Aquat Ecol (2021) 55:807–824
https://doi.org/10.1007/s10452-021-09862-5

Environmental association of heterotrophic microeukaryotes in the varying biogeochemical regimes of the Arabian Sea, resolved via high-throughput sequencing

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Received: 7 October 2020 / Accepted: 14 April 2021 / Published online: 10 May 2021

Abstract The diverse physicochemical conditions prevailing in the Arabian Sea are expected to result in marked spatial variations in heterotrophic flagellate (HF) and ciliate communities. Here, we report the environmental association of heterotrophic microeukaryotes, particularly the heterotrophic flagellates and ciliates, based on 18S rRNA gene survey in the region. High-throughput next-generation sequencing, using the V4 eukaryotic-specific primer, was employed to study the composition of these communities associated with low-O_2 waters in both coastal and offshore settings. Canonical correspondence analysis (CCA) revealed a preference of the heterotrophic flagellates for nitrate- and nitrite-rich zones. Notably, the heterotrophic nanoflagellate genus *Monosiga* showed a strong positive correlation with NO_3^-, which suggests its potential denitrifying capability. Shannon's entropy analysis revealed a higher HF diversity in the hypoxic waters of the open ocean (depth 103 m), whereas ciliates were more diverse at oxygenated coastal stations. The estuarine waters exhibited a low diversity of both ciliates and flagellates. The UPGMA clusters of heterotrophic flagellates and ciliates in suboxic waters of the open ocean oxygen minimum zone were distinct from those found at other sites. Overall, CCA revealed the important relationship between nitrite, nitrate, salinity and chlorophyll *a*, which could be important factors for the partitioning of different ecological niches for specific HF and ciliate communities in the Arabian Sea. The community of heterotrophic protists that can adapt to varying biogeochemical regimes has been identified.

Keywords Heterotrophic flagellates · Ciliates · Arabian Sea · Ecological niches · Next-generation sequencing

Handling Editor: Télesphore Sime-Ngando

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10452-021-09862-5.

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Introduction

Both autotrophic and heterotrophic microorganisms play key roles in organic matter cycling and pelagic microbial food web in the ocean (Sherr and Sherr 1994; Legendre and Rivkin 2009). While the vast majority of unicellular plankton ecology studies focused on pigmented protists (Irigoien et al. 2004; Ptacnik et al. 2008; Barton et al. 2010), heterotrophic

plankton organisms are comparatively less well-characterised on the basis of high-throughput sequencing studies. However, there is growing emphasis on studying diversity and abundance of heterotrophic protists in the ocean (de Vargas et al. 2015), for better understanding the ecological role of unpigmented planktonic protists. Among the planktonic protists, heterotrophic flagellates (HF), a diverse group in terms of morphology and biogeochemical functions, play important roles in predator–prey interactions in aquatic food webs (Weisse et al. 2016). In the pelagic microbial web, dissolved organic matter (DOM) serves as a substrate for bacteria, which, in turn, are the food source for heterotrophic nanoflagellates (HNF) and small ciliates (Azam et al. 1991). The HNF, smaller than 10 µm, are consumed by microflagellates and larger ciliates. Thus, the microbial loop makes the DOM available to the filter-feeding zooplankton (Kopylov et al. 1981).

In suboxic and anoxic water columns, bacterivorous protists have profound implications for the growth and mortality rates of bacteria (Anderson et al. 2012). These bacteria play significant roles in ocean nutrient cycles through anaerobic processes, including denitrification and production of potent greenhouse gases such as nitrous oxide. A prerequisite for a concrete understanding of these fundamental processes in aquatic ecosystems is the basic knowledge of the key players involved in these processes. However, despite the importance of heterotrophic protists in ecosystem functioning, relatively little is known about the (molecular) diversity of these organisms in the oxygen-minimum zones (OMZ) of the oceans.

The Arabian Sea experiences some of the most pronounced oxygen depletion in the water column anywhere in the oceans. Contrasting biogeochemical processes occurring in the open ocean and coastal waters over the Indian shelf result in different low-oxygen regimes (Naqvi et al. 2006). The large spatio-temporal variability arises primarily from monsoon-related processes (Naqvi et al. 2003). However, the oxygen deficiency in the open ocean differs from that over the Indian continental shelf. Most notably, the former is perennial, whereas the latter is seasonal, and sulphidic conditions develop only in coastal waters, but not in the open ocean (Naqvi et al. 2006). The coastal zone off western India also receives runoff from a large number of small rivers originating in peninsular India. The estuaries of these rivers are also subjected to marked monsoon-driven seasonal changes. The Mandovi and Zuari in Goa, typical of such estuaries (Qasim and Sen Gupta 1981), exhibit biogeochemical and ecological characteristics that are notably different from those of coastal and open ocean waters.

Hence, the wide diversity of biogeochemical regimes leads to the development of vastly different community structures of multicellular zooplankton, fungi and bacteria in the region (Morrison et al. 1999; Fuchs et al. 2005; Wishner et al. 2008; Jebaraj et al. 2010). However, the diversity of heterotrophic flagellates and ciliates in these heterogeneous pelagic environments is poorly understood. Previous studies applied microscopic observations for the enumeration of ciliates, heterotrophic flagellates and thraustochytrids in the central and north-eastern Arabian Sea (Gauns et al. 1996; Garrison et al. 2000; Raghukumar et al. 2001). These studies demonstrated the general occurrence of specific taxon groups related to oxygen gradients in the water column. However, no attempt has been made to relate these taxon groups to specific environmental conditions. Thus, the present study was aimed to investigate the spatial variations of heterotrophic flagellate and ciliate diversity in these unusual and globally important environmental settings of the Arabian Sea. Using high-throughput sequencing of heterotrophic marker genes (V4 region of the hypervariable SSU rDNA), we present here an analysis of community structures of these functional protistan groups in the open Arabian Sea, coastal region over the Indian shelf, and the Mandovi-Zuari estuarine system, varying in their oxygen levels and other hydrochemical characteristics.

Material and methods

Study sites and sampling analyses

Samples for the present study were collected during the 56th cruise of R/V *Sindhu Sankalp*, from 18th October 2013 to 2nd November 2013. The areas that were sampled included the open ocean (the Arabian Sea Time Series station, ASTS, located at 17°N, 68°E; water depth 3,600 m), the outer continental shelf (station G12 located at 15.24°N, 72.98°E; water depth 160 m), the inner continental shelf (station G5 located

at 15.50°N, 73.67°E; water depth 26 m), the Mandovi estuary (station M located at 15.49°N, 73.81°E; water depth 5 m) and the Zuari estuary (station Z located at 15.4°N, 73.9°E; water depth 6 m) (Fig. 1).

Water samples were taken from four depths in the open ocean (surface-oxic, 103 m-hypoxic, 134 m-upper suboxic and 190 m-lower suboxic). Three depths were sampled over the outer shelf (surface-oxic, 80 m-upper hypoxic, 120 m-lower hypoxic). At station G5, samples were collected from three depths (surface-upper oxic, 8 m-lower oxic, and 24 m-anoxic). The estuarine stations were only sampled at the surface and close to the bottom, where oxic conditions prevailed. The criterion used for defining the state of oxygenation was based on Winkler dissolved oxygen (DO) concentrations according to Naqvi et al. (2010): oxic ($DO > 62$ μM), hypoxic ($4.5 < DO \leq 62$ μM), suboxic ($0 < DO \leq 4.5$ μM) and anoxic ($DO = 0$ μM). The details of sampling sites and the environmental parameters are partly described in Das et al. (2019).

Samples were collected using 10-L Niskin bottles mounted on a Sea-Bird Electronics CTD (conductivity–temperature–depth)-Rosette system that also provided vertical profiles of temperature and salinity in the water column. Dissolved oxygen, nutrients (NO_3^-) and (NO_2^-) were measured on board the ship within a few hours of collection, following the titrimetric Winkler method and the automated colorimetric procedures adopted for a SKALAR autoanalyser, respectively (Grasshoff et al. 1983). One litre of the sample was collected for chlorophyll analysis and immediately filtered through a GF/F filter. Chlorophyll *a* (Chl *a*) was extracted from the filters with 90% acetone for 24 h in the dark at − 20 °C and fluorescence was measured using a fluorometer (Turner Designs, Model no. 10-AU). Lab measurements for chlorophyll were not considered for the depths 134 m and 190 m at the open ocean station (ASTS) due to higher depth. In this context, the measured value was taken from CTD.

