Brute force prey metabarcoding to explore the diets of small invertebrates

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Abstract

1. Prey metabarcoding has become a popular tool in molecular ecology for resolving trophic interactions at high resolution, from various sample types and animals. To date, most predator-prey studies of small-sized animals (<1 mm) have met the problem of overabundant predator DNA in dietary samples by adding blocking primers/peptide nucleic acids. These primers aim to limit the PCR amplification and detection of the predator DNA but may introduce bias to the prey composition identified by interacting with sequences that are similar to those of the predator. 2. Here we demonstrate the use of an alternative method to explore the prey of small marine copepods using whole-body DNA extracts and deep, brute force metabarcoding of an 18S rDNA fragment. 3. After processing and curating raw data from two sequencing runs of varying depth (0.4 and 5.4 billion raw reads), we isolated 1.3 and 52.2 million prey reads, with average depths of ˜15 900 and ˜120 000 prey reads per copepod individual, respectively. While data from both sequencing runs were sufficient to distinguish dietary compositions from disparate seasons, locations and copepod species, greater sequencing depth led to better separation of clusters. 4. As computation and sequencing are becoming ever more powerful and affordable, we expect the brute force approach to become a general standard for prey metabarcoding, as it offers a simple and affordable solution to consumers that are impractical to dissect or unknown to science.

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Code availability statement

Code for bioinformatics is openly available on github and shared on the Title page. For the review process the code is appended as an anonymous separate text file. Code for filtering and analyses in R-studio is appended as an anonymous rmarkdown file.

Data-availability statement

The raw data is openly available in the NIRD Research Data Archive. DOIs are shared in the Title page.

Keywords: Prey Metabarcoding, Sequence data, Blocking Primers, Species interactions

Introduction

Understanding ecosystems require construction and modelling of complex networks that represent various species interactions, and abiotic factors. In such ecosystems, small invertebrates form important components as consumers, prey, decomposers, pollinators and ecosystem engineers, and are thus critical to include (Kellert, 1993), but large and charismatic animals like birds and mammals have to date garnered the bulk of trophic ecologists attention (Eisenhauer & Hines, 2021). Traditionally, dietary studies have been conducted through direct observation of feeding behavior, or morphological identification of prey species in regurgitate, stomach or fecal contents (Pompanon et al., 2012; Sousa et al., 2019; Symondson, 2002), but these approaches are highly impractical for a large portion of invertebrates whose small bodies complicate stomach content extraction and produce comparatively small fecalia.

Microscopy has helped identify small prey from small consumers, but is laborious and biased in favor of big, well-preserved prey (Berg, 1979), and demands morphological and taxonomic expertise (Pompanon et al., 2012). Conversely, experimental monitoring of communities over time can detect trophic interactions, and even allow quantifying ingestion rates, but struggles in reproducing the natural variability. The species assortment would typically be limited to prey expected in advance, or that co-occurred with the consumer if a natural sample was used as the starting point. Prey metabarcoding has become popular because it allows identification of diverse prey from complex and partly digested material, and does not require considerable *a priori* knowledge of prey, or taxonomical or morphological knowledge from the researcher (Casper et al., 2007). In broad strokes, metabarcoding includes extraction of DNA from dietary material – most often regurgitate, feces or stomach content, PCR amplification of target DNA (the marker gene or barcode), sequencing of PCR amplicons, and culminates with taxonomic identification by comparing obtained sequences to those in a reference database (Santoferrara, 2019).

Deciding on a dietary material is an important step that depends on the logistics and ethics of sampling, or the nature of the species being studied, such as its size or tendency for violence (Pompanon et al., 2012). Medium-sized crustaceans like northern shrimp (*Pandalus borealis*) may be suitable for excision of stomach content (Urban et al., 2022), but small invertebrates ($<$ 1 mm) are challenging to dissect, and may require whole body extraction (e.g. Novotny et al., 2021; Zamora-Terol et al., 2020). This comes at a cost, however, because the majority of DNA in the sample will naturally stem from the consumer itself (Piñol et al., 2014, 2015). An overabundance of consumer DNA may also be a challenge when other materials (e.g., feces or gut content) are sampled (Kohn & Wayne, 1997), but its concentration becomes severely exalted in extracts of whole bodies (Piñol et al., 2014). Hence, the DNA of interest are in minority, while the unexciting consumer DNA will compose a competitive majority.

Conceptually different approaches have been developed to enable prey studies from such "mixed" DNA samples. Nowadays most popular approach was spurred when Nielsen et al., (1991) researched synthetic

analogs to DNA. A polymer with peptide instead of a sugar-phosphate backbone showed particular promise, because it formed stable hybrid duplexes with DNA (Nielsen et al., 1991). So-called peptide nucleic acids (PNAs) had higher melting temperatures than DNA (Egholm et al., 1993), went unrecognized by DNA polymerases, and could not initiate amplification by PCR (Orum et al., 1993). PNAs could be introduced prior to PCR to hybridize irreversibly with a target sequence and thereby suppress its amplification (Orum et al., 1993). Comparatively rare but interesting sequences would thus be allowed to replicate to detectable abundances (e.g. eukaryote parasites of blue crab, Troedsson et al., 2008). Other variants of blocking primers have also been put to the test, such as oligonucleotides modified with inhibitory C3 spacers (Deagle et al., 2009; Vestheim & Jarman, 2008). Since then, blocking primers have been used to study the prey of many different animals and with sample material ranging from whole body extracts of copepods (Cleary et al., 2016, 2017; Durbin & Casas, 2014; Novotny et al., 2021; Ray et al., 2016; Zamora-Terol et al., 2020), to dragonflies and apex canine predators (Morrill et al., 2021; Shi et al., 2021).

