Post-bioinformatic methods to identify and reduce the prevalence of artefacts in metabarcoding data

Running title: Dietary metabarcoding artefacts

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Abstract

Metabarcoding provides a powerful tool for investigating biodiversity and trophic interactions, but the high sensitivity of this methodology makes it vulnerable to errors, resulting in artefacts in the final data. Metabarcoding studies thus often utilise minimum sequence copy thresholds (MSCTs) to remove artefacts that remain in datasets; however, there is no consensus on best practice for the use of MSCTs. To mitigate erroneous reporting of results and inconsistencies, this study discusses and provides guidance for best-practice filtering of metabarcoding data for the ascertainment of conservative and accurate data. The most common MSCTs identified in the literature were applied to example datasets of Eurasian otter (*Lutra*

lutra) and cereal crop spider (Araneae: Linyphiidae and Lycosidae) diets. Changes in both the method and threshold value considerably affected the resultant data. Of the MSCTs tested, it was concluded that the optimal method for the examples given combined a sample-based threshold with removal of maximum taxon contamination, providing stringent filtering of artefacts whilst retaining target data. Choice of threshold value differed between datasets due to variation in artefact abundance and sequencing depth, thus studies should employ controls (mock communities, negative controls with no DNA and unused MID-tag combinations) to select threshold values appropriate for each individual study.

Keywords: contamination, diet, eDNA, environmental DNA, false positives

Introduction

Metabarcoding provides a powerful tool for ecological studies of biodiversity and trophic interactions (Deiner et al. 2017; Taberlet et al. 2018). By combining high throughput sequencing (HTS) with DNA barcoding, large volumes of high-resolution data can be generated from many samples simultaneously (Taberlet et al. 2018). As an accurate means of detecting and identifying not just common species, but also cryptic and rare species, metabarcoding has in many cases superseded traditional methods such as morphological analysis of prey remains in gut contents and faeces, and direct observation (Bowser et al. 2013; Roslin and Majaneva 2016; Elbrecht, Vamos, et al. 2017). The high sensitivity of metabarcoding does, however, render it vulnerable to error (Alberdi et al. 2018; Jusino et al. 2019), with differences in the treatment of samples producing distinct data, and thus conclusions, from the same samples (Alberdi et al. 2018; Alberdi et al. 2019). Better guidelines on best practice for data processing are thus required for metabarcoding studies as they become increasingly commonplace.

False positives, or 'artefacts', can be introduced at any stage of the metabarcoding process, from sample collection through to bioinformatic analysis (Alberdi et al. 2019; Jusino et al. 2019). These can occur through contamination from environmental or lab sources (Leonard et al. 2007; Siddall et al. 2009; Czurda et al. 2016), tag-jumping and sample mis-assignment (transfer of sample-specific tags between samples; Schnell et al. 2015) or PCR and sequencing errors (chimeras or mis-identified sequences; Shin et al. 2014; Bjornsgaard Aas et al. 2016). Artefacts may also be produced through errors in reference databases (such as GenBank and BOLD;

Valentini et al. 2009), resulting in sequences being assigned to the wrong taxon (Keskin et al. 2016; Rulik et al. 2017; Taberlet et al. 2018). Many of these artefacts can be limited through careful study design (e.g. pre- and post-PCR workstations; King et al. 2008; Murray et al. 2015) or the use of bioinformatics software to detect and remove erroneous sequences (e.g. UNOISE; Edgar 2016). However, it is likely that some artefacts will remain regardless of precautionary steps taken (Weyrich et al. 2019, Nakagawa et al. 2018), potentially inflating species richness (Schnell et al. 2015; Clare et al. 2016; Zinger et al. 2019) and distorting data interpretation.

Minimum sequence copy thresholds (MSCTs) are one adaptable method commonly used to reduce the prevalence of artefacts (e.g. Hänfling et al. 2016). The choice of threshold must be carefully considered as it can considerably impact the data; low thresholds will be unsuccessful at removing artefacts, leaving false positives in the resultant data, whereas high thresholds may remove too much data, resulting in false negatives (Hänfling et al. 2016). This is especially true for dietary studies in which DNA of the focal consumer can be present at much higher concentrations than that of the food items (i.e. prey) and is undegraded, often resulting in its greater degree of amplification, depending on the PCR primers used. The use of general primers that amplify the consumer will result in a lower proportion of each sample being assigned to food item DNA, whereas specific primers that avoid amplifying the consumer may reduce amplification of some food items over others due to primer bias (Piñol et al. 2014). This variation increases the risk of target sequences being excluded if inappropriate filtering thresholds are selected.

Experimental controls are valuable components for empirically assigning MSCT thresholds, as they provide a mechanism for estimating the proportion of artefacts within a dataset (Taberlet et al. 2018; Alberdi et al. 2019). Theoretically, negative controls (e.g. extraction blanks, PCR blanks and unused MID-tag (molecular identifier tag) combinations) should contain no DNA, and positive controls (e.g. mock communities) should only contain DNA from selected taxa. This is, however, rarely the case, and these unexpected reads facilitate effective determination of optimal thresholds for data clean-up. Reads in negative controls may be previously undetected contamination present in other samples (predominately identified using extraction and PCR blanks; Leonard et al. 2007; Czurda et al. 2016; Alberdi et al. 2019) or may occur due to tag-jumping or sequence mis-assignment (predominately identified using unused MID-tag combinations; Schnell et al. 2015). Such artefacts are impossible to identify with certainty without negative controls since they are mostly assigned to taxa that occur in high read abundances across many samples and are thus indistinguishable from environmental DNA (Carew et al. 2016; Jensen et al. 2016). Further artefacts are detected through the presence of positive control taxa in eDNA samples and eDNA taxa in positive controls, likely through tag-jumping, mis-assignment or sample cross-contamination. Unexpected reads in positive controls also allow low abundance artefacts from contaminants and PCR or sequencing errors, that may occur across eDNA samples too, to be identified. Control samples thus highlight artefacts prevalent throughout unfiltered data, with those identified through negative controls increasing the frequency of occurrence of taxa, those identified through positive controls inflating sample diversity, and both contributing to higher total read counts and, ultimately, false positives.

Application of MSCTs, and use of controls for assessing thresholds, remains ambiguous and non-standardised, with many studies employing entirely distinct methodologies and thresholds (e.g. Gebremedhin et al. 2016; Guardiola et al. 2016; McInnes et al. 2017). Here we compared common practices for removing artefacts from eDNA metabarcoding data using example datasets of Eurasian otter, *Lutra lutra* (Linnaeus, 1758), and cereal crop spider (Araneae: Linyphiidae and Lycosidae) dietary DNA. Samples were processed alongside experimental controls, allowing the practicality of controls for selecting filtering thresholds to be assessed. Through these examples, distinctions in the data outputs when using different techniques are highlighted, providing a basis for standardisation and outlining optimal solutions for the use of MSCTs on metabarcoding datasets. We hypothesised that; (i) data with MSCTs applied would still contain artefacts; (ii) the extent of artefact removal would differ depending upon the method of MSCT applied, with different MSCTs removing artefacts from different sources (e.g. artefacts in blanks vs. those in mock communities); (iii) low filtering thresholds would fail to remove many artefacts; (iv) high thresholds would remove too much data, resulting in the loss of target sequences and hence trophic relationships; (v) using multiple MSCTs simultaneously would remove more artefacts than MSCTs applied on their own; (vi) experimental controls would greatly benefit the choice of filtering method and threshold through identification of known target sequences and artefacts.

