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RAPID COMMUNICATION

Note on important and novel findings

Pre-centrifugation before DNA extraction mitigates extraction efficiency reduction of environmental DNA caused by the preservative solution (benzalkonium chloride) remaining in the filters

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Abstract

In the biomonitoring using environmental DNA (eDNA) analysis, the suppression of eDNA degradation has been an important issue to estimate accurately the presence of target species and their eDNA concentrations. A recently proposed eDNA preservation technique requires only the addition of benzalkonium chloride (BAC) to water samples and has been used for various studies as an alternative solution to on-site filtration. However, BAC remaining in filters may adsorb to and inhibit the function of the enzyme used to extract eDNA from filters. In this study, we tested whether eDNA yield is affected by BAC addition immediately before filtration and demonstrated that the BAC in water remaining in filters decreased the eDNA yield significantly. We found that simple pre-centrifugation is effective for removing the remaining water from the filters to prevent the eDNA yield reduction due to the use of BAC. This is the most important finding in the present study. Appropriate eDNA extraction techniques are needed when BAC is used to secure eDNA yields and expand the application of eDNA analysis for biomonitoring.

Keywords Benzalkonium chloride · Environmental DNA · Pre-centrifugation · Extraction · Proteinase K

Introduction

The use of environmental DNA (eDNA) analysis for biomonitoring is rapidly expanding because it potentially enables us to avoid the limitations of conventional capture and visual observations imposed by time, labour, monitoring biases and invasiveness etc. (Rees et al. 2014). The simplicity of sampling without the need for specialist knowledge, techniques or tools has helped expand the application of eDNA analysis (Thomsen and Willerslev 2015). Several countries have implemented eDNA analysis into nationwide biomonitoring or environmental education programmes (Minamoto et al. 2020). Thus, there are more opportunities for newcomers, citizen-scientists and private sector workers in environmental science to conduct eDNA analysis.

Suppression of eDNA degradation in water samples is essential in biomonitoring using eDNA analysis (Allan et al. 2020). The eDNA in water degrades over time and leads to false-negative results and under-estimation of eDNA concentrations. Further, eDNA degradation is accelerated at higher water temperatures (Tsuji et al. 2017a, b; Jo et al. 2019). Therefore, various techniques have been developed to suppress eDNA degradation, including cooling or freezing of water sample, on-site filtration and storage on ice, ethanol fixation and immersion in lysis buffers (Jerde et al. 2011; Goldberg et al. 2011, 2016; Renshaw et al. 2015). However, for non-experts, these methods are often difficult to implement because they require a large cooler box, portable freezer or specialised system and techniques for on-site filtration (Takahara

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et al. 2020). Non-experts of eDNA analysis can use disposable filter cartridges to filter water on-site; however, these cartridges are generally more expensive than filter discs. They are also not designed to filter a large amount of water because many of them have a small pore size (i.e. < 0.45 µm) or effective filtration area (i.e. < 4.5 cm^2). In terrestrial waters containing large amounts of algae or suspended solids, the amount of water that can be filtered may be limited because the filters clog easily (Tsuji et al. 2019).

To address these issues, an eDNA preservation technique was proposed that only requires the addition of benzalkonium chloride (BAC) to water samples. BAC is a cationic surfactant. BAC 0.01% halts or significantly decreases the degradation of eDNA in water samples even at ambient temperatures (Yamanaka et al. 2017; Takahara et al. 2020). Preservation of eDNA using BAC does not require a specialised system or technique. BAC is commonly used as an antiseptic in medical and sanitation work and can be easily and affordably obtained. For these reasons, BAC treatment has contributed significantly to expanding the application of eDNA analysis as a user-friendly technique.

Although BAC is useful for preserving eDNA in a water sample, its effect on the extraction of eDNA from filters has not been thoroughly explored (Yamanaka et al. 2017; Takahara et al. 2020). The antimicrobial activity of cationic surfactants containing BAC is attributed to its absorbance to the negatively charged cell membrane and disrupts cellular function (Ziani et al. 2011). Thus, the presence of BAC may also inhibit the function of the enzyme (proteinase K) used to extract eDNA from filters. Because eDNA has been suggested to exist as cells or organelles in water (Turner et al. 2014; Tsuji et al. 2017a, b), unintended inactivation of proteinase K during extraction would be expected to cause a reduction in eDNA yield. Therefore, examining the effect of BAC on the efficiency of eDNA extraction from filters would help to improve the protocol for eDNA extraction and to ensure the advantages of BAC as a preservative.

