Road deicing salt and copper: Transfer and effects on fertilization and early life history stages of Atlantic salmon (*Salmo salar*)

Veisalt og kopper: opptak og effekt på tidlige livsstadier på Atlantisk laks (*Salmo salar*)

Philosophiae Doctor (PhD) Thesis

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Preface and acknowledgements

The current thesis is based on research conducted during the period 2010-2015 at the Norwegian University of Life Sciences (NMBU), Department of Environmental Sciences (IMV) in collaboration with the Norwegian Public Road Administration (NPRA) and the Higher Education Commission Pakistan (HEC). The experimental work performed during the PhD period was designed and conducted under supervision and guidance of Professor Hans-Christian Teien, Professor Brit Salbu, and Professor Bjørn Olav Rosseland. The work associated with gene expression was supervised by Professor Knut Erik Tollefsen, NIVA. The doctoral thesis includes four scientific papers, which are either published (paper II) or submitted for publication (paper I, III and IV) to international peer reviewed journals.

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Urma Mahrosh, Ås, June 2015

Summary

Application of road deicing salt in the Nordic countries have increased considerably over the years, in order to enhance good friction and thereby increasing the traffic safety on icy roads during the winter period. The far most applied deicing salt in Norway is made from NaCl (99.5%), and over the past few years the total use of NaCl on the major roads has substantially increased. Road salt can mobilize a series of metals from road sand, silt and dust, and increasing concentrations of road deicing salt and metal mixtures have potentially negative impacts on environment. However, investigations associated with ecotoxicological effects of road deicers on aquatic organism such as fish are rather limited. The current thesis has focused on the ecotoxicological effects of road deicing salts and Cu towards early life stages of Atlantic salmon (*Salmo salar*).

Effects of road deicing salt and Cu on Atlantic salmon were studied under controlled laboratory conditions by exposing early life stages (from fertilization of egg until swim-up) of Atlantic salmon to different concentrations of road salt and Cu, separately and also in mixtures. Water quality parameters such as pH, O_2 and CO_2 in exposure water were not affected by road salt addition, while conductivity and the concentrations of major cations (Na, Ca, Mg and K) and anions (Cl, SO_4) increased by the road salt addition. Using fractionation techniques, results showed that about 50 % of Cu in control water (Lake Maridalsvannet water) as well as in the exposure water was present as colloids and particles, while the remaining fraction was present as mobile and potentially bioavailable LLM Cu species. The concentration of LMM Cu species increased after road salt addition due to mobilization.

Results demonstrated uptake of Na, Cl and Cu in the eggs of Atlantic salmon. The uptake was affected by the exposure concentrations and also the duration of exposure. High uptake during fertilization of egg and swelling is attributed to structural changes of the chorion, allowing the elements to be transported into the egg. After swelling, the uptake is attributed to the association with the surface of chorion. Dissection of eggs exposed to ^{22}Na tracer, stable Cu and Cl solutions demonstrated that Na and Cl were transferred into the egg mainly during the stage of swelling. Na and Cl uptake increased by increasing road salt concentrations. The Cu uptake was, however, not affected by the road salt addition. Episodic exposure of eggs to road salt ($\geq$5000 mg/L) during fertilization resulted in reduced swelling and low egg survival compared to control. The dose-response relationships between the road salt exposure and swelling ($p=0.001$) and mortality of exposed eggs were significant ($p<0.001$).

Based on quantitative rtPCR analysis of eyed eggs exposed to a range of road salt solutions (0, 50, 100, 500 and 5000 mg/L), a positive road salt concentration-dependent increase in Cyp4A14, a gene involved in lipid turnover and renal function, and Nav1, a gene involved in neuraxonal development were observed in exposed eggs. A NOEC value of 50 mg road salt/L is suggested for responses at the molecular level.

When alevins were exposed to road salt (100-1000 g/L), reduced survival and growth, increasing formation of deformities and delayed swim- up were observed, compared to control. Although Cu exposure (≥ 10 µg/L) resulted in reduced swelling of eggs, no effects on exposed alevins could be were observed. Exposure of egg and alevins to road salt and Cu mixtures, however, a series of endpoints were affected such as delayed hatching of eggs, increased formation of deformities, reduced growth, delayed swim-up and reduced survival.

The present results have demonstrated that the road salt and Cu represent a multiple stressor mixture, having additive or more than additive (synergistic) effects. The key stressor is the road salt containing especially NaCl (98 %), and a series of effects are observed when fertilized egg are exposed during swelling, and alevins are exposed from hatching to swim-up. The effect are more pronounced when Cu is added to the mixtures, although the effects induced by Cu alone is modest. Thus, the road salt and Cu mixture represent a multiple stressor exposure inducing additive or more than additive (synergistic) effects; more than additive (e.g., swelling of eggs, mortality of alevins). For exposed eggs, the most sensitive responses are: gene regulation ≥ delayed hatching ≥ reduced swelling and mortality (reduced survival). For exposed alevins, the most sensitive responses are: swim-up, growth and mortality.

The present results document that the early life history stages of Atlantic salmon, from fertilization to swim-up, are very sensitive towards road salt exposure containing additional stressors such as Cu. Such mixtures are expected under natural conditions as road run-off especially under spring snowmelt, when road salts are used for deicing purposes. Thus, aquatic organisms such as Atlantic salmon in downstream receiving waters could potentially be affected. As most of the affected endpoint are of relevance not only on the individual level, but also on the population level having an environmental impact of concern. It is therefore recommended that the use of high concentrations of road salt should be restricted. Furthermore, the road salt application close to downstream rivers or brooks should be avoided during the time of spawning (early October- December, depending on geography and strain differences in spawning period) and late winter-early spring to protect early life history stages of Atlantic salmon.

Sammendrag

Veisalt er i økende grad blitt benyttet til å avise snødekte og glatte veier i de nordiske land for å bedre friksjonen og derved øke trafikksikkerheten. Veisaltet som vanligvis benyttes i Norge inneholder for det meste NaCl (99.5 %), og over de siste årene har bruken av veisalt økt betraktelig. Da veisalt kan mobilisere metaller for eksempel Cu som er assosiert til sand, silt og veistøv, kan økende konsentrasjoner av veisalt i blanding med andre forurensninger potensielt ha negative effekter på miljøet. Undersøkelser knyttet til økotoksiske effekter av veisalt er imidlertid meget begrenset. Dette arbeidet fokuserer derfor på økotoksiske effekter av veisalt og Cu på tidlige livsstadier av Atlantisk laks (*Salmo salar*).

Effekter av veisalt og Cu på Atlantisk laks ble studert under kontrollerte laboratoriebetingelser ved å eksponere tidlige livsstadier (fra befruktning til starforing) av laks til veisalt og Cu, individuelt og i blandinger. Vannkvalitetsparametere som pH, O_2 and CO_2 i eksponeringsløsningene forble uendret ved tilsetning av veisalt, mens ledningsevnen og konsentrasjonen av hovedkationer (Na, Ca, Mg and K) og anioner (Cl, SO_4, tilsetningsstoff (E 535) $Na_4Fe(CN)_{6,}$) økte med tilsetting av veisalt. Ved hjelp av fraksjoneringsteknikker, viste resultatet at ca 50 % av Cu i kontrollvannet (Maridalsvannet) og i Cu eksponeringsløsningene var tilstede som kolloider og partikler, mens restfraksjonen var tilstede som mobile og presumtivt biotilgjengelig lavmolekylære (LLM) Cu spesier. Konsentrasjonen av LMM Cu spesier økte ved tilsetning av veisalt.

Resultatene viste at Na, Cl og Cu ble tatt opp i befruktede egg fra laks. Opptaket var påvirket av konsentrasjonen i eksponeringsløsningen, og eksponeringstiden. Høyt opptak under befruktning av egg antas å skylde strukturelle endringer i chorion som tillot elementene å trenge inn i egget. Etter svelling var opptaket knyttet til assosiasjoner/binding til overflaten av chorion. Disseksjon av egg som var eksponert med hensyn på ^{22}Na, stabilt Cu og Cl løsninger viste at ^{22}Na og Cl ble transportert inn i egg hovedsakelig under svellefasen. ^{22}Na og Cl opptaket økte med økende veisalt konsentrasjoner, mens Cu opptaket var uavhengig av salttilsetning. Episodisk eksponering med veisalt ($\geq$5,000 mg/L) på egg under befruktning medførte også redusert svelling og lav overlevelse sammenlignet med kontroll. Dose-respons relasjonene mellom eksponering (veisalt konsentrasjon) og svelling ($p=0.001$) og dødelighet av eksponerte egg ($p<0.001$) var signifikant.

Basert på kvantitativ rtPCR analyse av befruktet egg som var eksponert til ulike veisaltløsninger (0, 50, 100, 500 and 5000 mg/L), viste resultatene en positive veisalt konsentrasjons - avhengig økning i Cyp4A14, et gen som er involvert i fett omsetning og nyre utskillelse, og i Nav1, et gen som er involvert i utvikling av nervesystemet. NOEC (ingen-effekt konsentrasjon) verdi på 50 mg road salt er foreslått for responser på molekylært nivå.

Når plommesekkyngel ble eksponert med veisalt (100-1000 g/L), økte dødeligheten, veksten ble redusert, deformasjonene økte og utviklingen av starforingsklar yngel ble forsinket sammenlignet med kontroll. Selv om Cu eksponering ($\geq$ 10 μg/L) medførte redusert svelling av egg, hadde Cu eksponering ingen observerbare effekter på plommesekkyngel. Eksponering med veisalt og Cu på befruktede egg og startforingsklar yngel hadde imidlertid effekter på en rekke endepunkter som forsinket klekking av egg, økt andel med deformiteter, redusert vekst, forsinket utviklingen av starforingsklar yngel og økt dødelighet signifikant.

Resultater fra dette arbeidet har vist at veisalt og Cu utgjør en "multiple stressor" blanding som kan bidra til additive eller mer enn additive (synergistiske) effekter. Den viktigste «stresseren» er veisalt som spesielt inneholder NaCl (98 %), hvor en rekke effekter ble observert når egg ble eksponert under svelling og når plommesekkyngel ble eksponert fra klekking til utviklingen av starforingsklar yngel. Effekten var imidlertid langt mer uttalt hvis Cu ble tilsatt veisalt blandingen, selv om Cu eksponering alene medførte små til ubetydelige effekter. Veisalt og Cu i blanding representerer derfor en "multiple stressor" eksponering som induserte mer enn additive (synergistiske) effekter (e.g., redusert svelling, dødelighet for plommesekkyngel). For veisalteksponerte befruktede egg, var de mest sensitive responser: gen regulering > forsinket klekking > dødelighet. For veisalteksponert plommesekkyngel, var de mest sensitive responser: vekst og utvikling til starforingsklar yngel > vekst og dødelighet.

Resultatene fra dette arbeidet viser at tidlige livsstadier av Atlantisk laks, fra befruktning til startforing, er svært sensitive med hensyn på eksponering av veisalt som også kan inneholde andre miljøgifter som Cu. Slike blandinger forventes Under realistiske forhold som veiavrenning særlig under perioder med snøsmelting når veisalting har blitt benyttet til avising kan avrenning inneholde slike veisaltblandinger. Akvatiske organismer i nedstrøms vannforekomster kan derfor potensielt bli negativt påvirket. Da mange av de identifiserte responser har betydning, ikke bare på individnivå, men også på populasjonsnivå, kan miljøproblemene være betydelige. Det anbefaler derfor at høye konsentrasjoner av veisalt begrenses. I tillegg bør bruk av veisalt i områder med avrenning til gyteområder i vassdrag med laks og laksefisk begrenses i gyteperioder (tidlig oktober til desember, avhengig av geografi og artsforskjeller), samt senvinter/tidlig vår for å beskytte tidlige livsstadier av laks

List of papers

Current thesis is based on 4 individual Papers, which are referred to in the text by their Roman numerals:

Paper I

Mahrosh, U., Rosseland, B. O., Salbu, B., Teien, H. -C. Uptake and distribution of ^{22}Na, Cl and Cu in Atlantic salmon eggs exposed to road salt (NaCl). Submitted

Paper II

Mahrosh, U., Kleiven, M., Meland, S., Rosseland, B. O., Salbu, B., Teien, H. -C. Toxicity of road deicing salt (NaCl) and copper (Cu) to fertilization and early developmental stages of Atlantic salmon (*Salmo salar*). Journal of Hazardous Materials. 280(2014): 331-339. Published

Paper III

Tollefson, K. E., Song, Y., Kleiven, M., Mahrosh, U., Meland, S., Rosseland, B. O., Teien, H. -C. Transcriptional changes in Atlantic salmon after embryonic exposure to road salt (NaCl) by using microarray and qrtPCR. Submitted

Paper IV

Mahrosh, U., Rosseland, B. O., Salbu, B., Teien, H. -C. Impact of road deicing salt (NaCl) and copper (Cu) on Atlantic salmon (*Salmo salar*) alevins from hatching till swim-up. Manuscript

Abbreviations

LMM	Low molecular mass
PAHs	Polyaromatic hydrocarbons
EQS	Environmental quality standards
STF	Statens Forurensningstilsyn (Norwegian Pollution Control Authority)
USEPA	United States Environmental Protection Agency
CMC	Criteria maximum concentration
CCC	Criteria continuous concentration
BLM	Biotic ligand model
HMM	High molecular mass
PVF	Pervitelline fluid
PVS	Perivitelline space
mRNA	Messenger RNA
TOC	Total organic carbon
AADT	Annual average daily traffic
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
ICP-MS	Inductively Coupled Plasma-mass Spectrometry
TMAH	Tetremethylammonium Hydroxide
qrtPCR	Quantitative real-time polymerase chain reaction
MOA	Mode of action
dNTPs	Deoxynucleoside triphosphates
cDNA	Complementary DNA

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Paper I-IV has individual page numbers

1 Introduction

The traffic density has increased the last decades as a result of urbanization, which ultimately results in increase road network. For the road user's safety and ensuring the secure transportation of goods and services, a safe and well operational road network is essential (Meland, 2010). In Norway, from 1984 until today, the road network has increased from approximately 45,000 km to 93,000 km, while the transportation load increased from 2.5 to 60.6 million passenger/km in the same period (OVF, 2008). In order to enhance friction hence increasing the traffic safety, during harsh winter period within northern hemisphere, deicing salts such as sodium chloride (NaCl) are used (Environment Canada, 2001; Marsalek, 2003). Nearly 70,000 tons of NaCl was used on Norwegian roads in 2000/2001, increasing to approximately 20,0000 tons in 2008/2009 (Meland, 2010), while in North America, 14 million tons of salt are applied every year to roads as deicing agents (Environment Canada, 2001).

Road salts are easily transported with the road run off (Karraker *et al.*, 2008) and can persist in the aquatic environment for a long time period (Sanzo & Hecnar, 2006) ultimately causing toxicity towards aquatic organisms such as fish (Vosylienė *et al.*, 2006). In past few years the chloride (Cl) concentrations in freshwater systems of many northern countries have exceeded the background levels due to extensive use of road deicing salts (Kaushal *et al.*, 2005). The detrimental environmental effects of road salt are becoming of increased public concern worldwide and range from increased salt concentrations in groundwater (Kelly & Wilson, 2002), impairment of roadside vegetation (Kayama *et al.*, 2003; Viskari & Kärenlampi, 2000) and deleterious effects on the water quality in streams, rivers and drinking water wells located close to roads.

In addition to deicing salts, runoff water from roads contains a number of toxic pollutants such as hydrocarbons and metals including copper (Cu) (Norrström & Jacks, 1998) probably caused due to mobilization from surfaces of particles and colloids in the presence of elevated concentrations of road salt (Amrhein *et al.*, 1993; Backstrom *et al.*, 2004). The concentrations of these chemicals vary in road runoff as well as in receiving water bodies such as streams and lakes, due to different environmental conditions such as high flow events after heavy rainfall or snowmelt.

Heavy metals are able to persistently accumulate in animal tissues, therefore, they are considered the harmful substances in aquatic environment (Vinodhini & Narayanan, 2008).

Road deicing salts along with metals such as Cu may act a multiple stressors having an additive, synergistic or antagonistic effect on aquatic organisms. Aquatic freshwater organisms, such as fish are sensitive towards pollutants. Among freshwater fish species in Norway, Atlantic salmon (*Salmo salar*) are considered the most sensitive (Rosseland & Staurnes, 1994) that also has a great commercial value in aquaculture to several countries such as Norway. In addition, Jezierska *et al.* (2009) reported that early developmental stages soon after fertilization are mainly sensitive to metal intoxication and during that specific period most turbulences and the highest embryonic mortality may occur.

To evaluate the effects of road deicing salt and Cu on aquatic organisms and improve risk assessments associated with road salt exposure of aquatic ecosystem, information about exposure characterization, bioavailability and biological uptake, as well as biological effects of road deicing salt and Cu mixtures is required. Since the eggs are stationary and they cannot avoid pollutants compared to other free living life stages (Li *et al.*, 1989) road salt runoff might, therefore, be toxic if applied at the time of fertilization, swelling and during the early developmental stages such as alevins, from hatching to swim-up.

1.1 Hypothesis and objectives

Based on the present literature and many years research on trace metal effects on Atlantic salmon, it is hypothesized that:

Road salt will have a negative impact on early life history stages of Atlantic salmon.

Road salt in combination with metals can act as a multiple stressor exposure, and additive or more than additive (synergistic) effects should be expected.

The main objective of the present thesis was, to assess possible effects of road deicing salts and Cu exposure on early life history stages of Atlantic salmon from fertilization till swim-up. To investigate the presence, transfer, speciation, uptake and ecotoxicological effects of environmentally relevant concentrations of road salt and Cu in the road runoff on early developmental stages of Atlantic salmon, laboratory experiments are performed with focus on the following four sub-objectives:

1. Investigate the effect of road salt on mobilization of Cu from road dust.
2. Characterize the speciation of Cu in natural waters and influence of road salt on Cu speciation.

3. Follow the uptake of Na, Cl and Cu in eggs and alevins of Atlantic salmon to obtain information about their bioavailability and distribution pattern, separately and in the mixture.
4. Identify susceptibility of different early life stages (fertilization, swelling, eyed eggs, hatching and swim-up) of Atlantic salmon when exposed to road salt and Cu toxicity separately and in the mixture.
5. Investigate if road salt and Cu exert a multiple stressor effects on various developmental stages of Atlantic salmon when present in the mixture.

The thesis focuses on the area between environmental chemistry (exposure) and ecotoxicology (responses) which includes several scientific disciplines ranging from chemical processes to biological responses. Outline of all papers included in the thesis is given in Fig (1).

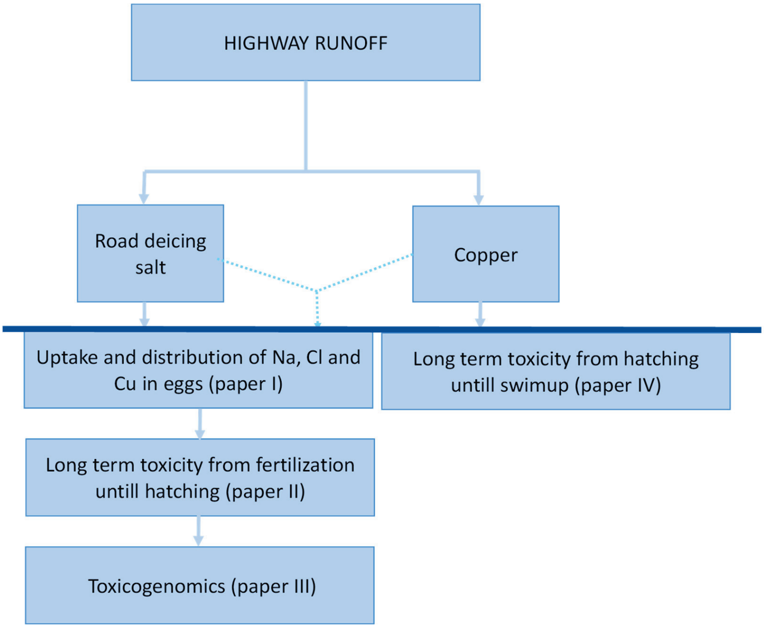

Figure 1. Outline of four Papers (I-IV) included in thesis.

2 Background theory

2.1 Road network in Nordic countries

Natural landscapes and ecosystems have been modified due to increase in human population (McDaniel and Borton, 2002) and resulted in the massive road network, which covers the huge area of many regions (Miller *et al.*, 1996). The increased traffic load has a significant negative effect on the environment in the form of soil and water pollution.

2.2 Road dust

Road dust is composed of several components such as soil, natural biogenic materials and metallic constituents produced from human activity (Ferreira-Baptista & De Miguel, 2005). In addition, all other components of road dust such as mineral constituents, heavy metals, organic matter (humus), water, vehicle exhaust particles, residues from lubrication oil, tire wear particles, organic carbon from plants, are transformed into powder form by heavy traffic (Howari *et al.*, 2004; Shi *et al.*, 2008). Dust particles originate from the traffic (vehicles, oil/petrol, road surface materials), from interactions of solid, liquid and gaseous metals and deposit from the atmosphere and will accumulate along roadsides (Akhter & Madany, 1993; Hjortenkrans *et al.*, 2006).

Heavy metals attach to the surface of soil and also bound with the roadside dust. High metal load leading to metal contamination of the surface environment nearby the road is the result of constant emissions of heavy metals and their deposition in soil over time (Sabiha *et al.*, 2009). Furthermore, road dust is washed away by the precipitation in the form of runoff enabling it to enter water bodies such as streams and lakes in the form of dissolved solids (Ferreira-Baptista, & De Miguel, 2005). Worldwide concentration values of Cu in road dust have been compiled from literature and are given in Table (1). Road dust containing various pollutants is an emerging problem throughout the world affecting both developing and developed countries (Faiz *et al*., 2009).

Table 1. Concentrations of Cu in road dust from different countries.

Country/City	Cu (µg/g road dust)	Reference
Norway (Oslo)	123	(De Miguel *et al.,* 1997)
Sweden	79	(Hjortenkrans *et al.,* 2006)
USA (Cincinnati/Ohio)	253	(Tong, 1998)
Canada (Ottawa)	65.84	(Rasmussen *et al.*, 2001)
France (Paris)	1075	(Pagotto *et al.,* 2001)

2.3 Winter conditions and snow removal

In Nordic countries, different management strategies are required in winter weather events due to severe winter conditions with heavy snowfall (Cuelho *et al.*, 2010). In order to make the roads and pathways easier and safer for travel, snow removal is essential after snow fall. Snow removal involves both chemical and mechanical methods. Different strategies are implemented for snow removal, which include (1) mechanical removal with or without friction enhancements, (2) deicing and (3) anti-icing. In deicing, ice-control products are applied to driving or walking surfaces to melt existing snow and ice. Deicing is performed after snow-removal operations to melt remaining snow and ice. However, proactive application of melting products to driving or walking surfaces before snow fall or storm is called anti-icing. Anti-icing prevents the bonding of snow and ice to the pavement, which further helps workers to clear the surfaces more easily. These strategies can either be used alone or also in combination with another (Blackburn *et al.*, 2004). While selecting an appropriate chemical for snow and ice control chemical, agencies should take into account various factors such as cost, availability, ease of use, corrosion impacts, environmental and health effects of such chemicals (Cuelho *et al.*, 2010).

2.3.1 Sand

Sand is one of the most common abrasives used in winter maintenance. It plays role in winter maintenance programs. Sand provides temporary friction improvement on snow/ice. Use of sand in winter time has been decreased over time due to a variety of limiting factors, such as efficacy, environmental impacts, cost and safety implications. Abrasive materials especially, sand can be used during high winds or storm conditions, which prevent the use of salt. Sand is also used when deicing agents cannot work effectively at roadway temperatures below -14 °C or less (TSR, 2010).

2.3.2 Road deicing with salt

Road salt was first introduced in 1930's for snow melting operations. In 1960's the use of road salt became widespread for the maintenance of highway during winter, (Paschka *et al.*, 1999). The most common chemical products used for winter maintenance activities are sodium chloride (NaCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), calcium magnesium acetate (CMA), and potassium acetate (CH_3CO_2K). Since the early 20th century, NaCl has been preferred over all other chemicals because of less cost, easy availability, easy storage, easy to handling, application, dispersion, and high efficacy (Cuelho *et al.*, 2010).

The amount of road deicing salt used in Norway from 1993-2007 is shown in Fig (2). In North America, approximately 14 million tons of road salt are used annually. It has also been estimated that in 1998, 4.9 million tons of road salts were applied to Canadian roads, which resulted in increased chloride concentration to the environment (Environment Canada, 2001).

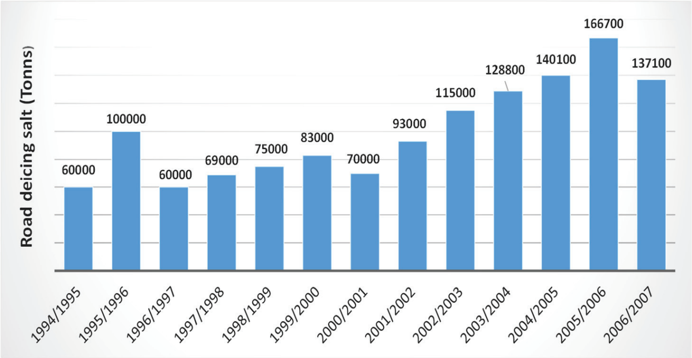

Figure 2. Total salt (included sand) used in Norway from winter 1993-2007 (after Bant, 2009).

2.4 Highway runoff processes

The pattern of runoff varies according to the type of area as the runoff on paved roads and highways is markedly different from the runoff occurring in vegetated pervious area. Highest amount of pollutants are carried by the first fraction of the discharged volume that is transported during one storm event, and is defined as first flush. During the initial phase of road runoff episode, first flush is designated by a rapid and high increase in the pollutant concentrations followed by a consecutive fast decline in concentrations probably due to dilution (Sansalone & Buchberger, 1997). First flush is transported in the first 30 % of the volume discharged by rainfall and contains at least 80 % of the total concentration of pollutants transported from the road during runoff processes (Bertrand-Krajewski *et al.*, 1998).

The concentrations of various pollutants fluctuate in the road runoff and is highly dependent on several factors e.g. weather, climate conditions, time between episodes, density of traffic, amount of studded tires during winter time, size and type of pavement and road maintenance activities such as road cleansing, deicing etc (Meland, 2010).

2.5 Chemical contaminants in highway runoff

Road runoff contains a variety of pollutants, *i.e.* suspended solids, heavy metals and hydrocarbons, ,which are originated by a wide range of sources such as, vehicle exhausts, vehicle and road wear, deicing operations, accidents, and soil erosion (Mangani *et al.*, 2005). Road dust comprises of several elements including Cu, Cd, Ni, Pb, Zn, Al and Be (Table 2), which ultimately become part of the road runoff (Howard, 1993). In addition, road salts are also the component in road runoff especially in the Northern countries where deicing salts are used during harsh winter season.

2.5.1 Organic compounds (PAH)

Road runoff usually contains elevated concentrations of polycyclic aromatic hydrocarbons (PAHs), which consist of 100 semi volatile compounds, which are comprised of two or more benzene rings fused to each other (Srogi, 2007). Different sources such as combustion, tire wears, bitumen from the asphalt and leakage and spill of petroleum products are mainly responsible for the production of these compounds (Kose *et al.*, 2008; Napier *et al.*, 2008).

Organic pollutants released by automobiles in the environment are increasing with time (Napier *et al.*, 2008) and they can be harmful to aquatic organisms resulting in high mortality of exposed animals, reduced growth, yolk sac edema, cardiac failure, larval deformities, formation of abrasions and tumors, impairment of immune system and estrogenic effects (Logan, 2007). Different molecular masses of PAHs are the reason for their different physical and chemical properties (Logan, 2007). In addition, PAHs can also easily be distributed the environment and they pose a risk to all living organisms due to their carcinogenic and mutagenic properties (Meland, 2010). Table (2) shows various pollutants and their sources.

Table 2. Information of heavy metals and road deicing components in the road runoff and their sources based on literature data (after Meland) 2010).

	Source	Pollutant	References
Vehicle	Brakes	Cu, Ba, Fe, Mo, Na, Ni, Pb, Sb	(Dongarra *et al.,* 2009; McKenzie *et al.,* 2009; Sternbeck et al., 2002; Thorpe and Harrison, 2008).
	Tires (incl. studded tires)	Cu, Al, Zn, Ca, Cd, Co, Mn, Pb, W, PAH	(McKenzie *et al.,* 2009; Sternbeck *et al.,* 2002)
Non- vehicle	Deicing salts	Na, Cl, Mg, Ca, Anticaking agents	(Thorpe and Harrison, 2008)

2.5.2 Road deicing salt

Road deicers are used in Norway, other countries on the northern hemisphere and temperate regions worldwide during harsh climatic conditions (Fig. 3) for maintenance and safety of road traffic in winter (Denoel *et al.*, 2010). The total use of NaCl on major highways of Norway has been increased over the past few years due to its abundant use in winter (NPRA, 2007).

Figure 3. Road salt application in winter (after Meland, 2010).

NaCl has extensively been used for snow and ice removal from roads, which has resulted in environmental impacts in many areas. Road salt application give rise to major problems including the erosion of vehicles, highway surfaces, bridges, and most importantly exerting toxicity towards vegetation and aquatic organisms inhabiting the water bodies which receive road runoff containing elevated road salt concentrations (Murray & Ernst, 1976). Earlier in 1960's road salts were extensively used for road maintenance (Paschka *et al.*, 1999), however, currently road deicing salts are generally used in order to increase the snow melting process in winter (Oberts, 1986). Table (2) presents the sources of Cu and road salt based on literature.

Road deicing salts enter into environment (Fig. 4) in following two possible alternative pathways (Ramakrishna & Viraraghavan, 2005).

1. Road salt may become part of road runoff after dissolving into the melting snow.
2. During application in winter the road salt may also be splashed into the surrounding roadside environment further affecting vegetation and aquatic organisms (Ramakrishna & Viraraghavan, 2005).

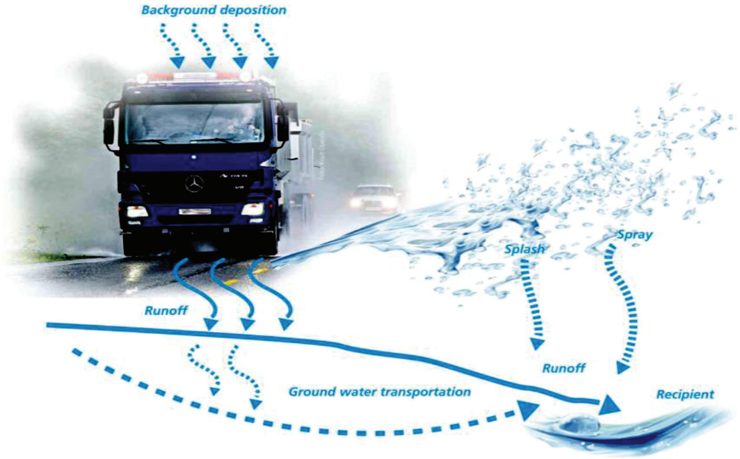

Figure 4. Conceptual drawing of highway runoff transportation (after Meland, 2010).

NaCl or rock salt is most commonly used deicer because it is easy to use, easy to handle and less costly. Rock salts efficiently work at temperatures down to −9 °C by softening the ice and also melting the water content of the snow and ice layer of the treated road surface (Ramakrishna & Viraraghavan, 2005). The concentration of salt in water bodies is increased in water bodies by addition of 39 kg road salt/km^2 daily on roads within the watersheds. Road salt application has resulted in increased concentration of Na^+ and Cl^- in surface waters. Road salt application changes the water chemistry of runoff receiving water bodies resulting in increased

Cl concentrations, change of the density gradient of water, which results in more algal growth. In addition, road salt also changes the soil chemistry and alter the ground water quality (Ramakrishna & Viraraghavan, 2005).

In past few years, research has been performed on highway runoff but limited attention has been given on ecological effects of road salt. Hofstra & Smith (1984) reported increased concentrations of Na^+ and Cl^- in the soil within 30 ft of the roadway but the concentrations decreased to background level at a distance of 90 ft from road. Road salts can spread into the environment in a variety of forms such as aerosol sprays, overland flow and also penetration into the groundwater (Marsalek, 2003). Since chloride is highly soluble in water , therefore, it can accumulate in the wetlands located close to heavily salted highways, where its concentrations can reach 100 times higher than the water bodies unaffected by salt (Kaushal *et al.*, 2005).

2.5.3 Road salt concentrations in environment

With increasing urbanization, the area requiring deicer application also increases, which further increases the amount of road salt transported to surface waters (Gardner & Royer, 2010). Na^+ and Cl^- entering water bodies in the form of road run-off are present in a molar ratio of 1:1. Chloride is considered to be the main toxic component of deicers and it can detrimentally affect aquatic life (Sanzo & Hecnar, 2006). Storms and overland flows in winter months increase the Cl concentration from road salt application. Table (3) shows the concentration of road salt in water bodies of different parts of the world. Road salt application increases the concentrations of Ca, Mg, K and the anticaking agent, which poses toxicity towards fish (Vosyline *et al.*, 2006). Kaushal *et al.* (2005) observed considerably high amount of road salt in the streams in Baltimore, Maryland and values were in the range of 4600 to 11,000 mg Cl/L.

Table 3. The concentrations of road salt in road runoff.

Road salt concentration (mg/L)	Location	Reference
>18,000 mg/L	Canada	(Environment Canada, 2001)
150 mg/L	Rural lakes Canada	(Environment Canada, 2001)
5000 mg/L	Urban lakes and snow cleared from streets Canada	(Environment Canada, 2001)
4000 mg/L	Ponds and wet lands Canada	(Environment Canada, 2001)
4300 mg/L	Other watercourses	
18-2700 mg/L	Water bodies adjacent to highways Michigan	(Benbow & Merritt, 2004)
12,463 mg/L	Wetland associated with a sand-salt storage facility	(Ohno, 1990)

2.5.4 Metals

Trace metals are probably the most frequently reported group of contaminants present in road runoff ultimately increasing the risk of metal toxicity towards aquatic organisms such as fish, (Meland, 2010). Sources of metals include brakes linings, wearing of tires included studded tires (e,g. Fe, Ni, Cu, Zn, and Cd), vehicle engines (e,g. Al, Cu and Ni) catalytic converters, fuels (Pb) vehicle body (e,g. Fe, Al and Zn), safety fences (Zn), and combustion of fuel (Legret & Pagotto, 1999). Metals are non-biodegradable therefore they accumulate in nature and after a long residence time may become ‘chemical time bombs’ (Shi *et al*., 2008).

2.5.5 Cu Concentrations

Cu is widely distributed in the environment in the form of naturally occurring element and also as a component of many minerals. It is involved in many vital functions of plants and animals and is also found in surface and drinking water (Faiz *et al*., 2009). Cu is produced from different vehicle sources such as automobile brake abrasion (Hewitt and Rashed, 1990) since the brake pad material also contributes Cu to the environment during brake ware (Davis *et al*., 2001).

Although Cu is an important essential metal but increasing concentrations may be toxic towards fish (Kazlauskiene & Vosyliene, 2008). In unpolluted freshwater systems, the concentration of Cu usually ranges from 2 to 4 µg/L, while in highly polluted aquatic systems the concentration may range up to several folds higher. Environmental quality standards (EQS) for Cu are given in Table (4). Lydersen *et al.* (2002) and Norwegian (SFT) classification ranges from insignificantly polluted (I) to very strongly polluted (IV) and (V) (Table 4). According to USEPA classification, criteria maximum concentration (CMC) is an estimate of the highest concentration of a material in surface water, which does not cause unacceptable effects to aquatic biota exposed acutely to such compounds, while criteria continuous concentration (CCC) is an estimate of the highest concentrations of a material in surface water,which does not cause adverse effects to aquatic organisms chronically exposed to such chemicals (Heier, 2010).

Table 4. Environmental quality standards for Cu applicable for surface water after (Heier, 2010).

Criteria	Class	Cu µg/L
Norwegian (SFT)*	I	<0.6
	II	0.6-1.5
	III	1.5-3
	IV	3-6
	V	>6
Lydersen *et al.,* 2002	I	<3
	II	3-15
	III	16-30
	IV	>30
US EPA	CMC	13
	CCC	9

*Statens Forurensningstilsyn (Norwegian Pollution Control Authority)

2.6 Metal speciation

Trace elements in water are present in different physico-chemical forms. Salbu (2009) suggested a more comprehensive definition of speciation. According to this definition “trace element species should be defined according to their physico-chemical properties; nominal molecular mass, charge properties and valence, oxidation state, structure and morphology, density, degree of complexation etc". In addition, speciation analysis was defined as "the analytical activity of fractionating, isolating, identifying and quantifying one or more individual trace element species in a sample, and should include in situ, at situ, online, in laboratory fractionation techniques applied prior to analysis".

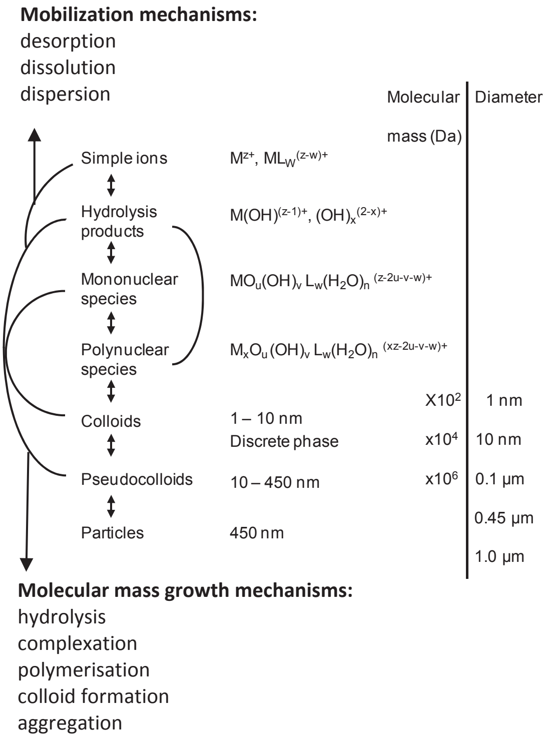

Figure 5. Transformation processes changing the distribution of Cu species (after Salbu, 2000)

Processes such as hydrolysis, polymerization and complexation and colloid formation will influence the physico-chemical forms of an element and will increase the molecular mass of the species (Fig. 5). On the other hand, processes such as desorption, dissolution and dispersion processes can mobilize the elements (Salbu, 2000). Thus, the system is dynamic and changes in the distribution of species occur. For instance in low water pH, Cu is present in low molecular mass (LMM) species, which is more mobil and potentially bioavailable than colloids and hence can easily be taken up by aquatic organisms (Fig. 6).

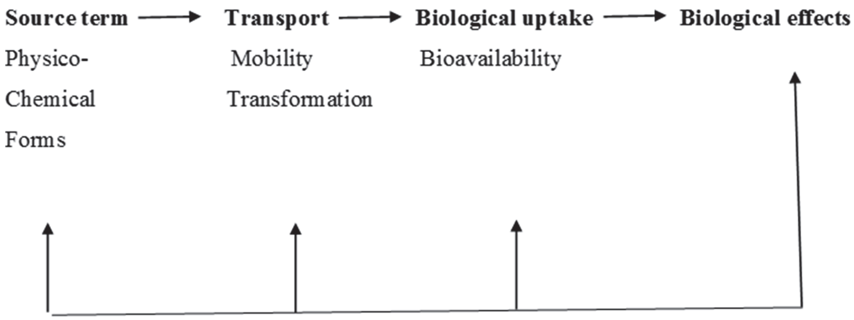

Figure 6. Processes influencing the biological uptake and effects of different physico-chemical forms (after Salbu, 2000).

2.6.1 Factors affecting speciation

Road salt and heavy rains may result in change of water quality such as increased ionic strength, reduced pH, and increased total metal concentration. Different physico-chemical forms of metals may change after such events, as reported by high flow events following sea salt episodes in natural catchments (Rosseland *et al.*, 1992; Teien, 2005). Hydrolysis, polymerization, aggregation, complexation and colloid formation changes physico-chemical forms, resulting in an increase in molecular mass. On the other hand, elements are mobilized by processes such as desorption, dissolution and dispersion (Salbu, 2000, Teien *et al.*, 2006).

Conductivity, pH, and presence of organic and inorganic ligands also affect metal speciation (Teien *et al*., 2004). Speciation of the metal is often related to the its bioavailability, therefore, speciation and bioavailability are interconnected to eachother (Peakall and Burger, 2003). Water pH strongly influences the chemical speciation of Cu. At pH 7 and higher, Cu is predominantly found as carbonate and hydroxide species, while at water pH below 7 there is a rapid increase of free Cu ions (Miwa *et al.*, 1989). At low pH most metals are present in low LMM species (Salbu, 2000).

2.7 Bioavailability and Biological uptake of metals

2.7.1 Bioavailability

Bioavailability of any substance has been defined in several ways. Hare (1992) defined bioavailability as a part of a chemical, which can possibly be taken by organisms. According to Fairbrother *et al.* (2007) bioavailability represents the degree to which various compounds cross the biological membrane under a certain time period. According to Chapman (2008) bioavailability is defined as the fraction of bioaccessible compound that is currently available for uptake by organisms. It includes both bioaccessible fractions that is bioavailable right away and also portions that may become bioavailable with the time. Bioavailability of compounds include physico-chemical availability, bioavailability and toxicological bioavailability (Landner & Reuther, 2004).

2.7.2 Bioavailability of Cu

According to the biotic ligand model (BLM) theory, metal ions compete for binding to biological surfaces (Grosell and Wood, 2002) such as fish gills, egg chorion and after crossing the membranes, they react with enzyme and other internal biomolecules. In natural waters, the metal bioavailability can be reduced due to high pH of water and presence of inorganic anions such as Cl^-, OH^- etc., and presence of organic matter by reducing the concentration of free metal ions and making complexes (Teien *et al.*, 2004). In addition, major cations such as Ca and Mg provide protection against metal toxicity (Fig. 7) by competing for active sites on biological surfaces (Paquin *et al.*, 2002). Environmental mobility and bioavailability depends on the chemical fraction of a metal (Norrstrom & Jacks, 1998). High molecular mass (HMM) species such as colloids, polymers, pseudocolloids and particles are assumed to be more nonreactive species, while LMM species such as ionic forms, are considered more mobile and hence potentially bioavailable (Salbu, 2009). In addition to other factors, such as pH plays an important role in speciation and hence bioavailability of metals. Different species of Cu with different bioavailability are found at various pH levels. Among different Cu species, free Cu ions (Cu^{2+} and Cu^+) are more bioavailable and hence more toxic in aquatic system (Petersen, 1982). Speciation of Cu is highly dependent on pH (Sauve *et al.*, 1997).

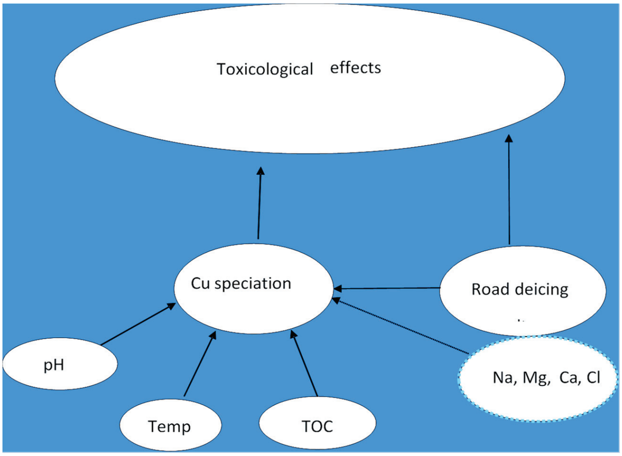

Figure 7. Factors affecting the Cu speciation and toxicological effects.

2.7.3 Uptake and accumulation

Different life history stages of fish (such as eggs and alevins) have different routes of metal uptake. Eggs, are always found in a stationary state and they cannot move, therefor cannot avoid or excrete pollutants (Xu *et al.*, 2009), however, freely moving life stages have more chances to avoid the chemicals. Early developmental processes of many aquatic organisms are believed to be most vulnerable to environmental pollution (Somasundaram *et al.*, 1984).

After spawning in fresh water, the eggs of Atlantic salmon may be subjected to various pollutants, which affect different life stages ultimately effecting the offspring's quantity and quality. Pollutants can easily be taken by fish eggs, especially, during fertilization and swelling (Jezeirska *et al.*, 2009). In addition, egg chorion contains carboxyl- and sulfhydryl rich groups, which provide the site for metal binding (Lacoue-Labarthe *et al.*, 2008; Rainbow, 1997). Metals may, therefore, attach with the chorion and passively move inside the eggs by accumulating into the perivitelline fluid surrounding inside the developing embryo (Xu *et al.*, 2009).

