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Trace Metal Speciation and Uptake in Atlantic Salmon (*Salmo salar*) in Coastal Water

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Acknowledgments

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Abstract

Estuarine environments have a high risk of metal pollution due to the influx from rivers. Freshwater from rivers mixed with seawater in coastal areas gives varied and complex water chemistry. In the estuarine environment of Kaldvellfjorden in Norway were the metals copper (Cu), zinc (Zn) and aluminum (Al) classified to have a high risk of impact. If these metals are bioavailable, based on their speciation, fish can accumulate the metals in tissue. To improve the knowledge of bioavailability and toxicity of metals in coastal waters are the main objectives of this thesis to identify the uptake of trace metals in fish in coastal water.

Atlantic salmon smolts (*Salmo salar*) were exposed to waterborne Cu, Zn, or Al in brackish water (20‰, pH 8) for 96-hours. The exposures were conducted with nine concentrations of Cu and Zn, and six concentrations of Al. Several of the concentrations were the same, to directly compare the uptake between the metals. To investigate the uptake in fish; gills, kidneys, and livers were sampled at the end of the exposure. Metal concentrations were quantified using Inductively Coupled Plasma Mass spectrometry (ICP-MS). Water samples of the exposure water were size- and charge- fractionated *in-situ* at 0h and 96h, and analyzed later using ICP-MS.

The results of size fractionation revealed that speciation of the Cu, Zn, and Al was relatively similar in brackish water, and was assumed to be bioavailable. Aluminum was found associated with the low molecular mass (LMM, <10kDa) fraction at >83% of the total, LMM-associated Zn at >78% of the total, while Cu had large uncertainties in this fraction. Copper was either associated with the colloidal or LMM fraction. Only a small percentage, <14%, of the metals were associated with particles. This low association is likely due to low organic content in the exposure water.

The uptake of metals on fish followed the order, Al>Cu>Zn, where gills were the tissue with the highest uptake, kidneys intermediate and the liver had no uptake within 96 hours of exposure. Significant uptake ($p<0.05$) was only found in the gills for Cu at high exposure concentrations, and for Al in the gills. No uptake was found for Zn. High concentrations of calcium (Ca) and other major ions in the exposure water likely acted as a competing compound and such a protective agent against the uptake of the metals. Based on the results gained from the present study there is no risk of uptake of Zn, and there is some risk associated with the uptake on gills at high concentrations of Cu and Al in estuarine waters at

20‰ salinity. Further studies are needed to look at chronic exposure to verify if the concentrations stay low in the tissue. Also, further studies with lower salinity in the exposure water are needed to investigate the effect of competing ions.

Sammendrag

Estuarine områder har en høy risiko for forurensing av metall fra elveløp i nærområdet. Når ferskvann fra elver møter sjøvann i kystområder gir dette en varierende og kompleks vannkjemi. I det estuarine området av Kaldvellfjorden i Norge ble metallene kopper (Cu), sink (Zn) og aluminium (Al) klassifisert som å ha høy risiko for påvirkning. Om disse metallene er biotilgjengelige, basert på deres tilstandsformer, kan fisk akkumulere metall i deres organer. For å forbedre kunnskapen om biotilgjengelighet og toksisitet av metall i kystvann er hovedmålene ved denne avhandlingen å identifisere opptak av spormetaller i fisk i kystvann.

Laksesmolt (*Salmo salar*) ble eksponert til vannbåren Cu, Zn eller Al i brakkvann (20‰, pH 8) i 96 timer. Eksponeringene var gjennomført med ni konsentrasjoner av Cu og Zn og seks konsentrasjoner av Al. Flere av konsentrasjonene var like for de tre metallene for å kunne sammenligne de direkte mot opptaket. For å undersøke opptaket i fisk ble gjeller, nyre og lever fra eksponert fisk prøvetatt ved endt eksponering. Metallene i fisk ble kvantifisert av Induktiv koblet plasmamasspektrometri (ICP-MS). I tillegg, ble vannprøver av eksponeringsvannet størrelse- og ladnings- fraksjonert *in-situ* ved start og slutt av eksponering, og analysert senere på ICP-MS.

Resultatene av størrelsefraksjonering avslørte at tilstandsformene av Cu, Zn og Al var relativt like i brakkvann, og ble antatt biotilgjengelige. Aluminium var assosiert med lav molekylær masse (LMM, <10kDa) fraksjonen ved >83% av totalt, LMM-assosiert Zn med >40%, mens Cu hadde høye variasjoner knyttet til denne fraksjonen. Kopper ble regnet som å være assosiert med enten kolloider eller LMM-fraksjonen. Bare en liten prosentandel av metallene var assosiert med partikler. Denne lave assosiasjonen er trolig på grunn av en lav konsentrasjon av organisk materiale i vannet.

Opptaket av metall i fisk fulgte rekkefølgen Al>Cu>Zn, hvor gjellene var organet med høyest opptak, nyrer var mellomliggende og lever hadde lavest opptak. Et signifikant opptak ($p<0.05$) var kun oppdaget for Cu ved høye konsentrasjoner, og for Al i gjeller. Det ble ikke oppdaget opptak av Zn. Kalsium (Ca) i vannet har trolig virket som en beskyttende agent mot opptak av metall hos fisk. Basert på resultatene fra denne avhandlingen er det noe risiko for opptak av Cu og Al i fisk ved høye konsentrasjoner i estuarine områder. Videre studier bør inkludere kronisk opptak for å redegjøre for at konsentrasjonen av metaller holder seg lavt i

organer. I tillegg, vil studier utført med lavere salinitet kunne undersøke effekten av konkurrende ioner i vannet.

List of abbreviations

Al - Aluminum

°C – Degree Celsius

Ca - Calcium

CF – Concentration factor

CRM – Certified reference material

Cu - Copper

DOC – Dissolved organic carbon

DOM – Dissolved organic material

dw – Dry weight

FW – Freshwater

HMM – High molecular mass

HNO_3 – Nitric acid

ICP-MS – Inductively coupled plasma mass spectrometry

In-situ – At site

K – Potassium

kDa – kilo Dalton

LMM – Low molecular mass

LOD – Limit of detection

LOQ – Limit of quantification

Mg – Magnesium

Na – Sodium

NaOH – Sodium Hydroxide

NMBU – Norwegian University of Life Sciences

NOK – Norwegian kroner

OECD - Organization for Economic Co-operation and Development

pH – logarithmic scale expressing acidity of a solution ($pH = -\log10^{[H+]}$)

RSD – Relative standard deviation

SW – Seawater

Zn – Zinc

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1 Introduction and background

Metal pollution in the aquatic environment is not a new phenomenon. It has been a viable problem for centuries, both from natural and anthropogenic sources. These sources being for example mining, wastewater treatment, or natural leaching from bedrock (Wood et al., 2012a). The aquatic systems include freshwater systems such as lakes and rivers, seawater, and brackish waters such as estuaries. These systems exhibit different chemistry and speciation. Speciation is used to evaluate if the metals are bioavailable and toxic to aquatic life. Even though the oceans keep uniform chemistry, estuaries are prone to complex chemistry due to the mixing of seawater and rivers (Day et al., 1989).

The concentration of cations, pH, salinity, and organic material in estuaries vary considerably. For example, salinity in coastal brackish water can range from 0.5-17 ‰ (Fondriest Environmental Inc, 2014). Estuaries and brackish water are often exposed to varied content of organic matter and different ion concentrations (Day et al., 1989). All that makes the speciation more complex. If the metals are bioavailable then aquatic organisms, such as fish, may accumulate the metals in tissue and cause harmful effects. The effects of metals on freshwater fish have been extensively studied, while the effects of metals in coastal waters have not gained the same amount of attention. This is linked to documented results of lower toxicity of metals in seawater compared to freshwater (Wheeler et al., 2002; Wood et al., 2012a). There is a need for further studies on metal uptake on fish in coastal and estuarine environments.

Kaldvellfjorden is a fjord located between the municipalities Lillesand and Grimstad in the county Agder, south in Norway. After the road construction of the European route, 18 (E18) elevated concentrations of trace metals were found in the acidic drainage from the rock landfill, M15/M16. The drainage entered the fjord through the tributary Stordalsbekken. A water treatment plant is situated by Stordalsbekken to treat the acidic drainage with NaOH, to avoid the input of metals to the fjord (Teien et al., 2017).

Analyses of water samples demonstrated a high concentration of trace elements in downstream tributaries in the following order Al>Mn>Fe>Ce>Ni>La>Nd>U>Ge>Cu, before

dilution in the coastal water (Teien et al., 2017). The exposure data from the sites monitored was subjected to a component-based Cumulative Risk Assessment (CRA) using the NIVA Risk Assessment database to predict site-specific impacts (NIVA, 2017). The risk for acute effects of these multi-component mixtures in fish was driven by a selection of metals, where zinc (Zn), aluminum (Al), and copper (Cu) were among the five assumed to have the most impact.

1.1 Aims of the study

To improve the knowledge of bioavailability and toxicity of metals in coastal waters are the main objectives of this thesis to identify the uptake of trace metals in fish in coastal water. The following hypothesizes for the study were set;

H_0: There is a difference in the speciation of copper, zinc, and aluminum in the water

H_1: The trace metals copper, zinc, or aluminum in coastal water can be taken up in fish.

$H_{2:}$ There is a difference in uptake of copper, zinc, and aluminum in different tissues.

2 Theory

2.1 Metal speciation in aquatic systems

2.1.1 Properties of natural waters

The different aquatic systems, freshwater, seawater, and brackish water all exhibit complex chemistry. This is due to a varying mixture of inorganic ligands and organic ligands. These systems are also in contact with soils and sediments which influence water chemistry.

Freshwater chemistry is connected to the geology and climate of the area and therefore vary not only with geographic areas but also seasons (Ellwood, 2004). Among the properties influencing is pH. In surface waters, such as rivers and lakes can pH range from 5-9 (Nikanorov & Brazhnikova, 2009). Cation concentrations are also influenced by the geology and climate found for the aquatic systems. Freshwater systems, in general, have lower

concentrations of cations than seawater (Day et al., 1989; Gibbs, 1970). The level of cations and anions influence the salinity of the systems. The salinity of freshwater is low (<5 ‰) as freshwater also has low concentrations of salts (Fondriest Environmental Inc, 2014). Another property influenced by the surrounding area of the aquatic systems is the level of organic material or organic carbon. Organic material consists of humic macromolecules that are acidic and complexes with metals (NORDTEST, 2003).

In comparison to freshwater, the general properties; pH, cations, salinity, and organic material are stable in seawater. The pH of seawater is reported at approximately 8 (Marion et al., 2011). The concentrations of cations and anions; chloride (Cl), sodium (Na), magnesium (Mg), calcium (Ca) and potassium (K), are high in comparison to the freshwater systems (Day et al., 1989; Duxbury et al., 2018; Gibbs, 1970). Due to these high concentrations of salt is the salinity of seawater high. The average salinity for the ocean is 35 ‰ (Duxbury et al., 2018; Fondriest Environmental Inc, 2014). Lastly, the level of dissolved organic carbon in the ocean is only a quarter of the input from the rivers, which is likely due to dilution or processes that destroy the organic carbon (Hedges, 1987). The content of organic material in seawater is only relevant for the uppermost part of the ocean and is generally not a concern for the lowermost parts (Duxbury et al., 2018).

The mixing of freshwater and seawater leads to different chemistry with different speciation for metals. The properties that influence estuarine or brackish water are high concentration of organic matter, major ion concentration, alkalinity, salinity, and pH (Wood et al., 2012a). With the input of freshwater to seawater can this reduce surface salinity from >20 to >10‰ (Bjerknes et al., 2003). Additionally, the mixing of freshwater and seawater is the influx from rivers important for coastal speciation.

Rivers input silt, clay, or colloidal humic acids to coastal systems. These components are negatively charged, making them attractive for most cations and metals (Day et al., 1989). When freshwater meets seawater will the different components, such as metals, cations, anions, and organic matter, be diluted or undergo physical, chemical, or biotic processes (Day et al., 1989). These processes include adsorption or desorption on particles, the components may coagulate, flocculate or precipitate, or undergo biotic assimilation (Day et al., 1989). The input from rivers also differs with season and climate. For example, after periods of heavy

snow melting in Norway was the input of aluminum to the river so severe that it resulted in fish kill of Atlantic salmons (Driscoll, 1985). The varying and unpredictable chemistry of estuarine/brackish water makes studies of this system important when there is a high risk of metal pollution. The speciation of the element is based on the chemistry of the environment.

2.1.2 Speciation of metals

Speciation is defined by the physico-chemical properties of the element of interest. These properties include density, size, electrical charge, oxidation state, and morphology (Lead et al., 1997; Salbu & Skipperud, 2009). The species or the fraction of the element, that resides in the system are based on the size of the species. These being: ions, molecules, complexes, colloids, and particles. The fractions are sorted into two groups; low molecular mass (LMM) and high molecular mass (HMM). The smallest species reside in the LMM fraction which consists of ions, molecules, and complexes. All smaller than 1nm. Due to the size of this fraction is it considered to be bioavailable to aquatic life through waterborne uptake (Salbu & Skipperud, 2009). The HMM fraction includes bigger complexes, colloids, and particles. Where colloids are defined in the size 1nm – 1 µm, while particles are bigger than >1µm (Lead et al., 1997). Another definition of colloids is constituents that do not settle and remains suspended (Gardner & Apul, 2009). The physical and chemical properties of metal-species are therefore crucial to determine the bioavailability of the element.

Freshwater is characterized by having pH in the range of 5-9, having low salinity, and varying levels of organic matter and major ions present depending on the area (Day et al., 1989; Fondriest Environmental Inc, 2014; Gibbs, 1970; Nikanorov & Brazhnikova, 2009; NORDTEST, 2003). Seawater, on the other hand, has high pH (pH around 8), high salinity, low levels of organic matter and high concentrations of major ions (Day et al., 1989; Duxbury et al., 2018; Fondriest Environmental Inc, 2014; Gibbs, 1970; Hedges, 1987). The aquatic systems exhibit different properties such as pH, cations, salinity, and content of organic material. All of which is crucial for determining the speciation of metals (Wood et al., 2012a).

Three main processes influence the mobility and bioavailability of metals in natural waters, these are complexation, precipitation, and adsorption (Flemming & Trevors, 1989). Metals in the aquatic systems may complex with two of the major complexing ligands present, OH^- and

CO_3^{2-}. Other ligands include the inorganic (Cl^-, SO_4^{2-}, PO_4^{2-}) and organic ligands (urea, organic acids, humic and fulvic acids) (Scoullos & Pavlidou, 2000). The chemical nature of the metals as well as the binding energy of the ligand's functional group determines how stable the complexes are (Flemming & Trevors, 1989). Metal ions can also precipitate, which removes soluble metals from surface waters. Sediments often retain and accumulate metals, working as a sink (Hu et al., 2018). Lastly, metals can be adsorbed to suspended matter, minerals, and living and dead cells. The adsorption can range between weak forces of van der Waals or strong covalent binding (Flemming & Trevors, 1989).

2.2 Copper

Copper occurs naturally in the aquatic systems, due to leaching of bedrock and rocks (USEPA, 2007). In addition to natural sources are anthropogenic sources; mining, metal production, electric equipment, wastewater, and fertilizers (Wood et al., 2012a). Copper is an essential micronutrient for organisms (USEPA, 2007). For example, is the element used as a cofactor for several enzyme processes (Blanchard & Grosell, 2005; Grosell et al., 2004).

Copper species found in the water are dependent on the water chemistry. This includes pH, hardness, major ions, dissolved organic matter, and suspended solids (Erickson et al., 1996). The species of copper found in water are usually carbonate-complexes; $CuCO_3$, $Cu(CO_3)_2^{2-}$, hydroxy-complexes; $CuOH^-$, $Cu(OH)_2$, or ionic copper; Cu^{2+} (Wood et al., 2012a). Complexes with dissolved organic matter, in the form of humic acid, are also likely (Mantoura et al., 1978; Moffett & Dupont, 2007). The distribution of these species varies with the different aquatic systems; freshwater, seawater, and brackish water.

In freshwater is the copper speciation dependent on pH, alkalinity, and dissolved organic matter (DOM). With high alkalinity and high pH dominates carbonate complexes, with only a small fraction of ionic copper and the hydroxy-complexes present (Blanchard & Grosell, 2005; Wood et al., 2012a). While the hydrolyzed copper species dominate systems with low alkalinity and high pH (Chakoumakos et al., 1979; Erickson et al., 1996). Systems with intermediate or low alkalinity and low pH have a higher concentration of ionic copper

(Chakoumakos et al., 1979). The free ionic copper concentration increases as pH decreases, figure 2.2 (Wood et al., 2012a). In addition to the inorganic species of copper is this element also largely complexed to natural dissolved organic matter (DOM) (Mantoura et al., 1978).

The speciation of copper in seawater is similar to freshwater, but the distribution is different. Seawater has high salinity and high pH, influencing the speciation. The carbonate complexes and organic matter complexes dominate in seawater (Moffett & Dupont, 2007). While a small portion occurs as ionic and hydrolyzed copper (Wood et al., 2012a). However, with the addition of freshwater to a coastal system changes the speciation of copper.

Brackish water is found in estuaries. Species in these systems are influenced by the mixing of seawater and freshwater. This influences the salinity, pH, and DOM of the water. Brackish water exhibits much of the same dominant copper species as seawater, that being the carbonate complexes (Blanchard & Grosell, 2005). However, with decreasing salinity increases the concentration of ionic copper and hydroxy- complexes. In addition to the inorganic species, is the input of organic matter from rivers and land to estuaries abundant. Humic acid in organic matter complexes easily with copper, which influences the speciation (Muller & Batchelli, 2013).

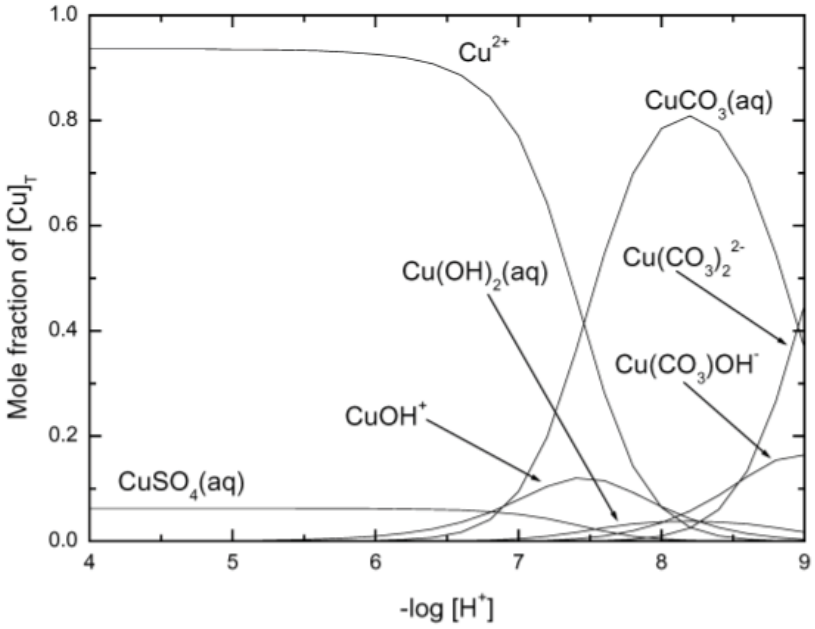

Figure 2.2: Speciation diagram for the Cu. With OH-, CO_3-, and SO_4^{2-}complexes. The system is at 25 °C. Printed from Powell et al., 2007. ©IUPAC

2.3 Zinc

Zinc occurs in abundance in the earth's crust and therefore exists naturally in aquatic systems (Wood et al., 2012a). However, high concentrations of zinc are always connected with human activities. Zinc is used for virtually all products. For example, in alloys, paper, paints, healthcare products, and galvanized tools and ships (Naito et al., 2010). Zinc is an essential element for organisms, due to its use in many biological processes (Wood et al., 2012a).

Speciation of zinc is crucial to determine the fraction it resides in, and therefore, determine its bioavailability. The most common inorganic species of zinc in aquatic systems are; the free ionic form Zn^{2+}, complexed with carbonates, sulfate or phosphate as $ZnCO_3$, $ZnSO_4$ or $ZnHPO_4$, hydroxy complexes $ZnOH^+$ and $Zn(OH)_2$, and chloro-complexes $ZnCl^+$, $Zn(Cl)_2$, $Zn(Cl)_3^-$ or $Zn(Cl)_4^2$ (Bervoets & Blust, 2000; Evans, 2000; Rainbow et al., 1993; Vega et al., 1995). Zinc may also complex weakly with organic matter and occur as organic species (Van Den Berg et al., 1986; Vega et al., 1995). The most dominant species present in the systems depends on the chemistry of the water. That being pH, salinity, and major ions present. This varies with the aquatic system in question.

The speciation of zinc in FW depends on pH, major ions present, and organic matter content. In general, the aquo complex of the free ion Zn^{2+} dominated the speciation of zinc in most waters, figure 2.3. Especially with the absence of dissolved organic matter and pH<8 (Bervoets & Blust, 2000). The concentration of carbonate and hydroxy- species increases with increasing pH and dominates the waters when reaching pH>8 (Bervoets & Blust, 2000; Evans, 2000). In oxygenated waters can the sulfate species be prevalent, depending on the concentration of sulfate (Evans, 2000).

Seawater speciation of zinc is similar to freshwater. The ionic form of zinc dominates the speciation in seawater (Wood et al., 2012a). However, the high content of salinity promotes the formation of chloro-complexes (Rainbow et al., 1993). In addition to the ionic form, the species $ZnOH^+$, $ZnCO_3$, Zn-organic, $ZnCl_n^{2-n}$ can occur in seawater (Eisler, 1993). The mixing of seawater and freshwater, however, makes for more complex chemistry in brackish

water.

In brackish waters varies salinity, pH, major ions, and organic matter content with the seasons (Ellwood, 2004). The ionic form of zinc is again the most dominant species for brackish water. In brackish water with low salinity occurs $ZnSO_4$ species as well (Eisler, 1993). With increased salinity increases the concentration of chloro-complexes of zinc (Eisler, 1993). For zinc, in general, the ionic form dominates for all water systems, some variation in other species are seen with different pH and different salinity in the system.

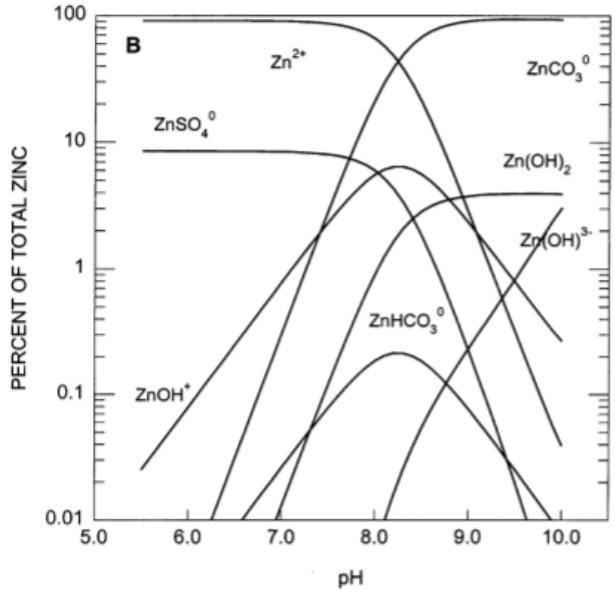

Figure 2.3: Speciation of zinc as a function of pH. Reprinted with permission from Bervoets & Blust, 2000.

2.4 Aluminum

Aluminum, as opposed to copper and zinc, is not an essential metal for organisms, as it has no known biological function (Gensemer & Playle, 1999). The metal is however abundant in the earth's crust (WHO, 2003). Aluminum is used in transport industries, for alloys, electric industry, cooking utensils, and food packaging (WHO, 2003). The main source of aluminum to aquatic systems is from natural processes, such as leaching from Al-rich rocks due to acidification (Driscoll et al., 2001). The species of aluminum present in the water, therefore,

depends on the chemistry of the water and the geology of the environment.

The speciation of aluminum is in general dependent on the pH and inorganic and organic ligands present in the aquatic system. Aluminum may occur as; hydroxy- complexes, $Al(OH)_2^+$, $AlOH^{2+}$, $Al(OH)_3$, or $Al(OH)_4^-$, as ionic aquo complex, Al^{3+}, or complexed with inorganic ligands of F^-, SO_4^{2-}, PO_4^{3-} or $Si(OH)_4$. Aluminum can also complex with organic compounds such as humic or fulvic acid. The distribution of these species depends on the chemistry of the system and their properties.

Aluminum-species in freshwater differ with pH, temperature, and ligands present. In general, aluminum is insoluble at pH 6-8. As it occurs as $Al(OH)_3$, also known as gibbsite (Wood et al., 2012b). In more acidic environments increases the solubility, and aluminum-species present are dominated by Al^{3+}, $AlOH^{2+}$, and $Al(OH)_2^+$. While in alkaline conditions dominates $Al(OH)_4^-$, figure 2.4. Aluminum can also complex with fluoride, sulfate, and phosphate, depending on pH, temperature, and ionic strength (Gensemer & Playle, 1999; Wood et al., 2012a). Aluminum also forms both weak and strong complexes with humic and fulvic acid in organic matter (Gensemer & Playle, 1999; Wood et al., 2012a).

The aluminum speciation in seawater and brackish water is derived from the pH and salinity of the water, figure 2.4. The source of aluminum to seawater and estuaries stem from freshwater rivers (Bjerknes et al., 2003; Wood et al., 2012a). The aluminum-influx from rivers quickly sediments to clay particles, but can at a later time be remobilized in estuaries (Hydes & Liss, 1977). Bjerknes et al. (2003) found that an increase in salinity transformed particulate/colloidal aluminum to reactive species. The speciation of aluminum in seawater is dominated by $Al(OH)_4^-$, $Al(OH)_3$, and $Al(OH)_2^+$ (Brown et al., 2010; Millero et al., 2009). A rapid change in chemistry occurs when freshwater and seawater mix, making the chemistry more complex (Bjerknes et al., 2003; Teien et al., 2006b). In contrast to freshwater, there are no analytical methods to measure the ionic and neutral dissolved forms of aluminum in seawater at present (Gillmore et al., 2016). Which is why size fractionation is used to obtain information on the fraction aluminum resides in.