This study aimed to examine the effect of residual BAC on the extraction efficiency of eDNA from filters. We prepared water samples with and without BAC. We performed filtration and extraction with the same technique and compared eDNA yield among the water samples. Moreover, to propose techniques for improving eDNA yield, we tested whether the reduction in extraction efficiency could be mitigated by removing the BAC by pre-centrifugation or rinsing. We quantified the eDNA yield of two freshwater species widely distributed in Japan, Ayu (*Plecoglossus altivelis altivelis,* fish) and a net spinning caddisfly (*Stenopsyche marmorata*, aquatic insect), using species-specific quantitative real-time PCR (qPCR). We also identified fish species and their eDNA yield simultaneously with a quantitative fish metabarcoding method using a universal fish primer set.

Materials and methods

Two experiments were performed in the present study. Experiment 1 (Exp. 1) examined the effect of residual BAC on the extraction efficiency of eDNA from filters. Experiment 2 (Exp. 2) tested whether the reduction in extraction efficiency could be mitigated by rinsing the filters. Details of the experimental design are shown in Fig. 1. All water sampling was performed in the Saba River, western Japan (34.063772°N, 131.558276°E) in November 2020. In the sampling site, Ayu and the caddisfly are dominant fish and aquatic insect species, respectively. In both experiments, the final concentration of BAC was set to 0.01%, because this concentration was suggested as optimal to preserve eDNA by Yamanaka et al. (2017). Details on sampling time and water quality are shown in Table S1. In both experiments, the water samples were cooled on ice and transported immediately to the laboratory (transit time ca. 1 h).

Experiment 1: Examination of the effect of BAC remaining in filters on the extraction efficiency of eDNA

Approximately 20 L of surface water was collected with a bucket as water sample and transported immediately to the laboratory. The water samples were mixed well in the laboratory and divided into subsamples: 12 L with BAC (final concentration, 0.01%; OSVAN S 10 w/v % benzalkonium chloride, Nihon Pharmaceutical, Tokyo, Japan) and 6 L without BAC. We added the BAC directly to water samples using a pipette. A portion of each sample, 9 L with BAC and 3 L without BAC was filtered using GF/F glass-fibre filters (1 L per filter; Cytiva, Tokyo, Japan). We started the filtration of water samples within 1.5 h from water sampling, and it was completed within 30 min. The remaining water from each subsample was dispensed into three 1 L plastic bags (DP16-TN1000; Yanagi, Nagoya, Japan) and stored in a refrigerator (ca. 4 °C). After 24 h, the stored water samples were filtered in the same manner. At each time point (0-h and 24-h), 1 L of ultrapure water with BAC (final concentration, 0.01%) was filtered as a negative control. All the filters were wrapped in aluminium foil and stored at − 20 °C until extraction.

In the eDNA extraction step, three methods were used to examine the effect of the water containing BAC remaining in the filters on eDNA extraction efficiency (Fig. 1, three filter replications for each method). A1 and A2 were the standard method described in the 'Environmental DNA Sampling and Experiment Manual (ver. 2.1)' (https://ednasociety.org/wp/wp-content/uploads/2020/09/

