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ORIGINAL ARTICLE

Through the eDNA looking glass: Responses of fjord benthic foraminiferal communities to contrasting environmental conditions

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Abstract

The health of coastal marine environments is severely declining with global changes. Proxies, such as those based on microeukaryote communities, can record biodiversity and ecosystem responses. However, conventional studies rely on microscopic observations of limited taxonomic range and size fraction, missing putatively ecologically informative community components. Here, we tested molecular tools to survey foraminiferal biodiversity in a fjord system (Sweden) on spatial and temporal scales: Alpha and beta diversity responses to natural and anthropogenic environmental trends were assessed and variability of foraminiferal environmental DNA (eDNA) compared to morphology-based data. The identification of eDNA-obtained taxonomic units was aided by single-cell barcoding. Our study revealed wide diversity, including typical morphospecies recognized in the fjords, and so-far unrecognized taxa. DNA extraction method impacted community composition outputs significantly. DNA extractions of 10 g sediment more reliably represented present diversity than of 0.5-g samples and, thus, are preferred for environmental assessments in this region. Alpha- and beta diversity of 10-g extracts correlated with bottom-water salinity similar to morpho-assemblage diversity changes. Sub-annual environmental variability resolved only partially, indicating damped sensitivity of foraminiferal communities on short timescales using established metabarcoding techniques. Systematically addressing the current limitations of morphology-based and metabarcoding studies may strongly improve future biodiversity and environmental assessments.

KEYWORDS
barcoding, biodiversity, coastal ecosystems, environmental DNA, foraminifera, hypoxia, metabarcoding

INTRODUCTION

CLIMATE and environmental changes pose risks to the marine realm worldwide, with negative effects on function and diversity of ecosystems and global economies dependent on them (Doney et al., 2012; Smale et al., 2019). Coastal and transitional environments are particularly affected due to anthropogenic stressors leading to eutrophication and deoxygenation (Harley et al., 2006; Rabalais et al., 2014). With the prevalence of human activities and contrasting influences of marine and terrestrial regimes, such systems are prone to high environmental variability. It is, therefore, critical to close knowledge gaps regarding baseline environmental

variability and anthropogenic drivers of ecosystem dynamics. Such may support early detection of declining ecosystem health and management efforts.

Direct measurements of physico-chemical conditions allow monitoring of relevant environmental parameters. However, for comprehensive evaluations of environmental and climatic changes on biota and ecosystem health, bioindicators are imperative (e.g., review by Alve et al., 2016; Borja et al., 2000; Cairns Jr. & van der Schalie, 1980). Benthic foraminiferal (Rhizaria) communities are useful for assessing ecosystem status and environmental trends, both in recent and past sediment records, with high sensitivity to stressors (Alve, 1995; Alve et al., 2016; Bouchet et al., 2012; Dolven et al., 2013). Recently, metabarcoding emerged as a potential time- and labor-efficient alternative to morphology-based observational studies (Cordier, Lanzén, et al., 2019; Deiner et al., 2017; Pawlowski et al., 2018, 2021). It relies on recognizing community composition and diversity by the presence of genomic and extracellular DNA in sedimentary or aqueous samples (i.e., environmental DNA, eDNA). This can be achieved by PCR amplification and high-throughput sequencing of short DNA barcodes known to be taxonomically informative (Pawlowski & Lecroq, 2010). Advantages include fast processing, potential of re-analysis, and higher taxonomic resolution than achieved by conventional, observation-based techniques (e.g., review by Pawlowski et al., 2022). To date, foraminiferal eDNA studies focused on the deep sea (abyssal to hadal depths: Cordier, Frontalini, et al., 2019; abyssal regions: Lejzerowicz et al., 2021) and, more recently, polluted areas adjacent to offshore fossil-fuel drilling sites (Frontalini et al., 2020; Laroche et al., 2018; Mauffrey et al., 2021), fish farms (Pawlowski et al., 2016; Pawlowski, Esling, et al., 2014), and former steel mills (Cavaliere et al., 2021). However, impacts of other anthropogenic activities, such as the increased prevalence of oxygen-deficient seawater, have rarely been assessed by metabarcoding approaches (Langlet et al., 2013), and studies focusing on natural variability are lacking particularly in near-coast environments.

Here we focus on three Swedish fjords, each characterized by distinct hydrographic conditions (incl. brackish to fully marine salinities), as well as intermittent oxygen deficiency. Foraminiferal eDNA from fjord surface sediments were collected over short- and long-term environmental fluctuations. The outputs of two commonly used eDNA extraction kits were compared with regard to community composition and community responses to natural environmental variability. Moreover, we genetically characterized typical fjord morphospecies to aid the taxonomic assignment of eDNA sequences and compared eDNA-defined communities with previous local morphology-based core-top studies (Gustafsson & Nordberg, 1999, 2000, 2001; Höglund, 1947).

We address the following questions: Can metabarcoding reliably assess foraminiferal biodiversity? Do eDNA-based foraminiferal assemblages from contrasting environments differ significantly, as known for morphospecies assemblages? Are eDNA diversity and community composition informative and, therefore, proxies of short- and/or long-term natural environmental variability?

MATERIALS AND METHODS

Study sites

Three fjords of the Swedish west coast were studied: Gullmar Fjord (GF), Havstens Fjord (HF), and Koljö Fjord (KF; Figure 1). The basins represent transitional settings influenced by marine waters of the Skagerrak (North Sea) and brackish waters of the Baltic Sea, as well as adjacent terrestrial waters. Fjord topography and water exchange connections to the open sea result in distinct hydrographic and environmental conditions, including a range of salinities and/or bottom-water oxygen concentrations. An increase in low-oxygen periods during the last century has been associated with variations in the North Atlantic Oscillation, and, potentially, human-induced environmental changes (Filipsson & Nordberg, 2004; Nordberg et al., 2000). Industrial, agricultural, and fishing activities are heavily regulated in the area, as Gullmar Fjord is classified as a nature reserve. The fjords have a long history of hydrographic measurements (since 1890, Swedish Meteorological and Hydrological Institute, SMHI) and morphology-based foraminiferal core-top studies (Gustafsson & Nordberg, 1999, 2000, 2001; Höglund, 1947), providing baselines for comparison.

Collection of sediment samples, water samples, and environmental data

Two sites in Gullmar Fjord and one site each in Havstens and Koljö Fjord (Table S1 and Figure 1) were sampled in September 2018, February and June 2019 for environmental DNA. The GF sites were additionally sampled in November 2017 (Choquel et al., 2021). All sites are located below sill depth of the respective fjord (Table S1 and Figure 1) at 43 m (GF), 20 m (HF), and 8 m (KF). For single-cell barcoding, further sites were sampled in GF between November 2013 and July 2019 (Table S1 and Figure 1).

The 2018–2019 GF cores are replicates (by repeated deployment) of those in Brinkmann et al. (2022), where core sampling methods are outlined in detail. Koljö- and Havstens Fjords were sampled during the same campaigns using the same equipment. Briefly, duplicate sediment cores were collected with a GEMAX twin-barrel corer (modified Gemini corer, 9-cm inner diameter) in two casts. Sediment samples for eDNA analysis were

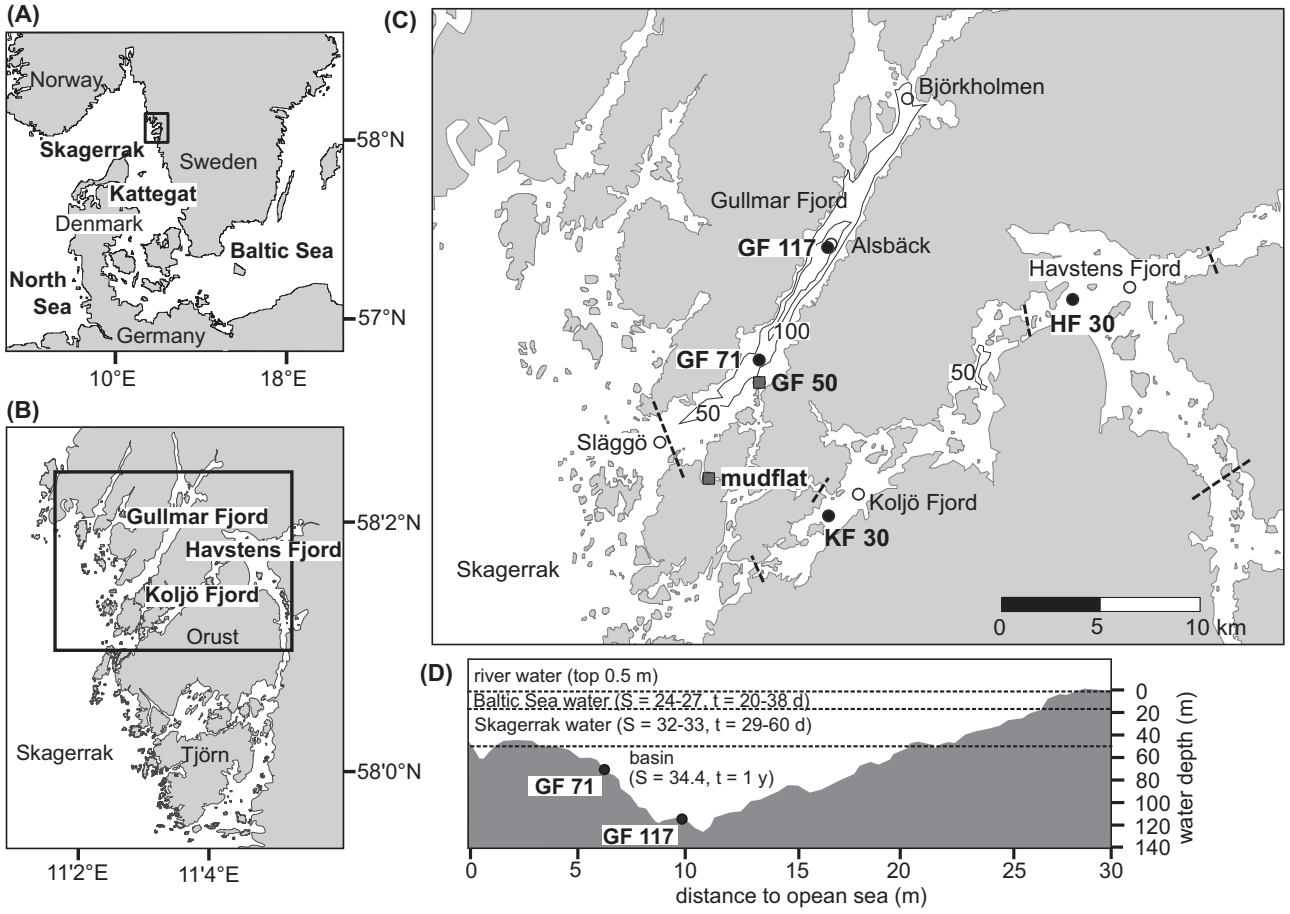

FIGURE 1 (A) Study location on Swedish west coast (marked by rectangle). (B) Overview of Gullmar Fjord (GF), Havstens Fjord (HF), and Koljö Fjord (KF) in the context of the Orust-Tjörn island system. (C) Study sites sampled for metabarcoding and single-cell barcoding GF 71, GF 117, HF 30, and KF 30 (black circle), for solely barcoding "mudflat" and GF 50 (gray square), and environmental monitoring sites Alsbäck, Björkholmen, and Släggö, as well as in monitoring sites in Havstens and Koljö Fjords (white circle). Sill locations marked by dashed lines. Fjord bathymetry indicated for water depths of 50 and 100 m. (D) Gullmar Fjord transect showing water-mass sources (S = salinity, t = typical residence time in days (d) or years (y); after Arneborg, 2004).

retrieved from one duplicate core for each station and season. The 2017 GF cores were collected as box cores, and the undisturbed surfaces sampled for sediment. On each occasion, surface sediments were subsampled (top 1 cm; 50–75 mL) with sterile equipment and placed in several sterile 25-mL centrifuge tubes. Cross-contamination was avoided by subsampling from the inner part of the cores and cleaning of equipment with 95% ethanol. The subsamples were frozen and transported to the Laboratory of Planetology and Geosciences (LPG), University of Angers, France, and stored at −20°C until DNA extraction.