Freshwater fish can accumulate metals through two main routes, which include the body surface (gills, skin) or by ingesting food. Due to high surface area, the gills are easily exposed to several pollutants and provide the major route of waterborne pollutants. Fish gills are involved in many functions such as respiration, acid-base regulation, ion-regulation and excretion of nitrogenous wastes (Wood, 2001). The surface area of gills is covered with mucous, which protects the gill surface and consists of glycoproteins, mycopolysaccarides, amino acids and water (Schlenk & Benson, 2001).

2.8 Multiple stressors

Almost all living organisms face stress in the environment in various forms at different stages of their life history and the process of natural selection is highly affected by such exposures. The process which can bring the organisms near to or over the edges of their fundamental ecological niche is called ecological stress (Van Straalen, 2003). Stressors refer to environmental factors that cause stress in an organism. A stressor can be abiotic (e.g. pollutants, organic compounds, trace metals or radionuclides and environmental factors e.g. temperature, pH or radiation). Stressors are produced by different sources which may include a natural event (e.g. volcano eruption, earthquake, flooding or cosmic activity) or by anthropogenic activities (e.g. use of chemicals etc) (Song, 2014).

In the environment pollutants occur usually as mixtures, and the toxicity of such mixtures may act additively (1+1=2), synergistically (1+1>2) or antagonistically (1+1<2) (Eggen *et al.*, 2004; Salbu *et al.*, 2005). Chronic exposure of low concentrations of contaminants may result in various biological end points leading from altered gene expression to physiological processes such as reduced growth and death (Salbu *et al.*, 2005).

2.9 Sensitivity of different life history stages of Atlantic salmon towards different stressors

Atlantic salmon undergoes various stages during its development. Early developmental stages of fish, compared to adults, are very sensitive toward various toxicants. Taking the sensitivity of early life stages into account, in addition to the yolk sac stage the embryonic stage prior to completion of gastrulation is also considered very sensitive towards the exposure of pollutants (Westernhagen von, 1988). Since the fish larvae have more surface to volume ratio and high metabolic activity, which makes them more in contact with the ambient toxicant and hence more sensitive (Flik *et al.*, 2002). Even very small concentrations such as submicromolar amounts of Cu have been found very effective in malformation of larvae of Common Carp by disturbing normal skeletal development and hydromineral balance, (Stouthart *et al.*, 1996). It is also assumed that high sensitivity of larval stages are due to undeveloped hypothalamopituitary interrenal (HPI) axis, which helps vertebrates to cope with environmental stressors (Flik *et al.*, 2002).

2.9.1 Biological effects

Biological endpoints, which are also called umbrella end points/biomarkers that include reduced egg swelling, high mortality, delayed hatching, delayed swim up, deformities, gene expression, ion regulation and gill toxicity. Biological endpoints are defined as the biological effects caused by any pollutant. These end points include mutations, morbidity and mortality. In addition to these negative end points some organisms activate a defensive mechanism that results in increasing survival rates, number of offspring and growth. Some organisms adapt themselves to condition and environment by changing their physiology, biochemistry or DNA (Copplestone *et al.*, 2008).

2.9.2 Fertilization success

Fertilization is the process of formation of diploid zygot by fusion of male and female gametes. In majority of fish species, sperms and eggs are produced in separate individuals and are expelled into external aquatic environment for fertilization, where they can be exposed to various pollutants. Fertilization success can thus be affected by these environmental pollutants. According to Jezierska *et al.* (2009) spermatozoa motility time can be affected by metals, which further disrupts the successful fertilization of fish eggs. Daye & Glebe (1984) observed that fertilization success and sperm motility is negatively affected by acidic waters. Stekoll *et al.* (2009) also reported reduced fertilization success of salmonids after exposing eggs to total dissolved salts (250 mg/L) during fertilization.

2.9.3 Degree of swelling

Fish eggs swell, when enter into water. Changes in the permeability of chorion occur, when the eggs are released into hypotonic solution (Coward *et al.*, 2002). A perivitelline space (PVS) is formed during swelling, which makes up 85 % of the total egg volume (Fig. 8). Embryonic volume increases during the development and space for this volume increase is provided by PVS (Li *et al.*, 1989). Swelling occurs due to breakdown of cortical alveoli, which releases colloidal material when they break down on egg activation. As soon as the eggs are exposed to the treatment water they start to swell by the process of hydration. Egg volume increase during swelling, depends upon the adsorption of water from the ambient media (Lønning & Davenport, 1980) and within this time period each liter of dry eggs give 6-9 liters of swollen eggs. Exposure to toxic compounds might reduce fertilization success and change the degree of swelling. Thus, a reduced PVS and a suboptimal PVF is not a good environment for the developing embryo. Reduction in PVS formation and hence reduced swelling of Atlantic salmon eggs was observed by Li *et al.* (1989) after exposing to different salinity levels (<1000 mg/L, and between 2700-3100 mg/L).

Chorion of egg is highly permeable during the process of swelling, but its structure and permeability can be changed due to metal ion exposure and uptake. In addition, soluble pollutants can easily enter into the egg during the process of swelling and may also affect the further embryonic development (Jezierska *et al.*, 2009). Jezierska *et al.* (2009) observed in their experiments that Cu, Cd and Pb reduced the swelling of Common carp (*Cyprinus carpio*) eggs in concentration-dependent way. Stouthart *et al.* (1994) observed that ion exchange disturbance between perivitelline fluid and external medium might be caused by a membrane permeability change due to pollutants binding.

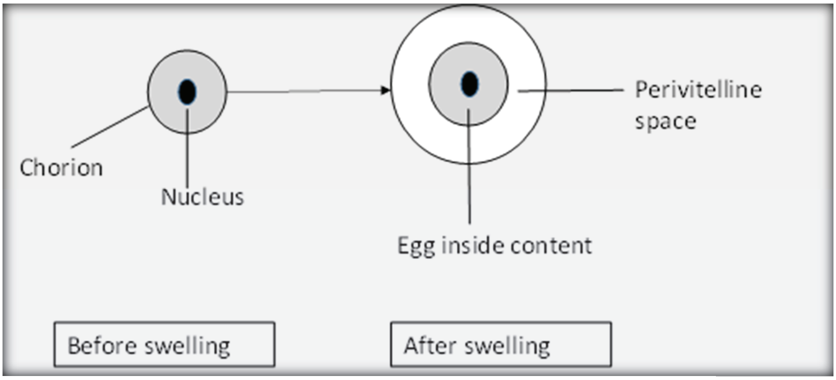

Figure 8. Process of swelling in fish egg immediately after fertilization.

2.9.4 Eyed embryo development and hatching time

Eyed embryo is the stage of embryonic development, when the eye get pigmented. Time of development of eyed embryo stage can thus be identified. Exposure to toxic compounds such as road salt and Cu might affect the development and thus the time of development of eyes in developing Atlantic salmon embryos. Lugowska & Jezierska (2000) observed developmental retardation at the stage of eye pigmentation after exposing the *Cyprinus carpio* embryos to Cu solution (200 µg Cu/L).

Embryos are hatched, when they reach the physiological condition, where available oxygen in the pvf and macroenvironment surrounding the egg is less than respiratory demands of embryo (Yamagami, 1988). Developing embryos may hatch earlier the normal due to certain factors such as insufficient water exchange around the egg, increase in water temperature and decrease in the oxygen concentration dissolved in the water (Keinanen, 2002). Change in time of hatching, expressed as degree days, thus gives information about the influence of pollutants on development of the embryo. Jezierska *et al.* (2009) reported that heavy metals may influence hatching by accelerating or inhibiting this process. Thus the rate of fish embryonic development is considered to be highly affected by metals. Lugowska & Jezierska (2000) observed delayed and extended hatching of *Cyprinus carpio* embryos incubated at 20 ºC with the exposure solution containing 20 µg Cu/L.

2.9.5 Swim-up time and growth

Yolk sac fry is defined as the embryo, which has freed itself from the chorion (Balon 1975) and swim-up stage can be identified when alevins have absorbed or utilized the yolk sac and the fry is ready to start eating. Temperature plays an important role in speed and efficiency of yolk absorption (Blaxter, 1991). At the time of hatching, the fish gills are not completely developed to perform the osmoregulatory functions and, therefore, exchange of respiratory gases occurs through the skin of alevins (Wells & Pinder, 1996). During early developmental stages (Fig. 9) the fish gills are mainly used for ion regulation and not the gaseous exchange (Li *et al.*, 1995). Newly hatched alevins have rather a very simple physiology including very simple gut and kidney, colorless and acellular blood, gills without filaments and skin lacking scales and pigments. All these features develop with time and growth (Blaxter, 1991). Time of swim up and changes due to exposure could give information about the influence of pollutants on development of the yolk sac fry.

Growth of alevins is presented by their length increment. The overall condition of alevins cannot easily be expressed by condition factor, which is defined as the relationship based on the length–weight ratio and is normally used to express the overall condition of a fish (Bolger & Connolly, 1989). Alevins condition factor will have a weight dominated by a yolk sac, which will be decreased during the development. Fish condition factor can also be inflouenced by various factors, which influence the metabolism of alevins, including different pollutants in the ambient media. All these factors may lead to changes in K-factor of exposed fish (De la Torre *et al.*, 2000; Dethloff *et al.*, 2001).

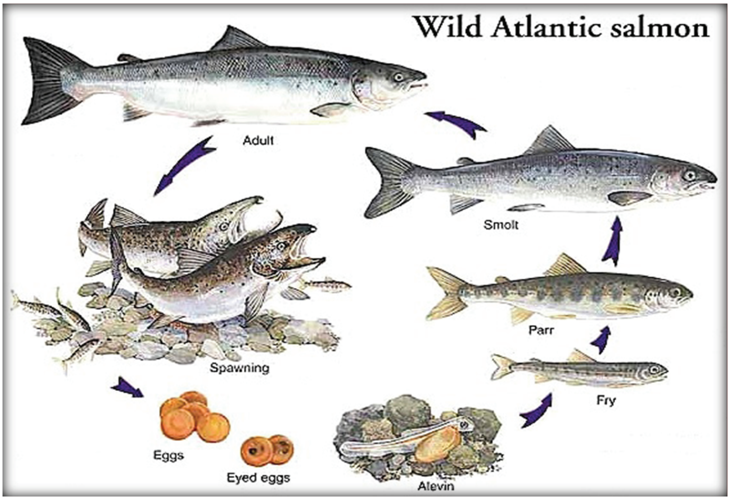

Figure 9. Different life stages of Atlantic salmon (http://www.nasco.int/atlanticsalmon.html).

2.9.6 Deformities

Developmental process of fish eggs and embryos are known to be affected by environmental factors such as salinity (Alderdice, 1988). Heavy metals such as Cu may cause developmental retardation such as morphological and functional anomalies, by adversely affecting various metabolic processes in developing embryos (Jezierska *et al.*, 2009). Environmental pollutants may affect embryos at different stages of development. Most commonly observed malformations observed in fish, include vertebral shortening, craniofacial anomalies, yolk sac malformation, axial (lordosis or kyphosis) or lateral (scoliosis) curvature of the spine in the abdominal or caudal region, body curvatures, tail curvatures, conjoined twins, yolk sac edema and cardiac anomalies, C-shaped larvae, deformed skull, deformed eye and cardiac edema (Jezierska *et al.*, 2009). Jezierska *et al.* (2000) observed high frequency and complexity of larval deformities affecting various parts of the body, after exposing fish embryo to solutions containing Cu. Deformities might be the result of disturbances caused by pollutants either during the process of development or stress faced during the process of hatching.

2.9.7 Gill toxicity and Ion regulation

As freshwater fish does not drink water, gills are believed to be the main action site for various toxicants including both inorganic and organic compounds (Rosseland & Staurnes, 1994). In freshwater fish the sodium-potassium ATPase (NaK-ATPase enzyme) present in chloride cells maintains the homoeostasis through active uptake of monovalent ions (Na^+ and Cl^-) across the gills (Perry, 1997; Wood, 1992). Exposure to metals such as Cu and Al are demonstrated to cause inhibition of NaK-ATPase, which leads to loss of plasma ions and osmoregulatory disturbances (McGeer *et al.*, 2000; Rosseland *et al.*, 1992). Gill tissues can be damaged by pollutants by various ways such as necrosis of chloride and pavement cells, uplifting of lamellar epithelium, breakage of epithelium and lamellar fusion. All these processes are accompanied with loss of Na^+ and Cl^- ions from blood plasma (Wendelaar Bonga & Lock, 1991; Wilson and Taylor, 1993).

2.9.8 Gene expression

one of the sensitive biomarker of toxic pollutants exposure and cellular metabolism of exposed organisms is characterized as the gene expression (Meland, 2010). Different techniques are applied, such as microarray and qtPCR, to identify responses: up/down regulation of a large number of genes extracted from (mRNA) in biological samples and it assumed to be a well-known and reliable method (Wang *et al.*, 2006). Polymerase chain reaction (PCR) is the advanced technology that is novel enough to manage copying the pieces of DNA in an exponential rate in only few hours by using easily available laboratory chemicals (Mullis, 1990).

Microarray technique is implied to identify the effects of environmental stressors on thousands of genes in a single experiment (Lettieri, 2006). Generally, a DNA microarray is a process where single stranded DNAs (probe) with various sequences coding for genes in interest is deposited on a glass slide. These probes hybridize with labelled single stranded DNAs (targets) that are obtained from the experimental samples. The target indicates the amount of isolated mRNA in the samples. Amount of mRNA produced from the gene having the corresponding DNA sequence is proportional with the amount of fluorescence emitted from each spot on the glass slide. After scanning the microarray, the fluorescence signals are converted to numerical values (raw data), which finally are statistically analyzed (Draghici, 2003).

Exposure to environmental stressors such as road salt may give rise oxidative stress in organisms by producing Reactive Oxygen Species (ROS). Cellular components such as protein, lipids and DNA may directly be damaged by ROS or other free radicals produced as a result of oxidative stress. In addition, they can also affect other biological pathways which might not necessarily be linked directly to oxidative stress (Lushchak, 2011). Exposure of fish eggs to high salinities may also affect the genes regulating osmoregulation ultimately giving rise to ion regulatory disturbances. The imbalance between ROS and antioxidant defense system may result in either excess of ROS or a shortage of antioxidants which causes oxidation of lipid, proteins, membranes, DNA and ultimately cell death (Felton & Summers, 1995). Environmental stressors may affect extended range of genes regulating several important functions such as development, growth and homeostasis resulting in delayed hatching of fish eggs or abnormal development giving rise to various deformities (Dave and Xiu, 1991). High salinity exposure to fresh water fish resulted in upregulation of genes associated with well-known osmoregulatory functions including ATP-fueled active transport of Cl^- across the gill and skin epithelium by Na^+/K^+ATPase (ATP1A2) (Hoar, 1988). Elevated concentrations of road salt such as NaCl, in previous studies have been reported to cause oxidative stress (Carlstrom *et al.*, 2009) and cell damage (Burg *et al.*, 2007) in living organisms other than fish.

2.10 Toxicity of highway runoff

2.10.1 Toxicity of road salt

According to USEPA (1988) the water quality criteria for Cl exposure for the protection of aquatic species specify the average concentration of Cl (≤230 mg/L) which is considered safe for aquatic species. Chronic exposure to Cl concentrations above 220 mg/L is toxic to approximately 10% of aquatic species. Community structures and food webs are altered by concentrations >220 mg/L by damaging primary producers and invertebrate communities (Environment Canada, 2001). NaCl quickly dissociates into the Cl anions and Na cations, thereby releasing Cl ions into solution. The concentration of Cl that affect the organisms depends on the taxa and life history stages exposed (Gardner & Royer, 2010). Evans and Frick (2001) found Cl concentration of 870 mg/L lethal to fathead minnow embryos. Benbow and Merritt (2004) observed for several aquatic invertebrates such as *Callibaetis fluctuans* and *Physella integra* the 96-h median lethal concentration (LC_{50}) values were between 5,000 and 10,000 mg Cl/L, respectively.

Acute toxicity tests performed by Collins and Russell (2009) showed that spotted salamanders and wood frogs were most sensitive to lowest median lethal Cl^- concentration of 1178 mg/L and 1721 mg/L, respectively. Inspite of harmful effects to the environment and corrosion of highway structures, chlorides are kept in use due to the benefits of traveler safety. Road salt shows its toxicological effects directly and indirectly by mobilizing more reactive forms of metals such as Cu (Backstrom *et al.*, 2004).

2.10.2 Cu toxicity

Environmental pollutants after entering into tissues, cells and organelles interact with biological structures leading to potential toxicity. Toxicity can be evaluated at various levels of biological and ecological organization ranging from molecular and cellular level to organisms, population and communities and finally ecosystem (Shea, 2004). Cu toxicity can be observed at various life history stages ranging from fertilization till adult fish. Li *et al.* (1998) have shown that water borne Cu is involved in necrosis and apoptosis of branchial chloride cells that are the primary ion transporting cells of the branchial epithelium. Metal concentrations at the site of action exceeding the critical concentration result in acute toxicity (Di Toro *et al.*, 2001). Exposure to pollutants may result in excess muous secretion, which may hinder the exhcnage of respiratory gases across the gill surface (Wood, 2001). The function of chloride cells, mucus cells, pavement cells and neuroepithelial cells found on gill epithelium (Perry, 1977) can also be affected by pollutants.

Lower Cu concentrations which do not disturb the larval physiology do not exclude the possibility that these concentrations would not have adverse effects on later life stages of fish (Flik *et al.*, 2002). For a particular trace metal, different chemical species have different toxicity than others. The presence of high concentrations of cations such as Ca^{2+} and Mg^{2+} may reduce trace-metal toxicity by cation competition (Pagenkopf, 1983).

3 Methodology

3.1 Road dust sampling and extraction

To obtain information about relevant concentrations of Cu in road run-off, mobilization of Cu from road dust due to addition of road salt was investigated by extraction experiment. Road dust was collected from the road shoulder of a four lane motorway (E6) in the city of Oslo having an annual average daily traffic (AADT) of 64 000 vehicles and a local road in the city of Ski. Fifteen g road dust was added into 150 mL Milli-Q water along with 5000 and 10,000 mg road salt/L and placed on the roller table for 24 h. Samples were centrifuged for 15 min at the speed of 5000 rpm and supernatant was carefully separated and fractionated to characterize the size distribution of Cu species in the experimental waters, according to the protocol described in Chapter (3.4.1). Finally, concentrations of Cu were determined in acidified samples (2 % HNO_3) using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (Perkin Elmer Optima 5300 DV).

3.2 Exposure study

Three independent exposure experiments were performed to study the single and combined effects of environmentally relevant doses of road deicing salt and Cu. In the first exposure, Atlantic salmon eggs were exposed during and post fertilization, to five nominal concentrations (50, 100, 500, 5,000 and 10,000 mg/L) of road salt and three nominal concentrations (10, 20 and 30 µg/L) of Cu separately and in mixtures until egg hatching in a flow throw system. Degree of fertilization, egg swelling, egg survival and delay in hatching were measured to identify any toxic effects of road salt and Cu (Paper II). Eggs from same exposure were used to assess transcriptional changes (genregulations) by microarray and qrtPCR analysis to characterize the key mode of action of road salt (Paper III).

In the second exposure study Atlantic salmon eggs were exposed during and post swelling to two nominal concentrations (500 and 5000 mg/L) of road salt and 30 µg/L of Cu, separately and in mixtures, in a static system for four weeks. Egg samples were collected in the form of total egg, egg inside content and chorion. ^{22}Na was used to determine the uptake of stable Na (Paper I). Third experiment was conducted to evaluate single and combined effect of nominal concentrations of road salt (100, 500, and 1000 mg/L) and Cu (5, 10 and 20 µg/L) on alevins of Atlantic salmon from hatching until swim-up. End points such as mortality, growth, formation of deformities, uptake of Cu and swim-up time were used to study the toxicological effects of road salt and Cu on alevins. Speciation of Cu was studied in Paper (II) and (IV) to investigate the effect of road salt on speciation of Cu.

3.2.1 Experimental setup/design

The setups for each exposure experiment was designed and performed in a temperature controlled room or cabinet at 6 ºC (Paper I, II and III) or 10 ºC (Paper IV).

3.2.1.1 Flow through system

Two flow through systems was built during the study (Fig. 10, 11 & 12). In flow through system, water was continuously pumped by peristaltic pumps from reservoirs to separately header tanks from where water was distributed to small exposure boxes. The overflow from the header tank was transported back to the reservoir tanks to keep the water level and the water flow to the exposure boxes constant. From the outflow of the exposure boxes, the water returned to the corresponding reservoirs by gravitation. Flow through system allow to use large water volume, but are bulky.

In flow through system A, two different reservoir tanks were used: small reservoir tanks with road salt of 30 L (one for each concentration tested) for episodic use 24 h weekly, and a large reservoir tank (600L) with control water for use between each episodic exposure. Thus, in flow through system A, there was a shift between two reservoir tanks of water. Schematic presentation of flow through system is presented in Fig (10).

In flow through system B (Fig. 12) each separate reservoir tank was continually filled with flowing water throughout the exposure experiment for each type of exposure (road salt only, Cu only and mixtures of road salt and Cu). Thus, in the flow through system B test organisms were continually exposed to road salt and Cu separately and in mixtures using one reservoir tanks of 70 liter and the header tank of 5 liter to each test.

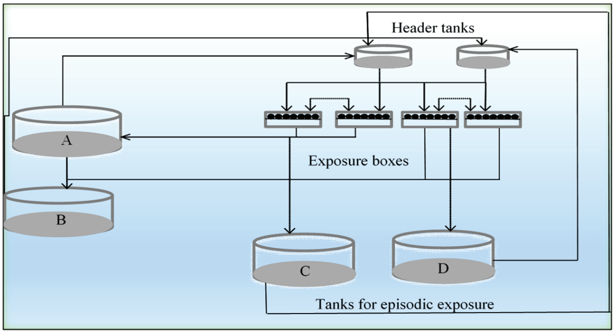

Figure 10. Schematic presentation of experimental setup flow through system A (Paper II) & B (Paper IV).

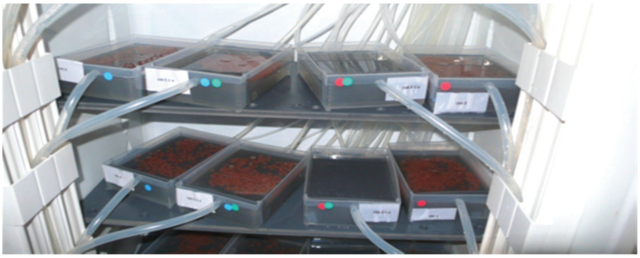

Figure 11. Experimental setup Flow through system A (Paper II) (Photo: U. Mahrosh).

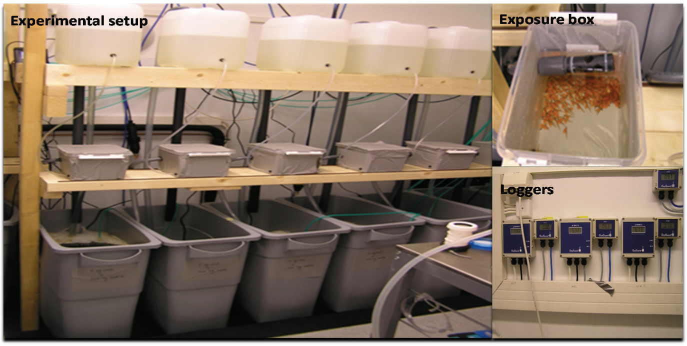

Figure 12. Experimental setup Flow through system B (Paper IV) (Photo: U. Mahrosh).

3.2.1.2 Static system

In addition to the flow through system one static exposure system was also used in the study. In the static system the exposure unit was only used without any reservoir tank. Thus, the volume of water was limited to the volume of the exposure unit and was only manually changed during the exposure period. The static design allows several exposure unit to be included in the study as the needed space are significantly smaller than the flow through design. The static system was used for short time experiments described in Paper (I).

3.2.1.3 Placement of eggs and alevins in exposure boxes

The fish eggs were placed on small grids in special designed exposure boxes (155 mm × 106 mm × 45 mm, 739 cm^3), while alevins were placed in exposure boxes of 2 L (15 cm × 20 cm × 10 cm, 3000 cm^3) without any grids (Fig. 11 & 12). The small exposure box used for eggs was selected to simplify the inspection of eggs (counting- removing dead eggs), while the comparatively bigger boxes were designed for alevins in order to avoid the alevins from jumping out of the exposure box. In each exposure boxes, the water flowed through the boxes from bottom from one side to top at the opposite side, at the point of overflow the water returned to the reservoir. During exposure the boxes were kept dark, and light were only limited used for inspection. In experiment I (Paper I) and experiment II (Paper II) the exposure unit were placed in temperature controlled cabinets, while in experiment (IV) the exposure units were placed in a temperature controlled room.

3.3 Water quality tested

3.3.1 Maridalswater (Control)

The control water, used in all the experiments, was obtained from the Lake Maridalsvannet in Oslo, which is a water supply to Oslo city, representing a typically water quality in Norway characterized by low conductivity and has been used in many previous exposure studies such as by Olvisk *et al.* (2010).

3.3.2 Road salt solution

To obtain the information of road salt toxicity towards eggs and alevins different road salt concentrations were added into Maridalswater. Road salt solutions were produced by dissolving NaCl Isbryter's rock salt into a small volume of Maridalswater before diluting to final volume. Following road salt concentrations were used in the experiment:

- 50 mg road salt/L
- 100 mg road salt/L
- 500 mg road salt/L
- 1000 mg road salt/L
- 5000 mg road salt/L
- 10,000 mg road salt/L

3.3.3 Cu solutions

To obtain information of Cu toxicity towards eggs and alevins, 5, 10, 20 and 30 µg Cu/L was added into Maridalswater. Cu solutions were produced by $CuCl_2.2H_2O$ stock solution (1 mg Cu/L) before addition to Lake Maridalsvannet water.

3.3.4 Mixtures of road salt and Cu

In order to determine the toxic effects of road salt and Cu in the mixtures, both NaCl Isbryter's rock salt and $CuCl_2.2H_2O$ stock solutions were mixed into control water (Lake Maridalsvannet) according to Chapter 3.3.2 and 3.3.3.

Following mixtures were included in the study:

- 500 mg road salt/L+ 5 and 10 µg Cu/L
- 1000 mg road salt/L+5 and 10 µg Cu/L
- 5000 mg road salt/L+5 and 10 µg Cu/L
- 10,000 mg road salt/L+ 10 µg Cu/L (Paper II only)

3.4 Measurement of water variables

To obtain information about the water quality and key influencing parameters a serious of measurements were performed. Temperature, oxygen and conductivity were continuously logged in one or two exposure units by an automatic electronic logger, in experiment I using Campbell CR200 and in experiment 4 (WTW 4301). In addition pH, dissolved oxygen, temperature and conductivity were measured in each exposure unit every week manually using WTW 3401 equipped with SenTix41 glass pH electrode, optic oxygen probe and tetracon 325 conductivity probe, respectively. Water CO_2 concentrations were analyzed using Phoenix 8000 UV-Persulfate TOC Analyzer (Tekmar Dohrman, Mason, OH, USA).

Inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer, Optima 5300 DV) was used to determine the major cations in collected water acidified with 5 % HNO3 (volume) from each exposure unit several times during the exposure period. Total organic carbon (TOC) was determined by using total organic analyzer (Shimadzu TOC cpn, Kyoto, Japan) and Iachat IC5000 ion chromatography was used to determine major anions in collected water without acidification.

To get information of the NH_4 (Ammonium) in treatment waters ammonium (NH_4) test kit Spectroquant® was used. 5 mL of the sample was added into the test tube followed by adding 0.60 mL of reagent NH_4-1 and both solutions were mixed. 1 level blue microspoon of reagent NH_4-2 was additionally added and mixed strongly until the reagent was completely dissolved. The solution was left to stand for 5 min approximately. Afterwards, 4 drops of reagent NH_4-3 was added and mixed. After leaving the solution to stand again for 5 min, the samples were added into the cell and measured in the spectrophotometer.

3.4.1 Fractionation techniques

Different analytical methods can be used for quantification of potential bioavailable metal species. In this work a combined size and charge fractionation method was applied as described by Teien *et al.* (2004). Fractionation was applied *in situ* in order to avoid any storage effect. The metal concentration in each fraction was quantified using ICP-OES.

Filtration and ultrafiltration techniques were performed to obtain information about size fractions utilizing membrane filters and hollow fibers. The greatest drawback using membrane filtration is connected to clogging of the filter. Filter clogging may change the actual pore diameter of the filter producing filtrate having undefined size classes. Millipore membrane of 0.45μm was used for excluding particles. Amicon H1P1-20 hollow fiber with a molecular mass cut-off level of 10KDa was used for ultrafiltration and excluding particles and colloids (Fig. 13). A cut-off of 10 KDa has been used in many toxicity tests (e.g. Rosseland *et al.*, 1992; Salbu *et al.*, 2008; Teien *et al.*, 2006) and also in highway runoff studies (e.g. Tuccillo, 2006). The retention of metal species in Amicon hollow fiber is low and it is characterized by insignificant clogging due to high tangential flow and low cross filter flow (Salbu, 2009). During ultrafiltration the filtering flow rate was approximately 50 mL/min at a pressure of 12 psi which prevents deformation of the filtered species.

Charge fractionation (Fig. 13) was based on ion-exchange chromatography technique using Chelex 100 (BioRad, Na-form, and 50-100 mesh size). The resin material (about 15 ml) was placed inside the glass column and elution rate through the resin was 20 ml/min, giving the specific contact time 0.75 ml resin/ml water/min. The fraction retained inside the resin includes charged species in solution and also the species dissociated from weak complexes or desrob from solid surfaces (Salbu, 2009; Teien *et al.*, 2004).

Following different physico-chemical fractions of Cu were obtained.

Size fractions:

- Total Cu, determined based on unfiltered sample after acidification (2 % HNO_3)
- $Cu_{Particulate} = Cu_{Total} - Cu_{0.45\ \mu m\ filtrate}$
- $Cu_{Colloidal} = Cu_{0.45\ \mu m\ filtrate} - Cu_{LMM\ (low\ molecular\ mass)}$
- $Cu_{LMM} = Cu \leq 10$ kDa.

Charge fractions:

Charge fractionations were obtained by ion-exchange chromatography using cation exchange Chelex-100 (BioRad, 50 – 100mesh, Na form).

- $Cu_{Cationic} = Cu_{Entering\ cation\ exchange\ column} - Cu_{Leaving\ cation\ exchange\ column}$
- LMM $Cu_{Cationic}$= passing 10 kDa ultrafilter and retained in Chelex-100 resin.

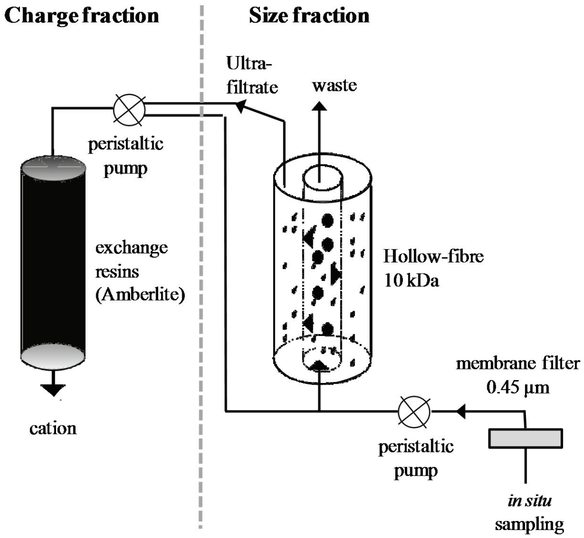

Figure 13. The applied size and charge fractionation system (Teien, 2005).

3.5 Choice of test animal

Atlantic salmon (*Salmo salar*) eggs and alevins were used as test model in all the experiments. This fish species was chosen primarily due to their environmental relevance, known biology and commercial value in aquaculture. It is assumed that early life stages such as eggs and alevins of fish are more susceptible to toxicants then adults. All life stages were handled very carefully to minimize stress. Whole experimental work including fish strictly followed the Norwegian Welfare Act and research animal legislation. Fish experiments were approved in advance by the local representative of the Norwegian Animal Research Authority (NARA ID: 3026).

3.5.1 Dry fertilization

The eggs and milt of Atlantic salmon were obtained by dry stripping from the Aquagen (AS) hatchery Norway and were immediately transported (within 24 h) in plastic bags on ice in a box of polystyrene, prior to fertilization. A drop of water was added to a tiny drop of milt and the viability of two milt batches was tested under the binocular microscope. The most viable group was selected for fertilization. Milt should be added to the dry eggs in the proportion of 10:100 i.e. 10 mL of milt is required for 1000 mL of dry eggs. Eggs were mixed with milt for dry fertilization (Fig. 14) and soon after it, the eggs were transferred to specially designed boxes with a good water quality in recirculation system (Fig. 11).

Before transfer to the exposure unit eggs were rinsed with corresponding water quality. Grids were placed in all boxes in order to provide the gravel environment to eggs and also to avoid the transfer of eggs into the outlet and recirculating water (Chapter 3.2.1.1). Eggs were placed in the boxes and continuously observed until hatching. In Paper (II) 200 eggs were placed in each exposure units (flow through recycling system A, Chapter 3.2), while in Paper (I) 100 eggs were used in the exposure unite (static system , Chapter 3.2.1.2). Fig (15) shows the handling of eggs after dry fertilization.

Figure 14. Dry fertilization of Atlantic salmon eggs (Photo: S. Meland).

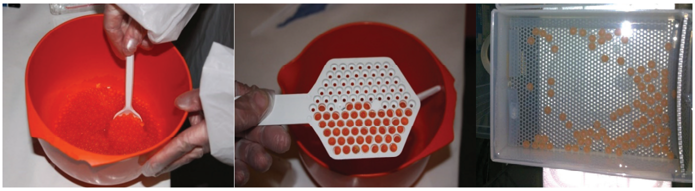

Figure 15. Handling of eggs after dry fertilization (Photo: H.-C. Teien).

3.5.2 Hatching till swim-up

In order to expose the alevins soon after hatching to the corresponding treatments, eyed eggs soon before hatching were placed in the boxes with a good water quality in a flow through recirculation system (system B, Chapter 3.2). All boxes were covered with black paper in order to avoid the light interference and the light was off between inspections. Alevins were observed carefully until the swim-up stage.

3.6 Biological uptake

3.6.1 Uptake in eggs, sorption to surface of chorion- transfer into eggs

To determine the uptake of Na, Cl and Cu, three types of samples were collected. Total eggs, content inside eggs and egg shell (chorion). Exposed eggs were dissected by injecting a syringe into the egg, and taking out all the content from inside the egg and transferring into Eppendorf tube, while the egg shell was stored in separate tubes. Samples were stored at -20 ^{o}C before analysis.

3.6.2 *Uptake in alevins*

To determine the accumulation of Cu in the gills of alevins the whole head of exposed alevins was dissected, transferred to Eppendorf tubes and stored at -20 ^{o}C before analysis. Uptake was studied only in the head region in order to see the accumulation on gills. Alevins have very small gills, which cannot be easily collected, therefore, whole head region was collected as it mainly involves the accumulation on gills but uptake by sensory organs can also not be omitted.

3.6.3 *Determination of ^{22}Na*

The ^{22}Na tracer (specific activity: 376.09 mci/mg Na) was produced by NEN^{TM} life sciences products, Inc Boston USA and 6.25 mL of tracer was added to control water and road salt solutions. Following the tracer exposure, egg samples were measured with respect to ^{22}Na by using NaI detector (Wallac WIZARD 3 1480 Automatic Gamma Counter Perkin Elmer) for 600 seconds. The activity was measured in dpm, which was further converted into Bq (taking measurement efficiency into account) indicating the amount of ^{22}Na activity in or at the surface of the egg. ^{22}Na activity determination in water samples was based on 5 mL sample collected from each box containing the ^{22}Na exposure, while ^{22}Na activity of the eggs was based on individual egg stored in Eppendorf tubes. ^{22}Na activity in eggs was presented in Bq/g wet weight of egg.

3.6.4 *Determination of Cl in eggs*

In order to determine the concentration of Cl in eggs, 0.5 ml of tetramethylammonium hydroxide solution (TMAH) was added in the Eppendorf tube containing the sample. Since the eggs were sticking inside the tube, therefore, tubes were placed inside the oven at 90 ^{o}C for 30 min until the sample was properly dissolved. Milli-Q water (2 ml) was added into the sample and transferred into another pre labelled 15 ml ICP tube. Samples were again placed in the oven at 90 ^{o}C for 2 h. Samples were diluted afterwards up to the final volume of 5 ml and measured on ICP-MS for Cl.

Concentration of Cl in the samples was calculated by the following formula:

µg Cl/g wet weight of egg = (A*V)/W

where A= µg/L of Cu, V= dilution volume of sample (L), and W= wet weight of sample (g).Values are given as µg/g egg wet weight.

3.6.5 Determination of Cu in eggs and alevins

In order to determine the concentration of Cu in eggs, weighed samples were transferred to Teflon tubes before digestion using ultraclave. One mL concentrated ultrapure HNO_3 was added into each tube with sample and left overnight to completely dissolve the whole sample content before transfer to teflon tubes. After transfer, 2mL Milli-Q water and 50µl internal standard (In) was added before digesting using ultraclave (Milestone, Leutkirch, Germany). After digestion, samples were diluted to 10 mL (10% HNO_3) with Milli-Q water before analysis of Cu using ICP-OES and ICP-MS (Inductive Coupled Plasma-mass Spectrometry, Agilent).

Cu level in the samples was calculated by the following formula:

µg Cu/g wet weight of egg = (A*V)/W

where A= µg/L of Cu, V= dilution volume of sample (L), and W= wet weight of sample (g). Values are given as µg/g egg wet weight, while Cu concentration of alevins are given in µg/g dry weight.

Control blank samples (3-5) were included to control contamination and determine quantification limits. In addition, standard reference material was also added for quality assurance.

3.7 Biological responses

3.7.1 Fertilization success

To obtain information about the influence of different exposures on fertilization success, 10 eggs were randomly collected 24 h after fertilization, from each of the treatment replicates. The collected eggs were examined under a binocular microscope after being treated in a fixation liquid for 2 min.

Fixation liquid was prepared by mixing following ingredients:

1) 7 g NaCl
2) 15 mL 35% vinegar
3) 1L Milli-Q water

Eggs were classified as *not fertilized*, if lacking cells in the center (Fig. 16).

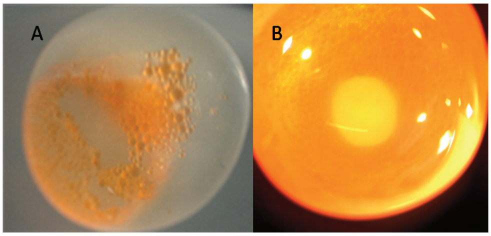

Figure 16. Difference between unfertilized (A) and fertilized egg (B) (Photo: U. Mahrosh)

3.7.2 *Egg swelling*

To obtain information about the effect of road salt and Cu exposures on swelling of the eggs, the diameter of 20 randomly collected eggs of each treatment was measured 24 h after fertilization in each exposure unit, and compared to a reference of 20 eggs exposed kept in control water and/or size of unfertilized eggs. The diameter of eggs was used as a measure of swelling, and the change in size of egg was used as a measure of effect.

3.7.3 *Gene expression*

In the present thesis two different techniques applied includes, the microarray technique and the quantitative real-time polymerase chain reaction (qrtPCR) (Papers III). The analyses of samples were performed at Norwegian Institute for Water Research (NIVA).

To study the gene expression six Atlantic salmon eggs with an average individual wet weight of 0.146±0.020 g were randomly selected (n=6). Total RNA was extracted using Direct-zol™ RNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA) according the instruction given by the manufacturer. The yield of RNA and its purity was quantified by using Nanodrop® spectrophotometer (ND-1000, Nanodrop Technologies, Wilminton, Delaware, USA) and RNA integrity was determined by Bio analyzer gelelectrophoresis with RNA 6000 Nano chips (Agilent technologies, Santa Clara, California, USA). The purified RNA samples were stored at -80°C until analysis for gene expression by microarray and qrtPCR.

Only two groups (5000 mg road salt/L and control) were selected for Microarray analysis. The changes in expression of genes from eggs exposed to 5000 mg road salt/L compared to control, were used to identify mode of action (MOA) and form the basis for selection of genes for the qrtPCR analysis. Bioinformatics and biostatics were used to investigate differential expression of genes, perform functional (gene ontology) enrichment analysis, and investigate affected pathways and gene-gene interactions.

Quantitative rtPCR (qrtPCR) analysis was performed on the mRNA from eyed eggs from control, 50 mg road salt/L, 100 mg road salt/L, 500 mg road salt/L and 5000 mg road salt/L to verify the concentration-response relationship and determine the No Transcriptional Effect Level (NOTEL) for a selection of differentially expressed genes. During the qrtPCR technique the mRNA was purified from the sample (tissue/cells) before two primers (forward and reverse), matching the genes of interest, reverse transcriptase, DNA polymerase and the four deoxynucleoside triphosphates (dNTPs) needed for DNA synthesis were added. The first round of synthesis was the reverse transcription of the mRNA into complementary DNA (cDNA) using one of the primers, which then were followed by a series of heating and cooling cycles, which finally amplified the RNA strand and introduced a fluorescent dye such as Sybr Green (Alberts *et al.,* 2008). The fluorescence signal generated is then detected for each cycle and will be used to determine the number of copies for the given gene compared to the control (e.g. gene expression). Adjustment of difference in starting material (mRNA) and differences in the amplification process is performed by normalizing against specific housekeeping genes, that are not regulated by the exposure to a stressor.

3.7.4 Eyed embryos and Hatching

Eggs were considered 'eyed' when having a black spot or pigmentation visible with naked eye. Time for the onset of hatching was noted for each exposure unit and the number of hatched and/or not hatched eggs in each box was counted daily. Both fully and partly hatched larvae were included as hatched in the observation according to Stouthart *et al.* (1996). Degree of hatching was presented as percentage compared to the total number of eggs in each exposure unit, and related to degree days of exposure. Degree day of exposure is actually the number of days since fertilization multiplied with the average temperature of exposure water.

3.7.5 Mortality

Dead eggs (Fig. 17) and larvae were counted daily and were immediately removed from the exposure boxes in order to prevent fungal growth. Eggs and larvae were considered dead when parts of their content turned opaque. Dead eggs were removed by plastic pipette/dropper with removed tip, without disturbing other living eggs (Fig. 18), while dead alevins were either removed with plastic pipette or forceps. Mortality was presented as percentage compared to the total number of eggs in each exposure at time of mortality.

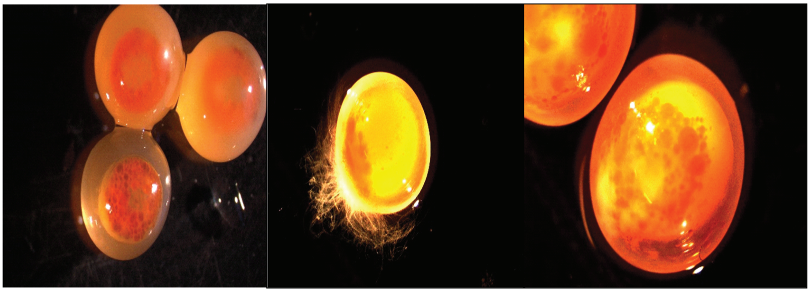

Figure 17. Dead eggs affected by different exposure solutions (Photo: U. Mahrosh; M. Kleiven).

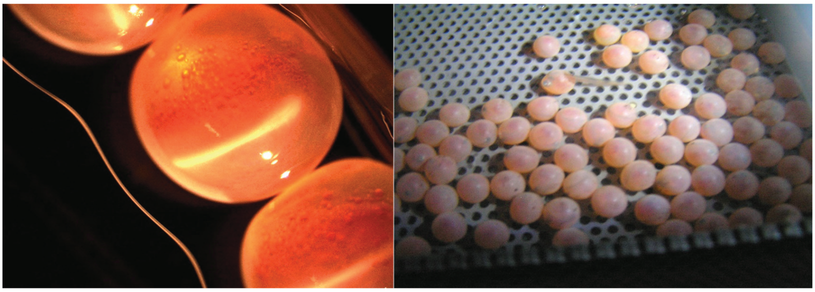

Figure 18. Living eggs not affected by exposure solutions (Photo: U. Mahrosh; M. Kleiven)

3.7.6 Growth

To determine the size of the alevins, both length and weight were determined every 7 or 10 days after using anesthesia. Alevins (n=10) were transferred from exposure units to a bottle with anesthetic (1 g Tricaine methanesulfonate/L) before determination of size.

