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# Distinct assemblage of planktonic ciliates dominates both photic and deep waters on the New England shelf

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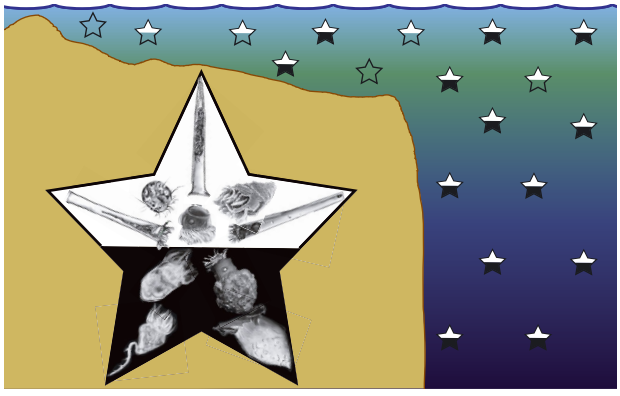
ABSTRACT: Microbes are critical members of marine ecosystems, given their roles as both primary producers and consumers in food webs. Despite their importance, data on biogeographical patterns of microbial eukaryotes are limited. Past studies have generally targeted either all eukaryotes or broad clades like Rhizaria and Alveolata. For this study, we focus more narrowly on oligotrich and choreotrich ciliates (both members of the class Spirotrichea) as these lineages play major roles in marine food webs. We assess distribution patterns of abundant ciliate community members along a 163 km transect off the coast of New England, USA. Over 3 d, we sampled ciliates at 23 stations from shallow waters (<30 m depth) to beyond the continental shelf (>800 m). We used a community DNA fingerprinting technique, denaturing gradient gel electrophoresis (DGGE), to assess patterns for abundant community members and found 2 overlapping assemblages of ciliates: one common in samples from inshore to offshore (up to 180 km from the coast) and from the surface to 850 m deep; and a second that is generally restricted to offshore waters. The distributions of these 2 assemblages correspond with distance from the coast but not with the environmental factors that we measured, including depth, temperature, degree of stratification, phytoplankton fluorescence and accessory pigment composition (a proxy for phytoplankton composition). The presence of these ciliate assemblages as deep as 850 m suggests they may have a broader impact on marine food webs than just photic zone herbivory.

KEY WORDS: Ciliate diversity · Oligotrich · Choreotrich · DGGE · Inshore-offshore · Surface-deep waters





Representative of ciliate assemblages off the New England coast. Ciliates in black portion of star are restricted to offshore; those in the white portion are present almost everywhere. Empty stars represent communities that do not match the assemblages.

*Image: Kelsie Maurer-Alcalá, Jean-David Grattepanche*

## INTRODUCTION

The spatial variability of microbial eukaryotes in marine systems has been explored in only a few studies (reviewed in Dolan 2005, Foissner 2006). Bates et al. (2013) showed that protist communities in general are not globally distributed since composition diverges considerably across large geographic distances. Conversely, Rodríguez-Martínez et al. (2013) showed that particular microbial lineages (e.g. MAST-4) can be found only under specific environmental conditions.

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Overall, the spatial scale of biogeographic patterns for microbial species is unclear (Fenchel et al. 1997, Dolan 2005) as some have argued for cosmopolitan distributions (e.g. Finlay 2002, Massana et al. 2004, Weisse et al. 2008, Bates et al. 2013), while others suggest there is considerable endemism (e.g. Hillebrand et al. 2001, Foissner 2006, Simon et al. 2008, Bik et al. 2012). The distribution of co-occurring assemblages of microbial eukaryotes is not well known either. By comparison, for microbial prokaryotes, DNA-based diversity studies have shown scales of community variability that correlate with ocean circulation and seasonality (e.g. Fuhrman et al. 2006, Hewson et al. 2006).

Ciliates in the clades Oligotrichia and Choreotrichia (Class Spirotrichea) are major components of marine food webs, where they feed upon phytoplankton and bacteria and in turn are fed upon by copepods and other small metazoans (Azam et al. 1983, Fenchel 1988). The diversity and spatial distribution of these marine grazers has been poorly studied despite their critical roles within the 'black box' of marine microbial food webs (Sherr & Sherr 2002, Calbet & Saiz 2005).

