PhD Thesis

"Study of phytoplankton as food resource and toxicity risk for human health in offshore bivalve aquaculture in the Basque Country"

Presented by

Oihane Muñiz Pinto

Thesis Directors

Marta Isabel Revilla Rodríguez

José Germán Rodríguez Patiño

Department

Plant Biology and Ecology

PhD Program

Marine Environment and Resources

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CONTENTS

LIST OF ACRONYMS

ANOVA: Analysis of Variance

ASP: Amnesic Shellfish Poisoning

AZA: Azaspiracid

AZP: Azaspiracid Shellfish Poisoning

CCA: Canonical Correspondence Analysis

CFP: Ciguatera Fish Poisoning

CTD: Conductivity, Temperature, Depth

DA: Domoic Acid

DHA: Docosahexaenoic Acid

DSP: Diarrheic Shellfish Poisoning

DTX: Dinophysistoxin

EC: European Commission

EPA: Eicosapentaenoic Acid

ESD: Equivalent Spherical Diameter

EU: European Union

FAO: Food and Agriculture Organization

HAB: Harmful Algal Bloom

HPLC: High Performance Liquid Chromatography

ICES: International Council for the Exploration of the Sea

INTECMAR: Technological Institute for the Monitoring of the Marine Environment

IOC: Intergovernmental Oceanographic Commission

LC-MS/MS: Liquid Chromatography-Mass Spectrometry

LSD: Least Significant Difference

MDS: Multidimensional Scaling

MSFD: Marine Strategy Framework Directive

NPOC: Non-purgeable Organic Carbon

NSP: Neurotoxic Shellfish Poisoning

NW: Northwest

OA: Okadaic Acid

PAR: Photosynthetically Active Radiation

PERMANOVA: Permutational Multivariate Analysis of Variance

PSP: Paralytic Shellfish Poisoning

PSU: Practical Salinity Unit

PTX: Pectenotoxin

QL: Quantification Limit

RE: Retention Efficiency

REPHY: French Phytoplankton and Phycotoxins Monitoring Network

SIMPROF: Similarity Profile Analysis

SST: Sea Surface Temperature

STX: Saxitoxin

TOC: Total Organic Carbon

TS: Temperature-Salinity

TTX: Tetrodotoxin

UI: Upwelling Index

UK: United Kingdom

UNESCO: United Nations Educational, Scientific and Cultural Organization

URA: Basque Water Agency

UV: Ultraviolet

WFD: Water Framework Directive

YTX: Yessotoxin

SUMMARY

Phytoplankton are the autotrophic component of the plankton community and, as the base of several food webs, they are a key element of both marine and freshwater ecosystems and contribute part or most of the organic carbon available to upper trophic levels. In this regard, phytoplankton are of essential importance for filter-feeding bivalves, among others, as they feed on the organic matter in suspension in the water and phytoplankton, in particular, are their main source of energy. However, phytoplankton can also be harmful for bivalve aquaculture, as some species produce potent biotoxins, which are ingested by filter-feeding organisms, accumulate within their flesh and get gradually transferred to the higher trophic levels, posing a threat to human health.

The interactions of mollusc bivalves with phytoplankton have been extensively studied in areas where bivalve aquaculture has been importantly developed. In the Basque Country (southeastern Bay of Biscay), an experimental bivalve farm was installed in open marine waters in 2012. The recent interest in developing offshore bivalve aquaculture on this coastal area has led to the need of studying and understanding the ecology and structure of phytoplankton community at local scale.

In this context, with the overall aim of improving the knowledge on phytoplankton community in open waters off the Basque coast, two main objectives have been defined in this thesis: (i) to describe the potential implications of phytoplankton attributes as food resource for mussels, and (ii) to evaluate the risk for offshore aquaculture associated with potential shellfish poisoning events.

To accomplish these goals, two information sources have been employed. On the one hand, a phytoplankton time series belonging to the "Littoral Water Quality Monitoring and Control Network" of the Basque Water Agency (URA) was analysed for the period 2003-2015, including information on both environmental variables and surface phytoplankton community along the Basque coast. On the other hand, a specific study was performed at the experimental bivalve farm from 2014 to 2017, including information on environmental variables and phytoplankton community throughout the water column, and also on toxin content in mussels.

For the first objective, phytoplankton composition, abundance and biomass were studied, together with the environmental conditions. Overall, appropriate phytoplankton attributes were found for mussel growth. The detected bloom events would be favourable for mussel culture, as previous studies have related phytoplankton blooms to increased growth and production of several mussel species. Similarly, the observed dominance of certain phytoplankton groups would also benefit the growth of mussels. In this sense, in the global study along the coast, diatoms revealed as the dominant group in surface waters, in terms of both number of bloom-forming species, spatial distribution and peaks of cell abundance. Similarly, diatoms were dominant during spring biomass peaks at the experimental farm, whereas dinoflagellates also showed a relevant contribution to the sub-superficial abundance and biomass. These two groups are important for mussel culture since they are known to synthesize some of the most important fatty acids for their

growth. Moreover, the observed high contribution of haptophytes would also favour mussel production, as they have been reported to contain, on average, the highest proportion of saturated fats. In relation to chlorophyll *a,* used as a proxy for phytoplankton biomass, the annual average was slightly above the threshold of 0.5 μg L^{-1} below which mussels have been reported not to filter. Finally, regarding the potential relationships between phytoplankton and the environment, phytoplankton community has been found to follow the seasonal cycle already described for temperate areas, which is driven by hydrographic conditions which depend, in turn, on atmospehric conditions. Depending on the season, between 21 and 29% of the species variability was explained by environmental parameters, mainly temperature and nutrients.

For the toxicity risk assessment, the presence of potentially toxic phytoplankton species was studied, together with their frequency, abundance and distribution. In the global study along the Basque coast, a special focus was given to the three genera more frequently associated to the three main shellfish poisonings that can affect human health (i.e., those produced by amnesic, paralytic or diarrheic toxins); whereas the specific study at the experimental farm included the concentration of these toxins (and any other one regulated by the European legislation) in mussels, together with the potential causative phytoplankton species and the environmental conditions. *Pseudo-nitzschia* spp., *Alexandrium* spp. and *Dinophysis* spp. (potential producers, respectively, of amnesic, paralytic and diarrheic toxins) showed a wide spatial distribution along the Basque coast, being detected in surface waters at most sampling stations. Their abundance was studied based on the trigger limits usually employed in European coastal zones. In global, these three genera exceeded the alert limits over most part of the Basque coast, but with frequencies usually low (<22% of the cases). Most of these cases occurred during spring. In the specific study at the experimental farm similar results were observed: *Pseudo-nitzschia* spp. and *Dinophysis acuminata* always showed the highest abundances in spring. In the present study, at least one of the analysed toxins was detected in mussels from the experimental site in almost 60% of the cases, although only 15% would have implied a ban on mussel harvest. All these cases occurred in spring and were given by okadaic acid and associated with *D. acuminata.* By contrast, the presence of toxins was not always linked to increased abundances of the causative phytoplankton taxa, and vice versa, which suggests that the established trigger limits cannot be recommended to ban bivalve harvest and new specific thresholds should be established. Finally, no general pattern was found between the studied toxic species and the environment. However, some specific associations were found: the greatest abundance increases of four target toxic species (i.e., *Pseudo-nitzschia* spp., *Dinophysis acuminata, Gonyaulax spinifera* and *Protoceratium reticulatum*) occurred in spring and within a very narrow temperature and salinity range throughout the water column. In addition, some *Dinophysis* species were found to be associated with high ammonium concentrations during summer and autumn, and with the presence of their prey *Mesodinium* during winter.

RESUMEN

El fitoplancton es el componente autotrófico de la comunidad del plancton y, en la base de las redes tróficas, es un elemento clave de los ecosistemas marinos y de agua dulce, donde contribuye en buena medida al carbono orgánico disponible para niveles tróficos superiores. A este respecto, el fitoplancton es de esencial importancia para los bivalvos filtradores, entre otros, ya que se alimentan de la materia orgánica en suspensión en el agua y el fitoplancton, en particular, es su principal fuente de energía. Sin embargo, el fitoplancton también puede ser un elemento de riesgo para la acuicultura de bivalvos, debido a que algunas especies producen potentes biotoxinas, que son ingeridas por organismos filtradores, se acumulan en su carne y son gradualmente transferidas a los niveles tróficos superiores, suponiendo una amenaza para la salud humana.

Las interacciones entre los moluscos bivalvos y el fitoplancton han sido ampliamente estudiadas en áreas donde la acuicultura de bivalvos ha tenido un gran desarrollo. En la costa vasca (sudeste del golfo de Vizcaya), en 2012 se instaló una planta piloto de cultivo de bivalvos en mar abierto. El reciente interés en desarrollar acuicultura *offshore* de bivalvos en esta área costera ha llevado a la necesidad de estudiar y entender la ecología y estructura de la comunidad de fitoplancton a escala local.

En este contexto, con el objetivo general de mejorar el conocimiento sobre la comunidad fitoplanctónica en la costa vasca, se han definido dos objetivos principales en esta tesis: (1) describir los atributos del fitoplancton que tienen implicaciones en su utilización como recurso alimenticio por parte de los mejillones, y (2) evaluar el riesgo potencial para la acuicultura *offshore* asociado a episodios de intoxicación por ingestión de marisco.

Para cumplir estos objetivos, se han utilizado dos fuentes de información. Por una parte, se ha analizado una serie temporal de fitoplancton perteneciente a la "Red de seguimiento del estado ecológico de las aguas de transición y costeras de la Comunidad Autónoma del País Vasco" para el periodo 2003-2015, que incluye información sobre las variables ambientales y sobre la comunidad de fitoplancton en superficie a lo largo de la costa vasca. Por otra parte, se ha llevado a cabo un estudio específico en la planta piloto de cultivo de bivalvos desde 2014 hasta 2017, incluyendo información sobre las variables ambientales y sobre la comunidad de fitoplancton a lo largo de la columna de agua, así como sobre el contenido de toxinas en mejillón.

Para el primer objetivo, se estudiaron la composición, abundancia y biomasa del fitoplancton, junto con las condiciones ambientales. En general, se encontraron atributos fitoplanctónicos favorables para el crecimiento de mejillones. Las floraciones ("blooms") detectadas serían favorables para el cultivo de mejillones, ya que estudios previos han relacionado las proliferaciones de fitoplancton con aumentos en el crecimiento y producción de varias especies de bivalvos. De manera similar, la dominancia observada de ciertos grupos de fitoplancton también beneficiaría el crecimiento de mejillones. En este sentido, en el estudio global a lo largo de la costa, las diatomeas aparecieron como el grupo dominante en las aguas superficiales, en términos de especies formadoras de floraciones, distribución espacial y picos de abundancia celular. Así mismo, las diatomeas fueron dominantes durante los picos de biomasa de primavera

en la planta piloto, mientras que los dinoflagelados también mostraron una contribución relevante a la abundancia y biomasa sub-superficial. Estos dos grupos son importantes para el cultivo de mejillones debido a su composición, rica en algunos de los ácidos grasos más importantes para su crecimiento. Además, la gran contribución observada de las haptofitas también favorecería la producción de mejillones, ya que se conoce que contienen, de media, la mayor proporción de grasas saturadas. En relación a la clorofila *a,* utilizada como una aproximación a la biomasa fitoplanctónica, la media anual en la planta piloto está ligeramente por encima del umbral de 0,5 $\mu g\ L^{-1}$ por debajo del cual se ha descrito que los mejillones no filtran. Finalmente, en cuanto a las relaciones entre el fitoplancton y el ambiente, se ha encontrado que la comunidad de fitoplancton sigue el ciclo estacional previamente descrito para áreas templadas, que responde a las condiciones hidrográficas, a su vez dependientes de las atmosféricas. Dependiendo de la estación del año, entre el 21 y el 29% de la variabilidad de las especies se vio que estaba explicada por las variables ambientales, principalmente temperatura y nutrientes.

Para la evaluación del riesgo de toxicidad, se estudió la presencia de especies de fitoplancton potencialmente tóxicas, junto con su frecuencia, abundancia y distribución. En el estudio global a lo largo de la costa vasca, se hizo especial hincapié en los tres géneros más frecuentemente asociados con las tres principales intoxicaciones por marisco que afectan a la salud humana (es decir, las producidas por toxinas de efecto amnésico, paralítico o diarreico); mientras que el estudio específico en la planta piloto incluyó la concentración de estas toxinas (y cualquier otra regulada por la legislación europea) en mejillones, junto con las especies de fitoplancton potencialmente causantes y las condiciones ambientales. *Pseudo-nitzschia* spp., *Alexandrium* spp. y *Dinophysis* spp. (potenciales productores de toxinas amnésicas, paralíticas y diarreicas, respectivamente) mostraron una amplia distribución a lo largo de la costa vasca, siendo detectados en las aguas superficiales de la mayoría de las estaciones de muestreo. Su abundancia se comparó con los umbrales de alerta comúnmente utilizados en zonas costeras europeas. En general, estos tres géneros superaron los límites de alerta a lo largo de la mayor parte de la costa vasca, pero normalmente con frecuencias bajas (22% de los casos). La mayoría de estos casos se dieron en primavera. En el estudio específico de la planta piloto se observaron resultados similares: *Pseudo-nitzschia* spp. y *Dinophysis acuminata* mostraron siempre las abundancias más altas en primavera. En casi el 60% de las campañas de análisis de mejillón de la planta piloto se detectó al menos una toxina, aunque sólo el 15% hubiera implicado una prohibición en la recolecta de mejillones para consumo. Estos últimos casos ocurrieron en primavera, se debieron a ácido ocadaico y pudieron asociarse a *D. acuminata.* Sin embargo, la presencia de otras toxinas no estuvo siempre asociada a aumentos en la abundancia del taxón de fitoplancton potencialmente causante, y viceversa, lo cual sugiere que los límites de alerta establecidos no pueden ser recomendados para prohibir la recolecta de bivalvos en esta zona y que se deberían establecer nuevos umbrales específicos para la costa vasca. Finalmente, no se encontró un patrón general de relaciones entre las especies tóxicas estudiadas y las variables ambientales. Sin embargo, se observaron algunas asociaciones específicas: los mayores aumentos de abundancia de cuatro taxones relevantes (i.e., *Pseudo-nitzschia* spp., *D. acuminata, Gonyaulax spinifera* and *Protoceratium reticulatum*) ocurrieron en primavera y en un rango muy estrecho de temperatura y salinidad a lo largo de la columna de agua. Además, algunas especies de *Dinophysis* se asociaron con altas concentraciones de amonio durante el verano y el otoño, así como con la presencia de su presa *Mesodinium* durante el invierno.

I. INTRODUCTION

1. Phytoplankton and marine aquaculture

Phytoplankton are the autotrophic component of the plankton community, and as these single-celled organisms form the base of several aquatic food webs, they are a key element of both marine and freshwater ecosystems. In the oceans, in particular, photosynthetic microalgae are the major primary producers, and as such they have an essential ecological role in maintaining the healthy structure and functioning of marine systems. Through photosynthesis, phytoplankton synthesize organic matter from solar energy, CO_2, macronutrients (nitrogen, phosphorous, silicate) and trace elements, and they contribute part or most of the organic carbon that is available to upper trophic levels and pelagic food webs (Reynolds 2006).

1.1. Factors that control phytoplankton communities in marine ecosystems

Phytoplankton assemblages depend on species succession, which is influenced in turn by environmental changes (e.g., Huisman *et al.* 1999), leading to a very dynamic interaction between this biologic component and the physico-chemical conditions in marine ecosystems. This dynamism is due to several factors such as their small size, rapid nutrient uptake, high growth rates and susceptibility to grazing (Stolte *et al.* 1994). Margalef (1978) and Margalef *et al.* (1979) proposed this now-classic mandala in which he related the life forms of diatoms and dinoflagellates to turbulence and nutrients, showing the importance of the balance between physical and nutritional forces. This mandala simplifies how different phytoplankton functional types cope with their environment, showing the differences in response between organisms that flourish under turbulent and nutrient-rich conditions, such as diatoms, and the organisms that are favoured by more nutrient-poor and less turbulent environments, such as dinoflagellates. However, these relationships are much more complex and the mandala has been revisited and extended (Smayda & Reynolds 2003; Wyatt 2014; Glibert 2016).

One of the most important requirements of these microorganisms are inorganic nutrients, such as nitrates, phosphates and sulphur, which they need to produce proteins, fats and carbohydrates. Hence, nutrients in adequate proportions and quantities are essential for their growth (Holligan 1992; Sigman & Hain 2012). There appear to be relatively uniform requirements for nitrogen and phosphorous among phytoplankton. As described by Redfield (1958), plankton build their biomass with C:N:P stoichiometric ratios of ~106:16:1, which we now refer to as the Redfield ratios. In situations of changes in nutrient availability, phytoplankton is usually the first autotrophic compartment to respond (Livingston 2000; Paerl *et al.* 2003). These responses of ecosystems to nutrient loading are variable; in some cases it may be an increase in the growth rate or turnover of one or more species, whereas in other cases the response may be an overall increase in algal biomass (Glibert *et al.* 2001).

It is widely recognized that both top-down regulation, such as grazing (Burkill *et al.* 1987), and bottom-up processes driven by meteorological and hydrographic factors play a major role in the control and dynamics of phytoplankton populations (Smayda 1998; Nogueira *et al.* 2000). On the

one hand, increasing evidence is given for the theory of trophic control in the oceans, suggesting that phytoplankton blooms are tightly controlled by microzooplankton grazing (Calbet & Landry 2004; Zarauz *et al.* 2009). On the other hand, nutrients and light are the main environmental factors controlling phytoplankton community structure, which depends in turn on physical processes related to temperature, salinity and turbulence (Troccoli *et al.* 2004; Álvarez-Góngora & Herrera-Silveira 2006).

In the same manner, environmental conditions are also important for the toxin-producing microalgae (Hallegraeff 2010; Röder *et al.* 2012; Glibert 2016). For instance, better growth rates for concrete toxic species have been associated with specific temperature or salinity ranges (Reguera *et al.* 1993; Röder *et al.* 2012). Similarly, anthropogenic coastal inputs alter the nutrient pool which may, in turn, imply favourable conditions for the proliferation of certain harmful species (Anderson *et al.* 2002).

Productivity in coastal ecosystems is often distinct from that of the open ocean. Along the coasts, the seafloor is shallow and sunlight can sometimes penetrate from the surface to the bottom, enabling benthic organisms to photosynthesize. The proximity to land and its nutrient sources, the essential processes of benthic recycling and the propensity for coastal upwelling all result in highly productive ecosystems (Sigman & Hain 2012). In these marine coastal areas, phytoplankton abundance and composition show a great spatio-temporal fluctuation, and in temperate areas in particular, they are characterized by seasonal variability, natural species succession and the occurrence of blooms (Berg & Newell 1986; Varela 1996). Many studies worldwide have highlighted the seasonal periodicity of phytoplankton assemblages linked to seasonal variations in the physical forcing of mixing dynamics, temperature and light regime (Diehl 2002; Diehl *et al.* 2002; Leterme *et al.* 2014; Agirbas *et al.* 2017).

1.2. Importance of phytoplankton for bivalve growth

As already mentioned, phytoplankton are at the base of marine food webs. In this regard, most bivalves are filter feeding organisms, and as such, they feed on the organic matter in suspension in the water. Phytoplankton, in particular, are the main source of energy for the growth of most filter-feeding bivalves (Shumway & Cucci 1987; MacDonald & Ward 1994; Grant 1996; Petersen *et al.* 2008). The nutritional value of microalgae varies depending on their size and shape, digestibility and biochemical composition together with the requirements of the animal feeding on the algae (e.g., Brown 2002). For instance, it is known that the quantity and cell-size of the phytoplankton can influence the recruitment of oysters as well as the survival of bivalve larvae (Robert & Trintignac 1997; Bourlès *et al.* 2009). Different phytoplankton retention efficiencies by filter-feeding bivalves have been observed depending on the cell-size, and although there is still considerable controversy about the most appropriate particle-size, the majority of the studies agree that the minimum particle-size for efficient retention is 4 μm (Møhlenberg & Riisgård 1978; Riisgard 1988; Jørgensen 1990), ranging up to 45 μm for high food depletion (Cranford *et al.* 2014). Similarly, not all phytoplankton species contribute equally to bivalve nutrition, as there are differences among groups in their edibility and nutritional value for higher trophic levels (Sterner & Elser 2002). Several bivalves (including mussels) have shown preferential utilisation of determinate species depending not only on their cell-size but also on their food value, such as their lipids content which is the main source of energy for bivalve larvae and which varies among different phytoplankton species or groups (Kiørboe & Mohlenberg 1981; Volkman *et al.* 1989;

Rouillon & Navarro 2003; Marshall *et al.* 2010). Additionally, phytoplankton blooms have been directly related to increases in mussel growth and condition index (i.e., the ratio between the dry weight of the meat and the shell) (Blanton *et al.* 1987; van der Veer 1989; Hickman *et al.* 1991).

1.3. Harmful Algal Blooms

Phytoplankton blooms are natural phenomena which contribute to the sustenance of bivalve and fish production. However, not all of these blooms are beneficial. The so-called *"Harmful Algal Blooms"* (HABs) can have deleterious effects on entire ecosystems and even cause important economic impacts (Anderson 2009). In the last decades, the increased frequency and geographical distribution of HAB events has been cited as one of the main problems in coastal regions worldwide (Masó & Garcés 2006). These occurrences are probably linked to a combination of factors, such as weather conditions impacting water parameters (i.e., salinity, temperature, currents and winds causing up- and downwelling) (Tan *et al.* 2006), improved methodologies for the detection of HABs and their toxins, increased dispersal of species as a consequence of anthropogenic activities (i.e., ballast waters, shellfish seeding) and eutrophication events leading to the stimulation of HABs (Hallegraeff 1993; Anderson 2009; Glibert & Burkholder 2011; Berdalet *et al.* 2014; 2015).

The term HAB is very broad and covers blooms of different types, but all of them have one unique feature in common: they cause harm (Anderson 2009). In terms of harmful effects, two main types of causative organisms can be considered within the phytoplankton group: the high-biomass producers and the toxin producers (Masó & Garcés 2006). The overgrowth of microalgae and its consequent accumulated biomass affects co-occurring organisms and alters food-web dynamics, causing effects such as the reduction of light penetration and anoxia, shading of sea grasses, oxygen depletion in the water from algal and bacterial respiration (especially upon death of the algal biomass) and suffocation of fish from the stimulation of gill mucus production (Glibert *et al.* 2001; Anderson *et al.* 2002; Landsberg 2002). But not all harmful algal events involve the development of significant accumulations of biomass because many HAB species can be harmful even at very low densities (Glibert *et al.* 2001; Masó & Garcés 2006). These species produce potent biotoxins which are ingested by filter feeding organisms, accumulate within their flesh (e.g., Wang 2008) and get gradually transferred to the higher trophic levels along the food web, posing a threat to human health (Shumway *et al.* 2003; Davidson & Bresnan 2009).

Among the 4000 marine phytoplankton species described worldwide, around 300 can occur in such high numbers that they cause damage, while at least 80 have the capacity to produce toxins (Hallegraeff 2003). Marine phytoplankton species, including toxic ones, present a wide geographic distribution (ICES 2017). Every year, the number of known toxic and harmful bloom-forming species increases. In the same way, new phytoplankton species are described yearly, showing that the actual values are underestimations of the real data (Zingone & Enevoldsen 2000; Smayda & Reynolds 2003). Globally, near 2000 cases of human intoxication occur every year through fish or shellfish consumption, with approximately 15% mortality (Hallegraeff 2014).

The main poisoning syndromes associated with the presence of phytoplankton toxins in shellfish' flesh are amnesic, paralytic, diarrheic, neurotoxic and azaspiracid shellfish poisoning (ASP, PSP, DSP, NSP and AZP, respectively) (FAO 2005). All of them are caused by different dinoflagellates except for ASP, which is caused by some species of the diatom genus *Pseudo-*

nitzschia H.Peragallo that are capable of producing the neurotoxin domoic acid (DA). PSP is associated with saxitoxins (STX) produced by some *Alexandrium* Halim species, as well as by *Gymnodinium catenatum* H.W.Graham and *Pyrodinium bahamense* var. *compressum* (Böhm) Steidinger, Tester & F.J.R.Taylor. Several species within the dinoflagellate genera *Dinophysis* Ehrenberg and *Phalacroma* Stein, together with *Prorocentrum lima* (Ehrenberg) F.Stein, can produce okadaic acid (OA) and are the main causative taxa for DSP. Most species of the genus *Karenia* Gert Hansen & Moestrup produce a variety of toxins that can result in the mortality of fish and other marine organisms when they bloom, and at least one species, *Karenia brevis* (C.C.Davis) Gert Hansen & Moestrup, produces brevetoxins, which can cause NSP (Brand *et al.* 2012). Finally, some species of the genus *Azadinium* Elbrächter & Tillmann produce azaspiracids (AZAs), which are lipophilic toxins that cause AZP (Hallegraeff 2003; 2014). Yessotoxin (YTX) and its analogues are lipophilic toxins that were initially associated with DSP (Visciano *et al.* 2013) but are now considered to make up a different group (Ferreiro *et al.* 2015; Visciano *et al.* 2016). Although YTX symptoms are still unknown in humans, mouse bioassays have shown paralytic effects on the cardiac muscle (Paz *et al.* 2008). YTXs are produced by the dinoflagellates *Protoceratium reticulatum* (Claparède & Lachmann) Bütschli, *Lingulodiniuim polyedra* (F. Stein) J.D.Dodge and *Gonyaulax spinifera* (Claparède & Lachmann) Diesing (Paz *et al.* 2004). Notwithstanding, all these events not only affect human health but the risk of intoxication produces great economic losses in the aquaculture industry, since the market must be banned when toxin concentrations are over the regulatory threshold (Hallegraeff 2003).

2. Case study of the Basque Country (southeastern Bay of Biscay)

2.1. The interest in bivalve offshore aquaculture

The interactions of mollusc bivalves with phytoplankton have been extensively studied, especially in areas where bivalve aquaculture is a strong socio-economic activity, such as China (e.g., Jiang *et al*. 2016; Li *et al*. 2016), the Atlantic French coast (e.g., Amzil *et al*. 2008; Batifoulier *et al*. 2013) and north-west Spain (e.g., Figueiras *et al*. 2002; Bravo *et al*. 2010). In the Basque Country, the recent interest in developing offshore bivalve aquaculture has led to the need for studying and understanding the ecology and structure of the phytoplankton community in Basque coastal waters.

According to the global context in which natural fish stocks are gradually depleted and fisheries capture falls short of world demand (FAO 2014, 2017), fishing over-exploitation and a decrease in the capture of commercial fisheries is also evident in the Bay of Biscay (DMAPTAP 2009). This situation has increased the need for searching for alternatives that diversify economic activities and create new products and employment in the aquaculture sector of the Basque Country (DMAPTAP 2009). In this context, an experimental bivalve farm was installed in 2012 in open waters off the Basque coast of Mendexa (Bizkaia). Previous to the installation of the experimental farm, a study was developed to assess the viability of submerged longlines for mollusc bivalve growth on the Basque coast. This study concluded that the Basque Country meets the appropriate hydrographic and environmental conditions as well as the market and socioeconomic aspects required for the start-up of these kinds of activities (Mendiola *et al.* 2011).

Some studies on mussel growth and quality in open waters off the Basque coast have already been developed. The first ecological study of mussel culture within the experimental farm off the

Basque coast observed positive results in terms of the growth, condition index and biochemical composition of mussels (Azpeitia *et al.* 2016). Thereafter, Azpeitia *et al.* (2017) compared the biometric parameters, nutritional content and sensory aspects between mussels from the experimental farm and mussels from the Galician Rias and concluded that the experimental mussels would compete with current commercial products.

2.2. Studies dealing with phytoplankton in Basque coastal waters

Despite the studies mentioned above, information on the phytoplankton community within the experimental bivalve farm is missing, and thus, research on the implications of phytoplankton for bivalve nutrition as well as on their associated toxicity in humans is needed.

The phytoplankton community and primary production within the southern Bay of Biscay was first studied more than 30 years ago (Estrada 1982; Flos 1982; Fernandez *et al.* 1991; Bode & Fernández 1992; Varela 1996). Since then, phytoplankton ecology has been studied in this zone, both in the open waters of the Bay (Rodriguez *et al.* 2003; Hartman *et al.* 2014; Smythe-Wright *et al.* 2014) and along the Spanish (Calvo-Díaz & Morán 2006; Granda & Anadón Álvarez 2008; Álvarez *et al.* 2009; Revilla *et al.* 2009; Garmendia *et al.* 2011; Seoane *et al.* 2012) and French (Lampert *et al.* 2002; Glé *et al.* 2008; Dupuy *et al.* 2011; Batifoulier *et al.* 2013) shelf and coastal waters.

A seasonal cycle has been described for phytoplankton dynamics in the Cantabrian Sea (southern Bay of Biscay) that corresponds with the hydrographic conditions (Varela 1996; Valdés & Moral 1998). Winter is characterized by water column mixing and high nutrient concentrations together with low irradiance levels that lead to low biomass and production of phytoplankton. During spring, the increasing irradiance heats the surface layers enhancing the stabilization of the water column and the proliferation of phytoplankton, giving rise to the spring bloom (Varela 1996). In summer, heating of the surface waters leads to a stratified water column; phytoplankton growth depletes surface nutrients and the thermocline acts as a physical barrier preventing the supply of nutrients from the deep reaching the surface, with the consequent low values of phytoplankton production and biomass (Varela 1996). Finally, in autumn, the thermocline is destroyed by surface cooling. The mixing of the water column causes nutrients to become available in the upper layers resulting in a second outburst of phytoplankton, though less important and persistent than that in the spring (Estrada 1982; Fernández 1990; Varela 1996; Calvo-Díaz *et al.* 2008).

However, in the Basque Country in particular, knowledge of coastal phytoplankton dynamics is more scarce, as most studies concerned with marine phytoplankton ecology have been undertaken in estuaries (Garcia-Soto *et al.* 1990; Orive *et al.* 1998; Trigueros & Orive 2001; Ansotegui *et al.* 2003; Orive *et al.* 2004; Seoane *et al.* 2005; 2006; Laza-Martinez *et al.* 2007; Butrón *et al.* 2009). Estrada (1982) described for the first time the annual cycle of phytoplankton in coastal waters off the Basque Country. There, the typical seasonal cycle previously described for temperate coastal areas was found: the highest total abundance was registered in spring, whereas the lowest values were observed in summer and early-autumn. The phytoplankton community showed a succession from diatoms to dinoflagellates, corresponding to the transition from water column mixing to stratification (Estrada 1982). More recently, Revilla *et al.* (2009) studied the bloom frequencies along the Basque coast, concluding that most of the bloom-forming taxa

belonged to the group of diatoms and finding a higher bloom frequency in nearshore areas compared to offshore (Revilla *et al.* 2009). Garmendia *et al.* (2011) studied the variability in phytoplankton biomass and composition and observed the same typical seasonal cycle previously described for the southern Bay of Biscay.

Despite the existing information on general phytoplankton dynamics in Basque coastal waters, there is a gap in the implications of the phytoplankton community for mussel aquaculture in this area. On the one hand, there are no studies relating phytoplankton attributes, such as composition and biomass, with mussel nutrition. On the other hand, although the toxicity risk for humans through shellfish consumption has been extensively studied in areas where strong bivalve aquaculture activities are developed (such as Galicia and Arcachon) (Fernández *et al.* 2006; Bravo *et al.* 2010; Maurer *et al.* 2010; Rodríguez *et al.* 2015), no information is available for Basque coastal waters. Seoane *et al.* (2012) studied the presence of toxic phytoplankton species on the neighbouring Cantabrian coast, but no additional information on toxin concentrations in mussels was included.

3. General aim and structure of the thesis

Considering all of the above, there is a lack of information on the phytoplankton community and its implications for mussel aquaculture within Basque coastal waters. In particular, there is a need to improve our knowledge of the distribution of HAB species and phycotoxins at the local scale in order to prevent human health problems, damage to aquaculture and economic losses. Hence, the present study aims to address (i) the participation of different phytoplankton attributes (i.e., composition, abundance, size and biomass) in mussel nutrition and (ii) the potential toxicity risk for humans associated with toxic phytoplankton species.

For this, two sources of information have been employed. One source is the data belonging to the "Littoral Water Quality Monitoring and Control Network" of the Basque Water Agency (URA). This programme has monitored Basque coastal and estuarine water quality since 1994, contributing to European Directives (e.g., Water Framework Directive 2000/60/EC, European Marine Strategy Framework Directive 2008/56/EC) (Borja *et al.* 2004; 2009a; 2011b). In addition to this and taking into account the limitations of the phytoplankton time series for this concrete investigation, new data have been obtained as a second source of information by means of specific sampling activities at the experimental bivalve farm over the period 2014–2017. It is important to remark that the identification of all phytoplankton samples from both data series has been carried out by taxonomists from the Department of Plant Biology and Ecology of the University of the Basque Country.

In regard to the time series, although it was not designed for the purpose of the present study, it can provide essential information. The global importance of time series is well-acknowledged, as they represent one of the most valuable tools available to characterize and quantify ocean cycles and fluxes, among other factors (Valdés & Lomas 2017). Thus, phytoplankton time series in particular are essential for the assessment of the ecological health status of the waters as well as the changes occurring as a consequence of both climatic and anthropogenic pressures (Zingone *et al.* 2015). Although this time series only provides information on a seasonal scale for surface waters, it contains very relevant information since it covers the whole Basque coast over a long

period (more than ten years), including both environmental and phytoplankton data with which spatial and temporal variability can be studied.

The specific study at the experimental farm has been designed with the aim of complementing the information provided by the time series. This complementary study employs a much higher sampling frequency and provides information on the environmental variables and phytoplankton community throughout the whole water column, as well as on the toxin content in mussels.

II. HYPOTHESIS AND OBJECTIVES

This thesis aims to get a better understanding of the phytoplankton community in open marine waters off the Basque coast, given the present interest in the Basque Country for the implementation of offshore bivalve aquaculture.

Previous studies that are commented in the General Introduction have been considered in order to formulate the hypotheses for this dissertation, which is based on the following findings:

- Mussels present good growth performance in an experimental farm located in this study area.
- Some phytoplankton species, including toxic ones, present a wide geographic distribution.
- Different phytoplankton species can have different responses to environmental conditions.

Hypothesis

"In open waters off the Basque coast, (i) the phytoplankton attributes involved in mussel nutrition present spatial and seasonal differences, (ii) the phytoplankton community implies the presence of biotoxins in these bivalves' flesh under specific environmental conditions."

Objectives

The general objective of this study is to describe the dynamics and structure of phytoplankton community along Basque coastal waters and, in particular, within the experimental bivalve farm located at 2 nautical miles off the coast of Mendexa (Bizkaia).

To achieve this principal goal, the next specific objectives have been defined:

1. Describe the bloom events in coastal waters of the Basque Country, together with their potential implications for bivalve nutrition.

2. Describe the phytoplankton community composition, including potentially toxic species, along coastal waters of the Basque Country in different seasons of the year.

3. Describe the relationships between environmental factors and phytoplankton community variability (in terms of abundance and biomass).

4. Assess the intra-annual variability of phytoplankton community throughout the water column within an experimental offshore bivalve farm and assess its potential implications for mussel aquaculture.

5. Assess the presence of phytoplankton biotoxins in mussels and identify the potential phytoplankton causative species in an experimental bivalve farm off the Basque coast.

III. STUDY AREA

1. The Bay of Biscay and the Basque coast: location and main hydrographic features

The Basque coast is located within the southeastern Bay of Biscay, at mid-latitude of the Northeast Atlantic Ocean (Figure III.1). The Bay of Biscay encompasses the area from NW France (offshore of Brittany) to NW Spain (Galicia), considering Cape Finisterre (43° N latitude) as the southern limit. Overall, the Bay of Biscay shows a strong thermal stratification in summer followed by a strong water column mixing in winter (Lavín *et al.* 2006). Continental freshwater discharges from numerous rivers contribute to the existence of buoyant low-salinity plumes, particularly during late winter and spring, along the Bay of Biscay (Puillat *et al.* 2004).

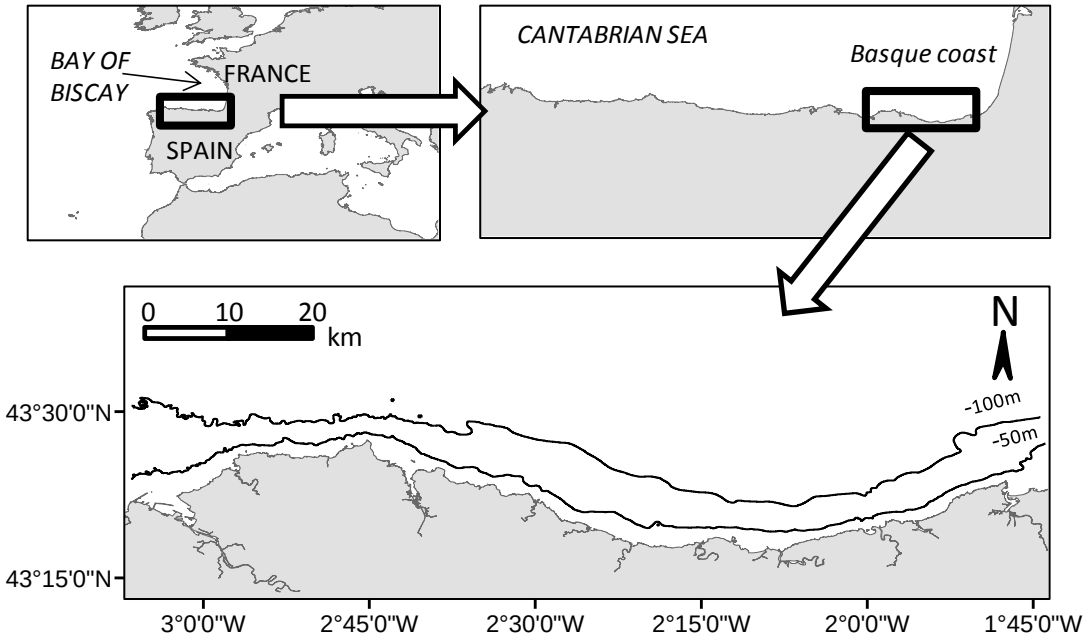

Figure III.1. Map of the location of the Basque coast, in the southeastern Bay of Biscay.

In the southeastern Bay of Biscay, the annual cycle corresponds to that of temperate sea areas. Winter is characterised by water column mixing, which is generated by a combination of cooling, turbulence and downwelling. This mixing process modifies the properties of the upper waters and leads to great nutrient input from deep waters to the surface. In spring, solar irradiance heats the surface resulting in an increase in the temperature of these waters and a relative stabilisation. However, the stratification of the water column depends also on the relaxation of wind, turbulence and downwelling. Summer is characterised by stratification resulting from greater solar irradiance. Finally, during autumn the surface waters cool down and the southerly and westerly winds prevail, resulting in the mixing of the water column (Valencia *et al.* 2004; Lavín *et al.* 2006; Fontán *et al.* 2008).

The Basque coast, located between the west-east oriented Cantabrian littoral and the north-south oriented French coast, extends approximately 100 km along the Cantabrian Sea (Figure III.1). The climate of the area is rainy, temperate and oceanic, with moderate winters and warm

summers (e.g., Fontán *et al.* 2009). It can be described as a littoral coast exposed to waves, mostly formed of cliffs and influenced by the input of 12 short rivers. In comparison to the French rivers, nutrient loads from the Cantabrian rivers to the Bay of Biscay are very low. As an example, nitrogen annual contribution from the Cantabrian basins to the Bay of Biscay are estimated to be 1.0×10^9 mol, whereas the Loire river annually transports 6.4×10^9 mol of nitrogen (Lavín *et al.* 2006).

Basque coastal waters have been classified as euhaline, exposed and fully mixed waters with negligible natural fertilization given by upwelling events or large river plumes (Carletti & Heiskanen 2009). Although no large coastal plumes are formed (Diez *et al.* 2000), this freshwater supply modifies the chemical composition of the shelf waters and often leads to increased nutrient levels in inner shelf waters (Valencia *et al.* 2004; Ferrer *et al.* 2009). Wind-driven upwelling events strongly affect the nearby Galician coasts (Fraga 1981), but this activity decreases eastward along the Cantabrian shelf and it is almost negligible on the Basque coast (Valencia *et al.* 2004; Lavín *et al.* 2006). As a result, the natural inputs of deep nutrient-rich waters also decrease in this area.

At station RF10 (Figure III.2), about 13 km off the Basque coast, sea surface temperature (SST) presents a distinct seasonal cycle, and chlorophyll *a* in surface waters (0–1 m) is inversely correlated to SST. The cold season, at this station on the mid shelf, can be defined as November–April, with monthly averaged SST ranging from 12.5 to 16.5°C and average surface chlorophyll ranging from 0.6 to 1.0 µg L^{-1}. The warm season can be defined as May–October (15.6–22.7°C, monthly averaged SST). During the warm months, the mean chlorophyll concentration is below 0.5 µg L^{-1} in surface waters (Morán *et al.* 2012).

2. Sampling stations in open marine waters off the Basque coast

2.1. Stations for surface waters along the coast: the "Littoral Water Quality Monitoring and Control Network"

Chapters 1, 2 and 3 of the present thesis draw on data from the "Littoral Water Quality Monitoring and Control Network" of the Basque Water Agency (URA) (Figure III.2). This program has monitored Basque coastal and estuarine water quality since 1994 (Borja *et al.* 2004; 2009a). For phytoplankton, the network includes 32 estuarine stations, 16 coastal stations and 3 offshore stations. The time series comprises data on hydro-morphological, physico-chemical (in water, sediment and biota) and biological elements (phytoplankton, macroalgae, benthos and fishes) under the requirements of the Water Framework Directive (Borja *et al.* 2010; 2011a).

From all the information available in the time series, this study focuses on coastal and offshore stations for the period 2003-2015 and includes information on surface phytoplankton community, chlorophyll *a* and water physico-chemical variables.

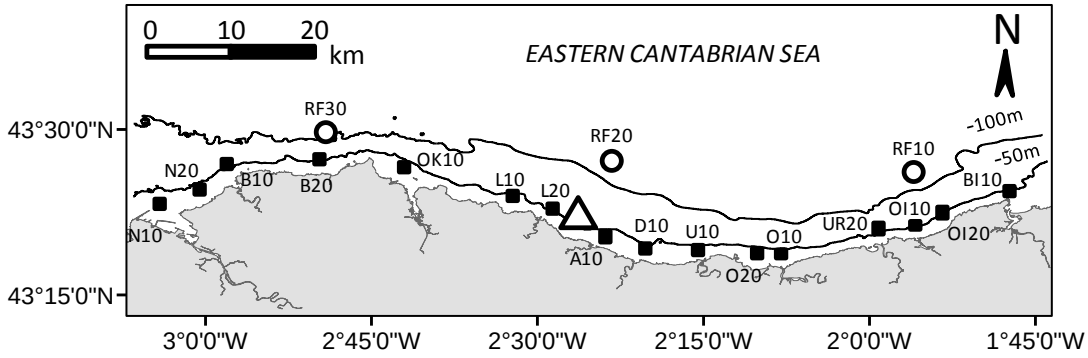

Figure III.2. Map of the Basque coast showing the locations of the 16 nearshore sampling sites (represented by squares) and the three offshore sampling sites (represented by circles) belonging to the "Littoral Water Quality Monitoring and Control Network" of the Basque Water Agency (URA). The triangle shows the location of the experimental bivalve farm, in front of the coast of Mendexa (Bizkaia), installed in 2012.

2.2. The water column sampling: stations at the experimental bivalve farm

Chapters 4 and 5 are based on data collected at an experimental bivalve farm installed in 2012 in open waters off the Basque coast, in front of the coast of Mendexa (Figure III.2). The experimental farm is located at 2 nautical miles (3-4 km) off the coast, at a depth of approximately 45-50 m, and it consists of longlines. This system is based on a subsurface structure maintained by buoys, from which bivalve ropes and lanterns are suspended, and anchored to the sea bottom (e.g., Sunila *et al.* 2004) (Figure III.3). Longline systems are especially appropriate in wave-exposed areas and thus, it is the preferred option for offshore bivalve culture (e.g., Morse and Rice 2010). The organisms currently cultured at the farm are mussels (*Mytilus galloprovincialis* Lamarck*)* and, to a lesser extent, oysters (*Crassostrea gigas* Thunberg and *Ostrea edulis* Linnaeus*)*.

Figure III.4 shows a scheme of the vertical location of the mussel culture, and the sampling depths for phytoplankton and physico-chemical variables, within a theoretical water column structure. Mussels were taken from the upper layers of the culture for toxin analysis. In addition, a single station, near but outside the mussel culture area, was employed for phytoplankton and physico-chemical variables (43° 21,411' N; 2° 26,918' W). The selection of the sampled discrete depths (3, 10, 17, 24, 33 and 42 m) was made based on the heterogeneity of the water column that is expected during the stratification period in this area (Valencia *et al.* 2004) and with the aim of obtaining a good representation of the water column. In order to know the approximated location of the thermocline and the effect of river plumes, together with the information given by Valencia *et al.* (2004), quarterly CTD casts of temperature and salinity from 1997 to 2011 were studied (Annex III.1 and Annex III.2). These had been obtained at two stations (L10 and A10, Figure III.2) in the vicinity of the experimental farm. Moreover, vertical profiles of chlorophyll *a* and oxygen saturation (%), as a proxy for primary production, were studied at both stations (Annex III.3 and Annex III.4). These data showed the presence of peaks of both chlorophyll and oxygen saturation in the discrete water depths selected for phytoplankton sampling.

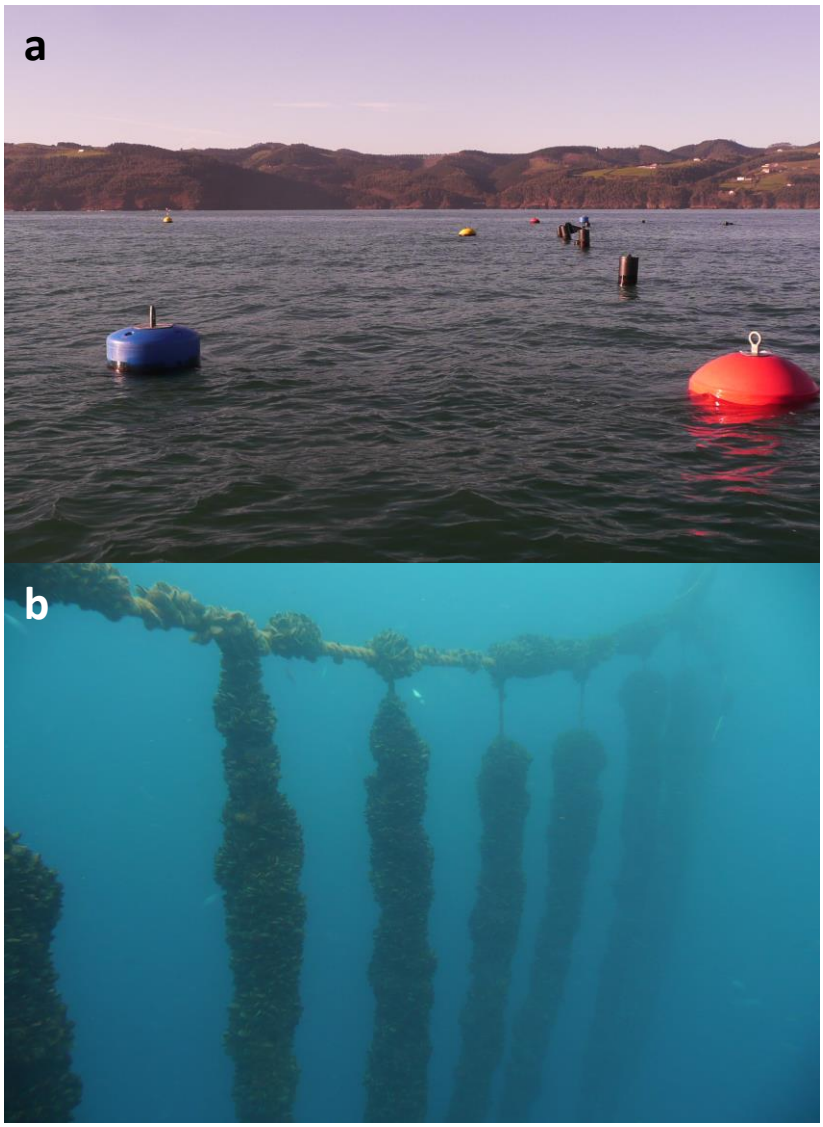

Figure III.3. Pictures of the experimental bivalve farm off the Basque coast showing: (a) a surface view with the buoys that maintain the structure, and (b) the subsurface structure with suspended mussel ropes.

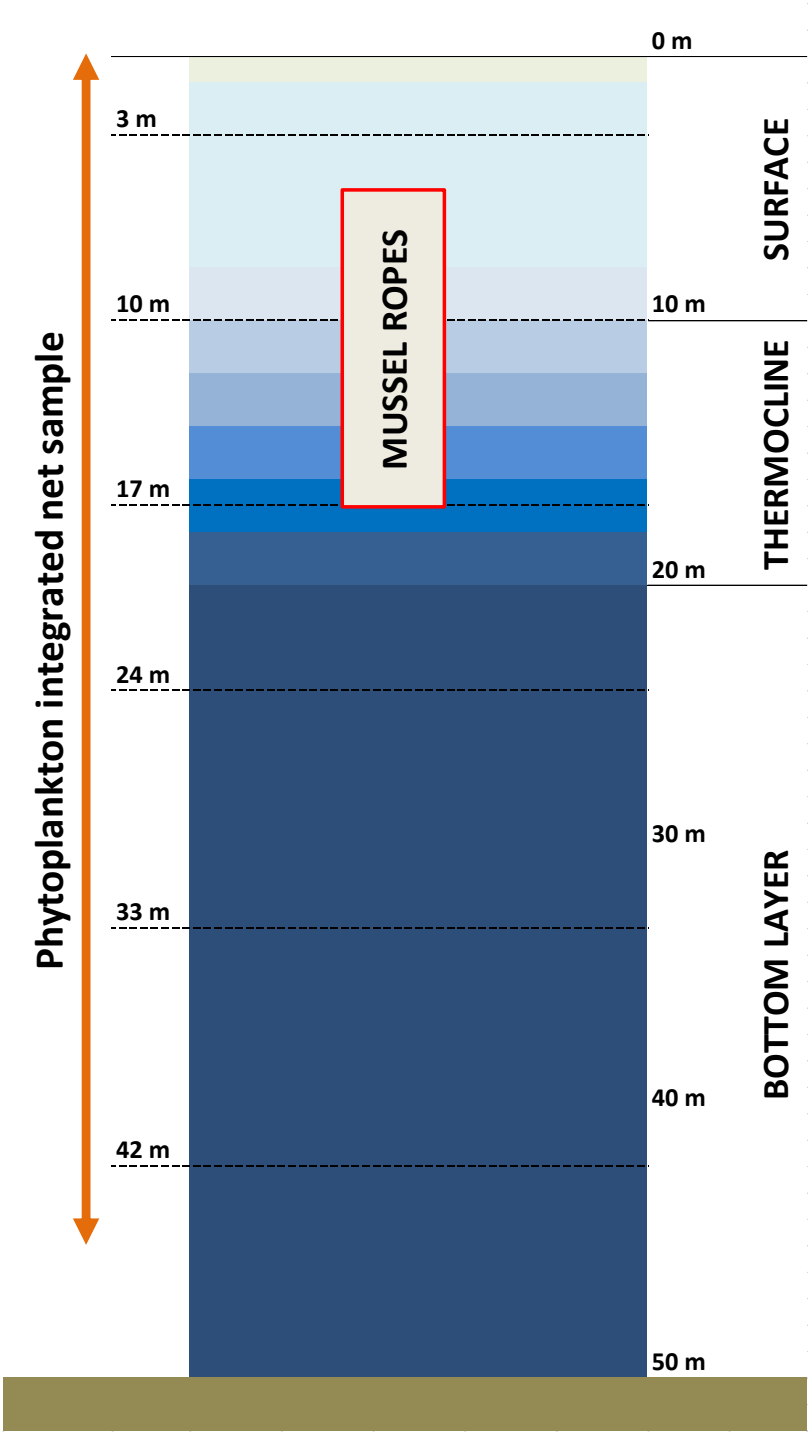

Figure III.4. Distribution of the mussel culture within the experimental bivalve farm in coastal waters off the Basque coast (modified from Azpeitia *et al*. 2016). The dotted lines indicate the depths at which water samples were taken. On the right a theoretical water column structure based on previous studies on the oceanography of the Basque Country is depicted. This structure illustrates a typical summer situation for mid shelf waters, although the depth and strength of the thermocline can vary, both intra- and interannually, in response to meteorological factors and currents (Valencia *et al*. 2004).

Chapter 1

Evaluation of phytoplankton quality and toxicity risk based on a long-term time series previous to the implementation of a bivalve farm (Basque coast as a case study)

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Abstract

In the last decades there has been a great development in aquaculture worldwide and, on the Basque coast (southeastern Bay of Biscay) in particular, there is a recent interest in implementing bivalve aquaculture in open marine waters. In this context, the study of phytoplankton is essential given that it is the main source of energy for bivalves and, at the same time, a main potential toxicity risk. Bivalves, as filter-feeding organisms, can accumulate phycotoxins and these can be transferred through the food-chain, posing a threat to humans. All this, together with a recently installed pilot-scale bivalve farming, motivated a study of the phytoplankton community. Here, 11-year phytoplankton time series from 16 nearshore and 3 offshore stations off the Basque Country are analysed, as a preliminary step for evaluating the potential of this region for aquaculture development. Special attention was given to bloom events and potentially toxic taxa. A total of 32 bloom-forming taxa were detected, mostly diatoms. In regard to harmful species, all stations presented many potentially toxic taxa, mostly dinoflagellates. The diatom genus *Pseudo-nitzschia* was the one blooming at more stations. *Pseudo-nitzschia* spp. as well as the dinoflagellates *Dinophysis* spp. and *Alexandrium* spp., which might be causative of Amnesic, Diarrheic and Paralytic Shellfish Poisoning, respectively, exceeded the abundance limits that would imply toxicity risk in several occasions, mostly during spring and summer. However, it occurred at a low frequency (in average, <15% for *Pseudo-nitzschia* spp. and <10% for the dinoflagellates). Overall, phytoplankton community composition and abundance, together with the low frequencies for the exceeded alert limits by the three main phycotoxin producing genera, suggest that the area presents appropriate conditions for bivalve aquaculture.

1. Introduction

World aquaculture production has greatly increased in the last 60 years, from about 20 million t in 1950 to almost 150 million t in 2010 (FAO 2012). Production of marine molluscs presently accounts for 75% of global marine aquaculture and it is expected to keep expanding given the depletion of natural stocks (Barg 1992; FAO 2014). Spain is amongst the biggest aquaculture mussel producers in a world scale and the first in the European Union. In Spain, almost the entire aquaculture mussel production is developed on the northwest coast, in Galicia. There, mussel cultivation is the most important socio-economic activity with an annual production above 250,000 t (Figueiras *et al.* 2002). Nevertheless, this activity has never been developed on the Basque coast (northern Spain).

Bivalves, as filter-feeding organisms, get the energy and nutrients necessary to grow from suspended microscopic food particles (e.g. Jørgensen 1990). Regarding shellfish aquaculture, phytoplankton is the main component of the diet of suspension feeding bivalves (Shumway & Cucci 1987; MacDonald & Ward 1994; Grant 1996; Petersen *et al.* 2008). The quantity and size of the phytoplankton can influence the recruitment of oysters for instance, as well as the survival of bivalve larvae (Robert & Trintignac 1997; Bourlès *et al.* 2009). Moreover, in field studies, *Wall et al*. (2013) observed that the growth rates of bivalves were more related to the density of certain cellular types than to the total phytoplankton biomass. Therefore, a good knowledge of phytoplankton composition and variability is essential to assess the appropriateness of an area to sustain bivalve aquaculture.

Phytoplankton can also be harmful: the so-called "*Harmful Algal Blooms*" (HABs) can have deleterious effects on entire ecosystems, and even cause important economic impacts (Anderson 2009). In fact, the increased frequency of HABs has been indicated as one of the main problems in coastal regions worldwide. In terms of harmful effects, two types of causative organisms can be considered within the phytoplankton: the high-biomass producers and the toxin producers. Although some taxa present both features, the last ones can be harmful even at very low densities (Masó & Garcés 2006). This is because phytoplankton toxins ingested by filter feeding organisms can accumulate within their flesh (e.g. Wang 2008) and get gradually transferred to the higher trophic levels along the food web, posing a threat to human health (Davidson & Bresnan 2009). Examples of toxic syndromes include ciguatera fish poisoning (CFP), and paralytic, diarrheic, neurotoxic, azaspiracid and amnesic shellfish poisoning (PSP, DSP, NSP, AZP and ASP, respectively) associated mostly with shellfish consumption (Glibert *et al.* 2001). Other less frequent toxins produced by microalgae have also been evidenced to produce damages to humans and/or shellfish, such as yessotoxins (Amzil *et al.* 2008), palytoxins (Aligizaki *et al.* 2011) and pectenotoxins (Fernández *et al*. 2006). In addition to the production of toxins, some phytoplankton species could cause mechanical stress to other organisms (Delegrange *et al*. 2015) implying also a damage to aquaculture.

Many studies have been carried out in the Basque estuaries regarding phytoplankton composition (Orive *et al*. 1998; Trigueros & Orive 2001; Ansotegui *et al*. 2003; Seoane *et al*. 2005; 2006; Laza-Martinez *et al*. 2007) and potentially toxic species (Orive *et al*. 2010; 2013). However, very few studies have addressed the composition and the size-structure of the phytoplankton communities in open coastal waters of the Basque Country. Furthermore, there is a limited

amount of information about toxic species from the neighbouring areas (e.g., the French Phytoplankton and Phycotoxins Monitoring Network – REPHY (Maurer *et al*. 2010)) and, to our knowledge, only the studies of Seoane *et al*. (2012) and Batifoulier *et al*. (2013) addressed it in open waters near the Basque coast.

In this context, research at the local scale is necessary in order to understand HAB dynamics and enhance the management of coastal ecosystems. This is of special concern in the Basque Country, since during the last years there is an increasing interest in developing shellfish aquaculture in open waters of this region, where a pilot-scale bivalve farming (longline system) was installed in 2012 (Azpeitia *et al*. 2016).

Taking all this into account, the present study aims to contribute to the evaluation of the potential of this region for the development of aquaculture activities in exposed marine areas from the perspective of the phytoplankton composition taking advantage of a long-term data series (2003-2013). For this, the specific objectives of this study are to evaluate (i) the quality of the phytoplankton to sustain bivalve growth, and (ii) the occurrence of phytoplankton species considered to have the capacity for toxin production, within open coastal waters of the Basque Country (southeastern Bay of Biscay).

2. Materials and methods

2.1. Study area

The Basque coast is located in the eastern Cantabrian Sea, southeastern Bay of Biscay (Figure 1.1). It extends approximately 100 km along the north of Spain. It can be described as an exposed littoral coast, mostly formed by cliffs and influenced by 12 short rivers, accounting for a total flow of about 150 $m^3 s^{-1}$ (annual mean). Although no large coastal plumes are formed (Diez *et al.* 2000), this freshwater supply modifies the chemical composition of the shelf waters and leads often to increased nutrient levels in inner shelf waters (Valencia *et al*. 2004; Ferrer *et al*. 2009). The upwelling activity is almost negligible on the Basque coast (Valencia *et al*. 2004). The climate of the area is rainy, temperate and oceanic, with moderate winters and warm summers. According to Köppen's classification it is described as marine west-coast and mild (Fontán *et al*. 2009).

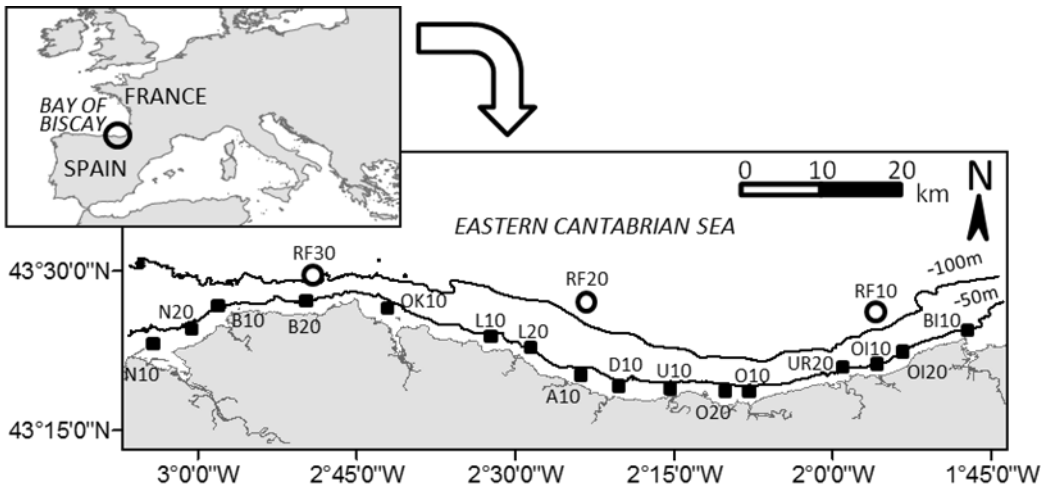

Figure 1.1. Map showing the study area and sampling stations. Squares correspond to nearshore sampling sites and circles to offshore sampling sites.

2.2. Sampling strategy and laboratory work

In this study, data from 19 stations of the "Littoral Water Quality Monitoring and Control Network" of the Basque Water Agency were employed (Figure 1.1) (Borja *et al*. 2004; Borja *et al*. 2016). Most of these stations (16 of them) are located in exposed coastal areas at a depth of 25-35 m. Three further stations are offshore, at 100-120 m depth.

The data set involves 11 years (from 2003 to 2013) except for two offshore stations that present a 5-year data set (RF20 and RF30, from 2009 to 2013). Two samplings per year (spring and summer) were conducted from 2003 to 2007 and four (winter, spring, summer and autumn) from 2008 to 2013. A summary of all the samplings performed is included in Annex 1.1.

Samples were taken in surface waters (0-1 m depth), preserved immediately and maintained in 125 ml borosilicate bottles in dark and cool conditions (4°C) until analysis. Glutaraldehyde was used for preservation until 2011 and acidic Lugol from then on. The taxonomic identification and cell counting were made on subsamples of 50 ml and 10 ml, depending on the density of particles settled, following the Utermöhl method (Utermöhl 1958; Hasle 1978; Edler & Elbrächter 2010). Therefore, the picoplankton fraction was not recorded. Most of the diatoms and armoured dinoflagellates were identified to the level of species. Smaller or more fragile forms were classified generally at the level of genus or class. The nanophytoplankton cells that could not be assigned to any taxonomic group were clumped together into a group named "unidentified forms <10 µm". A taxa list is provided in Annex 1.2.

2.3. Composition and size-structure of phytoplankton blooms

Historically, blooms have been inferred to be significant population increases. However, there is no universal criterion or specific cell abundance to define a bloom event (Smayda 1997). In this study the cell-size-based approach defined by Revilla *et al*. (2009) was followed to define bloom episodes. Two different thresholds were used based on the Equivalent Spherical Diameter (ESD): 7.5×10^4 cells L^{-1} for taxa >20 µm, and 7.5×10^5 cells L^{-1} for taxa 2-20 µm. The size-split is of special interest for this study given the size-dependent ingestion efficiency of mussels (Møhlenberg & Riisgård 1978; Cranford *et al*. 2014).

2.4. Toxicity risk

The occurrence of potentially toxic species was studied according to the Taxonomic Reference List of Harmful Micro Algae from the Intergovernmental Oceanographic Commission of the UNESCO (Moestrup *et al.* 2009 onwards) and contrasted with Algae Base database (Guiry & Guiry 2015). For genera that are known to contain toxic species, when it was not possible to identify the organism at species level, the whole genus was included in the toxicity list as a measure of precaution.

Alert levels of cell concentration taken from the literature were applied to the main causative genera of the three syndromes of greatest concern, ASP, DSP and PSP, e.g. *Pseudo-nitzschia* spp.*, Dinophysis* spp. and *Alexandrium* spp., respectively (Lawrence *et al*. 2011) (Table 1.1). For *Pseudo-nitzschia* spp. some differences can be found in the literature on the established alert limit and, hence, two thresholds were employed. In the case of *Dinophysis* spp., the lowest value indicates the limit for presence of toxins, and the highest one could imply a ban on mussel harvesting for

human consumption. Regarding *Alexandrium* spp., its mere presence would imply a risk. These limits were applied at genera level, summing up the abundances of the different registered species, as a precautionary measure for toxicity risk. The threshold levels employed here are common in European harmful phytoplankton monitoring programs (ICES 2015). Finally, although an alert limit of 5,000 cell L^{-1} was found for *Karenia brevis* (a NSP causative organism) (e.g., Etheridge 2010), this limit was not applied since this species was not detected in the dataset.

Table 1.1. Alert levels used in this study for phytoplankton taxa associated to risk of shellfish poisoning (ASP: Amnesic Shellfish Poisoning; DSP: Diarrheic Shellfish Poisoning; PSP: Paralytic Shellfish Poisoning).

Risk	Taxon	Alert level (cells L^{-1})	Reference
ASP	*Pseudo-nitzschia* spp.	50,000	Swan and Davidson (2012)
ASP	*Pseudo-nitzschia* spp.	100,000	Bates *et al*. (1998), Fillon *et al*. (2013)
DSP	*Dinophysis* spp.	100	Swan and Davidson (2012), Fillon *et al*. (2013)
DSP	*Dinophysis* spp.	500	Fillon *et al*. (2013)
PSP	*Alexandrium* spp.	presence	Swan and Davidson (2012)

2.5. Statistical analysis

All statistical analyses were performed with Statgraphics Centurion software. Temporal (season and year) and spatial differences in total cell abundance were analysed by means of ANOVA tests. Prior to the study of the differences, data were log transformed to fit a normal distribution.

3. Results

3.1. Phytoplankton size-structure, abundance and composition

Phytoplankton community abundance was dominated by cells ranging 2-20 µm (ESD), with average values between 90.1 % and 99.3 % at each sampling site in terms of contribution to the total phytoplankton cell abundance (Figure 1.2). Nevertheless, from that contribution, between 6.4 % and 78.8 %, depending on the sampling station, was given by chain-forming diatoms (Annex 1.3). In terms of total biomass, the contribution of the two cell-size ranges presented higher variability along the coast (Figure 1.3). The cells larger than 20 µm contributed between 15.5 and 84.4% to total biomass. Moreover, the contribution of the diatoms to total biomass was remarkable, ranging from 44 to 95% (Annex 1.4).

No clear W-E variation pattern was found in the mean total phytoplankton abundance nor in the mean total phytoplankton biomass distribution along the Basque coast (Figure 1.2 and 1.3). Total cell densities (geometric mean) at the nearshore stations varied from 2.7×10^5 to 4.7×10^5 cells L^{-1}, whereas at the offshore stations ranged from 2.6×10^5 to 2.9×10^5 cells L^{-1}. Total biomass was in the range 15.4-42.2 µg C L^{-1} (geometric mean), also with relatively low values at the offshore stations.

Significant differences ($p < 0.005$) were observed in mean values of both log transformed cell abundance and log transformed total biomass among the seasons and years, but not among sampling stations (Annex 1.5).

Taking into account sampling season, the highest geometric mean value for the total abundance was observed in spring (6.0 x 10^5 cells L^{-1}). Geometric means in summer, autumn and winter were of 3.6 x 10^5 cells L^{-1}, 2.4 x 10^5 cells L^{-1} and 2.7 x 10^5 cells L^{-1}, respectively (Figure 1.4A). Regarding the year sampled, the highest geometric mean value was registered in 2006 (9.8 x 10^5 cells L^{-1}) and the lowest in 2004 (1.8 x 10^5 cells L^{-1}) (Figure 1.4B).

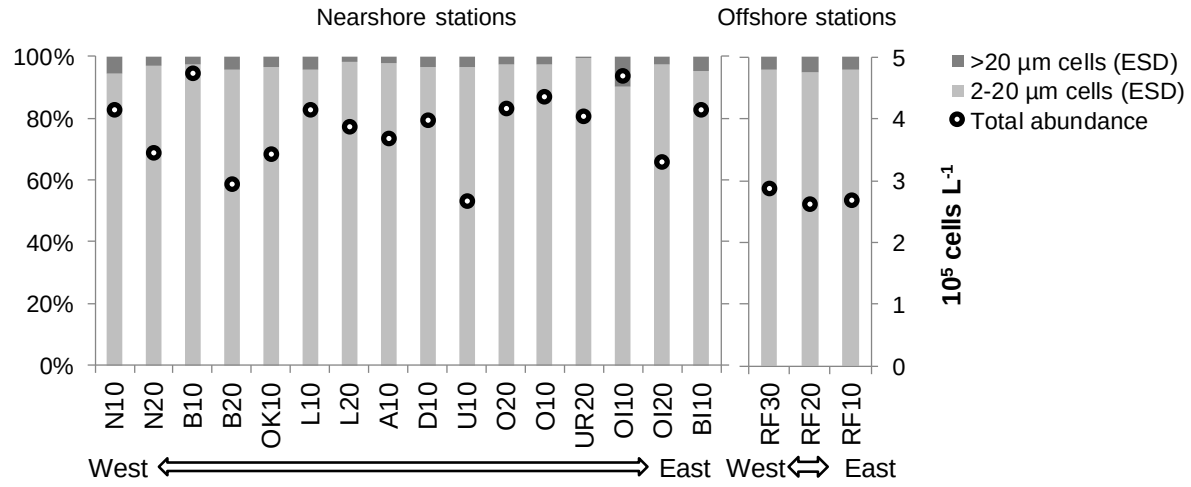

Figure 1.2. Average phytoplankton abundance during the study period (11 years for all sites, except stations RF20 and RF30, with a 5-year period). Right axis: total cell abundance (geometric mean). Left axis: percentage contribution of the two size-fractions considered (arithmetic mean). ESD: Equivalent Spherical Diameter.

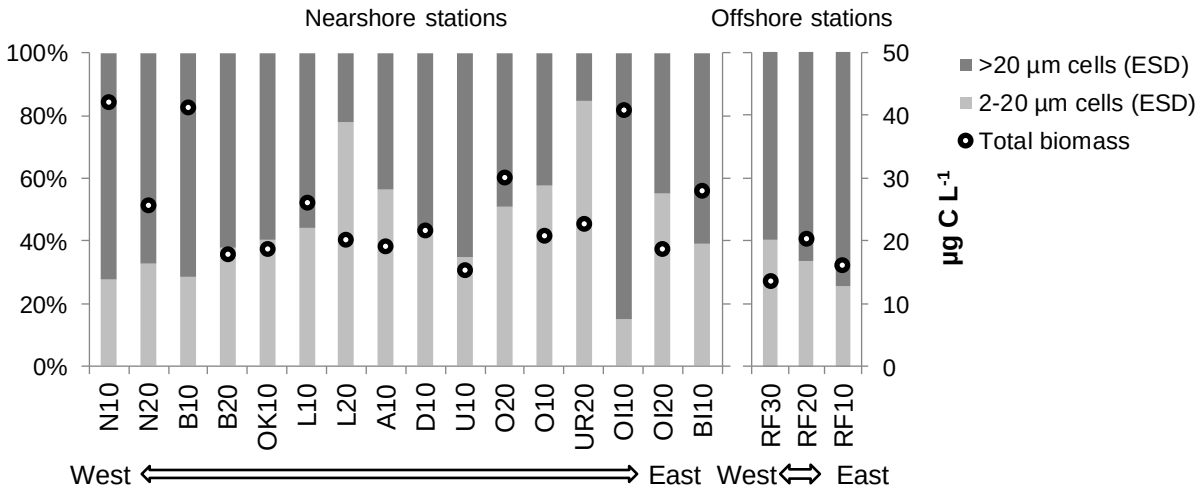

Figure 1.3. Average phytoplankton biomass during the study period (11 years for all sites, except stations RF20 and RF30, with a 5-year period). Right axis: total biomass (geometric mean). Left axis: percentage contribution of the two size-fractions considered (arithmetic mean). ESD: Equivalent Spherical Diameter.

The taxa responsible for the blooms are presented in Table 1.2. All stations presented at least one taxon that exceeded its corresponding bloom threshold. In total, 32 bloom-forming taxa were identified, belonging mostly to the group of diatoms (21 of them), but also to the dinoflagellates, haptophytes, cryptophytes and chlorophytes. 20 out of the 32 taxa belonged to the small cellular size fraction (2-20 µm).

Regarding mean bloom frequencies for the whole period and all stations, the most frequent taxon was the diatom *Pseudo-nitzschia* spp. (2.5 %) followed by another diatom, *Chaetoceros salsugineus* Takano (1.4 %). *Pseudo-nitzschia* spp. was also the most widely distributed taxon, blooming in 14 out of 19 sampling stations (Table 1.2).

In order to better characterize the blooms, the maxima in cell abundance have been identified within each of the major taxonomic groups (Annex 1.6). Some diatoms reached values around 10^7 cells L^{-1} (*Thalassiosira* Cleve, *Chaetoceros salsugineus* and *Asterionellopsis glacialis* (Castracane) Round complex). These were followed by some chlorophytes (*Tetraselmis* F.Stein), cryptophytes, unidentified small flagellates and haptophytes, with maxima in the order of 10^6 cells L^{-1}. The highest abundances within the dinoflagellates were in the order of 10^5 cells L^{-1} (*Heterocapsa* cf. *rotundata* (Lohmann) Gert Hansen*, Prorocentrum micans* Ehrenberg*, Scrippsiella* Balech-group and *Gyrodinium* cf. *flagellare* J.Schiller). On the other hand, the maxima within the raphidophyceans, autotrophic ciliates and euglenophytes were in the order of 10^3-10^4 cells L^{-1}.

The maximum abundances given by *Thalassiosira* spp., *Tetraselmis* spp. and *Heterocapsa* cf. *rotundata* corresponded to communities with a high dominance (contributions of 95.8 %, 97.1 % and 66.1 % to the total abundance of the sample, respectively) and were associated to winter or spring campaigns (Annex 1.6).

