



Comparison of markers for the monitoring of freshwater benthic biodiversity through DNA metabarcoding

Gentile Francesco Ficetola, Frédéric Boyer, Alice Valentini, Aurélie Bonin, Albin Meyer, Tony Dejean, Coline Gaboriaud, Philippe Usseglio-polatera, Pierre Taberlet

► To cite this version:

Gentile Francesco Ficetola, Frédéric Boyer, Alice Valentini, Aurélie Bonin, Albin Meyer, et al.. Comparison of markers for the monitoring of freshwater benthic biodiversity through DNA metabarcoding. *Molecular Ecology*, 2021, *Environmental DNA for Biodiversity and Ecosystem Monitoring*, 30 (13), pp.3189-3202. 10.1111/mec.15632 . hal-03100928

HAL Id: hal-03100928

<https://hal.univ-lorraine.fr/hal-03100928>

Submitted on 24 Jan 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Comparison of markers for the monitoring of freshwater benthic biodiversity through
DNA metabarcoding**

Gentile Francesco Ficetola^{1,2*}, Frédéric Boyer¹, Alice Valentini³, Aurélie Bonin^{1,2}, Albin
Meyer⁴, Tony Dejean³, Coline Gaboriaud³, Philippe Usseglio-Polatera⁴, Pierre Taberlet^{1,5}

¹ Univ. Grenoble Alpes, CNRS, Univ. Savoie Mont Blanc, LECA, Laboratoire d'Ecologie
Alpine, F-38000 Grenoble

² Department of Environmental Sciences and Policy; University of Milano, Milano, Italia.

³ SPYGEN, Le Bourget du Lac, France

⁴ Université de Lorraine, CNRS, LIEC, F-57000 Metz, France

⁵ UiT – The Arctic University of Norway, Tromsø Museum, Tromsø, Norway

* Corresponding author. Email: francesco.ficetola@gmail.com

Running title:

Markers for freshwater biomonitoring

Abstract

Metabarcoding of bulk or environmental DNA has great potential for biomonitoring freshwater environments. However, successful application of metabarcoding to biodiversity monitoring requires universal primers with high taxonomic coverage that amplify highly variable, short metabarcodes with high taxonomic resolution. Moreover, reliable and extensive reference databases are essential to match the outcome of metabarcoding analyses with the available taxonomy and biomonitoring indices. Benthic invertebrates, particularly insects, are key taxa for freshwater bioassessment. Nevertheless, few studies have so far assessed markers for metabarcoding of freshwater macrobenthos. Here we combined *in silico* and laboratory analyses to test the performance of different markers amplifying regions in the 18S rDNA (Euka02), 16S rDNA (Inse01), and COI (BF1_BR2-COI) genes, and developed an extensive database of benthic macroinvertebrates of France and Europe, with a special focus on key insect orders (Ephemeroptera, Plecoptera and Trichoptera). Analyses on 1514 individuals representing different taxa of benthic macroinvertebrates showed very different amplification success across primer combinations. The Euka02 marker showed the highest universality, while the Inse01 marker showed excellent performance for the amplification of insects. BF1_BR2-COI showed the highest resolution, while the resolution of Euka02 was often limited. By combining our data with GenBank information, we developed a curated database including sequences representing 822 genera. The heterogeneous performance of the different primers highlights the complexity of the identification of the best markers, and advocates for the integration of multiple metabarcodes for a more comprehensive and accurate understanding of ecological impacts on freshwater biodiversity.

43 Keywords: amplification rate; cytochrome c oxidase I; biomonitoring; biotic indices;
44 environmental DNA; freshwater biodiversity; macroinvertebrates; primer bias; taxonomic
45 resolution; universality.

46

47

1 INTRODUCTION

Freshwater environments are essential providers of clean water and other services for human society. They also host a substantial biodiversity, still they are globally subjected to the joint impact of multiple stressors such as pollution, eutrophication, climate change and hydrological and hydromorphological modifications (Noges *et al.* 2016; Iversen *et al.* 2019). As a consequence, numerous regulations have been adopted at both the national and international level for the protection of water resources, such as the European Water Framework Directive (Directive 2000/60/EC) and the Clean Water Act of the US Environmental Protection Agency (33 U.S.C. §§1251-1387 1972; Pawlowski *et al.* 2018). These regulations generally require the monitoring of freshwater environments through a combination of physical, chemical hydrological, and biotic parameters to obtain prompt measurements of water quality and of the ecological status of ecosystems.

Multiple approaches exist to assess freshwater quality using aquatic organisms. Benthic macroinvertebrates are perhaps the most frequently used biological group in aquatic bioassessment (Birk *et al.* 2012), because (i) they are taxonomically, biologically and functionally diverse (Usseglio-Polatera *et al.* 2000; Usseglio-Polatera *et al.* 2001), (ii) they are rather easy to identify at the genus or family levels (Tachet *et al.* 2010), (iii) they are often sedentary and react rapidly to anthropogenic pressures in all types of freshwater bodies (Hering *et al.* 2006b; Archaimbault *et al.* 2010; Hering *et al.* 2013), and (iv) their occurrence integrates the effects of environmental changes over several months (Floury *et al.* 2013). Macroinvertebrate assemblages are thus a tool of choice to assess the ecological status of water bodies (*e.g.* Marzin *et al.* 2012; Hering *et al.* 2013; Mondy & Usseglio-Polatera 2013) and to demonstrate environmental degradation (Miler *et al.* 2013; Mondy & Usseglio-Polatera

2013; Theodoropoulos *et al.* 2020) or restoration (Arce *et al.* 2014; Kupilas *et al.* 2016; Camargo 2017; Carlson *et al.* 2018).

Generally, bioassessment indices relying on benthic communities are based on the standardized collection of macroinvertebrate assemblages from monitored sites, followed by organism sorting and taxonomic identification using morphological criteria. Then, quality scores can be attributed on the basis of the presence and/or abundance of certain taxa (Friberg *et al.* 2006; Birk *et al.* 2012). As morphological identification is often challenging, in many case protocols do not require species-level identification, and identification at the genus or family level (and, in some cases, even at coarser levels) can be enough for the calculation of many biotic indices evaluating the ecological status of rivers (Bailey *et al.* 2001; Chessman *et al.* 2007; Birk *et al.* 2012). Nevertheless, the morphological identification of hundreds collected individuals, including young, small-sized, larval stages and organisms damaged during sampling, remains time-consuming and requires a substantial taxonomic expertise, increasing the cost and time required for in-depth assessment of water quality (Haase *et al.* 2004; Hering *et al.* 2018).

DNA metabarcoding and environmental DNA (eDNA) are revolutionizing the monitoring of biodiversity at all levels, because they circumvent the challenge of morphological identification and allow the efficient detection of many taxa that are difficult to capture and detect using traditional methods (Taberlet *et al.* 2018). Environmental DNA and metabarcoding are therefore extremely promising for the assessment of freshwater communities (Hering *et al.* 2018; Li *et al.* 2018; Kuntke *et al.* 2020). For metabarcoding, DNA can be extracted from the tissue of pooled macroinvertebrate communities, amplified using universal primers, sequenced, and identified on the basis of reference databases (Baird & Hajibabaei 2012; Yu *et al.* 2012; Andújar *et al.* 2018). This approach uses the same starting material than traditional biomonitoring, but allows skipping the complexity of morphology-

based taxonomy (Baird & Hajibabaei 2012). Alternatively, DNA can be obtained directly from the water (Ficetola *et al.* 2008). Environmental DNA extracted from freshwaters allows the detection of many taxa that are difficult to capture and detect using traditional methods, but also poses new challenges compared to metabarcoding performed on the tissues of captured individuals. In aquatic environments, DNA undergoes rapid degradation (Eichmiller *et al.* 2016; Buxton *et al.* 2017); therefore eDNA is generally characterized by short fragment sizes (Jo *et al.* 2017; Bylemans *et al.* 2018), but see also (Sigsgaard *et al.* 2017). Therefore, primers amplifying short regions can provide a better detection compared to "standard" barcode primers, which often amplify long DNA fragments (e.g. >300 bp in the most frequently used COI markers; Andújar *et al.* 2018). Furthermore, highly degenerated primers increase the risk of non-specific amplification, thus this kind of primers is not really suitable for the amplification of the complex mix of DNA extracted from the environment. As a consequence, the monitoring of benthic macroinvertebrates using eDNA requires the development and assessment of primers with appropriate features.