Methods

124 The methodologies discussed herein refer to techniques considered best practice for
125 environmental DNA metabarcoding, such as the use of negative controls (samples
126 included in DNA extraction and/or amplification steps), unused MID-tag combinations
127 (combinations of MID-tags that are not included in PCR amplification or sequencing),
128 positive controls (tissue extract DNA of a known species amplifiable by the selected
129 primers, but not expected to occur in eDNA samples) and mock communities
130 (mixtures of positive controls comprising DNA of several species). Theoretically,
131 blanks (i.e. negative controls and unused MID-tag combinations) should contain no
132 reads, and mock communities should contain reads only from selected taxa, with
133 these taxa only occurring within mock communities. Thus, unexpected reads in such
134 controls facilitate effective determination of optimal thresholds for data clean-up.
135 Therefore, inclusion of these controls throughout the metabarcoding process is
136 recommended for stringent data review, and is often necessary for the techniques
137 discussed.

138 To review existing artefact removal methodologies in use for DNA metabarcoding
139 data, the methods used in 154 studies conducting metabarcoding on eukaryotic DNA
140 for environmental monitoring or dietary analysis were tabulated (Table S1). Given the
141 focus of this study on the clean-up of dietary metabarcoding data, which presents
142 many unique challenges, each method was applied to four different datasets from
143 two dietary studies: a dietary study of the Eurasian otter, *Lutra lutra* (one COI and
144 one 16S dataset) and a dietary study of cereal crop money spiders (two COI
145 datasets).

146

Example dataset one: British otter diet

Faecal samples were collected during otter post-mortems by the Cardiff University Otter Project. Extracted faecal DNA was amplified using two metabarcoding primer pairs designed to amplify regions of the 16S rRNA and cytochrome *c* oxidase subunit I (COI) genes, each primer having ten-base-pair molecular identifier tags (MID tags) to facilitate post-bioinformatic sample identification. Extraction and PCR negative controls, unused MID tag combinations, repeat samples and mock communities were included alongside the focal eDNA samples. Mock communities comprised standardised mixtures of DNA of marine species not previously detected in the diet of Eurasian otters (Table S2; Supplementary Information 1). The resultant DNA libraries for each marker were sequenced on separate MiSeq V2 chips with 2x250bp paired-end reads. Greater detail regarding sample processing, amplification and sequencing is provided in Supplementary Information 2.

Example dataset two: cereal crop spider diet

Money spiders (*Bathyphantes*, *Erigone*, *Microlinyphia* and *Tenuiphantes*; Araneae: Linyphiidae) and wolf spiders (*Pardosa*; Araneae: Lycosidae) were visually located on transects through barley fields. Gut DNA, extracted from the whole spider abdomen, was amplified using two COI metabarcoding primer pairs. One primer pair was selected for broad amplification of all invertebrates present, including the predator, and the other designed to exclude spider DNA to avoid predator amplification, each primer having ten-base-pair MID tags to facilitate post-bioinformatic sample identification. Extraction and PCR negative controls, unused

MID tag combinations, repeat samples and mock communities were included alongside the focal eDNA samples. Mock communities comprised standardised mixtures of DNA of exotic species not previously recorded in Britain (Table S2; Supplementary Information 1). The resultant DNA libraries for each marker were sequenced on a MiSeq V3 chip with 2x300bp paired-end reads. Greater detail regarding sample processing, amplification and sequencing is provided in Supplementary Information 3.

Sequence analysis

Bioinformatic analyses were carried out using a custom pipeline. Sequences were first checked for truncation of MID-tags by determining the proportion of sequence files containing exactly 10bp before their respective primer. In all cases, the degree of truncation was deemed acceptable (≤10%).

FastP (Chen et al. 2016) was used to check the quality of reads, discard poor quality reads (<Q30, <125bp long or too many unqualified bases, denoted by 'N') and merge read pairs from MiSeq files (R1 and R2). Merged reads were assigned a sample ID based on the MID tags associated with each primer using the 'trim.seqs' function of Mothur (Schloss et al. 2009); this also removed the MID tag and primer sequences from the reads. Using the files created by Mothur, reads were demultiplexed to obtain one file per sample ID. Read headers were modified for each file to include the sample ID and reads were then concatenated back into one file. Sequences were denoised (removal of PCR and sequencing errors), clustered into zero-radius

operational taxonomic units (zOTUs) and an OTU table was created using the commands ‘fastx_uniques’, ‘unoise3’ and ‘otutab’ in Usearch (v. 11) (Edgar 2016; Edgar 2020). Taxonomic assignment for each zOTU was obtained using the ‘blastn’ command in BLAST+, using a threshold of 97% similarity and e-value of 0.00001, against a downloaded database of DNA barcoding sequences submitted to online databases (e.g. Genbank; National Center for Biotechnology Information 2008; Camacho et al. 2009).

Before assigning taxonomic identities to each zOTU, BLAST results were filtered using the ‘dplyr’ package in R [version 3.6.0] using R Studio [version 1.2.1335] (R Core Team 2019). This was used to retain only accession codes with the top BIT score for each zOTU. These data were then processed via MEGAN [version 6.12.3] (Huson et al. 2016) to assign taxonomic names to each zOTU. As erroneous entries on online databases can prevent species-level assignments, zOTUs for which the top BLAST hit (i.e. top BIT score) was not resolved to species-level were thus manually checked and assigned the most appropriate taxon. Taxonomic identity for each zOTU was added to the OTU table produced by Usearch and reads were aggregated by taxonomic identity for each sample in R using the ‘aggregate’ function with a sum base function. OTUs were allocated taxonomic identities to overcome issues such as over-splitting of taxonomic groups, and to facilitate ecological interpretation of the data, particularly regarding identification of artefacts (e.g. identifying marine species in non-coastal otters).

Minimum Sequence Copy Thresholds (MSCTs)

215 The seven most common MSCTs identified from the literature (Table 1) were tested
216 and their efficacy in cleaning all datasets compared. Filtering methods were enacted
217 in excel using IF formulae.

218

Table 1: Seven post-bioinformatic filtering methods often applied to eDNA metabarcoding datasets, selected from those identified in a review of 154 metabarcoding studies (Table S1). The 'method name', herein used to refer to these methods, is given alongside the description (how the methods are executed) and the aim of each.

