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Depth matters: Microbial eukaryote diversity and community structure in the eastern North Pacific revealed through environmental gene libraries

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ABSTRACT

Protistan community structure was examined from 6 depths (1.5, 20, 42, 150, 500, 880 m) at a coastal ocean site in the San Pedro Channel, California. A total of 856 partial length 18S rDNA protistan sequences from the six clone libraries were analyzed to characterize diversity present at each depth. The sequences were grouped into a total of 259 Operational Taxonomic Units (OTUs) that were inferred using an automated OTU calling program that formed OTUs with approximately species-level distinction (95% sequence similarity). Most OTUs (194 out of 259) were observed at only one specific depth, and only two were present in clone libraries from all depths. OTUs were obtained from 21 major protistan taxonomic groups determined by their closest BLAST matches to identified protists in the NCBI database. Approximately 74% of the detected OTUs belonged to the Chromalveolates, with Group II alveolates making up the largest single group. Protistan assemblages at euphotic depths (1.5, 20 and 42 m) were characterized by the presence of clades that contained phototrophic species (stramenopiles, chlorophytes and haptophytes) as well as consumers (especially ciliates). Assemblages in the lower water column (150, 500 and 800 m) were distinct from communities at shallow depths because of strong contributions from taxa belonging to euglenozoans, acantharians, polycystines and Taxopodida (*Sticholonche* spp. and close relatives). Species richness (Chao I estimate) and diversity (Shannon index) were highest within the euphotic zone and at 150 m, and lowest for protistan assemblages located in the oxygen minimum zone (500 and 880 m). Multivariate analyses (Bray–Curtis coefficient) confirmed that protistan assemblage composition differed significantly when samples were grouped into shallow ($\leq$150 m) and deep water assemblages ($\geq$150 m).

1. Introduction

Protistan assemblages play pivotal ecological roles in marine ecosystems as primary producers and consumers (Azam et al., 1983; Sherr and Sherr, 2002). Despite their importance, our knowledge is still rudimentary regarding total protistan species diversity and community structure in most natural environments, and how these features relate to ecosystem function. The acquisition of this information has been impeded in part by the enormous diversity of microbial eukaryote assemblages and the complex, morphology-based taxonomies that are employed for these species (Caron, 2009). Culture-independent approaches for investigating microbial diversity have begun to provide new tools for examining species richness of microbial eukaryote assemblages, as well as the biogeography and ecology of specific taxa (Caron et al., 2004; Caron and Schnetzer, 2007; Caron and Gast, 2008; López-García et al., 2003; Massana et al., 2006b). In particular, the use of DNA sequence analysis and fragment analysis are beginning to allow for more complete characterization of the protistan taxa that are present in aquatic and terrestrial ecosystems than have been possible using methods of microscopy and culture. Although still in their infancy, molecular analyses have already revealed several previously undetected, undescribed lineages of protists (Doherty et al., 2007; Guillou et al., 1999; López-García et al., 2001; Shalchian-Tabrizi et al., 2006; Stoeck and Epstein, 2003; Von Der Heyden et al., 2004), and provided new insights into protistan phylogeny and life histories (Berney and Pawlowski, 2003; Gast et al., 2007; Massana et al., 2006b; Moreira et al., 2007).

These studies have also demonstrated a recurring pattern of community structure for natural protistan assemblages. Rank abundance curves (graphical representations of the number of sequences within Operational Taxonomic Units; OTUs) have revealed protistan assemblages that are characterized by a relatively small number of abundant taxa and an extremely large

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number of taxa present at very low abundance, the so-called microbial 'Rare Biosphere' (Amaral-Zettler et al., 2009; Caron and Countway, 2009; Sogin et al., 2006). Thus far, the presence of these rare taxa has made it exceedingly difficult to estimate the total species richness of microbial communities. The significance of many of the taxa making up the Rare Biosphere is presently unknown, although it has been conjectured that at least some of these species can increase rapidly in abundance and play important ecological roles when environmental conditions change and favor their growth (Caron and Countway, 2009). If this is true, then rapid temporal shifts in protistan assemblage composition might be anticipated in nature, and dramatic differences in community structure might be expected across relatively small spatial scales.

Molecular investigations of protistan diversity to date have included protistan populations from a variety of habitats, including unique or extreme (mostly anoxic) environments, and have covered geographically distant locations (Amaral-Zettler et al., 2002; Dawson and Pace, 2002; Countway et al., 2007; Edgcomb et al., 2002; Moon-Van Der Staay et al., 2001; Stoeck et al., 2003; Zuendorf et al., 2006). Some of these studies have examined small-scale spatial or temporal patterns in protistan community structure and diversity (Behnke et al., 2006; Countway et al., 2005; Countway et al., 2007; Countway et al., 2010; Lovejoy et al., 2006; Medlin et al., 2006; Not et al., 2007; Vigil et al., 2009). Latter studies have begun to demonstrate that the dominant protistan taxa within an assemblage can differ markedly over spatial scales associated with common oceanographic features, and temporally in one location over time scales of several days. Even modest shifts in environmental parameters during the incubation of water samples can substantially alter the protistan community structure (Countway et al., 2005). These studies indicate the potential for rapid community reassembly in response to changes in environmental conditions, and the need to conduct surveys at higher spatial and temporal resolution.

Protistan community structure and diversity was examined at six depths in the San Pedro Channel, California, using 18S rDNA gene sequence information. Analyses included all protists $<200\,\mu m$ in size, in contrast to previous vertical distribution studies that specifically targeted small-sized eukaryotes ($<5\,\mu m$) (i.e., López-García et al., 2001; Not et al., 2007). Similarly to previous surveys this survey included the collection of protistan assemblages from within a minimum oxygen zone (i.e., Countway et al., 2007; Countway et al., 2010; Not et al., 2007). Overall, members of the alveolates (mainly ciliates, dinoflagellates and Groups I and II alveolates) outnumbered all other protists in each of our clone libraries. Lineages containing phototrophic taxa (e.g. chlorophytes, stramenopiles, haptophytes) and their predators (e.g. ciliates) were dominant in the euphotic zone, while euglenozoans, Taxopodida, polycystines and acantharians were almost exclusively found deeper in the water column (150, 500 and 880 m). Species richness and diversity estimates were highest for assemblages in surface waters and from 150 m, and were lowest for communities within the oxygen minimum zone from 500 and 880 m depth. Protistan assemblages from adjacent depths were not significantly different and shared up to 35% of their community structure, but significant differences in community makeup existed between samples grouped according to depth (<150 and ≥ 150 m). Surprisingly, only two OTUs were found at all six depths. However, it is unclear if this result indicates the presence of endemic assemblages at these different depths in the San Pedro Channel, or strong environmental selection for different dominant taxa from an extremely large number of ubiquitously dispersed taxa.

