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SHORT COMMUNICATION

WILEY

Effect of low dissolved oxygen on the viability of juvenile *Margaritifera margaritifera*: Hypoxia tolerance ex situ

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Abstract

1. The decline of endangered freshwater pearl mussel (FPM, *Margaritifera margaritifera*) has been attributed to juvenile mortality caused by low concentrations of dissolved oxygen in the stream substrate resulting from fine sediments (siltation) that impede water exchange in the interstitial microhabitat of juveniles.
2. If low oxygen concentration causes recruitment failure of FPMs, knowledge on the oxygen tolerance of juvenile FPMs is essential for the conservation of the species, as it will justify conservation efforts improving water exchange in the bottom gravel. However, the tolerance of low oxygen of FPM juveniles has not been directly studied.
3. Juvenile FPMs (9–11 months old) were exposed in individual chambers equipped with optical oxygen measurement spots to different levels of dissolved oxygen at 19 °C and their viability was monitored for 10 days to assess the acute oxygen tolerance of juvenile FPMs. Oxygen concentration ranged between 8.8 and 6.2 mg L^{-1} in the high oxygen treatment (control), 5.0–0.4 mg L^{-1} in the medium treatment, and 1.3–0.04 mg L^{-1} in the low oxygen treatment (near-anoxic conditions).
4. Viability of juvenile FPMs depended on the concentration of available dissolved oxygen, such that all juveniles exposed to near-anoxic conditions were classified as non-viable, whereas all mussels exposed to high and medium concentrations were viable at the end of the 10 day experiment. Juveniles differed in their ability to tolerate near-anoxic conditions, so that some individuals survived only 1 day and others survived up to 9 days.
5. This study provides the first direct experimental evidence on the oxygen sensitivity of FPM juveniles and suggests that >10-day events of very low dissolved oxygen at summer temperatures are fatal to juvenile FPMs, supporting the view that actions preventing low oxygen episodes in the substrate are essential for recruitment, and conservation, of FPMs.

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KEYWORDS
anoxia, Bivalvia, ex situ oxygen measurement, freshwater pearl mussel, hypoxia tolerance, river, stream

1 | INTRODUCTION

Freshwater mussels (Mollusca: Bivalvia: Unionida) are benthic macroinvertebrates that are among the most endangered animals in the world (Lydeard et al., 2004; Régnier, Fontaine & Bouchet, 2009; Lopes-Lima et al., 2017; Lopes-Lima et al., 2018). These remarkable molluscs have a life history that includes a parasitic larval stage, a juvenile stage that lives buried within the stream or lake sediment, and a filter-feeding adult stage that provides and contributes towards a number of ecosystem services (Vaughn & Hakenkamp, 2001; Howard & Cuffey, 2006; Vaughn, 2018). The decline of unionid mussels has been attributed to human disturbances, including fine sediment deposition, eutrophication, pollution, and loss of host fish as a result of damming and the impacts of invasive salmonids (Bauer, 1988; Österling, Greenberg & Arvidsson, 2008; Österling, Arvidsson & Greenberg, 2010; Taskinen et al., 2011; Österling & Högberg, 2014; Gosselin, 2015; Salonen, Marjomäki & Taskinen, 2016; Lummer, Auerswald & Geist, 2016; reviewed by Strayer, 2008 and Haag, 2012). Given their role as a keystone and umbrella species (Geist, 2010), the decline of freshwater mussels can significantly alter the functioning of aquatic ecosystems.

