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Speciation, uptake and toxic effects of Cadmium on developing stages of Atlantic salmon (*Salmo salar*)

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Speciation, Uptake and Toxic Effects of Cadmium on Developing Stages of Atlantic Salmon (*Salmo salar*)

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Abbreviations

LMM	**Low molecular mass**
PVM	**Perivitalline membrane**
PVS	**Perivitalline space**
TOC	**Total organic carbon**
HMM	**High molecular mass**
ICP-MS	**Inductively couple plasma-mass spectrometry**
Fpg	**Formamidopyrimidine glycosylase**
USEPA	**United states environmental protection agency**

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Abstract

Cadmium (Cd) is well known as one very toxic trace elements for aquatic organisms including fish. Uptake and toxic effects of Cd in embryo of Atlantic salmon was studied from fertilization to hatching stage. Eggs from Atlantic salmon were exposed from fertilization to hatching to four different concentrations of Cd (10µg/L, 30µg/L, 100µg/L including control, respectively). Cd uptake and distribution of Cd was determined in whole egg compared to inside egg content only, and in whole alevin compared to yolk sac only. The long-term body concentration of Cd was followed in swimup after end of exposure. Sub lethal and lethal effects were followed by swelling, time of hatching, DNA damage, growth, deformity and mortality, respectively. Cd was taken up in embryos, however surprisingly no significant lethal effects were identified at the relative high concentration tested. No toxic effect was observed on degree of swelling by Cd. However, the hatching time was altered by initiation of premature hatching which was followed by extended period of hatching in 10ugCd/L. However, 30ugCd/L and 100ugCd/L showed only extended hatching time. Comet assay results showed some DNA damage in Cd treated groups. More over Cd with all three concentrations did not show any significant effects on growth of alevins. Body malformations were also recorded low in number.

Compared to previous study this study demonstrate that the toxic effects of Cd is highly dependent upon life stage of exposure. Acute lethal effects of Atlantic salmon juveniles has been demonstrated at 0.5 µg/l while this study demonstrate significantly lower effects when exposure of Atlantic salmon embryos to Cd at 100 µg/L in similar water quality. Low chorion penetration of Cd could explain low effects.

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1. Introduction

Cadmium (Cd) is a xenobiotic and one of the most toxic heavy metals (Witeska *et al.*, 2014). Cd emission to water arise from the chemicals and metals industries, the transport sector and waste streams, including agriculture (EEA, 2011). Cd occurs at low concentration in aquatic systems. In freshwater total dissolved Cd is usually less than 0.5µg/L and it is even lower in seawter (0.02 µg/L) (Pan *et al.*., 2010). In a survey of European streams soluble Cd ranged from 0.002 µg/L in the most pristine sites to 1.25µg/L for more contaminated sites.

Speciation has important role in Cd toxicity and exposure to living organisms. Cd speciation influences its availability, accumulation, bio-modification and its transport inside the organisms. That is why speciation studies is vital to analyze how this element behaves in the environment (Crea *et al.,* 2013).

Cd can cross or be absorbed through biological membrane. Essential metals such as Ca^{2+}, Mg^{2+} Cu^{2+}, Zn^{2+} are under homeostatic control (Maret and Moulis, 2013). Since Cd is not an essential element therefore is not under homeostatic control and this is why Cd can accumulate inside the organisms. Cd has a high affinity for Ca binding sites in organisms, and which is assumed to be one of the reasons lead to Cd accumulation in tissues (Mcgeer *et al.*, 2012)

Vast range of sub lethal and lethal effects have been reported in different organisms concerning Cd exposure. For example, acute Cd effects has been associated with disruption of ion homeostasis, particularly Ca regulation (Mcgeer *et al.*, 2012). Chronic Cd effects in fish has been associated with inhibition of growth and disturbed sex maturation (Szczerbik *et al.,* 2006), effects on immune system (Witeska *et al.*, 2006), altered fish behavior (Almaida *et al.*, 2009), cellular damage and protein breakdown (De Smet and Blust. 2001). Toxicity can be altered by factors like fish species specific sensitivity, age, gender and life stage. The sensitivity of an organism to a toxicant varies during life time. (Belanger *et al.*, 2009). It is generally considered that early life stages of fish are more sensitive to toxicants than adult life stage. However, during early life stages early embryos which are protected by egg envelope (chorion) that may reduce the reduce the exposure to chemicals compared to free embryo and larva after hatch (Lammer *et al.,*2009). This supported by earlier study where Juvenile parr Atlantic salmon had 50% mortality exposed to 0.5µgCd/L (Giblin *et al.*, 2015) while severe effects in fish eggs have only been reported at significantly higher concentrations (Giblin *et al.*, 2015). However, the

difference could be due to different speciation of Cd and less uptake of Cd and not the embryos are less sensitive than juvenile.

Toxicity tests can provide essential information which are the basis for the assessment of the quality of the aquatic environment because they can determine adverse effects of contaminants on the aquatic organisms (Cao *et al.,* 2009). The sublethal effects of environmental pollutants may be difficult to detect in organisms again they produce useful information regarding long term consequences of their effects on growth, reproduction and survival (Sfakianakis. 2014).

Objectives and hypothesis

The main objectives of the current study were to investigate the speciation, uptake and effects of Cd exposure on developing stages of Atlantic salmon from fertilization till hatching. In this regard following objectives were focused;

1. Identify Cd speciation in exposure water.
2. Examine the uptake of Cd in eggs and alevins overtime.
3. Identify toxic effects of Cd exposure in developing embryo (swelling, hatching, DNA damage, growth, deformity) by Cd.

2. Background

Cadmium naturally is rare metal occur in earth crust associated with zinc with concentration between 0.1-0.2mg/kg (OECD 1994). However, important drivers of Cd into biosphere are volcanic activity, windblow, leaching of rocks, forest fire, and exudates from vegetation (Ravera 1984). Anthropogenic sources of cadmium mainly result from exploitation of naturally occurring ores which are smelters, incinerators, sewage sludge applied to the land and Phosphate fertilizers (OECD 1994).

Background level of cadmium in unpolluted surface water is reported to be 0-0.2µg/L. According to Norwegian limit of Cd in surface water, ≤0.04µg/L is considered safe (Lydersen 2002). In addition, critical limit of cadmium recorded in Norwegian lakes are from 0.1 to 1.0 µg/L (ICP waters report 67/2002).

Speciation and mobility

Cd can be found in different physico-chemical species in fresh water (Crea *et al.*, 2013). These species can be available as free cations Cd^{2+} or in association with inorganic anions and organic complexes. Inorganic anions that Cd complex with comprise of Cl^-, OH^-, CO_3^{2-}, HCO_3^{2-}. Organic ligands that complexes with Cd include humic and fulvic acid (Crea *et al.*, 2013). Therefore, all chemical species with certain sizes in a solution are called speciation. The speciation is influenced by water quality parameters such as TOC and pH (Gundersen and Steiness. 2003).

Determining speciation of an element based on size and charge is called fractionation (Salbu. 2009). In size fractionation technique an element is divided into defined sizes such as particle, colloidal, low molecular mass. Fractionation helps to figure out the distribution of species in water.

Adsorption and desorption to humic and fulvic acid substances (TOC) influence Cd speciation and is a rapid process. Adsorption limits the mobility of free Cd^{2+} ion if TOC available in high concentration (Lydersen. 2002). TOC may be found in particle and colloidal forms. PH is considered one of the most important parameters that significantly effects the speciation and mobility (Gundersen and Steniness. 2002). This happens in either way; First, PH ranges from 4-5 increases mobility of free Cd cations possibly due to release from colloids and particles. Second, indirectly H^+ ions may compete with Cd for inorganic ligands Cl^-, OH^-, CO_3^{2-}, HCO_3^2

etc. (Gundersen and Steiness. 2002). Here it can be concluded that the mobility is affected by TOC depending on PH (Meinelt *et a.,l* 2000).

Bioavailability

Bioavailability is defined as the fraction of a substance present in environment which may be available to organism for uptake. This fraction includes the portion which is currently available and also the portion which will become available overtime (Chapman. 2008). The bioavailability depends on speciation and water hardness (Concentration of Ca and Mg).

