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ORIGINAL RESEARCH ARTICLE

Inhomogeneity detection in phytoplankton time series using multivariate analyses

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Summary Phytoplankton communities have long been used as water quality indicators within environmental policies. This has fostered the development of national and international phytoplankton monitoring programs, but these networks are subject to sources of uncertainty due to laboratory issues. Nevertheless, studies regarding the interference associated with these aspects are not well-documented. Hence, a long time series (2003—2015) from the Basque continental shelf (southeastern Bay of Biscay) was analyzed to evaluate the uncertainty given by laboratory strategies when studying phytoplankton variability. Variability in phytoplankton communities was explained not only by environmental conditions but also by changes in fixatives (glutaraldehyde and acidic Lugol's solution) and laboratory staff. Based on Bray-Curtis distances, phytoplankton assemblages were found to be significantly dissimilar according to the effect of changes in the specialist handling the sample and the employed fixative. The pair-wise permutational multivariate analysis of variance (PERMANOVA) showed significant differences between the two fixatives utilized and also between the three taxonomists involved. Thus, laboratory-related effects should be considered in the study of phytoplankton time series.

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1. Introduction

Phytoplankton has long been studied as a key environmental quality indicator within several international policies including European directives, such as the Water Framework Directive (WFD, 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, 2008/56/EC) (Borja et al., 2008; Garmendia et al., 2013). These policies require large monitoring networks in order to assess water quality and involve indicators that reflect different phytoplankton attributes, such as composition (Devlin et al., 2009; Domingues et al., 2008). Additionally, bivalve mollusk culture areas worldwide require phytoplankton monitoring programs in order to manage potential toxicity (Bricelj and Shumway, 1998).

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

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

In this context, the aim of the present study is to investigate the detection of inhomogeneities in phytoplankton time series and assess how these differences can be caused by factors other than the environment. This work does not attempt to be a methodology or inter-laboratory comparison, but it shows the importance of a previous data analysis when studying long-term trends or patterns in phytoplankton composition and abundance. Phytoplankton time-series can contain relevant ecological information (e.g., to address the effect of climate change) (Martinez et al., 2009), but can also be subject to methodological interferences (Kahler et al., 2012; Menden-Deuer et al., 2001). Hence, a complete overview of the potential interference in phytoplankton inter-annual variability given by taxonomist experience and fixative type is addressed. We use a long time series (>10 years), which involves both coastal and offshore areas and takes into account the whole nano- and microplankton community.

2. Material and methods

2.1. Study area, sampling and laboratory strategies

This study draws on data from the Littoral Water Quality Monitoring and Control Network of the Basque Water Agency, which has been used for the implementation of the Water Framework Directive in the Northeast Atlantic ecoregion (Borja et al., 2004, 2016; Revilla et al., 2009). The dataset consists of 16 stations along the Basque coast and three offshore stations in the southeastern Bay of Biscay (Figure 1). The climate in the study area is temperate and oceanic with moderate winters and warm summers. Coastal water bodies are euhaline and exposed. A detailed description of hydrographical conditions is given in Valencia et al. (2004).

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

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

For phytoplankton, surface water was preserved immediately and maintained in 125 mL borosilicate bottles under dark and cool conditions (4°C) until analysis. Glutaraldehyde (0.1% v/v) was used for preservation until 2011 and acidic Lugolś solution (0.4% v/v) from then on. Taxonomic identification and cell counting were performed on subsamples of 50 mL (occasionally, particle density was too high and 10 mL samples were used instead), following the Utermöhl method (Edler and Elbrächter, 2010; Hasle, 1978; Utermöhl, 1958) under a Nikon diaphot TMD inverted microscope. Depending on the organism size, 100x or 400x

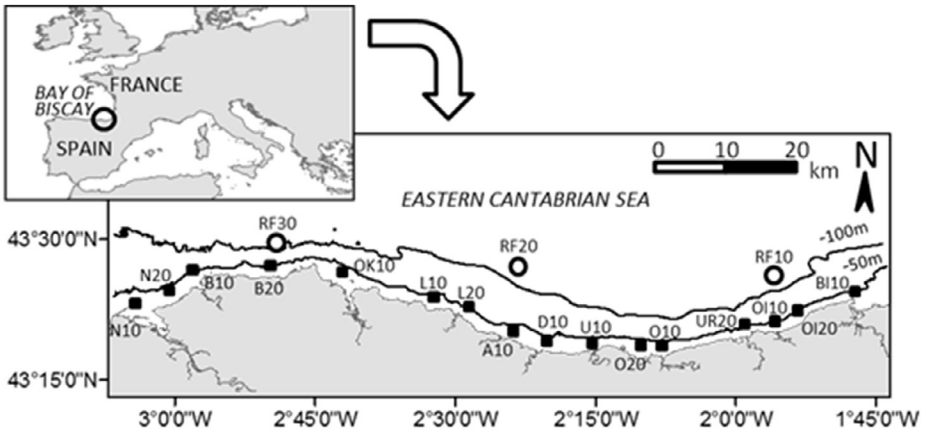

Figure 1 Map of the study area and sampling stations. Squares correspond to nearshore sampling sites and circles to offshore sampling sites.

