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Research Article

RESEARCH ARTICLE

Seasonal variations of marine protist community structure based on taxon-specific traits using the eastern English Channel as a model coastal system

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One sentence summary: The seasonal structure of the protistan community was examined from March 2011 to July 2013 in a temperate meso-eutrophic coastal system, using tag pyrosequencing of the 18S rRNA gene.

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ABSTRACT

Previous microscopy-based studies in the eastern English Channel have revealed it to be a productive meso-eutrophic coastal ecosystem, characterized by strong repeating patterns in microplankton succession. The present study examines the seasonal structure of the entire protistan community from March 2011 to July 2013, using tag pyrosequencing of the V2–V3 hypervariable region of the 18S rRNA gene. A total of 1242 OTUs and 28 high-level taxonomic groups, which included previously undetected taxa in the area, were identified. The detected OTUs were considered according to taxon-specific traits, which included their trophic role, abundance and specialization level. Taxa differentiation based on specialization level rather than abundance was more informative in describing community organization. While generalists were always abundant, numerous specialists that were either rare or absent in most samples, increased in abundance for short periods, appearing to be overall abundant. Statistical and network analyses showed that the protistan seasonal organization was influenced by environmental parameters. It also highlighted that in addition to grazers, fungi and parasites played potentially significant roles during phytoplankton blooms. Overall, while the protistan succession was mainly shaped by environmental variations, biotic interactions among co-occurring taxa were the main structural drivers of the temporal assemblages.

Keywords: unicellular eukaryotes; tag pyrosequencing; 18S rRNA gene; succession; generalists; specialists

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INTRODUCTION

In recent years, using next-generation molecular tools, investigations into the community structure of marine protists have revealed novel diversity, which play significant role in maintaining the functional stability of ecosystems (e.g. Behnke *et al.* 2010; Countway *et al.* 2010; Medinger *et al.* 2010). However, only a few studies have examined spatial (e.g. Stoeck *et al.* 2009; Edgcomb *et al.* 2011; Logares *et al.* 2014) and temporal community structure relative to the effects of environmental parameters (e.g. Countway *et al.* 2010; Steele *et al.* 2011). Seasonal investigations of the environmental effects on marine bacterial communities' structure are numerous and have shown that environmental parameters explain most of the seasonal variability of bacteria, suggesting that temporal changes in abiotic parameters are more important than taxa interrelationships (e.g. Fuhrman *et al.* 2006; Gilbert *et al.* 2012; Salter *et al.* 2015). Considering that abundant and rare marine bacterial subcommunities might have fundamental different characteristics and ecological roles (Pedrós-Alió 2006), few studies have investigated the response of these subcommunities to environmental variation (e.g. Caron and Countway 2009; Pedrós-Alió 2012). The level of taxa specialization in such studies has not yet been considered, even though it has been suggested that it can adequately describe community structure along environmental gradients (Székely and Langenheder 2014).

However, taxonomic identification alone is insufficient to assess the environmental functions and ecology of the community. Recent NGS studies of marine community composition, examining the statistical relationships among organisms and environmental parameters measured over various spatial and temporal scales, have shown not only the environmental conditions that influence individual microorganisms, but also the likely interactions among them (Fuhrman and Steele 2008; Logares *et al.* 2014; Shade *et al.* 2014; Székely and Langenheder 2014). In fact, molecular data of marine microbial communities provided by NGS combined with well-established working tools in macroecology (e.g. Prosser *et al.* 2007; Konopka 2009; Azovsky and Mazei 2013; Logares *et al.* 2014) enables us to investigate the 'natural history' of the various microbes in the same way we do with animals and plants, and to investigate the factors affecting their community structure (Steele *et al.* 2011; Faust and Raes 2012).

The eastern English Channel (EEC) is a productive mesoeutrophic marine ecosystem, characterized by strong repeating patterns in microplankton succession. Based on microscopical observations of microplanktonic communities, it has been established that every spring a *Phaeocystis globosa* bloom is preceded and followed by communities of colonial diatoms and dinoflagellate grazers (Schapira *et al.* 2008; Grattepanche *et al.* 2011a,b and references therein). The objective of the present paper was to complement the picture of the seasonal protistan community structure relative to environmental variation and taxa interactions through a whole community SSU rRNA gene sequencing study. Expectations were to describe the succession of the entire eukaryotic community by including ecologically important groups, such as parasites, degraders and nanoheterotrophs, otherwise unverifiable by conventional microscopy. Specifically, the three main questions were as follows: (i) How are protistan community assemblages affected by major biotic and abiotic variations, such as bloom events and nutrient fluctuations? (ii) Is protistan communities' structure influenced mostly by biological interactions or by variations in the physical and chemical environment? (iii) What is the importance of abundant/rare and generalist/specialist taxa in protistan communities' structure? In order to answer these questions, 32 samples (collected from March 2011 to July 2013, and covering three periods of *P. globosa* growth and senescence) were analyzed by tag pyrosequencing of the 18S rRNA gene. The detected taxa were sorted according to taxon-specific traits, which included their trophic status (autotrophs, microplankton and nanoplankton grazers, nanoheterotrophs and parasites), abundance (abundant/rare) and level of specialization (generalists/specialists). Based on these traits, their seasonal structure, relative to abiotic parameters and potential interactions between organisms, was analyzed with statistical and network analyses.

MATERIALS AND METHODS

Sample collection

The sampling site was located at the SOMLIT (French Network of Coastal Observatories) station (50° 40′ 75″ N, 1° 31′ 17″ E; 20–25 maximum depth) in the EEC. This site was chosen as the physical and hydrological properties encountered here are representative of the coastal water masses in the EEC. Subsurface samples (2–3 m water depth) were collected in 2.5 L sterile polyethylene bottles, at times of high tide, from 07 March 2011 to 09 July 2013 on a biweekly basis when local weather conditions allowed. After collection, each of the total 32 samples (12, 11 and 9 for each year, respectively) was kept in the dark at the *in situ* temperature, and filtered within 2 h. Before filtering, the samples were screened with a 150 μm mesh to retain larger particles and most metazoa. Next, sequential filtration through 10, 3 and 0.6 μm nucleopore filters (47 mm diameter) was performed using a very low filtration pressure peristaltic pump (15 rpm) in order to avoid filter clumping and minimize organism disruption (see also Monchy *et al.* 2012). The filters were immediately stored at –80°C until further molecular analysis.

Physical–chemical parameters and chlorophyll a

Seawater temperature (°C) and salinity were measured *in situ* using a conductivity-temperature-depth profiling system (CTD Seabird SBE 25). The level of oxygen was immediately analyzed in triplicate by Winkler microtitration (Aminot and Chaussepied 1983). Inorganic nutrient concentrations were determined from 100 mL samples with an Alliance Integral Futura Autoanalyzer II for nitrate (NO_3), nitrite (NO_2), phosphate (PO_4) and silicate (SiO_4) based on Strickland and Parsons (1972) and Aminot and Kerouel (2004). Chlorophyll *a* (Chl *a*) concentrations were measured on 90% (v/v) acetone-extracted particulate material isolated by filtration on GF/F glass fiber filters (Whatman). Concentrations were determined by fluorescence using a 10-AU Turner Designs fluorometer (Lorenzen 1966).

DNA extraction

The DNA of planktonic organisms was extracted and purified, after pooling collectively the 10, 3 and 0.6 μm filters, with the PowerWater DNA isolation kit (MoBio Laboratories Inc., CA, USA), following the manufacturer's protocol. The samples contained between 0.5 and 4.5 ng μL^{-1} of DNA as measured by the Qubit 2.0 Fluorometer (Thermo Fischer Scientific Inc., Massachusetts, USA).

PCR and tag pyrosequencing

The DNA samples were amplified using the two eukaryotic primers 18S-82F 18S-82F (5′-GAAACTGCGAATGGCTC-3′) (Lopez-Garcia *et al.* 2003) and Euk-516r (5′-ACCAGACTTGCCCTCC-3′) (Amann *et al.* 1990). These primers have been designed to amplify a domain around 470–480 bp corresponding to the hypervariable V2 and V3 eukaryote 18S rRNA gene regions and have been used in previous studies of the protist community in this site (Monchy *et al.* 2012; Christaki *et al.* 2014).

A 10 bp tag sequence specific to each sample, a 4 bp TCAG key and a 26 bp adapter for the GS FLX technology were added to the primers. Polymerase chain reaction (PCR) was carried out according to standard conditions for Platinum Taq High-Fidelity DNA Polymerase (Invitrogen, Carlsbad, CA, USA), with 5 ng of environmental DNA as template, using the GeneAmp PCR System Apparatus (Applied Biosystems, Foster City, CA, USA). After the denaturation step at 95°C for 5 min, 30 cycles of amplification were performed at 95°C for 30 s, 50°C for 30 s and 72°C for 1 min. A final extension step of 7 min at 72°C was included. Tag pyrosequencing was carried out by the GenoScreen company (Lille, France). The library was prepared following the procedures described by Roche (Basel, Switzerland) and used in one plate run on a 454 GS FLX Titanium Sequencer. Pyrosequences were submitted to GenBank-SRA under the accession number SRX768577.