Speciation of metal is primary determinant of bioavailability. For example, particles with higher molecular mass (HMM) are insoluble hence they settle down with time. However, colloids could be small complexes of Cd that may have low reactivity but generally they are assumed inert. On the other hand, Cd^{2+} free ions with low molecular mass (LMM) are soluble in water and highly reactive therefore they can cross biological membrane (Salbu 2009).

It is generally considered that free Cd^{2+} species are more bioavailable. Cd^{2+} compete with other major cations specially Ca in water because of close similarity (Cd^{2+} 109 picometer and Ca^{2+} 114 picometer) in ionic radius. for their reaction sites on biological surface (Maret and Moulis. 2013). Therefore, the competition between Ca and Cd is physiological in nature and does not affect water quality (Wright *et al.*, 1985).

Uptake

The process of uptake involves the sorption of dissolved metal ion from water throμgh the biological membrane (Das *et al.,* 2008). Uptake of Cd depends on bioavailabilty and water hardness. Cd has different routes of uptake during different life stages.

In later stages of life cycle, fish with functioning gills and digestive track Cd has two routes to enter. One with waterborne exposure and second via dietary exposure. In water borne exposure Cd is absorbed by gills throμgh passive diffusion or carrier mediated transport. In dietary exposure Cd can be ingested throμgh endocytosis in intestine (Kumar and singh. 2010). A small amount of Cd may also be taken up by skin. After the absorption throμgh gills or intestine Cd is transformed in assimilation. During assimilation Cd is bound to a metal binding protein known as metalothionein. With the help of this protein Cd can be excreted. If not excreted Cd is bound to lipids or proteins (Ravera. 1984). Binding to cellular macromolecules can result in accumulation.

The rout pathway of Cd absorption during early developmental life stages is chorion. Atlantic salmon's eggs have bigger surface to volume ratio which mean chorion with larger amount of anionic sites thus more binding of Cd (Burnison. 2006). From chorion Cd can eventually be taken up inside egg leaving developing embryo sensitive to Cd.

The toxic effects and sensitivity of early developmental life stages to Cd

Once taken up Cd may pose toxic effects on organism which involve alterations of normal physiological functions. This can threaten the long term fitness and survival of organisms. Generally early life stages are considered more sensitive to metals including those embryos protected by chorion than adult stage (Mohammad. 2013). The factors that make early life sensitive to metals may be;

1. It may take less time for metals to reach to target site because of shorter size (eggs, alevins) compared to adults.
2. Embryo and larva have poorly developed gills, kidney and liver. Therefore, in early life stages chorion and integument may be the primary site for ionic regulation. Thus chorion and integument provide larger surface area that for absorption of metals.
3. Kidney and liver have important role in detoxifying and eliminating metals but they are underdeveloped in embryo or larva.

Biomarkers

Biomarkers are defined as a change in biological response that may be because of an exposure to or toxic effect of any toxicant (Shμgart. 2000). Thus biomarkers related to effects of Cd in developing fish are swelling, hatchability, DNA damage, growth, deformity, mortality. The main underlying mechanism of toxicity related to these biomarkers may be osmotic disturbances and changes in enzyme synthesis and activity (Jezierska *et al.,* 2009).

Swelling

After spawning, eggs are exposed to hypotonic medium and eggs go under activation which takes 1-2 hours (Heath. 1995). During activation, two processes occur in eggs which bring chemical and physical changes into structure of eggs.1) swelling, 2) hardening of chorion.

Swelling is process of water uptake and formation of perivitalline fluid (PVF). During the formation of PVF, the macromolecules including enzymes of cortical alveoli are released across cell membrane in an outer space (Heath.1995). These macromolecules are broken down

by enzymes. This results osmotic colloid pressure which causes an influx of water in to egg and swells the space now called perivitalline space (PVS) increasing egg volume (Finn. 2007). PVS facilitates the free movement and growth of embryo (Li *et al.,* 1989)

During activation changes also occur in structure of chorion where it modifies from soft during ovulation to hard and resistant structure which is selectively permeable to external environment. Hardening takes place due to enzymatic activity that cross link the certain other proteins in to proteins of chorion (Oppen bersten 1990). Hardening of chorion also causes closure of micropyle (a structure on chorion where sperm enters into egg) that prevents polyspermy (Finn. 2007)

Cd can affect the synthesis of PVF which leads to reduction in swelling and incomplete hardening of chorion. The incompletely hardened chorion may permit an increased flow of hydrated metal ions such as Cd into the PVF. In this way in a higher amount of metal may be available for the future embryo (Gonzalez-Doncel *et al.*, 2003).

Time of hatching

After fertilization the process of hatching starts around 450 day degree (ºD) under normal conditions. The onset of hatching depends on the external environment of egg for example oxygen During early developmental stages, embryos have less oxygen demand that increases gradually with development. This tend to cause hypoxia in some parts of embryo which induces hatching. Hatching starts both with the help embryonic movement and hatching enzymes (chorionase) (Oppen-Berntsen *et al.*,1990). Chorionase are released from hatching glands which are synthesized during embryogenesis. Because of strong mechanical strength of chorion, it is not possible that chorion is torn only by embryonic movement. However, embryonic movement provokes the release of hatching enzymes into PVS which degrade the chorion from inner side and help to set embryo free (Oppen-Berntsen *et al.*, 1990). Heavy metals like Cd can affect the hatching process. Cd can delay or accelerate the hatching reported in (Jezierska *et al.*, 2009). (Zhang *et al.,* 2012) demonstrated the slow rate and delayed hatching in soldatov's catfish. In addition, Szczerbik *et al.,* (2008) observed accelerated hatching due to Cd.

DNA damage

Deoxyribonucleic acid (DNA) exist in all organisms and carry inherited or genetic information. If any unprogrammed changes occur in DNA molecule can have serious consequences. Cd is

classified in genotoxicants and it can disrupt cellular process resulting alterations in DNA structure causing problems for cell and can even threaten organism's survival (Shμgart. 2000). DNA damage has been reported in Japanese medaka larva exposed to Cd (Morin *et al.,* 2011).

Growth

Growth is measure of body length and weight. Growth is highly variable and a very sensitive biomarker during early fish development to heavy metals. Cd is known to inhibit growth Therefore, reduction in growth determines the reduction in fish fitness (Jezierska *et al.,* 2009).

Deformities and mortalities

When eggs are exposed to contaminants, the larvae may develop a variety of teratogenic deformities (Heath. 1995). Different types of body malformations observed are axial or lateral curvature of spine in the abdominal or caudal region, C shaped larva, deformed skull, deformed eye, deformed yolk sac and shortened body (Jezierska *et al.,* 2009). Cheng. (2000) reported deformities in embryos of Japanese medaka exposed to Cd. Metin. (2001) also reported head malformation and spinal curvature in larvae of mirror carp due to water borne Cd exposure.

Exposure of early developing eggs to toxicants can reduce the survival of embryo thus cause mortalities. Most of the deformed and normal embryos die during development (Jezierska *et al.,* 2009).

3. Material and Method

Exposure study

The present study was conducted to test the speciation, uptake and Biological effects (toxicity) of Cd on developing eggs of Atlantic salmon (*Salmo salar*). The eggs were exposed to four nominal concentrations of Cd, 0µg/L, 10µg/L, 30µg/L and 100µg/L. The exposure was initiated after fertilization and before swelling until hatching. I fractionated water samples before and after exposure to determine Cd species. To measure the Cd uptake, samples were collected from eggs and alevins. In addition, to test the biological effects of Cd, I measured the swelling, hatchability, deformities, DNA damage, growth and mortality during the developmental stages

Experimental design

The experimental design was set at FIGARO gamma irradiation facility in light and 6ºC temperature controlled room, Ås Norway. Four exposure reservoirs were aligned holding 24liter water in each. Water from reservoirs was pumped in to boxes placed on top of reservoirs. Each reservoir could pump water to three boxes for fish egg at a time. Pumping of water into boxes was facilitated by two small holes made on end of boxes. In this way exposure water could circulate in and out of the boxes. Each box was covered with black lid to avoid light (light can damage DNA in developing embryo). The lid was removed whenever samples had to be taken.

