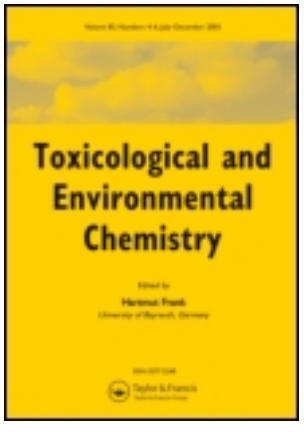

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J. Taskinen [a], P. Berg [a], M. Saarinen-Valta [a], S. Välilä [a], E. Mäenpää [b], K. Myllynen [b] & J. Pakkala [b]

[a] Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, FI-40014 Jyväskylä, Finland

[b] South Ostrobothnian Centre for Economic Development, Transport and Environment, PO Box 77, FI-67101, Kokkola, Finland

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Effect of pH, iron and aluminum on survival of early life history stages of the endangered freshwater pearl mussel, *Margaritifera margaritifera*

J. Taskinen[a]*, P. Berg[a], M. Saarinen-Valta[a], S. Välilä[a], E. Mäenpää[b], K. Myllynen[b] and J. Pakkala[b]

[a]*Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, FI-40014 Jyväskylä, Finland;* [b]*South Ostrobothnian Centre for Economic Development, Transport and Environment, PO Box 77, FI-67101, Kokkola, Finland*

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Glochidium larvae and juveniles of the endangered freshwater pearl mussel *Margaritifera margaritifera* may be sensitive to low pH and metal exposure, but to our knowledge, no tolerance tests have been performed. Therefore, we exposed glochidia, fish-attached glochidia, and juveniles of the pearl mussel to low pH and increased iron (Fe) and aluminum (Al) by using realistic pH (6.0–4.5), Fe (0.5–2.0 mg L^{-1}), and Al (0.25–1.0 mg L^{-1}) levels periodically observed in this study site. Survival of glochidia decreased with decreasing pH, increasing Fe, and increasing Al, as well as with increasing Fe + Al concentration in a 72 h exposure. All glochidia died within 24 h in pH 4.5 and Fe 2.0 mg L^{-1}. When infected trout, *Salmo trutta*, were exposed to increased Fe and Al from 4 days before to 76 days after infection, the numbers of encysted glochidia did not differ from unexposed control fish. In juvenile mussels, a slight decrease in survival was observed in lowered pH and increased Al and a combination of Al + Fe in a 168 h experiment. Results indicate that episodes of low pH and high metal concentrations may harm glochidia and potentially contribute to local decline of *M. margaritifera*.

Keywords: unionida; toxicity; metal; acidity; glochidium; juvenile; parasite

Introduction

Many freshwater mussel species and populations are threatened worldwide (Lydeard et al. 2004). Due to habitat alteration, climate change, pollution, and introduction of invasive species, unionid mussels have remarkably declined during the last decades so that, for example, in the North America about two-thirds of the mussel species are considered to be threatened (Strayer 2008). In Europe, the freshwater pearl mussel *Margaritifera margaritifera* has declined markedly or disappeared from many areas during the last century (Bauer 1986; Oulasvirta 2010). *Margaritifera margaritifera* is classified by the International Union for Conservation of Nature IUCN as endangered.

The freshwater pearl mussel has a parasitic life cycle stage in fish, Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*). *Margaritifera margaritifera* occurs in cool, running waters of the Holarctic region. It is extremely long-lived, living possibly more than

*Corresponding author. Email: jouni.k.taskinen@jyu.fi

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200 years in the north (Helama and Valovirta 2008). Most of the viable European *M. margaritifera* populations are located in the north, but many populations are also declining in the Northern Europe (Oulasvirta 2010). Alterations in the physicochemical characteristics of the streambed have caused problems for juvenile mussels (Geist and Auerswald 2007). Pearl mussel populations have suffered from pearl fishing and the decline of the host fish. Anthropogenic perturbations such as habitat alteration and fragmentation are probably the most important factors for the global decline of *M. margaritifera* (Geist 2010).

Studies on the possible impact of pollution on *M. margaritifera* are scarce. Frank and Gerstmann (2007) observed much higher concentrations of the organochlorine insecticide DDT and its metabolite DDE, as well as heavy metals, especially cadmium, in mussels from Bavaria, Germany, than in mussels from northern Finland. This contamination may have contributed to the occurrence of brittle shells in that Bavarian mussel population (Frank and Gerstmann 2007). However, to our knowledge, no toxicity tests on *M. margaritifera* glochidia or juveniles have been performed. For conservation and management purposes, it would be important to know how sensitive different life cycle stages of the pearl mussel are.

In Finland, acidic sulfate soils cause fluctuation in water pH of some pearl mussel rivers, such as the present study site, River Ähtävänjoki, where pulses of low pH release metals and cause periodic high concentrations of, e.g., iron (Fe) and aluminum (Al). Critically low pH values have been observed in other rivers with acid sulfate soils adjacent to River Ähtävänjoki (Saarinen et al. 2010). The recruitment of young pearl mussel individuals in River Ähtävänjoki is practically zero. It has been hypothesized that periods of low pH accompanied with high concentrations of Fe and Al may contribute to the reduced success of *M. margaritifera* in River Ähtävänjoki. Especially, it has been proposed that low pH and metal exposure would be detrimental to the early life cycle stages, as the adult mussels seem to tolerate water quality variation and produce glochidia in this river.

