopia, tradizionalki, fitoplanktonaren biomasa eta dibertsitatea ebaluatzeko gehien erabili izan den teknika izanik, eskuragarri dauden mota honetako ikerketa kopurua gainontzeko teknikena baino handiagoa da, erreferentzia gisa hartu edo/eta konparatzeko lagungarria dena. Horrek, Urdaibai estuarioan lortutako emaitzak perspektiban jartzen lagundu eta ikerketa aberastu du.

Hala ere, askotan, mikroskopia bidezko identifikazio taxonomikoak zehaztasun falta edo espezieen omisioa dakar (Agirbas et al., 2015). Hori dela eta, aztertutako maila taxonomikoa zenbat eta txikiagoa izan, orduan eta handiagoak dira mikroskopiaren eta metabarcoding-aren arteko ezberdintasunak Urdaibai estuarioko komunitatea deskribatzerakoan (*1. Kapitulua*). Mikroskopiaren bidezko fitoplankton taxonen identifikazio desegokiak edo omisioak bi arrazoi nagusi ditu: taxon batzuen zelula-tamaina txikia eta hauskortasuna (organismo pikoplanktoniko eta nanoplanktonikoak) (adibidez, Abad et al., 2016) edo/eta antzekoak diren espezieen ezaugarri morfologiko diagnostikoak identifikatzeko ezintasuna teknikaren bereizmen faltagatik (Kim et al., 2019; Santi et al., 2021). Honek azaltzen du Urdaibai estuarioko klorofito, prasinofito eta haptofito espezie askoren identifikazio-eza, baita genero/espezie mailara identifikatu ezinik hainbat diatomeo "identifikatu gabeko diatomeo zentrikoak" edo "identifikatu gabeko diatomeo pennatuak" bezalako taldeetan multzokatzea (*1. Kapitulua*). Are gehiago, saneamendu lanen ondorioz fitoplankton-komunitatean ikusitako aldaketa esanguratsuenetako bat prasinofita pikoplanktonikoen gorakada izan zen (*4. Kapitulua*), jarraipenetan mikroskopia bakarrik erabili izan balitz alde batera utziko zitekeena. Horrez gain, ikerketa askotan aipatu den bezala (Edler eta Elbrächter, 2010), mikroskopia bidez aztertutako bolumena (50 mL) baxua izan da beste teknikekin alderatuz. Gainera, emaitzek ikertzaileen gaitasunen menpekotasun handia erakusten dute (Muñiz et al., 2020) eta laginen analisirako denbora asko behar da (Wang et al., 2018), beraz, ez da guztiz egokia laginketa kopuru handia biltzen dituzten jarraipen-programa internazionaletan erabiltzeko.

eDNA metabarcoding-ak, araztegiak hondakin-urak isuri bitarteko (2019-2020) estuarioko fitoplankton-komunitatearen identifikazio taxonomiko zorrotzagoa egitea ahalbidetu zuen, mikroskopiak baino identifikazio-gaitasun nabarmen handiagoa eskainiz (*1. Kapitulua*). Teknika honek, DNAn oinarrituz, ingurumen-laginetako taxon guztien aldi-bereko identifikazioa ahalbidetzen du (Keck et al., 2017; Zimmermann et al., 2015), biodibertsitatearen informazio kantitate handia sortuz, espezieak edozein bizitzako fasetan identifikatzeko gai delako eta espezie kriptikoak eta metodo tradizionaletan aintzat hartzen ez diren espezieak identifikatzeko aukera ematen duelako (Comtet et al., 2015). Ikerketa honetan, metabarcoding-ak mikroskopia bidez identifikatu ez ziren 349 fitoplankton taxonen presentzia hauteman zuen, eta horietatik 273 (223 espezie) lehenengo aldiz identifikatu ziren Urdaibai estuarioan, besteak beste, 77 diatomeo espezie, 50 dinoflagelatu, 40 alga berde, 15 kriptofito, 13 haptofito. Hori dela eta, ikerketa honetan metodo hau erabili izanak Urdaibai estuarioko organismo pikoplanktonikoen eta espezie kriptikoen inguruko ezagutza baliotsua

eskaini zuen. Honek argi erakutsi du azterketa morfologikoek bakarrik ezin dutela fitoplankton dibertsitate handiaren deskribapen osoa eman (adibidez, Huo et al., 2020; Penna et al., 2017). Gainera, eDNA metabarcoding-aren emaitzak lortzeko aztertutako lagin-bolumena handiagoa izan da (2 L inguru) (adibidez, Gran-Stadniczeñko et al., 2019), horrek aberastasun taxonomiko handiagoa lortzen lagundu duelarik, eta emaitzak ez dira ikertzailearen taxonomia-ezagutzen hain menpekoak izan (Penna et al., 2017; Trebitz et al., 2017), Tara Oceans Expedition bezalako itsas-inguruneko nazioarteko proiektuetan izan duen aplikazio arrakastatsua azalduz (Malviya et al., 2016).

Hala ere, ikerketa honetan zehar, hainbat izan dira metabarcoding-aren esleipen taxonomiko osatugabek (200 inguru). Osatu gabeko identifikazio horien atzean hainbat arrazoi daude: markatzaileen bereizmen eskasa (Santoferrara, 2019), *primer* aukeraketa desegokia (Van der Loos eta Nijland, 2021), DNA metabarcoding-aren lan-fluxuan zehar sartutako alborapen teknikoak (Martin et al., 2022), bioinformatika metodo edo parametroen erabaki desegokiak (Santoferrara, 2019) edo datu-base osatugabeak edo zehaztugabeak (Rimet et al., 2021). Identifikazio osatugabe hauek Urdaibai estuarioan *K. foliaceum*, *Prorocentrum micans* eta *Gephyrocapsa huxleyi* bezalako espezien agerpenaren "negatibo faltsu"-ak eragin zituen, baina, hala ere, identifikatuta espezie kopuruak oso handia izaten jarraitzen du eta ezin da ahultasun moduan kontsideratu. Fitoplanktonaren jarraipenerako teknika bezala erabiltzeko metabarcoding-ak duen ahulgune nagusia kuantifikazio-ahalmen eza da, fitoplankton taldeen, generoen eta espezieen artean dagoen 18S rRNA genearen kopia-zenbakiaren aldakuntzaren ondorioz (Martin et al., 2022; Santi et al., 2021). Ikerketa honetan ikusi da metodo honek emaitza erdi-kuantitatiboak (ugaritasun erlatiboaren emaitza) eskaintzen dituela komunitatearen konposizioaren deskribapenerako, eta hauek, mikroskopia emaitzen nahiko antzekoak izan daitezkeela talde taxonomiko handiak kontuan hartuz gero (*1. Kapitulua*). Beraz, orokorrean, eDNA metabarcoding bidez zehazten diren ugaritasun erlatibo hauek erabilgarriak eta fidagarriak izan litezke interpretazio ekologikoak egiteko (Martin et al., 2022; Piwosz et al., 2020). Hala ere, ugaritasun erlatibo honek ez du kuantifikaziorako balio, ez baitute zelula ugaritasun daturik ematen, ikerketa askotan loraketak edo espezie toxikoak aztertzeko ezinbestekoa dena.

HPLC bidezko pigmentuen analisiak Urdaibai estuarioko fitoplanktonaren biomasaren, komunitatearen osaeraren eta talde ezberdin bakoitzak biomasa totalari egiten dion ekarpena deskribatzeko balio izan du. Metodo honek aipatutako gainerako tekniken aldean duen abantaila nagusietako bat Chl *a* modu fidagarrian zehaztuz biomasaren estimazio zehatza

lortzeko aukera da, hau izanik nazioarteko politika ezberdinetan uraren kalitatea eta eutrofizazio maila ebaluatzeko biomasaren aldagai ofiziala (Seoane et al., 2011). Ikerketa honetan, Chl *a* erabili da estuarioko fitoplankton biomasaren denboran eta espazioan zeharreko dinamikak deskribatzeko (*1. Kapitulua*), saneamendu lanen erantzuna aztertzeko (*2. Kapitulua* eta *4. Kapitulua*) eta saneamendu lanen aurretik eta ondoren estuarioaren egoera ekologikoa zehazteko (*4. Kapitulua*). Hau kontutan hartuz, fitoplankton-komunitatearen osaera aztertzeko, egokiagoa da talde bakoitzak Chl *a* honi egiten dio ekarpena zehazteko pigmentu diagnostikoak (PD) eta hauen PD:Chl *a* ratioak erabiltzea, zenbaketa mikroskopikoetan oinarritzea baino. Gainera, ikerketa honetan zehar, pigmentuen analisiak mikroskopia bidez baztertuak edo gutxietsiak izan diren taldeen identifikazio eta kuantifikazioa ahalbidetu du (Jeffrey et al., 1997), estuarioaren kanpoaldean haptofitoekin eta prasinofitoekin gertatu dena, adibidez. Metabarcoding-aren kasuan bezala, pigmentuen analisian ere emaitzak aztertutako bolumen handiago batean (2 L inguru) oinarritu dira eta, gainera, teknika azkarra eta erreproduzigarria da (Agirbas et al., 2015). Honek azaltzen du pigmentuen analisia fitoplanktonaren jarraipenerako maiz erabiltzea bai ekosistema itsastarretan (Lee et al., 2022; Zhang et al., 2023) baita estuarioetan (Cereja et al., 2022; Anil et al., 2023) eta ur gezatan ere (Marguš et al., 2023; Tanttu et al., 2023). Hala ere, pigmentuen bidez fitoplankton-komunitatearen osaketa deskribatzen laguntzen duten tresna kimiotaxonomiko gehienek (adibidez, Letelier et al., 1993; Mackey et al., 1996; Uitz et al., 2006), muga komun batzuk partekatzen dituzte (Higgins et al., 2011), emaitzen interpretazioaren xehetasun falta eragin dezaketenak.

Ahultasun horiei aurre egiteko asmoz, eta ikerketa honetan zehar aurkitutako beharrak asetzeko, tresna kimiotaxonomiko berri bat proposatu da, *PIGMENTUM* (*3. Kapitulua*). Tresna honen bidez 10 pigmentu-talde ezberdinek (fitoplankton talde guztiak biltzen dituztenak) Chl *a*-ri egiten dioten ekarpena zehazteko aukera dago, HPLC bidezko pigmentuen datu-multzo batean oinarrituta. *3. Kapituluan*, *PIGMENTUM* fitoplankton-komunitatearen deskribapen errealista lortzeko tresna azkarra eta erraza dela frogatu da, eta Chl *a* kontzentrazio eta gazitasun baldintza oso ezberdinak dituzten ur-masetan aplikatu daitekeela, eutrofizazio arazoak dituzten ekosistemetan bereziki eraginkorra delarik. Tresna honek eskaintzen duen nobedade nagusia PD-ak “pigmentu-taldeei" esleitzea izan zen, "talde taxonomikoen" ordez, "talde taxonomikoak" definitzeak eragin dezakeen gehiegizko sinplifikazioa, eta beraz akatsak, ekiditeko. Horrez gain, *PIGMENTUM*-ek, beste tresna kimiotaxomiko batzuen aldean abantaila ugari eskaintzen ditu (*3. Kapitulua*), hala nola, aztergai den fitoplankton-komunitatearen aurretiko ezagutzaren menpekotasun eza, laginak

modu independentean aztertzeko aukera eta PD:Chl *a* ratioa zuzentzeko aukera, behar duen laginari bakarrik aplikatuko zaiona, datu-multzo osoa aldatu gabe. Hau ikusita, Urdaibai estuarioko fitoplankton-komunitatea aztertzeko, eta batez ere, fitoplanktonak saneamendu lanen aurrean duen erantzuna aztertzeko *PIGMENTUM* erabiltzea erabaki zen (*4. Kapitulua*), tresnaren lehenengo aplikazioa izan dena. Metodo honek, estuarioko komunitatearen denboran eta espazioan zeharreko aldakortasuna deskribatu, saneamendu lanen ondoriozko aldaketak hauteman eta biomasa altuak edo/eta loraketa isolatuak eragiten zituzten taldeak zehaztea ahalbidetu du, teknika osagarrien beharrik gabe. Esaterako, *PIGMENTUM*-ek estuarioan saneamendu lanen ondoren egondako PrCAren areagotzea hauteman zuen, gainerako jarraipen-teknikak erabiliz ez litzatekeena ikusiko, talde honetako organismo gehienen izaera pikoplanktonikoagatik mikroskopiak ez dituelako identifikatu (ezta zenbatu) eta metabarcoding-ak (nahiz eta identifikatu) ez duelako hauen kuantifikazioa egiteko gaitasunik. Gainera, bestelako tresna kimiotaxonomiko batzuk erabiliz ere ez litzateke posible izango aldaketa hau hautematea, izan ere, PrCAen bilakaera aztertzeko derrigorrezkoa da prasinoxantina PD moduan aukeratzea (Yu et al., 2007), eta tresna kimiotaxonomiko askok ez dute kontuan hartzen (adibidez, Vidussi et al., 2001), prasinofitoak Chl *b* ekarpena egiten duten bestelako alga berdeekin batera multzokatuz (CbCA taldean). Honenbestez, *PIGMENTUM*-ek egiten duen 10 PDen aukeraketa (normalean erabiltzen diren 7 PDak gehi prasinoxatina, Dibinil Chl *a* eta baukeriaxantina) erabakigarria izan da ikerketa honetan. Horrez gain, *PIGMENTUM*-ek proposatutako paradigma-aldaketak garrantzia handia izan zuen *4. Kapituluan*, izan ere, Urdaibai estuarioaren barruko eremuko FCA taldean diatomeak ez ezik (Fuco-ari esleitu ohi zaion "talde taxonomikoa"), *K. foliaceum* dinoflagelatua ere bazegoen, aitzitik taldeko taxoi nagusia zena araztegiko hondakin-uren isurketak egon bitartea. Orain arte jarraitu den PDak eta talde taxonomikoak lotzeko ohitura hori mantendu izatekotan, dinoflagelatu honek egindako Chl *a*-ren ekarpena diatomeei esleituko litzaieke. Banakako ratioaren zuzenketa ere arrakastaz aplikatu zen hainbat aldiz 2020 eta 2022ko datuetan, batez ere kriptofitoen loraketen ondorioz, Urdaibai estuarioko barruko eremuan maiz gertatzen zirenak. Hala ere, gainerako teknikekin gertatzen den bezala, pigmentuen analisian lortutako informazioak muga batzuk ditu. Kasu honetan, desabantaila nagusia informazio taxonomiko eza da, izan ere, datuen interpretazioa zailtzen du eta fitoplanktonaren dibertsitateari edo espezie toxikoei buruzko jarraipenetan aplikazio mugatua izatea dakar. Horrek azaltzen du zergatik, nahiz eta *PIGMENTUM* teknika osagarrien beharrik gabe tresna eraginkorra izan, *4. Kapitulurako* pigmentuen analisian oinarritutako fitoplanktonaren jarraipena mikroskopia bidezko laginen behaketarekin osatu den.

Oro har, Urdaibai estuarioko fitoplankton-komunitatea aztertzeko teknika ezberdinen konbinazioak bi alderdi garrantzitsu agerian utzi ditu. Alde batetik, ikerketa honek frogatu du, fitoplanktona eutrofizazioaren adierazle gisa aztertzean erabili ohi den Chl *a* (adibidez, UEZean), fitoplankton-komunitatearen osaeraren aldaketekin batera ikertu behar dela (Niveditha et al., 2022). Ikerketa honetan, fitoplanktonak estuarioko saneamendu lanen aurrean izandako epe-ertaineko erantzuna aztertzeko Chl *a* bakarrik erabili izan balitz (*4. Kapitulua*), ikusitako biomasa igoeraren ondorioz emaitzak negatiboak zirela ondoriozta zitekeen, biomasa igoera hau estuarioaren egoeraren hobekuntza adierazi zezakeen komunitate osaeraren aldaketa batek eragin zuen arren. Honenbestez, eta hainbat ikertzailerekin bat eginez (Ferreira et al., 2011; Van Meerssche eta Pinckney, 2019), fitoplanktonaren ugaritasunean oinarritutako uraren kalitate-irizpideek komunitatearen osaera ere kontuan hartu beharko lukete. Bestalde, emaitzek baieztatu dute hiru metodoen erabilera bateratuak Urdaibai estuarioko fitoplankton komunitatearen, eta honen denboran eta espazioan zeharreko aldakortasunaren eta saneamendu lanekiko erantzunaren irudi askoz osatuagoa lortzeko aukera eman duela. Hala ere, argi geratu da teknika bakoitzak galdera ekologiko ezberdinei erantzuten diela eta, beraz, jarraipen-teknikaren aukeraketa, aukeratu behar izanez gero, jarraipenaren helburuaren araberakoa izan beharko litzatekeela.

Proposamen gisa, uraren kalitatearen kudeaketa helburu duten etorkizuneko fitoplankton-jarraipenetarako edo/eta helburu ekologikoak dituzten epe luzerako jarraipen-programentzako, non bereizmen taxonomiko altua derrigorrezkoa ez den, aukerarik onena *4. Kapituluan* deskribatutako metodologia jarraitzea izango liteke. Fitoplanktonaren azterketa HPLC bidezko pigmentuen analisian (aukerazko tresna kimiotaxonomikoarekin batera, adibidez, *PIGMENTUM*) oinarritu beharko litzateke, lortutako datuak mikroskopio bidezko identifikazioarekin osatuz (zenbaketarik gabe), komunitateko pigmentu-talde bakoitzaren taxon nagusiak ezagutzeko, honek emaitzen interpretazioan lagundu eta taxon toxiko edo espezie-aniztasunari buruzko informazio osagarria eskaintzen baitu (beharrezkoa izatekotan). Gainera, Chl *a* kontuan hartzeko modukoa bada (altuegia izateagatik adibidez), taxon nagusienak mikroskopio bidez ere zenbatu beharko lirateke. Metodologia honek aukera ematen du, fitoplanktonak, epe-laburrean gertatzen diren perturbazioekiko, urtarokotasunaren ondorioz edo klima-aldaketaren aurrean duen erantzuna aztertzeko, modu azkar eta erreproduzigarri batean. Aipatu behar da komunitatearen deskribapen kualitatiboa (taxonen identifikazioa) egiteko teknika eraginkorrena eDNA metabarcoding-a dela, identifikazio taxonomikorako gaitasun handiagoa duelako. Hala ere, teknika hau oraindik ez da mikroskopia bezain eskuragarria jarraipen arruntak egiteko, eta, beraz, fitoplankton

espezieen dibertsitatea aztertzeko helburu zehatza ez badago, mikroskopia bidezko identifikazioa egitea komeni da, kuantifikazioa ere ahalbidetzen duelako.

VI. ONDORIOAK ETA TESIA

Tesi honetan lortutako ondorioak hurrengoak dira:

1. Araztegiko isurketak egon bitartean (2019-2020), fitoplanktonaren biomasa (Chl *a*) handiagoa zen estuarioaren barrualdera hurbildu ahala eta udaberri-uda sasoian. Estuarioaren kanpoko eta erdiko eremuetan diatomeoak (hala nola, *Minutocellus polymorphus* eta *Chaetoceros tenuissimus*) nagusi ziren, eta barrualdean ordea, kriptofitoak (*Teleaulax acuta* eta *Urgorri complanatus,* adibidez) gailentzen ziren gehienbat, nahiz eta alga berdeak ere ugariak izan (*Eutreptiella* spp. eta organismo pikoplanktonikoak, esaterako). Gainera, araztegiaren inguruan, *Kryptoperidinium foliaceum* dinoflagelatuak eta diatomeo txikiek (adibidez, *Cyclotella* spp.) ekarpen garrantzitsua egiten zioten biomasa totalari.

2. Saneamendu lanen aurretik, Chl *a*-ren hazkuntza mantenugaiekin eta tenperaturarekin positiboki erlazionatua zegoen, eta gazitasunarekin ordea, negatiboki. Komunitatearen osaeraren aldaketak, luzetarako gazitasun-gradiente nabariak eta mantenugai ez-organikoen kontzentrazioek (batez ere araztegitik zetozenak) eragin zituzten gehienbat, argiaren eskuragarritasuna (edo uhertasuna) bezalako beste aldagai batzuekin batera.

3. Gernikako araztegiko isurketak estuariotik kanpo desbideratzeak amonio eta fosfato kontzentrazioen berehalako beherakada eragin zuen estuarioaren barruko eremuan. Hau, urte bat geroago (epe ertainera) nabarmenagoa bihurtu zen, mantenugai horien jaitsiera

estuario osoan zehar nabaritu baitzen. Horrek, mantenugaien ratio molarrak aldatzea eta estuarioko uraren kalitatea hobetzea eragin zuen, adierazle fisiko-kimikoen egoera "oso ona" lortuz (817/2015 Errege Dekretua).

4. Estuarioaren barruko eremuko fitoplankton-komunitateak saneamendu lanekiko berehalako erantzuna erakutsi zuen. *Eutreptiella* sp.-aren hazkunde azkarraren ondorioz, *b* klorofila eremu horretako pigmentu diagnostiko nagusia bihurtu zen, aloxantina eta fukoxantina ordezkatuz. Hala ere, komunitatearen osaera aldaketa hau ez zen denboran zehar mantendu, urtebete geroago (2022an) ez baitzen ikusi.

5. Saneamendu lanen ondoriozko fitoplanktonaren epe ertaineko erantzuna nabaria izan zen bai biomasan baita komunitatearen osaeran ere. Mantenugaien kargen murrizketak estuarioaren kanpoko eta erdiko eremuan Chl *a* gutxitzea eragin zuen (mantenugaien eskuragarritasuna murrizteagatik) eta araztegiaren inguruan handitu egin zen, ziurrenik gehiegizko amonio kargek fitoplanktonaren hazkuntza mugatzen zutelako. Komunitatearen osaerari dagokionez, prasinoxantinadun algen (PrCA) biomasa ekarpena handitu egin zen (URD1-URD4), eta barruko eremuan (batez ere URD5-URD6), diatomeoen ekarpena handitu (FCA) eta flagelatuena (ACA eta CbCA) murriztu egin zen, ziurrenik nitratoaren eskuragarritasun handiagoaren eta DIN:Si ratioaren txikitzearen ondorioz, eremuaren berreskurapenaren seinale izan zitekeena.

6. Mikroskopia, DNA metabarcoding-a eta pigmentuen analisia teknika osagarriak dira. Mikroskopiak, identifikazio taxonomikoa (orokorrean genero mailara arte) eta kuantifikazio egokiak ahalbidetu ditu, ezinbestekoa dena komunitateko zenbait taxon aztertzerakoan (adibidez, loraketak sortzen dituztenak edo/eta taxon toxikoak). Metabarcoding-ak, bereizmen taxonomiko handia eskaini du (mikroskopiak baino handiagoa, batez ere pikoplanktonarentzat) eta Urdaibai estuarioan aurretik identifikatuak izan ez diren 223 espezieren agerpenaren berri eman du. Pigmentuen analisiak, fitoplankton-komunitatearen deskribapen kualitatibo eta kuantitatibo azkar eta erreproduzigarria ahalbidetu du, nahiz eta bereizmen taxonomikoa baxua izan.

7. *PIGMENTUM*, fitoplankton-komunitatearen karakterizaziorako eta honek epe laburreko aldaketen, zein urtarokotasun aldaketen edo/eta inpaktu antropogenikoen aurrean erakusten duen erantzuna zehazteko tresna eraginkorra dela frogatu da, bereizmen taxonomiko handia derrigorrezkoa ez denean, teknika gehigarrien beharrik gabe eta bestelako tresna kimiotaxonomikoetan zeuden hainbat eragozpenei aurre eginez.

Tesia

Honenbestez, ikerketa honen ondorioak kontuan hartuta, lortutako tesia honako hau da:

Urdaibai estuarioko fitoplankton-komunitateak (i) biomasa eta osaeraren denboran eta espazioan zeharreko aldakortasuna erakusten du, gehienbat gazitasunak eta, mantenugaien kasuan, haien kontzentrazioak ez ezik, konposizio eta ratio molarrak ere eragindakoa, eta (ii) berehalako eta epe-ertaineko aldaketak jasan ditu, bai biomasan baita komunitatearen osaeran ere, Gernikako araztegiko hondakin-urak estuariotik kanpo desbideratzearen ondoriozko mantenugai kargen aldaketei erantzunez; (iii) komunitate aldaketa hauek jarraipen-tekniken konbinazioari esker hobeto ulertu direlarik.