The cores for DNA barcoding were collected directly from the sediment surface in the GF mudflat, and by GEMAX twin-barrel coring between November 2013 and July 2019 in three sites in GF and one site in HF (Table S1). (Sub-)samples of the top 2 cm of the sediment were stored with ambient seawater in bottles in a cooler, transported to the LPG, France, and kept at 4°C until further processing.

In 2018 and 2019, temperature, salinity (reported in practical salinity unit), and dissolved oxygen concentration of water-column and bottom waters (BWs; c. 0.5 m above sediment–water interface) were obtained from CTDO$_2$ measurements for each station (Brinkmann et al., 2022). Data for the 2017 GF sampling appears in Choquel et al. (2021). Data for HF and KF is reported in Figure S1. For 2018–2019, pH of the water–sediment interface and pore-water (PW) were obtained using a Unisense pH Microelectrode pH-200.

We used the data collection service Svenskt HavsARKiv (SHARK; https://sharkweb.smhi.se/, last access: February 2021) organized by SMHI to obtain monthly data on salinity, [O_2], and/or chlorophyll-*a* across 2017–2019, if available, from three stations in GF: Släggö (58°25.98′N, 11°43.57′E, 70 m), Alsbäck (58°19.40′N, 11°32.80′E, 120 m) and Björkholmen (58°23.26′N, 11°37.60′E, 70 m; Figure 1), and one site each in HF and KF (58°18.75′N, 11°46.40′E; 58°13.80′N, 11°34.80′E; 30 m each; Figures S2, S3). Surface-water

chlorophyll-*a* measurements preceding the sampling by 2 months were used for statistical analyses (see below), knowing that assemblages respond to increased chlorophyll-*a* production in surface waters approx. 2 months after spring/autumn blooms (based on GF; Gustafsson & Nordberg, 2000). Släggö data was used for the 71-m deep site of GF(GF 71), and Alsbäck for the 117-m deep site GF 117.

DNA barcoding of single cells

For DNA barcoding, sediment samples were sieved (>100 µm) with artificial seawater (ASW, Red Sea Salt, salinity=34) within a week after collection and examined under a stereomicroscope. Foraminifera with pigmented cytoplasm and an empty last chamber were collected in Petri dishes containing ASW and fine sediment. Vitality was confirmed by individual activity overnight (e.g., presence of feeding cysts, movement; Tables S2, S3). Live specimens were cleaned with a fine brush and imaged by stereomicroscopy (Leica S9i) or environmental Scanning Electron Microscopy (SEM; Zeiss EVOLS10; for organic walled and mineralized foraminifera, respectively). After imaging, specimens were individually placed in DOC (Deoxycholate) buffer for DNA extraction (Pawlowski, 2000). A ~500 bp region at the V9 region of the SSU rDNA, used for foraminiferal barcoding (Pawlowski & Holzmann, 2014), was amplified with foraminifera-specific primers s14F3-J2 and s14F1-N6 (Darling et al., 2016; Pawlowski, 2000) following the protocol described in Darling et al. (2016). Positive amplifications were directly sequenced with the Sanger method (GATC Biotech, Cologne). To assess intra-individual variability, four specimens (i.e., GF002, GF162, GF203, GF211) were amplified for a longer fragment (~1000 bp) with primers s14F3-sB (Pawlowski, 2000) for the first PCR and s14F1-J2 for the nested PCR following the same conditions as the other PCR. Positive amplifications were purified with the High Pure PCR Purification Kit (Roche Diagnostics) and cloned using the pGEM®-T Easy Vector System (Promega). One to four clones were sequenced with the Sanger method (GATC Biotech, Cologne). Specimens were taxonomically identified by morphological criteria and corresponding DNA sequences by BLAST (Basic Local Alignment Search Tool; Altschul et al., 1997). All sequences were deposited in the National Center for Biotechnology Information's database GenBank, accession numbers ON818317–ON818462.

The nonionid rDNA sequences were placed with a representative selection of sequences of *Nonionella* and *Nonionellina* in Seaview v.4 (Gouy et al., 2010), automatically aligned with MUSCLE (Edgar, 2004a, 2004b), implemented in Seaview and corrected manually. A total of 1187 nucleotide sites were selected to build a molecular phylogenetic tree with the PHYML program (Guindon & Gascuel, 2003) implemented in Seaview, choosing the GTR (General Time Reversible) evolutionary model (Tavaré, 1986) and the approximate Likelihood Ratio Test (aLRT) for branch support estimation (Anisimova & Gascuel, 2006).

We followed the high-rank classification of foraminifera defined by Pawlowski et al. (2013), with monothalamids (single-chambered, organic-walled foraminifera), the class of Globothalamea (globular, multi-chambered, mineralized tests)—including Textulariida (agglutinated tests) and Rotaliida (calcitic, hyaline tests) and the class of Tubothalamea (tubular, multi-chambered mineralized tests), including Miliolida (porcelaneous tests).

Extraction, amplification, and sequencing of environmental DNA

Total DNA was extracted from homogenized sediment subsamples of each site in three pseudo-replicates: one pseudo-replicate (A) with 10 g of sediment and two pseudo-replicates with 0.5–1 g (B, C; hereafter denoted '0.5-g'). DNA of pseudo-replicate A was extracted with DNeasy PowerMax Soil Kit (Qiagen) and DNA of B and C with NucleoSpin Soil Minikit (Macheret Nagel), following the manufacturer's instructions.

Initial PCR used AccuPrime™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific). Foraminifera-specific primers s14F1 (AAGGGCACCACAAG AACGC; Pawlowski, 2000) and s15r (CCACCTATCAC AYAATCATG; Lejzerowicz et al., 2014) were used to amplify a 135–190 bp fragment at the 3′ end of the SSU rRNA gene targeting the first variable region of the area amplified for foraminiferal DNA barcoding. PCR cycle conditions comprised an initial denaturation of 94°C for 1 min followed by 40 cycles at 94°C for 30 s (denaturation), at 50°C for 30 s (primer annealing), and at 72°C for 1 min (extension) plus final extension at 72°C for 3 min. For each sample, two PCR replicates were prepared and pooled. Afterward, amplicons were purified using Sera-Mag™ Magnetic carboxylate-modified particles (GE Healthcare). A second PCR amplification was performed to incorporate Illumina adapters and barcodes according to the manufacturers' protocol (GoTaq® G2 DNA Polymerase, Promega). Amplicon purification was repeated after the first PCR. Amplicons were quantified using Quant-iT™ PicoGreen™ dsDNA Assay kit (Invitrogen) and an equimolar pool was dosed by qPCR using the KAPA Library Quant Illumina Kit (Roche). The equimolar pool with 5% of phiX phage mix was finally diluted to 12 pM and 600 µL was added in the sequencer cartridge (MiSeq Reagent Kit v3 MS-102-3003). Library constructions and MiSeq sequencing were performed at the ANAN platform (SFR QUASAV, INRAE Beaucouzé, France).

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Bioinformatics and taxonomic assignment

Prior to sequence data processing, primer sequences were removed using cutadapt v. 3.4 (Martin, 2011). Sequence data were processed using DADA2 v. 1.16 (Callahan et al., 2016) in R (R Core Team, 2016), following the tutorial for paired-end data (https://benjjneb.github.io/dada2/tutorial.html). Raw reads were quality controlled by truncating the reads (forward and reverse length of 120 bp) and filtering to a maximum number of 'expected errors' of two. Amplicon sequence variants (ASV) were dereplicated if identical, clustered, and pair-end reads merged using a minimum overlap of 12 bp and maximum mismatch of 0 bp. Chimeras were removed using the 'pooled' method. The ASVs were taxonomically assigned using VSEARCH v. 2.18.0 (Rognes et al., 2016) and our custom foraminifera reference database based on GenBank.

Non-foraminiferal ASVs (i.e., percentage identity of <80%) were removed, as well as rare ASVs with read counts of less than 100 and those occurring in less than three samples, aiming to omit chimeras and/or contaminants. As molecular and morphological species concepts differ (e.g., review by Pawlowski, Lejzerowicz, & Esling, 2014), we checked the taxonomic assignment of each ASV manually by sequence alignment and comparisons with reference databases, and pooled ASVs related to the same taxon. The final number of reads versus number of taxa was computed (Pearson's R correlation, $r=-4.13$, $p<0.001$). As no positive correlation was found, we decided not to rarefy the data to retain diversity.

Statistical analyses

The community matrix was analyzed using vegan v. 2.5-7 (Oksanen et al., 2020) in R, if not indicated otherwise. Rarefaction curves were produced with the *rarecurve* function. Alpha diversity (taxon richness, Shannon index, Evenness) was estimated with *ddply* (plyr v. 1.8.6). Venn counts were calculated (*vennCounts*, LIMMA v. 3.44.3) and visualized (*vennplot*, VennDiagram v. 1.7.1) organized by extraction kits and sampling site.

