# Environmental DNA extraction method for a high and stable DNA yield

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June 15, 2022

**Abstract**

Environmental DNA measurement has been widely applied in organism biomonitoring. Different DNA extraction methods may cause changes in yield and stability, resulting in an inaccurate estimation of eDNA, especially when quantitative measurements are performed. This study focused on the DNA extraction method and compared its yield and stability for stream fish and spiked DNA samples. Samples were collected periodically over a year from river and lake water systems and eDNA was spiked into them. The samples were extracted and compared using three methods: using Buffer-AL for initial lysis with the DNeasy Blood and Tissue Kit (Qiagen); using Buffer-ATL for initial lysis and the microfluidic-channel method (BC method). The method using Buffer-ATL in the DNeasy Blood and Tissue Kit showed better stability and a higher yield than the Buffer-AL method. In addition, the BC method, despite being comparatively simple, performed the extraction stably and with relatively high yields. We showed that differences in DNA extraction methods based on the long-term evaluation of eDNA measurements with various methods may cause alterations in DNA yield and stability.

**Environmental DNA extraction method for a high and stable DNA yield**

Short running title: High and stable eDNA extraction method

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Abstract

Environmental DNA measurement has been widely applied in organism biomonitoring. Different DNA extraction methods may cause changes in yield and stability, resulting in an inaccurate estimation of eDNA, especially when quantitative measurements are performed. This study focused on the DNA extraction

method and compared its yield and stability for stream fish and spiked DNA samples. Samples were collected periodically over a year from river and lake water systems and eDNA was spiked into them. The samples were extracted and compared using three methods: using Buffer-AL for initial lysis with the DNeasy Blood and Tissue Kit (Qiagen); using Buffer-ATL for initial lysis and the microfluidic-channel method (BC method). The method using Buffer-ATL in the DNeasy Blood and Tissue Kit showed better stability and a higher yield than the Buffer-AL method. In addition, the BC method, despite being comparatively simple, performed the extraction stably and with relatively high yields. We showed that differences in DNA extraction methods based on the long-term evaluation of eDNA measurements with various methods may cause alterations in DNA yield and stability.

Keywords: eDNA, extraction, filtering, DNeasy extraction kit, and Biryu-chip

## Introduction

Environmental DNA (eDNA) in aquatic environments has been used to detect species distributions (Buxton et al., 2018; Doi et al., 2017; Ficetola et al., 2008; Katano et al., 2017; Takahara et al., 2012; Wang et al. 2021). eDNA has been detected in water from various ecosystems, including streams, lakes, ponds, reservoirs, canals, and oceans (Doi et al., 2017; Fornillos et al., 2019; Katano et al., 2017; Suter et al., 2021; Takahara et al., 2012), and its measurement has been mainly performed using quantitative real-time PCR (qPCR) (Doi et al., 2017; Fornillos et al., 2019; Katano et al. 2017; Preece et al., 2021; Takahara, et al., 2012).

The measurement of eDNA is conducted by water sampling, followed by filtration and extraction, and finally DNA detection by qPCR. To obtain accurate eDNA measurements, it is necessary to select a reliable method for each process. Water sampling and filtration processes can provide relatively precise results if the process conditions are set; however, few studies have reported on the stability of eDNA extraction. eDNA has been extracted from filter and sediment samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany; hereafter, DNeasy method) (Tsuji et al., 2019), PowerWater (Coster et al., 2021; Hinlo et al., 2017), and PowerSoil (Díaz et al., 2020; Eichmiller et al., 2016; Sakata et al., 2020). Among these, DNeasy is the most commonly employed (Tsuji et al., 2019), however there are two groups of methods. One method is based on blood or cells without the use of ATL (Miya et al., 2016; Yamamoto et al., 2016), while the other involves tissues that employ ATL (Coster et al., 2021; Djurhuus et al., 2017). These methods are described in the manual of the DNeasy Blood and Tissue Kit. Recently, many studies have referred to the procedure given by Miya et al. (2016), which does not use ATL, for the specific procedures of this method, since this is the method on which the manual from the eDNA Society was based (Minamoto et al., 2021; Wong et al., 2020). However, few studies have reported the stability of both methods.