Tag pyrosequencing quality filtering

All samples produced between 12 581 and 29 712 reads. Downstream read processing was performed using the mothur 1.28.0 software following the standard operating procedure (Schloss *et al.* 2009; Schloss, Gevers and Westcott 2011). First, the flowgrams from each sample were extracted and separated according to their tag. The resulting 32 flowgrams were denoised using the mothur 1.28.0 implementation of PyroNoise (Quince *et al.* 2009). Primer sequences, tag and key fragments were subsequently removed, and only reads above 200 bp long with homopolymers shorter than 8 bp were included in the analysis. The data set was dereplicated to the unique reads and aligned against the SILVA 108 database containing 62 587 eukaryotic SSU rRNA sequences. The reads suspected of being most likely chimeras were removed using the UCHIME software (Edgar 2010). Finally, the data set was normalized to the sample with the lowest number of reads using the subsample command in mothur, so that all samples contained 12 581 reads. These reads were clustered into operational taxonomic units (OTUs) at 97% similarity threshold, using the average neighbor method. Single singletons (i.e. unique amplicons that occurred only once in the whole data set) were removed as these are most likely erroneous sequencing products (Reeder and Knight 2009; Behnke *et al.* 2010; Kunin *et al.* 2010).

Data analysis

After tag pyrosequencing filtering and normalization, 1303 OTUs were produced. Taxonomic classification was assigned using BLASTN (Altschul *et al.* 1990), based on the PR2 curated database, containing 23 003 sequences. The PR2 database focuses on nuclear-encoded protists sequences (Guillou *et al.* 2013). All reads affiliated to metazoa were removed from the data set, thus the remaining 1242 OTUs belonged to protists. Alpha diversity estimators (the richness estimator S_{Chao1}; the heterogeneity of the diversity, and the Shannon, Simpson and Berger–Parker indexes) in all samples were calculated with the PAST 2.17c software (Hammer, Harper and Ryan 2001).

The protistan assemblages of the different sampling dates were compared using the Plymouth routines in the multivariate ecological research software package (PRIMER v.6; Clarke and Gorley 2006). The Bray–Curtis dissimilarity coefficients were calculated to build the matrix based on OTUs abundance in order to identify interrelationships between the samples. The similarity profile (SIMPROF) permutation test was conducted to determine the significance of the dendrogram branches resulting from cluster analysis. For the determination of the OTUs responsible for the within group similarities and between group dissimilarities, the similarity percentage analysis (SIMPER) was used (Clarke and Warwick 1994).

For the network analysis, it was decided to focus on the OTUs indicated by SIMPER analysis mainly because they were thought to be the most important for the formation of the groups, as these were considered the main 'regulators' of protist community structure, but also, in order to reduce the number of potential connections and improve visibility. These OTUs were sorted into major trophic groups, such as microplankton grazers, autotrophs, nanoheterotrophs, nanoplankton grazers and parasites. This grouping was based on their major trophic role in marine ecosystems as inferred by the literature. The relationship between these OTUs was characterized through MINE statistics by computing the maximal information coefficient (MIC) between each pair of OTUs (Reshef *et al.* 2011). MIC captures associations between data and provides a score that represents the strength of a relationship between data pairs. The matrix of MIC values corresponding to a *P*-value <0.05, based on pre-computed *p*-values of various MIC scores at different sample sizes, was used to visualize the networks of associations with Cytoscape 3.0 (Smoot *et al.* 2011).

Finally, the relationship among OTUs abundance in each sample and environmental variables (see Table 1) was explored with canonical correspondence analysis (CCA; CANOCO; Ter Braak and Smilauer 2002). The significance of the axes obtained by the CCA analysis was determined based on the Monte Carlo permutation test (Ter Braak and Smilauer 2002).

Definitions of abundant and rare OTUs, and generalists and specialists

OTUs were classified as abundant or rare in relation to their total and per sample relative abundance. Per sample abundant OTUs were defined as those with relative abundances >1%, and per sample rare OTUs, as those with abundances <0.2%, following studies on prokaryotes (Galand *et al.* 2009; Pedrós-Alió 2012; Hugoni *et al.* 2013) and protists (Mangot *et al.* 2013; Logares *et al.* 2014). The respective thresholds for defining total abundant and rare OTUs were set as the per sample threshold divided by a factor of 10 (i.e. >0.1% for abundant and <0.02% for rare; Logares *et al.* 2014).

Furthermore, OTUs were classified as generalists and specialists based on Levins index (1968). Levins proposed that niche breath could be estimated by measuring individuals' uniformity of distribution among the resource states (Levins 1968). For this, specialization of each individual OTU was calculated according to Pandit, Kolasa and Cottenie (2009), using Levins' niche width (*B*) index (Levins 1968):

$$B = \frac{1}{\sum_{i=1}^{N} p_{ij}^2},$$

Table 1. Number of OTUs, the richness estimator (S_{Chao1}) and the heterogeneity of the diversity indexes (dominance, Simpson, Shannon, Equitability and Berger–Parker) from March 2011 to July 2013 at the SOMLIT station in the EEC. Gray shadowing indicates samples during the peak of the *P. globosa* bloom.

	Nb OTUs	S_{Chao1}	Simpson (*D*)	Equitability (H/H_{max})	Berger–Parker
07/03/2011	194	201	0.06	0.69	0.16
21/03/2011	139	146	0.13	0.60	0.31
04/04/2011	85	90	0.53	0.32	0.72
18/04/2011	134	157	0.28	0.43	0.46
04/05/2011	186	189	0.13	0.61	0.31
06/06/2011	47	48	0.23	0.52	0.34
15/06/2011	196	203	0.13	0.58	0.3
04/07/2011	109	110	0.15	0.57	0.3
27/09/2011	257	265	0.02	0.79	0.08
25/10/2011	289	301	0.02	0.80	0.06
09/11/2011	375	394	0.02	0.79	0.11
23/11/2011	315	332	0.04	0.73	0.12
24/01/2012	213	218	0.03	0.78	0.09
20/03/2012	58	63	0.08	0.77	0.16
05/04/2012	46	46	0.13	0.68	0.24
09/05/2012	172	203	0.07	0.65	0.19
05/06/2012	85	88	0.13	0.59	0.25
21/06/2012	196	203	0.03	0.80	0.07
04/07/2012	101	119	0.13	0.71	0.34
23/07/2012	59	66	0.08	0.77	0.21
03/09/2012	300	313	0.02	0.80	0.06
03/10/2012	353	365	0.02	0.81	0.06
13/11/2012	317	326	0.03	0.77	0.1
11/02/2013	205	211	0.04	0.73	0.13
26/02/2013	80	86	0.19	0.60	0.41
26/03/2013	165	175	0.4	0.39	0.62
08/04/2013	59	60	0.37	0.46	0.59
24/04/2013	114	116	0.3	0.43	0.48
27/05/2013	183	190	0.13	0.57	0.23
10/06/2013	138	146	0.27	0.46	0.49
25/06/2013	205	231	0.36	0.43	0.59
09/07/2013	244	264	0.14	0.6	0.36

where p_{ij} is the proportion of OTU *j* in sample *i*, and *N* is the total number of samples. Therefore, *B* describes the extent of niche specialization based on the distribution of OTUs abundances without taking the environmental conditions in a local community into account. The values of the index were between 1 for singletons and 20 for the top generalist. OTUs with *B* index higher than 10 were arbitrary considered as generalists, while OTUs with *B* index lower than 5 were grouped as specialists (see Székely and Langenheder 2014).

RESULTS

Environmental parameters

Seawater temperature during the period of the study ranged from 5.4 to 18.5°C, and the salinity from 33.3 to 34.7 (Table S1, Supporting Information). The concentrations of inorganic nutrients exhibited typical seasonal patterns for the site (Fig. S1, Supporting Information). The highest values were recorded for all years during September–February, before the onset of the *P. globosa* bloom, reaching 15.1 μM for $NO_3 + NO_2$, 1.06 μM for PO_4 and 7.7 μM for $SiOH_4$ (Table S1, Supporting Information). During the *P. globosa* proliferation, the N/P ratio always dropped dramatically (as low as 0.1; Fig. S1 and Table S1, Supporting Information). Chl *a* fluctuated from 0.4 to 11.7 $\mu g\ L^{-1}$, with characteristic peaks during early March–April (Fig. S1, Supporting Information).