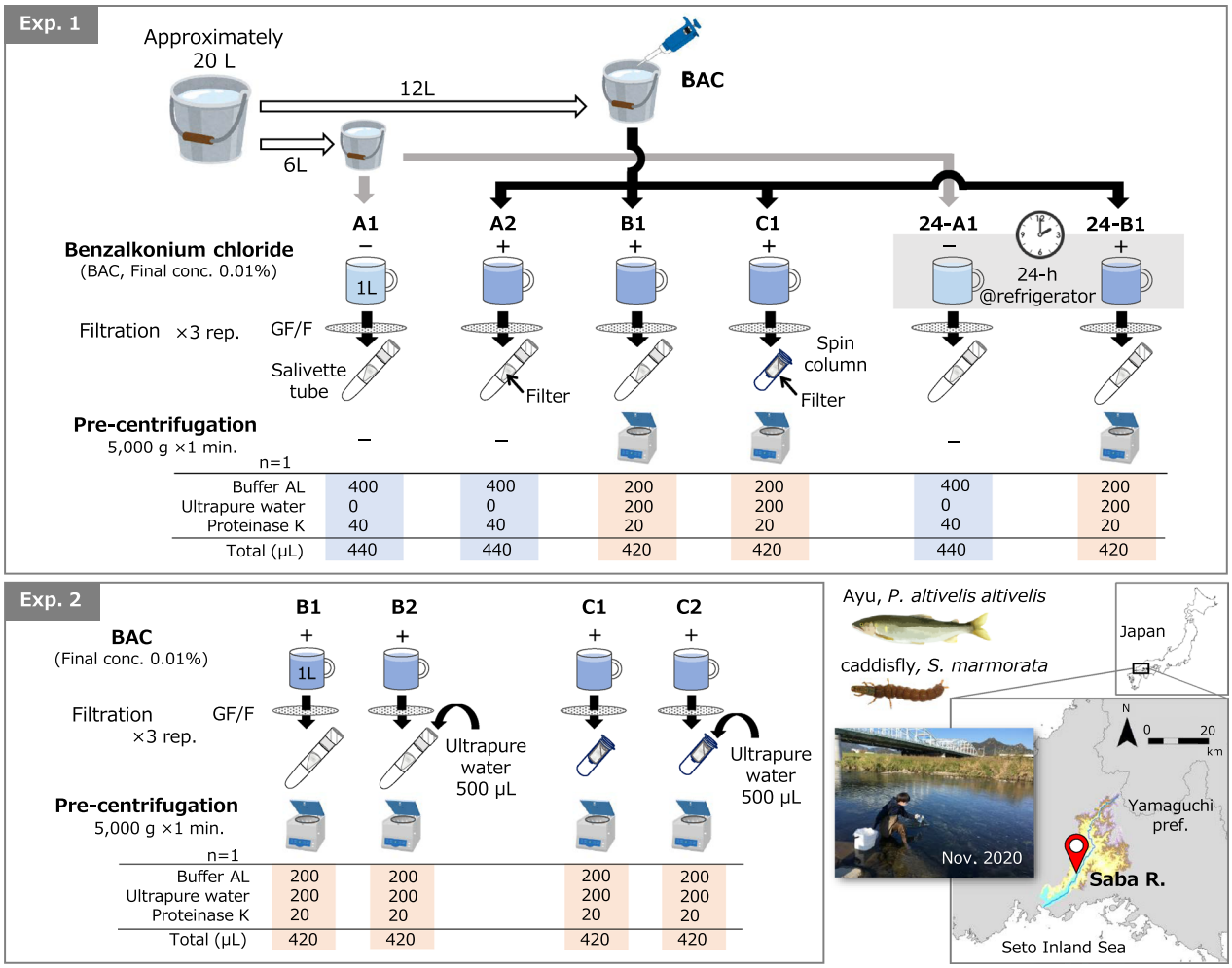

Fig. 1 Overview of the sampling site and experimental design

eDNA_manual_Eng_v2_1_3b.pdf), which was published by the eDNA Society of Japan. Briefly, a filter was placed in the upper part of a salivette tube (Sarstedt, Nümbrecht, Germany) and then soaked with 440 μL of a mixture composed of 400 μL Buffer AL and 40 μL of proteinase K. B1 was a slightly modified method in which the sample was centrifuged and a thinner buffer used. In B1, a salivette tube containing a filter was pre-centrifuged for 1 min at 5000 g to remove the remaining water, and the filtrate was discarded. The mixture is composed of 200 μL Buffer AL, 200 μL ultrapure water and 20 μL proteinase K. C1 was an alternative to A1 and A2, developed for researchers without access to large centrifuges. This method employs spin columns (EconoSpin EP-31201, Epoch Life Science) with the silica gel membranes removed in advance. All subsequent treatments were the same as in method B1. In all methods, samples were incubated after buffer addition for 30 min at 56 °C and centrifuged for 3 min at 5000 g. To retrieve the remaining eDNA from the filters, 220 μL Tris–EDTA buffer (pH 8.0) was added to the filters. After incubating for 1 min at room temperature, samples were re-centrifuged at 5000 g for 3 min, followed by disposal of the upper part of the salivette tubes or spin columns. Next, 400 μL ethanol was added to each filtrate and mixed by pipetting. The eDNA in the mixture was then purified using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) accordingly to manufacturer protocols. Finally, eDNA was eluted in 100 μL of Buffer AE. eDNA was extracted from the 24-h chilled samples with or without BAC using A1 and B1 method, respectively.

