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Metagenomic sequencing of environmental DNA reveals marine faunal assemblages from the West Antarctic Peninsula

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ABSTRACT

The West Antarctic Peninsula (WAP) is the fastest warming region in Antarctica where climate impact on the cold-adapted marine ecosystem is already visible. To monitor faunal changes in remote vast bodies of Antarctic waters, efficient and informative tools are essential. High-throughput sequencing of environmental DNA (eDNA) has emerged as one such tool for monitoring biodiversity and ecosystems, as it increases detection sensitivity of taxa, and sampling is often simpler and less costly than traditional collection methods. We collected water samples from four WAP shallow (≤ 300 m) shelf regions, recovered the eDNA therein, and performed metagenomic shotgun sequencing and analyses to determine the effectiveness of this method to assess marine benthic faunal diversity; this includes the detection of deep-water predatory king crabs whose potential shoreward expansion to warming shelves has sparked much concern. Using a customized bioinformatics pipeline, we identified abundant signatures of common benthic invertebrate fauna, endemic notothenioid fishes, as well as lithodid king crabs. We also uncovered species richness and diversity comparable to biological inventories compiled by the use of traditional survey methods, supporting the efficacy of the eDNA shotgun sequencing approach. As the rate of eDNA degradation affects faunal detection sensitivity, we also quantified mitochondrial ND2 gene copies in eDNA derived from a WAP icefish and found *ND2* copies persisted to at least 20 days in the cold WAP water, much longer than values reported for temperate environments. We propose that eDNA metagenomic sequencing complements traditional sampling, and combining both will enable more inclusive biodiversity detection and faunal change monitoring in the vast Southern Ocean.

1. Introduction

The Southern Ocean hosts a unique biota as a result of Antarctica's distinctive climatic and geologic history (Clarke and Crame, 1989; Clarke and Johnston, 2003). The marine fauna that had evolved in the cold stable Antarctic environment over millions of years now face climate warming trends that have broad influence on community composition and overall ecosystem functioning (Clarke et al., 2007; Schofield et al., 2010). Organisms can respond and adapt to slow increases in environmental temperatures, while rapid changes are likely to cause physiological stress, driving migrations, extinctions and invasions by non-native taxa (Frenot et al., 2005; Cheung et al., 2009; Trivelpiece et al., 2011; Peck et al., 2014). The effects of increasing temperatures are most pronounced at the West Antarctic Peninsula (WAP) and its adjacent waters, which are experiencing the fastest rates of warming in Antarctica (Vaughan et al., 2003; Meredith and King, 2005; Mulvaney et al., 2012). To understand the full impact of rapidly shifting climate on the WAP ecosystem, efficient and effective tools are required to record spatial and temporal variations in biological communities.

1.1. Benthic marine animal communities of the Southern Ocean

Contemporary Antarctic marine macrofaunal communities have intermediate levels of benthic diversity that are comparable to temperate and tropical "non-reef" habitats (Clarke, 2008). Similar to these warmer waters, the West Antarctic continental shelf is populated by a variety of macro-invertebrates that include porifera (sponges), cnidarians (hydroids, stylasterids and anthozoans), bryozoans, annelids (polychaetes), echinoderms (asteroids, ophiuroids and echinoids), arthropods (amphipods, isopods and pycnogonids), mollusks (gastropods and bivalves) and tunicates (*see*De Broyer and Koubbi, 2014 and references therein). The Southern Ocean has also been a remarkable "evolutionary hotspot" that witnessed the adaptive radiation of the morphologically and ecologically diverse Antarctic notothenioid fishes (Eastman, 2005). Notothenioids constitute a major component of the

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benthos; in the coastal waters of the WAP, many species of the red-blooded family Nototheniidae and the white-blooded Channichthyidae are frequently found occupying shallow to deep-water habitats (600 to ≥ 1000 m) (DeWitt et al., 1990; Iwami and Kock, 1990; Kock, 1992).

In contrast, durophagous "bone-crushing" predators including sharks, rays, benthic reptant (walking) crabs and lobsters are absent or rare south of the Polar Front (Dayton et al., 1994; Aronson et al., 2007a; Aronson et al., 2015a). Durophagous crabs were previously thought to have disappeared with the late Eocene cooling and completely excluded from Antarctic waters for at least 14 my (Aronson et al., 2007b). Durophagous brachyurans (true crabs) are indeed absent in Antarctic waters (Hall and Thatje, 2011; Griffiths et al., 2013); their exclusion was attributed to their inability in hypo-regulating hemolymph Mg^{2+} levels at subzero temperatures, limiting crucial muscular functions including heartbeat, ventilation and locomotion (Frederich et al., 2001; Thatje and Arntz, 2004; Thatje et al., 2005). Anomuran crabs of the family Lithodidae (king crabs) however, proved not to be Mg^{2+} limited (Wittmann et al., 2012) and various lithodid populations were discovered south of the Polar Front in the recent decades, although still limited to deep, salty (> 34.6 psu), non-freezing (> 0.5 °C) habitats of the Upper Circumpolar Deep Water (UCDW) (Klinck et al., 2004; Dinniman et al., 2011; Griffiths et al., 2013; Griffiths et al., 2014). The most recent lithodid populations have been observed within the UCDW, at Palmer Deep basin (Smith et al., 2012) and Marguerite Bay (Aronson et al., 2015b). Although the non-freezing UCDW periodically intrudes onto surface waters (Smith et al., 1999; Prézelin et al., 2000; Bentley et al., 2011), no lithodid crabs have yet been observed on shallow shelves of the WAP, or of the Weddell and Ross Seas where temperatures remain consistently colder than 0 °C (Hall and Thatje, 2011). Regardless of the current debate on the historical origin of Antarctic lithodids (Griffiths et al., 2013), there is a common concern that rising shallow water temperatures could enable the predatory king crabs to expand from their current bathyal niche onto shelf ecosystems and alter benthic community structures (Turner et al., 2014a). Efficient and informative tools for tracking their movement over time and space are essential to allow evaluation of the risk of lithodid range expansion.

1.2. Environmental DNA as an ecosystem monitoring tool

Traditional biomonitoring methods typically rely on the observation and collection of whole organisms, combined with morphology-based identifications. Major challenges that traditional methods face include logistic costs of collection, as well as difficulties in reaching remote environments, which would bias sample inventories towards organisms and locations that are easier to access (Baird and Hajibabaei, 2012; Valentini et al., 2016). Additionally, rare or elusive organisms may go unobserved using traditional sampling (Jerde et al., 2011; Rees et al., 2014). The obvious difficulties of monitoring marine biodiversity within large swaths of oceanic provinces calls for more efficient approaches to survey aquatic communities. The sequencing of environmental DNA (eDNA) – genetic material extracted from cells and tissue fragments shed by organisms into the environment (Thomsen and Willerslev, 2015; Taberlet et al., 2012a) – has emerged as a useful tool for assessing biodiversity. Compared to traditional surveys, eDNA has increased detection sensitivity of organisms, as animal remains or early life stages too small to identify by eye can be detected at the DNA sequence level (Bohmann et al., 2014). eDNA has been widely employed in aquatic ecosystems to assess overall community composition (Thomsen et al., 2012a,b; Kelly et al., 2014; Leray and Knowlton, 2015) as well as to identify rare, endangered or invasive species (Jerde et al., 2011; Thomsen et al., 2012a; Goldberg et al., 2013; Dougherty et al., 2016; Larson et al., 2017).

A common approach for eDNA analyses is metabarcoding, which uses one or more barcoding genes to elucidate the taxonomic composition of complex eDNA samples (Hebert et al., 2003; Taberlet et al., 2012b; Valentini et al., 2016). Metabarcoding relies on PCR amplification of gene fragments with inherent uncertainties of whether all taxa within the complex sample could be evenly amplified using a given primer set or sets, which would lead to gene-specific taxonomic biases and skewed biodiversity assessments (Coissac et al., 2012; Zhou et al., 2013; Cowart et al., 2015; Pedersen et al., 2015). In contrast, shotgun metagenomic sequencing that involves the direct sequencing of total eDNA could bypass the PCR limitations associated with metabarcoding while still providing insights into community composition (Tringe and Rubin, 2005; Taberlet et al., 2012b). For Antarctic organisms, the metagenomic approach has been applied at the level of targeted sequencing of small subunit rRNA-enriched libraries derived from marine bacterial and archaeal metagenomes to investigate seasonal variations in bacterioplankton community composition and functional diversity (Brown et al., 2012; Grzymski et al., 2012; Cavicchioli, 2015). No study to-date has utilized metagenomic shotgun sequencing of eDNA to investigate Antarctic marine macrofaunal community composition.

