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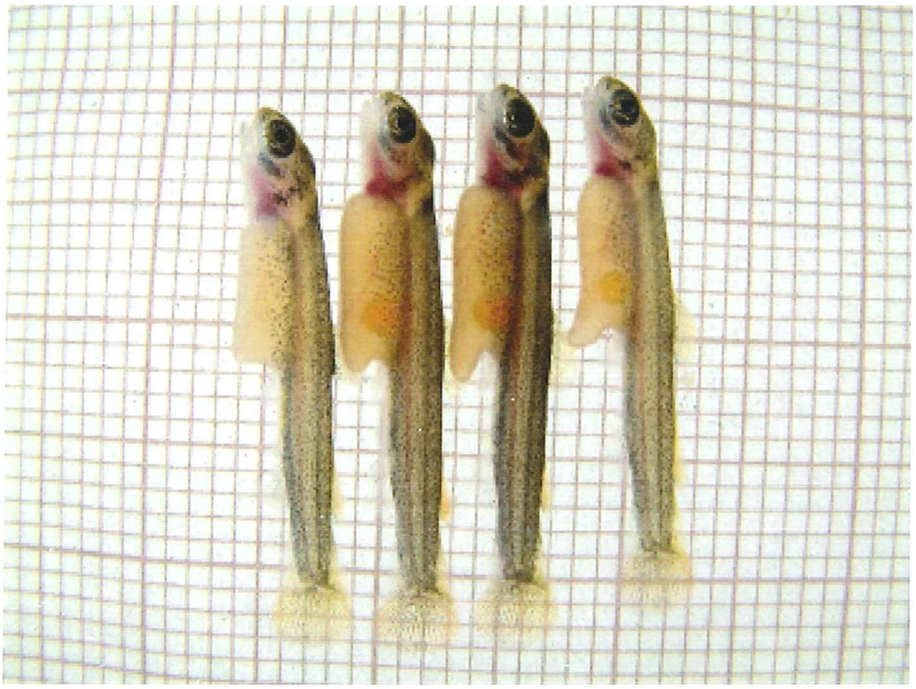
Effect of zinc on the early life stages of brown trout *(Salmo trutta)* at different levels of water hardness



Norwegian Institute for Water Research

REPORT

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Abstract

The effect of water hardness on the chronic toxicity of zinc to early life stages of brown trout has been studied. Separate tests were carried out in four dilution waters. Two of these were natural lake waters from Lake Maridalsvann (hardness 8.6 mg $CaCO_3$/l and Lake Store Sandungen (hardness 6.7 mg $CaCO_3$/l). High-hardness waters were prepared by adding calcium to these waters to achieve a hardness level of 100 mg $CaCO_3$/l. Eggs of brown trout were fertilised in the test waters and exposed for approximately 120 days in flow-trough channels. Hatching success, time to hatching and length and weight of larvae at termination of the test were observed. Signifcant delay of haching was observed at 100 µg/l nominal, added concentrations of Zn in both lake waters. The NOECs were 56 and 61 µg Zn/l respectively. With increase of water hardness to 100 mg $CaCO_3$/l, the effects of Zn was reduced in Lake Store Sandungen (NOEC=250 µg/l) but not in Lake Maridalsvann (NOEC=57 µg/l). The effects on length and weight of larvae on test termination were generally less pronounced.

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Effects of zinc on early life stages of brown trout (*Salmo trutta*) at different levels of water hardness

Preface

International Lead Zinc Research Organization contracted the Norwegian Institute for Water Research, NIVA, to perform a study of the effect of water hardness on the chronic toxicity of zinc to brown trout in December 2002. The study was started in December 2002 and terminated in April 2004.

Sigurd Hytterød and Torstein Kristiansen were responsible for the practical performance of the study and carried out the observations and measurenents during the tests. The chemical analyses were performed at NIVA's chemical laboratory.

Oslo, 09. May 2003

Torsten Källqvist

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Summary

The effect of water hardness on the chronic toxicity of zinc to early life stages of brown trout has been studied. The tests were designed in accordance with OECD Test Guideline 210; Fish, Early-life Stage Toxicity Test (OECD 1992). Separate tests were carried out in four dilution waters. Two of these were natural lake waters from Lake Maridalsvann (hardness 8.6 mg $CaCO_3$/l and Lake Store Sandungen (hardness 6.7 mg $CaCO_3$/l). High-hardness waters were prepared by adding calcium to these waters to achieve a hardness level of 100 mg $CaCO_3$/l. Five concentrations of zinc and one control without zinc were included in each test. The nominal added concentrations of zinc ranged from 10-250 µg/l in the low-hardness waters and 50-1000 µg/l in the high-hardness waters. Eggs of brown trout were fertilised in the test waters and exposed for approximately 120 days in flow-trough channels. Hatching success, time to hatching and length and weight of larvae at termination of the test were observed.

None of the endpoints investigated in the study showed drastic effects of zinc in the concentration range tested. The endpoint that appears to be most affected by the zinc treatment was the hatching time. In the low hardness waters a pronounced delay of hatching was observed as shown in figures 2 and 4. This was verified by the statistical analysis as significant deviations from the control.

Also in the two hard waters, a significant delay of hatching was observed at the highest zinc concentrations. This effect was most obvious in Lake Store Sandungen+Ca, where the two highest concentrations (500 and 1000 µg Zn/l) caused a pronounced delay of hatching. In Lake Maridalsvann+Ca, a significant delay in haching was found at the four highest concentrations of zinc (250-1000 µg/l).

Intermediate concentrations of zinc caused a small but statistically significant decrease in the time to hatching in three of the tests.

A negative effect of zinc exposure on the development of trout larvae was also shown in the length of larvae at test termination in Lake Store Sandungen, where the two highest concentrations of zinc (100 and 250 µg/l) gave significantly reduced length. In the other low-hardness water, Lake Maridalsvann, no significant reduction in length was found in any of the zinc-exposed groups. In the two hard waters, length of larvae was reduced only at the highest zinc concentration (1000 µg/l).

Negative effects of zinc on the weight of larvae were only found at the highest zinc exposure in Lake Store Sandungen and Lake Store Sandungen+Ca.

"Positive" i.e. or increased length and weight of larvae were observed at some zinc-exposed groups. The reason for these apparent stimulatory effects of zinc could not be explained.

The NOEC values, i.e. the highest concentrations in which no significant deviations from the control was observed for the three endpoints that could be statistically analysed are shown below. Zinc concentrations are the mean measured concentrations during exposure.

Dilution waters	NOEC hatching	NOEC length	NOEC weight
Lake Store Sandungen	56	56	106
Lake Maridalsvann	61	-*	-
Lake Store Sandungen + Ca	250*	496	-
Lake Maridalsvann +Ca	57	502	502

*"Positive" effects, i.e. shorter time to hatching and increased length, are ignored.

1. Introduction

The effect of water hardness on the toxicity of zinc to aquatic organisms has been discussed in connection with the production of a Risk Assessment Document for zinc and zinc compounds under the Directive EEC 793/93. In particular the generally soft water in the Nordic countries (Finland, Sweden and Norway) may require a hardness correction of the predicted no effect concentration (PNEC) which has been calculated for water of higher hardness. The available database on zinc toxicity contains very few effect concentrations for hardness below 10 mg $CaCO_3$/l. In order to provide more knowledge on effect of water hardness on the toxicity of zinc, a project to study chronic effects of zinc on organisms from three trophic levels (algae, daphnids and fish) was proposed at the Technical Meeting on Existing Chemicals.

The Norwegian Institute for Water Research, NIVA was invited to perform the fish tests in the project. It was decided to perform the tests on the early life stages of brown trout (*Salmo trutta* L).

The final protocol for the tests was approved by the International Lead Zinc Research Organization (ILZRO) on 2. December 2002. Two lakes with water hardness between 5 and 10 mg $CaCO_3$/l were selected as test media. In addition tests were carried out in the same two waters spiked with calcium to obtain a hardness of 100 mg $CaCO_3$/l.

The tests were designed in accordance with the OECD Test Guidline 210; Fish, Early-life Stage Toxicity Test, with a modification related to the swelling water quality and incubation temperature. Due to a late contract agreement in relation to the natural spawning period for brown trout, and changes in test conditions after start of the experiment, a non-resident brown trout population had to be used in the final experiment. In order to obtain results within the agreed time frame, the test was terminated before all individual control fish reached the free-feeding stage. The final tests were started on 12 December 2002 and terminated on 19 April 2003.

2. Materials and Methods

The test was performed in 24 channels with the dimensions:
- length: 52 cm
- whith: 8 cm
- depth: 8 cm

Each channel was divided into two chambers with a length of 26 cm. The volume of each chamber was 1.66 l.

Water was continuously added to one end of the tanks at a rate of approximately 5 ml/minute. An overflow in the opposite end kept the water level constant at 8 cm.
The tanks were placed in a thermostatically controlled room.

2.1 Dilution water

Dilution water was obtained from two lakes North of Oslo, Lake Maridalsvann and Lake Store Sandungen. Two additional hard dilution waters were obtained by addition of 37 mg Ca as $CaCl_2{\cdot}2H_2O$ to give a hardness of 100 mg $CaCO_3$/l. New batches of water were collected from the lakes and prepared on a weekly basis.