Method name	Method Description	Method Aim
1. No filter	No OTU or sample filtering.	No clean-up/maximum preservation of data.
2. Singletons	Remove any read counts of one.	Remove extremely low frequency artefacts (e.g. sequencing artefacts).
3. <10	Remove any read counts that are less than ten.	Remove low frequency artefacts (e.g. sequencing artefacts, low-lying PCR contamination)
4. Max Contamination	Remove any read counts within each OTU that are lower than the highest read count within a negative/blank control for that OTU.	Remove contamination detected by the negative controls (e.g. extraction/PCR contamination, tag-jumping)
5. Total %	Remove any read counts less than a proportion of the total dataset read count for all reads.	Remove low frequency artefacts (e.g. sequencing artefacts, PCR contamination)
6. Sample %	Remove any read counts within a sample that are less than a proportion of the total sample read count for that sample.	Remove sample contamination (e.g. environmental, extraction or PCR contamination)
7. Taxon %	Remove read counts with an abundance less than a proportion of the total OTU read count for that OTU.	Remove cross contamination (e.g. cross contamination, tag-jumping)

If the read count (i.e. number of reads per sample per taxon) did not pass the designated threshold, then it was converted to zero (rather than subtracting the threshold, thus not altering the remaining read counts). For proportional methods (5-7, Table 1), a variety of thresholds were tested to explore how choice of threshold can affect data output. The range of thresholds tested were chosen based upon artefacts identified in control samples; we started with a low threshold and increased

231 the value until most of the identifiable artefacts were removed. We also explored the
232 effectiveness of using different MSCTs in pairwise combinations; this involved
233 simultaneously applying ‘Max Contamination’ with each proportional threshold
234 method (5-7), and ‘Sample %’ with ‘Taxon %’.

235 Basic statistics were calculated to assess the effectiveness of each filtering method;
236 total read count was used to assess the loss of reads across the whole dataset,
237 presence of singleton reads was used to assess removal of PCR and sequencing
238 errors, reads in blanks (negative controls and unused MID-tags) were used to assess
239 levels of contamination and tag-jumping, and mock communities were used to
240 assess presence of false positives within samples. Artefacts could also be identified
241 through taxa unexpectedly occurring in samples, such as taxa from dietary samples
242 in controls, marine taxa associated with otters that did not have access to marine
243 habitats, exotic taxa in British spider samples and mock community taxa in negative
244 controls, unused MID tags or dietary samples.

245 To visualise the results of each method, tables of reads were converted into heat
246 charts using the ‘ggplot2’ package (Warnes et al., 2012) in R. Frequency of
247 occurrence for each taxon across all MID-tag combinations was also calculated for
248 each filtering method and used to create heat charts. Relative frequencies were
249 calculated by dividing frequency of occurrence by the total number of MID-tag
250 combinations; these values then underwent non-metric multidimensional scaling
251 (NMDS) to visualise dissimilarity between the taxa present following application of
252 each MSCT. This was conducted using the ‘metaMDS’ function in the ‘vegan’
253 package (Oksanen et al. 2013) with two dimensions (stress <0.1) and a Bray-Curtis

254 dissimilarity calculation (Bray and Curtis 1957). Ellipses were created using the
255 'ordiellipse' function with the default 'sd' setting (standard deviation).

256

257 **Results**

258 *Sequencing output*

259 Sequencing yielded 17.6, 13.7, 11.2 and 11.0 million paired-end reads, for the otter
260 16S and COI, and spider general and exclusion datasets, respectively, which
261 decreased to 11.7, 7.9, 7.9 and 7.4 million, respectively, following bioinformatic
262 analysis. Comparison of post-bioinformatic clean-up methods produced the same
263 general patterns across the four datasets (otter 16S, otter COI, spider general COI
264 and spider exclusion COI). We therefore used the simplest dataset (otter 16S) to
265 graphically represent artefact removal (Figures 1-2; Table 2), with supplementary
266 information presenting the same data for the other datasets (otter COI, spider
267 general COI and spider exclusion COI; Figures S1-3; Tables S2-4), as well as graphs
268 depicting read counts per sample (Figures S4-7) and the spatial distribution of otter
269 faecal samples with marine taxa presences (Figures S8-9). The effectiveness of each
270 clean-up method across all datasets is also summarised in Table 3.

271

272 *No filter applied ('No Filter')*

273 The highest read counts and occurrence of artefacts were observed in data with no
274 MSCT applied. False positives in mock communities, reads in blanks, mock

community taxa present in blanks and eDNA samples, taxa from eDNA samples occurring in control samples, and obviously erroneously present taxa (e.g. marine taxa occurring in faecal samples from otters with no access to marine habitats) all occurred frequently across the datasets (Figure 1; Table 2). Artefacts appeared to be much more prevalent for taxa with high total read counts (e.g. mock community taxa, taxa commonly consumed by the predator and the focal predator itself). Many low abundance reads, including singletons, were also observed in the unfiltered data, possibly representing rare species but likely also sequencing errors.

Remove singleton reads ('Singletons')

Removing singleton reads resulted in data very similar to that of unfiltered data in all cases, with only few artefacts removed (Figure 1; Table 2).

Remove read counts less than 10 ('< 10')

Removing reads with an abundance less than 10 reduced the occurrence of artefacts in blanks, mock communities and the presence of mock community taxa in other samples. However, artefacts persisted in all controls and samples, producing data very similar to unfiltered data (Figure 1; Table 2).

Remove maximum taxon contamination ('Max Contamination')

Removing reads less than or equal to the maximum read count in blanks per taxon removed no reads from some taxa and high values from others (otter 16S: minimum read removal = 0, maximum = 8757, average = 394; otter COI: minimum = 0, maximum 23413, average = 117; spider amplification: minimum = 0, maximum = 5851, average = 136; spider exclusion: minimum = 0, maximum = 10764, average = 155). Taxa experiencing high levels of read removal were often those with high total read counts. This cleared all reads from blanks (Tables 2-3), all mock community taxa from eDNA samples and taxa with high read abundances in eDNA samples from controls (Figure 1). False positives were still present in mock communities though (Figure 1), as were singleton reads. This method also cleared several erroneously located taxa, such as marine species associated with inland otters, but not all (Figure 1; Table 2).

Proportion of total read count ('Total %')

This method removed artefacts present in blanks (Table 2), false positives in mock communities and erroneously located taxa (Figure 1; Table 2). Mock community taxa were cleared from blanks and eDNA samples to an extent, but some were still present even at high thresholds (Table 2). Taxa from dietary samples with high read abundances were not filtered efficaciously though, with many occurring in controls even at high thresholds. Thresholds tested across the datasets ranged between removing reads that contributed to less than 0.0001% and 0.02% of the total read count. The lowest thresholds only filtered out a proportion of the artefacts, whilst the highest thresholds filtered out all false positives within mock communities and almost

all reads in blanks (Figure 1; Table 2); however, the latter also removed target reads, shown by the loss of mock community taxa within mock communities. A lower threshold was therefore necessary to give a balance between false positives and false negatives. The optimal threshold was identified as 0.003%, 0.0008%, 0.0005% and 0.005% for otter 16S, otter COI, spider general amplification and spider exclusion, respectively, removing reads with abundances less than 79, 352, 39 and 236, respectively.

