MBMG
Metabarcoding & Metagenomics

Metabarcoding and Metagenomics 3: 49–57
DOI 10.3897/mbmg.3.34735

Forum Paper

Advancing the use of molecular methods for routine freshwater macroinvertebrate biomonitoring – the need for calibration experiments

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Academic editor: *Dirk Steinke* | **Received** 21 March 2019 | **Accepted** 24 June 2019 | **Published** 11 July 2019

Abstract

Over the last decade, steady advancements have been made in the use of DNA-based methods for detection of species in a wide range of ecosystems. This progress has culminated in molecular monitoring methods being employed for the detection of several species for enforceable management purposes of endangered, invasive, and illegally harvested species worldwide. However, the routine application of DNA-based methods to monitor whole communities (typically a metabarcoding approach) in order to assess the status of ecosystems continues to be limited. In aquatic ecosystems, the limited use is particularly true for macroinvertebrate communities. As part of the DNAqua-Net consortium, a structured discussion was initiated with the aim to identify potential molecular methods for freshwater macroinvertebrate community assessment and identify important knowledge gaps for their routine

application. We focus on three complementary DNA sources that can be metabarcoded: 1) DNA from homogenised samples (bulk DNA), 2) DNA extracted from sample preservative (fixative DNA), and 3) environmental DNA (eDNA) from water or sediment. We provide a brief overview of metabarcoding macroinvertebrate communities from each DNA source and identify challenges for their application to routine monitoring. To advance the utilisation of DNA-based monitoring for macroinvertebrates, we propose an experimental design template for a series of methodological calibration tests. The template compares sources of DNA with the goal of identifying the effects of molecular processing steps on precision and accuracy. Furthermore, the same samples will be morphologically analysed, which will enable the benchmarking of molecular to traditional processing approaches. In doing so we hope to highlight pathways for the development of DNA-based methods for the monitoring of freshwater macroinvertebrates.

Key Words

Bulk DNA, community, DNAqua-Net, environmental DNA, experimental methods, fixative DNA, monitoring, Water Framework Directive

Introduction and background

Worldwide, DNA-based methods are advancing and can aid in the determination of ecological state of ecosystems. In Europe, the COST Action DNAqua-Net, consisting of more than 500 members and is working to utilise and improve molecular methods for monitoring Biological Quality Elements (BQEs, e.g. fish, macroinvertebrates, and phytoplankton-benthos) used to determine aquatic ecosystem status under the requirements of the Water Framework Directive (WFD, 2000/60/EC) and beyond (Leese et al. 2016; Hering et al. 2018; Pawlowski et al. 2018). Thus far, much of the focus has been on fish, due to their suitability for environmental DNA (eDNA) monitoring that allows for non-invasive sampling and increased monitoring of water bodies such as large rivers and deep lakes that are not optimally surveyed with current WFD accepted methods (Hänfling et al. 2016; Pont et al. 2018). However, as highlighted by Hering et al. (2018), freshwater macroinvertebrates are also particularly suitable for monitoring with molecular methods, due to the potential reductions in processing time, greater taxonomic resolution, and reduction in errors compared with current morphological monitoring methods. Macroinvertebrates are already the most widely used BQEs to assess biodiversity and ecological quality status (EQS) and further indices (e.g. Average Score per Taxa, ASPT; Number of taxa, N-taxa). However, the collection methods and taxonomic resolution to which specimens are identified vary widely across countries and hinders comparisons across geopolitical boundaries in the European Union (Birk et al. 2012). Molecular methods may overcome some of these limitations, and we propose their comparison across European countries before broader uptake is attempted.

On the 17th December 2018, members of the DNAqua-Net consortium met at Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO), Portugal, and discussed future developments of DNA-based methods for monitoring macroinvertebrates. Based on discussions and collating information from members within the COST Action, knowledge gaps were identified, and research priorities outlined focusing on two key areas: (1) current research needs and the short-term focus of method development and (2) issues not being addressed by current official monitoring methods and the potential for molecular methods to address these. In this article, we summarise the outcomes of these discussions, including areas of consensus, knowledge gaps, proposed experimental designs to fill those knowledge gaps, and future opportunities for molecular approaches to aid aquatic bioassessment using macroinvertebrates.