The aforementioned DNA extraction methods are difficult for non-researchers to perform because they require many experimental steps, and this challenge, along with the questionable reliability, creates a hurdle for the social implementation of eDNA techniques. In fact, a stable and simple filtration and extraction method for the social implementation of eDNA has been suggested (e.g.,, Fukuzawa et al., 2022).

In evaluating the reliability of several extraction methods, we found that the DNeasy without ATL described in the manual could cause large fluctuations in the measured values due to foreign substances in the water sample, whereas highly stable results can be obtained using ATL. The stability of the BC method, which we developed based on a simple method, was simultaneously evaluated and stable measurements were obtained.

## Materials and Methods

### The extraction method with Buffer AL (hereafter, the "AL method")

A method using the DNeasy Blood and Tissue DNA extraction kit was used according to the procedure described by Miya et al. (2016), and characterized by the use of Buffer AL as the lysate.

For the AL method using Sterivex:

1. The premixes were prepared using proteinase-K, buffer AL, and PBS at 20 μL, 200 μL, and 220 μL, respectively.

2. The premixes (440 μL) were added to a Sterivex and incubated at 56 °C for 30 min.
3. The liquid was transferred from the Sterivex to a 1.5 mL tube.
4. Ethanol (200 μL) was added to the tube, which was then stirred.
5. The solution was transferred to a spin column, centrifuged at 6000 × g for 1 min, and the solution in the collection tube was discarded.
6. The collection tube was replaced, and 500 μL of Buffer AW1 was added to the spin column, which was then centrifuged at 6000 × g for 1 min, and the solution in the collection tube was discarded.
7. The collection tube was replaced, and 500 μL of Buffer AW2 was added to the spin column, which was centrifuged at 20000 × g for 3 min, and the solution in the collection tube was discarded.
8. The collection tube was replaced with a new 1.5 mL tube and 200 μL of Buffer AE was added to the spin column; after for 1 min, the solution was centrifuged at 6000 × g for 1 min.

The extracted sample was now contained in the 1.5 mL tube.

For the AL method using the GF/F glass filter (GE Healthcare UK)

1. A glass filter was placed on the top of a Salivette tube (Zalstat), and a mixture of 40 μL of proteinase K and 400 μL of Buffer AL was poured onto the filter paper evenly, and incubated at 56 °C for 30 min in a thermostatic chamber.
2. The tube was placed in a centrifuge at 3000 × g for 3 min.
3. TE (220 μL) was poured onto the filter paper, and after waiting for 1 min, it was centrifuged at 3000 × g for 3 min.
4. The upper part of the Salivette tube containing the filter was removed and discarded, 400 μL of ethanol (99.5%) was added, and the solution was stirred.
5. Approximately 650 μL (approximately half) of the sample was transferred to the spin column and centrifuged at 6000 × g for 1 min.
6. The solution in the bottom of the collection tube was discarded, and the remaining solution was transferred to the spin column again, and centrifuged at 6000 × g for 1 min.
7. The collection tube was replaced, and 500 μL of Buffer AW1 was added to the spin column, which was centrifuged at 6000 × g for 1 min, and the solution in the collection tube was discarded.

Following this, the Sterivex filtration method was performed, as described previously.

The extraction method with Buffer ATL (hereafter the "ATL method")

We modified the method for eDNA following the extraction procedure from tissue in the DNeasy manual, with the use of Buffer ATL as the lysate. The detailed procedures for using the Sterivex and GF/F glass filters are shown below.

For the ATL method using Sterivex:

1. Buffer ATL (380 μL) mixed with 20 μL of proteinase K was placed in the Sterivex and incubated at 56 °C for 30 min.
2. The liquid was collected from the Sterivex in a 1.5 mL tube.
3. Buffer AL (400 μL) was added to the tube, stirred, and incubated at 56 °C for 10 min.
4. Ethanol (400 μL) was added to the tube, which was then stirred.
5. Approximately 650 μL (approximately half) of the solution was transferred to the spin column and centrifuged at 6000 × g for 1 min.
6. The solution from the bottom of the collection tube was discarded, and the remainder was transferred to the spin column again, and centrifuged at 6000 × g for 1 min.

