

Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment

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9 **assessment, *in silico* PCR, invertebrates**

10 Abstract

11 A central challenge in the present era of biodiversity loss is to assess and manage human impacts on
12 freshwater ecosystems. Macroinvertebrates are an important group for bioassessment as many taxa
13 show specific responses to environmental conditions. However, generating accurate
14 macroinvertebrate inventories based on larval morphology is difficult and error-prone. Here, DNA
15 metabarcoding provides new opportunities. Its potential to accurately identify invertebrates in bulk
16 samples to the species level, has been demonstrated in several case studies. However, DNA based
17 identification is often limited by primer bias, potentially leading to taxa in the sample remaining
18 undetected. Thus, the success of DNA metabarcoding as an emerging technique for bioassessment
19 critically relies on carefully evaluating primers.

20 We used the R package PrimerMiner to obtain and process cytochrome c oxidase I (COI)
21 sequence data for the 15 most globally relevant freshwater invertebrate groups for stream assessment.
22 Using these sequence alignments, we developed four primer combinations optimized for freshwater
23 macrozoobenthos. All primers were evaluated by sequencing ten mock community samples, each
24 consisting of 52 freshwater invertebrate taxa. Additionally, popular metabarcoding primers from the
25 literature and the developed primers were tested *in silico* against the 15 relevant invertebrate groups.

26 The developed primers varied in amplification efficiency and the number of detected taxa, yet
27 all detected more taxa than standard 'Folmer' barcoding primers. Two new primer combinations
28 showed more consistent amplification than a previously tested ribosomal marker (16S) and detected
29 all 42 insect taxa present in the mock community samples. *In silico* evaluation revealed critical
30 design flaws in some commonly used primers from the literature.

31 We demonstrate a reliable strategy to develop optimized primers using the tool PrimerMiner.
32 The developed primers detected almost all taxa present in the mock samples, and we argue that high
33 base degeneracy is necessary to decrease primer bias as confirmed by experimental results and *in*
34 *silico* primer evaluation. We further demonstrate that some primers currently used in metabarcoding
35 studies may not be suitable for amplification of freshwater macroinvertebrates. Therefore, careful
36 primer evaluation and more region / ecosystem specific primers are needed before DNA
37 metabarcoding can be used for routine bioassessment of freshwater ecosystems.

38 1 Introduction

39 Freshwater resources worldwide are threatened by anthropogenic activities and the pressure on these
40 sensitive ecosystems will intensify with the exponential increase of the human population (Dudgeon
41 et al., 2005; Vörösmarty et al., 2010). Ambitious water monitoring, management and restoration
42 projects have been launched globally in the last decades which relies heavily on Environmental
43 Impact Assessment (EIA) and to protect and restore freshwater ecosystems (EU Water Framework
44 Directive, US Clean Water Act). Macroinvertebrates are often a biological key component
45 ('biological quality element') for assessing stream health, as many taxa are sensitive to stressors.
46 While many bioassessment protocols only require identification at higher taxonomic level (family,
47 genus), it is highly beneficial to include precise species-level information, as even closely related
48 species can show different tolerances to environmental stressors (Macher et al., 2016). However,
49 accurate species-level identification of freshwater macroinvertebrates can be difficult for larval
50 specimens, often leading to low taxonomic resolution or misidentifications (Haase et al., 2010;
51 Sweeney et al., 2011). This in turn decreases the accuracy of the approach and may result in
52 imprecise bioassessment or even misguided management (Stein et al., 2014). Additionally,
53 identification accuracy is affected by different levels of taxonomic expertise amongst specialists,
54 limiting the comparability of assessments (Haase et al., 2010). With the decline of available
55 taxonomic expertise and much of the world's diversity not being properly described, morphology-
56 based monitoring cannot keep pace with current challenges of sustainable water management.

57
58 A promising alternative to morphological identification is DNA based determination of
59 macroinvertebrates, which has been demonstrated in multiple case studies (Hajibabaei et al., 2011;
60 Sweeney et al., 2011; Stein et al., 2013; Carew et al., 2013; Elbrecht & Leese, 2015). Prerequisite of
61 such 'DNA barcoding' techniques is an appropriate reference data base. In short, a fragment of a
62 standardised genetic marker sequence is obtained from well-determined invertebrate material
63 (typically male adult specimens, which can be determined to species level often) is obtained and
64 stored in a reference database. This reference database can then be used for the identification of larval
65 specimens. The cytochrome c oxidase I (COI) gene is typically used for this DNA barcoding
66 technique and extensive reference sequences are already available in online databases (Ratnasingham
67 & Hebert, 2007; 2013). However, identifying single specimens using DNA barcoding is still quite
68 expensive because each specimen has to be processed and sequenced individually (Cameron,
69 Rubinoff & Will, 2006; Stein et al., 2014). Recent advances in high throughput sequencing (HTS)
70 have made it possible to characterize the species composition for complete bulk samples often
71 containing hundreds to thousands of specimens. This technique, coined 'DNA metabarcoding', has
72 already been widely used to generate comprehensive taxa lists for many ecosystems and
73 environments (Taberlet et al., 2012). However, the utility of DNA metabarcoding remains limited
74 due to severe primer bias, which prevents the detection of all taxa present in a sample and hinders
75 precise quantification of taxon biomass and abundances (Piñol et al., 2014; Elbrecht & Leese, 2015).