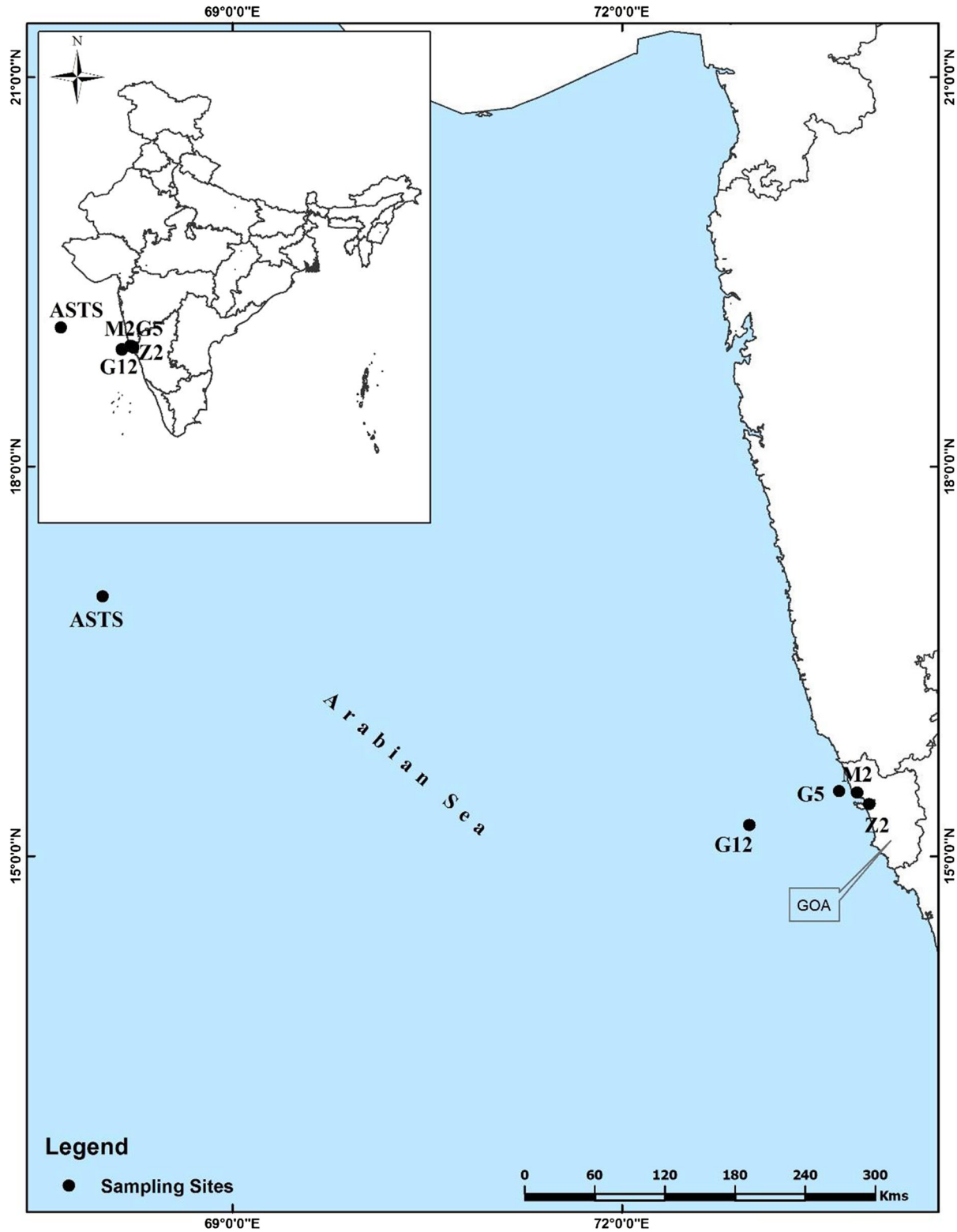
For DNA analyses, plankton samples were collected from up to 5 L of water on a Durapore membrane (47 mm, 0.65 μm, Millipore, Germany) using a peristaltic pump. Filters were immediately placed into cryovials, preserved with 3 ml RNAlater (Ambion, Germany) and stored at − 20 °C until DNA extraction.

Total DNA extraction

The DNA was extracted directly from the Durapore membrane filters, using Qiagen's All Prep DNA/RNA kit according to the manufacturer's guidelines. Three replicates of each sample were extracted and pooled. Bulk DNA was quantified spectrophotometrically (NanoDrop 2000, Thermo Scientific, Wilmington, DE, USA); the detailed quantification for each sampling site is provided in Table 1. The DNA extracts were amplified with PCR primers specific for the hypervariable V4 region of the 18S rRNA gene (TAReuk454FWD1: 5′CCAGCA(G/C)C(C/T)GCGGTAATTCC3′;TAReukREV3′5′ACTTTCG-TTCTTGAT(C/T)(A/G)A-3′, Stoeck et al. 2010). The PCR reaction included 50–100 ng of DNA template in a 50 μl final volume with 1 μl of Phusion High-Fidelity DNA polymerase (Finzymes, New England Biolabs, Ipswich, MA, USA), 1 × Phusion GC Buffer (New England Biolabs, Ipswich, MA, USA), 200 μM each of deoxynucleotide triphosphate and 0.5 μM oligonucleotide primer. The PCR protocol employed an initial denaturation (30 s at 9 °C) followed by 30 identical amplification cycles, denaturation (at 98 °C for 10 s, annealing at 59 °C for 10 s and extension at 72 °C for 30 s) and a final extension at 72 °C for 30 s. To reduce PCR bias, three individual reactions per filter were pooled during the PCR product purification using Qiagen's Min Elute PCR purification Kit. Paired-end sequencing of V4 Purified amplicons was processed on an Illumina Miseq platform by SeqIT, Kaiserslautern, Germany, following the Sequencing-by-synthesis (SBS) strategy. The paired-end (2 × 250 bp) reads produced from the same amplicon were merged using a custom script.

Amplicon data processing and OTU (operational taxonomic units) analysis

The total raw sequences were denoised with Acacia (Bragg et al. 2012). Denoising, data cleaning and chimera checking were performed using QIIME (Caporaso et al. 2010). The quality reads were taken into account with particular barcodes and primers having a minimum length of 300 bp. Phylotype clustering was performed using Uclust (Edgar 2010) at different sequence similarities (100–90%). The length distribution of the tags was plotted in R (R Core Team 2012). The core (longest and thus most


69°0'0"E
72°0'0"E
21°0'0"N
18°0'0"N
15°0'0"N
N
ASTS
M2
G5
Z2
G12
ASTS
Arabian Sea
M2
G5
Z2
G12
GOA
Legend
Sampling Sites
0
60
120
180
240
300
Kms

◀**Fig. 1** Map of the sampling sites located in different regions of the Arabian Sea ranging from estuarine to open ocean stations (Estuary: Mandovi-M2, Zuari-Z2; Open ocean-ASTS; Outer continental shelf-G12; Inner continental shelf-G5)

Table 1 The water sample filtered and the bulk DNA was quantified spectrophotometrically (NanoDrop 2000) from the four sampling sites: the open ocean (ASTS), outer continental shelf (G12), inner continental shelf (G5) and the Mandovi and Zuari estuaries

Sampling site	Sample filtered (L)	Measured DNA (ng/L)
ASTS (surface)	5	3.7
ASTS (103 m)	5	2.7
ASTS (134 m)	5	5.2
ASTS (190 m)	5	5.4
G12 (Surface)	2	16.2
G12 (80 m)	2	4
G12 (120 m)	2	2.3
G5 (surface)	2	8.6
G5 (8 m)	2	12.2
G5 (24 m)	2	7.3
M (surface)	1	8
M (5 m)	1	14.1
Z (surface)	1	8
Z (6 m)	1	7.5

informative) sequence for each phylotype at 97% was extracted in a FASTA file, which was analysed with JAguc software (Nebel et al. 2011). The JAguc employed BLASTn searches, with algorithm parameters adjusted for short (200–500 bp) reads (-m 7 -r 5 -q -4 -G 8 -E 6 -b 50). The output files as a custom script QIIME's OTUpipe (seq_otus.txt) and JAguc (taxonomic tree for analysed representative sequence) were merged to a biome file containing information about OTU IDs, number of sequences per OTU and per sample as well as taxonomic affiliations. Non-target OTUs (metazoans and embryophytes) were excluded, and the resulting file was used for statistical analysis. From this total eukaryotic data set, we analysed the HFs (genus-level, based on published articles, see Supplementary File S1) and ciliates as main contributors to the heterotrophic protistan plankton.

