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COMMENTARY

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An illustrated manual for environmental DNA research: Water sampling guidelines and experimental protocols

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Abstract

Environmental DNA (eDNA)-based assessments of macro-organisms have now become an essential approach for biomonitoring. eDNA survey methods have a number of advantages over conventional survey methods. However, the value of the data that will accumulate would be greatly enhanced by standardizing the analysis methods, which would allow us to compare data from multiple monitoring sites at different points in time. The eDNA Society (http://ednasociety.org/en/about), whose founding members consist of Japanese researchers conducting eDNA studies on macro-organisms, was established in 2018, with the aim of expanding eDNA technology and science. Here, we introduce our key publication, "Environmental DNA Sampling and Experiment Manual" (http://ednasociety.org/en/manual), which was published under the initiative of the eDNA Society. Detailed methods for the surveys and experiments are described in the manual, including the selection of sampling sites, sampling methods, filtration methods, DNA extraction, species-specific detection by real-time polymerase chain reaction, and fish eDNA metabarcoding. The manual assists users in conducting standardized surveys and quality experiments, and provides a basis for collecting comparable data. Given that the efficacy of methods can be context dependent and variable, and that procedures may sometimes conflict with standardization, it is difficult to ensure that all processes are equally effective. However, even in such cases, it is important to maintain sufficiently high data quality by setting the minimum standards to be followed. Implementation of such standardized methodologies will enable the systematic and frequent collection of flawless, comparable eDNA data from around the world; this will provide important fundamental information for biodiversity conservation, as well as the sustainable use of fisheries resources.

KEYWORDS

biological monitoring, environmental monitoring, filtration, laboratory manual, polymerase chain reaction, standardization

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The eDNA Society, established in 2018, has recently published a manual called the "Environmental DNA Sampling and Experiment Manual" (http://ednasociety.org/en/manual). In this commentary, we will introduce the purpose and content of this manual, with a brief summary of the current status of the implementation of environmental DNA (eDNA) surveys in biodiversity monitoring.

In an age of rapid biodiversity loss, the establishment of protected areas and their continuous monitoring are highly important for the conservation and management of ecosystems (Acreman, Hughes, Arthington, Tickner, & Dueñas, 2019). Twelve years have passed since the first application of eDNA method for aquatic macro-organisms (Ficetola, Miaud, Pompanon, & Taberlet, 2008). Since then, eDNA methods have become an indispensable tool for monitoring underwater macro-organisms. A variety of applications for these methods have been reported, including the collection of information directly related to conservation, such as habitat identification of rare and non-native species (Takahara, Minamoto, & Doi, 2013; Thomsen et al., 2012) and estimation of breeding seasons or habitats (Erickson et al., 2016; Sakata, Maki, Sugiyama, & Minamoto, 2017), as well as monitoring of the currently present fauna (Bylemans, Gleeson, Lintermans, et al., 2018; Stoeckle, Soboleva, & Charlop-Powers, 2017). Empirical quantitative studies of eDNA indicated its possible application to estimate biomass/abundance (Fukaya et al., 2020; Lacoursière-Roussel, Côté, Leclerc, & Bernatchez, 2016; Yamamoto et al., 2016). Their target organisms also varied, with many researchers targeting fish and amphibians, but applications seem to be possible for virtually any macro-organisms, including mammals (Ushio et al., 2017), birds (Ushio, Murata, et al., 2018), and invertebrates (Ruppert, Kline, & Rahman, 2019). eDNA-based monitoring is also expected to contribute to the conservation of ecosystems by recording regional fauna that have not been sufficiently investigated previously because of limited accessibility, and by promotion of environmental education through public participation in the monitoring program.

eDNA methods have advantages over conventional collection-based biomonitoring methods in many aspects, such as higher accuracy of species detection and nondestructivity. Among these advantages, the most important one is probably that it does not require specialist knowledge, techniques, or tools in field work. Generally, conventional sampling methods such as netting, trapping, kick-sampling, and scuba diving require specific skills and resources, and morphological identification of organisms needs a high level of knowledge and experience. As a result, conventional methods could produce results that vary from worker to worker with different skill levels. In contrast, with eDNA methods, although careful consideration is necessary with respect to sampling strategies and risk avoidance, the field survey can be done without field specialists because it is basically only water sampling, and organisms are identified based on genetic information. By maximizing these advantages, eDNA surveys can be labor-saving, resulting in more survey opportunities with low-cost budgets, allowing multiple-site and high-frequency surveys.