Analyses using microscopy have yielded evidence for correlations between species abundance and environmental parameters. Using mouth size as a parameter to group tintinnid ciliates, Dolan et al. (2013) showed that morphospecies distribution is related to the distribution of prey size in 2 Mediterranean gyres and in the California Current. Morphospecies richness in tintinnids has also been shown to correlate with phytoplankton concentration (Santoferrara & Alder 2012).

A number of studies have shown that the morphospecies concept is a biased proxy for biodiversity among ciliates given the frequent detection of cryptic species in analyses using molecular markers (e.g. Katz et al. 2005, Bickford et al. 2007). In oligotrich and choreotrich ciliates, molecular tools have been used to reveal changes in distributions of phylotypes at small spatial scales (~1 km between stations; Grattepanche et al. 2014). Analyses of these 2 clades within a large temperate estuary indicate that biogeographical patterns result from a complex interaction of abiotic and biotic factors (Tamura et al. 2011).

We analyzed common community members of the ciliate orders Oligotrichia and Choreotrichia (Class Spirotrichea) and assessed patterns along a 163 km transect off the coast of New England, USA, from Narragansett Bay (Rhode Island) to the shelf break, sampling at the surface and ~5 m above the ocean floor (for all except the 3 stations after the shelf break; see Fig. 1). Over 3 d, we sampled ciliates at 23 stations from shallow waters (<30 m) to beyond the continental shelf (>800 m). We assessed spatial variability using small subunit rRNA (SSU rDNA) amplicons and denaturing gradient gel electrophoresis (DGGE), and we generated DNA sequences for common community members by excising bands from DGGE gels. Given the structure of the water column during the summer, we hypothesized that ciliates would show a spatial gradient inshore to offshore and reduced diversity below the photic zone (given the potential hypoxia and lack of phytoplankton) as compared to the uppers layers.

## MATERIALS AND METHODS

### Sampling

We sampled on board the R/V 'Cape Hatteras' from just outside Narragansett Bay (RI, USA) to the shelf break, collecting at 23 stations, each 6 km apart (Fig. 1, see Table S1 in Supplement 1 at www.int-res.com/articles/suppl/m526p001_supp/), during 3 d from 6 to 8 July (around 2 h between samplings during the 40 h transect). For every station, 4 depths were sampled: surface, pycnocline, chlorophyll maximum depth and deep layers (Fig. 1 and Dataset S1 in the supplementary material). A CTD profiler (Sea-Bird Electronics) mounted on a rosette measured the temperature, salinity, chlorophyll fluorescence and oxygen, and allowed us to identify the pycnocline (strongest density gradient) and the chlorophyll maximum depth. On the shelf, the deep samples were taken ca. 5 m off the bottom. For stations beyond the shelf break (21, 22, 23), the deepest samples were between 100 and 200 m, with an extra sample at 850 m at the furthest station (Stn 23, ca. 100 m above the bottom).

### Pigment composition (HPLC)

To estimate the phytoplankton composition, 250 ml from each chlorophyll maximum depth sample was filtered on a GF/F filter and stored in the dark at −80°C until extraction and analysis by high-performance liquid chromatography (HPLC) according to Van Heukelem & Thomas (2001).

### Sample filtration and DNA extraction

For each sample, 1 l of seawater was collected in a Niskin bottle and filtered in series through 80 µm mesh to remove metazoans and other large organ-