Besides the length of the amplified region, three main characteristics are essential for satisfactory eDNA metabarcodes (Ficetola *et al.* 2010; Wilcox *et al.* 2013). First, the eDNA amplification success generally decreases with the number of mismatches between target fragments and primers. Primers must therefore be designed in order to have a consistently low number of mismatches within sequences of the target group (high universality or taxonomic coverage; Ficetola *et al.* 2010; Piñol *et al.* 2015; Marquina *et al.* 2019). Taxonomic coverage can be assessed through both *in silico* and laboratory analyses. *In silico* analyses can allow the rapid assessment of all the taxa for which information is publicly available in databases, but laboratory tests (hereafter, *in vitro* tests) are still needed to confirm the actual performance of primers. Second, the amplified region must be highly variable, to ensure the identification of amplified organisms at the desired taxonomic level (high resolution; Ficetola *et al.* 2010;

Tang *et al.* 2012; Marquina *et al.* 2019). Finally, extensive databases are essential if we want to assign the amplified sequences to known taxa. Even though attempts have been made for the assessment of environmental quality without a taxonomic assignment of DNA fragments (Ji *et al.* 2013; Apothéloz-Perret-Gentil *et al.* 2017; Cordier *et al.* 2018; Cordier *et al.* 2019), taxonomic assignment is essential if we want to produce data comparable with traditional indices of water quality, or if we want to combine eDNA data with information obtained through traditional methods (*e.g.* to analyze long-term series of water body surveys). Despite several attempts to assess freshwater quality using eDNA (Hering *et al.* 2018; Li *et al.* 2018; Serrana *et al.* 2019; Czechowski *et al.* 2020; Pont *et al.* 2020; Yang & Zhang 2020), so far limited formal comparisons have been performed among short primers suitable for eDNA metabarcoding of freshwater macrobenthos (but see Elbrecht *et al.* 2016; Elbrecht & Leese 2017). In addition, there is a pressing need for exhaustive reference databases for taxonomic assignment (see Moriniere *et al.* 2017; Morinière *et al.* 2019; Weigand *et al.* 2019 for remarkable examples).

In this study we combined *in vitro* and *in silico* analyses to compare the performance of three primer pairs potentially suitable for the metabarcoding of bulk or environmental DNA from freshwater macroinvertebrates (macrobenthos), and we developed an extensive reference database for benthic macroinvertebrates living in European freshwaters. We mostly focused on three insect orders (Ephemeroptera, Plecoptera and Trichoptera), which are among the most frequently used macroinvertebrates for the bioassessment of streams (*e.g.* Brabec *et al.* 2004; Hering *et al.* 2006a; Gabriels *et al.* 2010; Arman *et al.* 2019; but see also Cox *et al.* 2019). We also considered a broad range of organisms belonging to other orders of insects and other classes. We first used high-throughput DNA barcoding (Vivien *et al.* 2020) of reference individuals identified by experts to obtain sequences on the broadest available number of taxa from France, and then combined the obtained sequences with those available

in public database, to obtain extensive and reliable measures of the performance of markers, and to produce an extensive reference database for the monitoring of freshwaters through eDNA.

2 MATERIAL AND METHODS

We used the standardized database of European freshwater organisms (Schmidt-Kloiber & Hering 2015; download on 01 March 2018) as taxonomic reference for our analyses, considering all the benthic macroinvertebrates. Although in some cases this database considers non-monophyletic groups (*e.g.* Crustacea), it provides an exhaustive checklist of benthic macroinvertebrates that serve as an essential basis for bioassessment and monitoring.

2.1 Analyses of reference individuals

Most of the reference individuals were provided by OPIE-Benthos which is a working group of OPIE (Office Pour les Insectes et leur Environnement) especially dedicated to aquatic insect studies and aquatic ecosystem protection in France. OPIE-Benthos has developed a national inventory and reference collection of aquatic insects (<http://www.opie-benthos.fr/opie/insecte.php>), including Ephemeroptera, Plecoptera, Trichoptera, and more recently aquatic Coleoptera, aquatic and semi-aquatic Heteroptera, aquatic larval stages of Megaloptera, Neuroptera and Diptera (Ptychopteridae). Corresponding organisms, identified at the highest possible level (species, if possible) by experienced taxonomists, were provided in triplicates (*i.e.* three individuals per taxon, if possible). The collection was completed by

additional taxa (*e.g.* non-insect taxa) specifically sampled by the authors for this reference database.

Individuals were stored in 99% ethanol before DNA extraction. Total DNA was extracted from the entire organism. Samples (constituted of one individual) were initially incubated overnight at 56 °C in 0.5 ml of lysis buffer (Tris-HCl 0.1 M, EDTA 0.1 M, NaCl 0.01 M and N-lauroyl sarcosine 1%, pH 7.5–8.0). Extractions were then completed using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. DNA extracts were recovered in a total volume of 300 µl of elution buffer. Negative extractions without individuals were systematically performed to monitor possible contaminations. Three DNA amplifications were carried out for each sample using the following primer pairs: Inse01, amplifying a ~155 bp region of the 16S mitochondrial rDNA (Taberlet *et al.* 2018); Euka02, amplifying a ~123 bp region of the 18S rDNA (Guardiola *et al.* 2015; Taberlet *et al.* 2018); and the BF1 and BR2 primers, which amplify a ~316 bp region of the cytochrome c oxidase I (Elbrecht & Leese 2017). Inse01 has been developed mostly to amplify insects, Euka02 to amplify all eukaryotes, while BF1 and BR2 were designed to amplify freshwater macroinvertebrates (Elbrecht & Leese 2017; Taberlet *et al.* 2018). The DNA of each individual was amplified in one PCR replicate per each marker considered. DNA amplifications were performed in a final volume of 20 µL, using 2 µL of DNA extract as template. The amplification mixture contained 10 µL of Applied Biosystems™ Master Mix AmpliTaq Gold™ 360, 0.2 µg/µL of bovine serum albumin (BSA, Roche Diagnostic, Basel, Switzerland) and 0.5 µM of each primer for COI and Inse01, or 0.2 µM of each primer for Euka2. Forward and reverse primers were 5'-labeled with eight-nucleotide tags with at least three differences between any pair of tags, so that each PCR reaction was identified by a unique combination of tags. This allowed the assignment of each sequence to the corresponding sample during sequence analysis (Coissac 2012; Taberlet

et al. 2018). The PCR mixture was denatured at 95°C for 10 min, followed by 35 cycles of 30 s at 95°C, 30 s at 52°C for COI and Inse01 or 45°C for Euka2, and 1 min at 72°C (1m 30 s for COI), and followed by a final elongation at 72°C for 7 min. Ten negative DNA extraction and 18 PCR controls (ultrapure water) were analyzed in parallel with the samples to monitor possible contaminations during the experimental process.

Library preparation was performed using the MetaFast protocol by Fasteris (Geneva, Switzerland; <https://www.fasteris.com/dna/?q=content/metafast-protocol-amplicon-metagenomic-analysis>), which significantly limits the tag-jump problem (Taberlet *et al.* 2018). For Euka02 and Inse01, sequencing was performed by 2 × 125-bp paired-end sequencing on the Illumina HiSeq 2500 platform, while for BF1_BR2-COI sequencing was performed by 2 × 250-bp paired-end sequencing on the Illumina MiSeq platform using default settings at Fasteris (Geneva, Switzerland).

Sequencing data were processed using the OBITools (Boyer *et al.* 2016). All libraries were processed using the same pipeline whatever the primer pair used for amplification. Raw sequences were first aligned (program *illumina-paired-end*) to recover the amplicon sequence. Based on the alignment score, sequences were filtered for badly aligned paired-end, i.e. all alignment with a score of less than 40 were discarded (a score of 40 corresponds to 10bp of high sequencing quality perfectly aligned). Then sequences were demultiplexed (program *ngsfilter*) to assign them to the samples, primers with up to two errors were allowed whereas the tags used for demultiplexing had to be strictly conserved. The last step was dereplication (program *obiuniq*) keeping track for each unique sequence of its count in the different samples. For each sample, the most abundant sequence was kept as the most likely barcode. Sequences having a count lesser than 1000 or an abundance ratio with the second most abundant sequence above 1/10 were tagged as belonging to badly amplified samples or

samples where several products amplified. These sequences were given particular attention in the further manual selection of barcodes.

As a further validation step, all the retrieved metabarcodes were matched against NCBI using BLAST, to identify eventual cases in which the obtained metabarcode is a spurious amplification of a non-target organism (*e.g.* fungi or algae). For each taxon, the amplification success was measured as the proportion of individuals for which we obtained valid metabarcodes with a given marker. Such measure of amplification success includes the proportion of individuals that cannot be amplified for a given marker (*e.g.* because of mismatches in the primer region), and also possible lab failures that can reduce amplification even if the primers have a good match with the primer region (*e.g.* low DNA quality or other issues occurring during laboratory procedures), thus it provides a value comparable to what can occur in real-world biodiversity analyses performed using metabarcoding.

