Enhancing environmental DNA metabarcoding from marine ecosystems: Impact of filter type, storage method, and storage time on the assessment of fish alpha and beta diversity

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Abstract

The collection of environmental DNA (eDNA) and subsequent metabarcoding are useful tools for assessing marine fish biodiversity noninvasively. It is of particular importance to evaluate biodiversity in regions that are hard to access and thus less well studied. Sampling and preservation methods tailored to the specific circumstances are required. Aquatic eDNA is often captured on filters made of different materials and pore sizes, and subsequently stored under divergent conditions for varying periods of time. Previous studies on multispecies detection in marine systems have primarily focused on capture and extraction effects. Our study, in contrast, examined the effects of filter type, storage method, and storage time on DNA yield, alpha (i.e., ZOTU richness) and beta diversity (i.e., ZOTU composition) recovered from a marine ecosystem in Shark Bay, Western Australia. We compared two different filter types (cellulose-nitrate filters with pore sizes of 0.45 μm; glass-fiber filters with pore sizes of 0.1 μm), two storage methods (preservation in Longmire’s solution and drying, respectively), various storage times (30–68 days) on two metabarcoding assays using different fish-specific primers. Our results showed that storage time decreased DNA yield and affected alpha and beta diversity estimates. Cellulose-nitrate filters stored in Longmire’s solution proved to be the best combination with the smallest decrease in DNA yield, no effect on alpha diversity and consistent community compositions. Storing glass-fiber filters in Longmire’s solution led to a decrease in eDNA yield and alpha diversity estimates with increasing storage time. Furthermore, the largest change in beta diversity for each metabarc ode was found for glass-fiber filters regardless of storage method. Our results highlight the importance of considering storage time and interactions between storage method and filter when analyzing eDNA results, especially when storing samples for an extended time period or comparison of samples stored for different durations.

Keywords
biodiversity, environmental DNA, fishes, marine metabarcoding, preservation
Indirect monitoring of species diversity based on the detection of DNA traces in environmental samples such as soil, water, or air has gained popularity as a noninvasive, time, and money-efficient tool (Fraija-Fernández et al., 2020; Gaither et al., 2022; Hansen et al., 2018; Miyia et al., 2015; Yamamoto et al., 2017). The field of "environmental DNA" (eDNA, Pawlowski et al., 2020) was initially established to detect DNA of single-celled organisms, but has since been extended to detecting traces of macroorganisms such as amphibians (Ficetola et al., 2008), fish (Blackman et al., 2023; Jerde et al., 2011), cetaceans (Foote et al., 2012), and various other vertebrates and invertebrates (Cordier et al., 2021; Keck et al., 2022; Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012). In aquatic systems, genetic traces occur as free-floating DNA or are found in fragments of cells from mucus, feces, saliva, urine, or skin (Hansen et al., 2018; Taberlet, Coissac, Pompanon, et al., 2012). This genetic material can be used for targeted detection of a species, e.g., a rare or invasive species, using eDNA barcoding. Alternatively, eDNA metabarcoding approaches (Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012) allow to take a census of a broad range of species present in the sample (Deiner et al., 2017).

Earlier studies using eDNA to monitor macroorganisms in aquatic environments focused on freshwater bodies including wetlands (Ficetola et al., 2008), ponds, rivers, and streams. More recently, an increasing number of studies extended their scope to marine environments. Despite potential challenges due to larger water volume to species biomass ratio and different persistent times of eDNA in salt rather than freshwater (Díaz-Ferguson & Moyer, 2014; Fraija-Fernández et al., 2020; Hansen et al., 2018), eDNA studies in such systems have been successful in detecting rare (Boussarie et al., 2018; Sigsgaard et al., 2020) or invasive species (Borrell et al., 2017), or characterizing multispecies community assemblies (Andruszkiewicz et al., 2017; Djurhuus et al., 2020; Jeunen, Taylor, et al., 2019; Lacoursière-Roussel et al., 2018; Miyia et al., 2015; Stat et al., 2017).

With regard to fish diversity, numerous studies reported extensive to complete matches with traditional monitoring methods (Keck et al., 2022; Polanco Fernández et al., 2021; Port et al., 2016; Thomsen et al., 2012; Yamamoto et al., 2017). Some eDNA studies, however, detected species that were not found by traditional methods (Polanco Fernández et al., 2021; Yamamoto et al., 2017). Other work considered the relationship between DNA concentrations, or generated read counts, and abundance and/or biomass of aquatic taxa. While most studies found a positive correlation (Fraija-Fernández et al., 2020; Kelly et al., 2014; Pilliod et al., 2013; Port et al., 2016; Rouke et al., 2022; Takahara et al., 2012; Thomsen et al., 2016), some did not (Fraija-Fernández et al., 2020).

There are numerous challenges associated with molecular monitoring (for reviews, see Bowers et al., 2021; Darling et al., 2020; Hansen et al., 2018). Several systemic factors are known to influence the presence and abundance of eDNA (Lacoursière-Roussel et al., 2016; Altermatt et al., 2023; for review see: Barnes & Turner, 2016). The amount of eDNA traces in the water varies with abiotic and biotic factors (Goldberg et al., 2016), affecting the rates of shedding, degradation, and transport (for review see: Hansen et al., 2018). Similarly, the protocol used for barcoding or metabarcoding studies affects the detection and quantification of biodiversity (Deiner et al., 2015). Conclusions based on eDNA studies may be further influenced by a combination of sampling effort (Bessey et al., 2020), sampling depth (Alexander et al., 2023; Andruszkiewicz et al., 2017; Jeunen et al., 2020), sample substrate (Alexander et al., 2023), and the efficiency of the capture, storage, extraction, amplification, and sequencing protocol (Goldberg et al., 2011). These steps may act either singularly or in combination, and the effect of each step can be difficult to disentangle.