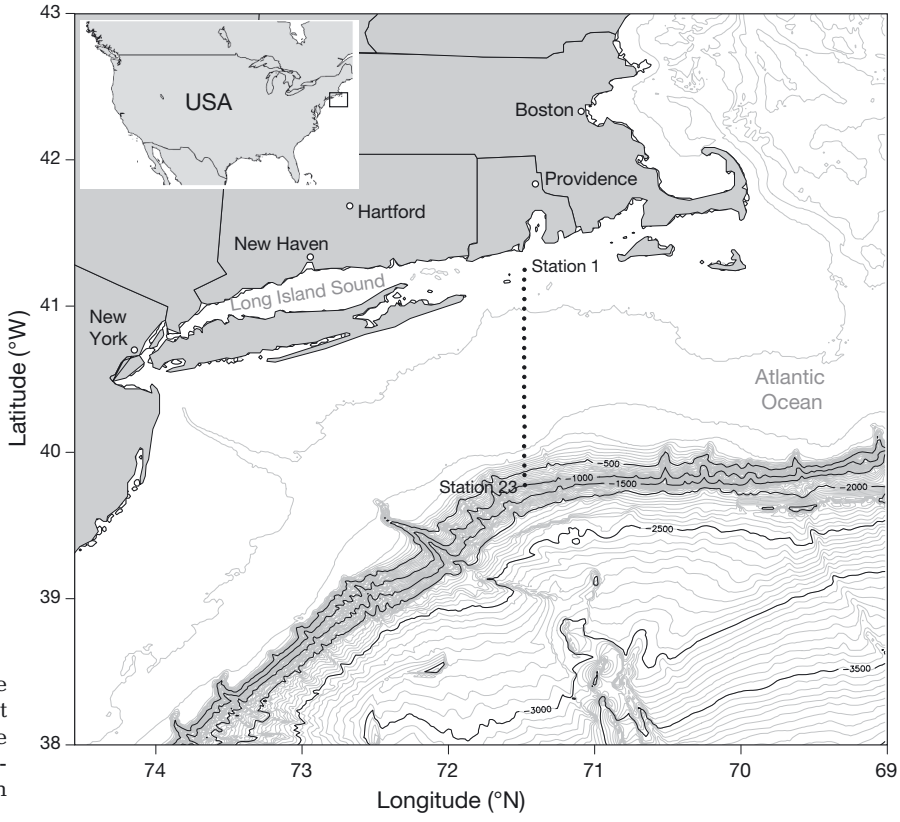


Fig. 1. Station locations during the cruise carried out in July 2012 from just outside Narragansett Bay (Stn 1) to the shelf break (Stn 23). Isobaths are represented with intervals of 50 m depth

isms, then onto 10 and 2 µm polycarbonate filters to separate the micro- and nanosize fractions. We used sequential filtration on 10 and 2 µm pore sizes to assess the smallest ciliates, which are generally underestimated in microscope-based studies. We also filtered water samples through separate 3 µm nitrocellulose filters to compare with the size fractionation. All filters were immediately placed in 0.5 ml of DNA preparation buffer (100 mM NaCl, Tris-EDTA at pH 8, and 0.5% sodium dodecyl sulfate [SDS]) and stored at 4°C until DNA extraction. A standard phenol-chloroform extraction protocol (Sambrook et al. 1989, Ausubel et al. 2002) was carried out to extract and purify the DNA using a method adapted to filters (Costas et al. 2007). The final DNA pellet was air dried and re-suspended in 50 µl Tris-EDTA (ph 8.0) and 0.1 µl RNase.

### DNA amplification

The DNA from the filters of each station was amplified under conditions aimed at minimizing PCR recombination. PCR was carried out using 20 µl of master mix, composed of 4 µl 5× GC buffer (NEB), 0.5 mM MgCl, 50 mM BSA, 50 µM of each deoxynucleoside triphosphate (dNTP), 0.25 pM of each primer, and 0.1 µl Phusion polymerase (NEB). We used SSU rDNA primers specific to choreotrich and oligotrich ciliates (152+ and 528-GC from Doherty et al. 2007, Tamura et al. 2011). These target regions were amplified by PCR under the following conditions: 98°C for 3 min, 30 cycles of 98°C for 15 s, 58°C for 15 s, and 72°C for 1 min, and a final extension at 72°C for 10 min.

### DGGE analysis

For each sample, 5 PCR products amplified from a 1/10 dilution of genomic DNA were pooled prior to DGGE. Each station (8 samples: 2 and 10 µm samples from surface, pycnocline, chlorophyll maximum, and deep layer, respectively) was run on an independent DGGE gel. DGGE gels (6% acrylamide gel) containing a linear denaturant gradient from 35 to 55% (100% denaturant corresponds to 7 M urea and 40% deionized formamide) were run at 245 V for 5 min followed by an incubation at 45 V for at least 15 h using a Dcode universal mutation system (Bio-Rad). We verified the robustness of DGGE by replicating several gels using PCR reactions that had been run on different days. The brightest bands and all common bands were excised from the gels, amplified by 10 cycles of PCR following the aforementioned cycling

conditions and sequenced by the Sanger method (sequences are available at GenBank under accession numbers KR056176-KR056216). We assigned taxonomy to each DGGE band sequenced using a BLAST approach and confirmed this assignment using a phylogenetic tree (Fig. 2). However, given the relative shortness of our sequences and the low interspecific variability within the oligotrich and choreotrich ciliates (e.g. Santoferrara et al. 2015), we recognize the uncertainty in these assignments, which should be viewed as the closest morphospecies from sequences in GenBank. In order to compare the DGGEs from each station, we created a set of markers representing known phylotypes to run on gels (Grattepanche et al. 2014).