2.2 Setting up the composite reference databases

For each species within the database of European freshwater organisms (Schmidt-Kloiber & Hering 2015), we matched the binomial name with the NCBI taxonomy database to retrieve their NCBI taxonomic code (taxid). All the available metabarcodes for the three regions of interest, together with their associated taxid, were extracted from the EMBL sequence data repository (release 136) using the ecoPCR program (Ficetola *et al.* 2010) by matching the primer sequences with up to three errors and restricting the metabarcodes to relevant lengths (lengths >30 bp for Euka02, lengths 70-270 bp for Inse01, lengths 100-500 for BF1_BR2-COI). The three composite reference databases (one for each metabarcoding region) were then built by aggregating metabarcodes for each genus with those obtained from analysed specimens. In order to obtain the most complete coverage of genera found in France, we

obtained the taxid of all metabarcodes produced through *in vitro* analyses as well as metabarcodes extracted from EMBL and associated to the taxid of a species found in France. For genera for which no such metabarcode existed, we included the metabarcodes extracted from EMBL and associated to the taxid of a species of the same genus found in Europe. If no such metabarcode existed, we included all the metabarcodes extracted from EMBL, and associated to a taxid belonging to this genus, also considering species that are not native in Europe.

2.3 Assessing the resolution of metabarcodes

We assessed the resolution of each metabarcoding region with the same procedure. First, the metabarcodes obtained as described above were compared against the ones of each specimen to find identical metabarcodes; this allowed producing a list of unique metabarcodes. For each unique metabarcode, we obtained the list of all the associated taxids. We tested taxonomic resolution at four levels: order, family, genus, and species. More specifically, we tested if, at a given taxonomic level, the list of associated taxids would collapse to a unique taxid or not (*i.e.* all taxids have the same ancestor taxid at that level). If a list collapsed to more than one taxid for the tested taxonomic level, it meant that this metabarcode was not discriminant for this taxonomic level. Consider for instance a given metabarcode associated to multiple species within multiple genera within one single family. This particular metabarcode showed a family-level resolution, but not a species- or a genus-level resolution. It should be noted that these measures of taxonomic resolution heavily depend on the available database (Weigand *et al.* 2019). For example, if the database includes the metabarcode of only one species within a

genus, this analysis could return a species-level resolution, even though it is possible that unanalyzed species within the same genus share the same metabarcode.

2.4 Statistical analyses

We used generalized linear mixed models (GLMMs) to test the significance of differences in amplification success and resolution among markers. GLMMs allowed taking into account non-independence, i.e. the fact that same individual and the same taxon were tested with multiple markers (Pineiro & Bates 2000). For the comparison of amplification success, we used binomial GLMMs; the amplification of each individual with each marker was the dependent variable, while marker and class identity were the independent factors. To take into account the possibility that markers can have a different performance across taxa, we also tested the interaction between marker and taxonomic class. We also considered the identity of the individual, and the finest taxonomic information available for each individual (e.g. species, or genus identity if species information was not available) as random effects. The main GLMM detected significant differences among markers and among taxa, therefore we repeated the GLMM for each of the 12 major taxonomic groups considered here (six classes and six insect orders; see Table S1). Significant GLMMs were followed by Tukey's post-hoc tests, to assess differences in performance of the three markers within each taxon (Hothorn *et al.* 2008).

For the comparison of taxonomic resolutions, we used GLMMs with Gaussian error. The taxonomic resolution of each marker within each taxonomic group was the dependent variable, marker identity was the independent factor, and taxonomic group was included as a random effect. Tukey's post-hoc tests were also used to compare the performance of the three

markers (Hothorn *et al.* 2008). GLMMs were built using the *lme4* package in R (Bates *et al.* 2015). Significance of GLMMs was assessed using likelihood ratio tests (binomial GLMMs) or using a F test with approximated degrees of freedom (lmerTest Package in R; Kuznetsova *et al.* 2017).

3 RESULTS

3.1 Analyses of reference individuals

We extracted and amplified DNA from 1514 individuals, belonging to 578 different taxa (species, genera or families, depending on the identification level; Table 1). The majority of individuals were insects, and three insect orders with macrobenthic larvae (Ephemeroptera, Plecoptera and Trichoptera) altogether accounted for 80% of all the analyzed individuals. Out of these individuals, 99% were morphologically identified at the family level or higher, 95% at the genus level or higher, and 62% at the species level. The average number of sampled individuals was 2.6 individuals per taxon (range: 1-12; median: 3). For Ephemeroptera, Plecoptera, Trichoptera and Megaloptera, the analyzed specimens covered well the diversity of French and European benthic fauna (100%, 74%, 78% and 100% of genera recorded in France for these orders, respectively; 70%, 52%, 65% and 100% of all the genera recorded in Europe; Table 2). Representation was relatively good for Coleoptera, Hemiptera and Neuroptera, whereas coverage was weaker for the remaining orders of insects and for non-insects.

Sequencing returned a total of 15 328 548 reads for Euka02, 14 872 950 reads for Inse01 and 6 023 376 reads for BF1_BR2-COI. After filtering, the average number of reads per individual was high for all the markers (Euka02: 6800; Inse01: 5951; BF1_BR2-COI: 1801 reads).

The amplification success was significantly different between the three markers and among classes (GLMM: differences between markers: $\chi^2_2 = 736.1$, $P < 0.0001$; differences among classes: $\chi^2_6 = 15.1$, $P = 0.020$). Furthermore, a significant interaction between class and metabarcodes indicated that the performance of markers was heterogeneous across taxonomic classes ($\chi^2_{12} = 164.5$, $P < 0.0001$). Differences in amplification success between markers were significant for all the classes except Crustacea (Fig. 1, Supplementary Table S1). Euka02 showed the highest average amplification success (88%), with consistently high amplification success in all the taxa except Malacostraca (Fig. 1). According to Tukey's post-hoc tests, Euka02 showed a particularly high amplification success for Gastropoda and Clitellata (Table S1). Within insects, Euka02 showed excellent amplification success in most of orders, with particularly good performance for Ephemeroptera and Trichoptera, while its amplification success was significantly lower than the other markers for Diptera (Fig. 1b, Table S1).

As expected, Inse01 showed good amplification success for insects (82%), while it showed a limited amplification for the remaining taxa (Fig. 1a). Within insects, Inse01 showed a particularly good performance for Coleoptera and Diptera, and an amplification success similar to Euka02 for Hemiptera and Plecoptera (Fig. 1b, Table S1). Within insects, the lowest amplification success for this marker was observed for Trichoptera (71%; Fig. 1b).

Finally, BF1_BR2-COI showed an average amplification success of 48%, with highly variable results among taxa (Fig. 1a; Table S1). BF1_BR2-COI showed a relatively good amplification success for Gastropoda, Clitellata and Malacostraca, while the rate was lower

for some orders of insects. Within insects, BF1_BR2-COI showed good performance for Coleoptera and Diptera (amplification success $\geq 74\%$, significantly better than Euka02; Table S1), while it amplified less than 50% of individuals from Ephemeroptera, Plecoptera and Trichoptera (Fig. 1b; Table S1).

3.2 Combined database

When we combined sequences of reference individuals with those obtained from GenBank, we obtained a total of 18 834 gene sequences (3 441 for Euka02, 9 715 for Inse01 and 5 678 for BF1_BR2-COI). Insects accounted for the majority of sequences, followed by Crustacea and Clitellata (Table 3). The combined database showed a good coverage of the diversity of European benthic fauna. For the Euka02 primer pair, the completeness of the database was particularly good ($>80\%$) for free-living Platyhelminthes, Coleoptera and Odonata. For instance, our database included Euka02 sequences for 35 out of 43 dragonfly genera living in Europe, i.e. 81% of the European fauna. For Inse01, the completeness was particularly good for Coleoptera, Ephemeroptera and Odonata, while BF1_BR2-COI showed a relatively homogeneous completeness across taxa, with values between 50 and 70% for most taxa (Fig. 2).

3.3 Taxonomic resolution of the different markers

The taxonomic resolution was strongly different among markers. At the species level, the best resolution was observed for BF1_BR2-COI, with just 3% of sequences associated with more

than one species, while Inse01 showed an intermediate resolution (10% of sequences associated with more than one species; Fig. 3a). For Euka02, 21% of sequences were associated with more than one species in the database (Fig. 3a). Differences in resolution between markers were strongly significant (linear mixed models: $F_{2,12} = 271.8$, $P < 0.001$). The resolution of BF1_BR2-COI was significantly better than the one of both Euka02 and Inse01, and the resolution of Inse01 was significantly better than the one of Euka02 (Tukey's post-hoc: both $P < 0.001$).

