



# Small-scale variability of protistan planktonic communities relative to environmental pressures and biotic interactions at two adjacent coastal stations

Savvas Genitsaris, Sébastien Monchy, Elsa Breton, Eric Lecuyer, Urania Christaki\*

Laboratoire d'Océanologie et Géosciences (LOG), UMR CNRS 8187, Université du Littoral Côte d'Opale (ULCO),  
32 av. Foch, 62930 Wimereux, France

**ABSTRACT:** The aim of this study was to analyze planktonic protistan assemblages under different environmental pressures at 2 adjacent coastal stations (inshore and offshore; ~6.4 km apart) in the eastern English Channel (EEC). Samples were collected between March 2012 and June 2013, and analyzed using bioinformatic analysis and tag pyrosequencing of the V2–V3 hypervariable region of the 18S rRNA gene. In addition to the taxonomic composition of the protistan communities, the detected operational taxonomic units (OTUs) were sorted into 6 major functional groups based on their trophic roles in marine systems. Comparisons of 13 environmental factors, including physical and chemical variables, indicated significant differences between the 2 stations. However, the only significant relationship between environmental pressures and protistan community structure was detected when the trophic role of the OTUs was considered at the inshore station. In order to interpret differences in community structure, the effect of biotic interactions was investigated through the examination of the co-occurrence networks of the protistan communities at both stations. In terms of the number of edges and connectivity of nodes, the analysis showed that the inshore station had more complex associations between OTUs than the offshore station. This strongly suggests that due to the higher and more variable environmental pressures the inshore station comparatively receives, its protistan community has developed a greater complexity of biotic connections. This in turn reflects the rapid responses of trophic interactions within the entire microbial community.

**KEY WORDS:** Unicellular eukaryotes · Community organization · Environmental parameters · Biotic interactions · 18S rRNA · Pyrosequencing · Bioinformatics · Ecological tools

## INTRODUCTION

A central question in marine ecology is how environmental variation affects microbial community dynamics. Because of the short generation times and rapid metabolic responses of aquatic microbes, pelagic food webs are considered to have maximum resilience to environmental change (Laws 2003). Next-generation sequencing (NGS) methods provide a comprehensive assessment of marine microbial diversity, including the detection of rare and 'invisible'

taxa (e.g. Medinger et al. 2010, Behnke et al. 2011). Recent NGS studies have examined the statistical relationships among the dynamics of marine microbes, and the influence of environmental parameters measured over various spatial and temporal scales. In doing so, they have determined the environmental conditions that influence individual microorganisms, and highlighted the significance of their biotic interactions. For example, existing studies on bacterial communities have indicated that environmental parameters (such as physical mixing, day length, temper-

\*Corresponding author: urania.christaki@log.cnrs.fr

ature, and nutrients) were more important in shaping community structure than taxa interrelationships (e.g. Gilbert et al. 2012, Salter et al. 2015). Other studies have suggested that the biotic interactions among co-occurring taxa have been the main structural drivers of spatial and temporal assemblages (e.g. Fuhrman & Steele 2008, Shade et al. 2014, Székely & Langenheder 2014). In bacterial communities, functional similarity among taxonomically distinct groups is common (e.g. Burke et al. 2011). Alternatively, in eukaryotes, morphological and phylogenetic relatedness does not necessarily correspond to ecological relatedness (e.g. reviewed in Sherr et al. 2007, Caron & Countway 2009, Caron et al. 2012). For instance, within the common high-level taxonomic group Alveolata, Dinophyceae are diatom grazers (e.g. Sherr & Sherr 1994, Grattepanche et al. 2011a, b), and marine alveolates (MALV) are most likely intracellular symbionts or parasites (e.g. Skovgaard et al. 2005, Harada et al. 2007, Massana & Pedrós-Alió 2008); within the marine stramenopiles (MASTs), Bacillariophyceae (diatoms) are known autotrophs, and MASTs have been identified as free-living bacterivorous heterotrophs (e.g. Massana et al. 2006); fungi are involved in the degradation of organic matter (e.g. Raghukumar 2004); and cercozoans are suggested to exhibit mainly parasitic behavior (e.g. Tillmann et al. 1999, Schnepf & Kühn 2000).

The novelty of the present study was in considering the trophic roles of planktonic eukaryotes detected by NGS in addition to their taxonomic position. The main questions we investigated were: (1) Is there an increased significance of inter-taxa relationships in counterbalancing environmental variability and shaping protistan community structure, and (2) Are eukaryotic functional roles (such as trophic traits) more informative than taxonomic affiliations in the interpretation of biotic/abiotic interactions? These questions were tested with a small-scale approach in a seasonal study (15 samples over a 15 mo period) at closely located stations in the eastern English Channel (EEC). In this area, physical and chemical variables (winds, currents, tides, and nutrient concentrations) exhibit strong contrasts. The 2 coastal stations, hereafter referred to as 'inshore' and 'offshore', were expected to show differences in the prevailing environmental forcings, providing a framework with which to test plankton communities' responses to environmental changes. The detected taxa (determined by tag pyrosequencing) and environmental parameters were analyzed by examining the co-occurrence networks of the protistan communities at each station.

## MATERIALS AND METHODS

### Sample collection

Sampling was carried out at 2 stations located 6.4 km apart in the EEC SOMLIT (French Network of Coastal Observatories). The inshore station ( $50^{\circ} 40.75' N$ ,  $1^{\circ} 31.17' E$ , 27 m max. depth) was 1.6 km from the coast, and the offshore station ( $50^{\circ} 40.75' N$ ,  $1^{\circ} 24.60' E$ , 56 m max. depth) was 8 km from the coast (Fig. 1). A total of 30 sub-surface samples (2 to 3 m water depth) were collected (15 from each site) in 2.5 l sterile polyethylene bottles twice a month during high tide, from 20 March 2012 to 25 June 2013 (16 and 14 for each year, respectively), when local weather conditions permitted. After collection, samples were kept in the dark at *in situ* temperature, and filtered within 2 h. Before filtering, samples were screened through a 150  $\mu m$  mesh to retain larger particles and most metazoa. Next, sequential filtration through 10, 3, and 0.6  $\mu m$  nucleopore filters (47 mm diameter) was performed using a low filtration pressure peristaltic pump

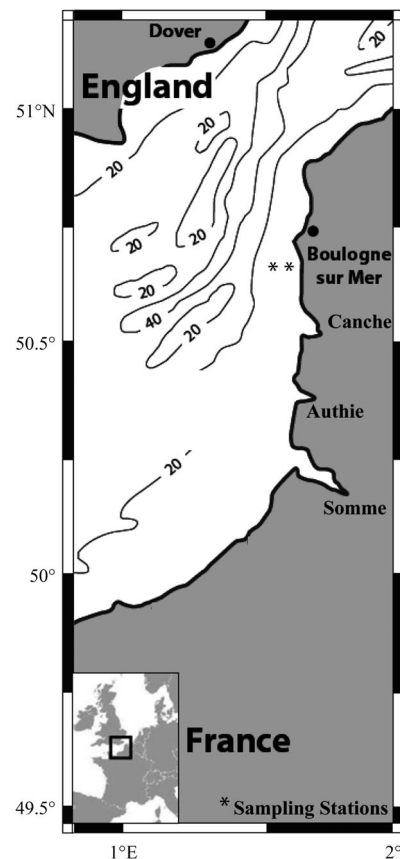


Fig. 1. Study area in the eastern English Channel, indicating major estuaries (Somme, Authie and Canche) and the location of the sampling stations (\*)

(15 rpm) in order to avoid filter clumping and minimize organism disruption. The filters were immediately stored at  $-80^{\circ}\text{C}$  until DNA extraction.

