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ORIGINAL ARTICLE

Salinity and resource availability as drivers of Baltic benthic fungal diversity

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Abstract

Marine biodiversity consists of a complex network of organisms responsible for keeping the ecosystem's balance. Fungi are an understudied group of organisms despite their recognized importance for ecosystem processes and diversity. How fungi respond to environmental change remains poorly understood, especially in marine benthic habitats. The Baltic Sea is a brackish coastal ecosystem with steep environmental gradients in a relatively limited geographical area and is therefore a particularly good system to investigate the impact of different abiotic factors on benthic fungal diversity. This study used environmental DNA (eDNA) metabarcoding to analyze the spatial dynamics of benthic fungal diversity in the Baltic Sea and quantify the environmental drivers that shape these dynamics. Based on 59 stations spreading over 1145 km the results showed that benthic fungal communities were dominated by the phylum Chytridiomycota, and the fungal species *Alphamyces chaetifer* and *Operculomyces laminatus* from this phylum were the main drivers of the community structure dissimilarities observed between regions. Water depth and salinity were the main predictors of the benthic fungal community composition. The impact of nutrient availability was also significant, possibly related to the known role of Chytridiomycota species such as *A. chaetifer* and *O. laminatus* in nutrient cycling. Our results indicate that the benthic fungal diversity of the Baltic Sea is shaped by salinity gradients and nutrient availability and highlights that the current fungal biodiversity is at risk of species shift or decline with predicted changes in salinity due to climate change and intensified eutrophication.

KEYWORDS
Baltic Sea, benthic, Chytridiomycota, environmental DNA, fungal diversity, salinity

Elias Broman and Francisco J. A. Nascimento are joint last authors of this work.

1 | INTRODUCTION

Marine biodiversity plays a vital role in ensuring numerous important ecosystem services, including nutrient cycling and supply of natural resources (Goulletquer et al., 2014). This biodiversity is distributed in the pelagic and benthic zones, both characterized by heterogenous environmental conditions, which leads to an equally heterogenous distribution and structure of the inhabiting communities (Matthiessen et al., 2018). The distribution of organisms in aquatic environments is related to their specific adaptations and tolerance limits to abiotic factors, such as salinity (Smyth & Elliott, 2016; Telesh et al., 2013), oxygen (Deutsch et al., 2020; Verberk et al., 2011), or nutrient availability (Tee et al., 2021; Wu et al., 2023).

According to the World Register of Marine Species database a total of 243,589 marine species from all kingdoms of life have been registered so far in 2023 (https://www.marinespecies.org). Among this vast fauna, microorganisms play a major role in ensuring ecosystems' balance through nutrient cycling (Falkowski et al., 2008). Marine fungi are eukaryotic osmotrophic microorganisms adapted to life in marine ecosystems (Raghukumar, 2017; Richards et al., 2012) that have been found living associated with a variety of organisms either as symbionts or parasites (Alker et al., 2001; Cawthorn, 2011; Gutiérrez et al., 2016; Higgins, 2000; Hyde et al., 1998; Wang et al., 2008). Fungi have also been found to be saprotrophs, degrading and remineralizing organic matter present in the water column and in sediments (Raghukumar, 2017; Sridhar, 2012). From the Fungi kingdom only 1378 marine species were accepted in the mentioned database until now in 2023, which corresponds to a reduced portion of the 10,000 fungal species estimated to exist in marine ecosystems (Jones, 2011a). A few reasons that explain this situation are the reduced exploration of possible fungi substrates, the incapacity to culture some fungi species, and difficulty in differentiating cryptic species and taxa with similar morphology (Jones, 2011a). In comparison to other taxonomic groups, our current knowledge of the diversity of fungi and the main environmental drivers that govern their distribution remains scarce (Jones, 2011b), especially in coastal benthic environments and even more so in brackish waters (Cuadros-Orellana, 2013; Manohar & Raghukumar, 2013).

The application of environmental DNA (eDNA) metabarcoding has already promoted the development of the existent knowledge on phylogeny, taxonomy, and ecology of terrestrial and aquatic fungi (Cuadros-Orellana, 2013; da Silva et al., 2022; Marčiulynienė et al., 2021). This was possible by targeting the internal transcribed spacer (ITS) region, which has been suggested as the fungal universal barcode, particularly useful in deeper taxonomic analysis of fungi from well-described taxonomic groups (Schoch et al., 2012). The use of recent high-throughput next-generation sequencing techniques in metabarcoding, which enables an efficient large-scale analysis of DNA samples (Taberlet et al., 2012), has led to a better understanding of fungal diversity (Picard, 2017; Polinski et al., 2019; Rämä et al., 2016). Environmental metabarcoding thus offers a powerful technique to analyze still unexplored benthic habitats (da Silva et al., 2022; Gonçalves et al., 2022; Li et al., 2016; Polinski et al., 2019; Wu et al., 2023). These techniques have rarely been employed in the study of coastal benthic fungal diversity, particularly in the brackish Baltic Sea and at large spatial scales (i.e. regional scale).

The Baltic Sea is a shallow brackish water ecosystem (Lehmann et al., 2022) where benthic diversity of groups, such as macrofauna (Gogina & Zettler, 2010), meiofauna (Broman et al., 2019), or bacteria (Broman et al., 2022), and their environmental drivers are relatively well known (Broman et al., 2019, 2022; Klier et al., 2018). The Baltic Sea is characterized by a long water residence time and horizontal and vertical salinity gradients mostly influenced by marine and freshwater inflows (Lehmann et al., 2022; Reissmann et al., 2009). Therefore, the Baltic Sea is a good study system to analyze the impact of a salinity gradient on biodiversity, through sampling in different regions within this geographical location. Such studies have described the development and survival of both marine, brackish, and freshwater species in the sediments of the Baltic Sea (Viitasalo et al., 2015). Besides salinity, oxygen levels, temperature, and nutrient availability have also been described as important drivers of Baltic Sea communities of benthic bacteria, zooplankton, phytoplankton, and benthic fauna (Broman et al., 2022; Viitasalo et al., 2015).

Among the few studies investigating Baltic Sea pelagic (see e.g. Hassett et al., 2019; Majaneva et al., 2012; Rojas-Jimenez et al., 2019; Vass et al., 2022) and benthic fungal diversity (see e.g. Miettinen et al., 2019; Polyak et al., 2014), only two described variation in pelagic fungal community structure between regions, mainly driven by environmental conditions such as salinity levels or nutrient concentration (Rojas-Jimenez et al., 2019; Vass et al., 2022). Therefore, there is still a significant knowledge-gap regarding benthic fungal diversity in the Baltic Sea and also on how environmental variables are shaping these benthic fungal communities. This study aimed to address this knowledge-gap and assess the spatial dynamics of benthic fungal communities along the Baltic north–south salinity gradient, covering approximately 1145 km. Based on the lack of literature on Baltic fungi and on information from studies on other taxonomic groups, it was hypothesized that: (1) community structure of fungal communities is dissimilar between regions and (2) salinity and nutrient availability (carbon (C) and nitrogen (N)) are the main environmental variables influencing benthic fungal community composition.

To test the stated hypotheses eDNA samples were extracted from sediments collected in 2019 from 59 stations of the Baltic Sea, from north to south, distributed along the salinity gradient. Water salinity, temperature, oxygen levels, and sediment C and N concentrations were measured. The samples were analyzed using DNA metabarcoding targeting the fungal ITS2 region. The PCR amplicons were sequenced, and the data was analyzed to quantify the main environmental drivers of Baltic benthic fungal diversity.

2 | METHODS

2.1 | Sediment sampling and study site abiotic conditions

Sediment surface samples (top 0–2 cm) of soft seafloor, coastal and offshore, clay-muddy habitats were collected by the Swedish National and Regional Benthic Monitoring program between May and June 2019 at 59 stations in the Baltic Sea. Following the same approach as Broman et al. (2019, 2022), the stations were divided into six main regions (Bothnian Bay, Bothnian Sea, Stockholm, Sörmland, Östergötland, and Southern Baltic). The stations were numbered by latitude from north to south as displayed in Table 1.

