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Population-level ecotoxicology: combined effects of POPs exposure and density stress on zebrafish populations

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Abstract

As oceans and freshwater ecosystems function as sinks for persistent contaminants, assessing effects of contaminant exposure on fish populations is important. This is true in particular for contaminants with ability to accumulate in the food chain, as there are generally many trophic levels in aquatic food webs and the potential for biomagnification in fish is great.

A challenge in ecotoxicology is the translation of individual-level effects of exposure, as measured in laboratory tests, into population-level effects that have more ecological relevance. It is not necessarily possible to predict effects of toxicant exposure on the population level directly from observations on individual-level endpoints. Density-dependent processes within natural populations is a major complicating factor.

In this study, I have exposed laboratory populations of zebrafish (*Danio rerio*) to a naturally occurring mixture of persistent organic pollutants, at three levels of population density and investigated the effects on demographic rates. I found that toxicant exposure decreased somatic growth in juveniles and adults, and increased time to sexual maturation. Increasing population density had the same effects. For fish populations, individual growth can be an important regulatory mechanism. I therefore developed a size-structured population model, which I used to synthesise the toxicant effects on vital rates into population-level endpoints. This model enabled me to interpret the effects on individual vital rates, as observed in the experiment, on the population level.

The modelling of toxicant-exposed and unexposed populations has demonstrated that population-level effects of exposure may differ considerably from the individual-level effects. Firstly, toxicant-induced reductions of demographic rates (larval survival, growth, and fecundity) may be ameliorated by relief from density stress. Secondly, the toxicant-induced reductions may in some cases (larval survival and growth) be more than compensated by density-dependent mechanisms. The effects of both toxicant and density on somatic growth rate were key elements in these mechanisms. This kind of size-structured population model can be a very useful tool for interpreting results from toxicological experiments in a larger ecological setting, such as an ecological risk assessment.

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1. Introduction

Assessing effects of exposure to pollution on fish populations is important for several reasons. Many types of contaminants eventually end up in lakes or in the sea, and oceans are considered a major sink for persistent chemicals, which are transported from continental areas by atmospheric and oceanic currents (Corsolini et al. 2002). In aquatic environments, there are generally many trophic levels and a potential for a great degree of biomagnification (SFT 2008)[1]. Along with overexploitation and habitat destruction, pollution is listed as one of the principle threats to marine biodiversity in a statement from the Second Conference on Parties on the Biological Diversity Convention (The Jakarta Mandate) (Lawrence & Elliot 2003). Moreover, fish is an important food source, and fisheries is an important economic resource. As fisheries management are struggling to control overfishing, pollution may be detrimental to already depleted stocks (Lawrence & Elliot 2003). The threat that pollution represents is no less severe for freshwater species, as the volume of water in which pollution can be diluted generally is smaller in a lake than in marine habitats.

Persistent organic pollutants (POPs) in Lake Mjøsa

The Stockholm Convention on Persistent Organic Pollutants (POPs) defines POPs as toxic organic substances that are highly resistant to degradation in the environment, accumulate in the fatty tissue of organisms and have long-range atmospheric transport potential (UN 2001). Among the more infamous POPs are dichloro-diphenyl-trichloroethan (DDT) and polychlorinated biphenyls (PCBs), with well-documented harmful effects. Brominated flame retardants such as polybrominated diphenyl ethers (PBDE) have recently been included among the POPs, and recent studies have shown that some PBDE congeners have a toxic potential similar to the PCBs (Mariussen et al. 2008). The properties of the particular contaminants will not be elaborated on in this thesis, but generally, neurodevelopmental and reproductive effects, immunotoxicity, and endocrine disruption are reported from studies of POPs (see e. g. Brouwer et al. (1999)).

The detection of persistent organic pollutants (POPs) in aquatic environments worldwide has raised concerns about the effects of these compounds on populations and ecosystems. In Norway, an alarming example of a contaminated lake is the Lake Mjøsa. Lake Mjøsa is the largest lake in Norway and covers an area of 365 km^2 and is situated 60° 53′ 56” N,