3.8 Data analysis

Water quality parameters were presented as mean±SD (standard deviation) based on the measurements throughout the each experimental period. The water quality measurements and biological end points were based on two replicates (Paper II), three replicates (Paper I) and one replicate (Paper IV). Since hundreds of eggs and alevins were placed in each replicate, one or two replicates were considered sufficient due to overload sampling work and space availability and potency of regression. All data are presented in degree days in order to take into account any small changes occurring in temperature between each exposure unit and to compare with previous published data.

One-way ANOVA followed by Tuckey's post hoc test (GraphPad prism 6.0) was used to see the significant differences in pH, temperature and O_2 between different treatments. Data was tested for normal distribution and equal variances. Data showing unequal variance was $\log_{10}$ transformed prior to analysis. Data was tested for normal distribution and equal variances by using D'Agostino & Pearson omnibus normality test. Data showing unequal variance was $\log_{10}$ transformed prior to analysis. A Kruskal–Wallis nonparametric test followed with Dunn's post hoc tests was performed for transformed data sets which could not meet the criteria of equal variance. Differences in significance between groups for ^{22}Na and Cu were analyzed by paired t-test. Activity of ^{22}Na in treatments with 500 and 5000 mg road salt concentrations was corrected by multiplying measured dpm with the Na dilution factor compare to ^{22}Na in control. In addition dpm measured in the water was normalized due to different activity in the treatments. Regression analysis (R-Comdr 2.14.1) was applied to identify significant effects of road salt and Cu exposure on various water quality parameters including Cu speciation and on different biological end points such as swelling. In Paper 2 Pearson correlation was performed to study the relationship between road salt and other factors such as conductivity and egg swelling.

Survival analysis was performed by using GraphPad prism 5.0 (Graphpad Software, Inc., San Diego, CA, USA) to determine the percentage survival and hatching of the fertilized eggs and alevins followed by Log-rank (Mantel-Cox) test to study the effect on survival and hatching and logrank test to identify the dose-response trends in the treatments. Data for survival analysis and hatching was coded as 0 for living and unhatched egg/alevins and 1 for dead and hatched egg/alevins and was pooled together from both replicates.

Significant differences or trends were defined by using the criterion $p \leq 0.05$ as significance level.

4 Summary of Scientific Papers

4.1 Paper I: Road salt and Cu uptake

Uptake and distribution of ^{22}Na, Cl and Cu in Atlantic salmon (*Salmo salar*) eggs exposed to road salt (NaCl).

Uptake of Na, Cl and Cu in developing Atlantic salmon eggs from water at different concentrations of road salt was demonstrated using ^{22}Na tracer, and stable Cl and Cu in a 30 days static exposure immediately post fertilization. The exposures were differentiated as during swelling and post swelling, in order to investigate the uptake during such critical time periods. Our results indicated that ^{22}Na, Cl and Cu was taken up by the eggs. The uptake was highest during the first 24 h of swelling, however, the uptake continued until 48 h post fertilization and swelling. However, uptake during swelling was significantly higher than the uptake in post swelling exposures. The uptake of ^{22}Na and Cl increased with increasing road salt concentration, while the Cu uptake remained unaffected by road salt addition. The uptake of ^{22}Na, Cl and Cu also increased with increasing duration of exposure. During the first 24 h of the exposure, most of the total accumulated ^{22}Na, Cl and Cu was found inside the egg and comparatively less accumulation was associated with the chorion (14-41 %). Accumulation inside the eggs and in the chorion increased also with time. The results demonstrated that exposure to both Na, Cl and Cu was most critical during the first 24 h post fertilization, and during this period the transport of Na, Cl and Cu into the egg fluid could affect the developing embryo and thus show the toxic effects in the form of mortality, delayed hatching or formation of various deformities.

4.2 Paper II: Road salt and Cu toxicity

Toxicity of road deicing salt (NaCl) and copper (Cu) to fertilization and early developmental stages of Atlantic salmon (*Salmo salar*).

In the present study the effect of road salt and Cu on fertilization and early life stages of Atlantic salmon was studied from fertilization till hatching. Following in vitro fertilization, eggs were exposed for first 24 h and episodic exposure 24 h post fertilization to different water qualities containing 50, 100, 500, 5000 and 10,000 mg road salt/L, respectively, without and with addition of 10, 20 and 30 µg Cu/L. Fertilization success was not affected by any of the treatment, while early developmental stages from swelling till hatching were vulnerable to such exposures. Reduced swelling and less percent survival indicated lethal effects of eggs exposure to high road salt concentrations (≥5000 mg/L) without and with Cu during fertilization. Larval deformities were found after exposing eggs to high road salt concentration (≥5000 mg/L) mixed with 10 µg Cu/L during fertilization. Delayed hatching was also observed at road salt concentrations (≥500 mg/L) mixed with Cu (≥10 µg/L) during fertilization and also in post fertilization episodic exposure. It appears that the sensitivity of early developmental stages of Atlantic salmon increased, when exposed to multiple stressors i.e., road salt mixed with Cu mimicking road runoff. Taking the sensitivity at fertilization and early life stages of salmonids into account, it is expected that stress experienced by these developmental stages of fish may also affect later life stages. It is, therefore, important to avoid road salt application before and during the spawning time of salmonids, if a road is located close to an adjacent brook or river that receives the runoff.

4.3 Paper III: Road salt effects at molecular level

Transcriptional changes in Atlantic salmon (*Salmo salar*) after embryonic exposure to road salt (NaCl).

Due to extensive road salt application in Northern countries, the water quality has deleteriously been affected, which also resulted in toxic effects on aquatic organisms such as fish inhabiting in water bodies receiving the road runoff. Early developmental stages of Atlantic salmon such as fertilization and swelling are vulnerable to road salt. Studies have shown the toxic effects of road salt on early life stages of Atlantic salmon at elevated salt concentrations. However, the aim of current studies was to study the effects of different road salt concentrations on molecular levels. In current study the molecular changes occurring in the eyed eggs of Atlantic salmon that were exposed to road salt (5000 mg/L) during fertilization, were studied. In order to investigate the gene expression, RNA was isolated form Atlantic salmon eggs. After isolation, the purity of RNA was quantified by using spectrophotometer. The global transcriptional changes were monitored by a 60 k salmonid microarray at the eyed egg stage. Microarray analysis was performed on two selected groups *i.e.* control and 5000 mg road salt/L. In order to verify the concentration-dependent regulation of genes, quantitative rtPCR analysis of biomarkers for exposure and effects was performed in the eyed egg stage embryos exposed to different concentrations of road salt (0, 50, 100, 500 and 5000 mg/L). Results from microarray analysis showed that 1002 of the total 60k features on the array were significantly regulated

(721 up-regulated and 281 down-regulated). Transcriptional (gene expression) analysis of eyed eggs exposed to 5000 mg road salt/L during fertilization identified the genes affected in developing embryos involve in controlling several function such as osmoregulation, ion regulation, oxidative stress, metabolism (energy turnover), renal function/cell death, acute renal failure, organ development and mitochondrial dysfunction (depolarization of mitochondria and mitochondrial membrane). Quantitative rtPCR revealed a positive concentration-dependent increase in some genes involved in lipid turnover and renal function, neuraxonal development, oxidative stress (Txndc9), and the gene regulating homeostasis. Although all of the four above mentioned genes displayed a concentration-dependency, only expression of Cyp14A4 and Nav1 at road salt concentrations of 100 mg/L and higher were significantly regulated. The current findings suggest that road salt concentrations (<100 mg/L) are not supposed to affect the developing Atlantic salmon embryo exposed during fertilization and swelling. Effects on corresponding genes indicate the toxicity of elevated road salt concentrations on eggs in the form of egg mortality, delayed growth and body malformations. Thus the current study has suggested that road salt concentrations lower than 100 mg/L were defined as No Transcriptional Effect Level (NOTEL=50 mg/L) which did not cause any significant transcriptional changes in the developing embryos.

4.4 Paper IV: Road salt and Cu uptake and effects

Impact of road deicing salt (NaCl) and copper (Cu) on Atlantic salmon (*Salmo salar*) alevins from hatching till swim-up.

During harsh winter season of Northern countries, due to deicing salt application on roads, the road salt is one of the major components of road runoff in addition to metals such as Cu. The environmental effects of each compound separately and in mixtures is of great concern. Toxicity of road salt and Cu, separately and in mixtures was, therefore, studied in alevins of Atlantic salmon (*Salmon salar*) from hatching till swim-up. Alevins were exposed to extended solutions of road salt (100, 500 and 1000 mg/L), Cu (5, 10 and 20 µg/L) and mixture of road salt and Cu, one day before hatching. High mortality of alevins, reduced growth, delayed swim up and various deformities were observed at all road salt concentrations (100-1000 mg/L) compared to control. No effect of Cu on alevins survival, growth, and swim-up was observed at any tested concentrations (5-20 µg/L). In addition, exposure to Cu did not result in formation of any deformities. No effects at any Cu concentration could probably be due to its less bioavailability and hence less toxic effects. Head-Cu concentration of alevins increased at high

Cu concentrations and also increasing road salt concentrations, which indicates that road salt releases more bioavailable forms of Cu. In addition, the alevins mortality, reduced growth, delayed swim-up and formation of deformities was observed in the mixtures of road salt and Cu. The results indicate that the road salt poses its toxic effects separately but additive or more than addivitve effects are obtained when mixed with metals such Cu. Thus, road salt application might seriously affect the sensitive life stages of Atlantic salmon and application of road salt should be avoided during the late winter-early spring period.

5 Results and discussion

5.1 Water quality and development of Atlantic salmon

In the present work (Paper I, II and IV) Lake Maridalsvannet water was used as control water in a temperature controlled system (6.0±1.0 °C, Paper I, 6.6±2.2 °C Paper II and 9.9±0.4 °C, in Paper IV). This water was characterized as very soft freshwater (5.7±0.9 mg Ca/L, 1.0±0.0 mg Mg/L, 3.2±1.0 mg Na/L, 3.2±2.0 mg K/L, 4.3±0.8 mg Cl/L, 2.7±0.4 mg SO_4/L, 1.4±1.4 mg NO_3/L) with pH 7.0±1.0 and concentration of TOC 4.5±0.5 mg/L. Thus, the lake Maridalsvannet water represents a typical Norwegian and Scandinavian water quality with low content of organic matter and low ionic concentrations as observed in the studies of Olsvik *et al.* (2010) and Salbu *et al.* (2008).

The development of Atlantic salmon embryos from fertilization until hatching and alevins from hatching until swim-up were followed in the present work (Fig. 19). Using Lake Maridalsvannet water at 6.6±2.2 °C, results demonstrated that the fertilization of Atlantic salmon eggs was successful (99 %, Paper II). The size of the eggs increased from 5.5±0.1 mm to 6.5±0.1 mm (Paper I) during the stage of swelling, i.e., the first 24 h after fertilization. Survival of developing eggs (98 %) and alevins (99 %) were high and ranged within the normal level given in the OECD guidelines (2013). The development of embryos was characterized as normal, with eyed embryo stage reaching at 255 degree days, started hatching at 498 degree days and completed hatching at 542 degree days. The period of hatching was, thus, 64 degree days in total from start to end. This is in agreement with Gorodilov (1996) and Rosseland & Skogheim (1984), who reported eyed embryo stage at about 250 degree days and hatching at about 400 degree days.

5.2 Effects of road salt on early life stages of Atlantic salmon

The transfer and effects of road salt on developing Atlantic salmon embryos from fertilization until hatching and alevins from hatching until swim-up (Fig 19) were followed in the present work (Paper I-IV).

Results of water chemistry analysis from our experiments showed that road salt addition did not affect the pH of any treatment water. The concentration of TOC and major anions (F^-, NO^{3-}, SO_4^{2-}) remained also unaffected by road salt addition. However, conductivity and the concentrations of Na^+ (2010-2100 µg/L) and Cl^- (2610-3100 µg/L) increased by road salt (5000 mg/L) addition (Paper I&II). The concentrations of other cations such as Mg^{2+}, Ca^{2+} and K^+ increased at high road salt addition (5000 and 10,000 mg road salt/L) (Paper 1, II and IV). Mason *et al.* (1999) have also reported that road salt application strongly affected the stream water chemistry, where the concentrations of Cl, Ca, Mg and Na increased considerably. Exposure waters contained relatively low concentrations of CO_2 (1.0-2.0 mg/L) and NH_4 (1.2-3.7 mg/L).

It is also assumed that high levels of Ca^{2+} and K^+ can be toxic towards fish (Stekoll *et al.*, 2009). Exposing developing embryos of several salmonid species to elevated concentrations of Ca^{2+} (≥520 mg/L) during water hardening resulted in increased mortality (Ketola *et al.*, 1988). Peterson *et al.* (1988) observed that early developmental stages of Atlantic salmon were more susceptible towards the toxicity of K^+ than Na^+ and Ca^{2+}. Little work has been done on study the toxicity of salts on salmonids (Weber-Scannell and Duffy, 2007), however, Craigie (1963) reported that exposure to elevated salinities resulted in decreased resistance to thermal stress of developing salmonids. In addition, exposure of salmonids to high salinities during fertilization resulted in ploidy modifications (Miller *et al.*, 1994), affected the normal development (Morgan *et al.*, 1992) and negatively influenced the process of osmoregulation (Shen & Leatherland, 1978).

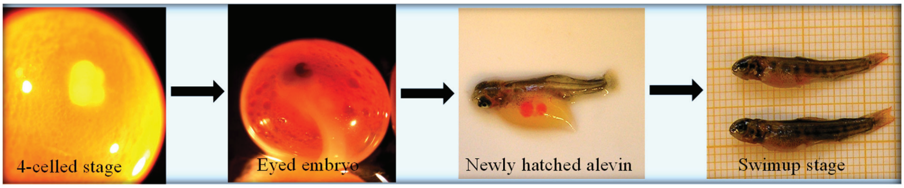

Figure 19. Different life stages of Atlantic salmon studied from fertilization until swim-up (Photo: U. Mahrosh; M. Kleiven).

5.2.1 Transfer of road salt from water into eggs

The uptake and distribution of Na and Cl in eggs were studied in Paper I. The results demonstrated that both Na and Cl were taken up by the eggs. The uptake was highest during the time of swelling and increased with increasing road salt concentrations and duration of exposure. Comparing the Na and Cl fractions associated with the chorion and inside the egg, results demonstrated that the uptake inside the egg was highest during swelling (60-75 % and 63-81 % of the total egg content of Na and Cl, respectively), while mainly associated with the chorion due to sorption post swelling (after 48 h of fertilization). The Cl concentration inside the egg during swelling was 678 and 1000 µg/g egg w.w after exposure to 500 and 5000 mg road salt/L, respectively. The bioaccumulation of Na and Cl increased until the end of experimental period (30 days). At the end of the experimental period the bioconcentration factors (BCF) demonstrated that the transfer of Cl, increased from 0.003 (L/Kg) in Lake Maridalsvannet water to 0.57 (L/Kg) in exposures to 5000 mg road salt/L. Thus, we assume that toxic effects of road salt could be a direct effects of enhanced level of Cl and/or Na inside the eggs during the stage of swelling.

The results are in accordance with previous findings, demonstrating high uptake of metals during the time of swelling, when the chorion is highly permeable (Lønning & Davenport, 1980). Most of the metal was associated with the chorion as the embryonic development progresses (Jezeirska *et al*., 2009). They reported, however, that the fraction within the egg, mainly was found in the PVS and very little entered the embryo itself. This could also be the case for Na and Cl, however, the distribution of the elements between the PVS and embryo was not investigated in the present study.

5.2.2 Toxic effects of road salt on early life stage of Atlantic salmon

The effects of road salt on early developmental stages from fertilization to hatching and from hatching to swim-up were studied in the present work (Paper II, III and IV).

Results demonstrated no negative effects on fertilization success when exposing dry fertilized eggs to road salt (100-10000 mg/L) during swelling stage since 99 % eggs were successfully fertilized as in control eggs (Chapter 5.1). However, Stekoll *et al*. (2009) observed reduced fertilization success of salmonids at exposure concentrations of 2500 mg total dissolved salt/L.

Road salt exposure (24 h) during swelling had no effects on the developmental rate until the eyed egg, while affected the degree of swelling, egg survival and time of hatching significantly.

Road salt exposure post swelling, did not affect survival but affected the hatching. Road salt exposure after hatching showed effects on survival and development of alevin until swim-up.

Eggs exposed to road salt during swelling were significantly affected by the road salt addition (r^2= -0.97, p= 0.0001, and R^2= 0.97, p = 0.001, in paper I and paper II, respectively) and were smaller in diameter compared to the eggs exposed to Lake Maridalsvannet water. The size of eggs was correlated (r^2= -0.97, p= 0.0001) to road salt concentration and decreased with increased concentration of salt. Thus, exposure to high road salt concentrations could suppress the formation of PVS and further reduce the egg volume and weight (Li *et al*., 1989). Fish eggs swell after shedding into the water (Eddy & Talbot, 1983) and hence PVS is formed (Li *et al*., 1989). Small PVS does not provide sufficient space for the expansion of the embryo, which can result in restriction of embryonic development (Li *et al*., 1989). Reduced swelling of Atlantic salmon eggs was observed in the present work, probably due to decrease in the PVS formation. The size of PVS measured in the experiment of Li *et al.* (1989) was 28 µL in fresh water and 20 µL, when eggs were exposed to the salinity levels of 1000-2000 mg/L. Swelling is considered to be one of the more critical stages for ambient pollutant uptake from ambient media. During the process of swelling, the chorion is highly permeable and influx of water occurs after breakage of cortical alveoli during egg activation (Alderdice, 1988; Gilkey, 1981; Guraya, 1982, Laale, 1980; Lønning & Davenport, 1980), which allows pollutants to enter into the egg. The structure and permeability of chorion might be severely affected if sorption to the chorion takes place (Jezierska *et al.,* 2009). Reduced PVS disrupts normal functions such as offering mechanical protection to developing embryo (Laale, 1980) and offering protection against ambient metal ions and acid waters (Eddy & Talbot, 1985).

Exposure to road salt during swelling, resulted in reduced survival of the eggs and embryos. Results demonstrated high egg mortality at high road salt concentration of 5000 (20-24 %) and 10,000 mg road salt/L (92-96 %), respectively, when exposed during fertilization and swelling (Fig. 20), while no significant changes in mortality (1-3 %) of eggs exposed 24 weekly post swelling to road salt was observed (Fig. 21). The mortality of eggs started at 30 degree days, reached the maximum at 132-174 degree days and continued until hatching. Only 4-8 % of eggs survived after exposure to 10,000 mg road salt/L during swelling (24 h exposure from the time of fertilization). Stekoll *et al.* (2009) observed also high post-hatch mortality of Coho salmon after exposure to elevated salts (>2000 mg/L) at or just after fertilization through hatch. Fridman *et al.* (2012) reported that exposure of fish eggs to high salinities (15,000, 20,000 and

25,000 mg/L) had a substantial effect on embryo viability. However, the present work demonstrated effects even at lower salinities.

The results demonstrated that road salt concentrations at 5000 mg/L or higher at the time of swelling are critical towards Atlantic salmon eggs, while not lethal as episodic exposures (24 h/week) post fertilization (Fig. 21). The toxic effects observed on eggs and embryonic development could be explained by reduced PVS formation and also uptake of salt inside the egg during the time of fertilization and swelling. Fridman *et al.* (2012) suggested that the time of exposing egg to increased salinities had a considerable effect on embryo viability. They also demonstrated higher effects on eggs being exposed immediately after fertilization to salinity, compared with exposure 4 h after fertilization.

All eggs, independent of the road salt exposure, reached the eyed embryos stage soon after 255 degree days. Eggs exposed to road salt during fertilization hatched during the period from 495 to 542 degree days, similar to the control eggs. The results (Paper II) demonstrated, therefore, that exposure to road salt during swelling had no effect on the development of eye pigmentation, and the eggs survived following the 5000 and 10000 mg road salt/L exposure. Therefore, no link can be established between the high egg mortality before the eyed egg stage and the delayed development, since all eggs reached the same developmental stage (eyed egg) at the same time as control eggs. Thus, hatching started and finished almost at the same time in all groups. The exposure of eggs to road salt during and post swelling affected, however, the hatching occurring within 400-621 degree days. Usually, salmon eggs hatch approximately after 510 degree days (Kittelsen, 1981). Results (Paper II) demonstrated that the dose-response relationship between road salt exposure and delayed hatching was significant ($p<0.01$) and that the hatching curves associated with the different treatments were significant different from each other ($p<0.01$).

Previous studies have demonstrated that salinity can influence hatching by affecting the buoyancy of fertilized eggs rather than directly affecting the metabolic processes inside the egg (Murashige *et al.*, 1991). It is, therefore, assumed that high road salt concentrations (≥ 500 mg/L) might be responsible for the observed delayed hatching of Atlantic salmon eggs by affecting their buoyancy. Buoyancy affects the movement of eggs in the water column further affecting the developmental processes and hatching etc. Process of hatching is characterized by chorion disintegration, which is performed by a protein called chorionase. The glands, which release this protein are produced in the mouth cavity of embryo soon before hatching

(Jezierska *et al.*, 2009). Road salt is believed to effect the development of this gland that will further affect the process of delaying.

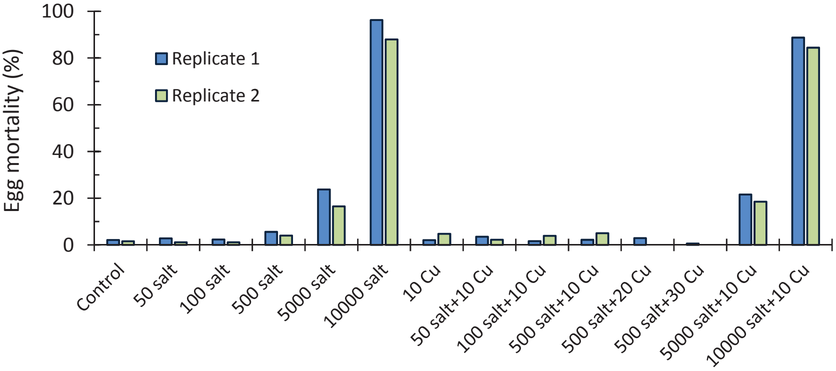

Figure 20. Relative (%) egg mortality in different road salt treatments during fertilization with and without Cu. Road salt concentrations are given in mg/L while Cu concentration are given in µg/L

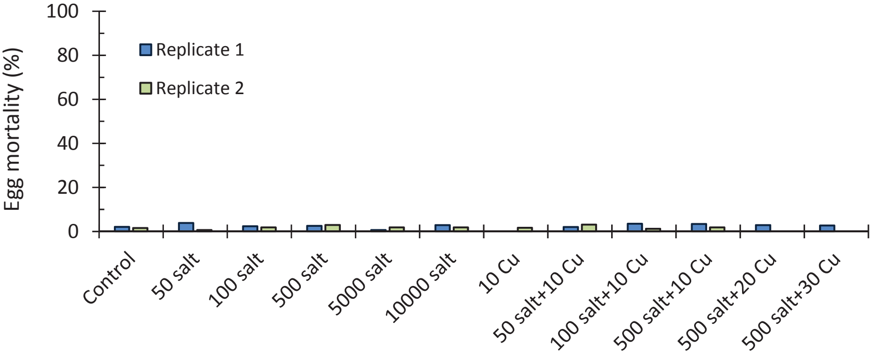

Figure 21. Relative (%) egg mortality associated with exposure to different road salt treatments post fertilization with and without Cu. Road salt concentrations are given in mg/L, while Cu concentration are given in µg/L (Paper II).

Microarray was performed on eyes eggs exposed to 5000 mg road salt/L during fertilization and swelling. Results indicated that out of total 60k features on the array, 1002 were significantly regulated. Among them 721 genes were up-regulated while 281 were down-regulated. Gene ontology (GO) functional enrichment analysis was performed to identify a certain number of biological processes and molecular functions which are particular among the selection of differentially expressed genes (DEGs). The genes affected in developing embryos involve in controlling several function such as osmoregulation, ion regulation, oxidative stress, metabolism (energy turnover), renal function/cell death, acute renal failure, organ development and mitochondrial dysfunction (depolarization of mitochondria and mitochondrial membrane), by the episodic exposure to 5000 mg/L road salt during fertilization. The differential regulation of genes regulating cell survival or death may be the reason for high egg mortality at 5000 mg road salt/L (Paper II). The results indicate that elevated road salt concentrations (5000 mg/L) during fertilization affect by changing the expression of several number of genes involved in vital cellular functions. When Atlantic salmon eggs are exposed to high salinity water (5000 mg/L) their swelling is significantly reduced (Li *et al.*, 1989) as a result of accumulation of ions from ambient media which ultimately give rise to oxidative stress to developing embryos and accumulation of ions and water inside the eggs the developing embryos may experience osmotic stress. The physiological (transcriptional) changes occurring in Atlantic salmon eggs were consistent with hypo-osmoregulation, e.g. the regulation of internal plasma and tissue ions to concentrations being lower than that of the surroundings as reported by Hoar (1988) in exposure of freshwater fish to high salinity. Osmotic stress and ion regulatory disturbances may lead to egg mortality at high road salt concentrations (Paper II). Thus, the results of Paper II are verified by Paper III indicating that stress experienced by Atlantic salmon eggs due to high road salt exposure resulted in altered expression of gene involved in osmoregulation and oxidative stress etc.

Quantitative rtPCR revealed a positive concentration-dependent relationship between road salt concentrations (50, 100, 500 and 5000 mg/L) and gene expression. The results indicated a positive concentration-dependent increase in some genes such as Cyp4A14 (a gene involved in lipid turnover and renal function) Nav1 (a gene involved in neuraxonal development), (ATP1A2), the main sodium/potassium ATP-fueled transporter for chloride ions and oxidative stress (Txndc9), a gene regulating redox homeostasis. Thus, qrtPCR analysis showed that the overall NOTEL value of 50 mg/L road salt has been suggested.

Alevins survival was also affected by road salt exposures. Continuous chronic exposure of road salt to alveins after hatching (Paper IV) significantly affected the survival and development (growth, swim-up and deformities) of alveins at intermediate salt levels (100-1000 mg/L). Thus, exposure to road salt at high levels (5000-10,000 mg/L) during swelling was most toxic and lethal, but not lethal when exposed episodically post swelling until hatching. Intermediate road salt concentrations (100-1000 mg/L) were, however, lethal to developing alevins. Thus it is suggested that different developmental life stages of Atlantic salmon have different sensitivity towards road salt. Salt toxicity towards eggs and alevins could be attributed to the combination of two separate mechanisms of toxicity; firstly toxic effect on eggs at the moment of fertilization and secondly chronic toxic exposure of alevins from hatching till the swim-up stage (Stekoll *et al.*, 2009).

The development of alevins was affected by the concentration of road salt in the exposures. Results (Paper IV) showed that survival of alevins was affected by all road salt concentrations tested (100, 500, 1,000 and 5,000 mg/L). Reduced growth of alevins and delayed swim-up were observed at intermediate road salt concentrations (100, 500 and 1,000 mg/L). Slow rates of yolk absorption and reduced lengths and increased weights (due to less yolk utilization) of swim-up fry of salmonids, when compared to control, were also seen in chronic exposures to elevated levels of dissolved salts by Stekoll *et al.* (2009). Alevins exposed to 5,000 mg road salt/L remained, however, surprisingly unaffected, and the growth and swim-up data were similar to control. It is difficult to explain why effects on growth and swim-up were seen in alevins exposed to intermediate salt concentrations, while no such effects were seen when alevins were exposed to 5,000 mg/L road salt. One possible explanation could be associated with the reduced osmolality difference between alevins and the external 5,000 mg/L medium, which will reduce the amount of energy needed for osmotic and ionic regulation and for maintenance of neutral buoyancy (Lam and Sharma, 1985). The results reflected well that the life history stage of an organisms during episodic events is essential for the type of effects caused.

The effects of road salt on developing eggs (embryos) and alevins increased the frequency of deformed embryos. The present results showed that exposure to road deicing salt resulted in formation of deformities, when eggs were exposed during fertilization (Paper II) and when alevins were exposed after hatching (Paper IV). Different types of deformities (Fig. 22) observed included coiled tail, twin bodies, twin heads, spinal cord deformation (eggs exposure during fertilization), blue sac disease, subcutaneous yolk sac edema, which is characterized by accumulation of fluid between the yolk sac and perivitelline membranes and scoliosis (Jezierskra *et al.*, 2009).

The mechanism by which road salts induce various forms of abnormalities is not presently known. However, similar exposures resulted in significantly smaller eggs (reduced swelling) and high mortality. Reduced swelling can result in reduced PVS formation (Li *et al*., 1989). Small sized PVS does not provide enough space for expansion during embryonic development, which resulted in deformed larvae, as studied by Li *et al.* (1989). In Paper (IV) scoliosis was the main deformity observed at various road salt concentrations. In addition, yolk sac edema and severe fluid accumulation in the yolk sac were also observed in the alevins. It is also suggested that edema is a possible consequence of a paralysis or lack of movement (Roman *et al.*, 2002). Severe larval body deformation caused by environmental stressors may reduce the movements (Sanzo & Hecnar, 2006) of alevins and, therefore, survival rates could probably be low.

Our results demonstrated that road salt exposure exerts negative effects towards early developmental stages of salmonids affecting vital functions such as swelling, hatching, growth, swim-up and survival etc. Stekoll *et al*., (2009) has also observed toxic effects of total dissolved salts on early developmental stages of slamonids. Previous studies have reported toxicity of road salts on differnet aquatic organisms such as fish (Vosyliene, 2006), frogs (Collins and Russell, 2009; Karraker *et al.*, 2008; Sanzo & Hecnar, 2006) and also macroinvertebrates (Benbow & Merritt, 2004). In addition to Na and Cl, the anti cacking agent potassium ferricyanide added to the road salt may also induce toxic effects in fish (Siegel, 2007; Vosyline *et al*., 2006). We have, however, not been able to identify specific effects (if any) associated with this additive in the present work.

Based on literature and the present results, it is assumed that road salts can affect Atlantic salmon physiologically in a variety of ways, such as interfering with osmotic regulation, respiration (Mahajan *et al.*, 1979), development causing deformities, circulatory effects and changes in hematological parameters, (Vosyline *et al.*, 2006).

Figure 22. Various forms of deformities observed after exposure of eggs to road salt with and without Cu in Paper II and IV (Photo: U. Mahrosh).

5.3 Effect of Cu on early life stages of Atlantic salmon

The transfer and effects of Cu on early life history stages of Atlantic salmon were studied (Paper I-IV)

- Determining the concentration of Cu mobilized from collected road dust by road salt extraction (Chapter 5.4.1 interaction of road salt on Cu).
- Exposing developing Atlantic salmon embryos during and post swelling to environmental relevant Cu concentrations, and mixtures of road salt and Cu.
- Exposing alevins from hatching until swim- up to environmental relevant Cu concentrations and mixtures of road salt and Cu.

5.3.1 Effect of Cu on water chemistry

Based on extraction of Cu from road dust samples by road salt solutions (10,000 mg/L), the concentrations range 5-60 µg Cu/L were considered as environmental relevant concentration of Cu in road salt run-off (Chapter 5.4.1). However, the Cu concentrations used in our experiments (5-30 µg/L) were based on initial extraction results performed in 2010.

The speciation of Cu in the Lake Maridalsvannet water and in test solutions containing Cu (5-20 ug/L) changes with increased Cu concentration were studied in Paper II and IV. The water quality used in during the egg exposure was characterized by (pH 7.0±0.5 and temperature 6.3±1.3) and used in the during alevins exposure by (pH 7.0±0.3 and temperature 9.9±0.05). Cu speciation is highly affected by pH of the exposure water and carbonate and hydroxide species of Cu are predominate at pH 7 or higher (Miwa *et al.*, 1989). Addition of 10 µg Cu/L into the control water resulted in formation of 35 % LMM Cu. Colloidal Cu was the predominant species (53 %), while particulate Cu was almost negligible (Paper II). After addition of various Cu concentrations (5-20 µg/L) from acid stock solutions (1 mg Cu/L from $CuCl_2$) to Lake Maridalsvannet water, both the concentration of colloidal Cu and LMM species increased (Paper IV). Complexation of Cu to organic material (4.4 –5.1 mg/L) was assumed to be the main reason (Andrew *et al.*, 1977; Meador, 1991; Winner, 1985) for the relative low fraction of the LMM Cu species and the fraction of added Cu transferred to colloidal Cu species.

5.3.2 Cu uptake by eggs and alevins

The uptake of Cu by eggs and alevins is dependent on many factors such as Cu speciation, bioavailability, concentration of competing compounds, exposure duration, the permeability of the eggs and the affinity towards biotic ligands.

The uptake and distribution of Cu in eggs (Paper I) and in alevins (Paper IV) was demonstrated in the study. Cu is an essential element and thus assumed to be present in aquatic organisms such as Atlantic salmon embryos and alevins (Lorentzen *et al.*, 1998). The concentration of Cu in control eggs was 1.4±0.2 µg/g egg w.w. while the head-Cu concentration in control alevins was 5.8±0.1 µg/g w.w. Unfertilized egg was, however, not analyzed, thus the transfer of Cu from Lake Maridalsvannet water (control) and that present from the female fish could not be distinguished. However, the Cu uptake in eggs observed after addition of Cu to the water was significant, which demonstrates the transfer from water. The uptake of Cu in eggs increased with increasing Cu concentration in the water and was especially high, when exposed during the swelling compared to post swelling exposures. The Cu concentration inside the eggs increased to 2.6±0.1µg/g egg w.w. during swelling stage exposure, while a slight change was observed in eggs exposed post swelling by the highest Cu concentrations tested (30 µg Cu/L). The relative distribution of Cu in chorion increasing with the exposure duration demonstrated that Cu was mainly sorbed to the chorion after swelling, increasing Cu in chorion with increasing time of exposure. The uptake was, however, relative low as the bioconcentration factor (BCF) was 0.08 L/kg for eggs exposed during swelling, and 0.04 L/kg for eggs exposed post swelling, based on the concentration of Cu in whole egg and in water.

The present results demonstrated that swelling was critical for uptake of Cu as also demonstrated for Cl and Na (Chapter 5.2.1). The chorion of egg changes its structure during fertilization (Nakano, 1956), which can allow pollutants to enter inside egg, especially during the process of swelling. Chorion of egg contains mucine-rich metal binding sites characterized by the presence of carboxyl- and sulfhydrylrich groups (Boletzky 1986; Guadagnolo *et al.* 2001; Lacoue-Labarthe *et al.*, 2008). Such binding sites and also the presence of chitin (Muzzarelli, 1997) are probably the basic reasons for Cu binding to chorion. Accumulation of Cu may also change the selective permeability of the chorion, leading to an impaired cation exchange capacity between the perivitelline fluid (PVF) and the surrounding water. The PVF contains a negatively charged colloid (Eddy and Talbot, 1985), which attracts cations such as Cu from the ambient water.

Jezierska *et al*. (2009) reported that during egg exposures towards metals, most of the metal was found to be associated with the chorion as the embryonic development progresses, while only a small fraction was found in the PVS and very little entered the embryo itself. However, our results demonstrated significantly higher uptake into the egg during swelling but the fraction associated with chorion increased after swelling. The concentration of Cu in embryo and in PVS was, however, not distinguished in the present work. The results also demonstrated uptake of Cu in the head of alevins. The Cu concentration in the heads increased also significantly with increasing concentration of positively charged LMM Cu in water.

The head area of alevins, in addition to sensory organs, mainly consists of the gills. Sorensen (1991) stated that fish gills are the major target for the acute toxic effects of water-born metals such as Cu. Fish gills are composed of a complex mixture of functional groups, which provide major sites for binding metal ions and hence toxicity (MacRae *et al.*, 1999). In the present study Cu associated with gills was, however, not distinguished from Cu sorption to the head or sensory organs, as the total Cu head content was determined. However, we assume that a large fraction of the Cu is associated with the gills of the alevins.

5.3.3 Toxicity of Cu towards early life history stages of Atlantic salmon

Cu toxicity towards fish depends on various factors such as pH, concentration of Ca, Mg in water, conductivity/salinity and organic matter content of water (Erickson *et al.*, 1996). The toxicity of Cu on early developmental stages from fertilization to hatching and from hatching to swim up was studied in the present work (Paper II and IV). The exposure solutions contained total Cu (5, 10 and 20 µg/L), with a relatively small fraction present as positively charged LMM Cu species (33-52 %). Exposure of eggs to 10 µg Cu/L was found to have no effects on degree of fertilization, development of eyed egg and survival, while the degree of swelling and time of hatching were significantly affected. However, no effects of Cu have been observed on development and survival of alevins until swim-up.

Paper (I) and (II) demonstrated significant inhibition of egg swelling due to Cu exposure at 30 µg Cu/L. Similar effects of Cu on fish eggs have been reported by Jezierska *et al.* (2009). The survival of eggs exposed during swelling (95-98 %) and post swelling (98 %) was not affected by Cu. All eggs reached the eyed embryos stage soon after 255 degree days, similar to control eggs that developed normally (Chapter 5.1). This illustrates that exposure to Cu at concentrations of 10 µg Cu/L, resulted in uptake of Cu in eggs and changed the swelling of eggs, but this Cu concentration did not cause any observed effects on survival or development of eyed embryos. Lugowska & Jezierska (2000) observed delay in eyed embryo development following the exposure to concentration of 200 µg Cu/L, which is far higher than the concentrations used in our experiments (10 µg Cu/L). Therefore, no effect of exposure to Cu concentrations on development of eye pigmentation was expected in our exposures studies.

However, Cu exposure was found to affect the time of hatching of eggs (paper II). Exposure to 10 µg Cu/L during and post fertilization caused a delay in the window of hatching, and the Cu effects seemed more pronounced in delaying hatching following post fertilization episodic exposures (Fig. 23 B). The effects following Cu exposures during fertilization were, however, also statistically significant (Fig. 23 A).

Exposure to Cu has previously been demonstrated to affect the fish embryos by delaying the time to hatch (Jezierska *et al.*, 2009). Cu may affect hatching via different mechanisms, either individually or in combination with road salt. Jezierska *et al.* (2009) also suggested that metal penetration into the egg might delay the hatching of eggs, and this is also in agreement with the present findings.

Cu might affect the development of hatching gland and hence the production of hatching enzyme (chorionase), which could result in delayed time of hatching (Jezierska *et al.*, 2009). Kapur & Yadav (1982) observed reduced synthesis of chorionase as a result of metal-induced instabilities of transcription and translation processes. Mis & Bigaj (1997) reported reduced production of chorionase, when the *Cyprinus carpio* embryos were exposed to Zn and Cu. Further examination showed that hatching glands of metal exposed embryos had smaller surfaces compared to the controls and showed intracellular granules, which resulted in lower chorionase production. In addition to affecting chorionase activity, Cu is assumed to cause osmotic disturbances that may also disturb the muscular movements necessary to break the chorion during the process of hatching (Dave & Xiu 1991).

The chorion provides protection to the developing embryo as demonstrated by limited uptake of Cu after swelling. Breakage of chorions during the process of hatching might expose developing embryo towards pollutant resulting in premature hatching of less developed larvae or mortality. The present work demonstrated uptake of Cu in head of alevins, but the level was too low to cause any effects on survival (96-98 %), development or growth of the exposed alevins (Paper IV).

It is reported that metal exposure to embryos of aquatic organisms causes abnormalities (Chow & Cheng, 2003; Devlin & Mottet, 1992; Speranza *et al.*, 1977) as observed in *Cyprinus carpio* embryo exposed to Pb, Cu or Cd (Jezierska *et al.*, 2009). They linked abnormal cell division to uneven and irregular distribution of blastomeres and deformation of the entire blastula. In addition, effects on organogenesis stage could be expected. No abnormalities were, however, observed in eggs and alevins exposed to Cu (5-20 µg/L) in our experiments.

Lydersen *et al.* (2002) has set the Cu concentration 6.5 µg/L as criteria of toxicity for freshwater species in soft water, which exceeded in almost all of our samples. Khangarot & Daas (2010) also observed delay in hatching of freshwater pulmonate snail *Lymnaea luteola* at Cu concentrations of 3.2, 5.6 and 10 µg/L. Thus, reduced swelling and delayed hatching seems to be the sensitive endpoints reflecting Cu toxicity towards fish. Our results also indicated that embryonic stage (using hatching as endpoint) was more sensitive to Cu exposure than the larval stage. Thus, hatching seems to be one of the most sensitive measures of Cu toxicity towards fish, as has also been suggested by Scudder *et al.* (1988). The present work indicates that Cu toxicity is highly dependent upon the bioavailability of Cu and on factors influencing the Cu speciation. In the present work only a small fraction of Cu in the water was present as positively charged LMMCu species, assumed to be bioavailable. The moderate concentration of TOC was assumed to be one of the main reason for reduced bioavailability (Meador, 1991; Winner, 1985). Thus, more pronounced effects of Cu are assumed in water at lower concentrations of TOC/DOC.

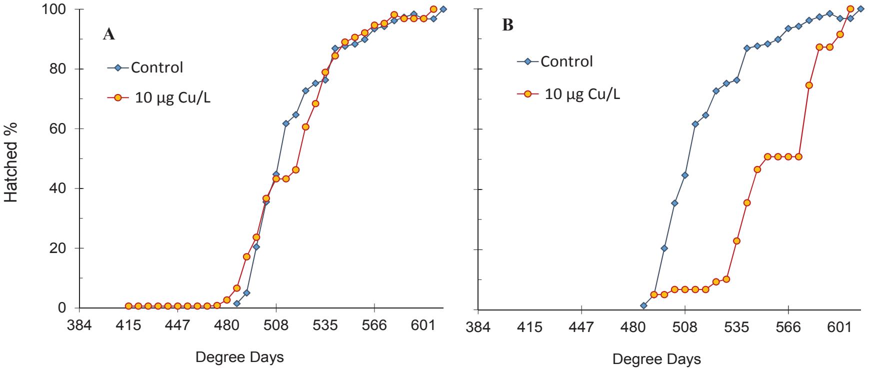

Figure. 23. Time of hatching following exposure of Cu to Atlantic salmon eggs (A) during fertilization and (B) post fertilization.

5.4 Effect of road salt and Cu in mixtures on early life stage of Atlantic salmon

In natural environment, developing eggs and embryos can potentially be exposed to several stressors simultaneously and the toxicity occurring through the interactions of multiple stressors is normally not taken into account (Heier *et al*., 2013). Studies on biological effects from mixtures have been performed e.g. combined effects of metals, ionizing radiations and organic pollutants (Eggen *et al*., 2004; Salbu *et al.*, 2005), however, little attention has been given to the combined effects of Cu and road salt on fish. A single stressor that is not capable to cause a significant effect alone, when combined with other stressors, may lead to substantial effects through additive or synergistic (more than additive) effects, or in some cases might show antagonistic (less than additive) effects (Norwood *et al.*, 2003). The interaction of road salt and Cu on toxicity towards early life stages of Atlantic salmon could also occur via different mechanisms. In the present work, the mobilization of Cu from road dust was studied to identify environmental relevant levels of Cu in road salt run-offs. Then, combined effect of road salt and Cu was studied with respect to bioavailability, uptake and effects on developing Atlantic salmon embryos from fertilization until hatching and on alevins from hatching until swim-up

5.4.1 Effects of Road salt on mobilization of Cu from road dust

The results illustrated potential leaching of Cu from road dust (5 samples collected from two cities in Norway, Fig. 24) when mixed with solution of 10,000 mg road salt/L. The leaching of Cu was at its highest (66 µg Cu/L) from road dust collected from nearby a car parking in Ski of Norway (Ski St.1). High concentration was also found leaching from the road dust in a highway with high traffic density (Oslo St. 1), lowest concentration (12 µg Cu/L) was, however, found from the highway with less traffic density (Ski St.2). The findings confirmed that Cu could be mobilized from road dust by road salt leaching, compared to control (dust water). Our results supported the hypotheses stated earlier by Amrhein *et al.*, (1992); Harrison *et al.* (1981) and Howard & Sova (1993) that metals such as Cd and Cu may be mobilized through a cation-exchange process.