Statistical data analysis

Indices of alpha and beta diversity were calculated using QIIME v.1.8.0 (Caporaso et al. 2010). We used Shannon entropy to avoid the biased abundant taxa and to obtain a meaningful comparison of microbial eukaryotic diversity with reference to molecular datasets (Haegeman et al. 2013). For this interpretation, we converted the Shannon index to true diversity in the form of effective number of species (ENS = equally common species), according to Jost (2006). For this purpose, data were first normalised and resampled 1000 times to account for uneven sample sizes (Filker et al. 2015). Jaccard distance dendrograms were drawn using the application of the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. Further multivariate statistical analysis was conducted on relative sequence abundance of HFs and ciliates, using the software package CANOCO 4.5 (ter Braak and Smilauer 2002). Prior to statistical analysis, data were logarithmically (log x + 1) transformed to meet the normality assumptions. The relationship between community composition patterns and associated environmental gradients was investigated by canonical correspondence analysis (CCA). Prior to CCA, detrended correspondence analysis (DCA) was performed to determine the variability within the dataset; the length of the first axis gradients for all data sets was > 2 standard deviation unit (SD), indicating the unimodal character of the data set. Due to the unimodal characteristics, CCA was performed (ter Braak and Smilauer 2002). Data were run under a reduced model. Monte Carlo significance tests of the first coordination axes and all canonical axes together were performed. All available environmental factors were involved in the model. Moreover, the correlations of individual HF and ciliate communities with the environmental factors were assessed through Spearman correlation analysis, using the software package SPSS 16 (Supplementary File S2). This tool was used to further confirm the correlation of community abundance with environmental parameters obtained through CCA.

Accession numbers

All sequences obtained from the study have been deposited in the National Center for Biotechnology

Information (NCBI), Sequence Read Archive (SRA), under accession numbers SRR5217061, SRR5217067, SRR5217068, SRR5217151 and SRR5217173.

Results

Environmental parameters

The results of environmental parameters from the study (Das et al. 2019) were used to evaluate the association of heterotrophic protists with the environmental status. The detail results of environmental variables are described here to draw the clear picture of environmental characteristics of each sampling sites. The DO concentrations varied widely. At the open ocean station (ASTS), the DO was 205 μM at the surface (designated as oxic), 43 μM at 103 m (hypoxic), 4.1 μM at 134 m (suboxic) and 4.4 μM at 190 m (suboxic). The outer continental shelf station (G12) had 197 μM at the surface (oxic), 14.6 μM at 80 m (hypoxic) and 6.9 μM at 120 m (hypoxic). The inner shelf station (G5) had 153 μM O_2 (oxic) at the surface and 82 μM at 8 m (oxic); DO was not detectable close to the bottom (24 m), indicating anoxia. The two estuarine stations did not experience hypoxic conditions, although the O_2 levels were below saturation values: 161 μM at the surface and 156 μM in near-bottom waters in the Mandovi estuary, and 158 μM at the surface and 145 μM in near-bottom waters in the Zuari estuary.

Chl *a* was conspicuously high in anoxic near-bottom waters at station G5 (3.48 μg l^{-1}). The shallow estuarine stations were well-mixed with similar Chl *a* levels in surface and near-bottom waters. Overall, in the oxic surface water, Chl *a* increased from the open ocean to the estuaries, with the highest concentration (7.5 μg l^{-1}) observed in the surface water of the Mandovi estuary.

Nitrite (NO_2^-) was found to accumulate in the suboxic waters at ASTS, with the highest concentration of 4 μM at the lower suboxic depth (190 m). At the other stations, nitrite concentrations were low or below the detection limit. Nitrate (NO_3^-) was below the detection limit in surface waters, except for the two estuarine stations. The highest concentration ($\sim$ 19 μM) at the ASTS station was recorded at the upper hypoxic depth. The concentration was even higher ($\sim$ 25 μM) in the hypoxic bottom waters of station G12. Nitrate was absent in the water column at the inner shelf station (G5). The surface and near-bottom waters of two estuarine stations contained low concentrations of NO_3^-, but well above the detection limit, with 2–threefold higher concentrations at the surface (1.4–1.6 μM) than at depth.

Surface salinity at the ASTS station (36.9 PSU) was considerably higher than at stations G12 (34.3 PSU) and G5 (34.2 PSU). The hypoxic water at the ASTS was also more saline (36.2 PSU) than the water over the outer continental shelf (34.5–34.6 PSU). However, suboxic waters of the open ocean (35.7–35.8 PSU) and the anoxic water at G5 (35.3 PSU) had comparable salinities. The estuarine surface water samples were brackish, but a large salinity gradient existed between the surface (26.1–27.9 PSU) and near-bottom (27.3–30.3 PSU) waters, with more stratified conditions occurring in the Mandovi (salinity difference 2.4 PSU) than in the Zuari estuary (salinity difference 1.2 PSU).

Sea surface temperatures (SST) at the open ocean and outer shelf stations were higher (29.1 °C) than those in the inner shelf and estuarine stations (25.5–28.9 °C). Hypoxic water at the ASTS was warmer (22.76 °C) than the waters over the outer shelf (18.1–18.7 °C). The deeper suboxic waters at ASTS were cooler (16.4–19.1 °C). At the inner shelf station G5, temperature varied from 27.16 °C at the surface to 21.89 °C close to the bottom. There was little difference between the temperatures of the surface and bottom waters at both estuarine stations, although the waters of the Zuari estuary were somewhat warmer (by $\sim$ 1.5 °C) than those of the Mandovi estuary.

Sequence data overview

Low-quality reads and the sequences assigned to metazoans and embryophytes were removed from the total eukaryotic sample. After the removal of non-targets, a total of 1,395,168 sequences remained. Subsequently, the singletons and doubletons were removed, and the remaining 1,387,818 high-quality sequences represented target eukaryotes, accounting for 51% of total raw sequences. Of these, 152,858 reads (6%) represented HF and 7,853 reads (0.3%) were assigned to ciliates. The detailed distribution of HF and ciliate V4 SSU rDNA sequences for the sampling sites under study are listed in Table 2.

Community composition

Overall, 15 genera of the HF community were documented at the open ocean site, among which *Gyrodinium* (45%) and *Amoebophyra* (27%) were dominant (Fig. 2). *Gyrodinium* was the dominant taxon (69% of HF) in oxic water, and accounted for 36% of HF in hypoxic water. The suboxic strata (134 m and 190 m) supported a different HF community structure, with *Polykrikos* dominating in the upper zone (35%) and *Amoebophyra* being most abundant (70%) in the lower zone. Interestingly, the *Monosiga* was present in the hypoxic (0.04%) and suboxic zones (0.3% at 134 m and 4% at 190 m) (Fig. 3).

The ciliate community at the open ocean station included 10 higher-level taxon groups, of which 53% were represented by Bryometopida; the lowest contribution was from Stichotrichia (0.7%) (Fig. 4). Oligotrichia was the dominant taxon (34%) in oxic waters, followed by Apostamatida (20%). Bursariomorphida was the dominant taxon (22%) in hypoxic water, whereas Scuticociliata (35%) was the most diverse subclass in the upper suboxic water column. Bryometopida was dominant in terms of diversity (88% of total Ciliophora community) at the lower suboxic depth (Fig. 5).

Overall, the HF community in waters of the outer continental shelf station, represented by 16 genera, included *Gyrodinium* (77%), followed by *Amoebophyra* (18%). *Protaspis* and *Rhizaria,* the latter accounting for less than 1% of the HF community composition (Fig. 2). The oxic layer was dominated by the genus *Gyrodinium* (80%). Both the upper and lower hypoxic water columns were characterised by the dominance (55%) of *Amoebophyra* (55%). Unclassified genera belonging to *Pfiesteriaceae, Monosiga* and *Abedinium* were present exclusively in hypoxic waters. The unclassified genus *Cryptomonas* (3%) was exclusive to the upper hypoxic water column (Fig. 3).