Preparation of soft water and Cd stock solution

Very soft US EPA (United states environmental protection agency) water was prepared in1000 L tanks by adding salt to reverse osmosis water (table). Very soft US EPA water was selected as it represents typical Norwegian water without interaction of organic matter. To prepare Cd stock solution for exposure water, 10mg of $CdCl_2$ was added in one liter of water. $CdCl_2$ was used because of its high solubility in water. The Cd exposure waters were prepared by adding Cd from the stock solution to 100 L of very soft US EPA water in three separate tanks, one for each of the Cd solution. Each exposure units were filled with 24L water from storage tank of exposure water. Bubblers were installed in the tanks to maintain homogeneity of Cd in water. In order to maintain the Cd concentration, water samples from exposure reservoirs were measured regularly in ICP-MS. In case of decrease in Cd concentration, excess Cd was added directly from stock solution in 24L exposure reservoirs.

Table (1) Concentrations of salts used in preparation of very soft US EPAwater

EPA very soft water	mg/L
NaHCO3	12
CaSO4x 2H20	7,5
MgSO4	7,5
KCl	0,5

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Measurement of general water quality and Speciation

Water quality parameters such as temperature, PH, concentration of major cations, TOC, dissolved oxygen, conductivity, were monitored.

Temperature was measured by temperature logger placed in reservoirs during experiment. The logger measured temperature with time interval of thirty minutes.

In exposure reservoirs pH and dissolved oxygen were measured using pH electrode and oxygen electrode respectively at different times during experiment. In addition, pH was logged in reservoir with control water only. Concentrations of major cations i-e. Na, Mg, K, Ca and TOC was also monitored on different times during exposure. Excessive Ca can promote fungal growth (Finn 2007) To get the total concentrations for major cations and TOC, unfiltered samples were measured in ICP-MS and TOC analyzer respectively.

Speciation of Cd

Cd was measured in acidified regularly collected unfiltered water samples. In order to determine the distribution of Cd species in exposure water, combined size and charge fractionation was applied *in situ*. Fractionation was performed twice, before and after exposure to analyze the changes in the dynamic of Cd species. The size fractionation was based on membrane filtration (0.45µm) and ultrafiltration (hollowfibre with 10kDa cutoff). The charge filtration included Amberlite ion exchange chromatography. Both procedures followed washing of filters and performed through peristaltic pump.

In size fractionation, at first 50ml water sample was taken for total unfiltered water. The Cd content in the sample gives an information about total concentration of Cd. The next step was followed by filtration through 0.45um (membrane filtration) and 50ml water was sampled. This tells us about the concentration of Cd species in form of particles and colloids. At the final stage, ultrafiltration was run using <10KDa and 100ml of water was collected. Ultrafiltration

determines the concentration of low molecular mass. In the procedure peristaltic pump was used and set for 115 round per minute. The pump created a pressure that made water run through filtration and ultrafiltration. The pressure is crucial and should be set around 115 rounds per minute. For ultrafiltration the pressure is crucial and should be less than 10 Psi.

In charge filtration, Amberlite resin was used. The sample filtered through hollow fiber was filtered again through Amberlite ion exchange chromatography. 15ml of water was sampled for this purpose. All samples were analyzed using ICP-MS. House standard of 6.7µgCd/L was used as control.

Test species

Atlantic salmon egg is good example for chronic exposure tests because of long developmental period. In the current experiment Atlantic salmon was used as model organism. Eggs from three different females and semen from a male fish were shipped overnight by Aqua Green hatchery Norway. Eggs were dry fertilized with semen *in situ* and transferred in to the exposure boxes after fertilization. The boxes contained grids to hold eggs (Figure1). Each box occupied five hundred eggs. Each box was assigned with eggs from one female and three exposure boxes one for each female were connected to one reservoir. In this way every exposure unit comprised of eggs from three females. The boxes were titled with females F1, F2, and F3. The selection of eggs from three different female replicate was made to observe intraspecific biological responses towards Cd.

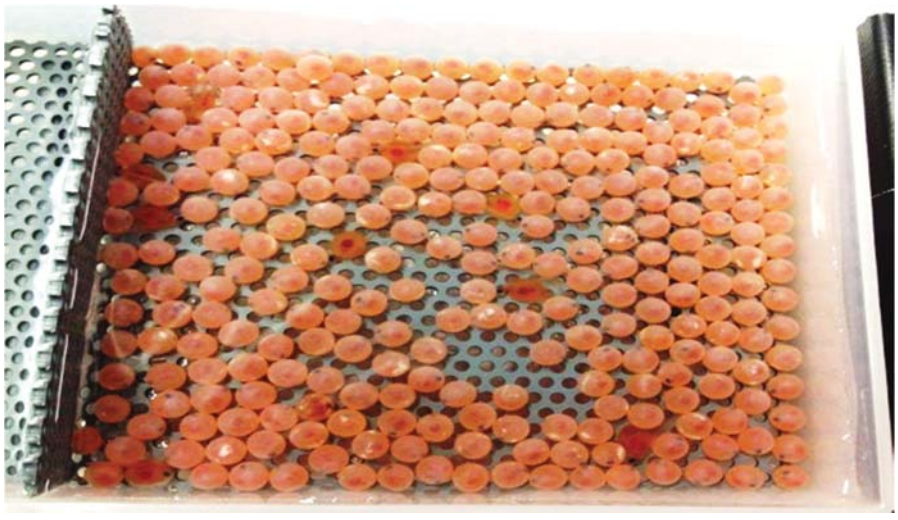


Figure (1); holding eggs in exposure box

Uptake of Cd

To measure the uptake of Cd over time, samples were taken; on day after fertilization, gastrula, eyed egg, approaching hatching and after hatching. In addition to determine Cd uptake in different locations, eggs were divided into whole egg, inside egg and alevins were divided in alevin and yolk sac.

Egg sampling for uptake mainly involved whole egg and inside egg content. In total six eggs were selected from each female separately; three for each inside egg and whole egg content. Prior sampling eggs were rinsed with distilled water and dried on paper towel. To extract the inside egg content, syringe was used by injecting the needle inside egg. Inside egg content was then transferred into pre-weighted 5ml tubes. For uptake in whole egg, eggs were collected in pre-weighted eppendrof.

Alevins and yolk sac were sampled after hatching. The number of alevins were kept same as for egg. Yolk content was yet again collected using syringe. Both alevins and yolk sac contents were collected separately in to 5ml tube. All samples were stored at -20ºC, freeze dried weighed before digestion.

An additional follow up measurement was performed to test the tissue Cd concentration of swim up after the termination of exposure. This time F1 and F3 were selected for sampling as it was observed during exposure that F2 had comparatively low fitness. Therefore, relying on results from F2 can be manipulative.

Digestion of egg and alevin samples in Ultraclave

For digestion in UltraClave egg samples were subjected to concentrated HNO_3 and MiliQ water. Ultra Clave uses high temperature and pressure during digestion. The temperature is rised to 260°C for thirty minutes. To control pressure H_2SO_4 and H_2O_2 were used. The samples were treated with acid and MiliQ water a day prior to digestion in ultraclave. This was specifically done with inside egg samples in order to dissolve contents stuck to tube walls. Samples from tubes were transferred in ultraclave Teflon tubes. In total, 5ml of HNO_3, 2ml of MiliQ water and 500ul of internal standard (2% of HCl+ HNO_3 + 8mg/L Rh) were added. Internal standard is used to correct the loss of sample during sampling or preparation. Three blank samples and certified reference material (DOLT4, DORM3) were also included. Blanks and certified reference material for information about background of trace elements and

validation of analysis at ICP-MS respectively. The samples were measured by Hans Christian Teien in ICP-MS.

Toxic effects of Cd

In order to obtain information toxic effects of Cd, following end points were analyzed.

Swelling

In order to measure effect of Cd on degree of swelling, ten unfertilized eggs from each female were selected randomly before the start of exposure and after 24 hours of fertilization. The diameter of these eggs were measured on scale. The degree of swelling was calculated as relative percentage in swelling. Relative percentage swelling is change in diameter of egg after fertilization.

Time of hatching

The time of onset and termination of hatching were determined. The number of hatchings were recorded every day. The hatching success was calculated as percentage hatching in each exposure groups relative to total fertilized eggs.

