Influence of regional oceanography and hydrothermal activity on protist diversity and community structure in the Okinawa Trough

Running Title: Protist diversity and biogeography in the Okinawa Trough

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The authors declare no competing interests.

Abstract

Microbial eukaryotes (protists) contribute substantially to ecological functioning in marine ecosystems, but the relative importance of factors shaping protist diversity, such as environmental selection and dispersal, remain difficult to parse. Water masses of a back-arc basin with hydrothermal activity provide a unique opportunity for studying the effects of dispersal and environmental selection on protist communities. In this study, we used metabarcoding to characterize protist communities in the Okinawa Trough, a back-arc spreading basin containing at least twenty-five active hydrothermal vent fields. Water was sampled from four depths at fourteen stations spanning the length of the Okinawa Trough, including three sites influenced by nearby hydrothermal vent sites. While significant differences in community structure reflecting water depth were present, protist communities were mostly homogeneous horizontally. Protist communities in bottom waters affected by hydrothermal activity were significantly different from communities in other bottom waters, suggesting that environmental factors can be especially important in shaping community composition under specific conditions. Amplicon sequence variants that were enriched in hydrothermally influenced bottom waters largely derived from cosmopolitan protists that were present, but rare, in other near-bottom samples, thus highlighting the importance of the rare biosphere.

Keywords: protists, diversity, microbial oceanography, hydrothermal vents, plankton, community structure

Introduction

Microbial unicellular eukaryotes (protists) are important contributors to all marine ecosystems, from the sunlit surface waters (Massana, 2011) to the deep, dark bathypelagic (Edgcomb, 2016). Extensive phylogenetic and functional diversity exist among protists, especially in extreme environments like the deep sea and hydrothermal vents (Sauvadet et al., 2010), but factors influencing protist community structure remain difficult to parse (de Vargas et al., 2015; Pernice et al., 2016). Physicochemical factors, such as temperature, light and nutrient availability, have historically been regarded as major influences on microbial community structure. These factors vary most substantially by depth and, correspondingly, sampling depth is a major determinant of protist community composition (Countway et al., 2007; Giner et al., 2020). Oceanographic features, including current systems (Richter et al., 2019), and geographic restrictions, such as land barriers between ocean basins (de Vargas et al., 2015) or water-mass boundaries (Agogué et al., 2011; Pernice et al., 2016), can also influence microbial community structure. Moreover, the relative contribution of different spatial and environmental factors shaping community structure can vary regionally, by ecosystem type (Giner et al., 2020; Pan et al., 2020), and depending on the scale at which microbial communities are investigated (Martiny et al., 2011; Kuhn et al., 2019; Richter et al., 2019; Pan et al., 2020).

The Okinawa Trough (OT), a deep back-arc spreading basin (> 2000 m) within the East China Sea, represents a unique setting to investigate factors shaping protist community structure on a regional scale (Figure 1). The Kuroshio Current, the western boundary current of the North Pacific subtropical gyre, enters the trough to the east of Taiwan, and transports warm, high-salinity water northward (Barkley, 1970) (Figure 2).

Fast-moving western boundary currents contribute to dispersal and can homogenize regional community structure, but this effect can also be modulated by latitudinal light and temperature gradients, eddy-mixing, and cross-jet exchange (Kuhn et al., 2019). As a result, protists communities in the surface waters of the Okinawa Trough are expected to be generally similar along the Kuroshio Current, but advection of water through the Kerama Gap or from the continental shelf may locally influence community composition.

Intermediate water (~600 m) in the Okinawa Trough is formed as a result of horizontal advection and diffusion of North Pacific Intermediate Water (NPIW) and South China Sea Intermediate Water (SCSIW) (e.g., Nitani, 1972; Guo and Morinaga, 1998; Chen, 2005). The salinity-minimum water of NPIW and SCSIW enters the trough through the channel east of Taiwan (Yonaguni Depression) and the channel south of Okinawa Island (Kerama Gap) (Nakamura et al., 2013) (Figure 2). Strong diapycnal diffusion increases the salinity of these incoming, mixed water masses (Nakamura et al., 2013). The deep water (> ~1,100 m) of the Okinawa Trough is isolated geographically, forming a closed basin. Overflow from the sill of the Kerama Gap and upwelling in the Southern Trough ventilate the deep water with residence times of 4.7–9.4 years (Nakamura et al., 2013). Horizontally, deep water circulation spreads water parcels throughout the entire basin relatively quickly, within months, connecting hydrothermal vent fields in the OT without particular directionality (Mitarai et al., 2016). As a back-arc spreading basin, there is extensive hydrothermal activity throughout the Okinawa Trough, with the majority of the hydrothermal vent fields being distributed in deep water. Twenty-five active hydrothermal vent sites are listed in The InterRidge Database v3.4 (Beaulieu and Szafranski, 2018). Hydrothermal vents in the Okinawa

Trough are distinct from those found at mid-ocean ridges, primarily due to thick layers of terrigenous sediments overlaying vent sites. Compared to sediment-starved vent systems, vent fluids from Okinawa Trough vents are typically low pH with high concentrations of CO_2, NH_4^+, boron, iodine, potassium, lithium (Toki et al., 2016) and methane (Mino et al., 2013). Unique physicochemical conditions associated with different vent systems give rise to heterogeneous biological communities (Dick, 2019), warranting careful study of diverse hydrothermal systems and their influence on protist communities. In addition, organic matter and inorganic nutrients resuspended by vents in the Okinawa Trough may locally increase microbial production in bottom waters (Nagata et al., 2010).

In this study, we present a comprehensive survey of protist communities in the Okinawa Trough and Kuroshio Current and investigate factors that could drive community structure, including environmental parameters and oceanographic features. We analyzed protist community composition in replicate samples collected from the sea surface, subsurface chlorophyll maximum, intermediate and deep waters at sites spanning the entire length of the Okinawa Trough and within the Kerama Gap. Deep water at three sampling sites was influenced by nearby hydrothermal vents, as evidenced by increased turbidity, CDOM (colored dissolved organic matter), NH_4^+, and total carbon concentrations—conditions typical of hydrothermal vent plumes in the Okinawa Trough (Toki et al., 2016; Zhang et al., 2019). Main objectives of the study were to: (i) assess broad patterns in protist diversity in the Okinawa Trough and Kuroshio Current, (ii) evaluate the extent to which Okinawa Trough protist diversity is affected by regional oceanography and environmental factors, and (iii) investigate the influence of hydrothermal activity on deep-sea protist communities.

Methods

Sampling locations

Water samples were collected from 14 sites spanning the Okinawa Trough and Kerama Gap during the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) MR17-03C cruise from May 29 to June 13, 2017 (Figure 1). The Okinawa Trough is divided into Southern (SOT) and Northern (NOT) regions based on the location of the Kerama Gap (KG), where surface and intermediate water is advected into the trough (Jin et al., 2010; Na et al., 2014) (Figure 2). Stations 8, 9, 10, and 11 are located in the Southern Okinawa Trough (SOT); stations 3, 4, 2, 5 make up a transect of the Kerama Gap (KG) from east to west; stations 12, 13, 14, 15, 17, and 18 are located in the Northern Okinawa Trough (NOT).

Five stations are close to known active hydrothermal vent sites; station 10 is at the Hatoma Knoll (Toki et al., 2016), station 2 is at the ANA vent site of the Daisan-Kume Knoll (Makabe A., Tsutsumi S., Chen C., Torimoto J., Matsui Y., Shibuya T., Miyazaki J., Kitada K., Kawagucci S., 2016), station 11 is near the Dai-Yon Yonaguni Knoll (Konno et al., 2006), station 5 is near the Higa vent site (Makabe A., Tsutsumi S., Chen C., Torimoto J., Matsui Y., Shibuya T., Miyazaki J., Kitada K., Kawagucci S., 2016), and station 12 is near the Iheya North vent field (Nakajima et al., 2015) (Figure 1). Deep water movement in the Okinawa Trough lacks consistent directionality (Mitarai et al., 2016) making it impossible to predict vent plume location when sampling near hydrothermal vents. As a result, physicochemical data collected by in-situ sensors and chemical analysis of water samples were used to discern whether or not samples were influenced by hydrothermal vent plumes, instead of proximity to vent sites. Vertical profiles from stations 2, 10, and 11 showed near-bottom turbidity and CDOM maxima (Supplemental Figure 1A), which is typical for OT vent plumes (Toki et al., 2016; Zhang et al., 2019). In addition, nutrient analysis showed elevated NH_4^+ concentrations in bottom water samples from stations 2 and 10, providing further evidence of hydrothermal influence (Toki et al., 2016; Zhang et al., 2019) (Supplemental Figure 1B). Total carbon was only measured for samples from stations 2, 10, 14, and 15; consistent with hydrothermal influence (Toki et al., 2016; Zhang et al., 2019), stations 2 and 10 had near bottom peaks in total carbon concentrations (Supplemental Figure 1C). Stations 2, 10 and 11 are, therefore, included in analyses aimed at determining how hydrothermal activity influences protist community structure in the Okinawa Trough, while the other sampling stations near vent sites are not.