76
77 A barcoding primer pair, which amplifies a marker sequence of suitable length for HTS for ideally all
78 taxa contained in the sample, is therefore the most critical component to assess macroinvertebrate
79 bulk samples with DNA metabarcoding. However, the COI barcoding gene region shows high codon
80 degeneracy throughout its sequence, making the design of such "truly" universal primers difficult
81 (Deagle et al., 2014; Sharma & Kobayashi, 2014). Several COI barcoding primers with different
82 levels of base degeneracy have been developed of which many are now used or could be suitable for
83 metabarcoding studies (Figure 1, e.g. (Folmer et al., 1994; Hebert et al., 2004; Meusnier et al., 2008;
84 Van Houdt et al., 2010; Zeale et al., 2011; Shokralla et al., 2011; Leray et al., 2013; Geller et al.,
85 2013; Gibson et al., 2014; Shokralla et al., 2015; Brandon-Mong et al., 2015). However, often these
86 primers were developed for a specific taxonomic group, purpose or ecosystem, for example the
87 primers by Zeale and co-authors (2011) which were originally developed for gut content analysis on

88 bats but are now more widely used. Thus, despite including several degenerate bases, metabarcoding
89 primers typically recover only 80-90% or even less of the taxa present in a sample (Leray et al.,
90 2013; Elbrecht & Leese, 2015; Brandon-Mong et al., 2015). Furthermore, many primers have not
91 been thoroughly evaluated for primer bias and the proportion of undetected taxa, making
92 development and testing of universal primers a pressing issue. Additionally, details on criteria for
93 primer design such as the used reference sequence data are often not described extensively (e.g.
94 (Hajibabaei et al., 2011; Shokralla et al., 2015). Typically, primers are developed either with aligned
95 reference barcode sequences for the taxonomic target groups available from NCBI or BOLD (Zeale
96 et al., 2011; Leray et al., 2013; Gibson et al., 2014), or alternatively only mitochondrial genomes or a
97 small subset of barcoding sequences are used (Geller et al., 2013; Deagle et al., 2014; Brandon-Mong
98 et al., 2015). These two approaches are typically biased, as sequences for certain taxa are
99 overrepresented in big datasets (e.g. from population genetic studies), while datasets containing only
100 mitochondrial genomes have an underrepresented number of reference sequences which is
101 insufficient to capture sequence variation for primer design.

102
103 In the study, we used the recently developed R package PrimerMiner to explore these two problems
104 for primer development and evaluated the suitability of existing primers for freshwater invertebrate
105 metabarcoding using computational, i.e. *in silico* analyses. Specifically, we downloaded available
106 sequences from public archives and checked, whether published primers showed obvious mismatches
107 to the references which limit their probability of amplification. Furthermore, we experimentally
108 evaluated own optimised primer sets using ten macroinvertebrate mock communities consisting of 52
109 freshwater species that were also used for method evaluation in in previous studies (Elbrecht & Leese
110 2015; Elbrecht et al. 2016).

111 **2 Material and Methods**

112 **2.1 Primer development and *in silico* evaluation**

113 The PrimerMiner package v0.7 was used to download and cluster COI sequences for the 15 most
114 relevant freshwater invertebrate groups for bioassessment (accessed September 2016, table S2,
115 (Elbrecht & Leese, 2016)). Sequences were aligned with MAFFT v7.017 (Kato et al., 2002) as
116 implemented in Geneious 8.1.7 (Kearse et al., 2012). PrimerMiner's "selectivetrim" function was
117 used to trim 26 bp in the HCO and 25 bp in the LCO binding sites, and the alignment for each group
118 was visualized with PrimerMiner to manually identify suitable primer binding sites. Two forward
119 (BF1, BF2) and two reverse primers (BR1, BR2) were designed with high base degeneracy. Fusion
120 primers were designed by adding Illumina adapters and inline barcodes, as described by (Elbrecht &
121 Leese, 2015), to increase per-base pair sequence diversity during sequencing and allow for a one step
122 PCR protocol.

123 PrimerMiner was also used to evaluate all primers shown in Figure 1 against alignments of the 15
124 freshwater invertebrate groups, using the default "Position_v1.csv" and "Type_v1.csv" table for
125 mismatch scoring (tables are included in the PrimerMiner example data). Primers that obtained a
126 penalty score of >120 were considered as inappropriate for metabarcoding.

127 **2.2 Testing of DNA metabarcoding primers on mock communities**

128 Amplification success of the BF / BR primers was evaluated using ten mock communities, each
129 containing a set of 52 freshwater invertebrates also used in previous studies (Elbrecht & Leese, 2015;
130 Elbrecht et al., 2016). The DNA aliquots and the one step PCR protocol as in (Elbrecht & Leese,
131 2015) was used for all four primer combinations, but the number of PCR cycles was increased from

132 30 to 35 and the annealing temperature increased to 50°C. As in the previous studies, each sample
 133 was uniquely tagged from both sides, but for half of the samples only 25 ng instead of 50 ng DNA
 134 was used in PCR (see Figure S1). For each primer combination, all ten samples were run in the same
 135 PCR setup, using one PCR replicate per sample. Ready-to-load products were purified with magnetic
 136 beads (left sided, 0.8x SPRIselect, Beckman Coulter, Brea, CA, USA) and quantified using the
 137 Qubit HS DNA Kit (ThermoFisher Scientific, Carlsbad, CA, USA). For each primer combination,
 138 equimolar amounts of amplicons were pooled into one library (amplicon concentrations had to be
 139 adjusted due to variation in amplicon length, see Figure S1). The library was sequenced on one lane
 140 of a HiSeq 2500 (rapid run, 2x250 bp) with 5% PhiX spike-in, carried out by the DNA Sequencing
 141 Center of Brigham Young University, USA.