Further analyses were performed on matrices retaining taxa with ≥1% relative abundance (RA) in at least one sample, to remove noise induced by the rare biosphere (Schiaffino et al., 2016). Matrices were created comprising all replicates, only A pseudo-replicates, and only B/C pseudo-replicates. Hellinger-transformation—adapted to datasets containing high occurrences of zeros (i.e., absences) and correcting for double-zero problem in similarity analyses (Legendre & Gallagher, 2001; Legendre & Legendre, 2012)—was applied prior to multivariate analyses. The RA of taxa >5% was visualized with *pheatmap* (ComplexHeatmap v. 2.9.4). One-way and two-way permutational multivariate analysis of variance (PERMANOVA) was performed to evaluate effects of extraction kits and habitat on community composition using the function *adonis2* with Bray–Curtis dissimilarity. Similarly, the ANOSIM test was applied to test for differences between eDNA communities (*anosim*). Pairwise post hoc comparisons were performed with pairwiseAdonis v. 0.4. Differences between assemblage compositions of samples were visualized by non-metric multidimensional scaling (nmMDS) analyses with Bray–Curtis distance (*metaMDS*). Mantel tests were conducted to identify correlations between eDNA assemblages of 10-g kit samples (Bray–Curtis dissimilarity) and environmental variables (Euclidian distance) with the function *mantel*. For non-normally distributed (Shapiro–Wilk's test) and heteroscedastic (Bartlett's test) parts of the data, we performed Kruskal–Wallis with Mann–Whitney pairwise post hoc (non-parametric tests) to test for differences. Sparse Partial Least Squares (sPLS) regression model (mixOmics v. 6.12.2; Lê Cao et al., 2008; Rohart et al., 2017) was performed and visualized with the *cim* function to assess correlations between taxa and recorded environmental conditions (correlation of $r<-0.5$ or $r>0.5$ to at least one tested parameter).

RESULTS

DNA barcoding of single foraminiferal cells

Among 395 foraminiferal specimens amplified for this study, 337 were imaged (Tables S2, S3; Figure 2; Files S1, S2). A total of 146 sequences belonging to 134 individuals were deposited in GenBank (Table S2), comprising monothalamids (8), miliolids (2), and globothalamids (19) listed in Table 1. Two other specimens were previously sequenced (GenBank accession numbers KY347798, MH011650–MH011654) and published (Jauffrais et al., 2018, 2019).

Six monothalamids were identified as published species in BLAST (100% similarity each). One monothalamid represented a so far not published species of *Bathysiphon* and was identified here as *Bathysiphon* sp. GF1. Three specimens morphologically identified as the 'silver monothalamid' did not match any GenBank sequence above 90% similarity (Table 1). Two sequenced miliolids were morphologically identified as *Quinqueloculina* species: the first identical to a *Quinqueloculina seminulum* sequence, whereas the closest match of the second specimen's sequence was <93% similar to any *Quinqueloculina* species in GenBank (Table 1). Most textulariids were morphologically identified as *Eggerelloides scaber* (Williamson, 1858), but formed three presently cryptic genetic species here called *Eggerelloides* sp. GF1, GF2, and GF3. Another agglutinated individual, morphologically identified as *Textularia porrecta*, was genetically identical to GenBank sequences (Table 1).

The most diverse foraminiferal group was the rotaliids with 17 recognized species (Table 1). Of these, four

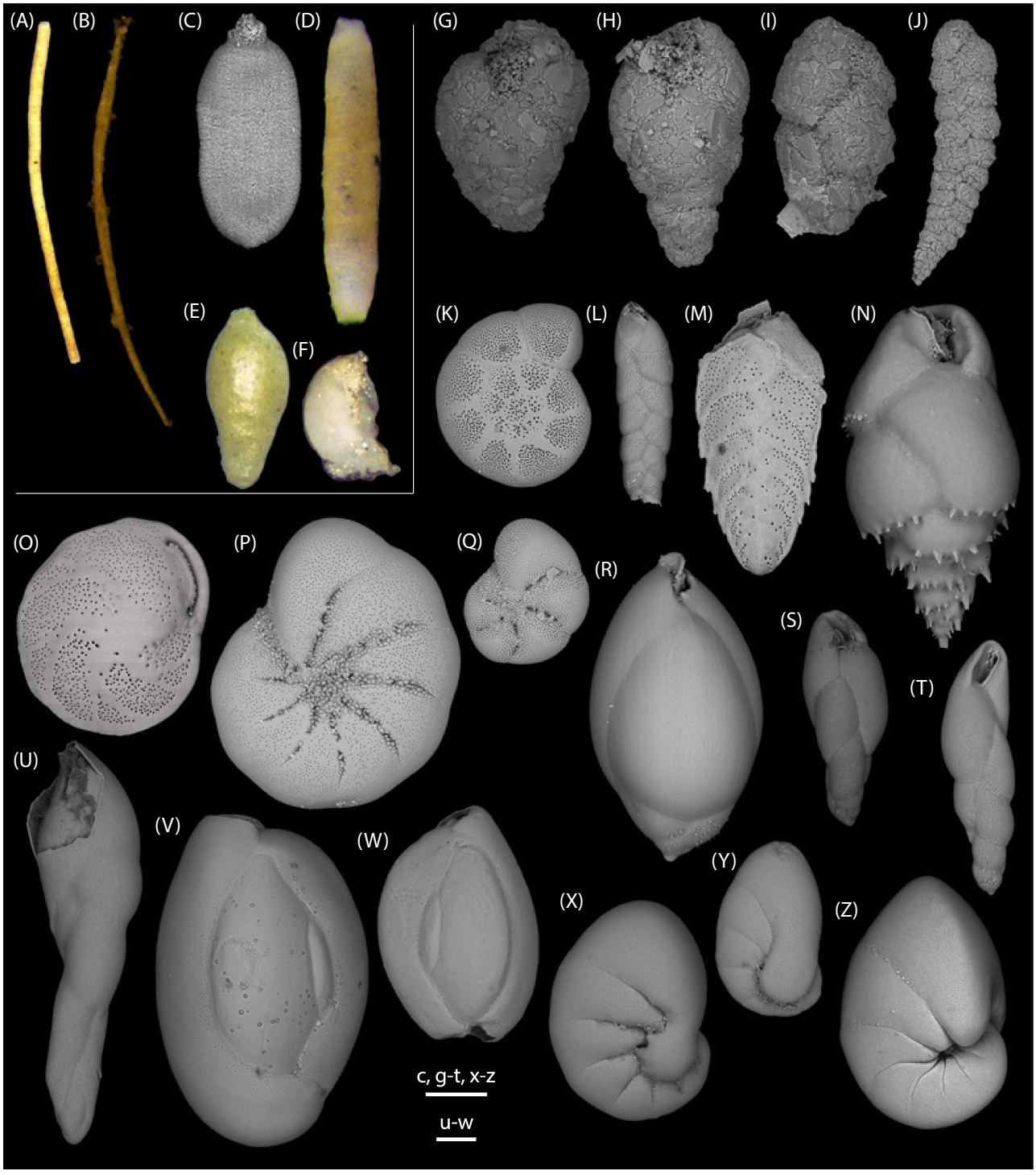

FIGURE 2 Light or scanning electron microscope images of foraminiferal species sequenced in this study. (A) *Bathysiphon flexilis* (GF736); (B) *Bathysiphon* sp. GF1 (GF396–397); (C) *Hippocrepinella alba* (GF185); (D) *H. hirudinea* (GF405); (E) undetermined monothalamid 'silver monothalamid' (GF707); (F) *Psammophaga crystalliphera* (GF717); (G) *Eggerelloides* sp. GF2 (GF508); (H) *Eggerelloides* sp. GF1 (GF510); (I) *Eggerelloides* sp. GF3 (GF623); (J) *Textularia porrecta* (GF520); (K) *Ammonia* sp. T6 (GF925); (L) *Bolivina pseudopunctata* (GF250); (M) *B. spathulata* (GF640); (N) *Bulimina marginata* (GF374); (O) *Cassidulina laevigata* (GF162); (P) *Elphidium* sp. S23 (GF922); (Q) *Elphidium magellanicum* (S24) (GF923); (R) *Globobulimina auriculata* (GF002); (S) *Stainforthia fusiformis* (GF897); (T) *Fursenkoina complanata* (GF880); (U) *Virgulina concava* (GF829); (V) *Quinqueloculina* sp. GF1 (GF824); (W) *Quinqueloculina seminulum* (GF822); (X) *Nonionella* sp. T1 (GF864); (Y) *Nonionella* sp. T7 (GF879); (Z) *Nonionellina labradorica* (T5) (GF367); DNA extraction number of specimens in brackets. Scale bars 100 μm; A–B, D–F not to scale.

have not previously been sequenced: *Bolivina pseudopunctata*, *Elphidium* sp. S23, *Elphidium magellanicum* (S24), and *Fursenkoina complanata*. Further, three morphospecies of nonionellids were sampled: *Nonionella* sp. T1, *Nonionellina labradorica* (T5), and *Nonionella turgida*. Among the presently sequenced specimens of *N.*

TABLE 1 Summary of imaged and sequenced single cells with species identification, link to DNA extraction numbers and ASV*s in environmental DNA dataset.

Species	Class/order	Imaged	Sequenced	DNA extractions	Identical ASVs
Astrorhiza limicola Sandahl, 1858	Monothalamids	1	1	GF439	ASV61*
Bathysiphon flexilis Höglund, 1947	Monothalamids	6	6	GF211, GF320, GF396, GF397, GF736, GF737	
Bathysiphon sp. GF1	Monothalamids	7	7	GF203-GF207, GF209, GF212	ASV10*
Hippocrepinella alba Heron-Allen & Earland, 1932	Monothalamids	1	1	GF185	ASV27*
Hippocrepinella hirudinea Heron-Allen & Earland, 1932	Monothalamids	1	1	GF405	ASV14*
Micrometula hyalostriata Nyholm, 1952	Monothalamids	0	1	GF449	ASV68*
Psammophaga crystallifera (Dahlgren, 1962)	Monothalamids	1	1	GF717	ASV18*
undetermined ('silver monothalamid')	Monothalamids	18	3	GF704, GF707, GF728	ASV35*
Quinqueloculina seminulum (Linnaeus, 1758)	Miliolida	1	1	GF822	
Quinqueloculina sp.	Miliolida	1	1	GF824	
Eggerelloides sp. GF1	Textulariida	8	8	GF505, GF506, GF509-GF513, GF577	
Eggerelloides sp. GF2	Textulariida	4	4	GF504, GF507, GF508, GF826	
Eggerelloides sp. GF3	Textulariida	1	1	GF623	
Textularia porrecta Brady, 1884	Textulariida	25	1	GF520	ASV44*
Ammonia confertitesta Zheng, 1978 (T6)	Rotaliida	17	1	GF925	ASV1*
Bolivina pseudopunctata Höglund, 1947	Rotaliida	5	1	GF250	
Bolivina spathulata (Williamson, 1858)	Rotaliida	5	5	GF640, GF874-GF877	ASV98*
Bulimina marginata d'Orbigny, 1826	Rotaliida	65	30	GF172, GF175, GF374, GF564-GF566, GF612, GF615, GF616, GF798-GF802, GF804, Gf805, GF841-GF843, GF846-GF849, GF865, GF867, GF869, GF870, GF904-GF906	ASV15*
Cassidulina laevigata d'Orbigny, 1826	Rotaliida	8	8	GF162, GF548, GF549, GF551, GF552, GF871-GF873	
Elphidium sp. S23	Rotaliida	17	3	GF885, GF886, GF922	
Elphidium magellanicum Heron-Allen & Earland, 1932 (S24)	Rotaliida	1	1	GF923	
Elphidium williamsoni Haynes, 1973 (S1)	Rotaliida	25	6	GF190-GF195	
Globobulimina auriculata (Bailey, 1894)	Rotaliida	74	2	GF002, GF878	
Nonionella sp. T1	Rotaliida	36	20	GF361, GF816, GF830-GF839, GF855-GF857, GF859-GF861, GF863-GF864	ASV20*
Nonionellina labradorica (Dawson, 1860) (T5)	Rotaliida	38	4	GF104, GF367, GF818, GF819	
Nonionella sp. T3	Rotaliida	5	5	GF820, GF851-GF854	ASV12*
Nonionella sp. T7	Rotaliida	2	2	GF821, GF879	ASV49*
Stainforthia fusiformis (Williamson, 1858)	Rotaliida	26	8	GF888, GF890-GF892, GF896-GF899	ASV7*
Virgulina concava Höglund, 1947	Rotaliida	3	3	GF807, GF808, GF829	
Fursenkoina complanata (Egger, 1893)	Rotaliida	2	1	GF880	