In the eDNA detection step, Ayu and the caddisfly eDNA was quantified by qPCR. A quantitative metabarcoding assay using internal standards and a universal fish primer set was conducted to identify fish species and quantify their eDNA concentrations simultaneously.

Experiment 2: Examination of the effect of rinsing on eDNA yield

Twelve litres of surface water was collected from the river, and BAC was added (final concentration, 0.01%). In the laboratory, the water samples were agitated well and then filtered using GF/F filter as in Exp. 1. In the eDNA extraction step, four treatments (B1, B2, C1 and C2) were used to examine whether the yield of eDNA is improved by rinsing off the BAC remaining in the filters with water (Fig. 1). B2 and C2 were the slightly modified subvariants of B1 and C1, respectively, in which 500 µL of ultrapure water was dropped onto the filter before pre-centrifugation to rinse. All subsequent treatments were as in the B1 and C1. Ayu and the caddisfly eDNA were quantified by qPCR.

Quantitative real-time PCR (qPCR)

The numbers of eDNA copies of Ayu and the caddisfly eDNA was quantified using the species-specific primer sets developed by Yamanaka and Minamoto (2016) and the present study, respectively (Table 1). The specificity of the newly developed primer set for the caddisfly was tested by in silico and in vitro tests. The eDNA of the target species was quantified using TaqMan probe (for Ayu) or SYBR-GREEN (for the caddisfly). The four-dilution series of the plasmid DNA containing each target region at known copies (3×10^1 to 3×10^4 copies per reaction) and negative (no-template) controls with ultrapure water were used in all qPCR runs. All reactions were performed in a 15-µL total volume, as described in Appendix 1. All assays were performed in triplicate using a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, MA, USA). Detailed information on qPCR run qualities was as follows: R^2 values, 0.997 to 1.000; Y-intercept, 34.687 to 40.252; Slope, − 3.533 to − 3.307; Efficiency (%), 91.898 to 100.606 (Table S3). No amplification was observed in any of the filtrations and PCR negative controls. Further methodological details are shown in Appendix S1.

Quantitative fish metabarcoding using internal standard DNAs and MiFish-U primers (qMiFish)

To identify fish species and quantify their eDNA concentration simultaneously, the quantitative fish metabarcoding method, qMiFish, using internal standards were adopted in this study. The eDNA samples obtained from the first replicate in each extraction method were used for the qMiFish study.

The qMiFish was initially described as 'qMiSeq' by Ushio et al. (2018). In general, the number of sequence reads obtained using high-throughput sequencing does not necessarily correspond to the eDNA quantity in a given sample because PCR efficiency differs. Thus, it is difficult to estimate the quantity of eDNA in a sample by the eDNA metabarcoding approach. In qMiFish, the number of sequence reads can be converted to DNA copies without being affected by differences in the PCR efficiency among the eDNA samples by obtaining and using sample-specific standard lines. To obtain sample-specific standard lines, internal standards were added to each eDNA sample and amplified along with the fish eDNA with a universal primer set (MiFish-U, Miya et al. 2015). The prepared DNA libraries were sequenced on the iSeq platform (Illumina, CA, USA). After the bioinformatic analysis, to obtain sample-specific standard lines, a linear regression analysis was performed for each sample using sequence reads of standard DNAs and their eDNA copies. The regression slope and R^2 of the sample-specific standard line are shown in Table S5. The R^2 of all standard lines were higher than 0.98. For each sample, eDNA copies per species were calculated as $Nc = iRs/S$. iRs is the number of iSeq sequence reads and S is the regression slope of the standard lines. The estimated Nc values are hereafter referred to as 'qMiFish DNA concentration'. Further methodological details are shown in Appendix S1 and Table S4. In PCR negative controls, *Nipponocypris temminckii* was detected at a level similar to the real samples (Table S5). Thus, it was considered to be contamination and eliminated from further analysis.

