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Research paper

Sulfate toxicity to early life stages of European whitefish (*Coregonus lavaretus*) in soft freshwaterJuha Karjalainen^{a,*}, Mikko Mäkinen^a, Anna K. Karjalainen^a^a University of Jyväskylä, Department of Biological and Environmental Science, Surfontie 9C, PO Box 35, FI-40014, Finland

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ABSTRACT

Sulfate occurs naturally in the aquatic environment but its elevated levels can be toxic to aquatic life in freshwater environments. We investigated the toxicity of sulfate in humic, soft freshwater to whitefish (*Coregonus lavaretus*) from fertilization of eggs to hatching i.e. during the critical phases of whitefish early development. Anadromous Kokemäenjoki whitefish eggs and sperm during fertilization, embryos and larvae were exposed in the long-term 175-day incubation to seven different sodium sulfate (Na₂SO₄) concentrations from 44 to 2 000 mg SO₄ L⁻¹. Endpoint variables were the fertilization success, offspring survival and larval growth. Egg fertilization and early embryonic development were the most sensitive developmental stages of whitefish to sulfate, although the fertilization success and survival of embryos decreased only in the highest concentration of 2 000 mg SO₄ L⁻¹. The survival during late embryonic period, hatching and the 5-day larval period was high and no difference between the control and sulfate treatments were observed. LC₅₀-values of sulfate for early embryonic period and for the entire embryonic and larval period was 1 413 and 1 161 mg L⁻¹, respectively. The NOEC (No-observed Effect Concentration) of sulfate for the both periods was 1 207 mg L⁻¹. The tolerance of whitefish early stages to sulfate toxicity seems to be on the same level as the tolerance of other salmonids' early stages.

1. Introduction

Sulfate (SO₄²⁻, hereinafter SO₄) is an anion, which occurs naturally in the aquatic environment. Seawater contains approximately 2 700 mg SO₄ L⁻¹ and brackish water such as the Baltic Sea water in northern Europe about 470 mg SO₄ L⁻¹ (Environment Canada, 1984; Finnish Environment Institute, 2020; Katz, 1977). In boreal inland waters, sulfate levels typically are around 0.1–1% of the levels in the seas and from less than 1–6% of the levels in brackish waters, between 3 and 30 mg L⁻¹ (Environment Canada, 1984; Finnish Environment Institute, 2020; Katz, 1977; Sahlin and Ågerstrand, 2018). Sulfates are widely used in industry as salts of sulfuric acid and sulfate levels can be elevated in solid wastes and wastewaters from mine drainage (Akcil and Koldas, 2006; Nordstrom et al., 2015), chemical industry (Wells, 1923), pulp production (Singh et al., 2019), power plants (Mohammadi et al., 2018), municipal wastewater treatment plants (Van den Brand et al., 2018) and agricultural runoff in acid sulfate soils area (Huang et al., 2016; Wallin et al., 2015). Other releases of SO₄ to the environment include consumer uses of a wide spectrum of products, e.g. washing and cleaning products, plant protection products, biocides and personal care products (ECHA,

2020).

Elevated levels of sulfate can be toxic to aquatic life in freshwater environments. Therefore, a variety of aquatic test organisms, including species of aquatic algae, moss, macrophytes, bivalves, crustaceans, rotifers, amphibians and fishes (Elphick et al., 2011; Lasier and Hardin, 2009; Simmons, 2012; Soucek, 2007; Soucek and Kennedy, 2005), have been tested for toxicity of sulfate to develop local water quality guidelines (WQGs). However, the majority of sulfate toxicity studies have been acute exposures with aquatic invertebrates and have been conducted in reconstituted deionized waters with varying compositions (Davies and Hall, 2007; Elphick et al., 2011; Lasier and Hardin, 2009; Mount et al., 1997; Soucek and Kennedy, 2005; USEPA, 2010), dechlorinated municipal tap waters (Elphick et al., 2011), well waters (Davies, 2007) or various combinations of these but not in natural soft freshwaters. Because the toxicity of sulfate is dependent on concentrations of other major ions, with a general decrease in toxicity associated with an increase in water hardness (Elphick et al., 2011), the additional data of sulfate toxicity in soft waters are urgently needed to develop further the sulfate WQGs for low water hardnesses (Elphick et al., 2011; Meays and Nordin, 2013; Sahlin and Ågerstrand, 2018). Especially in the

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boreal region, inland waters are typically humic and soft with a low buffer capacity. For example in 86% of Swedish rivers and lakes ($n = 33$ 865), the hardness of water is below $25 \text{ mg CaCO}_3 \text{ L}^{-1}$ (Sahlin and Ågerstrand, 2018).

In this study, we examined sulfate toxicity to the early life stages of anadromous European whitefish from Kokemäenjoki River (*Coregonus lavaretus* L.) in natural, soft and humic freshwater. Coregonid fishes (Coregonidae) are important species for commercial and recreational fishing and aquaculture with high economic and social value. However, many of the river-spawning whitefish stocks have declined or endangered due to power plant dams and wastewater loads into their spawning rivers. In Finland, anadromous whitefish stocks are classified as threatened species (Hyvärinen et al., 2019), and the vitality of whitefish stocks in many rivers is poorly known. In the toxicity tests, we concentrated to early life of whitefish because fertilization and consequent embryonic development and hatching are generally more sensitive to xenobiotic contamination than adults are (Hutchinson et al., 1998).