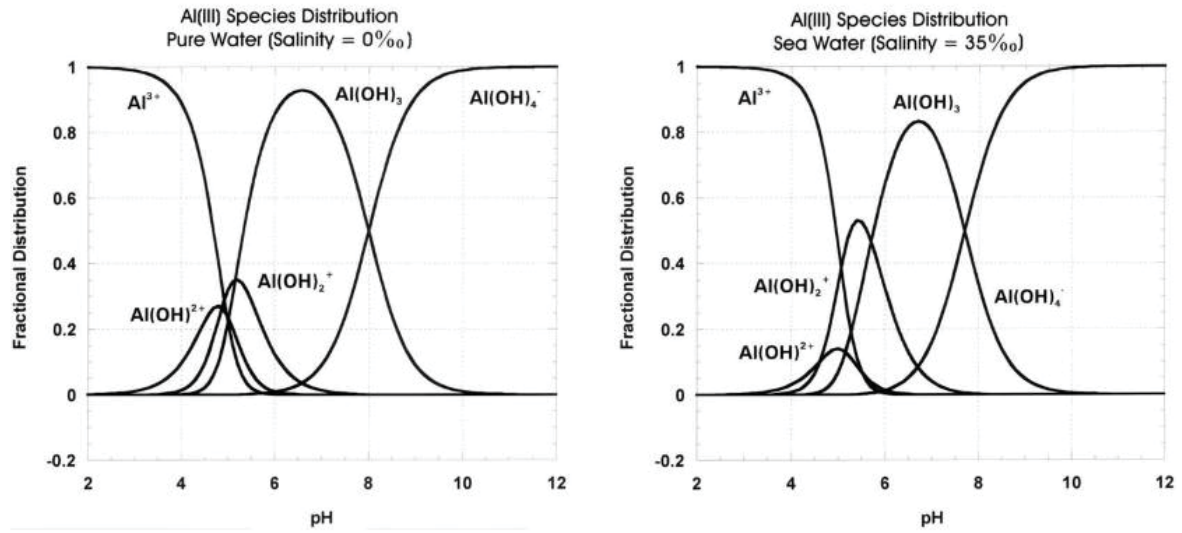

Figure 2.4: Distribution of Al species at salinity 0‰ and salinity 35‰ as a function of pH. Reprinted from Elkins & Nelson, 2002.

2.5 Uptake in fish

2.5.1 Main pathways

Uptake of metals in fish can occur through three pathways: gills, ingestion, and dermal contact. The main pathway is diffusion across the gills. This is due to a large surface area and the gills being sensitive to nutrients in the water (Wood et al., 2012a). The gills have three main uptake routes. These being a metal-specific carrier, mimicry uptake, and diffusion across the membrane. Metal-specific carriers are used for many essential metals. These are designed for the active transport of essentials metals from the water. Mimicry uptake occurs when metals are mistaken as an essential element and go through the active transport pathways. For example, Zn^{2+} can be mistaken as Ca^{2+}. The last uptake route is diffusion across the gill membrane. Due to the electrochemical gradient may metals simply be diffused from water to blood (Wood et al., 2012a).

Another pathway of uptake in fish is through ingestion. This affects the gastrointestinal system, that being the gut. This is dependent on the diet of the fish, but fish living in seawater drink water to keep themselves hypotonic (Grosell, 2006). As they drink the metals present in the water may end up in the gut. The same three uptake-mechanisms for gills; metal-specific

carrier, mimicry, and diffusion, are also applicable for the gut. The metals can also bind to amino acids and be transported through amino acid transporters (Wood et al., 2012a).

The last uptake pathway is through dermal contact, that being direct uptake through the skin. This is an unlikely uptake route, possibly due to the mucous acting as a protective layer (Dallinger et al., 1987). Though unlikely have some studies indicated that some calcium analogs, for example, Zn or cadmium (Cd), have some uptake through the skin (Wood et al., 2012a).

All three metals, copper, zinc, and aluminum have diffusion across the gills as their main uptake pathway. This is due to the size of the bioavailable species. Copper is taken up mainly through the gills, however, through diet or drinking may also the gut be at risk. The LMM species are generally thought to be bioavailable for uptake. That being the smallest of species. For copper are the bioavailable species Cu^{2+}, $CuOH^{-}$ and $Cu(OH)_2$ (Chakoumakos et al., 1979; Erickson et al., 1996; Wood et al., 2012a). Zinc uptake is proven to be primarily gills, though the intestine is not unlikely (Zhang & Wang, 2007). Zinc has been found in tissues such as gill and liver (Heier et al., 2009). The bioavailable species of zinc are the free ionic Zn^{2+} (Bervoets & Blust, 2000). The main uptake pathway of aluminum is through the gills (Wood et al., 2012b). The bioavailable species of aluminum are free ionic Al^{3+}, hydroxy-, fluoride-, and sulfate-complexes (Driscoll, 1985; Gensemer & Playle, 1999). Even though only the LMM species are available for uptake, are the bigger HMM species of aluminum more gill reactive, and can ultimately be more detrimental to the gills (Teien et al., 2006).

2.5.2 Concentration factor

The concentration factor is a useful tool to use when comparing the accumulation of different chemicals in aquatic organisms. Concentration factors are used to explain the extent to which the concentration of a chemical in an aquatic organism exceeds the concentration of the chemical in the aquatic environment. A few assumptions are made when using this factor, one being that exposure is long enough to obtain equilibrium, and the second being uptake is only waterborne (McGeer et al., 2003; Wood et al., 2012a). The relation between the toxicity of metals and CF is hard to determine. Due to aquatic organisms regulating metals internally.

2.5.3 Possible effects

The metal present in the water enters the fish and accumulates in the different tissues, which can cause harmful effects. The three metals of concern are copper, zinc, and aluminum. Copper is among the essential elements that are due to their redox properties important for many processes in the body. However, this property may also be a reason for its toxic effects. Copper inhibits of Na^+/K^+ - ATPase in freshwater aquatic organisms, which reduces sodium in the body (Wood et al., 2012a). Copper can also increase plasma ammonia and disrupt nitrogen metabolism (Blanchard & Grosell, 2006). For seawater, effects are not well known or proven. Some studies indicate that copper in seawater disturbs osmoregulation and alters nitrogenous waste excretion (Grosell et al., 2004).

High zinc concentration may lead to harmful effects on the gills of fish. For example, uptake of zinc can lead to inflammation of the gills leading to impaired gas exchange. This ultimately leads to insufficient oxygen, hypoxia. High concentrations of zinc can also inhibit branchial calcium uptake, leading to a lack of calcium, hypocalcemia. These effects have been proven in freshwater, while the effect mechanism in seawater is thought to be the same as freshwater (Wood et al., 2012a).

Aluminum is not an essential element, as opposed to the two other metals. Likewise, are the effects of aluminum been found mainly to affect the gills. Aluminum impairs gill ion regulation and leads to respiratory dysfunction. This metal also clogs the gills of the fish, leading to lowered plasma oxygen (Gensemer & Playle, 1999). Both, which ultimately have a high risk of mortality. Aluminum has been found to accumulate slowly in tissue and no internal toxic effects have been reported (Wood et al., 2012b).

2.5.4 Bioavailability and toxicity of metals

To predict if a metal is bioavailable for uptake, must the speciation of the metals be considered. The present study is investigating the speciation of metals in coastal waters. Copper is expected to be complexed with organic ligands in coastal waters, therefore, decreasing the bioavailability of the metal (Donat et al., 1994; Oldham et al., 2014). The toxicity of copper increased if the concentration of copper exceeds the concentrations of

organic ligands. Zinc is also predicted to be complexed with inorganic or organic complexes in coastal water, this decreases the bioavailability of zinc (Neff, 2002; Wood et al., 2012). For example, zinc can complex with chlorine in seawater. This complex has a lower affinity to gills than ionic zinc, therefore decreasing the toxicity of the metal (Bielmyer et al., 2012). In addition, the uptake of zinc is influenced by the concentration of cations present, as these compete for binding-sites on the gills of the fish. Aluminum is predicted to exist as complexes of hydroxides in coastal waters (Zhou et al., 2018). Marine organisms show high tolerance against aluminum in coastal waters as opposed to freshwater organisms (Zhou et al., 2018). However, increased concentrations of LMM-aluminum have been documented in estuarine areas with an influx from rivers (Kroglund et al., 2007; Kroglund et al., 2011). Based on the literature of metal speciation copper, zinc, and aluminum is predicted to have different speciation which influences if the metals are bioavailable.

2.5.5 Test species, *Salmo salar*

Salmo salar is a ray-finned teleost fish in the family Salmonidae. The Atlantic salmon is native to the north Atlantic Ocean but has been found in other oceans as well. The wild salmon spends most of its juvenile years in freshwaters before migrating to sea. The salmon are spawned in rivers and grow to the "parr" stage which they stay in for 1-4 years (Hansen & Quinn, 1998; Siriwardena, 2019). During these years they go through a transformation to survive the marine environment, called "smoltification". The wild salmon stay in freshwater for 2-3 years before they migrate to the sea, as "smolts" (Hansen & Quinn, 1998; McCormick et al., 1998; Siriwardena, 2019).

The salmon has huge economic importance in Norway. Especially regarding the farmed salmon. For instance, Norway exported salmon worth 72,5 billion NOK in 2019 (Norwegian Seafood Council, 2020). Possible release of metals to the marine or estuarine environment is likely for both for the native population and farmed salmon in fisheries and can have a negative impact on export. The smolting stage is in many cases the most sensitive life-stage for salmons. During this period the salmons need to travel through coastal waters before reaching the sea (McCormick et al., 1998). It is worth noting that the farmed salmon in cages cannot escape the water they reside in if metal pollution or changes in water chemistry occurs. The literature is extensive on the effects of metal uptake on freshwater fish. While the

literature on seawater and brackish water are further studies needed. The complex water chemistry of the brackish and estuarine environment is of high interest. More knowledge is needed regarding the metal uptake of salmons living in brackish or coastal waters.

3 Method and materials

3.1 Exposure

3.1.1 Exposure design

This study is a part of a larger Ph.D. study that is a part of the project Cumulative Hazard and Risk Assessment of Complex Mixtures and Multiple Stressors (MixRisk). The exposure experiment was designed to investigate the uptake and distribution of metals in fish. The fish exposure experiment was conducted during the period; October 2019 – December 2019 at NIVA Marine research station Solbergstrand at Drøbak. The exposure experiment followed the OECD Guideline 203 for acute toxicity testing on fish (OECD, 1992). The experiment was approved in advance by the National Food Safety Authority (NARA), FOTS ID 21058.

Tanks were lined with plastic wrap to limit contamination, figure 3.1, and figure 3.2. To the tanks were natural seawater pumped from 60-meter depth, collected at Solbergstrand from the Oslofjord. Additional freshwater was added to ensure a salinity of 20‰. The tanks contained 500 L of water in total. This follows the OECD guidelines (1992) of 1L/gram of biomass in each tank. The tanks were equipped with an air stone connected to an aquarium pump to ensure a constant high oxygen concentration. The tanks were kept at approximately 10-12°C with weak artificial light during the exposure. The exposure was conducted with nine different concentrations of the metals, Cu, and Zn. While Al had six different exposure groups, table 3.1. This is as recommended by OECD guidelines (1992) The control group consisted of the same type of water without any additional metal added.

Seven fish were randomly assigned to each of the exposure tanks and exposed to brackish water with metals for 96 hours. The exposure was conducted under a static procedure, meaning no water replacement and no feeding during the test period. The tanks were checked for adverse effects, like mortality, daily during the 96-hour exposure. The exposures were

conducted after each other in October, November, and in December, due to practical limitations.

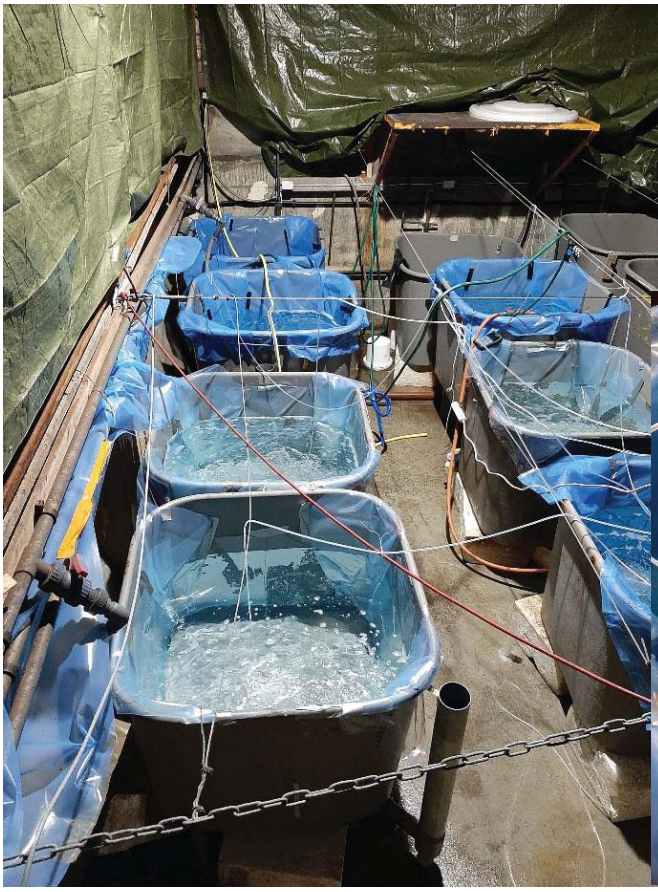

Figure 3.1: Set up of experimental units. Photo: Emil Jarosz.

Figure 3.2 Tank lined with plastic wrapping with aeration stone. Photo: Emil Jarosz.

3.1.2 Water quality

Brackish water made from natural seawater from the Oslofjord with additional freshwater was used as reference water. Natural seawater was chosen to obtain a realistic exposure experiment. Additional freshwater from the NIVA facility was added to obtain a salinity of 20‰. The stock metal solutions were made by dissolving salts of anhydrous $CuCl_2$, $ZnCl_2$, and $AlCl_2$ (Sigma-Aldrich, USA) in deionized water in plastic containers. The concentration of copper, zinc, and aluminum in the stock solutions was 4.5 mg/l, 18.4 mg/l, and 20 mg/l for each metal respectively. The stock solutions were added to the tanks 48 hours before exposure, to ensure stable metal speciation before the transfer of fish. The stock solutions

were added to obtain the concentrations of Cu in the range 0.09-7.78 µmol, of Zn in the range 0.4-15.6 µmol, and for Al in the range 1.37-11.86 µmol, table 3.1.

Final concentrations were recalculated from µmol/l to µg/l by eq. 1.

Eq. 1 $$concentration\ (\frac{\mu mol}{l}) \times molar\ mass(\frac{g}{mol}) = concentration\ (\frac{\mu g}{l})$$

Table 3.1 Expected nominal concentrations for each exposure metal group

	Cu	Zn	Al
Molar mass g/mol	63.546	65.38	26.98
	µMol	µMol	µMol
	0.09	N.A	N.A
	0.18	N.A	N.A
	0.38	0.4	N.A
	0.55	0.6	N.A
	0.76	0.8	N.A
	N.A	1.4	1.37
	2.37	2.4	2.37
	4.26	4.3	4.36
	7.78	7.8	7.78
	N.A	15.6	11.86

N.A Not analyzed

3.1.3 Holding and acclimation

Atlantic salmon smolts of both sexes were obtained from the Fish laboratory at NMBU (Ås, Norway). The fish were transferred to the NIVA research facility to holding tanks at least one week before exposure start, according to the standard method. Following the OECD Guideline, 203 (1992) were the fish held for at least nine days (2 days settling and 7 days acclimation) in brackish water with a salinity of 20‰. The acclimation is to ensure no further stress to the fish when transferred to exposure tanks. Any effects on stress is therefore a result of uptake of metals, and not holding. This also ensures the same optimized conditions for all the fish. The salmon smolts were fed daily up to 48 hours before exposure. This ensures that the fish has lower metabolism and therefore a lower need for oxygen when moved from holding tanks to exposure tanks. This also minimizes the amount of feces, which can affect

the experiment by metals adsorbing to particles.

3.2 Water sampling and analysis

3.2.1 Water quality parameters

Quality parameters; pH, temperature, salinity, ammonia, calcium (Ca), potassium (K), and magnesium (Mg) were recorded or measured during or after the exposure. The temperature was recorded continuously with data loggers during 96-hours exposure. The light of the environment around the tanks was measured to ensure that fish was not stressed due to light. For measurements of temperature and light were HOBO loggers used. Salinity and pH were measured once each day of the exposure. While ammonia, calcium, potassium, and magnesium were measured at the end of the exposure. Ammonia was not measured for tanks containing aluminum as exposure metal.

3.2.2 Water sampling and analysis of metal concentration

Water samples were collected at the start of (0h) and after the exposure period (96h). The samples were fractionated on site. The fractionation was performed by different operators at 0- and 96-hours. Water from the tanks was collected with a plastic jug and transferred to 2 L plastic containers. From the plastic container containing sampled water was unfiltered water transferred to a 50ml tube (Saarsted AG & Co, Germany) for the total fraction. Water from the plastic container was filtered with a syringe with a 0.45 µm filter, Acrodisc® 32 mm Syringe Filter with 0.45 Supor® Membrane (Pall Corporation, USA) and transferred to 50 ml tubes. This ensures that colloids and ions are the only species present in the sample, as filtration by the 0.45 µm Millipore or Nucleopore membranes retain particles.

The remaining water from the container was filtrated with a hollow fiber filter with a 10kDa Microza Hollow Fiber Ultrafiltration Modules (Pall Corporation, USA) and obtained in 50 ml tubes. The ultrafiltrated water was filtrated with a Chelex® 100 Resin (Bio-Rad Laboratories inc., USA) and collected in 50 ml tubes. Hollow fiber ultrafiltration interfaced with ion chromatography is used to fractionate species in a colloidal 1-10 kDa range based on their charge. The Chelex® cation resin retains cations and any neutral or anionic species pass

through.

Water fractionation was utilized to obtain information regarding the trace element speciation. The fractionation technique used a 0.45µm syringe membrane filter. This filter excludes particulates, while colloids and ions are included in the filtered fraction. A 10kDa ultrafilter was utilized to separate colloidal and LMM fractions. Ion-chromatography was used with Chelex® 100 Resin to obtain information regarding the charge.

From the fractionation analysis can these species be determined:

M_{total} : Total metal concentration in unfiltered water

$M_{particulate}$: Derived from subtracting metal concentration in 0.45 µm filtration from the concentration in unfiltered water, eq. 2.

Eq. 2 $$M_{particulate} = M_{total} - M_{0.45}$$

$M_{colloids}$: Derived from subtracting metal concentration in ultrafiltrated water from concentration in 0.45 µm filtration, eq.3.

Eq. 3 $$M_{colloids} = M_{0.45} - M_{LMM}$$

M_{LMM} : Metal concentration in ultrafiltrated water (cutoff 10kDa)

$M_{cations}$: Metal retained in Chelex® 100 Resin, from ultrafiltrated (cutoff 10kDa) water. Derived from subtracting the metal concentration in ion-exchanged water from the concentration in ultrafiltrated water, eq. 4.

Eq. 4 $$M_{cations} = M_{LMM} - M_{chelex}$$

After sampling were all tubes transferred to the Isotope laboratory (Ås, Norway). From the 50 ml tubes were 1ml of each water sample mixed with 1ml sub-boiled ultrapure 69% (w/w) EMSURE® HNO_3 (Merck, Germany) and 200µl of internal standard (100ugl^{-1} Rh/In) in one 15 ml tube (Saarsted AG & Co, Germany) and diluted to 10 ml with Milli-Q® (18 MΩ cm) water. Each sample had three replicates. The samples were measured on ICP-MS.

The results of water samples include the average total concentration of metal present in each

exposure group, given at the start of the exposure (0h) and the end of the exposure (96h). The values are given as mean concentration ± standard deviation µmol/l. All values are recorded in appendix A.5. The speciation of metals is given as a percentage of the total concentration.

3.3 Fish tissue samples and analysis

3.3.1 Sampling of fish

Fish were collected and euthanized after the exposure. The size and weight of the fish were recorded before dissection. The dissection was performed by several people to ensure higher efficiency and save time.

Blood samples were collected with a 1 ml syringe by the caudal vein. The blood samples were analyzed using I-STAT cassette EC8+ on an I-STAT analyzer on-site. The tissues were collected into 5 ml vials (Saarsted AG & Co, Germany). To ensure no contamination between fish in the same exposure group, and between the different exposure groups were all dissection equipment cleaned with Ethanol absolute ≥99.8%, AnalaR NORMAPUR® (VWR Chemicals, France) and scalpel blades were changed between each fish. To avoid contamination from tissues to the cutting board were the fish placed on a plastic bag. All tissues were collected into their respective vials immediately after dissection and the lids on the vials were closed after each dissection to avoid any contamination.

The tissues were stored at -20° before freeze-drying for 48 hours. After the samples were freeze-dried they were stored at room temperature. To restrict the work of the master thesis were only the results of the gill, kidney, and liver chosen for further discussion.

3.3.2 Digesting of tissues and analysis

The freeze-dried tissues were weighed into Teflon tubes used for digesting. To the tissue weighing less than 1.0 gram were 1ml HNO_3 and 200 µl internal standard (100ugl^{-1} Rh/In) added before digesting. For tissue weighing more than 1.0 gram were 5ml HNO_3 and 1 ml internal standard (100ugL^{-1} Rh/In) added. After digesting were the samples weighing less than 1.0 gram transferred to 15 ml tubes (Saarsted AG & Co., Germany) and diluted to 10 ml with deionized water. Samples weighing more than 1.0 gram were transferred to 50 ml tubes (Saarsted AG & Co., Germany) and diluted to 50 ml with deionized water. The fish tissue samples were measured using Agilent 8800 ICP-MS Triple Quad and Agilent 8900 ICP-MS

Triple Quad (Agilent Technologies, USA).

To ensure a homogenous and liquid sample before analysis was the fish tissue samples digested through microwave-assisted acid digestion. The fish tissue samples were digested using UltraClave® (Milestone Srl, Italy) or UltraWave® (Milestone Srl, Italy) at the Isotope laboratory at NMBU. Microwave-assisted acid digestion is based on the principle that microwaves cause friction and therefore generate enough heat to dissolve organic material. To the load is HNO_3, Sub-boiled ultrapure EMSURE, 69% (w/w) (Merck, Germany) added to absorb the microwaves. However, as this causes the formation of NO_x-gases is H_2O_2, Technical Quality (VWR International, USA) also added to the load to prevent this. In the chamber is the starting pressure 50 bar with the temperature at room temperature. The temperature increases to 260°C and stays at maximum temperature for 25 minutes.

3.4 ICP-MS Analysis

3.4.1 ICP-MS method

The water samples were measured on ICP-MS, Agilent 8900 ICP-MS Triple Quad (Agilent Technologies, USA). For this method was reaction modes ammonia and helium used for two masses of Cu and Zn, and one mass for Al, appendix A.12. One reaction mode and masses for each element were chosen, based on the accuracy of reference material and limit of detection, appendix A.1. The reaction mode, ammonia, were chosen for all three elements. An internal standard is used to control dilution and drift during the analysis. Using the internal standard can one correct for incorrect dilution or loss of sample.

The digested tissue samples were measured on the ICP-MS, Agilent 8800 ICP-MS Triple Quad (Agilent Technologies, USA). For this method were gas modes; oxygen and ammonia, used for Al. While gas modes; ammonia and helium were used for different masses of Cu and Zn, Appendix A.12. One reaction mode and mass for each element were chosen, based on the accuracy of reference material and limit of detection, appendix A.2. These being helium or ammonia Cu-63, ammonia or helium Zn-64, and oxygen Al-27.

3.4.2 Water samples – traceability

Certified reference material SRM 1640a was used to ensure the method's traceability for water samples. 1640a contains trace elements in natural water. The CRM undergoes the same sample preparation as the water samples, with the same amount of internal standard and dilution added. The reference material is produced and certified by the National Institute of Standards and Technology. The trace elements copper, zinc, and aluminum are certified for this CRM (NIST, 2010).

3.4.3 Fish tissue – traceability

To ensure the traceability of the method and ensure the samples were properly digested were certified reference materials (CRM) used. The CRM's undergo the same digesting with the same type and amount of acid and internal standard added as the fish tissue samples. For fish organ samples were ERM-BB422 and DOLT-5 used. Appendix A.3 summarizes the results of CRM.
The ERM-BB422 reference material is fish muscle from the species Saithe (*Pollachius virens)*. It is produced and certified by the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre. The material includes certified reference values for copper and zinc (IRMM, 2012). The DOLT-5 reference material is Dogfish liver (*Squalus acanthias)* is produces and certified by the National Research Council Canada. The material includes certified reference values for copper, zinc, and aluminum (National Research Council Canada, 2014).

3.5 Data processing and statistical analysis

3.5.1 Data treatment

All values were obtained as µg/l or mg/l before further treatment was calculated from µg/l or mg/l to mg/kg for fish tissue, eq. 5. Furthermore, were all values calculated from µg/l (water samples) to µmol/l, or mg/kg (fish tissue samples) to mmol/kg by equation 6. This is performed to compare the results to other studies, as well as compare the different metals on

a mole basis.

Eq. 5 $$\frac{mg}{kg} = \frac{Concentration(\frac{\mu g}{l}) \times Volume\ l}{Weight\ kg \times 1000 \mu g}$$

Eq. 6 $$\frac{mmol}{kg} = concentration\ (\frac{mg}{kg}) \div molar\ mass\ (\frac{g}{mol})$$

3.5.2 Treatment of outliers

Some values measured in this experiment did not fall into the range of expected values. These have been identified as outliers by three criteria; reviewing the standard deviations, plotting values in a scatterplot, and using GraphPad Prism function of "identify outlier". This function uses the ROUT method which detects outliers by fitting the values to a curve with nonlinear regression. Setting a false discovery rate at Q = 1%, meaning that no more than 1% of identified outliers to be false (Motulsky & Brown, 2006).
These values have been marked as outliers, with red in appendix A.8.