Consensus

There are three main sources of DNA from macroinvertebrates being assessed for use in biomonitoring programs (Fig. 1), each with its individual strengths and weaknesses, described below: (1) DNA sourced from whole collected individuals (subsequently called "bulk DNA"), (2) non-destructively extracted DNA sourced from the preservative used on a sample i.e. a kick-net sample and not individual specimens (subsequently called "fixative DNA"); and (3) environmental DNA sourced from water or sediment samples (subsequently called "eDNA").

(1) Bulk DNA metabarcoding of macroinvertebrates has proven successful in several studies so far (Hajibabaei et al. 2012; Yu et al. 2012; Ji et al. 2013; Gibson et al. 2014, 2015; Elbrecht et al. 2017a; Emilson et al. 2017; Lobo et al. 2017) and is even applicable to bioassessment in freshwater and marine environments alike (Aylagas et al. 2014, 2018; Elbrecht et al. 2017a). However, the most obvious technical challenges with bulk sample analysis are processing speed (i.e. the removal of specimens from the organic material they are collected with, for example with a kick-net sample) and both biomass and primer biases (Piñol et al. 2014, 2018; Elbrecht and Leese 2015, 2017).
(2) Fixative DNA metabarcoding builds on methods developed for barcoding precious or rare museum

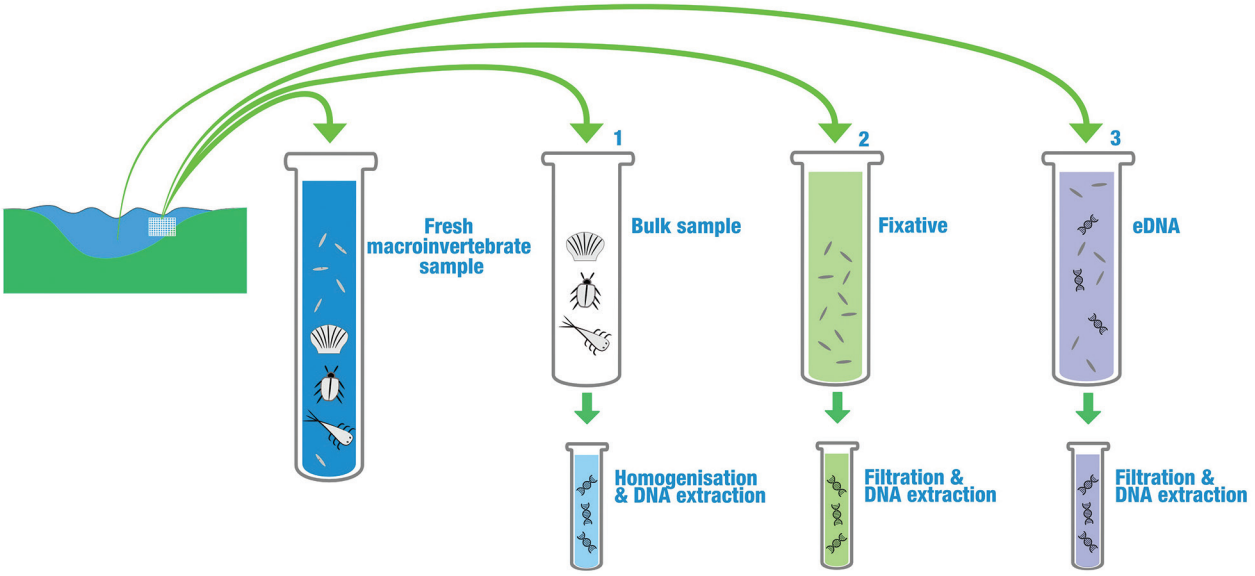

Figure 1. The three methods for DNA retrieval for macroinvertebrate biomonitoring: (1) DNA bulk samples from homogenised specimen samples, (2) DNA extracted from the fixative used to preserve or store a sample, and (3) DNA extracted from a water sample (eDNA).