The abundance of freshwater pearl mussel (FPM) *Margaritifera margaritifera* (Linnaeus, 1758) has severely declined, and the species is now highly endangered or threatened throughout most of its distribution range (Young & Williams, 1983; Young, Cosgrove & Hastie, 2001; Lopes-Lima et al., 2017; Moorkens et al., 2017; Lopes-Lima et al., 2018). The survival at the post-parasitic juvenile stage of FPM is considered to be critical for maintaining a viable population (Bauer, 1988). Juvenile FPMs burrow within the stream substrate for the first years of their life, during which they depend on a continuous exchange between the water body and the interstitial water of their microhabitats. Fine sediment deposition decreases substrate permeability, restricting the availability of oxygen to juvenile mussels (Munn & Meyer, 1988; Ryan, 1991; Wood & Armitage, 1997; Geist & Auerswald, 2007), which is considered to be the cause of recruitment failure in many declining FPM populations (Buddensiek et al., 1993; Hastie, Boon & Young, 2000; Geist & Auerswald, 2007; Österling & Högberg, 2014).

Low concentrations of dissolved oxygen have been found to cause surfacing and other stress behaviour and mortality in juvenile freshwater mussels (Polhill & Dimock, 1996; Sparks & Strayer, 1998). Conditions that are near anoxic have caused acute mortality in juvenile unionids (Dimock & Wright, 1993), and Bílý et al. (2020) observed that decreased dissolved oxygen within the test substrate was associated with a low survival rate of juvenile FPMs. In addition, fine substrate—which leads to low dissolved oxygen conditions (reviewed by Ryan, 1991)—has been associated both with low recruitment of FPMs (Geist & Auerswald, 2007) and surfacing behaviour in juvenile FPMs (Hyvärinen et al., 2021). Thus, both correlative evidence from field studies and the results of laboratory experiments indicate that juvenile freshwater mussels are sensitive to low dissolved oxygen, but to our knowledge the effect of low oxygen concentration on the survival or viability of FPM juveniles has not been studied experimentally. Knowing the hypoxia tolerance of juvenile FPMs would be a step towards understanding the habitat requirements of FPMs at their most vulnerable life-cycle stage. This information can be used to identify and conserve sites that can sustain FPM juveniles and to restore habitats affected by human activities. Information on the oxygen requirements of FPM juveniles can also be used in captive breeding programmes by ensuring sufficient levels of dissolved oxygen during breeding and by identifying suitable stream sites for the introduction of captive-bred individuals.

The aim of the present study was to investigate the effect of available dissolved oxygen on juvenile FPM viability. A laboratory experiment was conducted in which mussels that were almost 1 year old were exposed to a range of oxygen concentrations for 10 days. Based on the results reported by Bílý et al. (2020) and Černá et al. (2018) showing that decreases in oxygen within the substrate resulted in low viability rates in juvenile FPMs, and field observations indicating that poor FPM recruitment is associated with sites where hyporheal oxygen is assumed to be low (Buddensiek et al., 1993; Hastie, Boon & Young, 2000; Geist & Auerswald, 2007; Österling & Högberg, 2014), our hypothesis was that the viability of juvenile FPMs is poor in low dissolved oxygen concentration (0–2 mg L^{-1}) compared with higher oxygen levels.

2 | METHODS

To obtain juvenile FPMs, one-summer-old brown trout (*Salmo trutta* Linnaeus, 1758) and salmon (*Salmo salar* Linnaeus, 1758) sourced from Hanka-Taimen Oy (Hankasalmi, Finland) were infested at the Konnevesi Research Station (University of Jyväskylä) from 17 to 21 October 2019, with glochidia collected from adult mussels originating from the River Ähtävänjoki, Ostrobothnia, western Finland. On 16 June 2020, fish were moved to a juvenile collection tank where the water temperature was gradually increased from 13 °C to approximately 16 °C over a period of 1 week to trigger metamorphosis of FPM glochidia attached to the fish gills. In mid-July, juvenile mussels started dropping off the fish gills, and these were collected from the tank water three times per week using an Artemia sieve combination (mesh sizes 120, 300, 560, 900 μm), such that the tank water was poured through the sieve set from the largest mesh

size to the smallest. Collected juveniles were maintained in culture containers without substrate and fed with microalgae (ShellFish Diet© and Nanno 3,600©; Reed Mariculture, USA).

