



Title	Stable isotope ratios of emergent adult aquatic insects can be used as indicators of water pollution in the hyporheic food web
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3 Title: Stable isotope ratios of emergent adult aquatic insects can be used as indicators of water
4 pollution in the hyporheic food web

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18

19 **Abstract**

20 Bio-assessment protocols for the subsurface domain of river channels (i.e. hyporheic zone) are
21 scarce despite of known importance of its ecological function in river ecosystems. The larvae of the
22 *Alloperla ishikariana* Plecoptera species inhabit the hyporheic zone. Therefore, we examined
23 whether emergent adult *A. ishikariana* could serve as proxy indicators of the effects of pollution in
24 the hyporheic food web. The study site comprised a 15-km stretch of a gravel bed river in Hokkaido,
25 Japan, from which all invertebrates were collected from the riverbed at a depth of 30–50 cm, and
26 emergent *A. ishikariana* adults were sampled in riparian zones. Water quality changed gradually
27 along the river owing to the effects of a wastewater treatment plant and surrounding land uses.
28 Nitrogen and carbon stable isotope ratios (SIRs) of epilithic biofilms, hyporheic particulate organic
29 matter (POM), benthic POM, and invertebrates were used to determine the major basal carbon
30 sources, the effects of water pollution on the hyporheic zone, and the trophic positions of dominant
31 invertebrates. Generalized linear (mixed) models were used for statistical testing and modeling.
32 Amphipoda and the larvae of *A. ishikariana* were considered top predators and secondary consumers
33 in the hyporheic food web, respectively. The observed similarity in water quality between the
34 hyporheic and surface water suggested a large degree of hydrological exchange between the two
35 zones, and resulted in positive increases in the nitrogen SIRs of hyporheic invertebrates, including *A.*
36 *ishikariana* larvae, in proportion to nitrate levels in the surface water. The nitrogen SIRs of *A.*
37 *ishikariana* adults were significantly correlated with the nitrogen SIRs of their larvae within the
38 same location (adjusted $R^2 = 0.78$), indicating that emerged adults can provide information on the
39 longitudinal variability of the effects of synthetic nitrogen. In contrast, the carbon SIRs of adults did
40 not predict those of their larvae, possibly reflecting the diverse feeding habitats of larvae. Overall,
41 we demonstrated that adult aquatic insects emerging from the hyporheic zone can be used as indirect
42 indicators of pollution-associated nutrient assimilation as well as the spatial heterogeneity of dietary
43 carbon resources in hyporheic food web.

44

45 **Keywords:** Adult aquatic insects; bio-assessment; gravel-bed river; groundwater; stable isotope
46 ratios; trophic positions

47 **1. Introduction**

48 Benthic macroinvertebrates, particularly aquatic insect orders such as Ephemeroptera (E), Plecoptera
49 (P), and Trichoptera (T), are useful indicators of river conditions and are often used in bio-
50 assessment programs (Hodkinson and Jackson, 2005; Bonada et al., 2006; Beyene et al., 2009;
51 Fierro et al., 2017). Macroinvertebrates are a ubiquitous and diverse group that form a vital part of
52 the aquatic ecosystem as can they constitute intermediate and upper trophic levels (Wallace and
53 Webster, 1996; Rosi-Marshall and Wallace, 2002; Negishi et al., 2019a). Macroinvertebrates are
54 relatively sedentary, with variable levels of tolerance to changes in environmental variables such as
55 water quality, sedimentation, and temperature (Cox and Rutherford, 2000; Larsen et al., 2009).
56 Therefore, they can be used to detect a variety of perturbations coming from localized as well as
57 remote sources. Consequently, the effects of human activities, including agricultural practices,
58 mining, and water treatment plants, can be reflected in the community structure, tissue stable isotope
59 signatures, and other biological markers of macroinvertebrates (Kay et al., 2001; Fierro et al., 2017;
60 Rodrigues et al., 2019). Most bio-assessment programs aiming to examine river health using
61 macroinvertebrates adopt benthic sampling, thus failing to represent the conditions below the
62 riverbed within the subsurface boundary. That is, most programs fail to assess the hyporheic zone, in
63 which the mixing of surface and shallow groundwater takes place through porous sediments
64 (Robertson and Wood, 2010). Thus far, only limited attempts have been made to develop bio-
65 assessment protocols for the hyporheic zone (Moldovan et al., 2013).

66 The structure, function, and diversity of freshwater fauna is partially maintained through
67 interactions between the surface water and groundwater in the hyporheic zone (Stanford and Ward,
68 1993; Robertson and Wood, 2010). The hyporheic zone forms a dynamic ecotone in which surface
69 water interactions with ground water are controlled by hydrological exchanges and material
70 transfers, which can fluctuate in response to spatial and temporal variations in channel characteristics
71 (e.g., slope gradient, alluvium volume, hydraulic head) and sediment properties (e.g., porosity)
72 (Malard et al., 2002; Tonina and Buffington, 2009). The crucial functions of the hyporheic zone
73 include nutrient cycling, organic matter processing, and habitat provision for fauna such as insect
74 larvae (Dahm et al., 1998; Hancock, 2002; Lefebvre et al., 2004; Birgand et al., 2007; Boulton et al.,

75 2010; DelVecchia et al., 2016). The invertebrate community found within the hyporheic zone are
76 either permanent hyporheic dwellers (stygobites), or are temporary inhabitants of the hyporheic zone
77 (stygophiles), which spend only part of their life history in the hyporheic zone (Edwards, 1998;
78 Pacioglu, 2010; Vorste et al., 2016). Stygophiles include some insect taxa that rarely occur in or near
79 the surface as larvae, but that migrate to the surface to metamorphose, emerge, mate, and lay eggs
80 (known as amphibites) (Boulton, 2007; Dorff and Finn, 2019). Major challenges in developing
81 bioassessment indicators for the hyporheic environment include the labor-intensive nature of
82 sampling the hyporheic zone (Fraser and Williams, 1997) and the fact that information on the trophic
83 structure and sensitivity of hyporheic ecosystems is scarce (Pacioglu, 2010).

84 Surface water nutrient pollution caused by high nutrient inputs (e.g., nitrogen) from point or
85 non-point sources, such as agricultural fields and wastewater treatment plants, affects aquatic
86 biodiversity and biogeochemical processes and is a pressing environmental concern (Carpenter et al.,
87 1998; Mainstone and Parr, 2002; Hilton et al., 2006). Alterations to water physico-chemistry can
88 lead to changes in the abundances and distribution patterns of invertebrates because species differ in
89 their physiological tolerance capabilities and feeding traits (Townsend et al., 1997; Weijters et al.,
90 2009). The diverse consequences of nutrient pollution extend to changes in aspects of ecosystem
91 functioning, such as litter decomposition and nutrient retention efficiency (Gücker et al., 2006;
92 Woodward et al., 2012). Furthermore, the relative contributions of different basal resources to
93 consumers or energy pathways within the food web can be altered, thereby changing food web
94 structures (Davis et al., 2010; Negishi et al., 2019a). The hyporheic zone is potentially exposed to
95 these changes because it sustains the exchange of water and nutrients between the surface and sub-
96 surface areas (Hancock, 2002). Despite abundant information on the effects of water pollution on
97 benthic invertebrates, the responses of hyporheic organisms have been far less examined, with a
98 majority of studies focusing on the impacts of heavy metals (Nelson and Roline, 2003; Moldovan et
99 al., 2011) or organic pollutants (Williams and Fulthorpe, 2003; Atashgahi et al., 2015). Although an
100 increasing number of studies have demonstrated the impacts of nutrient pollution on community
101 structure of hyporheic organisms (Khebiza et al., 2006; Pacioglu and Moldovan, 2016; Sánchez-

102 Morales et al., 2018), few had examined the responses of food web (Negishi et al., 2019b).

103 Stable isotope ratios (SIRs) can be defined as the natural abundances of stable isotopes and
104 their proportional deviations from standards, and they have been widely-used in ecological studies to
105 determine the structure of, trophic interactions within, and energy and material flow within food
106 webs (Peterson and Fry, 1987; Cabana and Rasmussen, 1994; Vander Zanden et al., 1999). Stable
107 isotopes of carbon (C) can be utilized to identify consumers' carbon sources because of their
108 distinctive proportions in different basal resources, with little changes in the proportion when
109 propagating through multiple trophic levels (France and Peters, 1997). Nitrogen (N) SIRs are an
110 important tool for estimating trophic positions, as consumers' values increase in a step-wise manner
111 as they occupy higher trophic levels (Vanderklift and Ponsard, 2003). Mixing models have long been
112 incorporated along with C and N SIRs to quantify food web characteristics (Post, 2002; Kurle, 2009;
113 Negishi et al., 2019a, b). Nitrogen SIRs are also useful in detecting the influences of anthropogenic
114 activities. Inorganic or organic forms of N from point or non-point sources (e.g., fertilizer, animal
115 waste, effluents, and pulps) are often enriched with heavier isotopes, which can lead to higher
116 proportions of heavier N stable isotopes in consumer tissues (Cabana and Rasmussen, 1996;
117 Hartland et al., 2011; Negishi et al., 2019a,b). After corrections are made to accommodate the fact
118 that N SIRs may change slightly during arthropod metamorphosis (Doi et al., 2007; Tibbets et al.,
119 2008), the stable isotope signatures of adult aquatic insects have the potential to infer the structure of
120 food webs and the effects of nutrient pollution on aquatic macroinvertebrates.

