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ORIGINAL ARTICLE

Eight years of weekly eDNA monitoring in the North-Western Pacific

Sirje Sildever[1,2] | Noriko Nishi[3] | Satoshi Tazawa[3] | Hiromi Kasai[4] | Junya Hirai[5] | Akihiro Shiomoto[6] | Taisei Kikuchi[7,8] | Seiji Katakura[9] | Satoshi Nagai[1,10]

[1]Yokohama Field Station, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, Yokohama, Japan

[2]Department of Marine Systems, Tallinn University of Technology, Tallinn, Estonia

[3]Axiohelix Co. Ltd, Tokyo, Japan

[4]Kushiro Field Station, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, Kushiro, Japan

[5]Atmosphere and Ocean Research Institute, The University of Tokyo, Chiba, Japan

[6]Department of Bioindustry, Tokyo University of Agriculture, Abashiri, Japan

[7]Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan

[8]Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

[9]City of Mombetsu, Mombetsu, Japan

[10]Yokohama Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Yokohama, Japan

Correspondence
Satoshi Nagai, Yokohama Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, 2-12-4 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-8648, Japan.
Email: snagai@affrc.go.jp

Sirje Sildever, Department of Marine Systems, Tallinn University of Technology, Akadeemia tee 15A, 12618, Tallinn, Estonia.
Email: sirje.sildever@taltech.ee

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Abstract

Detection of marine organisms based on environmental DNA (eDNA) has expanded the knowledge of species richness, distribution, and appearance patterns. We used eDNA data from a single location sampled weekly over 8 years to detect as many species as possible, and investigate their appearance patterns and associations with biotic and abiotic factors. We detected >2500 operational taxonomic units (OTUs) associated with unique species from nine supergroups. The dominating currents that differ significantly in temperature, salinity, and nutrient concentrations structured the richness of the OTUs. We further detected sporadic and seasonal or continuous presence among the OTUs connected with a single species. This study shows that the long-term eDNA-based monitoring approach provides comprehensive knowledge of the species present as well as their associations with biotic and abiotic factors.

KEYWORDS
biodiversity, currents, metabarcoding, plankton, seasonality, time series

1 | INTRODUCTION

High-throughput sequencing (HTS) technologies have facilitated the simultaneous detection of a broad range of taxa based on environmental DNA (eDNA) (Taberlet et al., 2012). This has proven useful for capturing detailed information on the biodiversity present across the tree of life (Sawaya et al., 2019; Weigand et al., 2019; Zhang, Pavlovska et al., 2020b). It has also led to the exponential growth of the knowledge available both in terms of previously undescribed biodiversity as well as on the spatial and temporal distribution of the described species (Sawaya et al., 2019; Sevellec et al., 2021). This information is much needed as at the beginning of the last decade, 91% of the diversity present in the oceans was estimated to yet be described (Mora et al., 2011). This need is still reflected by the eDNA studies detecting sequences with no match available in the existing databases (Berry et al., 2019; Chavez et al., 2021; Lima-Mendez et al., 2015). eDNA-based methods are becoming even more accessible, which facilitates their use as universal monitoring tools. Supported by the advances in sequencing technology and extensive high-quality databases they will contribute to the development of a comprehensive global and operational ecosystems observation network (Chavez et al., 2021).

Combining environmental monitoring with metabarcoding and the eDNA approach also advances the knowledge of the influence of abiotic parameters on community dynamics (Berry et al., 2019; Hirai et al., 2017; Jacobs-Palmer et al., 2021; Robicheau et al., 2022). Also, the simultaneous detection of multiple taxa facilitates the understanding of potential interactions between species/groups (Djurhuus et al., 2020; Hirai et al., 2021; Sildever et al., 2022). Time series monitoring based on eDNA can be especially useful for determining the natural variability, fluctuations, and changes in community composition (Djurhuus et al., 2020). This knowledge would form a basis for a realistic understanding of human-induced changes in marine communities (Gallego et al., 2020; Smayda, 1998; Zingone et al., 2019). Further, it will serve as valuable input for achieving several of the desired outcomes of the United Nations Decade of Ocean Science (United Nations, 2022).

In this study, we focus on the diversity and seasonal patterns of eukaryotic planktonic communities based on weekly eDNA samples from 8 years. The samples originate from the north-western Pacific, more specifically from the southern part of the Sea of Okhotsk facing the northern coast of Hokkaido, Japan. Those waters are commercially important for scallop aquaculture (Imai et al., 2014) and as nurseries for juvenile salmon (Urawa et al., 2000). The area is influenced by two seasonally dominating current systems: Soya warm current (>7°C, salinity >33.6, April–October) and cold East Sakhalin current (<7°C, salinity <32, November–March; Itoh & Ohshima, 2000). The area also has the southernmost sea ice cover in the northern hemisphere (Nihashi et al., 2009). However, during the past 50 years, the air temperatures in the region have increased by approximately +2°C in winter months (Nakanowatari et al., 2007). This has resulted in reduced sea ice coverage (Ohshima et al., 2009), which has implications for local biological productivity (Mustapha & Saitoh, 2008). Through the reduction of water, nutrients, and organic carbon reaching the North Pacific, the local reduction in sea ice may have a broader impact on the regional biological production, fisheries resources, and carbon sequestration capacity (Ohshima et al., 2009; Yan et al., 2022).

Our main goal was to detect as much of the planktonic richness present in a single location as possible and investigate their appearance patterns and associations with biotic and abiotic factors. To achieve this goal, we targeted three different genes and utilized five marker pairs, including group-specific markers.

2 | METHODS

2.1 | Sampling and DNA extraction

Surface seawater was collected weekly between April 2012 and March 2020 ($n=445$) from the Okhotsk Tower off the coast of Mombetsu in northern Hokkaido, Japan (44°20.2′ N, 143°22.9′ E) using a bucket. The sampling was carried out around the same time (11 a.m.) throughout the years. A subsample of the surface water was used to analyze Chl *a*, nitrite (NO_2), nitrate (NO_3), phosphate (PO_4), and silica (SiO_2) concentrations. Chl *a* and nutrient analyses were conducted as described in the literature (Parsons et al., 1984; Welschmeyer, 1994). Water temperature and salinity were measured during sampling by a CTD (ASTD102, JFE Advantec Co. Ltd.) from the surface and bottom layers. For collecting eDNA samples, 200–1000 mL of seawater was filtered through different size fraction filters: 2012–2017: 8 and 1 μM pore-size filters (Nuclepore membrane, GE Healthcare) were used, and DNA extracted from both filters was pooled by mixing of equal volumes; from 2017 to 2019, the samples were filtered only through 1 μm pore-size filters, and from 2019 onward through 0.22 μm Sterivex filters (Merck KGaA). The filtration time was limited to 20 min to avoid damaging the cells and the filters were stored in a freezer (−80°C) until DNA extraction. DNA was extracted using a 5% Chelex buffer following the previously published protocol (Nagai et al., 2012).