The ciliate community over the outer shelf was composed of 10 taxon groups, dominated by Oligotrichia (55%) and Choreotrichia (31%). Stichotrichia and Scuticociliatia only slightly (< 1%) contributed to this community (Fig. 4). Oxic waters were dominated by Oligotrichia (57%), followed by Choreotrichia (32%). Apostomatida (25 and 20%) and Astomatida (25 and 20%) were most diverse in the upper and lower hypoxic zones, respectively. Scuticociliata and

Table 2 Overview of Illumina sequencing data sets for the open ocean, outer shelf, inner shelf and estuarine regions. % relates to total number of obtained raw sequences

Sample ID	Raw sequences paired	High quality target eukaryotes Sequence n (%)	High quality target eukaryotes without single and double tones Sequence n (%)	High quality target heterotrophic flagellates (HF) Sequence n (%)	High quality target ciliates Sequence n (%)
ASTS_surface	327,354	113,609 (35)	112,380 (34)	30,102 (9.2)	360 (0.1)
ASTS_103 m	252,205	172,875 (69)	171,919 (68)	8975 (3.6)	126 (0)
ASTS_134 m	227,350	111,497 (49)	110,890 (49)	11,844 (5.2)	197 (0.1)
ASTS_190 m	185,173	123,886 (67)	123,487 (67)	14,910 (8.1)	999 (0.5)
G12_surface	290,518	115,635 (40)	114,554 (39)	25,442 (8.8)	879 (0.3)
G12_80 m	111,567	5984 (5)	5849 (5)	308 (0.3)	8 (0.0)
G12_120 m	93,441	7364 (8)	7218 (8)	792 (0.8)	15 (0.0)
G5_surface	254,683	120,655 (47)	119,791 (47)	25,813 (10.1)	1859 (0.7)
G5_8 m	176,501	84,013 (48)	83,207 (47)	23,232 (13.2)	879 (0.5)
G5_24 m	156,937	29,819 (19)	29,544 (19)	7608 (4.8)	507 (0.3)
Mandovi_Surface	173,529	138,079 (80)	137,949 (79)	218 (0.1)	733 (0.4)
Mandovi_5m	138,098	105,881 (77)	105,755 (77)	1230 (0.9)	243 (0.2)
Zuari_surface	126,545	94,332 (75)	94,240 (74)	406 (0.3)	937 (0.7)
Zuari_6m	233,080	171,539 (74)	171,035 (73)	1978 (0.8)	111 (0.0)
Total	2,746,981	1,395,168 (51)	1,387,818 (51)	152,858 (6)	7853 (0.3)

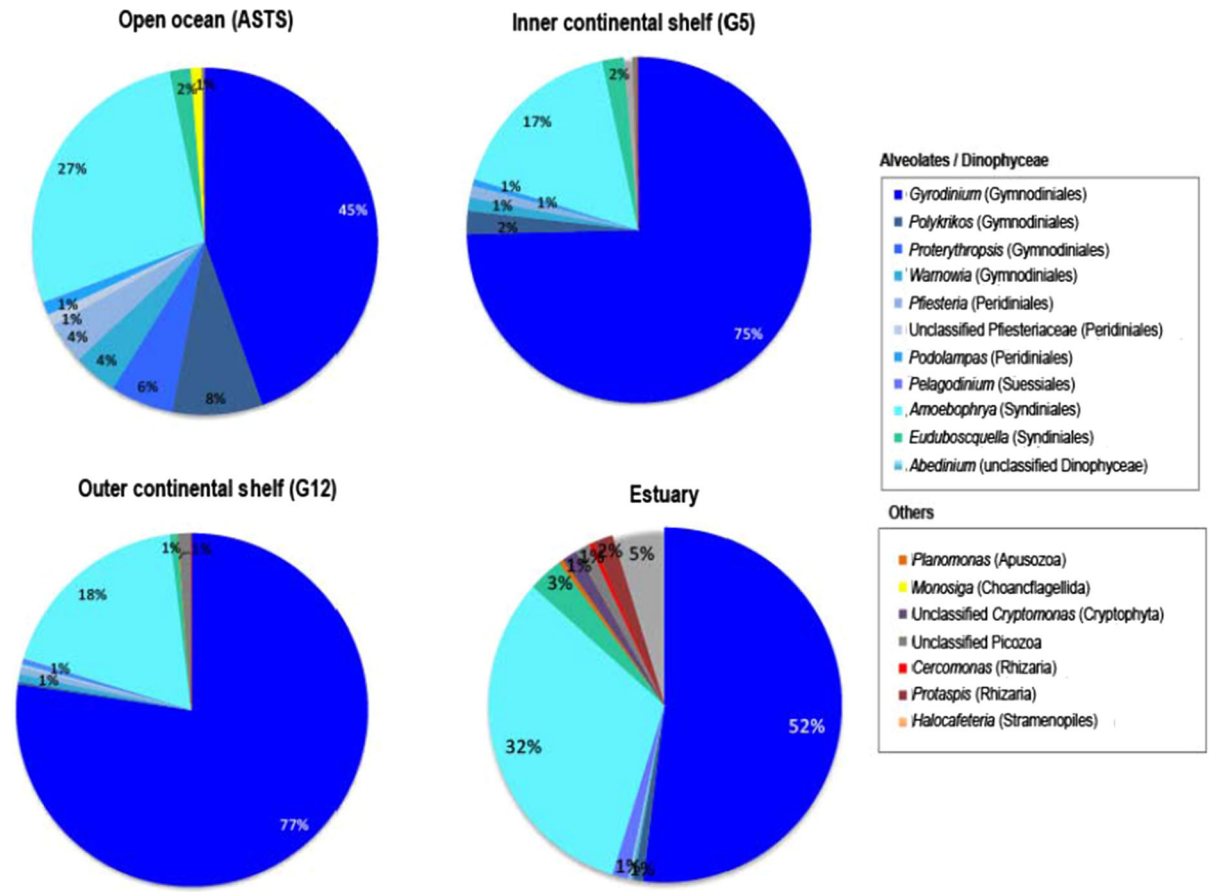


Fig. 2 Taxonomic assignment (relative distribution) of heterotrophic flagellate (HF) OTUs at the investigated open ocean, continental shelf, coastal and estuarine sites of the Arabian Sea

Stichotrichia were restricted to the lower hypoxic water column (Fig. 5).

The HF community at the inner shelf station was mainly represented by the genera *Gyrodinium* (75%) and *Amoebophyra* (17%) (Fig. 2). Even though the oxygen levels were very similar at the surface and 8 m depth, the HF community composition varied notably. In contrast, the unclassified *Picozoa* showed a gradual decrease as the oxygen concentration decreased with depth. The genus *Gyrodinium* was dominant throughout the water column (Fig. 3). The ciliate community at this shallow coastal station was dominated by Choreotrichia (61%) and Oligotrichia (23%). Astomatida was recorded in low abundance (< 1%) at this station (Fig. 4). The taxon group Choreotrichia showed a gradual increase with decreasing dissolved oxygen concentration with depth, unlike Oligotrichia, which gradually became less abundant with the depletion of oxygen. Choreotrichia was abundant (64%) in near-bottom water. Scuticociliata and Cyrtophoria were the distinct groups in lower oxic and anoxic waters (Fig. 5).

The water columns were oxygenated in the well-mixed and shallow Mandovi and Zuari estuaries. The HF community of the estuarine sites included the genera *Gyrodinium* (52%), *Amoebophyra* (32%), *Protaspis* (2%), *Halocafeteria* (5%), *Eudubosquella* (3%), unclassified *Cryptomonas* (1%) and unclassified *Picozoa* (1%) (Fig. 2). In the Mandovi estuary, the genus *Gyrodinium* (56%) was predominant in surface waters, but decreased by one order of magnitude in near-bottom waters (Fig. 3). In both estuaries, the HF community was less diverse in surface waters than close to the bottom.

The ciliate community at the estuarine sites was mainly composed of Choreotrichia (34%),