142
 143 Bioinformatic processing of HTS data was kept as similar as possible to previous studies (Elbrecht &
 144 Leese, 2015; Elbrecht et al., 2016). In short, reads were demultiplexed (script S1) and paired end
 145 reads merged using Usearch v8.1.1831 -fastq_mergepairs with -fastq_merge_maxee 1.0 (Edgar &
 146 Flyvbjerg, 2015). Where necessary, reads were converted into reverse complement. For each primer
 147 combination all ten replicates were pooled and sequences which were present only one single time in
 148 the dataset (singletons) were removed prior to clustering with Usearch (cluster_otus, 97% identity,
 149 strand plus, includes chimera removal) (Edgar, 2013). Dereplicated reads for each of the 40 samples
 150 (including singletons) were compared against the respective Operational Taxonomic Unit (OTU)
 151 dataset, using usearch_global with a minimum match of 97% and strand plus. As in previous studies,
 152 low abundance OTUs without at least one sample above 0.003% sequences assigned, were
 153 considered unreliable and excluded from the dataset. Taxonomy of the remaining OTUs were
 154 identified and manually verified using the BOLD and NCBI databases. To ensure that the same
 155 taxonomy was assigned across primer combinations and the reference COI study (Elbrecht & Leese,
 156 2015), the most abundant sequence for each OTU in each sample was extracted using an R script
 157 (Script S2) and the haplotype of all individual specimens assembled, if amplified by more than one
 158 primer combination.

159
 160

161 3 Results

162 3.1 Developed primers using PrimerMiner

163 We designed four primer pairs (Table 1) using the alignments of 15 major freshwater groups relevant
 164 for bioassessment (Figure S2). The two BF and two BR primers show high base degeneracy to
 165 amplify as many insect taxa as possible. Amplified regions range from 217 bp for internal barcodes
 166 and up to 421 bp for combinations using a degenerated version of the HCO2198 primer (Figure 1).
 167 While samples in this study were tagged uniquely from both sides using fusion primers (Figure S3),
 168 the inline barcodes allow for tagging of up to 72 samples for each primer combination (see Figure S4
 169 for recommended primer combinations).

170

171 **Table 1:** Newly developed universal primers targeting freshwater macroinvertebrates relevant for
 172 aquatic bioassessment.

| Primer name | Direction | Primer sequence (from 5' to 3') |
|-------------|-----------|---------------------------------|
| BF1 | Forward | ACWGGWTGRACWGTNTAYCC |
| BF2 | Forward | GCHCCHGAYATRGCHTTYCC |
| BR1 | Reverse | ARYATDGTRATDGCHCCDGC |
| BR2 | Reverse | TCDGGRTGNCCRAARAAAYCA |

173

174 All four BF / BR primer combinations were tested on ten invertebrate mock community samples on
175 an Illumina HiSeq sequencer. PCR efficiency varied across primer combinations, with PCRs
176 involving the BF2 primer showing good amplification whereas those with the BF1 primer always
177 showing decreased yields (Figure S5). Amplification efficiency with fusion primers was always
178 lower than in the positive control (standard COI Folmer primers without Illumina tail, data not
179 shown). Sequencing was successful for all samples, with very similar numbers of sequences obtained
180 for all replicates (on average 1.55 million reads per sample, SD = 0.2, Figure S1A). Cluster density
181 on the lane was low (402 k/mm²) yielding only 48.74% of the expected sequencing output, yet with
182 good sequence quality (Phred Q30 score \geq 92.17%, raw data deposited on SRA: SRX1619153). The
183 amplified read lengths had an influence on the number of sequences retained in bioinformatic
184 processing. Longer amplicons showed less overlap when paired-end merged and were thus excluded
185 more often due to expected errors > 1 (Figure S1B). Additionally, for primer combinations that used
186 the P5_BF1_2 primer more sequences were discarded than with other primer combinations, as $\sim 1/5$
187 of the reads had poor Phred quality scores (See Figure S1B). There were also issues with the BF1 and
188 BF2 primers which showed insertions or deletions on the 3' end affecting total sequence length by 1-
189 2 bp across all replicates (Figure S6). Some primer combinations also amplified up to 1.35% shorter
190 or longer fragments than expected (Figure S7).

191 3.2 Number of taxa recovered

192 All insect taxa present in the mock samples were detected with each primer combination, with
193 exception of the BF1 + BR1 combination that failed to amplify the Scirtidae (Coleoptera) specimens
194 (Table 2, raw OTU data table S3, haplotype sequences data Script S2). All primers failed for some of
195 the other metazoan taxa, with the BF1 + BR2 combination showing the lowest number of undetected
196 taxa. In comparison to the traditional Folmer primers (Folmer et al. 1994), all BF / BR freshwater
197 primers showed a more consistent and equal read abundance across the mock samples (Figure 2). As
198 in Elbrecht et al. (2016), the standard deviation from the expected abundance and precision for the
199 primer pairs was estimated, which summarizes the variance in amplification for each morphotaxon.
200 The primer combination BF1 + BR1 showed the highest inconsistencies in read abundance, while the
201 BF2 + BR1 and BF2 + BR2 combination showed even higher precision than a previously tested 16S
202 marker (Elbrecht *et al.* 2016). The proportion of detected non-insect metazoan taxa varied between
203 primer combinations, with the combination BF1+BR2 detecting all but one taxon.

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216 **Table 2:** Number of species recovered with the newly developed primers and data on 16S and
 217 Folmer primers from previous tests (Elbrecht & Leese, 2015; Elbrecht et al., 2016).

| Taxonomic group | Number of specimens | Number of specimens recovered with specific primer combination | | | | | |
|-----------------|---------------------|--|-----------|-----------|-----------|-----------|-----------|
| | | LCO1490+HCO2198 | 16S ins | BF2+BR2 | BF2+BR1 | BF1+BR2 | BF1+BR1 |
| | | 7 (88%) | 8 (100%) | 8 (100%) | 8 (100%) | 8 (100%) | 8 (100%) |
| Plecoptera | 4 | 4 (100%) | 4 (100%) | 4 (100%) | 4 (100%) | 4 (100%) | 4 (100%) |
| Trichoptera | 15 | 13 (86%) | 15 (100%) | 15 (100%) | 15 (100%) | 15 (100%) | 15 (100%) |
| Diptera | 8 | 7 (88%) | 7 (88%) | 8 (100%) | 8 (100%) | 8 (100%) | 8 (100%) |
| Other insects | 7 | 7 (100%) | 7 (100%) | 7 (100%) | 7 (100%) | 7 (100%) | 6 (86%) |
| Other metazoa | 10 | 5 (50%) | 2 (20%) | 7 (70%) | 6 (60%) | 9 (90%) | 6 (60%) |
| Σ All insects | 42 | 38 (91%) | 41 (98%) | 42 (100%) | 42 (100%) | 42 (100%) | 41 (98%) |
| SD* | | 1.01 | 0.62 | 0.54 | 0.65 | 0.71 | 0.84 |
| Precision** | | 0.72 | 0.37 | 0.28 | 0.35 | 0.49 | 0.58 |
| Σ All taxa | 52 | 43 (83%) | 43 (83%) | 49 (94%) | 48 (92%) | 51 (98%) | 47 (90%) |