Table 1 Primers and probe used for species-specific qPCR

Target species	Primers/probe	Sequence (5′→3′)	Length	Method
Plecoglossus altivelis altivelis	Paa-CytB-F	CCTAGTCTCCCTGGCTTTATTCTCT	131 bp	TaqMan
	Paa-CytB-R	GTAGAATGGCGTAGGCGAAAA		
	Paa-CytB-Pr	[FAM]–ACTTCACGGCAGCCAACCCCC–[TAMRA]		
Stenopsyche marmorata	Sma-COI-F	TGTAACAGCCCACGCGTTC	161 bp	SYBR
	Sma-COI-R	AGATTAAAGAAGGGGGGAGTAATCAA		

Statistical analysis

In Exp. 1, the eDNA yields of Ayu and the caddisfly for each eDNA extraction method were compared using the parametric Tukey honest significant differences (HSD) test ($\alpha = 0.05$). The Friedman test followed by Wilcoxon signed-rank test was performed ($\alpha = 0.008$, Bonferroni adjusted) to compare the qMiFish DNA copy numbers of each species among the extraction methods. In Exp. 2, for each species, the effect of filter rinsing on eDNA yield was analysed using the Mann–Whitney U test ($\alpha = 0.05$). All statistical analyses were performed in R ver. 3.6.0 software (R Core Team 2019).

Results and discussion

We found that remaining water containing BAC in the filters decrease the eDNA yield of Ayu and the caddisfly significantly (A1 and A2, $p < 0.01$ in both species; Fig. 2). In qMiFish, 34 freshwater fish species were detected, and their eDNA concentrations quantified (Fig. 3). The qMiFish DNA concentration for each species was significantly lower when remaining water containing BAC was present in the filters (A2 and all the other methods, $p < 0.0001$). The number of detected species was the lowest in A2 (A1, 28; A2, 26; B1, 29; C1, 29 species, respectively) as some species detected with lower qMiFish DNA concentrations in A1, B1 and C1 were not detected in A2 (Table S5). These findings support the hypothesis that BAC remaining in the filters inhibits the function of proteinase K. Our results also showed that simple pre-centrifugation is effective for removing remaining water

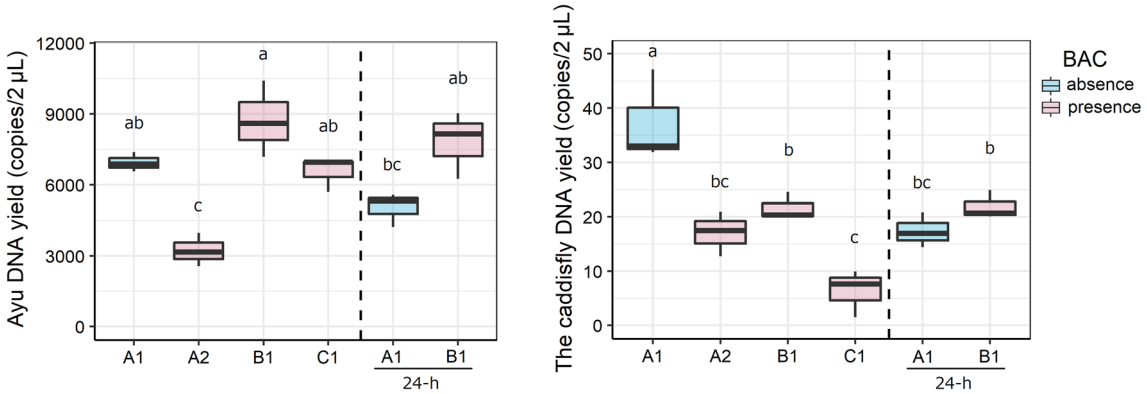

Fig. 2 Comparisons of eDNA yields among different extraction methods in Exp. 1. The blue and pink box plots indicate the presence and absence of BAC in the water sample, respectively. Significant differences were indicated by different letters (Tukey HSD test, $p < 0.05$)