Following this, the same procedure was performed as described for the AL method.

For the ATL method using a GF/F glass filter:

1. A glass filter was placed on the top of a Salivette tube, a mixture of 40 μL of proteinase K and 400 μL of Buffer ATL was poured onto the filter paper evenly, and incubated at 56 °C for 30 min in a

thermostatic chamber.
2. The tube was placed in a centrifuge at 3000 × g for 3 min.
3. Buffer ATL (220 μL) was poured onto the filter paper and then centrifuged at 3000 × g for 3 min.
4. The upper part of the Salivette tube containing the filter was removed and discarded. The 650 μL of Buffer AL was added to the top of the tube, which was vortexed for 10 s, and incubated in a thermostatic chamber at 56 °C for 10 min.
5. Ethanol (650 μL; 99.5%) was added and the solution was stirred.

The same procedure was then performed as for the AL method.

The Biryu-Chip (BC) method (Fukuzawa et al., 2022):

1. The water sample was injected using a 10 mL syringe into the BC inlet port and filtered. Using the same syringe, the excess water in the BC was then drained.
2. The tape attached to the BC extract port was removed, and 20 μL of the extraction solvent was injected into the BC. Solution A from the Kaneka Easy DNA Extraction Kit version 2 (Kaneka, Tokyo, Japan) was used as the extraction solvent.
3. Two minutes later, the extraction solvent was drawn from the extract port using a pipette and transferred to a microcentrifuge tube to obtain the extracted DNA sample.

Experiment 1

As a preliminary experiment, a comparison of the three extraction methods was conducted over four months from September to December 2020. First, rainbow trout (*Oncorhynchus mykiss* ) aquarium water was mixed well and divided into 40 mL aliquots in centrifuge tubes, which were frozen and stored as reference samples of eDNA (rainbow-trout-eDNA). Each week, we sampled 400 mL of surface water from the shores of the Sagami River (35.575099° N, 139.308802° E) and Kasumigaura Lake (36.035112° N, 140.257929° E) using a 1 L plastic bottle. The 40 mL rainbow-trout-eDNA was then spiked into the water sample and mixed thoroughly. The mixed sample (100 mL) was filtered using two Sterivex units with a 50 mL syringe and frozen at -20 °C. A second mixed sample (50 mL) was poured into a centrifuge tube and frozen to be used in the BC method. The time from water sampling to freezing was approximately 3 h. Subsequently, all accumulated Sterivex were extracted monthly using the AL and ATL methods. For the BC method, water sampling began in October in the Sagami River. Concurrent with water sampling, another 400 mL of surface water was collected using a 1 L plastic bottle to confirm the presence of rainbow-trout eDNA in the water samples. This non-spiked water sample was filtered using a Sterivex and extracted by the ATL method.

Experiment 2

We continued the sample water collection for approximately one year, starting in January 2021. Water from a second rainbow trout aquarium was dispensed and frozen, as described in Experiment 1. We collected 900 mL of surface water from the same site as that of Experiment 1 and then spiked 40 mL of rainbow-trout-eDNA to the sample. A GF/F glass filter was used for filtration for the AL and ATL methods. For the BC method, filtration and extraction were performed after sampling was completed. As in Experiment 1, the non-spiked water sample was filtered using a GF/F glass filter and extracted using the ATL method.

To confirm the stability of rainbow-trout-eDNA, we spiked 40 mL to the 900 mL of DNA-free pure water every month and extracted it using the ATL method.

DNA measurement with qPCR

Quantification of eDNA from all samples was performed using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Massachusetts, USA). Rainbow trout measurements were performed for all samples. In the summer season (June to September), we also conducted measurements of the inhabited species, sweetfish (*Plecoglossus altivelis* ) in the Sagami River and silver carp (*Hypophthalmichthys molitrix* ) in Kasumigaura.