Sample collection

A Niskin rosette with 30 bottles (10 L) and fitted with a conductivity-temperature-depth (CTD) probe (SBE 911plus, Sea-Bird Scientific, Bellevue, WA) was deployed at each station to collect water from the subsurface chlorophyll maximum (SCM; 50–100 m), mid water column (mid; 700, 1000, or 1500 m), and approximately 10 m above the seafloor (bottom; 772–2957 m) (Supplemental Table 1); surface water was collected by bucket alongside the research vessel. Mid water samples were collected from 700 m at all stations except stations 3 and 18, where mid water samples were collected from

1000 and 1500 m, respectively, reflecting much deeper total depths at these stations (2407 and 2957 m vs. < 2000 m). As a result of the varying sampling depths, and because mid-water samples at stations 3 and 18 were taken from the salinity layer that contributes to bottom waters at the other stations (Supplemental Figure 2), station 3 and 18 mid-water samples were excluded from mid-water analyses and replaced the near-bottom samples from these stations in bottom-water analyses instead.

Water samples were sequentially filtered under a gentle vacuum through 10.0-µm and 0.2-µm pore-size polytetrafluoroethylene (PTFE) filters (Millipore, Burlington, MA). Size-fractionation was applied to prevent large or colonial protists and zooplankton from dominating the signal from smaller cells. Sequences from filters with larger and smaller pore-size were pooled for each sample and analyzed together as a single community. Two replicates of 4.5 liters of surface seawater from separate bucket casts or 5 liters of seawater from separate Niskin bottles (SCM, mid, bottom) were filtered from each station. Filters were flash-frozen in liquid nitrogen and stored at −80°C until DNA was extracted. Temperature, salinity, dissolved oxygen, fluorescence, and turbidity profiles were recorded by CTD probe at each station. CDOM and chemical analyses for nitrate, nitrite, NH_4^+, phosphate, silicate and total carbon were performed by Marine Works Japan Ocean Chemistry Analysis Section onboard with water collected by Niskin bottle.

DNA extraction and sequencing library preparation

DNA was extracted from PTFE filters (n = 224, two replicates of two filter pore-sizes at four depths from 14 stations) following manufacturer's protocols for the DNeasy

PowerWater Kit (Qiagen, Hilden, Germany) including the optional heating step for 10 min at 65°C to fully lyse cells. Sequencing libraries were prepared following the Illumina 16S Metagenomic Sequencing Library Preparation manual, but with universal eukaryotic primers for the V4 region of the eukaryotic 18S rRNA gene (F: CCAGCASCYGCGGTAATTCC (Stoeck et al., 2010), R: ACTTTCGTTCTTGATYR (Mars Brisbin et al., 2018)) and 58°C annealing temperature in the initial PCR. Amplicon libraries were sequenced by the Okinawa Institute of Science and Technology DNA Sequencing Section on the Illumina MiSeq platform with 2x300-bp v3 chemistry. Amplification and sequencing were successful for 211 samples and at least one replicate succeeded for each sample type.

Sequence processing

Sequence data from each of four MiSeq flow-cells were denoised separately using the Divisive Amplicon Denoising Algorithm (Callahan et al., 2016) through the DADA2 plug-in for QIIME 2 (Bolyen et al., 2019). We analyzed amplicon sequence variants (ASVs) to maximize the amount of diversity included in the study (Callahan et al., 2017) and increase our ability to detect potentially small shifts in community composition. Denoised ASV tables were merged before taxonomy was assigned to ASVs with a naive Bayes classifier trained on the Protist Ribosomal Reference (PR^2) database v4.11 (Guillou et al., 2013) using the QIIME 2 feature-classifier plug-in (Bokulich et al., 2018). We imported results into the R statistical environment (R Core Team, 2018) for further processing with the R packages phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2019), and DESeq2 (Love et al., 2014). Sequences were initially filtered to remove ASVs that were not assigned taxonomy at the Kingdom level, which are likely data artifacts, and all ASVs classified as metazoan. We did not apply prevalence or minimum abundance filtering since samples were from varied locations and depths, but the DADA2 algorithm does discard singletons. Alpha diversity metrics (observed richness and Shannon indices) were calculated with the phyloseq function ‘estimate_richness’ and pairwise Wilcox tests were performed to determine if differences in alpha diversity between samples were statistically significant.

Community, Environmental, Geographic, and Oceanographic Distance

Aitchison distance, which is defined by transforming read counts with a centered-log ratio normalization before computing euclidean distances, minimizes compositional bias inherent in metabarcoding data (Gloor et al., 2017). The Aitchison distance was, therefore, used to calculate the distance between protist community compositions in samples from different depths at each station. Aitchison distances were calculated with the clr function from the package CoDaSeq (Gloor et al., 2016).

Environmental distance between sampling stations at each depth was calculated by first transforming environmental data using the Z-scale and then computing euclidean distance between stations at each depth. Measurements for environmental parameters—including temperature, salinity, dissolved oxygen, turbidity, fluorescence, colored dissolved organic matter, nitrate, nitrite, ammonium, silicate, and phosphate—were subset by depth before being converted to Z-scores (mean = 0, standard deviation = 1), and euclidean distance between each station pair was computed for each depth layer.

Geographic distance between stations was calculated simply as the length (in km) of a straight line connecting coordinates. Oceanographic distance was calculated as the mean connection time between station pairs. Mean connection times were estimated using the 3D hydrodynamic model of Mitarai et al. (2016), which simulates ocean circulation processes in the Okinawa Trough reasonably well. The model domain, covering all of the sampling sites with a 1-km numerical mesh, was forced by realistic heat, water and momentum fluxes through the sea surface as well as barotropic tides through lateral boundaries. The rotating primitive equations with a realistic equation of state were integrated by using the Regional Ocean Modeling System (ROMS) (Shchepetkin and McWilliams, 2005; Shchepetkin, 2015). Around 260,000 model floats were deployed from each of the sampling sites in the simulation (10 floats every hour from January 1, 2011 through January 1, 2014). These floats were passively transported in horizontal directions by simulated ocean currents at a constant depth (0 and 500 m below the sea surface) together with stochastic processes representing subgrid-scale (less than 1 km) turbulence. Float time series were tracked for 360 days, and saved every 15 minutes. Transport probabilities from one site to another were calculated from an ensemble of the model floats, based on the methods of Mitarai et al. (2009). The mean connection time was derived from the transport probabilities. Results from 0 m were used in analyses for surface and SCM samples and results from 500 m were used in analyses with mid-water samples. Oceanographic distance was not included in analyses for bottom waters because ROMS does not account for complicated processes induced by bottom topography, which strongly affect dispersal trajectories in the bottom layer (e.g., see (Xu et al., 2018)).

Effects of depth, geography, oceanography, and environmental factors on community structure

A principal coordinate analysis (PCoA) was first applied to all samples, followed by a Permutational Analysis of Variance (PERMANOVA, 999 permutations) by sampling depth. Data were then subset by depth for the remaining analyses. Spearman rank correlation tests were performed to evaluate the relationship between environmental, geographic, and oceanographic distance with community dissimilarity (Aitchison distance). The influence of geography and environmental factors on community composition was further investigated in Redundancy Analyses (RDA) of Aitchison distances between communities including all Z-scaled environmental factors that did not co-vary. We tested for collinearity of environmental variables by computing Pearson correlation coefficients between variables in each depth layer (Supplemental Figure 3). If the absolute value of correlation coefficients was greater than 0.8, only one of the collinear variables was included in the RDA for that depth (Ramette, 2007). The depth of the chlorophyll maximum was additionally included in SCM RDA and the distance in kilometers from the nearest known hydrothermal vent was included in the bottom water RDA. We applied ANOVA-like permutation tests to RDA results to test whether RDA model results were statistically significant and to test which environmental variables significantly contributed to variation in community composition (Legendre and Oksanen, 2011). Pairwise PERMANOVA (999 permutations) were additionally applied to test whether region (SOT, KG, NOT) significantly affected community composition for each depth.

Hydrothermal influence on protist communities in bottom waters

To evaluate the effect of hydrothermal activity on alpha diversity, we tested whether observed richness and Shannon indices were significantly different in bottom water samples from hydrothermally influenced sites (stations 2, 10, and 11, as defined by environmental parameters) using Wilcox tests. A PERMANOVA was used to test whether community composition was significantly different in samples from hydrothermally influenced sites. In addition, we used the DESeq function to test for ASVs that were differentially abundant in bottom water from sites influenced by hydrothermal activity compared to bottom water at other sites. ASVs were considered significantly differentially abundant when the False Discovery Rate adjusted *p*-value (p.adj) was < 0.01.