Whitefish spawn in autumn and eggs incubate almost 6 months over the winter on the river bed until the larvae hatch in spring in April or May. Water temperature in Kokemäenjoki during the egg incubation is low varying from $0.3 \text{ }^\circ\text{C}$ in winter to $10 \text{ }^\circ\text{C}$ in spring. After hatching, the larvae drift to the river estuary in the Baltic Sea. We investigated sulfate toxicity to whitefish in a 175-day incubation from the fertilization of eggs to the hatching and larval stage at natural temperatures. In our toxicity tests, the endpoint variables were: 1) fertilization success, 2) survival during early embryonic period, 3) survival during late embryonic period, 4) survival during hatching and yolk sac larvae and 5) hatching size of the larvae.

2. Materials and methods

2.1. Test species

Parental fish were caught from the spawning areas in Kokemäenjoki River in November 2019 and kept in the river water flowing through a pool in the riverbank before striping of the gametes. After striping of the eggs, parental fish were released to the river. Parental fish sampling was carried out by Pro Agria Satakunta Association and aimed to conservation support of whitefish stock by pisciculture and only small amount of eggs and sperm were taken to our experiment. Eggs and sperm of whitefish (*Coregonus lavaretus* L.) were transported from Kokemäenjoki (Harjavalta, N61° 20' 8043", E 22° 6' 17,927") to Ambiotica building at the University of Jyväskylä on 12 November 2019. During transport, the eggs and sperm of each parental fish were kept separately in plastic tubes without cover in a container with crushed ice. During striping of the parental fish, water temperature in Kokemäenjoki was $2 \text{ }^\circ\text{C}$. The eggs of 5 females were fertilized at $6 \text{ }^\circ\text{C}$ by the sperm of 5 males and fertilization was performed separately for each exposure concentration in random order within 2 h on the same day as the collection of gametes occurred. The length of the males was between 500 and 635 mm (median 570 mm) and the females between 490 and 575 mm (median 536 mm).

2.2. Test chemicals and setup

Exposure solutions were prepared by addition of sodium sulfate (Na_2SO_4 , Merck, purity $\geq 99.0\%$) to Lake Konnevesi water (KV) to achieve the 7 target sulfate concentrations in the 0.5–0.6-fold dilution series. Sodium sulfate was used rather than the sulfate salts of other cations (Ca, Mg, or K) because Na is expected to contribute the least to toxicity relative to the other cations (Mount et al., 1997). KV water was the control treatment and additional Kokemäenjoki River treatment was used in the experiment (Supplemental Material Table 1). Kokemäenjoki water (KJ) was taken from the Harjavalta at the same time as the gametes were collected and the water was kept at $6 \text{ }^\circ\text{C}$ in a cold storage

Table 1

The nominal and measured sulphate (SO_4) concentrations (mean \pm SD, $n = 28$) and pH in Kokemäenjoki (KJ) water, Konnevesi (KV) control water and the Na_2SO_4 exposure treatments during the entire experimental period.

| Test waters | $\text{SO}_4 \text{ mg L}^{-1}$ | pH |
|----------------------------------|---------------------------------|---------------|
| Kokemäenjoki | 20.8 ± 0.4 | 7.1 ± 0.2 |
| Konnevesi control water | 5.0 ± 0.5 | 7.0 ± 0.2 |
| Nominal 40 mg L^{-1} | 44.4 ± 0.9 | 7.1 ± 0.1 |
| Nominal 80 mg L^{-1} | 86.0 ± 1.6 | 7.0 ± 0.1 |
| Nominal 150 mg L^{-1} | 151.8 ± 3.9 | 7.0 ± 0.1 |
| Nominal 300 mg L^{-1} | 303.9 ± 6.3 | 6.9 ± 0.1 |
| Nominal 600 mg L^{-1} | 605.7 ± 10.3 | 6.9 ± 0.1 |
| Nominal 1200 mg L^{-1} | 1207.1 ± 26.2 | 6.9 ± 0.1 |
| Nominal 2000 mg L^{-1} | 2000.0 ± 27.2 | 6.8 ± 0.1 |

room and tempered to the temperature needed during the water change days. The experimental target sulfate concentrations were 40, 80, 150, 300, 600, 1200 and 2000 mg L^{-1} (Table 1). Stock solutions were prepared by dissolving the Na_2SO_4 at their highest exposure concentration and diluting portions of the stock solution with KV water to achieve desired exposure concentrations. Representative SO_4 concentrations were confirmed in the beginning, during and at the end of the treatments by independent accredited lab analysis. Measured concentrations were used in the statistical analysis.

2.3. Fertilization

About 100 eggs from each female was placed into a 500 ml glass jar. Approximately 50 μl of sperm from each male was added upon the eggs and distributed evenly on eggs of different females. 200 ml of water containing the different concentrations of Na_2SO_4 was added into the glass jar to active the gametes. A jar was gently stirred and water was replaced to remove the excess sperm until the liquid was clear. In the next day, the fertilization success (%) of eggs was examined by a microscope from 50 eggs per each treatment.

2.4. Embryonic and larval period

After fertilization, the jars were left undisturbed for 2 h for eggs to harden. Afterwards the eggs were transferred to 6-well plates with movable inserts (VWR 6-well plate type 734–2717 6). Each well was filled with 10 ml of treatment solution. The plates were covered with lids to prevent the evaporation. Each treatment had 8 plates ($8 \times 6 = 48$ fertilized eggs per treatment) divided on 2 aluminium trays. The trays were placed inside a growth chamber (HiPoint EH-1800) on two shelves. The order of trays on each shelf was randomized. Temperature was monitored during the experiment by using the growth chambers own thermometer as well as two separate temperature loggers, which were placed in separate plates filled with tap water on each shelf inside the chamber.

