

Collaborative Project (large-scale integrating project)  
Grant Agreement 226273  
Theme 6: Environment (including Climate Change)  
Duration: March 1<sup>st</sup>, 2009 – February 29<sup>th</sup>, 2012



## **Deliverable D4.1-1: Report on identification of type-specific phytoplankton assemblages for three ecoregions**

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Due date of deliverable: **Month 12**

Actual submission date: **Month 13**

Project co-funded by the European Commission within the Seventh Framework Programme (2007-2013)

Dissemination Level

PU	Public	X
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

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## Non-technical summary

The European Water Framework Directive requires the Member States to assess the ecological status of the marine coastal and estuarine waters. In this assessment, several aspects of the phytoplankton communities, such as composition, abundance and biomass, must be included.

A key step in the development of indicators for the assessment of the phytoplankton quality is the establishment of the reference conditions (the conditions that would exist under no or very minor anthropogenic impact). The aim of this study is to gain a better understanding of the reference conditions for phytoplankton composition in three different ecoregions: the Baltic, the Northeast Atlantic and the Mediterranean Sea ecoregion.

This technical report gives a description of the composition of the phytoplankton communities in several water bodies in Europe that are considered to be at high ecological quality status. These communities are representative of the reference conditions. In addition, data from the non-pristine Baltic Sea are evaluated to provide a characterisation of phytoplankton under good or high ecological status.

This report also provides information about different methodologies for the study of the phytoplankton communities. These methodologies involve a range of aspects: from the approach for selecting the most suitable data sets, to the laboratory techniques and the mathematical and statistical analyses employed.

## 0. Introduction

To combat eutrophication and to manage a healthy marine environment are some of the main objectives within different International Policies (e.g. HELCOM and OSPAR) and European Directives (e.g. Water Framework Directive, WFD (2000/60/EC); and Marine Strategy Framework Directive, MSFD (2008/56/EC)).

One of the key biological elements within these policies is the phytoplankton; however, the variability and complexity in terms of diversity and dynamics within the phytoplankton communities is very high (Jaanus et al., 2009). This fact is even more accentuated in estuarine and coastal waters (in comparison with offshore waters) because, apart from the environmental factors (e.g. hydrodynamics and nutrients availability) and the complex biological processes (e.g. grazing and competition) that affect the phytoplankton communities, the majority of the estuaries and coastal zones have been significantly influenced by anthropogenic factors worldwide (Buchanan et al., 2005; Borja et al., 2006; Dan Petersen et al., 2009). These anthropogenic factors consist mainly of: large population settlements, disposal of nutrients from diffuse sources (e.g. agriculture fields and farms), effluents from wastewater treatment plants, disposal of toxic compounds (e.g. industry and shipping), hydromorphological changes (e.g. dredging, channels and ports), and fishing. These pressures impose two major problems in the assessment of the water quality based upon the phytoplankton: on one hand, since the estuaries and coastal zones have supported the anthropogenic pressures historically, it is very difficult to find pristine zones (with no or very minor human impact) to establish reference conditions (Borja et al., 2006; Dan Petersen et al., 2009; Henriksen, 2009); on the other hand, the great variability due to the above-mentioned factors (environmental, biological and anthropogenic factors) makes the phytoplankton a difficult element to use for the assessment of the ecological status (Devlin et al., 2009).

The WFD considers phytoplankton composition, abundance and biomass among the biological indicators for the classification of the ecological quality status (European Commission, 2000). Also, frequency and intensity of phytoplankton blooms are mentioned in the normative definitions. However, although some investigators have proposed different potential indicators (e.g. Buchanan et al., 2005; Tett et al., 2008; Devlin et al., 2009; Jaanus et al., 2009; Lucena-Moya et al., 2009) and different multi-metric tools (e.g. Bricker et al., 2003; Lacouture et al., 2006; Devlin et al., 2007; Revilla et al., 2009, accepted) to assess it, nowadays there is not a regulatory process to assess the ecological status of the coastal and transitional water using the full phytoplankton quality element (Domingues et al., 2008; Devlin et al., 2009). Only the chlorophyll "a" as a proxy for phytoplankton biomass has been established as an accepted methodology to assess it between Member States (Carstensen and Henriksen, 2009; Henriksen, 2009).

In recent years, indicators capable of detecting changes in the community structure have attracted great attention, since shifts in species composition (e.g. shifts from diatoms to dinoflagellates, or from larger sizes to smaller ones) could indicate a change in the water quality.

In addition, some authors have suggested shifts in the phytoplankton community as indicator of eutrophication (Devlin et al., 2007, 2009; Ferreira et al., 2007; Bricker et al., 2008).

Due to the need of developing indicators of composition in order to fulfil the requirements of the WFD, this report aims at describing type-specific phytoplankton assemblages for three different ecoregions, as a first step for the development of composition-based metrics. Therefore, the phytoplankton communities identified from two of the ecoregions covered in this report refer to waters at high ecological status, and they can be regarded as reference phytoplankton communities. For the third ecoregion, the Baltic Sea, phytoplankton communities representing good or high ecological status are described from “the best samples” collected during recent monitoring.

The following ecoregions and water body types have been addressed:

- The Baltic ecoregion: Finnish national types in the Bothnian Bay, the Quark, the Archipelago Sea and the Gulf of Finland.
- The Northeast Atlantic ecoregion: Eastern Cantabrian coast (Spanish national type).
- The Mediterranean Sea ecoregion: MA-15/CW-M3.

In the next sections, each of the foregoing ecoregions is considered separately.

Regarding the Baltic ecoregion, the spatial variability of the summertime phytoplankton communities in Finnish coastal water types have been analysed.

As for the Northeast Atlantic ecoregion, the composition of the phytoplankton communities has been studied at a station located offshore on the Cantabrian shelf (southern Bay of Biscay). These offshore waters are near the Basque coastal waters, which are included within the common intercalibration type NEA1/26a (Carletti and Heiskanen, 2009).

The last section deals with pico-phytoplankton assemblages from the Mediterranean Sea ecoregion. These have been studied in coastal waters of the Balearic Islands (Mallorca, Spain).

## 1. Finnish coastal waters (Baltic ecoregion)

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### Study area

The national typology of Finnish coastal waters was initially based on the proposal by Kangas et al. (2003), the ecological relevance of which, however, was only tested by zoobenthos assemblages (Perus et al., 2004). The final typology consists of eleven types (Kauppila, 2007), of which four were included in this study, i.e. the outer coastal waters of the Bothnian Bay (BB), the outer archipelago of the Quark (Q), the south-western outer archipelago (here restricted to the Archipelago Sea, AS) and the outer archipelago of the Gulf of Finland (here restricted to the eastern part of the Gulf, GF). The coastal type located in the Quark is a part of the common intercalibration type CW\_B0 between Finland and Sweden, whereas the type in Archipelago Sea belongs to the common intercalibration type CW\_B3 carried out by the Geographical Intercalibration Group (GIG) of the Baltic Sea (Carletti and Heiskanen, 2009). The other national types (BB and GF) were not included in EU Baltic GIG intercalibration work. The chosen type areas differ from each other mainly in terms of exposure, salinity (1 to 6 psu) and the duration of ice cover. Along the Finnish coast, the Bothnian Bay is the only predominantly P-limited area, whereas the other parts are mostly N-limited (e.g. Pitkänen and Tamminen, 1995; Tamminen and Andersen, 2007). However, the types also share common features: the water is brackish, seasonally mixed and the residence time of the water ranges from weeks to months.

*The outer coastal zone of the north-eastern Bothnian Bay* is characterized by the strong influence of river waters, shallowness and the long duration of ice cover (over 150 days). The type is a harsh environment much like the open sea. There are scattered small islands and island groups. The water depth varies in general between 5 to 10 m but some deeper areas exist, as well. Salinity in the whole water column ranges from 1 to 3 psu, and Secchi depth from 3 and 5 m. River waters entering the sea contain large amounts of humus, but only small quantities of nutrients. Primary production is phosphorus limited. Two coastal monitoring stations (17 and 24 m water depth) off the cities of Oulu and Kemi were included in this study.

*The outer archipelago of the Quark* is a complex of small islands, stretching shallows and deep trenches. The area is moderately exposed due to the islands. The type is characterised by quick changes in water quality caused by the influx of more saline and nutrient-rich water from the Bothnian Sea. Salinity ranges from 3 to 5 psu. Acid river waters from the coast do not generally reach the outer archipelagic waters in summer. One 18 m deep, intensive monitoring station located in the southern part of the area was included in this study.

*The outer archipelago of the Archipelago Sea* is characterised by areas of open sea with scattered small islands, restricting the strength of the wind to some extent. The outer archipelago

area is basically deeper than the middle archipelago but shallows are still common. Faults across the area form deep trenches in places. Salinity varies from 5 to 6 psu, and Secchi depth from 4 to 5 m. In recent years, there has been a shift from production limitation by both nutrients to limitation by nitrogen alone (Kirkkala et al., 1998). The growth season is longer than in any other types around the Finnish coast (ice duration below 90 days). Two monitoring stations (both around 80 m depth) were included to represent conditions of the area.

*The eastern outer archipelago of the Gulf of Finland* is characterised by varied bottom topography, depth ranging from 15 to 30 m. Islands are small and cover a much smaller fraction of the overall area compared to the inner archipelago. Salinity ranges from 4 to 5 psu, and Secchi depth from 4 to 5 m, during summer. Deep nutrient-rich and saline water from the open sea occasionally extends into the outer archipelago zone. Hydrographical and meteorological factors have strong influence on the trophic status of the area (Pitkänen et al., 1993; Kauppila et al., 1995; Rantajärvi et al., 1998). Primary production is limited mainly by nitrogen (Tamminen and Andersen, 2007). Five monitoring stations, with water depths ranging from 42 to 65 m, were included in this study.

## Material and methods

### Data set

Phytoplankton and physico-chemical data originated from the coastal monitoring results of the Finnish Environment Institute (SYKE) from the mid-summer period (July to August) of 1968 to 2004. The dataset, comprising altogether 10 monitoring sites, was compiled for 62 water and phytoplankton samples to identify type-specific phytoplankton assemblages. The sites were located in the outer coastal waters of the Bothnian Bay, the Quark, the Archipelago Sea and eastern Gulf of Finland. The sites were located outside the direct influence of the coast.

### Biological and chemical analyses

Composite samples of phytoplankton (surface to twice the Secchi depth) were taken with a Ruttner sampler and preserved with acid Lugol's solution (Willén, 1962). Cells were counted with a Zeiss IM35 microscopy employing the technique by Utermöhl (1958). Cell numbers were converted to wet weight biomass (ww) using the volumes of the phytoplankton database maintained by SYKE, most of which have been calculated according to Edler (1979). Chlorophyll *a*, taken from composite samples of phytoplankton, was analyzed according to Lorenzen (1967). The chlorophyll samples were extracted with acetone until 1994, and ethanol thereafter. Total nitrogen (TN) and total phosphorus (TP) concentrations were determined from unfiltered samples following the Finnish standard methods (see Kauppila, 2007).

### Identification of type-specific phytoplankton assemblages

Type-specific phytoplankton assemblages were identified from present-day coastal monitoring data using "the best samples", which means samples which can be regarded as reference



phytoplankton communities that refer to waters at good or high ecological status. The criteria for phytoplankton samples chosen as "the best samples" were, at first, that in the concurrent water quality samples the concentrations of chlorophyll *a*, total nitrogen and total phosphorus should not exceed the type-specific reference values, estimated previously empirically for the Finnish coastal water types included in this study (Table 1.1; Kauppila, 2007; Vuori et al., 2009; Kauppila, unpublished data). In cases it turned out impossible to find any water quality samples representing near-reference conditions, i.e. high status, we applied the HELCOM EUTRO approach to define the acceptable deviation from the average reference values (Table 1.1). According to HELCOM (2009), the acceptable deviation of the average reference values is 50%, which is considered to represent the boundary between good and moderate status in the ecological classification of the WFD. However, due to high humic content in Finnish coastal waters as a whole, the reference and boundary values established for Secchi depth are only suggestive in this study. The humus present in river water impairs the transparency of the coastal waters especially in the Bothnian Bay, where euphotic layer is about twice as deep in the open sea as in the coastal waters (Alasaarela, 1979a). Anyway, according to the Guidance Document on the intercalibration process 2008-2011 (Anonymous, 2009), an alternative benchmark could be used in cases where near-natural conditions no longer exist. Consequently, we screened the samples of the coastal monitoring stations of Finland meeting abiotic criteria representing low level of impairment (see Birk and Hering, 2009). The results were compared to the reports on phytoplankton biomass and composition for the late 1960s and early 1970s – a period when many offshore water areas around Finland indicated undisturbed conditions.

*Table 1.1. Empirically estimated reference values of chlorophyll *a* (Kauppila, 2007; Vuori et al., 2009), total phosphorus and total nitrogen (Kauppila, unpubl. data) and the historical values of Secchi depth (see Launiainen et al., 1989) in the outer coastal types of the Bothnian Bay (BB), the Quark (Q), the Archipelago Sea (AS) and the eastern Gulf of Finland (GF). The acceptable deviation (50%) from the average reference values is presented in the brackets and represents the boundary between good and moderate status (see HELCOM, 2009).*

	BB	Q	AS	GF
Chl ( $\mu\text{g l}^{-1}$ )	1.6 (2.4)	1.4 (2.1)	1.4 (2.1)	2.1 (3.2)
TP ( $\mu\text{mol l}^{-1}$ )	0.19 (0.29)	0.26 (0.39)	0.45 (0.68)	0.55 (0.84)
TN ( $\mu\text{mol l}^{-1}$ )	18.8 (28.3)	17.2 (25.8)	17.5 (26.3)	19.3 (28.9)
Secchi depth (m)	6.7 (4.5)	8.6 (5.7)	8.9 (5.9)	5.6 (3.7)

## Results

### Physico-chemical conditions

As a result of the screening, only 7 out of 62 samples met the requirements of reference or at least near-natural conditions, based on the analyses of chlorophyll *a*, total nitrogen and total phosphorus. These "high status" samples were mainly taken from the Bothnian Bay during the 1990s and 2000s. A couple of samples originated from the Archipelago Sea taken in the early

1980s. The rest of the samples were considered to represent good status of water, based on the analyses applying the HELCOM EUTRO approach.

In these "best of samples", waters reflecting high or good status showed variation within and between the coastal water types (Tables 1.2, 1.3). A typical pattern was an increasing nutrient and trophic gradient from north to east along the Finnish coast. Nutrient and trophic status in July to August was lowest in the Gulf of Bothnia and the Archipelago Sea, where the average concentrations of chlorophyll *a* varied from 1.4 to 1.6  $\mu\text{g l}^{-1}$  and those of TN remained below 20.5  $\mu\text{mol l}^{-1}$ . Only in the Bothnian Bay, the average concentrations of TP were as small as 0.23  $\mu\text{mol l}^{-1}$ . However, in the Quark and the Archipelago Sea it was also possible to find single samples with TP below 0.32  $\mu\text{mol l}^{-1}$ . By contrast, in the Gulf of Finland, chlorophyll *a* was on average ca. 2  $\mu\text{g l}^{-1}$ , whereas TN and TP were around 22 and 0.52  $\mu\text{mol l}^{-1}$ , in respectively.

