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Hanna Arola

Effects of Bioheapleaching Technology Utilizing Metal Mine Emissions on Fish in Boreal Freshwaters

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ABSTRACT

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Diss.

Emissions from metal mining activities are known to deteriorate the quality of aquatic habitats and impair the condition and reproductive potential of fish. Metal extraction by biomining methods has been considered to cause fewer emissions, but for example in Finland, the impacts of a bioheapleaching technology utilizing metal mine on the local freshwaters have been substantial. In this thesis, the impacts of the bioheapleaching mine emissions on three native fish species, brown trout (*Salmo trutta*) European perch (*Perca fluviatilis*) and whitefish (*Coregonus lavaretus pallasi*), were investigated. Manganese and sulphate concentrations have been elevated in the mining impacted waters, and in our laboratory experiment with whitefish early life stages, a continuous exposure to manganese sulphate increased the early life stage mortality and impaired the larval growth and yolk consumption. In addition, the tolerance of the whitefish early life stages to manganese sulphate varied among the female parents, and the tolerance also seemed to be linked to larval metallothionein messenger RNA induction. In the long-term *in situ* egg incubation experiment, however, no mining impact related effect on brown trout and whitefish embryonic mortality or growth was observed. Although low water pH increased the embryonic mortality of both species, the low water pH was characteristic to the waterbodies at the study region in general. With wild male perch, the liver and testes size were lower in the mining impacted study lakes, indicating lower energy resources compared to the males from the reference lakes. The perch from the mining impacted lakes also had lower sperm counts, which seemed to have been compensated by elongated sperm motility. These results suggested that the condition and reproductive potential of fish may have been compromised in the bioheapleaching mine impacted lakes.

Keywords: Concentrations of elements in tissues; early life stages of fish; fish condition; gene expression; parental effect; sperm motility; tolerance.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-III.

The original ideas were formulated by Anna Karjalainen and Juha Karjalainen, but at the experiment planning stage the ideas were worked together by all the authors. The data collecting was mainly my responsibility, and besides myself Maija Hannula (II), Anna Karjalainen (I–III), Juha Karjalainen (I–III), Jukka Syrjänen (II), Jaana Wallin (III) and Eeva-Riikka Vehniäinen (I) participated in the collecting of the data. Most of the laboratory analyses were carried out by me, Ari Väisänen (I–III) and Maija Hannula (II). Also, Anna Karjalainen and Eeva-Riikka Vehniäinen provided valuable instructions for the laboratory analyses. I was responsible for the data analyses, which were supported by Juha Karjalainen and other supervisors. I have written the first drafts of all the papers, but all the co-authors have also participated in the writing process, and their thorough comments have substantially improved the manuscripts.

I Arola H.E., Karjalainen J., Vehniäinen E.-R., Väisänen A., Kukkonen J.V.K. & Karjalainen A.K. 2017. Tolerance of whitefish (*Coregonus lavaretus*) early life stages to manganese sulfate is affected by the parents. *Environmental Toxicology and Chemistry* 36: 1343–1353.

II Arola H.E., Karjalainen A.K., Syrjänen J.T., Hannula M., Väisänen A. & Karjalainen J. 2018. Fish embryo survival and growth in natural boreal streams and downstream a multi-metal mine using a bioheapleaching technique. Manuscript.

III Arola H.E., Karjalainen A.K., Wallin J., Vehniäinen E.-R., Väisänen A., & Karjalainen J. 2018. Condition and sperm characteristics of perch (*Perca fluviatilis*) inhabiting metal mining effluent contaminated lakes. Manuscript.

1 INTRODUCTION

1.1 Metal production and biomining

From 1984 to 2015, the global total production of iron and ferro-alloy metals, non-ferrous metals, precious metals, industrial minerals and mineral fuels has nearly doubled (Reichl *et al.* 2017). Although the majority of the global mineral production is by far mineral fuel production, the total production of different types of metals has more than tripled within the past three decades (Reichl *et al.* 2017). As high-grade ore deposits have been exhausted throughout the world, the pressure for feasible utilization of low-grade ore bodies has increased (e.g. Rawlings and Silver 1995, Rawlings *et al.* 2003). Biomining has been considered as an economic method for the extraction of the low-grade metal ores (Rawlings *et al.* 2003, Johnson 2013).

In biomining, naturally occurring chemolithoautotrophic acidophilic micro-organisms are utilized in extracting metals from sulphide minerals (Rawlings 2002). Biomining can be categorized into bio-oxidation and bioleaching (Johnson 2013). In bio-oxidation, micro-organisms are applied in pre-treatment of the ore for removing the metal sulphides before the chemical extraction of the target metal, such as gold (Brierley 2008, Johnson 2013). In bioleaching, the micro-organisms solubilize the target metals (e.g. copper, zinc and nickel) from insoluble metal sulphides or oxides (Rawlings and Silver 1995, Brierley 2008, Johnson 2013). The share of the biomining methods from global metal production has been most significant for copper and gold, being roughly 20 and 5 %, respectively (Brierley 2008, Johnson 2013). The gold bio-oxidation has mainly been utilized in Asia, Australia and Africa, whereas the majority of commercial copper bioleaching mines has been located in South America (Watling 2006, Brierley 2008).

By the end of 2007, also nickel extraction from nickel sulphide ores by different bioleaching methods had been tested in Australia, China, Finland and South Africa (Watling 2008). In 2008, the Talvivaara Mining Company plc (currently Terrafame Ltd.), started the commercial production of nickel as well

as copper, cobalt and zinc by utilizing the bioheapleaching technology in Talvivaara, Sotkamo, North-Eastern Finland (Riekkola-Vanhanen 2013). In the Talvivaara Sotkamo Mine, the ore processing starts with crushing, screening and agglomeration of the ore (Riekkola-Vanhanen 2013). Then, the ore is stacked to primary heap pads for leaching for 13–14 months (Riekkola-Vanhanen 2013). To maintain optimal conditions for the micro-organisms, the heaps have been aerated and irrigated with acidic solution (Riekkola-Vanhanen 2013). Following the leaching in the primary heaps, the ore is moved and stacked for further leaching to secondary heap pads that also are the final deposits for the barren ore (Riekkola-Vanhanen 2013). The metals are recovered as metal sulphides from the leachates (Riekkola-Vanhanen 2013).

1.2 Current knowledge of the impacts of bioheapleaching mines on freshwaters

Besides biomining has been suggested as an economic method for low-grade ore utilization, it has also been considered to cause fewer emissions than the conventional metal extraction methods (Rawlings and Silver 1995, Rawlings *et al.* 2003, Johnson 2013). In the boreal region, the utilization of bioheapleaching technology in metal mining has been scarce, and thus its impacts on boreal aquatic environment have not been extensively studied. In the Talvivaara region in Finland, the impacts of the Talvivaara Sotkamo Mine emissions have started to appear in the nearby waterbodies in 2010 for example as elevated manganese, nickel and zinc concentrations, as well as sulphate and sodium induced salinization (Kauppi *et al.* 2013). The increased water salinity has even led to ectogenic meromixis of some small nearby lakes (Anonymous 2016a). Additionally, in November 2012, a gypsum pond leakage occurred at the mine (Anonymous 2013, Kauppi *et al.* 2013). Besides elevated metal concentrations and salinization, the pH of the mining impacted waters has also fluctuated substantially, from acidic (4.7) to circumneutral (7.3), particularly during the accidental leakages (Kauppi *et al.* 2013, Salmelin *et al.* 2017).

Regarding the ecological impacts of the mine, a recent study indicated that the diversity and species richness of diatom and cladoceran communities have decreased due to mining effluent contamination in Lake Kivijärvi, which is one of the impacted lakes (Leppänen *et al.* 2017). On the other hand, the organismal responses can also be site specific, as shown by the variation in behavioural responses of mayfly (*Heptagenia dalecarlica*) larvae incubated in the bioheapleaching mine impacted streams (Salmelin *et al.* 2017). Although there has been some monitoring of the local fish populations, scientific studies about the impacts of the bioheapleaching mine emissions on boreal freshwater fishes have been lacking.

1.3 Effects of metal mining and production on freshwater habitats and fish

Freshwaters elsewhere impacted by historical or active metal mining or smelting activities have been described to have elevated concentrations of sulphate (Olías *et al.* 2004) and/or metals, such as Al, Fe, Cd, Cu, Mn, Ni or Zn (Soucek *et al.* 2000, Moiseenko and Kudryavtseva 2001, Eastwood and Couture 2002, Couture and Kumar 2003, Olías *et al.* 2004). The water pH at the impacted waters can be acidic (Soucek *et al.* 2000) with some fluctuation (Olías *et al.* 2004), or circumneutral (Soucek *et al.* 2000) or even alkaline (Eastwood and Couture 2002, Couture and Kumar 2003).

Regarding fish, metals can affect indirectly through food web alterations as well as directly via food and water (e.g. Campbell *et al.* 2003). There is evidence that in acidic or circumneutral waters with elevated metal concentrations, the aquatic food webs can become simplified (Iles and Rasmussen 2005, Hogsden and Harding 2012), and as demonstrated with yellow perch (*Perca flavescens*) for example, a change in macroinvertebrate community can lead to slower and stunted growth, if suitable prey for diet shift from zooplankton to larger invertebrates and fish is lacking (Sherwood *et al.* 2002, Iles and Rasmussen 2005). Metals (e.g. Zn, Cd or Cu) can also be accumulated in high concentrations into the tissues of aquatic organisms in metal mining impacted waterbodies and also be transferred in the food webs (Besser *et al.* 2001). In metal-contaminated environments, the accumulation of metals into various tissues of fish has been reported in several studies (Besser *et al.* 2001, Moiseenko and Kudryavtseva 2001, Eastwood and Couture 2002, Levesque *et al.* 2002, Rajotte and Couture 2002, Couture and Kumar 2003, Pyle *et al.* 2005, Pierron *et al.* 2009). In some of those studies, the elevated tissue metal concentrations have been linked to slower growth rates and/or lower condition of the fish (Eastwood and Couture 2002, Levesque *et al.* 2002, Rajotte and Couture 2002, Pyle *et al.* 2005) as well as to the impairment of reproductive potential (Levesque *et al.* 2002, Pyle *et al.* 2005). For example, Levesque *et al.* (2002) suggested that the normal energy cycling and intermediary metabolism processes could have been disturbed under chronic exposure to metals (Cd, Cu and Zn), and that may have impaired the condition of the fish.

1.4 Effects of acidity, metals and sulphate on early life stages of fish

The contaminants present in the mining impacted waters can also affect the viability of fish gametes as well as the early life stages of fish. A short-term exposure of fish sperm to acidic (pH < 5–6) water at the sperm activation stage has been shown to reduce sperm motility rate (Lahnsteiner *et al.* 2004), and in

highly acidic (pH: 3.5) conditions milt can coagulate (Keinänen *et al.* 2003). Also, a short-term exposure of sperm to high concentrations of metals (e.g. Cd, Cu, Hg, Ni, Pb or Zn) at the time of activation has shown to reduce the proportion of motile sperm cells and alter their swimming velocity (Lahnsteiner *et al.* 2004). Lahnsteiner *et al.* (2004) also demonstrated that the effective concentrations affecting the sperm motility rate as well as the sperm swimming velocity response patterns can differ among different fish species. They observed that among four different fish species; African catfish (*Clarias gariepinus*), brown trout (*Salmo trutta fario*), burbot (*Lota lota*) and chub (*Leuciscus cephalus*), the sperm cells of chub were the most sensitive to the tested metals, whereas those of African catfish were the most tolerant (Lahnsteiner *et al.* 2004). Acidic conditions (Sayer *et al.* 1991, Keinänen *et al.* 2003, 2004), elevated concentrations of metals (Sayer *et al.* 1991, Stubblefield *et al.* 1997, Meinelt *et al.* 2001, González-Doncel *et al.* 2003, Keinänen *et al.* 2003, 2004, Jezierska *et al.* 2009) as well as sulphate (Elphick *et al.* 2011, Wang *et al.* 2016) have been shown detrimental for the fish eggs, embryos and/or larvae as well. The exposure of eggs to acidic or metal-contaminated waters at fertilization can impair their water hardening process and disturb the perivitelline space formation (Gonzáles-Doncel *et al.* 2003, Keinänen *et al.* 2003). This has been shown to lead to restricted growth and increased mortality of the embryos (Keinänen *et al.* 2003), as well as increased metal uptake into the eggs (Gonzáles-Doncel *et al.* 2003). Regarding hatching, suboptimal water pH may delay the hatching (Kamler 2002), whereas metals may disturb the hatching gland development and functioning (Jezierska *et al.* 2009). Fish larvae have been suggested to be particularly sensitive to chemical stressors (Hutchinson *et al.* 1998), whereas at the embryonic stage the chorion (Michibata 1981) and/or perivitelline fluid (Stouthart *et al.* 1995) have been suggested to protect the embryo from excess metal uptake.

1.5 Factors affecting the toxicity of contaminants released by metal mining

The toxicity of metals and sulphate can be altered by interactions between natural or anthropogenic factors (e.g. Campbell and Stokes 1985, Wang 1987, Sayer *et al.* 1991, Stubblefield *et al.* 1997, Meinelt *et al.* 2001, Pyle *et al.* 2002, Keinänen *et al.* 2004, Elphick *et al.* 2011). For example, the impact of water pH on metal toxicity is very complex, since a decrease in water pH can either 1) increase the metal toxicity by increasing bioavailability of metals or 2) reduce the toxicity by increasing competition between the H^+ and metal ions on the cell membrane binding sites or by causing changes in the cell membrane potential (Peterson 1984, Peterson *et al.* 1984, Campbell and Stokes 1985). Also, increased water hardness is known to reduce the toxicity of metals (e.g. Stubblefield *et al.* 1997, Meinelt *et al.* 2001, Pyle *et al.* 2002), since metal ions and carbonates can be complexed, and also Ca and Mg may compete for binding sites with metal ions

(Wang 1987). With several aquatic species, including fish, the increased water hardness has also been shown to reduce the toxicity of SO_4 (Elphick *et al.* 2011). Additionally, metal toxicity to for example fish, is also known to be reduced by increased humic material concentration of the water, as humic material may bind metals (Vuorinen *et al.* 1998, Meinelt *et al.* 2001).

1.6 Factors affecting metal tolerance in fish

Metal contamination of fish habitats as well as metal accumulation into the fish have been associated with alterations in expressions of genes related to metal detoxification (e.g. metallothionein), protein protection (e.g. heat shock protein) and oxidative stress (e.g. superoxide dismutase) (Pierron *et al.* 2009). For example, induced metallothionein gene transcription has been shown to increase metal (Cd) tolerance in fish (George *et al.* 1996). Weis *et al.* (1981) also suggested that metal-exposed female fish may provide metal binding compounds, such as metallothioneins, into their eggs, and thus increase the metal resistance ability of their offspring. At the very early embryonic stages of fish, its development rests upon maternally derived products, such as maternal messenger ribonucleic acids (mRNAs) and proteins, until the embryo's own genome activates transcription during mid-blastula stage (Bobe and Labbé 2010). Indeed, Lin *et al.* (2000) demonstrated that the maternal transfer of acclimatory protein mRNA increased the metal (Cd) tolerance in the offspring of females that had been pre-exposed to Cd. However, the maternally derived metal tolerance may not be long-lasting, as demonstrated with Cu (Sellin and Kolok 2006).

Although the maternally derived non-genetic tolerance to metals has received some attention, comprehensive parental effect investigation has seldom been included into ecotoxicological studies with fish. With for example whitefish (*Coregonus* sp.), the male parent provides only genes, whereas the effect of the female parent is a combination of maternally derived environmental and genetic effects (e.g. Wedekind *et al.* 2001, Neff and Pitcher 2005, von Siebenthal *et al*. 2009). It is known that the offspring survival can differ intrinsically among the different parent fish, as well as among different parent combinations (Wedekind *et al.* 2001), indicating that also certain parent pairs can be more compatible than others (Neff and Pitcher 2005). Additionally, there is some evidence that under stressful conditions (e.g. suboptimal salinity), the role of the female effect on the survival of the early life stages in fish can be significant (Papakostas *et al.* 2012). Then, in cases when the tolerance has a genetic basis, and if natural selection would favour the more tolerant individuals, it could result into a more tolerant population (Klerks *et al.* 2011).

2 OBJECTIVES

The general objective of this thesis was to bring new and ecologically relevant information about the impacts of bioheapleaching technology utilizing metal mine emissions on three native boreal fish species; brown trout (*Salmo trutta* L.) (II), European perch (*Perca fluviatilis* L.) (III) and northern densely-rakered whitefish (*Coregonus lavaretus pallasi* L.) (I, II). The investigation methods included a laboratory experiment (I), as well as an *in situ* experiment (II) and field observations (III) (Table 1). Both the *in situ* and the field experiments were conducted in North-Eastern Finland, in waterbodies under the influence of the Talvivaara Sotkamo Mine. Additionally, the ecological relevance, as well as the novelty of the studies were increased by including the parental effect into the design of the laboratory experiment (I). The results obtained here are valuable for national risk assessment of the bioheapleaching mine. The specific objectives of the thesis were:

i. To investigate the impacts of continuously elevated $MnSO_4$ concentrations on the early life stages of whitefish, and also to estimate if certain parents or parent combinations would produce more $MnSO_4$ tolerant offspring than others.
ii. To investigate the embryonic mortality, growth and yolk consumption of brown trout and whitefish under natural incubation conditions by egg incubations in streams impacted by the bioheapleaching mine emissions as well as in reference streams.
iii. To investigate the impacts of metal and sulphate contamination on the morphology as well as reproductive potential of wild male perch that live in the mining impacted lakes.

TABLE 1 Studied species, experimental designs and measured endpoints in original papers.

	I	II	III
Species	Northern densely-rakered whitefish	Brown trout Northern densely-rakered whitefish	European perch
Life stage	Embryos, yolk-sac larvae	Embryos	Mature males, sperm cells
Experiment type	Laboratory $MnSO_4$ exposure	*In situ* incubation	Field sampling
Design specifi-cations	Control and 6 exposure concentrations Parental effect	6 reference and 6 mining impacted streams	2 reference and 3 mining impacted lakes
Endpoints	Fertilization success Mortality Growth and yolk consumption Embryonic and larval element concentrations Gene expressions	Mortality Growth and yolk consumption	Total length Carcass wet mass Testes wet mass Liver wet mass Sperm variables Muscle and liver ele-ment concentrations Gene expressions

3 MATERIALS AND METHODS

3.1 Study sites

The field investigations focused on streams (II) and lakes (III) impacted by the Talvivaara Sotkamo Mine, now known as Terrafame Mine, in North-Eastern Finland. The Talvivaara Sotkamo Mine area is located in the watersheds of Oulujoki and Vuoksi (Fig. 1). Metalliferous black shale occurs in the Talvivaara bedrock in the Oulujoki watershed, and thus the metal background concentrations are naturally elevated in the waters running from the black shale bedrock containing areas (Loukola-Ruskeeniemi *et al.* 1998, Gustavsson *et al.* 2012). From 2009 onwards, treated mining effluents have been discharged into both watersheds, and the total annual discharged effluent volumes have been from roughly 0.22 million m^3 (2009) up to 8.41 million m^3 (2015) (Anonymous 2010, 2016a). Additionally, in November 2012, a gypsum pond leakage occurred at the mine and within two weeks, roughly 20 000 m^3 and 216 000 m^3 of acidic effluents with high metal concentrations were accidentally released into the Oulujoki and Vuoksi watersheds, respectively (Anonymous 2013).

The reference sites in both experiments II and III included both humic, naturally acidic waters in the same geographical region as the mine, as well as less humic and less acidic waters in North-Eastern and/or Central Finland (Fig. 1) (II, III).

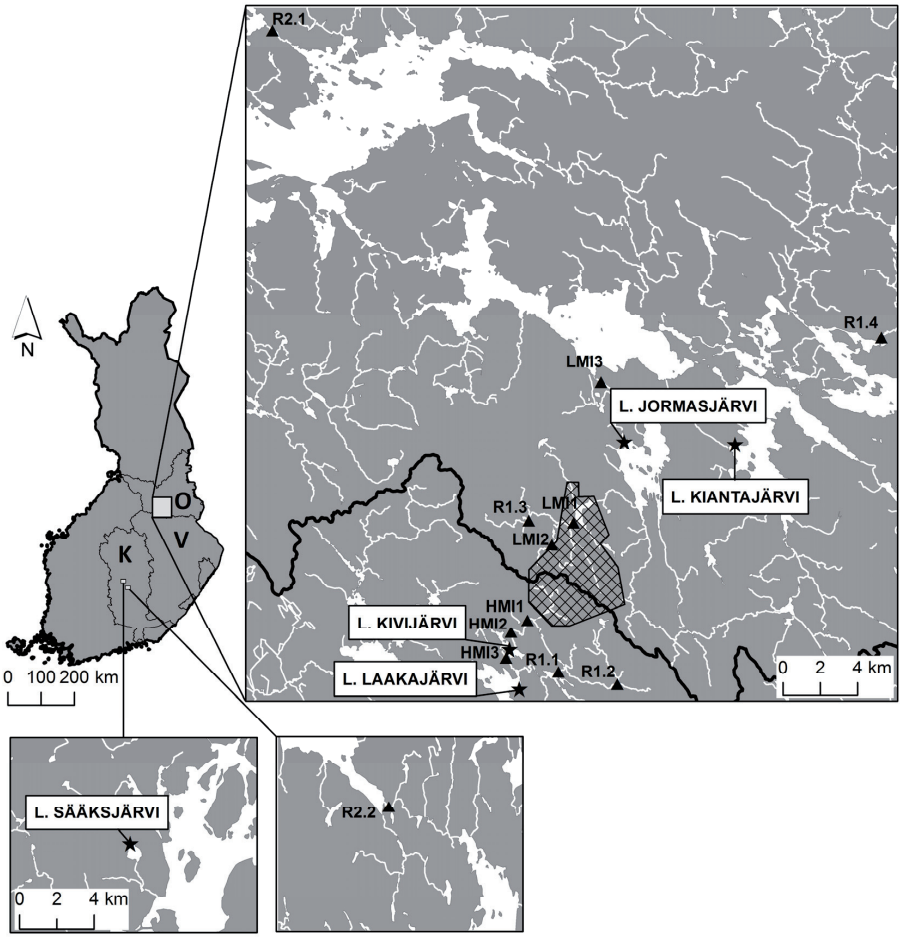

FIGURE 1 The study streams (II) and lakes (III) are indicated as triangles and stars, respectively. With the streams, the prefix R denotes the reference streams, LMI the low mining impacted streams and HMI the high mining impacted steams. With the lakes, Kiantajärvi and Sääksjärvi were the reference lakes, Kivijärvi was the high mining impacted study lake and Jormasjärvi and Laakajärvi the low mining impacted study lakes. The mining district of the Talvivaara Sotkamo Mine is depicted by a grid. Study stream R2.2 and Sääksjärvi were located in Central Finland in Kymijoki watershed (K), whereas all other study sites were located either in Vuoksi (V) or Oulujoki (O) watershed in North-Eastern Finland. Map construction: ArcGIS® v. 10.3.1 (ESRI Inc., Redlands, CA); Map data: General & Topographic maps of Finland, © National Land Survey of Finland, 2016; and Catchment Areas, National Database of Regional Land Use Plans, © Finnish Environment Institute, SYKE, 2016).

3.2 Study species

All the study species; brown trout, perch and whitefish are native to the northern hemisphere, inhabiting lakes, streams and brackish waters (e.g.

MacCrimmon *et al.* 1970, Thorpe 1977a,b, Svärdson 1979, Jonsson 1985, Kallio-Nyberg and Koljonen 1988, Elliott 1989, Säisä *et al.* 2008, Snickars *et al.* 2010), and sea-migrating brown trout occur as well (e.g. Jonsson 1985, Elliott 1989). With brown trout and whitefish, our focus was on the early life stages (I and II). Those two species have a long egg incubation period under low water temperature conditions. For example, under boreal conditions both brown trout and whitefish spawn in autumn and the larvae hatch mainly in spring (e.g. Syrjänen *et al.* 2008, Karjalainen *et al.* 2015). With perch, we focused on the mature males (III).