Filtration is the most commonly used approach to capture eDNA from aquatic environments (Kumar et al., 2020). Various filter membranes and pore sizes have been used (Deiner et al., 2018; Lacoursière-Roussel et al., 2016; Renshaw et al., 2015), and while some studies focussed on single-species detection and biomass estimations (e.g., Deiner et al., 2015; Eichmiller et al., 2016; Turner, Miller, et al., 2014), fewer studies have investigated how different filters may affect multispecies detection. In the marine environment, to our knowledge, only three studies (Deiner et al., 2018; Djurhuus et al., 2017; Jeunen, Knapp, et al., 2019) have investigated the effects of capture and extraction on metabarcoding results. All three studies find that capture methods, specifically pore size (Deiner et al., 2018; Jeunen, Knapp, et al., 2019) and filter membrane type (Deiner et al., 2018; Djurhuus et al., 2017; Jeunen, Knapp, et al., 2019) affect DNA yields. Djurhuus et al. (2017) used three broad-scale metabarcodes to target microorganisms, phytoplankton, and vertebrates. They found no differences between the filter materials used in downstream metrics such as community composition and operational taxonomic unit (OTU) richness. However, they reported differences in community composition based on the extraction protocol. In contrast, also using a broadscale metabarcode targeting eukaryotic eDNA, Deiner et al. (2018) reported differences in OTU richness based on filter material and extraction protocol. Similarly, when using a targeted fish metabarcode, Jeunen, Knapp, et al. (2019) observed that an optimized capture and extraction approach (i.e., cellulose-nitrate filters with DNeasy kit) led to higher OTU richness. Yet, they did not find such differences using universal metabarcoding assays.

While previous studies have investigated the effects of capture and extraction (Deiner et al., 2015, 2018), fewer studies have examined the effects of storage method and time on metabarcoding studies. In marine environments, it is of particular importance to assess biodiversity in regions that are hard to access and less well studied. Work in these regions requires sampling protocols and preservation methods appropriate for the task at hand. In remote areas, water samples are often collected by means of in situ filtration using a portable pump (Pilliod et al., 2013; Renshaw et al., 2015). Subsequent storage of the filters is usually done by freezing (Jerde et al., 2011), drying (Majaneva et al., 2018), as well as immersion in ethanol (Goldberg et al., 2011; Muri et al., 2020), or lysis buffers,
FIGURE 1 Setup of comparative experiments: Samples were collected using cellulose nitrate (CN) or glass-fiber (GF) filters, and then stored either dry (D) with silica beads or submerged in Longmire’s solution (L). Samples were stored between 30 and 68 days. Eight samples were used for each of the four treatments (CN-L, CN-D, GF-L, GF-D).

Most studies of filter storage have been conducted for short periods of time, and few studies have investigated the effects of long-term storage (150 days: Wegleitner et al., 2015; 8 months: Mauvisseau et al., 2021). While freezing filters at −20°C for storage might be the most common approach (Dickie et al., 2018), such storing is often not feasible in remote or tropical areas. Even if frozen, subsequent freezing and thawing cycles during sample transport can cause DNA degradation (Spens et al., 2017), diminish the eDNA detection rates (Takahara et al., 2015), and thus alter abundance estimates (Cardona et al., 2012). Drying or storing filters in a liquid preservative at room temperature or 4°C may thus be more apt for samples collected in remote areas (for review: Bowers et al., 2021). Specifically, using Longmire’s solution as a storage medium has been reported to be promising (Mauvisseau et al., 2021; Wegleitner et al., 2015), and it appeared to even enhance eDNA recovery (Wegleitner et al., 2015).

2 | MATERIALS AND METHODS

2.1 | Sampling and extraction

All eDNA samples were collected in Shark Bay, Western Australia from mid-September to mid-October 2019. We filtered the samples directly off the boat using a peristaltic pump (GeoPump™, Geotech Environmental Equipment, Inc., Denver, Colorado, USA). The pump was connected to a filter cup (Nalgene™ Analytical), which we held just below the water surface wearing gloves. We collected a total of 32 samples from the same location (E113.34238, S25.99598, Figure S1) on 4 different days (i.e., 8 samples per day; Appendix A: Table S1). We sampled only in low winds (Beaufort 0–2) under varying tidal and current conditions.

For each sample we filtered 3 L of surface water. We performed comparative experiments to establish which combination of capture and storage method is least affected by storage time. For the capture, we assessed cellulose-nitrate (CN, Nalgene™ Analytical) and glass-fiber filters (GF, Whatman™, UK) with pore sizes of 0.45 μm and 1.0 μm, respectively. We will refer to this combination of filter material and pore size simply as “filter” types or “filter”. The GF filters had to be manually inserted into the filter cup (Nalgene™ Analytical) in a sterile environment prior to use. The use of different pore sizes was driven by practical constraints and lack of prior knowledge. This study served as a pilot in a remote area, and all material decisions had to be made prior to fieldwork without detailed knowledge of water turbidity. Small pore sizes are known to yield most eDNA (Liang & Keeley, 2013; Turner, Barnes, et al., 2014), but they may clog easily which reduces the volume that can be filtered (Bowers et al., 2021). Testing different pore sizes and filter materials simultaneously thus allowed us to maximize the chances of success in this novel field campaign within our time constraints.

For storage, we tested submerging filters in Longmire’s solution (Longmire et al., 1997) or drying them using silica beads.
(Carim et al., 2016; Majaneva et al., 2018). On each sampling day, we collected a total of eight samples (each of 3L), yielding two biological replicates of each of the four treatments: CN filter stored in Longmire’s solution (CN-L), CN filter stored dry (CN-D), GF filter stored in Longmire’s solution (GF-L), GF filter stored dry (GF-D). The samples were stored from 30 to 68 days prior to extraction (Figure 1). Storage time was primarily dictated by practical considerations within the confines of the study setup, including access to a clean laboratory for sample extraction and logistical constraints such as sample collection timing, resource availability, and transporting the samples from Australia to Switzerland.