2.2 | Paired-end library preparation, sequencing, bioinformatics, and taxonomic assignment

Five different primer sets targeting the ribosomal RNA (rRNA) genes and internal spacer region (ITS; Table S1) were applied to detect as many taxa as possible. Two universal primer pairs targeting 18S (set 1; Table S1) and 28S (set 2; Table S1) rRNA genes were used to cover broader planktonic diversity. Group-specific primer pairs were utilized to target dinoflagellates (set 3: 28S_ dinoflagellate) and zooplankton (set 4: 28S_zooplankton). To capture marine fungi, the primers targeting ITS were also used (set 5: Table S1). Two-step PCR for the construction of paired-end libraries and HTS on the Illumina Miseq using the v3-600 reagent kit (Illumina) followed the previously described approach (Sildever

et al., 2022). Briefly, the first PCR was performed in a 25 μL reaction mixture containing 1.0 mL template DNA (<1 ng), 0.2 mM of each dNTP, 1 × PCR buffer, 1.5 mM Mg^{2+}, 1.0 U KOD-Plus-ver.2 (Toyobo), and 1.0 mM of each primer. The PCR cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30–32 cycles at 94°C for 15 s, 56°C for 30 s, and 68°C for 40 s. PCR amplification was verified by 1.5% agarose gel electrophoresis. The PCR products were purified using Agencourt AMPure XP (Beckman Coulter, Life Sciences) and eluted in 25 mL of TE buffer following the manufacturer protocol.

The second-round PCR used the first PCR products (diluted 10× with TE buffer) as a template and dual indexes and adapter sequences were added to the target sequences amplified during the first PCR. The second PCR was carried out in the same way as the first round PCR, except the volume of the reaction mixture was 50 mL with the addition of 2.0 mL of the diluted PCR product. The PCR cycling conditions were the same as for the first PCR, however, the number of cycles was reduced to 12 cycles. PCR amplification was again verified by agarose gel electrophoresis, and the PCR products were purified using an Agencourt AMPure XP (Beckman Coulter). The amplified PCR products were quantified using Qubit 2.0 Fluorometer (Life Technology) and were pooled in equal concentrations. The libraries were stored at −30°C until sent for sequencing to a sequencing facility.

Treatment of obtained sequences, selection of operational taxonomic units (OTUs), and taxonomic identification of OTUs were done according to the previously published workflow (Sildever et al., 2022). More specifically, the demultiplexing and trimming were performed using Trimmomatic version 0.35 (http://www.usadellab.org/cms/?page=trimmomatic). Nucleotide sequences were demultiplexed according to the 5′-multiplex identifier tag and primer sequences were demultiplexed according to the default format in MiSeq. Sections containing: (1) palindrome clips longer than 30 bp and (2) monopolymers longer than 9 bp were trimmed from the sequences at both ends. 3′ tails with an average quality score lower than 30 at the end of the final 25 bp window were also trimmed from each sequence. 5′ and 3′ tails with an average quality score lower than 20 at the end of the final window were also trimmed. The remaining sequences were merged into paired reads using Usearch version 8.0.1517 (http://www.drive5.com/usearch/) with default settings (≥16 bp overlap, ≥90% similarity and mismatch ≤5 bp; http://www.drive5.com/usearch/man-ual/merge_options_html) resulting in a maximum sequence length of 584 bp. Singletons were also removed. Sequences were aligned using Clustal Omega v. 1.2.0. (http://www.clustal.org/omega/) and only sequences that were contained in >75% of the read positions were extracted. Filtering and a part of the multiple alignment process were performed using the screen.seqs and filter.seqs commands in Mothur, as described in the MiSeq SOP (www.mothur.org/wiki/MiSeq_SOP; Schloss et al., 2011). Erroneous and chimeric sequences were detected and removed using the pre.cluster (diffs = 4) and chimera.uchime (minh = 0.1; http://drive5.com/usearch/manual/uchime_algo.html; Edgar et al., 2011) commands in Mothur, respectively. Using the unique.seqs command of Mothur, the same sequences were collected into OTUs. The contig sequences were counted as OTUs by count. seqs and used for the subsequent taxonomic identification analysis.

The sequence database used to identify OTUs was downloaded from GenBank on 23.08.2021. The raw sequence data are available from the DDBJ Sequence Read Archive (Accession numbers: DRA016522: 18S, DRA016523: 28S, DRA016526: 28S_dinoflagellate, DRA016521: 28S_zooplankton, DRA016527: ITS). Sequences were clustered to OTUs at ≥99.1% (18S), ≥98.8% (28S, 28S_dinoflagellate, 28S_zooplankton), and ≥98.6% (ITS) similarity levels. Only OTUs that had a ≥98% or ≥99% similarity with the match from the database were used in further analysis. The taxonomic assignment at a supergroup level for eukaryotes follows the latest division of eukaryotic taxa into of supergroups based on the Tree of Life model (Burki et al., 2020).

2.3 | Overview of the diversity present and associations with environmental parameters

To evaluate the number of samples required until the number of OTUs associated with unique species reaches a plateau (e.g., no or few new OTUs are detected), the cumulative number of individual OTUs identified based on the 18S and 28S universal primers was plotted for each sampling occasion. To evaluate the identification success based on different similarity levels with the best match from the database (≥98% or ≥99%), the number of OTUs connected with single species, multihits (match with several species from the database), or identified only to genus level were differentiated and visualized as stacked bar charts. The number of OTUs shared among different markers was analyzed by a Venn diagram. Species richness was calculated as the number of OTUs.

To investigate the differences in the environmental conditions and biodiversity between the Soya warm current and East Sakhalin current, the dataset was divided based on the bottom water salinity. There are transition periods at the end of May and October and the exact time of change in the dominant current varies depending on the year. To account for this, the samples associated with the Soya warm current were defined based on the period from when the salinity at the bottom layer was >33.0 on three successive days based on the daily monitoring in May or June (daily monitoring environmental monitoring data is not included in this study) until the decrease to <33.0 during three successive days in October or November. The remaining samples were defined as linked with the East Sakhalin current. To investigate changes in associated species diversity present during the domination of different currents, the Simpson's diversity index was calculated based on relative sequence abundances (non-rarefied data) using the "vegan" (v2.5–7) (Oksanen et al., 2020) package in R v. 4.0.3 (R Core Team, 2020). Mann–Whitney *U*-test/Wilcoxon rank-sum test was used to test for differences in species richness, diversity, and environmental parameters based on the data from sampling occasions related to the two different current

systems. The differences between the taxonomic divisions were also statistically analyzed by using a Chi-squared test in "*stats*" package in R (R Core Team, 2020).