DGGE gels were photographed and band intensity measured using Kodak molecular imaging software (Carestream Health) after staining for 30 min in 200 ml of Tris-acetate-EDTA (TAE) buffer with 20 µl of SYBR Gold (Invitrogen). The ciliate community structure obtained by DGGE was analyzed with Fast UniFrac software (Hamady et al. 2010) based on band pattern and intensity. For Fast UniFrac inputs, we used a star tree (phylogenetic tree with same distance between each phylotype or DGGE band and the root), a 'map' of phylotypes (i.e. presence/absence or abundance table for each DGGE band or phylotype at each sample or station; see Dataset S2 in the supplementary material) presented at each sample or station, and a 'map' of the environmental conditions for each sample (see Dataset S1 in the supplementary material). To compare environment patterns, we performed the same analysis using environmental parameters (temperature, salinity, oxygen and chlorophyll fluorescence) as starting points. In the DGGE bands cluster analysis, phylotypes are considered as variables of each sample/station. In the environment clustering analysis, the environmental parameters are considered as the variables of each sample/station. To avoid sample clustering related only to depth, we performed the clustering analysis without this parameter. The samples or stations were clustered using the unweighted pair group method with arithmetic mean (UPGMA). The robustness of the UPGMA clusters was tested with jackknife analysis based on 50 permutations. The phylotype distribution obtained by DGGE was analyzed with hierarchical clustering analyses of Euclidean distance using MATLAB (version R2012a). We confirmed the presence of the 2 ciliate assemblages by repeating DGGE with 1 representative sample from each station to yield results similar to those described by Grattepanche et al. (2014).

### Statistical analysis

To compare the structure given by the environmental parameters to the community structure, sample clustering analysis was performed using Fast UniFrac (Hamady et al. 2010), and hierarchical clustering of the Euclidean dissimilarity index in MATLAB was used to identify the ciliate assemblages.

### Repeatability of DGGE

We replicated PCR and DGGE gels multiple times to ensure that DGGE methods were robust enough to capture the ciliate community. Repeating the DGGE with the pooled PCR products generated the same band pattern for each sample replicated. We confirm here the robustness of DGGE, which we have also assessed in another study (Grattepanche et al. 2014).

## RESULTS AND DISCUSSION

### Overall patterns in the DGGE

DGGE allows the abundant members of a community to be quantified and subsequently identified by DNA sequencing. We used DGGE to analyze communities from a total of 190 samples (23 stations, at least 4 depths, 2 size fractions). Amplification with species-specific primers of samples collected sequentially on 10 and 2 µm filters generated a profile of common community members at stations 6 km apart (Fig. S1 and Dataset S2 in the supplementary material). We observed a total of 38 unique bands in DGGE gels, each corresponding to a single sequence (i.e. phylotype) of a common community member. Individual bands were present in as few as 2 and up to as many as 178 of the 190 total samples (91 of the 97 samples if pooling the 2 size fractions; Fig. S1, Dataset S2). Across all depths, the number of abundant phylotypes per station varied from 2 to 22, with generally more bands at offshore sites (Fig. S1, Dataset S2).

### Two assemblages of common ciliates: 'common' and 'offshore'

Our analyses revealed the presence of 2 assemblages of co-occurring ciliates. One consists of 6 ciliate species that are found in many, but not all,