DNA damage

To test effects of Cd on DNA, comet assay was performed on blood cells of alevins. Comet assay determines DNA damage in individual cells. The procedure was carried out in two phases. First phase was done at FIGARO and involved collection of blood samples. The second phase was completed at Public health institute Oslo which included scoring of cell for DNA damage.

The protocol for blood sampling was developed by Isotope Lab NMBU, Ås Norway. Four alevins were selected from female 1 (F1) and dissected immediately *in situ*. The dissection was performed on ice to keep alevins alive and minimize heat shock and stress. Factors like heat shock and stress can induce DNA damage which can manipulate results. Alevins were put in petri dishes and excess of water was dried around alevins. Needles were inserted on tail and head to limit their movement. For collecting blood, needle was punched on the artery running down into yolk sac. The blood was collected throμgh glass pasture pipette (figure 2B). Estimating 2μl of blood containing 10^6 cells was removed from pasture pipette and transferred into pre-cold Eppendrof with 50ml of merchant buffer in it. Blood cells were embeded in

agarose gel and was molded on films (figure 2C). Two films were produced: one for enzyme treatment and other with no enzyme treatment.

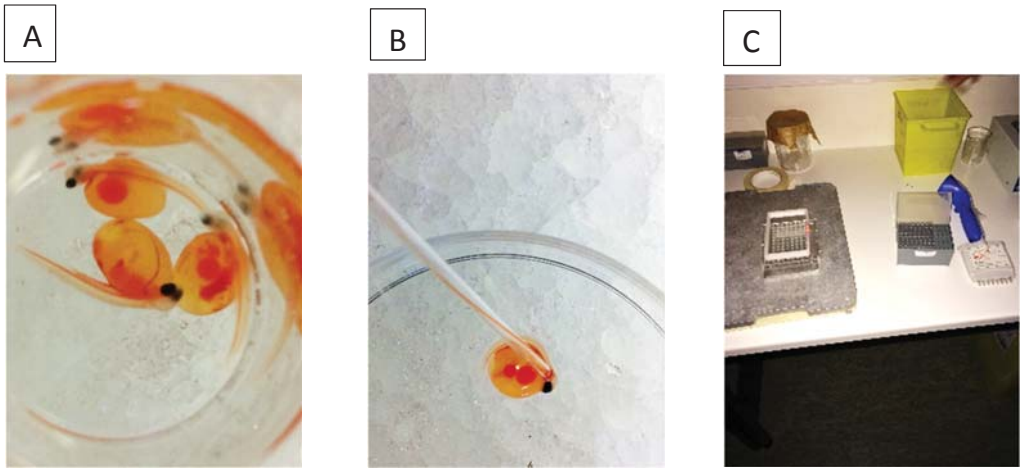


Figure (2): Blood sampling; A, hatched alevins. B, collecting blood through pasture pipette. C, molding of gel on films.

The purpose of enzyme treatment

The comet assay determines DNA damage as strand breaks in DNA. However, there may be other kinds of damage or lesions which may not be in the form of strand breaks. Other types of damage are quantitatively measured and can be converted to strand breaks with the use of lesion-specific endonucleases. In current study, Formamidopyrimidine DNA glycosylase (FPG)was used. This enzyme recognizes the oxidized purine 8-oxo-guanin but also ring opened purines or Formamidopyrimidine lesions and cleaves sµgar-phosphate backbone of DNA at sites of mentioned base lesions converting them into strand breaks.

In the second phase of the procedure films were lysed with lysis buffer. Lysis buffer destroys cell components living only cell nucleus. In addition, these films were subjected to electrophoresis with alkaline medium. The films were then stained and scored under microscope where fragmented DNA strands migrate away from the nuclei. Distance of migrated DNA strand from nuclei indicates the degree of damage and appear as comet under microscope The resulting score from film without enzyme treatment is subtracted from the scores resulted from enzyme treated film. Quantitative DNA damage was determined using %tail intensity which is the % migration of DNA from or nucleus.

Growth

As set by the OECD 210 guidelines, at the end of exposure the body length (mm) and body weight (g) of 6 alevins were recorded from all three females.

Deformities

Deform alevins with two heads and tails, retardation with curved body, smaller yolk sac, pale body were identified and the numbers were documented by the end of exposure.

Mortality

Dead eggs were counted and eliminated from boxes on daily basis. It was important in sense that dead eggs can decompose quickly and develop fungus. The dead eggs can be distinguished from living as dead ones turn white because of coagulation of protein inside egg. In the beginning of experiment, the dead eggs could not be distinguished if they were fertilized or not. With naked eyes fertilized eggs can be recognized when they develop eyes. Dead eggs were treated with vinegar. The treatment can dissolve the coagulated protein and makes it easy to differentiate between dead fertilized and unfertilized eggs.

Statistics

The general water quality and speciation of Cd are presented in table with mean and standard deviation (SD). The statistical tests were performed using R commander and all the figures were made in Microsoft excel 2013. Following tests were used; Shapiro test, Kruskal-Wallis test, one way ANOVA or analysis of variance, Tukey pos hoc tests and linear regression.

In all tests, Female type, Cd ambient concentration and °D were set as factors. Cd uptake, degree of swelling, DNA damage and growth and hatching time were set as response variables. First the response variables were subjected to Shapiro test which is performed to test the normality. In case if the data was not normally distributed, Kruskal-Wallis test which is non-parametric test was applied. However, since the number of samples (n) were low therefore Shapiro test would be not trust worthy. In order to test the difference in means of sample one way ANOVA was performed which was followed by Tukey post hoc to test the difference between the means. Inside egg Cd content was log transformed and then subjected to linear regression. Level of significance was set $p \leq 0.05$ as a criteria to define the differences.

4. Results and discussion

General water quality

The general water quality measurements are given in mean and SD in (table 2). The results indicate that measured temperature during the whole exposure was maintained and did not exceed from 6.0±1.5 standard temperature in any of the treatments. pH results before and after exposure are similar. The concentration of major cations and TOC did not change much in all four exposure units (table 1).

Speciation of Cd

Results from water samples based on before and after exposure demonstrate that Cd in total unfractionated water was close to nominal concentrations 0, 9.6±0.1, 27.4±0.4 and 113±33 µg/L respectively (table2). However, Cd was mainly present as LMM (<10kda) and cationic forms about 83% of total Cd (figure). On the other hand, colloidal and particulate made only 5% to 10% (<0.45µm) and 10% to 12% (>0.45) fractions of total Cd respectively (figure 3).

As stated earlier that pH is a crucial water quality parameter. Change in pH can dramatically change the distribution of Cd. However, results indicate that it is very unlikely that pH influenced the distribution of Cd because of the similar and close range of pH to neutral level (table). In addition, higher concentration of Cd as LMM compared to colloidal and particulate forms indicate that mostly TOC has not complexed with Cd.

Table (2) General water quality parameters

Parameters	Control	10µgCd(µg/L)	30µgCd(µg/L)	100µgCd(µg/L)
PH	6.8±0.07	7.0±0.3	7.0±0.3	7.0±0.3
Temperature	6.4±0.2	6.6±0.1	6.7±0.1	6.6±0.1
Na(mg/L)	4.3±0.4	4.2±0.2	4.2±0.3	4.1±0.2
Mg(mg/L)	1.6±0.1	1.6±0.1	1.6±0.1	1.6±0.1
K(mg/L)	1.1±0.5	0.8±0.4	0.7±0.4	0.7±0.4
Ca(mg/L)	2.1±0.1	1.9±0.1	1.9±0.3	2±0.3
TOC(mg/L)	1.4±1.0	0.4±0.5	1.1±0.5	1.3±0.6

Table (3) Speciation of Cd in water before and after exposure

	Total	particulate	colloidal	LMM	Cd cation
nominal Cd Concentration	unfiltered	>0,45µm	0,45µm-10Da	<10Kda	
control	0.05±0.07	0.05±0.07	0.1±0.1	0.1±0.1	0.1±0
10µgCd (µg/L)	9.6±0.1	0.5±0.4	1.15±0.3	7.95±0.2	7.1±0.7*
30µgCd (µg/L)	27.45±0.3	1.45±0.2	3.3±0.1	22.7±0.4	20.5±0.8*
100µgCd(µg/L)	113.4±33.3	8.15±1.6	11.3±7	93.75±22.1	88.65±15.9*