VI. CONCLUSIONS AND THESIS

The conclusions obtained in this thesis are following:

1. During wastewater discharges, phytoplankton biomass (Chl *a*) increased towards the inner estuary and spring-summer seasons. Diatoms (e.g., *Minutocellus polymorphus* and *Chaetoceros tenuissimus*) dominated the outer and middle estuary, while mostly cryptophytes (e.g., *Teleaulax acuta* and *Urgorri complanatus*), but also green algae (e.g., *Eutreptiella* spp. and picoplanctonic organisms), prevailed in the inner estuary; although in the surroundings of the WWTP the dinoflagellate *Kryptoperidinium foliaceum* and small diatoms (e.g., *Cyclotella* spp.) became important as well.

2. Before sewerage works, the increase of Chl *a* showed positive relationship with nutrients and temperature, and negative with salinity; while the changes in the community composition were promoted mainly by the strong longitudinal gradients of salinity and inorganic nutrient concentrations (mainly coming from the WWTP), together with other factors such as light availability (or turbidity).

3. The diversion of the Gernika WWTP effluent outside the estuary resulted in an immediate decrease of ammonium and phosphate concentrations in the inner estuary, which became more marked in the medium-term, when the decrease of these nutrients was noticeable thought to entire estuary. This altered the nutrient molar ratios and led to the improvement

of the water quality of the estuary, achieving "very good" status for the physicochemical indicators (Royal Decree 817/2015).

4. The phytoplankton community showed an immediate response to sewerage works in the inner estuary. Chlorophyll *b* became the dominant diagnostic pigment in the area (replacing alloxanthin and fucoxanthin) due to the rapid increase of *Eutreptiella* sp., but this community composition shift was not permanent a year later (in 2022).

5. The medium term effects of the sewerage works on the phytoplankton community were noticeable both in biomass and community composition. The decrease in nutrient loadings led to the decrease of Chl *a* in the outer and middle estuary (less nutrient availability) and the increase in the surroundings of the WWTP, since the excessive ammonium loadings were probably suppressing phytoplankton growth. Regarding community composition, the contribution of prasinoxanthin-containing algae (PrCA) to total biomass increased (URD1-URD4), and, in the inner estuary (especially URD5-URD6), there was a shift from flagellates (ACA and CbCA) to diatoms (FCA) probably as a result of higher nitrate availability over ammonium and the decrease of DIN:Si, which might be a sign of the recovery of the area.

6. Microscopy, DNA metabarcoding and pigment analysis were complementary techniques. Microscopy enabled adequate taxonomic identification (mainly until genus level) and quantification, this being essential when studying abundances of certain taxa in the community (e.g. bloom-forming and/or toxic taxa). Metabarcoding provided high taxonomic resolution (higher than microscopy, especially for picoplankton) and revealed the presence of 223 species that had not been previously identified in the Urdaibai estuary. Pigment analysis enabled the rapid and reproducible qualitative and quantitative description of the phytoplankton community, although with low taxonomic resolution.

7. *PIGMENTUM* was validated as a powerful tool for the characterization of the phytoplankton community and the determination of phytoplankton responses to short-term perturbations, seasonal changes or/and anthropogenic impacts, when high taxonomic resolution is not compulsory, without the need of using additional techniques and addressing several drawbacks present in previous chemotaxonomic approaches.

Thesis

Therefore, taking into account the conclusions of this study, the resulting thesis is:

The phytoplankton community of the Urdaibai estuary (i) shows spatio-temporal variability in both biomass and composition mostly prompted by salinity and, in the case of nutrients, not just their concentration but also their composition and molar ratios, and (ii) underwent immediate and medium term changes, both in biomass and community composition, in response to changes in nutrient loadings caused by the diversion of the wastewater discharges from the Gernika WWTP outside de estuary, which (iii) are better understood due to the combined application of different monitoring techniques.

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ACRONYMS AND ABBREVIATIONS

ACA: Alloxanthin Containing Algae / Aloxantinadun algak

Allo: Alloxanthin /Aloxantina

ANOSIM: Analysis of similarities

ASV: Amplicon Sequence Variants / Anplikon sekuentziaren aldaerak

BCA: 19'-butanoyloxyfucoxanthin Containing Algae / But-dun algak

BCE: Bayesian Compositional Estimator

But: 19'-butanoyloxyfucoxanthin / 19'-butanoiloxifukoxantina

CbCA: Chlorophyll *b* Containing Algae / *b* klorofiladun algak

CCA: Canonical Correspondence Analysis

Chl *a*: Chlorophyll *a* / *a* klorofila

Chl *b*: Chlorophyll *b* / *b* klorofila

CNV: Copy Number Variation / Kopia zenbakiaren aldakortasuna

DCA: Divinyl chlorophyll *a* Containing Algae/ Dibinil *a* klorofiladun algak

DIN: Dissolved Inorganic Nitrogen / Disolbaturiko nitrogeno inorganikoa

DO: Dissolved Oxygen / Disolbaturiko Oxigenoa

DP: Diagnostic Pigment

DV Chl *a:* Divinyl chlorophyll *a* / Dibinil *a* klorofila

eDNA: Environmental deoxyribonucleic acid / ingurumen DNA

ESD: Equivalent Spherical Diameter

FCA: Fuco Containing Algae / Fukoxantinadun algak

Fuco: Fucoxanthin /Fukoxantina

HAB: Harmful Algal Bloom / Alga loraketa kaltegarriak

HBBE: Hegaztientzako Babes Bereziko Eremu

HCA: 19'-hexanoyloxyfucoxanthin Containing Algae / Hex-dun algak

Hex: 19'-hexanoyloxyfucoxanthin / 19'-hexanoiloxifukoxantina

HPLC: High Performance Liquid Chromatography / Bereizmen altuko kromatografia likidoa

HTS: High Throughput Sequencing

HUA: Hondakin Uren Araztegia

IEEZ: Itsas Estrategiaren Esparru Zuzentaraua

IQR: Interquartile Range

ISE: Inverse Simultaneous Equations

MSFD: Marine Strategy Framework Directive

NGS: Next Generation Sequencing

nMDS: Non-metric Multidimensional Scaling

OTU: Operational Taxonomic Unit / Unitate Taxonomiko Operatiboa

PAR: Photosynthetically Active Radiation

PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

PD: Pigmentu Diagnostikoa

PeCA: Peridinin Containing Algae / Peridininadun algak

Peri: Peridinin / Peridinina

PERMANOVA: Permutational Multivariate Analysis of Variance

PR2: Protist Ribosomal Reference Database

Pras: Prasinoxanthin / Prasinoxantina

PrCA: Prasinoxanthin Containing Algae / Prasinoxantinadun algak

QL: Quantification Limit

rRNA: Ribosomal Ribonucleic Acid

SIM%: Similarity percentage

SIMPER: Similarity percentages analysis

UEZ: Uraren Esparru Zuzentaraua

Vauch: Vaucheriaxanthin /Baukeriaxantina

VCA: Vaucheriaxanthin Containing Algae / Baukeriaxantinadun algak

WFD: Water Framework Directive

WWTP: Wastewater Treatment Plants

ZCA: Zeaxanthin Containing Algae / Zeaxantinadun algak

Zea: Zeaxanthin /Zeaxantina

ANNEXES

1. Annexes for *Chapter 1*

Table A1.1. *Data of the physicochemical parameters measured during the study period along the Urdaibai estuary and in the Oka river.*

Station	Date	Secchi disck (m)	Salinity (PSU)	pH	Conductivity (µS cm^{-1})	Temperature (ºC)	Dissolved O_2 (mgL^{-1})	O_2 saturation (%)	Turbidity (NTU)	Ammonium (µmolL^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmolL^{-1})	Silicate (µmolL^{-1})
URD1	**26/09/2019**	2.5	34.89	8.02	48412.6	20.627	7.51	102.7	2.68	14.8	0.20	5.70	0.40	2.2
	10/10/2019	3.25	34.51	7.97	47236.7	19.918	7.71	103.8	1.84	13.5	0.20	2.5	0.60	2.4
	24/10/2019	1.75	30.71	7.84	39093	16.078	7.87	96.2	4.61	14.0	0.40	9.3	0.80	7.1
	20/11/2019	1.5	26.7	7.85	31583.4	12.465	8.89	98.5	5.27	13.2	0.50	17.0	0.90	23.4
	04/12/2019	1.25	20.37	7.7	23442.8	10.198	9.5	96.3	7.52	9.60	0.60	24.5	1.00	40.8
	19/12/2019	2	34.27	7.95	40879.6	13.714	8.13	97	2.76	2.90	0.45	3.65	0.25	3.60
	08/01/2020	3.25	34.19	7.94	40457.7	13.36	8.54	101	1.44	4.30	0.20	7.80	0.35	6.9
	21/01/2020	2.5	33.67	7.86	37500	10.78	8.73	97.5	2.3	3.30	0.20	7.4	0.20	5.95
	06/02/2020	3	35.99	7.85	42266	13.268	8.52	101.8	1.11	5.50	0.40	7.30	0.20	6.95
	20/02/2020	2.75	34.63	7.76	41554.4	14.015	8.54	102.7	2.1	8.3	0.6	7.4	0.5	2.7
	20/03/2020	1.5	28.07	7.85	35327.1	15.152	8.69	102.6	2.67	7.8	0.6	10.3	0.4	14.3
	17/04/2020	3	32.24	8.03	42817.2	18.182	7.93	101.9	3.59	4.9	0.2	4.0	0.2	6.8
	04/05/2020	3.3	35.05	8.88	47500.4	19.532	7.8	104.6	0.89	4.3	0.2	2.3	0.1	0.8
	18/05/2020	2.9	31.11	8.26	42414.2	19.224	7.99	104	0.76	4.8	0.2	3.4	0.1	5.9
	02/06/2020	3.1	33.56	8.93	48517.9	22.38	7.37	103.2	0.48	4.1	0.2	0.8	0.1	0.8
	16/06/2020	3	29.78	7.9	40799.7	19.189	7.63	98.5	1.18	7.9	0.5	3.9	0.3	7.9
	30/06/2020	3	35.1	8.09	48620	20.6	9.04	123.5	2.77	3.5	0.2	0.8	0.3	0.8
	17/07/2020	3	33.1	9.25	49539.2	24.058	7.07	101.6	1.79	5.9	0.4	0.8	0.5	1.8
	29/07/2020	3.3	35.05	8.11	51188	23.1	7.31	104.5	1.37	5.3	0.2	0.8	0.7	0.8
	17/08/2020	3.7	35.02	8.76	51234.5	23.214	6.86	98.3	0.82	4.0	0.2	0.8	0.5	0.8
	31/08/2020	3.3	34.69	9.45	50448.1	22.804	7.02	99.8	2.32	4.7	0.2	0.8	0.4	0.8
	14/09/2020	3	34.36	9.66	50093.7	22.938	7.22	102.4	0.91	4.30	0.20	0.80	0.35	1.75
	29/09/2020	2.5	33.44	9.77	45819.3	19.81	7.53	100.5	1.3	3.80	0.20	3.80	0.35	2.30
URD2	**26/09/2019**	1.75	33.36	8.02	46606.8	20.716	7.37	100	2.33	22.2	1.05	4.40	0.65	6.3
	10/10/2019	1.75	29.69	7.86	40846.6	19.414	7.43	96.3	3.15	35.0	2.30	7.7	1.25	19.4
	24/10/2019	0.6	12.61	7.86	16614.6	14.1	8.69	91.4	17.46	15.8	1.35	50.0	1.55	53.7
	20/11/2019	1	12.97	7.9	15821.4	11.046	9.69	95.5	7.32	5.05	0.60	37.6	1.20	60.5
	04/12/2019	0.6	6.29	7.92	7582.4	8.478	10.48	93.3	12.34	5.85	0.55	34.8	1.10	55.9
	19/12/2019	1.1	22.25	8.02	26620.2	12.093	8.89	95.1	7.24	7.60	0.55	12.3	0.55	38.9
	08/01/2020	1.25	24.89	7.92	28927.2	11.32	8.84	94.5	3.23	20.4	1.20	18.8	0.90	33.5
	21/01/2020	0.85	20.34	7.85	22692.2	8.972	9.5	93.7	9.87	10.8	0.80	25.5	0.55	44.0
	06/02/2020	1.85	27.49	7.77	32120.5	11.922	8.6	94.7	3.68	20.5	1.30	14.8	0.80	29.3
	20/02/2020	1.85	28.74	7.75	33778.7	12.338	8.66	97	3.8	22.3	1.3	10.8	0.9	16.5
	20/03/2020	1.2	14.8	7.86	19586.9	14.837	9.15	99	4.56	13.8	0.8	37.3	0.8	58.1
	17/04/2020	1.5	25.47	8.05	34681.8	18.24	8.34	103.1	5.29	15.6	1.2	10.7	0.5	27.2
	04/05/2020	1.6	29.64	8.55	41850.7	20.629	7.64	101.2	2.14	8.1	0.9	7.5	0.4	15.5
	18/05/2020	1.25	20.25	8.2	28862	19.342	8.36	102.3	3.37	10.8	0.9	11.9	0.1	23.6
	02/06/2020	1.6	28.36	8.48	42934.9	23.791	7.11	99	2.5	13.1	1.5	5.6	0.3	12.4
	16/06/2020	1.5	20.61	7.85	29320	19.337	7.74	94.9	2.27	18.2	1.1	14.5	0.8	23.0
	30/06/2020	1.25	29.08	8.08	42598	22.2	7.84	106.7	3.54	7.5	1.2	4.6	0.5	6.0
	17/07/2020	1.2	27.19	9.01	41118.5	23.529	6.91	95.1	3.34	22.3	1.6	6.1	0.8	14.8
	29/07/2020	1.5	32.37	7.96	48179	23.6	5.6	79.5	4.45	18.0	1.7	3.4	1.0	12.3
	17/08/2020	1.75	34.39	8.81	50271.3	23.076	7.06	100.4	1.85	6.0	0.6	0.8	0.5	3.3
	31/08/2020	1.1	30.09	9.18	43093.5	21.389	7.07	95.2	4.72	26.3	2.2	8.5	0.9	19.9
	14/09/2020	1.3	32.16	9.12	48611.3	24.375	7.08	101.8	1.15	13.10	2.15	5.10	0.45	13.55
	29/09/2020	0.6	24.16	9.33	33046.3	18.221	7.76	95.2	7.71	10.10	1.10	33.10	0.60	26.10

***Table A1.1.** (Continued).*

Station	Date	Secchi disck (m)	Salinity (PSU)	pH	Conductivity (µS cm^{-1})	Temperature (ºC)	Dissolved O_2 (mg L^{-1})	O_2 saturation (%)	Turbidity (NTU)	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmol L^{-1})	Silicate (µmol L^{-1})
	26/09/2019	1.25	30.46	7.91	43485.3	21.29	7.16	96.5	3.98	44.4	3.00	8.95	1.25	20.1
	10/10/2019	1.5	26.41	7.69	37062.6	19.79	6.93	88.8	3.48	76.1	5.35	15.6	2.40	38.8
	24/10/2019	0.25	2.89	7.79	4206.6	14.017	8.24	81.5	31.07	35.8	1.85	63.5	1.95	72.1
	20/11/2019	0.8	1.15	8.06	1612.9	10.563	10.33	93.4	8.86	13.8	0.65	57.4	0.70	83.1
	04/12/2019	0.25	0.5	8.15	705.5	9.033	10.43	90.6	32.83	16.1	0.50	53.7	0.80	89.4
	19/12/2019	1.2	14.39	8.02	17723.1	11.745	9.13	92.2	5.66	16.4	1.05	34.0	0.85	75.0
	08/01/2020	1.5	20.39	7.88	23907	10.947	9.03	93.1	2.92	30.5	1.65	24.7	1.30	59.1
	21/01/2020	0.75	13.65	7.83	15559.2	8.525	9.66	90.2	10.57	14.6	0.80	27.3	0.80	75.6
	06/02/2020	1.5	23.43	7.68	27917.9	12.12	8.18	80.1	3.9	33.3	1.95	21.1	1.40	44.7
	20/02/2020	1.75	24.12	7.69	28824.3	12.345	8.5	92.5	4.51	35.9	1.9	15.5	1.4	30.5
	20/03/2020	1.25	4.9	7.87	6978.7	14.46	9.4	94.9	4.42	23.6	0.7	30.4	1.0	84.1
URD3	**17/04/2020**	1	18.19	8.21	25671.5	18.455	9.76	115.9	5.61	23.2	1.6	14.0	0.9	43.4
	04/05/2020	1.25	23.65	8.44	34609.1	21.275	7.55	97.8	3.01	19.5	1.9	14.0	0.7	33.0
	18/05/2020	1.25	14.56	8.11	21607.8	19.924	8.27	99	3.9	21.4	1.3	21.0	0.6	38.6
	02/06/2020	1.4	23.95	8.31	37398.1	24.495	6.58	90.5	2.54	23.2	1.5	7.7	0.4	13.8
	16/06/2020	1.1	16.72	7.83	24210.8	19.299	7.83	89	3.63	24.7	1.5	21.9	1.0	35.7
	30/06/2020	1	24.98	7.88	37406	23.1	6.05	80.8	4.58	33.6	2.6	11.6	1.0	21.8
	17/07/2020	1.5	24.01	8.82	36946.9	23.77	6.04	82	2.69	56.1	4.5	11.1	1.6	35.6
	29/07/2020	1.25	29.21	7.74	45131	24.1	3.98	56	5.72	31.7	1.9	3.7	1.7	24.1
	17/08/2020	1.25	31.42	8.64	46374.2	23.066	6.98	97.7	3.05	24.1	2.5	4.8	1.0	18.9
	31/08/2020	1.1	24.54	8.98	35933.2	21.54	6.65	86.8	5.8	52.8	4.4	17.8	1.8	42.4
	14/09/2020	1.5	29.17	8.75	44712	24.566	6.82	96.8	2.83	50.15	5.60	11.05	1.65	30.05
	29/09/2020	0.6	18.47	9.01	25708.5	17.889	7.49	88.1	9.27	13.80	1.55	44.65	0.75	37.20
	26/09/2019	1	26.38	7.73	38838.6	22.076	6.33	84.5	5.6	86.8	6.30	15.2	2.55	37.2
	10/10/2019	1.15	21.8	7.54	31025.5	19.599	6.12	76	5.77	95.3	5.70	16.7	3.45	63.2
	24/10/2019	0.2	0.45	8.09	716.3	13.907	9.76	94.8	57.49	19.5	1.05	73.2	1.45	91.2
	20/11/2019	0.9	0.23	8.22	344.7	10.433	10.48	93.9	7.06	18.4	0.65	59.8	0.75	106
	04/12/2019	0.9	0.21	8.18	443.3	8.66	11.05	94.9	8.41	25.9	0.40	59.8	1.15	106
	19/12/2019	1.25	6.84	8.03	8855.3	11.487	9.39	89.9	5.41	34.1	1.15	37.2	1.10	91.3
	08/01/2020	1.3	13.48	7.84	16160	13.48	9.36	91.3	3.56	36.8	1.60	24.1	1.75	76.7
	21/01/2020	0.75	7.55	7.82	8922.3	8.215	9.77	87.1	10.51	38.9	1.10	34.2	1.30	76.1
	06/02/2020	1.15	17.05	7.55	21036.6	12.398	7.88	82.1	12.14	53.5	2.85	29.2	2.25	60.3
	20/02/2020	1.25	18.25	7.61	22389.9	12.414	8.34	87.5	5.44	59.5	2.9	23.9	2.2	39.1
	20/03/2020	1	1.24	7.98	1912.7	14.609	9.2	91.2	5.01	33.2	0.8	52.5	0.8	96.5
URD4	**17/04/2020**	0.6	12.81	8.41	18634	18.479	10.75	123.8	6.36	25.1	2.1	24.0	1.7	56.1
	04/05/2020	1.1	17.44	8.31	26205	21.205	7.1	88.6	3.34	39.2	3.1	21.5	1.1	49.5
	18/05/2020	1	7.15	8.08	11183.7	19.722	8.12	92.6	4.96	48.1	2.1	26.1	1.0	52.8
	02/06/2020	1.1	18.89	8.25	30333	24.773	6.31	84.6	4.25	57.5	3.8	14.8	1.2	36.0
	16/06/2020	0.65	9.36	7.7	14109.4	18.997	7.26	82.7	6.63	37.4	1.2	16.4	0.7	44.2
	30/06/2020	1.2	19.73	7.68	30327	22.8	4.22	54.9	3.99	61.8	3.3	14.5	2.1	42.0
	17/07/2020	1.1	18.92	8.7	20709.9	23.673	5.21	68.5	3.68	59.9	3.0	10.9	2.2	47.6
	29/07/2020	1.3	24.87	7.54	38744	24.2	2.58	35.4	4.58	106.9	5.6	10.8	4.1	51.8
	17/08/2020	1.2	26.6	8.38	40056.2	23.21	6.02	82.1	4.47	71.4	5.1	8.4	3.0	43.3
	31/08/2020	0.75	18.64	8.86	27725.5	20.987	6.17	77.2	8.84	64.5	5.5	26.2	2.4	61.4
	14/09/2020	1.25	25.02	8.64	39624.5	25.422	6.15	86.4	2.26	112.90	9.00	16.60	4.00	53.90
	29/09/2020	0.5	12.4	8.88	17780.1	17.704	7.73	87.4	10.89	21.50	2.40	66.55	0.85	56.40