Although blocking primers have enabled many prey studies there are issues that warrant attention. Like universal PCR primers typically used in metabarcoding, blocking primers can introduce bias during amplification (Elbrecht & Leese, 2015; Leray & Knowlton, 2017; Piñol et al., 2015). With universal primers, primer-template mismatches and stochasticity may result in skewed relative abundances of species of interest (Sipos et al., 2007). Blocking primer bias also relates to primer-template mismatches, but it is rather the lack of them that leads to unreliable results. A blocking primer should have zero mismatches with the consumer, and as many as possible mismatches with prey to limit off-target interactions. Piñol et al., (2015) showed that a blocking primer with four and five mismatches to interesting prey decreased their relative abundances. Hence, PNAs and blocking oligonucleotides may introduce strong taxonomic biases during amplification (Piñol et al., 2015, 2019). Furthermore, production of specific and ultimately successful blocking primers remains an expensive chemical procedure, and relies on both consumer and prey sequences before the design can begin.

We have explored a simple, cost-efficient, and versatile approach to prey metabarcoding of small invertebrate consumers. By sequencing deep to offset for the overabundance of consumer DNA, we show that one can obtain ample prey reads from mixed whole-body copepod extracts, while at the same time avoiding the costs and laborious design of potentially biased blocking primers. Conceptually, we argue that the brute force method holds a lot of promise because current sequencing and computation enable acquiring and processing large amounts of data, and continuous development will only improve these capabilities (Lightbody et al., 2019). Through two sequencing runs (a pilot and a full-scale) using two commercially available NGS platforms (Illumina HiSeq4000 and Illumina NovaSeq6000), we tested the brute force approach for the first time with marine invertebrates. We report on the effect of sequencing depth for resolving prey composition and discuss advantages and caveats of the brute force methodology for prey studies of small consumers.

Materials and Methods

Sample collection

Copepods were collected on four seasonal cruises from the central Barents Sea to the Arctic Nansen basin north-east of Svalbard, Norway (Table S2). Cruises occurred during Autumn (5 – 27 August 2019), early winter (28 November – 17 December 2019), late winter (2 – 24 March 2021) and early spring (27 April – 20 May 2021), and visited three stations on the Barents Sea central shelf (76.00N, 31.22E), northern shelf (79.72N, 34.32E) and Nansen basin (81.83-82.16N, 28.15-29.84E, positions varied due to sea-ice drift, Fig. S1). Small-sized mesozooplankton (<1 mm) were collected in vertical 64 µm Bongo-net hauls (to full depth or max. 1000 m, ascent = 0.3 m s^{-1}, descent = 0.5 m s^{-1}, 60 cm mouth diameter). All large and/or gelatinous animals (1-10 cm) were removed, and the remaining suspension sieved (64 µm) to discard seawater. Ice-cold ethanol (96%, -20 °C) was then used to rinse retained mesozooplankton, before transfer into a sample bottle. The container was topped up with ice-cold ethanol and stored at -20°C.

Microsetella norvegica(Boeck, 1865), *Microcalanus* spp. (*M. pygmaeus* or *M. pusillus* , Sars G. O., 1900-1903) and *Oithona similis* (Claus, 1866) were morphologically identified under a stereomicroscope (Table S2). Up to 14 individuals per species and station were picked where available. Each specimen was thoroughly rinsed individually three times in Milli-Q water, transferred to tissue lysis buffer (E.Z.N.A Tissue DNA kit, Omega Bio-tek). Surface sterilization with bleaching was excluded, since the minute body size (< 1 mm) of the copepods analyzed herein raised concerns regarding how the treatment could penetrate and potentially alter the dietary signal. Also, existing literature, mostly based on arthropods, are in disagreement regarding the efficacy of bleaching, with one study indicating little effect on the overall dietary signal (Miller-ter Kuile et al., 2021). DNA extraction was performed per manufacturer's protocol ("Tissue Spin Protocol", E.Z.N.A® Tissue DNA kit, Omega Bio-Tek), with a lowered elution volume of 2 x 50 µL elution buffer. One negative without material was included with every round of extraction.

We checked all copepod and negative DNA extracts by PCR amplification of a 18S V7 fragment (˜240 bp) using the universal eukaryotic primers 960F (5'-GGCTYAATTTGACTCAACRCG-3') and modified 1200R (5'-GGGCATCACAGACCTG-3') (Cleary & Durbin, 2016; Gast et al., 2004, Table S1). The amplifications were performed in 10 µL reaction volumes (4.8 µL MQ water, 1.0 µL DreamTaq buffer (10X), 0.2 µL DreamTaq Polymerase (5 U μL^{-1}), 1.0 µL dNTP (2.5 mM each), 0.5 µL forward-primer (960F, 10 µM), 0.5 µL reverse-primer (1200R, 10 µM) and 2.0 µL template). PCR negatives had 2 µL MQ instead of template. Thermal cycling consisted of an initial denaturation (2 min, 95°C), and 35 cycles of denaturation (30 s, 94°C), annealing (30 s, 54°C), and elongation (30 s, 72°C), with a final elongation step (15 min, 72°C). The success of the amplifications was inspected by 1% agarose gel electrophoresis, confirming single band PCR products in all samples and no amplification in all extraction negatives.

Library preparation and sequencing

For sequencing, we amplified a short hypervariable fragment of the 18S SSU rRNA V7 region (˜100 – 110 bp) with 18S_allshorts primers (Forward 5'-TTTGTCTGSTTAATTSCG-3', and Reverse 5'-GCAATAACAGGTCTGTG-3') (Guardiola et al., 2015). Broad coverage within Eukarya was verified with the Arb Silva TestPrime function (Klindworth et al., 2013). To enable pooling of samples after amplification, primers were pre-tagged with an 8-base oligonucleotide, wherein at least 3 nucleotides differed between tags. The primers also contained a leading 5'-end variable number of degenerate nucleotides (N, 2 [?] 4) to increase sequence variability and hence Illumina sequencing quality (Wangensteen et al., 2018). PCR amplification and all downstream processing from here and onwards were conducted twice. First as a pilot with a limited number of samples and negatives ($N_1 = 79$), and finally with a full set of samples and negatives ($N_2 = 456$). Samples were processed using the exact same protocol unless stated otherwise.