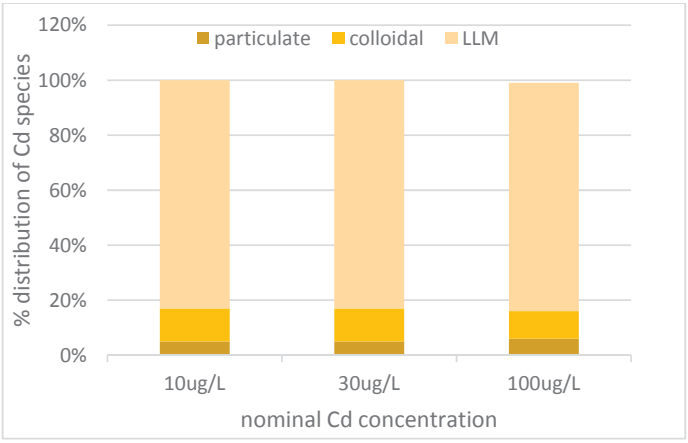


Figure (3): % distribution of Cd species in water before and after exposure.

Uptake of Cd

Results demonstrate the uptake of Cd. The uptake result in given in figure () which determines Cd uptake in whole egg, inside egg, alevin and yolk sac for each female. Results from regression model show that uptake was initially low ($p<0.0003$) which increased till gastrula stage in eggs of all females (figure 3 A, B, C). However, after gastrula stage Cd uptake remained unchanged until hatching in all ambient concentrations of Cd (figure 3A, B, C). This indicate an equilibrium state where the rate of uptake is equal to the rate of elimination. However, no significant differences were observed in pattern of uptake in any female type eggs.

According to our results, inside egg Cd uptake was low (figure 3D, E, F) after fertilization in eggs of each female in all treatments which is consistent with regression model giving out $p<0.01^*$. However, from the after fertilization stage till gastrula stage a dose dependent increase in uptake was observed in eggs from all females (figure). From gastrula stage until hatching no

difference was observed in uptake between life stages. In addition, eggs of three females do not show any difference in uptake.

Alevins also showed Cd uptake. However, Tukey-pos hoc test revealed that there was no significant difference (*p=0.2*) in pattern of uptake between 10µgCd and 30µgCd/L treated groups (figure 4A).There was significant difference however between 10ugCd/L to 100ugCd/L and 30ugCd/L to 100ugCd/L uptake (figure 4A). In addition, Cd uptake was found very low in yolk content 0.013±0 µg/g dry weight, independent of ambient Cd concentrations. In follow up sampling still low fractions of Cd found in swim up(figure 4B).

Cd uptake is dependent on bioavailability and water hardness. In many Norwegian fresh water lakes and river the water hardness level is below 10mg/L (Throng *et al.,* 2007). Therefore, in low water hardness more Cd can be taken up developing in eggs.

When the Cd is taken by eggs, it can be detected in different parts in different concentrations reflecting variable uptake within eggs (Burnison. 2006). Whole egg Cd uptake was from 70% to 90% compared to inside egg Cd uptake. However, alevin Cd uptake was 40% compared to inside egg only in 10µgCd/L but for 30µgCd/L and 100µgCd/L it was 4% and 10% respectively. In addition, Cd uptake was dose dependent both in whole and inside egg. An increase in uptake before swelling to gastrula stage both in whole egg and inside which reach equilibrium indicate that uptake mechanism was saturated but independent of ambient Cd concentrations. And also, it took 23 days to reach equilibrium.

Chorion is primary site for metal uptake in developing egg. It contains anionic sites such as sulfhydrylps that Cd can bind to (Petersen *et al.,* 1985). From Chorion Cd can gradually be taken up inside egg (Michibata. 1981). In addition, Cd uptake in chorion was not separately measured in current study. However, relative higher uptake of Cd in whole egg to inside egg shows that most of the Cd is bound to chorion.

From Chorion Cd can be absorbed inside egg (Michibata. 1981). It should be noted that in present study eggs were exposed to Cd after fertilization and before swelling. During swelling, while chorion is still greatly permeable facilitates the influx of water with Cd into PVS. The net negative charge of PVS has the tendency to attract Cd^{2+} from ambient water (Stouthart et al 1994). However, perivitalline Cd uptake was not measured in current study but higher uptake in inside egg compared to alevin and yolk show that most of Cd inside egg was retained in PVS.

Inside egg, from PVS Cd can gradually be absorbed by embryo primarily through integument (Daudt and Kennedy. 2008). Uptake in alevin and yolk sac at hatch indicate that Cd penetrated embryo in low fractions this is also supported by (Romboμgh and Garside 1982). This may be because most of Cd retained to the chorion and some in PVS (Beattie and Pascoe. 1978). The persistence of Cd after 200 days of exposure shows that the organs are not well developed to bioeliminate Cd (Osman *et al.*, 2007). However, it is unclear that concentration of Cd measured in alevins actually penetrated while they were still protected by chorion or after hatching.

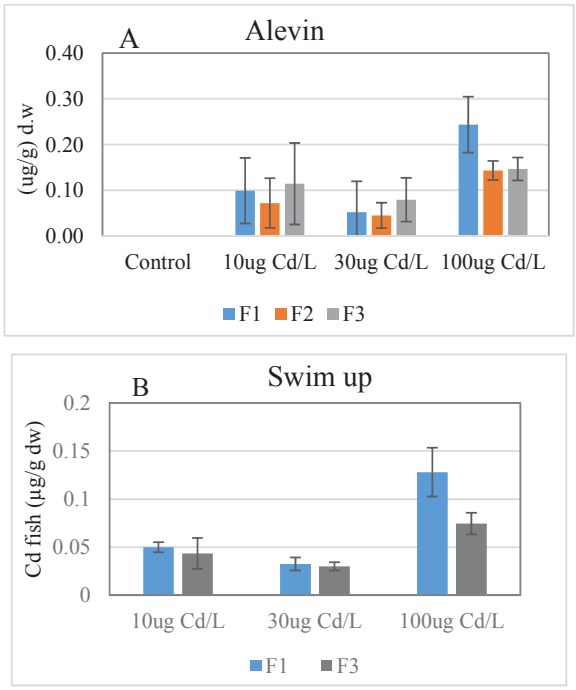


Figure (4): A, Cd uptake in alevins at hatch. B, the concentration of Cd persisted till swim up after the termination of Cd exposure.