The taxonomic resolutions of these markers were clearly better if we consider the identification at the genus level (Fig. 3b). Euka02 showed the weakest performance, with around 6% of sequences associated with more than one genus, while BF1_BR2-COI showed the best performance, with less than 1% of sequences associated with more than one genus. Inse01 showed a generally good performance, with less than 1% of sequences associated with more than one genus for most taxa. The performance was slightly poorer for Plecoptera and Trichoptera, with around 4% of sequences associated with more than one genus. Also in this case, differences in resolution among markers were significant ($F_{2,8} = 32.3$, $P < 0.001$). At the genus level, BF1_BR2-COI and Inse01 showed a similar resolution (Tukey's post hoc: $P = 0.20$), and both outperformed Euka02 (both $P < 0.001$). Family level identification was very good for all the metabarcodes, still Euka02 showed a slightly weaker performance than both BF1_BR2-COI and Inse01 (both $P \leq 0.05$). BF1_BR2-COI and Inse01 showed a comparable family-level resolution ($P = 0.24$; Fig. 3c). It should be noted that these values of resolution are calculated on an incomplete set of data, since our database did not include the sequences of many species and genera. For instance, our database only included sequences for ~60% of genera of European Trichoptera (Table 3), and all resolution estimates would probably be poorer if calculated on a complete database.

4 DISCUSSION

Metabarcoding-based biomonitoring requires the availability of primers with high performance, as they must amplify all the relevant target taxa, have sufficient resolution to identify them at the desired taxonomic level, and amplify short sequences usable with eDNA (Ficetola *et al.* 2010; Taberlet *et al.* 2018). Finding primers with all these features is challenging, and the identification of "perfect" regions for barcoding and metabarcoding has often been labelled as a "search for the Holy Grail" (Rubinoff *et al.* 2006). By combining an extensive high-throughput DNA barcoding analysis with the assessment of publicly available sequences, our study highlights the complexity of finding all these desired features in one single metabarcode. It also provides a comparison of performances, allowing the identification of most appropriate markers for different aims and taxonomic groups, and it produced a reference database for the taxonomic identification of a large number of benthic insects.

4.1 The importance of good reference databases

Metabarcoding enables biodiversity monitoring either with or without the taxonomic identification of the retrieved taxa. Taxonomic identification clearly requires appropriate reference databases that can be obtained *ad hoc* (e.g. by amplifying sequences from all the taxa from the target group; (Moriniere *et al.* 2017; Cilleros *et al.* 2019; Morinière *et al.* 2019) or by searching public databases such as GenBank or BOLD. Public databases offer an ever-growing resource, given that they combine the outcome of thousands of studies and produce a sheer amount of data that would be unreachable by *ad hoc* studies. Public databases are not error-free, still analyses showed that for animals, the error rate of GenBank for genus-level

identification is generally low ($\sim 0.7 / 3.5\%$), suggesting that it can be a formidable data source for applications relying on molecular data to understand the impact of environmental changes on biodiversity (Leray *et al.* 2019). However, public databases are opportunistic collections of the material from multiple studies, thus they do not have the ambition of taxonomic completeness. *Ad-hoc* databases (see also Ratnasingham & Hebert 2007) are thus essential resources to obtain the taxonomic coverage required if we want to identify most of benthic macroinvertebrates.

Several researchers advocated that COI-based markers should be favored for metabarcoding because they are standard barcodes for animals, and thus we can expect a very large availability of sequences in reference databases (Andújar *et al.* 2018; Leray *et al.* 2019). For benthic macroinvertebrates, a very large number of COI sequences is available in GenBank (Table 3). For instance, BF1_BR2-COI is largely the marker with the highest number of sequences of benthic Diptera, with nearly 3,000 sequences of BF1_BR2-COI available against just 1000 sequences of Inse01 (16S rDNA), still the number of available sequences is surprisingly variable across taxa. Nevertheless, a very large number of sequences does not necessarily allow a better taxonomic coverage. In fact, most of genera of benthic Diptera do not have GenBank sequences for COI, and Inse01 sequences represent slightly more genera than BF1_BR2-COI (25% for Inse01 against just 15% for BF1_BR2-COI; Fig. 3). The mismatch between number of sequences and database completeness could be related to the different scopes of studies employing the different markers. COI is the most used marker by standard barcoding studies, which often aim at unveiling diversity among closely related, cryptic taxa, thus studies often consider many individuals from closely related, morphologically similar species within genera (Hebert *et al.* 2004). Conversely, the 16S and 18S rDNA genes are often used to build phylogenies (*e.g.* Alvarez-Presas *et al.* 2008; Criscione & Ponder 2013), and many phylogenetic studies aim at representing the largest

number of genera and families. Such process could also explain the strong differences among taxa (*e.g.* a very high completeness for Euka02 with free-living Platyhelminthes, and the better coverage for Inse01 with Gastropoda; Fig. 2). If the aim is the species-level identification, databases should include all the species and markers should have a species-level resolution. Luckily, for freshwater biomonitoring a genus-level identification is often enough (Bailey *et al.* 2001; Mistri & Rossi 2001; Chessman *et al.* 2007; Birk *et al.* 2012), thus our database provides a good completeness that can allow the identification of most of genera, particularly with the markers Euka02 and Inse01. Here we tested our markers against the GenBank database only, because it includes sequences from our three genomic regions. However, an impressive number of additional COI sequences is available at the BOLD database. Integrating GenBank and BOLD data can certainly improve the resolution of COI-based metabarcoding studies.

4.2 Metabarcoding without taxonomic identification

Metabarcoding can provide ecological information even if reference databases are not available, as molecular taxonomic units can allow the comparison of communities among sites with environmental differences (Ji *et al.* 2013; Apothéloz-Perret-Gentil *et al.* 2017; Cordier *et al.* 2019). The taxonomy-free approach allows overcoming the fact that, despite intensive efforts, databases remain incomplete for many taxa (Fig. 3). Primers with high taxonomic coverage and resolution are essential also in this case. High taxonomic coverage is needed to avoid under-representation of some taxa, while resolution allows teasing apart related taxa. Related taxa can have very different ecological properties, and some widespread taxa, tolerant to human disturbance, can be closely related to highly sensitive specialists (Caro

et al. 2005). Therefore, ecological responses of communities can remain obscured if metabarcodes are not able to resolve related taxa with different ecology. Our study focused on European taxa, where taxonomic knowledge is particularly good (Moustakas & Karakassis 2005; Rodrigues *et al.* 2010; Brewer *et al.* 2012) and, with targeted studies, we could envisage an improvement of database completeness in the next years. However, our results on primer performance can be also useful in megadiverse, tropical areas, where taxonomy-free biomonitoring is a viable option (Andersen *et al.* 2019).

4.3 Universality and resolution of primers

Our analysis did not identify one single outperforming metabarcode. The universality of primers was variable among taxa, with Euka02 showing the highest performance for some phyla (platyhelminthes, molluscs, annelids and even some arthropods), and Inse01 showing a generally good performance for insects. However, each of these metabarcodes has some drawbacks. For instance, Euka02 amplifies very long sequences for some taxa of crustaceans (Isopoda and Amphipoda; Guardiola *et al.* 2015; Taberlet *et al.* 2018) thus their metabarcoding with this marker is problematic. Conversely, Inse01 is a marker developed specifically for insects, and fails to amplify key freshwater taxa such as free-living Platyhelminthes and molluscs (Fig. 1). In our analysis, BF1_BR2-COI showed a moderate amplification success, still for insects a relevant proportion of individuals was not amplified (Fig. 1). This is in contrast with previous analyses that successfully amplified 100% of tested insects using BF1_BR2-COI (Elbrecht & Leese 2017). Differences might be due to DNA quality, as this primer amplifies relatively long metabarcodes (>300 bp). Some of our >1500 specimens were old, and this can cause DNA degradation, while the starting material of

Elbrecht and Leese (2017) was probably of better quality. In fact, a few species (*Ephemerella mucronata*, *Torleya major* and *Odontocerum albicorne*) were successfully amplified by Elbrecht & Leese (2017), while failed amplification here. Furthermore, in several cases BF1_BR2-COI did not amplify the DNA of our target organisms, but amplified the DNA of contaminants, i.e. other organisms for which small body fragments were probably present in the tube, and that perhaps showed excellent match with the primers. Unfortunately, these conditions (degraded DNA, and simultaneous presence of many organisms) are typical of eDNA metabarcoding studies, stressing the complexity of finding appropriate primers.