The DNA yield from the AL method decreased considerably during the experiment. Therefore, we used a specific AL extraction sample to determine whether the decrease in yield was caused by PCR inhibition or extraction inhibition. Specifically, we prepared two samples, one with and one without the extraction sample added to the PCR reagent at a concentration of 10%, and added an equivalent concentration of horse mackerel (*Trachurus japonicus* ) template to each sample for qPCR.

The primer probe sets used are listed in Table 1. Each TaqMan reaction contained 900 nM of each primer (forward and reverse), 125 nM TaqMan-Probe, 0.025 U $\mu L^{-1}$ qPCR master mix (KAPA3G Plant PCR Kit, Kapa Biosystems), and 1.5 µL of the eDNA solution. The final volume for a single PCR assay was increased to 15 µL using distilled water (DW). The volume of eDNA solution added was 10% of the volume of the PCR mixture. The qPCR conditions were as follows: 95 °C for 20 s, followed by 55 cycles of 95 °C for 4 s and 60 °C for 20 s. Three replicates were performed for each sample and no-template control (NTC). Standard curves of qPCR measurements showed $R^2$= 0.992–1.000 and PCR efficiency = 94.3%–106.3%.

Statistical analysis

The data were plotted in a time series graph to confirm the variability of each extraction method and compare the mean value and coefficient of variation (CV; [standard deviation/mean value] × 100). We compared the differences in DNA concentrations among the methods using a paired t-test, multiple comparisons using "t.test", and controlled p-value for multiple comparisons using the Bonferroni method to avoid a Type I error. We performed Spearman's rank correlation for time-series data with the "cor. test" function. We set the significance criterion at a p-value of 0.05. All statistical analyses were performed using R ver. 4.2.0 (R Core Team 2022).

Results

Measurements of rainbow trout during two experiments

Weekly changes in the qPCR results of rainbow trout obtained using the three extraction methods are shown in Fig. 1. Although there was no continuity between Experiment 1 and Experiment 2 because of the different experimental conditions, an overall trend of variation can be observed. The results of the AL method fluctuated greatly even though the samples contained the same amount of eDNA, whereas the ATL and BC methods were stable.

The concentrations of rainbow trout DNA were significantly different among the three methods (paired t-test, $t < -4.565$, $p < 0.0009$). The ATL method yielded more than twice that of the AL method (Table 2), while the CV was approximately half that of the AL method. Despite its simplicity, the BC method was more stable and efficient than the AL method, although it was slightly inferior to the ATL method, which had more procedures. The results of rainbow trout measurements on non-spiked water samples extracted by the ATL method were less than 10% of those of spiked samples in all cases.

Spiked rainbow-trout-eDNA

The spiked rainbow-trout-eDNA showed no significant degradation in concentration during the one-year experiment (Fig. 2, Spearman's rank correlation, rho = -0.132, $p = 0.683$).

eDNA measurements of the inhabiting species

Aquarium water was spiked as a reference sample (rainbow-trout-eDNA). As shown in Fig. 1, the results of the reference sample by the AL method varied greatly; therefore, this behavior was compared to that of the inhabitant species. The results of the AL and ATL methods for sweetfish in the Sagami River and silver carp in Kasumigaura are shown in Fig. 3. A decreased yield obtained by the AL method, comparing to the ATL method, was observed for both fish species, with a similar trend between the reference sample and the inhabitant species. In addition, the ATL method resulted in significantly higher concentrations for both species than those of the AL method ($t < -6.56$, $p < 0.00000898$ for sweetfish; $t < -2.27$, $p < 0.0381$ for silver carp).

The amount of eDNA of the inhabitant species varied, thus the ratio of the copy numbers obtained by AL and ATL methods is shown in Fig. 4, where the denominator is the number of copies obtained by the ATL method, which is stable, and the numerator is that obtained by the AL method. The ratio was not significantly different between rainbow trout and the inhabiting fish DNA ($t = 0.571$, $p = 0.577$ for sweetfish; $t = -1.48$, $p = 0.171$ for silver carp). Thus, the ratio of both inhabiting fish showed the same tendency for spiked rainbow-trout-eDNA.