Brown trout spawn mainly in running waters (Jonsson 1989, Klemetsen *et al.* 2003), usually in their natal stream (Crisp 1989). At spawning, the female digs a nest into the stream bed for depositing the eggs (Crisp 1989, Crisp and Carling 1989). After depositing the eggs, the female starts digging upstream from the nest, creating a new nest and/or burying the eggs (Witzel and MacCrimmon 1983, Crisp 1989, Crisp and Carling 1989). During one spawning season, the female most commonly constructs only one redd that consists of one or more nests (Crisp and Carling 1989) and the eggs in one redd can be fertilized by one or several males (Garcia-Vazquez *et al.* 2001). Males also spawn with more than one female if possible (Klemetsen *et al.* 2003). Brown trout redds have been observed at sites with water velocity and depth ranges of 10.8 to 80.2 cm s^{-1} and 7.0 to 58.0 cm, respectively (e.g. Witzel and MacCrimmon 1983). The preferred gravel size of the redds has been reported to be in a range of 0.8 to 64 mm (Shirvel and Dungey 1983, Witzel and MacCrimmon 1983, Louhi *et al.* 2008), although finer as well as coarser particles can occur as well (e.g. Witzel and MacCrimmon 1983, Louhi *et al.* 2008).

Whitefish is an ecologically and morphologically diverse species, and the different whitefish forms (or ecotypes) differ in the number of gill rakers and spawning habitats (lake in deep or shallow water area, sea or stream) (e.g. Næsje *et al.* 2004, Säisä *et al.* 2008). Fairly little is known about the spawning behaviour of whitefish, but the reported water depths at the spawning sites at lakes have been in the range of < 20 m and > 20 m for shallow and deep water spawners, respectively (e.g. Sandlund and Næsje 1989, Næsje *et al.* 2004). The stream-spawning whitefish are suggested to select river inlets, outlets or deltas with water depth of 0.5 to 5 m (Sandlund and Næsje 1989, Næsje *et al.* 2004). Eggs of the northern densely-rakered ecotype have been found at sites having water velocity of 3 to 30 cm s^{-1} (Haakana and Huuskonen 2008).

With perch, our focus was on the condition and reproductive potential of mature males. Sexual maturity of male European perch is usually reached at the age of 1 or 2 years (females: 3 or 4 years) (e.g. Treasurer 1981, Viljanen and Holopainen 1982, Ceccuzzi *et al.* 2011). The gonad development in mature European perch occurs during the period from late summer to spring (Le Cren 1951). With males, the maximum size of the testes is reached in autumn, remaining such until spring as the spawning approaches (Le Cren 1951). European perch spawn in spring or early summer in freshwaters (Thorpe 1977b, Treasurer 1983, Gillet and Dubois 2007) or brackish water (Snickars *et al.* 2010). At spawning, the female sheds all her eggs in one ribbon-like structure

onto submerged vegetation or other underwater structures (e.g. Treasurer 1983) and at least two males participate in the spawning act (Treasurer 1981). The larvae hatch roughly after 1 to 4 weeks from fertilization, depending on the water temperature conditions (Treasurer 1983). Zooplankton is the main food source for the younger stages of perch, and later on their diet is composed increasingly of benthic macroinvertebrates (e.g. Rask 1986) and even fish (e.g. Ceccuzzi *et al*. 2011).

For the experiments in this thesis, the whitefish gametes were obtained from the Natural Resources Institute Finland hatchery at Laukaa in Central Finland (I) and fertilized eggs from Enonkoski in Eastern Finland (II). Fertilized eggs of brown trout were obtained from Natural Resources Institute Finland hatchery at Paltamo in North-Eastern Finland (II). Mature male perch were caught from lakes in North-Eastern Finland impacted by the Talvivaara Sotkamo Mine as well as from reference lakes in North-Eastern and Central Finland (III).

3.3 Study designs

3.3.1 Early life stages: laboratory experiment (I)

The concentrations of manganese (Mn) and sulphate (SO_4) have been elevated in the waterbodies impacted by the Talvivaara Sotkamo Mine and a need for information about the impacts of Mn and SO_4 on aquatic organisms was stated especially after the gypsum pond leakage (Kauppi *et al.* 2013). Additionally, as brought up in Chapter 1.6, parental effect can have a significant impact on the offspring survival in fish. Thus, the effects of manganese sulphate ($MnSO_4$) on whitefish early life stages were investigated by a laboratory exposure, and to increase the ecological relevance of the study, also the parental effect investigation was included into this experiment (I) (Table 1).

This laboratory experiment was conducted at Konnevesi Research Station from autumn 2013 to spring 2014. The exposure was started at fertilization, as the whitefish gametes were activated with either $MnSO_4$ ($MnSO_4 \cdot H_2O$, Emsure, ACS, Reag. Ph Eur, Merck KGaA; purity 98.8 %) spiked Lake Konnevesi water (filtered through 1 µm 155383-03, Model BP-410-1, Pentek), or with non-spiked pre-filtered L. Konnevesi water, i.e. the control water. Whitefish early life stages were exposed to six different $MnSO_4$ concentrations (5.6–965 mg $MnSO_4$ l^{-1}) and the fertilized eggs were incubated under semi-static exposure conditions from fertilization until the larvae were three days old.

To be able to evaluate the parental effect, the eggs of four females were fertilized with the milt of two males in a full-factorial design (i.e. producing all eight different parent pair combinations) (see Fig. 1 in paper I). Each parent pair had three egg batch replicates in each exposure concentration as well as in control. The eggs were incubated in plastic pools in 12.5 l volume of control or exposure water. There was one pool for each exposure concentration and

control, and the egg batches in each pool were randomly placed into separate compartments on a compartment grid. The compartments were later divided into four sections for the three-day larval incubations (i.e. one section containing the embryos and three others the hatchlings). The incubation water temperature conditions corresponded to the natural lake (Lake Konnevesi, Central Finland) water temperature conditions in winter, and in spring the temperature development was moderately accelerated from the natural spring time water temperature development to onset the hatching. Before the onset of spring temperature increase, embryo samples were collected to represent the winter period responses and in spring, the living three days old larvae were sampled at the end of the experiment.

3.3.2 Early life stages: *in situ* egg incubations (II)

The impacts of the bioheapleaching mine emissions were investigated *in situ* by incubating newly fertilized brown trout and whitefish eggs in six mining impacted and in six reference streams from autumn 2014 to the following spring (Table 1, Fig. 1). The study streams were grouped according to their specific conductance to high mining impact group (HMI) including the Ylä-Lumijoki (HMI1), Lumijoki (HMI2), and Kivijoki (HMI3), all from the Vuoksi watershed, and to low mining impact group (LMI), including the Kalliojoki (LMI1), Salmisenpuro (LMI2) and Jormasjoki (LMI3), all from the Oulujoki watershed (Fig. 1 and 2A). The reference group 1 composed from four streams including the Välijoki (R1.1) and Joutenjoki (R1.2) in the Vuoksi watershed and the Korentojoki (R1.3) and Tervajoki (R1.4) in the Oulujoki watershed (Fig. 1 and 2A). The reference group 2 composed from two streams: the Varisjoki (R2.1) in the Oulujoki watershed and the Rutajoki (R2.2) in the Kymijoki watershed (Fig. 1 and 2A). The reference stream groups also differed in their mean water pH, as the R1 streams were acidic and represented the catchment characteristics in the Talvivaara region without the mining impact (Fig. 2B). The R2 streams were closer to circumneutral pH (Fig. 2B). Of the study streams, the Kalliojoki catchment area partly contained black shale bedrock (Loukola-Ruskeeniemi *et al.* 1998). The mining impacted and reference streams are described in more detail in Materials and Methods of the paper II.

Fish eggs were incubated in plastic cylinders (2 mm mesh size, volume 2–2.7 dl) containing gravel (brown trout) or gravel and sand (whitefish). The whitefish cylinders were additionally covered with a nylon sock due to the relatively large cylinder mesh size compared to the size of the eggs. Six brown trout and six whitefish cylinders, each containing 50 eggs, were placed into each stream in plastic baskets, each basket containing either the six brown trout or the six whitefish cylinders. The baskets were filled with gravel and placed in such manner and into such places that corresponded as well as possible the known natural egg incubation microhabitats of these species (see 3.2 Study species). Three of the six cylinders in each basket were removed as samples in March 2015 and the experiment was finished by collecting the remaining three cylinders in April 2015, before the estimated onset of hatching.

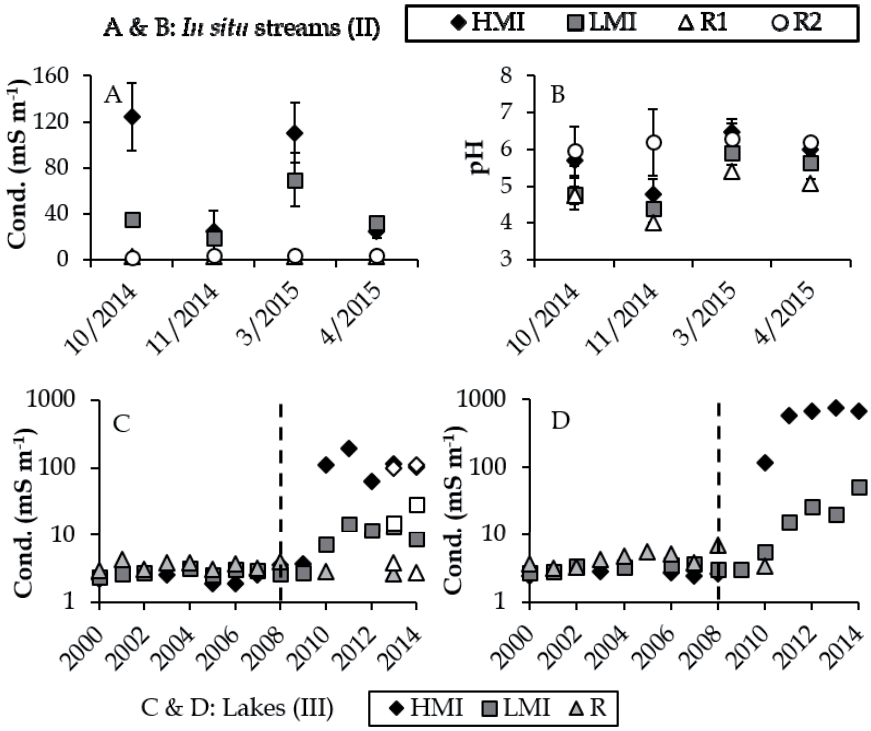

FIGURE 2 Mean (± SE) conductivity (A) and pH (B) of stream water in the *in situ* experiment (II) in the high (HMI) and low (LMI) mining impact and reference (R1 and R2) stream groups at each monitoring occasion, as well as the annual mean conductivity of the study lake groups (HMI: high mining impact; LMI: low mining impact; R: reference) from spring 2000 to spring 2014 in experiment III in water layers 0–2 m from the surface (C) and 0–2 m above the lake bottom (D). The dashed vertical line in C and D indicates the starting of the mining operations. In A and B the figures are based on our own data and in C and D our own observations are indicated as white symbols and the filled symbols in C and D represent the values obtained from Finnish Environment Institute Water Quality Database (Finnish Environment Institute 2017).

3.3.3 Mature perch males (III)

To investigate the impacts of the bioheapleaching mine emissions on the condition and reproductive potential of mature fish, male perch were caught with fish traps from three mining impacted and two reference lakes in spring in years 2013 and 2014 (Table 1, Fig. 1). The focus was on the males, since the number of perch females caught was low. To obtain uniform size range in the study lakes, only males with total fresh body mass less than 100 g were included in the analyses. The lake water conductivity had started to elevate in the mining impacted study lakes after the mine had started to operate (Fig. 2C and D), and thus the lake groups were based on the lake water conductivity: HMI group included Lake Kivijärvi (Vuoksi watershed), the LMI group included Lake Jormasjärvi (Oulujoki watershed) and Lake Laakajärvi (Vuoksi

watershed) (Fig. 1, 2C and 2D) (III). The reference group included a lake from North-Eastern Finland, Lake Kiantajärvi (Oulujoki watershed), and a lake from Central Finland, Lake Sääksjärvi (Kymijoki watershed) (Fig. 1, 2C and 2D) (III).

3.4 Measured endpoints

3.4.1 Early life stages (I, II)

In the laboratory experiment, the fertilization success, mortality, larval growth and yolk consumption, embryonic and larval tissue element concentrations and expressions of metallothionein-A and B (*mt-a* and *mt-b*), catalase (*cat*) and glutathione S-transferase (*gstt*) genes were analysed (I) (Table 1).

The fertilization success was inspected from 10 eggs from each egg batch replicate. The embryonic mortality was inspected two to three times per week and the larval mortalities daily. The fertilization success and mortality were compared among the exposure concentrations, female and male parent. The female parent specific NOEC (no observed effect concentration, i.e. the highest exposure concentration in which the test organism mortality does not significantly differ from the control mortality) and LC_{50} (median lethal concentration, i.e. here the waterborne concentration that is lethal to 50 % of the test organisms) values for $MnSO_4$ were defined. It should be noticed that the NOEC values depend on the selected exposure concentrations.

The larval growth and yolk consumption were analysed from the control, from two low $MnSO_4$ exposure concentrations (5.6 and 5.9 mg l^{-1}) as well as from one high $MnSO_4$ exposure concentration (41.8 mg l^{-1}). The analyses were done by separating the carcass and yolk sac, drying them and weighing thereafter. The carcass and yolk dry masses were compared among the exposure concentrations, female and male parent in relation to the degree-days (i.e. the sum of mean daily water temperature during the corresponding incubation period).

The concentrations of Al, As, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Ni, K, Na, P, Sr, S, U and Zn were analysed from the eggs and larvae with ICP-OES (Optima 8300 Inductively Coupled Plasma Optical Emission Spectrometer, Perkin-Elmer, Norwalk, CT, USA) and the limits of quantification (LOQ) were defined according to the method defined by United States Environmental Protection Agency (US-EPA) (Anonymous 2001). Before the analyses, the dried tissue samples were digested in aqua regia (1:3 HNO_3:HCl by volume) by sonication (ELMA Model Transonic 820/H,50 W, 35 kHz, or Bandelin Sonorex RK 512/H, 400 W, 35 kHz), filtered (41, Whatman) and diluted into final volume with ultrapure water (PURELAB Ultra water purification system, Elga, Buckinghamshire, UK). The egg and larval Mn and S concentrations were compared among the exposure concentrations and female parents. Also, egg and egg-to-larval median critical body residue (CBR_{50}: the tissue element concentration that is lethal to 50 % of the test organisms) values for Mn were

calculated according to the egg and larval Mn concentrations. The embryos were referred to as eggs concerning analyses of elements and CBR_{50} calculations from them, since they were not dechorionated. Additionally, as the incubation of the larval stage lasted only for three days and during that time the larval mortality was rather low, the CBR_{50} value for the concentrations of Mn in the larvae included the mortality during the whole incubation period and is called egg-to-larval CBR_{50}. Due to the low number of samples, the CBR_{50} analyses were made without parent or parent pair definitions.

The embryonic gene expressions were analysed from three parent pairs: one with low, a second with medium and a third with high offspring mortality. Regarding the larval gene expression analyses, only the low and medium mortality pair offspring had survived to the larval stage analysis, and thus the larval gene expression analyses included offspring of only those two parent pairs. The gene expressions among the exposure concentrations and the selected parent pairs were compared. The gene expressions were analysed with quantitative reverse transcription polymerase chain reaction (qRT-PCR). The primers for the target gene *mt-a* were obtained from a published study (Hansen *et al.* 2007). For other target genes (*cat*, *gstt* and *mt-b*) the primers were designed with Primer3 (Koressaar and Remm 2007, Untergasser *et al.* 2012) and those for reference genes ribosomal protein L2 (*rl2*) and *beta actin* with AmplifX 1.5.4 (Jullien 2008). The specificity of all the genes was checked with Primer-BLAST (Ye *et al.* 2012).

In the *in situ* incubation experiment, the mortality, growth and yolk consumption of the brown trout and whitefish embryos at each study stream were analysed and compared among the stream groups (II) (Table 1). Some of the cylinders had accumulated fine particles or organic material and this cylinder cleanliness was taken into account in the mortality analyses by dividing the cylinders into "clean" and "dirty". The mortality comparisons were made in relation to the observed minimum pH of the stream water. The growth and yolk consumption analysis method was the same as in the laboratory experiment (I). In the *in situ* incubation experiment (II), also the total lengths of the embryos were measured. With embryonic length, carcass and yolk masses, such division into clean and dirty cylinders was not done, since the cylinder cleanliness did not seem to have been affecting those variables. The comparisons of embryonic length, carcass and yolk masses were made in relation to the observed water minimum pH and degree-days.

3.4.2 Mature perch males (III)

From the perch males, the 1) total length, 2) wet mass of carcass, testes and liver, 3) sperm count, sperm motility and velocity, 4) muscle and liver element concentrations and 5) hepatic expressions of metallothionein (*mts*), Cu/Zn superoxide dismutase (*sod-1*), heat shock protein-70 (*hsp-70*) and glutathione peroxidase 3 (*gpox-3*) were analysed in 2013. In 2014, only total length, wet mass of carcass, testes and liver were measured (Table 1). The data from both years

was combined and the wet mass of carcass, testes and liver were each compared among the lake groups or lakes in relation to the total length of the fish.

The sperm count and motility analyses were conducted with ISASv1® CASA system (10 × phase contrast, Proiser). Three types of activation waters were used: artificial freshwater (0.1 mmol; Anonymous 1996), artificial freshwater spiked to a concentration of 50 mg Cd l^{-1} ($CdCl_2$, anhydrous, ACS min. 99%, Alfa Aesar) and filtered (48 µm mesh size) natal lake water of the male. The male-specific sperm counts were estimated as the mean number of sperm observed in the microscope frame of each activation water type. The proportion of motile sperm was estimated according to the curvilinear sperm head velocity, VCL (µm s^{-1}) and the sperm swimming velocity according to the straight line linear velocity of the sperm head, VSL (µm s^{-1}) (Quintero-Moreno *et al.* 2003) measured at 10 s and 20 s post-activation. The impact of the different activation waters on the proportion of motile sperm was tested. The mining impact investigations on the proportion of motile sperm and the swimming velocity of the sperm were done for the lake water activated sperm by comparing the lake groups or lakes.

The concentrations of As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, S, Sr, U and Zn were determined from the muscle of the perch males, and all the same elements, except Cu and Ni, from the liver as well. The analyses were mainly carried out with ICP-OES, except the muscle Cd, Ni and Pb concentrations were analysed with ETAAS (Electrothermal Atomic Absorption Spectrometer, Model AAnalyst 800 equipped with an AS-800 autosampler, Perkin-Elmer). These element concentration analyses from the muscle and liver were made in similar manner as those in the laboratory experiment (I). Certain elements (muscle: Cu, Ni, S and Zn; liver: Cd, Mn, S and Zn) were selected for the comparisons among the lake groups or lakes. Those selections were based on the reported elevated concentrations in the mining impacted waterbodies (Kauppi *et al.* 2013), and in the muscle and liver tissues those elements were mainly above the LOQs of the ICP-OES or ETAAS.

The gene expression analyses were made as in the laboratory experiment (I) and the primer sequences for target genes *hsp-70*, *mts* and *sod-1* were obtained from a study by Pierron *et al.* (2009). Primers for target gene *gpox-3*, and reference gene ribosomal protein L11 (*rpl11*) were designed with Primer3 (Koressaar and Remm 2007, Untergasser *et al.* 2012). The specificity of all the genes was checked with Primer-BLAST (Ye *et al.* 2012). The expressions of the target genes were compared among the lake groups or lakes and the correlation between hepatic Cd concentration and *mts* expression was examined as well.

3.5 Water quality measurements and elemental determinations

3.5.1 Water quality (I-III)

The water quality was monitored in each control and exposure water change (I) and at field visits (II, III). The water samples for dissolved element concentration analyses were filtered (25 mm GD/XP (II, III) or GD/X (II) syringe filters, 0.45 µm PVDF w/PP, Whatman) in the field immediately after sampling and all water samples for element determinations were conserved with nitric acid (SupraPur, 65 %, Merck) immediately after sampling.

The water temperature, oxygen concentration and saturation, specific conductance and pH were monitored as well (I: YSI Professional Plus YSI ProOdo, 744 pH meter Metrohm or SevenGo pH meter SG2 Mettler Toledo; II: YSI ProfessionalPlus and YSI ProOdo or YSI6600 Multiparameter sonde; III: YSI6600 Multiparameter sonde).

Dissolved organic carbon (DOC) was analysed from the control water (I) and from all the field study sites (II, III). For the DOC analysis (TOC-L, Total Organic Carbon Analyzer, Shimadzu), 20 ml of the sample water was filtered with 25 mm PES syringe filter w/0.45 µm, VWR (II) or Filtropur S (I, III) and acidified with 80 µl of 2 M HCl (PA quality).

3.5.2 Elemental determinations (I-III)

Total (I) or dissolved (II, III) element concentrations, Al, As, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Ni, K, Na, P (only in paper I), Sr, S, U, Zn, were analysed with ICP-OES and the LOQs were defined according to US-EPA (Anonymous 2001). Certain Mn concentration specifications in the laboratory experiment were done with ETAAS as well (I). In all three experiments, the water S concentrations were used to estimate the water sulphate (SO_4) concentrations, since the S was estimated to occur mainly as SO_4^{2-} in the oxygen saturation levels observed in the experiments.

4 RESULTS AND DISCUSSION

4.1 Early life stages

4.1.1 Mortality and growth (I, II)

Manganese has been considered as less toxic to aquatic organisms than other metals, such as Cd, Cu or Zn (Lewis 1978, Stubblefield *et al.* 1997), but there is evidence that Mn can reduce the survival of fish early life stages and impair the larval growth, as has been demonstrated in a 62 day exposure of brown trout early life stages (Stubblefield *et al.* 1997). Also, excessive SO_4 concentrations are known to reduce the early life stage survival and impair the larval growth of fish (Elphick *et al.* 2011). Our results showed that the continuously elevated $MnSO_4$ concentrations increased the mortality of the whitefish early life stages (I) (Table 2). However, no consistent $MnSO_4$ exposure related effect on fertilization success was observed. The $MnSO_4$ exposure did affect the larval carcass dry mass and yolk consumption, although the differences among the exposure concentrations were rather subtle (Table 2). However, the highest $MnSO_4$ concentration (41.8 mg $MnSO_4$ l^{-1}), from which the larval growth and yolk consumption were analysed, seemed to have been inhibiting the yolk consumption, as those larvae had larger yolk reserves left.

TABLE 2 Summary of the main observations in the thesis as well as the strengths and observed shortcomings of the experimental designs (I, II, III).

	Main observations	Strengths	Shortcomings
I	• $MnSO_4$ exposure increased mortality, caused impairment of growth and yolk consumption, induced *mt-a* and *mt-b* • Mn concentrated into the eggs and larvae • Particularly the female parent affected significantly the offspring $MnSO_4$ tolerance	• Effect characterisation of $MnSO_4$ • All the early life stages were included • Ecological relevance increased with parental effect investigation • Several endpoints included	• Only two male parents • Compromising between the parent numbers and exposure concentrations
II	• No obvious mining impact on embryonic mortality, growth or yolk consumption • Low water pH increased embryonic mortality • Mainly temperature regulated the growth and yolk consumption	• Natural incubation environment • Long-term incubation period • Two native fish species included	• Differentiating between the mining impacts and natural catchment characteristics is difficult • Fertilization and hatching not included • Only one incubation period
III	• Smaller liver size in the mining impacted lakes • Positive correlation between hepatic Cd concentration and *mts* expression levels • Smaller size of testes in the high mining impact lake males • Lower sperm count in the mining impacted lakes • Elongated sperm motility in the high mining impact lake males	• Indication of cumulative exposure throughout the lifetime of the fish • Several endpoints included	• Investigations focused on one season (spring) only • No data available before the mining activities • Females were not included

Besides Mn and SO_4, there are also other metals and major ions present in the mining impacted waters. Moreover, the environmental exposure conditions in the recipient waterbodies can fluctuate depending on the effluent quality and quantity. In the *in situ* experiment (II), the mining impacted streams clearly had higher ion concentrations than the reference streams, as indicated by the

elevated water conductivity (Fig. 2A). Our results, however, indicated that although the mean dissolved concentrations of metals, including Mn, and SO_4, were elevated in the mining impacted streams, those did not seem to have any consistent effect on brown trout and whitefish embryonic mortality, growth or yolk utilization (Table 2). Regarding mortality, the inorganic and organic particle accumulation into the incubation cylinders had a significant effect on both species, but the effect was inconsistent, as particle accumulation did not increase the mortality in all cases, and we did not observe any clear connection between the mining activities and cylinder particle accumulation either. When only the clean cylinders were included into the mortality analyses, the low pH of the stream water was observed to increase the embryonic mortality of both species (Table 2). Also others have observed that the low water pH is harmful for both brown trout (pH: 4.5) (Sayer *et al.* 1991) and whitefish (pH: 5.5) (Keinänen *et al.* 2003) embryos. However, in Talvivaara area the waters are naturally acidic (Fig. 2B, Anonymous 2016b), whereas the mining effluents are treated by liming before they are released into the environment, and thus the pH of the discharged effluent is mainly neutral or alkaline (Anonymous 2015, 2016c). Therefore, the impacts of low water pH on the embryonic mortality of brown trout and whitefish were not related to the mining impact in this study. Also, the increased water hardness in the mining impacted streams due to the liming of the effluents before they are released may have reduced the toxicity of metals as well as that of SO_4 (see e.g. Stubblefield *et al.* 1997, Meinelt *et al.* 2001, Pyle *et al.* 2002, Elphick *et al.* 2011). The embryonic growth of both species, as well as the whitefish yolk consumption were mainly controlled by water temperature, which we did not observe to be linked to the mining impact of the streams (Table 2).