Differences in performance were also strong when considering the resolution of the markers. BF1_BR2-COI clearly showed the best resolution while Euka02 showed a very poor performance, as in many cases it failed even at the family level (Fig. 3; see also Tang *et al.* 2012). COI is a highly variable region, and this has promoted its use as standard barcode for animals (Hebert *et al.* 2003; Hebert *et al.* 2004; Andújar *et al.* 2018). The excellent performance of BF1_BR2-COI can also be explained by the relatively long amplified region. Inse01 showed an intermediate performance, as its resolution was insufficient for species-level identification, while genus level identification was good for most of taxa (Fig. 3). It must be remarked that these are optimistic values of resolution, given that our database was far from complete, particularly at the genus-level and for some taxa, therefore a more complete database could yield poorer resolution values.

4.4 No Holy Grail for macrobenthos metabarcodes?

The heterogeneous performance of the different markers highlights the complexity of the identification of the best metabarcodes. No primer showed the best performance for all the

considered metrics, as the most "universal" marker (Euka02) showed a generally poor resolution, while the marker with the highest resolution (BF1_BR2-COI) did not successfully amplify several taxa. The selection of metabarcodes for biomonitoring is therefore a trade-off, depending on the aims of studies. Euka02 can allow a good assessment of overall biodiversity, but it is unable to tease apart closely related taxa, thus it might be not enough to define the ecological status of environments. Furthermore, the poor resolution would hamper the comparison with historical data for most of taxa. Conversely, the excellent resolution of BF1_BR2-COI could allow species-level identification, and might have more power to distinguish different communities. However, this comes at a cost. Several taxa did not amplify either because the level of DNA degradation compromises the amplification of a relatively long metabarcode, or because the poor match of the primer(s) with their target. In fact, the relatively long amplified region could limit its usefulness for application with environmental DNA extracted from water. Finally, Inse01 showed a generally good performance, but it is not appropriate for many non-insect taxa.

Given these limitations, it is unlikely that one single metabarcode will be able to fully replace the traditional biomonitoring using macrobenthic macroinvertebrates. Nevertheless, the data obtained through multiple metabarcodes can be integrated for a more comprehensive and accurate understanding of ecological impacts on freshwater biodiversity. For instance, highly universal markers, providing a complete but coarse picture of animal biodiversity (*e.g.* Euka02) can be combined with markers providing a specific focus on key taxa (*e.g.* Inse01) or a high-resolution level (*e.g.* BF1_BR2-COI). The integration of multiple metabarcodes certainly increases the cost and complexity of studies, still it has the potential to provide an unprecedented amount of data, thus opening unexplored avenues to biodiversity assessment.

Acknowledgments

532

533 This study was funded by Agence Nationale de la Recherche (ANR-13-ECOT-0002
534 aquaDNA). GFF and AB are funded by the European Research Council under the European
535 Community Horizon 2020 Programme, Grant Agreement no. 772284 (IceCommunities). We
536 thank three anonymous reviewers, who helped improve an early version of this manuscript.
537 We thank SPYGEN staff for its help in the laboratory, and S. Jolivet, M. Brulin, J. La Doaré,
538 G. Coppa, M. Ferran and OPIE volunteers for the collection of reference individuals.

539

540 **Competing interest**

541 A.V. and T.D. are research scientists at a private company specializing in the use of eDNA for
542 species detection.

543

544 **References**

545

- 546 Alvarez-Presas M, Baguna J, Riutort M (2008) Molecular phylogeny of land and freshwater
547 planarians (Tricladida, Platyhelminthes): From freshwater to land and back. *Molecular*
548 *Phylogenetics and Evolution*, **47**, 555-568.
- 549 Andersen JC, Oboyski P, Davies N, *et al.* (2019) Categorization of species as native or
550 nonnative using DNA sequence signatures without a complete reference library.
551 *Ecological Applications*, **29**, 11.
- 552 Andújar C, Arribas P, Yu DW, Vogler AP, Emerson BC (2018) Why the COI barcode should
553 be the community DNA metabarcode for the metazoa. *Molecular Ecology*, **27**, 3968-
554 3975.

555 Apothéloz-Perret-Gentil L, Cordonier A, Straub F, *et al.* (2017) Taxonomy-free molecular
556 diatom index for high-throughput eDNA biomonitoring. *Molecular Ecology*
557 *Resources*, **17**, 1231-1242.

558 Arce E, Archaimbault V, Mondy CP, Usseglio-Polatera P (2014) Recovery dynamics in
559 invertebrate communities following water-quality improvement: taxonomy- vs trait-
560 based assessment. *Freshwater Science*, **33**, 1060-1073.

561 Archaimbault V, Usseglio-Polatera P, Garric J, Wasson JG, Babut M (2010) Assessing
562 pollution of toxic sediment in streams using bio-ecological traits of benthic
563 macroinvertebrates. *Freshwater Biology*, **55**, 1430-1446.

564 Arman NZ, Salmiati S, Said MIM, Aris A (2019) Development of macroinvertebrate-based
565 multimetric index and establishment of biocriteria for river health assessment in
566 Malaysia. *Ecological Indicators*, **104**, 449-458.

567 Bailey RC, Norris RH, Reynoldson TB (2001) Taxonomic resolution of benthic
568 macroinvertebrate communities in bioassessments. *Journal of the North American*
569 *Benthological Society*, **20**, 280-286.

570 Baird DJ, Hajibabaei M (2012) Biomonitoring 2.0: a new paradigm in ecosystem assessment
571 made possible by next-generation DNA sequencing. *Molecular Ecology*, **21**, 2039–
572 2044.

573 Bates D, Maechler M, Bolker BM, Walker S (2015) Fitting Linear Mixed-Effects Models
574 Using lme4. *Journal of Statistical Software*, **67**, 1-48.

575 Birk S, Bonne W, Borja A, *et al.* (2012) Three hundred ways to assess Europe's surface
576 waters: An almost complete overview of biological methods to implement the Water
577 Framework Directive. *Ecological Indicators*, **18**, 31-41.

578 Boyer F, Mercier C, Bonin A, *et al.* (2016) obitools: a unix-inspired software package for
579 DNA metabarcoding. *Molecular Ecology Resources*, **16**, 176-182.

580 Brabec K, Zahradkova S, Nemejcova D, *et al.* (2004) Assessment of organic pollution effect
581 considering differences between lotic and lentic stream habitats. *Hydrobiologia*, **516**,
582 331-346.

583 Brewer MS, Sierwald P, Bond JE (2012) Millipede Taxonomy after 250 Years: Classification
584 and Taxonomic Practices in a Mega-Diverse yet Understudied Arthropod Group. *Plos*
585 *One*, **7**, 12.

586 Buxton AS, Groombridge JJ, Griffiths RA (2017) Is the detection of aquatic environmental
587 DNA influenced by substrate type? *Plos One*, **12**.

588 Bylemans J, Furlan EM, Gleeson DM, Hardy CM, Duncan RP (2018) Does Size Matter? An
589 Experimental Evaluation of the Relative Abundance and Decay Rates of Aquatic
590 Environmental DNA. *Environmental Science & Technology*, **52**, 6408-6416.

591 Camargo JA (2017) Multimetric assessment of macroinvertebrate responses to mitigation
592 measures in a dammed and polluted river of Central Spain. *Ecological Indicators*, **83**,
593 356-367.

594 Carlson PE, Donadi S, Sandin L (2018) Responses of macroinvertebrate communities to small
595 dam removals: Implications for bioassessment and restoration. *Journal of Applied*
596 *Ecology*, **55**, 1896-1907.

597 Caro T, Eadie J, Sih A (2005) Use of substitute species in conservation biology. *Conservation*
598 *Biology*, **1821-1826**.

599 Chessman B, Williams S, Besley C (2007) Bioassessment of streams with macroinvertebrates:
600 effect of sampled habitat and taxonomic resolution. *Journal of the North American*
601 *Benthological Society*, **26**, 546-565.

602 Cilleros K, Valentini A, Allard L, *et al.* (2019) Unlocking biodiversity and conservation
603 studies in high-diversity environments using environmental DNA (eDNA): A test with
604 Guianese freshwater fishes. *Molecular Ecology Resources*, **19**, 27-46.

605 Coissac E (2012) OligoTag: a program for designing sets of tags for nextgeneration
606 sequencing of multiplexed samples. *Methods in Molecular Biology*, **888**, 13–31.

607 Cordier T, Forster D, Dufresne Y, *et al.* (2018) Supervised machine learning outperforms
608 taxonomy-based environmental DNA metabarcoding applied to biomonitoring.
609 *Molecular Ecology Resources*, **18**, 1381-1391.

610 Cordier T, Lanzen A, Apotheloz-Perret-Gentil L, Stoeck T, Pawlowski J (2019) Embracing
611 Environmental Genomics and Machine Learning for Routine Biomonitoring. *Trends*
612 *in Microbiology*, **27**, 387-397.