To investigate appearance patterns (seasonal appearance: detection at the same season in different years, no pattern: appearance throughout the seasons, or sporadic appearance: detection in some years, but not in all sampling years), relative sequence abundance data based on the OTUs with ≥99% similarity with a single match from the databases (based on 18S universal primers) was used. The presence of different categories of appearance patterns was estimated by eye. No estimate on the proportion of OTUs coupled with the different types of appearance is provided as the seasonal appearance depends on various factors, such as current strength and conditions in the place of origin for the transported organisms, which could not be reliably considered within the framework of this study.

2.4 | Correlation and ordination analyses

To investigate the patterns in biodiversity and the association between various OTUs and environmental parameters correlation and ordination analyses were conducted in R using the "vegan" or "psych" (Revelle, 2020) packages. The biodiversity of eukaryotic communities in the samples was evaluated by applying the Bray–Curtis similarity index and visualized by nonmetric multidimensional scaling (NMDS) in "vegan." The Bray–Curtis similarity index was calculated based on summarized data for each month. The data were summarized as follows: for each OTU, the number of appearances per month was calculated for each month and year separately, and the number of occurrences per month was transformed into percentages by dividing the number of occurrences per month by the total number of sampling occasions per month. The goodness of fit of the resulting NMDS mapping was assessed by a Shepard diagram (Figure S3). Differences in biodiversity between the years and months were investigated by PERMANOVA using the Bray–Curtis similarity index and 999 permutations and the assumption for homogeneity of multivariate dispersions was tested by ANOVA (betadisper function in "vegan"). As a post hoc test pairwise PERMANOVA was conducted with *p*-values for multiple comparisons by Benjamini–Hochberg correction (Benjamini & Hochberg, 1995).

Spearman correlations between environmental parameters and the presence/absence of all OTUs as well as between OTUs were calculated. The *p*-value was adjusted for multiple comparisons by Benjamini–Hochberg correction (Benjamini & Hochberg, 1995). The correlation analysis results were visualized by heatmap.2 ("gplots" package, Warnes et al., 2020) and by Gephi (v. 0.9.2) (Bastian et al., 2009).

Only OTUs that had statistically significant correlations ($p < 0.05$) between them were used for network visualization using Gephi. The value for the strength of the correlation is based on the OTU showing the outgoing correlation with another OTU. For visualization, ForceAtlas 2 algorithm was used with the following built-in parameters: number of threads: 7, tolerance (speed): 1.0; approximate repulsion checked; approximation: 1.2. The algorithm was left to run until there were no major changes in the appearance of the network. To improve the visualization of the resulting network, a "degree range" filter was applied and the minimum number of connections was set to 60. The size of the nodes was adjusted according to the number of outgoing connections, that is, larger node size indicates more connections. This was done by using ranking based on out-degree with min. size set to 5 and max. 50. The color of the nodes represents different taxa. The color of the edges represents the strength of the correlation based on a chosen parameter (different environmental parameters or the correlation between OTUs).

3 | RESULTS

3.1 | Overview of the diversity detected

Based on the universal markers, the highest total number of operational taxonomic units (OTUs) was detected: 18S marker facilitated the detection of 7409 OTUs, 28S marker 5614 OTUs, and ITS marker 2188 OTUs. In comparison, the group-specific markers yielded a lower number of OTUs: 28S_zooplankton marker detected 1225 and 28S_dinoflagellate marker 1212 OTUs (Table S2). The total number of unique OTUs detected based on all the five markers was 3529 (≥98% similarity) or 2594 with more stringent criteria (≥99%; Table 1).

The detected OTUs belonged to nine supergroups (Table 1; Burki et al., 2020). The most species-rich supergroup was Amorphea (1565/2186 OTUs at ≥99% or ≥98% similarity), followed by TSAR (786/1018) and Archaeplastida (180/244). In the Amorphea and TSAR supergroups, Opisthokonta and Alveolata as well as Stramenopiles were the most species-rich sub-supergroups, respectively. Based on all five markers, the majority of the OTUs at ≥99% similarity level were coupled with Opisthokonta (33%–94%) and Stramenopiles (4%–25%; Table S2).

Of the OTUs matched with a single species at ≥99% similarity level, only two were shared among all five markers: a fungi *Cerrena unicolor* (Basidiomycota) and a yeast, *Glaciozyma antarctica* (Figure 1a). More than half of the OTUs coupled with individual species were unique to specific markers (62.60%; Figure 1a). The highest proportion of unique OTUs was detected based on 18S marker (29.96%), followed by 28S (15.46%) and ITS (12.66%) markers. The taxonomic identification success was the highest among Rhodophyta, Cryptophyta, and Opisthokonta and lowest in Discoba, Amoebozoa, and Alveolata based on the 18S marker (≥99%; Figure 1b). With the 28S universal primers, the highest proportion of OTUs related to a single species in Alveolata, Rhodophyta, and Stramenopiles, whereas the lowest identification success was found among Haptophyta, Rhizaria, and Viridiplantae (Figure 1b). Similar percentages were also present

TABLE 1 Overview of the richness detected based on all markers used at ≥99%/≥98% similarity level.

Supergroup	Sub-supergroup	Phylum	Species[a]
TSAR	Alveolata	Dinophyceae	221/250
(786/1018)	(368/460)	Ciliophora	134/188
		Apicomplexa	11/17
		Other Alveolata	2/5
	Stramenopiles	Bacillariophyceae	232/296
	(354/470)	Oomycota	37/55
		PX clade	29/44
		Chrysophyceae	11/17
		Dictyochophyceae	9/10
		Synurophyceae	11/13
		Other Stramenopiles	28/35
	Rhizaria	Cercozoa	38/53
	(62/86)	Other Rhizaria	24/33
	Telonemia		2/2
Amorphea	Opisthokonta	Fungi	1055/1455
(1565/2186)	(1553/ 2169)	Metazoa	475/684
		Choanoflagellida	16/21
		Other Opisthokonta	7/9
	Other Amoprhea		12/17
Archaeplastida	Viridiplantae		136/186
(180/244)	Rhodophyta		44/58
Haptista	Haptophyta		31/38
Cryptista	Cryptophyta		22/28
Excavata			5/7
CRuMs			2/5
Ancyromonadida			2/2
Picozoa			1/1
Total			2594/3529

[a]Species: no. of operational taxonomic units (OTUs) associated with a unique species.

for the 28S dinoflagellate-specific primers. At the same time, zooplankton-specific 28S primers had the highest identification success for Rhizaria and Opisthokonta and the lowest for Stramenopiles (Figure 1b). In the case of ITS, around 26%–32% of OTUs could be matched with a species (≥99% and ≥98%, respectively; Figure 1b). The number OTUs associated with unique species detected based on 18S and 28S universal primers (≥99%) continued to increase throughout the 7 years of monitoring (Figure S1).