Protist diversity and seasonality

A total of 1242 unique OTUs were identified in all samples. The ratio of observed to expected OTUs (S_{Chao1}) was >90% in all cases. The sample with the highest OTU richness was on 09/11/2011, when 375 OTUs were detected (Table 1). In general, OTU richness exhibited seasonality (Fig. 1) with September–February samples showing the highest number of OTUs (>200 OTUs). In April–May samples, the OTUs richness dropped considerably, reaching its lowest number (46 OTUs) on 05/04/2012 (Fig. 1, Table 1). As expected, Simpson index (*D*) was highest in the samples with the lowest richness, representing low diversity (Table 1). The equitability index fluctuated from 0.32 to 0.81. Higher values, reflecting low variation between species abundances within the community, were calculated between October and February along with high OTUs richness (Table 1).

The 1242 OTUs were affiliated to 28 high-level taxonomic groups (Fig. 2). Dinophyceae was the most diverse high-level group comprising 15% of the total number of OTUs, followed by MALV (13%), Bacillariophyta (8%) and Fungi (8%) (Fig. 2a). Dinophyceae was also the most dominant high-level group in terms of number of reads, comprising 38% of the total number of reads, followed by Bacillariophyta (10%), MALV (9%) and Haptophyta (8%) (Fig. 2b). Overall, the most diverse taxonomic groups (i.e. Dinophyceae, other Alveolates, Bacillariophyceae and other Stramenopiles) exhibited temporal variations

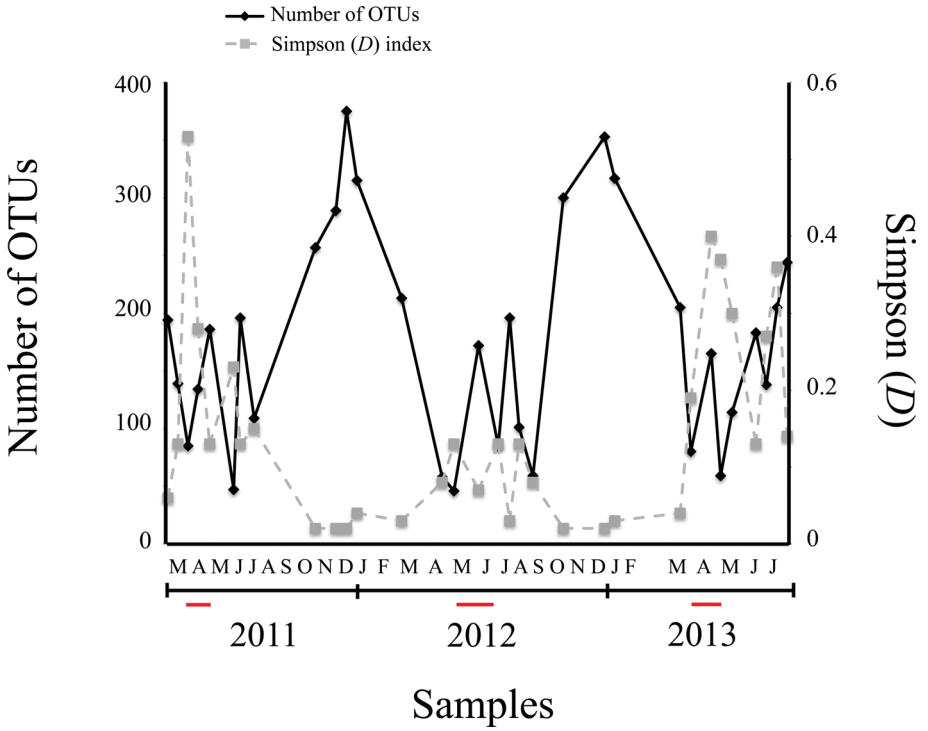

Figure 1. Temporal variation of OTUs richness and the Simpson (*D*) index during the study. The red line indicates samples during the peak of the *P. globosa* bloom.

regarding their OTUs richness throughout the study period. In particular, in most cases, the OTUs richness increased considerably between June and March (Fig. 2c). On the other hand, in less diverse taxonomic groups, such as Cercozoa, Chlorophyta and Fungi, low numbers of OTUs were constantly detected throughout the study period (Fig. 2c). Concerning the Haptophyta group, a *P. globosa*-related OTU exhibited a considerable increase in terms of read number between March and May in all years, contributing to the high relative abundance of the group (Fig. 2b).

The cluster analysis, based on all OTUs abundance, identified three major clusters at a similarity level >25% (Fig. 3a). Cluster A, consisted of July–August samples; cluster B, included September–February samples; and finally cluster C, included March–June samples, related to the *P. globosa* bloom. Cluster C was further separated into four subgroups, comprising of the pre-bloom (subcluster i), the bloom (subcluster ii), the post-bloom (subcluster iv) and early-summer samples (subcluster iii); (Fig. 3a). The SIMPROF significance test showed significant differences ($P < 0.05$) between the three clusters and also between the four subclusters. The Venn diagram including all OTUs, showed relatively low number of shared OTUs between the three main clusters (approximately 15% of the total number of OTUs), while cluster A had the lowest number of unique OTUs (71 OTUs; Fig. S2, Supporting Information).

SIMPER analysis indicated a different assemblage of OTUs responsible for the formation of each cluster; consisting of 21 OTUs for cluster A, 26 for cluster B and 100 for cluster C. The co-occurrence patterns between them were calculated according to MIC values. Network analysis was implemented on the OTUs exhibiting strong co-occurrence patterns (high MIC values, corresponding to a *P*-value >0.05) in each cluster. These OTUs were subsequently sorted out into major 'trophic groups' (Table 2). Network analyses provided a visualization of the OTUs associations in each cluster, indicating that different trophic groups showed strong associations among themselves in each seasonal cluster (Fig. 3b). In cluster A, microplankton grazers showed strong species-specific associations among themselves and with other groups—mainly autotrophs. In cluster B, autotroph-related OTUs dominated the eukaryotic community, both in terms of richness, number and strength of connections. Finally, in the bloom-related samples (cluster C), a mixture of parasites and grazers primarily interacted with each other, exhibiting strong connections (Fig. 3b).

Abundant and rare OTUs—generalists and specialists

The mean abundant: rare ratio was 20:80, except in three samples (20 March 2012, 05 April 2012 and 23 July 2012), where the number of the abundant OTUs was higher than the number of rare OTUs (mean ratio 60:40 Fig. 4a). In these three samples, corresponding to the pre-bloom and the early-summer period of 2012, the number of the abundant OTUs increased dramatically (Fig. 4a). However, the proportions of per sample generalists ($B > 10$) and specialists ($B < 5$) were always relatively constant across time (Fig. 4b). In fact, specialists always comprised of >80% of the taxa per sample. Subsequently, the total protistan community during the entire study consisted principally of specialist taxa (>1000 OTUs), and the average *B* index of the major high-level taxonomic groups was <5 (Table 3). The trophic groups recognized within the seasonal clusters (see Fig. 3b) had an intermediate average specialization index ($5 < B < 10$), except for the nanoplankton grazers (Table 3). The majority of taxa classified as specialists were observed in one to eight samples, while all generalist taxa were detected in >18 samples (Fig. S3, Supporting Information).

When the entire data set was considered, generalists were always abundant (i.e. their number of reads in all samples was >0.1% of the total number). However, specialists were not always overall rare (i.e. their number of reads was not always <0.02% of the total number). In particular, various specialists that were