Proportion of read count per sample ('Sample %')

This method removed false positives from mock communities (Figure 1) and erroneously located taxa (Table 2). Low abundance taxa (e.g. foreign taxa occurring through sequencing errors) were less prevalent (Figure 1), as were singletons. Taxa with high total read abundances (e.g. mock community taxa and common taxa in dietary samples) and reads present in blanks were only filtered to an extent (Figure 1; Table 2), resulting in artefacts from both being prevalent in filtered data regardless of the threshold utilised. This method removed fewer reads from samples with low total read counts, therefore these samples were more likely to still contain artefacts. Thresholds tested across the datasets included removing reads that contributed less than 0.01% to 8% of a sample's reads. The highest thresholds were required to remove all false positives from mock communities. A much higher threshold was required for some datasets (e.g. otter 16S) when they contained taxa with greater relative read counts. The high thresholds required to clear mock communities of false positives also removed many target reads (highlighted by the loss of mock

community taxa), thus lower thresholds effectively balanced false positives and false negatives. The optimal threshold was identified as 1%, 0.3%, 0.38% and 1% for otter 16S, otter COI, spider general amplification and spider exclusion, respectively. These thresholds removed reads to a varying degree (otter 16S: minimum read removal for a sample = 0, maximum = 8757, average = 394; otter COI: minimum = 0, maximum = 23413, average = 117; spider general amplification: minimum = 1, maximum = 240, average = 80; spider exclusion: minimum = 1, maximum = 1704, average = 199).

Proportion of read count per taxon ('Taxon %')

This method filtered out reads in blanks (Figure 1; Table 2), as well as artefacts from taxa with high read abundances, clearing most of these from the datasets when using sufficient thresholds. A large proportion of reads were removed using this method (Figure 1; Table 2), especially from taxa with high total read counts. Taxa with low read counts had fewer reads removed, resulting in these containing more artefacts, highlighted by the prevalence of singleton reads and taxa identified as PCR or sequencing errors (e.g. foreign taxa; Figure 1). This method proved insufficient at removing false positives from eDNA samples, with false positives prevalent in mock communities regardless of the threshold used, and erroneously located taxa were only removed when using a high threshold (Figure 1; Table 2). Thresholds tested included removing reads that contributed to less than 0.1% - 3% of a taxon's reads. With low thresholds applied, many more artefacts were observed in blanks, but a threshold of 3% cleared most of these artefacts from the datasets in most cases. The highest thresholds removed a high proportion of reads, therefore lower thresholds

were selected to give a balance between clearing out artefacts and not losing too many reads; this was 0.5%, 0.8%, 0.5% and 1% for otter 16S, otter COI, spider general amplification and spider exclusion, respectively. These thresholds removed reads to different extents (otter 16S: minimum read removal for a taxa = 0, maximum = 26039, average = 553; otter COI: minimum = 0, maximum = 2040, average = 49; spider general amplification: minimum = 0, maximum = 306, average = 28; spider exclusion: minimum = 0, maximum = 1286, average = 76).

Combining methods

Many of the thresholds tested for MSCTs based on read counts ('Total %', 'Sample %' and 'Taxon %') did not clear all artefacts, particularly regarding clearance of blanks. Proportional methods were thus also combined with 'Max Contamination' to overcome this issue. 'Sample %' thresholds were also combined with 'Taxon %' thresholds given their complementary removal of artefacts. Combining methods removed more artefacts than using just one method. 'Total %' thresholds or 'Sample %' thresholds combined with 'Max Contamination' left very few artefacts in the data. These methods were highly complementary, with proportional thresholds clearing most false positives from mock communities and erroneously located taxa (Figure 1; Table 2), whilst the contamination threshold cleared reads in blanks and artefacts from taxa with high read counts (e.g. mock community taxa in non-mock community samples and faecal taxa in controls; Figure 1; Table 2). These combinations also cleared singletons and taxa suspected to be PCR or sequencing errors (Figure 1; Table 2). Combining these methods sometimes allowed lower thresholds to be used

387 concurrently for optimal results, but in other cases did not change the thresholds
388 required (otter 16S: optimal sample % = 0.5%, optimal total % = 0.002%; otter COI:
389 optimal sample % = 0.2%, optimal total % = 0.0008%; spider general amplification:
390 optimal sample % = 0.38%, optimal total % = 0.005%; spider exclusion: optimal
391 sample % = 0.39%, optimal total % = 0.005%).

392 'Taxon %' thresholds combined with 'Max Contamination' still contained many
393 artefacts; all reads in blanks and singletons were removed, but false positives were
394 still present in mock communities as were erroneously located taxa (although in
395 lower abundances compared to either filter alone; Figure 1; Table 2). This is likely
396 due to the similar action of both filters. Combining 'Taxon %' thresholds with 'Sample
397 %' thresholds removed more artefacts and performed similarly to MSCTs combining
398 'Sample %' thresholds with 'Max Contamination'. Combining these methods cleared
399 the majority of reads from blanks, all singleton reads, artefacts from taxa with high
400 read counts and most false positives in mock communities (Figure 1; Table 2);
401 however, there were still artefacts present in the negative controls and erroneously
402 located taxa were still present (Table 2). Combining these methods also removed
403 many overall reads. The optimal combination of thresholds changed between
404 datasets (otter 16S: sample = 0.5%, taxon = 0.3%; otter COI: sample = 0.2%, taxon =
405 0.3%; spider general amplification: sample = 0.5%, taxon = 0.3%; spider exclusion:
406 sample = 0.5%, taxon = 0.3%). Lowering the sample threshold introduced more false
407 positives to the data, whilst increasing the threshold removed target reads. Lowering
408 the taxon threshold retained more reads in blanks and artefacts from taxa with high
409 total read counts, whilst increasing the taxon threshold greatly decreased the total
410 read count, resulting in loss of target reads.