Environmental DNA methods have already been, or will be, used in governmental biomonitoring programs in several countries and regions such as Japan, the EU, the UK, and the US (Biggs et al., 2014; Hering et al. 2018; Kitagawa, Muraoka, Yamada, & Nakamura, 2020; Pilliod, Goldberg, Laramie, & Waits, 2013). Moreover, in the EU, the European Committee for Standardization (CEN) and DNAqua-Net (Leese et al., 2018) are engaged in discussions relating to the standardization of eDNA methods in EU member countries. For such technology to be used on a large scale and treated as scientific evidence for political decision-making, proper application of the technology is essential. For example, if the sensitivity of the analysis varies from survey to survey, it could lead to misjudgment of the status of rare species. Additionally, if the experiments are performed without proper counter-contamination measures, the eDNA analysis is liable to produce false positives due to contamination, leading to incorrect judgments. This means that the development of standard methods for eDNA analysis is essential for providing consistent and reliable data for eDNA-based governmental biomonitoring. However, the reviewing of papers published regarding this topic so far has revealed large variations in the amount of water sampled, the method of concentrating DNA from the water, and the DNA extraction kit used (Tsuji, Takahara, Doi, Shibata, & Yamanaka, 2019), and differences in sensitivity between these methods have not been fully explored. One of the key factors accounting for these differences among methods is that of context dependency. For example, it is impracticable to filter large volumes of water containing excessive quantities of suspended material. Furthermore, if a sample contains substances that are inhibitory to PCR, a procedure for their removal should be incorporated. There can also be time, financial, or logistical constraints. However, it is still possible to achieve greater data comparability by unifying methods where possible or by setting minimum standards. Therefore, the eDNA Society, founded by researchers involved in eDNA studies of macro-organisms, has taken the initiative to publish a standardized protocol for use throughout the country (http://ednasociety.org/en/manual).

The manual, named "Environmental DNA Sampling and Experiment Manual", was prepared and published by the eDNA Society, whose purpose is to develop eDNA studies as a discipline that contributes to the well-being of human society, through the sustainable use of ecosystems and environmental conservation. To achieve this goal, an eDNA Methods Standardization Committee was organized, which took the role of editing the manual in cooperation with a wide range of Society members. The manual was released first in Japanese in April 2019 (http://ednasociety.org/

eDNA_manual_ver2_1_3.pdf), followed by the one in English released in March 2020 (http://ednasociety.org/eDNA_manual_Eng_v2_1_3b.pdf) (The eDNA Society, 2019). The manual (in English) consists of 93 pages with 172 figures, including 162 photos of the experimental process and many technical tips for the easy reproduction of the experimental procedures (Table 1; Figure 1). Detailed methods for surveys and experiments are described in the manual, including the selection of sampling sites, sampling methods, filtration methods, DNA extraction, species-specific detection by real-time polymerase chain reaction (PCR), and fish eDNA metabarcoding using MiFish primers (Miya et al., 2015), which have been shown to outperform other primers in recent studies (Bylemans, Gleeson, Hardy, & Furlan, 2018; Collins et al., 2019).

To make eDNA methods accessible for a wide range of users, a manual should be easily understandable, even for nonspecialists. To ensure this, many nonspecialists were also involved in the editing process. First, undergraduate students played an essential role in improving the descriptions in the manual. Students lacking experience of eDNA experiments were asked to carry out the experiments using just the manual and provide feedback to the committee. Second, government officials in a position to order survey works were also involved in the reviewing process. This feedback allowed the committee to make the manual more accessible to people who are not necessarily familiar with molecular biological analysis methods; however, of course, training in basic molecular biological experiments is required to actually work on the experiments. Following the release of the manual in April 2019, a series of technical seminars were held in seven cities to disseminate the information in detail for the people in charge of eDNA analysis in a wide range of sectors, such as the government, academia, and industry. Through these activities, eDNA analysis in Japan is approaching a high level of standardization. At present, many governmental and academic projects follow this manual for eDNA monitoring. For example, the Ministry of Land, Infrastructure, Transport and Tourism of Japan has already started to use eDNA analysis as a pilot study in a regular river survey called the National Census on River Environments (Kitagawa et al., 2020). In addition, meetings have been set up among ministries and agencies to obtain intercomparable data, as more governmental eDNA surveys will be conducted in the near future. The eDNA monitoring network, consisting of more than 50 field research sites, has been formed and has started frequent biodiversity monitoring of coastal sea, rivers, and lakes all over the Japanese archipelago under the same protocol. Thus, with the development and distribution of the manual, eDNA analysis in Japan is expected to be standardized further, and the data are comparable among surveys. Moreover, another important advantage of methodological standardization relates to the reuse of eDNA samples; the samples could be archived and then used for purposes different from the original ones for which the samples were used (Dysthe et al., 2018). Thus, the samples need to be collected via standardized or comparable methods. Although many excellent eDNA assays have been developed for multiple taxa, better assays will be developed in the future. Use of the manual would facilitate the effective reuse of eDNA samples in such situations.

