1 **Title: Scaling up freshwater macrozoobenthos metabarcoding with new BF2+BR2 fusion**

2 **primer sets in 96-well format**

3 **Running Title (45 char max):** Metabarcoding with fusion primer sets

4 **Word count:** _____

5 **Authors:** Vasco Elbrecht[1*], Dirk Steinke[1,2]

6

7 Affiliations:

8 1) Centre for Biodiversity Genomics, University of Guelph, 50 Stone Road East, Guelph, Ontario, N1G 2W1, Canada

9 2) Department of Integrative Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario, N1G 2W1, Canada

10 *Corresponding author: Vasco Elbrecht (elbrecht@uoguelph.ca),

11

12 **Abstract:**

13 The viability of DNA metabarcoding for assessment of freshwater macrozoobenthos has been demonstrated over the past

14 years. It matured to a stage where it can be applied to monitoring at a large scale, keeping pace with increased high

15 throughput sequencing (HTS) capacity. However, workflows and sample tagging need to be optimized to accommodate for

16 hundreds of samples within a single sequencing run. We here conceptualize a streamlined metabarcoding workflow, in

17 which samples are processed in 96-well plates. Each sample is replicated starting with tissue extraction. Negative and

18 positive controls are included to ensure data reliability. With our newly developed fusion primer sets for the BF2+BR2

19 primer pair up to three 96-well plates (288 samples) can be uniquely tagged for a single Illumina sequencing run. By

20 including Illumina indices tagging can by extended to thousands of samples. We hope that our metabarcoding workflow

21 will be used as practical guide for future large-scale biodiversity assessments involving freshwater invertebrates. However,

22 we also want to acknowledge that this is just one approach, and that we hope this article will stimulate discussion and

23 publication of alternatives and extensions.

24

25 **Key words:** Biomonitoring, high throughput sequencing, macrozoobenthos, multiplexing

26

27

28

29

30

1

Introduction

Reliable monitoring of freshwater macroinvertebrate diversity is a key component in the assessment and management of stream ecosystems (Dudgeon *et al.* 2005; Vörösmarty *et al.* 2010). DNA-based identification methods such as metabarcoding are promising alternatives (Baird & Hajibabaei 2012) to morphological identification, which is often limited in resolution (Sweeney *et al.* 2011). In addition to reducing human bias, DNA based identifications can also lead to improved stream assessment (Stein *et al.* 2013). Over the past few years several studies demonstrated the feasibility of metabarcoding based monitoring of freshwater macroinvertebrates (Hajibabaei *et al.* 2011; Carew *et al.* 2013; Andújar *et al.* 2017). Despite some methodological limitations (presence/absence data, primer bias (Elbrecht & Leese 2015)), assessment results are at least comparable if not superior to conventional morphology based stream monitoring approaches (Elbrecht *et al.* 2017b; Emilson *et al.* 2017). Some macroinvertebrate reference databases are already fairly comprehensive especially for common taxa (Carew *et al.* 2017; Elbrecht *et al.* 2017a), and recently many aspects of the metabarcoding approach have been thoroughly validated (Hajibabaei *et al.* 2012; Carew *et al.* 2013; Elbrecht & Leese 2015; 2017; Elbrecht *et al.* 2017a; Emilson *et al.* 2017; Andújar *et al.* 2017). Consequently, many countries are now actively working towards the use of DNA metabarcoding for routine monitoring of macroinvertebrates (Leese *et al.* 2016).

Routine stream monitoring requires the collection and identification of thousands of kick samples (Buss *et al.* 2015), however, current metabarcoding studies are typically limited to a few dozen samples. If DNA metabarcoding is to be used in routine large scale monitoring projects, a substantial scale up of laboratory protocols is needed in a way that ensures a high level of reliability and quality of data.

We propose a streamlined metabarcoding approach that runs up to 288 individual samples on a single Illumina sequencing run (Figure 1), using the BF2+BR2 fusion primer system (Elbrecht & Leese 2017) which has been shown to work well with macroinvertebrate monitoring samples (Elbrecht *et al.* 2017b). The extended primer set allows for flexible multiplexing of samples in up to three 96-well plates thereby simplifying sample handling and reducing the risk of cross-contamination. By incorporating replicates already at the tissue homogenization stage, as well as including positive and negative controls, samples affected by laboratory issues can be reliably detected and either excluded from subsequent analysis or re-extracted. Furthermore, by minimizing the number of validation steps throughout the protocol and the use of streamlined fusion primer tags in a 96-well format, we ensure practicality of the protocol. Rather than continuously validating or replicating every step of the workflow, we recommend utilizing controls and replicates in a manner that highlights samples affected by errors.