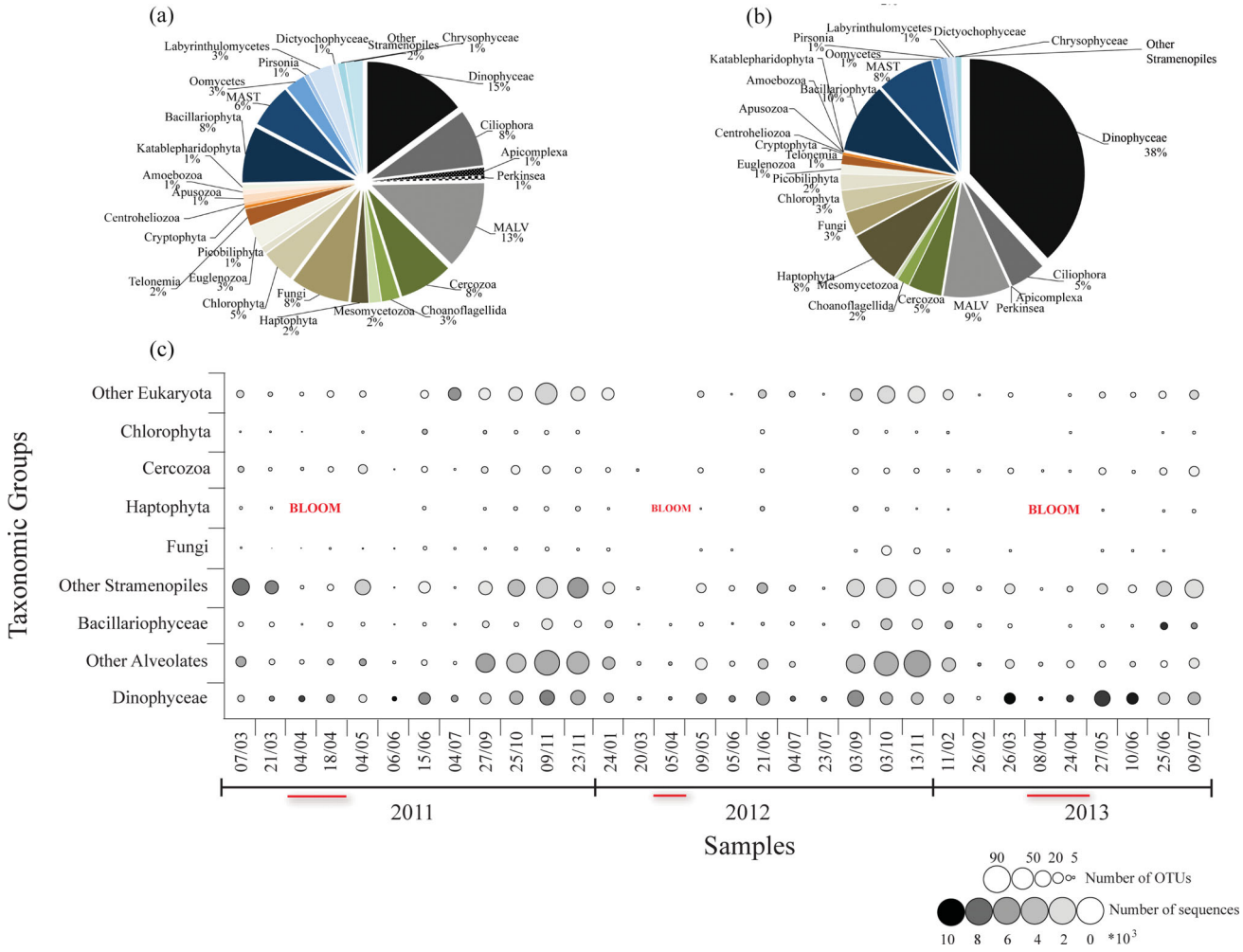

Figure 2. Relative number (**a**) and abundance (**b**) of OTUs belonging to major high-level protist taxonomic groups (the taxonomic affiliation was based on BLASTN searches against the PR2 database; to facilitate reading, the percentage of OTUs number/reads assigned to a specific group is given when ≥ 1%). Also, temporal variation of number (circle diameter) and abundance (circle opacity) of OTUs belonging to the dominant high-level taxonomic groups (**c**). The red line indicates samples during the peak of the *P. globosa* bloom.

rare or absent in the majority of samples, increased in abundance for short periods, appearing to be overall abundant. For example, among the 30 most abundant OTUs (>2000 reads in the entire data set), 11 OTUs characterized as specialists, were overall rare or absent in most of the samples. However, they showed high abundances in individual samples, and thus they were finally classified as abundant since their overall number of reads was >0 .1% of the total number (Fig. 5). An extreme example of this was the third most abundant OTU in the entire data set (>98% similarity with the centric diatom *Leptocylindrus danicus*), which was actually a specialist taxon, showing an abrupt and extreme increase in read number in May 2011, June and July 2012, and May–July 2013. Another example closely related to the underexplored nanoheterotrophic group of Marine Stramenopiles (MAST) was OTU07, which although rare or absent in the majority of the samples also showed abrupt read increase on certain dates (always between February and March, and July 2012).

Environmental variability and seasonal succession

The effects of physical and chemical parameters variability (Table S1, Supporting Information) on the grouping of samples, according to all OTUs abundance, were examined. According to the CCA ordination diagram, the samples exhibited clear seasonal succession (Fig. 6). The July–August samples (cluster A) were correlated with temperature. The September–February samples (cluster B), dominated by Alveolates and Stramenopiles (Fig. 2), were more closely related to high concentrations of silicate and nitrogen ($NO_3 + NO_2$). Bloom-related samples (cluster C) were separated from the other groups (Fig. 6), yet, estimation of the percentage of explained sample variation with the measured environmental variables was relatively low (approximately 30%).

Ordination diagrams of samples, and physical and chemical parameters, were also constructed for the abundant (Fig. 7a), rare (Fig. 7b), generalist (Fig. 7c) and specialist OTUs (Fig. 7d). Sample ordination based on abundant, rare and specialist OTUs followed a seasonal pattern similar to the one observed when the entire protistan community was considered (Fig. 6). Likewise, the seasonal clusters were generally separated from each other and associated with environmental parameters (Fig. 6). When only generalist OTUs were included in the analysis, there was no distinct separation of seasonal clusters, with most samples located in the center of the ordination diagram, seemingly not directly influenced by environmental variation (Fig. 7c). The

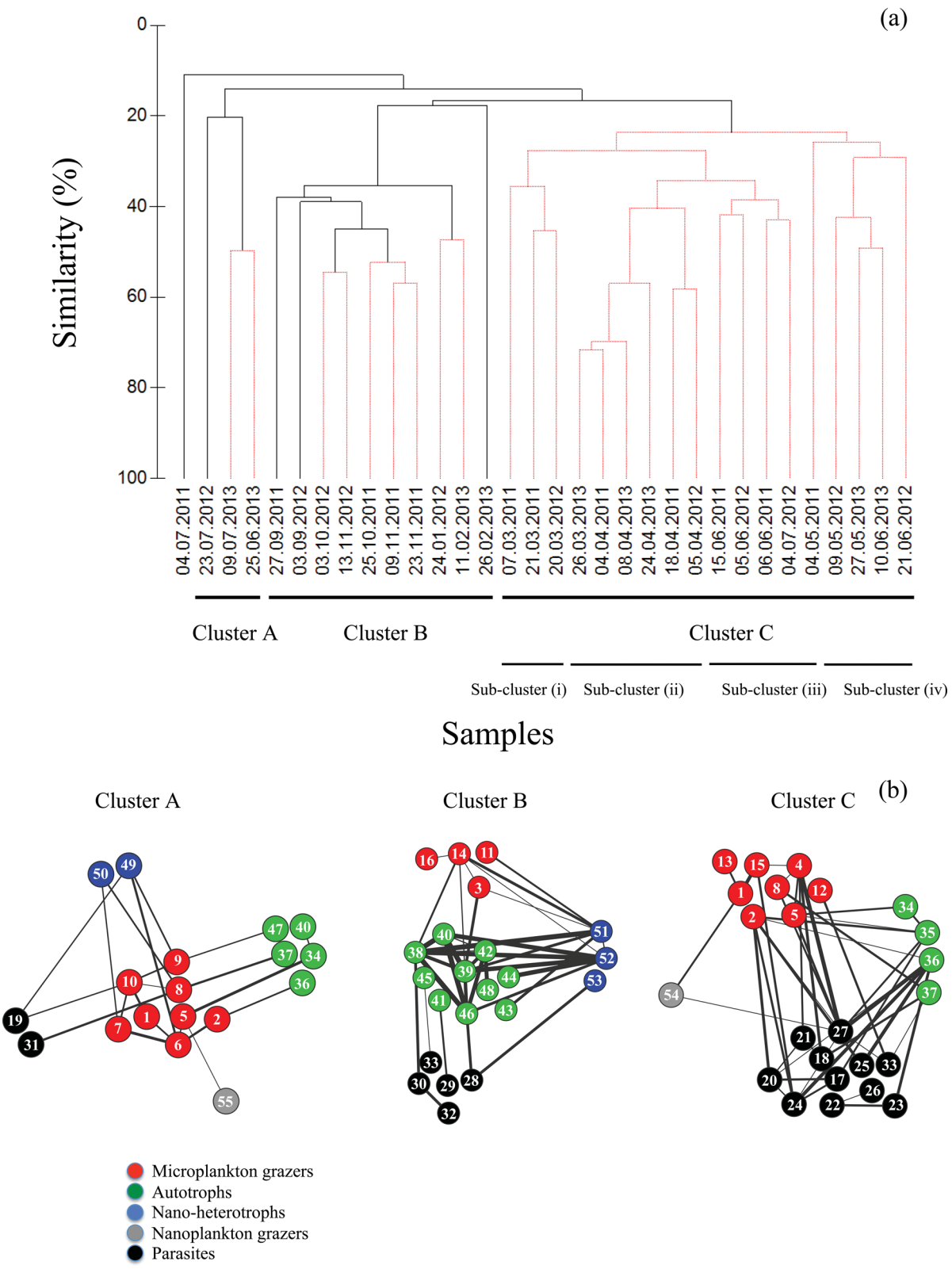

Figure 3. Cluster diagram based on Bray–Curtis dissimilarities calculated based on the non-transformed number of reads of OTUs found during the study. Red clades in the dendrogram indicate significant differences ($P < 0.05$) between bifurcations, based on the SIMPROF significance test (**a**). Network diagram of MIC correlations (edges) based on the abundance of the OTUs (nodes) responsible for the formation of the three clusters of the cluster analysis, according to SIMPER analysis. The different colors represent different ecological categories, based on the major ecological role of each OTU in a marine ecosystem as determined by the literature. The numbers within each node correspond to the serial number of each OTU in Table 2. The weight of the edges is analogous to the strength of the connection (MIC value) (**b**).