Although numerous laboratory toxicity tests on unionid glochidia and juveniles have been performed (Ingersoll et al. 2007), those with Fe and Al are rare. Previous results indicate that exposure to low pH (Huebner and Pynnönen 1992; Pynnönen 1995) and increased Al (Huebner and Pynnönen 1992) decrease the viability of glochidia of the unionids *Anodonta anatina* and *Anodonta cygnea*. Results on the unionid *Lampsilis fragilis* indicate that survival of glochidia can be affected negatively by iron exposure (Dunn, Farris, and Van Hassel 2007). In addition, the negative effect of low pH on survival of juvenile unionids has also been observed (Wang et al. 2008). Studies on the effect of acidic conditions or metal contamination on glochidia encysted in the gills of host fish are scarce, but results by Pettersen et al. (2006) suggest that the numbers of *A. anatina* glochidia decrease when the host fish is exposed to acidic water rich in Al.

Therefore, the aim of this study was to investigate how low pH, high Fe concentration, and high Al concentration may influence the different life cycle stages of the pearl mussel, i.e., the glochidium larvae released to the water by adult female mussels, glochidia encysted on the gills of host fish, and the recently metamorphosed juveniles of *M. margaritifera*. Moreover, the aim was to use realistic pH levels and metal concentrations that have been measured in river Ähtävänjoki. Our hypothesis was that exposure to low pH, increased Fe, and increased Al would have a negative impact on the survival of released glochidia, attached glochidia in host fish and juveniles of the endangered freshwater pearl mussel, *M. margaritifera*.

Materials and methods

Survival of free, released glochidia

In this experiment, the success of free *M. margaritifera* glochidia was monitored up to 72 h in different, realistic pH levels and in different, realistic concentrations of Fe, Al, and their combinations. Survival of glochidia was examined 24, 48, and 72 h from the beginning of the experiment. A reference group, originating from the same batch of glochidia and kept in equal conditions as the experimental glochidia, was sampled in the beginning (0 h) and after 8 days (192 h).

To obtain glochidia, 50 mature *M. margaritifera* mussels were collected by a scuba diver from River Ähtävänjoki on 30 September 1993. Mussels were transported to the laboratory in the river water in large buckets and allowed to release glochidia. After that, the water was filtered using a 50 µm filter to collect glochidia and the mussels were returned back to their original site. Glochidia were randomly collected from a freshly released glochidia suspension using a pipette and allocated to different treatments in 500 mL glass vials, about 1000 glochidia per vial.

In pH exposure, the treatment groups were (1) pH control, (2) pH 6.0, (3) pH 5.5, (4) pH 5.0, and (5) pH 4.5. In Fe exposure, the treatment groups were (1) Fe control, (2) Fe 0.5 mg L^{-1}, (3) Fe 1.0 mg L^{-1}, (4) Fe 1.5 mg L^{-1}, and (5) Fe 2.0 mg L^{-1}. In Al, the treatment groups were (1) Al control, (2) Al 0.25 mg L^{-1}, (3) Al 0.5 mg L^{-1}, (4) Al 0.75 mg L^{-1}, and (5) Al 1.0 mg L^{-1}. In combination of Al and Fe exposure, the treatment groups were (1) Al + Fe control, (2) Al 0.25 + Fe 0.5 mg L^{-1}, (3) Al 0.5 + Fe 1.0 mg L^{-1}, (4) Al 0.75 + Fe 1.5 mg L^{-1}, and (5) Al 1.0 + Fe 2.0 mg L^{-1}. To achieve the desired pH level, Fe concentration, and Al concentration, water from the upper part of River Ähtävänjoki, close to Lake Lappajärvi, the main source of the river, was modified with hydrochloric acid (HCL), $FeSO_4$, and $AlCl_3$, respectively. Unmodified river water, having pH value of 6.8, Fe concentration of 0.28 mg L^{-1}, and Al concentration of 0.07 mg L^{-1} was used as the control water. Half of the water was exchanged every day.

At each time point (24, 48, and 72 h), five random samples of about 30 glochidia were collected from each vial. One vial represented one treatment group, i.e., the control and the treatment groups for each test consisted of only one replicate jar. Dead and alive glochidia were counted for each sample so that a glochidium was classified as dead if it did not close its valves when repeatedly disturbed. Death of a given glochidium individual was checked by touching the glochidium gently with a pipette tip to ensure that the glochidium did not respond. Ability to close valves is essential for a glochidium to attach to host fish. Therefore, valve closure is an ecologically relevant measure of glochidia viability. The experiment was performed in 6°C temperature in a dark room. The study was conducted with a permission to collect, transport, and handle *M. margaritifera*.

Survival of attached glochidia in fish

In this experiment, the success of *M. margaritifera* glochidia in fish was monitored for 2.5 months post infection in a set up where fish were exposed to realistic concentrations of Al and Fe throughout the experiment so that the metal exposure started 4 days before the infection. The experiment lasted for 76 days, including nine time points of observation.