Doner (1978) showed that high concentrations of NaCl (5900 and 29,000 mg/L) leached 1.1-4 times more Ni, Cu, and Cd from a soil than the NaCl concentration equal to the weight of soil. In addition to destroying the soil structure, Na is reported to increases the mobilization of metals by complexing with organic matter and through colloid-assisted transport (Amrhein *et al.*, 1992).

5.4.2 Effect of road deicing salt on Cu speciation

The effects of road salt on Cu speciation was studied in Paper II and IV. The results demonstrated that the concentration of LMM Cu and cationic species increased at high road salt concentrations. Thus, high road salt concentrations increase the assumed bioavailable forms of Cu (Backstrom *et al.*, 2004) that can be toxic towards fish. Warren & Zimmerman (1994) stated that NaCl and water temperature are considered to be the most important environmental factors influencing the Cu speciation in the road runoff. Low temperatures and increase in road salt concentrations reduce the concentration of Cu associated with the particulate matter hence making them more bioavailable. Amrhein & Strong (1990); Amrhein *et al.* (1992) and Amrhein *et al.* (1993) demonstrated in their studies that NaCl produces more bioavailable forms of Cu by mobilizing the Cu associated with particulate and colloids.

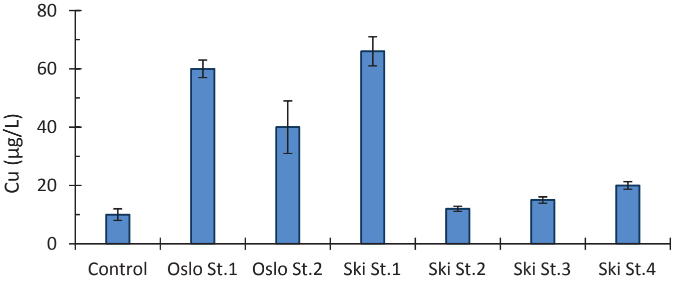

Figure 24. Concentrations of Cu mobilized from road dust samples after mixing with solution of 10,000 mg road salt/L.

5.4.3 Effects of road salt and Cu mixture on transfer towards Atlantic salmon embryos and alevins

The uptake and distribution of Na, Cl and Cu in eggs (Paper I) and Cu in alevins (Paper IV) have been investigated by using ^{22}Na tracer and stable Cu and Cl. Results demonstrated high uptake during the swelling and the uptake of Na and Cl increased by increasing road salt concentrations. The uptake of Na, Cl and Cu was higher, when the eggs were exposed during swelling compared to post swelling exposures. By increasing the exposure duration (1-30 days) Na, Cl and Cu were mainly associated with the surface of chorion. The uptake of Cu by egg was, in the present work, not effected by road salt concentration. It was shown that the road salt increased the concentration of LMM Cu species, which are considered to be more mobile and potentially bioavailable and hence easily taken up by eggs and alevins. However, Na and other cations in high concentrations in the road salt might compete with Cu for uptake (Grossel & Wood, 2002), and thereby reduce the total uptake of Cu. Our results demonstrated that exposure during fertilization and swelling was the critical for ambient uptake of Na, Cl and Cu.

5.4.4 Toxicity of road salt and Cu mixtures

In the present work combined effects of road salt and Cu exposure on toxicity towards Atlantic salmon eggs (embryos) and alevins were studied using several endpoints such as swelling, fertilization success, hatching and survival of eggs and on growth and survival of alevins, in addition to abnormalities.

Great emphasis has been given regarding the multiple stressors issue for a long time (Salbu *et al.*, 2005). The present results demonstrated that the fertilization success (degree of fertilization) was not affected by road salt and Cu, separately or in mixtures. The degree of swelling was, however, inhibited by both road salt and by Cu separately and also in mixtures.

The swelling of eggs in mixtures of road salt and Cu was more inhibited than by road salt separately, indicating a synergistic (more than additive) effects of road salt and Cu (Fig. 25).

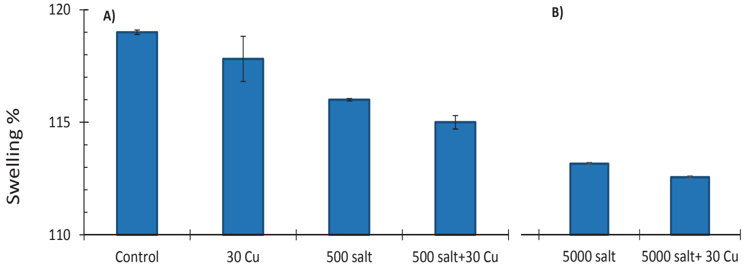

Figure 25. Combined effects of road salt (mg/L) and Cu (µg/L) exposure to eggs during swelling in exposure to A) 500 mg road salt/L and B) 5000 mg road salt/L. Swelling is determined by the relative (%) size of the egg.

Road salt and Cu mixture given during fertilization and swelling showed highest egg mortality compared to post fertilization exposure and control. Egg mortality in road salt exposures was, however, not significantly different ($p=0.3$) from the mortality observed during road salt and Cu mixture exposures. The exposure to Cu caused no mortality. Thus, it is assumed that the high mortality observed in road salt and Cu mixtures exposed during fertilization was mainly due to the toxicity of road salt. The interacting effects on survival seem, therefore, to be additive. Paper (II) showed that exposure to road salt and Cu, separately and in mixtures, had no effect on the development of eyed embryos since eggs in all the treatments developed eye pigmentation at the same time. Hatching of embryos (Paper II) was, however, affected by the exposure of road salt and Cu separately and mixtures, when eggs were exposed both during and post fertilization. Fig (26) shows the pronounced effect of the mixture on the hatching of developing embryos. The delay related to the window of hatching was more prominent at increasing Cu concentrations (20 and 30 µg/L).

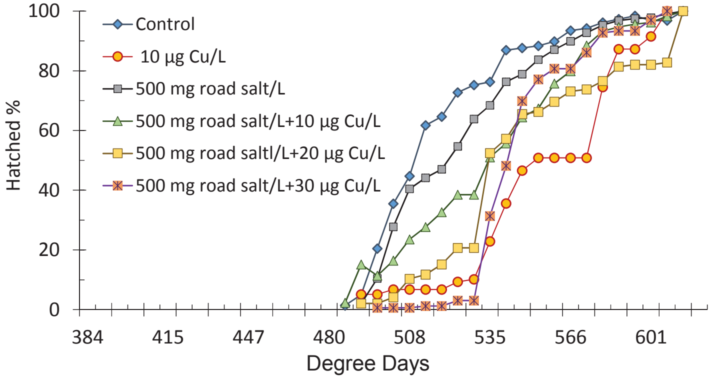

Figure 26. Time of hatching of Atlantic salmon eggs following exposure to the mixture of road salt and Cu post fertilization and post swelling.

The effects of road salt and Cu on body malformations of embryos are given in Paper II and IV. The present results showed that exposure to road salt and Cu mixtures resulted in formation of deformities, when eggs were exposed during fertilization and swelling (Paper II) and alevins were exposed during hatching (Paper IV). Different types of observed deformities (Fig. 22) include coiled tail, conjoined twins, twin heads, spinal cord deformation (eggs exposure during fertilization), blue sac disease, subcutaneous yolk sac edema which is characterized by accumulation of fluid between the yolk sac and perivitelline membranes and scoliosis (alevins exposure during hatching) (Jezierska *et al.*, 2009). A high frequency of deformed larvae was observed, when road salt was mixed with Cu and exposed during fertilization and swelling (Paper II). In Paper (IV) scoliosis was the main deformity observed at various road salt concentrations mixed with Cu. However, the frequency of deformities was higher in road salt and Cu mixtures compared to exposure in road salt alone. This indicates an additional effect of the mixtures compared to road salt separately. Road salt and Cu mixture might induce disturbances of cardiovascular functions in developing embryos, which may result in damage to the blood vessels and hemorrhages, as observed by Heisinger & Green (1975) after exposing the embryos of *Oryzias latipes* to Hg.

Alevins mortality was affected by road salt and Cu mixtures (Paper IV) and highest mortality (5-76 %) was observed at intermediate road salt concentrations (100, 500 and 1,000 mg/L) mixed with Cu (5 and 10 µg/L). Increased Cu uptake in the presence of road salt indicated the mobilization of LMM Cu (from colloidal Cu), which can easily be taken up by alevins and thereby also contribute might to alevin mortality. Exposure to road salt and Cu might weakens the developing embryo and such embryos cannot survive hatching due to lack of the energy required during the hatching process (Jezierska *et al.*, 2009).

Increased Cu uptake by alevins and exposure to road salt increased the mortality although the toxicity of Cu exposure alone had no effect on survival. Growth of alevins was affected by road salt separately (Chapter 5.2.2) and mixed with Cu (Paper II). The growth was not effected by exposure to Cu alone (Chapter 5.3.3). The results showed that growth of alevins was mainly affected by the road salt concentrations (100, 500 and 1,000 mg/L) as there were no difference between exposures to road salt separately and in mixture with Cu. Survived alevins showed delayed swim-up after exposure to mixtures of road salt and Cu and to road salt exposure alone (Chapter 5.2.2), while were not affected by the Cu exposure alone. Results indicated that the delay in swim-up could mostly be attributed to the road salt exposure as there were no difference between the time to swim-up between mixture exposures and exposure to road salt only. The increased concentrations of LMM positively charged Cu species in contact with road salt and increased Cu uptake can, however, not be excluded as a contributory cause not only to the reduced growth but also to the delayed swim-up at low road salt concentrations (100, 500 and 1,000 mg/L) mixed with Cu. As stated by Jezierska & Witeska (2001), the bioavailability and toxicity of chemicals towards aquatic organisms can be changed by variations in the salinity levels.

Early life stages of fish (embyo-larval) are often more susceptible to hazardous materials than seen in toxicity tests with juvenile and adult fish (Dave & Xiu, 1991, Chapman, 1978; Parker & McKeown, 1987; Weber, 1991; Rice *et al.*, 2001). In the present study toxic effects at early life stages such as egg mortality, delayed hatching, and deformities (Paper II and IV) could be explained by high uptake of Na, Cl and Cu during fertilization and swelling (Paper I). Toxic effects of Na, Cl and Cu might result in changing the permeability of chorion, causing disturbances in cation exchange between PVF and the ambient water (Stouthart *et al.*, 1996).

When Cu was added to high road salt concentration (5,000 mg/L), surprisingly, hardly any effects could be observed compared to control. The rate of survival was somewhat lower than control, while growth and time of swim-up for alevins was not affected. One plausible reason could be attributed to the reduced osmolality difference between the fish and the external medium, which will reduce the amount of energy needed for osmotic and ionic regulation and for maintenance of neutral buoyancy (Lam and Sharma, 1985). Grosell and Wood (2002) also reported that the presence of ambient Na suppressed the branchial Cu uptake by 50 % across the gills of juvenile Rainbow trout. This is in accordance with the present result, as the Cu uptake was reduced in the presence of high road salt concentrations. Furthermore, Blanchard and Grosell (2006) observed no apparent Cu-induced mortality of euryhaline fish *Fundulus heteroclitus* when exposed to mixtures of Cu (30 and 150 µg/L) and high concetrations of salt (5,000, 11,000 and 22,000 mg/L). Therefore, it is suggested that Cu does not disrupt the ionregulation in fish at high salinities. It is also assumed that excessive mucous secretion at high salt compared to lower salt concentration also will reduce the Cu uptake in gills of alevins exposed to 5,000 mg road salt/L mixed with Cu (Hansen *et al.*, 2007; Tao *et al.*, 2006).

5.5 Vulnerability

The vulnerability towards road salt and Cu separately and in mixtures is believed to be different for different life history stages for Atlantic salmon.

1. The present work showed a significant dose (road salt and Cu) - response (delayed hatching, increased deformations, higher mortality) relationship for exposed eggs.
2. Alevins seem to be more affected, when exposed to even low road salt concentrations. Unlike eggs, alevins have osmoregulatory system that allows control and regulation of the toxicity. However, compared to eggs, they have no chorion as a protecting barrier towards contaminants that also make them more vulnerable.
3. However, at high road salt concentrations less mortality and normal growth rate was, however, surprisingly observed.

The concentrations of the road salt also in combination with Cu that would influence different endpoints vary, as illustrated in Fig 27 A (eggs) and Fig 27 B (alevins). The vulnerability of Atlantic salmon alevin (from hatching to swim-up) with respect to road salt exposure is slightly different from that of embryos (eggs), with respect to endpoints considered. As illustrated in Fig 27 B, reduced growth and delayed swim- up occurs at the lowest road salt concentrations (100-1,000 mg/L), while mortality continued to occur at road concentrations even above 1,000 mg/L.

For eggs, exposure of road salt will upregulate the genes essential for development (100 µg/L), will induce delayed hatching (500 µg/L) and increase the egg mortality (≥5000 mg/L). As illustrated in Fig (28), the observed effects will depend whether the exposure takes place during or post-fertilization. For alevins the exposure to road salt affected the swim-up, growth and survival.

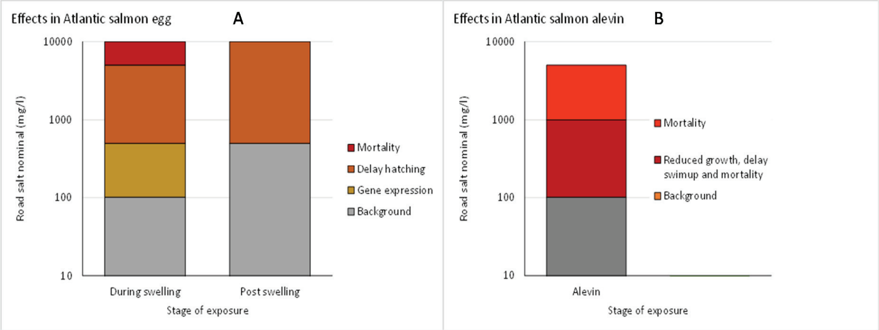

Figure 27. Different end points observed in Atlantic salmon eggs (A) and alevins (B) exposed to road salt solution.

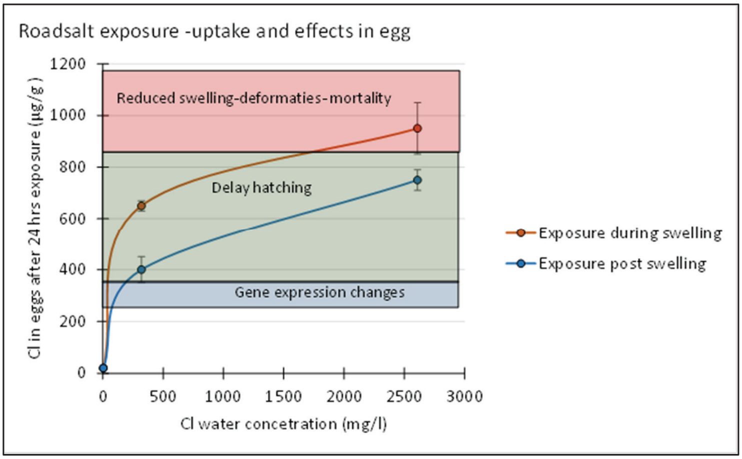

Figure 28. Different end points observed in Atlantic salmon eggs exposed to road salt during and post swelling.

6 Ecological impacts

Morphological abnormalities, reduced growth and low survival rates observed during the experiments suggest that the use of road deicing salt at the spawning time of Atlantic salmon would negatively impact not only individuals, but also on the population level by reducing larval recruitment to adult stocks.

After swim-up from the gravel in the spring, a stressed first feeding fry is less fit to start feeding on food organisms. If they do not immediately (within a few days) get food, they will die either from starvation or by being an easy prey for predators (Stekoll *et al*., 2009). Road deicing salts could also affect the development of motor and sensory functions (Sanzo & Hecnar, 2006), which possibly will interfere with predator avoidance and prey-capture behaviors during critical periods of larval development and lead, in turn, to a lower rate of survival in road salt-exposed fish.

The concentration of dissolved road salt and Cu in the natural environment is likely to be highly variable with elevated levels associated with the road salt application especially during snowmelt, distance to the downstream water bodies from the road, amount of salt used etc. Delayed hatching and swim-up of Atlantic salmon effects the population recruitment, since the alevins hatching later than normal degree days may not be suitable to survive in the environment. Fish reaching the hatching and swim-up stage later than normal may experience problems in growth and survival due to a mismatch in time with suitable prey organisms in their natural environment. It is also assumed that the environmental conditions for delayed hatched and delayed swim-up alevins are probably not suitable for growth and survival and they are more prone to predation. Reduced lengths and increased weights of fish at the time of swim-up make them less competitive in catching prey and more vulnerable to other predators (Stekoll *et al.*, 2009).

The issue of ecological exposure is further complicated by the possibility of avoidance behavior for free swimming fish. Therefore, it is assumed that the free swimming stage might be less susceptible to chemicals than other stationary early life stages (eggs) due to its free activity (not tested by us). Alevins depend on their yolk sac for energy, they have a finite store of antioxidants, which could be depleted under the conditions of oxidative stress, and therefore, they might be prone to mortality or predation. Results from the present work demonstrates that road deicing salts affect body size and weight, time to hatch, swim-up and activity of the alevins that can influence the outcome of competitive and predatory encounters, and thus affect the structure of Atlantic salmon populations and communities (Sanzo & Hecnar, 2006).

These effects can potentially have far ranging impacts on ecosystems. Na and Cl ions are known to affect muscle activity (Hill & Wyse, 1988) making alevins less active during swim-up. Many organisms might struggle hard, while swimming, as a result of bent tails, making it harder for those individuals to acquire food and orientate themselves, while feeding (Sanzo & Hecnar, 2006). Evaluation of toxicity data of any chemical depends on the life history stage of fish such as salmonids being exposed (Peterson *et al.*1988). Thus, road salt application at critical time of development may have deleterious effects on population (Sanzo & Hecnar, 2006).

7 Regulatory considerations

In an environmental regulatory context, the results obtained from the present work, both chemically and biologically, suggest that road deicing salt, and associated mobilized contaminants such as Cu also indirect effects such as due to leaching from sand, silt and road dust of more bioavailable forms of Cu, can pose a threat to Atlantic salmon population. This could be due to direct toxic effects of road deicing salts such as during spawning periods at the time of fertilization, leading to reduced egg swelling, delayed hatching, egg mortality, as well as mortality of alevins, and delayed development until swim-up and also indirect effects such as due to leaching of more bioavailable forms of Cu. High concentrations of LMM Cu species are being discharged during road runoff process due to road salt application. Thus in addition, the overall performance in terms of reducing ecotoxicological effects of road deicing salts can be questioned. It is, therefore, recommended that following solution might be taken into account:

1. The use of high concentrations of road salt should be restricted.
2. The road salt application on roads close to downstream rivers or brooks during the time of spawning should be avoided. Depending on geography and strain differences in spawning period, restrictions should be in place from early October through winter season.
3. Road runoff should, if possible, be treated as harmful chemicals, before entering downstream water bodies, especially during the spawning period, and during spring melt, when runoff waters can influence the gravel water quality with alevins as the main stream quality can affect the first feeding fry.
4. Road run-off network and outflow from storage ponds should be constructed sufficiently far from the downstream water bodies such as river and lakes, in order to dilute the chemicals before entering spawning places of aquatic organisms such as Atlantic salmon.

8 Significance and novelty of work

The work contributes to linking exposure to uptake and effects, and also demonstrates that Atlantic salmon eggs are more sensitive during the stage of swelling due to increased uptake of elements compared to after swelling stages (Paper I) and after hatching when the alevins no longer have protective chorion (Paper IV). Significantly higher uptake of road deicing salt and Cu can induce serious effects that may alter the Atlantic salmon population in downstream water bodies receiving road runoff (Fig. 29). The paper II identifies the risk and toxic effects posed by these road related chemicals on aquatic organisms such as the embryo stage (egg) of Atlantic salmon, which is similar to that observed by Collins and Russel (2009) in study of road salt toxicity towards Nova Scotia amphibians. Exposure of these chemicals in mixtures increased the sensitivity mainly in an additive or more than additively order. The results have also demonstrated that road salt influences the Cu speciation, thus increasing the concentration of the more bioavailable forms of Cu and the Cu uptake that may pose threat to Atlantic salmon. It has also been investigated that fertilization and swelling are more critical stages than other developmental stages. Exposure of road salt and associated contaminants during these stages could potentially alter the local Atlantic salmon population in downstream water bodies receiving the road runoff. Thus, the current study should contribute significantly to the field of environmental toxicology, risk assessment and management related to the use of road deicing.

Road salt toxicity tests using aquatic organisms are not new. Small scale toxicity tests have been performed under controlled laboratory conditions using aquatic organisms such as macro invertebrates (Benbow & Merritt, 2004), various amphibians species (Collins & Russell, 2009; Sanzo & Hecnar, 2006) and fish (Vosyline *et al.*, 2006). Compared to the previous studies the present toxicity test experiments were performed as chronic exposures (4-15 weeks) and biological effects were investigated using several end points, including gene expression (Paper III) reflecting sub-lethal and lethal effects, being a novelty in the scientific literature.

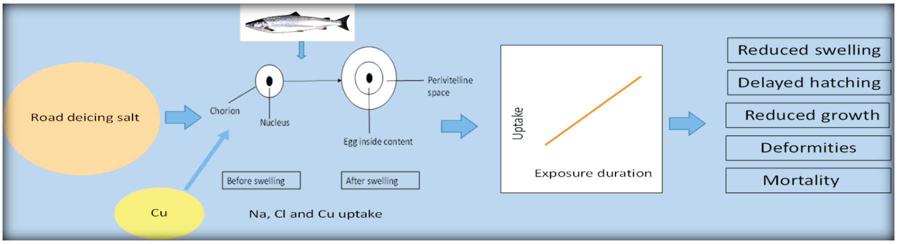

Figure 29. Summary of the findings in current study

9 Limitations of the study/experimental work

There were also a number of limitations and uncertainties affecting in the current project that should have been taken into account. In paper (II) and (IV) the number of replicates were limited. Hundreds of eggs and alevins were used in each treatment group, and individual egg was considered as biological replicate in all studies. Limited number of replicates may impact the statistical analysis and may give rise to the situation called ‘‘pseudoreplicates’’. However, they were kept in the same exposure box, due to limited technical exposure. Paper (I) lacks Cu speciation data, which could further explain the link between bioavailability and uptake of Cu by eggs. The gene expression analysis should have been more comprehensive, more eggs should in particular have been picked out to be able to include more qrtPCR analyses in order to document potential effects at low salinity levels. Especially the exposure of alevins experiment suffer also from a low number of replicates and surprising results with low effects at high salt concentration call for new experiments in the future. In addition, experiments including Na and Cl uptake data should have been performed on alevins as well (Paper IV).

10 Conclusion

Our results suggest that early life stages of Atlantic salmon experience stress, reduced survival, delayed hatching and altered development resulting from chronic exposure of road deicing salts. Our findings indicate of a general detrimental effects of environmentally realistic concentrations of road salts used every year in Northern countries. Results suggested that road deicing salts mobilize Cu from road dust and that road salt influences the speciation of Cu in water by increasing the fraction of LMM Cu species. Road salt, (both Na and Cl) and as well Cu is mainly taken up by eggs during swelling but some limited uptake was observed thereafter as well. However, the uptake of Cu was not affected by the presence of road salt. Increased Na concentration due to addition of road salt seems to compete with the increase in concentration of LMM Cu causing no change in Cu uptake.

Fertilization and swelling are critical stages for road salt and Cu uptake and hence affect in the form of reduce egg swelling, egg mortality, delayed hatching and formation of various deformities. Alevins exposure soon after hatching also resulted in mortality, reduced growth and formation of deformities at road salt concentrations lower than osmolality difference between alevins blood and ambient media. Road deicing salts showed direct toxicity towards different life stages of Atlantic salmon from fertilization until swim-up.

To protect salmon streams during embryo–larval stages, the maximum acceptable toxicant concentration for road salts (mainly NaCl) should not exceed 500 mg/L especially during spawning period, however, during hatching period even low road salt concentrations can be toxic towards newly hatched alevins. However, effects at molecular levels have been found at lower salt concentrations too (>50 mg/L). Results showed that road salt thus, had a toxic effect on Atlantic salmon eggs and alevins at environmentally realistic concentrations (100-10,000 mg/L). At high road salt concentrations the Cl ions might make complexation with metals, which can further reduce the metal toxicity. It is also expected that concentrations used in our study might be exceeded in more heavily populated areas of northern countries, where road deicing salts are frequently used for road maintenance especially in harsh winter.

The major findings of current thesis are:

1. Road salt mobilized bioavailable forms of Cu (LMM cationic Cu).
2. Degree of fertilization (fertilization success) was unaffected by road salt and Cu addition.
3. Hatching of alevins was affected by road salt and Cu, and effects of hatching were more pronounced, especially, when the eggs were exposed post fertilization and swelling.
4. Degree of swelling was reduced by high road salt concentrations and Cu.
5. Survival of eggs and alevins was highly affected by road salt concentrations, especially when eggs are exposed during fertilization and swelling.
6. Na, Cl and Cu were taken up by the eggs mainly during the process of swelling.
7. In exposure after swelling the relative fraction of Na, Cl and Cu was mainly associated with chorion.
8. The uptake of Cu, Cl and Na by eggs was dependent on concentrations of road salt and the duration of exposure.
9. Various deformities were observed, after exposing the eggs during fertilization and following the exposure of alevins during and after hatching, depending on the concentration of road salt and Cu.
10. Exposure of eggs to low road salt concentrations (≥100 mg/L) can also differentially regulate genes of importance for development, and a NOEC value of 50 mg road salt/L has been suggested.
11. Additive and more than additive effects of road salt and Cu were observed in some end points such as egg swelling, hatching and formation of deformities.

The application of salts for road maintenance may have devastating effects on Atlantic salmon populations. In order to gain insight into possible impacts of deicing chemicals on water quality, further research is recommended on long term monitoring of these chemicals, their fate during transportation, the impact of discharge on the concentration at the time of entry and the subsequent changes over a period of time due to exchanges with sediments and the contributions of base flows. Research is needed to develop eco-friendly alternative deicers and improved application technology for the deicers.

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Paper I

Uptake and distribution of ^{22}Na, Cl and Cu in Atlantic salmon (*Salmo salar*) eggs exposed to road salt (NaCl)

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Abstract

Early developmental stages of Atlantic salmon are susceptible to different environmental stressors such as road salt, Cu. Current study was performed to investigate the uptake and distribution of Na, Cl and Cu into the eggs of Atlantic salmon exposed to road salt and Cu during and post swelling. Sodium (Na), chloride (Cl) and copper (Cu) uptake from water in developing Atlantic salmon (*Salmo salar*) eggs at different concentrations of road salt and Cu was demonstrated during and post swelling by using ^{22}Na tracer and stable Cl and Cu. ^{22}Na, Cl and Cu uptake by eggs was higher during the first 24 h after fertilization, including the swelling stage. Some uptake continued until 48 h post fertilization. The uptake of ^{22}Na and Cl increased with increasing road salt concentration (5000 mg road salt/L), while the Cu (30 µg/L) uptake was not affected by road salt addition. During the first 24 h of the exposure, most of the total accumulated ^{22}Na, Cl and Cu was found inside the egg and comparatively less accumulation was associated with the chorion (14-41%). The fraction associated with the chorion increased, however, with time (43-70%). The results demonstrated that exposure to both Na, Cl and Cu was most critical during the first 24 h post fertilization, which includes the swelling stage and during this period the transport of Na, Cl and Cu into the egg fluid could affect the developing embryo.

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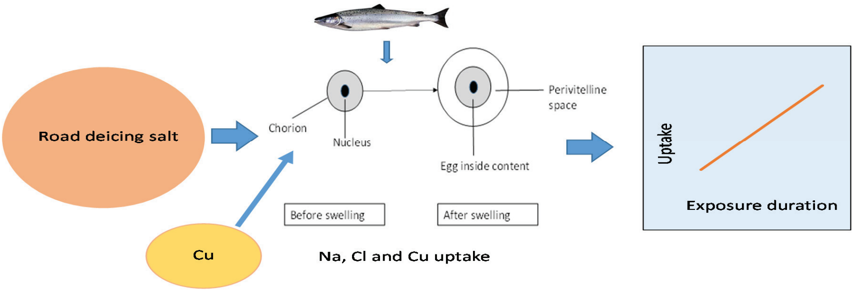

Keywords: Uptake, Distribution, Road salt, Cu, Atlantic salmon, eggs, fertilization

1 Introduction

Road salt (mainly NaCl) is an extensively used deicing agent in northern North America and Europe[1] and is a significant source of sodium (Na) and chloride (Cl) to the ecosystem, as the salt can easily spread into environmental compartments at measurable levels. Increased levels of deicing salts in the water bodies close to roads have been linked to defrosting periods, snow melting, snow storms and leaching of road side soil storing huge amounts of road salt.[2] Although road salt application is highly beneficial for safety purposes, however, surface water and groundwater quality has been shown to be severely affected by its application.[3] Thus, constant road salt applications over the last 50 years have caused deleterious impacts on fauna,[4] and flora.[5]

Increased concentration of Cl is assumed to be the main reason for the toxicity of road salt. Excessive amounts of Cl ions are known to produce toxic effects on organisms and also result in impairment of the function and structure of ecosystem.[6] Road deicing salts also show negative effects indirectly via leaching of road dust and soils that have been accumulating salts and other contaminants such as copper (Cu).[7] Cu is an crucial metal ion for human health, plants and animals, because it is involved in many vital functions of living cells and their organelles, particularly the mitochondria.[8] However, excessive Cu concentrations induce toxicity in exposed organisms due to the formation of reactive oxygen species (ROS), which can damage proteins, lipids and nucleic acids[9,10] resulting in altered gene expression.

Fertilization and early developmental stages of fish are particularly sensitive to water pollution such as road salt and Cu.[11] In addition, high salinity levels in ambient media may negatively impact the swelling of fish eggs.[12] Furthermore, eggs cannot avoid or excrete pollutants, compared to other freely moving life stages of fish (juveniles and adult fish), which if recognized, can avoid pollutants. Many aquatic organisms are considered to be most vulnerable to environmental stress during early developmental stages of their life hostory.[13] The eggs of Atlantic salmon (*Salmo salar*) spawning in fresh water systems may be subjected to road salt and Cu, which could affect various developmental processes during embryonic period and ultimately resulting in a decline of offspring quantity and quality. Growth, reproduction and osmoregulation, in different life stages of fish can be affected by xenobiotics[14] such as excessive NaCl and Cu. In addition, such chemicals may also affect organisms by affecting several biochemical processes and producing changes in the properties of the membrane.[15]

Although high concentrations of NaCl and Cu have been demonstrated to be toxic to the developing embryo and consequently reduce hatching success and induce various deformities.[11] It is not clear if the adverse effects can be attributed to sorption on the chorion hindering the transport of essential ions and oxygen, or accumulation in the embryo developing inside the egg.

The objective of this paper was, therefore, to investigate the uptake of road salt (Na and Cl) and Cu and their relative distribution to chorion and inside the eggs of Atlantic salmon, in exposure to the road salt and Cu separately and in mixtures after fertilization.

2 Materials and Methods

The experiment was designed to obtain information about the uptake of Na, Cl and Cu by Atlantic salmon eggs during and post swelling. After dry fertilization, eggs were exposed to the water containing ^{22}Na tracer and stable Cl and Cu respectively, in exposures to road salt during and post-swelling. Swelling period is defined as the first 24 h immediately after fertilization, while post swelling is defined as the time period from 24 h post fertilization until the end of the experiment. The experiment was conducted in a static exposure for 30 days (180 degree days) from March, 2012 until April, 2012 at Isotope Laboratories at Norwegian University of Life Sciences (NMBU), in a temperature controlled cabinet at 6°C. The distribution of ^{22}Na, Cl and Cu between the chorion and content inside the egg (including egg yolk) was determined at 1, 13 and 30 days of exposure.

Following five treatments were included in the study:

- Control (Lake Maridalsvannet, Oslo Norway)
- 25 Bq ^{22}Na/L
- 500 and 5000 mg road salt/L mixed with 25 Bq ^{22}Na/L.
- 30 µg Cu /L.
- 500 and 5000 mg road salt/L mixed with 30 µg Cu /L.

Eggs were given following three types of exposure regimes:

1. During swelling exposure:

   Eggs were kept in water qualities containing ^{22}Na and Cu separately and in mixture at variable road salt concentrations for first 24 h soon after fertilization (during swelling) until the end of the exposure.
2. Post swelling exposure:

   Eggs were kept in control water for first 24 h soon after fertilization and were exposed to ^{22}Na and Cu in water qualities separately and in mixture at variable road salt concentrations until the end of exposure.
3. Control group:

   Eggs were kept in control water for the whole experimental period.

2.1 In-vitro dry fertilization of eggs

Dry stripped eggs and sperms of Atlantic salmon, produced at Aquagen hatchery (Sunndalsøra, Norway), were transported overnight to (NMBU) Ås in plastic bags on ice in a box of polystyrene. Eggs and milt were mixed for fertilization, in pre washed dry equipment. Soon after dry fertilization, eggs were rinsed with corresponding treatment water to remove excess of milt and transferred to the same water qualities for swelling according to Kallqvist et al.[16]

2.2 Experimental setup

One hundred eggs were placed in plastic boxes containing 150 mL water. Water was changed after 13 days (78 degree days) in the Cu treatments, while the tracer solution was not changed in order to limit its usage. The Cu stock solution was made from reagent grade $CuCl_2.2H_2O$, while road salt solutions were produced by adding 500 and 5000 mg NaCl Isbryter's rock salt into one liter Maridalsvannet water. ^{22}Na with specific activity 376.09 mci/mg Na and radionuclide purity 99.90 % was used in the experiment. It was produced by NEN^{TM} life sciences products, Inc Boston USA with the concentration of 5000.00 µc/ml. In corresponding exposure boxes, 6.25 mL of ^{22}Na tracer was added into control water and road salt solutions, respectively. The corresponding solutions were mixed well before the placement of eggs into the boxes.

Each water quality included in the study consisted of three independent replicates. Eggs (n=15) were sampled at three different times after start of exposure for both ^{22}Na, Cl and Cu. For both during and post swelling exposure regimes, eggs were sampled 24 h after start of exposure (1 and 2 days after fertilization respectively), in addition 13 and 30 days after fertilization.

2.3 Water quality assessment

To control the exposure water, pH, temperature, and conductivity was measured weekly, using WTW 340i equipped with SenTix® 41 glass electrode and TetraCon® 325 conductivity probe respectively. Concentration of major cations (Na, Ca, Mg and K), major anions (Cl) and total organic carbon (TOC) was determined in unfiltered water samples. Organic analyzer (Shimadzu TOC cpn) was used to determine TOC and Lachat IC5000 Ion Chromatography was used to determine the anions in non-acidified samples. Major cations and Cu were analyzed by using Inductive Coupled Plasma Emission Spectroscopy (ICP-OES, Perkin Elmer, Optima 5300 DV) in acidified samples (5 % ultrapure HNO_3). Cations and TOC were not measured in ^{22}Na water to avoid the risk of instrumental contamination.

2.4 Increase in size of eggs after swelling

To obtain information about the degree of swelling, the mean diameter of 10 eggs was measured with a ruler pre-fertilization, and compared to 10 randomly collected eggs of each treatment after 24 h of swelling. Values are given as % swelling relative to unfertilized eggs.

2.5 ^{22}Na, Cl and Cu uptake

To determine the uptake of ^{22}Na, Cl and Cu, five eggs were randomly collected from each replicate. Eggs were sampled individually in the form of total egg (n=15), egg inside content (n=15) and chorion (n=15) into pre weighed Eppendorf tube and stored at -20 ^{o}C. Eggs were emptied by a syringe and the content inside the egg was transferred into the Eppendorf tubes.

2.6 ^{22}Na measurement

Following the tracer exposure, egg samples were measured with respect to ^{22}Na by using NaI detector (Wallac WIZARD 3 1480 Automatic Gamma Counter Perkin Elmer) for 600 seconds. The activity was measured in dpm, which was further converted into Bq (taking measurement efficiency into account) indicating the amount of ^{22}Na activity in or at the surface of the egg. ^{22}Na activity determination in water samples was based on 5 mL sample collected from each box containing the ^{22}Na exposure, while ^{22}Na activity of the eggs was based on individual egg stored in Eppendorf tubes. ^{22}Na activity in eggs was presented in Bq/g wet weight of egg.

2.7 Determination of Cl in eggs

To determine the concentration of Cl in Atlantic salmon eggs, 0.5 ml of tetramethylammonium hydroxide solution (TMAH) was added in the Eppendorf tube containing the sample. Since the eggs were sticking inside the tube, therefore, the tube was placed inside the oven at 90 ^{o}C for 30 min until the sample was properly dissolved. 2 ml Milli-Q water was added into the sample and transferred into another pre labelled 15 ml ICP tube. Samples were again placed in the oven at 90 ^{o}C for 2 h. Samples were diluted afterwards to the final volume of 10 mL before analysis.

2.8 Determination of Cu

Concentrated ultrapure HNO_3 (1 mL) was added into the samples of eggs and left overnight, to completely dissolve the whole sample content. Further, 2 mL Milli-Q water and 50 µl internal standard (Rh) was added before digesting using ultraclave (Milestone, Leutkirch, Germany). After digestion, samples were diluted to 10 mL (10 % HNO_3) with Milli-Q water before analysis of Cu using ICP-OES. In addition standard reference material and blank samples were included for quality assurance.

Cu and Cl level in the samples was calculated by the following formula:

μg Cu or Cl/g wet weight of egg = (A*V)/W

where A= μg/L of Cu or Cl, V= dilution volume of sample (L), and W= wet weight of sample (g). Values of Cu and Cl are given as μg/g egg wet weight.

2.9 Statistical analysis and data handling

Statistical analysis was performed using GraphPad prism 5.0 (Graphpad Software, Inc., San Diego, CA, USA). Data was presented as mean±SD based on three independent replicates. Paired t-test and One-way ANOVA followed by Tuckey's post hoc test was used to study the significant differences in the uptake between different exposures and change in various water quality parameters over time. Pearson correlation was performed to study the relationship between road salt and other factors such as conductivity and egg swelling. Statistical analysis was performed on normally distributed data, while the data not meeting the criteria of normality was $\log_{10}$ transformed prior to analysis. Activity of ^{22}Na in treatments with 500 and 5000 mg road salt concentrations was corrected by multiplying measured dpm with the Na dilution factor compared to ^{22}Na in control and converted to Bq (Table 3). Significant differences were defined by using the criterion $p \leq 0.05$ as significance level.

3 Results

3.1 Data quality

Quantification limit of ^{22}Na using NaI detector, Cl using ICP-MS and Cu using ICP-OES, determined by taking the sum of the average and three times the standard deviation of 10 blanks was 0.03 Bq/L for ^{22}Na, 0.011µg/L for Cl and 0.4 µg/L for Cu.

3.2 Water quality

The general water quality variables are presented in the supplementary Table 1 and 2. The ionic strength, measured as conductivity, showed a substantial increase ($r^2=0.95$, $p=0.95$) with road salt addition, from control to 5000 mg road salt/L. During the whole experimental period pH, conductivity and concentration of Cu in each exposure unit changed with the time. Similar trend was observed in all treatments. The activity concentration of ^{22}Na in exposure water compared to stable Na in each treatment is presented in Table (1).

The concentration of total Cu in exposure water decreased significantly ($p=0.002$) from day-1 to day-30, which showed an opposite trend to Na, as the major cations increased significantly (Ca, $p<0.01$; Na, $p=0.04$). Concentration of TOC increased significantly from day-1 to day-13 of exposure ($p=0.0002$).

3.3 Effect of road salt and Cu on swelling

Following dry fertilization, the size of eggs increased during the swelling period. The swelling is expressed relative (%) to the unfertilized egg (Fig. 1). High road salt concentrations (5000 mg/L) mixed with ^{22}Na (r^2= -0.75, p= 0.02) and Cu (r^2= -0.97, p= 0.0001) caused significant reduction in swelling compared to the eggs in control water.

3.4 ^{22}Na uptake during and post swelling

The rate of ^{22}Na uptake by whole egg increased throughout the exposure period (Fig. 2 A, B and C). The highest increase in the ^{22}Na uptake was observed in mixture of ^{22}Na and 5000 mg road salt/L. The results showed that ^{22}Na uptake increased significantly with increasing road salt concentration ($p<0.0001$). The ^{22}Na uptake in whole egg also increased significantly with exposure time and reached the maximum level at the end of the exposure in treatments with ^{22}Na in control water (p=0.0001), ^{22}Na mixed with 500 mg road salt/L (p=0.0001) and ^{22}Na mixed with 5000 mg road salt/L (p=0.0003).

Within first 24 h of exposure, ^{22}Na transferred mainly inside the egg was (6, 94 and 282 Bq/g egg w.w) in the treatments with ^{22}Na admixture with 0, 500 and 5000 mg road salt/L, respectively. Thus, during the first 24 h the uptake was comparatively higher than the sorption to chorion. Only 25, 34 and 35 % of total accumulated ^{22}Na was bound to the chorion after 24 h of exposure, while it increased to 45, 47 and 62 % on day-30 in treatments with ^{22}Na mixed with 0, 500 and 5000 mg road salt/L, respectively.

The ^{22}Na activity uptake by whole egg during swelling was significantly higher than the uptake post swelling (p=0.002) (Fig. 2 C, D and E). Pattern of ^{22}Na distribution on chorion and inside the egg in post swelling treatments was not different from exposure during swelling.

3.5 Cl uptake during and post swelling

Cl was taken up by eggs both during and post swelling (Fig. 3), however, the uptake was higher during swelling compared to post swelling exposures ($p=0.004$). Highest uptake was observed at high road salt concentration *i.e.* 500 and 5000 mg/L. Cl uptake was significantly affected by road salt concentration both during ($p=0.01$) and post swelling exposures ($p=0.04$). Highest Cl uptake in whole egg (2180-3122 µg/g egg w.w) was observed at high road salt concentration (5000 mg/L) from day-1 to day-30 in exposures during swelling. The rate of Cl uptake was also affected by increasing the exposure duration.

3.6 Cu uptake during and post swelling

Cu in water was taken up by the eggs (Fig. 4). After 24 h of exposure Cu concentration in the whole egg, chorion and content inside the egg was comparatively higher in all treatments during swelling ($p=0.03$) and post-swelling exposure ($p=0.04$) compared to control. There was no significant difference in Cu uptake in eggs with treatments including road salt compared to those without road salt ($p=0.07$). Cu concentration in whole egg samples increased by increasing exposure time, from day-1 to day-30 ($p=0.01$). During the first 24 h and 48 h the relative (%) Cu uptake in the inside egg content was about 50-60% and was higher than in chorion regardless of the road salt concentrations. However, the relative distribution of Cu associated with chorion increased by increasing exposure time ($p=0.004$).

A significant higher Cu uptake by whole egg was observed during swelling than post swelling exposures ($p=0.03$). The relative (%) fraction of the total accumulated Cu bound to the chorion in post swelling exposure, was 17, 18 and 14 % in treatments with 30 µg Cu mixed with 0, 500 and 5000 mg road salt/L, respectively. However, the sorption to the chorion increased significantly ($p=0.02$) and was 41, 34 and 40 % of total Cu content in the respective treatments after 30 days of exposure.

4 Discussion

4.1 Water chemistry

Lake Maridalsvannet water used in the experiment is characterized by low ionic strength, with low nutrient concentration and the cationic concentrations are within typical values for Norwegian freshwater.[18] Conductivity increased significantly in some treatments as a result of the addition of road salts (Na^+ and Cl^-), which appeared in high concentrations (197 mg Na/L, 1967 mg Na/L, 300 mg Cl/L and 3500 mg Cl/L) after addition of 500 and 5000 mg road salt/L, respectively.

Since the experiment was performed in a static system, the concentration of major ions and conductivity in all exposure waters slightly increased over time due to a small reduction in the water volume caused by evaporation. The reduction in Cu was mainly due to the uptake by eggs, but sorption to surfaces of the exposure box can also not be excluded. Relatively low TOC (4.1-5.0 mg/L) in the beginning of the experiment indicated that Cu and Na were assumed to be available for uptake by the eggs. TOC was approximately three times higher (14 mg/L compared to 4 mg/L) at the day-13 of the experiment, compared to the start probably due to the accumulation of egg residues. The TOC is known to influence the speciation and thus reduce the bioavailability of Cu, as metal ions associated with organic carbon are not easily taken up by the eggs.[15] Thus, the Cu uptake over time could be influenced by the increase in TOC or increase in major cations[19] due to complexation or competition for binding sites[8] on chorion.