2.2 Test design

5 concentrations of Zn were tested in each of the four water qualities. These (added nominal) concentrations were 10, 25, 50, 100 and 250 µg Zn/l at low hardness and 50, 100, 250, 500 and 1000 µg Zn/l at hardness 100 mg $CaCO_3$/l. Zn was added as $ZnSO_4 \cdot 7H_2O$. One control without Zn addition was included for each of the four water qualities. Thus, the number of treatments in the experiment was 24.

2.3 Preparation of test media

The 24 test solutions were prepared in batches of 50 litres, approximately once a week. Water from these batches was distributed by peristaltic pumps to the experimental channels at a rate of approximately 5 ml/min.

2.4 Test organisms

Brown trout of the Lake Bygland strain was used in the experiment. This strain is resident in Lake Byglandsfjorden, Valley of Setesdalen, County of Aust Agder, southern Norway. This strain has been used in a series of ecotoxicological experiments related to acid waters, and recognised as an acid tolerant species (Rosseland and Skogheim 1987, Dalziel *et al.* 1995). The local hatchery, Setesdalen Settefisk, located at the outlet of Lake Byglkandsfjorden, provided the egg and milt from their brood stock. The brood stock represent a first generation from wild brown trout (parents being wild fish caught in the lake).

2.5 Fertilisation and start of test

Eggs from three females and milt from two males, were dry stripped at the hatchery on 12 December 2002, and shipped separately in plastic bags onto cooling elements, from the hatchery to NIVA. After a transport period of approximatly 6 hours, the eggs were mixed and dry-fertilized with the mixed milt. In each of the 24 channels, two groups of dry fertilized eggs were placed in separate chambers within the channels for swelling. The swelling water quality was thus different for each channel. As the quality of the swelling water can be crucial to both fertilization success and survival until start feeding (Keinänen 2002), the new test procedure ensured ecological relevance, i.e., the test conditions simulated the situation where a natural population spawn occurs in a contaminated locality.

2.6 Exposure temperature

The temperature was gradually adjusted from approximately 3.5°C at the start of the test to 5.7 °C after one month. Thereafter, the temperature was held at 5.7-6.3 °C. In its natural habitat, as well as in the Hatchery at Byglandsfjorden, the embryos develop at temperatures between 2-5 °C from December to mid-May. An incubation temperature below 8 °C is very important to avoid any deformation due to temperature stress (Baeverfjord 1998).

2.7 Test duration

The test was terminated after 116-119 days, which was 34-36 days after the beginning of hatching in the controls. The length of the exposure period was adjusted in order to obtain approximately the same degree-days[1] in each treatment. Most of the fry had a typical "start feeding behaviour", being very active, well pigmented and most of the yolk sac resorbed. As practical startfeeding would have biassed the weight measurements of individual fish at the end of the experiment, and our experimental

[1] degree-days is defined as the product of time(days) and mean temperature (°C)

permission given by the authorities did not include start fed fish, we stopped the experiment before lack of food became a problem in the control group.

2.8 Analyses and observations

The water temperature in each channel was recorded daily throughout the experiment with a calibrated digital thermometer.

Each new batch of water from the two lakes was analysed for TOC and Ca. After addition of calcium chloride and zinc sulphate, each of the 24 batches was analysed for electrical conductivity and Zn.

Dissolved oxygen in the test chambers was measured with a WTW oxygen electrode on three occasions during the test.

The eggs/fry were inspected daily and dead (opaque) eggs and dead fry were removed. Opaque eggs were inspected under stereoscopic microscope after treatment in a solution "clearing" the eggs, too verify if the eggs had been sucessfully fertilised.

After termination of the exposure, all surviving fry were measured (length) and weighed blotted dry in pre-weighed plastic tubes. Finally, the fry were dried for 24±2 hours at 60 °C and weighed again.

3. Results

The treatments in the study are denoted as the *nominal added concentration of zinc.*

3.1 Test performance and water quality

The course of temperature in one set of channels (Lake Maridalsvann + Ca) is shown in Figure 1. The temperature was gradually increased from approximately 4.5 ℃ at the start to approximately 5.9 after one month. The temperatures recorded during the test are shown in Table 1. The maximum variation in temperature between channels within each sub-set was 0.2 ℃ (Lake Maridalsvann). In the other experimental sub-sets the variation between the channels was from 0.03-0.11 °C. Since the development of fish eggs are closely related to the temperature, the exposure time has been calculated as degree-days. The extension of the exposure in days and degree-days is shown in Table 1.
An extensive chemical characterisation of the lake waters was performed on three occasions from December 2003 to March 2003. The results of the analyses are shown in Table 2 (Lake Store Sandungen) and Table 3 (Lake Maridalsvann). The results indicate a slight increase in pH, alkalinity and hardness during the winter. Based on the mean values for calcium and magnesium, the hardness of Lake Maridalsvann is calculated at 8.6 mg $CaCO_3$/l and Lake Store Sandungen at 6.7 mg $CaCO_3$/l.

Results of the more frequent analysis of calcium and total organic carbon in the weekly batches of lake water are shown in Table 4. The mean values of calcium are 2.08 mg/l in Lake Store Sandungen and 2.61 mg/l in Lake Maridalsvann. The mean TOC concentrations were 4.05 mg/l in Lake Store Sandungen and 3.75 mg/l in Lake Maridalsvann.

The control analyses of zinc in all batches are compiled in Table 5. The results show that the mean background concentration of Zn was 6 µg/l in both lakes. The measured concentrations of Zn in the batches spiked with zinc sulphate in general were close to the nominal concentrations when the background concentrations were subtracted. The mean values were from 98-114% of the nominal (background subtracted). The largest deviation was for the treatment Lake Maridalsvann with 50 µg Zn/l, where one of the batches showed a concentration of 124 µg/l.

All batches were also analysed for electrical conductivity to verify that the spiking with calcium chloride was correct. The results show that all batches without Ca-spiking had an electrical conductivity around 23 mS/m in Lake Sandungen and 30 mS/m in Lake Maridalsvann. Batches spiked with Ca had conductivity around 330 mS/m (See Table 6).

Dissolved oxygen measured in the beginning, middle and end of the test are shown in Table 7. The concentrations were between 8.8 and 11.8 mg/l, which corresponds to between 70 and 95% saturation.

3.2 Analysis of fish data

The data for time of hatching (degree-days), which appeared to be the most sensitive endpoint, were used to analyse variation between the two replicate chambers at each treatment. No significant difference (t-test, 95% confidence level) was found in 23 of the 24 treatments. The only channel where a difference was observed was In Lake Maridalsvann with 50 µg Zn/l. A possible reason is that one of the chambers in this channel contained 67 eggs, while all of the other chambes had 38-49 eggs. Since no difference between the two replicate chambers was found in all but one of the channels, the data from the two replicates were pooled in the analysis.

Mortality and hatching successes of eggs

The number of dead eggs and fertilised dead eggs is presented in Table 8. A significant portion of the eggs was removed as dead during the test. Eggs that became opaque immediately after fertilisation and up to day 38 were classified as unfertilised. During the remaining 81 days of the test, dead eggs were inspected to confirm whether they were fertilised or not. The dead eggs were found to be mainly unfertilised. Since fertilisation occurred with exposure to the test solutions, effects of zinc on the fertilisation process could be expected to result in decreasing fertilisation with increasing zinc exposure within each group. However, the data shows a variation in fertilisation success of 67-93% with no obvious relation to the zinc exposure.

The number of eggs that died after fertilisation was between 0 and 9% of the number of fertilised eggs. A trend increasing lethality with increasing zinc concentration were found in Lake Sandungen, where lethality increased from 2.4% in the control to 6.3% at 100 µg Zn/l and 9.1% at 250 µg Zn/l. A less pronounced concentration/response relation was found in Lake Maridalsvann+Ca, where lethality increased from 2.5% in the control to 3.8% at 500 µgZn/l and 4.9% at 1000 µg Zn/l.

Deformation and lethality of larvae.

The number of larvae that were deformed or that died during the test is shown in Table 9. Presentation of percentages is given in Table 10. Between 0 and 4 deformed larvae (0-6%) were recorded in the channels. No obvious relationship with zinc exposure was found.

Except one dead larva in the Sandungen+Ca control, no lethality of larvae was observed in any of the four control channels (Table 9). In 12 of the 24 channels 1-7 dead larvae were recorded. A concentration/response relationship was found only in two of the series (Lake Maridalsvann with and without addition of Ca), where the highest lethality was observed at the highest concentrations, which may indicate a lethal effct of zinc.

Hatching period

A widly used measurement of biological and especially embryonic development in poikilothermic animals like fish is degree-days. It represents the product of time in days needed to reach a certain developmental stage, and the temperature in °C. It used to be thought of as being constant in a broad range of temperatures (Apstein 1909), but will be different when the temperature range is outside a rather narrow range of suitable temperatures Braum (1978). The very narrow range in temperatures of $\leq$ 0,2°C in our experiment, calls for degree-days as being most suitable for comparison of effect of zink on embryo development of brown trout.