Zero years old, farmed brown trout *Salmo trutta* (River Ähtävänjoki stock) – mean ± S.E. length and weight 74.7 ± 0.5 mm and 3.2 ± 0.1 g, respectively – were transported from a fish farm to the laboratory where they were kept in a 1000 L flow-through tank. After 1 week of adaptation to laboratory conditions, fish were

randomly distributed to four experimental 400 L tanks which were filled with (1) Lake Lappajärvi water (control), (2) Lake Lappajärvi water adjusted with additional $AlCl_3$ so that the concentration was aimed at Al 0.5 mg L^{-1}, (3) Lake Lappajärvi water adjusted with additional $FeSO_4$ so that the concentration was Fe aimed at 0.5 mg L^{-1}, and (4) Lake Lappajärvi water added with $FeSO_4$ so that the concentration was aimed at Fe 1.5 mg L^{-1}. Number of fish per tank was 55, except for Fe 0.5 mg L^{-1} treatment where it was 53, in total there were 218 fish. Water temperature was 8°C and the water was continuously aerated. Eighty liters of water in each tank was changed every 4–5 days. Levels of pH and metal concentrations were monitored throughout the study on the day following the water change. In Al 0.50 mg L^{-1} treatment, the actual Al concentration varied from 0.29 on day 8 to 0.64 mg L^{-1} on day 69, with the mean concentration of 0.43 mg L^{-1} ($n = 16$ measurements between days 1 and 69). In Fe 0.50 mg L^{-1} treatment, the actual Fe concentration varied from 1.00 on day 1 to 0.55 mg L^{-1} on day 24, with the mean concentration of 0.65 mg L^{-1} ($n = 16$ measurements between days 1 and 69). In Fe 1.50 mg L^{-1} treatment, the actual Fe concentration varied from 2.50 on day 1 to 0.80 mg L^{-1} on day 24, with the mean concentration of 1.26 mg L^{-1} ($n = 16$ measurements between days 1 and 69). In control water, the actual Fe concentration varied from 0.28 on day 1 to 0.16 mg L^{-1} on day 24, with the mean concentration of 0.20 mg L^{-1} ($n = 16$ measurements between days 1 and 69). The actual Al concentration in the control water varied from 0.04 on day 1 to 0.08 mg L^{-1} on day 67, with the mean concentration of 0.05 mg L^{-1}.

Fish were infected after being 4 days in the experimental tanks. To obtain glochidia, 50 *M. margaritifera* individuals were collected by a scuba diver from River Ähtävänjoki on 30 September 1993. Mussels were transported to laboratory and kept in 60 L buckets for 24 h in aerated ground water where they were allowed to release their glochidia. After that, the mussels were returned to their original site. In the laboratory, four separate 60 L glochidium suspensions with a density of 25,000 freshly released glochidia L^{-1} were prepared. Each treatment group was infected by placing the fish in the glochidium suspension for 5 min, after which the fish were returned to their experimental tanks.

Numbers of glochidia were studied at nine time points: 1, 4, 7, 14, 21, 28, 42, 56, and 76 days post infection. Fish were killed by sharp blow on the head, measured for wet weight and total length, and the gills were removed to be examined microscopically. At each time point, six fish were randomly collected from each treatment tank, except for the time point 76 days when the last 7, 7, 5, and 7 fish were collected from control, Al 0.5 mg L^{-1}, Fe 0.5 mg L^{-1}, and Fe 1.5 mg L^{-1} tanks, respectively. The study was conducted with a permission to use fish in experimental research and to collect, transport, and handle *M. margaritifera*.

Survival of juvenile mussels

The juvenile pearl mussels in this experiment originated from two populations, River Ähtävänjoki population, Western Finland, and River Iijoki population, Northern Finland. In late September 2006, zero years old, farmed brown trout (River Ähtävänjoki stock) were caged in a tributary of River Iijoki to get infected with the pearl mussel glochidia. After infection, fish were transported to the laboratory where they were kept in a 1000 L flow-through tank at 8°C. River Ähtävänjoki glochidia were obtained by collecting adult pearl mussels from the river and incubating them in buckets in the laboratory, as described above. After that, zero years old, farmed brown trout

(River Ähtävänjoki stock) were infected with River Ähtävänjoki glochidia in the laboratory and stored in a 1000 L flow-through tank at 8°C. Water temperatures in the maintenance tanks were gradually decreased to 2°C until the next spring when the temperatures were gradually increased to 17–18°C. Fish with the glochidia from both pearl mussel populations were maintained in equal conditions in the same laboratory until the glochidia were fully developed and started to excyst on 11 June 2007, when the cumulative sum of day-degrees approached 1200–1300°C.

The juvenile survival experiment was conducted between June 13 and 20, 2007. Excysted juvenile mussels were collected from the bottom of the fish tanks. Juveniles were pipetted to cell culture dishes (width 60 mm, height 15 mm), randomly chosen 10 juvenile pearl mussels $dish^{-1}$. The handling of the juvenile pearl mussels and the experiment took place in 17–18°C. pH levels and concentrations of Fe and Al were as in the glochidium survival experiment above. In the case of River Iijoki, there was one common control with six replicate dishes of 10 juvenile individuals. Otherwise, the treatment groups were exactly as in the above glochidium experiment with three replicate dishes of 10 juveniles per treatment. Thus, in total there were 540 juveniles for River Iijoki population. Due to the lower number of juveniles available for River Ähtävänjoki population, a lower number of treatment levels were applied. Thus, in the case of River Ähtävänjoki, there was one common control group with three replicate dishes of 10 individuals. In pH exposure, the only treatment group was pH 5.0. In Fe exposure, the treatment groups were Fe 0.5 mg L^{-1}, Fe 1.0 mg L^{-1}, and Fe 2.0 mg L^{-1}. In Al, the treatment groups were Al 0.25 mg L^{-1}, Al 0.5 mg L^{-1}, and Al 1.0 mg L^{-1}. In combination of Al and Fe exposure, the treatment groups were Al 0.25 + Fe 0.5 mg L^{-1}, Al 0.5 + Fe 1.0 mg L^{-1}, and Al 1.0 + Fe 2.0 mg L^{-1}. Each treatment group had three replicate dishes of 10 juveniles. Thus, the total number of juveniles for River Ähtävänjoki population was 330 individuals.