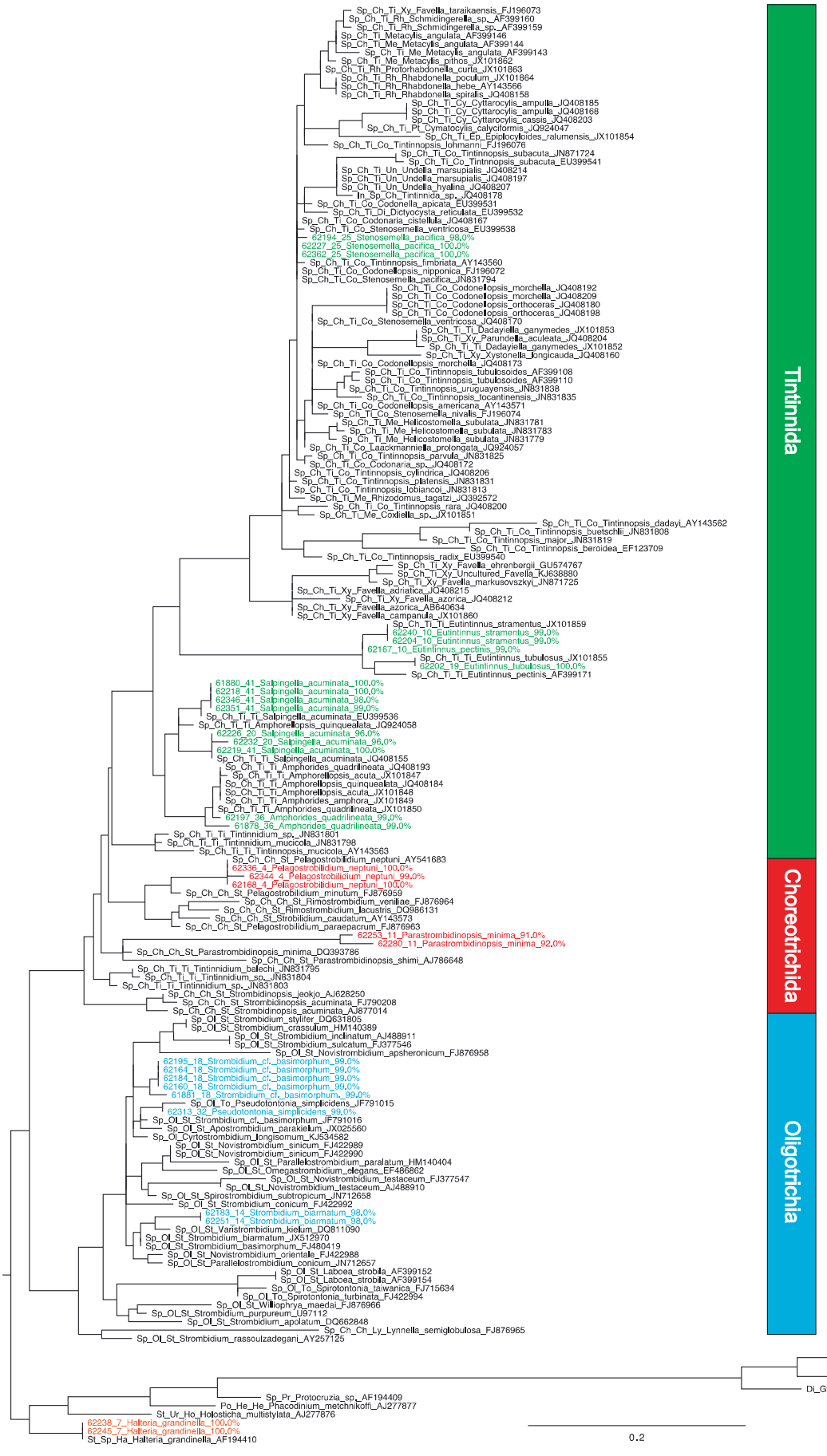


Fig. 2. Phylogenetic tree used to confirm the BLAST taxonomic assignment. The taxa in color represent the sequenced DGGE bands (Table S1 in Supplement 1). The scale bar represents the number of differences per base pair. The maximum likelihood tree built using RaxML and a Newick formatted version is available in the supplementary material (www.int-res.com/articles/suppl/m526p001_supp/)