411

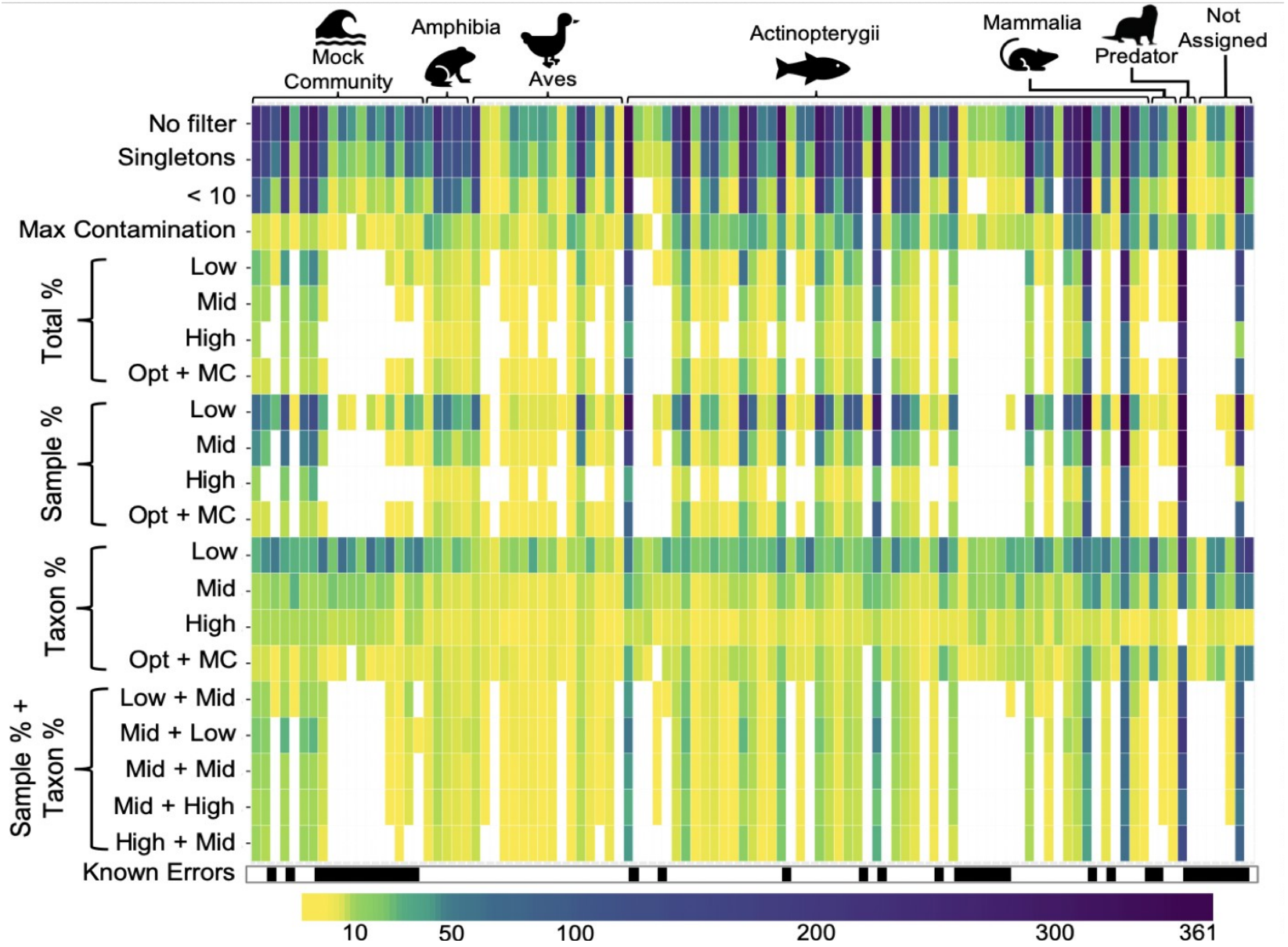

Mock Community
Amphibia
Aves
Actinopterygii
Mammalia
Predator
Not Assigned
No filter
Singletons
< 10
Max Contamination
Total %
Low
Mid
High
Opt + MC
Sample %
Low
Mid
High
Opt + MC
Taxon %
Low
Mid
High
Opt + MC
Sample % + Taxon %
Low + Mid
Mid + Low
Mid + Mid
Mid + High
High + Mid
Known Errors
10
50
100
200
300
361

Figure 1: Otter diet 16S counts. The number of presences of each taxon is displayed for each method (low count = yellow, high count = purple) along with the number of taxa in each dataset following clean-up. Differences in common taxa, mock communities, predator amplification and erroneous taxa can be observed. 'Low', 'Mid' and 'High' depict the context-dependent range of values utilised for proportional thresholds ('Total %', 'Sample %' and 'Taxon %'), with 'Opt + MC' denoting the threshold deemed 'optimal' combined with the 'Max Contamination' method (for specific values see Table S3). The same figure is available for three other datasets (otter COI, spider general COI and spider exclusion COI) in supplementary information (Figures S1-3).

Table 2: Performance of different minimum sequence copy thresholds on otter 16S data. 'Low', 'Mid' and 'High' depict the context-dependent range of values utilised for proportional thresholds ('Total %', 'Sample %' and 'Taxon %'), with 'Opt + MC' denoting the threshold deemed 'optimal' combined with the 'Max Contamination' method (for specific values see Table S3). Expected presences of marine taxa (~) were defined by the number of Eurasian otters (*Lutra lutra*) displaying reads for each marine taxon that was located along the coast or near an estuary. Similar tables were produced for three other datasets (otter COI, spider general COI and spider exclusion COI) and are presented in supplementary information (Tables S2-4).