TABLE 1 Contents of the Environmental DNA Sampling and Experiment Manual ver 2.1

Chapter	Contents
Chapter 1	Introduction
Chapter 2	Selection of sampling sites
	2–1. Selection of sampling sites in rivers
	2–2. Selection of sampling sites in ponds and lakes
	2–3. Selection of sampling sites on the coast
Chapter 3	Water sampling and filtration
	3–1. Water sampling and on-site filtration using a filter cartridge
	3–2. Water sampling and filtration using glass fiber filters in the laboratory
Chapter 4	Extraction of DNA
	4–1. DNA extraction from a filter cartridge
	4–2. DNA extraction from glass fiber filters
Chapter 5	DNA analysis
	5–1. Single species detection and quantification of eDNA
	5–2. Multiple species detection using MiFish primers

New techniques are developed and published for eDNA methods almost every day. Given this fact, methodological standardization, which potentially establishes a specific method, should be carried out with special care not to decelerate advances in the field. To strike a balance between standardization and progress of the field, we decided not to include some parts of the survey and analytical procedures in the manual. For example, strategies for deciding sampling locations and frequencies (timings) are not introduced despite the growing number of publications on this topic (e.g., Buxton, Groombridge, & Griffiths, 2018; Hinlo, Furlan, Suitor, & Gleeson, 2017). This is partly because it is not clear to what extent the presence of eDNA indicates a spatio-temporal distribution of the target organisms. Knowledge of the dynamics of eDNA is necessary to understand the range in which the eDNA of a species can be detected. Although many studies have attempted to elucidate this (Barnes & Turner, 2016), not enough knowledge has been accumulated to date. Moreover, downstream data processing after high-throughput sequencing, that is, bioinformatic analysis, has also not been presented. Bioinformatics processes for eDNA metabarcoding have highly diverse methodologies and tools, and this was found to be hard to summarize. With regard to fish eDNA metabarcoding using MiFish primers (Miya et al., 2015), researchers set up a portal site for analyzing sequence output files from Illumina sequencers and have made it available to the public (MiFish Pipeline, http://mitofish.aori.u-tokyo.ac.jp/mifish, Sato, Miya, Fukunaga, Sado, & Iwasaki, 2018). However, even in MiFish metabarcoding, each researcher uses different pipelines and databases (Bylemans, Gleeson, Lintermans, et al., 2018; Collins et al., 2019; Hayami et al., 2020).

(a)

5-2. Multiple species detection using MiFish primers

5-2-1. Library preparation—1: First-round PCR (1st PCR)

Before experiment: Reducing contamination risks

MiFish metabarcoding uses the polymerase chain reaction (PCR) to amplify the target eDNA to the extent that it can be analyzed with current molecular techniques, while it appends various adapters to both ends of the PCR products to analyze with a next-generation sequencing platform at the same time (library preparation). PCR, on the other hand, synthesizes exceptionally large amounts of DNA fragments and is likely to contaminate the experiments. Therefore, the laboratory for PCR preparation (pre-PCR room) and the laboratory for performing PCR and handling PCR products (post-PCR room) should be spatially separated. It is also necessary to implement measures to reduce contamination risks. Specifically, the personnel should not be engaged in DNA extraction or other experiments after handling PCR products during the same day. Furthermore, since MiFish metabarcoding employs two-step PCR, it is necessary to dilute the first-round PCR (1st PCR) product as a template for the second-round PCR (2nd PCR). Therefore, it is necessary to install a clean bench or an equivalent (KOACH T 500, Koken Co., Ltd.) that creates a clean (open) space in the latter laboratory (post-PCR room) in order to prevent contamination during this procedure. Micropipettes, tips, tubes, tube racks, and Milli-Q water should be decontaminated in advance using a UV sterilizer light bulb (e.g., NB-5, Nichiban Co., Ltd.). Experimental tables should also be decontaminated using the foaming bleach.