Table 1 Fixatives and taxonomists associated with the analyses in the time series. In both the spring and the summer data sets, the number of samples is shown, together with the arithmetic mean ± standard deviation for the total number of taxa, as well as for the number of taxa identified at species or genus levels and at higher level. The rare taxa are excluded (i.e., those occurring in less than 1% of the samples). In addition, the samples from stations RF20 and RF30 are not included, as these stations were monitored only from 2009 onwards.

Fixative	Taxonomist	Year	Season	Samples	Total taxa	Species or genus	Higher ranks
Glutaraldehyde	#1	2003, 2008, 2009	Spring	51	24.4 ± 7.1	20.2 ± 7.5	4.2 ± 1.1
			Summer	51	25.2 ± 8.3	20.9 ± 7.9	4.3 ± 1.0
	#2	2005, 2006, 2007,	Spring	83	21.4 ± 5.8	16.0 ± 5.6	5.5 ± 1.2
		2010, 2011	Summer	82	21.1 ± 5.0	15.4 ± 5.0	5.8 ± 1.0
	#3	2004	Spring	16	12.2 ± 3.4	11.9 ± 3.5	0.3 ± 0.5
			Summer	16	7.0 ± 5.2	6.8 ± 5.0	0.3 ± 0.4
Acidic Lugol	#1	2012, 2013, 2014,	Spring	68	36.3 ± 7.6	31.8 ± 7.2	4.6 ± 1.3
		2015	Summer	68	36.4 ± 11.5	31.6 ± 11.3	4.8 ± 0.9

magnification was used; the detection limit of microscope counts for microplanktonic organisms was 20 cells L^{-1}. Small nanophytoplankton cells that could not be assigned to any taxonomic group were clumped together into a group named "unidentified forms <10 μm". Three different taxonomists belonging to the same laboratory took part in the identification and counting of phytoplankton. Taxonomist #1 handled samples corresponding to years 2003, 2008, 2009 and from 2012 to 2015. Taxonomist #2 handled samples from 2005, 2006, 2007, 2010 and 2011, and Taxonomist #3 identified and counted samples from 2004. No changes in the staff took place within the year of analysis. The experience of the taxonomists increased from the beginning of the time series, reaching more specific taxonomic levels. In most of the identifications, and particularly in those made by Taxonomist #3, the levels of species or genus were reached (Table 1).

2.2. Data analysis

2.2.1. Environmental variables

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

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

2.2.2. Phytoplankton community

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

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

Finally, partial Canonical Correspondence Analyses (pCCA) (Borcard et al., 1992; Legendre and Legendre, 1998) were applied to test if the variability in the abundance of the phytoplankton taxa could be associated with changes in the environmental conditions and/or in the laboratory staff. Two pCCA were carried out for the period 2003—2011: one with spring data and another one with summer data. The pCCA were carried out with CANOCO for Windows 4.5 (Braak and Smilauer, 2002). The log-transformed phytoplankton abundance at each sampling site was used as the dependent data set. The independent data consisted of two sets of explanatory variables: (i) the environmental variables (temperature, salinity, Secchi depth, suspended solids, ammonium, nitrate, phosphate, and silicate), and (ii) the factor 'taxonomist', using dummy coding (Legendre and Legendre, 1998). Previously, the environmental variables were Box-Cox transformed and normalized. The pCCA parted the explained variance of the phytoplankton abundance into the following components: (i) the variance uniquely described by the environment (but not by the taxonomist effect), (ii) the variance uniquely described by the taxonomist effect (but not by the environment), (iii) the variance jointly described by the environment and the taxonomist effect, and (iv) the unexplained variance. The significance of the pCCA models was tested with the Monte-Carlo test.

3. Results

3.1. Environmental variables

All of the investigated environmental variables showed statistically significant differences in mean values among some years, both in spring and summer (ANOVA test, alpha = 0.05). Results for the individual environmental variables are summarized in Figure 2, which shows the means and standard deviations, and Table 1 of the Supplementary Electronic Material, which includes results of the multiple range tests.

