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Effects of multiple stressors on recruitment of long-lived endangered freshwater mussels

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Abstract

1. Multiple stressors can interactively affect populations. However, the process by which they affect recruitment remains unclear, especially in long-lived organisms with a complex life cycle.
2. This study explored the bottleneck stages of juvenile recruitment in the endangered freshwater pearl mussel *Margaritifera togakushiensis* and the interactive effects of nutrients and fine sediments on these stages. The proportion of ≤ 10 -year-old *M. togakushiensis* juveniles was investigated in 24 rivers from northeastern Japan. *Margaritifera togakushiensis* has a complex life cycle with an obligate parasitic larval stage. Gravid female density, glochidia density (the number of glochidia infections per stream area), and juvenile survival rate as indices for adult, parasitic, and post-parasitic juvenile stages, respectively, were obtained from 11 of these rivers.
3. Twenty-four population status assessments showed that the proportion of ≤ 10 -year-old juveniles ranged from 0.00 to 0.23 fraction, and they were absent in 12 rivers. Parasitic and post-parasitic juvenile stages were bottlenecks for recruitment; juvenile survival rates had a stronger positive effect on recruitment in rivers with high glochidia density. Juvenile survival rate was negatively associated with the synergistic effects of nutrients and fine sediments, although factors limiting glochidia density were not identified.
4. These findings suggest that the interaction between nutrients and fine sediments can synergistically reduce the recruitment of *M. togakushiensis*, indicating an underestimated impact of stressors on mussel recruitment if treated individually. Future mitigation measures should consider both nutrient levels and fine sediments to recover mussel recruitment. These results also emphasise the importance of considering both parasitic and post-parasitic juvenile stages of mussels to maximise the success of future restoration actions.

25 **Keywords**

26 catchment-scale management, complex life cycles, juvenile recruitment, sedimentation, stream

27 pollution, synergistic interaction

28

1. Introduction

Anthropogenic stressors have diminished the populations of various organisms worldwide (Hof et al., 2011; Reid et al., 2018). Many ecosystem types are interactively affected by multiple stressors, which makes it difficult to predict the responses of organisms to a single factor (Darling & Côté, 2008). This highlights the importance of further efforts to understand the causal mechanisms of habitat loss in the context of multiple stressors (Hof et al., 2011). Studies on multiple stressors have mostly focussed on abundance or presence/absence data (Toft et al., 2018). Recruitment failure is also an important process in determining population decline (Strayer & Malcom, 2012; Lopes-Lima et al., 2021). Low recruitment rates can severely increase the risk of local extinction; however, there may also be an opportunity to recover declined populations in situations where reproducing individuals are still present (Hylander & Ehrlén, 2013; Sousa et al., 2016, 2018). However, few studies have demonstrated the interactive effects of stressors on recruitment.

Recruitment of organisms can be regulated at multiple stages of life. Organisms with complex life cycles are particularly sensitive to human disturbances (Kingsolver et al., 2011). These organisms have distinct life stages and an abrupt ontogenetic change in the individual's morphology, physiology, and behaviour, which is usually associated with a change in habitat (Wilbur, 1980). Their highlighted sensitivity may be associated with each life stage contributing differently to recruitment and being constrained by different stressors (González-Varo, Nora & Aparicio, 2012). To effectively conserve organisms with complex life cycles, it is crucial to identify the life stages that experience recruitment bottlenecks by considering the interactive effects of multiple stressors. However, such empirical studies have been limited.

Freshwater mussels (Order Unionida) are among the most endangered groups of organisms (Lopes-Lima et al., 2018). Their vulnerability is largely derived from their complex life cycle, which requires host fish parasitisation during their larval phase (larva is called as “glochidium”) (Modesto et

al., 2018). Many mussel populations face a high risk of extinction due to recruitment failure (Varandas et al., 2013; Sousa et al., 2016; Miura et al., 2021). Their longevity (4–190 years) allows them to persist for a long time even in non-recruiting populations (Geist, 2010; Haag & Rypel, 2011). Understanding how stressors affect recruitment is needed to prevent local extinction of mussel populations.

Previous studies have suggested that habitat degradation is a major cause of recruitment failure in freshwater mussels and that benthic juveniles in their post-parasitic juvenile stage are considered to be the most sensitive (Österling, Greenberg & Arvidsson, 2008; Österling, Arvidsson & Greenberg, 2010; Strayer & Malcom, 2012; Haag et al., 2019). Most of these studies have focussed only on part of their life cycle, which may provide insufficient information for recovering their natural recruitment with actual conservation measures. This is because impaired recruitment may result from stressors at multiple life stages (Galbraith et al., 2018). A comprehensive test is necessary to determine the life stage that regulates recruitment and the mechanisms and drivers that cause recruitment failure. Increased nutrients and fine sediments delivered from catchments are major stressors that threaten freshwater mussels worldwide (Lopes-Lima et al., 2017). In particular, benthic juveniles of lotic mussel species are highly sensitive to eutrophication and siltation because they require silt-free and well-oxygenated substrates (Geist & Auerswald, 2007; Varandas et al., 2013; Lopes-Lima et al., 2017; Barák et al., 2022).

The present study examined 1) the key life stages limiting the recruitment of the freshwater pearl mussel *Margaritifera togakushiensis* and 2) the relationship between the concentration of nutrients and fine sediment loads and their combined effects on recruitment failure. In this study, the population status of *M. togakushiensis* was first determined in 24 rivers in northern Japan. Second, the key life stages that strongly limit mussel recruitment were identified using field observations and experiments. Finally, the impacts of nutrients and fine sediments and their combined effects on the

key life stages of *M. togakushiensis* were explored.

2. Methods

Target species

Margaritifera togakushiensis is distributed in cool-water rivers in Sakhalin, Russia, and in the Honshu and Hokkaido Islands of Japan (Miura et al., 2019b). They typically have a maximum shell length of 100 mm (Kondo, 2008). Their reproductive season is from April to May, and females are gravid once during a reproductive season (Kondo, 2008; Kobayashi & Kondo, 2009). The age of first reproduction in this species has not been previously investigated. The longevity of this species reaches 100 years by region (see “3. Results” section of the present study), and they mainly use white-spotted charr (*Salvelinus leucomaenis leucomaenis*) and perhaps Dolly Varden charr (*S. malma krascheninnikovi*) as the natural hosts (Kondo, 2008; Kurihara & Goto, 2011). *Margaritifera togakushiensis* parasitizes the gills of host fish for 40–50 days (Kondo, 2008). This species is threatened by anthropogenic disturbances, such as habitat fragmentation, river habitat modification, and land-use change, and is thus listed as an “Endangered” species on the Red List of Japan (Ministry of the Environment of Japan, 2014, 2020). Recently, this species has also been designated as the “Specified class II nationally rare species of wild fauna and flora” in Japan (Ministry of the Environment of Japan, 2023).

