Aquatic insect community structure revealed by eDNA metabarcoding derives indices for environmental assessment

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Environmental DNA (eDNA) analyses provide an efficient and objective way of monitoring and assessing biodiversity; however, only a few studies have explored the utility of eDNA approach for environmental indices targeting stream insects, which are useful indicators of river health. Here, we evaluated whether eDNA analyses of aquatic insect communities could derive indices that are currently used for environmental assessments. The structure of the aquatic insect community was investigated using eDNA metabarcoding, targeting the Cytochrome Oxidase subunit 1 gene in mitochondrial DNA, and a conventional Surber net sampling method. The surveys were conducted at six reaches located along an upstream to lowland gradient of two rivers in Japan in July and November 2016. eDNA metabarcoding detected 93 families of aquatic insects, which was three-fold more than that detected by the conventional Surber-net sampling method (especially families in Coleoptera, Diptera, and Hemiptera). The mean sensitivity from eDNA metabarcoding against the conventional method was 66.8% in July and 55.3% in November. Community dissimilarity analysis demonstrated that community structures were clustered by each season for both eDNA and conventional methods. Focusing on the Ephemeroptera, Plecoptera, Trichoptera, and Diptera, we found that spatial and seasonal trends in the ratio of sequence reads (eDNA metabarcoding) and individuals (conventional Surber net sampling) were mostly consistent. Our findings suggest that the number of sequence reads derived from eDNA metabarcoding is useful to obtain indices for environmental assessments for coarse spatial scale along rivers (up-, mid- and low land region) based on aquatic insects.

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Abstract

Environmental DNA (eDNA) analyses provide an efficient and objective way of monitoring and assessing biodiversity; however, only a few studies have explored the utility of eDNA approach for environmental indices targeting stream insects, which are useful indicators of river health. Here, we evaluated whether eDNA analyses of aquatic insect communities could derive indices that are currently used for environmental assessments. The structure of the aquatic insect community was investigated using eDNA metabarcoding, targeting the Cytochrome Oxidase subunit 1 gene in mitochondrial DNA, and a conventional Surber net sampling method. The surveys were conducted at six reaches located along an upstream to lowland gradient of two rivers in Japan in July and November 2016. eDNA metabarcoding detected 93 families of aquatic insects, which was three-fold more than that detected by the conventional Surber-net sampling method (especially families in Coleoptera, Diptera, and Hemiptera). The mean sensitivity from eDNA metabarcoding against the conventional method was 66.8% in July and 55.3% in November. Community dissimilarity analysis demonstrated that community structures were clustered by each season for both eDNA and conventional methods. Focusing on the Ephemeroptera, Plecoptera, Trichoptera, and Diptera, we found that spatial and seasonal trends in the ratio of sequence reads (eDNA metabarcoding) and individuals (conventional Surber net sampling) were mostly consistent. Our findings suggest that the number of sequence reads derived from eDNA metabarcoding is useful to obtain indices for environmental assessments for coarse spatial scale along rivers (up-, mid- and low land region) based on aquatic insects.

Introduction

Stream ecosystems are threatened by global anthropogenic impacts, including damming, water abstraction, and land-use changes (WWF, 2016). For sustainable development and resource use of freshwater, the management of stream environments requires effective methods and indicators to measure and assess the environmental impacts. To understand the status of waterbodies, the macroinvertebrate fauna is commonly used as an indicator due to their high sensitivity to deterioration of water quality. Furthermore, aquatic insect community is core of ecological food web in river ecosystem since they are major consumers of first production as well as energy source for higher consumers. Thus, monitoring the aquatic insect fauna is important both in ecological and bio-assessment aspect. However, conventional surveillance based on kick net or Surber net sampling is subject to several issues. Firstly, a field sampling process include errors between observers, e.g., experienced or untrained observers (Zurell et al., 2010). Subsequent processes, sorting and morphological identification, are the most time-consuming parts and requiring a trained person, and also often includes identification error (Haase et al., 2006). In addition, the direct sampling methods cannot avoid to destruct natural habitat and organism's bodies. These difficulties have become a bottleneck for sufficient surveys.

DNA-based approaches are prospected to overcome these difficulties and provides alternative tools for multiple taxa detection and identification (Baird and Hajibabaei, 2012; Hering et al., 2018; Leese et al., 2018). DNA metabarcoding examining bulk or tissue samples has great contribution to reveal taxonomic names with high resolution and small failure even if organisms are difficult to be identified morphologically (Carew et al., 2013; Elbrecht and Leese, 2015; Hajibabaei et al., 2011; Serrana et al., 2018). Hence, DNA-based identification of benthos is widely applied to biomonitoring applications (Aylagas et al., 2014). It is less time consuming because it does not require individual identification. In addition, the analysis skill can be acquired in a short period compared to morphological identification skills. However, several issues (e.g., sampling bias, disturbance of habitat, ethical problem etc.) still remain in the DNA-based approaches using bulk/ tissue samples because the approaches use the same sampling methods and treat the sampled organisms as traditional approaches.

Recently, environmental DNA (eDNA) is being paid attention and becoming more attractive due to its unique advantages (Rees et al., 2014; Smart et al., 2015). eDNA potentially reduces sampling bias due to its simple sampling method (e.g. grab sampling of water, soil, etc.). In addition, it can escape from the issues such as disturbance of habitat and ethics. Although the use of eDNA still remains lots of elusiveness especially in its ecology (e.g., production and degradation rates, transportation in a river (Roussel et al., 2015)), it provides ecological information which cannot be obtained from conventional methods. eDNA metabarcoding can detect taxa as many as or more than traditional methods (Fernández et al., 2018; Macher et al., 2018). Moreover, a community trait revealed by eDNA is corresponding to that revealed by conventional surveys (Deiner et al., 2016; Bista et al., 2017). However, only a few eDNA studies focus on aquatic insect community using metabarcoding while fish or amphibians are intensively investigated. Furthermore, the utility of eDNA towards environmental assessment indices focusing on aquatic insects in stream ecosystem has not been examined.