In April–June 2021, 9- to 11-month-old juvenile FPMs were exposed to three different dissolved oxygen concentration levels: low (close to zero), medium, and high (close to fully saturated) at 19 °C (Table 1). Juveniles were kept in permanent darkness except for the times they were monitored (approximately 1 h per monitoring day). Juveniles were not fed during the experiment to avoid additional respiration by organic matter and to prevent juvenile excrement from accumulating. The water used in these experiments was from Lake Konnevesi (see Supporting Information Table S1 for water quality information), filtered using an FSPT-WBW 114304-MR self-cleaning filter (Filterit, Helsinki, Finland) to remove excess particulate matter and algae, and ultraviolet (UV)-radiated to disinfect (ULTRAAQUA MR6–350 SS 316 LUVT; Filterit). Oxygen concentration was measured using a PreSens Microx 4 Fiber Optic Oxygen Transmitter (PreSens, Regensburg, Germany) with either a compatible oxygen dipping probe or non-invasive oxygen sensor spots (spot diameter 5 mm; resolution ±0.005 mg L^{-1} at 0.4 mg L^{-1} and ±0.025 mg L^{-1} at 9.06 mg L^{-1}). All measurements were corrected for the ambient temperature and calibrated for the measuring instruments applied. The average proportional random error of the corrected and calibrated oxygen concentration is <4% of the calculated concentration.

The water in the high dissolved oxygen treatment was untreated, apart from filtering and UV-treatment, as it was naturally high in oxygen. For the low and medium dissolved oxygen water, oxygen was removed from the water by bubbling nitrogen gas through it until a desired oxygen level was obtained. At this stage, oxygen was measured using a PreSens Microx 4 meter with a dipping probe. The dissolved oxygen concentrations that were used are representative of natural conditions that may occur in stream substrates supporting FPMs (Quinlan, Malcolm & Gibbins, 2014).

Oxygen sensor spots were placed inside 25 ml measuring flasks made of glass, with plastic stoppers, so that each flask contained a single sensor spot (Figure 1). When a desired oxygen level was reached, water was poured into the measuring flasks. When the flask was filled to the brim, a single juvenile was added with a pipette and the flask was sealed with a stopper. Juveniles were transferred directly from their culture containers and were maintained in the flasks, without added food, for up to 10 days. After all flasks had gone through the same process, dissolved oxygen was measured in each flask, to make sure the oxygen level was still at the level intended. At this stage the oxygen measurements were taken contactlessly by aligning the sensor spot with the beam projecting from the Microx 4 meter.

Once the oxygen measurements were taken, flasks were placed inside a chamber with an inlet and an outlet that allowed the exchange of gases to and from the chamber (Figure 1). Nitrogen gas was directed via a plastic tube to the chamber to remove oxygen from the chamber atmosphere for 5–10 min, after which the inlet and outlet were closed. The atmospheric oxygen removal was a precautionary measure to prevent oxygen from entering the flasks should they not be completely airtight. As the chamber wall was thicker than the flask wall, the same sensor spots could not be used for a non-invasive oxygen measurement from the chamber. In other words, it was not possible to confirm that the oxygen removal from the chamber was successful, but it was assumed that most, if not all, oxygen had been removed from the chamber atmosphere. As the flasks were taken out of the chamber for oxygen measurements and juvenile viability monitoring, the chamber was deoxygenated with nitrogen gas after the flasks were put back in.

For practical reasons, the experiments were conducted serially on several occasions to obtain the desired number of replicates per treatment. The high and low oxygen treatment experiments were performed on three separate occasions and the medium treatment on two occasions in April–June 2021. The viability of juveniles and the oxygen concentration in the flask was checked after 10 days in the high and medium oxygen treatments. Monitoring the flasks in the low oxygen treatment was performed more frequently, on days 1–3 and 6–10 (the first experiment: 10 replicates), on days 1–3 and 6, 8, and 10 (the second experiment: five replicates), except for the third experiment (three replicates) when monitoring was performed only on day 10 (at the end of the experiment). Each treatment constituted 18 replicate units in total and each replicate consisted of a flask with a single juvenile.