specimens (Rohland et al. 2004; Rowley et al. 2007; Gilbert et al. 2007). Five studies have tested the approach on real samples so far (Hajibabaei et al. 2012, Carew et al. 2018; Erdozain et al. 2018; Zizka et al. 2019; Martins et al. 2019). While not dependent on sorting organisms from the original sample, the invertebrates themselves still need to be collected. In addition to primer bias, some taxa specific challenges were encountered, for example underrepresentation or loss of small, sclerotized, or mollusc taxa. The method is faster than bulk sample processing and has shown to be sensitive enough to detect expected macroinvertebrate taxa. However, non-targeted taxa, such as potential prey items are also detected, likely arising from regurgitation of species once they are in the preservative. We must consider that this method, and also bulk DNA, can detect non-target species via eDNA on the exterior of organisms, unsorted debris and parasites.

(3) eDNA metabarcoding is yet less resolved for macroinvertebrate monitoring. So far, studies have reported that richness of macroinvertebrate communities can be measured from eDNA (Deiner et al. 2016, 2017; Li et al. 2018; Macher et al. 2018, 2019; Majaneva et al. 2018a), such that catchment level and local richness estimates are comparable or complimentary to morphologically identified kick-net samples (Deiner et al. 2017; Mächler et al. 2019). However, while there is great potential in this approach, other comparisons have revealed lower detection of invertebrate taxa from eDNA samples compared with kick-net sample metabarcoding (Macher et al. 2018). The difference in detection outcomes can be the result of several factors including sampling, laboratory or bioinformatic methods (e.g. volume of water, eDNA capture and extraction, or bioinformatic processing), inherent primer bias, and the effect of detecting greater amounts of non-targeted taxa such as bacteria and phytoplankton. Environmental DNA also fundamentally differs from the two other approaches in that it is free from the organism and can be transported in flowing waters (Deiner and Altermatt 2014), likely giving rise to a more spatially integrated rather than a single site assessment (Deiner et al. 2016). Thus, this form of DNA monitoring requires entirely new evaluations beginning at the sampling stage as to how we can use this type of DNA data from macroinvertebrates and further basic research is needed before applications can be standardised.

To retain comparability with current practices, but utilise the benefits of DNA-based methods, a consensus emerged that for the time being, DNA from a bulk sample and fixative DNA are the most comparable options to current methods used for applied biomonitoring of freshwater macroinvertebrates. These methods rely on the same field sampling methods used under current official monitoring practices and have the same spatial interpretation. The advantage of using DNA metabarcoding from sources (1) and (2) compared with current morphological identification of samples is the potential for increased time efficiency and identification resolution. Although not yet demonstrated with current bulk DNA processing methods, in the case of fixative DNA, removal of the time intensive step needed to sort the macroinvertebrate sample from organic material collected in the sampling process, while also avoiding destruction of the species is particularly important. However, both methods do not overcome possible limitations encompassed by the physical sampling of organisms, and this is where developing eDNA methods may be utilised (see *Future Application* for further discussion).

Knowledge gaps

We identified several gaps in our current understanding warranting further research. Primarily, research has focused on small scale comparisons within single systems or countries. This form of testing does not allow for variation in geographic range or among water quality classes and should be included within future experimental design. Secondly, current macroinvertebrate community indices require abundance or frequency classes for community assessment. Metabarcoding studies have so far been unable to relate read number precisely to abundance, relative abundance or biomass of macroinvertebrates. A recent study by Beentjes et al. (2018) demonstrated a strong correlation between ecological quality ratios of presence/absence data and abundance-based data on historical macroinvertebrate monitoring data from a wide range of waterbody types for the Netherlands. Such comparisons to validate the importance of abundance measures for EQS assessments are underway in the Nordic countries, which is important to determine if methods can be calibrated without including abundance information. However, progress with PCR-free methods suggests that inferring biomass and abundance data might be possible with metagenomic approaches in the future (e.g. Choo et al. 2017; Bista et al. 2018; Li et al. 2019) allowing for integration of molecular data with current abundance-based indices.