PCR amplifications were performed in 20 µL reactions with 10.00 µL AmpliTaq Gold Master Mix (Applied Biosystems), 0.16 µL Bovine Serum Albumin (BSA, 20 µg μL^{-1}), 5.84 µL ultrapure MQ water, 2.00 µL of 18S_allshorts Forward and Reverse primer mix (2.5 µM each), and 2.00 µL DNA template. Thermal cycling consisted of an initial denaturation step (10 min, 95°C), and 35 cycles of denaturation (30 s, 95°C), annealing (30 s, 45°C) and elongation (30 s, 72°C). For each PCR plate, a subsample of real samples (n = 9) and a PCR negative were tested on a 1% agarose gel to verify amplification and a lack thereof, respectively. PCR amplicons were then pooled, purified with MinElute spin-columns (Qiagen, Hilden, Germany) and quantified using the broad-range dsDNA assay on a Qubit 4 fluorometer (Invitrogen by ThermoFisher). Sequencing-ready libraries were prepared from purified pools in accordance with the NEXTflex PCR-Free DNA Sequencing Kit (Bioo Scientific, Austin, Texas, USA). Here, DNA templates (3000 ng total for each library) were first purified by size (retaining fragments >150 bp) with magnetic Agencourt AMPure XP beads (Beckman Coulter Genomics, California, USA), then adenylated, and ligated with Illumina-compatible adapters. We used several Illumina-compatible adapters (NEXTflex DNA Barcode Adapter, Bioo Scientific) to distinguish libraries of 96 samples. The libraries were quantified by qPCR using the NEBNext(r) Library Quant Kit for Illumina(r) (New England Biolabs, Massachusetts, USA) to verify successful preparation. For the pilot sequencing one library of 79 samples (75 real and 4 extraction negatives), were sequenced using 150 Paired-End (PE) chemistry on a HiSeq 4000 platform (Novogene Co., Ltd.). For the full sequencing run,

five libraries with a total of 456 samples (437 real and 19 extraction negatives), were sequenced using 150 PE chemistry on two lanes of a NovaSeq6000 platform (Novogene Co., Ltd.).

Bioinformatic processing

A custom bioinformatics pipeline based on the OBITools (v. 1.2.12, (Boyer et al., 2016)) and VSEARCH (v. 2.9.1, (Rognes et al., 2016)) software suites and the unoise3 algorithm (Edgar, 2016) was developed to process reads (available at: anonymized github-link). Forward and reverse reads were paired with the illuminapairedend function. The reads were then passed via criteria selecting aligned reads of high-quality (score $>$ 40.00), assigned to sample and trimmed based on the sequences of the primers and attached oligonucleotide tags (ngsfilter). Reads were further selected for sequence non-ambiguity and read lengths between 80 to 120 bp. To enable faster processing, the data were split by sample, and distributed over several CPUs whom in parallel performed dereplication (obiuniq), sorting (vsearch –sortbysize), denoising with removal of rare sequences (vsearch –cluster_unoise, –minsize 4, –unoise_alpha 8) and chimera removal (vsearch –uchime3_denovo). Resulting sequence variants are hereon referred to as zero-radius Operational Taxonomic Units (zOTUs). After the most computationally heavy processing, all sample subfiles were concatenated, and zOTUs were reassigned to sample (obiuniq). Finally, taxonomy was assigned to the Protist Ribosomal database (PR2, v.4.14.0, Guillou et al., 2013) using blastn (BLAST+, v. 2.8.1, Altschul et al., 1990; Camacho et al., 2009).

Curation of prey

To obtain datasets with putative prey only, the assigned reads were subjected to a two-step curation process in R studio (v. 4.1.3). First, the reads were manually filtered based on taxonomy using 'tidyverse' functions (Wickham et al., 2019). All Maxillopoda reads were discarded to remove consumer DNA. We acknowledge that maxillopods may compose a food-source for the species studied but the short read-length used to capture prey from partly digested materials, did not allow for distinguishing DNA from maxillopod prey and consumer. Taxa known to interact with copepods (any Copepoda) in symbiosis (parasitism, commensalism and mutualism) were recorded from current literature (Bass et al., 2021; Cleary et al., 2017; Cleary & Durbin, 2016; Zamora-Terol et al., 2021) and used to discard likely non-dietary interactions. By inspection, we discarded several zOTUs assigned to unlikely prey including seed-plants, insects and mammals. Putative contaminants from the marine environment were likewise discarded, notably large gelatinous organisms (Cnidaria, Ctenophora). We acknowledge that also these taxa may have a dietary origin, but we consider it more plausible that most of the sequences originated from the batch sample from which the copepods were picked. Gelatinous organisms are sticky and fragile, and have been suspected of contaminating other studies of copepods using similar methodologies (Cleary et al., 2017). Decontam was used to identify and discard remaining contaminants by comparing the prevalence of putative contaminant zOTUs in real samples and extraction negatives (the 'prevalence method', Davis et al., (2018)). Relatively few contaminants (2 and 38 for pilot and full datasets, respectively) were identified and discarded at this stage. The remaining zOTUs were considered putative prey, and were stored with metadata as phyloseq-objects (McMurdie & Holmes, 2013).

Sample metrics and analyses

Read metrics (counts and number of zOTUs) were acquired using different summarizing functions during bioinformatic processing (grep, obigrep, gawk), and selection functions (tidyverse, Wickham et al., 2019) during taxonomic filtration in R. To isolate the effect of differential sequencing, we generated an additional dataset (referred to as 'pilot full') by sub-setting – from the full dataset – the same real samples that were sequenced in the pilot (n = 75). To test how well the different datasets represent prey diversity, we performed rarefaction analyses with the rarecurve function (step = 10) of vegan (Oksanen et al., 2019), and calculated the average number of reads required to discover new zOTUs (rareslope, sample = sample total reads).