TABLE 1 The range of oxygen (O_2) concentrations and saturations throughout the experiments, mean O_2 concentrations and saturation at the beginning and end of the experiments, and the number of viable and non-viable juvenile mussels after exposure to varying concentrations of dissolved O_2 (mg L^{-1}) for up to 10 days

Treatment	O_2 concentration (mg L^{-1}) and saturation (%)						No. viable juveniles	No. non-viable juveniles
	Range		Mean, start of experiment		Mean, end of experiment			
	mg L^{-1}	%	mg L^{-1}	%	mg L^{-1}	%		
High O_2	8.8–6.2	98–70	8.4	95	7.3	83	18	0
Medium O_2	5–0.4	57–4	4.1	47	2.1	24	18	0
Low O_2	1.3–0.04	15–0	0.9	10	0.3	4	0	18

The average concentrations mark the lowest and highest O_2 concentration measured in a flask. O_2 concentrations tended to decrease towards the end of the experiment; thus, the lowest values were measured at the end of the experiment and the highest at the beginning of the experiment.

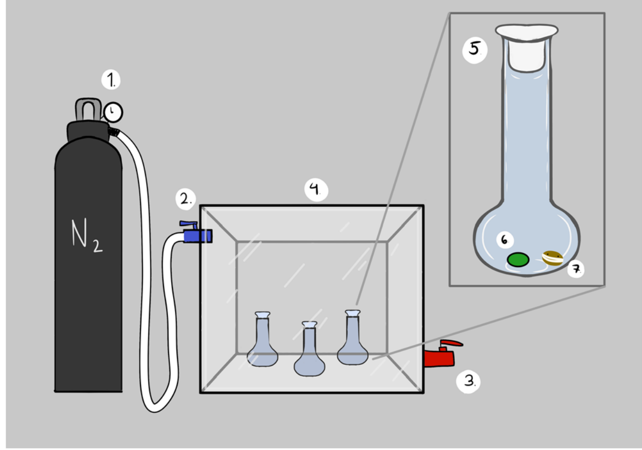

FIGURE 1 The experimental apparatus included (1) a nitrogen gas tank, used for oxygen removal from water and the chamber atmosphere. The chamber (4) had an inlet (2) and an outlet (3) allowing nitrogen to enter and oxygen to exit the chamber atmosphere; after deoxygenation, the chamber was sealed. The measuring flasks (5) containing an oxygen measuring spot (6) and a single juvenile freshwater pearl mussel (7) were kept in the chamber and taken out for monitoring oxygen and juvenile viability on monitoring days. After placing the flasks back in the chamber, the deoxygenation of the chamber was repeated. (Illustration: Tiia Penttinen)

To ensure that deoxygenation with nitrogen gas did not increase ammonia and lower pH to levels that would cause stress and increase the risk of mortality in juveniles, these parameters were measured. Measurements were made in flasks with a juvenile and without a juvenile (established for the purpose of these measurements) 10 days after the preparation of deoxygenated water to find out how the nitrogen bubbling and the presence of a juvenile affected ammonia and pH (see Supporting Information Table S2 for results). Ammonium nitrogen (NH_4-N) was used to evaluate the level of ammonia. Both parameters were within safe limits for aquatic life (US Environmental Protection Agency, 2013) in all treatments.

Healthy juveniles are typically active, extruding their foot and opening their valve. The viability of juveniles was determined by reference to foot or valve movement, similar to Bringolf et al. (2007). The visual inspection was done through the flask wall with a magnifying glass. Juveniles that did not display movement in a 10 min observation period were taken out of the flask and inspected on a petri dish with a microscope; if the juvenile did not move on the petri dish it was determined to be non-viable. Juveniles that had an immobile foot or did not open their valves and move their foot within 10 min after being placed on a petri dish were assumed to be either dead or non-viable. As the death of invertebrates is not easily distinguished from immobility, we could not be certain that unmoving juveniles were dead, and therefore we chose to use the term 'non-viable'. An individual that is non-viable is assumed to be stressed and weakened, such that it does not grow, develop, and function successfully.