2

Sample collection, homogenization and DNA extraction

After samples are collected using a standardized protocol (Figure 1A), invertebrate specimens should be separated from any debris such as substrate and non-target organic matter (Figure 1B). This increases the chance that some taxa and specimens will be overlooked (Haase *et al.* 2010), but homogenizing an entire sample can introduce PCR inhibitors and render standardization extremely difficult. Once specimens are separated from debris they can be dried (Figure 1C) and homogenized (Figure 1D). DNA of bulk samples has also been extracted directly from the preservation ethanol (Hajibabaei *et al.* 2012), through homogenization of the wet sample (Hajibabaei *et al.* 2011), or by lysing the complete sample (Braukmann *et al.* in prep). We, however, recommend the homogenization of dried bulk samples using e.g. beat mills, which allows DNA extraction of the entire community using just a small quantity of tissue powder (10-15 mg, (Elbrecht & Leese 2015; Elbrecht *et al.* 2017b)). Two replicates per sample should be used for DNA extraction (Figure 1E), both of which will be metabarcoded to facilitate the detection of insufficient tissue homogenization, as taxon composition will vary substantially between replicates if homogenization was incomplete. Any method of DNA extraction yielding high quality DNA can be used (Figure 1F). As tissue powder is easily electrically charged, direct transfer of powder into the 96-well plate should be avoided. Rather the powder should be incubated in individual 1.5 ml reaction tubes to reduce the risk of cross-contamination. For the same reason, a strong adhesive plate sealing tape (if necessary detergent resistant) should be used throughout the entire workflow to prevent spilling of samples. Additionally, plates should always be centrifuged before opening and sealed with fresh sealing tape (ideally tightened with a plastic squeegee). To enable detection of cross-contamination each row and each column needs to contain an extraction blank that will be included in PCR and sequencing (Figure 1). Tissue powder from a previous project or a mock sample can be used as a positive control throughout the metabarcoding workflow. It's recommended to homogenize the positive control sample with liquid nitrogen to ensure it's homogeneity if used across several experiments (Elbrecht & Leese 2015). To increase PCR success and for easier troubleshooting we recommend normalizing all DNA extracts to identical concentrations. Ideally, DNA is quantified by using a chromatogram-based approach (e.g. Fragment analyzer, Advanced Analytical, USA), which will also quantify DNA quality and verify that the negative controls are clean.

Amplification and tagging: Two step PCR protocol

After the DNA is extracted and normalized, the barcode marker can be amplified. For freshwater macrozoobenthos, the cytochrome oxidase subunit I (COI) gene is usually used, but some authors also recommended ribosomal markers (Deagle *et al.* 2014). We think ribosomal markers do not offer any advantages over well-designed degenerated COI primer sets

3

89   (Elbrecht & Leese 2017). Additionally, ribosomal markers often lack adequate reference data (Elbrecht *et al.* 2016). We

90   recommend the use of BF2+BR2 primer set as it was specifically designed for freshwater macrozoobenthos and has been

91   already evaluated using both mock and kick samples (Elbrecht *et al.* 2017b; Elbrecht & Leese 2017). Further PCR and

92   primer modifications are dependent on the strategy used to multiplex several uniquely tagged samples for a sequencing run.

93   We recommend the use of a two-step PCR protocol, in which the first PCR amplifies the target fragment utilizing universal

94   primers, while the second PCR uses fusion primer versions of the same primer sets, which include an inline tag and Illumina

95   sequencing tails (Figure 2). Fusion primers can be used directly in a single PCR approach, but a two-step PCR setup is less

96   susceptible to PCR inhibition (Schnell *et al.* 2015). Additionally, fusion primers greatly reduce the chance for tag switching

97   (Elbrecht *et al.* 2017b), which can become an issue with other more modular tagging approaches (Esling *et al.* 2015; Schnell

98   *et al.* 2015). Furthermore, inline tags of different length and parallel sequencing in forward and reverse direction can

99   substantially increase sequence diversity which in turn leads to better results on Illumina machines and allows for a reduced

100   spike-in of PhiX (Elbrecht & Leese 2015). That being said, fusion primers can be quite costly, as many versions with

101   different in-line tags are needed. They also need to be developed for each new primer set (thus using commercial indexing

102   kits for small projects might be more cost effective). However, if the same fusion primer set is used more frequently, it can

103   become highly cost effective. One primer costs around $50 and yields over 100 µl with a 100 pmol/µl concentration, of

104   which 25 pmol are used per 50 µl PCR reaction. Such a set (forward + reverse primer) can be used to tag 400 samples at a

105   cost of $100 ($0.25 per reaction).