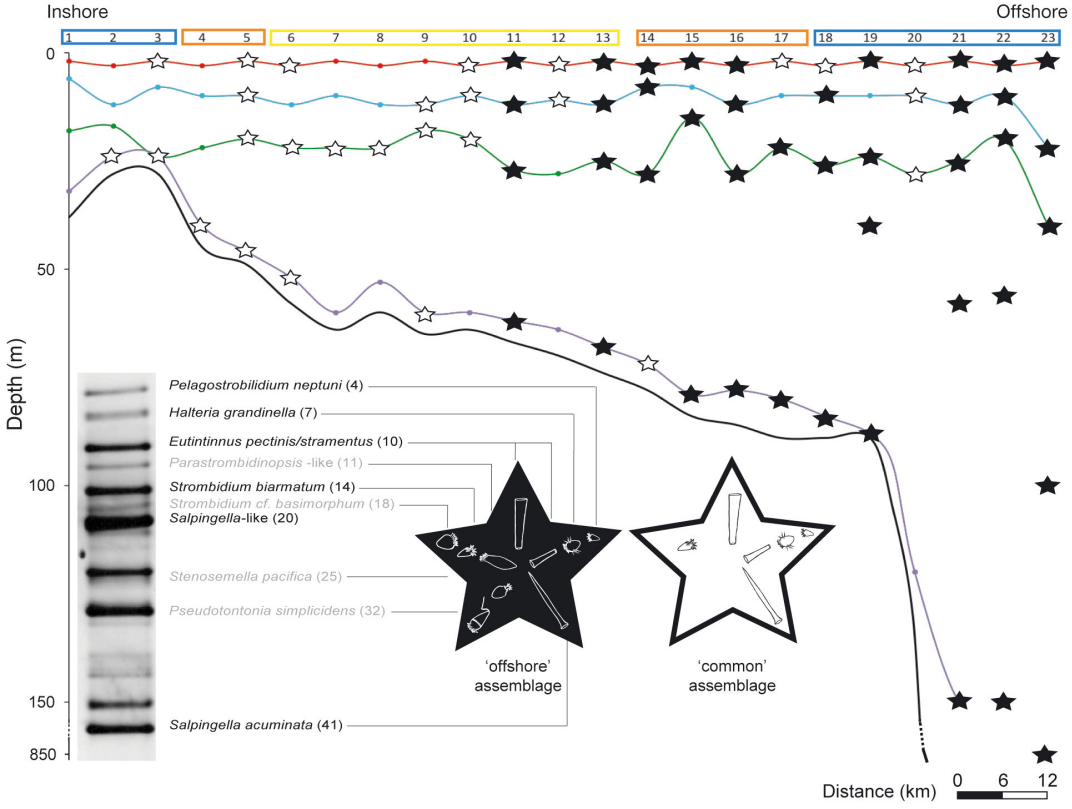


Fig. 3. Distribution of the 2 ciliate assemblages from inshore to the shelf break assessed by denaturing gradient gel electrophoresis (DGGE). The 'common' assemblage (open stars) was observed at many but not all locations, and was joined by an offshore assemblage (black stars) in many stations >90 km from the shore and greater than 67 m depth (Figs. S1 & S2 in Supplement 1 at www.int-res.com/articles/suppl/m526p001_supp/). The colored lines represent the different layers, and the dots, the samples: (red) surface, (turquoise) pycnocline, (green) chlorophyll maximum and (purple) deep (see Fig. 4 for more details). The boxes around the station numbers represent the stratification level: (yellow) stratified water, (blue) mixed water, and (orange) intermediate stratification levels. The closest morphospecies members of the 'common' and 'offshore' ciliate assemblages observed by DGGE (the 850 m sample lane for Stn 23 is used in this figure) are represented inside the stars. The 6 morphospecies listed in black print are the members of the 'common' ciliate assemblage; morphospecies listed in grey print are the 4 additional morphospecies found in the 'offshore' ciliates assemblage. The values in parentheses refer to the band numbers from DGGE gels (Table S1 in Supplement 1)

samples ranging from inshore to offshore and from surface to deep waters (i.e. 'common' assemblage). The second assemblage overlapped the first one at offshore stations and includes the 6 'common' species plus 4 additional species (Fig. 3, Figs. S1 & S2 in Supplement 1 at www.int-res.com/articles/suppl/m526p001_supp/). The presence of 2 assemblages is evident in hierarchical clustering analyses of DGGE bands (Fig. S2). For this study, we used previously designed primers that show considerable specificity for oligotrich and choreotrich ciliates as they capture representatives of most known genera (Doherty et al. 2007, Tamura et al. 2011, Grattepanche et al. 2014, Santoferrara et al. 2014). We recognize that no primer can capture all taxa, and that we likely missed some cryptic species (e.g. those not distinguished by variation in the targeted V1–V2 region), and unknown clades (those not included when we designed primers). Only 1 band (39) represented a non-target organism, being 99% similar to the dinoflagellate *Heterocapsa triquetra* GU594638, thus indicating some level of non-specificity in our primers (Doherty et al. 2007, Tamura et al. 2011, Grattepanche et al. 2014).