The number of OTUs detected differed significantly between the current systems for most of the markers, except for the marker targeting zooplankton (Table 2). No statistically significant difference was detected between the Simpson's diversity index values between the two current systems based on the 18S and 28S universal primers, however, based on the other three markers, statistically significant differences were found ($p < 0.05$; Table 2). In addition, there were statistically significant differences between all the environmental parameters in different current systems as well as between the number of OTUs associated with different genera detected during all sampling occasions between the two current systems (Figure S2 and Table 2).

3.2 | Influence of environmental parameters and coappearance patterns

Based on the 18S universal marker, three types of appearance patterns were detected: seasonal, throughout different seasons, or sporadical appearance (Figure 2). The seasonal appearance patterns were also reflected in the correlations with the environmental parameters. From all the OTUs associated with a single species (≥99%), the highest number of statistically significant correlations ($p < 0.05$) was found in association with the bottom water temperature, followed by surface water temperature and bottom water salinity (Figure 3 and Table S3). Those parameters had the highest number of correlations with OTUs detected based on all the makers, except ITS. Based on the ITS, the highest number of correlations were between the OTUs and SiO_2. When the significant associations between

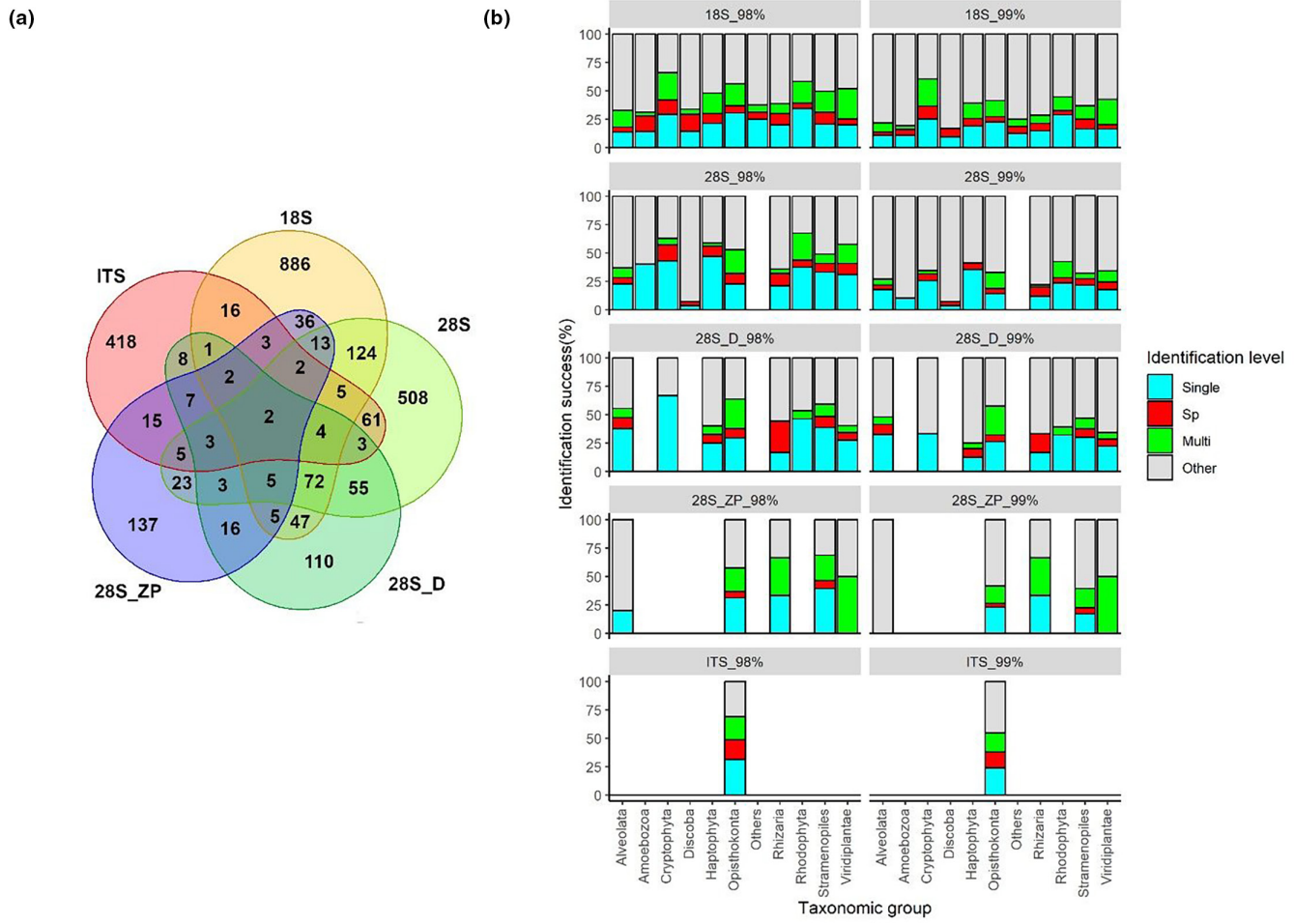

FIGURE 1 Overview of the eukaryotic plankton richness identification. (a) Number of unique and shared operational taxonomic units (OTUs) among the five markers used. (b) Identification success based on different markers, "Single" indicates identification at a species level; "Sp" at a genus level; "Multi" indicates a match with several species from the database; and "Other" includes all the OTUs, which similarity with the taxonomic database match was lower than the established criteria.

OTUs were visualized as correlation networks, it was apparent that the summer communities had more significant positive correlations with temperature, salinity, and Chl *a*, and negative significant correlations with nutrient concentrations, whereas an opposite pattern was detected for winter communities (Figure 4).

There was also a high number of statistically significant correlations between OTUs, for example, 1197 out of 1223 OTUs associated with single species (18S marker at ≥99% similarity level; Table S4). The majority of the OTUs displayed significant positive correlations (92.7%) with other OTUs and the correlation strength ranged from 0.13 to 0.86. In the case of significant negative correlations, the strength was between −0.13 and −0.55 (Table S3). The highest number of statistically significant correlations (249) was found between a diatom *Chaetoceros curvisetus* and OTUs from various taxonomic groups, for example, diatoms, dinoflagellates, cryptophytes, etc.

There was an overall significant difference in species diversity based on monthly data (Figure 5 and Table S5). Although, there was yearly variance between diversity detected in different months, the samples from July to October in each year were clustered on the positive side of the *X*-axis, whereas samples from December to April were present on the negative side of the *X*-axis. At the same time, samples from May, June, and November were more spread along the *X*-axis. The different placement of July–October and December–April communities was also supported by a statistically significant post hoc test (Table S6).

4 | DISCUSSION

Based on 8 years of weekly monitoring data, >2500 unique OTUs connected with a single species were detected from the Sea of Okhotsk, north-western Pacific. This is the first comprehensive study of planktonic diversity from the area capturing diversity from all nine eukaryotic supergroups (Burki et al., 2020).