[1] The Norwegian Pollution Control Authority

10° 41′ 31” E. With 20 species of fish, such as brown trout (*Salmo trutta*), pike (*Esox lucius*), perch (*Perca fluviatilis*), burbot (*Lota lota*), crucian carp (*Carassius carassius*), common whitefish (*Coregonus lavaretus*), grayling (*Thymallus thymallus*) and the nationally famous vendace (*Coregonus albula*), (“lågåsild” in Norwegian), it is one of the most species-rich lakes in Norway. Regrettably, the lake has a long history of pollution due to human activities. The major pollution events can be summed up as follows: In the 1950-60s DDT was used extensively as an insecticide in the surrounding area agriculture. Between 1960 and 1970, mercury was spilled from a cardboard factory located in Lillehammer. Effluents containing polychlorinated biphenyls (PCBs) from a railway workshop in Hamar were discovered in 1990, and in the period 1997-2003, brominated flame retardants of the type polybrominated diphenyl ethers (PBDEs), were spilled into the lake from a fabric manufacturer located in Lillehammer. Although DDTs was largely banned in Norway from 1970, the pollutant has occasionally leaked from landfills (Miljøstatus).

The levels of organic contaminants in fish from Lake Mjøsa have been monitored by the Norwegian Institute of Water Research (NIVA) since 1980 (Faafeng 2007). For the past two decades, the levels of PCBs and DDTs have been found to be high, and levels of PBDEs in burbot and trout liver close to the highest recorded worldwide were reported by the Norwegian Pollution Control Authority in 2001 (Fjeld 2001).

The ZEBPOP-project

The reports of high levels of POPs in general, and PBDEs in particular, in fish from Lake Mjøsa, was the background for the project “Zebrafish as a model for effect studies of persistent organic pollutants in aquatic ecosystems” (ZEBPOP), of which this thesis is a part. The ZEBPOP-project is an initiative of the Norwegian School of Veterinary Science, in collaboration with the University of Oslo, (Department of Biology) and the Norwegian Institute of Water Research (NIVA). The overarching aim of the ZEBPOP-project is to investigate effects of exposure to environmentally relevant concentrations of persistent organic pollutants (POPs), using zebrafish as a model organism. The endpoints evaluated in the project range from the suborganismal to the population level (Figure 1). The scope of my thesis is the transition from the individual to the population level, i. e. the effects of pollution in an ecological context.

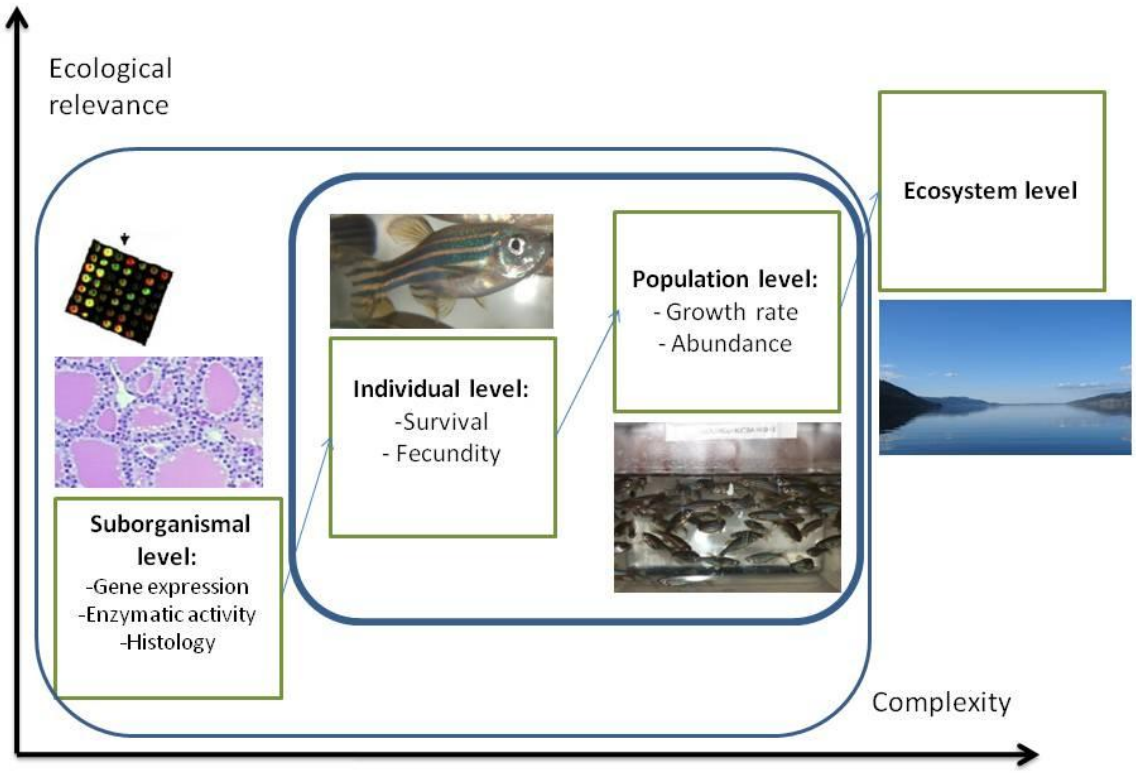

Figure 1: Large square: The scope of the ZEBPOP-project, small square: the scope of this thesis.