The water was changed weekly by filling out new plates and transferring the eggs via movable inserts from one plate to another. The water change schedule was divided into 5-week periods. A new set of treatment solutions were created after each period. The trays inside the chamber were rotated weekly during the water change and after a 5-week period the trays on upper shelf were swapped with the ones on the lower to even out possible temperature differences. During the water change, the number of dead eggs were recorded and they were removed from the trays.

The water temperature in the experiment simulated the natural temperature rhythm of R. Kokemäenjoki (mean of daily water temperatures in 2015–2018 in Pori, database of Southwest Finland Center for Economic Development, Transport and the Environment) from spawning to hatching of the larvae and the experiment lasted 175 days (Fig. 1). The eggs were incubated in dark for the first 142 days after which a light-dark-rhythm was formed to imitate the natural increase of light

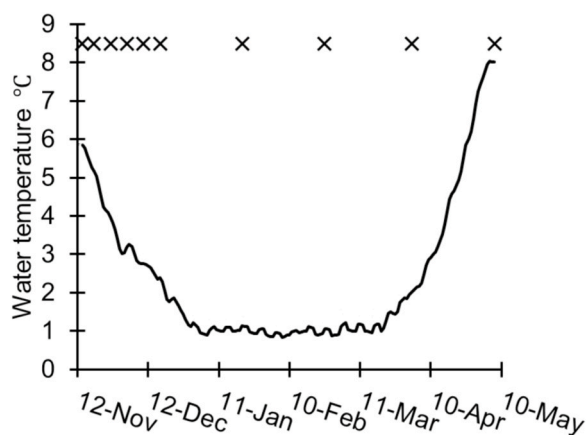


Fig. 1. Water temperature during the experimental incubation. The occasions, when the water samples were taken to control the sulfate (SO_4) concentrations of the exposure solutions, are represented by the crosses.

during spring. At the start the rhythm was 12 L:12D with the measured intensity of 120 lx right above the plates. After 7 days the rhythm was changed to 14 L:10D with 200 lx and after 14 days to 16 L:8D with 500 lx of intensity.

After the first hatched larvae the trays were inspected daily for additional hatchings. The yolk sac larvae were left in their wells for 5 days, counting the day of hatching as the first one. After 5 days the larvae were collected to concentrations specific tubes and preserved in 95% ethanol for further analysis.

2.5. Larval rearing

After 5-day exposure of yolk sac larvae, whitefish larvae were moved to the larval rearing in 8 flow-through aquaria. In the rearing, the larvae were not exposed anymore to the different SO_4 solutions but they were reared in the similar low-sulfate, soft groundwater in all treatments. The sulfate concentration (mg L^{-1}) and Ca-Mg hardness (mmol L^{-1}) in the water of larval rearing were 19 and 0.05, respectively. From the highest SO_4 exposure ($2\,000\text{ mg L}^{-1}$), there was not enough larvae for larval rearing. Thus, from eight treatments, 15–20 larvae were reared at $12.8 \pm 0.01\text{ }^\circ\text{C}$ (mean \pm SE) for 31 days and they were fed by *Artemia* nauplii ad libitum. Before transport, the larvae were acclimated to the rearing temperature for 3 days. After acclimation period the larvae were transferred to 20 L flow-through aquaria. Light rhythm in rearing was 16 L:8D with intensity of 2500 lx. Aquaria were inspected daily for any dead individuals and the temperature from each aquarium was measured. Aquaria were cleaned from excess food and feces regularly. In the end of the rearing, the larvae were collected and preserved in 95% ethanol.

2.6. Total length and mass measurements

Total length, fresh mass and dry matter (% dry mass divided by fresh mass) of preserved larvae were measured for growth analysis. Before measurements, larvae were put in a petri dish filled with water for 15 min to omit the ethanol and restore the size of tissues (Karjalainen, 1992). The excess moisture was removed from larvae by gently swiping it on a moist paper towel. Immediately after the measurement larvae was placed in a pre-weighted aluminum cup and was weighted in a microscale to determine the fresh mass. The larval samples were dried at $40\text{ }^\circ\text{C}$ for 24 h to determine the dry mass and subsequently the proportion of dry matter (%) by dividing dry mass by fresh mass.

2.7. Chemical analyses and quality control

The water quality was monitored during the experiment by measuring pH (MeterLab PHM220), oxygen (PreSens Microx 4 trace) and sulfate levels were measured in the days of water changes to the microplate wells i.e. weekly in the beginning of the experiment for 5 weeks and then after 5-week periods (Fig. 1). Sulfate analysis was analysed by liquid chromatography of ions according to SFS-EN ISO 10304-1 (2009). Uncertainty in the analysis was $\pm 10\%$. Sulfate concentrations in test waters were very stable during the test (Table 1). The mean oxygen concentration in the wells of microplates was $11.3\text{ mg L}^{-1} \pm 0.6$ (\pm SD, $n = 38$). pH varied from 6.8 to 7.1 between the test waters (Table 1).