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

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

MDS biplots represent the samples as points in low-dimensional space such that the larger the distance between two points in the plot, the more dissimilar they are with regard to the environmental variables and vice versa. Hence, when analyzing the variability of all environmental variables together, some years appeared substantially different from the others in the MDS (e.g., spring 2003 and summer 2003, 2005, 2013 and 2014) (Figure 3). The

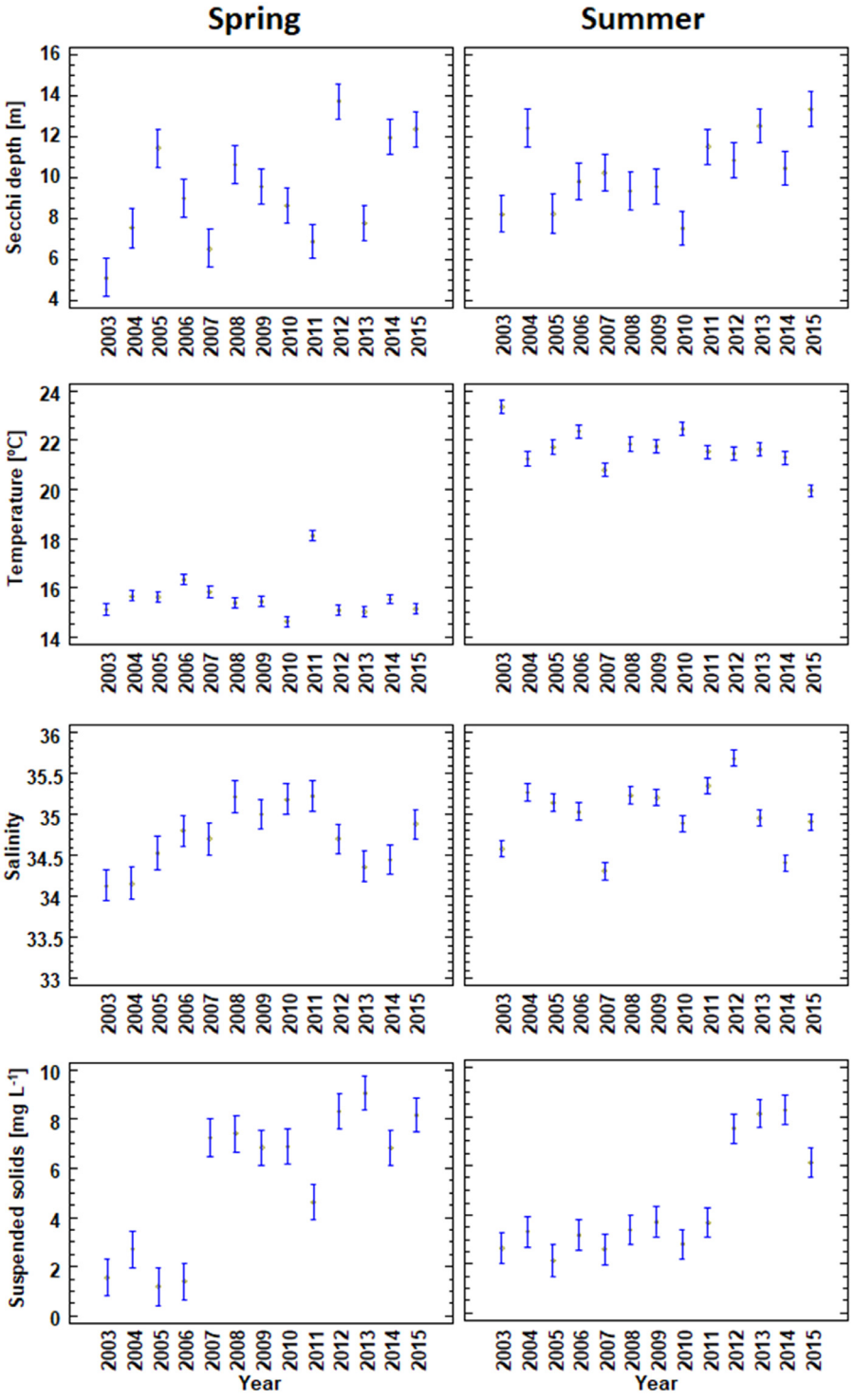

Figure 2 Mean plots for the environmental variables in each year during the period 2003—2015, with spring and summer shown in the left and right columns, respectively. Vertical error bars represent the standard deviation.

pair-wise PERMANOVA revealed significant differences between all years, both in spring and summer (Table 2 of the Supplementary Electronic Material).

In the MDS analysis of environmental variables using average values per season and year, the chronological trajectory showed great dissimilarities between some consecutive years, such as spring 2006—2007 or summer 2003—2004, 2012—2013 and 2014—2015 (Figure 4). In contrast, some years appeared close to each other indicating similar mean environmental conditions. However, cluster analyses (SIMPROF test, alpha = 0.05) for average values of environmental data did not find any significant group, either in spring or summer (Figure 1 of the Supplementary Electronic Material).