Testing the effect of sequencing depth on prey composition

Non-metric multidimensional scaling (NMDS) plots were used to visualize how depth of sequencing and data

transformation may influence prey composition resolution. Multiple variants were made to explore beta-diversity using both occurrence, and presence/absence-based dissimilarity metrics. All ordinations were computed from sample-wise dissimilarities from compositional data at zOTU-level. Relative abundances were used to compute Bray-Curtis dissimilarities (Bray & Curtis, 1957), whereas a presence/absence-table enforcing a 0.01% relative abundance threshold was used for Jaccard dissimilarities (Jaccard, 1901). We used Scree-plots to find the appropriate dimensions for a conservative acceptance threshold of [?]0.1 stress. Final NMDS plots were calculated iteratively (trymax = 100) with adequate dimensions (k = 4 or 5) using the metaMDS function of vegan (Oksanen et al., 2019). PERMANOVA (nperm = 10000) analyses were used to test if three explanatory variables (season, station and species) accounted for the observed variance in prey composition. We subsequently tested if the grouped samples had homogenous and comparable dispersions (p [?] 0.05), or if compositional differences in prey could be due to heterogenous dispersion among groups ($p < 0.05$, Betadisper).

Results

Detected prey

Identified prey zOTUs belonged to a broad range of eukaryote taxa including metazoans (e.g. Chaetognatha, Urochordata, Rotifera), fungi (Ascomycota, Basidiomycota), ciliates (e.g. Spirotrichea), dinoflagellates (Dinophyceae, Dinophyta_X), heterokonts (e.g. Bacillariophyta, Chrysophyceae, Labyrinthulea) and radiolarians (Acantharea). Mean relative abundances of prey are available in the supplementary files (Table S4), and prey compositions are presented in greater detail in an upcoming publication (Flo et al., unpublished data).

Sequencing metrics

The pilot sequencing (N = 79 including sequencing blanks) led to a total of 412 million paired end raw reads, which initial processing steps (pairing, filtering by length, quality and ambiguity, and demultiplexing) reduced to 342 million ("Pilot", Table 1). Removal of chimeric, erroneous and rare sequences further reduced the dataset, and after taxonomic identification 284 mill. reads (69% of raw) distributed over 49 697 zOTUs were isolated. Most of the reads that were subsequently filtered out were assigned to the consumer taxon Maxillopoda (98% of assigned reads), whereas reads identified as contaminants or symbionts accounted for 1.6% and 0.2% respectively. The final dataset of putative prey counted 1.2 million reads (0.4% of the assigned reads) in 1500 zOTUs. Distributed over 75 real samples, the pilot averaged 16 000 prey reads per copepod consumer.

The final sequencing, with an increased number of samples (N = 456 including sequencing blanks), yielded 5.4 billion paired end raw reads ("Full", Table 1). Of these, approximately 4.3 billion reads (79% of raw) in 130 000 zOTUs were subsequently assigned to taxonomy. After discarding zOTUs assigned to Maxillopoda (98% of assigned reads), contaminants (1.1%) and symbionts (0.2%), the putative prey counted 52.2 million reads in 22 391 zOTUs. This corresponded to 1.2% of the assigned reads, or 1.0% of the raw reads, and a mean depth of ˜120 000 prey reads per copepod consumer. Compared to dividends from relevant literature using dissection or blocking primers, the average prey reads per sample of both sequencing runs were more than two times greater (Table 2).

Table 1: Summary of read and zOTU abundances before and during bioinformatic processing (Step 1-4), according to sample type (real samples or extraction negatives), and according to taxonomic identity (consumer, symbiont, contamination, prey). The total number of samples (N) are presented for both sequencing runs, and the number of extraction negatives and real samples are indicated in parentheses for the pilot (n_p) and for the full sequencing (n_f). Sample types and identified taxa are also presented with percentage-wise contributions to the total of assigned reads (percentage of assigned; POA) or to assigned reads from real samples (POA^+).

	Pilot (HiSeq 150 PE, N = 79)	Pilot (HiSeq 150 PE, N = 79)	Pilot (HiSeq 150 PE, N = 79)	Full (2 x Novaseq 150 PE, N = 456)	Full (2 x Novaseq 150 PE, N = 456)	Full (2 x Novaseq 150 PE N = 456)
Step	Reads	zOTUs		Reads	zOTUs	
Raw PE reads	412 449 403	-	-	5 436 416 402	-	-
Paired, filtered, demultiplexed	342 785 829	-	-	4 857 351 483	-	-
Denoised, without chimeras or singletons (<4)	284 213 421	49 723	-	4 268 556 612	130 677	-
Assigned to taxonomy	284 212 399	49 697	-	4 268 371 437	129 940	-
Sample type			POA			POA
Extraction negatives (n_p = 4, n_f = 19)	10 801 345	1 986	3.80	8 269 055	5 046	0.19
Real samples (n_p = 75, n_f = 437)	273 411 054	48 252	96.20	4 259 081 079	129 182	99.78
Identified taxa in real samples			POA^+			POA^+
Consumer	267 342 579	43 766	97.78	4 153 774 204	90 725	97.53
Symbionts	587 313	599	0.21	8 160 170	4 266	0.19
Contaminants	4 291 765	2 387	1.57	44 980 919	11 800	1.06
Prey	**1 189 397**	**1500**	**0.44**	**52 165 786**	**22 391**	**1.22**

Rarefaction

We used rarefaction to infer whether the sampling depths were sufficient to describe the full prey composition (i.e. zOTU richness). In theory, when rarefaction curves approximate a plateau, only a few novel zOTUs will be found with increased sequencing depth, thus indicating that the samples represent the full diversity of prey. The datasets all displayed plateau-like curves (Fig. 1), but curves of from the full sequencing run (pilot full, full) were arguably less steep. The average slope (i.e. zOTU discovery rate) of samples in the full dataset were smaller (0.0045, i.e. 4.5 new prey zOTUs per 1000 reads, Fig. 1c) as opposed to 0.0103 for the pilot dataset (i.e. 10.3 new zOTUs per 1000 reads, Fig. 1a).

