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Title:

Linkage between spatiotemporal distribution of environmental DNA and phenological activity in an amphidromous fish, ayu *Plecoglossus altivelis altivelis*, in a river located in its northernmost distributional area

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Abstract

Environmental DNA (eDNA) is a promising tool for the continuous monitoring of fish ecology and diversity. However, its potential for describing the phenological activity of fish has rarely been examined. This study aimed to elucidate a linkage between the spatiotemporal distribution of eDNA and the phenology of an amphidromous fish, ayu *Plecoglossus altivelis altivelis*, in a river in Hokkaido, Japan, which is its northernmost distributional area. A significant positive correlation between eDNA concentration and catch per unit effort of *P. a. altivelis* in the river confirmed the use of eDNA as a surrogate for the abundance of *P. a. altivelis*. eDNA of *P. a. altivelis* was first detected in late April on a sandy beach adjacent to the river mouth. Subsequent to its first detection at the lowest site in the river in early May, eDNA spread throughout the river, indicating the upstream migration of *P. a. altivelis*. Spawning activity was also represented by a rapid increase in eDNA concentration and its surge at night in the lowest reaches of the river during September and October. These results suggest that upstream migration and spawning primarily commenced when the water temperature reached 10 °C and decreased below 20 °C, respectively. This observation is consistent with the behavioral responses observed in *P. a. altivelis* populations from other regions of Japan. Consequently, this study demonstrated that eDNA distribution was closely linked to the phenological activity of *P. a. altivelis* and that eDNA is a powerful tool for studying the phenology of migratory fishes.

Keywords

environmental DNA, northern limit of distribution, *Plecoglossus altivelis*, spawning, upstream migration

1. Introduction

Environmental DNA (eDNA), refers to the DNA extracted from environmental samples, is a promising tool for ecological monitoring. Genetic material in various forms, such as feces and mucus shed from aquatic animals (e.g., fish, marine mammals, amphibians, and crustaceans) into the surrounding water, is considered a source of eDNA (Barnes and Turner, 2016). Therefore, eDNA can be used as an indicator of the source organism's distribution (Ficetola *et al.*, 2008). Furthermore, previous studies have found positive correlations between eDNA concentration and parameters surrogating biomass (e.g., abundance and body weight) of source organisms under rearing conditions (e.g., Takahara *et al.*, 2012; Minegishi *et al.*, 2019). Although it is still controversial, the relationship has also been suggested in field studies (Thomsen *et al.*, 2016; Sato *et al.*, 2021). Consequently, eDNA has often been used to detect invasive or endangered species (Balasingham *et al.*, 2017; Kasai *et al.*, 2021) and obtain snapshots of fish diversity (Yamamoto *et al.*, 2016).

The eDNA technique facilitates the elucidation of the phenology of aquatic animals. A periodic eDNA survey successfully revealed the seasonal migration of the Shishamo smelt *Spirinchus lanceolatus* (Hikita 1913) in coastal regions (Yatsuyanagi & Araki, 2020) and the migration patterns of juvenile chum salmon *Oncorhynchus keta* (Walbaum 1792) in the bay (Minegishi *et al.*, 2019). Short-term fluctuations in eDNA can also be indicative of ecologically significant seasonal events such as reproduction (Thalinger et al., 2019; Tillotson *et al.*, 2018; Tsuji & Shibata, 2020). A phenological shift in the life history of fish, which consists of a succession of seasonal events, such as migration and spawning, was observed to be associated

66 with global warming and could potentially cause the collapse of the fish population (Rogers &
67 Dougherty, 2019; Tao *et al.*, 2018). Despite its importance for the conservation of biodiversity
68 and generalizing phenological responses to climate change across various ecosystems,
69 phenology in freshwater ecosystems at the species level remains understudied (Woods *et al.*,
70 2022). To precisely capture phenological shifts in freshwater ecosystems, it is essential to
71 establish an extensive and continuous monitoring system. However, conventional methods such
72 as visual censuses and net sampling are regarded as time-consuming and laborious approaches
73 with limited spatial and temporal scope for large-scale monitoring. Therefore, introduction of
74 the eDNA technique would be beneficial for phenological studies (Woods *et al.*, 2022).
75 However, there is a lack of empirical knowledge regarding the link between eDNA dynamics
76 and fish ecology.

77 The ayu *Plecoglossus altivelis altivelis* (Temminck & Schlegel 1846) is an appropriate model
78 species to assess the effectiveness of eDNA analysis in the context of phenological studies. *P.*
79 *a. altivelis* is amphidromous with an annual lifespan (Takahashi & Azuma 2006). It exhibits
80 clear seasonal migration between the river and sea. *P. a. altivelis* spawns during autumn and
81 winter in the lower reaches of the river, which are covered with fine gravel. The spawning
82 season generally spans from September to December. Fertilized eggs adhere to gravel and
83 typically hatch within 10–14 days at 15–20 °C. Hatched larvae passively drift down to the sea
84 where they consequently spend their juvenile period. After growth in the sea, juveniles ascend
85 to the river in spring when the riverine water temperature increases to the optimal range for
86 their survival. After their upstream migration, they continue to grow in the river and feed on
87 adhesive diatoms during the summer. Short photoperiods and decreasing water temperatures

88 stimulate their maturation, and they spawn and die in autumn in the lower reaches of rivers.
89 Therefore, it can be hypothesized that the amount of eDNA will show spatiotemporal
90 fluctuation that synchronizes with the phenology of *P. a. altivelis*. Furthermore, ecological
91 knowledge pertaining to *P. a. altivelis* has accumulated because it is an essential species for
92 inland and recreational fisheries in Japan, providing sufficient reference data for comparison
93 with eDNA results.

94 A species-specific eDNA assay for *P. a. altivelis* is currently available and has been used to
95 describe its riverine distribution (Yamanaka & Minamoto, 2016). This assay successfully
96 revealed the spatial and temporal distribution of *P. a. altivelis* on the basin scale (Nagayama *et*
97 *al.*, 2023b; Naito *et al.*, 2018; Tenma *et al.*, 2021) and on the stream scale during their spawning
98 season (Inui *et al.*, 2021). As transportation by river flow is a source of uncertainty in eDNA
99 detection (Deiner & Altermatt, 2014), the extent to which the eDNA of *P. a. altivelis* diffuses
100 in rivers has been experimentally determined (Yamaguchi *et al.*, 2018). The eDNA technique
101 also demonstrated its usefulness for detecting spawning behavior and spawning grounds based
102 on a surge in concentration because *P. a. altivelis* intensively spawns after sunset, aggregating
103 at a particular site (Inui *et al.,* 2021; Yoshida *et al.*, 2019). However, the linkage between
104 fluctuation in eDNA and the phenological activity of *P. a. altivelis* covering the whole life
105 history has not been fully clarified.

106 This study aimed to elucidate a linkage between the spatiotemporal distribution of eDNA and
107 the phenology of *P. a. altivelis* throughout the entire period of their riverine phase on the stream
108 scale. To achieve this aim, a spatially and temporally intensive eDNA survey was conducted in
109 the Hekirichi River, Hokkaido, Japan (Figure 1). Because the amphidromous form of *P. a.*

altivelis shows a wide latitudinal distribution from Vietnam (approximately 21°N) to Hokkaido (approximately 45°N), the river in our study almost corresponds to the northern limit of its distribution (Naito, 2011; Tran *et al.*, 2014). Thus, our secondary aim was to assess how environmental conditions relate to the life history of the northernmost population of *P. a. altivelis* based on eDNA results. Although the presence of *P. a. altivelis* populations in Hokkaido has long been known (Okada & Sakurai, 1939; Yamada, 2017), there have been few sporadic reports describing their riverine distribution, the season of upstream migration and spawning in the Shubuto River (Okada & Sakurai, 1939; Takahashi & Aino, 2022), and the possibility of multiple spawning (Sakai *et al.*, 1991). Population genetic analysis revealed that *P. a. altivelis* in Hokkaido is genetically distinct from those inhabiting mainland Japan (Takeshima *et al.*, 2016). Considering the recent warming of the sea, a phenological shift in the life history and a northward shift in the species range can be expected. The nationwide fisheries catch of *P. a. altivelis* in Japan has been gradually decreasing since the 1990s (Ministry of Agriculture, Forestry and Fisheries of Japan, 2023), requiring further understanding of their ecology. Applying the eDNA technique to the northernmost population of *P. a. altivelis* would also contribute to the conservation of this important resource for Japanese inland fisheries, in addition to advancing the applicability of eDNA.

2. Materials and Methods

2.1 Study area

This study was conducted in the Hekirichi River, which flows into Hakodate Bay in southern

Hokkaido, Japan (Figure 1). The Hekirichi river has a watershed area of 63.5 km^2 and a river length of 22.8 km (Shimoda *et al*. 2017). The Kamiiso Dam is located approximately 8 km upstream of the Hekirichi River mouth. A series of weirs and check dams for erosion control installed immediately below the Kamiiso Dam precluded the upstream migration of *P. a. altivelis*. Although releasing hatchery-reared *P. a. altivelis* for stock enhancement and promoting recreational fishing, which is mainly conducted by inland water fisheries cooperatives, is widespread in Japanese rivers, the Hekirichi River has not received any hatchery fish releases due to a lack of an entity conducting the release. Therefore, it is assumed that the population of *P. a. altivelis* in the Hekirichi River is composed solely of wild fish.

Five stations (Sts.) for periodic water sampling were set in the lower reaches of the Hekirichi River below the dam and its river mouth, based on a foot survey held in early April (Sts. 1–5; Figure 1). The width of the reach was < 20 m. St. 1 was located on a sandy beach adjacent to the river mouth. Sts. 2–5 were set along the mainstream of the river at intervals of approximately 2 km. We used distances described in Yamaguchi et al. (2018) to separate the stations, based on the assumption that eDNA of *P. a. altivelis* has a maximum persistence range of 1–2km downstream from a shedding source. An additional station, St. 3C, was set approximately 1.3 km above St. 3 as a replacement for St. 4 after August 2021 because St. 4 could not be accessed in that period owing to the heavy rain that occurred in July.