### Environmental variables

Seawater temperature ( $T$ ,  $^{\circ}\text{C}$ ), salinity (S), and photosynthetically active radiation (PAR) were measured *in situ* with a CTD (Seabird SBE 19) equipped with a biospherical PAR light sensor (QSP 2300, Biospherical Instruments). The diffuse attenuation coefficient for down-welling irradiance ( $k_d$ ,  $\text{m}^{-1}$ ) was assessed from instantaneous vertical CTD profiles. The average sub-surface daily light intensity ( $I$ ,  $\text{E m}^{-2} \text{d}^{-1}$ ) experienced by phytoplankton (over the last 6 d prior to sampling) was estimated using the formula based on Riley (1957):

$$I = \frac{I_0(1 - e^{-k_d z})}{k_d z} \quad (1)$$

where  $z$  is the depth at which samples were collected (2 m) and  $I_0$  is the daily incident light estimated from global solar radiation (GSR,  $\text{W m}^{-2}$ ) measured continuously every 5 min with a solar radiation sensor (Vantage Pro, Davis) mounted on the laboratory roof bordering the seashore, in close proximity to the sampling area. Prior to calculating the coefficient, GSR was converted to PAR under the assumption that  $\text{PAR} = 50\%$  of GSR, and  $1 \text{ W m}^{-2} = 0.36 \text{ E m}^{-2} \text{d}^{-1}$  (Morel & Smith 1974). The level of dissolved oxygen ( $\text{O}_2$ ,  $\text{mg l}^{-1}$ ) was analyzed in triplicate by Winkler micro-titration with a Titrino848 (Methrom) according to the methodologies outlined in Aminot & K erouel (2004). pH was measured with a pH1970i (WTW) pH meter. Nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), phosphate ( $\text{PO}_4^{3-}$ ), and silicate ( $\text{SiO}_4^{4-}$ ) concentrations ( $\mu\text{M}$ ) were determined from 100 ml samples with an Alliance Integral Futura Autoanalyzer II based on Aminot & K erouel (2004). Ammonium ( $\text{NH}_4^+$ ,  $\mu\text{M}$ ) was determined manually by fluorometry in accordance with Holmes et al. (1999), modified by Taylor et al. (2007). Chlorophyll *a* concentrations (chl *a*,  $\mu\text{g l}^{-1}$ ) were measured using a 10-AU Turner Designs<sup>®</sup> fluorometer in accordance with Lorenzen (1967), after extraction of pigment retained on Whatman GF/F glass fiber filters in 90% acetone for 12 h at  $4^{\circ}\text{C}$ . Particulate organic carbon (POC,  $\text{mg l}^{-1}$ ) was assessed with a NA2100 Frisons CHN analyzer after the GF/F filters were dried at  $60^{\circ}\text{C}$  for 24 h and exposed to vapors of 1 N HCl for 5 h. Suspended particulate matter (SPM,  $\text{mg l}^{-1}$ ) was estimated by weighing the particulate matter collected by filtra-

tion on a GF/F filter. Additional details on environmental data acquisition and sample analysis can be found at <http://somlit.epoc.u-bordeaux1.fr/fr/>.

### DNA extraction

After collectively pooling the 10, 3, and  $0.6 \mu\text{m}$  filters, the DNA of the planktonic organisms was extracted and purified using a PowerWater<sup>®</sup> DNA isolation kit (Mobio Laboratories), following the manufacturer's protocol. The samples contained between 0.5 and  $6 \text{ ng } \mu\text{l}^{-1}$  of DNA as measured by the Qubit<sup>®</sup> 2.0 fluorometer (Thermo Fischer Scientific).

### PCR and tag pyrosequencing

DNA samples were amplified using the 2 eukaryotic primers 18S-82F (5'-GAA ACT GCG AAT GGC TC-3') (L opez-Garc a et al. 2003) and Euk-516r (5'-ACC AGA CTT GCC CTC C-3') (Amann et al. 1990). These primers have been used successfully in previous studies of the protistan community at this site (Monchy et al. 2012, Christaki et al. 2014, Genitsaris et al. 2015); thus, they were selected in order to construct a dataset comparable with those studies. Polymerase chain reaction (PCR) was carried out according to standard conditions for Platinum Taq High-Fidelity DNA Polymerase (Invitrogen), with 5 ng of environmental DNA as a template, using the GeneAmp PCR System Apparatus (Applied Biosystems). Tag pyrosequencing was carried out by GenoScreen. The library was prepared following the procedures described by Roche Diagnostics, with all 30 samples sequenced on 1 plate run (15 samples per  $\frac{1}{2}$  plate run) on a 454 GS FLX Titanium Sequencer, with the long-read chemistry of Roche Applied Sciences supplied for the GS-FLX system. Pyrosequences were submitted to GenBank-SRA under accession number SRX768577.

### Tag pyrosequencing quality filtering

Pyroreads were subjected to quality filtering using mothur v.1.28.0, following standard operating procedures (Schloss et al. 2009, 2011). Flowgrams from each sample were extracted, separated according to their tag, and de-noised using the mothur implementation of 'PyroNoise' (Quince et al. 2009). The dataset was de-replicated to the unique reads and aligned against the SILVA 108 database. Reads suspected

of being chimeras were removed using UCHIME v.4.2.40 (Edgar 2010). Finally, the dataset was normalized to the sample with the lowest number of reads, so that all samples contained 12 581 reads. The reads were clustered into operational taxonomic units (OTUs) at 97% similarity threshold, using the average neighbor method in mothur. Unique amplicons that occurred only once in the dataset were removed, as these were most likely erroneous sequencing products (Kunin et al. 2010, Behnke et al. 2011). For more details of PCR and tag pyrosequencing procedures, see Genitsaris et al. (2015).

After tag pyrosequencing filtering and normalization, 1303 OTUs were produced. Taxonomic classification was assigned using BLASTN (Nucleotide–Nucleotide Basic Local Alignment Search Tool; Altschul et al. 1990) based on the Protist Ribosomal Reference (PR<sup>2</sup>) curated database (built on GenBank 203; October 2014) containing 23 003 sequences (Guillou et al. 2013). All reads affiliated with metazoa were removed from the dataset; accordingly, the remaining 1174 OTUs were protists.

### Data analysis

The 1174 OTUs belonging to 9 taxonomic supergroups were sorted into 6 groups according to their trophic status (Table 1, Table S1 in the Supplement at [www.int-res.com/articles/suppl/m548p061\\_supp.pdf](http://www.int-res.com/articles/suppl/m548p061_supp.pdf)). In eukaryotes, morphologically and phylogene-

tically similar taxa can exhibit many different types of metabolism. For example, dinoflagellates include grazers, autotrophs, mixotrophs, and parasites. Therefore, the strategy applied here was to individually examine the 1174 OTUs and annotate them to a trophic group using the highest level of information currently available. For many OTUs affiliated with microplankton and nanoplankton organisms, primarily dinoflagellates (e.g. *Gyrodinium*, *Gymnodinium*, and *Karodinium*), ciliates (e.g. *Strombidium*), diatoms (e.g. *Leptocylidrus*, *Rhizosolenia*), cryptophytes, coccolithophorids, chlorophytes, and the blooming *Phaeocystis globosa*, confidence about their valid affiliation in the dataset was high. This was because they had been previously observed by microscopy and/or flow cytometry in the same area (e.g. Grattepanche et al. 2011a,b, Monchy et al. 2012, Bonato et al. 2015, 2016). Conversely, for OTUs that were affiliated with taxonomic groups impossible to detect with microscopy, consideration of their annotation to higher taxonomic groups (e.g. family level) was taken into account, which is considered reliable at the OTU level (Bachy et al. 2013, Santoferrara et al. 2014). For example, most taxa belonging to the groups MALV and MAST are considered symbionts and nano-grazers, respectively, according to the existing literature (Skovgaard 2014, Massana et al. 2006, respectively for MALV and MAST) (see Table S1 in the Supplement)