3.5.3 Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using five blank samples for fish tissue and five blank samples for water samples measured on the ICP-MS. The fish tissue samples were measured in three batches. The highest LOD/LOQ was chosen for each metal, Cu, Zn, and Al, these are summarized in Appendix A.1 and A.2.
The standard deviation obtained from the samples is used to calculate the limit of detection and limit of quantification, equations 7 and 8. The limit of detection is defined as the lowest concentration of an analyte which can be detected. While the limit of quantification is defined as the lowest concentration of analyte which can be quantified (Shrivastava & Gupta, 2011). Appendix A.1 and A.2. All values which are below LOD or LOQ will be reported as <LOD or <LOQ.

Eq. 7 Limit of Detection: $3 \times Standard\ Devation\ (of\ blank\ samples)$

Eq. 8 Limit of Quantification: $10 \times Standard\ Deviation\ (of\ blank\ samples)$

3.5.4 Concentration factor calculation

The concentration factor is calculated using the relation between the difference in concentration of metal present in the fish tissue group and control group, divided by the difference dissolved metal concentration (<0.45µm) in the exposure water and water in the control group, by equation 9.

Eq. 9 $$CF = Diff.Conc.metal\ in\ tissue\ \left(\frac{mmol}{kg}\right) \times 1000\mu mol \div Diff.Conc.metal\ water\left(\frac{\mu mol}{l}\right)$$

3.5.5 Statistical analyses

Two statistical programs were used for the treatment of data, GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft Office Excel 2016 (Microsoft Corporation Redmond, WA, USA).

All fish tissue data for each exposure group have been checked for normality, appendix A.9. Using the Shapiro-Wilk test will any p-value less than α-value indicates that the exposure group did not pass the normality test. Therefore, rejecting the hypothesis that there is a normal distribution in the population.

A one-way ANOVA test was used to determine if there was a significant difference between the mean of the control group and the mean of the exposure group. To determine which groups are different from the control group was Dunnett's or Tukey's *post hoc* test for multiple comparisons used, with α=0.05. Any p-value less than α-value determine that the hypothesis can be rejected, and therefore there is a significant difference between means, appendix A.10.

4 Results and discussion

4.1 Quality of analysis

4.1.1 Limit of detection and limit of quantification

The limit of detection for water samples was $2.4*10^{-4}$ µmol/l for Cu, $5.2*10^{-5}$ µmol/l for Zn, and $1.1*10^{-5}$ µmol/l for Al. While the limit of quantification for water samples was measured to $7.8*10^{-4}$ µmol/l for Cu, 1.7 µg/l for Zn and $3.7*10^{-5}$ for Al, table 4.1.1

The limit of detection of fish tissue samples was 0.0015 mmol/kg for Cu, $4.5*10^{-3}$ mmol/kg for Zn, and $7.3*10^{-8}$ mmol/kg for Al. While the LOQ was measured to $5.0*10^{-3}$ mmol/kg for Cu, $1.5*10^{-2}$ mmol/kg for Zn, and $2.4*10^{-7}$ mmol/kg for Al, table 4.1.1

Table 4.1.1 Limit of detection and limit of quantification for copper, zinc, and aluminum of water samples and fish tissue samples. Given in µmol/l and mmol/kg, respectively.

	Water		Fish tissue	
	LOD (µmol/l)	LOQ (µmol/l)	LOD (mmol/kg)	LOQ (mmol/kg)
Cu	2.4E-04	7.8E-04	1.5E-03	5.0E-03
Zn	5.2E-05	1.7E-04	4.5E-03	1.5E-02
Al	1.1E-05	3.7E-05	7.3E-08	2.4E-07

4.1.2 Traceability of analysis

Certified reference material, CRM 1640a was used to obtain information on traceability of the method. Copper was measured in the range 82-97 µg/l. The results exhibited some variation, with an error in the range of 2-12%, Appendix A.3. The measured samples passed the normality test and no outliers were discovered. Zinc was measured in the range 52-58 µg/l, with an error not exceeding 6%. The lowest sample measured at 52µg/l was marked as an outlier. Removing this sample lowers the error to 4%. The measured values of Cu and Zn is therefore within the error accepted for the certified reference material. While Al was measured in the range 56-70 µg/l, with an error of 33% at most, table 4.1.2. Two of the measured 1640a samples had an error at 6%, while the rest all exceeded at least 11%. The

measured values of Al have a large variation and all values do not fall within the accepted range for the CRM. A high error and high variation indicate that there is uncertainty in the method, which must be taken into consideration when evaluating the results.

Using the certified reference material of tissues, e.g. ERM-BB422, results show that this CRM did not exceed an error from certified value by more than 11%, however, the same sample of both Cu and Zn exhibit higher error (10% and 11% respectively), when compared to other samples measured. It is possible that this sample was weighed in faulty or was contaminated before analysis. Removing this sample gives errors in the range 1-2% for Cu, and 1-6% for Zn, Appendix A.3. The measured values of Cu were found in a range of 1.63-1.86 mg/kg, while Zn was measured in the range 16.1-17.7 mg/kg, table 4.1.2.

Results from the analysis of certified reference material DOLT-5 shows that Cu and Zn did not exceed a difference of 15% from the certified value. The same sample for Cu and Zn has a high error. Removing this sample gives a maximum error of 6% for Cu, and 3% for Zn. Copper was measured in the range of 33-39.5 mg/kg and Zn was measured in the range 102-121 mg/kg, table 4.1.2. Aluminum on the other hand had overall only one of five CRM samples within acceptable error. This exceeded no more than 10% error, while the other samples had a maximum error of 40%. This indicate that the method is uncertain when analyzing for aluminum in the fish tissue samples.

The analysis of certified reference material shows that the method is uncertain for Al, for analysis of water samples. While for analyzing fish tissue samples are the method within reasonable error for the metals Cu and Zn. Analysis of Al in fish tissue samples is however uncertain with this method. Acid added to the samples was likely contaminated with Al, which increases the concentration of Al measured in the samples. This must be considered when evaluating the results from both water samples and fish tissue. All results are recorded in Appendix A.3.

Table 4.1.2. Certified reference materials, ERM-BB422, DOLT-5 and 1640a. Average measured values of Cu, Zn, and Al, with standard deviation, relative standard deviation, maximum and minimum values given.

Reference material		Certified value	Average	SD	RSD%	Minimum	Maxium
ERM-BB422							
Cu	mg/kg	1.67 ± 0.16 mg/kg	1.70	0.075	4 %	1.64	1.83
Zn	mg/kg	16 ± 1.1 mg/kg	16.9	0.57	3 %	16.1	17.7
DOLT-5							
Cu	mg/kg	35.0 ± 2.4 mg/kg	35	2.6	7 %	33	40
Zn	mg/kg	105.3 ± 5.4 mg/kg	108	7.4	7 %	102	121
Al	mg/kg	31.7 ± 4.2 mg/kg	22	7.7	36 %	17	35
1640a							
Cu	µg/l	85.75 ± 0.51 µg/L	89	5.8	7 %	82	97
Zn	µg/l	55.64 ± 0.35 µg/L	56	2.0	4 %	52	58
Al	µg/l	53.0 ± 1.8 µg/L	62	5.1	8 %	56	70

4.2 Water quality parameters

To obtain information on water quality and stability over time, were several parameters measured. Over the 96-hour exposure varied the pH of the water in the range 8.1-8.2. The water temperature varied in the tanks in the range 8.5-12.3 ° C. The salinity of the water varied from 19.9-20.3 ‰. While the ammonia measured after 96h exposure varied in the range 0.16-0.3, all values are reported in appendix A.4. No significant difference in pH, temperature, and salinity was found in the tanks between the different exposure metals, table 4.2.

The concentration of calcium, potassium, and magnesium in the water was measured to obtain information of salts present in the water. The concentration of Ca varied in all the tanks between 0.32-0.45 g/L. The concentration of K in all tanks varied between 0.14-0.35 g/L. While the concentration of Mg in all tanks varied between 0.70-1.06 g/L, appendix A.4. The high variation found of measured salts disagrees with the low variation found for measured salinity. A possible explanation behind the disagreement is uncertainty when measured by ICP-MS.

The concentration of Ca and Mg are found to be as significantly higher ($p<0.05$) for tanks containing exposure of aluminum. While the concentration of K was not found to be different for each exposure metal, table 4.2. The measured values of Ca, Mg, and K follow the relation

2:7:1. The concentrations found in natural seawater follow the relation 1:5:1 for Ca, Mg, and K, respectively (Besson et al., 2014). This indicates that the relation between Ca, Mg, and K is higher than expected. Calcium-rich groundwater used for mixing with seawater likely increased the Ca-concentrations in the exposure water.

The results suggest that no major difference between water quality parameters was found in the tanks for the different exposure metals. The variation for pH, temperature, and salinity is low, indicating that the conditions were kept stable over 96h.

Table. 4.2. Average values with a standard deviation of parameters pH, temperature, salinity, ammonia, calcium, potassium, and magnesium in exposure water of each metal group, copper, zinc, and aluminum.

	Cu	Zn	Al
	Average ± SD	Average ± SD	Average ± SD
pH	8.17 ± 0.03	8.17 ± 0.02	8.16 ± 0.03
Temp (C°)	10.3 ± 0.7	10.0 ± 0.7	10.8 ± 0.3
Salinity (‰)	20.1 ± 0.1	20.1 ± 0.1	20.1 ± 0.1
Ammonia (mg/L)	0.22 ± 0.06	0.23 ± 0.05	N.A
Calcium (g/L)	0.36 ± 0.02	0.36 ± 0.02	0.39 ± 0.01
Potassium (g/L)	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.02
Magnesium (g/L)	0.88 ± 0.04	0.88 ± 0.06	0.92 ± 0.03

N.A - Not analyzed

4.3 Metal concentration and speciation in water

4.3.1 Copper concentration and speciation in water

The average total concentration of Cu in the control group was measured to 0.04 ± 0.01 µmol/l. The expected concentrations of Cu in the different exposure groups were 0.09, 0.18, 0.38, 0.55, 0.76, 2.37, 4.26 and 7.78 µmol/l. The results show that the measured concentrations are all lower than expected, with 0.08, 0.14, 0.25, 0.42, 0.56, 2.08, 3.4 and 7.0 µmol/l, respectively, table 4.3. The concentration over time increased for some of the

exposure groups, these being for the three highest concentrations. It is, however, unlikely that the concentration increased over time and is most likely due to different sampling conditions.

Copper concentrations found in natural waters range from $3.15*10^{-3}$-0.47 µmol in FW systems to $1.57*10^{-3}$-1.57 nmol in SW (USEPA, 2007; Wood et al., 2012a). While in coastal waters have Cu been reported at $7.87*10^{-4}$-0.27 µmol (Kozelka & Bruland, 1998; van Geen & Luoma, 1993). This is natural systems void of metal pollution. In cases that metal pollution is a viable problem, for example in Kaldvellfjorden is the concentration much larger. In the tributary, "Stordalsbekken", was the concentration of copper reported in the range 0.14-0.83 µmol/l (Hindar & Nordstrom, 2015; Teien et al., 2017; Todt et al., 2015). While in the fjord, "Kaldvellfjorden", was Cu reported between 0.06-0.80 µmol/l (Todt et al., 2015). The Norwegian Environment Agency has set the limits of concentration, regarding the acute effects of Cu in coastal water to 0.08 µmol/l (5.2 µg/l) (Miljødirektoratet, 2016). This means that the Cu-concentrations measured in this exposure experiment and the tributary and the fjord, all exceeds the limit set by the Norwegian Environment Agency. The concentrations used for this experiment are within the range of what is naturally found in coastal systems but also exceeds the maximum measured concentrations found in both the tributary and the fjord.

The distribution of Cu-species varied considerably between the different exposure concentrations. The standard deviation for these results is based on the deviation between results measured at 0h and 96h. The speciation of Cu in the control group showed that Cu was found in the dissolved fraction; colloids and LMM, as 24% and 76% respectively. Of the LMM fraction was 22% found as cationic. In the exposure groups was more than 85% of Cu found in the dissolved fraction and less than 16% were found in the particulate fraction, figure 4.3.1. The colloidal fraction dominates overall the speciation of Cu for the intermediate concentrations, while the LMM fraction dominates the two highest concentrations. The LMM fraction is largely found as cationic for all exposure groups. The results of Cu in the colloidal fraction in this thesis range from 15-85%. Not only does this indicate a huge uncertainty in defining the speciation of copper but can also indicate that the results of LMM Cu are measured too low when compared to earlier studies. A pilot study conducted before the exposure experiment indicated that Cu filtered through hollow-fiber ultrafiltration decreased over time. This indicate that some of the copper was being retained by the filter. The actual

speciation of Cu in the exposure water is uncertain. It is therefore likely that there is a higher content of Cu associated with LMM.

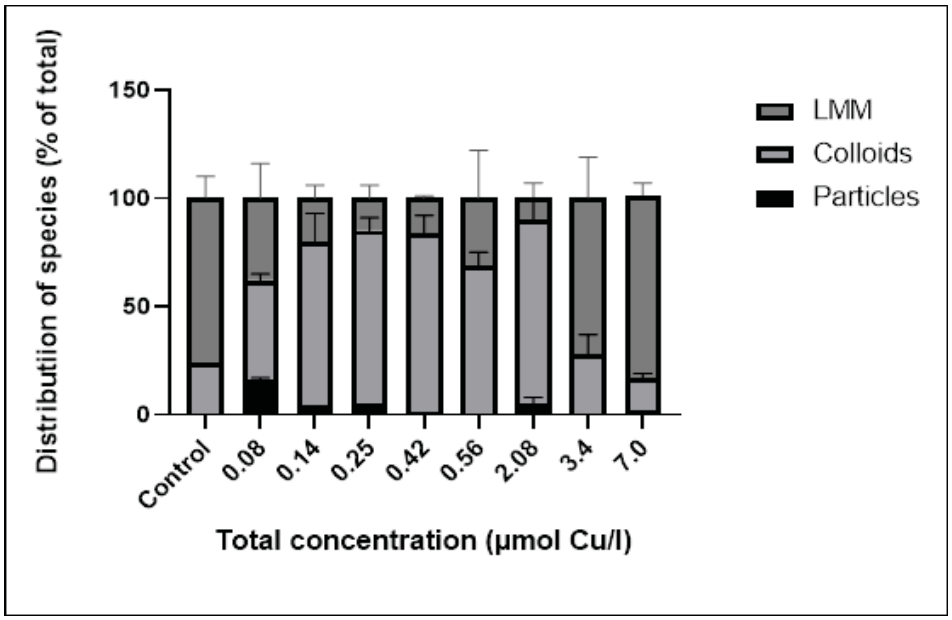

Figure 4.3.1. Mean distribution copper species (Particles, Colloids, and LMM) for control, and conc. 0.08-7.0 µmol Cu/l. Standard deviation is given from the difference in means between measured species at 0h and 96h.

Earlier studies give conflicting results on the speciation of Cu in coastal systems. The conflicting results are likely due to the low content of organic material present in the freshwater used in the experiment. This gives the system a lower content of particles which usually are found in natural systems. Wells et al. (2000) found that Cu occurred mainly (>70%) in the size range 1-8kDa at Narrangaset Bay, Rhode Island. The same study also found that particulate Cu was low, which agrees with this thesis results. Another study reports that Cu occurred as much as 47% in the LMM size class (1kDa – 10kDa), and only 8% at colloidal (10kDa – 0.45 µm) at Galveston bay (Wen et al., 1999). In contrast, Shafer et al. (2004) found Cu greatly associated with the colloidal fraction (in the size range 1kDa – 0.4µm) in several marine estuaries. It is worth noting that these studies were working with environments with fluctuating salinity compared to this thesis.

Despite the conflicting results are this thesis results on the speciation of Cu in line with the reported findings conducted on freshwater systems. Masresha et al. (2011) found that in three

different Ethiopian lakes was Cu associated with the HMM fraction, that being both colloids and particles. These were also found to mainly be associated with the non-cationic Cu, which conflicts with the results from this thesis. Other studies have reported Cu associated mostly to the colloidal fraction and some with the LMM fraction (Allan et al., 2007; Heier et al., 2009). The speciation of copper in freshwater agrees some with the results of this thesis. Studies on coastal systems are however conflicting, such that some agree with the thesis' results while others do not. This can indicate that Cu speciation is not stable and is easily influenced by the environment. This also reflects on the varying results found for the distribution of Cu species in this thesis experiment.

4.3.2 Zinc concentration and speciation in water

The average total concentration of Zn in the control group was measured at 0.10 ± 0.03 µmol/l. The expected concentrations of Zn in the exposure groups were 0.4, 0.6, 0.8, 1.4, 2.4, 4.3, 7.8, and 15.6 µmol/l. The measured concentrations of zinc were measured lower for all, except for the three highest concentrations. The measured values being 0.27, 0.45, 0.64, 1.02, 1.72, 4.4, 8.2 and 15.7 µmol/l, respectively, table 4.3. Some concentrations increased significantly ($p<0.05$) over time, these being concentrations 4.4 and 8.2 µmol/l. This difference can be due to uncertainties in the measurement or mixing of the water at sampling.

Zinc concentrations in natural water without pollution are reported much lower. In FW systems are Zn concentrations reported in the range $3.05*10^{-4}$-0.76 µmol/l and the range 0.02-0.92 nmol/l in SW systems (Eisler, 1993; Ellwood, 2004; Naito et al., 2010). However, in the case of Kaldvellfjorden was Zn reported at much higher concentrations. In the tributary, Stordalsbekken were zinc reported between 1.61-16.12 µmol/l (Hindar & Nordstrom, 2015; Teien et al., 2017; Todt et al., 2015). While in Kaldvellfjorden was the reported concentration in the range <0.03-0.15 µmol/l (Todt et al., 2015). The Norwegian Environmental Agency has set the concentration limit of zinc to 0.92 µmol/l (60 µg/l) (Miljødirektoratet, 2016). The reported concentration of zinc in the tributary and some concentrations used for this exposure study exceeds this limit.

The speciation of Zn is largely dominated by the dissolved fraction. In the control group were the species distributed almost equally between colloids and LMM fraction, with 56% and 44%, respectively. Where the LMM fraction consists of 17% cationic species.
The LMM fraction dominates the speciation of zinc overall (>78%), except for the lowest concentration group (40%), figure 4.3.2. The LMM fraction consists of mostly cationic species, with >85% of LMM off all concentration groups found as cationic. The highest concentration has a high standard deviation, this is due to a great difference in results gained at 0h and 96h. This is most likely due to either contamination, different sampling, or difference in fractionation.

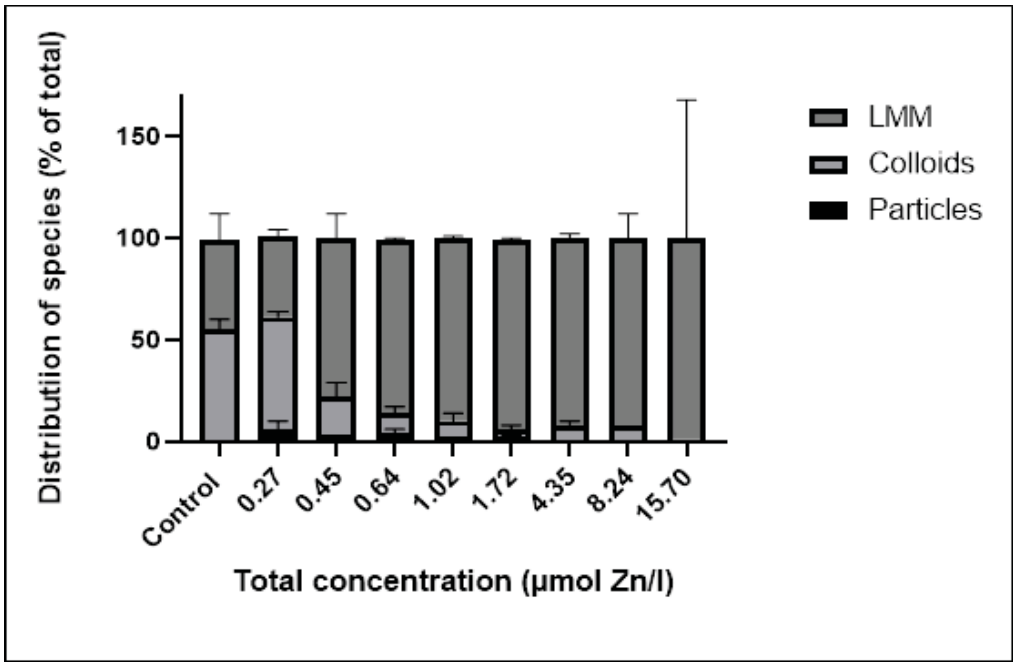

Figure 4.3.2. Mean distribution zinc species (Particles, Colloids, and LMM) for control, and conc. 0.27-15.70 µmol Zn/l. Standard deviation is given from the difference in means between measured species at 0h and 96h.

Previous studies exhibit some conflicting results. One study concluded that Zn had a low association with colloids in the dissolved phase (<0.2 µm) and high association to the soluble fraction (<1kDa) at Narranganset bay (Wells et al., 2000). At Galveston Bay was 40% of Zn found in the colloidal fraction (10kDa – 0.45 µm) (Wen et al., 1999). Shafer et al. (2004) reported the colloidal Zn association low to intermediate (5-30%) at three different estuaries.

The same study supports the low amount of Zn found in the particulate fraction, and a high amount of Zn found in the <1kDa fraction. Studies conducted on freshwater systems report conflicting results on Zn speciation. One study found Zn highly associated with particles in three different lakes (Masresha et al., 2011). While Heier et al. (2009) found Zn in a freshwater stream to be associated with the different fractions in the order LMM>colloidal>particles. Both studies concluded that Zn was cationic. Allan et al. (2007) support the latter study, where Zn was found associated with the LMM fraction. The conflicting results are probably due to the chemical compositions of the aquatic systems. Different pH, salinity, and inorganic and organic complexes can change the speciation of Zn. The results from this thesis agree with the studies that concluded that Zn had a low association with colloidal fraction and high association to cationic LMM fraction. These studies were conducted on both coastal/marine waters and freshwater studies.

4.3.3 Aluminum concentration and speciation in water

The average concentration of Al in the control group was measured to 0.8 ± 0.1 µmol/l, that being 22 ± 2 µg/l. This is a higher concentration than expected in control water. Screening tests of both seawater and freshwater used in the exposure did not exhibit high concentrations of Al. The acid added to the samples may contain elevated concentrations of Al, which pollutes the samples. The analysis of traceability revealed that the certified reference material was measured higher for Al than expected for the CRM. This can indicate that Al was measured higher due to pollution or faulty analysis.

The expected concentrations of Al for the different exposure groups were 1.37, 2.37, 4.26, 7.78, and 11.86 µmol/l. The measured concentrations were close to the expected concentrations. The measured average values were 1.7, 2.7, 4.3, 7.6, and 11.8 µmol/l, table 4.3. The concentration of Al was stable over time and decreased only slightly for the three lowest concentrations.

Aluminum found in natural waters is usually only high in FW systems. The concentrations are reported in the range 0.04-1.85 µmol/l, while SW systems are reported around $3.71*10^{-3}$

µg/l, with coastal Al found with <2 µg/l (Bjerknes et al., 2003; WHO, 2003). However, with a change of pH can Al concentrations increase rapidly. Elevated concentrations of Al was found in Stordalsbekken in the range of 0.04-0.19 mmol/l (Hindar & Nordstrom, 2015; Teien et al., 2017; Todt et al., 2015). These results are almost a hundred times higher than measured in non-contaminated waters. Aluminum concentrations in Kaldvellfjorden were reported in the range of 0.15-8.90 µmol. Which is within the range of Al-concentrations used in this exposure. In comparison was dissolved Al concentration in four different estuaries in China reported in the range 0.25-2.0 µmol (at 15 ‰) (Zhang et al., 1999).

The distribution of aluminum species in the exposure water was largely found in the dissolved fraction. In the control waters were aluminum species found in the dissolved fraction, as colloidal at 10%, LMM as 83%, while particulates were found as 7%.
The LMM fractions exhibit some high standard deviations due to high variation between measured samples at 0h and 96h. The aluminum species were mainly found in the LMM fraction (>83%), with most found as cationic LMM (>68%). The colloidal and particulate fraction varied between the different concentration groups. With colloidal being almost non-existent for all concentrations, except the lowest concentration of 1.7 µmol Al/l, where 33% of Al species were found in the colloidal fraction.

A low amount of Al was found in the particulate fraction, except for the highest concentration of 11.8 µmol Al/l, where 14% of the Al species were found as particulates, figure 4.3.3. A higher association with particles at water concentration 11.8 µmol Al/l can be due to Al reaching its solubility limit. Edzwald and Haarhoff (2011) calculated that the most common Al form in seawater, amorphous aluminum hydroxide ($AlOH_3$) had a solubility of 10 µmol/l at 10°C and pH 8.1.

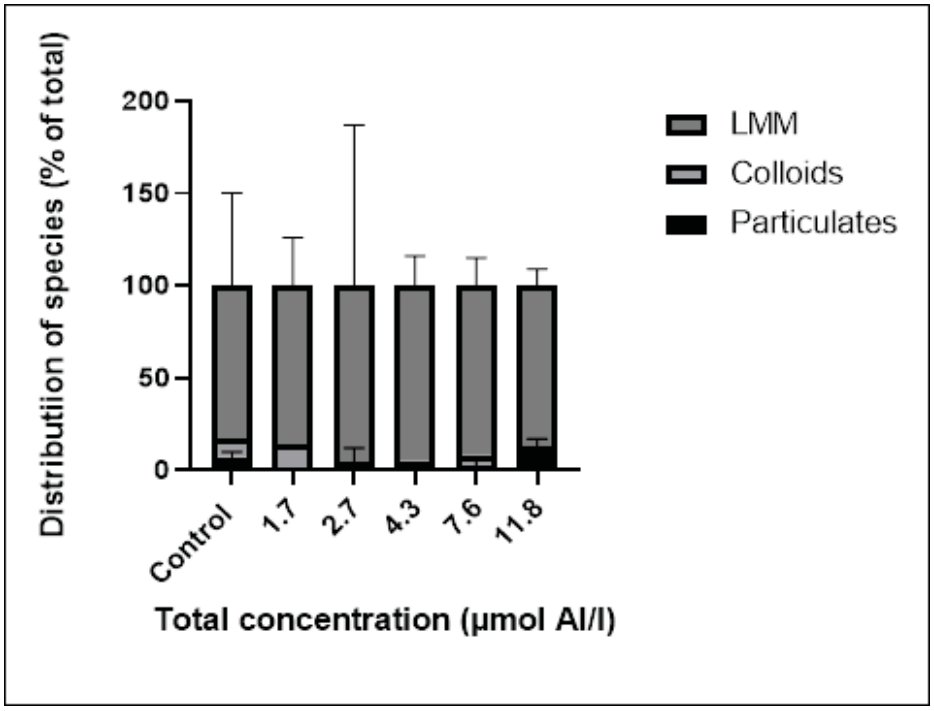

Figure 4.3.3. Mean distribution of aluminum species (Particles, Colloids, and LMM) for control, and conc. 1.7-11.8 µmol Al/l. Standard deviation is given from the difference in means between measured species at 0h and 96h.