It should also be kept in mind, that the timing of the exposure can be critical regarding the embryonic development of fish. Exposure to metals and/or acidic water at the time of fertilization can impair the egg hardening process and formation of perivitelline space (González-Doncel *et al.* 2003, Keinänen *et al.* 2003) and lead to increased embryonic metal uptake (González-Doncel *et al.* 2003) as well as restrict embryonic growth (Keinänen *et al.* 2003). Thus, the fact that the $MnSO_4$ exposure in the whitefish early life stage laboratory experiment was started at fertilization likely had an impact on the magnitude of the responses. In the *in situ* incubation experiment (II), as the eggs were fertilized in the hatchery rearing water, their chorion and perivitelline fluid were likely developed normally and protected the embryos from external stressors, such as metals. The protective role of those egg structures has been indicated in previous studies (Michibata 1981, Stouthart *et al.* 1995, González-Doncel *et al.* 2003, Keinänen *et al.* 2003). Also, the critical stage of hatching was not included into the *in situ* study (II) due to spring flood preventing the field work in the streams at the hatching time, although the development and functioning of the hatching glands can be interfered by metals (Jezierska *et al.* 2009).

4.1.2 Accumulation of manganese and sulphur (I)

In the laboratory experiment, it was shown that Mn accumulated into the eggs and newly hatched larvae of whitefish, and that Mn accumulation was higher as Mn concentration of the incubation water increased (I) (Table 2). The sulphur from the SO_4, however, did not seem to concentrate into the eggs or the larvae. Although the exposure was started at fertilization, our findings suggested that the egg structures may have given protection to the developing embryo, as the whitefish eggs had extremely high Mn concentrations compared to those measured from the hatched three-day-old larvae. Chorion can accumulate polyvalent cations (Peterson and Martin-Robichaud 1986), and the negatively charged colloids in the perivitelline fluid can concentrate cations as well (Rudy and Potts 1969). As stated earlier, these structures may have protected the developing whitefish embryos (see Michibata 1981, Stouthart *et al.* 1995). Also, the critical body residues (CBR) were thus higher with the eggs, as their CBR_{50} value was 9.08 (95 % CI: 7.13–12.81) μmol g^{-1} dry weight, whereas the egg-to-larval CBR_{50} value was clearly lower, 0.88 (95 % CI: 0.56–2.05) μmol g^{-1} dry weight.

4.1.3 Parental effect and tolerance (I)

The parent pair investigation indicated that there can be intrinsic differences in the reproductive success among different parents, and that especially the female can significantly affect the offspring survival under stressful conditions (I) (Table 2). We did not observe that the male effect alone would have had significant impacts on the fertilization success or on the early life stage mortality, growth or yolk consumption of the whitefish. It is worth to note here that parent fish had not been exposed to mining effluents.

Already from fertilization, the differences in the reproductive success among the four female parents were obvious, but no parent fish or parent pair related $MnSO_4$ tolerance differences regarding fertilization were observed. However, regarding mortality, the results showed that particularly one of the female whitefish had produced more $MnSO_4$-tolerant offspring than the three others. The mortality results indicated male related $MnSO_4$ tolerance differences as well, but this would need further research, because we only had two males in our experiments (Table 2). Indeed, if this experiment would be repeated, the focus could be turned even more into the parental effect investigation by increasing the number of parent fish and reducing the number of $MnSO_4$ exposure concentrations. The female parent specific LC_{50} values suggested that the more tolerant offspring could survive better under twice as high $MnSO_4$ concentrations than those having lower tolerance, as the lowest and highest female parent specific LC_{50} values (95 % CI) for $MnSO_4$ were 42.0 (33.9–50.9) mg l^{-1} and 84.6 (71.0–97.9) mg l^{-1}, respectively. The female parent specific NOEC values were from 5.9 to 41.8 mg $MnSO_4$ l^{-1}. The highest observed NOEC value, however, belonged to the female with the highest offspring mortality in the control.

Furthermore, the larval gene expression results suggested that this difference between the $MnSO_4$ tolerance may be linked to the metallothionein mRNA expression levels, as the larvae of the parent pair that were the most tolerant to $MnSO_4$ had significantly induced *mt-a* and *mt-b,* whereas the larvae of the pair with medium offspring survival had not (Table 2). The metallothionein mRNA induction has been previously connected to increased metal tolerance in fish (George *et al.* 1996). With the embryos, however, none of the target gene expressions were altered by the $MnSO_4$ exposure, but that could also suggest that the egg structures may have been protecting the embryos.

4.2 Mature perch males (III)

4.2.1 Body and liver size of perch males

The mining impacted lakes contained elevated concentrations of dissolved metals (e.g. Mn, Ni and Sr) as well as SO_4. Those multi-stressor conditions had affected the energy reserves of the males, as the perch males in the mining impacted lake groups had significantly lower liver size than the males in the reference group (Table 2).

Although we could not differentiate if certain factor(s) in the mining impacted lakes had caused the observed differences in the liver size, for example, starvation has been shown to increase the liver energy reserve utilization and decrease the hepatosomatic index in golden perch (*Macquaria ambigua*) (Collins and Anderson 1995). Also, the growth of yellow perch in metal contaminated lakes has been linked to increased activity costs due to the lack of suitable prey for diet shift (Sherwood *et al.* 2002, Iles and Rasmussen 2005), as well as reduced food conversion efficiency (Sherwood *et al.* 2000). However, mining impact related effect on the body mass of the perch males was not observed in our study (Table 2, see also Fig. 3A in paper III). In addition, although the lower condition, hepatosomatic index and growth rate of yellow perch have also been associated with elevated liver and muscle metal (e.g. Cd) concentrations (Rajotte and Couture 2002), in our study, we did not observe that the smaller liver size of the perch males would have been linked to the liver or muscle metal concentrations, as the metal concentrations in those tissues did not clearly follow the mining impact gradient.

It is also worth to note that with perch, the main lipid storage is in the visceral fat and in not the liver (Blanchard *et al.* 2005). In addition, there are also studies in which the hepatosomatic index has not been observed as a good mining impact gradient indicator, as there either has not been observed any mining impact gradient related patterns in it and/or the observed variations have been different between the seasons (Eastwood and Couture 2002, Levesque *et al.* 2002, Audet and Couture 2003). Thus, regarding our studies in Talvivaara, the effect of the seasonal variation on the size and composition of

perch hepatic tissue and body condition should be included in possible future studies (Table 2).

4.2.2 Metal accumulation and detoxification

A strong positive correlation between the hepatic Cd accumulation and *mts* expression was observed (Table 2). A similar pattern with hepatic metallothionein and Cd concentration of wild European perch has been observed by others as well (see Olsson and Haux 1986). Despite the positive correlation between the hepatic Cd concentration and *mts* induction, those variables did not seem to follow the defined mining impact gradient. The mean ± SE hepatic Cd concentration was the highest in LI lake Jormasjärvi perch males (23.4 ± 2.07 $mgkg^{-1}$ dry weight, dw), whereas in the other LI lake Laakajärvi the mean hepatic Cd concentration (3.87 ± 1.03 $mgkg^{-1}$ dw) was similar to those observed from the reference perch males (Kiantajärvi: 2.13 ± 0.45 $mgkg^{-1}$ dw, Sääksjärvi: 5.15 ± 1.52 $mgkg^{-1}$ dw). In HI lake Kivijärvi perch males, the corresponding Cd concentration was 12.34 ± 2.74 $mgkg^{-1}$ dw. The Cd concentrations in the male perch in the mining impacted lakes in this study were similar to those observed by Pyle *et al.* (2005) from livers of yellow perch inhabiting metal contaminated lakes. However, the Cd concentrations of the reference perch males were higher in our study compared to those in Pyle *et al.* (2005). Pyle *et al.* (2005) found a strong non-linear relationship between the hepatic Cd concentration and water hardness of the study lakes, the hepatic Cd concentrations being lower in perch collected from sites with higher water hardness. In our study the water hardness in the mining impacted lakes was the lowest in Jormasjärvi and highest in Kivijärvi (see Fig. S4 in paper III). Thus, our observations also suggested that the water hardness may have been affecting the Cd accumulation in a similar manner as observed by Pyle *et al.* (2005).

No Cd or mining impact related patterns were observed in the expressions of the other target genes. Also, none of the other metals analysed from muscle or liver clearly indicated that the mining contamination had increased their concentrations in those tissues.

4.2.3 Reproductive potential

The significantly lower size of the testes of the males in the high mining impact group suggested that the reproductive potential of the males in the most contaminated study lake may have been reduced. With postspawn male yellow perch, the observed lower gonadosomatic index has been suggested to be linked to feeding status of the males living in metal contaminated lakes (Pyle *et al.* 2005).

In addition, some of the sperm characteristics also suggested that the multi-stressor conditions in the mining impacted lakes had altered the male reproductive potential, as the males from the mining impacted lakes had lower sperm counts compared to the reference males (Table 2). The lower sperm

counts, however, seemed to have been compensated by elongated sperm motility (Table 2), whereas the swimming velocity of the sperm did not differ significantly among the males. In addition, the observed differences in the sperm motility was not linked to the sperm activation water according to the activation water comparisons as only the Cd spiked (50 mg Cd l^{-1}) artificial freshwater reduced the sperm motility significantly.

5 CONCLUSIONS

The results revealed that the continuously elevated high $MnSO_4$ concentrations are able to reduce the reproductive success of whitefish. Also, the female parent related differences in offspring $MnSO_4$ tolerance can be significant, as we observed that the offspring of one of the females tolerated roughly twice as high $MnSO_4$ concentrations compared to the offspring of the female parent with poorest offspring survival. Additionally, the larval $MnSO_4$ tolerance was higher in those larvae that had significantly induced the metal-binding genes.

However, as we moved from the laboratory to the mining impacted streams with the *in situ* brown trout and whitefish egg incubation experiment, it became clear that, rather than the mining activities, other catchment characteristic-related factors can significantly affect the outcome of the early life stage exposure under environmental exposure conditions. Particularly, low water pH increased the egg and embryonic mortality, whereas the alterations in the embryonic growth and yolk consumption were mainly controlled by water temperature. However, the water pH was naturally low in the study streams, and we did not observe that the water temperature differences among the streams would have been linked to the mining activities either. Thus, no clear link between the mining impact and increased embryonic mortality, growth or yolk utilization were observed in the *in situ* experiment. On the other hand, the critical life stages of fertilization, hatching and larval period were not included into the *in situ* incubation experiment. In addition, since our observations only covered one incubation period, it would be relevant to repeat the experiment in order to examine annual variability in the embryonic responses caused by possible water quality fluctuations.

With the wild male perch, the habitat metal contamination and/or salinization was linked to smaller size of the liver and testes as well as alterations in sperm characteristics, suggesting that the energetic demands may have been higher and/or the nutritional status of the males may have been impaired in the mining impacted lakes. However, the body size differences of the male perch among the different study lakes did not reflect the mining impact gradient. Additionally, the increased hepatic Cd concentrations

probably caused the induction of *mts* in the perch liver. Since there was no corresponding data regarding perch males before the mining activities, we cannot conclude if there had been any changes in the perch populations due to the mining activities. However, since we observed some indication of the perch males being stressed in the mining impacted lakes, it would be relevant to continue this kind of monitoring of perch in those study lakes, including the females and different seasons as well, in order to define and extend the observations made in this thesis.

By focusing on native fish species and conducting both laboratory and field experiments, these studies have provided ecologically relevant and comprehensive information for the national risk assessment regarding the impacts of metal mining (bioheapleaching in particular) on fish in the boreal region. These studies have also demonstrated the significance of conducting environmentally realistic experiments in addition to the laboratory-based effect characterisation when estimating how certain contaminant(s) may affect the fish populations in the wild.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Biokasaliuotustekniikkaa hyödyntävän metallikaivoksen päästöjen vaikutukset kaloihin pohjoisissa sisävesissä

Metallikaivostoiminnan tai metallien prosessoinnin vaikutuksen alaisissa vesistöissä metalli- ja sulfaattipitoisuudet voivat olla huomattavasti luontaisia pitoisuuksia suurempia. Aiemmissa tutkimuksissa kohonneiden metalli- tai sulfaattipitoisuuksien on osoitettu olevan haitallisia vesieliöille, kuten kaloille. Esimerkiksi metallien saastuttamissa vesistöissä elävien aikuisten kalojen kunnon, kasvun ja lisääntymispotentiaalin (eli sukurauhasten massan) on havaittu heikentyneen. Aikuisiin kaloihin verrattuna kalojen varhaiset kehitysvaiheet, alkiot ja poikaset, ovat yleensä herkempiä vierasaineille. Sukusolujen tai alkioiden metallialtistus voi häiritä alkioiden ja poikasten kehitystä aiheuttaen kuolleisuutta, epämuodostumia ja kasvun heikkenemistä. Metallien lisäksi suuret sulfaattipitoisuudet voivat olla myrkyllisiä kaloille. Kalojen varhaisten elinvaiheiden selviytymisessä ja kehityksessä on kuitenkin osoitettu olevan myös luontaista vaihtelua eri emojen ja emoyhdistelmien tuottamien jälkeläisten välillä. Emovaikutuksen määrittämistä on silti harvoin sisällytetty ekotoksikologisiin tutkimuksiin.

Väitöskirjatyöni päätavoitteena oli tuottaa uutta tietoa biokasaliuotusmenetelmää hyödyntävän metallikaivoksen päästöjen vaikutuksista kalojen lisääntymispotentiaaliin ja varhaiskehitykseen. Tutkimuslajeina olivat kolme Suomessa yleisesti tavattavaa kalalajia, ahven (*Perca fluviatilis*), siika (*Coregonus lavaretus pallasi*) ja taimen (*Salmo trutta*). Laboratoriokokeessa määritettiin mangaanisulfaatin vaikutuksia siian varhaisiin kehitysvaiheisiin ja samalla arvioitiin eri emokalojen ja emoyhdistelmien merkitystä jälkeläisten kuolleisuuteen, kasvuun ja ruskuaisravinnon käyttöön. Jotta metallikaivostoiminnan vaikutuksista saatiin laboratorioaltistuksen lisäksi todenmukaisempi ja moniulotteisempi kuva, tutkittiin siian ja taimenen alkioiden kuolevuutta ja kasvua biokasaliuotusteknologiaa hyödyntävän metallikaivoksen vaikutuksen alaisissa virtavesissä tehdyn haudontakokeen avulla. Erityisen herkkinä pidettyjen varhaisten kehitysvaiheiden ohella selvitettiin myös, oliko metallikaivoksen vaikutuksen alaisten järvien sukukypsien ahvenkoiraiden somaattisen kudoksen tuoremassassa, maksan koossa ja lisääntymispotentiaalissa nähtävissä merkkejä altistumisesta kaivoksen päästöille.

Laboratorioaltistuskokeessa, suuret mangaanisulfaattipitoisuudet (41.8–965.0 mg $MnSO_4$ l^{-1}) lisäsivät selkeästi siian varhaisten kehitysvaiheiden kuolevuutta. Mangaanisulfaattialtistus heikensi myös siianpoikasten kasvua ja ruskuaisen käyttöä, vaikkakin niissä havaitut erot altistuspitoisuuksien välillä olivat melko pieniä. Lisäksi mangaanisulfaattialtistuksen sietokyvyssä oli merkittäviä eroja eri naaraiden tuottamien jälkeläisten välillä. Parempi mangaanisulfaattialtistuksen sietokyky saattoi liittyä myös poikasten kykyyn tuottaa metalleja sitovia proteiineja. Taimenen ja siian alkioiden kuolevuuden ja kasvun kannalta muut kuin kaivostoimintaan liittyvät tekijät osoittautuivat kuitenkin merkittävämmiksi. Luonnonoloissa haudontasylintereihin kertynyt hiekka tai muu

hienojakoinen aines vaikutti merkittävästi alkioiden kuolevuuteen, vaikka vaikutukset eivät olleet yksiselitteisesti kuolevuutta lisääviä. Lisäksi partikkeleiden kertymisellä ei ollut selkeää yhteyttä kaivosvaikutukseen. Puhtaissa sylintereissä veden alhainen pH lisäsi alkioiden kuolevuutta merkittävästi. Jokiveden alhainen pH oli tyypillistä etenkin kaivosalueen lähistöllä oleville vertailujoille. Lämpötilaerot eri haudontapaikkojen välillä vaikuttivat eniten alkioiden kasvunopeuteen sekä siialla myös ruskuaisvararavinnon kulumiseen; kasvu ja ruskuaisen kuluminen olivat sitä nopeampia mitä lämpimämpää vesi oli. Eri havaintopaikkojen veden lämpötilalla ei tässä tutkimuksessa ollut yhteyttä kaivostoimintaan. Toisaalta luonnossa tehdyssä haudontakokeessa tutkittiin vain alkioita, kun taasen laboratorioaltistuskokeessa oli huomioitu myös muut herkät elinvaiheet, kuten hedelmöitys ja kuoriutuminen. Aiemmissa tutkimuksissa on havaittu, että heti hedelmöityksessä tapahtuva altistus esimerkiksi metalleille heikentää hedelmöitetyn mätimunan rakenteiden, kuten kuoren, normaalia muodostumista ja on siten haitallisempaa verrattuna siihen, jos altistus on aloitettu vasta hedelmöityksen jälkeen. Metallit voivat myös häiritä kuoriutumisrauhasten normaalia muodostumista ja toimintaa.

Kaikkinensa tässä väitöskirjatyössä tehtyjen haudontakokeiden havainnot korostavat sekä laboratorio- että maastokokeiden merkitystä arvioitaessa haitallisten aineiden vaikutuksia luonnossa esiintyviin kalapopulaatioihin. Siinä missä altistusolosuhteet laboratoriossa ovat kontrolloituja, luonnossa altistusolosuhteet vaihtelevat ja koostuvat monimutkaisista kokonaisuuksista erilaisia luonnollisia ja/tai ihmistoiminnan seurauksena muodostuneita tekijöitä, joiden keskinäinen vuorovaikutus voi myös vaikuttaa tutkittuihin vasteisiin eliöissä.

Metallikaivoksen vaikutuksen alaisten järvien sukukypsissä ahvenkoiraissa oli joitakin viitteitä ahvenkoiraiden kunnon ja lisääntymispotentiaalin heikkenemisestä. Ahvenkoiraiden maksan tuoremassat metallikaivoksen vaikutuksen alaisilla järvillä olivat merkittävästi pienempiä kuin vertailujärvien koirailla, ja tämän lisäksi voimakkaan kaivosvaikutuksen järven ahvenkoiraiden sukurauhasten massa oli merkittävästi pienempi kuin vertailujärvien ja vähäisen kaivosvaikutuksen järvien koirailla. Myös siittiöiden määrät olivat keskimäärin pienempiä kaivosvaikutuksen alaisten tutkimusjärvien koirailla, mutta toisaalta voimakkaan kaivosvaikutuksen järven ahvenilla siittiöt jaksoivat liikkua pidempään kuin vertailujärvien ahventen siittiöt. Metallikaivostoiminnan ei kuitenkaan havaittu vaikuttaneen ahvenkoiraiden somaattisen kudoksen tuoremassaan kalan kokonaispituuteen suhteutettuna. Ahvenkoiraiden maksasta havaittiin myös positiivinen yhteys metallien sitomiseen liittyvän geenin, metallotioneiinin, lähetti-RNA:n määrän ja kadmiumpitoisuuden välillä.

Tässä väitöskirjatyössä saavutettiin uutta ja ekologisesti olennaista tietoa biokasaliuotustekniikkaa käyttävän metallikaivoksen päästöjen vaikutuksista pohjoisen alueen sisävesiin sekä niissä esiintyvään kolmeen kalalajiin. Tutkimustulokset hyödyttävät kaivosteollisuuden ympäristövaikutusten ja -riskien arviointia.

REFERENCES

Anon. 1996. Water quality determination of the inhibition of the mobility of *Daphnia magna* Strauss (Cladocera, Crustacea). Acute toxicity test (SFS-EN ISO 6341:1996).

Anon. 2001. United States Environmental Protection Agency. US-EPA METHOD 200.7, Revision 4.4, Cincinnati.

Anon. 2010. Talvivaara Mine Monitoring report 2009. Part III: Effluent discharge monitoring. Pöyry Oy, Finland. (in Finnish).

Anon. 2013. Talvivaara Mine Monitoring report 2012. Part III: Effluent discharge monitoring. Pöyry Oy, Finland. (in Finnish).

Anon. 2015. Talvivaara Mine Monitoring report 2014. Part III: Effluent discharge monitoring. Ramboll Oy, Finland. (in Finnish).

Anon. 2016a. Terrafame Mine Monitoring report 2015. Part V: Surface water monitoring. Ramboll Oy, Finland. (in Finnish).

Anon. 2016b. Terrafame Mine Monitoring report 2015. Part I: Summary. Ramboll Oy, Finland. (in Finnish).

Anon. 2016c. Terrafame Mine Monitoring report 2015. Part III: Effluent discharge monitoring. Ramboll Oy, Finland. (in Finnish).

Audet D. & Couture P. 2003. Seasonal variations in tissue metabolic capacities of yellow perch (*Perca flavescens*) from clean and metal-contaminated environments. *Can. J. Fish. Aquat. Sci.* 60: 269–278.

Besser J.M., Brumbaugh W.G., May T.W., Church S.E. & Kimball B.A. 2001. Bioavailability of Metals in Stream Food Webs and Hazards to Brook Trout (*Salvelinus fontinalis*) in the Upper Animas River Watershed, Colorado. *Arch. Environ. Contam. Toxicol.* 40: 48–59.

Blanchard G., Druart X. & Kestemont P. 2005. Lipid content and fatty acid composition of target tissues in wild *Perca fluviatilis* females in relato hepatic status and gonad maturation. *J. Fish. Biol.* 66: 73–85.

Bobe J. & Labbé C. 2010. Egg and sperm quality in fish. *Gen. Comp. Endocr.* 165: 535–548.

Brierley C.L. 2008. How will biomining be applied in the future? *Trans. Nonferrous Met. Soc. China* 18: 1302–1310.

Campbell P.G.C. & Stokes P.M. 1985. Acidification and toxicity of metals to aquatic biota (a review). *Can. J. Fish. Aquat. Sci.* 42: 2034–2049.

Campbell P.M., Potfinger T.G. & Sumpter, J.P. 1992. Stress Reduces the Quality of Gametes Produced by Rainbow Trout. *Biol. Reprod.* 47: 1140–1150.

Campbell P.G.C., Hontela A., Rasmussen J.B., Giguère A., Gravel A., Kraemer L., Kovesces J., Lacroix A., Levesque H. & Sherwood G. 2003. Differentiating Between Direct (Physiological) and Food-Chain Mediated (Bioenergetic) Effects on Fish in Metal-Impacted Lakes. *Hum. Ecol. Risk Assess.* 9: 847–866.

Ceccuzzi P., Terova G., Brambilla F., Antonini M. & Saroglia M. 2011. Growth, diet, and reproduction of Eurasian perch *Perca fluviatilis* L. in Lake Varese, northwestern Italy. *Fish. Sci.* 77: 533–545.

Collins A.L. & Anderson T.A. 1995. The regulation of endogenous energy stores during starvation and refeeding in the somatic tissues of the golden perch. *J. Fish. Biol.* 47: 1004–1015.

Couture P. & Kumar P.J. 2003. Impairment of metabolic capacities in copper and cadmium contaminated wild yellow perch (*Perca flavescens*). *Aquat. Toxicol.* 64: 107–120.

Crisp D.T. 1989. Some impacts of human activities on trout, *Salmo trutta*, populations. *Freshwat. Biol.* 21: 21–33.

Crisp D.T. & Carling P.A. 1989. Observations on siting, dimensions and structure of salmonid redds. *J. Fish Biol.* 34: 119–194.

Eastwood, S. & Couture, P. 2002. Seasonal variations in condition and liver metal concentrations of yellow perch (*Perca flavescens*) from a metal-contaminated environment. *Aquat. Toxicol.* 58: 43– 56.

Elliott J.M. 1989. Wild brown trout *Salmo trutta*: an important national and international resource. *Freshwat. Biol.* 21: 1–5.