In the present study, we applied high-throughput shotgun metagenomic sequencing and analyses of eDNA extracted from seawater samples collected from shallow (≤ 300 m) shelf locations at four WAP regions. We evaluated the efficacy of this approach for assessing overall marine invertebrate and vertebrate metazoan biodiversity as a method of recording spatial and temporal variations in communities facing climatic shifts. Antarctic notothenioid fish distributions in WAP waters are well known (Duhamel et al., 2014), and thus detection of their sequences in the eDNA samples serve as positive controls for this assessment. Furthermore, we determined the usefulness of this approach for detecting DNA signatures of lithodid king crabs in these shelf locations, and thus a potentially simpler and less expensive sampling method than the current logistically expensive remotely operated vehicles and mechanical trapping.

The use of eDNA to detect organisms is predicated upon the presence of their DNA at the sampling locations, which is directly influenced by the rate of eDNA degradation and water transport within aquatic environments (Lindahl, 1993; Deiner and Altermatt, 2014; Strickler et al., 2015). Therefore, we also estimated the rate of eDNA degradation in coastal WAP water via controlled aquarium experiments using eDNA derived from a common WAP icefish (*Chionodraco rastrospinosus*), to gain insight on the effect of sub-zero temperatures and the persistence of eDNA in polar marine ecosystems.

2. Materials and methods

2.1. Water sampling for environmental DNA

Eleven seawater samples were collected during the austral winter (July and August) of 2014 from four coastal regions near Anvers and Brabant Islands, West Antarctic Peninsula (Table 1). These sites include Dallmann Bay, Gerlache Strait, Bismarck Strait/Port Lockroy, and Palmer Station vicinity, approximating a North-South transect within 150 km of Palmer Station (USA) (Fig. 1, Table 1). Collections were made using sterilized, standard 5 L oceanographic water sampling Niskin bottles (Model 1010, General Oceanics, Miami), deployed using nylon rope from the R/V *Laurence M. Gould* at ocean sites and from a Zodiac rubber boat at sites near Palmer Station. The cap of the tubular bottle at each end was held open under tension with the elastic cord/holding pin assembly on the side of the bottle. On vertical descend, the chamber was continuously and freely flushed by ambient water as it transited the water column. On reaching the bottom, a stainless steel weight (Devil messenger 1000-MG, General Oceanics) was sent from surface sliding down the rope to dislodge the pin and trip the tension, shutting the caps and sealing the tube before retrieval. The water captured within the bottle therefore consisted of bottom water. Sampling depths varied from about 5 m (near surface) to 300 m depending on sampling sites. Two bottles were deployed together from the ship at each ocean sampling location, and single collections were made when

Table 1
Water samples collected from four regions along West Antarctic Peninsula. Sample number is a discrete number assigned to each collected water sample. eDNA that was recovered from half of a filter membrane was used for Illumina sequencing. Extracted eDNA from multiple water collections at a given region were pooled prior to sequencing.

Region	Sample	Location	Latitude	Longitude	Filtered volume per membrane (L)	Sample depth (m)
1: Dallmann Bay	049	Dallmann Bay	63° 55′06.48″ S	62° 46′40.20″ W	5	180
2: Gerlache Strait	035	South Gerlache Strait	64° 48′33.42″ S	63° 09′51.06″ W	2.5	27
	041	South Gerlache Strait	64° 44′33.72″ S	63° 02′33.96″ W	5	300
	052	South Gerlache Strait	64° 47′06.78″ S	63° 07′04.08″ W	5	300
3: Bismarck Strait & Port Lockroy	043	Bismarck Strait	64° 52′23.16″ S	63° 39′17.28″ W	5	250
	045	Port Lockroy	64° 49′37.32″ S	63° 31′42.42″ W	5	65
4: Palmer Station vicinity	039	Palmer Station 1	64° 47′12.18″ S	63° 58′19.02″ W	1	27
	040	Palmer Station 2	64° 46′54.24″ S	64° 02′00.30″ W	1	23
	047	North Humble Island	64° 45′48.24″ S	64° 05′23.22″ W	5	27
	048	Litchfield Island	64° 45′59.94″ S	64° 05′59.94″ W	5	27
	053	Palmer Station Boat Ramp	64° 46′28.08″ S	64° 03′15.84″ W	5	≥5

sampling from the Zodiac around Palmer Station. Sampling from each location was performed days apart, and between collections, the bottle was thoroughly washed with a 10% bleach solution and Millipore Type 1 water, as per recommended decontamination protocols at the time, and up until more recently (Deiner and Altermatt, 2014; Eichmiller et al., 2014; Evans et al., 2016). We recognize that 50% bleach solution is now generally recommended, as per best practice guides for handling eDNA that were published following our collections (*see*Goldberg et al., 2016; Wilcox et al., 2016). Once brought on board, samples were processed in a restricted lab space, and surfaces were cleaned with the bleach solution. Seawater from each collection was vacuum filtered through 47 mm diameter 0.45-μm pore size Whatman nylon membrane or Advantec mixed cellulose esters membrane to retain eDNA. All membrane filters were placed in individual sterilized 1.5 mL tubes and immediately stored at − 20 °C (Goldberg et al., 2016). eDNA extractions were performed for some of the filters at Palmer Station, and the remaining filters were returned on dry ice to the laboratory at the University of Illinois and kept at − 80 °C until DNA extraction was carried out.

2.2. Water sampling for estimating eDNA degradation rate

To estimate the rate of eDNA degradation in the WAP marine environment, two sterilized 20 L Nalgene® HDPE carboys were filled with seawater from a 2000 L flow-through tank housing live Antarctic icefish *Chionodraco rastrospinosus*. The tank was part of the Palmer Station aquarium facility and had a flow-through rate of approximately 1.4 m^3/h. Each carboy (labeled “A” and “B”) was tightly capped to prevent leakage and submerged in the tank water to maintain internal seawater temperature as that of the ambient flow-through (mean of − 1.04 ± 0.12 °C) for the 20-day duration, as monitored with an Onset Hobo Water Temperature Pro v2 data logger (Fig. S1). Each day, the

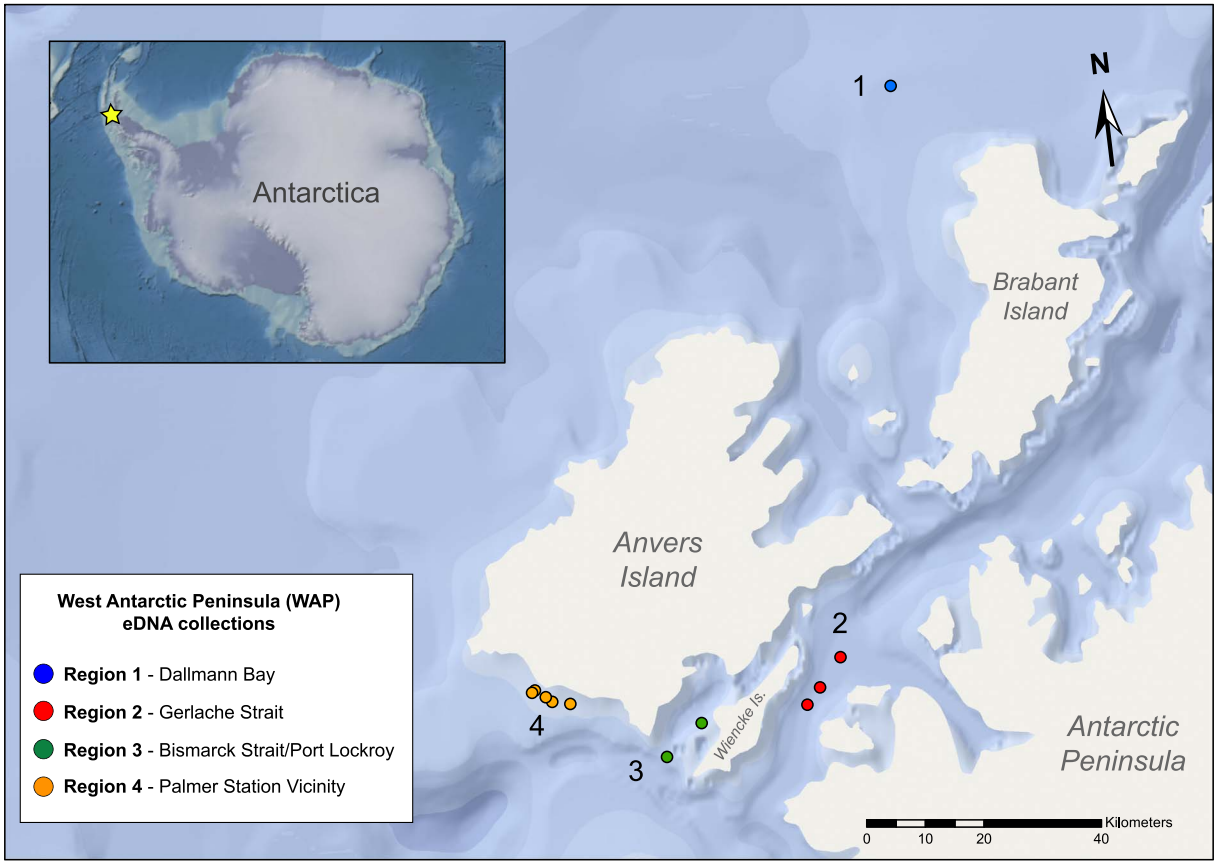

Fig. 1. Map of West Antarctic Peninsula (WAP) locations (n = 11) and regions (n = 4) from where water samples were collected for eDNA extraction and metagenomic sequencing.

carboys were removed briefly from the tank for sampling. The exteriors of the carboys were wiped with 70% ethanol and 10% bleach solution, then 1 L of seawater per carboy was carefully removed for vacuum filtration through 47 mm diameter, 0.45-μm pore size nylon membrane. Membranes were immediately stored at − 80 °C until DNA extraction in a separate laboratory.