2.8. Statistical analysis

The effect of sulfate exposure on the fertilization and survival of whitefish embryos and larvae in the different developmental stages was analyzed by the Kruskal-Wallis analysis and the paired comparisons between the exposure concentrations and Konnevesi control water was made by Conover test (Conover, 1999). Determination of the no-observed effect concentration of sulfate (NOEC) was based on these paired comparisons. The median lethal concentration (LC_{50}) of sulfate was determined by probit analysis. The effect of sulfate exposure on the total length and fresh mass was analysed by the analysis of variance (ANOVA) and on dry matter (%) by the Kruskal-Wallis analysis. All analyses were done by IBM SPSS statistics v. 24 software.

3. Results

3.1. Fertilization

Sulfate exposure affected statistically significantly the fertilization success of whitefish eggs (Kruskal-Wallis, $\chi^2 = 43.56$, $\text{DF} = 8$, $P < 0.001$, Fig. 2), but only in the highest exposure concentration, the fertilization differed statistically significantly from the KV control water (Conover test, $P < 0.01$). The median lethal concentration (LC_{50}) of sulfate for the fertilization was $2\,280\text{ mg L}^{-1}$ (95% confidence limits from 2 035–6 503, probit analysis). No-observed effect concentration of sulfate (NOEC) of whitefish was $1\,027\text{ mg L}^{-1}$.

3.2. Embryonic period

In the early embryonic period (Fig. 3A), survival of embryos differed statistically significantly between the exposure concentrations (Kruskal-

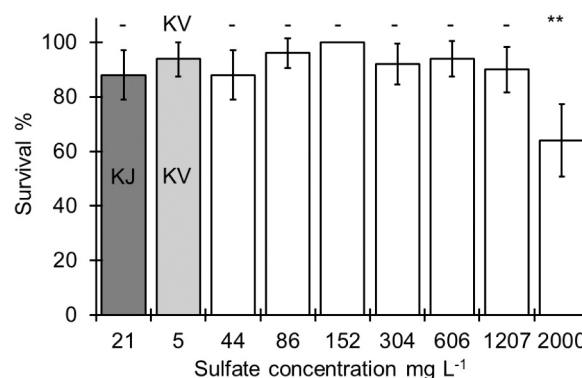


Fig. 2. Proportion of successfully fertilized eggs (%; $n = 50$ per treatment) in the seven Na_2SO_4 -exposures expressed as sulfate (SO_4) concentrations, and in Konnevesi (KV) control and Kokemäenjoki (KJ) water treatments with their natural background SO_4 concentrations. Vertical lines represent 95% confidence limits. Significance level of Conover test results above the bars: - $p > 0.05$, ** $p < 0.01$.

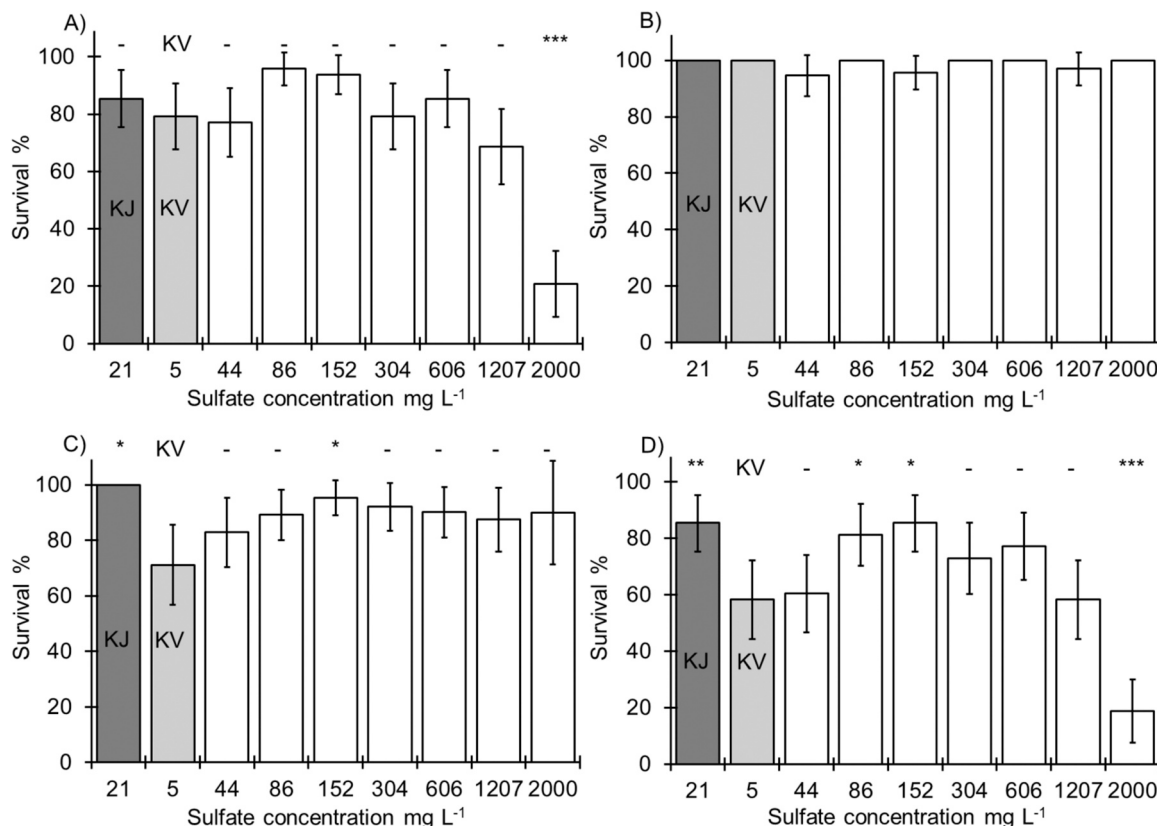


Fig. 3. Survival (%) of whitefish embryos during A) the early embryonic period (25 days from the fertilization, $n = 48$ per treatment) and B) the late embryonic period (107 days from the end of early embryonic period, $n = 48$). Survival of whitefish larvae during hatching and larval period (40 days from the end of late embryonic period, $n = 48$) and D) during the entire incubation period (175 days, $n = 48$ per treatments) in the seven Na_2SO_4 -exposures expressed as sulfate (SO_4) concentrations, and in Konnevesi (KV) control and Kokemäenjoki (KJ) water treatments with their natural background SO_4 concentrations. Vertical lines represent 95% confidence limits. Significance level of Conover test results above the bars: - $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$.