Distinguishing between PCR inhibition and extraction inhibition in the AL method

The Ct value of the AL method varied significantly. As indicated in Experiment 2, the DNA yield of the AL method at the Sagami River in the second week of July 2021 was approximately 10% that of the ATL method. This indicates the presence of a substance that prevents PCR or DNA extraction. The Ct values of PCR for horse mackerel with and without the DNA extraction samples added to the PCR reagent were 31.1 and 30.8, respectively. Since the addition of the extraction sample resulted in a slightly higher Ct value, we assumed that the lower yield was not a result of PCR contamination with foreign substances.

Discussion

From the results of the stability check of the reference sample (rainbow-trout-eDNA) and the comparison between the reference sample and eDNA of the inhabitant species, it was suggested that the results of Experiments 1 and 2 represent changes in the environmental DNA for three methods. Using this assumption, we evaluated the yields of the AL, ATL, and BC methods based on the results of Experiments 1 and 2.

The AL method showed a strong seasonal variation that greatly exceeded the variability of the reference sample (CV<50%), and in some cases, the yield was less than 10% that of the ATL method. Both the Sagami River and Kasumigaura results showed a tendency for the measured values to decrease substantially during the summer, probably because of the increase in foreign substances generated in this season. In the Sagami River, the water level rose considerably on a day in the first week of July, and a similar increase occurred in Kasumigaura in the second week of August (e.g., https://www.ktr.mlit.go.jp/sagami/en/sagami00668.html, Web site of Sagami River System Management Office), suggesting that the source of foreign substances is likely the discharge. In contrast, the ATL and BC methods had stable measurement results throughout the year.

The ATL method provided close to twice the DNA concentration than that of the AL method, and the CV value was approximately half that of the AL method. This indicates that the ATL method was more stable and reliable. Regarding the yield fluctuation of the AL method, the Sagami River water flows, while the Kasumigaura (the second largest lake in Japan) water is still, and the two water bodies are distant from each other. However, the yield decreased significantly during the summer. Thus, it can be inferred that this phenomenon occurs at many locations.

Djurhuus et al. (2017) reported that there can be differences in extraction yields depending on the filter material used, even with the same extraction method. The Sterivex (filter material PVDF) was used in Experiment 1 and a glass filter was used in Experiment 2 for the AL method, although our results showed similar fluctuations in the yield.

Regarding the newly developed BC method, the results show that the variation is within the same range as that of the reference sample, indicating the possibility of stable measurement using this simpler procedure. The yield was slightly lower than that of the ATL method, but higher than that of the AL method. Therefore, this method may be useful for future social implementation of eDNA technology.

The yield and stability were compared between two extraction methods (protocols) using the DNeasy Blood and Tissue DNA extraction kit, which is commonly used by researchers (Tsuji et al., 2019). However, from these results, the extraction yield and stability varied greatly depending on which reagents (i.e., Buffer AL or ATL) were used, and the results of this experiment suggest that the method using ATL was superior. The BC method has the potential to be an important extraction method for the development of eDNA technology.

In conclusion, we showed that differences in DNA extraction methods may cause differences in yield and stability using long-term evaluation of eDNA measurements with various methods. The AL method caused significant fluctuations in yield, whereas the ATL method could allow for extraction at a high yield. In addition, the BC method could provide a reliable extraction method for the development of eDNA technology. In this study, we tested one river and one lake, so further testing of the methods is needed to confirm their performance in other environments.

Data availability

All the data (Supplementary Table S1) are available at Zenodo (doi:10.5281/zenodo.6618648).

Acknowledgments

This study was supported by the Environment Research and Technology Development Fund (JPMEERF20204004).

Conflict of interest

The commercial affiliations of the authors [TF, HS, NN, HN, YK, and HS] did not alter their adherence to journal policies on sharing data and materials. TF, NN, HN, and HS were employed by the manufacturer of the equipment. However, none of the authors would directly benefit from the publication of this paper.