2.3. eDNA extractions, library construction and sequencing

For both sampling series, eDNA was extracted from membrane filters following published protocol (Thomsen et al., 2012b) with some modification (*see* File S1 - Protocol 1). Briefly, the filter was rolled up, cut into two halves, and each half was sliced into small pieces and placed in separate 1.5 mL tubes. About 0.2 g of Zirconia/Silica white beads (0.5 mm, Biospec Products, Bartlesville, USA) and 720 μL of ATL buffer from the Qiagen DNeasy Blood and Tissue Kit (Hildren, Germany) were added to each tube which was agitated in a Bullet Blender® Storm homogenizer (Next Advance, USA) at maximum speed for one minute, to free the eDNA into solution. The rest of the extraction process followed the Qiagen Kit protocol, with final elution of eDNA with 75 μL AE buffer for each sample. An aliquot of extracted DNA was electrophoresed on a 0.8% agarose gel and stained with ethidium bromide to visualize eDNA quality and size. Inclusion of extraction blanks showed no evidence of contamination. The concentrations of extracted eDNA were measured using a microplate (Epoch Take 3) spectrophomoter (BioTek, Winooski, VT). Extracted eDNA samples were then pooled by sampling region and used for library construction as follows: (1) Dallmann Bay, (2) Gerlache Strait, (3) Bismarck Strait and Port Lockroy and (4) Palmer Station vicinity (Table 1).

Construction of indexed shotgun metagenomic libraries and sequencing on the Illumina® HiSeq 2500 were carried out at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign (UIUC). The shotgun metagenomic DNA libraries were constructed from 250 ng of DNA. The eDNA samples were sonicated in a Covaris ME220 (Covaris, MA) to an average fragment size of 500 bp. Libraries were constructed with the Hyper Library Preparation Kit from Kapa Biosystems (Roche, CA). After ligation of uniquely barcoded adaptor to DNA fragments of each sample, the individual libraries were electrophoresed on a 2% agarose gel and DNA templates between 600 bp to 800 bp in length were recovered. The size selected libraries were amplified with 3 cycles of PCR and run on a Fragment Analyzer (AATI, IA) to confirm the absence of free primers and adaptor dimers, and the presence of DNA of the expected size range. Libraries were pooled in equimolar concentration and the pool was further quantitated by qPCR on a Bio-Rad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc. CA). The pooled shotgun libraries were sequenced on one lane on an Illumina HiSeq 2500 for 250 nt from each end using a rapid TruSeq SBS kit version 2. The fastq read files were generated and demultiplexed with the bcl2fastq v1.8.4 Conversion Software (Illumina, San Diego, CA).

2.4. Bioinformatics workflow

We customized a bioinformatics pipeline for data processing and analyses (*see*Fig. 2). A list of executed commands for the pipeline is given in File S1 - Workflow 1. Raw reads were first processed with Trimmomatic v0.32 (Bolger et al., 2014) to remove residual adaptor sequence, leading and trailing bases with low quality scores (< 8), and reads shorter than 36 bp after trimming. The cleaned reads were further evaluated for quality and adaptor contamination using FastQC (Andrews, 2010). Forward and reverse reads were merged with PEAR (Paired-End reAd mergeR) v0.9.6 (Zhang et al., 2014) using default parameters. To analyze the sequence datasets to the fullest extent, paired-end reads that did not overlap and merge were concatenated by joining the reverse complement of the reverse sequences to the end of their respective forward sequence.

Taxonomic information was then assigned based on BLAST matches to public sequence databases obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) GenBank, release 220 (June 2017). Sequences were mapped to NCBI nucleotide databases in a successive order, implementing the “ublast” (blastn) algorithm of the 64-bit version of the USEARCH sequence analysis tool (Edgar, 2010). Reads remaining unassigned at each database step were carried forward to query the next database in the pipeline. The search order of the databases was as follows: mitochondrial genomes (to leverage less bias across metazoan phyla), “Antarctic metazoan” (a custom-built database composed of nucleotide sequences from Antarctic animals), the full nucleotide “nt” collection (with mitochondrial genomes and “Antarctic metazoan” sequences removed), UniVec (for elucidating any additional vector contamination), and “env nt” (nucleotide sequences obtained from environmental sequencing projects). The “Antarctic metazoan” database was compiled from publically available nucleotide sequences to leverage the greater specificity of this group and contained nearly 600,000 sequences from 167 species across 13 phyla, including Antarctic notothenioid fishes and lithodid king crabs that are of particular interest in this study (see Fig. S2 and the complete database in File S2). Following the nucleotide assignments, reads remaining unassigned were mapped against protein databases “env nr” (protein sequences obtained from environmental sequencing projects), and “nr” (non-redundant database) using the double index alignment of next-generation sequencing data program (DIAMOND v0.7.9) implementing the “blastx” command (Bunchfink et al., 2015). All sequence assignments were performed using a fixed e-value threshold of $1e^{-5}$, and command parameters are detailed in Workflow 1. To provide a measure for the goodness of assignment for each sequence, the frequency of sequences versus resulting percent identities and e-values were plotted as histograms for nucleotide and protein datasets separately, using the ‘hist’ function in the R statistical program (R Development Core and Team, 2014). Reads remaining unassigned after the multi-database mapping were not analyzed further (see Fig. S3 for complete sequence mapping pipeline).

All sequence assignments were assessed with the aid of MEGAN6 (MEtaGenome Analyzer, Huson et al., 2007), using the lowest common ancestor (LCA) algorithm at stringent parameters to explore the taxonomic content of non-assembled metagenomic datasets. Sequences assigned to high taxonomic levels (Domain/Super Kingdom, Phylum, etc.) were counted from both nucleotide and protein assignments. However, due to the differences in reference database sensitivity (nucleotide vs. protein), and programs used for assignment (USEARCH vs. DIAMOND), we chose only the nucleotide assignments for downstream community analyses described below. All sequences grouped as metazoans in MEGAN were normalized by the regional dataset with the smallest number of sequences (Region 2 - Gerlache Strait) before being exported from MEGAN. Exported datasets were converted to presence or absence of taxonomically assigned putative molecular operational taxonomic units (MOTU) (Blaxter and Floyd, 2003), a definition for eukaryotic groups when only DNA sequence information is available. We acknowledge that MOTUs may not be unique to the taxon they match, and use the term “putative MOTU”, as determining whether an MOTU is unique is an inherent and persistent issue when dealing with environmental sequence data and incomplete reference databases for taxonomic assignment. We chose the presence/absence criterion, as the number of sequences ascribed to a given MOTU do not necessarily reflect species abundance. This is due to various factors other than the number of individuals, such as the concentration of cells from any given taxon present in the water column at the time of sampling, which may affect the number of times a sequence can be observed.