Here, we investigated the applicability of eDNA metabarcoding to environmental assessment indices based on aquatic insects. We compared the results of eDNA metabarcoding against conventional Surber-net sampling at different locations along two rivers in two seasons. We focused on detection sensitivity, richness of taxa, and relative abundance and evaluated the indices obtained by eDNA metabarcoding.

Materials & Methods

2.1. Aquatic insect sampling by surber net.

Field surveillance were conducted at Hirose River and Natori River, which are located in the Natori river basin, Miyagi Prefecture, northeast Japan. The length of Hirose River channel is 45.2 km and the catchment area is 315.9 km^2. Natori River is 55.0 km long and the catchment area is 623.0 km^2 (not including the Hirose River basin). Sampling was conducted in July and November 2016 at the six reaches from upland- to lowland-domains along the two rivers (site H1–H3 and N1–N3; see Figure 1 and Table S1). These are temperate rivers that originate in the mountains and flow through the hills at the middle reach and through urbanized flatlands at the lower reach, and finally output into the Pacific Ocean. Conventional aquatic insect collection was conducted using a Surber net of 250-µm mesh size, in a 30 cm x 30 cm quadrat at randomly selected a riffle and a pool habitat in river body (collection area in total: 0.18m^2 /reach). Collected invertebrates were placed in a 99.5% ethanol solution and morphologically identification using a stereomicroscope (Leica MZ APO, Leica, Germany) by referring to the identification key for the aquatic insects of Japan (Kawai and Tanida, 2018). Because morphological identification was difficult for some aquatic insects, particularly Chironomidae and some Baetidae individuals, population abundance and richness were summarized at family level.

2.2. eDNA sampling, filtration, and DNA extraction

Water samples for eDNA analysis were collected at the same sites and on the same days but before the conventional collection method. The plastic bottles for eDNA sampling were sterilized with 10% chlorine bleach (Kao corporation, Tokyo, Japan) and rinsed with tap water in the laboratory, and subsequently washed three times with river water at the collection site before sampling. At each site, 1 L of flowing surface water were collected (Mächler et al., 2016) and transported to the laboratory on ice in a cool box. Water samples were filtered on that day using vacuum filtration with 47-mm diameter glass-fiber filters with 0.7-µm pore size (GF/F, Whatman, 1 L/filter). These filtered samples were stored at −20°C until DNA extraction. DNA was extracted from the filters through lysis using proteinase K at 56°C for 30 min. After incubation, the supernatant was subjected to phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. Eventually, the elution was purified using the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA) with a final volume of 100 µl.

120 **2.3. Library preparation and sequencing**
121 Target regions (cytochrome oxidase subunit 1 gene (CO1) in mitochondrial DNA) of extracted
122 DNA were amplified using the universal primer for invertebrates developed by Folmer et al
123 (Folmer et al., 1994). The primer set of LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA
124 TTG G-3′) as forward primer and HC02198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT
125 CA-3′) as reverse primer, resulting in an amplification of a 658-bp fragment. For MiSeq library
126 preparation, a three step PCR was conducted. The first PCR was performed in a total volume 20
127 µl PCR mixture containing 10 µl of Taq™ HS Low DNA (TaKaRa, Kyoto, Japan), 0.4 µl each
128 of 10 µM forward and reverse primers, 17.2 µl ultra-pure water, and 2.0 µl of template DNA.
129 The PCR conditions were as follows: 35 cycles at 94°C for 5 s, 50°C for 5 s, 68°C for 10 s; and a
130 final extension at 68°C for 7 min. The fragment size of amplicons and concentrations were
131 verified by electrophoresis using the Agilent 2100 Bioanalyzer DNA7500 kit (Agilent, Santa
132 Clara, CA, USA). PCR products were purified using the Agencourt AMPure XP (Beckman
133 Coulter, Brea, CA, USA) and the purified products were used as template for the following. The
134 second PCR was performed to add the overhang sequences that required amplification with the
135 Nextera XT Index Kit v2 for Illumina MiSeq analysis using Ex Taq Hot Start Version (TaKaRa,
136 Kyoto, Japan). The PCR conditions were as follows: 94°C for 2 min; followed by 5 cycles of
137 94°C for 30 s, 50°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min. The
138 amplicons were electrophoresis verified and purified in the same way as those from the first
139 PCR, and the purified products were used as templates for the following. The third PCR was
140 performed using Ex Taq Hot Start Version and Nextera XT Indice Kit v2 set A (Illumina, San
141 Diego, CA, USA). The PCR conditions were follows: 94°C for 2 min; followed by 8 cycles of
142 94°C for 30 s, 50°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min. After
143 purification by AMPure XP and verification by BioAnalyzer, the final PCR amplicons were
144 quantified using the Qubit dsDNA High Sensitivity Kit. The sequencing of prepared libraries
145 was performed following the manufacturer's instructions for MiSeq.
146
147 **2.5. Bioinformatics**
148 The sequence lengths were 658 bp; therefore, the forward and reverse reads in our study could
149 not be merged while MiSeq Reagent Kit v3 (600 cycles) were adopted. Elbrecht and Leese
150 (Elbrecht and Leese, 2017) demonstrated that invertebrate species could be identified at the
151 reverse side of the CO1 region through an *in silico* PCR. Therefore, we conducted subsequent
152 analysis using the reverse side sequence. At first, raw sequence reads were subjected to the
153 Trimmomatic v0.36 software to discard low-quality sequences and read sequence lengths of
154 <150 bp. Filtered reads were clustered into operational taxonomic units (OTUs) with an identity
155 cut-off value of 97% which is common approach for invertebrate metabarcoding analyses
156 (Macher et al., 2018) using QIIME (Caporaso et al., 2010); subsequently, OTUs with singleton
157 sequences were removed. The most frequently occurring sequences in each OTU were extracted
158 as representative sequences. Taxonomic identification was performed by BLAST search using
159 the QIIME script "assign_taxonomy.py" with minimum percent identity of 85% (See Text S1)

and maximum e-value of 10^{-50} (Fernández et al., 2018). The assignment was performed against 3,433,026 sequences retrieved from the NCBI database by the following search criteria: cytochrome [all fields] AND oxidase [all fields] AND mitochondrion [filter]. After assignment, eight orders, namely Ephemeroptera, Plecoptera, Trichoptera, Diptera, Coleoptera, Odonata, Megaloptera, and Hemiptera, which mostly include aquatic insect species, were extracted using the QIIME script "filter_taxonomy_from_table.py." Subsequently, representative sequences of extracted OTUs were subjected to chimera check. To avoid unequal diversity comparison due to the differences of sequence depth among samples, either 250 or 2,500 reads were picked randomly because the smallest and the second smallest numbers of sequence reads were 290 (N3_November) and 2,610 (H1_Nov sample), respectively. Good's coverages were calculated based on OTUs to know what percent of the total taxa is represented in a sample using the QIIME script "alpha_diverisity.py". A flow of bioinformatics analysis is shown in Figure S1.