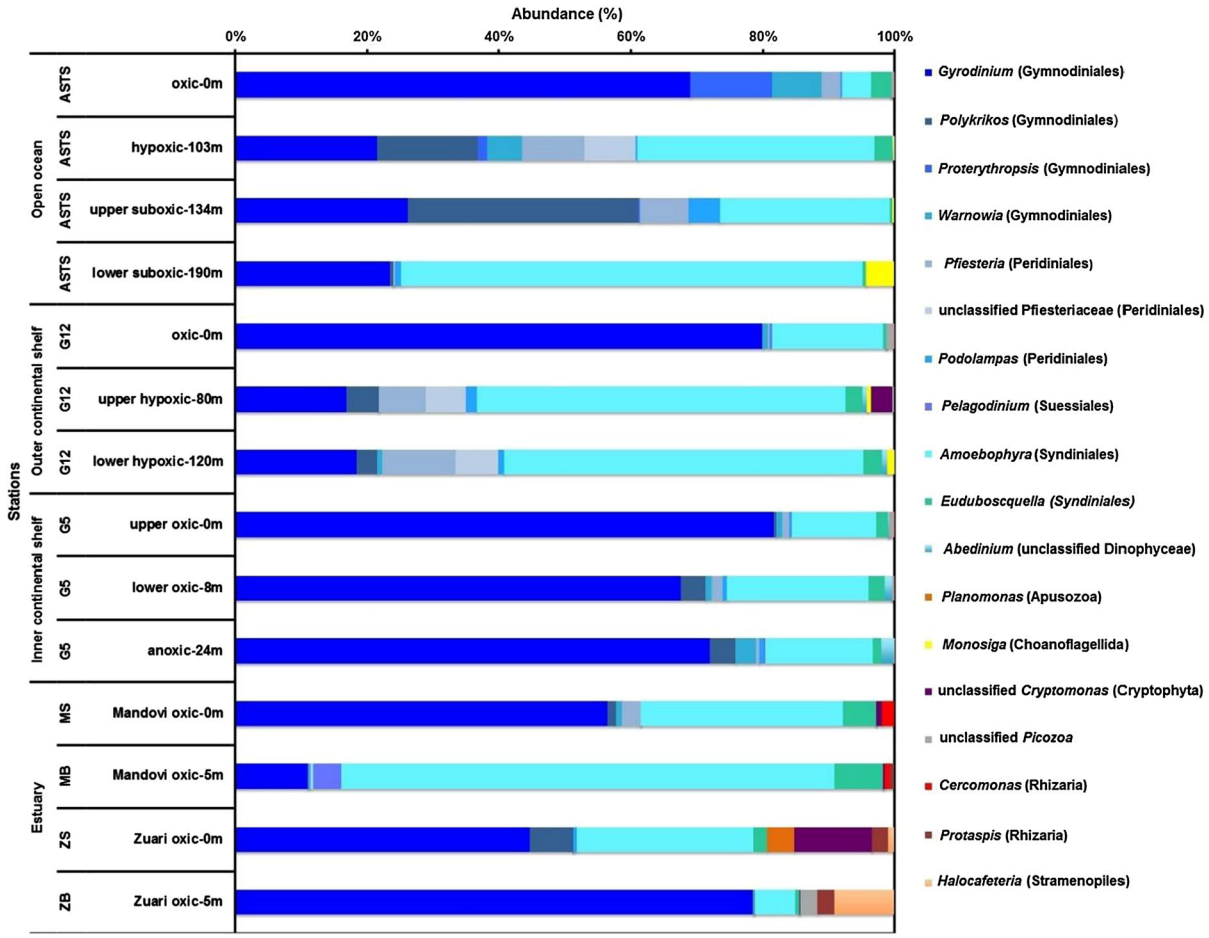


Fig. 3 Heterotrophic flagellate community composition with reference to the degree of de-oxygenation at the sampling sites

Oligotrichia (46%), Peritrichia (17%) and Haptoria (3%) (Fig. 4). Also, the ciliate community structure varied in the shallow water column. The surface waters of Mandovi were dominated by Oligotrichia (95%) and Prorodontida (4%), while in the bottom water, Oligotrichia (74%) and Choreotrichia (14%) were the major groups (Fig. 5). Surface waters at the Zuari estuary were dominated by Choreotrichia (62%) and Peritrichia (32%), while the bottom water had Peritrichia (38%), Choreotrichia (31%), Oligotrichia (26%) and Stichotrichia (5%) (Fig. 5).

Alpha diversity

In the open ocean and over the outer continental shelf, higher HF diversity was observed in the hypoxic strata, whereas at the inner shelf station, this was not the case (Fig. 6). In the estuaries, higher diversity was recorded in near-bottom waters of the Zuari estuary as compared to the surface water at this site. By contrast, in the Mandovi estuary the HF community was more diverse at the surface.

While the lowest ciliate diversity occurred within the lower suboxic layer (190 m) at the ASTS, it was the highest in the oxic and hypoxic waters at this open ocean site. Over the outer shelf, ciliate community was more diverse in the oxic water column than in hypoxic waters. Over the inner shelf, ciliate diversity was lower in the anoxic near-bottom waters. In the Mandovi and Zuari estuaries, ciliates were more diverse at the surface than at depth.

Partitioning of diversity

The Jaccard distance matrices (beta diversity) showed different HF community clusters at various sites

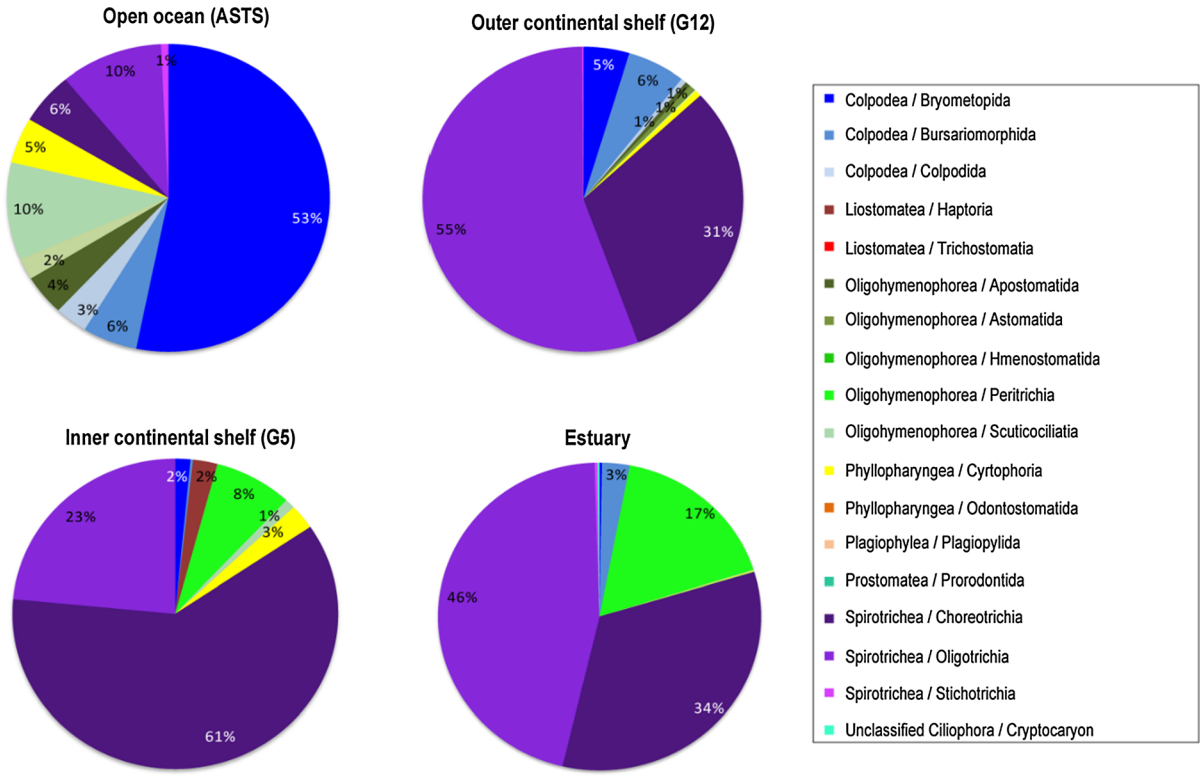


Fig. 4 Taxonomic assignment (relative distribution) of ciliate OTUs at the investigated open ocean, continental shelf, coastal and estuarine sites of the Arabian Sea

(Fig. 7). The UPGMA clustering based on OTU abundance identified four clusters. The upper suboxic and lower suboxic communities at the ASTS formed one cluster with close similarity, unlike the hypoxic and oxic water column. The upper hypoxic waters over the outer continental shelf exhibited close similarity with the lower hypoxic waters, but not with the oxic waters. At the inner shelf station, the upper oxic, lower oxic and anoxic strata formed a single cluster showing no clear difference between the three strata. By contrast, the HF communities in the Zuari and Mandovi estuaries were quite different. As per the Jaccard distance analysis, the surface and bottom waters in the Mandovi had a similar HF community, while in the Zuari estuary, different HF communities inhabited the surface and bottom waters (Fig. 7 a).

For the ciliate community, the UPGMA analysis yielded three clusters where lower suboxic and oxic waters of the open ocean station were different from the hypoxic and upper suboxic waters, which formed one cluster. The upper and lower hypoxic waters over the outer shelf were not included in the cluster analysis due to low sequence numbers. High community similarity was noticed over the inner shelf among the upper oxic, lower oxic and anoxic strata. In the Zuari estuary, the bottom water community structure was distinct from that of the surface water and the waters of the Mandovi estuary (Fig. 7 b).