4.1 | Species richness

The species detected in this study add more information to the findings of previous reports on zooplankton (Asami et al., 2007; Hikichi

TABLE 2 Analysis of differences between the current systems.

	Mean		SD		Wilcoxon rank-sum test with continuity correction	
Parameters	W	S	W	S	Test statistic W	*p*-Value
Chl *a*	1.14	2.07	1.12	1.27	39,374	**<0.0001**
Surface water salinity	31.20	32.00	1.98	2.07	36,023	**<0.0001**
Bottom water salinity	32.10	33.50	0.64	0.31	48,370	**<0.0001**
Surface water temperature	2.18	15.40	3.42	3.98	49,063	**<0.0001**
Bottom water temperature	2.02	14.60	3.32	4.12	48,881	**<0.0001**
NO_2	0.26	0.21	0.13	0.14	18,182	**<0.0001**
NO_3	7.05	2.10	4.14	2.54	6748	**<0.0001**
PO_4	0.80	0.21	0.34	0.13	3625.5	**<0.0001**
SiO_2	23.00	19.40	23.30	24.30	16,845	**<0.0001**
No. of OTUs_18S	259.58	283.59	82.78	96.73	29,239	**<0.001**
No. of OTUs_28S	118.61	150.05	44.40	51.54	34,252	**<0.0001**
No. of OTUs_ 28S_D	40.55	54.17	23.28	30.05	30,004	**<0.0001**
No. of OTUs_28S_ZP	14.31	15.46	12.43	12.60	26,548	0.09
No. of OTUs_ITS	25.83	39.30	22.19	29.12	14,984	**<0.0001**
Simpson's diversity_18S	0.88	0.90	0.11	0.10	23,340	0.29
Simpson's diversity_28S	0.85	0.88	0.12	0.07	23,004	0.2
Simpson's diversity_28S_D	0.72	0.69	0.20	0.18	27,402	**0.01**
Simpson's diversity_28S_ZP	0.38	0.44	0.24	0.24	20,692	**0.007**
Simpson's diversity_ITS	0.72	0.60	0.22	0.28	13,774	**0.0004**

Note: Statistically significant results ($p < 0.05$) are indicated in bold, number of OTUs and Simpson's diversity indices calculated based on all OTUs that have ≥99% similarity with the database match. 28S_D: 28S_dinoflagellate marker, 28S_ZP: 28S_zooplankton marker.

Abbreviations: S, summer; W, winter.

et al., 2018; Hirai et al., 2015, 2017; Pinchuk & Paul, 2000), harmful algal species (Sildever et al., 2019; Stonik & Orlova, 2013), winter microalgal communities (Yan et al., 2022), and protists in general (Matsumoto et al., 2021). Using zooplankton as an example, similar to previous studies from the same sampling location, most of the OTUs were associated with copepods (especially calanoids), Polychaeta, and Hydrozoa (Hirai et al., 2015, 2017). We also detected all zooplankton taxa reported in those studies (Hirai et al., 2015, 2017), as well as an additional copepod taxon (Monstrilloida) based on the 28S zooplankton-specific marker.

When comparing the detected diversity with other large-scale studies, such as the circumglobal Tara Oceans expedition (De Vargas et al., 2015) or the Tree-of-Life holistic metabarcoding approach in the Black Sea (Zhang, Pavlovska et al., 2020b), more eukaryotic diversity on a supergroup level (nine compared to four or three supergroups) was detected. This could be explained by the presence of sporadically appearing taxa, which might not be detectable based on a single sample and by a single molecular marker (Sildever et al., 2021). In addition, the high number of unique species detected here may also result from the presence of two well-defined current systems that can transport organisms from other areas (Shimada et al., 2016; Sildever et al., 2019) and facilitate distinct communities (summer vs winter) with different environmental requirements (Asami et al., 2007; Matsumoto et al., 2021). The influence of low environmental variability can be exemplified by the low number of OTUs (571) detected from Osaka Bay, eastern Seto Inland Sea, Japan, using the same 18S universal marker as in this study, with weekly sampling conducted for 4 years (February–May) (Nagai et al., 2019). The combination of environmental variability, the number of markers used as well as the number of samples taken may also explain the detection of almost twice the number of OTUs in this study compared to the global diversity from the Tara Oceans dataset (≥99% similarity with the best match from the database, 2466 OTUs, 18S rRNA gene V7–V9 region, compared to 1339 OTUs, V9 region; De Vargas et al., 2015). At the same time, we acknowledge that the number of OTUs is not directly comparable among different studies. On the other hand, the taxa associated with the highest number of OTUs is similar between this and other studies (De Vargas et al., 2015; Sildever et al., 2021, 2022) with the most species-rich groups being Alveolata (especially dinoflagellates), Opisthokonta (metazoa and fungi), Stramenopiles (especially diatoms), and Rhizaria. In fungi, most of the OTUs were linked with Ascomycota and Basidiomycota as also reported for other marine environments around the world (Tisthammer et al., 2016; Zhang, Pavlovska et al., 2020b).

Detection of the high species richness in a single sampling location was also facilitated by the five different primer pairs comprising universal and group-specific markers. The identification success varied between the markers and similarity level used for the taxonomic