The sediment sampling, transportation, and preservation of the samples have previously been described in Broman et al. (2022). Briefly, one sediment core at each station was collected with a Kajak gravity corer (surface area: 50 cm^2, one core per station), and, from each core, the top 2 cm layer was sliced into an autoclaved 215 mL polypropylene container (207.0215PP, Noax Lab, Sweden). The equipment required for sampling and slicing was cleaned between each sampling using deionized water. In this study, ecological replicates were collected at different stations within the same region to allow for a large-scale spatial study. After homogenization of the sediments inside the containers, the sediment samples were stored at −20°C on the boat and then transported in a cooling box with ice for approximately 2 h to the University where they were stored at −20°C until DNA extraction.

The abiotic variables (water salinity, depth, temperature, oxygen levels, and sediment nutrient availability (namely C and N concentration)) at each sampling site were measured as described in Broman et al. (2019, 2022). The water parameters were based on the analysis of collected bottom water samples, approximately located 20 cm above the sediment surface, with a Niskin bottle and measured on deck using a portable multimeter (HQ40D, Hach). Total C and N content in the surface sediment were measured from 1.5 mL of dried sediment (60°C) and then analyzed with an elemental analyzer (Europa EA-GSL, Sercon Ltd., Cheshire, UK). The respective concentrations are presented as % by mass. The results of these measurements are displayed in Table 1.

2.2 | DNA extraction, library preparation, and sequencing

DNA was extracted from 0.25 g of sediment using the DNeasy Power Soil Kit (QIAGEN) according to the supplied protocol. After the extraction process, the extracted DNA was measured for quality and quantity with a NanoDrop Microvolume Spectrophotometer (NanoDrop One, Thermo Fisher Scientific). For the amplicon library preparation, the DNA samples were sent to Novogene (UK, Cambridge), where a PCR amplification of the partial ITS gene, and library preparation, were performed using fungal-specific primers (ITS86F (5′-GTGAATCATCGAATCTTTGAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)) (Vancov & Keen, 2009). This primer pair targets the 5.8S region and the entire ITS2 region of a wide variety of fungal species (Op De Beeck et al., 2014) and has previously been used for metabarcoding of benthic fungi (Polinski et al., 2019). The final library pool was sequenced by Novogene using a 2×250 bp paired-end setup on the Illumina NovaSeq 6000 SP platform.

2.3 | Bioinformatic analysis

The raw sequence quality was checked using FastQC 0.11.9 (Andrews, 2023), and the reports were combined with MultiQC 1.9 (Ewels et al., 2016). This was followed by removing primers using cutadapt 4.0 (Martin, 2011) following the DADA2 1.8 ITS Pipeline Workflow as previously performed in other studies of fungi (Pauvert et al., 2019; Rolling et al., 2022). The data was analyzed with the R package DADA2 v.1.26.0 (Callahan et al., 2016) using R 4.2.2 (R Core Team, 2022). Filter and trimming of the sequences were conducted with the *filterAndtrim* function (*maxEE = c(2, 2), truncQ = 2, minLen = 50*). The sequence quality profile was verified once again using FastQC and MultiQC, followed by error rate analysis with the *learnErrors* function with default settings and merging of paired-end reads using the *mergePairs* function with default settings. Chimera sequences were filtered out leaving a total of 25,563 amplicon sequence variants (ASVs) remaining.

Taxonomic identification was performed using the *assignTaxonomy* function of the DADA2 package against the UNITE 9.0 database (Abarenkov et al., 2022), which has been progressively developed to improve the accuracy of fungi molecular identification when using high-throughput sequencing techniques (Nilsson et al., 2019). To complement the taxonomic identification, sequences alignment and annotations were also performed against the NCBI NT database using nucleotide BLAST (Altschul et al., 1990) with an *e-value threshold* of 0.001 and *max_target_seqs* 1 to only report the top hit (Broman et al., 2019). To obtain the taxonomy results based on the NCBI accession numbers, these BLAST results were imported into MEGAN6 (with default Lowest Common Ancestor (LCA) algorithm parameters; Huson et al., 2016), and NCBI accession numbers were linked to taxonomic labels with the MEGAN supplied database (megan-nucl-Feb2022.db). The BLAST results were used to improve the taxonomic identification performed with the UNITE database. The NCBI classification at the class level was used when the phylum attributed by both databases matched, and the UNITE database was not capable of assigning a class to an ASV. The same logic was applied to assign genus and species classifications to the ASVs but the classification had to match until the order or family level, depending on if a family was assigned or not. The complete taxonomic identification of the studied ASVs can be found in Data S1.

TABLE 1 Baltic Sea stations sampled between May and June 2019 and the respective environmental measurements performed during sampling and in the laboratory: water depth, temperature, salinity (practical salinity unit (PSU)), and oxygen levels were measured in the bottom water, while C and N content percentages were measured in the 0–2 cm sediment surface.

Novogene_ID	Station	Region	Year	Latitude	Longitude	Water depth (m)	Salinity (PSU)	Temperature (°C)	Oxygen (mg/L)	N (%)	C (%)
L157	01	Bothnian Bay	2019	64.70007	22.0654	120.86	3.66	0.83	10.06	0.50	5.23
L159	02	Bothnian Bay	2019	64.653	21.31315	26.64	2.6	4.91	10.28	0.12	1.37
L156	03	Bothnian Bay	2019	64.64943	22.115617	121.96	3.66	0.88	9.47	0.43	4.53
L158	04	Bothnian Bay	2019	64.63757	21.37195	32.09	2.69	3.91	10.52	0.06	0.71
L155	05	Bothnian Bay	2019	64.57255	21.8886	106.04	3.55	0.97	9.86	0.52	5.47
L154	06	Bothnian Bay	2019	64.53622	21.8054	89.82	3.47	1.31	9.44	0.46	4.85
L153	07	Bothnian Bay	2019	63.41208	20.371233	75.45	5.84	3.16	7.18	0.32	2.41
L152	08	Bothnian Bay	2019	63.39665	20.30987	84.66	5.88	3.3	6.93	0.42	3.32
L151	09	Bothnian Bay	2019	63.38173	20.2581	88.75	5.89	3.34	6.93	0.40	3.29
L150	10	Bothnian Bay	2019	63.35752	20.28697	86.75	5.87	3.28	6.84	0.38	3.14
L149	11	Bothnian Bay	2019	63.33335	20.2192	85.17	5.87	3.29	7.01	0.37	3.02
L145	12	Bothnian Sea	2019	62.89065	18.28877	51.03	5.49	2.26	8.12	0.14	1.18
L146	13	Bothnian Sea	2019	62.88428	18.255217	16.61	5.25	2.85	2.37	0.84	7.28
L147	14	Bothnian Sea	2019	62.8623	18.20875	46.43	5.44	2.21	7.61	0.40	3.50
L144	15	Bothnian Sea	2019	62.85603	18.259267	62.86	5.59	2.55	6.79	0.22	1.91
L143	16	Bothnian Sea	2019	61.99237	17.76027	84.45	5.51	2.56	7.14	0.30	2.66
L142	17	Bothnian Sea	2019	61.94002	17.74007	78.68	5.46	2.38	7.16	0.30	2.63
L141	18	Bothnian Sea	2019	61.90505	17.590217	67.95	5.36	2.07	7.81	0.32	2.81
L140	19	Bothnian Sea	2019	61.85877	17.57155	64.52	5.29	1.95	8.1	0.18	1.51
L139	20	Bothnian Sea	2019	61.8099	17.602383	66.55	5.27	1.88	9.34	0.34	2.93
L138	21	Bothnian Sea	2019	61.46057	17.31875	48.52	5.09	2.66	8.52	0.41	3.40
L137	22	Bothnian Sea	2019	61.39318	17.36855	70.05	5.15	2.13	10.01	0.38	3.54
L136	23	Bothnian Sea	2019	61.37588	17.36053	64.33	5.16	2.1	9.99	0.39	3.30
L135	24	Bothnian Sea	2019	61.3442	17.432783	76.89	5.15	2.27	10.05	0.37	3.25
L134	25	Stockholm	2019	60.24428	18.6332	23.46	5.2	5.15	8.88	0.62	5.17
L133	26	Stockholm	2019	60.24363	18.6704	26.44	5.13	5.3	9.78	0.63	5.24
L131	27	Stockholm	2019	59.96585	19.2189	121.21	6.78	2.79	10.75	0.44	3.43
L132	28	Stockholm	2019	59.9111	19.268633	110.09	6.75	2.81	8.01	0.49	3.86
L120	29	Stockholm	2019	59.52433	18.85331345	24	4.24	9.7	11.13	0.68	5.06
L122	30	Stockholm	2019	59.50808	19.00438118	62	5.7	4.7	7.82	0.23	1.56
L121	31	Stockholm	2019	59.47882	18.92152405	40	4.85	5.6	10.37	0.39	2.67