Wallis, $\chi^2 = 43.56$, $DF = 8$, $P < 0.001$), but only in the highest exposure concentration, the survival differed statistically significantly from the KV control water (Conover test, $P < 0.001$). The mortality of the early embryonic period included also unsuccessful fertilization. In the early embryonic period, LC_{50} was $1\ 413\ \text{mg L}^{-1}$ (95% confidence limits: from 1 024–2 293) and NOEC was $1\ 207\ \text{mg L}^{-1}$.

In the late embryonic period (Fig. 3B), the survival was high in all the exposure concentrations, and the survival did not differ statistically significantly between the exposure concentrations (Kruskal-Wallis, $\chi^2 = 11.78$, $DF = 8$, $P = 0.161$). Due to the low mortality, LC_{50} was not estimated for the late embryonic period.

3.3. Hatching and larval period

In hatching and larval periods, the exposure affected statistically significantly the survival of the larvae (Kruskal-Wallis $\chi^2 = 21.6$, $DF = 8$, $P = 0.006$, Fig. 3C) and the highest mortality was observed in KV control water with a low natural background sulfate concentration. Konnevesi treatment differed statistically significantly from the Kokemäenjoki treatment and the SO_4 exposure of $152\ \text{mg L}^{-1}$ (Conover test, $P < 0.05$), but did not differ from other exposures (Conover test $P > 0.05$). During the 5-day period after hatching, no larvae died in any of the treatments.

Due to the low mortality in the late embryonic and hatching/larval periods, LC_{50} -values were not estimated for these periods separately. For the entire embryonic and larval period (Fig. 3D), LC_{50} -value of sulfate was $1\ 161$ (95% confidence limits: from 663 to 3 333) and NOEC was $1\ 207\ \text{mg L}^{-1}$.

Total length and fresh mass of the hatched larvae after the 5-day

exposure (Fig. 4) did not differ statistically significantly between the treatments (ANOVA for total length, $F = 1.16$, $DF = 8$, $P = 0.332$ and ANOVA for fresh mass, $F = 1.57$, $DF = 8$, $P = 0.141$). Dry matter (%) differed significantly between treatments (Kruskal-Wallis $\chi^2 = 33.3$, $DF = 8$, $P < 0.001$) and Kokemäenjoki, 152 and 304 exposures differed from the KV control water (Conover test, $P < 0.05$).

3.4. Larval rearing

In the 31-day larval rearing after hatching in depuration (no sulfate exposure), the survival of larvae was high and no statistically significant difference between exposure concentrations was observed (Fig. 5). Neither the total length, fresh mass or dry matter % differed statistically significantly between the treatments (ANOVA for total length, $F = 0.633$, $DF = 7$, $P = 0.728$ and ANOVA for fresh mass, $F = 0.99$, $DF = 7$, $P = 0.435$ and Kruskal-Wallis for dry matter (%), $\chi^2 = 10.43$, $DF = 8$, $P = 0.166$). See Fig. 6.

4. Discussion

4.1. Sensitive periods and mechanisms of toxicity

The fertilization and pre-eyed embryos of rainbow trout (*Oncorhynchus mykiss*) have been identified as being more sensitive to sulfate than eyed embryos (Meays and Nordin, 2013). Similarly, in our experimental conditions in soft and humic water, the egg fertilization and early embryonic development were the most sensitive developmental stages of the R. Kokemäenjoki whitefish. However, harmful effects were observed only at the highest concentration of sulfate. Weis and Weis