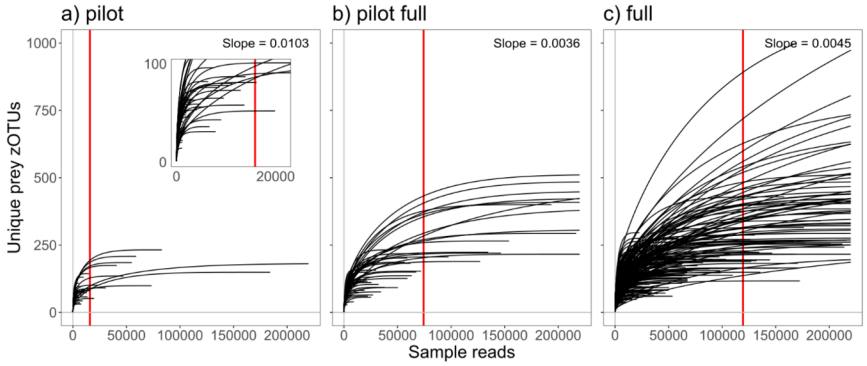

Figure 1: Rarefaction curves of prey data acquired from a) the pilot (n = 75), b) the upscaled sequencing run but with the copepod samples assessed in the pilot only (pilot full, n = 75), and c) the full upscaled sequencing (n = 437). Vertical red lines indicate in averages of a) 16 000, b) 74 000 and c) 119 000 prey reads per copepod. Slopes indicate the average number of prey zOTUs discovered with each new read sequenced.

Ordination and statistics

We prepared NMDS plots of all three datasets ("Pilot", "Pilot full" and "Full", see explanation below) with abundance and presence-absence based dissimilarity metrics to investigate the importance of depth and transformation for determining the composition of prey (Fig. 2). Depending on the dataset being used, ordination required 4 or 5 dimensions to reach a conservative and low stress-level of 0.1. The pilot prey reads required fewer dimensions (k = 4) than the full dataset and the dataset consisting of the full subset (k = 5). Regardless of dataset or metric, prey composition differed significantly between copepods from different seasons and stations (PERMANOVA, $p < 0.001$, Fig. 2). The most visually distinct clusters were found when using the season sampled for profiling prey compositions, and samples acquired during the pilot ("Pilot", Fig. 2a and d) formed less distinct clusters than those from the full sequencing. The same physical samples subset from the full dataset ("Pilot full", Fig. 2b and e) formed more divergent clusters. Ordination of the complete set of samples ("Full", Fig. 2c and f) returned a pattern typical of a seasonal transition, with prey compositions from successive seasons overlapping, and samples from disparate seasons (e.g. August and April/May) forming separate clusters. Successive Betadisper tests (Table S3) indicated however that the clusters observed may be influenced by heterogenous dispersion (e.g. Fig. 2f). The copepod species sampled was a less significant predictor of pilot and pilot full diets (Fig. 2a and b) when using Bray-Curtis as dissimilarity metric ($p = 0.003$ and $p = 0.03$, respectively), than with Jaccard. For both datasets with greater depth ("Pilot full", and "Full"), Jaccard dissimilarities led to visually greater separation of clusters.

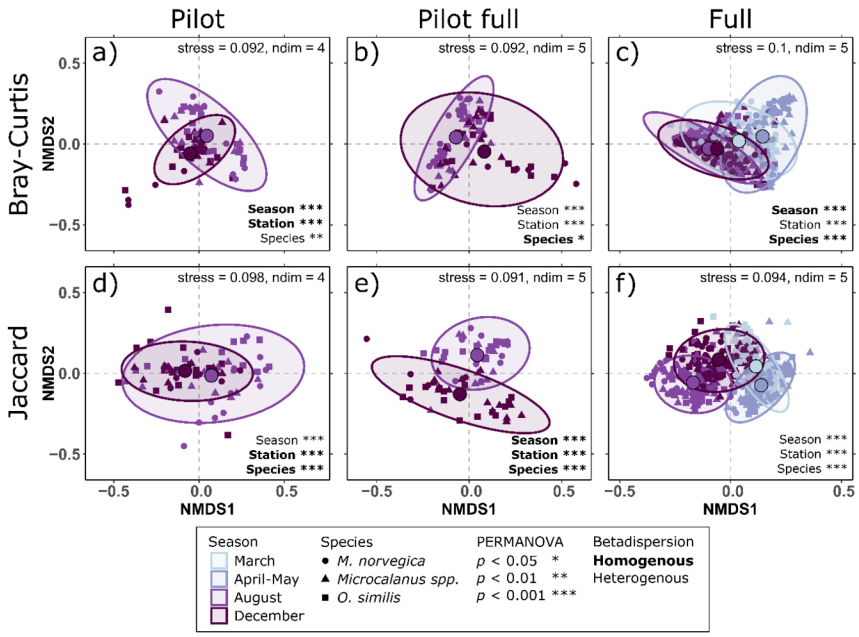

Figure 2: Non-metric multidimensional scaling (NMDS) of dietary samples from (a, d) the pilot dataset (n = 75), (b, e) the same samples sequenced during the pilot, but from the full sequencing run (n = 75) and (c, f) the full sequencing with all samples (n = 437). Ordinations in (a, b, c) are based on Bray-Curtis dissimilarities, whereas (d, e, f) are based on Jaccard-indexed dissimilarities computed from presence-absence data (zOTU presence = >0.01%). Colors distinguish the season of sampling, whereas shapes denote the identity of the consumer. Overlayed ellipses indicate 95% confidence levels, and accompanying centroids show the average within-group positions. Stress and number of dimensions (demanding a stress of ˜0.1 or less) are shown. Significant differences between levels of groups (seasons, stations, and species) are shown based on PERMANOVAs, and in bold if the group dispersion was found to be homogenous (betadisper).