*Table 1.2. Data on phytoplankton total biomasses, the number of taxa and the concurrent water quality of the monitoring sites reflecting near-reference or good status in the Gulf of Bothnia.*

		Bothnian Bay				Quark			
		mean	SD	min	max	mean	SD	min	max
Phytoplankton	Total Biomass ( $\text{mg l}^{-1}$ )	0.15	0.04	0.03	0.16	0.20	0.1	0.04	0.43
	Number of taxa	24	6	12	33	21	9	10	41
Water quality	Chl ( $\mu\text{g l}^{-1}$ )	1.5	0.3	0.7	2.1	1.6	0.5	0.5	2.4
	TP ( $\mu\text{mol l}^{-1}$ )	0.23	0.09	0.13	0.32	0.42	0.09	0.26	0.65
	TN ( $\mu\text{mol l}^{-1}$ )	18.7	1.5	15.7	20.7	19.1	3.5	13.6	26.4
	Secchi depth (m)	3.7	0.8	2	5	3.8	0.9	2.1	5.4
	Salinity (psu)	2.3	0.9	0.8	3	6	0.4	5.4	6.6

*Table 1.3. Data on phytoplankton total biomasses, the number of taxa and the concurrent water quality of the monitoring sites reflecting good status in the Archipelago Sea and the Gulf of Finland.*

		Archipelago Sea				Gulf of Finland			
		mean	SD	min	max	mean	SD	min	max
Phytoplankton	Total Biomass ( $\text{mg l}^{-1}$ )	0.22	0.17	0.04	0.60	0.25	0.06	0.17	0.31
	Number of taxa	18	7	8	31	15	4	10	22
Water quality	Chl ( $\mu\text{g l}^{-1}$ )	1.4	0.3	0.9	2	2	0.7	0.7	2.6
	TP ( $\mu\text{mol l}^{-1}$ )	0.39	0.11	0.23	0.65	0.52	0.10	0.36	0.65
	TN ( $\mu\text{mol l}^{-1}$ )	20.5	3.6	15.7	25.7	21.8	3.1	17.9	25.0
	Secchi depth (m)	6.5	1.3	3.5	8	4.8	0.3	4.5	5.2
	Salinity (psu)	6.6	0.1	6.4	6.7	4	0.2	3.6	4.2

## Phytoplankton communities

In mid-summer conditions, phytoplankton communities in the outer coastal water types showed variation in respect to total biomasses, species composition and taxa numbers. In the Bothnian Bay, total biomass was on average  $0.15 \text{ mg l}^{-1}$  and the number of taxa 24 (Table 1.2). The communities were dominated by cryptophytes and diatoms, that comprised on average 40 and 25% of the total biomasses, respectively (Figure 1.1). Among cryptophytes, the biomass of *Plagioselmis prolunga* was greatest, whereas *Chaetoceros wighamii* and *Thalassiosira baltica* were the dominant diatoms so that the common cold-water diatom *C. wighamii* gave way to *T. baltica* in mid- July and August. *Oocystis borgei*, an indicator of oligotrophy according to Tikkanen and Willén (1992), had greatest biomass among chlorophytes, the group of that comprised one fifth of the total biomasses on average. The photosynthetic ciliate (protozoan) *Myrionecta rubra* was common. Cyanobacteria, consisting mainly of *Anabaena lemmermannii*, and chrysophytes were rare.

In the Quark, phytoplankton total biomass was on around  $0.20 \text{ mg l}^{-1}$ , consisting on average of 21 taxa, the maximum number of taxa being as high as 41 (Table 1.2). The communities were dominated by cryptophytes, which accounted for on average 30% of the total biomass (Figure 1.1). *Plagioselmis prolunga* was the most common and dominating species of cryptophytes. The biomass of the order Ochromonadales was highest among chrysophytes whereas *Pyramimonas* spp. was the dominating taxa of chlorophytes. The proportion of diatoms was lower compared to the northernmost communities. Dinoflagellates such as *Dinophysis acuminata* and *Heterocapsa rotundata* had relatively high biomasses. Cyanobacteria comprised around five percent of total biomass. The ciliate *Myrionecta rubra* was common similar to the Bothnian Bay. Typical species indicating oligotrophy, such as the cyanobacterium *Merismopedia warmingiana* (Tikkanen and Willén, 1992) occurred occasionally.

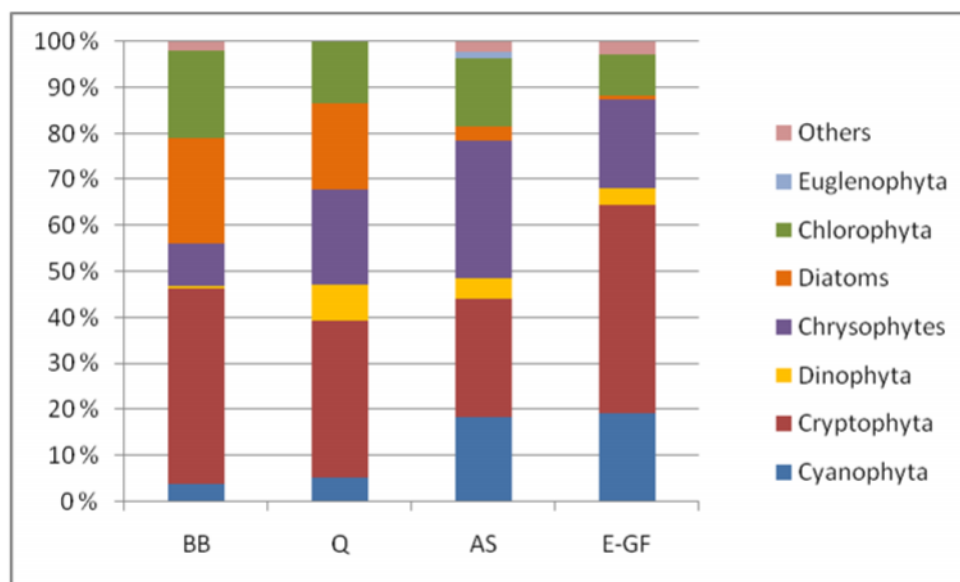


Figure 1.1. Relative biomasses of different phytoplankton groups estimated for the outer coastal water types of the Bothnian Bay, the Quark, the Archipelago Sea and the eastern Gulf of Finland.

In the Archipelago Sea, the average total biomass ( $0.22 \text{ mg l}^{-1}$ ) and total number of taxa (18) were on the same level as in the Bothnian Sea (Table 1.3). Chrysophytes, mainly from the order Ochromonadales, and cryptophytes belonged to the dominating groups, accounting on average 30 and 25% of the total biomass, respectively (Figure 1.1). *Chrysochromulina* sp. had also high biomass. Cyanobacteria accounted for on average one fifth of the total biomass, and typical cyanobacterial species in the area were the  $\text{N}_2$ -fixing *Aphanizomenon* sp. and *Nodularia spumigena*. *Pyramimonas* spp. and *Oocystis borgei* formed the major part of the biomass of chlorophytes. Dinoflagellates and diatoms were minor groups and were characterized by the dinoflagellate *Dinophysis acuminata* and the diatom *Stephanodiscus hantzschii*.

In the eastern Gulf of Finland, phytoplankton total biomass was on average  $0.25 \text{ mg l}^{-1}$  and the number of taxa 15 (Table 1.3). Cryptophytes comprised 40% of the total biomass, *Plagioselmis prolunga* being the dominating species. Cyanobacteria and chrysophytes were the second most abundant groups. *Aphanizomenon* sp. was the dominating cyanobacterial species. Nanoflagellates from the order Ochromonadales and *Pseudopedinella tricostata* were the most important chrysophytes. Among the chlorophytes, the most common species was *Pyramimonas* spp.

## Discussion

In this study, we demonstrated an alternative approach to attempt to identify type-specific phytoplankton assemblages based on "the best samples" of the present-day monitoring data, information from the literature and the use of the HELCOM EUTRO approach to determine the acceptable deviations from the reference values of chlorophyll *a* and nutrients (TN and TP). We succeeded to compile a small dataset, which gives very preliminary results on the near type-specific phytoplankton communities of Finnish coastal waters or at least on communities reflecting good ecological status.

"The best samples" representing the good or high ecological status from mid-summer communities around Finland showed similarities to those reported from the Baltic Sea offshore water areas in the late 1960s and 1970s. In the Bothnian Bay, our results on total biomasses ranging from  $0.03$  to  $0.16 \text{ mg l}^{-1}$  in the outer coastal waters matched those reported by Alasaarela (1979a,b) in the 1970s. It, in part, suggests that the samples selected from the dataset might represent even high status. In the open sea, cryptophytes and diatoms appeared to be the main groups, and the oligotrophic brackish-water diatoms *Chaetoceros wighamii* and *Thalassiosira baltica* characteristic species in the 1970s (Alasaarela, 1979a,b), which was also the case in our "best samples". In fact, the abundance of diatoms in the Bothnian Bay is a consequence of the great quantities of silicon ending up to the sea from the catchments through the large northern rivers (Kohonen, 1973). According to Niemi and Ray (1977), the proportion of diatoms varied from ca. 30 to 90% of the total biomass between July and August in the early 1970s, which shows that, depending on hydrographical conditions, the contribution from diatoms may vary considerably. Additionally, comparison to the open waters reveals that the

community structure is similar to that found in the outer coastal water type, in that cryptophytes and *Thalassiosira* spp. dominate together with the chlorophyte *Pyramimonas* spp. (cf. Huttunen et al., 1986). Furthermore, in the open sea dinoflagellates are poorly represented and there exist many freshwater species of chlorophytes, which was also evident in the "best samples" of our study. The small biomass of cyanobacteria in outer and offshore waters of the Bothnian Bay is mainly explained by phosphorus limitation (Buch, 1932; Voipio, 1976; Alasaarela, 1979a), which has also been proved by bioassay experiments (Tamminen and Andersen, 2007). The occasional occurrence of species indicating oligotrophy according, inter alia, to Järnefelt (1952), such as the mixotrophic chrysophyte (Bird and Kalff, 1987) *Dinobryon divergens*, suggested that at least some of the identified communities in the outer coastal water type of the Bothnian Bay may reflect high status.

In the Quark, total biomass (average  $0.20 \text{ mg l}^{-1}$ ) was higher than that (ca.  $0.05 \text{ mg l}^{-1}$ ) observed by Niemi and Ray (1975) in the northern Bothnian Sea. Cryptophytes were the dominating group similar to the Bothnian Bay but the proportion of diatoms was lower. A typical feature was also that dinoflagellates had higher biomasses than in the Bothnian Bay. Niemi and Ray (1975, 1977) showed that in the 1970s the dinoflagellates, mainly the genera *Dinophysis* and *Protoceratium*, could comprise up to 70% of the total biomass during mid-summer conditions. Furthermore, according to them, chrysophytes made up a few percentage of the total biomass in the early 1970s, but e.g. *Uroglena*, recorded also in our study, had occasionally a very high biomass. The small proportion of cyanobacteria was also noteworthy for the communities of the sea area. *Aphanizomenon* and *Composphaeria*, the important cyanobacterial genera in the Quark, dominate both near the sea zone (Niemi and Ray, 1977) and in the open Gulf of Bothnia (Huttunen et al., 1986). Some species indicating oligotrophy according to Tikkanen and Willén (1992), such as the cyanobacterium *Merismopedia warmingiana*, were also found among "the best samples".

In the Archipelago Sea, phytoplankton communities dominated by chrysophytes and cryptophytes resemble those reported for the middle Archipelago Sea (Kauppila and Lepistö, 2001). However in the middle Archipelago Sea the contribution of cyanobacteria was small even in the early 1990s. During the late 1990s their biomass started to increase as a result of eutrophication. The greater contribution of cyanobacteria in our "best samples" can be explained by nitrogen limitation during mid-summer conditions. This is true also in the open sea, where the filamentous *Aphanizomenon flos-aquae*, *Nodularia spumigena* and *Anabaena* spp. dominate from July to August (Seija Hällfors, pers. com.). According to Tamminen and Andersen (2007) the typical Baltic Sea surface salinity regions between 5 to 6 psu were clearly N-limited during the summer months. Phytoplankton communities in the outer Archipelago Sea may also be compared to those of the outer parts of the western Gulf of Finland, as these areas belong to the same national type (Kauppila, 2007). However phytoplankton communities in these two areas are not quite comparable (see Kauppila and Lepistö, 2001). The reason is that mixing conditions in the western Gulf are more unstable due to hydrological conditions, which, in part, determine whether phytoplankton communities will be exposed to the influence of river waters or more saline open sea waters. This also explains the more complex community structure and shifts

between the dominating phytoplankton species of the western Gulf of Finland (Gasi nait et al., 2005). Moreover, contrary to the Archipelago Sea, wind-driven coastal upwelling is an important phenomenon in the western Gulf; it changes the euphotic layer temperature and nutrient conditions, which in turn have an effect on the late summer phytoplankton communities (Vahtera et al., 2005).

In the eastern Gulf of Finland, the total biomass ( $0.25 \text{ mg l}^{-1}$ ) during July to August was smaller than on the average in the 1980s and 1990s (Kauppila and Lepistö, 2001) and on the same level as that recorded in the early 1970s (Niemi and Ray, 1975). Phytoplankton communities in "the best samples" were dominated by cryptophytes, which are still one of the main phytoplankton groups in the area (e.g. Pitkänen et al., 1990; Rantajärvi et al., 1998; Gasi nait et al., 2005). The study by Gasi nait et al. (2005) suggests that communities dominated by cryptophytes are more likely predicted by site-specific factors such as hydrodynamics than salinity and trophic status. Additionally, the small contribution of dinoflagellates to community structure seems not to have been changed during the mid-summer periods of the past three decades (Figure 1.1; Kauppila and Lepistö, 2001; Kauppila, unpublished data). By contrast, the share of cyanobacteria has increased especially in the 1990s. The dominance relationship between the  $\text{N}_2$ -fixing *Aphanizomenon* sp. and non- $\text{N}_2$ -fixing *Planktothrix agardhii* has been connected to variation in the N/P-ratio, determined not only by nutrient loading, but also, by meteorological and hydrographical factors (Pitkänen et al., 1993; Kauppila et al., 1995). Therefore, the contribution of cyanobacteria to total biomass and the species dominance relationships may be applicable in the indicator development work of the Water Framework Directive.

Considering these results as representative of the general conditions, it is clear that the data and the number of stations are too small to draw any extensive conclusions. However, the monitoring stations included in this study represent larger water bodies within the outer coastal types. Natural variation, both spatial within a type and temporal within the mid-summer season, is not sufficiently covered. The lesson of this study is that by screening "the best samples" of the present day monitoring data and analysing literature, it may be possible to identify phytoplankton assemblages revealing at least good status.

## 2. Basque coastal waters (Northeast Atlantic ecoregion)

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### Background and interest of this study

For the implementation of the WFD within the Basque country, the ecological status of the coastal and estuarine waters has been assessed using phytoplankton (Revilla et al., 2009; accepted). At present, the methodology is based upon indicators of biomass (chlorophyll “a” concentration, 90<sup>th</sup> percentile) and bloom frequency (any single taxon >750,000 cells L<sup>-1</sup>). However, a proper community composition indicator has not yet been developed for these waters. Therefore, once the water typologies have been defined for the different waters bodies (Borja et al., 2004, 2006; BOE, 2008) (Figure 2.1), the first and most important step in this process is the description of the reference phytoplankton communities.