Results

Protist diversity in the Okinawa Trough

Overall, 31.5 million sequencing reads were generated for this study, with 34,631–421,992 sequencing reads per sample (mean = 144,604). All sequences are available from the NCBI Sequencing Read Archive with accession PRJNA546472. Following denoising, 16.8 million sequences remained, with 1,724–215,842 sequences per sample (mean = 77,342). A total of 22,656 unique ASVs were identified in our dataset, with 49–1,906 observed ASVs per sample (mean = 730). Samples from the SCM had significantly higher observed ASV richness than surface, mid, and bottom water samples ($p < 0.001$, Supplemental Figure S4B). Shannon indices ranged from 2.1 to 6.4

for all samples and were highest for samples from the surface and SCM (mean = 5.3 for both). The Shannon indices for SCM and surface samples were not significantly different from each other but were both significantly higher than indices for mid and bottom water samples ($p < 0.001$, Supplemental Figure S4C).

Effect of depth on community structure

Samples clearly clustered by depth in PCoA with SCM with surface samples clustering separately from mid and bottom water samples along the primary axis, which accounted for 26.5% of variance (Figure 3). SCM and surface samples also formed separate clusters along the secondary axis, which explained an additional 10.6% of variance. The mid and bottom water samples, however, did not separate in the PCoA. PERMANOVA results were significant when performed by depth layer on all samples ($p = 0.001$, $R^2 = 0.15$, $F = 4.2$). Moreover, the relative abundance of major protist groups showed clear depth-related patterns (Figure 4). Protist communities in surface samples were dominated by Dinoflagellata ASVs (mean = 65% relative abundance), but also included substantial proportions of MAST (mean = 9%), Haptophyta (mean = 9%), Radiolaria (mean = 5%), and Ochrophyta (mean = 5%) ASVs. Dinoflagellata ASVs also had the highest relative abundance at the SCM (mean 50%), but other groups, including Chlorophyta (mean 17%) and Radiolaria (mean = 16%), had higher relative abundances at the chlorophyll maximum than at the surface. The mid and bottom water community compositions were made up almost completely by Radiolaria and Dinoflagellata ASVs with respective means of 50 and 48% relative abundance in mid water samples and 43 and 52% in bottom water samples. In contrast, there were not clear horizontal or regional patterns in protist community composition at the phylum level (Figure 4). Notably, though, bottom water samples from hydrothermally influenced stations (2, 10, 11) included higher proportions of Ciliophora, Ochrophyta, and MAST groups.

Relationships of environmental, geographic, and oceanographic distance with community distance

In the surface waters, Aitchison community distances correlated most strongly with geographic distance (r_s = 0.52, p = 0), followed by oceanographic distance (r_s = 0.2, p = 0.01) and environmental difference (r_s = 0.13, p = 0.02) (Figure 5). Both geographic distance (r_s = 0.51, p = 0) and oceanographic distance (r_s = 0.29, p = 0.01) were correlated with environmental distance in the surface waters (Supplemental Figure 5). The region (SOT; Southern Okinawa Trough, KG; Kerama Gap, NOT; Northern Okinawa Trough) from which surface samples were collected significantly influenced community composition based on PERMANOVA results (Table 1). In pairwise tests, communities in Northern Okinawa Trough samples were not significantly different from Kerama Gap samples, but Northern and Southern Okinawa Trough samples were significantly different and Southern samples were also significantly different from Kerama Gap samples (Table 2). These results are also apparent in the RDA ordination plot (Figure 6), where SOT samples cluster separately from NOT and KG samples. Temperature, salinity, turbidity, and nitrite, nitrate and silicate concentrations significantly contributed to the RDA results for surface water samples (Figure 6A, Supplementary Table 2). Surface waters in the Southern OT were warmer (26.8–27.9ºC, mean 27.3ºC) than in the Kerama Gap (25.3–26.2ºC, mean 25.8ºC) and

Northern OT (24.8–26.4ºC, mean 25.7ºC). Salinity was slightly higher in the Northern OT (34.6–34.8 PSU, mean 34.7 PSU) and the Kerama Gap (34.4–34.7 PSU, mean 34.6 PSU) compared to the Southern OT (34.3–34.7 PSU, mean 34.5 PSU) (Supplemental Figure 6).

At the SCM, Aitchison community distances most strongly correlated to environmental distance ($r_s = 0.62$, $p = 0$), followed by oceanographic distance ($r_s = 0.21$, $p = 0$) and geographic distance ($r_s = 0.17$, $p = 0.02$) (Figure 5). Similar to in the surface water, environmental distance at the SCM correlated with both oceanographic ($r_s = 0.22$, $p = 0.04$) and geographic distance ($r_s = 0.35$, $p = 0$) (Supplemental Figure 5). While the region from which samples were collected (SOT, KG, NOT) significantly influenced community composition (Table 1) and pairwise tests indicated all three regions were significantly different from each other (Table 2), samples from the different regions did not form separate clusters in the RDA ordination plot (Figure 6B). The RDA model results for the SCM were more robust than for the other depth layers ($R^2 = 0.43$) reflecting the strong correlation between environmental variables and community composition; salinity, temperature, SCM depth, turbidity, CDOM, and nitrite, nitrate, ammonium, and silicate concentrations all significantly contributed to RDA results (Supplemental Table 2). Nitrate and nitrite concentrations tended to be higher in the NOT and KG compared to the SOT, whereas salinity and temperature effects seemed to be driven primarily by conditions at station 13 (Supplemental Figure 6).

Community distances between samples collected from intermediate water (mid samples) correlated with environmental distance, but did not correlate with geographic or oceanographic distance (Figure 5) and environmental distance did not correlate with geographic or oceanographic distance for mid water samples (Supplemental Figure 5). Results from PERMANOVA by region and pairwise PERMANOVA for mid water samples were not statistically significant (Tables 1, 2). Like the SCM, RDA ordination did not show clustering by region among mid-water samples (Figure 6C), although the RDA model results were significant; salinity, temperature and turbidity significantly contributed to the RDA model. Since salinity and temperature were mostly very similar among mid water samples, the RDA results appear to be strongly affected by the higher salinity and temperature recorded at station 15 (Supplemental Figure 6).

In the bottom water samples, community distance correlated with environmental distance but not with geographic distance (Figure 5). Results from PERMANOVA by region were significant (Table 1), as were results for all three pairwise tests (Table 2). Southern Trough samples clustered separately from KG and NOT samples in the RDA ordination (Figure 6D) and environmental variables significantly contributing to results included temperature, salinity, dissolved oxygen, and ammonium and nitrite concentrations (Supplemental Table 2). Salinity and temperature were more variable among near-bottom samples than in intermediate waters, but the differences were small (Supplemental Figure 6). Distance to nearest hydrothermal vent did not significantly contribute to the RDA model, but it was 180º from turbidity and ammonium concentration in the ordination plot, both of which aligned with hydrothermally influenced sites (Figure 6D) and are associated with hydrothermal activity in the Okinawa Trough, where vents resuspend overlying terrigenous material creating turbid vent plumes (Toki et al., 2016).

Protist communities at sites influenced by hydrothermal activity

Hydrothermal influence did not increase or decrease observed ASV richness or Shannon indices in bottom water samples (Supplemental Figure 4D–E). Community composition at hydrothermally influenced sites, however, was significantly different from the other bottom water samples (Table 1). The differences in community composition were visible at the phylum level, with stations under hydrothermal influence having distinct relative abundance fingerprints (Figure 4). When investigated at the ASV level, 45 ASVs were found to have significantly different abundances in bottom water samples from hydrothermally influenced sites and the majority of these AVSs (30) were more abundant at sites with hydrothermal influence (Figure 7). The significantly more abundant ASVs included Dinophyceae, Syndiniales, Chrysophyceae, MAST, RAD-A and RAD-B Radiolarians, Oligohymenophorea and Spirotrichea ciliates, and Picozoa.