Each station in the river is in a section characterized by different river morphologies and substrata, which were classified based on Montgomery and Buffington (1997). St. 2 was set immediately above the tidal area, located in the lowest part of the pool riffle reaches, and covered by fine gravel. Sts. 3, 3C, and 4 were set up within the plane-bed reach. St. 3 had coarse

gravel and cobble substrata, and St. 3C and St. 4 had cobble and boulder substrata. The riverbanks of the reaches, including Sts. 2 and 3, are partially covered by concrete revetments. St. 5 was set in a drainage channel immediately downstream of the Kamiiso Dam. Because weirs that inhibit the migration of fish are presented between St. 4 and St. 5, we assumed that neither *P. a. altivelis* nor their DNA were present in St. 5.

2.2 eDNA sampling

eDNA sampling was performed at Sts. 1–5 and St. 3C from April to December 2021, two to four times per month at one to two weeks interval. As heavy rain increased river discharge in July and scoured the riverbank, eDNA sampling was conducted at St. 3C instead of St. 4 after August. eDNA sampling in December was conducted only at Sts. 1 and 2, due to the downstream spawning migration of *P. a. altivelis* in autumn (Nagayama et al., 2023a) and cessation of their spawning from November to December. Hydrographic conditions can affect the detection of eDNA. High stream flows dilute eDNA concentration, reducing the detectability of the target organism (Curtis et al., 2021). Highly turbid water can impair the efficiency of filtration, DNA recovery, and PCR amplification (Harper et al., 2019). Therefore, we conducted eDNA sampling on days when the water level was as close to normal as possible to avoid the timing of increased discharge and turbidity. When we could not wade into the river due to the high water level, the sampling was halted partially or completely. Water sampling started in the morning and ended before noon to minimize the effect of human activity (e.g., recreational fishing), except at St. 1. To avoid the effects of disturbances while wading the river, collection was conducted from downstream to upstream. At St. 1, water was collected at a time

close to high tide to minimize the influence of river water.

All equipment used for eDNA sampling, except for disposable ones, was decontaminated before use by soaking in a 2% bleach solution (approximately 0.12% NaOCl) or wiping with a bleach disinfectant spray (approximately 0.1% NaOCl). All the researchers participated in the sampling wore disposable gloves, which were changed at each station. Masks were used to prevent contamination from human saliva.

At each station, 1 L of surface river water was collected in duplicate from the downstream side of the riffle. At Sts. 1–4, to minimize the possible heterogeneity in eDNA concentration, approximately 330–340 mL of water was collected from the left, center, and right sides of the river using a 1 L plastic beaker and then pooled in a disposable plastic bag (DP16-TN1000, COWPACK CO., LTD., Aichi, Japan) to make a 1 L water sample. At St. 5, a 1 L water sample was collected in a similar manner, but using a plastic folding bucket lowered from the bridge without differentiating the position in the river flow. The beaker and bucket were rinsed with the onsite river water before use to reduce the risk of carryover contamination. To prevent eDNA degradation, 1 mL of 10% benzalkonium chloride solution (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) was added to each plastic bag (Yamanaka *et al.*, 2017). As a field blank, 500 mL of purified water was stored in a plastic bag onsite and transported along with the water samples. We assumed that the source of contamination during the water sampling and subsequent filtration processes was DNA present on the surfaces of the sampling apparatus that came into contact with water (e.g., the inside of the plastic bag or the tube) and an unintentional mixing of any droplets of river water. Thus, the total amount of contaminated DNA was supposed to be independent of the amount of water used to make the blank. Rinsing the inside

198 of the bag with 500 mL of purified water was probably sufficient to dissolve the possible
199 contaminant DNA.

200 The collected water and field blank were filtered using a GF/F glass fiber filter (47 mm in
201 diameter and 0.7 μm in particle retention; GE Healthcare Life Science, Chicago, IL, USA) for
202 each bag. From April to mid-July, the water samples were transported to the laboratory in a
203 cooling box with frozen ice packs. Subsequently, the water samples were filtered using a
204 vacuum pump (EZ-Stream; Merck, Rahway, NJ, USA) connected to a manifold. When we
205 found that the volume of collected water inadvertently deviated from 1 L, the filtration volume
206 was calculated by subtracting the final weight from the initial weight of the water sample. As
207 the river water was highly turbid in April, it was divided into two equal volumes (approximately
208 500 mL) and filtered separately to avoid clogging. After late July, the water samples were
209 filtered on-site using a homemade filtration device in a car while driving between stations
210 (Supplementary Figure 1). Briefly, the device applies suction filtering using a peristaltic pump
211 (WPX1 DM; WELCO, Tokyo, Japan) and filters two water samples in parallel. The filtered
212 samples were transported to the laboratory in a cooling box with frozen ice packs. Filtration
213 took < 15 min for each station. On-site filtration allows eDNA samples to be obtained under
214 better conditions and is time efficient (Yamanaka *et al.*, 2017). All filtered samples were stored
215 at -20 °C until DNA extraction.

216 Additional sampling aimed at detecting spawning events was conducted from August to
217 October at Sts. 2 and 3, which were downstream of the possible spawning grounds of *P. a.*
218 *altivelis*. As *P. a. altivelis* is known to spawn immediately after sunset (Takahashi & Azuma,
219 2006), additional water samples were collected approximately one hour after sunset. Water

sampling and filtration were performed on-site as described previously.

2.3 Simultaneous sampling of fish and eDNA

To examine the correlation between the abundance or biomass of *P. a. altivelis* and the abundance of its eDNA, *P. a. altivelis* was collected in parallel with the water sampling on August 1. During the survey, participants were divided into two groups, and each group separately collected water and *P. a. altivelis*. To avoid disturbance by wading and net casting, water sampling always preceded fish sampling. Water samples were collected at stations for periodic eDNA sampling (Sts. 1–3C) and additional stations (Sts. 2B and 2C) in the same manner as the periodic sampling. These stations were separated approximately 500–1,200 m from each other. Fish were collected using a cast net (7.5 mm mesh; HOGA, Kyoto, Japan) in a section approximately 500 m upstream from each station. We basically cast a net once per riffle to reduce the sampling bias among sections. Given that eDNA flux showed a better correlation with the estimated total body weight of *P. a. altivelis* observed in a section 400–800 m upstream (Akamatsu *et al.,* 2018), this sampling design was presumed not to confound the relationship between eDNA abundance and fish abundance. The same environmental conditions as the periodic sampling were measured at each station, except for water velocity and depth.

Prior to the simultaneous sampling, only fish sampling had been conducted on July 15 at Sts. 2 and 3 in the same manner described above. The results were considered the counterparts of eDNA concentrations obtained on July 19, which is the most recent eDNA sampling day.

Body weights of the collected fish were measured in the laboratory. Catch per unit effort (CPUE; mean number of fish captured in one cast) and $CPUE_{BW}$ (mean total body weight of

fish captured in one cast) were calculated as representatives of abundance and biomass.

2.4 Environmental condition

Water temperature, salinity, and turbidity were measured using a water quality profiler (AAQ-RINKO; JFE Advantech, Hyogo, Japan) or portable conductivity meter (YSI-Pro30; Xylem, Washington DC, USA) concurrently with every water sampling. Discharge was also calculated following the cross-sectional method, except at St.1, which was located on a beach. The river width was measured using a measuring tape or laser distance meter (DISTO X4; Leica Geosystems, St. Gallen, Switzerland) and then divided into multiple sections at 1 or 2 m intervals. Water velocity and depth in each section were measured using a propeller-type current meter (VR401; KENEK, Tokyo, Japan) consisting of a stainless-steel wading rod marked in increments of 10 cm. The total discharge (m^3/s) was calculated as the sum of the partial discharges computed from the water velocity and cross-sectional area of each section. When wading was impossible owing to a high water level, the total discharge was estimated based on velocity and depth measured at a few sections from a riverbank, assuming constant velocity and depth along a transect. Precipitation data measured by the weather station of the Automated Meteorological Data Acquisition System in Hokuto City, which is in the vicinity of the study river, were downloaded from the Japan Meteorological Agency website (https://www.data.jma.go.jp/gmd/risk/obsdl/index.php).

2.5 DNA extraction

DNA was extracted from the filtered GF/F using DNeasy Blood and Tissue kit (Qiagen,

Hilden, Germany) following the protocol described in the illustrated manual for environmental DNA research (The eDNA Society, 2019). First, the filter was placed in an insert of a Salivette tube (Sarstedt, Nümbrecht, Germany) and was soaked with a mixture of 400 µL of AL buffer and 40 µL of Proteinase K (Qiagen, Hilden, Germany). The filter was then incubated at 56 °C for 30 min and centrifuged at 3000 ×*g* for 3 min to obtain the lysate. Subsequently, 220 µL of TE buffer was added onto the filter which was centrifuged again at 3000 ×*g* for 3 min. The collected lysate was then mixed with 400 µL of ethanol and filtered using a DNeasy spin column by centrifugation. In case sampled water was filtered through two GF/F filters, the lysates were prepared separately and then centrifuged on a single column. After washing the column with AW1 and AW2 buffers, DNA was eluted into a DNA LoBind tube (Eppendorf, Hamburg, Germany) using 110 µL of AE buffer. The extracted DNA was stored at -30 °C.

2.6 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) assay was performed using a StepOne Plus real-time PCR system (Thermo Fisher Scientific, MA, USA). To specifically detect the eDNA of *P. a. altivelis*, we used primers and a probe developed by Yamanaka & Minamoto (2016).

Paa-CytB-Forward: 5'-CCTAGTCTCCCTGGGCTTTATTCTCTCT-3'

Paa-CytB-Reverse: 5'-GTAGAATGGCGTAGGCGAAAA-3'

Paa-CytB-Probe: 5'-FAM-ACTTCACGGGCAGCCAACCCCCCC-TAMRA-3'

Each 20 µL reaction contained 1x Probe qPCR mix, with UNG (Takara, Shiga, Japan), 1x ROX reference Dye (Takara, Shiga, Japan), 900 nM of each primer, 125 nM of a probe, and 2 µL of DNA template. To evaluate cross-contamination, a 2.0 µL of nuclease-free water

(UltraPure; Invitrogen, Waltham, MA, USA) was included in each PCR run as a no-template control (NTC) in place of the DNA template. The thermal cycling conditions were as follows: 25 °C for 10 min, 95 °C for 30 s, and 55 cycles of 95 °C for 5 s and 60 °C for 30 s. Three technical replicates were used for each sample and NTC.