Defining Al-speciation in aquatic systems has been and still is difficult. This is due to several factors, including the low concentration of aluminum in natural waters and complex matrixes. Especially is the analysis of seawater samples a major concern due to possible matrix effects from interfering ions (Tria et al., 2007). There are only a few studies on Al-speciation in estuaries, coastal or brackish waters. Studies on Al-speciation in seawater show that colloidal aluminum in the Pacific Ocean ranged between <1-11% (Reitmeyer et al., 1996). This is supported by Dammshauser and Croot (2012) who found that the dissolved fraction had a low colloidal association, while the soluble phase (LMM) dominated. Aluminum associated with particles was found to be low in marine surface waters (Brown et al., 2010). These studies agree with the findings of this thesis, concerning the speciation of Al in brackish waters. One study conducted on brackish water agrees with these findings, Teien et al. (2017) reported that Al was associated largely with the LMM fraction. The distribution of aluminum species in freshwater systems is heavily influenced by the pH and the complexes present in the system. In acidic freshwater with low organic content is Al associated with the LMM fraction, as pH increases will Al polymerize and hydrolyze to larger fractions (HMM) (Teien et al., 2004). The results of the present study are therefore in agreement with earlier studies conducted on SW systems.

4.3.4 Comparison of metal concentration and speciation in water

The measured total concentration of metals present in the control water followed the order Al>Zn>Cu, table 4.3. The concentrations of Cu and Zn does not exceed the concentration limit set by the Norwegian Environmental Agency (2016). All three metals had a good correlation between expected nominal concentration and measured concentration (r^2>0.98).

In general, are the concentrations for Zn higher than for Cu at the comparable nominal concentrations, except for the nominal concentration of 2.4 µmol. Copper and Zn exhibit similar measured concentrations due to their molar mass being similar (63.55 and 65.38 g/mol respectively). The measured nominal concentrations of Al are higher than both Cu and Zn at comparable nominal concentrations, except for at 4.3 µmol and 7.8 µmol, table 4.3. It is worth noting that the highest concentration of Al was expected to be 11.8 µmol but is included in this table at 15.6 µmol for practical reasons.

The distribution of species varies for the different metals. Both Zn- and Al-species are mainly associated with the LMM fraction. While Cu species are either found as colloidal or as LMM-species, figure 4.3.4. The metals found in the LMM fraction followed the decreasing order Al>Zn>Cu. Aluminum associated to the LMM fraction was >83% of the total fraction, with Zn associated with LMM were found in the range 40-100% of the total. Copper associated with LMM ranged greatly between 10-85% of the total. However, Cu is likely more associated with LMM than the results suggest, due to sorption to the filter. The speciation of metals is different. A one-way ANOVA test shows that there was a significant difference ($p<0.05$) between the means LMM-fractions for the metals Cu, Zn, and Al. A *post hoc* Tukey multiple comparison tests revealed that were no difference between LMM Zn and Al, but there was a difference between Cu and the other two metals. However, if Cu is more associated with LMM than the results suggest is the speciation between the metals not significantly different.

The metals found in the colloidal fraction followed the order Cu>Zn>Al. Due to possible sorption of LMM associated Cu is the actual colloidal fraction lower than the results suggest. A one-way ANOVA test revealed that there was a significant difference between the means

of colloidal Cu and the other two metals. Colloidal Cu was found in the range 15-85%, colloidal Zn in the range 1-55%, and colloidal Al was found <33%. The distribution of dissolved Cu- and Zn- species (colloidal and LMM) have a greater variation than Al-species, which indicates that the actual distribution of the metals is more uncertain. The particle fraction is overall low for all three metals, figure 4.3.4. No significant difference between means of metals was found for the particulate fraction. This indicate that the metals are associated with the dissolved fraction and therefore are bioavailable.

Table 4.3. Comparison of expected nominal concentration, with the measured concentration of Cu, Zn and Al given in µmol/l and µg/l in the exposure water.

Nominal water concentration	Measured concentration					
	Cu		Zn		Al	
µmol/l	µmol/l	µg/l	µmol/l	µg/l	µmol/l	µg/l
Control	0.04 ± 0.01	3 ± 1	0.10 ± 0.03	6 ± 2	0.8 ± 0.1	22 ± 2
0.4	0.30 ± 0.03	16 ± 2	0.30 ± 0.01	18 ± 1	N.A	N.A
0.6	0.40 ± 0.03	27 ± 2	0.50 ± 0.01	29 ± 1	N.A	N.A
1.4	N.A	N.A	1.0 ± 0.04	66 ± 2	1.7 ± 0.1	45 ± 4
2.4	2.1 ± 0.1	132 ± 3	1.7 ± 0.1	112 ± 4	2.7 ± 0.4	72 ± 10
4.3	3.4 ± 0.4	219 ± 27	4.4 ± 0.2	285 ± 12	4.3 ± 0.2	116 ± 4
7.8	7.0 ± 0.3	444 ± 20	8.2 ± 0.2	539 ± 12	7.6 ± 0.4	204 ± 9
15.6	N.A	N.A	15.7 ± 0.2	1027 ± 12	11.8 ± 0.2	318 ± 6

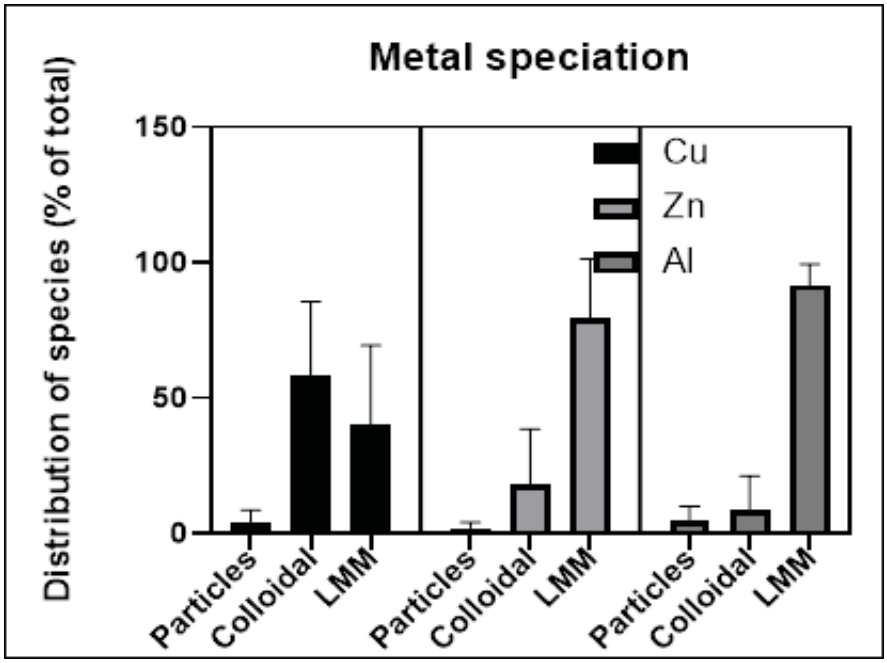

Figure 4.3.4. Comparison of average distribution with a standard deviation of species (particles, colloids, and LMM) for Cu, Zn, and Al.

4.4 Metal uptake in fish tissue

4.4.1 Fish characteristics

The fish (n=168) used for the exposure experiments had an average weight of 76 ± 12 gram, in the range 53-103 gram, and an average length of 20 ± 1.1 cm in the range 17-22 cm. The weight and length did not vary considerably between the different metal exposures, appendix A.7.

4.4.2 Stress levels in fish

To obtain information about stress in fish was an iSTAT analyzer with cassette EC8+ used. Results show that the glucose levels found in the fish exposed to Cu, Zn, or Al did not exceed normal levels, figure 4.4.2. Stress increases the plasma glucose in fish and is a good indication of stress levels (Gatica et al., 2010). For salmon, is the average value for unstressed fish at 5.5 mmol/l, while a stressed fish has glucose levels at 10-12 mmol/l (Evensen et. al 2008, as referenced in Olsen, 2013). When fish is stressed, they breathe more and therefore have a higher chance of uptake of metals. No significant difference was found between the control group for Cu and Zn and the exposure groups.

A significant difference ($p<0.05$) was found for the control group for Al and the exposure groups, figure 4.4.2. Further tests revealed that three exposure groups; 2.67, 4.31, and 11.79 µmol Al/l had a significant difference from the control group. However, these groups also exhibit higher standard deviations, indicating that there might be a few fish in these groups that have elevated glucose levels while the rest do not. No outliers were discovered. Comparing the glucose levels found for fish exposed to Al against glucose levels exposed to Cu and Zn reveals that they are similar. Glucose levels are influenced by the diet of the fish. Therefore, the elevated levels of glucose could be due to the fish being fed later up to transfer. This makes it more likely that the control group for Al had lower glucose levels due to their diet. However, it cannot be excluded that Al might have impacted the fish negatively as Al is a known stressor for fish (Rosseland et al., 1990)

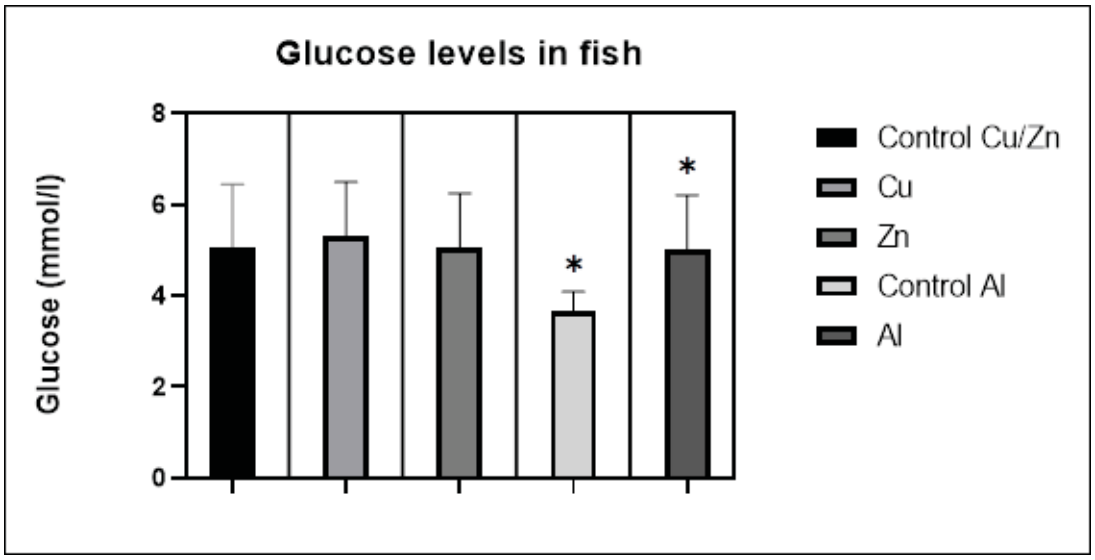

Figure 4.4.2 Average with standard deviation glucose levels in the blood of fish from exposure to Cu, Zn, and Al
** denotes a significant difference ($p<0.05$) between means.*

4.4.3 Copper uptake in fish tissue

The uptake of copper in fish varied with the tissue and the copper concentration in the water. The uptake of gill increased considerably as the concentration of copper increased. The control group of gills had an average measured value of copper at 0.028 ± 0.0083 mmol/kg

gills dw, figure 4.4.3a. One value in the control group was marked as an outlier but was included in the calculation. This outlier increases the average and the standard deviation slightly. All exposure groups passed the normality test.

A significant increase was found for the four highest concentrations ($p < 0.05$), figure 4.4.3a. The average measured values were found to be 0.06 ± 0.02, 0.08 ± 0.01, 0.10 ± 0.08 mmol Cu/kg gills dw, with increased concentration of 2.04, 3.41 and 6.69 µmol/l respectively. Significant uptake of copper in the gills agrees with the findings of Grosell et al. (2003) with 7-days Cu exposure conducted on clear nosed skates and sculpins at comparable Cu exposure levels as this thesis. The accumulation of copper was reported higher than this thesis's findings, despite both studies having similar copper concentrations in the water. The magnitude of uptake on clear nosed skate and sculpins was also much higher than for salmon. The difference in uptake can be due to fish species' sensitivity.

The uptake of copper in kidneys was overall not significant and no trend was discovered. The control group for kidneys was measured to an average value of 0.10 ± 0.01 mmol Cu/kg kidney dw, figure 4.4.3b. All groups, except for concentration 0.14 and 0.25 µmol/L, passed the normality test. Two values were marked as outliers, one for concentration 0.14 and one for concentration 0.25. Both have been included in the calculations. These outliers increased the average and standard deviation of their groups and hinder these groups from passing the normality test. The average values of Cu were not significantly higher for exposure groups compared to control groups, figure 4.4.3b. Average values were found in the range 0.09-0.12 mmol Cu/kg kidney dw, figure 4.4.3b. There have been reported uptake of copper in kidneys of sculpins at comparable Cu concentrations in the exposure water to this thesis (Grosell et al., 2003). As the study with sculpins was conducted over seven days it is possible that the salmon of this thesis could accumulate copper in the kidneys had the exposure prolonged longer than 96-hours.

The uptake of copper in the liver was not significant and did not follow any trend. The control group for the liver gave an average value of 4 ± 2 mmol Cu/kg liver dw, figure 4.4.3c. All groups passed the normality test. The average values varied between 3-5 mmol Cu/kg liver dw. The results on the lack of uptake of copper in the liver are supported by Blanchard and Grosell (2005). This study found that killifish exposed to copper at comparable salinity and Cu concentrations in the water also found the highest concentration of copper in the liver.

However, there was not a significant uptake of copper in the liver, which agrees with the results of this thesis. No internal elevated copper concentrations were found in the bodies of clear nosed skates and sculpins either (Grosell et al., 2003).

The concentration of copper found in the tissues naturally in control fish followed the order liver>kidney>gills. However, the uptake compared to the control groups were found to be the highest for gills, while the uptake in kidneys or liver were non-existent compared to the control groups. The results indicate that there is a naturally high concentration of copper in the liver of the salmons which is not influenced by the copper present in the water. Shukla et al. (2007) support the results of the degree of accumulation of copper in spotted snakeheads. However, this study was conducted in a freshwater system with much higher concentrations of copper in the exposure water. The significantly higher exposure groups, with nominal water concentrations 0.56-7.0 µmol Cu/l, increased the metal concentration in gills in the range 0.012-0.075 mmol Cu/kg from lowest to highest water concentration. No significant uptake was found for the kidney and liver.

The Norwegian Environment Agency (2016) has set the limit of Cu concentration in coastal water for acute exposure at 0.08 µmol/l. The results from the present study indicate that there is no uptake at this concentration limit. A significant uptake in the gills is only found at concentrations at least seven times higher than the limit. This indicates that there is a low risk of uptake of Cu in the fish at low concentrations of Cu in coastal waters. However, higher concentrations of Cu in coastal waters do impose a risk of uptake in fish.

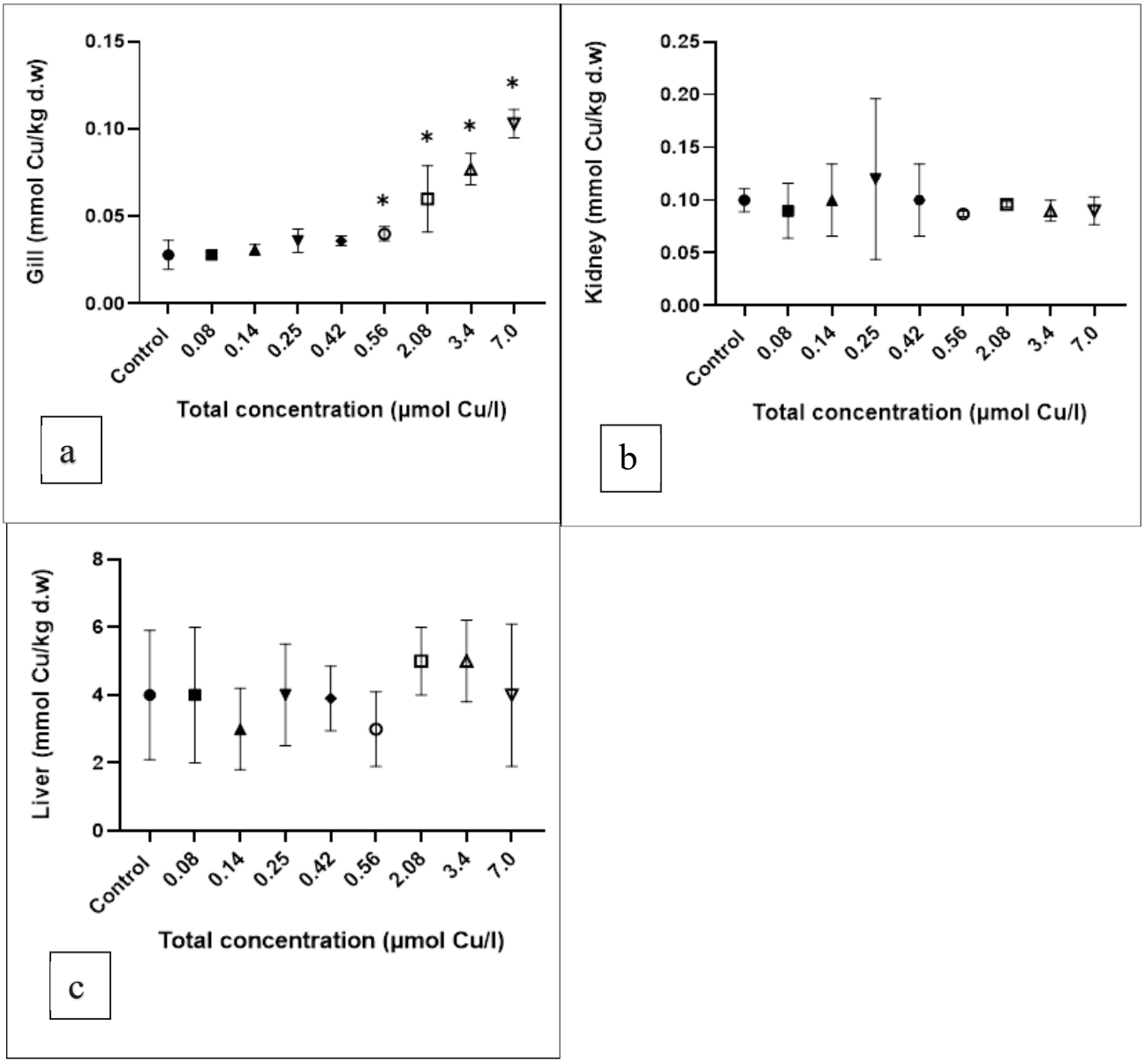

Figure 4.4.3. Concentration of copper in gills (a), kidney (b) and liver (c) at different exposure concentrations of Cu at 20 promille. Given as average ± SD mmol/kg.
** denotes a significant difference ($p<0.05$) from control group*

4.4.4 Zinc uptake in fish tissue

The uptake of zinc did not vary much with tissue or zinc content in the water. Some significant difference from control groups was found for gills and liver, figure 4.4.4. The control group of gills gave an average measured value of 10 ± 2 mmol Zn/kg gills dw. All groups passed the normality test. The average values for all concentrations ranged between 7-10 mmol Zn/kg gills dw, figure 4.4.4a. A significant difference was found for two

concentrations, though both are significantly lower than the control ($p<0.05$).
The observed results of gill Zn concentration are higher than reported for other fish species. A study conducted on *Fundulus heteroclitus* in SW with comparable Zn concentrations to this thesis reported lower Zn accumulation in the gills after seven days of exposure. However, no significant uptake on the gills was found in the study, in agreement with the results of this thesis (Shyn et al., 2012).

The concentration of zinc in kidneys increased slightly with an increase of zinc in the water. However, no significant difference from the control group was found. The control group for zinc in kidneys had an average measured value to 4.0 ± 1.8 mmol Zn/l kidney dw. All groups, except for the control group and concentration 0.64 µmol/L, passed the normality test. Two values were marked as outliers, one for the control group and one for concentration 0.64, which hinders the passing of normality tests. Average values were found between 3.0-6.0 mmol Zn/kg kidney dw, figure 4.4.4b. With the highest concentration found for concentration of 4.35 µmol/L at 6.0 ± 2.2 mmol Zn/kg kidney dw.

The control group for zinc in the liver had an average measured value at 1.0 ± 0.3 mmol Zn/kg liver dw. All groups passed the normality test. Average values were found between 1-2 mmol Zn/kg liver dw, figure 4.4.4c. A significant difference was found between the control group and concentration 1.0 µmol Zn/l and 1.7 µmol Zn/l ($p<0.05$), the former with a large standard deviation. This is consistent with other studies with the same Zn concentrations in the exposure water (Shyn et al., 2012). However, the same study also reported a significant increase at even higher concentration also used for this experiment (15.7 µmol Zn/l), in conflict with the results of this thesis.

The concentration of Zn found in the different tissue followed the order gills>kidney>liver. These results agree with other studies conducted with seawater and with similar Zn concentrations in the seawater (Shyn et al., 2012). However, the uptake of zinc did not increase significantly with an increase of zinc content in the water, despite high concentrations. For the two significantly higher exposure groups, 1.0 and 1.7 µmol Zn/l increased the metal concentration in the liver by 1 mmol/kg. It is uncertain why these two groups were significantly higher when no significant difference was found for higher water

concentrations. It is worth noting that there is a significant difference ($p<0.05$) between means at a molar level, µmol/l, but not when the test is performed with weight, mg/kg.

The Norwegian Environment Agency has set the concentration limit of Zn in coastal water for acute effects to 0.92 µmol/l. This limit is exceeded by the five highest concentration of Zn, from 1.0 to 15.7 µmol/l. There was no significant uptake of Zn in the fish in brackish water. Despite the concentrations of Zn exceeding the limit by a magnitude of 17 at most. This indicates that there is a low risk of uptake of Zn in fish in coastal waters.

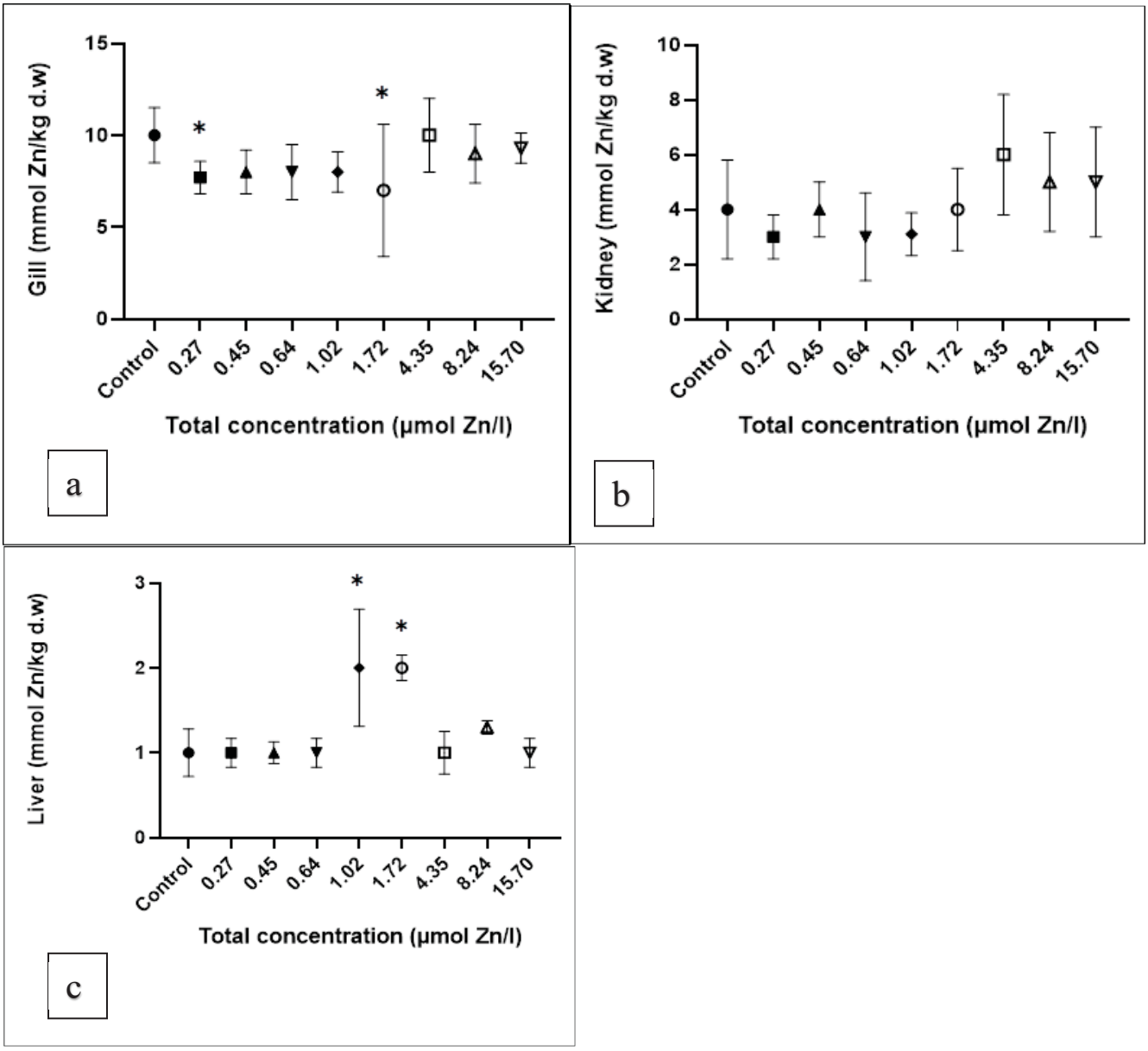

Figure 4.4.4. Concentration of zinc in gills (a), kidney (b) and liver (c) at different exposure concentrations of Zn in seawater at 20 promille. Given as average ± SD mmol/kg.
** denotes a significant difference ($p<0.05$) from control group*

4.4.5 Aluminum uptake in fish tissue

Uptake of aluminum in the different tissue varied with the tissue and concentration of metal present in the water. For the control group of gills were the average value given at 0.25 ± 0.04 mmol Al/kg gill dw. The average measured values of aluminum did increase with a higher concentration of Al in the water. With the highest three concentrations giving an average measured value; 0.5 ± 0.2 mmol Al/kg, 0.6 ± 0.2 mmol Al/kg, and 0.8 ± 0.2 mmol Al/kg dw, respectively with higher concentration, figure 4.4.5a. These three concentrations were significantly higher than control ($p<0.05$), figure 4.4.5a. All five exposure groups, including the control group, passed the normality test. Gills are proven to be the main organ of concern regarding the uptake of aluminum. Studies conducted on fish in freshwater systems report higher aluminum accumulation than the observed results found for this experiment. Even with lower concentrations of aluminum present in the exposure water (Monette et al., 2010; Nussey et al., 1999). These results indicate that there is an effect on speciation, as higher pH and higher salinity decrease the uptake of Al in fish.