Elphick J.R., Davies M., Gilron G., Canaria E.C., Lo B. & Bailey H.C. 2011. An aquatic toxicological evaluation of sulfate: the case for considering hardness as a modifying factor in setting water quality guidelines. *Environ. Toxicol. Chem.* 30: 247–253.

Finnish Environment Institute 2016. Catchment Areas and National Database of Regional Land Use Plans. Download Service LAPIO, Finnish Environment Institute. Data retrieved 11/2016.

Finnish Environment Institute 2017. Water Quality Database OIVA, Finnish Environment Institute. http://www.syke.fi/avointieto. Data retrieved 11/2017.

Garcia-Vazquez E., Moran P., Martinez J.L., Perez J., de Gaudemar B. & Beall E. 2001. Alternative mating strategies in Atlantic salmon and brown trout. *J. Hered.* 92: 146–149.

George S.G., Hodgson P.A., Tytler P. & Todd K. 1996. Inducibility of Metallothionein mRNA Expression and Cadmium Tolerance in Larvae of a Marine Teleost, the Turbot (*Scophthalmus maximus*). *Fund. Appl. Toxicol.* 33: 91–99.

Gillet C. & Dubois J.P. 2007. Effect of water temperature and size of females on the timing of spawning of perch *Perca fluviatilis* L. in Lake Geneva from 1984 to 2003. *J. Fish. Biol.* 70: 1001–1014.

González-Doncel M., Larrea M., Sánchez-Fortún S. & Hinton D.E. 2003. Influence of water hardening of the chorion on cadmium accumulation in medaka (*Oryzias latipes*) eggs. *Chemosphere* 52: 75–83.

Gustavsson N., Loukola-Ruskeeniemi K. & Tenhola M., 2012. Evaluation of geochemical background levels around sulfide mines – A new statistical procedure with beanplots. *Appl. Geochem.* 27: 240–249.

Haakana H. & Huuskonen H. 2008. The endangered whitefish (*Coregonus lavaretus pallasi*) population in the Koitajoki River, eastern Finland: the present state and threats. *Advanc. Limnol.* 63: 519–533.

Hansen B.H., Rømma S., Garmo Ø.A., Pedersen S.A., Olsvik P.A. & Andersen R.A. 2007. Induction and activity of oxidative stress-related proteins during waterborne Cd/Zn-exposure in brown trout (*Salmo trutta*). *Chemosphere* 67: 2241–2249.

Hogsden K.L. & Harding J.S. 2012. Anthropogenic and natural sources of acidity and metals and their influence on the structure of stream food webs. *Environ. Pollut.* 162: 466–474.

Hutchinson T.H., Solbé J. & Kloepper-Sams P.J. 1998. Analysis of the ECETOX aquatic toxicity (EAT) database III – comparative toxicity of chemical substances to different life stages of aquatic organisms. *Chemosphere* 36: 129–142.

Iles A.C. & Rasmussen J.B. 2005. Indirect effects of metal contamination on energetics of yellow perch (*Perca flavescens*) resulting from food web simplification. *Freshwat. Biol.* 50: 976–992.

Jezierska B., Ługowska K. & Witeska M. 2009. The effects of heavy metals on embryonic development of fish (a review). *Fish. Physiol. Biochem.* 35: 625-640.

Johnson D.B. 2013. Development and application of biotechnologies in the metal mining industry. *Environ. Sci. Pollut. Res.* 20: 7768–7776.

Jonsson B. 1985. Life history patterns of freshwater resident and sea-run migrant brown trout in Norway. *Trans. Am. Fish. Soc.* 114: 182–194.

Jonsson B. 1989. Life history and habitat use of Norwegian brown trout (*Salmo trutta*). *Freshwat. Biol.* 21: 71–86.

Jullien N. 2008. AmplifX 1.5.4. CNRS, Aix-Marseille Université, Aix-Marseille, France. Available from: http://crn2m.univ-mrs.fr/pub/amplifx-dist. Cited 3/2014.

Kallio-Nyberg I. & Koljonen M-L. 1988. A stock registry for Finnish whitefish. *Finnish Fisheries Research* 9: 49–60.

Kamler E. 2002. Ontogeny of yolk-feeding fish: an ecological perspective. *Rev. Fish. Biol. Fisher.* 12: 79–103.

Karjalainen J., Keskinen T., Pulkkanen M. & Marjomäki T.J. 2015. Climate change alters the egg development dynamics in cold-water adapted coregonids. *Environ. Biol. Fish.* 98: 979–991.

Kauppi S., Mannio J., Hellsten S., Nystén T., Jouttijärvi T., Huttunen M., Ekholm P., Tuominen S., Porvari P., Karjalainen A., Sara-Aho T., Saukkoriipi J. & Maunula M. 2013. Assessment of the potential impacts on water environment caused by the gypsum pond leakage at the Talvivaara mine. *Reports of the Finnish Environment Institute* 11/2013. 90 p. (in Finnish, English abstract).

Keinänen M., Tigerstedt C., Kålax P. & Vuorinen P.J., 2003. Fertilization and embryonic development of whitefish (*Coregonus lavaretus lavaretus*) in

acidic low-ionic-strength water with aluminum. *Ecotox. Environ. Safe.* 55: 314–329.

Keinänen M., Tigerstedt C., Peuranen S. & Vuorinen P.J. 2004. The susceptibility of early developmental phases of an acid-tolerant and acid-sensitive fish species to acidity and aluminum. *Ecotox. Environ. Safe.* 58: 160–172.

Klemetsen A., Amundsen P.-A., Dempson J.B., Jonsson B., Jonsson N., O'Connell M.F. & Mortensen E. 2003. Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpinus* (L.): a review of aspects of their life histories. *Ecol. Freshw. Fish* 12: 1–59.

Klerks P.L., Xie L. & Levinton J.S. 2011. Quantitative genetics approaches to study evolutionary processesin ecotoxicology; a perspective from research on the evolution of resistance. *Ecotoxicology* 20: 513–523.

Koressaar T. & Remm M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23: 1289–1291.

Lahnsteiner F., Mansour N. & Berger B. 2004. The effect of inorganic and organic pollutants on sperm motility of some freshwater teleosts. *J. Fish. Biol.* 65: 1283–1297.

Le Cren E.D. 1951. The length-weight relationship and seasonal cycle in gonad weight and condition in the perch (*Perca fluviatilis*). *J. Anim. Ecol.* 20: 201–219.

Leppänen J.J., Weckström J. & Atte Korhola. 2017. Multiple mining impacts induce widespread changes in ecosystem dynamics in a boreal lake. *Nature Scientific Reports* 7: 1–11.

Levesque H.M., Moon T.W., Campbell P.G.C. & Hontela A. 2002. Seasonal variation in carbohydrate and lipid metabolism of yellow perch (*Perca flavescens*) chronically exposed to metals in the field. *Aquat. Toxicol.* 60: 257–267.

Lewis M. 1978. Acute toxicity of copper, zinc and manganese in single and mixed salt solutions to juvenile longfin dace *Agosia chrysogaster*. *J. Fish Biol.* 13: 695–700.

Lin H.C., Hsu S.C. & Hwang P.P. 2000. Maternal transfer of cadmium tolerance in larval Oreochromis mossambicus. *J. Fish Biol.* 57: 239–249.

Louhi P., Mäki-Petäys A. & Erkinaro J. 2008. Spawning habitat of Atlantic salmon and brown trout: general criteria and intragavel factors. *River. Res. Applic.* 24: 330–339.

Loukola-Ruskeeniemi K., Uutela A., Tenhola M. & Paukola T. 1998. Environmental impact of metalliferous black shales at Talvivaara in Finland, with indication of lake acidification 9000 years ago. *J. Geochem. Explor.* 64: 395–407.

MacCrimmon H.R., Marshall T.L. & Gots B.L. 1970. World distribution of brown trout, *Salmo trutta*: further observations. *J. Fish. Res. Bd. Canada* 27: 811–818.

Meinelt T., Playle R.C., Pietrock M., Burnison B.K., Wienke A. & Steinberg C.E.W. 2001. Interaction of cadmium toxicity in embryos and larvae of zebrafish (*Danio rerio*) with calcium and humic substances. *Aquat. Toxicol.* 54: 205–215.

Michibata H. 1981. Uptake and distribution of cadmium in the egg of the teleost, *Oryzias latipes*. *J. Fish Biol.* 19: 691–696.

Moiseenko T.I. & Kudryavtseva L.P. 2001. Trace metal accumulation and fish pathologies in areas affected by mining and metallurgical enterprises in the Kola Region, Russia. *Environ. Pollut.* 114: 285–297.

Næsje T.F., Vuorinen A. & Sandlund O.T. 2004. Genetic and morphometric differentiation among sympatric spawning stocks of whitefish (*Coregonus lavaretus* L.) in Lake Femun, Norway. *J. Limnol.* 63: 233–243.

National Land Survey of Finland 2016. General and Topographic maps of Finland, File service of open data, National Land Survey of Finland. Data retrieved in 11/2016.

Neff B.D. & Pitcher T.E. 2005. Genetic quality and sexual selection: an integrated framework for good genes and compatible genes (a review). *Mol. Ecol.* 14: 19–38.

Olías M., Nieto J.M., Sarmiento A.M., Cerón J.C. & Cánovas C.R. 2004. Seasonal water quality variations in a river affected by acid mine drainage: the Odiel River (South West Spain). *Sci. Total Environ.* 333: 267–281.

Olsson P.-E. & Haux C. 1986. Increased hepatic metallothionein content correlates to cadmium accumulation in environmentally exposed perch (*Perca fluviatilis*). *Aquat. Toxicol.* 9: 231–242.

Papakostas S., Vasemägi A., Vähä J.-P., Himberg M., Peil L. & Primmer G.R. 2012. A proteomic approach reveals divergent molecular responses to salinity in populations of European whitefish (*Coregonus lavaretus*). *Mol. Ecol.* 21: 3516–3530.

Peterson R.H. 1984. Influence of varying pH and some inorganic cations on the perivitelline potential of eggs of Atlantic salmon (*Salmo salar*). *Can. J. Fish. Aquat. Sci.* 41: 1066–1069.

Peterson R.H. & Martin-Robichaud D.J. 1986. Perivitelline and vitelline potentials in teleost eggs as influenced by ambient ionic strength, natal salinity, and electrode electrolyte; and the influence of these potentials on cadmium dynamics within the egg. *Can. J. Fish. Aquat. Sci.* 43: 1445–1450.

Peterson H.G. Healey F.P. & Wagemann R. 1984. Metal toxicity to algae: a highly pH dependent phenomenon. *Can. J. Fish. Aquat. Sci.* 41: 974–979.

Pierron F., Bourret V., St-Cyr J., Campbell P.G.C., Bernatchez L. & Couture P. 2009. Transcriptional responses to environmental metal exposure in wild yellow perch (*Perca flavescens*) collected in lakes with differing environmental metal concentrations (Cd, Cu, Ni). *Ecotoxicology* 18:620–631.

Pyle G.G., Swanson S.M. & Lehmkuhl D.M. 2002. The influence of water hardness, pH, and suspended solids on nickel toxicity to larval fathead minnows (*Pimephales promelas*). *Water Air Soil Poll.* 133: 215–226.

Pyle G.G., Rajotte J.W. & Couture P. 2005. Effects of industrial metals on wild fish populations along a metal contamination gradient. *Ecotox. Environ. Safe.* 61: 287–312.

Quintero-Moreno, A., Miro J., Teresa Rigau A. & Rodriguez-Gil J.E. 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. *Theriogenology* 59:1973–1990.

Rajotte J.W. & Couture P. 2002. Effects of environmental metal contamination on the condition, swimming performance, and tissue metabolic capacities of wild yellow perch (*Perca flavescens*). *Can. J. Fish. Aquat. Sci.* 59: 1296–1304.

Rask M. 1986. The diet and diel feeding activity of perch, Perca fluviatilis L., in a small lake in southern Finland. *Ann. Zool. Fennici* 23: 49–56.

Rawlings D.E. 2002. Heavy metal mining using microbes. *Annu. Rev. Microbiol.* 56: 65–91.

Rawlings D.E. & Silver S. 1995. Mining with Microbes (Review). *Biotechnology* 13: 773–778.

Rawlings D.E., Dew D. & du Plessis C. 2003. Biomineralization of metal-containing ores and concentrates (Review). *Trends Biotechnol.* 21: 38–44.

Reichl C., Schatz M. & Zsak G. 2017. World Mining Data 2017. *Minerals Production*. 32: 1–255. Vienna (2017).

Riekkola-Vanhanen M. 2013. Talvivaara mining company – From a project to a mine. *Miner. Eng.* 48: 2–9.

Rudy P.P. Jr & Potts W.T.W. 1969. Sodium balance in the eggs of the Atlantic salmon, *Salmo salar*. *J. Exp. Biol.* 50: 239–246.

Säisä M., Rönn J., Aho T., Björklund M., Pasanen P. & Koljonen M-L. 2008. Genetic differentiation among European whitefish ecotypes based on microsatellite data. *Hereditas* 145: 69–83.

Salmelin J., Leppänen M.T., Karjalainen A.K., Vuori K.-M., Gerhardt A. & Hämäläinen H. 2017. Assessing ecotoxicity of biomining effluents in stream ecosystems by in situ invertebrate bioassays: a case study in Talvivaara, Finland. *Environ. Toxicol. Chem.* 36: 147–155.

Sandlund O.T. & Næsje T.F. 1989. Impact of a pelagic gill-net fishery on the polymorphic whitefish (*Coregonus lavaretus* L. s.l.) population in Lake Femund, Norway. *Fish. Res.* 7: 85–97.

Sayer M.D.J., Reader J.P. & Morris R. 1991. Embryonic and larval development of brown trout, *Salmo trutta* L.: exposure to aluminium, copper, lead or zinc in soft, acid water. *J. Fish Biol.* 38: 431–455.

Sellin M.K. & Kolok A.S. 2006. Maternally derived Cu tolerance in larval fathead minnows: how long does it persist? *J. Fish. Biol.* 69: 1570–1574.

Sherwood G.D., Rasmussen J.B., Rowan D.J., Brodeur J. & Hontela A. 2000. Bioenergetic costs of heavy metal exposure in yellow perch (*Perca flavescens*): in situ estimates with a radiotracer (^{137}Cs) technique. *Can. J. Fish. Aquat. Sci.* 57: 441–450.

Sherwood G.D., Kovecses J., Hontela A and Rasmussen, J.B. 2002. Simplified food webs lead to energetic bottlenecks in polluted lakes. *Can. J. Fish. Aquat. Sci.* 59: 1–5.

Shirvell C.S. & Dungey R.G. 1983. Microhabitats chosen by brown trout for feeding and spawning in rivers. *Trans. Am. Fish. Soc.* 112: 355–367.

Snickars M., Sundblad G., Sandström A., Ljunggren L., Bergström U., Johansson G. & Mattila J. 2010. Habitat selectivity of substrate-spawning fish: modelling requirements for the Eurasian perch *Perca fluviatilis*. *Mar. Ecol. Prog. Ser.* 398: 235–243.

Soucek D.J., Cherry D.S., Currie R.J., Latimer H.A. & Trent G.C. 2000. Laboratory to field validation in an integrative assessment of an acid mine drainage-impacted watershed. *Environ. Toxicol. Chem.* 19: 1036–1043.

Stouthart A.J.H.X., Spanings F.A.T., Lock R.A.C. & Wendelaar Bonga S.E. 1995. Effects of water pH on chromium toxicity to early lifestages of the common carp (*Cyprinus carpio*). *Aquat. Toxicol.* 32: 31–42.

Stubblefield W.A., Brinkman S.F., Davies P.H., Garrison T.D., Hockett J.R. & McIntyre M.W. 1997. Effects of water hardness on the toxicity of manganese to developing brown trout (*Salmo trutta*). *Environ. Toxicol. Chem.* 16: 2082–2089.

Svärdson G. 1979. Speciation of Scandinavian Coregonus. *Stockholm. Rep. Inst. Freshw. Res. Drottningholm* 57: 1–95.

Syrjänen J., Kiljunen M., Karjalainen J., Eloranta A. & Muotka T. 2008. Survival and growth of brown trout *Salmo trutta* L. embryos and the timing of hatching and emergence in two boreal lake outlet streams. *J. Fish Biol.* 72: 985−1000.

Thorpe J.E. 1977a. Morphology, physiology, bahviour and ecology of *Perca fluviatilis* L. and *Perca flavescens* Mitchill. *J. Fish. Res. Bd. Canada* 34: 1504–1514.

Thorpe J.E. 1977b. Synopsis of biological data on the perch, *Perca fluviatilis* Linnaeus, 1758 and *Perca flavescens* Mitchill, 1804. *FAO Fisheries Synopsis* 113.

Treasurer J.W. 1981. Some aspects of the reproductive biology of perch, *Perca fluviatilis* L. Fecundity, maturation and spawning behavior. *J. Fish Biol.* 18: 729–740.

Treasurer J.W. 1983. Estimates of egg and viable embryo production in a lacustrine perch, *Perca fluviatilis*. *Environ. Biol. Fish.* 8: 3–16.

Untergasser A., Cutcutache I., Koressaar T., Ye J., Faircloth B.C., Remm M. & Rozen S.G. 2012. Primer3—new capabilities and interfaces. *Nucleic Acids Res.* 40:15: e115 1–12.

Viljanen M. & Holopainen I.J. 1982. Population density of perch (*Perca fluviatilis* L.) at egg, larval and adult stages in the dys-oligotrofic Lake Suomunjärvi, Finland. *Ann. Zool. Fennici* 19: 39–46.

von Siebenthal B.A., Jacob A. & Wedekind C. 2009. Tolerance of whitefish embryos to *Pseudomonas fluorescens* linked to genetic and maternal effects, and reduced by previous exposure. *Fish Shellfish Immun.* 26: 531–535.

Vuorinen P.J., Keinänen M., Peuranen S. & Tigerstedt C. 1998. Effects of iron, aluminium, dissolved humic material and acidity on grayling (*Thymallus thymallus*) in laboratory exposures, and a comparison of sensitivity with brown trout (*Salmo trutta*). *Boreal Environ. Res.* 3: 405–419.

Wang W. 1987. Factors affecting metal toxicity to (and accumulation by) aquatic organisms - overview (a review). *Environ. Int.* 13: 437–457.

Wang N., Dorman R.A., Ingersoll C.G., Hardesty D.K., Brumbaugh W.G., Hammer E.J., Bauer C.R. & Mount D.R. 2016. Acute and chronic toxicity of sodium sulfate to four freshwater organisms in water-only exposures. *Environ. Toxicol. Chem.* 35: 115–127.

Watling H.R. 2006. The bioleaching of sulphide minerals with emphasis on copper sulphides — A review. *Hydrometallurgy* 84: 81–108.

Watling H.R. 2008. The bioleaching of nickel-copper sulfides. *Hydrometallurgy* 91: 70–88.

Wedekind C., Müller R. & Spicher H. 2001. Potential genetic benefits of mate selection in whitefish. *J. Evol. Biol.* 14: 980–986.

Weis J.S., Weis P., Heber M. & Vaidya S. 1981. Methylmercury tolerance of killifish (*Fundulus heteroclitus*) embryos from a polluted vs non-polluted environment. *Mar. biol*. 65: 283-287.

Witzel L.D. & MacCrimmon H.R. 1983. Redd-site selection by brook trout and brown trout in southwestern Ontario streams. *Trans. Am. Fish. Soc.* 112: 760–771.

Ye J., Coulouris G., Zaretskaya I., Cutcutache I., Rozen S. & Madden T. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134: 1–11.

ORIGINAL PAPERS

I

TOLERANCE OF WHITEFISH (*COREGONUS LAVARETUS*) EARLY LIFE STAGES TO MANGANESE SULFATE IS AFFECTED BY THE PARENTS

by

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TOLERANCE OF WHITEFISH (*COREGONUS LAVARETUS*) EARLY LIFE STAGES TO MANGANESE SULFATE IS AFFECTED BY THE PARENTS

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Abstract: European whitefish (*Coregonus lavaretus*) embryos and larvae were exposed to 6 different manganese sulfate ($MnSO_4$) concentrations from fertilization to the 3-d-old larvae. The fertilization success, offspring survival, larval growth, yolk consumption, embryonic and larval Mn tissue concentrations, and transcript levels of detoxification-related genes were measured in the long-term incubation. A full factorial breeding design (4 females × 2 males) allowed examination of the significance of both female and male effects, as well as female–male interactions in conjunction with the $MnSO_4$ exposure in terms of the observed endpoints. The $MnSO_4$ exposure reduced the survival of the whitefish early life stages. The offspring $MnSO_4$ tolerance also was affected by the female parent, and the female-specific mean lethal concentrations (LC50s) varied from 42.0 mg $MnSO_4$/L to 84.6 mg $MnSO_4$/L. The larval yolk consumption seemed slightly inhibited at the exposure concentration of 41.8 mg $MnSO_4$/L. The $MnSO_4$ exposure caused a significant induction of metallothionein-A (*mt-a*) and metallothionein-B (*mt-b*) in the 3-d-old larvae, and at the exposure concentration of 41.8 mg $MnSO_4$/L the mean larval *mt-a* and *mt-b* expressions were 47.5% and 56.6% higher, respectively, than at the control treatment. These results illustrate that whitefish reproduction can be impaired in waterbodies that receive Mn and SO_4 in concentrations substantially above the typical levels in boreal freshwaters, but the offspring tolerance can be significantly affected by the parents and in particular the female parent. *Environ Toxicol Chem* 2017;36:1343–1353. © 2016 SETAC

Keywords: Aquatic toxicology Embryonic development of fish Metal toxicity Mine effluents Parental effect

INTRODUCTION

Manganese (Mn) and sulfate (SO_4) occur naturally in the aquatic environment [1,2]. Median Mn and SO_4 concentrations in Nordic surface waters range from 3.2 μg/L to 65 μg/L and 1.3 mg/L to 3.8 mg/L, respectively [3–5]. Although Mn and sulfur (S) are essential nutrients [1,2], excessive concentrations of Mn and SO_4 can be toxic to aquatic organisms [6,7]. Mining and mineral processing are 2 of the major anthropogenic sources of Mn [1]. Similarly, SO_4 is often a prevalent contaminant in mine water, and it can contribute substantially to salinization of the waterbodies receiving the mine waters [8]. The metal mining industry has adopted and developed biomining processes, in which microorganisms are utilized in metal recovery, and biomining is considered to have economic and environmental advantages compared with conventional recovery methods [9]. In Europe, the first commercial application of biomining utilizing bioheap leaching technology was established in 2008 in northeastern Finland [10]. Since the mine started to operate, concentrations of Mn and other metals, as well as SO_4, in the waterbodies receiving the mine effluents have been elevated, and an accidental gypsum pond leakage at the mine in late 2012 caused deterioration of nearby water quality [5,11–13]. This has raised concern about the effects of Mn and SO_4 especially on the commercially important boreal freshwater fish.

The early life stages of fish, larvae in particular, are generally more sensitive to chemical toxicants than adults [14]. Offspring stress tolerance can depend on their genetic background and especially on the female parent [15,16]. Metal exposure during early development is known to disturb developmental processes, reduce hatching rate and larval body size, and cause both embryonic and larval malformation and mortality [17]. Compared with other metals, such as cadmium (Cd), copper (Cu), and zinc (Zn), the toxicity of Mn to aquatic organisms is suggested to be low [6,18]. The 25% inhibition concentration (IC25) of Mn on survival and growth of brown trout (*Salmo trutta*) early life stages has been reported to be 4.67 mg Mn/L to 8.68 mg Mn/L, Mn being more toxic in soft water than in hard water [6]. In soft water, Mn concentrations of 0.32 mg/L to 0.35 mg/L have disturbed the mineral uptake and skeletal calcification of brown trout larvae [19]. For SO_4, previously reported IC25 values affecting embryo development of coho salmon (*Oncorhynchus kisutch*), embryo-to-alevin development of rainbow trout (*Oncorhynchus mykiss*), and larval mortality of fathead minnow (*Pimephales promelas*) were 1264 mg/L, 501 mg/L, and 933 mg/L, respectively [7]. Mixture toxicity studies on aquatic organisms, such as salmonid embryos, tropical duckweed (*Lemna aequinoctialis*), green hydra (*Hydra viridissima*), and pulmonate snail (*Amerianna cumingi*), focusing on both SO_4 and a cationic metal, such as calcium (Ca^{2+}) or magnesium (Mg^{2+}), suggest that the cation is the toxic cause rather than the SO_4 [20,21].