121 The key goal of this study was to establish a method of indirectly assessing the hyporheic
122 environment by collecting and analyzing adult aquatic insects. If this is achieved, more efficient
123 assessments of hyporheic conditions over large spatial scales can be completed to enable sound
124 ecosystem management and to further address research questions concerning the hyporheic zone. We
125 tested whether adult aquatic insects emerging from the hyporheic zone could serve as a proxy
126 indicator of nutrient pollution in the hyporheic food web by analyzing water quality, as well as the
127 SIRs of macroinvertebrates and their basal food resources, in a gravel-bed river. *Alloperla*
128 *ishikariana* (Order: Plecoptera, Family Chloroperlidae) was chosen as a target species because it had

129 previously been identified as an amphibitic species (Negishi, 2019; Negishi et al., 2019b). We
130 hypothesized that the C and N SIRs of *A. ishikariana* adults would reflect the degree to which the
131 food web is exposed to nutrient pollution, and that they would also indicate any associated shifts in
132 dietary carbon sources in the hyporheic zone. The specific objectives of this study were 1) to
133 determine the food web structure, including the trophic position of *A. ishikariana*, in the hyporheic
134 zone, and 2) to examine the relationships between isotopic signatures in *A. ishikariana* larvae and *A.*
135 *ishikariana* adults in relation to variable levels of nutrient pollution exposure.

136 Chloroperlidae stoneflies are omnivores as they can be carnivores, shredders, or scavengers,
137 with species-specific variations in the relative contributions of different food items (Derka et al.,
138 2004; Rúa et al., 2011). Furthermore, the N SIRs of the larvae and the contributions of autotrophic
139 resources to dietary carbon, as revealed through C SIRs, have been shown to vary substantially
140 across sites with different levels of exposure to nutrient pollution (nitrate, NO_3^-) (Negishi et al.,
141 2019b). Therefore, we specifically predicted that 1) *A. ishikariana* larvae would consistently occupy
142 either the primary or secondary consumer trophic level in the hyporheic food web, regardless of
143 pollution level, and 2) spatial variations in both the C and N SIRs of adults would explain the C and
144 N SIRs in larvae (1:1 relationship). The experiment was also designed to test whether nitrate
145 concentrations were similar between the surface water and the hyporheic zone within the same
146 sample site, and whether there was a linear relationship between the spatial variation in nitrate
147 concentration and the N SIRs of larvae, in order to assess the validity of using tissue N SIRs of
148 hyporheic larvae as indicators of nutrient pollution in hyporheic zone.

149

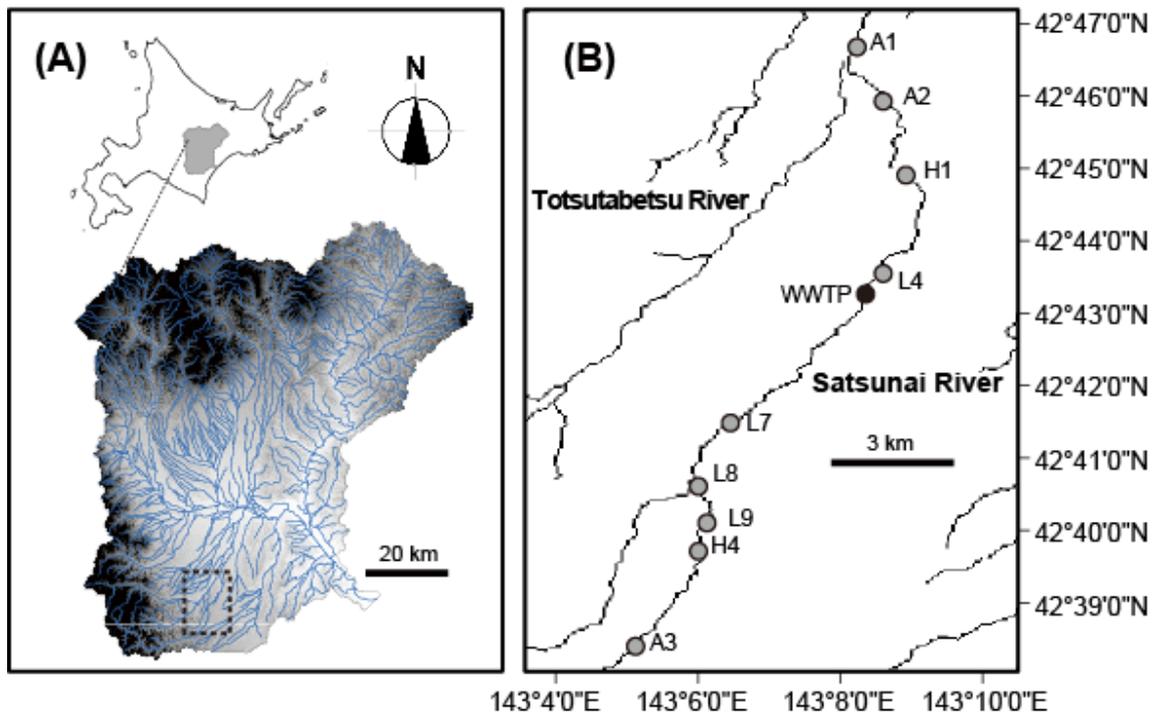
150 **2. Materials and Methods**

151 2.1 Study site

152 This study was conducted from June 2016 to September 2018 in the Satsunai River (catchment area,
153 725 km²), which runs from Mt. Satsunai (42°41' N, 142°47' E; 1,895 m above sea level) to the
154 Tokachi River, in eastern Hokkaido, Japan (**Fig. 1**). Nine study sites (A1, A2, H1, L4, L7, L8, L9,

155 H4, and A3) were selected, and all sites were considered typical gravel-bed river landscapes with
156 riparian areas, gravel bars, and braided river channels. The studied stretch of the river was mostly
157 diked on both sides, with the inter-dike width of the channel and riparian zones extending over
158 approximately 350 m and with a main channel width of 30–50 m (**S1**). The gravel bars were either
159 interconnected with riparian forest on one side and with the channel on the other side, or with the
160 channel on both sides. The regional climate is characterized as having lower air temperature and
161 precipitation in winter and higher temperature and precipitation in summer. The annual mean
162 (\pm standard deviation) precipitation from 1999–2018 was 1,181.3 (\pm 204.2) mm (Kami-satsunai
163 station, Japan Meteorological Agency), and the mean (\pm standard deviation) daily flow rate from
164 1999–2016 was 11.1 (\pm 17.6) m³/sec [Kami-satsunai station, Ministry of Land, Infrastructure,
165 Transport and Tourism (MLIT)]. A wastewater treatment plant discharges water enriched with
166 dissolved and particulate solids into the main channel through a small tributary (flow rate =
167 approximately 0.8 m³/sec) (**Fig. 1**). Together with the impact of surrounding agricultural fields,
168 water quality gradually degraded from upstream to downstream, thus forming a longitudinal gradient
169 in the level of surface water pollution by nutrients such as nitrate and dissolved phosphorous. More
170 detailed background information on the study site can be found in Negishi (2019) and Negishi et al.
171 (2019b).

172



173 **Fig. 1** Location of the Tokachi River watershed in Hokkaido, Japan, with the study area indicated by
 174 a dotted rectangle (A), and the study sites in the Satsunari River (B). The blue lines in (A) indicate
 175 the major river channels. The filled circle in (B) denotes the confluence of the study area and the
 176 tributary containing the effluent from the wastewater treatment plant (WWTP).