The time to start and end of hatching period expressed as days and degree-days is shown in Table 11. The progress of hatching is also displayed graphically in Figure 2 to Figure 5. The hatching commenced after 82-87 days or 459-490 degree-days. The figures indicate a delay of hatching at the highest concentrations of zinc. This effect is most pronounced in Lake Sandungen, where the onset of the hatching was delayed and the time span between the start and end of the hatching period was extended as compared to the control. A similar, but less pronounced pattern was seen in Lake Maridalsvann (see Figure 4). In Lake Sandungen+Ca (Figure 3), The haching was clearly delayed at 1000 µg Zn/l and to less extent at 500 µg/l. In this series, however, the lowest concentrations of Zn (50-250 µg/l) seemed to accelerate the hatching. The least significant effect of zinc exposure on the time of hatching was found in Lake Maridalsvann+Ca. Also in this case, however, the highest zinc treatment (1000 µg/l) showed the slowest hatching.

The time for hatching for individual fish showed uequal variances when analysed with Levene's test even after logarithmic transformation. The hatching time data were therefore analysed using a non-parametric pair-wise comparison of each group with the control using t-test assuming unequal variances with Bonferroni adjustment of alpha to 0.01. The results are summarised in Table 12. The statistical analysis verifies that significant increase in the hatching time was found at the two highest zinc treatments (100 and 250 µg/l) in both lake waters without calcium spiking.

In Lake Store Sandungen+Ca, significant increase of the hatching time was found at treatments 500 and 1000 µgZn/l. In this series, however the three lowest concentrations showed a significant decrease in hatching time as compared to the control. In Lake Maridalsvann+Ca, all treatments from 100-1000 µg/l caused a significant increase in hatching time.

Analysis of the hatching based on number of days to hatch shows the same results as for degree-days in all series except for Lake Maridalsvann (low hardness) (Table 13). In this series, no significant increase in the hatching time at 100 and 250 µg Zn was observed, while the hatching time was shorter than in the controls at 25 and 50 µg/l. This can be explained by the lower temperature in the control and 10 µg Zn/l channels (mean values 5.57 and 5.53 °C) compared to the other channels in the series (5.67-5.73 °C). These differences in temperature are compensated for when the data are analysed on the basis of degree-days.

Length of larvae

The difference in length of larvae at the end of the test was generally small (See Table 14). The mean values for the treatments were between 2.17 and 2.34 cm. Box plots of the data are shown in Figure 6 to Figure 9. Statistical analysis of the data was performed using Dunnett's test (alpha 0.05). In Lake Sandungen, the larvae exposed to 25 µg Zn/l were significantly longer than in the control, while those exposed to 100 and 250 µg Zn/l were shorter than in the control. In Lake Store Sandungen+Ca, larvae in the highest treatment of zinc (1000 µg/l) were significantly shorter than the control larvae. The same result was found in Lake Maridalsvann+Ca. In Lake Maridalsvann, no treatment gave significant reduction in length of larvae, but in two intermediate Zn treatments (25 and 50 µg/l), the larvae were significantly longer than in the control according to the Dunnett's test.

Weight of larvae

The mean values of weight (blotted dry) of larvae are presented in Table 15. The coefficient of variation within each group was approximately 10-12% and the difference in mean values between the treatments and the controls were between 6.1 and 8.3 %. Deviations from the control group were both positive and negative. A significant decrease in weight was observed at the highest concentration of zinc in Lake Store Sandungen and Lake Store Sandungen+Ca (Dunnett's test). No reduction of weight of larvae was found in the two series with Lake Maridalsvann. However, significant increase in weight was found at 250 μg Zn/l in Lake Maridalsvann and at 500 μg Zn/l in Lake Maridalsvann+Ca.

The dry weigh determinations turned out to be less accurate than the total weights because of the very low dry weight of larvae compared to the eppendorf tubes in which they were dried, and no significant difference between the groups could be detected.

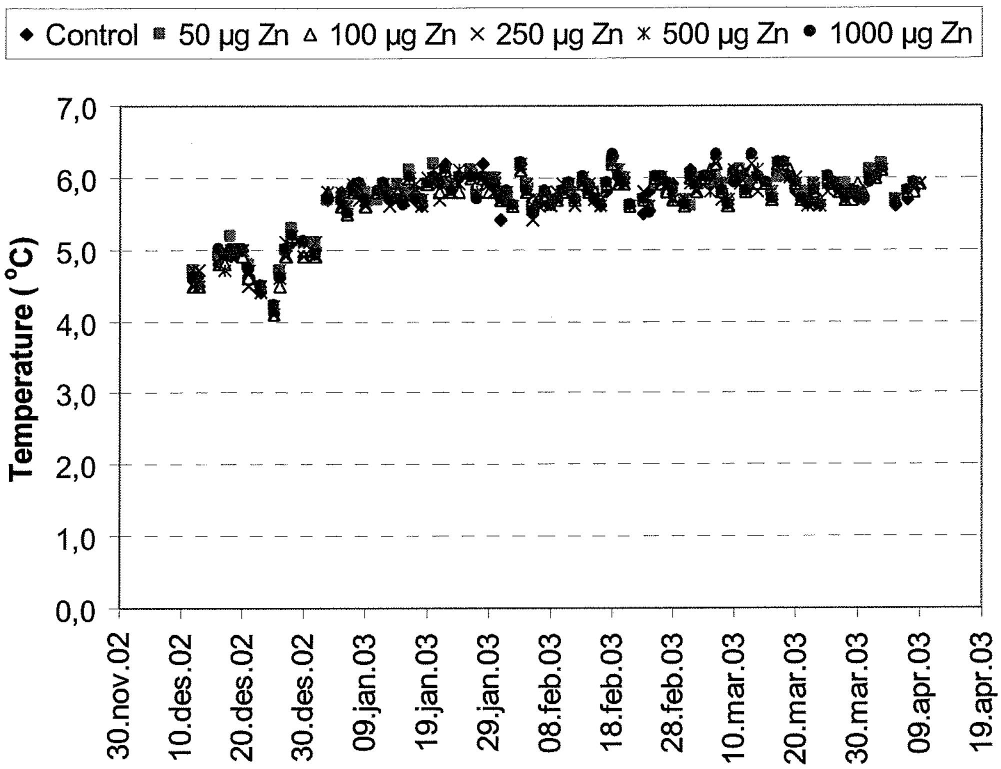

Figure 1. Temperature recordings during the exposure period (Lake Maridalsvann + Ca).

Table 1. Mean temperature, length of exposure and degree-days for all treatments

Water type	Treatment	Mean temp.	exposure (days)	exposure (degree-days)
St. Sandungen	Control	5.70	117	667
St. Sandungen	10 µg Zn/l	5.71	116	662
St. Sandungen	25 µg Zn/l	5.69	118	671
St. Sandungen	50 µg Zn/l	5.69	118	672
St. Sandungen	100 µg Zn/l	5.72	116	663
St. Sandungen	250 µg Zn/l	5.70	116	661
St. Sandungen + Ca	Control	5.69	117	666
St. Sandungen + Ca	50 µg Zn/l	5.69	118	671
St. Sandungen + Ca	100 µg Zn/l	5.70	116	662
St. Sandungen + Ca	250 µg Zn/l	5.68	118	670
St. Sandungen + Ca	500 µg Zn/l	5.59	119	666
St. Sandungen + Ca	1000µg Zn/l	5.60	119	666
Maridalsvann	Control	5.57	119	663
Maridalsvann	10 µg Zn/l	5.53	119	659
Maridalsvann	25 µg Zn/l	5.67	119	675
Maridalsvann	50 µg Zn/l	5.67	119	675
Maridalsvann	100 µg Zn/l	5.70	117	667
Maridalsvann	250 µg Zn/l	5.73	116	665
Maridalsvann + Ca	Control	5.69	117	660
Maridalsvann + Ca	50 µg Zn/l	5.72	116	663
Maridalsvann + Ca	100 µg Zn/l	5.68	118	671
Maridalsvann + Ca	250 µg Zn/l	5.67	118	670
Maridalsvann + Ca	500 µg Zn/l	5.71	116	663
Maridalsvann + Ca	1000µg Zn/l	5.69	117	666

Table 2. Chemical composition, Lake Store Sandungen

Date:	02.12.2002	12.12.2002	06.03.2003	10.04.2003
pH	6.22	6.54	6.60	6.59
Conductivity (mS/m)	1.88	1.96	2.00	2.04
Alkalinity (mmol/l)	0.093	0.094	0.099	0.095
Tot.-N (µg N/l)	245	285	270	280
NO_3 (µg N/l)	115	125	135	145
TOC (mg/l)	4.0	4.1	3.4	3.6
Cl (mg/l)	0.93	1.0	0.97	1.07
SO_4 (mg/l)	2.38	2.39	2.56	2.71
Al-reactive (µg/l)	39	37	26	30
Al/Il (µg/l)	36	35	21	29
Ca (mg/l)	1.93	1.91	2.56	2.67
K (mg/l)	0.28	0.31	0.26	0.26
Mg (mg/l)	0.317	0.317	0.38	0.39
Na (mg/l)	1.02	1.08	1.08	1.10
Zn µg/l)	7.5	8.6	5.7	

Table 3. Chemical composition Lake Maridalsvann

Date:	02.12.2002	12.12.2002	06.03.2003	10.04.2003
pH	6.59	6.72	6.85	6.57
Conductivity (mS/m)	2.47	2.62	2.61	2.76
Alkalinity (mmol/l)	0.104	0.110	0.114	0.111
Tot.-N (µg N/l)	385	415	380	415
NO_3 (µg N/l)	210	205	235	275
TOC (mg/l)	3.9	3.9	3.7	3.6
Cl (mg/l)	1.70	1.77	1.53	1.97
SO_4 (mg/l)	2.86	2.91	2.99	3.15
Al-reactive (µg/l)	33	31	36	29
Al/Il (µg/l)	30	25	32	26
Ca (mg/l)	2.57	2.49	3.13	3.25
K (mg/l)	0.33	0.37	0.30	0.33
Mg (mg/l)	0.424	0.413	0.47	0.49
Na (mg/l)	1.49	1.57	1.44	1.60
Zn µg/l)	11	7.9	6.4	

Table 4. Calcium and total organic carbon (TOC) in batches of lakewater used to prepare test solutions.