Alpha-diversity estimators (the richness estimator  $S_{Chao1}$ , and the Simpson, Equitability, and Berger-

Table 1. Functional groups presented in this study based on their trophic role and prey grazing size. For more details on representative taxonomic groups, see Table S1 in the Supplement at [www.int-res.com/articles/suppl/m548p061\\_supp.pdf](http://www.int-res.com/articles/suppl/m548p061_supp.pdf)

Functional groups	Trophic role	Example of representative taxonomic groups
Autotrophs (auto)	Primary producers: fix CO <sub>2</sub> to produce organic material using light energy	Bacillariophyta (diatoms) Chlorophyta
Mixotrophs (mixo)	Either autotroph or heterotroph that can use mix of sources of carbon and energy <sup>a</sup>	Cryptophyta, Haptophyta Ciliates, dinoflagellates
Picoplankton grazers (picoG) <sup>b</sup>	Graze on heterotrophic bacteria and other picoplankton	Apusozoa, Discoba MAST, Telonemia, Choanoflagellida
Nano- and microplankton grazers (nanoG, microG) <sup>b</sup>	Graze on nano- and microplankton (e.g. on nanoflagellates, diatoms)	Ciliates, dinoflagellates
Symbionts/parasites/decomposers (symbdec)	Infect hosts, decompose and remineralize organic matter	Fungi, MALV, Labyrinthulea, Perkinsea, Omycetes

<sup>a</sup>Mixotrophs can be autotrophs, which supplement photosynthesis by bacterivory and/or dissolved organic matter assimilation, or heterotrophs that use the chloroplasts of their ingested prey for photosynthesis, or have symbiotic algae (e.g. mixotrophic ciliates). A perfect mixotroph would use organic and inorganic carbon equally well. For more details, see Table S1 in the Supplement

<sup>b</sup>PicoG, nanoG, and microG are heterotrophic grazers

Parker indices) were calculated for all samples. These diversity estimators were compared for each date with a bootstrapping randomization procedure, and the p-value was computed based on 1000 random permutation pairs. The coefficients of variation (CVs) of the 13 environmental parameters measured were tested for each date with the Fligner-Killeen test statistic. The mean ranks from both stations were also compared using the Wilcoxon test. The above tests were calculated using PAST v.3c software (Hammer et al. 2001).

We used co-inertia analysis (COIA) to investigate the coupling between the 2 stations in terms of environmental variables and plankton assemblages, and also between the environmental variables and plankton assemblages at each station (Dolédéc & Chessel 1994, Dray et al. 2003). The principle of COIA consists of finding co-inertia axes by maximizing the covariance between the row coordinates of 2 matrices. It defines axes that simultaneously explain the highest possible variance in each of the 2 matrices and describes their closest possible common structure. A PCA (principal component analysis) was performed on each matrix, and then the COIA analysis was applied (Dray et al. 2003, see their Fig. 1). To estimate the strength of the coupling between the 2 matrices, a multidimensional correlation coefficient (RV) was calculated, and statistical significance was tested using a Monte Carlo permutation procedure with 1000 permutations. COIA was carried out with the 'ade4' package in R (Dray & Dufour 2007). Finally, in order to define the variables that were the most important in structuring the COIA scatterplot, Spearman's correlation coefficients were calculated between all variables and COIA coordinates. Environmental and abundance data were  $\log(x + 1)$  transformed to ensure normal distribution (Legendre & Legendre 1998), and environmental data were standardized to render them dimensionless (z-scores; Kenkel 2006).

For the network analysis, 2 matrices corresponding to offshore (881 OTUs) and inshore (919 OTUs) read numbers were integrated into a 'total' matrix containing 1800 rows (the sum of the offshore and inshore OTUs). In total, 626 OTUs were common between the 2 stations; thus all OTUs received a distinct coding for each station (e.g. oOTU0001 for offshore, and iOTU0001 for inshore). Consequently,  $n \times (n - 1) / 2$  pairings were analyzed; where  $n$  is the total number of OTUs in the analysis matrix (i.e. 1800 OTUs). Markedly, 626 pairings corresponded to the common OTUs. The relationship between OTUs was characterized through maximal information-based

nonparametric exploration (MINE) statistics by computing the maximal information coefficient (MIC) between each pair (Reshef et al. 2011). MIC captures associations between data and provides a score that represents the strength of the relationship between data pairs. OTU pairings where  $MIC > 0.8$  were used to visualize the co-occurrence relationships between OTUs within and between the different stations. The 0.8 MIC threshold was selected to depict the strongest OTU pairings (see Reshef et al. 2011); this was considered as the best compromise in order to clearly visualize the most representative co-occurrence patterns at different trophic levels during the study period. The network of MIC associations of OTUs was constructed and respective topological parameters were calculated using Cytoscape v.3.0 (Smoot et al. 2011).

## RESULTS

### Community composition and seasonal succession

A total of 898 296 raw reads were produced from the sequencing of all samples. After quality checking, de-noising, and chimera removal, 591 307 reads remained. Sub-sampling and removal of metazoan reads resulted in a total of 162 705 reads at the offshore station and 160 912 at the inshore station. Results in all samples identified 1174 unique OTUs affiliated with protists at the inshore (919 OTUs) and offshore (881 OTUs) stations. Among these, 626 OTUs were observed at both stations, while 293 and 255 were only found at the inshore or offshore station, respectively. The ratio of observed to expected OTUs ( $S_{\text{Chao1}}$ ) was  $>75\%$  in all cases, and  $>90\%$  in the majority of samples (Table 2). The 1174 OTUs were affiliated with 9 supergroups (Amoebozoa, Apusozoa, Alveolata, Archeplastida, Excavata, Hacrobia, Opisthokonta, Rhizaria, and Stramenopiles) and miscellaneous other protists (Fig. 2), which were further divided to 34 taxonomic groups (Table S1 in the Supplement at [www.int-res.com/articles/suppl/m548p061\\_supp.pdf](http://www.int-res.com/articles/suppl/m548p061_supp.pdf)). The relative OTU richness of the supergroups was similar at both stations over the sampling period (Fig. 2). The supergroup Alveolata had the highest OTU richness at both sites (341 OTUs at the inshore and 325 OTUs at the offshore station) followed by Stramenopiles (228 and 219 OTUs, respectively) (Fig. 2).

The OTUs were further sorted into 6 functional/trophic groups (Table 1, for more details see Table S1 in the Supplement): autotrophs (auto), mixotrophs

Table 2. Number of operational taxonomic units (OTUs), richness estimator ( $S_{\text{Chao1}}$ ) and the heterogeneity of the diversity indexes (Simpson's  $D$ , Equitability [ $H$ : Shannon diversity index] and Berger-Parker) at the SOM-LIT offshore (O) and inshore (I) stations in the eastern English Channel from March 2012 to June 2013 (dates are given as dd/mm/yyyy)