177

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180

181 2.2 Collection of hyporheic macroinvertebrates and *A. ishikariana* adults

182 Hyporheic macroinvertebrates were collected using colonization traps installed in the riverbed,
183 which were installed as a part of various parallel studies focusing on the hyporheic zone (S2; Negishi
184 et al., 2019b). The sediment collected *in situ* was rinsed thoroughly and was enclosed in 4 mm×3
185 mm mesh nylon netting. To estimate the abundance and taxonomic composition of hyporheic
186 invertebrates, quantitative traps were set by enclosing a mixture of sediment with diameters of 4–
187 22.4 and 22.4–64 mm so that the traps contained a total sediment volume of 3,800 cm³. Qualitative
188 traps were also constructed to obtain sufficient samples for SIR analyses. The volume of sediment in
189 the qualitative traps was not measured precisely or normalized, and the traps contained an estimated
190 7,000–10,000 cm³ of sediment. Within a 100-m section per site, a total of eight to 24 traps were
191 installed at a depth of 30–50 cm using a backhoe. Traps were collected from two sites in 2016 (H1
192 and H4), three sites in 2017 (A1, A2, and A3), and four sites in 2018 (L4, L7, L8, and L9). Traps
193 were manually excavated and retrieved using a hand-held D-frame net (375-µm mesh) within 30 to
194 60 days of installation (retrieval dates were 9th and 16th July 2016, 14th July and 30th August 2017, 9th
195 August and 4th November 2018). This retrieval procedure was done immediately after the removal of
196 sediment above the trap to ensure that macroinvertebrates and other detritus from the surface zone
197 did not contaminate the samples from the hyporheic zone. In total, we used samples obtained from
198 24 quantitative traps in 2016, six qualitative and 48 quantitative traps in 2017, and 32 quantitative
199 traps in 2018 for this study.

200 Within 30 minutes after traps were retrieved from the riverbed, the sediment was rinsed
201 thoroughly with water, and organic matter including macroinvertebrates (HPOM) was sieved
202 through a 500-µm stainless steel mesh. Additionally, *A. ishikariana* adults were collected from all
203 sites by handpicking or net-sweeping between 18th and 26th July, 2018 (S3). At each site, individuals
204 were visually spotted and caught from the edge of riparian forests within a 50 m lateral distance
205 from the river channel. All samples were kept in an ice chest and were transported to the laboratory
206 within two days of collection. Larval samples were immediately processed or kept frozen for later
207 analyses, while adult samples were immediately frozen.

208 2.3 Collection of benthic samples and measurements of abiotic factors

209 Our previous study suggested that epilithic biofilms on riverbed substratum and benthic particulate
210 organic matter (BPOM) were potential basal resources in the hyporheic zone (Negishi et al., 2019b).
211 Thus, biofilms and BPOM were collected twice (July and August 2017) at three sites for which food
212 web analyses were possible (i.e., A1, A2, and A3; see “Laboratory analyses”). Biofilms were
213 removed by scrubbing five to seven submerged cobbles that were collected from two areas (one pool
214 and one riffle) per site, and two composite slurry samples (one each for riffle and pool) were formed
215 per site per sampling occasion. Additionally, BPOM was collected by vigorously disturbing the
216 riverbed in two areas where biofilm samples were collected by foot for 10 min each, and debris was
217 immediately caught downstream using a D-frame net (375- μ m mesh); two samples per 10-min
218 collection were mixed together to represent each site. Biofilm and BPOM samples were transported
219 as other samples were, and were immediately frozen.

220 Water samples were collected in acid-washed polyethylene bottles to determine nitrate
221 concentrations. Hyporheic water was collected using PVC wells installed within 30 cm of
222 quantitative traps (Negishi et al., 2019b; **S4**). Hyporheic water collection was carried out
223 simultaneously with surface water collection so that the water quality in both zones could be
224 compared in a pair-wise manner. The electrical conductivity (EC) of the surface water served as an
225 indicator of water chemistry and was measured on the same day as hyporheic sampling as well as
226 during other field visits at intervals of twice a day to 30 days, using a portable probe (model WM-
227 32EP, DKK-TOA Co., Japan) (**S5**). To obtain further information on the relationship between nitrate
228 and EC, samples were occasionally collected to be used in nitrate measurements of these water
229 samples. All water samples were immediately transported to the laboratory in an ice chest.

230

231 2.4 Laboratory analyses

232 Both HPOM and thawed BPOM samples were passed through stainless steel sieves (1 mm and 500
233 μ m, respectively), and were examined using a stereo microscope (Model SZX7, Olympus

234 Corporation, Japan). All macroinvertebrates were removed. For HPOM, the following numerically-
235 dominant macroinvertebrates identified as hyporheic dwellers in Negishi et al. (2019b) were kept to
236 provide samples for food web analyses while other individuals were kept for other purposes (**S3**):
237 Amphipoda (*Pseudocrangonyx yezonis* Akatsuka and Komai, 1922), Isopoda (Asellota), *A.*
238 *ishikariana*, Oligochaeta, and Diptera (Chironomidae). Larvae of *A. ishikariana* were collected at all
239 sites, while sufficient numbers of individuals for other taxa were obtained at only three sites (i.e.,
240 A1, A2, and A3). All individuals were kept frozen until later analyses. The remainder of each
241 fraction void of invertebrates was kept frozen as samples of fine (500 µm to 1 mm) and coarse (>1
242 mm) HPOM and BPOM. Samples of HPOM from 2016 were not collected and unavailable for this
243 study.

244 Upon arrival at the laboratory, water samples were filtered using glass-fiber filters with a
245 pore size of 0.45 µm (GC-50, Advantec Co., Japan), and were kept refrigerated until further analyses
246 within a week of collection. Concentrations of nitrate were determined using ion chromatography
247 (IA-300, TOA-DKK Co., Japan).

248 Macroinvertebrate samples (including adult insects) were thawed within two months and
249 were dried at 60°C for 48 h. Whenever possible, individuals were ground to form independent
250 powder samples, although multiple individuals were pooled together if the amount of sample
251 obtained was insufficient. No *A. ishikariana* samples were pooled together and 3 – 15 and 4 – 10
252 individual larvae and adults were collected at each site for a total of 52 and 63 individuals,
253 respectively. Biofilm samples were thawed, centrifuged to discard the supernatant, dried at 60°C for
254 at least 48 h, and then ground into a fine powder using a mortar and pestle. The POM samples were
255 also dried and ground using the same method as for the biofilm samples. Both biofilm and POM
256 samples were then treated with 0.1 mol HCl to remove inorganic carbon before being dried once
257 again. These samples were used for SIR analyses.

258 Carbon and N SIRs are expressed using the following delta notation as the relative
259 differences between samples and conventional standards [Vienna Peedee Belemnite (VPDB) for C
260 and air N₂ for N, respectively,]: $\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1,000$. Where, X represents the isotope

261 of interest (e.g., ^{13}C) and R denotes the relative abundance of stable isotopes of the respective
262 element in samples and standards (e.g., $^{13}\text{C}/^{12}\text{C}$). The SIRs of C ($\delta^{13}\text{C}$) and N ($\delta^{15}\text{N}$) were
263 determined using two types of isotope ratio mass spectrometers each coupled with elemental
264 analyzers (Finnigan-MAT252, Thermo Fisher 227 Scientific, Florence, KY, USA and Delta V plus,
265 Thermo Fisher Scientific, Bremen, Germany). Standards with known delta values (such as tyrosine)
266 were analyzed at least every 10 runs to confirm reproducibility (standard deviations for C and N
267 were 0.10‰ and 0.14‰ using MAT252 and 0.13‰ and 0.27‰ using Delta V). The accuracy of the
268 measurements as minimum and maximum values were within the range of +0.28 to -0.23‰ (C) and
269 +0.33 to -0.34‰ (N) for MAT252, and +0.74 to -0.51‰ and +0.29 to -0.24‰ for Delta V compared
270 to the true values of the standards.

271

272 2.5 Statistical analyses

273 We examined whether surface water nutrient pollution affected hyporheic invertebrates and food
274 webs, as predicted, by examining the relationship between nitrate concentration in the hyporheic
275 zone and the N SIRs of hyporheic macroinvertebrates. The SIRs of animal tissues are relatively
276 long-term and temporally integrated indications of the diet (e.g., Overmyer et al., 2008). Therefore,
277 it is preferable to collect long-term water quality data in the hyporheic zone to infer the effects of
278 water quality on tissue SIRs. However, long-term continuous hyporheic water chemistry data were
279 unavailable in the present study due to logistical difficulties in maintaining sampling wells in the
280 relatively large unstable study rivers. Thus, we took the following two steps to ensure that surface
281 water EC measurements, which were taken much more frequently (**S5**), could be used as a proxy for
282 long-term nitrate concentration in the hyporheic zone. It was predicted that nitrate concentration
283 would be strongly correlated with EC, and that nitrate concentrations in hyporheic and surface water
284 would be comparable at each site despite of high variations among sites, according to previous
285 studies (Cañedo-Argüelles et al., 2013; Negishi et al., 2019b). If these two predictions were proven
286 true, we assumed that the average surface water EC values for each site would represent the long-
287 term average nitrate concentration in the hyporheic zone.

288 Therefore, using simultaneously measured paired EC and nitrate data, we firstly developed a
289 GLMM with EC as a response variable, nitrate concentration as a main factor, and sampling date as
290 a random factor. Second, we determined whether nitrate concentration differed among sites but not
291 between habitats by developing a generalized linear mixed model (GLMM) with nitrate
292 concentration as a response variable, site, habitat and their interaction as main factors, and sampling
293 date as a random factor. Third, using all of the available EC data, we tested whether surface water
294 EC differed among sites by developing a GLMM with sampling site as a main factor and sampling
295 date as a random factor.