Laboratory instruments, reagents, and consumables required for 1st PCR

- Thermal cyclers (e.g., GeneAmp PCR System 9700, Applied Biosystems)
- UV sterilizer light bulb (e.g., NB-5, Nippon Steel Industry Co., Ltd.)
- KAPA HiFi HS ReadyMix (KK2602, KAPA Biosystems Inc.)[1]
- MiFish primer stock solution (stock solution diluted to 100 μM with TE buffer is convenient, if it is available)

Primers for elasmobranchs (primers optimized for sharks and rays)

MiFish-E-F-v2 (5′–3′; 61 mer):
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNRGTTGGTAAATCTCGTGCCAGC
MiFish-E-R-v2 (5′–3′; 68 mer):
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNGCATAGTGGGGTATCTAATCCTAGTTTG

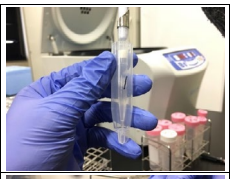
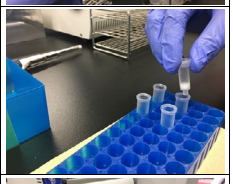
Primers for actinopterygians (i) (universal primer for ray-finned fishes)

MiFish-U-F (5′–3′; 60 mer):
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNGTCGGTAAAACTCGTGCCAGC
MiFish-U-R (5′–3′; 67 mer):
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNCATAGTGGGGTATCTAATCCCAGTTTG

Primers for actinopterygians (ii) (primers optimized for perch sculpin, which is a common species in the temperate coastal waters of Japan)

MiFish-U2-F (5′–3′; 60 mer):
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNGCCGGTAAAACTCGTGCC

(b)

Fig. 4-2-3-9 Once more, transfer the DNA solution from the Salivette tube to the column and centrifuge.	
Fig. 4-2-3-10 After centrifuging, transfer the column to a new 2 mL tube.	
Fig. 4-2-3-11 Pour buffer AW1.	
Fig. 4-2-3-12 After centrifuging, transfer the column to a new 2 mL tube.	

(c)

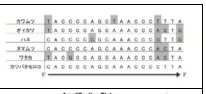
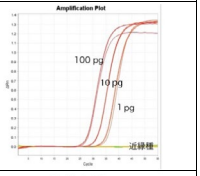
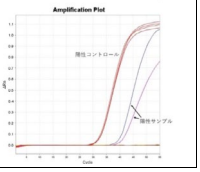
Fig. 5-1-1-1 Primer design. An example of primer design for *Hemigrammocypris rasborella* (Source: Fukuoka et al. 2016)	
Fig. 5-1-1-2 In vitro amplification check. Verify that the DNA of the species in question is amplified (red line) while the DNA of closely related species is not amplified (other colors).	
Fig. 5-1-2 An example of the result from real-time PCR.	

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FIGURE 1 Example pages from the Environmental DNA Sampling and Experiment Manual (The eDNA Society, 2019). The manual includes lists of required goods (a), photos (b) and illustrations (c) of the experimental processes [Colour figure can be viewed at wileyonlinelibrary.com]

There are some potential issues arising from the rapid advances in various methodologies. For example, if a better DNA recovery method is developed, many people would like to use it. As a result, it would become less straightforward to compare the data before and after changing the methods. To avoid such a situation, it is necessary to carefully determine what is to be included in the manual and when, and to describe correction methods between the results obtained by old and new techniques to ensure data comparability. The present manual does not cover everything on eDNA methods; thus, the eDNA Society has already started routine revision (once a year) of the manual by collecting comments from the members of the Society to keep it updated and practical, while trying to retain compatibility between methods in different versions of the manual. We believe that we have successfully obtained a practical platform to exchange ideas and disseminate and address them efficiently among the community through future revisions of the manual.

The eDNA Society decided to publish the manual promptly and seemingly succeeded in taking a timely lead toward establishing a standardization method for eDNA analysis in Japan before the rapid implementation of eDNA-based monitoring in Japanese society. Many newcomers and private sector workers in environmental science now follow the manual; we expect this to allow mutual exploitation of data and samples in the future and increase the efficiency of eDNA surveys throughout the nation.

Standardization would produce even more benefits if it was implemented on a global scale rather than on a limited national or regional basis. Global standardization would result in high-frequency, multiple-site, and comparative data collection on a global scale. A framework for the international standardization of eDNA methods, along with a global network for relevant discussion, such as the DNAqua-Net initiative of the EU (Leese et al., 2018), would thus be strongly recommended. Such comparable data may be used to detect species interactions using time-series analysis (Ushio, Hsieh, et al., 2018) or to predict future dynamics of fisheries resources at any scale. Thus, the standardization of eDNA methods has the potential to contribute to the conservation of ecosystems, sustainable use of resources, and consequently, to human well-being.

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CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTION

The first author wrote the first draft of the manuscript. All authors provided feedback for the manuscript.

DATA AVAILABILITY STATEMENT

No data was used for this commentary manuscript.

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