A key advancement for any new method to be adopted in EQS assessment requires a reduction in sample processing cost (both time and monetary); an issue repeatedly raised by regulators during DNAqua-Net stakeholder meetings. Recent studies using bulk DNA samples often include specimens being picked from the sample matrix, a form of size sorting of specimens, or removing legs from individuals prior to processing (Elbrecht et al. 2017b; Wangensteen et al. 2018; Cahill et al. 2018). These methods also do not remove errors associated with missed taxa during sorting steps (Haase et al. 2006; JI Jones pers. comm.) Therefore, it was agreed, that future developments of bulk DNA must remove any form of biomass sorting and considerably reduce any sample picking / cleaning steps for this method to be more cost effective than morphological identification. Homogenisation of whole bulk samples and subsampling is one way forward (Majaneva et al. 2018b), but this may result in missed taxa due to differing biomass (Elbrecht et al. 2017b). A faster and non-invasive alternative may be fixative DNA metabarcoding. Using fixative DNA versus tissue DNA may resolve the time issue of sorting (i.e. little or no processing of the specimens), but it has not been demonstrated if issues such as primer bias and biomass create bias in detection results. The first studies to assess macroinvertebrate taxa from fixative DNA suggest important comparisons need to be tested prior to implementation, including: preservative type, length of time the sample is preserved for, amount of preservative used for extraction and DNA extraction protocols (Hajibabaei et al. 2012; Carew et al. 2018; Shokralla et al. 2018; Zizka et al. 2019; Erdozain et al. 2019; Martins et al. 2019). However, for regulators to use such methods, the procedures need to be replicable, well documented, and preferably standardised (Comité Européen de Normalisation, CEN or International Standards Organisation, ISO) to be adopted in routine monitoring of large geographical areas such as the European Union. In this regard, many other parameters are also important, such as development of a standard metric to compare morphological and molecular results in calibration experiments, which and how many primer pairs should be used, and the sample number and replication number necessary to obtain meaningful results to enable standardisation. In the following, we outline an experimental validation strategy that could act as a part or precursor to the development of such standardised protocols.

Experimental validation strategy

With the goal to develop robust protocols for macroinvertebrate assessment from DNA extracted from samples collected using current official sampling methods, we outline a set of experiments (Fig. 2). As part of these experiments, we will carry out comparisons with traditional analysis by including morphological identification of all specimens used within the two experiments. It is our aim not only to carry out country specific WFD morphological identification but also specimen identification to the highest taxonomic level, i.e. species, where possible. This quality assurance step will form the basis for the comparisons of which taxa are found and conversely not found by molecular methods and why this may be (i.e. gut content, eDNA, or parasite). We will include using N-taxa and ASPT as these are standard WFD reporting metrics which have been intercalibrated between countries and as such are widely accepted as providing a uniform measure of comparison. These steps will also allow us to ground truth the results generated by the different labs and different methods. We will assess the performance of molecular methods across several labs and at two stages: pre-extraction (i.e. how specimen/fixative is sampled and compared to morphological analysis) and post extraction (i.e. how extracted DNA is amplified, sequenced and compared to morphological analysis). By carrying out these intercalibration experiments, we will be able to determine the variation in our data attributable to molecular source (bulk DNA vs fixative DNA), laboratories, replicates, and protocols (standard vs custom). This will enable us to initially identify and focus further development of best practice in terms of target sources of DNA, and in sample collection, processing and analysis steps.

Pre-extraction

The performance and comparability of bulk and fixative DNA metabarcoding should be assessed based on samples collected with an established sampling method, such as the multi-habitat kick-net sample (Meier et al. 2006).