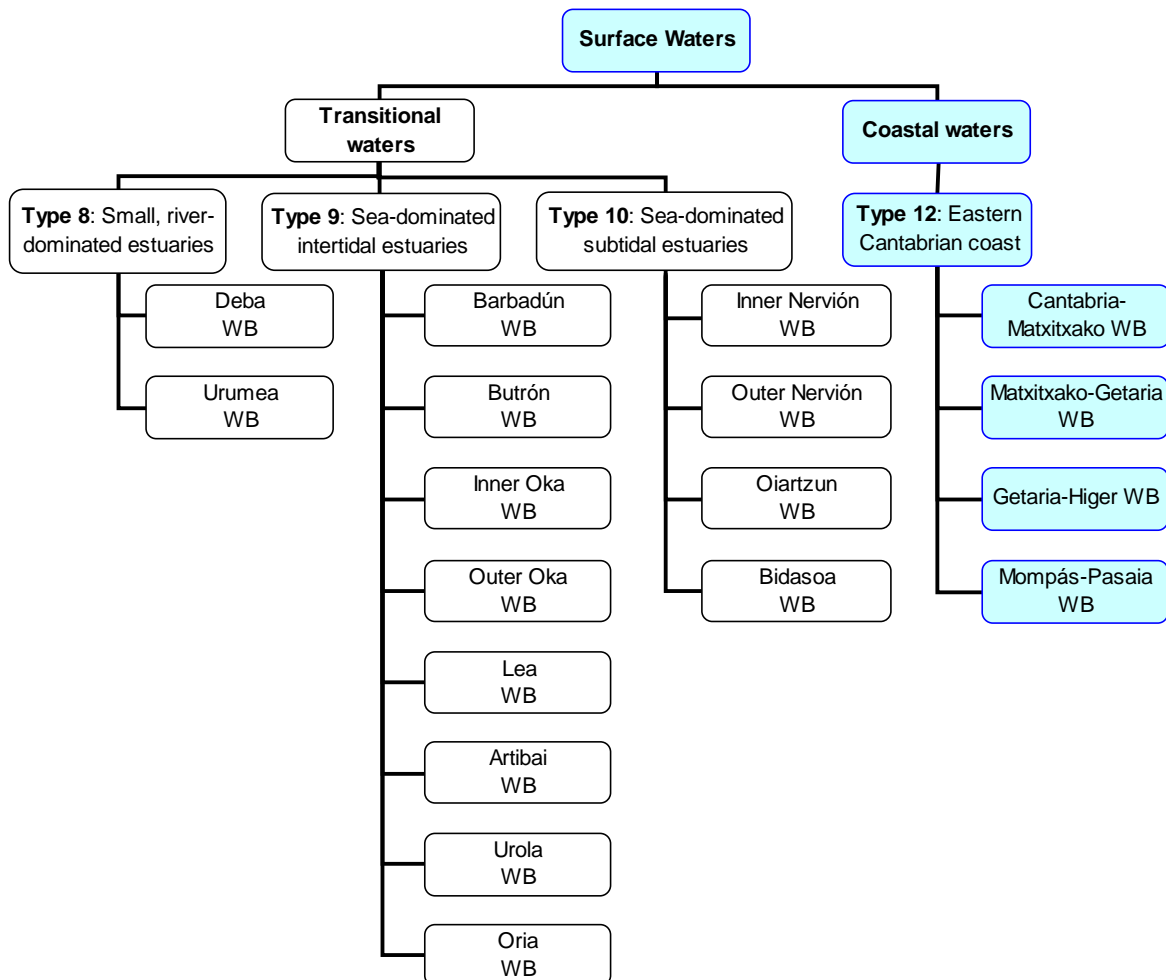


Figure 2.1. Diagram of the coastal and transitional water bodies (WB) in the Basque country (northern Spain). Types are those officially established in Spain (BOE, 2008). The coastal water bodies are highlighted.

The reference condition for a water type consists on the description of the biological quality elements (phytoplankton in this case) that exist, or would exist at high biological status (with no, or only very minor disturbance, from human activities) (Borja et al., 2004). Consequently, the objective of this study is to describe the reference community composition in the Basque coastal waters in order to fill the gap in the assessment based on phytoplankton, and to enhance further research to develop an appropriate community-based phytoplankton indicator.

In this first approach, the reference community composition will be studied only in the coastal waters, since reference stations are available. The Basque estuaries have been more affected by anthropogenic pressures and reference stations cannot be found within these systems. This fact, combined with the higher natural variability that exists within the estuaries, makes it more difficult to establish the phytoplankton reference community composition for transitional waters.

## Study area

### Location within the European WFD context

The Basque coast in the north of Spain is located within the Northeast Atlantic ecoregion (Figure 2.2). It extends along approximately 150 km on the Cantabrian shelf (southern Bay of Biscay). The Cantabrian shelf presents significant differences in hydrology and geomorphology with the neighbouring Atlantic shelves (i.e., those of the French coast and the west coast of Spain and Portugal).

The Cantabrian shelf is narrower (<20 km) than the French shelf. Also, continental water inputs (hence, nutrients) are lower in the Cantabrian coast, compared to the French coast, because rivers are comparatively smaller (Díez et al., 2000; OSPAR, 2000). For example, the main rivers discharging on the French coast, the Loire and Gironde have an annual flow of  $900 \text{ m}^3 \text{ s}^{-1}$  each (Lavín et al., 2006), whereas the annual average flow of the Cantabrian rivers amounts to  $561 \text{ m}^3 \text{ s}^{-1}$  in total (Prego et al., 2008).

Regarding the west coast of Spain and Portugal, the influence of the upwelling events is much stronger in that area than over the Cantabrian coast (Mason et al., 2005). During the Phase I of the intercalibration exercises of the WFD, two areas were distinguished along the Cantabrian shelf for the phytoplankton quality element: the Eastern Cantabrian coast and the Western Cantabrian coast (Carletti and Heiskanen, 2009) (Figure 2.2). This differentiation was established upon the criteria of the relative influence of the wind-driven upwelling events. The strength of upwelling events decrease eastward along the Cantabrian shelf and, consequently, the natural input of deep nutrient-rich waters also decrease in this direction.



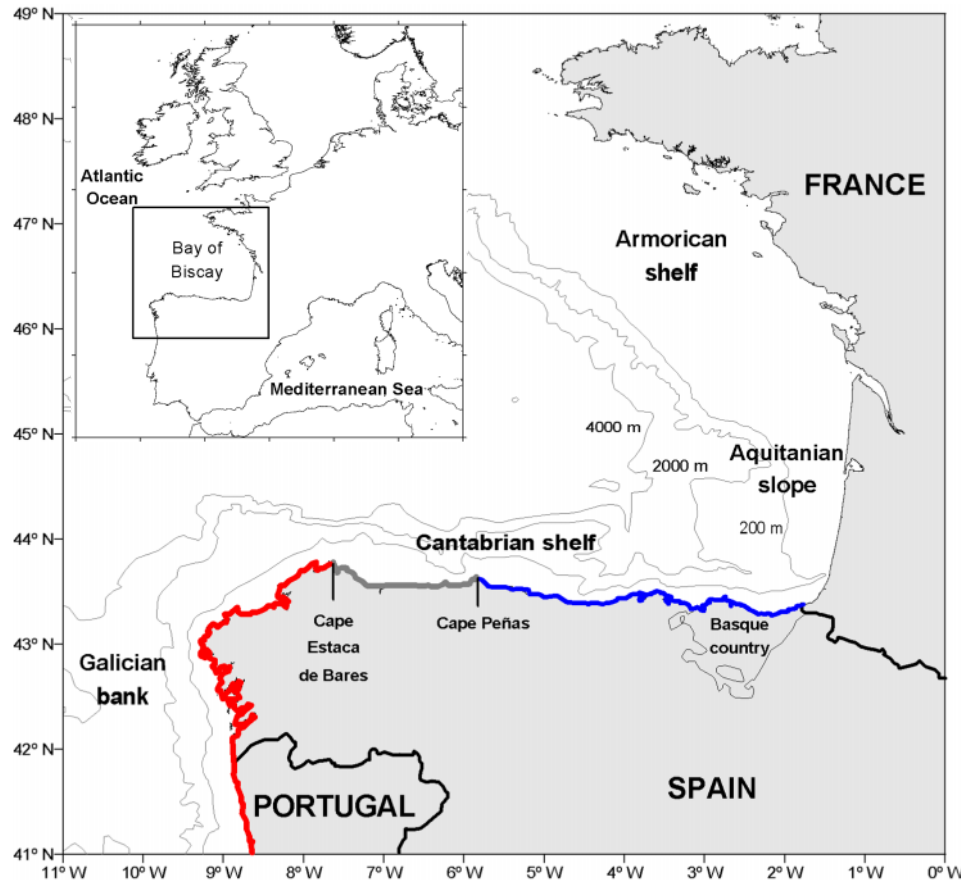


Figure 2.2. Location of the Basque coast within the context of the Bay of Biscay and the Northeast Atlantic ecoregion. Blue line: Eastern Cantabrian coast (NEA 1/26a type). Grey line: Western Cantabrian coast (NEA 1/26e type). Red line: Iberian upwelling coast (NEA 1/26e type).

The Basque coast is located within the Eastern Cantabrian coast (Figure 2.2). Therefore, the coastal waters in this study belong to the common intercalibration type NEA 1/26, which is basically defined as exposed, shallow, euhaline and fully-mixed waters. For the purposes of the intercalibration, they belong to the subtype NEA 1/26a, which means that the natural fertilization in these coastal waters, due to upwelling events or large river plumes, is negligible (Carletti and Heiskanen, 2009). In contrast, the Western Cantabrian coast, similarly to the west coast of Spain and Portugal, belong to the subtype NEA 1/26e, which means that it receives higher natural inputs of nutrients. Those inputs result from upwelling influence, being more intense along the west coast of Spain and Portugal (the Iberian upwelling coast, Figure 2.2).

### Geomorphological and hydrographical features

The Basque coast is exposed and mostly formed by cliffs of calcareous rocks, with small beaches, bays and estuaries. There are 12 main estuaries (Figures 2.1, 2.3), and some additional smaller estuaries (<0.5 km<sup>2</sup>). Basin areas are small, and the rivers are short and with high slopes. The total flow is about 150 m<sup>3</sup> s<sup>-1</sup> (annual mean). Along the Cantabrian coast, the nutrient fluxes are distributed among several small rivers, and no large coastal plumes are formed (Díez et al., 2000; Ferrer et al., 2009). In addition, loads of suspended solids from the rivers are relatively

modest and diffuse (Prego et al., 2008). The climate is temperate, oceanic, with moderate winters and warm summers. According to Köppen's classification, the area is associated with a Cfb climate (marine west coast- mild). Under average conditions, river flow is at its minimum during summer and early autumn. Nevertheless, freshets are relatively frequent throughout the year and they have considerable influence on the rivers flow (Valencia et al., 2004).

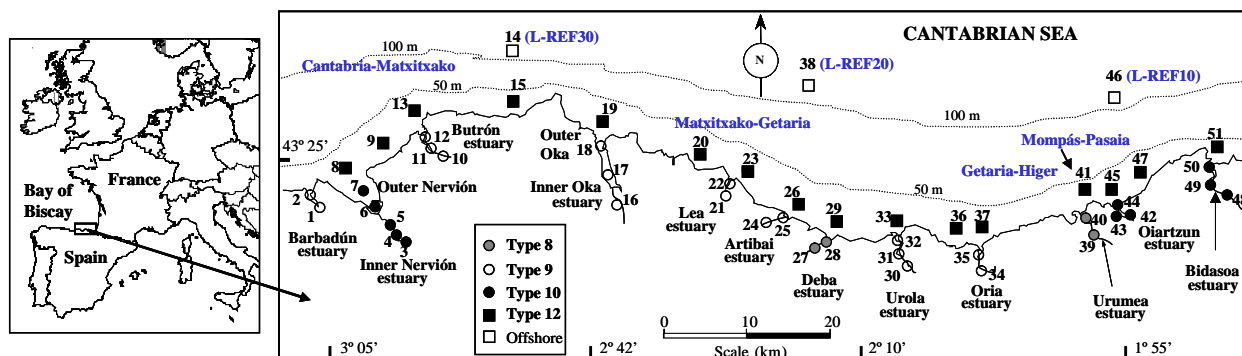


Figure 2.3. Sampling stations and water bodies within the Littoral Water Quality Monitoring and Control Network (LQM) of the Basque Country. Type 8: small, river-dominated estuaries; Type 9: estuaries with extensive intertidal flats; Type 10: estuaries with extensive subtidal areas; Type 12: Eastern Cantabrian coast. Offshore stations are considered as reference stations for the coastal water bodies.

The hydrographic conditions of the Basque coastal and offshore waters are well documented (e.g. Valencia et al., 2004; Fontán et al., 2008; Revilla et al., 2009). Along the Basque coast, as in other temperate areas located at mid-latitude, the annual cycle of the sea surface temperature (SST) shows a marked seasonality related strongly to atmospheric temperatures, at least for monthly averaged temperatures. There are two clearly defined seasons, winter and summer; and two transitional seasons, spring and autumn (Table 2.1). The SST differentiates the period of vertical mixing from the stratification period. Throughout the autumn and winter seasons the speed and regime of winds reactivates the east and northward current and the dominance of downwelling in the SE corner of the Bay of Biscay. A combination of winter cooling, turbulence and downwelling generates the winter mixed layer. This layer frequently exceeds the nominal depth of the continental shelf (Valencia et al., 2004). During winter, the mixing of the water column, together with the frequent rainfall events and freshwater discharges cause a maximum in nitrate and phosphate concentrations in nearshore as well as in offshore waters (Table 2.1). In early spring, a reduction in wind stress and a change in the prevailing direction of the winds allow some degree of stability and the initiation of the stratification. The decrease in density of the surface waters, due to warming and expansion of low salinity plumes, increase the probability of the permanence of these waters within the surface layer. Under these conditions, the surface waters receive a high percentage of the total heat flux from the atmosphere and the process is self-enhancing. If the stability is high enough, stratification of waters continues throughout the remainder of spring, all of the summer and the early autumn. Storm events, like summer *galernas* produce turbulent mixing and deepen the thermocline. In these cases, a

significant reduction in the SST can be observed. The depth of the thermocline and the associated layers depends also upon the balance between upwelling and downwelling. If downwelling prevails the thermocline moves deeper down. Conversely, if upwelling prevails the thermocline becomes shallower and more distinct, than in the neutral status. However, it rarely rises up and breaks into the surface layers (Valencia et al., 2004). In summer, ammonia is slightly higher (Table 2.1), which could be due to the biological remineralisation of organic matter, which is enhanced at higher temperatures. On the other hand, biological processes of uptake would explain the decrease in the surface concentrations of nitrate and phosphate in summer. However, nitrate depletion, which is observed in areas subjected to seasonal stratification and high biological activity over the North-West European shelf (e.g. Siddorn et al., 2007) is not observed in the surface waters of the Basque coast. This is probably due to the influence of summer storms that activate the export of nutrients from rivers and estuaries to the coastal waters. Moreover, thermal stratification allows freshwater inputs to stay in the surface layer (Revilla et al., 2009). In autumn, some increase in the concentration of nutrients can be observed in the surface waters (Table 2.1), which has been related to turbulent mixing processes and to the subsequent repartition of nutrients in the water column from subsurface waters (Valencia et al., 2004).