Ecotoxicology

Assessing the effect of contaminants on population-level endpoints is held as one of the ultimate goals in the field of ecotoxiology (Forbes & Calow 1999; Gleason & Nacci 2001; Moe 2008; Moe et al. 2001; Newman 2001; Nisbet et al. 1989; Power & Power 1995). Ecotoxicology was proposed as a new branch of toxicology by the Committee of the International Council of Scientific Unions in 1969 (Truhaut 1977), and was initially defined as "*..the branch of Toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context.*" (Truhaut 1977). At the time, it was emphasized that while classical toxicology is concerned with the study of toxic effects on individual organisms, ecotoxicology is concerned with the study of their impact on populations of living organisms.

Shortcomings of standard toxicity tests in ecotoxicology

A challenge for ecotoxicologists is that most ecotoxicological assays are still founded on traditional toxicological studies (Barnthouse et al. 2008; Elliot et al. 2003; Laskowski 2001). In a recent review, it was found that a bias in the toxicological literature is the use of acute mortality values, usually in the form of lethal dose or concentration (LD/LC) (Stark & Banks 2003). Classical toxicological tests were originally developed to compare relative toxicity among different chemicals, or to establish dose-response relationships for a certain chemical and a certain species (Laskowski 2001; Newman 2001). The outcomes of these tests may provide limited information about the potential population-level effect of a toxicant (Forbes & Calow 1999; Laskowski 2001; Newman 2001; Spromberg & Meador 2005). For instance the LC50 metric for a species – the concentration of a substance that is lethal for 50 % of the population after a certain time –does not provide information about sublethal effects of the toxicant. A certain concentration of a toxicant that does not kill an organism may still have adverse effects on growth or reproduction (Moe et al. 2001; Newman 2001). Furthermore, these tests are usually lasting for a short period of time compared to the lifespan of the test organism (Laskowski 2001; Roex et al. 2001). Toxicant exposure at one point in life may ultimately lead to reduced lifespan, or have other delayed effects.

Endpoints for assessing the impact of exposure on the population level

Measures of population growth rate have been suggested as better ecotoxicological endpoints than individual vital rates (Forbes & Calow 1999; Stark & Banks 2003), since measurements like these will summarise all sublethal and lethal effects throughout the life cycle (Moe et al. 2001; Munns jr. et al. 1997; Schäfers & Nagel 1991). Indeed, it is stated that "Population growth rate is central to our understanding of environmental stress: environmental stressors should be defined as factors which when first applied to a population reduce population growth rate" (Sibly & Hone 2002).

Ideally, measures of population growth rate should be obtained by monitoring exposed populations over several generations. However, running time series experiments over several generations can prove labour-intensive and costly, especially for vertebrates with long life-span. To accomplish the translation from a pollutant's effects on individual vital rates to its effects on the population growth rate, demographic models can be used. In life table response experiments, organisms are subjected to chronic exposure and monitored throughout their life-cycle. Stage- or age-specific vital rates appear as the response variables in an experimental design. The calculated vital rates are then synthesised into population growth

rate using demographic models such as for instance projection matrices (Caswell 2001; Gleason & Nacci 2001).

Density dependence

A potentially important limitation of many life cycle studies, is that the demographic rates are measured under conditions where population density does not influence population growth rate (Forbes et al. 2001). When contamination is studied at the population level, however, density-dependent responses to toxicant exposure could be expected (Beketov & Liess 2005; Elliot et al. 2003; Forbes et al. 2001; Moe 2008). Following Begon et al. (1990), factors that determine the abundance of a population are categorised as being dependent on or independent of population density. Regulation occurs when a population has a tendency to decrease in size when it is above a particular density, and increase in size when below that density (Begon et al. 1990). It is likely that for most natural populations, population abundance is determined in part by regulation (i. e. density dependence). Long term average rates of realised population growth are therefore likely to be close to zero, even if intrinsic (potential) population growth rate is positive (Begon et al. 1990; Forbes et al. 2001; Grant 1998).