The quantity of eDNA per reaction (copies/reaction) was determined based on a standard curve generated by measuring a standard solution consisting of artificially synthesized double-stranded DNA (gBlocks; Integrated DNA Technologies, Inc., Coralville, IA, USA) containing the target sequence. Ten-fold serial dilutions ranging from 10 to 10,000 copies/reaction or from 30 to 30,000 copies/reaction of the standards were used. The standards were included in triplicates for each qPCR run. To decrease the risk of false detection, the quantity of eDNA detected in the field blanks was subtracted from that in the sample collected on the same day. Subsequently, reactions that yielded < 1 copies/reaction were considered negative. The representative eDNA concentration for each sample was expressed as the mean of the triplicates. No NTCs were amplified.

The R^2 values, log-linear slopes, intercept, and PCR efficiency of the standard curves across 14 runs were 0.996 ± 0.005, -3.45 ± 0.09, 39.23 ± 1.33, and 94.9 ± 3.5% (mean ± SD), respectively (Supplementary Table 1).

2.7 Statistical analyses

All data handling and statistical analyses were performed using the R version 4.1.2. (R Core Team, 2021) in combination with the RStudio 2023.03.0+386 (available online: https://www.rstudio.com/). The significance level was set at 0.05 throughout the analysis.

The quantity of each sample was converted into eDNA concentration (copies/L) based on the filtration and elution volumes. The eDNA flux (copies/s) was calculated by multiplying the eDNA concentration by the river discharge. The concentration and flux at each sampling site were represented as the average of the results from the filtration duplicates.

The relationship between the abundance of *P. a. altivelis* (CPUE or $CPUE_{BW}$) and eDNA abundance (eDNA concentration or flux) was evaluated using the Pearson's correlation coefficient. The log-transformed eDNA concentration was used in the following analysis because eDNA concentration was positively correlated with $CPUE_{BW}$ and eDNA flux when log-transformed (see Results). The spatial and temporal distributions of the eDNA were visualized using a contour map after log10 transformation. To infer the origin of the eDNA detected in the estuary, correlations between eDNA concentrations at St. 1 and St. 2 were also evaluated using the Pearson's correlation coefficient based on the results from June to August, when *P. a. altivelis* was assumed to be absent from the estuary or sea. The spawning period of *P. a. altivelis* was estimated by finding an increase in eDNA concentration at night on the line plot during August to October by applying daytime eDNA concentration as a baseline.

To determine the timing of the upstream migration, the probability of eDNA detection was estimated using logistic regression analysis. The eDNA detection results for each filtration replicate were converted to presence/absence data and used as dependent variables. Upstream migration was expected to cease until August, and the results from April to July were used in the analysis. The data were regressed against water temperature or sampling date using the "glm" function in the "stats" package with a binomial error distribution and a logit link.

2.8 Ethical statement

Fish sampling in this study was approved by the prefectural governor of Hokkaido based on the Hokkaido Inland Water Fisheries Adjustment Regulation. All fish used in this study were treated in accordance with the current law of Japan.

3 Results

3.1 Environmental condition

The water temperature increased from approximately 6 °C to over 20 °C from April to July (Figure 2a). During this period, the temperatures of the river were almost the same among stations (Sts. 2–4), whereas the temperature at the sandy beach (St. 1) was similar to or slightly higher than that of the river. The temperature then decreased to below 20 °C in early August and fluctuated in a range of 15–20 °C until early October, followed by a gradual decrease to about 5 °C from October to December. During this period, the temperatures at the upstream stations tended to be lower than those at the downstream stations, whereas St. 1 had consistently higher temperatures than those at the river. Salinity indicated that St. 1 was covered with brackish water (Figure 2b). The temporal changes in turbidity were synchronized across stations in the river (St. 2–5), with moderate peaks in late April and November, except for some sporadic peaks in St. 5 in August and Sts. 3 and 3C in late September (Figure 2c). A similar temporal change was observed in St. 1, but there were moderate peaks in June and between August and September. The river discharge temporally and synchronously fluctuated in Sts. 2–4, with two large peaks in April and November and with three moderate peaks from late May to August

(Figure 2d). On the contrary, the discharge in St. 5 was stable during the sampling period. The peaks of turbidity and discharge appeared to be related to heavy rain (Figure 2e), although the high amount of discharge in April was probably dominated by snowmelt runoff.

3.2 Relationship between abundances of fish and eDNA

Log-transformed eDNA concentrations showed significant positive correlation with log-transformed CPUE (Figure 3a; $r = 0.805$, $p = 0.016$) and log-transformed $CPUE_{BW}$ (Figure 3b; $r = 0.884$, $p = 0.004$), whereas no significant relationship was observed without log-transformation. A significant positive correlation was also found between eDNA concentration and flux after log transformation (Figure 3c; $r = 0.960$, $p < 0.001$). Based on these results, we concluded that the log-transformed eDNA concentrations could replace the biomass of *P. a. altivelis*. Although correlations between eDNA flux and CPUE or $CPUE_{BW}$ could not be evaluated due to a lack of river discharge on the sampling day, the eDNA flux was also considered a reasonable proxy for *P. a. altivelis* biomass given the significant correlation against eDNA concentration.

3.3 Spatial and temporal distribution of eDNA

A clear seasonality was observed in the spatial and temporal distributions of *P. a. altivelis* eDNA in the river (Figure 4a–d). The eDNA of *P. a. altivelis* was first detected at St. 1 on April 15, at a low concentration (15 copies/L; Figures 4a, c). The concentration of *P. a. altivelis* eDNA gradually increased along the upstream river in May, with a concentration of approximately 15–2,000 copies/L, except at St. 5. The eDNA concentration at Sts. 2–4 exceeded that at St. 1 in

late May. The eDNA of *P. a. altivelis* was continuously detected at Sts. 1–3 until November. Although there was a lack of data in August, eDNA was detected until early October in St. 3C. On the other hand, the eDNA was sporadically detected at low concentrations at St. 5. The eDNA concentration gradually increased across the study area from May to August in synchrony with the expansion of the spatial distribution of *P. a. altivelis* and reached the order of 10^4 copies/L. However, the eDNA concentration at St. 1 did not exhibit such a remarkable increase and remained relatively low (26–8831 copies/L). The highest concentration was detected at St. 2 in early September (approximately 1.2–1.5 x 10^5 copies/L). After September, the eDNA concentration gradually decreased and disappeared from the upstream stations sequentially, while the eDNA concentration in Sts. 3C and 5 between late October and early November could not be interpolated on the contour map due to a lack of eDNA data. *P. a. altivelis* eDNA almost disappeared in November (< 200 copies/L), but a minute amount of eDNA (15 copies/L) was still detected in December at St. 2.

The spatial and temporal distribution of eDNA flux was mostly similar to that of eDNA concentration (Figures 4b, d), with some exception. The eDNA flux at Sts. 2 and 3 gradually increased from August to September without a temporary decrease in mid-August. The highest value of eDNA flux at St. 2 was observed in late August, being slightly earlier than the concentration.

3.4 Estimating the timing of upstream migration and spawning period

Temporal changes in eDNA concentrations were further analyzed to estimate the timing of the upstream migration and spawning period.

In April to July, logistic regression curves against water temperature indicated that the detection probability of *P. a. altivelis* eDNA at Sts. 1 and 2 increased rapidly over approximately 9 °C and reached its asymptote at 10–11 °C (Figure 5a). The water temperature with a 50% detection probability was similar between the two stations, it was predicted to be 9.9 °C and 10.6 °C at Sts. 1 and 2, respectively. However, the detection of *P. a. altivelis* eDNA at St. 2 was much later than that at St. 1 (Figure 5b). The date with a 50% detection probability was May 7 at St. 2, whereas it was April 22 at St. 1, which was 15 days earlier than at St. 2. In contrast, an increase in detection probability occurred at higher water temperatures in the later study period at the upstream stations (Sts. 3 and 4). The speed of range expansion in the river was estimated based on the differences in the date at 50% detection and the distance between stations. This speed was approximately 360 m/d from St. 2 to St. 3, and 140 m/d from St. 3 to St. 4.

The daytime eDNA concentration at St. 2 showed a gradual unimodal distribution from August to October, reaching a peak in early September. In contrast, the concentration at St. 3 remained almost constant at a lower level compared to St. 2 (Figure 6). Temporal changes in eDNA concentration at St. 2 indicated that obvious surges at night were found in mid-September and early October (approximately 6×10^5 copies/L). Notably, the eDNA concentration was similar between day and night in August; however, when the surge occurred, the concentration at night was two to ten times higher than that in the daytime when comparing the same day or between adjacent days.

3.5 Fluctuation of eDNA concentration in the estuary

Fluctuations in eDNA concentration at the estuarine site, St. 1, were analyzed in relation to

418 salinity, river discharge, and eDNA concentration at St. 2 from June to August. The log-
419 transformed eDNA concentration at St. 1 showed significant negative correlation with salinity
420 (Figure 7a, r = -0.816, p = 0.001), but not with discharge and concentration at St. 2. The ratio
421 of eDNA concentration was calculated between St. 1 and St. 2 after log transformation, and it
422 was found that the ratio showed a similar trend of significant negative correlation with salinity
423 (Figure 7b, r = -0.61, p = 0.035).
424
425 **4. Discussion**
426
427 The main finding of this study was that the eDNA of *P. a. altivelis* in the river showed drastic
428 fluctuations both spatially and temporally. The information from the eDNA analysis indirectly
429 represented the presence and abundance of the target species. Therefore, to convert the
430 distribution of eDNA into ecological information about the target species, it is necessary to
431 evaluate a link between the eDNA and its source organism. Previous studies have found that
432 the quantity of eDNA detected in rearing water is positively correlated with fish abundance and
433 body size under experimental conditions (Maruyama *et al.*, 2014; Minegishi *et al.*, 2019).
434 Furthermore, the eDNA concentration of *P. a. altivelis* in a river was found to correlate with its
435 biomass (g/m^2) and abundance (number of individuals/m^2) estimated by an underwater visual
436 census (Akamatsu *et al.*, 2018; Doi *et al.*, 2017). However, *P. a. altivelis* exhibits distinct
437 behavioral modes depending on its population density in the river (Kawanabe, 1957, 1970);
438 territorial fish with a larger body size, and schooling fish with a smaller body size. Such
439 differences in body size and behavior between the two forms confound the relationship between

440 eDNA concentration and the biomass of *P. a. altivelis*. In the present study, we found a positive
441 correlation between eDNA concentration and *P. a. altivelis* abundance (CPUE and $CPUE_{BW}$),
442 similar to the results of previous studies. This relationship confirmed that eDNA concentration
443 could serve as a surrogate for *P. a. altivelis* biomass, even in a small river in Hokkaido.