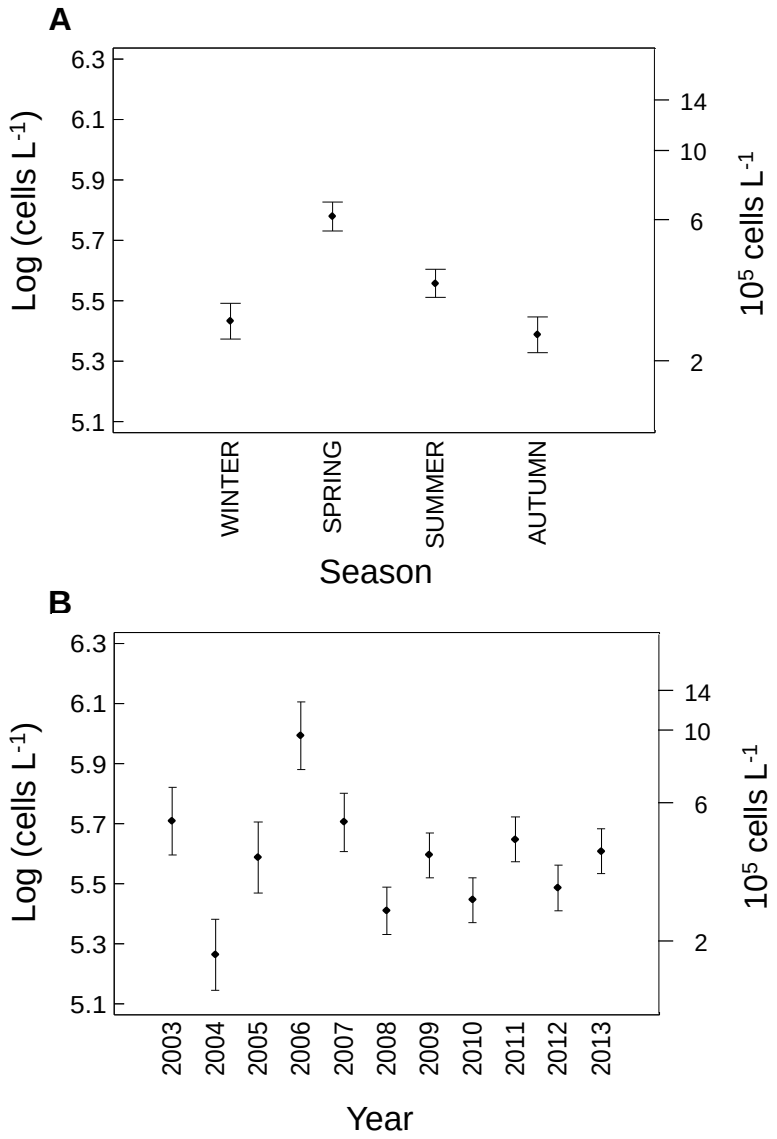

Figure 1.4. Arithmetic mean and standard errors of the log-transformed total cell abundances at different seasons (A) and years (B), considering the 19 sampling stations. The right axis shows the untransformed abundance values.

Table 1.2. Frequency (%) of bloom forming taxa per station (Chlo: chlorophyte, Cryp: cryptophyte, Diat: diatom, Dino: dinoflagellate, Hapt: haptophyte).

SIZE (µm)	GROUP	TAXON	N10	N20	B10	B20	OK10	L10	L20	A10	D10	U10	O20	O10	UR20	OI10	OI20	BI10	RF30	RF20	RF10	Mean
2-20	Chlo	*Tetraselmis* spp.	0	2.9	0	2.9	0	0	2.9	0	0	0	3.3	0	0	0	0	0	0	0	0	0.6
2-20	Cryp	*Hemiselmis* spp.	0	0	2.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1
2-20	Cryp	*Plagioselmis* spp.	0	0	2.8	0	0	0	0	0	2.9	2.8	0	0	0	0	0	0	0	0	0	0.4
2-20	Diat	*Asterionellopsis glacialis* complex	2.9	0	0	0	2.9	2.8	2.9	2.9	2.9	0	0	0	0	0	0	0	0	0	0	0.9
2-20	Diat	Solitary centrales ≤10 µm	0	0	0	0	0	0	2.9	0	0	0	0	0	0	3.0	0	0	0	0	0	0.3
2-20	Diat	*Chaetoceros salsugineus*	2.9	2.9	2.8	0	0	0	0	0	0	0	0	0	0	9.1	2.9	5.6	0	0	0	1.4
2-20	Diat	*Chaetoceros socialis*	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2
2-20	Diat	*Chaetoceros* spp. (solitary cells)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.9	0	0	0	0	0.2
2-20	Diat	*Chaetoceros* spp.	2.9	2.9	2.8	0	0	0	0	2.9	0	0	3.3	0	0	0	2.9	0	0	0	0	0.9
2-20	Diat	*Leptocylindrus danicus/hargravesii*	0	0	0	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0.2
2-20	Diat	*Leptocylindrus minimus*	0	0	0	0	0	0	0	0	0	0	0	0	2.8	0	0	0	0	0	0	0.1
2-20	Diat	Pennales ≤10 µm	0	0	0	0	0	0	0	0	0	0	0	0	0	3.0	0	0	0	0	0	0.2
2-20	Diat	*Pseudo-nitzschia* spp.	2.9	0	2.8	2.9	2.9	2.8	2.9	5.9	2.9	2.8	3.3	2.9	0	3.0	0	0	5.0	5.0	0	2.5
2-20	Diat	*Skeletonema* spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	3.0	0	0	0	0	0	0.2
2-20	Diat	*Thalassiosira* spp.	0	0	2.8	0	0	0	0	0	2.9	2.8	0	0	2.8	0	2.9	0	5.0	5.0	0	1.3
2-20	Hapt	*Emiliania huxleyi*	0	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2
2-20	Hapt	*Phaeocystis globosa*	0	0	0	0	0	2.8	0	2.9	0	0	0	0	2.8	0	0	0	0	0	0	0.4
2-20	Hapt	Prymnesiales	0	0	0	0	2.9	0	0	0	0	0	0	0	0	3.0	0	0	0	0	0	0.3
2-20	Other	Autotrophic coccoids	0	0	0	0	0	0	0	0	0	0	0	0	0	3.0	0	0	0	0	0	0.2
2-20	Other	Unidentified small flagellates	2.9	0	0	0	0	2.9	0	0	2.9	2.9	0	2.9	0	5.7	0	2.9	2.9	0	0	1.4
>20	Diat	*Cerataulina pelagica*	2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2
>20	Diat	*Dactyliosolen fragilissimus*	0	0	0	0	0	0	0	0	0	0	0	0	0	6.1	0	0	0	0	0	0.3
>20	Diat	*Guinardia delicatula*	2.9	0	0	0	2.9	0	0	0	0	0	0	0	0	3.0	2.9	0	5.0	5.0	2.9	1.3
>20	Diat	*Lauderia annulata*	0	0	0	0	0	0	0	0	0	2.8	0	0	0	0	0	0	0	0	0	0.1
>20	Diat	Pennales 10-50 µm	0	0	0	2.9	0	0	0	0	0	0	0	0	0	0	0	2.8	0	0	0	0.3
>20	Diat	*Proboscia alata*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.8	0	0	0	0.1
>20	Diat	*Rhizosolenia* spp.	2.9	2.9	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.6
>20	Diat	*Thalassiosira* cf. *mediterranea*	0	0	2.8	0	2.9	2.8	2.9	2.9	2.9	0	0	0	0	0	0	0	0	5.0	0	1.2
>20	Diat	*Thalassiosira* spp.	0	2.9	2.8	0	0	0	0	0	0	0	0	0	0	6.1	0	0	0	0	0	0.6
>20	Dino	*Gyrodinium* cf. *flagellare*	2.9	0	0	2.9	0	0	0	0	0	0	0	0	0	3.0	0	2.8	0	0	0	0.6
>20	Dino	*Prorocentrum micans*	0	0	0	0	0	2.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1
>20	Dino	*Scrippsiella*-group*	0	2.9	2.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.3
		number of blooming taxa	10	7	10	4	5	6	6	5	6	5	3	2	3	12	5	5	4	4	1	

*Dinoflagellates of the *Scrippsiella*-group gather species from the genera *Scrippsiella*, *Ensiculifera* and *Pentapharsodinium*

3.2. Potentially toxic phytoplankton

As it can be seen in Table 1.3, among the taxa that could have toxic activity, 14 out of 19 were dinoflagellates, the remaining were diatoms (*Pseudo-nitzschia* spp.), haptophytes (Prymnesiales) and raphidophyceans (*Heterosigma akashiwo* Y.Hada*)*. Most of them showed a wide distribution. Some taxa such as *Pseudo-nitzschia* spp., *Prorocentrum cordatum* (Ostenfeld) J.D.Dodge (= *P. minimum*)*,* and Prymnesiales were present at all stations.

The whole studied area presented several potentially toxic taxa, varying from 10 to 16 among the sampled stations. A spatial pattern was not evidenced (Table 1.3).

Figure 1.5 shows the frequency at which alert thresholds were exceeded by three main toxin-producing genera at each sampled station. In the case of *Pseudo-nitzschia* spp. the limit of 100,000 cells L^{-1} was exceeded at all the stations, except for station OI20. Nevertheless, at station OI20 the first threshold (50,000 cells L^{-1}) was surpassed. For this last limit, the frequency of exceedance among stations ranged from 2.9 to 22.2 %. It is also important to highlight that in spring 2010 most of the stations (17 out of 19) presented values exceeding the 50,000 cells L^{-1} threshold, and 14 of them surpassed also the limit of 100,000 cells L^{-1}. For both alert limits, the vast majority of the exceedance events occurred in spring and summer.

For *Dinophysis* spp. the limit for presence of toxins (100 cells L^{-1}) was surpassed at most of the sampled sites (16 stations) with frequencies ranging from 2.8 % to 10.0 % (Figure 1.5). The limit of 500 cells L^{-1}, which would imply the banning of the bivalve-culture harvesting, was exceeded at 9 stations with frequencies that ranged from 2.8 to 5.9 %. The 75 % of the cases that exceeded the banning limit were observed in spring. At least five different species of this genus were observed: *Dinophysis acuminata* Claparède & Lachmann*, Dinophysis. acuta* Ehrenberg*, Dinophysis caudata* W.S.Kent*, Dinophysis fortii* Pavillard and *Dinophysis tripos* Gourret*.*

Alexandrium spp., whose mere presence implies the alert, was registered at 12 stations with a maximum frequency of 8.3 %. All of the cases occurred in spring and summer (Figure 1.5). Although in some cases it reached values near 1 to 2 x 10^3 cells L^{-1}, its abundance was usually close to the limit of detection.

Finally, although *Karenia brevis* was not recorded, *Karenia* spp., which might be toxic, exceeded four times the limit of 5,000 cells L^{-1} set for *K. brevis* (data not shown)*.*

Table 1.3. Presence of potentially toxic taxa. Taxa identified at genus level could contain both toxic and non-toxic species. Diat: diatom, Dino: dinoflagellate, Hapt: haptophyte, Raph: raphidophyceae, ASP: Amnesic Shellfish Poisoning, PSP: Paralytic Shellfish Poisoning, DSP: Diarrheic Shellfish Poisoning.

Group	Syndrome/ Toxin	Potentially toxic taxa	N10	N20	B10	B20	OK10	L10	L20	A10	D10	U10	O20	O10	UR20	OI10	OI20	BI10	RF30	RF20	RF10	Stations
Diat	ASP	*Pseudo-nitzschia* spp.	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	19
Diat	ASP	*Pseudo-nitzschia galaxiae*	x	x	x		x		x	x		x	x	x	x	x	x			x	x	14
Diat	ASP	*Pseudo-nitzschia multistriata*	x	x	x						x	x	x	x			x			x	x	10
Dino	DSP	*Dinophysis acuminata*	x	x	x	x	x	x	x		x	x		x	x	x	x	x	x	x	x	17
Dino	DSP	*Dinophysis acuta*	x		x		x										x	x				5
Dino	DSP	*Dinophysis caudata*		x	x	x	x	x	x	x				x	x						x	10
Dino	DSP	*Dinophysis fortii*	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x		x	17
Dino	DSP	*Dinophysis* spp.	x		x	x	x			x	x	x		x	x	x		x	x	x	x	14
Dino	DSP	*Dinophysis tripos*	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x		x	x	17
Dino	DSP	*Phalacroma rotundatum*		x	x				x	x	x			x		x		x			x	9
Dino	Not confirmed	*Prorocentrum cordatum*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	19
Dino	PSP	*Alexandrium* spp.	x		x	x			x	x	x		x		x	x	x	x	x			12
Dino	Brevetoxin	*Karenia papilionacea*			x			x			x	x	x		x	x			x			8
Dino	Brevetoxin	*Karenia* spp.		x	x	x	x	x	x	x	x			x		x	x		x		x	13
Raph	Ichtyotoxic	*Heterosigma akashiwo*	x	x			x	x	x			x	x		x	x		x	x	x	x	13
Hapt	Ichtyotoxic	Prymnesiales*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	19
Dino	Palytoxin	*Ostreopsis* cf. *siamensis*	x								x	x			x							4
Dino	Yessotoxin	*Gonyaulax* spp.**	x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	17
Dino	Yessotoxin	*Lingulodinium polyedra*												x	x							2
		Total toxic taxa	14	12	16	10	12	10	13	12	14	13	11	14	15	14	12	12	11	10	14	

*Prymnesiales is a diverse group (e.g. *Chrysochromulina* spp., *Phaeocystis* spp.) which could include some toxic species, such as *Chrysochromulina leadbeateri* or *Prymnesium polylepis*.

***Gonyaulax* spp. could include the toxic species *Gonyaulax spinifera*.

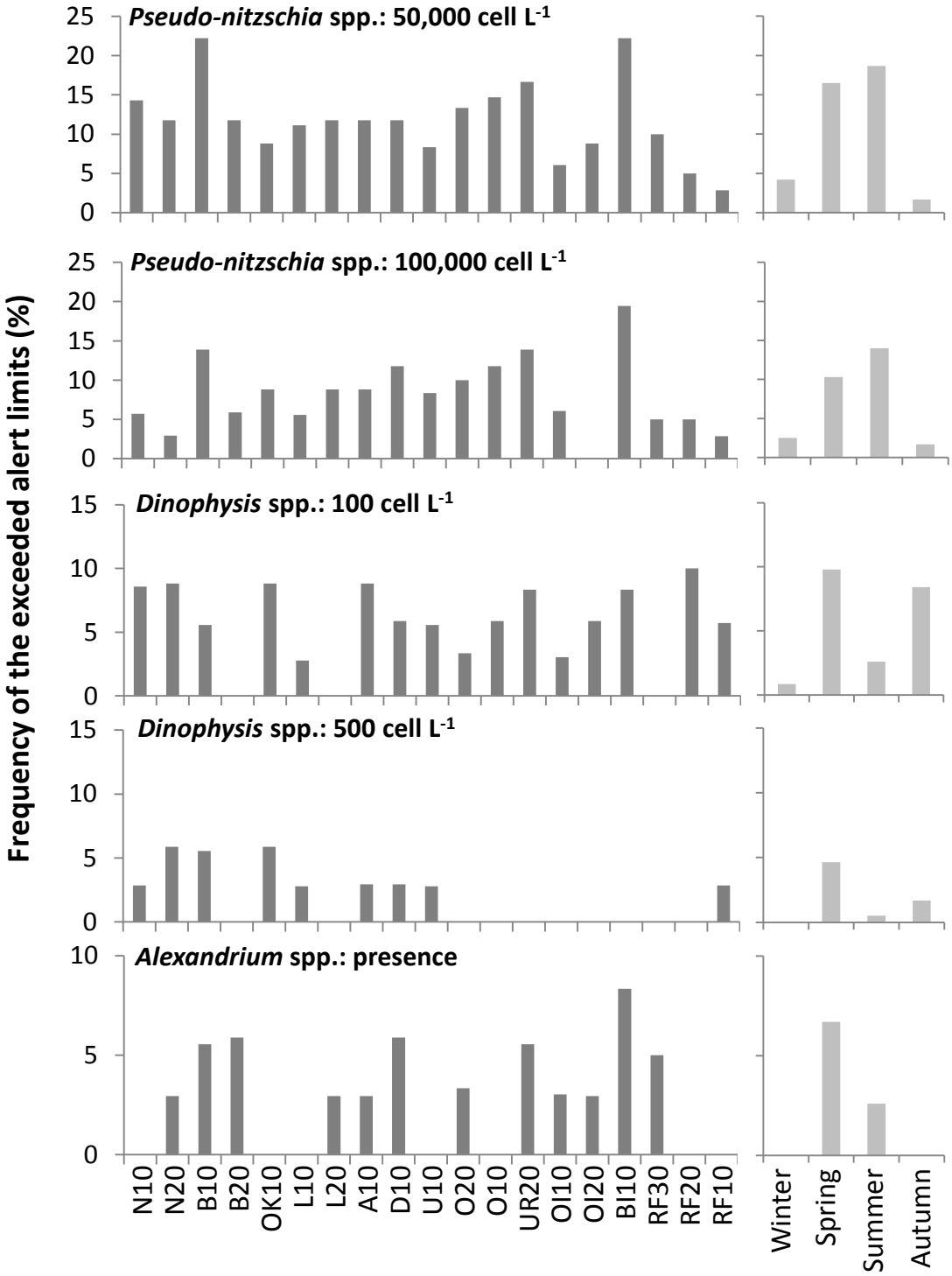

Figure 1.5. Frequencies (%) for each station at which the different alert limits were exceeded by three taxa considered potential phycotoxin producers. On the left side, from the top to the bottom: *Pseudo-nitzschia* spp. (two thresholds were applied as a precautionary measure, see main text for details), *Dinophysis* spp. (the lowest threshold indicates the limit for presence of toxins and the highest one would imply the banning of mussel harvesting for human consumption) and *Alexandrium* spp. (whose mere presence could imply a toxicity risk). On the right side, the frequency of occurrences in each season is shown.

4. Discussion

Some characteristics of the phytoplankton community of the Basque coastal waters suggest favourable conditions for bivalve growth. Regarding community cell-size, there is still considerable controversy about the most appropriate particle-size in relation with the retention efficiency (RE) in mussels. While some studies set the 100 % RE for particles up to 35-45 µm (Strohmeier *et al*. 2012; Cranford *et al.* 2014), some other investigations establish the size of 15-20 µm as the maximum particle-size for an efficient retention (Lucas *et al*. 1987; Stenton-Dozey & Brown 1992). In any case, the majority of the studies agree that the minimum particle size for an efficient retention is 4 µm (Møhlenberg & Riisgård 1978; Riisgard 1988; Jørgensen 1990) and the particle size range of 4 to 45 µm seems to be appropriate for a high food depletion (Cranford *et al*. 2014). In the present study, cells ranging 2-20 µm (ESD) were much more abundant than larger ones. Nevertheless, within that dominant cell-size range there was an important contribution of chain-forming diatoms at some stations (up to a maximum of 78.8 %). This means that although the cell-size for those organisms is in the range 2-20 µm, the chains are larger, contributing to that 4 to 45 µm appropriate range for bivalve nutrition.

The low efficiency of the Utermöhl technique to detect cells below approximately 2 µm (Padisák *et al*. 1993) did not represent a direct problem for the interpretation of our results since, as said before, many bivalve filter feeders have a reduced retention efficiency for particles below 3-5 µm (Jørgensen 1990; Safi & Gibbs 2003; Cranford *et al*. 2014). Nevertheless, although we lack data on the smallest cells, such as picophytoplankton and heterotrophic bacteria, such information would be of interest as these components of the microbial community are consumed by larger predators, such as nanoflagellates and ciliates (Lenz 1992) and, consequently, could indirectly affect bivalve growth (Kamiyama 2015).

Considering the mean values of total abundance at each station, the whole studied area would present similar conditions for bivalve growth in terms of phytoplankton density. However, the sampling year and season had a significant influence on total cell abundance. Year to year variation in phytoplankton communities might occur as a result of variation in climatic factors (Lehman 2000) or can be also related to differences between taxonomists counting the samples (Dromph *et al*. 2013).

The differences related to the sampling season show some similarities with the seasonal pattern previously described for the nano- and microphytoplankton in the southern Bay of Biscay: highest blooms are usually found from late winter to spring, when the transition from mixing to thermal stratification occurs in the water column (Estrada 1982; Fernández & Bode 1994; Varela 1996). In the present study, high peaks of cell density were observed during winter and spring campaigns, and the highest mean abundance corresponded to samples collected in spring. However, the summer mean value was not too low, which would indicate that phytoplankton growth is not limited along the entire Basque coast during the warmest months. Previous studies in this area have shown that the nutrient depletion associated to the stabilization of the thermocline is neither a strong, nor a permanent feature in the surface waters of the Basque coast. Although nutrient concentrations do decrease in these waters during summer (Valencia *et al*. 2004), rainfall pulses activate sporadically the exportation of nutrients from rivers and estuaries (Revilla *et al.* 2009; Garmendia *et al*. 2011).

The detection of bloom events at all the sampled stations would imply a favourable condition for bivalve culture. This interaction between mussel population and phytoplankton as food source has long been documented: phytoplankton blooms have been related to increased growth and production and improved condition index of several mussel species (Blanton *et al*. 1987; van der Veer 1989; Hickman *et al.* 1991; Benemann 1992). Indeed, diatoms revealed as the dominant group in the Basque surface waters, considering the number of bloom-forming species, as well as their spatial distribution and peaks of cell abundance. This would also be favourable for the development of bivalve farming in this area, since many studies have found a significant positive correlation between diatoms and bivalve growth (Beukema & Cadée 1991; Weiss *et al*. 2007; Pernet *et al*. 2012; Wall *et al*. 2013).

Regarding the potentially toxic phytoplankton, special attention was paid to the genera causative of the three main human syndromes. For *Pseudo-nitzschia* three different taxa were registered. On the one hand, *Pseudo-nitzschia galaxiae* N.Lundholm & Moestrup and *Pseudo-nitzschia multistriata* H.Takano were identified, which have been evidenced to produce domoic acid (Moschandreou & Nikolaidis 2010; Ajani *et al*. 2013). And on the other hand, a third group was identified at genus level. Light-microscopy does not allow identifying some harmful taxa (e.g., *Pseudo-nitzschia delicatissima* (Cleve) Heiden and *Pseudo-nitzschia pungens* (Grunow) Hasle) at species level so, as a measure of precaution, *Pseudo-nitzschia* spp. was taken as a harmful taxon. The alert level of 100,000 cells L^{-1} was exceeded at all stations excepting one, implying a risk for bivalve culture. Nevertheless, it occurred at low frequency (in average, less than 15%) during the studied 11 years.

Amongst the species found for *Dinophysis* spp., special attention should be given to *D. acuminata, D. acuta* and *D. caudata*. These taxa are described as toxin producers (Faust & Gulledge 2002; Fernández *et al*. 2006) and have been found to be the most frequent and abundant *Dinophysis* species in other areas of the Bay of Biscay, such as the West French coast (Batifoulier *et al*. 2013) and the Northwest Iberian peninsula (Moita *et al*. 2016). In the neighbouring Arcachon Bay, *Dinophysis* spp. have caused several events of okadaic acid intoxication in bivalves (Maurer *et al*. 2010) and cells have been demonstrated to originate from the open shelf (Batifoulier *et al*. 2013). This suggests that registered *Dinophysis* spp. in the Basque waters may have a similar origin as those found in Arcachon Bay.

At last, *Alexandrium* spp., whose mere presence implies a risk, was detected in several occasions. This genus is known to be very widespread globally and can develop in very different habitats (Lilly *et al*. 2007). Despite its high adaptability, we did not find high frequencies or elevated abundances of this genus in the Basque open coastal waters. However, sporadic accumulation of PSP toxins in shellfish cannot be discarded as, although relatively scarce, these dinoflagellates were present along the Basque coast, both in nearshore and offshore waters. Particular attention must be paid to spring and summer, where the totality of the occurrences happened. A more detailed analysis of the thecal plates (cells stained with Fluorescent Brightener 28 and examined under the epifluorescence microscope Leica DMRB following Fritz and Triemer (1985)) performed on some cells from coastal samples from 2014 revealed the presence of the *Alexandrium tamarense* (Lebour) Balech species complex (unpublished data). The presence of *Alexandrium minutum* Halim has also been documented in one of the estuaries of the Basque Country (Orive *et al*. 2008). The *A. tamarense* complex contains both toxic (e. g. *Alexandrium*

catenella (Whedon & Kofoid) Balech) and non-toxic species (e. g. *A. tamarense*). Thus, a thorough characterization of the *Alexandrium* populations from the Basque coastal waters stands as a necessary task, due to its potential impact on the development of local aquaculture.

Other taxa have also been considered here as included in the IOC list of toxic microalgae for precaution, although for some of them their actual threat potential in the study area seems low. The raphidophycean *Heterosigma akashiwo* has been reported to bloom in confined areas of an estuary on the Basque coast (Laza-Martinez *et al.* 2007), a typical habitat for this species, in contrast to the highly hydrodynamic open coast (Smayda 1998), where its potential to bloom seems low. The group Prymnesiales, which includes diverse taxa such as *Phaeocystis* Lagerheim, *Chrysochromulina* Lackey, *Imantonia rotunda* N.Reynolds or *Prymnesium* Massart, is a common and numerically abundant component of the phytoplankton community. But, only some of the species in this group are known to have toxic capacity (Ulitzer & Shilo 1966; Johnsen *et al*. 1999; Bertin *et al*. 2012) and they have not been identified in the 2003-2013 data set. The benthic dinoflagellate *Ostreopsis* cf. *siamensis* Johs.Schmidt has been recorded in the plankton samples. *Ostreopsis* is very frequent and well-documented in the Mediterranean Sea (Vila *et al*. 2001; Turki 2005; Aligizaki & Nikolaidis 2006) and the species *O.* cf. *siamensis* has been registered before within Basque coastal waters (Laza-Martinez *et al*. 2011). Nevertheless, it has never been observed in bloom proportions so far and, as it is associated to the benthos, it is not expected to pose a risk in open water farming.

However, other dinoflagellates are of more concern. This is the case of *Gonyaulax* Diesing and *Lingulodinium polyedra*, as recent analyses conducted in mussels collected at the pilot-scale farm revealed the presence of yessotoxins. In addition, pelagic species from the genera *Prorocentrum* and *Karenia* also should be carefully monitored, taking into account modelling studies from Glibert *et al*. (2014) that projected a more suitable habitat for these HAB taxa in the NW European Shelf-Baltic Sea region, as a consequence of climate change. *Prorocentrum cordatum* has been found widely distributed along the Basque coast. Although a specific toxin has not been characterized yet, certain clones of *P. cordatum* have demonstrated lethal and sub-lethal effects on shellfish (Saba *et al*. 2011). Recently, Vlamis *et al*. (2015) have linked the presence of *P. cordatum* with tetrodotoxin found in shellfish from Greek production areas, but there is still much controversy around the origin of this neurotoxic compound. Finally, *Karenia papilionacea* A.J.Haywood & K.A.Steidinger was identified in the present study; this is a widely distributed neritic species that had already been detected on the seaward end of an estuary from the study area (Orive *et al*. 2008) and on the French Atlantic coast (Nézan *et al*. 2014). Fowler *et al*. (2015) indicate that strains of *K. papilionacea* from the western Atlantic coast (Delaware) and from New Zealand produce brevetoxins (neurotoxic compounds), and may pose a threat to humans through consumption of contaminated shellfish.

Hence, there is a need to improve our knowledge at the local scale on the distribution of HAB species and phycotoxins, in order to prevent human health problems, damages to farmed bivalves and economic losses. Besides the limitation of the analysed time series due to the low sampling frequency, which implies that short-term dynamics cannot be addressed, this study contributes importantly to the knowledge of phytoplankton composition in the eastern Cantabrian Sea. It provides with novel information regarding nutritional quality and potential toxicity of phytoplankton in this area.

5. Conclusions

Focusing on the potential for aquaculture production, in open marine waters of the Basque Country the occurrence of bloom events, together with the dominance of diatoms, suggests favourable conditions for bivalve growth, especially in spring. However, it is precisely in this season when the toxicity risk increases. Potentially toxic species could represent a key factor in limiting aquaculture development or even questioning its sustainability in specific areas. In the present study, although *Pseudo-nitzschia* spp., *Dinophysis* spp. and *Alexandrium* spp. exceeded the alert limits in several occasions, the frequencies were low considering an 11-year time series. Potentially toxic taxa should be carefully monitored paying special attention to spring and summer, when most of the cases that could imply the closure of the production area occurred. It would be advisable to carry out a short-periodicity sampling at least during 2-3 years at the pilot-scale bivalve farm, to better determine the potential risks of HABs to occur.

Chapter 2

Inhomogeneity detection in phytoplankton time series using multivariate analyses

Submitted as: **Muñiz O.,** Rodríguez J.G., Revilla M., Laza-Martínez A., Seoane S. and Franco J. Inhomogeneity detection in phytoplankton time series using multivariate analyses. *Oceanology.*

Abstract

Phytoplankton communities have long been used as water quality indicators within environmental policies and are also an essential element of mollusc culture area management. This has fostered the development of national and international phytoplankton monitoring programs, but these networks are subject to sources of uncertainty due to laboratory issues. Nevertheless, studies regarding the interference associated with these aspects are scarce. Hence, a long time series (2003–2015) from the Basque shelf (southeastern Bay of Biscay) was analysed to evaluate the uncertainty given by laboratory strategies when studying phytoplankton variability. Inter-annual variability in phytoplankton communities was explained by changes in fixatives (glutaraldehyde and acidic Lugol's solutions) and laboratory staff. Based on Bray-Curtis distances, phytoplankton assemblages were found to be significantly dissimilar according to the effect of changes in the specialist handling the sample and the employed fixative. The pair-wise permutational multivariate analysis of variance (PERMANOVA) showed significant differences between the two fixatives utilized and also between the three taxonomists involved. Thus, laboratory-related effects should be considered in the study of phytoplankton time series.

1. Introduction

Phytoplankton, as the base of marine food webs, are of essential importance to maintain and understand marine ecosystem functioning (e.g., Arrigo 2005). This biological element has long been studied as a key environmental quality indicator within several international policies including European directives, such as the Water Framework Directive (WFD, 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, 2008/56/EC) (Borja *et al*. 2008; Garmendia *et al*. 2013). These policies require large monitoring networks in order to assess water quality and involve indicators that reflect different phytoplankton attributes, such as composition (Domingues *et al.* 2008; Devlin *et al*. 2009). Additionally, bivalve mollusc culture areas worldwide require phytoplankton monitoring programs in order to manage potential toxicity (e.g., Bricelj and Shumway 1998).

Phytoplankton assemblages depend on species succession, which is influenced in turn by environmental changes (e.g., Huisman *et al*. 1999). However, there are also several sources of variation associated with the monitoring techniques of phytoplankton communities (Dromph *et al*. 2013). The microscope-based method following the Utermöhl technique is standard for phytoplankton identification and counting within the European Union (EN 15204 2006). This method requires highly specialized taxonomists, yet most studies show a bias due to variation in the level of expertise exercised by each taxonomist counting phytoplankton (Culverhouse *et al.* 2003; Wiltshire & Dürselen 2004; Peperzak 2010; Dromph *et al*. 2013; Straile *et al*. 2013; Jakobsen *et al*. 2015). An exception was found for diatom indices for which some studies have concluded that, as long as a harmonized methodology is followed, the error associated with taxonomist variation has little effect (Kahlert *et al*. 2009; 2012). The preservation of plankton samples can also introduce artefacts on species abundance, as well as cell volume estimates. Traditional fixatives, such as Lugol's iodine and glutaraldehyde, have been reported to produce shrinkage, swelling, or even breakage of phytoplankton cells, which can bias estimates of abundance and biomass (Booth 1987; Verity *et al*. 1992; Menden-Deuer *et al*. 2001; Yang *et al*. 2016).

In order to develop more accurate monitoring programs and be able to interpret their results, it is essential to estimate the variability given by each source of uncertainty. To the best of our knowledge, such studies are scarce. Some of the existing literature focused on specific issues, such as the need of a harmonized methodology (Kahlert *et al.* 2009; 2012; 2016), or specifically on a concrete taxonomic group (Heino & Soininen 2007), or on the influence of taxonomic resolution (Carneiro *et al*. 2010; 2013).

In this context, the aim of the present study is to investigate the detection of inhomogeneities in phytoplankton time series and assess how these differences can be caused by factors other than the environment. This work does not attempt to be a methodology or inter-laboratory comparison, but it shows the importance of a previous data analysis when studying long-term trends or patterns in phytoplankton composition and abundance; phytoplankton time-series can contain relevant ecological information (e.g., to address the effect of climate change), but can also be subject to methodological interferences. Hence, a complete overview of the potential interference in phytoplankton inter-annual variability given by different sources of uncertainty (e.g., taxonomist experience, fixative type) is addressed. We use a long time series (>10 years),

which involves both coastal and offshore areas and takes into account the whole nano- and micro-phytoplankton community.

2. Materials and methods

2.1. Study area, sampling and laboratory strategies

This study draws on data from the "Littoral Water Quality Monitoring and Control Network" of the Basque Water Agency, which has been used for the implementation of the Water Framework Directive in the Northeast Atlantic ecoregion (Borja *et al*. 2004; Revilla *et al*. 2009; Borja *et al*. 2016). The dataset consists of 16 stations along the Basque coast and three offshore stations in the southeastern Bay of Biscay (Figure 2.1). The climate in the study area is temperate and oceanic with moderate winters and warm summers. Coastal water bodies are euhaline and exposed. A detailed description of hydrographical conditions is given in Valencia *et al*. (2004).

The analysed time series was collected over 13 years (from 2003 to 2015), except for two offshore stations with seven-year datasets (RF20 and RF30, from 2009 to 2015). Although phytoplankton samples have been obtained quarterly since 2007, only the spring and summer data were analysed (i.e., two surveys per year) as these were the seasons sampled during the complete time series.

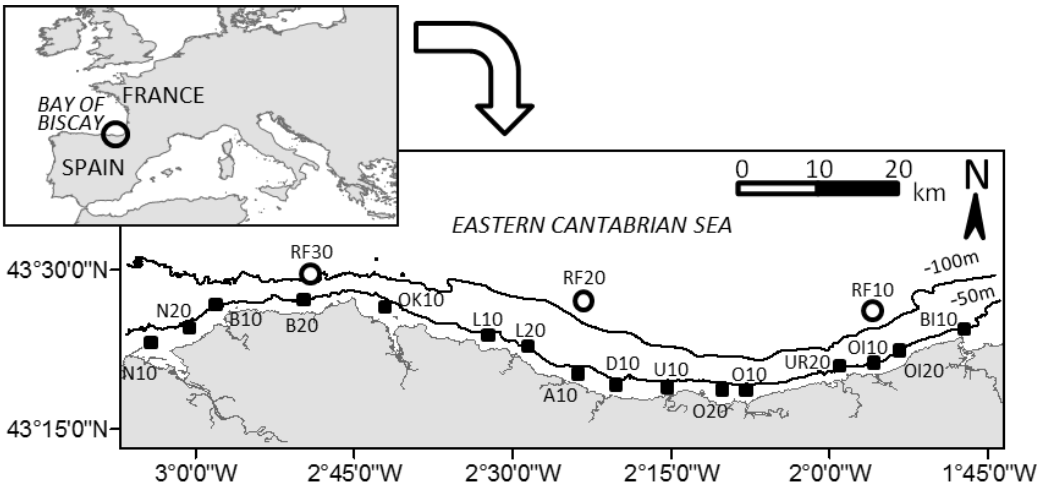

Figure. 2.1. Map of the study area and sampling stations. Squares correspond to nearshore sampling sites and circles to offshore sampling sites.

The following environmental variables were used in the analysis: temperature; salinity; Secchi depth; suspended solids; ammonium; nitrate; phosphate; and silicate. In the field, the temperature and salinity were recorded in surface waters using a conductivity, temperature and depth device (CTD) (Seabird25), the Secchi disc depth was measured as an estimator of the water transparency, and surface water samples were taken for subsequent laboratory analyses. The concentration of suspended solids was estimated following the procedure described in *Clesceri et al*. (1989) after the filtration of water through Whatman GF/C filters. Inorganic nutrients (ammonium, nitrate, silicate, phosphate) were measured using a continuous-flow autoanalyzer (Bran + Luebbe Autoanalyzer 3, Norderstedt, Germany) according to colorimetric methods described in Grasshoff *et al*. (1983). When nutrient concentrations were below the quantification limit (1.6 µmol L^{-1} for ammonium, nitrate or silicate; 0.16 µmol L^{-1} for phosphate), the value used for statistical analyses was equal to one half of that limit.

For phytoplankton, water was preserved immediately and maintained in 125 mL borosilicate bottles under dark and cool conditions (4°C) until analysis. Glutaraldehyde (0.1% v/v) was used for preservation until 2011 and acidic Lugol´s solution (0.4% v/v) from then on. Taxonomic identification and cell counting were performed on subsamples of 50 mL (occasionally, particle density was too high and 10 mL samples were used instead), following the Utermöhl method (Utermöhl 1958; Hasle 1978; Edler & Elbrächter 2010) under a Nikon diaphot TMD inverted microscope. Depending on the organism size, 100x or 400x magnification was used; the detection limit of microscope counts for microplanktonic organisms was 20 cells L^{-1}. Small nanophytoplankton cells that could not be assigned to any taxonomic group were clumped together into a group named "unidentified forms <10 µm". Three different taxonomists belonging to the same laboratory took part in the identification and counting of phytoplankton. Taxonomist #1 handled samples corresponding to years 2003, 2008, 2009, and from 2012 to 2015. Taxonomist #2 handled samples from 2005, 2006, 2007, 2010, and 2011, and Taxonomist #3 identified and counted samples from 2004. No changes in the staff took place within the year of analysis. The experience of the taxonomists increased from the beginning of the time series, reaching more specific taxonomic levels.

2.2. Data analysis

2.2.1. Environmental variables

Environmental data were transformed and standardized in order to achieve the assumptions of normality and homoscedasticity. All analyses were performed separately for spring and summer. Each individual variable was subjected to one-way analysis of variance (ANOVA) and a multiple range test (95% least significant difference, LSD) to check for significant differences among years. Additionally, based on Euclidean distance matrices, nonmetric multidimensional scaling (MDS) ordination and cluster analyses were performed to study the variability of all environmental variables together. Similarity profile analysis (SIMPROF) at alpha = 0.05 was included to test for significant differences at each cluster dendrogram node (Clarke & Gorley 2006).

The MDS analyses were carried out with the (i) 19 sampling sites and (ii) average values of each variable per season and year (i.e., average between the sampling stations), excluding stations RF20 and RF30 because they were only sampled from 2009 on. Additionally, for the analysis of the 19 sampling sites, permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences between years. A PERMANOVA with 9999 permutations was carried out with "year" as a fixed factor. A second PERMANOVA, applying the same settings, was used as a post-hoc test for pair-wise comparisons between the 13 different years. Statgraphics Centurion XVI was used for ANOVA, PRIMER 6 statistical software (Primer-E Ltd., UK) for cluster analyses and MDS, and RStudio (R Core Team 2015) for PERMANOVA.

2.2.2. Phytoplankton community

Prior to mathematical analysis, the phytoplankton species list was standardized according to AlgaeBase (Guiry & Guiry 2015). Rare taxa, defined here as those occurring in less than 1% of the samples, were excluded in the analyses to reduce noise in the data. A total of 129 of the 336 taxa were left out of the analysis.

Phytoplankton abundance data (cell L^{-1}) were log (x + 1) transformed. Separate analyses were performed for spring and summer. MDS and cluster analyses were performed equally to the environmental data but based on zero-adjusted Bray-Curtis matrices (Clarke *et al*. 2006). These matrices were used to study the inter-annual variability of community assemblages. MDS is a powerful ordination method for ecological community analysis that allows a large presence of zero values and does not assume a linear relationship between variables (McCune *et al*. 2002). Similar to the environmental data, analyses were carried out with the (i) 19 sampling sites and (ii) average cell density values per season and year. At the level of virtual sampling units, analyses were performed based on densities of (i) the lowest taxonomic level available and (ii) major taxonomic groups (i.e., autotrophic coccoids, chlorophytes, *Mesodinium* Stein, cryptophytes, diatoms, dinoflagellates, euglenophytes, haptophytes, ochrophytes, and unidentified forms). Moreover, a PERMANOVA (9999 permutations) was performed to test for significant differences associated with "fixative" as a fixed factor. The dataset was then split into two subsets based on the two fixatives. The first subset, which corresponded to glutaraldehyde and included data for the three taxonomists (i.e., period 2003–2011), was subjected to a second PERMANOVA (9999 permutations) with "taxonomist" as a fixed factor. An additional PERMANOVA was used as a post-hoc test for pair-wise comparisons between the three different taxonomists. The second subset (i.e., period 2012–2015), where the acidic Lugol´s solution was used, could not be subjected to a second PERMANOVA since it only included information for a single taxonomist.

3. Results

3.1. Environmental variables

All of the investigated environmental variables showed statistically significant differences in mean values among some years, both in spring and summer (ANOVA test, alpha = 0.05). Results for the individual environmental variables are summarized in Figure 2.2, which shows the means and standard deviations, and Annex 2.1, which includes results of the multiple range tests.

Secchi disc depth showed seven homogeneous groups (i.e., statistically significant different groups) both in spring and summer. The groups with the lowest values were obtained from data collected in spring 2003, 2007, and 2011, and summer 2003, 2005, and 2010. The highest values occurred in 2012 and 2015 in spring, and 2004, 2013, and 2015 in summer. Mean Secchi depths ranged from 5.1 to 13.7 m. Temperature minimum (mean: 14.6 °C) and maximum (mean: 18.1 °C) values in spring were represented by the years 2010 and 2011, respectively, whereas in summer, the minimum occurred in 2015 (20.0 °C) and the maximum in 2003 (23.4 °C). Each of these years formed a separate homogeneous group, statistically different from the others. Salinity mean values ranged from 34.1 to 35.7 PSU. In spring, minimum mean values were given by the homogeneous group formed by the years 2003, 2004, 2005, 2013, and 2014, whereas the maximum was represented by the group from years 2008, 2010, and 2011. In summer, maximum values occurred during 2012. Suspended solids mean concentrations ranged from 1.2 to 9.1 mg L^{-1} with a general increasing trend from the beginning towards the end of the time series, both in spring and summer.

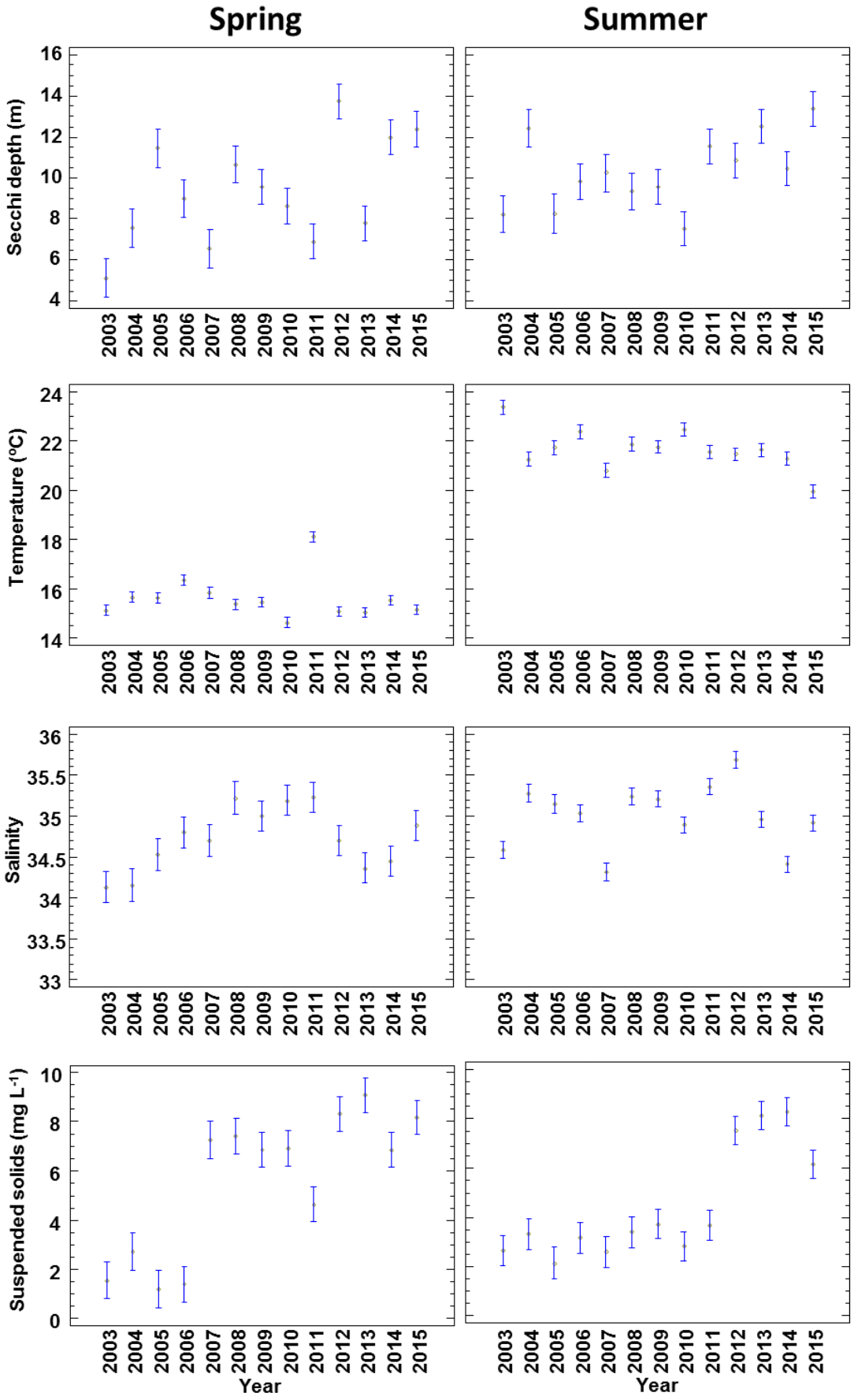

Figure 2.2. Mean plots for the environmental variables in each year during the period 2003–2015, with spring and summer shown in the left and right columns, respectively. Vertical error bars represent the standard deviation.

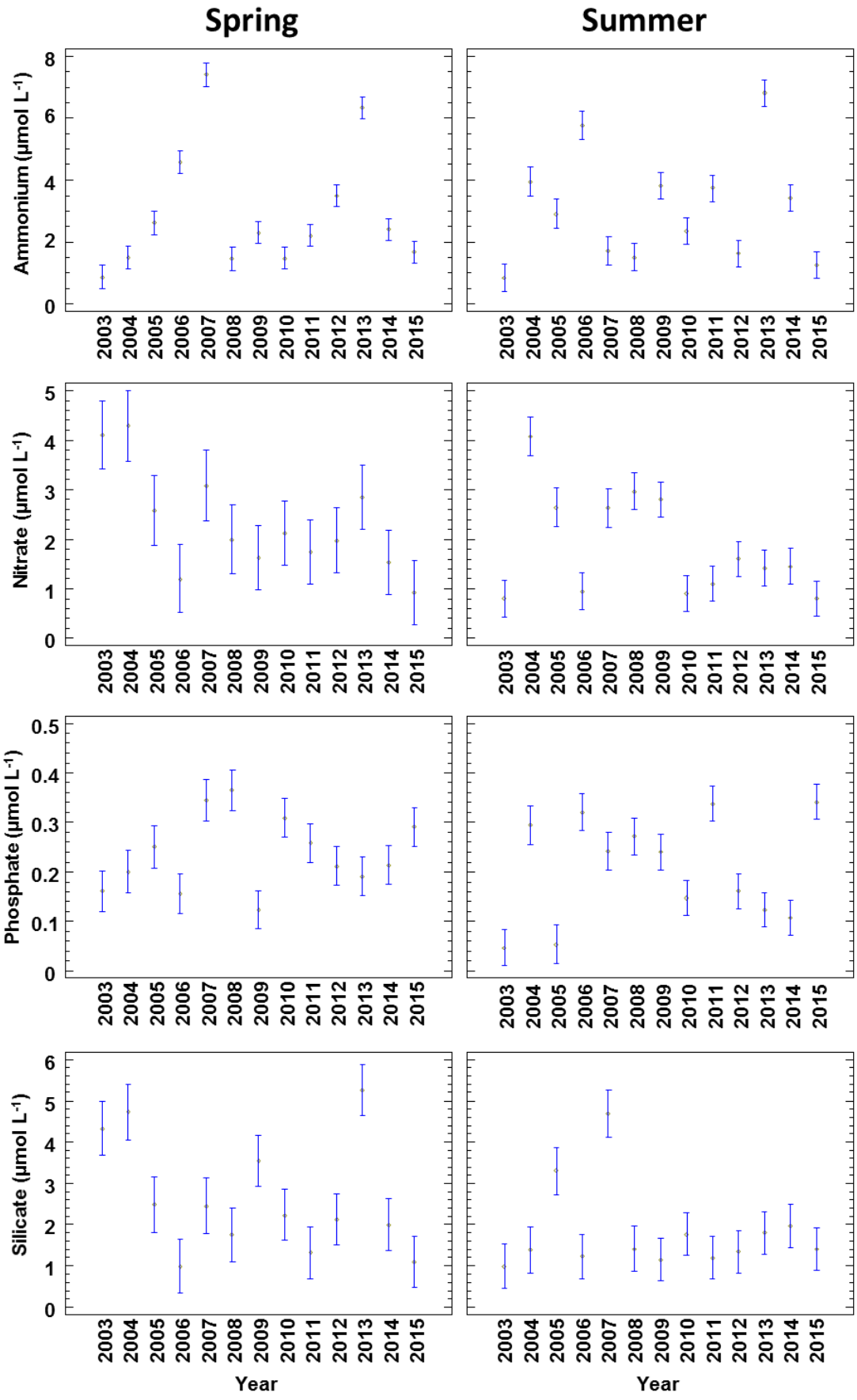

Figure 2.2 (continued). Mean plots for the environmental variables in each year during the period 2003–2015, with spring and summer shown in the left and right columns, respectively. Vertical error bars represent the standard deviation.

With regard to nutrients, mean ammonium values were significantly lower during 2003. In spring, the years 2007 and 2013 formed the group with the highest ammonium concentrations, whereas in summer, 2006 and 2013 were the years with the highest values. Mean nitrate concentrations ranged from 0.8 to 4.3 µmol L^{-1}. Compared with spring, where six significant groups of years were found, mean summer values showed lower variability, as shown by the four groups of years. Phosphate concentrations presented mean values between 0.05 and 0.37 µmol L^{-1}. Maxima were found in spring during 2007–2008. 2003 and 2005 presented especially low concentrations in summer. Silicate showed five significantly different homogeneous groups of years. In spring, mean concentrations ranged from 1.0 to 5.3 µmol L^{-1} and in summer from 1.0 to 4.7 µmol L^{-1}.

MDS biplots represent the samples as points in low-dimensional space such that the larger the distance between two points in the plot, the more dissimilar they are with regard to the environmental variables and vice versa. Hence, when analysing the variability of all environmental variables together, some years appeared substantially different from the others in the MDS (e.g., spring 2003 and summer 2003, 2005, 2013, and 2014) (Figure 2.3). The pair-wise PERMANOVA revealed significant differences between all years, both in spring and summer (Annex 2.2).

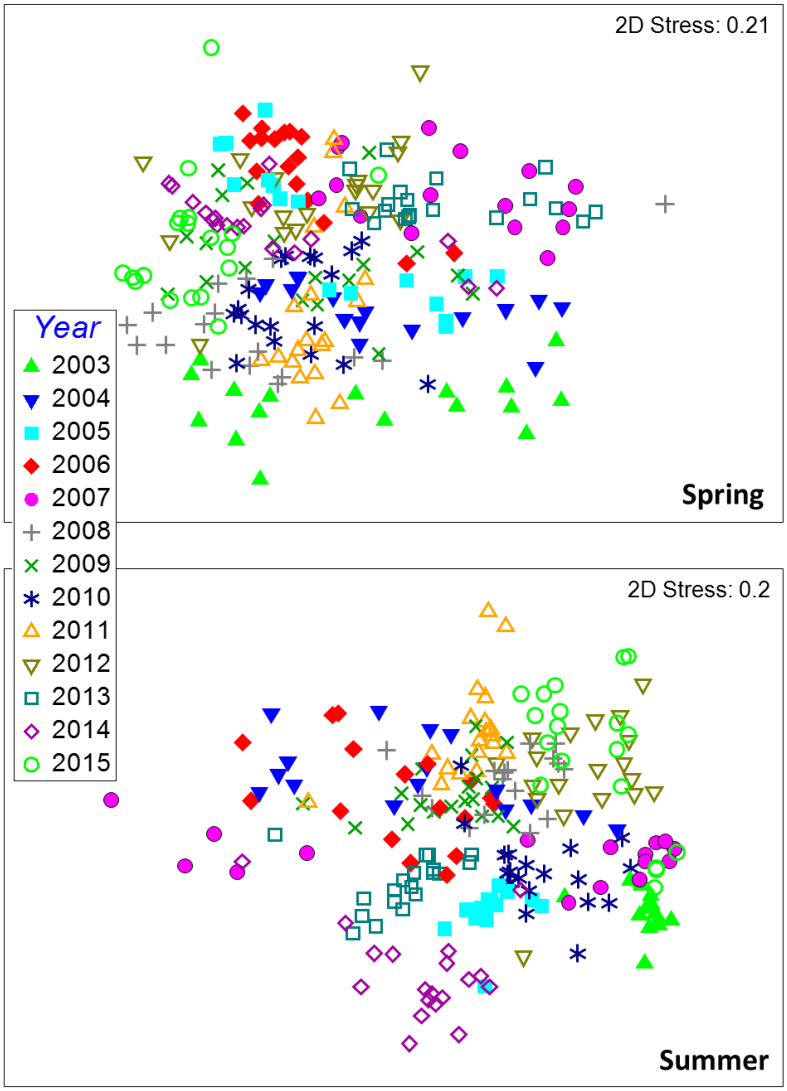

Figure 2.3. Multidimensional scaling (MDS) of the transformed environmental data in spring and summer using Euclidean distances for the period 2003–2015.

In the MDS analysis of environmental variables using average values per season and year, the chronological trajectory showed great dissimilarities between some consecutive years, such as spring 2006–2007 or summer 2003–2004, 2012–2013 and 2014–2015 (Figure 2.4). In contrast, some years appeared close to each other indicating similar mean environmental conditions. However, cluster analyses (SIMPROF test, alpha = 0.05) for average values of environmental data did not find any significant group, either in spring or summer (Annex 2.3).

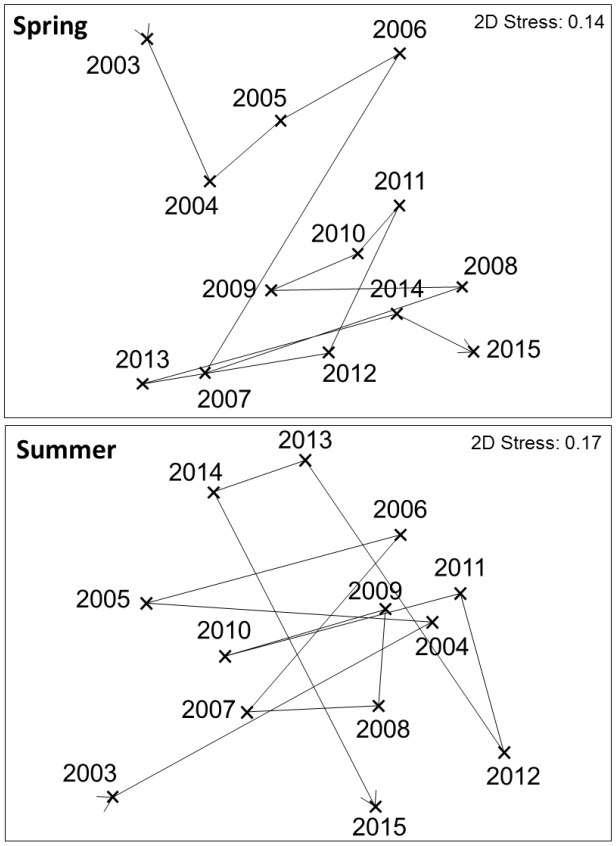

Figure 2.4. Multidimensional scaling (MDS) of the transformed environmental data (mean values of 17 sampling sites) using Euclidean distances. Cluster analyses did not find any significant group of years (SIMPROF test, alpha = 0.05).

3.2. Phytoplankton assemblages

When the complete dataset (19 sites) was analysed, the MDS showed two separate groups with regard to inter-annual variability of community composition: one referring to the year 2004 and the other referring to the remaining years (Annex 2.4). Separate MDS were conducted for spring and summer considering, firstly, the influence of the fixative (Figure 2.5). In the MDS biplots, a separation based on the type of fixative used can be observed in both seasons. Moreover, the PERMANOVA analysis indicated that phytoplankton variability was explained by the utilized fixative (p = 0.0001).

The influence of the taxonomist was then studied in the subset where one unique fixative was employed (i.e., glutaraldehyde during the period 2003–2011). The MDS biplots showed two main groups: one associated with Taxonomist #1 and Taxonomist #2 and the other associated with Taxonomist #3 (Figure 2.6). The pair-wise PERMANOVA for this subset revealed significant differences between the three different taxonomists handling the samples (Annex 2.5). Similar results were obtained for spring and summer.

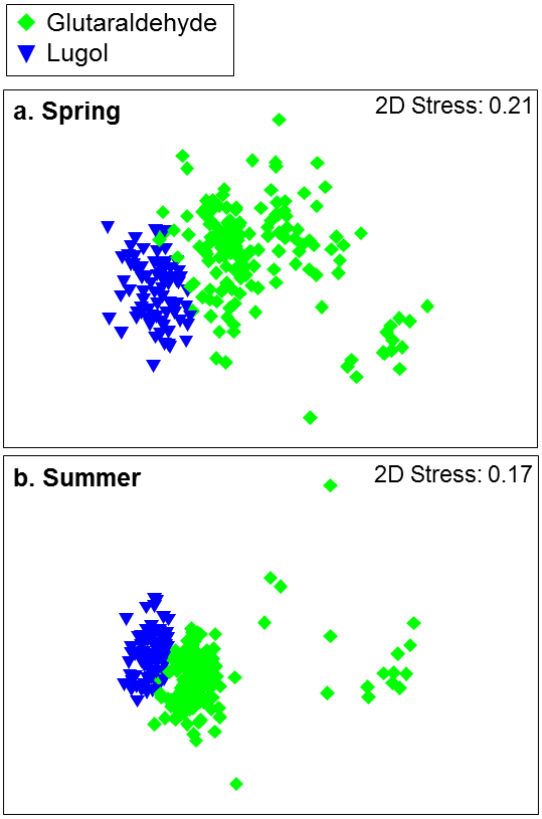

Figure 2.5. Multidimensional scaling (MDS) for phytoplankton abundance (log (*x* + 1) transformed data using zero-adjusted Bray-Curtis distances) for the period 2003–2015. Data are shown separately for spring (a) and summer (b). Different symbols represent the different fixatives employed.

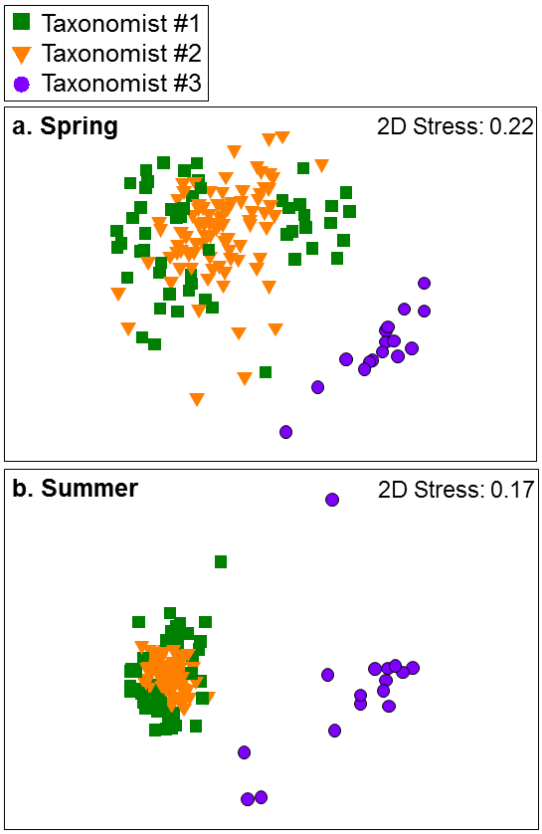

Figure 2.6. Multidimensional scaling (MDS) for phytoplankton abundance (log (*x* + 1) transformed data using zero-adjusted Bray-Curtis distances) for the period 2003–2011. Data are shown separately for spring (a) and summer (b). Different symbols represent different taxonomists handling the samples.

Inter-annual variability was also studied based on average values per season and year. Here, the MDS and cluster analyses for phytoplankton assemblages showed several significant groups according to changes both in the utilized fixative and taxonomist handling the samples (Figure 2.7).

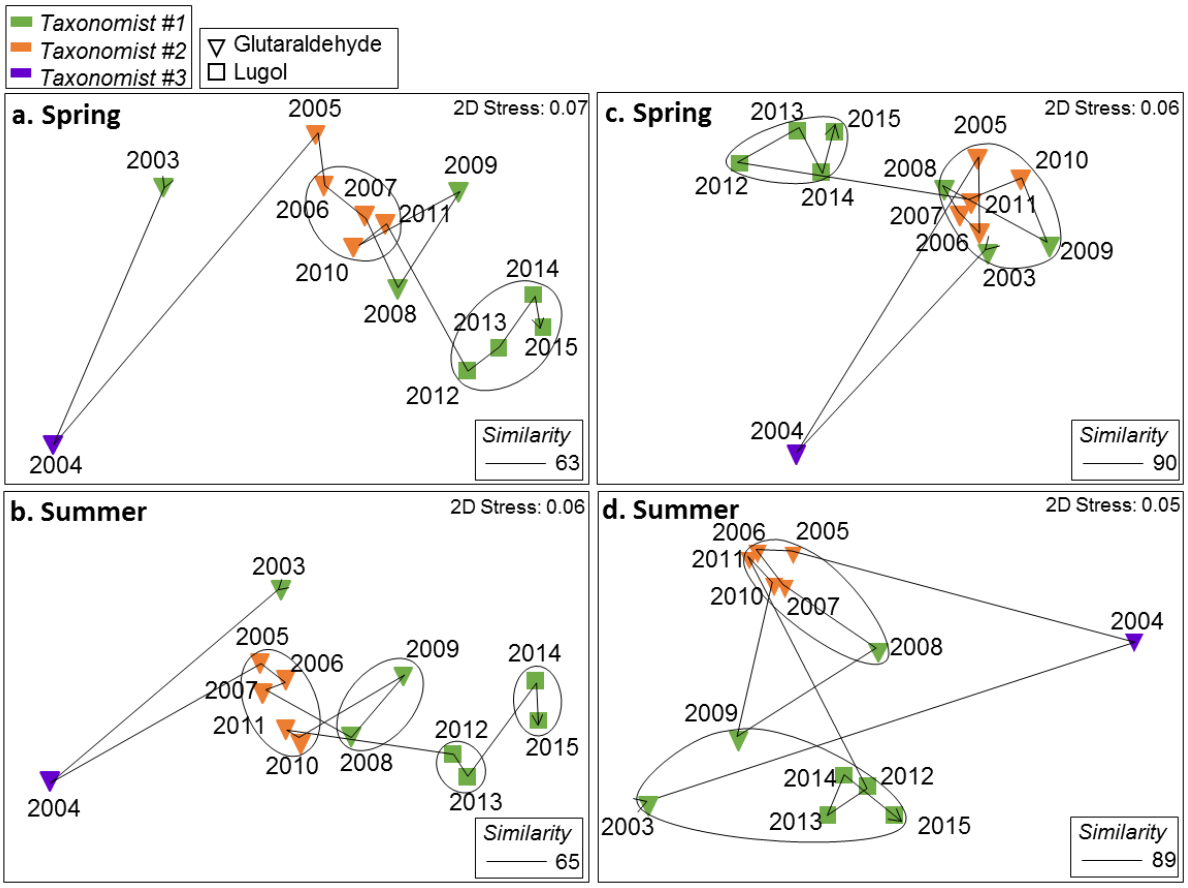

Figure 2.7. Multidimensional scaling (MDS) of the annual phytoplankton community assemblages (log (*x* + 1) transformed data using zero-adjusted Bray-Curtis distances). Average values per year and season (i.e., mean values of 17 sampling sites) are shown for spring (a, c) and summer (b, d). Panels a and b show data at the lowest taxonomic level available, and panels c and d at the major group level. Symbols represent different fixatives, colours show different taxonomists, and contour lines indicate significantly different groups (SIMPROF test, alpha = 0.05). See Annex 2.6 for cluster analyses.

At the lowest taxonomic level, 2004 (associated with Taxonomist #3) was the most different (Figure 2.7a, 2.7b). In spring, significant groups formed between years associated to the same fixative, such as the period 2012–2015 (Figure 2.7a). In summer, years were grouped not only according to the fixative but also to the taxonomist, as shown by the group formed by the years identified by Taxonomist #2. The similarity of the significant groups of years was approximately 60%.

At the level of major taxonomic groups, the year 2004 also showed different phytoplankton assemblages compared to other years. At this taxonomic level, spring in all years appeared significantly grouped in accordance to the utilized fixative, except for 2004 that was also associated with a change in the taxonomist (Figure 2.7c). In summer, except for 2008, years were grouped in agreement with the specialist doing the identification, even if the employed fixative was different (Figure 2.7d). The observed groups of years presented a similarity of around 90%.

Not only were differences among taxonomists observed, but also among different years with the same taxonomist. However, when looking at the years identified by Taxonomist #1 and Taxonomist #2 separately, the dissimilarities in community assemblages between years become smaller, particularly for Taxonomist #2. Cluster analyses of phytoplankton data are described in further detail in Annex 2.6.

4. Discussion

Yearly variation in phytoplankton communities can be explained not only by changes in nutrient concentrations and climatic factors (Cloern & Jassby 2010; Cloern *et al*. 2013), but also by the employed fixative (e.g., Zarauz and Irigoyen 2008) and uncertainty introduced by the taxonomists even if the methodology was similar (Peperzak 2010). This study presents evidence of the effect of these two factors.

The results presented here show evidence of the bias introduced by changes in the utilized fixative. Different fixatives have been found to produce several effects on phytoplankton cells, such as diameter shrinkage, size changes, and reduction in the abundance of detected cells (Leakey *et al*. 1994; Zarauz & Irigoien 2008; Mukherjee *et al*. 2014). Thus, the identification and counting of cells can be biased and lead to distorted results. In the analysis of phytoplankton communities from 19 sampling sites, a clear differentiation was found from the year 2012 onwards (i.e., when the change from glutaraldehyde to Lugol's solutions occurred).

Environmental conditions in surface waters were studied to check if they could explain the inter-annual variability of phytoplankton community, but the main changes observed in environmental variables (e.g., temperature, salinity, optical properties, inorganic nutrients) were not in accordance with those observed in the phytoplankton assemblages. Therefore, these preservation-induced artefacts are significant factors in introducing uncertainty to the study of phytoplankton communities. Along the chronological trajectory, the largest dissimilarities in environmental conditions between years, with respect to average values per season and year, did not reflect such changes in community assemblages for the same years. In fact, some of the largest dissimilarities in phytoplankton communities between consecutive years, apart from 2004, are associated with changes in the fixative and taxonomist (i.e., 2011 to 2012).

Additionally, evidence of interference arising from changes in the taxonomist performing the identification was identified. This could be explained in part by the risk of misidentification of small and cryptic species that is likely when using traditional techniques, such as that of Uthermöhl, which require a high level of expertise of the taxonomist (Mouillot *et al*. 2006). The clearest finding was observed for phytoplankton assemblages from 2004, which appeared notably differentiated from the others in the MDS plots. These results could not be linked to the previously mentioned effect of the fixative because the same fixative was employed in other years and such differences were not observed. Similarly, the phytoplankton community in 2004 was not explained by environmental conditions such that no extreme values were detected for individual environmental variables or for all variables together. Thus, the observed phytoplankton assemblages for 2004 were probably artefacts of the change in the taxonomist.

In general, dissimilarities found in the environmental conditions did not explain the main dissimilarities observed in the phytoplankton communities. As an example, apart from the above

explanation regarding 2004, 2003 was found to be one of the most different years in terms of environmental variables, both in spring and summer. This year was characterized by relatively low values in most studied variables (e.g., Secchi depth, salinity, suspended solids, ammonium, nitrate, phosphate, silicate), together with the maximum summer temperature. However, these findings were not consistently accompanied by great dissimilarity in phytoplankton assemblages between 2003 and other years.

Although data obtained by different taxonomists in the same samples were not compared in this study, Taxonomist #1 and Taxonomist #2 took part in a previous study that assessed the variability in total cell counts within a similar set of samples analysed by different taxonomists (Dromph *et al*. 2013). That study involved several localities, including the Basque coast, and concluded that in all cases, important differences were observed due to the taxonomists' effect.

It is also interesting to assess this effect not only at the lowest taxonomic level available, but also at other taxonomic levels. At the level of major taxonomic groups, the bias due to the experience of the taxonomist was found to be much lower compared with that of species level, as shown by the similarity percentages of significant groups (Figure 2.3c, 2.3d). Consequently, for studies or monitoring networks in which a high taxonomic detail is not required, it would be desirable to work at a higher taxonomic level in order to minimize identification errors. However, interpretation of this finding should be taken with care as Straile *et al*. (2015) found that, at least in lakes, taxonomic aggregation does not always imply more robust results.

It should be noted that studies focused on inhomogeneity detection in phytoplankton time series are relatively scarce. This is not the case for climate datasets, for which several methodologies have been developed for the detection of inhomogeneities (e.g., Buishand 1982; Costa *et al*. 2008; Ribeiro *et al*. 2016). Thus, it is necessary to test the usefulness of the methodology employed in the present study (i.e*.,* detection of multivariate changes in biological assemblages by means of multivariate analyses, such as PERMANOVA and SIMPROF tests) to other long-term phytoplankton datasets.

5. Conclusions

Evidence of the uncertainty due to laboratory issues (i.e*.,* changes in fixatives, experience or changes in the taxonomist) is demonstrated and should be considered when studying long-term phytoplankton time series. Interference introduced by changes in the taxonomists was lower at the level of major taxonomic groups and thus, we suggest that community studies be conducted at higher taxonomic levels when possible. Continuous learning should be combined with detailed protocols and strict standards, and further research should be done regarding the detection of inhomogeneities in phytoplankton time series.

Chapter 3

Seasonal variations of phytoplankton community in relation to environmental factors in an oligotrophic area of the European Atlantic coast (southeastern Bay of Biscay)

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Abstract

In the present study, seasonal variability of physico-chemical variables and phytoplankton community as well as their relationships were studied for oligotrophic coastal waters of southeastern Bay of Biscay. During a 4-year period (2012-2015), a total of 265 phytoplankton taxa were identified, mainly represented by dinoflagellates and diatoms. The highest cell abundances were usually found in spring, mainly attributed to diatoms. Similarly, the biggest contribution to total biomass was given by diatoms: highest values (geometric mean) were found in winter and spring. Although phytoplankton abundance was mostly composed of small cells (2-20 µm), biomass was similarly represented in the 2-20 µm and >20 µm size ranges. Between 21 and 29% of total species variability was significantly explained by different physico-chemical variables. However, this percentage was notably lower at the level of major taxonomic groups. In general, nutrients (mainly ammonium and phosphate) and temperature explained the highest percentage of species variability, whilst salinity played an important role in the summer months. Among the potentially toxic taxa, *Dinophysis* and *Phalacroma* species in summer and autumn appeared associated with relatively high ammonium concentrations.

1. Introduction

As main primary producers, phytoplankton play an essential role in maintaining the structure and functioning of marine coastal systems (Malone *et al.* 2016). Marine phytoplankton sustain pelagic food webs (Fenchel 1988) and directly affect biogeochemical cycles and climate (Holligan 1992). Phytoplankton abundance and composition show a great spatio-temporal fluctuation in marine coastal areas. In temperate areas in particular, they present a seasonal variation and a natural species succession, together with the occurrence of blooms (Berg & Newell 1986; Varela 1996). Although some blooms are beneficial to food-web processes (Smayda 1997), the so-called "*Harmful Algal Blooms"* (HAB) can cause damage on entire ecosystems, resulting even in important economic losses (Anderson 2009).

Phytoplankton communities are highly sensitive to environmental changes, which leads to a very dynamic interaction between this biologic component and the physico-chemical conditions in marine ecosystems. This dynamism is given by several factors such as their small size, rapid nutrient uptake, high growth rates and susceptibility to grazing (Stolte *et al.* 1994). The main environmental factors controlling phytoplankton community structure are light, nutrients and physical processes related to temperature, salinity and turbulence (Troccoli *et al.* 2004). In situations of change in nutrient availability, phytoplankton is usually the first autotrophic compartment responding (Livingston 2000; Paerl *et al.* 2003). Therefore, the study of the effect of environmental factors on phytoplankton abundance, species composition and biomass may be useful to better predict ecological responses to future environmental changes.

Phytoplankton dynamics are also of great importance for shellfish aquaculture, since this community is the main source of energy for filter-feeding bivalves (Grant 1996; Petersen *et al.* 2008). Several studies have reported positive correlations between phytoplankton blooms and increased growth and improved condition index of mussels (Blanton *et al.* 1987; van der Veer 1989; Hickman *et al.* 1991). All phytoplankton species may not be equal in terms of food quality, as bivalves might prefer specific groups or taxa (Weiss *et al.* 2007; Pernet *et al.* 2012). For instance, Beukema and Cadée (1991) found faster growth and better condition in clams, associated with higher diatom abundances. With regard to HAB species, those able to produce biotoxins can be harmful even at very low cell densities (Masó & Garcés 2006). These phytoplankton species are of concern for human health because biotoxins accumulated in seafood cause different acute symptoms, usually shellfish poisonings, and could also cause long-term effects at low-level exposure (Munday & Reeve 2013; Visciano *et al.* 2016). In addition, some toxic species cause detrimental effects on bivalves (e.g., Galimany *et al.* 2008; Mu and Li 2013).

Numerous studies on dynamics of phytoplankton communities that include toxic species have been carried out in coastal waters associated to mollusc production areas, mainly in estuaries (e.g. Ball *et al.* 1997; Anderson *et al.* 2010; Batifoulier *et al.* 2013) and other enclosed nutrient-rich zones, such as those influenced by upwelling systems (e.g., Bravo *et al.* 2010; Anderson *et al.* 2014). These studies are less abundant in open marine waters (e.g*.,* Rhodes *et al.* 2001; Loureiro *et al.* 2005; Vale *et al.* 2008; David *et al.* 2012; Smythe-Wright *et al.* 2014), where aquaculture activities have been considerably less developed. However, there is an increasing interest in developing bivalve aquaculture in open waters in regions, such as the Basque coast (southeastern Bay of Biscay), where sheltered coastal areas are scarce or sustain activities incompatible with

aquaculture (Azpeitia *et al.* 2016). Nevertheless, phytoplankton ecology in this oligotrophic area of the North East Atlantic coast has been very little studied (Estrada 1982; Bode & Fernández 1992; Fernández & Bode 1994; Varela 1996; Garmendia *et al.* 2011).

All this motivated the analysis of the phytoplankton community in open waters off the Basque coast and its relationship with environmental variables. The results here obtained can be useful to better define the factors explaining phytoplankton community variability in non-eutrophic coastal areas, where studies are scarcer than in highly productive zones.