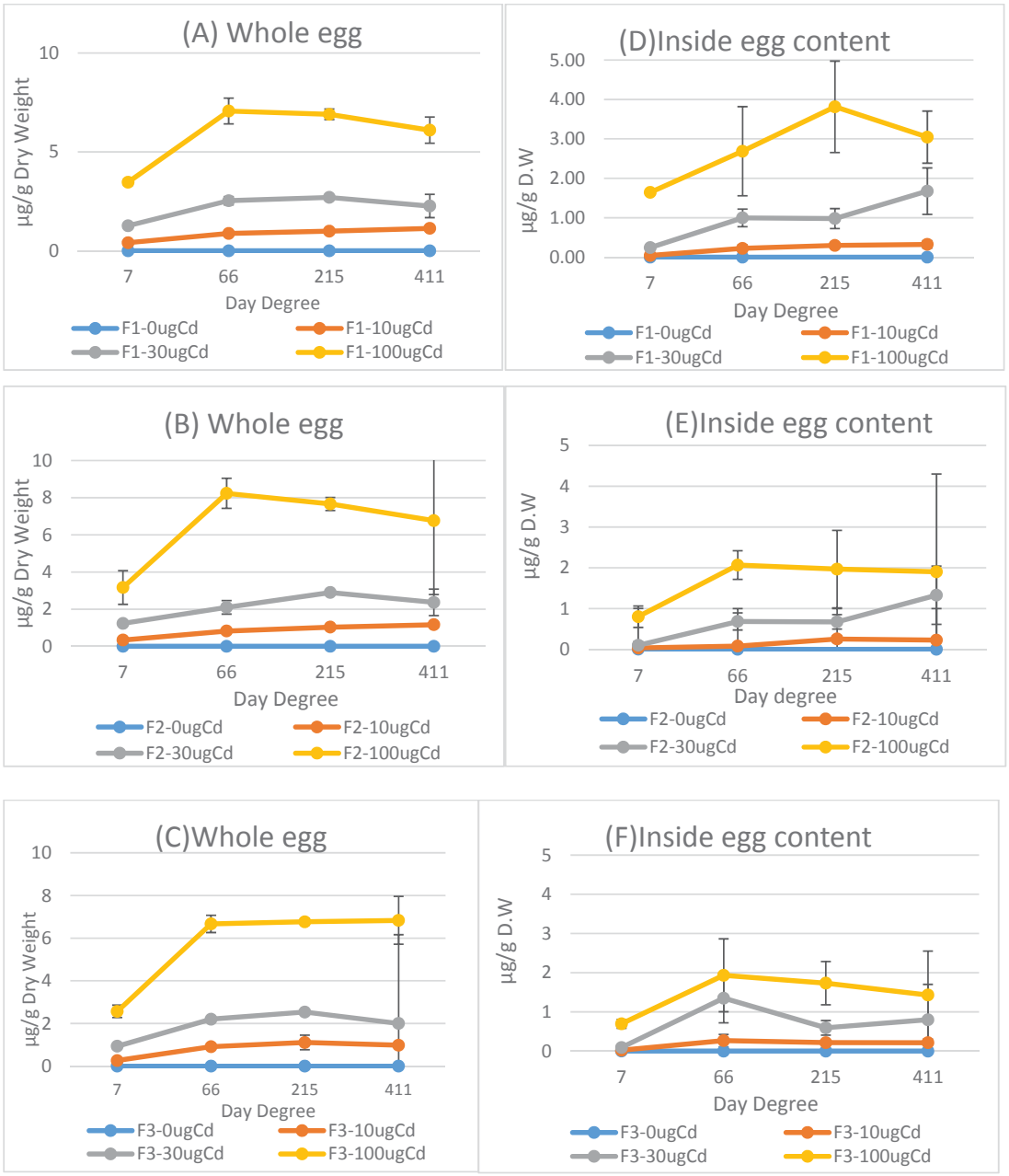


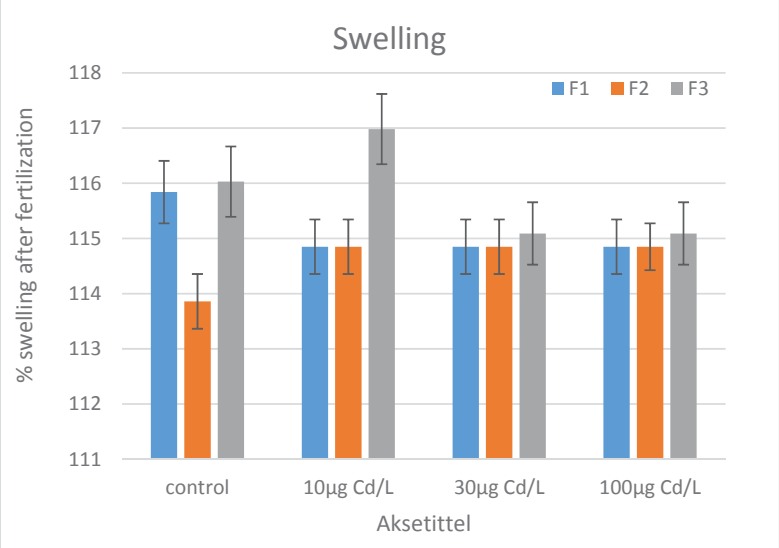
Figure (5) Cd uptake in whole egg (A, B, C) and inside egg (D, E, F) collected from each female type eggs after fertilization, gastrula, eyed egg and approaching hatching which are expressed in day degrees. The Cd concentration is given in µg/g dry weight.

Toxic effects of Cd

Toxicity of Cd is dependent on speciation, bioavailability and uptake**.** Toxicity of Cd is dependent on speciation, bioavailability and uptake**.** According to our results, higher concentration of Cd was bound to chorion which shows the protective role of chorion (Beattie and Pascoe. 1978, Witeska *et al.*, 1995). However, in this section we will see the concentration of Cd that was measured inside egg and in alevin causes toxic effects or not.

Swelling

The ANOVA type one results revealed that there is no significant difference (*P>0.93*) in egg swelling between control and Cd treated groups. However, there is slight difference (*P<0.01*) in egg swelling between the female group where F3 eggs diameter was bigger after swelling in all ambient concentrations compared to F1 and F2 eggs (figure 5).



Figure(5): % relative swelling after fertilization of three female type eggs in ambient concentrations.

It is quite clear based on the present result that Cd did not affect the degree of swelling after fertilization. However, all three female eggs show slight endogenous difference in degree of swelling independent of Cd concentration. However, previously Cd has been reported to affect swelling in concentrations of 3µgCd/L, 10µgCd/L and 30µgCd/L (Giblin *et al.,* 2015). In addition, common carp (fresh water fish) eggs showed reduced swelling from 40% to 26% in concentration from 1µgCd/L to 10µgCd/L (Witeska *et al.,* 1995). On the other hand, (Metin. 2001) reported that Cd significantly reduced swelling in eggs of mirror carp in 5-50 µgCd/L.

Time of hatching

In control group hatching started at 469 ºD (day degree) and finished at 550 ºD day degree. In Cd treated groups eggs hatching onset was from 383 to 580 ºD day degree. Eggs in 10µgCd/L showed the earliest hatching which was followed by 100µgCd/L and 30 µgCd/L respectively (figure 6). The early onset of hatching in 10µgCd/L however, was extended compared to control (figure 6). Same phenomenon was observed in 30µgCd/L and 100µgCd/L despite they hatched close to time of control group (figure 6). However, eggs of each female type showed differences in time to hatch not only between Cd treated groups but also within group. Surprisingly, the hatching success remained 74% to 96 % in control and Cd treated groups.

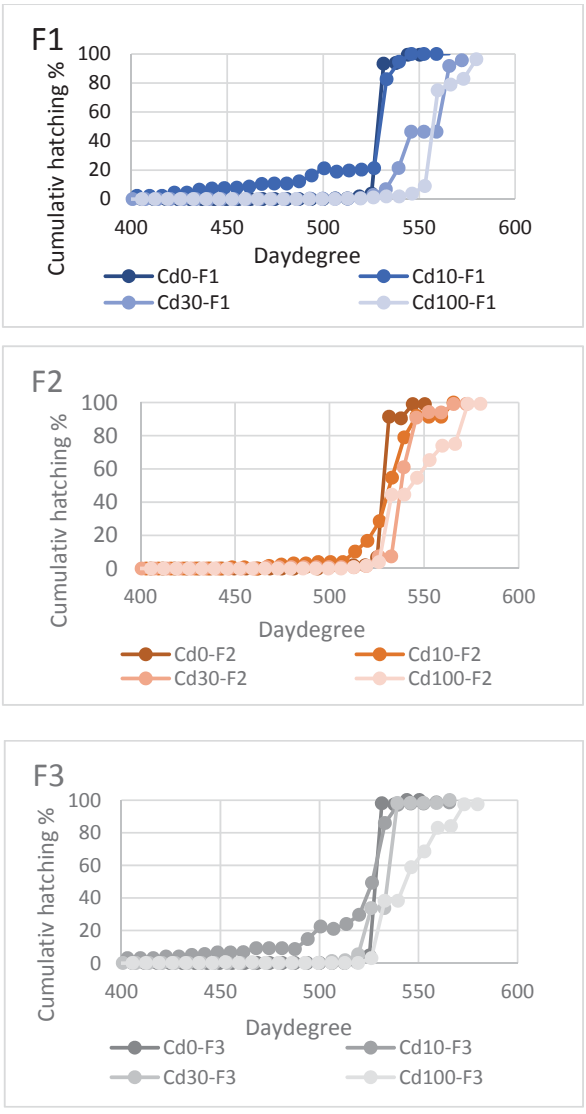


Figure (6): cumulative hatching % of fertilized eggs for each female eggs separately against ºD.

