

Longitudinal and Seasonal Profiles of Concentration of Stream Environmental DNA for Stream Invertebrates in Fresh Water

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Abstract

Environmental DNA (eDNA) is a novel molecular technique to detect presence of target species from a water sample. This paper applies the eDNA approach to invertebrates to reveal longitudinal and seasonal profiles of the eDNA along a river passing through diverse land-uses. We examined a relationship between the target eDNA concentration and wet weight of invertebrates concurrently sampled at three reaches along the Hirose River in Japan. As a result, no significant correlations between the target eDNA and the wet weight was observed. Concentration of extracted eDNA (non-specific) displayed significant difference between the studied localities and significant correlation with water-temperature.

1. Introduction

Anthropogenic impacts such as land-use alteration and climate change have resulted in habitat deterioration of wild animals. Hence, understanding the distribution of wildlife, especially critical or rare species, is required (Hurlbert and Jetz, 2007, Arai *et al.*, 2014). Monitoring of aquatic fauna has been carried out by visual observation and fishing in various manners. However, when assessing on a broader scale or longer term, these conventional methods are time- and effort- consuming. In addition, they require special skills to identify species and involve observer errors or bias.

Environmental DNA (eDNA) is a novel monitoring method to address these problems. The eDNA is a small fragment of DNA originated from animal bodies and metabolites such as skin cells, feces, saliva, urine of animals occupying a water body and has been extracted from water bodies such as lakes, ponds, oceans, or rivers (Rees *et al.*, 2014). By detecting species specific genome sequences in a given water sample using molecular biological methods (e.g. PCR: Polymerase Chain Reaction, Next Generation Sequencing) presence / absence of a target species can be determined with high accuracy. Darling and Mahon (2011) reported that the eDNA

method is more efficient way of evaluating species composition or existence than conventional monitoring methods. The eDNA method is expected to optimize biological monitoring, make monitoring results more objective, and help to reduce cost. Furthermore, Pilliod *et al.* (2013) and Takahara *et al.* (2012) found a positive correlation between biomass and amount of eDNA collected from closed aquatic habitats such as aquariums, lakes, and lagoons. However, only a few attempts have been made to examine this correlation in rivers and oceans because these are more complicated environments, as they are subject to various chemical degradation factors and physical transportation via streamflow. In a freshwater ecosystem, invertebrate species supply energy sources for animals living in the upper-level, and are sensitive to environmental changes, such as climate changes, the modification by man-made structures or deterioration of water quality. For this reason, aquatic invertebrates are often used to evaluate stream health and biodiversity (Heino, 2009)

We aim to quantify temporal variation of eDNA collected from 3 sites along Japanese rivers using qPCR (quantitative Polymerase Chain Reaction, a real-time PCR technique). We first developed a methodology to detect the eDNA and quantify the concentration of the target taxonomic group (i.e. invertebrates). We examined the relationship between the target eDNA concentration and wet weight of invertebrates concurrently sampled at the three reaches. Finally, we assessed relationship between the quantified eDNA concentrations and ambient environmental factors such as land-use and water temperature.

2. METHODOLOGY

2.1 Field survey

Freshwater invertebrate taxa were used for revealing longitudinal and seasonal profiles of eDNA concentration. Most taxa can be found over a large area and the biomass is found in large quantities along the rivers. Therefore, it is best to detect invertebrate eDNA at various locations with different land uses such as forest, agriculture, or urban. Sampling of eDNA was carried out at 3 sites, which are longitudinally located along the Hirose River, with when possible equal intervals (Figure 1), in northeast Japan. Sampling was done monthly from August to December 2015 except St.1 on August. We wore gloves and collected 2L of water from the water surface using sterilized plastic containers. For field survey and experimental procedures, we mainly followed the earlier reports (Ficetola *et al.* 2008, Fukumoto *et al.* 2015). The sampled stream water was transported to the laboratory the day they were collected and kept in a cool box before being filtered using vacuum filtration with 90mm diameter glass-fiber filters (normal pore size, 0.7 μ m; Whatman) and GF/D filter