444 As eDNA is transported downstream by flowing water, there is a risk of false-positive
445 detections in rivers (Deiner & Altermatt, 2014). In this study, a small amount of eDNA was
446 continuously detected in the estuary (St. 1) from June to August, when *P. a. altivelis* is
447 considered to exclusively inhabit the river. Upon incomplete amphidromy *P. a. altivelis* has
448 been observed to spend its entire life in tidal areas without migrating into rivers in mainland
449 Japan (Abe *et al.*, 2001; Takasaki & Otake, 2003). Moreover, given the general trend of
450 increasing dependency of diadromous fish on marine environments at higher latitudes (Chalant
451 *et al.*, 2019), it cannot be ruled out that *P. a. altivelis* may inhabit estuaries without entering
452 rivers in Hokkaido, which is the northern limit of their distribution. However, the eDNA
453 concentrations at St. 1 were significantly influenced by salinity, which is an indicator of the
454 mixing ratio of freshwater in the estuary. Therefore, it is reasonable to conclude that the
455 detection of eDNA in the estuary was a false-positive result of eDNA transported from the river
456 and did not indicate the presence of *P. a. altivelis*. On the other hand, the sampling stations in
457 the river (Sts. 2–5) were sufficiently separated and were to be presumed independent of one
458 another. Therefore, false positives and overestimates due to transportation and accumulation of
459 eDNA were ignored in this study.

460 The spatiotemporal variation in eDNA was clearly synchronized with the generally known
461 seasonality in the life cycle of *P. a. altivelis*. In early April, prior to its detection in the river,

462 eDNA was first detected at St. 1, located on a sandy beach adjacent to the river mouth. The
463 estuary and surf zones are the principal habitats of *P. a. altivelis* during the larval and juvenile
464 periods (Hamada and Kinoshita 1988; Takahashi *et al.*, 1998). After spending early growth in
465 the surf zone, the juveniles shift their habitats to more saline and deeper water and subsequently
466 migrate to the estuary after growing in the sea (Tsukamoto *et al.*, 1989). The eDNA detected in
467 the estuary in April suggests the aggregation of *P. a. altivelis* juveniles prior to upstream
468 migration.
469 The subsequent expansion of eDNA detection in the river from May to June clearly corresponds
470 to the upstream migration and expansion of the distributional range of *P. a. altivelis*. The timing
471 of upstream migration was estimated to be early May, when the riverine water temperature
472 reached approximately 10 °C, based on logistic regression analysis between eDNA
473 concentration and water temperature. This timing also suggested that *P. a. altivelis* in Hokkaido
474 showed a similar response to that in mainland Japan with regard to the water temperature when
475 they were motivated to commence upstream migration (≥10 °C; Tago 2002). On the other hand,
476 the duration of the upstream migration remained unknown. The disappearance of individuals
477 that have recently migrated into the river (migrants) indicates the cessation of migration.
478 However, the current eDNA technique cannot distinguish between DNA from migrants and
479 DNA from individuals that have already inhabited the river (river residents). To precisely
480 reconstruct the phenology of *P. a. altivelis* from an environmental sample, a novel method is
481 required to supplement the limitations of the eDNA technique.
482 One possible approach to overcome the limitations of biological monitoring using eDNA is
483 to use environmental RNA (eRNA), which is extra-organismal RNA found in an environmental

484 medium (Yates *et al.*, 2021). Because RNA is a less stable molecule compared to DNA, it has
485 been assumed that eRNA cannot be detected in environmental samples in biologically
486 meaningful quantities (Cristescu, 2019). However, recent laboratory experiments have
487 demonstrated that eRNA can be readily extracted and detected (Tsuri *et al.*, 2021). Particularly,
488 targeting eRNA derived from mRNA may provide physiological information about the source
489 organism, indicating its developmental stage (Yates *et al.*, 2021). When compared to eDNA,
490 eRNA has the potential to significantly improve spatial and temporal resolution in biological
491 monitoring due to its rapid turnover and enhanced diagnostics of population and community
492 characteristics (Yates *et al.*, 2021). Previous research has shown that ayu undergo changes in
493 hormonal levels (prolactin and thyroxine) in accordance with the onset of upstream migration
494 and following riverine residency (Tsukamoto & Uchida, 1992; Yada *et al.*, 2014). This
495 physiological change can be tracked by the quantification of mRNA (Yada *et al.*, 2014). These
496 findings imply that specific detection of eRNA derived from mRNA regarding hormone
497 synthesis might be able to differentiate groups with different behaviors (i.e., sea residents,
498 upstream migrants, and river residents). Although eRNA is still in its infancy, its application in
499 biological monitoring is expected to advance the understanding of the population dynamics of
500 *P. a. altivelis* and other diadromous fish.

501 Fish spawning behavior and spawning grounds can be detected as an increase in eDNA
502 concentration owing to the release of eggs and sperm, which are sources of eDNA (Inui *et al.*,
503 2021; Tsuji *et al.*, 2020). In this study, eDNA concentrations increased significantly at St. 2,
504 which was estimated to be the primary spawning site, at the beginning of September, when the
505 water temperature was below 20 °C. Short photoperiods stimulate the maturation of *P. a.*

506 *altivelis*, and successive decrease in water temperature triggers spawning behavior (Chang *et*
507 *al.*, 1992, 1993; Ishida, 1964; Shiraishi, 1961). Decreasing water temperatures also cause
508 downstream migration to suitable spawning grounds (Nagayama *et al.*, 2023a). Therefore, the
509 increase in eDNA concentration and flux at St. 2 may indicate the aggregation of *P. a. altivelis*
510 on spawning grounds. Diel fluctuation in eDNA concentration is also a signal of spawning in *P.*
511 *a. altivelis* because they are known to spawn at sunset (Inui *et al.*, 2021). In this study, an abrupt
512 increase in eDNA concentration was observed at night, indicating that the spawning period of
513 *P. a. altivelis* in the Hekirichi River started during late August to mid-September and lasted until
514 early October. The water temperature during this period ranged from 16–19 °C. This
515 relationship between spawning and water temperature corroborates with a previous report that
516 described that their spawning begins when the water temperature falls below 15–20 °C
517 (Takahashi & Azuma, 2006).

518 A decreasing trend in both the concentration and flux of eDNA was observed from September
519 to November. It is generally recognized that *P. a. altivelis* dies after reproduction from
520 exhaustion and a lethal low water temperature below 10°C in winter (Tachihara & Kimura,
521 1991; Takahashi & Azuma, 2006). Given that the spawning season in the Hekirichi River was
522 estimated to have commenced between late August and mid-September and that the water
523 temperature decreased below 10°C in November, the decreasing trend of eDNA was most likely
524 synchronized with the decrease in the number of individuals due to death after reproduction.
525 However, a minute amount of eDNA was unexpectedly detected at the downstream site in
526 December. Tenma et al. (2021) also reported that the eDNA of *P. a. altivelis* was successively
527 detected in January and February, even though *P. a. altivelis* at any developmental stage had

528 disappeared. Based on this result, they presumed the presence of overwintering individuals that
529 survived after the spawning season. Although the overwintering of *P. a. altivelis* would be
530 unlikely because of the lethal water temperature during winter in Hokkaido, it is possible to
531 presume that adult fish are still viable after cessation of reproduction, assuming that multiple
532 maturation and spawning were observed in a population of *P. a. altivelis* in Hokkaido (Sakai et
533 al., 1991).

534 Tillotson *et al.* (2018) investigated the eDNA of the sockeye salmon *Oncorhynchus nerka*
535 (Walbaum, 1792) and suggested that post-spawning carcasses could be a source of eDNA,
536 providing uncertainty for the quantitative estimation of salmon spawning. Hatched larvae and
537 juveniles are also possible sources of eDNA, whereas fertilized eggs do not release sufficient
538 eDNA for detection in rearing water (Takeuchi *et al.*, 2019). Fertilized eggs of *P. a. altivelis*
539 hatched within 10–16 days at 15–20 °C, with higher water temperatures yielding shorter egg
540 periods (Kashiwagi *et al.*, 1986). A possible overlap between the spawning periods of adult fish
541 and emergence of hatched larvae may result in a composite of eDNA from multiple sources.
542 Furthermore, eggs spawned at the end of the estimated spawning period might experience low
543 water temperatures around 10 °C, requiring approximately 20–30 days to hatch. It was
544 speculated that such late-hatched larvae caused the unseasonable detection of eDNA in
545 December. Therefore, in the case of *P. a. altivelis*, the eDNA detected after the spawning season
546 likely originated from living adult fish that survived after the cessation of spawning, dead fish
547 after reproduction, and hatched larvae drifting down to the sea.

548 Because water temperature is the primary environmental condition that determines the timing
549 of upstream migration and spawning season, a latitudinal cline has been found in the phenology

550 of *P. a. altivelis* (Kishino, 2009). For example, the northern population of *P. a. altivelis* typically
551 migrates into the river in later seasons than the southern population: early March at the
552 Shimanto River (32°56’03"N 132°59’42"E; Takahashi, 2004), late March to early April in the
553 Nagara River (35°01'55"N 136°42'54"E; Suzuki *et al.*, 2014), early April in the Sho River
554 (36°47'23"N 137°04'34"E; Tago, 2002), and late April in the Nou River (37°06'00"N
555 137°58'49"E; Okaji *et al.*, 2020). In contrast, spawning of *P. a. altivelis* occurs earlier in the
556 northern population than in the southern population: the main spawning season has been
557 reported in early to middle November in the Shimanto River (Takahashi, 2004), early to middle
558 October in the Sho River (Tago, 1999), and late September to late October in the Nou River
559 (Okaji *et al.*, 2020). Although there is insufficient ecological information about *P. a. altivelis* in
560 Hokkaido to discuss the latitudinal cline, upstream migration and spawning are estimated to
561 occur in late May to mid-July and late August to late September, respectively, at the Shubuto
562 River (42°45'57"N, 140°15'20"E), located in the southern part of Hokkaido (Okada & Sakurai,
563 1932; Takahashi & Aino, 2022). Our study also found that *P. a. altivelis* migrated to the river in
564 early May, and the most conservative estimate of the spawning season was between mid-
565 September and early October, suggesting that the latitudinal cline in phenology is applicable to
566 *P. a. altivelis* in Hokkaido. This congruence also suggests that *P. a. altivelis* in Hokkaido has
567 similar physiological responses to environmental conditions (e.g., water temperature and
568 photoperiod) as those inhabiting other parts of Japan, despite distinct genetic differences
569 (Takeshima *et al.*, 2016).

