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Suitability of two areas of the Basque coast to sustain shellfish aquaculture according to both the presence of potentially toxic phytoplankton and the biotoxins regulated by the European Union

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ABSTRACT

The composition and dynamics of microalgae play an important role in shellfish aquaculture, since phytoplankton is the main source of energy for filter-feeding bivalves as well as the main potential toxicity risk. Together with the increase in world aquaculture production in the last decades, there is recent interest in the implementation of shellfish aquaculture on the Basque coast (southeastern Bay of Biscay). In this context, the study of the potentially toxic phytoplankton abundance and dynamics has become essential, since the viability of shellfish aquaculture in the area could be compromised by biotoxins. In the present study, two euhaline sites of the Basque shelf, one inshore (Mutriku) and the other offshore (Mendexa), were compared during a one-year period. The main aim was to determine which site was more suitable for the development of shellfish aquaculture, from the perspective of exposure to toxic phytoplankton, by comparing the composition and abundance of the potentially toxic phytoplankton community and the concentrations of toxins in mussel flesh. The mussels that grew offshore presented a higher amount of okadaic acid (OA), in accordance with the fact that this site (Mendexa) also presented a higher cell abundance of *Dinophysis acuminata*, a potential producer of OA. In addition, although *Dinophysis* spp., *Pseudo-nitzschia* spp. and *Alexandrium* spp. exceeded their cell alert thresholds several times at both studied sites, the dinoflagellates presented a higher frequency of exceedance at Mendexa. Moreover, the percentage of samples with toxin concentrations that exceeded quantification limits was higher at Mendexa as well. Therefore, from the perspective of the currently regulated biotoxins, in the Basque Country, inshore euhaline waters seem to be more suitable for mussel aquaculture than offshore waters.

1. Introduction

Phytoplankton composition and dynamics are critical factors for shellfish aquaculture, with microscopic algae being the main source of energy for the growth of most filter-feeding bivalves (Shumway and Cucci, 1987; MacDonald and Ward, 1994; Grant, 1996; Petersen et al., 2008). Therefore, the proliferation of phytoplankton in the marine environment is in most cases beneficial for aquaculture operations. However, at least 80 marine phytoplankton species have the capacity to produce toxins and are consequently considered harmful (Hallegraeff, 2003). Toxic microalgae species can be found among diatoms, haptophytes, dinoflagellates, raphidophyceans, dictyochophyceans, pelagophyceans, and cyanobacteria (Moestrup et al., 2009). Some of them can contaminate shellfish, even at very low cell concentrations, because when the toxic cells are filtered as food by molluscs, toxins are accumulated actively and concentrated in their flesh (Masó and Garcés, 2006). This becomes a serious risk to humans, who can be affected by different poisoning syndromes (e.g., Backer et al., 2004; Lawrence et al., 2011). Nowadays most coastal countries are threatened by toxic phytoplankton species and many aquaculture companies all over the world are forced to shut down operation for long periods (Anderson, 2009; Davidson and Bresnan, 2009; Reguera et al., 2016).

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World aquaculture production has increased considerably in the course of half a century (FAO, 2012). Mussel production, specifically, has registered an upward trend in the period between 2007 and 2016, exceeding two million tons for the first time in 2016. Within the European Union (EU), Spain is the country with the highest mussel production, amounting to more than 200,000 tons per year (FAO, 2018). Most of this production takes place on the northwest coast of Spain, in the Atlantic waters of Galicia (e.g., Ferreira et al., 2014; Rodríguez et al., 2015). There, mussels are cultivated by means of floating rafts in coastal inlets (Rias), where phytoplankton production is enhanced by the influence of upwelling processes and estuarine circulation (e.g., Moncoiffé et al., 2000; Figueiras et al., 2002; Varela et al., 2005). Nevertheless, globally, there is increasing interest in expanding shellfish aquaculture to offshore areas (e.g., van den Burg et al., 2017). The potential advantages compared to inshore areas point to greater sanitary safety, better use of space, and lower visual and ecological impacts (Mizuta et al., 2019). Offshore aquaculture is aligned with the European Blue Growth Strategy (European Commission, 2012), which aims at developing technology innovations that contribute to economic progress whilst safeguarding biodiversity and protecting the marine environment. In a global context, FAO promotes blue growth as "a cohesive approach for environmentally compatible integrated and socio-economic sensitive management of aquatic resources including marine, freshwater and brackish water environment" (Soma et al., 2018).

Offshore shellfish aquaculture has also attracted the attention of local authorities and investors in the north of Spain, which has led to the establishment of an experimental farm off the Basque coast in the southeastern Bay of Biscay (Azpeitia et al., 2016, 2017, 2018; Muñiz et al., 2019). However, taking into account that some phytoplankton species are toxigenic, the viability of shellfish aquaculture in the Basque Country could be compromised by biotoxins. In order to prevent illness in humans due to shellfish consumption, European food legislation focuses on bivalve molluscs and provides maximum limits for several marine biotoxins (Visciano et al., 2016). Regulation (EC) No 853/2004 considers the toxins associated with amnesic and paralytic shellfish poisonings (ASP and PSP, respectively) and several lipophilic toxins. These last ones are okadaic acid (OA), dinophysistoxins (DTXs), pectenotoxins (PTXs), azaspiracids (AZAs), and yessotoxins (YTXs). A group that includes the OA, the DTXs, and their esters is associated with diarrheic shellfish poisoning (DSP), whereas the AZAs are associated with azaspiracid shellfish poisoning (AZP). The PTXs and YTXs were initially considered DSP toxins, but recent studies indicate that these groups do not cause diarrhoea when fed via the oral route (FAO, 2014). Although the symptoms of YTXs in humans are still unknown, paralytic effects on the cardiac muscle have been confirmed in mice (Paz et al., 2008; Ferreiro et al., 2015). Most of these biotoxins are produced by dinoflagellates, although cyanobacteria have also been reported to produce PSP toxins, whereas ASP is produced by some species of the diatom genus *Pseudo-nitzschia* (Lawrence et al., 2011). Nevertheless, new toxigenic algal species and more toxic compounds are continually being discovered (Toyofuku, 2006; Munday and Reeve, 2013). Previous studies have indicated the presence of phytoplankton species that could synthesize some of the above mentioned biotoxins in open marine waters (Muñiz et al., 2017) as well as in estuaries of the Basque Country (Orive et al., 2010, 2013). However, studies dealing with biotoxins in this region are still very scarce and, up to now, have only focused on azaspiracids (Blanco et al., 2017) and pinnatoxins (Lamas et al., 2019).

Due to the prospect of new aquaculture operations, in the Basque Country there is now a special interest in assessing the likelihood of phytotoxin levels being above the regulatory limits in bivalves. Methods for the prediction of toxic outbreaks usually require the use of data on physical, chemical, and biological variables (Mateus et al., 2019). This is based on the fact that environmental factors, mainly light and nutrients, regulate phytoplankton composition (e.g., Adolf et al., 2006). Therefore, ocean-meteorological variables that modify the supply of these resources (i.e., temperature, wind, depth of the mixing layer, day-length, river runoff) determine phytoplankton assemblages (Reynolds, 2006). Anthropogenic pressures such as nutrient loads from sewage also alter the communities by stimulating the growth of certain organisms, among which are toxic plankton (GEOHAB, 2006). However, the relationships with environmental variables cannot be fully generalized to a whole taxonomic group (e.g., dinoflagellates) or genus (Muñiz et al., 2018); even different strains of the same species have been observed to show different responses (Burkholder et al., 2006). In addition, biotic factors like grazing, competition, parasitism, and microbial attack also influence phytoplankton populations (Granéli and Turner, 2006).

In this context, a question that remains is whether the link between anthropogenic nutrient inputs and harmful microalgae events can be generalized, or if, on the contrary, given the variety of life strategies among the phytoplankton and the complexity of the ecological interactions (Huisman and Weissing, 2001; Smayda and Reynolds, 2003; Glibert, 2016), this is not always the rule (Davidson et al., 2014). On the Basque shelf, anthropogenic nutrient enrichment usually decreases from inshore to offshore waters (Borja et al., 2011; Garmendia et al., 2011). Shellfish aquaculture areas are generally established inshore, but in the Basque Country, longline mussel farms are being set up in open marine waters. Therefore, it is important to know whether or not this decision will be favourable from the perspective of exposure to toxic phytoplankton.