It can thus be argued that the most appropriate indicator of the impact of toxicants on a population with a stable equilibrium is their effects on equilibrium population size (Grant 1998). For a population at a stable equilibrium, the realised population growth rate is zero. If the population is then exposed to a contaminant or pesticide which impairs the performance of individuals there will usually be a reduction in population numbers, so the realised population growth rate will become negative. If the exposure continues, the population will stabilize at a different equilibrium and the population growth rate will return to zero. So if the abundance of a population is determined by density dependence, population growth rate is not a very helpful indicator of its behavior (Grant 1998). Beketov & Liess (2005) and Forbes et al. (2001) argue along the same line. In fact, density dependence has been termed “an unwelcome complication for ecotoxicologists“ (Walker et al. 2001).

Grant (1998) recognises four ways in which density dependence can reduce population growth: (1) Reducing the rate of offspring production (either directly, or by reducing adult size); (2) Reducing the rate of offspring survival; (3) Reducing the rate of adult survival; (4) Extending development times. Conceivably, toxicant exposure can also affect any of these demographic rates. There is thus the possibility that the effects of toxicant exposure may

substitute the effects of density stress. For instance, say an increase in a population at its carrying capacity is compensated by mortality due to competition for food. If the population is exposed to toxicants, and exposure kills some of the individuals, this will relieve density stress and there will be less competition for food. The effect of exposure may thus be ameliorated to a greater or lesser degree. However, if there is no competition at the outset, the mortality due to an exogenous factor such as exposure will add to the total mortality in the population (Sutherland 2001). This implies that the effect of exposure on the population level may be dependent on population density. Furthermore, the exposure effects on individual-level endpoints may be dependent on density as well. For instance, food limitation might physiologically impair individuals such that they become increasingly susceptible to toxicant exposure (Forbes et al. 2001; Lawrence & Elliot 2003)

There are long traditions for population models with density-dependent compensation in fisheries and natural resource management literature. For example, classical population models such as Ricker and Beverton-Holt describe density-dependent recruitment in fish populations. Such models can include harvesting as an additional mortality factor, in order to estimate e.g. the maximum sustainable yield. If toxicant exposure affects only mortality, then effects of exposure may be compared to harvesting. Effects of toxicant exposure on population level may then be modelled in a similar way as effects of harvest in fisheries population models. For toxicant exposure, however, sublethal effects such as reduced growth or delayed effects imply that the parallel to harvest is no longer valid. For modeling such more subtle effects of exposure, it may be necessary to incorporate e. g. effects on individual growth and reproduction (Moe 2008).

In summary, as there may be complex interactions between effects of toxicant exposure and population density, there is not necessarily a straightforward relationship between the effects of a toxicant on the individual-level and the effects on the population-level.

Aims

In this study, I addressed the problem of translating the effects of toxicant exposure on individuals into effects on the population level. More specifically, I wanted to investigate effects of exposure and density on growth, since growth is of particular importance in fish as there can be strong correlations between size and both survival probability and fecundity (Wootton 1998). As previous studies in the ZEBPOP project had demonstrated effects of exposure on somatic growth in the fish (Almås 2007), it was of interest to study effects of

both exposure and population density. Testing effects of exposure and density in a balanced 2 x 3 factorial design would make it possible to model growth as a function of density and exposure. Moreover, in this type of experimental design, possible interactions between density and exposure could be detected.

The results presented in this thesis were obtained in two parts. First individual-level effects were tested in experiments in the laboratory, and then these results were used in population modelling to estimate population-level effects.

1. *Laboratory experiments*

The purpose of the laboratory experiments was to test effects of exposure on the individual-level endpoints survival and batch fecundity, and to test the combined effect of density stress and toxicant stress on the endpoints growth and time to sexual maturation.

In the discussion of the results from the laboratory, the emphasis will be on the implications of the effects of exposure that may be relevant for the population-level effects.

2. *Population models*

The purpose of the population modeling was twofold: to synthesise individual-level effects of toxicant exposure into population-level endpoints, and to explore whether negative effects on the individual level could be ameliorated or exacerbated by density dependence on the population level.