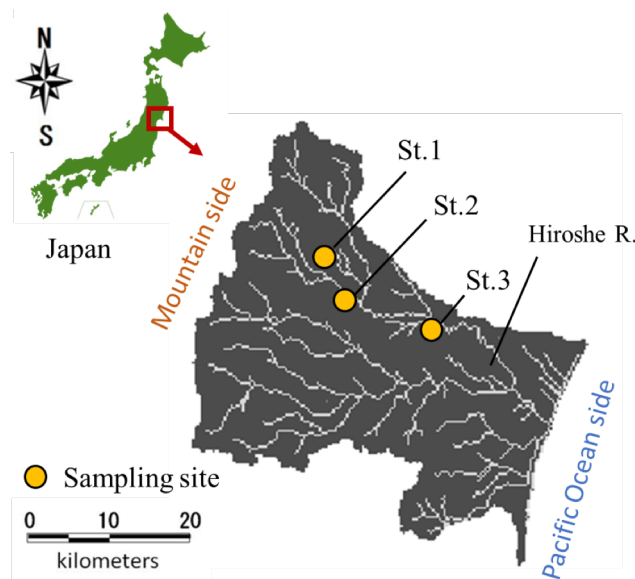


Figure1: Sampling sites in the Natori River basin in northeast Japan

(Whatman, diameter 90mm, normal pore size 2.7 μ m) as a pre-filter. After that, these filter samples were wrapped in commercial aluminium foil and stored in -20°C until DNA extraction. DNA was extracted from filters using the DNeasy Blood & Tissue Kit (QIAGEN) and followed the manufactural protocol. Finally, the concentration of the extracted DNA was measured using PicoGreen (Thermo Fisher Scientific Inc). In addition, stream invertebrates were sampled using a surber-net with 30cm x 30cm quadrat at a fast-flowing habitat (e.g. riffle) and in a pool at the same site (n=2/site). Then, the collected invertebrates were placed in a 99.5% ethanol solution and the wet weight of the samples was measured. Catchable insects were mostly Ephemeroptera, Trichoptera, Plecoptera, Chironomidae, and some Coleoptera. At the same time, environmental conditions, namely water temperature, electric conductivity (EC), pH, turbidity, average water depth, average flow velocity, total phosphorus (TP) were also measured. To calculate average water depth and flow velocity, we measured depth and velocity from 10 points at a cross section of each reach.

2.2 DNA analysis

Mitochondrial DNA (mtDNA) was employed as the target genetic marker because the number of mtDNA in stream water could be larger than that of nuclear DNA per cell so that detection rate increases. We used a universal primer targeting mtDNA cytochrome *c* oxidase subunit 1 (CO1), which is divergent among all animal phyla (Folmer *et al.* 1994, Hebert *et al.* 2005). The target DNA fragments of the samples were amplified using the PCR primers designed to amplify invertebrate-

specific loci from the CO1 region. A 658 bp fragment from CO1 region was amplified with the following primer pair; LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.* 1994). We initially validated the primers on tissue samples for a larva of the caddis fly species (*Stenopsyche marmorata*) which was also sampled in the study catchment.

The qPCR was performed using SYBR®Premix Ex Taq (TaKaRa) and LightCycler®2.0 (Roche). The condition of qPCR consists of an initial incubation at 95°C for 20 seconds followed by 40 cycles of 95°C for 5 seconds, 53°C for 30 seconds, 72°C for 1 minute. Fragment size of amplicons was verified with agarose gels electrophoresis, and qPCR products were purified using a MicroSpin S-400 Columns (GE Life Science). Further, we set a template DNA which was extracted from tissue of *S. marmorata* and amplified using the above- mentioned primer pair. Then we created a dilution series from the PCR products of a standard DNA through 1.0×10^7 to 1.0×10^1 (copies/ μ l).