Consequently, this work performs a study comparing two euhaline sites on the Basque shelf, one located inshore (within a harbour) and the other offshore, with the aims of (i) determining differences in environmental factors, (ii) assessing the occurrence of potentially toxic phytoplankton in water as well as biotoxins in mussel flesh, and (iii) determining which environmental variables are the most significantly related to both the abundance of toxic phytoplankton and the amount of toxins.

The hypothesis tested is that, on the Basque shelf, mussels growing offshore will be exposed to lower abundances of toxic phytoplankton and will contain lower amounts of phytotoxins than mussels growing inshore, assuming that anthropogenic nutrients boost toxic events and that there is a decreasing gradient of nutrient pressure from the inshore to the offshore waters.

2. Material and methods

2.1. Study area and sampling stations

The Spanish Basque coast is located in the southeastern Bay of Biscay and extends along approximately 100 km (Fig. 1). This area is an exposed littoral coast, of high energy and mainly erosional, with large cliffs (Cearreta et al., 2004). The tide is semi-diurnal. The tidal range is, on average, 3.5 m. The region is defined as 'low meso-tidal' during neaps and 'high meso-tidal' during spring tides. In the open sea, wind-induced currents are important, particularly in the upper layers of the water column. Port entrances are exposed to the combined action of waves and tidal currents (González et al., 2004).

The climate is rainy, temperate, and oceanic, with warm summers and moderate winters (Fontán et al., 2009). The Spanish Basque coast is influenced by 12 short rivers with big slopes, which are torrential in character (Ferrer et al., 2009) and provide,

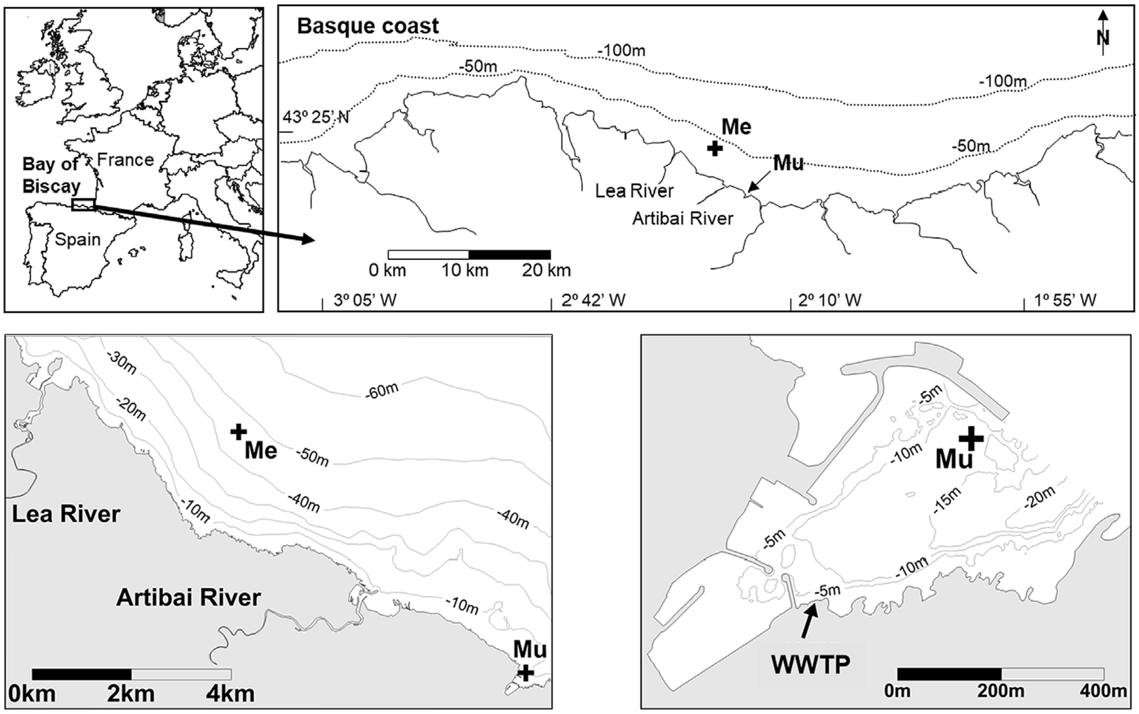

Fig. 1. Study area and sampling stations. Upper panels: on the left, the Basque coast within the Bay of Biscay; on the right, the location of the inshore station "Mutriku" (Mu) and the offshore station "Mendexa" (Me). Lower panels: on the left, a closer look at the locations of the stations; on the right, Mutriku harbour is depicted with the inshore sampling station and the discharge point of the Wastewater Treatment Plant (WWTP).

annually, 150 m^3 s^{-1} of freshwater to the coastal water bodies. Although no large river plumes are formed, this freshwater supply alters the physico-chemical composition of the shallow waters and often leads to an increase in nutrient concentrations in inner shelf waters. The influence of upwelling events as natural fertilization processes is almost negligible in these waters (Valencia et al., 2004).

This study draws on data from two sampling sites (an inshore station and an offshore station) separated by a distance of 7.5 km (Fig. 1). The inshore station, "Mutriku", is located in the outer part of a marina (43°18.7′N, 2°22.6′W); this site is partially protected from the wave action by a jetty and its depth is approximately 15 m. Although rivers do not discharge into this harbour, it receives the effluents from a Wastewater Treatment Plant (WWTP) that serves the surrounding population (approximately 5300 inhabitants). The offshore station, "Mendexa", is located 2 km off the coast (43°21.4′N, 2°26.9′W), at a depth of about 45 m, immediately outside an experimental bivalve farm. The organisms cultured during this study were mainly mussels (*Mytilus galloprovincialis*), in longlines in the offshore farm (Azpeitia et al., 2016; Muñiz et al., 2019) and in a floating raft in the harbour.

2.2. Sampling and laboratory work

This study extended over 12 months (from June 2016 to May 2017). The sampling was carried out at each station on a monthly basis (except for August at Mutriku and April and May at both sites, when samples were taken twice a month). Data and water samples for the characterization of physico-chemical conditions and toxic phytoplankton were obtained at two depths (3 and 10 m). Mussels were sampled for the analysis of their toxin content in both study areas, the offshore farm and the harbour, usually by diving; growing ropes were 12 m long, but in most cases, the collected organisms were from approximately the upper 3 m of the water column. Although oysters were also cultured, they were not present during the whole study period in both areas, which was a requisite for making the statistical comparisons. The sampling dates and type of samples collected are summarized in Appendix A.

In the field, several in situ measurements were undertaken. Secchi disk depth was measured as an estimate of water transparency, and a Seabird 25 CTD (conductivity, temperature, and depth) device was employed for the measurement of temperature, salinity, density (sigma-theta), Light Transmission (LT), Photosynthetically Active Radiation (PAR), chlorophyll "a", oxygen concentration, oxygen saturation, and pH.

Water samples were collected using Niskin bottles at two discrete depths: 3 and 10 m. These samples were used for the analysis of turbidity, Suspended Solids (SS), Total Organic Carbon (TOC), dissolved inorganic nutrients, and phytoplankton identification and counting.

In the laboratory, the turbidity of seawater was measured using a turbidimeter (2100 Turbidimeter, HACH; Loveland, Colorado, USA). The concentration of SS was measured as described in Clesceri et al. (1989) after filtration of the water through Whatman GF/C filters. For TOC, an analyser (TOC-V CSH/CSN, Shimadzu Corporation, Kyoto, Japan) was used in non-purgeable organic carbon (NPOC) mode as described in Grasshoff et al. (1983). Regarding nutrients (ammonium, nitrite, nitrate, silicate, and phosphate), the measurements were carried out using a Continuous-Flow Autoanalyser (Bran + Luebbe Autoanalyzer 3, Norderstedt, Germany) following the colorimetric methods described in Grasshoff et al. (1983). The quantification limit was 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 average concentrations, for the measurements that did not reach these quantification limits, a quantity equal to 50% of the limit was assumed.

Water samples used for phytoplankton identification were stored in 125-ml topaz borosilicate bottles, fixed with acidic Lugol's solution, and preserved in the dark and cool (4 °C) until analysis. Subsamples of 50 ml were analysed following the Utermöhl sedimentation method (Utermöhl, 1958; Hasle, 1978; Edler and Elbrächter, 2010) and using a Nikon diaphot TMD inverted microscope. The whole sedimentation chamber was analysed at low magnifications (100×) to count the microplankton-sized cells (for example, *Dinophysis* spp. and *Alexandrium* spp.). If the cell concentration was high enough (for example, for *Pseudo-nitzschia* spp. in some samples), transects were analysed and a minimum of 100 cells were counted. For nanoplankton-sized cells, transects at lower magnifications (up to 10 cm at 400×) were analysed.