Control water was from the uppermost part of River Ähtävänjoki, close to Lake Lappajärvi, the main source of the river, as in the experiment testing the survival of free glochidia. The control water was modified for each treatment to achieve the desired concentrations. The pH of water was modified with hydrochloric acid (HCl). Fe concentration was modified using $FeSO_4$ and Al concentration using $AlCl_3$. In the beginning, each dish contained 4.5 mL of water. The test waters were replaced with fresh ones at 120 h after the beginning of the experiment. The juvenile mussels were not fed.

Only alive juveniles were included in the experiment. Survival of the pearl mussel juveniles was monitored at 24, 48, 72, 120, and 168 h. Every pearl mussel in every dish was monitored with microscope and the number of living and dead juveniles was counted. Every time when the mussels were examined, the dish was gently moved to make the mussels close their shells. After such a disturbance, alive mussels quite quickly opened their valves and pushed their foot out. Dead mussels did not react to disturbance and their shells remained open. The study was conducted with a permission to use fish in experimental research and to collect, transport, and handle *M. margaritifera*.

Chemical and statistical analyses

Chemical analyses were performed in the Laboratory of West Finland Environment Centre, Kokkola. Effects of metal exposure and pH treatment on the survival of free glochidia and juvenile mussels were analyzed with 1-way ANOVA, or if the assumptions of ANOVA were violated, with a non-parametric Kruskall–Wallis test. Replicate-specific survivals were used as the statistical units. When analyzing the survival of attached

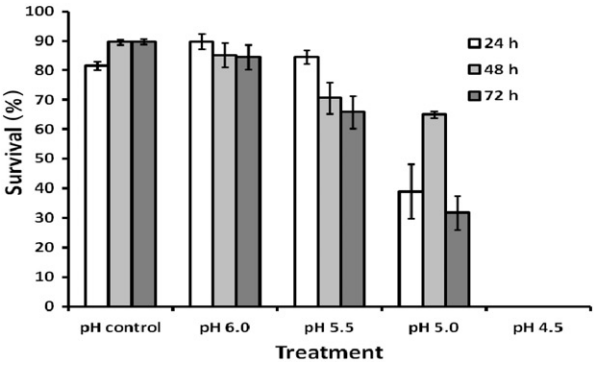

Figure 1. Mean ± S.E. survival of *M. margaritifera* glochidia larvae in different pH levels in a 72 h experiment. In pH 4.5 all glochidia were dead at 24 h time point.

glochidia in fish, we divided the monitoring time into two periods. The first period included the time points 1 and 4 days. This early stage of infection included the attachment of glochidia to the gills of fish and the development of glochidial cyst within the gill tissue (Fustish and Millemann 1978). Following the infection, this early stage is usually characterized by a rapid decline of glochidia numbers in gills (Bauer and Vogel 1987). The second period included time points 7, 14, 21, 28, 42, 56, and 76 days. This corresponds to time when the initial high mortality of glochidia is leveled off (Bauer and Vogel 1987). Effect of metal treatment and time on the number of glochidia in fish was analyzed using ANCOVA with treatment and time as fixed factors and fish weight as the covariate. Statistical analyses were performed using SPSS statistical package.

Results

Survival of free, released glochidia

Mean ± S.E. survival of free glochidia in the reference group in the beginning of the experiment (0 h) was 97.4 ± 0.7 %. After 8 days (192 h), 80.6 ± 4.0% of the glochidia were still alive in the reference group.

Survival of free glochidia went down with decreasing pH so that none of the glochidia survived 24 h in pH 4.5 (Figure 1). For example, at the time point 48 h, the differences between control/treatment groups in the mean survival were statistically significant (Kruskall–Wallis test, test value = 20.594, df = 4, $p < 0.001$). Paired comparisons revealed that the 48 h survival of the control group was higher than that of pH 4.5 ($p < 0.001$) and pH 5.0 ($p = 0.007$) but did not differ significantly from the other treatment groups. Survival of glochidia seemed also to decrease with time (Figure 1). In pH 5.5, for example, differences between 24, 48, and 72 h in the mean survival of glochidia were statistically significant (1-way ANOVA, $F_{2,12} = 4.373$, $p = 0.037$). LSD multiple comparisons revealed that 24 h survival was significantly higher than that of 72 h survival ($p = 0.015$), but 48 h did not differ from either 24 h ($p = 0.056$) or 72 h ($p = 0.476$).