Table 2: A summary of recent studies using prey metabarcoding to assess copepod trophic interactions. The list includes the consumers studied, the dietary source of DNA extractions, total numbers of assigned 18S reads from all sources (e.g. consumer, prey, symbiont, contaminant) and prey. In addition, we report the average prey reads per sample (calculated from reported sample size and total prey reads), the 18S fragment and its putative length (bp). The use of blocking primers is listed with concentrations of blocking primer per concentration of template DNA. Putative methodological biases are listed, and we outline the conceptual requirements and suitable consumers for each approach. NA = Not Available. + = prey reads are calculated from the reported proportions of total reads. ++ = unknown if reads are assigned or raw. +++ = unknown if reads are prey or all assigned.

Ref.	Copepod consumers	Dietary source	Assigned 18S reads	18S prey reads	Avg. prey reads per sample	Fragment (bp)	[Blocking primer] per [DNA]	Bias	Requireme
This study (pilot)	*O. similis*, *M. norvegica*, *Microcalanus* spp.	Whole bodies	284 212 399	1 189 397	16 000	18S V7 (˜100-110)	No	PCR primers, CNV	Upscaled sequencing
This study (full)	*O. similis*, *M. norvegica*, *Microcalanus* spp.	Whole bodies	4 268 371 437	52 165 786	120 000	18S V7 (˜100-110)	No	PCR primers, CNV	Upscaled sequencing
Hirai et al., (2018)	*Calanus sinicus*	Dissected guts	2 919 386	$^{+}$ 106 266	2 678	18S V9 (˜130)	No	PCR primers, CNV	Dissection
Yeh et al., (2020)	*Calanus finmarchicus*	Dissected foregut	NA	NA	NA	18S V4 (NA)	No	PCR primers, CNV	Dissection
Cleary et al., (2017)	*Calanus glacialis*	Whole bodies	$^{++}$ 11 266 639	638 231	7 975	18S V7 (˜250)	20 µM PNA per ˜0.5 ng µL^{-1} DNA	PCR primers, CNV, blocking primers	PNA design and costs
Cleary et al., (2016) *P. newmani*, *P. acuspes*	*Whole bodies*	*NA*	*28 456*	*618*	*18S V7 (˜250)*	*20 µM ΠNA περ ˜0.5 νγ µΛ^{-1} ΔNA*	*PCR primers, CNV, blocking primers*	*PNA design and costs*	*Any invertebrate whose target sequence is known*

Ref.	Copepod consumers	Dietary source	Assigned 18S reads	18S prey reads	Avg. prey reads per sample	Fragment (bp)	[Blocking primer] per [DNA]	Bias	Requireme
Ho et al., (2017)	Calanus sinicus	*Dissected anterior digestive tracts*	*+++ 2 183 773*	*NA*	*NA*	*18S V4 (˜300-350)*	*2 mM PNA per ˜1.7 νγ μΛ^{-1} DNA*	*PCR primers, CNV, blocking primers*	*PNA design and costs, dissection*
Novotny et al., (2021)	Temora longicornis, Acartia *spp.*, Pseudocalanus *spp.*, Centropages hamatus	*Whole bodies*	*NA (>37 mill. 16S + 18S reads)*	*NA*	*NA*	*18S V4 (˜400)*	*Yes, NA [PNA] per NA [DNA]*	*PCR primers, CNV, blocking primers*	*PNA design and costs*
Zamora-Terol et al., (2020)	T. longicornis, Acartia *spp.*, Pseudocalanus *spp.*, C. hamatus, Eurytemora affinis	*Whole bodies*	*12 800 000*	*1 200 000*	*NA*	*18S V4 (˜400)*	*Yes, NA [PNA] per NA [DNA]*	*PCR primers, CNV, blocking primers*	*PNA design and costs*

Discussion

The pragmatic approach applied here facilitated prey studies of small-sized copepods despite an overabundance of non-informative consumer DNA. By scaling up the sequencing to offset losses in consumer reads, we avoided using expensive and potentially biased blocking primers. While the pilot sequencing resulted in approximating the full diversity of our samples, deeper sequencing reduced the zOTU discovery rate from 10.3 in the pilot to 3.6 and 4.5 new prey zOTUs per 1000 reads in the full pilot and full datasets, respectively. Our results from small-sized marine copepods, which are known to consume a wide diversity of prey, thus suggest that upscaled brute force sequencing is a suitable general method for determining the prey of small consumers, regardless of *a priori* access to the consumer sequence or possibility of dissection.

Sequencing; how deep is deep enough?

The brute force concept was initially tested by Piñol et al., (2014) to determine the prey of the *Oedothorax*

fuscus spider. Using a single pooled sample of 109 individual extracts they acquired what they called 'ample' prey reads. Most sequence reads originated from the consumer, although the percentage of *O. fuscus* reads to the total was not reported. Their overall recovery was 61 000 prey reads, translating to 6% of all assigned reads. In our study the prey recovery rates were much lower, with prey sequences accounting for only 0.44% and 1.22% of the assigned reads in the "pilot" and "full" sequencing runs, respectively. Thus, deeper sequencing was needed to acquire enough reads to cover the full diversity of prey species.

Nonetheless, if comparing prey read metrics with recent studies using PNA or other blocking primers (Table 2), it becomes clear that the applied brute force approach yielded a high absolute prey read output (1.2 and 52.2 mill. prey reads), regardless of high consumer losses. Reaching similar numbers with the use of blocking primers, a recent study of Baltic Sea copepods reported 1.2 million prey reads from an unknown number of samples (Zamora-Terol et al., 2020). The use of a PNA blocker also did not eliminate the consumer DNA problem completely, given that the total read count before discarding Maxillopoda ASVs was 12.8 million reads. Hence, despite using blocking primers, 89% of the sequences were uninformative and discarded. Cleary et al., (2017) also acquired a large dataset with a total of 11.3 million reads from 80 samples of *Calanus glacialis* copepods. Although a PNA probe also here was used to block amplification of *Calanus* spp., only a smaller portion of the dataset belonged to prey taxa (638 231 reads, 7 975 prey reads per sample). Whether the discarded reads were assigned to contaminant, symbiont or consumer sources was not reported, but we find it likely that the majority was sourced from the consumer DNA. Likewise, a study of *Pseudocalanus* spp. copepods found 28 000 prey sequences in 46 samples (Cleary et al., 2016). PNA-PCR was used also here to block amplification of the consumer, but as the authors did not report the initial read counts, the success rate of blocking is not known.