Life cycle indices

Recruitment of freshwater mussels can be slowed or stopped (i.e., decreasing the proportion of young individuals or the absence of young individuals) when any stage of their life cycle is disrupted (Österling, Greenberg & Arvidsson, 2008). In particular, the three life-cycle stages; adult, parasitic, and post-parasitic juvenile stages are considered critical stages in their life cycles (Österling,

Greenberg & Arvidsson, 2008; Strayer & Malcom, 2012).

At the adult stage, sufficient larval production is necessary to maintain the recruitment of freshwater mussels (McLain & Ross, 2005). Larval production is a function of the abundance of mature gravid females. Gravidity rate (the proportion of gravid individuals within a population) has been used as an index of fertilisation success and the potential for the production of mature glochidia (Österling, Greenberg & Arvidsson, 2008). The gravid abundance of the population can be described as the product of the gravidity rate and mature adult density; this is defined as the “gravid density” and is used as an index of the potential amount of larval production in this study.

Glochidia released from female mussels attach to host fish tissues and proceed to the parasitic stage (Modesto et al., 2018). In the early parasitic period, glochidia that fail to encyst or resist host immunity perish within several days to two weeks (Ziuganov et al., 1994). Therefore, the maximum number of mussels that proceed to the post-parasitic juvenile stage is limited by the glochidia surviving this initial parasitisation period. The potential abundance of mussels that proceed to the post-parasitic juvenile stage in a population can be described as the product of host fish density and the mean glochidia load (mean number of glochidia infections per fish in a population) on a host fish. This index is referred to as “glochidia density” (i.e., the number of glochidia infections per stream area) (Österling, Greenberg & Arvidsson, 2008) and was used to evaluate the bottleneck for the parasitic stage in the present study.

Mussels detached from host fish become post-parasitic juveniles. These juveniles live in riverbed sediments for several years to decades (Geist, 2010). The survival rate of post-parasitic juveniles directly affects their recruitment performance within mussel populations. This early juvenile stage is generally considered the life stage most sensitive to habitat degradation (Österling, Arvidsson & Greenberg, 2010). Thus, this study used the “juvenile survival rate” shortly after the parasitic period as an index of this bottleneck stage.

The above index for each life stage can exhibit a positive association with the recruitment of young individuals if recruitment is limited by one or more stages. Therefore, by examining the relationships between recruitment and the three indices, it is possible to identify the key life-cycle stage(s) that regulate recruitment (Österling, Greenberg & Arvidsson, 2008; Stoeckl, Taeubert & Geist, 2015). This study used the proportion of ≤ 10 -year-old individuals of *M. togakushiensis* within the population (unit: fraction, “the proportion of ≤ 10 -year-old juveniles” hereafter) as an index of successful recruitment (Österling, Arvidsson & Greenberg, 2010). This age could be considered as sexually immature (see third paragraph of “Intensive field observations and experiments” section in “2. Methods” below).

Study site

The study was conducted in 24 rivers (sites) across eight catchments encompassing >1500 km² of eastern Hokkaido, Japan, where several populations of *M. togakushiensis* were confirmed by Miura et al. (2019a) (Fig. 1). Sites *a* to *o* and *p* to *x* drain into the Nemuro Strait and the Pacific Ocean, respectively. No sites were established upstream of each site to ensure the independence of sites as much as possible because *Margaritifera* individuals in upstream sites can be transported to downstream (Terui et al., 2014). There were no large dams or weirs inhibiting fish migration. The stone loach (*Noemacheilus barbatulus toni*), white-spotted charr, and sticklebacks (*Pungitius* spp.) are the most abundant fish species in the studied streams (Ishiyama et al., 2021). Another congeneric species, *Margaritifera laevis*, also occurs in the study region (Miura et al., 2019a, 2023). *Margaritifera laevis* has different life history traits from *M. togakushiensis* in terms of the reproductive season (mid-June to August) and suitable host fish (Masu salmon *Oncorhynchus masou masou*) (Akiyama, 2007; Terui et al., 2015). In addition, they are morphologically different; therefore, the two species can be non-lethally identified with high accuracy using the relationship between shell length and height

(Miura et al., 2019b).

Population status assessment

Field sampling was conducted from June to September 2017. Two study reaches (length: mean $\pm$ SD, 26.86 ± 6.18 m) were established in glides at each site and were separated from each other by >500 m. Quadrat-based quantitative and semi-quantitative protocols were used to collect *M. togakushiensis* individuals. For each study reach, five transects were laid out at equal intervals. Three 0.09-m^2 quadrats were placed in each transect, one at the mid-channel and two at both sides separately, near the bank, for a total of 15 quadrats per reach. Mussels were collected from each quadrat as follows. First, all visible mussels were collected using a glass-bottomed viewing bucket. Second, the bed sediment was excavated to a depth of $\sim 10\text{-cm}$ using a trowel and immediately sieved all material through a 2-mm mesh sieve to reduce the size bias of mussels. From a single site, at least 100 *Margaritifera* individuals were collected to reach at least 50 individuals of *M. togakushiensis*, except for site *c*, where only 57 *Margaritifera* individuals were collected because a previous study revealed only *M. togakushiensis* occurrence (Miura et al., 2019a). When the number of *Margaritifera* individuals collected by quantitative quadrat sampling was less than 100 individuals in a study site, mussel individuals were additionally collected to be a total of 100 or more individuals. Then, one investigator visually found mussel individuals using a glass-bottomed viewing bucket by random walking in the study reach, and a single quadrat was laid out with found individuals as the centre. Mussels at each additional quadrat were collected in the same manner as described above. The collected mussels were photographed for size measurement and released to the original study reach. The shell length and height of each individual were estimated using ImageJ software (Schneider, Rasband & Eliceiri, 2012), except for 29 individuals from site *r* which were measured using callipers.

Species identification of each mussel individual was conducted using non-lethal identification criteria (Miura et al., 2019b).

Age determination of *Margaritifera togakushiensis*

Development of growth models and age determination were performed following the protocol of Kawajiri et al. (2021) to visualise the age structure of the *M. togakushiensis* populations and identify the threshold size of 10-year-old individuals. The age of margaritiferids can be estimated from the relationship between shell length and the number of growth rings engraved onto the shell ligament (e.g. Hastie, Young & Boon, 2000). Live mussels were collected from 23 to 83 individuals (mean: 41) from each catchment, including the study sites, between 2017 and 2018. The shell length and height of the collected individuals were measured and identified based on the criteria of Miura et al. (2019b) (N = 314), inner-shell morphology (N = 2), and the presence of mature glochidia in the gills of female individuals during the reproductive season of *M. togakushiensis* (N = 15) (Kondo, 2008). After measuring shell morphologies, the number of growth rings on the ligament of each mussel was counted under a stereomicroscope. Some ligaments of freshwater mussels are usually abraded due to erosion. Thus, the number of growth rings on the abraded part of the ligament was estimated based on the relationship between ligament length and the number of growth rings obtained from 29 non-eroded shells (shell length, range: 7.6–22.0 mm) collected from the study region, as described by Kawajiri et al. (2021). This study fitted four non-linear models: von Bertalanffy growth function, Hyperbolic saturation function, Logistic function, and Gompertz function, to individuals of each catchment using the measured shell length and age of the individual (San Miguel et al., 2004; Akiyama & Iwakuma, 2010; Haag & Rypel, 2011; Nakamura et al., 2018). The four models are expressed as the following functions:

von Bertalanffy growth function

$$L_t = L_{\infty}(1 - e^{-k(t-t_0)})$$

Hyperbolic saturation function

$$L_t = \frac{L_{\infty}k(t - t_0)}{1 + k(t - t_0)}$$

Logistic function

$$L_t = \frac{L_{\infty}}{1 + e^{-k(t-b)}}$$

Gompertz function

$$L_t = L_{\infty}e^{-ae^{-kt}}$$

where L_{∞} is the theoretical maximum length (or asymptotic length, mm), k is the growth coefficient (year^{-1}), t_0 is the theoretical age at zero-length (years), and a and b (years) are constants (Akiyama & Iwakuma, 2010). The best-fitting model was selected by comparing RSS (residual sum of squares) (Akiyama & Iwakuma, 2010). All growth model construction was performed using R 4.2.2 (R Core Team, 2022) with a package of nlsMicrobio v. 0.0–3 (Baty & Delignette-Muller, 2013).

As a result of fitting the growth functions and comparing the fitting results of the four growth models, the Hyperbolic saturation function and Gompertz function were the most fitted functions in seven study catchments (A , B , C , D , E , G , and H) and one catchment (F), respectively, which was supported by the lowest RSS of the comparison of four functions in each catchment (Table S1). The shell length of individuals estimated as 10-year old in each catchment was 18.86–26.20 mm calculated from the length-age relationship (Table S1). The age of individuals collected by population status assessments was estimated for each catchment using seven growth models (six Hyperbolic saturation functions and one Gompertz function). The age frequencies of each population were drawn, and then, the data from the two reaches were combined.

Intensive field observations and experiments

Intensive field observations and experiments were conducted in one reach of 11 selected sites (Fig. 1) because they were easily accessible and had wide ranges in the proportion of ≤ 10 -year-old juveniles (0.00–0.23) and adult mussel density (2.22–38.52 individuals m^{-2} , see the definition of “adult” individuals below). The proportion of ≤ 10 -year-old juveniles and adult mussel density was not correlated ($r = 0.06$, $P = 0.87$).

The gravidity rate of *M. togakushiensis*, which is a factor of gravid density, was measured on 3–6 and 9–11 May 2018. These periods coincided with the peak of *M. togakushiensis* glochidia release (Kobayashi & Kondo, 2009). Additional observations on 17–18 May 2018 confirmed the approximate end of mussel gravidity and helped decide the timing of host fish sampling (see next paragraph). One investigator randomly collected visible mussels using a glass-bottom viewing bucket for 15–30 min and at least 50 *M. togakushiensis* individuals in each study reach, except site c. Each individual was checked for the presence of mature glochidia in the gills by slightly opening the shell valves (< 5 mm) using a shell opener. Mussels with swollen and cream-coloured outer demibranchs were considered gravid individuals (Hastie & Young, 2003). However, it was difficult to non-lethally check the gravid condition (presence/absence of mature glochidia) below a shell length of 30 mm. Therefore, individuals with > 30 -mm shell length were opened to check for gravid conditions. Each mussel was photographed on a white tray with a ruler and level gauge in relation to the gravid condition. After the photography, each individual was marked with a small sign on the shell surface using white paint or a hand drill (Negishi & Kayaba, 2009; Miura et al., 2018). Each mussel was immediately released into the original study reach after marking. If marked mussels were captured during later observations, they were excluded from the observation targets to avoid handling effects. Later, the shell length and height of each individual were estimated from the images using the same protocols in

the “Population status assessment” section. Species identification of each mussel was conducted using the criteria of Miura et al. (2019) or the presence of mature glochidia in the gills.

A total of 1,265 *M. togakushiensis* individuals (shell length, range: 31.10–97.50 [mean $\pm$ SD, 66.13 ± 10.14] mm) were checked for gravid conditions on the two sampling occasions. The minimum shell size of the mature individuals was 35.80 mm. Thus, mussel individuals that exceed 35.80 mm shell length were defined as “adult” individuals and were used for further analysis because this size could be considered an approximate threshold size of sexual maturity of *M. togakushiensis* in the study region. The gravidity rate of *M. togakushiensis* at the first monitoring 0.00–0.51 (0.31 ± 0.17) fraction was higher than at the second 0.00–0.45 (0.13 ± 0.14) fraction. The lower gravidity rate on the 2nd monitoring means that glochidia release had already occurred, and thus measured values did not necessarily represent the true gravidity rate. Therefore, the gravidity rate values from the first monitoring were used to calculate the gravid density. Adult mussel density ($N\ m^{-2}$) at each site was calculated using quadrat-based quantitative data obtained during the population status assessments. The gravid density ($N\ m^{-2}$) of each site was calculated by multiplying the gravidity rate by adult mussel density.

Quantitative and qualitative electrofishing was conducted using a backpack electrofisher (Model 12 B, Smith-Root Inc., Vancouver, WA, USA) between 6–11 June 2018 to examine glochidia density, approximately two weeks after the end of their gravidity period in this region (see above). At each site, one reach that was more than 10 times its mean wetted width was established near the mussel monitoring area. The host fish (white-spotted charr and Dolly Varden charr) density ($N\ m^{-2}$) was estimated by the 2-pass electrofishing method (quantitative electrofishing) in reaches with a length of more than 10 times the mean wetted-width in each study river. When the number of captured individuals of each age class (Young-of-the-year [YOY] or $\geq 1^+$ individuals) in each study reach did not reach 10 individuals during quantitative electrofishing, additional electrofishing was performed 1–

2 h to qualitatively collect host fish individuals within the fish collection reach and 50–100 m upstream and/or downstream of the reach. The fork length (FL) of each fish was measured in the field after anaesthetizing (FA100, DS Pharma Animal Health Co., Osaka, Japan), but the FL of each fish brought back for dissection (see next paragraph) was measured in the laboratory. To calculate the collection area, five transects were laid at the fish collection reach in equal intervals, and the wetted-width was measured on each transect using a 50-m tape. The host density ($N\ m^{-2}$) of YOY and $\geq 1^+$ individuals was calculated using quantitative electrofishing data.