Sequencing common community members revealed that 7 of the 10 bands are 99% identical to known morphospecies. The remaining 3 are no closer than 97% to any previously reported sequences. The common assemblage (bands 4, 7, 10, 14, 20 and 41) consists of 3 tintinnids, 1 aloricate choreotrich, and 2 oligotrichs (Fig. 3, Table S1). The offshore assemblage incorporated 4 additional members, 1 tintinnid, 1 aloricate choreotrich, and 2 oligotrichs. Surprisingly, 1 phylotype of the common assemblage (band 7) is

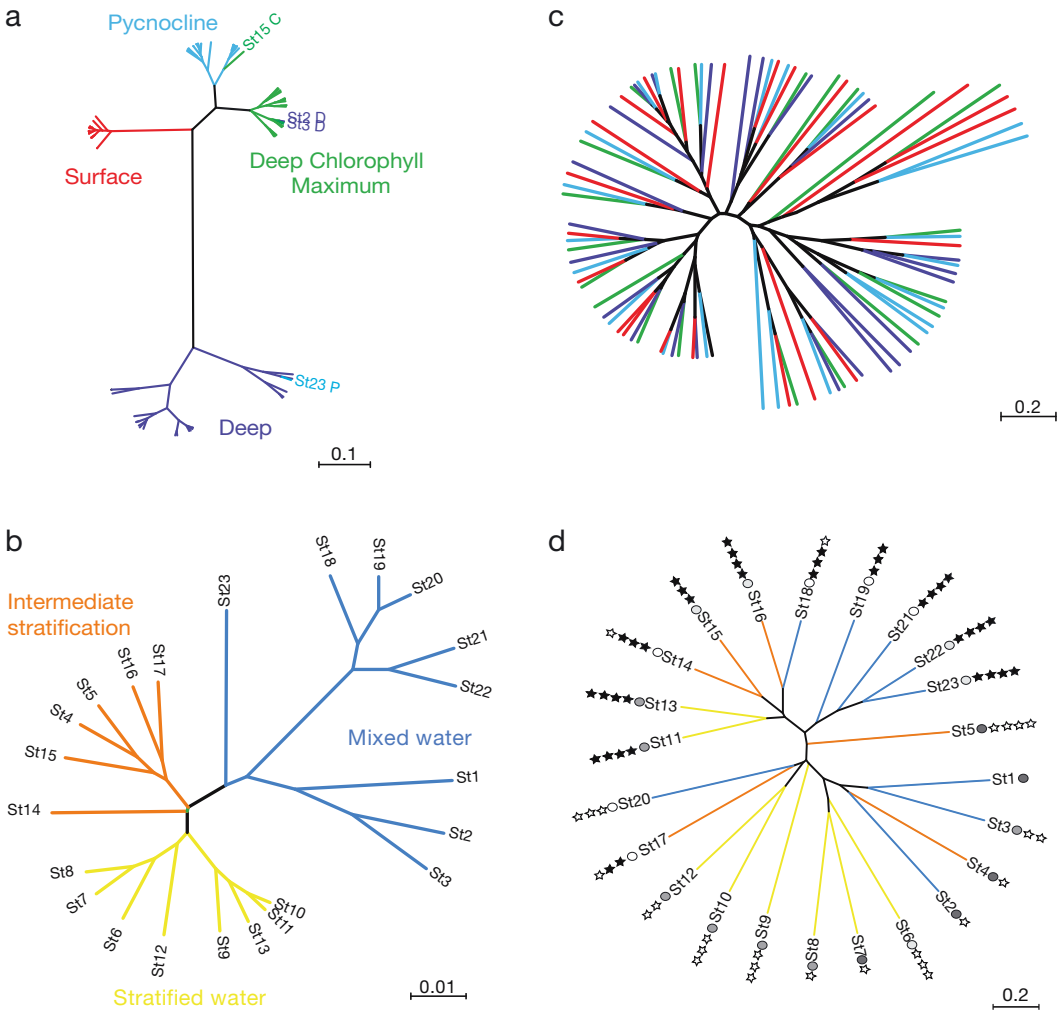


Fig. 4. Clustering of samples (station and depth) using Fast UniFrac (Hamady et al. 2010) by environmental variables and ciliate diversity shows different patterns. (a) Samples clustered according to water layers (surface, pycnocline, chlorophyll maximum and deep layers) using environmental parameters (temperature, salinity, oxygen and fluorescence), (b) stations (i.e. all layers from the same station grouped in 1 'sample') clustered by nature of the water mass (mixed or stratified) using the same environmental parameters, (c) samples clustered by DGGE band pattern do not correlate with layers and (d) clustering of DGGE bands by station is also unrelated to stratification level or pigment composition. The presence of the 'common' (white star) and the 'offshore' (black star) communities (Fig. 3, and Fig. S1 in Supplement 1) and the different phytoplankton communities (dark gray dot: inshore; gray dot: middle shore; light gray and white dots: 2 offshore communities) assessed by HPLC pigment composition (Fig. S5 in Supplement 1) are indicated. Networks drawn to scale; the scale bar shows the distance between clusters in UniFrac units (distance matrix where 0 = identical samples, 0.5 = samples composed of different ciliates)