TABLE 1 (Continued)

Novogene_ID	Station	Region	Year	Latitude	Longitude	Water depth (m)	Salinity (PSU)	Temperature (°C)	Oxygen (mg/L)	N (%)	C (%)
L92	32	Sörmland	2019	58.84083	17.55184746	21.5	5.76	8.1	11.34	0.82	5.97
L91	33	Sörmland	2019	58.82609	17.57608795	39.5	6.17	4.6	9.53	0.77	5.62
L94	34	Sörmland	2019	58.81092	17.60691643	37	6.11	4.8	10.28	0.80	5.71
L95	35	Sörmland	2019	58.79023	17.72844505	37.5	6.31	4	9.26	0.63	4.37
L90	36	Sörmland	2019	58.77397	17.69137955	44	6.31	4.2	10.43	0.22	1.52
L127	37	Sörmland	2019	58.74395	17.81402588	47	7.41	4	7.37	0.64	4.19
L93	38	Sörmland	2019	58.71892	17.84226036	58.5	6.67	4.2	9.59	0.50	3.49
L112	39	Östergötland	2019	58.39609	16.88535309	13	5.83	11	10.09	0.94	7.15
L124	40	Östergötland	2019	58.37907	16.97112656	13	6.06	11.6	10.73	0.90	6.50
L108	41	Östergötland	2019	58.37626	16.98077583	13	5.64	11.5	10.77	1.06	7.62
L110	42	Östergötland	2019	58.37392	16.94440079	11	5.66	12.4	10.59	1.06	7.76
L111	43	Östergötland	2019	58.36969	16.96035576	16.5	5.65	11.6	10.46	0.97	7.42
L109	44	Östergötland	2019	58.36209	16.94333649	20	5.68	10.2	8.71	1.07	7.83
L113	45	Östergötland	2019	58.32343	16.93635	20	6.08	9.5	10.86	0.69	5.15
L107	46	Östergötland	2019	58.32203	16.97151756	20	6.06	9	10.61	1.16	8.29
L118	47	Östergötland	2019	58.25433	16.78655434	38.5	5.65	5.9	11.49	0.98	7.13
L117	48	Östergötland	2019	58.22487	16.81530762	25	5.58	8.4	11.14	0.80	6.14
L116	49	Östergötland	2019	58.21693	16.84322739	32	5.58	7.9	11.34	0.82	5.99
L123	50	Östergötland	2019	58.2095	16.93776894	33	5.72	7.8	11.04	1.21	8.08
L114	51	Östergötland	2019	58.20266	16.91519928	11	5.68	10	11.54	0.88	6.51
L115	52	Östergötland	2019	58.198	16.8501091	29	5.71	8.7	11.24	0.81	5.91
L97	53	Southern Baltic	2019	55.2334	13.333375	41	12.28	5.8	5.28	0.23	1.72
L101	54	Southern Baltic	2019	55.22505	13.633525	43	12.46	6.5	7.55	0.81	6.11
L98	55	Southern Baltic	2019	55.22481	13.26669788	41	12.75	6.8	6.42	0.38	2.93
L100	56	Southern Baltic	2019	55.22461	13.41825	41	11.67	5.9	5.24	0.41	3.12
L96	57	Southern Baltic	2019	55.13332	13.666575	45	11.93	7.4	8.62	0.82	6.73
L99	58	Southern Baltic	2019	55.12389	13.26145833	39	12.35	9	9.58	0.25	1.85
L102	59	Southern Baltic	2019	55.00903	14.07380676	48	15.02	8.1	8.54	0.74	6.15

Note: A Novogene identification number (Novogene ID), attributed by the sequencing company, was associated with the samples collected in each station.

2.4 | Statistical analysis

Taxonomical analysis of the fungal communities from each station was based on relative abundance values calculated from the ASV counts and plotted in stacked bars and a heatmap using Explicet 2.10.5 (Robertson et al., 2013). Differences in relative abundance of phyla, classes, and ASVs between regions were statistically tested with a Kruskal–Wallis test, followed by a pairwise Dunn test using the *dunn.test* function (*method* = *"bh"*, *altp* = *TRUE*, *list* = *TRUE*).

Alpha diversity was analyzed by plotting the ACE and Shannon H diversity indices, based on the ASVs counts in R using *vegan* 2.6-4 (Oksanen et al., 2022) and *phyloseq* 1.42.0 (McMurdie & Holmes, 2013) packages. Before alpha diversity analysis, to reduce sequencing biases, the counts for each station were subsampled to the lowest sample size (28,995 reads). The results were tested statistically with a one-way analysis of variance (ANOVA) after confirmation that the data met the normality and homogeneity of variation requirements through the Shapiro–Wilks and Levene tests, respectively. The functions used for each respective test were *shapiro.test* and *leveneTest*. If main effects were detected by the ANOVA tests, multiple comparisons between regions were performed with post hoc Tukey test using the *TukeyHSD* function (95% confidence level).

Community structure of the benthic fungal communities from each region was analyzed in Past 4.12b (Hammer-Muntz et al., 2001) using a non-parametric multidimensional scaling (NMDS) ordination based on the Bray–Curtis dissimilarity index. To test if there were statistical differences between regions a permutational multivariate analysis of variance (PERMANOVA, 9999 permutations) was performed using the software Past. This was followed by pairwise PERMANOVA (9999 permutations) tests with Bonferroni corrected *p*-values to test differences between pairs of regions. The similarity percentages (SIMPER) test, using the *simper* function (*trace* = *FALSE*, *permutations* = *999*, *parallel* = *2*) included in the *vegan* package, was performed to determine the top 10 ASVs that contributed to the dissimilarities in community structure observed between regions. A distance-based redundancy analysis (dbRDA) test was performed to quantify the impact of the measured environmental variables on the benthic fungal community structure dissimilarities observed between regions. The dbRDA was performed in R, using the Bray–Curtis dissimilarity index (999 permutations), with the *capscale* and *anova.cca* functions from the *vegan* package.

3 | RESULTS

Sampling was conducted at 59 stations (Figure 1) and sediments were collected at 11–122 m water depth, with water temperatures ranging from 0.83°C to 12.4°C and salinity interval between 2.6 to 15.02 PSU (Table 1, Figures S1 and S2). The registered oxygen levels in the bottom water ranged from 2.37 to 11.54 mg/L and the C and N percentages in the sediments varied from 0.71% to 8.29% and 0.06% to 1.21%, respectively (Table 1, Figure S2).

3.1 | Alpha and community structure of benthic fungi

There were no statistically significant differences between the sampled Baltic Sea regions regarding fungal alpha diversity measured as the Shannon's H index was found not to be significantly different between the sampled Baltic Sea regions (one-way ANOVA, $F_{5,53} = 1.898$, p-value = 0.11; Figure 2a). However significant statistical dissimilarity was observed for the ACE alpha diversity (one-way ANOVA, $F_{5,53} = 2.522$, p-value = 0.0404; Figure 2b). This main effect of region on fungal alpha diversity was driven by the significantly higher alpha diversity observed for benthic fungal communities in Bothnian Bay when compared to the Bothnian Sea (Tukey HSD post hoc, p-value = 0.017 for ACE index; Figure 2b), which was also reflected by the higher number of unique ASVs observed in the Bothnian Bay compared to Bothnian Sea (one-way ANOVA $F_{5,53} = 5.536$, p-value = 0.0394; Tukey HSD post hoc, p-value = 0.0158; Figure S3).