Note: ASV* refers to pooled ASVs (see High-throughput sequencing output and data quality section).

turgida, two clades emerged comprising T3 and a newly described clade T7. Both clades are well separated and indicate the presence of (pseudo-)cryptic species within the morphospecies *N. turgida* (Figure 3).

High-throughput sequencing output and data quality

A total of 1,969,428 reads were obtained from 14 sediment samples (up to three pseudo-replicates per sediment sample, >30,000 reads per pseudo-replicate) and have been deposited in the Sequence Read Archive (SRA) at NCBI. Three pseudo-replicates were removed from consideration due to low read number (<6000 reads; HF 30 Febr. 2019 A, KF 30 Febr. and June A), to avoid biases in proportional abundance introduced by potentially low amplification rates. During bioinformatic processing, 206,396 reads were removed (10.48%). The retained reads were clustered into 236 ASVs assigning to 139 taxa (Table S4). The taxa were renamed as ASV*, numbered according to overall abundance. All samples reached asymptotic taxa accumulation values within their given read numbers, suggesting sequencing depth reliably described sample diversity (Figure S4). Sixty-eight ASV* in our eDNA data set were assigned to monothalamids, 50 to Globothalamea, and only one to Tubothalamea. Twenty ASV*s remained unclassified at class level.

Community composition by extraction-kit type

The 0.5-g extract output varied highly in taxon numbers grouped by mono- and globothalamids in temporal - and pseudo-replicates, although averaged by site the contributions of mono- and globothalamid taxa were c. 40%–50% each (Figure 4A). In 10-g extracts, on the contrary, relative class-level taxon richness varied between sites, and monothalamids dominated (up to 76%, $\bar{x}=58\%$, Figure 4B). The eDNA samples obtained by 10-g extractions had, on average, higher taxon richness (72±8 in Gullmar Fjord 117, 87±1 in Gullmar Fjord 71, 37±2 in Havstens Fjord, 21 in Koljö Fjord) than samples from the 0.5-g kit (24±18, 28±19, 16±11 and 13±8, respectively). In total, 118 of 139 taxa were shared by outputs of both extraction kits. Resolved by site, Havstens- and Koljö Fjord, which were represented by fewer replicates, comprised a higher degree of unique taxa, particularly in 0.5-g extracts (Figure 5A).

Despite 85% shared taxa, the metabarcoding output of 0.5- and 10-g extracts differed significantly (one-way ANOSIM of abundance data, Bray–Curtis, $r=0.95$, $p<0.001$). This difference was also expressed in read numbers at class level. In the 0.5-g output, most samples comprised >85% Globothalamea independent of sites (average 93±5% RA; Figure 4B), albeit driven by the high abundance of two globothalamid taxa representing on average 83% of the assemblages (ASV1*: *Ammonia confertitesta*, ASV2*: *Nonionella* sp. T4). In 10-g outputs, the contributions of globo- and monothalamids were more variable, although on average monothalamid reads dominated (71±18% RA). Further, a contrasting pattern emerged where highly abundant taxa (>5% RA) derived by one extraction-kit type was minor or absent in extracts of the other (Figure 6). Indeed, extraction-kit type caused more variability in the community composition than the sampling site (two-way ANOSIM: sampling kit: $r=0.95$, $p<0.001$, sites: not significant; PERMANOVA, 9999 permutations, sampling kit: $F=58.4$, $p<0.001$, sites: $F=5.9$, $p<0.001$, kit × sites: $F=2.8$, $p=0.01$).

Visualization by nmMDS distinctly separated the two extraction communities along the first two dimensions (Figure 5B). This dissimilarity suggests methodological influences on the output. Hence, the results are discussed separately. The 0.5-g extracts produced a comparatively tight cluster, whereas the 10-g output showed compositional differences between Gullmar Fjord and Havstens/Koljö Fjords. Additionally, the communities of the 0.5-g extracts did not show significant differences in diversity indices by study site, nor correlations to the majority of environmental variables of interest (Tables S5, S6). Due to this higher degree of site-specific community differentiation (Hellinger-transformed RA data; one-way PERMANOVA: $F=6.0$, $p<0.001$; vs. 0.5-g extractions: $F=2.3$, $p=0.02$), we retained only 10-g extracts for the interpretation of environmental trends. Further arguments for focusing on the 10-g extracts are discussed in the section 'Extraction-kit type impacts foraminiferal metabarcoding output'.

ASV1* (*Ammonia confertitesta*), ASV2* (*Nonionella* sp. T4), and ASV6* (*Elphidium oceanense* (S3)) were dominant and/or exclusive to 0.5-g samples across all fjords (Figure 6), driving the differences between extraction-kit outputs (Figure S5). While representing on average 86% of the 0.5-g assemblage, these taxa were rare or absent in 10-g extracts. Conversely, ASV3* (*Hippocrepinella acuta*), ASV5* (*Cibicidoides lobatulus* clade B), ASV7* (*Stainforthia fusiformis*) and ASV8* (unassigned monothalamid) were on average most abundant in the 10-g samples, although occurrences were not homogeneous across all fjords (Figure 6) and sampling occasions.

eDNA assemblage differentiation by fjords (10-g kit output)

Gullmar Fjord and Havstens/Koljö Fjords differed in taxon richness and Shannon Index (Mann–Whitney *U* test $p=0.02$ each), with the highest values recorded in GF sites (Table 2; data for 0.5-g kit output shown in Table S5). Evenness showed no site-specific differentiation. At each site, neither index showed temporal trends across subsequent sampling occasions. Nine of 56 abundant taxa

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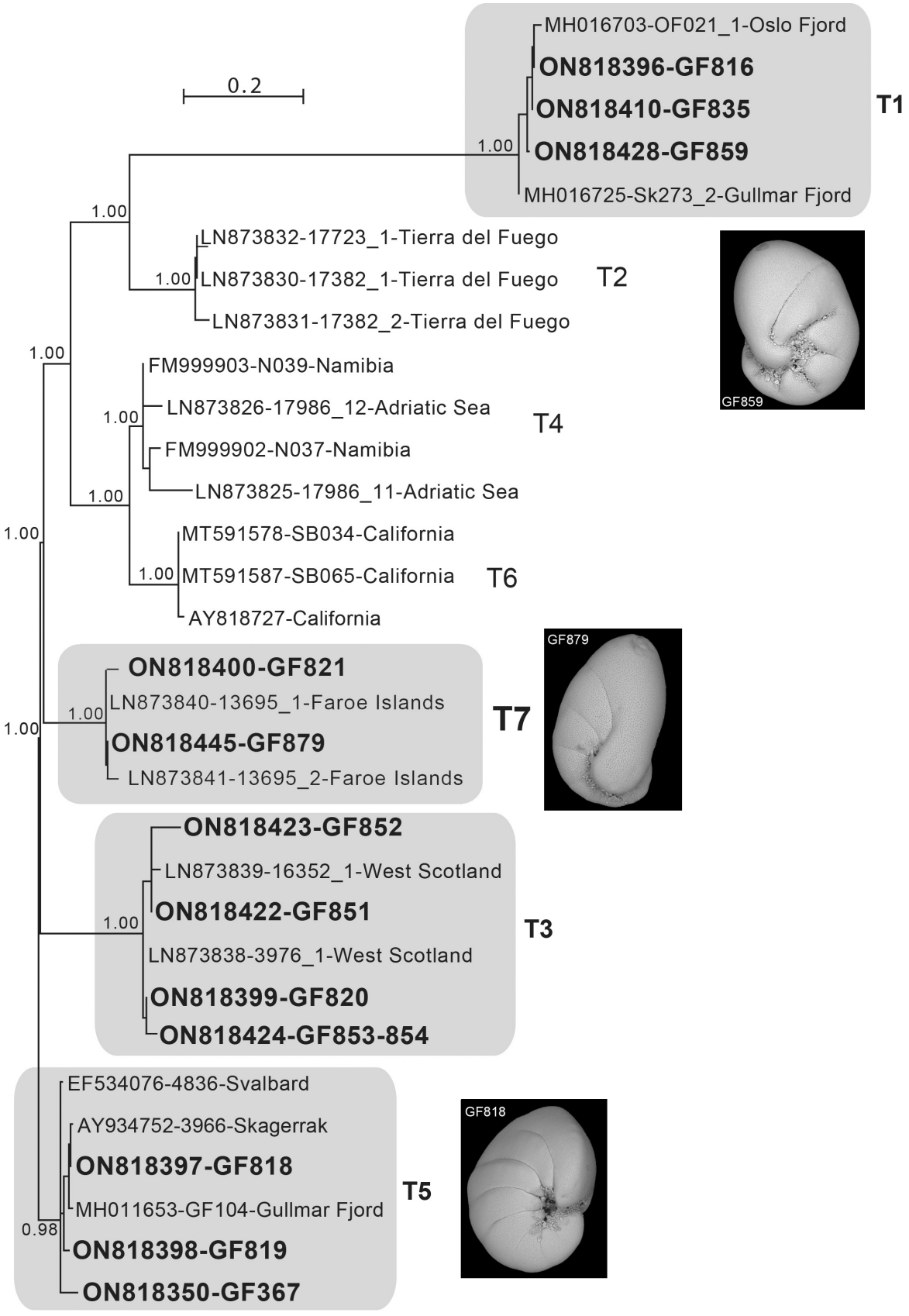

FIGURE 3 Phylogenetic tree of nonionellids based on partial SSU rDNA sequences inferred using Maximum Likelihood (ML) with the GTR+G model.