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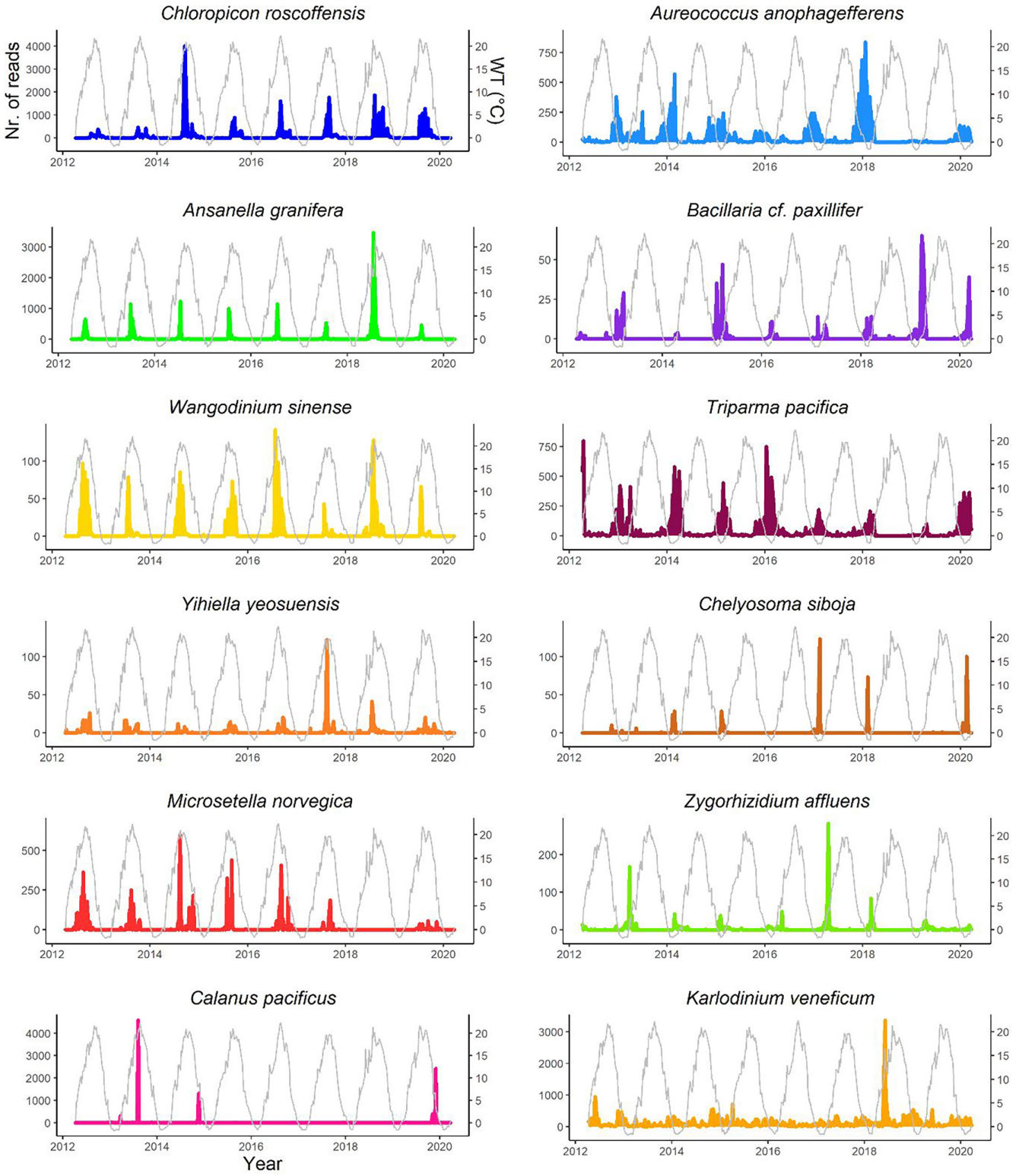

FIGURE 2 Appearance patterns of selected operational taxonomic units (OTUs) associated with a single species. Colored lines represent relative sequence abundances and gray line sea surface temperature. OTUs associated with *Chloropicon roscoffensis* to *Zygorhizidium affluens* display seasonal appearance patterns, OTU associated with *Calanus pacificus* is an example of sporadical appearance and *Karlodinium veneficum* for appearance throughout different seasons.

assignment (≥98% or 99%). The identification success is influenced by the sequences available in the database (Ekrem et al., 2007) and the resolution of the target gene and region (Pawlowski et al., 2012; Tanabe et al., 2016). At the ≥99% similarity level with a single species from the database, the highest identification success (30.27%) was shown by the 28S universal primer pair. This can be explained

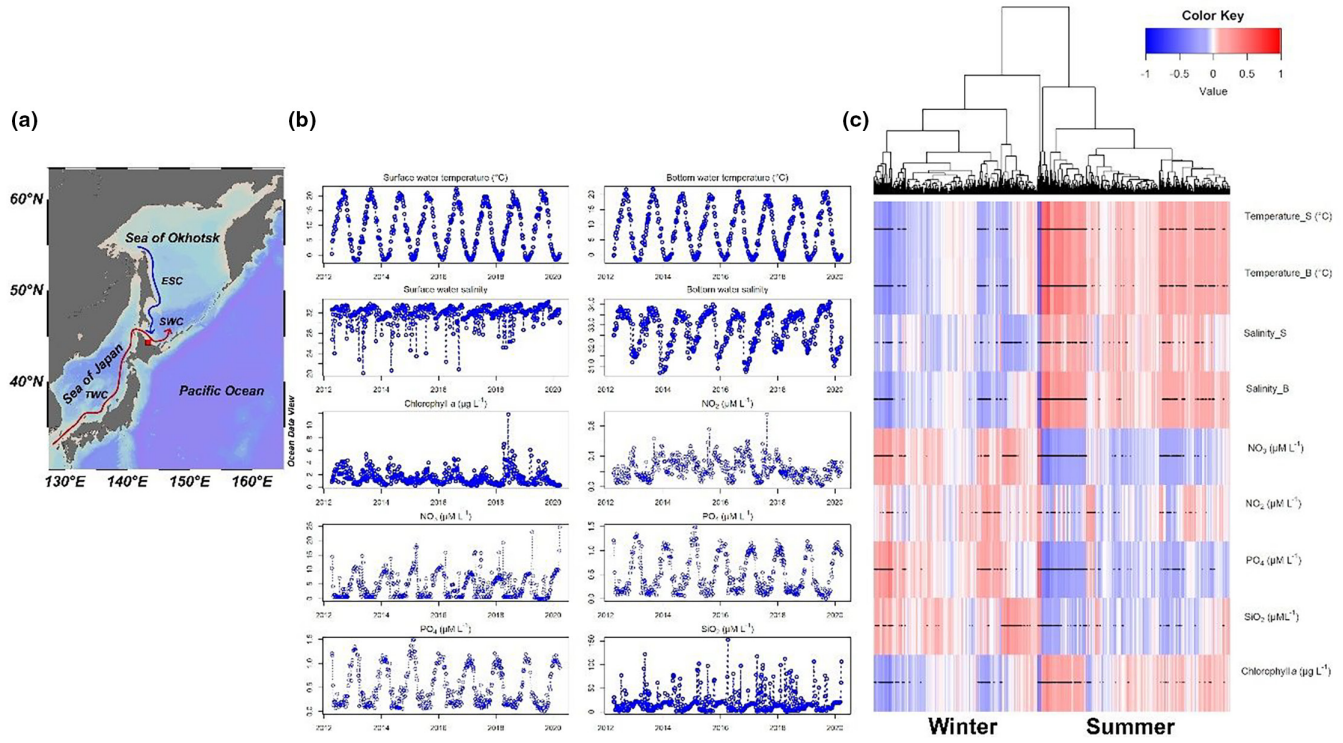

FIGURE 3 Overview of the environment at the sampling location and association between the operational taxonomic units (OTUs) and environmental parameters. (a) Sampling location and the dominating current systems. (b) Changes in different environmental parameters during the sampling period. (c) Correlations among OTUs associated with single species at ≥99% similarity level based on the 18S universal primers and environmental parameters. Colors in the heatmap represent the correlation strength (*r* values).