External stressors can also activate defense mechanisms in aquatic organisms, and oxidative stress is often associated with a strong stress [22]. Metals and salinity changes are known to modulate oxidative stress responses in fish [23,24], and the main antioxidant enzymes protecting organisms from oxidative damage are catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and superoxide dismutase (SOD) [25,26]. Metal-binding proteins, metallothioneins (MTs) [27], have been considered as suitable biomarkers for

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metal exposure [25]. Although the exact role of MTs is still unclear [27], they are known to regulate the availability of essential and nonessential metals [28]. Also, the induction of MT gene transcription can correlate with metal tolerance, as observed with Cd-exposed turbot (*Scophthalmus maximus*) larvae [29].

The present study was designed to specifically assess the critical mixture concentration of Mn and SO_4 that reduces survival of a native boreal fish species, European whitefish (*Coregonus lavaretus*) embryos and larvae and disturbs their yolk utilization for growth. We conducted a continuous laboratory-scale manganese sulfate ($MnSO_4$) exposure with whitefish embryos and hatched larvae to investigate the effect of the parental combination on the sensitivity of whitefish early life stages to $MnSO_4$; the Mn body residues of the whitefish eggs and the 3-d-old larvae; and the transcript abundance of *cat, gstt*, *mt-a*, and *mt-b* in the embryos and larvae under the $MnSO_4$ exposure. These results bring new information for assessing the effects of SO_4-induced salting and Mn on the reproduction of European whitefish stock, and they allow comparison of the species sensitivity with the effects of salting and Mn in freshwaters worldwide.

MATERIALS AND METHODS

Test species and chemicals

Newly stripped whitefish eggs of 4 females and milt of 2 males (Rautalampi stock from the Finnish Game and Fisheries Research Institute, Laukaa, Finland) were transported from the hatchery to the laboratory for fertilization. The eggs were kept in plastic boxes and on ice until fertilization. Milt was transported (40 min) in oxygen-filled Minigrip plastic bags and kept on ice. Before fertilization, the milt was pipetted into microcentrifuge tubes and placed into a cool block (Echtotherm Chilling/Heating dry bath, Torrey Pines Scientific) at 5 °C.

Manganese sulfate monohydrate, $MnSO_4 \cdot H_2O$ (Emsure, ACS, Reag. PhEur; Merck; purity 98.8%), was weighed into 4 $MnSO_4 \cdot H_2O$ stock solutions of 6.4 mg/L, 160 mg/L, 4000 mg/L, and 100 000 mg/L into ultrapure water (Ultra Clear UV UF TM; Evoqua), and the solutions were stored at 4 °C in the dark prior to use. New stock solutions were made 2 times (6.4–4000 mg $MnSO_4 \cdot H_2O$/L) to 3 times (100 000 mg $MnSO_4 \cdot H_2O$/L) during the course of the experiment. Suprapur HNO_3 (65%, Merck) was used in the water sample acidification, and acid washes were done with analytical grade HNO_3 (Merck). The reagents (HNO_3 and HCl) used in tissue sample digestion were of analytical grade (Sigma-Aldrich), and only high-purity water of 18.2 MΩ cm resistivity produced by a PURELAB Ultra water purification system supplied by Elga was used throughout with the tissue samples.

Test setup and fertilization

Prefiltered (1 μm; 155383-03, model BP-410-1; Pentek) Lake Konnevesi (Konnevesi, Central Finland) water was used as the control water and spiked with $MnSO_4$ to 6 nominal $MnSO_4$ exposure concentrations of 0.06 mg/L, 0.29 mg/L, 7.2 mg/L, 35.7 mg/L, 179 mg/L, and 893 mg/L, respectively (element-specific concentrations are presented in Table 1). The continuous laboratory-scale $MnSO_4$ exposures were started from fertilization and ended 3 d after the hatching of the larvae. The experiment lasted for 160 d, starting on 7 November 2013 and ending on 16 April 2014, when nearly all the embryos had either died or hatched (with 2 alive but unhatched embryos in the concentration of 41.8 mg $MnSO_4$/L). During the winter period (experiment days 1–108), the water temperature development followed natural Lake Konnevesi water temperature. The water temperature elevation was started on experiment day 109, resulting in an approximately 0.2 °C daily mean water temperature increase. The mean, minimum, and maximum water temperatures of all the pools during the whole experiment period were 3.6 °C, 1.0 °C, and 10.3 °C, respectively. The light rhythm followed the local natural light rhythm (Konnevesi, Central Finland), resulting in approximately 7:17-h, 5:19-h, 6:18-h, 9:15-h, 12:12-h, and 14:10-h mean monthly light:dark cycles during the experiment time. The embryos were sampled at the end of the winter period to represent the embryonic development during winter period, and, correspondingly, the 3-d-old larvae represent development during the spring period.

A full-factorial breeding design was applied by fertilizing the eggs of each female (F1, F2, F3, and F4) with the milt of both males (M1 and M2) separately to produce all 8 different female–male combinations in 3 replicates for each $MnSO_4$ exposure concentration and the control (Figure 1). The sperm motility of the males was inspected with an Integrated Semen Analysis System (ISASv1 Casa; Proiser) before fertilization. Approximately 50 eggs to 200 eggs per replicate were fertilized on plastic Petri dishes with 5 μL to 10 μL of milt using the corresponding exposure or control water as the sperm activation water. The activation water temperature was 5 °C. A few minutes after fertilization, each replicate egg batch was placed

Table 1. The nominal and the mean measured $MnSO_4$, Mn, and SO_4 concentrations (mg/L) of the whole experiment period[a]

Nominal concentration added to control			Measured concentrations[a]		
$MnSO_4$	Mn	SO_4	$MnSO_4$	Mn	SO_4[b]
Control	Control	Control	5.5 ± 0.1 47/55	1.5[c] ± 0.3[c] 47/55	5.5 ± 0.1 55/55
5.7[c]	2.1[c]	3.6[c]	5.6 ± 0.1 46/46 (102.2 ± 0.9) (41)	1.0[c] ± 0.2[c] 46/46 (27.9 ± 4.1) (41)	5.6 ± 0.1 46/46 (102.1 ± 0.8) (46)
28.6[c]	10.4[c]	18.2[c]	5.9 ± 0.1 50/50 (102.0 ± 0.8) (42)	4.5[c] ± 0.6[c] 50/50 (35.1 ± 4.9) (42)	5.8 ± 0.1 50/50 (102.9 ± 0.8) (47)
7.2	2.6	4.6	12.8 ± 0.1 44/44 (102.5 ± 1.7) (36)	2.1 ± 0.1 44/44 (81.6 ± 5.0) (36)	10.7 ± 0.1 44/44 (107.4 ± 1.5) (41)
35.7	13.0	22.7	41.8 ± 0.6 48/48 (100.4 ± 1.8) (38)	12.5 ± 0.3 48/48 (96.2 ± 2.9) (38)	29.3 ± 0.5 48/48 (102.7 ± 2.0) (43)
178.7	65.0	113.7	197.8 ± 1.4 56/56 (106.2 ± 0.7) (44)	70.2 ± 0.4 56/56 (107.6 ± 0.6) (44)	127.6 ± 1.2 56/56 (105.9 ± 0.9) (51)
893.4	325.0	568.4	965.0 ± 5.9 49/49 (107.7 ± 0.7) (42)	341.6 ± 1.6 49/49 (105.1 ± 0.5) (42)	623.4 ± 5.5 49/49 (108.6 ± 1.0) (49)

[a]The values shown for measured concentrations are mean ± standard error and $n >$ limit of quantification and relative standard deviation $< 10\%/n$ total; the mean proportion (% ± SE and no.) of the measured $MnSO_4$, Mn, and SO_4 exposure concentrations compared with the nominal concentrations including the background levels of the corresponding time are presented in parentheses.
[b]Calculated from S.
[c]Values are $\times 10^{-2}$.

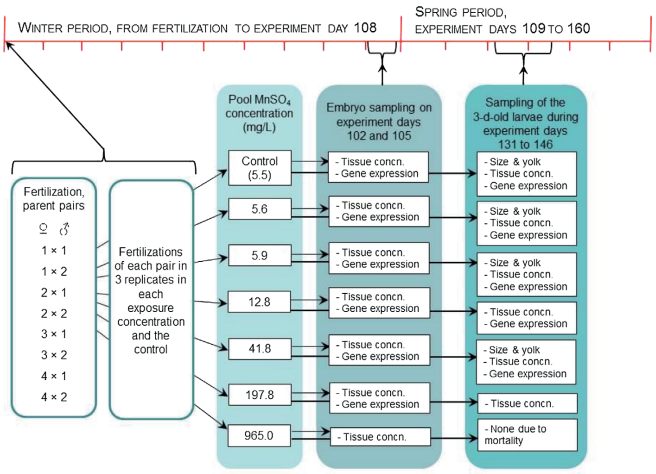

Figure 1. A schematic illustration of the experiment setup with a timeline and procedures. The exposures were started at fertilization and continued until all larvae were hatched. Fertilization success was estimated 4 d to 5 d after the fertilization from 10 eggs per replicate from each exposure concentration and the control. In the spring period, water temperature was elevated gradually and hatching started at experiment day 119. On average, 50% of all the embryos had hatched on experiment day 136 and larval samples were collected after 3-d incubation of the larvae. In the highest exposure concentration, none of the embryos hatched. See Supplemental Data, Table S4 for more detailed information about the number of samples of each sample type in each concentration and parent pair.

into a plastic box (350 mL, polypropylene; Greiner) containing 100 mL of the corresponding exposure or control water and taken immediately into the experiment room at 5 °C temperature. Eggs that were left over from the fertilization were stored for size analysis at –20 °C. Fertilization success was estimated under a light microscope from 10 eggs/replicate 4 d to 5 d after fertilization; after investigation, those eggs were not returned for further incubation.

The egg batches were moved into plastic (LE-marked, Robusto; OKT) incubation pools (inside size: 565 mm × 365 mm × 220 mm) containing 12.5 L of the corresponding test water 6 d after fertilization. There was 1 pool for each $MnSO_4$ exposure and 1 control pool; the eggs were placed into compartment grids, 1 replicate egg batch per 1 randomly selected compartment. The compartment grids (350 mm × 350 mm × 70 mm) containing 36 compartments (50 mm × 50 mm × 40 mm) were made of plexiglass, with a 750-μm mesh (PETP, 07-750/53; Sefar Petex) glued (Acrifix 192) to the bottom of the compartments. Water depth both underneath and above the eggs was 30 mm (total water depth 60 mm), allowing sufficient water circulation for the eggs. Before the onset of hatching, the compartments were divided into 4 sections with thin plexiglass slides allowing 3-d incubation of the larvae with 24-h accuracy of the individual hatching time. The grids and plexiglass slides were acid washed (10% HNO_3), and the grids and incubation pools were soaked in the corresponding exposure or control water before the eggs were placed into them. Pool waters were aerated with glass Pasteur pipets (10% HNO_3 acid washed) from 2 opposite sides of the pool into opposite directions to enhance adequate water circulation. Pools were protected from contamination with loosely placed clear plastic film covers on top, and polystyrene covers were placed on the pools for 3 mo in early December to mimic the ice cover typical of boreal regions at that time of the year.

Quality control of the exposure

The water renewal intervals were 3 d to 4 d, and 4 L from each pool was changed at a time. At every water renewal time, both new exposure and control waters for the next water renewal were prepared and left to aerate and stabilize to the incubation temperature.

Incubation water temperature, pH (744 pH meter, Metrohm, Professional Plus, YSI; and SevenGo pH meter SG2, Mettler Toledo), conductivity (Professional Plus, YSI), and oxygen concentration (ProOdo, YSI) were monitored at both the beginning and the end of the experiment and before and after water renewals. Mean water oxygen concentration (± standard error [SE]) of all the pools during the experiment was 12.6 ± 0.1 mg/L (min 11.0 mg/L and max 15.2 mg/L), and pH was 6.66 ± 0.01 (min 5.46 and max 7.49) (Supplemental Data, Table S1). Degree days (cumulative sum of mean daily temperatures during the whole incubation period) were calculated for each pool with linear interpolation using the water temperature before every water renewal. Dissolved organic carbon of prefiltered Lake Konnevesi water (i.e., newly made control water) was determined at the beginning of the experiment and during embryo and larval sampling (TOC-L, Total Organic Carbon Analyzer; Shimadzu), and the result was a mean dissolved organic carbon concentration of 7.7 ± 0.2 mg/L. Ammonium concentrations of the pool waters were analyzed in an accredited laboratory (FINAS T142; EN ISO/IEC 17025) according to a standard method [30] once after 2 mo of incubation. The mean ammonium concentrations (± SE) of all the pools before and after the water renewal were 361 ± 35.1 μg/L (min 260 μg/L and max 500 μg/L) and 253 ± 23.6 μg/L (min 180 μg/L and max 340 μg/L), respectively.

Manganese (Mn) and sulfur (S) concentrations in the incubation waters were monitored, and other common elements such as aluminum (Al), arsenic (As), Cd, calcium (Ca), chrome (Cr), cobalt (Co), Cu, iron (Fe), lead (Pb), magnesium (Mg),

nickel (Ni), phosphorus (P), potassium (K), sodium (Na), strontium (Sr), uranium (U), and Zn were analyzed as well (Supplemental Data, Table S2). Sulfate concentrations were estimated based on S concentrations, assuming that all the S was present as SO_4 in the well-aerated exposure and control waters. Filtered (50-mL sterile syringe, BD Plastipak, 25-mm GD/XP syringe filters, 0.45-μm polyvinylidene fluoride [PVDF] with polypropylene, Whatman) and unfiltered water samples from the newly made control and $MnSO_4$-spiked waters were collected twice at the beginning of the experiment, and unfiltered pool water samples were collected twice, 1 d and 3 d after the eggs were placed into the pools. Afterward, unfiltered pool water samples were collected just before and after every water renewal and at the end of the experiment. Water samples were collected into metal-free plastic tubes (polypropylene, 50 mL or 15 mL; VWR), acidified immediately after sampling by adding 6 (50-mL samples) or 2 (15-mL samples) drops of HNO_3, and stored at 4 °C in the dark until the analyses. The total numbers of analyzed samples are shown in Supplemental Data, Table S2.

The chemical element concentrations of the waters (Supplemental Data, Table S2) were analyzed with inductively coupled plasma–optical emission spectrometry (ICP–OES; Optima 8300, PerkinElmer); in case of low Mn concentrations, the Mn analysis was performed with electrothermal atomic absorption spectrometry (AAnalyst 800; PerkinElmer). Water concentration results above the limits of quantification (LOQs) with a relative standard deviation (RSD) below 10% were accepted. The LOQs were defined according to US Environmental Protection Agency method 200.7 [31].

The control (i.e., background) water mean total concentrations (± SE) of $MnSO_4$, Mn, and SO_4 for the whole experiment period were 5.5 (± 0.1) mg/L, 1.5×10^{-2} ($\pm 0.3 \times 10^{-2}$) mg/L, and 5.5 (± 0.1) mg/L, respectively (Table 1). The measured total $MnSO_4$, Mn, and SO_4 exposure concentrations varied between 100.4% and 107.7%, 27.9% and 107.6%, and 102.1% and 108.6% of the nominal concentrations that included the corresponding background levels (Table 1; Supplemental Data, Table S3). During the first month of the experiment, the Mn concentrations in 2 of the lowest exposure concentrations fell below the nominal levels and remained there until the end of the experiment. It is probable that Mn could have been oxidized in the well-aerated pool water and thus adsorbed onto the pool and compartment grid surfaces and/or onto the developing eggs. In this case, if both the Mn was oxidized and the amount of Mn adsorbed was uniform in each pool, the difference between the measured and nominal Mn concentrations would thus have been the most extreme in the low-concentration pools compared with the higher-concentration pools.

Most of the control water Mn was not in dissolved form (mean Mn dissolved 7.5%), whereas the $MnSO_4$ concentration increase in exposure waters gradually increased the proportion of dissolved Mn (range of the mean Mn dissolved 78−99%). Sulfur was in dissolved form in the $MnSO_4$ exposure (range of the S dissolved 99−100%) and control (mean S dissolved 99%) waters.

Mortality and overview of embryo and larval sampling

Dead embryos were counted and removed 3 times per week during the first month of the experiment and twice per week afterwards. Hatching started at 119 d after fertilization, and the mean hatching peak of all parent pairs was reached 136 d after fertilization. During the hatching period, hatched and dead larvae were counted daily.

Detailed information on the embryo and larval samples of every sample type is given in Supplemental Data, Table S4 according to $MnSO_4$ exposure concentration and parent pair. Both embryos and larvae were sampled for tissue element concentration and gene expression analyses, whereas only larvae were sampled for growth and yolk consumption analyses. Embryo samples were collected before the beginning of the water temperature elevation at the end of the winter period, on experiment days 102 (for tissue concentration analyses) and 105 (for gene expression analyses). The hatched larvae were incubated for 3 d under exposure conditions before larval sampling. For the 3-d-old larvae, samples were collected from experiment day 131 to experiment day 146.

Growth, yolk consumption, and egg size

One growth and yolk consumption sample from each replicate contained 1 to 10 larvae from the same hatching day and a maximum of 4 larvae per replicate was measured. The samples were collected into 1.5-mL microcentrifuge tubes (StarLab), excess water was removed, and approximately 1 mL of 10% neutralized formalin (1:9 v/v of 37% formalin and Na_2HPO_4 3.55 g/L, $NaH_2PO_4 \cdot H_2O$ 7.3 g/L dissolved in ultrapure H_2O) was added. The samples were stored at −20 °C in the dark until analysis. Thereafter, the samples were thawed on ice and rinsed with ultrapure water (Ultra Clear UV UF TM); then, yolk and carcass were separated and placed into preweighed aluminum cups. The samples were dried at 40 °C for 24 h and weighed. The initial egg size of each female was measured by analyzing the dry weight from 16 to 20 eggs per female. The initial egg size samples were stored at −20 °C in the dark without formalin fixation.

Tissue concentration analysis

The embryo tissue concentration samples were analyzed as whole eggs (no dechorionation), and thus those samples are referred to as eggs. A maximum of 4 eggs (∼48.4 mg wet wt resulting in 8.7 mg dry wt) from each replicate was collected into each tissue concentration sample. To get enough material for the analysis, the egg tissue concentration samples of the 3 replicates for each parent pair in each concentration were pooled (see Supplemental Data, Table S4). With the 3-d-old larvae, the larval samples were replenished until 10 3-d-old larvae/sample/replicate were obtained. The sample replicates of the larval tissue concentration were not pooled. The egg and larval tissue concentration samples were collected into 1.5-mL microcentrifuge tubes (StarLab), washed 3 times with filtered (50-mL sterile syringe, BD Plastipak, 25-mm GD/XP syringe filters, 0.45-μm PVDF with polypropylene, Whatman) Lake Konnevesi water, blotted dry, placed into preweighed 1.5-mL microcentrifuge tubes (StarLab), and stored at −20 °C in the dark prior to analysis. The sampled larvae were anesthesthetized with sodium hydrogen carbonate containing fruit salt (Samarin, Cederroth), and some of the larvae were photographed under a microscope (SteREO, Discovery V8, AxioCam ERc 5s, Zen lite 2011, Zeiss) for malformation investigation before washing and storing. In the course of photography, the larvae at 197.8 mg $MnSO_4$/L were observed to be opaque, but because no proper malformations were observed from any of the larvae at any exposure concentration, the malformation data were not analyzed further.

Freeze-dried and weighed egg and larval samples were digested in aqua regia (HNO_3:HCl, 1:3 v/v solution) by ultrasound (ELMA model Transonic 820/H [650 W, 35 kHz] or Bandelin Sonorex RK 512/H [400 W, 35 kHz]). Eggs were

digested in 1.5-mL microcentrifuge (StarLab) tubes by moisturizing the sample with ultrapure water and adding 8 drops of aqua regia, followed by sonication in 2-min to 3-min cycles at 30 °C to 40 °C. Larval samples were digested in 13-mL tubes (polypropylene, Sarstetd) by moisturizing the sample with ultrapure water and adding 10 drops of aqua regia, followed by sonication in 3-min cycles at 55 °C. The sonication was repeated 2 times to 12 times until the samples were fully digested. Samples were shaken, and sample tube pressure was released by opening the caps between each sonication cycle. The digested samples were filtered (Whatman no. 41, GE Healthcare Life Sciences) and filled to a final volume of 5 mL with ultrapure water.

The element concentrations of the eggs (Supplemental Data, Table S5) and the larvae (Supplemental Data, Table S6) were analyzed with ICP-OES (Optima 8300, PerkinElmer). The same LOQ and RSD limit requirements were used for both the water and the tissue concentrations, and the tissue concentration results are presented as upper bound concentrations (values below LOQ and/or RSD > 10% are replaced with LOQ), unless noted otherwise.

Gene expression

Gene expression samples were collected at the incubation temperature to avoid sudden temperature changes that may affect embryo and larval gene expressions. A maximum of 3 embryos per sample from each replicate was collected into 1.5-mL microcentrifuge tubes (StarLab), washed 3 times with filtered (50-mL sterile syringe BD Plastipak, 25-mm GD/XP syringe filters, 0.45-μm PVDF with polypropylene; Whatman) Lake Konnevesi water, blotted dry, and placed into 1.5-mL microcentrifuge tubes (StarLab). With the 3-d-old larvae, 1 sample from each replicate contained 5 larvae from the same hatching day. The larvae were sampled into 1.5-mL microcentrifuge tubes (StarLab), and the excess water was removed with a needle and a syringe. Both the embryonic and larval samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

The embryonic and larval expression of the target genes *mt-a*, *mt-b*, *gstt*, and *cat* was analyzed with quantitative reverse transcription−polymerase chain reaction (qRT−PCR) using ribosomal protein L2 (*rl2*) and *beta actin* as reference genes (Supplemental Data, Table S7). The selected reference genes had the most stable expression among treatments from all the reference genes tested. The *mt-a* gene was obtained from Hansen et al. [32]. Other target gene primers were designed with Primer3, Ver 4.0.0 [33,34], and the reference gene primers were designed with AmplifX Ver 1.5.4 [35]. The specificity of the genes was checked with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) [36]. The RNA extraction and integrity analysis, deoxyribonuclease treatment, complementary DNA synthesis, and amplification reactions were done as described in Vehniäinen and Kukkonen [37]. The qPCR run was done using CFX96 Real-Time PCR cycler (Bio-Rad), and the protocol was 95 °C for 3 min; 40 cycles of 95 °C (10 s), 58 °C (10 s), and 72 °C (30 s); 95 °C for 10 s; and melt curves from 65 °C to 95 °C with 0.5 °C intervals. Samples were run in duplicate using clear 96-well PCR plates (Bio-Rad). A no-template control was always run for each gene.

Mortality calculations and statistics

Fertilization loss (Z_F; Equation 1), instantaneous mortality of the winter period (Z_W; Equation 2) and spring period (Z_S; Equation 3), and instantaneous total mortality (Z_T; Equation 4), excluding the fertilization loss of the cross-fertilizations, were estimated according to the following equations

$$Z_F = -\ln(N_F/N_0) \quad (1)$$

$$Z_W = -\ln(N_W/N_F + 0.01) \quad (2)$$

$$Z_S = -\ln(N_L/N_S + 0.01) \quad (3)$$

$$Z_T = Z_W + Z_S \quad (4)$$

where N_0 is the number of live eggs before fertilization, N_F is the number of successfully fertilized eggs, N_W is the number of embryos that had survived by the end of the winter period just before embryo sampling, N_S is the number of embryos in the beginning of the spring period just after embryo sampling, N_L is number of live larvae 3 d after hatching, and 0.01 is a constant added to avoid 0 values when taking the natural logarithm (ln). Instantaneous mortality (Z) was assumed to be a normally distributed variable. A full-factorial general linear model (GLM), including the main effects of male, female, and $MnSO_4$ exposure and their interactions was used to test for the effect of $MnSO_4$ exposure on Z_F and Z_T.

The highest $MnSO_4$ concentration at which the female-specific total survival did not differ significantly from the control values (no-observed-effect concentration [NOEC]) were tested with analysis of variance (ANOVA) and, depending on the homogeneity of variances, the pairwise comparisons were done with a one-sided Dunnett's test or Dunnett's T3 test. The lethal $MnSO_4$ concentrations resulting in 50% mortality of the offspring (LC50) with 95% confidence limits were calculated for each female separately by pooling the data of both males per female. The female-specific LC50 values for $MnSO_4$ were calculated according to total offspring mortality and the mean measured total $MnSO_4$ concentrations of each pool during the whole experiment period using each female's mean total mortality in the control pools as natural response rates. Data analyses were made with the Probit model using an ln–transformed covariate. A heterogeneity factor was used in the confidence limit calculations because the significance level of the Pearson goodness-of-fit chi-square test was less than 0.15.