613 Cox B, Oeding S, Taffs K (2019) A comparison of macroinvertebrate-based indices for
614 biological assessment of river health: A case example from the sub-tropical Richmond
615 River Catchment in northeast New South Wales, Australia. *Ecological Indicators*,
616 **106**.

617 Criscione F, Ponder WF (2013) A phylogenetic analysis of rissooidean and cingulopsoidean
618 families (Gastropoda: Caenogastropoda). *Molecular Phylogenetics and Evolution*, **66**,
619 1075-1082.

620 Czechowski P, Stevens MI, Madden C, Weinstein P (2020) Steps towards a more efficient use
621 of chironomids as bioindicators for freshwater bioassessment: Exploiting eDNA and
622 other genetic tools. *Ecological Indicators*, **110**, 11.

623 Eichmiller JJ, Best SE, Sorensen PW (2016) Effects of Temperature and Trophic State on
624 Degradation of Environmental DNA in Lake Water. *Environmental Science and*
625 *Technology*, **50**, 1859–1867.

626 Elbrecht V, Leese F (2017) Validation and Development of COI Metabarcoding Primers for
627 Freshwater Macroinvertebrate Bioassessment. *Frontiers in Environmental Science*, **5**,
628 11.

629 Elbrecht V, Taberlet P, Dejean T, *et al.* (2016) Testing the potential of a ribosomal 16S
630 marker for DNA metabarcoding of insects. *Peerj*, **4**, 12.

631 Ficetola GF, Coissac E, Zundel S, *et al.* (2010) An In silico approach for the evaluation of
632 DNA barcodes. *BMC Genomics*, **11**, 434.

633 Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental
634 DNA from water samples. *Biology Letters*, **4**, 423-425.

635 Floury M, Usseglio-Polatera P, Ferreol M, Delattre C, Souchon Y (2013) Global climate
636 change in large European rivers: long-term effects on macroinvertebrate communities
637 and potential local confounding factors. *Global Change Biology*, **19**, 1085-1099.

638 Friberg N, Sandin L, Furse MT, *et al.* (2006) Comparison of macroinvertebrate sampling
639 methods in Europe. *Hydrobiologia*, **566**, 365-378.

640 Gabriels W, Lock K, De Pauw N, Goethals PLM (2010) Multimetric Macroinvertebrate Index
641 Flanders (MMIF) for biological assessment of rivers and lakes in Flanders (Belgium).
642 *Limnologia*, **40**, 199-207.

643 Guardiola M, Uriz MJ, Taberlet P, *et al.* (2015) Deep-Sea, Deep-Sequencing: Metabarcoding
644 Extracellular DNA from Sediments of Marine Canyons. *Plos One*, **10**, e0139633.

645 Haase P, Lohse S, Pauls S, *et al.* (2004) Assessing streams in Germany with benthic
646 invertebrates: development of a practical standardised protocol for macro invertebrate
647 sampling and sorting. *Limnologia*, **34**, 349-365.

648 Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one:
649 DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes*
650 *fulgurator*. *Proceedings of the National Academy of Sciences of the United States of*
651 *America*, **101**, 14812-14817.

652 Hebert PDN, Ratnasingham S, deWaard JR (2003) Barcoding animal life: cytochrome c
 653 oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal*
 654 *Society B-Biological Sciences*, **270**, S96-S99.

655 Hering D, Borja A, Carvalho L, Feld CK (2013) Assessment and recovery of European water
 656 bodies: key messages from the WISER project. *Hydrobiologia*, **704**, 1-9.

657 Hering D, Borja A, Jones JJ, *et al.* (2018) Implementation options for DNA-based
 658 identification into ecological status assessment under the European Water Framework
 659 Directive. *Water Research*, **138**, 192-205.

660 Hering D, Feld CK, Moog O, Ofenbock T (2006a) Cook book for the development of a
 661 Multimetric Index for biological condition of aquatic ecosystems: Experiences from
 662 the European AQEM and STAR projects and related initiatives. *Hydrobiologia*, **566**,
 663 311-324.

664 Hering D, Johnson RK, Kramm S, *et al.* (2006b) Assessment of European streams with
 665 diatoms, macrophytes, macroinvertebrates and fish: a comparative metric-based
 666 analysis of organism response to stress. *Freshwater Biology*, **51**, 1757-1785.

667 Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models.
 668 *Biometrical Journal*, **50**, 346-363.

669 Iversen LL, Winkel A, Bastrup-Spohr L, *et al.* (2019) Catchment properties and the
 670 photosynthetic trait composition of freshwater plant communities. *Science*, **366**, 878.

671 Ji Y, Ashton L, Pedley SM, *et al.* (2013) Reliable, verifiable and efficient monitoring of
 672 biodiversity via metabarcoding. *Ecology Letters*, **16**, 1245-1257.

673 Jo T, Murakami H, Masuda R, *et al.* (2017) Rapid degradation of longer DNA fragments
 674 enables the improved estimation of distribution and biomass using environmental
 675 DNA. *Molecular Ecology Resources*, **17**, e25-e33.

676 Kuntke F, de Jonge N, Hesselsoe M, Nielsen JL (2020) Stream water quality assessment by
677 metabarcoding of invertebrates. *Ecological Indicators*, **111**.

678 Kupilas B, Friberg N, McKie BG, *et al.* (2016) River restoration and the trophic structure of
679 benthic invertebrate communities across 16 European restoration projects.
680 *Hydrobiologia*, **769**, 105-120.

681 Kuznetsova A, Brockhoff B, Christensen HB (2017) lmerTest Package: Tests in Linear Mixed
682 Effects Models. *Journal of Statistical Software*, **82**, 1-26.

683 Leray M, Knowlton N, Ho SL, Nguyen BN, Machida RJ (2019) GenBank is a reliable
684 resource for 21st century biodiversity research. *Proceedings of the National Academy
685 of Sciences of the United States of America*, **116**, 22651-22656.

686 Li FL, Peng Y, Fang WD, *et al.* (2018) Application of Environmental DNA Metabarcoding
687 for Predicting Anthropogenic Pollution in Rivers. *Environmental Science &
688 Technology*, **52**, 11708-11719.

689 Marquina D, Andersson AF, Ronquist F (2019) New mitochondrial primers for
690 metabarcoding of insects, designed and evaluated using in silico methods. *Molecular
691 Ecology Resources*, **19**, 90-104.

692 Marzin A, Archaimbault V, Belliard J, *et al.* (2012) Ecological assessment of running waters:
693 Do macrophytes, macroinvertebrates, diatoms and fish show similar responses to
694 human pressures? *Ecological Indicators*, **23**, 56-65.

695 Miler O, Porst G, McGoff E, *et al.* (2013) Morphological alterations of lake shores in Europe:
696 A multimetric ecological assessment approach using benthic macroinvertebrates.
697 *Ecological Indicators*, **34**, 398-410.

698 Mistri M, Rossi R (2001) Taxonomic sufficiency in lagoonal ecosystems. *Journal of the
699 Marine Biological Association of the United Kingdom*, **81**, 339-340.

700 Mondy CP, Usseglio-Polatera P (2013) Using conditional tree forests and life history traits to
 701 assess specific risks of stream degradation under multiple pressure scenario. *Science of*
 702 *the Total Environment*, **461**, 750-760.

703 Morinière J, Balke M, Doczkal D, *et al.* (2019) A DNA barcode library for 5,200 German
 704 flies and midges (Insecta: Diptera) and its implications for metabarcoding-based
 705 biomonitoring. *Molecular Ecology Resources*, **19**, 900-928.

706 Moriniere J, Hendrich L, Balke M, *et al.* (2017) A DNA barcode library for Germanys
 707 mayflies, stoneflies and caddisflies (Ephemeroptera, Plecoptera and Trichoptera).
 708 *Molecular Ecology Resources*, **17**, 1293-1307.

709 Moustakas A, Karakassis I (2005) How diverse is aquatic biodiversity research? *Aquatic*
 710 *Ecology*, **39**, 367-375.

711 Noges P, Argillier C, Borja A, *et al.* (2016) Quantified biotic and abiotic responses to multiple
 712 stress in freshwater, marine and ground waters. *Science of the Total Environment*, **540**,
 713 43-52.

714 Pawlowski J, Kelly-Quinn M, Altermatt F, *et al.* (2018) The future of biotic indices in the
 715 ecogenomic era: Integrating (e)DNA metabarcoding in biological assessment of
 716 aquatic ecosystems. *Science of The Total Environment*, **637-638**, 1295-1310.