2. Materials and methods

2.1. Study area

The Basque coast is located in the eastern Cantabrian Sea, north of Spain, southeastern Bay of Biscay (Figure 3.1). It extends approximately 100 km. It can be described as an exposed littoral coast, mostly formed by cliffs and influenced by 12 short rivers. The total flow of these rivers is about 150 m^3 s^{-1} (annual mean). This freshwater supply leads often to increased nutrient levels and turbidity in inner shelf waters (Valencia *et al.* 2004; Ferrer *et al.* 2009), although no large coastal plumes are formed (Diez *et al.* 2000). The upwelling activity is almost negligible on the Basque coast (Valencia *et al.* 2004; Lavín *et al.* 2006). The climate of the area is rainy, temperate and oceanic, with moderate winters and warm summers. According to Köppen's classification it is described as marine west-coast and mild (Fontán *et al.* 2009).

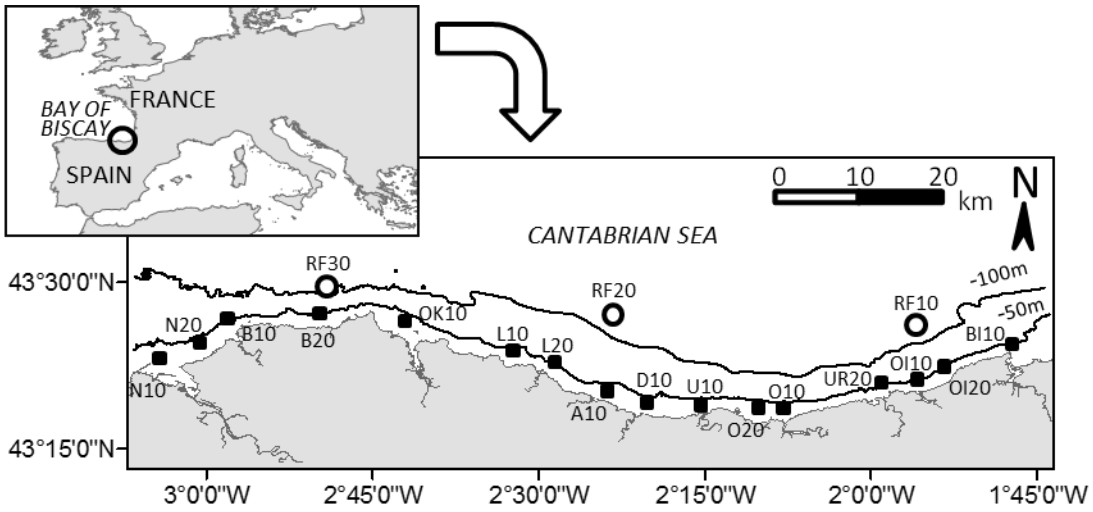

Figure 3.1. Map showing the study area and sampling stations. Squares correspond to nearshore sampling sites, circles to offshore sampling sites.

2.2. Sampling strategy and laboratory work

The study draws on data from the "Littoral Water Quality Monitoring and Control Network" of the Basque Water Agency, which has been used for the implementation of the European Water Framework Directive in the North East Atlantic ecoregion (Borja *et al.* 2004; Borja *et al.* 2016). The dataset consists of 16 nearshore stations (depth around 25-35 m) and 3 offshore stations (depth around 100-120 m) (Figure 3.1). Data from 2012 to 2015 were analysed, corresponding to a unique taxonomist and fixative utilized. Four samplings per year were carried out (winter, spring, summer and autumn).

Samples were taken in surface waters (0-1 m depth), preserved immediately with acidic Lugol´s solution (0.4% v/v) and maintained in dark and cool conditions (4°C) until analysis. The taxonomic identification and cell counting were made following the Utermöhl method (Hasle 1978) under a Nikon diaphot TMD inverted microscope. Depending on the organism's size and abundance 100x, 200x or 400x magnification was used (for more details see Muñiz *et al.* 2017). In addition to strictly functionally phytoplanktonic cells, heterotrophic dinoflagellates, some heterotrophic flagellates (*Ebria tripartita* (J.Schumann) Lemmermann, *Katablepharis remigera* (N.Vørs) B.Clay & P.Kugrens, *Leucocryptos* Butcher, *Telonema* Griessmann) traditionally considered in phytoplankton studies, and the kleptoplastic ciliates of the genus *Mesodinium* were also included in the study. Cells were differentiated into two size groups according to their Equivalent Spherical Diameter (ESD): 2-20 µm and larger than 20 µm. The list of identified taxa was standardized according to AlgaeBase (Guiry & Guiry 2015).

Surface water temperature and salinity were measured in the field using a CTD (Seabird25). The Secchi disc depth was measured as an estimator of the water transparency. The concentration of suspended solids was estimated as described in Clesceri *et al.* (1989) after filtration of the water through Whatman GF/C filters. Inorganic nutrients (ammonium, nitrate, silicate and phosphate) were measured by a Continuous-Flow Autoanalyzer (Bran + Luebbe Autoanalyzer 3; Norderstedt, Germany), using the colorimetric methods described in Grasshoff *et al.* (1983). When nutrient concentration was below the quantification limit (QL) (1.6 µmol L^{-1} for ammonium, nitrate or silicate; 0.16 µmol L^{-1} for phosphate), the used value for statistical analyses was equal to one half of that limit.

2.3. Biomass calculation and assignation of potential toxicity

Phytoplankton data were analysed considering cell concentrations and biomass. In order to calculate the latter, first, the biovolume of each lowest level taxon was calculated from its ESD using the equation of the sphere's volume. Information on phytoplankton cell size was collected from different sources: the ESD measured in phytoplankton species from the northwest Spanish coast by investigators from other institutions (M. Huete from the Spanish Institute of Oceanography - A Coruña Centre, and M. Varela, L. Mene and J. Lorenzo from the University of Vigo) and the report by Olenina *et al.* (2004). Then, biomass was obtained using the equation given by Montagnes *et al.* (1994) for marine phytoplankton: $Biomass=0.109 x Volume^{0.991}$, where *Biomass* is expressed in pg C $cell^{-1}$ and *Volume* is expressed in μm^3. For the data analyses, the specific results on abundance and biomass were summed to obtain total data for the following groups: chlorophytes, kleptoplastic ciliates (*Mesodinium* spp.), cryptophytes, cyanobacteria (filaments), diatoms, dinoflagellates, euglenophytes, haptophytes, heterotrophic flagellates and ochrophytes (chrysophyceans, dictyochophyceans, raphidophyceans and xanthophyceans).

Potentially toxic species were determined according to the Taxonomic Reference List of Harmful Micro Algae from the Intergovernmental Oceanographic Commission of the UNESCO (Moestrup *et al.* 2009 onwards). For genera that are known to contain toxic species, when it was not possible to identify the organism at species level, the whole genus was considered potentially toxic as a measure of precaution (Annex 3.1).

2.4. Statistical analysis

Canonical Correspondence Analysis (CCA, Hill's scaling, downweighting of rare taxa) was undertaken to ascertain the relationships between phytoplankton and the environmental variables. Prior to analysis, all taxa appearing in less than 1% of the samples were excluded. Also, the cells that could not be assigned to any taxonomic group ("unidentified forms <10 µm") were not included. A total of 66 out of 265 taxa were left out (Annex 3.1). Among them, the case of *Lingulodinium polyedra* is noteworthy, as evidenced by Paz *et al.* (2004) as a yessotoxin producer.

The CCAs were performed independently for each season, and for abundance and biomass data. In addition, the analyses were applied at three levels within the phytoplankton community. Firstly, data for the major taxonomic groups listed above were analysed, except for the group "cyanobacteria (filaments)", that was left out of the analysis following the exclusion criteria for those taxa with frequency <1%. Secondly, data for the phytoplankton individual taxa were analysed. In this case, only species with a minimum fit and weight of 15-20% for abundance, and 18-25% for biomass were represented in the corresponding biplots. Finally, a third CCA was undertaken for the potentially toxic taxa. In this case, the taxon *Mesodinium rubrum* Leegaard complex was included as an explanatory variable, as the essential prey for the toxic genus *Dinophysis* spp. (Park *et al.* 2006). Before performing the CCAs, the frequency of the taxa in each season was checked back and those species appearing in one unique sample were excluded.

Species data were *log (x + 1)* transformed and environmental data were normalized and standardized. Only those environmental variables that significantly explained phytoplankton community variability were included (Monte Carlo test at alpha=0.05, 1999 permutations). A second Monte Carlo permutation test was undertaken to determine the statistical significance of all canonical axes together. CCAs and the Monte Carlo permutation tests were performed using CANOCO for Windows 4.5 (Ter Braak & Smilauer 1998).

3. Results

3.1. Environmental variables

Annex 3.2 shows the range and the arithmetic mean of the environmental variables in each season for the study period 2012-2015. Similarly, Figure 3.2 shows the seasonal variability and data distribution of the environmental variables. Secchi disc depth ranged from 2 to 21 meters. Mean values were higher in spring and summer (close to 12 m), than in autumn and winter (around 8 m). Mean surface temperature showed a seasonal pattern and ranged from 12.0°C (winter) to 21.1°C (summer). Mean surface salinity varied little among seasons, between 34.5 and 35.1 PSU; minima were detected in winter and spring, around 31 PSU. Suspended solids concentration usually ranged between 4 and 13 mg L^{-1}, despite some exceptional values up to 29.6 mg L^{-1} in winter. Ammonium and phosphate presented little variation in their median seasonal values. Nevertheless, ammonium reached maxima in summer and autumn (close to 11 µmol L^{-1}), and phosphate in spring (around 1 µmol L^{-1}). Nitrate and silicate median values in surface waters presented a seasonal variation opposite to that of temperature (Figure 3.2). In winter, the median value for nitrate concentration was notably higher than in the rest of the seasons, when it was below the QL. Similarly, silicate reached the maxima in winter, when it was 6-fold higher than in summer.

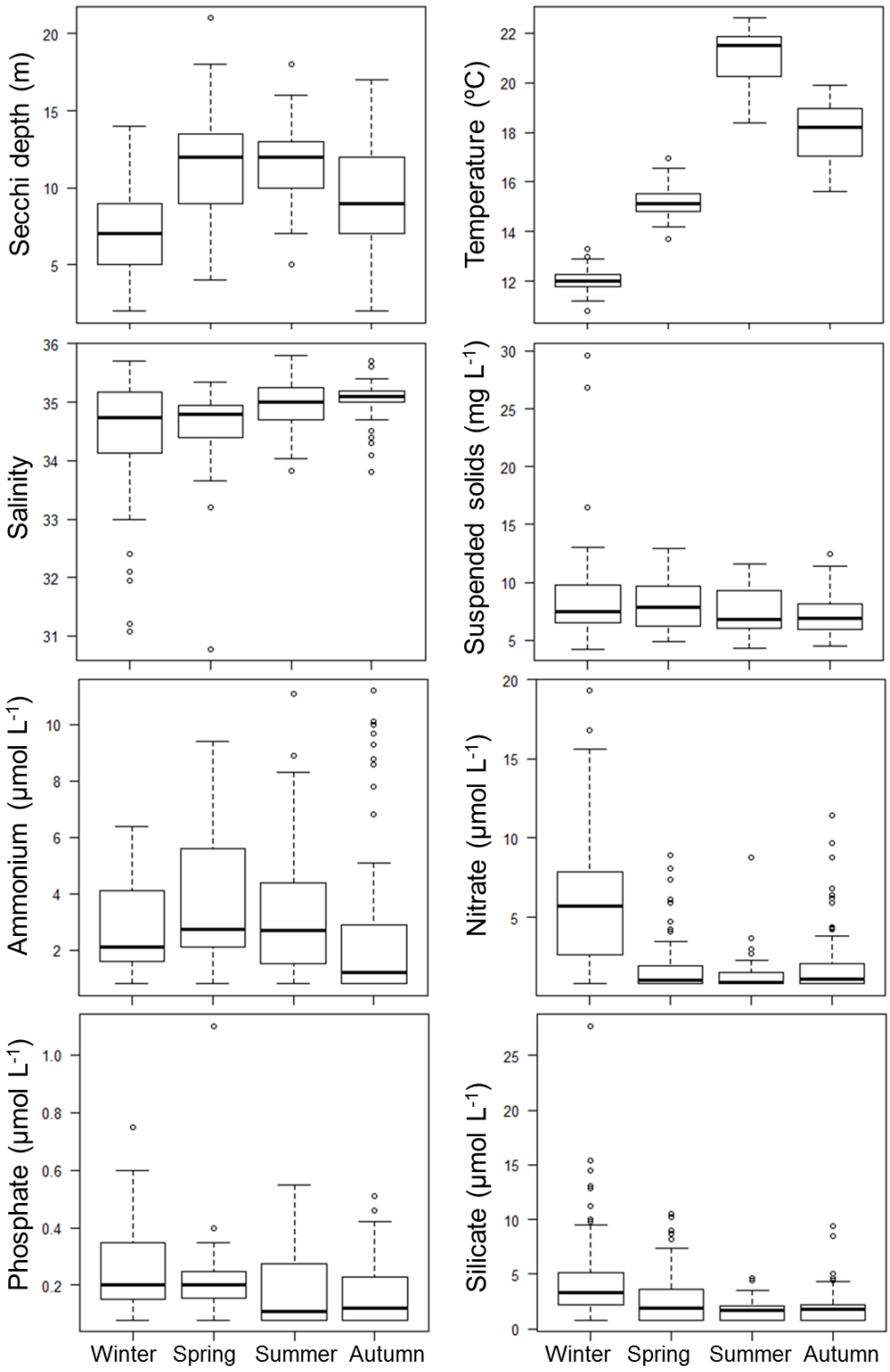

Figure 3.2. Boxplots showing the seasonal variability of environmental variables along the Basque coast for the period 2012-2015. The bold line in the box shows the median. The upper part of the box represents the 75th percentile, and the lower part, the 25th percentile. The points outside the whiskers are the outliers. In the case of nutrients, when concentration was below the limit of quantification (1.6 µmol L^{-1} for ammonium, nitrate or silicate; 0.16 µmol L^{-1} for phosphate), the used value was equal to one half of that limit.

3.2. Phytoplankton community

The total number of phytoplankton taxa (265) was primarily represented by dinoflagellates and diatoms, including 45.5% and 32.6% of the taxa, respectively. The rest of the taxa belonged to ochrophytes (6.1%), chlorophytes (5.7%), cryptophytes (4.5%), euglenophytes (1.9%), haptophytes (1.9%), heterotrophic nanoflagellates (1.1%), kleptoplastic ciliates (0.4%) and cyanobacteria (0.4%). In addition, a separate group of unidentified forms <10 µm was counted. A complete list of the recorded taxa is provided in Annex 3.1.

Table 3.1 shows a brief description of phytoplankton community. Total abundances per sample differed between seasons, with the highest values occurring in spring. Phytoplankton cells larger than 20 µm were less important numerically than nanophytoplankton (2-20 µm ESD), contributing to a mean of 4-23% to total phytoplankton abundance depending on the season. In contrast, phytoplankton biomass was similarly represented by the two size fractions (Figure 3.3).

The highest abundances, in the order of magnitude of 10^6-10^7 cells L^{-1}, were usually given by different diatom taxa (data not shown). The maximum registered was 1.2 x 10^7 cells L^{-1} (*Chaetoceros salsugineus)*. Other diatoms, such as *Minutocellus polymorphus* (Hargraves & Guillard) Hasle, Stosch & Syvertsen*, Thalassiosira* spp. and *Pseudo-nitzschia* spp., presented some values in the range 1-8 x 10^6 cells L^{-1}. The dinoflagellate *Heterocapsa* F.Stein, the cryptophyte *Plagioselmis* Butcher and the haptophyte *Phaeocystis globosa* Scherffel also showed occasionally high abundances, around 1-2 x 10^6 cells L^{-1}. Detailed information on the variability of total abundance, as well as the contribution of the two studied size fractions is shown in Figure 3.3.

The most frequent taxa in each season were “dinoflagellates (athecata)” and “prymnesiales”, which are groups of low specificity present in nearly all the samples. Among the taxa identified at least at genus level, the most frequent were represented by cryptophytes: *Teleaulax* D.R.A.Hill in winter, *Plagioselmis* spp. in spring and autumn, and the katablepharid *Leucocryptos* sp. in summer (Table 3.1).

Regarding biomass, the highest value in each season was always given by species larger than 20 µm (Figure 3.3). Most of these occurrences were usually represented by the genus *Thalassiosira*, up to a maximum biomass of 6.3 x 10^3 µg C L^{-1}. Other species such as *Cerataulina pelagica* (Cleve) Hendey*, Minutocellus polymorphus* and *Proboscia alata* (Brightwell) Sundström also showed high biomass values. In terms of major taxonomic groups, the highest biomass per sample was given by diatoms, in the four studied seasons, presenting values in the order of 10^2-10^3 µg C L^{-1}.

And finally, in relation to potentially toxic species, a total of 25 taxa were identified (Annex 3.1). Most of them were dinoflagellates, except for 3 diatoms (*Pseudo-nitzschia* spp., *P. galaxiae* and *P. multistriata*), one haptophyte (*Phaeocystis globosa*) and one ochrophyte (*Heterosigma akashiwo*).

Table 3.1. Phytoplankton total abundance and biomass ranges and geometric means per sample, geometric means for each cell size range, most frequent taxa, total number of taxa and potentially toxic taxa in each season.

		Winter	Spring	Summer	Autumn
Abundance per sample	range	1.8×10^4 - 5.2×10^6	1.3×10^5 - 1.4×10^7	2.4×10^4 - 1.0×10^7	4.2×10^4 - 1.5×10^6
(cells L^{-1})	mean	4.4×10^5	7.1×10^5	2.8×10^5	2.6×10^5
2-20 µm	mean	3.0×10^5	6.8×10^5	2.7×10^5	2.5×10^5
>20 µm	mean	3.3×10^4	1.2×10^4	5.8×10^3	7.0×10^3
Biomass per sample	range	0.9 - 6907	6.5 - 1671	3.2 - 2640	1.9 - 448
(µg C L^{-1})	mean	46.8	47.7	15.4	17.8
2-20 µm	mean	5.4	17.7	6.8	5.8
>20 µm	mean	24.4	21.0	6.0	9.0
Most frequent taxa		Prymnesiales	Dinoflagellates (Athec.)	Prymnesiales	Prymnesiales
		Dinoflagellates (Athec.)	*Plagioselmis* spp.	Dinoflagellates (Athec.)	Dinoflagellates (Athec.)
		Teleaulax spp.	*Pseudo-nitzschia* spp.	*Leucocryptos* sp	*Plagioselmis* spp.
Number of taxa		159	167	193	189
Number of toxic taxa		13	17	25	21

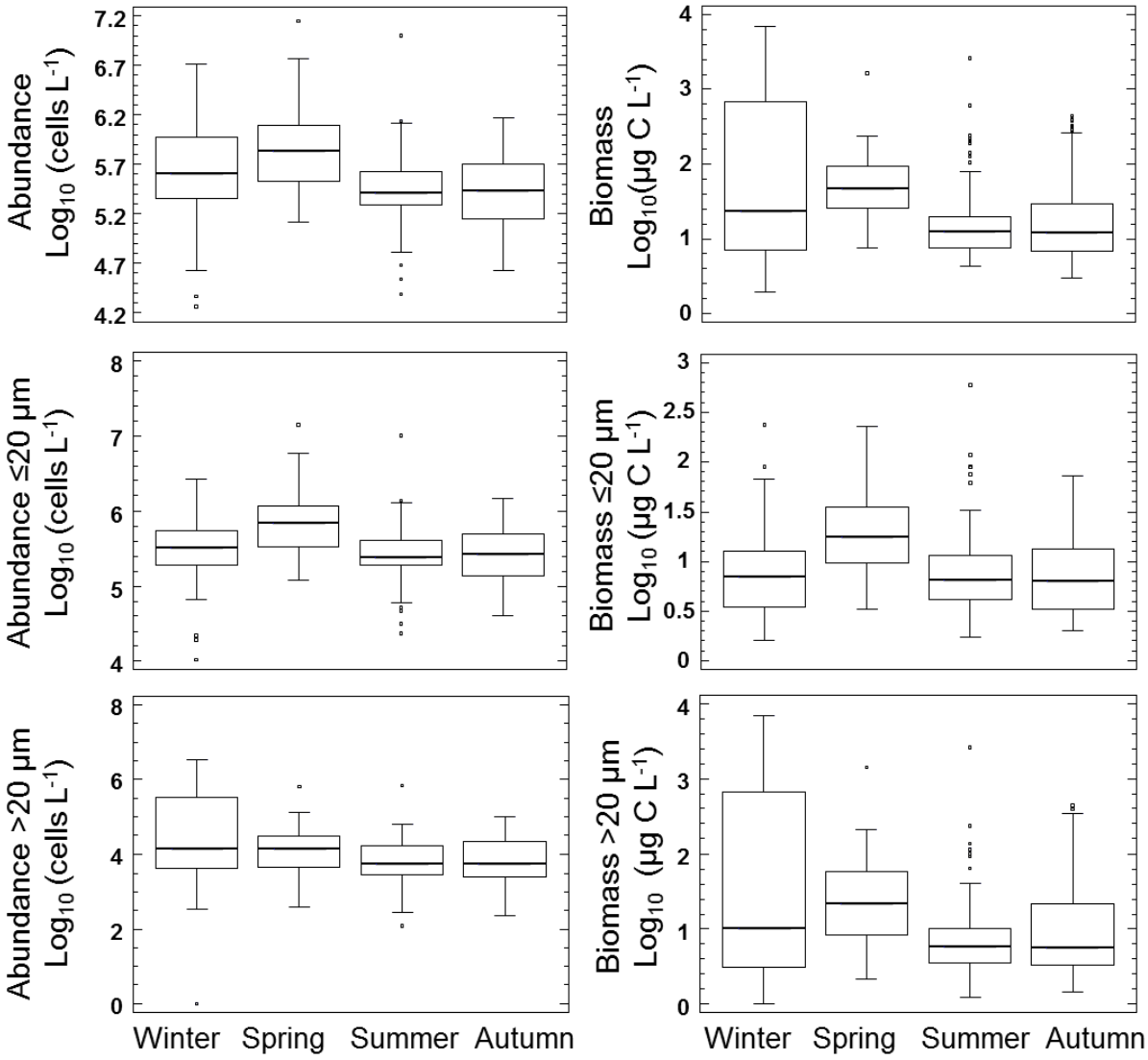

Figure 3.3. Boxplots showing the seasonal variability of total phytoplankton abundance and biomass per sample, as well as the contribution of the two cell size fractions (2-20 µm and >20 µm) along the Basque coast for the period 2012-2015. The bold line in the box shows the median. The upper part of the box represents the 75th percentile, and the lower part, the 25th percentile. The points outside the whiskers are the outliers.

3.3. Relationship between phytoplankton community and environmental variables

Annex 3.3 shows the statistical significance of the physico-chemical variables that significantly explained phytoplankton abundance variability. Annex 3.4 includes detailed information on the statistics for the first two axes of all the CCAs for phytoplankton abundance. Similarly, Annex 3.5, Annex 3.6 and Annex 3.7 show the corresponding information for analyses of phytoplankton biomass.

3.3.1. Abundance and biomass of major taxonomic groups

Figure 3.4 shows the ordination biplot for the CCA obtained for abundance data. Generally, most of the taxonomic groups appeared very close to each other and to the origin.

In winter, phosphate was the variable explaining most of the abundance variability, followed by salinity (Annex 3.3A). Euglenophytes were associated with higher phosphate concentrations and lower ammonium concentrations, whereas the identified heterotrophic nanoflagellates

showed an opposite pattern in regard to phosphate. The abundance of these two groups was higher at lower salinity values. The variability of the rest of the groups was little explained by environmental variables.

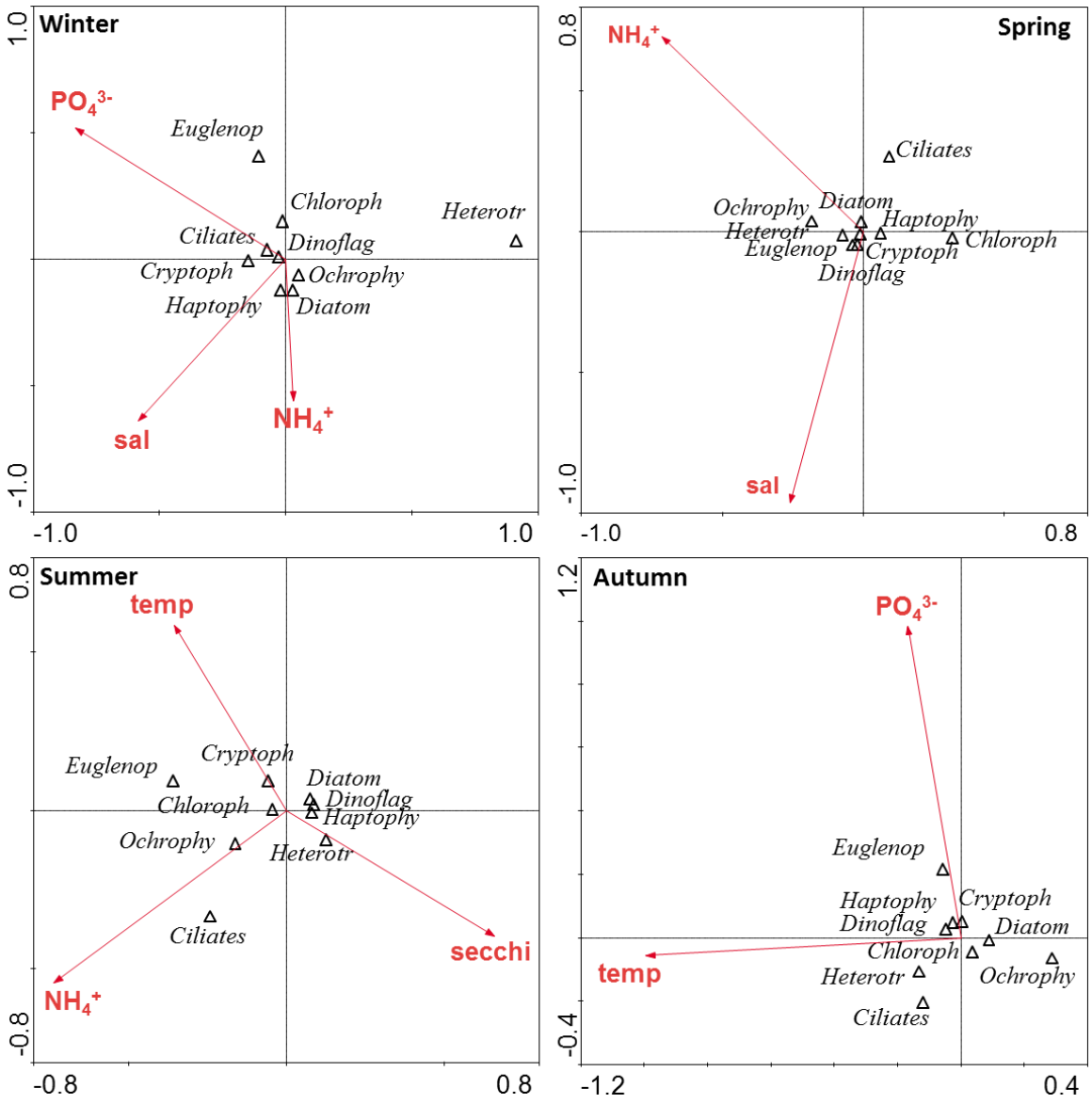

Figure 3.4. Ordination biplots resulting from the CCA performed for the abundance of phytoplankton major taxonomic groups. Information for 76 samples is included for each season (19 sampling sites, 4 years). Arrows represent the correlation of the environmental variables with the axes, and their direction show whether those relations are positive or negative. PO_4^{3-}: phosphate, sal: salinity, NH_4^+: ammonium, temp: temperature, secchi: Secchi disc depth. Ciliates: kleptoplastic ciliates, Cryptoph: cryptophytes, Dinoflag: dinoflagellates, Chloroph: chlorophytes, Haptophy: haptophytes, Heterotr: heterotrophic nanoflagellates, Diatom: diatoms, Euglenop: euglenophytes, Ochrophy: ochrophytes.

In spring, abundances of kleptoplastic ciliates (hereinafter referred to as ciliates) and ochrophytes appeared with higher ammonium concentrations, whereas chlorophytes were associated with lower ones. Moreover, ciliates were registered at lower salinity values.

In summer, ciliates, ochrophytes and euglenophytes were the groups whose variability was most explained by the environment, with greater presence at higher concentrations of

ammonium. Furthermore, euglenophytes were associated with higher temperatures and low Secchi depth values, while ciliates showed greater presence at lower temperatures.

Finally, in autumn temperature and phosphate were the significant explanatory variables (Annex 3.3A). Ciliates and ochrophytes were associated with lower concentrations of phosphate, whereas euglenophytes were linked to higher ones, similarly than in winter. Ciliates and heterotrophic nanoflagellates showed higher presence with higher temperatures, whereas ochrophytes showed the opposite pattern (Figure 3.4).

For winter, spring, summer and autumn the ordination along all axes together explained 24.0%, 7.6%, 15.9% and 10.6% of the abundance variance, respectively (Table 3.2A).

Table 3.2. Summary of the CCAs performed for the abundances of the three different datasets and variance explained by environmental variables in phytoplankton community season by season.

A. Abundance of major taxonomic groups

	Winter	Spring	Summer	Autumn
Sum of all eigenvalues	0.250	0.211	0.157	0.151
Sum of all canonical eigenvalues	0.060	0.016	0.025	0.016
Variance explained (%)	24.0	7.6	15.9	10.6
p-value	<0.005	<0.005	<0.005	<0.005

B. Abundance of individual taxa

	Winter	Spring	Summer	Autumn
Sum of all eigenvalues	1.906	1.669	1.630	1.701
Sum of all canonical eigenvalues	0.492	0.375	0.467	0.477
Variance explained (%)	25.8	22.5	28.7	28.0
p-value	<0.005	<0.005	<0.005	<0.005

C. Abundance of potentially toxic taxa

	Winter	Spring	Summer	Autumn
Sum of all eigenvalues	2.135	2.023	2.431	1.974
Sum of all canonical eigenvalues	0.368	0.315	0.459	0.412
Variance explained (%)	17.2	15.6	18.9	20.9
p-value	<0.005	<0.005	<0.005	<0.005

Regarding biomass of major taxonomic groups, in general, results obtained in the CCA differed little from those described for abundance (Figure 3.5A). The variance explained by environmental variables was slightly lower in the case of the biomass, between 3.2% and 21.0% (Annex 3.5A). The variables that significantly explained the variability of the biomass of major groups during the year were the same found in the analysis of abundance variability, except some dissimilarities in concrete seasons (Annex 3.6A). Annex 3.7A shows the summary statistics for the first two axes of CCA on the biomass of phytoplankton major groups and environmental variables.

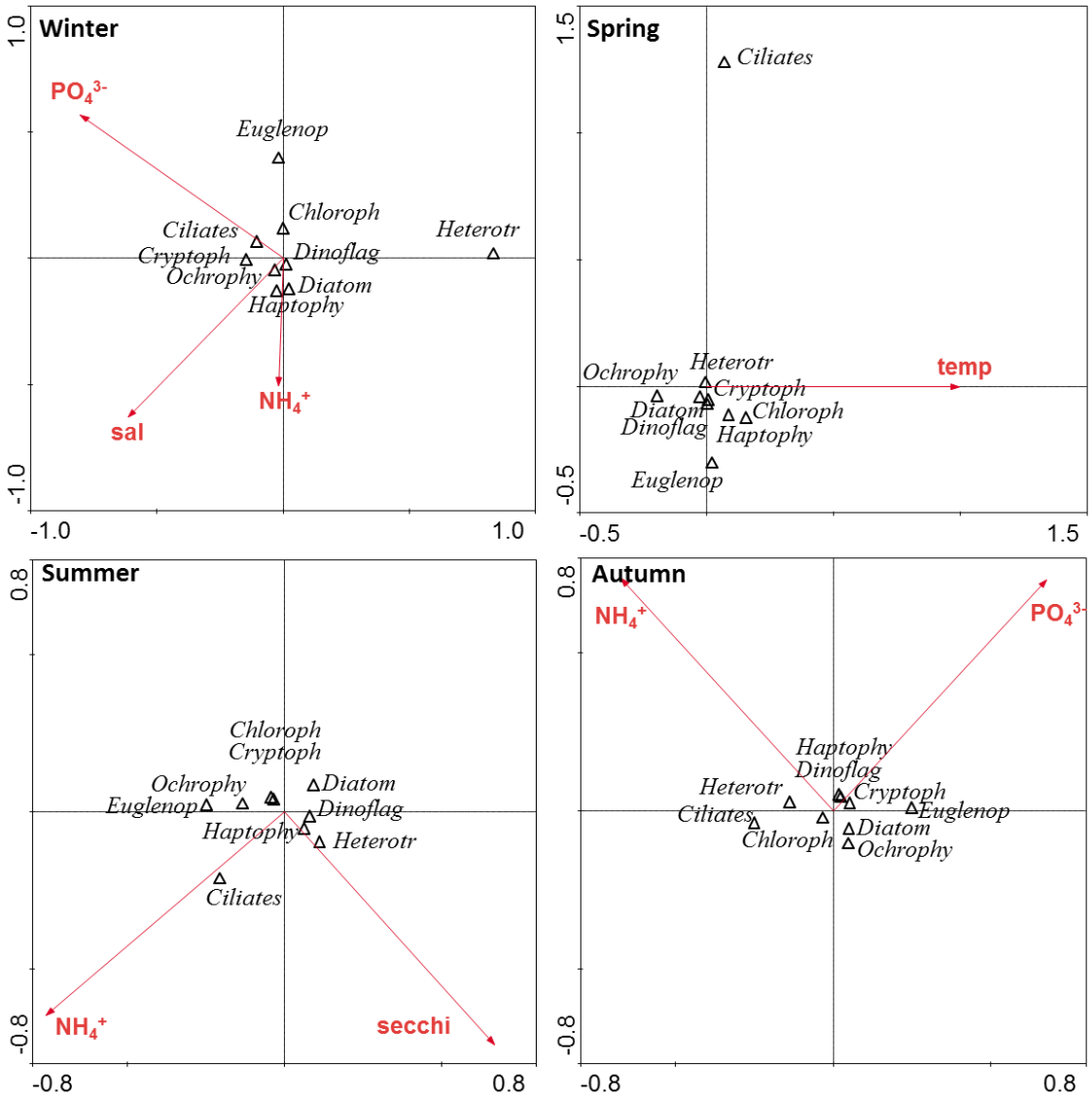

Figure 3.5. Ordination biplots resulting from the CCA performed for the biomass of phytoplankton major taxonomic groups. Information for 76 samples is included for each season (19 sampling sites, 4 years). Arrows represent the correlation of the environmental variables with the axes, and their direction show whether those relations are positive or negative. PO_4^{3-}: phosphate, sal: salinity, NH_4^+: ammonium, temp: temperature, secchi: Secchi disc depth. Ciliates: kleptoplastic ciliates, Cryptoph: cryptophytes, Dinoflag: dinoflagellates, Chloroph: chlorophytes, Haptophy: haptophytes, Heterotr: heterotrophic nanoflagellates, Diatom: diatoms, Euglenop: euglenophytes, Ochrophy: ochrophytes.

3.3.2. Abundance and biomass of individual taxa

At the level of individual taxa there were more environmental variables that significantly explained the variability compared to the level of major taxonomic groups.

Regarding abundance, in winter, phosphate was the variable explaining most of the variance (Annex 3.3.B). Higher cell abundances of the small dinoflagellate *Gyrodinium* cf. *flagellare,* and several small flagellates (*Plagioselmis* spp., *Pyramimonas* Schmarda, *Teleaulax amphioxeia* (W.Conrad) D.R.A.Hill and *Teleaulax gracilis* Laza-Martinez*)* were found with higher values of phosphate and suspended solids and lower values of Secchi depth. The opposite pattern was found for several diatoms (*Thalassiosira* spp., *Thalassiosira* cf. *mediterranea* (Schröder) Hasle*,*

Asterionella glacialis sp. complex and *Guinardia delicatula* (Cleve) Hasle), other small flagellates *(Leucocryptos* sp. and *Tetraselmis* sp.) and *Phaeocystis globosa.* At the same time, the represented diatoms (i.e., species fit and weight between 15 and 20%) occurred at higher temperatures and ammonium concentrations together with lower silicate values (Figure 3.6).

In spring, ammonium was the most explanatory variable, followed by Secchi depth and silicate (Annex 3.3B). Abundances of the taxa coccolithaceae and *Lessardia elongata* Saldarriaga & F.J.R.Taylor, as well as the diatoms *Cerataulina pelagica* and *Rhizosolenia* Brightwell., were linked to higher silicate and ammonium concentrations and lower water transparency and salinity. Most of the representative diatoms were associated with relatively higher temperatures (*Cerataulina pelagica*, *Chaetoceros salsugineus, Leptocylindrus* Cleve. and *Nitzschia longissima* (Brébisson) Ralfs) (Figure 3.6).

In summer, salinity and temperature were the environmental factors that most explained phytoplankton abundance variability (Annex 3.3B). The most remarkable taxon was coccolithaceae, which was associated with higher temperatures and ammonium concentrations, together with lower values of phosphate. The species *Dinobryon faculiferum* (Willén) Willén and *Tripos furca* (Ehrenberg) F.Gómez followed a similar trend. The opposite pattern was found with a group of diatoms, such as *Pseudo-nitzschia* spp., *Pseudo-nitzschia galaxiae, Nitzschia longissima,* unidentified pennales and *Chaetoceros* spp. (solitary cells). Unlike in spring, the most representative diatoms were registered at lower temperatures (Figure 3.6).

During autumn, temperature and ammonium were the main explanatory variables (Annex 3.3B). The dinoflagellates *Torodinium robustum* Kofoid & Swezy and *Gyrodinium* Kofoid & Swezy. appeared associated with the highest ammonium concentrations. As in summer, a group of diatoms (*Chaetoceros decipiens* Cleve, *Proboscia alata* and *Dactyliosolen fragilissimus* (Bergon) Hasle) were present at lower temperatures, being at the opposite side several dinoflagellates and small flagellates (Figure 3.6).

In the case of abundance analysis, variance explained by the environmental variables ranged from 22.5% to 28.7% among the different seasons (Table 3.2B).

On the other hand, CCA results regarding biomass of individual taxa were very similar to those obtained for abundance (Figure 3.7). The variance explained by environmental variables was slightly lower in the case of the biomass, between 21.4% and 26.8% (Annex 3.5B). The variables that significantly explained the variability of the biomass of individual taxa were the same found in the analysis of abundance variability, except for silicate and nitrate during summer (Annex 3.6B). Annex 3.7B shows the summary statistics for the first two axes of CCA on the biomass of phytoplankton individual taxa and environmental variables.

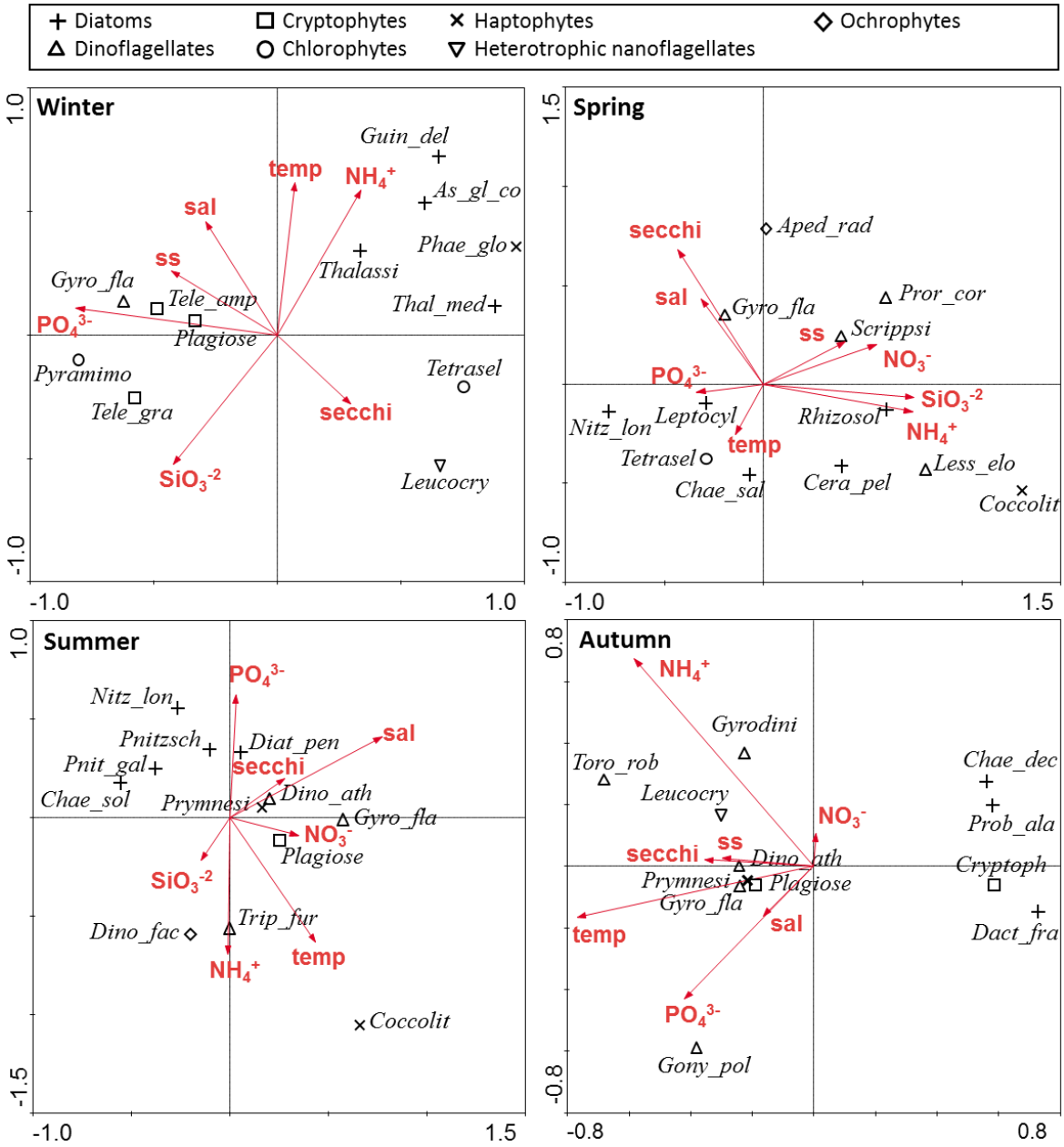

Figure 3.6. Ordination biplot resulting from the CCA performed for the abundance of phytoplankton community. Information for 76 samples is included for each season (19 sampling sites, 4 years). Arrows represent the correlation of the environmental variables with the axes, and their direction show whether those relations are positive or negative. Symbols refer to different taxonomic groups. Only species with a minimum fit and weight of 15-20% are represented. PO_4^{3-}: phosphate, ss: suspended solids, sal: salinity, temp: temperature, NH_4^+: ammonium, SiO_3^{-2}: silicate, secchi: Secchi disc depth, NO_3^-: nitrate. Species and their corresponding abbreviations are shown in Annex 3.1.

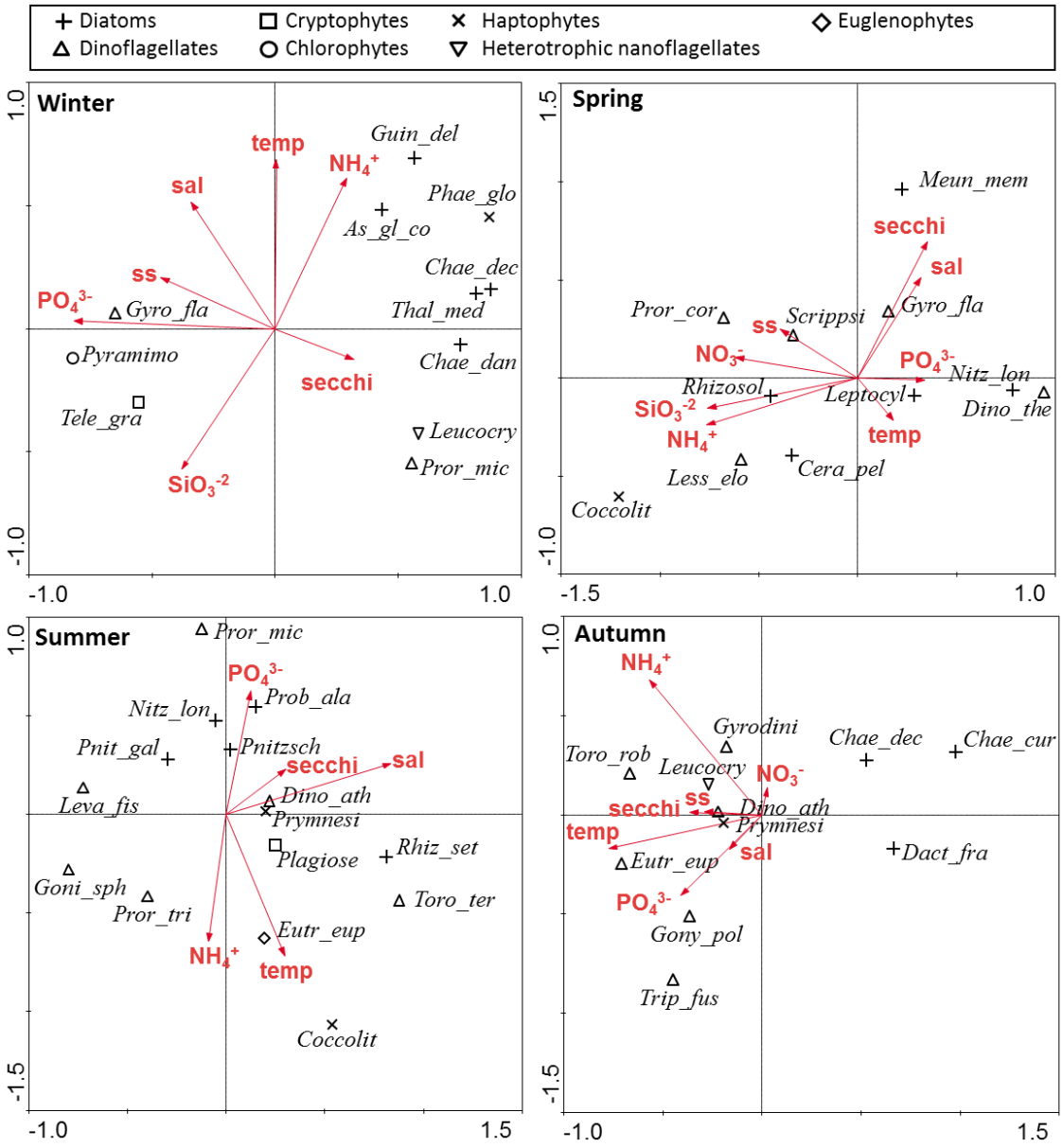

Figure 3.7. Ordination biplot resulting from the CCA performed for the biomass of phytoplankton community. Information for 76 samples is included for each season (19 sampling sites, 4 years). Arrows represent the correlation of the environmental variables with the axes, and their direction show whether those relations are positive or negative. Symbols refer to different taxonomic groups. Only species with a minimum fit and weight of 18-25% are represented. PO_4^{3-}: phosphate, ss: suspended solids, sal: salinity, temp: temperature, NH_4^+: ammonium, SiO_3^{-2}: silicate, secchi: Secchi disc depth, NO_3^-: nitrate. Species and their corresponding abbreviations are shown in Annex 3.1.

3.3.3. Abundance and biomass of potentially toxic taxa

Figure 3.8 shows the ordination biplot resulting from the CCA for abundance in each season. In winter, phosphate was the most explanatory variable, followed by temperature (Annex 3.3.C). Abundances of *Prorocentrum cordatum* and *Karenia papilionacea* were related to higher phosphate concentrations while *Protoceratium reticulatum,* cf*. Karlodinium* J.Larsen. and *Dinophysis* cf. *ovum* T.H.Avé were associated with lower ones (Figure 3.8).

In spring, the most explanatory variable of abundance variability was nitrate, followed by suspended solids concentration (Annex 3.3C). The dinoflagellates *Prorocentrum cordatum, Karenia mikimotoi* (Miyake & Kominami) Gert Hansen & Ø.Moestrup and *Dinophysis tripos* were associated with the highest nitrate concentrations. In contrast, other dinoflagellates such as *Gonyaulax spinifera* and *Phalacroma rotundatum* (Claparède & Lachmann) Kofoid & Michener appeared in samples with relatively low nitrate concentration (Figure 3.8).

In summer, *Takayama* M.F.Salas, Bolch, Botes & Hallegraeff. and *Karenia* cf. *mikimotoi* were found at high salinity and temperature values, the variables that explained most of the variability of potentially toxic phytoplankton abundance in this season (Annex 3.3C). In addition, several species of the genus *Dinophysis* and *Phalacroma* (diarrheic toxins producers) were found positively related to ammonium.

Finally, in autumn ammonium and temperature explained most of the variability (Annex 3.3C). As in summer, some *Dinophysis* and *Phalacroma* species were found associated with higher ammonium concentrations, in particular: *D. tripos, D. fortii* and *Phalacroma mitra* F.Schütt. The potentially producer of clupeotoxin poisoning, *Ostreopsis* cf. *siamensis*, showed a similar distribution (Figure 3.8).

Regarding abundance, the variance explained by the environmental variables ranged from 15.6% to 20.9% (Table 3.2C).

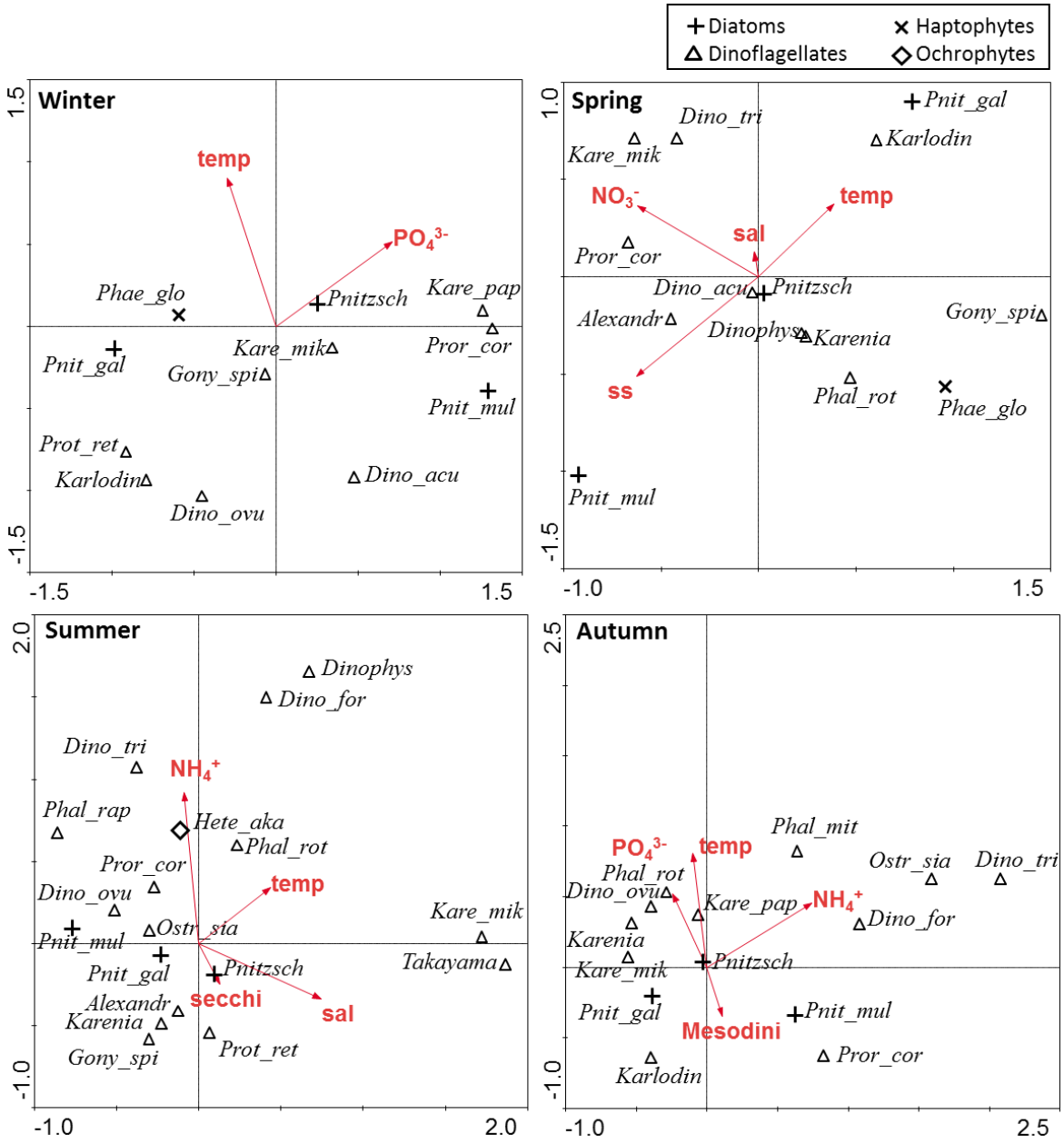

Figure 3.8. Ordination biplot resulting from the CCA performed for the abundance of potentially toxic taxa of phytoplankton. Information for 76 samples are included for each season (19 sampling sites, 4 years). Arrows represent the correlation of the environmental variables with the axes, and their direction show whether those relations are positive or negative. Symbols refer to different taxonomic groups. PO_4^{3-}: phosphate, ss: suspended solids, sal: salinity, temp: temperature, NH_4^+: ammonium, secchi: Secchi disc depth, NO_3^-: nitrate, Mesodini: *Mesodinium rubrum* sp. complex. Species and their corresponding abbreviations are shown in Annex 3.1.

In terms of biomass, CCA biplots showed similar results compared to results for abundance (Figure 3.9). The variance explained by environmental variables was also very similar, between 14.8% and 20.4% (Annex 3.5C). Despite some exceptions, significant explanatory variables were usually the same for biomass and abundance (Annex 3.6C). The most remarkable fact found in the analysis of biomass variability was that in winter *Mesodinium rubrum* spp. complex was significantly explaining part of the variability: *Dinophysis acuminata* and *Dinophysis* cf. *ovum* were found in association with the presence of this taxon. Annex 3.7C shows the summary statistics for the first two axes of CCA on the biomass of potentially toxic taxa and environmental variables.

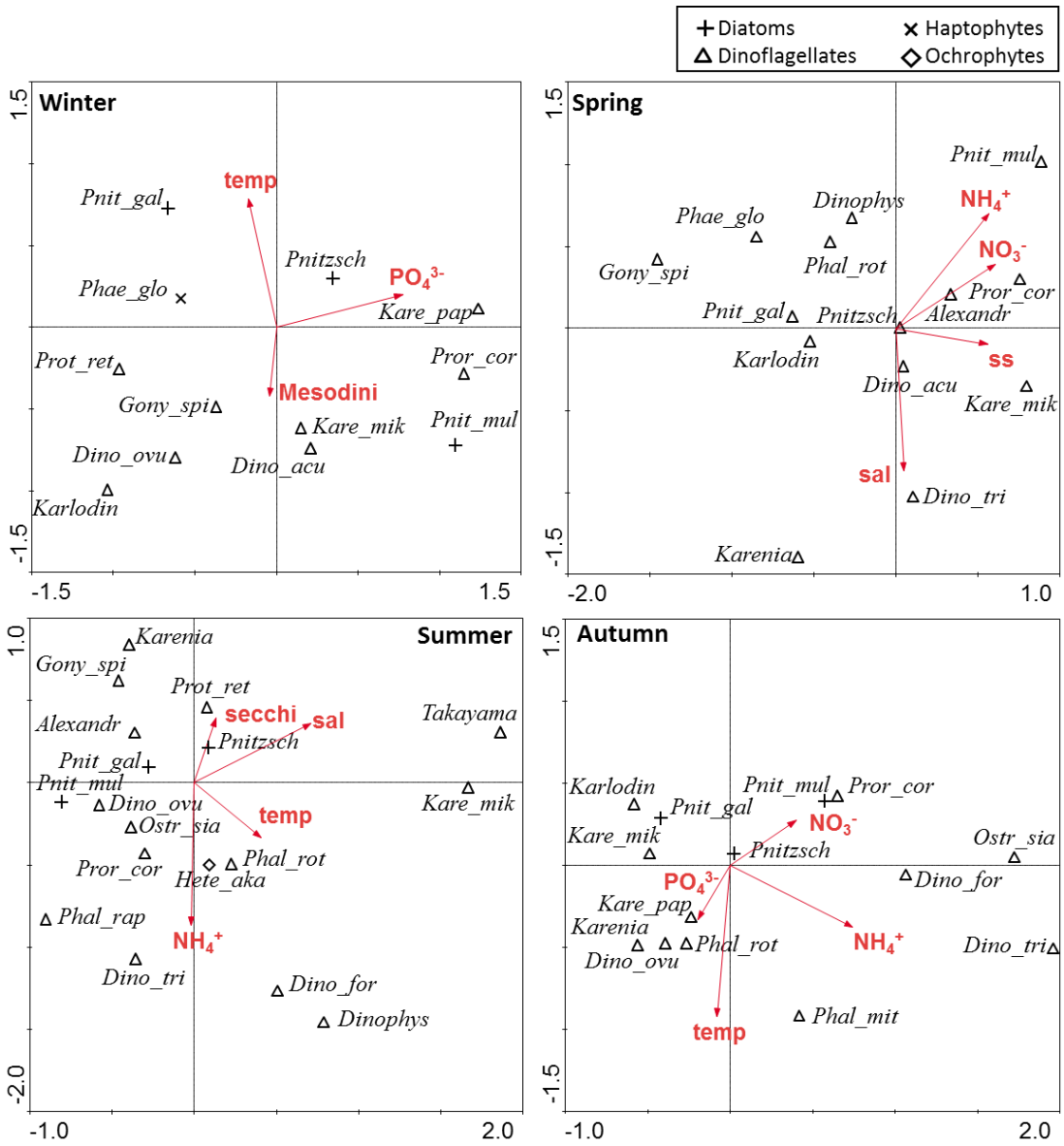

Figure 3.9. Ordination biplot resulting from the CCA performed for the biomass of potentially toxic taxa of phytoplankton. Information for 76 samples are included for each season (19 sampling sites, 4 years). Arrows represent the correlation of the environmental variables with the axes, and their direction show whether those relations are positive or negative. Symbols refer to different taxonomic groups. PO_4^{3-}: phosphate, ss: suspended solids, sal: salinity, temp: temperature, NH_4^+: ammonium, secchi: Secchi disc depth, NO_3^-: nitrate, Mesodini: *Mesodinium rubrum* sp. complex. Species and their corresponding abbreviations are shown in Annex 3.1.

4. Discussion

Surface temperature showed the seasonal warming and cooling pattern previously described for the southern Bay of Biscay (e.g., Lavín *et al.* 1998), which Valencia *et al.* (2003) found to be highly related to air temperature in the Basque shelf waters. In the study area, the annual cycle of dissolved nutrients in the upper layers is related with the thermal cycle and the succession of homogeneity and stratification conditions (Valencia & Franco, 2004). As indicated by these authors, although during summer stratification nutrient concentrations in shelf waters off the Basque coast rarely reach "zero" values, the residual concentrations in the water layer above the thermocline are comparable to those found in oligotrophic areas. Within Basque coastal waters, wind-driven upwelling is almost negligible and river inputs together with the effect of phytoplankton production are the main factors modulating the annual nutrient cycle (Valencia & Franco 2004).

In the present study, some similarities were found in nutrient concentration patterns with the neighbouring area of Arcachon Bay (southwest coast of France) (Glé *et al.* 2008). Nitrate and silicate showed notably higher values in winter, according to higher freshwater discharge periods as well as the turbulent mixing processes that generates a nutrient input from the deeper waters to the surface (Prego & Vergara 1998; Valencia & Franco 2004). Further, ammonium and phosphate levels did not follow the same pattern of nitrate and silicate. As suggested for other nearshore shallow areas, the main source of ammonium might come from exchange at the water-sediment interface, due to the organic matter mineralization (e.g., Glé *et al.* 2008). At the deeper offshore sites, the mineralization processes occurring in the water column would have more influence. The activity of heterotrophic bacteria is strongly affected by temperature (Li 1998), which would explain more frequent peaks of ammonium in surface waters off the Basque coast during summer and autumn. Moreover, it is well known that sewage discharges from urban origin are rich in ammonium and phosphate. Although the load of nutrients from anthropogenic origin has decreased considerably in the area during the last two decades, some sporadic inputs might still occur (García-Barcina *et al.* 2006; Garmendia *et al.* 2011; Borja *et al.* 2016).

Despite these similarities in nutrient patterns, concentrations were much lower along the Basque coast compared to those described for the French basin, such as those related to Gironde and Loire rivers, due to the great differences in freshwater inputs (e.g., Meybeck *et al.* 1988; Labry *et al.* 2002; Glé *et al.* 2008). Moreover, compared to the semi-enclosed embayment of Arcachon, the observed relatively low nutrient concentrations might also be influenced by a higher dilution effect created by the higher exchange with oceanic waters.

Regarding the community size structure, cells ranging 2-20 µm (ESD) were much more abundant than larger ones, in agreement with the general pattern observed in culture and field studies of phytoplankton (Chisholm 1992). Small phytoplankton cells have smaller diffusion boundary layers and higher surface-volume ratio (Raven 1986; Kiørboe 1993; Raven 1998), which gives a competitive advantage over larger cells due to diffusion limitation, which constrains nutrient uptake (Fogg 1991). However, within the nano- and micro-plankton, other factors become important for growth, such as the ability of the cells to store nutrients, the use of alternative nutritional strategies (e.g., mixotrophy or symbioses), the swimming capacity, the resistance to zooplankton grazing, etc. (Chisholm 1992). Marañón *et al.* (2013) in culture

experiments found that maximum growth rates peak at intermediate cell sizes, and attributed it to the dependence that not only the nutrient uptake rates, but also the nutrient requirements and the assimilation rates, have with cellular size.

Phytoplankton abundance in Basque coastal waters was dominated by diatoms: highest cell densities were mainly represented by species belonging to the genera *Chaetoceros* (*Hyalochaetae*) and *Thalassiosira*. These results are in agreement with previous studies in other areas within the Bay of Biscay (Gohin *et al.* 2003; Lunven *et al.* 2005) as well as in the western English channel (Widdicombe *et al.* 2010).

Phytoplankton are highly sensitive to environmental changes, responding not only with shifts in total biomass but also in composition (Li *et al.* 2009). In fact, differences in tolerance to environmental conditions between different species have been reported (Heino & Soininen 2006; Fariñas *et al.* 2015). In the present case, between 21.4% and 28.7% of the species variability was explained by the available environmental parameters. The rest of the variability might be explained by other factors not taken into account in this study, such as micronutrients, competition, grazing or parasite pressure (Litchman & Klausmeier 2008).

In terms of abundance of major taxonomic groups, the environmental variables here studied explained little about the variance (usually <16%, except in winter that was 24%), which might be given by species-specific ecology that cannot be generalized to a whole taxonomic group. For example, different dinoflagellates species have diverse habitat preferences (Smayda & Reynolds 2003). Except ciliates, euglenophytes and heterotrophic flagellates, most of the groups were found to occur together in the CCA biplots, indicating that their response to environmental factors in the study area is very similar. It should be noted that these three groups were formed by a much lower number of taxa in comparison to the others, which have conferred them more homogeneity. The group of kleptoplastic ciliates, which includes species belonging to the genus *Mesodinium*, was one of the most distant in spring, summer and autumn. Opposite results were described for heterotrophic ciliates by other authors, who found that the distribution of ciliates in temperate coastal ecosystems is usually closely associated with nanophytoplankton, as these constitute their main preys (Verity 1987; Lynn & Montagnes 1991).

The main environmental parameters shaping the phytoplankton community were temperature and nutrients, as found in previous studies for other areas within the Bay of Biscay (Fariñas *et al.* 2015). Furthermore, salinity was the factor explaining the largest part of both abundance and biomass variability in summer. Temperature and nutrients show a combined effect on phytoplankton community. The interaction between these two elements is well recognized: increased surface temperatures influence water column stratification which, in turn, affects the mixing between the surface and deeper nutrient-rich waters reducing the transport of inorganic nutrients to the euphotic zone (Varela 1996). At species level and focusing on temperature, different relationships were found depending on the season. In summer and autumn, the most relevant diatoms were associated with low temperatures (which usually implies lower nutrient limitation), unlike dinoflagellates. However, this divergence between these two groups was not found in winter and spring. In fact, some of the large diatoms (*Guinardia delicatula, Cerataulina pelagica, Nitzschia longissima)* were linked to relatively higher temperatures, which could indicate higher atmospheric stability and insolation. In spring, autumn and especially in summer, when

salinity was the variable explaining most of the variability, some of the represented diatoms (*Chaetoceros* spp. (solitary cells) and the toxic *Pseudo-nitzschia galaxiae*) were linked to lower salinity values. This could be explained by (i) sporadic freshwater inputs due to storms (typical of the area) that prevent from a total depletion of nutrients (Valencia & Franco 2004) or (ii) cells exported from the estuaries, given that in summer phytoplankton concentration is much higher in estuaries than in coastal waters (Garmendia *et al.* 2011). However, despite some associations, a general pattern was not found. Even within the same genus, different responses to environmental conditions were found.

Regarding potentially toxic taxa, species appeared highly dispersed in the CCA biplots indicating a high heterogeneity in their responses to environmental variables. In summer and autumn species belonging to the genus *Dinophysis* were found to be usually associated with high ammonium concentrations. Similar phenomena have been reported in other areas (Carpenter *et al.* 1995; Koike *et al.* 2001; Nishitani *et al.* 2005), revealing the importance of monitoring ammonia levels to predict these events. However, further research is needed on the relationship between ammonium concentrations and *Dinophysis* spp. presence. Additionally, the need of *Mesodinium* preys to sustain the growth of *Dinophysis* has been shown *in vitro* (Park *et al.* 2006). In NW coast of Iberian peninsula, *Dinophysis* blooms usually occur after *Mesodinium* blooms, with a time-lag of 2-3 weeks, showing the potential of these ciliates as predictors of the toxic blooms (Moita *et al.* 2016). In this regard, when *Mesodinium* was introduced as a variable in the CCA, it explained part of the variability of *Dinophysis* biomass in winter. Toxic species have been largely studied in Galicia (northwest of Iberian Peninsula), given the important problem that they pose for bivalve aquaculture (Fernández *et al.* 2006; Bravo *et al.* 2010; Rodríguez *et al.* 2015). There, upwelling together with the estuarine circulation leads to a very high primary production (Fraga 1981; Figueiras *et al.* 2002). It is widely acknowledged that nutrient loading fuels high biomass algal blooms, including those considered toxic or harmful (Anderson *et al.* 2002). However, the mentioned ecosystem dynamics for Galician rias are very different to the planktonic system functioning in the Basque coast and thus, further research on *Dinophysis* spp. (among others) dynamics in relatively oligotrophic areas, such as the Basque waters, are needed.

5. Conclusions

Summarizing, variations in phytoplankton community were significantly explained by different environmental variables in each season. The variability of the phytoplankton community at the level of major taxonomic groups was much less explained by the environment compared to that at the lowest taxonomic level. In most cases, the variability of individual taxa was mainly explained by temperature and nutrients (mostly ammonium and phosphate). Potentially toxic taxa also showed heterogeneity and different responses to environment, even within the same genus. However, an association between ammonium concentrations and several potentially toxic dinoflagellates was found.

Chapter 4

Annual cycle of phytoplankton community throughout the water column: study applied to the implementation of bivalve offshore aquaculture in the southeastern Bay of Biscay

Submitted as: **Muñiz O.**, Revilla M., Rodríguez J.G., Laza-Martínez A. and Fontán A. Annual cycle of phytoplankton community throughout the water column: study applied to the implementation of bivalve offshore aquaculture in the southeastern Bay of Biscay. *Oceanologia*.

Abstract

This study describes, for the first time, the annual variability of the phytoplankton community in different layers of the water column in open waters off the Basque coast (southeastern Bay of Biscay). Phytoplankton composition, abundance and biomass, together with size-fractionated chlorophyll *a*, nutrients, and optical and hydrographic conditions were measured in an experimental bivalve culture area from May 2014 to June 2015. Water column conditions showed the typical dynamics previously described for temperate areas, characterised by winter homogeneity and summer stratification. Phytoplankton temporal variability was studied at depths of 3, 17 and 33 m, and was found to be related to those processes. In particular, temperature and nutrients (mostly nitrate and silicate) were the environmental variables which significantly explained most of the variability of chlorophyll concentration, whereas river flow was the main driver of abundance variability. Total chlorophyll was generally low (0.6 µg L^{-1} on average). Of the 194 registered taxa, 47.4% belonged to dinoflagellates and 35.1% to diatoms. In addition, diatoms showed the highest biomass values, and haptophytes represented the greatest contribution to cell-abundance. This fact, despite the low chlorophyll values indicate low phytoplankton biomass, could favour mussel growth given the high fatty acid content reported for diatoms and haptophytes.

1. Introduction

Phytoplankton constitute an important component of the diet of suspension feeding bivalves (Shumway & Cucci 1987; MacDonald & Ward 1994; Grant 1996; Petersen *et al.* 2008). In fact, microalgae have long been used as food resource for mollusc bivalves at all growth stages (Brown 2002). This interaction of mollusc bivalves with phytoplankton as a food source has been studied extensively. For instance, it is known that the quantity and size of the phytoplankton can influence the recruitment of oysters, as well as the survival of bivalve larvae (Robert & Trintignac 1997; Bourlès *et al.* 2009). Moreover, phytoplankton blooms have been directly related to the increase of mussel growth and condition index (i.e., the ratio between the dry weight of the meat and the shell) (Blanton *et al.* 1987; van der Veer 1989; Hickman *et al.* 1991). However, not all phytoplankton species are equal in terms of nutritional quality for bivalves. Several bivalves (including mussels) have shown a preferential utilisation of phytoplankton species which depends on both their food value and cell size (Møhlenberg & Riisgård 1978; Kiørboe & Mohlenberg 1981; Cucci *et al.* 1985; Rouillon & Navarro 2003). In this sense, lipids are the main source of energy for larvae and lipid content of phytoplankton varies depending on the species or group (Volkman *et al.* 1989; Volkman *et al.* 1991; Marshall *et al.* 2010). Feeding experiments on *Mytilus galloprovincialis* carried out by Pettersen *et al.* (2010) showed that alterations in phytoplankton species composition can produce variations in mortality and settlement rates. Also, in field studies, Wall *et al.* (2013) found that the growth rates of bivalves were more related to the density of certain cellular types than to the total phytoplankton biomass. Therefore, the study of phytoplankton community composition is essential from the standpoint of bivalve nutrition in shellfish production areas.

Currently, there is an increasing interest in developing offshore aquaculture in regions where sheltered coastal areas are scarce or sustain activities incompatible with aquaculture (Azpeitia *et al.* 2016). This interest prompted the installation of an experimental bivalve farm in open waters off the Basque coast (southeastern Bay of Biscay). However, temporal variability of phytoplankton nutritional attributes and their relationships with environmental conditions need further investigation. It is widely recognised that both top down regulation, such as grazing (Burkill *et al.* 1987), and bottom up processes driven by meteorological and hydrographic factors play a major role in the control and dynamics of phytoplankton populations (Smayda 1998; Nogueira *et al.* 2000).

The Bay of Biscay is located at mid-latitude of the Northeast Atlantic Ocean and thus, here, the annual cycle corresponds to that of temperate sea areas. Winter is characterised by water column mixing, which is generated by a combination of cooling, turbulence and downwelling. This mixing process modifies the properties of the upper waters and leads to great nutrient input from deep waters to the surface. In spring, solar irradiance heats the surface resulting in an increase in the temperature of these waters and a relative stabilisation. However, the stratification of the water column depends also on the relaxation of wind, turbulence and downwelling. Summer is characterised by stratification resulting from greater solar irradiance. Finally, during autumn the surface waters cool down and the southerly and westerly winds prevail, resulting in the mixing of the water column (Valencia *et al.* 2004; Fontán *et al.* 2008).

Many studies worldwide have highlighted the seasonal periodicity of phytoplankton assemblages linked to seasonal variations in physical forcing of mixing dynamics, temperature and light regime (Diehl 2002; Diehl *et al.* 2002). In the Bay of Biscay in particular, according to the seasonal cycle of hydrographic conditions, phytoplankton biomass shows two main periods related to two main events: winter mixing and summer stratification (Varela 1996; Valdés & Moral 1998). On the one hand, the nutrient input caused by the winter mixing leads to favourable conditions for the proliferation of the phytoplankton community and, thus, biomass peaks are usually recorded during late winter and spring. On the other hand, heating of the surface waters during summer leads to a stratified water column. The thermocline acts as a physical barrier that prevents the supply of nutrients, and phytoplankton production and biomass show the lowest values (Fernandez & Bode 1991; Varela 1996; Calvo-Díaz *et al.* 2008).

Although previous studies on phytoplankton communities have been carried out in the southern Bay of Biscay (Bode & Fernández 1992; Fernández & Bode 1994; Varela 1996) and, in particular, in the Basque coast (Estrada 1982; Garmendia *et al.* 2011; Muñiz *et al.* 2017), further research is needed. The relevance of the present study is based on the inclusion of novel issues, such as the importance of phytoplankton community composition as a food resource for bivalves in waters off the Basque coast, which was not addressed before, and the variability throughout the water column, since most of the previous studies were limited to surface waters.

In this context, our study aims to evaluate for the first time the implication of phytoplankton community as a food resource for bivalves within an experimental aquaculture farm. Recent studies developed in that experimental site indicate that mussels present good growth rates, biometry and nutritional quality (Azpeitia *et al.* 2016; 2017). Although chlorophyll values in the area are known to be relatively low (Estrada 1982; Revilla *et al.* 2009; Garmendia *et al.* 2011), we hypothesise that the composition and contribution of the different major taxonomic groups could be favourable for bivalve growth. To this end, we examined phytoplankton community composition, abundance and biomass, as well as environmental conditions, throughout the whole water column from May 2014 to June 2015. Since the period of study covered more than one year, a complete seasonal cycle was investigated.

2. Materials and methods

2.1. Study area

The Basque coast extends 100 km along the Cantabrian Sea (southeastern Bay of Biscay) (Figure 4.1). The climate of the area is rainy, temperate and oceanic, with moderate winters and warm summers (Fontán *et al.* 2009). The Basque coast can be described as a littoral coast exposed to waves, mostly formed of cliffs and influenced by 12 short rivers. Although no large coastal plumes are formed (Diez *et al.* 2000), this freshwater supply modifies the chemical composition of the shelf waters and often leads to increased nutrient levels in inner shelf waters (Valencia *et al.* 2004; Ferrer *et al.* 2009).