Each juvenile mussel was classified as alive or non-viable (after 10 days exposure or when monitored during the experiment). The statistical significance of the differences between treatments in the distribution of number of observations of viable and non-viable individuals (after 10 days exposure) was not estimated asymptotically with, for example, a chi-square-test, but calculated exactly based on probabilities because the number of classes with zero observations was high.

In the low oxygen treatment, the time of non-viability was coded so that it was at the midpoint between the last observation when living and the observation when non-viable for the periods when the viability was not checked daily. In those cases, the oxygen concentration when an individual was found to be non-viable was assumed to be the average of the last observation when living and the observation when non-viable.

In the low oxygen treatment, the association between the index of viability time (day when determined non-viable) and (i) oxygen concentration at the beginning, (ii) the rate of decline of concentration, and (iii) the absolute decrease in oxygen concentration during the first day of the experiment was analysed using a Spearman's rank correlation coefficient ρ with one-tailed tests; the time of viability is short if the oxygen concentration is low at the beginning (a positive association) or if the rate of decline or decrease in concentration during the first day is high (a negative association). The rate of decline in oxygen b was estimated by regression by fitting the model

$$\mathrm{Ox} = a \exp(-bt)$$

where Ox is oxygen concentration and t is time (days from the beginning of the experiment).

3 | RESULTS

3.1 | Dissolved oxygen content

In the high oxygen treatment, flask-specific oxygen concentrations ranged between 8.8 and 6.2 mg L^{-1}, in the medium treatment

between 5.0 and 0.4 mg L^{-1}, and in the low treatment between 1.3 and 0.04 mg L^{-1} (for mean oxygen concentrations, see Table 1). Corresponding oxygen saturation values were calculated based on temperature and oxygen values in milligrams per litre and were 98–70% in high, 57–4% in medium, and 15–0% in low oxygen treatments (calculated using a nomogram from Särkkä, 1996). The oxygen concentration tended to decrease towards the end of the experiment (Table 1, Figure 2). The concentration decreased more ($t = 3.80$, $df = 28$, $P = 0$ 0.001) in medium (mean decrease 2.0 mg L^{-1}, $SE = 0.11$) oxygen treatments than in the high treatments (mean decrease 1.2 mg L^{-1}, $SE = 0.19$).

3.2 | Viability of FPM juveniles

There was a significant difference in viability between oxygen treatments ($P < 0.001$): every juvenile FPM survived in high and medium oxygen concentration treatments, but all individuals were found non-viable within 10 days in the low oxygen concentration (Table 1). Foot movements of juveniles in the control treatment (high oxygen) were observed within 1 min after being placed on a petri dish. In the low oxygen treatment, about 50% of individuals were determined as non-viable within 3 days. Three juveniles were found non-viable on the first monitoring day (24 h from the start of the experiment), three on day 2, two on day 3, one on day 7, and two on day 10. One juvenile in the low oxygen treatment (replicate 13, see supporting information Appendix S1 for oxygen concentrations) was taken out of the flask on day 6 after being immobile for 10 min. After being placed on a Petri dish, this juvenile was determined alive based on movement and was put back in the flask in newly prepared low oxygen water. This individual was determined non-viable on day 10, having succumbed within days 9–10. As the low oxygen treatment experiments were carried out on three separate occasions, not all replicates were monitored daily. Thus, the exact day these juveniles succumbed to hypoxia and became non-viable is not known. One juvenile was found non-viable on day 6 and had succumbed within days 4–6, one on day 7 succumbed within days 5–7, and two on day 10 succumbed within days 9–10.

There was no significant association between the time of viability and oxygen concentration at the beginning of the experiment (Spearman's $\rho = +0.12$, $P = 0.33$, one-tailed test, positive association expected), the rate of decline in oxygen concentration (Spearman's $\rho = +0.03$, $P = 0.54$, one-tailed test, negative association expected) or the rate of oxygen decline during the first day (Spearman's $\rho = +0.40$, $P = 0.93$, one-tailed test, negative association expected).