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219

220 * Mean standard deviation (SD) of \log_{10} sequence abundance from each insect taxon that was
 221 detected (specimens with $< 0.003\%$ read abundance discarded)

222 ** Precision defined as the SD of the mean \log_{10} distance to the expected abundance, calculated for
 223 each morphotaxon (all taxa).

224

225 3.3 *In silico* evaluation of primers

226 Performances of the 11 forward and 12 reverse primers were computationally evaluated against
 227 OTUs of all insect orders (Figure 3). Reference data for binding sites of the standard Folmer primers
 228 HCO and LCO were very limited and Megaloptera and Turbellaria had below 100 OTUs. Primer
 229 efficiencies were very similar across orders but varied slightly between primers. However, Bivalvia,
 230 Turbellaria and Hirudinea showed higher penalty scores than other groups, while the high penalty
 231 scores for Amphipoda are likely due to the low sequence coverage and one mismatching sequence in
 232 the binding region (Figure 3). *In silico* and PCR (mock community samples) amplification success of
 233 BF/BR primer combinations were similar, but not always consistent. For example, while the BR1
 234 primer shows a mean *in silico* amplification of only 77% (Figure 3), the BF2+BR1 primer
 235 combination performed well with actual samples (Figure 2). In general, primers incorporating wobble
 236 bases (jgLCO1490, BF1, BF2, BR1, BR2, jgHCO2198, H2123d) or inosine (Ill_B_F, ArF5, Il_C_R,
 237 ArR5) at the 3'-end performed better than primers with no or just few wobble bases (linear regression
 238 mean penalty scores against \log_{10} primer degeneracy: $p = 0.004$, adj. $R^2 = 0.296$).

239

240 It should be noted that some primers from the literature are not only poorly matching because they
 241 lack wobble bases, but are rather affected by additional problems (see Figure S2, "critical
 242 mismatches"). For instance, near the 3'-ends, the EPT-long-univR has a completely unnecessary
 243 second inosine at a conserved position, while the Uni-MinibarF1 has a "T" at a position where more

244 than half of the reference OTUs have an "A". Furthermore, the L499 primer targets a highly variable
245 region. The mlCOIintR primer incorporates S (= C or G) leading to many mismatches (Figure S2),
246 while the forward version of the same primer uses W (= A or T) wobble bases which match better.
247 The reverse primers listed in the supplementary information of (Gibson et al., 2014) are not written in
248 reverse complement, and will not work if ordered as provided (we evaluated the ArR5 primer in the
249 reverse complement *in silico*). Finally, certain primers show mismatches to particular groups, e.g. the
250 ZBJ-ArtF1c primer does not match well to sequences of Bivalvia and the BR1 primer shows an
251 unambiguous mismatch to Turbellaria and Hirudinea at the fifth position (Figure S2).

252 4 Discussion

253 4.1 Amplification success of mock communities

254 Aquatic bioassessments require standardized and reliable data on biological quality elements such as
255 macroinvertebrate communities. Metabarcoding holds the potential to assess biodiversity of
256 freshwater ecosystems quickly and more reliably, if suitable primers are available. We used
257 PrimerMiner to obtain freshwater invertebrate specific sequence information based on OTU sequence
258 alignments generated from mitochondrial and COI barcodes obtained from the NCBI and BOLD
259 databases. Using this well-balanced dataset as a reference, we developed and experimentally tested
260 four primer sets targeting freshwater invertebrates. We deliberately decided to not factor-in
261 nucleotide variability present in only a few groups (mostly non-insect Metazoa) to limit the
262 degeneracy of the primers to a reasonable level.

263
264 All four BF / BR primer combinations amplified the ten mock communities successfully, especially
265 for insect taxa. By factoring-in the different amplicon lengths in library pooling, we obtained similar
266 numbers of reads for each sample. All degenerated COI primers showed superior detection rates (up
267 to 100% of insects and 98% of all morphotaxa) and more consistent read abundances compared to the
268 standard Folmer barcoding primers that lacked any base degeneracy (Folmer et al., 1994; Elbrecht &
269 Leese, 2015). The primer BF2 in combination with BR1/BR2 even showed better detection rates and
270 higher precision than a previously used primer targeting a more conserved region of the
271 mitochondrial 16S rRNA gene, which was tested on the same communities (Elbrecht et al., 2016).
272 An *in silico* analysis of the BF / BR primers against 15 freshwater groups obtained from the NCBI
273 and BOLD databases confirmed their good detection rates (especially the BF2+BR2 combination).
274 However, other primer sets from the literature are also suitable for amplification of insect taxa based
275 on our *in silico* testing (e.g. the primers by (Geller et al., 2013; Gibson et al., 2014; Shokralla et al.,
276 2015). Deagle and co-authors argued strongly against the use of degenerated primers in DNA
277 metabarcoding and instead proposed the use of ribosomal markers with more conserved binding
278 regions ((Deagle et al., 2014). However, using a highly standardized approach with 10 independent
279 taxa-rich mock communities, we clearly show that the application of highly degenerated COI primers
280 is not only feasible but even superior to ribosomal metabarcoding of animals with respect to primer
281 performance and available reference databases. Additionally, ribosomal markers often have limited
282 taxonomic resolution, which is less of an issue for the COI barcoding marker (Meusnier et al., 2008;
283 Clarke et al., 2014; 2017).