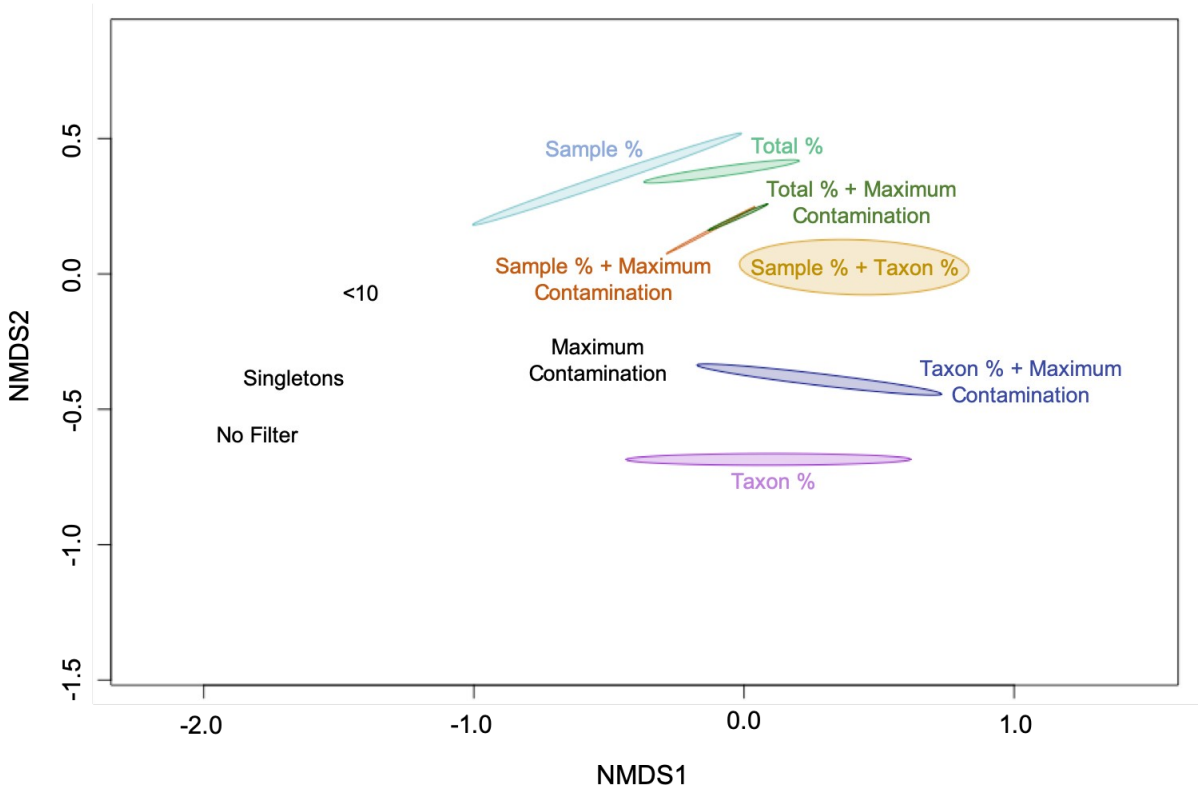
Minimum Sequence Copy Threshold	Total		Singletons	Blanks		Mock Communities			Marine taxa presences		
	Summed read count	Taxa	Number of presences	Summed read count	Average read count	Average false positive read count	Average false positive presences	Presences in eDNA samples/ blanks	*T. bubalis* (~1-3)	Pleuronectidae (~10-15)	*E. viperia* (~1)
No filter	11723871	105	2767	117460	1864	3121	38	295	166	324	37
Singletons	11721104	105	0	117032	1858	3113	30	259	84	291	28
< 10	11705943	99	0	114675	1820	3066	19	198	38	194	7
Maximum Contamination	10938496	102	63	0	0	314	4	0	36	14	7
Low Total %	11534535	71	0	96869	1538	2498	5	38	11	36	1
Mid Total %	11349821	60	0	78023	1238	2018	2	11	1	14	1
High Total %	10733900	46	0	35916	570	220	0	2	1	10	0
Opt Total % + MC	10874148	63	0	0	0	115	0	0	1	14	1
Low Sample %	11659268	89	218	116737	1853	3290	10	126	40	172	3
Mid Sample %	11478669	68	0	113804	1806	2113	2	51	6	38	1
High Sample %	10631707	46	0	86797	1378	0	0	21	1	8	1
Opt Sample % + MC	10875890	65	0	0	0	96	0	0	3	14	1
Low Taxon %	11031736	105	742	45985	730	376	13	21	36	27	22
Mid Taxon %	8669244	105	267	30812	489	163	8	2	19	12	5
High Taxon %	3660086	104	25	30645	486	99	5	1	1	7	1
Opt Taxon % + MC	8569029	102	0	0	0	96	2	0	19	12	5
Low Sample % + Mid Taxon %	10187214	72	2	30851	490	15	0	2	12	13	1
Mid Sample % + Low Taxon %	10959369	68	0	44471	706	140	0	19	4	16	1
Mid Sample % + Mid Taxon %	10177475	67	0	30434	483	124	0	2	4	13	1
Mid Sample % + High Taxon %	8647191	67	0	29865	474	124	0	2	4	12	1
High Sample % + Mid Taxon %	10155032	60	0	29886	474	0	0	2	2	13	1

436
437

Table 3: Success of different filtering methods in achieving the key objectives of post-bioinformatic data clean-up. Green, orange and red denote positive, neutral and negative outcomes, respectively. 'Low', 'Mid' and 'High' depict the value utilised for proportional thresholds ('Total %', 'Sample %' and 'Taxon %'), with 'Opt + MC' denoting the 'optimal' threshold combined with 'Max Contamination' methods (for specific values see Table S3).

NMDS analysis

Choice of MSCT method greatly affected the final composition of the data across all four datasets, as shown by NMDS (Figure 3). Application of ‘No Filter’, ‘Singletons’ and ‘< 10’ MSCTs produced similar outcomes, with the ‘< 10’ threshold also appearing to elicit similar effects to MSCTs based on ‘Total %’ and ‘Sample %’. ‘Sample %’ and ‘Total %’ thresholds were the most similar and gave results distinct from those of taxon MSCTs (‘Taxon %’ and ‘Maximum Contamination’). By combining taxon MSCTs with either ‘Sample %’ or ‘Total %’ thresholds, an intermediate result was obtained. All combinations of taxon filters with ‘Sample %’ or ‘Total %’ thresholds performed similarly to one another; however, with the otter 16S data those that combined ‘Sample %’ or ‘Total %’ with ‘Maximum Contamination’ were more dissimilar to taxon methods than combinations between ‘Sample %’ and ‘Taxon %’.



459

460 **Figure 2: Otter 16S non-metric multidimensional scaling of relative frequency of occurrence for each taxon following**
461 **application of different minimum sequence copy thresholds, including different methods and thresholds where possible.**
462 **Ellipse colours denote each method with None, Singletons, <10 and Maximum Contamination not having ellipses given**
463 **the lack of modifiable threshold. The same figure is available for three other datasets (otter COI, spider general COI and**
464 **spider exclusion COI) in supplementary information (Figures S7-9).**

Discussion

Here we have illustrated the efficacy of different filtering methods and thresholds for removal of artefacts from metabarcoding data, allowing us to identify an optimal method for artefact removal; utilising a threshold that removes a proportion of read counts per sample, combined with a threshold that removes reads with a count less than the maximum contamination identified per taxon ('Opt sample % + MC'; Table 3). For optimisation of thresholds, previous studies have disproportionately emphasised the importance of mock communities (e.g. Elbrecht and Leese 2017; Jusino et al. 2019); however, since the biases affecting true unknown mixtures of eDNA are almost impossible to experimentally replicate (Alberdi et al. 2018), data cannot be adequately filtered using only mock communities. By sequencing and analysing mock communities, blank samples and eDNA together, it was possible to fully assess which filters and thresholds were optimal in cleaning metabarcoding data of this nature.