4 | DISCUSSION

The results indicate that FPM juveniles do not survive even 10 days in very low oxygen conditions (1.3–0.04 mg L^{-1}). Thus, although some FPM individuals were able to resist hypoxia for several days, these results support the view that substrate quality preventing low oxygen episodes is essential for the recruitment of FPMs, emphasizing the importance of actions to improve the river-bed conditions in the conservation of FPMs.

The results showed that juvenile FPMs may tolerate rather low oxygen conditions (medium oxygen concentration 5.0–0.4 mg L^{-1}) for up to 10 days. It should be noted that oxygen was measured only at the beginning and at the end of the 10-day experiment. Thus, it is not known how long juveniles tolerated oxygen concentrations as low as <1 mg L^{-1} in the medium oxygen treatment.

The hypothesis that the viability of juvenile FPMs is poor in low dissolved oxygen concentrations was supported, as 100% of the juveniles exposed to near-anoxic conditions were determined non-viable within 10 days. In contrast, all mussels exposed to high (control)

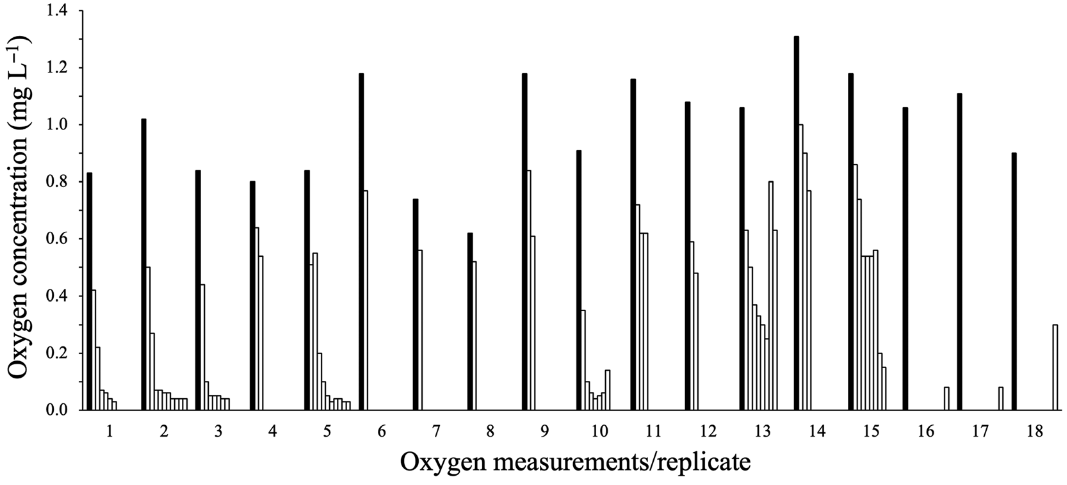

FIGURE 2 Oxygen concentration (mg L^{-1}) for each juvenile mussel flask in the low oxygen experiment. The columns correspond to day-specific oxygen measurements; the black column marks the first day of the experiment. Oxygen measurements were performed on days 1–3 and 6–10 (the first experiment: 10 replicates) and on days 1–3 and 6, 8, and 10 (the second experiment: five replicates), but for the third experiment (three replicates) the monitoring was performed only on day 10 (at the end of the experiment)

and medium concentrations of oxygen were viable at the end of the 10-day experiment. The potential delayed lethality or sublethal effects, such as decreased growth, were not monitored after this experiment. The possible long-term effects of acute oxygen stress on juvenile FPMs, as well as reasons for the observed large individual variation in tolerance of low oxygen, remain to be investigated in the future.