570 Our findings also implicate that the prediction that global warming will lead to a phenological
571 shift in *P. a. altivelis* populations on Honshu Island, Japan (Nakamura & Kasuya, 2004; Suzuki

et al., 2014; Takahashi, 2004) can be applied to *P. a. altivelis* populations on Hokkaido. The average sea surface temperature around Japan has exhibited an increasing trend over the last 100 years (Japan Meteorological Agency, 2023). It can be speculated that future warming in the sea may cause *P. a. altivelis* in Hokkaido to migrate into the river earlier than in the past, extending their growth period in the river. In contrast, water temperatures in Japanese rivers and lakes have not necessarily shown an increasing trend since the 1980s (Ye & Kameyama, 2021). This inconsistency makes it difficult to forecast its future impact on the growth, survival, and reproduction of *P. a. altivelis* during the riverine period. With such a possible asynchrony between the fluctuations of sea and river, it is unclear how global warming influences population dynamics and its persistence via phenological shift for *P. a. altivelis*.

Various methods, including visual censuses, fish sampling, otolith analysis, and stable isotope analysis, have been used to study the phenology of diadromous fish (Fukuda *et al.*, 2016; Fuji *et al.*, 2014; Holt & Cox, 2008; Murase *et al.*, 2019). Each method had a different spatial and temporal scope. Visual censuses and fish sampling can directly observe target species, and thus provide real-time data on their presence. However, these methods can only provide temporally fragmented results that require frequent and continuous surveys to comprehend the phenology of the target species. In contrast, otolith and stable isotope analyses provide retrospective data about past environments, feeding habits, and physiological conditions, and can thus reconstruct life history at the individual level. In particular, the combined use of otolith increment analysis and microchemistry has frequently been used to reveal the migration patterns and survival of *P. a. altivelis* (Hata *et al.*, 2016; Murase *et al.*, 2019). However, preparing specimens for otolith or stable isotope analyses is time-consuming,

making it difficult to conduct large-scale research.

Since eDNA is dispersed through the surrounding water after shedding, it is advantageous to accumulate more comprehensive, less biased, and near-real-time data with less effort than conventional sampling approaches. This implies that it is possible to establish a large eDNA monitoring network (e.g., the All Nippon eDNA Monitoring Network [ANEMONE], https://db.anemone.bio/). One disadvantage of the eDNA technique is that it does not provide biological information on the target organism (e.g., size, age, sex, maturation, and growth). However, recent technical developments regarding eDNA-based population genetic analysis and the utilization of nuclear eDNA, eRNA, and epigenetic modifications for determining biological information will expand the field of eDNA study (Sigsgaard *et al.*, 2020; Yates *et al.*, 2021). The incorporation of the eDNA technique into the study of diadromous fish would substantially contribute to the understanding of how these organisms respond to ongoing global climate change and evaluate the intensity of the cascading effect in river ecosystems caused by the phenological shift of these fish.

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Supporting Information

Supplementary Figure 1 Schematic illustration of the on-site filtration devise. 1 Sealed plastic bag (DP16-TN1000; COWPACK CO., LTD., Aichi, Japan); 2 One-way stopcock (S75-4037-30; Narika, Tokyo, Japan); 3 Silicon tube (internal diameter 6.5 mm); 4 Filter holder (PP-47; ADVANTEC, Tokyo, Japan); 5 Silicon tube (internal diameter 5 mm); 6 Y-shape tubing connector; 7 Female luer lock (VRF606, Nordson MEDICAL, OH, USA); 8 Male luer lock (VRM606, Nordson MEDICAL, H、US AO); 9 Pump tube (internal diameter 4.8 mm); 10 Tubing pump (WPX1; WELCO, Tokyo, Japan); 11 Drain tank; 12 Conductor wire; 13 Cigarette lighter plug DC 12V (No. 1538, Amon Industry, Hyogo, Japan); 14 Stepped tubing connector; 15 Silicon tube (internal diameter 9 mm); 16 Coupler (JF-02W; Joplax, Osaka, Japan); 17 Filter holder (top); 18 Glass fiber filter (Whatman 47 mm GF/F; Cytiva, Tokyo, Japan); and 19 Filter holder (bottom).

Supplementary Table 1 Summary of standard curves for the TaqMan probe qPCR assay for species-specific detection of eDNA of *Plecoglossus altivelis altivelis*. Curves were created by measuring the dilution series of artificially synthesized double-stranded DNA corresponding to the target region of the assay.

Author contributions

T.K. conceived and designed the study. T.Y., T.K., and A.K. collected the fish and eDNA

samples. T.Y. and T.K. performed the molecular work, statistical analysis, and visualization. T.Y. wrote the manuscript under the supervision of T.K. and A.K. All the authors critically reviewed the manuscript, contributed to the final version, and approved the submitted version.

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876

877 **Figure captions**

878

879 **Figure 1** Map showing stations for water sampling to capture environmental DNA of ayu
880 *Plecoglossus altivelis altivelis* in the Hekirichi River, which flows into Hakodate Bay in
881 southern Hokkaido. Surface water samples were routinely collected at five stations (Sts. 1, 2, 3,
882 4, and 5) during April and December 2021, with St. 4 being replaced by St. 3C after August
883 2021. Collection of *P. a. altivelis* was conducted in July and August at Sts. 2, 2B, 2C, 3, and 3C,
884 in parallel with water sampling. Star indicates the location of a weather station of the Automated
885 Meteorological Data Acquisition System, maintained by the Japan Meteorological Agency.

886

887 **Figure 2** Environmental condition in the stations for water sampling to capture environmental
888 DNA of ayu *Plecoglossus altivelis altivelis* during the study period (April to December 2021).
889 (a) water temperature; (b) salinity; (c) turbidity; (d) river discharge; (e) precipitation.
890 Precipitation data are obtained from a weather station of the Automated Meteorological Data
891 Acquisition System in the vicinity of the study river, maintained by the Japan Meteorological
892 Agency.

893

894 **Figure 3** Correlation between environmental DNA (eDNA) concentration and catch per unit
895 effort (CPUE) of ayu *Plecoglossus altivelis altivelis* in the Hekirichi River. (a) log-transformed
896 eDNA concentration and log-transformed CPUE; (b) log-transformed eDNA concentration and
897 log-transformed $CPUE_{BW}$; (c) log-transformed eDNA flux and log-transformed eDNA
898 concentration. CPUE and $CPUE_{BW}$ are defined as the number of individuals caught per one net

casting and the total body weight per one net casting, respectively. Pearson's correlation coefficient (r), t-value, and p-value are indicated in figures.

Figure 4 Spatiotemporal variation in environmental DNA of ayu *Plecoglossus altivelis altivelis* in the Hekirichi River. Contour maps shows the spatiotemporal distribution of eDNA, which is indicated as a log-transformed eDNA concentration (a) and flux (b). Line graphs shows the temporal fluctuations of a log-transformed eDNA concentration (c) and flux (d). For visibility, the results at Sts. 4 (April–July 2021) and 3C (August–December 2021) are merged in the line graph. Color scales in panels (a) and (c) indicate eDNA concentration and flux using a base-10 logarithmic scale, respectively. White spaces in contour maps are the regions that could not be interpolated due to a lack of eDNA results.

Figure 5 Logistic regression curves showing changes in detection probability of environmental DNA of ayu *Plecoglossus altivelis altivelis* against (a) water temperature and (b) date. A horizontal dotted line indicates 50% detection. Each data point is slightly jittered along x axis for visibility.

Figure 6 Temporal changes in environmental DNA concentration of ayu *Plecoglossus altivelis altivelis* observed in daytime and nighttime at St. 2 and daytime at St. 3 from August to October.

Figure 7 Correlation between salinity and (a) environmental DNA (eDNA) concentration of ayu *Plecoglossus altivelis altivelis* and (b) the ratio of eDNA concentration between St. 1 and

921 St. 2. The results obtained between June and August was included in the analysis. eDNA
922 concentration is used after log-transformation. Pearson's correlation coefficient (r), t-value, and
923 p-value are indicated in figures.
924