A full-factorial GLM, including the main effects of male, female, and $MnSO_4$ exposure, with their interactions and degree days as a covariate, was used to test for the effect of $MnSO_4$ exposure on growth and yolk consumption of the 3-d-old larvae. The mean carcass and yolk dry mass of the larvae in each replicate were used in the analyses.

A full-factorial GLM, including the main effects of female and $MnSO_4$ exposure and their interactions, was used to test for the effect of $MnSO_4$ exposure on the egg and larval Mn and S tissue concentrations. Because of mortality, the number of samples was small, and thus the male effect was not included in the data analysis. The concentrations below the LOQ or with RSD above 10% were replaced with upper bound concentrations.

The critical body residues of Mn taken up by the eggs and larvae causing 50% mortality of the observed individuals (CBR50) with 95% confidence limits were calculated separately for eggs and egg-to-larvae stages. Because of mortality, the number of samples was small, and thus the CBR50 values could not be distinguished according to the parent pairs or females. In all the egg samples, the Mn concentrations were above LOQ, with an RSD less than 10%, whereas for the larvae, an upper bound (concentrations below the LOQ or with RSD above 10%

replaced with LOQ) estimate for the CBR50 value was calculated. Data analyses were made with the Probit model using an ln-transformed covariate. A heterogeneity factor was used in the confidence limit calculations because the significance level of the Pearson goodness-of-fit chi-square test was less than 0.15. Because the egg tissue concentration replicates of each exposure concentration and control were pooled according to the parent pair, the corresponding mean winter embryonic mortality values were used in the egg Mn CBR50 analysis, and the mean mortality of the control samples was used as a natural response rate. The total mortality values were used in the egg-to-larval analysis, and thus the egg-to-larval CBR50 values represent the entire exposure period from fertilization until the larvae were 3 d old. The natural response rate in the egg-to-larvae analysis was the mean total mortality of the control samples.

Embryonic and larval gene expression differences of 3 and 2 parent pairs, respectively, were analyzed, and thus the female and male effect could not be tested. The pair selection was based on the offspring total mortality: a pair with low (F4 × M2), intermediate (F2 × M1), and high (F3 × M2) offspring total mortality. The degree days of the analyzed replicates were taken into account, and thus no effects of developmental differences were expected. The gene expression differences between the parent pairs and treatments were analyzed with log10-transformed normalized expressions using the GLM full-factorial effect model structure. In addition, to distinguish the differences in embryonic gene expression among the 3 parent pairs, the exposure concentration was excluded from the factors and only the parent pairs were compared with one-way ANOVA including Tukey's honest siginficant difference (HSD) or Tamhane's T2 post hoc tests depending on the homogeneity of the variances. Also, the differences in gene expression of the offspring of each parent pair separately were analyzed for both embryos and larvae with one-way ANOVA, including Tukey's HSD or Tamhane's T2 post hoc test depending on the homogeneity of the variances.

All statistical analyses were done with SPSS (IBM SPSS Statistics 22).

RESULTS

Fertilization, mortality, NOEC, and LC50 values

The Z_F of the whitefish eggs was significantly affected by both the $MnSO_4$ exposure concentration and female parent but male effect or interactions between the variables were not found (Table 2 and Figure 2A). Similarly, $MnSO_4$ exposure and female, but not male, parent had a significant effect on the Z_T (Figure 2B). The significant interaction between $MnSO_4$ exposure and female effects indicates that some of the females had produced more $MnSO_4$-tolerant embryos than others, and this was also evident from the female-specific NOEC and LC50 values (Table 3). Although there was a significant interaction between $MnSO_4$ exposure and male effects as well, the differences between the males were not as clear as those between the females at different concentrations.

Growth and yolk consumption

The carcass dry weight of the 3-d-old whitefish larvae was significantly affected by $MnSO_4$ exposure and female parent (Table 4). The male effect was not significant, but the interaction between exposure and male effects were. Degree days, $MnSO_4$ exposure, and female parent had a significant effect on the yolk dry weight of the 3-d-old larvae (Table 4). The interactions between female and $MnSO_4$ exposure effects, and between parent pair and $MnSO_4$ exposure, were also significant. On average, the larvae of each female had more yolk left at the 41.8 mg $MnSO_4$/L concentration compared with the control larvae (Figure 3).

Table 2. General linear model analysis results of the $MnSO_4$ exposure concentration, female and male significance in fertilization loss and instantaneous total mortality

Z	*df*	*F*	*P*
Z_F, Concn.	6	4.478	<0.001
Z_F, F	3	206.356	<0.001
Z_F, M	1	0.117	0.733
Z_F, F × Concn.	18	1.329	0.183
Z_F, M × Concn.	6	0.274	0.948
Z_F, F × M	3	2.253	0.086
Z_F, F × M × Concn.	18	0.998	0.467
Z_T, Concn.	6	358.180	<0.001
Z_T, F	3	9.182	<0.001
Z_T, M	1	1.341	0.249
Z_T, F × Concn.	18	5.419	<0.001
Z_T, M × Concn.	6	2.375	0.034
Z_T, F × M	3	1.183	0.319
Z_T, F × M × Concn.	18	1.665	0.056

Z_F = fertilization loss; Z_T = instantaneous total mortality; Concn. = $MnSO_4$ exposure concentration; F = female; M = male.

Mn and S concentrations in the eggs and the larvae

The Mn concentrations of the eggs were significantly affected by $MnSO_4$ exposure (GLM, $F = 207.137$, $df = 6$,

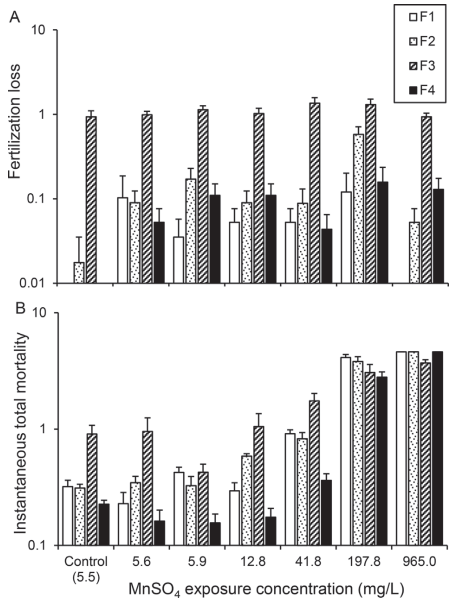

Figure 2. (**A**) Mean instantaneous fertilization loss (± standard error [SE], $n = 6$ per female and concentration) and (**B**) mean instantaneous total mortality (± SE, $n = 6$ per female and concentration) for each female (F1–F4) in Lake Konnevesi control water and the different $MnSO_4$ exposure concentrations (note: y-axes are log10 scale). The *Z* values of 1.0 and 4.6 are approximately equal to 63.2% and 100% mortality, respectively.

Table 3. Female-specific NOEC and LC50 values of $MnSO_4$ (mg/L) according to total mortality of the offspring of each female, and the natural response rate according to the control mean total mortality of the female's offspring

Female	NOEC	$LC50_{MnSO4}$	95% CI	Natural response rate (%)
1	12.8	45.6	39.1–53.0	28.1
2	5.9	42.0	33.9–50.9	27.6
3	41.8	76.3	36.1–147.1	57.8
4	12.8	84.6	71.0–97.9	21.2

NOEC = no-observed effect concentration; LC50 = median lethal concentration; CI = confidence limit.

Table 4. General linear model analysis results of the $MnSO_4$ exposure concentration, female and male significance in the growth and yolk consumption analysis of the 3-d-old larvae

	df	*F*	*P*
Dry wt C, Degree days	1	1.074	0.304
Dry wt C, Concn.	3	8.709	<0.001
Dry wt C, F	3	27.761	<0.001
Dry wt C, M	1	0.174	0.678
Dry wt C, Concn. × F	9	1.894	0.070
Dry wt C, Concn. × M	3	3.037	0.036
Dry wt C, F × M	3	1.537	0.214
Dry wt C, Concn. × F × M	8	1.808	0.093
Dry wt Y, Degree days	1	38.902	<0.001
Dry wt Y, Concn.	3	16.330	<0.001
Dry wt Y, F	3	19.163	<0.001
Dry wt Y, M	1	2.315	0.133
Dry wt Y, Concn. × F	9	3.691	0.001
Dry wt Y, Concn. × M	3	1.732	0.170
Dry wt Y, F × M	3	1.193	0.320
Dry wt Y, Concn. × F × M	8	3.290	0.004

Dry wt C = carcass dry weight; Concn. = $MnSO_4$ exposure concentration; F = female; M = male; Dry wt Y = yolk dry weight.

$p < 0.001$), and the Mn concentrations of the eggs of the different females differed significantly as well (GLM, $F = 3.098$, $df = 3$, $p = 0.049$; Table 5; Supplemental Data, Table S8). The $MnSO_4$ exposure also had a significant effect on the larval Mn concentrations (GLM, $F = 58.810$, $df = 5$, $p < 0.001$), but the female effect was not significant (GLM, $F = 1.069$, $df = 3$, $p = 0.368$). Significant interactions between $MnSO_4$ exposure and female effects were not found with either the eggs or the larvae (GLM, $p > 0.05$; Supplemental Data, Table S8). Compared with the eggs, the 3-d-old larvae had roughly 8 to even 60 times lower Mn body burdens. The egg CBR50 value for Mn was 9.08 μmol/g dry weight, with 95% confidence limits of 7.13 μmol/g to 12.81 μmol/g dry weight and a natural response rate of 30.5%. For the egg-to-larvae period, the CBR50 value was 0.88 μmol/g dry weight, with 95% confidence limits of 0.56 μmol/g to 2.05 μmol/g dry weight and a natural response rate of 29.1%. Sulfur was not concentrated in either eggs or larvae (GLM, $p > 0.05$; Supplemental Data, Table S8).

Gene expression

The $MnSO_4$ exposure did not have a significant effect on the transcript abundance of any of the target genes in the embryos (Figure 4 and Table 6). However, *cat*, *mt-a*, and *mt-b* expression differed significantly between the embryos of the 3 different parent pairs (Table 6). As the exposure concentration was excluded from the analysis and only the embryonic gene expression differences between the parent pairs were analyzed, the significant differences in *cat*, *mt-a*, and *mt-b* expression between the parent pairs were still observed (ANOVA, *cat*: $p = 0.001$, $F = 8.060$, $df = 2$; *gstt*: $p = 0.062$, $F = 2.945$, $df = 2$; *mt-a*: $p = 0.010$, $F = 5.052$, $df = 2$; *mt-b*: $p = 0.027$, $F = 3.925$, $df = 2$). Pairwise comparison showed that *cat* expression of the embryos of the parent pair with high offspring total mortality (F3 × M2) was significantly higher than that of the embryos of the other 2 pairs (Tukey's HSD, F2 × M1: $p = 0.015$; F4 × M2: $p = 0.001$). The *mt-a* expression of the embryos of the parent pair with the lowest offspring total mortality (F4 × M2) was significantly lower than that of the embryos of the 2 other parent pairs (Tukey's HSD, F2 × M1: $p = 0.028$; F3 × M2: $p = 0.018$), whereas *mt-b* expression of the embryos of the parent pair with intermediate offspring total mortality (F2 × M1) was significantly lower than that of the embryos of the parent pair with high

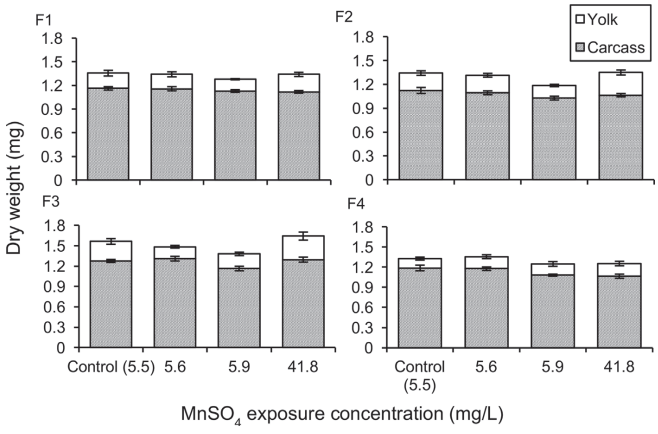

Figure 3. Mean larval carcass and yolk dry weights (± standard error, $n = 6$ per female and concentration, except for F3 in 41.8 mg $MnSO_4$/L, $n = 2$) of each female (F1–F4) in Lake Konnevesi control water and the 3 different $MnSO_4$ exposure concentrations.

Table 5. Mean Mn tissue concentrations ± standard error of the eggs and the 3-d-old larvae of each female in different $MnSO_4$ exposure concentrations and the control[a]

	Eggs (Mn mg/kg dry wt)				Larvae (Mn mg/kg dry wt)			
$MnSO_4$ (mg/L)	Female 1 ($n=1-2$)	Female 2 ($n=1-2$)	Female 3 ($n=1-2$)	Female 4 ($n=2$)	Female 1 ($n=5-6$)	Female 2 ($n=5-6$)	Female 3 ($n=1-2$)	Female 4 ($n=3-6$)
Control (5.5)	10.7 ± 1.0	11.1 ± 1.7	9.5 ± 2.3	18.1 ± 1.8	0.3 ± 0.04	0.5 ± 0.3	<LOQ	<LOQ
5.6	17.4	10.8 ± 0.05	12.8 ± 1.8	19.0 ± 6.1	<LOQ	0.5 ± 0.3	<LOQ	<LOQ
5.9	36.8 ± 3.2	36.0	33.4 ± 7.0	53.2 ± 3.5	<LOQ	0.6 ± 0.3	1.0 ± 0.7	<LOQ
12.8	131.5 ± 19.0	112.5 ± 8.5	74.6 ± 5.7	137.0 ± 7.4	3.3 ± 0.8	2.4 ± 0.3	1.6 ± 0.7	2.8 ± 0.4
41.8	210.5 ± 4.3	223.0 ± 41.2	201.8	299.1 ± 18.9	23.2 ± 3.7	21.2 ± 2.7	18.7	33.9 ± 5.1
197.8	474.2 ± 57.0	394.1 ± 1.1	354.8	397.5 ± 4.3	—	—	—	46.7 ± 11.3
965.0	—	—	—	661.9 ± 67.3	—	—	—	—

[a]All embryo samples were above the limit of quantification (LOQ) with relative standard deviation (RSD) < 10%. The larval samples of each female in each concentration are presented as upper bound values and if none of the replicates exceeded the LOQ and/or had RSD below 10%, they are presented as <LOQ. A dash indicates that there were no samples because of mortality.

offspring total mortality (F3 × M2; Tukey's HSD, $p = 0.028$). According to the individual parent pair analyses, only the pair F2 × M1 had significant differences (ANOVA, $p = 0.023$, $F = 4.344$, $df = 5$) in their offspring's *cat* expression. However, only in the $MnSO_4$ concentration of 5.6 mg/L was the embryonic *cat* expression significantly lower than in the control (Tamhane's T2, $p = 0.011$).

The 3-d-old larvae showed induction of both *mt-a* and *mt-b* under $MnSO_4$ exposure (Figure 4 and Table 6). According to the GLM results, *mt-b* was differently induced among the 2 pairs, F2 × M1 and F4 × M2, and the joint effect between $MnSO_4$ exposure and the parent pair was significant as well (Table 6). Even though the GLM results revealed significant differences only in *mt-b* induction between the parent pairs, the individual parent pair analyses showed that both *mt-a* and *mt-b* were significantly induced only in the larvae of the pair with the lowest offspring total mortality (F4 × M2; ANOVA, *mt-a*: $F = 10.569$, $df = 4$, $p = 0.003$; *mt-b*: $F = 7.176$, $df = 4$, $p = 0.009$), whereas the larvae of the pair with an intermediate offspring total mortality (F2 × M1) had no *mt* induction (ANOVA, *mt-a*: $F = 0.931$, $df = 4$, $p = 0.484$; *mt-b*: $F = 1.038$, $df = 4$, $p = 0.435$). In pairwise comparisons, larval *mt-a* and *mt-b* expression of the pair F4 × M2 was significantly higher in $MnSO_4$ exposures of 5.9 mg/L (*mt-a*: Tamhane's T2, $p = 0.035$; *mt-b*: Tukey's HSD, $p = 0.038$) and 41.8 mg/L (*mt-a*: Tamhane's T2, $p = 0.043$; *mt-b*: Tukey's HSD, $p = 0.020$) than in the control larvae. However, *mt-b* expression of the larvae reared at the 12.8 mg $MnSO_4$/L concentration also differed significantly from expression in the larvae reared at 41.8 mg $MnSO_4$/L (Tukey's HSD, $p = 0.032$). According to the GLM results, $MnSO_4$ exposure or parent pair did not have a significant effect on *gstt* or *cat* expression of the larvae (Table 6), and the individual parent pair analyses did not reveal any differences either. The numbers of valid replicates for both embryos and larvae for each target gene and parent pair are given in the Supplemental Data, Table S9.

DISCUSSION

Under experimental conditions, early life stages of whitefish were sensitive to $MnSO_4$, and the variation in their tolerance

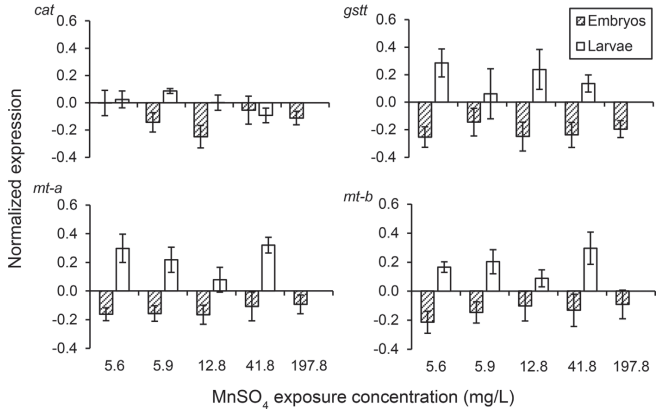

Figure 4. Mean embryonic ($n = 6-9$) and larval ($n = 3-6$) *cat*, *gstt*, *mt-a*, and *mt-b* normalized expressions ± standard error under different $MnSO_4$ exposure concentrations after subtracting the mean expressions of the control samples. The number of valid observations is given in more detail in Supplemental Data, Table S9.

Table 6. General linear model analysis results of the $MnSO_4$ exposure concentration and parent pair significance in gene expression of the embryos and the 3-d-old larvae

	Embryos			Larvae		
Gene	*df*	*F*	*p*	*df*	*F*	*p*
cat						
Concn.	5	1.553	0.206	4	1.214	0.339
PP	2	8.987	0.001	1	0.277	0.605
Concn. × PP	10	1.174	0.348	4	0.996	0.435
gstt						
Concn.	5	0.583	0.712	4	0.480	0.751
PP	2	2.655	0.085	1	0.005	0.946
Concn. × PP	10	0.375	0.949	4	0.462	0.763
mt-a						
Concn.	5	0.388	0.853	4	3.794	0.021
PP	2	3.867	0.031	1	1.245	0.279
Concn. × PP	10	0.343	0.962	4	1.413	0.270
mt-b						
Concn.	5	0.620	0.685	4	3.805	0.021
PP	2	3.582	0.040	1	5.559	0.030
Concn. × PP	10	0.339	0.963	4	3.028	0.045

cat = catalase; Concn. = $MnSO_4$ exposure concentration; PP = parent pair; *gstt* = glutathione-S-transferase; *mt-a* = metallothionein-A; *mt-b* = metallothionein-B

was significantly affected by the parent fish, the female in particular. The female had a significant effect on fertilization success, offspring total mortality, and growth and yolk consumption of the larvae, whereas the male alone did not have a significant effect on those endpoints. The $MnSO_4$ exposure caused a significant induction of *mt-a* and *mt-b*, but only in the larvae of the pair with the lowest total offspring mortality.

The female-dependent differences in offspring tolerance to toxic chemicals or unfavorable environmental conditions could be because of genetic or environmentally induced variability. In methylmercury-exposed mummichog (*Fundulus heteroclitus*) embryos, some females living in an unpolluted environment produced more tolerant offspring than other females of the same population, and it was suggested that the variability in offspring methylmercury tolerance was linked to genetic differences between the females [15]. Also, survival of freshwater-adapted European whitefish embryos under chronic osmotic stress has been shown to depend significantly on their female parents [16]. However, there is evidence that if the parent fish is exposed to metals before spawning, the metal tolerance can be maternally transferred to the offspring as well. For example, female fathead minnows that had been exposed to Cu produced larvae with a higher Cu tolerance [38]. In the present study, however, the parent fish were hatchery-reared and thus likely had a uniform life history and had been reared in an unpolluted environment. Thus, the female effect observed in the present study was more likely the result of natural individual variation. The male effect was not observed or remained vague because of a low number of males.

The higher $MnSO_4$ tolerance of the offspring of F4 could also be related to the larval *mt-a* and *mt-b* induction observed in parent pair F4 × M2. The ability of Cd-exposed turbot larvae to induce MT gene transcription has previously been correlated with their Cd tolerance [29], and thus the activation of detoxification processes could have been the reason for the higher $MnSO_4$ tolerance of the offspring of F4 as well. However, the offspring gene expression of the parent pair F4 × M1 was not investigated, and only the larvae had induced expression of *mt-a* and *mt-b*, whereas in the embryos, the observed differences in gene expression between the parent pairs did not seem to be connected to offspring survival. Also, induction of *mt-a* and *mt-b* in the whitefish larvae did not show a consistent concentration-related pattern. Previously, a concentration-related pattern of MT induction has been observed from several tissue types of Cd-exposed juvenile river pufferfish (*Takifugu obscurus*) [39]. On the other hand, the expression patterns of MT have also been shown to depend on exposure duration [39], and different metals can cause very different induction patterns as well [40].

The $MnSO_4$ exposure slightly inhibited yolk consumption of all the larvae in the 41.8 mg $MnSO_4$/L exposure concentration and with nearly all females, exposure also reduced the dry weight of the larval carcass. Metal exposure can cause an inability to utilize yolk reserves, as has been demonstrated with Cu-exposed common carp (*Cyprinus carpio*) larvae [41], and the larval growth of brown trout also has been shown to be reduced after Mn exposure [6]. In addition, increased osmoregulatory cost can reduce the larval length of whitefish [16].

Because Mn accumulation in the eggs was substantially higher than in the larvae, it is most likely that Mn was blocked by the chorion and/or perivitelline fluid, as previously demonstrated with fish embryos exposed to Cd [42] and Cr [43]. Such a conclusion is also supported by the finding that, in the embryos, none of the target genes were significantly affected by $MnSO_4$ exposure. The observed difference between Mn concentrations in eggs of the different females is most likely because of the differences in the size of the offspring, as F3 had the largest eggs and larvae but nearly always had accumulated the least Mn. This was most likely because the surface area to body mass ratio was smaller for the eggs and larvae of F3 than in the other females. Thus, such female-related differences also support the view that Mn had accumulated in the chorion and/or pervitelline fluid.

Also, the egg and egg-to-larval Mn CBR50 values (95% confidence limits) of 9.08 (7.13−12.81) μmol/g dry weight and 0.88 (0.56−2.05) μmol/g dry weight, respectively, suggest that the chorion and/or perivitelline fluid had protected the developing embryo from Mn. According to the CBR50 values, the eggs seemed to be far more tolerant to Mn compared with the whole development period from egg to 3-d-old larva. However, we used total offspring mortality instead of larval mortality alone when estimating the egg-to-larval CBR50 value, because the actual larval mortality during the 3-d rearing was rather negligible. The egg-to-larval CBR50 value thus represents the entire exposure period from fertilization until the larvae were 3 d old, which may better represent real-life conditions in the water bodies affected by the mining effluents.

In the present experiment, we were unable to determine whether the observed responses were because of Mn or SO_4 alone or because of their interaction. However, if it is assumed that the mixture toxicity effect of $MnSO_4$ in the exposures would have been solely additive for the 2 substances, and so back-calculating the $MnSO_4$ NOEC and LC50 values to the respective Mn and SO_4 exposure concentrations, we can make some comparisons with the previously reported toxicity values of Mn and SO_4. The female-specific NOEC range for Mn and SO_4 would thus be 0.04 mg/L to 12.5 mg/L and 5.8 mg/L to 29.3 mg/L, respectively. The female-specific $MnSO_4$ LC50 values varied from 42.0 mg/L to 84.6 mg/L, with a 95% confidence limit of 33.9 mg/L to 147.1 mg/L. Such values are within the $MnSO_4$ exposure concentration range of 12.8 mg

$MnSO_4$/L to 197.8 mg $MnSO_4$/L, and the mean proportions of Mn and SO_4 in the water samples of those exposure concentrations were 28% and 72%, respectively. These proportions thus result in LC50 values of 11.8 mg to 23.7 mg Mn/L and 30.2 mg to 60.9 of mg SO_4/L, with 95% confidence limits of 9.5 mg to 41.2 mg Mn/L and 24.4 mg to 105.9 mg SO_4/L. Compared with previously reported Mn [6,18] and SO_4 [7] levels, the toxicity values of the early life stages of other fish species, whitefish embryos and larvae seemed to be slightly more or equally sensitive to Mn than those of other fish species, whereas the whitefish early life stages were far more sensitive for SO_4. However, the difference in the water hardness between the different experiments makes comparison of the toxicity values uncertain.