Table 2. OTUs identified in the seasonal clusters and showed strong interrelationships according to the MIC. The attributes were given based on previous microscopic and experimental observations in the area and literature data.

OTUs	Trophic Group	Taxonomic affiliation	Closest Relative (% similarity) [Accession Number]	Cluster A	Cluster B	Cluster C
1. OTU001	**Microplankton grazers**	Dinophyceae	*Gyrodinium* sp. (100%) [AB120001]	√		√
2. OTU011		Dinophyceae	*Gymnodinium* sp. (98%) [AF274260]	√		√
3. OTU012		Dinophyceae	*Wornowia* sp. (97%) [FJ947040]		√	
4. OTU015		Dinophyceae	Dinophyceae sp. (95%) [AY434686]			√
5. OTU022		Dinophyceae	*Wornowia* sp. (95%) [FJ947040]	√		√
6. OTU023		Dinophyceae	*Lessardia elongata* (94%) [AF521100]	√		
7. OTU025		Dinophyceae	*Blastodinium galatheanum* (97%) [FJ541188]	√		
8. OTU029		Dinophyceae	Dinophyceae sp. (100%) [AM503930]	√		√
9. OTU042		Dinophyceae	*Gymnodinium catenatum* (97%) [AY421784]	√		
10. OTU055		Dinophyceae	*Gyrodinium rubrum* (99%) [AB120003]	√		
11. OTU061		Dinophyceae	*Gymnodinium* sp. (97%) [AF274260]		√	
12. OTU065		Dinophyceae	*Wornowia* sp. (100%) [FJ947040]			√
13. OTU095		Dinophyceae	*Heterocapsa pygmaea* (85%) [AF274266]			√
14. OTU099		Dinophyceae	Dinophyceae sp. (93%) [AY434686]		√	
15. OTU118		Dinophyceae	*Piscinoodinium* sp. (92%) [EF016919]			√
16. OTU173		Dinophyceae	*Karlodinium micrum* (100%) [JF791045]		√	
17. OTU004	**Parasites**	Syndiniales	MALV-I (100%) [EF172954]			√
18. OTU020		Fungi	*Tritirachium* sp. (100%) [AB003951]	√		
19. OTU024		Syndiniales	MALV-II (98%) [AJ402344]			√
20. OTU028		Cercozoa	Ebrida sp. (100%) [AB275053]			√
21. OTU030		Cercozoa	*Protaspa* sp. (99%) [DQ314810]			√
22. OTU038		Cercozoa	*Cryothecomonas aestivalis* (100%) [AF290541]			√
23. OTU045		Cercozoa	*Cryothecomonas* sp. (100%) [DQ314811]			√
24. OTU048		Cercozoa	Cercozoan sp. (100%) [DQ314814]			√
25. OTU062		Syndiniales	MALV-II (100%) [DQ186533]			√
26. OTU075		Discoba	*Ichthyobodo* sp. (89%) [AY255800]			√
27. OTU080		Syndiniales	MALV-I (98%) [DQ103798]			√
28. OTU082		Syndiniales	MALV-I (99%) [DQ186529]		√	
29. OTU087		Syndiniales	MALV-II (99%) [EU793221]		√	
30. OTU094		Syndiniales	MALV-II (100%) [EU793383]		√	
31. OTU111		Labyrinthulomycetes	Thraustochytriaceae sp. (91%) [FJ800622]	√		
32. OTU113		Syndiniales	MALV-II (93%) [AY129038]		√	
33. OTU169		Cercozoa	*Protaspa* sp. (100%) [DQ314809]		√	√
34. OTU003	**Autotrophs**	Bacillariophyta	*Leprocylindrus danicus* (98%) [AJ535175]	√		√
35. OTU005		Haptophyta	*Phaeocystis globosa* (100%) [GQ118979]			√
36. OTU017		Picobiliphyta	Picobiliphyta sp. (100%) [DQ060524]	√		√
37. OTU018		Haptophyta	*Chrysochromulina strobilus* (100%) [FN599060]	√		√
38. OTU031		Chlorophyta	*Ostreococcus* sp. (100%) [AF525864]		√	
39. OTU032		Chlorophyta	*Micromonas* sp. (100%) [AY425319]		√	
40. OTU034		Chlorophyta	*Bathycoccus prasinos* (100%) [AF525879]	√	√	
41. OTU050		Picobiliphyta	Picobiliphyta sp. (99%) [DQ222878]		√	
42. OTU078		Chlorophyta	*Micromonas pusilla* (100%) [AY425316]		√	
43. OTU081		Cryptophyta	*Teleaulax amphioxeia* (100%) [AJ421146]		√	
44. OTU092		Bacillariophyta	*Chaetoceros rostratus* (97%) [X85391]		√	
45. OTU129		Haptophyta	*Chrysochromulina* sp. (100%) [AB199882]		√	
46. OTU142		Haptophyta	Haptophyta sp. (100%) [AF107085]		√	
47. OTU147		Picobiliphyta	Picobiliphyta sp. (96%) [AY426835]	√		
48. OTU161		Picobiliphyta	Picobiliphyta sp. (100%) [DQ222877]		√	
49. OTU037	**Nanoheterotrophs**	Choanoflagellida	Stephanoecidae sp. (90%) [EU446411]	√		
50. OTU068		Telonemia	Telonemia sp. (100%) [EF526860]	√		
51. OTU085		MAST	MAST-IV (100%) [AY129060]		√	
52. OTU109		MAST	MAST-VII (96%) [AY381207]		√	
53. OTU219		MAST	MAST-III (97%) [EF526878]		√	
54. OTU014	**Nanoplankton grazers**	Ciliophora	*Strombidium* sp. (100%) [DQ103842]			√
55. OTU040		Ciliophora	Strombidiidae sp. (100%) [EF527098]	√		