Lake	Parameter	n	Min.	Max.	Mean	St.dev.
Maridalsv.	Ca (mg/l)	14	2.38	3.08	2.61	0.18
Maridalsv.	TOC (mg/l)	14	3.50	4.00	3.75	0.15
St. Sandungen	Ca (mg/l)	14	1.91	2.19	2.08	0.07
St. Sandungen	TOC (mg/l)	14	3.50	5.30	4.05	0.57

Table 5. Concentration of Zn (µg/l) in batches of water used for exposure.

Lake/Treatment	Min.	Max.	Mean	St.dev.
St.Sandungen Control	5	8	6	1,0
St.Sandungen Zn-10µg/L	15	26	17	2,8
St.Sandungen Zn-25µg/L	29	37	32	1,9
St.Sandungen Zn-50µg/L	54	64	56	3,1
St.Sandungen Zn-100µg/L	103	110	106	2,4
St.Sandungen Zn-250µg/L	241	265	253	6,6
St.Sandungen + Ca Control	5	12	7	2,0
St.Sandungen + Ca Zn-50µg/L	51	62	56	3,4
St.Sandungen + Ca Zn-100µg/L	99	112	104	3,4
St.Sandungen + Ca Zn-250µg/L	243	261	250	6,5
St.Sandungen + Ca Zn-500µg/L	478	510	496	9,4
St.Sandungen + Ca Zn-1000µg/L	978	1050	997	21,6
Maridalsvann Control	5	10	7	1,2
Maridalsvann Zn-10µg/L	16	18	17	0,8
Maridalsvann Zn-25µg/L	30	37	32	1,8
Maridalsvann Zn-50µg/L	54	124	61	18,8
Maridalsvann Zn-100µg/L	102	123	108	5,2
Maridalsvann Zn-250µg/L	250	269	256	5,3
Maridalsvann + Ca Control	5	9	6	0,9
Maridalsvann + Ca Zn-50µg/L	53	70	57	4,4
Maridalsvann + Ca Zn-100µg/L	103	114	108	3,8
Maridalsvann + Ca Zn-250µg/L	249	270	255	5,2
Maridalsvann + Ca Zn-500µg/L	475	519	502	10,3
Maridalsvann + Ca Zn-1000µg/L	970	1020	1003	13,6

Table 6. Conductivity (mS/m) in batches of water used for exposure

Lake/Treatment	Min.	Max.	Mean	St.dev.
St.Sandungen Control	20	25	23	1.5
St.Sandungen Zn-10µg/L	20	25	23	1.5
St.Sandungen Zn-25µg/L	21	26	23	1.4
St.Sandungen Zn-50µg/L	22	25	24	1.0
St.Sandungen Zn-100µg/L	22	25	23	1.0
St.Sandungen Zn-250µg/L	22	25	24	1.1
St.Sandungen + Ca Control	310	332	320	6.8
St.Sandungen + Ca Zn-50µg/L	312	337	323	7.6
St.Sandungen + Ca Zn-100µg/L	315	340	327	7.6
St.Sandungen + Ca Zn-250µg/L	316	334	325	5.7
St.Sandungen + Ca Zn-500µg/L	312	336	323	6.7
St.Sandungen + Ca Zn-1000µg/L	313	335	325	6.3
Maridalsvann Control	29	30	30	0.4
Maridalsvann Zn-10µg/L	29	30	30	0.4
Maridalsvann Zn-25µg/L	29	30	30	0.4
Maridalsvann Zn-50µg/L	29	30	30	0.4
Maridalsvann Zn-100µg/L	29	30	30	0.3
Maridalsvann Zn-250µg/L	29	32	30	0.7
Maridalsvann + Ca Control	320	334	327	4.7
Maridalsvann + Ca Zn-50µg/L	323	340	331	4.4
Maridalsvann + Ca Zn-100µg/L	321	335	331	4.3
Maridalsvann + Ca Zn-250µg/L	328	336	333	2.5
Maridalsvann + Ca Zn-500µg/L	329	344	333	4.1
Maridalsvann + Ca Zn-1000µg/L	323	347	336	6.1

Table 7. Dissolved oxygen (mg/l) in all channels measured on three occasions during the test

Lake/Treatment	06.01.2003	12.03.2003	29.03.2003
St.Sandungen Control	11.6	10.3	10.4
St.Sandungen Zn-10µg/L	11.2	10.5	9.9
St.Sandungen Zn-25µg/L	10.5	9.3	9.0
St.Sandungen Zn-50µg/L	10.9	9.8	9.3
St.Sandungen Zn-100µg/L	11.1	10.7	9.0
St.Sandungen Zn-250µg/L	11.9	10.5	9.9
St.Sandungen + Ca Control	11.0	10.7	8.8
St.Sandungen + Ca Zn-50µg/L	11.1	9.7	8.9
St.Sandungen + Ca Zn-100µg/L	10.7	10.8	9.0
St.Sandungen + Ca Zn-250µg/L	11.0	9.8	8.9
St.Sandungen + Ca Zn-500µg/L	11.8	10.4	9.8
St.Sandungen + Ca Zn-1000µg/L	11.6	10.8	9.6
Maridalsvann Control	11.4	11.1	10.3
Maridalsvann Zn-10µg/L	11.5	11.0	10.9
Maridalsvann Zn-25µg/L	11.5	10.9	11.5
Maridalsvann Zn-50µg/L	11.6	10.4	10.0
Maridalsvann Zn-100µg/L	11.2	11.2	11.6
Maridalsvann Zn-250µg/L	11.8	10.7	10.7
Maridalsvann + Ca Control	11.8	10.6	10.1
Maridalsvann + Ca Zn-50µg/L	11.6	10.6	10.1
Maridalsvann + Ca Zn-100µg/L	11.5	11.0	10.4
Maridalsvann + Ca Zn-250µg/L	11.6	11.1	10.3
Maridalsvann + Ca Zn-500µg/L	11.7	10.6	10.1
Maridalsvann + Ca Zn-1000µg/L	11.5	10.5	10.1

Table 8. Number of eggs, fertilised eggs and accumulated lethality of eggs during the exposure period.

Treatment	No of eggs	Number fertilised	Dead fertilised eggs	Fertilisation (%)	Dead fertilised (%)
St.Sandungen Control	85	72	2	85	2.4
St.Sandungen Zn-10µg/L	83	77	2	93	2.5
St.Sandungen Zn-25µg/L	83	69	2	83	2.5
St.Sandungen Zn-50µg/L	84	66	2	79	2.4
St.Sandungen Zn-100µg/L	84	73	5	87	6.3
St.Sandungen Zn-250µg/L	84	75	7	89	9.1
St.Sandungen + Ca Control	85	70	0	82	0.0
St.Sandungen + Ca Zn-50µg/L	83	62	1	75	1.2
St.Sandungen + Ca Zn-100µg/L	90	75	4	83	4.7
St.Sandungen + Ca Zn-250µg/L	83	60	2	72	2.5
St.Sandungen + Ca Zn-500µg/L	83	60	2	72	2.5
St.Sandungen + Ca Zn-1000µg/L	84	70	3	83	3.7
Maridalsvann Control	85	66	2	78	2.4
Maridalsvann Zn-10µg/L	79	58	2	73	2.6
Maridalsvann Zn-25µg/L	82	62	3	76	3.8
Maridalsvann Zn-50µg/L	110	78	4	71	3.8
Maridalsvann Zn-100µg/L	77	55	2	71	2.7
Maridalsvann Zn-250µg/L	79	53	3	67	3.9
Maridalsvann + Ca Control	82	56	2	78	2.5
Maridalsvann + Ca Zn-50µg/L	82	59	2	79	2.5
Maridalsvann + Ca Zn-100µg/L	79	62	2	81	2.6
Maridalsvann + Ca Zn-250µg/L	79	62	2	84	2.6
Maridalsvann + Ca Zn-500µg/L	81	51	3	69	3.8
Maridalsvann + Ca Zn-1000µg/L	85	73	4	91	4.9

Table 9. Number of fertilised and hatchhed eggs, deformed larvae and dead larvae during the exposure period.