The phytoplankton considered potentially toxic was identified and enumerated. Since the toxic character of some microalgae species is under debate, the Taxonomic Reference List of Harmful Micro Algae from the Intergovernmental Oceanographic Commission of UNESCO was used as a checklist (Moestrup et al., 2009; http://www.marinespecies.org/hab/, accessed on 30 June 2018) to determine which taxa had to be considered potentially toxic and to standardize the nomenclature. As a precautionary measure, when a genus contained both toxic and non-toxic species, the whole genus was considered as potentially toxic if species-level identification could not be achieved.

As an approach to determine events of risk of shellfish poisoning, alert levels of cell concentration were considered for the genera causing the three main syndromes of concern in this study area (ASP, DSP, and PSP): *Pseudo-nitzschia* spp., *Dinophysis* spp., and *Alexandrium* spp., respectively. Since some differences can be found in the literature, the threshold levels employed here were the most restrictive ones among those previously used for Basque marine waters (Muñiz et al., 2017). Thus, following Swan and Davidson (2012), the alerts levels were 50 000 cells L^{-1} for *Pseudo-nitzschia* spp., 100 cells L^{-1} for *Dinophysis* spp., and "presence" for *Alexandrium* spp. Moreover, these threshold levels are commonly used in European monitoring programmes for harmful phytoplankton (ICES, 2015).

Regarding the concentration of marine biotoxins in mussel flesh, only the ones regulated by the EU legislation were analysed: domoic acid (DA) (ASP causative), saxitoxin (STX) and derivatives (PSP causatives), and several groups of lipophilic toxins, which include OA and DTXs (DSP causatives) together with PTXs, AZAs (AZP causatives), and YTXs (cardiotoxicity causatives). The regulatory limits implemented in Regulations 853/2004 and 786/2013 were applied (European Commission, 2004, 2013). The analyses were performed by INTECMAR (Technological Institute for the Monitoring of the Marine Environment in Galicia, Spain) using internationally recognized validated methods (http://www.intecmar.gal/intecmar/Biotoxinas.aspx?sm=f), as summarized in Table 1.

2.3. Statistical analysis

The main statistical parameters (range, median, and/or arithmetic mean and standard deviation) were calculated for physico-chemical variables, phytoplankton cell abundance, and amount of toxins for each study site and depth.

It was assumed that the variables (physico-chemical parameters, phytoplankton cell abundance, and toxin concentration) were not all normally distributed, as is typical for environmental data (Legendre and Legendre, 1979). Consequently, non-parametric methods were applied to detect significant differences and relationships.

The Wilcoxon signed-rank test was used to determine whether there were significant differences (alpha = 0.05) in the median values of physico-chemical parameters, phytoplankton cell abundance, and toxin concentration between the two studied sites (Mutriku and Mendexa). The data from January 2017 were excluded from this analysis because the sites were not sampled on the same day (Appendix A). In addition, the data from the second sampling of August was also eliminated because only data from Mutriku were available. This test was also applied to determine whether there were significant differences in the median values between 3 and 10 m at each station. This last analysis used the whole data set (June 2016–May 2017).

Spearman rank correlation was carried out to measure the degree of association between phytoplankton cell abundance or the amount of toxins detected in mussel flesh and physico-chemical variables. In the case of the biotoxin data, which refer to mussels collected at just one sampling depth (approximately 3 m), separate analyses were applied to look for relationships with the environmental data obtained at each of the two depths (3 and 10 m). The whole data set was used for the correlation analyses. However, the analysis only included the environmental variables that, a priori, could have an effect on the phytoplankton variability: Secchi disk depth, temperature, salinity, light transmission, chlorophyll "a", turbidity, TOC, and nutrient concentration. In addition, in order to avoid a "Type I" error, Bonferroni correction was applied (alpha = 0.05).

All statistical analyses were carried out using PAST 3.2 (PAleontological STatistics), a software package for data analysis (Hammer et al., 2001).

3. Results

3.1. Environmental conditions

In order to describe the general conditions of the water, Table 2 shows the arithmetic mean and the standard deviation values for each sampling site and depth. The range (minimum–maximum), the medians, and the p-values resulting from the Wilcoxon signed-rank test can be viewed in Appendix B.

Considering the first 10 m of the water column, physico-chemical conditions showed a more homogeneous vertical distribution at the offshore station (Mendexa) than at the inshore station (Mutriku). Thus, at Mendexa, only chlorophyll "a" was significantly different between 3 and 10 m, whereas at Mutriku, temperature, salinity, density, and turbidity differed with depth. While Mendexa showed higher chlorophyll "a" values at 10-m depth, the waters at Mutriku were slightly colder, saltier, denser, and more turbid at this depth than at 3 m.

When stations were compared, significant differences were found in the optical variables and oxygen conditions at both 3 and 10 m depth (Fig. 2). The water at the offshore station was clearer than that at the inshore station, since the median values of Secchi disk depth and LT were higher and the median turbidity was lower at Mendexa (Table B1, Supplementary Electronic Material). Concerning dissolved oxygen, Mendexa showed significantly higher median values of both concentration and saturation than Mutriku at both 3 m ($p < 0.05$) and 10 m ($p < 0.01$).

Regarding temperature, salinity, and density, there were no significant differences in the median values between stations during the study period. Chlorophyll "a", pH, SS, TOC, and nutrient median values did not show significant differences either (Table B4, Supplementary Electronic Material). When it came to nutrient concentrations, nitrate and ammonium were the most abundant inorganic nitrogen forms, but while the annual means of these forms were almost the same at Mutriku, nitrate was less abundant than ammonium at Mendexa (Table 2). When using their annual mean concentrations, the ratio of ammonium to nitrate was slightly lower at Mutriku (0.9) than at Mendexa (1.4). As for the N:P ratio, summing up the annual mean concentrations of the three nitrogen forms, it was close to 16 (the Redfield ratio) at both sites (15.8 at Mutriku and 15.5 at Mendexa). In contrast, due to the relatively low concentration of silicate, the mean N:Si

Table 1
The methods applied for the analysis of the biotoxins in this study according to the regulations in force (European Commission, 2005, 2011), together with the quantification limits used for each analyte. The regulatory limits are also indicated (European Commission, 2004, 2013). For lipophilic toxins, the regulatory limits apply to the sum of several analytes[a,b,c]. HPLC: High Performance Liquid Chromatography; MBA: mouse bioassay (it implies the death of at least two out of three inoculated mice in 24 h); LC-MS/MS: Liquid Chromatography–Mass Spectrometry.

Methods	Toxin groups	Analytes	Quantification limits	Regulatory limits	Units
HPLC	Amnesic shellfish poisoning toxin	Domoic acid (DA)	2	20	mg DA kg^{-1}
MBA	Paralytic shellfish poisoning toxins	Saxitoxin (STX) and derivatives	380	800	μg STX diHCl eq. kg^{-1}
LC-MS/MS	Okadaic acid (OA) group	OA	40	160[a]	μg OA eq. kg^{-1}
		DTX1	40		
		DTX2	24		
	Pectenotoxin (PTX) group	PTX1	40		
		PTX2	40		
	Azaspiracid (AZA) group	AZA1	40	160[b]	μg AZA eq. kg^{-1}
		AZA2	42		
		AZA3	41		
	Yessotoxin (YTX) group	YTX	0.06	3.75[c]	mg YTX eq. kg^{-1}
		homo-YTX	0.06		
		45-hydroxy-YTX	0.06		
		45-hydroxyhomo-YTX	0.03		

[a]Sum of OA, dinophysistoxins (DTXs), and PTXs.
[b]Sum of AZAs.
[c]Sum of YTXs.

Table 2
Description of the water column conditions at the inshore station (Mutriku) and at the offshore station (Mendexa), for the period June 2016–May 2017. The values of the arithmetic mean and standard deviation are shown for the two discrete sampled depths; the Secchi disk depth refers to the whole water column. The number of samples is 15 and 14, for Mutriku and Mendexa, respectively.