Field samplings were carried out at a station (43° 21,411’ N; 2° 26,918’ W) immediately outside an experimental bivalve farm located at 2 nautical miles off the Basque coast, at a depth of approximately 45 m. The experimental farm used a longline system, based on a subsurface structure, from which bivalve ropes and lanterns were suspended. In particular, the installation

consisted of three longlines, occupying a total area of 1 ha. Each longline sustained 100 vertical hanging ropes. The organisms cultured at the farm during the study were mainly mussels (*Mytilus galloprovincialis*) and, to a lesser extent, oysters (*Crassostrea gigas* and *Ostrea edulis*).

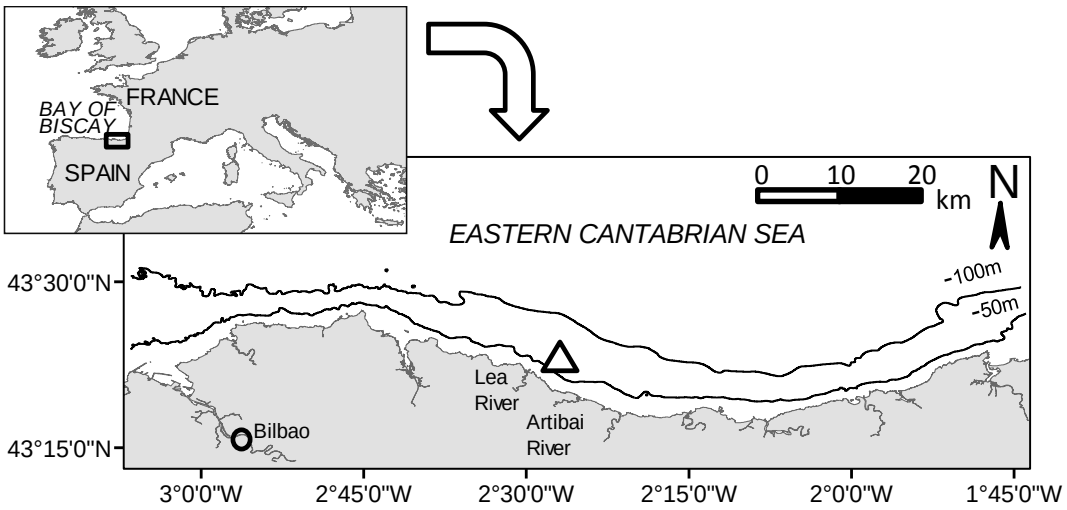

Figure 4.1. Map of the study area. The triangle shows the location of the experimental bivalve farm.

2.2. Sampling/laboratory strategy and data acquisition

Samplings took place from May 2014 to June 2015. CTD (conductivity, temperature and depth device) casts and Secchi disc measurements were usually performed twice per month, whereas water samples were collected monthly, except for February when sampling could not be carried out due to meteorological conditions.

In the field, a Seabird25 CTD was employed for the measurement of temperature, salinity, chlorophyll *a* and photosynthetically active radiation (PAR) at every meter of the water column. The Secchi disc depth was measured as an indicator of the water transparency. Water samples were collected using Niskin bottles at six discrete depths throughout the water column: 3, 10, 17, 24, 33 and 42 m.

Water samples were used for the analysis of nutrients and fractionated chlorophyll *a*, as well as phytoplankton identification and counting. Inorganic nutrients (ammonium, nitrite, nitrate, silicate and phosphate) were measured using a continuous-flow autoanalyzer (Bran + Luebbe Autoanalyzer 3, Norderstedt, Germany), according to colorimetric methods described in Grasshoff *et al*. (1983).

In order to obtain the concentrations of the different chlorophyll *a* fractions, sequential filtrations were performed. Three size fractions were differentiated: smaller than 3 µm, between 3 and 20 µm, and larger than 20 µm, to quantify the chlorophyll contained in the pico-, nano- and microphytoplankton. Whatman Nuclepore track-etched membrane filters (pore size 3 and 20 µm) and Whatman GF/F glass microfiber filters were used, diameter 47 mm. Firstly, approximately 4.5 L of water was filtered through the polycarbonate 20 µm filter to retain the largest fraction. Then, the filtrate was passed through the polycarbonate 3 µm pore size filter to obtain the 3–20 µm fraction. Finally, a final filtration was undertaken using the Whatman GF/F filter to retain the smallest fraction. The nominal pore size of GF/F filters is 0.7 µm, but the effective pore size of the

glass-fibre filters is substantially smaller (Sheldon 1972) and these are routinely used for picophytoplankton (Morán *et al.* 1999). Pigments were extracted in 10 ml of 90% acetone for 48 h in dark and cold conditions. The absorbance of the extract was measured using a UV-visible spectrophotometer (UV-2401PC Spectrophotometer, Shimadzu Corporation, Kyoto, Japan). The chlorophyll concentration was estimated according to the equations of Jeffrey and Humphrey (1975). The sum of the three fractions was used to determine if the total chlorophyll concentration was above 0.5 µg L^{-1}; this is the established threshold below which bivalves do not filter (Dolmer 2000; Riisgård 2001; Riisgård *et al.* 2011).

Phytoplankton identification and counting was conducted for three depths: 3, 17 and 33 m. Samples were preserved immediately after collection with acidic Lugol's solution (0.4% v/v) and maintained in 125-mL borosilicate bottles under dark and cool conditions (4 °C) until analysis. Taxonomic identification and cell counting were performed on subsamples of 50 mL, following the Utermöhl method (Utermöhl 1958; Hasle 1978; Edler & Elbrächter 2010) under a Nikon diaphot TMD inverted microscope. Depending on the organism size, 100× or 400× magnification was used; the detection limit of microscope counts for microplankton organisms was 20 cells L^{-1}. Small nanophytoplankton cells that could not be assigned to any taxonomic group were assigned to a group named "unidentified forms <10 µm". The minimum cell size that could be detected was 2–3 µm; therefore, picophytoplankton could not be identified and counted.

Three variables were used to describe hydrographic conditions: light extinction coefficient, depth of the photic zone and river flow. Light extinction coefficient (*k*) was estimated from the PAR measured by the CTD using the equation derived from the Beer-Lambert law:

$$I_z = I_f \cdot e^{-kz}$$

where I_z (E m^{-2} d^{-1}) is the radiation received at a specific depth, I_f is the radiation right below the surface, and z is the specific depth (m).

The *k* was then used to calculate the depth of the photic layer using the following equation: photic zone (m) = 4.605/*k*. Information on the flow rate of one of the rivers closest to the experimental site, Artibai river (Figure 4.1), was obtained from a regional website ("Diputación Foral de Bizkaia", http://www.bizkaia.eus). Information on the other river surrounding the farm, Lea river, was not included due to missing data on the time series. To account for a delay in the influence of river flow on the water column conditions, flow rates were averaged for the seven days prior to the sampling day.

2.3. Data analysis

The variability of temperature and salinity was represented using a temperature-salinity (TS) diagram. The temporal variation of chlorophyll *a* throughout the water column (up to 45 m depth) was presented as a contour map.

Regarding phytoplankton data, the species list was standardised prior to statistical analysis according to AlgaeBase (Guiry & Guiry 2015). The phytoplankton community was analysed according to cell concentration (cell L^{-1}) and biomass (µg C L^{-1}). In order to calculate the latter, the biovolume of each taxon was first calculated from its equivalent spherical diameter (ESD) using the equation of the sphere's volume. Information on phytoplankton cell size was collected from

two sources: i) the ESD measured in phytoplankton species from the northwest Spanish coast by investigators from other institutions (M. Huete from the Spanish Institute of Oceanography - A Coruña Centre, and M. Varela, L. Mene and J. Lorenzo from the University of Vigo) and ii) the report by Olenina *et al*. (2004). Then, biomass was determined using the equation reported by Montagnes *et al.* (1994) for marine phytoplankton: $Biomass = 0.109 \times Volume^{0.991}$, where *Biomass* is expressed in pg C $cell^{-1}$ and *Volume* is expressed in μm^3. For the data analyses, the specific results on abundance and biomass were combined to obtain total data for the following groups: chlorophytes, kleptoplastic ciliates (*Mesodinium* spp.), cryptophytes, diatoms, dinoflagellates, euglenophytes, haptophytes, ochrophytes (chrysophyceans, dictyochophyceans, raphidophyceans and xanthophyceans), heterotrophic nanoflagellates (including the taxa *Ebria tripartita*, *Katablepharis remigera*, *Leucocryptos* sp. and *Telonema* sp., traditionally considered in phytoplankton studies) and unidentified forms <10 µm. For the description of phytoplankton abundance and biomass, some of these groups were merged into a group called "others". This group was primarily comprised of unidentified forms, but also included the following minority groups (i.e. those contributing less than 6.5% to total abundance and biomass): chlorophytes, euglenophytes, ochrophytes and heterotrophic nanoflagellates.

For the study of relationships between the environment and phytoplankton community, exploratory analysis was conducted by means of biplots representing environmental variables against phytoplankton. Correlation matrices (Pearson correlation coefficient, alpha = 0.05) were also performed. Two separate analyses were undertaken: one for abundance of phytoplankton groups and a second one for chlorophyll *a* fractions, as a proxy for phytoplankton biomass. The group "unidentified forms" was excluded from the correlation analysis due to its heterogeneity.

Among the environmental variables, only those that *a priori* could be considered most explanatory of phytoplankton variability were included in the analysis, namely Secchi disc depth, light extinction coefficient, temperature, salinity, Artibai river flow and nutrient concentration (ammonium, nitrite, nitrate, phosphate and silicate). Environmental variables were previously transformed in order to attain a distribution close to normal.

Phytoplankton data were also pre-treated. Prior to analysis, phytoplankton rare taxa, defined here as those occurring in less than 10% of the samples, were removed to avoid noise in the data (Austin & Greig-Smith 1968). A total of 78 of the 194 taxa were excluded from the analysis. Phytoplankton abundance data were log-transformed (after adding one to avoid taking the log of zero values) and relationships with the environment were studied at depths of 3, 17 and 33 m.

Finally, chlorophyll *a* was also log-transformed prior to analysis and relationships between the three size fractions of chlorophyll and environmental variables were studied at depths of 3, 10, 17, 24, 33 and 42 m.

In ecological research, when multiple statistical tests are undertaken, each at the same significance level (alpha), the probability of achieving at least one significant result is greater than that significance level. In this context, to avoid a "Type I" error, one strategy is to correct the alpha level when performing multiple tests. The most well-known correction is called Bonferroni correction; in this study, Bonferroni sequential correction, described by Holm (1979), was applied. Statgraphics Centurion XVI software was used for the correlation matrices.

3. Results

3.1. Hydrographic, physico-chemical conditions and bulk chlorophyll a

The TS diagram shows the prevalence of thermohaline stratification due to spring warming and the presence of waters of continental origin in May 2014 (Figure 4.2). The thermal stratification prevailed from June to October in relation to the progression of the summertime warming. Moreover, more or less extended haline stratification was present throughout this period. In November, a reduction of the vertical gradients of temperature and salinity was observed induced by vertical mixing and cooling. December was characterised by thermohaline homogeneity of the water column and, more importantly, by high water column temperatures (above 16 °C) associated with extremely warm conditions of the previous months. Conversely, in January 2015, the entire water column cooled due to extremely cold winter months. This change, together with high precipitation, resulted in the prevalence of haline stratification and thermal inversion in January. The haline stratification was especially enhanced in March and April. Again, the thermal stratification was observed in May and June, induced by an extremely warm spring in 2015. Again, relatively strong haline stratification could be observed in spring 2015 (additional information on temporal and vertical variability of both temperature and salinity can be found in Chapter 5, Section 3.1, Figure 5.3).

Overall, relatively strong thermohaline stratification could be observed throughout the period, with a few exceptions in November-December 2014 and January-April 2015 where homogeneity and haline stratification of the water column prevailed, respectively. Additional information on river flows is included in Annex 4.1.

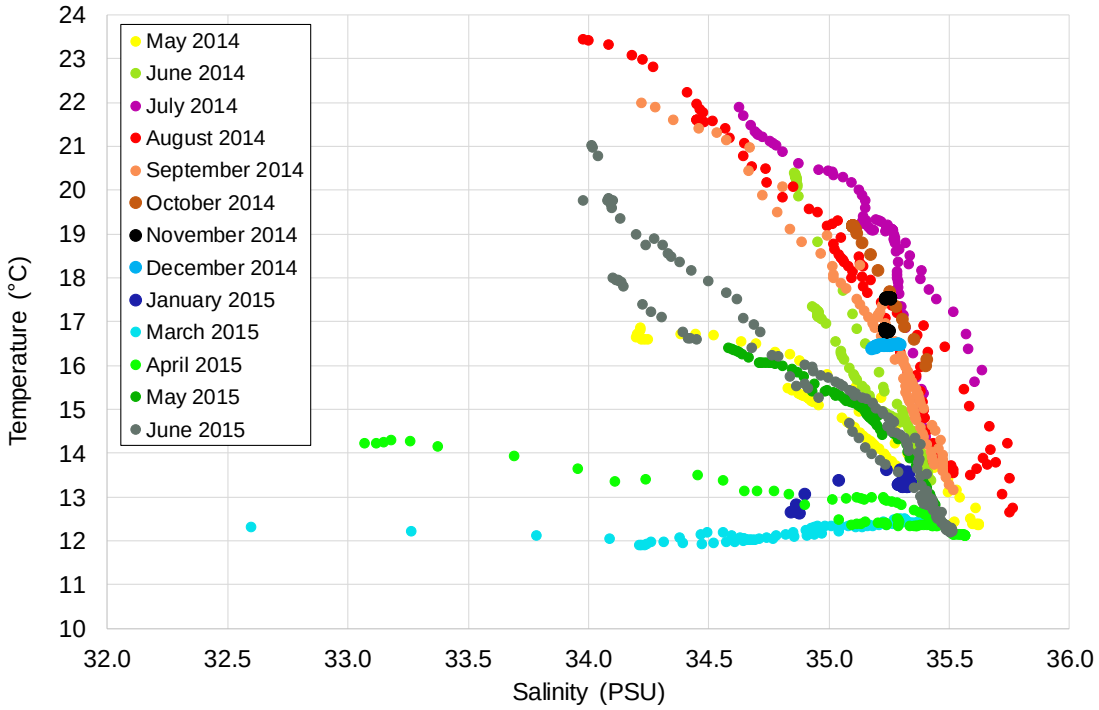

Figure 4.2. TS diagram of the waters off the Basque coast, in the SE Bay of Biscay, from May 2014 to June 2015.

Chlorophyll *a* (obtained from the fluorescence measured by the CTD) showed several peaks during the study period (Chapter 5, Section 3.1, Figure 5.3). At the end of May and beginning of June 2014, two deep chlorophyll peaks were observed at depths of 34 and 41 m, respectively, with

values between 1.6 and 2.0 µg L^{-1}. Three other sub-surface chlorophyll increases were then detected at the end of July, beginning of August and mid-September, ranging from 1.1 to 1.4 µg L^{-1}. These were followed by a period with low values (<0.8 µg L^{-1}) from October to March. The maximum value reported was detected in March at approximately 12 m depth, reaching 2.6 µg L^{-1}. In April, a surface peak was observed (2 µg L^{-1}). Subsequently, chlorophyll concentrations decreased reaching the lowest surface values during spring 2015, although higher concentrations were detected around 20-35 m depth in June.

Table 4.1 shows the mean values and standard deviations of the parameters relating to the physico-chemical conditions of the study area at the different depths studied and for the whole water column. Secchi disc depth annual mean value was 11 m. Mean light extinction coefficient (*k*) was 0.1 m^{-1}. Photic layer depth had a mean value of 43.7 m. Mean temperature values for each depth ranged from 17.4 to 14.3 °C, showing a decreasing trend from the surface to the deeper waters. In contrast, salinity increased towards the deeper water, with mean values ranging from 34.5 to 35.4 PSU. The mean chlorophyll concentrations measured by the CTD were very similar between the six depths, approximately 0.6–0.7 µg L^{-1}. The concentration of several inorganic nutrients did not present great dissimilarities between the mean values of the different sampled depths, showing ranges of 1.4–1.8 µmol L^{-1} (ammonium), 0.3–0.4 µmol L^{-1} (nitrite), 0.2–0.3 µmol L^{-1} (phosphate) and 0.9–1.5 µmol L^{-1} (silicate). However, nitrate concentration varied more throughout the water column, with mean values close to 1 µmol L^{-1} within the shallower and intermediate layers (3, 10, 17 and 24 m) to a maximum of 3.0 µmol L^{-1} at 42 m depth (additional information on nutrient concentrations is shown in Chapter 5, Section 3.1, Figure 5.5).

3.2. Phytoplankton composition, abundance and biomass

With regard to phytoplankton richness, a total of 194 phytoplankton taxa were identified during these surveys. Dinoflagellates and diatoms represented the most diverse groups, comprising 47.4% and 35.1% of the total taxa described, respectively.

Phytoplankton total abundance ranged from 3.4×10^4 cells L^{-1} to 5.1×10^6 cells L^{-1}. Differences were found in relation to the different taxonomic groups. Putting aside the group of "unidentified forms", which in several samplings was the most abundant due to its heterogeneity, haptophytes were the most abundant group in 46% of the samples, followed by dinoflagellates (26%), cryptophytes (15%) and diatoms (13%).

The phytoplankton community differed in composition as well as in total cell density between the three sampled depths (Figure 4.3). Firstly, the depth of 3 m, where the highest abundance values were found, showed a maximum of approximately 5×10^6 cells L^{-1} in May 2014 (Figure 4.3a), which was characterised by a large proportion of the group called "others" (i.e., chlorophytes, euglenophytes, ochrophytes, unidentified forms and heterotrophic nanoflagellates). During June and July, the abundance at 3 m depth dropped to just over half of that registered in May, followed by a period of low densities from August 2014 to January 2015, ranging from 1.8×10^5 to 5.0×10^5 cells L^{-1}. The end of the study period was characterised by a peak that was first dominated by diatoms, contributing to more than 50% of the total abundance in March 2015, followed by an increase of the haptophyte community representing 60% of the total abundance in April 2015. The maximum abundance value in that peak (2.8×10^6 cells L^{-1}) occurred in April 2015.

Similarly, at the intermediate depth (17 m) the highest cell densities were found at the beginning of the study period, from May to July 2014 (Figure 4.3b). However, here maximum values were much lower compared to those at the 3 m depth, with the highest value of 1.3×10^6 cells L^{-1} occurring in July. This peak was dominated by the group "others". Two more increases in abundance were detected in October 2014 and April 2015, with very low values during the intervening period. The three peaks observed at 17 m depth involved an important contribution from the haptophytes, ranging from 40% to 47% of the total abundance. Dinoflagellates also showed increased presence during these three reported peaks.

The greatest depth (33 m) produced the lowest total abundance values, with a maximum of approximately 8.4×10^5 cells L^{-1} (Figure 4.3c). The cell density increases observed in July and October 2014 were concurrent with the first two peaks observed at the 17 m depth. Very low abundances were registered from December 2014 to May 2015, between 1.1×10^5 and 1.4×10^5 cells L^{-1}, followed by a six-fold increase in June 2015. As with the intermediate depth (17 m), dinoflagellate abundance slightly increased during the peaks.

Table 4.1. Description (mean values and standard deviations) of the water column conditions in a bivalve culture experimental site off the Basque coast for the period May 2014–June 2015. Water-column weighted mean values, as well as the values for the six discrete sampled depths, are shown. *k*: light extinction coefficient; Chl *a* CTD: chlorophyll *a* obtained from the fluorescence measured by the CTD.

	Mean ± SD						
Variable	**Water column**	**3 m**	**10 m**	**17 m**	**24 m**	**33 m**	**42 m**
Secchi disc depth (m)	11.0 ± 3.5	-	-	-	-	-	-
k (m^{-1})	0.1 ± 0.0	-	-	-	-	-	-
Photic layer depth	43.7 ± 9.3	-	-	-	-	-	-
Temperature (°C)	15.6 ± 2.6	17.5 ± 3.3	16.8 ± 2.7	16.1 ± 2.4	15.4 ± 2.1	14.7 ± 2.0	13.9 ± 1.7
Salinity	35.1 ± 0.4	34.6 ± 0.5	34.9 ± 0.3	35.1 ± 0.2	35.2 ± 0.1	35.3 ± 0.1	35.4 ± 0.1
Chl *a* CTD (µg L^{-1})	0.6 ± 0.4	0.6 ± 0.5	0.6 ± 0.6	0.7 ± 0.5	0.7 ± 0.3	0.7 ± 0.4	0.6 ± 0.3
Ammonium (µM)	1.5 ± 0.6	1.4 ± 0.7	1.4 ± 0.7	1.8 ± 0.9	1.7 ± 1.2	1.4 ± 0.5	1.4 ± 0.8
Nitrite (µM)	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.3	0.4 ± 0.2	0.4 ± 0.2
Nitrate (µM)	1.4 ± 1.5	1.0 ± 1.7	0.9 ± 1.6	0.9 ± 1.3	1.1 ± 1.4	1.9 ± 2.2	3.0 ± 2.6
Phosphate (µM)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Silicate (µM)	1.1 ± 0.6	1.5 ± 1.1	1.2 ± 0.9	1.0 ± 0.6	0.9 ± 0.5	1.1 ± 0.7	1.5 ± 0.8

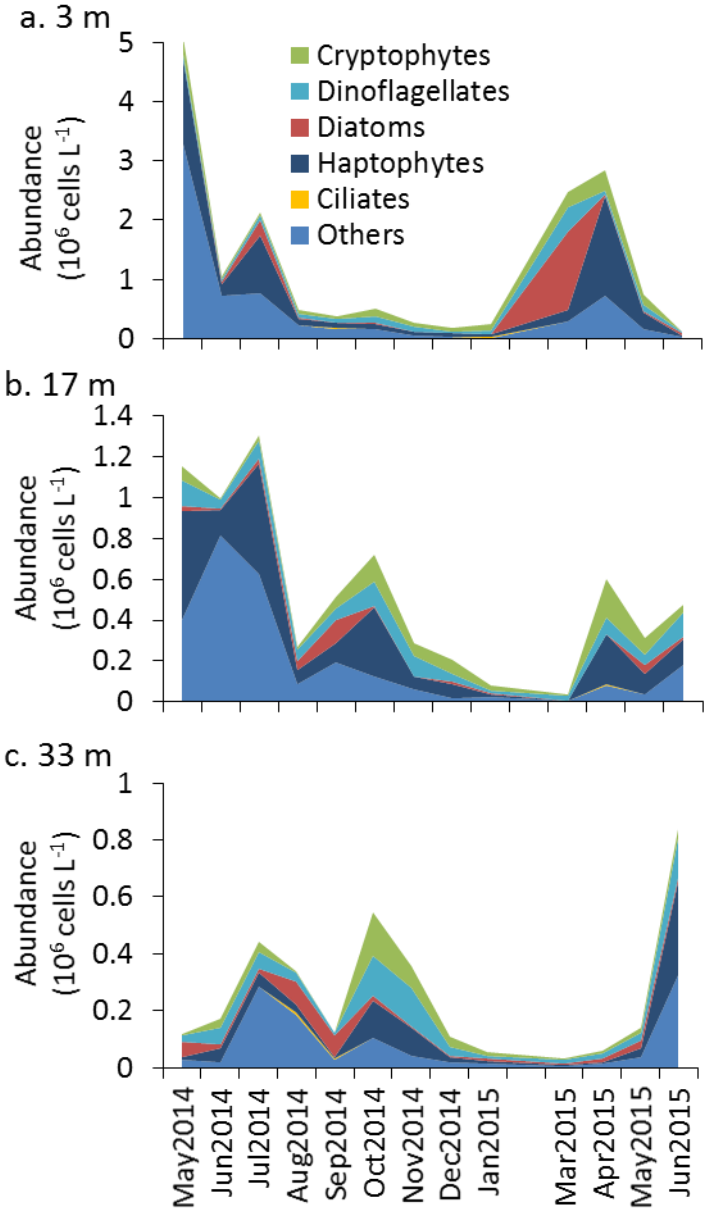

Figure 4.3. Contribution of each of the major phytoplankton groups to the total abundance per sample at three different depths (3, 17 and 33 m). The group "others" consisted of chlorophytes, euglenophytes, ochrophytes, heterotrophic nanoflagellates and unidentified forms. Note that plots have different scaled y axes.

The comparison of biomass variability with abundance variability showed that most of the peaks or increases were in accordance with time and space (Figure 4.4). However, the greatest difference was the contribution of each of the groups.

The highest biomass values were observed at 3 m, with a maximum of 435 µg C L^{-1}. The contribution of the different phytoplankton groups to the peaks of May and July 2014 was similar compared to abundance values, being dominated by the groups "others" and haptophytes. Nevertheless, from December 2014 to March 2015 diatoms dominated the community, representing between 54% and 78% of the total biomass (Figure 4.4a).

At the intermediate depth (17 m), biomass values were notably lower than at the 3 m depth, ranging from 9 to 104 µg C L^{-1} (Figure 4.4b). Similar to the shallower depth studied, diatoms were the dominant group from December 2014 to March 2015 (44–79% of the total biomass). The

occasional and significant contribution of ciliates (represented by the genus *Mesodinium*) during the peak of April 2015 was also notable, representing 30% of the total biomass. Dinoflagellates gained importance during the biomass increases, especially in September when they represented 33% of the total biomass.

Finally, the range of biomass values at the 33 m depth was similar to that at 17 m, with the exception of the occurrence of a larger peak which reached 153 µg C L^{-1} in May 2014 (Figure 4.4c). Diatoms dominated the community in May and June 2014 and from January to May 2015, representing 74–95% of the total biomass. In August 2014, ciliates contributed 44% of the total biomass.

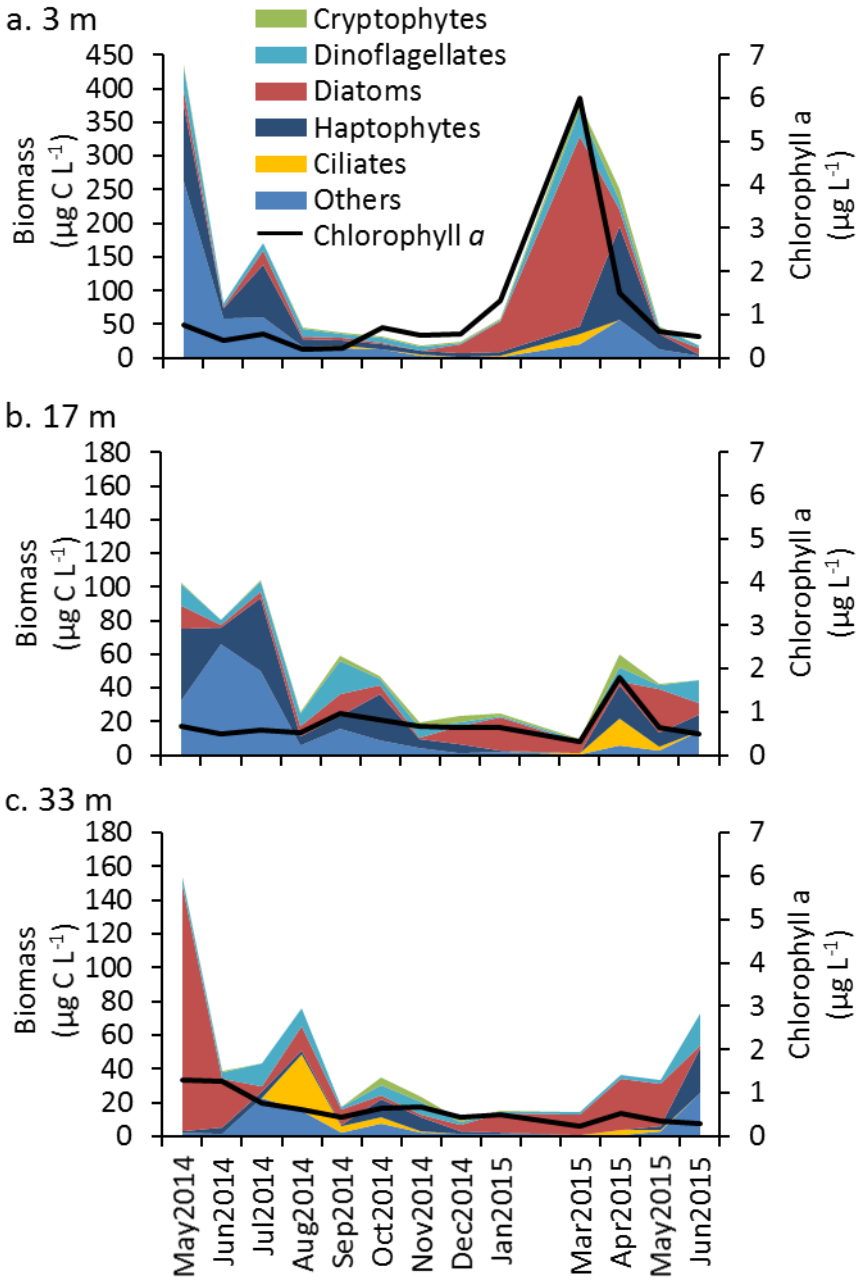

Figure 4.4. Contribution of each of the major phytoplankton groups to the total biomass per sample at three different depths. The group "others" consisted of chlorophytes, euglenophytes, ochrophytes, heterotrophic nanoflagellates and unidentified forms. The black line represents total chlorophyll *a* concentration (µg L^{-1}) obtained by means of chemical analysis (right axis).

3.2. Size-fractionated chlorophyll a

The relative contribution of the three chlorophyll size fractions was studied at six depths (Figure 4.5). Overall, the picophytoplankton made the greatest contribution. However, an increase in the nanophytoplankton was observed towards the greatest depths (33 and 42 m). Results for May 2014 at the 33 m depth were remarkable, with 58% of the total chlorophyll provided by the microphytoplankton (Figure 4.5e).

Total chlorophyll *a* concentrations, estimated from the sum of the three size fractions studied, showed values lower than 1 µg L^{-1} in most of the samples. The highest concentrations were observed in March 2015, with approximately 6 µg L^{-1} at the 3 m depth and 2.5 µg L^{-1} at the 10 m depth, although values were still low in deeper samples. One month later, in April 2015, secondary peaks were found at depths of 3 to 24 m. Similar peaks were also detected in late spring 2014 at depths of 33 m and 42 m.

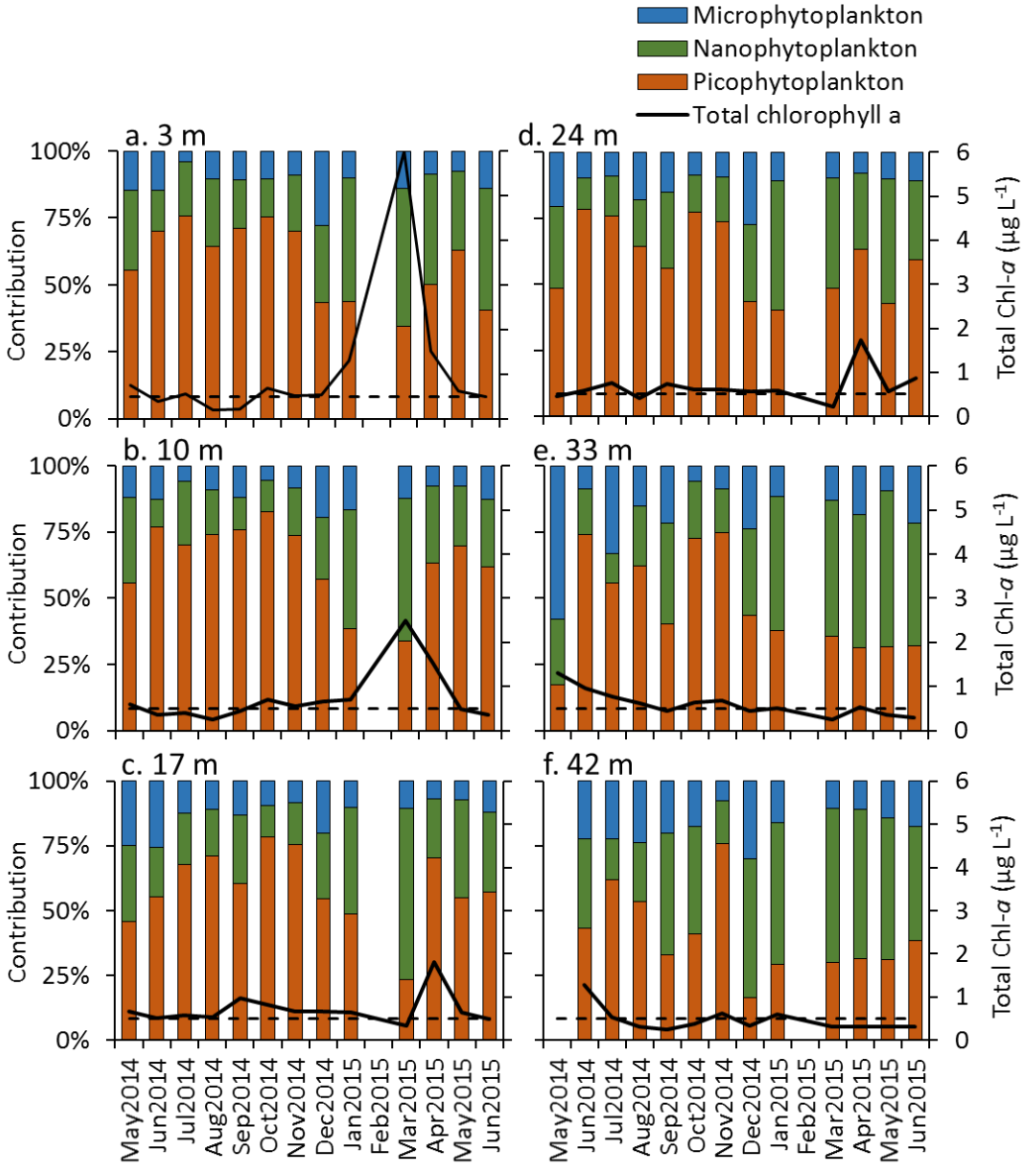

Figure 4.5. Chlorophyll *a* size fraction (<3 µm, 3–20 µm and >20 µm; i.e. pico-, nano- and micro-phytoplankton, respectively) contribution at the six depths for the period May 2014 to June 2015. Total chlorophyll *a* (sum of fractions) is shown on the right axis. The dotted line shows the chlorophyll threshold below which mussels do not filter (Dolmer 2000; Riisgård 2001; Riisgård *et al.* 2011). This threshold should be viewed with caution since it was not developed for open waters (see Discussion).

Total chlorophyll concentration was above the 0.5 µg L^{-1} threshold in 62% of the samples. The depth of 42 m showed the highest proportion of values below that value (67% of the samples). Overall, chlorophyll concentrations below 0.5 µg L^{-1} were found during the summer.

3.3. Relationship between environmental variables and phytoplankton community

Several strong linear relationships were found between some environmental variables and both phytoplankton abundance and chlorophyll *a* measured in the laboratory (as a proxy for phytoplankton biomass).

Firstly, the relationships between environment and abundance of phytoplankton groups at each depth were studied (Table 4.2). Biplots for each significant correlation are shown in Annex 4.2.

At 3 m, total abundance of phytoplankton was not significantly correlated with any environmental variable. Some of the minor groups, such as chlorophytes and heterotrophic nanoflagellates, showed inverse correlations with different environmental variables. Ciliates (*Mesodinium* spp.) appeared to reach higher abundance at higher values of light extinction coefficient.

At a depth of 17 m, overall, nutrients were the main variable which significantly explained variability in phytoplankton abundance. Ammonium concentration significantly explained the variability of chlorophytes and dinoflagellates, showing a direct correlation. Nitrate showed a strong inverse relationship with total abundance of phytoplankton and, in particular, with dinoflagellates and haptophytes. Finally, silicate partly explained the variability of heterotrophic nanoflagellate abundance (inverse correlation). In addition, Artibai river flow showed inverse correlation with total abundance.

Finally, the greatest number of significant linear correlations was found at a depth of 33 m. However, some of these correlations should be viewed with caution since there were several ‘zero’ values in the dependent variable. Similar to the pattern observed at the 17 m depth, Artibai river flow showed inverse correlation with total abundance of phytoplankton and, in particular, with diatom abundance. Cryptophytes showed greater abundance at higher temperature and lower salinity.

Table 4.2. Significant correlations (alpha = 0.05, adjusted by sequential Bonferroni correction) between abundance of major phytoplankton groups and environmental variables at depths of 3 m, 17 m and 33 m. The Pearson coefficient (*r*) and the probability (*p*) are shown. *k*: light extinction coefficient estimated for the whole water column. Correlations with an * should be viewed with caution since there were several 'zero' values in the dependent variable.

Depth	Phytoplankton abundance	Environmental variable	*r*	*p*
3 m	Chlorophytes	Temperature	-0.5536	0.0497
	Ciliates	*k*	0.6862	0.0096*
	Heterotrophic nanoflagellates.	Nitrate	-0.7448	0.0035
		Phosphate	-0.6378	0.0190
17 m	Chlorophytes	Ammonium	0.6207	0.0236
	Dinoflagellates	Ammonium	0.6581	0.0145
		Nitrate	-0.6284	0.0214
	Haptophytes	Nitrate	-0.6512	0.0159
	Heterotrophic nanofl.	Silicate	-0.5589	0.0471
	Total abundance	Artibai flow	-0.6840	0.0099
		Nitrate	-0.7486	0.0032
33 m	Chlorophytes	Phosphate	0.7494	0.0032*
	Cryptophytes	Temperature	0.6881	0.0093
		Salinity	-0.7552	0.0028
	Diatoms	Artibai flow	-0.6867	0.0095
	Euglenophytes	Nitrite	-0.6865	0.0095*
	Heterotrophic nanoflagellates	*k*	-0.6442	0.0175*
		Ammonium	0.7053	0.0071*
	Ochrophytes	Secchi disc depth	0.5895	0.0340*
	Total abundance	Artibai flow	-0.5702	0.0419

Similarly, relationships between environment and different chlorophyll size fractions were ascertained at six depths: 3, 10, 17, 24, 33 and 42 m (Table 4.3). Biplots for each significant correlation are shown in Annex 4.3 to Annex 4.8.

At the 3 m depth, temperature, nitrate and silicate concentration were the variables explaining most of the variability of the different chlorophyll fractions: higher chlorophyll values were found at lower temperatures and higher nitrate concentrations. Higher concentrations of the chlorophyll fraction of 3–20 µm were found at lower Secchi disc depths and at higher silicate concentrations. Similar results were obtained at the 10 m depth: higher chlorophyll values were observed at lower temperatures and at higher nitrate and silicate concentrations. In addition, the chlorophyll fraction of 3–20 µm was associated with lower Secchi disc depths, whereas the larger fraction (>20 µm) was directly related to Artibai river flow.

At the 17 m depth, variability of chlorophyll was explained to a lesser extent by environmental variables compared to the shallower depths. Only the chlorophyll fraction of 3–20 µm showed significant correlation with the environment, with higher values at lower temperatures and higher silicate concentrations. At the 24 m depth, silicate was the only variable explaining chlorophyll variability: the 3–20 µm fraction was directly correlated with silicate concentration.

At the 33 m depth, temperature, nitrate and silicate concentrations significantly explained the variability of the small chlorophyll fraction, but with the opposite pattern to that observed at 3,

10, 17 and 24 m: higher chlorophyll values were found at higher temperatures and lower nutrient concentrations. The large fraction (>20 µm) was directly related to salinity. Finally, at the 42 m depth, the small (<3 µm) and large (>20 µm) chlorophyll fractions were inversely correlated with silicate concentration. Higher concentrations of the intermediate chlorophyll fraction (3–20 µm) were found at lower Secchi disc depths. In contrast, the large fraction presented lower values as the light extinction coefficient increased.

Table 4.3. Significant correlations (alpha = 0.05, adjusted by sequential Bonferroni correction) between chlorophyll *a* size fractions analysed at the laboratory and environmental variables at depths of 3, 10, 17, 24, 33 and 42 m. The Pearson coefficient (*r*) and the probability (*p*) are shown. *k*: light extinction coefficient estimated for the whole water column.

Depth	Chlorophyll *a*	Environmental variable	*r*	*p*
3 m	Chl <3µm	Temperature	-0.8327	0.0004
		Nitrate	0.6206	0.0236
	Chl 3–20µm	Secchi disc depth	-0.7595	0.0026
		Temperature	-0.8636	0.0001
		Nitrate	0.7832	0.0015
		Silicate	0.8386	0.0003
	Chl >20µm	Temperature	-0.8141	0.0007
		Nitrate	0.8057	0.0009
		Silicate	0.6960	0.0082
10 m	Chl <3µm	Temperature	-0.6544	0.0152
	Chl 3–20µm	Secchi disc depth	-0.7959	0.0011
		Temperature	-0.8697	0.0001
		Nitrate	0.7850	0.0015
		Silicate	0.8986	0.0000
	Chl >20µm	Temperature	-0.8636	0.0001
		Artibai flow	0.6871	0.0095
		Nitrate	0.8299	0.0004
		Silicate	0.8126	0.0007
17 m	Chl 3–20µm	Temperature	-0.8005	0.0010
		Silicate	0.7352	0.0042
24 m	Chl 3–20µm	Silicate	0.7144	0.0061
33 m	Chl <3µm	Temperature	0.6766	0.0111
		Nitrate	-0.7180	0.0057
		Silicate	-0.8952	0.0000
	Chl >20µm	Salinity	0.6333	0.0201
42 m	Chl <3µm	Silicate	-0.7555	0.0045
	Chl 3–20µm	Secchi disc depth	-0.7470	0.0052
	Chl >20µm	*k*	-0.6623	0.0189
		Silicate	-0.6484	0.0226

4. Discussion

The study area showed the typical hydrographic conditions of temperate coastal zones (Mann & Lazier 1991), previously described for the Bay of Biscay (Varela 1996; Valdés & Moral 1998; Valencia *et al.* 2004). The sea surface appeared stratified during summer months, due to heating by solar irradiation (Varela 1996). Late autumn and winter were mostly characterised by vertical mixing, which might be generated by a combination of cooling, turbulence and downwelling processes (Valencia & Franco 2004; Valencia *et al.* 2004). The low surface salinity values observed in the present study were explained by river discharges mostly during late winter and spring.

Mixing processes are usually accompanied by changes in light and nutrient availability and, thus, growth performance of phytoplankton species within the water column is partly defined by vertical mixing (Diehl 2002; Huisman *et al.* 2004). In this study, a well-mixed homogeneous water column was observed in November, December and January, when phytoplankton abundance and biomass showed the lowest values or a decreasing trend. As described by Fernandez and Bode (1991), during this period, although an upward flux of nutrients from deep water layers occurs as a consequence of the mixing, phytoplankton biomass is expected to be low due to limited light. From January onwards, surface phytoplankton abundance and biomass, as well as chlorophyll concentration, started to increase. This increase notably coincided with nutrient input, reaching a maximum in March-April. In particular, these peaks in surface waters were characterised by a high contribution of diatoms, as shown in other late winter blooms previously described in the southern Bay of Biscay (Labry *et al.* 2001; Guillaud *et al.* 2008). This fact also agrees with Margalef (1978), who found that strong vertical mixing favours the dominance of diatoms. According to Margalef's mandala, dinoflagellates are expected to be favoured in stratified water columns, where they show competitive advantage over other groups based on their ability to swim to zones rich in light and nutrients (Margalef 1978). Here, a slight increase in the contribution of dinoflagellates was detected during August–September 2014, when the water column was stratified.

Among the studied environmental variables, temperature and nutrients (mostly nitrate and silicate) seemed to be the variables that explained most of chlorophyll annual variability. The results at depths of 3 m and 10 m coincided with the winter conditions, when deeper cold and nutrient-rich water is mixed with surface waters leading to increase in phytoplankton biomass (Varela 1996; Valdés & Moral 1998). According to this, the observed chlorophyll peak at these depths in March 2015 might be explained by the contemporaneous increase in nitrate and silicate concentrations and low temperatures.

In contrast, different results were obtained for phytoplankton abundance. Neither temperature nor silicate explained the variability in total abundance. Among the significant correlations between environmental variables and abundance of phytoplankton groups, the fewest number of correlations was found at the 3 m depth. In fact, previously it has been found that environmental variables explained little about phytoplankton group variability (usually <16%, except in winter when this was 24%) in surface waters off the Basque coast, although the explained variability was higher at the species level (Muñiz *et al.* 2018). At 17 m, a reduced total abundance coincided with higher river flow and nitrate concentrations. Nitrate has been found to be linked to river discharges into the Basque coastal waters (Borja *et al.* 2016). This situation would

reflect winter conditions, when river flows are high and phytoplankton abundance is low. Indeed, the low abundance of dinoflagellates during winter conditions (and its inverse relation with nitrate) is consistent again with the reported preference of this group for summer stratified waters. Variability in the abundance of dinoflagellates at 17 m was also explained by ammonium concentrations. This observed direct relationship is in accordance with the well-established concept that ammonium is the preferred nitrogen source for marine phytoplankton, with the exception of diatoms, that have shown higher nitrate uptake rates (Walsh & Dugdale 1971; Heil *et al.* 2007). Specifically in the case of dinoflagellates, Li *et al*. (2010) found higher acquisition of reduced forms of nitrogen, such as ammonium.

As mentioned before, the variability explained by the environment was different for chlorophyll concentration and for phytoplankton abundance. Although chlorophyll *a* has long been used as a proxy for phytoplankton biomass, it is well known that chlorophyll *a* concentration, phytoplankton biomass (in carbon units) and cell abundance are three different attributes of the phytoplankton community (Domingues *et al.* 2008). Therefore, different results can be expected from each of them. In the present study, marked differences were found between chlorophyll concentrations and biomass (determined from biovolumes and cell densities). It should be considered that there is an associated error when biomass is calculated from the ESD and the abundance. In addition, the ratio of carbon biomass to chlorophyll in the cell is highly variable, both at intra- and inter-specific levels, and also depending on environmental conditions, mainly light and nutrients (Taylor *et al.* 1997; Ríos *et al.* 1998; Domingues *et al.* 2008).

Overall, chlorophyll values were low compared to adjacent areas, such as the Atlantic French coast with median values from 1.2 to 3.2 µg L^{-1} (Fariñas *et al.* 2015); the euhaline zone of Basque estuaries with median values about 2 µg L^{-1} from spring to autumn (Garmendia *et al.* 2011), or the Galician Rias with values up to 20 µg L^{-1} (Varela *et al.* 2008). For two stations off the Basque coast located at a depth of nearly 50 m, similar to the one studied here, Estrada (1982) found similar results to the ones described above: overall, chlorophyll values ranged between 0 and 1 µg L^{-1} during the year, showing occasional peaks in the winter. In the present study, during most of the year phytoplankton biomass was dominated by picophytoplankton. However, at the time of maximum biomass, a relative decrease in the contribution of the smallest fraction compared to the larger ones could be noticed. This is in accordance with the findings by Calvo-Díaz *et al*. (2008) reported for the central Cantabrian Sea.

In relation to mussel filtration, not all of the seston is available as food for these bivalves. Although controversy still exists, it has been reported by some authors that mussels do not filter below a chlorophyll threshold of around 0.5 µg L^{-1} (Dolmer 2000; Riisgård 2001). This threshold should be viewed with caution since it was not developed for open waters. Although on some occasions chlorophyll concentrations were below this limit, the annual average value was slightly above this value. Nevertheless, despite chlorophyll concentrations being not very high in comparison to other areas where bivalve aquaculture has traditionally developed (Figueiras *et al.* 2002; Varela *et al.* 2008), it has previously been reported that mussels from the experimental site off the Basque coast show good growth and biochemical performance, with similar mean chlorophyll values to the ones described here (Azpeitia *et al.* 2016; 2017).

In addition, the dominance of the diatoms during spring peaks in biomass, together with the relevant contribution of dinoflagellates to the sub-superficial abundance and biomass, suggests favourable conditions for mussel culture, since some of the important fatty acids for bivalve growth (EPA and DHA) are known to be synthesised by these two groups (e.g., Azpeitia *et al*. 2016). Experiments on mussel nutrition, in terms of carbon biomass, have also shown highest retention of diatoms and dinoflagellates, together with ciliates, compared to other phytoplankton groups (Trottet *et al.* 2008). Moreover, direct correlations have been reported between diatoms and bivalve growth (Beukema & Cadée 1991; Weiss *et al.* 2007; Pernet *et al.* 2012; Wall *et al.* 2013). Thompson *et al*. (1993) found that diets containing high levels of saturated fats were more nutritious for oyster larvae. The observed high contribution of haptophytes also suggests favourable conditions for bivalve growth, since they have been reported to contain, on average, the highest proportion of saturated fats (33%), followed by diatoms (27%) (Volkman *et al.* 1989; Volkman *et al.* 1991). In this study, one genus of ciliates (*Mesodinium* spp.) and four taxa of heterotrophic nanoflagellates (*Ebria tripartita*, *Katablepharis remigera*, *Leucocryptos* sp. and *Telonema* sp.) were taken into account. However, for future studies it would be of interest to account for all the heterotrophs and ciliates, given their significant role as a food source for mussels (Trottet *et al.* 2008).

Some of the observed results from the water column, such as the higher phytoplankton abundance and biomass registered at shallower depths in comparison to the greater depths, suggest that bivalves would grow better in shallower waters. Furthermore, abundance and biomass of diatoms, dinoflagellates and haptophytes (i.e. the groups with the highest fatty acid content) were lower at the 33 m depth. In contrast, some subsurface chlorophyll maxima were found during the summer. Also, as previously mentioned, the chlorophyll size fractions above 3 µm (corresponding to nano- and micro-phytoplankton) appeared to increase slightly towards the greatest depths that were sampled. These size fractions are the ones of interest for the correct growth of bivalves as, although there is still considerable controversy, the majority of the studies indicate that the minimum particle size for efficient retention is 4 µm (Møhlenberg & Riisgård 1978; Riisgard 1988; Jørgensen 1990). Azpeitia *et al*. (2016) analysed mussels from the same experimental site to compare whether there were differences between two culture depths. They found significant differences between mussels cultured at 5 m and at 15 m in terms of dry weight, length, shell shape and density, but not for any of the biochemical parameters analysed, such as fatty acid content. They finally concluded that a depth difference of 10 m might not be sufficient to cause differences in product quality.

5. Conclusions

In summary, the water column conditions in open waters off the Basque coast are characterised by the classical seasonal cycle of temperate areas at mid-latitudes of the Northeast Atlantic. These hydrographic and environmental conditions influence to a great extent the phytoplankton community in terms of vertical distribution, composition and temporal variability. The overall phytoplankton community found throughout the water column in the experimental site seems to be suitable for bivalve aquaculture, based on the dominance of diatoms, dinoflagellates and haptophytes, and a chlorophyll concentration that was above the stablished threshold for bivalve filtration in most of the samples collected. Composition and contribution of the major groups are in accordance with the reported requirements for mussel growth. Although

chlorophyll values were found to be relatively low during some periods, this may not be a problem for the good performance of mussels, as other authors who found very similar average chlorophyll values have previously reported good growth and biochemical composition in mussels from the experimental site.

Chapter 5

Toxicity risk assessment in an experimental bivalve farm off the Basque coast: presence of toxins in mussels and potentially causative phytoplankton

Abstract

Chapter 5 presents a complete report on all the legislated toxins registered in mussels from the experimental bivalve farm off the Basque coast, together with probable causative phytoplankton species. During the study period (2014–2017), at least one toxin was above quantification limits in almost 60% of the cases (from a total of 39 sampling campaigns); however, only 15% would have implied a risk for human health if the shellfish were consumed, which would have resulted in a ban on mussel harvest. All these cases (i.e., concentrations above regulatory limits) were associated with lipophilic toxins, okadaic acid in particular, with *Dinophysis acuminata* as the causative species. The unique case of the amnesic toxin above the quantification limit coincided with a bloom of *Pseudo-nitzschia* spp.; which included low densities of *P. pungens.* PSP-toxins (paralytic shellfish poisoning-toxins) were quantified on three occasions, but only one occurrence could be associated with a causative phytoplankton species, tentatively *Gymnodinium catenatum.* Yessotoxins were quantified frequently, especially during 2016, with *Lingulodinium polyedra* and, to a lesser extent, *Protoceratium reticulatum* as the potential causative organisms. Other relevant potentially toxic taxa were recorded, such as *Azadinium* spp., *Karenia* spp. and *Prorocentrum cordatum*, although they did not seem to pose a threat for shellfish aquaculture or human health during the study period. The abundance trigger limits obtained from the literature for *Dinophysis, Pseudo-nitzschia* and *Alexandrium* cannot be recommended to predict toxic events in bivalves in this study area as they were not always directly related to the presence of toxins. No clear general pattern was found between all these species and the environmental conditions, however, the main abundance peaks for *Dinophysis acuminata* and *Pseudo-nitzschia* spp. always occurred in a very narrow range of both temperature and salinity. With all this, future recommendations would be to increase the sampling frequency in spring, when all the toxin events above the regulatory limit occurred, and to pay special attention to the temperature and salinity conditions all year round. Moreover, it would be of interest to include more environmental variables (such as turbulence or currents) that could allow prediction of toxic outbreaks and also to include brevetoxins in the routine monitoring analyses.

1. Introduction

Inasmuch as world population increases, natural fish stocks are gradually depleted and fisheries capture falls short of world demand. Annual consumption of seafood has been rising, doubling over the period from the 1960s to 2016, and aquaculture currently accounts for over a quarter of the world's seafood supply (Barg 1992; Tidwell & Allen 2001; FAO 2009, 2016). In this context, there is an increasing interest in developing offshore aquaculture in regions where sheltered coastal areas are scarce or sustain activities that are incompatible with aquaculture (Azpeitia *et al.* 2016). This interest prompted the installation of an experimental bivalve farm in open waters off the Basque coast (southeastern Bay of Biscay).

A significant fraction of aquaculture activities is focussed on molluscs and, more specifically, on bivalves. Several species of bivalves are filter feeding organisms and, as such, they feed on the organic matter in suspension in the water. In particular, phytoplankton are one of the main sources of energy for most bivalve growth (Grant 1996; Petersen *et al.* 2008). Therefore, it is essential to understand the relationships between phytoplankton and these filter feeders. On one hand, it is well known that phytoplankton constitute an important component of the diet of suspension-feeding bivalves as microalgae have long been used as food resource for these molluscs at all growth stages (Brown 2002). Different attributes of phytoplankton, such as cell size or lipid content, are key to the growth of filter-feeding bivalves (Robert & Trintignac 1997; Marshall *et al.* 2010). On the other hand, special attention should be given to harmful algal blooms (HABs). Phytoplankton blooms are natural phenomena that contribute to the sustenance of bivalve and fish production. However, some blooms are not beneficial as they can impair ecosystems, water uses and/or human health (Masó & Garcés 2006). All negative phenomena caused by planktonic species are considered HABs, and among them, some are caused by species that can be dangerous at very low densities due to the potent toxins they produce (Anderson 2009). As an example, cell densities as low as 100–200 cells L^{-1} of the toxic genus *Dinophysis* have been associated with poisoning incidents in humans caused by seafood consumption (Escalera *et al.* 2007).

HABs are phenomena that occur naturally as a result of the combination of physical, chemical and . However, in recent decades the frequency and geographical distribution of HAB events seem to have increased, including those related to toxicity. Some of the reasons explaining this expansion are (i) improved methodologies for the detection of HABs and their toxins, (ii) increased dispersal of species as a consequence of anthropogenic activities (i.e., ballast waters, shellfish seeding) and (iii) the intensification of eutrophication processes in coastal areas (Hallegraeff 1993; Anderson 2009; Glibert & Burkholder 2011).

Among the 4000 phytoplankton species described, around 80 have the ability to produce toxins (Hallegraeff 2003). These biotoxins are ingested by filter feeding organisms, accumulating within their flesh, and then they are gradually transferred to the higher trophic levels within the food web, posing a threat to human health if shellfish are consumed (Shumway *et al.* 2003; Wang 2008; Davidson & Bresnan 2009). Every year, nearly 2000 cases of human intoxication occur worldwide through fish or shellfish consumption, with a mortality rate of approximately 15% (Hallegraeff 2014). The main poisoning syndromes related to shellfish consumption are amnesic, paralytic, diarrheic, neurotoxic and azaspiracid shellfish poisoning (ASP, PSP, DSP, NSP and AZP,

respectively). All are caused by different dinoflagellates, except for ASP. ASP is caused by some species of the diatom genus *Pseudo-nitzschia* that are capable of producing the neurotoxin domoic acid (DA). PSP is associated with saxitoxins (STX) produced by some *Alexandrium* species, as well as by *Gymnodinium catenatum* and *Pyrodinium bahamense* var. *compressum.* Several species within the dinoflagellate genera *Dinophysis* and *Phalacroma,* together with *Prorocentrum lima,* can produce okadaic acid (OA) and are the main causative taxa of DSP. Most species of the genus *Karenia* produce a variety of toxins that can result in the mortality of fish and other marine organisms when they bloom; and at least one species, *K. brevis,* produces brevetoxins which can cause NSP (Brand *et al.* 2012). Finally, some species of the genus *Azadinium* produce azaspiracids (AZAs), which are lipophilic toxins that cause AZP (Hallegraeff 2003; FAO 2005; Hallegraeff 2014). Yessotoxin (YTX) and its analogues have also been included within the DSP-group toxins, although their symptoms are still unknown in humans (Visciano *et al.* 2013). These toxins are produced by the dinoflagellates *Protoceratium reticulatum, Lingulodiniuim polyedra* and *Gonyaulax spinifera* (Paz *et al*. 2004). Notwithstanding, these events not only affect human health, but the risk of intoxication produces great economic loses in the aquaculture industry since the sale of shellfish must be banned when toxin concentrations are over the regulatory threshold (Hallegraeff 2003).

As already mentioned, most of the toxin-producing phytoplankton species belong to the group of dinoflagellates. Vertical migrations of some of these organisms are well known and, sometimes, they accumulate in so-called "thin layers," which are extremely important in terms of toxic phytoplankton distribution (Farrell *et al.* 2012; Berdalet *et al.* 2014; Raine *et al.* 2014). This fact, among others, demonstrates the importance of monitoring phytoplankton not only in surface waters, but also throughout the water column.

The interaction of phytoplankton with the environment is highly dynamic, due to several factors such as their small size, rapid nutrient uptake, high growth rates and susceptibility to grazing (Stolte *et al.* 1994). In this regard, the study of the temporal variation of both the abundance of toxic species and the environmental variables is an essential step to predict the occurrence of toxic events.

Studies of toxic phytoplankton are very scarce in the open waters off the Basque coast. The neighbouring Galician (e.g., Rodríguez *et al.* 2015) and French Atlantic coasts (e.g., Maurer *et al.* 2010; Batifoulier *et al.* 2013) are better studied. However, those zones present morphologic and oceanographic conditions that differ significantly from those of the Basque coast, which is naturally more eutrophic (Revilla *et al.* 2009). Galician waters, as in other areas of the west and northwest coast of the Iberian Peninsula, are influenced by an upwelling system which makes them very productive, especially at the rias (Varela *et al.* 2005). The continental shelf becomes wider in the French zone of the southeastern Bay of Biscay, and there it receives the inputs of larger river plumes (Diez *et al.* 2000). Moreover, most of the studies conducted in both Galician and French areas have focussed specifically on the genus *Dinophysis* (Pizarro *et al.* 2009; Reguera *et al.* 2012; Moita *et al.* 2016).

Some previous studies on phytoplankton communities included potentially toxic taxa in the adjacent Cantabrian coast (Seoane *et al.* 2012) and along the Basque coast (Muñiz *et al.* 2018). However, those studies did not include information on toxins in mussels and considered only surface waters. Therefore, to our knowledge, this is the first research study in a relatively

oligotrophic area within the surroundings of the Bay of Biscay that examines the presence of both toxic phytoplankton species and toxins in mussels, together with oceanographic conditions. Two complete annual cycles are covered and information throughout the water column within an experimental bivalve farm off the Basque coast (southeastern Bay of Biscay) is presented. The objectives are (i) to determine the toxins of main concern in the area, from the viewpoint of human health; (ii) to assign the toxin presence to a causative species of microalgae; (iii) to describe the vertical distribution of the potentially toxic phytoplankton in the water column; and (iv) to identify phytoplankton dynamics and/or environmental factors that can help to predict the occurrence of toxic events.

2. Material and methods

2.1. Study area

The Basque coast extends ca. 100 km along the Cantabrian Sea (southeastern Bay of Biscay) (Figure 5.1). It can be described as a littoral coast exposed to waves, mostly formed of cliffs and influenced by 12 short rivers. Although no large coastal plumes are formed (Diez *et al.* 2000), this freshwater supply modifies the chemical composition of the shelf waters and often leads to increased nutrient levels in inner shelf waters (Valencia *et al.* 2004; Ferrer *et al.* 2009). However, the short-term response of the phytoplankton biomass to these fertilization events is not proportional to the input loads, mainly due to the advection of the phytoplankton by spreading plumes, the water turbidity caused by them and the atmospheric instability (Valencia & Franco 2004). The upwelling activity is almost negligible in the area (Valencia *et al.* 2004), and the climate is rainy, temperate and oceanic, with moderate winters and warm summers (Fontán *et al.* 2009).

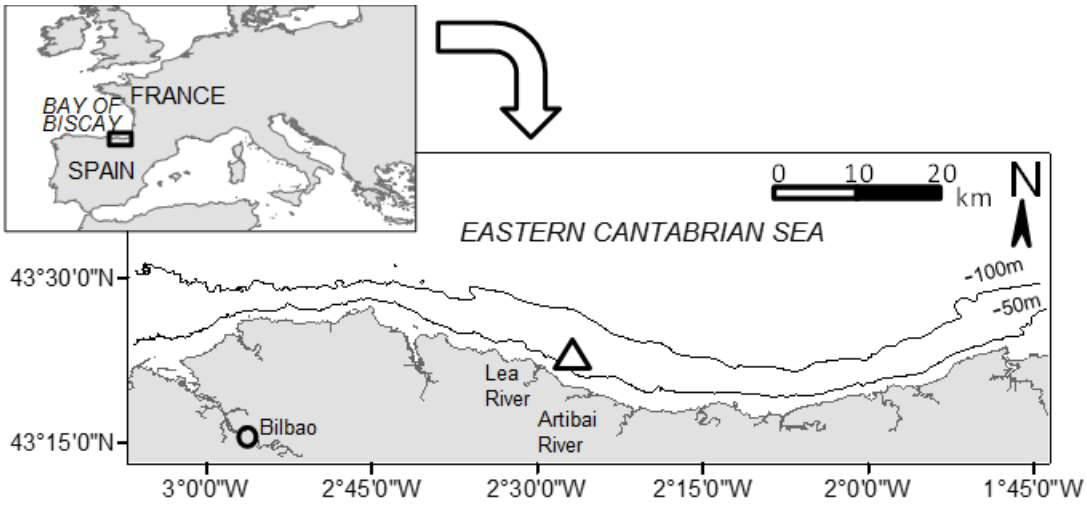

Figure 5.1. Map of the study area, located in the southeastern Bay of Biscay. The triangle shows the location of the experimental bivalve farm.

Field sampling was carried out at a station (43° 21.411' N, 2° 26.918' W) immediately outside an experimental bivalve farm, located 2 nautical miles off the Basque coast, at a depth of approximately 45 m. The experimental farm used a longline system based on a subsurface structure from which bivalve ropes and lanterns were suspended. In particular, the installation consisted of three longlines, occupying a total area of 1 ha. Each longline sustained 100 vertical hanging ropes. The organisms cultured at the farm during the study were mainly mussels (*Mytilus galloprovincialis*) and, to a lesser extent, oysters (*Crassostrea gigas* and *Ostrea edulis*).

2.2. Sampling/laboratory strategy and data acquisition

Sampling took place in two separate periods: from May 2014 to June 2015 and from April 2016 to September 2017. During the first period, CTD (conductivity, temperature, depth) casts and Secchi disc depth measurements were usually performed twice per month, whereas water samples and mussels were collected monthly, except for February 2015 when the sampling could not be carried out due to rough seas. During the second period the sampling frequency was increased: CTD casts, Secchi disc depth measurements, water samples and mussels were usually collected fortnightly and, if not possible, at least monthly. More information on the sampling dates can be found in Table 5.1.

In the field, a Seabird25 CTD was employed for the measurement of temperature, salinity, chlorophyll *a*, and photosynthetically active radiation (PAR) at a depth increment of one metre within the water column. The Secchi disc depth was measured as an estimate of water transparency. Water samples were collected using Niskin bottles at several discrete depths in the water column (3 m, 10 m, 17 m, 24 m, 33 m and 42 m). Water samples were analysed for dissolved inorganic nutrients, total organic carbon (TOC) and for phytoplankton identification and counting (during the first period, phytoplankton was studied at only three depths: 3 m, 17 m and 33 m). Additionally, for a semiquantitative study of the phytoplankton community, a plankton net (mesh opening size: 20 µm) was used to take an integrated sample of the water column, from the bottom up to the surface.

Inorganic nutrients (ammonium, nitrite, nitrate, silicate, phosphate) were measured using a continuous-flow autoanalyser (Bran + Luebbe Autoanalyzer 3, Norderstedt, Germany) according to colourimetric methods described in Grasshoff *et al.* (1983). Quantification limits were 1.6 µmol L^{-1} for ammonium, nitrate and silicate, 0.4 µmol L^{-1} for nitrite and 0.16 µmol L^{-1} for phosphate. In order to calculate the average value for each nutrient when nutrient concentrations were below the quantification limit, half of the limit was used. TOC was estimated with a TOC Analyzer (TOC-V CSH/CSN, Shimadzu Corporation, Kyoto, Japan) in non-purgeable organic carbon (NPOC) mode as described in Grasshoff *et al.* (1983).

Table 5.1. Summary of all sampling carried out at the experimental bivalve farm off the Basque coast from May 2014 to September 2017.

			Phytoplankton identification		
Sampling date	**CTD cast**	**Water analysis**	**water samples at discrete depths**	**integrated net samples**	**Toxin analysis**
20/05/2014	x	x	x	x	x
27/05/2014	x				
09/06/2014	x	x	x	x	
23/06/2014	x				x
01/07/2014	x	x	x	x	
23/07/2014	x				
05/08/2014	x	x	x	x	x
20/08/2014	x				
01/09/2014	x	x	x	x	x
15/09/2014	x				x
23/10/2014	x	x	x	x	x
10/11/2014	x	x	x	x	x
18/11/2014	x				
03/12/2014	x	x	x	x	x
26/01/2015	x	x	x	x	x
12/03/2015	x	x	x	x	x
17/03/2015	x				
16/04/2015	x				
21/04/2015	x	x	x	x	x
18/05/2015	x	x	x	x	
28/05/2015	x				x
01/06/2015	x				
08/06/2014	x				
24/06/2015	x	x	x	x	x
15/04/2016	x	x	x		x
02/05/2016	x	x	x		x
17/05/2016	x	x	x		x
07/06/2016	x	x	x	x	x
20/06/2016	x	x	x	x	x
06/07/2016	x	x	x	x	x
26/07/2016	x	x	x	x	x
16/08/2016	x	x	x	x	x
29/08/2016	x	x			x
20/09/2016	x	x	x	x	x
17/10/2016	x	x	x	x	x
16/11/2016	x	x	x	x	x
01/12/2016	x	x	x	x	x
13/12/2016	x	x	x	x	x
25/01/2017	x	x	x	x	x
21/02/2017	x	x	x	x	x
08/03/2017	x	x	x	x	x
20/03/2017					x
03/04/2017	x	x	x	x	x
25/04/2017	x	x	x	x	x
03/05/2017	x	x	x	x	x
23/05/2017	x	x	x	x	x
12/06/2017	x	x			x
04/07/2017	x	x			x
01/08/2017	x	x			x
04/09/2017	x				x

Water samples for phytoplankton identification and counting were preserved immediately after collection with 0.5 mL of acidic Lugol's solution (0.4% v/v) and maintained in 125 mL borosilicate bottles under dark and cool conditions (4°C) until analysis. Similarly, net samples were preserved in 250 mL borosilicate bottles with 1 mL of the same Lugol's solution (depending on the cell density of the sample, up to 2.5 mL of fixative were used). Taxonomic identification and cell counting were performed on 50 mL subsamples, following the Utermöhl method (Utermöhl 1958; Hasle 1978; Edler & Elbrächter 2010) under a Nikon diaphot TMD inverted microscope. Depending on the organism size, 100x or 400x magnification was used; the detection limit of microscope counts for microplanktonic organisms was 20 cells L^{-1}. The minimum cell size that could be detected was 2–3 µm, and thus, picophytoplankton could not be identified and counted. For this study, only those species described as "potentially toxic taxa" were considered (see Section 2.3). In the case of the net samples, a relative abundance index was applied, as it was not possible to determinate an abundance value, i.e., the abundance of each toxic taxon was semiquantitatively estimated on a scale from 1 (one unique observation) to 5 (dominant taxon).

At the same time, mussels (*Mytilus galloprovincialis)* from the upper 5 m of the culture were collected for toxin analysis. According to European legislation (EC 853/2004, EU 15/2011 and EU 786/2013), the analysed toxins were domoic acid (ASP causative), saxitoxin and derivatives (PSP causative) and the group of lipophilic toxins, i.e., okadaic acid, dinophysistoxins and pectenotoxins (DSP causatives), azaspiracids (AZP causatives) and yessotoxins (cardiotoxicity). Lipophilic toxins were analysed together until December 2014 (mouse bioassay), and from 2015 onwards separate analyses for each of them were performed by means of chemical methods. Toxin content analyses were performed by INTECMAR (Technological Institute for the Monitoring of the Marine Environment in Galicia, Spain) using reference methods for which the institute is duly accredited (http://www.intecmar.gal/intecmar/Biotoxinas.aspx?sm=f). The analytical techniques for each toxin are summarized in Table 5.2.

Table 5.2. Summary of the analysed toxins together with the analytical technique and concentration limits established by European legislation. ASP: Amnesic Shellfish Poisoning; PSP: Paralytic Shellfish Poisoning; HPLC: High Performance Liquid Chromatography; Mouse bioassay implies the death of at least 2 out of the three 3 inoculated mice in 24 hours; LC-MS/MS: Liquid Chromatography-Mass Spectrometry.

Toxin group	Method	Toxin	Quantification limit	Legal limit**	Units
ASP	HPLC	Domoic acid (DA)	2	20	DA mg kg^{-1}
PSP	Mouse bioassay	Saxitoxin (STX) and derivates	380	800	STX di HCL equiv. µg kg^{-1}
Lipophilic*	LC-MS/MS	Okadaic acid (OA), dinophysistoxins, pectenotoxins	40	160	OA equiv. µg kg^{-1}
		Azaspiracids (AZA)	40	160	AZA equiv. µg kg^{-1}
		Yessotoxins (YTX)	0.06	3.75	YTX equiv. mg kg^{-1}

*Until December 2014 lipophilic toxins were analysed together by means of mouse bioassay.
**EC 853/2004; EU 15/2011 and EU 786/2013.

Three more variables were used to describe hydrographic conditions: light extinction coefficient, river flow and upwelling index. Light extinction coefficient (*k*) was estimated from the PAR measured by the CTD using the equation derived from the Beer-Lambert law:

$$I_z = I_f \cdot e^{-kz}$$

where I_z (E m^{-2} d^{-1}) is the radiation received at a specific depth, I_f is the radiation right below the surface, and z is the specific depth (m). Information on the flow rates of the two rivers closest to the experimental site, the Artibai and Lea rivers (Figure 1), was obtained from a regional website ("Diputación Foral de Bizkaia," http://www.bizkaia.eus). Upwelling indices (UI) off the coast of the Bilbao area (Figure 5.1) were obtained from the "Instituto Español de Oceanografía" (2017) and correspond to data modelling. For river flows and UI, daily average values were used.

2.3. Toxicity risk

The occurrence of potentially toxic phytoplankton taxa was studied according to the Taxonomic Reference List of Harmful Micro Algae from the Intergovernmental Oceanographic Commission of UNESCO (Moestrup *et al.* 2009 onwards). For genera that are known to contain toxic species, when it was not possible to identify the organism at the species level, the whole genus was considered potentially toxic as a precautionary measure.

Alert levels for phytoplankton cell concentrations taken from the literature were applied to the main causative genera for the three syndromes of greatest concern, ASP, DSP and PSP, i.e., *Pseudo-nitzschia* spp., *Dinophysis* spp. and *Alexandrium* spp., respectively (Lawrence *et al.* 2011) (Table 5.3). These trigger levels have been determined by comparing phytoplankton count data with biotoxin analyses in shellfish tissue (Swan & Davidson 2012). For *Pseudo-nitzschia* spp. some differences were found in the literature on the established alert limit and, hence, two thresholds were employed. In the case of *Dinophysis* spp. two trigger levels were employed: the lowest value indicates the potential limit for the presence of toxins and the highest value could imply a ban on mussel harvesting for human consumption. Regarding *Alexandrium* spp., its mere presence would imply a risk. These limits were applied at the genus level, summing up the abundances of the different registered species, as a precautionary measure for toxicity risk. The threshold levels employed here are common in European harmful phytoplankton monitoring programs (ICES 2015).

Table 5.3. Alert levels used in this study for phytoplankton taxa associated with the risk of shellfish poisoning (ASP: Amnesic Shellfish Poisoning; DSP: Diarrheic Shellfish Poisoning; PSP: Paralytic Shellfish Poisoning).

Risk	Taxon	Alert level (cells L^{-1})	Reference
ASP	*Pseudo-nitzschia* spp.	50000	Swan and Davidson (2012)
ASP	*Pseudo-nitzschia* spp.	100000	Bates *et al.* (1998), Fillon *et al.* (2013)
DSP	*Dinophysis* spp.	100	Swan and Davidson (2012), Fillon *et al.* (2013)
DSP	*Dinophysis* spp.	500	Fillon *et al.* (2013)
PSP	*Alexandrium* spp.	presence	Swan and Davidson (2012)

Toxicity risk in terms of toxin content in mussels was assessed according to European legislation. The European Union has set a regulatory limit of 20 mg kg^{-1} for DA; 800 µg kg^{-1} for STX; 160 µg kg^{-1} for the sum of OA, dinophysistoxins (DTXs) and pectenotoxins; 160 µg kg^{-1} for AZAs; and 3.75 mg kg^{-1} for YTXs (Table 5.2) (EC 853/2004, EU 15/2011 and EU 786/2013).

3. Results

3.1. Hydrographic, physico-chemical conditions and chlorophyll a

As previously described in Chapter 4 (Section 3.1), the water column within the experimental bivalve farm showed typical hydrographic conditions for temperate coastal zones during the first study period (May 2014 – June 2015). Overall, thermohaline stratification developed from spring to autumn 2014. This was followed by mixing conditions in November – December 2014. Afterwards, high precipitation resulted in the prevalence of strong haline stratification during the next winter and spring months, together with thermal stratification in May and June 2015.