4.2 Swelling

Swelling is influenced by ionic and osmotic composition of the ambient environment. High road salt doses (≥500 mg/L) can inhibit the swelling.[11] In addition to the road salt, egg swelling was also affected by Cu as previously demonstrated.[11] Due to additive effects, considerable suppression in swelling was also observed in Cu and road salt admixture. When Atlantic salmon eggs enter into the medium with high salinity and ionic concentrations, the resulting imbalance of ions may affect the swelling process causing embryonic deformities and egg mortality as reported by Fridman et al.[20] High road salt and Cu concentrations can inhibit the formation of perivitelline space (PVS), which restricts the embryonic development.[12] Jezierska[21] suggested that during swelling phase of eggs, metals may accumulate inside the egg indicating that the chorion does not fully protect the embryo against metal penetration.

4.3 ^{22}Na, Cl and Cu uptake during and post swelling

^{22}Na, Cl and Cu were mainly taken up during the time of swelling but uptake also continued first 24 h of exposure post swelling (48 h post fertilization). Results demonstrated uptake from water into the egg and also sorption on the egg chorion. The uptake inside the egg was mainly due to the exposure during swelling from 0-24 h after fertilization, but also post swelling from 24-48 h after fertilization. ^{22}Na, Cl and Cu accumulation inside the egg increased with time, however, the relative distribution on chorion also increased, which was probably due to increased uptake on chorion with increasing exposure, with limited changes inside the egg. High uptake of road salt and Cu during swelling is probably due to the changes in chorion structure after contraction at the time of fertilization.[22]

Egg membrane is torn soon before fertilization, therefore, it loses its spherical shape and results in formation of an irregular mass. This is the critical time for metal uptake from ambient environment by the egg as found by Nakano.[22]

The ^{22}Na and Cu binding to chorion is probably due to the presence of carboxyl- and sulfhydrylrich groups, the mucine-rich metal binding sites[23,24,25,26] and also the presence of chitin.[27] It is assumed that high sorption by the chorion reduces the penetration into perivitelline fluid (PVF) and further hinders the transfer into the developing embryo.[28] It is also expected that the number of active sites on chorion for the binding of pollutants are limited and thus the sorption will reach a maximum (saturations of binding sites) and stable level will be reached at a given time of exposure. This was, however, not demonstrated during the 30 days of exposure. Results indicated that the first 24 h soon after fertilization, which includes the swelling period, were crucial for uptake of road salt. Cl uptake confirms the toxicity of Cl towards aquatic organisms as documented in previous studies.[11,29,30]

More Cu accumulation with increasing exposure duration is also important in metal toxicity since the egg sensitivity is also dependent upon the duration of exposure. Responses of eggs to various environmental pollutants change due to its structural and physiological changes as the development progresses.[31] Khangarot and Daas[32] reported increase in embryonic mortality, deformity and developmental delay in snail embryo by increasing Cu concentrations and exposure duration. Some other factors such as pH, organic content, chemical speciation of Cu, movement patterns and physiology of organisms[33] also play role in Cu uptake and effects.

Increased sorption of road salt and Cu to the chorion occurred although the concentration of TOC and major cations increased in the water. It is uncertain if metal sorption to the chorion influences the embryonic development, however, the first 24 and 48 h of exposure seem to be the most critical period for the main Cu transport inside the egg. Cu and ^{22}Na sorption by the chorion was followed by transport, or possibly by passive diffusion into developing embryo, which is in agreement with previous reported observation with uptake and distribution of ^{65}Zn in Atlantic salmon eggs.[34] It is also assumed that the chorion provides protection to the developing embryo by binding the pollutants such as Cu found in ambient environment.[35] However, high concentrations of Cu and other metal species associated with the chorion might accumulate in the embryo by passively moving into the egg interior as observed by Xu et al.[36]

4.4 Uptake of Cu in road salt mixtures

Road salt at high concentrations is believed to mobilize Cu from soil or dust surfaces[7,37] and thereby increases the bioavailable fraction of Cu that is taken up by the eggs. Comparatively high accumulation in the chorion with time might result in over saturation due to ^{22}Na and Cu binding, which possibly would reduce further uptake. Grosell and Wood[9] reported that Cu uptake is affected by the presence of various ions in the ambient environment. Our results showed that Cu uptake was not significantly ($p=0.2$) affected by road salt addition. It is assumed that increase in road salt concentration may give rise to more cation competition[9], therefore, the Cu uptake remains unaffected even at high road salt concentration (5000 mg/L). Furthermore, pH of the test solution could also influence the Cu uptake by eggs. At low pH increasing concentrations of H^+ leads to a competition between protons and cationic metals such as Cu for the binding sites on the biological surface[38] such as chorion and gills.

5 Conclusion

Current study has shown that first 24 h of swelling were important for road salt and metal uptake from the ambient media, but the uptake continued post swelling until 48 h after fertilization. Increase in road salt concentration resulted in increased uptake of Na and Cl by Atlantic salmon eggs. Road salt addition up to 5000 mg/L had no effect on Cu uptake of eggs, at 30 µg/L or lower. Results showed that the Na, Cl and Cu were taken up by Atlantic salmon eggs, which could further affect the embryonic development and thereby affecting the offspring's quantity and quality. A short exposure (1-2 min) to high salt concentrations during fertilization and swelling is long enough to produce toxic effects on later developmental stages of fish.[39] Thus, the use of road salt, especially, in spawning period of Atlantic salmon may lead to deformation or mortality of developing embryos probably by high uptake of road salt and Cu.

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Competing interests

The authors declare they have no competing interests.

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Table 1. The ^{22}Na activity concentrations in different treatment waters during and post swelling exposures.

	Nominal Na conc. (mg/L)		Actual Na conc. (mg/L)			^{22}Na Activity (Bq/L)					
						Day-1		Day-13		Day-30	
Treatment	DS	PS	DS	PS	Dilution factor	DS	PS	DS	PS	DS	PS
Control	4	4	4.4±9.0	5.0±2.1	-	<0.03*	<0.03	<0.03	<0.03	<0.03	<0.03
^{22}Na	4	4	3.2±0.2	4.1±0.4	-	27	19	10	12	6.0	9.0
^{22}Na+500 mg road salt/L	197	197	265±61	270±21	22	26	21	12	12	7.0	7.0
^{22}Na+5000 mg road salt/L	1967	1967	3100±231	2830±330	217	31	27	14	14	11	9.0

DS: During swelling PS: Post swelling * Detection limit

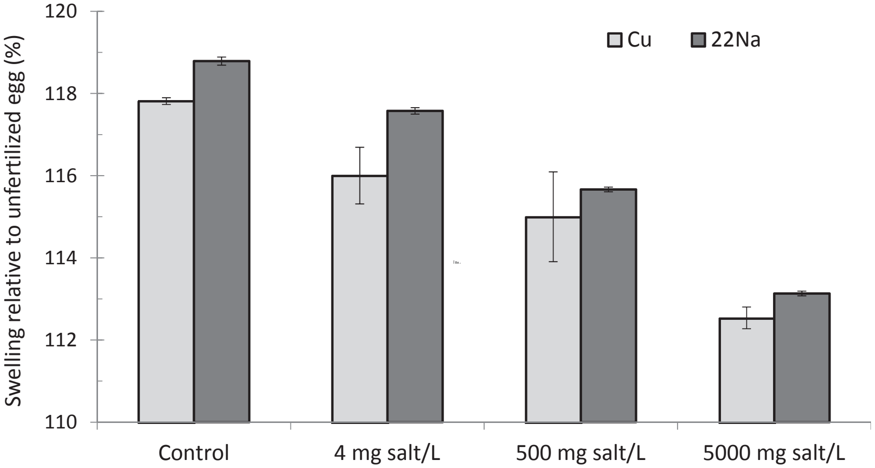

Figure 1. Increase in diameter of Atlantic salmon eggs after swelling relative (%) to unfertilized eggs, exposed to different water qualities with the addition of 30 µg Cu/L and 25 Bq ^{22}Na/L along with different road salt concentrations.

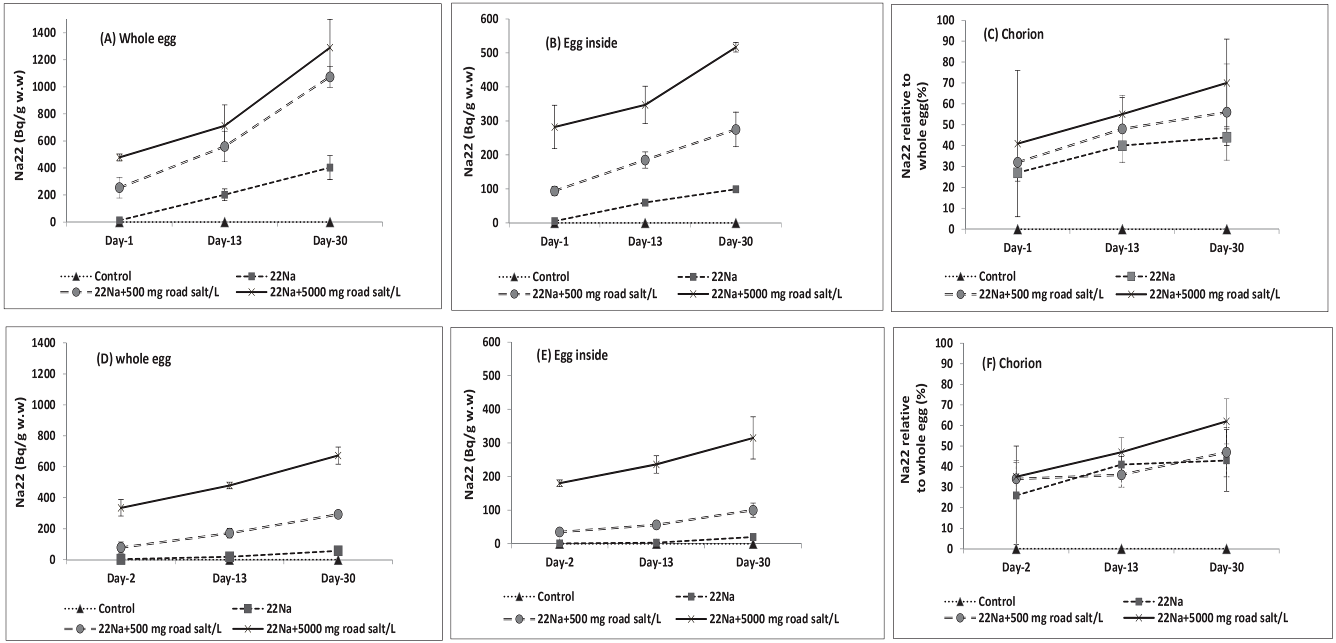

Figure 2. Activity concentration of ^{22}Na as function of time in whole egg, egg inside content and chorion, collected on day-1, 13 and 30 after fertilization in the eggs exposed during swelling (A, B and C) and post swelling (D, E and F) (n=15).

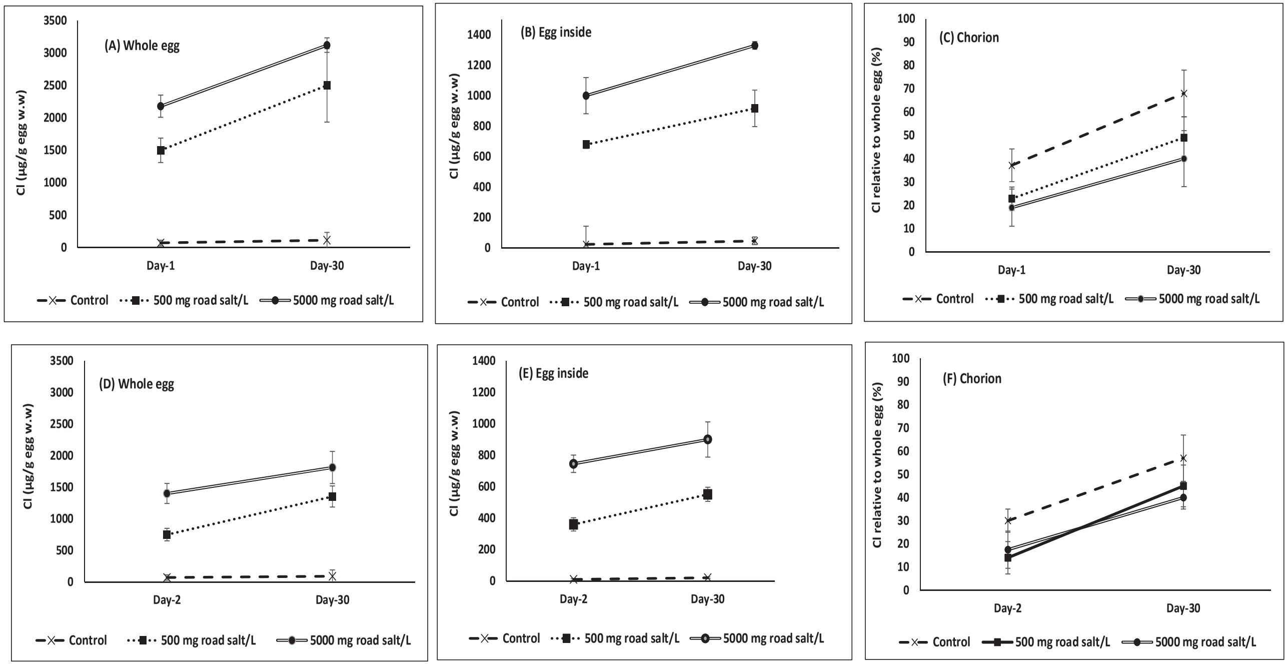

Figure 3. Cl concentration in whole egg, chorion and egg inside content, collected on day-1 and 30 after fertilization in the eggs exposed during swelling (A, B and C) and post swelling (D, E and F) (n=15).

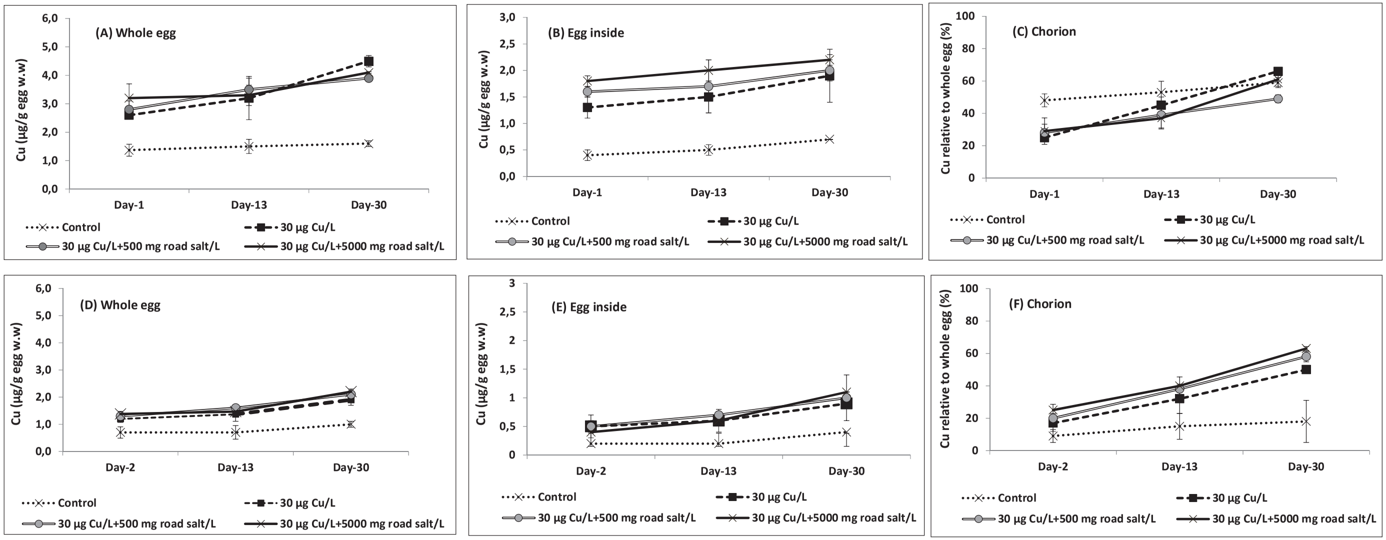

Figure 4. Cu concertation in whole gg, egg inside and chorion, collected on day-1, 13 and 30 after fertilization in the eggs exposed during swelling (A, B and C) and post swelling (D, E and F) (n=15).

Supplementary **Table 1**. Physical and chemical water variables in different treatments during egg swelling (n=5)

Water variables	Exposure duration	Control	30 µg Cu/L	30 µg Cu/L+500 mg road salt/L	30 µg Cu/L+5000 mg road salt/L	25 Bq ^{22}Na/L	25 Bq ^{22}Na/L+500 mg road salt/L	25 Bq ^{22}Na/L+5000 mg road salt/L
pH	day-1	7.4±0.0	7.4±0.0	7.3±0.0	7.4±0.4	7.6±0.0	7.3±0.1	7.3±0.0
	day-13	7.7±0.1	7.8±0.0	7.6±0.0	7.4±0.2	7.5±0.0	7.3±0.1	7.4±0.1
	day-30	7.5±1.1	7.6±0.3	7.8±0.4	7.6±1.2	7.7±0.2	7.4±0.0	7.4±0.1
Cond. µs/cm	day-1	48±13	43±29	810±225	7954±560	42±12	767±37	8113±614
	day-13	53±3	61±4.0	952±69	8310±231	57±3.0	790±608	9500±172
	day-30	62±1.2	59±4.1	997±54	8732±342	51±4.0	716±312	9160±230
Cu µg/L	day-1	3.2±1.2	27±5.4	26±0.4	23±2.5	N.A	N.A	N.A
	day-13	4.0±1.5	13±3.0	11±4.0	20±11	N.A	N.A	N.A
	day-30	3.2±1.0	17±2.1	15±3.3	19±3.9	N.A	N.A	N.A
Ca mg/L	day-1	2.7±0.6	2.5±0.7	4.0±1.0	7.0±0.1	N.A	N.A	N.A
	day-13	8.6±0.3	8.9±9.0	8.8±4.3	10±0.4	N.A	N.A	N.A
	day-30	6.5±1.2	9.3±0.3	7.5±0.1	8.3±1.4	N.A	N.A	N.A
Na mg/L	day-1	4.4±8.9	3.2±0.2	365±61	2100±231	N.A	N.A	N.A
	day-13	14±2.0	11±2.3	432±25	1722±671	N.A	N.A	N.A
	day-30	16±3.1	14±2.2	347±21	1942±432	N.A	N.A	N.A
K mg/L	day-1	4.5±0.6	4.1±0.7	5.0±0.9	7.3±0.1	N.A	N.A	N.A
	day-13	5.1±0.1	9.0±0.5	10±0.3	10±1.0	N.A	N.A	N.A
	day-30	6.8±1.1	11±2.2	9.5±3.1	9.8±0.1	N.A	N.A	N.A
Mg mg/L	day-1	2.0±4.0	2.0±0.3	2.1±0.6	2.3±0.2	N.A	N.A	N.A
	day-13	9.0±1.3	11±4.2	5.0±1.0	6.3±2.2	N.A	N.A	N.A
	day-30	9.9±2.4	12±2.2	6.1±0.4	9.0±1.3	N.A	N.A	N.A
Cl mg/L	day-1	4.1±0.9	5.2±2.4	300±24	2611±45	N.A	N.A	N.A
	day-13	4.1±1.1	4.9±1.7	352±13	3021±56	N.A	N.A	N.A
	day-30	4.4±0.6	5.4±0.8	410±75	3500±84	N.A	N.A	N.A
TOC mg/L	day-1	4.1±0.4	3.9±0.3	4.2±0.6	5.1±1.0	N.A	N.A	N.A
	day-13	14±0.3	11±1.2	8.5±3.0	10±4.4	N.A	N.A	N.A
	day-30	-	-	-	-	N.A	N.A	N.A

*Cond.: Conductivity

Supplementary **Table 2**. Physical-chemical water variables in different treatments 24 h post swelling (n=5)

Water variables	Exposure duration	Control	30 µg Cu/L	30 µg Cu/L+500 mg road salt/L	30 µg Cu/L+5000 mg road salt/L	25 Bq ^{22}Na/L	25 Bq ^{22}Na/L+500 mg road salt/L	25 Bq ^{22}Na/L+5000 mg road salt/L
pH	day-1	7.3±3.0	7.7±1.4	7.2±0.1	6.9±0.2	8.1±1.0	7.3±0.1	7.3±0.0
	day-13	7.6±0.1	7.9±0.0	7.6±0.0	7.1±0.3	8.3±1.1	7.3±0.1	7.4±0.1
	day-30	7.4±0.4	7.6±0.1	7.9±0.0	7.6±0.1	8.1±0.4	-	-
Cond. (µs/cm)	day-1	39±4.0	45±12	506±69	6100±25	45±8.0	767±37	8113±614
	day-13	60±13	52±8.3	846±625	7887±560	63±12	790±608	9500±172
	day-30	54±9.5	61±5.5	921±233	7226±342	-	-	-
Cu µg/L	day-1	6.0±3.1	28±4.7	25±2.6	27±0.5	N.A	N.A	N.A
	day-13	2.0±0.1	24±4.1	21±2.1	23±0.6	N.A	N.A	N.A
	day-30	3.5±1.2	21±2.2	18±3.1	20±0.4	N.A	N.A	N.A
Ca mg/L	day-1	3.1±0.1	4.0±1.0	4.1±0.5	6.3±1.0	N.A	N.A	N.A
	day-13	7.5±2.2	8.2±1.6	6.4±1.2	9.3±0.6	N.A	N.A	N.A
	day-30	7.5±2.2	7.5±2.2	7.5±2.2	7.5±2.2	N.A	N.A	N.A
Na mg/L	day-1	5.0±1.4	4.2±2.2	370±34	2010±450	N.A	N.A	N.A
	day-13	8.0±1.1	11±1.0	461±25	2193±920	N.A	N.A	N.A
	day-30	9.4±0.4	10±1.3	510±34	2500±523	N.A	N.A	N.A
K mg/L	day-1	6.1±10	14±2.0	541±42	6.0±3.1	N.A	N.A	N.A
	day-13	6.8±5.0	7.3±0.8	14±6.6	8.1±1.4	N.A	N.A	N.A
	day-30	6.8±5.0	7.1±5.0	±5.0	±5.0	N.A	N.A	N.A
Mg mg/L	day-1	2.2±0.1	2.0±1.1	3.2±1.4	4.1±3.2	N.A	N.A	N.A
	day-13	5.4±4.0	6.1±3.2	9.1±6.3	8.4±3.0	N.A	N.A	N.A
	day-30	-	-	-	-	N.A	N.A	N.A
Cl mg/L	day-1	4.2±1.2	3.8±1.2	320±20	2610±520	N.A	N.A	N.A
	day-13	4.5±0.04	4.1±0.04	361±14	2852±741	N.A	N.A	N.A
	day-30	5.1±0.6	4.4±0.6	410±63	3110±438	N.A	N.A	N.A
TOC mg/L	day-1	5.0±0.3	4.2±0.1	4.5±1.8	4.6±2.0	N.A	N.A	N.A
	day-13	14±0.8	14±0.2	9.1±5.0	24±3.0	N.A	N.A	N.A
	day-30	-	-	-	-	N.A	N.A	N.A

Cond.: Conductivity

Paper II

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Toxicity of road deicing salt (NaCl) and copper (Cu) to fertilization and early developmental stages of Atlantic salmon (*Salmo salar*)

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HIGHLIGHTS

- Road salt and Cu exposure to Atlantic salmon eggs simulated road runoff episodes.
- Road salt exposed during fertilization inhibited swelling and reduced egg survival.
- Post fertilization exposure to Cu ($\geq$10 µg/L) caused delayed hatching.
- Mixture of road salt and Cu exposed during fertilization caused larval deformities.
- Fertilization and swelling stage was most sensitive to road salt and Cu.

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Toxicity

ABSTRACT

In many countries, salting of ice or snow covered roads may affect aquatic organisms in the catchment directly or indirectly by mobilization of toxic metals. We studied the toxicity of road deicing salt and copper (Cu) on the vulnerable early life stages of Atlantic salmon (*Salmo salar*), from fertilization till hatching. Controlled episodic exposure to road salt ($\geq$5000 mg/L) during fertilization resulted in reduced swelling and less percent egg survival. Exposure to Cu both during and post fertilization caused delayed hatching. Larval deformities were, however found as an additional effect, when eggs were exposed to high salt concentration ($\geq$5000 mg/L) mixed with Cu (10 µg Cu/L) during fertilization. Thus, it appears that the sensitivity of early developmental stages of Atlantic salmon increased when exposed to these stressors, and road salt application during spawning can pose threat to Atlantic salmon in water bodies receiving road runoff. The study gives insight on assessment and management of risks on Atlantic salmon population posed by road related hazardous chemicals.

1. Introduction

Sodium chloride (NaCl) is used as an effective deicing agent at temperatures within 0 to −9 °C. Since 1940, large quantities of NaCl have been applied to roads in cold climate regions for the purpose of winter road maintenance [1]. Deicing salts are widely applied to roads in the northern hemisphere in winter, and can be transported as runoff at least 170 m from roads into the wetlands [2]. Studies have confirmed that a high proportion of deicing salts such as NaCl is actually removed from the roads as surface runoff and finally distributed to rivers and streams [3]. Road salt concentrations can vary from 150 to 5000 mg road salt/L in rural lakes, urban impoundment lakes and snow cleared from streets. Concentrations of road salt in aquatic environment such as ponds, streams and rivers can reach up to 4000 and 4300 mg road salt/L [4]. In addition, seasonal inputs and environmental persistence of road salt may result in elevated concentrations that may be present during critical life history stages of Atlantic salmon e.g., fertilization and later development stages.

Copper (Cu) is naturally present in surface waters at concentrations ranging from 0.2 to 30 µg/L in fresh water systems [5].

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Elevated Cu concentrations in surface waters can also be attributed to automobile traffic and by urban waste on the roadways [6]. The main sources of Cu in this respect are tire wear, fluid leakage, engine and brake parts, vehicle component wear, atmospheric deposition and road surface abrasion [7]. Thus, Cu discharged into the aquatic compartment from roads is related to factors such as traffic patterns, road construction, drainage system, weather conditions as well as properties of soils situated in the vicinity of roads and finally different environmental factors such as temperature, pH and conductivity [8].

NaCl has a mobilizing effect on several metals associated with roadside soils [7]. Amrhein et al. [9] showed in laboratory studies that the addition of NaCl mobilized different metals including Cu associated with organic matter and colloids. In Norwegian fresh waters, the background concentration of Cu is normally less than 1–2 μg/L [10]. However, elevated Cu concentrations are more likely to be found in road salt impacted lakes located close to roads compared to more distant lakes [11].

Studies indicate that road salt affects early life stages (ELS) of different aquatic organisms. The impact of road deicing salt on larval wood frogs (*Rana sylvatica*) was observed in the form of higher mortality, reduced weight and activity, and increased physical abnormalities at concentrations ranging from 2636 to 5109 mg road salt/L [12]. The community structure of Nova Scotica amphibians was influenced by chloride concentrations in ponds due to road deicing salts application [13].

Although Cu is an essential metal for nearly all organisms, including fish [14] and is involved in many physiological functions [15]. Elevated Cu concentrations may cause toxic effects [16] such as, interference with branchial ion transport and enzyme activities [17]. Fertilization and ELS of aquatic organisms such as fish are also sensitive towards Cu. Cu may cause various developmental effects such as morphological deformity, low hatchability and survival, delayed time in hatching and growth reduction [18], craniofacial alterations, yolk sac abnormalities, oedemata, cardiovascular disturbances, lack of pigmentation and spinal deformities [19].

Road salt may also interact with the Cu toxicity, but normally elevated Na^+ and Cl^- concentrations would have a mitigating effect e.g., ion competition following the Biotic Ligand Model (BLM) [20]. Alternatively, road salt and metals may act as multiple stressors, where synergistic effects may also occur.

In Norway, the first ice and snow leading to salting of roads often occur during spawning season of salmonids such as Atlantic salmon and Sea trout/Brown trout (*Salmo trutta*) the runoff can affect the water quality of nearby rivers and streams. Therefore our objective was to identify the toxicity of road runoff salt and Cu mixtures and compare the susceptibility of fertilization and early developmental stages to exposure. The effects on early development followed from fertilization till hatching were investigated by exposing the eggs of Atlantic salmon during and post fertilization. Our findings are discussed in the light of potential risk caused by the road salt and Cu towards different developmental stages of fish under environmental relevant exposure scenarios.

2. Materials and methods

The experiment was designed to simulate the episodic runoff from roads, and the subsequent exposure of fish eggs.

2.1. Concentration of Cu in road dust

To determine environmentally relevant concentrations of mobilized Cu in road dust due to road salt addition, we conducted an extraction experiment. Road dust was collected from the road shoulder of a four lane motorway (E6) in the city of Oslo having an annual average daily traffic (AADT) of 64,000 vehicles. Fifteen g road dust was added into 150 mL milliQ water along with 5000 and 10,000 mg road salt/L and placed on the roller table for 24 h. Samples were centrifuged for 15 min at the speed of 5000 rpm, and supernatant was carefully separated and the concentrations of total Cu was determined in acidified samples (2% HNO_3) using ICP-OES (PerkinElmer Optima 5300 DV).

2.2. Experimental setup

To determine road salt and Cu exposure effects on eggs during and post fertilization episodic exposures, the following two exposure regimes were included:

1. Exposure to road salt without and with Cu during fertilization of eggs (24 h) and exposure to control water thereafter.
2. Episodic (24 h/week) post fertilization exposure of eggs by road salt without and with Cu. The first episode started 24 h after fertilization.

The experiment was conducted from November 2010 till March 2011 in Fish Laboratory at Norwegian University of Life Sciences (NMBU) in a temperature controlled system (6 °C). The control water was a typical low conductivity water quality for Norway used in many exposure studies such as by Olvisk et al. [21]. Different water quality for each exposure regime was continuously distributed to exposure boxes containing eggs placed in dark climate chambers. Different water qualities used are as follows:

- Control water (Lake Maridalsvannet).
- 10 μg Cu/L.
- 50,100, 500, 5000 and 10,000 mg road salt/L without and with 10 μg Cu/L.
- 500 mg road salt/L mixed with 20 and 30 μg Cu/L.

Road salt and Cu solutions were produced by mixing road salt (NaCl Isbryter's rock salt) and $CuCl_2{\cdot}2H_2O$ with Lake Maridalsvannet water. Road salt (NaCl Isbryter's rock salt), obtained from GC Rieber Salt AS, was used in the study instead of laboratory fine grade NaCl because the former was considered to be more environmentally realistic. According to the fact sheet the road salt consisted of 98.5% NaCl, 0.30% Ca and Mg, 0.70% SO_4, 0.30% H_2O humidity and 70–100 mg anticaking agent (E 535). The anticaking agent, which is sodium ferrocyanide, may produce free cyanide after reacting with UV.

2.3. Fertilization

Dry stripped Atlantic salmon eggs and sperms from the Aquagen hatchery Norway, were transported and stored in plastic bags on ice in polystyrene. After fertilization, eggs were transferred to specially designed boxes (155 mm × 106 mm × 45 mm, 739 cm^3) with specific water quality for swelling eggs in climate chambers according to Kallqvist et al. [22].

2.4. Water quality parameters

Temperature, pH and conductivity were measured weekly by using WTW 340i equipped with SenTix® 41 glass electrode and TetraCon® 325 conductivity probe. Dissolved oxygen was measured by using optic probe WTW 4301. These parameters were also logged continuously with an automatic electronic logger Campbell CR200.

2.5. Water fractionation

A combined filtration/ultrafiltration and ion exchange technique was used to obtain information of different physico-chemical forms of Cu species [23].

2.5.1. Size fractionation

Size fractions of Cu were obtained by filtration and ultrafiltration:

- Total Cu, determined based on unfiltered sample after acidification (2% HNO_3).
- $Cu_{Particulate} = Cu_{Total} - Cu_{0.45\,\mu m\ filtrate}$.
- $Cu_{Colloidal} = Cu_{0.45\,\mu m\ filtrate} - Cu_{LMM\ (low\ molecular\ mass)}$.
- $Cu_{Low\ molecular\ mass} = Cu \leq 10\,kDa$.

2.5.2. Charge fractionation

Charge fractionations were obtained by ion-exchange chromatography using cation exchange Chelex-100 (BioRad, 50–100 mesh, Na form).

- $Cu_{Cationic} = Cu_{Entering\ cation\ exchange\ column} - Cu_{Leaving\ cation\ exchange\ column}$
- LMM $Cu_{Cationic}$ = passing 10 kDa ultrafilter and retained in Chelex-100 resin.

2.6. Chemical analysis

Concentrations of Cu fractions and the total concentration of major cations such as calcium (Ca), potassium (K), magnesium (Mg) and sodium (Na) in unfiltered water samples were measured using ICP-OES. Water samples (15 mL) were acidified with 2% ultrapure nitric acid (HNO_3) prior to analysis. Total organic carbon (TOC) in unfiltered samples was determined by using Shimadzu TOC cpn Total organic analyzer and major anions including chloride (Cl^-), flouride (F^-), nitrate (NO_3^-), and sulfate (SO_4^{2-}) were determined by using Lachat IC5000 Ion Chromatography.

2.7. Biological endpoints

2.7.1. Fertilization

To study the influence of different exposures on fertilization success, 10 eggs randomly collected 24 h post fertilization, were examined under a binocular microscope after being treated in a fixation liquid. Eggs were classified as *not fertilized*, if lacking cells in the center.

2.7.2. Egg swelling

To obtain information about the effect of road salt and Cu exposures on swelling of the eggs, the diameter of 20 randomly collected eggs of each treatment was measured 24 h after exposure, and compared to a reference of 20 eggs exposed to control water. The diameter of eggs was used as a measure of swelling.

2.7.3. Egg survival

Egg survival data was obtained by subtracting the number of dead eggs from the total number of eggs added into each treatment. Dead eggs were counted and removed on daily basis. Eggs were considered dead when turned opaque and white.

2.7.4. Eyed embryos

The time period for eggs to reach the eyed stage was noted and the number of eggs reaching this stage was counted. Eyed embryo stage was defined, when the black eyes of the embryo were noticeable with the naked eye through the chorion.

2.7.5. Hatching success

Time for the onset of hatching was noted and the number of hatched and/or not hatched eggs in each box was counted daily. Both fully and partly hatched larvae were included in the observation according to Stouthart et al. [24].

2.7.6. Deformities

The percentage of larval deformation was determined with microscope before hatching and with the naked eye after hatching. Both deformed embryos within non-hatched eggs and alevins (after hatching) were included in the calculation of total percentage of deformities.

2.8. Statistics and data handling

Water quality parameters were presented as mean ± 1 SD (standard deviation) based on the measurements in two replicates throughout the experimental period. Regression analysis (R-Comdr 2.14.1) was applied to identify significant effects of road salt and Cu exposure on various water quality parameters including Cu speciation and on different biological end points such as swelling.

Survival analysis by using GraphPad prism 5.0 (Graphpad Software, Inc., San Diego, CA, USA) was performed to determine the percentage survival and hatching of the fertilized eggs followed by Log-rank (Mantel-Cox) test to study the effect on survival and hatching and logrank test to identify the dose response trends in the treatments. One-way ANOVA followed by Tuckey's post hoc test (GraphPad prism 5.0, Graphpad Software, Inc., San Diego, CA, USA) was used to reveal significant differences in pH, temperature and O_2 between different treatments. Data was tested for normal distribution and equal variances by using D'Agostino & Pearson omnibus normality test. Data showing unequal variance was $\log_{10}$ transformed prior to analysis. A Kruskal–Wallis nonparametric test followed with Dunn's post hoc tests was performed for transformed data sets which could not meet the criteria of equal variance. Significant difference or trend was defined by using the criterion $p \leq 0.05$ as significance level.

3. Results and discussion

3.1. Data quality

Limits of detection (LOD) determined by taking the sum of the average and three times the standard deviation of 10 blanks were 0.3 μg/L for Cu and 0.012, 0.2, 0.003 and 0.05 mg/L for Ca, K, Mg and Na, respectively. Instrumental uncertainty was estimated to 0.9–5.3%.

3.2. Field concentrations of Cu

At high road salt concentration (10,000 mg/L) the total amount of Cu leached from the road dust was 28 μg/L. Thus, Cu concentration in the range of 1–30, as applied in the present work, was environmentally relevant.

3.3. Water quality parameters

The temperature during the whole experimental period was 5.7 ± 0.7 based on the logger data. The measured pH values were within 6.7–7.2 in all water qualities (Table 1). During the whole experimental period independent of exposure type, no significant differences in pH ($p = 0.8$) or temperature ($p = 0.9$) were observed between different treatments based on the weekly measurements. In addition the concentration of TOC and major anions (F^-, NO_3^-, SO_4^{2-}) was similar in different treatments regardless of road salt and Cu concentrations. The concentration of TOC and major anions

Table 1
Physical and chemical variables measured in exposure water ($n = 9$).

Nominal road salt (mg/L)	Control	50	100	500	5000	10,000	0	50	100	500	500	500	5000	10,000
Nominal Cu (µg/L)	Control	0	0	0	0	0	10	10	10	10	20	30	10	10
Temperature (°C)	6.6 ± 2.2	6.1 ± 3.0	6.3 ± 2.5	6.4 ± 2.5	6.3 ± 2.7	6.8 ± 2.9	6.3 ± 1.3	6.7 ± 2.8	6.6 ± 2.7	7.0 ± 2.7	6.4 ± 2.8	6.5 ± 2.7	7.0 ± 2.2	7.0 ± 3.0
Conductivity (µS/Cm)	54 ± 9.5	141 ± 11	208 ± 35	878 ± 187	8313 ± 2891	13,258 ± 3843	48 ± 8.5	180 ± 39	219 ± 34	902 ± 128	1865 ± 1934	923 ± 64	6883 ± 2372	15,079 ± 3966
pH	7.0 ± 1.0	7.2 ± 0.4	6.9 ± 0.4	6.9 ± 0.4	6.9 ± 0.2	7.0 ± 0.1	7.0 ± 0.5	7.0 ± 2.3	7.0 ± 0.3	6.9 ± 0.4	6.7 ± 1.6	7.0 ± 0.3	7.0 ± 0.1	7.0 ± 0.2
O_2 (mg/L)	12 ± 1.0	12 ± 1.0	12 ± 0.8	12 ± 0.8	12 ± 1.1	12 ± 1.0	12 ± 0.6	12 ± 0.9	12 ± 1.0	12 ± 0.9	12 ± 0.9	12 ± 0.8	12 ± 1.0	12 ± 1.0
Na (mg/L)	3.2 ± 1.0	23 ± 4.2	37 ± 7.0	209 ± 16	N.A	N.A	4.0 ± 1.0	21 ± 4.7	41 ± 4.1	192 ± 37	392 ± 138	273 ± 87	N.A	N.A
Ca (mg/L)	5.7 ± 0.9	5.7 ± 1.0	5.9 ± 1.0	6.0 ± 0.6	8.7 ± 1.0	10 ± 1.1	5.7 ± 1.0	5.1 ± 2.0	6.0 ± 2.0	5.0 ± 0.0	8.4 ± 3.3	9.1 ± 1.2	8.1 ± 1.0	11 ± 1.0
Mg (mg/L)	1.0 ± 0.0	1.0 ± 0.3	1.0 ± 0.0	1.0 ± 0.1	4.2 ± 6.0	9.0 ± 3.0	1.2 ± 0.0	1.0 ± 0.0	1.2 ± 0.0	1.5 ± 0.0	2.0 ± 0.0	1.0 ± 0.0	5.0 ± 1.1	10 ± 2.1
K (mg/L)	3.2 ± 2.0	4.0 ± 0.2	3.2 ± 1.0	6.0 ± 2.1	13 ± 18	23 ± 11	4.4 ± 1.0	4.4 ± 0.4	4.0 ± 0.1	4.3 ± 1.2	8.1 ± 0.0	6.3 ± 0.0	16 ± 3.9	24 ± 9.0
TOC (mg/L)	4.5 ± 0.5	4.3 ± 0.4	4.5 ± 0.3	4.4 ± 0.2	4.7 ± 0.2	4.9 ± 0.3	4.3 ± 0.2	4.2 ± 0.4	4.3 ± 0.4	N.A	N.A	N.A	N.A	N.A
F^- (mg/L)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	N.A	N.A	N.A	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	N.A	N.A	N.A	N.A	N.A
Cl^- (mg/L)	4.3 ± 0.8	31 ± 2.5	52 ± 7.4	104 ± 19	N.A	N.A	4.6 ± 1.1	30 ± 2.8	52 ± 11	450 ± 54	N.A	460 ± 97	N.A	N.A
NO_3^- (mg/L)	1.4 ± 1.4	0.8 ± 0.3	1.3 ± 0.7	N.A	N.A	N.A	0.9 ± 0.3	1.0 ± 0.6	0.6 ± 0.4	N.A	N.A	N.A	N.A	N.A
SO_4^{2-} (mg/L)	2.7 ± 0.4	2.6 ± 0.1	2.8 ± 0.1	N.A	N.A	N.A	2.7 ± 0.1	3.1 ± 0.7	3.0 ± 0.1	N.A	N.A	N.A	N.A	N.A

N.A: not analyzed.

such as F^-, NO_3^-, SO_4^{2-} remained constant in all water qualities throughout the experimental period, while significant effect ($R^2 = 0.98$, $p < 0.0001$) of road salt was observed on increasing Cl^- concentrations. The mean TOC levels varied from 4.2–4.9 mg/L and were not significantly different ($p = 0.6$) in different treatments. In this study TOC concentration was relatively low indicating high bioavailability of Cu.

The concentration of dissolved O_2 in all water qualities was about 12 mg/L ($p = 0.9$), indicating that the water flow through boxes and circulating tanks had similar normoxic concentration. This indicates similar O_2 consumption rate in eggs of Atlantic salmon in all the treatments regardless salinity. Similar results were found in the different salinity exposures of eggs and alevins of Steelhead trout (*Oncorhynchus mykiss*) and Chinook salmon (*Oncorhynchus tshawytscha*) by Morgan et al. [25]. The conductivity and the concentration of Na^+ and Cl^- was significantly affected ($R^2 = 0.98$, $p = 0.0001$) by increasing road salt concentration. The concentrations of other cations such as Mg, Ca and K increased only at the addition of 5000 and 10,000 mg road salt/L.

The concentration of total Cu in the control water was 1.4 ± 0.4 µg/L and due to addition of Cu the final concentration ranged between 7.5–14 µg/L and 24–28 µg Cu/L (Table 3). At 10,000 mg road salt/L mixed with 10 µg Cu/L, the Cu was predominantly present as LMM (47%) and particulate (35%) species (Table 2).

Results indicated that the concentration of colloidal Cu was reduced significantly from 41% to 6% ($R^2 = 0.30$, $p = 0.02$) in the treatments with road salt and Cu, while the concentration of particulate Cu was significantly increased from 1% to 35% ($R^2 = 0.80$, $p = 0.02$) probably due to aggregation. In addition, the concentration of positively charged Cu species increased with increasing road salt concentration ($R^2 = 0.80$, $p = 0.03$), being higher at 10,000 mg road salt/L than at lower salt concentration. This showed that increasing road salt concentrations changed the Cu speciation towards a more mobile and presumed bioavailable form which confirms the results of Backstrom et al. [7] showing that road salt mobilizes reactive forms of Cu.

3.4. Biological end points

3.4.1. Fertilization

Fertilization success was found to be approximately 99–100% in all exposures and was not affected by any of the exposure treatments during the swelling process.

3.4.2. Increase in size after swelling

Swelling of Atlantic salmon eggs was significantly affected by the road salt addition ($R^2 = 0.97$, $p = 0.001$). The results showed that high road salt concentrations (≥5000 mg/L), regardless the presence of Cu, reduced the swelling (Fig. 1). In mixtures with elevated Cu concentrations (>20 µg/L), inhibition of swelling was also observed even at lower salt concentration (500 mg road salt/L). Exposure of road salt and Cu thus inhibited swelling, when given individually and also as mixtures, especially at higher Cu concentrations (>10 µg/L).

Swelling of fish eggs occurs upon spawning and fertilization and finally they increase in weight and volume. During the inflow of water a perivitelline space (PVS) is formed between chorion and zona pellucida of the egg. Embryonic volume increases during the development and space for this volume increase is provided by PVS [26]. Results indicated that high salinity and Cu inhibit the formation of PVS in exposed Atlantic salmon eggs. Small PVS did not provide sufficient space for the expansion of the embryo, which resulted in restriction of embryonic development [26] which can lead to increased number of deformities [27]. Since the Atlantic salmon eggs have a similar osmotic concentration with respect to

Table 2
Concentrations of different Cu-fractions measured in exposure water ($n \geq 3$).