***Table A1.1.** (Continued).*

Station	Date	Secchi disck (m)	Salinity (PSU)	pH	Conductivity (µS cm^{-1})	Temperature (ºC)	Dissolved O_2 (mg L^{-1})	O_2 saturation (%)	Turbidity (NTU)	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmol L^{-1})	Silicate (µmol L^{-1})
	26/09/2019	1.15	20.81	7.6	31118.8	21.73	6.15	78.9	6.64	147	7.65	21.2	4.95	59.9
	10/10/2019	0.75	16.62	7.56	24665.3	20.426	6.45	78.9	8.84	146	7.10	31.0	5.90	78.4
	24/10/2019	0.2	0.18	8.31	289.5	13.851	9.74	94.3	35.4	8.60	0.60	75.9	0.80	99.0
	20/11/2019	0.9	0.18	8.27	264.3	10.166	10.83	96.4	6.15	22.5	0.55	62.2	0.80	108
	04/12/2019	1	0.18	8.17	254.5	8.299	11.25	95.8	6.92	37.2	0.20	58.6	1.10	103
	19/12/2019	1.1	0.43	8.38	638.2	11.163	10.28	93.8	5.79	45.6	0.70	53.8	1.15	88.8
	08/01/2020	1.25	5.47	7.9	8150.2	10	10.47	96.3	4.71	70.5	1.90	46.3	2.25	90.8
	21/01/2020	0.8	0.46	8.27	620.2	7.554	10.66	89.3	7.86	68.9	1.00	58.2	2.10	105
	06/02/2020	0.9	8.01	7.65	10386.4	12	7.9	77.1	7.05	98.4	2.40	36.4	3.60	64.5
	20/02/2020	0.75	11.79	7.63	15097.6	11.79	8.25	83.7	10.47	82.9	3.2	43.9	3.2	62.3
	20/03/2020	1	0.21	8.24	350.1	14.575	10.28	101.2	4.69	30.3	0.5	54.1	0.4	104.6
URD5	**17/04/2020**	0.6	8.81	8.12	12914	17.488	9.36	103.2	7.56	46.9	1.6	14.5	2.3	60.2
	04/05/2020	0.75	11.63	8.31	17995.2	20.957	7.13	85.5	6.22	57.9	3.3	30.3	2.2	71.8
	18/05/2020	0.6	1.13	8.38	1916.3	18.159	8.99	95.9	7.48	38.5	1.3	27.4	1.1	74.1
	02/06/2020	0.75	13.79	8.3	22862.3	24.986	6.07	79.5	4.93	67.4	3.4	17.1	2.0	44.9
	16/06/2020	0.5	2.11	8.03	3429.2	17.996	8.04	86	9.46	47.9	1.6	28.8	1.3	55.7
	30/06/2020	0.6	14.45	7.66	22870	22.9	4	50.6	5.87	64.7	2.6	15.2	2.5	54.0
	17/07/2020	0.8	13.32	8.78	21521.5	23.588	6.05	77	5.24	99.0	3.4	18.8	4.2	62.6
	29/07/2029	0.9	20.76	7.52	32611	24.1	1.83	24.5	6.03	94.8	3.4	9.9	5.3	64.5
	17/08/2020	1	20.72	8.32	32080.2	23.389	5.61	74.2	3.91	139.4	5.7	11.1	6.2	70.4
	31/08/2020	0.5	13.15	8.98	20060.4	20.775	6.5	78.4	16.33	64.1	5.0	35.7	2.7	62.2
	14/09/2020	0.75	19.26	8.75	32199.2	25.664	7.14	97.9	8.35	166.20	8.55	21.40	8.05	66.35
	29/09/2020	0.5	6.39	9.03	9522.4	17.222	8.04	86.9	10.76	42.75	2.25	65.55	1.25	79.45
	26/09/2019	0.75	18.66	7.62	28355.1	22.04	6.17	78.7	10.16	197	7.00	23.4	7.70	66.2
	10/10/2019	0.6	14.37	7.55	21375.9	19.947	6.83	81.7	10.06	163	6.65	33.7	6.80	85.0
	24/10/2019	0.2	0.15	8.11	247.8	13.981	9.56	92.8	36.53	47.6	1.10	72.3	1.25	101.4
	20/11/2019	1	0.16	8.25	244.3	10.093	10.95	97.3	5.91	30.6	0.80	61.9	1.05	112
	04/12/2019	1	0.16	8.12	222.7	8.246	11.22	95.4	6.63	27.9	0.20	58.4	1.05	102
	19/12/2019	0.85	0.24	8.36	371.1	11.575	9.71	89.3	7.08	148	0.70	51.2	2.10	93.2
	08/01/2020	0.9	4.57	7.87	6833.7	-	9.31	97.8	4.13	125	1.80	46.5	4.10	100.0
	21/01/2020	0.6	0.27	8.23	376.5	8.11	10.24	86.8	8.62	228	0.80	46.6	5.90	105
	06/02/2020	0.5	8.79	7.59	11267.9	11.832	7.65	74.8	39.5	113	2.95	42.3	3.95	78.4
	20/02/2020	0.5	8.4	7.62	11016.2	12.641	7.78	77.1	11.38	423.0	3.4	33.9	21.6	79.7
	20/03/2020	0.75	0.21	8.12	347.8	14.416	10.16	99.6	5.77	189.6	0.6	49.0	2.4	86.1
URD6	**17/04/2020**	0.5	7.73	7.73	13785.9	17.327	7.38	81.5	10.59	207.2	2.3	18.4	8.2	50.2
	04/05/2020	0.6	9.37	8.3	14595.9	20.511	7.26	85.2	9.71	65.7	2.4	18.7	2.4	68.5
	18/05/2020	0.55	0.31	8.38	543	17.36	9.12	95.2	6.72	140.3	0.7	24.5	2.6	70.8
	02/06/2020	0.7	11.84	8.26	19662.9	24.438	6.51	83.4	6.07	90.2	3.8	21.9	3.6	47.9
	16/06/2020	0.5	1.19	7.94	1971.4	17.376	7.54	79.2	9.91	334.5	1.4	26.2	7.0	80.3
	30/06/2020	0.5	12.88	7.61	20355	22.3	3.95	49	5.62	73.4	2.1	18.6	2.6	52.9
	17/07/2020	0.5	11.19	8.71	18201.8	23.203	5.72	71.3	9.36	137.6	4.8	25.3	5.8	53.0
	29/07/2020	0.6	20.13	7.45	31357	24.1	1.35	18	7.92	153.4	5.2	16.6	8.3	76.2
	17/08/2020	0.75	17.51	8.28	27494.2	23.327	5.74	74.5	6.18	220.4	5.1	12.0	11.4	80.3
	31/08/2020	0.4	9.95	8.82	15428.4	20.49	7.13	84	23.03	170.5	4.0	35.3	6.1	85.6
	14/09/2020	0.5	18.68	8.65	30335.4	25.3	6.36	86	6.41	197.35	8.35	22.45	10.00	76.40
	29/09/2020	0.5	3.39	9	5241.1	16.944	7.98	84.2	10.89	80.25	2.40	77.55	2.50	89.25

Table A1.1. *(**Continued**).*

Station	Date	Secchi disck (m)	Salinity (PSU)	pH	Conductivity (µS cm^{-1})	Temperature (ºC)	Dissolved O^2 (mg L^{-1})	O_2 saturation (%)	Turbidity (NTU)	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmol L^{-1})	Silicate (µmol L^{-1})
Oka River	**26/09/2019**	-	0.22	7.85	390	17.47	7.78	81.4	2.92	2.90	2.15	52.9	1.25	142
	10/10/2019	-	0.22	7.83	367.4	15.658	8.08	81.4	2.21	3.40	1.80	48.7	1.20	123
	24/10/2019	-	0.15	7.9	252.1	14.422	9.43	92.5	52.05	2.65	0.70	65.5	0.40	94.3
	20/11/2019	-	0.19	8.12	288.6	10.509	10.76	96.6	29.44	0.80	0.20	66.4	0.20	88.4
	04/12/2019	-	0.2	8	295.1	9.23	11.12	96.8	5.59	0.80	0.20	64.1	0.25	94.7
	19/12/2019	-	0.22	8.18	339.6	12.22	10.08	94.1	3.5	3.35	0.20	59.1	0.35	96.8
	08/01/2020	-	0.23	8.11	317	8.549	11.71	100.3	1.72	3.15	0.80	59.8	0.35	91.4
	21/01/2020	-	0.22	8.13	304.7	7.499	11.49	95.9	3.21	4.65	0.45	59.6	0.35	101
	06/02/2020	-	0.24	8.05	334.5	8.62	11.73	100.7	1.86	0.8	0.80	52.4	0.35	75.1
	20/02/2020	-	0.22	8.05	320.7	10.087	12	106.7	1.9	2.4	0.8	50.4	0.3	65.1
	20/03/2020	-	0.22	8.21	348.5	12.704	11.04	104.2	2.8	5.0	0.2	61.5	0.1	89.2
	17/04/2020	-	0.2	8.03	349.1	15.997	9.1	92.2	4	2.1	1.4	50.9	0.1	67.1
	04/05/2020	-	0.22	8.69	388.7	18.223	8.88	94.4	2.91	3.4	2.0	57.5	0.2	95.4
	18/05/2020	-	0.22	8.37	364.3	14.789	9.72	96.1	3.17	4.8	1.3	57.6	0.1	75.9
	02/06/2020	-	0.23	8.43	416.4	18.817	7.39	79.5	3.52	6.8	3.2	52.8	0.4	58.5
	16/06/2020	-	0.23	8.26	387.6	16.165	8.92	90.8	3.01	4.4	0.8	48.5	0.1	84.2
	30/06/2020	-	0.22	7.77	384.5	17.3	8.38	87.9	-	4.9	2.8	57.6	0.4	92.4
	17/07/2020	-	0.22	9.2	402.7	18.413	7.77	82.9	3.12	4.4	2.8	55.2	0.5	88.8
	29/07/2020	-	0.22	7.81	415.9	19.7	6.57	73	-	4.8	2.8	60.4	1.0	142.6
	17/08/2020	-	0.22	9.15	412.2	19.729	6.52	71.4	2.54	3.8	2.4	65.8	0.9	156.3
	31/08/2020	-	0.19	9.51	327.8	16.825	8.25	85.1	29.1	0.8	0.7	76.0	0.5	106.2
	14/09/2020	-	0.22	9.88	408.8	19.746	6.73	73.7	1	3.90	5.10	65.45	0.75	141.20
	29/09/2020	-	0.24	9.82	400	15.115	9.17	91.3	2.42	0.80	0.50	88.20	0.40	100.00

Table A1.2. *Data of the hydro-meteorological parameters considered during the study period, provided by the Basque Agency of Meteorology (Euskalmet) and the Provincial Council of Bizkaia.*

Date	Hours of sunshine per month	Average daily accumulated rainfall (L mm^{-2})		
		Muxika	Arteaga	Average
sep-19	175.1	103.6	80.5	92.05
oct-19	123.9	144.7	128	136.35
nov-19	38.8	484.6	423	453.8
dec-19	95.2	157.3	143.2	150.25
jan-20	106.0	50	40	45
feb-20	121.3	72.8	53.9	63.35
mar-20	109.6	173.3	155.9	164.6
apr-20	105.1	68.9	55.3	62.1
may-20	211.7	52.9	64	58.45
jun-20	153.4	115	86.4	100.7
jul-20	210.9	25.2	19.9	22.55
aug-20	185.5	74.7	49.1	61.9
sep-20	203.9	101.6	100.2	100.9

Table A1.3. *Data of the Oka River flow provided by the Provincial Council of Bizkaia.*

Sampling dates	Oka river flow (m3 s^{-1})						
	6 days before	5 days before	4 days before	3 days before	2 days before	1 day before	Sampling day
26/09/2019	0.071	0.079	0.149	0.082	0.074	0.064	0.062
10/10/2019	0.070	0.081	0.063	0.061	0.050	0.092	0.061
24/10/2019	0.064	0.059	1.220	0.362	0.369	0.819	1.454
20/11/2019	4.401	3.318	6.026	4.281	3.978	1.919	1.319
04/12/2019	0.827	0.843	2.215	1.526	4.696	1.745	1.217
19/12/2019	6.631	1.820	1.140	0.896	0.798	0.683	0.622
08/01/2019	0.347	0.328	0.358	0.328	0.306	0.291	0.288
21/01/2020	0.255	0.246	0.267	0.275	1.414	0.487	0.414
06/02/2020	0.249	0.246	0.233	0.227	0.276	0.253	0.238
20/02/2020	0.210	0.209	0.203	0.231	0.210	0.200	0.198
20/03/2020	0.563	0.636	**7.414**	3.107	1.346	0.952	0.789
17/04/2020	0.258	0.283	0.236	0.223	0.215	0.225	0.262
04/05/2020	0.442	0.310	0.269	0.251	0.235	0.217	0.211
18/05/2020	0.353	0.802	0.445	0.380	0.317	0.281	0.269
01/06/2020	0.180	0.172	0.164	0.158	0.152	0.147	0.150
16/06/2020	0.369	0.855	0.897	0.511	0.426	0.310	0.291
30/06/2020	0.812	0.363	0.512	0.217	0.221	0.175	0.377
17/07/2020	**0.001**	0.003	0.003	0.003	**0.001**	0.005	**0.001**
29/07/2020	0.095	0.114	0.101	0.106	0.107	0.113	0.117
17/08/2020	0.105	0.126	0.091	0.065	0.065	0.057	0.052
31/08/2020	0.044	0.047	0.046	0.056	0.188	0.266	0.117
14/09/2020	0.068	0.064	0.066	0.067	0.063	0.061	0.062
29/09/2020	0.058	0.081	0.350	1.966	1.449	0.190	0.132

Table A1.4. *Loading values (correlations) of the first two PCs of the PCA done for 11 physico-chemical parameters.*

	PC 1	PC 2
Salinity	**-0.92633**	-0.03254
pH	-0.27815	-0.17981
Temp (ºC)	-0.66187	0.51633
O2 (mg L^{-1})	0.52833	**-0.77594**
O2 saturation (%)	-0.12686	**-0.80419**
Turbidity (NTU)	0.63543	0.037615
Ammonium (µmol L^{-1})	0.37148	**0.77708**
Nitrate (µmol L^{-1})	**0.91274**	-0.1605
Phosphate (µmol L^{-1})	0.30179	**0.80951**
Silicate (µmol L^{-1})	**0.92264**	0.17248

Table A1.5. *Taxa list identified by microscopy (Micro) and eDNA metabarcoding (Metabar) in Urdaibai estuary in the present study, indicating if they have been previously described in the estuary in research papers or technical reports.1: Ansotegui et al. (2001); 2: Ansotegui et al. (2003); 3: Iriarte et al. (1997); 4: Madaria and Orive (1989); 5: Madariaga (1995); 6: Madariaga et al. (1989); 7: Orive (1988); 8: Orive et al. (1998); 9: Sarobe (2009); 10: Trigueros (2001); 11: Trigueros and Orive (2000); 12: Trigueros and Orive (2001); 13: Trigueros et al. (2000b).*

	Micro	Metabar	Published research papers	Tech. reports (URA)
Green algae				
Bathycoccus prasinos W. Eikrem & J. Throndsen		X		
Carteria lunzensis Pascher & Jahoda		X		
cf. *Ankistrodesmus* Corda	X			
cf. *Closterium* Nitzsch ex Ralfs	X			X
Chlamydomonas kuwadae Gerloff		X		
Chlamydomonas spp. Ehrenberg	X		2	X
Chlorochytrium lemnae Cohn		X		
Chlorococcum hypnosporum R.C. Starr		X		
Chlorococcum spp. Meneghini		X		
Chlorococcum tatrense P.A. Archibald		X		
Chlorogonium spp. Ehrenberg		X		
Chloroidium saccharophilum (W. Krüger) Darienko & al.		X		
Chloromonas kasaiae Matsuzaki, Nakada, Y. Hara & Nozaki		X		
Chloroparva pannonica Somogyi, Felfoldi & Voros		X		
Chloroparvula japónica Lopes dos Santos, M.H. Noël & Eikrem		X		
Chloroparvula pacifica Lopes dos Santos, M.H. Noël & Eikrem		X		
Chloropicon laureae Lopes dos Santos & Eikrem		X		
Chloropicon sieburthii Lopes dos Santos & Eikrem		X		
Coccomyxa sp. Schmidle		X		
Crustomastix didyma T. Nakayama, M. Kawachi & Inouye		X		
Desmodesmus abundans (Kirchner) E.H. Hegewald		X		
Dolichomastix tenuilepis J. Throndsen & A. Zingone		X		
Euglena sp. Ehrenberg	X		5, 9	X
Euglenophyceae Schoenichen		X	8	X
Eutreptiella eupharyngea Moestrup & R.E. Norris		X		X
Eutreptiella spp. A.M. Cunha	X		1, 2, 6, 9	X
Marsupiomonas pelliculata H.L.J. Jones, Leadbeater & J.C. Green		X		
Marsupiomonas spp. H.L.J. Jones, Leadbeater & J.C. Green		X		
Microglena monadina Ehrenberg		X		
Micrinomonas pusilla (R.W. Butcher) Doweld		X	1	
Microglena reginae Demchenko, Mikhailyuk & Proschold		X		
Micromonas bravo N. Simon, Foulon & B. Marin		X		
Micromonas commoda Baren, Bachy & Worden		X		
Micromonas spp. Manton & Parke		X	1, 2	
Monoraphidium contortum (Thuret) Komárková-Legnerová	X			
Nannochloris sp. Naumann		X		
Neospongiococcum gelatinosum Ettl & Gärtner		X		
Nephroselmis pyriformis (N. Carter) Ettl		X	1	
Nephroselmis sp. Stein	X		1, 2	
Oedogonium sp. Link ex Hirn		X		
Oltmannsiellopsis viridis M. Chihara & Inouye		X		X
Ostreococcus sp. C. Courties & M.-J. Chrétiennot-Dinet		X		
Ostreococcus lucimarinus Palenik et al.		X		
Ostreococcus mediterraneus B. Marin & N.H. Grimsley		X		
Ostreococcus tauri C. Courties & M.-J. Chrétiennot-Dinet		X		
Pachysphaera sp. Ostenfeld	X		1	X
Pediastrum duplex Meyen	X			X
Petalomonas plana Won J. Lee & D.J. Patterson		X		
Picochlorum spp. W.J Henley et al.		X		
Polytoma uvella Ehrenberg		X		
Prasinoderma coloniale T. Hasegawa & M. Chihara		X		
Prasinoderma singularis Jouenne		X		

Table A1.5. (Continued).

	Micro	Metabar	Published research papers	Tech. reports (URA)
Green algae				
Prasinoderma spp. T. Hasegawa & M. Chihara		X		
Pyramimonas australis Andreoli & Moro		X		
Pyramimonas gelidicola McFadden, Moestrup & Wetherbee		X		
Pyramimonas obovata N. Carter		X		
Pyramimonas propulsa Moestrup & D.R.A. Hill		X		
Pyramimonas spp. Schmarda	X	X	1, 2	X
Pyrobotrys squarrosa (Korshikov) Korshikov		X		
Sarcinofilum mucosum (Broady) Darienko & Pröschold		X		
Scenedesmus sp. Meyen	X		9	X
Spermatozopsis similis H.R. Preisig & M. Melkonian		X		
Tetradesmus obliquus (Turpin) M.J. Wynne		X		
Tetraselmis spp. F. Stein	X	X	1, 2	X
Trichosarcina sp. H.W. Nichols & Bold		X		
Tupiella akineta (Tupa) Darienko & Pröschold		X		
Ulothrix zonata (F. Weber & Mohr) Kützing		X		
Unkown flagellate chlorophyte	X			
Cryptophytes				
Chroomonas coerulea Geitler (Skuja)		X		
Chroomonas dispersa Butcher		X		
Chroomonas sp. Hansgirg		X	1, 2	X
Cryptomonas marssonii Skuja		X		
Cryptomonas obovate Skuja		X		
Cryptomonas obovoidea Pascher		X		
Cryptomonas paramaecium Hoef-Emden & Melkonian		X		
Cryptomonas pyrenoidifera Geitler		X		
Cryptomonas spp. Ehrenberg	X	X	2	X
Falcomonas daucoides (W. Conrad & H. Kufferath) D.R.A. Hill		X		
Geminigera cryophila (D.L. Taylor & C.C. Lee) D.R.A. Hill		X		
Goniomonas amphinema J. Larsen & D.J. Patterson		X		
Goniomonas sp. F. Stein		X		
Goniomonas truncata (Fresenius) F. Stein		X		
Guillardia theta D.R.A. Hill & R. Wetherbee		X		
Hemiselmis cryptochromatica C.E. Lane & J.M. Archibald		X		X
Hemiselmis sp. Parke	X	X	1, 2, 9	X
Hemiselmis virescens Droop		X		
Plagioselmis spp. Butcher, G. Novarino, I.A.N. Lucas, & S. Morrall	X			X
Rhodomonas pusilla (Bachmann) Javornický		X		
Rhodomonas spp. G. Karsten		X	1	X
Teleaulax acuta (Butcher) D.R.A. Hill	X	X	1	X
Teleaulax amphioxeia (W.Conrad) D.R.A. Hill		X		
Teleaulax gracilis Laza-Martínez	X	X		X
Teleaulax spp. D.R.A. Hill		X	9	X
Urgorri complanatus Laza-Martínez	X	X		X
Diatoms				
Achnanthes armillaris (O.F. Müller) Guiry	X		10, 12	
Achnanthes sp. Bory		X	10	
Actinoptychus splendens (Shadbolt) Ralfs		X		
Arcocellulus cornucervis Hasle, Stosch & Syvertsen		X		
Arcocellulus mammifer Hasle, Stosch & Syvertsen		X		
Arcocellulus sp. Hasle, Stosch & Syvertsen		X		
Asterionella formosa Hassall	X	X		
Asterionellopsis glacialis (Castracane) Round	X		1, 10, 12	X
Asteromphalus sp. Ehrenberg		X		
Bacillaria paxillifera (O.F. Müller) T. Marsson		X		
Bacteriastrum hyalinum Lauder		X	10	
Bacteriastrum jadranum Godrijan, Maric & Pfannkuchen		X		
Bacteriastrum parallelum D. Sarno, A. Zingone & D. Marino		X		
Bacteriastrum spp. Shadbolt	X		10	X
Berkeleya rutilans (Trentepohl ex Roth) Grunow		X		

Table A1.5. (Continued).

	Micro	Metabar	Published research papers	Tech. reports (URA)
Diatoms				
Brockmanniella brockmannii (Hustedt) Hasle, Stosch & Syvertsen		X		
Caloneis amphisbaena (Bory) Cleve		X		
Campylodiscus thuretii Brébisson		X		
Cerataulina pelagica (Cleve) Hendey	X	X	9, 10, 12	X
Chaetoceros spp. Ehrenberg		X		X
Chaetoceros affinis Lauder	X	X	1, 10, 12	X
Chaetoceros anastomosans Grunow		X		
Chaetoceros brevis F. Schütt		X	10, 12	
Chaetoceros calcitrans (Paulsen) H.Takano		X		
Chaetoceros cf. *compressus* Lauder	X		10, 12	
Chaetoceros cf. *coronatus* Gran	X			
Chaetoceros cf. *densus* (Cleve) Cleve	X			X
Chaetoceros cf. *laciniosus* F. Schütt	X		10	X
Chaetoceros costatus Pavillard	X	X		X
Chaetoceros curvisetus Cleve	X	X	9, 10, 12	X
Chaetoceros danicus Cleve		X	10, 12	X
Chaetoceros debilis Cleve		X		X
Chaetoceros decipiens Cleve	X	X	9, 10, 12	X
Chaetoceros didymus Ehrenberg	X			X
Chaetoceros eibenii Grunow		X	10, 12	
Chaetoceros jonquieri		X		
Chaetoceros lauderi Ralfs ex Lauder	X	X	9, 10	X
Chaetoceros lorenzianus Grunow	X	X		
Chaetoceros muelleri Lemmermann		X		
Chaetoceros protuberans Lauder		X		
Chaetoceros pseudocurvisetus Mangin	X	X		X
Chaetoceros sociales H.S. Lauder	X	X	5, 9	X
Chaetoceros sp. *P. quinquecorne* endosymbiont		X		
Chaetoceros sporotruncatus Gaonkar, Kooistra & Lange		X		
Chaetoceros tenuissimus Meunier	X	X	1, 10, 11, 12	X
Chaetoceros throndsenii Marino, Montresor & Zingone		X		
Cocconeis placentula Ehrenberg		X		
Conticribra guillardii (Hasle) Stachura-Suchoples & D.M. Williams	X	X	2, 9 –12	X
Conticribra weissflogii Stachura-Suchoples & D.M. Williams	X	X		X
Corethron hystrix Hensen		X	10, 12	
Coscinodiscus granii L.F. Gough		X		
Coscinodiscus sp. Ehrenberg	X	X	10,	X
Coscinodiscus wailesii Gran & Angst		X		
Cyclotella atomus Hustedt		X	2, 10, 11, 12	X
Cyclotella choctawhatcheeana Prasad		X		
*Cyclotella*spp. (Kützing) Brébisson		X	3, 8,	X
Cyclotella striata (Kützing) Grunow		X		
Cylindrotheca closterium (Ehrenberg) Reimann & J.C. Lewin	X	X	1, 7, 9, 10, 12	X
Cylindrotheca spp. Rabenhorst	X	X		
Cymbella cistuliformis Krammer		X		
Cymbella spp. C. Agardh	X	X	10	X
Dactyliosolen blavyanus	X	X	12	X
Delphineis sp. (H. Peragallo) Hasle		X		
Detonula pumila (castracane) Gran	X		10, 12	X
Diatoma sp. Bory	X			
Diatoma vulgaris var. *Linearis* Grunow		X	9, 10	
Discostella sp. V. Houk & R. Klee		X		
Ditylum brightwellii (T. West) Grunow	X	X	2, 10, 12	X
Ellerbeckia sol (Ehrenberg) R.M. Crawford & P.A. Sims		X	10, 12	
Encyonema formosum (Hustedt) D.G. Mann		X		
Entomoneis sp. Ehrenberg		X	10	
Entomoneis alata Ehrenberg (Ehremberg)		X		
Eucampia sp. Ehrenberg	X		10, 12	X
Eupyxidicula turris (Greville) S. Blanco & C.E. Wetzel		X	10	
Fragilaria sp. Lyngbye	X			

Table A1.5. (Continued).