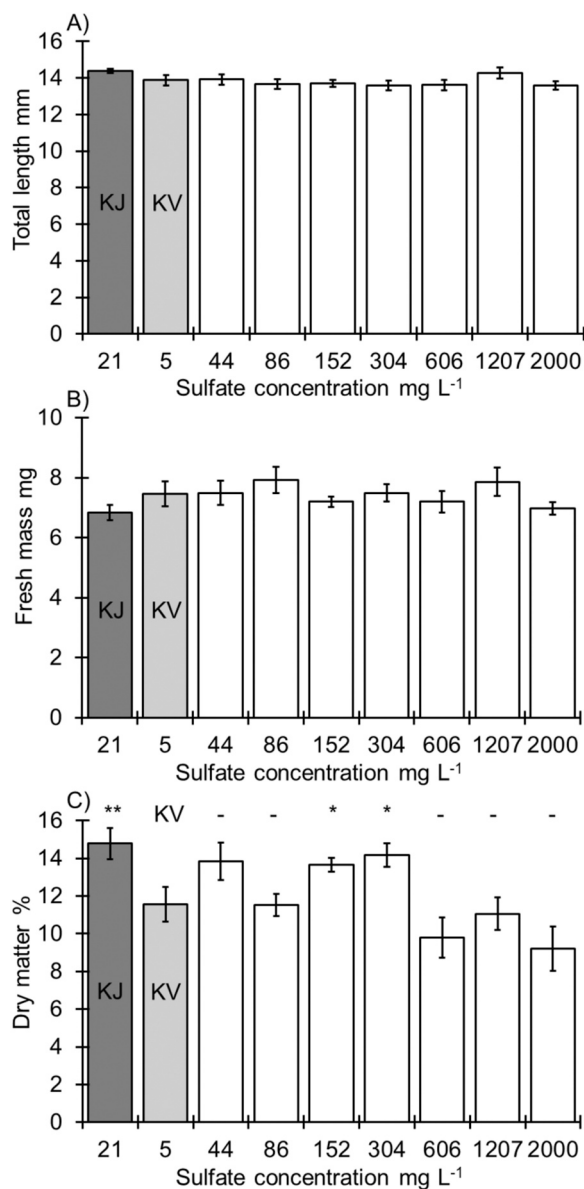


Fig. 4. A) Total length (mm), B) fresh mass (mg) and C) dry matter (%) of the 5-day old whitefish larvae in the seven Na₂SO₄-exposures expressed as sulfate (SO₄) concentrations, and in Konnevesi (KV) control and Kokemäenjoki (KJ) water treatments with their natural background SO₄ concentrations. Vertical lines represent standard error. Significance level of Conover test results above the bars: - p>0.05, * p<0.05, ** p<0.01.

(1987) stated that “embryos in the natural environment can be exposed to pollutants in two ways: via yolk, which is synthesized during oogenesis by exposed females, and during the brief period between shedding of the gametes and elevation of the chorion.” Thus, harmful substances may be transferred into the eggs during the swelling process of the eggs by water during fertilization. Following perivitelline space formation perivitelline space, the eggs are hydrated and the chorion is formed. The chorion can act as a barrier, which partially protects the developing embryo from the toxic effects of the pollutant (Weis and Weis, 1987). The general mechanism of salt toxicity is usually divided into two main categories: osmotic stress and specific ion toxicity (Davies and Hall, 2007). In osmotic stress, the regulation of water balance is disturbed, and specific ion toxicity occurs when the ions have entered the cells and cause adverse effect on the normal cellular functions (Davies and Hall, 2007).

In freshwater fish, sperm activation in mating occurs when male

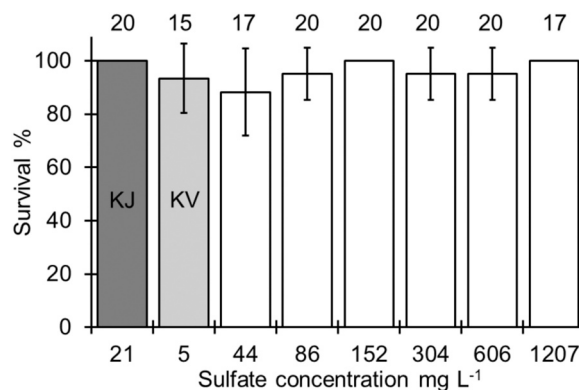


Fig. 5. Survival (%) of whitefish larvae in the larval rearing in depuration in low-sulfate, soft groundwater after the Na₂SO₄-treatments. Vertical lines represent 95% confidence limits. From the highest SO₄ exposure (2 000 mg L⁻¹), there was not enough larvae for larval rearing.

release the milt to the water and hypo-osmotic pressure activates the sperm motility. Sperm motility is affected by ion concentrations, osmotic pressure, pH, temperature, and dilution rate of sperm (Alavi and Cosson, 2006). Generally, potassium (K⁺) ions inhibit sperm activation and other ions such as sodium (Na⁺), calcium (Ca²⁺) and magnesium (Mg²⁺) induce sperm motility (Alavi and Cosson, 2006; Bozkurt et al., 2011), but sperm motility of fish is controlled ultimately by the interplay of ions in the external environment in combination with osmotic pressure. Optimal NaCl concentrations at 30 mM in sperm activation solutions improved the sperm motility of whitefish compared to natural hatchery water (Dziewulska et al., 2015), but on the other hand, at high Na⁺ concentration (125 mM), sperm motility of muskellunge (*Esox masquinongy*) decreased primarily due to the increasing osmotic pressure (Lin and Dabrowski, 1996). Concentration of 60 mM NaCl decreased the sperm motility of vendace (*Coregonus albula*) (Dietrich et al., 2010). Indeed, high external osmotic pressure (400 mOsmol kg⁻¹) compared to that of the seminal plasma at 300 mOsmol kg⁻¹ inhibits sperm activation in salmonids (Alavi and Cosson, 2006). The osmolality of seminal plasma of coregonids seem to be slightly lower than other salmonids being ca. 250 mOsmol kg⁻¹ (Dietrich et al., 2010). The Na⁺ concentration in our highest test water was 42 mM and the salinity 3‰ and thus, osmotic stress inhibiting the sperm activation seemed not to be the reason for the low fertilization of the eggs. (Jäger et al., 1981) has observed that anadromous whitefish eggs fertilized successfully in brackish water with salinity up to 10.2‰.