Of the collected host fish, more than 10 individuals of each age class were brought back to the laboratory and frozen. However, site *d* had only two $\geq 1^+$ individuals, and only two sites had more than ten samples of YOY individuals (Table 1). In the laboratory, all gills (a total of eight gill arches per fish) were removed from each individual, and the total number of glochidia infections on each fish was counted under a stereomicroscope. YOY host fish are generally important for the recruitment of *Margaritifera* because they do not acquire immunity (Österling, Greenberg & Arvidsson, 2008). However, preliminary observations showed encystment and growth of glochidia on the gills of $\geq 1^+$ host fish; therefore, both YOY and $\geq 1^+$ host individuals were collected and distinguished by differences in FL (Fig. S1). Glochidia density was calculated as follows. First, the mean glochidia load (N per fish) was calculated separately for the two age classes (YOY and $\geq 1^+$). Second, the host density ($N\ m^{-2}$) of each size class was multiplied by the mean glochidia load for each age class. Third, glochidia density ($N\ m^{-2}$) was calculated as the sum of the products of host density and mean glochidia load in each of the two age classes. When only one YOY individual was captured, the glochidia load was estimated from that individual.

Juvenile survival rate was measured using a modified Buddensiek mesh cage (Buddensiek, 1995) with a 130 mm long axis, 90 mm short axis, 10 mm thickness, 5 mm cell diameter, and 250 μm mesh nets. The juveniles were propagated in the indoor aquaria of Tomakomai Experimental Forest of

Hokkaido University (42°40'35"N, 141°35'59" E) by artificial infection using glochidia of six gravid mussels (shell length: 59.58 ± 6.71 mm) from site *u* and approximately 500 YOY white-spotted charr individuals. Detailed procedures for artificial glochidia infection and indoor aquarium culturing of infected hosts are described in Ito (2020). The peak of juvenile excystment occurred from 23 to 28 July 2018, and individuals were gently collected using Pasteur pipettes under a stereomicroscope. Only juveniles collected during the peak period were used in the experiments to avoid bias caused by the use of incompletely developed juveniles. From 29 to 31 July 2018, five live juveniles were placed in a single cell, giving a total of 30 individuals per cage. Triplicate mesh cages were installed on the riverbed in similar environments in each reach (a total of 90 individuals per site) between 30 July and 1 August 2018. All mesh cages were set directly above the riverbed surface. Surviving juveniles in each mesh cage were counted on three occasions: 31 August–3 September (1st monitoring), 27–29 September (2nd monitoring), and 22–23 October (3rd monitoring). Juvenile survival was determined based on visible activities (valve movement and/or foot activity) using a stereomicroscope in the field.

The experiment was conducted in accordance with the Hokkaido University legislation on animal experimentation.

Environmental factors

Inorganic fractions of suspended substances ("fine sediments", hereafter) were measured as an indicator of the fine sediment load. Surface water samples (4 L) were collected from each site on 27–29 September 2018. The collected samples were transported to the laboratory for subsequent treatment. Water samples were filtered using precombusted and preweighed microfiber glass filters (0.7 μ m pore size). Fine sediments (mg L^{-1}) were measured by loss on ignition after drying for >24 h at 60 °C and combustion for 3 h at 450 °C.

Electrical conductivity (EC, mS m^{-1} , corrected to 25°C) had a strong positive correlation

with total nitrogen (TN) and total phosphorus (TP) in the surface water of the study rivers (Fig S2, $r = 0.88$ and 0.44 , $P < 0.01$; $N = 24$ sites), suggesting that EC could be used as an indicator of nutrient concentration in the study rivers. EC was measured (WM-32EP, TOA DKK Co., Japan) at the uppermost part of each reach during the monitoring of mussel gravidity, at fish sampling, and at each monitoring of the field experiment.

Statistical analyses

A generalised linear model (GLM) was constructed to test the effects of three life-cycle stage indices on the proportion of ≤ 10 -year-old juveniles as the response variable. In addition, the interaction between glochidia density and juvenile survival rate was included as an explanatory variable because they exhibited a positive interaction with the proportion of ≤ 10 -year-old juveniles in the preliminary plots. For juvenile survival rate, the mean values at the three monitoring times were used as explanatory variables. The juvenile survival rate during the 2nd and 3rd monitoring at site i was excluded from further analyses because all experimental cages were buried in the streambed by > 30 cm, which was too deep for juvenile survival. The significance of each explanatory variable in the model was tested using Wald Z-statistics.

Based on the model construction and the identification of influential parameters, it was found that there was a positive interactive effect between glochidia density and juvenile survival rate on the proportion of ≤ 10 -year-old juveniles among the significant variables (see “3. Results” section). GLM and generalised linear mixed model (GLMM) were constructed to determine the limiting factors of glochidia density and juvenile survival rate, respectively. To explain glochidia density, EC values measured on fish sampling occasions, fine sediments, and the interaction between EC and fine sediments were used. Gravid density was also included as an explanatory variable because the abundance of gravid female mussels (i.e., the potential amount of larval production) can potentially

affect the glochidia load (N per fish) (Taeubert & Geist, 2013). To determine the juvenile survival rate, fine sediments, EC values measured on each occasion of juvenile monitoring, and the interaction between the two variables were used as explanatory variables. The timing of monitoring (1st, 2nd, or 3rd) was used as a random factor. The influential parameters were determined as described above.

In GLMs and a GLMM, binomial error distribution was performed using a log link function for ratio data as a response variable, whereas a Gaussian error distribution was used for other data types. When constructing each model, multicollinearity was tested among the explanatory variables, and there was no significant combination of variables ($r < 0.60$ with $P > 0.05$, in all cases). All statistical analyses were conducted using R 4.2.2 (R Core Team, 2022) with a significance level of $\alpha = 0.05$. The glmmTMB v. 1.1.5 package (Brooks et al., 2017) was used for model construction.

3. Results

A total of 2,462 *M. togakushiensis* individuals were collected. More than 50 individuals were caught (N = 55–211) at each site, with the exception of sites *l*, *m*, *r*, and *s* (N = 15–47). The proportion of ≤ 10 -year-old juveniles ranged from 0.00 to 0.23. Signs of recruitment within the last 10 years were absent at 12 sites (sites *c*, *e*, *f*, *h*, *j*, *k*, *m*, *o*, *p*, *q*, *s*, and *w*) (Fig. 2).

On two monitoring occasions of gravid condition, the number of checked adult *M. togakushiensis* individuals at each site ranged between 31 and 85 (57.27 ± 11.69) individuals per site. The gravid density of each site ranged between 0.00 and 17.82 (6.78 ± 6.38) individuals m⁻².

In total, 287 host fish (99 YOY and 188 $\geq 1^+$ individuals) were collected. Of the host fish collected, three were $\geq 1^+$ Dolly Varden charr. Infection with glochidia was confirmed in host samples from all sites and on both host fish species. A total of 190 host fish (47 and 143 individuals of YOY and $\geq 1^+$, respectively) were examined for glochidia load. The mean glochidia load on YOY individuals ranged between 15.00 and 163.00 (71.40 ± 51.01) per fish among the six sites and $\geq 1^+$ individuals

between 130.24 and 2045.31 (830.36 ± 753.84) per fish among the 11 sites (Table 1). The glochidia density at each site ranged between 3.17 and 65.79 (30.44 ± 22.54) m⁻².