	Micro	Metabar	Published research papers	Tech. reports (URA)
Diatoms				
Gomphonema parvulum (Kützing) Kützing		X		
Guinardia delicatula (Cleve) Hasle	X	X	4, 6, 10, 12	X
Guinardia flaccida (Castracane) H. Peragallo	X	X	4, 10, 12	X
Guinardia striata (Stolterfoth) Hasle	X	X	10, 12	X
Gyrosigma limosum Sterrenburg & Underwood		X		
*Halamphora*spp. (Cleve) Mereschkowsky		X	10	
Halamphora coffeiformis (C. Agardh) Mereschkowsky		X		
Halamphora semperpalorum Rimet & R. Jahn		X		
Haslea nipkowii (Meister) M. Poulin & G. Massé		X		
Haslea pseudostrearia Massé, Rincé & E.J. Cox		X		
Lauderia annulata Cleve	X	X	4, 9, 10, 12	X
Leptocylindrus convexus D. Nanjappa & A. Zingone		X		X
Leptocylindrus danicus Cleve	X	X	1, 4, 5, 7–12	X
Leptocylindrus minimus Gran	X		1, 9, 10, 12	X
Leptocylindrus sp. Cleve		X		
Licmophora communis (Heiberg) Grunow		X		
Licmophora paradoxa (Lyngbye) C. Agardh		X		
Licmophora sp. C. Agardh	X		9, 10, 12	X
Lithodesmium variabile H. Tanako		X		
Luticola sp. D.G. Mann		X		
Mediolabrus comicus (H.Takano) Yang Li		X		
Melosira cf. *arctica* Dickie	X			
Melosira cf. *moniliformis* C. Agardh	X		10, 12	X
Melosira nummuloides C. Agardh	X	X	10, 12	X
Melosira spp. C. Agardh		X		
Melosira varians C. Agardh	X	X		X
Meuniera membranacea (Cleve) P.C. Silva	X	X	9, 10, 12	X
Minidiscus sp. Hasle		X		
Minidiscus spinulatus (H.Takano) J.S. Park & J.H. Lee		X		
Minidiscus variabilis Kaczmarska		X		
Minutocellus polymorphus Hasle, Stosch, & Syvertsen	X	X		X
Navicula cryptocephala var. *veneta* (Kützing) Rabenhorst		X	10	
Navicula cryptotenella Lange-Bertalot		X		
Navicula gregaria Donkin		X	10	
Navicula lanceolata (C. Agardh) Kützing		X	10	
Navicula phyllepta Kützing		X	10	
Navicula pulchripora Kaczmarska & A.M. Chan		X		
Navicula radiosa Kützing		X	10	
Navicula reinhardtii (Grunow) Grunow		X		
Navicula salinicola Hustedt		X	10	
Navicula spp. Bory		X	9, 10, 12	X
Navicula tripunctata (O.F. Müller) Bory		X		
Neidium affine (Ehrenberg) Pfitzer		X		
Neobrightwellia alternans (Bailey) M.P. Ashworth & P.A. Sims		X	10, 12	
Nitzschia communis Rabenhorst		X		
Nitzschia dissipata (Kützing) Rabenhorst		X	10, 12	
Nitzschia draveillensis Coste & Ricard		X		
Nitzschia dubiiformis Hustedt		X		
Nitzschia inconspicua Grunow		X		
Nitzschia linearis W.Smith		X		
Nitzschia microcephala Grunow		X		
Nitzschia palea (Kützing) W. Smith		X		
Nitzschia sigma (Kützing) W. Smith		X	10, 12	
Nitzschia spp. Hassall		X	9, 10, 12	X
Nitzschia supralitorea Lange-Bertalot		X		
Nitzschia thermalis (Ehrenberg) Auerswald		X		
Odontella sp. C. Agardh	X			X
Papiliocellulus sp. Hasle, Stosch & Syvertsen		X		

Table A1.5. (Continued).

	Micro	Metabar	Published research papers	Tech. reports (URA)
Diatoms				
Pierrecomperia catenuloides K. Sabbe, N. Vyverman & L. Ribero		X		
Pleurosigma spp. W. Smith	X	X	10, 12	X
Proboscia alata (Brightwell) Sundström	X	X	4, 10, 12	X
Proboscia indica (H. Peragallo) Hernández-Becerril		X		X
Psammodictyon sp. D.G. Mann		X		
Pseudo-nitzschia cuspidata (Hasle) Hasle		X		
Pseudo-nitzschia fraudulenta (Cleve) Hasle		X		
Pseudo-nitzschia galaxiae Lundholm & Moestrup	X	X		X
Pseudo-nitzschia multiseries (Hasle) Hasle		X		
Pseudo-nitzschia seriata (Cleve) H. Peragallo		X	4, 7, 9, 10, 12	
Pseudo-nitzschia sp. H. Peragallo		X		
Pseudo-nitzschia spp. <3 µm	X			X
Pseudo-nitzschia spp. >3 µm	X			X
Pseudostriatella pacifica		X		
Rhizosolenia fallax B.G. Sundström		X		
Rhizosolenia formosa H. Peragallo		X		
Rhizosolenia imbricata Brightwell	X	X	9, 10, 12	
Rhizosolenia setigera Brightwell	X		10, 12	X
Rhizosolenia spp. Brightwell	X	X	12	X
Skeletonema costatum (Greville) Cleve	X		1, 3, 8, 10–12	
Skeletonema marinoi Sarno & Zingone		X		
Skeletonema menzelii Guillard, Carpenter & Reimann		X		
Skeletonema pseudocostatum L.K. Medlin		X		
Skeletonema spp. Greville		X		X
Skeletonema tropicum Cleve		X		
Stauroneis kriegeri R.M. Patrick		X		
Stellarima microtrias (Ehrenberg) G.R. Hasle & P.A. Sims		X		
Stephanocyclus meneghinianus Kulikovskiy, Genkal & Kociolek		X	10,	
Stephanopyxis sp. (Ehrenberg) Ehrenberg	X		10	
Striatella unipunctata (Lyngbye) C. Agardh	X		10, 12	X
Sundstroemia similoides Medlin, Lundholm, Boonprakob, Moestrup		X		
Surirella angusta Kützing		X	10, 12	
Surirella sp. Turpin		X	10, 12	
Synura petersenii Korshikov		X		
Thalassiosira aestivalis Gran		X		
Thalassiosira angustelineata (A.W.F. Schmidt) G. Fryxell & Hasle		X		
Thalassiosira delicatula Ostenfeld		X		
Thalassiosira eccentrica (Ehrenberg) Cleve		X		
Thalassiosira gessneri Hustedt		X		
Thalassiosira gravida Cleve		X	7, 9, 10, 12	X
Thalassiosira minima Gaarder		X		
Thalassiosira minuscula Krasske		X		
Thalassiosira oceanica Hasle		X		
Thalassiosira profunda (Hendey) Hasle		X		
Thalassiosira pseudonana Hasle & Heimdal		X		
Thalassiosira spp. Cleve		X	1, 2, 7, 9, 12	X
Thalassiosira spp.<20 µm	X			X
Thalassiosira spp.>20 µm	X			X
Thalassiosira tenera Proshkina-Lavrenko		X		
Trieres mobiliensis (Bailey) Ashworth & E.C. Theriot	X	X	10	X
Tryblionella apiculata W. Gregory		X	10, 12	
Ulnaria ulna (Nitzsch) Compère	X	X		X
Unidentified centric diatoms <5 µm	X			
Unidentified centric diatoms >20 µm	X			
Unidentified centric diatoms 10-20 µm	X			
Unidentified centric diatoms 5-10 µm	X			
Unidentified pennated diatoms <20 µm	X			
Unidentified pennated diatoms 20-50µm	X			
Unidentified pennated diatoms >50 µm	X			

Table A1.5. (Continued).

	Micro	Metabar	Published research papers	Tech. reports (URA)
Dinoflagellates				
Akashiwo sanguinea (K. Hirasaka) Gert Hansen & Moestrup	X			
Alexandrium hiranoi Kita & Fukuyo		X		
Alexandrium margalefii Balech		X		
Alexandrium minutum Halim		X		
Alexandrium spp. Halim	X	X		X
Amphidinium crassum Lohmann	X			X
Amphidinium sp. Claparède & Lachmann	X	X	2, 11	
Amylax triacantha var. *buxus* (Balech) Gárate-Lizárraga		X		
Ankistrodinium semilunatum Hoppenrath, Murray, Sparman, Leander		X		
Ansanella granifera H.J. Jeong, S.H. Jang, Moestrup & N.S. Kang		X		
Asulcocephalium miricentonis Takahashi, Moestrup, Iwataki		X		
Azadinium cf. *dexteroporum* Percopo & Zingone	X			X
Balechina gracilis (Bergh) F. Gómez, Artigas & Gast		X		
Biecheleria cincta (Siano, Montresor & Zingone) Siano		X		
Biecheleria spp. Moestrup, K. Lindberg & Daugbjerg		X		
Blixaea quinquecornis (T.H. Abé) Gottschling	X	X	2, 3, 4, 6–13	X
Borghiella tenuissima Moestrup, Gert Hansen & Daugbjerg		X		
Cryptoperidiniopsis brodyi Steidinger, Landsberg, Mason,Tester		X		
Dinophysis acuminata Claparède & Lachmann	X	X	10, 12	X
Dinophysis acuta Ehrenberg	X		10	X
Dinophysis caudata Kent	X		7, 10, 12	X
Dinophysis norvegica Claparède & Lachmann	X	X	1,	
Dinophysis spp. Ehrenberg		X	1, 7, 13	X
Dissodinium pseudolunula Swift ex Elbrächter & Drebes		X		
Fragilidium sp. Balech		X		
Gonyaulax ellegaardiae Mertens, Aydin, Takano, Yamaguchi		X		
Gonyaulax polygramma F. Stein		X		X
Gonyaulax sp. Diesing		X	10	X
Gonyaulax spinifera (Claparède & Lachmann) Diesing	X	X	10, 12	X
Gymnodinialesspp. < *20* µm Apstein	X			X
Gymnodiniales spp. > 20 µm	X			X
Gymnodinium dorsalisulcum Shauna Murray, Salas & Hallegraeff		X		
Gymnodinium sp. F. Stein		X	7, 9	X
Gyrodinium dominans Hulburt		X		
Gyrodinium fusiforme Kofoid & Swezy		X		
Gyrodinium guttula J. Larsen		X		
Gyrodinium helveticum (Penard) Y. Takano & T. Horiguchi		X		
Gyrodinium heterogrammum J. Larsen		X		
Gyrodinium rubrum (Kofoid & Swezy) Y. Takano & T. Horiguichi		X		
Gyrodinium spirale (Bergh) Kofoid & Swezy		X		
Gyrodinium spp. Kofoid & Swezy		X	7	X
Gyrodinium spp. > 50 µm	X			X
Gyrodinium spp. 20-50 µm	X			X
Gyrodinium spp. < 20 µm	X			X
Hematodinium sp. Chatton & Poisson		X		
Heterocapsa niei (A.R. Loeblich) L.C. Morrill & A.R. Loeblich		X		
Heterocapsa pygmaea Lobelich III, R.J. Schmidt & Sherley		X	1, 9–13	X
Heterocapsa rotundata (Lohmann) Gert Hansen		X	1, 2, 9–13	X
Heterocapsa spp. F.Stein		X	2, 10, 12	X
Heterocapsa spp. <20 µm	X			
Heterocapsa spp. >20 µm	X			
Ichthyodinium sp. Hollande & J. Cachon		X		
Islandinium tricingulatum E. Potvin, A. Rochon & C. Lovejoy		X		
Karenia brevis (C.C. Davis) Gert Hansen & Moestrup		X		
Karenia spp. Gert Hansen & Moestrup		X		
Karlodinium spp. J. Larsen		X		X
Karlodinium veneficum (D. Ballantine) J. Larsen		X		
Kofoidinium pavillardii J. Cachon & M. Cachon		X		
Kryptoperidiniaceae Er. Lindemann		X		

Table A1.5. (Continued).

	Micro	Metabar	Published research papers	Tech. reports (URA)
Dinoflagellates				
Kryptoperidinium foliaceum (F. Stein) Lindemann	X		2, 3, 5, 8–13	X
Kryptoperidinium triquetrum Tillmann, Gottschling, Elbrächter		X		
Lepidodinium chlorophorum Gert Hansen, Botes & Salas		X		
Lepidodinium spp. M.Watanabe, S. Suda, Inouye, T. Sawaguchi		X		
Levanderina fissa (Levander) Moestrup, Hakanen, Gert Hansen,		X		
Lingulodinium polyedra (F. Stein) J.D. Dodge		X		X
Luciella sp. P.L. Mason, Jeong, Litaker, Reece & Steidinger		X		
Margalefidinium fulvescens M. Iwataki, H. Kawami & Matsuoka		X		
Margalefidinium polykrikoides F. Gómez, Richlen		X		
Noctiluca scintillans (Macartney) Kofoid & Swezy		X		X
Nusuttodinium poecilochroum (J.Larsen) Y. Takano & T. Horiguchi		X		
Ostreopsis siamensis Johs. Schmidt	X	X		X
Oxyrrhis marina Dujardin		X	2, 13	
Oxyrrhis spp. F. Dujardin		X		
Oxytoxum caudatum Schiller	X			X
Oxytoxum gracile Schiller	X		10	X
Oxytoxum laticeps J. Schiller	X			X
Oxytoxum scolopax F. Stein	X			
Oxytoxum sp. Stein	X		10	X
Paragymnodinium shiwhaense N.S. Kang, H.J. Jeong, Moestrup		X		
Pelagodinium beii (H.J. Spero) Siano, Montresor, Probert & Vargas		X		
Pelagodinium spp. Siano, Montresor, Probert & Vargas		X	2	
Pellucidodinium psammophilum R. Onuma & T. Horiguchi		X		
Pentapharsodinium sp. Indelicato & A.R. Loeblich		X		
Pfiesteria shumwayae Glasgow & J.M. Burkholder		X		
Phalacroma favus Kofoid & J.R. Michener	X			
Phalacroma rotundatum Kofoid & J.R. Michener	X		10, 12	X
Podolampas palmipes Stein	X		10	X
Polykrikos geminatus (F.Schütt) D.X. Qiu & Senjie Lin		X		
Polykrikos kofoidii Chatton		X		
Prorocentrum balticum (Lohmann) Loeblich III	X		10, 12	X
Prorocentrum cordatum (Ostenfeld) J.D. Dodge		X		X
Prorocentrum micans Ehrenberg	X		1, 6, 9, 10, 12	X
Prorocentrum nanum J. Schiller		X		
Prorocentrum spp. Ehrenberg	X	X	1, 8, 10, 13	X
Proterythropsis sp. Kofoid & Swezy		X		
Protoceratium reticulatum (Claparède & Lachmann) Bütschli		X		X
Protodinium simplex Lohmann		X		
Protoperidinium bipes (Paulsen) Balech	X	X	10, 12	X
Protoperidinium depressum (Bailey) Balech	X	X	10, 12	X
Protoperidinium divergens (Ehrenberg) Balech	X		10, 12	X
Protoperidinium elegans (Cleve) Balech		X		
Protoperidinium monovelum (T.H. Abé) Balech		X		
Protoperidinium pallidum (Ostenfeld) Balech		X	10	
Protoperidinium pellucidum Bergh		X		
Protoperidinium stenii (Jørgensen) Balech	X		10	X
Pseliodinium fusus (F. Schütt) F. Gómez		X		
Scrippsiella acuminata (Ehrenberg) Kretschmann, Elbrächter		X		
Scrippsiella spp. Balech	X	X	1, 10, 12	X
Sourniaea diacantha H. Gu., K.N. Mertens, Zhun Li & H.H. Shin		X		
Spatulodinium pseudonoctiluca (Pouchet) J. Cachon & M. Cachon		X		
Symbiodinium spp. Gert Hansen & Daugbjerg		X		
Syndinium sp. Chatton		X		
Togula britannica Flø Jørgensen, Shauna Murray, & Daugbjerg		X		
Torodinium robustum Kofoid & Swezy	X	X		X
Triadinium polyedricum (Pouchet) J.D. Dodge	X	X	10, 12	X
Tripos cf. *macroceros* (Ehrenberg) Hallegraeff & Huisman	X		10, 12	X
Tripos digitatus (F.Schütt) F. Gómez		X		
Tripos furca (Ehrenberg) F. Gómez	X	X	7, 10, 12	X

Table A1.5. (Continued).

	Micro	Metabar	Published research papers	Tech. reports (URA)
Dinoflagellates				
Tripos fusus (Ehrenberg) F. Gómez	X	X	7, 10, 12	X
Tripos hexacanthus (Gourret) F. Gómez		X	10	
Tripos lineatus (Ehrenberg) F. Gómez	X		9, 10, 12	X
Tripos massiliensis (Gourret) F. Gómez		X		
Tripos muelleri Bory	X		10, 12	X
Tripos spp. Bory		X	7, 13	X
Warnowia sp. Lindemann		X	9	
Haptophytes				
Algirosphaera robusta (Lohmann) R.E. Norris		X		
Cf. *Calyptrosphaera* Lohmann	X			X
Chrysochromulina leadbeateri Estep, Davis & Sieburth		X		
Chrysochromulina rotalis Eikrem & Throndsen		X		
Chrysochromulina scutellum Eikrem & Moestrup		X		
Chrysochromulina sp. Lackey		X	1	X
Chrysochromulina spinifera (Fournier) Pienaar & R.E. Norris		X		
Chrysochromulina throndsenii Eikrem		X		
Chrysotila sp. P.L. Anand		X		
Diacronema sp. Prauser		X		
Dicrateria rotunda (N. Reynolds) El M. Bendif & I. Probert		X		
Gephyrocapsa huxleyi (Lohmann) W.W. Hay & H. Mohler	X		1, 2	X
Gephyrocapsa oceanica Kamptner		X		
Haptolina sp. Edvardsen & Eikrem		X		
Pavlomulina ranunculiformis S. Sym, R. Pienaar & M. Kawachi		X		
Phaeocystis cordata A. Zingone & M.J. Chrétiennot-Dinet		X		
Phaeocystis globosa Scherffel	X	X	1	
Phaeocystis sp. Lagerheim		X	2	X
Prymnesiales Papenfuss	X			
Prymnesium neolepis Edvardsen, Eikrem & Probert		X		
Prymnesium polylepis Edvardsen, Eikrem & Probert		X		
Prymnesium spp. Massart		X	2	
Rhabdosphaera clavigera G.Murray & V.H. Blackman	X			X
Syracosphaera mediterranea Lohmann		X		
Unknown cocolithophorids	X			X
Others				
Bolidophyceae				
Triparma eleuthera Ichinomiya & Lopes dos Santos		X		
Triparma laevis B.C. Booth		X		
Triparma mediterranea Ichinomiya & Lopes dos Santos		X		
Triparma pacifica Ichinomiya & Lopes dos Santos		X		
Triparma spp. B.C. Booth & H.J. Marchant		X		
Chrysophyceae				
Apoikia sp. Euns. Kim, Yubuki, B.S. Leander & L.E. Graham		X		
Chlorochromonas sp. I.F. Lewis		X		
Chromophyton rosanoffii Woronin		X		
Chrysamoeba spp. G.A. Klebs		X		
Chrysamoeba tenera Matvienko		X		
Chrysocapsa vernalis Starmach		X		
Chrysosaccus sp. Pascher		X		
Chrysosphaerella spp. Lauterborn		X		
Cladophora striolata Kützing		X		
Hydrurus foetidus (Villars) Trevisan		X		
Monas sp. O.F. Müller		X		
Naegeliella flagellifera Correns		X		
Ochromonas sp. Vysotskii	X	X	1	X
Poterioochromonas malhamensis (Pringsheim) Péterfi		X		
Poterioochromonas spp. Scherffel		X		
Uroglena sp. Ehrenberg		X		

Table A1.5. (Continued).

	Micro	Metabar	Published research papers	Tech. reports (URA)
Others				
Dictyochophyceae				
Apedinella radians (Lohmann) P.H. Campbell		X	1	X
Ciliophrys infusionum Cienkowski		X		
Dictyocha fibula var. *robusta* Schrader & Murray	X			
Dictyocha sp. Ehrenberg		X		X
Florenciella parvula Eikrem		X		
Florenciella sp. Eikrem		X		
Helicopedinella sp. Sekiguchi, Kawachi, Nakayama & Inouye		X		
Octactis speculum F.H. Chang, J.M. Grieve & J.E. Sutherland	X	X		X
Pedinella elastica Skuja		X		
Pseudochattonella sp. Hosoi, Honda, Fukaya, Inagaki & Sako		X		
Pseudochattonella verruculosa Y. Hara & M. Chihara		X		
Pseudopedinella sp. N. Carter		X		X
Pteridomonas danica D.J. Patterson & Fenchel		X		
Rhizochromulina marina D.J. Hibberd & Chrétiennot-Dinet		X		
Rhizochromulina sp. D.J. Hibberd & Chrétiennot-Dinet		X		
Vicicitus globosus (Y. Hara & Chihara) F.H. Chang		X		
Olisthodiscophyceae				
Olisthodiscus luteus N. Carter		X	1	
Pelagophyceae				
Ankylochrysis sp. C. Billard		X		
Aureococcus anophagefferens Hargraves & Sieburth		X		
Pelagomonas calceolata R.A. Andersen & G. Saunders		X		
Ectocarpus siliculosus (Dillwyn) Lyngbye		X		
Heribaudiella fluviatilis (Areschoug) Svedelius		X		
Raphidophyceae				
Heterosigma akashiwo (Y. Hada) Y. Hada ex Y. Hara & M. Chihara		X		
Synurophyceae				
Mallomonas annulata (D.E. Bradley) K. Harris		X		
Mallomonas cratis K. Harris & D.E. Bradley		X		
Tessellaria sp. Playfair		X		
Rhodophyta				
Corynoplastis japonica A. Yokoyama, J.L. Scott, G.C. Zuccarello		X		
Dixoniella grisea (Geitler) J.L. Scott, S.T. Broadwater		X		
Glaucosphaera vacuolata Korshikov		X		

Table A1.6. *Phytoplankton taxa identified by eDNA metabarcoding that have been described for the first time in the Urdaibai estuary in the present study.*

Diatoms	
Actinoptychus splendens	*Minidiscus variabilis*
Arcocellulus cornucervis	*Navicula cryptotenella*
Arcocellulus mammifer	*Navicula pulchripora*
Arcocellulus sp.	*Navicula reinhardtii*
Asteromphalus sp.	*Navicula tripunctata*
Bacillaria paxillifer	*Neidium affine*
Bacteriastrum jadranum	*Nitzschia communis*
Bacteriastrum parallelum	*Nitzschia draveillensis*
Berkeleya rutilans	*Nitzschia dubiiformis*
Brockmanniella brockmannii	*Nitzschia inconspicua*
Caloneis amphisbaena	*Nitzschia linearis*
Campylodiscus thuretii	*Nitzschia microcephala*
Chaetoceros anastomosans	*Nitzschia palea*
Chaetoceros jonquieri	*Nitzschia supralitorea*
Chaetoceros muelleri	*Nitzschia thermalis*
Chaetoceros protuberans	*Papiliocellulus* sp.
Chaetoceros calcitrans	*Pierrecomperia catenuloides*
Chaetoceros sporotruncatus	*Psammodictyon* sp.
Chaetoceros throndsenii	*Pseudo-nitzschia cuspidata*
Cocconeis placentula	*Pseudo-nitzschia fraudulenta*
Coscinodiscus granii	*Pseudo-nitzschia multiseries*
Coscinodiscus wailesii	*Pseudo-nitzschia* sp.
Cyclotella choctawhatcheeana	*Pseudostriatella pacifica*
Cyclotella striata	*Rhizosolenia fallax*
Cymbella cistuliformis	*Rhizosolenia formosa*
Delphineis sp.	*Sundstroemia similoides*
Discostella sp.	*Skeletonema marinoi*
Encyonema formosum	*Skeletonema menzellii*
Entomoneis alata	*Skeletonema pseudocostatum*
Gomphonema parvulum	*Skeletonema tropicum*
Gyrosigma limosum	*Stauroneis kriegeri*
Halamphora coffeiformis	*Stellarima microtrias*
Halamphora semperpalorum	*Synura petersenii*
Haslea nipkowii	*Thalassiosira anguste-lineata*
Haslea pseudostrearia	*Thalassiosira aestivalis*
Leptocylindrus sp.	*Thalassiosira delicatula*
Licmophora communis	*Thalassiosira eccentrica*
Licmophora paradoxa	*Thalassiosira gessneri*
Lithodesmium variabile	*Thalassiosira minima*
Luticola sp.	*Thalassiosira minuscula*
Mediolabrus comicus	*Thalassiosira oceanica*
Melosira spp.	*Thalassiosira profunda*
Minidiscus sp.	*Thalassiosira pseudonana*
Minidiscus spinulatus	*Thalassiosira tenera*
Cryptophytes	
Chroomonas coerulea	*Geminigera cryophila*
Chroomonas dispersa	*Goniomonas amphinema*
Cryptomonas marssonii	*Goniomonas* sp.
Cryptomonas obovoidea	*Goniomonas truncata*
Cryptomonas obovata	*Guillardia theta*
Cryptomonas paramaecium	*Hemiselmis virescens*
Cryptomonas pyrenoidifera	*Rhodomonas pusilla*
Falcomonas daucoides	*Teleaulax amphioxeia*
Haptophytes	
Algirosphaera robusta	*Dicrateria rotunda*
Chrysochromulina leadbeateri	*Gephyrocapsa oceanica*
Chrysochromulina spinifera	*Haptolina* sp.
Chrysochromulina rotalis	*Pavlomulina ranunculiformis*
Chrysochromulina scutellum	*Phaeocystis cordata*
Chrysochromulina throndsenii	*Prymnesium neolepis*
Chrysotila sp.	*Prymnesium polylepis*
Diacronema sp.	*Syracosphaera mediterranea*

Table A1.6. (Continued).