Treatment	Fertlised eggs	Hatched eggs	Deformed larvae	Dead larvae
St.Sandungen Control	72	70	1	0
St.Sandungen Zn-10µg/L	77	75	1	1
St.Sandungen Zn-25µg/L	69	67	2	2
St.Sandungen Zn-50µg/L	66	64	3	0
St.Sandungen Zn-100µg/L	73	68	4	4
St.Sandungen Zn-250µg/L	75	68	0	3
St.Sandungen + Ca Control	70	70	2	1
St.Sandungen + Ca Zn-50µg/L	62	61	2	0
St.Sandungen + Ca Zn-100µg/L	75	71	2	4
St.Sandungen + Ca Zn-250µg/L	60	58	1	0
St.Sandungen + Ca Zn-500µg/L	60	58	2	0
St.Sandungen + Ca Zn-1000µg/L	70	67	3	3
Maridalsvann Control	66	64	0	0
Maridalsvann Zn-10µg/L	58	56	0	0
Maridalsvann Zn-25µg/L	62	59	0	0
Maridalsvann Zn-50µg/L	78	74	2	0
Maridalsvann Zn-100µg/L	55	53	1	0
Maridalsvann Zn-250µg/L	53	50	2	2
Maridalsvann + Ca Control	56	54	1	0
Maridalsvann + Ca Zn-50µg/L	59	57	1	3
Maridalsvann + Ca Zn-100µg/L	62	60	0	2
Maridalsvann + Ca Zn-250µg/L	62	60	1	1
Maridalsvann + Ca Zn-500µg/L	51	48	0	0
Maridalsvann + Ca Zn-1000µg/L	73	69	1	7

Table 10. Percentages of fertilised and hatched eggs, deformed larvae and dead larvae during the exposure period.

Treatment	Fertlised eggs (%)	Hatched eggs (% of fertilised)	Deformed larvae (% of hatched)	Dead larvae (% of hatched)
St.Sandungen Control	86	97	1.4	0.0
St.Sandungen Zn-10µg/L	93	97	1.3	1.3
St.Sandungen Zn-25µg/L	89	97	3.0	3.0
St.Sandungen Zn-50µg/L	82	97	4.7	0.0
St.Sandungen Zn-100µg/L	90	93	5.9	5.9
St.Sandungen Zn-250µg/L	90	91	0.0	4.4
St.Sandungen + Ca Control	89	100	2.9	1.4
St.Sandungen + Ca Zn-50µg/L	83	98	3.3	0.0
St.Sandungen + Ca Zn-100µg/L	93	95	2.8	5.6
St.Sandungen + Ca Zn-250µg/L	78	97	1.7	0.0
St.Sandungen + Ca Zn-500µg/L	76	97	3.4	0.0
St.Sandungen + Ca Zn-1000µg/L	86	96	4.5	4.5
Maridalsvann Control	86	97	0.0	0.0
Maridalsvann Zn-10µg/L	77	97	0.0	0.0
Maridalsvann Zn-25µg/L	79	95	0.0	0.0
Maridalsvann Zn-50µg/L	80	95	2.7	0.0
Maridalsvann Zn-100µg/L	79	96	1.9	0.0
Maridalsvann Zn-250µg/L	80	94	4.0	4.0
Maridalsvann + Ca Control	78	96	1.9	0.0
Maridalsvann + Ca Zn-50µg/L	79	97	1.8	5.3
Maridalsvann + Ca Zn-100µg/L	81	97	0.0	3.3
Maridalsvann + Ca Zn-250µg/L	84	97	1.7	1.7
Maridalsvann + Ca Zn-500µg/L	69	94	0.0	0.0
Maridalsvann + Ca Zn-1000µg/L	91	95	1.4	10.1

Table 11. Time to start and end of hatching period.

	Start of hatching		End of hatching	
Lake/Treatment	days	degree-days	days	degree-days
St.Sandungen Control	82	459	87	489
St.Sandungen Zn-10µg/L	83	467	88	497
St.Sandungen Zn-25µg/L	82	460	86	483
St.Sandungen Zn-50µg/L	82	459	89	501
St.Sandungen Zn-100µg/L	84	474	93	528
St.Sandungen Zn-250µg/L	87	490	95	544
St.Sandungen + Ca Control	82	459	89	501
St.Sandungen + Ca Zn-50µg/L	82	459	90	506
St.Sandungen + Ca Zn-100µg/L	82	461	87	491
St.Sandungen + Ca Zn-250µg/L	82	459	86	483
St.Sandungen + Ca Zn-500µg/L	84	462	91	502
St.Sandungen + Ca Zn-1000µg/L	84	462	94	521
Maridalsvann Control	83	454	91	500
Maridalsvann Zn-10µg/L	84	462	89	485
Maridalsvann Zn-25µg/L	82	458	87	488
Maridalsvann Zn-50µg/L	82	459	88	494
Maridalsvann Zn-100µg/L	84	472	91	514
Maridalsvann Zn-250µg/L	85	482	92	524
Maridalsvann + Ca Control	82	461	86	485
Maridalsvann + Ca Zn-50µg/L	82	463	86	487
Maridalsvann + Ca Zn-100µg/L	82	459	90	507
Maridalsvann + Ca Zn-250µg/L	82	459	88	494
Maridalsvann + Ca Zn-500µg/L	82	463	89	505
Maridalsvann + Ca Zn-1000µg/L	82	461	91	514