284
285 While our developed primers showed very reliable amplification results, we also identified problems
286 associated with the primers and the metabarcoding protocol. First, while the use of fusion primers
287 potentially decreases the chance of tag switching and reduces the laboratory work needed, it also
288 reduces PCR efficiency substantially (Schnell, Bohmann & Gilbert, 2015). Primer combinations
289 involving BF2 primers were less affected by this issue, but it was more pronounced with the BF1

290 primer (especially in combination with BR1). Concerns have also been raised by amplification biases
291 associated with use of tagged primers (O'Donnell et al., 2016). While we could not directly test for
292 this bias due to the lack of replicates we did not observe any obvious effects in our current dataset
293 (most taxa were detected to equal proportions regardless of primer tag), there was a decrease in
294 sequence quality when using the P5_BF1_2 primer. Whether this was a systematic effect associated
295 with the tag of the P5_BF1_2 primer or a problem in primer synthesis / quality could not be
296 determined from this dataset. Independently of the source of this possible bias, no effects on the
297 number of detected taxa was observed. Further, 17% of reads from the BF2+BR2 primer
298 combinations were discarded due to low read quality, as the paired end read show only little overlap
299 of ~ 35bp. Additionally, with highly degenerated primers the specificity of the primers decreases
300 (Deagle et al., 2014), potentially amplifying non-target regions. This effect was often minimal, with
301 few sequences deviating from the expected length (below <0.5 % for most primer sets). These
302 numbers were potentially inflated by PCR / sequencing errors and pseudogenes (Bensasson et al.,
303 2001; Eren et al., 2013). More problematically, the BF1 and BF2 primers were affected by
304 insertion/deletion ('indel') effects making up to 40% of the sequences 1-2 bp shorter or longer at the
305 primer binding site. The reasons for these effects, which were also observed to a lesser degree in
306 datasets from previous studies (Elbrecht & Leese, 2015; Elbrecht et al., 2016), are unclear. It is
307 possible that the high degeneracy of the forward primers in combination with low diversity
308 nucleotides at the primer's 3'-end (e.g. C[cta]TT[tc]CC in BF2) makes this effect particularly
309 pronounced. Therefore, we recommend designing primers with two unique nucleotides at the 3'-end
310 e.g. CG and additionally considering common primer design guidelines (Kwok et al., 1994;
311 Mülhardt, 2008; Shen et al., 2010). The effect of this minimal shifting, shortens read length by 1-2 bp
312 while having no effect on the detection of taxa (OTUs will still match the same reference taxon,
313 regardless of 1-2 bp being clipped from the sequence). However, when calculating OTU based
314 biodiversity indices, the small shift might lead to a bias in these metrics due to inflated OTU
315 numbers. While this might be solved by aligning OTU sequences and trimming them to the same
316 length, we still advise that OTU-based diversity measures should be taken with caution when using
317 the BF / BR primer set. Finally, we must acknowledge that the BF / BR primer sets showed poor
318 performance on non-insect Metazoans like Bivalvia, Turbellaria, Amphipoda and Hirudinea, which
319 are genetically distant to insects, making the development of a universal primer difficult.

320
321 While the primer sets developed and thoroughly evaluated in this study provide enhancements to
322 existing primer resources, they are by no means perfect. While we can recommend using the
323 BF2+BR2 or BF2+BR1 primer set for targeting freshwater taxa with DNA metabarcoding, we
324 explicitly express that for routine monitoring further improved primers would be desirable. This can
325 be achieved by testing additional degenerated primer pairs or develop multiplex primer sets (targeting
326 the same or similar regions), while the latter have the disadvantage of adding additional laboratory
327 costs (Mülhardt, 2008; Shen et al., 2010; Hajibabaei et al., 2012; Gibson et al., 2014).

328

329 **4.2 Primer success is determined by base degeneracy and reference data**

330 *In silico* analysis of 23 potentially suitable primers for COI DNA metabarcoding showed that high
331 primer degeneracy leads to the best amplification of freshwater and insect taxa. We verified this also
332 experimentally with the tested macroinvertebrate mock communities that showed high primer bias
333 with standard Folmer primers (Elbrecht & Leese, 2015) but a very consistent amplification with
334 higher detection rates with the BF/BR primers developed in this study. It is possible that other
335 primers (Gibson et al., 2014; Shokralla et al., 2015) may lead to equally good amplification.
336 However, a lack of degeneracy can lead to substantial bias in many of the other evaluated primers.

337 These biases might not strongly affect PCR for DNA barcoding on single organisms, but they may
338 substantially skew detection rates of complex multispecies bulk samples and lead to taxa remaining
339 undetected (Piñol et al., 2014; Elbrecht & Leese, 2015). For example, the mlCOIint primers which
340 have a maximum degeneracy of two nucleotides at each position (Leray et al., 2013), were previously
341 tested with two mock communities and up to 35% of taxa remained undetected (Leray & Knowlton,
342 2015). Probably even more problematic are primers that lack base degeneracy. Despite primer bias
343 associated with the high variation of the COI gene having been well-documented (Clarke et al., 2014;
344 Deagle et al., 2014; Sharma & Kobayashi, 2014; Piñol et al., 2014; Elbrecht & Leese, 2015), primers
345 without base degeneracy like ZBJ-Art by (Zeale et al., 2011) are widely used e.g. for gut content
346 analysis (153 citations as of March 2017). It is critical therefore that degenerate primers optimized
347 for the ecosystems and organism groups under study are employed. If using primers derived from the
348 literature, these should be tested *a priori* to investigate if they are suitable for the planned
349 metabarcoding project.