Juvenile survival rates ranged between 0.00 and 0.78 (0.40 ± 0.30), 0.00 and 0.74 (0.35 ± 0.27), and 0.00 and 0.66 (0.28 ± 0.24) on the 1st, 2nd, and 3rd monitoring, respectively. Juvenile survival rate decreased by an average of 0.60 from the beginning of the experiment to the 1st monitoring, and this decreasing trend continued to the end of the experiment (Fig. 3).

The proportion of ≤ 10 -year-old juveniles was modelled using three life-stage indices and the interaction of glochidia density and mean juvenile survival rate. Among the four explanatory variables, the effect of the interaction between glochidia density and mean juvenile survival rate was significant, and the sign of the estimated coefficient was positive ($P < 0.05$) (Table 2a). The positive effect of glochidia density on recruitment success was stronger at sites with higher juvenile survival rates (Fig. 4a). The effect of gravid density was also statistically significant ($P < 0.001$) but was negative, contrary to the a priori prediction (Table 2a, see the “Life cycle indices” section). Gravid density did not appear to be a bottleneck for juvenile recruitment.

The determination of the influential parameters using the generalised linear model with Wald Z statistics results for explaining the glochidia density showed that there was no significant effect of all variables. This result indicates that the parameters had non-limiting effects on the glochidia density (Table 2b).

Among the three explanatory variables, the effect of the interaction between EC and fine sediments on the juvenile survival rate was negative ($P < 0.001$) (Table 2c). Juvenile survival rate did not change when EC or sediments were at low levels, but drastically decreased when both EC and fine sediment values increased (Fig. 4b).

4. Discussion

Despite the known effects of multiple stressors on population responses, as measured by abundance or presence/absence data (e.g., Shea et al., 2013; Cope et al., 2021), the effect of multiple stressors on juvenile recruitment remains unclear. This knowledge gap is particularly wide for organisms with complex life cycles (Lopes-Lima et al., 2021). To the best of our knowledge, this is the first study to show that increases in nutrient concentrations and fine sediments can interactively cause recruitment failure of freshwater mussels by synergistically reducing the survival rate of post-parasitic juveniles. In addition, the present study showed that a low glochidia density could cause recruitment failure interacting with the juvenile survival rate. Recruitment failure is hypothesised to be a major cause of freshwater mussel decline (Strayer & Malcom, 2012; Ferreira-Rodríguez et al., 2019). Previous studies have suggested that habitat degradation from excessive nutrients and fine sediments causes recruitment failure of freshwater mussels via impacts on juvenile survival rates (Geist & Auerswald, 2007; Österling, Arvidsson & Greenberg, 2010; Strayer & Malcom, 2012). The present study expanded on these preceding important studies by clearly distinguishing the effects of the two stressors on the post-parasitic juvenile stage and earlier life stages, and quantifying the level of their interactions.

The negative effects of nutrients and fine sediment on mussels were consistent with previously reported independent effects of each. Nutrients, except for unionised ammonia (NH_3), generally have no strong acute toxicity but can have weak or chronic toxicity to post-parasitic juveniles. For example, nitrate (NO_3^-) can reduce juvenile survival rates (Douda, 2010). Concentrations of NO_3^- had a strong positive relationship with EC at our study sites ($r = 0.86$, $P < 0.01$; $N = 24$ sites; unpublished data of K. Miura and T. Inoue). In addition, excessive fine sediment in the water column can directly decrease the survival rate of post-parasitic juveniles by clogging their gills (Österling, Greenberg & Arvidsson, 2008). However, the mechanisms underlying the synergistic

effects of these two stressors on juvenile survival rates remain unclear. Possible mechanisms may involve modifications to the biogeochemistry of benthic habitats. Sustained high levels of fine sediments in water could cause sediment deposits on the riverbed and clogging of interstitial spaces (Fung & Ackerman, 2019), which potentially disrupts water exchange at the surface–hyporheic interfaces. This, in turn, may cause hypoxic conditions and higher concentrations of NH_3 , which are highly toxic to benthic mussel juveniles (Wood & Armitage, 1997; Strayer & Malcom, 2012; Roley & Tank, 2016). Furthermore, increasing nutrient concentrations may exacerbate this condition because loading with reactive nitrogen as the source of ammonia also increases (Strayer & Malcom, 2012; Roley & Tank, 2016; Barák et al., 2022).

Glochidia density was found to be important for the recruitment of *M. togakushiensis*, together with juvenile survival rate. Low glochidia density may cause a low potential number of mussel individuals to proceed to the post-parasitic juvenile stage. On the other hand, glochidia density had a weak relationship with abiotic and biotic factors, suggesting that it was not strongly limited by these factors. Glochidia density is a function of host density and mean glochidia load per fish. Low habitat quality can reduce host fish availability and the infection success of glochidia larvae (Kawajiri et al., 2021). However, host fish (white-spotted charr) density in the study region was mainly limited by catchment size and habitat complexity, and not by habitat degradation (Ishiyama et al., 2021). In addition, the relationships between the mean glochidia load of both age classes (YOY and $\geq 1^+$) and fine sediment loads and nutrient concentrations were not correlated (GLM with log-likelihood ratio tests, YOY: $P = 0.40$ and $P = 0.06$; $\geq 1^+$: $P = 0.26$, and $P = 0.42$). Therefore, the indirect effects of habitat degradation in the study region on the recruitment of *M. togakushiensis* through its effects on host population size were considered small.

The results of this study showed that low glochidia density can also cause recruitment failure interactively with juvenile survival rate. This finding also has meaningful management implications,

namely, that parasitic and post-parasitic juvenile stages should be considered simultaneously when recovering freshwater mussel recruitment. Previous studies that have attempted to recover mussel recruitment by considering only one stage have not always been successful. For example, Galbraith et al. (2018) attempted to recover the recruitment of Eastern Elliptio (*Elliptio complanata*) by reintroducing American eels (*Anguilla rostrata*), which are migratory hosts of Eastern Elliptio, at two target creeks where American eels were absent due to fragmentation by past dam installation. As a result of the reintroduction of American eels, the recruitment of Eastern Elliptio recovered both target creeks, but the sign of the recovered recruitment was extremely limited in one of the two creeks. The creeks where the recruitment of Eastern Elliptio was not effectively recovered had a more agricultural catchment and lower water quality characterised by high turbidity, suspended solids, and nutrients. This discrepancy might be a result, at least partially, of the decreased juvenile survival rate owing to habitat degradation.