Green algae	
Bathycoccus prasinos	*Nannochloris* sp.
Carteria lunzensis	*Neospongiococcum gelatinosum*
Chlamydomonas kuwadae	*Oedogonium* sp.
Chlorochytrium lemnae	*Ostreococcus* sp.
Chlorococcum hypnosporum	*Ostreococcus lucimarinus*
Chlorococcum spp.	*Ostreococcus mediterraneus*
Chlorococcum tatrense	*Ostreococcus tauri*
Chlorogonium spp.	*Petalomonas plana*
Chloroidium saccharophilum	*Picochlorum* spp.
Chloromonas kasaiae	*Polytoma uvella*
Chloroparva pannonica	*Prasinoderma coloniale*
Chloroparvula japonica	*Prasinoderma singularis*
Chloroparvula pacifica	*Prasinoderma* spp.
Chloropicon laureae	*Pyramimonas australis*
Chloropicon sieburthii	*Pyramimonas gelidicola*
Coccomyxa sp.	*Pyramimonas obovata*
Crustomastix didyma	*Pyramimonas propulsa*
Desmodesmus abundans	*Pyrobotrys squarrosa*
Dolichomastix tenuilepis	*Spermatozopsis similis*
Marsupiomonas pelliculata	*Sarcinofilum mucosum*
Marsupiomonas spp.	*Tetradesmus obliquus*
Microglena monadina	*Trichosarcina sp.*
Microglena reginae	*Tupiella akineta*
Micromonas bravo	*Ulothrix zonata*
Micromonas commoda	
Dinoflagellates	
Alexandrium hiranoi	*Kryptoperidinium triquetrum*
Alexandrium margalefii	*Lepidodinium chlorophorum*
Alexandrium minutum	*Lepidodinium* spp.
Amylax triacantha var. buxus	*Levanderina fissa*
Ankistrodinium semilunatum	*Luciella* sp.
Ansanella granifera	*Margalefidinium fulvescens*
Asulcocephalium miricentonis	*Margalefidinium polykrikoides*
Balechina cincta	*Nusuttodinium poecilochroum*
Biecheleria gracilis	*Oxyrrhis* spp.
Biecheleria spp.	*Paragymnodinium shiwhaense*
Borghiella tenuissima	*Pelagodinium beii*
Cryptoperidiniopsis brodyi	*Pellucidodinium psammophilum*
Dissodinium pseudolunula	*Pentapharsodinium* sp.
Fragilidium sp.	*Pfiesteria shumwayae*
Gonyaulax ellegaardiae	*Polykrikos geminatus*
Gymnodinium dorsalisulcum	*Polykrikos kofoidii*
Gyrodinium dominans	*Prorocentrum nanum*
Gyrodinium fusiforme	*Proterythropsis* sp.
Gyrodinium guttula	*Protodinium simplex*
Gyrodinium helveticum	*Protoperidinium elegans*
Gyrodinium heterogrammum	*Protoperidinium monovelum*
Gyrodinium rubrum	*Protoperidinium pellucidum*
Gyrodinium spirale	*Pseliodinium fusus*
Hematodinium sp.	*Scrippsiella acuminata*
Heterocapsa niei	*Sourniaea diacantha*
Ichthyodinium sp.	*Spatulodinium pseudonoctiluca*
Islandinium tricingulatum	*Symbiodinium* spp.
Karenia brevis	*Syndinium* sp.
Karenia spp.	*Togula britannica*
Karlodinium veneficum	*Tripos digitatus*
Kofoidinium pavillardii	*Tripos massiliensis*

Table A1.6. (Continued).

Others	
Bolidophyceans *Triparma eleuthera* *Triparma laevis* *Triparma mediterranea* *Triparma pacifica* *Triparma* spp. **Chrysophyceans** *Apoikia* sp. *Chlorochromonas* sp. *Chromophyton rosanoffii* *Chrysamoeba* spp. *Chrysamoeba tenera* *Chrysocapsa vernalis* *Chrysosaccus sp.* *Chrysosphaerella* spp. *Cladophora striolata* *Hydrurus foetidus* *Monas* sp. *Naegeliella flagellifera* *Poterioochromonas malhamensis* *Poterioochromonas* spp. *Uroglena* sp. **Rhodophyta** *Corynoplastis japonica* *Dixoniella grisea* *Glaucosphaera vacuolata*	**Dictyochophyceans** *Ciliophrys infusionum* *Florenciella parvula* *Florenciella* sp. *Helicopedinella* sp. *Pedinella elastica* *Pseudochattonella* sp. *Pseudochattonella verruculosa* *Pteridomonas danica* *Rhizochromulina marina* *Rhizochromulina* sp. *Vicicitus globosus* **Pelagophyceans** *Ankylochrysis* sp. *Aureococcus anophagefferens* *Pelagomonas calceolata* *Ectocarpus siliculosus* *Heribaudiella fluviatilis* **Raphidophyceans** *Heterosigma akashiwo* **Synurophyceans** *Mallomonas annulata* *Mallomonas cratis* *Tessellaria* sp.

Table A1.7. *Phytoplankton species identified by microscopy analysis that have not been identified by eDNA metabarcoding in the present study. Differentiation among species that were not available in the database and those available but not detected by metabarcoding.*

Species not available in PR2	**Species available in PR2 but not identified by metabarcoding**
Achnanthes armillaris	*Akashiwo sanguinea*
Amphidinium crassum	*Asterionellopsis glacialis*
Chaetoceros cf. *compressus*	*Azadinium* cf. *dexteroporum*
Chaetoceros cf. *coronatus*	*Chaetoceros didymus*
Chaetoceros cf. *densus*	*Detonula pumila*
Chaetoceros cf. *laciniosus*	*Dinophysis acuta*
Dictyocha fibula var. robusta	*Dinophysis caudata*
Melosira cf. *moniliformis*	*Gephyrocapsa huxleyi*
Oxytoxum caudatum	*Kryptoperidinium foliaceum*
Oxytoxum gracile	*Leptocylindrus minimus*
Oxytoxum laticeps	*Melosira* cf. *arctica*
Oxytoxum scolopax	*Monoraphidium contortum*
Prorocentrum balticum	*Pediastrum duplex*
Protoperidinium stenii	*Phalacroma favus*
Rhabdosphaera clavigera	*Phalacroma rotundatum*
Tripos cf. *macroceros*	*Podolampas palmipes*
Tripos lineatus	*Prorocentrum micans*
Tripos muelleri	*Protoperidinium divergens*
	Rhizosolenia setigera
	Skeletonema costatum
	Striatella unipunctata

Table A1.8. *Two-way PERMANOVA analysis results. Permutation N: 9999. Sum of sqrs: sum of squares; df: degrees of freedom; MS: mean squares; F: ratio; p: significant levels.*

		Source	Sampling station	Season	Interaction
Microscopy	**Statistics**	**Sum of sqrs**	4.258	7.921	1.086
		df	5	3	15
		MS	0.852	2.640	0.072
		F	3.373	10.456	0.287
		p	0.0001	0.0001	0.116
Metabarcoding	**Statistics**	**Sum of sqrs**	8.689	8.756	1.915
		df	5	3	15
		MS	1.738	2.919	0.128
		F	7.477	12.558	0.549
		p	0.0001	0.0001	0.0007

Table A1.9. *One-way PERMANOVA pairwise test analysis results. Permutation N: 9999. p: significant levels.*

			Microscopy	Metabarcoding
Pairwise test, p	**Sampling station**	URD1, URD2	0.351	0.347
		URD1, URD3	0.020	0.020
		URD1, URD4	0.002	0.003
		URD1, URD5	0.002	0.002
		URD1, URD6	0.002	0.002
		URD2, URD3	1	1
		URD2, URD4	0.410	0.458
		URD2, URD5	0.045	0.048
		URD2, URD6	0.020	0.020
		URD3, URD4	1	1
		URD3, URD5	1	1
		URD3, URD6	0.905	0.902
		URD4, URD5	1	1
		URD4, URD6	1	1
		URD5, URD6	1	1
	Season	Autumn, Winter	0.009	0.009
		Autumn, Spring	0.002	0.001
		Autumn, Summer	0.0006	0.0006
		Winter, Spring	0.0006	0.0006
		Winter, Summer	0.0006	0.0006
		Spring, Summer	0.0006	0.0006

Table A1.10. *SIMPER analysis results. Top 15 taxa for each of the analysis performed: a) Microscopy analysis by sampling point; b) Microscopy analysis by season; c) Metabarcoding analysis by sampling point; d) Metabarcoding analysis by season.*

a) Microscopy-spatial variability

Taxa	Av. dissim	Contr. %	Cum. %	Mean abundance (cells L^{-1})					
				URD1	URD2	URD3	URD4	URD5	URD6
***Plagioselmis* spp.**	19.17	24.52	24.52	$3.13x10^5$	$9.75x10^5$	$1.68x10^6$	$1.49x10^6$	$1.35x10^6$	$1.14x10^6$
M. polymorphus	17.18	21.98	46.49	$1.63x10^6$	$3.38x10^6$	$1.88x10^6$	$3.63x10^5$	$8.63x10^4$	$4.78x10^4$
T. gracilis	8.376	10.71	57.21	$1.17x10^5$	$4.89x10^5$	$5.10x10^5$	$2.69x10^5$	$2.86x10^5$	$1.20x10^5$
T. acuta	5.819	7.444	64.65	$6.76x10^3$	$5.79x10^4$	$2.35x10^5$	$4.47x10^5$	$5.02x10^5$	$2.87x10^5$
***Tetraselmis* spp.**	5.283	6.758	71.41	$8.08x10^4$	$3.58x10^5$	$3.67x10^5$	$2.00x10^5$	$2.34x10^5$	$3.24x10^5$
C. tenuissimus	5.223	6.682	78.09	$4.45x10^5$	$1.44x10^6$	$6.91x10^5$	$2.12x10^5$	$3.92x10^4$	$2.15x10^4$
U. complanatus	4.059	5.192	83.28	0	$7.45x10^3$	$1.28x10^5$	$4.01x10^5$	$4.39x10^5$	$3.12x10^5$
Centric <5 µm	2.268	2.901	86.19	$9.56x10^3$	$5.30x10^3$	$9.07x10^3$	$2.88x10^4$	$2.51x10^5$	$3.77x10^5$
Pennated <20 µm	2.167	2.773	88.96	$4.89x10^4$	$4.47x10^4$	$4.47x10^4$	$7.20x10^4$	$1.14x10^5$	$9.55x10^4$
Prymnesiales	1.955	2.501	91.46	$2.13x10^5$	$3.17x10^4$	$7.45x10^3$	$4.49x10^3$	$2.83x10^3$	$2.40x10^3$
Centric <10 µm	1.424	1.821	93.28	$6.77x10^3$	$8.71x10^3$	$1.04x10^4$	$2.24x10^4$	$1.49x10^5$	$1.91x10^5$
Heterocapsa <20 µm	0.8105	1.037	94.32	$4.41x10^4$	$2.35x10^4$	$1.13x10^4$	$9.91x10^3$	$4.51x10^4$	$5.74x10^4$
K. foliaceum	0.7746	0.9909	95.31	35	503	$3.38x10^3$	$1.16x10^4$	$7.90x10^4$	$7.61x10^4$
***Eutrepriella* spp.**	0.7397	0.9462	96.25	395	899	$2.81x10^2$	$9.54x10^2$	$9.40x10^4$	$7.70x10^4$
***Chaetoceros* spp.**	0.6334	0.8102	97.07	$5.17x10^4$	$1.35x10^5$	$7.93x10^4$	$1.91x10^4$	$9.74x10^3$	$2.73x10^3$
Average dissim.				78%					

b) Microscopy-seasonal variability

Taxa	Av. dissim	Contr. %	Cum. %	Mean abundance (cells L^{-1})			
				Autumn	Winter	Spring	Summer
***Plagioselmis* spp.**	19.84	24.75	24.75	$6.01x10^5$	$3.38x10^5$	$1.10x10^6$	$2.29x10^6$
M. polymorphus	17.91	22.34	47.08	$3.67x10^5$	0	$3.73x10^5$	$3.63x10^6$
T. gracilis	8.138	10.15	57.24	$1.43x10^5$	$5.48x10^5$	$5.34x10^5$	$3.83x10^4$
T. acuta	5.908	7.37	64.61	$7.35x10^4$	$1.22x10^5$	$3.64x10^5$	$4.19x10^5$
C. tenuissimus	5.699	7.109	71.71	$1.57x10^3$	912	$3.63x10^4$	$1.64x10^6$
***Tetraselmis* spp.**	5.567	6.944	78.66	$2.43x10^4$	$2.06x10^4$	$3.73x10^5$	$5.40x10^5$
U. complanatus	4.14	5.164	83.82	$5.03x10^3$	177	$7.86x10^5$	$1.05x10^4$
Centric <5 µm	2.455	3.062	86.89	$3.46x10^4$	$5.50x10^3$	$3.88x10^3$	$3.84x10^5$
Pennated <20 µm	2	2.495	89.38	$7.42x10^4$	$7.98x10^4$	$8.18x10^4$	$4.97x10^4$
Prymnesiales	1.766	2.203	91.58	$5.68x10^4$	$2.60x10^4$	$5.00x10^4$	$3.79x10^4$
Centric <10 µm	1.477	1.842	93.42	$3.55x10^4$	$5.82x10^3$	$1.01x10^4$	$1.93x10^5$
***Eutrepriella* spp.**	0.8074	1.007	94.43	$1.01x10^4$	90	957	$9.74x10^4$
Heterocapsa <20 µm	0.7972	0.9945	95.43	$2.31x10^4$	$7.52x10^3$	$2.43x10^4$	$6.61x10^4$
K. foliaceum	0.7644	0.9535	96.38	$3.67x10^4$	$1.58x10^3$	$3.23x10^4$	$3.78x10^4$
***Chaetoceros* spp.**	0.6832	0.8523	97.23	$7.13X10^3$	22	$1.95X10^3$	$1.67X10^5$
Average dissim.				80%			

c) Metabarcoding-spatial variability

Taxa	Av. dissim	Contr. %	Cum. %	Mean abundance (reads L^{-1})					
				URD1	URD2	URD3	URD4	URD5	URD6
T. acuta	16.33	19.87	19.87	391	$3.34x10^3$	$5.01x10^3$	$6.00x10^3$	$1.03x10^4$	$5.05x10^3$
C. tenuissimus	10.9	13.26	33.13	$2.56x10^3$	$5.50x10^3$	$5.08x10^3$	$2.10x10^3$	$1.29x10^3$	702
***Cyclotella* spp.**	6.822	8.301	41.43	19.4	114	427	$1.42x10^3$	$4.59x10^3$	$6.28x10^3$
U. complanatus	6.416	7.807	49.23	5.16	109	$1.09x10^3$	$1.87x10^3$	$6.03x10^3$	$3.96x10^3$
H. cryptochromatica	5.347	6.506	55.74	184	$1.12x10^3$	$1.78x10^3$	$2.16x10^3$	$3.13x10^3$	$2.29x10^3$
N. draveillensis	4.23	5.147	60.89	1.71	38.5	185	$1.46x10^3$	$4.19x10^3$	$3.52x10^3$
C. striolata	3.052	3.714	64.6	28.9	124	253	561	$2.41x10^3$	$2.56x10^3$
O. tauri	2.94	3.577	68.18	162	654	$1.16x10^3$	$1.34x10^3$	$1.17x10^3$	757
N. phyllepta	1.929	2.347	70.53	18.9	76.7	171	365	$1.32x10^3$	$1.57x10^3$
C. guillardii	1.823	2.218	72.74	38.9	259	578	734	693	454
M. polymorphus	1.59	1.935	74.68	344	$1.16x10^3$	273	282	62.2	84.8
S. angusta	1.29	1.57	76.25	1.1	2.81	68.7	180	$1.11x10^3$	380
O. lucimarinus	1.18	1.436	77.69	588	295	133	52.1	62.3	27.7
O. mediterraneus	1.117	1.36	79.04	22.2	127	175	429	758	438
***Surirella* sp.**	1.012	1.232	80.28	5.27	68.3	137	368	503	201
Average dissim.					82%				

d) Metabarcoding-seasonal variability

Taxa	Av. dissim	Contr. %	Cum. %	Mean abundance (reads L^{-1})			
				Autumn	Winter	Spring	Summer
T. acuta	16.43	19.64	19.64	$2.28x10^3$	$8.50x10^3$	$6.45x10^3$	$3.95x10^3$
C. tenuissimus	12.39	14.81	34.45	381	23.3	$1.05x10^3$	$8.79x10^3$
***Cyclotella* spp.**	6.677	7.982	42.43	$1.97x10^3$	117	104	$5.88x10^3$
U. complanatus	6.424	7.679	50.11	712	53.1	$6.59x10^3$	872
H. cryptochromatica	5.362	6.409	56.52	$1.69x10^3$	44.7	$2.35x10^3$	$2.49x10^3$
N. draveillensis	4.01	4.794	61.31	$2.62x10^3$	372	$1.70x10^3$	$1.35x10^3$
O. tauri	3.292	3.935	65.25	119	1.8	60.3	$2.96x10^3$
C. striolata	3	3.587	68.83	219	434	681	$2.51x10^3$
C. guillardii	1.945	2.326	71.16	51.8	290	$1.35x10^3$	96.7
N. phyllepta	1.879	2.246	73.41	360	$1.10x10^3$	898	217
M. polymorphus	1.681	2.01	75.42	158	43.1	141	982
S. angusta	1.341	1.603	77.02	8.87	$1.22x10^3$	256	1.32
O. lucimarinus	1.16	1.386	78.41	207	350	248	20.2
O. mediterraneus	1.149	1.374	79.78	0.858	117	878	250
***Surirella* sp.**	1.042	1.246	81.02	55.8	852	145	10.6
Average dissim.				83%			

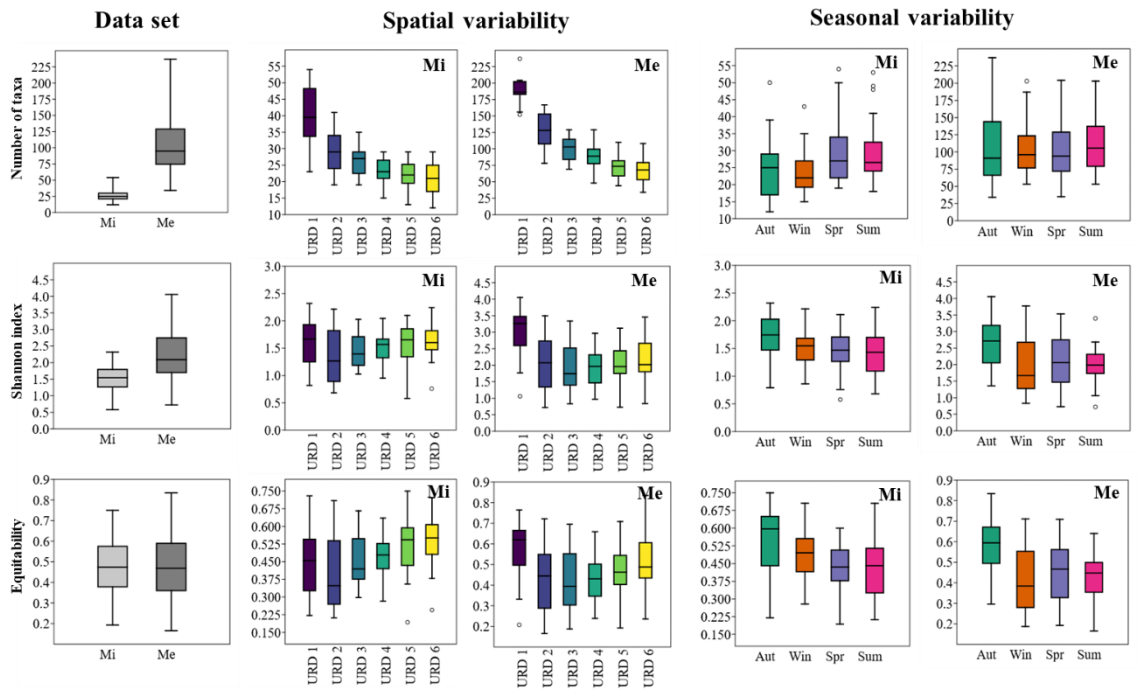
Table A1.11. *Spearman correlation analysis results for microscopy cell counts. rho (ρ) and p values.*

Microscopy		**Salinity**	**Temperature**	**Dissolved O_2**	**O_2 saturation**	**pH**	**Turbidity**	**Ammonium**	**Nitrate**	**Phosphate**	**Silicate**
Tetra (Mi)	ρ	0.26	0.70	-0.63	-0.16	0.26	-0.24	0.16	-0.37	0.10	-0.29
	p	0.65	<0.01	<0.01	1	0.38	1	1	0.01	1	0.22
Cen<*10 (Mi)*	ρ	-0.17	0.27	-0.32	-0.36	0.17	0.23	0.25	0.21	0.30	0.25
	p	1	0.46	0.06	<0.01	1	1	0.44	1	0.06	0.56
Cen>*10 (Mi)*	ρ	-0.30	-0.28	0.29	0.03	-0.23	0.08	0.03	0.17	-0.05	0.10
	p	0.15	0.26	0.14	1	1	1	1	1	1	1
M. poly (Mi)	ρ	0.56	0.76	-0.69	-0.02	0.32	-0.35	-0.10	-0.55	-0.08	-0.50
	p	<0.01	<0.01	<0.01	1	0.04	0.01	1	<0.01	1	<0.01
C. ten (Mi)	ρ	0.45	0.60	-0.53	-0.03	0.31	-0.37	-0.19	-0.51	-0.17	-0.47
	p	<0.01	<0.01	<0.01	1	0.12	<0.01	1	<0.01	1	<0.01
B. quin (Mi)	ρ	0.58	0.72	-0.64	0.04	0.29	-0.49	-0.18	-0.60	-0.21	-0.57
	p	<0.01	<0.01	<0.01	1	0.29	<0.01	1	<0.01	1	<0.01
Plagio (Mi)	ρ	0.15	0.73	-0.73	-0.37	0.22	0.01	0.27	-0.25	0.24	-0.18
	p	1	<0.01	<0.01	<0.01	1	1	0.32	1	0.84	1
T.acu (Mi)	ρ	-0.19	0.48	-0.53	-0.51	0.00	0.18	0.56	0.08	0.53	0.17
	p	1	<0.01	<0.01	<0.01	1	1	<0.01	1	<0.01	1
U. comp (Mi)	ρ	-0.31	0.28	-0.21	-0.21	0.25	0.32	0.38	0.14	0.35	0.23
	p	0.05	0.42	1	1	0.47	0.02	<0.01	1	<0.01	1
Prym *(Mi)*	ρ	0.79	0.11	-0.06	0.54	0.04	-0.58	-0.73	-0.67	-0.66	-0.79
	p	<0.01	1	1	<0.01	1	<0.01	<0.01	<0.01	<0.01	<0.01

Table A1.12. Spearman correlation analysis results for metabarcoding. rho (ρ) and p values.