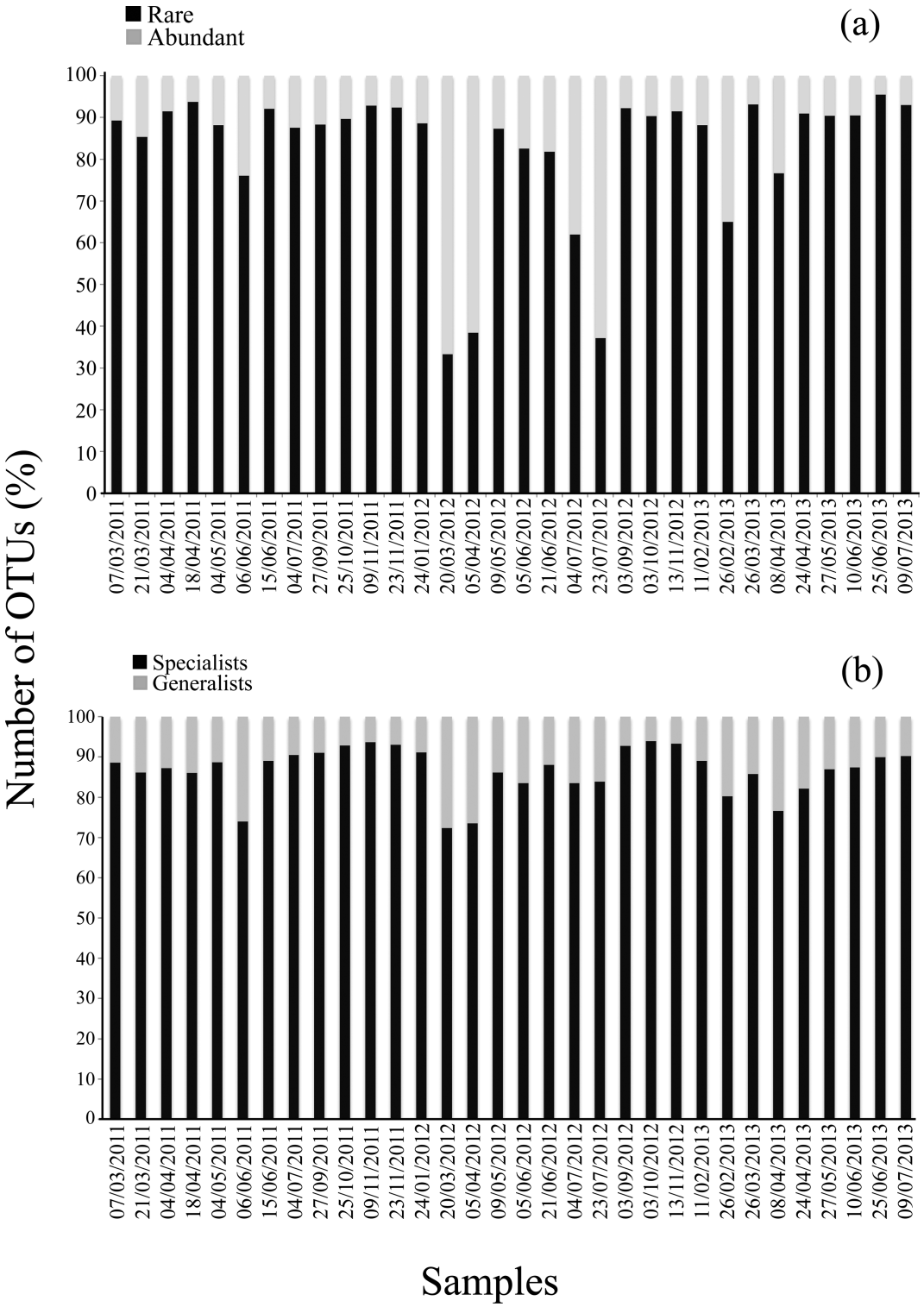

Figure 4. Proportions of per sample abundant (>1%) and rare (<0.2%) OTUs (**a**), and proportions of per sample generalists ($B > 10$) and specialists ($B < 5$) (**b**) across time.

explained variation based on the measured variables was about 30% in all cases.

Finally, the ordination diagrams of the OTUs forming the three seasonal networks were also constructed. These were based on the abundance data of these OTUs and the environmental data, during the entire study period (Fig. S4, Supporting Information). It was expected that OTUs appearing in each network would correlate with the same environmental variable as the samples comprising the cluster. For example, OTUs responsible for the formation of the cluster including hot months (cluster A; July–August) were expected to be associated with temperature (as on Fig. 6); yet this was not observed, even

Table 3. Number of OTUs, relative number of OTUs, relative abundance and the average specialization index (B) of the total protist community, the ecological groups, the major taxonomic groups and the ecological groups that were identified in the seasonal clusters.

	Number of OTUs	Number of OTUs/total number	Abundance/total abundance	Average B
Total community	1242	1	1	2.5
OTU Groups				
Abundant	142	0.11	0.87	5.7
Rare	901	0.73	0.04	1.7
Generalists	15	0.01	0.29	12.6
Specialists	1097	0.88	0.41	1.9
Abundant and specialists	73	0.06	0.32	3.1
Rare & specialists	881	0.71	0.03	1.6
Major Taxonomic Groups				
Dinophyceae	182	0.15	0.38	2.6
MALV	155	0.13	0.09	2.4
Fungi	103	0.08	0.03	1.6
Bacillariophyta	99	0.08	0.1	2.5
Cercozoa	94	0.08	0.05	2.8
MAST	77	0.06	0.08	3.5
Chlorophyta	59	0.05	0.03	2.3
Labyrinthulomycetes	41	0.03	0.01	2.3
Oomycetes	35	0.03	0.01	1.9
Haptophyta	30	0.02	0.08	3.8
Trophic Groups in Clusters				
Microplankton grazers	16	0.3	0.51	7.5
Parasites	17	0.31	0.18	5.8
Autotrophs	14	0.26	0.26	7.1
Nanoheterotrophs	5	0.09	0.02	5.4
Nanoplankton grazers	2	0.04	0.03	12.4

though a number of OTUs showed a slight correlation with temperature (Fig. S4, Supporting Information).

DISCUSSION

Protistan community structure, environmental parameters and potential biological interactions

Three distinct seasonal clusters were observed during this study, including July–August, September–February and March–June (*P. globosa* bloom related) samples. The *P. globosa*-related OTU showed an increase in abundance in April and May samples in each of the years. Previous studies have shown that during the bloom the strong top-down control on microzooplankton by copepods slows down their grazing effect and promotes *P. globosa* biomass accumulation (e.g. Grattepanche *et al.* 2011a,b). In addition, because of top-down control by nanoheterotrophs, the heterotrophic bacteria cannot efficiently process the large amounts of accumulated organic matter during the phytoplankton bloom senescence (Lamy *et al.* 2009). At the end of the bloom, a shift in the protistan community assemblage was observed, including OTUs previously undetected with microscopy and related to known decomposers (e.g. Fungi) and parasitic taxa (e.g. MALV, Cercozoa). This was apparently related with the phytoplankton decay and the process of the excess of the organic matter.

During the bloom, the overall protistan OTUs richness dramatically decreased, and the dominance reached its highest values, as only few phylotypes dominated the community. The protistan community seemed to 'reset' to a higher richness state during the autumn–winter period (Table 1). Marine microbial communities are dynamic and resilient, implying that despite external forces that alter the community (such as temperature, nutrient supply and physical mixing), there are internal feedback mechanisms (Fuhrman, Cram and Needham 2015). During this study, canonical analysis of samples and environmental parameters explained approximately only one third of the community variation (Fig. 6). In addition, when examining the effects of the abiotic parameters on individual OTUs, no patterns could be detected in the data set (Fig. S4, Supporting Information). Against expectations that OTUs responsible for the formation of each seasonal cluster would correlate with the same environmental variable as the samples comprising the cluster, such a pattern was not observed (Fig. S4, Supporting Information). It should be noted that since samples were taken at biweekly intervals, the lack of a clear correlation between individual OTUs and environmental parameters could be attributed to a 'lag time' in the response of the community to a perturbation in an environmental factor (e.g. increased nutrients). However, it could also indicate that these OTUs might not be significantly influenced by variations in environmental parameters. We suggest that while environmental parameters can explain partially the protistan seasonal succession picture, inter-taxa relations were the main drivers of the structure within temporal assemblages. This is in contrast to what has been previously proposed for marine bacterial communities, where environmental parameters (such as physical mixing, day length, temperature and nutrients) are more important in shaping bacterial community structure than taxa interrelationships, such as trophic interactions (e.g. Gilbert *et al.* 2012; Chow *et al.* 2013).

The possible factors shaping the community seasonal structure were further investigated by examining taxa interrelations based on co-occurrence patterns. For this, different attributes were assigned to the OTUs responsible for the