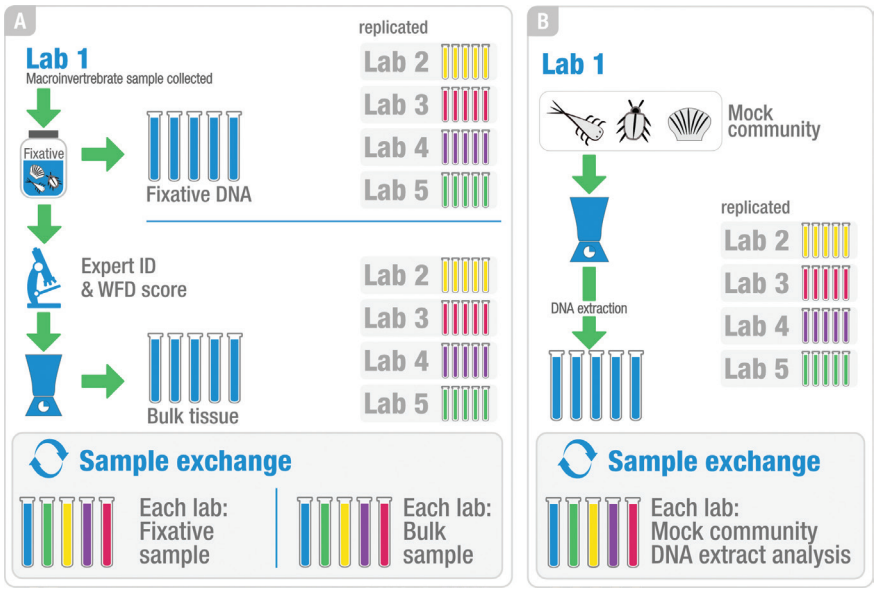

Figure 2. Outline of the proposed intercalibration experiments: **A.** Pre-extraction: Each lab will process one standard WFD macroinvertebrate sample to send to the other four labs. Each lab will work with five fixative samples and five blended bulk samples, four of which are unknown. Each lab will run their custom lab and bioinformatic pipelines and an agreed standard pipeline for the extraction and downstream analysis. **B.** Post-extraction: Each lab will create one macroinvertebrate bulk DNA mock sample to send to the other four labs, thus each lab will work with five mock community samples, four of which are unknown to analyse. Each lab will run their custom lab and bioinformatic pipelines and an agreed standard pipeline.

Traditional samples will be collected by WFD trained collaborators in several countries and subject to a short on-site cleaning step, where only large organic debris is removed. Environmental data required to determine EQR will be collected according to national standards. The sample shall then be preserved using an agreed upon preservative (Fig. 2A). Following an agreed minimum time, the preservative will be removed, divided into replicates and sent to participating labs for processing. The sample itself shall be subject to traditional morphological identification to the country specific requirement (e.g. in the UK, the Nordic countries, Germany, and Switzerland this is mixed taxon level). The morphologically identified sample shall then be homogenised, divided into replicates and sent to participating labs for processing. In different labs, replicates from each material type (preservative and homogenised tissue) shall be subject to DNA extraction, using an in-house protocol alongside an agreed "standard" method. Downstream processing steps will depend on the outcome of a separate Post-extraction experiment (described in next section).

Post-extraction

The DNA source is not the only factor determining variation in results, a key component of variance arises from differing laboratory protocols used to amplify and sequence the DNA. Laboratories use a wide variety of approaches for DNA amplification, PCR primers, library preparation, and bioinformatic pipelines. We therefore propose to establish a set of "blind mock community" samples, each to be tested by contributing labs (Fig. 2B). This would require labs contributing a DNA sample extracted from a known mock community of macroinvertebrate specimens. This sample will then be divided and sent to all other labs to follow in-house protocols for lab processing. A standard approach will also be run in parallel to ensure comparability. By performing this experiment, we will be able to identify variation in laboratory approaches and establish a best practice. The most important parameters we consider for establishing best practice are: the proportion of false negatives (i.e. species that are not detected when they are present) and the proportion of false positives (i.e. species that are detected when they are absent), when comparing results from metabarcoding against morphology and the variation of these error rates across sample replicates and laboratories. As results of the post-extraction experiments affect the choice of pre-extraction workflow, in terms of primer choice, library preparation, sequencing and bioinformatics analysis, we plan to start with the analysis of post-extraction analyses and then proceed with the pre-extraction experiment outlined above.