2.6. Community structure analysis

Binary classification was conducted to measure the sensitivity and positive predictive value in the detection. The presence/absence of taxa obtained from the surber net sampling method were used as condition positive/negative, and the presence/absence of taxa obtained from the eDNA metabarcoding were used as test outcome positive/negative (Text S2).

Shannon's diversity index was used to represent abundance and evenness of species in a community (α-diversity). The Chao-1 estimator was used to show community dissimilarities among samples (β-diversity) since the Chao estimator provides robust results when handling the samples containing rare occurrence species (Chao and Chiu, 2016; Olds et al., 2016). These diversity indices were calculated based on abundance at family level (R ver. 3.4.0, package"vegan" (Friendly et al., 2018)). As abundance data, the number of individuals for the samples obtained from the surber net sampling and the number of read counts for samples obtained from eDNA metabarcoding were used, respectively. Using the Chao dissimilarity measure, non-metric multidimensional scaling (nMDS) was performed to show the similarity in community structures between eDNA and conventional methods (R ver. 3.4.0, package library "MASS" and library "labdsv"). A phylogenetic tree was constructed using the eDNA sequences in the ARB software (Ludwig, 2004) and UniFrac distances were measured from the tree. Principal coordinate analysis (PCoA) was conducted to identify factors explaining differences among samples with and without considering the number of read counts of each sequence (weighted or unweighted analysis) using QIIME.

2.7. Environmental assessment indices

To examine the consistency with the conventional Surber-net sampling, biological information obtained from environmental DNA was applied to existing environmental assessment indicators. One of the indicators is the average score per taxon method (ASPT) which use presence/absence data of taxa identified at family level. The ASPT was developed in 2016 by Japanese Ministry of the Environment (Ministry of the Environment Government of Japan, 2017) in accordance with

the biota in Japan based on the BMWP (Biological Monitoring Working Party) score method developed in 1976 by UK Ministry of the Environment. These score methods are used to assess water quality. The scores assigned to each taxon are shown in the Table S2.
Another indicator is EPT indices or Diptera indices. For samples obtained using the conventional surber net sampling method, the EPT index (Net %EPT) was calculated using the relative abundance or the richness at family level of Ephemeroptera, Plecoptera, and Trichoptera, i.e., the ratio of the number of individuals/taxonomic-richness of EPT taxa to the total number of individuals/taxonomic-richness of the eight orders observed in the sample (Ephemeroptera, Plecoptera, Trichoptera, Diptera, Coleoptera, Odonata, Megaloptera, and Hemiptera). The Diptera index (Net %Diptera) uses the same method as the EPT index, but using Diptera instead of Ephemeroptera, Plecoptera, and Trichoptera. For eDNA-analyzed samples, EPT and Diptera indices (eDNA %EPT and eDNA %Diptera) were calculated using the number of reads, OTU richness, and assigned family richness. Here, the OTU richness refers to the number of OTUs included in the sample, and the assigned family richness refers to the number of families included in the sample. Same as the net sampling method, eDNA %EPT and eDNA %Diptera were the ratio of the number of reads/OTU-richness/assigned-family-richness of the eight aquatic insect orders (See Text S3 for formula).

Results

3.1. Community structure of aquatic insects revealed by eDNA analysis

Overall, 1,235,176 sequences (50,728–168,413 sequences/sample) passed through the sequence quality filter (Table 1). These sequences were used to create OTUs (Operational Taxonomic Unit) based on 97% sequence identity. As a result, 90,948 OTUs were formed. Out of these, 66,175 OTUs included just one sequence (singletons), which were excluded from the analysis. Therefore, a total of 1,169,000 sequences (47,443–161,461 sequences/sample), generating 24,773 OTUs, were analyzed.
After a BLAST search (at the threshold of a minimum identity of 85% and e-value of 1.0E-50 against the database that we constructed), we found that only 8.1% of the total sequences was assigned to aquatic insect taxa (Table 1 and Figure S2). The aquatic insects identified from all 12 eDNA samples were 93 families, including eight Ephemeroptera families, four Plecoptera families, 15 Trichoptera families, three Odonata families, one Megaloptera family, 13 Hemiptera families, nine Coleoptera families, and 40 Diptera families. The community structure varied for each sample (Table S3). The mean number of assigned families was mostly the same in both seasons (mean ± S.D.:35.2 ± 6.6 taxa in July and 36.7 ± 13.2 taxa in November); however, the mean of the Shannon's diversity index was slightly higher in July (1.48 ± 0.39 taxa in July and 0.93 ± 0.48 taxa in November). The common taxa found in both seasons included 11 families found at more than five locations. Eighteen and 21 families were only detected in July and November, respectively. Seasonal taxa in July included five Trichoptera and five Hemiptera families. Seasonal taxa in November included nine Diptera families.

Thirty-five aquatic insect families were collected by the conventional Surber net sampling method. Of these, 30 families were common with eDNA detections (Table S4A and S4B). Five families were not detected in any eDNA samples, but were found with net-sampling, including Isonychiidae (Ephemeroptera), Hydrophilidae (Coleoptera), Ptilodactylidae (Coleoptera), Psephenidae (Coleoptera), and Blephariceridae (Diptera).