106   Previously developed BF2+BR2 fusion primer sets were limited to tagging a maximum of 72 samples (Elbrecht & Leese

107   2017), which will quickly become insufficient for large-scale metabarcoding projects. Therefore, we developed new fusion

108   primer sets that allow unique tagging and multiplexing of up to 288 samples on three 96-well microplates within the same

109   run (Figure 3, see Fig S1 for full primer sequences and Tab S1 for plate layouts). These new tags use a 7 bp sequence for

110   both forward and reverse primers, while avoiding inline tags of 0 - 1 bp length which are easily affected by insertions or

111   deletions caused by sequencing errors. Because the manual development of large numbers of different tags is difficult, we

112   employed an R script that we used to randomly generate 500 tagging sets (Script S1). Eight previously developed primer

113   pairs were incorporated into the design process, but the overall base composition was kept similar where possible (Fig S2).

114   The similarity between tags of each generated set was subsequently visualized (Fig S3), and the primer set with most

115   divergent tags was chosen in order to reduce potential tag switching through sequencing errors. All resulting tags differed

116   by at least 3 bp, with the exception of four fusion primers that had only a 2 bp insert. For PCR we recommend using a

117   reaction volume of 50 µl with a high quality standard *Taq*. It is our experience that proof reading *Taq's* often struggle with

118   degeneracy and long primer tails. For the first PCR (Figure 2), a master mix using the standard BF2+BR2 primers is added

4

119   to each 96-well plate. As the extracted DNA (including negative/positive controls) is already present in a 96-well format,

120   ~25 ng DNA can be easily transferred to the PCR plate (Figure 1H). After the initial PCR 1 µl amplicon is used as template

121   for the second PCR which individually tags each sample (Figure 1I). The number of cycles needed in each PCR might have

122   to be optimised depending on how strongly samples are inhibited. While the cycle number should be kept as low as possible,

123   studies on barcoding data show that a high number of cycles is not necessarily compromising data quality (Vierna *et al.*

124   2017). PCR success of the first and second PCR can be verified by electrophoresis, however, bands might only be visible

125   after the second PCR depending on cycle number. Amplicons from failed PCR reactions should be excluded from

126   sequencing.

127

Library Preparation and Pooling

129   Amplicons of the second PCR can be directly used for sequencing after chromatographic quantification (Figure 1J) and

130   cleanup (to remove residual primers and other PCR components). As long as it is possible to measure the concentration of

131   amplicons independently from primer dimers, samples can be pooled first and then subjected to cleanup. Otherwise, each

132   individual sample will need to be cleaned separately before quantification. Usually, all samples are pooled with identical

133   amplicon concentration to ensure similar sequencing depth across all of them. However, in some cases sample

134   concentrations can be adjusted, e.g. if amplicons of different length are sequenced on the same run (Elbrecht & Leese 2017)

135   or if the number of specimens across samples is highly variable (Beerman et al. in prep). It should also be stressed that both

136   the quantification and pooling step are absolutely essential for the desired sequencing depth across samples, and the

137   accuracy of any used quantification method should be verified prior to any experiments (Elbrecht *et al.* 2017b). As negative

138   controls are difficult to quantify due to low concentration any adjustment to the concentration of other samples would lead

139   to a strong overrepresentation. We therefore recommend to add each negative control to the library in volumes equal to the

140   average volume of the samples used for pooling.

141   An effective solution for cleanup is magnetic bead purification as it also allows for removal of amplicons that do not match

142   the targeted marker length (Figure 1L). Usually a left sided size selection is sufficient as long as no strong double bands are

143   present. Alternative cleanup methods will be needed if BSA was included as a PCR enhancer, as it can prevent re-

144   suspension of magnetic beads (Elbrecht *et al.* 2017a). The clean library can then be directly loaded onto an Illumina

145   sequencer. As only inline barcodes are used for sample tagging, both Illumina indexing read steps can be skipped (Figure

146   1M). Following sequencing, reads are demultiplexed using the first 7 bp of read one and two (e.g. implemented in the R

147   package "JAMP", http://github.com/VascoElbrecht/JAMP).