The test chemical was $MnSO_4$ with 98.8% purity, and especially at the highest exposure concentration, elevated levels of Cu, Ni, and Pb were measured, with the Cr concentration being already elevated at 41.8 mg $MnSO_4$/L (Supplemental Data, Table S2). However, compared with a previous experiment with rainbow trout embryos and larvae [44], the effect of the Cu impurity in the present experiment was likely negligible, as was the effect of Ni when estimated according to the annual average environmental quality standard concentration for Ni (20.0 μg/L) [45]. Also, the egg and larval tissue concentrations of Cu did not increase as the exposure concentration increased, and the tissue concentrations of Ni were below the LOQs. Exposure water Pb concentrations above the annual average environmental quality standard (7.2 μg/L) [45] were observed once at 41.8 mg $MnSO_4$/L, and increasingly from 197.8 mg $MnSO_4$/L and 965.0 mg $MnSO_4$/L concentrations, but the egg and larval Pb concentrations were below the LOQ. For Cr, a 499.2 μg/L concentration has been reported to increase the larval mortality of common carp [43], and the mean water Cr concentration (± SE) at the highest $MnSO_4$ exposure concentration (422.2 ± 45.0 μg Cr/L) was close to that value. According to other studies, only a Cr concentration of several mg/L is toxic to fish embryos and larvae [46,47]. In the present study, however, the egg Cr concentrations were elevated at 4 of the highest exposure concentrations. Thus, a possible interference of Pb and Cr especially at the highest exposure concentration cannot be completely excluded. In addition, after approximately 1 mo of incubation, brown precipitates and/or bacterial growth was observed on the walls of some of the pools and compartment grids (visually observed in pools containing 5.9−41.8 mg $MnSO_4$/L), which may have affected the Mn balance of those pools.

Finally, although the present study does not allow us to distinguish the individual roles of Mn or SO_4, the results give relevant insights into the field conditions of freshwaters under the impact of metal mining, as the concentrations of both Mn and SO_4 are often elevated in water bodies receiving mining effluents [5,48]. In the case of the bioheap leaching mine in northeastern Finland, from approximately 2010 onward, the annual mean Mn and SO_4 concentrations have increased in the waterbodies impacted by the mine, with the highest SO_4 concentrations being even several thousand mg/L and the highest Mn concentrations several hundred mg/L [5,12]. Whitefish is still caught from some of the less impacted lakes, and in those lakes the highest annual mean values of Mn and SO_4 have been found to be 2022 μg/L and 257 mg/L, respectively [12,49]. When these concentrations are compared with the findings from the present study, it can be seen that the observed concentrations of the impacted lakes may have adverse effects on whitefish early life stages. However, as has been demonstrated in earlier experiments, fish populations can also adapt to chemical stressors [50,51].

CONCLUSIONS

Continuous exposure to $MnSO_4$ decreased whitefish embryonic survival in relation to the $MnSO_4$ exposure concentration. Offspring tolerance to $MnSO_4$ exposure depended on the female parent in particular, resulting in substantial differences in offspring instantaneous total mortality already at moderate $MnSO_4$ concentrations. Also, larval expression of metal-regulating genes indicated that better offspring $MnSO_4$ tolerance is linked to the induction of metal-regulating genes. The present study has shown that whitefish reproduction success can be impaired in populations under waterborne $MnSO_4$ concentrations of approximately 40 mg/L in boreal soft waters. As a relevant continuation of the present experiment, we will perform a corresponding field study to assess the implications of mining effluents on whitefish reproductive success in field conditions.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3667.

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Data availability—Readers can access the data and associated metadata by contacting the corresponding author directly (hanna.e.arola@jyu.fi).

REFERENCES

1. Howe PD, Malcolm HM, Dobson S. 2004. Manganese and its compounds: Environmental aspects. WHO Concise International Chemical Assessment Document 63. World Health Organization, Geneva, Switzerland.
2. Meays C, Nordin R. 2013. British Columbia ambient water quality guidelines for sulphate. Technical Appendix. Ministry of Environment, Water Protection & Sustainability Branch, Environmental Sustainability and Strategic Policy Division. Victoria, BC, Canada.
3. Henriksen A, Skjelkvåle BL, Mannio J, Wilander A, Jensen JP, Moiseenko T, Harriman R, Traaen T, Fjeld E, Vuorenmaa J, Kortelainen P, Forsius M. 1997. Results of national lake surveys (1995) in Finland, Norway, Sweden, Denmark, Russian Kola, Russian Karelia, Scotland and Wales. Report 3645-97. Norwegian Institute for Water Research, Oslo, Norway.
4. Lydersen E, Löfgren S, Arnesen LT. 2002. Metals in Scandinavian surface waters: Effects of acidification, liming, and potential reacidification. *Environ Sci Technol* 32:73–295.
5. Kauppi S, Mannio J, Hellsten S, Nystén T, Jouttijärvi T, Huttunen M, Ekholm P, Tuominen S, Porvari P, Karjalainen A, Sara-Aho T, Saukkoriipi J Maunula M. 2013. Assessment of the potential impacts on water environment because of the gypsum pond leakage at the Talvivaara mine. Reports of the Finnish Environment Institute 11/2013. Helsinki, Finland (in Finnish).
6. Stubblefield WA, Brinkman SF, Davies PH, Garrison TD, Hockett JR, McIntyre MW. 1997. Effects of water hardness on the toxicity of manganese to developing brown trout (*Salmo trutta*). *Environ Toxicol Chem* 16:2082–2089.
7. Elphick JR, Davies M, Gilron G, Canaria EC, Lo B, Bailey HC. 2011. An aquatic toxicological evaluation of sulfate: The case for considering hardness as a modifying factor in setting water quality guidelines. *Environ Toxicol Chem* 30:247–253.

8. Bowell RJ. 2000. Sulphate and salt minerals: The problem of treating mine waste. *Min Environ Manage* 5:11–13.
9. Johnson DB 2013. Development and application of biotechnologies in the metal mining industry. *Environ Sci Pollut Res* 20:7768–7776.
10. Riekkola-Vanhanen M. 2013. Talvivaara mining company—From a project to a mine. *Miner Eng* 48:2–9.
11. Finnish Environment Institute. 2013. Lausunto vesistön pilaantumisen merkittävyyden arvioimiseksi. Helsinki, Finland (in Finnish).
12. Terrafame Oy. 2016. Terrafame mine monitoring report 2015 - Part V: Surface water quality. Ramboll Oy, Finland (in Finnish).
13. Salmelin J, Leppänen MT, Karjalainen AK, Vuori K-M, Gerhardt A, Hämäläinen H. 2016. Assessing ecotoxicity of biomining effluents in stream ecosystems by in situ invertebrate bioassays: A case study in Talvivaara, Finland. *Environ Toxicol Chem* (in press). DOI: 10.1002/etc.3511.
14. Hutchinson TH, Solbé J, Kloepper-Sams PJ. 1998. Analysis of the ECETOC aquatic toxicity (EAT) database III—Comparative toxicity of chemical substances to different life stages of aquatic organisms. *Chemosphere* 36:129–142.
15. Weis JS, Weis P, Heber M. 1982. Variation in response to methylmercury by killifish (*Fundulus heteroclitus*) embryos. In Pearson JG, Foster R, Bishop WE, eds, *Aquatic Toxicology and Hazard Assessment: Fifth Conference*. ASTM STP 766. ASTM International, Philadelphia, PA, USA, pp 109–119.
16. Papakostas S, Vasemägi A, Vähä J-P, Himberg M, Peil L, Primmer GR. 2012. A proteomics approach reveals divergent molecular responses to salinity in populations of European whitefish (*Coregonus lavaretus*). *Mol Ecol* 21:3516–3530.
17. Jezierska B, Ługowska K, Witeska M. 2009. The effects of heavy metals on embryonic development of fish (a review). *Fish Physiol Biochem* 35:625–640.
18. Lewis M. 1978. Acute toxicity of copper, zinc and manganese in single and mixed salt solutions to juvenile longfin dace *Agosia chrysogaster*. *J Fish Biol* 13:695–700.
19. Reader JP, Everall NC, Sayer MDJ, Morris R. 1989. The effects of eight trace metals in acid soft water on survival, mineral uptake and skeletal calcium deposition in yolk-sac fry of brown trout, *Salmo trutta* L. *J Fish Biol* 35:187–198.
20. Ketola HG, Longacre D, Greulich A, Phetterplace L, Lashomb R. 1988. High calcium concentration in water increases mortality of salmon and trout eggs. *Prog Fish Cult* 50:129–135.
21. van Dam RA, Hogan AC, McCullough CD, Houston MA, Humphrey CL, Harford AJ. 2010. Aquatic toxicity of magnesium sulfate, and the influence of calcium, in very low ionic concentration water. *Environ Toxicol Chem* 29:410–421.
22. Lushchak VI. 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol* 101:13–30.
23. Choi CY, An KW, An MI. 2008. Molecular characterization and mRNA expression of glutathione peroxidase and glutathione S-transferase during osmotic stress in olive flounder (*Paralichthys olivaceus*). *Comp Biochem Physiol A* 149:330–337.
24. Woo S, Yum S, Park H-S, Lee T-K, Ryu J-C. 2009. Effects of heavy metals on antioxidants and stress-responsive gene expression in Javanese medaka (*Oryzias javanicus*). *Comp Biochem Physiol C* 149:289–299.
25. Di Giulio RT, Meyer JN. 2008. Reactive oxygen species and oxidative stress. In Di Giulio RT, Hinton DE, eds, *The Toxicology of Fishes*. CRC/Taylor & Francis, Boca Raton, FL, USA, pp 273–324.
26. Sevcikova M, Modra H, Slaninova A, Svobodova Z. 2011. Metals as a cause of oxidative stress in fish: A review. *Veterinarni Medicina* 11:537–546.
27. Coyle P, Philcox JC, Carey LC, Rofe AM. 2002. Metallothionein: The multipurpose protein (Review). *Cell Mol Life Sci* 59: 627–647.
28. Roesijadi G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals—Review. *Aquat Toxicol* 22:81–114.
29. George SG, Hodgson PA, Tytler P, Todd K. 1996. Inducibility of metallothionein mRNA expression and cadmium tolerance in larvae of a marine teleost, the turbot (*Scophthalmus maximus*). *Fund Appl Toxicol* 33:91–99.
30. Finnish Standard Association. 1976. Determination of ammonia-nitrogen of water. *SFS* 3032:1976 (in Finnish).
31. US Environmental Protection Agency. 2001. Method 200.7, revision 4.4: Determination of metals and trace elements in water and wastes by inductively coupled plasma–atomic emission spectrometry. Washington, DC.
32. Hansen BH, Rømma S, Garmo ØA, Pedersen SA, Olsvik PA, Andersen RA. 2007. Induction and activity of oxidative stress-related proteins during waterborne Cd/Zn-exposure in brown trout (*Salmo trutta*). *Chemosphere* 67:2241–2249.
33. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3—New capabilities and interfaces. *Nucleic Acids Res* 40:e115 1–12.
34. Koressaar T, Remm M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23:1289–1291.
35. Jullien N. 2008. AmplifX 1.5.4. CNRS, Aix-Marseille Université, Aix-Marseille, France. [cited 2014 March 11]. Available from: http://crn2m.univ-mrs.fr/pub/amplifx-dist
36. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden T. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134:1–11.
37. Vehniäinen E-R, Kukkonen JVK. 2015. Multixenobiotic resistance efflux activity in *Daphnia magna* and *Lumbriculus variegatus*. *Chemosphere* 124:143–149.
38. Peake EB, Locke JC, Tierney LL, Kolok AS. 2004. Copper tolerance in fathead minnows: II. Maternal transfer. *Environ Toxicol Chem* 23:208–211.
39. Kim J-H, Wang S-Y, Kim I-C, Ki J-S, Raisuddin S, Lee J-S, Han K-N. 2008. Cloning of a river pufferfish (*Takifugu obscurus*) metallothionein cDNA and study of its induction profile in cadmium-exposed fish. *Chemosphere* 71:1251–1259.
40. Cheung APL, Lam TH-J, Chan KM. 2004. Regulation of *Tilapia* metallothionein gene expression by heavy metal ions. *Mar Environ Res* 58:389–394.
41. Stouthart XJHX, Haans JLM, Lock RAC, Wendelaar Bonga SE. 1996. Effects of water pH on copper toxicity to early life stages of the common carp (*Cyprinus carpio*). *Environ Toxicol Chem* 15:376–383.
42. Michibata H. 1981. Uptake and distribution of cadmium in the egg of the teleost, *Oryzias latipes*. *J Fish Biol* 19:691–696.
43. Stouthart AJHX, Spanings FAT, Lock RAC, Wendelaar Bonga SE. 1995. Effects of water pH on chromium toxicity to early life stages of the common carp (*Cyprinus carpio*). *Aquat Toxicol* 32:31–42.
44. Stasiūnaitė P. 2005. Toxicity of copper to embryonic development of rainbow trout (*Oncorhynchus mykiss*). *Acta Zoologica Lituanica* 15:3:259–265.
45. European Union. 2008. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. *Official J Eur Union* L348:84–97.
46. Nguyien LTH, Jansen CR. 2001. Comparative sensitivity of embryo–larval toxicity assays with African catfish (*Clarias gariepinus*) and zebra fish (*Danio rerio*). *Environ Toxicol* 16:566–571.
47. Domingues I, Oliveira R, Lourenço J, Grisolia CK, Mendo S, Soares AMVM. 2010. Biomarkers as a tool to assess effects of chromium (VI): Comparison of responses in zebrafish early life stages and adults. *Comp Biochem Physiol C* 152:338–345.
48. Olías M, Nieto JM, Sarmiento AM, Cerón JC, Cánovas CR. 2004. Seasonal water quality variations in a river affected by acid mine drainage: The Odiel River (South West Spain). *Sci Total Environ* 333:267–281.
49. Terrafame Oy. 2016. Terrafame mine monitoring report 2015 - Part VII: Fisheries monitoring. Ramboll Oy, Finland (in Finnish).
50. Weis JS, Weis P, Heber M, Vaidya S. 1981. Methylmercury tolerance of killifish (*Fundulus heteroclitus*) embryos from a polluted vs non-polluted environment. *Mar Biol* 65:283–287.
51. Angus RA. 1983. Phenol tolerance in populations of mosquitofish from polluted and nonpolluted waters. *Trans Am Fish Soc* 112:794–799.

Supplemental Data to the article:

H.E. Arola *et al.* "Tolerance of whitefish (*Coregonus lavaretus*) early life stages to manganese sulfate is affected by the parents"

Table S1. Cumulative degree days at the beginning and finish of the embryo sampling, and at the mean hatching peak. Mean pool water pH, specific conductivity, oxygen levels and NH_4 with ± SE (min-max) and *n*, before and after water renewal, and the mean ± SE (min-max) and *n* of pre-filtered L. Konnevesi dissolved organic carbon (DOC).

	Exposure concentrations, mg $MnSO_4$/L						
	Control (5.5)	5.6	5.9	12.8	41.8	197.8	965.0
Degree days, embryo sampling (°C)	283/291	284/289	281/289	287/294	301/308	286/293	269/276
Degree days, hatching peak (°C)	401	397	399	407	424	405	393
pH	6.64 ± 0.04 (5.87 - 7.49) *90*	6.73 ± 0.02 (6.22 - 7.35) *88*	6.72 ± 0.02 (6.24 - 7.30) *92*	6.67 ± 0.02 (6.21 - 7.22) *92*	6.55 ± 0.02 (5.46 - 7.38) *94*	6.70 ± 0.02 (6.40 - 7.34) *94*	6.64 ± 0.02 (6.34 - 7.12) *88*
Cond. (µS/cm)	48.5 ± 0.7 (26.2 - 63.2) *66*	47.9 ± 0.7 (23.8 - 61.2) *64*	49.2 ± 0.6 (26.9 - 65.2) *68*	56.3 ± 0.5 (34.3 - 61.2) *67*	97.0 ± 0.8 (66.6 - 102.8) *70*	291.1 ± 0.6 (275.0 - 301.4) *70*	1016.2 ± 4.0 (979.0 - 1209.0) *64*
O_2 (mg/L)	12.7 ± 0.2 (11.5 - 15.2) *25*	12.7 ± 0.2 (11.2 - 15.1) *23*	12.6 ± 0.2 (11.2 - 15.1) *26*	12.6 ± 0.2 (11.2 - 15.1) *26*	12.5 ± 0.2 (11.0 - 15.0) *28*	12.5 ± 0.2 (11.1 - 15.0) *28*	12.8 ± 0.2 (11.2 - 15.2) *23*
O_2 (%)	100.6 ± 0.7 (95.3 - 108.3) *25*	100.7 ± 0.7 (95.2 - 107.9) *23*	100.6 ± 0.7 (95.4 - 107.8) *26*	100.6 ± 0.7 (95.3 - 107.7) *26*	100.6 ± 0.6 (95.5 - 107.7) *28*	100.6 ± 0.6 (95.5 - 107.9) *28*	100.7 ± 0.7 (95.7 - 108.0) *23*
NH_4 (µg/L), before water change[a]	390	500	460	290	280	350	260
NH_4 (µg/L), after water change[a]	270	340	320	180	200	260	200
DOC (mg/L)[b]	7.7 ± 0.2 (7.3 - 8.0) *4*	-	-	-	-	-	-

[a]Samples collected only once
[b]Samples collected from the newly prepared waters, not from pool waters

Table S2. The mean ±SE (min - max) of the pool water elemental concentrations and the *n* samples above LOQ with RSD below 10 % during the whole experiment period. Only values above limit of quantification (LOQ) with RSD below 10 % were included into the calculations. The LOQs are presented below each element abbreviation and Ca, K, Mg, Mn, Na, S and SO_4 are in mg/L whereas all other are in µg/L.

	Exposure concentrations, $MnSO_4$ mg/L						
	Control (5.5)	5.6	5.9	12.8	41.8	197.8	965.0
	Number of analyzed samples						
	55	46	50	44	48	56	49
Al	27.7 ± 4.3	26.5 ± 4.1	27.1 ± 5.6	27.0 ± 5.3	24.7 ± 4.2	25.6 ± 3.4	21.6 ± 1.4
LOQ	(10.1 - 145.8)	(11.7 - 142.4)	(6.9 - 150.7)	(10.1 - 155.6)	(8.1 - 113.6)	(11.1 - 115.8)	(9.8 - 34.7)
6.0	37	34	26	28	26	33	29
As	< 8.2	< 8.2	< 8.2	< 8.2	< 8.2	< 8.2	< 8.2
LOQ	0	0	0	0	0	0	0
8.2							
Ca	4.2 ± 0.03	4.2 ± 0.04	4.2 ± 0.03	4.3 ± 0.05	4.1 ± 0.04	4.2 ± 0.04	4.2 ± 0.03
LOQ	(3.8 - 4.7)	(3.8 - 4.8)	(3.8 - 4.7)	(3.8 - 5.0)	(3.8 - 4.8)	(3.9 - 5.0)	(3.9 - 4.7)
1.6[a]	55	46	50	44	48	56	49
Cd	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8
LOQ	0	0	0	0	0	0	0
0.8							
Co	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6
LOQ	0	0	0	0	0	0	0
1.6							
Cr	7.6 ± 1.2	10.1 ± 0.7	9.1 ± 1.1	7.5 ± 1.0	23.2 ± 1.0	119.9 ± 1.3	422.2 ± 45.0
LOQ	(2.0 - 12.6)	(8.2 - 12.5)	(1.7 - 12.4)	(3.1 - 16.9)	(8.6 - 37.4)	(105.3 - 142.2)	(358.1 - 501.7)
1.5	13	9	10	24	48	56	49
Cu	< 2.7	4.6	< 2.7	4.0 ± 1.1	< 2.7	3.8 ± 0.2	8.5 ± 0.5
LOQ	0	1	0	(2.9 - 5.0)	0	(3.1 - 4.4)	(4.6 - 21.2)
2.7				2		5	42

Fe LOQ 0.5	49.1 ± 5.5 (6.6 - 190.4) 52	46.0 ± 5.4 (6.2 - 198.1) 42	41.0 ± 5.1 (7.6 - 203.0) 47	41.5 ± 6.4 (1.9 - 217.7) 41	40.8 ± 5.9 (6.4 - 174.8) 39	38.2 ± 4.6 (2.1 - 158.3) 48	37.6 ± 4.3 (10.1 - 145.4) 39
K LOQ 5.6[a]	1.3 ± 0.1 (0.8 - 2.3) 55	1.2 ± 0.1 (0.7 - 2.2) 46	1.3 ± 0.1 (0.7 - 5.6) 50	1.3 ± 0.1 (0.8 - 2.3) 44	1.2 ± 0.1 (0.7 - 6.0) 48	1.4 ± 0.1 (0.9 - 2.9) 56	1.3 ± 0.1 (0.3 - 3.1) 46
Mg LOQ 8.0[a]	1.5 ± 0.01 (1.3 - 1.7) 55	1.5 ± 0.02 (1.3 - 1.7) 46	1.5 ± 0.02 (1.4 - 1.7) 50	1.5 ± 0.02 (1.3 - 1.8) 44	1.4 ± 0.02 (1.3 - 1.7) 48	1.2 ± 0.01 (1.1 - 1.6) 56	0.2 ± 0.03 (0.02 - 0.42) 18
Mn LOQ 0.6[b]	14.5[b] ± 3.1[b] (0.7[b] - 89.0[b]) 47	10.2[b] ± 1.5[b] (0.9[b] - 53.7[b]) 46	44.9[b] ± 5.6[b] (1.1[b] - 153.0[b]) 50	2.1 ± 0.1 (0.01 - 2.9) 44	12.5 ± 0.3 (6.3 - 15.8) 48	70.2 ± 0.4 (64.0 - 75.6) 56	341.6 ± 1.6 (322.1 - 372.3) 49
Na LOQ 3.0[a]	2.0 ± 0.02 (1.5 - 2.4) 55	2.0 ± 0.03 (1.7 - 2.6) 46	2.0 ± 0.03 (1.6 - 2.7) 50	2.0 ± 0.03 (1.5 - 2.6) 44	1.8 ± 0.04 (1.5 - 2.6) 48	2.1 ± 0.03 (1.8 - 2.8) 56	1.9 ± 0.1 (1.3 - 2.9) 49
Ni LOQ 2.1	4.9 ± 0.6 (3.2 - 9.1) 9	4.1 ± 0.1 (4.0 - 4.2) 2	3.5 ± 0.3 (3.0 - 4.0) 3	3.7 ± 0.8 (2.1 - 4.6) 3	5.1 ± 0.6 (4.3 - 6.3) 3	7.8 ± 0.6 (2.2 - 9.9) 12	16.5 ± 0.9 (9.7 - 43.0) 49
P LOQ 16.0	33.7± 12.1 (19.9 - 57.9) 3	65.7 ± 11.4 (42.6 - 115.7) 6	121.9 ± 55.8 (32.4 - 339.7) 5	36.5 ± 8.4 (28.1 - 44.8) 2	150.6 ± 75.0 (42.2 - 447.8) 5	111.5 ± 14.7 (53.2 - 165.9) 8	129.3 ± 30.4 (98.9 - 159.7) 2
Pb LOQ 6.0	< 6.0 0	< 6.0 0	< 6.0 0	< 6.0 0	9.4 1	9.8 ± 0.6 (8.0 - 14.0) 9	37.7 ± 0.4 (29.4 - 44.0) 37

S LOQ 2.6[a]	1.8 ± 0.03 (1.3 - 2.2) 55	1.9 ± 0.03 (1.3 - 2.3) 46	1.9 ± 0.03 (1.3 - 2.3) 50	3.6 ± 0.04 (3.1 - 4.1) 44	9.8 ± 0.2 (5.8 - 11.8) 48	42.6 ± 0.4 (38.1 - 51.7) 56	208.1 ± 1.8 (190.7 - 236.5) 49
SO_4[c]	5.5 ± 0.1 (3.9 - 6.7) 55	5.6 ± 0.1 (3.9 - 6.7) 46	5.8 ± 0.1 (4.0 - 6.9) 50	10.7 ± 0.1 (9.4 - 12.2) 44	29.3 ± 0.5 (17.3 - 35.3) 48	127.6 ± 1.2 (114.2 - 154.9) 56	623.4 ± 5.5 (571.2 - 708.6) 49
Sr LOQ 0.6	28.5 ± 0.3 (25.5 - 32.4) 55	28.5 ± 0.3 (25.5 - 32.3) 46	28.8 ± 0.2 (25.9 - 32.5) 50	28.8 ± 0.4 (22.4 - 34.0) 44	29.0 ± 0.3 (26.3 - 33.3) 48	29.2 ± 0.3 (26.5 - 33.8) 56	28.4 ± 0.4 (23.6 - 33.5) 49
U LOQ 7.8	< 7.8 0	< 7.8 0	< 7.8 0	< 7.8 0	< 7.8 0	< 7.8 0	< 7.8 0
Zn LOQ 0.5	6.0 1	< 0.5 0	1.1 ± 0.2 (0.9 - 1.3) 2	32.5 ± 0.5 (32.0 - 33.0) 2	< 0.5 0	1.7 ± 0.4 (0.6 - 2.6) 5	3.5 ± 0.7 (0.9 - 9.4) 13

[a] $\times 10^{-2}$

[b] $\times 10^{-3}$

[c] Calculated from S

Table S3. The mean measured $MnSO_4$, Mn and SO_4 concentrations (mg/L ±SE and *n*) during winter and spring period. The mean proportion (% ±SE and *n*) of the measured exposure concentrations compared to the control sample of the corresponding time including the added nominal concentration ((measured concentration/(measured control sample concentration + nominal concentration))*100) are presented in parentheses. Also the mean difference ±SE and *n* between measured $MnSO_4$, Mn and SO_4 concentrations of the old and newly changed water (%, calculated as 100-(concn. new water/concn. old water*100)) for both winter and spring period are presented.