With respect to read counts for each taxon, the number of reads in all subsampled reads showed that Chironomidae had the largest number of sequence reads in each sample, except for July H1 (Figure 2). The top five families in the number of reads in July were Chironomidae, Simuliidae, Drosophilidae, Baetidae, and Heptageniidae, accounting for 80% of reads in all subsampled reads. The top five taxa differed between July and November, with these being Chironomidae, Simuliidae, Ephemerellidae, Baetidae, and Empididae in November, and also accounted for 85% of taxa. The families that were detected in many eDNA samples were also observed at high frequency by the Surber net sampling, regardless of season. These families included Baetidae and Ephemereliidae (order: Ephemeroptera); Stenopsychidae (Trichoptera); and Simulidae, Chironomidae, Tabanidae, and Tipulidae (Diptera).

3.2. Comparing presence/ absence of taxa between two methods

We compared presence/ absence of taxa between two methods using sensitivity (S, %) and positive predictive value (PPV, %). For subsampled 250 reads analysis, the mean sensitivity of eDNA against net-sampling at six locations was higher in July than the mean positive predictive value (mean ± S.D.: S; 53.0 ± 9.9% > PPV; 35.2 ± 8.9%) but reversed in November (S; 34.7 ± 15.5% < PPV; 38.0 ± 9.2%). In contrast, for subsampled 2,500 reads analyses, the mean sensitivity was higher than the mean positive predictive values both in July (S; 65.5 ± 11.0% > PPV; 26.6 ± 5.2%) and November (S; 51.9 ± 21.4% > PPV; 25.4 ± 2.9%). Therefore, eDNA was mostly able to detect taxa by detected by Surber-net sampling but it might be attributed to a greater number of subsampling read counts, with broader coverage resulting in a higher rate of sensitivity (mean of Good's coverage = 83.6 ± 4.6% for 250-read subsamples and 96.4 ± 1.7% for 2,500-read subsamples; Table 1).

3.3. Community structure analysis

The Shannon diversity index was 0.80–1.82 based on the eDNA analysis at the family level, and was 1.39–2.43 for individual counts based on the conventional method (Table 1). Because the dominant taxa from the eDNA analyzed samples had the great number of reads, the evenness of taxa at the community level was reduced, resulting in a lower Shannon diversity index from eDNA analysis. In addition, there was a significantly positive correlation between Shannon diversity indices obtained by eDNA metabarcoding and by the conventional method, supporting that samples with a greater Shannon diversity in the Surber net sampling approach had greater diversity in the eDNA analysis (Spearman's rank correlation coefficient $\rho = 0.66$, $p = 0.02$, see Figure S3).

Community dissimilarities among all samples using the Chao index as β-diversity were plotted on nMDS coordinate axes (Figure 3). Visually, the eDNA data showed that plots were separately distributed according to seasonal differences. The occurrence of specific families in July and November possibly made clusters be separated. In addition, the uppermost site of the Hirose River (H1) was clearly isolated from other sites. This is acceptable because the catchment area of site H1 has been covered only by forested area and absence of human impacts. Moreover, the habitat type of H1 was classified as plane-bed reach but the others were as pool-riffle reaches. Therefore, the species appearance is largely different from other sites. UniFrac analysis was carried out based on patterns from the 250 reads (Figure 3) and 2,500 reads (Figure S4) but there was no difference in trends between the 250- and 2,500-reads. Using both weighted and unweighted approaches, clusters were divided by season and same as the results obtained from the Chao estimator of dissimilarity. In summary, the community dissimilarity relationships were not modified markedly if we used assigned taxa data (Chao index), presence/absence (unweighted UniFrac) data or relative read counts (weighted UniFrac) data.

3.4. Environmental assessment indices

ASPT by eDNA indicated always lower than conventional net sampling (Figure S5, mean ± S.D. of ASPT by Surber-net; 7.76 ± 0.20, eDNA; 6.95 ± 0.51). For eDNA, the scores from Plecoptera and Coleoptera were smaller than Surber-net sampling because of its lower detectability of Plecoptera and aquatic Coleoptera. As a result, most of ASPT calculated by the conventional sampling method indicated “very good” water quality (ASPT >7.5) but ASPT calculated by eDNA indicated “good” water quality (7> ASPT >=6.0). In addition, the spatial variation was consistent only in Natori river in July. Since the present ASPT assessment is constructed by aquatic invertebrate list, it does not match the taxonomic list from eDNA which includes terrestrial taxa and led different water quality evaluation.

Most %EPT indices derived by the three metrics (i.e. relative read counts, richness, and OTU richness) from eDNA were lower than the %EPT indices derived from Surber-net sampling. Most %Diptera derived from all eDNA metrics were higher than %Diptera derived from Surber-net sampling (Figure 4). This difference was obtained because, out of all aquatic insect taxa detected by eDNA, the read count and taxonomic richness for Diptera were consistently higher than those of the other taxonomic groups. These trends were similar, regardless of the subsampled read numbers, i.e., 250- versus 2500- reads (Figure S6).

The trend in %EPT from Surber-net for the abundance metrics was consistent with the trends of %EPT from eDNA based on read counts metrics along river locations in both seasons. This trend was the same for %Diptera. The richness-based %EPT from Surber-net was consistent with richness-based %EPT from eDNA, except in November N1-N3. However, these results were not consistent with OTU richness-based %EPT from eDNA. Richness-based %Diptera from Surber-net was not consistent with richness-based or OTU richness-based %Diptera from eDNA, except for November N1-N3. Thus, bio-assessment indices derived from relative population abundance

of EPT or Diptera using Surber-net sampling showed a similar trend along stream location with indices derived from the relative read counts of eDNA metabarcoding.

Discussion

Long-term and large-scale monitoring is required to evaluate the impacts of anthropogenic activities and climate change, and transitions in monitoring methods that use molecular-based approaches are expected (Bush et al., 2019; Hering et al., 2018; Leese et al., 2018). In particular, there is interest in how biological information can be obtained using eDNA, which does not require the collection of whole organisms for analysis; and thus, eDNA analysis has been tested in various environments, including oceans, rivers, and terrestrial habitats (Cristescu and Hebert, 2018; Deiner et al., 2017).