296 We estimated contributions (fraction) of major dietary carbon sources to consumers, as well
297 as the trophic positions of each taxon, for the three sites (A1, A2, and A3) where resource and
298 consumer data were both available. Five types of end-members, fine and coarse fractions of both
299 HPOM and BPOM, and biofilms, were considered as potential food resources. In preliminary
300 analyses, HPOM and BPOM were distinguishable in terms of C SIRs, whereas POM size did not
301 affect the values (**S6**). Thus, the POM samples from the hyporheic and benthic zones were pooled
302 for each site, irrespective of size class. Bayesian stable isotope mixing models were used to calculate
303 the contribution of biofilm and POM to each consumer taxon at each site (Parnell et al., 2010). Only
304 C SIRs were included in the model without fractionation factors because insufficient information on
305 the accurate trophic position was available a priori and the C fractionation factor can be considered
306 minimal (Post, 2002). Then, consumers' trophic positions were estimated based on the N SIRs of
307 basal resources using contribution fractions obtained from the mixing model as follows:

308 Trophic position of consumer = $(\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{baseline}}) / f + \lambda$

309 Where, $f = 3.4$, $\lambda = 1$ (primary producer) (Post, 2002), and

310 $\delta^{15}\text{N}_{\text{baseline}} = \text{mean biofilm contribution} \times \text{mean biofilm } \delta^{15}\text{N} + \text{mean HPOM}$
311 $\text{contribution} \times \text{mean HPOM } \delta^{15}\text{N} + \text{mean BPOM contribution} \times \text{mean BPOM } \delta^{15}\text{N}.$

312 We then tested whether the N SIRs of *A. ishikariana* larvae were correlated with surface
313 water EC (as a proxy for long-term nitrate concentration in the hyporheic zone) by developing a

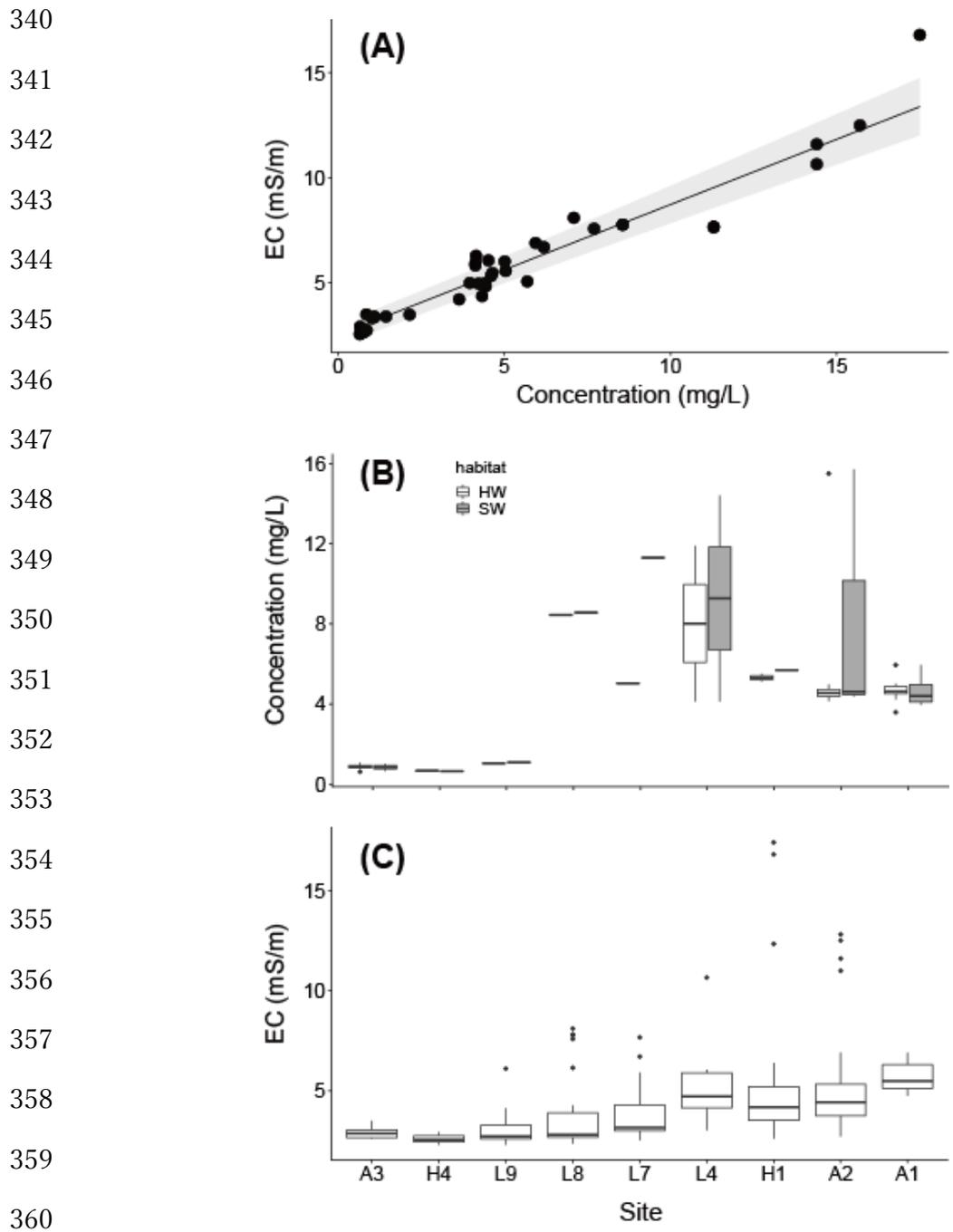
314 generalized linear model (GLM) with mean N SIRs of *A. ishikariana* larvae at each site as a
315 response variable and mean EC values as a main factor. A comparable model was also run using
316 mean nitrate in the hyporheic zone as a main factor, in order to examine whether the less frequently-
317 measured but direct measurement of nitrate resulted in the same interpretation. Lastly, we examined
318 whether the C and N SIRs of adults could predict those of larvae by developing GLMs with the
319 mean SIRs of larvae as the response variable and the mean SIRs of adults collected at the
320 corresponding sites as the main factor.

321 All statistical analyses were performed in R (version 3.5.2, R Core Team, 2018). All
322 GL(M)Ms adopted Gaussian error distribution. The “glmmADMB” package was used for GLMMs.
323 The effects of factors were tested by comparing them with models without variables of interest
324 sequentially with likelihood-ratio tests. The statistical significance level was set to $p=0.05$. Model
325 accuracy (the goodness-of-fit) was reported according to Nakagawa & Schielzeth (2013), who
326 proposed a further generalization of R^2 to generalized linear mixed effects models (GLMMs). The
327 “MuMIn” package was used to estimate marginal R-squared values, which were associated with
328 fixed effects as well as the conditional R-squared values, which were further associated with fixed
329 effects plus the random effects. For GLMs, ordinary R^2 and adjusted R^2 values were reported.

330

331 **3. Results**

332 Electrical conductivity (EC) was positively predicted by nitrate concentration in the surface water
333 (**Fig. 2A; Table 1**). Nitrate concentration differed among sites but did not differ between habitat
334 types, as seen by the presence of the significant effect of site without the effects of either habitat or
335 interaction (**Fig. 2B; Table 1**). There were differences in surface water EC among sites, as indicated
336 by the significant effect of site (**Table 1**). Site differences in both nitrate and EC were reflected
337 in higher values in downstream sites, particularly those located downstream of the wastewater
338 treatment plant (**Fig. 1; Fig. 2B and C**). The R^2_m and R^2_c values of the EC–nitrate relationship
339 were similar, indicating that the relationship was negligibly affected by sampling date.



361 **Fig. 2** Relationship between nitrate concentration and the corresponding electrical conductivity (EC)
 362 of surface water (A), nitrate concentrations in surface water (SW) and hyporheic water (HW) (B), ,
 363 and EC of surface water at each study site (C).