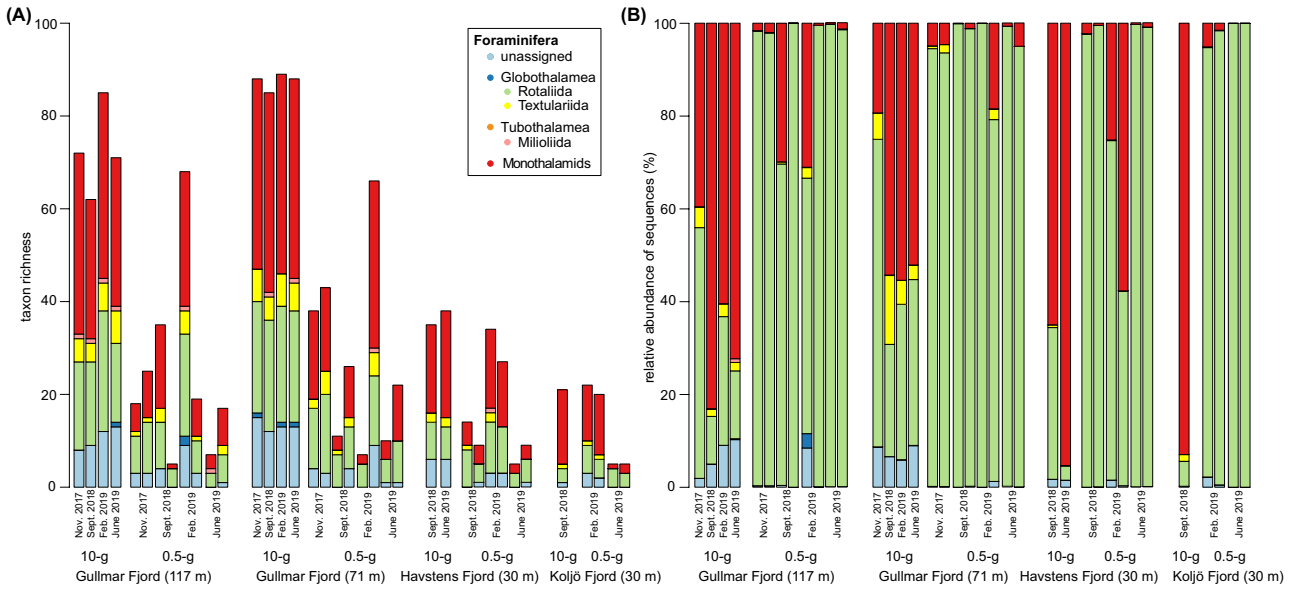

FIGURE 4 Class- and order-level taxonomic composition resolved per site, sampling occasion, and extraction-kit type. (A) Number of taxa. (B) Relative abundance of sequence reads.

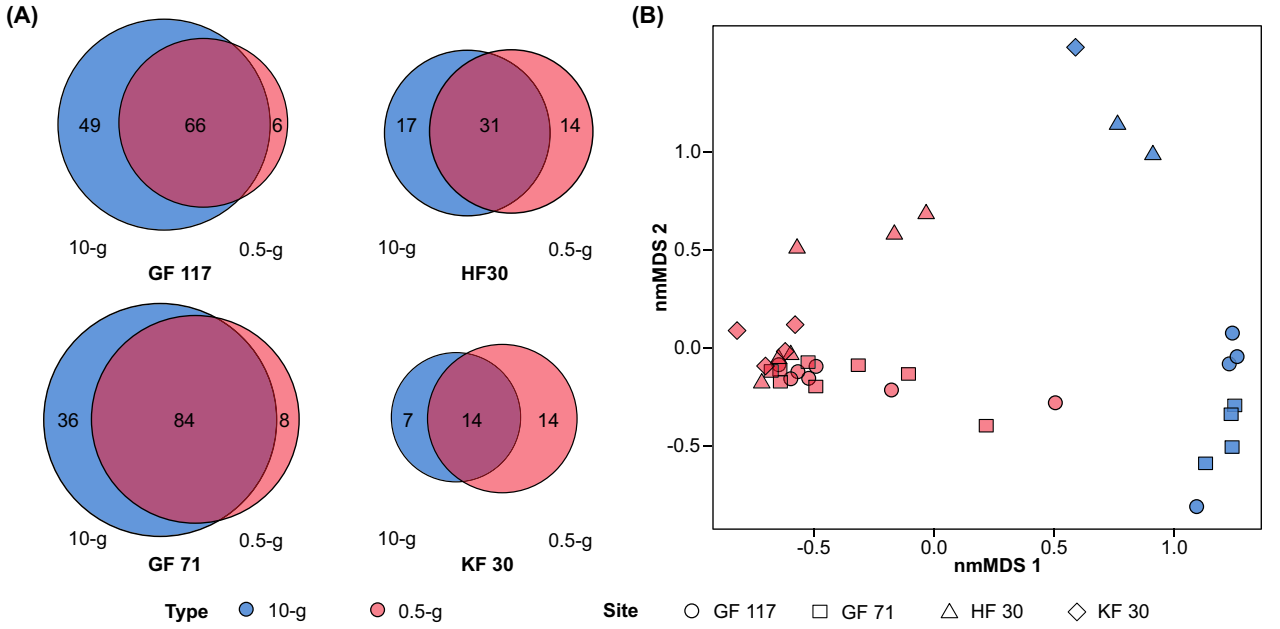

FIGURE 5 (A) Venn diagrams of 0.5-g (sum of B and C; red) and 10-g (A; blue) samples for each fjord site showing number of shared and unique taxa. B. nmMDS of Bray-Curtis dissimilarities matrix of Hellinger-transformed abundance data (with relative abundance > 1% in at least one sample). Stress = 0.079.

(> 1% average RA per fjord site) were shared between all sites, in all or some temporal replicates, explaining the assemblage differentiation by beta diversity. Seven of these shared taxa represent monothalamids. Further 11 taxa occurred in both GF and HF, of which ASV7* (*Stainforthia fusiformis*) was most abundant (on average 5%–15% RA). The Havstens and Koljö Fjords shared also ASV4* (unassigned monothalamid) and ASV8* (monothalamid HE998686). The two GF sites shared 32 abundant taxa during at least one sampling occasion, with ASV5* (*Cibicidoides lobatulus* clade B), ASV11* ('orange long pod' (EU213258)), ASV25* (unassigned saccaminid) and ASV29* (*Cylindrogullmia alba*) being, on average, most abundant. However, the dominance of ASV5* (*Cibicidoides lobatulus* clade B) was solely driven by its high abundance in November 2017 (25%–42% RA).

Environmental trends and assemblage responses (10-g kit output)

While sediment organic carbon content showed no correlation to the fjords' foraminiferal community, bottom-water salinity, sand-sized sediment fraction and PW

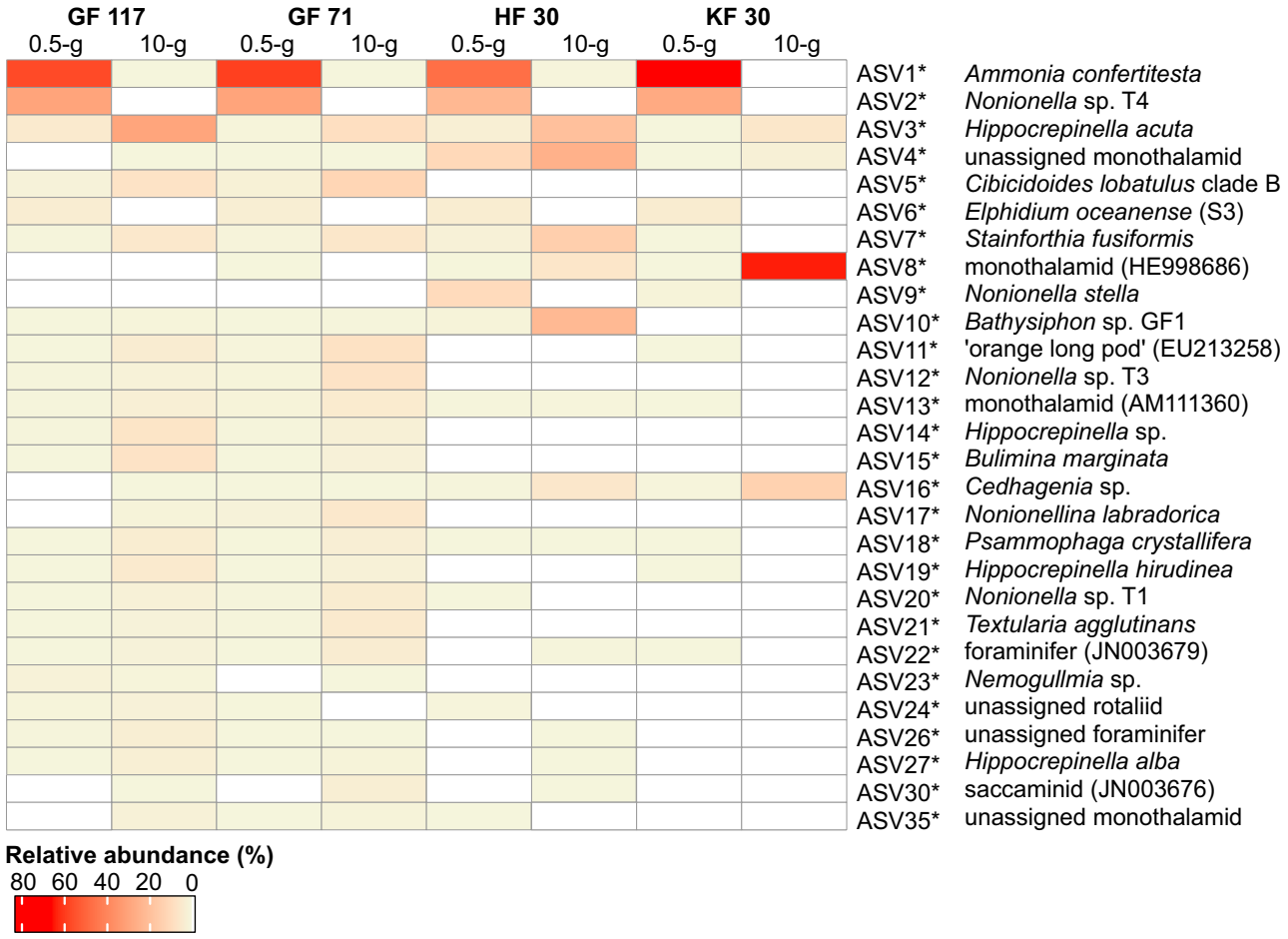

FIGURE 6 Heatmap of the most abundant taxa (>5% relative abundance in at least one sample) and their relative abundance by sampling site and extraction-kit type.