The eggs and embryos of freshwater fishes develop in hypo-osmotic conditions leading to slow osmotic water gain and possible loss of ions (Fyhn et al., 1999), although chorion and egg membranes inhibit the dilution to some extent. In our experiment, the survival of embryos during the 175-day period was higher in the sulfate concentrations of 86 and 152 mg L⁻¹ than in the control treatment. Slight increase in salinity in the environment of eggs seemed to decrease the osmotic stress of whitefish embryos. Davies and Hall (2007) have suggested that sulfate may interfere with cell permeability in aquatic organisms and our results imply that mechanisms other than direct osmotic stress only could cause sulfate toxicity to whitefish early stages. Elphick et al. (2011) have stated that sulfate uptake to fish likely occurs by passive diffusion through specific ion channels of the gill epithelium and that competitive exclusion by other ions may alter the uptake and thus sulfate toxicity. In activation of eggs to fertilization, the ionic currents at the plasma membrane play an important role (Carvalho et al., 2018). Ionic conductance at the plasma membrane encompass transporters in addition to Cl⁻, K⁺ and Ca²⁺ channels and other channels and have connections to the function of intracellular channels, too (Carvalho et al., 2018). High sulfate concentration may disturb the egg activation, but also the formation of chorion after fertilization and weaken the

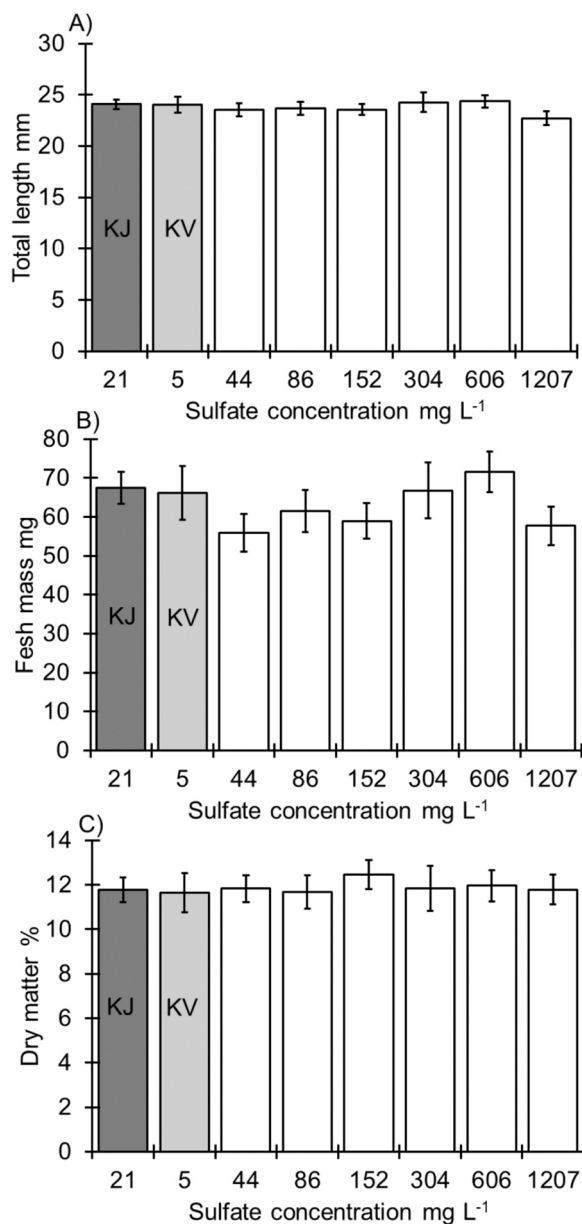


Fig. 6. A) Total length (mm), B) fresh mass (mg) and C) dry matter (%) of the whitefish larvae in the larval rearing in depuration in low-sulfate, soft groundwater. Vertical lines represent standard error. From the highest SO₄ exposure (2 000 mg L⁻¹), there was not enough larvae for larval rearing.

polyspermy block (Wozniak and Carlson, 2019).

In the late embryonic period, hatching and larval period the mortality of whitefish was low. Jäger et al., (1981) showed that whitefish larvae tolerated salinity of 15‰ without harmful effects. In our study, the size of hatched yolk sac larvae was at the same level in all treatments, but the proportion of dry matter indicated similar unimodal response pattern between the test waters as the total mortality: in the low-sulfate, soft Konnevesi control water and in the highest sulfate treatment, the dry matter percentage differed from the medium-sulfate treatments showing statistically significant differences in the body composition of newly hatched whitefish larvae. In sulfate treatments up to 304 mg L⁻¹ and in Kokemäenjoki treatment the larvae gained more dry mass during the egg incubation than in the KV control water and in the highest sulfate concentration. In our 31-d rearing experiment in depuration in the low sulfate, soft water, the larvae from all treatments started to feed without problems and achieved an average growth rate of

0.05 mg FM d⁻¹. Thus, sulfate exposure during early life had no delayed effects on the feeding and growth of larvae and the difference in dry matter percentage observed in newly hatched larvae disappeared during the larval rearing.