5

148

Sequencing depth

150 The number of samples (or plates) per sequencing run depends on the number of sequences a device produces as well as on

151 the desired sequencing depth. The lower the sequencing depth the more taxa will remain undetected, especially those with

152 low abundance, low biomass, and those strongly affected by primer bias (Elbrecht *et al.* 2017a). For macrozoobenthos bulk

153 samples we recommend a sequencing depth of at least 100000 sequences per replicate. As the BF2+BR2 primer set

154 amplifies a 421 bp region, paired end sequencing with at least 250 bp sequence length is necessary. Table 1 shows an

155 overview of currently available Illumina sequencers that meet these criteria (end of 2017) and the expected sequencing

156 depth they can produce per well. A library can be easily re-sequenced when sequencing depth turns out to be insufficient.

157 Additionally, sequencing depth between samples might vary depending on quantification accuracy for individual samples.

158 Samples with insufficient sequencing depth can be recovered, e.g. by adding additional PCR product to the affected samples

159 in a library for a re-run (or exclude respective samples from the dataset if only a few are affected).

160

161 **Table 1:** Sequencing depth per well with different Illumina sequencing platforms suitable for the BF2+BR2 fusion primers

162 (k = 1.000 spots).

Sequencer	MiSeq			HiSeq (1 of 2 lanes)
Sequencing Kit	250 PE v2 Nano*	250 PE v2	300 PE v3	250 PE v2 rapid run
Throughput (max)	1 000k	15 000k	25 000k	150 000k
Number of plates sequenced:				
One (96 wells)	10.4k	156.2k	260.4k	1 562.5k
Two (192 wells)	5.2k	78.1k	130.2k	781.3k
Three (288 wells)	3.5k	52.1k	86.8k	520.8k

163 * 2/3 the cost of the 250 PE v2 kit, too expensive

164

Bioinformatics processing and troubleshooting

166 The choice of bioinformatics pipelines and clustering settings can drastically affect the resulting taxon list, especially when

167 it comes to rare taxa (Figure 1N, (Kopylova *et al.* 2016)). However, as long as data is strictly filtered (removal of singletons,

6

168   abundance based filtering of Operational taxonomic units (OTUs)) and an appropriate OTU clustering algorithm is used for

169   the pool of all samples, results should be reliable (see e.g. (Elbrecht *et al.* 2017b)). However, only samples with sufficient

170   sequencing depth should be used in such analysis, and if samples vary strongly in sequencing depth, rarefaction should be

171   applied across all samples to ensure equal sequencing depth. If a single replicate is of insufficient sequencing depth, the

172   sample should be removed from the dataset. Both replicates for each sample should be very similar in OTU composition.

173   Any discrepancies could indicate e.g. problems caused by insufficient tissue homogenization or cross-contamination

174   (Zepeda-Mendoza *et al.* 2016). Low abundance OTUs that are not shared among replicates should be removed, or the

175   complete sample should be discarded (Figure 1O). However, these samples and OTUs should still be included and

176   highlighted when reporting the raw data, ideally in form of an OTU table. Strong cross-contamination can also be detected

177   by discrepancies between the replicates, especially if the contamination is patchy and not systematic (Kelly *et al.* 2005). The

178   positive control can be used to confirm consistency of the metabarcoding protocol between plates and sequencing runs.

179   Additionally, the 12 negative controls should be inspected for potential cross-contamination and severe tag switching. Tag

180   switching with very low abundance might be observed, but is not a concern (Elbrecht *et al.* 2017b). The sum of the

181   abundance of each OTU in the negative controls can be subtracted from all other samples in order to reduce the effects of

182   low abundance tag switching on the data set. However, if severe tag switching or cross-contamination is detected, the entire

183   metabarcoding run might have to be repeated (ideally starting from the DNA extraction stage).

184

Discussion and conclusions

186   Our proposed metabarcoding strategy is based on sufficiently validated laboratory methods, while still keeping the

187   workflow simple and scalable. By working with 96-well microplates high sample throughput can be easily achieved while

188   at the same time reducing the risk of cross-contamination. By running two replicates starting at the DNA extraction stage,

189   together with negative and positive controls, we ensure that errors are still detectable despite the reduced need to validate

190   each individual laboratory step. The BF2+BR2 fusion primer sets which are extended here (Fig S1) are well tested for

191   macroinvertebrate communities (Elbrecht *et al.* 2017b; Elbrecht & Leese 2017), enabling the tagging and sequencing of up

192   to 288 wells in a single sequencing run. We are confident that this metabarcoding workflow will produce reliable results for

193   up to 123 replicated samples per sequencing run (Figure 1) utilizing a simplified fusion primer based sample tagging

194   process.