(a)	N	R ² m	R ² c	logLik	AICc	p-value
Full model						
Nitrate concentration	40	0.912	0.930	-48.8	107	<0.001
Null model	40			-96.2	199	

364

(b)	N	R ² m	R ² c	logLik	AICc	p-value
Full model						
Habitat (H), site (S), H×S	61	0.604	0.866	-124.6	310.2	0.29
1 st Reduced model						
H, S	61	0.587	0.834	-129.4	289.2	<0.001 ^{H, 0.31} ^S
2 nd Reduced model						
H	61	0.033	0.326	-162.6	334.0	0.07
S	61	0.582	0.831	-129.9	287.1	<0.001
Null model	61			-163.9	334.4	

365

(c)	N	R ² m	R ² c	logLik	AICc	p-value
Full model						
Site	173	0.156	0.740	-319	662	<0.001
Null model	173			-357	710	

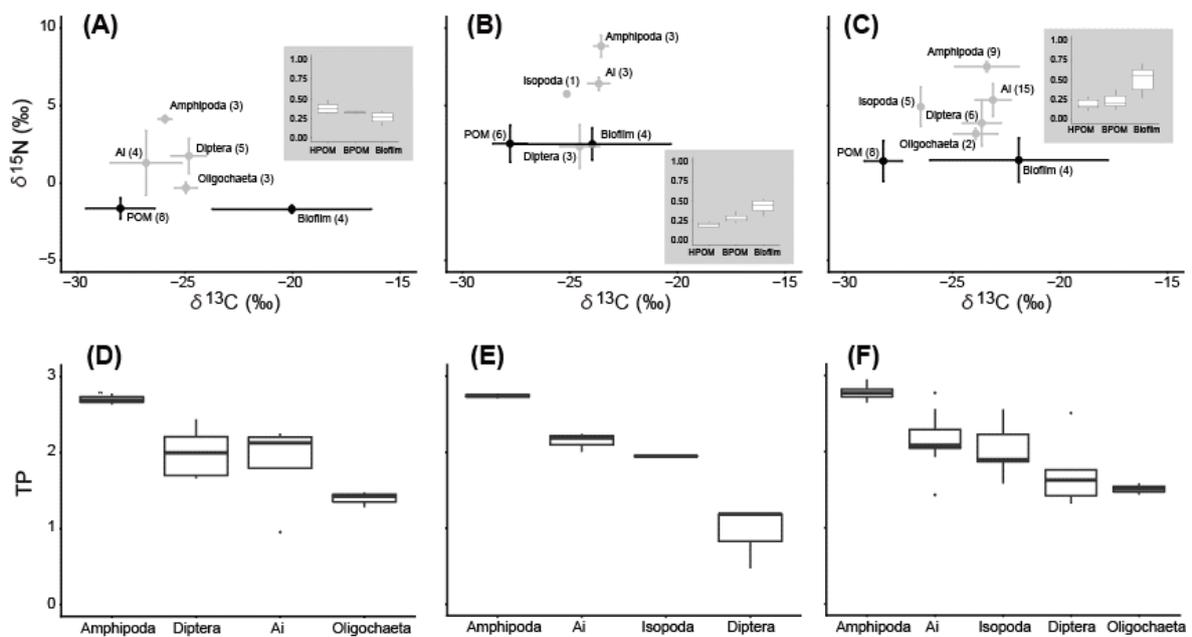
366

367 **Table 1** Results of generalized linear mixed models testing the effects of nitrate concentration on
368 electrical conductivity of surface water collected simultaneously with sampling date as a random
369 factor (a), the effects of habitat (benthic or hyporheic), site and their interactions on nitrate
370 concentration of water, with sampling date as a random factor (a), and the effects of sampling sites
371 on electrical conductivity of surface water with sampling date as a random factor (c). Gaussian error
372 distribution was adapted in all the models. The full model and reduced model were compared using a
373 likelihood ratio test N denotes sample size. For (b), when full model was insignificant, 1st reduced
374 models were compared to 2nd reduced models and sequentially to the null models; Superscripts of p-
375 values indicate the variables removed from the model to test with those from reduced models by one
376 level. R²m and R²c denote marginal R squared values, which were associated with fixed effects, as
377 well as the conditional R squared values, which were associated with fixed effects plus the random
378 effects, respectively. “logLik” and “AICc” are log-likelihood value and Akaike information criterion
379 corrected for small sample sizes. Bold letters for p-value denote statistical significance.

380

381

382 Consumer isotope values were within those of biofilms and POM, indicating that these two
 383 dietary C sources were likely food resources for consumers (**Fig. 3**). The C SIRs of resources varied
 384 among resource types, whereas N SIRs were similar among types at all sites. The mean N SIRs of all
 385 consumers and resources used in the analyses were much higher at sites A1 (5.5‰) and A2 (5.9‰)
 386 compared to A3 (1.7‰). The relative contributions of dietary C sources differed among sites, with
 387 biofilm being more than or as important as POM at sites A1 and A2, and with POM being more
 388 important with greater contributions at A3 (**Fig. 3**). The relative contributions of HPOM and BPOM
 389 were comparable across all sites. The trophic positions of consumers were generally consistent
 390 among sites, with Amphipoda occupying the highest trophic position and *A. ishikariana* occupying a
 391 position between Amphipoda and other consumers, at approximately the 2nd trophic position (**Fig. 3**).



392

393 **Fig. 3** Mean (\pm standard deviations) carbon and nitrogen stable isotope ratios of resources and
 394 consumers (A, B, and C, for sites A3, A2, and A1, respectively,) and estimated trophic positions of
 395 consumers (D, E, and F, for sites A3, A2, and A1, respectively,). The inset plots in the upper three
 396 panels denote the estimated relative contributions (fractions) of resources to total dietary carbon
 397 sources for the food web at respective sites; HPOM and BPOM represent hyporheic POM and
 398 benthic POM, respectively. “Ai” denotes *Alloperla ishikariana*, and POM in upper panels represents
 399 data including both HPOM and BPOM. Bracketed numbers in the upper panels are sample sizes.

400

401 The N SIRs of larvae significantly increased with increasing EC values (**Fig. 4A; Table 2**).

402 Although the N SIRs of larvae did not increase with increasing levels of nitrate when data from all

403 sites were included (**Fig. 4B; Table 2**), the relationship became significant ($p < 0.01$) when data from

404 L8 (a single measurement on August 9, 2018; **S4**) was removed. The L8 measurement (7.77 mS/m)

405 represented the EC of surface water measured simultaneously with nitrate in the hyporheic zone at

406 the same site. This was dramatically higher than other measurements at the same site (mean \pm

407 standard deviation, sample size; 3.48 ± 2.22 , 32) and was considered an anomalous measurement

408 possibly caused by the relatively large contribution of locally-upwelling groundwater rich in nitrate

409 to the surface water under low flow conditions (the lowest hourly summer water level in the period

410 2016-2018, Kami-satsunai station, MLIT, unpublished data). Adult N SIR values significantly

411 predicted those of larvae, whereas adult C SIR values were not able to predict those of larvae within

412 the same site (**Fig. 5; Table 2**).

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429 **Fig. 4** Relationship between electrical conductivity (EC) and stable nitrogen isotope ratios of *A.*

430 *ishikariana* larvae (A) and the relationship between nitrate concentration and stable nitrogen isotope

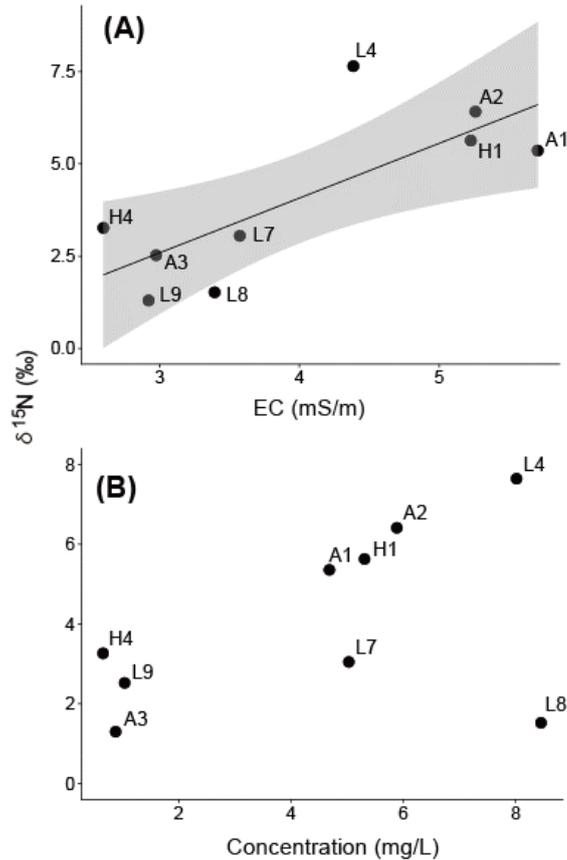
431 ratios of *A. ishikariana* larvae (B). Each data point represents the means for each sampling site, with

432 site names indicated by labels. The solid and gray zones in (A) denote a statistically significant

433 regression line developed from a generalized linear model and a 95% confidence interval of the

434 mean.