As the eDNA samples were pooled by region for sequencing, the lack of replicates per region limited our ability to estimate species richness within regions, as well as to determine if significant differences in communities exist between regions using conventional analyses (AMOVA and PERMANOVA). Therefore, we used the Chao1 index

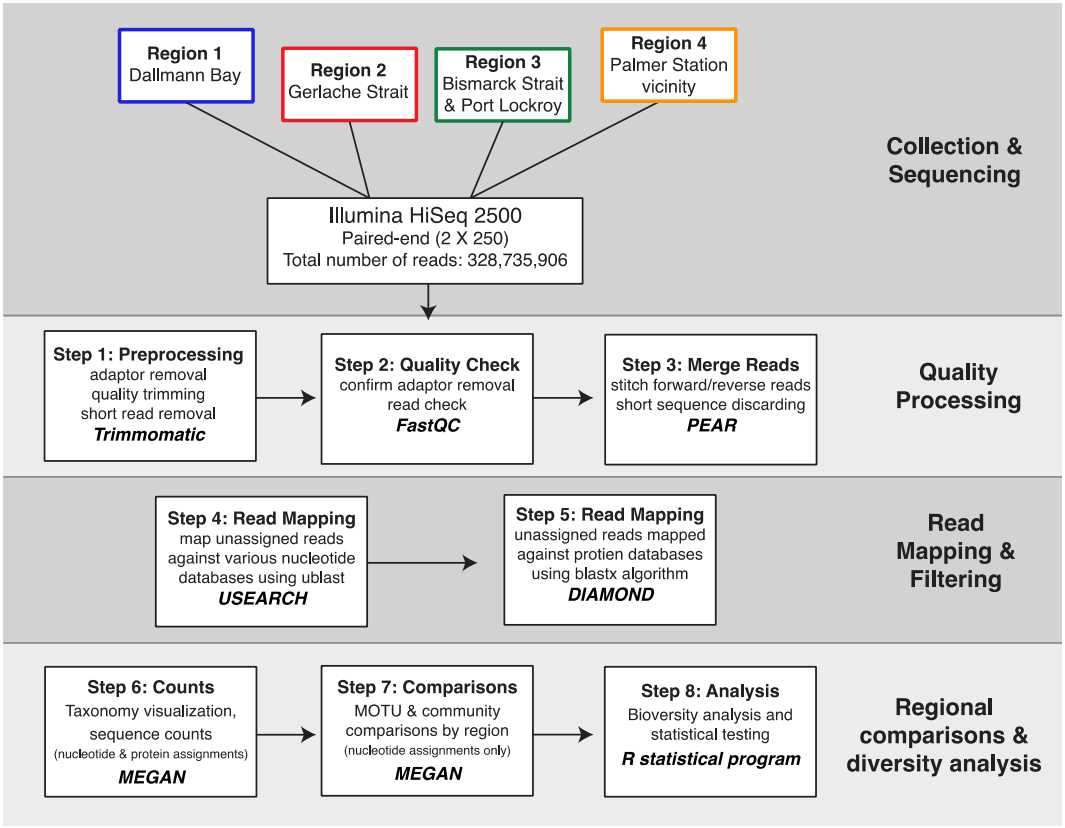

Fig. 2. Custom-designed bioinformatics pipeline for analysis of eDNA metagenomic data. The main steps are shown in bold, followed by the performed tasks and software used to execute tasks (italicized).

(Chao, 1984) to estimate overall species richness as the total number of putative MOTUs present in the entire community. The Chao1 index was calculated across all four regions combined using the *specpool* function within the vegan community ecology package (version 2.3-5, Oksanen et al., 2016), executed in R. To account for the proportion of species abundance in each region, the Shannon-Weaver index for diversity was calculated using the *diversity* function in vegan. To further assess species richness and determine whether the sequencing depth was sufficient to accurately characterize the metazoan community at the time of water collections, rarefaction curves were produced via the *rarefy* function. Finally, potential dissimilarities between regions in terms of putative MOTU composition was estimated using Slatkin's linearized Jaccard distances to minimize the weight given to "absence" values, and implemented using the *vegdist* function. The mid-point between the multiple sampling locations for Regions 2, 3, and 4 was calculated, prior to generating geographic distances between regions. Next, Slatkin's and geographic distances were used to generate a dissimilarity dendrogram based on UPGMA method via the *hclust* function, and the *mantel* function was used to assess (Pearson) correlation between community dissimilarities and geographic distances, at a significance threshold of 0.05.

2.5. Quantitative PCR (qPCR) assays to estimate eDNA degradation rate

Since the starting water was obtained from tank water holding the Antarctic icefish *C. rastrospinosus*, we estimated the decay rate of the mitochondrial ND2 (NADH dehydrogenase subunit 2) gene of this species as proxy for decay rate of total eDNA derived from the fish during the 20-day sampling time course. Candidate qPCR primers were designed against available full length *ND2* sequences of *C. rastrospinosus* using Primer 3.0 v.0.4.0 (Untergasser et al., 2012) and synthesized (Integrative DNA Technologies, Coralville, IA). Candidate pairs were tested via end-point PCR amplification using genomic DNA of *C. rastrospinosus* in 50 μL reactions containing 1 μL of DNA, 1 μL of each primer (10 μM), 1 μL of dNTPs (10 mM), 5 μL of 10 × buffer, 0.25 μL of Taq Polymerase and 40.75 μL of ddH_2O. Cycling conditions were as follows: 3 min at 95 °C, 35 cycles for 50 s at 94 °C, 50 s at 55 °C, and 50 s at 72 °C, and a final extension of 3 min at 72 °C. To verify amplicon sequences as *C. rastrospinosus ND2*, PCR products were sequenced with ABI BigDye ver.3.1 chemistry (Applied Biosystems, Waltham, MA) and read on an ABI 3730xl sequencer at UIUC Keck Center for Comparative and Functional Genomics. The best primer pair (ant_eDNA_F5: 5′-TCG CTGTGTCAGTGTATGTCGC-3′ and ant_eDNA_R5: 5′-GGGCGGGTGCTT TTGCTCA-3′) produced a 70 bp amplicon, and was subsequently used for qPCR estimation of *ND2* copy numbers.

To avoid cross-contamination, all qPCR preparations and assays took place in a different laboratory from where DNA extractions and end-point PCR assays were performed. Reactions were run in triplicate for all samples. Each 10 μL reaction contained 5 μL of iTaq™ Universal SYBR® Green Supermix (BIO-RAD®, Hercules, CA), 0.4 μL primer mix (200 nM of each primer), 4.1 μL of ddH_2O and 0.5 μL of eDNA extract (none for negative controls). Reaction plates were run on a CTX Connect™ Real Time System (Optics Module, BIO-RAD®) thermal cycler under the following cycling conditions: initial DNA denaturation and polymerase activation for 30 s at 95 °C, 40 cycles of denaturation at 3 s for 95 °C and annealing/extension for 40 s at 60 °C, followed by a melt curve analysis from 60 to 95 °C with a stepwise increase of 0.5 °C for 5 s. Full length (1047 bp) *C. rastrospinosus ND2* sequence (accession HM165956) was synthesized (gBlock synthesis, Integrated DNA Technologies) and used as template for generating a standard curve. The 70 bp target amplicon starts at nucleotide position 627. Eight 10-fold serial dilutions of *ND2* template (starting concentration of 10 ng/μL) were produced and assayed in the same qPCR plate with the eDNA samples to provide a range of copy numbers (standards) for

quantification of the samples. Reaction efficiencies were between 98 and 99%, and R^2 values for the calibration curves were > 0.98. None of the negative controls showed evidence of contamination among samples.

Final qPCR products from eDNA samples were size verified on agarose gel and were confirmed to be *C. rastrospinosus ND2* by sequencing 10 reactions from each biological replicate (carboy A and B) with BigDye ver.3.1 as described above. Quantification cycle (C_q) values for each triplicate set were averaged and measured against the calibration curves to determine the number of *C. rastrospinosus ND2* copies in the daily water eDNA samples, and the data were initially plotted to produce a decay curve (*nls* function in R, Fig. S4). A rate of *ND2* degradation was estimated from the change in copy number per liter of sampled water over the 20-day time course, by using the slope of a linear regression line via the *lm* function in R. The linear model was generated after log-transforming the mean copy number, which showed a linear relationship between copy number and day. The *predict* function was implemented to estimate the number of environmental *ND2* copies from the icefish that would be present at 25, 30 and 35 days, based on the model. Finally, eDNA half-life ($t_{1/2}$) was calculated using the following equation (Maruyama et al., 2014; Lance et al., 2017), where b is the rate of decay:

$$t_{1/2} = \frac{\ln(2)}{b}$$

3. Results

3.1. Sequence assignments

Our metagenomic eDNA sequencing produced a total of over 328 million raw reads from the four regions, with > 98% of reads from each region retained after quality processing (Table S1). The full metagenomic dataset for this project has been deposited at NCBI under SRA accession SAMN05421426. For each region, between 27% and 33% of the sequences matched entries in one of the seven databases (Table S2), representing approximately 10 to 15 million sequences taxonomically assigned per region. Histograms detailing the goodness of assignments for each region are detailed in Figs. S5 and S6.

Sequences were assigned to broad taxonomic groups for each region (Fig. 3A, Table S2). Bacteria were the most frequently assigned domain, representing between 64% (Region 3, Bismarck Strait & Port Lockroy) to 70% (Region 1, Dallmann Bay) of all assignments (Table S2). Assignments to Eurkarya were the next most frequent, with 8 to 13% (Regions 2 and 3, respectively) of all identified sequences, followed by Archaeal assignments ranging between 6 and 10% (Region 4, and Region 1, respectively). Prior to normalization, the number of metazoan reads for each region totaled between 407,000 and 581,500 (Table 2). In addition to the three domains of life, another prominent group – marine metagenomic sequences – emerged from each regional dataset, representing uncultured marine organisms obtained from environmental sampling of seawater (NCBI). The marine metagenomic group represented between 8 and 12% (Regions 1 and 2, respectively, Fig. 3A, Table S2) of all assigned reads.