195   The number of samples that can be multiplexed with our tagging system is optimized for the currently available Illumina

196   platforms. However, the throughput of sequencers continues to increase with new sequencers and kits being introduced

197   frequently. Already today a shorter COI fragment could be used to amplify macrozoobenthos bulk samples (Meusnier *et al.*

198   2008), which would allow for sequencing at ~50x increased throughput (e.g. HiSeq vs. NovaSeq). Such an approach would

199   require thousands of samples being uniquely tagged and multiplexed for a single sequencing run. Although our inline tags

200   are only able to tag 288 wells, they could easily be extended to several thousand tagging combinations by incorporating

201   Illumina indexing into the fusion primers.

202   While we are convinced that our metabarcoding approach is efficient and reliable it needs to be validated in practice and

203   thoroughly compared to other protocols. We hope that this manuscript will encourage discussion and helps to find better

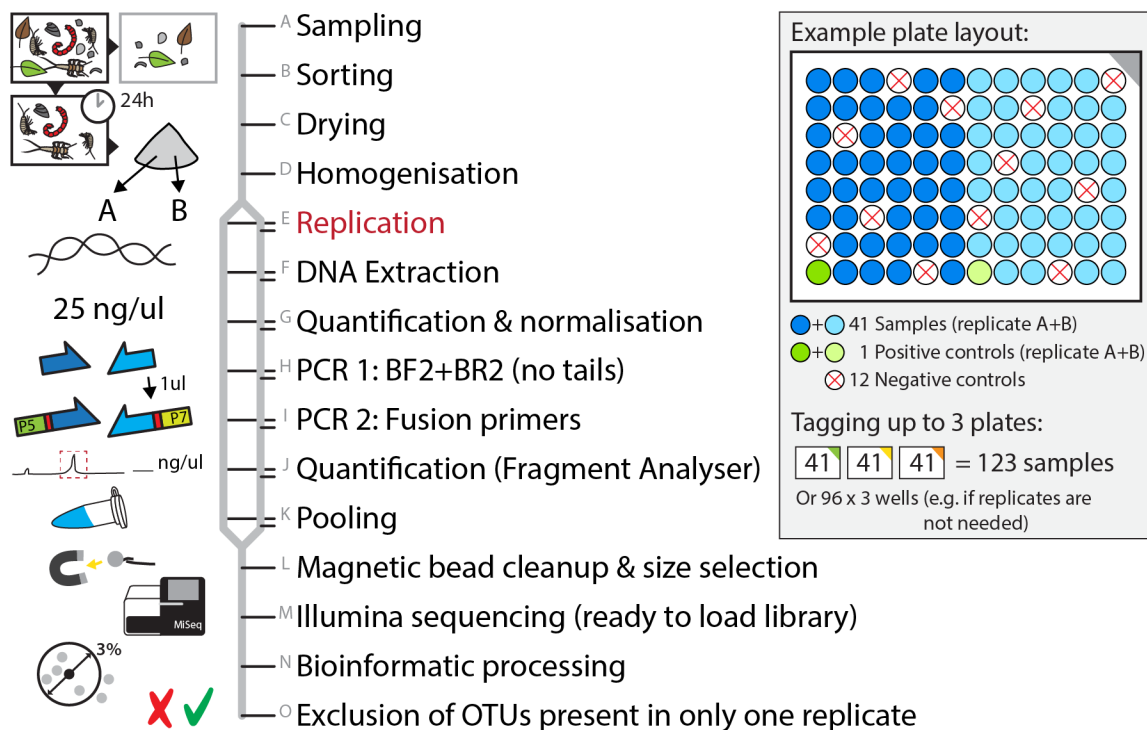204   approaches for the scale-up of metabarcoding for biodiversity assessment.