Identifying artefacts

Despite all appropriate precautionary steps being taken to reduce contamination (e.g. screening negative controls, pre- and post-PCR workstations), and bioinformatic programmes used to remove erroneous sequences, artefacts were still observed in the unfiltered data. Such contamination is, however, largely unavoidable when using a method so broad-spectrum and sensitive (Alberdi et al. 2018; Jusino et al. 2019). Artefacts primarily manifested as unexpected reads in control samples, but also as erroneous taxa and misassigned reads. Erroneous taxa, usually existing in low read counts in the unfiltered data (De Barba et al. 2013; Ficetola et al. 2015) are,

in this case, taxa produced through PCR or sequencing errors that are ecologically highly unlikely to appear in their respective samples (e.g. foreign species), thus rendering them easy to identify and eliminate. Mis-assigned reads were more difficult to identify, primarily detected through mock community taxa occurring in eDNA samples and vice versa; however, some datasets also allow detection of mis-assignment between eDNA samples through the presence of, for example, marine taxa in land-locked sites (Figures S1-6). In such cases, reads were assumed to be derived from other samples through cross-contamination, tag-jumping or mis-assignment (Schnell et al. 2015; Alberdi et al. 2019). If easily identifiable, this can be fortuitous for threshold determination, but where samples share taxa that could theoretically co-occur, they will remain undetected.

Detection of artefacts is facilitated through the presence of unexpected reads in controls. Such reads in negative controls may occur due to low levels of contamination (e.g. from reagents or samples; Leonard et al. 2007; Czurda et al. 2016; Alberdi et al. 2019) that went undetected during screening of samples and may be present throughout only a few, or potentially all samples. Reads present in blanks may also occur due to tag-jumping or mis-assignment (Schnell et al. 2015), which are primarily identifiable through unused MID-tag combinations. These artefacts are hard to detect without blanks because they are frequently assigned to taxa that legitimately occur in high read abundances across many samples (Jensen et al. 2015; Carew et al. 2018), such as mock community taxa and common taxa in eDNA samples (e.g. commonly consumed taxa or the consumer itself). Further artefacts were detected through the presence of mock community taxa in eDNA samples and common eDNA taxa in mock communities; these were concluded to be primarily due to tag-jumping or mis-assignment rather than sample cross-

514 contamination because eDNA and mock community samples were processed
515 separately. Unexpected reads in mock communities also allowed low abundance
516 artefacts from contaminants and PCR or sequencing errors to be identified, which
517 may have occurred across the eDNA samples. Control samples showed artefacts
518 were prevalent throughout the unfiltered data, with those identified through blanks
519 increasing the frequency of occurrence of taxa, those identified through mock
520 communities inflating sample diversity and both contributing to higher total read
521 counts and, ultimately, false positives

522 The composition of mock communities is of great importance to the process of
523 identifying artefacts. If the mock communities are comprised of species that may
524 feasibly occur in the eDNA samples taken from the focal study system, the utility of
525 those controls is reduced. Although the mock communities in this study comprised
526 species considered highly unlikely to appear in the corresponding eDNA samples,
527 distinct problems were encountered for all datasets. For the otter dietary analysis,
528 the mock communities contained marine taxa unlikely to have been consumed by
529 otters, yet high read counts were observed in the COI mock communities for brill
530 (*Scophthalmus rhombus*), a species known to be consumed by otters and not
531 included in the mock community mixtures. The marine samples from which DNA was
532 extracted were collected as part of a larger marine surveying initiative and, whilst
533 care was taken by the practitioners responsible for the collection, cross-
534 contamination between species was possible. Since this taxon could legitimately
535 occur in both mock communities and eDNA samples, false presences are harder to
536 confirm, but its marine origin meant that in areas lacking access to marine prey by
537 otters, reads could still be identified as artefacts. The mock community mixtures
538 used for the spider dietary analysis included exotic species from Round Island,

539 Mauritius, collected as part of a separate study. These were selected for their
540 absence in Britain and taxonomic relevance to the expected prey species (also small
541 invertebrates). Given the poorly described entomological fauna of Round Island,
542 Mauritius, the identities of a minority of these species were not resolved in the
543 bioinformatics process, resulting in their designation as 'not assigned' and thus their
544 exclusion from the filtering process alongside other unassigned taxa.

545

546 *Performance of Minimum Sequence Copy Thresholds (MSCTs)*

547 Artefacts were removed to varying extents depending on the filtering method and
548 threshold utilised. Basic MSCTs commonly used in the literature, such as removing
549 singletons (e.g. Oliverio et al. 2018) or reads with an abundance less than 10 (e.g.
550 Gebremedhin et al. 2016), removed very few artefacts. This will, however, vary with
551 sequencing depth, with relatively greater depths increasing the likelihood of artefacts
552 having more than 10 occurrences (De Barba et al. 2014; Elbrecht & Leese 2015).
553 MSCTs removing reads with an abundance below a proportion of the total read
554 count performed better, reducing abundance of all detectable artefacts; however,
555 applying one threshold across all read counts potentially indiscriminately removes
556 target reads with low abundances and retains abundant artefacts. This bias can be
557 overcome by using MSCTs based on sample read counts, as the read count will
558 inevitably vary between samples despite best efforts to facilitate consistent sample
559 read depths (Deagle et al 2019). Sample MSCTs efficaciously removed artefacts
560 from within samples, with lowered levels of cross-contamination and erroneous taxa,
561 but did not clear artefacts from blanks, nor abundant taxa.

MSCTs that removed reads less than the maximum read count present in the blanks for each taxon ('Max Contamination'), and those which removed reads less than a given proportion of the total read count for that taxon ('Taxon %'), removed artefacts from blanks and abundant taxa, but not mock communities or erroneous taxa. Of these two methods, removal of maximum taxon contamination was more suitable as it removed all artefacts from negative controls and taxa with high read counts without removing too many reads overall. To achieve the same result using thresholds based on taxon read counts resulted in much greater read losses, increasing the likelihood of false negatives. Proportional taxon thresholds also showed a strong bias towards removing reads from abundant taxa. Whilst helping to remove artefacts produced through tag-jumping, this would potentially produce false negatives if taxa legitimately occurred in many samples. Comparing proportional taxon thresholds to others that cleared out similar amounts of artefacts revealed that proportional taxon thresholds produced the highest loss of reads, thus making this method more likely to lead to false negatives. Removal of maximum taxon contamination is logically superior given that the taxa for which the greatest number of reads will be removed will be based on those that are verifiably contaminating the blanks. Care must, however, be taken to ensure that the protocols followed to produce the blanks are sufficiently stringent but not unnecessarily conservative (e.g. negative control volumes included being based on the average volume pooled per plate, vs. the maximum volume pooled per plate), since this will cause this filtering method to produce many false negatives through overly strict removal of data.

Combining MSCTs

Combining different MSCTs improved the performance of all filters, leading to a greater reduction in artefact presence. The weakest combination used proportional taxon thresholds with removal of maximum taxon contamination ('Taxon %' with 'Maximum Contamination'); these analogous methods removed artefacts in similar ways (i.e. removal based on reads present across taxa, rather than across samples), with neither sufficiently mitigating artefacts within samples. Artefacts persisting in blanks, following application of total read count thresholds, were removed by combining this method with removal of maximum taxon contamination; however, this combination may introduce biases by not accounting for read depth variation between samples, thus providing overly conservative filtering to some samples and insufficient filtering to others. Taxon-based thresholds were complementary to sample-based thresholds, with one removing artefacts identified through blanks and abundant taxa and the other removing artefacts within samples, including erroneous taxa. Combining sample-based thresholds with removal of maximum taxon contamination performed better than combinations with proportional taxon thresholds, as a greater proportion of artefacts were removed with a lower total read loss, reducing the likelihood of false negatives. Due to its consistently improved performance over other MSCTs across all four metabarcoding datasets, we conclude that combining a sample-based threshold with removal of maximum taxon contamination is the optimal method for stringent filtering of metabarcoding data whilst retaining target data.