Nominal road salt (mg/L) Nominal Cu (μg/L)	Control Control	50 0	100 0	500 0	5000 0	10,000 0	0 10	50 10	100 10	500 10	500 20	500 30	5000 10	10,000 10
Cu total	1.4 ± 0.4	2.8 ± 1.8	1.7 ± 0.8	1.1 ± 0.4	2.0 ± 0.6	3.2 ± 1.0	11 ± 0.7	7.5 ± 3.4	11 ± 1.01	8.6 ± 1.4	24 ± 6.4	28 ± 12	11 ± 13	14 ± 7.9
Cu particulate	<LD[a]	<LD	<LD	<LD	<LD	0.4 ± 0.8	<0.3	0.2 ± 3.9	1.4 ± 1.3	0.3 ± 1.4	2.9 ± 3.7	0.5 ± 4.5	3.4 ± 2.3	4.9 ± 1.4
Cu Colloidal	0.6 ± 0.2	0.1 ± 0.1	0.9 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.9 ± 0.0	5.8 ± 0.0	3.7 ± 0.1	1.2 ± 0.7	2.2 ± 0.1	8.0 ± 0.7	<0.3	<0.3	0.8 ± 0.0
Cu LMM	1.0 ± 0.1	1.0 ± 0.3	0.9	0.9 ± 1.4	1.1 ± 0.4	1.4 ± 0.0	3.9 ± 3.1	3.8 ± 4.0	3.7 ± 3.5	3.6 ± 1.0	5.8 ± 0.8	11 ± 6.0	6.9 ± 0.8	6.6 ± 1.7
Cu cationic	1.3 ± 0.2	2.2 ± 1.4	1.1 ± 0.5	1.3 ± 0.6	1.4 ± 0.7	2.1 ± 0.4	4.1 ± 0.2	4.4 ± 1.1	5.9 ± 1.1	6.6 ± 2.6	14 ± 3.5	16 ± 7.1	7.1 ± 1.3	8.9 ± 1.8
LMM Cu cationic	0.9 ± 0.0	0.7 ± 0.2	0.7 ± 1.2	1.3 ± 0.0	1.1 ± 0.0	1.3 ± 0.0	1.2 ± 0.6	1.9 ± 1.4	2.0 ± 1.4	2.9 ± 0.8	6.6 ± 1.0	8.4 ± 0.2	3.3 ± 0.0	3.7 ± 0.0

[a] Limit of detection 0.3.

their surrounding natural water, reduced swelling will be observed when placed in high salinity medium, as found in the experiment.

Jezierska et al. [19] reported that Cu can easily be taken up and hence accumulate inside the egg during swelling phase. Their data on *Cyprinus carpio* eggs showed reduced swelling when exposed to 50, 100, 200 and 500 μg Cu/L in a concentration-dependent way. Our data indicated reduced swelling in Atlantic salmon eggs at Cu concentrations of 20 and 30 μg/L. The level of swelling plays an important role in the whole embryonic development. The developing embryo occasionally changes position in appropriately swollen eggs. Due to improper egg swelling, the developing embryos will have a very little space to move, which may result in hatching of abnormal larva [27].

3.4.3. *Egg survival*

Exposure during fertilization showed relatively low survival especially at the highest road salt concentrations i.e. 5000 and 10,000 mg road salt/L (Fig. 2A and B), while relative high survival was observed in the episodic post fertilization exposure treatments (Fig. 2C and D) compared to control. The survival of fertilized eggs exposed during fertilization to different road salt concentrations mixed with Cu ($p < 0.01$) was significantly different from those exposed by road salt only ($p < 0.01$). No significant effect on the survival of eggs was observed in episodic post fertilization exposure treatments without ($p = 0.05$) and with Cu ($p = 0.6$).

Significant dose response effect ($p < 0.01$) on survival of fertilized eggs was found in the groups exposed to high road salt concentrations during fertilization regardless of the Cu concentration, while no such trend was found in episodic post fertilization exposure treatments ($p = 0.6$). At high road salt concentration such as 5000 and 10,000 mg road salt/L, most of the egg mortality occurred at blastula stage, while there was comparatively less at organogenesis and hatching.

Compared to the control group, no significant difference in the egg survival was observed at 500 mg road salt/L or at lower concentrations ($p = 0.07$). Yang and Chen [28] observed lower egg survival in obscure puffer (*Takifugu obscurus*), a euryhaline species, after exposure to ≥12,000 mg synthetic seasalt/L compared to the control.

The 24 h weekly episodic exposure of eggs to 10,000 mg road salt/L without and with Cu, did not affect egg survival (98%) compared to exposure during fertilization (2%). Regarding the egg survival, it seems that high road salt concentrations at the time of fertilization are toxic towards Atlantic salmon eggs, while not lethal as episodic exposures (24 h/week) post fertilization. This reflects that episodic exposure to a developing embryo 24 h post fertilization is probably less hazardous than exposure during fertilization (first 24 h).

It is well established that salinity exerts a strong influence on development and growth in early life stages of freshwater fish [29]. Exposure of eggs to increased salinities (15, 20 and 25 ppt) might have a substantial effect on embryo viability as suggested by Fridman et al. [29]. In our experiment high road salt concentrations resulted in inhibition of PVS formation and reduced swelling, and subsequently reduced egg survival (Fig. 1). In the present work exposure with road salt (500 mg/L) mixed with Cu (10–30 μg Cu/L) resulted in minor reduction of the egg survival (98%). However, it cannot be excluded that Cu may reduce the egg survival at concentrations higher than 30 μg Cu/L applied in the present experiment.

3.4.4. *Eyed embryos and time of hatching*

All eggs independent of the exposure treatment reached the eyed embryos stage soon after 255 degree days. Hence, none of the treatment had delaying effect on the development of eyed embryos.

Table 3
Deformities (%) of Atlantic salmon alevins after exposure to different levels of road salt with and without Cu during and post fertilization.

	Exposure during fertilization						Episodic exposure post fertilization					
Nominal road salt concentration (mg/L)	Control	50	100	50	100	5000	100	500	5000	100	500	5000
Nominal Cu concentration (μg/L)	Control	0	0	10	10	10	0	0	0	10	10	10
Deformity types												
Axial spine curvature	0	0.4	0.4	0.4	0.4	3	0.4	0	0.4	0	0.4	0.4
Coiled tail	0	0.4	0.4	0.4	0.4	4	0.4	0.4	0.4	0.4	0	0
Two headed embryo	0	0	0	0	0	1	0	0	0	0.4	0	0
Two bodied embryo	0	0	0	0	0	1	0	0	0	0	0	0
Total	0	0.8	0.8	0.8	0.8	7	0.8	0.8	0.8	0.8	0.4	0.4

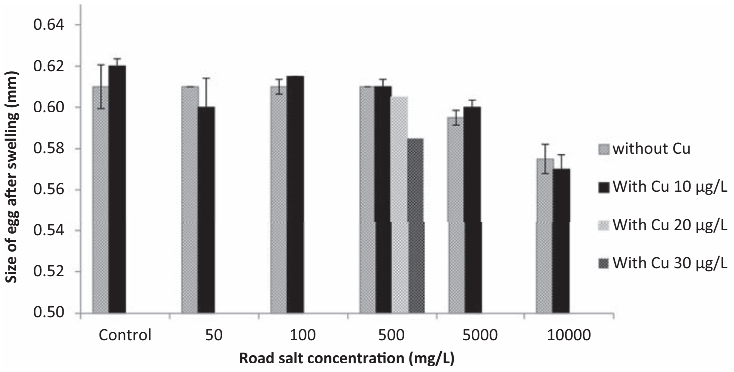

Fig. 1. Size of individual egg of Atlantic salmon after swelling in different road salt concentrations (mg/L) added without and with Cu (μg/L)

All eggs in the control group hatched between 498 and 542 degree days. For the remaining exposed eggs, hatching started from approximately 400 degree days and was completed at 621 degree days. Regardless of the treatment, hatching started and finished almost at the same time in all groups. However, within the “window of hatching”, differences could be seen between different exposure treatments (Fig. 3). Delay within the window of hatching time was, however, observed for eggs exposed during fertilization (Fig. 3A and B) and also exposed episodically post fertilization to different road salt concentrations without and with Cu (Fig. 3C and D).

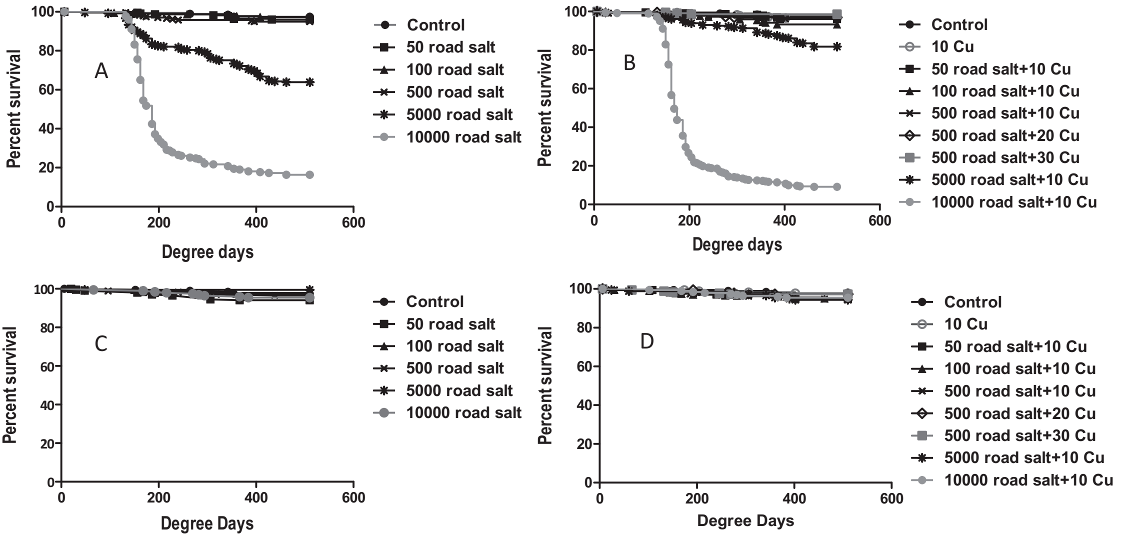

Fig. 2. Relative (%) survival of fertilized eggs exposed to different road salt and Cu concentrations during fertilization and in episodic post fertilization treatments (A) Exposures during fertilization without Cu (B) Exposures during fertilization with Cu (C) Episodic post fertilization exposures without Cu (D) Episodic post fertilization exposures with Cu. Concentration of road salt is given in mg/L and the concentration of Cu is given in μg/L.

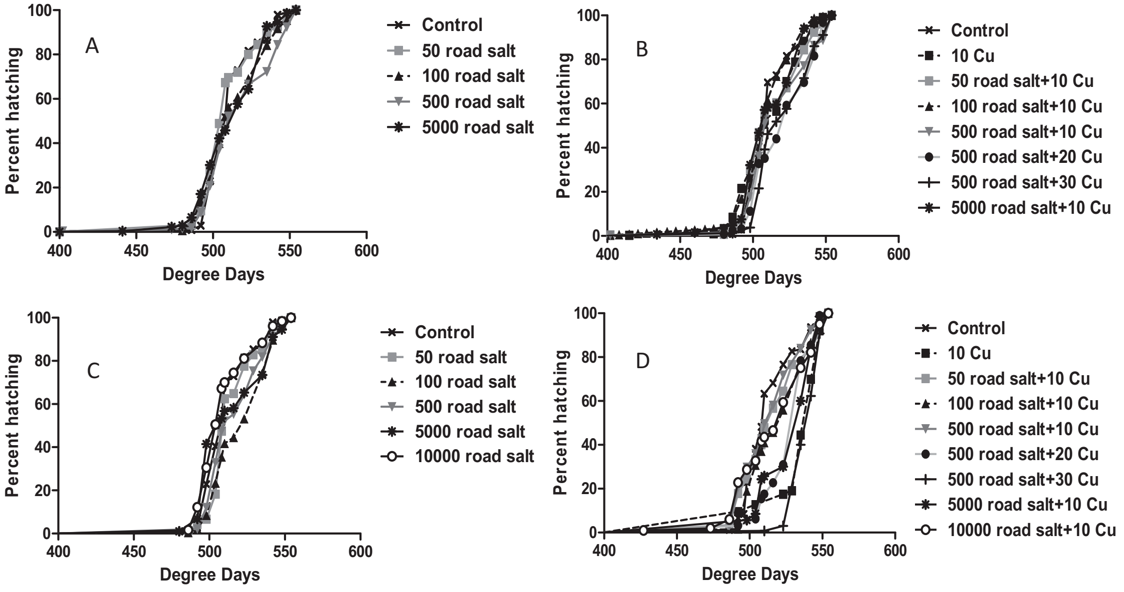

Fig. 3. Relative (%) hatching of fertilized eggs exposed to different road salt and Cu concentrations during fertilization and in episodic post fertilization treatments. (A) Exposures during fertilization without Cu. (B) Exposures during fertilization with Cu. (C) Episodic post fertilization exposures without Cu. (D) Episodic post fertilization exposures with Cu. Concentration of road salt is given in mg/L and the concentration of Cu is given in µg/L.

In exposures during fertilization the hatching of the eggs exposed to road salt only and of eggs exposed to road salt mixed with Cu were significantly different ($p < 0.01$). In addition significant dose response relationship was found between road salt exposure and delayed hatching ($p < 0.01$). In episodic post fertilization exposure treatments with road salt only, the hatching curves were significant different from each other ($p < 0.01$), while no significant dose response trend was observed ($p = 0.07$). In addition, a significant difference in hatching curves ($p < 0.01$) and also a significant dose response trend ($p < 0.01$) was obtained for eggs episodically exposed to road salt mixed with Cu.

The salinity can also influence hatching by affecting the buoyancy of fertilized eggs rather than affecting the metabolic processes as reported by Murashige et al. [30]. It is therefore assumed that high road salt concentrations (≥500 mg/L) in episodic exposure given post fertilization might be responsible for the observed delayed hatching of Atlantic salmon eggs. The total hatch rate of obscure puffer (*T. obscurus*) was affected by salinity (≥12 ppt) as reported by Yang and Chen [28].

Cu exposure at the egg stage may have significant effects on fish embryos, including delay in time to hatching [19]. Khangarot and Daas [31] found delay in hatching of freshwater pulmonate snail *Lymnaea luteola* L. at Cu concentration of 3.2, 5.6 and 10 µg Cu/L. Delayed hatching seems to be one of the sensitive endpoints reflecting Cu toxicity towards fish. Episodic post fertilization exposure with 10 µg Cu/L also resulted in delayed hatching. Hence, the effects of delayed hatching at higher Cu concentrations can also be expected.

Typical dose response trends in episodic post fertilization exposure groups of 500 mg road salt/L only and in combination with Cu were seen (Fig. 3D). This is in agreement with the work by Johnson et al. [18], who reported that exposure with 19 and 50 µg Cu/L at pH 6.3 resulted in considerable decline in hatching success after 75 h of the exposure, compared to controls. Our results indicated that road salt mixed with Cu had a negative effect on hatching.

Hatching is not a one-step phenomenon, but a combination of different biochemical and behavioral processes involving hatching glands and enzymes [32]. Waterborne pollutants such as road salt and Cu may therefore affect the development and functioning of hatching glands which produce the protein chorionase responsible for egg shell disintegration during hatching [19].

3.5. Deformities

The most frequently larval deformation observed included (a) coiled tail (b) two headed embryo (c) two bodied embryo and

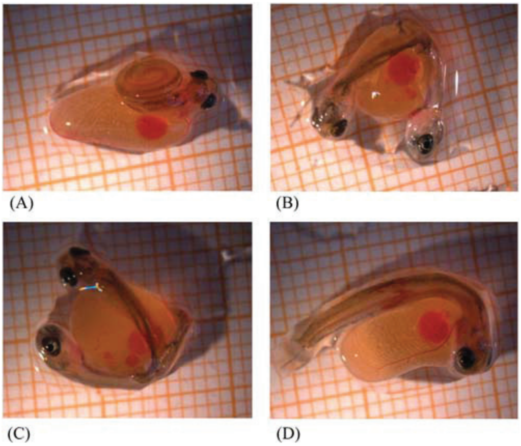

Fig. 4. Different types of deformities observed in Atlantic salmon alevins due to exposure to road salt and Cu. Photos: Merethe Kleiven (1 square box = 1 mm).

(d) axil spinal curvature-lordosis (Table 3). Photographic images (Fig. 4) along with the details of different deformity types show the effects of various environmental stressors, including road salt and Cu, on body deformations of newly hatched Atlantic salmon larvae. Due to severe body deformation, alevins were not able to swim properly and their survival rates are probably quite low.

A high frequency of deformed larvae resulted when road salt was mixed with Cu. No deformity type was observed in control group. Deformities such as curvature of the spine and tail flexure were observed due to road salt exposure. However, no deformity was found at high road salt concentrations (5000 mg road salt/L) in the eggs exposed during fertilization. Yang and Chen [28] found larval deformities in obscure puffer (*T. obscurus*) after exposure to salinity higher than 12 ppt.

Highest relative deformities (7%) were found in 5000 mg road salt/L mixed with 10 μg Cu/L. Inhibition of embryonic development and growth can take place at many different developmental stages [19]. Since road salt resulted in reduced swelling (Fig. 1), a reduced PVS will not provide proper space for embryonic development and movement which may cause larval anomalies [26,27]. Road salt and Cu may also cause defects in myotomes of the somites which results in alteration of axial curvature as observed by Fraysse et al. [32].

The results indicated multiple stressor effects of road salt exposure in combination with other stressors such as Cu in runoff from roads. As found in our study, road salt mobilizes LMM Cu from road dust and thereby may increase bioavailability, uptake and effects. A variety of studies have shown that early developmental stages of fish are mainly sensitive to waterborne Cu [33].

Increased numbers of larval abnormalities characterized by swollen and opaque yolk sacs, opaque white head region, spinal cord deformations and deformed heads were also demonstrated by Johnson et al. [18]. Heavy metals are assumed to adversely disrupt several metabolic processes in developing embryos (influencing degree days to hatch), which can result in developmental retardation, morphological and functional abnormalities and finally death of the sensitive individuals [19].

4. Conclusion

The experiment was designed to simulate road run off exposure during and post fertilization episodic exposures to Atlantic salmon eggs at environmentally relevant concentrations. The effects of road salt and Cu on Atlantic salmon were studied from fertilization till hatching. Exposure during fertilization resulted in reduced swelling, less egg survival, delayed hatching and high percentage of deformities, while the episodic post fertilization exposures resulted only in delayed hatching. These findings suggest that the spawning and fertilization period seems to be the most sensitive period for Atlantic salmon at the earliest life history stage when exposed to multiple stressors i.e., road salt mixed with Cu found in the road runoff. In addition our study documented road salt concentrations exceeding threshold levels for the protection of Atlantic salmon. In an environmental context, the spawning period seems to be the most sensitive period for Atlantic salmon at the earliest life history stage. Fish inhabiting waters near roads may be particularly susceptible to road related multiple stressors (salt and Cu).

Acknowledgement

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Paper III

Transcriptional changes in Atlantic salmon (*Salmo salar*) after embryonic exposure to road salt (NaCl)

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Highlights

Exposure to 5000 mg/L road salt (NaCl) during the first 24h after fertilization caused global transcriptional changes detected at the eyed egg stage in Atlantic salmon.

- Main transcriptional changes indicate interference with osmoregulation, ion regulation, oxidative stress, metabolism, renal function and development in the embryos.
- Effects in selected biomarker for exposure and effect occurred as low as 100 mg/L road salt (NOTEL 50 mg/L)

Key words

Road salt, Atlantic salmon, transcriptional effects, osmoregulation, ion regulation, oxidative stress, metabolism, renal function, development.

Abstract

Road salt (mainly NaCl) is extensively used as a deicing chemical for road maintenance during winter and has in certain areas of the world led to density stratifications in lakes and ponds, and adversely impacted aquatic organisms in the recipients of the road run-off. Aquatic vertebrates such as fish have been particularly sensitive during fertilization, as the fertilization of eggs involves rapid uptake of the surrounding water, reduction in egg swelling and in ovo exposure to high road salt concentrations. The present study aimed to identify the persistent molecular changes occurring in Atlantic salmon (*Salmo salar*) eggs after 24h exposure to high concentrations (5000 mg/L) of road salt at fertilization. The global transcriptional changes were monitored by a 60k salmonid microarray at the eyed egg stage (Clarvay stage, 255 day degrees after fertilization) and identified a high number of transcripts being differentially regulated. Functional enrichment, pathway and gene-gene interaction analysis identified that the differentially expressed genes (DEGs) were mainly associated with toxiciologically relevant processes involved in osmoregulation, ion regulation, oxidative stress, metabolism (energy turnover), renal function and developmental in the embryos. Quantitative rtPCR analysis of biomarkers for exposure and effects in the eye stage embryos exposed to an extended range of road salt concentrations (0, 50, 100, 500 and 5000 mg/L) revealed a positive concentration-dependent increase in Cyp4A14, a gene involved in lipid turnover and renal function, and Nav1, a gene involved in neuraxonal development. Biomarkers for osmoregulatory effects (ATP1A2), the main sodium/potassium ATP-fueled transporter for chloride ions and oxidative stress (Txndc9), a gene involved in regulation of cell redox homeostasis, displayed apparent concentration-dependency with exposure, although large variance in the control group precluded robust statistical discrimination between the groups. A No Transcriptional Effect Level (NOTEL) of 50 mg/L road salt was found to be several orders of magnitude lower than the adverse effects documented in developing fish embryos elsewhere, albeit at concentrations realistic in lotic systems receiving run-off from road salt.

1 Introduction

In northern North America and Europe road salt (mainly NaCl) is extensively used as a deicing chemical for road maintenance during winter (Kelly *et al.*, 2008). In Norway there has been a significant increase in the amount of annually applied road salt, and since year 2000 this amount has more than tripled (Kronvall, 2013). This is of concern as prolonged application of road salt may cause negative effects on the aquatic environment (Sanzo and Hecnar, 2006). For example, increase in road salt due to deicing operations has led to density stratification in lakes and ponds, reduced water circulation and subsequently oxygen depletion in the hypolimnion (Kjensmo, 1997; Novotny *et al.*, 2008). Although road salt concentrations are quickly diluted in running waters, it is now evident that also streams may be severely affected (Corsi *et al.*, 2015; Ramakrishna and Viraraghavan, 2005). Road salt concentrations can vary considerably in different waterbodies, and concentrations in the range of 150-5000 mg/L NaCl have been reported in rural lakes and urban impoundment lakes (Environment Canada, 2001). Such elevated concentrations of NaCl have been reported to cause negative impact on survival of rainbow trout at concentrations ranging from 2000-4560 mg/L (Vosylienė *et al.*, 2006), reduction in biodiversity in water bodies and wetland areas by affecting primary producers and invertebrate communities at concentrations above 220 mg Cl^-/L (Environment Canada, 2001), and enhanced the toxicity of other stressors such as Cu, Zn, Ni, Pb, Cr, Cd, and Hg (Anderson *et al.*, 1995; Ramakrishna and Viraraghavan, 2005). Early life stages of different aquatic organisms such as Atlantic salmon are particularly susceptible to road salt (Mahrosh *et al.*, 2014) and are at risk during periods of the year when emissions are large. Increased mortality, reduced weight, reduced activity, and increased physical abnormalities are some of the effects observed by high road salt concentrations (2636-5109 mg/L) in other aquatic vertebrates such as wood frog (*Rana sylvatica*) larvae (Sanzo and Hecnar, 2006).

In Norway, the initial need for deicing of roads often co-occurs with sensitive periods such as spawning of salmonids. Atlantic salmon (*Salmo salar* L.) is highly sensitive to road salt, especially during the early developmental stages, from fertilization until hatching (Mahrosh *et al.*, 2014). Episodic salting activities and run-offs to nearby rivers and streams are thus expected to affect the water quality at critical windows of salmon development. Road salt have been reported to reduce egg swelling and hence inhibit the formation of the perivitelline space (Li *et al.*, 1989) that ultimately may lead to egg mortality (Mahrosh *et al.*, 2014; Yang and

Chen, 2006). Reduction in embryo viability due to osmoregulatory stress has been demonstrated in Nile tilapia (*Oreochromis niloticus*) at salinities ranging from 15,000-32,000 mg/L (Fridman *et al.*, 2012), and thus shows that road salt may have many toxic mode of action (MOA) in fish. Road salts are believed to also influence hatching of fish eggs by affecting the buoyancy in water (Murashige *et al.*, 1991), and cause deformities when exposed during fertilization and swelling (Mahrosh *et al.*, 2014). Different abnormalities such as spinal curvature and tail flexure have also been reported after salt exposure (Haddy and Pankhurst, 2000; Mahrosh *et al.*, 2014). As developing fish embryo undergoes a number of different processes involved in organ development and differentiation, these organisms may be susceptible to external stressors during critical windows of development. Perturbations of any of these processes beyond the boundaries of homeostasis may potentially lead to adverse effects such as loss of viability, delayed maturation, malformations and non-functional organs (Dave and Xiu, 1991; Johnson *et al.*, 2007).

Although adverse effects are often directly associated with regulatory-relevant endpoints such as survival, growth, development and reproduction, initial interactions between the stressors and their biological targets normally occur at the molecular level (Miracle and Ankley, 2005). The current study aimed to characterize the transcriptional changes occurring in Atlantic salmon eggs at the eyed egg stage (Clarvay stage, typically at 255 day degrees after fertilization) after a 24h exposure to environmentally relevant road salt concentrations during fertilization. Global transcriptomics analysis by a 60k oligonucleotide salmonid microarray was used to characterize the MoA and assemble information about toxicity pathways affected by the exposure. Concentration-response curves for a selection of biomarker representing exposure and effect responses to road salt-induced stress were monitored by quantitative rtPCR to derive the No Observed Transcriptional Effect Level (NOTEL) as an indicator of the no effect concentration (NOEC) in the embryos.

2 Materials and methods

2.1 Experimental setup

Dry stripped Atlantic salmon eggs and sperms were obtained from the Aquagen hatchery (Sunndalsøra, Norway) and subjected to *in vitro* dry fertilization according to method described in Mahrosh *et al.,* (2014). Eggs were exposed from fertilization to 24h post fertilization to Lake Maridalsvannet water (Oslo Norway) as control, and 50, 100, 500, and 5000 mg/L road salt (Isbryter'n rock salt, kindly donated from GC Rieber Salt AS, www.gcrieber-salt.no). The road salt (containing 98.5 % NaCl, 0.30 % Ca and Mg, 0.70 % SO_4, 0.3 % H_2O and 70-100 ppm anticaking agent E 535) was used in the studies as considered to be more ecologically relevant than pure NaCl. Temperature-controlled flow-through exposures (40-60 mL/min) with control and road salt-spiked water were conducted in duplicate with specially designed exposure boxes (155×106×45 mm, 739 cm^3) containing 200 eggs in each. Following 24h exposure during the fertilization and swelling stage (Gorodilov, 1996), all groups were receiving control water under the same experimental conditions as described above until the end of the experimental period. The temperature and water qualities were monitored throughout the study. After 255 degree days, when the eggs reached the eyed embryo stage, eggs were sampled, snap-frozen in liquid nitrogen and stored at -80 ºC for subsequent RNA extraction and gene expression analysis.

2.2 RNA isolation

Six Atlantic salmon eggs with an average individual wet weight of 0.146±0.020g were used for gene expression analysis (n=6). Total RNA was extracted using Direct-zol™ RNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA) according the manufacturer's instructions. Briefly, the whole egg was lysed with TRIzol® reagent (Sigma-Aldrich, St. Louis, MO, 100 mg tissue/1 mL TRIzol) and homogenized (3 x 10 sec at 6000 rpm) using ZR BashingBead™ (Zymo) in a Precellys orbital shaker bead mill (Bertin, Montigny-le-Bretonneux, France). The homogenate was centrifuged (12,000 g, 1 min) and 500 µL supernatant was carefully transferred to a 1.5 mL RNase-free centrifuge tube. The remaining homogenate was kept in a -80 ℃ ultrafreezer for further analysis. One volume of ethanol (95%-100%) was added directly to the supernatant, vortexed well, and transferred into a Zymo-Spin™ IIC Column in a collection tube. The column was centrifuged (12,000 g, 1 min) and transferred into a new collection tube. To remove genomic DNA, the column was first washed once with 400 µL RNA wash buffer (12000 g, 1 min), and added with a mixture of 3 µL RNase-Free DNase I (1 U/µL), 3 µl 10X reaction buffer and 24 µL RNA wash buffer, incubated at 37℃ for 20 min and centrifuged (12,000 g for 30 s). The DNase treated RNA was then washed once with 400 µL RNA Prep Buffer (12,000 g, 1 min), once with 800 µL RNA wash buffer

(12000 g, 30 s) and a final wash with 400 µL RNA wash buffer (12,000 g, 2 min). Nuclease-free water (12 µL) was added to the column and centrifuged (10,000 g, 30 s) to elute RNA into a DNase/RNase-Free tube. The RNA yield and purity were quantified by photometric analyses (260/280 and 230/260 ratios> 1.8, yield >50 ng/µL) using Nanodrop® spectrophotometer (ND-1000, Nanodrop Technologies, Wilminton, Delaware, USA) and RNA integrity determined by Bioanalyzer gelelectrophoresis with RNA 6000 Nano chips (Agilent technologies, Santa Clara, California, USA). All samples with exception of the individuals exposed to 50 mg/L road salt (RIN =6.1, passed the quality criteria set (RIN>7.5, 260/280 ratio >1.8). The purified RNA samples were stored in -80℃ freezer for further analysis.

2.3 Microarray analysis

A 60k high density custom salmonid oligonucleotide microarray was manufactured by Agilent Technologies (Santa Clara, CA, USA). The microarray probes were designed based on Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) consensus sequences (contigs) and single ESTs from the cGRASP project (http://web.uvic.ca/grasp) and NCBI Unigenes (http://www.ncbi.nlm.nih.gov/ unigene) for the two species (*S. salar*: build 31 and *O. mykiss*: build 27) as described in Song *et al*. (2014). The array design can be accessed at Gene Expression Omnibus (GEO, accession number: GPL18864).

The global gene expression was determined in the control and the highest exposure group (5000 mg road salt/L) by a standard one-color protocol (Agilent Technologies, v6.5, Santa Clara, CA, USA) using commercially available kit and kit components from Agilent Technologies, as described previously in Song *et al.* (2014). After hybridization, the slides were washed and scanned using an Agilent Technologies C scanner (scan region 61x21.6 mm, resolution 3 µm, Tiff image 20 bit) and raw expression data was extracted using Agilent Feature Extraction (v10.6) software.

2.4 Quantitative rtPCR

Quantitative rtPCR were performed using RNA from the control and all road salt exposed eggs (50, 100, 500 and 5000 mg/L road salt), essentially as described by Song *et al.* (2014). In brief, the complementary DNA (cDNA) was reverse transcribed from 2 µg total RNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions. A total reaction volume of 10 µL containing 10 ng cDNA template, Quanta PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, Maryland, USA) and 300 nanomoles forward/reverse primer was used for quantitative rtPCR using a CFX384™ detection system (Bio-Rad, Hercules, CA, USA). The *Salmo salar* primer sequences (Table 1) were designed using Primer 3 (v0.4.0, http://frodo.wi.mit.edu/primer3), based on the cGRASP contigs, Genbank ESTs or Unigene sequences. The starting quantity of cDNA template and the amplification efficiency were determined based on a standard curve made from 0.6, 3, 15 and 75 ng input cDNA. The expression of target gene was normalized against total RNA and fold change was then calculated by comparing the normalized treatment gene expression to the control samples.

2.5 Statistics and Bioinformatics

Scanned images were analyzed with Agilent Feature Extraction, Ver 7.3 (Agilent Technologies). Resulting raw data were normalized (25 Quantile, median to baseline of all samples), features filtered on expression (20-100 %), outlier (non-uniform and saturated features) flagged and significantly regulated genes across treatments identified by a moderated T-test (Storey with Curve Fitting FDR multiple testing correction, $p \leq 0.1$, two-fold cut-off) by GeneSpring version 12.5 (Agilent Technologies). Significantly regulated genes were clustered (Squared Euclidean, Ward's linkage rule) by treatment and gene regulation, and subjected to functional gene enrichment analysis. Protein-protein networks, canonical pathways and relationships to well-characterized toxicological processes were identified by Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/products/ipa) using ortholog mapping to *Danio rerio*, *Homo sapiens*, *Mus musculens* and *Rattus norwegicus* as proxies for *S. salar*. Orthologs were identified by a reciprocal two-pass slightly modified blast (BLAST+ binaries instead of BLASTALL) to the RefSeq database (http://www.ncbi.nlm.nih.gov/refseq) using an Inparanoid algorithm (http://inparanoid.sbc.su.se/cgi-bin/index.cgi) according to specification provided by the developer (Ostlund *et al.*, 2010).

Statistical analysis of the quantitative rtPCR data were performed using JMP Pro, Ver. 10.0.0 (SAS Institute Inc, Cary, NC, USA). A one-way ANOVA test was conducted followed by a Tukey-Kramer post-hoc test to identify significantly regulated single gene responses ($p \leq 0.05$). The assumptions of normally distributed data with equal variance were met in all data with the exception of one gene (Cyp4A14) which were log transformed using Johnsons transformation available in Minitab16 (Minitab Inc., State College, PA, USA). Regression analysis (GraphPad prism 5.0, Graphpad Software, Inc., San Diego, CA, USA) was applied to study the significant effect of road salt in combination with water quality parameters such as pH, conductivity and TOC. Significant effect was defined by using the criterion $p \leq 0.05$ as level of significance.

3 Results

3.1 Water chemistry

The water quality was different between groups during the experimental 24h exposure period (Table 2). Thereafter, all groups were placed in the control lakewater until the end of the experimental period. During the experimental 24h exposure period a significant increase in conductivity ($R^2=0.98$, P=0.0001) was observed by road salt addition, mainly due to increased concentration of Na and Cl. Post exposure and swelling, all eggs received the same low ionic water (48±6 µS/cm) with pH 7.2±0.2 The pH was in range 6.9 –7.5 and dissolved oxygen was 12.6±0.6 mg/L, and unaffected by road salt addition. During the experimental period the temperature was in average 5.4±0.6 ^{o}C, similar for all groups and eyed egg stage was reached after 255 degree days not significantly different between the exposure units.

3.2 Global gene expression analysis

The microarray analysis showed that 1002 of the total 60k features on the array were significantly regulated (721 up-regulated and 281 down-regulated) after exposure to 5000 mg/L road salt (Fig. 1), whereof the majority of the features were identified to have high-quality BLAST hits and about 50 % were successfully identified as orthologs to zebrafish, humans, rats or mice. Supplementary Table S1 displays the differentially expressed genes in detail.

Gene ontology (GO) functional enrichment analysis identified a number of biological processes and molecular functions being particularly enriched among the selection of differentially expressed genes (DEGs). A total of 16 GOs were identified as significant when considering both up- and down-regulated genes (results not shown), whereas differentiation between direction of regulation led to unique enrichment of GOs associated with up-regulated transcripts (Table 3). Of the GOs identified, a majority of the GOs were associated with general development processes and development of the sensory system in particular. A few genes associated with the response to superoxide (PRDX2, APOA4, UCP3), were identified being relevant when considering only the up-regulated transcripts.

Mapping to mammalian orthologs and analysis of enrichment in well-annotated (curated) toxicological pathways revealed that the exposure to high road salt concentrations caused differential regulation of genes associated with a number of biological processes (Table 4, supplementary Table S2). As seen for the GO enrichment analysis, enrichment analysis on toxicological pathways were exclusively related to up-regulated transcripts. Genes associated with renal function/cell death and acute renal failure, mitochondrial dysfunction (depolarization of mitochondria and mitochondrial membrane), oxidative stress and nuclear receptor signaling (LXR/RXR and TR/RXR activation) were clearly up-regulated by the episodic exposure to 5000 mg/L road salt.

A number of canonical pathways were identified as being enriched among the differentially expressed transcripts (Table 5, supplementary Table S3). Of the 17 pathways affected, nuclear receptor signaling (LXR/RXR activation, TR/RXR activation, and MIF-mediated glucocorticoid regulation), cellular immune response (clathrin-mediated endocytosis signaling, agranylocyte adhension and diapedesis, MIF-mediated glucocorticoid regulation and granzyme B signaling), neurosignaling (regulation of actin-based motility by Rho, neurotransmitter and other nervous system signaling), cellular growth, proliferation and development (epithelial adherens junction signaling, ILK and Notch signaling), human disease-specific pathways (Parkinson's and artherosclerosis signaling), intercellular and second messenger signaling (calcium signaling), apoptosis (April-mediated signaling) and cancer (Notch signaling) were affected.

Protein-protein (gene-gene) interaction network analysis revealed a number of biological functions being affected by treatment to road salt (Supplementary Table S4). A selection of toxicological relevant gene-interactions and corresponding DEGs are shown in Fig. 2, including gene-networks related to energy production and lipid metabolism, organ and embryonic development, cell signaling and interaction, and nervous system development and function.

3.3 Quantitative rtPCR verification

Quantitative rtPCR (qrtPCR) analysis of gene expression was performed to establish a concentration-response relationship and determine the NOTEL for a selection of differentially expressed genes that were central in the toxicological pathways and gene-interaction networks. Significant differences between the exposed groups and the control were detected in two out of the four genes, Cyp4A14 and Nav1 (Fig. 3). In both cases, the groups exposed to 100 and 500 mg road salt/L displayed higher expression than the control group and an apparent concentration-dependent relationship was observed. An apparent concentration-response was observed also for ATPase1A2 and Txndc9, albeit the high variance in the control group precluded any statistical differences to be detected. The qrtPCR analysis showed that the overall NOTEL of the selected DEGs, based on the induction of Cyp4A14 and Nav1, were 50 mg/L road salt.

4 Discussion

The use of road salt has great societal benefits allowing deicing of roads, reducing car accidents, human damage and casualties due to traffic accidents (Bjørnskau, 2011). Although having an advantageous role in this respect, use of high amounts of road salt has led to extensive run-off of chlorides to nearby land and surface waters. Chloride concentrations in urban impoundment lakes and snow clearings from streets can reach as high as 5000 mg/L, whereas Cl-concentrations in ponds, stream and rivers may range up to 4300 mg/L (Environment Canada, 2001). In addition, chloride concentrations up to 18,000 mg/L have been reported in various runoff waters due to increased use of road salt (Sanzo and Hecnar, 2006). These large increases in aquatic salt concentrations, mainly due to the increase in NaCl but also minor contributions from Ca^{2+}, Mg^{2+}, SO_4^{2+} and anticaking agents (<2 % w.w), is potential stressors to aquatic organisms living in the receiving fresh waters. Observations of high mortality, reduced weight and activity, and increased physiological abnormalities in larval wood frogs exposed to road deicing salt at concentrations ranging from 2636 to 5109 mg/L (Sanzo and Hecnar, 2006) suggest that high concentrations of road salt may be hazardous to aquatic organisms. Reduced salmon egg swelling during fertilization and reduction in egg survival confirm that exposure to high concentrations (5000 mg/L) of road salt also affect fish (Mahrosh *et al.*, 2014). Increase in salmon larval deformities after exposure to high road concentrations suggest that exposure during critical windows of salmon embryo development may also give rise to adverse effects later in life (Li *et al.*, 1989; Mahrosh *et al.*, 2014). Although not measured directly, results may indicate that some of these effects may be related to higher uptake of Na and Cl in the egg fluid during swelling (Mahrosh *et al.*, unpublished). But reduced swelling also causes a reduction in the perivitelline space (PVS), the space between the zona pellucida and the cell membrane of the fertilized ovum (Li *et al.*, 1989). Addition of saltwater with salt concentrations around 1000 mg/L during swelling have been shown to reduce the PVS by as much as 50 % (Li *et al.*, 1989), and exposure to other stressors such as low pH and Al^{3+}, Zn^{2+}, Mg^{2+} and SO_4^{2-} may also reduce the PVS (Eddy and Talbot, 1983). The results from the present study verify that road salt exposure affected the developing salmon egg by modulating the gene expression of a high number of genes. As many as 721 genes were up-regulated, whereas 281 were down-regulated by the exposure to 5000 mg/L road salt. Although a selection of single DEGs could be associated with the direct response to road salt exposure, functional (GO) enrichment and pathway analysis identified a number of potential mechanisms that may provide more in-depth understanding of the toxic MoA of road salt in the developing embryos. The potential major toxic MoA has been reviewed in detail below and is supported by supplementary information (Supplementary Tables S1, S2, S3 and S4).

4.1 Ion- and acid-base regulation

Fertilization of salmon eggs involve uptake of considerable amount of surrounding water (nearly 25 to 30 % of the original volume of the egg) through the permeable chorion within the first hours (Davenport *et al.*, 1981) and the internal environment will thus resemble the major composition of the surrounding water (Li *et al.*, 1989). However, in environments with increased salinities up to 1 %, reduced PVS has been observed in Atlantic salmon eggs during swelling, thus leading to a limited space for the developing embryos (Li *et al.*, 1989). It should therefore be expected that the developing embryos exposed to high concentrations of road salt were experiencing osmotic stress due to a combination of accumulation of water with high road salt concentrations in the PVS and reduced swelling of the eggs themselves. To cope with this high electrolyte challenge, developing fish eggs and larvae regulate the salt balance (Lasker and Theilacker, 1962), perhaps as early as the blastula stage in certain fish species (Holliday and Jones, 1965). The present data suggest that developing salmon embryos exposed to 5000 mg road salt/L were indeed experiencing osmotic stress, and that they were not able to successfully excrete the road salt efficiently across the chorion in the period up to the eyed egg stage. As seen for freshwater fish exposed to high salinity (Hoar, 1988), the salmon eggs exhibited physiological (transcriptional) changes consistent with hypo-osmoregulation, e.g. the regulation of internal plasma and tissue ions to concentrations being lower than that of the surroundings. Genes associated with well-known osmoregulatory functions in freshwater fish including ATP-fueled active transport of Cl^- across the gill and skin epithelium by Na^+/K^+ATPase (ATP1A2) was clearly upregulated. Interestingly, several genes associated with regulation of the embryo acid-base balance (Genz *et al.*, 2011) were also up-regulated including carbonic anhydrase 4 (CA4) and solute carrier family proteins (SLC43A3, SLC22A7, SLC9A3 and SLC9A1). A number of additional DEGs, primary related to ion transport (SLC39A9, SLC39A14, and SLC30A1) were also up-regulated, although their primary roles are more clearly associated with the transport of sugars, bile salts, organic acids, amines and metals such as zinc (Guh *et al.*, 2015). Reports that specific blockers of Na^+/K^+ATPase activity and transmembrane transport of solutes and ions enhance the adverse effect of high salt exposure in zebrafish embryos suggest that osmoregulation is a key mechanism also in road salt toxicity (Lahnsteiner, 2009). Interestingly, dechorionation of the zebrafish embryos did not alleviate the adverse effects observed by exposure to hyper-saline solutions (Lahnsteiner, 2009), thus indicating that the ion transport from the embryo out to the PVS is likely the crucial step in reducing osmotic stress in developing embryos. Quantitative rtPCR measurements of transcriptional changes in ATP1A2 identified a concentration-dependent increase in expression, albeit high variance in the control group precluded determination of whether these changes were significantly different from the control group.

4.2 Metabolism and energy turnover

A number of DEGs, associated with the activation of nuclear receptors pathways (e.g. the LXR/RXR and TR/RXR) were differentially expressed and the majority of these seemed to be associated with increasing the cellular turnover of energy. As stress responses, such as increase in regulation of ions and acid-base balance, may cause increased energy demand to maintain physiological homeostasis, these transcriptional changes are likely associated with activation of cellular pathways leading to increased lipid, steroid, amino acid, and carbohydrate metabolism (Martinez-Alvarez *et al.*, 2002; Tseng and Hwang, 2008). Although not studied specifically in this work, modulation of different nuclear receptor pathways (Table 4 and 5) should be expected to cause modulation of the mobilization of numerous macromolecules as source for increasing cellular energy turnover. Up-regulation of DEGs such as APOE, APOA4, NCOR1, ACACA and MMP9 are all associated with the action of LXR/RXR by increasing the transport and ensuring homeostasis of lipids, lipopolysaccharides and cholesterol during stress-induced energy depletion (Cordier *et al.*, 2002; Lu *et al.*, 2010; Prunet *et al.*, 2007). Modulation of the DEGs COL6A3, SLC16A2 in the TR/RXR pathway indicate that mobilization of amino acids is additionally altered to accommodate an increased energy demand, whereas upregulation of SLC2A6 and GLUT1 indicate that glucose transport were also activated. Modulation of a number of DEGs in the Clathrin-mediated Endocytosis Signaling suggest that internalization of nutrients, hormones and other signaling molecules over the cellular plasma membrane into intracellular compartments were induced to accommodate a more rapid cellular metabolic turnover. Although not determined directly, increased metabolic turnover was coherent with the observation of up-regulation of CA4 that catalyzes the reversible reaction of CO_2 and water to facilitate CO_2 excretion or HCO_3^- transformation due to increased CO_2 production during cellular metabolism (Gilmour *et al.*, 2009).