Table 2.1. Average water temperature, salinity, inorganic nutrients (N, P) and chlorophyll "a" (Chl-a), calculated for each season and annually in the Basque coast, with data from surface waters (0-2 m) at several coastal stations during the 2001-2006 period, and from one offshore station during the 2002-2006 period. From Revilla et al. (2009), with some modifications.

	Sampling season	Temp (°C)	Salinity (psu)	Ammonia (µM)	Nitrate (µM)	Phosphate (µM)	Chl-a (µg L <sup>-1</sup> )
Coastal waters <sup>1</sup>	Winter (Feb-Mar)	12.0	34.7	2.77	4.57	0.37	0.61
	Spring (Apr-May)	15.2	34.3	2.29	2.68	0.18	0.86
	Summer (Aug-Sep)	21.7	34.7	2.91	2.54	0.21	0.62
	Autumn (Oct-Nov)	16.2	35.0	2.12	2.50	0.38	0.72
	<u>Annual</u>	<u>16.3</u>	<u>34.7</u>	<u>2.52</u>	<u>3.07</u>	<u>0.28</u>	<u>0.70</u>
Offshore waters <sup>2</sup>	Winter (Feb-Mar)	12.0	34.6	2.50	3.35	0.34	1.11
	Spring (Apr-May)	15.4	34.4	2.21	2.90	0.17	0.45
	Summer (Aug-Sep)	22.5	34.8	3.28	2.13	0.18	0.17
	Autumn (Oct-Nov)	16.0	35.1	1.75	2.86	0.16	0.89
	<u>Annual</u>	<u>16.5</u>	<u>34.7</u>	<u>2.44</u>	<u>2.81</u>	<u>0.21</u>	<u>0.72</u>

<sup>1</sup> Sampling sites are the stations named as 8, 9, 13, 15, 19, 20, 26, 29, 33, 36, 41, 45 and 51 in Figure 2.3.

<sup>2</sup> Sampling site is the station named as 46 (L-REF10) in Figure 2.3.

## Phytoplankton biomass

The seasonal variability of phytoplankton biomass (measured as chlorophyll “a” concentration) in the Basque coastal and offshore waters is described in detail in Orive et al. (2004), Revilla et al. (2009, 2010) and Garmendia et al. (accepted).

In offshore waters, the surface chlorophyll “a” concentration shows a large interannual variability but, it can be adjusted to the general seasonal cycle described for phytoplankton biomass in the shelf waters of the Cantabrian Sea (e.g. Varela, 1996; Calvo-Díaz et al., 2008). The seasonally averaged concentration reaches its maximum values during late winter-early spring (Table 2.1, Figure 2.4). The surface chlorophyll “a” decreases during spring, and it reaches the minimum during summer, being inversely correlated to the warming of the sea surface layers (Table 2.1, Figures 2.4, 2.5). In late autumn, as SST drops, chlorophyll “a” reaches a secondary peak in the surface waters (Table 2.1, Figures 2.4, 2.5). Therefore, the seasonal variability of the surface chlorophyll “a” in the offshore waters is mainly explained by the annual cycle of mixing and stratification (indicated by the SST, as above explained), which determines the nutrient concentrations and the retention of the phytoplankton biomass in the surface layers of the water column.

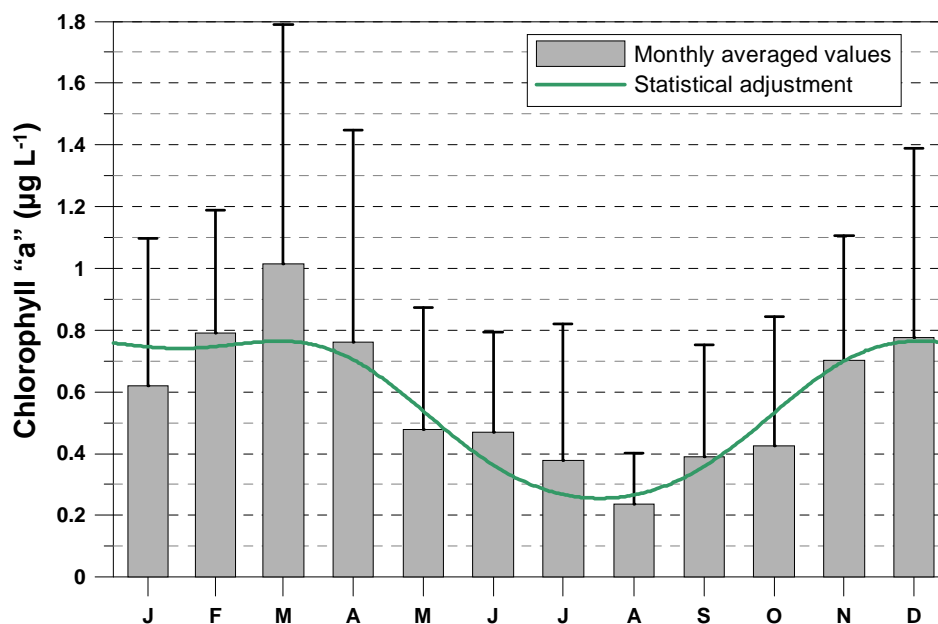


Figure 2.4. Monthly average and standard deviation of surface chlorophyll “a” (0-2 m) at offshore waters in the Basque coast. Data have been collected from 1986 to 2009 (185 surveys, total) at station L-REF10 (Figure 2.3). The line indicates the statistical adjustment of the annual variation (Revilla et al., 2010).

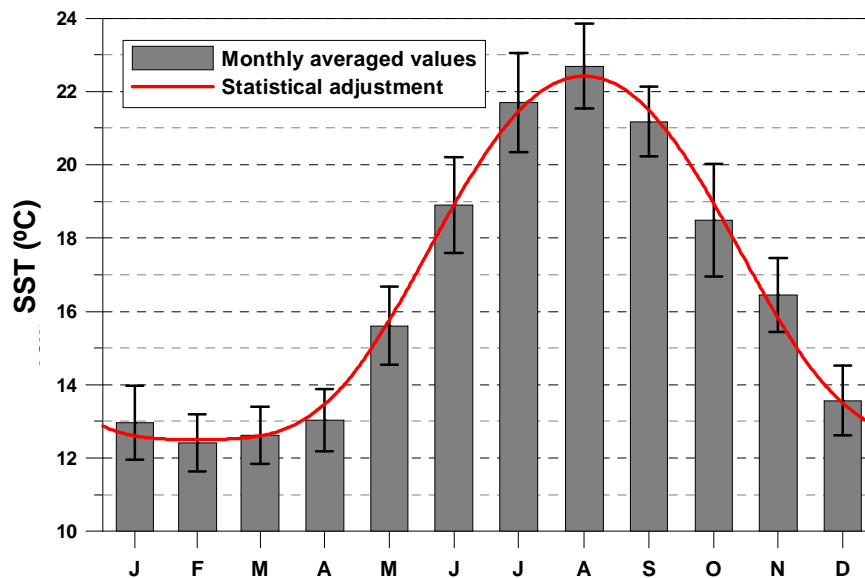


Figure 2.5. Monthly average and standard deviation of sea surface temperature (SST) at offshore waters in the Basque coast. Data have been collected from 1986 to 2009 (185 surveys, total) at station L-REF10 (Figure 2.3). The line indicates the statistical adjustment of the annual variation (Revilla et al., 2010).

In the coastal waters (nearshore), the surface chlorophyll “a” concentration shows little seasonal variability (Table 2.1). Although a relatively higher concentration is found in mid-spring (April-May), the seasonal pattern is less defined in comparison to the offshore waters. Other differences can be observed: (i) in summer, the chlorophyll concentration is higher in coastal waters compared to offshore waters; (ii) during late winter-early spring, the maximum is somewhat delayed in coastal waters. It can be hypothesised that the relative importance of nutrients and light availability, as control factors for primary production in these two environments, determines the different seasonal patterns. Coastal waters are relatively more turbid, but richer in nutrients. Therefore, during summer higher phytoplankton biomass could be maintained in the coastal than in the offshore waters; but, in contrast, during winter turbidity would be a limiting factor for phytoplankton production in the nearshore waters.

Furthermore, the short-term variability of the meteorological and hydrographical factors can overlap with the typical seasonal cycle and influence the phytoplankton dynamics. For example, windows of stability during winter are usually associated with low intensity blooms. Also, inputs of nutrient-rich continental waters, caused by short periods of atmospheric instability, may act as modulation factors of the bloom decay, during late spring and summer. However, the response of the phytoplankton to these events of fertilisation is not expected to be rapid and proportional to the loads, or to the resulting nutrient concentrations, due to the turbidity and the advection of the phytoplankton that the spreading plumes produce.

## Eutrophication risk

The European Directive 91/271/EEC on urban wastewater treatment defines eutrophication as ‘the enrichment of water by nutrients, especially compounds of nitrogen and/or phosphorus, causing an accelerated growth of algae and higher forms of plant life to produce an undesirable disturbance to the balance of organisms present in the water and to the quality of the water concerned’ (European Commission, 1991). Although the assessment of eutrophication is not the main objective of the WFD (European Commission, 2000), this Directive has an implicit requirement to assess eutrophication caused by anthropogenic pressure, when classifying the ecological quality status of surface water bodies (Borja et al., 2006).

Historically, the Basque coast has been conditioned strongly by different anthropogenic pressures. Reclamation of estuarine areas, for agricultural purposes and subsequent occupation of those areas for urban, industrial and port developments have provoked a dramatic reduction in size and the degradation of water quality in the estuaries (Franco et al., 2004). This situation began to improve during the last decade, due to the closure of many industries and the implementation of sewerage treatment schemes (García-Barcina et al., 2006; Borja et al., 2009a). However, although nutrient inputs to the Basque coast have reduced dramatically in recent years, some of the estuaries still receive discharges. Thus, their inner and middle reaches could present risk of eutrophication, at least during the summer season (Garmendia et al., accepted).

Regarding the coastal waters, the situation is very different. Borja et al. (2006) calculated the nutrient loads in terms of N ( $\text{kg d}^{-1} \text{km}^{-2}$ ), from the main anthropogenic sources of contamination, which included: urban, industrial, agricultural and aquaculture discharges. Subsequently, by comparing N loads with the sensitivity of the water body, a pressure level was derived. The sensitivity of the coastal water body was determined by the flushing time and dilution potential, as it is explained in their study (Table 2.2, in Borja et al., 2006). By this assessment, two areas with different level of pressure were identified: (i) the central area of the Basque coast, with no pressure ( $74 \text{ kg N d}^{-1} \text{km}^{-2}$ ); and (ii) the western and eastern areas of the coast, with low pressure ( $163$  and  $171 \text{ kg N d}^{-1} \text{km}^{-2}$ , respectively). Therefore, along the Basque coast, the Matxitxako-Getaria water body (Figure 2.3) could be considered the closest water body to the reference condition for the physico-chemical and phytoplankton quality elements.

In the Basque estuaries and coastal waters, physico-chemical conditions have been exhaustively studied by Bald (2005) and Bald et al. (2005), in order to develop assessment tools for the WFD implementation. By means of expert judgement and modelling, these authors established the reference condition for inorganic N- and P-nutrients. Thus, the annual average concentrations representative of high physico-chemical status in the coastal waters were:  $2.06 \mu\text{mol L}^{-1}$  (ammonia),  $6.14 \mu\text{mol L}^{-1}$  (nitrate) and  $0.45 \mu\text{mol L}^{-1}$  (phosphate). By taking into account nutrient concentrations together with oxygen saturation and optical conditions (Secchi depth, suspended solids and turbidity), these authors classified the physico-chemical status of the Basque coastal waters as “good” for the period 1995-2003. Subsequent evaluations have indicated a similar status, and no or low risk of eutrophication in the Basque coastal waters (e.g. Borja et al., 2009b; Garmendia et al., accepted).

The low risk of eutrophication in the Basque coastal waters is also indicated by the low phytoplankton biomass, measured as chlorophyll “a” concentration (Table 2.1, Figure 2.6). The reported chlorophyll concentrations in other areas of the European continental shelf are higher than in the Basque coastal waters, both in terms of annual means and maximum values (e.g. Siddorn et al., 2007). These differences in phytoplankton biomass levels are, to some extent, caused by natural differences in morphological and hydrographical conditions along the European shelves. Low intensity of upwelling activity, relatively small river loads and a narrow shelf characterise the Basque coast, which can explain the low level of phytoplankton biomass (Revilla et al., 2009). The dilution potential of this exposed and high-energy coast is an additional factor that precludes nutrient loads from anthropogenic origin to accumulate in the nearshore waters, and decreases the risk of eutrophication (Borja et al., 2006, 2009b).

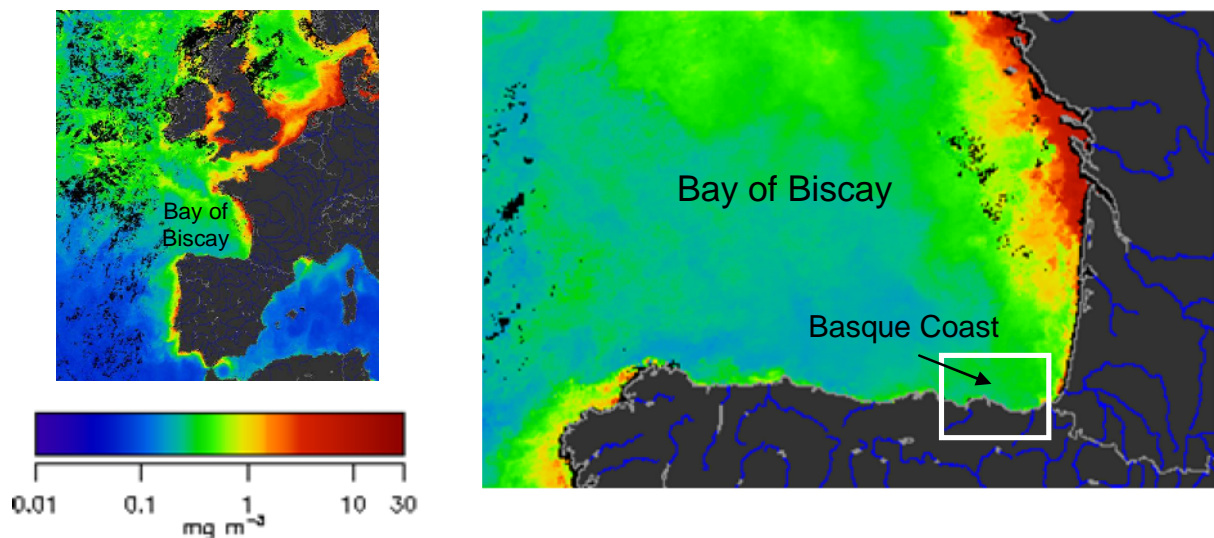


Figure 2.6. Monthly average of chlorophyll “a” concentration in August 2007. Images from the Ocean Colour Dataset for the NE Atlantic (<http://marine.jrc.cec.eu.int/cgi-bin/OC/select.pl?NADR>).