Survival of mussel glochidia was negatively affected by Fe exposure so that none of the glochidia survived up to 72 h if the Fe concentration was 1.5 mg L^{-1} or higher (Figure 2). When the between-treatment differences were analyzed for the time point 48 h, for instance, they appeared to be statistically significant (Kruskall–Wallis test, test

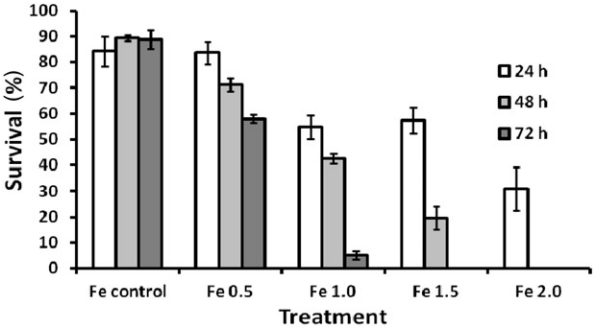

Figure 2. Mean ± S.E. survival of *M. margaritifera* glochidia larvae in different Fe concentrations in a 72 h experiment. In concentrations of Fe 1.5 mg L^{-1} and Fe 2.0 mg L^{-1} all glochidia died within 72 and 48 h, respectively.

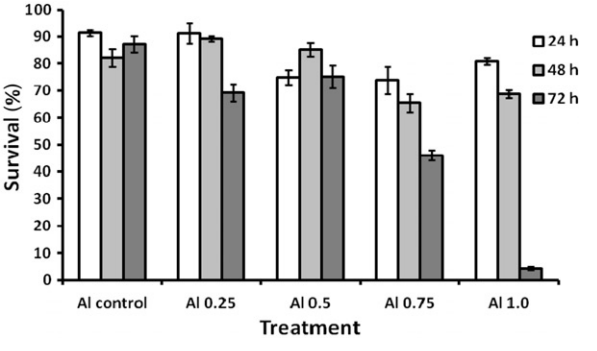

Figure 3. Mean ± S.E. survival of *M. margaritifera* glochidia larvae in different Al concentrations in a 72 h experiment.

value = 23.274, df = 4, $p < 0.001$). Paired comparisons indicated that the 48 h survival of the control group was higher than that of Fe 1.0 mg L^{-1}, ($p = 0.031$), Fe 1.5 mg L^{-1} ($p = 0.001$), and Fe 2.0 mg L^{-1} ($p < 0.001$), but did not differ from the treatment group of Fe 0.5 mg L^{-1}. There was also a clear time effect so that survival of glochidia decreased with time (Figure 2). In the treatment group Fe 1.0 mg L^{-1}, for example, differences between 24, 48, and 72 h in the mean survival of glochidia were statistically significant (1-way ANOVA, $F_{2,12} = 74.108$, $p < 0.001$). LSD multiple comparisons revealed that all the three time points differed significantly from each other by their mean glochidial survival ($p = 0.014$ or lower).

Also Al exposure affected negatively to glochidia survival so that the survival approached zero in the highest concentration at 72 h time point (Figure 3). Results of 1-way ANOVA suggested that differences between the groups were statistically highly significant after 72 h exposure ($F_{4,20} = 131.19$, $p < 0.001$). Multiple comparisons indicated that at this time point the survival of control glochidia was significantly higher than that of any Al exposure group ($p = 0.008$ or lower). A clear time effect can also be seen at least in the highest Al exposures (Figure 3). In aconcentration of 0.75 mg L^{-1}, for instance, differences between 24, 48, and 72 h in the mean survival of glochidia were statistically

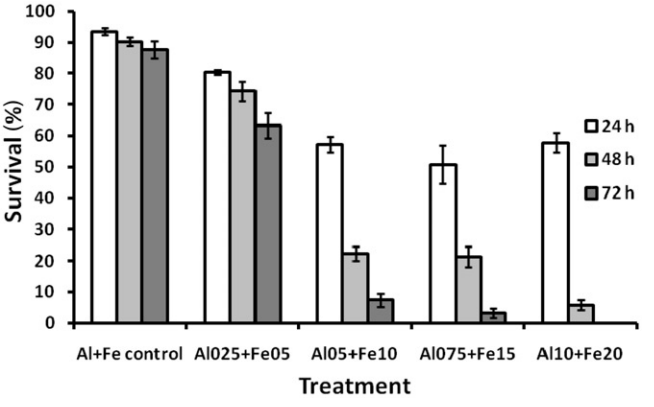

Figure 4. Mean ± S.E. survival of *M. margaritifera* glochidia larvae in different concentrations of combined Al and Fe in a 72 h experiment. All glochidia died in concentration of Al 1.0 mg L^{-1} + Fe 2.0 mg L^{-1}.

significant (1-way ANOVA, $F_{2,12} = 15.133$, $p = 0.001$). LSD multiple comparisons revealed that 72 h survival was lower than at either 48 h ($p = 0.003$) or 24 h ($p < 0.001$).