Regarding the second study period, Figure 5.2 shows the temperature–salinity diagrams. April and May 2016 presented some haline stratification together with relatively homogeneous temperatures, around 13–14°C, throughout the water column. The period from the end of May to October 2016 was characterized by thermohaline stratification, due to summertime warming and the presence of waters with a continental origin. From November 2016 on, a reduction in the vertical gradients of both temperature and salinity was observed, induced by vertical mixing and cooling. These conditions prevailed until the following spring, with slight surface salinity decreases in January and May. January was also characterized by thermal inversion. Finally, from the end of May to September 2017 thermal stratification prevailed due to solar heating of the surface waters. September was also characterized by a slight decrease in the surface salinity.

For the period 2014–2015, the vertical distribution of temperature, salinity and chlorophyll *a* is shown in Figure 5.3. The range of variation was wider in the first 20 m of the water column. Chlorophyll *a* presented values below 1 µg L^{-1} during most of the period and throughout the water column (Figure 5.3c). Nevertheless, some peaks were found. From May to September 2014, several chlorophyll increases were detected at depths between 25 and 45 m, ranging from 1.1 to 2.0 µg L^{-1}. In March 2015 the maximum value was reported reaching 2.6 µg L^{-1}at 12 m depth. Finally, a slight increase was observed in subsurface waters during June 2015. In both years, summer chlorophyll peaks were located below the thermocline.

For the period 2016–2017, information on the vertical variability of temperature and salinity is presented in Figure 5.4a and 5.4b, respectively. During summer and early autumn, the thermocline was located between 10 and 25 m depth in both 2016 and 2017. However, higher inter-annual variability was found in the vertical distribution of salinity. For example, salinity values below 35.2 PSU could affect more than half of the water column in 2016, but these salinity decreases were limited to the first 10 m in 2017.

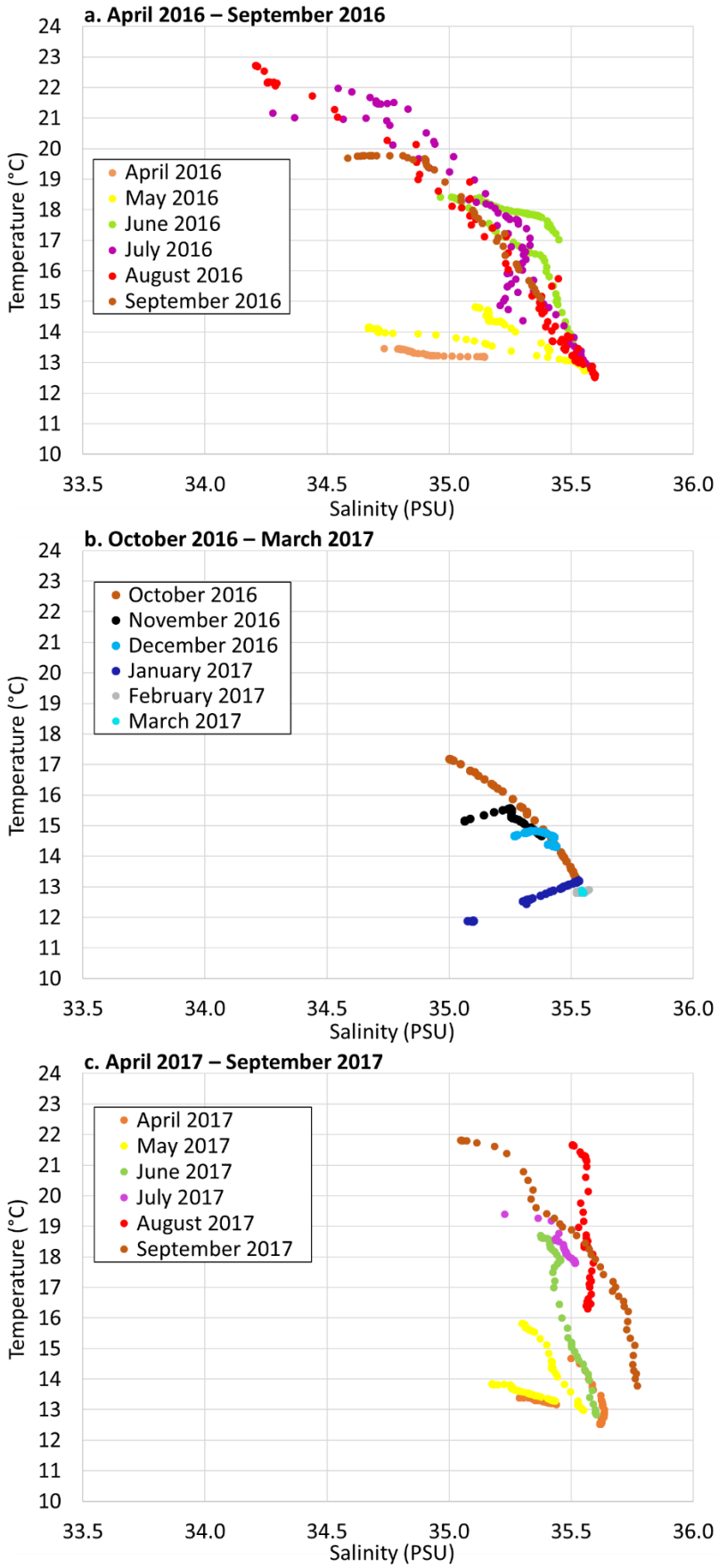

Figure 5.2. Temperature-salinity diagrams of the waters of the experimental bivalve farm off the Basque coast, in the SE Bay of Biscay. The period from April 2016 to September 2017 is shown, separated in three diagrams (a, b and c) chronologically ordered to facilitate the visualization. The previous period, i.e., May 2014 – June 2015, is described in Chapter 4 (Section 3.1).

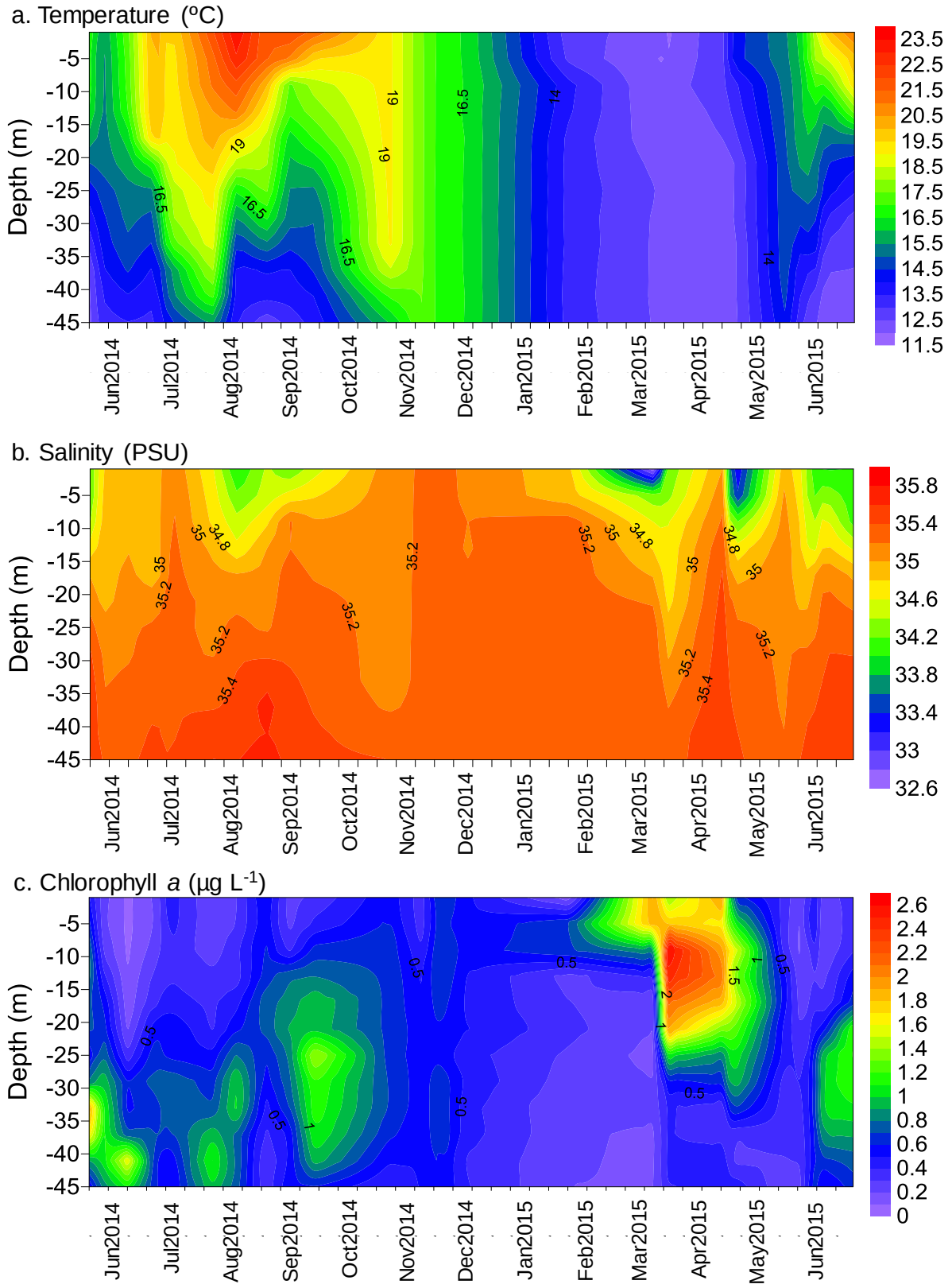

Figure 5.3. Contour maps of temperature, salinity and chlorophyll *a* concentration in the waters of the experimental bivalve farm off the Basque coast from the surface to 45 m in depth. The period from May 2014 to June 2015 is represented. Data were obtained from CTD casts.

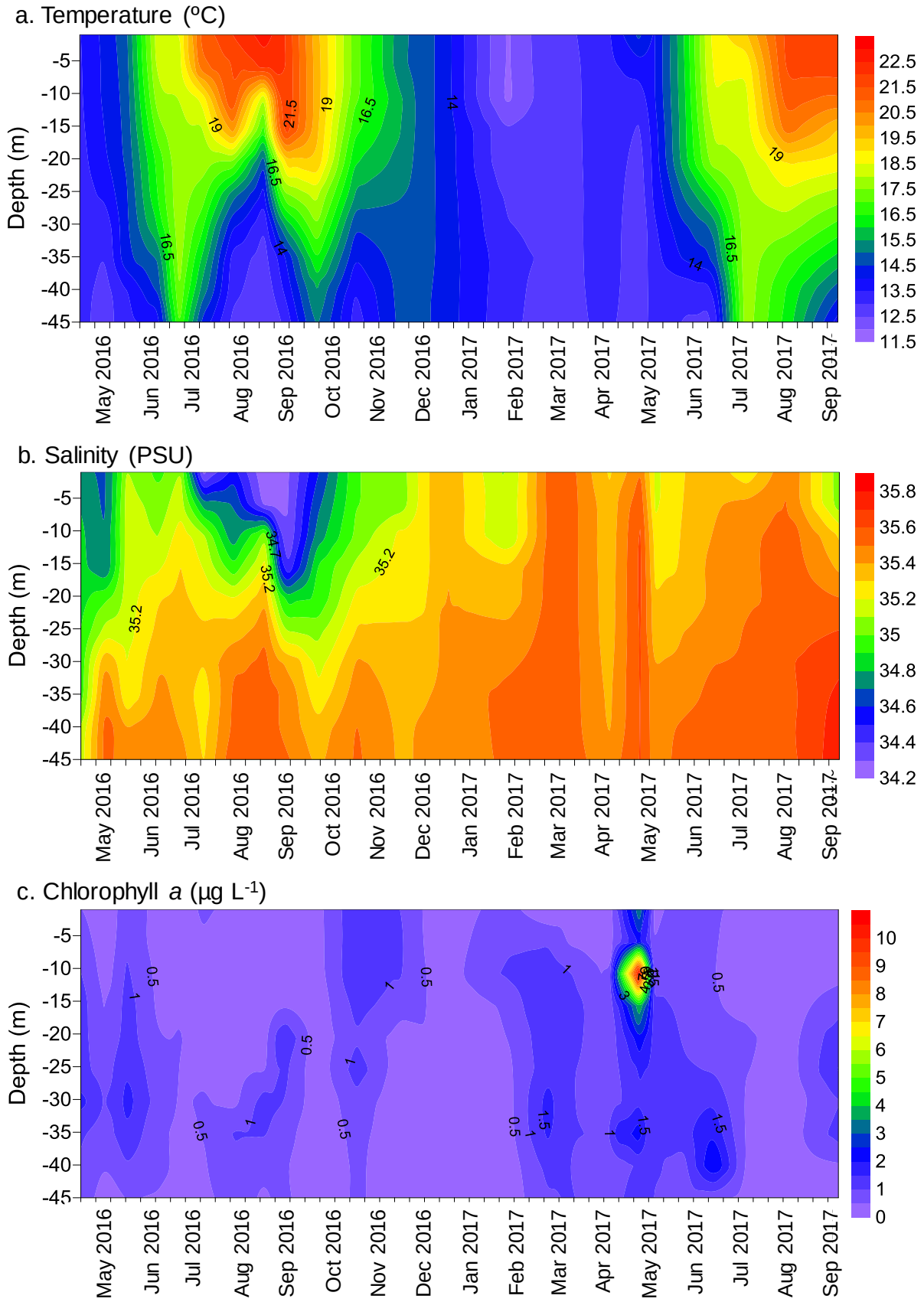

Figure 5.4. Contour maps of salinity, temperature and chlorophyll *a* concentration in the waters of the experimental bivalve farm off the Basque coast from the surface to 45 m in depth. The period from April 2016 to September 2017 is represented. Data were obtained from CTD casts.

As indicated in Figure 5.4c, during the last period, chlorophyll *a* concentration was usually below 1 µg L^{-1}, with some occasional deep (20–40 m) peaks with values between 1.6 and 2.8 µg L^{-1} in 2016 (April, May and August) as well as in 2017 (February, June and September). An exceptional maximum of 11.1 µg L^{-1} was detected in April 2017 at 11 m depth, coinciding with a well-mixed water column characterized by low temperatures and relatively high salinity values (Figures 5.4a and 5.4b). At that time, relatively high values of nitrate, phosphate and silicate were observed (Figure 5.5), together with some of the highest registered upwelling index values (Figure 5.6). Due to the observed high chlorophyll peak in this case, not only were the phytoplankton toxic species counted in the water samples, but also those taxa showing a high contribution to the total community (see Section 3.2).

Inorganic nutrient concentrations are shown in Figure 5.5. Average values (calculated with data from both periods and all sampled depths) were used as a reference threshold for each nutrient to describe the most extreme cases.

Ammonium concentrations were usually low during most of the study period (below the average of 3.2 µmol L^{-1}) except for some higher values observed at intermediate depths during summer surveys and throughout the water column in late spring 2017.

Nitrite concentrations were very low in surface waters, usually below the quantification limit (0.4 µmol L^{-1}); the maxima were observed at several depths during December 2014 – January 2015, agreeing with vertical mixing conditions (Figure 5.3).

Nitrate concentrations were usually below the quantification limit during the first period, although some peaks were observed in March (surface and intermediate depths) and in April and June 2015 (deepest samples). Relatively higher nitrate values were found in summer during the period 2016–2017, when the maxima were always found at 42 m depth.

As for phosphate, concentrations were generally below the average (0.3 µmol L^{-1}) during the first period but showed highly variable values with no temporal pattern during the next period. The highest phosphate values were usually found at 42 m depth, with a maximum of 0.6 µmol L^{-1} in January 2017.

Finally, silicate concentrations were generally below the quantification limit during the first study period, except for some samples in winter and spring; the maximum (4.3 µmol L^{-1}) was found at 3 m depth in March 2015. During the second period, relatively high silicate values were found between July and October 2016 in the deepest waters, coinciding with strong thermohaline stratification.

In summary, the highest concentrations of ammonium were usually found during the summer months and occasionally in late spring. Nitrate and silicate concentrations showed a similar temporal pattern. During the first period, surface peaks of both nutrients were observed in February–March 2015 coinciding with the highest river flow rates (Figure 5.6) and the strongest salinity decreases (Figure 5.3b). The response of nitrate and silicate to freshwater inputs in winter was not so conspicuous during the second period, agreeing with more moderate drops in salinity (Figure 5.4b). Although there was high variability throughout the water column, nitrate, phosphate and silicate generally showed the highest concentrations in the deepest waters (42 m).

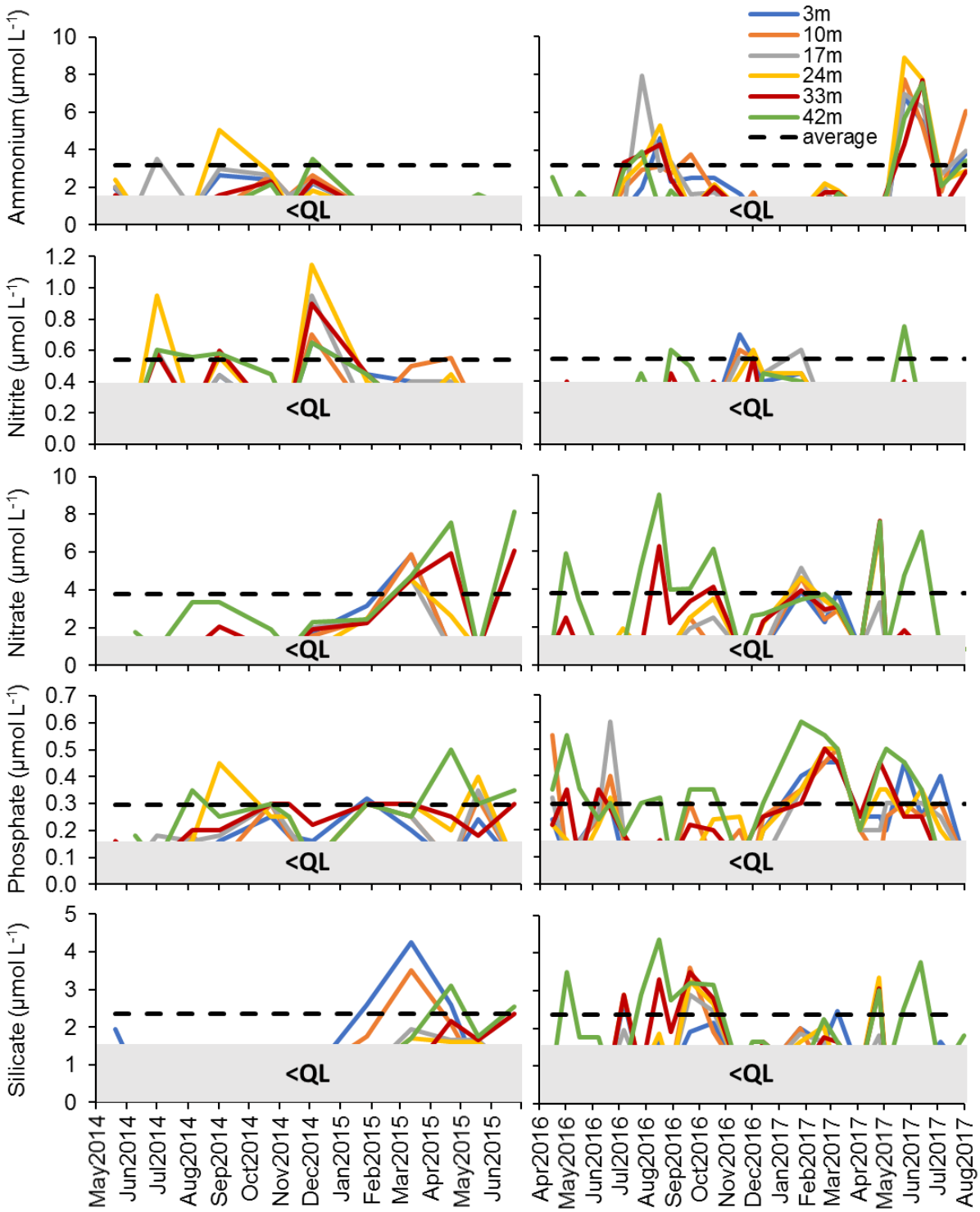

Figure 5.5. Temporal variability of nutrient (ammonium, nitrite, nitrate, phosphate and silicate) concentrations at six depths in the experimental bivalve farm off the Basque coast during the two study periods. QL stands for quantification limit and the dotted line is the average value for each nutrient, used to describe the most extreme conditions.

The upwelling indices and river flow rates were also studied. Upwelling index values were generally below 1000 $m^3 s^{-1} km^{-1}$, with some occasional maxima over 2000 $m^3 s^{-1} km^{-1}$ (Figure 5.6a). In terms of extreme values, downwelling was found to be more relevant than upwelling in the area, showing a maximum value of -6500 $m^3 s^{-1} km^{-1}$. Every annual cycle, long downwelling periods

were observed between November and March, coinciding with vertical mixing conditions. Flow rates of the Artibai and Lea rivers followed the same pattern as the downwelling: periods of high flow rates coincided with vertical mixing, with the highest values ranging between 47 and 78 $m^3 s^{-1}$ (Figure 5.6b).

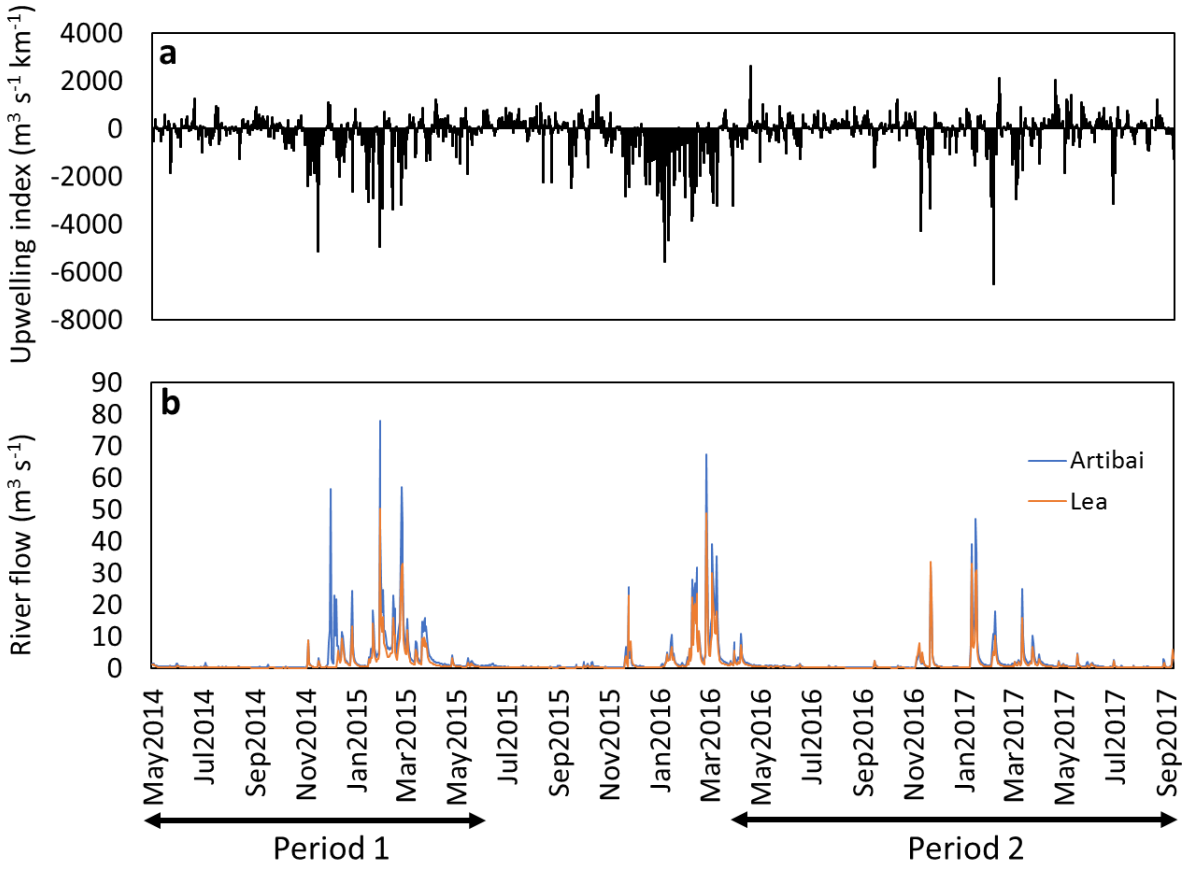

Figure 5.6. Daily means of upwelling index off the coast of Bilbao (a) and flow rates for the Lea and Artibai rivers (b) (see map in Figure 5.1 for locations).

3.2. Potentially toxic phytoplankton

Table 5.4 shows the complete list of all the potentially toxic taxa detected in the water samples collected at discrete depths and their associated risk. A total of 38 taxa were registered and the vast majority belonged to the group of dinoflagellates (76%). The most frequent taxa, present in 88–99% of the samples, were *Pseudo-nitzschia* spp., Gymnodiniales (potentially containing *Karlodinium*) and the Prymnesiales group (a diverse group that could include some toxic species, such as *Chrysochromulina leadbeateri* Estep, Davis, Hargreaves & Sieburth or *Prymnesium polylepis* (Manton & Parke) Edvardsen, Eikrem & Probert). In terms of risk for human health, the group of ASP-producers were the most frequent (95% of the samples) followed by DSP- (60%) and AZP-producers (54%). Finally, potential producers of PSP toxins or YTX were less frequent, appearing in 14% and 19% of the samples, respectively. During the abovementioned chlorophyll peak (11 µg L^{-1}) found in April 2017, the phytoplankton community was dominated by a particularly noteworthy bloom of the potentially toxic taxon *Prorocentrum cordatum* (2.3 x 10^6 cells L^{-1}) and also a bloom of *Pseudo-nitzschia* spp. (4.8 x 10^5 cells L^{-1}), together with a notable contribution of the non-toxic diatoms *Guinardia delicatula* (1.0 x 10^5 cells L^{-1}) and *Leptocylindrus convexus* D.Nanjappa & A.Zingone (1.2 x 10^5 cells L^{-1}) (data not shown).

Table 5.4. Complete list of the potentially toxic taxa detected in discrete depth water samples and integrated net samples taken at the bivalve experimental farm off the Basque coast during the period May 2014 – May 2017. Information on toxicity was obtained from The Taxonomic Reference List of Harmful Algae from the Intergovernmental Oceanographic Commission of UNESCO (Moestrup *et al.* 2009 onwards). Taxa identified at genus or higher taxonomic level could contain both toxic and non-toxic species. ASP: amnesic shellfish poisoning, DSP: diarrheic shellfish poisoning, DTX: dinophysistoxin PSP: paralytic shellfish poisoning, AZP: azaspiracid shellfish poisoning.

			Frequency of presence (% of sampling campaigns)	
Group	Taxon	Risk/toxin	Water samples	Net samples
Diatom	*Pseudo-nitzschia americana/brasiliana*	ASP	15	0
Diatom	*Pseudo-nitzschia galaxiae*	ASP	45	0
Diatom	*Pseudo-nitzschia multistriata*	ASP	27	7
Diatom	*Pseudo-nitzschia pungens*	ASP	6	3
Diatom	*Pseudo-nitzschia* spp.	ASP	97	83
Dinoflagellate	*Alexandrium ostenfeldii*	PSP	15	13
Dinoflagellate	*Alexandrium* sp.	PSP	3	3
Dinoflagellate	*Alexandrium* sp. *(A. tamarense* group*)*	PSP	6	0
Dinoflagellate	*Dinophysis acuminata*	DSP	58	77
Dinoflagellate	*Dinophysis acuta*	DSP	6	40
Dinoflagellate	*Dinophysis caudata*	DSP	15	53
Dinoflagellate	*Dinophysis fortii*	DSP	24	50
Dinoflagellate	*Dinophysis infundibulum*	DSP	18	30
Dinoflagellate	*Dinophysis ovum*	DSP	6	17
Dinoflagellate	*Dinophysis* spp.	DSP	0	3
Dinoflagellate	*Dinophysis tripos*	DSP	18	67
Dinoflagellate	*Phalacroma mitra*	DSP	3	10
Dinoflagellate	*Phalacroma rapa*	DSP	6	20
Dinoflagellate	*Phalacroma rotundatum*	DTX-1	76	93
Dinoflagellate	*Prorocentrum lima*	DSP	6	0
Dinoflagellate	*Prorocentrum cordatum*	not confirmed	52	0
Dinoflagellate	Potentially solitary cells of *Gymnodinium catenatum*	PSP	6	0
Dinoflagellate	*Azadinium* spp.	AZP	42	0
Dinoflagellate	Small thecate dinoflagellates *(Heterocapsa/Azadinium*-like)	AZP	58	0
Dinoflagellate	*Gonyaulax spinifera*	Yessotoxin	21	30
Dinoflagellate	*Lingulodinium polyedra*	Yessotoxin	24	23
Dinoflagellate	*Protoceratium reticulatum*	Yessotoxin	21	20
Dinoflagellate	*Karenia* cf. *papilionacea*	Brevetoxin	12	0
Dinoflagellate	*Karenia mikimotoi*	Brevetoxin	3	0
Dinoflagellate	*Karenia* spp.	Brevetoxin	42	7
Dinoflagellate	Gymnodiniales potentially containing *Karlodinium*	Karlotoxin	100	10
Dinoflagellate	cf. *Karlodinium* spp.	Karlotoxin	70	0
Raphidophyceae	*Heterosigma akashiwo*	Ictiotoxic	12	0
Dinoflagellate	cf. *Pfiesteria*	Ictiotoxic	3	0
Haptophyte	Prymnesiales*	Ictiotoxic	100	10
Dictyochophyceae	cf. *Pseudochatonella* sp.	Ictiotoxic	6	0
Dictyochophyceae	cf. *Vicicitus globosus*	Ictiotoxic	3	0
Dinoflagellate	*Takayama* sp.	Ictiotoxic	12	20
Dinoflagellate	*Ostreopsis* cf. *siamensis*	Ostreocin D	9	17

*Prymnesiales is a diverse group (e.g., *Chrysochromulina* spp., *Phaeocystis* spp.) which could include some toxic species, such as *Chrysochromulina leadbeateri* or *Prymnesium polylepis.*

Fewer species of toxic phytoplankton taxa were observed in the net samples compared to the complete taxa list obtained from water samples for the whole period, as only those larger than 20 µm could be retained by the plankton net (Table 5.4). Overall, no additional species were detected in the net samples. However, these integrated samples (45 m depth water column) were essential to complement the information given by the water samples at discrete depths, as differences between bottle and net samples were detected in some instances. Moreover, the differences in the frequency of presence of each taxon between discrete depth water samples and integrated net samples must be highlighted (Table 5.4). Net samples permitted us to detect higher frequencies of some target toxic species. For instance, different species of *Dinophysis* were detected with a frequency range of 6–58% in the discrete depth water samples, whereas those same species were recorded with frequencies of 17–77% in the integrated net samples. Similarly, *Phalacroma rotundatum* was recorded in 76% of the discrete depth water samples but in 93% of the net samples. Complete information regarding the net samples is included in Annex 5.1 and Annex 5.2.

3.3. Toxin content in mussels

The presence of different toxins in mussels (*Mytilus galloprovincialis)* could be confirmed in several samplings (Table 5.5). DA was always below the quantification limit, except for one unique occasion (spring 2017), when it presented a low concentration, far below the legal limit for human consumption. Saxitoxins and their analogues were quantified only on three occasions out of 38, which took place in summer and autumn; these values did not exceed the legal limit, ranging from 400 to 420 µg eq. kg^{-1}. Lipophilic toxins, when the mouse bioassay was applied (from May to December 2014), were detected on one occasion (spring), which would have implied a ban on harvesting. This event was more likely associated with OA (see Section 3.4). OA was the main toxin affecting mussels from the experimental farm. In addition to the positive bioassay result for lipophilic toxins previously mentioned, OA was detected on 12 occasions (accounting for 39% of the samples analysed by chemical methods); OA occurred always in spring and occasionally in autumn. The most remarkable finding was that six of the spring occurrences would have implied a ban on harvesting, as the OA concentration was above the legal limit. Azaspiracids were always below the quantification limit. Finally, the presence of YTX was also relatively frequent, appearing in 39% of the samples analysed by chemical techniques, but always below the regulatory limits. The concentrations of these lipophilic toxins exceeded quantification limits in samples collected in spring, summer and autumn, although they were detected multiple times only during 2016; the maximum concentration was observed in late spring.

Summarizing, in terms of mussel aquaculture, the registered toxic samples would have implied the closure of the farm in 15.4% of the cases. OA was always the causative toxin and all the banning events would have occurred in spring.

Table 5.5. Presence and concentration of toxins in mussels (*Mytilus galloprovincialis)* from the experimental bivalve farm off the Basque coast. QL: quantification limit, DA: domoic acid, STX: saxitoxin, OA: okadaic acid, AZA: azaspiracid, YTX: yessotoxin, NA: not analysed. Presence of toxins is shown in bold, and values above the legal limit are shown in red. For lipophilic toxins, a "POSITIVE" result in the mouse bioassay means that at least two out of three inoculated mice died in 24 h.

	DA (mg kg^{-1})	STX (µg eq. kg^{-1})	Lipophilic toxins	OA (µg eq. kg^{-1})	AZA (µg eq. kg^{-1})	YTX (mg eq. kg^{-1})
Legal limit	20	800	-	160	160	3,75
LOQ	2	380	-	40	40	0.060
27/05/2014	<QL	<QL	**POSITIVE**	-	-	-
23/06/2014	<QL	<QL	NEGATIVE	-	-	-
05/08/2014	<QL	**420**	NEGATIVE	-	-	-
01/09/2014	<QL	<QL	NEGATIVE	-	-	-
15/09/2014	<QL	<QL	NEGATIVE	-	-	-
23/10/2014	<QL	<QL	NEGATIVE	-	-	-
10/11/2014	<QL	<QL	NEGATIVE	-	-	-
03/12/2014	<QL	<QL	NEGATIVE	-	-	-
26/01/2015	<QL	<QL	-	<QL	<QL	<QL
12/03/2015	<QL	<QL	-	<QL	<QL	<QL
21/04/2015	<QL	<QL	-	**165.7 ±34**	<QL	<QL
18/05/2015	<QL	<QL	-	<QL	<QL	<QL
24/06/2015	<QL	<QL	-	<QL	<QL	<QL
15/04/2016	<QL	<QL	-	**99.3**	<QL	<QL
02/05/2016	<QL	<QL	-	**107.9**	<QL	**0.097**
17/05/2016	<QL	<QL	-	**56.4**	<QL	**0.1**
07/06/2016	<QL	<QL	-	<QL	<QL	**0.193**
20/06/2016	<QL	<QL	-	<QL	<QL	**1.056**
06/07/2016	<QL	<QL	-	<QL	<QL	**0.45**
26/07/2016	<QL	<QL	-	<QL	<QL	**0.395**
16/08/2016	<QL	<QL	-	<QL	<QL	**0.201**
29/08/2016	<QL	<QL	-	<QL	<QL	**0.154**
20/09/2016	<QL	<QL	-	<QL	<QL	**0.11**
17/10/2016	<QL	<QL	-	<QL	<QL	**0.069**
16/11/2016	<QL	**410**	-	**58.4**	<QL	**0.081**
01/12/2016	<QL	<QL	-	**46.1**	<QL	<QL
13/12/2016	<QL	**400**	-	<QL	<QL	<QL
25/01/2017	<QL	<QL	-	<QL	<QL	<QL
21/02/2017	<QL	<QL	-	<QL	<QL	<QL
08/03/2017	<QL	<QL	-	**77.1**	<QL	<QL
20/03/2017	<QL	<QL	-	**508.3**	<QL	<QL
03/04/2017	NA	NA	-	**340.9**	<QL	<QL
25/04/2017	**6.1**	<QL	-	**994.2**	<QL	<QL
03/05/2017	<QL	<QL	-	**570.9**	<QL	<QL
23/05/2017	<QL	<QL	-	**120.9**	<QL	**0.089**
12/06/2017	<QL	<QL	-	<QL	<QL	<QL
04/07/2017	<QL	<QL	-	<QL	<QL	<QL
01/08/2017	<QL	<QL	-	<QL	<QL	<QL
04/09/2017	<QL	<QL	-	<QL	<QL	<QL

3.4. Presence of toxins in mussels and potentially causative phytoplankton taxa

During the study period, DA was detected once in April 2017, agreeing with the highest peaks of *Pseudo nitzschia* spp. at depths of 3, 10 and 17 m (reaching up to 5.6 x 10^5 cells L^{-1}), but also with a small contribution of *Pseudo-nitzschia pungens* (maximum abundance of 2.0 x 10^4 cells L^{-1} at 3 m depth) (Figure 5.7). The detection of DA also coincided with the peak chlorophyll of 11 µg L^{-1}. The rest of the abundance peaks were not in line with the presence of DA in mussel flesh. The density of other DA-producers exceeded the alert limits obtained from the literature on several occasions. Two peaks in *Pseudo-nitzschia galaxiae* exceeding the 100000 cells L^{-1} threshold were registered at 3 m depth in July 2014 and August 2016. Another large peak (5.3 x 10^5 cells L^{-1}) was observed for *Pseudo-nitzschia* spp. in May 2016 at 17 m depth.

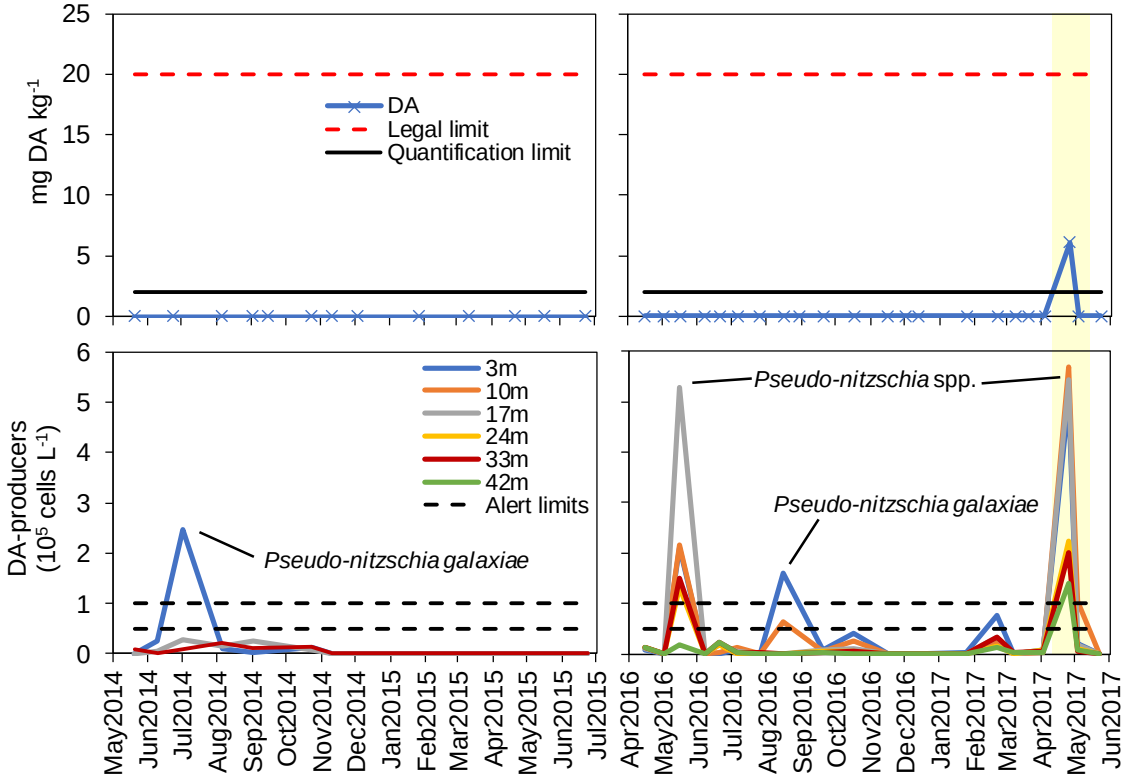

Figure 5.7. Domoic acid (DA, causative toxin of Amnesic Shellfish Poisoning) concentration in mussels (*Mytilus galloprovincialis)* and cell density of the DA-producing phytoplankton taxa (i.e., the sum of all the DA-producers included in Table 5.4) at different depths for the period May 2014 – May 2017. The yellow shaded area highlights the period of toxin presence. The dominant taxon is shown in the abundance peaks. *Pseudo-nitzschia* spp. includes the organisms that could not be identified at the species level.

Saxitoxin in mussels was quantified on three occasions (Figure 5.8). The first was in August 2014, when no STX-producing phytoplankton taxa were registered in any of the bottle or net samples, and then in November and December 2016, agreeing with the presence of very low densities (10 cells L^{-1}) of potentially *Gymnodinium catenatum* in November. However, this last result should be taken with caution (see Discussion). Similarly, *Alexandrium* spp. (*A. tamarense* complex) was recorded several times, with a maximum of 210 cells L^{-1} at 3 m depth in July 2016, but no STX presence was observed in molluscs during those occasions.

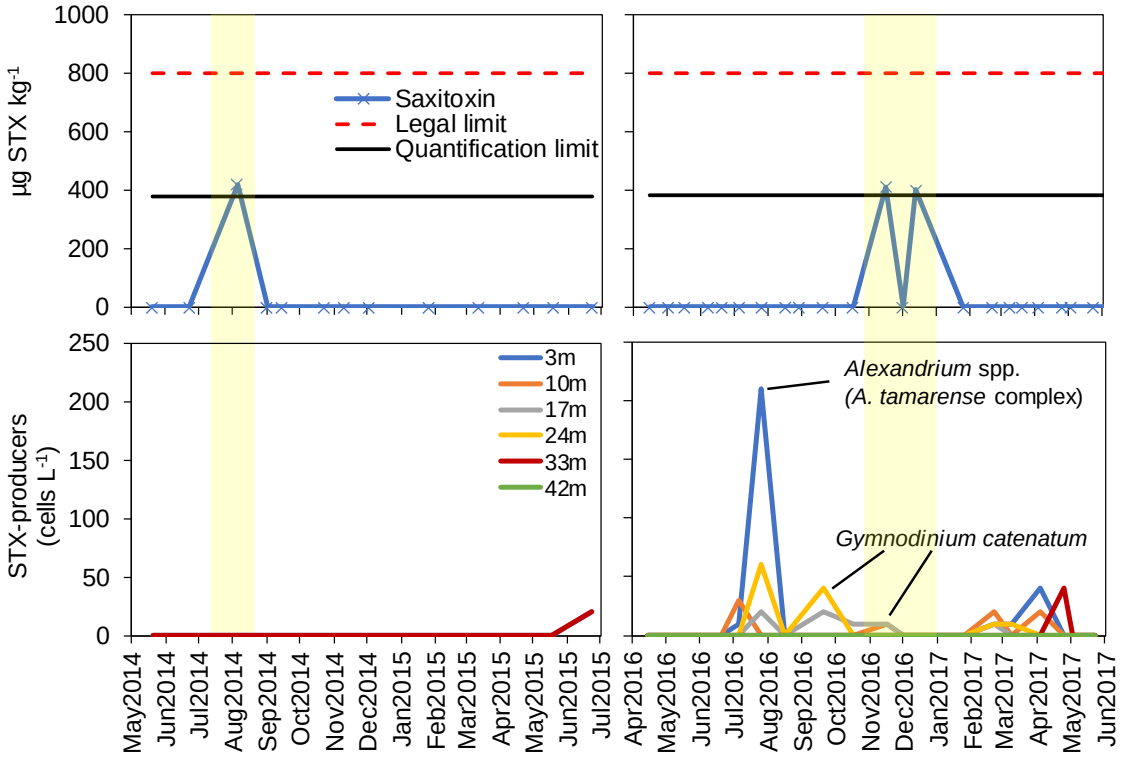

Figure 5.8. Saxitoxin (STX, causative toxin of Paralytic Shellfish Poisoning) concentration in mussels (*Mytilus galloprovincialis)* and cell density of the STX-producing phytoplankton taxa (i.e., the sum of all the STX-producers included in Table 5.4) at different depths for the period May 2014 – May 2017. The yellow shaded areas highlight the periods of toxin presence.

Although OA in bivalves was not analysed from May to December 2014, the presence of the group of lipophilic toxins was detected in May 2014 by mouse bioassay. These lipophilic toxins might be associated with OA since, although very low abundances (maximum of 100 cells L^{-1}) of the potential causative species were found in water samples from discrete depths, several OA-producers were recorded in the integrated net sample. Concretely, *Dinophysis acuminata, Dinophysis tripos* and *Dinophysis caudata* were registered with relative abundance indices of 4, 3 and 1, respectively, in the semiquantitative scale from 1 (one unique observation) to 5 (dominant taxon) (Annex 5.1). During the first study period, two abundance peaks exceeding the alert thresholds were recorded: in July 2014 *D. tripos* peaked at 33 m depth showing a maximum of 2.1 x 10^3 cells L^{-1} and *D. acuminata* showed a maximum of 500 cells L^{-1} at 3 m depth in April 2015 (Figure 5.9). This latter increase in the abundance of *D. acuminata* coincided with the presence of OA. From then on, three periods of OA presence in bivalves occurred somewhat in accordance with the presence of *D. acuminata*: in April–May 2016, November–December 2016 and March–May 2017. The latter was the most remarkable given the high values observed, with a maximum of 1000 µg OA kg^{-1} in mussels and maximum density of *D. acuminata* of 2000 cells L^{-1}. This last and greatest peak in OA coincided with the described chlorophyll maximum. Moreover, *D. acuta* was found in all the net samples from April and May 2017, when OA reached maximum values, with relative abundances of 2–3 on the semiquantitative scale from 1 to 5. Net samples provided additional information as several species that were not registered in the bottle samples were recorded: *D. fortii* and *D. acuta* for the period 2014–2015 and *Phalacroma mitra* for the period 2016–2017 (Annex 5.1 and Annex 5.2, respectively).

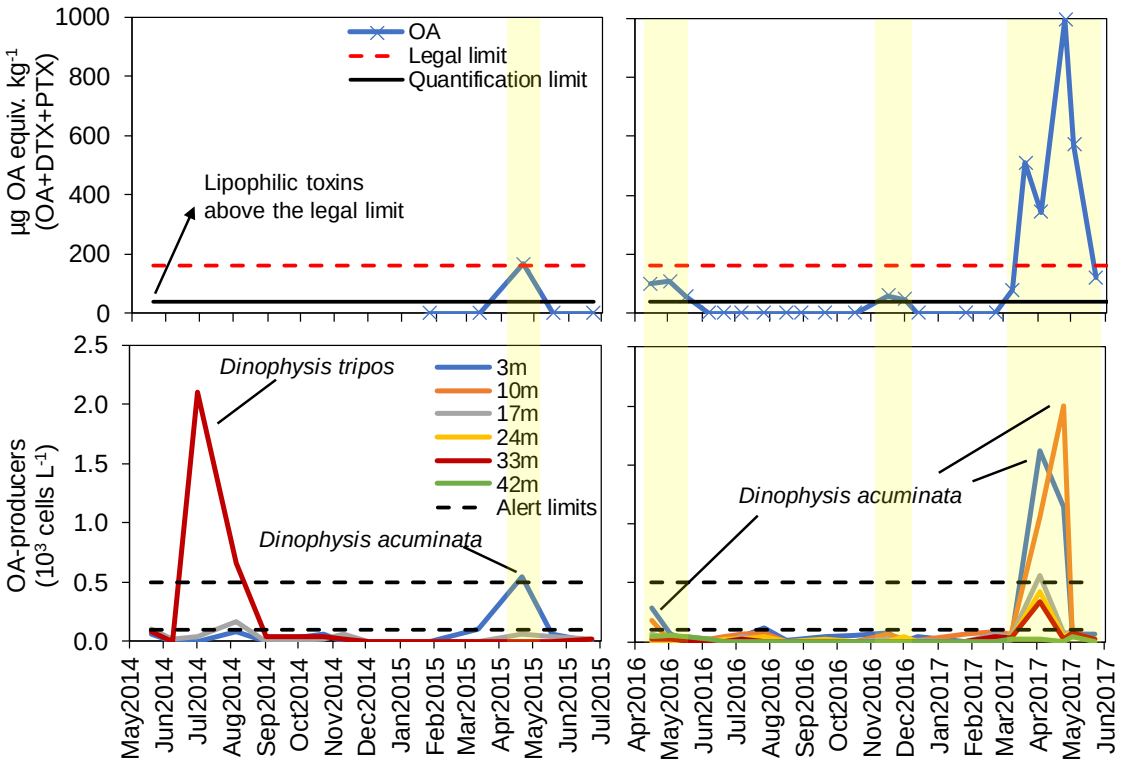

Figure 5.9. Concentration of okadaic acid (OA, causative toxin of Diarrheic Shellfish Poisoning) and its derivatives (DTX: dinophysistoxin, PTX: pectenotoxin) in mussels (*Mytilus galloprovincialis)* and cell density of the OA-producing phytoplankton taxa (i.e., the sum of all the OA-producers included in Table 5.4) at different depths for the period May 2014 – May 2017. The yellow shaded areas highlight the periods of toxin presence. The represented alert limits apply only for the sum of *Dinophysis* species and not for all the OA-producers. All the cases in which alert limits were exceeded were given by *Dinophysis* spp. From May to December 2014 OA and its derivatives were analysed altogether within the lipophilic toxins group by means of mouse bioassay, which did not allow its quantification.

Presence of the YTX-producing taxa was registered in four periods (Figure 5.10). On one hand, *G. spinifera* was recorded in May 2014 with very low densities (20 cells L^{-1}), but specific analyses for YTX were not conducted until January 2015. Then, *G. spinifera* was detected again in March 2015, in similar low densities, with YTX remaining below the quantification limit. On the other hand, *G. spinifera, L. polyedra* and *Protoceratium reticulatum* were present from April to July 2016 throughout the water column, with *L. polyedra* as the taxon with the highest density at 3 m depth. In addition, during spring and summer 2016, the abundance of *L. polyedra* in surface waters followed a trend very similar to the concentration of YTX in mussels. In April 2017 a maximum abundance of YTX-producing taxa of 640 cells L^{-1} was found, associated mostly with *L. polyedra* and, to a lesser extent, with *G. spinifera*. Immediately after that peak in May 2017, YTX appeared in a low but quantifiable concentration, coinciding again with the detection of *L. polyedra* (80 cells L^{-1}) and *P. reticulatum* (20 cells L^{-1}) at 3 m depth.

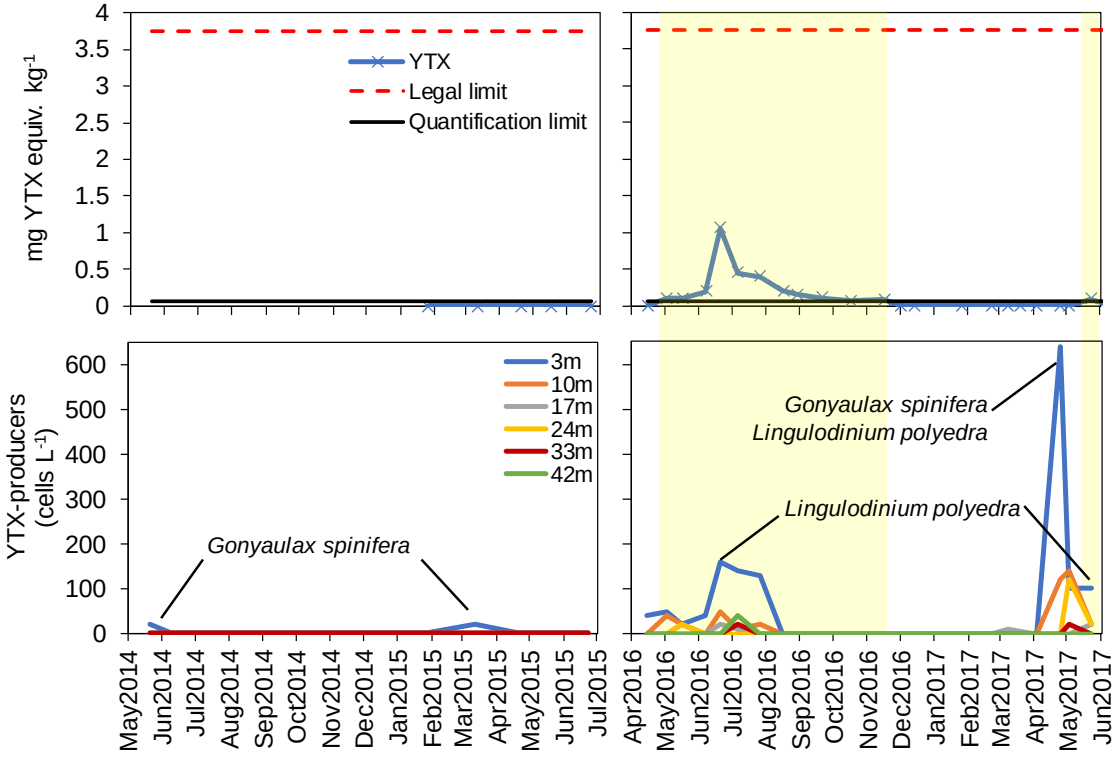

Figure 5.10. Yessotoxin (YTX) concentration in mussels (*Mytilus galloprovincialis)* and cell density of the STX-producing phytoplankton taxa at different depths for the period May 2014 – May 2017. The yellow shaded areas highlight the periods of toxin presence. From May to December 2014 YTX were analysed altogether within the lipophilic toxins group by means of mouse bioassay, which did not allow its quantification.

3.5. Toxicity risk and environmental parameters

The temporal variation of the abundance of the main toxic taxa as well as that of the environmental variables were studied to ascertain whether relationships existed between them. For this purpose, those species or genera that have been found to be associated with the presence of toxins in mussels or those that are more likely to pose a risk in the experimental bivalve farm have been studied.

Pseudo-nitzschia spp. (which does not represent the sum of all *Pseudo-nitzschia* species, but the sum of those that could not be identified at the species level) showed lower abundance values during the period 2014–2015, with a maximum on the order of 10^3 cells L^{-1}. During the second study period, two peaks were observed, in May 2016 and April 2017, both on the order of 10^5 cells L^{-1} (Annex 5.3a, Annex 5.3b and Annex 5.3c). These peaks coincided with concentrations of ammonium and nitrite below the quantification limit (Annex 5.3a) and partially mixed waters, with temperature and salinity throughout the water column ranging between 12.5–14.8°C and 35.1–35.6 PSU, respectively (Annex 5.3b). In addition, the second peak coincided with the maximum chlorophyll value observed during the whole study (Annex 5.3b), together with the presence of DA and a relatively high upwelling index (Annex 5.3c). During these two *Pseudo-nitzschia* spp. peaks, low values of river flow were observed (Annex 5.3c). However, a decrease in surface salinity (Figure 5.4b) was coherent with previous freshwater inputs during winter.

Alexandrium spp. showed one principal peak of 210 cells L^{-1} at 3 m depth (Annex 5.4a, Annex 5.4b and Annex 5.4c), but saxitoxin in mussels did not exceed the quantification limit at that time.

This event occurred in July 2016 with some of the highest registered ammonium concentrations throughout the water column (Annex 5.4a) and a strong thermohaline stratification (Annex 5.4b). Moreover, the upwelling index and river flow rates were near zero at that time (Annex 5.4c). The detection of saxitoxins (August 2014, November and December 2016) was not found to be associated with specific oceanographic conditions; however, the hydrographic conditions in August 2014 (detection of saxitoxin) and July 2016 (peak of *Alexandrium* spp.) were very similar, with the presence of strong thermohaline stratification and flow rate values near zero. Different conditions were found in November and December 2016, characterized by the beginning of vertical mixing and downwelling.

D. acuminata, the taxon posing the main risk for DSP in this area, showed two main abundance increases, both in spring (April 2015 and April 2017) (Annex 5.5a, Annex 5.5b and Annex 5.5c). These events occurred when ammonium and nitrite concentrations were very low (below quantification limit) and nitrate, phosphate and silicate concentrations were relatively high (Annex 5.5a). Peaks in *D. acuminata* occurred with homogenous temperature throughout the water column (i.e., no thermal stratification), ranging between 12.3°C and 14.7°C (Annex 5.5b).

L. polyedra, which probably was the cause of the presence of YTX during summer 2016, was only recorded during the second study period (April 2016 – May 2017). This species presented two notable abundance increases, in June 2016 and April 2017, both at 3 m depth (Annex 5.6a, Annex 5.6b and Annex 5.6c). These events reached values between 160–200 cells L^{-1} and coincided with minimum values of ammonium and nitrite concentrations (Annex 5.6a) and with flow rates near zero (Annex 5.6c). As already described, YTX was present in all the samples from May to November 2016, which coincides with the thermohaline stratification period (Annex 5.6b) and the lowest flow rate values (Annex 5.6c).

Another potential YTX-producer, *G. spinifera,* was registered on some occasions, but usually with densities below 20 cells L^{-1} (Annex 5.7a, Annex 5.7b and Annex 5.7c). An increase was registered in the shallowest layers in April 2017, reaching 440 cells L^{-1}. This peak occurred with homogeneous temperature and salinity throughout the water column and with low flow rates (Annex 5.7b and Annex 5.7c, respectively).

Similarly, very low densities (20–40 cells L^{-1}) of *Protoceratium reticulatum* were recorded during May 2016, coinciding with the first quantifiable values of YTX. *P. reticulatum* was usually below the detection limit but was present in the spring during both 2016 and 2017 at depths of 3–10 m, coinciding with low values of ammonium and nitrite and homogeneous temperatures throughout the water column (Annex 5.8a, Annex 5.8b and Annex 5.8c).

Finally, although azaspiracids were always below the quantification limit, the temporal variability of *Azadinium* spp. was also studied due to its potential risk in the area of study (see Discussion). *Azadinium* spp. presented two main peaks, in June 2015 and July 2016 (Annex 5.9a, Annex 5.9b and Annex 5.9c). The organisms observed in June 2015 in particular were very similar, given their proportions, to the toxic species *A. spinosum*. Most of the cells registered during the second peak were similar to *Azadinium dexteroporum* I.Percopo & A.Zingone. Both abundance increases occurred with strong thermohaline stratification (Annex 5.9b) and with low values of river flow and upwelling index (Annex 5.9).

4. Discussion

4.1. ASP toxins and potential producers

The genus *Pseudo-nitzschia* was frequently registered during this study. Furthermore, on several occasions it exceeded the alert thresholds usually employed in European coastal zones, such as Scotland, the Netherlands or Spain (ICES 2016). In contrast, none of the mussel samples exceeded the regulatory limit for DA, and in only one case was the concentration of this toxin above the quantification limit. Thus, the abundance of this potentially toxic genus cannot be necessarily linked to the presence of the amnesic toxin in the study area. On the northwest coast of Portugal, as an example, several harvesting closures occurred with cell concentrations of *Pseudo-nitzschia* species below the alert thresholds (ICES 2016). This can be explained by factors such as toxin content per cell, which may vary up to one order of magnitude even within the same species and same location (Pizarro *et al.* 2009), or the presence of toxic as well as non-toxic species within this genus as, for instance, the taxon here identified as *Pseudo-nitzschia* spp. encompassed several species that could not be differentiated under the inverted microscope. In this sense, the cell abundances used as threshold levels for the main toxic genera of concern are currently being reviewed by the European Union National Reference Laboratory for Biotoxins Working Group on Monitoring Toxic Phytoplankton (ICES 2017).

As DA was present in mussels in only one of the two blooms given by *Pseudo-nitzschia* spp., some differences between those abundance peaks need to be highlighted. The first bloom (May 2016), with no associated toxin presence in mussels, was characterized by cells smaller than 5 µm, whereas in the second one (April 2017) the contribution of cells larger than 5 µm increased, together with the presence of low but non-negligible abundances of *P. pungens.* Although *P. pungens* is usually non-toxic, toxic clones have been reported from New Zealand and the USA (Moestrup *et al.* 2009 onwards). In the farm area, spring seemed to be the most susceptible season for the occurrence of *Pseudo-nitzschia* spp. blooms, although this affirmation should be taken with caution as only two annual cycles were studied. This result coincides, in part, with what Orive *et al.* (2010) found in the outer reaches of the Nervión estuary (Basque coast), where *Pseudo-nitzschia* spp. blooms were usually found in spring and summer, and is similar to the dynamics described by Smythe-Wright *et al.* (2014) for the Bay of Biscay, where blooms of diatoms, in general, occurred in mid-April with little timing variation from year to year.

Different dynamics have been described for different *Pseudo-nitzschia* species worldwide. For instance, *P. delicatissima* usually blooms in late spring in the Bay of Naples (Mediterranean Sea) (Orsini *et al.* 2004). Along the Catalan coast, the highest cell abundances of *Pseudo-nitzschia* spp. were found in winter–early spring (Quijano-Scheggia *et al.* 2008), whereas blooms of *Pseudo-nitzschia australis* Frenguelli were more common and persistent during late summer to autumn in Monterey Bay (USA) (Bates *et al.* 1998). Similarly, Fehling (2004) found that the *Pseudo-nitzschia seriata* (Cleve) H.Peragallo group formed blooms only during summer months in Scottish waters. Although none of the environmental variables studied for the Catalan coast seemed to play an important role in either the spatial or temporal distribution of *Pseudo-nitzschia* spp. (Quijano-Scheggia *et al.* 2008), similar conditions, characterized by high water temperatures and thermal stratification, were described for the Bay of Naples and Monterey Bay during the blooms (Buck *et al.* 1992; Walz *et al.* 1994; Orsini *et al.* 2004). In the Bay of Biscay, the spring diatom bloom also

coincides with surface stabilization (Smythe-Wright *et al.* 2014). In the experimental bivalve farm, the two blooms of *Pseudo-nitzschia* spp. were found when the thermocline was still not developed. However, the observed slight temperature increase and salinity decrease in surface waters could have contributed to some stabilization of the upper layer. In addition, river flows increased during the previous months which would involve the input of nutrients, specially nitrate and silicate. These findings suggest that different *Pseudo-nitzschia* species are able to exploit different environmental conditions.

Although the influence of physical variables such as turbulence and currents has not been addressed in this study, it would be of interest to investigate them as possible predictors for toxic species abundance and/or toxicity in bivalves. In this regard, it is noteworthy that the *Pseudo-nitzschia* spp. bloom that coincided with the amnesic toxin presence in mussels occurred when the upwelling index was relatively high.

4.2. PSP toxins and potential producers

Alexandrium spp., whose mere presence may imply a risk (Swan & Davidson 2012), was present during long periods and was relatively widespread throughout the water column, especially during the second study period. Most of the specimens belonged to the *A. tamarense* complex, which can contain either toxic (e.g., *A. catenella)* or non-toxic species (e.g., *A. tamarense*). Thus, a more accurate identification at the species level would be necessary to assess the potential impact of *Alexandrium* on shellfish aquaculture in this area. In any case, saxitoxin was always below the regulatory limit, and the frequency of samples in which it exceeded the quantification limit was very low.

Alexandrium spp. has been previously recorded in open waters along the Basque coast during the period 2003–2013, but only in less than 10% of the samplings and only in spring and summer (Muñiz *et al.* 2017). Here, conversely, this genus was registered in all four seasons (note that the sampling frequency of the present study was much higher, and the previous one only included surface samples). Although the highest concentration of PSP toxins was found in summer and autumn, no seasonal pattern was observed for the presence of *Alexandrium.* Laboratory experiments have reported a strong influence of temperature on the content and composition of PSP-toxins in *Alexandrium* spp (Etheridge & Roesler 2005; Navarro *et al.* 2006); however, since PSP-toxins in this study were only quantified in three occasions and in a wide range of seawater temperatures (13.5–23.5°C), an association with temperature could not be concluded.

In November 2016, solitary cells of *Gymnodinium catenatum* were tentatively identified. It is important to note that, due to the presence of saxitoxins in November without the detection of *Alexandrium* in the immediately previous samples, in pursuit of a possible causative of the toxin, it was speculated that some solitary athecate dinoflagellate cells could be *G. catenatum.* These cells were recorded at the beginning of the water column mixing period and coincided with an intensification of downwelling. The last is in line with previous findings for Galician waters, where blooms of *G. catenatum* have been associated with upwelling relaxation or downwelling together with the sudden reversal of the wind direction (Fraga *et al.* 1993; Sordo *et al.* 2001; Bravo *et al.* 2010).

4.3. DSP toxins and potential producers

Among the OA-producing phytoplankton, the prominent peak in *D. tripos* in summer 2014 (at 33 m depth), although it did not result in toxin presence in mussels, cannot be neglected. Thin subsurface layers of species of *Dinophysis* have been previously described (Moita *et al.* 2006; Velo-Suarez *et al.* 2008), demonstrating the importance of sampling throughout the whole water column and not only at discrete depths.

The production and accumulation of toxins in microalgal cells is affected not only by genetically controlled factors, but also by their response to environmental (physical, chemical and biological) conditions and their ecophysiology (Cembella & John 2006), which explains the fact that a bloom of a toxic species is not always linked to the presence of toxin. Morton *et al.* (1994) observed that OA production by a *Prorocentrum* species drastically changed as a function of temperature and light, suggesting a higher OA content with higher environmental stress. Moreover, toxin content per cell depends on the balance between toxin production rate, excretion and cell division (Reguera *et al.* 2014). The variations in toxin content as a function of different growth stages in *Dinophysis,* in particular, are well-documented (Nagai *et al.* 2011; Smith *et al.* 2012; Nielsen *et al.* 2013). Both temperature and growth phase affect toxin production by *D. acuminata*, which may lead to a wide variation in cell toxicity in natural populations (Kamiyama *et al.* 2010; Reguera *et al.* 2014). In conclusion, toxin content from different strains of the same *Dinophysis* species have been found to be as different as those between different species (Reguera & Pizarro 2008). Based on these findings and on the results obtained in the present study, abundance trigger limits established at the genus level should be taken with caution.

In the experimental bivalve farm studied here, *D. acuminata* was found to be the most troubling species as it, most likely, occasionally resulted in very high values of OA, well above the legal limit. Spring was the season when OA was detected more frequently, although the risk of DSP should not be ruled out at other times of the year as some events could have been missed by the sampling frequency employed here (once or twice a month). As described previously, OA is the predominant toxin in Europe (Otero 2014). In particular, in the nearby areas of Galicia and Arcachon, OA is the main toxin contaminating molluscs and is the causative species of most harvesting area closures (Moroño *et al.* 2004; González & Mariño 2008; Maurer *et al.* 2010; Batifoulier *et al.* 2013). In Galicia, the presence of lipophilic toxins poses a great threat to shellfish aquaculture implying long periods of production site closure in some areas. During 2016, for instance, one of the Galician mussel production sites stayed closed the entire 365 days of the year, whereas the rest of the areas registered closures in at least 6 months with the number of closure days (monthly average) ranging between 3 and 31 (information available at the INTECMAR website, http://www.intecmar.gal/intecmar/). It has been suggested that transport of cells alongshore and their intrusion towards shore prompted by downwelling and favourable wind forcing are the phenomena causing recurrent blooms of *Dinophysis acuta* in Galician rias (Escalera *et al.* 2010).

Similar results have been described for the neighbouring Arcachon Bay, where the highest abundances of *Dinophysis* generally occur in spring, with *D. acuminata* as the dominant taxon during these events and also the causative species for the high OA concentrations in oysters and mussels during the typical spring events (Maurer *et al.* 2010). *Dinophysis* events within Arcachon

Bay may have originated in the Capbreton area and, driven by strong westerlies that cause intense northward currents, are transported along the coast up to Arcachon (Batifoulier *et al.* 2013).

All of these findings, together with the OA-producers recorded in the integrated net sample, suggest that the presence of the group of lipophilic toxins detected in May 2014 was most likely associated with OA. Finally, the observed decrease in the abundance of *D. acuminata* in April 2017, followed by a slight increase in cell density of *D. acuta* in May 2017, suggests the same species succession that has been previously described in Galicia and Portugal (Moita *et al.* 2016).

Reguera *et al.* (1993) found that in Galician waters, *D. acuminata* occurred at a temperature range of 12.5–22.8°C and salinity range of 28–34.5 PSU. Similarly, other studies found that *Dinophysis* spp. are tolerant to a wide range of salinity, temperature and light conditions (Koukaras & Nikolaidis 2004; Setälä *et al.* 2005; Batifoulier *et al.* 2013). Also, although some *Dinophysis* species have been found to be favoured by strong thermal and haline stratification on the French Atlantic coast (Delmas *et al.* 1992) and in Galicia (Reguera *et al.* 1995), the opposite pattern was observed on the Basque coast. Muñiz *et al.* (2017) found that along the Basque coast, the highest abundances of *Dinophysis* spp. were usually registered in spring, followed by autumn. In the study area, near the experimental farm, the abundance maximum observed for *D. acuminata* occurred with more homogeneity of temperature and salinity throughout the water column, blooming in a narrow range of both parameters.

4.4. YTX and potential producers

Among all the countries worldwide included in the ICES National Report on HAB events for the period 2014–2016, YTXs in shellfish were always below the regulatory limit except for one unique reported closure of a shellfish production area. This event occurred in the Balearic Islands (Mediterranean Sea) in April 2015, where YTX concentration in shellfish was over the regulatory levels, although the potential toxic phytoplankton species causing this event were under the detection limit (ICES 2017).

On the Basque coast, although YTXs in mussels were always below the regulatory limit, their presence was recorded frequently, usually showing a similar pattern to that of the abundance of *L. polyedra*. Similar results have been found on the French Mediterranean coast, where the detection of YTX in mussels coincided with the presence of both *L. polyedra* and *G. spinifera*. The detection of YTXs in May 2016 coincided with very low densities of *P. reticulatum.* The production of YTX by this species has been found to be 10 times higher than that of *L. polyedra* in cultures (Paz *et al.* 2004), which means that even very low abundances of *P. reticulatum* during a short period can cause accumulation of the YTX-group in mussels at levels above the sanitary thresholds (Aasen *et al.* 2005).

The highest YTX concentrations in the bivalve farm were found during summer 2016, when stratification prevailed. In terms of hydrographic conditions, this finding is in line with the first report of YTXs in French shellfish, occurring during summer as well (year 2007) (Amzil *et al.* 2008).

4.5. AZA and potential producers

Apart from the three main genera of concern, other toxic species were registered. As mentioned in the results, although azaspiracids were always below the quantification limit, their

potential risk on the Basque coast has already been described. Blanco *et al.* (2017) found azaspiracids above the detection limit twice in the experimental bivalve farm studied here. One of those events, in July 2016, was in line with the prominent peak of 2.5 x 10^4 cells L^{-1} of *Azadinium* spp. at 33 m depth described here. Notwithstanding, it is important to note that there are difficulties in the identification of this taxon by means of the Utermöhl technique due to the small size of the cells and the similarity of their morphology to other genera (e.g., *Heterocapsa)* (Maurer *et al.* 2010). Among the marine shellfish poisoning syndromes of concern, AZP is the most recently discovered, and from the species belonging to the genus *Azadinium,* two have the ability to produce toxins (Tillmann *et al.* 2014). The toxic *Azadinium spinosum* Elbrächter & Tillmann has been extensively recorded from the Irish Atlantic coasts (Salas *et al.* 2011), eastern Scotland (Tillmann *et al.* 2009) and the North Sea (Krock *et al.* 2009) to the Atlantic shelf waters of Argentina (Akselman & Negri 2012). This wide distribution worldwide would support its potential identification on the Basque coast as well.

4.5. Toxicity risk associated with other taxa

The genus *Karenia* was also identified. Some species of this genus (e.g., *K. brevis)* are known to produce brevetoxins, which are the cause of NSP (Plakas *et al.* 2002). *Karenia* cells can be difficult to differentiate at the species level by means of standard light microscopy, especially using standard fixatives such as Lugol's solution (Brand *et al.* 2012). In the French Atlantic waters, five *Karenia* species were identified, with no records for *K. brevis* (Nézan *et al.* 2014). Despite the taxonomic confusion and controversy concerning *K. brevis*, Steidinger *et al.* (2008) cited a distribution limited to America and thus, most likely, the *Karenia* spp. cells identified in the present study do not belong to *K. brevis.* However, in this study, the whole genus has been considered as potentially toxic as a precautionary measure. Based on the indications given by Nézan *et al.* (2014), most of the cells identified here would agree with the morphological range that comprises *Karenia brevisulcata* (F.H.Chang) Gert Hansen & Ø. Moestrup*, K. mikimotoi, Karenia* sp.1 and *Karenia* sp.2. Although brevetoxins are not included in European legislation, they are regulated in the National Shellfish Sanitation Program's Guide for the Control of Molluscan Shellfish of the United States (http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FederalStatePrograms/NationalShellfishSanitationProgram/ucm046988.htm). There, it is stated that shellfish harvesting is banned not only when brevetoxins are above the limit of 80 µg 100 g^{-1} in mussels, but also when *K. brevis* abundance exceeds 5000 cells L^{-1}. In the present study, species of *Karenia* were usually present at low cell densities*,* however, there was an occasional peak of 5680 cells L^{-1} of *Karenia* spp., which may or not contain toxic species. In this context, it might be advisable to consider including these organisms and/or their associated toxins in the monitoring as, for instance, recurrent blooms of *K. mikimotoi* cause fish and invertebrate mortalities in the coastal waters of Ireland and France (Atlantic coast) (Brand *et al.* 2012; Nézan *et al.* 2014).

Bringing all of the previous information together, the particularly noteworthy case of April 2017 must be highlighted, when several relevant events coincided: the maximum chlorophyll peak observed during the whole study period, the unique occasion when DA in mussels was quantified, the maximum concentration of OA in mussel flesh and the great bloom of *P. cordatum.* Although there is still great controversy, certain clones of *P. cordatum* have demonstrated lethal and sub-lethal effects on shellfish (Saba *et al.* 2011). Moreover, its presence has been linked with the

presence of tetrodotoxin (TTX) in shellfish from Greece (Vlamis *et al.* 2015); although Turner *et al.* (2017) concluded that there was no link between *P. cordatum* and TTX in bivalve molluscs from the UK.

5. Conclusions

During the study period (2014–2017), lipophilic toxins were the unique group exceeding regulatory limits. All of these cases were associated with OA, occurred in spring and had *D. acuminata* as the causative organism. Therefore, closures due to DSP risk are very probable in this area if commercial production is implemented. ASP- and PSP-toxins were below the quantification limits in most of the samplings. However, genus that are potential producers of ASP (*Pseudo-nitzschia*) and PSP (mainly, *Alexandrium*) were detected more frequently. The abundance trigger limits obtained from the literature for *Dinophysis, Pseudo-nitzschia* and *Alexandrium* cannot be recommended to predict toxic events in bivalves in this study area. Although no clear general pattern was found between all these species and environmental conditions, the main abundance peaks given by the genera *Dinophysis* and *Pseudo-nitzschia* always occurred in a narrow range of both temperature and salinity. With all this, future recommendations would be to give special attention to the phytoplankton composition in spring and to monitor these hydrographical variables at high frequency all year round. A coincidence of the amnesic toxin with a relatively high upwelling index leads to the recommendation of including more environmental variables (such as turbulence or currents) in future investigations focussed on predictions of toxic outbreaks. Other potentially toxic taxa, relevant for human health, were recorded. Among them, *Azadinium* spp. did not seem to pose a threat for shellfish aquaculture in the area, as azaspiracids were always below the quantification limit. In contrast, YTXs could be quantified several times, together with potential causative species. Finally, it would also be advisable to include brevetoxins in routine monitoring analyses due to the observed presence of *Karenia* spp., which may or may not contain brevetoxin-producing species.