Metabarcoding		**Salinity**	**Temperature**	**Dissolved O_2**	**O_2 saturation**	**pH**	**Turbidity**	**Ammonium**	**Nitrate**	**Phosphate**	**Silicate**
Tetra (Me)	ρ	0.18	0.49	-0.35	0.01	0.25	-0.27	0.05	-0.28	-0.02	-0.16
	p	1	<0.01	0.01	1	0.91	0.46	1	0.30	1	1
O. viri (Me)	ρ	0.53	0.76	-0.68	-0.04	0.28	-0.48	-0.05	-0.56	-0.08	-0.46
	p	<0.01	<0.01	<0.01	1	0.28	<0.01	1	<0.01	1	<0.01
C. ato (Me)	ρ	-0.08	0.45	-0.49	-0.43	0.11	0.20	0.38	0.05	0.44	0.19
	p	1	<0.01	<0.01	<0.01	1	1	<0.01	1	<0.01	1
Cycl (Me)	ρ	-0.50	0.26	-0.33	-0.63	0.15	0.58	0.63	0.51	0.66	0.62
	p	<0.01	0.89	0.03	<0.01	1	<0.01	<0.01	<0.01	<0.01	<0.01
T. gui (Me)	ρ	-0.42	-0.22	0.19	-0.17	-0.20	0.24	0.15	0.33	0.09	0.22
	p	<0.01	1	1	1	1	1	1	0.04	1	1
M. poly (Me)	ρ	0.69	0.67	-0.64	0.03	0.23	-0.44	-0.23	-0.65	-0.17	-0.58
	p	<0.01	<0.01	<0.01	1	1	<0.01	1	<0.01	1	<0.01
C. ten (Me)	ρ	0.58	0.76	-0.68	0.02	0.37	-0.44	-0.21	-0.64	-0.21	-0.61
	p	<0.01	<0.01	<0.01	1	<0.01	<0.01	1	<0.01	1	7.2E-12
B. quin (Me)	ρ	0.38	0.56	-0.53	-0.08	0.09	-0.30	-0.13	-0.42	-0.13	-0.44
	p	<0.01	<0.01	<0.01	1	1	0.12	1	<0.01	1	<0.01
H. crypto (Me)	ρ	0.01	0.70	-0.72	-0.46	0.18	0.08	0.34	-0.09	0.33	-0.03
	p	1	<0.01	<0.01	<0.01	1	1	0.02	1	0.03	1
T.acu (Me)	ρ	-0.25	0.10	-0.22	-0.43	-0.23	0.29	0.43	0.22	0.41	0.20
	p	1	1	1	<0.01	1	0.23	<0.01	1	<0.01	1
U. comp (Me)	ρ	-0.46	0.17	-0.16	-0.35	-0.03	0.44	0.53	0.34	0.48	0.40
	p	<0.01	1	1	0.01	1	<0.01	<0.01	0.02	<0.01	<0.01
Chryso (Me)	ρ	0.83	0.10	-0.08	0.54	0.16	-0.68	-0.70	-0.71	-0.67	-0.82
	p	<0.01	1	1	<0.01	1	<0.01	<0.01	<0.01	<0.01	<0.01
Prymne *(Me)*	ρ	0.73	0.14	-0.06	0.57	0.27	-0.65	-0.72	-0.67	-0.69	-0.77
	p	<0.01	1	1	6.5E-10	0.45	<0.01	<0.01	<0.01	<0.01	<0.01

Figure A1.1. *Diversity indices for the microscopy (Mi) and eDNA metabarcoding (Me) analysis of the phytoplankton community composition for the entire Urdaibai estuary data set (left), by sampling station (spatial variability) (middle) and by season (right). The box represents the Interquartile Range (IQR), data between Q1 (25th percentile) and Q3 (75th percentile); the line inside the box is the median. The lower whisker shows the Q1-1.5*IQR, and the upper Q3+1.5*IQR. Circles and asterisks represent outliers. Mi: microscopy; Me: metabarcoding. Aut: autumn; Win: winter; Spr: spring; Sum: summer.*



2. Annexes for *Chapter 2*

Table A2.1. *Data of the analysed environmental parameters.*

Station	Date	Secchi disk depth (m)	Salinity (PSU)	pH	Conductivity (µS cm^{-1})	Temperature (°C)	Dissolved oxygen (mg L^{-1})	Oxygen saturation (%)	Turbidity (NTU)
URD 1	30/06/2021	3.3	34.16	7.87	45174.7	18.274	7.58	98.8	0.1
	06/07/2021	2.6	33.81	7.88	46985.7	20.539	7.48	101.4	0.12
	09/07/2021	2.7	34.07	8	48502.6	21.734	7.5	104	0.23
	14/07/2021	3.1	34.31	7.61	45649.6	18.569	7.48	98	0.8
	19/07/2021	3.5	34.34	9.62	50405.5	23.317	7.28	104	0.13
	05/08/2021	2.7	34.17	9.72	51421.1	24.615	7.16	104.5	0.53
URD 2	30/06/2021	1.25	26.52	7.7	36393.8	18.772	6.87	86.4	1.61
	06/07/2021	1.25	27.1	7.87	39855.6	22.151	7.07	95	1.12
	09/07/2021	1	29.97	7.96	43869.3	22.421	7.85	107.7	2.82
	14/07/2021	1.25	28.72	7.53	39453	19.188	7.13	91.5	1.54
	19/07/2021	1.25	29.6	8.91	44765.8	23.96	7.25	102	1.41
	05/08/2021	1.6	27.81	9.17	42287.7	23.912	7.2	100.1	0.9
URD 4	30/06/2021	1	16.62	7.6	23808.4	18.786	6.58	77.9	2.3
	06/07/2021	0.75	17.69	7.65	27342.1	22.6	6.39	81.9	2.63
	09/07/2021	1	19.77	7.71	30160.7	22.472	7.17	92.7	2.48
	14/07/2021	1	17.49	7.46	25209.7	19.259	6.84	82.2	2.91
	19/07/2021	1	20.87	8.56	33551	25.302	6.4	87.6	2.26
	05/08/2021	0.8	18.52	8.61	29238	23.844	6.51	85.7	3
URD 6	30/06/2021	0.75	10	7.64	14784.8	18.268	7.03	79.3	3.5
	06/07/2021	0.4	9.31	7.63	14948.1	21.895	6.08	73.2	7.25
	09/07/2021	0.75	12.1	7.8	19153.3	22.216	8.05	99.1	4.25
	14/07/2021	0.8	10.54	7.43	15600.2	18.556	7.45	84.4	3.37
	19/07/2021	0.6	13.07	8.82	21378.2	24.115	7.98	102.4	4.39
	05/08/2021	0.7	14.11	8.41	22771	23.744	5.81	74.4	3.46
OKA	30/06/2021	-	0.21	8.15	378.4	17.2	7.99	83.1	1.57
	06/07/2021	-	0.21	7.94	387.1	19.304	7.31	79.4	1.88
	09/07/2021	-	0.21	8.13	371	17.443	8.56	89.5	1.12
	14/07/2021	-	0.19	7.97	336.5	16.79	7.98	82.3	2.69
	19/07/2021	-	0.21	8.02	390.8	19.116	6.82	73.8	1.5
	05/08/2021	-	0.19	8.15	352.5	18.834	7.35	79	2.72

Table A2.2. *Data of the analysed nutrient concentrations.*

Station	Date	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmol L^{-1})	Silicate (µmol L^{-1})
URD 1	30/06/2021	2.20	0.2	0.8	0.15	2.1
	06/07/2021	2.30	0.2	0.8	0.08	0.8
	09/07/2021	0.80	0.2	0.8	0.08	0.8
	14/07/2021	3.70	0.2	0.8	0.08	0.8
	19/07/2021	2.70	0.2	0.8	0.08	0.8
	05/08/2021	2.15	0.2	0.8	0.15	2.6
URD 2	30/06/2021	19.2	1.5	10.3	0.70	28.35
	06/07/2021	10.5	1.75	5.95	0.45	13.85
	09/07/2021	2.25	0.6	0.8	0.08	4
	14/07/2021	3.85	0.6	3.5	0.35	19.6
	19/07/2021	2.80	0.55	1.7	0.40	9.6
	05/08/2021	8.35	0.95	4.6	0.50	22.8
URD 4	30/06/2021	44.4	2.4	16.75	1.95	64.65
	06/07/2021	49.8	4.9	16.6	1.90	58.45
	09/07/2021	15.3	2.2	10.75	1.15	52.9
	14/07/2021	11.3	1.5	16.35	1.15	53.9
	19/07/2021	12.7	1.55	10.7	1.20	42.75
	05/08/2021	28.0	2.85	18.7	1.45	75.4
URD 6	30/06/2021	89.6	3.45	33.35	3.15	58.65
	06/07/2021	54.9	2.35	13.5	3.75	85.1
	09/07/2021	16.8	1.9	13.65	2.10	79.15
	14/07/2021	17.9	2	22.85	2.10	79.4
	19/07/2021	6.65	1.95	17.35	1.50	65.65
	05/08/2021	31.4	2.4	20.1	2.15	91.65
OKA	30/06/2021	5.30	1.8	52.5	0.65	76.05
	06/07/2021	3.20	2.05	43.25	0.70	96.65
	09/07/2021	2.30	1.7	56	0.70	90.9
	14/07/2021	2.55	0.9	32.35	0.70	96.7
	19/07/2021	4.05	2.9	54.35	0.90	101.55
	05/08/2021	2.25	0.85	29.95	0.80	103.45

3. Annexes for *Chapter 3*

Table A3.1. *Summary table of the Sim% obtained by PIGMENTUM for the Urdaibai estuary dataset before (B) and after (A) correction was applied. Median Sim% are displayed for the entire dataset and individually for each water mass and trophic status categories. Additionally, the percentage of samples belonging to each Sim% category was determined*.

		Median SIM%		% OF SAMPLES IN EACH SIM% CATEGORY									
				<45%		45-60%		60-75%		75-90%		>90%	
		B	A	B	A	B	A	B	A	B	A	B	A
	Sample set	79	82	3	1	8	6	28	25	43	42	18	25
Trophic status (Chl *a*)	**Ultraoligotrophic**	72.8	75.4	6	2	8	8	47	39	35	45	4	6
	Oligotrophic	80.9	82.2	5	2	9	5	21	23	49	54	16	16
	Mesotrophic	79.7	82.1	1	1	9	8	25	22	44	44	21	26
	Eutrophic	77.2	84.0	3	2	9	3	31	29	34	31	22	34
	Hypertrophic	78.5	90.3	0	0	4	0	21	17	58	29	17	54
Water masses (salinity)	**Freshwater**	70.4	76.5	5	0	25	15	40	25	30	50	0	10
	Oligohaline	70.8	74.4	6	6	6	0	61	50	17	28	11	17
	Mesohaline	79.2	83.7	3	0	9	5	24	23	44	35	20	37
	Polihaline	80.1	82.4	2	2	7	6	26	24	44	45	20	23
	Euhaline	79.6	80.9	3	1	7	5	26	26	49	49	16	19

4. Annexes for *Chapter 4*

Table A4.1. *Data of the hydrometeorological parameters during 2020 and 2022 in the study area.*

Date	Mean temperature (ºC)	Mean Humidity (%)	Monthly accumulated rainfall (L m^{-2})	Mean Oka River flow ($m^3 s^{-1}$)	Mean insolation (h day^{-1})	Radiation (10 kJ m^{-2})
Mar-20	10.15	81.00	164.60	0.71	3.5	-
Apr-20	14.70	83.00	62.10	0.28	3.5	44875
May-20	17.85	78.50	58.45	0.22	6.8	64034
Jun-20	17.40	81.00	100.70	0.32	5.1	58586
Jul-20	20.15	78.50	22.55	0.00	6.8	61549
Aug-20	20.75	80.50	61.90	0.10	6	52402
Sep-20	18.58	79.75	100.90	0.07	6.8	44232
Mar-22	11.45	72.50	73.75	0.49	3.1	33956
Apr-22	12.85	79.75	128.30	0.52	4.7	47071
May-22	15.95	81.50	35.45	0.25	6.3	60067
Jun-22	18.25	83.50	58.35	0.14	5.5	56940
Jul-22	20.85	77.50	9.35	0.08	9.2	69094
Aug-22	21.35	82.00	24.00	0.07	6.9	55252
Sep-22	18.60	80.25	105.60	0.07	5.5	41284

Table A4.2. *Data of the Oka River Flow-index during 2020 and 2022.*

Date	Oka River Flow index
20/03/2020	4.67
17/04/2020	0.63
04/05/2020	0.64
18/05/2020	0.90
02/06/2020	0.38
16/06/2020	1.11
30/06/2020	0.65
17/07/2020	0.01
29/07/2020	0.26
17/08/2020	0.19
31/08/2020	0.35
14/09/2020	0.16
29/09/2020	1.39
25/03/2022	0.92
26/04/2022	4.32
06/05/2022	0.98
23/05/2022	0.53
06/06/2022	0.44
20/06/2022	0.27
06/07/2022	0.25
20/07/2022	0.19
03/08/2022	0.20
18/08/2022	0.16
05/09/2022	0.14
21/09/2022	0.17
04/10/2022	0.80

Table A4.3. *Data of the physicochemical parameters measured during 2020 and 2022 along the Urdaibai estuary and in the Oka River.*

Sampling site	Date	Secchi disck (m)	Salinity (PSU)	pH	Conductivity (µS cm^{-1})	Temperature (ºC)	Dissolved O_2 (mg L^{-1})	O_2 saturation (%)	Turbidity (NTU)	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmol L^{-1})	Silicate (µmol L^{-1})
URD 1 2020	**20/03/2020**	1.5	28.07	7.85	35327.1	15.152	8.69	102.6	2.67	7.8	0.6	10.3	0.4	14.3
	17/04/2020	3	32.24	8.03	42817.2	18.182	7.93	101.9	3.59	4.9	0.2	4.0	0.2	6.8
	04/05/2020	3.3	35.05	8.88	47500.4	19.532	7.8	104.6	0.89	4.3	0.2	2.3	0.1	0.8
	18/05/2020	2.9	31.11	8.26	42414.2	19.224	7.99	104	0.76	4.8	0.2	3.4	0.1	5.9
	02/06/2020	3.1	33.56	8.93	48517.9	22.38	7.37	103.2	0.48	4.1	0.2	0.8	0.1	0.8
	16/06/2020	3	29.78	7.9	40799.7	19.189	7.63	98.5	1.18	7.9	0.5	3.9	0.3	7.9
	30/06/2020	3	35.1	8.09	48620	20.6	9.04	123.5	2.77	3.5	0.2	0.8	0.3	0.8
	17/07/2020	3	33.1	9.25	49539.2	24.058	7.07	101.6	1.79	5.9	0.4	0.8	0.5	1.8
	29/07/2020	3.3	35.05	8.11	51188	23.1	7.31	104.5	1.37	5.3	0.2	0.8	0.7	0.8
	17/08/2020	3.7	35.02	8.76	51234.5	23.214	6.86	98.3	0.82	4.0	0.2	0.8	0.5	0.8
	31/08/2020	3.3	34.69	9.45	50448.1	22.804	7.02	99.8	2.32	4.7	0.2	0.8	0.4	0.8
	14/09/2020	3	34.36	9.66	50093.7	22.938	7.22	102.4	0.91	4.30	0.20	0.80	0.35	1.75
	29/09/2020	2.5	33.44	9.77	45819.3	19.81	7.53	100.5	1.3	3.80	0.20	3.80	0.35	2.30
URD 1 2022	**25/03/2022**	2.9	32.76	7.85	38258.6	12.622	8.46	97.7	2.26	2.75	0.20	5.60	0.40	1.6
	26/04/2022	1.5	25.47	7.94	33367.3	16.509	8.49	101.2	2.74	2.9	0.20	2.7	0.08	2.4
	06/05/2022	2.2	32.5	7.82	38258.6	14.668	9.29	100.4	1.11	2.3	0.20	0.80	0.08	3.1
	23/05/2022	2.8	33.5	7.93	-	17.757	9.45	100.4	0.75	1.75	0.20	0.80	0.08	0.80
	06/06/2022	3.2	33.7	7.9	-	18.603	7.68	98.6	1.25	2.15	0.20	0.80	0.08	0.80
	20/06/2022	2.9	35.23	7.9	48551.5	20.325	7.32	99.8	1.84	0.80	0.20	0.80	0.20	0.8
	06/07/2022	2.2	34.85	7.99	48165.3	20.435	7.19	97.9	1.68	2.45	0.20	0.80	0.20	2.60
	20/07/2022	2.2	34.27	7.96	49592.5	22.563	6.94	97.9	1.36	3.40	0.20	0.8	0.20	2.55
	03/08/2022	2.8	34.37	7.97	49383.5	22.224	7.04	98.7	1.03	2.00	0.20	0.80	0.20	0.8
	18/08/2022	2.5	35.66	8.02	50403.5	21.63	7.02	98.1	1.3	1.95	0.20	0.80	0.20	0.8
	05/09/2022	3.2	35.79	8.06	52061.6	23.051	7.01	100.6	1	1.80	0.20	0.80	0.08	1.75
	21/09/2022	3	34.96	8.02	48366.5	20.495	7.39	100.7	0.61	1.95	0.20	0.80	0.15	0.8
	04/10/2022	3.2	33.15	7.57	44689.3	19.019	7.54	99	0.72	2.30	0.20	3.15	0.20	2.65
URD2 2020	**20/03/2020**	1.2	14.8	7.86	19586.9	14.837	9.15	99	4.56	13.8	0.8	37.3	0.8	58.1
	17/04/2020	1.5	25.47	8.05	34681.8	18.24	8.34	103.1	5.29	15.6	1.2	10.7	0.5	27.2
	04/05/2020	1.6	29.64	8.55	41850.7	20.629	7.64	101.2	2.14	8.1	0.9	7.5	0.4	15.5
	18/05/2020	1.25	20.25	8.2	28862	19.342	8.36	102.3	3.37	10.8	0.9	11.9	0.1	23.6
	02/06/2020	1.6	28.36	8.48	42934.9	23.791	7.11	99	2.5	13.1	1.5	5.6	0.3	12.4
	16/06/2020	1.5	20.61	7.85	29320	19.337	7.74	94.9	2.27	18.2	1.1	14.5	0.8	23.0
	30/06/2020	1.25	29.08	8.08	42598	22.2	7.84	106.7	3.54	7.5	1.2	4.6	0.5	6.0
	17/07/2020	1.2	27.19	9.01	41118.5	23.529	6.91	95.1	3.34	22.3	1.6	6.1	0.8	14.8
	29/07/2020	1.5	32.37	7.96	48179	23.6	5.6	79.5	4.45	18.0	1.7	3.4	1.0	12.3
	17/08/2020	1.75	34.39	8.81	50271.3	23.076	7.06	100.4	1.85	6.0	0.6	0.8	0.5	3.3
	31/08/2020	1.1	30.09	9.18	43093.5	21.389	7.07	95.2	4.72	26.3	2.2	8.5	0.9	19.9
	14/09/2020	1.3	32.16	9.12	48611.3	24.375	7.08	101.8	1.15	13.10	2.15	5.10	0.45	13.55
	29/09/2020	0.6	24.16	9.33	33046.3	18.221	7.76	95.2	7.71	10.10	1.10	33.10	0.60	26.10
URD2 2022	**25/03/2022**	2.1	25.25	7.98	30622.1	13.286	8.8	98.3	2.55	2.8	0.45	8.90	0.30	22.0
	26/04/2022	0.6	16.466	7.97	20542.8	16.466	8.82	97.7	8.41	2.8	0.20	15.95	0.35	38.6
	06/05/2022	1.5	29.61	7.92	37616.1	15.797	8.15	98.5	2.62	1.6	0.20	4.8	0.20	26.3
	23/05/2022	1.75	27.7	7.9	-	19.569	8.51	93.8	2.92	4.75	0.65	7.65	0.20	22.9
	06/06/2022	1.6	27.3	7.92	-	20.253	6.95	92.3	2.67	4.6	0.80	7.15	0.15	20.9
	20/06/2022	1.75	33.86	7.85	48215.2	21.714	6.3	87.3	2.91	3.9	0.20	1.60	0.50	13.3
	06/07/2022	1.25	29.51	7.97	42242.4	21.272	6.99	93.7	3.21	4.35	0.60	3.35	0.45	14.9
	20/07/2022	1.6	33.38	7.89	49773.6	23.899	6.22	89.3	3.85	4.00	0.45	0.80	0.50	12.4
	03/08/2022	1.5	33.63	7.9	49936.6	23.731	6.26	89.7	3.89	4.05	0.45	0.8	0.45	8.95
	18/08/2022	1.75	35.32	7.94	49585.7	21.247	6.55	90.7	2.37	5.40	0.65	3.10	0.55	6.0
	05/09/2022	1.75	34.51	8.02	50873.7	23.524	6.69	95.9	1.73	4.65	0.65	2.75	0.40	12.5
	21/09/2022	2	33.12	7.99	46441.1	20.854	7.13	96.8	1.93	6.30	0.85	4.2	0.45	14.2
	04/10/2022	1.75	25.27	7.93	35228	19.293	7.24	91.3	3.33	8.25	1.00	26.8	0.75	29.5

Table A4.3. (Continued).

Sampling site	Date	Secchi disck (m)	Salinity (PSU)	pH	Conductivity (µS cm^{-1})	Temperature (ºC)	Dissolved O_2 (mg L^{-1})	O_2 saturation (%)	Turbidity (NTU)	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmol L^{-1})	Silicate (µmol L^{-1})
URD3 2020	**20/03/2020**	1.25	4.9	7.87	6978.7	14.46	9.4	94.9	4.42	23.6	0.7	30.4	1.0	84.1
	17/04/2020	1	18.19	8.21	25671.5	18.455	9.76	115.9	5.61	23.2	1.6	14.0	0.9	43.4
	04/05/2020	1.25	23.65	8.44	34609.1	21.275	7.55	97.8	3.01	19.5	1.9	14.0	0.7	33.0
	18/05/2020	1.25	14.56	8.11	21607.8	19.924	8.27	99	3.9	21.4	1.3	21.0	0.6	38.6
	02/06/2020	1.4	23.95	8.31	37398.1	24.495	6.58	90.5	2.54	23.2	1.5	7.7	0.4	13.8
	16/06/2020	1.1	16.72	7.83	24210.8	19.299	7.83	89	3.63	24.7	1.5	21.9	1.0	35.7
	30/06/2020	1	24.98	7.88	37406	23.1	6.05	80.8	4.58	33.6	2.6	11.6	1.0	21.8
	17/07/2020	1.5	24.01	8.82	36946.9	23.77	6.04	82	2.69	56.1	4.5	11.1	1.6	35.6
	29/07/2020	1.25	29.21	7.74	45131	24.1	3.98	56	5.72	31.7	1.9	3.7	1.7	24.1
	17/08/2020	1.25	31.42	8.64	46374.2	23.066	6.98	97.7	3.05	24.1	2.5	4.8	1.0	18.9
	31/08/2020	1.1	24.54	8.98	35933.2	21.54	6.65	86.8	5.8	52.8	4.4	17.8	1.8	42.4
	14/09/2020	1.5	29.17	8.75	44712	24.566	6.82	96.8	2.83	50.15	5.60	11.05	1.65	30.05
	29/09/2020	0.6	18.47	9.01	25708.5	17.889	7.49	88.1	9.27	13.80	1.55	44.65	0.75	37.20
URD3 2022	**25/03/2022**	1.5	19.55	8.04	24943.3	13.55	9.29	101	3.69	1.8	0.65	13.0	0.30	36.7
	26/04/2022	0.5	8.82	8	12548.3	16.205	9.07	97.4	8.38	3.5	0.40	26.8	0.50	64.9
	06/05/2022	1.25	14.47	8.05	19705	15.998	9.2	101.8	3.69	0.80	0.60	8.1	0.25	52
	23/05/2022	1.75	23.2	7.84	-	20.169	6.7	89.5	2.77	7.5	1.10	13.80	0.25	39.4
	06/06/2022	1.6	23.7	7.88	-	21.068	6.6	89.3	2.5	8.2	1.30	13.7	0.30	32.4
	20/06/2022	1.4	30.89	7.74	45437.1	22.798	5.97	82.3	3.18	5.6	0.55	2.9	0.60	23.0
	06/07/2022	1.25	25.53	7.93	36968.5	21.116	6.81	88.9	3.25	9.15	1.35	9.90	0.60	36.7
	20/07/2022	1.4	31.42	7.79	47836.5	24.624	6.28	90.2	3.59	7.10	0.80	3.6	0.65	24.1
	03/08/2022	1.1	31.67	7.79	47922	24.346	6.34	90.8	7.99	5.30	0.60	2.4	0.70	12.8
	18/08/2022	1.6	34.2	7.78	48247.9	21.313	6.64	91.5	3.65	7.00	0.65	3.20	0.75	21.7
	05/09/2022	1.6	31.84	7.89	47837.1	23.982	6.3	89.9	1.81	10.20	1.65	8.25	0.70	29.5
	21/09/2022	1.75	30.8	7.84	43817.1	20.615	6.78	90.4	2.26	14.8	2.15	10.6	0.80	32.8
	04/10/2022	1.25	21.01	7.88	30183	19.867	7.11	88.3	5.52	6.45	0.75	20.8	0.75	33.9
URD4 2020	**20/03/2020**	1	1.24	7.98	1912.7	14.609	9.2	91.2	5.01	33.2	0.8	52.5	0.8	96.5
	17/04/2020	0.6	12.81	8.41	18634	18.479	10.75	123.8	6.36	25.1	2.1	24.0	1.7	56.1
	04/05/2020	1.1	17.44	8.31	26205	21.205	7.1	88.6	3.34	39.2	3.1	21.5	1.1	49.5
	18/05/2020	1	7.15	8.08	11183.7	19.722	8.12	92.6	4.96	48.1	2.1	26.1	1.0	52.8
	02/06/2020	1.1	18.89	8.25	30333	24.773	6.31	84.6	4.25	57.5	3.8	14.8	1.2	36.0
	16/06/2020	0.65	9.36	7.7	14109.4	18.997	7.26	82.7	6.63	37.4	1.2	16.4	0.7	44.2
	30/06/2020	1.2	19.73	7.68	30327	22.8	4.22	54.9	3.99	61.8	3.3	14.5	2.1	42.0
	17/07/2020	1.1	18.92	8.7	20709.9	23.673	5.21	68.5	3.68	59.9	3.0	10.9	2.2	47.6
	29/07/2020	1.3	24.87	7.54	38744	24.2	2.58	35.4	4.58	106.9	5.6	10.8	4.1	51.8
	17/08/2020	1.2	26.6	8.38	40056.2	23.21	6.02	82.1	4.47	71.4	5.1	8.4	3.0	43.3
	31/08/2020	0.75	18.64	8.86	27725.5	20.987	6.17	77.2	8.84	64.5	5.5	26.2	2.4	61.4
	14/09/2020	1.25	25.02	8.64	39624.5	25.422	6.15	86.4	2.26	112.90	9.00	16.60	4.00	53.90
	29/09/2020	0.5	12.4	8.88	17780.1	17.704	7.73	87.4	10.89	21.50	2.40	66.55	0.85	56.40
URD4 2022	**25/03/2022**	0.75	14.92	8.09	19181.3	13.64	9.87	104.2	5.13	2.8	0.80	13.85	0.50	38.0
	26/04/2022	0.4	2.26	8.13	3448.9	15.435	9.54	96.8	9.25	4.35	0.20	31.6	0.70	66
	06/05/2022	1.25	10.71	8.12	14972	16.082	9.5	102.9	3.39	1.60	0.95	17.40	0.30	53.9
	23/05/2022	1.25	18.8	7.79	-	20.469	6.34	86	3.77	7.5	1.15	15.0	0.40	53.8
	06/06/2022	1	18.4	7.84	-	21.339	6.47	88.6	4.41	10.2	1.70	16.9	0.50	48.2
	20/06/2022	0.75	26.98	7.65	40834.8	23.505	5.64	77.5	5.46	10.4	1.20	7.65	0.80	43.1
	06/07/2022	1	20.88	7.91	31005	21.407	6.88	87.9	4.22	8.65	1.30	10.2	0.60	53.6
	20/07/2022	1.1	28.48	7.68	44227.3	25.103	5.96	84.9	4.54	5.95	0.75	3.4	0.70	19.2
	03/08/2022	1	28.67	7.65	44160.6	24.711	5.52	78.2	5.28	16.4	2.10	9.65	1.20	39.8
	18/08/2022	1.25	31.64	7.61	45340.7	21.668	5.95	81.3	4.57	11.9	1.30	7.90	0.85	39.2
	05/09/2022	0.8	27.56	7.8	42092.1	24.095	6.25	87	4.2	14.30	3.15	16.6	0.85	46.4
	21/09/2022	1	27.73	7.7	398522.9	21.115	6.31	83.4	5.54	20.8	3.45	16.2	0.95	50.4
	04/10/2022	0.75	15.38	7.79	22841	20.123	6.87	82.9	7.36	7.25	1.00	27.5	0.95	49.2

Table A4.3. (Continued).