Figure

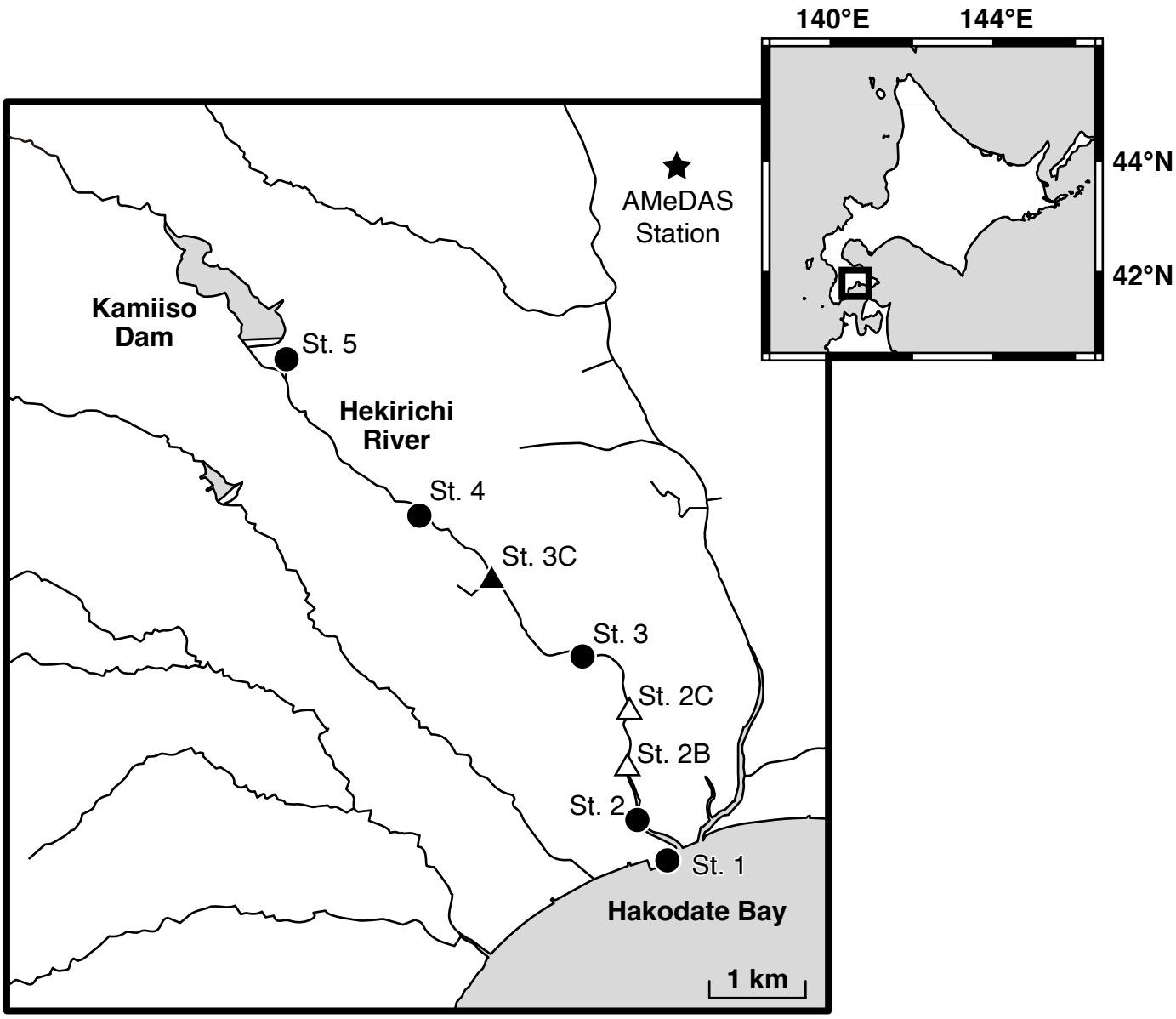


Figure 1 Map showing stations for water sampling to capture environmental DNA of ayu *Plecoglossus altivelis altivelis* in the Hekirichi River, which flows into Hakodate Bay in southern Hokkaido. Surface water samples were routinely collected at five stations (Sts. 1, 2, 3, 4, and 5) during April and December 2021, with St. 4 being replaced by St. 3C after August 2021. Collection of P. a. altivelis was conducted in July and August at Sts. 2, 2B, 2C, 3, and 3C, in parallel with water sampling. Star indicates the location of a weather station of the Automated Meteorological Data Acquisition System, maintained by the Japan Meteorological Agency.

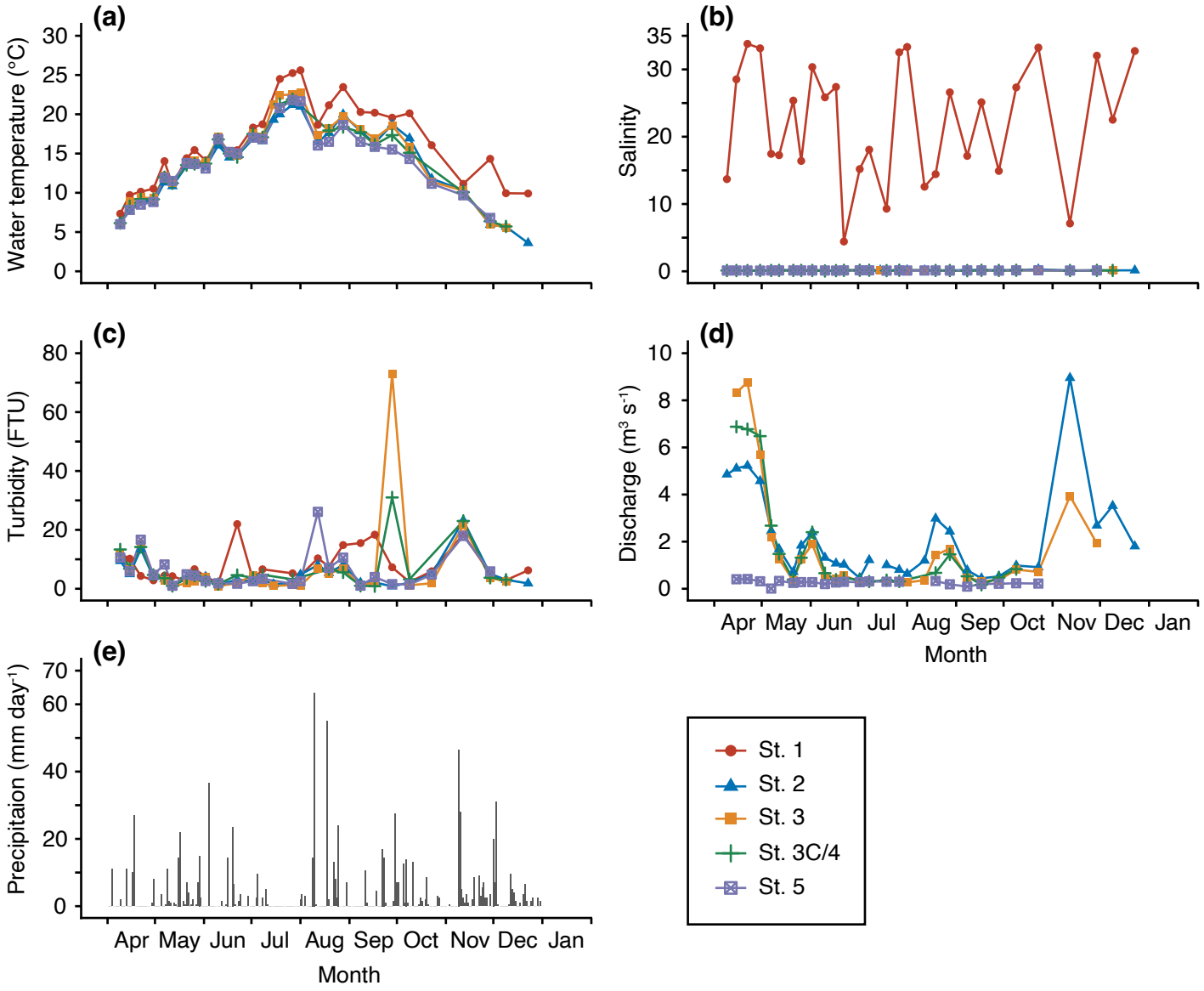


Figure 2 Environmental condition in the stations for water sampling to capture environmental DNA of ayu *Plecoglossus altivelis altivelis* during the study period (April to December 2021). (a) water temperature; (b) salinity; (c) turbidity; (d) river discharge; (e) precipitation. Precipitation data are obtained from a weather station of the Automated Meteorological Data Acquisition System in the vicinity of the study river, maintained by the Japan Meteorological Agency.

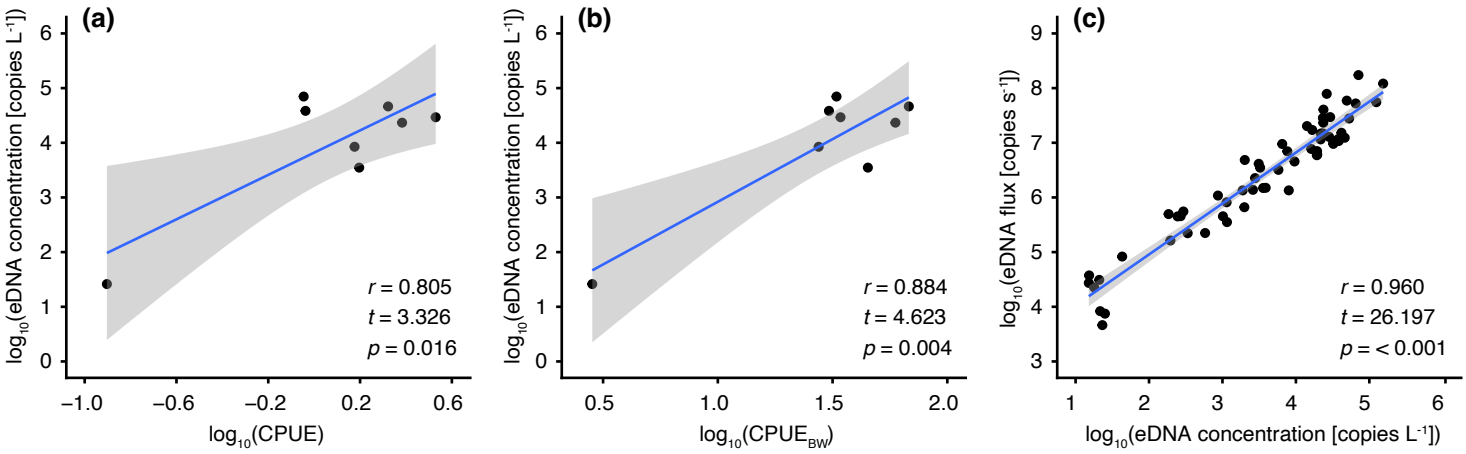


Figure 3 Correlation between environmental DNA (eDNA) concentration and catch per unit effort (CPUE) of ayu *Plecoglossus altivelis altivelis* in the Hekirichi River. (a) log-transformed eDNA concentration and log-transformed CPUE; (b) log-transformed eDNA concentration and log-transformed $CPUE_{BW}$; (c) log-transformed eDNA flux and log-transformed eDNA concentration. CPUE and $CPUE_{BW}$ are defined as the number of individuals caught per one net casting and the total body weight per one net casting, respectively. Pearson's correlation coefficient (r), *t*-value, and *p*-value are indicated in figures.