What influences prey sequence recovery?

There are many factors that can influence the recovery of prey sequences and thus the success of the brute force approach. The length of the target sequence is important, with small amplicons being favored due to a longer half-life and hence an increased detectability (Kamenova et al., 2018). Whether the extracts are based upon recently ingested or heavily digested materials (e.g. feces) will also have an impact on recovery (Kamenova et al., 2018). For small invertebrates, whole-body extraction is us usually the only feasible alternative. With whole-body samples, we find it probable that the consumer DNA has the greatest impact on prey sequence recovery as it competes for amplification and detection during PCR and sequencing. The severity of the overabundance problem is difficult to predict in advance, however, given that different consumers have variable genome sizes, cell numbers and target gene copy-numbers. Indeed, the ratio of the prey to consumer sequence may also vary from one season to the next, between different sexes, feeding or life stages. The exact ratios acquired here may thus not be representative for studies of other invertebrates although the study design is otherwise identical. Nonetheless, we show that the problem of consumer DNA overabundance may be overcome by sequencing deeper.

With a great overabundance of putative consumer DNA (98%), our results underline the problem of dietary samples from small animals. In fact, over 3.8 billion of the sequence reads belonged to the top three abundant zOTUs, recruiting 1.6, 1.4 and 0.8 billion reads, respectively. Although we could not resolve the taxonomy of these zOTUs beyond Maxillopoda, we are confident that these represent the three sampled copepod species just based on the abundance of their read counts. The first sequence variant dominated samples of *Oithona similis* , the second dominated samples from *Microcalanus* spp., and the third dominated samples from *Microsetella norvegica* . Other abundant maxillopod zOTUs with similar distribution patterns were also identified. These may be artifacts of the dominant sequences, real variants of the gene sequenced, or remnants of copepod prey. As we are unable to distinguish between these alternatives, we conservatively chose to discard all Maxillopoda sequence reads from our analyses.

It is however not just consumer DNA that is problematic as contaminants may further dilute samples, making finding enough prey data more difficult. In the datasets analyzed in this study, we observed more reads from the four extraction negatives sequenced for the pilot dataset (10.8 mill. reads) than in the combined twenty extraction negatives from the full dataset (9.3 mill. reads). This holds also at the level of real samples, given

that the pilot dataset contains a higher contaminant percentage (1.6%) than the full dataset (1.1%). If we somehow "worked cleaner" during preparation of the second sequencing run is not known, but we hypothesize that increased contamination could explain some of the observed gap in prey recovery (0.4% in pilot versus 1.2% in full dataset), since also contaminant sequences may compete for amplification and sequencing. A lot of non-prey sequences would inevitably lead to a lower output of prey, and the more contaminated samples are, the greater the problem. Future dietary studies may arguably increase the yield of prey sequences by paying attention to lab routines and making them as clean as possible.

Other possible influences on prey sequence recovery could be technical – for instance that deeper sequencing leads to a greater number of rare prey zOTUs surpassing a set cutoff-value (we denoised each sample individually with a four reads cutoff), or related to the biology of the consumer. Several Arctic copepod species may for example enter diapause prior to the polar night. A dormant state ensues, where energy spent on motility, reproduction and feeding is drastically lowered (Conover, 1988). Because internal wax ester storages are used for energy, feeding activities are no longer required. Consequently, some copepod species may be expected to contain little or no prey DNA during certain periods of the year. The copepods studied here are not expected to enter diapause, but may to some extent reduce metabolic rates during winter (Conover & Huntley, 1991).

Advantages of the brute force method

We argue that for metabarcoding of dietary samples, the brute force method is superior to blocking primers by being simpler, cheaper, faster and offering a less biased result. It is simpler because, theoretically, the trophic interactions of any animal may be sampled and studied regardless of whether it is well known to science or just recently discovered. With a blocking-primer approach, the mandatory first step would be to have the targeted barcode of the consumer sequenced (unless it already exists). With sequence in hand, a site must be found that is unique and targets the consumer only. Site selection is difficult, however, because the blocking primer must be designed based on the sequences of both consumer (should match blocking primer perfectly) and putative prey (should be different enough to not bind the blocking primer). Ideally, one should also test the blocking primers against putative prey to make sure that the mismatches hinder hybridization. Services like TestProbe have been used to test blocking primers against broad databases like Silva (e.g. Ray et al., 2016), but it would be naive to expect that all putative prey sequences are available, especially for little known organisms sampled from frontier environments. Exactly how many nucleotide mismatches should be demanded, and at what point the blocking primers start affecting the amplification of other relevant sequences is unknown. Piñol et al., (2015) found that amplification of non-target DNA was blocked even when the number of mismatches were 4 and 5 base pairs. Also, prey studies often utilize extra short sequences that sacrifice on taxonomic resolution to allow detection of prey from partly digested material. These factors make the blocking primer approach particularly problematic. Designing primers that solely block the consumer within a short stretch of DNA can be difficult, and there are no guarantees of finding out whether the study is doable before investments in sanger sequencing, blocking primer design or both have been made.

Costs of sequencing versus blocking primers

It is not straightforward to directly compare the costs of designing and applying blocking primers to that of ordering greater depth from a sequencing provider. We nonetheless argue that the potential for upscaling sequencing depth is already great – and will continue to improve, as sequencing technology becomes ever more sophisticated and cost-effective. Primer manufacture, and especially that of PNA primers remains a time-consuming and complicated chemical procedure that is less amenable to change or development. Due to high demand and a growing customer base, sequencing providers are also forced to compete amongst themselves for customers, often leading to offers of discounts or even help with post-sequencing computation.