Samples stored in Longmire’s solution were kept in a 2mL LoBind Tube (Eppendorf®) at room temperature (Parsons et al., 2018; Renshaw et al., 2015). Dried filters were kept in 5mL Cryo-tubes layered from bottom to top: filter, lint-free tissue (KIMTEC®), and silica beads. In the first week after sampling, we exchanged the beads every day to optimize drying. The lint-free tissue did not touch the filter and served as a barrier between the filter and the silica beads. Dried samples were stored at 4°C and transported at room temperature for 2 days.

We extracted the DNA using Qiagen’s DNeasy Blood & Tissue Kit following published modifications of the manufacturer’s protocol (Jeunen, Knapp, et al., 2019). We chose this kit as it had been shown to outperform other commercial kits in terms of DNA yield (Deiner et al., 2015; Djurhuus et al., 2017; Hinlo et al., 2017; Jeunen, Knapp, et al., 2019). For extraction from dry filters, we adhered to the protocol, except for adding 40μL Proteinase K (Sigma-Aldrich®) to the ATL Buffer and incubating overnight. For the filters stored in Longmire’s solution, we followed a protocol extracting eDNA from filter and storage solution (Spens et al., 2017), but only used 40μL Proteinase K. For both protocols, we added 3μL of RNAse A (Thermo Scientific™) to the AL buffer with an additional incubation step for 20min at 37°C.

All samples were eluted in 200μL Elution Buffer (Qiagen). After extraction, we removed potential PCR Inhibitors using the OneStep PCR Inhibitor Removal Kit (ZYMO Research Goldberg et al., 2016). We then measured the eDNA concentration of the extracts twice employing a Qubit dsDNA BR assay (Thermo Scientific™) on a Spark 10M Multimode Microplate Reader (Tecan Group Ltd) and used the mean of both measurements for all downstream analyses of eDNA yield. We stored the eDNA extracts at −20°C until further analysis.

2.2 | PCR, library preparation, and sequencing

We amplified the samples using two commonly used fish-specific markers: a hypervariable ~203bp long 16S rRNA gene fragment (“MiFish125”; original name MiFish-U/F-R, forward primer sequence: 5'-GTCGGTAAAAGCTGACGCACG-3'; reverse primer sequence: 5'-CATAGTGAGTTACCAATCCAGTTTG-3'; Miyai et al., 2015).

We generated two libraries, one for each metabarcode, which we pooled only after sequencing. Each library was constructed using the Illumina MiSeq dual-index two-step PCR amplicon sequencing protocol (see Appendix B for details). In the first PCR (PCR1), the target sequence was amplified using the fish specific primers with an overhang specific to the Illumina indexing primers. A heterogeneity spacer was introduced at the 5’-end (Blackman et al., 2021; Fadroz et al., 2014; Galan et al., 2018). In the second PCR (PCR2), Nextera XT indices were incorporated using a reduced number of amplification cycles. The primers in PCR2 target the Illumina overhang of the primers added in PCR1. This two-step approach prevents the generation of between sample chimeras due to tag-jumps, as reported in Schnell et al. (2015).

After indexing, we measured all samples twice with the Spark 10M Multimode Microplate Reader using a Qubit dsDNA BR assay. We used the mean concentration values of each sample to generate an equimolar pool using a Liquid Handling Station (BRAND). In this step, we pooled Fish16S and MiFish12S fragments. The final pool was purified with 0.8x Agencourt AMPure XP beads, and we checked the concentration and the length of the pooled fragments using the 2200 TapeStation (Agilent Technologies) with a Qubit dsDNA HS assay (Thermo Scientific™). We loaded the library at 8 pM concentration with 10% PhiX control. Paired-end (2×150nt) sequencing with 300 cycles was performed on an Illumina MiSeq (MiSeq Reagent Kit v2) following the manufacturer’s run protocol (Illumina).

We strictly adhered to sampling and laboratory protocols to prevent contamination with alien DNA and PCR products throughout all experiments (see Appendix B for a summary of all precautions taken). We checked for possible contaminants at each stage using different negative controls. During every round of extraction (NC<sub>ext</sub>3), PCR1 (NC<sub>PCR1</sub>), and PCR2 (NC<sub>index</sub>); an unused dual-index combination), we added negative controls which were processed simultaneously with the samples. To test the storage solution (Longmire) for potential contaminants, we extracted and sequenced DNA from 1mL Longmire’s solution (NC<sub>C</sub>). In the pooling step, the negative controls were conservatively added to the final pool undiluted in equal volume. As positive controls, we added two mock communities to PCR1 (MC<sub>1</sub>, MC<sub>2</sub>; Table S2) and a positive index control (PC<sub>index</sub>); DNA from Salmo trutta, an alien species to Shark Bay. The PC<sub>index</sub> was kept separate from all samples up until PCR2 according to Galan et al. (2018).

No field blanks were employed, but DNA extraction was performed from the Longmire’s solution used in the field. Employing field blanks with sterile water would primarily indicate the general cleanliness of the filter assembly. The cellulose-nitrate (CN) filters used were pre-packaged and PCR clean, while glass-fiber (GF) filters were inserted into CN filter housings in our DNA clean laboratory. Any contamination in our laboratory would have been detected in...
extraction blanks or the Longmire’s solution blank, as the latter was prepared concurrently.

2.3 | Data processing

We filtered the paired-end reads produced by Illumina sequencing through a series of data processing and quality control steps before taxonomic assignment. Data processing and filtering was done separately for the MiFish12S and the Fish16S region using the USEARCH software (Edgar, 2010) and a customized workflow. In short, after removing remaining PhiX (usearch:filter_phix) and low complexity sequences (usearch:filter_lowc) and trimming low-quality read ends (usearch:fastx_truncate), we merged the paired-end reads into amplicons (usearch:fastq_mergepairs). We then trimmed off the primer sites using in-silico PCR (usearch:search_pcr). This approach ensures that only the amplicons targeted by the primers used are retained and that potential contaminants from previous sequencing runs are excluded. Subsequently, we filtered the remaining amplicons, only keeping sequences in the size range of 100–250bp, with a GC content between 30%–70%, no ambiguous nucleotide, and with a mean phred score equal to or better than 20 (PRINSEQ-lite 0.20.4). We then dereplicated the amplicon reads and obtained read abundance counts for each unique sequence (usearch:fastx_uniques).