Up-regulation of TF and IL1RAPL1, which is associated with the mobilization of divalent ions such as iron and calcium during acute-phase responses to stress, suggest that other pathways involving in more broadly acting intracellular signaling were also affected. In fact, differential expression of a number of genes coding for Ca^{2+} pumps (e.g. calcium-binding mitochondrial carrier protein s-1 (SLC25A24), Ca^{2+} exchangers (e.g. anoctamin calcium activated chloride channel (ANO1) and transmembrane Ca-ion channels (e.g. calcium homeostasis endoplasmic reticulum protein (CHERP), orai calcium release-activated calcium modulator 1 (ORAI1), soluble calcium binding proteins such as Striatin (STRN) involved in maintaining intracellular calcium levels and regulating the activities of different proteins, enzymes and transcription factors (e.g. HDAC10, HDAC4, and calmodulin-dependent protein kinases), support the hypothesis that modulation of Ca-signaling were likely occurring (Boeuf and Payan, 2001; Evans *et al.*, 2005). Interestingly, up-regulation of UCP3 has also been associated with Ca-signaling and is additionally linked to regulation of cellular export of fatty acids, mitochondrial oxidation capacity and uncoupling of the transmembrane proton transport in the mitochondria (Shabalina and Nedergaard, 2011). The potential depolarization of the mitochondria by UCP3 was found to be consistent with the up-regulation of DEGs such as HTT, CYR61 and GZMB (Table 3) that is associated with the mitochondrial membrane permeability, membrane potential and incorporation of proteins in the mitochondrial membrane (Ismailoglu *et al.*, 2014;

Jacquemin *et al.*, 2015; van Waveren *et al.*, 2006). Mitochondrial depolarization has frequently been reported as the primary cause for cell injury or death for a number of stressors including natural toxins and environmental pollutants (Song *et al.*, 2014; Tiano *et al.*, 2001).

4.3 Oxidative stress

Formation of Reactive Oxygen Species (ROS) or other free radicals may result in direct damage of cellular components such as protein, lipids and DNA, or may affect other biological pathways which might not necessarily be linked directly to oxidative stress (Lushchak, 2011). Although the aforementioned up-regulation of UCP3 is suggested to provide some level of protection towards mitochondrial oxidative stress (Cannon *et al.*, 2006), several cellular antioxidant defenses were activated after exposure to high concentrations of road salt. The up-regulation of GGT1 and PRDX2 to increase the antioxidant capacity were consistent with increase in antioxidant responses in vertebrates exposed to high salinity (Martinez-Alvarez *et al.*, 2002). However, down-regulation of NFE2L1 and NQO1 seemed to contrast existing knowledge of ROS-induced antioxidant defense in fish (Mukhopadhyay *et al.*, 2015; Sarkar *et al.*, 2014; Wang and Gallagher, 2013). The rationale for salt-induced oxidative stress responses in fish is not entirely clear, but it has been reported in other organisms that high NaCl causes oxidative stress (Carlstrom *et al.*, 2009; Zhang *et al.*, 2004; Zhou *et al.*, 2005) and cellular damage (Burg *et al.*, 2007). Quantitative rtPCR analysis of thioredoxin domain containing genes (Txndc9), which is believed to play an important role in the defense against oxidative stress by directly reducing hydrogen peroxide and certain radicals and by serving as a reductant for peroxiredoxins (Palanisamy *et al.*, 2014; Wei *et al.*, 2012), displayed an apparent concentration-dependent increase in expression. Although a modest up-regulation (approximately 2-fold) was observed for Txndc9 by qrtPCR, as much as a 25-fold upregulation was observed for Txndc9 when reviewing the microarray data (Supplementary Table S1). In-depth studies to characterize whether high road salt concentrations cause oxidative stress beyond the protective antioxidant capacity of the developing salmon embryo and whether adverse cellular effects were occurring, is clearly warranted to determine if exposure to the developing embryo may lead to adverse effects later in life.

4.4 Renal function

Exposure of developing salmon embryos to high salt concentrations were associated with differential regulation of a number of DEGs involved in renal necrosis, cell death and acute renal failure. Lack of proper regulation of a number of these DEGs may ultimately lead to apoptosis and necrosis of renal tissues. Modulation of a number of genes (e.g. APOE, MAP2K7, BGN, HBEGF, TNFAIP3, C1QA, PRSS1, SDHC, P2RX7, TP53BP2, VOPP, RHOG, HTT, BNIP3L, SNCA, and PLAT) predominantly involved in stimulation, but also to a limited degree negative regulation of apoptosis suggest a large recruitment of genes involved in termination of damaged cells (Table 4). Observation of enriched DEGs in well-known apoptotic pathways such as April-mediated signaling and Granzyme B signaling (Table 4) confirm that cells were potentially adversely affected. Several DEGs (e.g. TNFAIP3, HBEGF, CIQA, CD68) involved in removal of cell debris as well as inflammatory and immune responses support that some level of tissue damage were likely occurring after exposure to high road salt concentrations. Differential regulation of genes involved in the migration of leukocytes from the vascular system to sites of pathogenic exposure (e.g. the Agranulocyte Adhesion and Diapedesis signaling pathway) and induction of ROS production to degrade damaged tissues, additionally suggest that cellular responses to injury through activating inflammatory responses were occurring. Concentration-dependent increase in Cyp4A14, which is proposed to be involved in arachidonic acid metabolism, icosanoid biosynthesis and kidney development in response to endogenous and exogenous stimulus (Capdevila *et al.*, 2015), suggest that renal function may be affected even at lower concentrations than 5000 mg/L.

4.5 Cellular growth, proliferation and development

The analysis performed clearly identified that many of the DEGs were associated with cellular growth, proliferation and development. This applied in particular to organ and embryonic development such as the sensory system (eye morphogenesis and eye development), the cardiovascular system (Trehalose degradation II, GDP-glucose biosynthesis, NOTCH signaling, MIF-mediated glucoroticoid regulation), the hematological system (Agranulocyte adhension and diapesis, MIF-mediated glucoroticoid regulation, atherosclerosis signaling, April-mediated signaling) and the reproductive system (Serotonin and melatonin biosynthesis, TR/RXR activation). Although the interference with these developmental processes were not studied in detail in the present paper, observations that a number of DEGs such as TCF4,

MYL3, MYL6, MYL7, SSX2IP, IQGAP1, NOTCH1, HEY2, RhoG, and RhoH are involved in the maturation, polarization and migration of cells in organ development and morphogenesis (Table 4), may suggest that prolonged hypertension due to road salt exposure may interfere with normal development of organs involved in hepatic and hematological processes (Vosylienė *et al.*, 2006), sensory processes (Sanzo and Hecnar, 2006), acid-base balance (Bentley and Schmidt-Nielsen, 1971), hepatic and neural development (Klein *et al.*, 2011; Yonkers and Ribera, 2009). Concentration-dependent up-regulation of the neuron navigator (Nav1), a gene expressed predominantly in the nervous system and believed associated with axonal guidance (Novak *et al.*, 2006) by qrtPCR suggest that neuronal development and regeneration in the developing embryos was affected by high road salt concentrations.

4.6 No Transcriptional Effect Level (NOTEL)

The knowledge of adverse effects of road salt exposure is limited and biomarkers to determine potential adverse effects are poorly developed. The present work have developed a better characterization of potential genes being candidate markers for more thorough assessment, including proposing a suite of genes to determine the NOTEL for osmotic stress (*ATP1A2*), oxidative stress (*Txndc9*), changes in lipid processing and renal function (*Cyp4A14*) and neuroaxonal development (*Nav1*). Although all of these DEGs showed a concentration-response relationship, NOTEL values were only obtained for *Cyp4A14* and *Nav1* due to the high variance in the control group for the other genes determined. The current assessment suggests that concentrations lower than 100 mg/L (NOTEL=50 mg/L) were not causing any transcriptional responses in the embryos and were found to be several orders of magnitude lower than the concentrations causing adverse effects in salmonids (Mahrosh *et al.*, 2014) and amphibians (Sanzo and Hecnar, 2006). A more thorough assessment to identify additional biomarker candidates for exposure, cellular response and adverse effects of road salt based on the existing data is expected to yield a better basis for future hazard and risk assessments.

4.7 Ecological consequence

The fertilization stage is very sensitive for pollutant uptake as the eggs may accumulate stressors such as salt and pollutants from the ambient environment (Nakano, 1956). As seen herein, substantial transcriptional responses were identified at high road salt concentrations, whereof some may be of relevance for adverse outcomes important for environmental risk. A NOTEL of 50 mg/L road salt were established, based on changes in marker genes for osmotic stress and neuroaxonal developmental changes are typically in the low range of road salt concentrations in surface waters receiving run-off from roads where deicing salts have been used. However, such concentrations are likely to occur in small streams that receive diluted run-off from road maintenance operations. For example, Winter *et al.* (2011) reported Cl^- concentrations way above 50 mg/L in tributaries to lake Simcoe (Canada) in the years 2004-2007 and Betts *et al.*(2014) reported annual Cl^- concentrations ranging from 118-765 mg/L in urban rivers within the City of Toronto (Canada). Despite lack of data, we believe that such concentrations may also be common in small Norwegian streams. In Norway, small streams along the coast line are known to be important for salmonids. For example, in Østfold county approximately 40 small streams, some having discharge on an average level less than 0.04 m^3/sec, are inhabited by brown trout (Karlsen, 2012). The current GIS-based environmental impact assessment method for road salt in Norway is so far only considering chemical stratification and biological effects in lentic systems (Kronvall, 2013). However, the present results indicate that fish inhabiting lotic systems close to roads may be affected by road salt exposures, although potential adverse effects of such transcriptional changes are still unknown. Future initiatives should therefore aim to provide better links between mechanistical data obtained herein and adverse effects relevant for regulatory decisions, and by doing so supporting identification of vulnerable recipients according to intention of the EU WFD.

5 Conclusions

Large transcriptional changes were observed in eyed egg stage embryos exposed to 5000 mg/L road salt during the first 24h after fertilization. These transcriptional changes were mainly associated with a number of potentially toxicity mechanisms including osmoregulation, ion regulation, oxidative stress, metabolism, renal function and development in the embryos. A number of DEGs were identified to be affected by the exposure and selection of these were used to derive the NOTEL for osmoregulation (ATP1A2), oxidative stress (Txndc9), renal function (Cyp14A4) and organ development (Nav1). Although all of the genes displayed a concentration-dependency, only expression of Cyp14A4 and Nav1 (100 mg/L and higher) were significantly regulated due to large data variation in the control group. The present data propose that road salt concentrations below 100 mg/L should not be expected to affect developing embryos when exposed during fertilization and these concentrations were found to be several orders of magnitude lower than the adverse effects reported elsewhere for Atlantic salmon and other aquatic vertebrates. The concentrations causing transcriptional responses were typically in the concentrations range of that expected to occur in lotic systems receiving dilute run-off from roads using road salt, and suggest using biomarker approaches to assist the risk assessment and risk management process of these types of exposure scenarios. Further research should aim to provide better links between mechanistical data obtained with more regulatory relevant chronic endpoints such as effects on growth, development and reproduction.

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Figure caption

Figure 1. Combined (genes and treatments) hierarchical clustering (Squared Euclidian, Ward's linkage) of differentially regulated features in Atlantic salmon (*S. salar*) embryos after exposure to control water (left columns) and 5000 mg/L Road salt (right columns) from fertilization to 24h post-fertilization. The data (n=4) represent differential gene expression determined at the eyed egg stage. Extended list of differentially expressed genes is provided in supplementary Table S1.

Figure 2. Protein-protein interaction networks in Atlantic salmon (*S. salar*) eyed embryos affected by exposure to 5000 mg/L road salt from fertilization to 24h post fertilization: A) energy production and lipid metabolism; B) organ and embryonic development, C) cell signaling and interaction and D) nervous system development and function. Color intensity indicates fold regulation of the genes at the eyed egg stage. (red=up, green=down, white=not applicable). Extended list of Regulatory networks is provided in supplementary Table S4.

Figure 3. Expression of selected genes in eyed eggs of Atlantic salmon (*S. salar*) after exposure to lake water (control) and lake water added different concentrations of road salt from fertilization to 24h post-fertilization. Asterics denote groups being significantly different from control ($p \leq 0.05$). Data represent minimum 5 individual replicates per group: cytochrome P450 4A14 precursor (Cyp4A14), thioredoxin domain-containing protein 9 (Txndc9), sodium potassium-transporting atpase subunit alpha-2 precursor (ATP1A2) and Neuron navigator 1 isoform 2 (Nav1).

Table 1. Atlantic salmon primer sequences (5’-3’) for the quantitative rtPCR analysis

Gene	Gene name	GenBank Acc.	Forward	Reverse
Stmn3	stathmin-like 3	DY700722	CTCACAACCACATCCCAACA	GCCTCCTGAGACTTCCTCCT
Cyp4A14	cytochrome P450, family 4, subfamily a, polypeptide 14	BT058728	TGATCAGGAAAAACCCAAGG	GTAGGGAAAGATCCGGAAGC
Txndc9	thioredoxin domain containing 9	BT043664	GGAGCAGTTGGACAAAGAGC	CACACGATTGCTCTCCTTCA
Atp1A2	ATPase, Na+/K+ transporting	EG826654	TGTGATCCTTGCTGAGAACG	AGATCGGCCCACTGTACAAC
Nav1	neuron navigator 1	GT297797	AATTAGCTGCTCAGCCCGTA	GCACTGGCAAGACCTACCTC

Table 2. Physical and chemical parameters of the rearing water in the 24 hrs exposure period (during swelling) and in the post-swelling period until the eyed egg stage (Clarvay stage to 255 day degrees).

	During swelling stage					Post-swelling
Parameter	Road salt concentration (mg/L)					All groups
	Control	50	100	500	5000	
Temperature	7.1	5.5	4.6	5	5.5	5.4±1.6
*Cond. µS/cm	35	145	240	850	1870	48±6.5
pH	6.9	7.2	7.5	7.3	7.2	7.2±0.2
Na mg/L	3.0	22	34	190	NA	3.0±1.5
Ca mg/L	6.0	6.0	6.0	8.4	9.0	4.8±0.1
Mg mg/L	1.2	1.0	1.0	1.0	5.0	1.2±1.0
K mg/L	2.1	2.0	2.8	5.0	12	2.1
TOC mg/L	4.25	4.04	4.03	3.94	4.01	4.25±1.0
F^- mg/L	< 0.1	< 0.1	< 0.1	NA	NA	< 0.1
Cl^- mg/L	5.46	32	59	450	NA	5.46
SO_4^{2-} mg/L	2.43	2.57	2.65	NA	NA	2.43
NO_3^- mg/L	0.21	0.45	0.10	NA	NA	0.21

NA = not analyzed

**Cond.: Conductivity*

Table 3. Biological processes associated with up-regulated genes in Atlantic salmon (*S. salar*) embryos after exposure to 5000 mg/L road salt from fertilization to 24h post-fertilization. The data represent differential gene expression determined at the eyed egg stage.

GO ID	Term	Genes
GO:0048592	eye morphogenesis	SIX3, AXIN1, DSCAM, GS-1, GM2, CRYGB, CRYGN, CRYGNB
GO:0000303	response to superoxide	PRDX2, APOA4, UCP3
GO:0070309	lens fiber cell morphogenesis	CRYGNB, GS-1, CRYGN, CRYGB, GM2
GO:0001654	eye development	SIX3, JMJD6, AXIN1, CRYGNB, CRYBA1, GS-1, CRYBA4, DSCAM, CRYGN, CRYGB, GM2

Abbreviations:
SIX3: Homeobox protein SIX3
AXIN1: Axin-1
DSCAM: Down syndrome cell adhesion molecule
GS-1: β-crystallin S-1
GM2: γ-crystallin M2
CRYGB: γ-crystallin B
CRYGN: γ-crystallin N
CRYGNB: γ-crystallin N-B
PRDX2: peroxiredoxin 2
APOA4: Apoplipoprotein A-IV precursor
UCP3: Mitochondrial uncoupling protein
JMJD6: Histone arginine demethylase
GRYBA1: β-crystallin A-2
GRYBA4: β-crystallin A-4

Table 4 Toxicity pathways associated with differentially expressed genes in Atlantic salmon (*S. salar*) embryos after exposure to 5000 mg/L road salt from fertilization to 24h post-fertilization. The arrows indicate direction of gene regulation at the eyed egg stage (↑=up-regulation, ↓= down-regulation). Extended list of toxicity pathways is provided in supplementary Table S2.

Toxicity Pathway	p-value	Ratio	Molecules
Renal Necrosis/Cell Death	0.002	0.044	↑APOE,↑TCF4, ↓MAP2K7, ↑CA4, ↑BGN, ↑HBEGF, ↑MST1, ↑TNFAIP3, ↑C1QA, ↓PRSS1, ↑SDHC, ↑P2RX7, ↑TP53BP2, ↓HK1, ↑VOPP1, ↑RHOG, ↑HTT, ↑BNIP3L, ↑SNCA, ↓PLAT
LXR/RXR Activation	0.005	0.065	↑APOE, ↑APOA4 ,↑TF, ↓NCOR1, ↑ACACA, ↑IL1RAPL1, ↑MMP9, ↑IL36B
Increases Depolarization of Mitochondria and Mitochondrial Membrane	0.005	0.176	↑HTT, ↑CYR61, ↑GZMB
Oxidative Stress	0.033	0.070	↓NQO1, ↑GGT1, ↓NFE2L1, ↑PRDX2
TR/RXR Activation	0.036	0.058	↑UCP3, ↑COL6A3, ↓SLC16A2, ↓NCOR1, ↑ACACA
Acute Renal Failure Panel (Rat)	0.043	0.065	↑CD68, ↑SLC9A3, ↑Cyp4a14, ↑SLC30A1

Abbreviations: LXR: liver X receptor; RXR: retinoic X Receptor; TR: Thyroid hormone receptor

Genes:

ACACA: acetyl-CoA carboxylase alpha
APOE: apolipoprotein E
BGN: biglycan
BNIP3L: BCL2/adenovirus E1B 19kDa interacting protein 3-like
C1QA: complement component 1, q subcomponent, A chain
CA4: carbonic anhydrase IV
CD68: CD68 molecule
COL6A3: collagen, type VI, alpha 3
Cyp4a14: cytochrome P450, family 4, subfamily a, polypeptide 14
CYR61: Cysteine-rich, angiogenic inducer, 61
GGT1: Gamma-glutamyltransferase 1
GZMB: granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)
HBEGF: heparin-binding EGF-like growth factor
HTT: huntingtin
HK1: hexokinase 1

IL1RAPL1: interleukin 1 receptor accessory protein-like 1
IL36B: interleukin 36, beta
MAP2K7: mitogen-activated protein kinase kinase 7
MMP9: matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
MST1: macrophage stimulating 1 (hepatocyte growth factor-like)
NCOR: nuclear receptor corepressor 1
NFE2L1: nuclear factor, erythroid 2-like 1
NQO1: NAD (P)H dehydrogenase, quinone 1
P2RX7: purinergic receptor P2X, ligand-gated ion channel, 7;
PLAT: plasminogen activator, tissue
PRDX2: peroxiredoxin 2
PRSS1: protease, serine, 1 (trypsin 1) RHOG: ras homolog family member G
SDHC: succinate dehydrogenase complex, subunit C
SLC16A2: solute carrier family 16, member 2 (thyroid hormone transporter)
SLC30A1: solute carrier family 30 (zinc transporter), member 1
SLC9A3: solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3), member 3
SNCA: synuclein, alpha (non A4 component of amyloid precursor)
TCF4: transcription factor 4
TNFAIP3: tumor necrosis factor, alpha-induced protein 3
TF: transferrin
TP53BP2: tumor protein p53 binding protein, 2
UCP3: uncoupling protein 3 (mitochondrial, proton carrier)
VOPP1: vesicular, overexpressed in cancer, prosurvival protein 1

Table 5. Canonical pathways being affected in Atlantic salmon (*S. salar*) embryos after exposure to 5000 mg/L road salt from fertilization to 24h post fertilization. The FDR-corrected p-value and ratio of genes identified being differentially expressed versus total number of genes on the pathway is depicted in detail. The arrows indicate direction of gene regulation at the eyed egg stage (↑=up-regulation, ↓= down-regulation). Extended list of canonical pathways is provided in supplementary Table S3.

Canonical Pathways	Signalling pathway category	Top functions and disease	p-value	Ratio	Genes
LXR/RXR Activation	Nuclear Receptor Signaling	Lipid Metabolism; Small Molecule Biochemistry; Molecular Transport	0.006	0.059	↑APOE, ↑APOA4, ↑TF, ↓NCOR1, ↑ACACA, ↑IL1RAPL1, ↑MMP9, ↑IL36B
Clathrin-mediated Endocytosis Signaling	Cellular Immune Response; Organismal Growth and Development; Pathogen-Influenced Signaling	Cellular Function and Maintenance; Infectious Disease; Cellular Assembly and Organization	0.007	0.051	↑AP2B1, ↑APOE, ↑APOA4, ↓SNX9, ↓EPS1, ↑TF, ↑CSNK2A1, ↓SH3GL2, ↑ITGB7, ↓HIP1R
Trehalose Degradation II (Trehalase)	Trehalose Degradation	Cardiovascular System Development and Function; Cell Morphology; Embryonic Development	0.012	0.250	↓HK1, ↑GCK
Agranulocyte Adhesion and Diapedesis	Cellular Immune Response	Cell-To-Cell Signaling and Interaction; Cellular Movement; Hematological System Development and Function	0.016	0.048	↑PODXL, ↑CLDN8, ↑MYL6, ↑CXCL6, ↑ITGB7, ↑MMP9, ↓MYL3, ↑IL36B, ↓MYL7
Regulation of Actin-based Motility by Rho	Neurotransmitters and Other Nervous System Signaling	Cell Signaling; Cellular Assembly and Organization; Cellular Function and Maintenance	0.034	0.056	↑RHOG, ↑MYL6, ↑RHOH, ↓MYL3, ↓MYL7
MIF-mediated Glucocorticoid Regulation	Cellular Immune Response; Nuclear Receptor Signaling	Hematological Disease; Infectious Disease; Organismal Injury and Abnormalities	0.038	0.071	↑NFKBIE, ↑PLA2G4F, ↓CD74

Canonical Pathways	Signalling pathway category	Top functions and disease	p-value	Ratio	Genes
Epithelial Adherens Junction Signaling	Cellular Growth, Proliferation and Development	Cellular Assembly and Organization; Cellular Function and Maintenance; Cell Morphology	0.038	0.048	↑TCF4, ↑MYL6, ↓SSX2IP, ↓IQGAP1, ↓NOTCH1, ↓MYL3, ↓MYL7
GDP-glucose Biosynthesis	Sugar Nucleotides Biosynthesis	Molecular Transport; Protein Trafficking; Cardiovascular System Development and Function	0.040	0.118	↓HK1, ↑GCK
Granzyme B Signaling	Cellular Immune Response	Cell Death and Survival; DNA Replication, Recombination, and Repair; Cell Morphology	0.045	0.125	↑PRF1, ↑GZMB
Serotonin and Melatonin Biosynthesis	Hormones Biosynthesis	Reproductive System Development and Function; Endocrine System Development and Function; Small Molecule Biochemistry	0.045	0.125	↑TPH1, ↑ASMT
Parkinson's Signaling	Disease-Specific Pathways; Neurotransmitters and Other Nervous System Signaling	Cell Death and Survival; Hereditary Disorder; Neurological Disease	0.045	0.125	↓PARK2, ↑SNCA
Atherosclerosis Signaling	Cardiovascular Signaling; Disease-Specific Pathways	Cell-To-Cell Signaling and Interaction; Tissue Development; Hematological System Development and Function	0.045	0.044	↑APOE, ↑APOA4, ↑PLA2G4F, ↑ALOXE3, ↑MMP9, ↑IL36B
April Mediated Signaling	Apoptosis	Cellular Development; Cellular Growth and Proliferation; Hematological System Development and Function	0.047	0.070	↓MAP2K7, ↑TNFSF13, ↑NFKBIE
Notch Signaling	Cancer; Organismal Growth and Development	Hair and Skin Development and Function; Organ Development; Organ Morphology	0.047	0.070	↑HEY2, ↓NOTCH1, ↑DTX2
TR/RXR Activation	Nuclear Receptor Signaling	Amino Acid Metabolism; Drug Metabolism; Endocrine System Development and Function	0.047	0.052	↑UCP3, ↑COL6A3, ↓SLC16A2, ↓NCOR1, ↑ACACA

Canonical Pathways	Signalling pathway category	Top functions and disease	p-value	Ratio	Genes
ILK Signaling	Cellular Growth, Proliferation and Development	Cellular Movement; Cellular Development; Cellular Growth and Proliferation	0.047	0.042	↑RHOG, ↑MYL6, ↑PPP1R14B, ↑RHOH, ↑ITGB7, ↑MMP9, ↓MYL3, ↓MYL7
Calcium Signaling	Intracellular and Second Messenger Signaling	Cell Signaling; Molecular Transport; Vitamin and Mineral Metabolism	0.047	0.038	↑HDAC4, ↑MYL6, ↓TNNC1, ↑RYR3, ↑HDAC10, ↓MCU, ↓MYL3, ↓MYL7

Abbreviations: LXR: liver X receptor; RXR: retinoic X Receptor; TR: Thyroid hormone receptor; MIF: Macrophage migration inhibitory factor; GDP-glucose: Guanosine 5-diphosphoglucose; ILK: Integrin-linked kinase;

Genes:

ACACA: acetyl-CoA carboxylase alpha
ALOXE3: arachidonate lipoxygenase 3
AP2B1: adaptor-related protein complex 2, beta 1 subunit
APOA4: apolipoprotein A-IV
APOE: apolipoprotein E
ASMT: acetylserotonin O-methyltransferase
CD74: CD74 molecule, major histocompatibility complex, class II invariant chain
CLDN8: claudin 8
COL6A3: collagen, type VI, alpha 3
CSNK2A1: casein kinase 2, alpha 1 polypeptide
DTX2: deltex homolog 2 (Drosophila)
EPS15: epidermal growth factor receptor pathway substrate 15
GCK: glucokinase (hexokinase 4)
GZMB: granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)
HDAC10: histone deacetylase 10
HDAC4: histone deacetylase 4
HEY2: hes-related family bHLH transcription factor with YRPW motif 2
HIP1R: huntingtin interacting protein 1 related
HK1: Hexokinase 1
IL1RAPL1: interleukin 1 receptor accessory protein-like 1
IL36B: interleukin 36, beta
IQGAP1: IQ motif containing GTPase activating protein 1
ITGB7: integrin, beta 7
MAP2K7: mitogen-activated protein kinase kinase 7
MCU: mitochondrial calcium uniporter
MMP9: matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)

MYL3: myosin, light chain 3, alkali; ventricular, skeletal, slow

MYL6: myosin, light chain 6, alkali, smooth muscle and non-muscle

MYL7: myosin, light chain 7, regulatory

NCOR1: nuclear receptor corepressor 1

NFKBIE: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon

NOTCH1: notch 1

PARK2: parkin RBR E3 ubiquitin protein ligase

PLA2G4F: phospholipase A2, group IVF

PODXL: podocalyxin-like

PPP1R14B: protein phosphatase 1, regulatory (inhibitor) subunit 14B

PRF1: perforin 1 (pore forming protein)

RHOG: ras homolog family member G

RHOH: ras homolog family member H

RYR3: ryanodine receptor 3

SH3GL2: SH3-domain GRB2-like 2

SLC16A2: solute carrier family 16, member 2 (thyroid hormone transporter)

SNCA: synuclein, alpha (non A4 component of amyloid precursor)

SNX9: sorting nexin 9

SSX2IP: synovial sarcoma, X breakpoint 2 interacting protein

TCF4: transcription factor 4

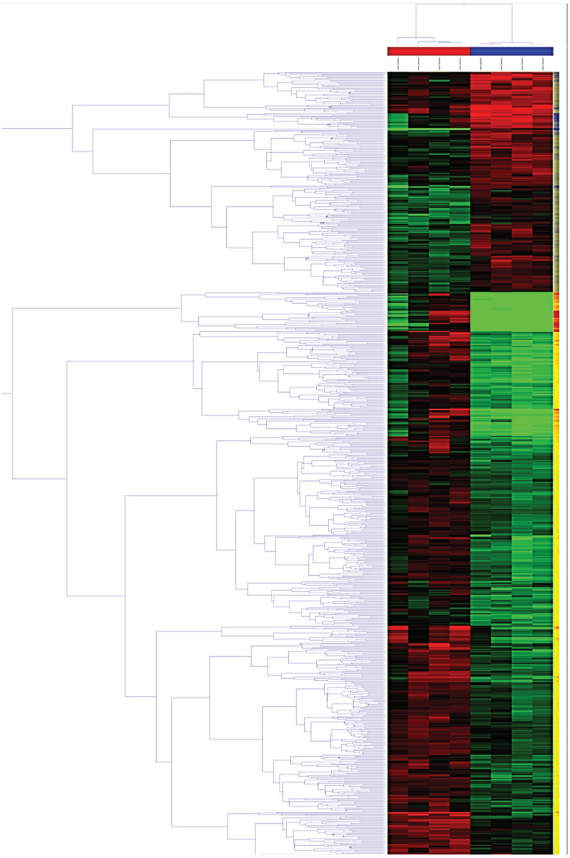
TF: transferrin

TNFSF13: tumor necrosis factor (ligand) superfamily, member 13

TNNC1: troponin C type 1 (slow)

TPH1: tryptophan hydroxylase 1

UCP3: uncoupling protein 3 (mitochondrial, proton carrier)



Down-regulated
Features: 281
GOs: 0
Pathways: 7
Networks: 11

UP-regulated
Features: 721
GOs: 13
Pathways: 10
Networks: 23

All
Features: 1002
GOs: 16
Pathways: 14
Networks: 25

Figure 1. Tollefsen *et al.*

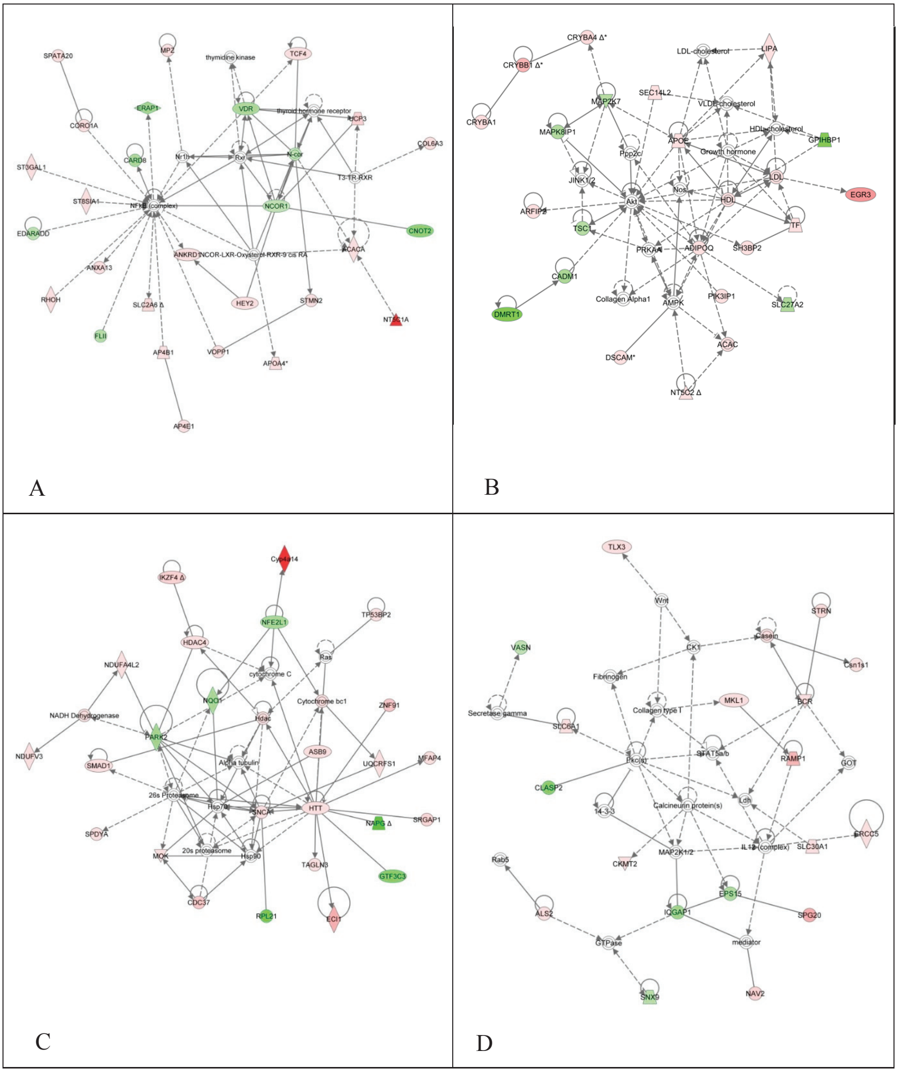


Figure 2. Tollefsen *et al.*

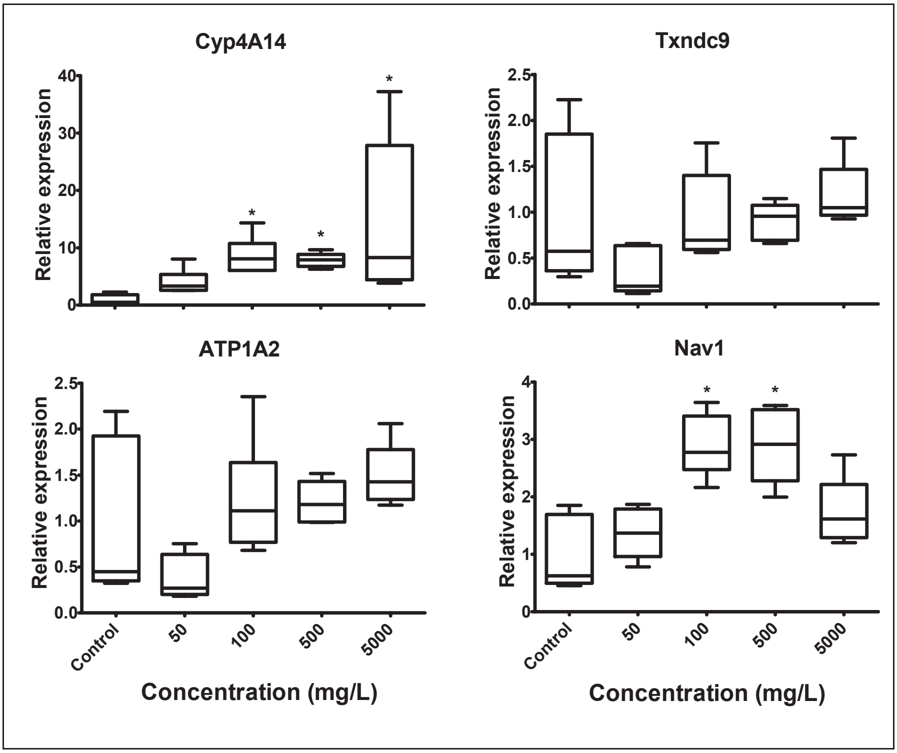


Figure 3. Tollefsen *et al.*

Paper IV

Impact of road deicing salt (NaCl) and copper (Cu) on Atlantic salmon (*Salmo salar*) alevins from hatching till swim-up

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Abstract

Road salts are frequently used for deicing of roads in the Nordic countries. Especially during snow melt, the road run-off containing high concentrations of road salt and a series of metals such as Cu remobilized from sand, silt and dust may negatively influence organisms in downstream receiving lakes and rivers. The present work focuses on the impact of road salt (NaCl) and Cu at environmentally relevant concentrations, separately and in mixtures, on an early life history stage of Atlantic salmon (*Salmo salar*). In the experimental work alevins, from hatching till swim-up, were continuously exposed (47 days) to the test solutions.

The results showed that high road salt concentrations could induce a series of negative effects in alevins such as reduced growth, deformities, delayed swim-up and mortality. For alevins exposed to all tested road salt concentrations (100-1000 mg/L), mortality was significantly higher than in control. Highest mortality (46 %) was observed, when alevins were exposed to solutions containing the highest road salt concentrations (1000 mg/L) tested.

When alevins were exposed to Cu solutions (5-20 µg Cu/L), no effects on growth, morphology, swim-up or mortality compared to control were observed. In mixture solutions (road salt and Cu), ultrafiltration of the exposure water demonstrated that Cu was present as colloids (10-40%) and low molecular mass (LMM) species (40-60 %). Only 20-40 % were present as positively charged LLM Cu species assumed to be bioavailable, and the Cu concentration in the head of alevins correlated with LMM Cu species in solution. When exposed to road salt and Cu mixtures, negative effects in alevins such as reduced growth, deformities, delayed swim up and mortality were also observed. When exposed to the most critical mixtures (1000 mg road salt/L+5 and 10 µg Cu/L), the mortality increased to 80 % compared to control. The overall results indicated that the road salt application could seriously affect sensitive life stages of Atlantic salmon, and application of road salt should be avoided during the late winter-early spring period.

Keywords: Road salt, Copper, Atlantic salmon, Alevins, biological effects

1 Introduction

During the winter period in the Nordic countries, road salts are frequently used for deicing roads. According to Environment Canada (2001) sodium chloride (NaCl) accounts for 98 % of all deicing salts used on the roads in winter throughout the northern hemisphere due to their ease of application, low cost and usefulness in reducing the freezing point of water (Collins and Russell, 2009). Due to road management and increased application of road salt during recent decades, large amounts of deicer elements are introduced to the environment (Ramakrishna and Viraraghavan, 2005) and concentrations up to ≥18,000 mg/L have been recorded in road runoffs (Environment Canada, 2001). Thus, seasonal applications of road deicing salt will result in increased concentrations of Na and Cl in downstream surface waters (Sanzo and Hecnar, 2006), and the water quality can be critical to sensitive life history stages of aquatic organisms such as Atlantic salmon (Mahrosh *et al.*, 2013). Road deicing salts are therefore considered as a source of pollution to lakes and streams inhabiting aquatic organism such as fish (Mason *et al.*, 1999), and this issue has created a great environmental concern in recent years (Environment Canada, 2001; Mayer *et al.*, 1999).

High concentrations of road salt may also cause indirect effects due to interaction with other components in the road runoff e.g., mobilization of metals such as cupper (Cu) due to desorption from surfaces of solid phases like sand, silt and road dust (Amrhein and Strong, 1990) or due to cation exchange processes (Mason *et al.*, 1999). The road run-off may, therefore, contains high concentrations of not only the road salt (NaCl) but also a series of chemicals such as hydrocarbons (Norrstrom and Jacks, 1998) and Cu. During spring snowmelt the combined mixture has also the potential of harming exposed organisms in downstream receiving lakes and rivers. Taking the toxicity of Cu in to account, the concentrations of 10-20 µg/L have been reported to adversely affect reproduction and survival in teleosts fish (Hodson, 1975). Furthermore, Cu is known to exert a wide range of toxic effects on fish such as changes in blood chemistry, altered synthesis of metallothionein in hepatocytes, and histopathology of gills and skin (Iger *et al.*, 1994). Mahrosh *et al.* (2014) have also demonstrated that the combination of road deicing salts and Cu are toxic towards early life stages of Atlantic salmon, from fertilized eggs until hatching, while little attention has been given to the newly hatched alevins.

Atlantic salmon undergoes various physiological and morphological changes during their development in freshwaters from fertilized egg to parr and smolt (migrating to the marine environment). To assess the environmental impact of road deicing salt practice, information is needed on the sensitivity and resistance of different developmental stages, when exposed to road salt separately and in combination with metals that can be mobilized from sand, silt and dust during run-off. Thus, the objective of the present work was to identify the responses in sensitive life history stage of salmon such as alevins, from hatching to the swim-up stage, when exposed to environmentally relevant concentrations of road salt separately and in combination with Cu being abundant in the road dust. Focus is given on responses such as growth, morphology (formation of deformities) and delayed or pre-mature swim-up as well as mortality, compared to control.

2 Methodology

The experiment was conducted in a temperature controlled room (10°C) at the Fish Laboratory at the Norwegian University of Life Sciences (NMBU) from May 14 until June 30, 2013. During the experiments, responses in newly hatched alevins of Atlantic salmon were followed until the swim-up stage (after resorption of the yolk sac) was reached. The alevins of Atlantic salmon, from hatching till swim-up, were chronically exposed to the following water qualities**:**

- Control water (pH 6.9, 4 mg Na/L)
- 100, 500 and 1000 mg road salt/L, respectively (pH 7.0)

To be environmentally realistic, commercially available road salt (NaCl Isbryter rock salt) was used. This road salt was obtained from GC Rieber Salt AS, Norway, and according to the fact sheet the road salt contained 98.5 % NaCl, 0.30 % Ca and Mg, 0.70 % SO_4, 0.3 % H_2O humidity and 70–100 ppm anticaking agent (E 535). The E535 refers to sodium ferrocyanide $Na_4Fe(CN)_6$, i.e., yellow prussiate of soda. In test solutions with 1000 mg road salt/L the concentration of $Na_4Fe(CN)_6$ is 100 µg/L.

To obtain information of Cu toxicity on Atlantic salmon alevins, additional exposure solutions were included:

- 5, 10 and 20 µg Cu/L, respectively (pH 7, Na 4-5 mg/L).

To obtain information about effects due to mixed exposures (road salt and Cu), alevins of Atlantic salmon were exposed to the mixtures of road salt and Cu:

- 500 and 1000 mg road salt/L, respectively, mixed with 5 µg Cu/L (pH 6.8).
- 500 and 1000 mg road salt/L, respectively, mixed with 10 µg Cu/L (pH 7.0).

Control water was obtained from Lake Maridalsvannet, Oslo. The road salt test solutions were prepared by well mixing of accurately weighed amounts of NaCl Isbryter rock salt in containers with 70 L Lake Maridalsvannet water. The Cu stock solution was produced by mixing analytical grade chemical $CuCl_2.2H_2O$ in containers with Milli-Q water (1 mg Cu/L from $CuCl_2.2H_2O$). The solutions were always freshly prepared when needed.

2.1 Experimental setup

Eyed eggs of Atlantic salmon were obtained from Aquagen hatchery Sunndalsøra, Norway and transported to NMBU (24 h) in sealed bags containing ice. Two hundred eyed embryos were placed in separate exposure units with corresponding test water one day before hatching. Each exposure box was entirely blackened from all the sides in order to shield from all light sources and reduce disturbance. The experiment was completed at the swim-up stage, when alevins had absorbed the yolk sac. At the end of the experiment the alevins were killed immediately with anesthesia. Anesthetic solution was prepared by adding 1 g of MS 222 (Tricaine methanesulfonate) in one liter water.

In each exposure unit (15 cm x 20 cm x 10 cm=3000 cm^3) the test water was delivered from the reservoir tank (70 L) to the header tank by pump and then returned back to the reservoir tank by overflow in such a way that the water level was constant in the header tank. From the header tank a small water flow was led to the exposure box. Water returned to the reservoir tanks by gravitation from the outflow of the exposure boxes. Water was continuously aerated in the reservoir tank to avoid impact of high CO_2 levels. Water in the reservoir tank was changed twice during the experimental period, i.e., replacement at 70 and 250 degree days after hatching.

2.2 Water quality and fractionation of Cu

Temperature, pH, conductivity and O_2 were continuously logged with an automatic electronic logger Campbell CR200 in control water and measured weekly in each experimental water quality. Temperature and pH were also measured weekly by using WTW 340i equipped with SenTix® 41 glass electrode and conductivity with TetraCon® 325 conductivity probe. Optic probe WTW 4301 was used to measure oxygen (O_2) dissolved in water. Water CO_2 concentrations were analyzed using Phoenix 8000 UV-Persulfate TOC Analyzer (Tekmar Dohrman, Mason, OH, USA).