Discussion

Factors influencing protist community structure in the Okinawa Trough

The vast diversity extant among marine protists contributes to their importance in sustaining marine ecosystem function, but to what extent different factors shape protist diversity under varying conditions remains challenging to study. Here, we investigated the effects of environmental variables, geography, and oceanography on the structure of protist communities in the Okinawa Trough. Consistent with previous studies (Countway et al., 2007; Canals et al., 2020; Giner et al., 2020), depth, which determines key environmental conditions (i.e. temperature, light), played the largest role in shaping protist communities; communities in samples collected from the same depth were more similar to each other than to samples from other depths, regardless of the geographic distance separating sampling sites (Figure 3). Depth-dependent diversity patterns in the Okinawa Trough were similar to results from other regions, with higher diversity in the surface and SCM than in the meso- and bathypelagic (Countway et al., 2007; Canals et al., 2020) (Supplemental Figure 4). In addition, overall community structure by depth was comparable to global patterns: Dinoflagellata, Haptophyta, Ochrophyta, Picozoa, Radiolaria and Marine Stramenopile (MASTs) sequences had high relative abundances at the surface and SCM while Dinoflagellata and Radiolaria sequences dominated in the meso- and bathypelagic (Countway et al., 2007; Pernice et al., 2016). Within depth layers, protist communities were mostly horizontally homogeneous when evaluated at a high taxonomic level (Figure 4), but some regional variation associated with both spatial and environmental factors emerged when communities were examined at the ASV level (Figure 6). Most notably, protist communities in bottom waters affected by nearby hydrothermal activity were significantly different from communities in other bottom water samples (Table 1).

We observed a strong distance-decay relationship for community compositions in the surface waters of the Okinawa Trough, where the fast-flowing Kuroshio Current causes primarily unidirectional dispersal (Figure 2, 5). Strong distance-decay relationships have been found for protist communities in a variety of ecosystems, including shallow ocean regions over the continental shelf (Wu et al., 2018), in marine sediments (Pan et al., 2020), and in terrestrial systems (Lentendu et al., 2018). The strength of microbial distance-decay relationship is related to the scale at which communities are sampled and stronger distance-decay relationships have been detected at regional scales compared to larger (i.e. continental) scales (Martiny et al., 2011; Pan et al., 2020). Geographic distances between sampling sites in this study ranged from ~30–840 km, representing a regional scale. The relationship between oceanographic distance (mean connection time) and protist community dissimilarity also varies in strength depending on scale. Richter et al. (2019) found that community dissimilarity correlated with oceanographic distance between sites if connection times were less than 1.5 years, as they were in our study area, and that oceanographic distance correlated more strongly with community dissimilarity than geographic distance (Richter et al., 2019). In contrast, geographic distance correlated more strongly with community dissimilarity in our study (Figure 5). This difference likely results from the sampling scheme; when geographic distance corresponds to a latitudinal gradient it will have a larger effect on community community composition than if it corresponds to a longitudinal gradient (Richter et al., 2019).

Ecosystem modelling predicts that strong western boundary currents, like the Kuroshio Current, have homogenizing effects on community structure, but also that boundary currents will have relatively high overall diversity due to advection from distinct water masses on either side of the current (Clayton et al., 2013; Kuhn et al., 2019). The homogenization effect is expected to be moderated by varying light and temperature conditions experienced by communities within currents traversing large distances across latitudinal gradients (Kuhn et al., 2019). Although largely similar, the differences in community composition in surface waters collected along the Kuroshio Current in the Okinawa Trough were best explained by variation in temperature (Figure 6), which vary along a latitudinal gradient (Figure 2). Advection of surface waters through the Kerama Gap into the Okinawa Trough (Na et al., 2014) also influenced community composition in upstream samples; communities in Kerama Gap surface water were significantly different from Southern Trough samples but not from Northern Trough samples (Figure 6, Table 2).

At the chlorophyll maximum, which experiences similar current conditions to the surface waters, community dissimilarity correlated with geographic and oceanographic distance to a lesser extent than at the surface (Figure 5), environmental conditions played a larger role in explaining variation in community structure (Figure 6), and regional differences were less pronounced (Figure 6). The significant difference in community composition between Northern Trough and Kerama Gap SCM samples (Tables 1, 2), despite advection also occurring through the Kerama Gap at this depth, was likely driven by Changjiang (a.k.a. Yangtze) Diluted Water (CDW) being advected and mixing with Kuroshio water near station 13. The Changjiang/Yangtze is the longest river in China and transports terrestrial nutrients and anthropogenic pollutants into the East China Sea, causing eutrophication that regularly triggers diatom blooms (Lin et al., 2014). CTD and nutrient measurements at station 13 showed decreased subsurface salinity and increased nutrient concentrations and chlorophyll fluorescence compared to other sampling sites (Supplemental Figure 7). Accordingly, we also observed an increased contribution of diatom sequences (Ochrophyta, Figure 4) to the community composition at station 13.

The distance-decay relationship observed in the surface and at the SCM broke down for the mid and near-bottom waters, where community dissimilarity correlated with environmental distance but not with geographic or oceanographic distance.

Intermediate waters entering the Okinawa Trough through the Yonaguni Depression and the Kerama Gap are well mixed throughout the trough (Nitani, 1972; Guo and Morinaga, 1998; Chen, 2005). The mid water samples were collected from this depth layer (700 m) and showed limited regional variation (Figure 6, Tables 1, 2). Apart from station 15, there was very little variability in temperature (< 1ºC) or salinity (~0.1 PSU) in mid water samples (Supplemental Figure 6 and also cf. Figure 2) and the majority of environmental parameters co-varied with temperature (Supplemental Figure 3). Bottom waters in the geographically confined basin of the Okinawa Trough mainly derive from intermediate water entering through the Kerama Gap (Nakamura et al., 2013). Bottom waters in the trough are considered well-mixed and there is minimal temperature variation (Figure 2). Deep water parcels spread throughout the basin on the order of months (Nakamura et al., 2013), which is longer than the generation times for most protists (Laws et al., 1987; Rose and Caron, 2007) and, therefore, could contribute to regional differences seen in bottom water samples (Tables 1, 2). Alternatively, the relatively more shallow depth of the Northern Trough compared to the Southern Trough (Figure 1, Supplemental Table 1) influences bottom water temperatures (Figure 2, Supplemental Figure 6) and may explain regional differences in community composition between the Southern Trough and Northern Trough (Figure 6, Table 2).

Protist communities at hydrothermal vent sites

The protist community compositions in samples from hydrothermally influenced sites were significantly different from communities in bottom waters at sites without hydrothermal influence (Table 1). Syndiniales, Ciliophora, and MAST (Marine

Stramenopiles) ASVs were enriched in hydrothermally influenced OT bottom waters, which is consistent with previous work comparing protist communities in bottom waters with varying proximity to hydrothermal vents (Sauvadet et al., 2010). We additionally found Ochrophyta, environmental clades of Radiolaria, and a Picozoa ASV to be enriched at hydrothermally influenced sites in this study (Figure 7). ASVs that were more abundant at hydrothermally influenced bottom-water were not completely absent from other sites and depths, and several were often present at low background levels. Deep-water circulation models indicate that vents in back-arc basins, such as the Okinawa Trough, are well-connected, but that basin to basin dispersal may be limited (Mitarai et al., 2016). This is consistent with our results showing the same taxonomic groups and ASVs becoming more abundant near vents in both the Southern Okinawa Trough (stations 10, 11) and the Kerama Gap (station 2), although results would be more conclusive if we had also sampled hydrothermally influenced bottom water in the Northern Trough. An important question that emerges from these results is whether or not protists enriched near vents in the Okinawa Trough are also found in other hydrothermal systems or other types of marine ecosystems. If so, it would support wider dispersal and opportunism among protists enriched in hydrothermal systems as opposed to restricted dispersal and more specific adaptation.

Dinoflagellate ASVs made up the majority of differentially abundant ASVs at hydrothermally influenced sites (22 out of 45, Figure 7) and half of these were more abundant near vents. Among the enriched ASVs, eight are in the parasitic order Syndiniales, including group I (clades 2 and 7) and group II (clades 1, 6, 12, 13, 14, and 16). The remaining enriched dinoflagellate ASVs belong to the Order Dinophyceae but could not be further classified. Group II Syndiniales are common in sunlit, surface waters, whereas group I is common in suboxic and anoxic ecosystems. However, both groups I and II are found in surface and deep water and have clades that have only been recovered from suboxic or anoxic ecosystems (Guillou et al., 2008). The majority of the enriched Syndiniales ASVs shared 100% identity with GenBank sequences (nr/nt, accessed 5/16/2019, (Camacho et al., 2009; Clark et al., 2016)) from a wide variety of ecosystems throughout the global ocean, including surface waters near the poles, mesopelagic water near the California coast, 100 and 200 m oxygenated and micro-oxic North Atlantic waters, and at the sediment interface near the Juan de Fuca Ridge vent system (Edgcomb et al., 2011; Lie et al., 2014); the Syndinales ASVs enriched in hydrothermally influenced samples clearly derive from cosmopolitan organisms. Although they are not restricted to vent systems, parasitic Syndiniales do seem to thrive in vent systems and may take advantage of increased host availability in such regions (Moreira and López-García, 2003).