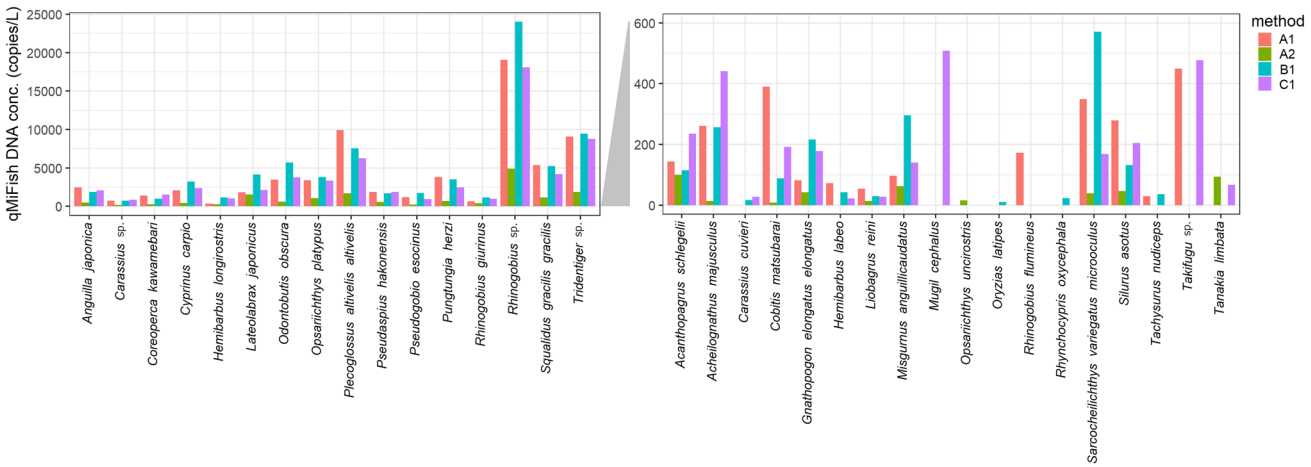

Fig. 3 The 34 freshwater fishes and their DNA concentrations were detected and quantified by qMiFish assay for Exp. 1 samples. The eDNA samples obtained by the first replicate of each extraction method (A1, A2, B1 and C1) were used for qMiFish. The number of DNA copies differed significantly between A2 and other methods (Friedman test, $p < 0.0001$; Wilcoxon signed-rank test, $p < 0.0001$)

containing BAC from the filters to prevent the decrease in eDNA yield reduction due to the use of BAC (A2, B1 and C1; Fig. 2). This is the most important finding of the present study.

In the caddisfly, the samples without BAC showed a significant decrease in eDNA yield after 24 h (A1 and 24-h A1, $p < 0.01$; Fig. 2). On the other hand, the eDNA was preserved when BAC was added to water samples (B1, $p = 0.99$; Fig. 2). In Ayu, there was no significant decrease in eDNA yields after 24 h both in A1 and B1 (A1 and 24-h A1, $p = 0.28$; B1 and 24-h B1, $p = 0.87$; Fig. 2); however, eDNA tended to be more conserved when BAC was added (B1 and 24-h B1). In this study, the water samples were kept at low temperature (approximately 4 °C) by using ice and a refrigerator throughout the experiment. Thus, it is likely that the result in this study shows what happens when DNA is conserved under a very ideal condition. Besides, it is not always possible to filter refrigerated water samples within 24 h. When BAC is not added, eDNA continues to be degraded even when stored at 4 °C. In contrast, when BAC is added, DNA is preserved at almost the same concentration as the initial concentration even after 20 days (Takahara et al. 2020). Considering these facts, the usefulness of BAC as eDNA preservative is remarkable and suggests that the use of BAC ensure the strict measurement of the eDNA concentration for various taxa even when on-site filtration is difficult. Thus, we recommend adding BAC to preserve eDNA in water samples. We also recommend that remaining water containing BAC should be removed by pre-centrifugation prior to adding eDNA extraction reagents to improve extraction efficiency.

We also found that the use of BAC may cause a decrease in eDNA extraction efficiency depending on the target taxa even if pre-centrifugation was performed before extraction. For Ayu, there was no significant difference in the eDNA yield of A1, B1 and C1 (Fig. 2). Similarly, a comprehensive quantitative analysis of fishes using the qMiFish also showed no differences in eDNA yields among these methods (A1 and B1, A1 and C1, $p = 1.00$; C1 and B1, $p = 0.46$; Fig. 3). On the other hand, for the caddisfly, the addition of BAC caused a significant decrease in the eDNA yield in all treatments compared with A1, even when pre-centrifugation was performed (all $p < 0.01$; Fig. 2). If the composition of materials mainly shed by fishes as eDNA differs from that of the caddisfly, the difference in the extraction efficiency of eDNA may be caused depending on the denaturing state of proteins by BAC. In fact, the primary source of eDNA is much more likely to differ between Ayu and the caddisfly because they have entirely different body forms and physiological characteristics. However, the present study was not focussed on this perspective and is thus left for future studies.