For more efficient DNA detection, pre-amplification (pre-Amp) process was conducted using a PCR device because water samples might contain only a few target DNA copies. The pre-Amp is used to amplify fewer template DNA in the reaction solution and help to amplify DNA copies easily in subsequent qPCR. It was processed using ABI® Pre-Amplification Master Mix and Thermal Cycler (TaKaRa). PCR conditions consisted of an initial incubation at 95°C for 10 minutes followed by 10 cycles of 95°C for 15 seconds, 60°C for 4 minutes. After the pre-Amp, qPCR was performed by the same process as the initial setting (see above).

3. RESULTS AND DISCUSSION

3.1 Detection of eDNA from stream water samples

An accurate standard curve, which is necessary to determine unknown DNA concentration of samples in the qPCR process, was obtained by amplified standard DNA series from 1.0×10^7 to 1.0×10^3 (copies/ μ l). Where the concentration of the sample DNA was larger than 1.0×10^3 (copies/ μ l), the concentrations were detected because they were within the provided range of standard curve derived from our setting. However, for the samples from St.1 to St.3 the concentrations were not detected as they did not fall within this range. This implied that detecting eDNA from river water requires further accuracy in a range of low concentrations, by building an optimized standard curve or using a more sophisticated technique such as the digital PCR. After the pre-Amp process was performed, a standard curve was expanded from 1.0×10^7 to 1.0×10^2 (copies/ μ l) with better efficiency than the initial qPCR

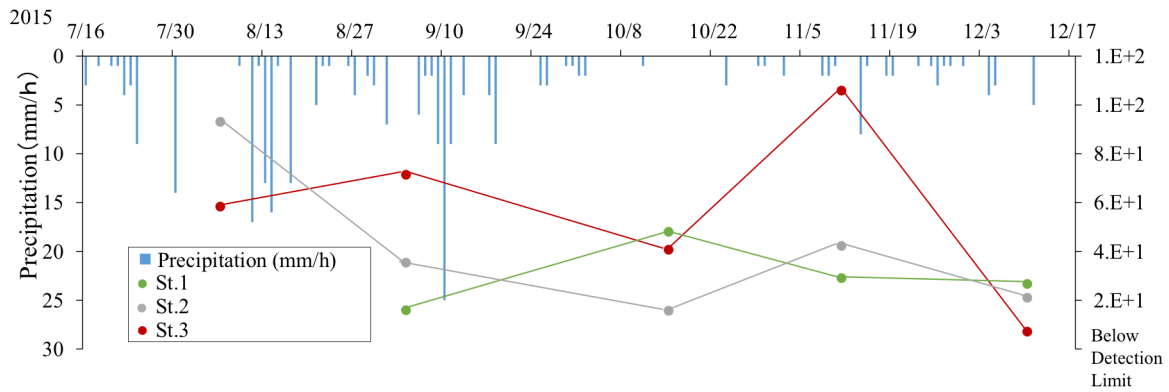


Figure2: Temporal variation in concentration of extracted DNA (ng/L) and precipitation (mm/h)

procedure. The series of extracted DNA concentrations were low (min.: 17.8 ng/L, max.: 264.6 ng/L, mean: 109 ng/L) (Figure 2). Because of these low concentrations of template DNA, the qPCR process may have resulted in low detection rates. However, in a previous paper, which studied eDNA of common carps (Takahara *et al.*, 2012), the concentration of eDNA was approximately 1.0×10^6 (copies/L) in 9L aquarium per carp. Comparing this approach, the eDNA of invertebrates in this study exhibited low- to equivalent concentrations from water samples even though they were taken from open and flowing water. This implies that eDNA released there is from a large number of invertebrate species in the freshwater ecosystems studied.