Ochrophyta accounted for the second-most enriched ASVs in hydrothermally influenced samples (Figure 7). The enriched Ochrophyta ASVs belong to several clades within the order Chrysophyceae. Two belong to clades with unknown morphology composed from environmental sequences (clades EC2H and EC2I) that were recovered from a diverse array of marine and freshwater environments (Scoble and Cavalier-Smith, 2014). The remaining ASVs belong to the genus *Paraphysomonas*, which are colorless phagotrophs that are globally distributed in freshwater, marine, and soil ecosystems and have previously been recovered from vent sites (Atkins et al., 2000). Although several of the enriched ASVs share 99%–100% identity with previously published vent-associated sequences (Atkins et al., 2000), they are equally similar to sequences recovered from many other marine environments, indicating that these enriched ASVs also derive from cosmopolitan organisms.

Five of the ASVs enriched in hydrothermally influenced samples belong to marine stramenopile lineages—MAST-1, -7, -8 (Figure 7). Most of what is known about MAST lineages has been learned through environmental molecular surveys. Aggregate analyses of sequences collected through such studies have found that MAST-1, -7, and -8 are highly diverse, globally-distributed, and abundant in surface waters (Massana et al., 2004, 2014). Culture-independent techniques have further demonstrated that MAST-1 (Massana et al., 2006), MAST-7 (Frias-Lopez et al., 2009), and MAST-8 (Massana et al., 2014) are bactivorous flagellates. Therefore, it is likely that the MAST ASVs enriched in hydrothermally influenced samples represent cosmopolitan bacterivores. Indeed, MAST ASVs from this study each share 99% (MAST-7) to 100% (MAST-1, -8) identity with multiple sequences recovered from surface waters, including from (anoxic) Saanich Inlet, Vancouver, Canada (Orsi et al., 2012), the Scotian Shelf in the North Atlantic (Dasilva et al., 2014), and the Southern Ocean (Massana et al., 2002).

The RAD-A and RAD-B radiolarian groups represent environmental clades that have no morphological description—other than being picoplankton—or known ecological roles (Not et al., 2007). The RAD-A ASV enriched in hydrothermally influenced samples (Figure 7) shares > 99% identity with many GenBank sequences recovered from oxic and anoxic waters globally, including the Cariaco basin in the Caribbean Sea (Edgcomb et al., 2011), the Gulf Stream (Lie et al., 2014), and the South

East Pacific (Shi et al., 2009). The enriched RAD-B ASV shares 100% identity with multiple sequences recovered from the East Pacific Rise (1500 m and 2500 m), the Arctic Ocean (500 m), oxygenated water in the Cariaco Basin (Edgcomb et al., 2011), the Juan de Fuca Ridge (Jungbluth et al., 2013), and the Southern Ocean (Clarke et al., 2019). While the radiolarian ASVs enriched in hydrothermally influenced samples appear to be cosmopolitan, it is notable that identical and similar sequences have repeatedly been isolated from vent fields and anoxic regions.

Previous studies indicate that Ciliophora are abundant in sediments and bacterial mats near hydrothermal vents (López-García et al., 2007; Coyne et al., 2013; Pasulka et al., 2019). It is unsurprising, then, that two Ciliophora ASVs, belonging to the classes Spirotrichea and Oligohymenophorea, were significantly more abundant in hydrothermally influenced samples (Figure 7). The enriched Spirotrichea ASV was classified to genus level by our classifier as belonging to Leegaardiella, a recently described genus collected from the North Atlantic (Santoferrara et al., 2017). The Leegardiella ASV shared 100% coverage and identity with one sequence in GenBank—an uncultured eukaryote clone recovered from 2500 m at the East Pacific Rise—and had > 99% shared identity with another sequence recovered from the East Pacific Rise, also from 2500 m (Lie et al., 2014). However, the Leegardiella ASV also had 100% coverage and > 99% identity matches with several sequences recovered from Arctic surface water (Terrado et al., 2013). This Leegardiella ASV may, therefore, represent a widely distributed organism that opportunistically becomes more abundant near vent sites. The Oligohymenophorea ASV was classified as belonging to the globally abundant Oligo5 environmental clade (Canals et al., 2020), which is defined only by sequences from environmental surveys and lacks any morphological or physiological descriptions (Boscaro et al., 2018). The Oligo5 ASV did not match any sequences in GenBank with > 96% identity; the closest match was to an uncultured eukaryote clone recovered from 500 m depth off the coast of California (Lie et al., 2014). While the Oligo5 clade lacks formal descriptions, it is grouped with the subclass Scuticociliatia in the EukRef-Ciliophora curated Oligohymenophorea phylogeny, which includes many formally described species (Boscaro et al., 2018). Zhao and Xu (2016) also found both Spirotrichea and Scuticociliatia enriched among ciliates found near a vent site in the Northern Okinawa Trough (Zhao and Xu, 2016). Both Spirotrich and Scuticociliatia ciliates are generally bacterivorous (Christaki et al., 1998; Coyne et al., 2013), but Scuticociliatia are also commonly found as symbionts, parasites, or pathogens of aquatic organisms (Umehara et al., 2003; Lynn and Strüder-Kypke, 2005; Bourne et al., 2008; Fan et al., 2011), including giant hydrothermal bivalves (Sauvadet et al., 2010). If the Oligo5 ASV represents a symbiotic or parasitic ciliate, it could provide an explanation as to why more similar sequences were not recovered elsewhere, since coevolution with a dispersal-limited host can also limit symbiont dispersal (Peek et al., 1998). However, it is equally likely that organisms with high sequence similarity to this ASV have not yet been detected elsewhere simply due to undersampling (Canals et al., 2020).

A single ASV enriched near vent sites belonged to Picozoa (Figure 7). The first Picozoa species was described in 2013 (Seenivasan et al., 2013) and Picozoa ecology remains poorly resolved. Information regarding their distribution derives from environmental molecular surveys, which have established five clades within Picozoa.

While all Picozoa are marine, biogeographical patterns vary between clades: clade BP2 has only been found in surface waters, group 2 is mostly found in deep waters, and the other three groups are globally distributed throughout the water column (Moreira and López-García, 2014). The enriched Picozoa ASV from this study belongs to one of the cosmopolitan clades (Group 1). The sequence shares 100% identity with three others in Genbank, from a variety of environments—surface water in the Arctic (Terrado et al., 2011) and Southern (Díez et al., 2001) oceans, as well as mesopelagic water near southern California (Lie et al., 2014).

Overall, all but one of the ASVs enriched in hydrothermally influenced bottom waters in the Okinawa Trough were at least 99% identical to sequences already recovered elsewhere. Therefore, it is likely that the enriched protists are widely dispersed and opportunistically become more abundant near vents in response to additional resource availability (e.g. hosts, prey, organic matter). However, the v4 hypervariable region of the 18S ribosomal RNA gene cannot capture all the diversity extant in protist populations, and a metagenomic approach may better resolve protists specifically adapted to certain conditions or locations in the future (e.g. (Obiol et al., 2020).

Conclusions and future directions

Disentangling factors that structure microbial communities remains a challenge, particularly since many environmental variables, such as temperature, covary with geography and oceanography. At the regional scale studied here, environmental factors linked to regional oceanography, such as surface currents and advection, played a role in shaping protist community structure. In the surface waters and at the SCM distance-decay relationships between protistan community compositions and geographic distance were also detected.

We identified distinct protist communities associated with hydrothermally influenced sites in the Okinawa Trough, with bacterivorous, parasitic, and potentially symbiotic protists enriched in hydrothermally influenced bottom waters. The enriched protists were mostly globally distributed, and many were previously found at vent sites in other parts of the world. These results emphasize the importance of the rare biosphere; most protists seem to be widely distributed, even if rare, and can become opportunistically more abundant under certain conditions. A major limitation of metabarcoding studies with DNA is that it is impossible to know whether sequences derive from actively metabolizing cells, dead or inactive cells, or even environmental DNA; future studies will benefit from incorporating methods that better delineate metabolic state. In the future, comparisons between the Okinawa Trough and other hydrothermal regions will continue to advance our understanding of important protists in hydrothermal ecosystems and how hydrothermal activity influences protist diversity and biogeography in the global ocean.

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Code Availability

The data and code necessary to reproduce the statistical analyses for this study are available on GitHub: https://github.com/maggimars/OkinawaTroughProtists.