Spatial dynamics of Baltic benthic fungal community structure based on NMDS ordination showed that fungal community structure was significantly different in the Baltic regions here studied (one-way PERMANOVA, *pseudo* $F_{5,53} = 4.637$, p-value = 0.0001; Figure 3). This significant effect of the region was found to be driven by the benthic fungal community structure in the Bothnian Bay and Southern Baltic regions, which were both different from all other studied regions (pairwise PERMANOVA, Bonferroni adjusted p-values < 0.05). Similarly, even regions with smaller differences in salinity showed significant differences in benthic fungal community structure, namely Bothnian Sea, Sörmland, and Östergötland that were significantly different from all the remaining regions (pairwise PERMANOVA, Bonferroni adjusted p-values < 0.05), except when compared to the Stockholm region (pairwise PERMANOVA, Bonferroni adjusted p-values = 1, 0.183 and 0.069, respectively). Consequently, the Stockholm region fungal community structure was only significantly dissimilar to the communities from Bothnian Bay and Southern Baltic (pairwise PERMANOVA, Bonferroni adjusted p-values < 0.05).

3.2 | Baltic benthic fungi community composition

The classification of the obtained ASVs against the UNITE database showed that Chytridiomycota was the phylum with the highest average relative abundance in the sampled region (45.03% ± 13.32%; Figure 4a), followed by Ascomycota and Basidiomycota (16.21% and 5.95% ± 13.32%, respectively; Figure 4a). A significantly higher relative abundance of Chytridiomycota was observed in the Bothnian Bay when compared to Bothnian Sea and Sörmland (Figure 4a; Kruskal-Wallis (KW) test, df = 5, $H = 12.222$, pairwise Dunn test, p-values < 0.05 for all comparisons between the mentioned regions). In contrast, a significantly higher relative abundance of Ascomycota was observed in Sörmland region compared to Bothnian Sea and Östergötland (Figure 4a; KW test, df = 5,

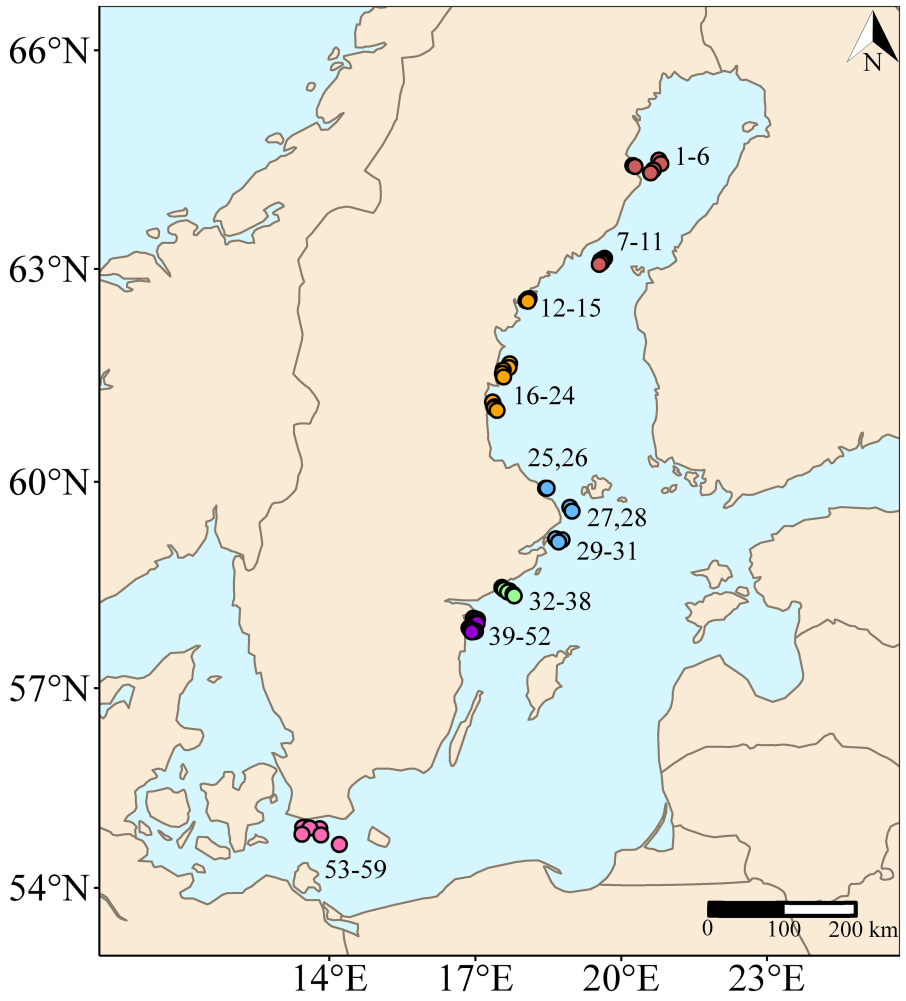

FIGURE 1 Map of 59 stations in the west and south coastal regions of Sweden where sediments were sampled between May and June 2019. The station numbers (displayed next to the dots) are grouped (X-X) or enumerated (X,X). The colors of the dots correspond to the region the stations belong to (Red – Bothnian Bay; Orange – Bothnian Sea; Blue – Stockholm; Green – Sörmland; Purple – Östergötland; Pink – Southern Baltic).

$H = 17.8175$, pairwise Dunn test, p-values < 0.01 for all comparisons between the mentioned regions).

At a lower taxonomic level, the two most abundant classes in our study Chytridiomycetes and Rhizophydiomycetes belonged to the Chytridiomycota phylum (average relative abundance: Chytridiomycetes = 24.95% ± 5.84% and Rhizophydiomycetes = 9.98% ± 5.84 %; Figure 4b). After these, Sordariomycetes and Dothideomycetes, from the Ascomycota phylum, were the most abundant classes in the sampled regions (6.50% and 4.39% ± 5.84%, respectively; Figure 4b).

A high percentage of unclassified ASVs was detected at the phylum level (32.65% total relative abundance; Figure 4a), and it increased at the class level (40.77% total relative abundance; Figure 4b). This value also varied between stations ranging from 15% to 70% relative abundance of unclassified ASVs (Figure 4).

To perform a deeper analysis of the diversity patterns of the most abundant fungal ASVs in the Baltic Sea, a heatmap of the ASVs with a total relative abundance higher than 0.5% was assembled (Figure 5, Data S2). The three most abundant ASVs were all classified as Chytridiomycota, from the Chytridiomycetes class (Figure 5), which were the most abundant phylum and class detected in the Baltic sediments, respectively (Figure 4). These three Chytridiomycota ASVs were present in the various sampled regions of the Baltic Sea with characteristic abundance patterns between regions. The *Alphamycetes chaetifer* (ASV_1) was observed to have an increasing abundance gradient, from north to south, with a higher relative abundance in the most southern regions (Östergötland and Southern Baltic) compared to Bothnian Sea (Figure 5; KW test, df = 5, $H = 20.1255$, pairwise Dunn test, p-values < 0.05 for all comparisons between the mentioned regions).

In contrast, for the *Operculomyces laminatus* (ASV_2), also from the Chytridiomycetes class and the Chytridiomycota phylum, an inverse abundance pattern along the salinity gradient was observed since it had a higher relative abundance in Bothnian Bay and Sea regions compared to the Östergötland and Southern Baltic regions (Figure 5; KW test, df = 5, $H = 20.962$; pairwise Dunn test, p-values < 0.05 for all comparisons between the mentioned regions). In this case, it was also observed a significantly higher abundance in Stockholm than in Southern Baltic (Figure 5; pairwise Dunn test,