Data analysis

It is important for the final assessment of pre- and post-extraction variation among the protocols that all data is analysed using the same bioinformatic pipeline to avoid differences due to pre-filtering of sequence reads, clustering algorithm, or taxa assignment. A standard protocol will be decided upon prior to the start of the experiments. However, the data generated from our calibration of methods

will lend itself well to further tests of how results in DNA metabarcoding protocols and DNA sources interact with bioinformatic processing decisions. Data generated from these experimental designs will be archived with full metadata to allow for such bioinformatic comparisons.

Future applications

Over the past century, the response of macroinvertebrates to pollution has been well studied (Metcalfe 1989); however, focus has only been on single or very few stressors (e.g. nutrients, sedimentation, drought). In much of the world, biomonitoring with macroinvertebrates has led to greater understanding of these types of pollution and action has resulted to reverse their negative impacts. Today, we face pollution in novel multi-stressor environments (e.g. climate change, land use, micro-plastics, and micro-pollution from agricultural, pharmaceuticals, and personal care products) in addition to unsolved pollution challenges of the past. Understanding the response of macroinvertebrates in this new paradigm is in its early stages (Macher et al. 2016, 2018; Beermann et al. 2018; Burdon et al. 2018).

Over the next year, the DNAqua-Net community has a distinct advantage to make use of its resources and plans to organise large-scale natural experimental studies, documenting ecological community responses in multi-stressor environments. Importantly, the network will focus on the goal of developing DNA-based methods for monitoring macroinvertebrates and compare them with morphometric identification methods based on macroinvertebrate sampling with kick-nets, (i.e. method alignment). However, we will also look to develop a monitoring approach which differs from the traditional approaches to sample collection (i.e. method independency). The former may allow us to link existing data but may be guided and optimised by past limitations inherent in the traditional approach. While the latter may make comparisons with past samples impossible, it will start without the historic constraints of macroinvertebrate indices. For example, using either DNA from bulk samples or fixative DNA still harbours the limitations of traditional sample methods and is driven by the restrictions caused by the traditional and invasive collection of organisms (i.e. missing locally low abundant taxa including rare, elusive, or invasive species), while this could be bypassed by alternative approaches, such as eDNA.

The use and application of eDNA may be able to resolve these latter issues in part, but is, as a method, not yet standardised for sampling or processing and may give a more complementary measure when compared with the classic macroinvertebrate sampling. The use of eDNA for community detection has rapidly developed in recent years and has been successfully applied to a number of groups, notably fish (Hänfling et al. 2016; Port et al. 2016). However, some issues remain, with the most important of which is that an eDNA sample is less representative of the very local, site specific fauna in lotic ecosystems, as it integrates a greater geographic area due to the transport and spatial integration of DNA (Deiner et al. 2016; Pont et al. 2018). Secondly, no suitable primers have been identified for amplification of macroinvertebrate DNA without avoiding non-target taxa, which is more prevalent for eDNA compared with bulk DNA, thereby reducing the likelihood of their detection from a sample (Macher et al. 2018). Therefore, consensus was reached that eDNA is currently less suitable for site specific classification of macroinvertebrates (a requirement of the WFD). Its value is in its spatial integration and the provision of complementary information, including microscopic taxonomic groups (bacteria, phytoplankton), from the same sample (e.g. Deiner et al. 2016; Li et al. 2018; Macher et al. 2018). Thus, collecting eDNA from water samples according to a standardised practice (see CEN/TC230/WG2 NWIP 1156 proposed from within DNAqua-Net) and storing DNA is recommended. Importantly, we must also consider the necessity of our current focus on site-specific estimates to infer status of a (riverine) ecosystem (e.g. to identify point source pollution) as only one aspect of biomonitoring. The dependency of the local ecological state on regional patterns is well documented both from a theoretical but also an applied perspective (Sundermann et al. 2011; Altermatt 2013; Tonkin et al. 2018), and eDNA may allow us to explore whole catchment-based monitoring further. While we acknowledge that such a shift towards this perspective is beyond the current monitoring goals, and would require major legislative shifts, we should not inherently limit the development of new methods by such constraints. This shift in perspective towards a catchment-scale view has been reached already when it comes to pure chemical water quality status (McGuire et al. 2014; Burdon et al. 2019). Thus, a similar shift for biological data collected on the same scale may allow the discovery of previously unknown cause and effect relationships. As further research into the dynamics of eDNA is understood (see Barnes and Turner 2016), we may look to eDNA as a complementary method for biomonitoring of entire ecosystems in catchments and to detect species missed with other molecular and morphological approaches.