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Figures

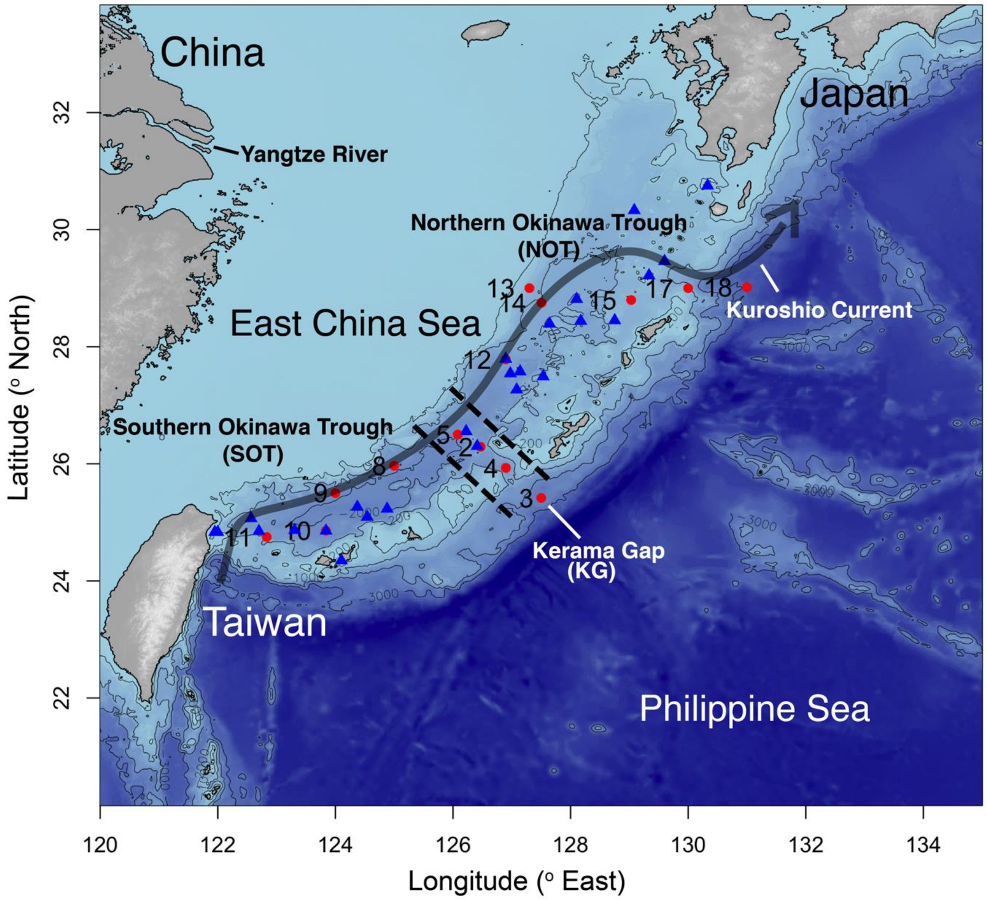

Figure 1. Sampling locations in the Okinawa Trough. Numbered red circles denote sampling stations where water was collected during the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) MR17-03C cruise in May and June 2017. Blue triangles indicate active hydrothermal sites according to the InterRidge Vents Database v3.4 (https://vents-data.interridge.org/ventfields-osm-map, accessed 05/08/2019).

Bathymetry data was accessed and plotted through the function getNOAA.bathy in R package marmap. The 200, 1000, 2000, and 3000 m isobaths are plotted and labeled. Station 2 is at the ANA site of the Daisan-Kume Knoll, station 10 is at the Hatoma Knoll, station 11 is near the Dai-Yon Yonaguni Knoll, station 5 is near the Higa vent site, and station 12 is near the Iheya North vent field.

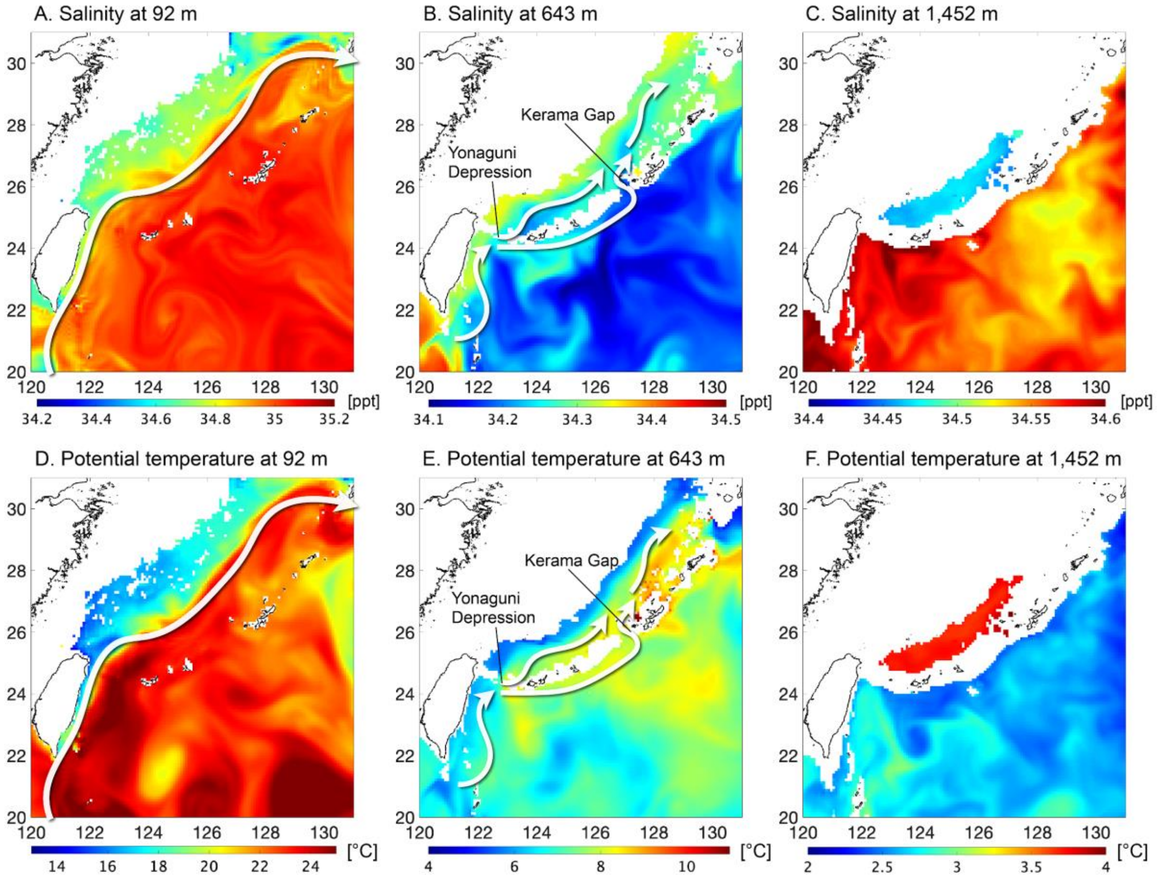

Figure 2. Water properties around the Okinawa Trough. Color contours indicate salinity at 92 m (A), 643 m (B) , and1,452 m (C) below the sea surface, and potential temperature at 92 m (D), 643 m (E), and 1,452 m (F) below the sea surface for June 6, 2017, obtained from Mercator Ocean data assimilation product, PSY4R3V1 (Gasparin et al., 2018). White arrows indicate the paths of known ocean currents in the surface and intermediate layers. Salinity-minimum intermediate water masses enter the Okinawa Trough through the Kerama Gap and Yonaguni Depression, which are labelled in panels B and E.

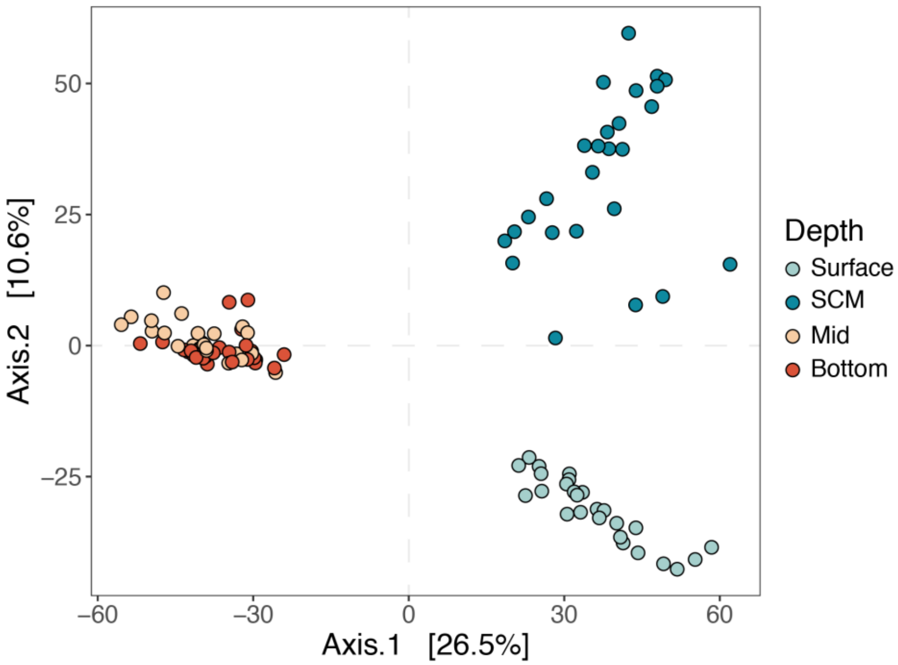

Figure 3. Principal coordinates analysis of Aitchison distances between protist communities from four depths in the Okinawa Trough. Point color indicates the depth layer from which samples were collected. Samples cluster in three main groups by depth: surface, subsurface chlorophyll maximum (SCM), and mid/bottom waters. Community composition was significantly different by depth layer (PERMANOVA, 999 permutations, $p = 0.001$, $R^2 = 0.15$, $F = 4.2$).