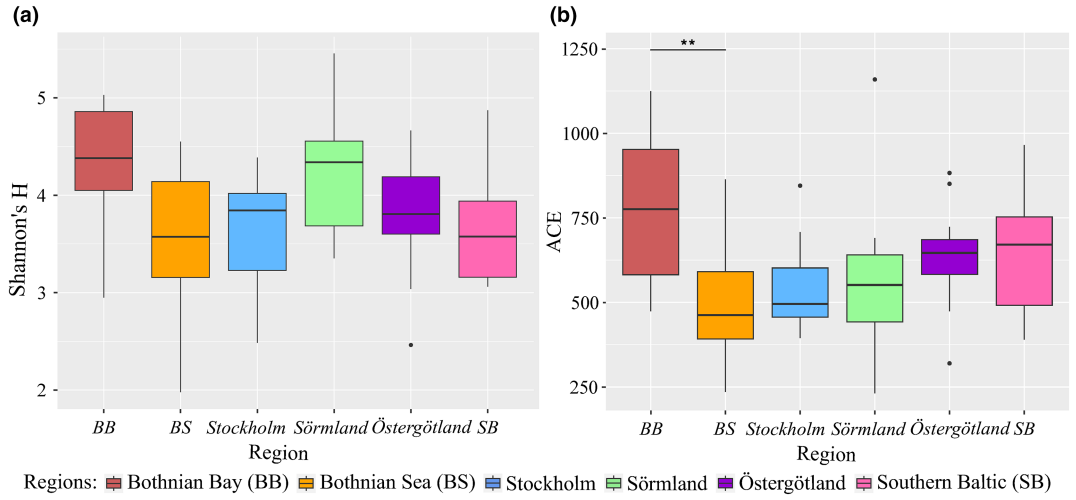

FIGURE 2 Box plot representing the fungi alpha diversity variation in each region in 2019 based on the Shannon's H (a) and ACE (b) diversity indices. The y-axis shows the index value and the *x*-axis shows the Baltic Sea regions where the sediments were collected from. The legend indicates the colors corresponding to each region. The central line represents the median value of the diversity index for each region, while the top and bottom lines of the box represent the upper and lower quartiles, respectively. The vertical lines above and below the box correspond to the whiskers and indicate the maximum and minimum values, respectively. The dots located after the whiskers are outlier values that are positioned 1.5 times the interquartile range above or below the upper and lower quartile, respectively. The line and stars above some of the boxes represent the existence of significant statistical dissimilarities in alpha diversity between the regions underneath each end of the line (two starts indicate $0.01 < p\text{-value} < 0.01$).

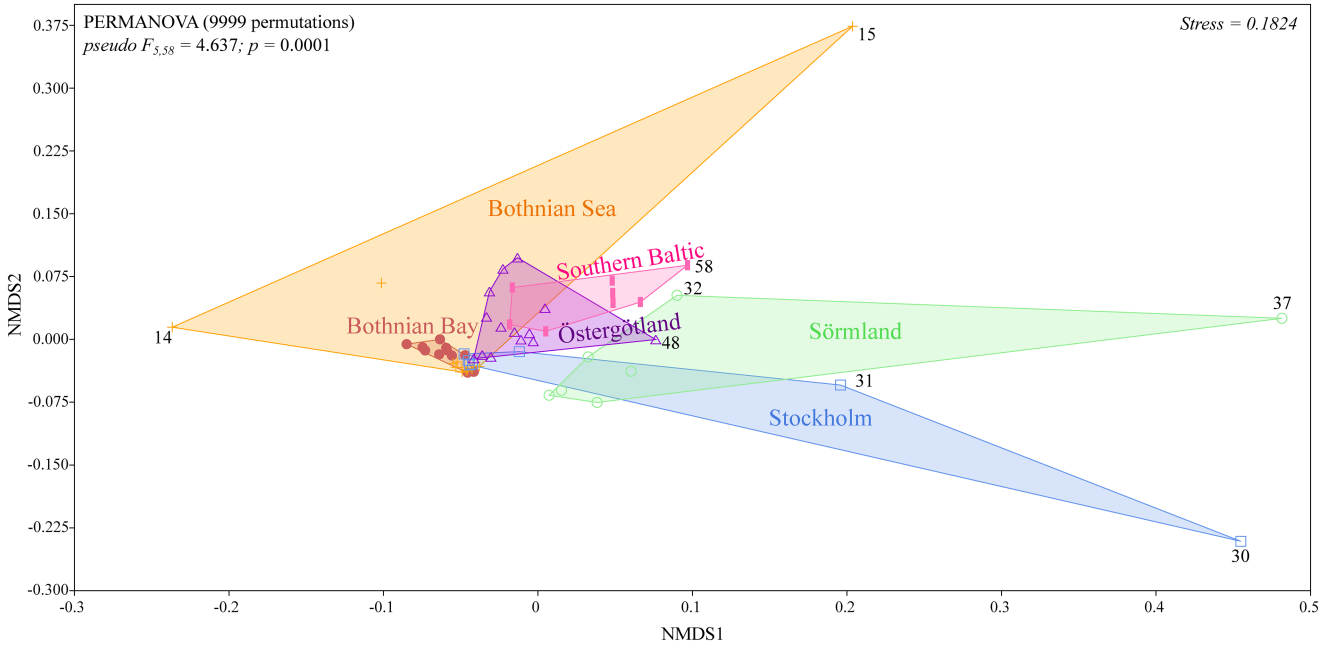

FIGURE 3 Non-metric multidimensional scaling (NMDS) plot of fungal community structure (Bray–Curtis dissimilarity) based on the ASVs amplified from the top 0–2 cm sediment layer of the Baltic Sea seafloor in 2019. The colors filling and outlining the hulls denote a specific region identified by the name with the same color. Each symbol forming the hull and inside of it corresponds to the stations of that region. Stations identified as outliers in this dataset present the corresponding station number next to its symbols. The NMDS ordination stress level is presented in the top right corner of the graph and the PERMANOVA (9999 permutations) *pseudo F* and *p* values are displayed in the top left corner.

p-values < 0.05), but not when compared to Östergötland (Figure 5; pairwise Dunn test, *p*-values > 0.05).

A clear abundance pattern like the ones described for the two previously mentioned ASVs was not observed for the third most abundant ASV classified as *A. chaetifer* (ASV_4). The relative abundance of this ASV was significantly higher in the Bothnian Bay, Bothnian Sea, Stockholm, and Östergötland regions in comparison to Sörmland (Figure 5; KW test, df = 5, $H = 24.8077$; pairwise Dunn test, *p*-values < 0.05 for all comparisons between the mentioned regions), and also higher in the Bothnian Bay region compared to the Bothnian Sea, Östergötland, and