V. GENERAL DISCUSSION

This study integrates information obtained from a phytoplankton time series along the whole Basque coast over more than 10 years, together with information derived from a specific study conducted in an experimental bivalve farm in recent years (2014–2017).

For the first time, potential implications of phytoplankton community attributes in offshore mussel aquaculture have been assessed along the Basque coast.

Despite the limitations of the analysed long time series (data acquired only in surface waters, and on a quarterly basis), essential information has been obtained from the dataset. The information provided on the phytoplankton community complements and updates the extant scarce studies in the area; this could be of high value as a baseline for future interdisciplinary investigations, such as an examination of the long-term variability or an estimation of the carrying capacity for bivalve production, among others, which require data on phytoplankton biomass and cell size (Dame & Prins 1997; Newell 2007).

Moreover, this study provides novel information regarding the implications of the phytoplankton community in mussel nutrition and its associated toxicity risk.

1. Potential implications of phytoplankton community attributes on mussel nutrition

1.1. Temporal and spatial variability

The phytoplankton community in Basque coastal waters presented a seasonal variability in line with the annual cycle previously described in the southern Bay of Biscay (Varela 1996; Valdés & Moral 1998). This seasonal pattern is related to hydrographic conditions and, from the standpoint of phytoplankton dynamics, is characterised by the usual occurrence of blooms, mainly during the spring (Varela 1996). This was also observed in the Basque coast, where cell abundance, biomass (calculated from abundance and size) and chlorophyll *a* showed maximum values in the spring, suggesting better food availability for mussels during this season (Chapters 1 and 4). In fact, growth studies in mussels from the experimental site have found that condition indices are affected by sampling time, showing lower values during winter and highest mean values, as well as a rapid increase in lipid and protein, during summer (Azpeitia *et al.* 2016). This might be fuelled, at least in part, by phytoplankton spring blooms, as increases in the lipid content have been associated not only with proximity to spawning (Prato *et al.* 2010) but also with favourable conditions of phytoplankton (Freites *et al.* 2002).

In terms of total phytoplankton abundance, significant differences were also found between years along the coast. Year to year variation in phytoplankton communities can occur as a consequence of changes in climatic or environmental factors (Lehman 2000) or can also be related to changes in the methodology among years, such as differences between taxonomists counting the samples (Dromph *et al.* 2013) or between the utilised fixatives (Menden-Deuer *et al.* 2001), as described in Chapter 2. These two sources of uncertainty should be taken into consideration when conducting phytoplankton time series studies.

Regarding spatial variability in the phytoplankton community, considering an 11-year dataset (2003–2013), no significant differences were found in total phytoplankton abundance nor in total phytoplankton biomass between sampling stations along the Basque coastal waters, as indicated in Chapter 1. Similarly, the participation of the two cell size ranges to the total abundance did not show great differences between the different stations. Bloom events were detected at all the sampled stations. In the same manner, bloom frequencies as well as the contribution of chain-forming diatoms to the small fraction did not show any concrete trend along the coast. All this suggests that, overall, the whole Basque coast would present similar conditions for mussel growth in terms of phytoplankton characteristics.

Phytoplankton vertical spatial variability throughout the water column in the experimental farm also was assessed (Chapter 4). There, despite the slight increase in the concentration of chlorophyll fractions larger than 3 µm (i.e., nano- and microphytoplankton) towards the greatest sampled depths (33 and 42 m), total cell abundance and carbon biomass (calculated from the abundance and the ESD of each taxon) did not follow the same trend. In fact, maximum abundance and biomass values were recorded at 3 m depth. Although chlorophyll *a* has long been used as a proxy for phytoplankton biomass, it is well known that chlorophyll *a* concentration, phytoplankton biomass (in carbon units) and cell abundance are three different attributes of the phytoplankton community (Domingues *et al.* 2008), and therefore, different results can be expected from each of them. As phytoplankton growth depends not only on nutrients but also on light, it is expected to decrease with depth inasmuch as light attenuates (e.g., Reynolds 2006). However, this situation could have relatively reversed during the period of thermal stratification (from May to October 2014, approximately). During this period, a progressive decrease in surface abundance and biomass occurred, together with a simultaneous increase at the deeper sampled depths (especially at 17 m, but also at 33 m). These processes may have occurred as a result of nutrient depletion by phytoplankton growth in surface waters, in addition to the thermocline acting as a physical barrier, which impedes the supply of nutrients towards surface waters (Varela 1996). All these findings suggest that, in terms of food availability given by phytoplankton, bivalves would not necessarily find better growth conditions in deeper levels if an annual scale is considered. In this sense, Azpeitia *et al.* (2016) studied the growth of mussels cultured at the experimental farm and concluded that no significant differences were detected between two culture levels (i.e., 5-17 m and 15-27 m).

In summary, temporal variability was considered more determinative than spatial variability in the study area. Inter-annual variability in phytoplankton abundance and composition was found to be affected by artefacts inherent to the data acquisition technique (fixation of the samples and taxonomist-related effects). Therefore, no conclusions on long-term variability for phytoplankton communities could be drawn. Regarding seasonal variability, in terms of food availability, spring stands out as the season showing the best conditions for mussel growth whereas the surface waters of summer-autumn, in contrast, would be less suitable. However, in an annual scale, no differences were evidenced in phytoplankton biomass within the different depths of the photic layer.

1.2. Influence of environmental parameters on phytoplankton community

Phytoplankton communities are highly sensitive to environmental changes (Stolte *et al.* 1994). In the present study, overall, the seasonal cycle found for phytoplankton community was found to be related to physico-chemical variables which, in turn, depend on hydrographic conditions (Chapters 3 and 4). In short, winter water column mixing in the study area occurs as a result of

cooling, turbulence and downwelling processes (Valencia *et al.* 2004); these subsequently lead to the observed phytoplankton proliferations in the spring. In the same manner, summer stratification is generated by solar irradiation heating the sea surface (Valencia *et al.* 2004).

In the global study along the whole Basque coast, depending on the season, between 21 and 29% of the species assemblages' variability was explained by the studied environmental variables (Chapter 3). Other factors not examined in this study might explain the rest of the variability (e.g., micronutrients, competition, grazing or parasite pressure) (Litchman & Klausmeier 2008). The main environmental parameters shaping the phytoplankton community within each season were temperature and nutrients (mainly ammonium and phosphate). Similar results were obtained in the French Atlantic coast for the period 1999–2012 on an annual basis, where temperature and nutrients were the principal physico-chemical factors influencing phytoplankton dynamics and community structure, together with light (Fariñas *et al.* 2015). During the last two decades the load of nutrients from anthropogenic origin has decreased in the area (Borja *et al.* 2009b); however, it is possible that some sporadic urban sewage discharges still occur, which are rich in ammonium and phosphate (García-Barcina *et al.* 2006; Butrón *et al.* 2009; Garmendia *et al.* 2011; Borja *et al.* 2016). Globally, different responses to environmental conditions were found, even within the same genus; thus, no general pattern could be concluded. However, in summer and autumn a divergence was found between diatoms and dinoflagellates: the most relevant (i.e., higher abundance and biomass) diatoms were associated with low temperatures (which usually implies lower nutrient limitation), opposite to dinoflagellates. In addition, except in winter, some of the most relevant diatoms were linked to lower salinity values, which could be associated with i) typical storms of the area that contribute to sporadic freshwater inputs and lead to nutrient enrichment (Valencia & Franco 2004) or ii) cells transported from the estuaries, where phytoplankton densities during summer are much higher than in coastal waters (Garmendia *et al.* 2011).

Within the experimental farm, chlorophyll annual variability was explained mainly by temperature and nutrients, mostly nitrate and silicate (Chapter 4). In the shallower sampled depths (mostly 3-10 m), higher chlorophyll concentrations were found at lower temperatures and higher nitrate and silicate concentrations, agreeing with winter conditions. However, different results were obtained for phytoplankton abundance. At 3 m depth, very few significant correlations were found between phytoplankton group abundances and the environment. However, at 17 and 33 m depth, a reduced total abundance was associated with higher values of the Artibai river's flow. In addition, at 17 m depth total abundance was also inversely related to nitrate concentration. This situation would coincide with winter conditions, when phytoplankton abundance is low and river flows are high (Varela 1996), with the latter linked to nitrate inputs into the Basque coastal waters (Borja *et al.* 2016).

1.3. Implications on bivalve aquaculture

Phytoplankton blooms have been related to increased growth and production and an improved condition index of several mussel species (Blanton *et al.* 1987; van der Veer 1989; Hickman *et al.* 1991; Benemann 1992); thus, the detection of bloom events at all the sampled stations along the Basque coast would imply a favourable condition for bivalve culture (Chapter 1).

Particle selection in filter feeding bivalves is well established (Kiørboe & Mohlenberg 1981; Riisgard 1988; MacDonald & Ward 1994). Among others, particle size is one of the proposed factors influencing preferential retention and ingestion by bivalves (Defossez & Hawkins 1997;

Petersen *et al.* 2008; Riisgård *et al.* 2011; Jiang *et al.* 2016). In this regard, considerable controversy exists about the most appropriate particle size for the most efficient retention by mussels. Some studies have set the 100% retention efficiency for particles up to 35–45 µm (Strohmeier et al. 2012; Cranford et al. 2014), while others have established the size of 15–20 µm as the maximum particle size for an efficient retention (Lucas et al. 1987; Stenton-Dozey & Brown 1992). In any regard, the majority of the studies agree that the minimum particle size for an efficient retention is 4 µm (Møhlenberg & Riisgård 1978; Riisgard 1988; Jørgensen 1990) and the particle size range of 4 to 45 µm seems appropriate for a high food depletion (Cranford et al. 2014).

Along Basque coastal waters, cells ranging from 2 to 20 µm (ESD) were much more abundant than larger ones, although the important participation of chain-forming diatoms at some stations contributed to that 4 to 45 µm appropriate range for bivalve nutrition (Chapter 1). In terms of phytoplankton biomass (estimated from the abundance and the ESD), the two size fractions studied (2-20 µm and >20 µm) contributed similarly to the total community (Chapter 3).

The observed dominance of certain phytoplankton groups in the study area would also benefit the growth of mussels, since it has been observed, in field studies, that the growth rates of bivalves are more related to the density of certain cellular types than to the total phytoplankton biomass (Wall *et al.* 2013). In the global study along the whole Basque coast, diatoms were revealed as the dominant group in surface waters, in terms of both number of bloom-forming species, spatial distribution (i.e., appeared forming blooms in a higher number of stations) and peaks of cell abundance (Chapter 1). This may benefit the growth of mussels in this area since many studies have found a significant positive correlation between diatoms and bivalve growth (Beukema & Cadée 1991; Weiss *et al.* 2007; Pernet *et al.* 2012; Wall *et al.* 2013). Similarly, in the experimental farm in particular, the observed dominance of the diatoms during spring peaks in biomass, together with the relevant contribution of dinoflagellates to the sub-superficial abundance and biomass, suggest favourable conditions for mussel culture (Chapter 4). Diatoms and dinoflagellates seem to be important phytoplankton groups for mussel growth since they are known to synthesise some of the important fatty acids for bivalve growth (EPA and DHA) (e.g., Azpeitia *et al*. 2016) and experiments on mussel nutrition have also shown highest retention of these two groups, together with ciliates, compared to other phytoplankton groups (Trottet *et al.* 2008). Moreover, the observed high contribution of haptophytes in the experimental bivalve farm also suggests favourable conditions for bivalve growth since, in laboratory experiments, they have been reported to contain, on average, the highest proportion of saturated fats (33%), followed by diatoms (27%) (Volkman *et al.* 1989; Volkman *et al.* 1991).

In regard to chlorophyll *a,* used as a proxy for phytoplankton biomass, it has been reported that mussels do not filter below a chlorophyll threshold of around 0.5 µg L^{-1} (Dolmer 2000; Riisgård 2001). This threshold should be viewed with caution since it was not developed for open waters. Although on some occasions chlorophyll concentrations were below this limit in the experimental bivalve farm off the Basque coast, the annual average value was slightly above this value (Chapter 4). Nevertheless, despite chlorophyll concentrations being not very high in comparison to other areas where bivalve aquaculture has traditionally developed (Figueiras *et al.* 2002; Varela *et al.* 2008), it has previously been reported that mussels from the experimental site off the Basque coast show good growth and biochemical performance, with similar mean chlorophyll values to the ones described here (Azpeitia *et al.* 2016; 2017).

In conclusion, from the standpoint of the potential implications of phytoplankton community on mussel nutrition, although chlorophyll concentration cannot be considered high in comparison

with other areas where bivalve aquaculture has traditionally been developed (such as French estuaries and Galician Rias) (Varela *et al.* 2008; Fariñas *et al.* 2015), the main findings of the present study suggest favourable conditions for mussel growth in terms of phytoplankton attributes (i.e., composition, abundance, cell-size), especially in the spring.

2. Human health toxicity risk associated with phytoplankton

In this thesis, a study of the potential toxicity risk associated with phytoplankton along the Basque coastal waters is presented for the first time. Moreover, a study on all the legislated toxins registered in the experimental bivalve farm off the Basque coast is included, together with the associated phytoplankton causative species.

2.1. Spatial and temporal variability of phytoplankton toxic taxa

This study evidences that the three main toxic genera of concern, i.e., *Pseudo-nitzschia, Dinophysis* and *Alexandrium*, are present in open waters off the Basque coast. This has been found not only in surface waters along the whole Basque coast (Chapters 1 and 3), but also throughout the water column within the experimental farm (Chapter 5). Although toxic marine HABs are natural phenomena that have historically occurred, their presence is evidenced to have greatly increased, not only in frequency and intensity, but also in geographic distribution (Hallegraeff 1993; Hallegraeff 2003). In this regard, analysing the data set of surface waters (2003–2013), the genera *Pseudo-nitzschia* and *Dinophysis* showed a wide spatial distribution along the whole Basque coast, being recorded at all the sampled stations, whereas *Alexandrium* spp. were detected in 12 out of the 19 sampled stations. These results are in line with previous research, as the three genera had previously been recorded in neighbouring areas within the southeastern Bay of Biscay, such as the Cantabria region coast (Seoane *et al.* 2012) and Arcachon Bay (Maurer *et al.* 2010).

According to the alert levels for phytoplankton abundance drawn from the literature (Swan & Davidson 2012), these three genera exceeded the abundance limits over most part of the coast, but usually with low frequencies (on average, <15% for *Pseudo-nitzschia* spp. and <10% for the dinoflagellates). These results, however, should be considered with caution, as the 2003–2013 data series involved only four samplings per year. Most of these events occurred during spring, although summer and autumn were also considerably susceptible to *Pseudo-nitzschia* and *Dinophysis* events, respectively (Chapter 1).

In the specific study at the experimental farm (2014–2017), temporal variability also pointed to spring as the most susceptible season for the occurrence of phytoplankton toxic events (Chapter 5). However, further research covering more years is recommended to confirm this pattern. Maximum abundance for *Pseudo nitzschia* spp. occurred in spring, agreeing with the described dynamics for diatoms in the Bay of Biscay, which were found to peak in mid-April, with little timing variation from year to year (Smythe-Wright *et al.* 2014). *D. acuminata*, which is known to be the causative species of most closures in Galicia and Arcachon (Moroño *et al.* 2004; González & Mariño 2008; Maurer *et al.* 2010; Batifoulier *et al.* 2013), always peaked in the spring (April–May), which is in line with the dynamics described for *D. acuminata* in Arcachon Bay (Maurer *et al.* 2010). Similarly, the YTX-producers *L. polyedra* and *G. spinifera* showed the greatest peak in spring, although their presence was also recorded during summer.

With regard to the spatial variability of potentially toxic phytoplankton species throughout the water column, roughly all the described blooms or abundance increases occurred within the upper studied depths (3, 10 and 17 m), most likely at 3 m. Even so, phytoplankton monitoring cannot be limited to surface waters as the occurrence of subsurface thin layers, including those characterised by toxic species, are ubiquitous phenomena in coastal ecosystems (Sullivan *et al.* 2010). In fact, two abundance maxima were observed in the experimental farm at 33 m depth, characterised by *Dinophysis tripos* and *Azadinium* spp.

2.2. Influence of environmental parameters on phytoplankton toxic taxa

The first approach to investigating the potential relationships between toxic phytoplankton and environmental variables was performed only for the last four years of the time series (i.e., 2012–2015), as these years were found to be unaffected by the uncertainty introduced by changes in the taxonomist and in the fixative. This study, carried out separately for the different seasons, revealed that potentially toxic taxa showed heterogeneity in their relationships with the environment, even within the same genus; thus, no general patterns could be concluded (Chapter 3). However, some associations were found regarding the genus *Dinophysis.* During summer and autumn, species belonging to this genus were found to be usually associated with high ammonium concentrations, which agrees with similar phenomena reported in other areas, such as the Baltic Sea and Japanese coastal waters (Carpenter *et al.* 1995; Koike *et al.* 2001; Nishitani *et al.* 2005). Although further research is needed on the relationship between the presence of *Dinophysis* and ammonium concentrations, these findings reveal the importance of monitoring ammonium levels, as it could help predict these events. Moreover, the ciliate *Mesodinium,* on which *Dinophysis* is known to predate (e.g., Park *et al*. 2006), was found to explain part of the variability of *Dinophysis* biomass in winter. In this regard, *Dinophysis* blooms have been found to usually occur after *Mesodinium* blooms in NW coast of Iberian Peninsula, suggesting the potential of these ciliates as predictors of the toxic blooms (Moita *et al.* 2016).

Using a shorter sampling frequency (once or twice a month), Chapter 5 addressed the association between the environment and those taxa associated with the presence of toxins in mussels (or those that were more likely to pose a risk). Although, in the experimental bivalve farm, no general pattern was found between these species and environmental conditions (i.e., inorganic nutrients, optic conditions, temperature, salinity, TOC, upwelling index and river flow), some specific associations were detected. As above mentioned, the largest observed blooms or abundance increases given by *Pseudo-nitzschia* spp., *D. acuminata, G. spinifera* and *P. reticulatum* occurred in spring and within a very narrow temperature range throughout the water column, immediately before the beginning of the stratification process. In contrast, *Alexandrium* spp. and *Azadinium* spp. presented higher abundances in situations of strong termohaline stratification.

It seems to exist controversy about the potential relationships between certain phytoplankton species and the environmental conditions. As an example, some *Dinophysis* species have been found to be favoured by strong thermal and haline stratification on the French Atlantic coast (Delmas et al. 1992) and in Galicia (Reguera et al. 1995), whereas the opposite pattern was observed on the Basque coast. In the same manner, different dynamics have been described for different *Pseudo-nitzschia* species worldwide, blooming in late spring in the Bay of Naples (Orsini *et al.* 2004), in winter-early spring along the Catalan coast (Quijano-Scheggia *et al.* 2008) or in late

summer-autumn in Monterey Bay (Bates *et al.* 1998). These findings suggest that different species within the same genus in different areas are able to exploit different environmental conditions.

The observed results in the experimental farm area, such as the abundance maxima of some target toxic species coinciding with low values of river flow rates and upwelling index, or with the described conditions of homogeneous temperature and salinity throughout the water column, suggest that special focus should be put on the periods with those conditions, especially in spring.

In terms of damage to the local aquaculture, in Galicia, Álvarez-Salgado *et al.* (2011) found that the percentage of closure days in summer could be predicted by the average continental runoff observed during the previous spring. In the Basque coast, the number of toxic events was relatively low and thus, solid conclusions about the influence of the environment cannot be reached. Further research covering more annual cycles would be needed, to assess the relationships between harmful algal bloom dynamics and environmental conditions in the area. In the same manner, it would be of interest to include information on more environmental variables. For instance, data on currents could give information on the origin of certain phytoplankton species, which could help predict toxic outbreaks.

2.3. Implications on bivalve aquaculture and human health

In the experimental farm, during the study period (2014-2017), very frequently, at least one of the toxins was detected. Specifically, in almost 60% of the cases (from a total of 39 toxin analyses in mussels) quantification limits were exceeded. However, only 15% would have implied a risk for human health if shellfish had been consumed, what would have resulted in a ban on mussel harvest. All these cases were associated with lipophilic toxins, with okadaic acid in particular, being the unique toxin exceeding the European regulatory limits in mussel flesh. This could be *a priori* a relatively positive result for the local farmers compared to the usual situation of some mollusc production areas in Galicia, the most important European producer (Ferreira *et al.* 2014). However, it should be noted that sampling frequency in Galicia is much higher (daily toxin analyses) and inasmuch as sampling frequency of this study was increased, the presence of toxins in samples was also higher. Thus, the comparation with the situation in Galicia should be taken with caution.

Although many potentially toxic taxa were registered at the experimental farm during most of the study period, these species very rarely coincided with toxin presence in mussels. Similarly, Maurer *et al.* (2010) in the French Atlantic coast found that toxin presence in mussels was not necessary linked to an abundance increase of the associated toxic taxon, and vice versa. This can be explained by the fact that toxin content per cell may vary up to one order of magnitude even within the same species and same location (Pizarro *et al.* 2009). The production and accumulation of toxins in microalgal cells is affected not only by genetically controlled factors, but also by their response to environmental conditions and their ecophysiology (Cembella & John 2006). Toxin content per cell depends, among others, on the balance between toxin production rate, excretion and cell division, and thus, it is significantly affected by growth phase (Reguera *et al.* 2014). Hence, the previously mentioned trigger levels established for the abundance of toxic phytoplankton are just guide values and cannot be recommended to ban bivalve harvesting. In fact, those cell abundances used as threshold levels are currently being reviewed by the European Union National Reference Laboratory for Biotoxins Working Group on Monitoring Toxic Phytoplankton (ICES

2017). Either way, in the future it would be recommendable to establish specific thresholds for the Basque open marine waters, that would help implement an early warning system for the presence of toxins in mussels, using phytoplankton abundance among other variables. For this, higher sampling frequency would be required, together with complementary sampling techniques (e.g., integrated samples throughout the water column (Lindahl 1986), which are considered more appropriate than samples taken at discrete depths for species that concentrate in thin-layers).

In regard to the analysed toxins in mussels, the amnesic toxin (DA) was always below the regulatory limit, and just in one unique occasion above the quantification limit. This event agreed with one of the blooms of *Pseudo-nitzschia* spp. and with the presence of *P. pungens*. Although *P. pungens* is usually non-toxic, toxic clones have been reported from New Zealand and the USA (Moestrup *et al.* 2009 onwards).

STXs, paralytic toxins, were also always below the regulatory limit. In three occasions they were above the quantification limit, but none of these cases were in accordance with abundance increases of *Alexandrium* spp. However, one of the STX detection in mussels coincided with a tentative identification of *G. catenatum.* PSP-causing organisms have been extensively reported showing a wide geographic distribution, such as Ireland, Scotland, Denmark, Poland, Norway and Canada, among others (ICES 2017). As an example, in NW coast of Portugal and south and NW of Spain (Andalucía and Galicia, respectively) shellfish farm closures have been reported in the last years associated with *G. catenatum* and the consequent presence of PSP-toxins in mussels. Similarly, *Alexandrium* spp. has also caused harvest bans in Galicia and Brest Bay, for instance, with PSP-toxins above the regulatory limits (ICES 2017).

OA (a lipophilic toxin associated with the diarrheic syndrome) was the most frequent toxin in mussels from Basque waters, which is in line with the described findings for Europe (Otero 2014). In the areas of Galicia and Arcachon (Atlantic coast of France), OA is the main toxin contaminating molluscs and is the causative species of most harvesting area closures (Moroño et al. 2004; González & Mariño 2008; Maurer et al. 2010; Batifoulier et al. 2013). In Basque coastal waters, as in Arcachon Bay (Maurer *et al.* 2010), *D. acuminata* was found to be the most troubling species as it, most likely, occasionally resulted in very high values of OA, being the unique toxin appearing above the regulatory limit.

Other lipophilic toxins, such as YTXs were always below the regulatory limit, however, their presence was recorded frequently. YTXs usually followed a pattern similar to that of the abundance of *L. polyedra*, although the potential implication of *G. spinifera* and *P. reticulatum* on these events cannot be neglected. Despite the frequent detection of YTXs in mussels within Basque waters, these toxins do not seem to pose a great risk for the aquaculture industry, as among all the countries worldwide included in the ICES National Report on HAB events for the period 2014–2016, YTXs in shellfish appeared always below the regulatory limit except for one unique reported closure of a shellfish production area in the Balearic Islands (Mediterranean Sea) (ICES 2017).

Azaspiracids were always below the quantification limit in this study. However, their presence within Basque waters cannot be discarded, as Blanco *et al.* (2017) found azaspiracids above the detection limit twice in the experimental bivalve farm studied here.

Finally, although the European legislation does not include regulatory limits for brevetoxins, the relevance of these neurotoxins and their causative organisms need to be highlighted. In fact, brevetoxins are regulated in the National Shellfish Sanitation Program's Guide for the Control of Molluscan Shellfish of the United States, which provides not only a limit for toxin concentration in mussels but also an abundance limit for *K. brevis* (http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FederalStatePrograms/NationalShellfishSanitationProgram/ucm046988.htm). *Karenia* cells can be difficult to differentiate at the species level by means of standard light microscopy (Brand et al. 2012), and thus, the taxon *Karenia* spp. recorded along Basque coastal waters may contain toxic and non-toxic species. Occasional relatively high abundances of *Karenia* spp. have been found (Chapters 1 and 5) and therefore it might be advisable to consider including these organisms and/or their associated toxins in the monitoring. In fact, recurrent blooms of *K. mikimotoi* have caused fish and invertebrate mortalities in nearby areas, such as coastal waters of Ireland and the Atlantic French coast (Brand *et al.* 2012; Nézan *et al.* 2014).

Based on the obtained results, it seems that special attention should be given to spring, when all the hypothetical closures of the bivalve farm would have happened. This might facilitate the forecast of these events and thus, help the mussel farmers manage their cultures. However, further research covering more annual cycles would be needed to confirm this pattern.

3. Further recommendations

In the present thesis we provide a deep insight of the phytoplankton community along the whole Basque coast over more than 10 years, together with its potential relationships with the environment. Moreover, the specific study within the experimental bivalve farm gives information, for the first time, on phytoplankton community throughout the water column in this area and, especially, on the potential risk that toxic phytoplankton could imply for bivalve aquaculture. In order to evaluate future trends of HAB events, there is a need to establish the present baseline, which underlies the relevance of the present study.

Based on both the observed limitations and findings of this work, we provide some suggestions for future research on phytoplankton monitoring:

a) Prior to the study of phytoplankton long time series, the uncertainty introduced by, at least, changes in the taxonomists handling the sample and in the utilized fixative must be analysed. For this, a previous analysis of the dataset needs to be performed, such as that described in Chapter 2.

b) In regard to the sampling strategy within the experimental bivalve farm, evidence is given on the importance of sampling throughout the whole water column and not only in surface waters. Moreover, the qualitative study by means of net sampling from the sea bottom to surface waters highlights its importance given the new information provided, complementary to that given by the discrete depth water samples, since some potentially toxic species might occur in concentrations too low to be detected with quantitative methods (Reguera *et al.* 2016). In the same manner, integrated hose sampling would be recommendable (Lindahl, 1986), as it would provide quantitative information throughout the water column, avoiding losing information on phytoplankton thin layers, for instance. Moreover, since the occurrence of HAB events or presence of biotoxins in mussels is *a priori* relatively low in the study area (as only two annual cycles have been studied), a longer study period covering more years would be required in order to provide a strong model or tool for the early warning of HABs or presence of toxins in mussels. In the same way, it would be also of interest to increase the temporal sampling frequency, at least weekly.

c) Including data on more environmental variables could provide essential information on phytoplankton dynamics and, concretely, on the occurrence of HAB events. For instance, data on currents would be essential to study whether the observed blooms or toxic species originate in the open ocean or adjacent areas and then are transported to the Basque coast and, this way, predict the blooms or toxic outbreaks.

d) Based on the observed mismatches between toxin in mussels and abundance of toxic phytoplankton, it is concluded that the trigger levels established in other regions for the abundance of toxic phytoplankton are just guide values and cannot be generalized to ban bivalve harvesting, at least within Basque coastal waters. In addition to the revision currently being carried out by the European Union National Reference Laboratory for Biotoxins Working Group on Monitoring Toxic Phytoplankton (ICES 2017), in the future it would be recommendable to establish specific thresholds for this concrete area, with the aim of predicting to some extent the presence of toxins in mussels through phytoplankton abundance.

e) According to the observed presence of *Karenia* spp. along Basque coastal waters and to the existing legislation in other countries, it might be advisable to consider including these organisms and/or their associated toxins in the monitoring.

f) Based on the observed susceptibility of spring for the presence of toxins in mussels above the regulatory limit, and on the increased abundances of some target toxic species mainly in spring, special attention should be given to this season, being recommended to increase the sampling frequency in this period.

VI. CONCLUSIONS AND THESIS

The conclusions obtained in this thesis are the following:

1. Bloom events (including both harmless and harmful taxa) were recorded at all the sampled stations along the Basque coast. These blooms were characterized by the dominance of diatoms and cells ranging 2-20 µm (ESD), with a high contribution of chain-forming diatoms to this cell-size range, which imply potentially favourable conditions for bivalve nutrition.

2. In regard to phytoplankton composition, along the Basque coast, diatoms revealed as the dominant group in surface waters, in terms of number of bloom-forming species, spatial incidence of blooms and peaks of cell abundance, suggesting favourable conditions for mussel culture. Similarly, in the experimental farm in particular, the observed high contribution of diatoms, dinoflagellates and haptophytes would also benefit mussel production due to the biochemical composition of these groups.

3. Along the coast, several potentially toxic species were recorded and the three main genera of concern (i.e., *Pseudo-nitzschia* spp., *Dinophysis* spp. and *Alexandrium* spp.) showed a wide geographic distribution. These toxic genera exceeded the abundance alert limits in several occasions (especially in spring and summer) and in most sampling stations.

4. Variations in phytoplankton community were significantly explained by different environmental variables in each season. The variability of the phytoplankton community at the level of major taxonomic groups was much less explained by the environment compared to that at the lowest taxonomic level. The variability of individual taxa was mainly explained by temperature and nutrients (mostly ammonium and phosphate).

5. Potentially toxic taxa showed heterogeneity and different associations with the environment, even within the same genus. However, an association between ammonium concentrations and some potential DSP producers was found in summer and autumn; as well as an association between the biomass of several species of *Dinophysis* and their prey *Mesodinium* spp. in winter.

6. Within the experimental bivalve farm, the seasonal cycle found for phytoplankton community coincided with the previously described pattern for the southern Bay of Biscay. Accordingly, phytoplankton abundance and biomass showed the highest values in surface waters during spring, which suggests better conditions for mussel growth in terms of food availability during this season. Then, this situation relatively reversed during thermal stratification period, when a progressive decrease in surface abundance and biomass occurred with the simultaneous increase at the deeper layers.

7. Among the toxins included in the European legislation, it has been possible to detect the causative species of the most troubling toxin in this area: the okadaic acid. The increase in this diarrheic toxin concentration was observed recurrently during spring and it can be associated with the presence of the dinoflagellate *Dinophysis acuminata.* ASP, PSP and YTX were detected less frequently and showing relatively lower concentrations, as none of these detections would have implied a ban on mussel harvest.

8. The abundance peaks given by toxic species were registered more frequently in the first 17 m of the water column, which coincides with the depth at which mussel cultures are usually located.

Thesis

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

"In open waters off the Basque coast, (i) the phytoplankton attributes involved in mussel nutrition present seasonal differences, and regarding spatial variability, the vertical heterogeneity throughout the water column was proven but not the variability along the coast, and (ii) the phytoplankton community implies the presence of biotoxins in these bivalves' flesh under specific environmental conditions."

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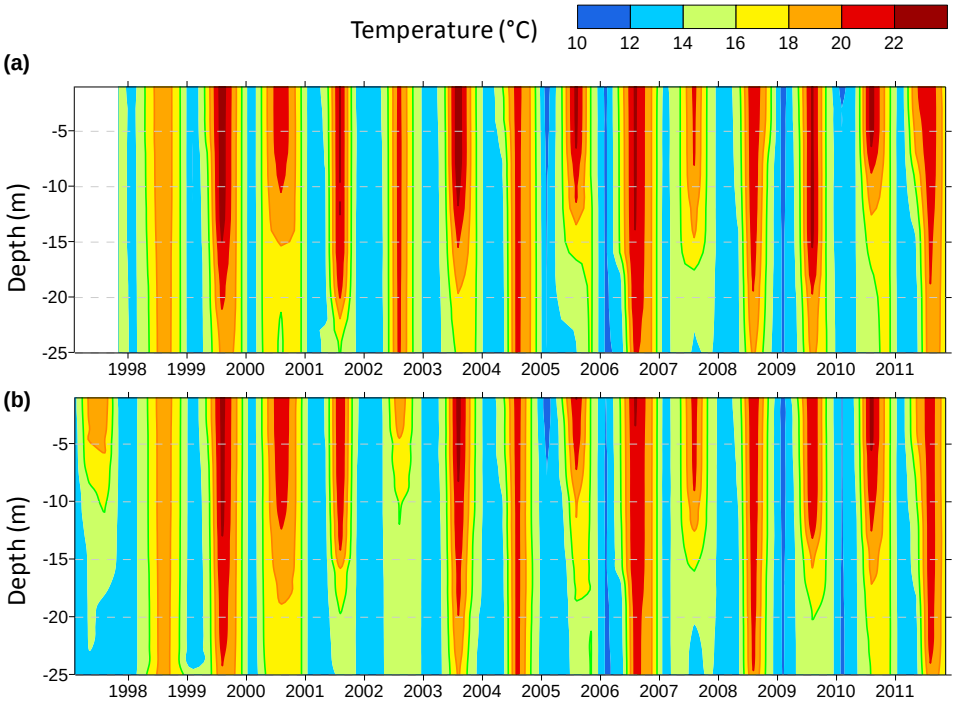
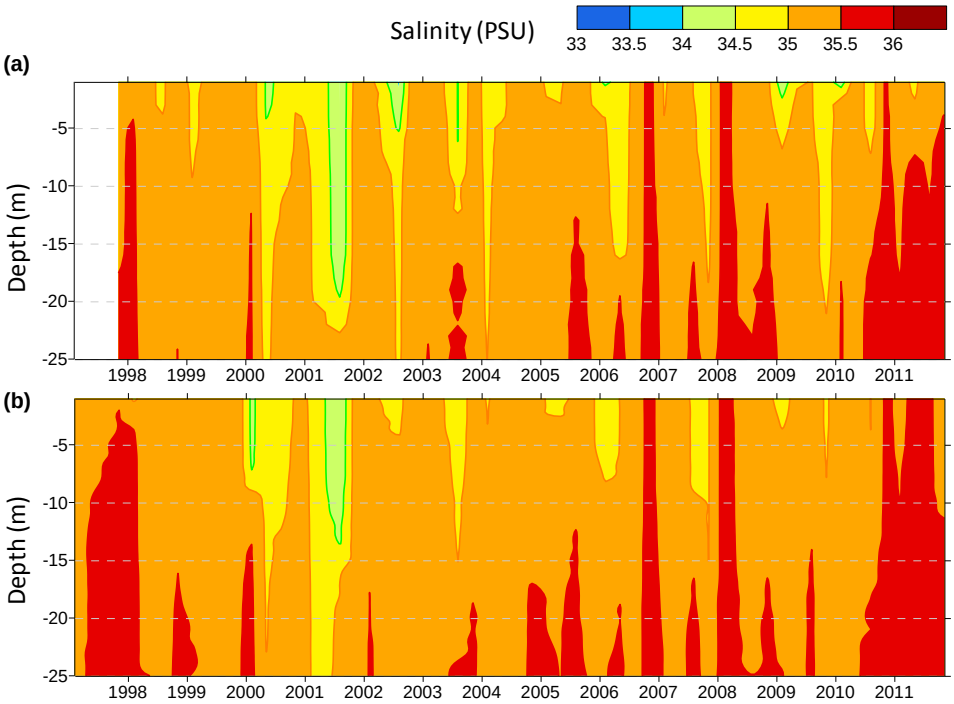
VIII. ANNEXES

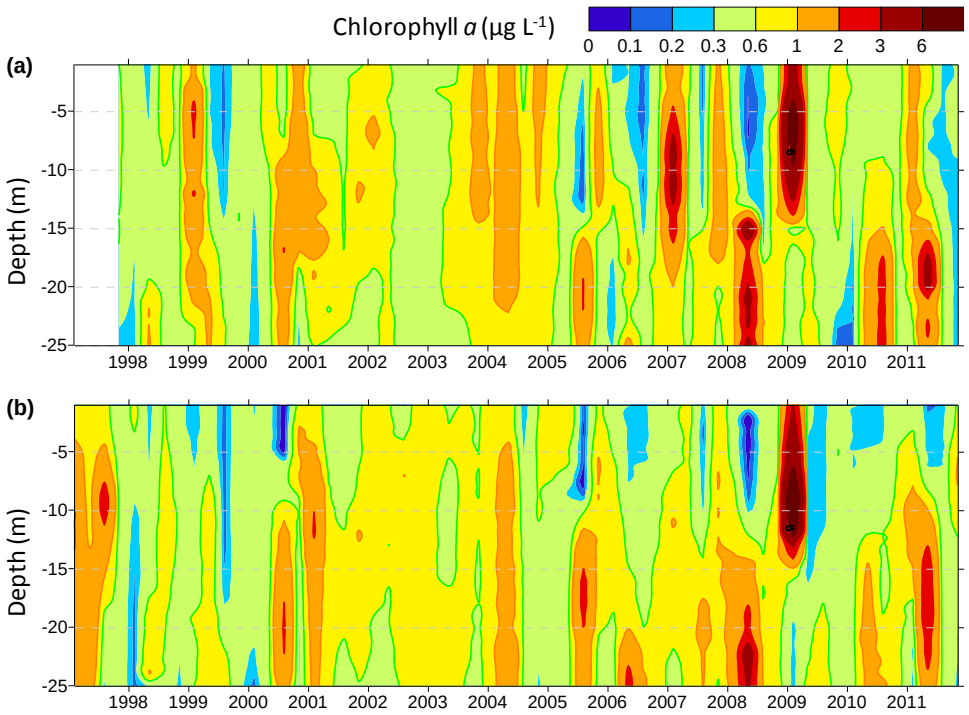
ANNEXES FOR THE STUDY AREA



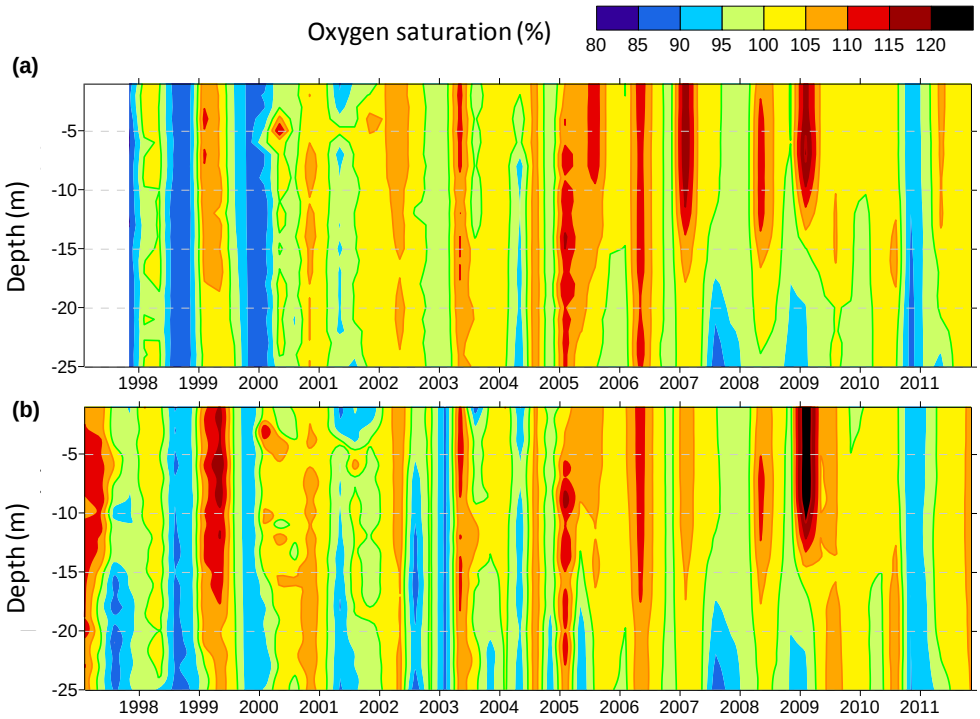
Annex III.1. Contour maps of temperature throughout the water column obtained from quarterly CTD casts at the stations A10 (a) and L10 (b) off the Basque coast. The period from 1997 to 2011 is represented.



Annex III.2. Contour maps of salinity throughout the water column obtained from quarterly CTD casts at the stations A10 (a) and L10 (b) off the Basque coast. The period from 1997 to 2011 is represented.



Annex III.3. Contour maps of chlorophyll *a* concentration throughout the water column obtained from quarterly CTD casts at the stations A10 (a) and L10 (b) off the Basque coast. Notice that the employed scale is approximately logarithmic (Log_{10}).



Annex III.4. Contour maps of oxygen saturation throughout the water column obtained from quarterly CTD casts at the stations A10 (a) and L10 (b) off the Basque coast.

ANNEXES FOR THE SPECIFIC INVESTIGATIONS

Annex 1.1. Summary of all the samplings performed for phytoplankton at the different stations along the Basque coast during the period 2003-2013.

STATION		2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
N10	**Winter**					Feb.	Feb.	Feb.	Jan.	Mar.	Feb.	Feb.
	Spring	May		May	May	May	May	May	May	May.	May.	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Sep.	Sep.	Aug.	Sep.	Sep.	Sep.
	Autumn					Nov.	Nov.	Nov.	Oct.	Nov.	Oct.	Oct.
N20	**Winter**						Feb.	Feb.	Jan.	Mar.	Feb.	Feb.
	Spring	May	May	May	May	May	May	May	May	May.	May.	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Sep.	Aug.	Sep.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
B10	**Winter**					Mar.	Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May.	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn					Nov.	Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
B20	**Winter**						Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
OK10	**Winter**						Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
L10	**Winter**					Mar.	Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn					Nov.	Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
L20	**Winter**						Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
A10	**Winter**						Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
D10	**Winter**						Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Oct.	Oct.

Annex 1.1 (continued). Summary of all the samplings performed for phytoplankton at the different stations along the Basque coast during the period 2003-2013.

STATION		2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
U10	**Winter**					Mar.	Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn					Nov.	Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
O20	**Winter**						Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May		May	May	May	May.	May	May.
	Summer	Sep.			Sep.		Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
O10	**Winter**						Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Sep.	Aug.	Aug.	Sep.	Sep.	Aug.	Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Oct.	Oct.
OI10	**Winter**						Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May		May	May	May	May	May	May.	May	May.
	Summer	Aug.	Aug.	Aug.	Sep.	Aug.	Aug.	Aug.	Aug.	Aug.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Nov.	Nov.
OI20	**Winter**						Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Aug.	Aug.	Aug.	Sep.	Aug.	Aug.	Aug.	Aug.	Aug.	Sep.	Sep.
	Autumn						Oct.	Nov.	Oct.	Nov.	Nov.	Nov.
BI10	**Winter**					Feb.	Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May.	May	May.
	Summer	Aug.	Aug.	Aug.	Sep.	Aug.	Aug.	Aug.	Aug.	Aug.	Sep.	Sep.
	Autumn					Oct.	Oct.	Nov.	Oct.	Nov.	Nov.	Nov.
UR20	**Winter**					Feb.	Jan.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May.	May	May.	May.	May.
	Summer	Aug.	Aug.	Aug.	Sep.	Aug.	Aug.	Aug.	Aug.	Aug.	Sep.	Sep.
	Autumn					Oct.	Oct.	Nov.	Oct.	Nov.	Nov.	Nov.
RF30	**Winter**							Feb.	Feb.	Mar.	Feb.	Mar.
	Spring							May.	May	May.	May.	May.
	Summer							Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn							Nov.	Oct.	Nov.	Oct.	Oct.
RF20	**Winter**							Feb.	Feb.	Mar.	Feb.	Mar.
	Spring							May.	May	May.	May.	May.
	Summer							Aug.	Aug.	Sep.	Sep.	Sep.
	Autumn							Nov.	Oct.	Nov.	Oct.	Oct.
RF10	**Winter**					Feb.	Feb.	Feb.	Feb.	Mar.	Feb.	Mar.
	Spring	May	May	May	May	May	May	May	May	May	May	May
	Summer	Aug.	Aug.		Sep.	Aug.	Aug.	Aug.	Aug.	Aug.	Sep.	Sep.
	Autumn					Oct.	Oct.	Nov.	Oct.	Nov.	Nov.	Nov.

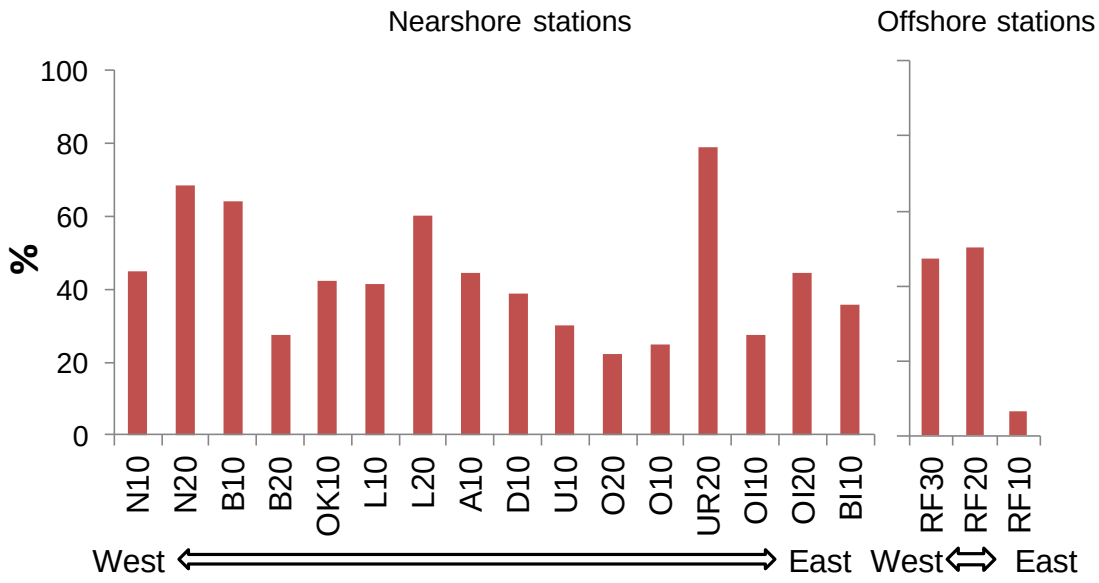
Annex 1.2. Complete list of the registered taxa along the 19 sampled stations of the Basque coastal waters for the period 2003-2013. Chain-forming diatoms are pointed with *.

Alexandrium sp. Halim
Amphidinium acutissimum J.Schiller
Amphidinium acutum Lohmann
Amphidinium crissum Lohmann
Amphidinium sp. Claparéde & Lachmann
Amphisolenia globifera F.Stein
Apedinella spinifera (Throndsen) Throndsen
Asterionellopsis glacialis (Castracane) Round*
Aulacoseira italica (Ehrenberg) Simonsen*
Autotrophic cocoids
Azadinium caudatum var. *caudatum* (Halldal) Nézan & Chomérat
Azadinium caudatum var. *margalefii* (Halldal) Nézan & Chomérat
Bacteriastrum furcatum Shadbolt*
Bacteriastrum hyalinum Lauder*
Bacteriastrum sp. Shadbolt*
Biddulphia sp. S.F.Gray
Braarudosphaera bigelowi (Gran & Braarud) Deflandre
Calciopappus caudatus K.R.Gaarder & E.Ramsfjell
Calciopappus sp. K.R.Gaarder & E.Ramsfjell
Calciosolenia murrayi Gran
Calciosolenia sp. Gran
Calycomonas ovalis Lohmann
Calyptrosphaera sp. Lohmann
Centrales >20 µm
Centrales ≤10 µm
Centrales 10-20 µm
Cerataulina pelagica (Cleve) Hendey*
Ceratocorys horrida Stein
cf. *Fragilidium* Balech ex Loeblich
cf. *Gloeodinium marinum* Bouquaheux
cf. *Haslea* Simonsen
cf. *Lioloma* Hasle
Chaetoceros affinis Lauder*
Chaetoceros anastomosans Grunow*
Chaetoceros brevis F.Schütt*
Chaetoceros compressus Lauder*
Chaetoceros constrictus Gran*
Chaetoceros costatus Pavillard*
Chaetoceros crinitus F.Schütt*
Chaetoceros curvisetus Cleve*
Chaetoceros danicus Cleve
Chaetoceros debilis Cleve*
Chaetoceros decipiens Cleve*
Chaetoceros densus (Cleve) Cleve*
Chaetoceros diadema (Ehrenberg) Gran*
Chaetoceros didymus Ehrenberg*
Chaetoceros furcellatus Yendo*
Chaetoceros laciniosus F.Schütt*
Chaetoceros lorenzianus Grunow*
Chaetoceros peruvianus Brightwell
Chaetoceros pseudocurvisetus Mangin*
Chaetoceros rostratus Ralfs*
Chaetoceros salsugineus Takano*
Chaetoceros similis Cleve*
Chaetoceros socialis H.S.Laud*er
Chaetoceros (*Chaetoceros*) spp. Ehrenberg*
Chaetoceros (*Hyalochaete*) spp.*
Chaetoceros spp. (solitary cells) Ehrenberg
Chaetoceros tenuissimus Meunier
Chaetoceros teres Cleve*
Chlamydomonas sp. Ehrenberg ≤ 5 µm
Chlamydomonas spp. Ehrenberg
Chlorophycota (cocoids)
Chlorophycota (flagellates)
Chroomonas sp. Hansgirg
Chrysochromulina hirta Manton
Chrysochromulina lanceolate Chrétiennot-Dinet, Nezan & Puigserver
Chrysochromulina parkeae J.C.Green & Leadbeater
Coccolithophores
Coolia monotis Meunier
Corethron criophilum Castracane
Corethron hystrix Hensen
Corymbellus aureus J.C.Green
Corythodinium michaelsarsii (Gaarder) Taylor
Coscinodiscus sp. Ehrenberg
Crucigenia tetrapedia (Kirchner) Kuntze
Cryptophycophyta <5 µm
Cryptophycophyta 5-10 µm
Cyanophycota (filaments)
Cyclotella meneghiniana

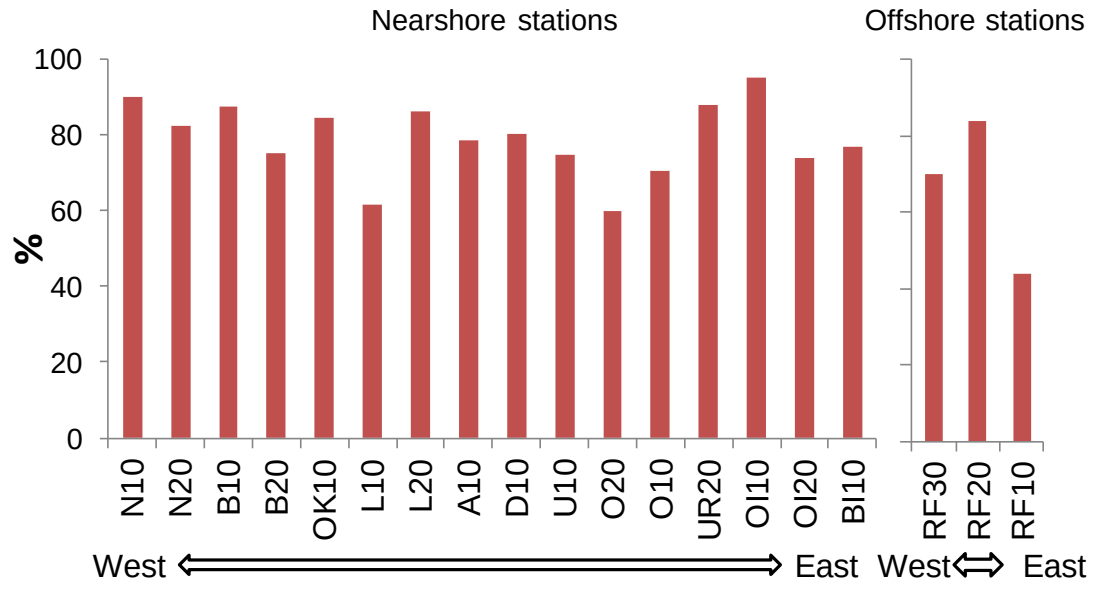
Cyclotella spp. (Kützing) Brébisson
Cylindrotheca closterium Kützing
Cymbomonas sp. J.Schiller
Cymbomonas tetramitiformis Schiller
Dactyliosolen fragilissimus (Bergon) Hasle*
Dactyliosolen phuketensis (B.G.Sundström) G.R.Hasle*
Detonula pumila (Castracane) Gran*
Dictyocha crux Ehrenberg
Dictyocha fibula Ehrenberg
Dictyocha sp. Ehrenberg
Dictyocha speculum Ehrenberg
Dinobryon faculiferum (Willén) Willén
Dinobryon sp. Ehrenberg
Dinophyceae >20 µm
Dinophyceae ≤20 µm
Dinophysis acuminata Claparède & Lachmann
Dinophysis acuta Ehrenberg
Dinophysis caudata W.S.Kent
Dinophysis cf. *ovum* T.H.Avé
Dinophysis fortii Pavillard
Dinophysis sp. Ehrenberg
Dinophysis tripos Gourret
Diplopsalis R.S.Bergh group
Dissodinium sp. Klebs
Ditylum brightwellii (T.West) Grunow
*Ebria tripartita (*J.Schumann) Lemmermann
Emiliania huxleyi (Lohmann) W.W.Hay & H.P.Mohler
Eucampia zodiacus Ehrenberg*
Euglena sp. Ehrenberg
Euglenophycota
Eutreptiella eupharyngea Moestrup & R.E.Norris
Eutreptiella gymnastica Throndsen
Eutreptiella spp. A.M.da Cunha
Goniodoma polyedricum (Pouchet) Jørgensen
Goniodoma sphaericum Murray & Whitting
Gonyaulax cf. *digitale* (C.H.G.Pouchet) Kofoid
Gonyaulax polygramma Stein
Gonyaulax sp. Diesing
Gonyaulax spinifera (Claparède & Lachmann) Diseing
Guinardia delicatula (Cleve) Hasle*
Guinardia flaccida (Castracane) H.Peragallo
Guinardia sp. H.Peragallo*
Guinardia striata (Stolterfoth) Hasle*
Gymnodiniales >20 µm
Gymnodiniales ≤20 µm
Gymnodinium elongatum Hope
Gymnodinium impudicum (S.Fraga & I.Bravo) Gert Hansen & Ø.Moestrup)
Gymnodinium spp. F.Stein
Gyrodinium biconicum Kofoid & Swezy
Gyrodinium cf. *flagellare* J.Schiller
Gyrodinium sp. Kofoid & Swezy >50 µm
Gyrodinium sp. Kofoid & Swezy ≤20 µm
Gyrodinium sp. Kofoid & Swezy 20-50 µm
Gyrodinium spirale (Bergh) Kofoid & Swezy
Halosphaera sp. F.Schmitz
Haptophyta 10-15 µm
Helicotheca sp. M.Ricard*
Helicotheca tamesis (Shrubsole) M.Ricard*
Hemiaulus hauckii Grunow*
Hemiaulus sp. Heiberg*
Hemiselmis spp. Parke
Heterocapsa cf. *minima* A.J.Pomroy
Heterocapsa cf. *pygmaea* Lobelich III, R.J.Schmidt & Sherley
Heterocapsa cf. *rotundata* (Lohmann) Gert Hansen
Heterocapsa sp. F.Stein
*Heterocapsa triquetra (*Ehrenberg) Stein
Heterosigma akashiwo Y.Hada
Imantonia rotunda N.Reynolds
Isochrysis galbana Parke
Karenia cf. *mikimotoi (*Miyake & Kominami) Gert Hansen & Ø. Moestrup
Karenia cf. *papilionacea* A.J.Haywood & K.A.Steidinger
Karenia sp. Gert Hansen & Moestrup
Katablepharis remigera (N.Vørs) B.Clay & P.Kugrens
Katodinium glaucum (Lebour) Loeblich III
Katodinium sp. B.Fott
Kofoidinium velleloides Pavillard
Kryptoperidinium foliaceum (F.Stein) Lindemann
Lauderia annulata Cleve*
Lepidodinium chlorophorum (M.Elbrächter & E.Schnepf) Gert Hansen, Botes & Salas
Leptocylindrus convexus D.Nanjappa & A.Zingone*

Leptocylindrus danicus/hargravesii Cleve/D.Nanjappa & A.Zingone*
Leptocylindrus minimus Gran*
Leptocylindrus sp. Cleve*
Leucocryptos marina (Braarud) Butcher
Leucocryptos sp. Butcher
*Licmophora gracilis (*Ehrenberg) Grunow
Licmophora sp. C.Agardh
Lingulodinium polyedra (F. Stein) J.D.Dodge
Lithodesmium undulatum Ehrenberg*
Mamiella gilva (Parke & Rayns) Ø.Moestrup
Mamiella sp. Ø.Moestrup
Melosira nummuloides C.Agardh*
Melosira varians C.Agardh*
Meringosphaera mediterranea Lohmann
Meringosphaera sp. Lohmann
Merismopedia sp. F.J.F.Meyen
Mesodinium rubrum Leegaard
Mesoporos perforatus (Gran) Lillick
Meuniera membranacea (Cleve) P.C.Silva*
Monoraphidium sp. Komárková-Legnerová
Nephroselmis sp. Stein
Nitzschia longissima (Brébisson) Ralfs
Nitzschia sp. Hassall
Oblea sp. Balech
*Octactis octonaria (*Ehrenberg) Hovasse
Odontella mobiliensis (Bailey) Grunow
Ollicola vangoorii (W.Conrad) Vørs
Oltmannsiellopsis sp. M.Chihara & I.Inouye
Oltmannsiellopsis viridis (P.E.Hargraves & R.L.Steele) M.Chihara & I.Inouye
*Ophiaster hydroideus (*Lohmann) Lohmann
Oscillatoria sp. Vaucher
Ostreopsis cf. *siamensis* Johs.Schmidt
Oxytoxum cf. *milneri* Murray & Whitting
Oxytoxum gracile Schiller
Oxytoxum laticeps Schiller
Oxytoxum scolopax Stein
Oxytoxum sp. Stein
Oxytoxum sphaeroideum Stein
Oxytoxum tesselatum (Stein) F.Schütt
Pachysphaera pelagica Ostenfeld
Pachysphaera sp. Ostenfeld
Palaeophalacroma unicinctum Schiller
Paralia sulcata (Ehrenberg) Cleve*
Pediastrum sp. Meyen
Pedinellales
Pennales >50 µm
Pennales ≤10 µm
Pennales 10-50 µm
Peridinium quinquecorne Abé
Peridinium sp. Ehrenberg
Petalomonas sp. F.Stein
Phaeocystis globose Scherffel
Phaeocystis sp. Lagerheim
Phalacroma mitra F.Schütt
Phalacroma rotundatum (Claparède & Lachmann) Kofoid & Michener
Plagioselmis spp. Butcher ex G.Novarino, I.A.N.Lucas & S.Morrall
Planktoniella sol (G.C.Wallich) Schütt
Pleurochrysis carterae (Braarud & Fagerland) T.Christensen
Pleurosigma sp. W.Smith
Podolampas bipes Stein
Polykrikos sp. Bütschli
Prasinophyceae
Proboscia alata (Brightwell) Sundström
Pronoctiluca pelagica Fabre-Domergue
Pronoctiluca sp. Fabre-Domergue
Prorocentrum balticum (Lohmann) Loeblich III
Prorocentrum compressum (Bailey) Abé
Prorocentrum dentatum Stein
Prorocentrum gracile F.Schütt
Prorocentrum micans Ehrenberg
Prorocentrum minimum (Pavillard) J.Schiller
Prorocentrum rhathymum A.R.Loeblich III, Sherley & Schmidt
Prorocentrum sp. Ehrenberg
Prorocentrum triestinum J.Schiller
Prorocentrum vaginulum (Ehrenberg) Dodge
Protoperidinium bipes (Paulsen) Balech
Protoperidinium conicum (Gran) Balech
Protoperidinium curtipes (Jørgensen) Balech
Protoperidinium diabolus (Cleve) Balech
Protoperidinium oblongum (Aurivillius) Parke & Dodge
Protoperidinium oceanicum (Vanhöffen) Balech
Protoperidinium sp. R.S.Bergh
Protoperidinium steinii (Jørgensen) Balech
Protoceratium reticulatum (Claparède & Lachmann) Bütschli

Prymnesiales
Pseudo-nitzschia galaxiae N.Lundholm & Moestrup
Pseudo-nitzschia multistriata H.Takano*
Pseudo-nitzschia pungens (Grunow ex Cleve) Hasle*
Pseudo-nitzschia sp. H.Peragallo <3 µm*
Pseudo-nitzschia sp. H.Peragallo >3 µm*
Pseudopediastrum boryanum (Turpin) E.Hegewald
Pseudopedinella pyriformis N.Carter
Pseudopedinella sp. N.Carter
Pseudoscourfieldia marina (J.Throndsen) Manton
Pterosperma sp. Pouchet
Pyramimonas octopus Moestrup & Kristiansen
Pyramimonas sp. Schmarda
Rapaza viridis A.Yamaguchi, N.Yubuki & B.S.Leander
Rhabdosphaera clavigera G.Murray & Blackman
Rhizosolenia hebetata Bailey
Rhizosolenia setigera Brightwell
Rhizosolenia spp. Brightwell
Rhodomonas sp. G.Krasten
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot
Scenedesmus sp. Meyen
Scrippsiella cf. *minima* Gao & Dodge
Scrippsiella group Balech
Skeletonema cf. *menzelii* Guillard, Carpenter & Reimann
Skeletonema sp. Greville*
Striatella unipunctata (Lyngbye) C.Agardh
Syracosphaera sp. Lohmann
Takayama sp. M.F.Salas, Bolch, Botes & Hallegraeff
Tasmanites marshalliae (M.Parke) G.T.Boalch & D.Guy-Ohlson
Teleaulax acuta (Butcher) D.R.A.Hill
Teleaulax amphioxeia (W.Conrad) D.R.A.Hill
Teleaulax gracilis Laza-Martinez
Teleaulax minuta Laza-Martinez
Teleaulax spp. D.R.A.Hill
Telonema sp. Griessman
Tenuicylindrus belgicus (Meunier) D.Nanjappa & A.Zingone
Tetraselmis sp. F.Stein
Thalassionema nitzschioides (Grunow) Mereschkowsky*
Thalassionema sp. Grunow*
Thalassiosira cf. *mediterranea** (Schröder) Hasle
Thalassiosira oceanica Hasle*
Thalassiosira rotula Meunier*
Thalassiosira sp. (chain-forming <10 µm)*
Thalassiosira sp. (chain-forming >20 µm)*
Thalassiosira sp. (chain-forming 10-20 µm)*
Thalassiosira spp. Cleve*
Thalassiothrix longissima Cleve & Grunow
Torodinium robustum Kofoid & Swezy
Torodinium sp. Kofoid & Swezy
*Torodinium teredo (*Pouchet*)* Kofoid & Swezy
Tripos arietinus (Cleve) F.Gómez
Tripos azoricus (Cleve) F.Gómez
Tripos belone (Cleve) F.Gómez
Tripos candelabrum (Ehrenberg) F.Gómez
Tripos carriensis (Gourret) F.Gómez
Tripos cf. *pentagonus* (Gourret) F.Gómez
Tripos falcatus (Kofoid) F.Gómez
Tripos furca (Ehrenberg) F.Gómez
Tripos fusus (Ehrenberg) F.Gómez
Tripos gibberus (Gourret) F.Gómez
Tripos hexacanthus (Gourret) F.Gómez
Tripos horridus (Cleve) F.Gómez
Tripos lineatus (Ehrenberg) F.Gómez
Tripos longipes (Bailey) F.Gómez
Tripos macroceros (Ehrenberg) F.Gómez
Tripos massiliensis (Gourret) F.Gómez
Tripos minutus (Jørgensen) F.Gómez
Tripos muelleri Bory
Tripos sp. Bory
Tripos trichoceros (Ehrenberg) F.Gómez
Unidentified small flagellates
Urgorri complanatus Laza-Martinez
Warnowia sp. Lindemann



Annex 1.3. Contribution (%) to total abundance of chain forming diatoms that belong to the cell-size range 2-20 µm. Abundance data for the whole period 2003-2013 are included for each sampling station.



Annex 1.4. Contribution (%) of diatoms to total biomass at each sampling station for the period 2003-2013.

Annex 1.5. ANOVA results testing differences in mean values of log transformed (a) cell abundance and (b) biomass of phytoplankton along the Basque coast for the period 2003-2013.

(a)	**Sum of squares**	**df**	**Mean square**	**F-ratio**	***p*-value**
Season	13.2617	3	4.42057	21.77	<0.0001
Year	13.2021	10	1.32021	6.50	<0.0001
Sampling station	3.11606	18	0.173114	0.85	0.6378
Residual	120.235	592	0.203099		
Corrected total	151.055	623			
(b)					
Season	47.9879	3	15.996	39.53	<0.0001
Year	11.5139	10	1.1514	2.85	<0.005
Sampling station	11.3112	18	0.6284	1.55	0.0671
Residual	239.575	592	0.4047		
Corrected total	313.196	623			

Annex 1.6. Highest cell densities recorded within the major phytoplankton groups, in the open coastal waters of the Basque country (2003-2013 sampling period). Diat: diatoms. Chlo: chlorophytes. Cryp: cryptophytes. Unid: unidentified forms < 10µm. Hapt: haptophytes. Dino: dinoflagellates. Raph: raphidophyceans. Auto: autotrophic ciliates. Eugl: euglenophytes.