Amplicons were clustered into zero-radius operational taxonomic units (ZOTUs) using a denoising algorithm (usearch:uninoise3). A combined minimum of eight identical reads was required to be considered for cluster definition (abundance threshold). As opposed to the classical 97% OTUs, ZOTUs aim to report correct biological sequences at a higher resolution (Callahan et al., 2017; Edgar, 2016). Furthermore, in the process of ZOTU clustering, chimeric sequences are discarded directly, and no extra step is required. After clustering, the amplicons were mapped back to the previously defined clusters to generate a ZOTU count table (usearch:chutab). For back mapping, an identity threshold of 97% was used, to allow up to 3% errors due to sequencing and PCR.

2.4 | Taxonomic assignment

For the annotation of the ZOTU of the MiFish12S data set, we used MIDORI srRNA (GB240). For the Fish16S data set, no suitable references were available and we created a database based on nucleotide data from NCBI. In a first step we downloaded all 16S rRNA sequences related to BonyFish, CartilaginousFish, Hapfish, Lamprey, Lungfish, Turtle (date: 25.05.21, n = 52,995). Subsequently, we loaded the ZOTU against these sequences and all hits were filtered (biscore >300; alignment length >200nt; identity >98%). The R packages retrez and taxize were used to adjust the sequence names for usearch:SINTAX. Missing taxonomic labels were added manually. The 16S reference used for the annotation of the ZOTU included 891 sequences.

2.5 | Post processing using negative and positive controls

We used the negative controls to estimate false positives originating from contaminations caused by laboratory work, or from sequence assignment error during the Illumina sequencing, i.e., the generation of mixed clusters (Kircher et al., 2012). We examined all samples, negative controls, and mock communities (Table S2) for external contamination stemming from laboratory work and reagents by identifying unexpected taxa (Homo sapiens, Felis catus, Sus scrofa, Orcella heinsohni, Gobio spp., Alburnus alburnus, Sardinella aurita) and excluded them. We further identified all ZOTUs that had their maximum abundance in negative controls instead of samples as external contaminants and excluded them (Taberlet et al., 2018). To account for cluster assignment errors, we used a threshold above which a given ZOTU in a sample may be considered as a true presence. We used the PC_index to define a false assignment threshold (0.02%) per ZOTU (Galan et al., 2018). ZOTUs that had fewer read counts in a sample than the set threshold were removed from that sample. Lastly, we visually assessed the samples for dysfunctional PCRs as proposed in Taberlet et al. (2018), but did not identify any.

2.6 | Statistical analyses

All statistical analyses were conducted in Rstudio V2022.07.2 (RStudio Team, 2022), using R 4.3.0 (R Core Team, 2023). We aimed to compare different combinations of capture and storage methods and the effect of storage over 30 to 68 days. We analyzed the effect of different filters (CN or GF), different storage methods (D or L), and the storage time (number of days a sample was stored before extraction) on the eDNA yield, alpha diversity (i.e., ZOTU richness), and beta diversity (i.e., ZOTU composition). Analyses on ZOTU richness and composition were conducted separately for the MiFish12S and Fish16S metabarcodes. In all analyses, we controlled for different environmental conditions on different sampling dates.

To model the eDNA yield (i.e., eDNA concentration [ng/μL] after extraction), and ZOTU richness for each metabarcode per sample, we used three linear mixed-effects models (R package nlme; Pinheiro et al., 2023). We transformed the response variable using a natural logarithm as eDNA is known to decay exponentially (Collins et al., 2018). For ZOTU richness, to account for unequal sequencing depths of the samples, we extrapolated values with the estimateR function using the Chao estimator as suggested in Taberlet et al. (2018) and implemented in the vegan package (Oksanen et al., 2007; Figures S4 and S5). The Chao estimator yields continuous estimates rather than discrete interval data, rendering in our case linear mixed-effects models more appropriate for modeling richness compared to models with a Poisson distribution. For all models, we included storage time, filter type, and storage method as fixed effects and investigated all interactions as well. To account for pseudo-replication, we included sampling date as a random factor, and when heteroscedasticity was detected, we...
incorporated an identity variance function for filter type to correct for it. Multicollinearity was assessed using the `vif` function from the car package (Fox & Weisberg, 2019) excluding interaction terms. No values of concern were observed (vif > 10; Field, 2012). Model selection was based on the Akaike Information Criterion (AIC), following recommendations by Zuur et al. (2009).

We employed Canonical Correspondence Analysis (CCA) with the R package vegan (Oksanen et al., 2007) (function `ordinate`) to assess the influence of filter type, storage method, and storage time on ZOTU composition. To account for random variability across different days, we conditioned the CCA on sampling date. Prior to analysis, raw read counts were Hellinger transformed (Legendre & Gallagher, 2001) using the decostand function, as suggested by Laporte et al. (2021). To assess collinearity (vif, cca > 10; Field, 2012) between storage method and storage time, we created two separate models: one with filter type and storage time, and another with filter type and storage method. Model selection employed the backward selection procedure implemented in the function `RsquareAdj` (Oksanen et al., 2007) in which the preferred model was determined on the basis of adjusted $R^2$ values. Lastly, significance tests relied on within-block (i.e., sampling date) permuted ANOVAs ($n_{permutations} = 9999$).