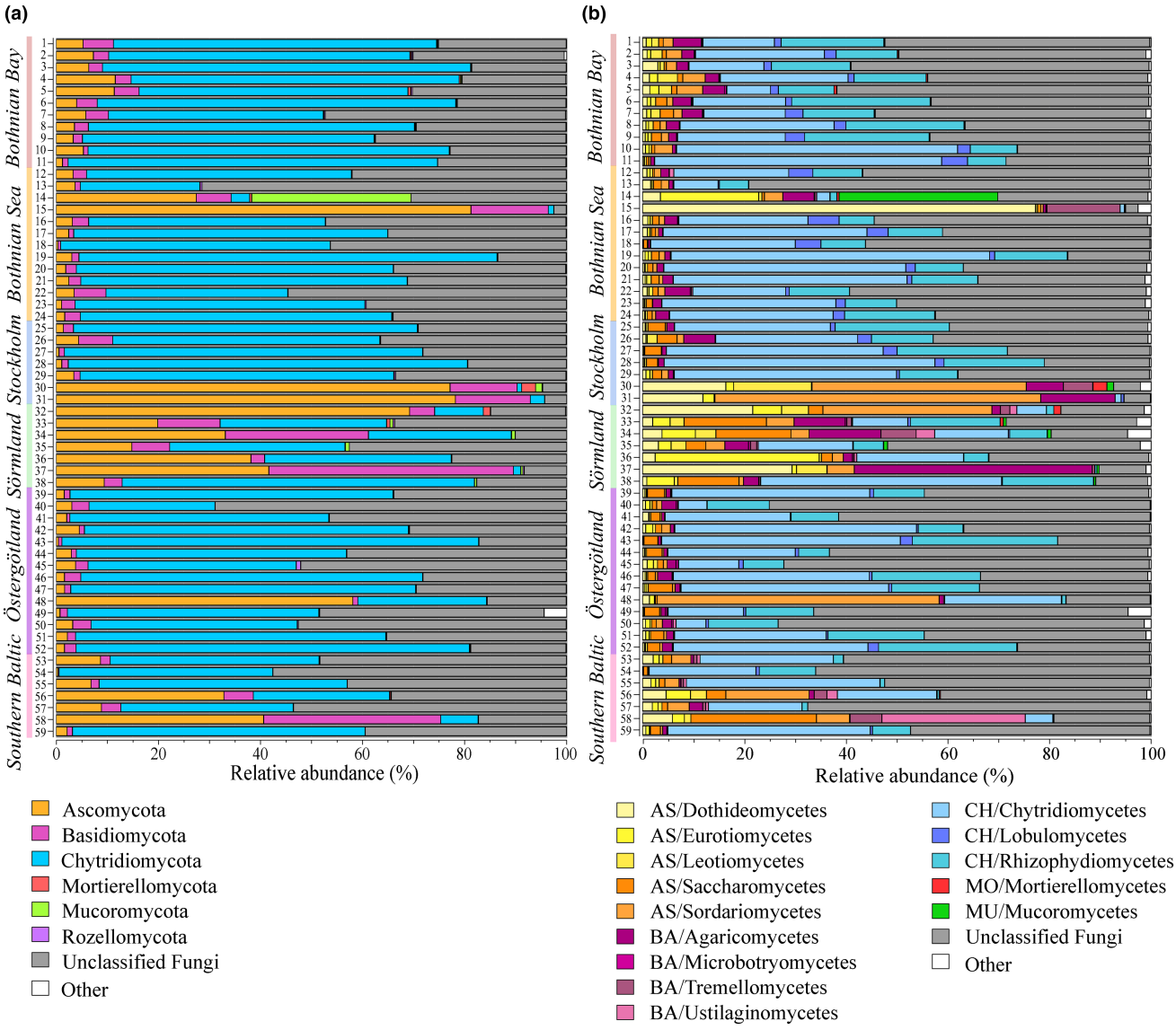

FIGURE 4 Stacked bar plots of the fungi phyla (a) and classes (b) identified in the ITS dataset obtained by amplification of environmental DNA extracted from the top 0–2 cm sediment layer of 59 stations in the Baltic Sea. The phylum taxonomic classification was only based on the UNITE database, while the class identification was complemented with the results from the NCBI database. On the *y*-axis of both plots, the stations were numbered following a descending order of latitude values, as presented in Figure 1, and the region where each station is located is highlighted by a vertical bar on the left of the graph with a characteristic color and the respective region name. The *x*-axes show the relative abundance of the phyla and classes, respectively. Below each plot the legend presents the observed phyla and class and the respective color (the classes' colors were based on the color attributed to the phylum they are included in). The classes' names follow an abbreviation of the phylum they are included in (AS – Ascomycota; BA – Basidiomycota; CH – Chytridiomycota; MO – Mortierellomycota; MU – Mucoromycota).

Southern Baltic regions (Figure 5; pairwise Dunn test, p-values < 0.05 for all comparisons between the mentioned regions).

A SIMPER analysis showed that the ASVs that significantly contributed to the dissimilarities in community structure between the benthic fungal communities belonged to the phyla Chytridiomycota, Ascomycota, or Basidiomycota (SIMPER test, all p-values < 0.05; Data S3). Multiple ASVs from the Chytridiomycota phylum were found to significantly contribute to the dissimilarity in fungal community structure between the Bothnian Bay community and the communities from all other regions (SIMPER test, all p-values < 0.05, contribution 1.09%–8.46%; Data S3). The higher relative abundance of two ASVs classified as *A. chaetifer* (one of the dominant taxa in our dataset) in the Bothnian Bay drove the dissimilarities in community structure between this region and both Östergötland and Southern Baltic regions (SIMPER test, all p-values < 0.05, contribution 4.08%–8.46%; Data S3). This same fungal species also significantly contributed to the dissimilarities observed between the communities of both Bothnian Sea and Sörmland with Östergötland and Southern Baltic regions (SIMPER test, all p-values < 0.01, contribution 8.20%–8.91%; Data S3) and between Bothnian Bay and

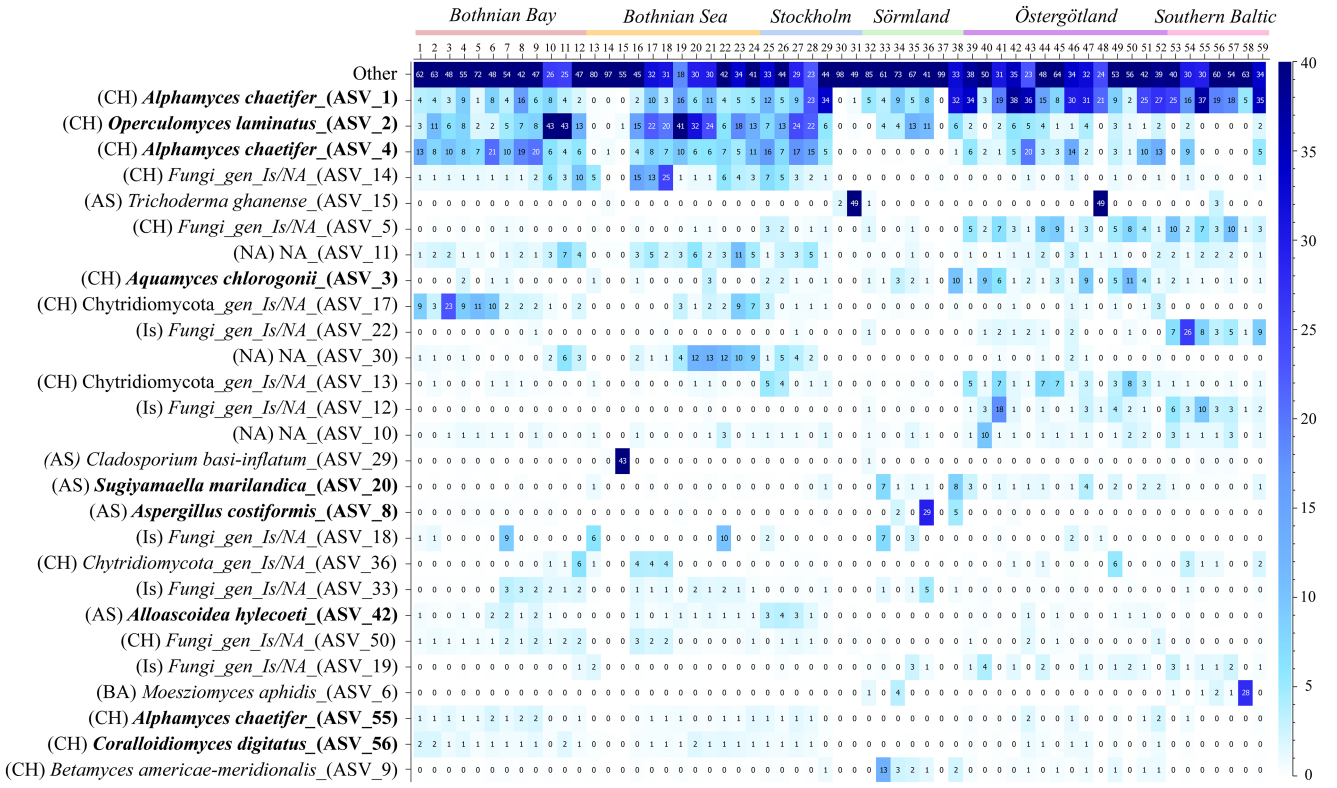

FIGURE 5 Heatmap of the ASVs with a relative abundance higher than 0.5% detected in the sediment samples collected in 2019. The ASVs' taxonomic classification at the phylum (abbreviated in parenthesis), genus, and species level, followed by the ASV number, is displayed on the *y*-axis in decreasing order of relative abundance from top to bottom. Phylum abbreviations: Chytridiomycota (CH), Ascomycota (AS), and Basidiomycota (BA) (The complete taxonomic classifications can be found in Data S2). ASVs classified with the UNITE database are in normal font and the ones classified with the NCBI database are in bold. The fungi ASVs with an unclassified phylum, genus, and species are presented as *Incertae sedis* (Is) or Not assigned (NA). In the top part of the heatmap the sampled stations are grouped according to the respective region and on the right a color gradient displays the relative abundance of the ASVs, ranging from 0 to 40%.

Sörmland (SIMPER test, all p-values < 0.05, contribution 5.72%; Data S3). The second most abundant ASV in our study, belonging to the species *Operculomyces laminatus* from the Chytridiomycota phylum, significantly contributed to the dissimilarities in community structure between the Bothnian Sea community and the Bothnian Bay, Östergötland, and Southern Baltic communities (SIMPER test, all p-values < 0.02, contribution 7.63%, 7.16%, and 7.71%, respectively; Data S3).