Hatching is a complex process that involve embryonic movement, hatching enzyme and hatching gland (Oppen-Berntsen *et al.*, 1990). Naturally after fertilization chorion becomes thinner which continues approaching hatching (Jaramillo *et al.,* 2015). In current study two phenomenon were observed; Premature and extended period of hatching.

Premature hatching due to Cd was reported in rainbow trout by Woodworth and Pascoe (1981) and Daudt, Kennedy (2008). In current study, premature hatching in 10µgCd/L may be due to binding of Cd to chorion which causes physico chemical changes possibly making chorion more soft and fragile which lead to early start of hatching (Beattie and Pascoe. 1979). Cd can also stimulate chorionase activity consequently setting up premature hatching (Daudt and Kennedy. 2008).

Premature hatching can also reduce the embryonic developmental time. Early and less developed embryos contain smaller amount of hatching enzymes (Oppen-Berntsen *et al.,* 1990). Therefore, it may require longer time to digest or degrade chorion causing extended hatching which was observed in 10µgCd/L group (Oppen-Berntsen *et al.,* 1990).

Extended hatching was observed in 30µgCd/L and 100µgCd/L. This was also reported by Brungs (1969) and Klein-Macphee *el al.,* (1984) when fathead minnow and winter flounder were exposed to Zn and Ag respectively. They concluded that decreased metabolic activity could trigger both premature and extended hatching. In addition, reduced activity of chorionase in perivitalline space may also be the reason of prolonged hatching (Metin. 2001).

From present results of hatching it could be seen that developing embryos behave differently to different concentrations of Cd. Further investigations are required to analyze the underlying mechanism of this behavior.

Assessment of DNA Damage using comet assay

Comet assay was used to determine quantitative DNA damage by Cd. Results show the mean % tail intensity in film without enzyme treatment was 28% for control group. However, in Cd treated groups the mean % tail intensity remained 32, 18, 34 % for 10µgCd/L, 30µgCd/L and 100µgCd/L respectively. The reason for so high background damage in control might have developed because of lack of skills during sampling which was improved with preparing second film.

On the other hand, one way ANOVA results from the film treated with enzyme show that Cd has induced DNA damage to significant level ($p<0.007$). In control group the mean % tail

intensity was 11.6 while for the Cd treated groups it was from 30 to 33%. However, there is no significant difference in mean % tail intensity between Cd treated groups. In addition, it should be well noted that in enzyme treated film the %DNA damage appears higher due to induced strand breaks at lesion sites. However, due to high background damage in film without fpg enzyme treatment, it was not possible to subtract the scores of fpg treated film from fpg non treatment film.

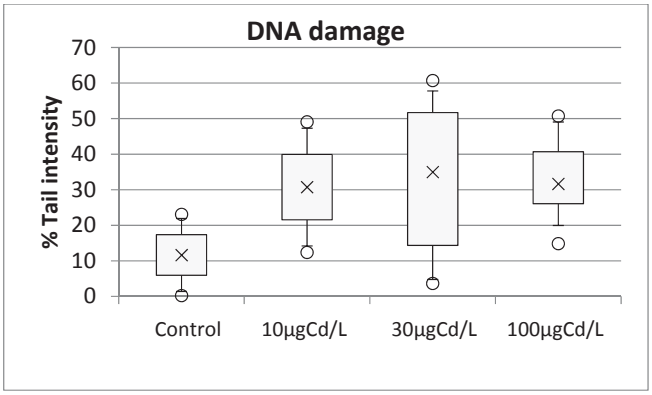


Figure (7): The median % tail intensity showing DNA damage in control and Cd treated groups

In comet assay, previously whole fish organism (embryo or larva) had been used due to small size to detect DNA damage in single cells which would give high intra-individual variability due to presence of different cell types and tissues. This could manipulate statistical analysis and sensitivity of the test (Morin *et al.,* 2011). However, the method of blood sampling in alevins that has been used in current study developed for the first time. It gives an advantage to determine DNA damage in specific tissue or cell types in developing embryo.

Results from enzyme treated film indicate that Cd have induced DNA damage to some degree well above control. DNA damage by Cd has been reported in Japanese medaka where Cd cause around 30% damage when exposed to 1.9µg/g d.w and 8.5µg/g d.w spiked sediment (Barjhoux *et al.,* 2012). A similar level of damage was also recorded in African catfish exposed to 100µgCd/L of lead nitrate (Osman. 2007).

Cd is known to induce DNA damage but indirectly throµgh producing reactive oxygen free radicals (ROS) or species (Joseph. 2009). These species are super oxide anion ($^{*}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{*}OH$). ROS can bind to proteins, lipids, DNA and oxidize these molecules (Barzilai and Yamamoto. 2004).

The effect of excessive production of ROS where cellular anti-oxidant defense mechanism cannot neutralize them is termed oxidative stress (Barzilai and Yamamoto. 2004). DNA damage occurring due to oxidative stress are single strand breaks and double strand breaks, with later more dangerous. In addition, under normal conditions DNA damage can be repaired by repair enzymes. However, if not repaired or repaired incorrectly can lead to chromosomal aberrations, mutation and cell death (Kienzler. 2013). Reportedly, Cd can inhibit the recruitment of DNA repair enzymes which can lead to accumulation of a lot of single and double strand breaks (Pereira. 2013). However, alkaline comet assay can detect both single and double strand breaks but cannot distinguish them. Therefore, it is difficult to determine the relative amount of double strand breaks in current study. Also, both ROS and the degree of DNA repair was not measured. Hence, ROS and DNA repair can be useful tools to be used in further investigations.

Another factor should also be considered here, DNA damage was measured after alevins were hatched and were in direct contact with water. Therefore, it is difficult to say if the damage occur before or after hatching.

Growth

To investigate the effect of Cd on growth, body length and weight were measured at hatching. And results demonstrate no significant difference between control and Cd treated alevins at hatching for both body length ($p=0.63$) and weight ($p=0.67$). However, in both parameters female type eggs show significant difference . In case of body length F2 type eggs were found smaller ($p<0.004$) than F1 and F3 type eggs. However, the body weight of F3 type eggs were found bigger than F1 and F2 type eggs ($p=0.05$).

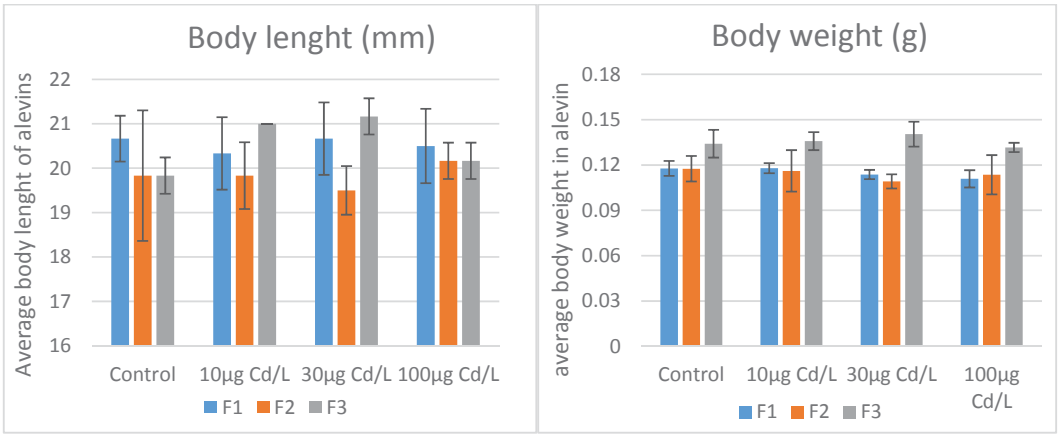


Figure (8): The length and weight of alevins at hatch from control and Cd treated groups.