2. Material and methods

2.1. Study site and sample collection

Seawater was collected from the central San Pedro Channel approximately 32 km off Long Beach/San Pedro, California (33°33′N and 118°24′W) on June 28, 2001 to investigate changes in eukaryotic community structure (Fig. 1). This location is the study site of the USC Microbial Observatory, which focuses on microbial diversity (bacteria, archaea and protists) in the coastal waters of the eastern North Pacific (http://www.usc.edu/dept/LAS/biosci/Caron_lab/MO/). Data on water column properties were obtained as a part of the San Pedro Oceanographic Time-series

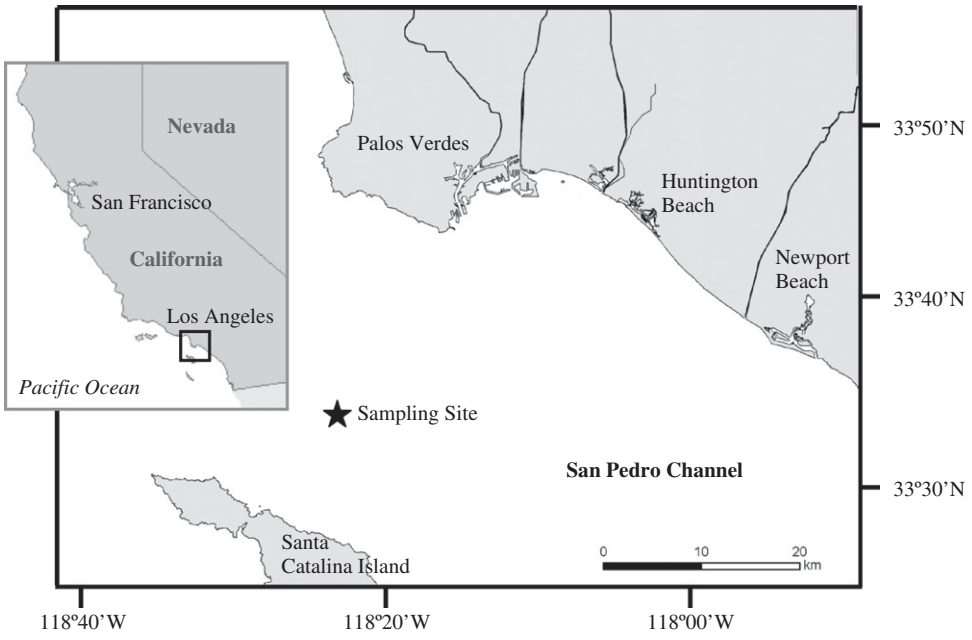

Fig. 1. Sampling location in the central San Pedro Channel of the Southern California Ocean. The study site was located approximately midway between the Los Angeles Harbor and Santa Catalina Island.

program (SPOT; http://wrigley.usc.edu/research/spot.html). Temperature, oxygen, salinity and chlorophyll measurements were determined using a Seabird CTD sensor package. Water samples for silicate, orthophosphate and nitrate were collected at multiple depths using 10 L Niskin bottles attached to a CTD rosette and frozen. Analyses were conducted on thawed samples using an Alpkem RFA AutoAnalyzer (Gordon et al., 1993). Two liters of seawater from 6 discrete depths (1.5, 20, 42 (location of a deep chlorophyll maximum or DCM), 150, 500 and 880 m) were passed through 200 μm Nitex screening to remove most metazoa, and then filtered onto 47 mm GF/F Whatman filters (0.65 μm pore size) for biomass collection and molecular analyses. Filters were immediately transferred into 15 mL centrifuge tubes, 2 mL of 2X lysis buffer were added (40 mM EDTA, pH 8; 100 mM Trizma-Base, pH 8; 100 mM NaCl; 1% SDS), and the samples stored at −20 °C.

2.2. DNA extraction, cloning and sequencing

Samples were processed to create clone libraries of small subunit ribosomal RNA genes (18S). Eukaryote community structure based on partial sequencing (450–700 bp) was determined for samples collected from 1.5, 20, 42 (DCM), 150, 500 and 880 m depth (see detailed methods in Countway et al., 2007). Briefly, the centrifuge tubes containing the GF/F filters were thawed in a water bath and then subjected to three cycles of bead-beating (vortexed with silica beads for 30 s) and heating at 70 °C for 5 min. The lysates were transferred into sterile centrifuge tubes, CTAB (1% final concentration) and NaCl (0.7 M final concentration) were added to the extracts and the tubes were incubated at 70 °C for 10 min. DNA cleanup was achieved by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), followed by DNA precipitation (1X volume of ice-cold 95% ethanol and 0.1X volume of 10.5 M ammonium acetate) and resuspension of dried DNA pellets in 50 μL sterile TE (10 mM Tris, 1 mM EDTA, pH=7.5) (Countway et al., 2007).

The 18S ribosomal genes were amplified by polymerase chain reaction (PCR) using eukaryotic specific primers Euk A (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and Euk B (5′-GAT CCT TCT GCA GGT TCA CCT AC-3′; Medlin et al., 1988). The PCR products were run on agarose gels, bands of the appropriate size were cut out and cleaned using a DNA recovery kit (Zymo Research, Orange CA). The purified PCR products were cloned using a commercially available cloning kit (pGEM®-T Easy Vector System I from Promega, Madison WI), Stratagene ElectroTen-Blue Competent cells (Agilent, La Jolla CA) and the Gene Pulser Xcell™ Electroporation System (Bio-Rad, Heracules CA). Plasmids were purified using the Wizard SV-96 Plasmid Purification System from Promega and stored frozen at −20 °C for later sequencing using a Beckman Coulter CEQ 8800 system. Partial sequence was obtained for all clones (450–700 bases) using a universal eukaryotic primer 575 F (5′-GTAATTC-CAGCTCCAATAGC-3′; Weekers et al., 1994) in sequence reactions. A total of 1231 clones were sequenced from six clone libraries of 137–233 clones per library.

2.3. Sequence data processing

Processing of sequence information for community analyses entailed a series of quality controls, pairwise alignments of all sequences, and the use of an OTU-calling algorithm as described in Caron et al. (2009). Sequence reads were quality trimmed on both ends using a medium stringency setting within the CEQ™8800 Sequence Analyses Software. This was followed by manual inspection of chromatogram files and chimera checking provided through the Ribosomal Database Project (Cole et al., 2003). The libraries from all depths were then combined and pairwise sequence alignments performed using ClustalW 1.83 (Thompson et al., 1994). The number of Operational Taxonomic Units (OTUs) was determined using an automated program for assigning OTUs at approximately species-level distinctions (95% sequence similarity) based on an analysis of a large protistan sequence dataset (MESA: Microbial Eukaryote Species Assignment; Caron et al., 2009). Finally, sequence similarities to protists in the National Center for Biotechnology Information database (http://www.ncbi.nih.gov) were examined to provide taxonomic information on the OTUs (Altschul et al., 1997). This taxonomic assignment was based on the best match to a well-described protist returned by the BLAST search, which commonly followed matches to uncultured and/or unidentified eukaryote sequences in NCBI. Sequence data from this study was deposited in GenBank (accession numbers GQ382277–GQ383513).