205

Figure 1: Overview of the proposed metabarcoding work flow for macroinvertebrates using a 96-well plate format and

replication for each sample. Twelve negative controls are included at DNA extraction to detect potential cross

contamination as well as tag switching. One positive control (replicated as well) can be used to estimate the overall

performance of the metabarcoding run. Failed extractions or PCRs can be excluded from the sequencing run, and moved to

a new plate. With the newly designed BF2+BR2 fusion primers developed in this publication up to three 96-well plates can
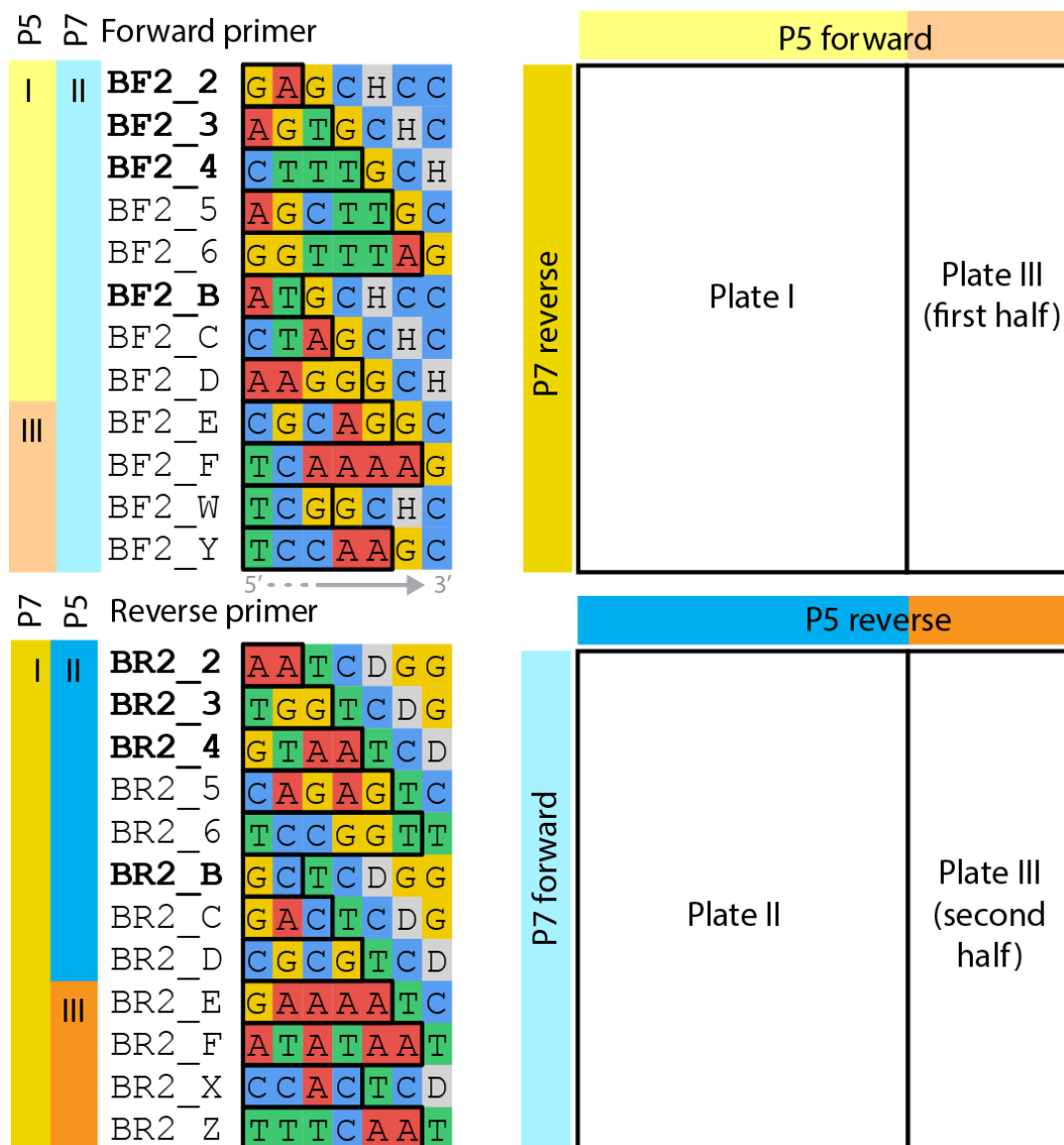
be multiplexed for a single run.

Figure 2: Overview of the two-step metabarcoding PCR protocol (using HotMaster *Taq*, QuantaBio, USA). The first PCR uses the standard BF2+BR2 primers without modifications, thereby increasing amplification efficiency. Subsequently, 1 µl of amplicon product from the first PCR is used (without cleanup) as template for the second PCR step utilizing fusion primers, which adds inline tags as well as Illumina sequencing adaptors. Note that the extension time is increased for the second PCR in order to ensure the entire fusion primer gets amplified. After the second PCR the product can be prepared for sequencing (quantification, pooling with other amplicons and clean-up).