TABLE 2 Alpha diversity indices for 10-g kit outputs across sampling occasions (i.e., replicates).

Site (number of replicates)	ASV richness	Shannon index	Evenness
GF 117 (no=4)	97±10	2.99±0.43	0.18±0.02
GF 71 (no=4)	117±5	3.50±0.38	0.19±0.01
HF 30 (no=2)	45	1.95±0.10	0.20±0.01
KF 30 (no=1)	24	1.46	0.18

pH correlated positively (Mantel test: $r=0.77$, $p=0.002$; $r=0.65$, $p=0.005$; $r=0.54$, $p=0.016$, respectively). These four parameters co-vary between the three fjords and can be considered approximately constant across all sampling occasions within each fjord. Further, the same water depth was sampled consistently in each fjord site. As a factor, depth correlated weakly ($r<0.5$) with the assemblages. Additionally, several environmental parameters varied both between basins, as well as temporally on monthly to annual timescales (Figures S3, S4; SMHI): BW oxygenation, primary productivity (i.e., chlorophyll-*a*), and BW temperature. None of them correlated significantly with the eDNA assemblages.

Comparing individual ASV* read abundance with environmental factors, the same major division by water depth, BW salinity, PW pH and sand-sized sediment fraction was confirmed, as well as BW organic carbon content (hierarchical clustering of sparse Partial Least Squares [sPLS] regression; Figure 7). Based on contrasting correlations to these parameters, two main taxon clusters formed (Cluster A and B in Figure 7). No significant correlations were found for chlorophyll-*a*.

In cluster A, taxa correlated positively to the sites' sediment organic carbon content value, in particular, ASV54* (*Trochammina hadai*), ASV86* (*Psammophaga* sp.), ASV8* (monothalamid HE998686), ASV4* (unassigned monothalamid), ASV16* (*Cedhagenia* sp.) and ASV39* (*Cedhagenia saltata*). Relations were negative with sand-sized sediment fraction, BW salinity, water depth, as well as PW pH ($r<-0.5$). Cluster A comprised dominantly monothalamid taxa abundant in or exclusive to HF and/or KF.

Cluster B comprised taxa with correlations to environmental parameters in opposition to correlations of Cluster A taxa.: correlations were negative to organic carbon content and positive to sand-sized sediment fraction, BW salinity, and water depth. Cluster B taxa

were nearly exclusively present in the two GF sites. Hence, the differentiation by clusters A and B likely represents the contrasting environments of HF/KF vs. GF. Further, a pattern of taxa being either positively correlated to BW temperature solely or PW pH and BW oxygenation was resolved (i.e., subclusters B.1 and B.2; Figure 7). Cluster B.1 contained monothalamids, as well as ASV5* (*Cibicidoides lobatulus* clade B) and ASV15* (*Bulimina marginata*), with positive correlations to BW temperature ($r>0.5$, $p<0.05$; Figure 7). Considering GF samples alone, the same trend persisted for all but ASV19* (*Hippocrepinella hirudinea*; Figure S6). While only ASV28* (*Vellaria pellucida*) was negatively correlated to the fjords' BW oxygenation, the relationship was evident for all cluster B.1 taxa when considering only GF samples (Figure S6). ASV68* (*Micrometula hyalostriata*), ASV5* (*C. lobatulus* clade B) and ASV15* (*B. marginata*) correlated negatively with BW oxygen ($r<-0.5$). In Cluster B.2, 16 taxa correlated positively with solely PW pH or PW pH and BW oxygenation. However, the positive relationships to pH could only be confirmed as significant for ASV26* (unassigned foraminifera) within GF (Figure S6). In contrast, positive correlations with BW oxygenation persisted for ASV13* (unassigned monothalamid), ASV63* (*Psammosphaera* sp.), ASV55* (*Bolivina* sp.), ASV18* (*Psammophaga crystallifera*), and ASV31* (*Psammophaga* sp.).

DISCUSSION

Conformity of species' presence between single-cell, eDNA, and previous morphology-based data

Extensive reference databases built with high sequencing coverage, and linking sequences with image documentation of taxon morphology, aid reliable eDNA sequence identification and comparisons of assemblages on a global scale. Further, establishing a database of locally or regionally known species can allow distinguishing between active individuals vs. dormant specimens or such in resting stages, referred to as propagules (Alve & Goldstein, 2010; Goldstein & Alve, 2011), which is not possible with eDNA data alone.

Most recently, a morphology-based assemblage study observed 29 common mineralized taxa in Gullmar Fjord (GF 50 and GF 117, sampled in Nov. 2017; Choquel et al., 2021). Of these, eight have been previously sequenced (i.e., *Ammonia falsobeccarii* (Rouvillois, 1974), *Bolivina skagerrakensis* Qvale & Nigam, 1985, *Elphidium clavatum* Cushman, 1930, *E. selseyense* (Heron-Allen & Earland, 1911), *Globobulimina turgida* (Bailey, 1851), *Hyalinea balthica* (Schröter, 1783), *Leptohalysis scottii* (Chaster, 1892), *Liebusella goesi* Höglund, 1947; in Darling et al., 2016; Holzmann et al., 2018; Schweizer et al., 2008, 2009, 2011); 12 more species were barcoded

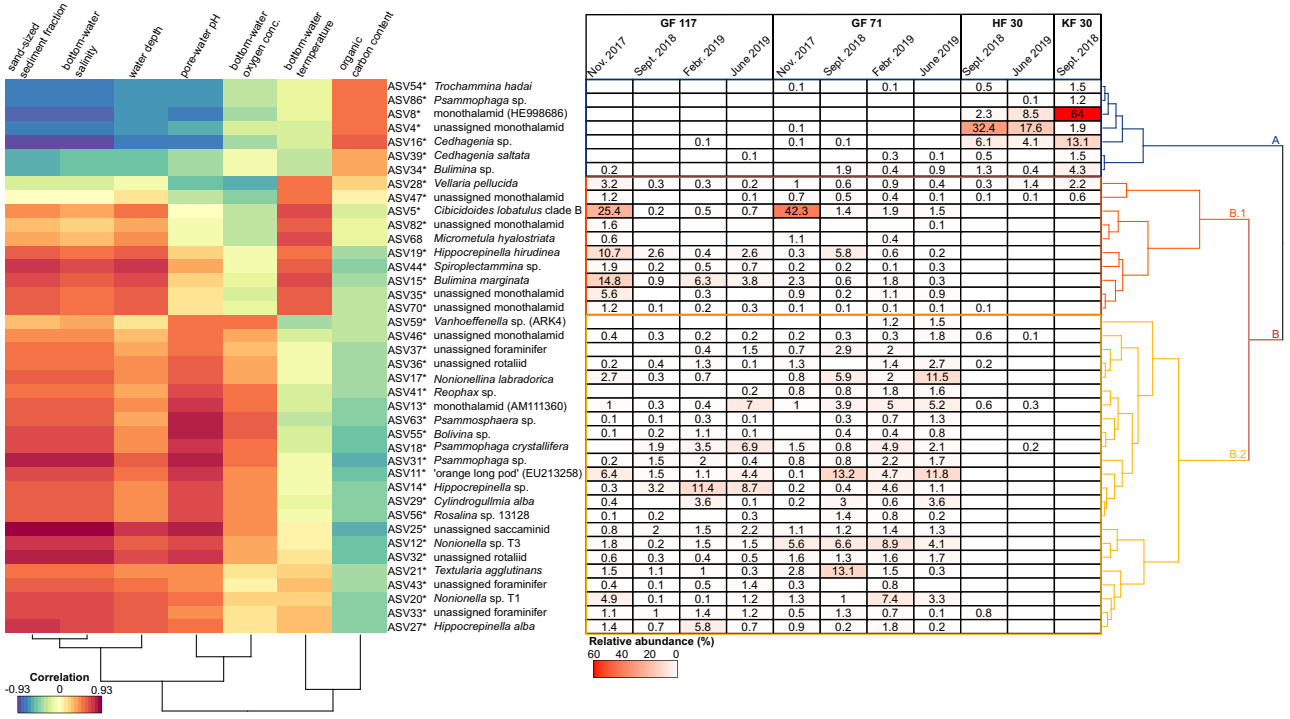

FIGURE 7 Clustered Image Map (CIM) of the first two sparse Partial Least Squares (sPLS) dimensions showing pairwise correlations between taxa and environmental parameters. Red colors in CIM heatmap depict positive correlations and blue colors negative correlations, with darkness indicating correlation strength. The relative abundance (in %) for each taxon is given per sampling site and occasion (relative abundance ≥0.1% indicated by numbers).

in our study. Despite the high sequence coverage of species known from the fjords, only nine of 22 mineralized morphospecies were identified by eDNA in GF 117 (this study; Choquel et al., 2021); the same trend was true for Havstens and Koljö Fjords (compare Gustafsson & Nordberg, 1999, 2000). Some variances in the presence of species can be expected, as sampling efforts may not reveal the total diversity of any given site (Bouchet et al., 2012 for morphological approaches; review by Pawlowski et al., 2022 for metabarcoding approaches). Still, in GF, the abundance of ASV*s identified as *Bulimina marginata*, *Stainforthia fusiformis*, *Nonionella* sp. T1, *Nonionella* sp. T3 and T7 (both morphologically recognized as *N. turgida*), and *Nonionellina labradorica* agrees with morphological observations of these species (Choquel et al., 2021; Gustafsson & Nordberg, 2001). In contrast, some common fjord species have not been identified by eDNA, such as *Quinqueloculina seminulum* and *Cassidulina laevigata*. Because these species were single-cell sequenced in this or previous studies, insufficient reference databases and/or discrepancies between molecular- and morphological species concepts (e.g., review by Pawlowski, Lejzerowicz, & Esling, 2014) did not hinder the taxonomic identification of specific ASVs and detection of species in these cases.