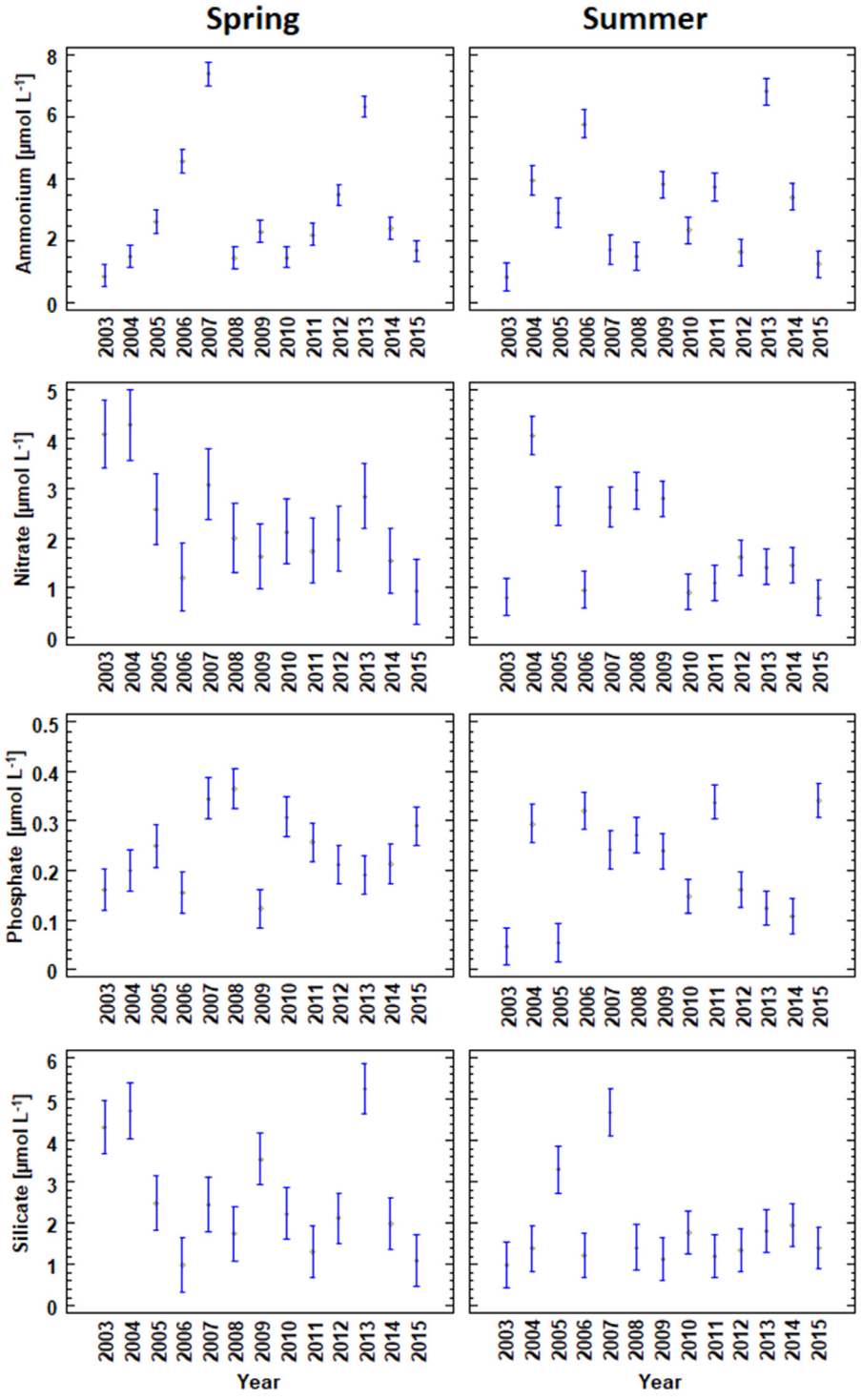

Figure 2 *Continued.*

3.2. Phytoplankton assemblages

As shown in Table 1, the samples fixed with glutaraldehyde differed little in the number of taxa if analyzed by Taxonomist #1 or by Taxonomist #2. The average richness of these samples (21—25 different taxa, approximately) was very similar between spring and summer. However, the glutaraldehyde-fixed samples analyzed by Taxonomist #3 resulted in a much smaller number of taxa (7—12), especially in summer. The highest number of taxa corresponded to the samples fixed with Lugol and analyzed by Taxonomist #1 after increased experience (36, in average, in spring as well as in summer).