Within the Eukarya, ten most frequently assigned metazoan phyla emerged (Fig. 3B). At Region 1, over 60% of all sequences assigned to metazoans were identified as chordates (Table S2). Chordates were followed by Mollusca, Arthropoda, Nematoda, and Platyhelminthes. Region 2 had the highest percentage of sequences matching to mollusks (46.50%), while Region 3 had the highest percentage of reads matching to arthropods (7.15%) and nematodes (2.62%). Region 4 had the highest percentage of reads matching to Platyhelminthes flatworms at 2.07% (Table S2). The remaining eight phyla (Priapulida, Nemertea, Rotifera, Brachiopoda, Hemichordata, Bryozoa, Ctenophora, and Nematomorpha) combined accounted for 0.04% of assigned sequences within each region.

Investigations at lower taxonomic levels revealed the presence of several classes and families at each region (Table S3). Chordates exhibited the highest number of classes, followed by arthropods, cnidarians, mollusks, and nematodes. At the familial level, arthropods and mollusks showed highest richness after the chordates. Within Chordata, Actinopterygii (boney fishes) was by far the largest class represented in the dataset, with over 600,000 sequences assigned across all four regions combined. For non-chordates, some notable Antarctic families identified included Syllidae (*Exogone* worms; > 11,000 sequences), Echinidae (*Sterechinus* urchin; > 7000 sequences), Spionidae (*Spiophanes* worms; > 3000 sequences), and Actiniidae (*Isosicyonis* anemonae; > 700 sequences).

3.2. Diversity and community comparisons

The number of putative MOTUs (molecular operational taxonomic units) uncovered was similar among regions, with Region 1 having a greater number (409) after sequence normalization while the maximum number of MOTUs across all four regions was 563 (Table 2). Community diversity was also similar across the regions, ranging between 5.81 at Region 3 to 6.01 at Region 1 (Table 2). Overall species richness (Chao1) across the entire geographic area was 750 ± 37.47 after normalization, providing a conservative estimate of the total number of species present in the community. Rarefaction curves quickly neared asymptotic values with the increasing number of sequences (Fig. S7), indicating that this dataset achieved extensive characterization of communities at each region at the time of sampling. Table S4 illustrates the number of putative MOTUs by phyla per region, with chordates, arthropods, nematodes and mollusks having the most assigned.

Regions 3 (Bismarck Strait/Port Lockroy) and 4 (Palmer Station vicinity) were found to be the most similar in terms of overall biological communities (Fig. S8), while Region 1 (Dallmann Bay) was identified as the most dissimilar community, in line with being the most geographically distant from the other three regions (Fig. 1). Despite these findings, mantel testing failed to identify a significant relationship between community dissimilarities and geographic distances of the sampling regions ($p = 0.125$, Fig. S8).

3.3. Antarctic notothenioid fishes and lithodid king crabs

Taxonomic dendrograms produced with the aid of MEGAN6 illustrate the number of sequences and the percentage of those assigned, by region, to several Antarctic genera of the Notothenioidei sub-order (Fig. 4) and the decapod family Lithodidae (Fig. 5). Nearly 150 sequences were assigned to the family Channichthyidae, including the icefish genus *Chionodraco*; most of the sequences originated from Regions 1 (Dallmann Bay) and 3 (Bismarck Strait/Port Lockroy). Additional families represented in the dataset include Artedidraconidae, Bathydraconidae, and Harpagiferidae, with a total of ~150 sequences assigned, predominately originating from Regions 1, 2 and 3. The remaining sequences were assigned to the red-blooded fishes of Nototheniidae: *Notothenia* ($n = 556{,}358$), followed by *Trematomus* ($n = 108$) and *Dissostichus* ($n = 168$). Sequences assigned to *Notothenia* were well represented across each region, though those originating from Region 3 (Palmer Station vicinity) were the most common. For the crustacean order Decapoda, nearly 900 sequences were assigned across all regions combined. From these, sequences specific to king crabs included a total of 50 sequences assigned to the genus *Neolithodes*, originating from Regions 1, 2 and 4.

3.4. eDNA degradation rate

The use of a 70 bp DNA sequence of the ND2 gene from the icefish *C. rastrospinosus* provided a proxy for estimating a rate of eDNA degradation in Antarctic WAP marine shelf environment. The *ND2* fragment derived from eDNA samples from carboys A and B exhibited a

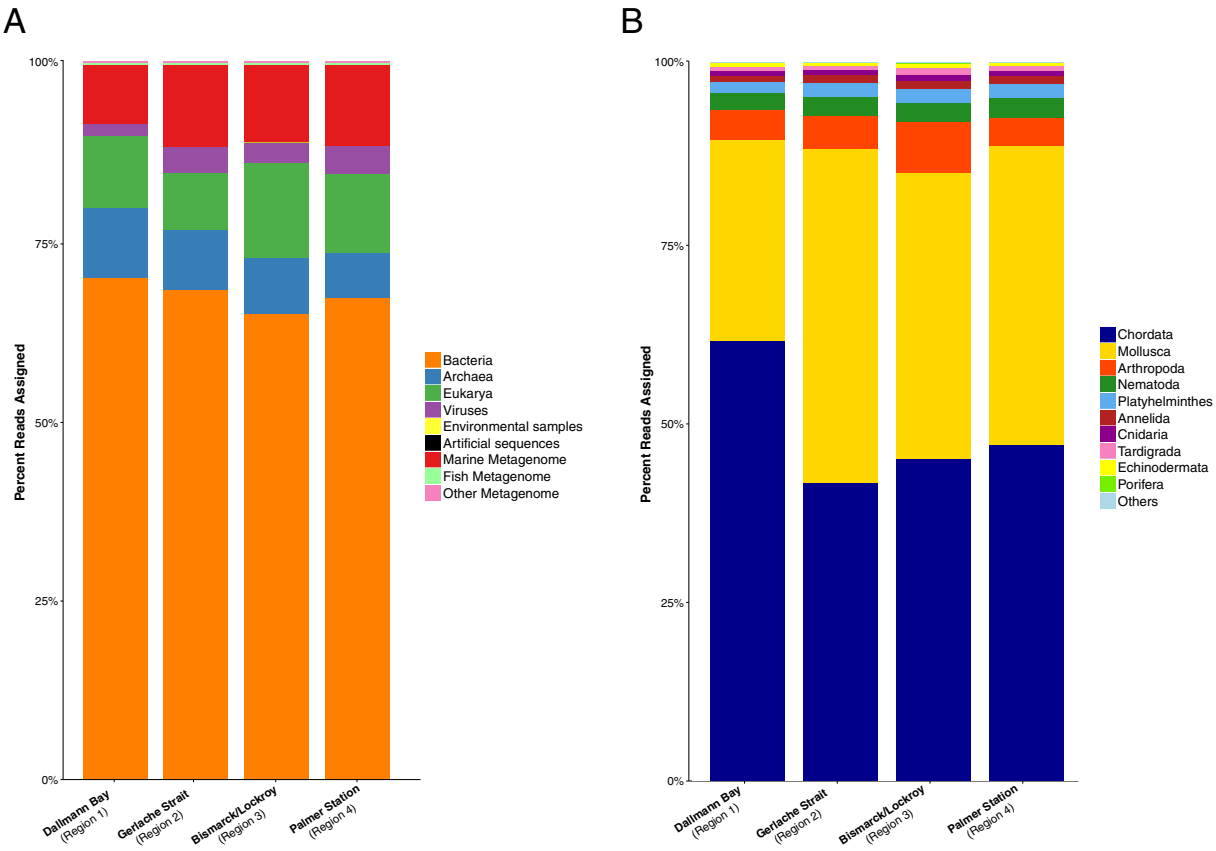

Fig. 3. (A) Percentage of sequence reads assigned to domain or other categories, by sampling region. The 'Environmental samples' category refers to reads matching uncultured organisms from various environmental samples in the database, while 'Artificial' refers to vector sequences and synthetic constructs. Reads assigned to 'Marine metagenome" represent uncultured organisms originating from marine environmental samples, while 'Other metagenome' represent those specific to sediment, microbial mats, freshwater, mine drainage, saltern, and sand stromatolites. (B) Percentage of sequence reads assigned by region, to the ten common metazoan phyla. The 'Others' category includes the following less common phyla: Priapulida, Nemertea, Rotifera, Brachiopoda, Hemichordata, Bryozoa, Ctenophora, and Nematomorpha.

pattern of exponential decay over time (Fig. S4), with initial copy numbers at 25,374 and 46,314, respectively, decreasing to 161 and 196 by day 20 (Table S5). The linear regression model identified an estimated rate of degradation of 0.234 day^{-1} (Fig. 6), as well as a significant relationship between copy number and time in number of days ($p < 0.001$). Finally, an eDNA half-life of 37.2 h was calculated, based on the exponential decay model (Fig. S4).