	Depth (m)	Mutriku (inshore station)	Mendexa (offshore station)
Secchi disk depth (m)	–	7.5 ± 1.7	10.2 ± 2.5
Turbidity (NTU)	3	0.8 ± 0.3	0.6 ± 0.1
	10	0.9 ± 0.4	0.6 ± 0.2
Light transmission (%)	3	81.9 ± 4.2	84.4 ± 2.8
	10	80.9 ± 4.2	84.7 ± 3.8
Temperature (°C)	3	16.3 ± 3.6	16.0 ± 3.3
	10	15.8 ± 3.0	15.5 ± 2.6
Salinity (PSU)	3	35.0 ± 0.4	35.1 ± 0.4
	10	35.2 ± 0.3	35.2 ± 0.2
Density (sigma-theta)	3	25.6 ± 1.1	25.8 ± 1.1
	10	25.9 ± 0.9	26.0 ± 0.8
Chlorophyll “a” (μg L^{-1})	3	0.6 ± 0.4	0.7 ± 0.7
	10	0.8 ± 1.0	1.4 ± 2.5
Dissolved oxygen (ml L^{-1})	3	5.4 ± 0.6	5.6 ± 0.5
	10	5.4 ± 0.5	5.7 ± 0.4
Oxygen saturation (%)	3	96.2 ± 8.5	101.0 ± 7.0
	10	95.9 ± 4.6	101.0 ± 4.9
pH	3	8.1 ± 0.1	8.1 ± 0.1
	10	8.1 ± 0.1	8.1 ± 0.1
Suspended Solids (mg L^{-1})	3	7.1 ± 2.6	7.6 ± 3.6
	10	8.0 ± 3.2	7.0 ± 3.6
Total Organic Carbon (μmol C L^{-1})	3	89.0 ± 25.8	97.7 ± 33.1
	10	90.7 ± 15.4	86.5 ± 21.8
Ammonium (μmol L^{-1})	3	1.7 ± 1.4	1.8 ± 1.8
	10	1.8 ± 1.3	2.0 ± 1.9
Nitrite (μmol L^{-1})	3	0.24 ± 0.11	0.28 ± 0.16
	10	0.24 ± 0.11	0.27 ± 0.15
Nitrate (μmol L^{-1})	3	2.0 ± 1.7	1.3 ± 1.1
	10	1.9 ± 1.6	1.5 ± 1.2
Phosphate (μmol L^{-1})	3	0.25 ± 0.14	0.23 ± 0.15
	10	0.25 ± 0.16	0.23 ± 0.14
Silicate (μmol L^{-1})	3	1.4 ± 0.9	1.4 ± 0.7
	10	1.5 ± 1.0	1.2 ± 0.8

and Si:P ratios (about 3 and 6, respectively) deviated from the Redfield ratios (1 and 16, respectively). Inorganic nutrients did not show very high peaks, with the maxima being 6.4 μmol L^{-1} for nitrate (registered at Mutriku, 3 m), 7.8 μmol L^{-1} for ammonium (Mendexa, 10 m), and 3.6 μmol L^{-1} for silicate (Mendexa, 10 m); phosphate did not exceed 0.5 μmol L^{-1} at either site. The maximum value of chlorophyll “a” was recorded at Mendexa,

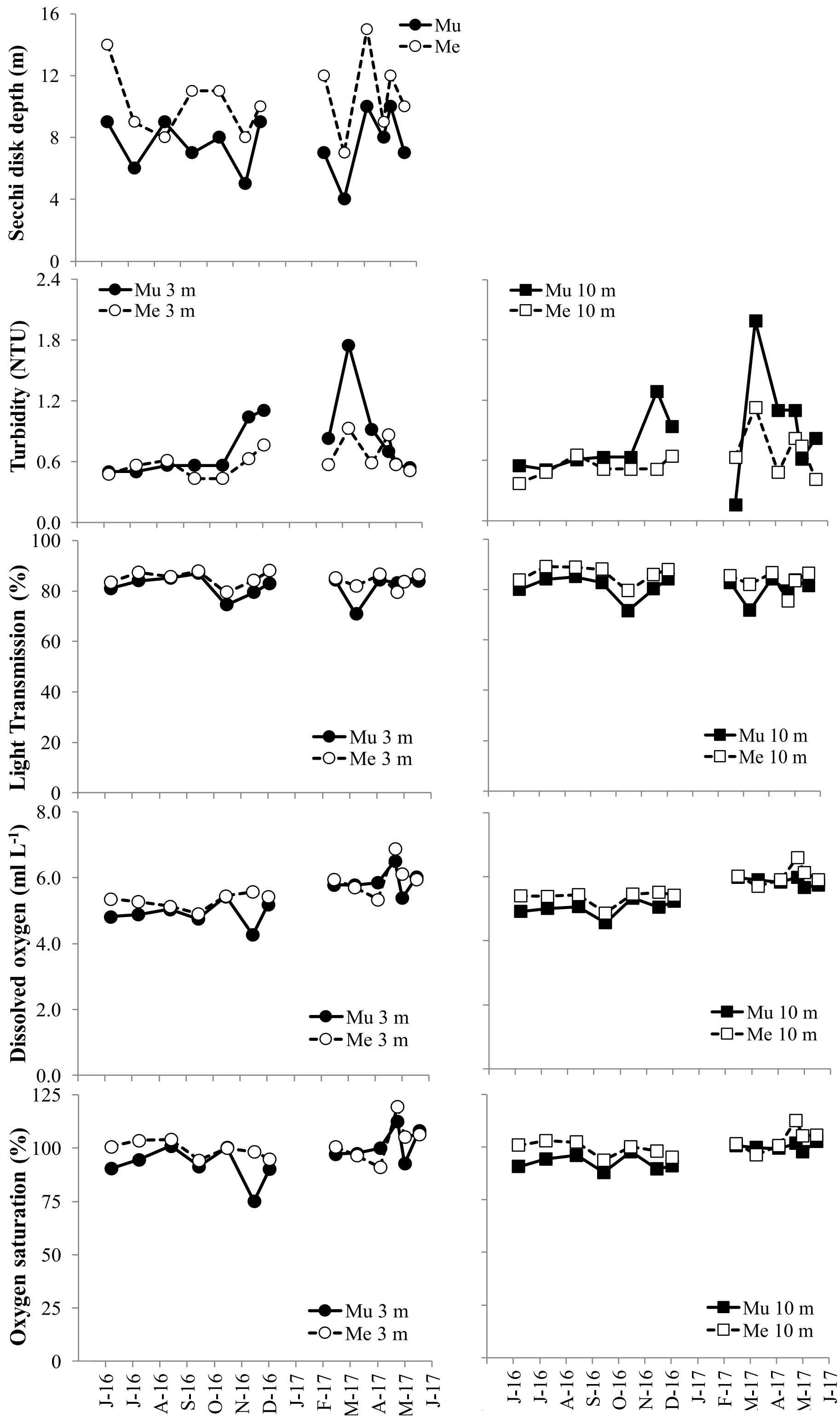

Fig. 2. Physico-chemical variables that showed statistically significant differences between the inshore station Mutriku (Mu) and the offshore station Mendexa (Me) during the study period (June 2016–May 2017).

where it was 9.9 μg L^{-1} at 10 m depth (Table B2, Supplementary Electronic Material).

3.2. Potentially toxic phytoplankton

At the inshore station, Mutriku, 14 potentially toxic phytoplankton taxa were identified during the study period. Among them, *Pseudo-nitzschia* spp. was the only diatom, whereas 12 taxa were dinoflagellates (Table 3). From other groups, only a prymnesiophycean (*Phaeocystis globosa*) was identified as a potentially toxic taxon at the inshore station. *Pseudo-nitzschia* spp. were present in all sampling campaigns and this taxon also showed the highest contribution to the total cell abundance of the toxic taxa, 94% on average. The second most frequent (∼67%) and abundant

Table 3
Frequency of presence of the potentially toxic phytoplankton taxa and their maximum abundance and date and depth of its registration at each of the stations during the study period (June 2016–May 2017). The toxins that could be associated with these taxa have been indicated. ASP: Amnesic Shellfish Poisoning; PSP: Paralytic Shellfish Poisoning; OA: Okadaic acid; YTXs: Yessotoxins; AZAs: Azaspiracids. The number of samples is 30 and 28, for Mutriku and Mendexa, respectively.