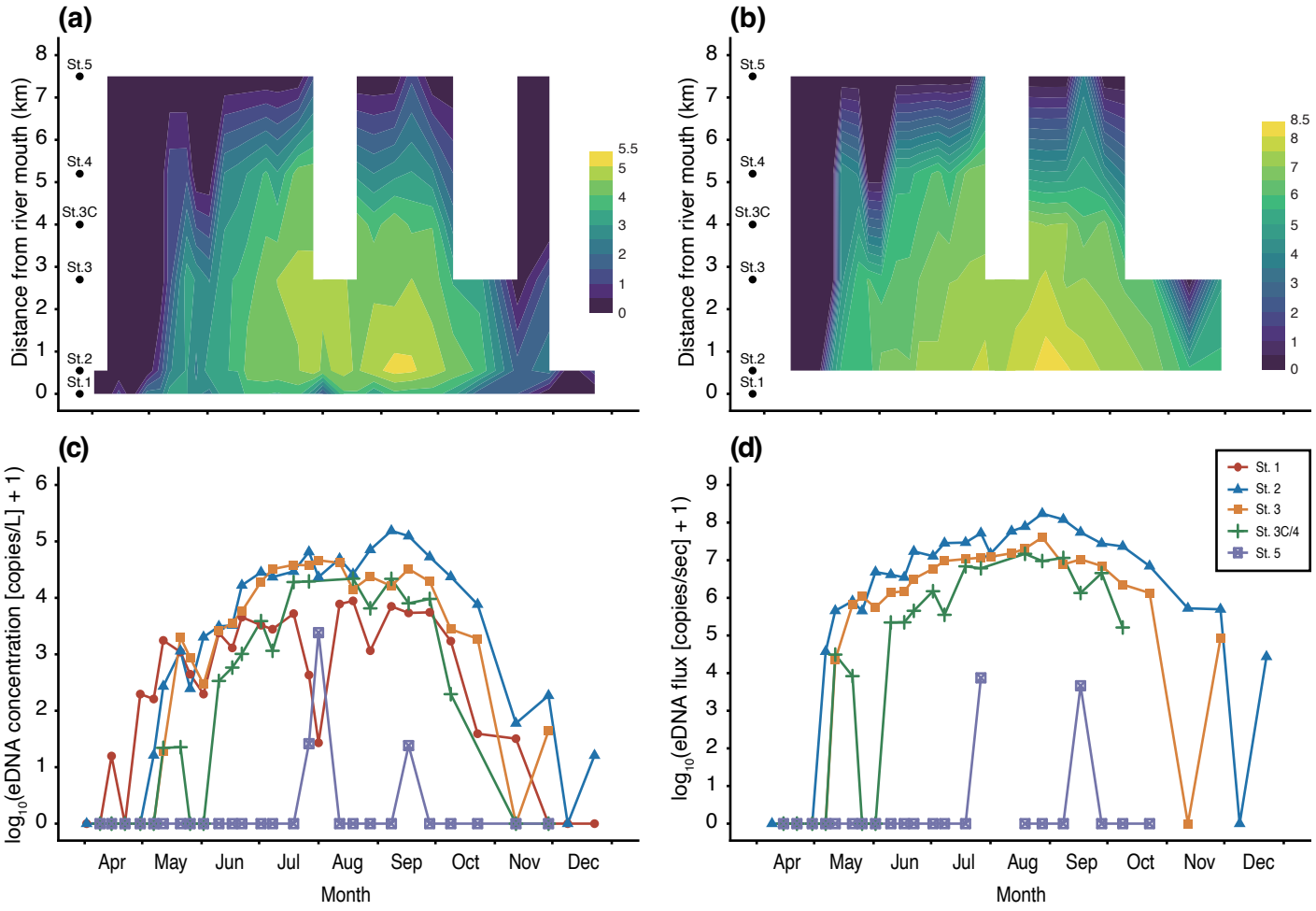


Figure 4 Spatiotemporal variation in environmental DNA of ayu *Plecoglossus altivelis altivelis* in the Hekirichi River. Contour maps shows the spatiotemporal distribution of eDNA, which is indicated as a log-transformed eDNA concentration (a) and flux (b). Line graphs shows the temporal fluctuations of a log-transformed eDNA concentration (c) and flux (d). For visibility, the results at Sts. 4 (April–July 2021) and 3C (August–December 2021) are merged in the line graph. Color scales in panels (a) and (c) indicate eDNA concentration and flux using a base-10 logarithmic scale, respectively. White spaces in contour maps are the regions that could not be interpolated due to a lack of eDNA results.

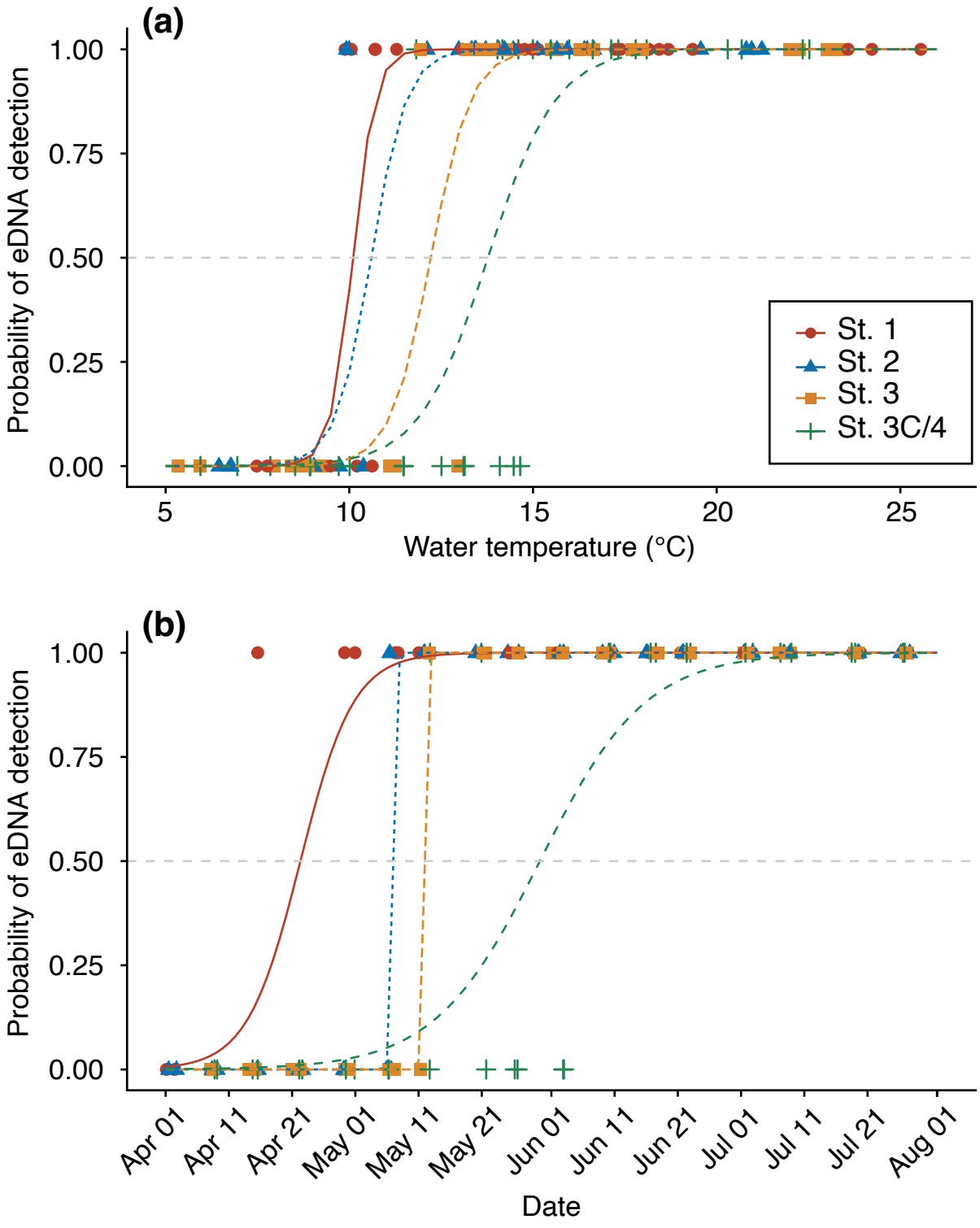


Figure 5 Logistic regression curves showing changes in detection probability of environmental DNA of ayu *Plecoglossus altivelis altivelis* against (a) water temperature and (b) date. A horizontal dotted line indicates 50% detection. Each data point is slightly jittered along x axis for visibility.

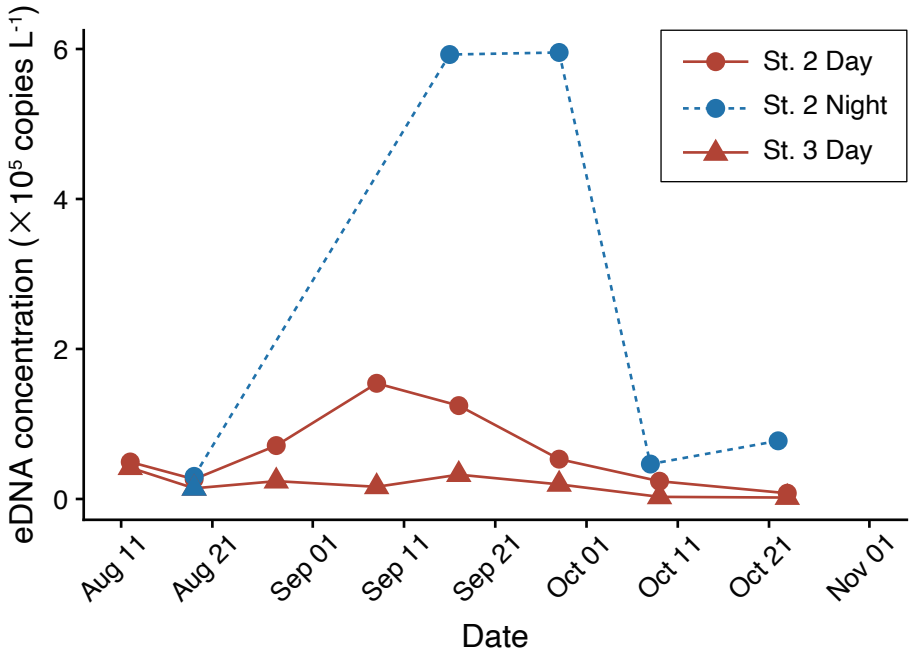


Figure 6 Temporal changes in environmental DNA concentration of ayu *Plecoglossus altivelis altivelis* observed in daytime and nighttime at St. 2 and daytime at St. 3 from August to October.