4. Discussion

The inherent logistic difficulties of accessing the remote Antarctic ecosystems calls for cost-effective and comprehensive tools for the appraisal of community biodiversity facing a changing climate. The relative ease in which eDNA sampling can be performed aids researchers seeking to understand how biological communities may be impacted by environmental changes. The West Antarctic Peninsula (WAP) is the fastest warming region in Antarctica where the need to assess effects of ocean warming on local biological communities is urgent, making this region a compelling candidate for biodiversity assessment and monitoring by means of eDNA surveys. In the present study, we applied – to our knowledge – the first metagenomic shotgun sequencing of eDNA recovered from seawater at four WAP coastal regions and analyzed the massive sequence datasets to assess overall macrofaunal community composition at these sites. We compiled a bioinformatics pipeline that integrated multiple data filtering steps and assignment thresholds to limit false positive detection of animal taxa.

Our metagenomic sequence datasets allowed for the detection of several signature resident fauna including members of the predominant notothenioid clade of fishes (Fig. 4), as well as common benthic invertebrate groups (Fig. 3B). However, < 3% of reads assigned to metazoans matched to animal phyla known to have high abundance and species richness in the Antarctic benthos, including echinoderms,

Table 2
Comparisons of the number of metazoan sequence reads, putative Molecular Taxonomic Units (MOTUs) and Shannon-Weaver diversity across regions. Total number (n) of putative MOTUs across all regions was 563. Regional datasets were normalized by the dataset with the smallest number of metazoan sequences (Region 2), and N_{abs} and N_{norm} refer to the absolute and normalized values, respectively.

	Region 1 Dallmann Bay		**Region 2** Gerlache strait		**Region 3** Bismarck strait/Port Lockroy		**Region 4** Palmer station vicinity	
	N_{abs}	N_{norm}	N_{abs}	N_{norm}	N_{abs}	N_{norm}	N_{abs}	N_{norm}
Reads	581,488	408,015	407,967	407,967	563,923	407,976	495,038	407,974
Putative MOTUs ($n = 563$)	409	409	340	339	334	334	389	389
Shannon-Weaver	6.01	6.01	5.83	5.83	5.81	5.81	5.96	5.96

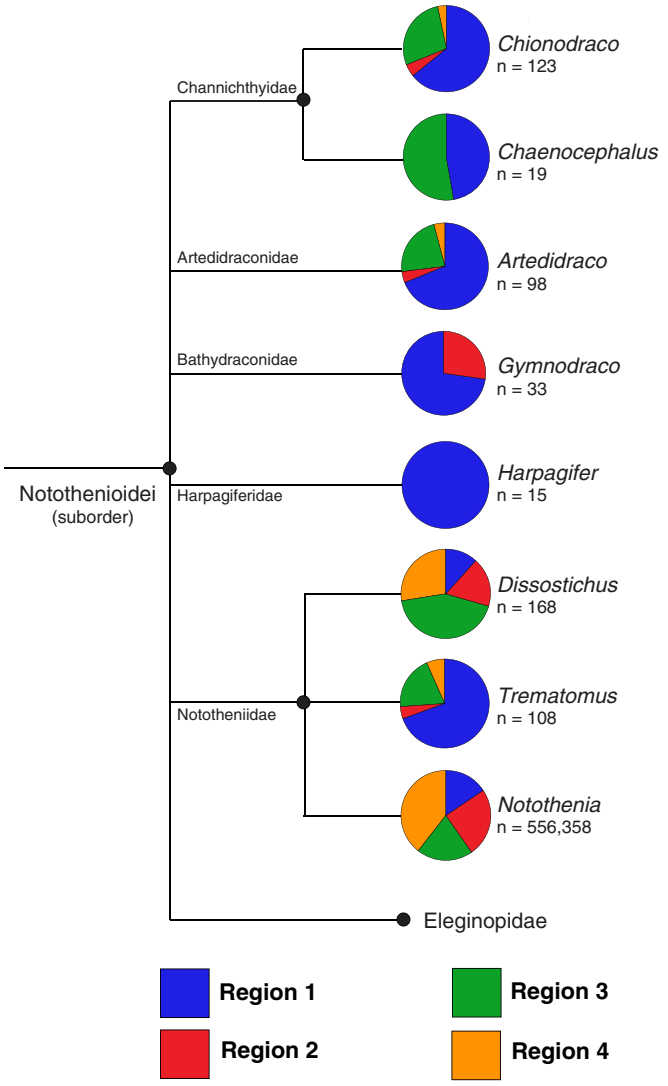

Fig. 4. Taxonomic dendrograms identifying the percentage of sequence reads assigned by region to genera of Notothenioid fishes. Number of assigned sequence reads (n) are given below each genus. Note: circle sizes are not proportional to the number of sequence reads.

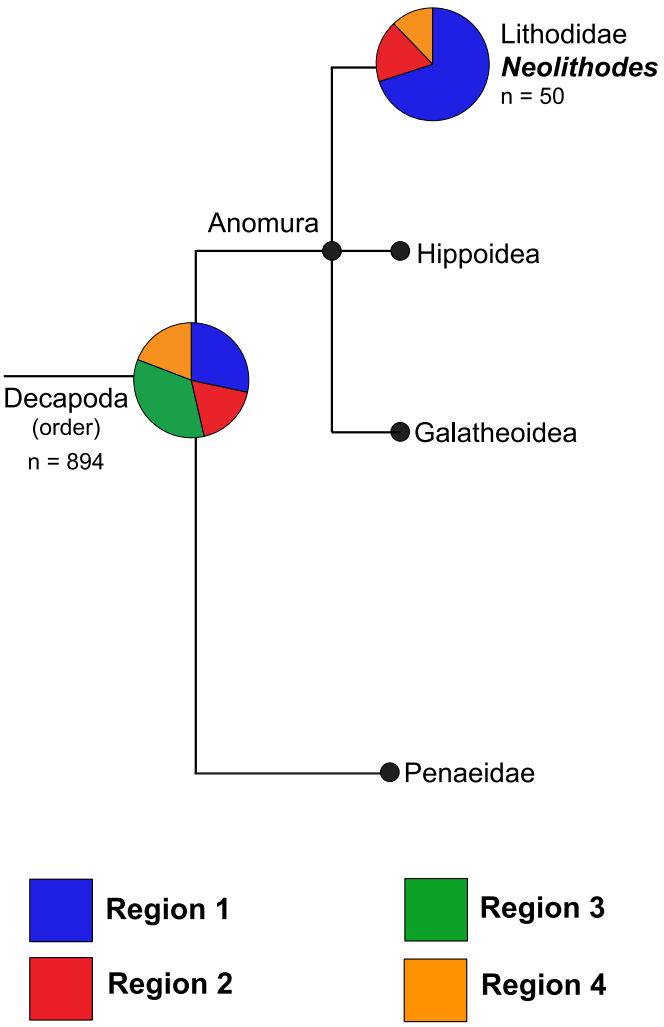

Fig. 5. Taxonomic dendrograms identifying the percentage of sequence reads assigned by region to Decapods and family Lithodidae (king crabs). Number of assigned sequence reads (n) are given below the order and genus. Note: circle sizes are not proportional to the number of sequence reads.

annelids, and porifera sponges (Fig. 3B, Table S2). The poor representation of these groups might have resulted from three possible causes: (1) their eDNA was present in low concentrations at locations sampled at the snapshot in time; (2) their eDNA was present in sufficient quantity, but resulting sequence reads were either not recognized as matching to taxa in databases (due to absence of closely related taxa in the database) or not informative enough to assign proper matches (due to absence of homologous fragments in the sample); and (3) eDNA was present in sufficient quantity, but not captured by our sample processing methodology (i.e. DNA not retained on the filters or fragments too degraded to be captured in constructed libraries).