Sampling site	Date	Secchi disck (m)	Salinity (PSU)	pH	Conductivity (µS cm^{-1})	Temperature (ºC)	Dissolved O_2 (mg L^{-1})	O_2 saturation (%)	Turbidity (NTU)	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmol L^{-1})	Silicate (µmol L^{-1})
URD5 2020	**20/03/2020**	1	0.21	8.24	350.1	14.575	10.28	101.2	4.69	30.3	0.5	54.1	0.4	104.6
	17/04/2020	0.6	8.81	8.12	12914	17.488	9.36	103.2	7.56	46.9	1.6	14.5	2.3	60.2
	04/05/2020	0.75	11.63	8.31	17995.2	20.957	7.13	85.5	6.22	57.9	3.3	30.3	2.2	71.8
	18/05/2020	0.6	1.13	8.38	1916.3	18.159	8.99	95.9	7.48	38.5	1.3	27.4	1.1	74.1
	02/06/2020	0.75	13.79	8.3	22862.3	24.986	6.07	79.5	4.93	67.4	3.4	17.1	2.0	44.9
	16/06/2020	0.5	2.11	8.03	3429.2	17.996	8.04	86	9.46	47.9	1.6	28.8	1.3	55.7
	30/06/2020	0.6	14.45	7.66	22870	22.9	4	50.6	5.87	64.7	2.6	15.2	2.5	54.0
	17/07/2020	0.8	13.32	8.78	21521.5	23.588	6.05	77	5.24	99.0	3.4	18.8	4.2	62.6
	29/07/2029	0.9	20.76	7.52	32611	24.1	1.83	24.5	6.03	94.8	3.4	9.9	5.3	64.5
	17/08/2020	1	20.72	8.32	32080.2	23.389	5.61	74.2	3.91	139.4	5.7	11.1	6.2	70.4
	31/08/2020	0.5	13.15	8.98	20060.4	20.775	6.5	78.4	16.33	64.1	5.0	35.7	2.7	62.2
	14/09/2020	0.75	19.26	8.75	32199.2	25.664	7.14	97.9	8.35	166.20	8.55	21.40	8.05	66.35
	29/09/2020	0.5	6.39	9.03	9522.4	17.222	8.04	86.9	10.76	42.75	2.25	65.55	1.25	79.45
URD5 2022	**25/03/2022**	0.75	4.98	8.45	5646.2	12.997	12.03	117	9.54	0.8	1.30	23.2	2.90	55.4
	26/04/2022	1	0.33	8.32	552.9	15.263	9.88	98.7	6.44	3.15	0.20	31.20	0.60	95.6
	06/05/2022	0.4	3.38	8.06	4900	14.209	9.13	90.9	9.46	0.8	1.75	29.70	2.45	84.1
	23/05/2022	0.8	10.7	7.86	-	19.829	4.43	91.6	6.04	6.8	1.45	21.85	0.60	81.1
	06/06/2022	0.75	11.3	7.87	-	20.679	4.65	87.4	12.79	12.6	2.40	30.0	0.95	72.5
	20/06/2022	1	21.86	7.68	33986.4	23.857	6.47	86.9	5.46	15.6	2.45	16.9	1.15	70.3
	06/07/2022	0.5	15.8	7.87	23830.4	20.977	7.28	89.5	5.8	11.1	2.90	25.9	1.40	79.5
	20/07/2022	0.75	23.35	7.72	36890	24.958	6.26	86.5	7.8	16.4	2.70	15.4	1.15	54.9
	03/08/2022	0.7	23.05	7.64	36362.9	24.823	5.82	80	7.93	24.0	3.60	17.7	2.00	64.3
	18/08/2022	0.6	26.71	7.62	39263.3	22.052	7	93.6	8.33	7.50	1.25	7.10	0.90	56.0
	05/09/2022	0.5	23.5	7.79	36424.4	24.034	6.29	85.5	9.11	9.10	2.10	10.9	1.05	57.3
	21/09/2022	0.5	24.71	7.76	36721.2	22.209	7.07	93.7	9.5	21.1	4.25	19.4	1.40	64.5
	04/10/2022	0.5	11.5	7.78	17159	19.242	8.15	94.5	8.6	8.85	1.30	38.4	1.25	64.1
URD6 2020	**20/03/2020**	0.75	0.21	8.12	347.8	14.416	10.16	99.6	5.77	189.6	0.6	49.0	2.4	86.1
	17/04/2020	0.5	7.73	7.73	13785.9	17.327	7.38	81.5	10.59	207.2	2.3	18.4	8.2	50.2
	04/05/2020	0.6	9.37	8.3	14595.9	20.511	7.26	85.2	9.71	65.7	2.4	18.7	2.4	68.5
	18/05/2020	0.55	0.31	8.38	543	17.36	9.12	95.2	6.72	140.3	0.7	24.5	2.6	70.8
	02/06/2020	0.7	11.84	8.26	19662.9	24.438	6.51	83.4	6.07	90.2	3.8	21.9	3.6	47.9
	16/06/2020	0.5	1.19	7.94	1971.4	17.376	7.54	79.2	9.91	334.5	1.4	26.2	7.0	80.3
	30/06/2020	0.5	12.88	7.61	20355	22.3	3.95	49	5.62	73.4	2.1	18.6	2.6	52.9
	17/07/2020	0.5	11.19	8.71	18201.8	23.203	5.72	71.3	9.36	137.6	4.8	25.3	5.8	53.0
	29/07/2020	0.6	20.13	7.45	31357	24.1	1.35	18	7.92	153.4	5.2	16.6	8.3	76.2
	17/08/2020	0.75	17.51	8.28	27494.2	23.327	5.74	74.5	6.18	220.4	5.1	12.0	11.4	80.3
	31/08/2020	0.4	9.95	8.82	15428.4	20.49	7.13	84	23.03	170.5	4.0	35.3	6.1	85.6
	14/09/2020	0.5	18.68	8.65	30335.4	25.3	6.36	86	6.41	197.35	8.35	22.45	10.00	76.40
	29/09/2020	0.5	3.39	9	5241.1	16.944	7.98	84.2	10.89	80.25	2.40	77.55	2.50	89.25
URD6 2022	**25/03/2022**	0.8	4.96	8.12	6829.6	13.096	10.24	100.5	6.39	0.80	1.20	25.8	1.55	45
	26/04/2022	1	0.24	8.37	404.3	14.799	10.24	101.3	5.28	3.5	0.50	49.30	0.50	91.8
	06/05/2022	0.6	0.74	8.28	1135.7	13.229	9.34	89.5	7.29	1.6	1.90	44.0	1.45	87.3
	23/05/2022	0.6	10	7.81	-	19.696	5.21	87.4	7.79	9.5	2.20	32.8	0.80	82.0
	06/06/2022	0.5	12.3	7.74	-	20.917	5.79	92.8	6.9	10.9	1.85	20.1	0.90	61.8
	20/06/2022	1	20.39	7.68	32061.6	24.083	6.12	81.8	5.64	14.60	2.35	16.0	1.20	75
	06/07/2022	0.4	13.67	7.8	20812.6	20.822	6.74	81.5	9.44	9.70	2.35	21.0	2.15	88.8
	20/07/2022	0.6	22.19	7.7	35362.1	25.151	6.59	90.7	9.11	10.7	1.75	9.35	1.35	45.6
	03/08/2022	0.6	22.09	7.6	35068.2	24.931	5.44	75.2	8.48	24.2	3.45	17.4	2.10	64.9
	18/08/2022	0.5	25.69	7.61	37864.2	21.99	6.39	84.8	9.18	12.0	2.10	13.4	1.05	62.8
	05/09/2022	0.6	23.05	7.71	38725.8	24.226	6.1	83.9	8.79	18.45	4.35	23.2	1.30	58.6
	21/09/2022	0.5	23.62	7.74	35364.4	22.366	7.05	93.1	11.36	18.0	3.15	15.0	1.45	64.8
	04/10/2022	0.5	12.1	7.68	17848	18.906	6.69	77.4	10.55	8.05	1.00	27.1	1.10	66.6

Table A4.3. (Continued).

Sampling site	Date	Secchi disck (m)	Salinity (PSU)	pH	Conductivity (µS cm^{-1})	Temperature (ºC)	Dissolved O_2 (mg L^{-1})	O_2 saturation (%)	Turbidity (NTU)	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmol L^{-1})	Silicate (µmol L^{-1})
OKA 2020	**20/03/2020**	-	0.22	8.21	348.5	12.704	11.04	104.2	2.8	5.0	0.2	61.5	0.1	89.2
	17/04/2020	-	0.2	8.03	349.1	15.997	9.1	92.2	4	2.1	1.4	50.9	0.1	67.1
	04/05/2020	-	0.22	8.69	388.7	18.223	8.88	94.4	2.91	3.4	2.0	57.5	0.2	95.4
	18/05/2020	-	0.22	8.37	364.3	14.789	9.72	96.1	3.17	4.8	1.3	57.6	0.1	75.9
	02/06/2020	-	0.23	8.43	416.4	18.817	7.39	79.5	3.52	6.8	3.2	52.8	0.4	58.5
	16/06/2020	-	0.23	8.26	387.6	16.165	8.92	90.8	3.01	4.4	0.8	48.5	0.1	84.2
	30/06/2020	-	0.22	7.77	384.5	17.3	8.38	87.9	-	4.9	2.8	57.6	0.4	92.4
	17/07/2020	-	0.22	9.2	402.7	18.413	7.77	82.9	3.12	4.4	2.8	55.2	0.5	88.8
	29/07/2020	-	0.22	7.81	415.9	19.7	6.57	73	-	4.8	2.8	60.4	1.0	142.6
	17/08/2020	-	0.22	9.15	412.2	19.729	6.52	71.4	2.54	3.8	2.4	65.8	0.9	156.3
	31/08/2020	-	0.19	9.51	327.8	16.825	8.25	85.1	29.1	0.8	0.7	76.0	0.5	106.2
	14/09/2020	-	0.22	9.88	408.8	19.746	6.73	73.7	1	3.90	5.10	65.45	0.75	141.20
	29/09/2020	-	0.24	9.82	400	15.115	9.17	91.3	2.42	0.80	0.50	88.20	0.40	100.00
OKA 2022	**25/03/2022**	-	7.99	381.1	12.442	9.26	87	1.07	0.80	0.60	36.40	0.35	35.9	7.99
	26/04/2022	-	8.08	385.8	10.633	10.06	90.5	1.63	0.8	0.20	34.20	0.25	34.7	8.08
	06/05/2022	-	8.26	447.7	13.361	10.35	99.2	4.33	3.3	0.20	52.9	0.35	63.0	8.26
	23/05/2022	-	8.11	436.4	14.079	9.23	89.9	1.82	2.8	0.65	45.85	0.35	71.5	8.11
	06/06/2022	-	7.94	-	17.724	7.72	81.5	2.14	3.35	1.80	41.0	0.60	86	7.94
	20/06/2022	-	7.96	-	16.829	7.45	77.6	2.05	3.70	2.30	57.35	0.70	81.50	7.96
	06/07/2022	-	7.83	407.3	19.873	6.52	71.6	3.32	4.10	3.15	58.5	0.90	169	7.83
	20/07/2022	-	7.97	406	18.156	7.47	79.2	2.61	4.20	2.60	60.8	0.75	159	7.97
	03/08/2022	-	7.77	411.6	21.401	6.39	72.3	1.65	3.50	2.80	56.3	0.80	168	7.77
	18/08/2022	-	7.76	412.4	21.057	6.37	71.6	2.38	2.95	1.95	60.7	0.95	174	7.76
	05/09/2022	-	7.79	386	18.892	6.41	69	4.02	2.50	1.65	56.5	1.00	181	7.79
	21/09/2022	-	7.93	407.9	19.029	6.66	71.9	3.64	1.85	1.40	57.6	1.15	188	7.93
	04/10/2022	-	7.8	380.2	15.863	6.86	69.3	1.21	2.50	2.25	66.2	1.00	176	7.8

Table A4.4. *Data of the phytoplankton community composition calculated with PIGMENTUM during 2020 and 2022 along the Urdaibai estuary. Values indicate the contribution percentage (%) of each pigment-group to the total biomass (Chl a). PeCA: Peridinin Containing Algae; BCA: 19'–butanoyloxyfucoxanthin Containing Algae; FCA: Fucoxanthin Containing Algae; PrCA: Prasinoxanthin Containing Algae; HCA: 19'–hexanoyloxyfucoxanthin Containing Algae; ACA: Alloxanthin Containing Algae; ZCA: Zeaxanthin Containing Algae; CbCA: Chl b Containing Algae.*

Sampling site	Date	PeCA	BCA	FCA	PrCA	HCA	ACA	ZCA	CbCA	Chl *a* (μgL^{-1})
URD 1 2020	20/03/2020	9.309	0.000	42.921	18.917	9.748	12.418	1.508	5.180	0.880
	17/04/2020	3.753	0.000	20.787	4.522	3.444	61.618	2.215	3.661	2.038
	04/05/2020	7.123	2.898	19.693	7.563	38.947	15.051	1.887	6.839	0.905
	18/05/2020	0.000	0.000	11.212	0.000	12.225	19.347	0.000	57.216	1.353
	02/06/2020	7.122	0.000	21.395	6.802	39.843	18.173	1.776	4.888	1.675
	16/06/2020	0.000	2.879	21.970	42.390	8.622	24.139	0.000	0.000	1.611
	30/06/2020	0.000	0.642	82.242	5.408	2.947	5.934	0.534	2.292	2.238
	17/07/2020	2.407	1.013	57.219	11.850	4.591	13.954	1.234	7.733	2.971
	29/07/2020	4.528	1.681	69.122	2.627	8.057	5.251	3.581	5.153	2.840
	17/08/2020	4.395	0.000	33.654	3.742	34.845	9.822	7.205	6.337	1.179
	31/08/2020	2.422	0.000	56.447	5.268	14.170	10.687	5.188	5.818	1.874
	14/09/2020	1.575	2.123	34.694	7.171	22.981	10.680	11.459	9.317	1.501
	29/09/2020	0.000	2.713	32.226	11.428	19.364	13.660	10.377	10.232	1.142
URD 1 2022	25/03/2022	0.000	2.718	54.829	10.684	0.000	20.506	0.848	10.416	0.659
	26/04/2022	0.000	0.000	37.497	0.000	12.826	17.254	3.852	28.571	0.694
	06/05/2022	4.579	0.000	33.620	9.355	14.020	28.720	0.000	9.707	0.904
	23/05/2022	0.000	0.000	6.015	19.706	54.397	9.169	0.724	9.990	0.638
	06/06/2022	2.102	2.283	0.000	52.678	21.555	21.383	0.000	0.000	1.548
	20/06/2022	6.533	2.874	5.696	23.746	39.067	9.569	1.001	11.515	0.902
	06/07/2022	3.680	0.000	50.464	13.814	6.958	9.540	4.362	11.182	2.144
	20/07/2022	0.000	0.000	55.916	11.223	12.048	5.319	3.032	12.461	1.658
	03/08/2022	8.403	0.000	25.561	20.627	21.913	5.827	17.670	0.000	0.825
	18/08/2022	5.914	0.000	34.141	0.000	15.794	2.914	14.579	26.657	1.037
	05/09/2022	0.000	0.000	34.523	15.110	11.683	10.636	15.761	12.287	1.218
	21/09/2022	4.679	0.000	24.835	23.390	14.789	23.245	6.118	2.944	1.268
	04/10/2022	0.000	4.094	14.625	36.580	26.715	12.913	4.414	0.658	1.037
URD2 2020	20/03/2020	0.000	0.000	44.898	38.059	0.000	7.816	0.268	8.959	1.292
	17/04/2020	0.000	0.000	3.515	1.010	0.000	93.871	0.000	1.604	10.888
	04/05/2020	0.000	0.000	7.812	5.086	5.256	51.236	0.000	30.610	2.774
	18/05/2020	0.000	0.000	5.103	0.000	0.000	39.536	0.000	55.361	5.649
	02/06/2020	0.000	0.000	33.014	3.251	1.177	40.614	0.000	21.943	5.451
	16/06/2020	0.000	0.000	15.685	24.456	0.000	53.151	0.000	6.709	2.959
	30/06/2020	0.000	0.582	88.356	2.468	0.000	5.864	0.000	2.731	10.756
	17/07/2020	0.000	7.073	28.903	16.317	0.000	25.178	0.000	22.529	4.201
	29/07/2020	0.000	2.103	80.207	3.613	0.000	7.599	0.579	5.899	6.492
	17/08/2020	0.000	0.000	72.681	8.664	6.489	7.009	0.866	4.292	5.077
	31/08/2020	0.000	0.000	84.408	4.307	0.000	5.534	0.148	5.603	5.586
	14/09/2020	0.000	3.798	41.879	10.053	0.000	19.735	0.000	24.534	5.036
	29/09/2020	0.000	0.000	52.506	14.459	5.381	14.881	0.000	12.773	3.880
URD2 2022	25/03/2022	0.000	0.000	3.499	0.000	0.000	93.354	0.311	2.836	3.274
	26/04/2022	0.000	0.000	44.249	0.000	0.000	33.820	6.152	15.779	0.671
	06/05/2022	0.000	0.000	5.820	19.381	0.000	72.601	0.000	2.198	4.151
	23/05/2022	0.000	0.000	12.310	46.655	0.000	32.300	0.000	8.736	3.017
	06/06/2022	0.000	0.000	5.440	70.041	0.000	24.518	0.000	0.000	5.404
	20/06/2022	0.000	0.000	27.903	34.099	0.000	22.885	0.000	15.113	2.246
	06/07/2022	0.000	1.124	69.787	12.526	0.000	11.991	0.032	4.540	6.959
	20/07/2022	0.000	0.000	64.542	14.900	0.000	8.805	0.633	11.120	5.019
	03/08/2022	0.000	0.000	50.973	24.990	0.805	9.264	2.226	11.742	3.035
	18/08/2022	0.000	0.000	56.390	20.543	0.000	2.208	2.199	18.661	2.609
	05/09/2022	0.000	0.000	42.870	34.195	0.000	15.361	1.631	5.944	3.329
	21/09/2022	0.000	0.000	10.219	41.349	0.000	47.825	0.607	0.000	2.652
	04/10/2022	0.000	5.319	19.435	42.529	0.000	30.110	0.000	2.607	1.879

Table A4.4. (Continued).

Sampling site	Date	PeCA	BCA	FCA	PrCA	HCA	ACA	ZCA	CbCA	Chl *a* (μgL^{-1})
URD3 2020	**20/03/2020**	0.000	0.000	54.142	0.000	0.000	0.000	29.116	16.742	0.436
	17/04/2020	0.000	0.000	1.738	0.000	0.000	96.413	0.000	1.849	25.155
	04/05/2020	0.000	0.000	6.296	4.588	0.000	56.558	0.000	32.558	4.852
	18/05/2020	0.000	0.000	5.009	0.000	0.000	85.324	0.000	9.667	8.661
	02/06/2020	0.000	0.549	17.046	4.104	0.000	59.597	0.000	18.704	5.571
	16/06/2020	0.000	0.000	6.543	15.990	0.000	77.467	0.000	0.000	6.872
	30/06/2020	0.000	0.000	57.560	8.251	0.000	25.706	0.000	8.483	4.890
	17/07/2020	0.000	2.502	15.315	18.279	0.000	50.127	0.000	13.777	3.617
	29/07/2020	0.000	0.000	53.769	11.461	0.000	18.207	0.000	16.563	3.855
	17/08/2020	0.000	0.000	58.449	19.548	0.000	13.794	0.000	8.209	8.905
	31/08/2020	0.000	0.000	73.380	9.666	0.000	12.441	0.000	4.513	3.588
	14/09/2020	0.000	4.678	11.669	11.611	0.000	41.322	0.000	30.720	3.926
	29/09/2020	0.000	0.000	49.818	12.228	3.631	19.000	0.000	15.323	5.543
URD3 2022	**25/03/2022**	0.000	0.000	1.965	0.000	0.000	96.427	0.161	1.446	7.857
	26/04/2022	0.000	0.000	37.062	0.000	0.000	56.537	6.401	0.000	0.773
	06/05/2022	0.000	0.000	2.241	0.000	0.000	93.316	0.000	4.444	9.613
	23/05/2022	0.000	0.000	7.104	43.789	0.000	42.536	0.000	6.572	4.860
	06/06/2022	0.000	0.000	4.236	69.645	0.000	26.119	0.000	0.000	5.790
	20/06/2022	0.000	0.000	33.986	31.440	0.000	23.885	0.000	10.690	3.440
	06/07/2022	0.000	1.823	47.862	25.355	0.000	23.279	0.000	1.681	4.237
	20/07/2022	0.000	0.000	48.621	21.739	0.000	13.386	0.564	15.691	4.384
	03/08/2022	0.000	0.000	56.411	28.066	0.000	10.360	1.415	3.748	4.152
	18/08/2022	0.000	0.000	52.667	28.533	0.000	5.529	0.000	13.271	3.100
	05/09/2022	0.000	0.000	29.619	36.058	0.000	27.411	2.170	4.742	3.578
	21/09/2022	0.000	0.000	9.664	44.984	0.000	45.351	0.000	0.000	4.136
	04/10/2022	0.000	3.484	17.036	23.382	0.000	48.813	0.916	6.370	1.929
URD4 2020	**20/03/2020**	0.000	0.000	65.815	0.000	0.000	0.000	21.446	12.739	0.490
	17/04/2020	0.000	0.000	4.419	0.000	0.000	94.502	0.000	1.079	54.607
	04/05/2020	0.000	0.000	5.310	0.000	0.000	80.428	0.000	14.262	7.027
	18/05/2020	0.000	0.000	12.090	0.000	0.000	82.013	0.000	5.898	3.683
	02/06/2020	0.000	0.000	6.187	5.200	0.000	78.486	0.000	10.127	7.892
	16/06/2020	0.000	0.000	4.731	3.432	0.000	90.744	1.094	0.000	7.489
	30/06/2020	0.000	0.000	39.173	18.559	0.000	39.167	0.000	3.101	3.957
	17/07/2020	0.000	0.000	9.581	8.993	0.000	76.278	0.000	5.148	4.716
	29/07/2020	0.000	0.000	44.430	13.888	0.000	25.899	0.000	15.783	2.479
	17/08/2020	0.000	0.000	17.368	30.970	0.000	28.749	0.000	22.913	6.190
	31/08/2020	0.000	0.000	33.010	0.000	0.000	45.319	0.142	21.529	2.006
	14/09/2020	0.000	0.000	4.733	12.104	0.000	54.137	0.000	29.026	8.253
	29/09/2020	0.000	0.000	45.380	0.000	0.000	29.821	0.000	24.800	4.548
URD4 2022	**25/03/2022**	0.000	0.000	1.348	0.000	0.000	98.269	0.000	0.383	41.117
	26/04/2022	0.000	0.000	24.264	0.000	0.000	73.566	2.171	0.000	2.651
	06/05/2022	0.000	0.000	10.656	0.000	0.000	87.608	0.000	1.736	14.633
	23/05/2022	0.000	0.000	4.401	33.259	0.000	61.673	0.000	0.667	5.411
	06/06/2022	0.000	0.000	5.615	68.586	0.000	25.799	0.000	0.000	6.084
	20/06/2022	0.000	0.000	47.131	20.500	0.000	17.653	0.054	14.662	3.836
	06/07/2022	0.000	0.000	44.541	19.087	0.000	27.675	0.437	8.259	3.383
	20/07/2022	0.000	0.000	44.186	21.475	0.000	14.268	1.454	18.617	4.768
	03/08/2022	0.000	0.000	51.036	25.886	0.000	9.913	1.961	11.204	3.320
	18/08/2022	0.000	0.000	53.739	24.621	0.000	5.725	0.000	15.916	4.036
	05/09/2022	0.000	0.000	61.708	12.659	0.000	17.310	1.640	6.683	5.508
	21/09/2022	0.000	0.000	2.444	30.518	0.000	66.283	0.756	0.000	7.003
	04/10/2022	0.000	0.000	17.313	0.000	0.000	61.700	1.709	19.277	1.730

Table A4.4. (Continued).