Toxins	Potentially toxic taxa	Mutriku (inshore station)			Mendexa (offshore station)		
		Presence[a]	Maximum abundance		Presence[a]	Maximum abundance	
		(%)	(cells L^{-1})	Date and depth	(%)	(cells L^{-1})	Date and depth
ASP toxin	*Pseudo-nitzschia* spp.	100	400 180	Apr. 2017 (10 m)	92.9	569 391	Apr. 2017 (10 m)
PSP toxins	*Alexandrium* spp.	13.3	40	Sept. 2016 and Jan. 2017 (3 m)	28.6	40	Apr. 2017 (3 m)
OA group toxins	*Dinophysis* spp.	33.3	640	Apr. 2017 (3 m)	92.9	2000	Apr. 2017 (10 m)
	Dinophysis acuminata	20	640	Apr. 2017 (3 m)	57.1	1980	Apr. 2017 (10 m)
	Phalacroma spp.	20	40	Oct. 2016 (3 m)	57.1	40	Jul. 2016 and May 2017 (3m); Nov. 2016 (10 m)
YTXs	*Gonyaulax spinifera*	20	80	Apr. 2017 (3 m)	14.3	440	Apr. 2017 (3 m)
	Lingulodinium polyedra	26.7	140	May 2017 (3 m)	35.7	200	Apr. 2017 (3 m)
	Protoceratium reticulatum	6.7	20	Jun. 2016 (10 m)	14.3	40	May 2017 (3 m)
AZAs	cf. *Azadinium* spp.	20	5522	Aug. 2016 (10 m)	50	8496	Jun. 2016 (3 m)
Others	Gymnodiniales p.p.[b]	66.7	5947	Aug. 2016 (3 m)	92.9	33 994	Nov. 2016 (10 m)
	Karenia spp.	20	120	Jun. 2016 (3 m)	21.4	160	Jun. 2016 (3 m)
	cf. *Karlodinium* spp.	33.3	2124	Jul. and Aug. 2016 (3 m)	71.4	8496	Nov. 2016 (10 m)
	Ostreopsis cf. *siamensis*	33.3	480	Aug. 2016 (10 m)	7.1	20	Sept. 2016 (10 m)
	Takayama sp.	6.7	120	Oct. 2016 (3 m)	14.3	20	Dec. 2016 (3 m); May 2017 (10 m)
	Phaeocystis globosa	20	17 040	Feb. 2017 (10 m)	14.3	20 390	Feb. 2017 (3 m)
	cf. *Pseudochattonella* sp.	0	–	–	14.3	2974	Feb. 2017 (10 m)

[a]Presence was assumed when the taxon was recorded at, at least, one of the two sampling depths.
[b]Unidentified gymnodinioid forms that could include small Kareniaceae.

taxon was the one referred to as Gymnodiniales *pro parte* (created for cells that could include unidentified kareniaceans), although its contribution to the total cell abundance was very low, less than 3%. Each of the other taxa contributed less than 1%.

At the offshore station, Mendexa, 15 potentially toxic phytoplankton taxa were identified, the same found at Mutriku, plus cf. *Pseudochattonella* sp. (a dictyochophycean species) (Table 3). Similarly to the inshore station, *Pseudo-nitzschia* spp. were present very frequently (∼93%) and were the dominant genus among the potentially toxic taxa at both depths, with a mean contribution of 90% to the cell abundance. Gymnodiniales p.p. were observed with an identical frequency to the diatoms, but contributed very little to the abundance (approximately 5%). It must be mentioned that the appearance frequency of several dinoflagellates was more than double at Mendexa compared to Mutriku (in particular, *Dinophysis* spp.).

Most of the taxa that are considered potential producers of the regulated biotoxins presented their maxima in April, May, or June (Table 3). Moreover, *Pseudo-nitzschia* spp., *Dinophysis* spp., *D. acuminata*, and *Gonyaulax spinifera* peaked in April 2017 at both sites. Other taxa presented some of their maxima out of the spring season, such as *Alexandrium* spp. (summer and winter), *Phalacroma* spp. (summer and autumn), and cf. *Azadinium* spp. (summer).

Regarding the differences with depth, at the inshore station (Mutriku) the taxa that can be producers or vectors of the OA group toxins (i.e., DSP causatives), *Dinophysis* spp. and *Phalacroma* spp., were only detected at 3 m, so their abundance was significantly higher than at 10 m according to the Wilcoxon signed-rank test ($p < 0.05$). On the contrary, at Mendexa no significant differences were found in the median values between 3 and 10 m, as far as the cell density of the potentially toxic phytoplankton was concerned (Table C1, Supplementary Electronic Material).

Several statistically significant differences were observed between the stations (Table C2, Supplementary Electronic Material). The offshore waters (Mendexa) presented a higher median abundance of the taxa potentially linked to the OA group toxins (*Dinophysis* spp. and *Phalacroma* spp.) at both depths ($p < 0.01$). This is explained by the higher abundance of *Dinophysis* spp. at 3 m ($p < 0.05$) and at 10 m ($p < 0.01$). More specifically, a significantly higher abundance of *Dinophysis acuminata* was detected in the offshore waters at both depths ($p < 0.05$). Apart from this, the stations showed one more difference: at 10 m, the abundance of Gymnodiniales p.p. was significantly higher at Mendexa.

Values above the cell alert limits for shellfish poisoning were observed in both stations (Fig. 3). *Pseudo-nitzschia* spp. showed a higher concentration than the threshold several times, but it only occurred at both sites and both depths in August and April. *Dinophysis* spp. exceeded their threshold in April; this was observed at both stations, but only at Mendexa for the two sampling depths. Since the mere presence of *Alexandrium* spp. implies risk

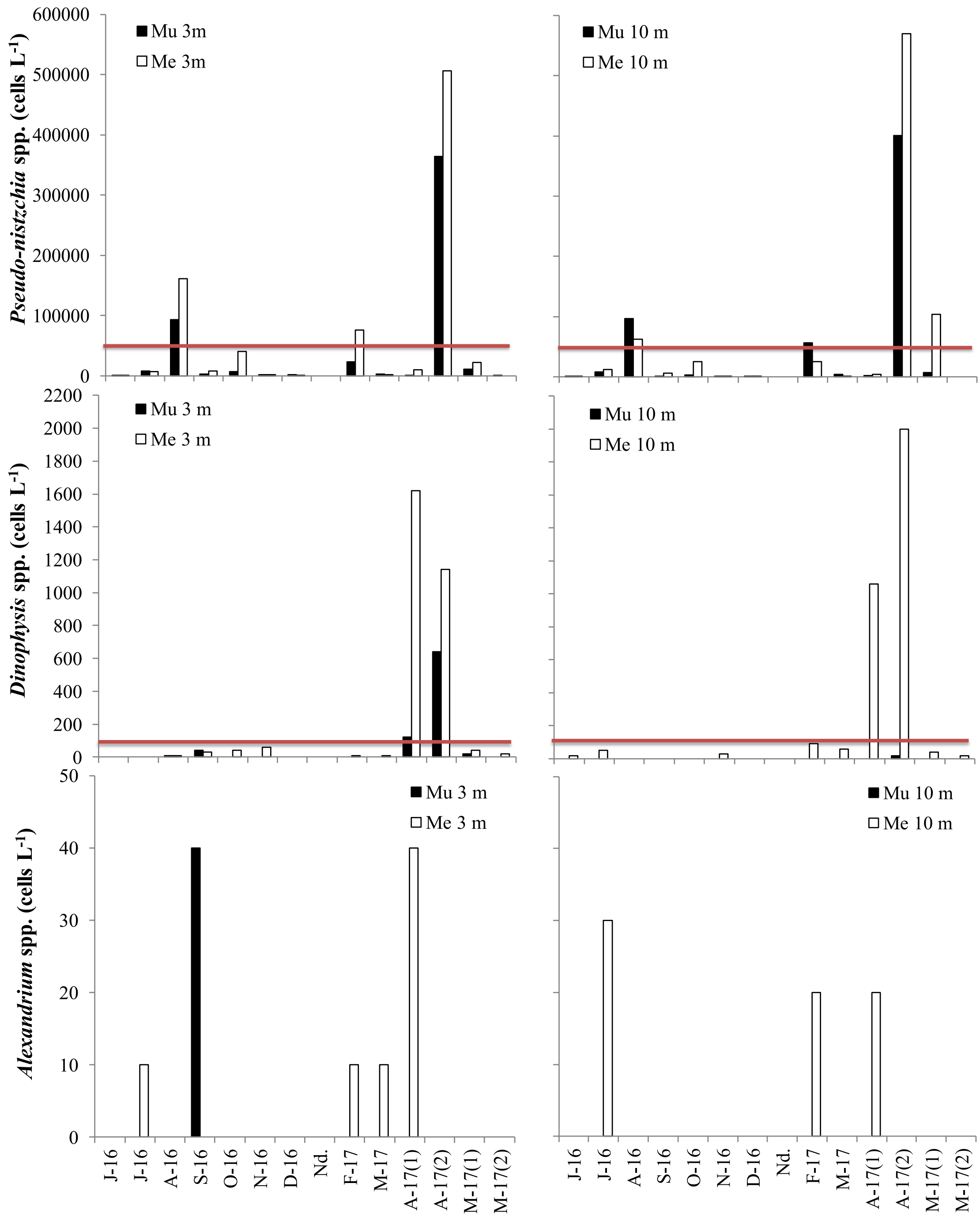

Fig. 3. Abundance of the main genera of concern for being responsible for causing shellfish poisonings, at the inshore station Mutriku (Mu) and the offshore station Mendexa (Me) at 3 and 10 m, sampled during the same days during the study period (June 2016–May 2017). The red line indicates the cell alert threshold (for *Alexandrium* spp. no line is shown as their mere presence indicates risk). Nd: No data.

of poisoning, a threatening situation at Mutriku was detected in September at 3 m. On the other hand, at Mendexa, *Alexandrium* spp. were recorded in July, February, March, and April (generally at both depths). Overall, taking into account the three genera of concern and both depths studied, Mutriku registered abundances above the alert thresholds for shellfish poisoning 8 times and Mendexa did so 17 times on comparable sampling days. *Pseudo-nitzschia* spp. exceeded the threshold in 23.1% of the sampling campaigns at Mutriku and in 30.8% at Mendexa; for *Dinophysis* spp. the frequency of exceedance was 15.4% at both sites; *Alexandrium* spp. were detected in 7.7% and 30.8% of the surveys at Mutriku and Mendexa respectively.