435



(a)	N	R ²	R ² _{Adj}	logLik	AICc	p-value
Full model						
Electrical conductivity	9	0.589	0.530	-15.6	41.9	<0.05
Null model				-19.6	45.1	

436

(b)	N	R ²	R ² _{Adj}	logLik	AICc	p-value
Full model						
Nitrate concentration	9	0.221	0.110	-18.4	297	0.134
Null model				-19.6	295	

437

(c)	N	R ²	R ² _{Adj}	logLik	AICc	p-value
Full model						
Carbon SIR	9	0.0845	-0.0462	-15.4	41.7	0.448
Null model				-15.8	37.7	

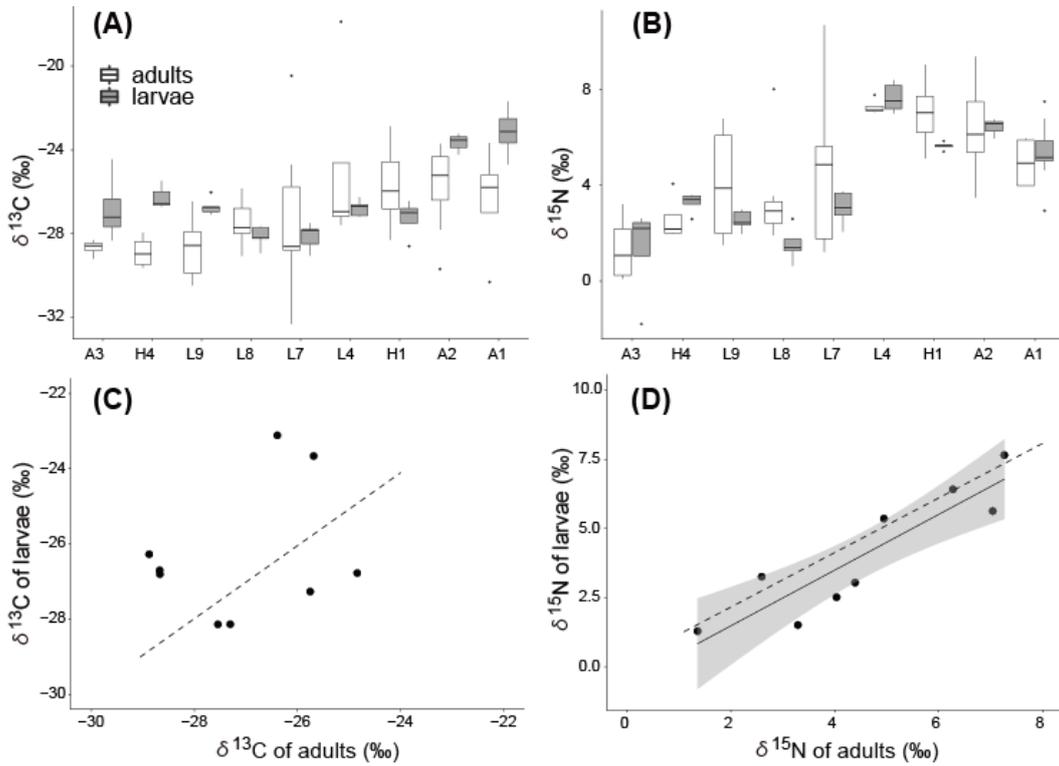
438

(d)	N	R ²	R ² _{Adj}	logLik	AICc	p-value
Full model						
Nitrogen SIR	9	0.809	0.782	-11.1	33.0	<0.001
Null model				-18.6	43.1	

439

440 **Table 2** Results of generalized linear models testing the effects of electrical conductivity (means of
441 all the measurements at each site) on means of stable isotope ratios (SIRs) of nitrogen for larvae of
442 *A. ishikariana* (a), the effects of nitrate concentration (means of all the measurements in the
443 hyporheic zone at each site) on means of SIRs of nitrogen for larvae of *A. ishikariana* (b), and the
444 predictability of adults of *A. ishikariana* for their larvae in terms of SIRs of carbon (c) and nitrogen
445 (d). Gaussian error distribution was adapted in all the models. The full model and reduced model
446 were compared using a likelihood ratio test N denotes sample size whereas R² and R²_{Adj} denote R-
447 squared and adjusted R-squared, respectively. “logLik” and “AICc” are log-likelihood value and
448 Akaike information criterion corrected for small sample sizes. Bold letters for p-value denote
449 statistical significance.

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452

453 **Fig. 5** Boxplots showing the carbon (A) and nitrogen (B) stable isotope ratios of *A. ishikariana*
 454 larvae and adults at each study site. Relationships between the mean carbon (C) and nitrogen (D)
 455 stable isotope ratios between adults and larvae are also shown. The solid and gray zones in (D)
 456 denote a statistically significant regression line developed from a generalized linear model and a
 457 95% confidence interval of the mean. Dotted lines in the lower panels denote 1:1 relationships
 458 between the values of adults and larvae.

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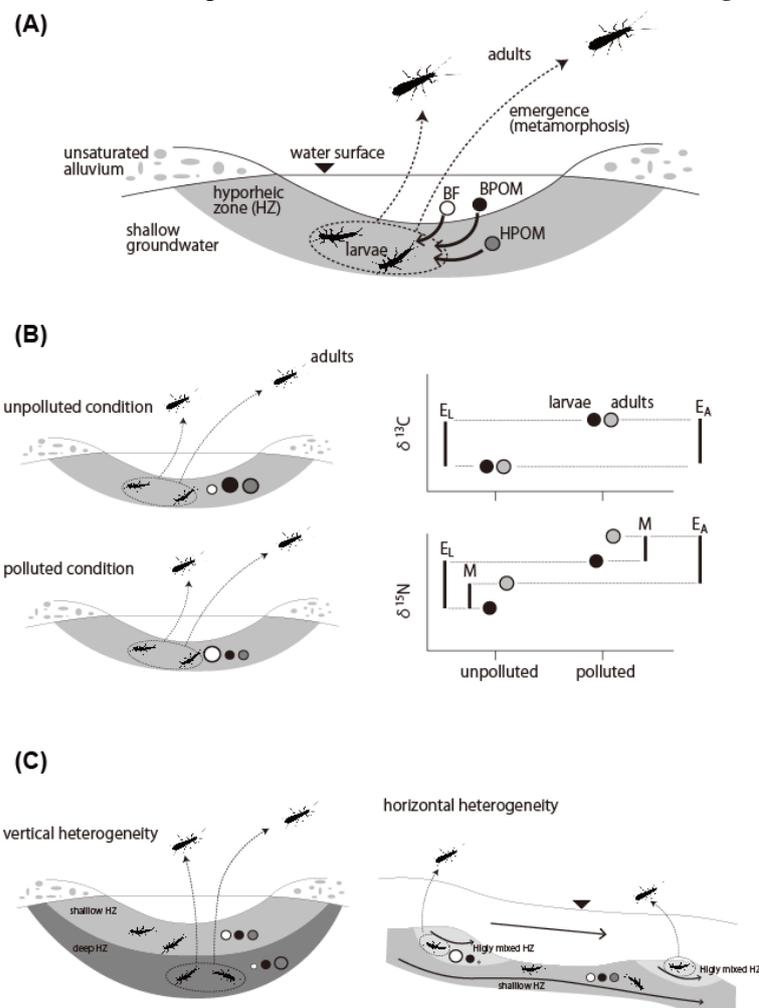
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462 **4. Discussion**

463 We examined whether adult aquatic Plecopteran insects (*Alloperla ishikariana*) emerging from the
464 hyporheic zone can act as a proxy indicator of nutrient pollution in the hyporheic food web in a
465 gravel-bed river. Hyporheic food webs consisting of invertebrates were found to contain *A.*
466 *ishikariana* larvae acting as secondary consumers, regardless of the variations in major dietary
467 carbon sources associated with nutrient pollution levels. Furthermore, the variations in nitrogen SIRs
468 of *A. ishikariana* larvae across the study sites, which were attributable to a gradient of nutrient
469 pollution, were linearly predicted by those of adults collected in adjacent riparian zones. These
470 results supported our predictions. In contrast, the carbon SIRs of adults did not predict those of
471 larvae, partially rejecting the initial hypothesis. Overall, this study reports a novel system in which
472 emergent adults of amphibitic Plecopteran insects can provide indirect measurements of food web
473 structure and the effects of water pollution on consumers in the hyporheic zone.

474 The spatial variation in surface water quality within the study stretch was consistent with
475 commonly-reported gradual longitudinal changes in nutrient content along rivers in response to
476 human activities in watersheds (Carey and Migliaccio, 2009; Morrissey et al., 2013). We relied on
477 EC data as a long-term proxy of nitrate content, and EC can be also affected by other water
478 characteristics such as salinity or total dissolved solids (Cañedo-Argüelles et al., 2013). However,
479 the strong correlation between EC and nitrate content justifies the initial hypothesis that nitrate,
480 which gradually increased along the river, was one of the key compounds characterizing water
481 quality changes. As for how surface water pollution affects water quality in the hyporheic zone,
482 Negishi et al. (2019b) showed the differences in hyporheic zone water quality between two sites
483 (data collected at H1 and H4 in 2016) and inferred that hydrological exchanges with surface water
484 were taking place at high rates based on the temporal synchrony of temperature, comparable mean
485 temperatures, and similar concentrations of chemical constituents including nitrate. The present
486 findings clearly demonstrated that water quality, at least in terms of nitrate, was comparable between
487 surface and subsurface zones throughout the study stretch, which suggests that the upper 30–50 cm
488 of the riverbed was persistently highly hydrologically interactive with the surface water.