Sampling site	Date	PeCA	BCA	FCA	PrCA	HCA	ACA	ZCA	CbCA	Chl *a* (μgL^{-1})
URD5 2020	**20/03/2020**	0.000	0.000	48.893	0.000	0.000	0.000	23.202	27.904	0.216
	17/04/2020	0.000	0.000	2.323	0.000	0.000	96.090	0.000	1.586	42.513
	04/05/2020	0.000	0.000	24.715	0.000	0.000	66.930	3.751	4.605	4.165
	18/05/2020	0.000	0.000	13.397	0.000	0.000	83.696	0.000	2.907	10.132
	02/06/2020	0.000	0.000	7.739	5.443	0.000	82.707	0.097	4.014	10.653
	16/06/2020	0.000	0.000	15.707	0.000	0.000	73.387	0.314	10.592	8.665
	30/06/2020	0.000	0.000	13.248	4.413	0.000	70.444	0.000	11.895	11.621
	17/07/2020	0.000	0.000	6.837	0.000	0.000	21.868	0.000	71.294	16.451
	29/07/2029	0.000	0.000	10.041	1.203	0.000	17.066	0.000	71.690	6.455
	17/08/2020	0.000	0.000	10.108	10.621	0.000	27.864	0.000	51.407	4.918
	31/08/2020	0.000	0.000	13.887	0.000	0.000	19.528	0.000	66.585	4.407
	14/09/2020	0.000	0.000	5.562	5.757	0.000	38.290	0.000	50.391	17.180
	29/09/2020	0.000	0.000	67.537	0.000	0.000	24.430	0.000	8.033	9.939
URD5 2022	**25/03/2022**	0.000	0.000	66.062	0.000	0.000	32.264	0.000	1.674	161.286
	26/04/2022	0.000	0.000	41.106	0.000	0.000	41.295	1.906	15.693	0.736
	06/05/2022	0.000	0.000	31.096	0.000	0.000	67.998	0.000	0.907	251.511
	23/05/2022	0.000	0.000	37.038	13.843	0.000	48.107	0.110	0.902	11.734
	06/06/2022	0.000	0.000	22.759	20.268	0.000	49.628	0.000	7.345	7.859
	20/06/2022	0.000	0.000	27.892	15.757	0.000	43.418	0.884	12.049	3.047
	06/07/2022	0.000	0.947	19.699	0.000	0.000	13.513	0.000	65.840	23.199
	20/07/2022	0.000	0.000	60.144	0.000	0.000	13.881	2.762	23.213	7.214
	03/08/2022	0.000	0.000	25.458	35.817	0.000	16.061	0.898	21.766	3.299
	18/08/2022	0.000	0.000	71.072	0.000	0.000	5.818	0.000	23.110	13.127
	05/09/2022	0.000	0.000	65.638	0.000	0.000	22.326	0.848	11.188	10.056
	21/09/2022	0.000	0.000	7.375	1.243	0.000	89.358	0.000	2.023	31.358
	04/10/2022	7.858	0.000	13.900	0.000	0.000	66.759	1.865	9.619	4.209
URD6 2020	**20/03/2020**	0.000	0.000	89.009	0.000	0.000	0.000	10.991	0.000	0.504
	17/04/2020	0.000	0.000	2.892	0.000	0.000	94.981	0.000	2.128	45.817
	04/05/2020	0.000	0.000	30.239	0.000	0.000	60.471	3.344	5.946	8.534
	18/05/2020	0.000	0.000	28.506	0.000	0.000	49.884	0.000	21.610	1.854
	02/06/2020	0.000	0.000	19.153	10.708	0.000	66.393	0.000	3.745	7.567
	16/06/2020	0.000	0.000	36.399	0.000	0.000	35.795	2.995	24.810	2.558
	30/06/2020	0.000	0.000	27.118	0.000	0.000	34.579	0.000	38.303	7.082
	17/07/2020	0.000	0.000	31.742	0.000	0.000	18.439	0.000	49.819	16.756
	29/07/2020	0.000	0.000	29.858	0.000	0.000	17.209	0.000	52.933	7.094
	17/08/2020	0.000	0.000	7.199	3.204	0.000	12.725	0.000	76.872	8.883
	31/08/2020	0.000	0.000	30.573	0.000	0.000	19.707	0.000	49.720	7.670
	14/09/2020	0.000	0.000	9.424	4.825	0.000	42.570	0.000	43.181	13.887
	29/09/2020	0.000	0.000	70.465	0.000	0.000	24.224	2.053	3.259	7.245
URD6 2022	**25/03/2022**	0.000	0.000	37.708	0.000	0.000	62.292	0.000	0.000	73.399
	26/04/2022	0.000	0.000	47.557	0.000	0.000	4.122	0.000	48.321	0.902
	06/05/2022	0.000	0.000	5.159	0.000	0.000	94.841	0.000	0.000	151.567
	23/05/2022	0.000	0.000	38.053	16.641	0.000	41.025	0.000	4.280	14.090
	06/06/2022	0.000	0.000	37.417	4.847	0.000	32.722	0.000	25.014	16.803
	20/06/2022	0.000	0.000	35.918	10.156	0.000	43.768	0.447	9.711	5.713
	06/07/2022	0.000	1.352	33.419	0.000	0.000	18.322	0.000	46.907	41.834
	20/07/2022	0.000	0.000	90.769	0.000	0.000	3.468	0.051	5.712	33.603
	03/08/2022	0.000	0.000	31.082	29.047	0.000	12.643	0.000	27.228	3.732
	18/08/2022	0.000	0.000	72.601	0.000	0.000	4.790	0.000	22.610	17.676
	05/09/2022	0.000	0.000	61.808	0.000	0.000	29.755	0.710	7.726	13.831
	21/09/2022	0.000	0.000	7.995	1.871	0.000	89.602	0.000	0.532	26.178
	04/10/2022	4.686	0.000	17.974	0.000	0.000	53.292	0.000	24.048	4.436

Table A4.5. *Two-way PERMANOVA analysis results for hydrometeorological data. Permutation N: 9999. Sum of sqrs: sum of squares; df: degrees of freedom; MS: mean squares; F: ratio; p: significant levels.*

		Source	Season	Year	Interaction
Meteorological data	**Statistics**	**Sum of sqrs**	0.025681	0.01444	-0.034861
		df	1	1	1
		Mean square	0.025681	0.01444	-0.034861
		F	13.954	0.78461	-18.942
		p	0.2741	0.4556	0.8193
Oka River data	**Statistics**	**Sum of sqrs**	0.047887	0.013249	0.0008165
		df	1	1	1
		Mean square	0.047887	0.013249	0.0008165
		F	65.475	18.115	0.11164
		p	0.0053	0.1512	0.1508

Table A4.6. *Mann-Whitney U test for equal medians, with Monte Carlo permutation, for the determination of differences in the hydrometeorological data between 2020 and 2022.*

Tested variables	p values
Air temperature	0.8033
Humidity	1
Monthly accumulated rainfall	0.5278
Insolation hours	0.9104
Radiation	1
Oka river flow	1
River Flow Index	0.5511

Table A4.7. *Two-way PERMANOVA analysis results for physicochemical data. Permutation N: 9999. Sum of sqrs: sum of squares; df: degrees of freedom; MS: mean squares; F: ratio; p: significant levels.*

		Source	Sampling station	Year	Interaction
March-October	**Statistics**	**Sum of sqrs**	11.472	1.6883	0.80656
		df	5	1	5
		Mean square	2.2943	1.6883	0.16131
		F	90.948	66.923	6.3945
		p	0.0001	0.0001	0.0001
Spring	**Statistics**	**Sum of sqrs**	35432	0.53692	0.30375
		df	5	1	5
		Mean square	0.70864	0.53692	0.06075
		F	35272	26725	30238
		p	0.0001	0.0001	0.0042
Summer	**Statistics**	**Sum of sqrs**	37842	0.74194	0.24167
		df	5	1	5
		Mean square	0.75684	0.74194	0.048334
		F	53672	52615	34277
		p	0.0001	0.0001	0.0025

Table A4.8. *Mann-Whitney U test for equal medians, with Monte Carlo permutation, for the determination of differences in the physicochemical conditions between 2020 and 2022 at each sampling station. Table shows p values.*

	URD 1	URD 2	URD 3	URD 4	URD 5	URD 6	Oka
Salinity	0.390	0.184	0.234	0.131	0.197	0.059	0.9051
Temperature	0.104	0.389	0.413	0.727	0.614	0.758	0.8808
pH	0.0004	0.002	0.0004	0.0001	0.0001	0.0001	0.0007
DO	0.486	0.354	0.155	0.431	0.570	0.311	0.1823
O_2 sat.	0.029	0.001	0.402	0.821	0.609	0.551	0.0417
Secchi disc	0.051	0.010	0.037	0.691	0.555	0.416	-
Turbidity	0.623	0.961	0.569	0.921	0.318	0.922	0.1842
Ammonium	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.1103
Phosphate	0.006	0.048	0.001	0.001	0.022	0.000	0.0214
Nitrite	0.2243	0.0001	0.0005	0.0035	0.1525	0.1133	0.6932
Nitrate	0.2098	0.1121	0.0812	0.1759	0.8394	0.6176	0.3895
Silicate	0.6483	0.9918	0.7752	0.2432	0.6239	0.7099	0.1529
DIN	0.0003	0.0007	0.0004	0.0001	0.0001	0.0001	0.2543
DIN:P	0.6916	0.0771	0.0491	0.154	0.1686	0.2673	-
DIN:SI	0.1696	0.0001	0.0001	0.0001	0.0001	0.0001	-
Si:P	0.47	0.0001	0.0583	0.0192	0.0384	0.0001	-

Table A4.9. *Median contribution percentage (%) of ammonium, nitrite and nitrate to DIN, in each sampling station, in 2020 and 2022.*

		Ammonium	Nitrite	Nitrate
2020	**URD1**	77.8	3.5	17.8
	URD2	56.8	5.2	34.5
	URD3	70.4	5.1	24.3
	URD4	68.0	4.1	27.2
	URD5	74.5	3.1	23.1
	URD6	82.1	2.1	15.1
2022	**URD1**	66.1	6.3	27.1
	URD2	52.4	6.4	40.4
	URD3	44.9	6.1	48.5
	URD4	44.9	6.1	48.5
	URD5	27.9	7.0	64.9
	URD6	33.2	7.1	61.1

Table A4.10. *Median nutrient ratios, in each sampling station, in 2020 and 2022.*

		DIN:P	DIN:Si	Si:P
2020	**URD1**	22.3	3.9	6.6
	URD2	45.2	1.6	30.1
	URD3	48.0	1.6	35.7
	URD4	40.1	1.6	31.3
	URD5	38.8	1.5	23.0
	URD6	34.7	2.4	13.5
2022	**URD1**	22.0	2.4	10.0
	URD2	25.2	0.6	33.1
	URD3	34.3	0.6	45.2
	URD4	34.3	0.6	45.2
	URD5	29.9	0.5	54.5
	URD6	27.5	0.5	59.8

Table A4.11. *Toxic phytoplankton blooms recorded in 2020 and 2022 according to the cell abundance limits established by Swan and Davidson (2012).*

Year	Date	Sampling point	Bloom forming taxa	Cell abundance (cells L^{-1})
2020	17th April	URD1	*Dinophysis* spp.	360
		URD2	*Pseudonitzschia* spp.	4.4 x 10^5
	4th May	URD1	*Dinophysis* spp.	1,220
		URD2	*Dinophysis* spp.	440
	18th May	URD1	*Dinophysis* spp.	220
	14th September	URD2	*Pseudonitzschia* spp.	1.4 x 10^5
2022	6th May	URD1	*Dinophysis* spp.	1,200
	20th July	URD2	*Pseudonitzschia* spp.	1.2 x 10^5

Table A4.12. *Two-way PERMANOVA analysis results for phytoplankton community composition data. Permutation N: 9999. Sum of sqrs: sum of squares; df: degrees of freedom; MS: mean squares; F: ratio; p: significant levels.*

		Source	Sampling station	Year	Interaction
March-October	**Statistics**	**Sum of sqrs**	3.9297	0.64971	0.55566
		df	5	1	5
		Mean square	0.78594	0.64971	0.11113
		F	6.4544	5.3356	0.91264
		p	0.0001	0.003	0.5498
Spring	**Statistics**	**Sum of sqrs**	22686	0.33251	0.26891
		df	5	1	5
		Mean square	0.45372	0.33251	0.053782
		F	37437	27435	0.44376
		p	0.0002	0.054	0.9611
Summer	**Statistics**	**Sum of sqrs**	30456	0.4127	0.62692
		df	5	1	5
		Mean square	0.60913	0.4127	0.12538
		F	74238	50298	15281
		p	0.0001	0.0032	0.0989

Table A4.13. *Mann-Whitney U test for equal medians, with Monte Carlo permutation, for the determination of differences in the phytoplankton community composition between 2020 and 2022 at each sampling station. Table shows p values for the entire study period (March-October).*

	URD1	URD2	URD3	URD4	URD5	URD6
PeCA	0.6692	-	-	-	-	-
BCA	0.4886	0.4451	0.7313	-	-	-
FCA	0.3652	0.3953	0.4837	0.6456	0.0085	0.1227
PrCA	0.0487	0.0193	0.0096	0.0608	0.6351	0.3347
HCA	0.5113	0.0982	-	-	-	-
ACA	0.5117	0.6819	0.8416	0.2158	0.4473	0.8785
ZCA	0.607	0.0581	0.0754	0.0672	0.3045	0.3911
CbCA	0.1998	0.1511	0.0126	0.1555	0.2635	0.3133
Chl *a*	0.0142	0.0343	0.2688	0.804	0.7228	0.1004

Table A4.14. *Mann-Whitney U test for equal medians, with Monte Carlo permutation, for the determination of differences in the phytoplankton community composition between 2020 and 2022 at each sampling station. Table shows p values for each season. SPR: spring; SUM: summer.*

	URD1		URD2		URD3		URD4		URD5		URD6	
	SPR	**SUM**	**SPR**	**SUM**	**SPR**	**SUM**	**SPR**	**SUM**	**SPR**	**SUM**	**SPR**	**SUM**
PeCA	0.260	0.610	-	-	-	-	-	-	-	-	-	-
BCA	1.000	0.101	-	0.392	-	0.857	-	-	-	-	-	-
FCA	0.821	0.073	0.815	0.159	0.939	0.259	0.936	0.257	0.066	0.071	0.311	0.256
PrCA	0.323	0.055	0.594	0.004	0.480	0.001	0.314	0.204	0.189	0.469	0.423	1.000
HCA	0.491	0.807	-	0.455	-	1	-	-	-	-	-	-
ACA	0.698	0.536	0.589	0.457	0.694	0.804	0.588	0.163	0.094	0.321	0.690	0.717
ZCA	0.555	0.320	0.458	0.060	1.000	0.071	0.844	0.006	0.567	0.071	0.185	1.000
CbCA	0.259	0.628	0.238	0.534	0.173	0.070	0.306	0.263	0.696	0.133	0.809	0.037
Chl *a*	0.043	0.090	0.493	0.037	0.822	0.317	0.938	0.899	0.699	0.902	0.245	0.323

Table A4.15. *Spearman correlation analysis results for 2020 and 2022. rho (ρ) and p values. DO:dissolved oxygen.*

2020		Salinity	Temperature	pH	DO	Turbidity	Ammonium	Phosphate	Nitrate	Silicate
PeCA	ρ	0.507	-0.019	0.237	0.040	-0.437	-0.509	-0.441	-0.491	-0.516
	p	0.0004	1	1	1	0.012	0.0004	0.010	0.001	0.0002
BCA	ρ	0.513	0.241	0.369	-0.199	-0.491	-0.417	-0.385	-0.511	-0.525
	p	0.0003	1	0.168	1	0.001	0.028	0.095	0.0003	0.0002
FCA	ρ	0.215	-0.049	0.412	-0.033	-0.036	-0.262	-0.210	-0.080	-0.163
	p	1	1	0.034	1	1	1	1	1	1
PrCA	ρ	0.604	0.337	0.187	-0.330	-0.492	-0.279	-0.215	-0.462	-0.515
	p	0.0001	0.491	1	0.602	0.001	1	1	0.004	0.0003
HCA	ρ	0.648	-0.080	0.320	0.098	-0.559	-0.719	-0.659	-0.567	-0.681
	p	0.0001	1	0.813	1	0.0001	0.0001	0.0001	0.0001	0.0001
ACA	ρ	-0.365	-0.009	-0.417	0.096	0.225	0.301	0.232	0.231	0.189
	p	0.194	1	0.028	1	1	1	1	1	1
ZCA	ρ	0.081	-0.320	0.024	0.234	-0.155	-0.319	-0.323	-0.095	-0.093
	p	1	0.819	1	1	1	0.843	0.741	1	1
CbCA	ρ	-0.037	0.269	0.226	-0.282	0.090	0.297	0.278	0.123	0.263
	p	1	1	1	1	1	1	1	1	1
Chl *a*	ρ	-0.312	0.260	-0.079	-0.183	0.436	0.440	0.478	0.216	0.286
	p	1	1	1	1	0.013	0.011	0.002	1	1

2022		Salinity	Temperature	pH	DO	Turbidity	Ammonium	Phosphate	Nitrate	Silicate
PeCA	ρ	0.294	-0.128	0.084	0.196	-0.282	-0.285	-0.293	-0.284	-0.327
	p	1	1	1	1	1	1	1	1	0.666
BCA	ρ	0.124	-0.177	-0.002	0.174	-0.186	-0.083	-0.080	-0.043	-0.187
	p	1	1	1	1	1	1	1	1	1
FCA	ρ	0.144	0.442	-0.184	-0.340	0.215	0.168	0.258	-0.132	0.004
	p	1	0.009	1	0.447	1	1	1	1	1
PrCA	ρ	0.517	0.303	-0.233	-0.389	-0.497	0.198	-0.289	-0.386	-0.434
	p	0.0002	1	1	0.082	0.001	1	1	0.092	0.014
HCA	ρ	0.558	-0.073	0.196	0.245	-0.597	-0.477	-0.607	-0.620	-0.635
	p	0.0001	1	1	1	0.0001	0.002	0.0001	0.0001	0.0001
ACA	ρ	-0.604	-0.523	0.217	0.316	0.296	-0.050	0.164	0.563	0.438
	p	0.0001	0.0002	1	0.920	1	1	1	0.0001	0.012
ZCA	ρ	0.357	0.235	0.119	-0.008	-0.245	-0.150	-0.263	-0.311	-0.372
	p	0.254	1	1	1	1	1	1	1	0.154
CbCA	ρ	0.111	0.336	-0.335	-0.295	0.134	0.215	0.153	-0.089	0.057
	p	1	0.506	0.521	1	1	1	1	1	1
Chl *a*	ρ	-0.487	0.104	-0.183	-0.212	0.576	0.315	0.606	0.444	0.568
	p	0.001	1	1	1	0.0001	0.94915	0.0001	0.009	0.0001

Table A4.16. *Threshold values in each haline section for the different physicochemical indicators (a) and Chl a (b) used in the water quality and ecological status assessment of coastal and transitional waters of the Basque Country. Modified from the Royal Decree 817/2015.*

a)

	Salinity	O_2 saturation (%)	Turbidity (NTU)	Ammonium (µmol L^{-1})	Nitrate (µmol L^{-1})	Phosphate (µmol L^{-1})
Good/Moderate threshold	**Euhaline**	≥83	≤7	≤9.1	≤19.6	≤0.88
	Polihaline	≥79	≤9	≤18.6	≤52.3	≤1.82
	Mesohaline	≥71	≤11	≤34.3	≤121.3	≤3.39
Very good/Good threshold	**Euhaline**	≥92	≤6	≤3.7	≤5.5	≤0.35
	Polihaline	≥88	≤8	≤7.5	≤14.8	≤0.72
	Mesohaline	≥82	≤10	≤13.7	≤34.3	≤1.33

b)

Salinity	Units	Chl *a* Reference condition	Chl *a* Class limits			
			very good/ good	good/ moderate	moderate/ deficient	deficient/ bad
Euhaline	**µg L^{-1}**	1.30	1.95	3.90	5.85	7.80
	RCE		>0.67	>0.33	>0.22	>0.17
Polihaline	**µg L^{-1}**	2.20	3.30	6.60	9.90	13.20
	RCE		>0.67	>0.33	>0.22	>0.17
Mesohaline	**µg L^{-1}**	3.40	5.10	10.20	15.30	20.40
	RCE		>0.67	>0.33	>0.22	>0.17
Oligohaline	**µg L^{-1}**	4.40	6.60	13.20	19.80	26.40
	RCE		>0.67	>0.33	>0.22	>0.17

Table A4.17. *Water quality/ecological status of the different sampling stations of Urdaibai estuary in 2020 (a) and 2022 (b) according to the criteria in the Royal Decree 817/2015 (Table A4.16).*

a)

2020	Salinity	O_2 saturation	Turbidity	Ammonium	Nitrate	Phosphate	Chl *a*
URD1	**Euhaline**	Very good	Very good	Good	Very good	Good	Good
URD2	**Polihaline**	Very good	Very good	Good	Very good	Very good	Moderate
URD3	**Polihaline**	Very good	Very good	Moderate	Good	Good	Moderate
URD4	**Mesohaline**	Very good	Very good	Moderate	Very good	Good	Good
URD5	**Mesohaline**	Very good	Very good	Moderate	Very good	Good	Deficient
URD6	**Mesohaline**	Very good	Very good	Very bad	Very good	Very bad	Deficient

b)

2022	Salinity	O_2 saturation	Turbidity	Ammonium	Nitrate	Phosphate	Chl *a*
URD1	**Euhaline**	Very good	Very good	Very good	Very good	Very good	Very good
URD2	**Polihaline**	Very good	Very good	Very good	Very good	Very good	Good
URD3	**Polihaline**	Very good	Very good	Very good	Very good	Very good	Moderate
URD4	**Mesohaline**	Very good	Very good	Very good	Very good	Very good	Moderate
URD5	**Mesohaline**	Very good	Very good	Very good	Very good	Very good	Bad
URD6	**Mesohaline**	Very good	Very good	Very good	Very good	Very good	Bad