Sampling date (station)	No. OTUs	$S_{\text{Chao1}}$	Simpson ( $D$ )	Equitability ( $H/H_{\text{max}}$ )	Berger-Parker
20/03/2012 (O)	124	136	0.24	0.54	0.47
20/03/2012 (I)	58	63	0.08	0.77	0.16
09/05/2012 (O)	122	126	0.09	0.67	0.21
09/05/2012 (I)	172	203	0.07	0.65	0.19
05/06/2012 (O)	148	150	0.11	0.62	0.27
05/06/2012 (I)	85	88	0.13	0.59	0.25
21/06/2012 (O)	230	246	0.06	0.69	0.14
21/06/2012 (I)	196	203	0.07	0.65	0.19
04/07/2012 (O)	96	102	0.25	0.57	0.49
04/07/2012 (I)	101	119	0.13	0.71	0.34
03/09/2012 (O)	184	192	0.12	0.60	0.25
03/09/2012 (I)	300	313	0.02	0.80	0.06
03/10/2012 (O)	298	311	0.03	0.78	0.11
03/10/2012 (I)	58	63	0.08	0.77	0.16
13/11/2012 (O)	146	148	0.05	0.77	0.18
13/11/2012 (I)	317	326	0.03	0.77	0.10
11/02/2013 (O)	199	207	0.05	0.72	0.16
11/02/2013 (I)	205	211	0.04	0.73	0.13
26/02/2013 (O)	101	119	0.13	0.71	0.34
26/02/2013 (I)	80	86	0.19	0.60	0.41
26/03/2013 (O)	164	167	0.14	0.58	0.33
26/03/2013 (I)	165	175	0.40	0.39	0.62
08/04/2013 (O)	158	175	0.09	0.63	0.21
08/04/2013 (I)	59	60	0.37	0.46	0.59
27/05/2013 (O)	296	335	0.05	0.71	0.20
27/05/2013 (I)	183	190	0.13	0.57	0.23
10/06/2013 (O)	230	260	0.06	0.66	0.15
10/06/2013 (I)	138	146	0.27	0.46	0.49
25/06/2013 (O)	128	169	0.74	0.18	0.86
25/06/2013 (I)	205	231	0.36	0.43	0.59

(mixo), picoplankton grazers (picoG), nanoplankton grazers (nanoG), microplankton grazers (microG), and a group mainly represented by parasites and/or symbionts. However, since parasitism defines a very specific mechanism of symbiosis, parasitic groups such as MALV, Apicomplexa, and Perkinsea can be also considered as symbionts (Table S1). With the exception of 1 OTU belonging Hyphochytriomyceta, the other OTU-rich group was the fungi, which included parasites and decomposers (56 and 76 OTUs offshore and inshore, respectively; Table S1). Historically, marine fungi have been poorly investigated, and the OTU affiliation here contained a low percentage, indicating no definite phylogenetic distance between the different fungi groups. Accordingly, although there was no binding connection between parasitic and decomposer fungi, they were allocated here into a symbiont/decomposer group, hereafter referred to as 'symbdec' (see Table 1).

In general, symbdec was the predominant group in most samples in terms of OTU richness; reaching 124 OTUs in the inshore sample collected on 3 Oct 2012 (Fig. 3a) and contributing >20% of the total number of OTUs in 28 of the 30 samples (Fig. 3b). This was followed by picoG, which also had the highest OTU richness (64 OTUs) in the 3 Oct 2012 sample (Fig. 3a). The least dominant group

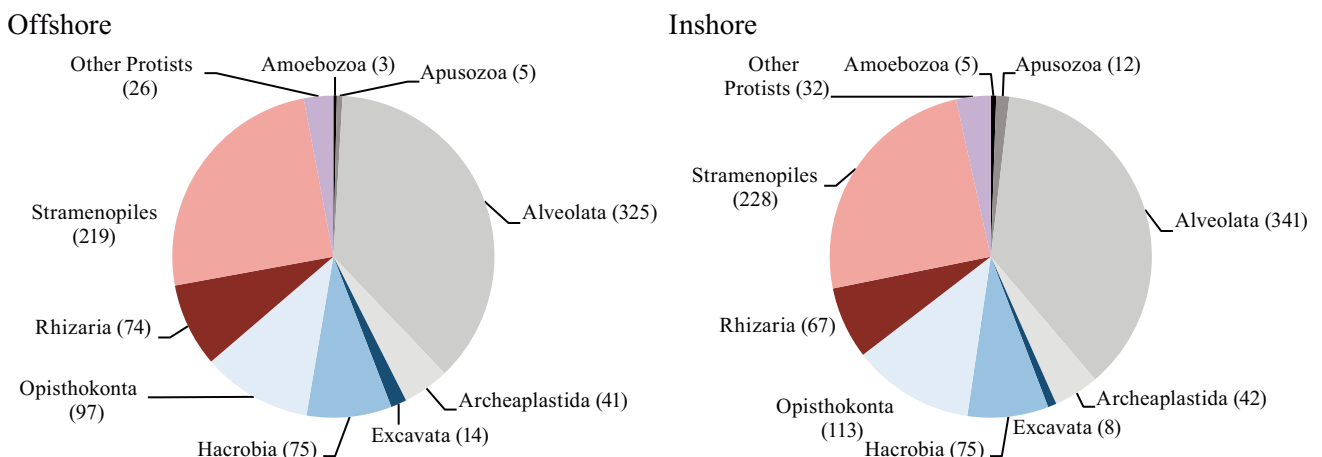


Fig. 2. Number of operational taxonomic units (OTUs) in the 9 protistan supergroups and other protists detected at the inshore and offshore sampling stations based on comparisons against the Protist Ribosomal Reference (PR<sup>2</sup>) database using the 'blastn' function (see also Table 1, Table S1 in the Supplement at [www.int-res.com/articles/suppl/m548p061\\_supp.pdf](http://www.int-res.com/articles/suppl/m548p061_supp.pdf))

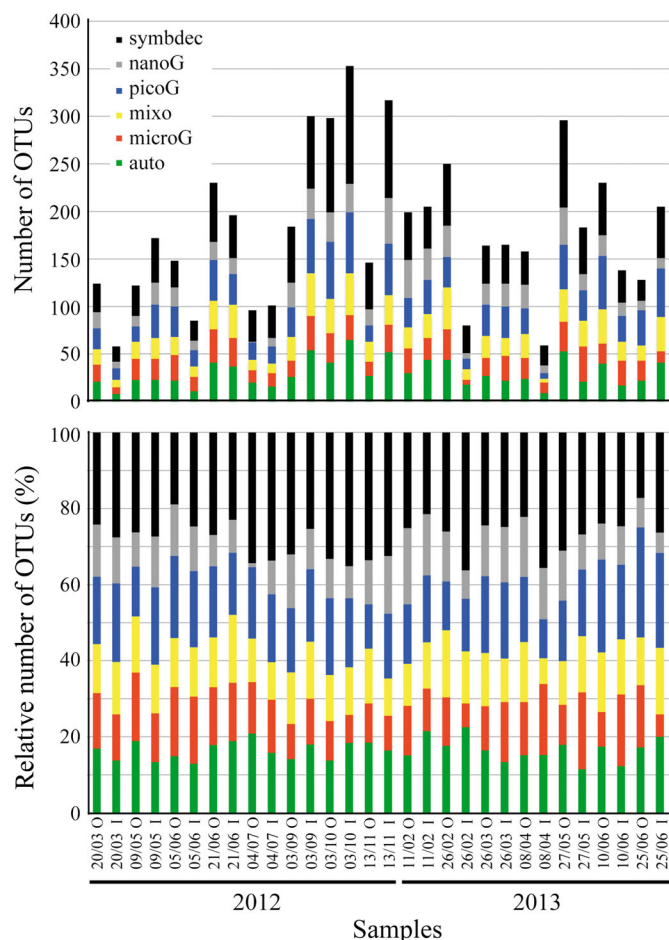


Fig. 3. Absolute and relative number of operational taxonomic units (OTUs) in the trophic groups detected on each sampling date at the 2 sampling stations (inshore [I] and offshore [O]). The trophic groups were formed based on the trophic status of the detected OTUs in marine systems as inferred from the literature (see Table 1, Table S1 in the Supplement). Dates are dd/mm

was nanoG, which contributed ~10% of the total number of OTUs in most samples (Fig. 3b), and in an extreme case (4 July 2012) were represented by only 1 OTU (Fig. 3a).