The growth in alevins has not been affected by Cd. A similar result has been reported on Atlantic salmon and ide exposed to Cd (Petersen *el al*.,1985, Witeska *et al.,* 2014). In contrast, (Woodworth and Pascoe. 1982) and Romboµgh and Garside. 1980 observed significant growth reduction due to Cd in newly hatched alevins of brown trout and Atlantic salmon respectively. Following assumptions can be produced regarding retarded growth as result of Cd exposure;

- Premature hatching increases the longer exposure of Cd to alevins that can in turn reduce growth (Woodworth and Pascoe. 1982). Because after hatching alevins are in direct contact with contaminated water.
- Partial swelling or hardened egg can also limit the growth of embryo (Li *et al.* 1989).
- Cd is antagonistic to Ca (see section 1.0). Cd can bind to proteins of yolk that can interfere with yolk utilization and affect growth (Petersen *et al.,* 1983, Romboµgh and Garside. 1982)
- Cd induces detoxifying process which is energy consuming. Thus in Cd exposed embryo less energy is available for growth (Jezierska *et al.,* 2009)
- Cd can also disturb ion balance developing fish. The cost of ion balance can reduce growth as compensation of osmoregulatory function (Witeska *et al.,* 2010).

Linking the first three assumptions to our finding, we assume that premature hatching had no impact on growth. The degree of swelling was also normal which mean that there was enough space for embryonic growth. Yolk sac were also not bigger than alveins and this indicate that yolk utilization was normal. However, yolk utilization was not measured in current study. The last two assumptions are out of scope of this study. However, fish respond in different ways

towards different concentrations of toxicants. For example, Romboμgh and Garside (1982) found retarded growth in 0.47μgCd/L ambient concentration but not in higher concentrations.

Deformity and mortality

There were very few deformities recorded after they hatched in Cd treated groups which were close to number of deformities in control group table (4). The types of the deformities were observed are as follows;

Table (4): number of deformed alevins at hatch.

Female	Control	10μgCd/L	30μgCd/L	100μgCd/L
F1	0	0	0	0
F2	1	3	0	1
F3	3	0	1	4

Fish deformities are easy to distinguish macroscopically and is advantageous over other biomarkers of heavy metal exposure (Sfakianakis et al 2014). In unpolluted water there are several reasons for deformities such as unfavorable abiotic conditions, inappropriate nuitrition and gentic factors (Boglione et al 2013, Mckay and Gjerde 1986). However, the pathway throμgh which Cd exerts deformities in fish is not quite clear (Sfakianakis et al 2014). Most of the deformities in fish are related to vertebral column or notochord. Notochord is a primary axial structure that differentiates into vertebral column or other tissues during early development. Metal like toxicant can disrupt notochord development and differentiation which can lead to skeletal, muscular and neural deformities (Sfakianakis 2014) As stated earlier that Cd can depress Ca and P level (Romboμgh and Garside 1983). Consequently, to compensate the loss, Ca can be released from bone tissue leaving spinal column fragile and sensitive to deformities. Otherwise, Cd can also directly affect bone tissue may be throμgh disturbing bone mineralization (Sfakianakis et al 2014). Fish deformity can also be linked to the genotoxic effect of Cd (Sfakianakis 2014). The extent of retardation is related to tissue Ca level (Romboμgh and Garside 1983).

In the beginning after fertilization rapid mortalities were observed. During that time dead eggs could not be distinguished if they were fertilized or not. However, upon the eyed stage eggs it became clear to identify fertilized dead eggs. The number of mortalities were relatively low. This may be due to the fact that Cd did not affect growth rate and did not cause body

malformations. Therefore, it seems unlikely that Cd cause mortality in the absence of any severe sub lethal effects.

5. Conclusion

Atlantic salmon eggs and newly hatched alevins were less sensitive than juvenile parr towards Cd exposure. The uptake was low during the whole exposure. Yolk sac showed the least uptake. The most pronounced sublethal effects of Cd were premature, prolong hatching and DNA damage. Other biomarkers did not show any sensitivity towards Cd exposure.

From the results of fractionation, it was revealed that Cd was predominantly present as LMM and cationic forms which determines that it was potentially bioavailable. The concentrations of particulate and colloidal forms were relatively low.

Higher concentration of Cd was found to be bound with chorion while some was absorbed inside egg. Cd uptake was also observed in alevins at hatch but it is difficult to predict if Cd was taken up inside embryos while they were still protected by chorion or they absorbed Cd mainly after they were hatched. Both in whole egg and inside egg, Cd uptake reached saturation after gastrula stage.

The degree of swelling remained unaffected by any of the Cd treated groups. On the other hand, the time of hatching was affected. In eggs in 10µgCd/L Cd showed both premature and extended period of hatching while eggs in 30µgCd/L and 100µgCd/L only showed extended period of hatching. This mean that Cd has different effects on different concentrations.

Comet assay results revealed that there was about 30% damage in DNA but in all Cd treated groups. In addition, it is also difficult to state that the DNA damage occurred to alevins before or after they were hatched.

The rate of growth remained unaffected by Cd in all ambient concentrations. This can be linked to normal degree of swelling because normal swelling in eggs provide space for embryonic growth. No effect on growth can be related to low uptake of Cd. Very few deformities were observed and low rate of mortalities were observed. This mean that chorion may have an important role in protecting early embryo in egg.

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7. Appendix

The detailed description of procedures involved performing comet assay. This steps were performed at Norwegian Public Health institute.

Lysis buffer

Concentrations of constituting chemicals were calculated for two GelBond® Films.

200ml stock solutions+ 22.2ml DMSO+ 2.22ml Triton.

The lysis buffer was stirred with magnet.

LMP agarose Gel

0.150g of agarose and 20ml PBS are added in pre-weighted flask. The mixture was dissolved in oven by heating it only for few seconds (the boiling point is low and crucial). The flask was put in pre-heated 37 ^{0}C heating block with covered lid to cool down. In addition 8 strips of 0.2ml of PCR tubes are placed to keep them warm.

Eppendrof tubes were placed on ice to keep them cool. 50µl of merchant buffer for cell suspension was added in 16 tubes. A group of 4 tubes for control, 10µg, 30µg and 100µg of cadmium respectively.

Preparation of Gelbond® films

Two films with cut right down corner edge was kept attached and kept ready to embed the blood cells. One film for enzyme treatment and the other for no enzyme treatment.

Pre-cooled cooling plate for films to place on while molding the gel.

Dissection of alevins

Molding of gel

10µl of cell suspension was added with 90µl of agarose in 0.2ml of PCR tubes. The time is crucial between adding cell suspension with agarose in pre warmed PCR tubes and molding them on films. This is the time where most background damage is expected to occur to cells or DNA. 90µl of gel was then molded on one of the films and the same step repeated for other film.

Both the films for put in lysis buffer at 4 ^{0}C separately for two days quickly after they are molded.

The second phase for electrophoresis and scoring was performed at Folkhelse Oslo.

Enzyme treatment

FPG enzyme was used. The optimum enzyme concentration 5.2µl was determined by titration before treating with enzyme.

When taken out of the lysis buffer the films were rinsed with distilled water briefly.The films were put in two different trays with cold collins buffer (no BSA) for 10 min in the cold room at 4 ^{0}C. For another 50 minutes the films were transferred in to trays with fresh Collins buffer. Meanwhile incubator was pre warmed up to 37 ^{0}C. Trays with Collins buffer+ BSA+ (one with FPG enzyme and other without) were kept in incubator to be pre warmed. The films were then transferred into trays in incubator for one hour.

Unwinding

2 litres of Electrophoresis solution was prepared by adding 1.8 L of distilled water+ 200 ml of electrophoresis stock solution+ 12ml of conc. HCl. 1.4 litre of electrophoresis solution was used for running electrophoresis and rest was used for unwinding. In order to perform unwinding the films were kept in tray with 600ml of electrophoresis solution for 40 minutes in cold room (4 ^{0}C).

Electrophoresis

1.4 litre of the remaining solution was poured in electrophoresis tank in refrigerator (10 ^{0}C). The films were then transferred into electrophoresis tank and run for 25 minutes.

Neutralization

From tank the films were put into tray with distilled water in it for 1 minute. Films were placed in neutralization buffer for 2×5 min. Rinsing the films were distilled water further they were treated with stock ethanol for 5 minutes and afterwards for 90 min with fresh ethanol. Completing this step both the films were put for drying out.

Staining and scoring

After the films were dried, they are treated with SYBR Gold and stained to be scored. The films are scored and analysed using Comet Macros.