Group	**Taxon**	**Abundance (cells L^{-1})**	**Contribution to total abundance (%)**	**Station**	**Year**	**Season**
Diat	*Thalassiosira* spp.	5.1×10^7	95.8	UR20	2011	winter
Chlo	*Tetraselmis* spp.	4.1×10^6	97.1	O20	2004	spring
Cryp	*Hemiselmis* spp.	2.5×10^6	7.3	B10	2009	spring
Unid	Flagellates	1.8×10^6	15.3	OI10	2011	summer
Hapt	*Phaeocystis globosa*	1.7×10^6	3.3	UR20	2011	winter
Dino	*Heterocapsa* cf.	4.7×10^5	66.1	RF10	2009	winter
Raph	*Heterosigma akashiwo*	5.1×10^4	3.9	OI10	2009	summer
Auto	*Mesodinium rubrum*	2.1×10^4	4.0	N10	2013	summer
Eugl	*Petalomonas* spp.	5.3×10^3	1.0	N10	2007	autumn

Annex 2.1. Results of the Multiple Range Tests (95% least significant difference, LSD) for the environmental variables by year over the studied period 2003-2015. The "X" aligned in columns represent the different homogeneous groups. There are no statistically significant differences between the levels that share a column of X's.

a. Secchi depth

Spring

Year	*Cases*	*Mean*	*Groups*
2003	17	1.88939	X
2007	16	2.30292	XX
2011	19	2.39801	XX
2004	16	2.58913	XX
2013	19	2.65409	XX
2010	19	2.89511	XX
2006	17	3.00055	XXX
2009	19	3.16625	XX
2008	17	3.47195	XX
2005	16	3.69818	X
2014	19	3.85918	X
2015	19	3.96462	XX
2012	19	4.35627	X

Summer

Year	*Cases*	*Mean*	*Groups*
2010	19	2.57877	X
2003	17	2.78169	XX
2005	15	2.79067	XX
2008	17	3.10157	XX
2009	19	3.16625	XX
2006	17	3.23625	XXX
2007	16	3.35831	XX
2014	19	3.42233	XX
2012	19	3.52778	XXX
2011	19	3.7236	XXX
2004	16	3.98439	XXX
2013	19	4.00981	XX
2015	19	4.25083	X

b. **Temperature**

Spring

Year	*Cases*	*Mean*	*Groups*
2010	19	2.35142	X
2013	19	2.46365	X
2012	19	2.47519	XX
2003	17	2.48199	XX
2015	19	2.49596	XXX
2008	17	2.5537	XXXX
2009	19	2.57673	XXXX
2014	19	2.59549	XXX
2005	16	2.62426	XX
2004	16	2.63454	XX
2007	16	2.67925	X
2006	17	2.82425	X
2011	19	3.30401	X

Summer

Year	*Cases*	*Mean*	*Groups*
2015	19	3.8116	X
2007	16	4.04511	X
2004	16	4.16758	XX
2014	19	4.17899	XX
2012	19	4.22573	XX
2011	19	4.24612	XX
2013	19	4.2729	XX
2005	15	4.2965	XX
2009	19	4.30561	XX
2008	17	4.33358	XX
2006	17	4.47459	XX
2010	19	4.49836	X
2003	17	4.74865	X

c. Salinity

Spring

Year	*Cases*	*Mean*	*Groups*
2003	17	1.98358	X
2013	19	2.09336	X
2004	16	2.1683	XX
2005	16	2.31538	XXX
2014	19	2.4187	XXX
2012	19	2.54121	XXX
2007	16	2.60645	XXX
2006	17	2.74472	XXX
2015	19	2.88444	XX
2009	19	3.12511	XX
2010	19	3.52091	XX
2008	17	3.61006	X
2011	19	3.67931	X

Summer

Year	*Cases*	*Mean*	*Groups*
2014	19	2.09628	X
2007	16	2.29143	X
2003	17	2.3243	X
2010	19	2.85323	X
2015	19	2.88434	XX
2013	19	2.97431	XX
2006	17	3.16679	XX
2005	15	3.38868	XX
2009	19	3.58256	X
2008	17	3.63175	X
2004	16	3.71553	XX
2011	19	3.96983	X
2012	19	4.98698	X

d. Suspended solids

Spring

Year	*Cases*	*Mean*	*Groups*
2005	16	1.28115	X
2006	17	1.35961	X
2003	17	1.43403	X
2004	16	2.01055	X
2011	19	2.58392	X
2010	19	3.22214	X
2009	19	3.24615	XX
2014	19	3.27755	XX
2007	16	3.29707	XX
2008	17	3.37739	XX
2015	19	3.58962	XXX
2012	19	3.62947	XX
2013	19	3.82094	X

Summer

Year	*Cases*	*Mean*	*Groups*
2005	15	1.73715	X
2007	16	1.92845	XX
2003	17	1.9456	XX
2010	19	2.03619	XXX
2011	19	2.14892	XX
2006	17	2.16113	XX
2004	16	2.2392	XX
2008	17	2.25189	XX
2009	19	2.37051	X
2015	19	3.10066	X
2012	19	3.44157	XX
2013	19	3.59044	X
2014	19	3.61706	X

e. Ammonium

Spring

Year	*Cases*	*Mean*	*Groups*
2003	17	1.63465	X
2008	17	2.14877	X
2010	19	2.18875	X
2015	19	2.37366	X
2004	16	2.4346	XX
2011	19	2.79715	XX
2009	19	2.9484	XX
2014	19	3.12143	XXX
2005	16	3.20705	XX
2012	19	3.421	X
2006	17	3.95495	X
2013	19	4.36404	X
2007	16	4.53314	X

Summer

Year	*Cases*	*Mean*	*Groups*
2003	17	1.61636	X
2015	19	2.09303	X
2007	16	2.29624	X
2008	17	2.3364	X
2012	19	2.41436	X
2010	19	2.94189	X
2005	15	3.31826	XX
2004	16	3.54301	X
2014	19	3.57159	X
2009	19	3.59598	X
2011	19	3.70716	X
2006	17	4.14113	X
2013	19	4.43906	X

f. Nitrate

Spring

Year	*Cases*	*Mean*	*Groups*
2015	19	1.8672	X
2014	19	2.13187	XX
2006	17	2.16002	XX
2009	19	2.44468	XX
2008	17	2.56646	XXX
2012	19	2.90557	XXX
2011	19	2.92041	XX
2005	16	2.96755	XX
2010	19	3.07353	XX
2003	17	3.09468	XX
2013	19	3.10482	XX
2007	16	3.50134	X
2004	16	3.55821	X

Summer

Year	*Cases*	*Mean*	*Groups*
2003	17	1.7701	X
2015	19	1.7701	X
2010	19	1.94035	XX
2006	17	1.94635	XX
2014	19	2.10093	X
2011	19	2.10161	X
2013	19	2.54801	X
2007	16	2.74243	X
2012	19	2.76633	X
2005	15	3.3976	X
2009	19	3.474	X
2008	17	3.56363	X
2004	16	3.71679	X

g. Phosphate

Spring

Year	*Cases*	*Mean*	*Groups*
2009	19	2.07023	X
2003	17	2.44509	XX
2006	17	2.53759	X
2013	19	2.81665	XX
2004	16	2.97802	XX
2012	19	3.02463	XX
2014	19	3.11616	XXX
2015	19	3.38515	XXX
2005	16	3.39495	XXXX
2011	19	3.45238	XXX
2007	16	3.73843	XX
2010	19	3.75374	XX
2008	17	3.81807	X

Summer

Year	*Cases*	*Mean*	*Groups*
2005	15	1.41995	X
2003	17	1.49095	X
2014	19	1.75656	XX
2013	19	2.1533	XX
2010	19	2.2988	XX
2012	19	2.47247	XX
2007	16	2.72999	X
2009	19	3.29358	X
2004	16	3.44995	XX
2008	17	3.46482	XX
2015	19	3.64671	XX
2006	17	3.69631	XX
2011	19	3.91326	X

h. Silicate

Spring

Year	*Cases*	*Mean*	*Groups*
2006	17	1.91276	X
2015	19	1.95397	XX
2014	19	2.37461	XX
2011	19	2.40431	X
2008	17	2.43274	X
2012	19	3.07613	X
2010	19	3.08119	X
2007	16	3.12126	X
2005	16	3.22877	X
2003	17	3.72963	X
2009	19	3.73137	X
2004	16	3.73766	X
2013	19	4.13939	X

Summer

Year	*Cases*	*Mean*	*Groups*
2003	17	1.87864	X
2011	19	2.1131	XX
2009	19	2.2283	XXX
2006	17	2.27609	XXX
2004	16	2.33819	XXX
2015	19	2.35365	XXX
2012	19	2.3561	XXX
2008	17	2.56216	XXX
2014	19	2.75682	XX
2013	19	2.88656	X
2010	19	2.94764	X
2007	16	3.44296	X
2005	15	3.77807	X

Annex 2.2. Results from the pairwise PERMANOVA for environmental data with "year" as fixed factor for the period 2003-2015. P-values indicate the significance of the differences between pairs of years.

a. Spring

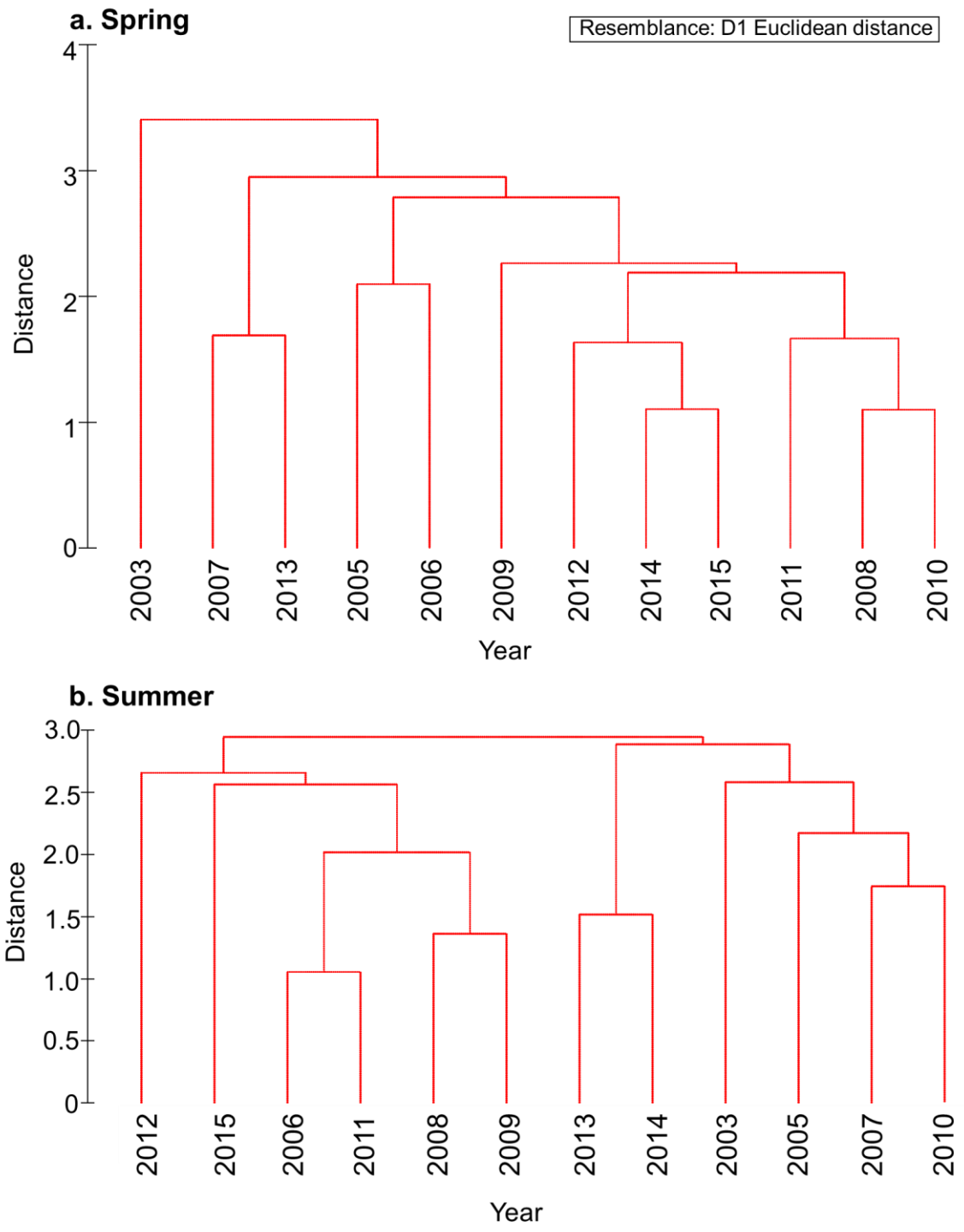
Groups	t	*p*-value	Unique perms
2003, 2004	1.7928	0.0416	9929
2003, 2005	3.2783	0.0001	9934
2003, 2006	4.863	0.0001	9937
2003, 2007	4.8516	0.0001	9938
2003, 2008	4.7611	0.0001	9953
2003, 2009	3.7173	0.0001	9945
2003, 2010	4.2478	0.0001	9943
2003, 2011	4.1369	0.0001	9948
2003, 2012	5.314	0.0001	9939
2003, 2013	5.499	0.0001	9958
2003, 2014	4.6001	0.0001	9944
2003, 2015	5.3865	0.0001	9945
2004, 2005	2.4273	0.0021	9944
2004, 2006	4.7302	0.0001	9934
2004, 2007	3.7675	0.0001	9939
2004, 2008	4.5093	0.0001	9945
2004, 2009	3.1866	0.0001	9942
2004, 2010	3.5535	0.0001	9939
2004, 2011	3.7185	0.0001	9950
2004, 2012	4.1611	0.0001	9952
2004, 2013	4.4694	0.0001	9947
2004, 2014	3.8948	0.0001	9934
2004, 2015	5.1403	0.0001	9941
2005, 2006	3.6551	0.0001	9951
2005, 2007	4.0179	0.0001	9951
2005, 2008	4.3771	0.0001	9949
2005, 2009	3.8634	0.0001	9942
2005, 2010	4.4918	0.0001	9945
2005, 2011	3.9308	0.0001	9941
2005, 2012	3.9679	0.0001	9937
2005, 2013	5.1947	0.0001	9942
2005, 2014	3.4931	0.0001	9942
2005, 2015	4.9564	0.0001	9946
2006, 2007	4.966	0.0001	9947
2006, 2008	6.315	0.0001	9947
2006, 2009	4.9285	0.0001	9938
2006, 2010	6.5351	0.0001	9946
2006, 2011	4.9949	0.0001	9963
2006, 2012	5.5233	0.0001	9944

2006, 2013	7.0895	0.0001	9950
2006, 2014	4.1084	0.0001	9955
2006, 2015	5.8186	0.0001	9939
2007, 2008	4.9455	0.0001	9941
2007, 2009	4.367	0.0001	9937
2007, 2010	4.8311	0.0001	9948
2007, 2011	4.1514	0.0001	9946
2007, 2012	4.1403	0.0001	9940
2007, 2013	2.4965	0.002	9945
2007, 2014	4.2934	0.0001	9939
2007, 2015	5.8991	0.0001	9929
2008, 2009	4.1678	0.0001	9953
2008, 2010	3.6983	0.0001	9938
2008, 2011	2.9268	0.0001	9949
2008, 2012	4.7332	0.0001	9952
2008, 2013	6.2703	0.0001	9938
2008, 2014	3.7644	0.0001	9942
2008, 2015	3.8151	0.0001	9938
2009, 2010	3.4943	0.0001	9945
2009, 2011	3.9503	0.0001	9944
2009, 2012	3.1416	0.0001	9941
2009, 2013	4.2502	0.0001	9942
2009, 2014	2.9551	0.0001	9945
2009, 2015	4.0344	0.0001	9944
2010, 2011	3.5278	0.0001	9947
2010, 2012	4.1445	0.0001	9936
2010, 2013	6.3641	0.0001	9936
2010, 2014	3.5794	0.0001	9953
2010, 2015	3.7831	0.0001	9942
2011, 2012	5.1617	0.0001	9938
2011, 2013	6.071	0.0001	9935
2011, 2014	4.0203	0.0001	9945
2011, 2015	4.7389	0.0001	9948
2012, 2013	4.5476	0.0001	9942
2012, 2014	2.1027	0.0045	9945
2012, 2015	3.687	0.0001	9933
2013, 2014	5.0732	0.0001	9932
2013, 2015	7.1928	0.0001	9936
2014, 2015	2.0092	0.0046	9938

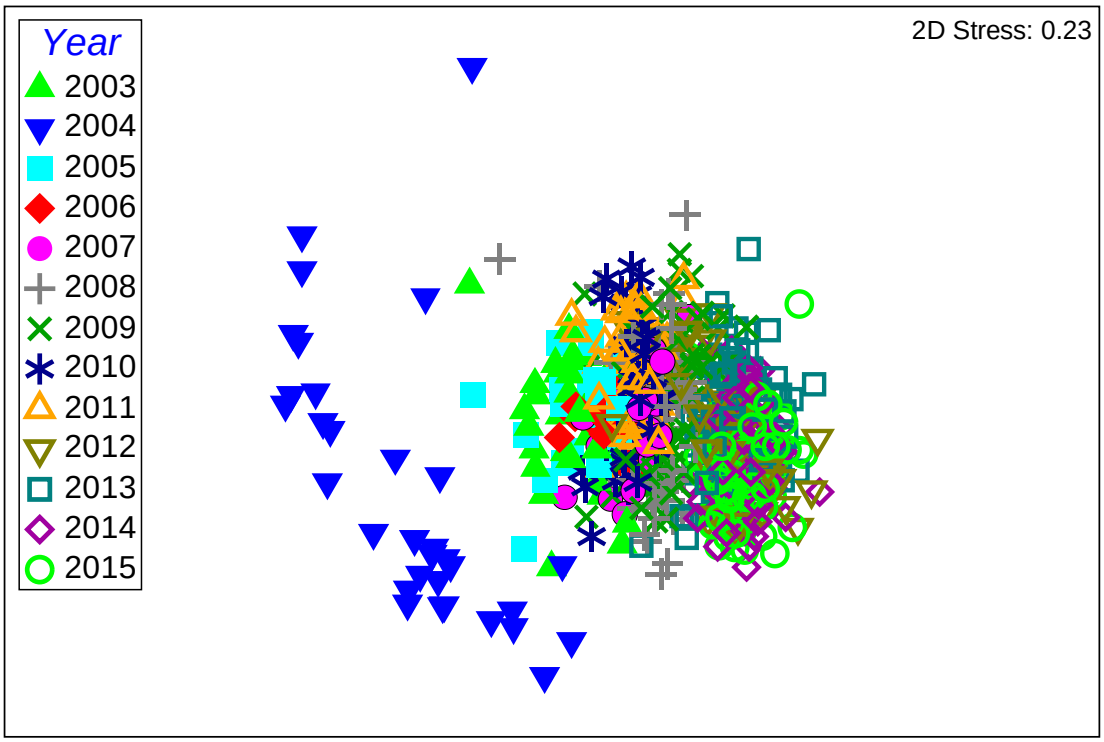
b. Summer

Groups	t	p-value	Unique perms
2003, 2004	7.6294	0.0001	9937
2003, 2005	9.4237	0.0001	9937
2003, 2006	8.1106	0.0001	9928
2003, 2007	3.2635	0.0001	9927
2003, 2008	8.0082	0.0001	9937
2003, 2009	8.8741	0.0001	9929
2003, 2010	4.7617	0.0001	9947
2003, 2011	7.7662	0.0001	9952
2003, 2012	7.6662	0.0001	9937
2003, 2013	11.227	0.0001	9936
2003, 2014	7.791	0.0001	9948
2003, 2015	6.2372	0.0001	9952
2004, 2005	4.6314	0.0001	9949
2004, 2006	2.9499	0.0001	9955
2004, 2007	3.0265	0.0007	9949
2004, 2008	3.0242	0.0005	9944
2004, 2009	2.0019	0.0166	9949
2004, 2010	5.0174	0.0001	9933
2004, 2011	3.0292	0.0001	9956
2004, 2012	4.5651	0.0001	9952
2004, 2013	4.1496	0.0001	9953
2004, 2014	4.9309	0.0001	9949
2004, 2015	5.1907	0.0001	9946
2005, 2006	6.1021	0.0001	9952
2005, 2007	2.7004	0.0011	9951
2005, 2008	5.6289	0.0001	9940
2005, 2009	5.1875	0.0001	9955
2005, 2010	4.4632	0.0001	9937
2005, 2011	6.1404	0.0001	9941
2005, 2012	6.3989	0.0001	9937
2005, 2013	6.7799	0.0001	9947
2005, 2014	6.403	0.0001	9943
2005, 2015	7.8516	0.0001	9945
2006, 2007	3.391	0.0002	9950
2006, 2008	4.9562	0.0001	9952
2006, 2009	3.6647	0.0001	9941
2006, 2010	4.6847	0.0001	9947
2006, 2011	2.8985	0.0001	9943
2006, 2012	6.1025	0.0001	9942
2006, 2013	4.5689	0.0001	9944
2006, 2014	4.5392	0.0001	9945
2006, 2015	5.596	0.0001	9924
2007, 2008	2.4267	0.0052	9951

2007, 2009	3.1925	0.0002	9937
2007, 2010	1.9649	0.0217	9947
2007, 2011	3.4176	0.0001	9937
2007, 2012	3.8782	0.0001	9937
2007, 2013	4.1307	0.0001	9942
2007, 2014	3.7089	0.0001	9939
2007, 2015	2.8786	0.0003	9954
2008, 2009	2.8058	0.0002	9940
2008, 2010	4.6138	0.0001	9943
2008, 2011	3.8407	0.0001	9942
2008, 2012	4.3274	0.0001	9950
2008, 2013	7.2331	0.0001	9941
2008, 2014	6.9706	0.0001	9942
2008, 2015	5.0094	0.0001	9947
2009, 2010	4.8896	0.0001	9949
2009, 2011	3.0144	0.0001	9942
2009, 2012	4.9478	0.0001	9934
2009, 2013	5.2258	0.0001	9942
2009, 2014	6.2754	0.0001	9958
2009, 2015	5.97	0.0001	9945
2010, 2011	4.5505	0.0001	9947
2010, 2012	5.451	0.0001	9950
2010, 2013	6.484	0.0001	9942
2010, 2014	5.6246	0.0001	9943
2010, 2015	4.9825	0.0001	9940
2011, 2012	4.581	0.0001	9931
2011, 2013	5.475	0.0001	9940
2011, 2014	6.4103	0.0001	9942
2011, 2015	4.4066	0.0001	9946
2012, 2013	6.8085	0.0001	9938
2012, 2014	7.0006	0.0001	9950
2012, 2015	4.9586	0.0001	9956
2013, 2014	4.0742	0.0001	9946
2013, 2015	7.3283	0.0001	9938
2014, 2015	6.5188	0.0001	9941



Annex 2.3. Cluster dendrograms (Euclidean distance, group-average linkage) of environmental variables in spring and summer for the different years. Samples connected by red lines cannot be significantly differentiated (SIMPROF test at alpha = 0.05).



Annex 2.4. Multidimensional scaling (MDS) for phytoplankton abundance (*log (x + 1)* transformed) using zero-adjusted Bray-Curtis distances for the period 2003-2015 (symbols represent different years).

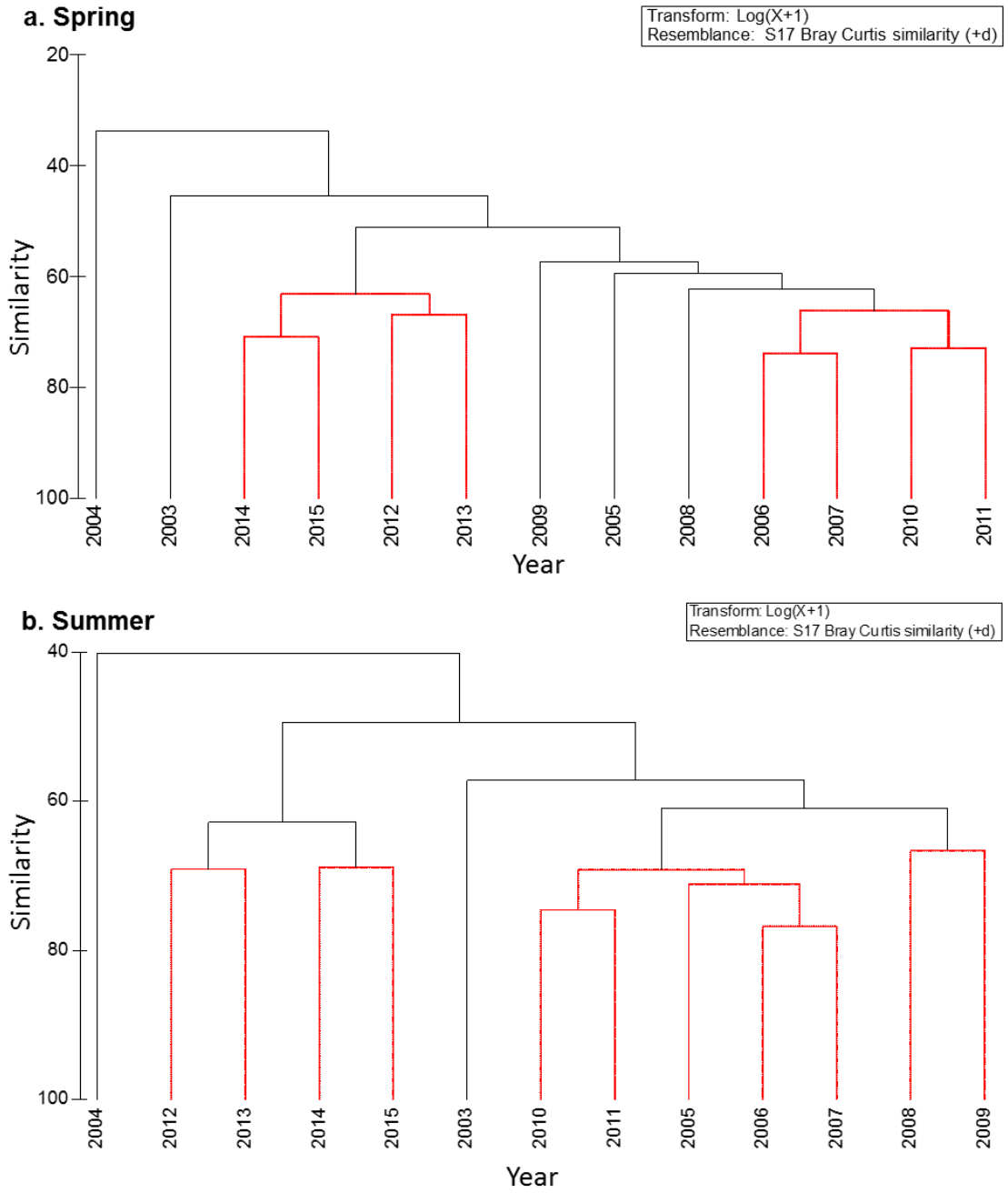
Annex 2.5. Results from the pairwise PERMANOVA for phytoplankton community with "Taxonomist" as fixed factor for the period 2003-2011. *P*-values indicate the significance of the differences between pairs of taxonomists.

a. Spring

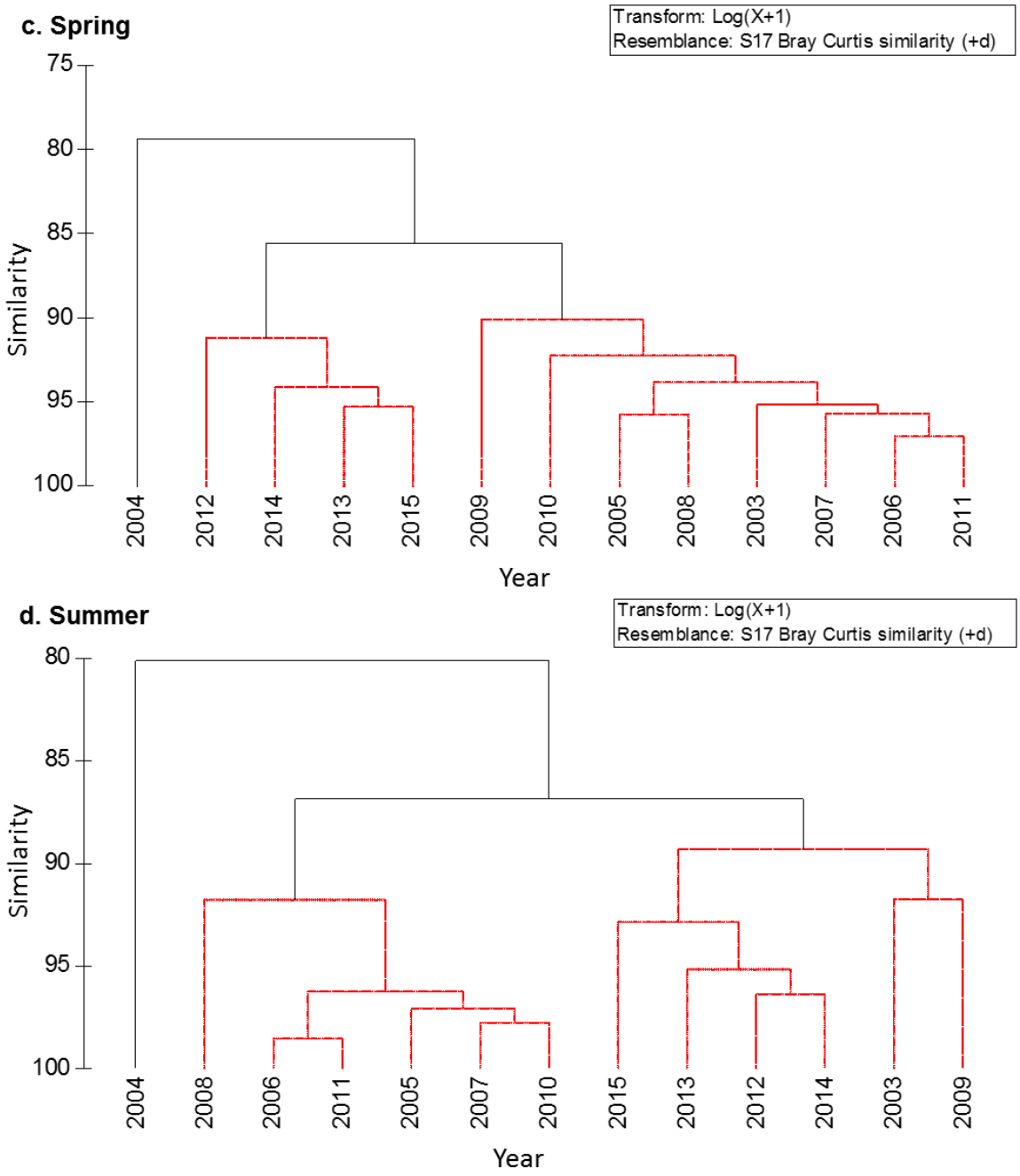
Groups	t	*p*-value	Unique perms
Taxonomist #1, Taxonomist #3	5.2724	0.0001	9943
Taxonomist #1, Taxonomist #2	3.5030	0.0001	9926
Taxonomist #3, Taxonomist #2	6.5680	0.0001	9939

b. Summer

Groups	t	*p*-value	Unique perms
Taxonomist #1, Taxonomist #3	5.5403	0.0001	9927
Taxonomist #1, Taxonomist #2	4.1294	0.0001	9930
Taxonomist #3, Taxonomist #2	7.3913	0.0001	9944



Annex 2.6. Cluster dendrograms (Bray–Curtis index, group-average linkage) of phytoplankton assemblages at the lowest taxonomic level available in spring (a) and summer (b), and at the level of major taxonomic groups in spring (c) and summer (d). Samples connected by red lines cannot be significantly differentiated (SIMPROF test at alpha = 0.05).



Annex 2.6 (continued). Cluster dendrograms (Bray–Curtis index, group-average linkage) of phytoplankton assemblages at the lowest taxonomic level available in spring (a) and summer (b), and at the level of major taxonomic groups in spring (c) and summer (d). Samples connected by red lines cannot be significantly differentiated (SIMPROF test at alpha = 0.05).

Annex 3.1. Complete list of the phytoplankton taxa registered along the Basque coast for the period 2012-2015. Potentially toxic taxa are pointed with *. Taxa appearing in less than 1% of the samples, and thus excluded from the analyses, are shown in red. For those species represented in the CCA biplots, the corresponding abbreviation is shown in brackets.

Alexandrium sp.* [Alexandr]
Amphidinium crassum
Amphidinium sphenoides
Apedinella radians [Aped_rad]
Asterionella formosa
Asterionellopsis glacialis sp. compl. [As_gl_co]
Asteromphalus flabellatus
Aulacoseira granulata
Aulacoseira italica
Azadinium caudatum var. *caudatum*
Azadinium caudatum var. *margalefii*
Azadinium sp.
Bacteriastrum sp.
Brachiomonas sp.
Centrodinium pavillardii
Cerataulina pelagica [Cera_pel]
Ceratocorys armata
Ceratocorys horrida
cf. *Fragilidium*
cf. *Gloeodinium marinum*
cf. *Haslea*
cf. *Karlodinium* spp. 10-20 µm* [Karlodin]
cf. *Levanderina fissa* [Leva_fis]
Chaetoceros affinis
Chaetoceros anastomosans
Chaetoceros atlanticus
Chaetoceros compressus/contortus
Chaetoceros constrictus
Chaetoceros costatus
Chaetoceros crinitus
Chaetoceros curvisetus [Chae_cur]
Chaetoceros danicus [Chae_dan]
Chaetoceros debilis
Chaetoceros decipiens/lorenzianus [Chae_dec]
Chaetoceros densus
Chaetoceros diadema
Chaetoceros didymus
Chaetoceros laciniosus
Chaetoceros peruvianus
Chaetoceros pseudocurvisetus
Chaetoceros rostratus
Chaetoceros salsugineus [Chae_sal]
Chaetoceros similis
Chaetoceros socialis
Chaetoceros (Chaetoceros) spp.
Chaetoceros (Hyalochaetae) spp.
Chaetoceros spp. (solitary cells) [Chae_sol]
Chaetoceros teres/lauderi
Chlamydomonas spp.
Chroomonas sp.
Coccolithaceae [Coccolit]
Coolia monotis
Corethron hystrix
Corymbellus aureus
Corythodinium frenguellii
Corythodinium tesselatum
Coscinodiscus sp.
Cryptophycophyta [Cryptoph]
Cyanobacteria (filaments)
Cyclotella meneghiniana
Cylindrotheca closterium
Dactyliosolen blavyanus
Dactyliosolen fragilissimus [Dact_fra]
Dactyliosolen phuketensis
Dactyliosolen mediterraneus
Detonula pumila
Diatoms (centric)
Diatoms (pennate) [Diat_pen]
Dictyocha crux
Dictyocha fibula
Dictyocha sp.
Dictyocha speculum
Dinobryon faculiferum [Dino_fac]
Dinobryon sp.
Dinoflagellates
Dinoflagellates (Athecata) [Dino_Ath]
Dinoflagellates (Thecata) [Dino_the]
*Dinophysis acuminata** [Dino_acu]
*Dinophysis caudata** [Dino_cau]
Dinophysis cf. *ovum** [Dino_ovu]
*Dinophysis fortii** [Dino_for]

Dinophysis spp.* [Dinophys]
*Dinophysis tripos** [Dino_tri]
Diplopsalis group
Ditylum brightwellii
Ebria tripartita
Emiliania huxleyi
Eucampia sp.
Eucampia zodiacus
Euglenophyceae
Eutreptiella eupharyngea [Eutr_eup]
Eutreptiella gymnastica
Eutreptiella spp.
Goniodoma polyedricum
Goniodoma sphaericum [Goni_sph]
Gonyaulax cf. *polygramma/digitale* [Gony_pol]
Gonyaulax sp.
*Gonyaulax spinifera** [Gony_spi]
Guinardia delicatula [Guin_del]
Guinardia flaccida
Guinardia striata
Gyrodinium cf. *flagellare* [Gyro_fla]
Gyrodinium sp. [Gyrodini]
Gyrodinium spirale
Helicotheca tamesis
Hemiaulus hauckii
Hemiaulus sp.
Hemiselmis spp.
Heterocapsa cf. *minima*
Heterocapsa cf. *rotundata*
Heterocapsa sp.
Heterocapsa triquetra
*Heterosigma akashiwo** [Hete_aka]
Karenia cf. *mikimotoi** [Kare_mik]
Karenia cf. *papilionacea** [Kare_pap]
Karenia sp.* [Karenia]
Katablepharis remigera
Katodinium sp.
Kofoidinium velleloides
Kryptoperidinium foliaceum
Lauderia annulata
Lebouridinium glaucum
Leptocylindrus minimus
Leptocylindrus spp. [Leptocyl]
Lessardia elongata [Less_elo]
Leucocryptos sp. [Leucocry]
Licmophora sp.
*Lingulodinium polyedra**
Lithodesmium undulatum
Mamiella gilva
Melosira borreri
Melosira nummuloides
Melosira varians
Meringosphaera mediterranea
Meringosphaera sp.
Mesodinium rubrum sp. compl.
Mesoporos perforatus
Meuniera membranacea [Meun_mem]
Minidiscus sp.
Minutocellus polymorphus
Monoraphidium sp.
Neocalyptrella robusta
Nitzschia longissimi [Nitz_lon]
Noctiluca scintillans
Octactis octonaria
Ollicola vangoorii
Oltmannsiellopsis sp.
Ostreopsis cf. *siamensis** [Ostr_sia]
Oxytoxum areolatum
Oxytoxum cf. *milneri*
Oxytoxum constrictum
Oxytoxum gracile
Oxytoxum laticeps
Oxytoxum longiceps
Oxytoxum sceptrum
Oxytoxum scolopax
Oxytoxum sp.
Oxytoxum sphaeroideum
Pachysphaera pelagica
Pachysphaera sp.
Palaeophalacroma unicinctum
Paralia sulcata
Pediastrum sp.
Pedinellales
Peridinium quinquecorne
*Phaeocystis globosa** [Phae_glo]
*Phalacroma mitra** [Phal_mit]
*Phalacroma rapa** [Phal_rap]
*Phalacroma rotundatum** [Phal_rot]
Plagioselmis spp. [Plagiose]
Pleurosigma sp.
Podolampas bipes
Podolampas palmipes
Podolampas spinifera
Polykrikos schwartzii
Polykrikos sp.

Proboscia alata [Prob_ala]
Proboscia truncata
Pronoctiluca pelagica
Pronoctiluca sp.
Prorocentrum balticum
Prorocentrum compressum
*Prorocentrum cordatum** [Pror_cor]
Prorocentrum dentatum
Prorocentrum gracile
Prorocentrum micans [Pror_mic]
Prorocentrum sp.
Prorocentrum triestinum [Pror_tri]
Prorocentrum vaginulum
Protoceratium areolatum
*Protoceratium reticulatum** [Prot_ret]
Protoperidinium bipes
Protoperidinium claudicans
Protoperidinium curtipes
Protoperidinium depressum
Protoperidinium diabolum
Protoperidinium divergens
Protoperidinium latidorsale/oblongum
Protoperidinium pallidum
Protoperidinium pellucidum
Protoperidinium pyriforme
Protoperidinium sp.
Protoperidinium steinii
Prymnesiales [Prymnesi]
*Pseudo-nitzschia galaxiae** [Pnit_gal]
*Pseudo-nitzschia multistriata** [Pnit_mul]
Pseudo-nitzschia spp.* [Pnitzsch]
Pseudopediastrum boryanum
Pseudoscourfieldia marina
Pterosperma sp.
Pyramimonas sp. [Pyramimo]
Rapaza viridis
Rhizosolenia hebetata f. *semispina*
Rhizosolenia setigera [Rhiz_set]
Rhizosolenia setigera f. *pungens*
Rhizosolenia setigera f. *setigera*
Rhizosolenia spp. [Rhizosol]
Rhodomonas sp.
Rhoicosphenia abbreviata
Scenedesmus sp.
Scrippsiella group [Scrippsi]
Skeletonema cf. *menzelii*
Skeletonema sp.
Spiraulax kofoidii
Striatella unipunctata
Takayama sp.* [Takayama]
Tasmanites marshalliae
Teleaulax acuta
Teleaulax amphioxeia [Tele_amp]
Teleaulax gracilis [Tele_gra]
Teleaulax minuta
Teleaulax spp.
Telonema sp.
Tenuicylindrus belgicus
Tetraselmis sp. [Tetrasel]
Thalassionema nitzschioides
Thalassiosira cf. *mediterranea* [Thal_med]
Thalassiosira rotula/grabida
Thalassiosira spp. [Thalassi]
Thalassiothrix longissima
Torodinium robustum [Toro_rob]
Torodinium teredo [Toro_ter]
Trieres mobiliensis
Tripos arietinus
Tripos azoricus
Tripos belone
Tripos candelabrus
Tripos carriensis
Tripos cf. *inflatum*
Tripos cf. *pentagonus*
Tripos falcatus
Tripos furca [Trip_fur]
Tripos fusus [Trip_fus]
Tripos gibberus
Tripos hexacanthus
Tripos horridus
Tripos lineatus
Tripos macroceros
Tripos massiliensis
Tripos minutus
Tripos muelleri
Tripos sp.
Unidentified forms (≤10 µm)
Urgorri complanatus
Warnowia sp.

Annex 3.2. Range of environmental variables in surface waters (0-1 m), in each season for the period 2012-2015. Arithmetic means for Secchi disc depth, temperature, salinity and suspended solids are included, as well as medians for ammonium, nitrate, phosphate and silicate. QL (quantification limit) for ammonium, nitrate and silicate is 1.6 µmol L^{-1}, and for phosphate 0.16 µmol L^{-1}.

Variable		Winter	Spring	Summer	Autumn
Secchi (m)	range	2 - 14	4 - 21	5 - 18	2 - 17
	mean	7.3	11.5	11.8	9.3
Temperature (ºC)	range	10.8 - 13.3	13.7 - 17.0	18.4 - 22.6	15.6 - 19.9
	mean	12.0	15.2	21.1	18.1
Salinity (PSU)	range	31.1 - 35.7	30.8 - 35.3	33.8 - 35.8	33.8 - 35.7
	mean	34.5	34.6	35.0	35.1
Suspended solids (mg L^{-1})	range	4.2 - 29.6	4.8 - 12.9	4.3 - 11.6	4.5 - 12.5
	mean	8.6	8.1	7.5	7.4
Ammonium (µmol L^{-1})	range	<QL - 6.4	<QL - 9.4	<QL - 11.1	<QL - 11.2
	median	2.1	2.8	2.7	<QL
Nitrate (µmol L^{-1})	range	<QL - 19.3	<QL - 8.9	<QL - 8.8	<QL - 11.4
	median	5.7	<QL	<QL	<QL
Phosphate (µmol L^{-1})	range	<QL - 0.75	<QL - 1.10	<QL - 0.55	<QL - 0.51
	median	0.20	0.20	<QL	<QL
Silicate (µmol L^{-1})	range	<QL - 27.7	<QL - 10.5	<QL - 4.6	<QL - 9.4
	median	3.3	1.9	1.7	1.8

Annex 3.3. Statistical significance of the environmental variables explaining the variance of the abundance for phytoplankton major taxonomic groups (A), individual taxa at the lowest taxonomic level (B) and potentially toxic taxa (C) in the Canonical Correspondence Analysis (Monte Carlo permutation test, after 1999 permutations) in each season.

A	Winter	Spring	Summer	Autumn
Variable	F-value	F-value	F-value	F-value
Phosphate	11.556***			3.361**
Salinity	7.042***	2.994*		
Ammonium	2.932**	3.041**	5.345**	
Secchi			4.671**	
Temperature			3.093*	5.142**

B	Winter	Spring	Summer	Autumn
Variable	F-value	F-value	F-value	F-value
Phosphate	9.284***	1.722**	2.059**	3.068***
Temperature	2.787***	1.874**	5.307***	7.760***
Secchi	2.425***	2.933***	4.254***	1.690**
Ammonium	2.293***	4.091***	4.645***	5.415***
Salinity	2.257***	1.993**	5.848***	2.056***
Silicate	1.819**	2.532***	1.378*	
Suspended solids	1.398*	1.625*		2.359***
Nitrate		1.645*	1.479*	2.104***

C	Winter	Spring	Summer	Autumn
Variable	F-value	F-value	F-value	F-value
Phosphate	6.663***			3.096**
Temperature	4.015***	1.926*	3.484***	4.144***
Nitrate		4.053***		
Suspended solids		3.595***		
Salinity		3.003**	6.783***	
Ammonium			2.846**	7.392***
Secchi			2.427*	
Mesodinium sp.				2.307**

*, ** and *** correspond to p-value <0.05, <0.01 and <0.001, respectively.

Annex 3.4. Summary statistics for the first two axes of CCA on the abundance of phytoplankton community (different levels of study: A, B, C) and environmental variables.

A. Abundance of major taxonomic groups

Season	Winter		Spring		Summer		Autumn	
Axes	1	2	1	2	1	2	1	2
Eigenvalues	0.040	0.017	0.013	0.003	0.015	0.006	0.010	0.006
Species-environment correlations	0.724	0.553	0.489	0.272	0.625	0.452	0.563	0.418
Cumulative percentage variance								
of species data	16.2	22.8	6.2	7.7	9.3	13.5	6.5	10.6
of species-environment relation	66.9	94.3	80.5	100.0	58.5	84.3	61.3	100.0

B. Abundance of individual taxa

Season	Winter		Spring		Summer		Autumn	
Axes	1	2	1	2	1	2	1	2
Eigenvalues	0.239	0.088	0.116	0.087	0.132	0.116	0.199	0.104
Species-environment correlations	0.882	0.865	0.888	0.726	0.887	0.933	0.896	0.928
Cumulative percentage variance								
of species data	12.5	17.2	7.0	12.2	8.1	15.2	11.7	17.8
of species-environment relation	48.6	66.4	31.0	54.1	28.3	53.2	41.8	63.6

C. Abundance of potentially toxic taxa

Season	Winter		Spring		Summer		Autumn	
Axes	1	2	1	2	1	2	1	2
Eigenvalues	0.301	0.067	0.161	0.066	0.253	0.084	0.228	0.094
Species-environment correlations	0.725	0.539	0.648	0.494	0.818	0.576	0.827	0.645
Cumulative percentage variance								
of species data	14.1	17.3	7.9	11.2	10.4	13.9	11.6	16.3
of species-environment relation	81.7	100.0	51.1	72.0	55.2	73.5	55.4	78.3

Annex 3.5. Summary of the CCAs performed for the biomass of the three different datasets and variance explained by environmental variables in phytoplankton community season by season.

A. Biomass of major taxonomic groups

	Winter	Spring	Summer	Autumn
Sum of all eigenvalues	0.252	0.221	0.176	0.166
Sum of all canonical eigenvalues	0.053	0.007	0.016	0.013
Variance explained (%)	21.0	3.2	9.1	7.8
p-value	<0.005	<0.05	<0.005	<0.01

B. Biomass of individual taxa

	Winter	Spring	Summer	Autumn
Sum of all eigenvalues	2.106	1.789	1.838	1.933
Sum of all canonical eigenvalues	0.509	0.383	0.453	0.518
Variance explained (%)	24.2	21.4	24.7	26.8
p-value	<0.005	<0.005	<0.005	<0.005

C. Biomass of potentially toxic taxa

	Winter	Spring	Summer	Autumn
Sum of all eigenvalues	2.341	2.107	2.760	2.211
Sum of all canonical eigenvalues	0.454	0.311	0.562	0.452
Variance explained (%)	19.4	14.8	20.4	20.4
p-value	<0.005	<0.005	<0.005	<0.005

Annex 3.6. Statistical significance of the environmental variables explaining the variance of the biomass for phytoplankton major taxonomic groups (A), individual taxa at the lowest taxonomic level (B) and potentially toxic taxa (C) in the Canonical Correspondence Analysis (Monte Carlo permutation test, after 1999 permutations) in each season.

A	Winter	Spring	Summer	Autumn
Variable	F-value	F-value	F-value	F-value
Phosphate	9.574***			2.875*
Salinity	6.556***			
Ammonium	2.224*		3.869*	3.272*
Temperature		2.463*		
Secchi			3.518*	

B	Winter	Spring	Summer	Autumn
Variable	F-value	F-value	F-value	F-value
Phosphate	8.215***	1.556*	1.861**	2.787***
Temperature	2.754***	1.800**	5.067***	7.154***
Ammonium	2.229**	3.882***	4.369***	5.181***
Salinity	2.202***	2.304***	5.765***	1.858**
Secchi	1.934**	2.665***	4.078***	1.682**
Silicate	1.780**	1.832**		
Suspended solids	1.419*	1.850**		2.403***
Nitrate		1.520*		2.031***

C	Winter	Spring	Summer	Autumn
Variable	F-value	F-value	F-value	F-value
Phosphate	5.925***			2.724**
Temperature	3.613**		3.634***	4.261***
Mesodinium sp.	2.435*			
Ammonium		3.775***	3.429**	7.567***
Nitrate		3.021**		2.040*
Suspended solids		2.879**		
Salinity		2.150*	6.987***	
Secchi			2.864*	

*, ** and *** correspond to p-value <0.05, <0.01 and <0.001, respectively.

Annex 3.7. Summary statistics for the first two axes of CCA on the biomass of phytoplankton community and environmental variables.

A. Biomass of major taxonomic groups

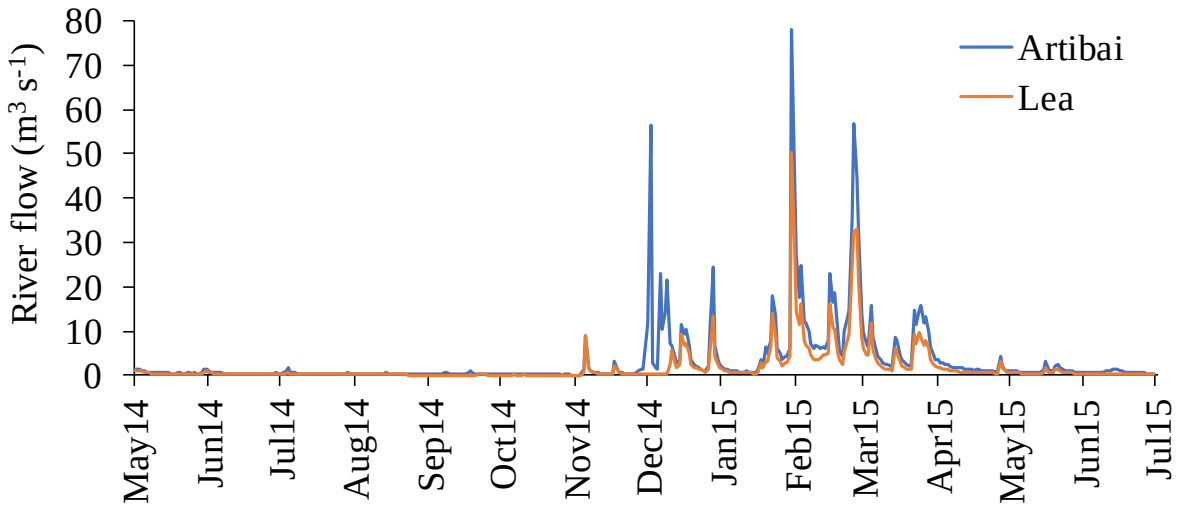
Season	Winter		Spring		Summer		Autumn	
Axes	1	2	1	2	1	2	1	2
Eigenvalues	0.035	0.017	0.007	0.081	0.011	0.005	0.011	0.002
Species-environment correlations	0.686	0.571	0.396	0.000	0.525	0.369	0.464	0.350
Cumulative percentage variance								
of species data	13.9	20.4	3.2	39.6	6.5	9.3	6.5	7.9
of species-environment relation	65.4	96.5	100.0	0.0	69.9	100.0	82.4	100.0

B. Biomass of individual taxa

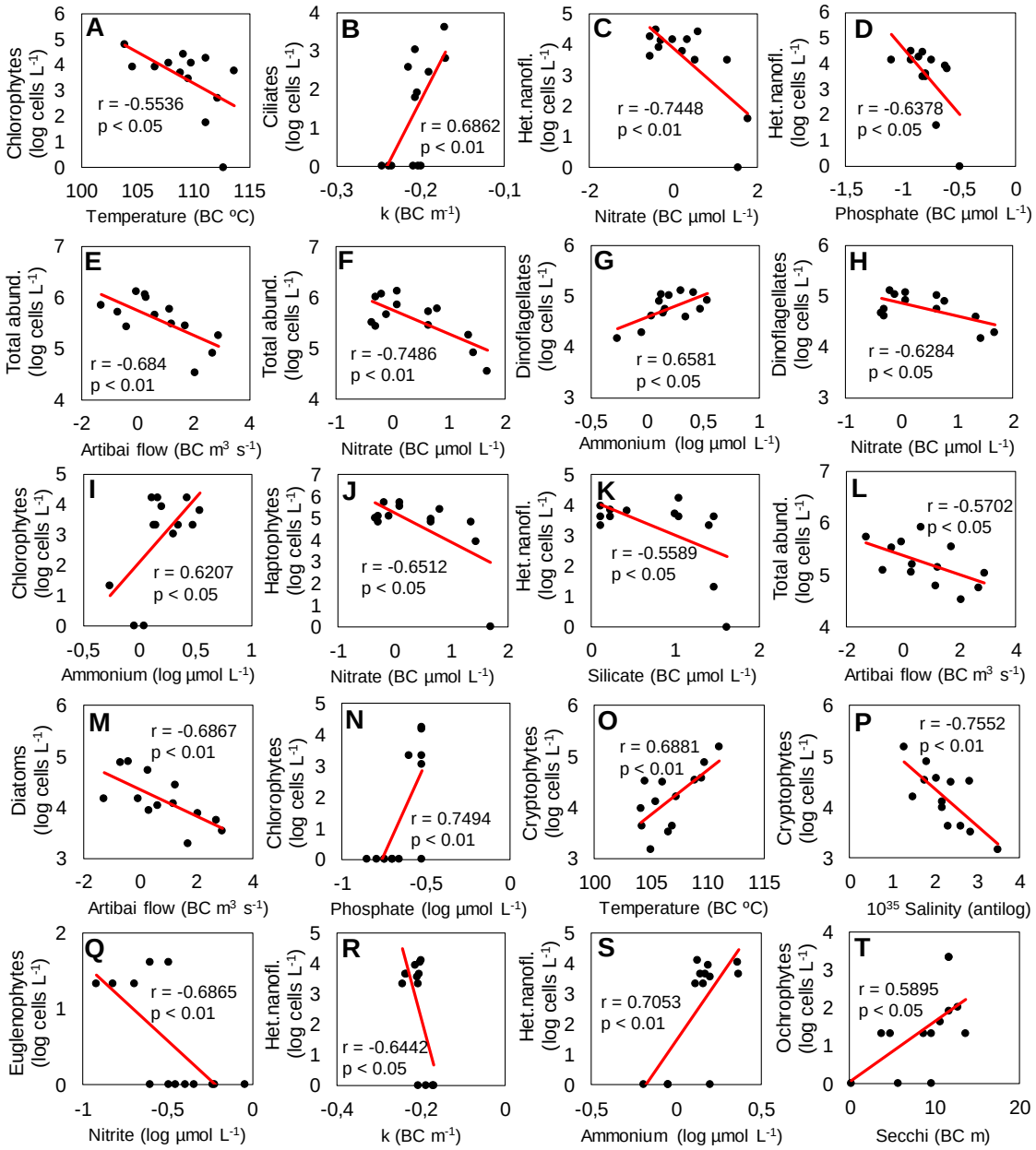
Season	Winter		Spring		Summer		Autumn	
Axes	1	2	1	2	1	2	1	2
Eigenvalues	0.236	0.093	0.114	0.085	0.142	0.128	0.205	0.118
Species-environment correlations	0.881	0.856	0.881	0.728	0.896	0.931	0.897	0.927
Cumulative percentage variance								
of species data	11.2	15.6	6.4	11.2	7.7	14.7	10.6	16.7
of species-environment relation	46.3	64.6	29.8	52.1	31.2	59.5	39.6	62.5

C. Biomass of potentially toxic taxa

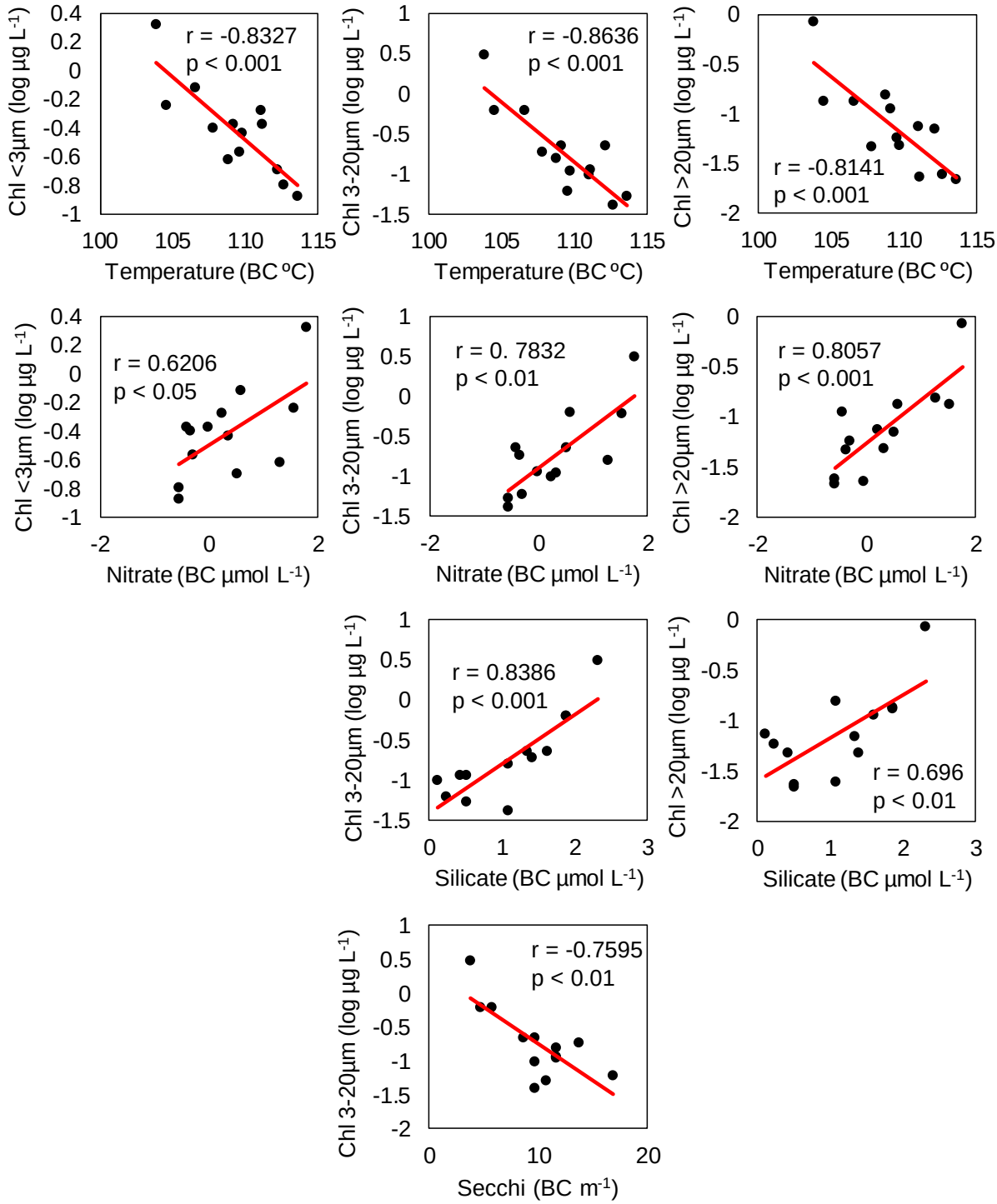
Season	Winter		Spring		Summer		Autumn	
Axes	1	2	1	2	1	2	1	2
Eigenvalues	0.266	0.129	0.170	0.066	0.297	0.114	0.259	0.103
Species-environment correlations	0.724	0.640	0.683	0.543	0.844	0.610	0.837	0.632
Cumulative percentage variance								
of species data	11.3	16.9	8.1	11.2	10.7	14.9	11.7	16.4
of species-environment relation	58.4	86.9	54.6	75.8	52.7	73.0	57.3	80.2



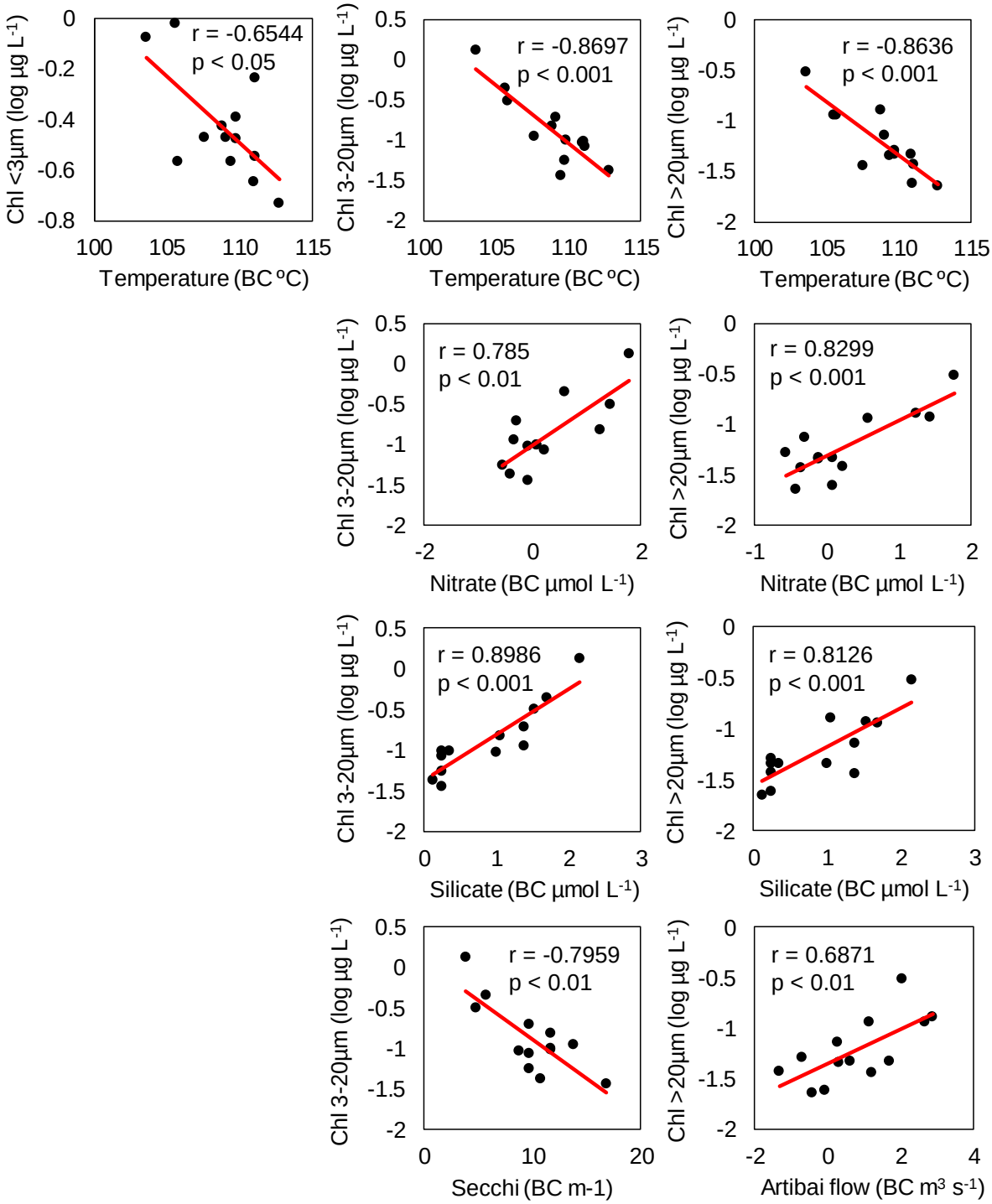
Annex 4.1. Flow values for the rivers Lea and Artibai, the two rivers surrounding the experimental bivalve farm. Daily average values are represented. Information obtained from a regional website ("Diputación Foral de Bizkaia", http://www.bizkaia.eus). Notice that there are some missing values for Lea river's flow.



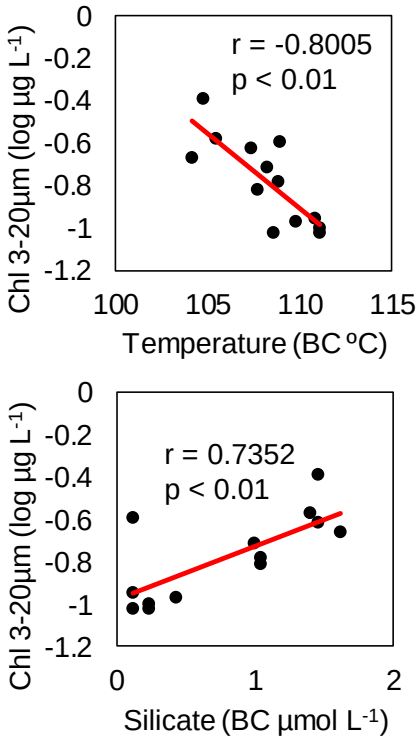
Annex 4.2. Statistically significant linear correlations (alpha = 0.05, adjusted by sequential Bonferroni correction) between environmental variables and abundance of major phytoplankton groups at 3 m depth (A to D), 17 m depth (E to K) and 33 m depth (L to T). Het. nanofl.: heterotrophic nanoflagellates, Total abund.: total abundance, k: light extinction coefficient, Secchi: Secchi disc depth, log: log transformed variable, antilog: antilog transformed variable, BC: Box-Cox transformed variable. Results for the following pair of variables should be taken carefully since there are several 'zero' values in the dependent variable: ciliates abundance vs. k; clorophytes abundance vs. phosphate concentration; euglenophytes abundance vs. nitrite concentration; heterotrophic nanoflagellates abundance vs. k; heterotrophic nanoflagellates abundance vs. ammonium concentration; ochrophytes abundance vs. Secchi disc depth.



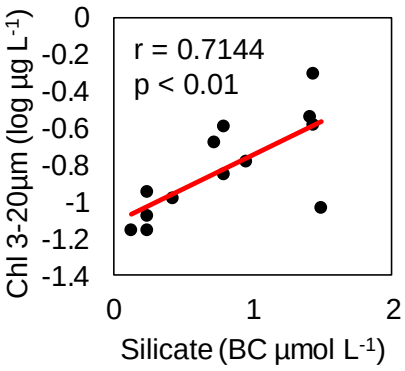
Annex 4.3. Statistically significant linear correlations (alpha = 0.05, adjusted by sequential Bonferroni correction) between environmental variables and three different chlorophyll size fractions at 3 m depth. Chl: chlorophyll *a,* Sechi: Secchi disc depth, BC: BC: Box-Cox transformed variable. Each column corresponds to one chlorophyll fraction: <3 µm in the left, 3-20 µm in the middle and >20 µm in the right.



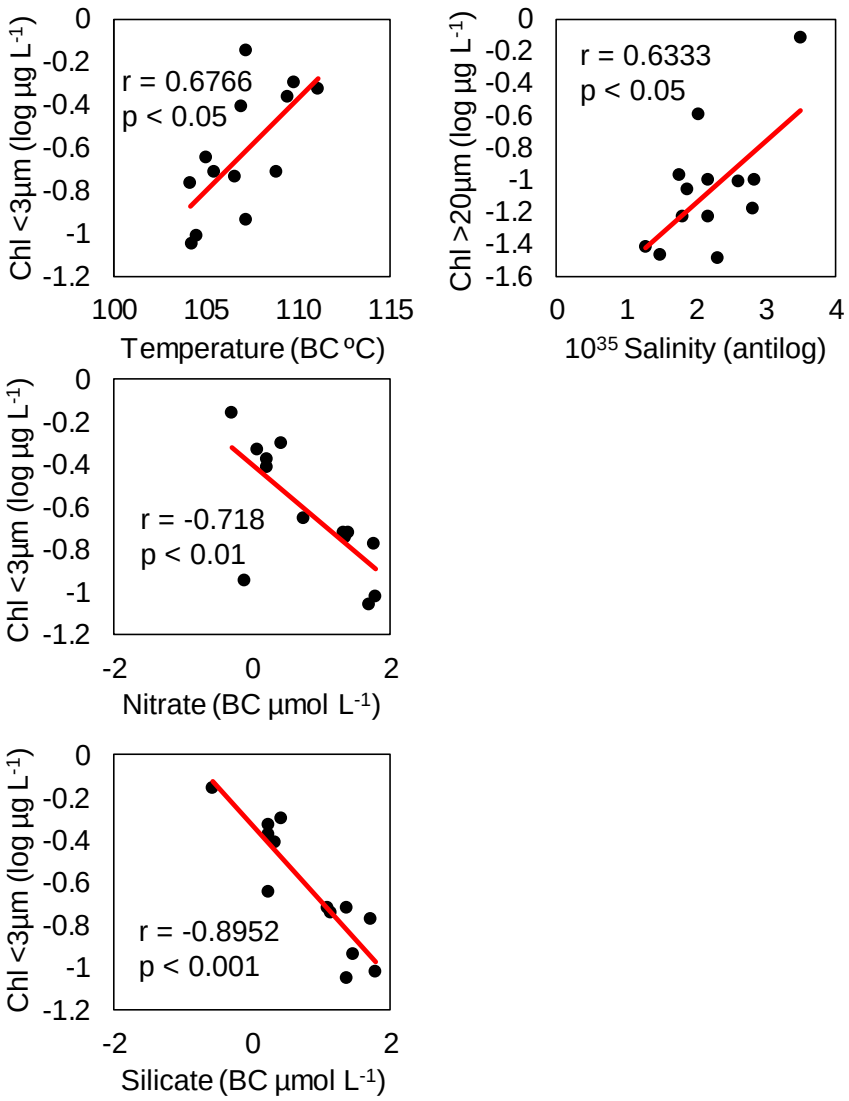
Annex 4.4. Statistically significant linear correlations (alpha = 0.05, adjusted by sequential Bonferroni correction) between environmental variables and three different chlorophyll size fractions at 10 m depth. Chl: chlorophyll *a,* Secchi: Secchi disc depth, Artibai flow: Artibai river's flow, BC: Box-Cox transformed variable. Each column corresponds to one chlorophyll fraction: <3 µm in the left, 3-20 µm in the middle and >20 µm in the right.



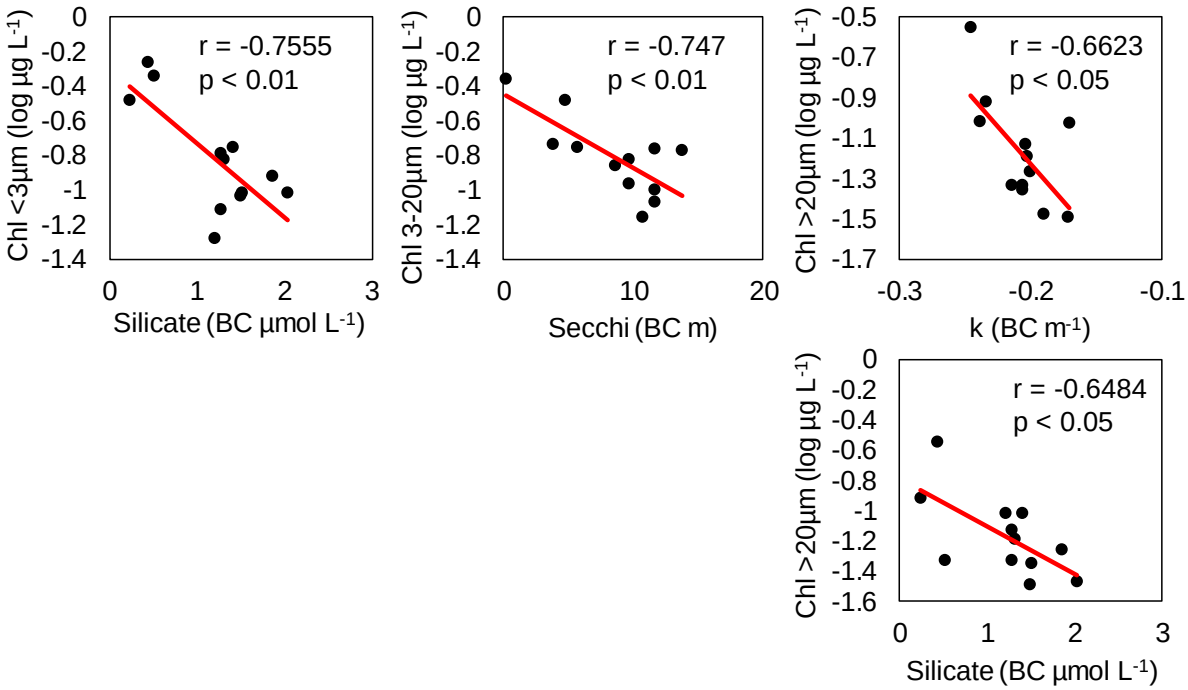
Annex 4.5. Statistically significant linear correlations (alpha = 0.05, adjusted by sequential Bonferroni correction) between environmental variables and three different chlorophyll size fractions at 17 m depth. Chl: chlorophyll *a*, BC: Box-Cox transformed variable.



Annex 4.6. Statistically significant linear correlations (alpha = 0.05, adjusted by sequential Bonferroni correction) between environmental variables and three different chlorophyll size fractions at 24 m depth. Chl: chlorophyll *a*, BC: Box-Cox transformed variable.



Annex 4.7. Statistically significant linear correlations (alpha = 0.05, adjusted by sequential Bonferroni correction) between environmental variables and three different chlorophyll size fractions at 33 m depth. Chl: chlorophyll *a*, antilog transformed variable, BC: Box-Cox transformed variable. Each column corresponds to one chlorophyll fraction: <3 µm in the left and >20 µm in the right.



Annex 4.8. Statistically significant linear correlations (alpha = 0.05, adjusted by sequential Bonferroni correction) between environmental variables and three different chlorophyll size fractions at 42 m depth. Chl: chlorophyll *a*, Secchi: Secchi disc depth, k: light extinction coefficient, BC: Box-Cox transformed variable. Each column corresponds to one chlorophyll fraction: <3 µm in the left, 3-20 µm in the middle and >20 µm in the right.

Annex 5.1. Phytoplankton potentially toxic taxa observed in the net sample (20 µm) from the experimental bivalve farm off the Basque coast during the period May 2014 – June 2015. The values are a semiquantitative estimate in a scale from 1 (one unique observation) to 5 (dominant taxon).

TAXON	Risk	May 2014	Jun 2014	Jul 2014	Aug 2014	Sep 2014	Oct 2014	Nov 2014	Dec 2014	Jan 2015	Mar 2015	Apr 2015	May 2015	Jun 2015
Pseudo-nitzschia multistriata	ASP					2.0								
Pseudo-nitzschia sp. >3 µm	ASP	3.5	3.0	1.0	3.5	3.0		2.5	3.0	2.5	2.5			2.0
Pseudo-nitzschia sp. <3 µm	ASP					4.0	2.0							
Dinophysis acuminata	DSP	4.0	3.0	1.0		2.0	1.0	1.0			1.0	3.5	2.5	2.0
Dinophysis acuta	DSP											2.5		2.0
Dinophysis caudata	DSP	1.0												1.0
Dinophysis fortii	DSP											1.0		
Dinophysis cf. *ovum*	DSP					1.0	2.0	3.0	1.0			1.0		
Dinophysis tripos	DSP	3.0	5.0	5.0	4.0	3.0	2.0	2.0	1.0	1.0		2.0	2.0	2.5
Dinophysis sp.	DSP							1.0						
Phalacroma mitra	DSP				1.0		1.0							
Phalacroma rotundatum	DSP	2.0	2.0	2.0	1.0	3.0	2.0	2.5	2.0		1.0	2.0		2.0
Karenia spp.	NSP							2.0						
Gonyaulax spinifera	YTX	2.0	1.0											
Ostreopsis cf. *siamensis*	Ostreocin D						1.0							
Takayama sp.	Ictiotoxic				1.0		1.0							1.0
Prymnesiales *(Phaeocystis globosa)*	Hemolysin										3.0			
Gymnodiniales (could contain toxic species)	Karlotoxin				2.0	3.0	5.0							

ASP: amnesic shellfish poisoning, DSP: diarrheic shellfish poisoning, DTX: dinophysistoxin, NSP: neurotoxic shellfish poisoning, YTX: yessotoxin.

Annex 5.2. Phytoplankton potentially toxic taxa observed in the integrated (from the bottom up to surface) net sample (20 µm) from the experimental bivalve farm off the Basque coast during the period June 2016 – May 2017. The values are a semiquantitative estimate in a scale from 1 (one unique observation) to 5 (dominant taxon).

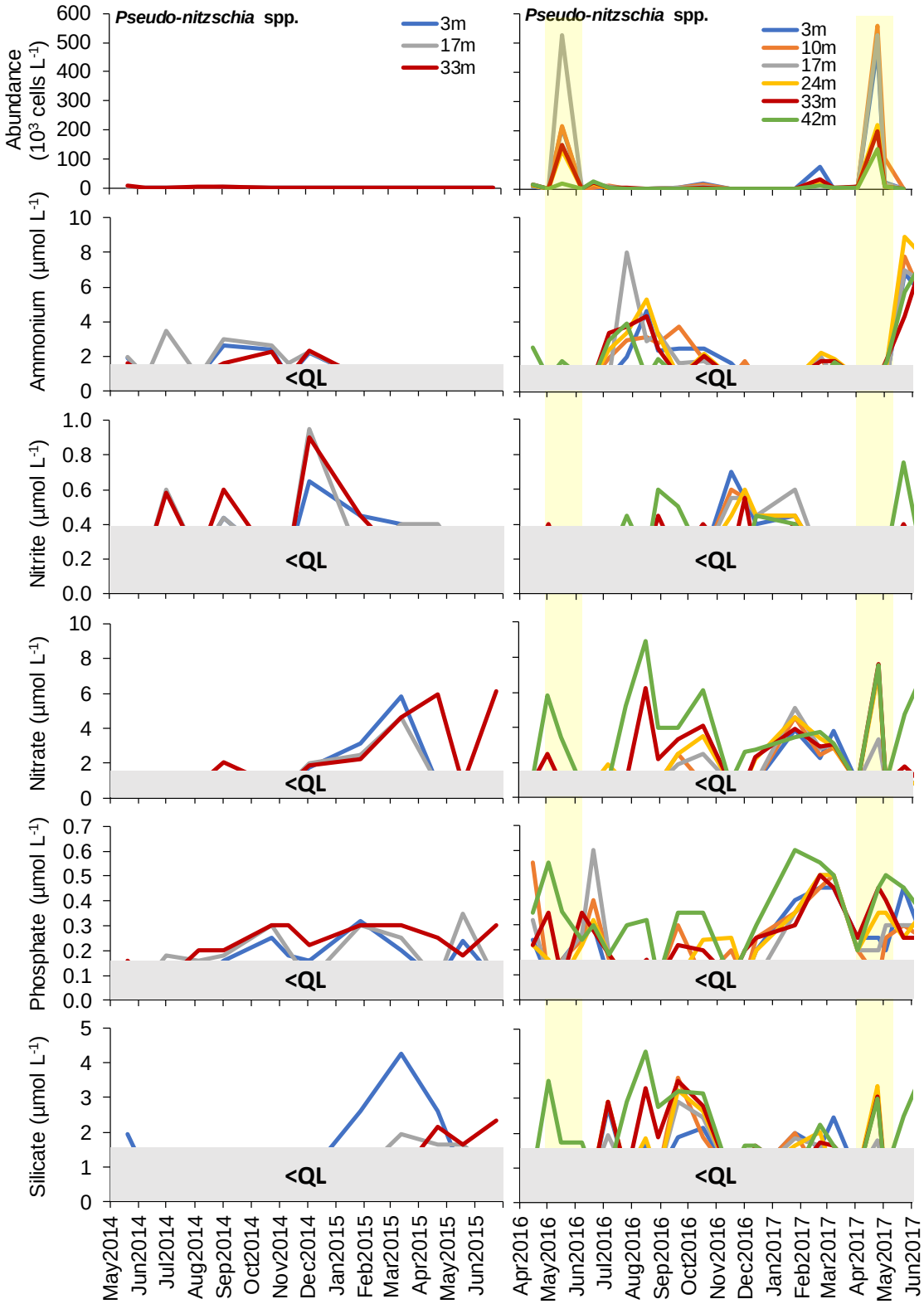
Taxon	Risk	7-Jun 2016	20-Jun 2016	6-Jul 2016	26-Jul 2016	16-Aug 2016	20-Sep 2016	17-Oct 2016	20-Nov 2016	1-Dec 2016	13-Dec 2016
Pseudo-nitzschia spp. (<5 µm)	ASP	2	2.5	2	2	2	3	4.5	2		2.5
Pseudo-nitzschia spp. (6-8 µm)	ASP	2.5	2	2			2	3	2		2
Pseudo-nitzschia multistriata	ASP							4			
Pseudo-nitzschia pungens	ASP										
Dinophysis acuminata	DSP	3	2		2	1	1		2		
Dinophysis acuta	DSP	2		2.5	2		1		1		
Dinophysis caudata	DSP	2	2	2.5	2.5	3	1	2.5	2	2	
Dinophysis fortii	DSP	2	2.5	2	2	1	2		2	2	2.5
Dinophysis infundibulum	DSP			1	2	2	2	2	3		2.5
Dinophysis tripos	DSP	2	2	2.5	2.5	1			1		
Phalacroma mitra	DSP			1							
Phalacroma rapa	DSP			2	2	2		1	2		
Phalacroma rotundatum	DSP	2.5	3	3	3	2	2.5	2.5	3	2.5	3
Alexandrium sp. (*A. tamarense* group)	PSP		2	2	3.5	2	2				
Alexandrium sp.	PSP										
Alexandrium ostenfeldii	PSP							1			2
Karenia spp.	NSP				2						
Gonyaulax spinifera	YTX	2	1			2	2		2		
Lingulodinium polyedra	YTX	2	3.5	3	2.5						
Protoceratium reticulatum	YTX	1	2.5	2					2		
Ostreopsis cf. *siamensis*	Ostreocin D				2		3	2		1	
Takayama sp.	Ictiotoxic			2	2					1	
Prymnesiales (*Phaeocystis globosa*)	Hemolysin										

ASP: amnesic shellfish poisoning, DSP: diarrheic shellfish poisoning, PSP: paralythic shellfish poisoning, NSP: neurotoxic shellfish poisoning, YTX: yessotoxin.