The highest number of OTUs in the offshore station was observed on 3 Oct 2012 (298 OTUs) and at the inshore station on 3 Sep 2012 (300 OTUs). The lowest OTUs richness was observed at the offshore station on 4 July 2012 (96 OTUs) and at the inshore station on 20 Mar 2012 and 3 Oct 2012 (both 58 OTUs) (Table 2). In certain cases, a large variation in OTU richness was observed between the 2 stations (the most extreme example occurred on 3 Oct 2012, when the difference in OTU richness was 240 OTUs). The Simpson index ( $D$ ) was highest in the samples with

the lowest richness, and vice versa. Fluctuations ranged from 0.02 on 3 Sep 2012 at the inshore station (the sample with the highest species richness in the entire dataset) to 0.74 on 25 Jun 2013 at the offshore station (Table 2). This was when the protistan community was dominated by a single OTU affiliated with *Phaeocystis globosa* (OTU0005), which comprised 86% of the total number of reads (Berger-Parker index = 0.86.). The Berger-Parker index showed large variations, ranging from 0.06 on 3 Sep 2012 at the inshore station to 0.86 on 25 Jun 2013 at the offshore station. These values were the same as those of the Simpson index, while the Shannon Equitability index fluctuated from 0.18 to 0.80, with higher values reflecting low variation between species abundances within the community. A comparison of these diversity estimators indicated significant statistical differences among all indices for all dates between the 2 stations ( $p < 0.05$ ), except for the Equitability index on 13 Nov 2012 and 11 Dec 2012 ( $p = 0.17$  and  $0.11$ , respectively).

#### Environmental parameters and community structure

Overall, higher mean values of  $k_d$ , pH,  $\text{NO}_3^- + \text{NO}_2^-$ , POC, SPM, and chl  $a$ , and lower values of S,  $\text{SiO}_4^{4-}$  and  $\text{PO}_4^{3-}$  were found at the inshore relative to the offshore station (Fig. 4, Table S2). These higher values were reflected in significant differences of the mean ranks (Wilcoxon test) for  $k_d$ , S, pH,  $\text{NO}_3^- + \text{NO}_2^-$ , POC, and SPM. The comparison of CVs of the 13 environmental variables showed significant differences for  $k_d$ , S, and  $\text{SiO}_4^{4-}$  (Table 3). When COIA was applied to test the seasonal evolution of the environmental variables of the 2 stations, it showed a significant environmental coupling between offshore and inshore stations over the study period ( $\text{RV} = 0.80$ ,  $p = 0.001$ ).

COIA analysis indicated that neither taxonomic ( $\text{RV} = 0.33$ ,  $p = 0.36$ ), nor trophic groups ( $\text{RV} = 0.27$ ,  $p = 0.22$ ), displayed any significant coupling between the 2 stations over the sampling period (Table 4). Furthermore, no significant relationship between the environmental variables and the 9 taxonomic supergroups was found at either station ( $p = 0.067$  and  $0.075$  for offshore and inshore, respectively; Table 4). The only significant coupling between environmental variables and trophic groups was found for the inshore station ( $\text{RV} = 0.41$ ,  $p = 0.033$ ; Fig. 5). Accordingly, the first 2 axes explained 89% of the total variance (Table 4). The environmental variables  $k_d$ , PAR,

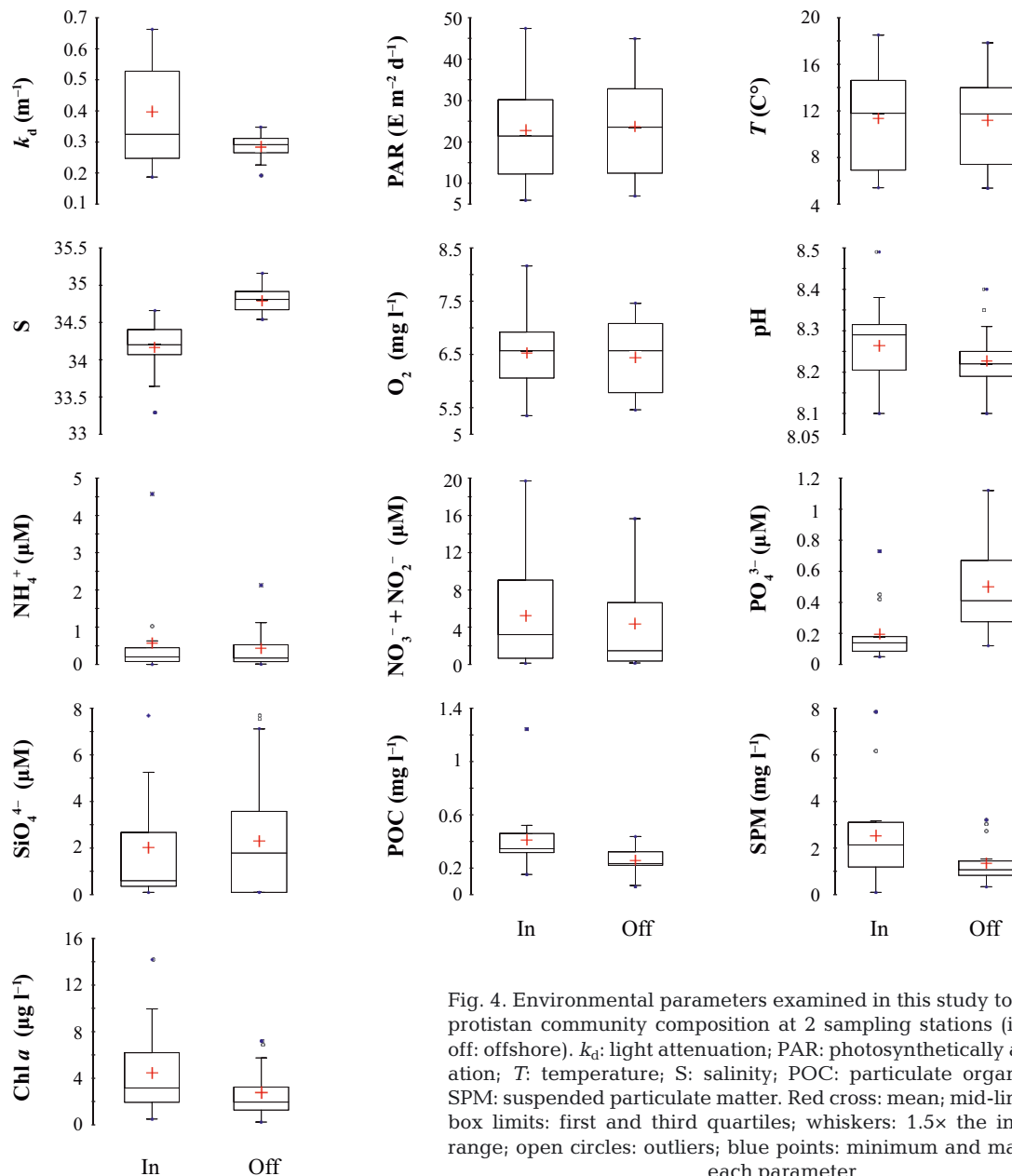


Fig. 4. Environmental parameters examined in this study to determine protistan community composition at 2 sampling stations (in: inshore; off: offshore).  $k_d$ : light attenuation; PAR: photosynthetically active radiation;  $T$ : temperature;  $S$ : salinity; POC: particulate organic carbon; SPM: suspended particulate matter. Red cross: mean; mid-line: median; box limits: first and third quartiles; whiskers: 1.5 $\times$  the interquartile range; open circles: outliers; blue points: minimum and maximum for each parameter

$T$ ,  $O_2$ ,  $NH_4^+$ ,  $NO_3^-+NO_2^-$ ,  $PO_4^{3-}$ , and SPM showed the strongest correlations with the 2 COIA axes ( $r_s$ ,  $p < 0.005$ , and  $p < 0.001$  for SPM; Table S3 in the Supplement). Among the trophic groups, microG and nanoG showed the strongest correlations with the COIA co-ordinates ( $r_s$ ,  $p < 0.001$  and  $0.005$  respectively; Table S3), followed by mixo, auto, and picoG ( $r_s$ ,  $p < 0.01$  and  $0.05$  respectively; Table S3). The parasites were not correlated with any of the axes. The scatterplot of the COIA analysis also indicated how far apart the stations were relative to their explana-

tory and response variables, and showed a relatively clear seasonal pattern with the autumn–winter and summer–spring stations grouped together (Fig. 5). The trophic group microG was associated with waters exhibiting relatively low nutrients but high PAR and high salinity, while the inverse was true for the auto group. The trophic groups mixo, picoG, and nanoG were all associated with relatively warm (high  $T$  values) and clear (high PAR but low  $k_d$  values) waters, but that were relatively poor in  $O_2$ , chl  $a$ , and SPM (Fig. 5).