350
351 We also demonstrated that several popular primers from the literature contain critical design flaws,
352 possibly introduced by accident (e.g. EPT-long-univR, mlCOIintR, Uni-MinibarF1). It has to be kept
353 in mind that a typographical error, or just one mismatching base at the 3'-end can make or break a
354 primer (Stadhouders et al., 2010; Piñol et al., 2014). Additionally, primers are often developed on a
355 small set of taxa, and thus might not work well for the ecosystem, geographic region or taxa under
356 study. For example, Clarke and co-authors evaluated the L499+H2123d as a metabarcoding primer
357 (Clarke et al., 2014), but it was originally only developed to target tephritid fruit flies and probably
358 was never intended to be used beyond this dipteran family (Van Houdt et al., 2010). Therefore,
359 careful *in silico* evaluation and mock community testing of newly developed primers or primers from
360 the literature against the specific taxa of interest is crucial for metabarcoding projects. We highly
361 recommend evaluation primers not only *in silico* but also using mock communities of known
362 composition, to validate that the primers work well for the targeted groups and purpose.
363 Unfortunately, resources are limited and metabarcoding primers are not always tested and validated
364 before being used in larger scale ecological or monitoring studies.

365

366 4.3 Recommended approaches for freshwater bio-assessment using macroinvertebrates

367 The success of DNA metabarcoding for bioassessment and specific Environmental Impact
368 Assessment of freshwater ecosystems depends on well-designed primers that reliably amplify the
369 target communities. The more conserved primer binding regions, the greater the amplification
370 efficiency (Deagle et al., 2014). Therefore, 18S and 16S ribosomal markers have been proposed as
371 suitable alternative markers to the COI gene, despite lacking comprehensive reference databases for
372 animal taxa and potential limitations in taxonomic resolution (Clarke et al., 2014; Deagle et al., 2014;
373 Elbrecht et al., 2016). However, the *in silico* evaluations and documented good performance of the
374 BF2+BR1 and BF2+BR2 primer sets of the COI gene shown in this study suggest clearly that
375 ribosomal markers are not necessary for reliable DNA metabarcoding on animal species tested here
376 (see also Clarke et al. 2017). The COI marker can lead to equally good results or better detection
377 rates (Elbrecht et al., 2016; Clarke et al., 2017), but already has large reference databases available
378 for animals. Therefore, we strongly encourage focusing efforts on developing optimized ecosystem
379 or community-specific COI primers.

380 When using DNA metabarcoding approaches for bioassessment, protocols from the literature should
381 be critically evaluated as success may be flawed by unsuitable primer design. Additionally, we
382 recommend that replicates are included to reduce the chance of tag switching and exclude false OTUs
383 from the dataset (Lange et al., 2015). While we have previously encouraged the use of fusion primers

384 due to their ease of use (single step PCR, (Elbrecht & Leese, 2015)), we have to acknowledge that
385 they decrease PCR efficiency (Schnell, Bohmann & Gilbert, 2015). Additionally, environmental
386 samples often contain PCR inhibitors, further decreasing amplification efficiency. In these cases, two
387 step PCR which is the recommended approach by Illumina (e.g. (Miya et al., 2015; Carvalho et al.,
388 2017)) might lead to more reliable amplification results, even though two step PCR can be more
389 prone to tag switching (Esling, Lejzerowicz & Pawlowski, 2015; Schnell, Bohmann & Gilbert,
390 2015).

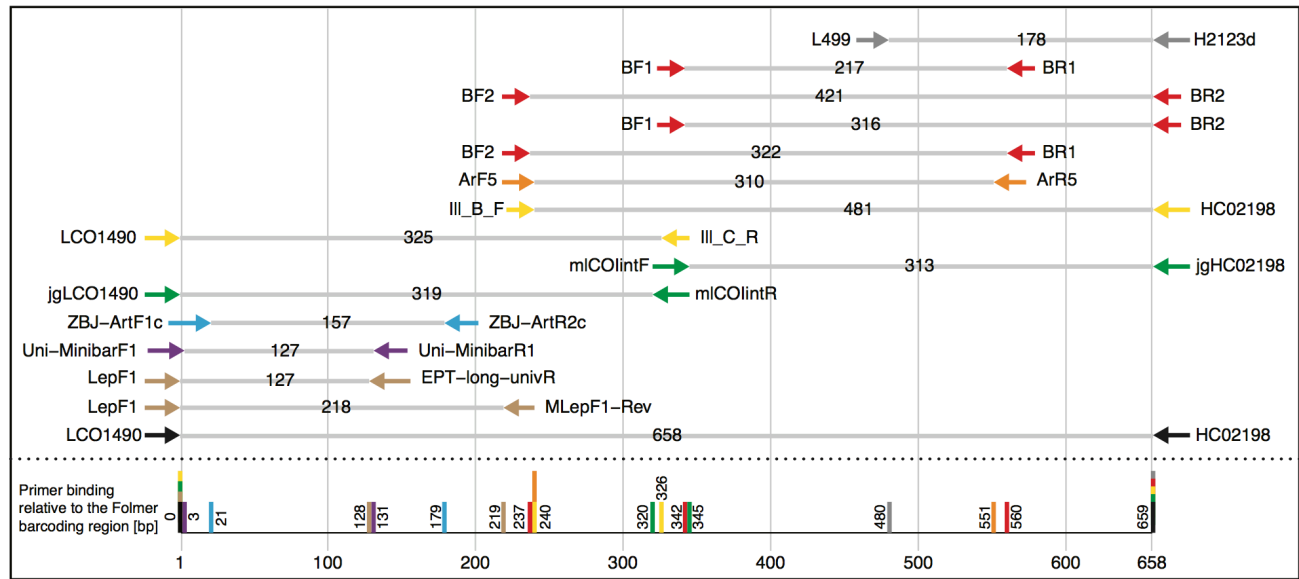
391
392 Besides metabarcoding, metagenomic approaches using enrichment for mitochondrial genomes may
393 also become suitable for bio-assessment, with potentially less bias as the PCR amplification step can
394 be omitted (Liu et al., 2016). However, as briefly discussed in (Elbrecht et al., 2016), metagenomic
395 methods have to be further validated and mitochondrial reference genome libraries ideally need to be
396 completed (Dowle, Pochon & Banks, 2015; Papadopoulou, Taberlet & Zinger, 2015).