4.1. Aquatic insect taxa detected by eDNA analysis from rivers

We detected taxa that are mostly distributed in riparian/terrestrial habitats (e.g., Hemiptera, Diptera (e.g., Culicidae) and Coleoptera (e.g., Staphylinidae)), as well as lentic habitats (e.g., Aeshnidae and Epiophlebiidae in Odonata). Thus, our eDNA outputs provided larger taxa richness with three-fold more taxa and higher sensitivity of taxa detection than the conventional survey, as similar with the earlier report of eDNA from river systems (Macher et al., 2018). eDNA metabarcoding detect more taxa than sampling through conventional benthos capturing surveys (Macher et al., 2018) in contrast with pond systems where transportation is very small (Hajibabaei et al., 2019). This is because DNA is transported to downstream, with eDNA metabarcoding can result in the additional detection of upstream community, which is not sampled by conventional methods. In addition to the detection of aquatic insect in main streams, eDNA also can detect taxa that usually difficult to capture through Surber-net sampling in lotic locations (Deiner et al., 2016). In summary, eDNA sampled from river ecosystem have different traits from the conventional Surber-net sampling.

To understand what community eDNA describes in rivers, we tried to figure out how much spatial range eDNA covers with referring earlier reports about ecology of eDNA. eDNA is decomposed and transported after release from organisms, with eDNA concentrations decreasing by 73% within 900 m of flowing downstream of a source (Nukazawa et al., 2018). Even 50–250 m downstream of the source, eDNA is not detected when target organisms' abundance or biomass is small (Jane et al., 2015; Pilliod et al., 2014). Thus, DNA sampled in rivers probably includes some DNA of large abundant organisms inhabiting up to around 1 km upstream. Adopting to our study, our samples may include negligible contamination of eDNA between samples, due to 3-5 km sampling site intervals.

4.2. Difficulties in eDNA metabarcoding analysis

The sensitivities of eDNA detection against the Surber-net sampling were mostly larger than positive prediction value, but the sensitivities themselves were not large (17.6-71.4%, Table 2). It

was possibly due to various difficulties in eDNA metabarcoding data analysis. One of the difficulties is insufficient reference libraries for the local aquatic insects' community. Even for two samples that were registered as belonging to the same species in the NCBI database, the sequence identity was markedly reduced when they were collected from geographically distant regions. For example, the identity of the CO1 gene of *Drusus discolor* (Trichoptera: Limnephilidae) collected from Montenegro and Germany is only 51.7% (Text S1). Second issue is the threshold for taxonomic identification. Considering the discrepancy between the reference library and the query sequence, we investigated interspecific, intergeneric, and interfamilial genetic identity (Text S1). As a result, 85% identity threshold was employed for taxonomic assignment in this study. However, some species might not even reach this threshold and went undetected. Therefore, the accumulation of genetic information of local aquatic insects and the construction of a database is necessary to improve the assignment ratio of sequence data obtained from eDNA metabarcording. Third, a primer mismatches at the 3' end, resulting in failed PCR primer binding. We found that the five families (i.e. Isonychiidae, Hydrophilidae, Ptilodactylidae, Psephenidae and Blephariceridae) not detected by our eDNA metabarcoding, had mismatches to the primers used in this study. Therefore, to analyze these five families, the primer should be modified or a new primer developed. Some refined primer sets for the metabarcoding of aquatic invertebrates have been developed (Elbrecht and Leese, 2017; Hajibabaei et al., 2012). The new primers success to reduce amplification bias between taxa compared to the primers used in the current study (Elbrecht and Leese, 2017) and led more aquatic insect taxa detection; thus, these primers could be used to improve the ability to measure taxa richness (alpha diversity). By these above reasons, the current study could not detect some specific taxa. Missing the detection of some specific taxonomic groups possibly affected the assessment results based on taxa richness directly.

4.3. Applicability of eDNA towards environmental assessments

For EPT or Diptera indices, we observed that the spatial trend of indices was more similar to that of the conventional method when these indices were based on relative abundance metrics, rather than taxa (p/a) metrics (Fig. 4). There are critical issues to use read counts derived by metabarcoding analysis. It is mainly due to PCR amplification bias with universal primers (Elbrecht and Leese, 2015). Therefore, relative read counts are regarded not to reproduce the original relative abundance straightforward. In case of our study, popular taxa revealed by Surber net sampling were also detected by eDNA metabarcoding. The popular taxa included that Chironomidae, Simuliidae, Epheremerellidae, Baetidae, and Heptageniidae (see Table S5). Additionally, we obtained large number of read counts of these taxa by metabarcoding (Figure. 2). Thus, taxa with large number of reads by metabarcoding were consistent with taxa with large population detected by the conventional sampling. As numerous studies indicated, eDNA concentration or eDNA detection success rate is higher when the source organism is abundant, and spatially close (Doi et al., 2017; Pilliod et al., 2013) or temporally close (Thomsen et al., 2012) to source organisms. Not many but a few previous eDNA metabarcoding studies reported

that the relative abundance of individuals or biomass was correlated with the relative read abundance derived from metabarcoding for mesocosms (Evans et al., 2016) and deep seawater environments (Thomsen et al., 2016). We have to care to analyze using read counts data, but according to our observation results, the read counts may provide information of popular taxa as well as the conventional Surber net sampling, even though eDNA metabarcoding cannot completely represent the original community composition. Thus, environmental assessment indices based on abundant taxa i.e. EPT or Diptera indices were able to indicate similar spatial variation among eDNA metabarcoding and the conventional Surber net sampling.

Conclusions

We compared aquatic insect communities revealed by the conventional Surber-net sampling and eDNA metabarcoding analysis. eDNA revealed community variations between seasons. Furthermore, taxa which is highly relative abundant based on individuals were likely to show highly relative abundance based on read counts. Consequently, environmental assessment indices using relative abundance such as EPT indices or Diptera indices indicated same spatial variations between two methods. Even though the use of read counts data obtained from metabarcoding should be carefully concerned, our findings suggest that read count data derived from eDNA metabarcoding analyses are able to use for the calculation of environmental assessment indices.