3.3. Toxins detected in mussel flesh

During the sampling campaigns conducted at Mutriku, the regulated biotoxins did not exceed the quantification limits very often (considering all analytes together, this occurred in 6 surveys out of 15). Appendix D presents the concentration range for those analytes that could be quantified on at least one occasion and Fig. 4 shows the spatial and temporal variability of the more frequent toxin groups (i.e., some lipophilic toxins). The OA exceeded the quantification limit three times (once in April and twice in May) and the YTX also did so three times (June, July, and August). A congener, the 45-hydroxy-YTX, could be quantified only in June. However, DTXs, PTXs, and AZAs were never above the quantification limits. The OA group exceeded the regulatory limit in May with 185.7 µg OA eq. kg^{-1}, due solely to the OA. The amnesic toxin was recorded above the quantification limit just once (in April, with 3.0 mg DA kg^{-1}), but it was far from the regulatory limit (20 mg DA kg^{-1}). Saxitoxins were not found above the quantification limit at the inshore station during this period.

At Mendexa, the regulated biotoxins were above the quantification limits in most sampling campaigns (considering all analytes together, in 12 surveys out of 14). The OA and the YTX each exceeded the quantification limits on seven occasions during the studied year. The 45-hydroxy-YTX was also above the quantification limit once (July). Although for the YTXs the concentration values remained low, the OA group exceeded the regulatory limit in April and May (Fig. 4). Similarly to Mutriku, no DTXs, PTXs, or AZAs were quantified. The amnesic toxin was found to be above the quantification limit on the same date as at Mutriku (April), but with a slightly higher concentration (6.1 mg DA kg^{-1}). Regarding the paralytic toxins, at Mendexa offshore station, they could be quantified in one sample out of the 13 analysed (in November, with 410 µg STX eq. kg^{-1}), although this concentration did not exceed the regulatory limit (800 µg STX eq. kg^{-1}).

Comparing the amount of toxins quantified in mussel flesh between Mutriku and Mendexa, a significant difference was observed in the median values according to the Wilcoxon signed-rank test (Table D2, Supplementary Electronic Material). The OA, a toxin responsible for DSP, presented a higher concentration at Mendexa, $p < 0.05$. These results agreed with those for potentially toxic phytoplankton, since the potential producers (or vectors) of the OA group toxins showed a significantly higher abundance at Mendexa as well (Table C2, Supplementary Electronic Material).

3.4. Relationships between the environmental parameters and the amount of potentially toxic phytoplankton and biotoxins

At Mutriku, no significant correlations were detected either between environmental parameters and phytoplankton or between environmental parameters and toxins. Therefore, none of the hydrographic, optical, and trophic parameters studied could explain the temporal variability of the potentially toxic phytoplankton or the toxins detected in mussel flesh at the inshore station.

At Mendexa, however, some significant correlations were found. A positive strong correlation ($p < 0.01$) was observed between the concentration of the YTX group in mussel flesh and the water temperature at both 3 m ($r_s = 0.914$) and 10 m ($r_s = 0.894$). In addition, at 10 m the abundance of *Phalacroma* spp. were positively correlated to nitrite ($r_s = 0.898$, $p < 0.01$), although this last correlation should be taken with caution as the majority of values in both variables did not reach quantification limits. The rest of the phytoplankton taxa and toxins did not show any significant correlations with the environmental parameters at Mendexa. The significant correlations are represented as graphs in Appendix E.

4. Discussion

4.1. Hydrography, optical variables, and trophic conditions of surface waters

Despite the proximity between the inshore (Mutriku) and offshore (Mendexa) sampling stations, significant differences were found in the optical variables and oxygen conditions. These differences could be explained by the fact that the coastal area is a transitional zone that is directly affected by the usage and characteristics of the bordering lands (e.g., Gazeau et al., 2004), but also by the different morphological features of these sites (depth and confinement).

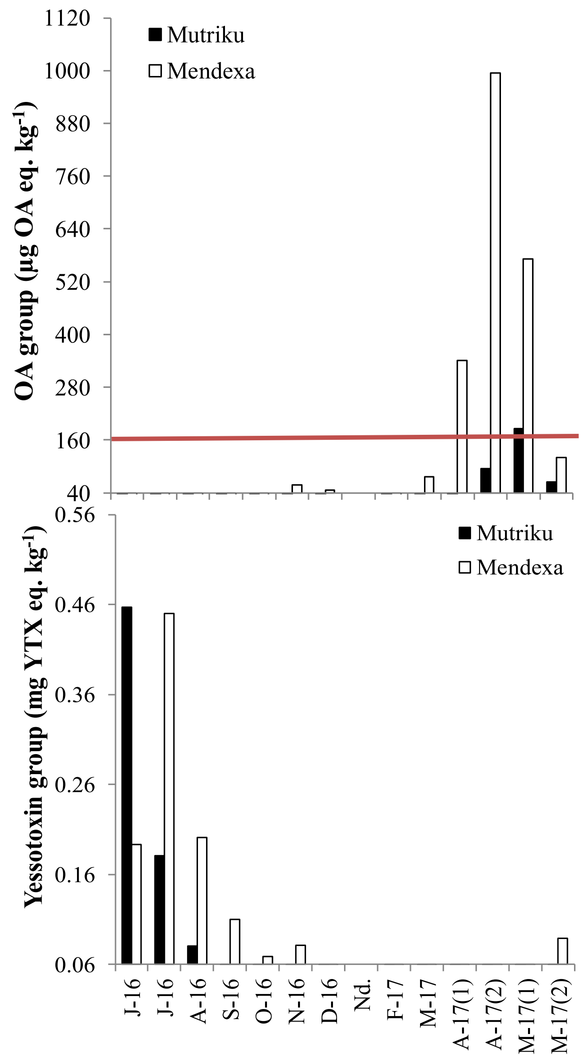

Fig. 4. Concentration of the lipophilic toxins that exceeded the quantification limits in mussel flesh at the inshore (Mutriku) and offshore (Mendexa) stations sampled on the same days during the study period (June 2016–May 2017). OA: okadaic acid; YTX: yessotoxin. The red line indicates the regulatory limit for toxin concentration (the limit for the yessotoxin group is 3.75 mg YTX eq. kg^{-1} and is therefore outside the figure). Nd: No data.

The lower availability of light that Mutriku presented in the first 10 m could be caused by the shallower depth of this site compared to Mendexa, since with a maximum depth of about 15 m in the harbour, the bottom sediments were closer to the surface waters than they were at the offshore site (45 m depth). This probably leads to higher resuspension of particles from the bottom in the harbour, mainly due to tidal currents, which would increase the turbidity (González et al., 2004).

Regarding oxygen conditions, Mutriku showed lower dissolved oxygen concentration and oxygen saturation values compared to the surface waters of Mendexa. These parameters are known to be controlled by air–sea fluxes, ocean mixing, internal advection, and biochemical processes (upper ocean primary production and deep ocean bacterial breakdown of organic matter) (Gupta and McNeil, 2012). In addition, dissolved oxygen can be reduced after the discharge of wastewaters or through eutrophic events fuelled by nutrient additions (Mudge et al., 2007). Mutriku is less exposed to the waves and therefore the exchange of oxygen with the atmosphere is expected to be more limited there than in the open sea. In addition, the lower light availability at Mutriku could lead to the limitation of primary production and therefore oxygen production.