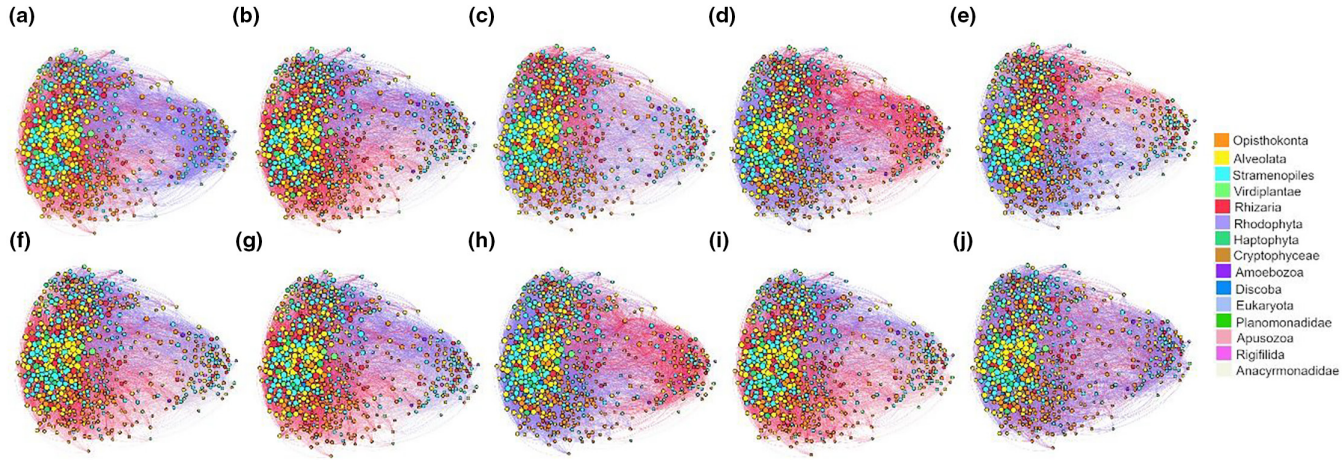

FIGURE 4 Network analysis based on correlations between operational taxonomic units (OTUs) and environmental parameters. Filled circles represent various taxa. Blue and red colored lines indicate the strength of the correlation (*R*-value with blue indicating negative and red positive correlation). In each subfigure, the colors of the lines represent the strength of the correlation with selected environmental parameters: (a) surface salinity, (b) surface temperature, (c) nitrite concentration, (d) nitrate concentration, (e) phosphate concentration, (f) bottom salinity, (g) bottom temperature, (h) silicate concentration, (i) Chl *a* concentration, and (j) with other OTUs. Only OTUs having statistically significant correlations ($p > 0.05$) with other OTUs are displayed. The OTUs on the left side of each subfigure are assumed to represent summer and the OTUs on the right side winter communities based on the correlations with the environmental parameters.

by the higher variability of the 28S rRNA gene compared to the 18S rRNA gene (Hillis & Dixon, 1991) with the D1–D2 region being the most informative (based on dinoflagellates; Ki & Han, 2007). This region has been targeted in only a few eDNA-based plankton diversity studies (Elferink et al., 2017; Grzebyk et al., 2017), mainly due to the length of the region and the maximum read length of the commonly used MiSeq platform (2×300 bp paired-end; Grzebyk et al., 2017). However, the 28S universal primers (Sildever et al., 2019), utilized in this study, and the Baltic Sea (Sildever et al., 2021), will promote targeting this region for surveying planktonic diversity. Until long-read

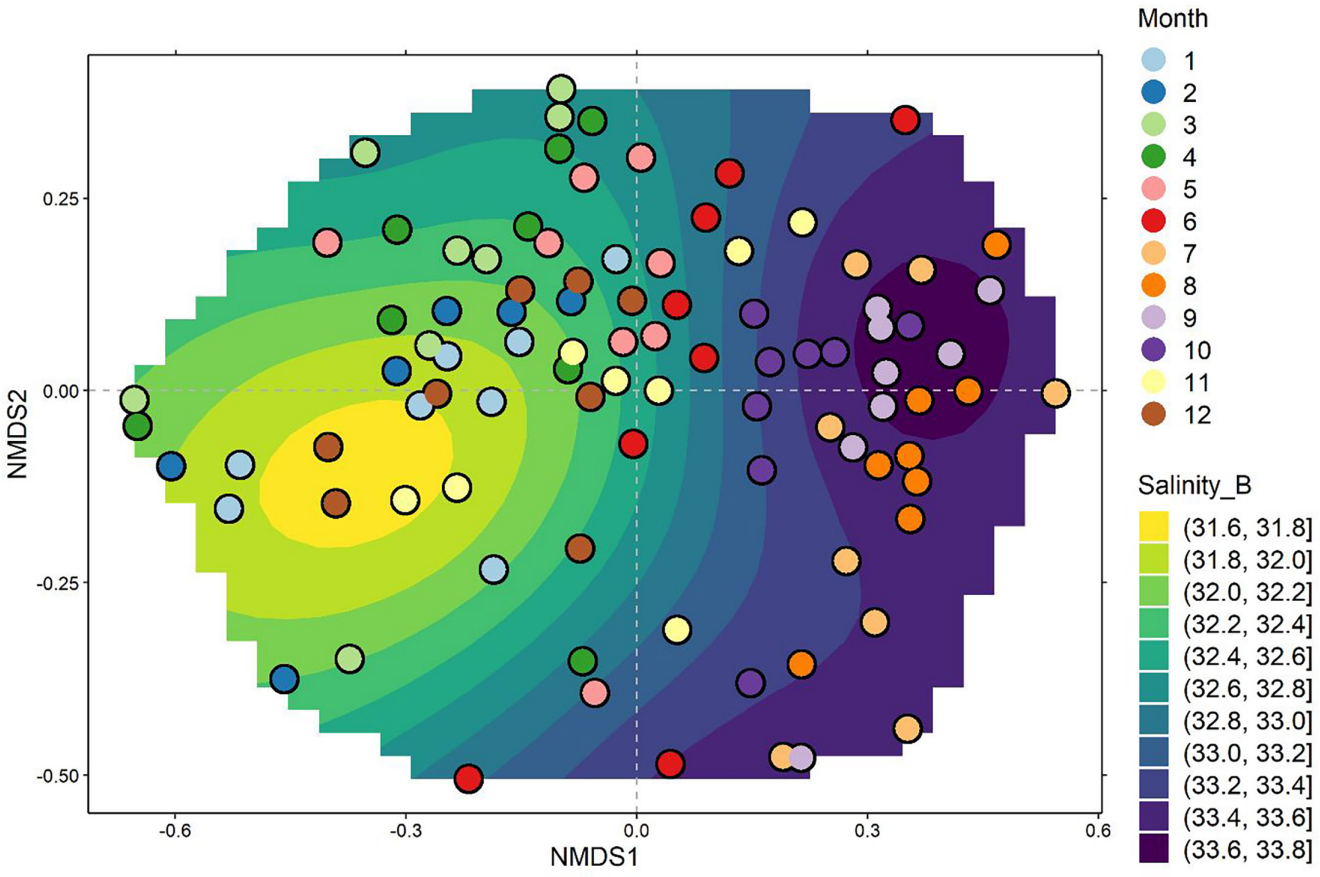

FIGURE 5 Non-metric multidimensional scaling (NMDS) analysis of biodiversity data based on 18S rRNA gene (≥99% similarity) blotted together with the bottom salinity (Salinity_B).

sequencing becomes more accessible and the current technological limitations have been eliminated (Latz et al., 2022), the use of multiple target genes and primer pairs is recommended for improving the detection of the diversity present (Djurhuus et al., 2018; Sildever et al., 2021; Zhang, Li et al., 2020a). This is further supported by our findings as each primer pair detected >100 unique OTUs associated with single species at ≥99% similarity level and universal primers for 18S and 28S rRNA genes detected >500 individual OTUs each.