2.4. Species diversity and species richness

Eukaryotic assemblages at the six depths were first compared by determining the relative contribution of metazoan and protistan taxa to each clone library. All metazoan sequences were removed from the dataset and the remaining sequences and OTUs were used to estimate species diversity and species richness EstimateS 7.51 (Colwell, 2004). The inverse Simpson diversity index (Ds^{-1}) which takes the number of species present and their relative abundance into account (Simpson, 1949) was calculated, as was the non-parametric richness estimator Chao1 (Chao, 1984, 1987). The Chao1 estimator was employed to predict total species richness (95% CI) within each individual library and within the complete dataset (all depths combined) based on the observed number of OTUs (Hughes et al., 2001). This estimate is based on the abundance of 'rarer' organisms which are represented as singletons and doubletons (OTUs that contain 1 or 2 clones, respectively).

2.5. Similarity among protistan assemblages from varying depths

The similarity of the protistan communities at the six depths was examined using the Bray–Curtis coefficient within the statistical software package PRIMER v.6 following the normalization of OTUs to relative abundances within each library (Clarke and Warwick, 2001). A square-root transformation of the data was performed to reduce the influence of highly represented OTUs. This transformation prevents a few well represented OTUs from driving the similarity analysis (Bray and Curtis, 1957; Clarke and Warwick, 2001). The Bray–Curtis coefficient is not affected by joint absences of OTUs between clone libraries (Clarke and Warwick, 2001). The transformed results for the Bray–Curtis test were further used in cluster analysis and non-metric multidimensional scaling (MDS) to examine if dissimilarities among the assemblages were significant (PRIMER v.6; Clarke and Warwick, 2001).

3. Results

3.1. Hydrological data

Biological, chemical and physical water column properties in the central San Pedro Channel exhibited different environmental conditions at each of the depths sampled (Fig. 2). The water column was characterized by a well defined pycnocline with the steepest gradients in salinity, temperature and dissolved oxygen between approximately 20 and 100 m (Fig. 2A, B). An oxygen minimum layer was well established within the lower water column (Fig. 2B). Salinity of >34.25, temperature of <7 °C and oxygen levels <1 mL O_2 L^{-1} were recorded at depths below 300 m (Fig. 2A, B). The deep chlorophyll maximum (DCM) on the day of sampling was

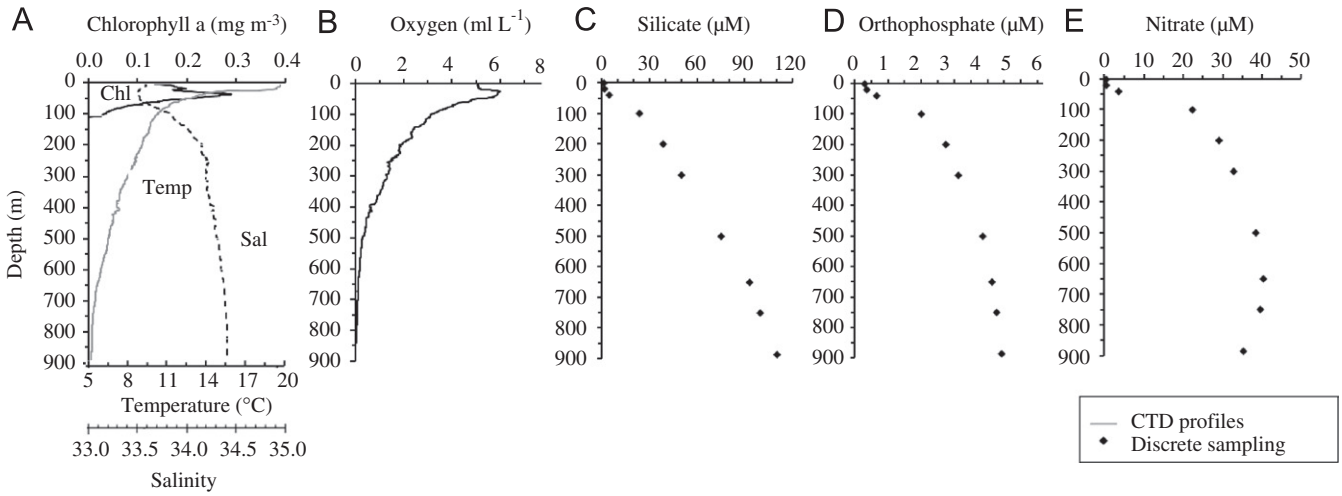

Fig. 2. Physical, chemical and biological profiles of the water column at the study site collected on June 28, 2001. Temperature, salinity, chlorophyll-*a* and oxygen concentrations were documented using a CTD sensor package (panels A and B) and 10 L Niskin bottles were used to collect discrete water samples to measure inorganic nutrient concentrations (panels C–E). Note different scale on *y*-axis for panel D.

Table 1
Physicochemical parameters of the water column measured in the central San Pedro Channel on June 28, 2001. bd: below detection.

Depth (m)	1.5	20	42	150	500	880
Salinity (ppt)	33.6	33.6	33.5	34.0	34.3	34.4
Temperature (°C)	19.5	17.8	12.9	9.5	6.4	5.1
Diss. oxygen (ml L^{-1})	5.1	5.3	5.7	2.4	0.3	bd
Chlorophyll *a* (μg L^{-1})	0.1	0.2	0.3	bd	bd	bd
NO_3^- (μM)	0.5	0.6	3.7	25.7[a]	38.4	35.2
PO_4^{3-} (μM)	0.3	0.4	0.7	2.4[a]	3.9	4.4
$Si(OH)_4$ (μM)	1.1	1.4	4.4	30.9[a]	75.0	110.0

[a] No discrete water sample was available for inorganic nutrient analyses from 150 m. Values are averaged from concentrations measured at 100 and 200 m depth.

detected at approximately 42 m (Table 1 and Fig. 2A). Inorganic nutrient concentrations in the upper mixed layer were less than 5 μM for $Si(OH)_4$ and NO_3^- and 1 μM for PO_4^{3-}, and increased substantially with depth (Table 1 and Fig. 2C–E).

3.2. Relative abundance of eukaryotic taxa

A total of 1237 partial-length 18S rDNA eukaryotic sequences were analyzed (all depths combined). A total of 381 reads across all libraries had close phylogenetic affinities to metazoan phyla including Arthropoda (mainly copepods), Cnidaria (mainly hydrozoans), Ctenophora and Appendicularia (Fig. 3A). The relative contribution of metazoa to each of the clone libraries generally decreased with depth from 55% at 1.5 m to 15% at 880 m (data not shown). Copepods dominated the metazoa observed in upper water column libraries (1.5, 20 and 150 m) with the exception of the DCM (42 m) where ctenophore sequences outnumbered all other metazoan taxa. Hydrozoans became increasingly dominant in the lower water column (150, 500–880 m). Appendicularia were most significant at 880 m depth (Fig. 3A).