Apart from that, the WWTP of Mutriku discharges its waters inside the port and such inputs from inland usually increase light attenuation in response to the supply of particulate matter and/or nutrients (e.g., Devlin et al., 2008; Abdelrhman, 2017) and might have also contributed to lowering the oxygen concentration at this site (Johannessen et al., 2015). For the euhaline coastal waters of the Basque Country, reference conditions (i.e., conditions representative of no or only very minor impact from human activities) were established by Bald et al. (2005) in order to assess the physico-chemical status in accordance with the Water Framework Directive (2000/60/EC). These conditions were ≥12 m (Secchi disk), ≥100% (oxygen saturation), ≤2.06 μmol L^{-1} (ammonium), ≤6.14 μmol L^{-1} (nitrate), and ≤0.45 μmol L^{-1} (phosphate), and they can be interpreted as annual mean values (Revilla et al., 2009). If the mean values obtained in this study were compared with them, no large deviations (>20%) would be observed except at Mutriku for the Secchi disk (37.5%). Regarding chlorophyll "a", the reference condition has been set as the 90th percentile value calculated over a period of several years (European Commission, 2018) and therefore in the present study it is not possible to infer deviations.

Nevertheless, the two stations showed similarities in the chlorophyll "a" concentration as well as in several physico-chemical factors. Although salinity values increased with distance from the shore, being slightly higher at Mendexa, this difference was not significant and both points can be considered euhaline. This indicates that the freshwater content of the two stations differs very little and, therefore, that the discharges from the WWTP are quite diluted at Mutriku. In addition, the WWTP performs biological treatment, which targets residual organic matter and suspended solids present in wastewater after the primary treatment stage and includes the removal of dissolved nutrients (Carey and Migliaccio, 2009). This could explain the lack of statistically significant differences in TOC, suspended solids, and nutrient concentration between Mutriku and Mendexa. Therefore, the inshore site overall did not seem to present symptoms of eutrophication.

Although it is not within the scope of the present study, the risk of microbial contamination (European Commission, 2019) should be considered if bivalve cultures are to be installed in inshore areas of the Basque Country, as these are generally near wastewater discharge points and rivers. In this region, the semi-enclosed marine areas are mostly estuaries and, historically, most of them have been heavily populated and industrialized, which has caused the degradation of their environmental quality (Valencia and Franco, 2004). In recent years, the chemical status, the general physico-chemical conditions, and the biological communities have improved considerably in many of these water bodies in response to the implementation of clean-up measures (Borja et al., 2016). However, sporadic events such as overflows from wastewater treatment plants and rivers could cause microbial quality to fail, preventing the use of some of these systems as bivalve culture areas (OSPAR Commission, 2009).

4.2. Toxic phytoplankton and biotoxin levels

Worldwide, the frequency and locations affected by toxic phytoplankton taxa have increased and therefore most coastal countries are threatened by toxic phytoplankton species (Anderson, 2009). The Basque coast is also a threatened area and, in the present study, 15 different potentially toxic phytoplankton taxa were identified, almost all of them registering at both the inshore and the offshore station. These taxa were previously described in different studies addressing phytoplankton taxonomic composition and dynamics in coastal waters of the southeastern Bay of Biscay (e.g., Orive et al., 2010; Laza-Martinez et al., 2011; Seoane et al., 2012; Batifoulier et al., 2013; Muñiz et al., 2017, 2018). The concentration and seasonality in which some of these taxa were found agreed with previous researches in the area (Orive et al., 2004, 2010; Seoane et al., 2012; Muñiz et al., 2018).

The taxa that can cause ASP and PSP have been considered the most relevant in this study, due to the severity of these syndromes, which can lead to death (Lawrence et al., 2011). In addition, although DSP does not cause fatalities, it is of large concern considering the economic losses that it causes in the shellfish industry worldwide (Blanco et al., 2005; Rodríguez et al., 2015). Therefore, for *Dinophysis* spp. (DSP producers), *Pseudo-nitzschia* spp. (ASP producers), and *Alexandrium* spp. (PSP producers), alert levels based on cell abundance established by Swan and Davidson (2012) were applied.

Regarding *Dinophysis* spp., the frequency of exceedance of the alert limit was low at both sampling stations (15%) and was very similar to those reported by Muñiz et al. (2017) in surface waters along the Basque coast (3%–10%). However, these sporadic concentrations above cell alert limits should not be underestimated at any of these sites, since this genus is considered one of the major threats for shellfish aquaculture production and the health of shellfish consuming public (Moita et al., 2016). Special attention should be paid to *D. acuminata*. Similarly to Mutriku and Mendexa, it is reported as one of the most abundant *Dinophysis* species on the neighbouring west French coast and is likely to be the main one responsible for the high concentrations of OA in oysters and mussels of that area (Batifoulier et al., 2013; Maurer et al., 2010). In addition, this species is also one of the most recurrent toxic taxa on the northwest Iberian Peninsula (Galician and Portuguese coasts) (Moita et al., 2016). Indeed, the Galician Rias and shelf suffer long harvesting closures and the consequent strong socioeconomic impacts mainly caused by *Dinophysis acuminata* and *Dinophysis acuta* (Ruiz-Villarreal et al., 2016). This is in accordance with the fact that OA is the main toxin contaminating Galician molluscs (Regueiro et al., 2011; Rodríguez et al., 2015) and the predominant toxin in Europe (Gestal-Otero, 2014). The closures in the Galician Rias mostly occur during spring and summer, but, in the present study, at Mutriku and Mendexa the OA regulatory limit was exceeded only in April and May. A longer data series (2016–2019), which is currently being analysed, shows that OA events only occur in Mendexa from March to June, although the toxin was occasionally detected outside the spring period (Revilla et al., 2019). The absence of OA concentrations over the regulatory limit during summer in the offshore waters of the Basque coast in comparison with the Galician Rias could be due to differences in phytoplankton dynamics in response to the hydrographic and physico-chemical conditions of the water masses. Galician Rias are within an upwelling area that differs considerably from the open marine waters of the southeastern Bay of Biscay (e.g., Muñiz et al., 2019).

Pseudo-nitzschia spp. were the most abundant potentially toxic taxa at both stations. Some species of this genus are the cause of ASP due to their capacity to produce DA (Fehling et al., 2005). The threshold established for the alert limit of this genus (50 000 cells L^{-1}) (Swan and Davidson, 2012) was exceeded in several samples throughout the year; however, DA was recorded in mussel flesh just in April and its concentration was far from the regulatory limit. The main reason for this might be that, even though toxic species like *P. australis*, *P. galaxiae*, and *P. multistriata* have been identified in Basque waters (Orive et al., 2010; Muñiz et al., 2017, 2018), the *Pseudo-nitzschia* spp. cells determined in the present study may also contain species that are not DA producers. Domoic acid has been previously detected in shellfish from many European countries, particularly the UK, Ireland, and France (EFSA, 2009), which resulted in closures of aquaculture sites every year. Moreover, Muñiz et al. (2017), considering a 10-year data series analysing surface waters (1 m depth) along the Basque coast, also stated that *Pseudo-nitzschia* spp. exceeded the abundance limits, implying a toxicity risk with a frequency ranging from 3 to 22% among 19 stations. The frequency calculated in the present study for the offshore station was slightly higher (31%), but this difference could be caused by the different sampling strategy, as in the present study the samples were taken at two depths.

Alexandrium spp., which are considered one of the major threats to human health as are able to produce STX and cause PSP (Yasumoto and Murata, 1993), were also identified at both Mutriku and Mendexa sites. This genus is known to be very widespread worldwide (Lilly et al., 2007), but despite its high adaptability, it is found in low frequencies and abundances in surface waters along the Basque coast (Muñiz et al., 2018), which agrees with the results of the present study. In addition, the STX concentration in mussels was always below the regulatory limit during the studied year, probably due to the low abundance at which *Alexandrium* spp. were registered (maximum of 40 cells L^{-1}). However, since the mere presence of the genus implies toxicity risk for shellfish aquaculture (Swan and Davidson, 2012), both stations recorded shellfish poisoning alert conditions. Previous studies in surface waters of the area (Muñiz et al., 2017) recorded the presence of *Alexandrium* spp. at lower frequencies (0%–8%) in comparison with those observed in the present study at Mendexa station (31%) using two sampling depths. Although the genus is not considered a common threat in this study area, these sporadic appearances cannot be disregarded, since PSP is the most widespread and severe HAB-related shellfish poisoning syndrome (Hallegraeff, 2003). The occurrence of paralytic toxins above their quantification limit in one of the samples from Mendexa (November 2017) confirms the presence of PSP toxin-producer species in the Basque offshore waters.