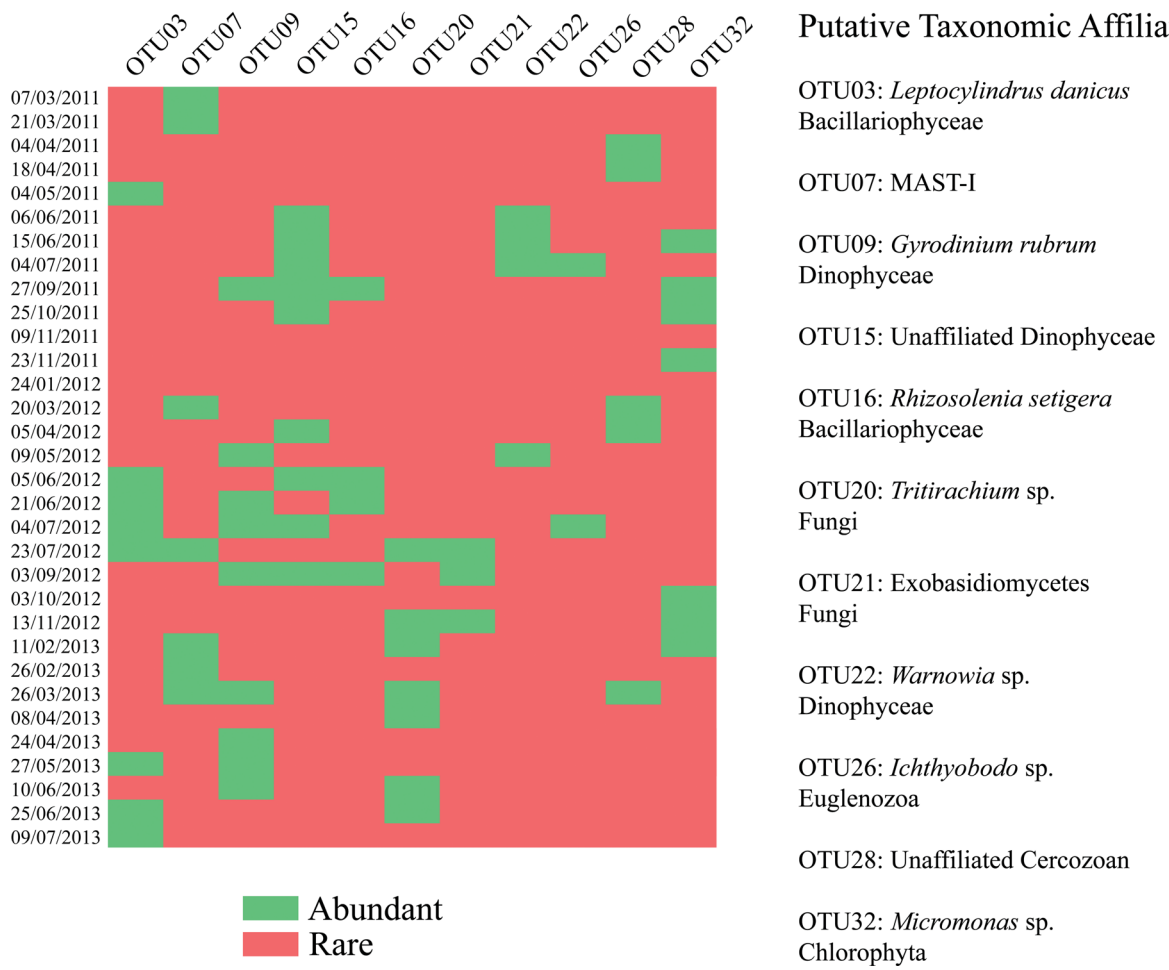

Figure 5. Heatmap of 11 out of the 30 most abundant OTUs (>2000 reads in the entire data set) that were found to be specialists with temporally high abundances (abundant specialists).

formation of the three seasonal clusters, based on their major trophic role. The OTUs associations network showed that in each cluster different trophic groups were exhibiting strong associations between them (Fig. 3b). Moreover, a small number of shared OTUs were detected between clusters (Fig. S2, Supporting Information). These observations suggest a different community structure in each temporal protistan assemblage, with different major processes dominating in each assemblage. For example, during July–August not very strong connections between OTUs were detected, with microplankton grazers dominating the protistan assemblages. The oligotrophic summer phytoplankton community in the EEC is characterized by large fine walled diatoms and their dinoflagellate grazers. On the contrary, in the other two clusters stronger connections were evident, but among different groups in each cluster. During September–February, strong connections within autotrophs were detected, while grazers and parasites seem to play a less important role, suggesting that autotrophy was the major trophic process (Fig. 3b). This reflects the onset of the colony forming diatom bloom, exploiting winter nutrient stocks and increasing irradiance. During March–June (within the bloom cluster), parasites were strongly connected with autotrophs, and a number of microplankton grazers appeared to play a significant role (Fig. 3b). The protistan community assemblage including OTUs previously undetected with microscopy and related to known decomposers (e.g. Fungi) and parasitic taxa (e.g. MALV, Cercozoa) suggests the predominance of alternate carbon pathways in the food web that could also shape the distribution of species (Hudson, Dobson and Lafferty 2006; Faust and Raes 2012; Christaki *et al.* 2014; Rasconi *et al.* 2014).

A plausible explanation for the increased significance of inter-taxa relationships in shaping protistan communities in comparison to bacteria could be associated with the complexity of trophic traits among different protistan taxonomic groups. In bacterial communities, functional similarity among taxonomically distinct groups is common (Burke *et al.* 2011). On the contrary, in eukaryotes, phylogenetic relatedness does not necessarily correspond to ecological relatedness. For example, within the common high-level taxonomic groups detected in our study: within Alveolata, Dinophyceae are almost all diatom grazers in the area (Grattepanche *et al.* 2011a,b), and Marine Alveolates (MALV) were found to be most likely intracellular symbionts or parasites (e.g. Skovgaard *et al.* 2005; Harada, Ohtsuka and Horiguchi 2007; Massana and Pedrós-Alió 2008); within Stramenopiles, Bacillariophyceae (diatoms) are known autotrophs, and MAST have been identified as free-living bacterivorous heterotrophs (e.g. Massana *et al.* 2006); Fungi are related to the degradation of organic matter (e.g. Raghukumar 2004); and cercozoans are suggested to exhibit mainly parasitic behavior (e.g. Tillmann, Hesse and Tillmann 1999; Schnepf and Kühn 2000).

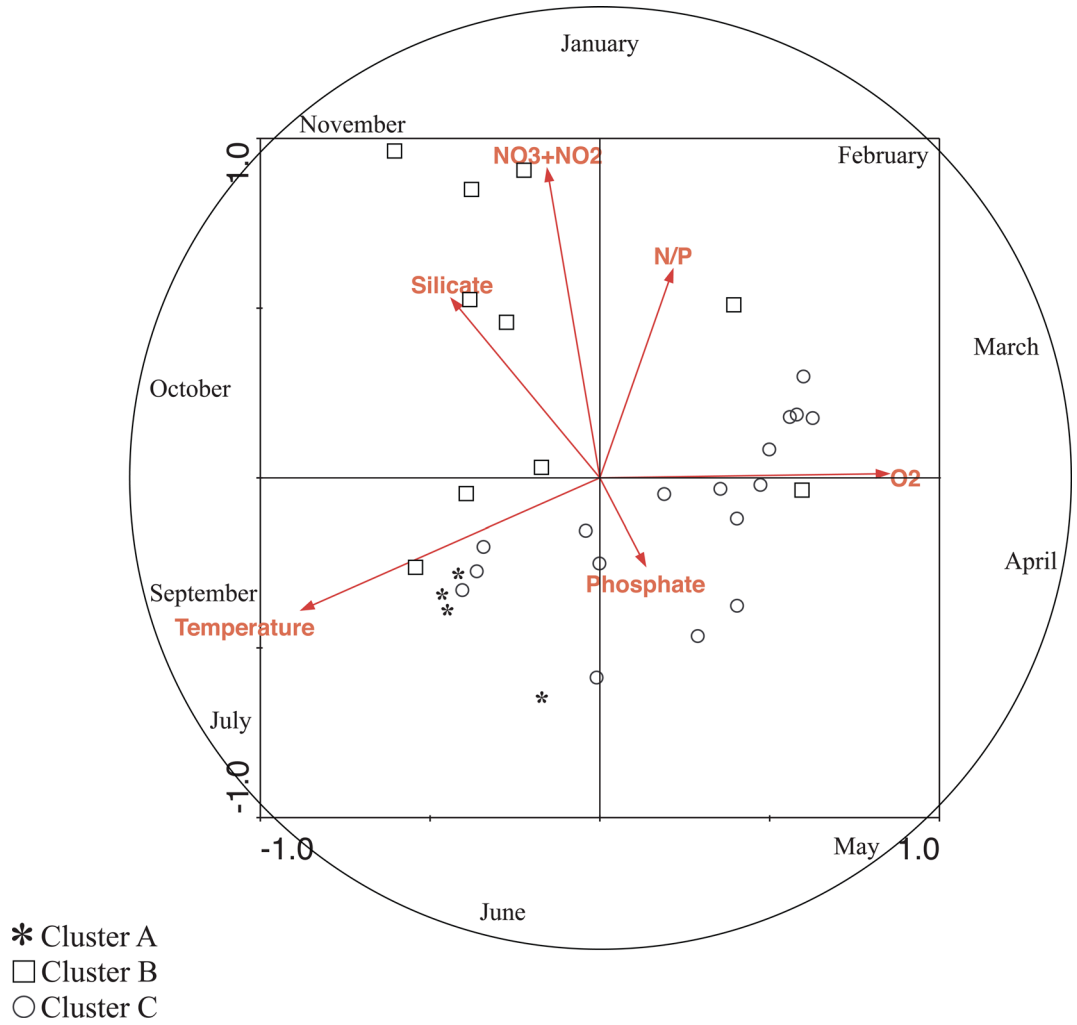

Figure 6. Biplot of physical and chemical parameters (see Table 1) and all samples, based on OTUs abundance.

Abundant/rare and generalist/specialist taxa, and their relevance for community structure

Most studies that have differentiated between abundant and rare marine taxa have concerned bacteria. This taxa sorting according to abundance, although somewhat arbitrary, has provided some indications on the functional structure of marine bacterial communities. For example, it was suggested that abundant bacteria contribute mainly to carbon flow and nutrient cycling (Pedrós-Alió 2012), while rare microorganisms might contribute to community stability by acting as a reservoir that can rapidly respond to environmental variation (Shade *et al.* 2014). Compared to bacteria, less is known about abundant and rare marine protist subcommunities. In a recent study, Logares *et al.* (2014) proposed that marine planktonic protistan assemblages incorporate metabolically active abundant and rare subcommunities, with contrasting structuring patterns but with regular proportions, across space and time.