Uptake of Al in the kidneys increased some at higher water concentrations. For the control group of kidneys were the average measured value given at 0.057 ± 0.010 mmol Al/kg kidney dw, figure 4.4.5b. One value was marked as an outlier for concentration 1.66 µmol/L. All groups passed the normality test, except for concentration 1.66 µmol/L. The average values for Al concentration in kidneys were found in the range of 0.08-0.12 mmol Al/kg kidney dw, figure 4.4.5b. Two concentrations, 1.66 and 1.56 µmol Al/l were found to have a significant difference from the control group ($p<0.05$). There is a lack of studies reporting Al concentrations in kidneys. However, studies indicate that the uptake of Al in the kidneys is low (Gensemer & Playle, 1999).

Uptake in the liver did not increase considerably with a higher concentration of Al in the exposure water. For the control group of liver were the average measured value given at 0.08 ± 0.02 mmol Al/kg liver dw Al, figure 4.4.5c. One value, for concentration 4.31, was marked as an outlier. All groups, except for concentration 4.31, passed the normality test. This group passes the normality test when removing the outlier. The concentrations of Al for the five groups varied between 0.10-0.11 mmol Al/kg liver dw. Studies on Al uptake in liver on freshwater fish *(Labeo umbratus*) reports higher Al concentrations than this study (Nussey et

al., 1999).

The accumulation of aluminum in the fish followed the order gill>kidney>liver. The results of the uptake of aluminum indicate that there was a significant uptake in the gills for all concentrations, except for the lowest, 1.7 µmol. The increase in natural Al concentration in the control group in the gills was in the range 0.11-0.52 mmol Al/kg. The measured value of aluminum of gills at the highest concentrations can be due to the speciation in the water. Particles can be stuck to the gills, and not transferred into the body of the fish. However, the speciation analysis indicates that Al associated with particles is low. The uptake in gills is therefore likely due to Al associated with the LMM fraction. Two groups of Al in the kidney had a significant increase in uptake, that being 1.7 and 7.6 µmol/l. These increased by 0.061 and 0.041 mmol Al/kg, respectively. No uptake of Al in the liver was discovered.

At present, there exists no proposed concentration limit for Al in coastal or marine waters. Golding et al. (2015) proposed a water quality guideline of 0.9 µMol total Al/l (24 µg/l) for marine waters for 95% species protection. The same study noted that the exposed juvenile fish did not exhibit adverse effects, even at the highest concentrations. This agrees with the present study. The concentration of Al in the control water was close to the proposed limit, and all exposure concentrations exceeded this limit as well. Despite this was significant uptake only registered in the gills from concentrations 2.7 to 11.8 µmol Al/l. There is a risk of uptake of Al on the gills of fish living in coastal waters at elevated concentrations of Al.

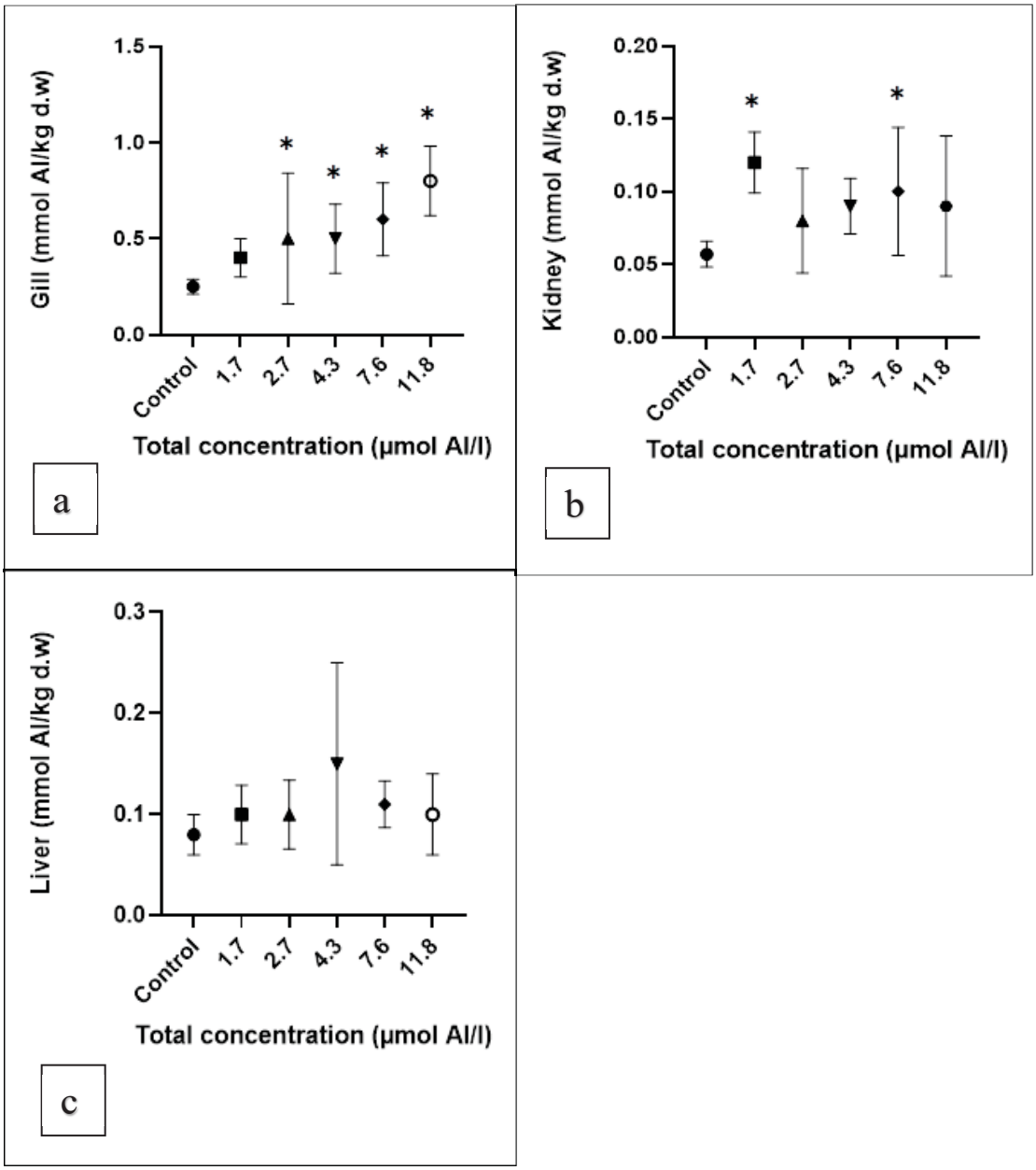

Figure 4.4.5. Concentration of aluminum in gills (a), kidney (b) and liver (c) at different exposure concentrations of Al. Given as average ± SD mmol/kg
** denotes a significant difference ($p<0.05$) from control group.*

4.4.6 Comparison of metals in tissue

The natural concentrations for the three tissues were found in the order Zn>Cu>Al. This is in agreement with other studies were zinc was most abundant out of several metals for some freshwater fish species (Gilbert & Avenant-Oldewage, 2014; Orata & Birgen, 2016). With the liver being the tissue with the highest concentration of Cu, while Zn and Al-concentrations found highest in the gills.

Table 4.4 summarized the increase (in mmol/kg) from the control group tissue of metal concentration measured in the tissues for the different exposure groups. It is worth noting that the table does not consider the standard deviation in the groups. In the gills was Zn found to have the highest concentrations in the control group, however, the results indicate that there was no increase. In comparison increases Cu and Al at comparable nominal concentrations in the water. Aluminum increases most of the three metals at the comparable nominal water concentrations 2.4-7.8 µmol/l. At nominal water concentration 7.8µmol/l increased the metal concentration in gills by 0.30 mmol Al/kg from the control group. Where in comparison Cu increased by 0.075 mmol/kg.

In the kidneys was Zn once again found the be the most abundant metal. There was no increase of Cu in the kidneys or of Zn at the lower nominal water concentrations. At nominal water concentration 4.3µmol/l increased the metal concentration in the kidneys of Zn by 2 mmol Zn/kg. However, this value was not proven significantly higher ($p<0.05$). At the same nominal water concentrations increased the metal concentration by 0.032 mmol Al/kg. Comparing the increase to the concentrations found in the control group tissue shows that the concentration of Zn increased by a magnitude of 0.9, where concentration of Al increased by a magnitude of 1.6.

In the liver was Cu found to have the highest concentrations of the three. Some increase, by 1 mmol/kg, in metal concentrations in the liver was found for Cu but was proven to not be significantly higher. The nominal water concentration 1.7 µmol/l for Zn and 4.3 µmol/l for Al was proven to have a significant increase. Zinc concentration in the liver increased by 1 mmol Zn/kg, this was significantly higher ($p<0.05$). Aluminum concentration in the liver increased by 0.07 mmol Al/kg at 4.3 µmol/l nominal water concentration.

The results indicate that there is a naturally high concentration of Cu in the liver, and Zn in all three tissues. This could be due to the Cu- and Zn-rich diet of the salmon smolt before exposure. In addition, Cu and Zn are essential metals for organisms, while Al is not. One reason behind an observed lower uptake could be due to the high concentration of major ions, such as Ca in the exposure water. As Ca competes for metal-binding sites on the gills, high concentration of calcium could hinder the metal uptake (Pagenkopf, 1983).

The results indicate that there is a difference in the uptake of metals. Using a one-way ANOVA test with Tukey's *post hoc* test was a significant difference ($p<0.05$) between means discovered for the uptake of metals in fish. For gills was a significant difference found between Zn vs Cu/Al. The difference between the metals and Zn is due to the lack of uptake of Zn on the gills. The uptake of Cu and Al, however, is not significantly different.
A significant difference between means was found between Al vs Cu/Zn on the uptake in kidneys. Again, no actual uptake was discovered in the kidneys of Cu or Zn, and only some uptake was found for Al for two groups. No uptake or significant difference in means of metals in the liver was discovered.

The results indicated that an acute 96 hours test, with the water parameters set for this exposure in 20 ppt seawater, is the internal uptake of Al, Cu, and Zn not an issue. There is an uptake on the gills of Al and Cu. This could be due to the sorption of particles to gills that does not travel further into the body of the fish. However, since the particle fraction associated with Cu and Al is low is this unlikely.

Table 4.4: Increase in mean tissue metal concentration in gill, kidney, and liver for metals Cu, Zn, and Al given in mmol metal/kg tissue. The increase is based on the average concentration of metal found for the exposure group subtracted by the average concentration found for control group (mean conc. in grey). Comparison between different nominal concentrations.

	Increase in tissue metal concentration (mmol/kg)								
	Gill			Kidney			Liver		
µmol metal/l water	Cu	Zn	Al	Cu	Zn	Al	Cu	Zn	Al
Control	0.028	10	0.25	0.10	4	0.057	4	1	0.08
0.4	0.008	-1	N.A	0.01	0	N.A	0	0	N.A
0.6	0.012	-1	N.A	-0.01	-1	N.A	-1	0	N.A
1.4	N.A	-2	0.11	N.A	-1	0.061	N.A	1	0.01
2.4	0.036	-3	0.24	0.00	0	0.026	1	0	0.02
4.3	0.049	0	0.29	0.00	2	0.032	1	0	0.07
7.8	0.075	0	0.30	0.00	1	0.041	0	0	0.03
15.6	N.A	0	0.52	N.A	0	0.034	N.A	0	0.01

4.4.7 Concentration factor

To obtain information on the accumulation of metals in fish tissue was a concentration factor calculated. The concentration factor is the relation between the difference in uptake of metal in the fish between the exposure group and the control group and the concentration of metals present in the water. From earlier observations was uptake only found for Cu and Al in the gills and Al in the kidneys.

The CF values found for Cu in gills exhibit a decreasing trend as water concentration of Cu increases, figure 4.4.7. The highest CF value, 22, is found at the lowest water concentration, 0.4 µmol Cu/l and lowest, 11, found for 7.8 µmol Cu/l, table 4.5. In comparison are the CF values of Al in gills not following a clear trend, figure 4.4.7. The highest CF value is found at the second-lowest concentration, 1.4 µmol Al/l at CF value 94. While the lowest CF value, 45, is found at the second-highest water concentration at 7.8 µmol/l, table 4.5.

The CF values found for Al in the kidney do not follow a decreasing trend, figure 4.4.8. The highest concentration is found for 1.7 µmol Al/l with a CF of 50, and lowest for 11.8 µmol with CF-value of 4, table 4.5. The low CF values for Al in kidneys are due to the low concentration measured in this tissue, compared to the concentration of Al in the water. Based on the results of CF is Al in the gills of the highest risk, and Cu in the gills of some risk for accumulation of in the tissues.

In general, biota regulates bioaccumulation of metals by bioaccumulation-controlling processes. This either by elimination, detoxification, or storage. They adapt to the environment based on the nutrients which are available to them (McGeer et al., 2003). The CF values are not solely based on exposure to the pollutant. Fish regulate the accumulation of metals based on the deficiency of an essential metal, or by eliminating or detoxifying the metal. A certain degree of accumulation is therefore normal and could be unrelated to the potential pollution.

It is worth noting that the CF is calculated using the <0.45 concentration of the metals present in the water. This is due to the uncertainty in the measured samples of LMM Cu. The results obtained from the 0.45 µm filter exhibit different trends in CF than for LMM for Cu. Among these results are overall higher CF found much lower at 0.45 than LMM for Cu. The error between the LMM fraction and 0.45 fraction was found in the range 15-91%, Appendix A.11. The CF for Al was found slightly lower at <0.45µm-fraction compared to the LMM fraction. The error between the fractions was found between 3-38% for both gills and liver, Appendix A.11.

The difference between LMM and <0.45µm fraction for Cu is large. The <0.45µm fraction was measured higher for Cu than the LMM fraction. This influences the CF as a higher concentration of Cu in the water decreases CF. However, it was earlier discussed that Cu may have been absorbed when ultrafiltrated, so the actual concentration of LMM Cu is uncertain. The CF results found for Cu would not be reported correctly if the LMM fraction were used. The <0.45µm fraction was measured similar to the measured LMM fraction for Al in the exposure water. The fraction 0.45 and LMM for Al in gills and kidney follows the same trend, with some exceptions. The error between 0.45 and LMM is the same for each group of gill and kidney. As the trend for Al does not vary considerably with a change fractions, and the CF only changes slightly is it highly likely that Al is found in the LMM fraction. Using the LMM fraction for calculating Al-CF does not change the results considerably.

Table 4.5. CF for Cu and Al in gills and Al in the kidney at comparable nominal concentrations.

Nominal water concentration	CF		
	Gill		Kidney
µmol/l	Cu	Al	Al
0.4	22	N.A	N.A
0.6	21	N.A	N.A
1.4	N.A	94	50
2.4	18	132	14
4.3	14	83	9
7.8	11	45	6
15.6	N.A	56	4

N.A – Not analyzed

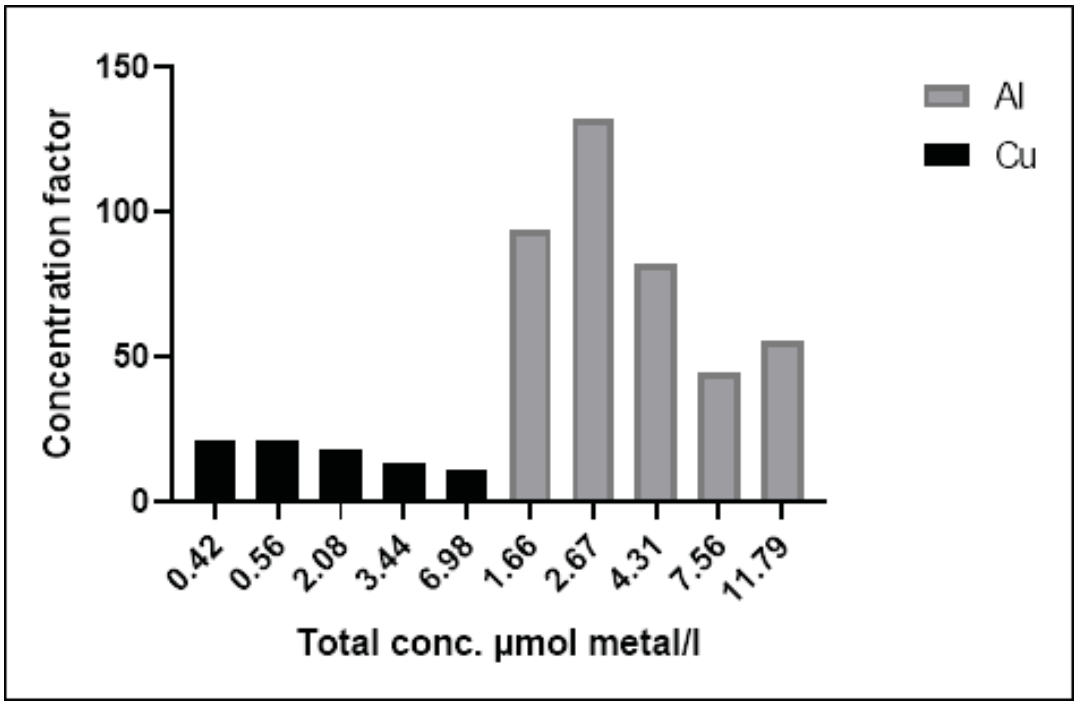

Figure 4.4.7: Concentration factor of Cu and Al in gills for different metal exposure concentrations

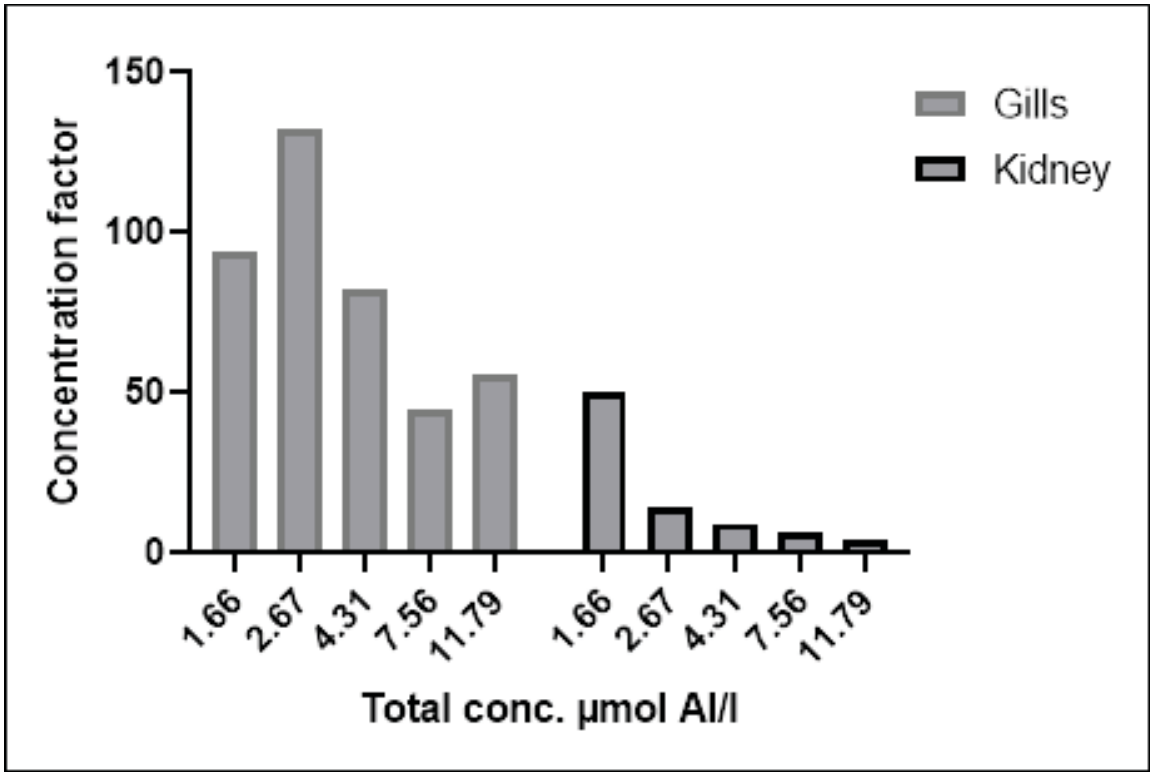

Figure 4.4.8. Concentration factors for Al in gills and kidney for different exposure concentrations.

4.4.8 Comparison of metal speciation on the uptake of metals in tissue

The speciation of Cu was earlier concluded to be uncertain. Comparing the uptake of Cu in tissue against the speciation may further prove this point. A significant difference from the control group was found for the four highest concentrations of copper; 0.56, 2.1, 3.4, and 7.0 µmol, figure 4.4.3a. The magnitude of which they increased from the control group was 1, 2, 3, and 4-times, respectively. The speciation of Cu indicated that of the four concentrations in question was copper associated with the colloidal fraction for the two lowest and the LMM fraction for the two greater concentrations. Either is the colloidal fraction more bioavailable than assumed, or the fractionation of copper species is faulty. If the former is true, a greater concentration of copper is assumed in the kidney and liver , and not just in the gills.

The distribution of Zn-species was largely found in the LMM fraction. Except for the lowest concentration, which had an almost even split of Zn associated with colloidal and LMM

fraction. This indicated that Zn was bioavailable. A significant difference from the control group was found in the liver for concentrations 1.02 and 1.72 µmol. However, the increase of which these increased from the control group was low. The uptake of Zn in the fish was therefore concluded to be non-existent, despite the speciation indicating that Zn was bioavailable. The lack of uptake of Zn could be due to the competition with salts in the water. It is highly likely that Ca present in the water essentially protected the fish against the uptake of the metal.

The speciation of Al, on the other hand, was overall found to be in LMM fraction for all exposure concentrations. This also reflects on the uptake of Al in the fish. A significant difference from the control group was found for several concentrations in both the gills and the two exposure concentrations in the kidneys. A clear trend can be seen for the gills, in which the increase of Al in the water gave a significant increase in Al concentration in the tissue as well. However, the same clear trend was not found for kidneys. It is not clear as of why the two concentrations; 1.7 and 7.6 µmol Al/l had such a significant increase. It is worth noting that the variation of Al concentration in the tissue is large. The increase from the control group for gills and kidneys was of a magnitude of 2 or 3 times for all concentrations, except for kidneys concentration 2.7 µmol Al/l.

The metals, Cu, Zn, and Al are bioavailable based on the results on speciation. However, due to the low content of organic material is this conclusion only applicable to coastal waters with low organic content. Despite the metals being bioavailable was internal uptake not an issue. In general, there was no uptake of Zn at acute exposure in brackish waters. High concentrations of Ca likely competed with binding-sites, therefore, mitigating the uptake. There was some uptake of Cu on the gills at high acute exposure concentrations and based on the trend of the results the uptake increases with Cu concentrations in the environment. There was uptake of Al at acute exposures in the gills, and some in the kidneys. Based on the results of this thesis can the following be concluded; the metals are bioavailable and uptake at acute exposure are plausible. The internal uptake at acute exposures are negligible, but longer exposure could accumulate metals in internal tissues. Coastal waters contaminated with high concentrations of Cu or Al, therefore, pose some risk for fish at acute exposure.

5 Conclusion and further work

This study set out to identify the speciation of trace metals in coastal systems and uptake of these metals in fish.

H_0: There is a difference in the speciation of copper, zinc, and aluminum in the water.
Based on the literature the study predicted that there is a difference in metal speciation in coastal water. The results showed that the speciation of zinc and aluminum was mostly associated with the low molecular mass-species in brackish water, >78%, and >83% of the total, respectively. While the speciation of copper varied greatly with different copper concentrations. In general, was Cu associated with the colloidal or LMM-fraction in brackish water. A low association with particles was found for all three metals, <14% of the total. Based on these results are the metals predicted to be bioavailable. A difference in speciation was found between the LMM fraction for Cu and the LMM fraction for the two other metals. However, due to possible sorption of Cu associated with LMM this fraction could be much higher than measured for this thesis. The results, therefore, reveal that all three metals had relative similar speciation and were assumed bioavailable for uptake.

H_1: The trace metals copper, zinc, or aluminum in coastal water can be taken up in fish.
The results demonstrated that copper and aluminum can be taken up in Atlantic Salmon smolts in brackish waters. While the uptake of Zn was not considered significant. The overall internal uptake was negligible for the water parameters set for this experiment (e.g. seawater, 20ppt, pH 8). One reason behind a low internal uptake could be due to salts (Ca and Mg) present in the water, which competes for binding sites. The uptake of metals on fish followed the order, Al>Cu>Zn, where gills were the tissue with the highest uptake, kidneys intermediate and the liver had no uptake. The uptake of Cu was only significantly higher for the four highest concentrations in the exposure water. The results indicate that the trace metals copper and aluminum can be taken up in fish at high water concentrations.