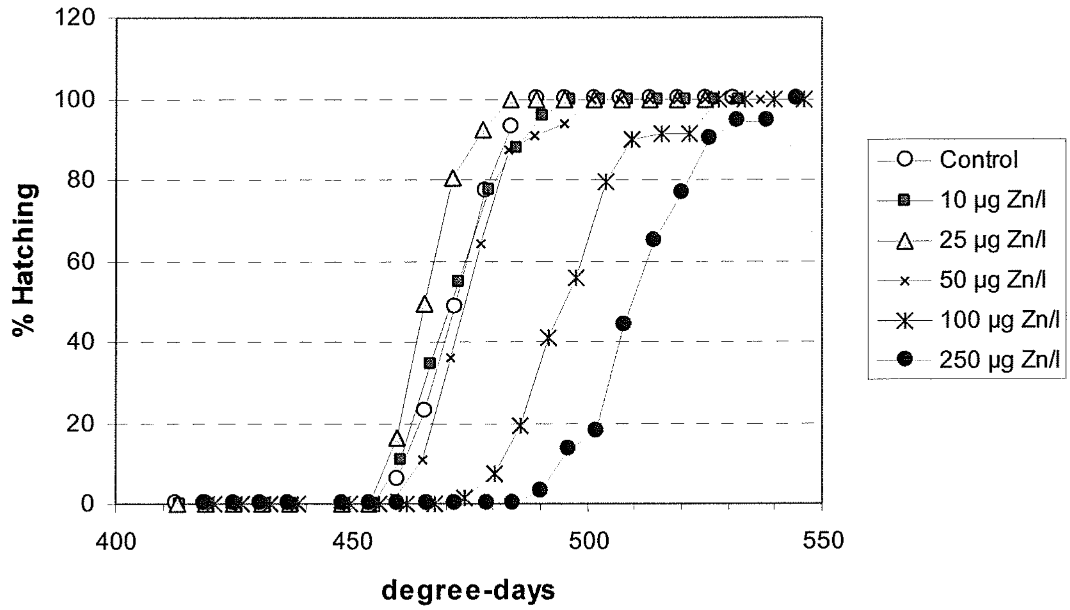

Figure 2. Progress of hatching in Lake Sandungen

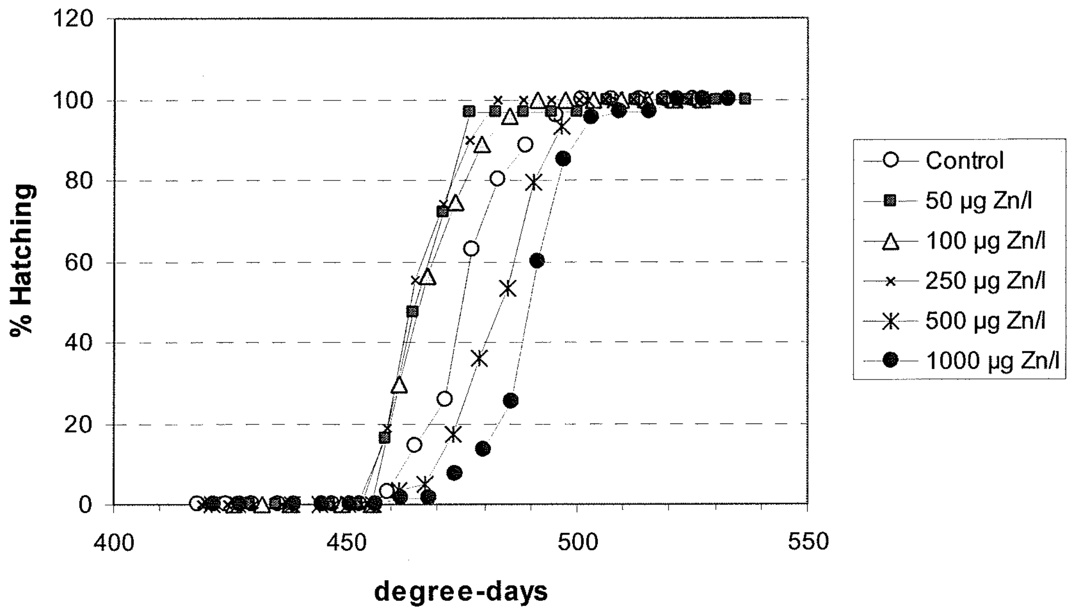

Figure 3. Progress of hatching in Lake Sandungen + Ca

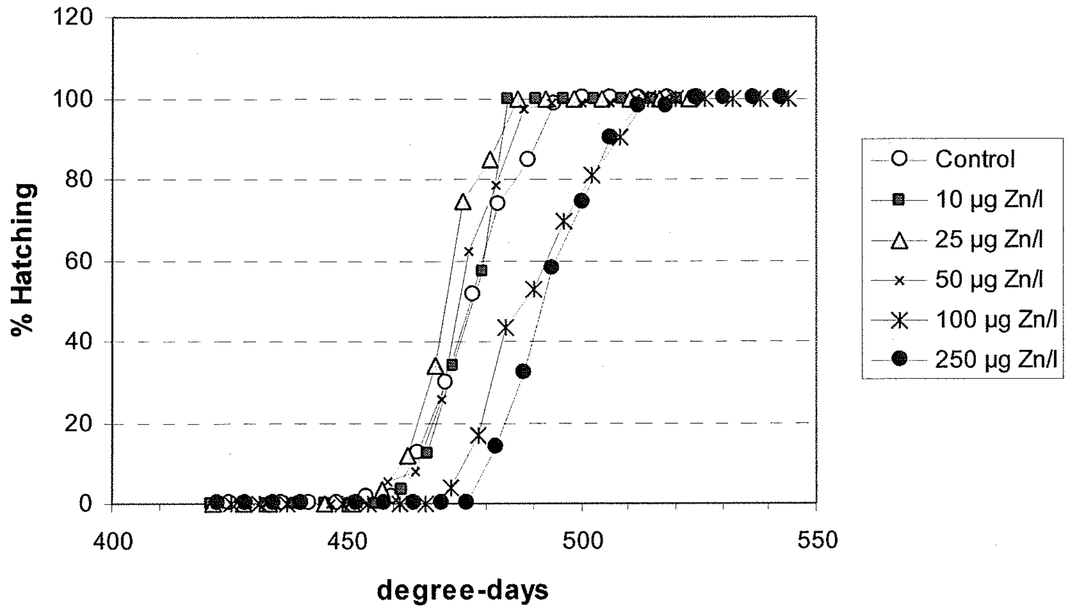

Figure 4. Progress of hatching in Lake Maridalsvann

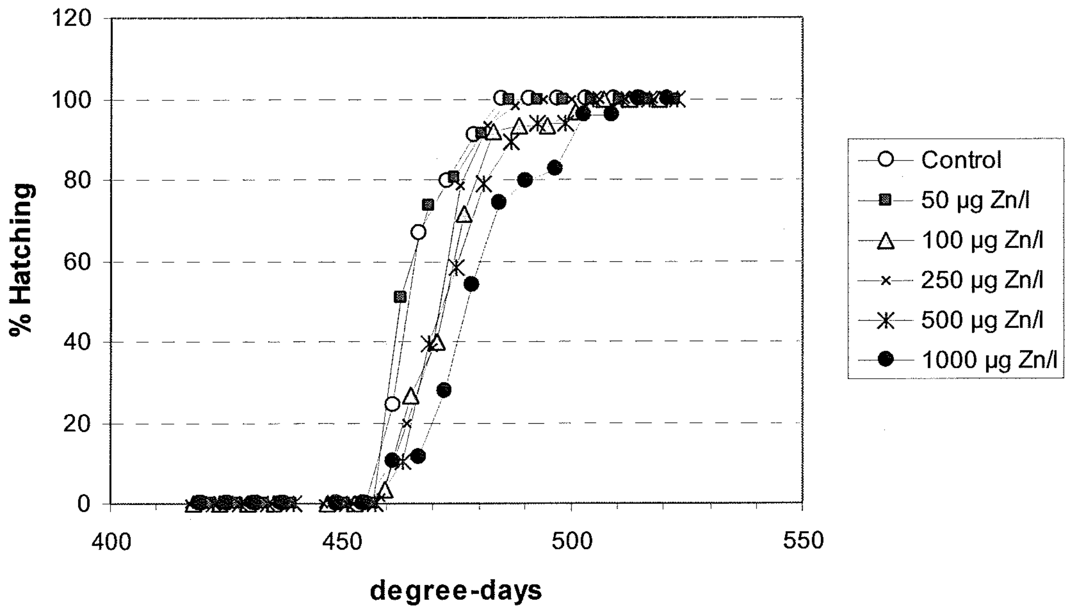

Figure 5. Progress of hatching in Lake Maridalsvann + Ca

Table 12. Mean time to hatch (degree-days) and comparison with control (Dunnett's test)

Treatment	Mean	st.dev.	Significant increase (+)/decrease (-)
St.Sandungen Control	475	7.9	
St.Sandungen Zn-10µg/L	475	9.7	
St.Sandungen Zn-25µg/L	469	6.7	-
St.Sandungen Zn-50µg/L	478	9.3	
St.Sandungen Zn-100µg/L	500	13.0	+
St.Sandungen Zn-250µg/L	514	12.7	+
St.Sandungen + Ca Control	479	9.6	
St.Sandungen + Ca Zn-50µg/L	470	9.2	-
St.Sandungen + Ca Zn-100µg/L	471	8.5	-
St.Sandungen + Ca Zn-250µg/L	469	7.3	-
St.Sandungen + Ca Zn-500µg/L	485	9.8	+
St.Sandungen + Ca Zn-1000µg/L	492	9.9	+
Maridalsvann Control	480	9.8	
Maridalsvann Zn-10µg/L	478	6.7	
Maridalsvann Zn-25µg/L	474	7.4	-
Maridalsvann Zn-50µg/L	478	8.8	
Maridalsvann Zn-100µg/L	493	12	+
Maridalsvann Zn-250µg/L	496	9.9	+
Maridalsvann + Ca Control	469	7.3	
Maridalsvann + Ca Zn-50µg/L	469	8	
Maridalsvann + Ca Zn-100µg/L	476	10.3	+
Maridalsvann + Ca Zn-250µg/L	474	7.2	+
Maridalsvann + Ca Zn-500µg/L	478	10.7	+
Maridalsvann + Ca Zn-1000µg/L	483	13.5	+

Table 13. Mean time to hatch (days) and comparison with control (Dunnett's test)

Treatment	Mean	st.dev.	Significant increase (+)/decrease (-)
St.Sandungen Control	84.5	1.30	
St.Sandungen Zn-10µg/L	84.4	1.60	
St.Sandungen Zn-25µg/L	83.6	1.28	-
St.Sandungen Zn-50µg/L	85.2	1.56	
St.Sandungen Zn-100µg/L	88.3	2.17	+
St.Sandungen Zn-250µg/L	91.0	2.12	+
St.Sandungen + Ca Control	85.3	1.62	
St.Sandungen + Ca Zn-50µg/L	83.8	1.55	-
St.Sandungen + Ca Zn-100µg/L	83.5	1.43	-
St.Sandungen + Ca Zn-250µg/L	83.6	1.27	-
St.Sandungen + Ca Zn-500µg/L	88.1	1.69	+
St.Sandungen + Ca Zn-1000µg/L	89.2	1.68	+
Maridalsvann Control	87.5	1.72	
Maridalsvann Zn-10µg/L	87.9	1.16	
Maridalsvann Zn-25µg/L	84.9	1.26	-
Maridalsvann Zn-50µg/L	85.3	1.50	-
Maridalsvann Zn-100µg/L	87.4	2.00	
Maridalsvann Zn-250µg/L	87.4	1.64	
Maridalsvann + Ca Control	83.3	1.23	
Maridalsvann + Ca Zn-50µg/L	82.9	1.17	
Maridalsvann + Ca Zn-100µg/L	84.8	1.74	+
Maridalsvann + Ca Zn-250µg/L	84.7	1.23	+
Maridalsvann + Ca Zn-500µg/L	84.4	1.79	+
Maridalsvann + Ca Zn-1000µg/L	85.7	2.26	+

Table 14. Length of larva

Treatment	Mean	st.dev.	Significant increase (+)/decrease (-)
St.Sandungen Control	2.30	0.080	
St.Sandungen Zn-10µg/L	2.30	0.085	
St.Sandungen Zn-25µg/L	2.33	0.074	+
St.Sandungen Zn-50µg/L	2.28	0.071	
St.Sandungen Zn-100µg/L	2.20	0.081	-
St.Sandungen Zn-250µg/L	2.17	0.086	-
St.Sandungen + Ca Control	2.32	0.074	
St.Sandungen + Ca Zn-50µg/L	2.34	0.078	
St.Sandungen + Ca Zn-100µg/L	2.31	0.097	
St.Sandungen + Ca Zn-250µg/L	2.32	0.058	
St.Sandungen + Ca Zn-500µg/L	2.29	0.076	
St.Sandungen + Ca Zn-1000µg/L	2.30	0.081	-
Maridalsvann Control	2.27	0.089	
Maridalsvann Zn-10µg/L	2.28	0.070	
Maridalsvann Zn-25µg/L	2.32	0.077	+
Maridalsvann Zn-50µg/L	2.31	0.071	+
Maridalsvann Zn-100µg/L	2.26	0.071	
Maridalsvann Zn-250µg/L	2.28	0.082	
Maridalsvann + Ca Control	2.31	0.075	
Maridalsvann + Ca Zn-50µg/L	2.32	0.075	
Maridalsvann + Ca Zn-100µg/L	2.32	0.083	
Maridalsvann + Ca Zn-250µg/L	2.32	0.065	
Maridalsvann + Ca Zn-500µg/L	2.29	0.065	
Maridalsvann + Ca Zn-1000µg/L	2.26	0.064	-