Dissolved oxygen was observed to decrease proportionately faster in medium and low oxygen treatments than in high oxygen treatments. The steep decrease in the medium oxygen treatments caused the dissolved oxygen concentrations of medium and low oxygen treatments to overlap, such that the lowest oxygen concentration measured in medium oxygen treatments was as low as 0.4 mg L^{-1} (4% oxygen saturation). Because the water was filtered and UV-radiated, a large part of the oxygen consumption in the flask is likely to have been by the juvenile mussels and not by microbial respiration. Some individuals may be able to acclimatize better to a decrease in oxygen by slowing down their metabolism, which may explain the varying rates of oxygen decrease among replicates and the survival of some individuals in <1 mg L^{-1} oxygen in the medium oxygen treatment.

Sparks & Strayer (1998) found that juvenile *Elliptio complanata* (Lightfoot, 1786) exposed to hypoxia exhibited various changes in behaviour (e.g. surfacing, gaping, extending their siphons and foot) before their death occurred. The authors suggested that the display of these behaviours indicated stress and exposed juveniles to an elevated risk of predation. In the present study, the behaviour of juveniles was not systematically monitored, but a general trend of decreased movement in juveniles placed in the low oxygen treatment was observed as the experiment progressed.

The concentrations of dissolved oxygen used in the present study reflect those that may occur in the interstitial microhabitats of juvenile mussels. Quinlan, Malcolm & Gibbins (2014) used optode sensors buried in the river sediment to measure dissolved oxygen and water temperature for a year, in a riffle in the regulated River Ehen, north-west England, which supports an FPM population. Mean oxygen concentration at 5 cm depth in the sediment was 10.2 mg L^{-1} (this value in milligrams per litre was converted using the dissolved oxygen saturation and temperature figures in Quinlan, Malcolm & Gibbins, 2014), the mean subsurface temperature being 11 °C. The highest dissolved oxygen concentration was 13.5 mg L^{-1}, and the lowest was 0 mg L^{-1}. Generally, dissolved oxygen concentration stayed above 5.2 mg L^{-1} throughout the year, but between July and November values ranging from 0 to 8.5 mg L^{-1} were measured on several occasions with one of the five optodes. Quinlan, Malcolm & Gibbins (2014) suggested that anoxic conditions were probably connected to discharge events resulting from river regulation. This shows that low oxygen (or even anoxic) events may occur in a substrate that appears to be well oxygenated.

Information on the interstitial temperatures of FPM streams is lacking, but the 19 °C temperature used in this study is likely to represent the highest end in the range of interstitial temperatures juvenile FPMs are exposed to during the year. Quinlan, Malcolm & Gibbins (2014) documented maximum subsurface temperatures of 19 °C at 5 cm depth in the River Ehen. As poikilothermic animals, bivalves reach their maximal metabolic rate at high temperatures (Pörtner, 2012); that is, their oxygen consumption is much higher during summer than in winter (Lurman, Walter & Hoppeler, 2014). Further studies are needed to investigate the temperature-dependent effects of intermediate and low dissolved oxygen at lower temperatures.

Poor oxygen conditions of the substrate caused by fine sediment loads have been regarded as one of the main reasons for the decline of the endangered FPM throughout its distribution range (Buddensiek et al., 1993; Hastie, Boon & Young, 2000; Geist & Auerswald, 2007; Österling & Högberg, 2014). The present study provides the first direct, experimental approach to oxygen tolerance of juvenile FPMs, and to the assumed link between habitat degradation and recruitment failure.

Stream substrate restoration is costly, but it is possibly the most essential conservation measure for restoring threatened FPM populations. In providing information about the tolerance limits of juvenile FPMs regarding hypoxia, the results of this study can be used to justify (i) substrate restoration in mussel habitats where siltation has lowered substrate permeability and (ii) structural restoration (stones and wooden structures) to increase hydrological variability and water pressure to enhance penetration of water into the bottom gravel. Our findings also support the inclusion of dissolved oxygen in monitoring programmes for FPMs, in concordance with the European Committee for Standardization standard protocol for monitoring FPM populations (Boon et al., 2019).

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available as supplementary material.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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