Previous studies have attested to the incompleteness and uneven representation of some taxa in public nucleotide databases as causes for skewed taxonomic assignment (Berney et al., 2004; Dayrat, 2005; Kvist, 2013). For instance, Kvist (2013) investigated the number of unique taxon labels associated to the mitochondrial Cytochrome c Oxidase subunit 1 (COI) gene fragment, a widely used barcoding marker (Hebert et al., 2003), as a proxy for estimating biodiversity coverage in two major databases − NCBI and Barcode of Life Data System (BoLD). Kvist identified that bryozoans, platyhelminthes worms and nematodes were highly underrepresented in these databases when compared to their numbers of recognized species, while other phyla with Antarctic

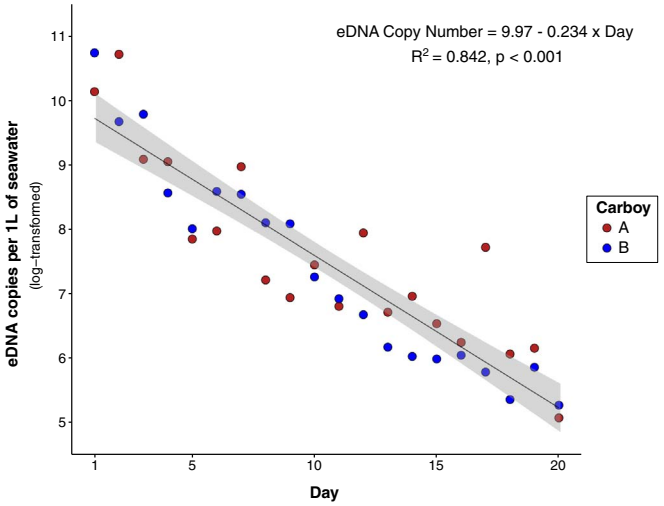

Fig. 6. Linear regression illustrating the relationship between the number of *C. rastrospinosus* eDNA copies per liter of seawater (log-transformed) and Day, for two carboys. The regression line shows a significant trend ($p < 0.001$) and gray shading indicates 95% confidence intervals. See Table S5 for cycle quantification (C_q) and eDNA copy numbers.

representatives (echinoderms, cnidarians and porifera sponges) had moderate barcode coverage. In contrast, platyhelminthes worms and nematodes were some of the most commonly assigned phyla in our dataset, though it should be mentioned that reads assigned to each group likely originated from genomic DNA fragments rather than mitochondrial *COI*. Several phyla that are known to be speciose in Antarctic waters (annelids, echinoderms, bryozoans, and poriferans, Clarke and Johnston, 2003) were either moderately or poorly represented in our dataset (Table S4). This indicates these particular groups are likely lacking high sequence representation in public databases, which are more heavily populated with specific gene regions of taxa that are easier to collect and/or better studied. The problem is further amplified by the holistic nature of the whole metagenomics approach in which both coding and non-coding DNA are present in the complex eDNA sample, yet not all have complements in public databases. In 2010, the Census of Antarctic Marine Life (CAML) initiative was launched to survey Antarctic fauna for the purposes of augmenting taxonomic databases. Additionally, the Register of Antarctic Marine Species (RAMS, De Broyer et al., 2011) and Polar Barcode of Life (PolarBOL) databases were created to provide inventories of species occurring in the Antarctic. Of these three repositories, PolarBOL is the sole sequence archive and it contains only *COI* barcodes of Antarctic organisms. This situation limits the amount of informative assignments that can be made using metagenomic datasets, demonstrating that the continued augmentation of reference databases beyond barcode sequences is necessary to bring us closer to assigning identifications to unknown sequences.

With regard to the recovery of eDNA that also affects taxonomic detection outcome, Deiner et al. (2015) found that the choice of eDNA capture method (i.e. water filtering vs precipitation) and eDNA extraction protocols can impact the detection rates of macrofaunal biodiversity in freshwater systems. Notably, a combination of filtration and the use of the DNeasy kit (Qiagen) allowed for the highest detection of eukaryotic diversity (Deiner et al., 2015), which was the protocol implemented in our study. This was also used for seawater samples (Thomsen et al., 2012b; Thomsen et al., 2016), thus we have used a currently best-supported method for capturing biodiversity in marine samples.

4.1. Antarctic marine metazoan community assessments

Within the present dataset, we identified sequences matching to several members of the Notothenioidei sub-order, including the red-blooded notothenioid genera *Notothenia*, *Trematomus* and *Dissostichus*, as well as the icefish genera *Chionodraco* and *Chaenocephalus* (Fig. 4), each of which have species occurring along the WAP coast (Duhamel et al., 2014). The largest number of sequence reads was assigned to the genus *Notothenia*, possibly from the species *Notothenia coriiceps*, which are plentiful along the WAP shelf at shallow depths, and from *N. rossii*, the next most common species (DeWitt et al., 1990). While the large number sequences assigned to *Notothenia* could indicate high concentrations of *Notothenia* eDNA in the water samples, it may also reflect a bias arising from the presence of a draft genome for *N. coriiceps* (RefSeq NC_015653.1, Shin et al., 2014), the only notothenioid whole genome sequence in the public domain. When sequences from other notothenid taxa within the eDNA samples are poorly represented or absent in the database, homologous matches would become assigned to the nearest relative having most abundant available data, i.e. *N. coriiceps*. Nonetheless, the definitive detection of eDNA of both red-blooded and white blooded notothenioids known to occur along WAP coastal locations is positive support of the utility of eDNA to capture signatures of endemic fauna.

Our metagenomics data additionally revealed between 339 and 409 putative MOTUs, as well as high levels of species diversity at the four WAP regions (Table 2). While within community inferences cannot be made due to lack of sampling replicates within each region, rarefaction curves approached the asymptote (Fig. S7), indicating that the metagenomic sequencing approach was extensive enough for characterizing marine metazoan communities at the specific time of collection.

Responding to the need for a modern database compiling Antarctic species, Griffiths et al. (2011) introduced SCAR-Marine Biodiversity Network (SCAR-MarBIN, www.scarmarbin.be), a centralized inventory for Antarctic marine biodiversity. In a biogeographic analysis of fauna, Griffiths and colleagues provided numbers of known species within 3° latitude by 3° longitude grid cells. Within the same geographic range and depth encompassing our sampling locations, 443 species have been reported thus far (De Broyer and Koubbi, 2014; Ocean Biogeographic Information System, OBIS: http://www.iobis.org/). Comparatively, our metagenomics approach identified a total of 563 putative MOTUs (Table 2) in addition to a species richness estimate of 750 ± 37.47 across the four regions. Thus, it appears that even within the very limited time frame when water samples were collected, our eDNA sequences recovered similar levels of known diversity. Furthermore, we identified 27–31 possible MOTUs assigned to Nematoda (Table S4), a phylum known to be speciose but under-sampled in the Antarctic (De Broyer et al., 2011). Our numbers compared to < 16 known species from the same geographic sector (Ingels et al., 2014), supporting the use of this method for uncovering hidden biodiversity of small organisms. Prior underestimates of species diversity are likely due in part to small, cryptic and/or invertebrate taxa (Bringloe et al., 2016), but molecular-based tools such as metabarcoding have helped to improve discovery of previously missed taxa by traditional/morphological approaches (Hajibabaei et al., 2007; Valentini et al., 2008).

4.2. Signatures of king crab eDNA: actual presence or influence by currents?

The cooling of the waters surrounding the Antarctic continent that began 35 my was hypothesized to have led to the extinction and subsequent exclusion of durophagous crabs due to limitations on their physiological performance imposed by low temperatures (Dayton et al., 1994; Aronson et al., 2007a; Hall and Thatje, 2011). However, populations of lithodid king crabs have been found to occur in the non-freezing deep waters of Ross, Amundsen and Bellingshausen Seas south of the polar front (Ahyong and Dawson, 2006), including most recent observations at > 800 m bottoms along the Western Antarctic continental slope (Smith et al., 2012; Aronson et al., 2015a; Smith et al., 2016). Whether lithodids reinvaded or endured cold Antarctic waters is an ongoing debate (Griffiths et al., 2013). Regardless, contemporary trends of rising sea temperature have spurred general concerns that these benthic predators may invade warming shallow shelf habitats, upending rich benthic communities that developed in their absence (Thatje et al., 2005).

We identified sequences matching to lithodid crabs in the eDNA isolated from our shallow (≤ 300 m) shelf water samples at four regions within 124 km from Palmer Station (Figs. 1 and 5). Reads identified as *Neolithodes* originating from Palmer Station vicinity (Region 4) suggests the potential presence of *Neolithodes yaldwini*, which had been observed and collected from the Palmer Deep basin (Smith et al., 2012), approximately 15 km from Region 4. In addition to sequences matching specifically to Lithodidae, a larger number of sequences assigned to the order Decapoda (Fig. 5) may contain additional lithodid sequences that failed to become assigned to king crabs as a result of fragmented/degraded sequence or the shortage of lithodid homologs in public databases and were thus assigned to the nearest decapod taxa. Targeted testing by implementing genetic markers designed specifically for king crabs would help increase the accuracy of eDNA detection and help to provide estimates of biomass and abundance, as demonstrated recently in fish, (Takahara et al., 2012; Yamamoto et al., 2016) and crayfish (Dougherty et al., 2016). It is unclear whether the presence of lithodid sequences in our WAP eDNA indicates that Palmer Deep king crab is present in the shallower shelf regions sampled in this study, or more likely, that their eDNA fragments were transported by ocean currents to

our sampled locations and depths. A combination of known crab sightings, estimates of how long eDNA may persist in Antarctic waters, as well as knowledge of oceanic current dynamics of WAP that would contribute to transporting eDNA (discussed below) is necessary to evaluate these two alternate hypotheses. Additional regular water collection campaigns for eDNA analyses could be integrated as a part of ongoing monitoring programs such as the Long Term Ecological Research (LTER), to provide corroborating data and help boost the reproducibility and reliability of species discovery by eDNA analyses.