H_2: There is a difference in uptake of copper, zinc, and aluminum in different tissues.
A difference in uptake in the tissues was found between the different metals. Uptake of Cu and Al in gills was significantly higher than Zn. No uptake of Zn was determined in the gills. The uptake of Cu on gills increased by 0.036-0.075 mmol/kg from the control group for the

three highest concentrations. At comparable molar concentrations increased the uptake of Al on gills by 0.24-0.30 mmol/kg from the control group. No uptake of Cu and Zn in kidneys was found. Uptake of Al increased by 0.026-0.061 mmol/kg from the control group in the kidneys. However, due to the large variation in these groups the results are uncertain. No metal uptake was discovered for the liver.

To conclude; the metals Cu, Al, and Zn were determined to be bioavailable for uptake in brackish waters. The results indicated that the internal uptake of these metals was not an issue at acute 96 hours exposure. There was significant uptake of Cu and Al on the gills at the highest exposure concentrations. At high concentrations of Cu and Al there is a risk of uptake in fish in coastal waters. The study successfully determined the distribution of metal species in water and successfully measured and quantified the concentration and uptake of metals in tissues of Atlantic salmons smolts. The results of this study provide further insight and knowledge of trace metal speciation in brackish water. This study also adds to the literature on the uptake of trace metals in fish in brackish waters.

5.1 Further work

The work of the thesis was limited both due to practical limitations and lack of time. Future work includes deeper analysis and some new proposals.

1. The speciation of copper in the exposure water was concluded to be uncertain, as the LMM fraction was measured lower than expected. This can be due to the adsorption of copper to the filter used for ultrafiltration. To verify if there was adsorption of Cu in the filter could an additional analysis of ultrafiltrated exposure water with wash water samples reveal if this was the case. This supports or undermine the results used for the speciation of copper in this thesis.

2. There seldom are only one pollutant in natural systems. Interaction effects are therefore highly likely to occur. These effects include synergistic, antagonistic, or additive interactions. Further work could develop further on this study to include exposure with mixtures of metals. Based on the work of this thesis, Cu and Al are viable options at similar molar concentrations for further experiments.

3. This study was conducted with a salinity of 20‰. Salinity in estuarine environments varies considerably. There is a possible protective effect of salts on the uptake of metals in fish. Further work could develop further on this study to investigate the uptake of metals in brackish water with lower salinity. This investigates the protective effect of salinity on the uptake.

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Appendix

Appendix A.1 – LOD/LOQ Water samples

Five blank samples were used to estimate for calculation of the limit of quantification (LOQ) and limit of detection (LOD). An example is given in eq. 7 for LOD, and eq. 8 for LOQ. The data used to calculate to LOD and LOQ are presented in Table A.1.

Eq. 7 $$LOD = 3 \times Standard\ deviation\ of\ Blanks$$

$$LOD = 3 \times 0.0050 = 0.01\ \mu g/l$$

Eq. 8 $$LOQ = 10 * STD\ Blanks$$

$$LOQ = 10 \times 0.0050 = 0.050$$

Table A.1: Measured blank water samples of Cu, Zn, and Al with Average, and standard deviation. LOD/LOQ given in µg/l and µmol/l.

	Cu		Zn		Al	
Sample Name	µg/l	µmol/l	µg/l	µmol/l	µg/l	µmol/l
blank 1	0.1		0.1342352		N.A	
blank 2	0.01274009		0.0033364		0.0625	
blank 3	0.01102237		0.0036854		0.0625	
blank 4	0.00225489		0.0012031		0.0623	
blank 5	0.01254992		0.0021162		0.0625	
Average	0.010	0.00015	0.003	0.00004	0.06245	0.002315
Standard Deviation	0.0050	0.000078	0.0011	0.000017	1E-04	0.0000037
Limit of detection, LOD (w/V) (mol/V)	**0.01**	**0.00024**	**0.003**	**0.00012**	0.0003	**0.000011**
Limit of quantification, LOQ (w/V) (mol/V)	**0.050**	**0.00078**	**0.011**	**0.00017**	0.001	**0.000037**

Appendix A.2 – LOD/LOQ Fish tissue

Five blank samples were used to estimate for calculation of the limit of quantification (LOQ) and limit of detection (LOD). An example is given in eq. 7 for LOD, and eq. 8 for LOQ. The data used to calculate to LOD and LOQ are presented in Table A.2. Fish tissue samples were measured in three batches therefore measuring blank samples three times. From the three batches was one LOD/LOQ chosen for each metal based on the highest LOD/LOQ.

Eq. 7 $$LOD = 3 \times Standard\ deviation\ of\ Blanks$$

$$LOD = 3 \times 0.0075 = 0.022$$

Eq. 8 $$LOQ = 10 * STD\ Blanks$$

$$LOQ = 10 \times 0.0075 = 0.075$$

Table A.2: Measured blank samples of fish tissue with average and standard deviation. LOD/LOQ are given in mg/kg.

Al + CuZn control	Cu	Zn	Al	CuZn C1-C5	Cu	Zn	CuZn C6-C8	Cu	Zn
Sample Name	mg/kg	mg/kg	mg/kg	Sample Name	mg/kg	mg/kg	Sample Name	mg/kg	mg/kg
Blank 1	2.37E-07	3.15E-07	1.22E-07	Blank 1	0.02413	0.02877	Blank 1	0.07457	-0.234
Blank 2	1.36E-07	3.93E-07	1.61E-07	Blank 2	0.03590	0.05540	Blank 2	0.01429	-0.099
Blank 3	9.35E-08	1.13E-07	7.79E-08	Blank 3	0.02055	0.07890	Blank 3	0.03453	-0.082
Blank 4	1.19E-07	2.29E-07	2.41E-07	Blank 4	0.01711	0.02109	Blank 4	0.01509	-0.317
Blank 5	1.43E-07	2.63E-07	1.61E-06	Blank 5	0.02996	0.01603	Blank 5	-0.01183	-0.186
Average	1E-07	3E-07	4E-07	Average	0.026	0.04	Average	0.03	- 0.18
SD	5.5E-08	1.0E-07	6.6E-07	SD	0.0075	0.026	SD	0.032	0.097
Limit of detection, LOD (w/w)	**1.6E-07**	**3.1E-07**	**2.0E-06**	**Limit of detection, LOD (w/w)**	**0.022**	**0.079**	**Limit of detection, LOD (w/w)**	**0.096**	**0.29**
Limit of quantification, LOQ (w/w)	**5.5E-07**	**1.0E-06**	**6.6E-06**	**Limit of quantification, LOQ (w/w)**	**0.075**	**0.265**	**Limit of quantification, LOQ (w/w)**	**0.32**	**0.97**

Appendix A.3 – Traceability of analysis

The traceability of the analysis was checked using CRM's 1640a for water samples and ERM-BB422 and DOLT-5 for fish tissue samples. All measured samples with average, standard deviation, maximum and minimum values are given in table A.3.1 and A.3.2

Table A.3.1: Measured samples of CRM 1640a, maximum, minimum, average, and standard deviation values are given. Error in percentage from certified values is given. All values are given in µg/l.

CRM 1640a	Cu µg/L	Error %	Zn µg/L	Error %	Al µg/L	Error %
Sample 1	96.6	12 %	58.0	4 %	65	19 %
Sample 2	92.7	7 %	57.6	3 %	61	11 %
Sample 3	91.7	6 %	57.2	2 %	62	13 %
Sample 4	82.1	-4 %	52.1	-6 %	56	2 %
Sample 5	82.4	-3 %	56.8	1 %	70	28 %
Sample 6	83.9	-2 %	56.6	1 %	56	2 %
Sample 7	92.1	7 %	57.0	2 %	61	11 %
Minimum	82		52		56	
Maxium	97		58		70	
Average	89		56		62	
SD	5.8		2.0		5.1	

Table A.3.2: Measured samples of CRM ERM-BB422 and DOLT-5. Average, SD, Maximum and minimum values are given. Error in percentage from certified values for each sample is given. All values are reported as mg/kg.

ERM-BB422	Cu mg/kg	Error %	Zn mg/kg	Error %	DOLT-5	Cu mg/kg	Error %	Zn mg/kg	Error %	Al mg/kg	Error %
Sample 1	1.64	-2 %	16.8	5 %	Sample 1	34.1	-3 %	105	0 %	16.5	-40 %
Sample 2	1.66	-1 %	16.1	1 %	Sample 2	33	-6 %	102	-3 %	22.2	-19 %
Sample 3	1.83	10 %	17.7	11 %	Sample 3	39.5	13 %	121	15 %	34.8	10 %
Sample 4	1.71	2 %	16.8	5 %	Sample 4	33.8	-3 %	106	1 %	16.7	-39 %
Sample 5	1.68	1 %	16.9	6 %	Sample 5	34.4	-2 %	106	1 %	17.9	-35 %
Average	1.70		16.9		Average	35		108		22	
SD	0.075		0.57		SD	2.6		7.4		7.7	
Min	1.64		16.1		Min	33		102		16.5	
Max	1.83		17.7		Max	39.5		121		34.8	

Appendix A.4 Water Quality Parameters

The temperature of the water in the tanks was logged each hour of the exposure, each 24h-interval of temperature is recorded in table A.4.1. Salinity and pH were measured once daily, and ammonia was measured at the end of the exposure, table A.4.1. Ca, K, and Mg was measured after exposure in unfiltered water samples for the total concentration of each metal. Due to the number of samples measured (three replicates of each sample) are the average of each given in table A.4.2.

Table A.4.1: Water quality parameters; Temperature (in Celsius), salinity (in %), pH, and ammonia (g/l) measured in each tank at 24h-intervals.

	28.10.2019	29.10.2019	30.10.2019	31.10.2019	01.11.2019	28.10.2019	29.10.2019	30.10.2019	31.10.2019	01.11.2019	28.10.2019	29.10.2019	30.10.2019	31.10.2019	01.11.2019	01.11.2019
	0h	24h	48h	72h	96h	0h	24h	48h	72h	96h	0h	24h	48h	72h	96h	96h
Exposure group	T 'C	T 'C	T 'C	T 'C	T 'C	salinity	salinity	salinity	salinity	salinity	pH	pH	pH	pH	pH	ammonia
Al control	11139	10944	10553	10553	10553	20.0	20.0	20.1	20.1	20.2	8.16	8.08	8.16	8.19	8.14	not meassured
Al C1	10944	10944	10651	10553	10455	20.0	20.1	20.1	20.1	20.1	8.16	8.1	8.15	8.19	8.17	not meassured
Al C2	11236	11041	10748	10651	10553	20.1	20.0	20.0	20.3	20.2	8.18	8.15	8.2	8.19	8.17	not meassured
Al C3	11236	11041	10651	10651	10553	19.9	20.0	20.1	20.1	20.1	8.14	8.14	8.15	8.17	8.16	not meassured
Al C4	11139	10944	10553	10553	10553	19.9	19.9	20.0	20.0	20.1	8.16	8.09	8.17	8.19	8.18	not meassured
Al C5	11236	11041	10651	10651	10553	19.9	20.0	20.0	20.1	20.1	8.12	8.11	8.13	8.19	8.21	not meassured
	04.11.2019	05.11.2019	06.11.2019	07.11.2019	08.11.2019	04.11.2019	05.11.2019	06.11.2019	07.11.2019	08.11.2019	04.11.2019	05.11.2019	06.11.2019	07.11.2019	08.11.2019	08.11.2019
	0h	24h	48h	72h	96h	0h	24h	48h	72h	96h	0h	24h	48h	72h	96h	96h
Exposure group	T 'C	T 'C	T 'C	T 'C	T 'C	salinity	salinity	salinity	salinity	salinity	pH	pH	pH	pH	pH	ammonia
Zn/Cu control	10846	10846	10651	10259	10161	20	20	20.1	20.1	20.3	8.13	8.18	8.17	8.2	8.17	0.21
Zn C1	10748	10748	10553	10161	9965	20.1	20	20.1	20.2	20.2	8.15	8.16	8.15	8.18	8.18	0.16
Zn C2	10846	10846	10651	10259	10063	20.2	20.2	20.1	20.2	20.2	8.15	8.17	8.19	8.17	8.2	0.19
Zn C3	10846	10846	10651	10259	10063	20	20.1	20.1	20.2	20.2	8.12	8.18	8.19	8.17	8.18	0.23
Zn C4						20	20	20	20.1	20.1	8.16	8.17	8.17	8.18	8.2	0.22
Zn C5	10846	10846	10748	10357	10063	20	20	20.1	20.1	20.3	8.17	8.14	8.17	8.2	8.17	0.19
Cu C1		10846	10748	10553	10063	20	20.1	20	20.1	20.2	8.17	8.18	8.2	8.18	8.2	0.2
Cu C2	10846	10846	10651	10259	10063	20.1	20.1	20.2	20.1	20.2	8.16	8.16	8.18	8.16	8.21	0.17
Cu C3	10944	10944	10748	10357	10063	20	20	20.1	20.2	20.1	8.17	8.18	8.18	8.22	8.2	0.17
Cu C4	11139	11041	10944	10553	10259	19.9	20	20.1	20.2	20.3	8.15	8.14	8.19	8.17	8.19	0.18
Cu C5	11139	11139	10944	10455	10259	19.9	19.9	19.9	20	20.1	8.16	8.18	8.17	8.16	8.18	0.19
	12.12.2019	13.12.2020	14.12.2020	15.12.2020	16.12.2020	12.12.2020	13.12.2020	14.12.2020	15.12.2020	16.12.2020	12.12.2020	13.12.2020	14.12.2020	15.12.2020	16.12.2020	16.12.2020
	0h	24h	48h	72h	96h	0h	24h	48h	72h	96h	0h	24h	48h	72h	96h	96h
Exposure group	T 'C	T 'C	T 'C	T 'C	T 'C	salinity	salinity	salinity	salinity	salinity	pH	pH	pH	pH	pH	ammonia
Cu/Zn control	8779	9077	9373	9571	9669	20	19.9	20	20	20.1	8.17	8.18	8.16	8.21	8.17	0.27
Cu C6	8680	8978	9275	9472	9571	20	20	20	20.1	20.1	8.17	8.18	8.11	8.17	8.1	0.28
Cu C7	8481	8779	9077	9275	9275	20	20	20.1	20.2	20.3	8.17	8.19	8.17	8.1	8.17	0.29
Cu C8	8.7	8879	9176	9373	9472	20.1	20	20	20.1	20.2	8.18	8.17	8.16	8.19	8.18	0.29
Zn C6	8.581	8.879	9.176	9.373	9.472	19.9	20	20.2	20.1	20.2	8.16	8.18	8.16	8.17	8.19	0.29
Zn C7	8.581	8.879	9.176	9.373	9.472	19.9	20	20.1	20.1	20.1	8.18	8.18	8.17	8.18	8.17	0.32
Zn C8	8.68	8.978	9.275	9.472	9.571	19.9	20.1	20.1	20.1	20.1	8.19	8.19	8.22	8.18	8.18	0.25

Table A.4.2: Average measured values (n=3) of K, Ca, and Mg in water samples collected at 0-96h for different metal exposure.

Zn		K	Ca	Mg
Time	Sample Name	Conc. [mg/l]	Conc. [mg/l]	Conc. [mg/l]
start 0h	Cu/Zn control	202.264	378.499	889.529
start 0h	Zn C1	202.252	383.892	883.912
start 0h	Zn C2	199.006	372.414	877.794
start 0h	Zn C3	200.908	360.900	895.590
start 0h	Zn C4	200.180	365.429	893.541
start 0h	Zn C5	199.376	358.111	874.472
exp. 24h	Cu/Zn control	180.509	352.160	892.420
exp. 24h	Zn C1	195.516	363.118	893.898
exp. 24h	Zn C2	195.019	352.466	900.963
exp. 24h	Zn C3	194.665	359.062	900.928
exp. 24h	Zn C4	193.974	356.829	897.953
exp. 24h	Zn C5	195.862	354.538	908.778
exp. 48h	Cu/Zn control	209.167	396.607	970.739
exp. 48h	Zn C1	204.132	376.671	936.254
exp. 48h	Zn C2	212.188	401.373	973.153
exp. 48h	Zn C3	205.931	395.819	942.930
exp. 48h	Zn C4	205.913	382.496	935.187
exp. 48h	Zn C5	210.684	399.874	977.646
exp. 72h	Cu/Zn control	188.572	333.924	844.606
exp. 72h	Zn C1	192.394	337.649	855.123
exp. 72h	Zn C2	188.791	339.403	844.742
exp. 72h	Zn C3	189.263	346.490	857.093
exp. 72h	Zn C4	187.517	336.527	850.215
exp. 72h	Zn C5	189.752	344.947	855.342
end 96h	Cu/Zn control	192.681	354.034	870.038
end 96h	Zn C1	190.590	349.688	856.985
end 96h	Zn C2	189.684	351.300	851.071
end 96h	Zn C3	190.225	349.540	850.833
end 96h	Zn C4	187.027	339.579	833.327
end 96h	Zn C5	185.162	335.671	819.750
end 96h	Zn C6	180.732	333.722	721.368
end 96h	Zn C8	193.285	358.402	777.429

Al		K	Ca	Mg
Time	Sample Name	Conc. [mg/l]	Conc. [mg/l]	Conc. [mg/l]
start 0h	Al ccontrol	201.054	386.061	883.128
start 0h	Al C1	199.067	390.570	881.836
start 0h	Al C2	199.175	398.484	871.935
start 0h	Al C3	196.271	377.726	895.205
start 0h	Al C4	193.956	371.556	864.552
start 0h	Al C5	194.496	385.638	861.341
exp. 24h	Al ccontrol	197.536	411.864	924.621
exp. 24h	Al C1	195.040	400.028	921.581
exp. 24h	Al C2	194.956	385.766	919.631
exp. 24h	Al C3	196.574	400.076	924.350
exp. 24h	Al C4	192.996	390.701	914.827
exp. 24h	Al C5	199.082	383.609	939.408
exp. 48h	Al ccontrol	199.720	409.066	953.717
exp. 48h	Al C1	202.487	419.201	970.098
exp. 48h	Al C2	196.288	384.500	922.073
exp. 48h	Al C3	197.859	395.302	940.286
exp. 48h	Al C4	199.786	395.079	945.220
exp. 48h	Al C5	199.895	391.954	951.245
exp. 72h	Al ccontrol	198.360	397.461	954.983
exp. 72h	Al C1	200.309	397.162	961.127
exp. 72h	Al C2	246.357	395.215	935.564
exp. 72h	Al C3	197.868	388.444	947.095
exp. 72h	Al C4	197.443	393.372	933.306
exp. 72h	Al C5	195.987	374.765	930.122
end 96h	Al ccontrol	193.815	385.867	921.286
end 96h	Al C1	196.910	394.176	954.148
end 96h	Al C2	196.787	396.160	935.039
end 96h	Al C3	197.722	393.388	945.333
end 96h	Al C4	193.077	374.717	922.768
end 96h	Al C5	192.899	382.338	915.027

Cu		K	Ca	Mg
Time	Sample Name	Conc. [mg/l]	Conc. [mg/l]	Conc. [mg/l]
start 0h	Cu C1	203.144	369.285	902.035
start 0h	Cu C2	202.317	367.834	903.490
start 0h	Cu C3	203.736	369.826	903.702
start 0h	Cu C4	202.785	376.333	903.933
start 0h	Cu C5	202.200	366.577	909.603
exp. 24h	Cu C1	198.011	357.481	923.746
exp. 24h	Cu C2	194.668	358.786	907.059
exp. 24h	Cu C3	194.292	359.199	895.926
exp. 24h	Cu C4	191.447	354.239	904.198
exp. 24h	Cu C5	193.315	350.960	896.140
exp. 48h	Cu C1	209.346	412.387	964.085
exp. 48h	Cu C2	197.207	387.771	907.373
exp. 48h	Cu C3	193.362	374.604	882.478
exp. 48h	Cu C4	190.532	381.539	870.601
exp. 48h	Cu C5	197.396	379.898	892.153
exp. 72h	Cu C1	190.826	344.840	861.675
exp. 72h	Cu C2	189.770	347.024	865.426
exp. 72h	Cu C3	190.366	341.834	853.535
exp. 72h	Cu C4	191.090	357.525	864.690
exp. 72h	Cu C5	192.427	354.104	878.053
end 96h	Cu C1	192.910	353.751	859.123
end 96h	Cu C2	185.789	335.105	814.015
end 96h	Cu C3	188.148	339.081	838.540
end 96h	Cu C4	188.835	347.177	857.156
end 96h	Cu C5	190.094	344.197	847.504

Appendix A.5 – Water samples analysis

Each water sample had three replicates. The mean and standard deviation of each sample unfiltered, 0.45 µm filtered, hollow-fiber ultrafiltration (HF), and chelex filtration, at 0h and 96h, are given in table A.5.1 (Cu), table A.5.2 (Zn), and table A.5.3 (Al).

Table A.5.1: Average, SD, and n of Cu measured in water samples for each exposure group, at 0h and 96h. Mean values are given in µg/l.

	Unfiltered (µg/l)			0.45µm (µg/l)			HF (µg/l)			Chelex (µg/l)		
0 h	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Control	2.3	0.64	6	2.9	0.58	6	1.60	0.984	5	1.06	0.185	6
Cu C1	5.8	0.96	3	4.9	0.42	3	2.4	0.44	3	<LOD		0
Cu C2	9.1	0.49	3	9.1	0.12	3	1.4	0.20	3	<LOD		0
Cu C3	17.0	0.31	3	16.3	0.17	3	1.9	0.071	2	<LOD		0
Cu C4	28	1.7	3	29.2	0.36	3	4.3	0.15	3	<LOD		0
Cu C5	39.0	0.21	2	45.4	11	3	18.2	0.91	3	<LOD		0
Cu C6	130	1.5	3	121	1.5	3	20.3	0.15	3	3.6	0.21	3
Cu C7	194.3	0.58	3	217	1.0	3	168	1.5	3	1.63	0.035	3
Cu C8	425	2.0	3	427	9.3	3	372	1.2	3	0.10	0.031	3
	Unfiltered (µg/l)			0.45µm (µg/l)			HF (µg/l)			Chelex (µg/l)		
96h	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Control	2.9	0.52	6	2.6	0.56	6	2.2	0.76	6	1.96	1.461	6
Cu C1	4.4	0.044	3	3.6	0.23	3	1.5	0.02	3	0.73	0.034	2
Cu C2	8.3	0.38	3	7.6	0.12	3	2.0	0.09	3	<LOD		0
Cu C3	14.2	0.52	3	13.5	0.10	3	2.7	0.02	3	<LOD		0
Cu C4	25.1	0.21	3	23.4	0.32	3	4.0	0.07	3	<LOD		0
Cu C5	33.2	0.40	3	31.2	0.49	3	5.3	0.40	3	<LOD		0
Cu C6	135	1.2	3	130.67	0.577	3	7.28	0.059	3	2.80	0.049	3
Cu C7	243	1.2	3	239.00	4.6	3	146	1.0	3	4.0	0.45	3
Cu C8	462	3.6	3	443.67	3.5	3	370.7	0.58	3	4.18	0.079	3

Table A.5.2: Average, SD, and n of Zn measured in water samples for each exposure group, at 0h and 96h. Mean values are given in µg/l.

	Unfiltered			0.45			HF			Chelex		
0h	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Control 1	5.6	0.94	6	5.8	0.72	6	3.1	0.89	6	2.96	2.138	6
Zn C1	18.4	0.78	2	17.6	0.12	3	8	2.5	3	1.0	0.000	1
Zn C2	29.6	0.60	3	30	2.8	3	26	6.3	3	<LOD		0
Zn C3	43.7	0.40	3	42	1.4	3	37	1.0	3	<LOD		0
Zn C4	68.5	0.67	3	68.5	0.76	3	61	2.5	3	<LOD		0
Zn C5	116	1.0	3	114	5.2	3	107	6.1	3	<LOD		0
Zn C6	274	6.0	3	275	1.5	3	249	1.0	3	28.3	0.25	3
Zn C7	527.3	0.58	3	536	1.5	3	528	1.0	3	5	4.1	3
Zn C8	1030	10	3	1067	5.8	3	1060	10	3	1.4	0.11	3
	Unfiltered			0.45			HF			Chelex		
96h	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Control 1	7.0	2.96	6	6.29	1.670	5	2.40	1.13	4	1.02	0.066	3
Zn C1	17.0	0.46	3	15.40	0.62	3	5.80	0.43	3	<LOD		0
Zn C2	28.8	0.35	3	27.00	0.10	3	20.07	0.68	3	<LOD		0
Zn C3	40.7	0.12	3	38.30	1.9	3	35.03	0.90	3	<LOD		0
Zn C4	64	1.2	3	62.00	1.2	3	59	3.5	3	<LOD		0
Zn C5	108	2.5	3	105.00	1.0	3	102	1.0	3	<LOD		0
Zn C6	294.7	0.58	3	294	2.1	3	275.9	0.47	3	29.5	0.15	3
Zn C7	550	1.5	3	546	2.3	3	458	3.1	3	8.58	0.092	2
Zn C8	1023	15	3	1008	17	3	998	13	3	10.5	0.82	3

Appendix A.5 – Cont.

Table A.5.3: Average, SD, and n of Al measured in water samples for each exposure group, at 0h and 96h. Mean values are given in µg/l.