Ways forward and conclusion

This article has aimed to highlight the status of macroinvertebrate community analysis via DNA-based methods. It is our hope that the experiments designed as part of our discussions and workshop provide immediate areas of research to be undertaken. By carrying out comparison of workflows both within (in-house vs standardised) and across laboratories we will establish key points of the methods which influence the results and we will be able to form a basis for best practice. Bulk DNA samples have been the focus of efforts thus far, and shortly will be looked at on a large geographic scale (e.g. by SCANDNAnet, a project funded by the Nordic Council of Ministers; Joint Danube Survey 4; GeDNA, a project fund-

ed by the German Federal Environmental Agency [FKZ 3719242040]; and FRESHING, funded by FCT-Portugal together with EnvMetaGen funded by H2020). However, implementation using this DNA source still requires further assessment at the individual lab scale and streamlining of sample processing methods. Fixative DNA, a promising DNA source, remains largely unexplored and should be further compared with bulk DNA and current monitoring methods. The chances of future uptake of the described molecular methods by regulators into official, mandatory routine monitoring programs such as the WFD and MSFD will be greatly increased by conducting large experimental validation studies and by agreeing on standardised procedural protocols amongst scientists. We encourage the exploration and research of whole catchment-based approaches (via eDNA, including its degradation and transport) and working towards gaining an understanding of macroinvertebrate community responses to new and varied pollutants. For example, eDNA is currently being sampled and analysed in parallel with large ongoing aquatic monitoring programs in Switzerland (Kunz et al. 2016). Moving forward it is important to address the questions raised in this article in a systematic way and work towards developing standardised protocols such as CEN or ISO approved standards.

Acknowledgements

This article is a direct outcome of the European Cooperation in Science and Technology (COST) Action CA15219 DNAqua-Net. RB, FA, and EM are supported by Swiss National Science Foundation Grants No PP00P3_179089 and 31003A_173074 and the University of Zurich Research Priority Programme "URPP Global Change and Biodiversity" (to FA). FA and EM are also supported by the Velux Foundation. PB was supported by EDP-Biodiversity Chair (EDP/FCT). BE is supported by the European Union's Horizon 2020 research and innovation programme under grant agreement No 668981. VE is supported by funding through the Canada First Research Excellence Fund. AFF is supported by the FRESHING project funded by FCT (Portugal) and COMPETE (PTDC/AAGMAA/2261/2014). MM is supported by the Research Council of Norway (project no. 243791/E50) and the Norwegian Environment Agency (project no. 15040013). FMSM is supported by FCT PhD grant SFRH/BD/104703/2014. KM is supported by BONUS FUMARI; BONUS (art. 185), which is jointly funded by the European Union, the Academy of Finland, and the Swedish Research Council Formas and by the Nordic Council of Ministers to SCANDNA-net, grant no.18103. JP is supported by the Swiss National Science Foundation grant (31003A_179125). FL and VZ are supported by the German Barcode of Life project (GBOL) funded through the German Federal Ministry of Education and Research. We thank Jörg Strackbein (University of Duisburg-Essen) for generating the figures and Sandra Aresta funded by the European Union's Horizon 2020 research and innovation programme under grant agreement No 668981 (ERA Chair in Environmental metagenomics, EnvMetaGen) and colleagues at CIBIO for facilitating our meeting. We also thank all those that participated in discussions both online and at the meeting. We thank Alex Bush, Erik Pilgrim, and an anonymous reviewer for comments on the manuscript.

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