717 Pinheiro P, Bates D (2000) *Mixed-effect models in S and S-PLUS* Springer, New York.

718 Piñol J, Mir G, Gomez-Polo P, Agustí N (2015) Universal and blocking primer mismatches
 719 limit the use of high-throughput DNA sequencing for the quantitative metabarcoding
 720 of arthropods. *Molecular Ecology Resources*, **15**, 819-830.

721 Pont D, Valentini A, Rocle M, *et al.* (2020) The future of fish-based ecological assessment of
 722 European rivers: from traditional EU Water Framework Directive compliant methods
 723 to eDNA metabarcoding-based approaches. *Journal of Fish Biology*, **in press**,
 724 <https://doi.org/10.1111/jfb.14176>.

725 Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System
 726 (www.barcodinglife.org). *Molecular Ecology Notes*, **7**, 355-364.

727 Rodrigues ASL, Gray CL, Crowter BJ, *et al.* (2010) A global assessment of amphibian
 728 taxonomic effort and expertise. *Bioscience*, **60**, 798-806.

729 Rubinoff D, Cameron S, Will K (2006) Are plant DNA barcodes a search for the Holy Grail?
 730 *Trends in Ecology & Evolution*, **21**, 1-2.

731 Schmidt-Kloiber A, Hering D (2015) www.freshwaterecology.info – An online tool that
 732 unifies, standardises and codifies more than 20,000 European freshwater organisms
 733 and their ecological preferences. *Ecological Indicators*, **53**, 271-282.

734 Serrana JM, Miyake Y, Gamboa M, Watanabe K (2019) Comparison of DNA metabarcoding
 735 and morphological identification for stream macroinvertebrate biodiversity assessment
 736 and monitoring. *Ecological Indicators*, **101**, 963-972.

737 Sigsgaard EE, Nielsen IB, Bach SS, *et al.* (2017) Population characteristics of a large whale
 738 shark aggregation inferred from seawater environmental DNA. *Nature Ecology &*
 739 *Evolution*, **1**, 0004.

740 Taberlet P, Bonin A, Zinger L, Coissac E (2018) *Environmental DNA for biodiversity*
 741 *research and monitoring* Oxford University Press, Oxford.

742 Tachet H, Richoux P, Bournaud M, Usseglio-Polatera P (2010) *Invertébrés d'eau douce.*
 743 *Systématique, biologie, écologie* CNRS Editions, Paris.

744 Tang CQ, Leasi F, Obertegger U, *et al.* (2012) The widely used small subunit 18S rDNA
 745 molecule greatly underestimates true diversity in biodiversity surveys of the
 746 meiofauna. *Proceedings of the National Academy of Sciences of the United States of*
 747 *America*, **109**, 16208-16212.

- Theodoropoulos C, Karaouzas I, Vourka A, Skoulikidis N (2020) ELF - A benthic macroinvertebrate multi-metric index for the assessment and classification of hydrological alteration in rivers. *Ecological Indicators*, **108**, 11.
- Usseglio-Polatera P, Bournaud M, Richoux P, Tachet H (2000) Biological and ecological traits of benthic freshwater macroinvertebrates: relationships and definition of groups with similar traits. *Freshwater Biology*, **43**, 175-205.
- Usseglio-Polatera P, Richoux P, Bournaud M, Tachet H (2001) A functional classification of benthic macroinvertebrates based on biological and ecological traits: application to river condition assessment and stream management. *Archiv für Hydrobiologie*, **139 Suppl.1 Monogr. Stud.**, 53-83.
- Vivien R, Apothéloz-Perret-Gentil L, Pawlowski J, *et al.* (2020) High-throughput DNA barcoding of oligochaetes for abundance-based indices to assess the biological quality of sediments in streams and lakes. *Scientific Reports*, **10**, 2041.
- Weigand H, Beermann AJ, Ciampor F, *et al.* (2019) DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. *Science of the Total Environment*, **678**, 499-524.
- Wilcox TM, McKelvey KS, Young MK, *et al.* (2013) Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. *Plos One*, **8**, 9.
- Yang JH, Zhang XW (2020) eDNA metabarcoding in zooplankton improves the ecological status assessment of aquatic ecosystems. *Environment International*, **134**, 9.
- Yu DW, Ji Y, Emerson BC, *et al.* (2012) Biodiversity soup: metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring. *Methods in Ecology and Evolution*, **3**, 613-623.

773 **Data Accessibility:**

774 The complete database is available at figshare:

775 <https://doi.org/10.6084/m9.figshare.12046242.v1>

776

777 The filtered sequences generated by the project with length >100 bp are also available on
778 GenBank (Euka02: accession numbers LR824819 to LR826155; Inse01: LR826156 to
779 LR827357, BF1-BR2 COI: LR827358 to LR828086).

780

781 **Author Contributions**

782 Designed the research: Pierre Taberlet, Tony Dejean, Gentile Francesco Ficetola, Frédéric
783 Boyer, Alice Valentini, Aurélie Bonin, Albin Meyer, Philippe Usseglio-Polatera

784 Performed research: Alice Valentini, Aurélie Bonin, Albin Meyer, Coline Gaboriaud, Pierre
785 Taberlet, Tony Dejean, Gentile Francesco Ficetola, Frédéric Boyer, Philippe Usseglio-
786 Polatera

787 Analyzed data: Gentile Francesco Ficetola, Frédéric Boyer, Aurélie Bonin

788 Wrote the paper: Gentile Francesco Ficetola wrote the first draft of the paper, with subsequent
789 contribution of all the authors

790

Figure legends

Figure 1. Amplification success of the three markers across benthic macroinvertebrate taxa. A): all taxa; B): insects only. Error bars are standard errors of the mean; * indicate significant differences between markers. See Table S2 for the raw values used to build the plot.

Figure 2. Completeness of the combined database, combining the sequences produced in this study with sequences retrieved from public databases. For each taxon, the plots report the proportion of European genera of macroinvertebrates with at least one sequence in the database.

Figure 3. Resolution of the three markers at the species, genus and family levels. The resolution is measured as the proportion of metabarcodes that are associated with a) at least two species; b) at least two genera; c) at least two families (non-identification), therefore low values of non-identification indicate a better performance of the markers.

807 Table 1. Inventory of macrobenthos individuals from which we extracted and amplified DNA.

808

Class / subphylum / phylum		<i>N</i> individuals	% identified at the genus level by taxonomists	<i>N</i> genera
Platyhelminthes		7	100%	2
Bivalvia		12	100%	5
Gastropoda		29	97%	11
Clitellata		35	69%	8
Arachnida		11	9%	1
Crustacea		9	100%	4
Insecta	Coleoptera	117	97%	40
	Diptera	54	20%	6
	Ephemeroptera	338	100%	35
	Hemiptera	24	100%	14
	Lepidoptera	2	100%	2
	Megaloptera	4	100%	1
	Neuroptera	2	100%	1
	Odonata	9	78%	2
	Plecoptera	210	100%	20
	Trichoptera	651	100%	84

809

810

811 Table 2. Representativeness of reference individuals used for analyses, relative to European
 812 and French genera of benthic macroinvertebrates.

813

Class / subphylum / phylum	Order (insects only)	<i>N</i> genera Europe	<i>N</i> Genera France	<i>N</i> genera for <i>in vitro</i> analyses
Platyhelminthes		29	23	2
Bivalvia		18	11	5
Gastropoda		65	34	11
Clitellata		102	64	8
Arachnida*		1	1	1
Hydracnida		56	52	0
Crustacea		72	34	4
Insecta	Coleoptera	127	95	40
	Diptera	522	323	6
	Ephemeroptera	50	35	35
	Hemiptera	30	28	14
	Lepidoptera	8	5	2
	Megaloptera	1	1	1
	Neuroptera	3	3	1
	Odonata	43	36	2
	Plecoptera	38	27	20
	Trichoptera	130	108	84