Nominal concentration added to control			Measured concentrations, winter			Measured concentrations, spring			Difference between old and newly changed water, winter			Difference between old and newly changed water, spring		
$MnSO_4$	Mn	SO_4	$MnSO_4$	Mn	SO_4[a]	$MnSO_4$	Mn	SO_4[a]	$MnSO_4$	Mn	SO_4	$MnSO_4$	Mn	SO_4
Ctrl	Ctrl	Ctrl	5.4 ± 0.1 31	2.0^{b} ± 0.4^{b} 31	5.4 ± 0.1 39	5.7 ± 0.2 16	0.4^{b} ± 0.1^{b} 16	5.7 ± 0.2 16	-0.3 ± 3.3 10	16.8 ± 33.5 10	-0.4 ± 2.3 17	-9.3 ± 3.7 8	-8.8 ± 41.7 8	-9.4 ± 3.7 8
5.7^{b}	2.1^{b}	3.6^{b}	5.4 ± 0.1 32 (100.3 ± 1.0) (27)	1.3^{b} ± 0.2^{b} 32 (33.8 ± 5.6) (27)	5.4 ± 0.1 32 (100.3 ± 0.8) (32)	5.9 ± 0.2 14 (105.7 ± 1.5) (14)	0.4^{b} ± 0.1^{b} 14 (16.6 ± 3.9) (14)	5.9 ± 0.2 14 (106.2 ± 1.5) (14)	1.7 ± 2.8 13	69.0 ± 6.8 13	1.5 ± 2.8 13	-9.3 ± 6.7 7	-13.6 ± 89.4 7	-9.4 ± 6.7 7
28.6^{b}	10.4^{b}	18.2^{b}	5.7 ± 0.1 32 (100.7 ± 0.9) (27)	6.3^{b} ± 0.7^{b} 32 (48.2 ± 6.2) (27)	5.6 ± 0.1 32 (101.5 ± 0.8) (32)	6.2 ± 0.2 18 (104.3 ± 1.7) (15)	1.4^{b} ± 0.3^{b} 18 (11.5 ± 2.9) (15)	6.2 ± 0.2 18 (106.0 ± 1.7) (15)	0.5 ± 3.1 13	51.5 ± 10.2 13	0.1 ± 3.1 13	-8.5 ± 5.7 9	62.5 ± 15.7 9	-9.0 ± 5.7 9
7.2	2.6	4.6	12.9 ± 0.1 30 (104.2 ± 1.3) (25)	2.5 ± 0.04 30 (95.8 ± 1.6) (25)	10.4 ± 0.1 30 (105.9 ± 1.4) (30)	12.4 ± 0.3 14 (98.8 ± 4.6) (11)	1.2 ± 0.3 14 (49.2 ± 11.3) (11)	11.2 ± 0.2 14 (111.6 ± 4.0) (11)	0.9 ± 2.2 11	6.8 ± 2.2 11	-0.6 ± 2.3 11	4.5 ± 1.9 7	58.8 ± 14.0 7	-4.3 ± 2.3 7
35.7	13.0	22.7	42.2 ± 0.8 28 (102.4 ± 2.4) (23)	13.7 ± 0.1 28 (105.7 ± 0.9) (23)	28.5 ± 0.8 28 (101.7 ± 2.8) (28)	41.3 ± 0.9 20 (97.4 ± 2.8) (15)	10.9 ± 0.6 20 (81.7 ± 5.5) (15)	30.4 ± 0.6 20 (104.6 ± 2.2) (15)	1.0 ± 2.6 9	-0.2 ± 2.0 9	1.7 ± 3.3 9	3.6 ± 1.8 10	15.6 ± 3.0 10	-1.1 ± 2.8 10
178.7	65.0	113.7	197.3 ± 1.7 36 (106.9 ± 1.1) (29)	69.8 ± 0.5 36 (107.3 ± 0.9) (29)	127.5 ± 1.4 36 (107.1 ± 1.1) (36)	198.6 ± 2.7 20 (105.0 ± 0.6) (15)	70.9 ± 0.5 20 (108.3 ± 0.7) (15)	127.7 ± 2.4 20 (103.2 ± 0.8) (15)	-2.1 ± 1.5 14	-0.1 ± 1.2 14	-3.2 ± 1.9 14	-4.0 ± 1.8 10	-3.3 ± 0.8 10	-4.5 ± 2.7 10
893.4	325.0	568.4	949.5 ± 6.0 35 (105.8 ± 0.8) (28)	343.2 ± 1.9 35 (105.7 ± 0.7) (28)	606.3 ± 4.7 35 (105.7 ± 0.8) (35)	1003.8 ± 7.0 14 (111.7 ± 0.8) (14)	337.5 ± 3.0 14 (103.9 ± 0.9) (14)	666.2 ± 7.6 14 (116.1 ± 1.3) (14)	-0.6 ± 0.9 13	-1.0 ± 0.5 13	-0.5 ± 1.4 13	-1.7 ± 1.2 7	-1.6 ± 1.7 7	-1.8 ± 2.0 7

[a]Calculated from S

[b] $\times 10^{-2}$

Table S4. Number of embryo/larval growth and yolk consumption, qPCR and tissue concentration samples analyzed from each parent pair in different $MnSO_4$ exposure concentrations and control.

Growth and yolk consumption[a]	$MnSO_4$ (mg/L)						
Parent pair	Control (5.5)	5.6	5.9	12.8	41.8	197.8	965.0
F1 x M1	-/3	-/3	-/3	-	-/3	-	-
F1 x M2	-/3	-/3	-/3	-	-/3	-	-
F2 x M1	-/3	-/3	-/3	-	-/3	-	-
F2 x M2	-/3	-/3	-/3	-	-/3	-	-
F3 x M1	-/3	-/3	-/3	-	-	-	-
F3 x M2	-/3	-/3	-/3	-	-/2	-	-
F4 x M1	-/3	-/3	-/3	-	-/3	-	-
F4 x M2	-/3	-/3	-/3	-	-/3	-	-
Tissue concentration[b]	$MnSO_4$ (mg/L)						
Parent pair	Control (5.5)	5.6	5.9	12.8	41.8	197.8	965.0
F1 x M1	1/3	1/2	1/3	1/2	1/3	1/-	-
F1 x M2	1/3	-/3	1/3	1/3	1/3	1/-	-
F2 x M1	1/3	1/3	1/3	1/3	1/3	1/-	-
F2 x M2	1/3	1/2	-/3	1/3	1/3	1/-	-
F3 x M1	1/2	1/1	1/1	1/-	1/-	-	-
F3 x M2	1/-	1/-	1/1	1/2	-/1	1/-	-
F4 x M1	1/1	1/3	1/3	1/3	1/3	1/2	1/-
F4 x M2	1/2	1/3	1/3	1/3	1/3	1/2	1/-
qPCR[c]	$MnSO_4$ (mg/L)						
Parent pair	Control (5.5)	5.6	5.9	12.8	41.8	197.8	965.0
F1 x M1	-	-	-	-	-	-	-
F1 x M2	-	-	-	-	-	-	-
F2 x M1	3/3	3/3	3/3	3/3	3/3	3/-	-
F2 x M2	-	-	-	-	-	-	-
F3 x M1	-	-	-	-	-	-	-
F3 x M2	3/-	3/-	3/-	3/-	3/-	1/-	-
F4 x M1	-	-	-	-	-	-	-
F4 x M2	3/2	3/3	3/3	3/3	3/3	3/-	-

[a] Samples contained1 to 10 larvae of which a maximum of 4 larvae were analyzed

[b] Maximum of 4 embryos per parent pair replicate in each concentration were pooled and each embryo sample contained 4 to 12 embryos and larval samples contained 1 to 10 larvae

[c] Embryo samples contained 3 embryos and larval samples 5 larvae

Table S5. The mean ± SE sample dry weights (mg), total number of samples analyzed, upper bound tissue concentrations (mg/kg dry weight) of the eggs in each $MnSO_4$ exposure concentration and control. The minimum and maximum tissue concentrations are presented in parentheses and numbers of samples above the LOQ with RSD < 10 %. The LOQ values for each element are the same as for water data and in cases when all analyzed samples were below the LOQ or had RSD above 10 % are indicated as < LOQ.

$MnSO_4$ concentration (mg/L)	Control (5.5)	5.6	5.9	12.8	41.8	197.8	965.0
Mean dry weight ± SE (mg)	26.4 ± 0.5	26.3 ± 0.6	26.2 ± 0.5	26.3 ± 0.5	25.8 ± 0.4	21.4 ± 2.4	23.9 ± 0.8
Total *n* analyzed	8	7	7	8	7	7	2
Al	11.5 ± 1.9 (4.5 - 19.0) 8	8.5 ± 1.3 (4.3 - 15.3) 7	11.8 ± 2.2 (6.3 - 21.3) 7	4.4 ± 0.9 (1.2 - 7.1) 6	4.2 ± 1.3 (1.2 - 10.1) 4	9.3 ± 1.5 (3.3 - 14.6) 6	15.0 ± 0.6 (14.4 - 15.9) 2
As	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ca	686.7 ± 46.5 (391.2 - 784.0) 8	602.9 ± 36.9 (415.3 - 737.8) 7	686.2 ± 20.6 (617.8 - 757.7) 7	667.7 ± 24.6 (536.0 - 761.9) 8	491.9 ± 31.7 (408.9 - 656.3) 7	500.5 ± 37.2 (365.1 - 600.8) 7	450.6 ± 27.2 (423.5 - 477.8) 2
Cd	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Co	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cr	< LOQ	< LOQ	< LOQ	0.3 ± 0.01 (0.3 - 0.3) 2	0.4 ± 0.1 (0.3 - 0.8) 2	0.8 ± 0.1 (0.3 - 1.1) 5	1.8 ± 0.2 (1.6 - 1.9) 2

Cu	1.0 ± 0.3 (0.5 - 3.2) 4	0.6 ± 0.1 (0.5 - 1.1) 2	< LOQ	0.8 ± 0.1 (0.5 - 1.2) 4	0.6 ± 0.1 (0.5 - 0.8) 2	1.0 ± 0.2 (0.5 - 2.2) 1	1.0 ± 0.4 (0.5 - 1.4) 1
Fe	30.2 ± 4.0 (10.0 - 45.7) 8	20.9 ± 2.9 (11.9 - 32.8) 7	25.9 ± 3.4 (15.5 - 39.6) 7	15.2 ± 1.2 (11.1 - 19.9) 8	15.9 ± 3.0 (6.1 - 30.0) 7	27.4 ± 5.0 (0.3 - 39.1) 6	39.3 ± 7.8 (31.5 - 47.1) 2
K	5273.5 ± 305.3 (3294.1 - 6136.3) 8	4822.6 ± 238.2 (3677.4 - 5729.9) 7	5235.6 ± 66.3 (4972.2 - 5429.0) 7	5355.5 ± 181.8 (4310.6 - 5856.9) 8	4647.1 ± 224.2 (3983.6 - 5634.4) 7	4945.0 ± 229.3 (4117.9 - 5674.8) 7	5217.7 ± 212.4 (5005.2 - 5430.1) 2
Mg	710.2 ± 50.3 (401.2 - 859.3) 8	640.5 ± 37.5 (457.2 - 770.5) 7	721.7 ± 15.6 (679.1 - 790.6) 7	714.4 ± 23.5 (566.0 - 795.1) 8	584.2 ± 27.9 (473.9 - 707.0) 7	620.4 ± 22.9 (530.5 - 683.3) 7	684.0 ± 25.0 (659.0 - 708.9) 2
Mn	12.4 ± 1.4 (7.2 - 19.9) 8	14.7 ± 2.0 (10.7 - 25.1) 7	40.4 ± 3.8 (26.4 - 56.7) 7	113.9 ± 10.2 (68.8 - 150.5) 8	238.1 ± 18.8 (181.8 - 317.9) 7	412.3 ± 21.0 (354.8 - 531.2) 7	661.9 ± 67.3 (594.5 - 729.2) 2
Na	1770.0 ± 111.5 (1067.7 - 2035.8) 8	1568.0 ± 94.6 (1107.3 - 1964.6) 7	1752.6 ± 45.4 (1595.1 - 1925.2) 7	1854.6 ± 75.4 (1508.3 - 2147.8) 8	1571.3 ± 80.6 (1287.3 - 1850.7) 7	1693.4 ± 78.2 (1405.0 - 1908.4) 7	1830.6 ± 44.8 (1785.8 - 1875.4) 2
Ni	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
P	4559.7 ± 315.4 (2538.9 - 5459.5) 8	4149.3 ± 276.6 (2115.4 - 5077.7) 7	4621.4 ± 130.2 (4124.1 - 5013.5) 7	4608.8 ± 180.4 (3500.9 - 5192.5) 8	4000.3 ± 228.1 (2988.7 - 4903.1) 7	4410.5 ± 195.0 (3709.8 - 5003.6) 7	4869.1 ± 326.3 (4542.8 - 5195.4) 2

Pb	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
S	3236.5 ± 200.7 (1995.9 - 3780.1) 8	3035.6 ± 169.5 (2115.4 - 3405.3) 7	3275.0 ± 132.6 (2629.8 - 3660.5) 7	3367.1 ± 176.5 (2567.8 - 3907.2) 8	2901.4 ± 200.8 (2385.5 - 3958.8) 7	3145.4 ± 147.0 (2585.0 - 3679.8) 7	3444.2 ± 55.7 (3388.5 - 3499.9) 2
Sr	4.4 ± 0.3 (2.6 - 5.3) 8	3.9 ± 0.2 (2.8 - 4.7) 7	4.4 ± 0.1 (3.8 - 4.8) 7	4.2 ± 0.2 (3.2 - 4.8) 8	3.0 ± 0.2 (2.3 - 3.7) 7	2.9 ± 0.1 (2.3 - 3.3) 7	2.9 ± 0.03 (2.9 - 2.9) 2
U	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Zn	40.6 ± 2.5 (26.6 - 49.9) 8	39.7 ± 1.9 (35.6 - 50.2) 7	41.7± 1.4 (35.5 - 47.0) 7	43.8 ± 1.8 (32.6 - 50.1) 8	37.9 ± 3.4 (26.6 - 51.7) 7	39.9 ± 1.5 (36.0 - 46.1) 7	45.2 ± 4.5 (40.7 - 49.8) 2

LOQ: Limit of quantification

Table S6. The mean ± SE sample dry weights (mg), total number of samples analyzed, upper bound tissue concentrations (mg/kg dry weight) of the 3-d-old larvae in each $MnSO_4$ exposure concentration and control. The minimum and maximum tissue concentrations presented in parentheses and numbers of samples above the LOQ with RSD < 10 %. The LOQ values for each element are the same as for water data and in cases when all analyzed samples were below the LOQ or had RSD above 10 % are indicated as < LOQ.

$MnSO_4$ concentration (mg/L)	Control (5.5)	5.6	5.9	12.8	41.8	197.8
Mean dry weight ± SE	13.2 ± 1.0	14.2 ± 0.8	14.4 ± 0.7	14.2 ± 1.0	13.2 ± 0.8	1.8 ± 0.4
Total *n* analysed	17	17	20	18	19	3
Al	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
As	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ca	531.4 ± 35.9 (196.0 - 692.0) 17	499.2 ± 39.8 (260.0 - 833.4) 17	501.5 ± 37.4 (44.2 - 701.2) 19	506.2 ± 52.1 (55.1 - 861.7) 18	399.9 ± 27.1 (159.1 - 590.2) 19	< LOQ
Cd	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Co	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cr	0.8 ± 0.2 (0.5 - 4.7) 2	< LOQ	0.7 ± 0.2 (0.4 - 4.1) 1	0.7 ± 0.1 (0.4 - 2.3) 1	0.7 ± 0.1 (0.5 - 1.5) 1	< LOQ
Cu	3.5 ± 1.0 (0.8 - 13.8) 5	2.5 ± 0.7 (0.8 - 10.2) 5	3.5 ± 0.8 (0.8 - 12.4) 8	2.9 ± 1.3 (0.8 - 24.7) 7	2.6 ± 0.7 (0.9 - 10.2) 5	< LOQ

Fe	8.5 ± 1.1 (1.6 - 14.1) 16	8.5 ± 1.4 (0.2 - 22.6) 15	9.0 ± 1.1 (1.4 - 18.6) 19	9.1 ± 1.5 (0.2 - 20.4) 16	10.4 ± 1.4 (0.5 - 19.9) 18	< LOQ
K	7970.4 ± 398.2 (4976.2 - 9943.4) 17	7650.2 ± 468.0 (4545.0 - 11681.5) 17	7980.5 ± 397.8 (4768.1 - 10795.9) 20	7709.9 ± 577.0 (2918.7 - 12169.1) 18	7552.6 ± 365.3 (3934.2 - 9571.0) 19	6632.0 ± 391.3 (5849.5 - 7032.5) 3
Mg	734.3 ± 34.9 (451.5 - 905.5) 17	710.2 ± 42.6 (430.4 - 1032.2) 17	701.8 ± 48.5 (49.7 - 954.3) 19	735.2 ± 51.2 (293.8 - 1107.4) 18	750.3 ± 35.7 (381.9 - 925.7) 19	532.5 ± 33.2 (481.1 - 594.7) 3
Mn	0.3 ± 0.1 (0.2 - 1.9) 2	0.4 ± 0.1 (0.2 - 1.5) 1	0.4 ± 0.1 (0.2 - 2.2) 4	2.7 ± 0.3 (0.8 - 5.2) 17	25.7 ± 2.4 (11.9 - 50.4) 19	46.7 ± 11.3 (34.6 - 69.3) 3
Na	2457.4 ± 113.9 (1531.6 - 3145.2) 17	2462.7 ± 160.0 (1433.2 - 3717.5) 17	2395.8 ± 113.8 (1316.5 - 3131.4) 20	2486.0 ± 194.4 (938.9 - 4202.7) 18	2645.8 ± 168.9 (1204.0 - 4708.0) 19	1695.4 ± 258.5 (1341.6 - 2198.7) 3
Ni	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
P	6206.0 ± 285.5 (3874.0 - 7627.2) 17	6087.0 ± 359.7 (3529.4 - 9065.1) 17	6026.5 ± 297.2 (3611.1 - 7805.6) 20	6199.6 ± 416.3 (2502.2 - 9313.3) 18	6413.9 ± 300.0 (3308.1 - 7991.6) 19	4672.4 ± 459.9 (4096.9 - 5581.5) 3
Pb	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

S	3271.0 ± 169.2 (2111.4 - 4123.3) 17	3298.7 ± 203.9 (1781.1 - 4856.4) 17	3268.9 ± 170.8 (1903.1 - 4221.1) 20	3379.8 ± 232.0 (1169.2 - 4985.9) 18	3529.6 ± 170.4 (1675.2 - 4544.8) 19	2551.6 ± 300.6 (2064.0 - 3099.9) 3
Sr	2.6 ± 0.1 (1.5 - 3.4) 16	2.4 ± 0.2 (1.4 - 3.7) 17	2.5 ± 0.1 (1.3 - 3.5) 19	2.5 ± 0.2 (1.0 - 4.2) 18	2.4 ± 0.1 (1.0 - 3.4) 19	< LOQ
U	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Zn	43.0 ± 2.1 (29.0 - 53.6) 17	43.2 ± 2.6 (22.1 - 65.4) 17	42.5 ± 2.5 (22.3 - 56.3) 20	43.3 ± 2.7 (15.3 - 57.0) 18	47.1 ± 2.4 (20.6 - 65.3) 19	32.2 ± 5.0 (23.9 - 41.3) 3

LOQ: Limit of quantification

Table S7. Target and reference gene primer information and reference.

Gene	Accesion no.	Forward primer (5'-3')	Reverse primer (5'-3')	Product length (bp)	Efficiency (%)	Ref.
MT-A	X97274	CCT TGT GAA TGC TCC AAA ACT G	CAG TCG CAG CAA CTT GCT TTC	101	93.4	[32]
MT-B	BT059884.1	GAT CCT GTA AGT GCG CCA AC	CTT GCC CTT ACA CAC ACA GC	110	93.1	-
Cat	BT059457.1	CAT GCT GTT CCC ATC CTT CG	CTC CAG AAG TCC CAC ACC AT	87	95.2	-
GstT	BT050013.1	CCC ATG GCT CAA AGG TGT TC	CCA CCA GGT CTG CTA GTG AG	186	94.3	-
Beta actin	NM_001124235.1	AGC GTA AAT ACT CCG TCT GGA TCG	CCT GCT TGC TGA TCC ACA TCT GTT	81	96.9	-
RL2	NM_001165173.1	TTG AGA CAT GCA TGG CCA CAG T	CGG TTC CTG CCA GCT TTA CCA AT	82	98.8	-

Table S8. The significance of the exposure concentration and female on the egg and larval Mn and S tissue concentrations.

Eggs	df	F	P
Mn, Concn.	6	207.137	< 0.001
Mn, F	3	3.098	0.049
Mn, Concn.*F	15	1.331	0.268
S, Concn.	6	0.795	0.584
S, F	3	1.093	0.374
S, Concn.*F	15	0.694	0.764
3-days-old larvae			
Mn, Concn.	5	58.810	< 0.001
Mn, F	3	1.069	0.368
Mn, Concn.*F	12	1.361	0.205
S, Concn.	5	0.833	0.531
S, F	3	0.102	0.959
S, Concn.*F	12	0.948	0.505

Mn: Tissue Mn concentration

S: Tissue S concentration

Concn.: $MnSO_4$ exposure concentration

F: Female

Table S9. The number of valid embryo/larval qPCR samples for each target gene and parent pair.

Gene and parent pair	$MnSO_4$ (mg/L)					
	Control (5.5)	5.6	5.9	12.8	41.8	197.8
cat						
F2 x M1	2/3	3/3	3/3	3/3	2/3	3/-
F3 x M2	3/-	2/-	3/-	2/-	3/-	1/-
F4 x M2	3/2	3/2	3/3	2/3	3/3	2/-
gstt						
F2 x M1	2/2	3/3	3/3	3/2	3/1	3/-
F3 x M2	3/-	3/-	3/-	3/-	3/-	1/-
F4 x M2	3/2	3/1	3/1	2/2	3/2	3/-
mt-a						
F2 x M1	3/3	3/3	3/3	3/3	3/3	3/-
F3 x M2	3/-	3/-	3/-	3/-	3/-	1/-
F4 x M2	3/2	3/2	3/3	2/3	3/3	3/-
mt-b						
F2 x M1	3/3	2/3	2/3	3/3	3/3	3/-
F3 x M2	2/-	3/-	3/-	3/-	3/-	1/-
F4 x M2	3/2	3/2	3/3	2/3	3/3	3/-

II

FISH EMBRYO SURVIVAL AND GROWTH IN NATURAL BOREAL STREAMS AND DOWNSTREAM A MULTI-METAL MINE USING A BIOHEAPLEACHING TECHNIQUE

by

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III

CONDITION AND SPERM CHARACTERISTICS OF PERCH (*PERCA FLUVIATILIS*) INHABITING METAL MINING EFFLUENT CONTAMINATED LAKES

by

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