Based on comparisons between single-cell sequences, metabarcoding output, and reported morphospecies in the fjords (Choquel et al., 2021; Gustafsson & Nordberg, 1999, 2000, 2001), three dominant taxa in our eDNA data set could potentially be present as propagules in the sediment: *Ammonia confertitesta* (T6), *Nonionella* sp. T4 and *Elphidium oceanense* (S3). Previous morphology-based observations of the fjords' foraminiferal communities have been limited to specimens >63 µm size, hence, overlooking smaller specimens and propagules (typically <32 µm). Propagules are easily dispersed (Alve & Goldstein, 2002, 2010) and, thus, detection of atypical or unrecognized species may represent false positives with respect to the active "adult" community. *Ammonia confertitesta* was identified morphologically in HF and the mudflat of GF, but not in GF 117 (based on Choquel et al., 2021). Although common in intertidal flats (Saad & Wade, 2016), this species also occurs in subtidal environments of low salinities such as the Baltic Sea (Bird et al., 2020). In eDNA, it was the most abundant ASV, representing 42% of the total read number. According to its ecology, we, therefore, infer that *A. confertitesta* sequences in subtidal (i.e., fully marine) sites of GF are restricted to propagules. Similarly, three nonionellid morphospecies common in GF were retrieved as single cells, *Nonionella* sp. T1, *Nonionellina labradorica* (T5) and *Nonionella turgida* (T3 and T7; Figure 3), and also present in the eDNA data. In contrast, the most abundant nonionellid in the metabarcoding data was an ASV identical to T4 (i.e., ASV2*, 19% of total number of reads). *Nonionella* sp. T4 is known from off Namibia (Grimm et al., unpublished) and the Adriatic Sea (Holzmann & Pawlowski, 2017), but has not been recognized morphologically in GF, indicating it may not be present there in adult life stages. Alternatively, it may represent a cryptic species not recognizable by morphological criteria. We infer the same for the less abundant ASV9* (2% of total number of reads), which is identical to *Nonionella stella* (T6) from off California (Brinkmann et al., 2021), but not identified morphologically in GF (Deldicq et al., 2019). Lastly, *E. oceanense* (ASV6*) may also represent a propagule signal. This species has been identified morphologically in the Kattegat (Darling et al., 2016) and in the mudflat of the Gullmar Fjord (M. Schweizer, personal observation), but not in the fjord's depth.

Monothalamids, in contrast, are not routinely examined in morphology-based studies, and therefore comparisons are limited. A broad spectrum of monothalamids was obtained via molecular methods, and six of seven barcoded morphospecies were also identified by eDNA: *Astrorhiza limicola* (ASV61*), *Hippocrepinella alba* (ASV27*), *Hippocrepinella hirudinea* (ASV14*), *Micrometula hyalostriata* (ASV68*), *Psammophaga crystallifera* (ASV18*) and the unidentified monothalamid 'silver monothalamid' (ASV35*). *Bathysiphon flexilis* was not recognized by eDNA, but ASV10* is identical to the newly sequenced *Bathysiphon* sp. GF1. The genera *Hippocrepinella* (Höglund, 1947), *Cylindrogullmia* (Nyholm, 1974) and *Micrometula* (Nyholm, 1952), recorded by eDNA, are characteristic monothalamids in Gullmar Fjord.

Extraction-kit type impacts foraminiferal metabarcoding output

To assess the performance of metabarcoding asproxy of foraminiferal communities, potential influences of extraction methods on the resolution of sedimentary eDNA signals must be considered. Indeed, the extraction methods used—DNeasy PowerMax Soil Kit (Qiagen) requiring 10 g of sediment and NucleoSpin soil minikit requiring 0.5 g of sediment—introduced biases affecting both perceived eDNA diversity and taxonomic composition. Despite a large overlap of present taxa (up to 68%, site-wide), extraction-kit type was the largest cause of differences observed in the foraminiferal eDNA assemblages and relative taxon abundances, demonstrating the importance of deliberate DNA extraction procedure choice. This methodological bias partially overwhelmed the expected natural variation of the fjord environments in the 0.5-g output. In contrast, 10-g extracts exhibited clear grouping by fjords (Gullmar Fjord and Havstens/Koljö Fjords), indicating strong local eDNA signals with potential for environmental inferences.

A previous study of benthic eukaryotic diversity of abyssal sediments suggested more diversity being captured if DNA is extracted from larger amounts of

sediment (2-g RNeasy PowerSoil Kit vs. 10-g PowerMax Kit; Brandt et al., 2020). At least 10 g of sediment might be necessary for the sample's DNA content to be representative of an environment's microbial community, including microeukaryotes such as foraminifera. Indeed, our 0.5-g extracts showed heterogeneity even between pseudo-replicates. This is conceivable, particularly if only entire specimens contribute to the foraminiferal DNA pool resolved by metabarcoding, as opposed to DNA extruded by living cells (extracellular DNA). While currently it is not known if extracellular foraminiferal DNA can be captured by metabarcoding, it has been hypothesized that intracellular DNA constitutes the majority of total foraminiferal DNA extracted from sediments (Siano et al., 2021). The underperformance by the 0.5-g sample analyses may be resolvable with the collection of several (pseudo-) replicate samples (i.e. >2), or subsampling homogenized samples of larger volume (Hestetun, Lanzén, & Dahlgren, 2021; Hestetun, Lanzén, Skaar, & Dahlgren, 2021; van der Loos & Nijland, 2021). Currently, there is no consensus regarding the appropriate level of replication for assessment of total diversity, especially when considering effort/cost vs. benefit (e.g., review by Pawlowski et al., 2022).

While the extraction kits' sample volume conceivably explains biodiversity fidelity or lack thereof, it cannot explain the systematic differences in composition. We propose that the 0.5-g kit, specifically, extracts DNA derived from propagules, as indicated by the abundance of *Ammonia confertitesta* (T6), *Nonionella* sp. T4 and *Elphidium oceanense* (S3; see Conformity of species' presence between single-cell, eDNA, and previous morphology-based data section). These species were dominant and/or exclusive to 0.5-g outputs only, while neither of these three taxa is known from morpho-assemblages of the four sites (Choquel et al., 2021; Gustafsson & Nordberg, 1999, 2000, 2001). The influence of propagules, potentially dispersed from shallower sites of the fjord, neighboring fjord systems, or the open sea through water exchange connections, can also explain the homogeneity across sampling sites and dates in the 0.5-g output (Figure 5B). Conversely, 10-g extracts resolving propagule DNA less efficiently may be advantageous for environmental assessments based on active "adult" community compositions, because only organisms viable under the ambient conditions are expected to grow beyond the propagule stage (Alve & Goldstein, 2010; Goldstein & Alve, 2011).

Both tested extraction kits use a physical approach (bead beating), in addition to chemical methods, to release cell material from tests, considered most effective for the recovery of microbial eDNA from sediment and soil samples (Carrigg et al., 2007). While bead beating for both types of extracts was achieved by vortexing, there was a difference in handling: Tubes of 0.5-g extracts were mounted in a rubber-foam adapter, whereas 10-g sample tubes were hand-held, resulting in higher and lower intensities, respectively. Bead beating intensity, as well as varying bead sizes and/or materials, may play a role in lysis efficiency (compare Hestetun, Lanzén, Skaar, & Dahlgren, 2021). Specifically, we propose that smaller beads and/or higher bead beating intensity during the 0.5-g extraction aids more effective cell lysis of small specimens such as propagules and should therefore be avoided in community composition studies for environmental assessments. A standardized protocol defining the use of a specific extraction method—sample volume, extraction material, and homogenization techniques—will aid comparability between foraminiferal eDNA metabarcoding studies (see also review by Pawlowski et al., 2022).

Benthic foraminiferal eDNA communities as ecosystem indicators (10-g extracts)

Convergence of eDNA communities with morphological data in the context of environmental drivers

A pre-requisite for evaluating foraminiferal eDNA metabarcoding as environmental assessment tool in biomonitoring applications is eDNA diversity and taxonomic composition changing with environmental conditions. Secondly, such changes (i.e., positive or negative correlations) must be consistent with established, morphology-based indices, for metabarcoding data to be environmentally informative at a rate comparable to morphology-based approaches.

Molecular and morphological assemblages of benthic foraminifera most commonly agree in terms of diversity trends, rather than species composition (Frontalini et al., 2020). In foraminiferal morphospecies' assemblages, decreasing diversity is commonly associated with naturally- or pollution-stressed conditions (Alve, 1995; Murray, 2006). The same was found for eDNA and eRNA diversity (i.e., taxonomic richness, Shannon diversity) comparing anthropogenically stressed environments to unstressed sites (e.g., fish-farming in Pawlowski, Esling, et al., 2014; Pochon et al., 2015; gas and oil exploitation in Laroche et al., 2016; Laroche et al., 2018; Cordier, Frontalini, et al., 2019; Frontalini et al., 2020; industry in Cavaliere et al., 2021). Similarly, our foraminiferal eDNA diversity was comparable to previous morphology-based studies, particularly in taxon richness of mineralized species (Choquel et al., 2021; Gustafsson & Nordberg, 1999, 2000, 2001), but not in terms of species' presence. The eDNA diversity decreased from Gullmar Fjord to Havstens Fjord and Koljö Fjord, and sites were differentiated clearly by alpha and beta diversities (Table 2; Figure 5B). The clear differentiation between GF and HF/KF confirms that also foraminifera that are traditionally overlooked in morphology-based studies (e.g., monothalamids) respond to environmental variability

as is known from customarily studied mineralized taxa (Cordier et al., 2017). The probable driver was BW salinity, differing by several units between the three fjords (28–34.5, i.e., brackish to fully marine; Table 3), and, in association, PW pH (Saraswat et al., 2015). Salinity and pH affect the calcium carbonate saturation state of seawater (Zeebe & Wolf-Gladrow, 2001), and can impair calcifying foraminifera (Berkeley et al., 2007). Indeed, in KF, foraminifera are frequently observed with dissolved tests, and few occurrences are known from the shallower, less-saline sites of GF (Brinkmann et al., unpublished data; Gustafsson & Nordberg, 1999). Despite significant correlation, we exclude sediment-size fraction playing an important role for our assemblages, as the percentage of sand-sized sediment fraction was arguably low at all sites (≤1%; Gustafsson & Nordberg, 1999, 2000, 2001). Data on the percentage contribution of finer sediment fractions such as silt or clay are not available.

Class-level compositions of our eDNA communities reflected salinity stress, with Globothalamea—considered mainly marine—being most diverse in samples from fully marine conditions. In GF (i.e., normal marine conditions) our taxa included typical marine species, such as *Stainforthia fusiformis*, *Bulimina marginata*, and *Cibicidoides lobatulus*. These were absent in KF and partly in HF, where typical brackish (below euhaline, i.e., <30; Anon., 1959) taxa, such as *Trochammina hadai* (Eichler et al., 2018; McGann, 2014), *Vellaria pellucida* and *Cedhagenia* sp. (Gooday et al., 2011), were common. Thus, abundant taxa of our eDNA communities represent assemblage components conceivably active at the studied sites, as opposed to DNA deriving from resting (inactive) cells or such transported to the studied sites (i.e., allochthonous).