Chlorophyll “a” is used, as an indicator of phytoplankton biomass, to assess the quality status of the phytoplankton element according to the WFD in the Basque coastal waters. The reference condition and some of the class boundaries for the metric (90<sup>th</sup> percentile over six-year periods) were established during the first phase of the European intercalibration exercises (Carletti and Heiskanen, 2009). Thus, the reference was set at 2.33  $\mu\text{g L}^{-1}$ , and the high/good status boundary at 3.5  $\mu\text{g L}^{-1}$ . This was for the whole Eastern Cantabrian coast, which includes the Basque coastal waters (Figure 2.2). By applying this metric, during recent periods, the quality status of the Basque coastal waters is classified as “high” (Revilla et al., 2009).

## Materials and methods

### Data set

The Basque Water Agency, by means of the *Littoral Water Quality Monitoring and Control Network* (LQM), has monitored the Basque coastal and estuarine water quality since 1994. In the coastal waters, the LQM includes 16 sampling stations. In recent years, two offshore stations have been added (L-REF20 and L-REF30) to the offshore station L-REF10, which has been also surveyed since 1986, providing continuous vertical profiles of oceanographic variables, including chlorophyll concentration (Revilla et al., 2010). These three stations are sampled for the Marine Strategy Framework Directive implementation (Figure 2.3). These offshore stations can be considered as reference stations due to their distance from the main pollution sources on land (~10 km).

Among several other variables, physico-chemical data and chlorophyll “a” are recorded quarterly within the LQM (Borja et al., 2004, 2009a). Phytoplankton abundance and composition started to be measured in 2002 (since 2006 at stations L-REF20 and L-REF30). The phytoplankton composition has been studied generally in spring and in summer (two sampling efforts per year). Data from winter and autumn are available only since 2007.

The objective of this study is to describe the phytoplankton communities that are representative of the reference condition in the Northeast Atlantic ecoregion for the type NEA 1/26a and, in particular, for the area of the Eastern Cantabrian coast. For this purpose, the data collected by the LQM at the reference station L-REF10 (43°27'N 01°55'W), in the Basque coast, were used (Table 2.2). This offshore station was selected among the three reference stations of the LQM because it represents the longest phytoplankton data series available among them.

*Table 2.2. Sampling schedule for phytoplankton composition at the reference offshore station L-REF10.*

	2002	2003	2004	2005	2006	2007	2008	2009
Winter	-	-	-	-	-	Feb	Feb	Feb
Spring	May	May	May	May	May	May	May	May
Summer	Aug	Aug	Aug	-	Sep	Aug	Aug	Aug
Autumn	-	-	-	-	-	Oct	Oct	Nov

### Field work and taxonomical analyses

Phytoplankton samples were collected at surface waters (0-1 m) by means of clean buckets, and were stored in borosilicate bottles. Each water sample (125 ml) was immediately preserved with 1 ml of 25% glutaraldehyde solution. It was maintained cold (4°C) and dark until its analysis, which was undertaken within 3 months after collection. Inverted microscopy and standard methods were used for identification and counting (Utermöhl, 1958). Therefore, the abundance of the organisms whose size was less than approximately 5 µm was not considered. For example, in the case of the cyanobacteria only some filaments could be counted and identified. Other techniques such as epi-fluorescence microscopy or flow cytometry are more suitable for



counting the small sized phytoplankton. Although the pico-phytoplankton (<2 µm) can be an important contributor to both phytoplankton abundance and biomass in the Cantabrian Sea (Calvo-Díaz et al., 2008), counts of pico-phytoplankton are not performed within the LQM, because this variable does not indicate anthropogenic impact in the Basque coastal waters. Orive et al. (2004) concluded that the nanoplankton (2-20 µm) is usually the most important fraction, in terms of chlorophyll concentration.

Most of the diatoms and armoured dinoflagellates were generally identified to the level of genus or species. The smaller and/or more fragile forms were classified at the level of phylum or class. In order to verify the nomenclature, the lists of the identified taxa were checked with the European Register of Marine Species (ERMS) of the European Network of Excellence *Marbef* (Marine Biodiversity and Ecosystem Functioning), by using the ERMS taxon search (<http://www.marbef.org/data/aphia.php?p=search>). In some cases, the identified taxa could not be found in the ERMS system (e.g. *Heterosigma akashiwo*). Then, the web site of AlgaeBase (Guiry and Guiry, 2010) was used to check the currently accepted names. The taxonomy browser of AlgaeBase was also used to assign higher ranks (phylum or class). In the above example, *Heterosigma akashiwo* was classified within the class Rhaphidophyceae and the phylum Heterokontophyta Moestrup (equivalent to Ochrophyta T. Cavalier-Smith). The major groups indicated in Table 2.3 comprised all the identified phytoplankton taxa.

Table 2.3. Major groups established with the identified taxa in the Basque coastal and estuarine waters between 2002 and 2009. For taxonomy hierarchy see AlgaeBase (<http://www.algaebase.org/>).

Coding	Common name	Phylum	Class
Cha	-	Charophyta Cavalier-Smith	-
Chl	-	Chlorophyta Pascher	Chlorophyceae Wille Prasinophyceae Christensen ex Silva Ulvophyceae Mattox & Stewart
Cry	-	Cryptophyta Cavalier-Smith	-
Cya	-	Cyanobacteria Stanier ex Cavalier-Smith	-
Dia	Diatoms	Bacillariophyta Engler & Gilg	-
Din	Dinoflagellates	-	Dinophyceae Fritsch
Eug	-	Euglenozoa Cavalier-Smith	-
Hap	-	Haptophyta Hibberd ex Edvardsen & Eikrem	Prymnesiophyceae Hibberd
Het	-	<sup>1</sup> Heterokontophyta Moestrup	Dictyochophyceae Silva Chrysophyceae Pascher Raphidophyceae Chadeffaud ex Silva Synurophyceae Andersen
Cil	Autotrophic ciliates	-	-
Coc	Coccolids	-	-
Nan	Nanoflagellates	-	-
Uni	Unidentified forms	-	-

<sup>1</sup>Equivalent to Ochrophyta T. Cavalier-Smith.

## Mathematical and statistical analyses

In order to describe the phytoplankton assemblages, a range of methods, including univariate, graphical and multivariate methods, were applied (Clarke and Warwick, 2001).

Firstly, *univariate indices* were calculated. These indices try to collapse the full set of species counts from a sample into single coefficients. The indices included: (i) total abundance; (ii) chlorophyll “a” concentration; (iii) richness (taxa number); and (iv) diversity (Shannon index).

Secondly, *k-dominance cumulative plots* were performed. These plots summarise the set of species counts for a single sample by means of a curve or histogram. The objective of these analyses was to determine the degree of dominance of a single taxon within the community. Data at the highest taxonomic level achievable was used (genus or species, when possible). The *k-dominance cumulative plots* were performed in Primer v6 (Clarke and Gorley, 2006).

Finally, *multivariate analyses* were used. As described by Clarke and Warwick (2001), these analyses can reduce the high dimensionality in the community data, taking a particular view of the structure it exhibits. The multivariate analyses were applied to the whole set of samples (Table 2.3). Major taxonomic groups were employed (diatoms, dinoflagellates, haptophytes, chlorophytes, cryptophytes; heterokontophytes, unidentified nanoflagellates, unidentified coccoids, autotrophic ciliates, euglenozoa and unidentified forms-10 µm). A square-root transformation was applied in order to smooth the high variability of the data. The analyses were performed in Primer v6 (Clarke and Gorley, 2006), as follows:

- Dendrograms from hierarchical agglomerative clustering (CLUSTER) with group average mode (based upon the Bray-Curtis similarity index). The objective was to identify groups of samples on the extent to which these samples share a particular taxonomic structure (in this case, based upon the relative importance of the major taxonomic groups). In addition, the null hypothesis that the groups of samples displayed in the diagram had no meaningful internal structure was tested by a similarity routine profile (SIMPROF) (Clarke et al., 2008).
- Non-metric multi-dimensional scaling (NMDS, usually shortened to MDS) based upon the Bray–Curtis similarity index. The MDS represents the samples as points in low-dimensional space. The points that are displayed close together correspond to the samples that are very similar in composition; the points that are far apart correspond to very different samples. As described by Clarke and Warwick (2001), the combination of both CLUSTER and MDS analyses can be a very effective way of checking the adequacy and mutual consistency of both representations. Furthermore, in those cases where the stress (a useful value to study the adequacy of the MDS representation) is > 0.1, the authors recommend the use of the two techniques in combination.
- A similarity percentage analysis (SIMPER) was used to examine the contribution of each major taxonomic group to the average resemblances between samples. A 90% cut off in the cumulative contribution (%) was applied to exclude the taxa that contributed very low to the average similarity.

## Results

### Bulk phytoplankton characteristics

All the winter samples for the study period were collected in February. The total phytoplankton abundance was  $>200 \cdot 10^3$  cells  $L^{-1}$  (Figure 2.7a). A peak of  $\sim 700 \cdot 10^3$  cells  $L^{-1}$  was observed in 2009. It was caused primarily by a dinoflagellate (*Heterocapsa* cf. *rotundata*), and to a minor extend by a cryptophyte (*Teleaulax* sp.). The chlorophyll “a” concentration in February is usually at its annual maximum (Figure 2.4). In the set of samples analysed, the chlorophyll was within the range expected, and its interannual variability was very low ( $0.8 \pm 0.1$   $\mu g L^{-1}$ , avg.  $\pm$  std. dev.) (Figure 2.7c). The species richness in the winter samples was in the range 13-21 (Figure 2.7b). The diversity index ranged from 1.5 to 3.2 bit  $ind^{-1}$  (Figure 2.7d). Both, the species richness and the diversity index were within the ranges observed during other seasons.

All the spring samples were collected in May. However, a high interannual variability was observed in the community descriptors. At the beginning of the data series, relatively low values of total abundance ( $<200 \cdot 10^3$  cells  $L^{-1}$ ) and richness (10-20) were observed (Figures 2.8a, b). In contrast, from 2005 to 2009, these variables were close to or above the average. During this last period, total cell densities near  $10^6$  cells  $L^{-1}$ , and richness values up to 39 were observed. A maximum of  $1.3 \cdot 10^6$  cells  $L^{-1}$  was registered in 2007, which was composed mainly by small flagellates. The chlorophyll “a” concentration in spring ranged from 0.1 to 1.0  $\mu g L^{-1}$  (Figure 2.8c). It was not significantly correlated with the total cell abundance ( $r=-0.347$ ;  $p>0.05$ ; Pearson’s coefficient). This could have resulted from differences in phytoplankton composition and cell size among the spring samples. The diversity index in spring was in the range 1.3-3.5 bit  $ind^{-1}$  (Figure 2.8d).

In the summer samples (August and September), the total abundance was always at low levels ( $<300 \cdot 10^3$  cells  $L^{-1}$ ) (Figure 2.9a). The species richness ranged broadly (5-27) (Figure 2.9b). The chlorophyll concentration was low ( $0.2 \pm 0.2$   $\mu g L^{-1}$ , avg.  $\pm$  std. dev.) (Figure 2.9c). The Pearson’s correlation coefficient between chlorophyll “a” concentration and total phytoplankton abundance ( $r=0.709$ ) was close to the statistical significance ( $r=0.755$ ;  $p<0.05$ ). This would reflect a lower variability in the cell size (due to the dominance of small sized organisms) in the summer samples, when compared to the spring samples. The diversity index in summer ranged from 1.8 to 3.7 bit  $ind^{-1}$  (Figure 2.9d).

In autumn (October and November), the total abundance was slightly higher than in summer. However, high peaks were not observed (Figure 2.10a). The maximum (October 2007) was  $\sim 500 \cdot 10^3$  cells  $L^{-1}$ , dominated mainly by dinoflagellates (*Heterocapsa* sp.) and small non-siliceous species. The chlorophyll “a” was also higher than in summer,  $0.6 \pm 0.2$   $\mu g L^{-1}$  (avg.  $\pm$  std. dev.) (Figure 2.10c). The species richness was in the range 17-28 (Figure 2.10b). The diversity ranged from 2.5 to 3.0 bit  $ind^{-1}$  (Figure 2.10d). The richness and diversity values were within the highest observed during other seasons.

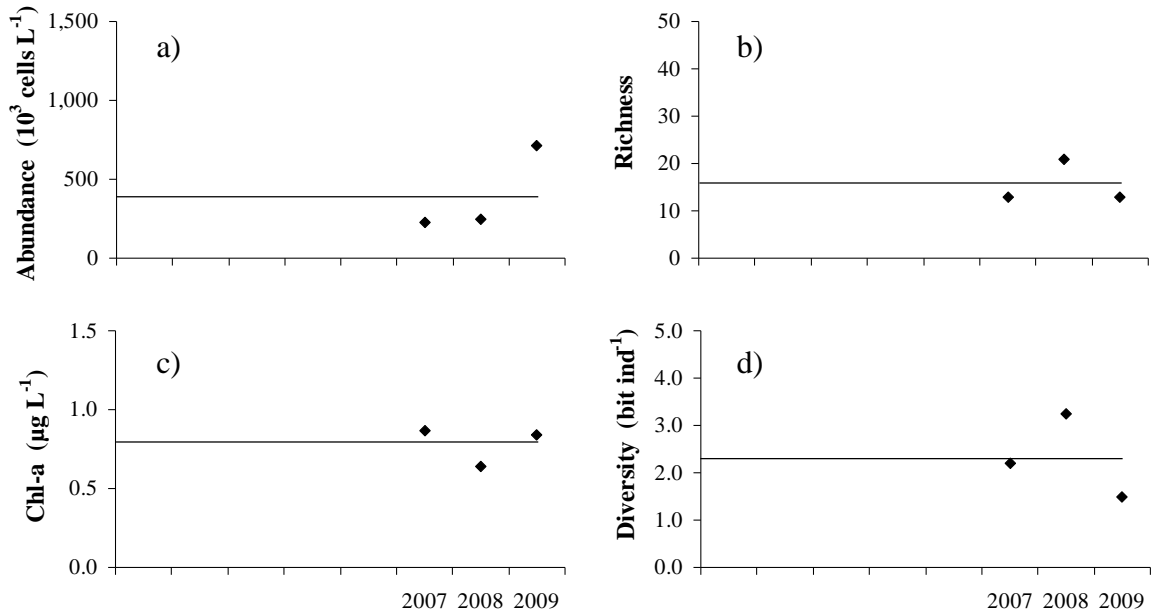


Figure 2.7. The descriptors of the phytoplankton community at the offshore station L-REF10 in winter. (a) Total abundance; (b) Species richness; (c) Chlorophyll "a" concentration; and (d) Diversity. The lines show the seasonal average for each descriptor.