Combined exposure of glochidia to both Al and Fe also appeared to lower the survival of glochidia, as the mortality rose to 100% after 72 h in the highest combined metal exposure (Figure 4). Between-group differences were statistically significant when analyzed at 72 h time point (Kruskall–Wallis test, test value = 21.458, $p < 0.001$). Paired comparisons suggested that after 72 h of exposure, survival of the control group was significantly higher than that of Al 1.0 + Fe 2.0 mg L^{-1} ($p < 0.001$), Al 0.25 + Fe 0.5 mg L^{-1}, ($p = 0.001$) and Al 0.5 + Fe 1.0 mg L^{-1} ($p = 0.012$), but did not differ from Al 0.25 + Fe 0.5 mg L^{-1} ($p = 0.274$). Survival also decreased by time in the combined exposure to Al and Fe (Figure 4). When analyzed for the treatment group Al 0.25 + Fe 0.5 mg L^{-1}, results of 1-way ANOVA indicated highly significant differences between time points ($F_{2,12} = 33.385$, $p < 0.001$). LSD multiple comparisons revealed that all time points differed from each other by their mean survival ($p = 0.009$ or lower).

Survival of attached glochidia in fish

When examined 1 day post infection, all the 24 fish studied were infected with minimum and maximum number of 36 and 232 glochidia $fish^{-1}$, respectively. Numbers of glochidia in the gills of fish varied remarkably between fish individuals. Results of ANCOVA indicated the main effect of treatment (Al 0.5 mg L^{-1}, Fe 0.5 mg L^{-1}, Fe 1.5 mg L^{-1}, and control) on glochidia was not significant ($F_{3,214} = 1.107$, $p = 0.347$). Moreover, there was no significant interaction between treatment and time ($F_{3,214} = 0.555$, $p = 0.645$), suggesting that the relationship between treatments and glochidium number did not differ between the initial (1–4 days) and later (7–76 days) phases of infection. However, the initial and later phases of infection differed significantly by the number of glochidia per fish (main effect 'time', $F_{1,214} = 19.622$, $p < 0.001$) (Figure 5). In addition, the effect of the covariate, fish weight, was also significant ($F_{1,214} = 12.610$, $p < 0.001$). Abundance of glochidia infection increased with fish weight and the mean (±S.E.) weight-adjusted

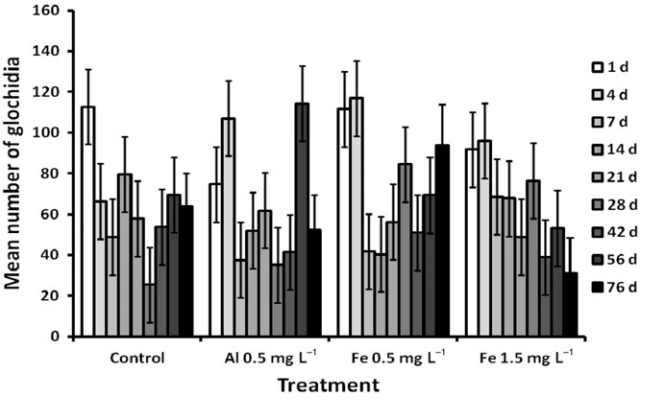

Figure 5. Mean ± S.E. numbers of *M. margaritifera* glochidia attached to the gills of host fish in different concentrations of Al and Fe in a 76 days experiment.

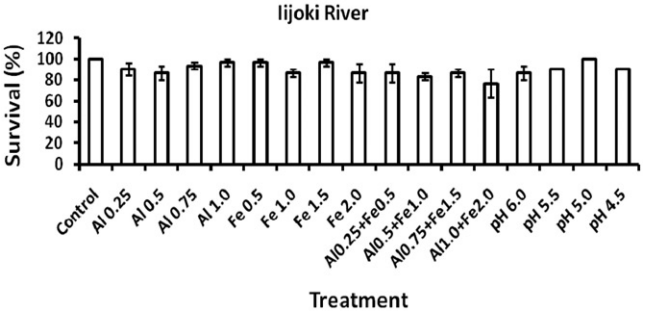

Figure 6. Mean ± S.E. survival of juvenile *M. margaritifera* in different pH levels and Fe and Al concentrations at the end of a 168 h experiment, results for River Iijoki population.

abundances of infection were 92.6 ± 6.6 and 58.6 ± 3.5 glochidia per fish in the initial (1–4 days) and later (7–76 days) stages of infection, respectively.

Survival of juvenile mussels

In general, mortality among the juvenile mussels was not high during the 168 h experiment. Mean ± S.E. survival at the end of the experiment over the replicate dishes was 90.7 ± 1.4% ($n = 54$ dishes) in River Iijoki and 92.7 ± 1.7 % ($n = 33$ dishes) in River Ähtävänjoki mussels. Due to the quite high survival rate, the statistical differences were analyzed only for the last time point, 168 h. Results of the non-parametric Kruskall–Wallis test indicated that any differences between groups were insignificant both in River Iijoki (test value = 25.145, df = 16, $p = 0.067$) and River Ähtävänjoki (test value = 15.907, df = 16, $p = 0.102$) (Figures 6 and 7). Similarly, the overall survival did not differ between populations in River Iijoki and River Ähtävänjoki (1-way ANOVA, $F_{1,67} = 0.535$, $p = 0.467$). As the next step, since the populations did not differ from each other by their mean survival, data from the two populations were combined. When using the combined

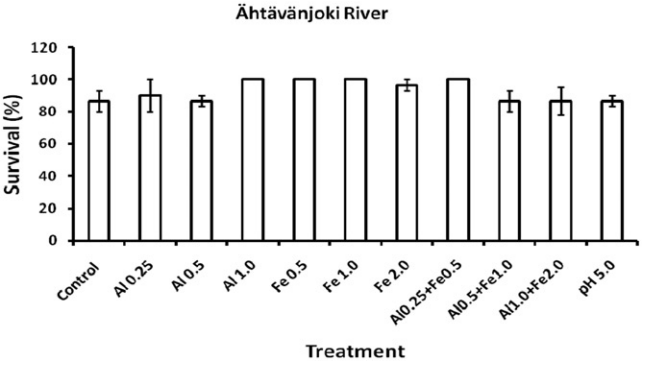

Figure 7. Mean ± S.E. survival of juvenile *M. margaritifera* in different pH levels and Fe and Al concentrations at the end of a 168 h experiment, results for River Ähtävänjoki population. C means control and numbers 1–10 denote the same treatments as in Figure 8, respectively.