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Table 1. The qPCR primer-probe sets used in this study

| Target | Sequence | Ref. |
|---|---|---|
| *Oncorhynchus mykiss* (Rainbow trout) | Forward: 5'- AGTCTCTCCCTG-TATATCGTC -3' Reverse: 5'-GATTTAGTTCAT-GAAGTTGCG*A*GAGTA -3' Probe: 5'-[FAM]-CCAACAACTCTTTAACCATC-[NFQ]-[MGB]-3' | The underlined base "A" was modified from (Wilcox et al., 2015) |
| *Plecoglossus altivelis* (Sweetfish) | Forward: 5'-CCTAGTCTCCCTGGCTTTATTCTCT-3' Reverse: 5'-GTAGAATGGCGTAGGCGAAAA-3' Probe:5'-[FAM]-ACTTCACGGCAGCCAACCCCC-[TAMRA]-3' | (Doi et al., 2017) |
| *Hypophthalmichthys molitrix* (Silver carp) | Forward: 5'-GCAATTAACTTCATCACCACAACTATTA-3' Reverse: 5'-TCCAGCAGCTAAAACTGGTAAGG-3' Probe:5'-[FAM]-AAACACCTCTCTTTGTTTGAGCTGTGC-[TAMRA]-3' | Farrington et al. (2015) |
| *Trachurus japonicus* (Horse mackerel) | Forward: 5'-CAGATATCGCAACCGCCTTT-3' Reverse: 5'-CCGATGTGAAGGTAAATGCAAA-3' Probe:5'-[FAM]-TATGCACGCCAACGGCGCCT-[BHQ1]-3' | Yamamoto et al. (2016) |

Table 2. Mean, SD, and CV of rainbow-trout-DNA concentration when extracted by each method from each location sample

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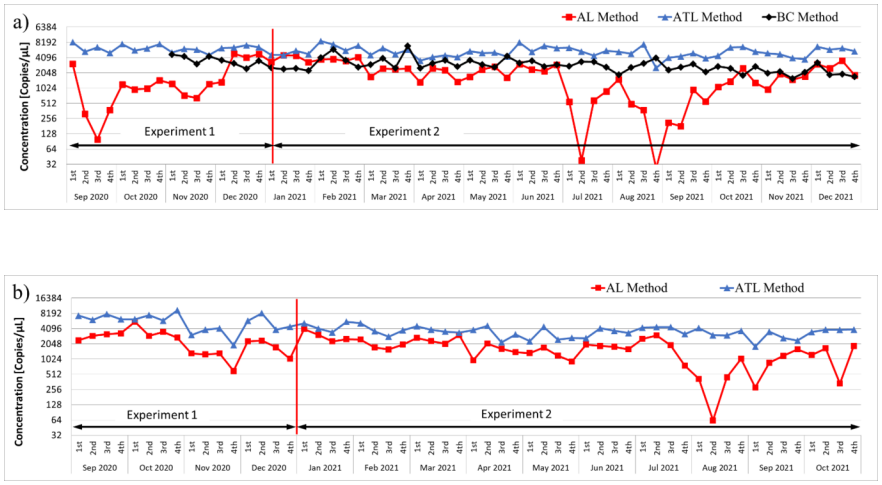


Fig. 1 Trends in rainbow-trout eDNA concentration obtained from three extraction methods for a) the Sagami River and b) Kasumigaura samples. Each point represents the three-replicate average.

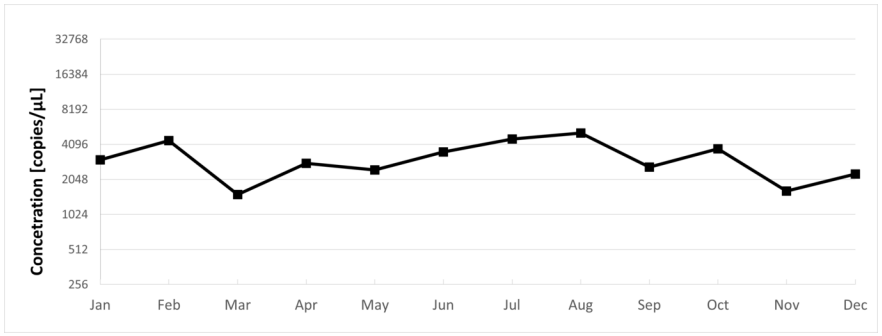


Fig. 2 Time series of spiked rainbow-trout-eDNA concentration. Each point represents the three-replicate average.