closely related to *Halteria grandinella*, an exclusively freshwater ciliate. This suggests the possibility of previously undiscovered biodiversity in marine systems. While the ciliate *H. grandinella* has not been documented in marine samples (Agatha 2011), we observed a phylotype with a V1–V2 sequence among our DGGE sequences with 99% similarity (Table S1); this phylotype might be another Halteriidae such as *Pelagohalteria cirrifera*, which has been reported in the North Atlantic (Agatha 2011) and which lacks a DNA sequence in GenBank.

### Environmental correlates for presence/absence of assemblages

The presence of the 2 ciliate assemblages does not correlate with any of the environmental parameters we measured, even though the environmental parameters themselves are structured by both water layer and location (Fig. 4). A Fast UniFrac cluster analysis (Hamady et al. 2010) using temperature, oxygen, chlorophyll fluorescence and salinity for all samples revealed that

each depth layer is distinct, with few exceptions (Fig. 4a). Similarly, the samples from each station (i.e. the temperature, chlorophyll fluorescence, salinity and oxygen across all depths) cluster on the basis of stratification level: inshore and offshore stations are marked by mixed water masses, while intermediate stations are stratified, i.e. dynamic versus more stable environment (Fig. 4b, and Fig. S3 in Supplement 1).

In contrast to environmental parameters, no clear patterns emerged in analyses of the community structure based on DGGE when we considered relative abundances (brightness of bands; Dataset S2 in the supplementary material), presence/absence of phylotypes as related to the water layers (Fig. 4c), level of stratification (Fig. 4d) or size fraction (Fig. S4 in Supplement 1). In other words, the 'common' and 'offshore' assemblages are not clearly related to the water masses (temperature, oxygen and pressure/depth) or to the estimated phytoplankton biomass and composition (chlorophyll concentration and accessory pigment composition; Fig. 4, and Fig. S5 in Supplement 1). Our data suggest that there is a widespread ciliate community offshore and that some members are excluded from nearshore waters, giving us the distinct 'offshore' versus 'common' assemblages.

Given that the oligotrich and choreotrich ciliates are believed to feed principally on phytoplankton, the detection of a highly diversified ciliate assemblage in deep samples, even at 850 m, is unexpected. Wickham et al. (2011) also observed oligotrich and choreotrich ciliates in morphological analyses of deep samples (up to 500 m), but these were less diverse than the upper layers community. This suggests that these diverse ciliates may also feed on bacteria (Sherr & Sherr 1987) or other heterotrophic protists (Sherr & Sherr 2002) below the photic zone. Alternatively, we may be detecting cysts at deep levels, though it would be surprising to find the offshore assemblage containing relatively constant abundances of both cysts and feeding stages throughout the water column, as is observed at all depths for Stn 23 (Fig. 3, and Fig. S1 in Supplement 1). It is also possible that the presence of these ciliate assemblages in deep samples is due to sinking organic particles or, given that we sampled so near the shelf break (Stns 21 to 23), to circulation patterns. Due to the structure of our sampling (40 h outbound), we were unable to assess patterns relating to the tidal cycles, unlike in our previous study, that followed a single water mass (Grattepanche et al. 2014).

## Synthesis

The presence of the common ciliate assemblage in our analyses of ciliate communities along a 163 km transect suggests that some phylotypes are widely adaptable to various environments and/or have a large niche space. The common assemblage occurs at various stations and layers representing gradients in light, temperature, phytoplankton abundance, nutrients, etc. Similarly, the distribution of the offshore assemblage does not correspond with the environmental parameters that we measured (e.g. depth, phytoplankton fluorescence, temperature), except for distance from the coast. An alternative explanation could be that these ciliate assemblages follow the unified neutral theory of biodiversity, as observed by Dolan et al. (2007) for tintinnid morphospecies. The unified neutral theory holds that local assemblages are put together from species randomly selected from a larger metacommunity of ecologically similar organisms. Open questions here include whether the common community is stable over time and in regions well beyond the shelf, and what drives the loss of assemblage members in some areas.

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