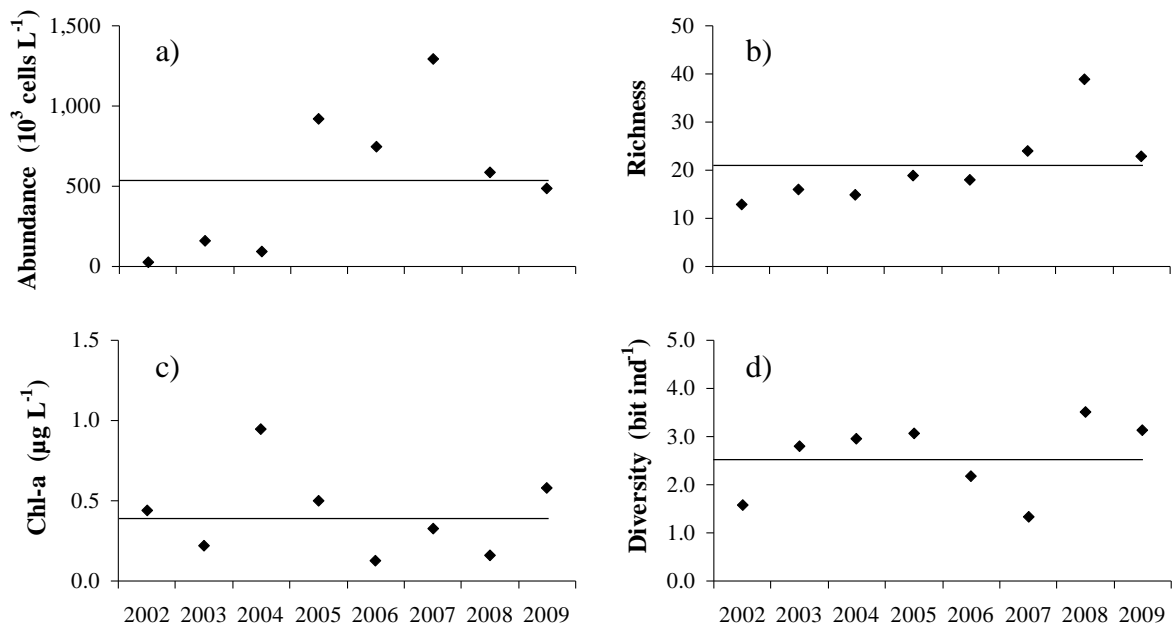


Figure 2.8. The descriptors of the phytoplankton community at the offshore station L-REF10 in spring. (a) Total abundance; (b) Species richness; (c) Chlorophyll "a" concentration; and (d) Diversity. The lines show the seasonal average for each descriptor.

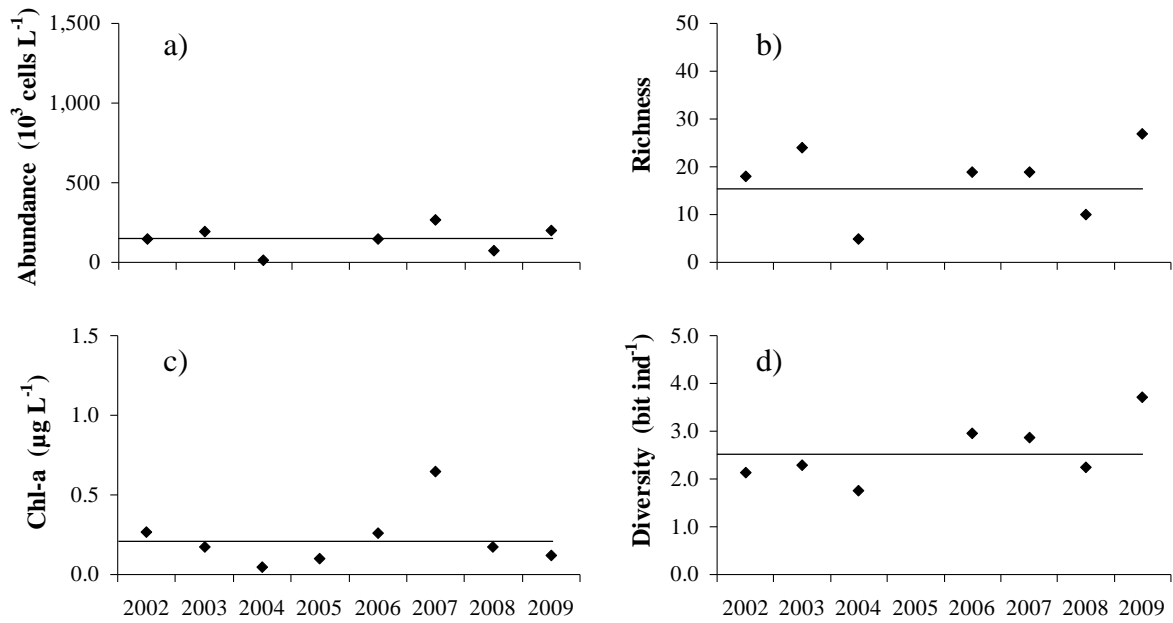


Figure 2.9. The descriptors of the phytoplankton community at the offshore station L-REF10 in summer. (a) Total abundance; (b) Species richness; (c) Chlorophyll "a" concentration; and (d) Diversity. The lines show the seasonal average for each descriptor.

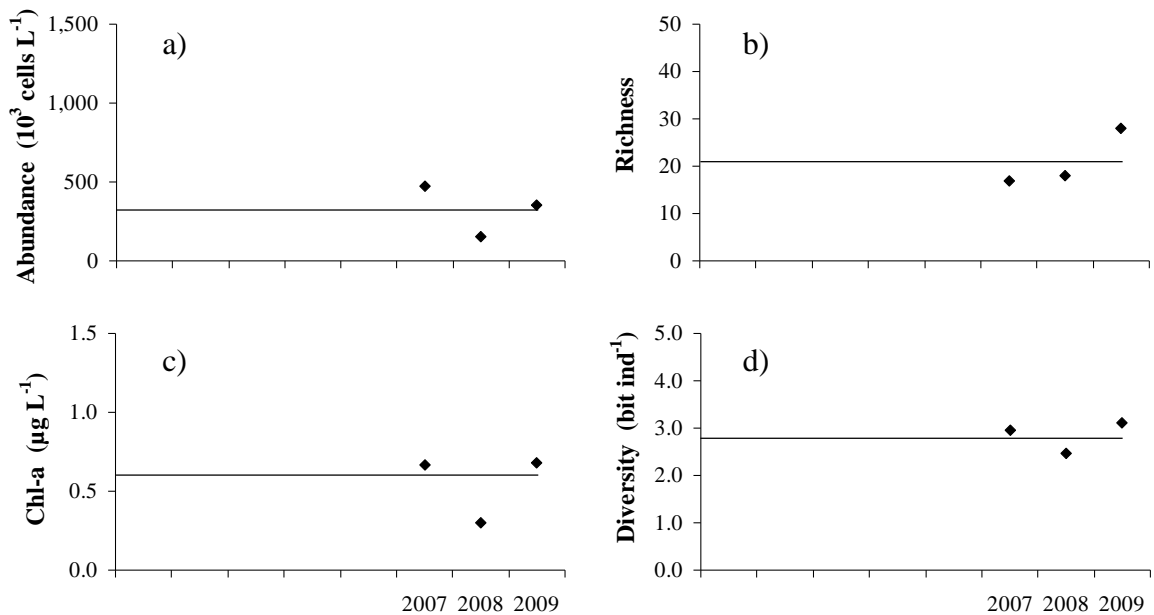


Figure 2.10. The descriptors of the phytoplankton community at the offshore station L-REF10 in autumn. (a) Total abundance; (b) Species richness; (c) Chlorophyll "a" concentration; and (d) Diversity. The lines show the seasonal average for each descriptor.

### Community structure: evenness *versus* dominance

The analysis of the winter samples by  $k$ -dominance plots showed communities with different structure, based upon the relative contribution of the species to the total abundance (Figure 2.11a).

The sample from 2008 (in blue colour) presented a mixed and evenly distributed community. Several species contributed with low percentages (5-20% approximately) to the total abundance, and many taxonomic groups were represented (haptophytes, small flagellates, cryptophytes, diatoms, dinoflagellates and chlorophytes).

In contrast, some samples presented one or two dominant species that together accounted for 70% of the total abundance (Figure 2.11a, in red colour). These dominant species could belong to different taxonomic groups (small flagellates and haptophytes, in 2007; dinoflagellates and cryptophytes, in 2009) (Table 2.4).

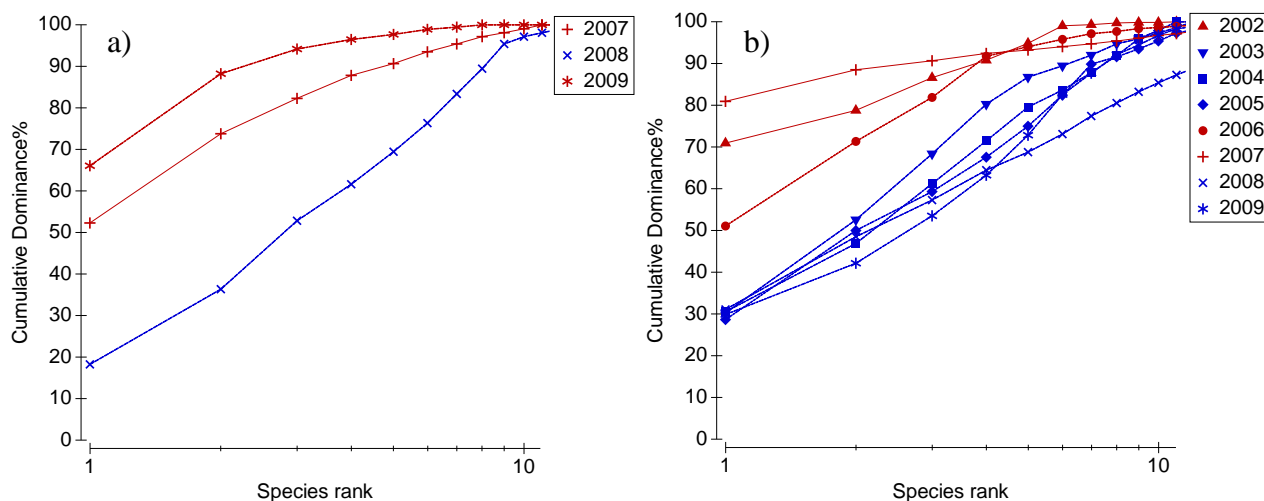


Figure 2.11. The  $k$ -dominance cumulative plots with species from a) winter; b) spring. The species rank ( $x$ -axis) shows only the first 10 species in logarithmic scale.

The analysis of the spring samples by  $k$ -dominance cumulative plots is showed in Figure 2.11b. Similarly to winter, differences in the community structure were observed. The low part of the graph (in blue colour) represents the samples where many different taxa contributed in an approximate equitable basis to the total cell counts (the 2003, 2004, 2005, 2008 and 2009 samples). In these cases, the highest contribution was approximately 30%. These taxa consisted of unidentified nanoflagellates, haptophytes (*Chrysochromulina* spp.) or diatoms (*Chaetoceros* sp. and *Proboscia alata*).

In contrast, in some of the spring samples the communities were distributed less evenly. These samples are depicted in red colour (Figure 2.11b). The highest dominance was found in spring 2007 during a bloom of nanoflagellates (80%). In 2002, unidentified coccolithophorids

accounted for 70%. In this case the phytoplankton abundances were very low. In 2006, *Chrysochromulina* spp. contributed 50% to the total cell abundance (Table 2.4).

The summer samples showed a continuous gradient from evenness to dominance (Figure 2.12a). The most even structure was found in 2009 (at the lowest part of the graph). Other summer samples with relatively low dominance could be distinguished (2006, 2007 and 2008, in blue colour). The contribution of the most abundant taxon in these samples ranged 19-42%. The first ranked species were small flagellates or cryptophytes (*Plagioselmis* sp.).

The summer samples that showed the lowest evenness (2002, 2003 and 2004) are depicted in red, at the top of the graph (Figure 2.12a). The dominant species accounted for 50-57% of the total abundance (Table 2.4). In 2002, the community was composed mainly by several small-sized diatoms (*Chaetoceros anastomosans*, *C. wighamii* and *C. costatus*). In 2003, another small-sized diatom (*Pseudo-nitzschia* spp.) showed the highest abundance. Similarly, in 2004 small-sized diatoms (such as unidentified pennales and *Thalassiosira oceanica*) dominated.

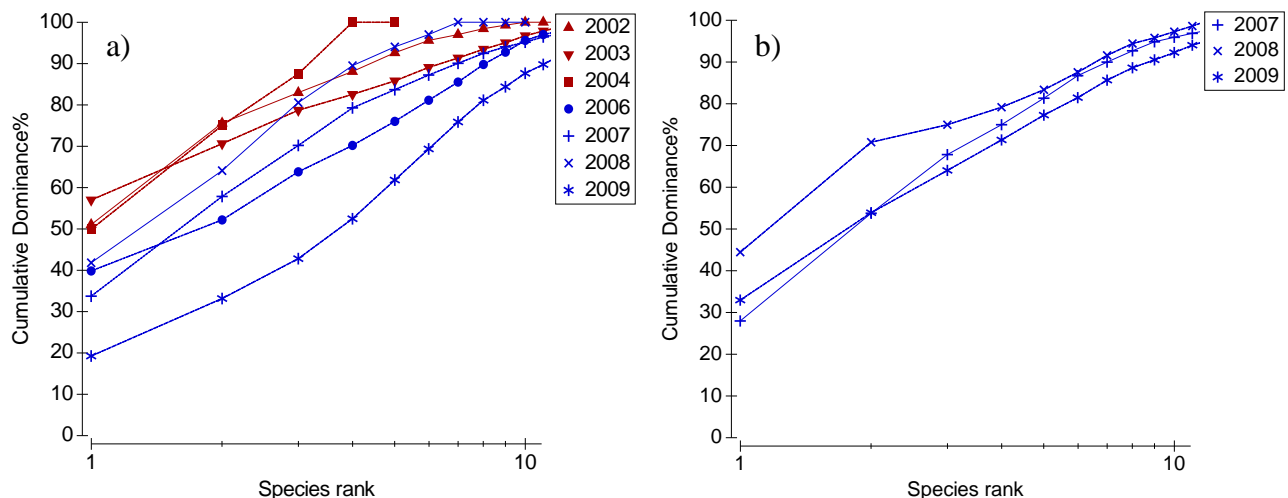


Figure 2.12. The  $k$ -dominance cumulative plots with species from a) summer; b) autumn. The species rank ( $x$ -axis) shows only the first 10 species in logarithmic scale.

In autumn, the degree of dominance within the three studied samples was, in general, low (Figure 2.12b). Nevertheless, the 2008 sample could be considered an intermediate case (70% of the total abundance was contributed by haptophytes and unidentified nanoflagellates). The first ranked species contributed 28-44% and consisted of haptophytes (*Chrysochromulina* spp.), cryptophytes (*Teleaulax* sp.) and nanoflagellates. Other taxa with relatively high contribution (21-26%) were dinoflagellates (*Heterocapsa* sp.) and cryptophytes (*Plagioselmis* sp.).

In summary, the communities dominated by only one or two species were more frequent in the winter, than in the rest of the seasonal surveys. The total cell abundance varied broadly ( $10^4$ - $10^6$  cells  $L^{-1}$ ) among the communities with the highest degree of dominance (Table 2.4). The most dominant species were represented by small-sized diatoms in the summer, and by non-siliceous species (nanoflagellates, small dinoflagellates and haptophytes) during the rest of the seasons.

Table 2.4. First and second rank species and their relative contribution (%) to total cell abundance within the phytoplankton samples classified as cases of dominance at station L-REF10.