Choosing an appropriate threshold

In metabarcoding studies, removal of false positives tends to be prioritised over false negatives due to the assumption that reads prove taxon presence whilst a lack of reads does not prove absence because false negatives can occur due to experimental biases (e.g. sampling or primer bias; Oehm et al. 2011; Pinol et al. 2015). A trade-off exists whereby removal of false positives leads to an increase in false negatives (Zepeda-Mendosa et al. 2016; Alberdi et al. 2019), observed here when utilising high thresholds which removed many artefacts but also removed target reads, biasing results to taxa with high read abundance. Ultimately though, not all false positives are identifiable, meaning some artefacts may persist despite appropriate filtering removing all known artefacts. A balance can be achieved by which a high proportion of false positives are removed whilst retaining only very few false negatives that are easily disregarded (Clare et al. 2016; Hanfling et al. 2016; Zizka et al. 2019), thus better reflecting the true diversity within samples. The threshold at which this balance is achieved varies between studies depending on the sequencing depth and breadth of taxa. Appropriate thresholds should be chosen based on artefact removal from control samples. The aim of the study should, however, also be considered. Studies concerning commonly-detected taxa can employ more stringent filters that remove more artefacts at the expense of losing rare taxa that may not be of interest anyway (e.g. studies of major sources of nutrition to a predator). However, studies concerning rare taxa should consider refining their thresholds to optimally remove artefacts whilst retaining the greatest amount of sequencing data (e.g. surveys of species richness).

In this study, we chose to assess the effectiveness of different thresholds using taxa read counts as well as occurrences (count data converted to presence or absence). Occurrence data is often assumed to be a conservative method of assessing

metabarcoding data, as recovery biases (e.g. primer bias, starting amount of DNA) have a lower impact on such data (Deagle et al. 2019). Although occurrence data can inflate the importance of taxa that occur at low read counts (e.g. rare taxa or taxa consumed in small amounts; Deagle et al. 2019), and therefore also artefacts, we found it provided a simple and concise method for assessing artefact prevalence. Other methods, such as relative read abundance (RRA), may provide an alternative method for assessing abundance of artefacts and their impact on metabarcoding datasets by considering the proportion of reads each taxon contributes to a sample's total read count (this is analogous to the 'Sample %' MSCT). However, conversion of reads to RRA can produce misleading results due to biases such as differential digestion rates or primer amplifications (Pompanon et al. 2012; Clare 2014; Piñol et al. 2014; Thomas et al. 2014; Elbrecht and Leese 2015; Elbrecht et al. 2017; Alberdi et al. 2018), whilst the loss of read count data can potentially obscure interpretations of overall data loss. For these reasons we chose not to convert read count data into RRA in this study but instead use raw read counts to assess the use of different MSCTs, thus allowing both artefact abundance and overall loss of reads to be assessed and directly compared. Future developments may make RRA a useful tool for artefact detection and removal though, allowing identification of artefacts that are having a proportionally large impact on metabarcoding data.

Previous studies

A review of the relevant literature (154 DNA metabarcoding papers; Table S1) revealed a large proportion of eDNA studies did not employ MSCTs (29%) and those which did often used entirely distinct methodologies and thresholds, with no optimal

658 method apparent. Studies utilising one threshold across all read counts were
659 commonly used (18% of studies; Table S1), but often employed largely arbitrary
660 thresholds (e.g. removal of reads with an abundance of less than 10) that did not
661 consider the variation in artefact prevalence that can occur through differences in
662 sequencing depth (De Barba et al. 2014; Elbrecht and Leese 2015). Whilst some
663 studies circumvent this issue by using relative thresholds (2% of studies used
664 thresholds based on total read abundance, 18% sample read abundance and 9%
665 taxon abundance; Table S1), each of these methods is likely to have removed
666 artefacts to a different extent, introducing inconsistencies between datasets as a
667 consequence. This study shows how using different MSCTs can drastically affect
668 metabarcoding data, and in turn ecological interpretations of such data, therefore
669 highlighting the need for more stringent removal of artefact across metabarcoding
670 studies. Furthermore, the disparity in terminology and methodological descriptions
671 between studies identified in the literature search obviates confident inter-study
672 comparison and undermines an overall requirement for scientific transparency. By
673 comparing existing filtering methodologies, this study thus also provides effective
674 descriptions for such methods which can be applied to mitigate this disparity.

675

676 *Conclusions*

677 Here we have shown that artefacts persist in metabarcoding data even following
678 stringent lab and bioinformatic procedures. Although artefacts often occur in low
679 abundances, they can create a disproportionate representation of biodiversity and
680 produce misleading results, highlighting the need for read count filters. MSCTs
681 removed artefacts to differing extents, but combining sample-based thresholds with

682 removal of maximum taxon contamination provided an optimal outcome. Whilst the
683 optimal method was the same for all four datasets, thresholds applied differed due to
684 variation in sequencing depth and differential taxon amplification. The choice of
685 thresholds must thus depend on the individual study, taking into consideration the
686 sequencing depth, breadth of taxa amplified, artefact abundance and the
687 fundamental question under investigation. Control samples were crucial in assessing
688 filters and selecting appropriate thresholds, providing a means for assessing removal
689 of artefacts and target reads. We recommend that future metabarcoding studies
690 include mock communities and blanks, and, if possible, use taxa detected within
691 eDNA samples that can be used to identify artefacts in the resultant metabarcoding
692 data (e.g. marine taxa in inland samples) to facilitate identification of appropriate
693 thresholds. Given the broad variation in MSCTs applied to metabarcoding studies,
694 inconsistent results between these studies are inevitable. To mitigate erroneous
695 reporting of results and inconsistencies, effective guidance for best-practice filtering
696 of metabarcoding data for the ascertainment of conservative and accurate data
697 should be followed.

698

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717

718 **Author contributions**

719 L.E.D, J.P.C, E.A.C and W.O.C.S conceived the ideas and oversaw the project;
720 L.E.D and J.P.C generated the data; A.M. carried out the sequencing and advised on
721 hypothetical implications for different data management strategies; L.E.D, J.P.C and
722 R.E.Y analysed the data; L.E.D led writing the manuscript. All authors commented
723 upon and contributed to the drafts and approved the final manuscript for publication.

724

725 **Data Accessibility**

726 The data relevant to this publication will be made publicly available via Dryad
727 following acceptance of the manuscript.

728

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