Overall, 856 protistan sequences (range = 106–166 per depth) with affiliations to 21 major taxonomic groups were analyzed to examine protistan community structure in the San Pedro Channel (Tables 2 and 3). Collectively, members of the Alveolata, which was comprised primarily of dinoflagellates, ciliates and novel alveolate Groups I and II, contributed more than half of the sequences to each of the libraries (55–77% of protistan clones; Table 2 and Fig. 3B). Sequences with closest BLAST matches to the ciliates, dinoflagellates

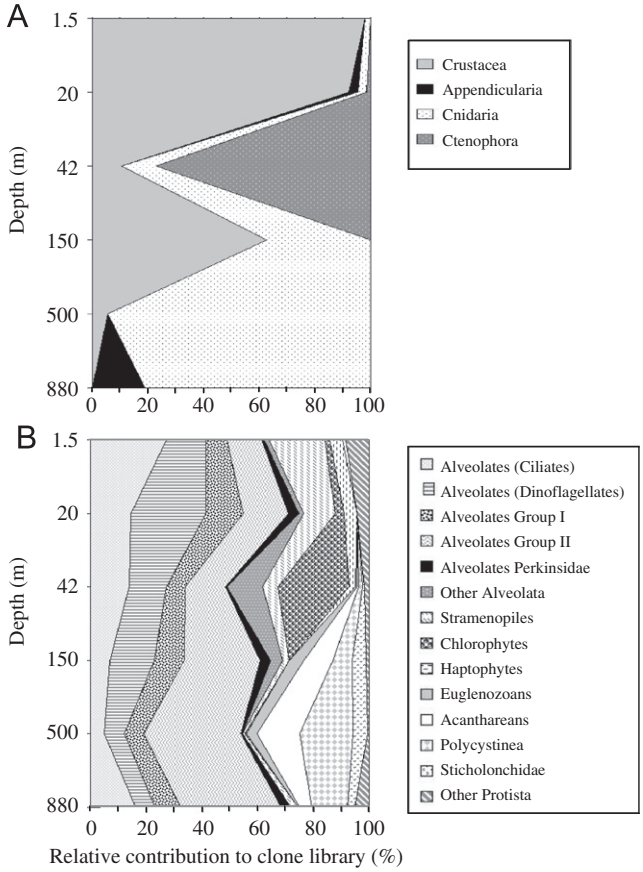

Fig. 3. Relative (%) contribution of metazoan (A) and protistan (B) sequences in clone libraries from each of six depths in the San Pedro Channel. Taxonomic identification was based on closest matches to available gene sequences in the NCBI database (see detailed information in Material and Methods). *Note*: Distances between depths on the *y*-axis are disproportionate.

and stramenopiles exhibited high relative abundances in clone libraries from the upper water column (1.5, 20 and 42 m) compared to depths at or below 150 m. The relative contribution of Group II alveolates increased with depth. Chlorophytes contributed most significantly at the depth of the DCM (25% of all clones at 42 m;

Table 2
Number of sequences and Operational Taxonomic Units of major protistan taxa for six depths in the San Pedro Channel. Taxonomies were based on BLAST similarity matches to the NCBI database. A total of 856 partial protistan sequences from six discrete depths were employed to establish OTUs based application of the MESA program (Caron et al., 2009).

	1.5	20	42	150	500	880	Total
Sequences per depth							
Alveolata Ciliophora	29	24	23	11	7	19	113
Alveolata Dinophyceae	15	45	22	26	10	8	126
Alveolata Group I	8	22	11	17	10	11	79
Alveolata Group II	13	27	24	44	49	42	199
Alveolata Perkinsidae	1	6	1	6	1	4	19
Other Alveolata	2	3	21	7			33
Stramenopiles	21	19	9	4	1	3	57
Chlorophyta	2	5	42		1		50
Haptophyceae	5	8	4				17
Euglenozoa			1	8	5	1	15
Polycystinea	1		1	17	22	5	46
Acantharea			2	11	27	15	55
Taxopoda			1	8	7	3	19
Fungi	1	3	1		1	1	7
Cercozoa	3	2				1	6
Choanoflagellida	4		1			1	6
Katablepharidophyta	1	1	1				3
Apusomonadidae						2	2
Telonema		1		1			2
Ichthyosporea						1	1
Rhodophyta				1			1
Total	106	166	165	161	141	117	856
OTUs per depth							
Alveolates Ciliophora	11	12	11	9	5	4	34
Alveolates Dinophyceae	6	6	6	14	4	4	30
Alveolates Group I	4	5	6	11	4	4	17
Alveolates Group II	9	18	18	10	6	12	62
Alveolata Perkinsidae	1	4	1	4	1	2	8
Other Alveolata	2	3	4	4			9
Stramenopiles	15	14	6	4	1	3	32
Chlorophyta	2	2	5		1		8
Haptophyceae	2	4	1				4
Euglenozoa			1	7	4	1	12
Polycystinea	1		1	9	1	3	11
Acantharea			2	2	3	3	7
Taxopoda			1	4	2	3	8
Fungi	1	3	1		1	1	5
Cercozoa	2	1				1	3
Choanoflagellida	2		1			1	4
Katablepharidophyta	1	1	1				1
Apusomonadidae						1	1
Telonema		1		1			1
Ichthyosporea						1	1
Rhodophyta				1			1
Total	59	74	66	80	33	44	259

Table 3
Simpson (Ds^{-1}) diversity indices and species richness estimates (non-parametric Chao1 at a 95% CI) for each of 6 clone libraries from different water column depths. The number of sequences (*N*) that were sequenced per depth and the number of OTUs represented in each of the libraries are shown.

Sample depth (m)	*N*	OTUs	Ds^{-1}	Chao I (95% CI)
1.5	106	59	40.62	185 (109–375)
20	166	74	17.03	148 (108–233)
42	165	66	20.22	167 (110–299)
150	161	80	46.33	276 (165–534)
500	141	35	8.4	68 (46–139)
880	117	44	17.18	117 (69–261)
Total	856	259	37.8	567 (457–740)

Fig. 3B) and, together with the haptophytes, were restricted to the euphotic zone (the only exception was a single chlorophyte sequence detected at 500 m, Table 2). Some groups were entirely absent from libraries obtained at 1.5 and 20 m and were encountered only at depths $\geq$150 m. The latter included the Euglenozoa, Acantharea, Taxopodida (*Sticholonche*-like organisms) and with one exception the Polycystinea (Fig. 3B and Table 2). No trend was apparent for protistan groups which consisted of 10 or less sequences such as Fungi, Cercozoa, Choanoflagellates, Kathablepharidophytes, Apusomomadidae, Telonema, Ichthyosporeans and Rhodophytes (shown as 'other protista' in Fig. 3B; Table 2).

3.3. Protistan community structure

3.3.1. Number of detected OTUs and their taxonomic affiliation

An analysis of all protistan sequences using the MESA program (Caron et al., 2009) resulted in the detection of 259 distinct OTUs based on a 95% sequence similarity cut-off (Table 2). The highest number of OTUs was detected for the protistan assemblage from 150 m depth (80 OTUs), the lowest at 500 m depth (33 OTUs) (Tables 2 and 3). The number of clones per OTU varied from 1 to 83, with the majority comprised of one or two sequences (62% and 16% of OTUs, respectively). Clones that were placed into separate OTUs but had closest BLAST matches to the same genus were assumed to be congeners. OTUs that contained sequences that matched closest to 'unknown' or 'uncultured' types at sequence similarities $\geq$90% were categorized as 'unclassified' species. Overall, alveolates made the highest contribution to the clone libraries forming 160 out of 259 OTUs (Table 2 and Fig. 3B). Stramenopile sequences were assigned to 32 OTUs, Euglenozoa to twelve, and Polycystines to eleven OTUs (Table 2). Acantharea and Taxopodida grouped into eight and seven OTUs, respectively, and rare taxa with five or fewer OTUs included Fungi, Cercozoa, Choanoflagellates,