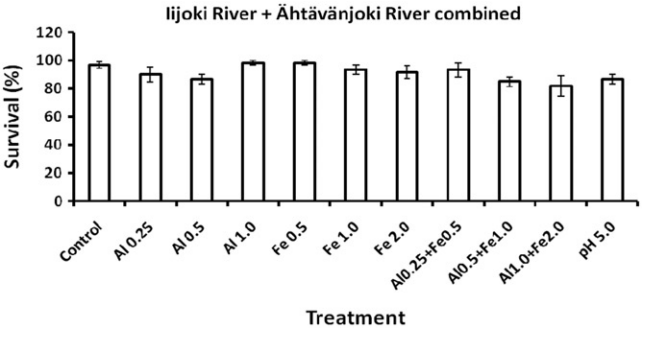

Figure 8. Mean ± S.E. survival of juvenile *M. margaritifera* in different pH levels and Fe and Al concentrations at the end of a 168 h experiment, results for combined data of River Iijoki and River Ähtävänjoki populations.

data, between-treatment differences were statistically significant (Kruskall–Wallis test, test value = 20.224, df = 10, $p = 0.027$). Paired comparisons revealed that the survival of juvenile mussels was significantly lower in the aluminum exposure Al 0.5 mg L^{-1} ($p = 0.017$), and in combined aluminum + iron exposures Al 0.5 mg L^{-1} + Fe 1.0 mg L^{-1} ($p = 0.008$) and Al 1.0 mg L^{-1} + Fe 2.0 mg L^{-1} ($p = 0.013$), as well as in pH 5.0 exposure ($p = 0.043$), than in the control group (Figure 8)

Discussion

Water drainage area of River Ähtävänjoki contained acid sulfate soils which increase the risk of acid discharge in the river. We have observed pH values less than 5.0 in the pearl mussel sites of River Ähtävänjoki when the river is flooding (unpublished). Recent results by Saarinen et al. (2010) from three adjacent rivers also having acid sulfate soils showed periods of critically low pH levels with minimum pH values as low as 4. Moreover, the worst acidity problems in those rivers occurred during the autumn–winter runoff period

(Saarinen et al. 2010). Thus, the low pH levels in River Ähtävänjoki may take place during the time when *M. margaritifera* release their glochidia.

Present results indicate that low pH has a negative effect on *M. margaritifera* glochidia – all glochidia died in the lowest pH value of 4.5 within 24 h. When compared to control group, survival of glochidia was significantly lower in pH 4.5 and pH 5.0 at the 48 h time point suggesting that pH values 5.0 or lower may remarkably decrease the success of *M. margaritifera* glochidia. Previous results on the unionids *A. anatina* and *A. cygnea* indicate that a simultaneous exposure to low pH and increased Al decrease the viability of glochidia (Huebner and Pynnönen 1992). Low pH also increased the negative effect of cadmium and copper on the viability of *A. cygnea* glochidia (Pynnönen 1995).

The highest Fe concentrations lead to a complete mortality of *M. margaritifera* glochidia within 48 h. In addition, the 48 h survival of glochidia in Fe 1.0 mg L^{-1}, Fe 1.5 mg L^{-1}, and Fe 2.0 mg L^{-1} concentrations was significantly lower than in the control group, suggesting that Fe concentrations of 1.0 mg L^{-1}, or higher, may considerably reduce the success of *M. margaritifera* glochidia. Dunn, Farris, and Van Hassel (2007) suggested that even as high concentration as 5.6 mg L^{-1} of Fe found in a mine waste site is below the acute lethal concentration of glochidia of the unionid *Lampsilis fragilis*. Thus, results on the unionid *L. fragilis* indicated that the survival of glochidia is affected negatively by Fe if the concentrations are very high (Dunn, Farris, and Van Hassel 2007).

Al exposure also decreased *M. margaritifera* glochidia survival, but a remarkable decrease was observed only at the end of the 72 h experiment in the highest concentration of Al 1.0 mg L^{-1}. Huebner and Pynnönen (1992) observed a negative impact of Al on glochidia of the unionids *A. anatina* and *A. cygnea* in low pH conditions. In addition, combined exposure to Fe and Al was also detrimental to *M. margaritifera* glochidia in this study. Survival in Al 0.5 + Fe 1.0 mg L^{-1}, or higher concentrations was lower than in the control water at 72 h time point, but the effect of combined Fe and Al seemed not to differ from Fe, only. In a toxicity test conducted with the unionid *A. anatina*, Hanstén, Heino, and Pynnönen (1996) observed that Fe ameliorated toxicity of cadmium, zinc, and copper to glochidia. Thus, the effect of combined metal exposures may not be the sum of individual effects.