397
398 Thus, DNA metabarcoding using the COI marker for DNA based monitoring of stream ecosystems,
399 is currently the most cost-effective approach for reliable bulk sample assessment. However, primers
400 for DNA metabarcoding of macroinvertebrates ideally need to be further optimized and primers from
401 the literature should be tested more extensively on mock communities.
402

403 **4.4 Conclusions**

404 Reliable and quick bioassessments are of critical importance for biomonitoring and Environmental
405 Impact Assessment of aquatic ecosystems. DNA metabarcoding has the potential to meet this
406 challenge if suitable primers can be obtained. Through computational evaluations as well as
407 experimental data, we showed that almost the entire aquatic macroinvertebrate community can be
408 reliably detected with COI metabarcoding. We provide novel degenerated primer sets with high
409 detection rates and greatly reduced primer bias. As databases are still incomplete, we encourage
410 further such *in silico* and *in vivo* evaluations of existing primers as well as the development of
411 improved metabarcoding primers to unlock the full potential of metabarcoding for bioassessment.
412 However, our data already suggests that for freshwater ecosystems, DNA metabarcoding is ready to
413 complement biomonitoring programs on a large scale.

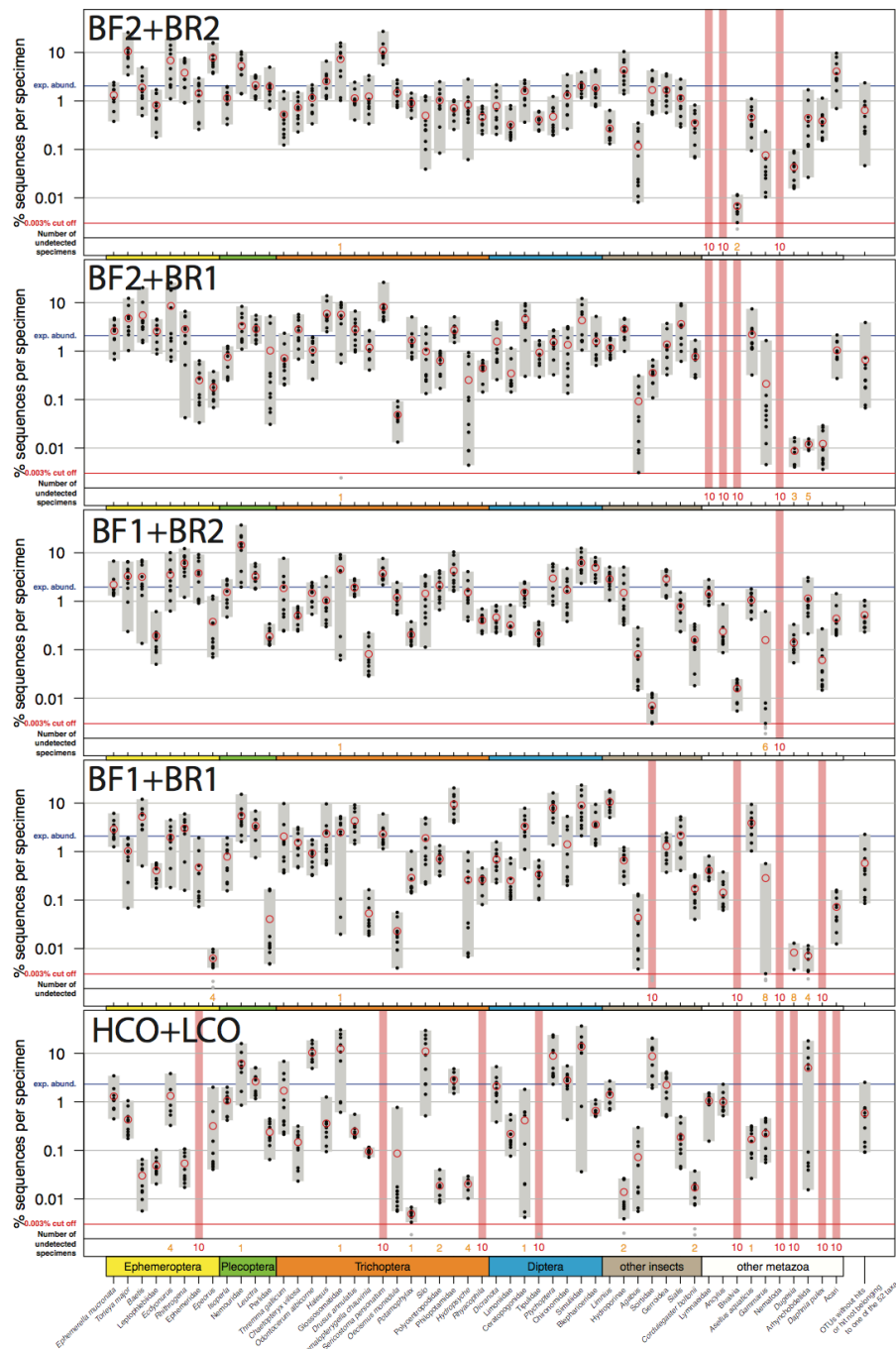
414
415

416 **5 Figures**

417

418 **Figure 1:** Selection of potential COI primer sets for DNA metabarcoding of insects, targeting the
 419 Folmer region. Primer pairs shown are typically used / suggested combinations from the literature.
 420 Table S1 gives an overview of the exact primer sequences and references.