	Unfiltered			0.45			HF			Chelex		
0h	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Control 1	22.1	0.93	3	19.9	0.35	3	21	1.7	3	5.1	0.30	2
Al C1	47	2.1	3	64	18	3	42	1.7	3	6.6	0.25	3
Al C2	79	9.9	3	71	5.3	3	67	4.5	3	10.6	0.12	3
Al C3	119	1.7	3	116	6.4	3	117	2.3	3	23.5	0.53	3
Al C4	200	13	3	201	3.5	3	203.2	0.27	3	46	2.7	3
Al C5	319	5.7	3	283	17	3	299	2.6	3	71	4.5	3
	Unfiltered			0.45			HF			Chelex		
96h	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Control 1	21	3.0	3	20	3.0	3	15.8	0.26	3	15.2	0.67	3
Al C1	42	2.7	3	42	0.6	3	34	4.0	3	18.0	1.84	3
Al C2	65.1	0.23	3	67	0.6	3	157	2.8	2	30.5	5.58	3
Al C3	113	4.5	3	112	8.4	3	103	2.1	3	39.2	1.81	3
Al C4	208	1.2	3	199	12	3	173	9.3	3	54.7	0.07	2
Al C5	318	7.8	2	262	18	3	268	3.6	3	82.7	2.61	3

Correlation	A X vs. Cu	B X vs. Zn	C X vs. Al
Pearson r			
r	0.9978	0.9985	0.9908
95% confidence interval	0.9649 to 0.9999	0.9894 to 0.9998	0.8621 to 0.9994
R squared	0.9955	0.9970	0.9816

Figure A.5: R-squared and correlation R of expected nominal concentration and the measured concentration of Cu, Zn and Al in the exposure water. Screenshot from Graphpad Prism.

Appendix A.6 – Metal speciation

The speciation of metals was determined by calculating the different fractions by eq. 2-4. The species were then calculated to a percentage of the total concentration. In the case when the total sum of all fraction surpassed 100% was the percentage colloidal fraction determined by subtracting the particle and LMM- fraction from total 100%. In the case of LMM results surpassing total, they adjusted down to 100%. The LMM cations are calculated as the percentage of the LMM fraction. The percentage for each average fraction of each metal at 0h and 96 hours are given in table A.6 (Cu, Zn, and Al).

Table A.6: Percentage of total for species of Cu, Zn and Al for each exposure group given in total conc. (µmol/l). Bold letters denotes the results have been modified to fit a sum of 100% in total. LMM cations are calculated as a percentage of LMM fraction.

Cu µmol/l	Particles	Colloids	LMM	LMM cations
Control	0 %	**24 %**	76 %	22 %
0.08	16 %	46 %	38 %	62 %
0.14	4 %	76 %	20 %	100 %
0.25	5 %	80 %	15 %	100 %
0.42	1 %	83 %	16 %	100 %
0.56	0 %	69 %	31 %	100 %
2.08	5 %	85 %	10 %	77 %
3.44	0 %	32 %	72 %	98 %
6.98	2 %	15 %	84 %	99 %
Zn µmol/l	**Particles**	**Colloids**	**LMM**	**LMM cations**
Control	0 %	51 %	44 %	17 %
0.27	6 %	55 %	40 %	85 %
0.45	3 %	19 %	78 %	100 %
0.64	4 %	10 %	85 %	100 %
1.02	2 %	8 %	90 %	100 %
1.72	2 %	4 %	93 %	100 %
4.35	0 %	8 %	92 %	89 %
8.24	0 %	9 %	92 %	99 %
15.70	0 %	1 %	100 %	99 %
Al µmol/l	**Particles**	**Colloids**	**LMM**	**LMM cations**
Control	7 %	10 %	83 %	38 %
1.66	0 %	**14 %**	86 %	68 %
2.67	4 %	0 %	**100 %**	73 %
4.31	2 %	3 %	95 %	72 %
7.56	2 %	6 %	92 %	74 %
11.79	14 %	0 %	89 %	73 %

Appendix A.7 – Fish characteristic

Fish were weighed and measured before dissection, all weights and lengths for each fish are summarized in table A.7. Any missing fish are marked with grey spaces.

Table A.7: Weight (grams) and length (cm) of each fish.

Metal exposure	Fish nr	Weight (g)	Length (cm)	Metal exposure	Fish nr	Weight (g)	Length (cm)	Metal exposure	Fish nr	Weight (g)	Length (cm)
Cu C5	119	69.1	19.1	Zn C5	84	82.3	21	Cu/Zn Control	49	64	18.5
Cu C5	118	71.9	20	Zn C5	83	55.5	18.2	Cu/Zn Control	48	74.9	19.7
Cu C5	117	71.4	19.8	Zn C5	82	73.7	19.5	Cu/Zn Control	47	69.2	19.3
Cu C5	116	75	20.3	Zn C5	81	79.9	21	Cu/Zn Control	46	84.5	20.5
Cu C5	115	84.2	20.5	Zn C5	80	76.9	20.5	Cu/Zn Control	45	94.4	21.2
Cu C5	114	73.9	19.5	Zn C5	79	77.4	20	Cu/Zn Control	44	76.1	19.5
Cu C5	113	81.3	20.5	Zn C5	78	63.1	19.3	Cu/Zn Control	43	60.8	18.5
Cu C4	112	82.1	20.4	Zn C4	77			Cu/Zn Control	120	68.08	18.5
Cu C4	111	69.6	19.6	Zn C4	76	87	21.5	Cu/Zn Control	121	83.08	20
Cu C4	110	65.7	19.2	Zn C4	75	84.8	20.8	Cu/Zn Control	122	91.34	21
Cu C4	109	58	18	Zn C4	74	75.6	20.5	Cu/Zn Control	123	92.4	21
Cu C4	108	78	20.3	Zn C4	73	87.5	21.3	Cu/Zn Control	124	85.77	20.7
Cu C4	107	88.9	20.7	Zn C4	72	76.4	20.5	Cu/Zn Control	125	72.75	19.6
Cu C4	106	87	20.6	Zn C4	71	61.1	19.2	Cu/Zn Control	126	82.82	20.5
Cu C3	105	88.5	20.7	Zn C3	70	79.8	19.8	Al Control	7	89.3	20.7
Cu C3	104	79.8	20.2	Zn C3	69	69.5	19.5	Al Control	6	69.3	18.9
Cu C3	103	66.2	19.5	Zn C3	68	91.5	21.7	Al Control	5	79.8	19.8
Cu C3	102	60.8	18.5	Zn C3	67	61.1	18.5	Al Control	4	85.2	20.5
Cu C3	101	62.4	18.7	Zn C3	66	93.8	21.8	Al Control	3	74.8	19.4
Cu C3	100	61.8	19.4	Zn C3	65	68.8	19.5	Al Control	2	95.3	21
Cu C3	99	96	22	Zn C3	64	60	18.7	Al Control	1	78.3	18.5
Cu C2	98			Zn C2	63	83.4	20	Al C5	14	55.9	17.4
Cu C2	97	84.7	21.1	Zn C2	62	92.7	21.3	Al C5	13	57.6	17.8
Cu C2	96	75.8	20.4	Zn C2	61	55.9	17.5	Al C5	12	67.9	19
Cu C2	95	81.3	21.3	Zn C2	60	82.9	20.6	Al C5	11	64.3	18.4
Cu C2	94	63.2	18.6	Zn C2	59	80.7	20.4	Al C5	10	69.3	19
Cu C2	93	69	19.5	Zn C2	58	82	19.8	Al C5	9	62.5	17.4
Cu C2	92	70.9	19.8	Zn C2	57	80	19.5	Al C5	8	68.5	19
Cu C1	91			Zn C1	56	73.8	20.7	Al C4	21	73	19.6
Cu C1	90	83.9	20.1	Zn C1	55	62.1	18.8	Al C4	20	65.1	18.8
Cu C1	89	61.7	19	Zn C1	54	74.3	19	Al C4	19	88.1	19.6
Cu C1	88	86.8	21.7	Zn C1	53	82	20	Al C4	18	56.5	17.8
Cu C1	87	87.5	21.3	Zn C1	52	73.2	19.3	Al C4	17	79.7	19
Cu C1	86	62.7	18.5	Zn C1	51	58.2	18.2	Al C4	16	63.1	18.5
Cu C1	85	88.6	21.5	Zn C1	50	56.3	18.5	Al C4	15	104.5	21.3
Cu C6	127	84.52	20.1	Zn C6	148	106.36	21.4	Al C3	28	81.8	20.3
Cu C6	128	64.06	19.1	Zn C6	149	77.97	19.9	Al C3	27	84.8	20.4
Cu C6	129	70.58	18.9	Zn C6	150	82.7	21.2	Al C3	26	61.4	18.1
Cu C6	130	93.24	21.5	Zn C6	151	88.78	20.5	Al C3	25	103.5	22
Cu C6	131	97.15	21.3	Zn C6	152	53.29	17.5	Al C3	24	74.2	18.7
Cu C6	132	74.06	19.6	Zn C6	153	53.21	17.1	Al C3	23	82.1	20
Cu C6	133	89.96	21.4	Zn C6	154	80.7	20	Al C3	22	72.8	18.6
Cu C7	134	76.87	20.7	Zn C7	155	92.9	21.5	Al C2	35	73	18.7
Cu C7	135	58.77	18	Zn C7	156	82.81	20.2	Al C2	34	65.9	18.4
Cu C7	136	83.66	20.5	Zn C7	157	75.43	19.6	Al C2	33	75.7	19.6
Cu C7	137	70.16	19.8	Zn C7	158	92.89	20.4	Al C2	32	59.5	17.5
Cu C7	138	66.7	19	Zn C7	159	69.18	19	Al C2	31	85	20.1
Cu C7	139	66.01	18.9	Zn C7	160	64.16	18.4	Al C2	30	80.8	20
Cu C7	140	89.79	20.9	Zn C7	161	83.53	20.2	Al C2	29	60.5	17.7
Cu C8	141	63.67	19	Zn C8	162	96.1	21.3	Al C1	42	61.6	18.3
Cu C8	142	95.66	21.7	Zn C8	163	75.76	19.7	Al C1	41	65.6	18.6
Cu C8	143	78.43	20.2	Zn C8	164	96.82	21.6	Al C1	40	52.6	16.8
Cu C8	144	66.8	18.8	Zn C8	165	74.74	19.9	Al C1	39	79.7	20.1
Cu C8	145	68.64	18.5	Zn C8	166	78.74	19.9	Al C1	38	66.6	18.5
Cu C8	146	90.03	21.1	Zn C8	167	84.94	20	Al C1	37	78	19.7
Cu C8	147	71.95	18.9	Zn C8	168	79.62	20.1	Al C1	36	83.9	19.8

Appendix A.8 - Fish tissue metal concentration

Fish tissue (gill, kidney, liver) was measured after dissection. The measured concentration of metals in each tissue of each fish is given in table A.8.1 (Cu), A.8.2 (Zn), and table A.8.3 (Al). All values are given in both µg/l and mg/kg.

Table A.8.1: Measured Cu concentration in tissues (gill, kidney, liver) for each individual fish in each exposure group. Concentration was measured in µg/l and converted to mg/kg.

Exposure group	Fish nr	Tissue	weight, g	Conc. [ug/l]	mg/kg
Cu control	F43	Gill	0.024	4.16564439	1.74
Cu control	F44	Gill	0.0287	4.80642362	1.67
Cu control	F45	Gill	0.0422	6.89045039	1.63
Cu control	F46	Gill	0.0311	4.68450034	1.51
Cu control	F47	Gill	0.0266	4.623944	1.74
Cu control	F48	Gill	0.0218	4.08170404	1.87
Cu control	F49	Gill	0.0207	3.80969823	1.84
Cu Control 2	F120	Gill	0.0307	5.47090685	1.78
Cu Control 2	F121	Gill	0.0361	6.42604954	1.78
Cu Control 2	F122	Gill	0.0338	5.76713809	1.71
Cu Control 2	F123	Gill	0.0232	4.49107152	1.94
Cu Control 2	F124	Gill	0.0242	4.77107471	1.97
Cu Control 2	F125	Gill	0.0267	5.22182805	1.96
Cu Control 2	F126	Gill	0.0351	12.9281566	3.68
Cu conc 1	F85	Gill	0.0338	5.7351165	1.7
Cu conc 1	F86	Gill	0.0289	5.40093895	1.87
Cu conc 1	F87	Gill	0.0367	6.4060933	1.75
Cu conc 1	F88	Gill	0.0338	5.7562487	1.7
Cu conc 1	F89	Gill	0.0222	3.86011698	1.99
Cu conc 1	F90	Gill	0.0348	5.73437115	1.65
Cu conc 2	F92	Gill	0.0306	6.17231982	2.02
Cu conc 2	F93	Gill	0.0251	5.28402533	2.11
Cu conc 2	F94	Gill	0.0335	5.74215074	1.71
Cu conc 2	F95	Gill	0.0302	5.70278386	1.89
Cu conc 2	F96	Gill	0.0224	4.97358249	2.22
Cu conc 2	F97	Gill	0.035	6.38497861	1.82
Cu conc 3	F99	Gill	0.0292	6.68004262	2.29
Cu conc 3	F100	Gill	0.0141	3.48677034	3.14
Cu conc 3	F101	Gill	0.0245	5.11004058	2.09
Cu conc 3	F102	Gill	0.0228	4.27074123	1.94
Cu conc 3	F103	Gill	0.0277	5.82772609	2.1
Cu conc 3	F104	Gill	0.0233	5.73354963	2.46
Cu conc 3	F105	Gill	0.0325	6.26219457	1.93
Cu conc 4	F106	Gill	0.0322	8.42433726	2.62
Cu conc 4	F107	Gill	0.0388	8.23228958	2.12
Cu conc 4	F108	Gill	0.031	6.84631661	2.21
Cu conc 4	F109	Gill	0.0194	4.03441038	2.28
Cu conc 4	F110	Gill	0.0263	5.69866957	2.17
Cu conc 4	F111	Gill	0.0277	6.71162069	2.42
Cu conc 4	F112	Gill	0.0311	6.81652601	2.19
Cu conc 5	F113	Gill	0.0322	8.11549065	2.52
Cu conc 5	F114	Gill	0.027	5.87995874	2.18
Cu conc 5	F115	Gill	0.0311	8.47558635	2.73
Cu conc 5	F116	Gill	0.0302	7.43894278	2.46
Cu conc 5	F117	Gill	0.025	6.82409636	2.73
Cu conc 5	F118	Gill	0.0281	7.96658755	2.84
Cu conc 5	F119	Gill	0.0257	5.71054938	2.22
Cu conc 6	F127	Gill	0.0222	5.1875422	2.34
Cu conc 6	F128	Gill	0.0205	8.07166505	3.94
Cu conc 6	F129	Gill	0.0234	13.9536326	5.96
Cu conc 6	F130	Gill	0.0365	13.8416724	3.79
Cu conc 6	F131	Gill	0.032	11.0967456	3.47
Cu conc 6	F132	Gill	0.0248	13.2956104	5.36
Cu conc 6	F133	Gill	0.0355	12.2350467	3.45
Cu conc 7	F135	Gill	0.0213	10.0167334	4.7
Cu conc 7	F136	Gill	0.0263	14.1539836	5.38
Cu conc 7	F137	Gill	0.0288	14.4200942	5.01
Cu conc 7	F138	Gill	0.0228	12.6809311	5.56
Cu conc 7	F139	Gill	0.0252	9.92750628	3.94
Cu conc 7	F140	Gill	0.0317	15.2116764	4.8
Cu conc 8	F141	Gill	0.0241	13.8618206	5.75
Cu conc 8	F142	Gill	0.0404	28.7468055	7.12
Cu conc 8	F143	Gill	0.029	20.6986327	7.14
Cu conc 8	F144	Gill	0.0254	16.3211026	6.43
Cu conc 8	F145	Gill	0.0258	15.6883429	6.08
Cu conc 8	F146	Gill	0.0324	22.0623864	6.81
Cu conc 8	F147	Gill	0.0286	18.7027174	6.54

Exposure group	Fish nr	Tissue	weight, g	Conc. [ug/l]	mg/kg
Cu control	F43	Kidney	0.0456	21.7280889	4.76
Cu control	F44	Kidney	0.0478	28.0522162	5.87
Cu control	F45	Kidney	0.0549	34.5828993	6.3
Cu control	F46	Kidney	0.0537	31.7495867	5.91
Cu control	F47	Kidney	0.0272	19.3339383	7.11
Cu control	F48	Kidney	0.026	16.0744987	6.18
Cu control	F49	Kidney	0.0322	19.411751	6.03
Cu Control 2	F120	Kidney	0.0364	25.3222935	6.96
Cu Control 2	F121	Kidney	0.0484	33.0779543	6.83
Cu Control 2	F123	Kidney	0.077	43.8838549	5.7
Cu Control 2	F124	Kidney	0.0656	42.3627275	6.46
Cu Control 2	F125	Kidney	0.0493	31.1887327	6.33
Cu Control 2	F126	Kidney	0.0605	30.3390412	5.01
Cu Control 2	F127	Kidney	0.0586	37.3954197	6.38
Cu conc 1	F85	Kidney	0.0453	22.7486711	5.02
Cu conc 1	F86	Kidney	0.0449	27.116434	6.04
Cu conc 1	F87	Kidney	0.0754	39.2842475	5.21
Cu conc 1	F88	Kidney	0.0738	23.4818119	3.18
Cu conc 1	F89	Kidney	0.0416	30.712269	7.38
Cu conc 1	F90	Kidney	0.0577	43.2957469	7.5
Cu conc 2	F92	Kidney	0.04	42.5629069	10.6
Cu conc 2	F93	Kidney	0.052	26.6610303	5.13
Cu conc 2	F94	Kidney	0.0315	17.7157049	5.62
Cu conc 2	F95	Kidney	0.0505	24.8439076	4.92
Cu conc 2	F96	Kidney	0.0722	39.9535777	5.53
Cu conc 2	F97	Kidney	0.066	34.4182941	5.21
Cu conc 3	F100	Kidney	0.042	22.6096328	5.38
Cu conc 3	F101	Kidney	0.0343	19.3678836	5.65
Cu conc 3	F102	Kidney	0.0295	51.6454871	17.5
Cu conc 3	F103	Kidney	0.0526	33.0272446	6.28
Cu conc 3	F104	Kidney	0.0416	25.7307356	6.19
Cu conc 3	F105	Kidney	0.0728	36.5386877	5.02
Cu conc 4	F106	Kidney	0.0308	31.6853988	10.3
Cu conc 4	F107	Kidney	0.0403	35.7459456	8.87
Cu conc 4	F108	Kidney	0.0593	32.4400166	5.47
Cu conc 4	F109	Kidney	0.051	22.8058495	4.47
Cu conc 4	F110	Kidney	0.0414	23.3358988	5.64
Cu conc 4	F111	Kidney	0.0442	23.9530931	5.42
Cu conc 4	F112	Kidney	0.0607	32.1718893	5.3
Cu conc 5	F113	Kidney	0.055	30.1738295	5.49
Cu conc 5	F114	Kidney	0.0595	34.4494828	5.79
Cu conc 5	F115	Kidney	0.0672	36.7440901	5.47
Cu conc 5	F116	Kidney	0.0594	33.5079029	5.64
Cu conc 5	F117	Kidney	0.0477	26.1462675	5.48
Cu conc 5	F118	Kidney	0.0588	31.1910238	5.3
Cu conc 6	F128	Kidney	0.0464	26.1269798	5.63
Cu conc 6	F129	Kidney	0.0524	31.145423	5.94
Cu conc 6	F130	Kidney	0.0731	49.2850157	6.74
Cu conc 6	F131	Kidney	0.0875	49.2753377	5.63
Cu conc 6	F132	Kidney	0.0532	31.9152722	6
Cu conc 6	F133	Kidney	0.0625	39.7590659	6.36
Cu conc 6	F134	Kidney	0.0499	30.9998438	6.21
Cu conc 7	F135	Kidney	0.0433	19.8387571	4.58
Cu conc 7	F136	Kidney	0.0536	31.8378022	5.94
Cu conc 7	F137	Kidney	0.0546	34.3233301	6.29
Cu conc 7	F138	Kidney	0.0496	30.6624605	6.18
Cu conc 7	F139	Kidney	0.0447	28.5776113	6.39
Cu conc 7	F140	Kidney	0.0564	32.7873491	5.81
Cu conc 8	F141	Kidney	0.0382	22.417651	5.87
Cu conc 8	F142	Kidney	0.0953	56.5068484	5.93
Cu conc 8	F143	Kidney	0.0677	35.8821797	5.3
Cu conc 8	F144	Kidney	0.0489	32.163862	6.58
Cu conc 8	F145	Kidney	0.0537	28.3155134	5.27
Cu conc 8	F146	Kidney	0.0698	52.2975894	7.49
Cu conc 8	F147	Kidney	0.0458	24.9752714	5.45

Exposure group	Fish nr	Tissue	weight, g	Conc. [ug/l]	mg/kg
Cu control	F43	Liver	0.053	1172.6316	221
Cu control	F45	Liver	0.096	4850.0669	505
Cu control	F46	Liver	0.0973	1531.26619	157
Cu control	F44	Liver	0.0834	2545.93636	305
Cu control	F47	Liver	0.082	2290.98276	279
Cu control	F48	Liver	0.0889	1942.21584	218
Cu control	F49	Liver	0.0709	1260.54854	178
Cu Control 2	F120	Liver	0.0758	392.942519	186
Cu Control 2	F121	Liver	0.1059	1333.65075	488
Cu Control 2	F122	Liver	0.1366	216.6529	101
Cu Control 2	F124	Liver	0.1076	496.672348	281
Cu Control 2	F125	Liver	0.0884	586.236336	323
Cu Control 2	F126	Liver	0.0907	379.815928	170
Cu conc 1	F85	Liver	0.0509	1016.18346	200
Cu conc 1	F86	Liver	0.044	1632.85685	371
Cu conc 1	F88	Liver	0.0844	3269.12215	387
Cu conc 1	F89	Liver	0.0445	352.759599	79.3
Cu conc 1	F90	Liver	0.102	2441.03588	239
Cu conc 2	F92	Liver	0.058	982.879417	169
Cu conc 2	F93	Liver	0.0576	983.264163	171
Cu conc 2	F94	Liver	0.056	1853.61533	331
Cu conc 2	F95	Liver	0.0999	1732.2442	173
Cu conc 2	F96	Liver	0.0525	784.467242	149
Cu conc 2	F97	Liver	0.0798	2167.53751	272
Cu conc 3	F99	Liver	0.1003	2997.24911	299
Cu conc 3	F100	Liver	0.0729	2213.1908	304
Cu conc 3	F101	Liver	0.068	2489.64859	366
Cu conc 3	F102	Liver	0.0706	1430.16201	203
Cu conc 3	F103	Liver	0.0494	421.538361	85.3
Cu conc 3	F104	Liver	0.0713	2094.67228	294
Cu conc 3	F105	Liver	0.0922	1510.87707	164
Cu conc 4	F107	Liver	0.0965	2921.43879	303
Cu conc 4	F109	Liver	0.0489	997.634606	204
Cu conc 4	F110	Liver	0.052	959.807062	185
Cu conc 4	F111	Liver	0.0815	2610.70058	320
Cu conc 4	F112	Liver	0.0834	1903.69667	228
Cu conc 5	F113	Liver	0.0764	1120.7356	147
Cu conc 5	F114	Liver	0.1005	2897.06351	288
Cu conc 5	F115	Liver	0.0766	2531.72648	331
Cu conc 5	F116	Liver	0.0788	1588.68953	202
Cu conc 5	F117	Liver	0.0836	1324.68044	158
Cu conc 5	F118	Liver	0.0756	1338.27099	177
Cu conc 5	F119	Liver	0.0755	1678.04459	222
Cu conc 6	F127	Liver	0.1119	664.35641	253
Cu conc 6	F128	Liver	0.1311	831.054835	298
Cu conc 6	F129	Liver	0.1396	1075.71176	394
Cu conc 6	F130	Liver	0.1366	683.044947	290
Cu conc 6	F131	Liver	0.1178	850.828968	378
Cu conc 6	F132	Liver	0.1124	829.66324	334
Cu conc 6	F133	Liver	0.1243	500.293504	224
Cu conc 7	F134	Liver	0.1115	403.896043	196
Cu conc 7	F135	Liver	0.103	534.651647	258
Cu conc 7	F137	Liver	0.1036	1080.84698	396
Cu conc 7	F139	Liver	0.1366	673.828376	336
Cu conc 7	F140	Liver	0.1004	887.178491	326
Cu conc 8	F142	Liver	0.1362	824.72815	362
Cu conc 9	F143	Liver	0.1139	756.524591	383
Cu conc 8	F145	Liver	0.0988	228.68512	123
Cu conc 8	F146	Liver	0.0926	927.230692	348
Cu conc 8	F147	Liver	0.1333	294.363987	121

Appendix A.8 – Cont.

Table A.8.2: Measured Zn concentration in tissues (gill, kidney, liver) for each individual fish in each exposure group. Concentration was measured in µg/l and converted to mg/kg.