The series of eDNA concentrations in different seasons were compared with the wet weight of invertebrate sampled concurrently (Figure 3). However, no

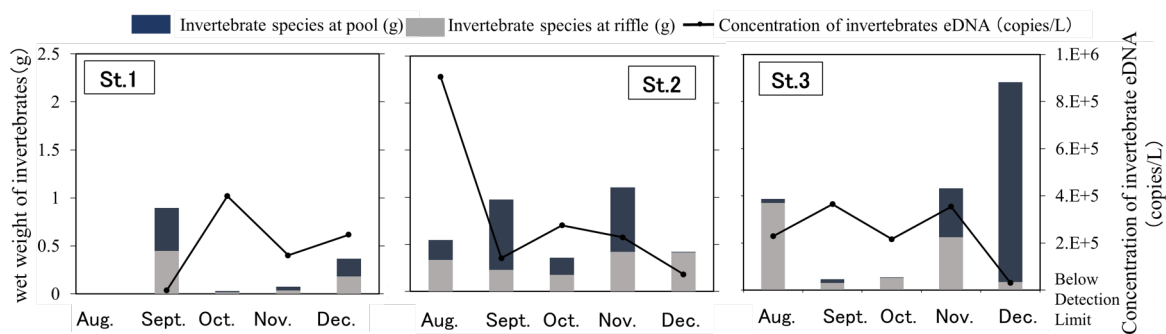


Figure3: The concentration of invertebrates eDNA (copies/L) and the wet weight of stream invertebrates

correlation between the eDNA concentration and the wet weight was observed, while earlier studies have reported positive correlations but in different organisms (Takahara *et al.* 2012, Thomsen *et al.* 2012). This result may be attributed to two

causes. First, amplified eDNA may include other invertebrate species than focused on. The PCR primer is primarily used for detecting DNA of Crustacea, Annelida, or Mollusca (Folmer *et al.*, 1994). Given our sampling of stream invertebrates does not cover whole taxa that are detectable by the primer, sampling method should be improved. Second, eDNA of invertebrates may be transported from upstream of each study site. In the case of freshwater plankton *Daphnia longispina* or bivalve *Unio tumidus*, eDNA can be detected over 10km from their habitat (i. a. dam reservoir) (Deiner and Altermatt, 2014). Accordingly, to obtain positive correlation in streamflow conditions, it might be necessary to consider standing crops upstream.

The wet weight of invertebrates decreased after September. As shown in figure 2, this region experienced heavy rain (Max: 163.5 mm/day) which disturbed river habitat. Through October to December, the wet weight of invertebrates was gradually restored. Comparing with other months, the concentration of invertebrates eDNA in October was not so small even though the sample was taken after the serious disturbances had occurred. It indicates that eDNA observations can reflect invisible or uncachable species such as the first stages of larvae or egg of aquatic insects.

3.2 Relationship between eDNA and environmental variables

In examining correlations between stream eDNA and environmental variables, we focused on the following eDNA measures: concentration of extracted DNA (ng/L), concentration of invertebrates eDNA (copies/L), and ratio of invertebrates eDNA (ng). Environmental conditions are water temperature ($^{\circ}\text{C}$), EC (mS/m), pH, turbidity (NTU), daily mean discharge: Q (m^3/s), average water height: H (cm), average velocity: V (m/s), and total phosphorus (TP) (mg/L). There are significantly positive correlations between the concentration of extracted DNA and water temperature ($R=0.594$, $p<0.05$); the concentration of invertebrates eDNA and water temperature ($R=0.602$, $p<0.05$); and the concentration of invertebrates eDNA and TP ($R=0.743$, $p<0.01$). Figure 4 shows scatter diagrams of these environmental measures. Most metabolite activity of organisms is promoted by water temperature, hence, concentrations of eDNA increased in seasons with high water temperature. Then, analysis of covariance (ANCOVA) was used to examine differences of the 3 eDNA variables (see above) between the study sites. For explanatory variables, water temperature, TP, and average discharge Q are quantitative data, and sampling site is a factor (3 levels). Concentration of the extracted eDNA was significantly correlated with water

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temperature but differed between the studied sites ($p < 0.05$). This indicates that temperature influences the eDNA concentration differently depending on land-use. It indicates that different land characteristics such as land-use, soil properties, flux of insolation might affect eDNA concentration.