Figure 3: Overview of the newly developed inline tags for the BF2+BR2 primer set. Names of previously published primers are highlighted in bold (Elbrecht & Leese 2017) and the inline tag for each primer is indicated by a black box (the full 7 bp sequence has to be used for demultiplexing). The pipetting schema for three 96-well plates is shown on the right. All three plates can be pooled and used for the same sequencing run, or just plate I + II if two plates are sufficient, or only plate III if tagging for only one plate is desired.

232

Acknowledgements

238

Author contributions

V.E. developed the laboratory workflow, fusion primer set, and wrote the manuscript. D.S. revised the manuscript.

241

Supporting information

Figure S1: Newly developed fusion primer sets (BF2+BR2), suitable for tagging 288 individual wells.

Figure S2: Base composition of the inline tagging region.

Figure S3: Similarity between inline barcodes across all fusion primers.

Script S1: R script used for randomly generating inline barcodes for the given primer sets (includes visualization, as shown

in Figure S3 and Figure 3).

Table S1: Table providing an overview of proposed tagging combinations (as shown in Figure 3).

249

Andújar, C., Arribas, P., Gray, C., Bruce, K., Woodward, G., Yu, D.W. & Vogler, A.P. (2017). Metabarcoding of freshwater invertebrates to detect the effects of a pesticide spill. *Molecular Ecology*.

Baird, D.J. & Hajibabaei, M. (2012). Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. **21**, 2039–2044.

Beermann, A., Zizka, V., Elbrecht, V., Baranov, V., Leese, F. (in prep). Under the cloak of taxonomic family level. DNA metabarcoding of Chironomidae in a multiple stressor mesocosm experiment manipulating salinity, fine sediment and flow velocity

Braukmann, T.W.A, Ivanova, N., Prosser, S., Sones, J., deWaard, J., Zakharov, E., Ratnasingham, S., Steinke, D., Hebert, P.D.N. (in prep). Understanding arthropod diversity using a mock community.

Buss, D.F., Carlisle, D.M., Chon, T.-S., Culp, J., Harding, J.S., Keizer-Vlek, H.E., Robinson, W.A., Strachan, S., Thirion, C. & Hughes, R.M. (2015). Stream biomonitoring using macroinvertebrates around the globe: a comparison of large-scale programs. *Environmental monitoring and assessment*, **187**, 4132.

Carew, M.E., Nichols, S.J., Batovska, J., St Clair, R., Murphy, N.P., Blacket, M.J. & Shackleton, M.E. (2017). A DNA barcode database of Australia's freshwater macroinvertebrate fauna. *Marine and freshwater research*, 1–15.

Carew, M.E., Pettigrove, V.J., Metzeling, L. & Hoffmann, A.A. (2013). Environmental monitoring using next generation sequencing: rapid identification of macroinvertebrate bioindicator species. *Frontiers in zoology*, **10**, 1–1.

12

266   Deagle, B.E., Jarman, S.N., Coissac, E., Pompanon, F. & Taberlet, P. (2014). DNA metabarcoding and the cytochrome c
267   oxidase subunit I marker: not a perfect match. *Biology Letters*, **10**, 20140562–20140562.

268   Dudgeon, D., Arthrington, A.H., Gessner, M.O., Kawabata, Z.-I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard,
269   A.-H., Soto, D., Stiassny, M.L.J. & Sullivan, C.A. (2005). Freshwater biodiversity: importance, threats, status and
270   conservation challenges. *Biological Reviews*, **81**, 163.

271   Elbrecht, V. & Leese, F. (2015). Can DNA-Based Ecosystem Assessments Quantify Species Abundance? Testing Primer
272   Bias and Biomass—Sequence Relationships with an Innovative Metabarcoding Protocol (M. Hajibabaei, Ed.). *PloS*
273   *one*, **10**, e0130324–16.

274   Elbrecht, V. & Leese, F. (2017). Validation and development of freshwater invertebrate metabarcoding COI primers for
275   Environmental Impact Assessment. *Frontiers in Freshwater Science*.

276   Elbrecht, V., Peinert, B. & Leese, F. (2017a). Sorting things out: Assessing effects of unequal specimen biomass on DNA
277   metabarcoding. *Ecology and Evolution*, **7**, 6918–6926.

278   Elbrecht, V., Taberlet, P., Dejean, T., Valentini, A., Usseglio-Polatera, P., Beisel, J.-N., Coissac, E., Boyer, F. & Leese, F.
279   (2016). Testing the potential of a ribosomal 16S marker for DNA metabarcoding of insects. *PeerJ*, **4**, e1966–12.