Although this study has significant management implications, it is important to acknowledge two limitations when interpreting our findings. First, this study found that gravid density, which is an index of the adult stage, had a negative relationship with the proportion of ≤ 10 -year-old juveniles. If this life-cycle stage (i.e., adult stage) limits the recruitment of *M. togakushiensis*, gravid density would have a positive relationship with the proportion of ≤ 10 -year-old juveniles, as expected by the biological trait of the index (see the “Life cycle indices” section). We believe that the adult stage could not be a recruitment bottleneck in the studied rivers because the negative relationship between the proportion of ≤ 10 -year-old juveniles and gravid density was found. This unexpected negative relationship cannot be explained logically based on our dataset and remains unresolved. One possible explanation for this is the disproportionately high host physiological responses to glochidia infection at sites where gravid density was high, as implied in Doua et al. (2012) in the context of invasive unionid species. Specifically, the rate of successful host attachment and juvenile

transformation might be kept relatively low when glochidia in the environment are at a high abundance due to hyperplastic tissue reactions to early attaching glochidia, leading to substantially lower rates for late-attaching glochidia. Second, we collected most data for a single year, which might not thoroughly capture the extent of variability in biotic and abiotic factors across different years. It is important to note that the factors studied can fluctuate from year to year, and our findings should be interpreted within the context of this potential temporal variability. This highlights the need for future investigations and long-term monitoring programs regarding mussel recruitment and environmental factors, including host-fish-population dynamics.

Implications for conservation measures

Given the significant interaction between nutrients and fine sediments, several management options can be proposed to overcome the bottleneck of low survival rates in early juveniles. The positive effects of reduced nutrients on juvenile survival rate could be maximised in rivers with a higher fine sediment load. Both nutrients and fine sediments are generally related to land use, such as agricultural land cover in catchments (Wood & Armitage, 1997; Howarth et al., 2012). In the present study, conductivity had a strong positive relationship with agricultural and urban land cover in catchments (Fig. S3, $r = 0.73$ and 0.86 , respectively; $P < 0.01$, $N = 24$ sites), indicating that nutrients originated from agricultural land and urban areas. Restoring wetlands and vegetated buffer strips may help capture nutrients before they reach streams (Weller et al., 2011). In addition, increasing the percentage of sewered populations may help reduce the nutrient input from urbanised areas. The latest reports showed that the percentage of the sewered population (including establishing the septic tank) of four towns comprising the study region was, on average, 86.1% (range: 81.5–92.8%) (Hokkaido Prefecture, 2023). The remaining population drains domestic and industrial wastewater directly into the environment without effective treatment. Establishing drainage and wastewater

treatment plants or septic tanks can effectively reduce the pollution of domestic and industrial wastewater. Regarding fine sediment loads, there was no clear relationship with agricultural land cover in the catchments ($r = 0.02$, $P = 0.92$, $N = 24$ sites). This might indicate that fine sediments originate from point sources rather than from diffuse sources. Large amounts of fine sediment enter rivers after localised events, including landslides, riverbank erosion, and deforestation (Wood & Armitage, 1997). Restoring wetlands and vegetated buffer strips can be effective in reducing inputs of fine sediment to streams and nutrient control in general (Hansen et al., 2021). Determining the sources of fine sediments is needed to establish more effective riparian management in the future.

The present results suggest that strategies for enhancing glochidia density may also be important for the success of future restoration actions for *M. togakushiensis* recruitment. In particular, managing fine sediments and nutrients can be most effective in streams with high glochidia densities but low juvenile survival rates. Glochidia density can be increased by enhancing the biological control of both host density and glochidia load on a single host fish. To increase host density, installing large wood structures in rivers may be an effective way to improve fish habitats (He, Wu & Shields, 2009). The installation of these structures can enhance fish density by providing covers and enhancing habitat heterogeneity, including greater variation in water depth, current velocity, and the size of riverbed sediments (Nagayama et al., 2009). In addition, the (re-)introduction of host fish can increase host fish density. In particular, (re-)introduction during the reproductive period of *M. togakushiensis* can be a more effective way to increase host fish density and glochidia loads on host fish. Furthermore, artificial infection of host fish with glochidia larvae may also be a more practical way to increase the glochidia load on individual fish, because a larger number of glochidia can be attached more reliably (Preston, Keys & Roberts, 2007). This artificial propagation may also be an important conservation measure for recovering *M. togakushiensis* recruitment in the future.

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Author contribution statement

Kazuki Miura: conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; software; validation; visualization; writing – original draft preparation; writing – review & editing. **Nobuo Ishiyama:** conceptualization; investigation; methodology; writing – original draft preparation; writing – review & editing. **Junjiro N. Negishi:** conceptualisation; funding acquisition; methodology; project administration; resources; supervision; writing – original draft preparation; writing – review & editing. **Daisetsu Ito:** conceptualization; investigation; methodology; resources; writing – review & editing. **Keita Kawajiri:** conceptualization; investigation; methodology; writing – review & editing. **Hokuto Izumi:** conceptualization; investigation; methodology; resources; writing – review & editing. **Takahiro**

Inoue: conceptualization; investigation; methodology; resources; writing – review & editing.
Masahiro Nakaoka: conceptualization; funding acquisition; methodology; project administration;
resources; writing – review & editing. **Futoshi Nakamura:** conceptualization; funding acquisition;
methodology; project administration; resources; supervision; writing – review & editing.

Data Availability Statement

Data are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare no potential conflicts of interest, financial or otherwise.

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Table 1 Summary of the number of fish samples and mean glochidia load of two age classes(YOY[†] individuals and individuals aged $\geq 1^+$ year) in 11 intensively studied rivers (sites).

River	The number of collected host fish aged $\geq 1^+$ year (the number of collected YOY host fish)	The number of dissected host fish aged $\geq 1^+$ year (The number of dissected YOY host fish)	Mean glochidia load (YOY)	Mean glochidia load ($\geq 1^+$)
<i>c</i>	11 (0)	11 (0)	-	384.00
<i>d</i>	3 (8)	2 (8)	80.88	1689.50
<i>e</i>	24 (0)	14 (0)	-	1282.79
<i>f</i>	18 (25)	15 (16)	43.81	131.47
<i>i</i>	17 (0)	13 (0)	-	2045.31
<i>n</i>	17 (0)	17 (0)	-	130.24
<i>p</i>	14 (1)	9 (1)	15.00	534.22
<i>q</i>	23 (1)	17 (1)	48.00	303.53
<i>u</i>	19 (63)	16 (20)	77.70	173.13
<i>w</i>	15 (0)	15 (0)	-	533.60
<i>x</i>	27 (1)	14 (1)	163.00	1926.14

[†]YOY: Young of the year

Table 2 Parameter estimates, standard error, Z value, and *P* values for the Wald Z statistics of the best models explaining the proportion of ≤ 10 -year-old juveniles (a), glochidia density (b), and juvenile survival rate (c). Bold styles in the column of *P* value indicate statistical significance at $\alpha = 0.05$.