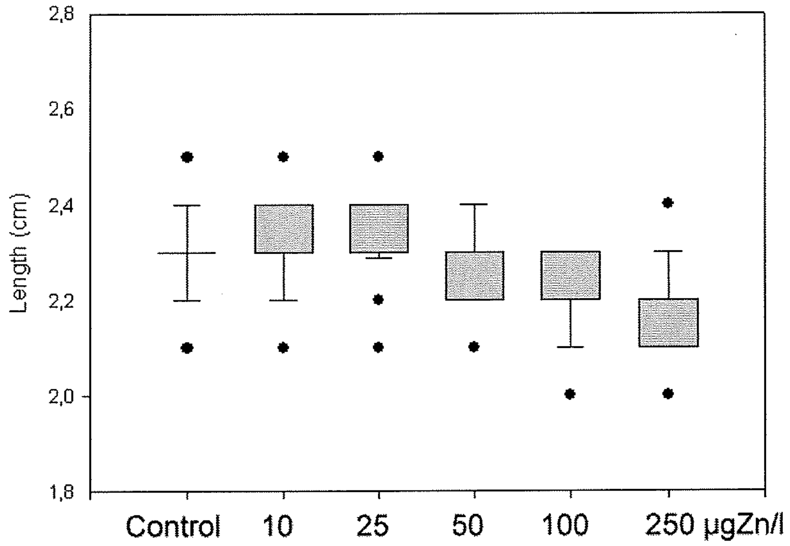

Figure 6. Length of fish, Lake St. Sandungen

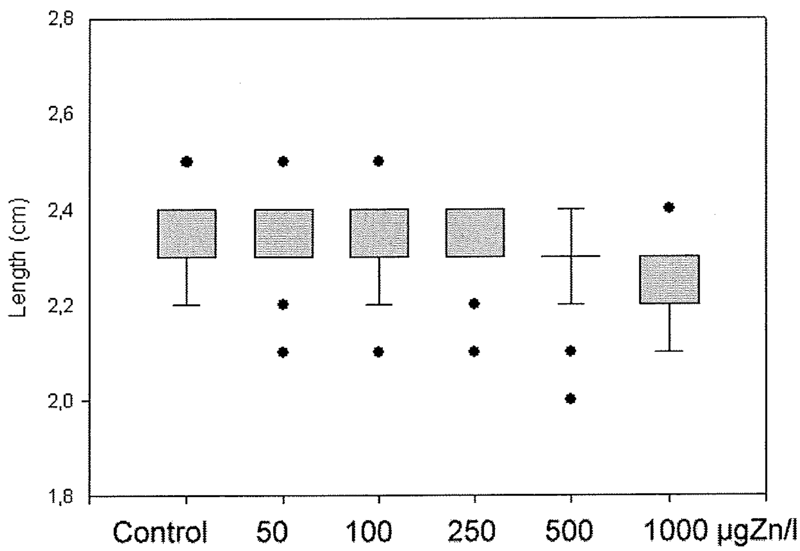

Figure 7. Length of fish, Lake St. Sandungen+Ca

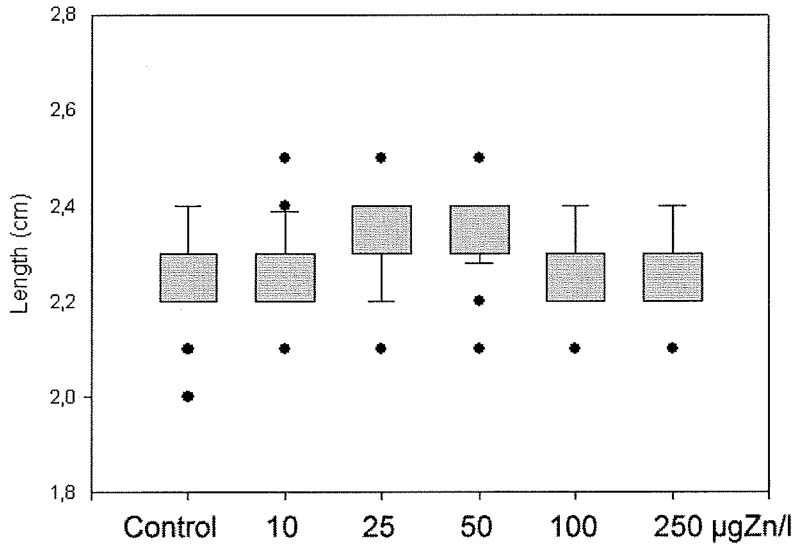

Figure 8. Length of fish, Lake Maridaklsvann

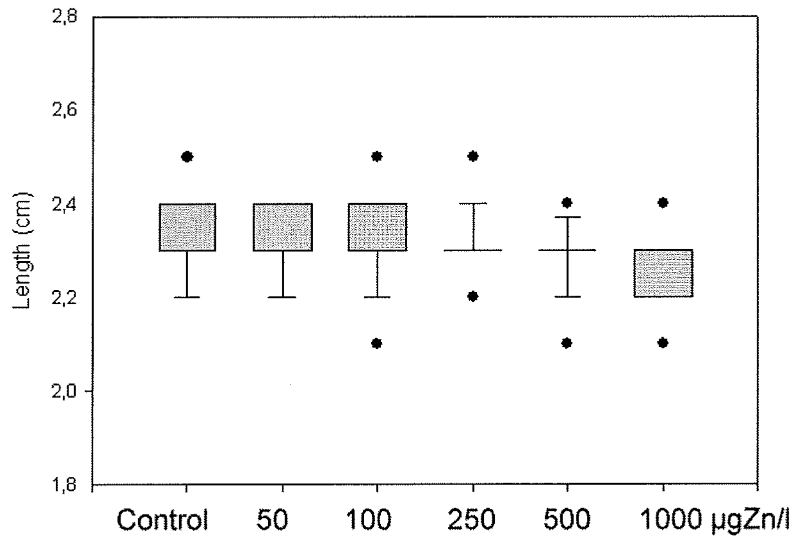

Figure 9. Length of fish, Lake Maridalsvann+Ca

Table 15. Weight of larvae

Treatment	Mean	st.dev.	Significant increase/decrease
St.Sandungen Control	90	8.53	
St.Sandungen Zn-10µg/L	91	9.31	
St.Sandungen Zn-25µg/L	94	9.74	
St.Sandungen Zn-50µg/L	88	11.0	
St.Sandungen Zn-100µg/L	90	10.6	
St.Sandungen Zn-250µg/L	84	9.44	-
St.Sandungen + Ca Control	94	11.52	
St.Sandungen + Ca Zn-50µg/L	97	13.11	
St.Sandungen + Ca Zn-100µg/L	100	11.29	+
St.Sandungen + Ca Zn-250µg/L	95	10.51	
St.Sandungen + Ca Zn-500µg/L	93	11.95	
St.Sandungen + Ca Zn-1000µg/L	86	10.67	-
Maridalsvann Control	90	10.78	
Maridalsvann Zn-10µg/L	92	12.51	
Maridalsvann Zn-25µg/L	93	10.48	
Maridalsvann Zn-50µg/L	91	10.36	
Maridalsvann Zn-100µg/L	92	10.55	
Maridalsvann Zn-250µg/L	97	12.44	+
Maridalsvann + Ca Control	94	9.36	
Maridalsvann + Ca Zn-50µg/L	96	10.39	
Maridalsvann + Ca Zn-100µg/L	96	10.08	
Maridalsvann + Ca Zn-250µg/L	98	11.27	
Maridalsvann + Ca Zn-500µg/L	100	12.11	+
Maridalsvann + Ca Zn-1000µg/L	91	8.44	