Effects of short-term environmental variability on foraminiferal eDNA assemblages

The studied fjords experience strong seasonal environmental variations, of which primary productivity and BW oxygenation particularly affect the foraminiferal communities (Gustafsson & Nordberg, 1999, 2000, 2001). Phytoplankton blooms occur typically bi-annually in spring (diatoms) and summer/autumn (dinoflagellates; Gustafsson & Nordberg, 2000, 2001; Harland et al., 2004). Phytodetritus deposition on the fjords seafloor triggers foraminifera reproductive events, with species-specific timing (Gustafsson & Nordberg, 2001). Further community changes relate to the BW oxygenation cycle and thus, water exchanges (Filipsson & Nordberg, 2004; Nordberg et al., 2000; Polovodova Asteman & Nordberg, 2013). Water renewals occur approximately annually in Gullmar Fjord and less often in Havstens/Koljö Fjords, resulting in a wide gradient and cyclicity of BW dissolved oxygen concentrations. Episodes of BW oxygen deficiency can last for months to years in HF and KF but are less severe, less frequent, and of shorter duration in GF (SMHI).

In our eDNA community, responses to temporally variable environmental parameters (i.e., BW temperature, oxygenation, PW pH, and chlorophyll-*a*) were minor, particularly when compared across the three fjords (Figure 7). Indeed, in the studied fjords, seasonal variations in foraminiferal morpho-assemblages mainly affect changes in abundance, as opposed to presence/absence. Similarly, in a morphology-based study of the saltwater Lake Grevelingen, Netherlands, extirpation of foraminiferal populations was only observed after 1–2 months of surface-sediment anoxia whereas shorter low-oxygen periods only affected abundances (Richirt et al., 2020). The validity of sequence abundance in eDNA metabarcoding reflecting species abundance is under active discussion (Frontalini et al., 2020; Weber & Pawlowski, 2013), although some studies showed congruence for dominant morpho- and molecular assemblage components (Cavaliere et al., 2021; Pawlowski, Esling, et al., 2014). Factors postulated to affect read abundance include biological biases, such as specimen biovolume, gene copy number, or divergence in primer regions, as well as amplification or sequencing artifacts (André et al., 2014; Elbrecht et al., 2017; Pawlowski, Esling, et al., 2014; Weber & Pawlowski, 2013). Indirectly, intragenomic polymorphisms within a species (Borrelli

TABLE 3 Hydrographic and environmental conditions in the three fjords.

Fjord	Data points	Water depth (m)	BW salinity	Sand-sized sediment fraction (%)[a]	Organic carbon content (%)[a]	BW temperature (°C)	BW oxygenation (μmol/L)	PW pH (top 1 cm)	Chlorophyll-*a*[b]
GF	4	117	~34.5	~1	~3	6.6–7.6	9–217	7.5–7.6	0.6–1.6
	4	71	~34.5			6.6–7.8	54–211	7.4–7.6	0.7–5.0
HF	2	30	~32	0.02	~3	6.5–6.9	10–176	7.1–7.3	0.8–2.9
KF	1	30	~28	0.20	~7	6.7	1	7.3	0.7

Note: If not stated otherwise, the data covers four data points from sampling occasions in Gullmar Fjord (14.11.2017, 05.09.2018, 26.02.2019, and 11.06.2019), two in Havstens Fjord (06.09.2018, 12.06.2019) and one in Koljö Fjord (06.09.2019). Pore-water pH was only measured during the 2018–2019 sampling occasions.

Abbreviations: BW, Bottom-water; PW, pore-water.

[a]Data deriving from literature (Filipsson & Nordberg, 2004; Gustafsson & Nordberg, 1999, 2000, 2001).

[b]Data deriving from monitoring sites (SMHI).

et al., 2018; Pillet et al., 2012), can affect its "molecular abundance" if variants are recognized as distinct ASVs (Weber & Pawlowski, 2014), and incomplete reference data prevents pooling such ASVs. Due to the semi-quantitative nature of eDNA abundance data, the metabarcoding approach may lack the sensitivity necessary to detect environmental shifts that modify foraminiferal communities in terms of RA. In contrast, our study design of sampling three to four time points may not have allowed the capture of seasonal dynamics and/or recognition of associated responses; several replicate samples may be necessary to represent local diversity and abundance in their entirety (shown e.g., for foraminifera by Lejzerowicz et al., 2014; for other protists by Lanzén et al., 2017).

Furthermore, the contribution of subfossil DNA (i.e., ancient DNA; Willerslev & Cooper, 2005) to the DNA pool can blur shorter-term environment-driven trends, as both current and recent biodiversity are captured by metabarcoding (Torti et al., 2018). The preservation potential of eDNA is driven by ambient environmental factors, including oxygen availability, organic matter content, temperature, sedimentation rate, and sediment composition (Corinaldesi et al., 2018; Lorenz & Wackernagel, 1987, 1994; Nielsen et al., 2007; Romanowski et al., 1992). Of these, particularly the presumably anoxic sediment conditions during hypoxia in BWs (i.e., $<63\,\mu mol/L$ $[O_2]$) in November 2017 in GF, as well as in KF, may have favored DNA preservation potential (Corinaldesi et al., 2007, 2008; Mejbel et al., 2022). While foraminiferal eRNA-based metabarcoding was shown to outperform eDNA in detecting environmental changes (Greco et al., 2022; Pawlowski, Esling, et al., 2014; Pochon et al., 2015), particularly with regard to species' diversity and abundance (Greco et al., 2022; Laroche et al., 2017), RNA also has preservation potential under anoxic conditions (Orsi et al., 2013). Even DNA preservation over weeks to months may significantly distort short-term diversity and/or abundance trends and increase spatial and temporal homogeneity. Marine sediments reportedly act as long-term repositories of foraminiferal DNA over millennial time scales (Lejzerowicz et al., 2013; Pawłowska et al., 2014, 2020), although in surface sediments the signal of living specimens appears to be stronger than of allochthonous or ancient DNA (Barrenechea Angeles et al., 2020), at least in some environments. Thus, our filtering of rare sequences with counts of <100 reads may have addressed the issue of ancient and extracellular DNA contributions. Finally, environmental DNA—in form of the propagules, grown specimens, and/or extracellular DNA—can be transported over large distances by currents (Goldberg et al., 2016). Particularly the fjords' deep basins may be a sink for dispersed material, which could distort signals of the living community. A study comparing shallower regions to deeper sites, and considering water circulation patterns and residence times, may elucidate dispersal mechanisms within the fjords.

While eDNA-based assemblage analyses are suitable for discerning contrasting environments, analytical techniques currently may not be sufficiently sensitive to reveal short-term environmental variability by community-level changes; hence, the use of eDNA-based biodiversity indices for evaluating short-term single- and multi-stressor impacts should be used with due caution. Seasonal studies with higher temporal resolution are necessary to address the potential of eDNA metabarcoding for assessing environmental conditions on seasonal scales.

Proxy potential of foraminiferal taxa detected by metabarcoding

For biomonitoring, an application's sensitivity to subtle and/or short-term environmental changes is crucial. We could not confirm this sensitivity on the foraminiferal community level. Individual taxa, on the other hand, did show strong correlations to environmental factors with shorter-term variability (i.e., sub-annual; Figure 7), specifically BW oxygen concentrations. These correlations were investigated solely for GF to exclude potential interactive influences of multiple environmental stressors, in particular the prominent BW salinity gradient across our sites.

Nevertheless, the correlation to BW oxygenation with the given foraminifera taxa could not be confirmed by known ecology of these particular species:

For instance, *Psammosphaera* spp. reportedly survive anoxic treatments in culturing studies (Bernhard, 1993), and certain representatives of the genus *Bolivina* increase in RAs under oxygen-depletion (Bernhard et al., 1997; Bernhard & Bowser, 1999), opposing the here observed positive correlation to BW oxygenation. Conversely, *Micrometula hyalostriata* is generally observed occurring in oxygenated conditions (Alve, 2010; Bouchet et al., 2018; Fossile et al., 2021), whereas our data suggested proxy potential for low-oxygen conditions. Generally, cibicidids are considered as oxic indicators (Kaiho, 1994), although recent observations found high abundances of a cibicidid species at below $45\,\mu mol/L$ $[O_2]$ (i.e., *Cibicidoides wuellerstorfi*; Rathburn et al., 2018). Similarly, *Cibicidoides lobatulus* sequences are dominant in BW hypoxia in our data (i.e., Gullmar Fjord samples November 2017). Also, previous observations are contradictory for *Vellaria pellucida*. In coastal areas of the Black Sea abundance maxima were correlated to higher oxygen availability (i.e., deeper sulfidic layer and redoxcline; Sergeeva et al., 2015), although the same study also reported high abundances of *Vellaria* spp. in sediments underlying severely hypoxic and sulphidic BWs at greater depths (150–230 m). In an eDNA-based study, Pochon et al. (2017) identified *V. pellucida* as a proxy for high organic enrichment sites, while our study

shows it correlated negatively with BW oxygenation but positively with BW temperature.

Conversely, known opportunistic morphospecies in environments of oxygen depletion, such as *Stainforthia fusiformis* and *Nonionella* spp. (Alve, 1990; Alve & Bernhard, 1995; Bernhard et al., 1997; Cedhagen, 1991; Fontanier et al., 2014; Leiter & Altenbach, 2010), did not correlate significantly in our eDNA data with environmental parameters. We infer that generalizations of potential proxy relationships in our study should be evaluated critically, as the ecological functioning of identified taxa to implied environmental correlations could not be confirmed.

CONCLUSIONS

Our study demonstrates the usefulness of eDNA metabarcoding in surveying community differentiation between ecosystems of certain contrasting environmental conditions. Nevertheless, several aspects of eDNA metabarcoding require further analyses before standardized protocols can be implemented for routine applications. Such analyses include dedicated comparisons of technical as well as biological replicates with regard to diversity, and the relation to in-situ foraminiferal morpho-communities. This may allow to elucidate the function of extraction-kit type and targeted foraminiferal groups. We found that metabarcoding data of 10-g extractions were suitable for discerning between contrasting environments, with recognized foraminiferal eDNA assemblages specific to each fjord environment. The trends in diversity from our study corresponded well with results from morpho-assemblage studies despite the predominance of different taxa in the eDNA assemblage taxa. However, insufficient sensitivity restricted the reliable identification of short-term environmental perturbations in our study region. Both species' coverage and sensitivity to environmental variability may be improved by optimizing sample replication. If methods are further developed to address current biases and limitations, implementation of eDNA metabarcoding in existing monitoring programs could be a valuable option to complement traditional approaches.

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