We examined whether the eDNA yield is improved by rinsing off the BAC remaining in the filters using water,

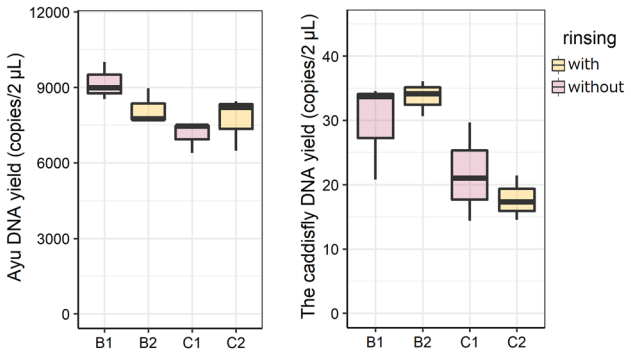

Fig. 4 Comparisons of eDNA yields between treatments in Exp. 2. The pink and yellow box plots indicate protocols with and without rinsing of filters before pre-centrifugation. No significant differences were observed between the protocols with and without the rinsing step (Mann–Whitney *U* test, $p > 0.05$)

but no significant increases in Ayu and the caddisfly eDNA yield were observed (Fig. 4). The most likely causes are (1) the pre-centrifugation was sufficient for removing BAC from the filters, or (2) a portion of eDNA was rinsed away with the BAC. To narrow down these possibilities, we carried out a preliminary test. The pre-centrifuged filtrate was incubated with the extraction reagents and purified with the eDNA extracted from the filter samples. However, no significant increases in eDNA yield were observed (Fig. S1). This result suggests that the first hypothesis is more likely to be supported if further investigations were made in future studies. In the present study, pre-centrifugation was performed for 1 min at 5000 g. After this process, a larger amount of water was removed from C1 than B1 (Mann–Whitney *U* test, $p < 0.01$; Fig. S2 A). This result was probably due to differences in the size and structure of salivette tubes and spin columns (Fig. S2 B). The amount of water remaining in the filters after filtration is not constant and varies with the degree of filter clogging and filtration technique. Although the amount of water remaining in filters after pre-centrifugation was not examined here, pre-centrifugation would reduce variability in the amount of water remaining in the filters. Thus, removing remaining water containing BAC by pre-centrifugation is also expected to reduce the variability of DNA extraction efficiency among filters. In addition, it is also worth mentioning that the difference in the volume of reagents may produce a difference in final DNA yield. The composition of the reagents was different between A1, A2, 24-A1 and B1, C1, 24-B1. However, at least the DNA yield was not lowered by reducing the volume of the reagents because there was no significant difference in the eDNA yields of Ayu and other fishes among A1, B1 and C1. Although it was not examined in this study because the aim to mitigate the reduction in extraction efficiency has been achieved, further experiments on the optimal composition

of reagents for extraction will be an important area in future studies.

Conclusions

The present study demonstrates the importance of removing BAC from filters by pre-centrifugation before extracting eDNA. The BAC remaining in filters inhibits the function of the enzyme such as proteinase K and reduces the efficiency of DNA extraction. Our result suggests that, at least in fishes, as long as BAC removal by pre-centrifugation is performed, the use of BAC will allow us to obtain eDNA yields comparable to those obtained by on-site filtration. The development of appropriate eDNA extraction techniques when BAC was used to preserve eDNA in a water sample would ensure the applicability of BAC and accelerate the implementation of eDNA analysis for biomonitoring.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10201-021-00676-w.

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Author contributions ST, RN, and YA conceived and designed research. ST, RN, and MS performed sampling and experiments. ST and TM performed data analysis. ST wrote the early draft and completed it with significant inputs from all authors.

Data availability Full details of the results for each experiment of the present study are available in the supporting information (Table S2, S3 and S5). All raw sequences obtained in the qMiFish analysis were deposited in the DDBJ Sequence Read Archive (accession number: DRA011253).

Declarations

Conflict of interest TM is an inventor of the patent for the use of BAC to eDNA preservation. The other authors have no conflicts of interest.

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