\section{RESULTS}

\subsection{Environmental DNA yield}

The preferred model for eDNA yield contained a significant three-way interaction term of “storage time x filter x storage method” (Table 1, Appendix E: Figure S6 for model diagnostics). The model with the three-way interaction removed scored significantly worse according to the AIC score (delta AIC = 10.88). To aid interpretation of this result, we dissected the interaction by performing a post-hoc simple effects analysis (Appendix E: Figure S9) with storage time set constant and means derived using the `Effect` function from the package effects (Fox & Weisberg, 2019). This revealed that CN filters always exhibited higher eDNA yield than GF filters. More precisely, the CN-L treatment showed the highest eDNA yield, followed by CN-D, GF-L, and GF-D. Storage time was significantly negatively associated with eDNA yield across all treatment groups, although the extent to which, varied depending on the specific filter-storage combination (Figure 2a).

\subsection{Library}

All samples yielded DNA sequences, regardless of filter, storage method and storage time. Our library generated 7,192,338 sequence reads (MiFish12S: 4,144,926; 16SFish: 3,047,412). After quality control, a total of 7,021,110 reads (MiFish12S: 4,021,115; Fish16S: 2,999,995) were used for creating the ZOTU tables. The median number of reads per sample, excluding controls, was 112,785.5 (min = 31,053, max = 144,320) for the MiFish12S and 63,010 (min = 17,802, max = 192,845) for the Fish16S. The average length was 170 and 203bp for MiFish12S and Fish16S, respectively.

\subsection{Taxonomic assignment}

Of the 216 ZOTUs for the MiFish12S metabarc ode, 88 ZOTUs (41%) were assigned to the species level, 32 ZOTUs (15%) to the genus, 42 ZOTUs (19%) to the family, 19 ZOTUs (9%) to the order, and 35 ZOTUs (16%) to the class. For the Fish16S, of the 227 ZOTUs, 132 ZOTUs (59%) were assigned to the species level, 24 ZOTUs (11%) to the genus, 5 ZOTUs (2%) to the order, and 65 ZOTUs (29%) to the class level (Appendix D: Table S3 for complete species list). Of the 71 assigned species for MiFish12S, 17 (14.5%) were also assigned with the Fish16S primer (Appendix D: Figure S3).

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
 & eDNA concentration & ZOTU richness MiFish12S & ZOTU richness Fish16S \\
 & [ln(ng/mL)] & [ln(richness)] & [ln(richness)] \\
\hline
Intercept & 4.690 (0.714)* & 5.073 (0.317)* & 4.579 (0.673)* \\
Storage time & -0.051 (0.014)* & -0.026 (0.006)* & -0.022 (0.013) \\
Filter: CN vs. GF & 0.452 (0.372) & -0.518 (0.317) & -0.657 (0.509) \\
Storage Method: L vs. D & 1.061 (0.383)* & 0.991 (0.317)* & 0.701 (0.513) \\
Storage time \times Filter & 0.012 (0.007) & 0.021 (0.006)* & 0.026 (0.010)* \\
Storage time \times Storage method & -0.013 (0.007)* & -0.015 (0.006)* & -0.009 (0.010) \\
Filter \times Storage method & -1.525 (0.372)* & -0.663 (0.317)* & -1.043 (0.509)* \\
Storage time \times Filter \times Storage method & 0.028 (0.007)* & 0.012 (0.006)* & 0.020 (0.010)* \\
Observations (df) & 32 (21) & 32 (21) & 32 (21) \\
Random intercept (Sampling date) & SD = 0.26, residuals = 0.20 & SD = 0.005, residuals = 0.13 & SD = 0.19, residuals = 0.24 \\
\hline
\end{tabular}
\caption{Model statistics for eDNA yield (extract concentration) and ZOTU richness (MiFish12S and Fish16S).}
\end{table}

Note: "p < 0.1, "p < 0.05; numbers show estimates and SD in brackets. The different outcome variables are given as columns, explanatory variables in rows.
For the MiFish12S metabarcode, 59, 8, 3, and 1 species were assigned to one, two, three and four ZOTUs, respectively. For the Fish16S metabarcode, 44, 16, 1, 1, species were assigned to one, two, four, and five ZOTUs, respectively. One species (Cymbacephalus staigeri) was assigned to 47 ZOTUs. These 47 ZOTUs were distributed over several samples and several treatments excluding the possibility of a PCR error. To ensure that the many ZOTUs assigned to C. staigeri did not substantially affect our results, we repeated the following analyses with this species removed in the supplementary information (Appendix F: Tables S9 and S10, Figure S10).

Of the assigned 71 species with the MiFish12S metabarcode, 36 had been previously documented directly in Shark Bay and eight additional species had been reported in the transitional waters around Shark Bay (see Appendix C: Figure S2 for bioregions), and 27 have never been documented in Shark Bay before (GBIF, 2001). Of these 27 species, 11 species are found in northern Australia and Indonesia (FishBase, 2021); Rhinoptera javanica, Lepadichthys frenatus, Plotosus canius, Ophichthus zophistius, Pentapodus setosus, Neopomacentrus bankieri, Valenciennea randalli, Yongeichthys criniger, Argyrosomus amoyensis, Planiliza macrolepis; Sillago ingenua. Thirteen species have distributions in Japan: Plotosus japonicus, Sardinops melanostictus, Pempheris japonica, Callogobius hasseltii, Engraulis japonicus, Sillago aequulae, Saurida uemyoshii, Synodus ulae, Parapercis cylindrica, Gerres equulus, Gerres akazakii, Cymbacephalus beauforti, Platycéphalus indicus. The other three species have been documented in China (Cynoglossus nanhaiensis), the Red Sea (Rhabdosargus haffara), and in eastern Australia (Opistognathus jacksoniensis).

Of the 63 assigned species for the Fish16S metabarcode, 52 have been documented in Shark Bay, seven more species in the transitional waters around Shark Bay, and four have never been observed in Shark Bay before (GBIF, 2001). These species are known to occur in northern Australia (Onigocia oligolepis), Indonesia (Tylosurus punctulatus), Japan (Gerres japonicus), and in the Gascyne River that discharges into Shark Bay (Leiopotherapon aheneus) (FishBase, 2021).