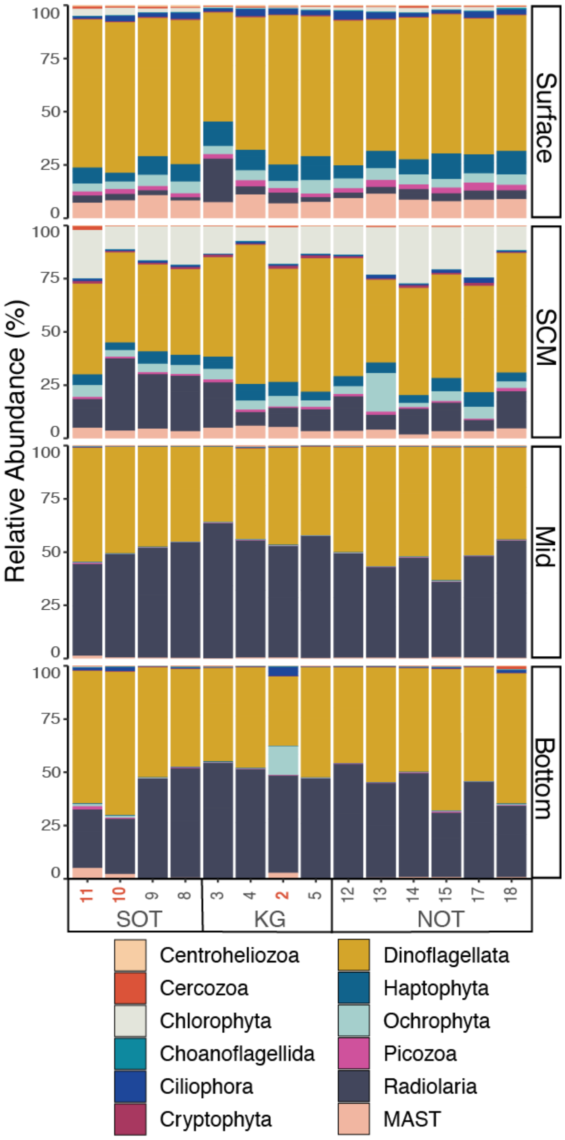

Figure 4. Relative abundance of major protist phyla in samples collected from four depths in the Okinawa Trough (OT). Sampling stations are grouped on the x-axis based on oceanographic region: Southern Okinawa Trough (SOT) stations include 11, 10, 9, and 8; Kerama Gap (KG) stations include 3, 4, 2, and 5; Northern Okinawa Trough (NOT) stations include 12, 13, 14, 15, 17, and 18. The plot is faceted by sampling depth, so that surface samples make up the top panel and bottom water samples make up the bottom panel. Replicates were merged and are represented by a single stacked bar. Regional community differences are not visible at the high taxonomic level represented in the plot, but there are clear differences in community composition by sampling depth. Bottom waters at hydrothermally influenced sites (11, 10, and 2; highlighted in red) have visibly increased relative abundance of Ciliophora, Ochrophyta, and MAST sequences.

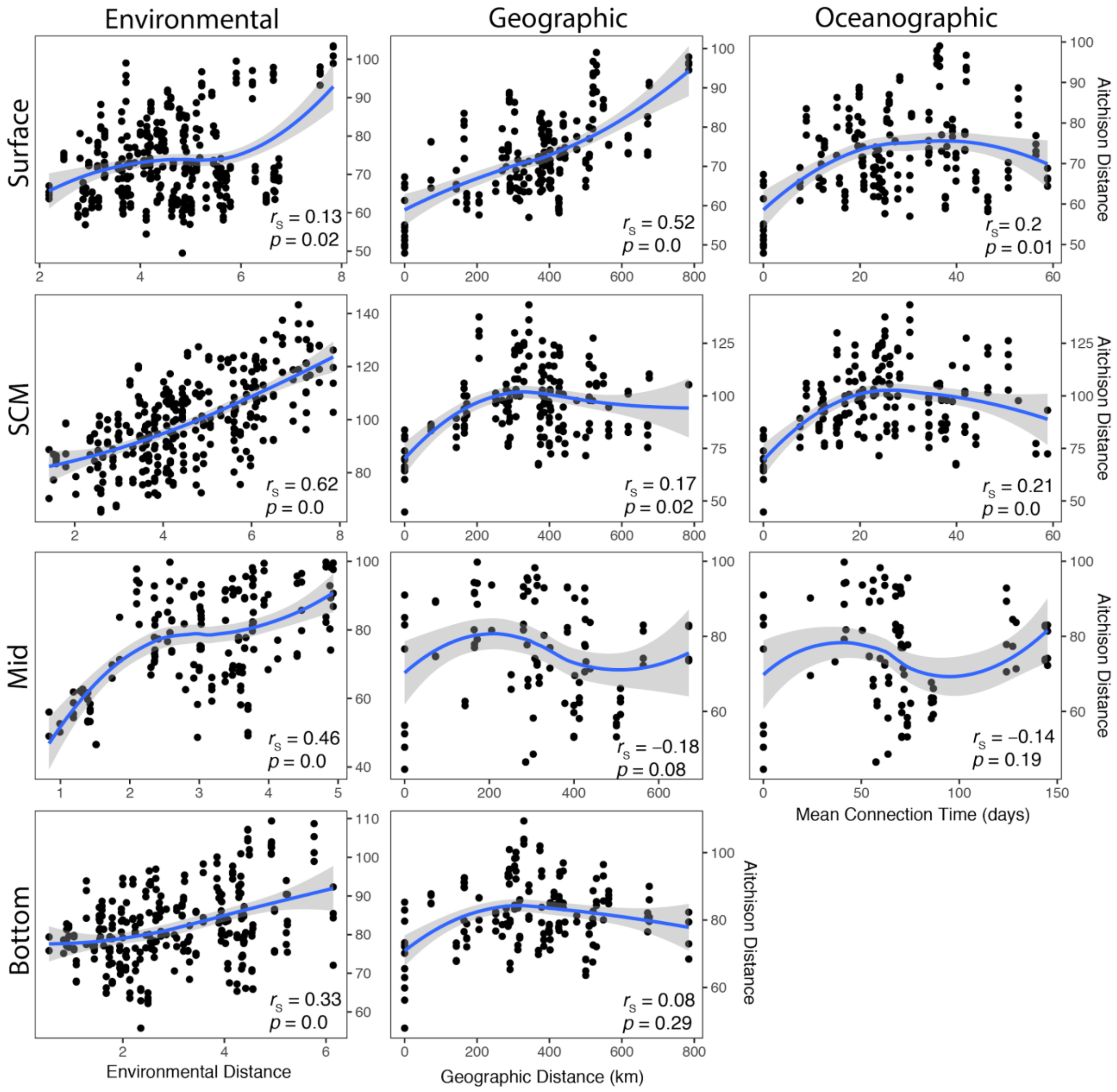

Figure 5. Relationships between the environmental, geographic, and oceanographic distances between sampling locations and the Aitchison distance between protist community compositions. Environmental distance between sampling locations was calculated as a Euclidean distance from Z-scaled environmental variables (temperature, salinity, dissolved oxygen, turbidity, fluorescence, colored dissolved organic matter, and nitrate, nitrite, ammonium, silicate, phosphate concentrations). Geographic distance was calculated as the direct distance between coordinates for each sampling location. Oceanographic distance is the mean connection time between points as estimated from simulated model floats. Curves were fit with local regression (loess) and shaded gray regions represent 95% confidence intervals. Spearman correlation coefficients (r_S) and p-values are included within plot panels.

Station 15 was excluded from mid and bottom water analyses because environmental conditions (relatively high salinity and temperature) made it an outlier.