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Table 1(on next page)

Sampling results from surbernet-collection and eDNA analysis outputs.

Sampled month	July						November					
sampling site	H1	H2	H3	N1	N2	N3	H1	H2	H3	N1	N2	N3
Surber net sampling												
sample size (individuals)	16	170	311	317	230	143	100	929	529	275	457	379
Identified family	8	16	13	17	18	14	17	19	16	17	14	18
Shannon's diversity	1.93	2.37	1.91	2.05	2.37	2.29	2.43	1.93	1.89	2.20	1.77	1.37
Metabercoding Reads												
Raw	58,130	124,870	148,614	60,913	54,330	62,916	168,136	171,748	149,205	159,015	51,939	54,613
Filtered	56,697	122,384	145,593	59,396	52,681	61,479	163,677	168,413	145,639	155,025	50,728	53,464
Removed Singletons	53,803	116,649	136,700	56,121	50,001	58,273	153,815	161,461	137,835	146,232	47,443	50,667
Assigned as aquatic insect.	4,616	11,936	6,232	5,672	8,929	4,423	5,344	31,740	5,742	38,169	7,715	399
Chimera Removed	3,231	7,654	4,847	3,643	6,905	2,914	2,619	28,952	4,518	31,275	4,988	290
A. Insect /Total	6.0%	6.6%	3.5%	6.5%	13.8%	5.0%	1.7%	17.9%	3.3%	21.4%	10.5%	0.6%
Metabercoding taxonomy assigned as aquatic insect												
Clustered OTUs	106	307	381	283	286	181	177	762	349	811	360	63
good's coverage (250)	88.8%	87.6%	76.0%	76.4%	86.0%	86.8%	83.6%	84.8%	77.6%	82.0%	86.4%	87.2%
good's coverage (2500)	99.1%	96.3%	95.5%	97.6%	96.7%	97.7%	93.9%	94.6%	98.6%	94.9%	95.2%	-
Families	31	32	44	39	39	26	42	51	33	45	36	13
Shannon's diversity	2.17	2.04	1.75	1.99	2.03	1.29	2.29	0.83	1.69	1.09	0.98	0.89

1

Table 2(on next page)

Sensitivity (S, %) and positive predictive value (PPV, %) by eDNA and Net sampling of each samples.

	250 reads				2,500 reads			
season	July		November		July		November	
sites	S (%)	PPV (%)	S (%)	PPV (%)	S (%)	PPV (%)	S (%)	PPV (%)
H1	71.4	20.8	57.1	36.4	85.7	19.4	78.6	26.8
H2	50.0	42.1	22.2	36.4	56.3	31.0	27.8	21.7
H3	53.8	38.9	40.0	40.0	69.2	23.1	46.7	23.3
N1	46.7	29.2	25.0	33.3	60.0	23.7	37.5	26.1
N2	52.9	45.0	46.2	54.5	64.7	31.4	69.2	29.0
N3	42.9	35.3	17.6	27.3	57.1	30.8	ND	ND
mean	53.0	35.2	34.7	38.0	65.5	26.6	51.9	25.4
SD	9.9	8.9	15.5	9.2	11.0	5.2	21.4	2.9

1

Figure 1

Study field

Sampling sites in Hirose River (from upmost site, H1, H2, H3, in orange circle) and in Natori River (N1, N2, N3, in blue circle) in the northeast part of Japan.

This map was modified by Uchida using a digital map provided by Geospatial Information Authority of Japan.

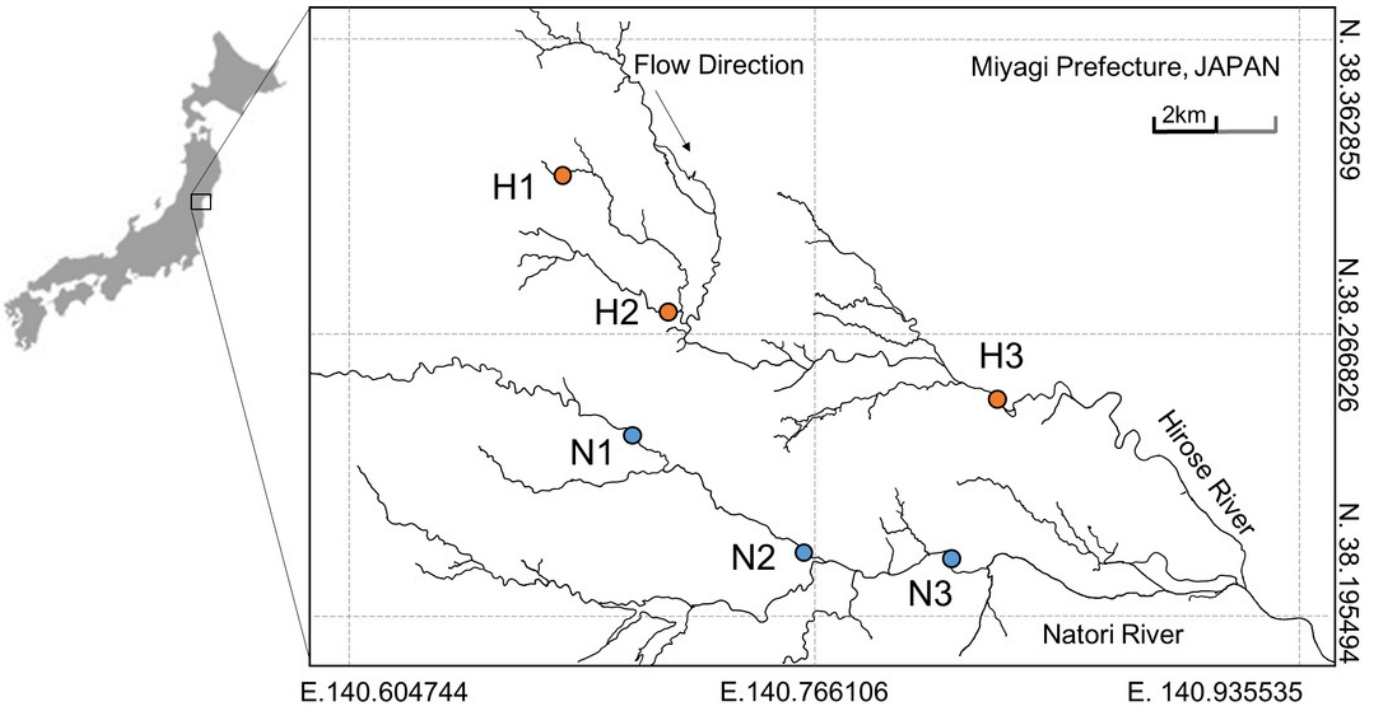

Figure 2

Component of aquatic insect community by eDNA, subsampled 250 reads in metabarcoding