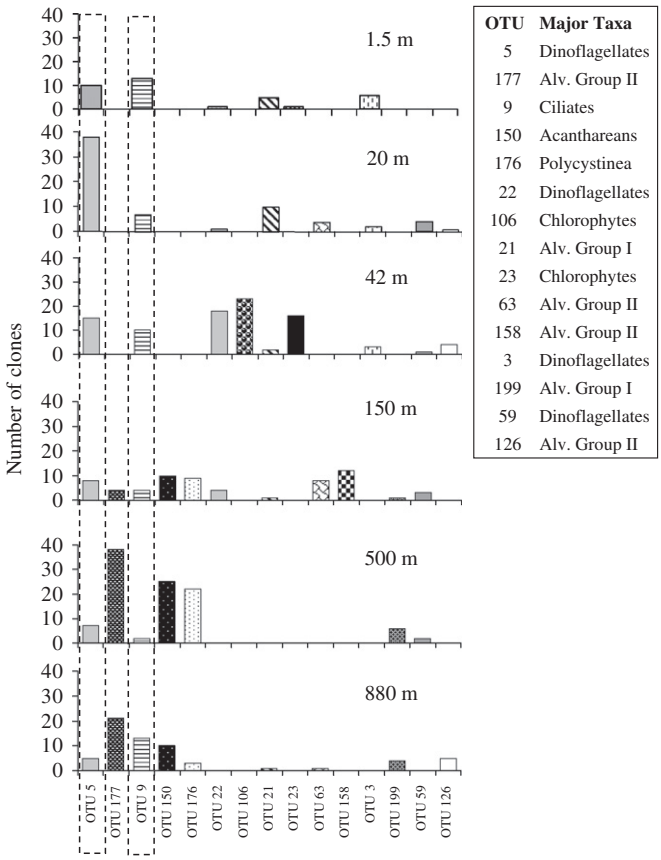

Fig. 4. Depth distribution of the fifteen most common OTUs (most clones per OTU). Alveolate sequences (mainly Group II alveolates and dinoflagellates) comprised eleven of the fifteen OTUs. Only two OTUs (5 and 9) were represented in clone libraries from all six depths.

Kathablepharidophytes, Apusomomadidae, Telonema, Ichtyosporeans and Rhodophytes (Table 2). Out of a total of 259 OTUs only two OTUs (a dinoflagellate and a ciliate) were represented at all depths (indicated in Fig. 4). Most OTUs (75%) were found in only one of the six clone libraries (as shown for chlorophyte OTU106 at 42 m and Alveolate Group II OTU158 at 150 m in Fig. 4).

3.3.2. *Protistan community structure at different depths*

Protistan communities from the upper water column (1.5, 20 and 42 m) were characterized by higher abundances of ciliate OTUs and stramenopile OTUs compared to communities from the lower water column (150, 500 and 880 m) (Fig. 5A, B). In contrast, dinoflagellate OTUs and members of alveolate Groups I and II contributed strongly to all six libraries (Fig. 5C, D), while chlorophyte and haptophyte OTUs were almost exclusively in samples collected from depths $\leq$42 m (Table 2). Ciliate OTUs that were identified only from shallow depths belonged to the genera *Laboea* (sequence similarities between 95–98%) and *Strobilidium* (97–98%) as well as tintinnids *Tintinnopsis* (96–100%) and *Codonellopsis* (97–98%) (Fig. 5A). The most common identifiable ciliate genus, *Strombidium* (92–100%; ten out of 34 OTUs), was present in libraries from both the upper and lower water column and included one of only two OTUs that were observed at all examined depths (Fig. 5A).

Stramenopile OTUs that were found only at 1.5 and 20 m belonged to the raphidophytes (93–99%), bolidophytes (98–99%), chrysophytes (93%), dictyochophytes (96–99%), eustigmatophytes (93–97%), bacillariophytes (90–99%), pinguiophytes (98%) and several novel stramenopile clades (96–100%) (Fig. 5B). The majority of novel stramenopile OTUs (6 out of 8) were restricted to the upper 2 water depths and matched closest to members of clusters MAST-1 and MAST-3 (Massana et al., 2004). The sample from the DCM (42 m) was distinct in the makeup of its stramenopile assemblage due to the detection of exclusively bacillariophyte and pelagophyte (91–100%) species (four and two OTUs, respectively), while pelagophyte OTUs were recovered only at 42 m (Fig. 5B). Chlorophytes showed a peak in abundance at the DCM, and the majority of sequences fell into two OTUs whose best matches were to species of *Ostreococcus* (95–99%) and *Bathycoccus* (90–99%). Finally, haptophytes were found only at depths within the upper water column ($\leq$42 m) and three out of four of these OTUs most closely matched sequences of *Chrysochromulina* (90–99%).

Dinoflagellates and the novel alveolates Groups I and II, in comparison to other major groups, showed no apparent pattern in OTU abundance or taxonomic affiliation related to depth. Overall, dinoflagellate OTUs showed strong similarity to thirteen known genera, many of which were represented only by a single clone and were observed in only one library. Four genera that were represented by more than one OTU included *Prorocentrum* (90–99%), *Pfiesteria* (93–99%), *Gyrodinium* (92–98%) and *Gymnodinium* (90–93%), with *Prorocentrum* being the most common (seven out of 30 OTUs; Fig. 5A). A considerable number of dinoflagellate sequences (83) were grouped within a single OTU (OTU5) that was found at all depths (Figs. 4 and 5C). Sequences from that OTU did not match to a single genus but sequences within this OTU showed sequence similarities (96–100%) to genera within the *Gymnodiniales* and *Prorocentrales*. Alveolate Groups I and II contributed