814 * except Hydracnida

815

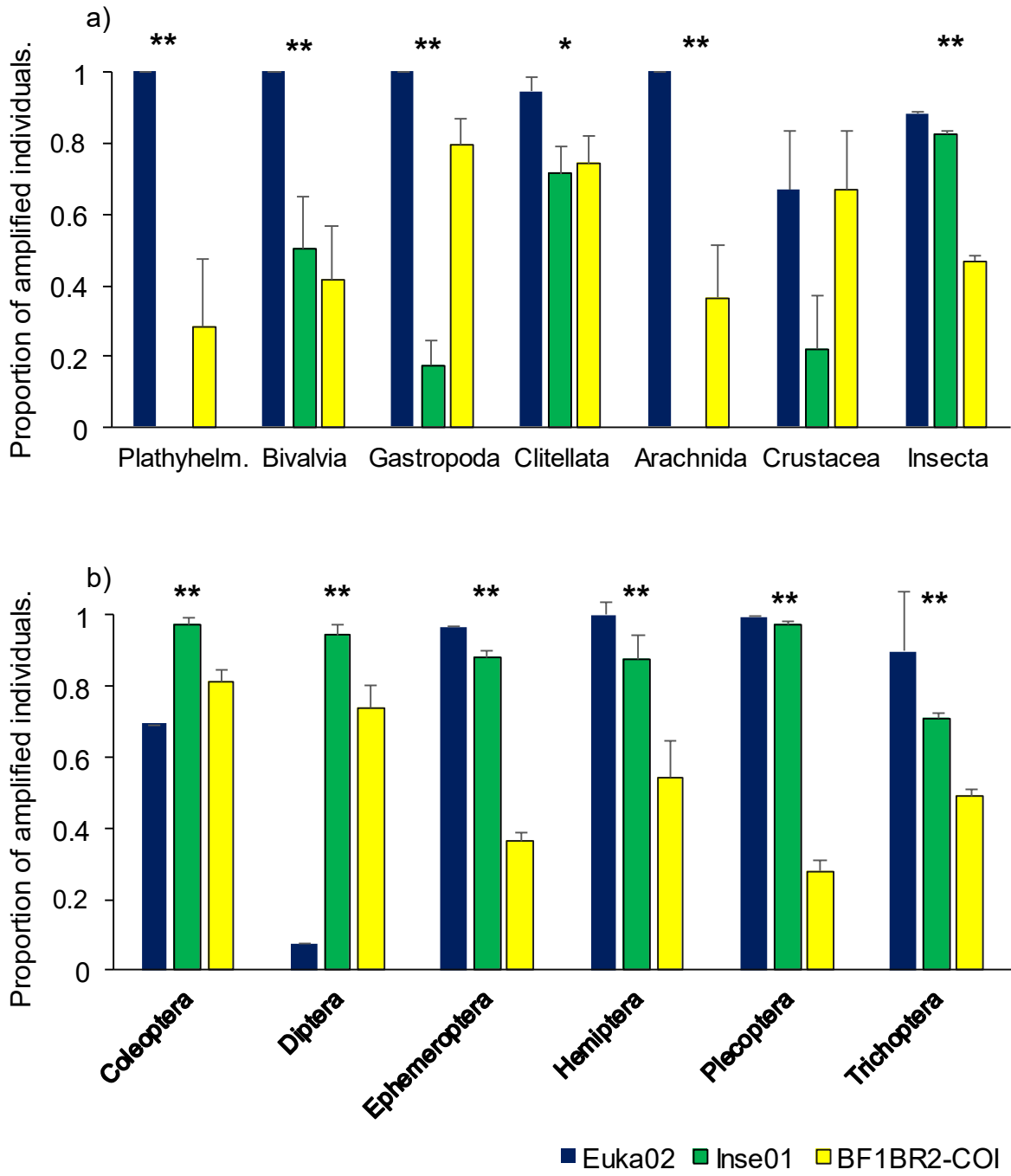
816

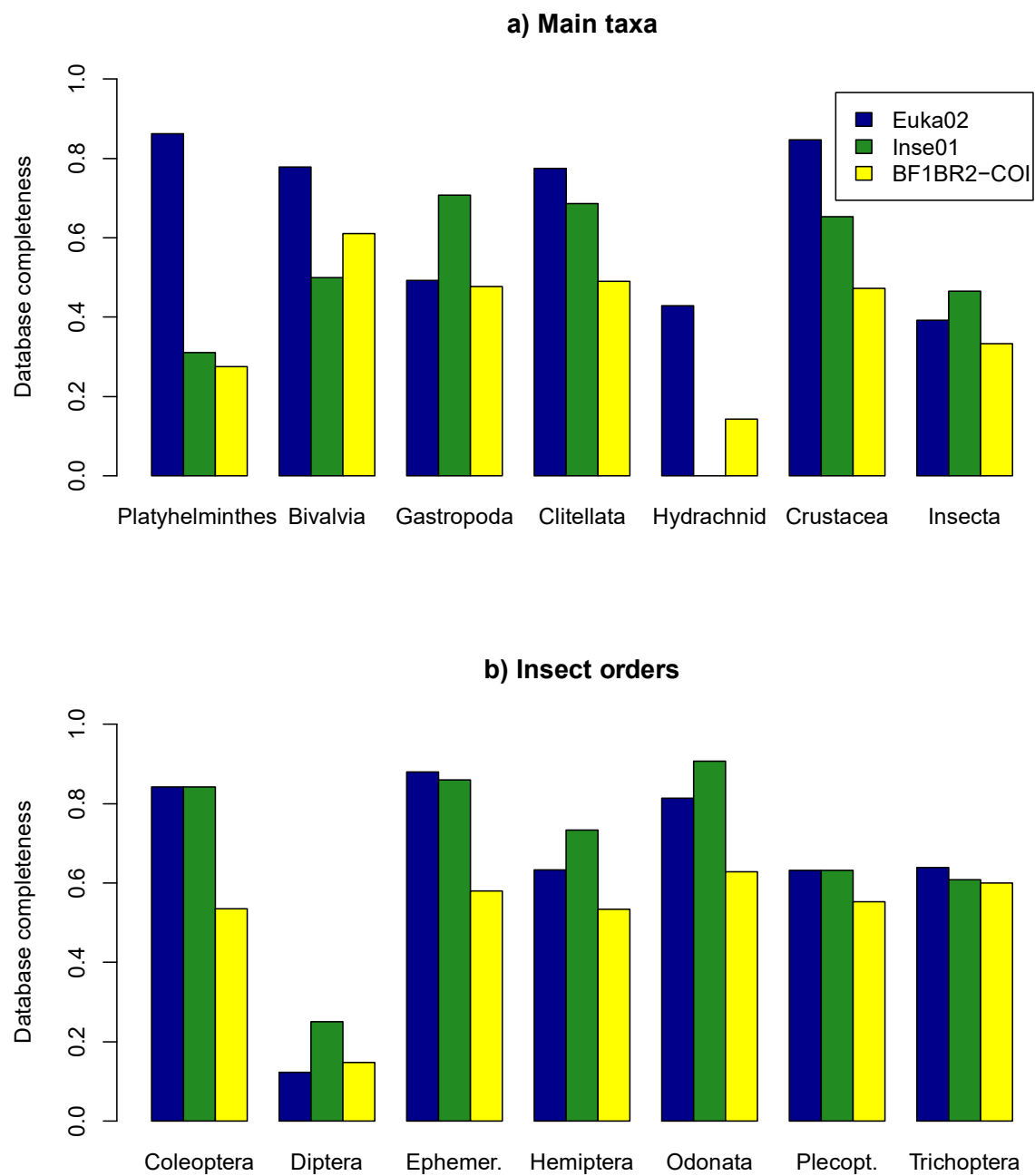
Table 3. Number of sequences and genera represented in the combined database, across taxa.
Taxa for which >70% of European genera are represented in the database are highlighted in bold.

Class / subphylum / phylum	Order (insects only)	N sequences in the database			N genera Europe	N genera in the database			
		Euka02	Inse01	COI		Euka02	Inse01	COI	Total
Hydrozoa		31	134	21	6	5	5	3	5
Enopla (Nemertini)		4	1	-	1	1	1	0	1
Platyhelminthes		217	25	316	29	25	9	8	26
Gordioda (Nematomorpha)		9	-	-	1	1	-	-	1
Bivalvia		66	453	125	18	14	9	11	16
Gastropoda		81	1147	102	65	32	46	31	51
Clitellata		414	838	170	102	79	70	50	86
Polychaeta		33	74	64	11	8	4	2	8
Gymnolaemata (Bryozoa)		23	44	-	3	2	2	-	2
Phylactolaemata (Bryozoa)		17	36	1	6	6	6	1	6
Arachnida*		2	2	4	1	1	1	1	1
Hydracnida		70	-	15	56	24	0	8	24
Crustacea		325	1980	303	72	61	47	34	65
Insecta		2149	4981	4557	981	385	456	327	530
	Coleoptera	450	1809	333	127	107	107	68	115
	Diptera	228	1078	2839	522	64	131	77	170
	Ephemeroptera	396	507	397	50	44	43	29	44
	Hemiptera	46	206	70	30	19	22	16	24
	Hymenoptera	4	21	33	29	3	4	5	9
	Lepidoptera	2	4	5	8	2	3	3	3
	Megaloptera	9	10	6	1	1	1	1	1
	Neuroptera	6	12	5	3	3	3	2	3
	Odonata	164	537	294	43	35	39	27	41
	Plecoptera	243	233	118	38	24	24	21	26
	Trichoptera	601	564	457	130	83	79	78	94

* except Hydracnida

Figure 1





830

831