Season	Year	First rank species		Second rank species		Total Abundance (Cells L <sup>-1</sup> )
Winter	2007	Nanoflagellates	52%	<i>Chrysochromulina</i> spp.	21%	227,270
	2009	<i>Heterocapsa</i> cf. <i>rotundata</i>	66%	<i>Teleaulax</i> sp.	22%	713,137
Spring	2002	Coccolithophorids	71%	<i>Prorocentrum minimum</i> , <i>Scrippsiella trochoidea</i>	16%	26,770
	2006	<i>Chrysochromulina</i> spp.	51%	Nanoflagellates	20%	747,563
	2007	Nanoflagellates	81%	Coccolithophorids	8%	1,291,394
Summer	2002	<i>Chaetoceros anastomosans</i>	51%	<i>Chaetoceros wighamii</i>	24%	143,378
	2003	<i>Pseudo-nitzschia</i> spp.	57%	<i>Chrysochromulina</i> spp.	14%	195,419
	2004	Pennate diatoms	50%	<i>Thalassiosira oceanica</i>	25%	15,361
Autumn <sup>2</sup>	2008	<i>Chrysochromulina</i> spp.	44%	Nanoflagellates	26%	152,934

<sup>2</sup>Intermediate case

### Phytoplankton assemblages established on the basis of similarity

Six different types of phytoplankton assemblages were identified based on their composition at the level of major taxonomic groups (phylum or class), when the composition was analysed by hierarchical agglomerative clustering (Figure 2.13). The resemblance level was set at 58%.

Among these types, three of them were represented by a single sample (“a”, “b”, and “c”). These were distinguished from the rest of the samples with a high statistical significance ( $\alpha = 0.05$ , SIMPROF test). However, the separation of the other clusters (“d”, “e” and “f”) was not statistically significant, at this level of significance.

In addition, a MDS analysis was performed with the whole set of samples, and the clusters that had been established in the dendrogram were overlaid (Figure 2.14). As indicated in the methodology, the points that lie far apart in the MDS diagram correspond to samples whose composition is very different from the rest. The three single-sample clusters (“a”, “b”, and “c”) presented a high distance among them and with the rest of the samples, in the MDS ordination. In order to reduce the range of variability within the data set, these three samples were removed and a second similar CLUSTER analysis was applied (58% resemblance). In this second analysis, although none of the clusters could be statistically separated by the SIMPROF test at  $\alpha = 0.05$ , the cluster “d” was statistically significant at  $\alpha = 0.10$ . When the composition of the clusters “e” and “f” was studied in more detail, important differences of ecological relevance were found. Therefore, it was decided to describe them separately, in spite of the lack of statistical significance when it was tested by SIMPROF. Also, it must be taken into account the high variability that is inherent to the phytoplankton communities and the small degree of overlapping among the clusters in the MDS ordination (Figure 2.14).



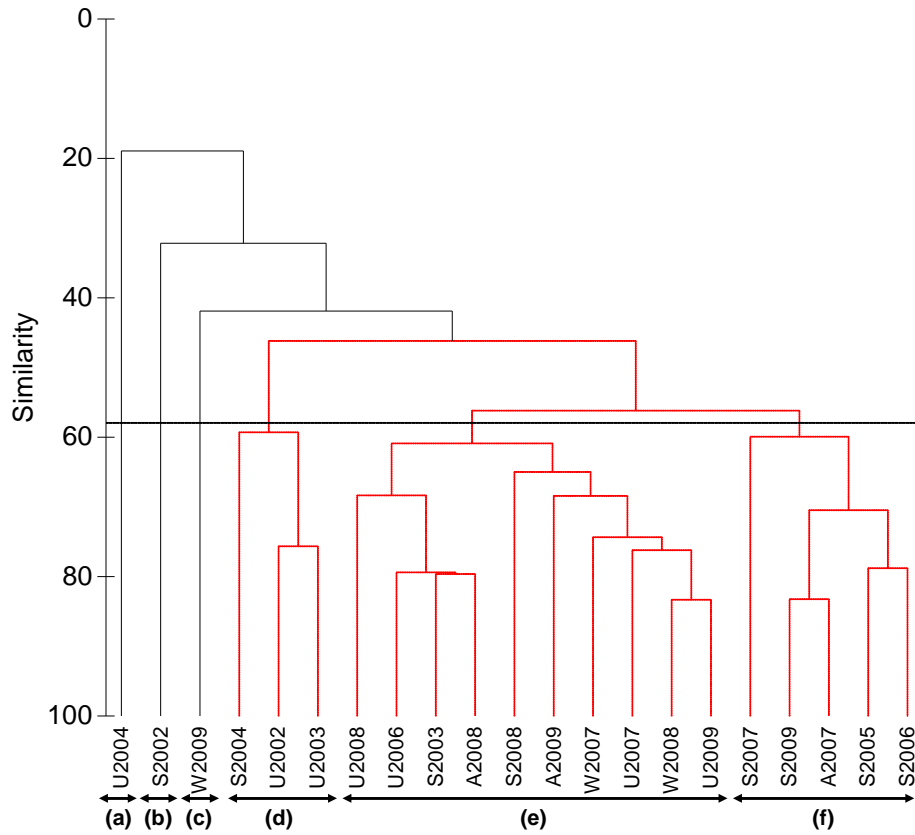


Figure 2.13. Dendrogram from the CLUSTER analysis of the samples collected at the reference offshore station L-REF10. The horizontal black line shows the level of resemblance of 58%. The red lines indicate the branches that are not separated by SIMPROF at  $\alpha = 0.05$ . Key (sample labels): W-Winter, S-spring; U-Summer; A-Autumn. Clusters: “a”, “b”, “c”, “d”, “e” and “f”.

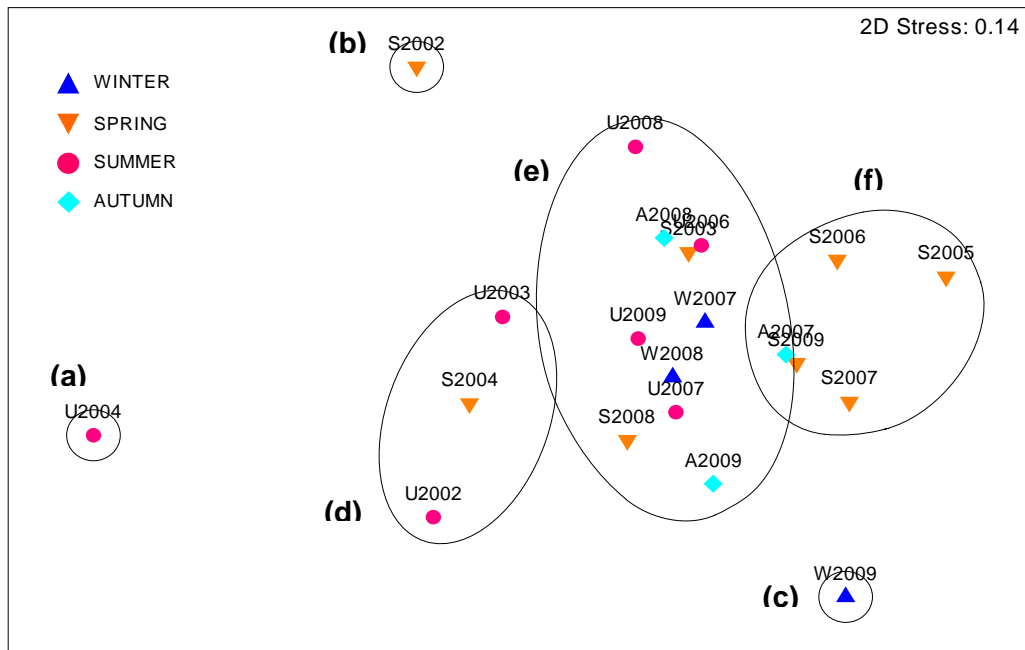


Figure 2.14. Non-metric multi-dimensional scaling (MDS) ordination of the samples collected at the reference offshore station L-REF10. The ellipses identify the groups that result from the above CLUSTER analysis (Figure 2.13). The seasons are represented by different symbols and colours.

The clusters presented distinct characteristics of abundance, composition and seasonality. Very low phytoplankton abundance ( $<200 \cdot 10^3$  cells  $L^{-1}$ ) characterized the clusters “a”, “b” and “d”, which involved some of the summer and mid-spring samples (Table 2.5). In summer, small diatoms such as, unidentified pennales, *Pseudo-nitzschia* spp., *Chaetoceros anastomosans* and *C. wighamii* were the dominant taxa (data not shown). In mid-spring, a higher contribution of non-siliceous taxa (haptophytes, dinoflagellates and cryptophytes) was observed in the very low-abundance communities.

Cluster “c” was constituted by a winter sample with high abundance ( $\sim 700 \cdot 10^3$  cells  $L^{-1}$ ), dominated by the dinoflagellate *Heterocapsa* cf. *rotundata* (Table 2.5). Cluster “e” presented usually low abundances, and included samples from any season that could show different taxonomic composition (mixed communities of diatoms together with non-siliceous taxa as well as communities dominated by flagellates). Cluster “f” showed generally high abundances of non-siliceous species, with the majority of the samples collected in mid-spring (Table 2.5).

Table 2.5. SIMPER analysis of the phytoplankton composition at the offshore station L-REF10. N: number of samples within each cluster; Abund.- cell abundance; Simil.- similarity; Dissimil.- dissimilarity.

Cluster (N)	Total Abund. ( $\times 10^3$ cells $L^{-1}$ )	Season	Taxonomic composition	Contrib. to Total Abund. (%)	Contrib. to Avg. Simil. (%)	Avg. Simil.	Avg. Dissimil.
a (1)	15	Summer	Diatoms	75	-	-	71
			Chlorophytes	25	-		
b (1)	27	Mid-spring	Haptophytes	75	-	-	63
			Dinoflagellates	16	-		
c (1)	713	Winter	Dinoflagellates	67	-	-	55
			Cryptophytes	26	-		
d (3)	94-195	Summer and mid-spring	Diatoms	39-84	51	65	49
			Dinoflagellates	4-22	18		
			Cryptophytes	3-16	17		
			Haptophytes	1-20	8		
e (10)	71-587	All seasons	Nanoflagellates	4-52	26	66	44
			Haptophytes	6-59	24		
			Dinoflagellates	3-36	18		
			Cryptophytes	0-64	11		
			Diatoms	1-58	9		
f (5)	470-1,291	Usually mid-spring	Heterokontophytes	0-7	5		
			Nanoflagellates	20-81	31	68	44
			Haptophytes	8-64	27		
			Dinoflagellates	2-32	18		
			Small coccoids	0-14	8		
			Chlorophytes	0-6	6		
			Diatoms	0-6	6		

## Summary

Phytoplankton assemblages in the surface waters were studied at a reference offshore station on the Basque shelf (Northeast Atlantic ecoregion, Eastern Cantabrian Sea). At this station, the anthropogenic influence is negligible, and the natural geomorphological and hydrographical conditions cause chlorophyll “a” concentrations to be generally low (monthly average 0.2-1.0  $\mu\text{g L}^{-1}$ ).

Phytoplankton biomass (chlorophyll “a”) and total cell abundance showed a high temporal variability. In autumn and winter, phytoplankton biomass was relatively high. The abundance showed moderate values during these seasons. In mid-spring, both chlorophyll “a” and total abundance presented a high interannual variability where peaks as well as minimum annual values could be observed. In summer, both phytoplanktonic variables were generally low. The seasonal pattern of the chlorophyll has been described previously. It responds, principally, to the annual cycle of the water temperature and the mixing and stratification processes, which in turn influence the nutrients and light availability in the surface waters.

The correlation between chlorophyll concentration and total cell abundance was weak. It was probably due to the high variability in phytoplankton composition and cellular size among the samples. This has important implications for the use of phytoplankton indicators for ecological status assessment. Thus, a unique index based upon only one of these variables (either chlorophyll “a” or total cell abundance) would not be appropriate for these coastal waters.

The richness (total species number) and diversity (Shannon index) did not show any pattern of seasonal variation. The structure of the communities was further studied by graphical methods (*k*-dominance cumulative plots). In some cases, the communities presented a low level of dominance (the first ranked taxon accounted for, approximately, 20-30% of the total cell abundance). In other cases, just one or two species contributed with a high percentage (70%) to the total abundance. The dominant taxa were small-sized organisms (2-20  $\mu\text{m}$ ), that could belong to different taxonomical groups. The dominant taxa in summer were always diatoms. During the rest of the year, small flagellates, haptophytes (such as, *Chrysochromulina* spp. or unidentified coccolithophorids), or the dinoflagellate *Heterocapsa* cf. *rotundata* could dominate.

Six different types of communities were distinguished on the basis of the total cell abundance and the taxonomic similarity. Two of these types were characterized by very low cell abundance ( $\sim 10^4$  cells  $\text{L}^{-1}$ ), as well as, by the dominance of a single taxon (either small pennate diatoms, or haptophytes); these were rare cases. The communities with relative high abundance ( $0.5\text{-}1.3 \cdot 10^6$  cells  $\text{L}^{-1}$ ) comprised two different types: (i) blooms of small non-siliceous taxa, most of them found in mid-spring; (ii) dinoflagellate blooms (*Heterocapsa* cf. *rotundata*), that were observed in a winter sample. Other community types involved low-moderate abundances ( $0.2\text{-}0.5 \cdot 10^6$  cells  $\text{L}^{-1}$ ), with either a relative high contribution of small diatoms (*Pseudo-nitzschia* spp. and *Chaetoceros* spp.) or small non-siliceous taxa (mainly, unidentified nanoflagellates and haptophytes). This last case was the most frequent community type being found in all seasons.

### 3. Balearic Islands (Mediterranean Sea ecoregion)

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#### Introduction

Pico-phytoplankton, the photosynthetic planktonic cells with cell diameter smaller than 2  $\mu\text{m}$ , are important contributors to the biomass of phytoplankton and to the primary production in warm, nutrient depleted waters (Agawin et al., 2000). Pico-phytoplankton, including the cyanobacteria *Synechococcus* sp., and *Prochlorococcus* sp. and small pico-eukaryotes, are the dominant autotrophic components of the oligotrophic Mediterranean Sea (Vaulot et al., 1990; Agawin et al., 1998). Pico-phytoplankton is present throughout the year in the Mediterranean waters, although as a temperate sea its abundance is larger during the summer time (Mura et al., 1996; Vaulot et al., 1990). Summer blooms dominated by the cyanobacteria *Synechococcus* sp. are described to occur in the NW Mediterranean Sea (Mura et al., 1996; Agawin et al., 1998; Agustí and Sánchez, 2002). Growth rates of Mediterranean *Synechococcus* sp. showed a positive relationship with water temperature (Agawin et al., 1998), growing as fast as expected from its size at the seawater temperature reached during the summer time in the Mediterranean Sea (Agawin et al., 1998), indicating that summer conditions are optimum for this species.

#### Counting Mediterranean pico-phytoplankton cells using flow cytometry

The introduction of flow cytometry to biological oceanography has clearly expanded the horizons of plankton research. Flow cytometry represents an important tool for the identification and counting of the smallest phytoplanktonic groups. Prochlorophytes, the most abundant photosynthetic cells in the ocean, could be only well identified and counted using a flow cytometer. Cell enumeration by flow cytometry is more rapid and less tedious than microscopy. In a flow cytometer, a frequency distribution of cells is generated and the variable over which the cells are distributed is relative fluorescence emission or light scattering of individual cells.