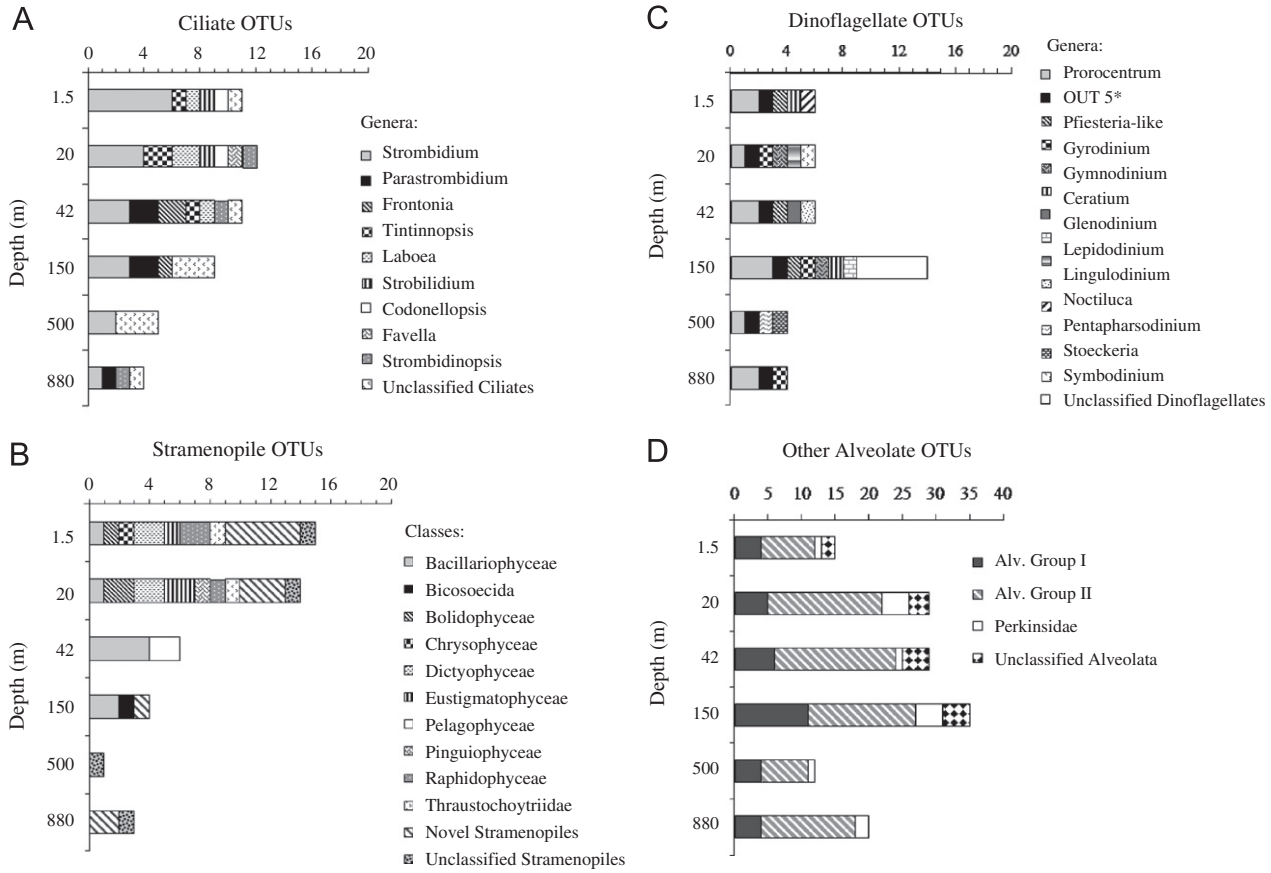

Fig. 5. Depth distribution of OTUs within the alveolates (A, C and D) and stramenopiles (B) in clone libraries from each of six depths in the San Pedro Channel. Taxonomic affiliations for OTUs were based on the nearest BLAST matches in the NCBI database (see text for details). *OTU 5 is listed separately since it included reads that showed sequence similarity to members of both the *Gymnodiniales* and *Prorocentrales*. *Note*: Distances between depths on the *y*-axis are disproportionate.

significantly to all depths. A total of 17 and 62 OTUs were inferred for alveolate Groups I and II, respectively. Group II alveolates included a considerable number of OTUs (14 out of 62 OTUs) that matched most closely to *Amoebophrya* taxa (91–99%) (Groisillier et al., 2006; Guillou et al., 2008). Alveolate sequences most similar to *Perkinsidae* (89–99%) were comprised of a total of eight OTUs that were distributed throughout the water column. One OTU comprised of a single sequence exhibited high similarity to *Apicomplexa* (96%) (grouped with Other Alveolata in Fig. 5D).

Protistan assemblages within the lower water column ($\geq$ 150 m), were relatively distinct from assemblages within the euphotic zone in part due to the presence of Euglenozoa (90–98%), Acantharea (84–100), Polycystines (84–98%) and Taxopodida (88–94%) (Gilg et al., 2010; López-García et al., 2001; López-García et al., 2002; Yuasa et al., 2005). Representatives of these taxa were rarely found in samples from the upper water column ($\leq$ 42 m) (Table 2 and Fig. 3B). The protistan assemblage at 150 m was characterized by high relative abundances of dinoflagellates, euglenozoans, polycystines and the Taxopodida. Notably, the relative contribution of unclassified OTUs ($<$ 90% sequence similarities to known genera or well-defined novel groups) within the ciliate, dinoflagellate and stramenopile assemblages was greater at depths $\geq$ 150 m compared to the upper water column (Fig. 5A–C). This trend was most apparent for the dinoflagellate community at 150 m for which six out of fourteen OTUs were unclassified, and for the ciliates at 500 m where more than half of the OTUs had low sequence similarity to any known genera. Ciliate genera from the lower water column that did exhibit sequence similarities $\geq$ 90% to any described/sequenced species included *Strombidium*, *Strombidinopsis* (94–95%), *Parastrombidinopsis* (95–97%) and *Frontonia* (92–96%) (Fig. 5A). These genera were also detected within the euphotic zone. Finally, stramenopile OTUs from the lower water column mainly belonged to novel stramenopile clades (2 OTUs that matched closest to MAST-4 and MAST-7 sequences) (Diez et al., 2001; Massana et al., 2004) or were unclassified, especially at 500 and 880 m (Fig. 5B).

3.4. Protistan diversity

Protistan species diversity (richness and evenness based on the Simpson diversity index) was highest in assemblages from 150 and 1.5 m (D_s^{-1} = 46 and 40, respectively; Table 3). Assemblages from 500 m were the least diverse (D_s^{-1} = 8), while samples from 20 and 880 m had intermediate values (D_s^{-1} = 20 and 17). These trends were apparent when rarefraction curves of the observed number of OTUs were plotted for each clone library (Fig. 6). Generally, rarefaction curves for the upper water column assemblages rose more steeply than curves for 500 and 880 m which indicated higher evenness within these communities (more equal abundances of OTUs). The Chao1 species richness estimator predicted a total of 567 OTUs for the entire dataset (95% CI = 457–740). According to this estimate, observed clone library coverage (259 OTUs) accounted for ~46% of the total estimated OTUs (Table 3). Chao1 estimates for the upper water column libraries ($\leq$ 42 m) ranged from 148 to 185 OTUs and were exceeded only by the most diverse library with an estimate of 276 OTUs at 150 m (Table 3). OTU richness predicted for the 500 m sample was considerably lower at 68, while richness predicted for the 880 m sample (117) was intermediate between values obtained for 500 m and the upper water column (Table 3). The 95% confidence intervals for all values were relatively high. The only richness estimates with non-overlapping confidence intervals were the 150 and 500 m samples (Table 3) suggesting statistically significant differences in protistan richness at these depths.

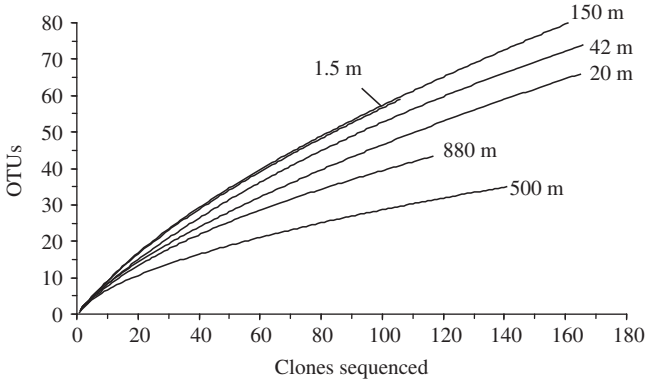

Fig. 6. Observed OTU richness in each of six clone libraries from six depths in the San Pedro Channel. Rarefraction curves were produced using EstimateS 7.5. and 100 randomizations and sampling without replacement.