In the present glochidium experiment, the control and the treatment groups for each test consisted of only one replicate jar. Therefore, it was not possible to assess the jar effect. In addition, despite of the five replicate samples examined for each time point, sometimes a higher mean survival rate was observed for a later time point although the real survival rate can only decrease with time. This could be due to a large random variation in the proportion of survived glochidia between samples of about 30 individuals. The sample size should probably have been higher to reduce the variation. However, the differences in survival rates between control and treatment groups, and with respect to time were so clear that the present results should be considered at least indicative of negative effect of low pH, high Al, and high Fe on the survival of *M. margaritifera* glochidia. The results are also in line with the previous observations on unionids, suggesting together a negative effect of low pH, high Fe, and high Al on unionid glochidia survival. Moreover, it is important to note that the pH levels and metal concentrations used in this study were within the range of their natural occurrence in River Ähtävänjoki. Therefore, the results suggest that the periodical low pH combined with high Fe and Al concentrations observed regularly in River Ähtävänjoki may deteriorate the survival of *M. margaritifera* glochidia if occurred at the same time with glochidia release of the pearl mussel. As the negative effects of low pH, high Fe, and high Al on the infectivity of glochidia can be assumed to be more pronounced than on survival, it may be concluded

that low pH, high Fe, and high Al may potentially contribute to the low recruitment success of *M. margaritifera* in this river.

Unlike the free glochidia, the encysted glochidia in the gills of host fish seemed not to be harmed by the Fe and Al treatments. In the present experiment, the glochidia were not directly exposed to metals before the infection but only after attachment to host fish gills. However, metal exposure can cause swelling of the gill flamella and increase their mucous production (Ledy, Giambérini, and Pihan 2003) which can be expected to lower the chances of glochidia to attach to gills and further inhibit glochidial development within the gills. In addition, during the first days, glochidia can be exposed to contaminants from water as the glochidial cyst wall formation is not completed (Cope et al. 2008). After that, encysted glochidia can be exposed to contaminants contained by the host tissues (Cope et al. 2008). Indeed, it has been shown that gill tissue of fish absorbs Al quickly from the water (Handy and Eddy 1989). On the other hand, metal exposure has been shown to impair the immune defense of fish (Dautremepuits et al. 2009) which should increase the success of glochidia (Bauer and Vogel 1987; Constance and Dimock 2003). However, the water temperatures used in this study probably are too low for the fish adaptive immune system to mount an effective response against the developing glochidia (Bly and Clem 1992; Roberts and Barnhart 1999). As the number of glochidia in metal treatments did not differ from control fish, it can be assumed that the peaks of high Fe and Al concentrations observed in River Ähtävänjoki may not harm the development of *M. margaritifera* glochidia attached to the gills. The experiment lasted for 72 days, but the glochidia are attached to brown trout gills in River Ähtävänjoki for about 270 days. Thus, the harmful effects of metal exposure in long run, and during the last part of glochidial development cannot be completely ruled out.

Effect of pH and metal exposure on the survival of juvenile *M. margaritifera* was relatively low. Statistically significant effects were found only when the two study populations were combined. In that way, survival of glochidia was lower in the highest concentrations of combined Fe + Al exposures than in the control juveniles, suggesting that simultaneous exposure to those two metals may be more detrimental than to either metal alone. Survival of juveniles was also lower in the aluminum exposure Al 0.5 mg L^{-1} than in the control group. It is hard to explain why juvenile survival did not decrease in the higher Al concentrations in the present study, but the decreased survival in low pH was expected. Previous studies on the effect of low pH, high Fe, and high Al on juvenile unionid survival are scarce, but the negative effect of low pH on juvenile survival was illustrated in the unionid *Lampsilis siliquoidea* – survival decreased with decreasing pH in toxicity tests with ammonia (Wang et al. 2008). As the freshwater pearl mussels grow very slowly (Hastie, Young, and Boon 2000) and are very long-lived (Helama and Valovirta 2008), the duration of juvenile stage is long. Therefore peaks of low pH, high Fe, and high Al may harm the survival of pearl mussel juveniles in River Ähtävänjoki and thereby contribute to the low recruitment of *M. margaritifera* in this river.

In conclusion, the current results indicate that glochidia of *M. margaritifera* may be sensitive to acid conditions and metal contamination. Free glochidia of *M. margaritifera* were clearly more sensitive than the encysted glochidia or juvenile mussels. This is in accordance with previous observations on unionid species in various toxicity tests (Farris and Van Hassel 2007). Extremely high age of adult *M. margaritifera* (Helama and Valovirta 2008) indicate that the adult stage is probably the least sensitive life history phase of *M. margaritifera*. Occurrence of old, reproductive individuals in River Ähtävänjoki also suggests that the episodic peaks of low pH, high Fe, and high Al probably do not affect markedly the survival and reproduction of adult mussels in the

river. Previously, to our knowledge, there was only very limited, if any information on the effect of water quality and pollution on the success of early life history stages of *M. margaritifera*. Our results indicate that low pH, as well as Fe and Al contamination can potentially have a negative impact on local pearl mussel population. This finding emphasizes the role of water quality as one of the factors that are important in the conservation of this endangered mollusc species.

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