4.2. Lethal and non-effective concentrations of sulfate

Elphick et al. (2011) have published EC₅₀-values of 1755 (1607–1921) mg L⁻¹ for embryos of coho salmon (*Oncorhynchus kisutch*) in 10-day exposure period and 734 (640–823) mg L⁻¹ for rainbow trout embryos and hatched larvae (*O. mykiss*) in 31-day exposure period in dechlorinated municipal waters. The CaCO₃ hardness of Elphick's et al. (2011) test water (15 mg L⁻¹) was low and similar to our study (14.3 mg L⁻¹). On an average, these EC₅₀-values (arithmetic mean of EC₅₀-values is 1244 mg L⁻¹) are on the same level as LC₅₀-values of whitefish during the 28-day early embryonic period (1413 mg L⁻¹) in our experiments. The 21-day eyed-egg-to-alevin test with rainbow trout (Kennedy's unpublished experiment in Meays and Nordin, 2013) however indicated higher sulfate toxicity: LC₅₀-values being between 484 (459–511) and 761 (724–799) mg L⁻¹ in the water hardness of 6 and 50 mg CaCO₃ L⁻¹, respectively. The corresponding NOEC-values of sulfate for coho salmon (*O. kisutch*) and rainbow trout (*O. mykiss*) were 825 and 205 mg L⁻¹, respectively (Elphick et al., 2011). No NOEC point estimates were reported by Meays and Nordin (2013) for the rainbow trout embryo and larvae test, but instead they represented LC₂₀-values of 255 (238–274) and 435 (408–464) mg L⁻¹, and LC₁₀-values of 176 (161–192) and 315 (290–341) mg L⁻¹ in the water hardness of 6 and 50 mg CaCO₃ L⁻¹, respectively. This comparison to earlier studies highlights the relationship that the toxicity of sulfate to aquatic invertebrates and fish are significantly affected by higher water hardness (Davies and Hall, 2007; Elphick et al., 2011; Mount et al., 1997). In all, the tolerance of the whitefish early life stages to sulfate toxicity seem to be at the same level as the tolerance of other salmonids. Thus, their early stages are not more sensitive as suggested by Arola et al. (2017) earlier.

Earlier we have determined manganese sulfate (MnSO₄) toxicity to European whitefish (*Coregonus lavaretus*) early life stages in a long-term 160-day incubation and LC₅₀ of MnSO₄ was between 42 and 98 mg MnSO₄ L⁻¹ depending on the parent pairs used in the egg fertilizations (Arola et al., 2017). In our earlier and present studies, the exposures were conducted in the same natural and soft L. Konnevesi freshwater with whitefish. Mount et al. (1997) stated that the toxicity of Na₂SO₄ salt is primarily due to the SO₄²⁻ anion and Na⁺ ions have only a minor role in the toxicity of this salt depending the total concentration. The same is not obvious for MnSO₄ salt. Manganese Mn²⁺ is a micronutrient (Terlech-Majewska et al., 2016) with a much lower intake requirements and homeostatic regions in organisms. Our studies together indicate that SO₄ as a mixture with Mn is 10–30 times more toxic to whitefish early life stages compared to the SO₄ with its LC₅₀-value for the entire embryonic period being 1161 mg L⁻¹ in this study.

During the entire 175-day incubation, survival of embryos and larvae in Konnevesi control water and natural Kokemäenjoki water was 58.3% and 85.4%, respectively. The ion composition of Kokemäenjoki water (e. g. higher hardness, higher Na⁺, K⁺ concentration) seemed to be more suitable for whitefish early development. On the other hand, we used sperm of 5 males to fertilize the eggs of 5 females which produce some random variability to the fertilization success and early development between treatments. In all the treatments, the same amount of eggs and sperm from each parental fish was measured out into the fertilization jars, but finally different amounts of eggs from different combinations of females and males may have been examined when the fertilization was determined and further, when the eggs were set to the microplates. It is a well-known fact in aquaculture that early mortality of whitefish depends on suitable female-male combinations demonstrating the partial incompatibilities of whitefish individuals (Wedekind et al., 2010). It is important to note that at least females, but also parental combination may affect the contaminant tolerance of whitefish offspring. Arola et al.

(2017) found that embryos of different whitefish females had even two-fold difference in the LC₅₀ of MnSO₄. Ecological variation in reproductive biology of fishes highlights the need for precautionary approach applying assessment factors in the determination of WQGs of contaminants.

5. Conclusions

Our long-term ca. 6-month incubation experiment from autumn to spring, from fertilization to hatching, suggest that the fertilization and early development of whitefish embryos are the most sensitive phases to sulfate. This is new observation and the further research are needed to study the effects of sulfate on the performance of the gametes before fertilization (e.g. sperm motility vs. activation of eggs to fertilization) and to clarify the mechanisms of the ion toxicity on the early embryonic development (osmotic stress vs. specific ion toxicity). In the late embryonic development and hatching, the mortality was low in all our exposures. Altogether, the tolerance of the whitefish early life stages to sulfate toxicity are at the same level as the tolerance of other salmonids in soft waters and medium-level sulfate concentrations seem to even improve the survival and growth of embryos. Sulfate loading from industry to freshwaters are increasing in boreal region and it is important to recall, that in addition to standard lab tests, toxicity studies with organisms from the natural populations (here endangered migratory whitefish) and different trophic levels under simulated natural conditions (here natural temperature conditions) in soft waters are needed to develop the present WQGs of sulfates.

CRedit authorship contribution statement

Juha Karjalainen: Planning, Experimental work, Data analysis and statistics, Writing-review & editing, Supervision, Project administration, Funding acquisition. **Mikko Mäkinen:** Planning, Methodology, Experimental work, Data collection and management, Writing-review & editing. **Anna K. Karjalainen:** Planning, Methodology, Quality control, Writing-review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Animal care

Sampling and handling of parental fish, embryos and larvae have been done according to the current permits and guidelines for experimental animals. Fishing right owners have permitted the catching of the fish.

Declaration of competing interest

There are no conflicts to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2020.111763](https://doi.org/10.1016/j.ecoenv.2020.111763).

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