### 3.4 Alpha diversity: ZOTU richness

Consistent with the results for the eDNA yield, the AIC-selected models for the MiFish12S and Fish16S metabarcodes both included the three-way interaction between “storage time × filter × storage method” (Table 1, Figure 2, Appendix E: Figures S7 and S8 for model diagnostics), despite it just failing to achieve statistical significance. The models with the three-way interaction removed scored significantly worse according to the AIC scores (MiFish12: delta AIC = 2.48; Fish16S: delta AIC = 2.45). Post-hoc simple effects analyses revealed that CN filters generally retained a greater amount of ZOTU information compared to GF filters. Specifically, the CN-L treatment exhibited the highest ZOTU richness, followed by the CN-D, GF-L, and GF-D treatments (Figure S9). Storage time affected CN and GF filters differently, with only GF filters showing a marked decline of ZOTU richness over time (Table 1, “filter × storage time”). Another set of post-hoc analyses rerunning the initial model for each treatment individually (Appendix E: Tables S5 and S6) suggested that storage time had the most pronounced impact on the GF-L treatment (although the statistical power of these tests is limited due to the small sample size). Overall, there was a tendency of each treatment being impacted differently by storage time.
3.5 | Beta diversity: ZOTU composition

The CCA model with the highest $R^2_{adjusted}$ included both filter type and its interaction with storage time for both metabarcodes (Table 2, all other tested models are shown in Appendix E: Tables S7 and S8). The results showed significant variation in ZOTU composition among the different filter types used (Figures 2d,e and 3a,b), indicating that CN and GF filters captured different ZOTU compositions. Within filter types, GF-L and GF-D treatments resulted in more heterogeneity in ZOTU composition compared to CN-D and CN-L, as shown by the wider spread of GF-L and GF-D samples in the CCA plot (Figure 2d,e). Samples collected by CN filters yielded similar ZOTU compositions (Figure 2d,e). When combining samples that received the same treatment per metabarcode, disparities in the top 20 most abundant ZOTUs were more evident among GF filters that were stored under different conditions and between GF and CN filters (Figure 3a,b), especially for the Fish16S metabarcode.

In addition to filter type, storage time also had a significant impact on ZOTU composition in the Fish16S metabarcode, as evidenced by the significant “Filter × Storage time” interaction in the Fish16S data (Table 2). The effect of storage time was mostly captured by the second axis of the CCA plots (Figure 2e), with GF and CN filters exhibiting opposite trends in response to storage time. The wider variation of the samples captured with GF filters suggests a stronger influence of storage time on these filters. In conclusion, these analyses suggest that GF filters may not be as reliable as CN filters in capturing the same ZOTU composition, and storage time can have a significant impact on ZOTU composition, particularly for GF filters.

**Table 2** Permutation ANOVAs for the adjusted $R^2$-selected MiFish12S and Fish16S CCA models.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>Chi-square</th>
<th>$F$</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MiFish12S</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filter</td>
<td>1</td>
<td>0.12</td>
<td>1.52</td>
<td>0.002*</td>
</tr>
<tr>
<td>Storage time</td>
<td>1</td>
<td>0.08</td>
<td>0.95</td>
<td>0.953</td>
</tr>
<tr>
<td>Filter × Storage time</td>
<td>1</td>
<td>0.09</td>
<td>1.11</td>
<td>0.17</td>
</tr>
<tr>
<td>Residual</td>
<td>25</td>
<td>2.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fish16S</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filter</td>
<td>1</td>
<td>0.19</td>
<td>1.69</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Storage time</td>
<td>1</td>
<td>0.11</td>
<td>1.00</td>
<td>0.291</td>
</tr>
<tr>
<td>Filter × Storage time</td>
<td>1</td>
<td>0.18</td>
<td>1.61</td>
<td>0.002*</td>
</tr>
<tr>
<td>Residual</td>
<td>25</td>
<td>2.74</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: *p<0.1, *p<0.05; MiFish12S: filter × storage time + Condition (Sampling date), $\chi^2 = 0.29$, $F = 1.19$, $p = 0.059$; $R^2_{adjusted} = 0.021$; Fish16S: filter × Storage time + Condition (Sampling date), $\chi^2 = 0.47$, $F = 1.44$, $p = 0.001$; $R^2_{adjusted} = 0.044$.

4 | DISCUSSION

With the advent of eDNA metabarcoding based monitoring in marine and potentially remote environments, it is crucial to address the lack of knowledge regarding the combined effects of filter type, storage method, and storage time on eDNA yield and alpha and beta diversity estimates. The objective of this study was to compare combinations of capture and storage methods for marine eDNA samples and to assess the effects of 30- to 68-days of storage on eDNA yield, ZOTU richness and composition.

4.1 | Environmental DNA yield

In summary, we found that eDNA yield was affected by a combination of filter type, storage method, and storage time. In general, CN filters had higher eDNA yield compared to GF filters and CN filters appeared to be less affected by storage time than GF filters. Notably, the GF-D treatment resulted in the lowest eDNA yield, while the GF-L treatment experienced the most pronounced negative effects of storage time. In this context it is important that our model does not suggest a consistently lower baseline of eDNA yield for GF filters, as the GF-L treatment exhibited similar eDNA levels to CN filters after 30 days. However, the GF-L treatment also exhibited the highest variability in eDNA yield, making it challenging to extrapolate our findings. A study including eDNA extraction immediately after collection and with more replicates is recommended to gain further insight into this phenomenon. The most plausible explanation for our results is that GF filters captured comparable amounts of eDNA, and eDNA on GF filters stored in a dry state may have experienced faster decay in the 30-day period prior to DNA extraction due to less effective preservation. Nonetheless, we cannot exclude the possibility that GF filters initially had a lower eDNA yield.