4. CONCLUSIONS

The results are summarized as follow:

- 1) Invertebrate eDNA was successfully detected from up-, middle-, down-stream of Hirose River, Japan. Pre -amplification for qPCR could be useful to detect some target DNA fragments which have lower concentration in water samples.
- 2) There are no significant correlation between the target eDNA and the wet weight while earlier studies have reported positive correlations but in different organisms. This may be attributed to additional input of eDNA from upstream.
- 3) From seasonal observation, the range of eDNA concentration of invertebrates were varied in the River. Especially, the amount eDNA remain at a certain level even after the flood disturbance. It indicates that the number of of invertebrate species are not significantly reduced or eradicated by serious disturbances.

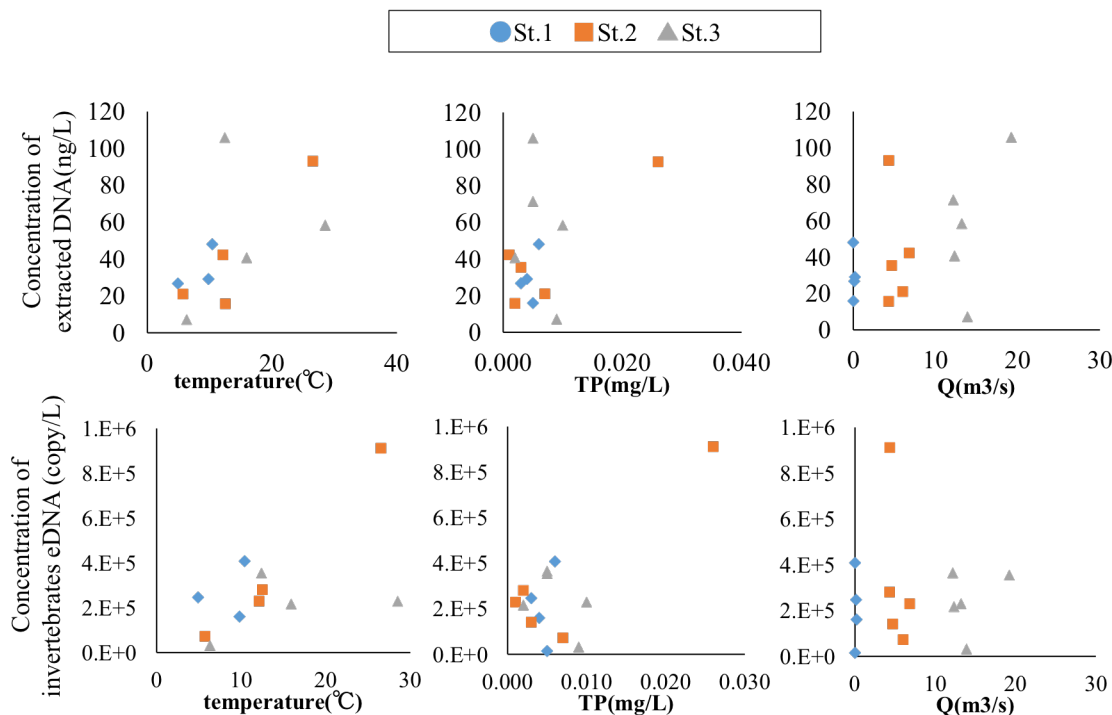


Figure4: Scatter diagrams showing the concentration of extracted DNA and the concentration of invertebrates eDNA and environmental variables

- 4) Concentration of extracted eDNA (non-specific) differed significantly between the studied localities and was significantly correlated with water temperature. This indicates that the eDNA concentration varies between different land-uses and the effects of water temperature varied between landuses.

This study also implies that by combining eDNA and traditional biological monitoring, minimum biomass restored after natural/ manmade disturbance, and recovery powers of organisms will be detectable. Such information would be useful when making decisions about developing rivers and conservation planning of freshwater ecosystems. Further studies should be conducted that covers conventional monitoring of biomass upstream. Also, another promising study to quantify environmental constrains of eDNA is the use of experimental flumes.

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