As for the cell density, in general, it was higher in spring compared to summer (Figure 2 of the Supplementary Electronic Material). However, some exceptions can be noticed

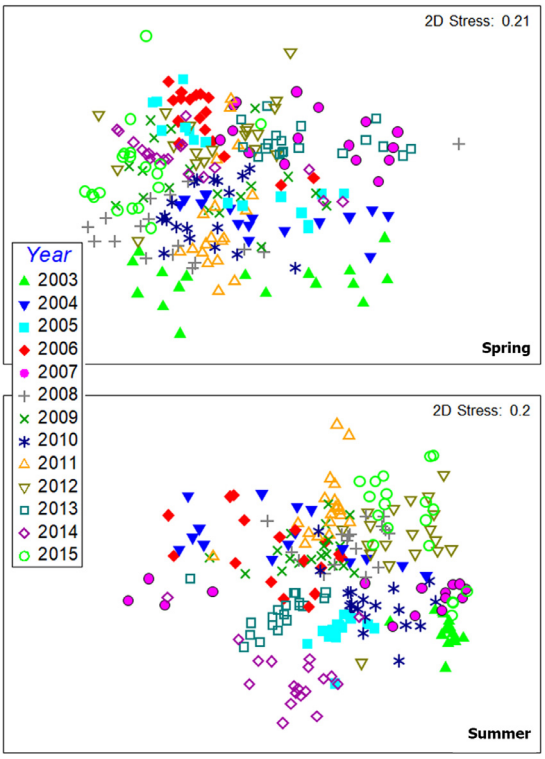

Figure 3 Multidimensional scaling (MDS) of the transformed environmental data in spring and summer using Euclidean distances for the period 2003–2015.

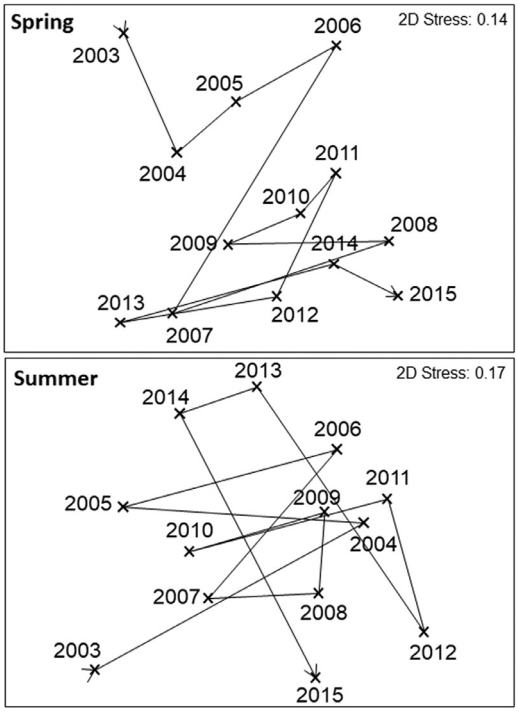

Figure 4 Multidimensional scaling (MDS) of the transformed environmental data (mean values of 17 sampling sites) using Euclidean distances. Cluster analyses did not find any significant group of years (SIMPROF test, alpha = 0.05).

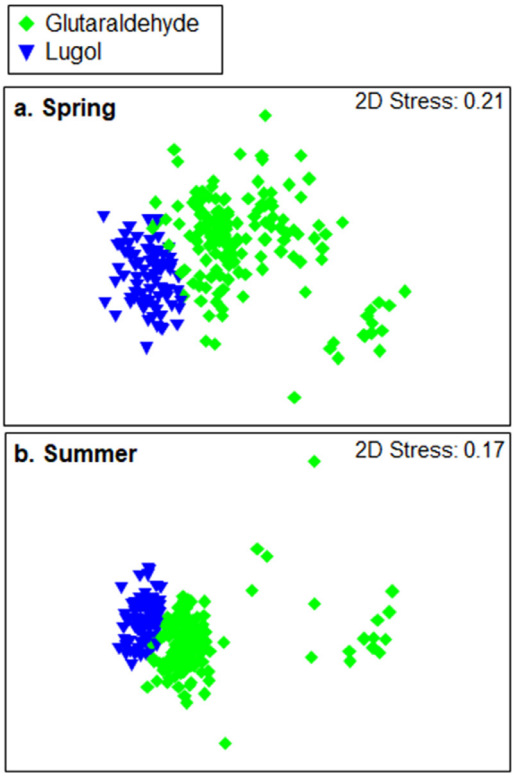

Figure 5 Multidimensional scaling (MDS) for phytoplankton abundance (log (x + 1) transformed data using zero-adjusted Bray-Curtis distances) for the period 2003–2015. Data are shown separately for spring (a) and summer (b). Different symbols represent the different fixatives employed.