3.3 | Environmental drivers of fungal diversity

In terms of water depth, the sediments collected in Sörmland, Östergötland, and Southern Baltic were on average shallower than 50 m water depth, while samples collected in the Bothnian Bay and Bothnian Sea were deeper than 50 m water depth. Average salinity levels increased gradually from Bothnian Bay to Östergötland and then presented a significant increase (>4 PSU) in the Southern Baltic region (Figure S2; KW, df = 5, $H = 34.82$; pairwise Dunn test, p-values < 0.05, except when compared to Sörmland).

To try to explain the biodiversity patterns observed in the previous sections, the environmental variables were analyzed and statistically related to the dissimilarities in community structure observed between the fungal communities from different regions. The distance-based redundancy analysis (dbRDA) results (Figure 6, Data S4) indicated that all the tested environmental variables significantly contributed to the benthic fungal community structure dissimilarities observed among the communities from the sampled regions (ANOVA.cca, p-values < 0.05; Data S4). Water depth and salinity were the two variables that contributed the most to the observed benthic fungal community structure dissimilarities (ANOVA.cca, Sum of Sqs 1.6101 and 1.2406, respectively; Data S4). Oxygen and nutrient concentrations (N and C) had a similar impact on the fungal community structure between regions (ANOVA.cca, Sum of Sqs 0.5289–0.5371; Data S4), followed by temperature (ANOVA.cca, Sum of Sqs 0.4244; Data S4).

4 | DISCUSSION

We show here for the first time that DNA metabarcoding analyses of benthic fungal communities in various regions of the Baltic Sea made it possible to describe their spatial distribution and to assess which main abiotic variables are driving their community structure.

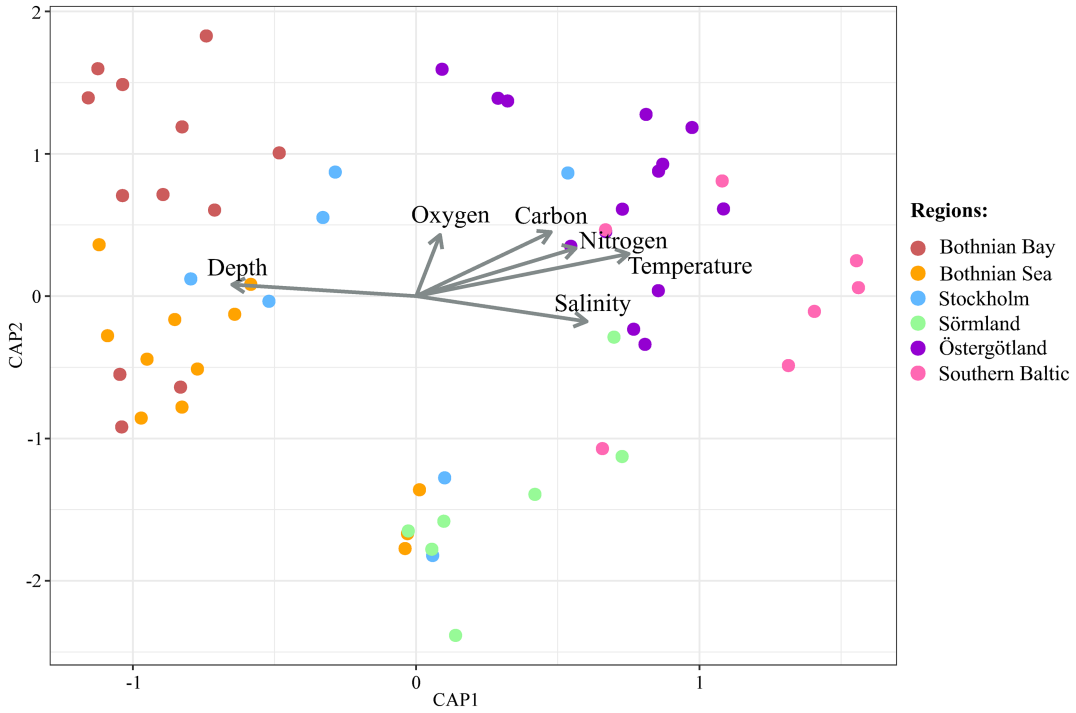

FIGURE 6 Distance-based redundancy analysis (dbRDA) plot (Bray–Curtis dissimilarity) showing the relation between fungal communities community structure and the environmental variables measured in 2019 during sampling (water depth, temperature, salinity, and oxygen levels were measured in the bottom water, while N and C content was measured in the 0–2 cm top sediment layer). The dots represent the community structure of the fungal communities sampled in each station, and they are colored according to the region they were collected in (see legend on the right side of the graph). As all environmental variables under analysis significantly contributed to the dissimilarities in community structure between the different regions (Data S4), no symbol was used to highlight the statistically significant variables.

Our results show significant differences in benthic fungi community structure between regions, mainly driven by water depth, salinity and sediment C and N availability. The Baltic benthic fungal communities are dominated by Chytridiomycota fungi, followed by Ascomycota and Basidiomycota.

4.1 | Relation of environmental variables to spatial dynamics of Baltic Sea benthic fungal diversity

We detected differences in alpha diversity between the two Bothnian regions, even though this was not consistent for all tested indices. The significantly higher alpha diversity was observed with the ACE index and for Observed ASVs, which indicated the presence of a higher number of rare taxa and species richness in Bothnian Bay than in the Bothnian Sea. These differences could be related to an addition to the Baltic Sea of freshwater taxa that can live in the lower salinity waters of Bothnian Bay, as studies have previously shown for bacteria (Kisand et al., 2005). Additionally the influx of dissolved organic carbon from the high number of freshwater systems that flow into this region of the Baltic could have led to an increase in benthic fungi diversity, as previously seen for marine fungi in the Mediterranean Sea when comparing areas near and far from river estuaries (Barone et al., 2018). The higher species richness in the Bothnian Bay seems to be driven by the significantly higher abundance and diversity of fungi from the Chytridiomycota phylum compared to the Bothnian Sea. These results are in accordance with studies describing taxa from this phylum in freshwater ecosystems (Calabon et al., 2022; Monchy et al., 2011). Additionally, the significantly higher abundance of one of the ASVs identified as *A. chaetifer* in the Bothnian Bay compared to the Bothnian Sea could have contributed to the described differences in alpha diversity. Fungi from this genus have been identified as obligate and facultative parasites of plankton inhabiting freshwater environments (Frenken et al., 2017) and described as composing approximately 30% of the Chytridiomycota identified in the freshwater Lake Tahoe (Comeau et al., 2016).

In the remaining Baltic Sea regions, the benthic fungal alpha diversity did not vary significantly for any indices, in alignment with previous studies that investigated Baltic fungal (Rojas-Jimenez et al., 2019) and bacterial (Herlemann et al., 2011) communities in the water column. In this last study, Herlemann et al. (2011) suggest that the lack of variation in alpha diversity is caused by the adaptation of marine and freshwater bacteria to the Baltic brackish conditions, enabling them to thrive in most Baltic regions.

In contrast, community structure was significantly distinct among the various regions indicating that alpha diversity and community structure in benthic fungal communities are not necessarily coupled, as previously hypothesized for pelagic fungi in the Baltic Sea (Rojas-Jimenez et al., 2019).

In terms of community structure, Bothnian Bay benthic fungal community was shown to be significantly dissimilar from the communities from all the remaining regions, as was previously observed for Baltic benthic bacteria (Klier et al., 2018). The community from the Southern Baltic region was also dissimilar to all other studied regions, which is in accordance with available literature on other benthic communities in the Baltic Sea. For example meiofauna community structure showed significant dissimilarities between the communities identified in Bornholm and Arkona regions (with Arkona corresponding to Southern Baltic in this study) when compared to communities from Stockholm, Sörmland, and Östergötland (Broman et al., 2019). Both community structure patterns found in our study could be explained by the differences in salinity and depth between these regions, which were shown to significantly impact the sampled fungal communities from the Baltic Sea. Therefore, these two abiotic variables could have promoted the formation of unique communities in the regions of Bothnian Bay (deep sediments and low salinity waters) and Southern Baltic (shallower sediments but high salinity waters).