4.2 | Associations between environmental parameters and OTUs detected

In this study, the detection of statistically significant associations between OTUs and environmental parameters revealed a clear differentiation between the dominating current systems and the associated environmental parameters, such as water temperature, salinity, and nutrient concentrations. Contrasting characteristics of the current systems were also visible in the ordination analysis based on OTU diversity from different months, where data from distinct seasons were placed separately along the first axis with data from transition periods spread along the axes. This was further supported by the statistically significant differences in the OTU richness between the two current systems. Interestingly, when testing average Simpson's diversity values for different seasons, the datasets obtained by the universal markers (18S and 28S) did not show any significant differences. This may be related to the usage of relative sequence abundances that do not reflect the true number of organisms present per species (Lamb et al., 2019).

At the same time, Simpson's diversity based on ITS, 28S dinoflagellate- and zooplankton-specific markers varied significantly between the current systems. Based on previous studies from the area, phyto- and zooplankton communities differ depending on the dominating current (Asami et al., 2007; Hikichi et al., 2018; Matsumoto et al., 2021), and in the case of zooplankton, higher species richness is present in the summer with the water temperature as the main driver of zooplankton richness and diversity (Hirai et al., 2017). This coincides with the findings of this study, where OTUs detected based on the 28S zooplankton-specific marker had the highest number of significant associations with bottom and surface water temperatures as well as with the salinity in the bottom layer. At the same time, fungi had the highest number of statistically significant ($p < 0.05$) correlations with silicate concentrations, bottom water temperature, and salinity, further confirming the influence of the dominant current. The presence of mainly positive associations with SiO_2 concentrations and mainly negative associations with surface salinity indicate the potential association with increased freshwater input/rainfall that

might increase the presence of various substrates for colonization (Jones, 2000). Salinity has also been reported as an important driver for the diversity of pelagic fungi (Rojas-Jimenez et al., 2019) and spatiotemporal changes in OTU abundances have been significantly connected with nutrient concentrations and phytoplankton detritus (Wang et al., 2018).

4.3 | Appearance patterns

The influence of different current systems was also visible in the appearance patterns of OTUs displaying seasonal appearance patterns from which the majority had statistically significant correlations with the water temperature and salinity. Appearance patterns detected based on eDNA approach have also been reported for several planktonic groups from other sampling locations around the world (Di Capua et al., 2021; Dzhembekova et al., 2017; Hirai et al., 2017; Sildever et al., 2021) and this information has proven useful for detecting departures from the regular appearance patterns in association with extreme weather events (Berry et al., 2019). The OTUs displaying sporadical appearance patterns may have been also influenced by the strength of the dominant current, for example, OTU connected with *Alexandrium catenella* appears in the sampling area only when the Soya warm current is weak (Shimada et al., 2012). In addition, their appearance may be further influenced by the community dynamics and environmental parameters at a different location as some of the detected species can originate from elsewhere, for example, warm-water species may be transported from the Sea of Japan to the coastal waters of Hokkaido (Shimada et al., 2016).

The high-resolution dataset also facilitated the detection of statistically significant associations between different OTUs. Similar approaches have been used for investigating potential interactions across several trophic levels in the marine community (Djurhuus et al., 2020; Zhang, Pavlovska et al., 2020b) as well as between harmful algal species and bacteria (Sildever et al., 2022). The majority of the OTUs that were associated with a single species at ≥99% similarity based on 18S universal primer had more than one significant association with another OTU. The high number of statistically significant associations with other taxa has been considered an indicator of how important the species is in an ecosystem (keystone species) (Berry & Widder, 2014). However, it is not possible to remove the influence of other factors, for example, environmental parameters (Freilich et al., 2018) and spatial heterogeneity (Banerji et al., 2018). At the same time, associations detected based on presence–absence data or association rule-based analysis can identify potentially interesting and relevant coappearance patterns in the environment (Sildever et al., 2022). In this study, an OTU linked with the genus *Chaetoceros* (centric diatom) had the highest number of statistically significant correlations with other OTUs. As the data used here are presence–absence data, the detected correlations probably reflect appearance patterns (Djurhuus et al., 2020). This coincides well with previously known information as the genus usually dominates the plankton community when the Chl *a* concentration, air, and sea surface temperatures are high, but nutrient concentrations are low (Matsumoto et al., 2021).

In summary, the weekly dataset from 8 years allowed the detection of thousands of OTUs associated with unique species from a single sampling location. Thanks to the high-frequency time series data, it was also possible to determine the general appearance patterns of different taxa as well as to characterize the associations among them. Various OTUs also displayed significant associations with environmental parameters that characterize the two major current systems dominating the area. The high-frequency time series monitoring is valuable for understanding the direction of future changes in the marine environment (Smayda, 1998; Zingone et al., 2019). Combined with metatranscriptomics data, changes in the plankton community composition in association with biotic and abiotic factors can be detected on a finer scale and the knowledge can serve as an input to model the scenarios in relation to climate change and shifts in anthropogenic pressures (Elferink et al., 2020; Semmouri et al., 2020). To improve the identification success in metabarcoding studies, further effort is needed to increase the quality and availability of reference sequences in the international nucleotide databases.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology: SN and SK; sampling: SK; wet-laboratory experiments: SK, HK, AS, TK, and SN; metabarcoding pipeline: NN and SN; data analysis and visualization: SS and SN; writing—original draft preparation: SS; writing—review and editing: JH and SN; supervision, project administration, and funding acquisition: SN. All authors have read and commented on the submitted version of the article.

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CONFLICT OF INTEREST STATEMENT

None.

DATA AVAILABILITY STATEMENT

The raw sequence data is available from the DDBJ Sequence Read Archive (Accession numbers: DRA016522: 18S, DRA016523: 28S, DRA016526: 28S_dinoflagellate, DRA016521: 28S_zooplankton, DRA016527: ITS).

ORCID

Sirje Sildever https://orcid.org/0000-0002-4847-936X

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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