421

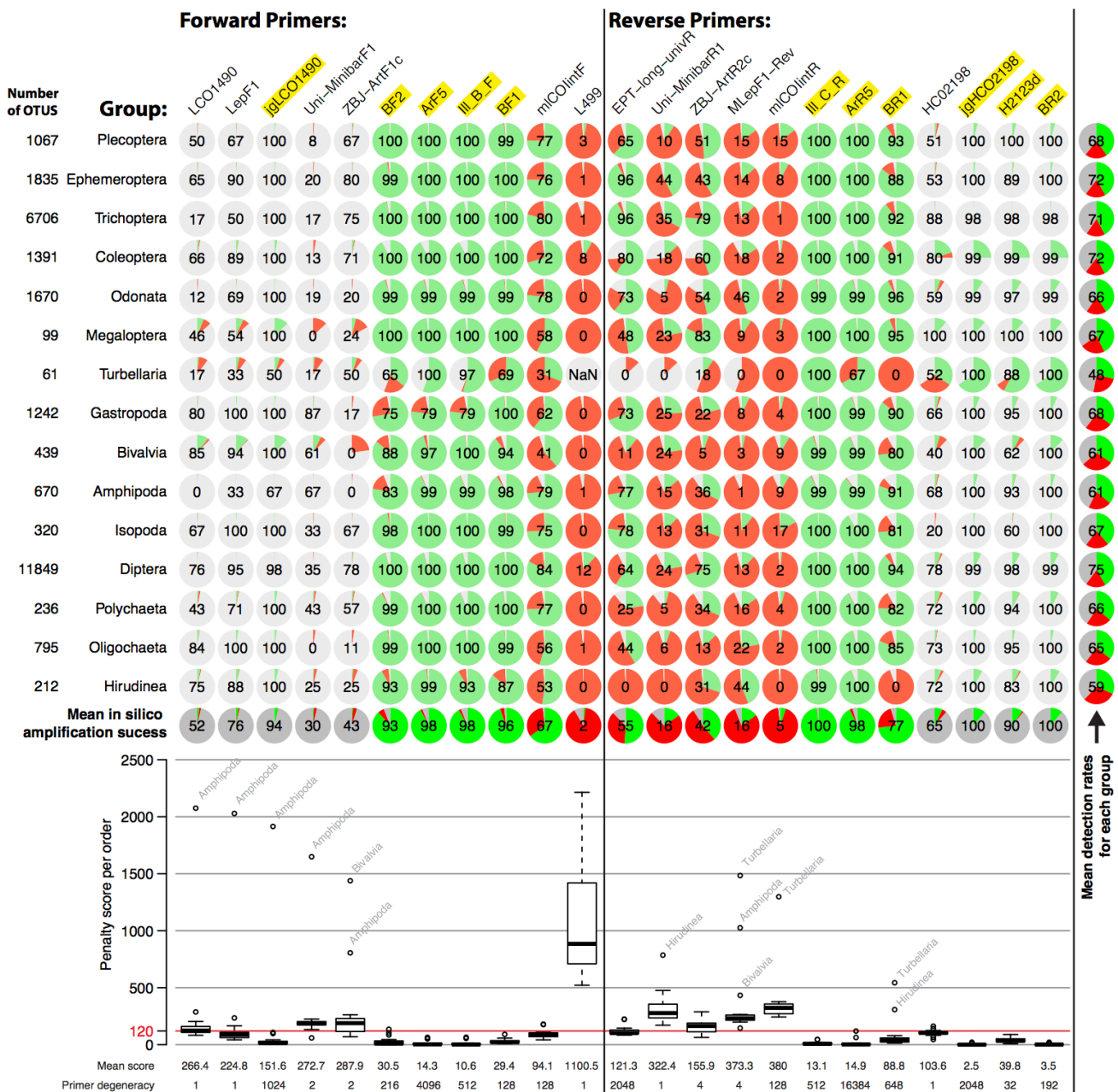


422

423 **Figure 2:** Comparison of the COI Folmer primer performance and the four tested newly developed
 424 primer combinations. All primer combinations were tested with the same ten bulk samples each
 425 containing 52 morphologically distinct macroinvertebrate taxa. The 52 taxa are shown on the x-axis
 426 with the relative number of reads obtained for each morphotaxon by black dots on the logarithmic y-
 427 axis (mean read abundance indicated by red circles), for each respective primer combination.
 428 Sequence abundance was normalized across the ten replicates and the amount of tissue used in each
 429 DNA extraction. Only OTUs with a minimum read abundance of 0.003% in at least one of the ten
 430 samples were included in analyses. Number of samples for which a morphotaxon was not detected is
 431 indicated by orange and red numbers in each plot. A thick vertical line in light red indicates if a
 432 morphotaxon was not detected.

433

12



434

435 **Figure 3:** Overview of *in silico* evaluation of primer performance using PrimerMiner v0.7 with OTU
 436 data from 15 freshwater assessment relevant invertebrate groups. Primer performance is shown for
 437 each group in pie charts (red = failure, green = working, grey = missing data / gaps). Every primer
 438 sequence match with a mismatch penalty score of above 120 is considered a failure, and the
 439 amplification success displayed in each circle (excluding missing data). The box plot is based on the
 440 mean penalty scores for each group, with the mean penalty score and degeneracy given for each
 441 primer. For metabarcoding, potentially suitable primers have a yellow background. For detailed
 442 evaluation parameters, see scripts S2. The L499 primer for the Turbellaria group could not be
 443 evaluated due a 3 bp deletion in the reference sequences, but the primer is not likely to amplify well.

444

445 5 Conflict of Interest

446 The authors declare that the research was conducted in the absence of any commercial or financial
447 relationships that could be construed as a potential conflict of interest.

448 6 Author Contributions

449 VE and FL conceived the ideas and designed methodology, VE carried out the laboratory work and
450 analyzed the data, VE led the writing of the manuscript. All authors contributed critically to the drafts
451 and gave final approval for publication.

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460 <https://peerj.com/preprints/2044/> .
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