Represent top 20 taxa for read counts through (A) sample series in July, (B) sample series in November. Graph legends are shown with “taxonomic order: family name (relative abundance (%) through 6 samples).” Taxonomic order used the following abbreviations: Ephemeroptera (E), Plecoptera (P), Trichoptera (T), Diptera (D), Coleoptera (C), Hemiptera (H).

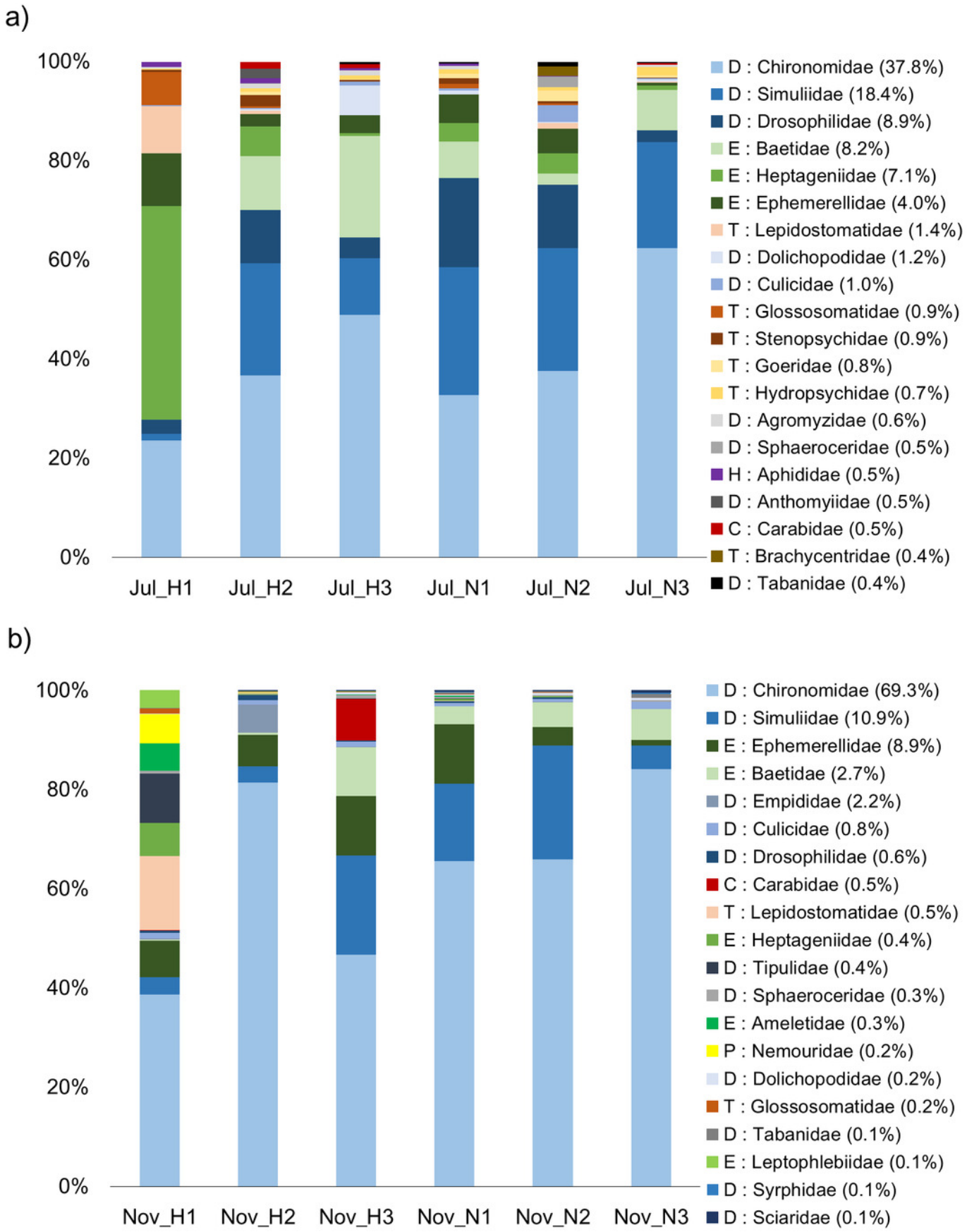
a)
100%
80%
60%
40%
20%
0%
Jul_H1
Jul_H2
Jul_H3
Jul_N1
Jul_N2
Jul_N3
D : Chironomidae (37.8%)
D : Simuliidae (18.4%)
D : Drosophilidae (8.9%)
E : Baetidae (8.2%)
E : Heptageniidae (7.1%)
E : Ephemerellidae (4.0%)
T : Lepidostomatidae (1.4%)
D : Dolichopodidae (1.2%)
D : Culicidae (1.0%)
T : Glossosomatidae (0.9%)
T : Stenopsychidae (0.9%)
T : Goeridae (0.8%)
T : Hydropsychidae (0.7%)
D : Agromyzidae (0.6%)
D : Sphaeroceridae (0.5%)
H : Aphididae (0.5%)
D : Anthomyiidae (0.5%)
C : Carabidae (0.5%)
T : Brachycentridae (0.4%)
D : Tabanidae (0.4%)
b)
100%
80%
60%
40%
20%
0%
Nov_H1
Nov_H2
Nov_H3
Nov_N1
Nov_N2
Nov_N3
D : Chironomidae (69.3%)
D : Simuliidae (10.9%)
E : Ephemerellidae (8.9%)
E : Baetidae (2.7%)
D : Empididae (2.2%)
D : Culicidae (0.8%)
D : Drosophilidae (0.6%)
C : Carabidae (0.5%)
T : Lepidostomatidae (0.5%)
E : Heptageniidae (0.4%)
D : Tipulidae (0.4%)
D : Sphaeroceridae (0.3%)
E : Ameletidae (0.3%)
P : Nemouridae (0.2%)
D : Dolichopodidae (0.2%)
T : Glossosomatidae (0.2%)
D : Tabanidae (0.1%)
E : Leptophlebiidae (0.1%)
D : Syrphidae (0.1%)
D : Sciaridae (0.1%)

Figure 3

Community dissimilarity plots.