Conclusions

Our results show that brute force sequencing can serve as a simple and efficient approach for prey metabarcoding of consumers that are less known to science or difficult to dissect. Exactly how many extra reads one should ask for to offset the losses to consumer sequencing depends on multiple often unknown factors like copy number variation, life cycle and seasonal parameters. We therefore suggest sequencing deeper than what is strictly required based on an arbitrary ratio (e.g. with expectancy of 99% consumer reads), as this approach is more cost-efficient than resequencing samples with low coverage.

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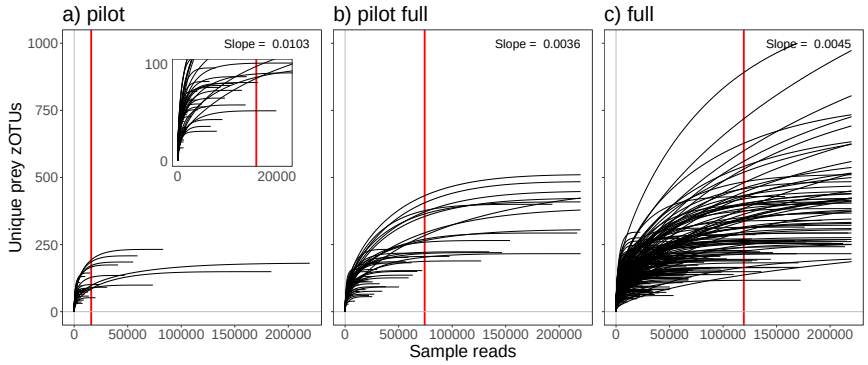
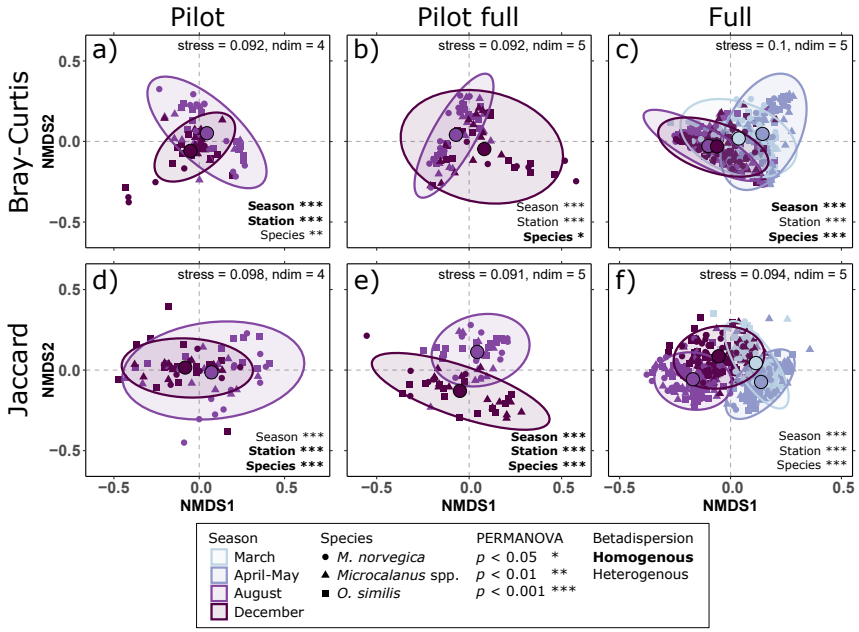


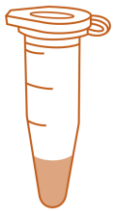


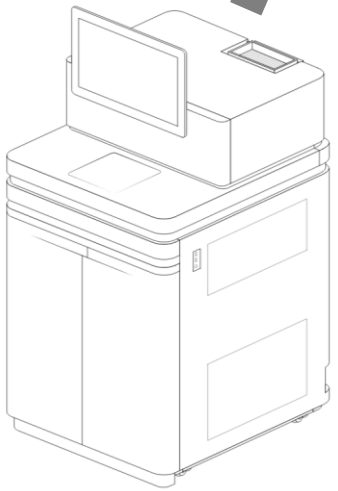
Unknown prey

Whole body DNA extraction

In situ feeding

Small invertebrates





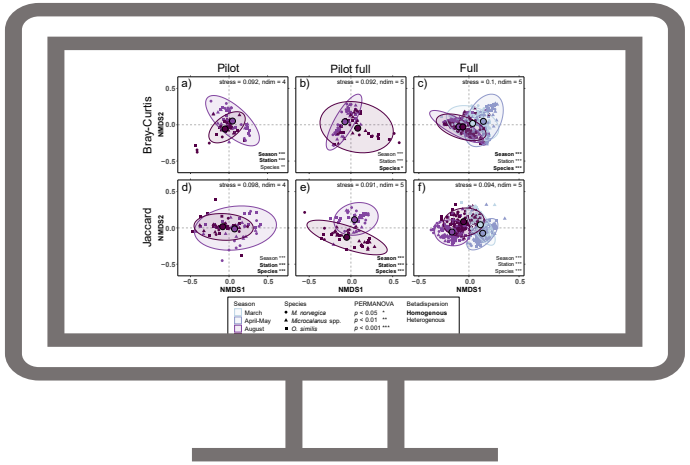
Deep sequencing

```
@AOCPOL1
ACGTCGGAGTGATGCTGATG
CTAGCATGTATGTCCATGAG
TCCCAAAGTCA
+
HHHHHHAACCAAHHIII
```

Billions raw reads

```
id tax s1 s2 s3 s4 …
zotu9 Bac… 13 99 99 …
zotu14 Cha… 0 15 80 …
zotu21 Bac… 0 14 90 …
…
```

Prey reads



Prey interpretation