Impact of environmental variables on heterotrophic protist assemblages

The first two CCA axes explained 65% of the cumulative variance of HF communities (Fig. 8 a). Nitrate and salinity appear to be significant environmental variables for the structuring of HF communities ($p < 0.05$; Table 3). Unclassified *Picozoa*, *Polykrikos*, *Podolampas* and *Amoebophyra* preferably occurred at low-oxygen sites of the open ocean and outer shelf stations. Over the inner shelf, unclassified Picozoa, *Pelagodinium* and *Warnoia* occurred at elevated DO concentrations and temperatures. *Protaspis*, *Halocafeteria* and unclassified *Cryptomonas* preferred lower salinities and occurred at sites with

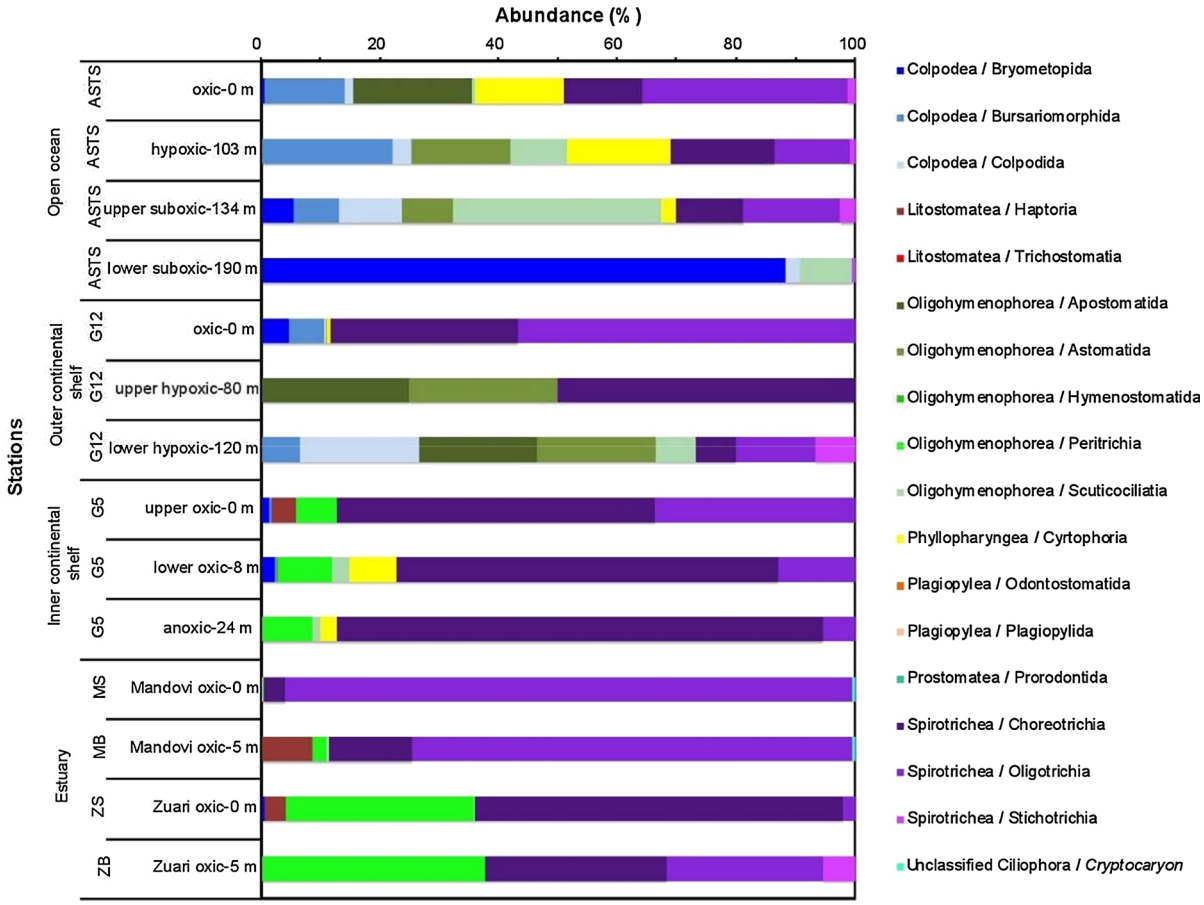


Fig. 5 Ciliate community composition with reference to the degree of de-oxygenation at the sampling sites

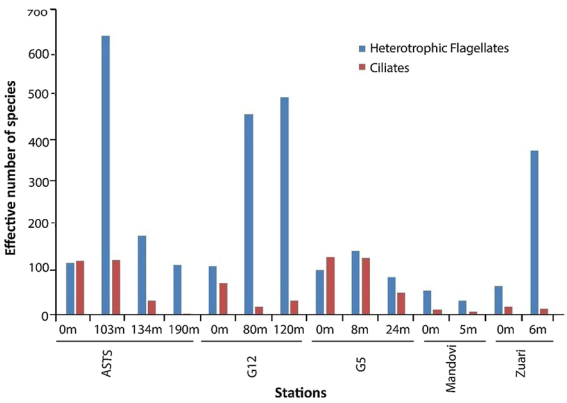


Fig. 6 Shannon diversity index for heterotrophic flagellates (HF) and ciliates at the sampling sites under study. Diversity values calculated are based on 97% similarity of ciliate and flagellate OTUs

higher Chl *a* values. *Cercomonas* and *Planomonas* were positively related to Chl *a*, temperature and DO. The HNF genus *Monosiga* correlated more strongly with NO_3^- ($r = 0.78$; $p = 0.001$) preferring suboxic conditions of the open ocean water column (Supplementary File S2).

The first two CCA axes explained 66% of the cumulative variance of the ciliate communities. Chl *a* was significantly related to axis 1 and NO_2^- to axis 2 ($p < 0.05$; Table 4). Specifically, Haptoria, Peritrichia and Prorodontida correlated positively with Chl *a* at less-saline estuarine stations. Bryometopida, Scuticociliata, Stichotrichia and Colpodida were recorded in suboxic waters of the open ocean, which were characterised by high NO_2^- levels (Fig. 8b). Cyrtophoria, Bursariomorphida, Astomatida and Apostomatida, were present in the open ocean samples (surface and upper hypoxic waters) and in all samples of the continental shelf, which were characterised by higher salinity and lower Chl *a*.