280   Elbrecht, V., Vamos, E., Meissner, K., Aroviita, J. & Leese, F. (2017b). Assessing strengths and weaknesses of DNA
281   metabarcoding based macroinvertebrate identification for routine stream monitoring. *Methods in Ecology and*
282   *Evolution*, 1–21.

283   Emilson, C.E., Thompson, D.G., Venier, L.A., Porter, T.M., Swystun, T., Chartrand, D., Capell, S. & Hajibabaei, M. (2017).
284   DNA metabarcoding and morphological macroinvertebrate metrics reveal the same changes in boreal watersheds
285   across an environmental gradient. *Scientific Reports*, 1–11.

286   Esling, P., Lejzerowicz, F. & Pawlowski, J. (2015). Accurate multiplexing and filtering for high-throughput amplicon-
287   sequencing. *Nucleic acids research*, **43**, 2513–2524.

288   Haase, P., Pauls, S.U., Schindehütte, K. & Sundermann, A. (2010). First audit of macroinvertebrate samples from an EU
289   Water Framework Directive monitoring program: human error greatly lowers precision of assessment results. *Journal*
290   *of the North American Benthological Society*, **29**, 1279–1291.

291   Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G. & Baird, D.J. (2011). Environmental Barcoding: A Next-Generation
292   Sequencing Approach for Biomonitoring Applications Using River Benthos. *PloS one*, **6**, 1–7.

293   Hajibabaei, M., Spall, J.L., Shokralla, S. & van Konynenburg, S. (2012). Assessing biodiversity of a freshwater benthic
294   macroinvertebrate community through non-destructive environmental barcoding of DNA from preservative ethanol.
295   *BMC ecology*, **12**, 1–1.

296   Kelly, P., Pereira-Maxwell, F., Carnaby, S. & White, I. (2005). Confidence in polymerase chain reaction diagnosis can be
297   improved by Bayesian estimation of post-test disease probability. *Journal of Clinical Epidemiology*, **58**, 252–260.

298   Kopylova, E., Navas-Molina, J.A., Mercier, C., Xu, Z.Z., Mahé, F., He, Y., Zhou, H.-W., Rognes, T., Caporaso, J.G. &
299   Knight, R. (2016). Open-Source Sequence Clustering Methods Improve the State Of the Art. *mSystems*, **1**.

300   Leese, F., Altermatt, F., Bouchez, A. & Ekrem, T. (2016). DNAqua-Net: Developing new genetic tools for bioassessment
301   and monitoring of aquatic ecosystems in Europe. *Research Ideas and Outcomes*.

302   Meusnier, I., Singer, G.A., Landry, J.-F., Hickey, D.A., Hebert, P.D. & Hajibabaei, M. (2008). A universal DNA mini-
303   barcode for biodiversity analysis. *BMC genomics*, **9**, 214.

304   Schnell, I.B., Bohmann, K. & Gilbert, M.T.P. (2015). Tag jumps illuminated - reducing sequence-to-sample
305   misidentifications in metabarcoding studies. *Molecular ecology resources*, **15**, 1289–1303.

306   Stein, E.D., White, B.P., Mazor, R.D., Jackson, J.K. & Battle, J.M. (2013). Does DNA barcoding improve performance of

13

307   traditional stream bioassessment metrics? *Freshwater Science*, **33**, 302–311.

308   Sweeney, B.W., Battle, J.M., Jackson, J.K. & Dapkey, T. (2011). Can DNA barcodes of stream macroinvertebrates improve
309   descriptions of community structure and water quality? *Journal of the North American Benthological Society*, **30**, 195–
310   216.

311   Vierna, J., Doña, J., Vizcaíno, A., Serrano, D. & Jovani, R. (2017). PCR cycles above routine numbers do not compromise
312   high-throughput DNA barcoding results. (F. Chain, Ed.). *Genome*, **60**, 868–873.

313   Vörösmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan,
314   C.A., Liermann, C.R. & Davies, P.M. (2010). Global threats to human water security and river biodiversity. *Nature*,
315   **467**, 555–561.

316   Zepeda-Mendoza, M.L., Bohmann, K., Baez, A.C. & Gilbert, M.T.P. (2016). DAMe: a toolkit for the initial processing of
317   datasets with PCR replicates of double-tagged amplicons for DNA metabarcoding analyses. *BMC Research Notes*, 1–
318   13.

319