Closed circles represent Hirose river samples (H1-H3) and open rhombuses represent Natori river samples (N1-N3). Orange characters show July samples and blue characters show November samples. a) Chao dissimilarity for surber net sampled community, b) Chao dissimilarity for eDNA community, c) unweighted Unifrac analysis with 250 reads, d) weighted Unifrac analysis with 250 reads. For panels c) -d), x axis showed PC1 (17.4% for variation explain) and y axis showed PC2 (15.8%).

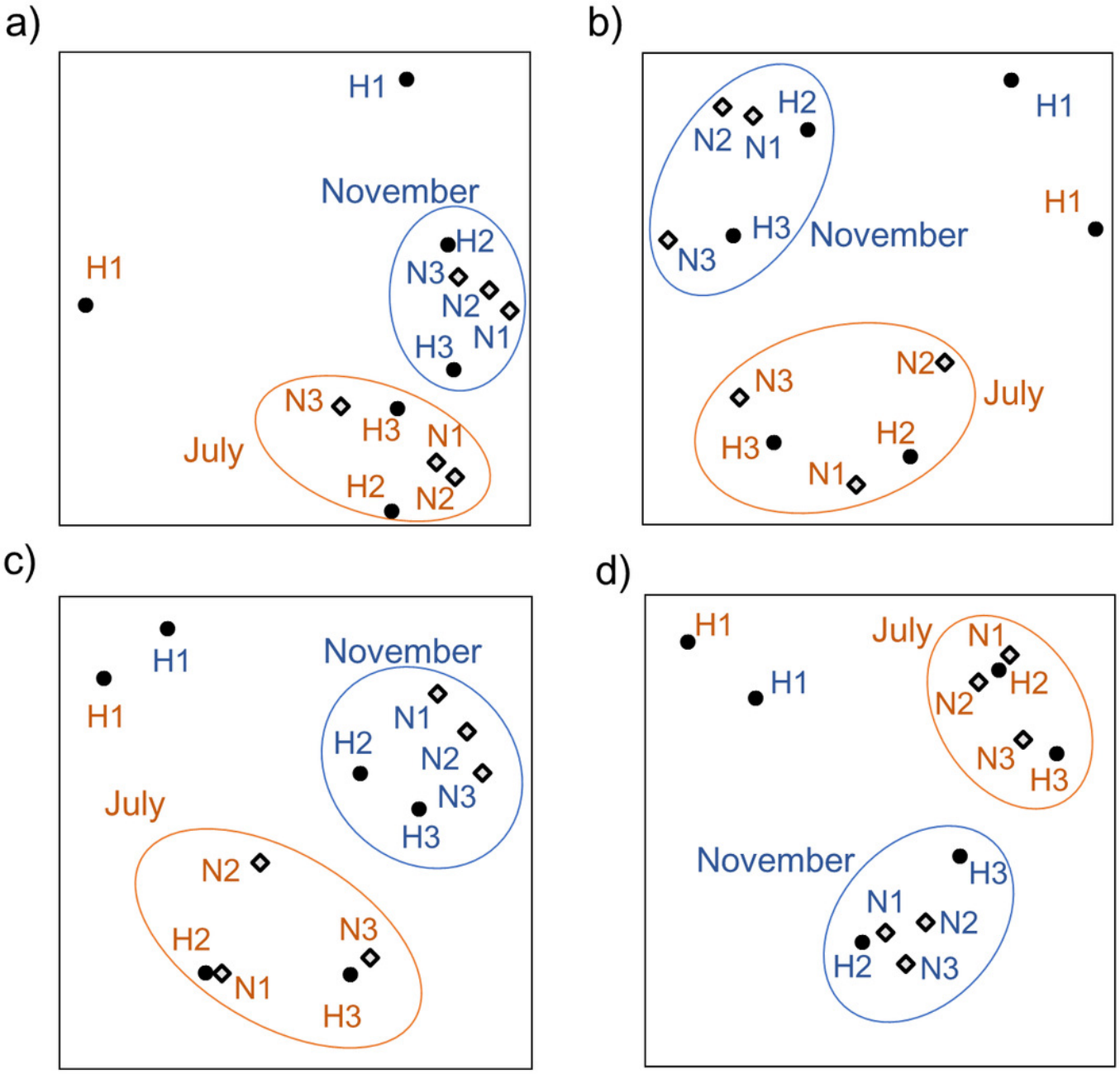
a)
H1
November
H2
N3
N2
N1
H3
H1
N3
H3
N1
July
H2
N2
b)
H2
N2
N1
H1
H1
H3
November
N3
N2
N3
July
H3
H2
N1
c)
H1
November
H1
N1
H2
N2
N3
July
H3
N2
H2
N3
N1
H3
d)
H1
July
N1
H1
H2
N2
N3
H3
November
H3
N1
N2
H2
N3

Figure 4

The spatial change of EPT index and Diptera index for two rivers in July and November.

The spatial changes of EPT index are shown in panel a) Hirose in July, b) Natori in July, c) Hirose in November and d) Natori in November. These of Diptera index are shown in panel e) - f) by same order as %EPT. Black and blue marks represent the Surber-net results and eDNA results (subsampled for 250 reads), respectively. Closed circles/rectangles with solid lines represent relative abundance-based indices, and open circles/rectangles with dotted lines and broken lines represent richness-based indices. For in the case of subsampled for 2,500 reads, please see Figure S6.