Salinity and depth also seemed to explain why the Stockholm fungal community structure was similar to the communities collected from Bothnian Sea, Sörmland, and Östergötland, since salinity and water depth ranges seen in Stockholm region were the same as in Sörmland and Östergötland. Therefore, taxa that can thrive in these ranges of conditions should be found in all three regions. Broman et al. (2019) also described a lack of significant dissimilarity between the meiofauna communities of Stockholm and Östergötland, similar to what was observed for the benthic fungi analyzed in this study. On the other hand, the dissimilarities in community structure between the benthic fungal communities from Bothnian Sea, Sörmland, and Östergötland seem to be related to the significantly higher relative abundance of Ascomycota fungi observed in Sörmland in comparison to Bothnian Sea and Östergötland. However, it was not possible to find an explanation as to why fungi from this phylum were more abundant in the Sörmland region in comparison to the other two mentioned regions.

Water depth was shown to have the strongest effect on community structure of benthic fungal communities in this study (Figure 6, Data S4) and has also been previously described to shape community structure of benthic fungi (Li et al., 2016), planktonic fungi sampled through a river continuum and adjacent marine waters (Ilicic et al., 2022), benthic macroinvertebrates (Baumgärtner et al., 2008), and benthic bacteria (Green et al., 2012). However, as in our study, the effect of water depth found in these studies was often seen to act on ecological communities together with other environmental variables, such as water level fluctuations (Baumgärtner et al., 2008), oxygen concentration (Green et al., 2012), or multiple variables: salinity, temperature, N concentration and C/N ratio (Li et al., 2016); salinity, temperature and C, ammonium, phosphate, and chlorophyl a concentrations (Ilicic et al., 2022). Interestingly, while water depth was found to be a strong driver of benthic fungal communities in the Baltic, the same was not true for Baltic pelagic fungi (Rojas-Jimenez et al., 2019). These contrasting evidence could be due to the larger range of water depth studied here. However, the differences in lifestyle of pelagic and benthic organisms should be important too. For example, some benthic fungi may require to be attached to particles to thrive and their growth may be promoted by the higher concentration of dissolved organic matter in the seafloor, while pelagic fungi don't usually require particle attached growth and have less access to dissolved organic matter in the water column (Orsi et al., 2021).

Salinity, which was shown to have the second strongest impact on benthic fungal community structure in this study, has been demonstrated to be a major variable shaping the community structure of Baltic pelagic fungi (Rojas-Jimenez et al., 2019), similar to what was observed in our results. The impact of salinity on community structure has been described for benthic fungal communities outside of the Baltic Sea such as fungi inhabiting surface sediments in Chinese coastal areas (Wu et al., 2023) and sediments of tidal marshes with different salinity levels (Mohamed & Martiny, 2011). Salinity has also been shown to have an effect on other benthic communities in the Baltic Sea as meiofauna (Broman et al., 2019) and bacteria (Broman et al., 2022; Dupont et al., 2014; Herlemann et al., 2011).

In addition to water depth and salinity C and N availability in the sediment was another significant predictor of benthic fungi community structure.

The effect of nutrient availability on community structure has been described for benthic fungi in other coastal environments (Wu et al., 2023). Additionally, the impact of C concentration on fungal diversity has also been shown in soils, both in studies based on analyses of DNA extracted from forest soils (Vasco-Palacios et al., 2020) and proteins extracted from semi-arid soils, to reflect the active fungal community (Bastida et al., 2016). Furthermore, in accordance to our study Rojas-Jimenez et al. (2019) also found nutrient availability (N, silicate, and phosphate) to significantly impact Baltic pelagic fungal community structure, although the authors could not conclude how these abiotic factors shaped the communities.

4.2 | Baltic benthic fungal community composition

Fungi from the Chytridiomycota phylum had the highest relative abundance among all the identified phyla in the sampled benthic fungal communities, which has also been shown for pelagic fungi in the Baltic Sea (Rojas-Jimenez et al., 2019). These are zoosporic fungi that have a widespread presence in both terrestrial (Freeman et al., 2009) and aquatic habitats, either as symbionts (Gutiérrez et al., 2016) or saprobes in the water column or seafloor (e.g. Comeau et al., 2016; Gonçalves et al., 2020). Therefore, the observed ubiquitous and high relative abundance of Chytridiomycota in the Baltic Sea indicated that fungi from this phylum have a preference for brackish conditions.

The three most abundant ASVs in the dataset were classified as *A. chaetifer* or *Rhizophydium chaetiferum* and *Operculomyces laminatus*, all belonging to the Rhizophydiales order which had approximately

20% relative abundance in the Baltic Sea water column during spring (Rojas-Jimenez et al., 2019). These taxa have been described as saprotrophs in the literature (Letcher et al., 2008; Powell et al., 2011) so their high relative abundance in the sediments of the Baltic Sea, and the significant impact of nutrient availability on benthic fungal community structure, could be related to the involvement of these fungi taxa in C and N cycling in the benthic environment (Orsi et al., 2013, 2021; Wu et al., 2023).

It is possible that some other taxa found in our samples might have been present in Baltic sediments through the sedimentation of phytoplankton blooms. Various species within the *Rhizophydium* genus have been shown to establish a parasitism relation with *Planktothrix* cyanobacteria commonly present in algal blooms (Agha et al., 2018; McKindles et al., 2021) and various other plankton species and genera found in freshwater environments (Frenken et al., 2017; Kagami et al., 2007). A portion of phytoplankton aggregates sink down to the seafloor (Kagami et al., 2014), and this might partly explain some of our findings. Similarly, zooplankton DNA was identified in Baltic Sea sediments (Broman et al., 2019). Therefore the identified benthic fungi could also correspond to parasitic pelagic fungi or particle-attached fungi that were transported to the seafloor attached to organic matter aggregates and accumulated in the sediments, or possibly even parasitic fungi of benthic diatoms (Ilicic et al., 2022).

4.3 | Limitations of the study

The DNA metabarcoding technique is associated with some limitations (Jurburg et al., 2022; Ruppert et al., 2019) that were carefully considered and attempted to be minimized during the development of this study. One of them is related to the use of eDNA that is heterogeneously distributed in the environment and is susceptible to degradation (Mauvisseau et al., 2022). It can also include extracellular DNA from dead or inactive fungi (relic DNA; Carini et al., 2016, 2020; Miettinen et al., 2019), despite some literature stating that relic DNA did not affect the biodiversity assessment of bacterial communities (Gustave et al., 2019; Lennon et al., 2018). The use of a single primer pair targeting only one section of the ITS region could also create a PCR amplification bias (Amend et al., 2019); however, this is a common practice in fungal diversity studies using metabarcoding (da Silva et al., 2022; Gonçalves et al., 2020; Polinski et al., 2019). The taxonomical classification is still limited by the existing reference databases and fungi diversity studies, as observed by the percentages of unclassified fungi at the phylum and class levels observed in this study (32.65% and 40.77%, respectively).

Despite the described caveats of using DNA metabarcoding to analyze eDNA extracted from sediments, this method has many advantages and is currently one of the most used methods to perform an in-depth analysis of microbial diversity, justifying its vast use in studies of various organisms, including fungi (Hassan et al., 2022; Ruppert et al., 2019).

5 | CONCLUSION AND IMPLICATIONS

In this study it was shown that the community structure of benthic fungi was significantly dissimilar between regions in the Baltic Sea, especially when comparing the Bothnian Bay and Southern Baltic with the other studied regions. These diversity dynamics were mainly associated with the variation in water depth and salinity, but also with differences in C and N availability in the sediments and water oxygen levels and temperature between the different regions. Changes in salinity and nutrient availability in the environment are expected in the Baltic Sea in the future, so the benthic fungal diversity and their role in the ecosystem may be impacted, as supported by our results. As these only represent a part of all the fungal diversity inhabiting the sediments of the Baltic Sea, further studies could focus on these understudied organisms, and their impact on the food web dynamics, organic matter degradation rates, and contribution to benthic-pelagic recycling of nutrients.

AUTHOR CONTRIBUTIONS

LQL analyzed the data and drafted the manuscript. DIS conducted laboratory work. FJAN and EB designed the study and assisted in data analysis and drafting the manuscript. FJAN financed the study. CR, JA, and JG assisted in field sampling.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support these findings are available in the manuscript and supplemental files. The raw sequence data can be found online on the NCBI database with the BioProject ID number: PRJNA1008059.

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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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