Response/Explanatory variable	Estimate	SE	Z value	<i>P</i> value
(a) The proportion of ≤ 10 -year-old juveniles				
Gravid density	-0.12	0.03	-4.39	<0.001
Glochidia density	0.02	0.01	1.17	0.24
Mean juvenile survival rate	-2.30	1.79	-1.29	0.20
Glochidia density $\times$ Mean juvenile survival rate	0.09	0.04	2.48	<0.05
(b) Glochidia density				
Gravid density	1.75	1.00	1.74	0.08
EC	0.24	1.79	0.14	0.89
Fine sediments	0.59	12.38	0.05	0.96
EC $\times$ Fine sediments	-0.20	0.65	-0.30	0.76
(c) Juvenile survival rate				
EC	0.00	0.02	0.01	0.99
Fine sediments	0.20	0.10	1.94	0.05
EC $\times$ Fine sediments	-0.05	0.01	-6.392	<0.001

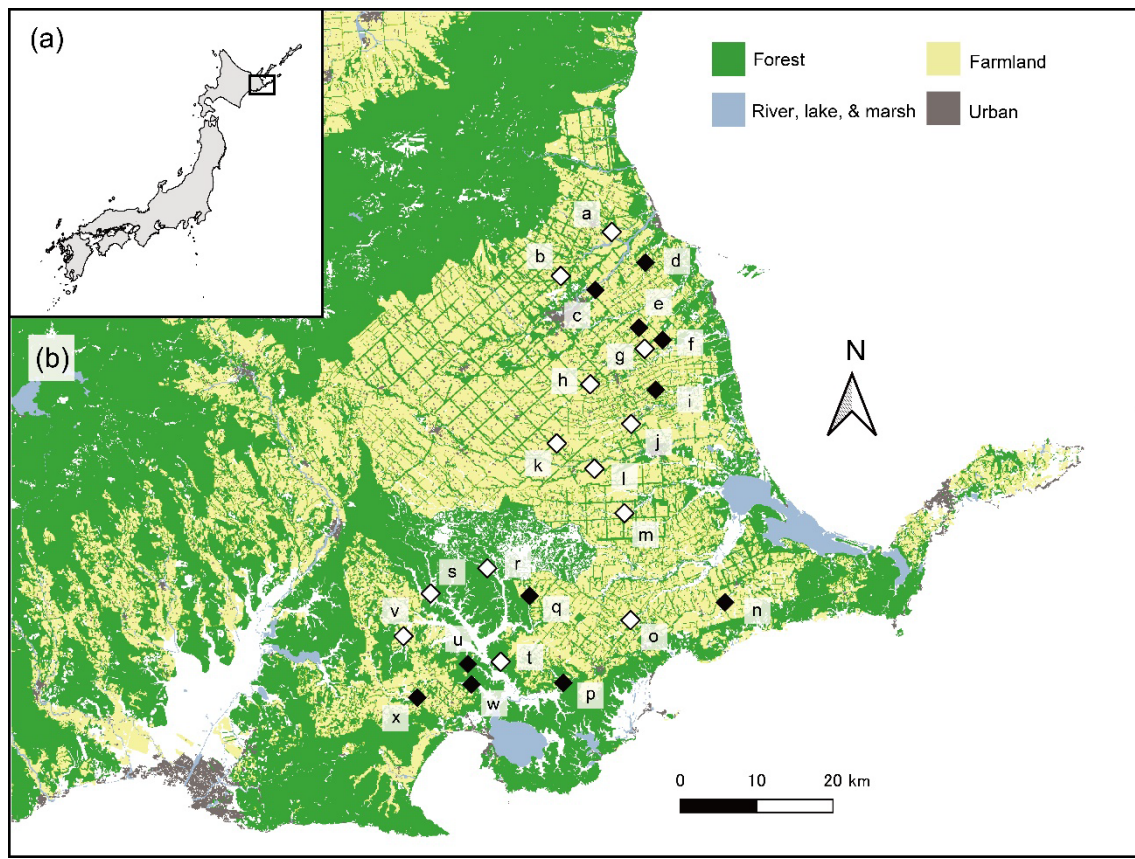


Fig. 1 Geographical locations of the study region (a) and 24 study rivers (sites), including 11 intensively studied sites and land-use types (100-m mesh resolution and data in 2016) around the study sites (b). Open and filled diamonds denote non-intensively and intensively studied sites, respectively. The detailed locations of the sites along the streamlines and each stream name remain undisclosed to assist in the conservation of *Margaritifera togakushiensis*. Land use data were obtained from the WEB “<https://nlftp.mlit.go.jp/ksj/index.html>” of the National Land Information Division, National Spatial Planning and Regional Policy Bureau, MLIT of Japan.