for dinoflagellates and for other non-diatom taxa. Although the dinoflagellates usually contributed very little to the total cell abundance, their almost zero presence in several samples analyzed by Taxonomist #3 is remarkable (Figure 2b of the Supplementary Electronic Material). The other non-diatom cells, all together, were the most abundant in the data sets associated with Taxonomists #1 and #2, but not in many of the summer samples analyzed by Taxonomist #3 (Figure 2c of the Supplementary Electronic Material). In the groups of samples analyzed by Taxonomist #1, the cell abundance (estimated as geometric mean) was very similar between the two types of fixatives (Figure 2 of the Supplementary Electronic Material).

When the complete dataset (19 sites) was analyzed, the MDS showed two separate groups with regard to interannual variability of community composition: one referring to the year 2004 and the other referring to the remaining years (Figure 3 of the Supplementary Electronic Material). Separate MDS were conducted for spring and summer considering, firstly, the influence of the fixative (Figure 5). In the MDS biplots, a separation based on the type of fixative used can be observed in both seasons. Moreover, the PERMANOVA analysis indicated that phytoplankton variability was explained by the utilized fixative ($p = 0.0001$).

The influence of the taxonomist was then studied in the subset where one unique fixative was employed (i.e., glutaraldehyde during the period 2003–2011). The MDS biplots showed two main groups: one associated with Taxonomist #1

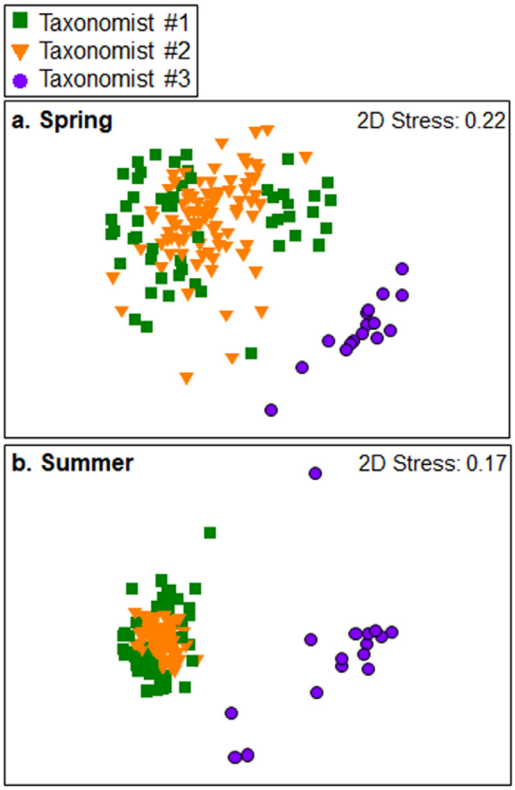

Figure 6 Multidimensional scaling (MDS) for phytoplankton abundance (log ($x + 1$) transformed data using zero-adjusted Bray-Curtis distances) for the period 2003–2011. Data are shown separately for spring (a) and summer (b). Different symbols represent different taxonomists handling the samples.

and Taxonomist #2 and the other associated with Taxonomist #3 (Figure 6). The pair-wise PERMANOVA for this subset revealed significant differences between the three different taxonomists handling the samples (Table 3 of the Supplementary Electronic Material). Similar results were obtained for spring and summer.

Inter-annual variability was also studied based on average values per season and year. Here, the MDS and cluster analyses for phytoplankton assemblages showed several significant groups according to changes both in the utilized fixative and taxonomist handling the samples (Figure 7).

At the lowest taxonomic level, 2004 (associated with Taxonomist #3) was the most different (Figure 7a, 7b). In spring, significant groups formed between years associated to the same fixative, such as the period 2012–2015 (Figure 7a). In summer, years were grouped not only according to the fixative, but also to the taxonomist, as shown by the group formed by the years identified by Taxonomist #2. The similarity of the significant groups of years was approximately 60%.

At the level of major taxonomic groups, the year 2004 also showed different phytoplankton assemblages compared to other years. At this taxonomic level, spring in all years appeared significantly grouped in accordance to the utilized fixative, except for 2004 that was also associated with a change in the taxonomist (Figure 7c). In summer, except for 2008, years were grouped in agreement with the specialist doing the identification, even if the employed fixative was different (Figure 7d). The observed groups of years presented a similarity of around 90%.

Not only were differences among taxonomists observed, but also among different years with the same taxonomist. However, when looking at the years identified by Taxonomist #1 and Taxonomist #2 separately, the dissimilarities in community assemblages between years become smaller, particularly for Taxonomist #2. Cluster analyses of phytoplankton data are described in further detail in the Electronic Supplementary Material (Figure 4 of the Supplementary Electronic Material).