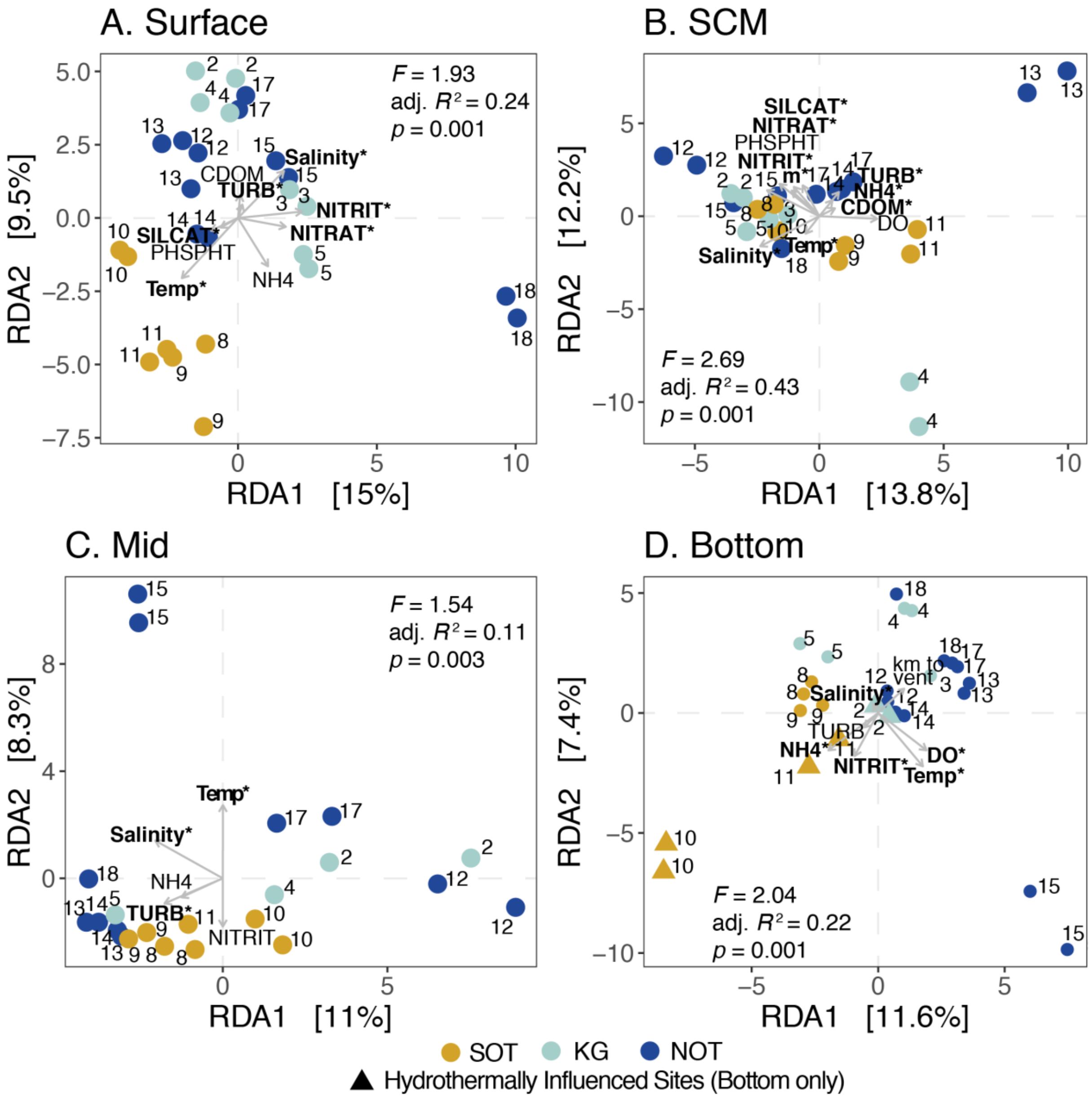

Figure 6. Distance based redundancy analysis (RDA) of protist communities and environmental variables from four depth-layers in the Okinawa Trough. RDA were performed on Aitchison distances between samples at each depth with Z-scaled environmental variables that were not collinear (Pearson correlation coefficient < |0.8|). Results from ANOVA-like permutation tests for RDA are displayed on each plot panel. Environmental variables that significantly contributed to RDA results (p <0.05, Supplementary Table 2) are bolded and marked with an asterisk. Points are colored by oceanographic region; KG is the Kerama Gap (light blue), NOT is the Northern Okinawa

Trough (dark blue), and SOT is the Southern Okinawa Trough (gold). For bottom water samples (D), hydrothermally influenced sites are demarcated as triangles.

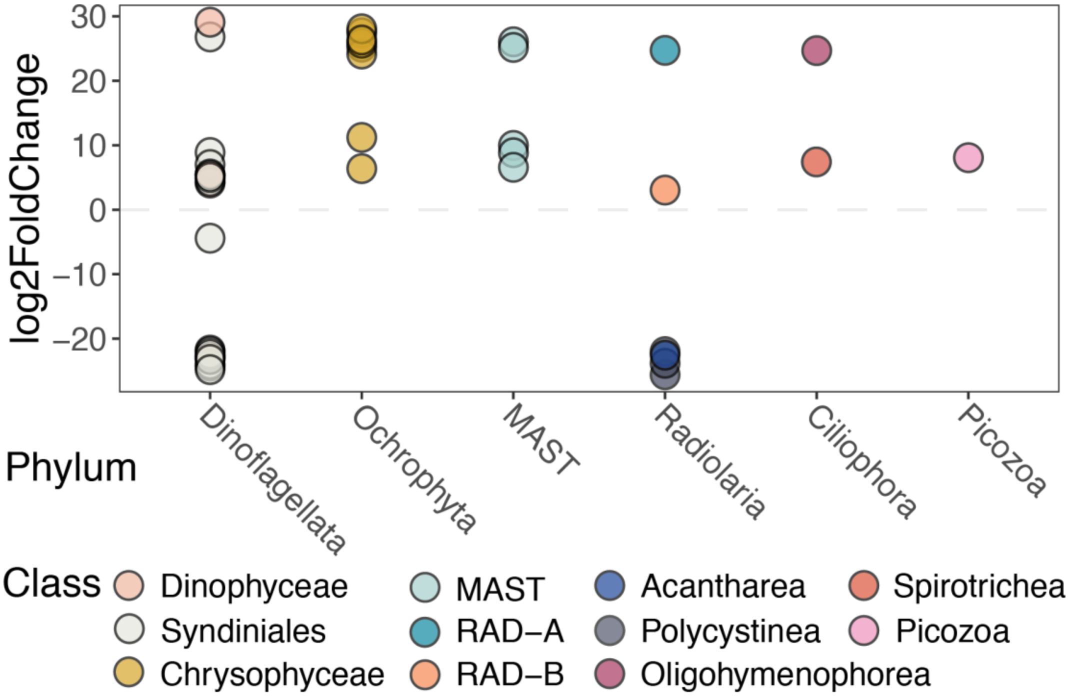

Figure 7. Log2 fold-change of ASVs with significant differential abundance in bottom waters influenced by hydrothermal activity. The DESeq function in the DESeq2 package was used to test whether ASVs were significantly more (positive log2 fold-change) or less (negative log2 fold-change) abundant in hydrothermally influenced bottom water samples from Stations 10, 11, and 2 compared to bottom water samples from the other sites. Each point represents a single significantly differentially abundant ASV and taxonomic groupings are indicated on the x-axis (Phylum) and by color (Class). ASVs were considered significantly differentially abundant if the False Discovery Rate adjusted *p*-value was < 0.01.

Table 1. PERMANOVA results by oceanographic region and presence of hydrothermal influence (999 permutations).

Test	R^2	F	p
Surface ~ region	0.15	2.30	**0.001***
SCM ~ region	0.15	2.10	**0.001***
Mid ~ region	0.11	1.22	0.092
Bottom ~ region	0.16	2.22	**0.001***
Bottom ~ hydrothermal influence	0.04	2.05	**0.001***

*** statistically significant ($p < 0.05$)**

Table 2. Results from pairwise PERMANOVA by oceanographic region for each depth (999 permutations).

A. Surface

Comparison	R^2	F	p	*p*.adj
KG.NOT	0.07	1.34	0.118	0.118
KG.SOT	0.17	2.74	0.001	**0.002***
NOT.SOT	0.14	2.75	0.001	**0.002***

B. SCM

Comparison	R^2	F	p	*p*.adj
KG.NOT	0.10	1.81	0.012	**0.012***
KG.SOT	0.15	2.30	0.001	**0.002***
NOT.SOT	0.12	2.23	0.001	**0.002***

C. Mid

Comparison	R^2	F	p	p.adj
KG.NOT	0.07	1.01	0.36	0.36
KG.SOT	0.11	1.13	0.19	0.28
NOT.SOT	0.08	1.45	0.04	0.12

D. Bottom

Comparison	R^2	F	p	p.adj
KG.NOT	0.10	1.75	0.002	**0.002***
KG.SOT	0.13	2.00	0.001	**0.002***
NOT.SOT	0.15	2.88	0.001	**0.002***

*** statistically significant (*p*.adj < 0.05)**