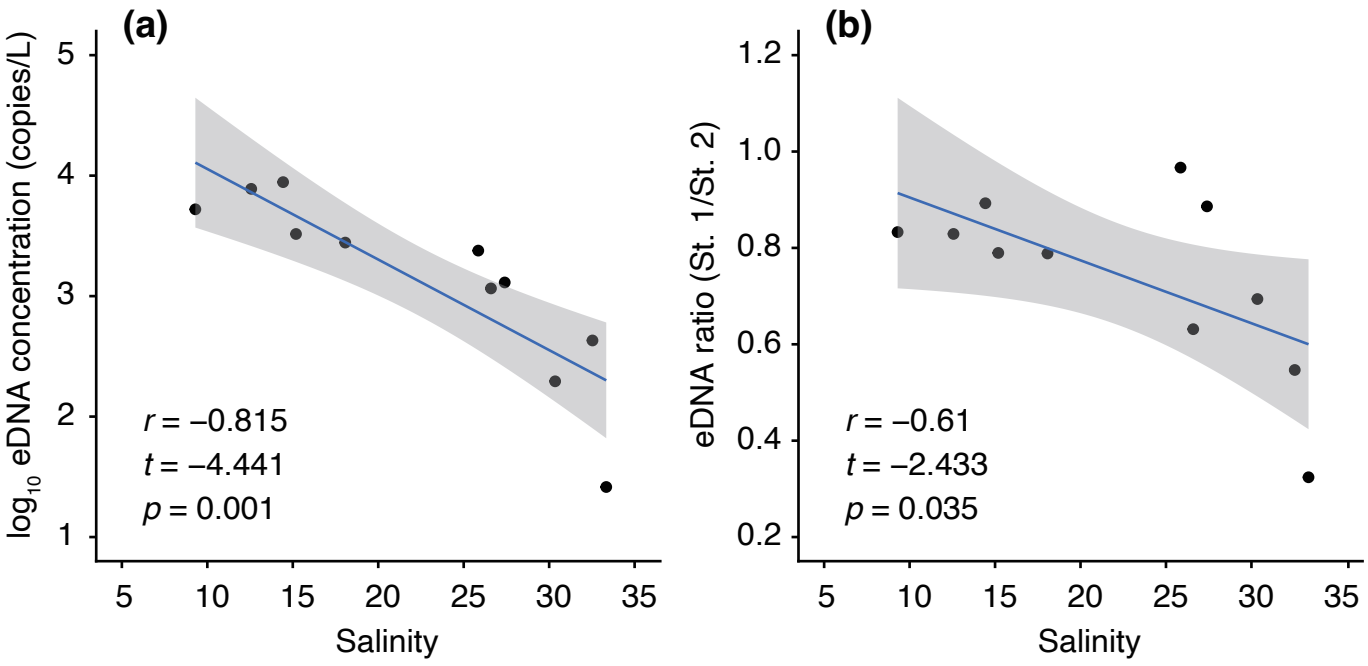
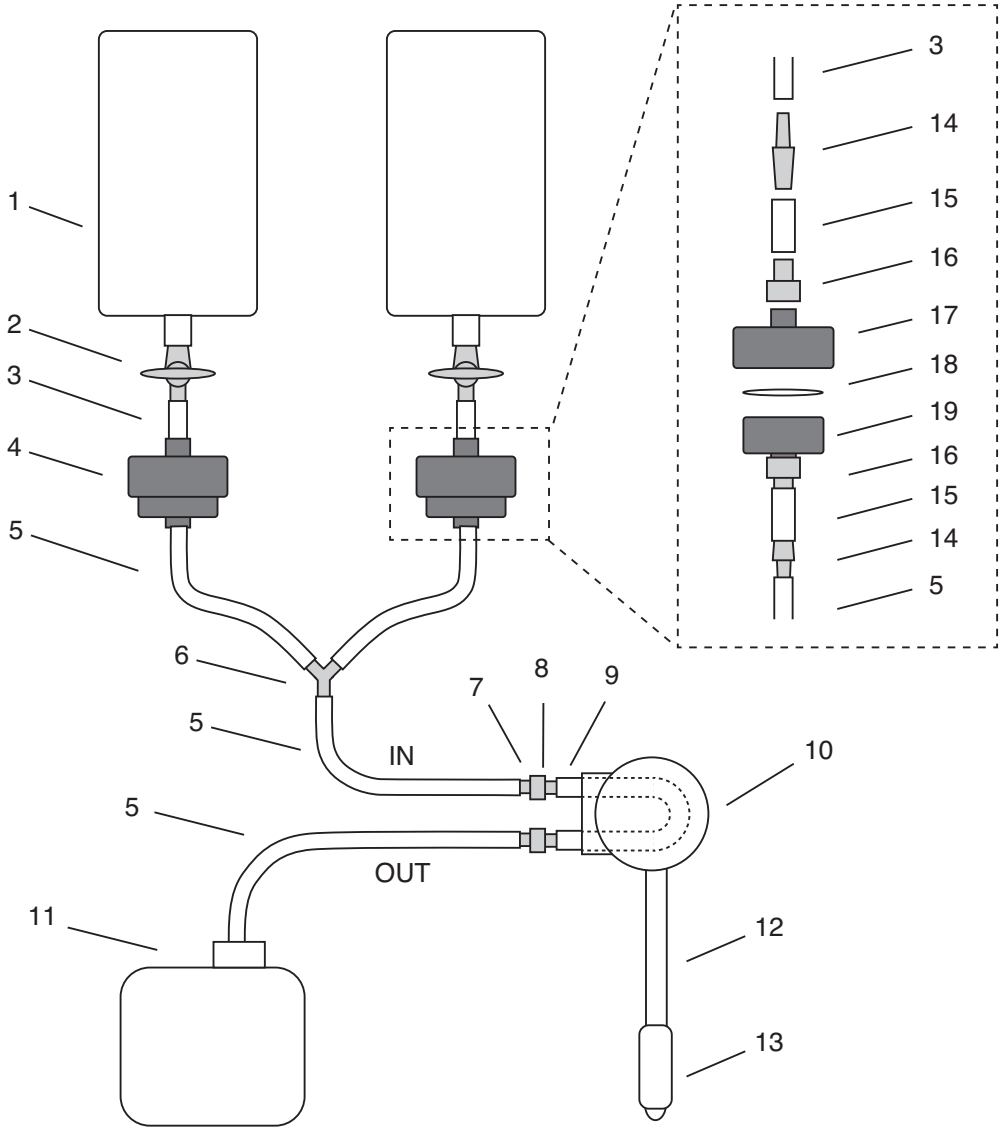


Figure 7 Correlation between salinity and (a) environmental DNA (eDNA) concentration of ayu *Plecoglossus altivelis altivelis* and (b) the ratio of eDNA concentration between St. 1 and St. 2. The results obtained between June and August was included in the analysis. eDNA concentration is used after log-transformation. Pearson's correlation coefficient (r), *t*-value, and *p*-value are indicated in figures.



Supplementary Figure 1 Schematic illustration of the on-site filtration devise. 1 Sealed plastic bag (DP16-TN1000; COWPACK CO., LTD., Aichi, Japan); 2 One-way stopcock (S75-4037-30; Narika, Tokyo, Japan); 3 Silicon tube (internal diameter 6.5 mm); 4 Filter holder (PP-47; ADVANTEC, Tokyo, Japan); 5 Silicon tube (internal diameter 5 mm); 6 Y-shape tubing connector; 7 Female luer lock (VRF606, Nordson MEDICAL, OH, USA); 8 Male luer lock (VRM606, Nordson MEDICAL, H、US AO); 9 Pump tube (internal diameter 4.8 mm); 10 Tubing pump (WPX1; WELCO, Tokyo, Japan); 11 Drain tank; 12 Conductor wire; 13 Cigarette lighter plug DC 12V (No. 1538, Amon Industry, Hyogo, Japan); 14 Stepped tubing connector; 15 Silicon tube (internal diameter 9 mm); 16 Coupler (JF-02W; Joplax, Osaka, Japan); 17 Filter holder (top); 18 Glass fiber filter (Whatman 47 mm GF/F; Cytiva, Tokyo, Japan); and 19 Filter holder (bottom).

Supplementary Table 1 Summary of standard curves for the TaqMan probe qPCR assay for species-specific detection of eDNA of *Plecoglossus altivelis altivelis*. Curves were created by measuring the dilution series of artificially synthesized double-stranded DNA corresponding to the target region of the assay.

Run ID	Concentration of standards	R^2	Intercept	Slope	Efficiency (%)
20210910_ayu_eDNA	$10–10^4$	0.993	40.04	-3.52	92.2
20211006_ayu_eDNA	$10–10^4$	0.997	39.80	-3.48	93.9
20211026_ayu_eDNA	$10–10^4$	0.999	39.24	-3.33	99.6
20211029_ayu_eDNA	$10–10^4$	0.997	40.28	-3.55	91.2
20211030_ayu_eDNA	$10–10^4$	0.996	40.32	-3.60	89.6
20211119_ayu_eDNA	$10–10^4$	0.988	40.00	-3.51	92.8
20211217_ayu_eDNA	$3 \times 10–3 \times 10^4$	0.997	39.25	-3.41	96.4
20211225_ayu_eDNA	$3 \times 10–3 \times 10^4$	0.999	39.83	-3.44	95.3
20220310_ayu_eDNA	$3 \times 10–3 \times 10^4$	0.999	39.74	-3.44	95.5
20220311_ayu_eDNA	$3 \times 10–3 \times 10^4$	1.000	36.45	-3.48	93.8
20220314_ayu_eDNA	$3 \times 10–3 \times 10^4$	0.998	35.98	-3.32	100.1
20220315_ayu_eDNA	$3 \times 10–3 \times 10^4$	0.998	39.42	-3.34	99.5
20220319_ayu_eDNA	$3 \times 10–3 \times 10^4$	0.999	39.47	-3.39	97.2
20220320_ayu_eDNA	$3 \times 10–3 \times 10^4$	0.999	39.47	-3.40	96.8
Mean ± SD		0.997 ± 0.003	39.23 ± 1.33	-3.44 ± 0.09	95.3 ± 3.2