By using the pCCA (Table 2), the variability in the species densities explained by the sum of the environmental conditions and the taxonomist effect was 29.1% (spring) and 25.9% (summer). The percentage of variability uniquely explained by the environment was 15.8% and 12.8%, whereas that of the taxonomist effect was 10.8% and 9.6% (for spring and summer, respectively). The part of the variation that was explained jointly by the environment and the taxonomist effect was 2.5% (spring) and 3.5% (summer). Hence, the two sets of independent variables were not very redundant in explaining the spatio-temporal variability of species densities (i.e., each set of independent data was largely explaining different aspects of the observed variability in the phytoplankton).

4. Discussion

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

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

Additionally, evidence of interference arising from changes in the taxonomist performing the identification was identified. This could be explained in part by the risk of misidentification of small and cryptic species that is likely when using traditional techniques, such as that of Uthermöhl, which require a high level of expertise of the taxonomist (Mouillot et al., 2006). The clearest finding was observed for phytoplankton assemblages from 2004, which appeared notably differentiated from the others in the MDS plots. These results could not be linked to the previously mentioned effect of the fixative because the same fixative was employed in other years and such differences were not

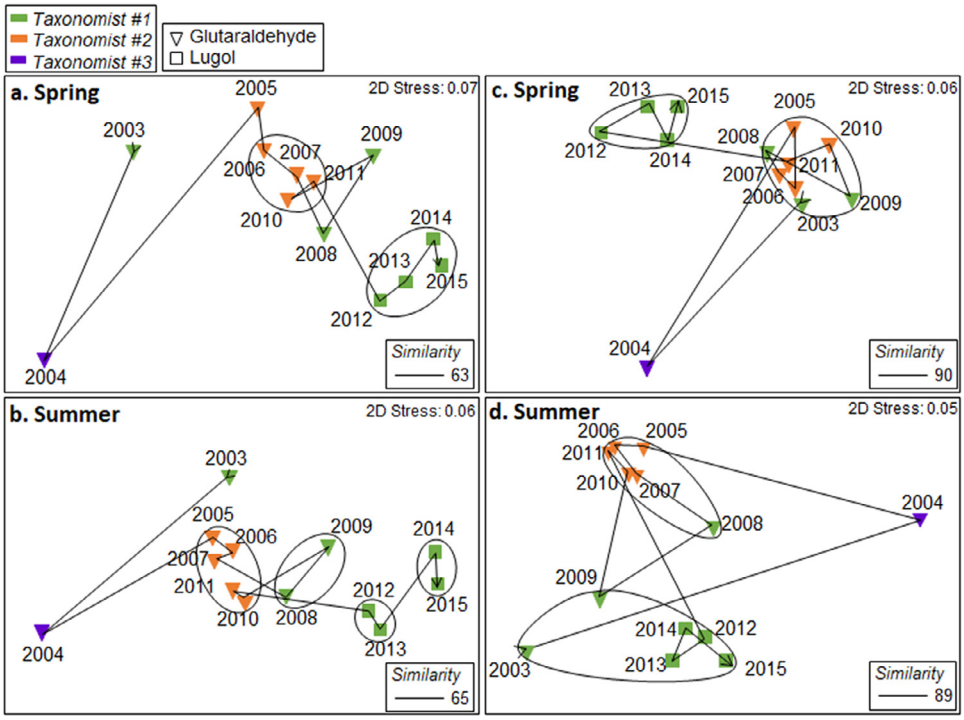

Figure 7 Multidimensional scaling (MDS) of the annual phytoplankton community assemblages (log ($x + 1$) transformed data using zero-adjusted Bray-Curtis distances). Average values per year and season (i.e., mean values of 17 sampling sites) are shown for spring (a, c) and summer (b, d). Panels a and b show data at the lowest taxonomic level available, and panels c and d at the major group level. Symbols represent different fixatives, colors show different taxonomists, and contour lines indicate significantly different groups (SIMPROF test, alpha = 0.05). See Figure 4 of the Supplementary Electronic Material for cluster analyses.

Table 2 Results of two partial Canonical Correspondence Analyses (pCCA) carried out within the period 2003–2011; one for spring data and other one for summer data.