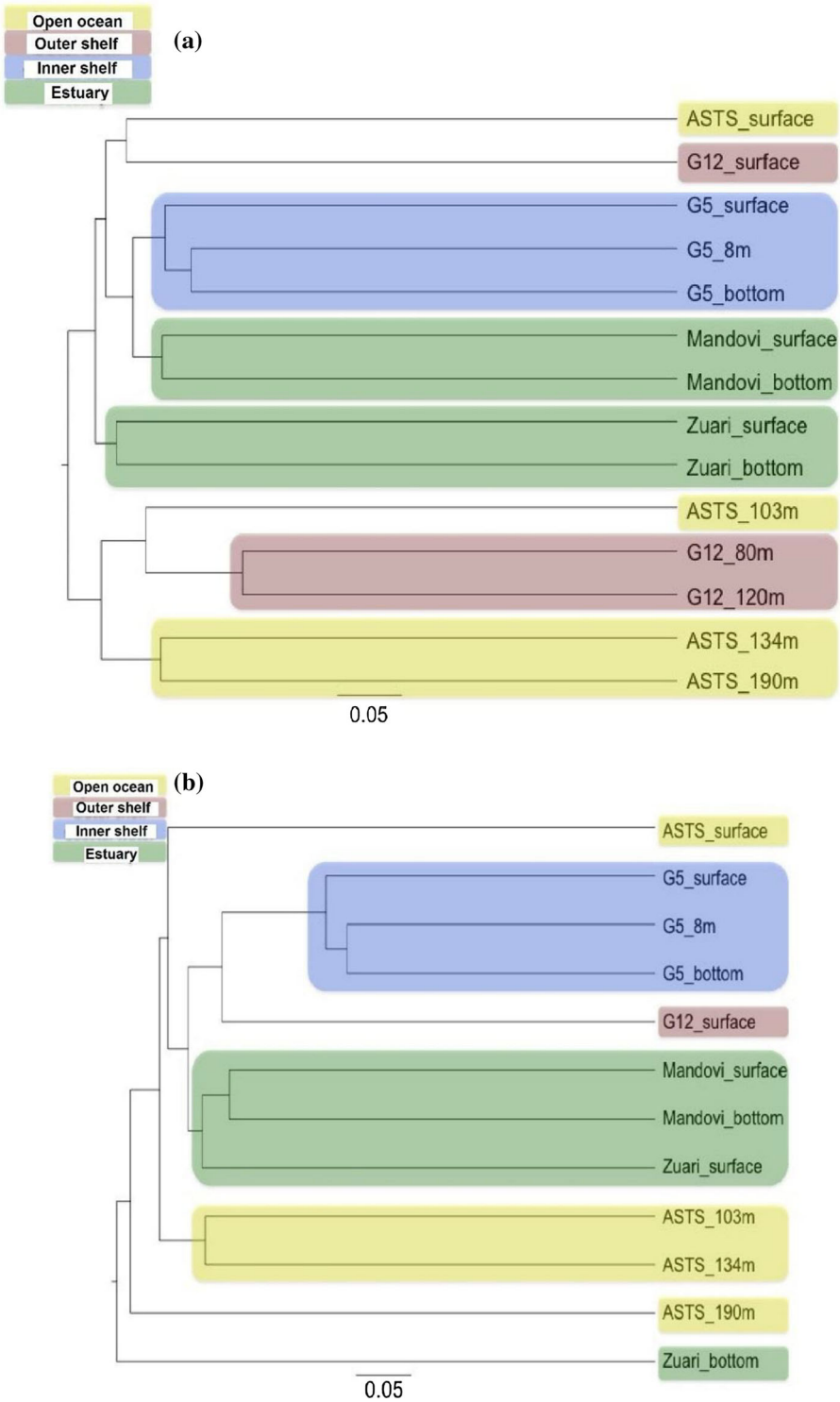


Fig. 7 **a** UPGMA clustering of Jaccard beta diversity for heterotrophic flagellates (HF, OTUs called at 97% sequence similarity); **b** Clustering of Jaccard beta diversity for ciliates (OTUs called at 97% sequence similarity) in different regions of Arabian Sea

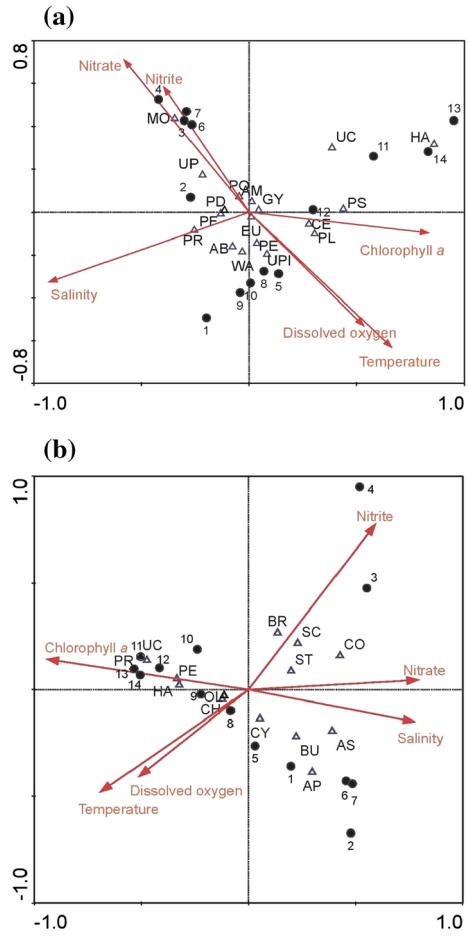


Fig. 8 Canonical correspondence analysis of **a** heterotrophic flagellates (HF) communities up to genera at four sites with respect to six environmental factors. HF communities represented as GY: *Gyrodinium*, PO: *Polykrikos*, PR: *Proterythropsis*, WA: *Warnowia*, PF: *Pfiesteria*, UP: Unclassified Pfisteriaceae, PD: *Podolampas*, PE: *Pelagodinium*, AM: *Amoebophyra*, EU: *Eudubosquella*, AB: *Abedinium*, PL: *Planomonas*, MO: *Monosiga*, UC: Unclassified *Cryptomonas*; UPI: Unclassified Picozoa, CE: *Cercomonas*, PS: *Protaspis*, HA: *Halocafeteria*; **b** Ciliate communities (subclasses) at 4 different sites with respect to six environmental factors. Ciliate communities are represented by BU: Bursariomorphida, CO: Colpodida, HA: Haptoria, AP: Apostomatida, AS: Astomatida, PE: Peritrichia, SC: Scuticociliata, CY: Cyrtophoria, PR: Prorodontida, CH: Choreotrichia, OL: Oligotrichia, ST: Stichotrichia, UC: Unclassified Ciliophora; 1 = Open ocean (ASTS-0 m); 2 = Open ocean (ASTS-103 m); 3 = Open Ocean (ASTS-134 m); 4 = Open ocean (ASTS-190 m); 5 = Outer continental shelf (G12-0 m); 6 = Outer continental shelf (G12-80 m); 7 = Outer Continental shelf (G12-120 m); 8 = Inner continental shelf (G5-0 m); 9 = Inner continental shelf (G5-8 m); 10 = Inner continental shelf (G5-10 m); 11 = Mandovi (M-0 m); 12 = Mandovi (M-5 m); 13 = Zuari (Z-0 m); 14 = Zuari (Z-6 m); (a, b)

Discussion

Environmental selection and community structure

Recently, taxonomic variations of flagellate and ciliate groups in OMZ waters have been studied in the Eastern Tropical South Pacific (ETSP) and the Eastern Tropical North Pacific (ETNP) (Parris et al. 2014; Duret et al. 2015). The composition of HF and ciliates exhibited a patchy distribution in the epipelagic and mesopelagic waters of the eastern Arabian Sea. In oxygenated waters, the HF *Gyrodinium* showed its dominance at all sampling sites, except the subsurface water of the Mandovi estuary during the fall intermonsoon. This genus is a major component of heterotrophic community in oxygenated surface and subsurface waters due to its wide range of prey (picoplankton to large diatoms) and its adaptability towards different environmental conditions (Jyothibabu et al. 2008). Other studies also concluded that this genus grazes actively on nano- and microphytoplankton cells (Gaines and Elbrachter 1987). In the open ocean, the parasitoid dinoflagellate genus *Amoebophyra*, belonging to Syndiniales (alveolates), was dominant in the lower suboxic water at 190 m, close to the core of the OMZ. This observation is consistent with previously reported abundance of the Syndiniales within the OMZ of ETSP (Parris et al. 2014). *Amoebophyra* infects bloom-forming algae (Miller et al. 2012; Chambouvet et al. 2008), thereby regulating algal blooms. It also produces lumps of active dinospores, which could be the reason behind the dominance of this genus. Its high abundance in suboxic waters supports the earlier reported function as endoparasite of macrozooans and other protists (Chambouvet et al. 2008). This genus could be vertically transported along with the sinking organic particles or may exist within free-floating host eukaryotes. The high grazing pressure of predators on flagellates may be the reason for their lower abundance in oxic and hypoxic strata, and the limited grazing might account for the enhanced abundance within the OMZ core. The observed distribution pattern suggests that niche specialisation is influenced mainly by DO-dependent biological interactions. Previous work on eukaryotic microbial communities in the ETNP revealed taxonomically different protists in the OMZ water column, although oxygen

Table 3 CCA summary for heterotrophic flagellate (HF) communities with different environmental factors

Marginal effects	Conditional effects				
Variable	Var. N	Lambda 1	Lambda A	P	F
Salinity	2	0.19	0.19	0.001	4.41
Nitrate	4	0.14	0.12	0.001	3.12
Nitrite	5	0.1	0.07	0.047	2.14
Temperature	1	0.14	0.05	0.118	1.88
Dissolved oxygen	3	0.11	0.04	0.326	1.2
Chlorophyll *a*	6	0.15	0.03	0.529	0.84

Table 4 CCA summary for ciliate communities with different environmental factors

Marginal effects	Conditional effects				
Variable	Var. N	Lambda 1	Lambda A	P	F
Chlorophyll *a*	6	0.31	0.31	0.001	4.37
Nitrite	5	0.21	0.15	0.013	2.32
Nitrate	4	0.24	0.09	0.149	1.49
Temperature	1	0.21	0.08	0.27	1.3
Dissolved oxygen	3	0.16	0.06	0.345	1.13
Salinity	2	0.23	0.04	0.719	0.59

concentration is not the sole parameter influencing the community structure (Duret et al. 2015).