Finally, several lipophilic toxins (DTXs, PTXs, and AZAs) were below their quantification limits in all of the samples collected during this study. However, in a recent study, Blanco et al. (2017) reported the detection of AZAs in some areas of the northern Spanish coast, including offshore and inshore stations along the Basque coast. It must be indicated that those cases were always below the quantification limits. Taking into account that the detection limits are lower and that the laboratories usually do not provide other information than the amounts that can be quantified, the presence of lipophilic toxins other than OA and YTXs in the mussels from the Basque coast cannot be rejected.

4.3. Factors influencing the risk of toxicity: environmental conditions and physical transport

It is well known that phytoplankton is highly sensitive to environmental changes (Li et al., 2009). Moreover, differences in tolerance to environmental conditions have been identified between different species (Fariñas et al., 2015), leading to variations in the total abundance and composition of phytoplankton. However, this variability is not fully explained by physico-chemical parameters and several other factors need to be taken into account, such as competition, grazing, or parasite pressure (Litchman and Klausmeier, 2008). Differences in the abundance and concentration found for *Dinophysis* spp. and the OA concentration in mussels between Mutriku and Mendexa could be explained by some of the factors named above.

Most harmful events arising from infestation by *Dinophysis* spp. are due to transport of cells from offshore into bays used for shellfish aquaculture (Reguera et al., 2012), as has been seen in southwest Ireland (Raine et al., 2010), northwest Spain (Escalera et al., 2010), and Texas (Campbell et al., 2010). More specifically, a study conducted in the neighbouring Arcachon Bay by Batifoulier et al. (2013) demonstrated that *Dinophysis* spp. originated outside the bay in the open ocean and were transported by northward currents from Capbreton to Arcachon Bay. In addition, Blanco et al. (2017) described the same behaviour for azaspiracids (AZAs) in northern Spain. The study showed that the detection of AZAs in bivalves on the northern Spanish coast was linked to downwelling or upwelling relaxation and, in the Galician Rias, took place in the outer (more oceanic) part, concluding that the responsible species developed in the open sea and that the populations were transported to the shore. These observations are in accordance with the pattern observed on the Basque coast, where higher *Dinophysis* spp. concentrations were observed at the offshore station (Mendexa), where the growth of the populations of this dinoflagellate is expected to occur. A spatially wider sampling could help to find out whether *Dinophysis* spp. growth occurs in this coastal area or in adjacent areas with the peaks detected being the product of the transport. Therefore, it might be helpful to consider the transport processes over the continental shelf in order to predict the presence of *Dinophysis* spp. in shellfish aquaculture areas.

The greater water depth of the offshore station could also lead to a higher abundance of dinoflagellates that perform vertical migration, like *Dinophysis* spp. Several studies (Villarino et al., 1995; Velo-Suárez et al., 2009) described the daily vertical migration of *D. acuminata* between 5 and 10 m; however, subsurface peaks have also been recorded (Hällfors et al., 2011; Batifoulier et al., 2013) and *Dinophysis* spp. can occur at any depth in the photic layer (Reguera et al., 2012). The photic layer at Mendexa has an average annual depth of 44 m (Muñiz et al., 2019). Therefore, and knowing that *D. acuminata* divides at the same rate throughout the euphotic layer (Velo-Suárez et al., 2009), the fact that Mendexa has a greater depth might facilitate the accumulation of higher cell abundances in surface waters compared to Mutriku, which has a shorter vertical profile. Consequently, this could be another possible reason for the higher abundance of *Dinophysis* spp. at Mendexa.

Apart from that, at Mendexa a statistically significant positive correlation was found between the concentration of YTXs in mussels and the water temperature, reflecting the fact that this toxin group presented the highest concentrations in late spring and summer. Yessotoxins are lipophilic toxins produced by *Protoceratium reticulatum*, *Lingulodinium polyedrum*, and *Gonyaulax spinifera* (Visciano et al., 2016). Since temperature is considered to significantly influence the biogeography of microalgae and the dynamics of HABs, some studies have been carried out to determine its influence on yessotoxin production. Paz et al. (2006) studied the influence of temperature on a *Protoceratium reticulatum* strain from Spain and determined that temperature increased the production of YTX, showing that temperature had a positive effect when maintained between 15 and 23 °C. Later, Guerrini et al. (2007) studied the effect of temperature on YTX production by *Protoceratium reticulatum* strains from the Adriatic Sea and, in agreement with Paz et al. (2006), found that a higher temperature resulted in more toxic cells based on the percentage of toxin released. Therefore, even if there are just a couple of previous studies on this topic, the results of this study are in agreement with what has been described previously. Consequently, although the concentration of the YTXs in mussels from Mendexa did not reach the regulatory limit during the one-year period studied, temperature could be a parameter to take into account in order to predict closures in bivalve production areas due to these lipophilic toxins.

Toxin concentrations may also vary with the shellfish species and it is important to choose the sentinel species properly. Some bivalves can prevent the ingestion of toxic dinoflagellates through mechanisms such as the closure of valves or the cessation of filtration. For example, under similar conditions it seems that, in general, mussels accumulate PSP toxins faster than oysters; however, the depuration times are considerably longer in oysters, which can remain toxic for several months (FAO, 2004). In the present study, due to their availability, mussels were chosen to compare the inshore with the offshore site. In addition, there was a higher interest in this commercial species for the local aquaculture sector. However, if other shellfish species were to be consumed in the future, biotoxins should be controlled specifically in those species due to the different dynamics that can affect these compounds in different molluscs.

Finally, it must be noted that the present study did not address the vertical variability of biotoxins along the mussel ropes due to the sampling strategy employed. Therefore, the conclusions about toxicity refer to mussels growing in the upper level of the water column (approximately the first 3 m). As commented before, dinoflagellates do perform vertical migrations daily and, in this regard, a similar chance of contamination throughout the length of the mussel ropes could be assumed (at least for some biotoxins). However, some authors have found important vertical differences (Viviani, 1992; Botana et al., 1996). Taking this into account, on the Basque coast, it would be advisable to conduct further research covering more sampling depths for biotoxins in order to confirm or not the patterns found in this study in surface waters.

5. Conclusion

The hypothesis proposed in this study was rejected, as the offshore mussels presented a statistically higher amount of OA than the inshore mussels. Indeed, this DSP toxin was above the regulatory limit three times during two spring months at the offshore Mendexa farm, whilst just one toxic event affected the Mutriku harbour, in spring as well. Furthermore, the offshore site presented a statistically significant higher cell abundance of *Dinophysis acuminata*, a dinoflagellate with potential to produce OA, in accordance with the offshore origin of this species observed at other European sites. Like *Dinophysis* spp., *Pseudo-nitzschia* spp. and *Alexandrium* spp. exceeded their cell alert thresholds in some samples at both studied sites, with a higher frequency of exceedance of potentially toxic dinoflagellates at Mendexa (*Alexandrium* spp. in particular). The percentage of samples with toxin amounts over quantification limits was also higher at Mendexa for the OA, the STXs (analysed by the MBA), and the YTX group toxins (mainly YTX). Therefore, in the Basque Country, inshore euhaline waters seem to be more suitable for mussel aquaculture than offshore waters from the perspective of the currently regulated biotoxins. Trophic conditions (i.e., TOC, inorganic nutrients, and chlorophyll "a" concentration) did not present significant differences between the studied sites, and the anthropogenic impact was very slight except for the water transparency in the harbour. This suggests that the inshore site was not under strong eutrophication pressure. However, in this region, other aspects such as sanitary quality (i.e., risk of faecal bacteria and viruses) could prevent the development of aquaculture activities inshore, even in euhaline waters.

Declaration of competing interest

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

CRediT authorship contribution statement

Jone Bilbao: Resources, Writing - original draft, Conceptualization. **Oihane Muñiz:** Resources, Writing - review & editing. **Marta Revilla:** Resources, Methodology, Writing - review & editing. **José Germán Rodríguez:** Resources, Formal analysis, Writing - review & editing. **Aitor Laza-Martínez:** Resources, Conceptualization, Writing - review & editing. **Sergio Seoane:** Conceptualization, Supervision, Validation.

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Appendix. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.rsma.2020.101279.

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Further reading

Muñiz, O., 2018. Study of Phytoplankton as Food Resource and Toxicity Risk for Human Health in Offshore Bivalve Aquaculture in the Basque Country (Ph.D. Thesis). University of the Basque Country, p. 176, + annexes.