4.2 | Effect of filter type on eDNA yield

Comparative analysis of filter types showed that CN filters consistently outperformed other filter types, such as polycarbonate track-etch (Liang & Keeley, 2013; Renshaw et al., 2015; Spens et al., 2017), polyethylene sulfone (Liang & Keeley, 2013; Majaneva et al., 2018), polyvinylidene fluoride (Liang & Keeley, 2013), and GF filters (Renshaw et al., 2015; Spens et al., 2017). The potentially superior efficiency of CN filters in our study may be attributed to a combination of pore size and material properties (Deiner et al., 2018). We used CN filters with a pore size of 0.45 μm, while GF filters had a pore size of 1.0 μm. Previous studies comparing pore sizes have shown that smaller pores are more effective in capturing DNA (Jeunen, Knapp, et al., 2019; Liang & Keeley, 2013; Turner, Barnes, et al., 2014), while others found larger pore sizes to be more efficient in GF filters, but not in CN filters (Deiner et al., 2018). However, the nominal pore size of different materials may not accurately reflect their effective sizes, making direct comparisons between different filter materials
difficult (Turner, Barnes, et al., 2014). Moreover, different materials exhibit varying DNA retention capacities. Cellulose-nitrate membranes, used in CN filters, are known for their high affinity for DNA and protein binding, which explains their historical use in DNA fixation during DNA Southern blotting (Towbin et al., 1979; Van Oss et al., 1987). Another potential explanation for lower yield on GF filters could be a less favorable interaction with the extraction protocol. Previous studies have emphasized the interactions between capture and extraction protocols (Deiner et al., 2015; Jeunen, Knapp, et al., 2019). Specifically, one study found that using CN filters with the DNeasy extraction method resulted in a higher eDNA yield compared to GF filters combined with the same extraction method (Spens et al., 2017).

4.3 | Effect of storage method on eDNA yield

When comparing the average eDNA yield on filters stored in Longmire's solution to the corresponding filters stored dry, we observed that dry storage resulted in a lower eDNA yield. For CN filters, this could be attributed to the washing eDNA off the filter and/or increased cell lysis efficiency releasing DNA into the solution which may have facilitated extraction and led to higher yields, as suggested in previous studies (Mauvisseau et al., 2021; Renshaw et al., 2015). Consistent with this, CN filters combined with DNeasy extraction were found to be more efficient than other filter-extraction combinations (Jeunen, Knapp, et al., 2019). Additionally, dried DNA may be less stable and thus more prone to spontaneous decay (Lindahl, 1993). Few studies have compared the eDNA yield from dry storage versus storage in buffer solutions. One study (Majaneva et al., 2018) reported highest eDNA concentrations for filters preserved in ethanol compared to dried filters after 1 week, but another study found that silica dried filters preserved eDNA equally well as filters stored in ethanol for up to 1 month (Allison et al., 2021). While there was an overall lower eDNA yield on dried filters it should be noted that such an effect may depend on how long filters were stored and how the filters interact with the storage medium. In the case of the GF filters, eDNA yield of filters stored in Longmire’s solution dropped below that of GF filters stored dry after 60 days. These findings highlight the need for more studies comparing storage methods considering various filter types and storage times.

4.4 | Effect of storage time on eDNA yield

The eDNA yield of all treatments was influenced by the storage time. The CN-L treatment exhibited the best preservation of eDNA during the 30- to 68-day period. Previous studies showed that eDNA on CN filters submerged in Longmire’s solution can be effectively stored in the short term (up to 2 weeks; Renshaw et al., 2015; Spens et al., 2017). Only two studies examined long-term storage (80 days: Mauvisseau et al., 2021; 150 days: Wegleitner et al., 2015) of eDNA in Longmire’s solution at room temperature and found no decrease in yield after storage when comparing to immediate eDNA extraction. However, it is important to note that the filters used in
these studies were polyvinylidene fluoride (Mauzisseau et al., 2021) or polycarbonate track etched filters (Wegleitner et al., 2015), respectively. To the best of our knowledge, this is the first study to show steady loss of eDNA on CN filters stored in Longmire’s solution over a relatively short amount of time.

In contrast, eDNA yields from GF filters stored in Longmire’s solution were most affected by storage time. Although these filters initially had comparable eDNA yields to the CN filters, their eDNA yields dropped below those of the other treatments after 68 days. In line with this observation, another study found that eDNA on GF filters was more affected by storage time than eDNA on Sterivex-GP capsule filter (Spens et al., 2017). During the eDNA extraction process, we observed that in contrast to CN filters, GF filters stored in Longmire’s solution appeared to swell, expand, and absorb most of the solution. Previous research on the interaction between borosilicate glass (contained in GF filters) and Tris, which is part of Longmire’s solution, suggests that Tris may have caused the dissolution of the borosilicate glass (Stone-Weiss et al., 2021; Tournié et al., 2013). This dissolution process leads to the formation of Tris-boron complexes and the release of B, Na and Si elements (Tournié et al., 2013) which may have interacted with the DNA present or the extraction process, resulting in a lower eDNA yield during extraction. We speculate that the dissolution of the borosilicate glass may have gradually increased over time, thus causing the decline of eDNA yield over time. This phenomenon has not been reported in other eDNA studies, highlighting the need for further detailed investigation. While GF filters have previously been recommended for eDNA sampling in freshwater (Eichmiller et al., 2016; Lacoursière-Roussel et al., 2016; Minamoto et al., 2016), and marine environments (Takahashi et al., 2020), our findings point to the importance of filter interactions with storage media that need to be considered when developing eDNA protocols.

4.5 Downstream effects on alpha and beta diversity

To assess the impact of filter type, storage method, and time on alpha and beta diversity, we examined the ZOTU richness and ZOTU composition for different treatment types over time. In our study, the interaction between filter and storage method, and between filter and storage time, consistently affected ZOTU richness for both filter types, thereby reflecting the patterns observed on eDNA yield. The CN-L treatment showed the highest ZOTU richness, followed by CN-D, GF-L, and GF-D, mirroring the eDNA yield results. Storage time had a minimal effect on the ZOTU richness of CN filters, but a stronger one on GF filters, particularly GF filters stored in Longmire’s solution. The ZOTU compositions found on CN filters exhibited higher consistency between the different samples than the compositions on GF filters. The composition on GF filters changed with storage time, especially with the Fish16S metabarcode.