The essence of a flow cytometer is a flow cell, which consists of a capillary containing a flowing sheath fluid. When a sample is injected into the centre of the flowing fluid, the cells are diluted and fluid focused such that they are carried through the capillary single file in a thin optical plane. A laser beam source is focused on the capillary and each particle cells is illuminated as it passes through the beam (Chisholm et al., 1986). Light emitted as fluorescence, and scattered by the particle can be detected simultaneously from each cell. The emitted fluorescence could be auto-fluorescence from the cell (e.g. from photosynthetic pigments) or induced fluorescence from added fluorochromes. In a typical system, forward angle and perpendicular scattering, yielding information about cell size and complexity, are detected

simultaneously to three different wavelengths emitted fluorescence, at a rate of about 50,000 cells per minute (Chisholm et al., 1986; Shapiro, 1995).

### Apparatus and sample handling

1. A flow cytometer provided with a blue light (488 nm) laser is required, as well as simultaneous detection of red (from chlorophyll a) and orange (from phycoerythrin pigment) emission fluorescence, green emission fluorescence, and side scattering (SSC), and forward scattering (FSC) from each cell.
2. An aliquot of a calibrated solution of 1  $\mu\text{m}$  diameter high-green fluorescent beads (Polysciences Inc.) in distilled water, added to the samples could be used as an internal standard for the quantification of cell concentration. Beads concentration in the standard solution should be calculated by filtering replicated aliquots onto black nuclepore filters and counting the beads under an epi-fluorescence microscope (Agustí, 2004).
3. Determination of cell concentration. Measurement of the sample volume analyzed is accomplished using the stock of beads of known concentration. By adding a known volume of calibrated beads solution to the sample, the cell concentration could be calculated as:

$$\text{Cells ml}^{-1} = (\text{cells counted/ beads counted}) * \text{beads ml}^{-1}$$

Where beads  $\text{ml}^{-1}$  is the concentration of beads in the sample

4. Sheath fluid. For best precision the refractive index of the sheath fluid should be matched that of the sample, thus, for seawater samples, the use of 0.2  $\mu\text{m}$  filtered sea water was recommended (Olson et al., 1993). However, the use of fresh distilled water has been proved to be as good as seawater in terms of getting excellent cells signals, avoiding problems of strong oxidation by salts. Hence, fresh MilliQ water was used in the analysis.
5. Fresh samples could be used for the quantification resulting in the best signals at low parasite noise at the flow cytometer. However, there is not always the opportunity for analysing immediately, and it is most convenient to fix the samples. Samples can be preserved with glutaraldehyde (final concentration of 1%), frozen in liquid nitrogen and maintained at  $-80^{\circ}\text{C}$  until further analysis.

### Identification of populations

The red, green and orange fluorescence emissions and the forward and side scattering of the cells and beads were used to detect different cell populations and to differentiate them from the fluorescent beads. Here only procedures that may be specific to analysis of pico-phytoplankton (*Synechococcus*, *Prochlorococcus* and eukaryotes) are covered in detail.

- Sensitivity is of critical importance when analyzing oceanic picoplankton. *Prochlorococcus* cells are very dim in surface oligotrophic waters and may be easily

missed if the sensitivity of the instrument is poor or if instrument settings are not optimal.

- The use of *Synechococcus* or *Prochlorococcus* cultures previous to run natural samples could help as a test samples for a rough adjustment of settings (sensitivities of the fluorescence and scatter detectors) previous to the identification of natural populations.
- The use of logarithmic rather than lineal amplifiers on all signals extends the dynamic range of measurements to 3 to 4 decades. This is important in dealing with natural populations where signal sizes encountered span over several orders of magnitude.
- Data acquisition is triggered by red fluorescence to reduce interferences from non fluorescent particles.
- Settings for a FACSort (Becton Dickinson) flow cytometer for a natural sample collected at low depth used by Marie et al (1997): FSC= E02, SSC= 450, Green Fluorescence= 650, Orange fluorescence = 650, Red fluorescence= 600. The discriminator was set on the red fluorescence and the threshold at 0.
- Multiparameter analysis is required for natural pico-phytoplankton analysis that is accomplished by the use of multiple scatter plots. Subpopulations are interactively defined by gates and identified by the combination of all recorded parameters.
- After the samples are run in the flow cytometer, built a dot plot with SSC (side scattering) in the X axis and FL3 (red fluorescence) in the Y axis. *Prochlorococcus* cells are the smallest and less fluorescent and appear at the left of the axis well differentiated from *Synechococcus* and eukaryotes (Figure 3.1). *Synechococcus* population is identified in a FL3 (red fluorescence) vs FL2 (orange fluorescence) as the population showing both orange and red fluorescence (Figure 3.2). Fluorescent microspheres were best identified as small cells showing FL3 (red fluorescence), FL1 (green fluorescence) and orange fluorescence (FL2).

### Sampling of pico-phytoplankton in the WISER campaign

Sampling was performed in the summer of 2009 in the Mediterranean Sea at three locations in the Island of Majorca. The three stations (Table 3.1) were sampled following the WISER protocol. Three replicated 1 ml sample were processed at the flow cytometer from each replicated bottle sampled at each station.

Table 3.1. Stations sampled in the WISER campaign.

Station	Date	Latitude	Longitude
Es caragol	27.07.09	39° 16.394 N	3° 2.476 E
S'estanyol	28.07.09	39° 21.38 N	2° 55.00 E
Colònia de Sant Jordi	30.07.09	39° 18.562 N	2° 59.958 E

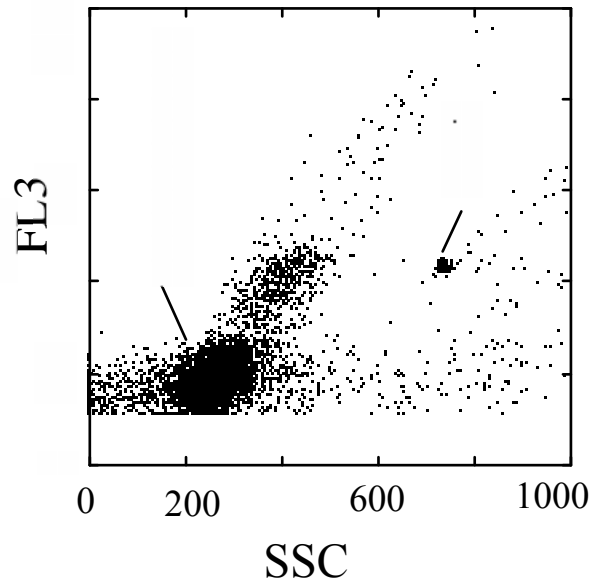


Figure 3.1. Flow cytometer scatter plot of red fluorescence (FL3) vs side scattering (SSC) of a sample from the sub-surface of the Mediterranean Sea.

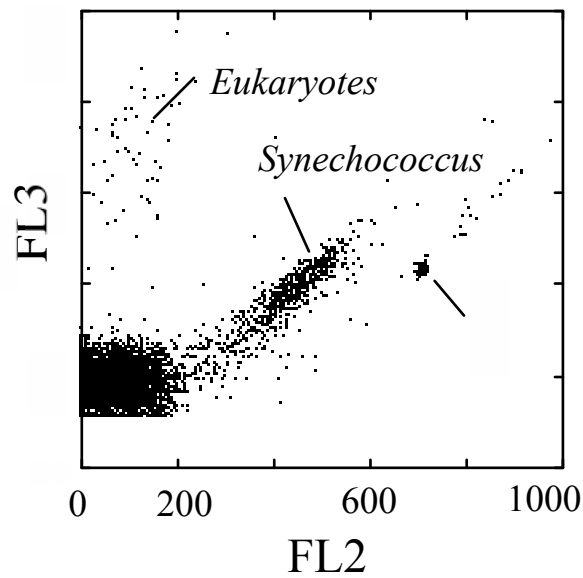


Figure 3.2. Scatter plot of red fluorescence (FL3) vs orange fluorescence (FL2) from flow cytometer data of a sample from the sub-surface of the open Mediterranean Sea.

### Seasonality of Mediterranean pico-phytoplankton

The pico-phytoplankton community observed in the Bay of Palma in previous studies was composed by *Synechococcus* sp., *Prochlorococcus* sp. and pico-eukaryotes (Alonso-Laita et al., 2005) and showed abundances comparable to those found in other Mediterranean areas (e.g. Vaultot et al., 1990, Agawin et al., 1998). *Synechococcus* sp. is the major component of the pico-phytoplanktonic community during all the seasons (Figure 3.3). The three pico-phytoplankton groups present in the Bay of Palma showed important seasonality and differed in the period of peak abundance, which were observed in winter, summer and spring for *Prochlorococcus* sp., *Synechococcus* sp. and pico-eukaryotes, respectively (Figure 3.3). Despite the differences found in the timing of the occurrence of peak abundance, *Prochlorococcus* and pico-eukaryote abundances were positively correlated to that of *Synechococcus* sp. ( $r = 0.53$ ,  $P < 0.0001$ ,  $r = 0.52$ ,  $P < 0.0001$  for *Prochlorococcus* sp. and pico-eukaryotes, respectively (Alonso-Laita et al., 2005).

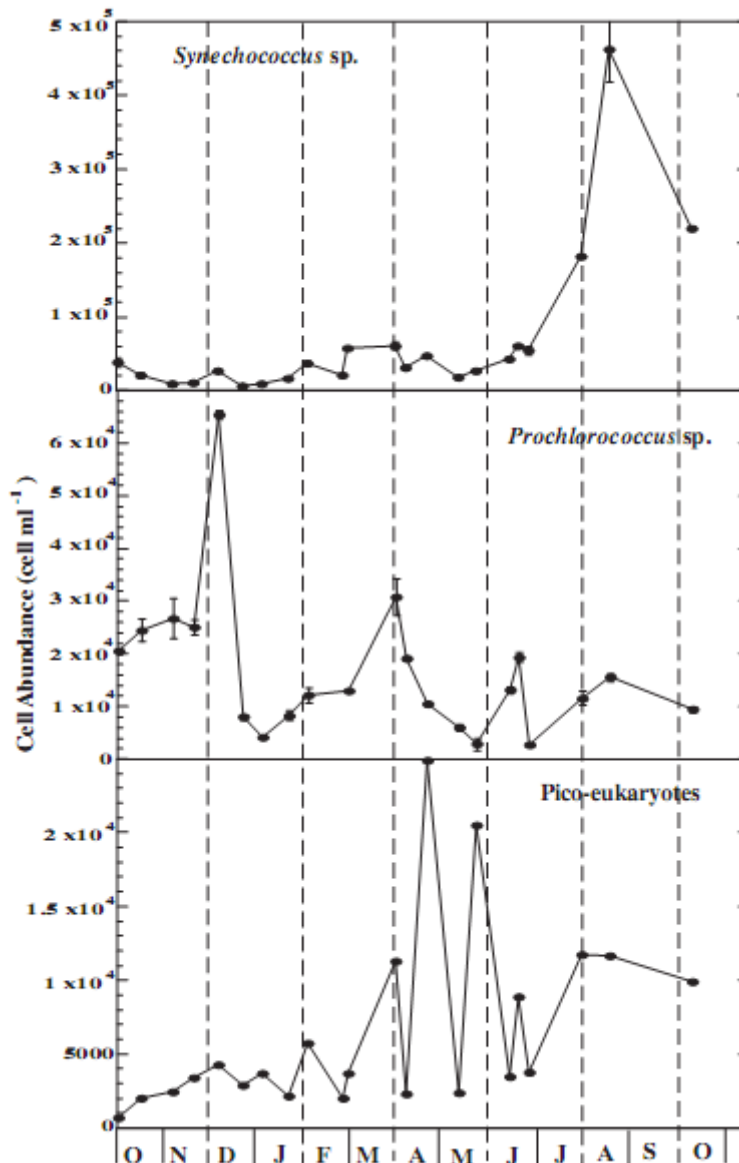


Figure 3.3. Seasonal variation in the abundance (solid line, full circles  $\pm$  std. dev.) for *Synechococcus* sp. *Prochlorococcus* sp. and pico-eukaryotes) from October 2001 to October 2002 in the Bay of Palma.



## Reproducibility of Mediterranean pico-phytoplankton counting

Cell concentration is determining the accuracy in the estimates of pico-phytoplankton abundance. Flow Cytometry techniques had the advantage against classical epi-fluorescence microscopy counts of pico-phytoplankton that is more rapid and less tedious, but also allows increasing the total number of cells counted, which could reach thousands of cells observed in a single measurement.

Reproducibility was found to be great in the abundance of cells counted in the Mediterranean Sea (Table 3.2). The sampling performed in the WISER campaigns included 3 stations performed in the Mediterranean Sea at three locations in the Island of Majorca. As observed previously, the abundance of *Prochlorococcus* was so low, even lower than in previous samplings, and precluded their quantification.

Table 3.2. Abundance of *Synechococcus* and pico-eukaryotes encountered in the Mediterranean Sea during the WISER campaign.

Station	Bottle number	<i>Synechococcus</i> Cells ml <sup>-1</sup>	Pico-Eukaryotes Cells ml <sup>-1</sup>
Es caragol	B1	1,84E+04	1,05E+03
Es caragol	B1	1,91E+04	1,29E+03
Es caragol	B1	2,11E+04	1,29E+03
Es caragol	B2.1	2,16E+04	2,26E+03
Es caragol	B2.1	2,43E+04	1,70E+03
Es caragol	B2.1	2,32E+04	1,78E+03
Es caragol	B2.2	2,73E+04	1,70E+03
Es caragol	B2.2	2,91E+04	1,70E+03
Es caragol	B2.2	2,62E+04	1,05E+03
S'estanyol	B1	1,16E+04	6,46E+02
S'estanyol	B1	1,19E+04	1,05E+03
S'estanyol	B1	1,30E+04	9,69E+02
S'estanyol	B2.1	1,15E+04	1,21E+03
S'estanyol	B2.1	1,18E+04	8,88E+02
S'estanyol	B2.1	1,11E+04	9,69E+02
S'estanyol	B2.2	7,75E+03	8,07E+02
S'estanyol	B2.2	1,05E+04	9,69E+02
S'estanyol	B2.2	1,04E+04	8,07E+02
Colònia de Sant Jordi	B1	2,38E+04	1,21E+03
Colònia de Sant Jordi	B1	1,98E+04	1,05E+03
Colònia de Sant Jordi	B1	2,02E+04	1,45E+03
Colònia de Sant Jordi	B2.1	1,27E+04	1,37E+03
Colònia de Sant Jordi	B2.1	1,40E+04	6,46E+02
Colònia de Sant Jordi	B2.1	1,57E+04	5,65E+02
Colònia de Sant Jordi	B2.2	2,20E+04	1,45E+03
Colònia de Sant Jordi	B2.2	2,16E+04	1,29E+03
Colònia de Sant Jordi	B2.2	2,37E+04	1,05E+03

## 4. Acknowledgements

M. Garmendia was funded by a PhD grant of the Department of Education, Universities and Investigation of the Basque Government. We wish to thank Javier Franco, Germán Rodríguez and Ángel Borja, from AZTI-Tecnalia, and Peter Henriksen from Aarhus University, for kindly advising us on some details of the manuscript.

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