The distribution of the heterotrophic nanoflagellate genus *Monosiga* (Choanoflagellida) appears to be considerably influenced by low oxygen conditions. The gradual increase in the abundance of this genus from hypoxic to deeper suboxic depths in the open ocean suggests its preference for oxygen-depleted waters. The abundance of nanoflagellate genera at deeper suboxic depths may be supported by a rich bacterial biomass (Naqvi et al. 1993) in addition to lower grazing pressure from predators. Thus, the nanoflagellates may serve as important participants in carbon cycling through the microbial loop. It should be noted that these organisms were far less abundant in hypoxic waters over the outer shelf and completely absent at the inner shelf and estuarine stations, which shows that oxygen gradients create a boundary separating organisms that can adapt to different levels of oxygen (Borcard et al. 2004). Low oxygen marine waters support diverse microeukaryote communities with a complex pattern of taxonomic structures (Parris et al. 2014). Moreover, the positive correlation ($r = 0.78$; $p = 0.001$) of *Monosiga* abundance with NO_3^- indicates that this species is a potential denitrifier in the suboxic ambience. Other HF species showed either a weak or no correlation with NO_3^- and NO_2^-, which implies that they may not play a significant role in denitrification, and their distribution is not controlled by the vertical variability of NO_3^-/NO_2^- concentrations. There is, as yet, no experimental evidence of HF carrying out denitrification, but in view of their occurrence in functionally anoxic waters an anaerobic mode of respiration cannot be ruled out.

Ciliates are one of the dominant members of the heterotrophic community in the south-eastern Arabian Sea, and their abundance varies spatially during the late summer monsoon (Jyothibabu et al. 2008). In the present study, the dominance of ciliate taxonomic groups varied in different niches, with the dominance of Oligotrichia in oxic water columns of the open ocean, outer shelf and the Mandovi estuary. Choreotrichia was dominant in inner shelf waters and the Zuari estuary. Food availability in the oxic water column, as well as other environmental factors such as temperature and DO, could be controlling variables. The CCA identified Chl *a* as the most important parameter for the dominance of Peritrichia, Oligotrichia and Choreotrichia, many of which feed on smaller pigmented algae. In addition to Chl *a*, variations in DO and temperature further add to changes in the community structure in oxic waters at

oceanic and inner shelf stations. In a recent study of global ocean ciliate plankton patterns, Gimmler et al. (2016) also identified these environmental parameters as important factors controlling the presence (and abundance) of numerous specific ciliate genera. Earlier studies in the Arabian Sea during the late summer monsoon reported that the dominance of ciliates at the inshore stations was promoted by the abundance of pigmented flagellates and diatoms in the water column (Jyothibabu et al. 2008). As in the open ocean and outer shelf stations, the Mandovi estuary (both at the surface and close to the bottom) was dominated by Oligotrichia. In the Zuari estuary, Choreotrichia dominated in the surface water, while Peritrichia was most abundant in the bottom water. These contrasting patterns between the two estuaries were possibly because tidal currents are stronger in the Zuari estuary than in the Mandovi estuary (Manoj et al. 2009).

The diversity of protists (heterotrophic flagellates as well as total ciliates) was high in the oxygen-deficient strata (hypoxic and suboxic waters) of the open ocean and inner shelf waters. Previous studies have shown that the particulate organic carbon (POC) content is highest during the southwest monsoon, which supports a high heterotrophic biomass in the Arabian Sea (Gauns et al. 2005). It is likely that heterotrophic protists favour low oxygen water, where the abundant organic matter promotes a high biomass of bacteria which, in turn, serve as a rich food source for HF and ciliates (Azam et al. 1983).

Partitioning of diversity

The Jaccard incidence-based cluster analysis shows that the HF as well as the ciliate community exhibit similarity in oxic, suboxic and anoxic waters of the inner shelf station, whereas over the outer shelf communities at the oxic and two suboxic depths are different. The open ocean station showed a higher variability in ciliate community composition across the oxygen gradient as compared to the inner shelf and outer shelf stations. The observed community variation could be attributed to the effect of changing environmental conditions at intermediate distance scales (3–10,000 km), as suggested for microbes (Martiny et al. 2006). In the present study, a clear difference in environmental conditions was noticed from the oceanic to estuarine stations. Surprisingly, the two estuaries showed different patterns: while in the Mandovi the community structure was similar in surface and bottom waters, such was not the case in the Zuari estuary. This dissimilarity of heterotrophic communities (ciliate and flagellates) may be attributed to hydrographical differences (e.g. tidal currents); in addition, differences in runoff and the width of the mouths of these two estuaries may also contribute to contrasting community patterns (Varma et al. 1975; Qasim and Sen Gupta 1981).

Among the ciliates, Bryometopida, Scuticociliata and Bursariomorphida dominated in the suboxic and hypoxic strata in the open ocean. The community pattern showed distinct variability, presumably driven by changes in the ambient oxygen concentration. The high abundance of Bryometopida at the lower suboxic depth is apparently related to high NO_2^- concentration. This dominant ciliate community may coincide with secondary nitrite maxima, which is a prominent feature in the suboxic waters of the Arabian Sea (Lam et al. 2011; Morrison et al. 1999). This indicates the possible role of ciliates in the denitrification process, as they may be able to respire NO_3^- in the absence of oxygen. The symbiotic association between ciliates and bacteria is a common feature in oxygen-depleted waters (Gast et al. 2009; Edgcomb et al. 2011). Earlier reports identified the freshwater ciliate *Loxodes* as a significant contributor to denitrification in eutrophic lakes (Aleya et al. 1992; Finlay 1985). Overall, the ciliate community showed a positive correlation with NO_2^- in our study. However, on the individual species level, none of the ciliate species showed a strong correlation with NO_2^-. It is also possible that the high bacterial abundance within the secondary nitrite maximum (Naqvi et al., 1993) supports a high ciliate population thereby accounting for a correlation between ciliate abundance and NO_2^- concentration. Most of these bacteria could be denitrifiers (Jayakumar et al. 2009). For sustained presence in functionally anoxic water, these communities should not only be adapted to live in low-oxygen waters, but should also be capable of anaerobic respiratory pathway(s). Within hypoxic waters, the ciliate community composition varied notably among the sampled sites. Besides oxygen, other environmental parameters, such as salinity, are also expected to influence the community composition. In fact, the results of the CCA suggest a strong influence of salinity on the distribution of Apostomatida, Astomatida and Bursariomorphida in hypoxic waters of the open ocean and outer

shelf. This is consistent with earlier reports of salinity being a major factor in determining the composition of the ciliate communities (Forster et al. 2012). The presence of Scuticociliata and Stichotrichia, albeit in lower numbers, in the lower hypoxic and upper hypoxic water column of the outer shelf suggests that they are also adapted to live in low-oxygen waters. Our observations agree with the results of earlier molecular surveys showing that scuticociliates are diverse and abundant members of the ciliate communities in oxygen-depleted marine habitats such as the Saanich Inlet, the Framvaren Fjord and the Cariaco Basin (Behnke et al. 2006; Edgcomb et al. 2011; Orsi et al. 2012).

At the inner shelf station, a clear effect of oxygen on community composition was not apparent, even though this station was distinguished by the presence of hydrogen sulphide in the bottom water. This lack of variability may be due to the shallow station depth, because of which sporadic wind-mixing events can homogenise the water column thus preventing the sustained presence of specialised communities and/or exposing the organisms to an environment they are not normally expected to be found in. Other biotic factors may also play a role, particularly autotrophic picoplankton proliferating under low-oxygen conditions (M. Gauns; unpublished data) that, may serve as the food source for these protist communities in coastal waters.

Conclusion

Spatial differences in the beta diversity between the open ocean, outer shelf, inner shelf and estuarine waters, experiencing different hydrographic and biogeochemical conditions, reflect diverse communities of protists, particularly ciliates and heterotrophic flagellates. Gradients in chlorophyll *a*, temperature, salinity, nitrate, nitrite and dissolved oxygen apparently control the distribution of these protists, enabling adaptability of distinct protist communities. Some HF and ciliate genera, living in functionally anoxic waters, could potentially play important role in nitrogen cycling by respiring nitrate. The present study provides the baseline information from the North-western Indian Ocean that allows a comparison of diversity metrics with other oceanic areas.

Acknowledgements The authors express gratitude to the Director, CSIR-NIO for providing logistics and laboratory facilities for execution of this work. P.B. Das is grateful to the CSIR for providing doctoral research fellowship. Also we are grateful to Professor Dr. Thorsten Stoeck, HOD, Division of Ecology, and Kaiserslautern University, Germany, for allowing first author to do the molecular analysis in their laboratory. We are thankful to Dr. Hema Naik for providing nutrient and dissolved oxygen data. We acknowledge the support extended by Dr. Siby Kurian and Dr. Damodar Shenoy for execution of this work. We gratefully acknowledge Dr. Anil Pratihary for the valuable suggestions, discussion, and correction of the manuscript. We are thankful to Sh. H. Dalvi and Sh. A. Methar for their support onboard. This work forms a part of doctoral research of P.B. Das and was carried out under of the SIBER-India programme funded by the Ministry of Earth Sciences (MoES) and the Network Project PSC0108 funded by Council of Scientific and Industrial Research (CSIR). This is NIO's contribution no. 6733.

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