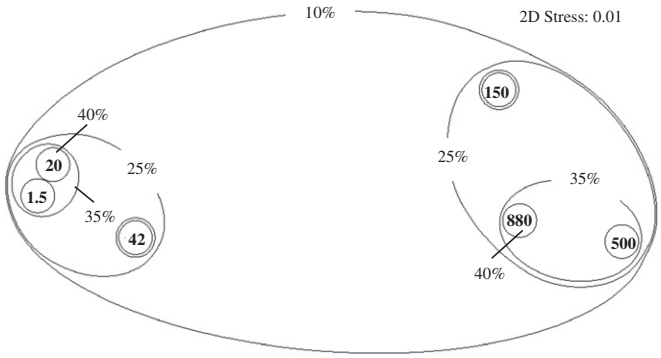

Fig. 7. Cluster diagram showing Bray–Curtis similarity for protistan communities from 6 different depths. The results are illustrated in a non-metric 2-dimensional scaling plot. Ellipses designate the level of similarity between the individual clone libraries contained therein. Analyses and plots were completed using PRIMER v.6.

3.5. Similarity among protistan assemblages at different depths

Comparison of community structure among all libraries revealed similarity values of $\leq$ 35%. Bray–Curtis similarity matrix data was plotted in a 2-dimensional MDS plot to illustrate these community structure similarities/differences between depths (Fig. 7). A stress value of 0.01 indicated that distances between the samples in the Bray–Curtis similarity matrix corresponded well to distances in the MDS plot. Overlay of the results of the cluster analyses on the MDS plot further highlighted community structure similarities (shown by ellipses in Fig. 7). Protistan assemblages in the upper water column (1.5, 20 and 42 m) and those at depth (150, 500 and 880 m) shared approximately 25% of their community structure, a value that decreased to 10% if all libraries were included (Fig. 7). The uppermost adjacent depths (1.5 and 20 m) shared more than a third but never more than 40% of their protistan assemblages, as did the deepest adjacent depths (500 and 880 m). Overall, differences in community structure among the 6 depths were not statistically significant except when testing followed a grouping into shallow ($\leq$ 150 m) and deep water assemblages ($\geq$ 150 m) (SIMPROF, $p < 0.05$; PRIMER 6).

4. Discussion

4.1. Depth-related changes in protistan community structure

Genetic surveys have become powerful tools for examining the spatiotemporal distributions of protistan taxa as well as the structure and diversity of these assemblages (Countway et al.,

2005; Countway et al., 2007; López-García et al., 2001; Not et al., 2007; Stoeck et al., 2007; Vaulot et al., 2008). These surveys have been conducted through the cloning and sequencing of specific genes (primarily ribosomal RNA genes) and more recently through the use of high-throughput sequencing approaches such as massively parallel tag sequencing that target these same genes (Amaral-Zettler et al., 2009; Stoeck et al., 2009). The latter approaches offer the potential for extensive coverage that will be necessary for documenting the tremendous number of rare taxa that are typically present in natural microbial assemblages (Bent and Forney, 2008; Caron and Countway, 2009; Epstein and López-García, 2007; Hughes et al., 2001; Pedrós-Alió, 2006; Podar et al., 2007; Schloss, 2008), but it is presently unclear how these very short sequences (typically <100 bp) extrapolate to protistan species. In the present study we employed an automated OTU-calling program that allowed the grouping of partial sequences (450–700 bp) into roughly species-level OTUs based on 95% sequence similarity (Caron et al., 2009). We then used these groupings to examine differences in community structure and diversity for the dominant members of protistan assemblages from each of six depths from the surface to near bottom in the San Pedro Channel. Depth distribution patterns varied among major taxa, and protistan assemblages within the euphotic zone (1.5, 20 and 42 m) were distinct from those observed below euphotic depths (150, 500 and 880 m). These findings are complementary to previously published studies that indicate that these depth-dependent trends consist independently of season (Countway et al., 2010) or even geographical location (i.e., Countway et al., 2007; Lopez-García et al., 2007; Not et al., 2007).

The protistan assemblages within the euphotic zone (1.5, 20 and 42 m) of the San Pedro Channel harbored, not surprisingly, many phototrophic taxa and species and their potential microzooplankton consumers. Microalgal communities at 1.5 and 20 m depths were composed primarily of stramenopile (i.e., bacillariophytes, dictyochophytes and raphidophytes) and haptophyte species from well-known phototrophic lineages (Fig. 5B). We identified several genera known to bloom in Southern California waters based on BLAST matches including the diatom *Pseudo-nitzschia* spp. and dinoflagellates *Ceratium* spp. and *Lingulodinium* spp. ($\geq$95% sequence similarities). Diatoms typically dominate phytoplankton assemblages during spring and are succeeded by dinoflagellate consortia by the time our survey was conducted (Moorthi et al., 2006; Schnetzer et al., 2007; Shipe et al., 2008;Venrick 2009). In our study, however, no dominance of any algal group was indicated based on contributions to clone libraries. This result and the fact that Countway et al. (2010) did not detect any dominance trends in their seasonal clone libraries seems to indicate potential PCR and sequencing biases which can lead to discrimination against (e.g, stramenopiles and haptophytes) or overrepresentation of certain taxa (e.g., Rhizaria and alveolates) (Liu et al., 2009; Not et al., 2009; Potvin and Lovejoy, 2009). Studies that employ quantitative detection methods free of PCR-bias will help with addressing these issues (Countway and Caron, 2006; Fitzpatrick et al., 2010; Gilg et al., 2010; Moorthi et al., 2006).

Stramenopile OTUs at 1.5 and 20 m were 3–8 times more abundant than at lower depths (Fig. 5B). Moreover, stramenopiles collected at depths below the euphotic zone generally were most closely related to novel MAST lineages that are believed to possess heterotrophic modes of nutrition (Massana et al., 2006a; Massana et al., 2006b; Massana et al., 2009). The presence of novel MAST clades down to 880 m supports the contention of a heterotrophic mode of nutrition.

Chlorophytes (mainly *Ostreococcus* and *Bathycoccus* spp.) made their greatest contribution in the relatively lower-light, higher-nutrient regime of the DCM (42 m; Tables 1 and 2; Fig. 3). The prevalence of minute eukaryotic algae and *Ostreococcus* specifically within the euphotic zone of this coastal ecosystem has been previously demonstrated (Countway and Caron, 2006; Countway et al., 2010).

Ciliate assemblages were dominated by well-known herbivorous taxa belonging to the genera *Strombidium*, *Tintinnopsis*, *Codonellopsis* and *Favella*. Loricate forms together with *Laboea* spp., a genus that includes mixotrophic species capable of retaining functional chloroplasts from its prey (Putt, 1990; Stoecker et al., 1988), were observed only in libraries from the upper water column while significant numbers of ciliate OTUs were present at all depths (Fig. 5A). *Strombidium* spp., the most common ciliate genus, was present in clone libraries from throughout the water column, a finding that is in good agreement with studies that have indicated that *Strombidium* species exploit a wide variety of ecological niches and trophic modes (Bernard and Fenchel, 1994; Doherty et al., 2007; Stoecker and Crawford, 1996).