To obtain information of road salt and Cu concentrations in test solutions, water samples (15 mL) were collected three times from each test solution unit. Information of Cu associated with different size fractions were obtained by using membrane filtration (0.45µm) and ultrafiltration (Pall hollow fiber with cutoff 10 kDa) according to Teien *et al.* (2006).

The following size fractions of Cu were obtained:

- Total Cu, determined in unfiltered sample after acidification (10 % HNO_3)
- $Cu_{\text{particles}}$ = Cu retained by 0.45µm filter. Based on subtraction: Total Cu minus Cu in 0.45µm filtered samples, $Cu_{\text{total}}-Cu_{0.45\mu m\ \text{filtrate}}$
- $Cu_{\text{colloidal}}$ = Cu penetrating the 0.45µm filter and retained by 10 kDa ultramembrane. Subtraction: Cu in 0.45µm filtered samples minus Cu in ultrafiltered sample. $Cu_{0.45\mu m\ \text{filtrate}} - Cu_{\text{LMM (low molecular mass)}}$
- $Cu_{\text{Low molecular mass}}$ = Cu in ultrafiltered sample, Cu ≤10kDa

Information of cationic Cu species was obtained by using ion chromatography by using 30 mL of Chelex-100 resin (BioRad, Na-form, and 100-mesh size) contained in the glass column. The elution rate through the resin was 20 mL/min.

The following positively charged Cu fractions were obtained:

- Cu_{cationic}= Cu passing 0.45µm filter and retained in Chelex-100 resin. Subtraction: Cu in 0.45µm filtered sample (input to column) minus Cu in the cation exchange elute, $Cu_{\text{entering cation exchange column}} - Cu_{\text{leaving cation exchange column}}$
- LMM Cu_i= Cu passing the 10 kDa ultrafilter and retained in Chelex-100 resin. Subtraction: Cu in ultrafiltered sample (input to column) minus Cu in the cation exchange elute.

2.3 Chemical analysis

The concentrations of Cu and other major cations such as (Na) magnesium (Mg), potassium (K) and calcium (Ca) in acidified water and digested biological samples were determined by using Inductive Coupled Plasma Emission Spectroscopy (ICP-OES) (Perkin Elmer, Optima 5300 DV). Water samples were prepared for analysis by adding internal standard (Rh) and acidification using 10 % ultrapure nitric acid (HNO_3). Organic analyzer (Shimadzu TOC cpn) was used to determine total organic carbon (TOC) and major anions such as chloride (Cl^-), nitrate (NO_3^-) and sulfate (SO_4^{2-}) were analyzed by Iachat IC5000 Ion Chromatograph.

Ammonium (NH_4) test kit Spectroquant® was used to measure the concentration of NH_4 (ammonium) in treatment waters. Reagent NH_4-1 (0.60 mL) was added to five mL of the sample and both solutions were thoroughly mixed. One level blue micro spoon of reagent NH_4-2 was then added and the sample was shaken vigorously until the reagent was completely dissolved. After 5 min of interval, 4 drops of reagent NH_4-3 were added and mixed. After leaving for 5 min, the samples were added into the cell and measured by the spectrophotometer.

2.4 Biological analysis

To obtain information about the concentration of Cu in the head area (containing gills and sensory organs) of alevins, the alevins were dissected and the head was collected. Samples were stored into pre weighed Eppendorf tubes and frozen (-20 °C) before freeze drying. Samples were weighed and then digested by adding 1 mL concentrated HNO_3 before transferring into Teflon tubes, with further addition of 2 mL Milli-Q water and 50 µl internal standard before digestion in ultraclave (Milestone, Leutkirch, Germany). After digestion, the samples were diluted with Milli-Q water to the final volume of 10 mL and analyzed for Cu. Samples were analyzed on an Agilent 8800 QQQ ICP-MS in He mode. Cu was measured on both mass 63 and 65 with In (Indium) as internal standard. In addition standard reference material and blank samples were included for quality assurance. Metal concentration of tissues, expressed as µg/g d.w was calculated by following formula:

µg Cu/g dry weight = (A*V)/W, where A= µg/L of metals, V= dilution volume of sample (L) and W= dry weight of sample.

2.5 Biological endpoints

2.5.1 Growth and size of alevins

Every 7 to 10 days, alevins (n=10) were anesthetized (1 g Tricaine methanesulfonate/L) and the weight and total body length was measured.

2.5.2 Deformities

Alevins were inspected with regards to various deformities and the relative (%) number of alevins affected by deformities were calculated. All deformed individuals were photographed.

2.5.3 Swim-up

Alevins were daily inspected with regards to their swim-up ability. Swimming activities were only observed visually.

2.5.4 Alevin survival

Alevins were inspected daily and dead alevins were counted and removed. Alevins were considered dead, when part of the body turned white (protein coagulation) and no activity or movement detected.

2.6 Statistical analysis and data handling

Data was presented as mean±SD (standard deviation) based on the measurements throughout the experimental period. All statistical analysis was performed by using GraphPad prism 5.0 (Graphpad Software, Inc., San Diego, CA, USA). Survival analysis by using GraphPad prism 5.0 was performed to determine the relative (%) survival of the alevins followed by Log-rank (Mantel-Cox) test to study the effect of road salt and Cu on survival, and logrank test to identify the dose response trends in the different treatments. One way ANOVA followed by Tuckey's post hoc test was used to study the differences of water quality parameters and length and weight of alevins. Pearson correlation was performed to study the relationship between head-Cu concentration of alevins and LMM Cu in water. Paired t-test was used to study the significant differences of swim-up time between individual treatment and control. Data was tested for normal distribution and equal variances by using D'Agostino & Pearson omnibus normality test. Data not fulfilling the criteria of normality was $\log_{10}$ transformed prior to analysis. Significant differences or trends were defined by using the criteria $p=\leq 0.05$.

3 Results

3.1 Water chemistry

The mean water temperature in the different test solutions (treatments) ranged from 9.6-11 °C and was within ±2 °C of nominal temperature during the experimental period (Table 1). Thus, no significant difference in temperature ($p=0.2$), dissolved O_2 ($p=0.6$), CO_2 ($p=0.08$) was found between different treatments throughout the experimental period. A significant increase in water pH ($p<0.0001$) was, however, observed from start to the end of the experiment (6.7-7.3). The concentration of NH_4 measured at the end of the experiment ranged within 1.8-3.7 mg/L in the different treatments (Table 1). The concentration of TOC and NO_3 remained unaffected by road salt and Cu addition, while the concentration of Cl ($p=0.0004$, $r^2= 0.73$) and SO_4 ($p<0.01$, $r^2= 0.71$) increased linearly with the road salt addition. The mean concentration of total Cu was 2.0±0.1 µg/L in control water and increased to 21 µg/L due to the addition of nominal 20 µg Cu/L (Table 2). Cu was mainly present as LMM Cu species (33-52 %) in Cu test solutions and (40-74 %) in the mixed test solutions (road salt and Cu mixtures). In addition, the concentration of particulate Cu (0-18 %) was modest in all treatments. The fraction of colloidal species ranged within (0-42 %), while positively charged Cu species ranged from 0-40 % (Table 3).

3.2 Cu concentration associated with the head of alevins

Limit of detection (LOD) determined by taking the sum of the average and three times the standard deviation of 10 blanks for Cu was 0.2 µg/L.

The concentration of Cu in the head of the alevins was dependent upon the Cu concentration in the exposure water (Fig. 1). Linear correlation ($p=0.02$, $r^2= 0.36$) was observed between head-Cu concentration of alevins and the concentration of LMM Cu species in water (Fig. 2)

The head-Cu concentration was significantly higher in test solutions with added Cu (10 and 20 µg/L), and also in the road salt and Cu admixture. However, the highest head-Cu concentration (9.2 and 8.1 µg/g d.w. head) was observed in mixed exposures (1000 mg road salt/L mixed with 5 or 10 µg Cu/L) compared to control (5.8 µg/g d.w. head). There was a significant difference between head-Cu concentration of alevins exposed to road salt and mixtures of road salt and Cu ($p=0.04$).

3.3 Growth

Compared to control, *Atlantic salmon alevins* suffered reduced growth when exposed to all tested road salt concentrations (i.e., 100, 500 and 1000 mg road salt/L), as seen in Fig. 3. The growth was at its lowest when alevins were exposed to 1000 mg road salt/L, where the average weight of alevins after 470 degree days of chronic exposure was 0.153±0.01 g and standard length was 2.24±0.11 cm compared to 0.149±0.01 and 2.71±0.10 in control, respectively. Exposure to Cu did not affect length ($p=0.6$) and weight ($p=0.3$) of alevins compared to control. However, the exposure of alevins to the mixture of road salt and Cu resulted in decreased length ($p<0.0001$) and increase weight ($p=0.01$) compared to control. Alevins exposed to all road salt concentrations with and without Cu exhibited shorter length and more weight (less yolk utilization) compared to control (Fig. 4).

3.4 Morphological abnormalities

Approximately (5-18 %) of the alevins (Table 3) exhibited scoliosis during the first three weeks post hatch (210 degree days), when exposed to 100, 500 and 1000 mg road salt/L, respectively (Fig. 5). No abnormalities were observed in the control group. In addition, blue sac disease was observed in alevins, which is characterized by subcutaneous yolk sac edema (accumulation of fluid between the yolk sac and perivitelline membrane). At two weeks post hatch (140 degree days) the prevalence of yolk sac edema indicated an exposure threshold value of between 100 and 1000 mg road salt/L. Exposure to Cu did not result in formation of deformities in exposed alevins. Exposure to road salt and Cu mixtures, however, resulted also in scoliosis, yolk sac edema and blue sac disease.

3.5 Swim-up

Alevins swim-up was observed at approximately 400 degree days after hatching in the control group. Exposure to road salt concentrations (100, 500 and 1000 mg/L) resulted in delayed swim-up ($p=0.02$, 0.001 and 0.04) compared to control (Fig. 6 A). Reduced swimming activity was observed in alevins exposed to all road salt concentrations compared to control, and most of these alevins were found lying on their sides at the bottom of the exposure boxes. No effect of Cu on swim-up was observed ($p=0.6$) compared to the control group. For alevins exposed to the mixtures of road salt and Cu, the swim-up was significantly delayed ($p=0.03$) as shown in Fig. 6 C.

3.6 Survival

In the control group mortality was very low (1 %) compared to all other treatments (Fig. 7). Alevin mortality started at 180 degree days and increased over time for alevins exposed to all road salt test solutions. Alevin survival decreased significantly (high mortality) by increasing the road salt addition (Fig. 7 A). Survival rates were significantly different from each other in all treatments ($p<0.01$). Lowest survival (54 %) was observed at 1000 mg road salt/L. The dose-response relationship between alevin mortality and road salt concentrations was significant ($p<0.01$).

No significant mortality was observed in exposures with Cu compared to control ($p=0.3$). In addition, no dose-response relationship between any treatment with Cu and mortality, compared to control, could be found ($p=0.3$) (Fig. 7 B). In the mixtures of road salt and Cu (Fig. 7 C), however, the survival was affected, as observed in exposure of alevins to road salt alone. Lowest relative survival (20 %) was found in mixture of 1000 road salt mg/L + 5 and 10 µg Cu/L. In road salt and Cu mixtures, survival rates were significantly lower than observed in all other exposures ($p<0.01$).

4 Discussion

4.1 Water quality and Cu bioaccumulation

Water quality (pH 6.8-7.0 and temperature 9.6-11ºC, on average) was relatively stable throughout the experiment and was considered suitable for efficient growth of alevins, in accordance with recommended levels for the growth of Atlantic salmon *alevins* (Marr, 1966; OECD, 2013). Road salt addition did not cause any change in pH or Cu concentrations, but increased the concentration of major cations (Na, Ca, K and Mg) and anions (Cl and SO_4) that could affect the speciation of trace metals such as Cu (Mahrosh *et al.*, 2013). Increased concentrations of Ca and K can be toxic towards fish (Stekoll *et al.*, 2009). In addition, elevated concentrations of K in exposure water were toxic to early developmental stages of Atlantic salmon (Peterson *et al.*, 1988). The total Cu concentrations in road salt solutions ranged from 1.9-2.5 µg/L and ranged from 5.5 to 9.6 µg/L in road salt and Cu mixtures, close to the nominal concentrations. The Cu concentrations remained about constant throughout the experiment. In the control water (pH 6.9) the total Cu concentration was 2.0 µg/L, where a major fraction was associated with particles larger than 0.45 µm and only a small fraction (10 %) was present as

LMM Cu species. The control water contained also 5 mg TOC/L, which probably indicates that particulate and colloidal Cu were associated with organics. In road salt and Cu mixtures, however, the LMM Cu was about 73 % of the total Cu concentration, probably due mobilization of Cu from surfaces at high concentration of Na (Amrhein *et al.*, 1992: Backstrom *et al.*, 2004). Thus, a larger fraction of Cu was present as LMM species, assumed to be bioavailable, in road salt test solutions compared with control.

The concentration of Cu in the head of exposed alevins ranged from 5.8±1.1 (control) to 9.8±2.5 µg/g in road salt and Cu mixtures. When exposed to road salt, the concentration of Cu in the head of alevins increased with increasing road salt concentrations. The increased Cu uptake in alevins exposed to road salt is attributed to the elevated fraction of LMM Cu species (mobilization), compared to control where the LMM Cu fraction was low.

4.2 Effects of road salt on alevins

Chronic exposure of Atlantic salmon alevins to road salt from hatching to swim-up reduced the growth and the rate of yolk utilization, delayed the time of swim-up, reduced the swim activity, and reduced the survival (increased mortality). Thus, the results demonstrated that the use of road salt could have a negative effect on the development and survival of alevins. The effects were pronounced, when alevins were exposed to all road salt levels tested (100-1000 mg/L) compared to control.

Fish larvae growth is a continuous process and it depends on various environmental factors such as photoperiod (Boeuf and Le Bail, 1999), temperature, salinity and pH, etc. Lam and Sharma (1985) reported increasing rates of growth, development and larval survival in *Cyprinus carpio* at increasing salinities (0-3000 mg/L). High salinity levels may increase the fish growth by creating iso-osmotic conditions (Boeuf and Payan, 2001; Lam and Sharma, 1985). Growth of alevins in the present experiment decreased, however, when exposed to road salt concentrations (100, 500 and 1000 mg/L). The reduction in growth rate can directly be linked with reduced swim activity and the size of yolk sac. Reduced growth could be associated with lower rates of utilization of yolk sac nutrient stores and slower yolk sac absorption, which gives more weight and reduced length to exposed alevins. Yolk provides the main source of nutrients to early larval stages of fish and yolk material is subdivided to provide for growth and metabolism (Kamler *et al.*, 1998). Slow rate of yolk sac resorption was observed in alevins exposed to all tested road salt concentrations (100, 500 and 1000 mg/L). Compared to the control, most of the alevins exposed to road salt had not resorbed their yolk sac completely.

Furthermore, the continuous exposure to road salt solutions significantly increased the abnormalities in alevins. Spinal cord flexure and scoliosis were the main physical abnormalities observed, and the response occurred at all road salt concentrations tested (100, 500 and 1000 mg/L). In addition, utilized yolk sac was replaced by edematous fluid giving rise to the condition called yolk sac edema. Abnormalities associated with yolk sac edema and severe fluid accumulation in the yolk sac were also observed by Spitsbergen *et al.* (1991), after exposing Lake trout (*Salvelinus namaycush* to 2,3,7,8-tetrachlorodibenzo-p-dioxin as fertilized eggs. It is also suggested that such alevins could starve to death because yolk reserves were not utilized, as observed by Billiard *et al.* (1999) in Rainbow trout exposed to retene, which is a polycyclic aromatic hydrocarbon. Road salts may also cause yolk sac coagulation, which could result in the reduction of yolk utilization as observed by Stouthart *et al.* (1996) in study of Cu toxicity towards common carp. Exposure to road salt could also induce oxidative stress to alevins, which may deplete the antioxidants contained in the yolk sac (Billiard *et al.*, 1999). Accordingly, Vosyliene *et al.* (2006) also observed reduced erythrocyte count and increased hematocrit in Rainbow trout after exposure to road salt concentration of 180 mg/L, which indicates that even low concentrations of road salt might have substantial effects on fish.

Following the continuous exposure to road salt solutions (100–1000 mg/L), the swim-up was delayed (Fig. 6), compared to control. Reduced swim activity was also observed when compared to control, most apparent at high road salt concentrations. Reduced swim activity of alevins may have several ecological implications as such alevins would be more susceptible to predation as suggested for frog tadpoles exposed to road salt (Alford, 1999). Na and Cl ions are also assumed to affect the muscular activity (Hill and Wyse, 1988) which may also affect the swimming behavior of Atlantic salmon alevins.

Chronic exposure of alevins to road salt after hatching (100-1000 mg/L) reduced the survival (high mortality) of *Atlantic salmon* alevins significantly ($p<0.01$). Thus, road salt exposure resulted in lethal effects on *Atlantic salmon* alevins at environmentally realistic concentrations (Environment Canada, 2001). Chronic exposures to low levels of road salt may result in change in populations (Sanzo and Hecnar, 2006). Stekoll *et al.* (2009) also observed high salmonid alevins mortality after exposure to 2500 mg total dissolved salt/L during hatching. Furthermore, high mortality of Atlantic salmon eggs was observed by exposing eggs to rather high road salt concentrations (5000 and 10,000 mg/L) during fertilization (Mahrosh *et al.*, 2014). The alevins mortality at 100 mg road salt/L indicates that effects could be significant even at levels lower than set by the guidelines from USEPA (1988) (≥230 mg Cl/L). In addition to the toxicity of Cl ions potaasium ferrocyanide present as anticaking agent in the road deicing salt can also pose toxicity towards fish (Vosyline *et al.*, 2006).

4.3 Effects of Cu

When fish in freshwaters are exposed to Cu, it may exert negative effects by elevating plasma ammonia concentrations and also by disrupting the ion regulation via the inhibition of Na^+/K^+ adenosine triphosphatase (Na^+/K^+ ATPase) in the gill (Lauren and McDonald 1987a,b). However, the present results demonstrated that the growth, time of swim-up and survival of Atlantic salmon alevins was not affected by any of the Cu concentration tested (5, 10 and 20 µg/L). The absence of negative effects in Cu exposed alevins is attributed to Cu speciation being essential for biological uptake, as only 20 % of the total Cu was present as positively charged LMM Cu species, being potentially bioavailable.

Although the uptake of Cu increased with increasing LMM Cu concentrations in water, toxic levels were never reached. Thus, the results demonstrated that concentrations of LMM Cu in water up to 11±8.0 µg Cu/L and accumulating 9.8±2.5 µg Cu/g d.w. in the head of alevins did not cause any effects on development or survival of alevins. Chapman (1978) has reported that the LC_{50} values (96-h) for Cu for Chinook salmon ranged from 17-38 µg/L, *i.e*., higher than the highest concentration (11 µg/L) of LMM Cu in the present experiment. Although the test solutions were categorized as soft water (OECD, 2013), the relative high pH in combination with TOC (5.1-5.2 mg/L) are assumed to be the main factors limiting the bioavailability (Andrew et al, 1977; Meador, 1991), uptake and effects of the Cu exposures.

4.4 Effects of the road salt and Cu mixtures

In mixtures of road salt and Cu, the LMM Cu fraction in the exposures increased (Mahrosh *et al.*, 2013) probably by mobilization from organic matter and colloids (Amrhein and Strong, 1990), hence increasing the Cu uptake in the head of alevins compared to control. By adding 10 µg Cu/L to road salt (1000 mg/L), the head-Cu concentration of alevins increased from 6.4 to 8.1 µg/g d.w. Chronic exposure of Atlantic salmon alevins to the road salt and Cu mixtures from the time of hatching until swim-up reduced the larval growth and rate of yolk utilization, delayed the swim-up and the swim activity, as well as decreased the survival. Reduced larval growth, inhibition of yolk utilization and delayed swim-up was also observed at road salt concentrations lower than 1000 mg road salt/L mixed with Cu, compared to alevins exposed to road salt solutions only. Thus, the results demonstrated adverse and toxic effects in alevins exposed to road salt separately and the effects were more pronounced when Cu was added.

In addition, morphological abnormalities were observed at 500 and 1000 mg road salt/L mixed with 5 and 10 µg Cu/L. Mahrosh *et al.* (2013) also reported various deformities, when Atlantic salmon eggs were exposed to the mixture of road salt and Cu during fertilization and swelling. Kazlauskiene & Vosyliene (2008) observed high mortality of hatching embryos and changes in gill ventilation frequency of Brown Trout alevins in exposure to the mixture of Cu and Zn.

Although the uptake of Cu in the head of alevins increased by addition of Cu in road salt solutions, results demonstrated that the main effects should be attributed to the road salt and not to Cu. The increasing Cu uptake in the head of fish exposed to road salt concentrations increasing up to 1000 mg/L indicated changes in the toxicokinetic of Cu in alevins; either by increased bioavailability and uptake of LMM Cu species from water, by inhibition/reduction of uptake due to cation competition (Grossel and Wood, 2002) or by physiological changes caused by road salt. Higher Cu concentrations should be included in future tests to demonstrate underlying and interacting effects.

High salt concentrations can also reduce the Cu-induced toxicity as observed by Blanchard and Grosell (2006) in exposure of euryhaline fish *Fundulus* heteroclitus to the mixture of Cu (30 and 150 µg/L) at higher salinity levels (5000, 11,000 and 22,000 mg salt/L), where no mortality was observed. They also suggested that Cu did not disrupt the ionregulation in fish at high salinities. Reduced growth, increase in deformities and reduced survival have, however, important ecological implications for Atlantic salmon population, since these factors can change the population structure and may result in small population size (Akcakaya *et al.*, 1999), which can further result in extinction (Soule, 1987).

Different growth rates in relation to the swim-up time reflects the effects at later developmental stages of Atlantic salmon. Length of larvae at swim-up stage was dependent on the different exposure solutions. Fish reaching the swim-up stage later than normally expected may experience problems in further growth and survival, as proper feed may be difficult to reach (e.g., too large insects) . If environmental conditions are suboptimal or not suitable for the growth and survival, alevins could also be more prone to predation (Stekoll *et al.*, 2009).

The survival of alevins exposed to road salt mixed with Cu decreased with increased concentration of road salt until 1000 mg/L. The survival was at its lowest (highest mortality) when alevins were exposed to mixtures (1000 mg road salt/L and Cu). Compared to separate road salt exposures the survival decreased from 40 % to 80 %, when Cu was mixed in the exposure solutions. Thus, the results demonstrated that the use of road salt could have significant negative effects on the development and survival of alevins, and that the effects will be more pronounced when metals are mobilized in the surface run-off.

5 Conclusion

The present work demonstrates that the application of road salt as a deicing agent of roads during winter may have ecological consequences. Exposure to road salt (100-1000 mg/L) resulted in lower growth and reduced yolk sac resorption, delayed swim-up, deformities and relatively high mortality of Atlantic salmon alevins. The effects increased with the road salt concentrations, *i.e.*, concentrations of Na, K Ca, Mg, and Cl.

High road salt concentrations mobilized bioavailable species such as cationic LMM Cu, and the uptake of Cu in the head of alevins increased with increasing concentration of LMM Cu species.

Under real situations a series of metals could be mobilized by the high road salt concentrations, thus, the run-off during spring snow-melt could contain a soup of stressors; road salt, metals and hydrocarbons. Although no negative effects of Cu exposure on alevins could be observed in the present work, the mixed exposure (road salt and Cu) induced negative effects that were more pronounced than observed in corresponding road salt exposure experiments. Thus, additive or more than additive effects could be expected when real conditions are considered (Horne and Dunson, 1995).

In the present work, exposure experiments were performed with alevins being a sensitive life history stage of Atlantic salmon, an organisms of high commercial value. The biological effects observed; lower growth, delayed swim-up, deformities and relatively high mortality, are ecological relevant and can influence the population structure. As road salt concentrations far exceeding the tested road salt concentration (1000 mg/L) often are applied under real situations, more severe effects should also be expected. In accordance with the present results, demonstrating that the road salt application could seriously affect sensitive life stages of

Atlantic salmon, it is recommended that the application of road salt should be avoided during the late winter-early spring period.

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Table 1. Physical and chemical variables measured in exposure water during the experimental period (n=7).

Treatment	pH	Conductivity µs/cm	Temperature °C	O_2 mg/L	CO_2 mg/L	*NH_4 mg/L	TOC mg/L	Na mg/L	Ca mg/L	Mg mg/L	K mg/L	Cl^- mg/L	NO_3^{-1} mg/L	SO_4^{-2} mg/L
Control	6.9±0.3	45±16	9.9±0.4	12±0.0	1.5±0.7	1.8	5.1±0.2	4.3±0.5	3.3±0.5	1.9±0.2	3.1±0.1	3.6±0.2	1.9±1.1	2.5±0.3
100 mg road salt/L	6.9±0.4	305±40	10±0.2	11±0.0	1.0±1.0	1.5	4.9±0.3	54±3.1	3.6±0.2	1.2±0.4	4.1±1.2	77±14	1.4±1.2	2.8±0.4
500 mg road salt/L	7.0±0.2	1044±60	10±0.3	11±0.1	2.0±2.0	1.8	5.1±0.5	210±22	3.8±0.1	1.0±0.1	5.9±0.9	298±5	1.2±1.4	3.5±0.3
1000 mg road salt/L	7.0±0.2	2146±111	10±0.3	11±0.1	2.0±2.0	1.2	5.0±0.1	400±51	4.2±0.4	3.2±0.3	8.6±3.0	617±30	0.9±1.0	4.5±0.5
5 µg Cu/L	7.0±0.3	48±18	9.9±0.2	11±0.0	1.0±0.0	2.7	5.1±0.9	4.0±0.9	3.4±0.4	1.1±0.1	3.2±0.2	4.2±2.2	1.0±1.2	2.5±0.4
10 µg Cu/L	7.0±0.3	42±13	10±0.2	11±0.6	1.5±0.7	2.6	5.2±0.9	5.3±1.1	3.7±0.6	1.5±0.1	2.9±0.5	2.2±0.1	0.4±0.4	2.6±0.4
20 µg Cu/L	7.0±0.3	46±10	10±0.3	11±0.0	1.0±0.0	2.7	5.2±0.8	5.1±0.6	4.6±0.1	2.1±1.3	3.1±0.6	3.2±1.0	1.4±1.6	3.9±1.0
500 mg road salt/L +5 µg Cu/L	6.9±0.3	1069±58	10±0.3	11±0.0	2.0±0.7	2.1	5.3±0.3	213±24	3.8±0.5	1.1±0.2	6.1±1.4	307±8.5	1.4±1.7	3.6±0.4
500 mg road salt/L +10 µg Cu/L	7.0±0.3	1129±81	9.6±0.3	11±0.0	1.5±0.7	2.7	5.4±0.1	182±32	4.1±0.3	0.9±0.1	5.2±2.1	310±16	1.6±2.0	3.8±0.4
1000 mg road salt/L +5 µg Cu/L	6.8±0.1	2310±108	10±1.0	11±0.1	1.5±0.7	-	5.1±0.4	401±75	3.8±1.1	2.9±1.0	7.4±1.3	658±58	0.2±0.1	4.6±0.5
1000 mg road salt/L +10 µg Cu/L	7.0±0.3	2188±156	11±1.3	11±0.1	1.6±0.4	3.7	5.0±0.1	386±44	3.7±0.1	3.5±0.9	8.1±1.1	630±51	0.2±0.0	4.8±0.8

*Measured at the end of exposure

Table 2. Concentration of different Cu-fractions measured in exposure water (n=3)

Treatment	Total Cu µg/L	Particulate Cu µg/L	Colloidal Cu µg/L	LMM Cu µg/L	LMM $Cu_{cationic}$ µg/L
Control	2.0±0.1	1.9±1.0	0.2±0.1	0.2±0.2	<LD*
100 mg road salt/L	2.3±0.9	<LD	0.2±0.9	1.8±1.3	0.3±0.1
500 mg road salt/L	2.5±1.1	<LD	0.4±2.3	2.1±4.1	0.4±0.5
1000 mg road salt/L	2.3±0.1	<LD	<LD	1.6±2.3	0.3±0.4
5000 mg road salt/L	1.9±1.0	<LD	<LD	1.4±2.0	0.4±0.5
5 µg Cu/L	6.6±2.2	<LD	2.0±1.2	2.2±1.3	2.4±1.1
10 µg Cu/L	9.9±1.6	0.2±0.2	2.2±0.5	5.2±2.9	1.8±0.4
20 µg Cu/L	21±0.7	1.0±0.1	4.5±0.3	11±8.0	4.1±1.3
500 mg road salt/L + 5 µg Cu/L	7.3±1.3	<LD	2.0±0.0	3.7±0.0	1.4±0.1
500 mg road salt/L + 10 µg Cu/L	11±0.5	2.0±0.0	4.0±1.0	4.5±0.5	4.0±0.1
1000 mg road salt/L + 5 µg Cu/L	5.5±2.5	0.6±0.8	0.2±0.2	4.1±0.2	1.2±0.9
1000 mg road salt/L + 10 µg Cu/L	9.6±0.8	<LD	4.1±0.1	3.9±0.3	1.4±0.4

*Limit of detection

Table 3. Deformities (%) of Atlantic salmon alevins after exposure to different concentrations of road salt with and without Cu in chronic exposure from hatching till swim-up

Nominal road salt concentration (mg/L)	Control	100	500	1000	500	500	1000	1000
Nominal Cu concentration (µg/L)	Control	0	0	0	5	10	5	10
Deformity types								
Scoliosis	0	5.4	10	18	5	3.4	15	7
Conjoined twins	0	0.5	-	-	-	-	-	-
Yolk sac edema	0	4.3	5.4	5.3	0.8	-	10	1.0
Coiled tail	0	-	-	2.7	-	0.5	-	0.5
Deformed yolk sac	0	-	-	0.9	1.1	0.5	-	0.5
Total	0	10	15	27	6.9	4.4	25	9

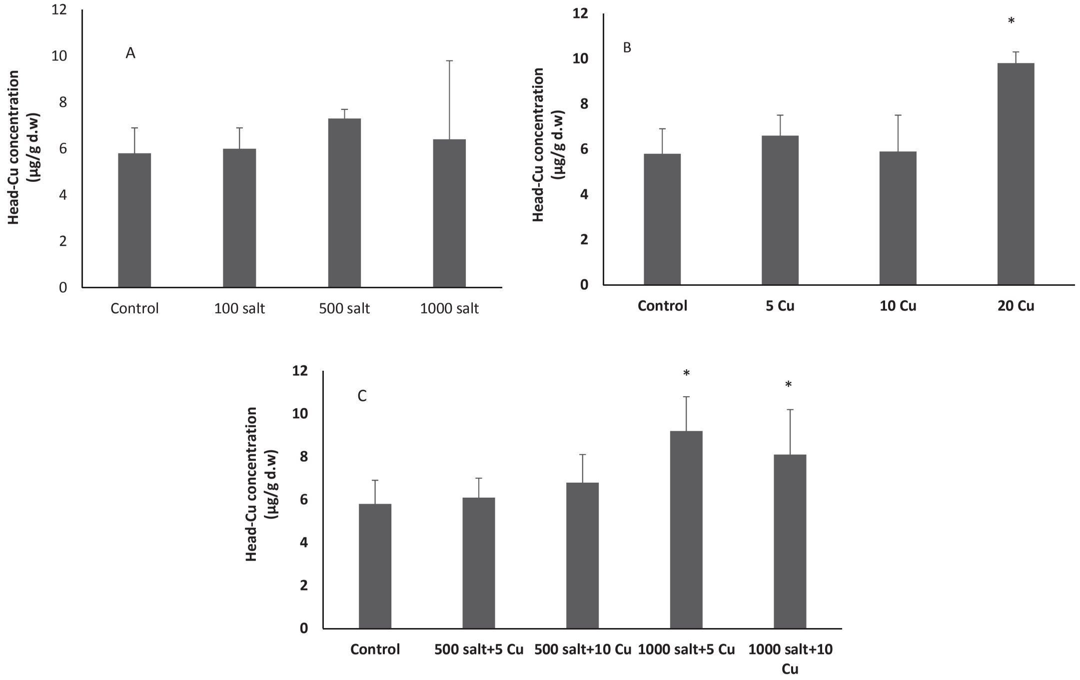

Figure 1. Cu concentration in the heads of Atlantic salmon alevins exposed to (A) road salt (B) Cu and (C) road salt and Cu admixture. In exposure solutions the concentrations of road salt are given in mg/L, while the concentrations of Cu are given in µg/L.

*Significant different from control.

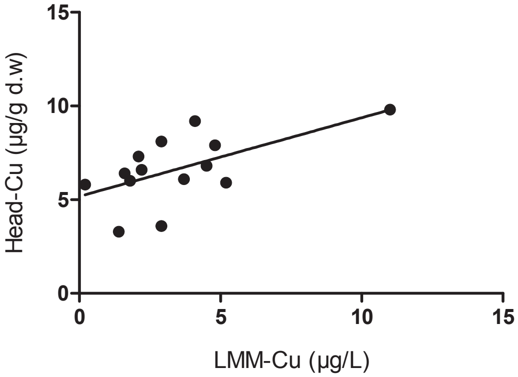

Figure 2. Relationship between LMM Cu and Head-Cu concentration in different treatments.

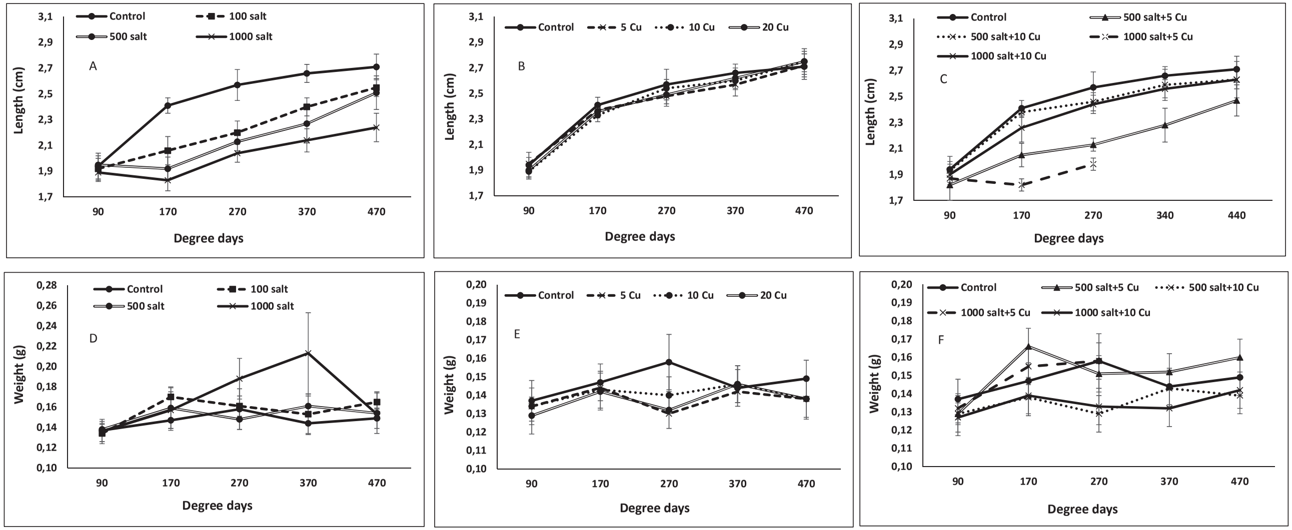

Figure 3. The length (A, B, C) and weight (D, E, F) of surviving Atlantic salmon alevins during chronic exposure to road salt and Cu separately and in admixtures. The concentrations of road salt are given in mg/L while the concentrations of Cu are given in µg/L.

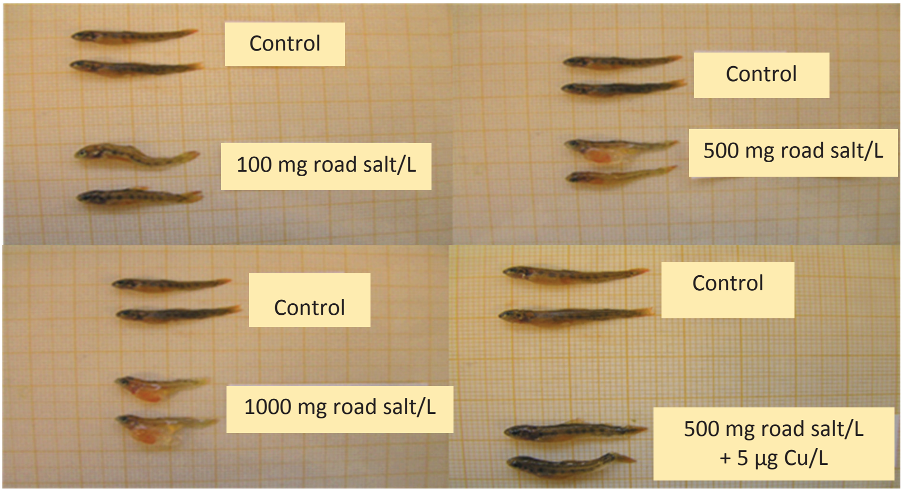

Figure 4. Atlantic salmon alevins at 400 degree days chronic exposed to road salt with and without Cu (Photo: Urma Mahrosh).

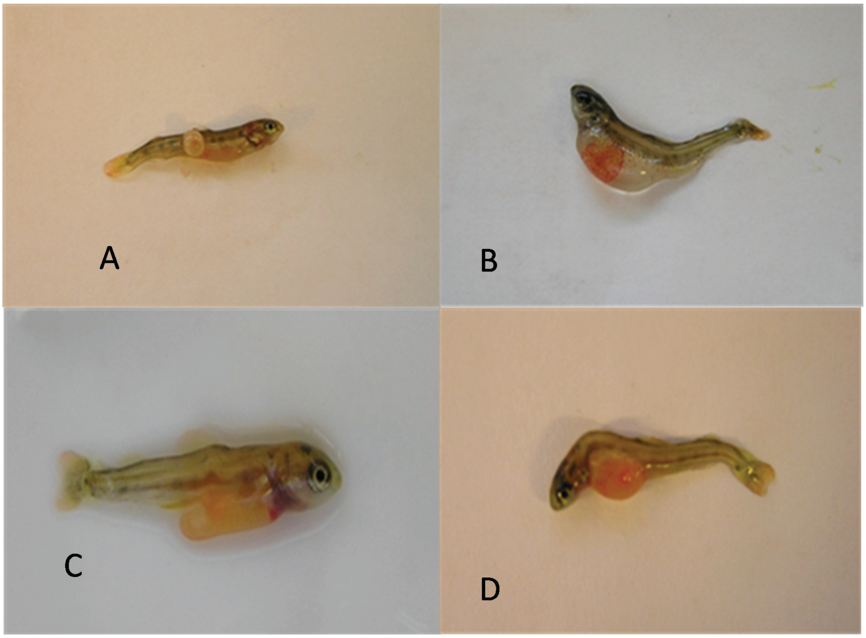

Figure 5. Different types of deformities observed in Atlantic salmon alevins due to exposure of road salt and Cu. (A, B and C) Blue sac disease and (D) Scoliosis. Photos: Urma Mahrosh

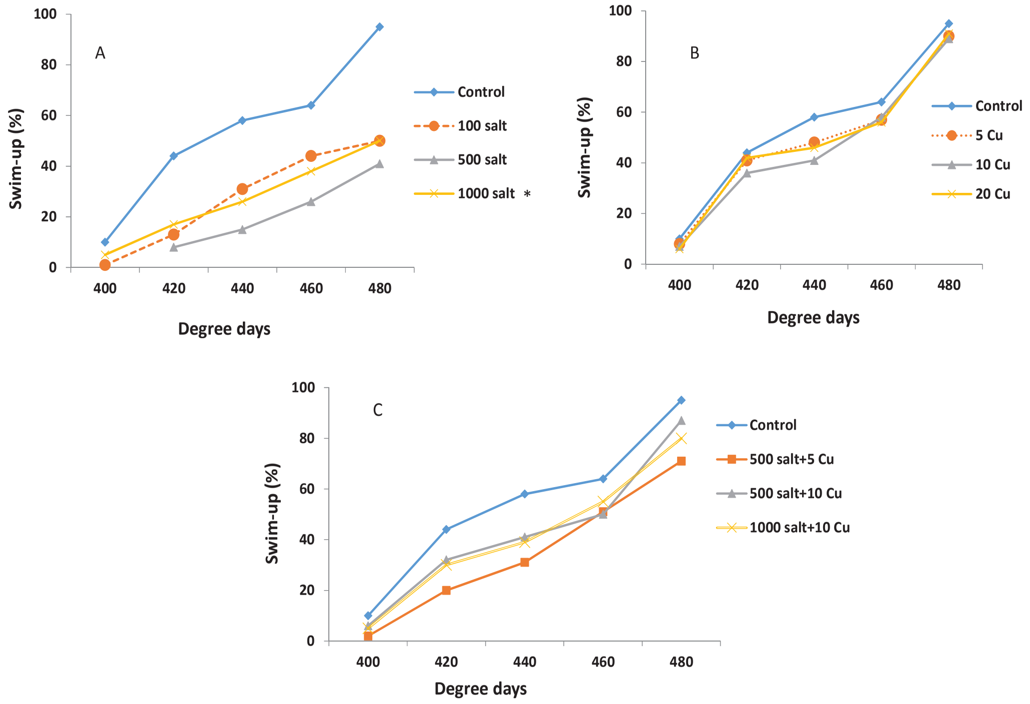

Figure 6. Time at swim-up (400-440 degree days) of Atlantic salmon alevins after chronic exposure of (A) road salt (B) Cu and (C) mixtures of road salt and Cu. The concentrations of road salt are given in mg/L while the concentrations of Cu are given in µg/L.

*Significant different from control

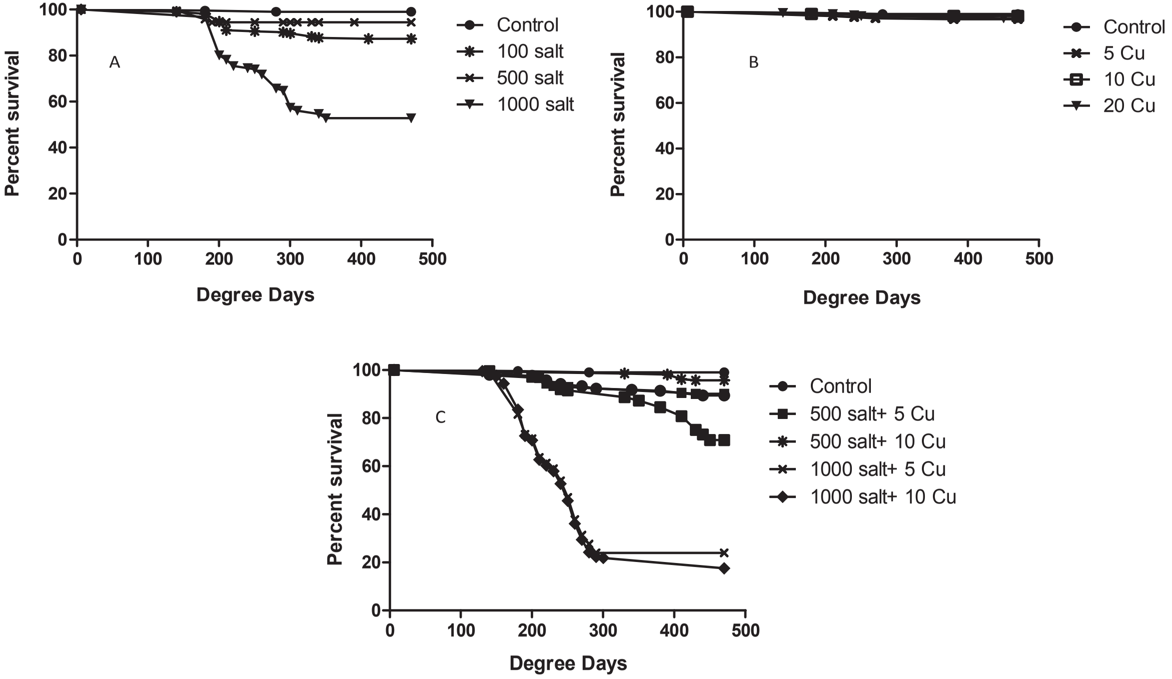

Figure 7. Percentage of surviving Atlantic salmon alevins during chronic exposure from hatching until swim-up of (A) road salt (B) Cu and (C) road salt and Cu admixtures. The concentrations of road salt are given in mg/L while the concentrations of Cu are given in µg/L.