Exposure group	Fish nr.	Tissue	Weight, g	Conc. [ug/l]	mg/kg	Exposure group	Fish nr.	Tissue	Weight, g	Conc. [ug/l]	mg/kg	Exposure group	Fish nr.	Tissue	Weight, g	Conc. [ug/l]	mg/kg
Zn/Cu control	F43	Gill	0.024	1421.90574	592	Zn/Cu control	F43	Kidney	0.0456	682.262772	150	Zn/Cu control	F43	Liver	0.053	438.040818	82.6
Zn/Cu control	F44	Gill	0.0287	1308.28856	456	Zn/Cu control	F44	Kidney	0.0478	1170.26146	245	Zn/Cu control	F44	Liver	0.0834	921.926388	111
Zn/Cu control	F45	Gill	0.0422	2976.3927	705	Zn/Cu control	F45	Kidney	0.0549	1886.51128	344	Zn/Cu control	F45	Liver	0.096	1022.29214	106
Zn/Cu control	F46	Gill	0.0311	2012.36512	647	Zn/Cu control	F46	Kidney	0.0537	1647.1973	307	Zn/Cu control	F46	Liver	0.0973	575.204872	59.1
Zn/Cu control	F47	Gill	0.0266	1606.3778	604	Zn/Cu control	F47	Kidney	0.0272	860.530695	316	Zn/Cu control	F47	Liver	0.082	1080.8294	132
Zn/Cu control	F48	Gill	0.0218	1386.32487	636	Zn/Cu control	F48	Kidney	0.026	756.86483	291	Zn/Cu control	F48	Liver	0.0889	828.868126	93.2
Zn/Cu control	F49	Gill	0.0207	1089.74472	526	Zn/Cu control	F49	Kidney	0.0322	655.614565	204	Zn/Cu control	F49	Liver	0.0709	628.694237	88.7
Zn/Cu control	F120	Gill	0.0307	1817.47749	592	Zn/Cu control	F120	Kidney	0.0364	1023.88622	281	Zn/Cu control	F120	Liver	0.0758	128.439604	84.7
Zn/Cu control	F121	Gill	0.0361	1979.934	548	Zn/Cu control	F121	Kidney	0.0484	3192.16737	660	Zn/Cu control	F121	Liver	0.1059	190.750262	90.1
Zn/Cu control	F122	Gill	0.0338	2205.1731	652	Zn/Cu control	F123	Kidney	0.077	2116.46544	275	Zn/Cu control	F122	Liver	0.1366	277.231733	101
Zn/Cu control	F123	Gill	0.0232	1983.49142	855	Zn/Cu control	F124	Kidney	0.0656	2063.5149	315	Zn/Cu control	F124	Liver	0.1076	164.404778	76.4
Zn/Cu control	F124	Gill	0.0242	1657.25832	685	Zn/Cu control	F125	Kidney	0.0493	1348.67458	274	Zn/Cu control	F125	Liver	0.0884	154.72291	87.5
Zn/Cu control	F125	Gill	0.0267	1918.08838	718	Zn/Cu control	F126	Kidney	0.0605	1474.83101	244	Zn/Cu control	F126	Liver	0.0907	199.10051	110
Zn/Cu control	F126	Gill	0.0351	2027.32632	578	Zn conc. 1	F50	Kidney	0.0314	721.683094	230	Zn conc. 1	F50	Liver	0.0522	410.769929	78.7
Zn conc. 1	F50	Gill	0.0218	1183.72557	543	Zn conc. 1	F51	Kidney	0.0319	873.197349	274	Zn conc. 1	F51	Liver	0.056	549.720685	98.2
Zn conc. 1	F51	Gill	0.0187	948.37336	507	Zn conc. 1	F52	Kidney	0.0345	800.012145	232	Zn conc. 1	F52	Liver	0.056	517.696603	92.4
Zn conc. 1	F52	Gill	0.0217	1255.85628	579	Zn conc. 1	F53	Kidney	0.0386	701.660755	182	Zn conc. 1	F53	Liver	0.0588	627.879082	107
Zn conc. 1	F53	Gill	0.0255	1233.22022	484	Zn conc. 1	F54	Kidney	0.0634	1267.08334	200	Zn conc. 1	F54	Liver	0.058	610.265054	105
Zn conc. 1	F54	Gill	0.0289	1496.09437	518	Zn conc. 1	F55	Kidney	0.0581	938.627114	162	Zn conc. 1	F55	Liver	0.0444	442.305208	99.6
Zn conc. 1	F55	Gill	0.0312	1536.73932	493	Zn conc. 1	F56	Kidney	0.0398	457.079899	115	Zn conc. 1	F56	Liver	0.0749	619.080439	82.7
Zn conc. 1	F56	Gill	0.0236	928.846486	394	Zn conc. 2	F57	Kidney	0.0484	1118.85985	231	Zn conc. 2	F57	Liver	0.0615	521.620183	84.8
Zn conc. 2	F57	Gill	0.0327	1698.5468	519	Zn conc. 2	F58	Kidney	0.0637	1187.16162	186	Zn conc. 2	F58	Liver	0.0653	591.426768	90.6
Zn conc. 2	F58	Gill	0.0308	1904.85401	618	Zn conc. 2	F59	Kidney	0.0532	731.741382	138	Zn conc. 2	F59	Liver	0.0369	355.916725	96.5
Zn conc. 2	F59	Gill	0.023	908.488351	395	Zn conc. 2	F60	Kidney	0.0452	1456.57294	322	Zn conc. 2	F60	Liver	0.0688	719.699097	105
Zn conc. 2	F60	Gill	0.0357	2127.58549	596	Zn conc. 2	F61	Kidney	0.0317	978.489175	309	Zn conc. 2	F61	Liver	0.0388	331.737753	85.5
Zn conc. 2	F61	Gill	0.0268	1664.95914	621	Zn conc. 2	F62	Kidney	0.0624	1480.05765	237	Zn conc. 2	F62	Liver	0.082	851.055035	104
Zn conc. 2	F62	Gill	0.0342	1786.74263	522	Zn conc. 2	F63	Kidney	0.0591	1541.339	261	Zn conc. 2	F63	Liver	0.0787	706.737447	89.8
Zn conc. 2	F63	Gill	0.0295	1646.37023	558	Zn conc. 3	F64	Kidney	0.0291	589.536424	203	Zn conc. 3	F64	Liver	0.0373	329.558073	88.4
Zn conc. 3	F64	Gill	0.0261	1239.52457	475	Zn conc. 3	F65	Kidney	0.0461	1006.60838	218	Zn conc. 3	F65	Liver	0.0509	579.562279	114
Zn conc. 3	F65	Gill	0.0292	1572.02319	538	Zn conc. 3	F66	Kidney	0.0503	951.937236	189	Zn conc. 3	F66	Liver	0.0772	717.917737	93
Zn conc. 3	F66	Gill	0.053	2349.09003	443	Zn conc. 3	F67	Kidney	0.0352	1717.95533	488	Zn conc. 3	F67	Liver	0.051	423.724275	83.1
Zn conc. 3	F67	Gill	0.0287	2040.77534	711	Zn conc. 3	F68	Kidney	0.0757	1707.11832	226	Zn conc. 3	F68	Liver	0.0694	637.350529	91.8
Zn conc. 3	F68	Gill	0.0386	2057.75128	533	Zn conc. 3	F69	Kidney	0.0503	1219.37402	242	Zn conc. 3	F69	Liver	0.0609	496.151071	81.5
Zn conc. 3	F69	Gill	0.0277	1202.66778	434	Zn conc. 3	F70	Kidney	0.0546	1514.77998	277	Zn conc. 3	F70	Liver	0.0702	609.845542	86.9
Zn conc. 3	F70	Gill	0.0321	1999.14107	623	Zn conc. 4	F71	Kidney	0.0437	529.108754	121	Zn conc. 4	F71	Liver	0.0363	360.958998	99.4
Zn conc. 4	F71	Gill	0.0239	983.603926	412	Zn conc. 4	F72	Kidney	0.0465	1178.71609	253	Zn conc. 4	F72	Liver	0.0833	1050.70225	126
Zn conc. 4	F72	Gill	0.0306	1599.21886	523	Zn conc. 4	F73	Kidney	0.0487	993.665031	204	Zn conc. 4	F73	Liver	0.0589	717.124052	122
Zn conc. 4	F73	Gill	0.0282	1391.54168	493	Zn conc. 4	F74	Kidney	0.0468	1004.24432	215	Zn conc. 4	F74	Liver	0.0418	481.01708	115
Zn conc. 4	F74	Gill	0.0293	1638.57519	559	Zn conc. 4	F75	Kidney	0.0602	1564.35064	260	Zn conc. 4	F75	Liver	0.0633	954.638317	151
Zn conc. 4	F75	Gill	0.0294	1581.3884	538	Zn conc. 4	F76	Kidney	0.0656	1193.00184	182	Zn conc. 4	F76	Liver	0.0488	1098.95728	225
Zn conc. 4	F76	Gill	0.0311	1921.83682	618	Zn conc. 5	F78	Kidney	0.0544	1404.61277	258	Zn conc. 5	F78	Liver	0.0664	842.66764	127
Zn conc. 5	F78	Gill	0.0334	2794.56574	837	Zn conc. 5	F79	Kidney	0.0513	2084.90393	406	Zn conc. 5	F79	Liver	0.0642	682.883437	106
Zn conc. 5	F79	Gill	0.0342	1746.23375	511	Zn conc. 5	F80	Kidney	0.0487	1263.06377	259	Zn conc. 5	F80	Liver	0.0586	681.814862	116
Zn conc. 5	F80	Gill	0.0286	1429.66755	500	Zn conc. 5	F81	Kidney	0.0545	881.438566	162	Zn conc. 5	F81	Liver	0.0816	826.324928	101
Zn conc. 5	F81	Gill	0.0816	1270.01713	156	Zn conc. 5	F82	Kidney	0.0369	989.197196	268	Zn conc. 5	F83	Liver	0.0542	592.323446	109
Zn conc. 5	F82	Gill	0.0288	1441.05028	500	Zn conc. 5	F83	Kidney	0.0374	1059.75844	283	Zn conc. 5	F84	Liver	0.0753	770.284054	102
Zn conc. 5	F83	Gill	0.0542	1476.60853	272	Zn conc. 5	F84	Kidney	0.0694	642.073421	92.5	Zn conc. 6	F148	Liver	0.1217	159.012454	65.3
Zn conc. 6	F148	Gill	0.0332	2699.07665	813	Zn conc. 6	F148	Kidney	0.0725	4160.10095	574	Zn conc. 6	F149	Liver	0.1235	279.085472	113
Zn conc. 6	F149	Gill	0.0264	2073.98371	786	Zn conc. 6	F149	Kidney	0.0589	2604.42063	442	Zn conc. 6	F150	Liver	0.0912	166.964039	91.5
Zn conc. 6	F150	Gill	0.0298	1503.48365	505	Zn conc. 6	F150	Kidney	0.0608	1391.43777	229	Zn conc. 6	F151	Liver	0.051	82.8355405	81.2
Zn conc. 6	F151	Gill	0.0377	2632.38431	698	Zn conc. 6	F151	Kidney	0.0637	3418.38661	537	Zn conc. 6	F152	Liver	0.0683	112.680291	82.5
Zn conc. 6	F152	Gill	0.0192	1225.7344	638	Zn conc. 6	F152	Kidney	0.0384	1306.20194	340	Zn conc. 6	F153	Liver	0.1146	167.091719	72.9
Zn conc. 6	F153	Gill	0.0238	1180.36064	496	Zn conc. 6	F153	Kidney	0.0399	1303.9395	327	Zn conc 7	F155	Liver	0.1146	199.314741	87
Zn conc. 6	F154	Gill	0.0284	1527.88623	538	Zn conc. 6	F154	Kidney	0.0667	1458.39568	219	Zn conc 7	F156	Liver	0.1625	241.878851	74.4
Zn conc 7	F155	Gill	0.0363	1849.3347	509	Zn conc 7	F155	Kidney	0.0702	1131.19711	161	Zn conc 7	F157	Liver	0.1377	226.586089	82.3
Zn conc 7	F156	Gill	0.0304	2399.7914	789	Zn conc 7	F156	Kidney	0.0577	2679.33246	464	Zn conc 7	F158	Liver	0.1152	178.401625	77.4
Zn conc 7	F157	Gill	0.0264	1408.82208	534	Zn conc 7	F157	Kidney	0.0567	1300.79774	229	Zn conc 7	F159	Liver	0.0965	161.69473	83.8
Zn conc 7	F158	Gill	0.0342	1776.35043	519	Zn conc 7	F158	Kidney	0.063	1818.44325	289	Zn conc 7	F160	Liver	0.0847	151.154639	89.2
Zn conc 7	F159	Gill	0.0247	1484.83726	601	Zn conc 7	F159	Kidney	0.0661	2100.74491	318	Zn conc 7	F161	Liver	0.1106	184.819713	83.6
Zn conc 7	F160	Gill	0.0213	1504.36101	706	Zn conc 7	F160	Kidney	0.0536	2575.07949	480	Zn conc 8	F162	Liver	0.1243	257.348093	104
Zn conc 7	F161	Gill	0.0301	2041.17684	678	Zn conc 7	F161	Kidney	0.0565	1935.65344	343	Zn conc 8	F163	Liver	0.1065	203.371709	95.5
Zn conc 8	F162	Gill	0.0365	2353.95036	645	Zn conc 8	F162	Kidney	0.0656	1815.75707	277	Zn conc 8	F164	Liver	0.1339	252.147734	94.2
Zn conc 8	F164	Gill	0.0363	2197.85918	605	Zn conc 8	F163	Kidney	0.0582	1389.28611	239	Zn conc 8	F167	Liver	0.1269	199.171964	78.5
Zn conc 8	F165	Gill	0.0264	1327.28675	503	Zn conc 8	F164	Kidney	0.0596	3272.60706	549	Zn conc 8	F168	Liver	0.1074	170.204698	79.2
Zn conc 8	F166	Gill	0.0249	1539.05296	618	Zn conc 8	F165	Kidney	0.051	1047.79352	205						
Zn conc 8	F167	Gill	0.0319	2018.01259	633	Zn conc 8	F166	Kidney	0.0688	2126.39824	309						
Zn conc 8	F168	Gill	0.029	1860.82759	642	Zn conc 8	F168	Kidney	0.0544	1214.37563	223						

Appendix A.8 – Cont.

Table A.8.3: Measured Al concentration in tissues (gill, kidney, liver) for each individual fish in each exposure group. Concentration was measured in µg/l and converted to mg/kg.

Exposure group	Fish nr.	Tissue	weight, g	Conc. µg/l	mg/kg	Exposure group	Fish nr.	Tissue	weight, g	Conc. µg/l	mg/kg	Exposure group	Fish nr.	Tissue	weight, g	Conc. µg/l	mg/kg
Al control	F1	Gill	0.03	18.1911819	6.06	Al control	F1	Kidney	0.0368	6.61283889	1.8	Al control	F3	Liver	0.075	19.056438	2.54
Al control	F2	Gill	0.0498	35.3433168	7.1	Al control	F2	Kidney	0.0624	8.73816497	1.4	Al control	F4	Liver	0.0822	15.2371123	1.85
Al control	F3	Gill	0.0248	20.5865654	8.3	Al control	F3	Kidney	0.0421	7.64215866	1.82	Al control	F5	Liver	0.0992	28.8411098	2.91
Al control	F4	Gill	0.0336	22.9622087	6.83	Al control	F4	Kidney	0.0783	9.39342912	1.2	Al control	F6	Liver	0.0691	16.6045799	2.4
Al control	F5	Gill	0.0317	16.6743611	5.26	Al control	F5	Kidney	0.0355	5.61335544	1.58	Al control	F7	Liver	0.1639	25.9653622	1.58
Al control	F6	Gill	0.0295	23.5282266	7.98	Al control	F6	Kidney	0.0608	9.94384881	1.64	Al conc. 1	F8	Liver	0.0587	16.8329639	2.87
Al control	F7	Gill	0.0412	27.0293317	6.56	Al control	F7	Kidney	0.0698	9.16562277	1.31	Al conc. 1	F9	Liver	0.0359	10.3527554	2.88
Al conc. 1	F8	Gill	0.0281	22.4794423	8	Al conc. 1	F8	Kidney	0.0503	16.2173548	3.22	Al conc. 1	F10	Liver	0.0495	8.93111785	1.8
Al conc. 1	F9	Gill	0.0206	22.2331041	10.8	Al conc. 1	F9	Kidney	0.0647	21.2169588	3.28	Al conc. 1	F11	Liver	0.0357	12.7121371	3.56
Al conc. 1	F10	Gill	0.0282	39.0415799	13.8	Al conc. 1	F10	Kidney	0.0966	31.2607599	3.24	Al conc. 1	F12	Liver	0.0538	14.91979	2.77
Al conc. 1	F11	Gill	0.0294	16.1941677	5.51	Al conc. 1	F11	Kidney	0.0763	22.9065043	3	Al conc. 1	F13	Liver	0.0348	10.5433957	3.03
Al conc. 1	F12	Gill	0.0299	28.8955993	9.66	Al conc. 1	F12	Kidney	0.0777	26.3112264	3.39	Al conc. 1	F14	Liver	0.0383	4.9688364	1.3
Al conc. 1	F13	Gill	0.0226	27.4850109	12.2	Al conc. 1	F13	Kidney	0.0451	13.2093293	2.93	Al conc 2	F15	Liver	0.1208	25.9034303	2.14
Al conc. 1	F14	Gill	0.0231	22.4678659	9.73	Al conc. 1	F14	Kidney	0.0635	11.0605162	1.74	Al conc 2	F16	Liver	0.1395	16.1384505	1.16
Al conc 2	F15	Gill	0.0393	40.3136222	10.3	Al conc 2	F15	Kidney	0.0462	12.6518855	2.74	Al conc 2	F17	Liver	0.1123	28.1464892	2.51
Al conc 2	F16	Gill	0.0226	36.1054952	16	Al conc 2	F16	Kidney	0.0359	13.8953855	3.87	Al conc 2	F18	Liver	0.0682	21.7375002	3.19
Al conc 2	F17	Gill	0.0338	39.9426182	11.8	Al conc 2	F17	Kidney	0.0385	9.42900594	2.45	Al conc 2	F19	Liver	0.0855	22.8645803	2.67
Al conc 2	F18	Gill	0.0258	80.5546246	31.2	Al conc 2	F18	Kidney	0.0679	6.82978933	1.01	Al conc 2	F20	Liver	0.06	24.6233985	4.1
Al conc 2	F19	Gill	0.1149	45.9670671	4	Al conc 2	F19	Kidney	0.0748	8.92871703	1.19	Al conc 2	F21	Liver	0.05	15.7454648	3.15
Al conc 2	F20	Gill	0.0675	98.0329809	14.5	Al conc 2	F20	Kidney	0.0364	8.76462874	2.41	Al conc 3	F22	Liver	0.0767	28.3371251	3.69
Al conc 2	F21	Gill	0.0819	43.7313677	5.34	Al conc 2	F21	Kidney	0.0406	8.13581834	2	Al conc 3	F23	Liver	0.0876	87.9267038	10
Al conc 3	F22	Gill	0.0317	71.4942858	22.6	Al conc 3	F22	Kidney	0.0437	9.79430391	2.24	Al conc 3	F24	Liver	0.068	18.9835892	2.79
Al conc 3	F23	Gill	0.0295	46.7520757	15.8	Al conc 3	F23	Kidney	0.056	10.1297865	1.81	Al conc 3	F25	Liver	0.1283	37.0721905	2.89
Al conc 3	F24	Gill	0.0263	49.7177894	18.9	Al conc 3	F24	Kidney	0.0532	9.88653212	1.86	Al conc 3	F26	Liver	0.0274	11.5837676	4.23
Al conc 3	F25	Gill	0.0472	51.5884665	10.9	Al conc 3	F25	Kidney	0.0466	13.626253	2.92	Al conc 3	F27	Liver	0.1207	31.8121484	2.64
Al conc 3	F26	Gill	0.0231	24.5568115	10.6	Al conc 3	F26	Kidney	0.0295	6.42680593	2.18	Al conc 3	F28	Liver	0.092	17.4782254	1.9
Al conc 3	F27	Gill	0.0259	23.7074266	9.15	Al conc 3	F27	Kidney	0.054	15.6950658	2.91	Al conc. 4	F29	Liver	0.0589	19.8216409	3.37
Al conc 3	F28	Gill	0.0301	42.8344298	14.2	Al conc 3	F28	Kidney	0.0405	11.9490335	2.95	Al conc. 4	F30	Liver	0.0642	26.0237714	4.05
Al conc. 4	F29	Gill	0.0198	47.4627873	24	Al conc. 4	F29	Kidney	0.0335	5.72107262	1.71	Al conc. 4	F31	Liver	0.0922	28.9695975	3.14
Al conc 4	F30	Gill	0.0279	24.8981427	8.92	Al conc. 4	F30	Kidney	0.0451	14.8517916	3.29	Al conc. 4	F32	Liver	0.0589	13.4255682	2.28
Al conc 4	F31	Gill	0.0268	32.2916607	12	Al conc. 4	F31	Kidney	0.0555	9.33319245	1.68	Al conc. 4	F33	Liver	0.0625	18.2928234	2.93
Al conc. 4	F32	Gill	0.0224	45.6279542	20.4	Al conc. 4	F32	Kidney	0.0193	9.73583895	5.04	Al conc. 4	F34	Liver	0.0832	21.0201534	2.53
Al conc. 4	F33	Gill	0.0252	31.5170527	12.5	Al conc. 4	F33	Kidney	0.0439	10.4367463	2.38	Al conc. 4	F35	Liver	0.0834	20.5923736	2.47
Al conc. 4	F34	Gill	0.0262	28.183889	10.8	Al conc. 4	F34	Kidney	0.0508	10.799242	2.13	Al conc. 5	F36	Liver	0.0764	26.9569384	3.53
Al conc. 4	F35	Gill	0.028	35.8938946	12.8	Al conc. 4	F35	Kidney	0.0477	11.1795357	2.34	Al conc. 5	F37	Liver	0.1005	23.188564	2.31
Al conc. 4	F36	Gill	0.0322	56.907428	17.7	Al conc. 5	F36	Kidney	0.055	8.05541472	1.46	Al conc. 5	F38	Liver	0.0766	15.9427745	2.08
Al conc. 5	F37	Gill	0.027	63.494669	23.5	Al conc. 5	F37	Kidney	0.0595	13.2405926	2.23	Al conc. 5	F39	Liver	0.0788	32.6641464	4.15
Al conc. 5	F38	Gill	0.0311	60.9167688	19.6	Al conc. 5	F38	Kidney	0.0672	15.6414237	2.33	Al conc. 5	F40	Liver	0.0836	10.0551157	1.2
Al conc. 5	F39	Gill	0.0302	78.8700536	26.1	Al conc. 5	F39	Kidney	0.0594	16.6150211	2.8	Al conc. 5	F41	Liver	0.0756	25.6260589	3.39
Al conc. 5	F40	Gill	0.025	31.0178518	12.4	Al conc. 5	F40	Kidney	0.0477	24.5564561	5.15	Al conc. 5	F42	Liver	0.0655	11.5848859	1.77
Al conc. 5	F41	Gill	0.0281	65.390649	23.3	Al conc. 5	F41	Kidney	0.0588	9.51545226	1.62						
Al conc. 5	F42	Gill	0.0257	53.9786756	21	Al conc. 5	F42	Kidney	0.0521	7.79430502	1.5						

Appendix A.9 Normality in fish tissue

Shapiro-Wilk test was used to analyze normality for the fish tissues. Data output with p-value > α = 0.01 indicates that the data is, with 99% probability, normally distributed.

Hypothesis at α=0.01
H0: data is normally distributed vs H1: data is not normally distributed

In GraphPad Prism
1. Select, **Analyze> Normality and Lognormality test**

One example of the Shapiro-Wilk test on normality in liver tissue of fish exposed to copper is given below.

Normality and Lognormality Tests Tabular results	A Control	B 0.08	C 0.14	D 0.25	E 0.42	F 0.56	G 2.08	H 3.4	I 7.0
Shapiro-Wilk test									
W	0.9467	0.9331	0.6098	0.5918	0.7878	0.9427	0.9405	0.7862	0.8666
P value	0.5112	0.6046	0.0006	0.0004	0.0310	0.6812	0.6431	0.0440	0.1734
Passed normality test (alpha=0.01)?	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	***	***	*	ns	ns	*	ns

Figure A.9: Screenshot of Normality test on Cu liver samples, using Graphpad Prism.

Appendix A.10 – One-way ANOVA test

One-way ANOVA with Dunnett's post hoc was used to determine the significant difference between means of the control group and other exposure groups. Data output with p-value > α = 0.05 implies that the mean of the exposure group is significantly different from the control group, with a 95% probability.

Hypothesis at α=0.05

H0: mean is significant different vs H1: mean is not significantly different

In GraphPad Prism

1. Select, **Analyze>One-way ANOVA>Multiple Comparisons>Compare each mean with mean of control group**

One example of a one-way ANOVA and Dunnett's multiple comparison test on gill tissue of fish exposed to aluminum is given below.

Table Analyzed	Al Gill
Data sets analyzed	A-F
ANOVA summary	
F	10.46
P value	<0.0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R squared	0.5606

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Control vs. 1.7	-0.1500	-0.3769 to 0.07688	No	ns	0.3240
Control vs. 2.7	-0.2500	-0.4769 to -0.02312	Yes	*	0.0255
Control vs. 4.3	-0.2500	-0.4652 to -0.03477	Yes	*	0.0169
Control vs. 7.6	-0.3500	-0.5652 to -0.1348	Yes	***	0.0005
Control vs. 11.8	-0.5500	-0.7652 to -0.3348	Yes	****	<0.0001

Figure A.10: Screenshot of one-way ANOVA test, with Dunnett's multiple comparisons test using Graphpad Prism. Test conducted on aluminum in gills.

Appendix A.11 – Concentration factor

The concentration factor was calculated using the measured concentration of the LMM fraction of metals in the water. The CF was calculated using the <0.45µm fraction. The following results for CF calculated using LMM fraction and <0.45µm fraction is given below. The error is calculated by eq. 9

Eq. 9 $$\%Error = (CF_{LMM} - CF_{<0.45}) \div CF_{LMM}$$

Table A.11: CF for Cu gills, and Al gills and kidney using <0.45 fraction and LMM fraction. With error% between the fractions.

Nominal concentration	CF								
	Cu Gill			Al Gill			Al kidney		
µmol	LMM	<0.45µm	%error	LMM	<0.45	%error	LMM	<0.45	%error
0.4	553	87	84 %						
0.6	228	66	71 %						
1.4				260	188	28 %	83	60	28 %
2.4	294	32	89 %	176	193	-10 %	30	33	-10 %
4.3	31	21	31 %	133	128	3 %	22	21	3 %
7.8	18	15	15 %	79	75	6 %	14	13	6 %
15.6				74	77	-4 %	9	9	-4 %

Appendix A.12 – ICP-MS method

Different methods for the analysis of fish tissue and water samples were made before the experiment. On the ICP-MS were several gas modes, oxygen, ammonia, and helium used for different masses of the metals. The water samples were analyzed with the following gas modes and atomic masses, table A.12.1. The fish tissue samples were analyzed using the following gas modes and atomic masses, table A.12.2.

Table A.12.1: Gas modes, and atomic masses for corresponding metals analyzed on ICP-MS for water samples.

Reaction mode	Ammonia, NH3	Helium, H
Element	atomic mass	atomic mass
Copper, Cu		63
	65	65
Zinc, Zn	64	64
	66	66
Aluminium, Al	27	

Table A.12.2: Gas modes, and atomic masses for corresponding metals analyzed on ICP-MS for fish tissue samples.

Reaction mode	Oxygen, O	Ammonia, NH3	Helium, H
Element	atomic mass	atomic mass	atomic mass
Copper, Cu		63	63
		65	65
Zinc, Zn		64	64
		66	66
Aluminium, Al	27	27	

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