In marine environments, to our knowledge, three studies (Deiner et al., 2018; Djurhuus et al., 2017; Jeunen, Knapp, et al., 2019) have examined the effects of protocol choice on metabarcoding assays to date. However, these studies primarily focused on the interaction between capture and extraction methods. In contrast to our study, Djurhuus et al. (2017) found no differences in downstream metrics such as community assemblages and species richness between the different filter types using three broadscale metabarcoding assay. However, Jeunen, Knapp, et al. (2019), focusing on a targeted fish metabarcode, found that CN filters extracted with the DNeasy kit exhibited the highest OTU richness and better consistency between replicates, supporting our results. Similarly, and also supporting our results, Deiner et al. (2018) reported differences OTU richness based on filter material, but not pore size used.

The overall differences in ZOTU composition between the two filter types may be attributed to either the filter material or the different pore sizes. Sassoubre et al. (2016) showed that eDNA exists in a range of particle sizes (i.e., pieces of skin, single cells, free floating DNA) in marine ecosystems. Having larger pore sized GF filters may have biased the species composition towards fish species that shed DNA in larger particles. This could also be reflected in the overall lower ZOTU richness recovered from the GF filters between 30 and 68 days. Yet, investigating the species absent from GF filters compared to CN filters in our samples did not reveal such a conclusive pattern. To test this hypothesis, a study comparing varying filter materials and pore sizes, and extraction of eDNA on day zero would be needed.

We observed minimal effects of a 30- to 68-days storage period on the alpha and beta diversity of CN filters stored in Longmire’s solution or dry and extracted with the DNeasy extraction kit, validating the use of these protocols for eDNA metabarcoding in marine environments. Even though we observed a decrease in eDNA yield, the collected, stored, and extracted samples remained comparable for downstream analyses. Potential data loss within the first 30 days of storage cannot be excluded, but previous studies (Muri et al., 2020; Renshaw et al., 2015; Spens et al., 2017) and consistent community compositions on CN filters regardless of storage method do not suggest such an effect.

In a freshwater setting, and with a storage time of up to 1 week, previous work investigated the effects of different filter and storage methods on eDNA community assays (e.g., Majaneva et al., 2018). The study compared 0.2 μm polyether sulfone and 0.45 μm mixed cellulose ester filters that were either dried, frozen, or stored at room temperature in ethanol or Qiagen’s ATL lysis buffer. Consistent with our study, storing the filters dry or in lysis buffer produced the most OTUs with the least replicate variation. Therefore, our study supports extending these findings to CN filters stored in Longmire’s solution or dry in marine settings, and we recommend this approach also for longer storage periods of one to three months.

The reduction in eDNA yield observed for the GF filters had a direct impact on the alpha and beta diversity. We observed a significant decrease in ZOTU richness between 30 and 68 days for the GF-L treatment, along with consistently lower ZOTU richness for the GF-D treatment. This loss of ZOTUs likely also contributed to greater variability in ZOTU composition on the GF filters. In the case
of the Fish16S metabarc ode, we observed a gradual shift in composition during the storage period. This can most likely be attributed to the gradual loss of low abundance reads, which changed the composition of the detected community.

Differing storage times for GF filters affected the comparability of the samples’ alpha and beta diversity. Our findings indicate that long- or even short-term storage of GF filters, whether dry or submerged in Longmire’s solution, is not recommended. If immediate freezing is not feasible, alternative storage methods such as ethanol preservation (Goldberg et al., 2011; Pilliod et al., 2013) have shown successful results and have been tested for GF filter storage up to 6 days (Minamoto et al., 2016).

4.6 | Limitations

A limitation of our study was the small sample size, which reduced the power to detect effects, especially given the complexity of our models. The larger variation observed, particularly with GF filters, resulted in higher uncertainties in our results, rendering our models inappropriate for extrapolating effects beyond the observed 30–68 days. Increasing the sample size and conducting extractions at regular time intervals would have provided more robust insights. Furthermore, our samples had varying sequencing depths, requiring correction in our analyses on alpha diversity using the Chao estimator for richness per sample. Deeper sequencing would have allowed the use of observed richness, thus enhancing the robustness of our results. Nonetheless, our findings offer valuable preliminary insights and serve as a foundation for inspiring more comprehensive research in this area. Lastly, it is worth noting that the species assignment in this study should be interpreted with caution. Species not previously identified in Shark Bay may indeed be genuine biological signals, but they could also be artifacts resulting from incomplete reference databases and misassignments (Keck et al., 2022). Further investigation into these species identifications is warranted, but it is beyond the scope of this study.

5 | CONCLUSIONS

Our findings demonstrate that filter type, storage method, and storage time have a significant influence on eDNA yield, as well as alpha and beta diversity measurements. Submerging CN filters with pore sizes of 0.45 μm in Longmire’s solution or dry storage proved to retain and preserve the highest amount of eDNA from 3L of seawater after 30–68 days of storage. CN filters with pore sizes of 0.45 μm also captured the highest ZOTU richness and recovered more consistent community compositions. Conversely, GF filters with pore sizes of 0.1 μm retained less eDNA, and ZOTU richness and community composition were less consistent and more affected by storage time. While storage in Longmire’s solution may have had a positive effect on CN filters with pore sizes of 0.45 μm due to the solution of DNA into the buffer, it may have had a negative effect on GF filters with pore sizes of 0.1 μm due to dissolution of the filters. Our results highlight the importance of considering filter-storage medium interactions and storage time as critical factors when optimizing eDNA protocols and comparing samples stored for different durations.

AUTHOR CONTRIBUTIONS

MRB, MK, and FA conceived the study. MRB and RC acquired and processed the data in the laboratory. MRB, JCW, and EPW analyzed and interpreted the data bioinformatically and statistically. MRB, MK, FA, RC, JCW, and EPW wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study is openly available in the European Nucleotide Archive (ENA), reference number PRJEB71101.

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