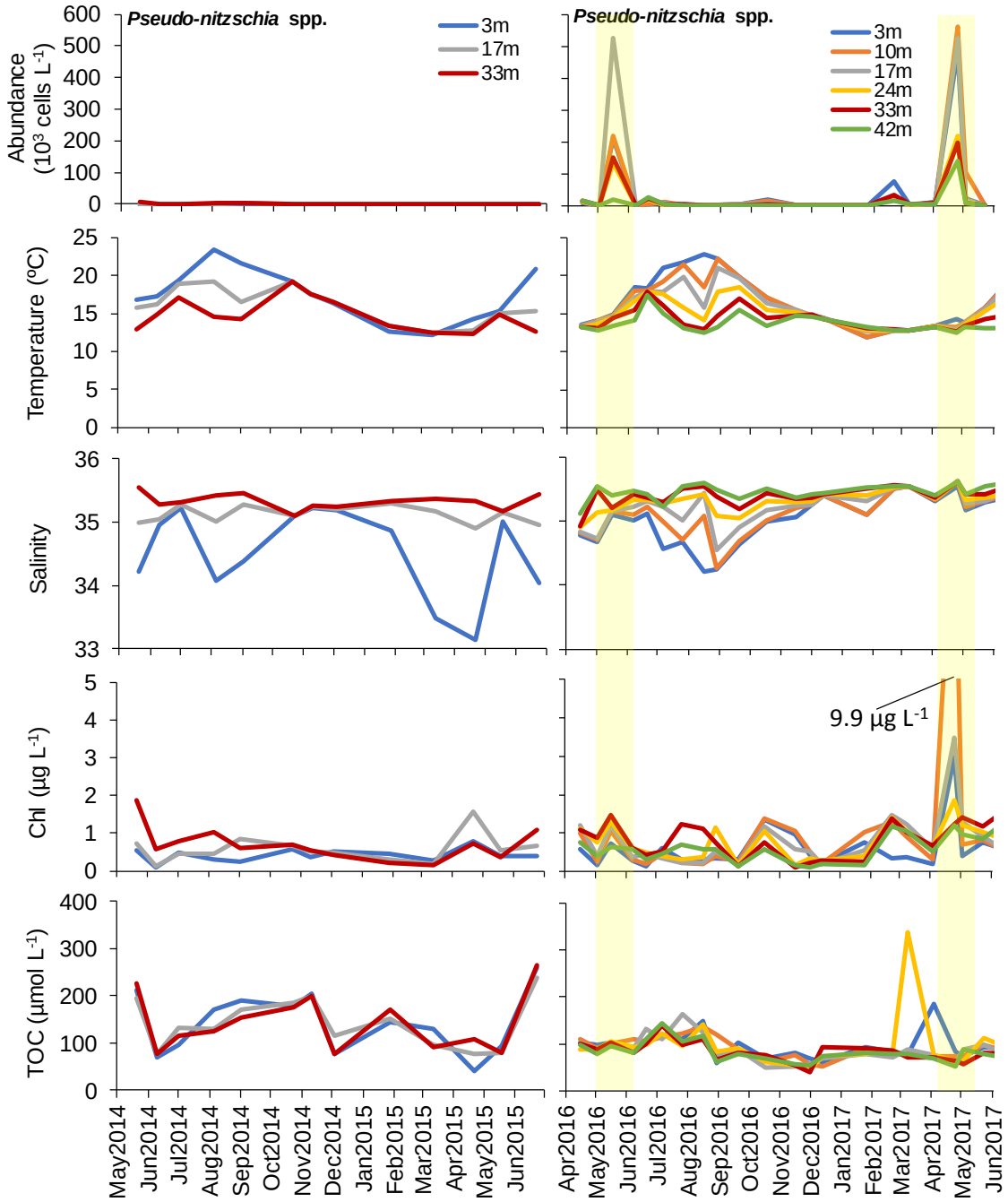
Annex 5.2 (continued). Phytoplankton potentially toxic taxa observed in the integrated (from the bottom up to surface) net sample (20 µm) from the experimental bivalve farm off the Basque coast during the period June 2016 – May 2017. The values are a semiquantitative estimate in a scale from 1 (one unique observation) to 5 (dominant taxon).

Taxon	Risk	25-Jan 2017	21-Feb 2017	8-Mar 2017	3Apr 2017	25Apr 2017	3May 2017	23May 2017
Pseudo-nitzschia spp. (<5 µm)	ASP	2	4			3.5	3	
Pseudo-nitzschia spp. (6-8 µm)	ASP	2	2.5		4	5	2.5	
Pseudo-nitzschia multistriata	ASP							
Pseudo-nitzschia pungens	ASP					2.5		
Dinophysis acuminata	DSP	2.5	3	3.5	4.5	3.5	3	3
Dinophysis acuta	DSP	2			2	2.5	3	3
Dinophysis caudata	DSP	2			1	1	1	2.5
Dinophysis fortii	DSP	3	2.5		2		2	2.5
Dinophysis infundibulum	DSP	2.5			1			
Dinophysis tripos	DSP						2	2.5
Phalacroma mitra	DSP							
Phalacroma rapa	DSP							1
Phalacroma rotundatum	DSP	2	3	3	2.5	2	2	2
Alexandrium sp. (*A. tamarense* group)	PSP							
Alexandrium sp.	PSP		1					
Alexandrium ostenfeldii	PSP		3			2		
Karenia spp.	NSP							
Gonyaulax spinifera	YTX	2				1		
Lingulodinium polyedra	YTX					3	2.5	2.5
Protoceratium reticulatum	YTX	1					2	
Ostreopsis cf. *siamensis*	Ostreocin D							
Takayama sp.	Ictiotoxic							
Prymnesiales (*Phaeocystis globosa*)	Hemolysin		3		3.5			

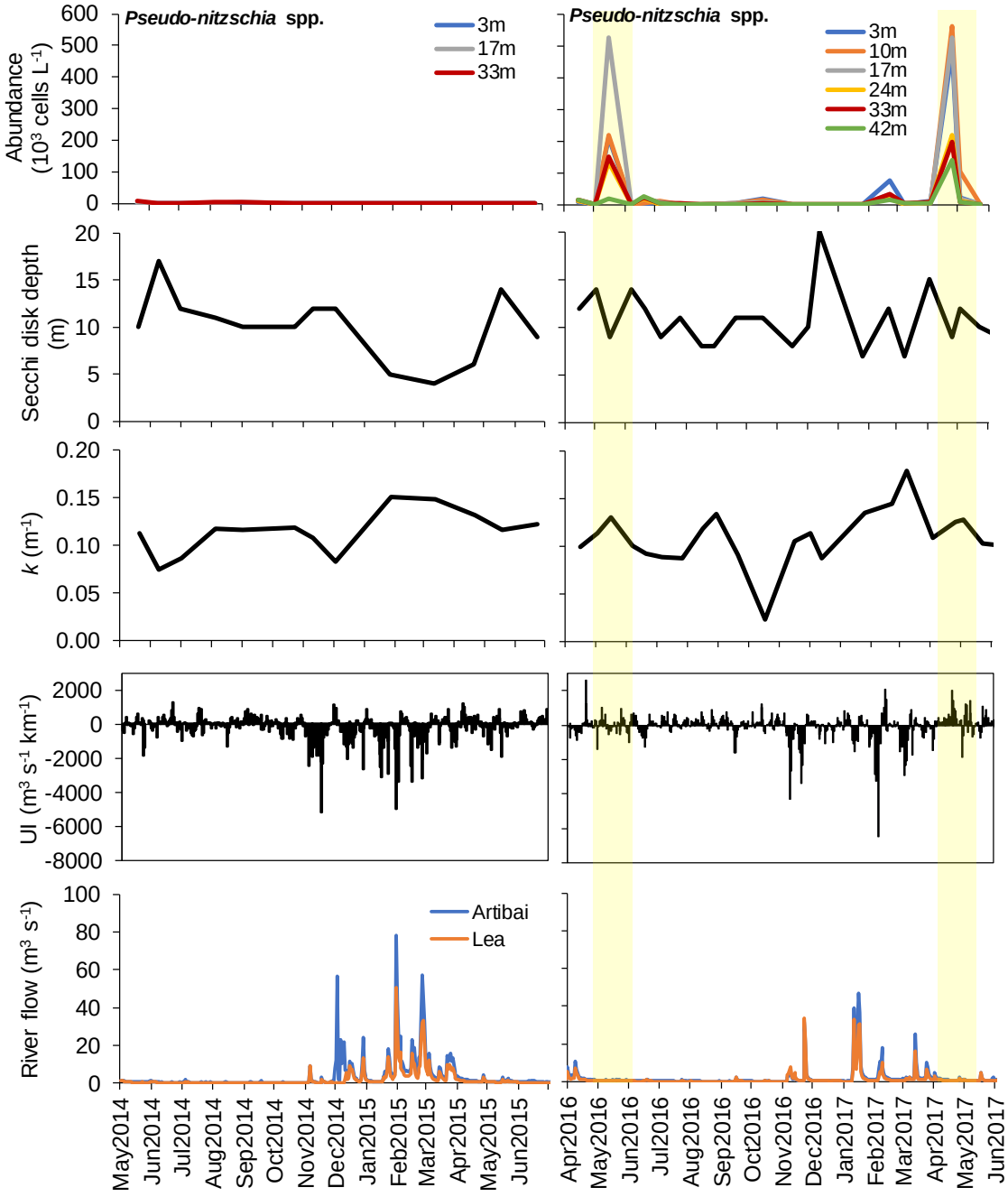
ASP: amnesic shellfish poisoning, DSP: diarrheic shellfish poisoning, PSP: paralythic shellfish poisoning, NSP: neurotoxic shellfish poisoning, YTX: yessotoxin.



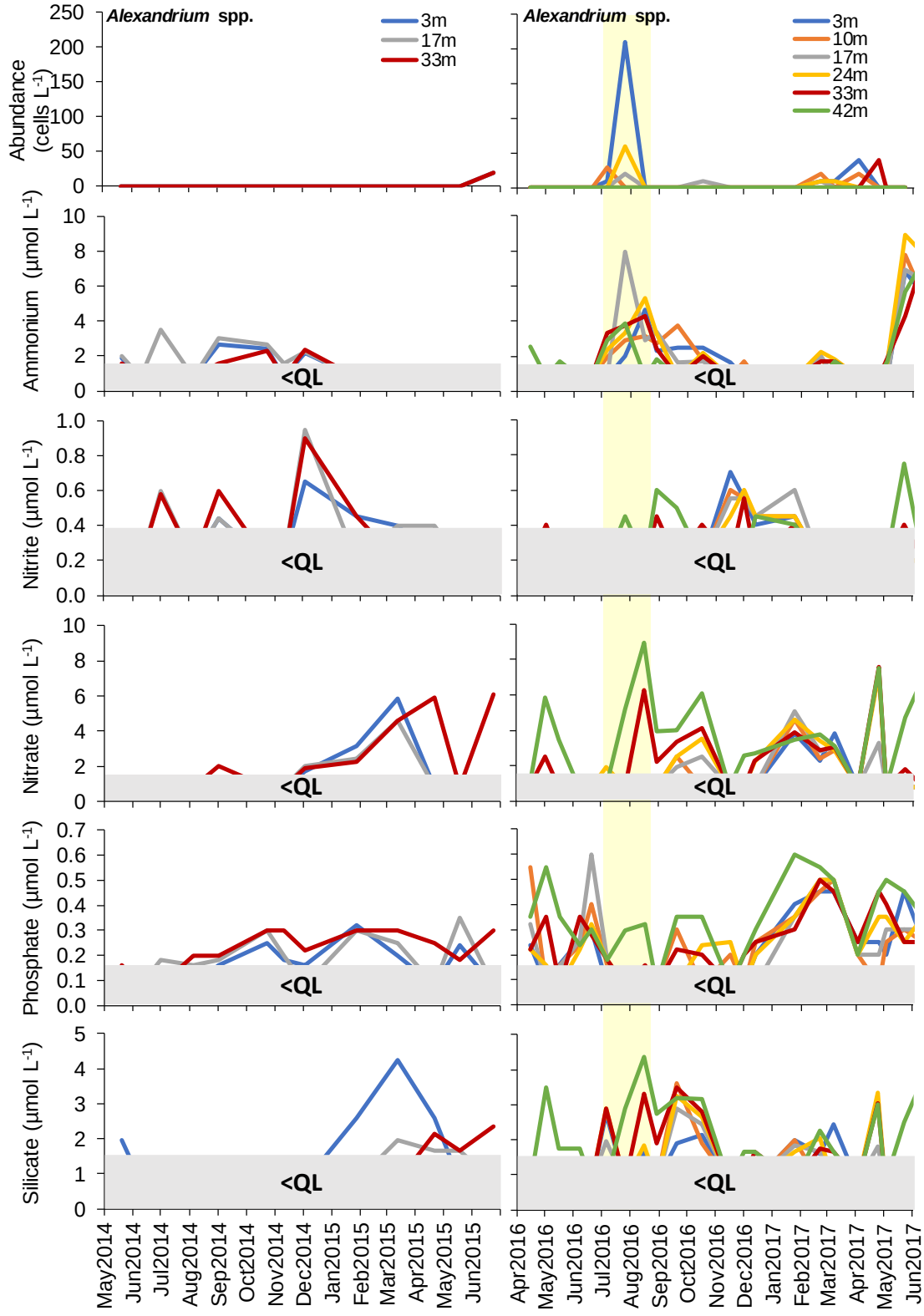
Annex 5.3a. Temporal variability in the abundance of the toxic genus *Pseudo-nitzschia* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. *Pseudo-nitzschia* spp. does not account for the sum of all the species, but for those that could not be identified at species level. QL stands for quantification limit: 1.6 µmol L^{-1} for ammonium, nitrate and silicate, 0.4 µmol L^{-1} for nitrite and 0.16 µmol L^{-1} for phosphate.



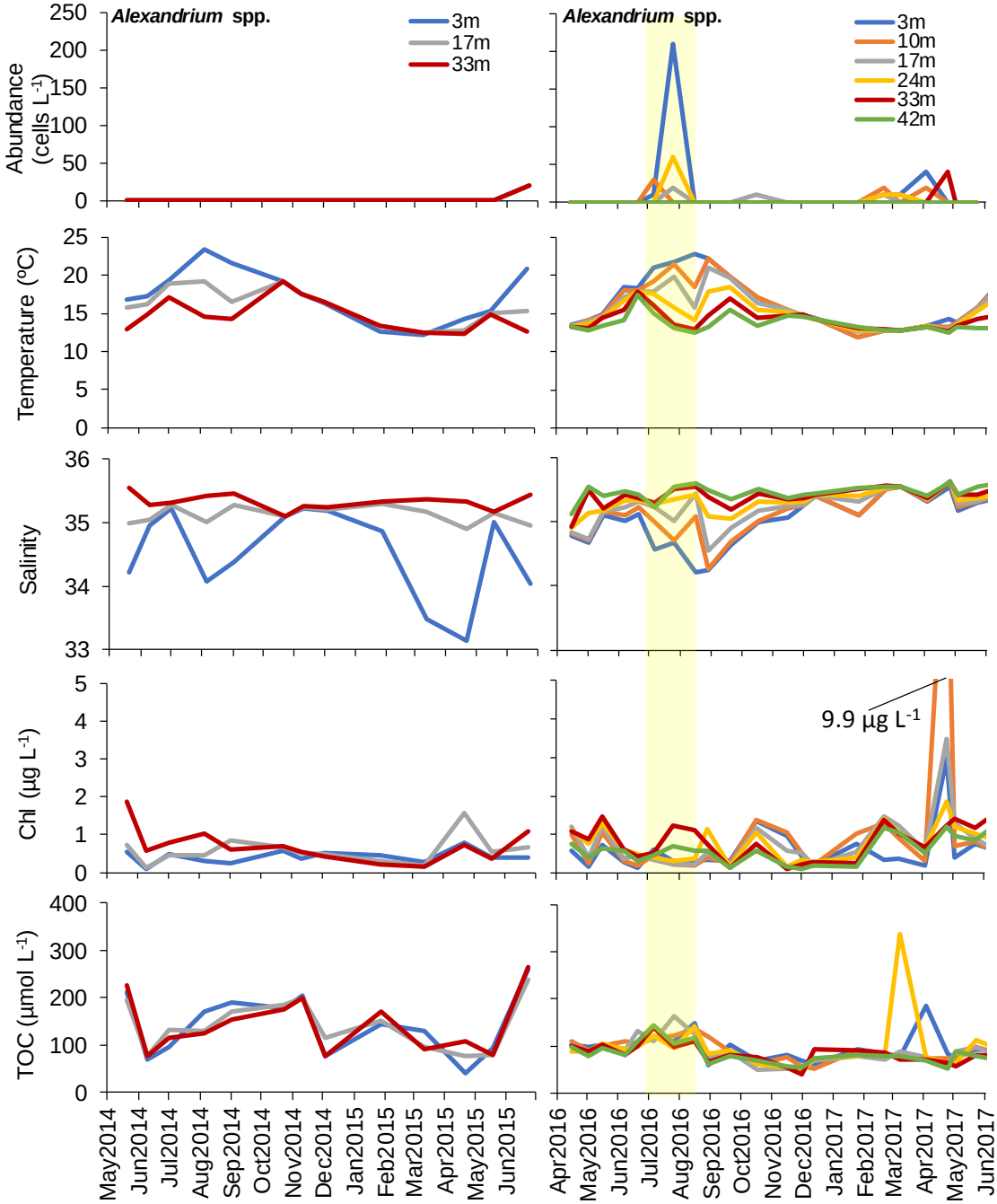
Annex 5.3b. Temporal variability in the abundance of the toxic genus *Pseudo-nitzschia* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. *Pseudo-nitzschia* spp. does not account for the sum of all the species, but for those that could not be identified at species level. Chl: chlorophyll *a,* TOC: total organic carbon.



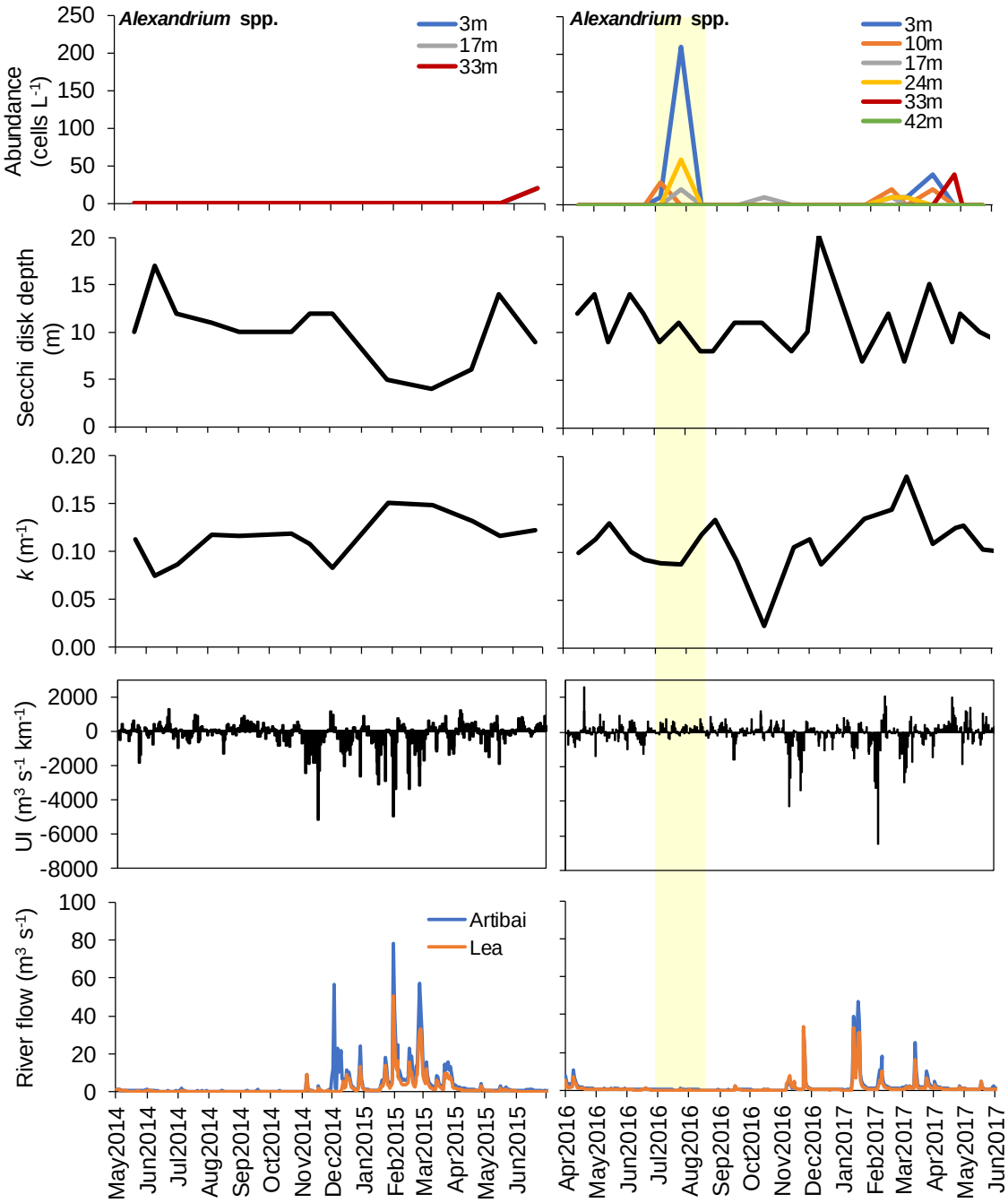
Annex 5.3c. Temporal variability in the abundance of the toxic genus *Pseudo-nitzschia* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. *Pseudo-nitzschia* spp. does not account for the sum of all the species, but for those that could not be identified at species level. *k:* light extinction coefficient, UI: upwelling index.



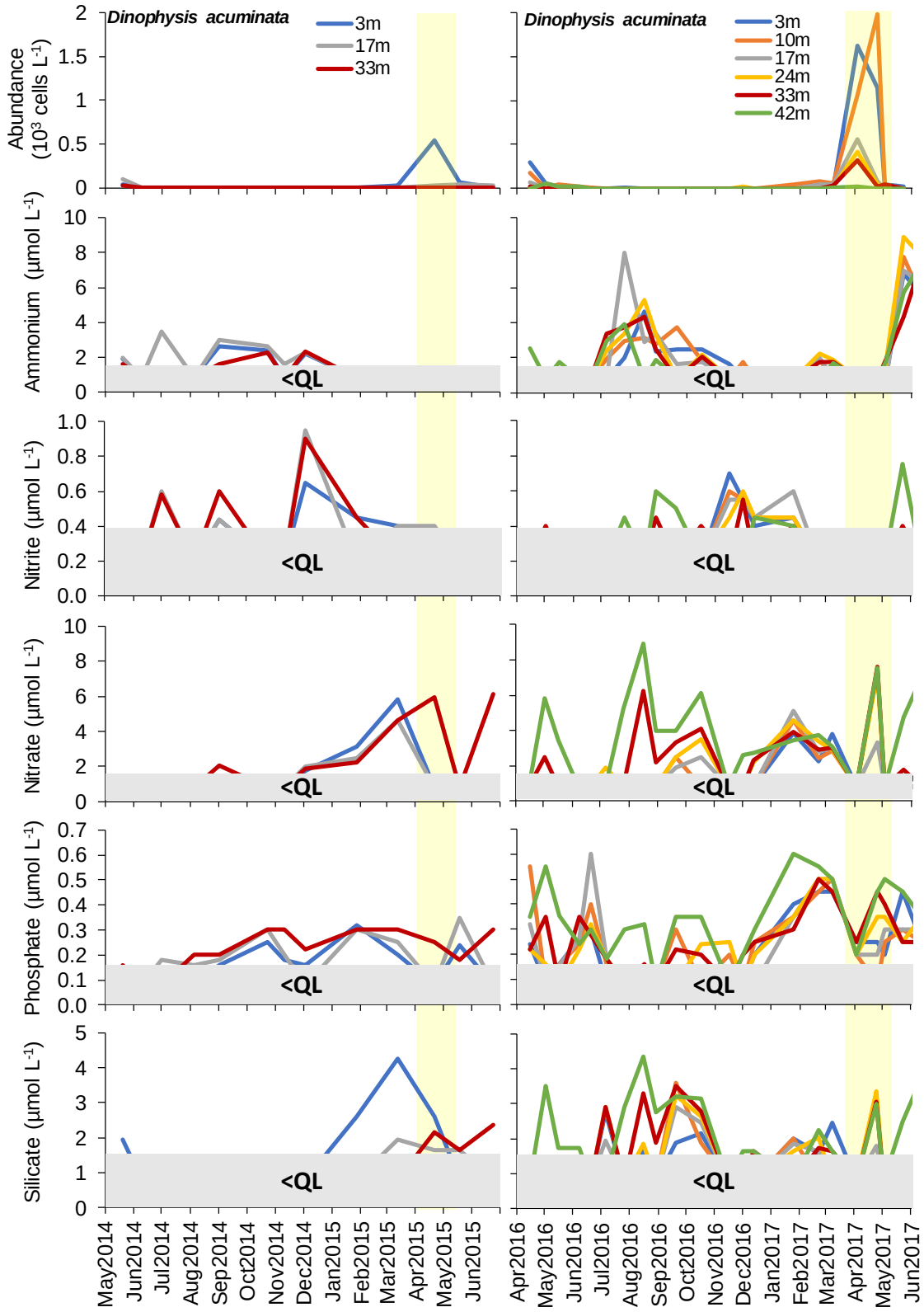
Annex 5.4a. Temporal variability in the abundance of the toxic genus *Alexandrium* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. QL stands for quantification limit: 1.6 µmol L^{-1} for ammonium, nitrate and silicate, 0.4 µmol L^{-1} for nitrite and 0.16 µmol L^{-1} for phosphate.



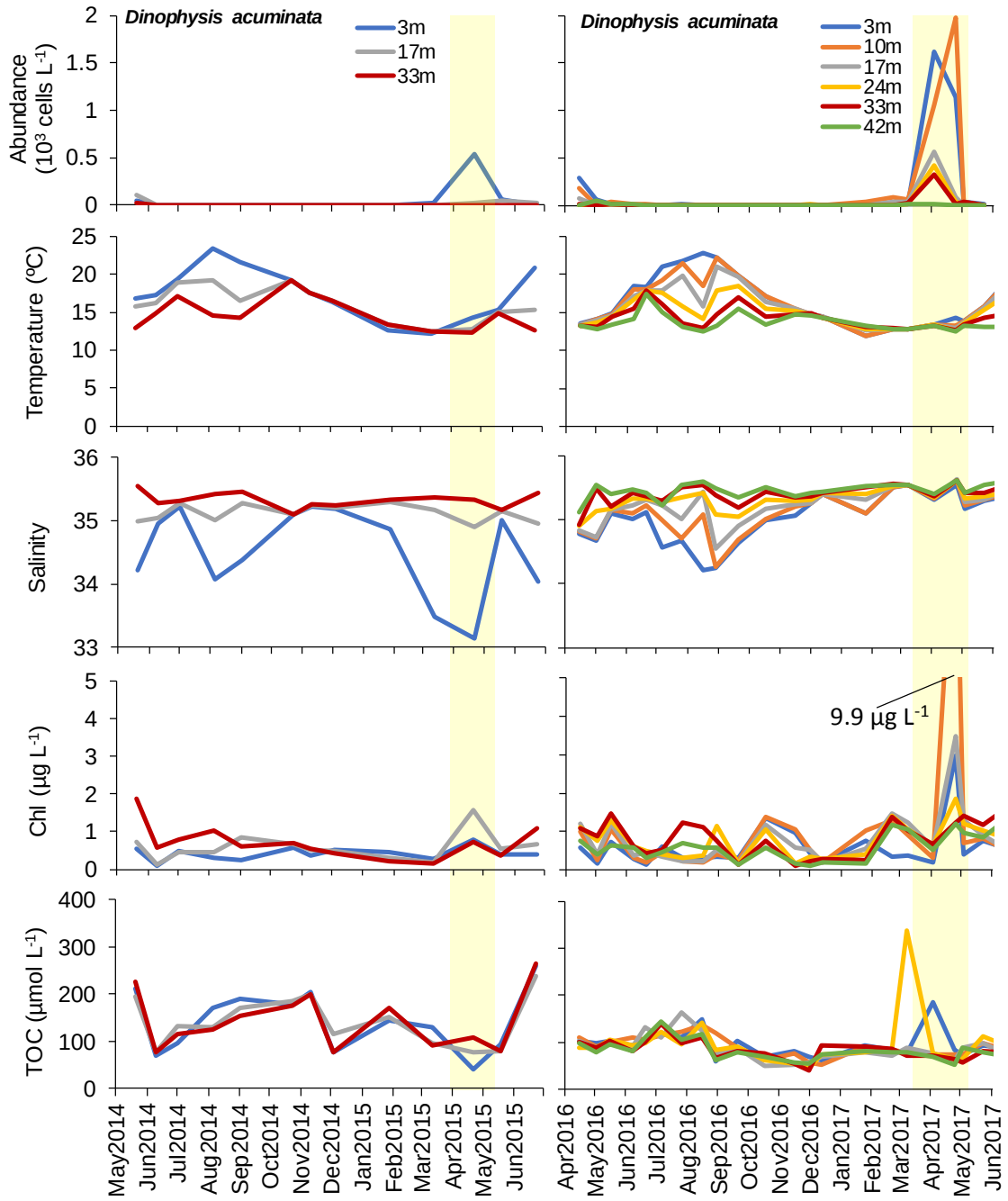
Annex 5.4b. Temporal variability in the abundance of the toxic genus *Alexandrium* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. Chl: chlorophyll *a,* TOC: total organic carbon.



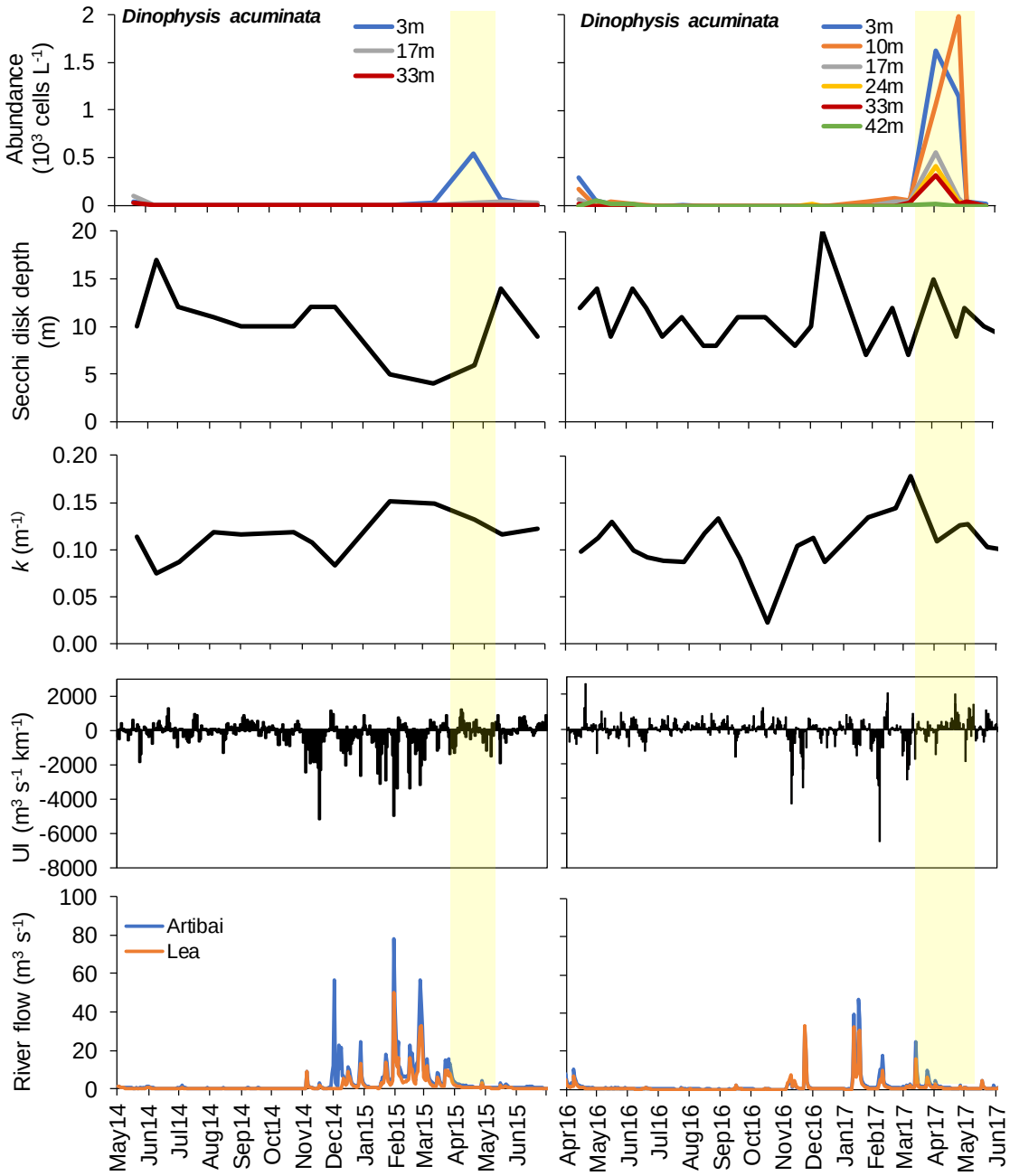
Annex 5.4c. Temporal variability in the abundance of the toxic genus *Alexandrium* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. *k:* light extinction coefficient, UI: upwelling index.



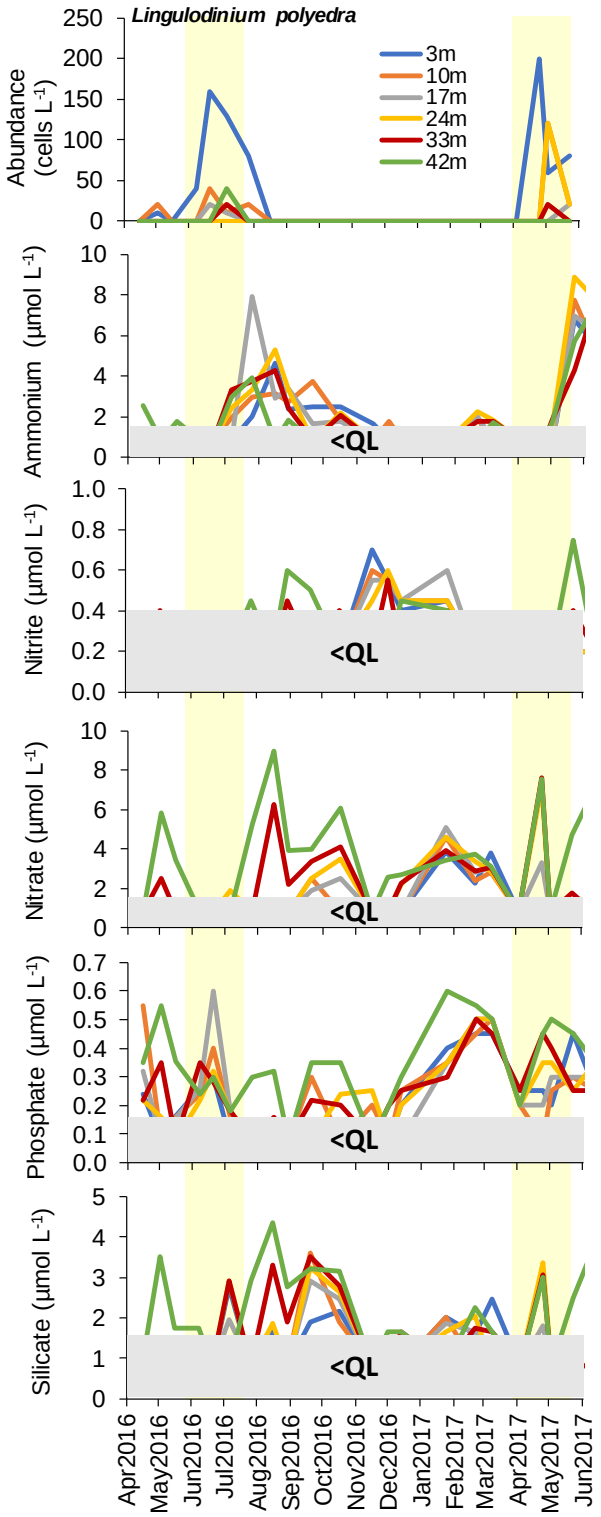
Annex 5.5a. Temporal variability in the abundance of the toxic species *Dinophysis acuminata* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. QL stands for quantification limit: 1.6 µmol L^{-1} for ammonium, nitrate and silicate, 0.4 µmol L^{-1} for nitrite and 0.16 µmol L^{-1} for phosphate.



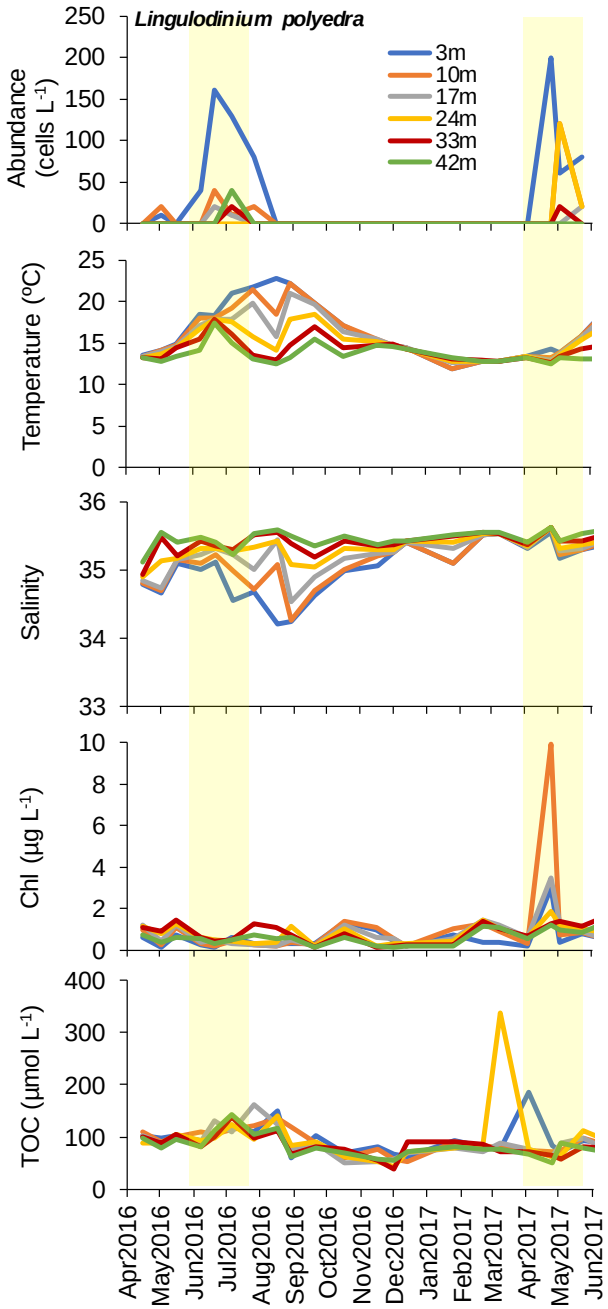
Annex 5.5b. Temporal variability in the abundance of the toxic species *Dinophysis acuminata* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. Chl: chlorophyll *a,* TOC: total organic carbon.



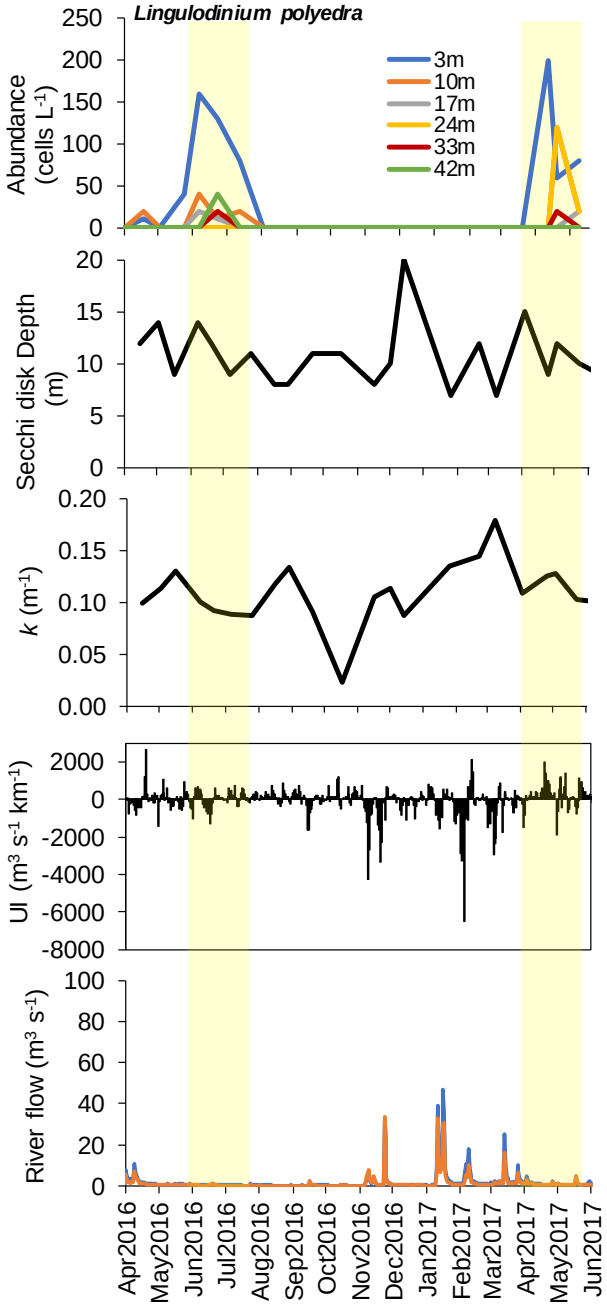
Annex 5.5c. Temporal variability in the abundance of the toxic species *Dinophysis acuminata* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. *k:* light extinction coefficient, UI: upwelling index.



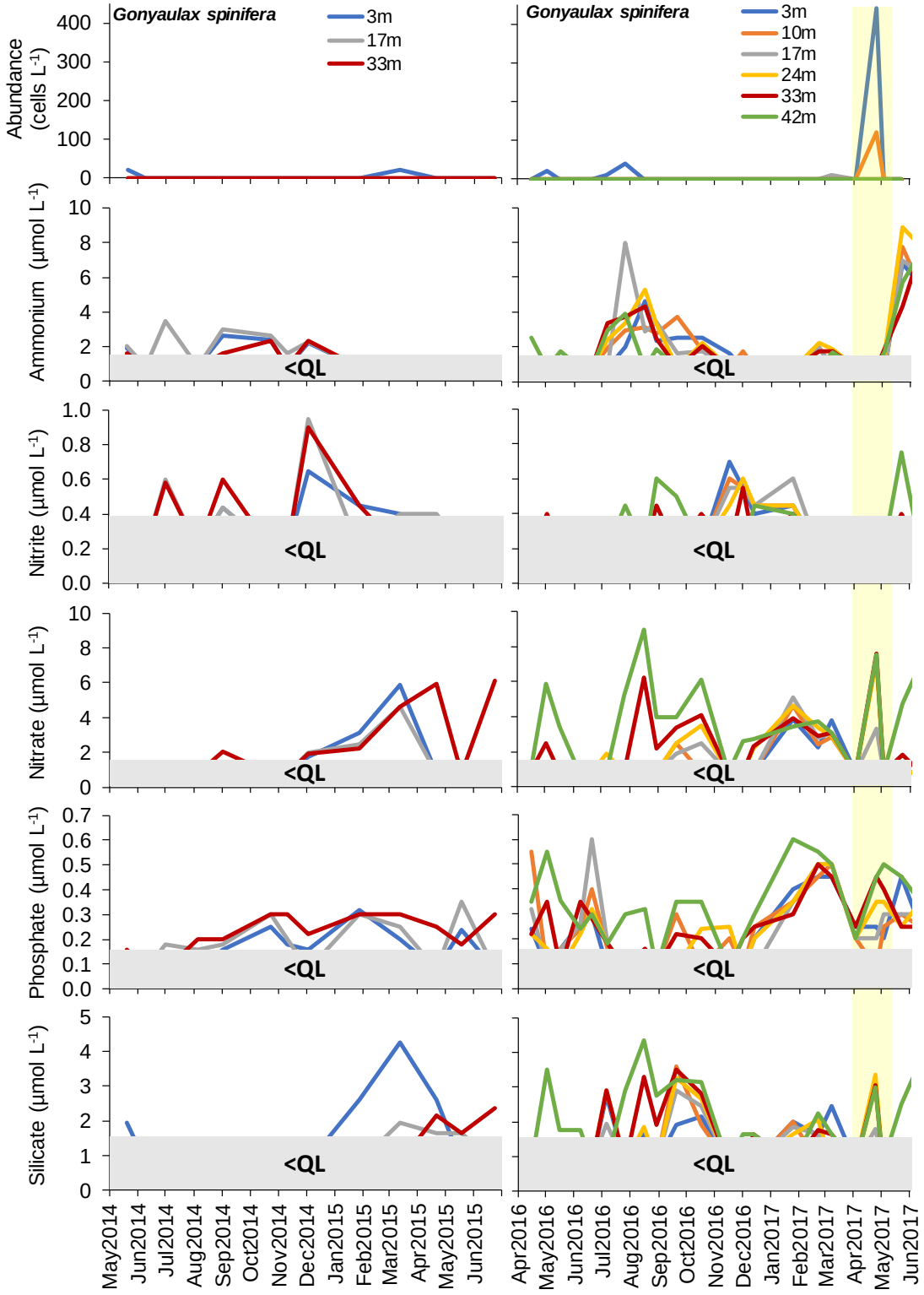
Annex 5.6a. Temporal variability in the abundance of the toxic species *Lingulodinium polyedra* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. QL stands for quantification limit: 1.6 µmol L^{-1} for ammonium, nitrate and silicate, 0.4 µmol L^{-1} for nitrite and 0.16 µmol L^{-1} for phosphate. *L. polyedra* was not registered during the period 2014-2015 and thus, only the period 2016-2017 is represented.



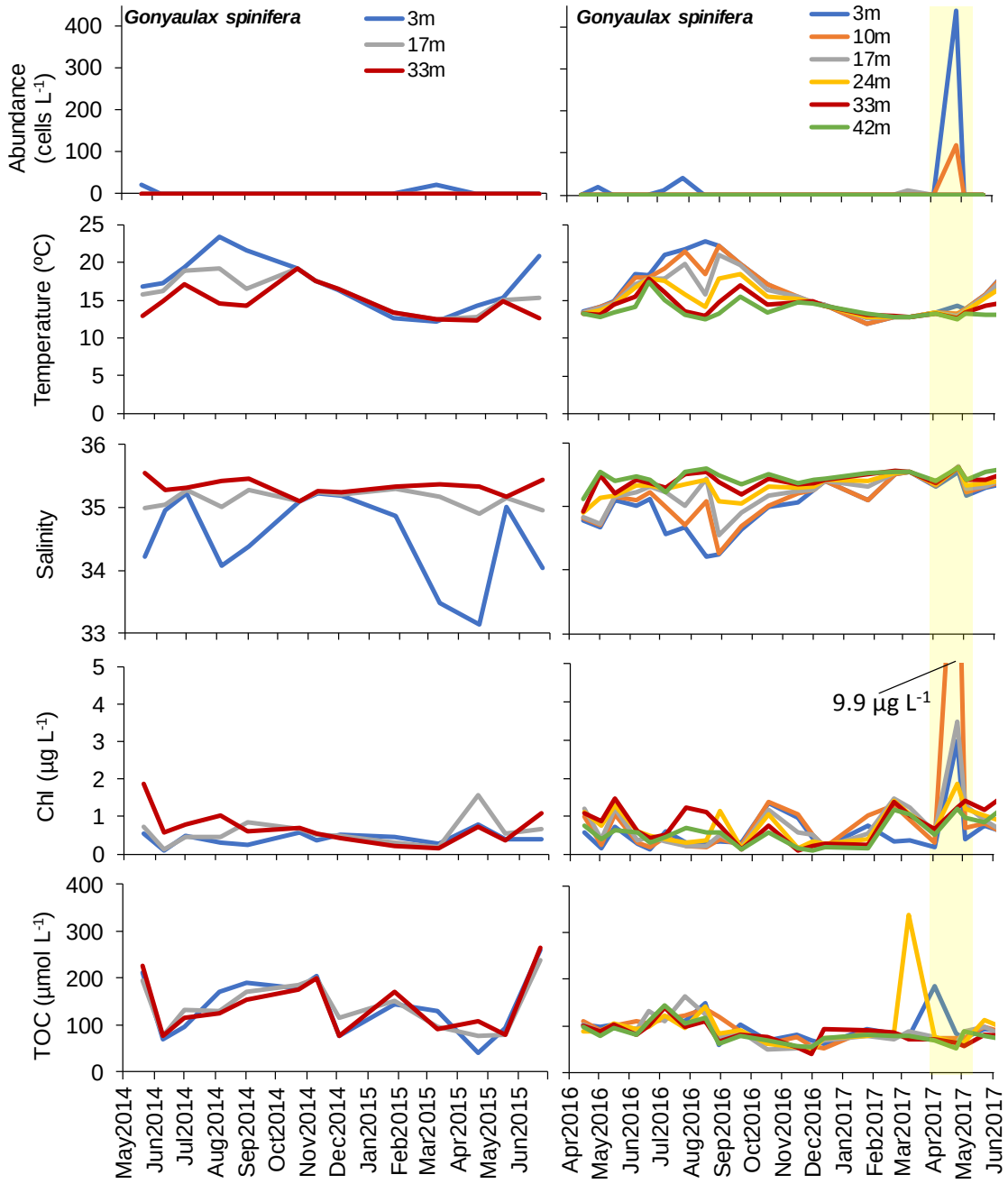
Annex 5.6b. Temporal variability in the abundance of the toxic species *Lingulodinium polyedra* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. Chl: chlorophyll *a,* TOC: total organic carbon. *L. polyedra* was not registered during the period 2014-2015 and thus, only the period 2016-2017 is represented.



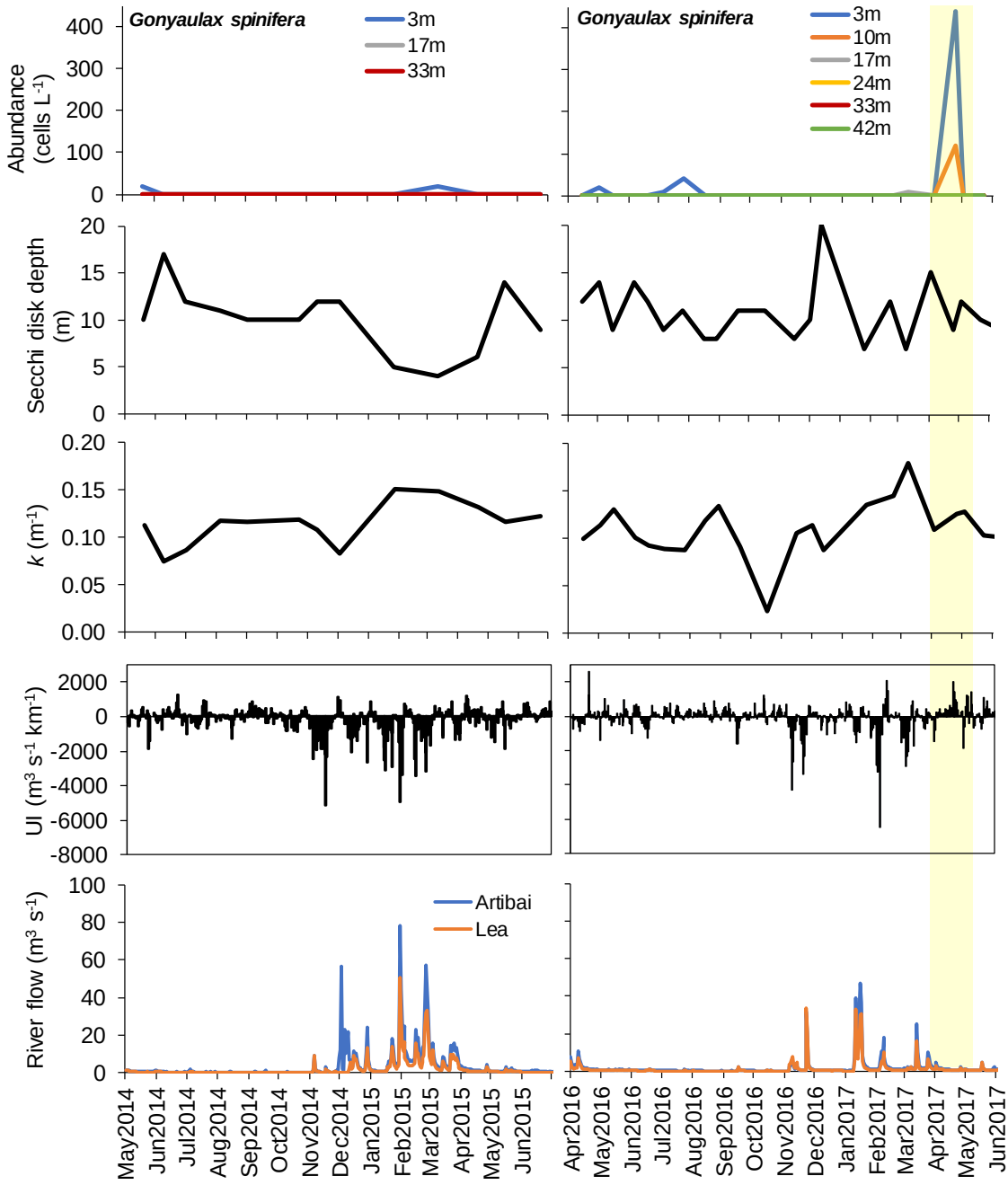
Annex 5.6c. Temporal variability in the abundance of the toxic species *Lingulodinium polyedra* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. *k*: light extinction coefficient, UI: upwelling index. *L. polyedra* was not registered during the period 2014-2015 and thus, only the period 2016-2017 is represented.



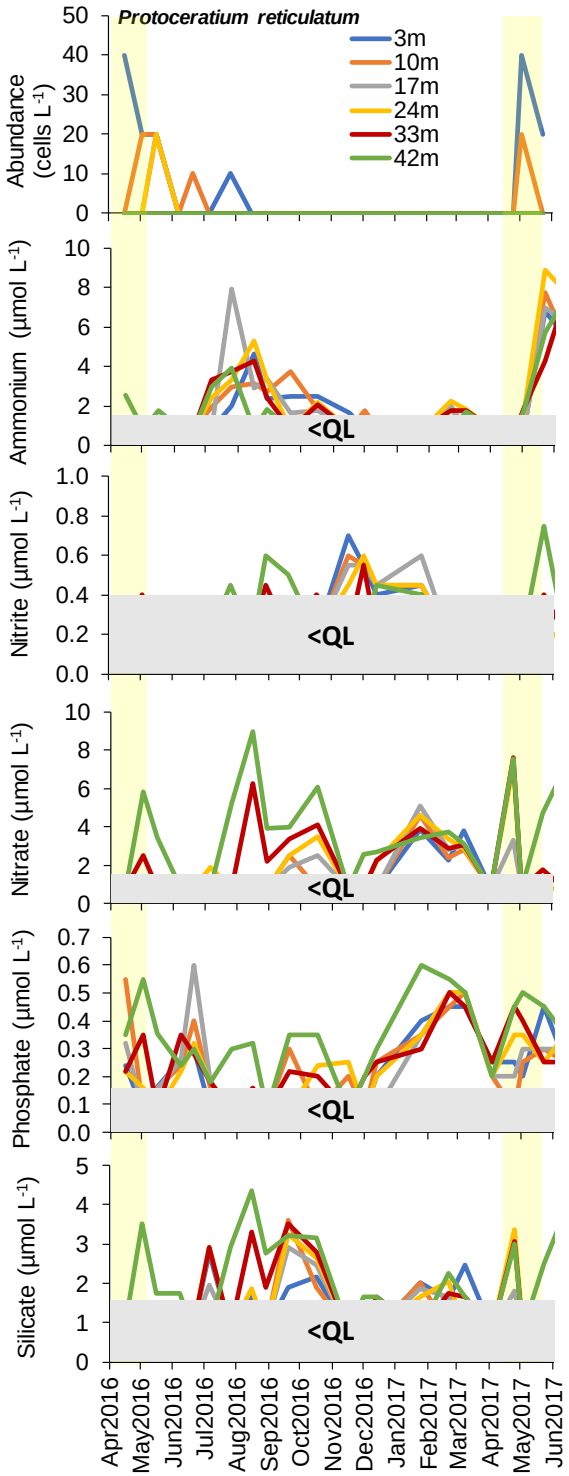
Annex 5.7a. Temporal variability in the abundance of the toxic species *Gonyaulax spinifera* and in the environmental variables through the water column. The yellow shaded area shows the peak in cell density. QL stands for quantification limit: 1.6 µmol L^{-1} for ammonium, nitrate and silicate, 0.4 µmol L^{-1} for nitrite and 0.16 µmol L^{-1} for phosphate.



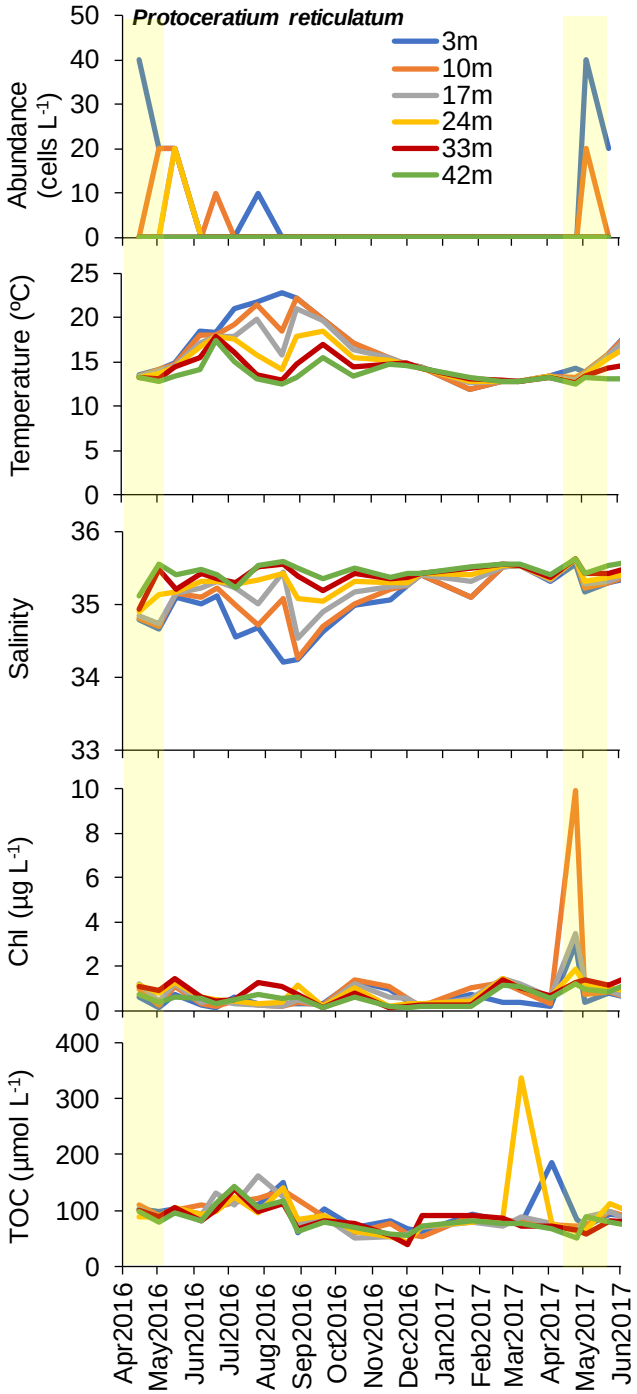
Annex 5.7b. Temporal variability in the abundance of the toxic species *Gonyaulax spinifera* and in the environmental variables through the water column. The yellow shaded area shows the peak in cell density. Chl: chlorophyll *a,* TOC: total organic carbon.



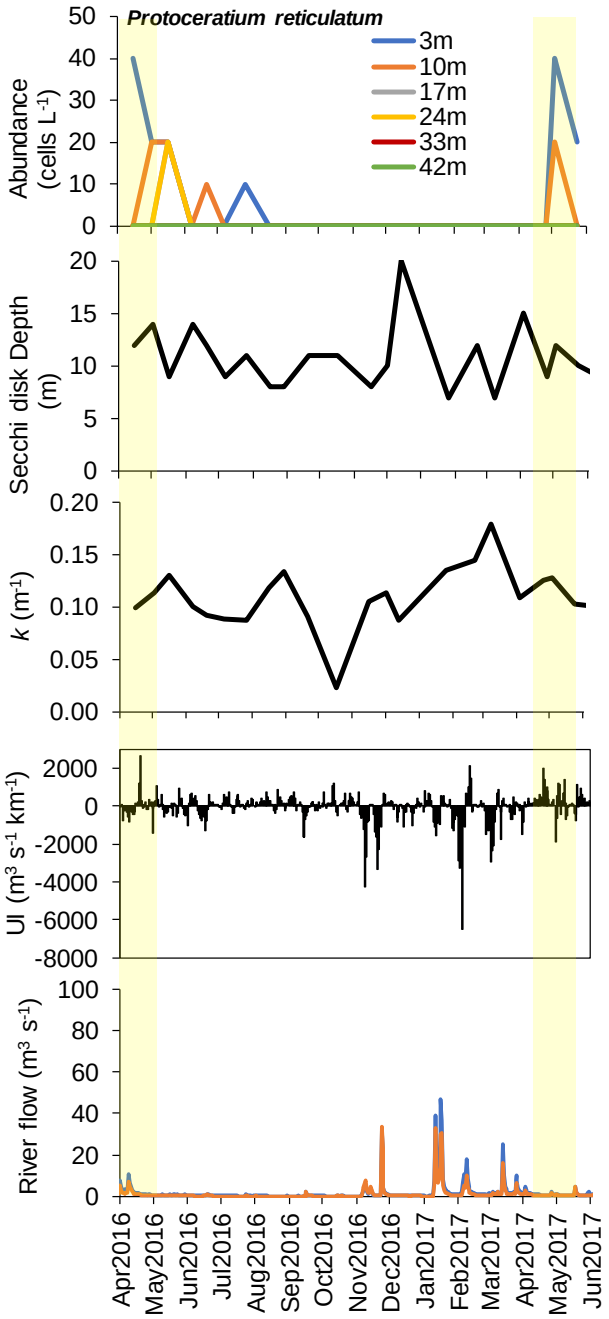
Annex 5.7c. Temporal variability in the abundance of the toxic species *Gonyaulax spinifera* and in the environmental variables through the water column. The yellow shaded area shows the peak in cell density. *k:* light extinction coefficient, UI: upwelling index.



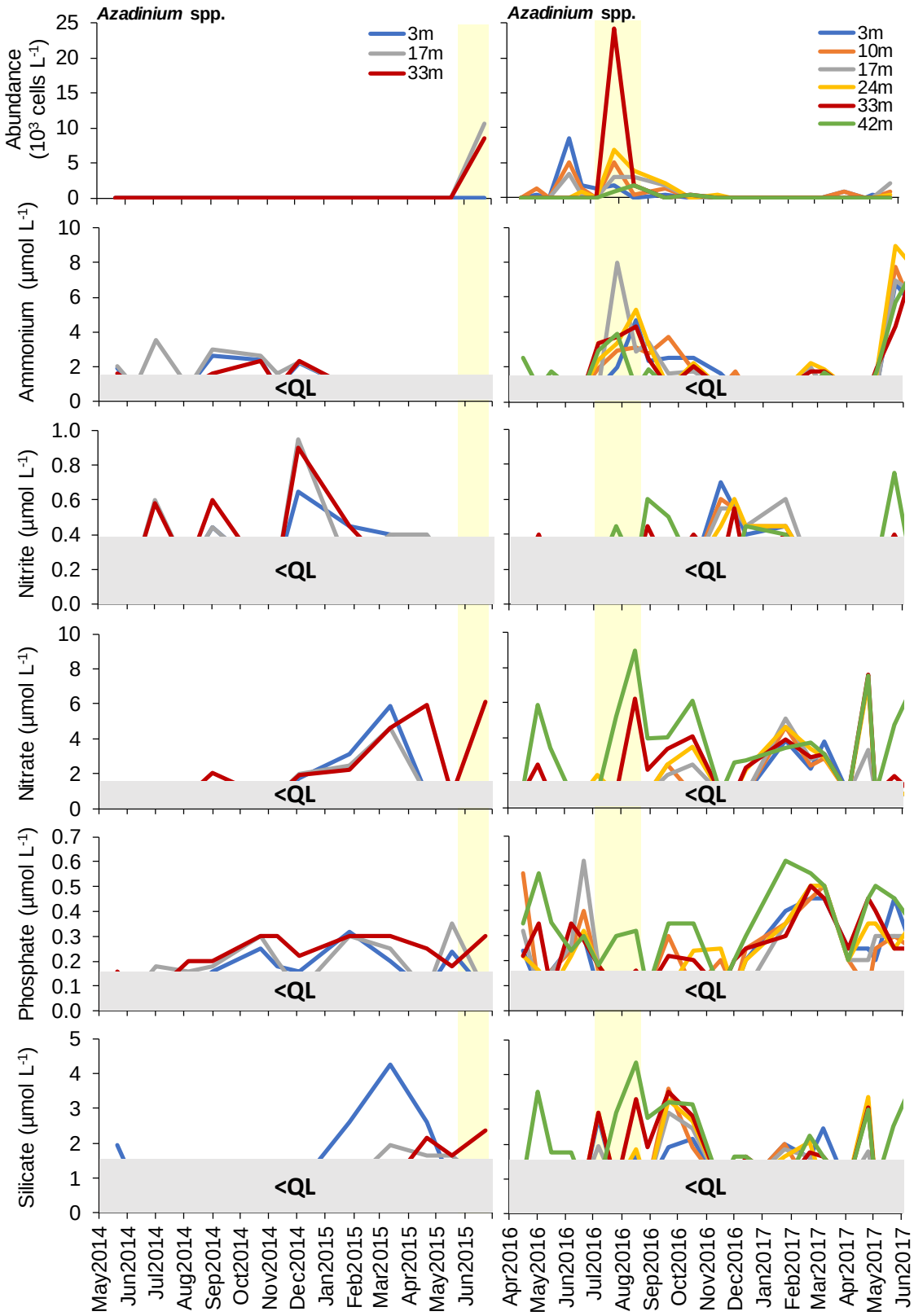
Annex 5.8a. Temporal variability in the abundance of the toxic species *Protoceratium reticulatum* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. QL stands for quantification limit: 1.6 µmol L^{-1} for ammonium, nitrate and silicate, 0.4 µmol L^{-1} for nitrite and 0.16 µmol L^{-1} for phosphate. *P. reticulatum* was not registered during the period 2014-2015 and thus, only the period 2016-2017 is represented.



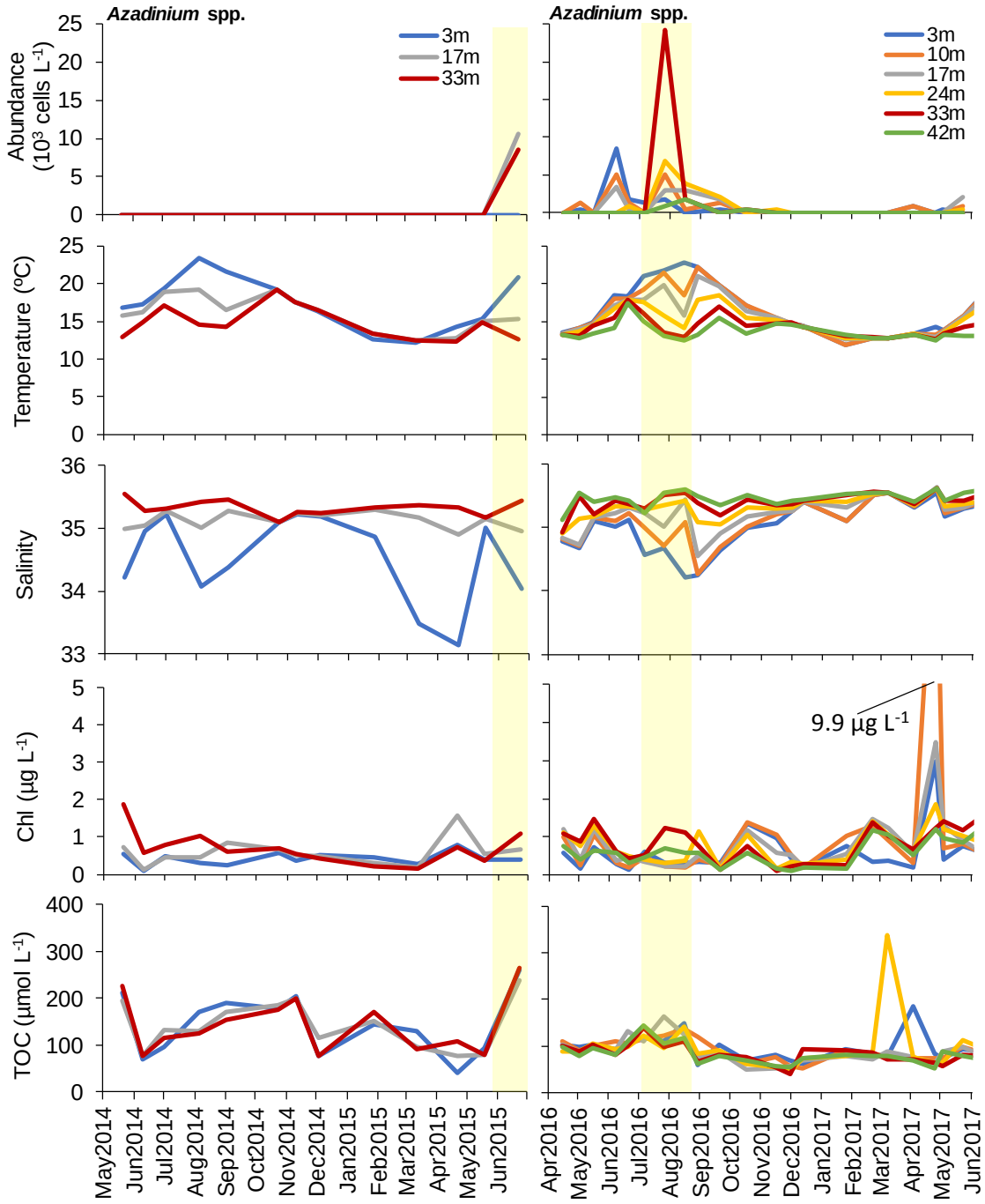
Annex 5.8b. Temporal variability in the abundance of the toxic species *Protoceratium reticulatum* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. Chl: chlorophyll *a,* TOC: total organic carbon. *P. reticulatum* was not registered during the period 2014-2015 and thus, only the period 2016-2017 is represented.



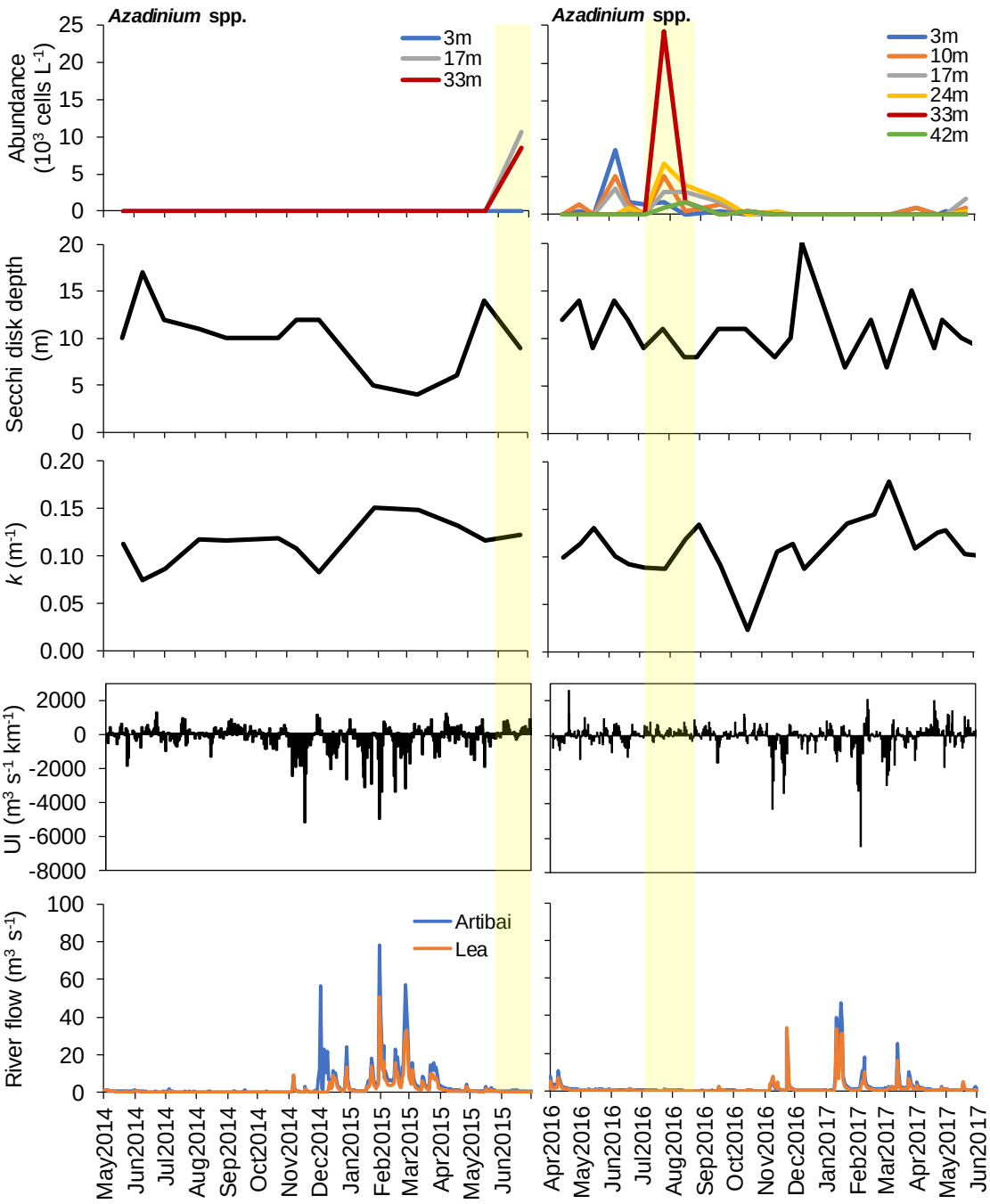
Annex 5.8c. Temporal variability in the abundance of the toxic species *Protoceratium reticulatum* and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. *k*: light extinction coefficient, UI: upwelling index. *P. reticulatum* was not registered during the period 2014-2015 and thus, only the period 2016-2017 is represented.



Annex 5.9a. Temporal variability in the abundance of the toxic genus *Azadinium* spp. and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. QL stands for quantification limit: 1.6 µmol L^{-1} for ammonium, nitrate and silicate, 0.4 µmol L^{-1} for nitrite and 0.16 µmol L^{-1} for phosphate.



Annex 5.9b. Temporal variability in the abundance of the toxic genus *Azadinium* spp. and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. Chl: chlorophyll *a,* TOC: total organic carbon.



Annex 5.9c. Temporal variability in the abundance of the toxic genus *Azadinium* spp. and in the environmental variables through the water column. The yellow shaded areas show the peaks in cell density. *k:* light extinction coefficient, UI: upwelling index.