Table 3. p-values of the Flinger-Killeen test for equal coefficients of variation (CV) and the Wilcoxon test between the environmental variables of the inshore versus offshore stations. Significant p-values ( $p < 0.05$ ) are shown in **bold**; ns: not significant.  $K_d$ : diffuse attenuation coefficient for down-welling irradiance; PAR: photosynthetically active radiation; POC: particulate organic carbon; SPM: suspended particulate matter

Parameters	Flinger-Killeen test	Wilcoxon test
$K_d$ ( $m^{-1}$ )	<b>0.0008</b>	<b>0.0344</b>
PAR ( $E\ m^{-2}\ d^{-1}$ )	0.4170	ns
$T$ ( $^{\circ}C$ )	0.3117	ns
Salinity	<b>0.0225</b>	<b>0.0007</b>
$O_2$ ( $ml\ l^{-1}$ )	0.3920	ns
pH	0.1497	<b>0.01707</b>
$NH_4$ ( $\mu M$ )	0.3644	ns
$NO_3^-+NO_2^-$ ( $\mu M$ )	0.2464	ns
$PO_4^{3-}$ ( $\mu M$ )	0.1368	<b>0.0006</b>
$SiO_4^{4-}$ ( $\mu M$ )	<b>0.0118</b>	ns
POC ( $mg\ l^{-1}$ )	0.3737	<b>0.0012</b>
SPM ( $mg\ l^{-1}$ )	0.1957	<b>0.0105</b>
Chl a ( $\mu g\ l^{-1}$ )	0.4658	ns

### Biotic interactions

The next step was to investigate the degree of biotic interactions based on OTU co-occurrence patterns by network analysis according to the MIC values. The network of OTU associations for both stations based on  $MIC > 0.8$  is shown in Fig. 6. The OTUs were presented according to their trophic role (see Table 1, Table S1 in the Supplement). The network indicated that the inshore station had more complex associations between OTUs, in terms of number of edges and connectivity of nodes (size of the node), than the offshore station. Additionally, a low number of connections between the 2 stations were detected since only 5% of the possible connections exhibited  $MIC > 0.8$ , and were therefore presented in the network (Fig. 6). MicroG and nanoG provided a modest contribution to the number of

nodes, since a low number of OTUs belonging to these trophic groups showed connections with  $MIC > 0.8$  (27 and 20 OTUs, respectively). In addition, these groups showed low connectivity between themselves and other groups included in the network, as indicated by the relatively small size of the nodes (Fig. 6). Furthermore, symbdec, auto, mixo, and picoG appeared to be more important at the inshore station, where they were represented by a higher number of OTUs (nodes), which also had more connections (edges) between themselves and other OTUs. The OTUs belonging to these groups comprised  $>75\%$  of the total number of nodes and  $>80\%$  of the total number of edges.

The calculated topological parameters indicated that the inshore station had more complex OTU associations than the offshore station (Fig. 6). In particular, the clustering coefficient (representing the ratio of existing links connecting a node's neighbors to each other divided by the maximum possible number of such links), the shortest path (lowest number of edges needed to pass through all nodes), the average path length (average number of edges along the shortest paths for all possible pairs of network nodes), and the average number of neighbors were, in all cases, about 2 times higher at the inshore station. Furthermore, the inshore station had a higher centralization value (an expression of how 'tightly' the graph organized around its most central point; 0.11 vs. 0.03), indicating the prevalence of focal points at the inshore station around which the rest of the nodes were organized. Finally, the inshore station showed a higher number of nodes (108 vs. 98), higher values of network density (which describes the portion of the potential connections in a network that are actual connections; 0.04 vs. 0.02), network diameter (the longest of all the calculated shortest paths between 2 nodes in a network; 10 vs. 9), and higher heterogeneity (1.08 vs. 0.64) (Fig. 6).

Table 4. Co-inertia analysis (PCA-PCA COIA) between the environmental variables, taxonomic, and trophic groups at the 2 sampled stations (offshore and inshore) in the eastern English Channel. The p-value (randomization test with 1000 permutations) shows the significance (in **bold**) of the relationship between the 2 matrices in each case. The correlation between the matrices is given by the RV-coefficient. Percentage of covariation is explained by the first 2 COIA axes (% Axis 1 and Axis 2, respectively)

	Offshore/ inshore vs. Environmental	Offshore/ inshore vs. Taxonomic	Offshore/ inshore vs. Trophic	Offshore vs. Environmental/ taxonomic	Offshore vs. Environmental/ trophic	Inshore vs. Environmental/ taxonomic	Inshore vs. Environmental/ trophic
p-value	<b>0.001</b>	0.36	0.27	0.067	0.189	0.075	<b>0.033</b>
RV	<b>0.8</b>	0.33	0.22	0.40	0.34	0.37	<b>0.41</b>
% Axis 1	67.3	60.4	72.4	69.5	47.4	40.4	63.8
% Axis 2	91.0	77.8	96.5	14.6	27.5	18.8	25.4