4.3. eDNA degradation and transport in the Antarctic marine environment

The rate of eDNA degradation in aquatic environments depends on the complex interactions of various abiotic and biotic factors including salinity, pH, intensity of UV radiation, temperature, microbial activity and the density of individuals, making the estimation of actual rates of degradation difficult (Strickler et al., 2015; DeJean et al., 2011). In the absence of controlled mesocosm experiments designed to mimic the natural environment, we kept two closed carboys of aquarium water that previously housed the icefish *C. rastrospinosus* in ambient flow through aquarium to maintain natural environmental temperatures. As a proxy for estimating eDNA degradation in the Antarctic, we followed the copy number of an amplified *ND2* fragment from *C. rastrospinosus* eDNA over a 20-day time course. The icefish *ND2* decay kinetics is exponential (Fig. S4, Table S5) similar to eDNA degradation in non-freezing fresh and marine environments (Thomsen et al., 2012b; Strickler et al., 2015; Lance et al., 2017; Barnes et al., 2014). The icefish *ND2* had a degradation rate of 0.234 day^{-1}, with quantifiable copies at day 20 (Fig. 6, Table S5). Predictive calculations show that at days 25, 30 and 35, approximately 57, 17, and 5 copies of *ND2* would still remain in the environment. In contrast and using similar methodology, Thomsen et al. (2012b) identified degradation rates of 0.701and 0.322 for two temperature marine fish, whose eDNA was no longer detectable after only one and seven days, respectively. Further, we obtained an eDNA half-life of 37.2 h. These findings support that DNA degradation is substantially slower in the subzero Antarctic waters than in temperate waters, likely due to longer preservation of DNA at colder temperatures (Lindahl, 1993; Shapiro, 2008). Factors that influence eDNA transport and degradation in the natural environment have major implications for biodiversity monitoring efforts that rely on eDNA protocols, as natural processes could disperse eDNA, limiting the ability to detect specific taxa within a narrow window of time (DeJean et al., 2012). Additionally, the chances of encountering "false positive" data (eDNA detected where the target species is not present, Bohmann et al., 2014) further complicates these investigations and underscores the necessity of understanding water mass movement as it pertains to eDNA assessments. At the WAP, water mass circulation patterns are complex, owing to the unique bathymetric terrain that resulted from ice scouring (Hofmann et al., 1996). Below the cold Antarctic Surface Water (AASW) lies the Circumpolar Deep Water (CDW), whose upper layer frequently intrudes onto shallower shelf environments and mixes with the AASW (Howard et al., 2004; Moffat et al., 2009). This frequent intrusion of CDW onto the shelf could entrain eDNA of king crabs from their deep bathyal habitats such that it could be detected at much shallower ($\leq$ 300 m) depths of our sampled sites even if the animals themselves are not physically present.

Current velocities and directions at the WAP vary with location, depth and season (Savidge and Amft, 2009). At our sampling locations, the Gerlache Strait Current is the main surface current ($<$ 400 m) that flows over the shelf past Anvers and Brabant Islands (Fig. 1) at speeds that can exceed 30 cm s^{-1} (Zhou et al., 2002). Using our estimated rate of degradation, we predicted that 57 copies of eDNA would be present after 25 days, which is above the 25 copy limit of detection identified by Thomsen et al. (2012b). Coupling this information with known current velocities suggests that detectable amounts of eDNA traveling at 30 cm s^{-1} may be dispersed as far as 648 km from its source in 25 days. Moreover, our four locations have a maximum distance of 124 km between them (Dallmann Bay and Palmer Station vicinity), which suggests that eDNA could be transported to these locations in as little as 5 days. We note that our experimental decay rate estimate involves higher initial concentrations of eDNA (from aquarium water holding many fish) than what may typically be found in the natural environment, and it also did not fully account for the degradation potential of differing DNA types, nor the complexities of the water currents directions at slower speeds. For example, nuclear DNA has been found to degrade faster than mitochondrial DNA (Murgia et al., 1992; Allentoft et al., 2012), as the double mitochondrial membrane may resist lysis (Turner et al., 2014b). Estimating degradation rates of nuclear eDNA fragments to compare against mitochondrial DNA rates would provide a wider understanding of factors controlling the persistence of different eDNA types in the aquatic environment. Finally, it is necessary to continue to integrate hydrodynamic data and mathematical modeling techniques into eDNA studies to provide more accurate estimates of eDNA movement and residence time at the Palmer Archipelago and elsewhere in the Southern Ocean.

4.4. Relative merits of eDNA sequencing vs. traditional survey approaches

Whole metagenomic shotgun sequencing is envisioned to circumvent taxonomic biases and amplification artifacts of genetic marker based PCR methods, as well as to expand taxonomic detection ability beyond the limit of single gene regions. In the present study, we applied metagenomic shotgun approach to identify a wide range of organisms, focusing on metazoan animal groups that are known to the Antarctic (Fig. 3, Table S2). Our approach uncovered several thousand sequences assigned to the endemic Notothenioid fishes (Fig. 4), supporting the usefulness of the approach for detecting taxa known to be present in the ecosystem. However, metagenomic studies are also subject to certain limitations, most notably the reliance upon properly curated and comprehensive reference databases for taxonomic identifications (discussed above). This dependence reveals the inextricable link between molecular-based and traditional sampling efforts (Pedersen et al., 2015), and that metagenomic sequencing of eDNA can first and foremost serve as informative and corroborative complement to traditional monitoring programs, particularly at remote ecosystems such as the Antarctic and deep-sea.

Environmental DNA as a tool for biodiversity monitoring has additional benefits over traditional approaches in enabling discovery of species diversity and richness independent of morphology-based taxonomic assignments (Blaxter and Floyd, 2003). The analysis of distinct taxonomic units (OTUs or MOTUs), rather than Linnaean species assignments, may uncover small (i.e. meiofaunal) or rare taxa that may be missed by surveys that require observation and physical collection of whole organisms (Thomsen et al., 2015). For example, through our sequence assignments we identified several putative MOTUs assigned to the nematodes (Table S4), a group that typically falls within the meiofaunal size range and is under-sampled in the Southern Ocean. It must be stated, however, that traditional surveys typically provide less ambiguity with regards to the presence and the identity of a particular organism and therefore, the risk of detecting false positives is less than with eDNA methods (Bohmann et al., 2014). Additionally, eDNA methods do not currently provide supplementary information on the size, developmental stages or sex of the organism being detected (Valentini et al., 2016).

The costs associated with high-throughput sequencing of eDNA can also be less than traditional methods; however, this is largely dependent upon the ecosystem of interest (Sigsgaard et al., 2015). The detection of eDNA from animal groups likely to be present in low concentrations in aquatic ecosystems requires high sequencing depth that can produce hundreds of millions of reads, as shown in the present study. Processing the resulting data is computationally demanding and can be limiting for research groups without extensive bioinformatics resources (Yu et al.,

2012; Pedersen et al., 2015). Nonetheless, rapid advances in the efficiency of computing software, as well as the decreasing cost of computational resources, allow for the increased accessibility for users. Despite these demands, the overall cost of collection, processing, sequencing and analysis are still likely to be lower than costs associated with traditional surveying methods in remote marine regions (i.e. ship operations, deploying of ROVs) and incorporating eDNA collections into already established sampling programs would help further reduce costs.

5. Conclusions

As far as we know, our study is the first to apply metagenomic shotgun sequencing and analyses of Antarctic marine eDNA to detect both endemic macro/meio-fauna, as well as the potentially invasive benthic predator (king crab) in shallow shelf habitats of the West Antarctic Peninsula. As the metagenomic shotgun sequencing approach relies upon adequate sequence homologs in public databases to achieve inclusive taxonomic assignments, continued collaboration between traditional and molecular approaches is necessary to provide holistic descriptions of Antarctic marine ecosystems. Regular water eDNA sampling accompanying ongoing long term ecological monitoring programs would allow for collection of replicates to boost the reproducibility and reliability of biodiversity assessment via high-throughput eDNA sequencing methods. Additionally, collaborations between biologists and oceanographers would allow the incorporation of physical environmental factors (currents, temperatures, bathymetry) with estimates of eDNA residence time for more precise assessment of the source of organisms such as the king crab.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.margen.2017.11.003.

Data accessibility

Metagenomic dataset is available online at NCBI, SRA accession: SAMN05421426.

Author contributions

DAC: Performed molecular experiments, contributed reagents/materials/analysis tools, performed statistical and bioinformatic analyses, interpretation of data and wrote the manuscript.
KRM: Designed the experiments, performed collections, molecular experiments and edits to the manuscript.
CCHC: Conceived the experiments and provided oversight of the study, performed collections, provided laboratory infrastructure and contributed to analysis tools, interpretation of data and edits to the manuscript.

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