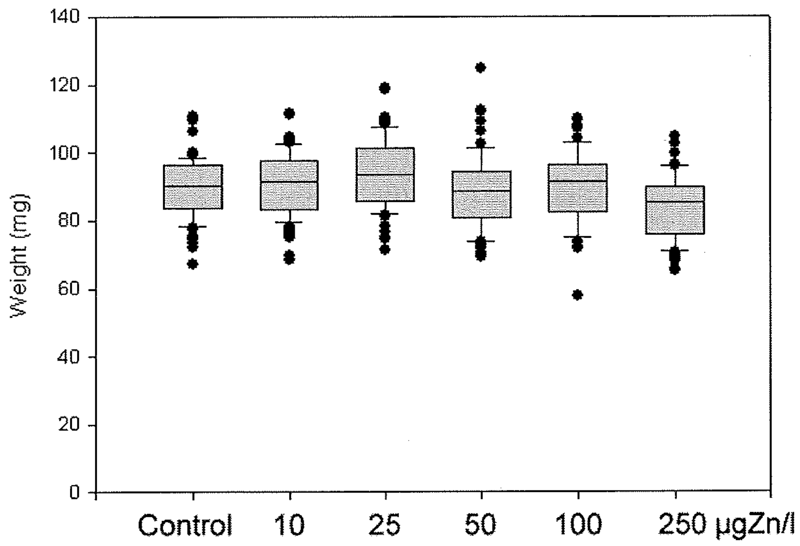

Figure 10. Weight of fish, Lake Sandungen

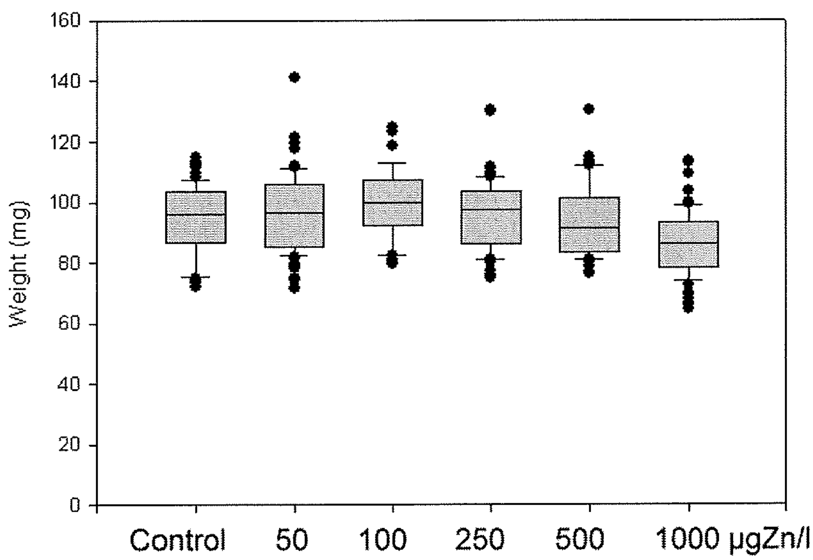

Figure 11. Weight of fish, Lake Sandungen+Ca

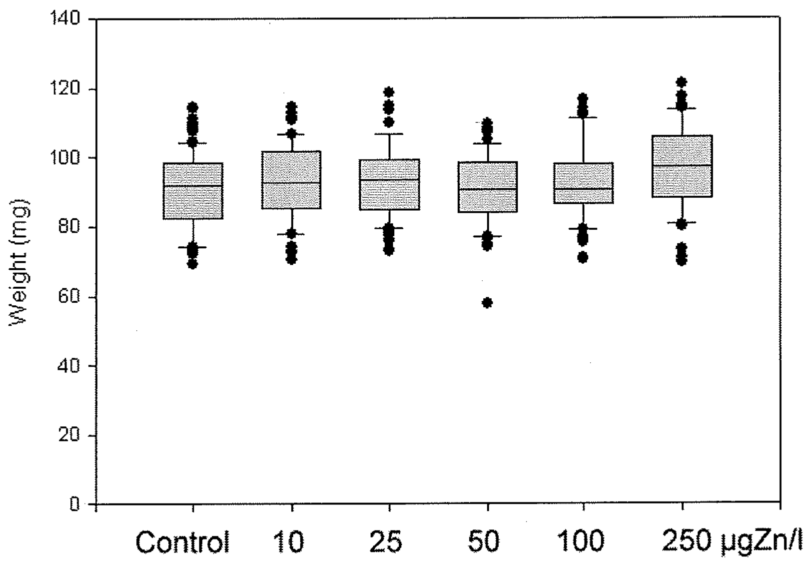

Figure 12. Weight of fish, Lake Maridalsvann

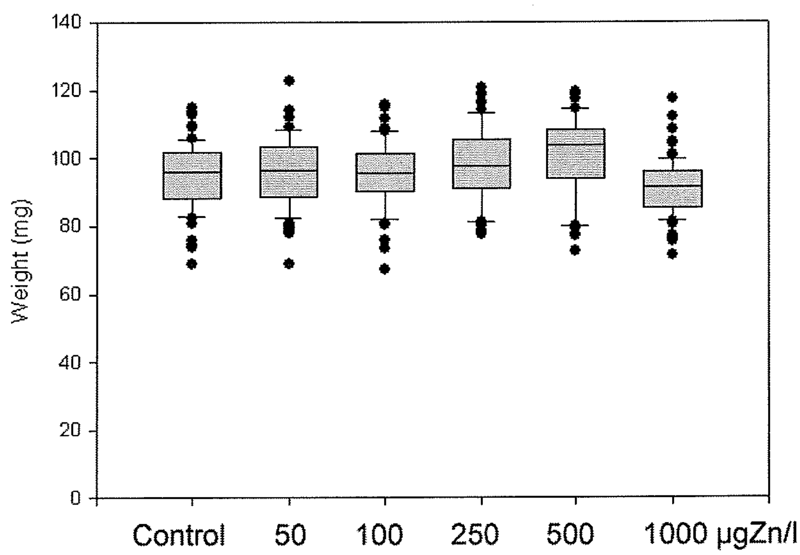

Figure 13. Weight of fish, Lake Maridalsvann+Ca

4. Conclusions

None of the endpoints investigated in the study showed drastic effects of zinc in the concentration range tested. The endpoint which appears to be most affected by the zinc treatment is the time to hatch.. In the low hardness waters a pronounced delay of the time to hatching was observed as shown in Figures 2 and 4. This was verified by the statistical analysis as significant deviations from the control. In both low hardness waters, however, also a small but statistically significant decrease in hatching time was found at the treatment 25 µg Zn/l.

Also in the two hard waters, a significant delay of hatching was observed at the highest zinc concentrations. This effect was most obvious in Lake Store Sandungen+Ca, where the two highest treatments (500 and 1000 µg Zn/l) caused a pronounced delay of hatching (See Figure 3). Also in this water, intermediate treatments of Zn (50-250 µg/l) seemed to stimulate hatching, with sigificant shorter times to hatching than in the control. In Lake Maridalsvann+Ca, no such "stimulation" was observed, but a significant delay in haching was found at the four highest treatments of zinc (250-1000 µg/l).

A negative effect of zinc exposure on the development of trout larvae was also shown in the length of larvae at test termination, where the two highest treatmentss of zinc (100 and 250 µg/l) gave significantly reduced length in Lake Store Sandungen. In the other low-hardness water, Lake Maridalsvann, significant reduction in length was not found in any of the zinc-exposed groups. Increased length of larvae was observed at intermediate concentrations of zinc in both low-hardness waters. In the two hard waters, length of larvae was reduced only at the highest zinc treatment (1000 µg/l).

Negative effects of zinc on the weight of larvae were only found at the highest zinc exposure in Lake Store Sandungen and Lake Store Sandungen+Ca.

The NOEC values, i.e. the highest concentrations in which no significant deviations from the control was observed for the three endpoints that could be statistically analysed, are shown in

Table 16. Two of these NOECs would be lower than indicated if also appearent "positive" or stimulatory effects of the zinc exposure, i.e. shorter time to hatching and increased length, would be considered. We have no suggestion on mechanisms that could explain these appearent "positive" effects. Zinc is an essential element that is required as an enzymatic component, but the levels of Zn in the ambient water required for normal embryo development are very low. As an example, the median concentrations of Zn in a total of 2267 freshwater lakes in Scandinavia range from 1,3 to 2,2 µgZn/l (Norway 1,54 µg/l (N=985), Sweden 1,27-2,20 µg/l (N= 820) and Finland 1,80 µg/l (N=462), respectively), se Skjelkvåle *et al.* (1999) and Lydersen *et al* (2002). If a true "positive" effect of an increase in zinc concentration above the natural background levels in the two lakes would occur, this would probably be observed already at the lowest zinc exposures and not only at intermediate concentrations in the tests. No other external factor in the experimental setup that can explain the "positive" responses has been found.

Table 16. NOEC values (μg Zn/l) for different endpoints in four dilution waters. The zinc concentrations are reported as the mean measured concentrations during exposure.

Dilution waters	NOEC hatching	NOEC length	NOEC weight
Lake Store Sandungen	56	56	106
Lake Maridalsvann	61	-*	-
Lake Store Sandungen + Ca	250*	496	-
Lake Maridalsvann +Ca	57	502	502

*"Positive" effects, i.e. shorter time to hatching and increased length are ignored.

The results are not conclusive as regards the effect of water hardness on the toxicity of zinc to early lifestages of trout. If only the data for Lake Sandungen are considered, the toxic effects appear to occur at concentrations that are a factor 5-10 higher at the high hardness level (100 mg $CaCO_3$/l) as compared to the low hardness level (6.7 mg/l). The results for Lake Maridalsvann also indicate a reduced effect of zinc at the high hardness level, but a factor can not be derived. The reason for differences in the results in the two lake waters is not known. The main factors that can be expected to affect the bioavailability of zinc are hardness, pH and dissolved organic matter. The hardness is approximately 2 mg $CaCO_3$/l or approximately 30% higher in Lake Maridalsvann than in Lake Store Sandungen. Ambient calcium concentration has a profound effect on toxicity of inorganic monomeric aluminium (Labile Al) on Atlantic salmon and brown trout, with a reduced toxicity when increasing from a $Ca_w < 1$, between 1-2 and > 2 mgCa/l (Rosseland 2000). If the same narrow Ca dependant toxicity of Zink exist for the low concentration range of Ca, this might result in lower toxicity of zinc in Lake Maridalsvann (Ca 2,61 mg/l) than in Lake Store Sandungen (Ca= 2,08 mg/l). No such difference was found in the NOEC for time to hatching, but reduction in length and weight of larvae were more pronounced in Lake Store Sandungen than in Lake Maridalsvann The differences in pH and TOC between the two lakes were minor and are not expected to have influenced the toxicity of zinc significantly.

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