489 Hydrological connectivity is the driver by which material exchange and associated biogeochemical
 490 processes such as nitrogen species transformations occur at the interface between surface and
 491 subsurface zones (Dahm et al., 1998; Hartwig and Borchardt, 2015). Such connectivity has
 492 important effects on the types of dietary carbon resources that fuel the hyporheic food web. In
 493 groundwater ecosystems, primary production is absent, and thus microbial biofilms and/or buried
 494 organic matter comprise the base of the food web (Baker et al., 2000; Craft et al., 2002). If high
 495 hydrological connectivity allows down-welling transport of organic matter from the surface zone,
 496 hyporheic organisms can also have access to food resources such as algae-derived carbon provided
 497 by surface zones with access to sunlight (Jones et al., 1995; Malard et al., 2002; **Fig. 6A**).



513 **Fig. 6** Conceptual diagrams showing food resource variations of *A. ishikariana* larvae in relation to
 514 environmental heterogeneity and source habitats of emergent adults. (A) represents a cross-sectional
 515 profile of a gravel-bed river channel with larvae of *A. ishikariana* inhabiting the hyporheic zone;
 516 larvae are able to utilize biofilms (BF), benthic POM (BPOM), or hyporheic POM (HPOM) as their

517 sources of dietary carbon (DC). (B) demonstrates possible variations in the dependences of *A.*
518 *ishikariana* larvae on different C sources in relation to nutrient pollution when adults originate from
519 the studied larvae. The sizes of the bubbles represent the relative contribution to the diet of *A.*
520 *ishikariana*. With stimulated BF primary productivity under polluted conditions, the contribution of
521 BF to the hyporheic food web increases. These changes can be reflected by changes in C and
522 nitrogen (N) stable isotope ratios (SIRs) in *A. ishikariana* tissues, with both C and N SIRs
523 increasing; an increased contribution of biofilm results in more ¹³C-enriched C SIRs of both adults
524 and larvae whereas anthropogenic N assimilation results in more ¹⁵N-enriched N. “M” refers to
525 possible isotope fractionation during metamorphosis, which is expected to be consistent regardless
526 of water quality. E_A and E_L are the SIRs enrichments in adults and larvae, respectively. In this case,
527 both the C and N SIRs of adults indirectly indicate the spatial variations in the C and N SIRs of
528 larvae. (C) presents two hypothetical cases in which adults do not represent the trophic niches or
529 effects of pollution on larvae due to disparity between the source areas of adults and sampling
530 locations of larvae. Vertical and horizontal heterogeneity in hyporheic habitat conditions can cause
531 errors in the predictability larvae C SIRs from the C SIRs of adults because food item variations can
532 alter C SIRs but not N SIRs (see the upper panels in Fig. 3).

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534

535

536 The food web structure in the hyporheic zone is poorly understood, with very little
537 information available on the functional roles of invertebrates, and resolving this remains a priority in
538 understanding hyporheic processes (Pacioglu, 2010; Marmonier et al., 2012). The present study
539 identified a food web with multiple trophic levels in the hyporheic zone, which consistently
540 contained Amphipoda as a top predator. Crustaceans, including Amphipoda, are among the most
541 commonly-reported organisms in groundwater aquifers and hyporheic zones, and are known to
542 possess flexible feeding modes, including scavenging (Mori et al., 2012; Moldovan et al., 2013).
543 Furthermore, *A. ishikariana* larvae appeared to act as secondary consumers approximately
544 occupying trophic level 2, which is consistent with the polyphagous feeding habits reported for
545 epigeal species in the same family (Fuller and Stewart, 1977), and were an important trophic link as
546 they constituted prey for Amphipoda. These two taxa occupied similar trophic positions at sites H1
547 and H4 in 2016 (Negishi et al., 2019b). Realistic representations of food web structures depend on
548 the correct identification of potential basal sources for consumers. The isotope mixing approach
549 adopted in the present study was incomplete in two aspects. First, the hyporheic community
550 generally contains microinvertebrates such as meiofauna, which can only be collected using finer
551 mesh nets, typically < 200 μm (Pacioglu, 2010; Hartland et al., 2011; Mori et al., 2012; Lencioni and
552 Spitale, 2015). A 500 μm mesh sieve was used, thus resulting in a possible failure to reflect this
553 potentially crucial trophic link. Second, POM and benthic biofilms were incorporated into analyses,
554 but hyporheic biofilms were not measured, which are important food resources for hyporheic
555 consumers (Craft et al., 2002). Nevertheless, consumers' carbon SIR signatures were within the
556 ranges of the carbon sources measured, and the estimated trophic positions of consumers did not
557 reveal conspicuously missing trophic positions. Overall, it is conceivable that these results
558 characterize the food web of hyporheic macroinvertebrates. In addition, the observed trophic shifts
559 in the carbon bases of consumers under polluted conditions are in agreement with shifts in dietary
560 carbon pathways reported in other studies (Negishi et al., 2019a, b). The increased importance of
561 biofilms under polluted conditions was likely due to the increased productivity of primary producers,
562 which was stimulated by nutrient pollution, and increased influxes into the hyporheic zone.

563 The assimilation of human-originated nitrogen enriches the tissue of aquatic organisms in
564 the form of heavy stable nitrogen isotopes. Benthic invertebrates, largely epigeal taxa, and fish
565 dependent on invertebrate prey, generally show a linear increase in nitrogen SIRs in response to
566 water pollution involving increased nitrogen inputs (Morrissey et al., 2013; Negishi et al., 2019a).
567 The observations that community-level nitrogen SIRs were greater in the polluted sites than in the
568 upstream sites and that there was a positive correlation between *A. ishikariana* larvae nitrogen SIRs
569 and EC are both consistent with these known trends. The weak relationship achieved when using
570 infrequent and anomalous nitrate data can be explained by the theory that tissue SIRs represent time-
571 integrated conditions; nitrate concentration was not representative of the long-term environment
572 simply due to infrequent measurements. Few studies have examined the effects of nutrient water
573 pollution using measurements of water quality from riverbeds (Khebiza et al., 2006; Negishi et al.,
574 2019b). The present study extends an earlier report focusing on Crustacea (largely Amphipods) and
575 Chloroperlidae (largely *A. ishikariana*) in two sites (Negishi et al., 2019b), and demonstrates that the
576 hyporheic food web is affected by the assimilation of nitrogen from anthropogenic sources via high
577 hydrological exchanges.