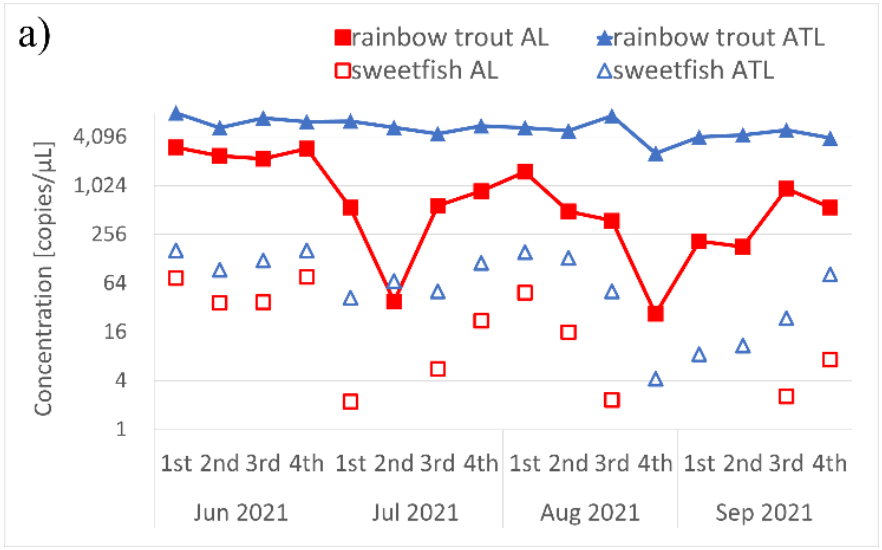

Fig. 3 The qPCR results of the reference sample (rainbow trout) and the inhabiting species. a) sweetfish at Sagami River, b) silver carp at Kasumigaura. Each point represents the three-replicate average.

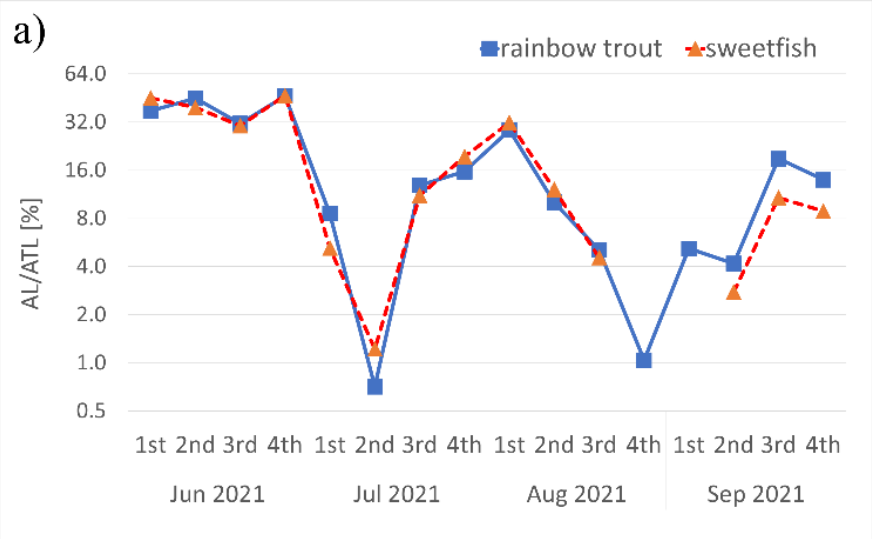


Fig. 4 Comparison of eDNA measurements of rainbow-trout-eDNA and inhabitant species. a) Sagami River, b) Kasumigaura