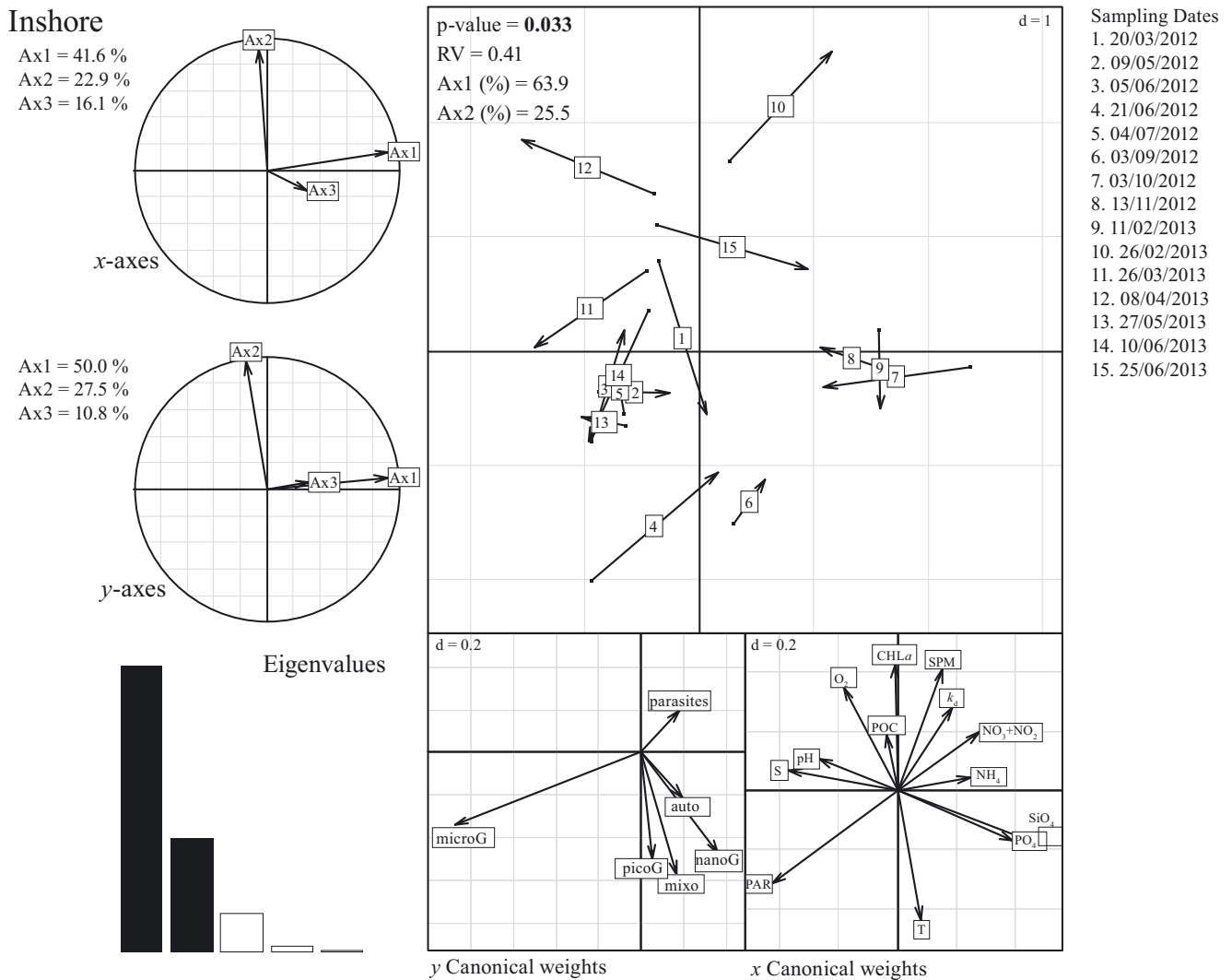


Fig. 5. Co-inertia analysis (PCA-PCA COIA) between environmental variables and operational taxonomic unit (OTU) abundance sorted into trophic groups. The x-axes show projections of the first 3 PCA components from the explanatory variables (environmental variables); y-axes show those of the response variables (trophic groups). The circles represent a view of the rotation needed to associate the 2 datasets. p-values were calculated using the Monte Carlo permutation test (1000 permutations). The sample scatterplot shows how far apart the samples were relative to their explanatory and response variables. The beginning of the arrow shows the position of the sample described by the explanatory variables, and the end by the response variables. Variables were  $\log(x + 1)$  transformed, and environmental variables were additionally standardized. RV: 'correlation coefficient between the 2 tables ('R' for correlation and 'V' for vectorial)

## DISCUSSION

In this era of substantial global change, a growing interest in ecosystem function and response (e.g. Duffy & Stachowicz 2006) has stimulated the development of methods for assessing change in communities and measuring their response to disturbance (Magurran 2011). Here, NGS was used to determine the composition of protistan communities in 2 closely located coastal stations and to understand the main factors (both biotic and abiotic) controlling their community structure.

The EEC has a megatidal regime in which strong tidal currents alternate and remain essentially parallel to the coast; the coastal water drifts nearshore, separated from the open sea. The lowest salinity values measured in all inshore station samples confirmed that the continental inputs from the bays of the Somme, Authie, and Canche (which consist of less saline and more turbid coastal waters) are restricted to the coastal area and are separated from the offshore waters by an unstable tidal front (Brylinski & Lagadeuc 1990, Lagadeuc et al. 1997). This hydrological regime is responsible for the different

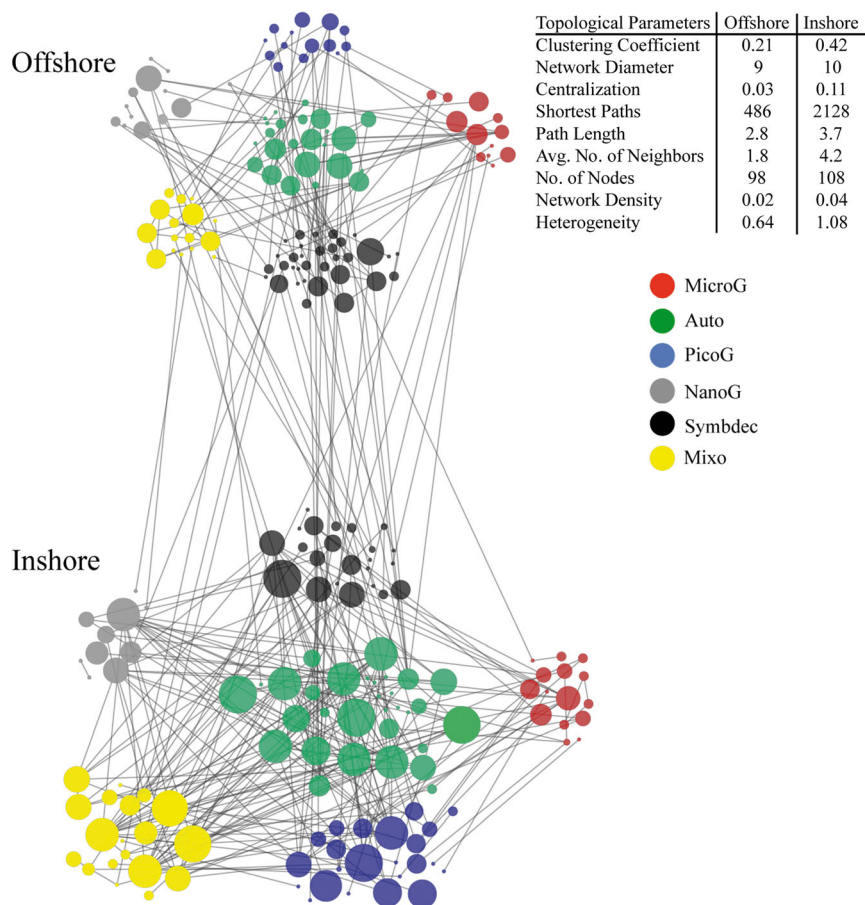


Fig. 6. Network diagram of the operational taxonomic units (OTUs; nodes) showing correlations (edges) with maximal information coefficient (MIC) values  $>0.8$ . Different colors: different trophic groups, based on the trophic status of the detected OTUs in marine systems as inferred from the literature (see Table 1, Table S1 in the Supplement). The size of the nodes is analogous to the clustering coefficient for each OTU. Table shows the topological parameters characterizing the individual network of each sampling station (inshore and offshore). Avg.: average; Nb.: number

environmental forcing that prevails at the inshore and offshore stations. Indeed, the measurements and analyses of 13 different parameters (see Table S2 in the Supplement at [www.int-res.com/articles/suppl/m548p061\\_supp.pdf](http://www.int-res.com/articles/suppl/m548p061_supp.pdf)) showed that although the environmental variables presented a quasi-identical seasonal evolution over the duration of the study at the 2 stations (COIA analysis; Table 4), statistical differences relative to their variability and mean ranks were detected. Several parameters reflecting the quantity of the suspended material in the water (live, detritus, or inorganic material) such as chl *a*, POC, SPM, and  $k_d$  were significantly higher at the inshore station (Fig. 4), while the concentrations of nutrients such as  $\text{SiO}_4^{4-}$  and  $\text{NO}_3^- + \text{NO}_2^-$ , were significantly different between the 2 stations (Table 3).

In a previous study dealing with mesozooplankton communities at the same site, Brylinski & Aelbrecht

(1993) reported that distinct zooplankton populations were maintained despite the close proximity of the stations sampled. This was attributed to the different environmental pressures exhibited at the 2 stations caused by the hydrochemical characteristics in the area (Brylinski & Aelbrecht 1993). Indeed, various studies have suggested that the spatiotemporal dynamics in microbial planktonic composition are often related to variability in environmental parameters (Hullar et al. 2006, Stomp et al. 2011, Read et al. 2015, Wang et al. 2015), although coherent patterns are often not apparent.