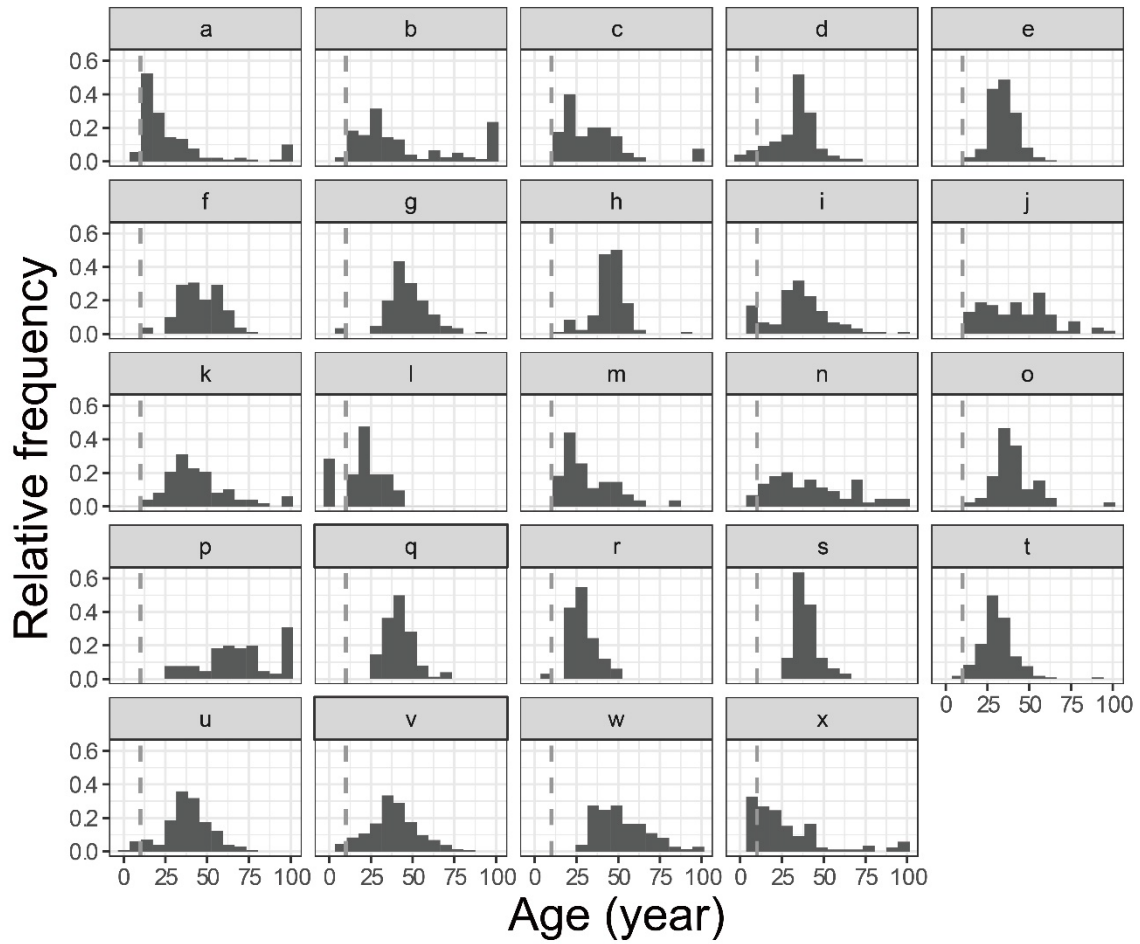


Fig. 2 Relative frequencies of the age of *Margaritifera togakushiensis* populations in 24 study rivers (sites) indicating variations in ≤ 10 -year-old-juvenile proportions and reproduction of *M. togakushiensis* among sites. All individuals aged > 100 years were categorised into the same category. Grey dashed lines indicate the age of a 10-year-old individual.

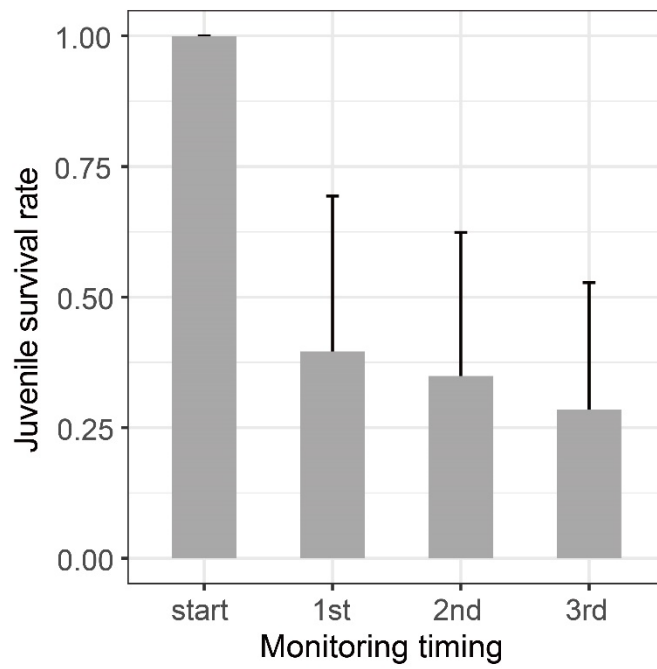


Fig. 3 Bar plots of time changes in juvenile survival rate of *Margaritifera togakushiensis*. The “start” means the beginning timing of the field exposure experiments on juveniles. Error bars indicate the standard deviation.

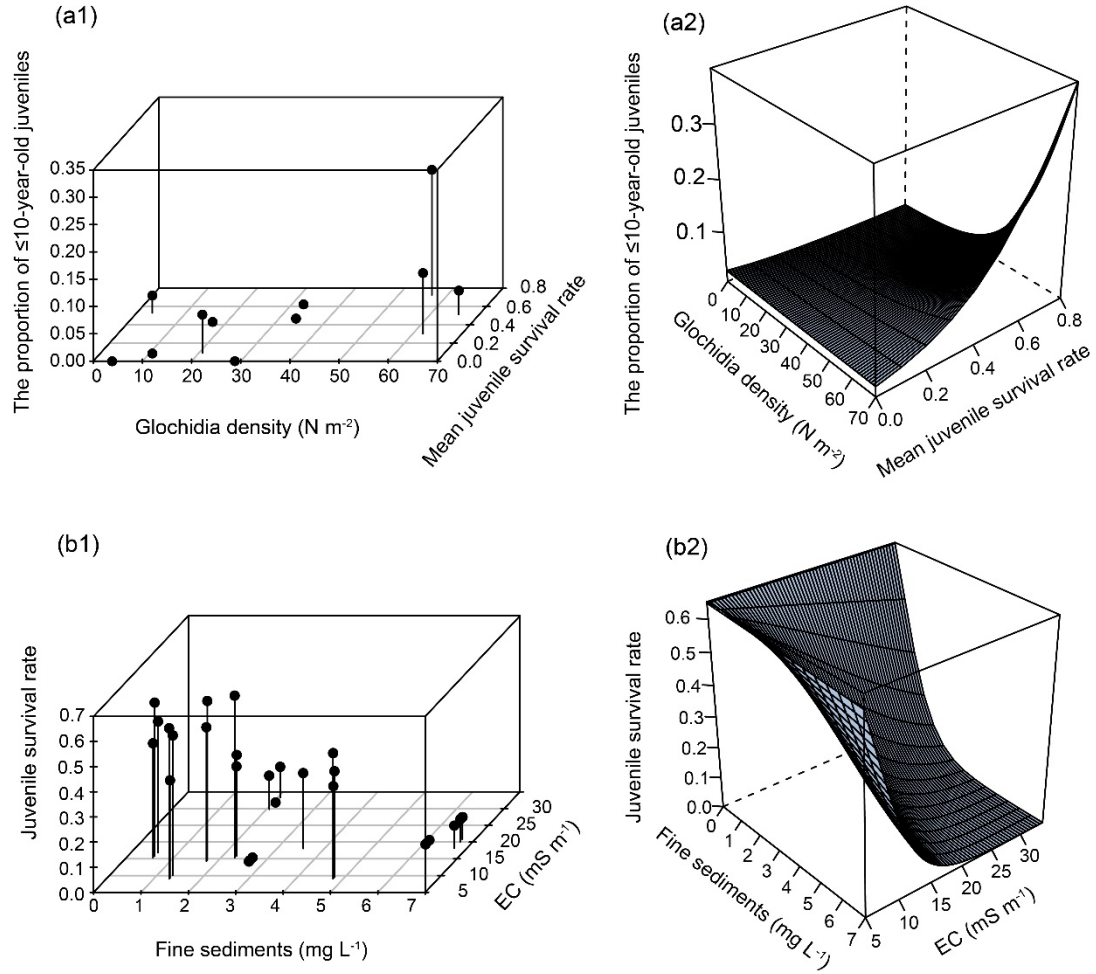


Fig. 4 Interactive effects of the mean juvenile survival rate and glochidia density (N m⁻²) on the proportion of ≤10-year-old juveniles (a1 and a2) and interactive effects of electrical conductivity (EC, mS m⁻¹) and fine sediments (mg L⁻¹) on juvenile survival rate (b1 and b2). The actual measurement variables are shown in a1 and b1, respectively. Estimates of the proportion of ≤10-year-old juveniles using the generalised linear model (GLM) with a binomial error distribution, and estimates of juvenile survival rate using the generalised linear mixed model (GLMM) with a binomial error distribution are shown in a2 and b2.