Protistan communities within the euphotic zone ($\leq$42 m) also included considerable numbers of dinoflagellate sequences, but these sequences constituted relatively few OTUs at euphotic zone depths (Fig. 5C). Genera that include known heterotrophic (*Gyrodinium* spp.) or potentially mixotrophic forms (*Prorocentrum* spp., *Gymnodinium* spp., *Ceratium* spp.) appeared to be randomly distributed throughout the water column. Interestingly, the highest number of dinoflagellate OTUs was observed at 150 m. Sequences belonging to alveolate Groups I and II, Perkinsidae and other alveolates constituted a fairly diverse array of OTUs that also were not exclusively associated with either the upper ($\leq$42 m) or lower ($\geq$150 m) water column (Fig. 5D). Similar to the dinoflagellates, these taxa showed a maximum in the number of OTUs at 150 m.

Four major taxa, the Euglenozoa, Polycystinea, Taxopodida and Acantharea, contributed strongly to assemblages at 150, 500 and 880 m, while these taxa were detected infrequently in libraries produced from depths within the euphotic zone ($\leq$42 m). This trend seemed independent of seasonal changes at our study site (Countway et al., 2010). The unexpected occurrence of significant numbers of Rhizaria, more specifically Acantharea, at depth has also been documented in Antarctic waters (López-García et al., 2001) or the North Atlantic (Countway et al., 2007; Not et al., 2007). Surface-dwelling species of Acantharea are generally large and often form siliceous or celestite skeletons, but reproductive stages (swarmer cells) of these individuals can be as small as several micrometers in diameter (Anderson, 1983). Not et al. (2007) examined libraries from samples prescreened through 3 μm filters, and yet still detected acantharian-like sequences. This finding coupled with absence of these sequences from surface samples appears to indicate the existence of either significant numbers of reproductive cells at depth, or the presence of previously undescribed, deep-dwelling, minute taxa within these lineages. Gilg et al. (2010) recently examined the phylogeny of a number of these taxa and employed fluorescent in situ hybridization in an attempt to link these acantharian sequences to morphological forms. The latter study supports the existence of novel lineages at our study site that are closely related to, but possibly distinct from the Acantharea.

The euglenozoa have also been recognized as common components of some samples from the deep-sea (Buck et al., 2000; Buck and Bernhard, 2001; Lopez-García et al., 2007). Taxa within this lineage have been implicated in protistan-prokaryotic symbiosis in deep sulfidic sediments (Buck et al., 2000), but the ecology of most of these deep-sea forms is presently unknown. Their absence from surface waters within the San Pedro Channel and their prevalence at depths within the oxygen minimum zone (Countway et al., 2010, this study) supports the possibility of a unique ecological niche for these taxa.

Several recent gene surveys have reported that novel alveolates are important contributors to overall protistan diversity

throughout distinct marine habitats. Examples include surveys conducted in the North Atlantic (Countway et al., 2007; Medlin et al., 2006), North and Equatorial Pacific (Countway et al., 2010; Moon-Van Der Staay et al., 2001; Worden, 2006), Antarctic waters (López-García et al., 2001), Arctic ocean (Lovejoy et al., 2006), Mediterranean Sea (Massana et al., 2004), and extreme habitats such as an anoxic basin in the Caribbean Sea (Stoeck et al., 2003) and a hydrothermal vent site (Edgcomb et al., 2002). Groups I and II alveolates in the present study constituted the largest number of sequences with a combined total of 278 sequences distributed among 79 OTUs (Table 2 and Fig. 3B). While there is some indication that high gene copy numbers might contribute to higher clone abundances by these alveolate groups (Not et al., 2009), their prevalence also implies that these organisms play important (albeit presently unknown) roles within protistan assemblages. Group I alveolates are believed to be an ecologically diverse lineage, in general agreement with their recovery at all examined depths (Countway et al., 2007; Groisillier et al., 2006; Harada et al., 2007). Group II alveolates are phylogenetically related to parasitic forms in the *Syndiniales*, organisms that are known to parasitize dinoflagellates, radiolarians, ciliates, crabs and copepods (Chambouvet et al., 2008; Coats and Park, 2002; Groisillier et al., 2006; Guillou et al., 2008).

4.2. Depth-related changes in protistan diversity

A surprising finding of this study was the high species richness observed at 150 m relative to other depths sampled. Species richness in the euphotic zone samples was expected to be high relative to samples from deep water because of the ability of photosynthesis to support large numbers of phototrophic taxa in lighted waters that should be absent from aphotic depth. However, the dinoflagellates, Group I alveolates, euglenozoans, polycystines, and Taxopodida exhibited their highest number of OTUs at 150 m (Table 2; Fig. 5). The number of OTUs for these taxa was lower at shallower and deeper depths, even though the number of sequences among these taxa may have been greater at other depths (Table 2 and Fig. 5C, D). As a result, species diversity and richness estimators appeared to be highest for the protistan assemblage at 150 m, particularly compared to the assemblages at 500 m depths (Table 3 and Fig. 6). Similarity between the assemblage at 150 m and other depths was also relatively low (Fig. 7).

Presumably, environmental features unique to 150 m explain this pattern. This depth represents a depth of major transitions of temperature, dissolved oxygen, salinity and major nutrients (Fig. 2). We speculate that these gradients, and perhaps the delivery of sinking particulate organic matter from productive surface waters above this depth, create a niche-rich environment that can support unique ecological roles for protistan taxa. In contrast, the extremely low oxygen concentrations at 500 and 880 m and the presence of less labile organic matter may have precluded the growth of some protistan species at those depths.

It is not clear if the higher apparent species richness observed at 150 m is a result of truly greater protistan diversity at that depth, or rather the ability to observe a greater percentage of the species richness that is present because of the absence of highly dominant taxa. We speculate that the sharp chemical/physical gradients (e.g. declining oxygen) at 150 m may prevent strong dominance by any particular protistan taxon. Greater evenness among the taxa present, as typically indicated by a steeper rarefraction curve (Fig. 6), would allow cloning and sequencing to reveal more of the rare OTUs in the assemblage. In this scenario, the prevalence of phytoplankton blooms (common in surface waters), or highly constant or selective conditions (such as might be the case for our samples $\geq$500 m) may yield assemblages that are strongly dominated by a few taxa and thus problematic for assaying total species richness.

This scenario in turn raises the possibility that many of the protistan OTUs observed in this study were present at all depths, but that dominance of small subsets of these taxa at specific depths masked the presence of many rare taxa. The relatively low number of clones produced and sequenced in genetic surveys employing cloning and sequencing provide relatively low coverage of the vast diversity that might be present because of the cost and labor-intensive nature of these studies. For this reason, new high-throughput sequencing approaches that can provide much greater coverage of the total microbial diversity within natural samples will be necessary to fully understand the nature and potential ecological significance of the protistan 'Rare Biosphere' (Caron and Countway, 2009). Nonetheless, the information presented here indicates substantial changes in the dominant taxa making up the protistan assemblages within this water column.

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