In addition, strong spatial variability in the taxonomic composition of protistan communities has been documented at distant oceanic (Not et al. 2009, Pernice et al. 2013) and coastal locations (Logares et al. 2012, 2014, Massana et al. 2015), reflecting different environmental pressures. Furthermore, a striking consistency in the relative proportions of protistan assemblages among closely located sites (2 to 21 km apart) has been shown (Lie et al. 2013, Logares et al. 2014, Massana et al. 2015). In this study, the protistan communities of the 2 sampling sites shared  $>60\%$  of the total number of OTUs over the whole sampling period, and had similar taxonomic composition (Fig. 2). However, comparison of several diver-

sity estimators (shown in Table 2) revealed that the indices differed significantly between the 2 sites for all dates. The  $\alpha$ -diversity estimators showed consistent patterns with previous studies conducted in the area with NGS tools (Genitsaris et al. 2015); for example, the lowest equitability and highest dominance indices (Simpson and Berger-Parker indices) that were calculated during the *Phaeocystis globosa* peaks. However, these estimators have a relative weight in comparisons among samples analyzed within the same pipeline, as they are based on the number of sequences, not the number of detected individuals. Among eukaryotes, the number of rRNA gene copies depends on cell size, and varies from one to several thousand (Zhu et al. 2005), providing biased estimates of taxa abundance.

Our results confirm differences in environmental parameters and community structure between the 2

stations. In a seasonal succession investigation of the protistan communities in the EEC at a single site, Genitsaris et al. (2015) showed with canonical analysis that the measured environmental parameters explained only 30% of the protistan seasonal variation, and that biotic interactions among co-occurring taxa appeared to be the main structural drivers of the temporal assemblages detected. A similarly low percentage of protistan variation explained by environmental parameters was reported by Chow et al. (2014), in a study that attempted to characterize ecological relationships between viruses, bacteria, and protists in the ocean. That study found that the predictable variability within protists was approximately 10% according to environmental factors (especially day length). The advantage of the analysis used here (COIA) is that it is based on partial least-squares regression; consequently, it places no restrictions on the number of variables that can be analyzed (unlike classic canonical models). The only significant coupling between the 13 environmental parameters and the protist community was found at the inshore station, after the protists were sorted into trophic groups (Table 4).

The trophic group symbdec included organisms involved in organic matter degradation, i.e. parasites, symbionts, and decomposers (Table 1). Most of the sequences belonged to MALV I, which have a wide host spectrum, whereas MALV II (which were also abundant) are considered dinoflagellate parasites (Massana et al. 2011). The considerable abundance and diversity of MALV suggests interactions with various hosts (Skovgaard et al. 2005, Massana & Pedrós-Alió 2008), such as dinoflagellates (Christaki et al. 2015). However, direct evidence that most MALV clades are parasitic is lacking (Worden et al. 2015). The associations observed between MALV and their hosts are equally consistent with symbiotic relationships (Bråte et al. 2012), and environmental factors could potentially shift a relationship from commensal to pathogenic (Worden et al. 2015). In the present study, the absence of a correlation between symbdec and the COIA co-ordinates indicates that the variability of this trophic group at the inshore station cannot be explained by any of the variables chosen in this study (Fig. 5). This could be because the organisms included in this trophic group are associated with other organisms, and thus, there is a lag-time in their response to perturbations in the environment. Statistical analysis based on lagged associations can be used to predict interactions in time series including several seasonal cycles and with equidistant sampling points (e.g. Steele et al. 2011, Xia et al. 2011, 2013, Chow et al. 2014, reviewed in Faust et al. 2015).

The trophic group mikroG was associated with waters exhibiting relatively low nutrients but high PAR and high salinity, while the inverse was true for the auto group. This pattern is consistent with the plankton succession evidenced in the EEC (Grattepanche et al. 2011a, Christaki et al. 2014, Genitsaris et al. 2015). Within the autotroph-affiliated OTUs, a *P. globosa*-related OTU and various diatom-related OTUs were among the dominant groups. Every spring, a *P. globosa* bloom is preceded and followed by communities of colonial diatoms. MicroG, consisting mainly of heterotrophic dinoflagellates, account for a large fraction of grazing on phytoplankton, and appear at the end of the diatom blooms when nutrients are depleted (Grattepanche et al. 2011b). The trophic groups mixo, picoG, and nanoG were all associated with relatively warm (high  $T$  values) and clear (high PAR, but low  $k_d$  values) waters, but which were relatively low in  $O_2$ , chl  $a$ , and SPM (Fig. 5). Pico- and nanograzers consume heterotrophic bacteria and small phytoplankton cells, while the 'mixo' group includes many of these small phytoplankton and several small dinoflagellates. Ciliates, which dominated the nanoG group, are known to increase at the end of the *Phaeocystis* bloom (between May and July) each year, and graze on the free *P. globosa* cells liberated by the senescent colonies (Grattepanche et al. 2011a,b).

Overall, the environmental parameters explain only a small part of the community structure, and only at the inshore station. Therefore, in order to provide additional information on the protistan communities' complexities, the next step was to investigate the effect of biotic interactions by examining the co-occurrence networks of the protistan communities at each site. These networks reveal elements of the natural history of various microbes (Steele et al. 2011). Our data analysis revealed 2 sub-networks corresponding to each site, highlighting differences between the inshore and offshore communities' organization (Fig. 6). In particular, the inshore station exhibited more complex interactions among the connected OTUs. This was shown by the higher values of almost all topological parameters calculated for each sub-network corresponding to each sampling site. Furthermore, the inshore station had a higher centralization value, indicating the prevalence of focal points at this station, around which the rest of the nodes were organized. The higher centralization value, and most importantly the higher clustering coefficient of the inshore station, suggests that it may have 'small world' properties, with a few highly connected nodes (e.g. the autotroph cluster) compared to

the offshore station (Watts & Strogatz 1998). This property would render the inshore protistan community more resilient to environmental pressures, and more susceptible to removal of highly connected species/nodes (Montoya et al. 2006). Indeed, it has been shown that in trophic webs, robustness increases with connectivity independent of taxa abundance (Dunne et al. 2002) in the same way that increasing species richness can stabilize community structure in the face of perturbation (Tilman et al. 1997). Although classical theory of ecosystem resilience predicts that the stability of food webs should decrease with increasing complexity (May 1974, McCann et al. 1998), Vallina & Le Quéré (2011) demonstrated that with an increase of food web complexity, the overall stability of the ecosystem in fact increases by increasing resistance to climatic perturbations. Undoubtedly, the use of molecular data is foreseeable in the future exploration of ecological questions (e.g. Faust & Raes 2012, Faust et al. 2015, Fuhrman et al. 2015 and references therein). However, the terminology and metrics used in macroecology must be applied with greater caution because the methods available with which to characterize microbial communities remain at 'intermediate resolution'. This is because there is still no means to obtain accurate quantitative data and to determine the accuracy of extraction, bioinformatic analysis, and detection methods (Øvreås & Curtis 2011).

In summary, our results suggest that due to the higher and more variable environmental pressures acting on the inshore station, its protistan community has differentiated from that of the offshore site. This has resulted in the development of more complex connections that are reflected the rapid response of trophic interactions within the whole microbial community. Network analysis demonstrated different degrees of complexity in community structure between the sites, and highlighted the potential importance of microbial interactions in counterbalancing environmental variability at these 2 closely located coastal stations. In addition, the analysis based on functional/trophic roles was found to be more informative than that based solely on taxonomic affiliation.

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