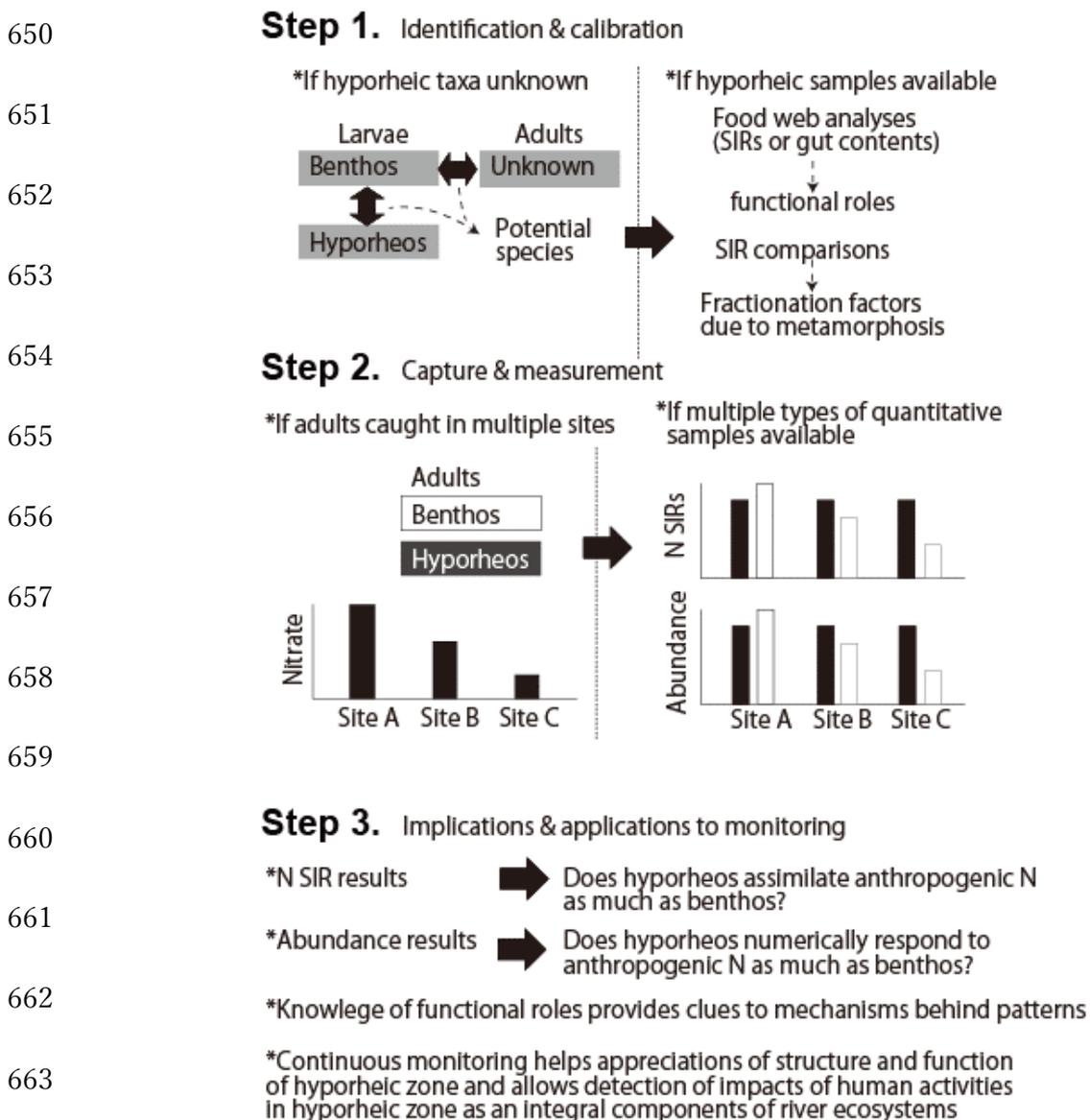
578 Nitrogen SIRs in *A. ishikariana* adults were similar to those in larvae collected in the
579 adjacent riparian zone despite a sharp contrast in larval values along the longitudinal gradient,
580 suggesting that emerged adults transmitted signals from the hyporheic zone to nearby land. Two of
581 our initial predictions disagreed with the regression model, that is, the intercept of the regression
582 model reflected lower than a 1:1 relationship and there were deviations in the observed values
583 relative to the predicted values. These errors are attributable to two processes. First, the
584 holometabolous metamorphosis process potentially causes nitrogen isotope fractionations, which can
585 enrich the SIRs of adults relative to the larvae (Mihuc and Toetz, 1994; Doi et al., 2007; Tibbets et
586 al., 2008; Krause et al., 2014). Second, observations deviating from the regression model could be
587 due to longitudinal flight dispersals of some adults (Briers et al., 2004). Because of the site-specific
588 nitrogen SIRs of the larvae, the dispersal of adults could cause spatial mismatches between their
589 SIRs and the SIRs of larvae in their capture location.

590 The measured nitrogen SIRs suggested that the collected *A. ishikariana* adults largely
591 originated from the same locality as larvae, and thus it was initially predicted that carbon SIRs
592 would also be correlated between adults and larvae (**Fig. 6B**). This was not the case, and the
593 following explanations that may reconcile these contradictions. First, metamorphosis may have
594 changed the carbon SIRs of adults compared to larvae through fractionation. This explanation is
595 unlikely because existing literature regarding this fractionation process has reported negligible
596 effects for aquatic insects (Doi et al., 2007; Wesner et al., 2014; Krause et al., 2014). Furthermore,
597 there is no logical reason to assume inconsistent fractionation across the sites, and fractionation were
598 the cause we would still expect to observe a linear relationship in the values between adults and
599 larvae. A more likely explanation is the heterogeneity in dietary carbon sources available to
600 hyporheic consumers at each site (**Fig. 6C**). River channels are vertically and laterally
601 heterogeneous, with the former referring to zones at different depths, and the latter referring to
602 different habitat types such as riffles and pools (Dahm et al., 1998; Malard et al., 2002; Boulton et
603 al., 2010). Larvae were collected from glides at a depth of 30 to 50 cm due to logistical constraints in
604 the installation and retrieval of colonization traps, whereas adults may have emerged from more
605 spatially-diverse source areas. The presence of variations in the vertical extent of the hyporheic zone
606 within the study stretch has also been suggested by Negishi (2019). It is known that different habitat
607 types can have different retention times, pathways, and impacts from surface water (Malard et al.,
608 2002; Mori et al., 2012), which can affect the types and compositions of organic matter available for
609 consumers. The results of the present study clearly demonstrated that different types of resources
610 possessed different carbon SIRs. This argument does not contradict the patterns observed for
611 nitrogen SIRs because different carbon sources possessed similar nitrogen-SIR values; thus, variable
612 relative dependences on dietary carbon resources would not cause substantial disparity between
613 larvae and adults. Nevertheless, the fact that these observed relationships were based on adults
614 collected in one year at a relatively small number of sites must be taken into consideration.
615 Therefore, increasing the number temporal and spatial replicates of will help to further our collective
616 understanding.

617 In conclusion, we established a method of indirectly assessing the hyporheic environment
618 by collecting and analyzing adult aquatic insects. Adult aquatic insects emerging from the hyporheic
619 zone can help us to understand how and to what extent nutrient pollution affects the hyporheic food
620 web. In particular, emergent *A. ishikariana* adults can indicate the status of secondary consumers in
621 the hyporheic food web. The nitrogen SIR of *A. ishikariana* adults can be used to infer the degree of
622 anthropogenic nitrogen assimilation in larvae if considered together with water quality data. In
623 contrast, the mismatches and matches in the carbon SIR signatures of adults and larvae could
624 provide valuable information to better our collective understanding of the variations in carbon
625 pathways in the hyporheic zone. For instance, a weak correlation between the carbon SIRs of larvae
626 and adults suggests the presence of high spatial heterogeneity in hyporheic habitats not represented
627 by the environment in which larvae were collected. Future studies involving meiofauna, hyporheic
628 biofilms, and flux estimates for emergent adults of amphibitic insect larvae to the land as well as
629 along the longitudinal continuum will deepen our collective understanding of food webs and
630 resource subsidies linking benthic, hyporheic, and riparian ecosystems

631 Based on these findings, we outline a technique for assessing the hyporheic environment
632 indirectly through the collection and analyses of adult aquatic insects (**Fig. 7**). Identification of
633 hyporheos followed by analyses of biochemical markers and population dynamics of the adults is
634 likely to be a promising procedure for monitoring the hyporheic environment. Chloroperid
635 Plecopteran insects have been reported to be hyporheic dwellers in other locations (e.g., DeWalt and
636 Stewart, 1995). Thus, the approach outlined in the current study could be applied to other locations
637 where amphibitic taxa have been identified. Economic feasibility also needs to be considered when
638 applying this technique to river bio-assessment programs, because stable isotope measurements
639 require an isotope ratio mass spectrometer (IRMS) coupled to an elemental analyzer. Currently, the
640 costs of obtaining C and N SIRs for animal tissues are not prohibitively expensive, and are
641 comparable to other modern chemical or molecular analyses (Hood-Nowotny and Knols, 2007). In
642 the specific case of the present study, one sample cost less than 300 Japanese yen (approximately
643 equivalent to US \$3) when consumables are estimated. Furthermore, depending on the machine used
644 and its configuration, analyses take 5–10 min per sample. More direct conventional methods such as

645 excavating pits or installing a network of wells for sampling can be costly in terms of both expenses
 646 and physical effort, and are often limited in terms of the spatial scale of observations. For these
 647 reasons, the proposed technique has its own advantages and could prove a powerful alternative
 648 approach for assessing the hyporheic zone that can be used in combination with conventional
 649 assessment procedures.



664 **Fig. 7** A conceptual flow chart describing the three steps taken to use adult aquatic insects to infer
 665 information about the hyporheic zone. Step 1 involves the identification of macroinvertebrates,
 666 including larvae of stygophile insects (in particular amphibites), in the hyporheic zone, as well as
 667 calibration to elucidate functional aspects and food web structure. Creating a taxonomic inventory of
 668 larvae and adult insects in both the benthic and hyporheic zones identifies taxa whose larvae are

669 disproportionately more abundant in the hyporheic zone. Existing literature can facilitate this step.
670 Analyzing the SIRs (or gut contents) of taxa of interest, as well as their potential food resources,
671 provides insight into their functional roles and metamorphosis fractionation factors. Step 2 involves
672 the capture and measurement of various properties of adult individuals, such as abundance and SIRs,
673 possibly across different sites with variable levels of exposure to nitrate pollution. Based on these
674 measurements, it is possible in Step 3 to deduce how similarly or differently consumers in the two
675 zones respond to pollution and the underlying mechanisms behind these patterns. Continuous
676 measurements help to detect the responses of hyporheic ecosystems to changes in the environment,
677 such as land use change and climate change.

678

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687

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