	Spring			Summer		
	explained inertia (%)	F-Ratio	p-value	explained inertia (%)	F-Ratio	p-value
Environmental data (1)	18.3	4.12	<0.01	16.4	3.57	<0.01
Taxonomist (2)	13.3	11.7	<0.01	13.1	11.43	<0.01
Environmental data − [taxonomist] (3)	15.8	4.05	<0.01	12.8	3.12	<0.01
Taxonomist − [environmental data] (4)	10.8	11.02	<0.01	9.6	9.27	<0.01
Environmental + taxonomist (shared variance)	2.5			3.5		
Total (5)	29.1	5.95	<0.01	25.9	5.03	<0.01

(1) CCA carried out with environmental data as independent data, (2) CCA carried out with taxonomist data as independent data, (3) CCA carried out with environmental data as independent data and taxonomist data as covariable; (4) CCA carried out with taxonomist data as independent data and environmental data as covariable, (5) CCA carried out with taxonomist data and environmental data as independent data. Note: the results of (1) and (2) are not part of the pCCA.

observed. In addition, the environmental variables in 2004 did not present extreme values that could explain such differentiation in the phytoplankton assemblages. Thus, the observed phytoplankton assemblages for 2004 were suspected to be artifacts of the change in the taxonomist (e.g., Dromph et al., 2013; Peperzak, 2010).

However, it must be noted that not only extreme values can shape the composition of phytoplankton communities (e.g., Remy et al., 2017). As shown in previous studies (Bode et al., 2015; Devlin et al., 2019; Hernández et al., 2015), phytoplankton variability is also influenced by gradual changes of several variables, such as water temperature, turbidity, salinity or nutrient concentration, at the long-term. In the present study, environmental conditions in surface waters were studied to check if they could explain the inter-annual variability of phytoplankton community. In general, dissimilarities found in the environmental conditions did not explain the main dissimilarities observed in the phytoplankton communities. As an example, apart from the above explanation regarding 2004, 2003 was found to be one of the most different years in terms of environmental variables, both in spring and summer. Spring 2003 was characterized by minimum values in water transparency (Secchi depth) and salinity, and relatively high values in

nitrate and silicate. Summer 2003 presented the maximum water temperature and the minimum in all nutrients (ammonium, nitrate, nitrite, phosphate and silicate). However, these findings were not consistently accompanied by great dissimilarity in phytoplankton assemblages between 2003 and other years. In addition, along the chronological trajectory, the largest dissimilarities in environmental conditions between years, with respect to average values per season and year, did not reflect such changes in community assemblages for the same years. In fact, one of the largest dissimilarities in phytoplankton communities between consecutive years, apart from 2004, was associated with changes in the both the fixative and the taxonomist (i.e., 2011 to 2012). Therefore, these laboratory-induced artifacts are confirmed as significant factors in introducing uncertainty to the study of phytoplankton communities.

It could be possible that the error in the taxonomic determinations and counts derived from the taxonomist change caused (at least partially) the lack of correlation of the environmental variables with the structure of the communities observed in the MDS results. Muñiz et al. (2018) found that, for this same studied area, the spatio-temporal variability in the phytoplankton densities was significantly explained by environmental data in the period 2012—2015, in which the taxonomist handling the samples and the fixative used were the same the same. Considering a longer period (2003—2011) with the same fixative, but including three different taxonomists, the pCCA results show that the variability in the phytoplankton densities was also significantly explained by environmental data, once removed the taxonomist effect (Table 2). Nevertheless, it is important to remark that the percentage of the variability explained by the environmental variables in the 2003—2011 period increases considerably when the taxonomist effect is taken into account. This implies that, at least in our case study, it is relevant to take into account appropriate measures when phytoplankton time series involving different taxonomists are studied.

Although data obtained by different taxonomists in the same samples were not compared in this study, Taxonomist #1 and Taxonomist #2 took part in a previous study that assessed the variability in total cell counts within a similar set of samples analyzed by different taxonomists (Dromph et al., 2013). That study involved several localities, including the Basque coast, and concluded that in all cases, important differences were observed due to the taxonomists' effect. When data from different monitoring programs are integrated, inter-laboratory biases are added to intra-laboratory ones. Intercomparison exercises among laboratories (for example, the International Phytoplankton Intercomparison, https://www.iphyi.org) arise as a good strategy to reduce uncertainty related to taxonomists and other analytical protocols.

It is also interesting to assess this effect not only at the lowest taxonomic level available, but also at other taxonomic levels. At the level of major taxonomic groups, the bias due to the experience of the taxonomist was found to be much lower compared with that of species level, as shown by the similarity percentages of significant groups (Figure 3c, 3d). Consequently, for studies or monitoring networks in which a high taxonomic detail is not required, it would be desirable to work at a higher taxonomic level in order to minimize identification errors. However, interpretation of this finding should be taken with care as Straile et al. (2015) found that, at least in lakes, taxonomic aggregation does not always imply more robust results.

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

5. Conclusions

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.oceano.2020.01.004.

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