However, when looking at protists, it is not straightforward to separate between abundant and rare taxa according to pyrosequence abundance, since PCR biases are magnified in protistan taxa with multiple 18S rRNA gene copies, such as alveolates (Medinger *et al.* 2010). In addition, according to our results, several taxa that were rare in most samples were classified as abundant, due to their abundance increase in a limited number of samples (Fig. 5). It is known that rare marine protists may include ecologically redundant taxa or dormant species that could considerably increase in abundance after environmental perturbation (Caron and Countway 2009). Conversely, many rare taxa may contribute a greater amount to microbial community dynamics than is apparent from their low proportional abundances (Shade *et al.* 2014).

Furthermore, it has been shown that for bacterial populations, taxa differentiation according to specialization traits can successfully address community structure questions (e.g. Székely and Langenheder 2014). A generalist species that is able to thrive in a wide variety of environmental conditions can make use of a variety of different resources and therefore can occur throughout the year, while a specialist species can only thrive in a narrow range of environmental conditions (Székely and Langenheder 2014). When environmental conditions change, generalists are able to adapt, while specialists tend to decrease dramatically in abundance, or even disappear (Townsend, Begon and Harper 2013). Indeed, the majority of taxa classified as specialists were observed in one to eight samples, while generalist taxa were detected in >18 samples.

For example, the generalist OTUs closely affiliated to the Dinophyceae *Gyrodinium* sp. and *Gymnodinium* sp., which were

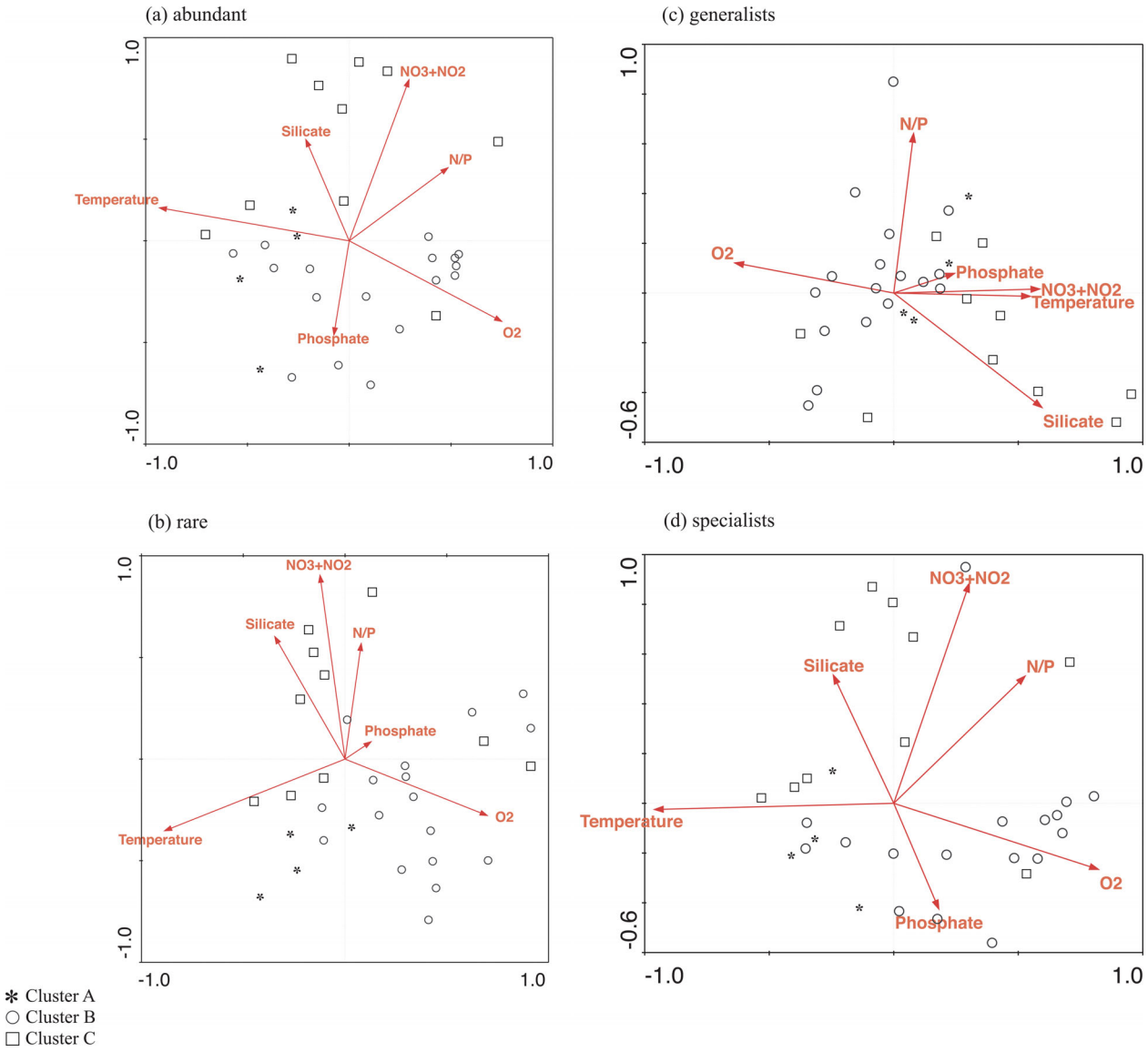

Figure 7. Biplot of physical and chemical parameters (see Table 1) and all samples, based on the abundant (**a**), rare (**b**), generalist (**c**) and specialist (**d**) OTUs abundance.

detected in the majority of the samples, are known grazers of diatoms in general, and consumers of *P. globosa* in the EEC (Grattepanche *et al.* 2011a,b). Other generalists belonged to ciliates (nanoplankton grazers), haptophytes (autotrophs) and Cercozoa and MALV (parasites). Ciliates, mainly comprised of *Strombidium* spp., are known to consume small-sized prey such as nanoflagellates (e.g. Calbet *et al.* 2008) and small diatoms (e.g. Nejstgaard *et al.* 2007). It is worth noting that other groups apart from diatoms seem to play an important role as autotrophs in the EEC, in contrast to what was believed. The OTU closely related to the haptophyte *Chrysochromulina* was present in high abundance, possibly contributing to the primary production throughout the study period. On the other hand, no diatoms were found to be generalists, indicating the strong bottom-up and top-down control imposed on these protists.

Furthermore, various specialist OTUs belonging mainly to parasites (Cercozoa and MALV) played a significant role in the separation of the seasonal clusters. These OTUs were thriving in specific sampling periods while abruptly increasing their abundance, and were found to participate in the complex network relative to the *P. globosa* bloom cluster (March–June). They were found to have strong interactions with both dinoflagellates and diatoms, reinforcing the hypothesis of alternate carbon pathways during the bloom period. An interesting strong connection with the generalist *Gymnodinium* sp. was exhibited by a specialist affiliated to an uncultured cercozoan, exclusively within the bloom cluster. Although *Gymnodinium* was observed constantly throughout the study period, the cercozoan was only present in high abundances during the March–April samples (in the beginning of the bloom). Overall, Cercozoa, both as generalists and specialists, were an important part of the protistan community, forming relations with a variety of other protists and influencing the trophic pathways, through parasitism.

CONCLUSIONS AND PERSPECTIVES

NGS-based investigation of the seasonal succession of marine protist communities complemented previous microscopical studies limited to specific microplankton groups. The major findings of this study, and opening new research

perspectives were as follows: (i) the potential role of degraders and parasites occurring every year at the late stage of the bloom; and (ii) while almost all molecular studies use taxa differentiation based on 'rare' versus 'abundant' taxa, it was shown here that taxon-specific traits such as specialization level and trophic role provided a better understanding of the seasonal protistan community organization. It was also shown that although environmental parameters could explain 30% of protistan succession, inter-taxa relations based on trophic traits were the main factors affecting seasonal community organization.

As novel sequencing technologies and bioinformatic tools develop rapidly, environmental sequencing studies combined with ecological tools can benefit in acquiring firm answers on the 'who, what, when, where, why and how' of marine protistan communities (Knight *et al.* 2012). In addition, the active fraction of microbial communities can now be accessed by the use of RNA, instead of DNA, as a template. Finally, future studies could benefit from more comprehensive metadata collection coupled with replicated sampling, which is valuable if sequence data are to be interpreted in an ecological context.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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Conflict of interest. None declared.

DATA ACCESSIBILITY

Pyrotags: GenBank-SRA accession number SRX768577.

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