2. Materials and methods

In the ZEBPOP-project, a total of three experiments were carried out with three successive generations (F0, F1, F2) of zebrafish (Figure 2). The experiments were performed from June 2006 until June 2008 in the Alestrøm Zebrafish Laboratory, Department of Basic Sciences and Aquatic Medicine at the Norwegian School of Veterinary Science (F0: June 2006 - December 2006; F1: January 2007 – June 2007; F2: October 2007 - June 2008). The results presented in this thesis were obtained from the F2 generation, except the proportion of spawning females and the relationship batch fecundity – female size, which was tested on individuals from the F1 generation (Table 1). The other results from the F0 and F1 generations are reported by Stavik (2007) and Almås (2007). In the following, the term "Mjøsa" refers to the mixture of toxicants obtained from Lake Mjøsa.

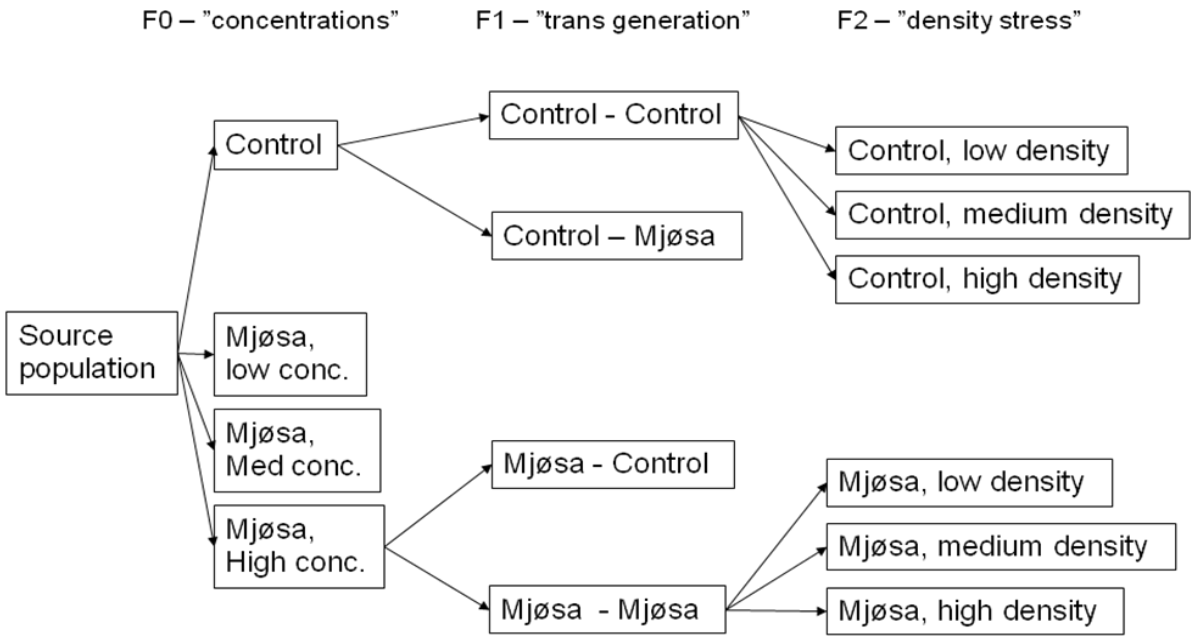

Figure 2: Overview of the different exposure groups in the F0, F1 and F2 generations. The purpose of the F0-generation was to test different concentrations of the Mjøsa mixture. The purpose of the F1-generation was to assess effects of transgenerational exposure, and the names of the treatments in this generation indicate the exposure of the parents of the fish (first name) and the exposure of the fish itself (second name).

Table 1: Overview of endpoints reported from the two experiments (F1 and F2). All results were incorporated in the population model (Chapter 4), except effects of exposure on larval survival in the F2 generation.

	Survival	*Growth*	*Time to maturity*	*Batch fecundity*	*Proportion of spawning females*
F1 generation	Effects of exposure			Effects of exposure and length	Effects of exposure
F2 generation	Effects of exposure	Effects of exposure and density	Effects of exposure and density	(Experiment terminated)	(Experiment terminated)

2.1. Experimental design

2.1.1. The F1-generation

Although the focus in this thesis will be on the results obtained from the F2-generation, for practical reasons, some endpoints had to be measured on individuals from the F1-generation. These individuals were taken from the Control-Control and Mjøsa-Mjøsa exposure groups (Figure 2). The F1-generation was established by breeding seven pairs from each of Control and Mjøsa exposure groups in the F0-generation according to the breeding procedure described in 2.4.1. The eggs from the females in each of the exposure groups were pooled, and 130 eggs were transferred to each of 2*5 replicate tanks of Mjøsa eggs and 2*5 replicates of Control eggs. The larvae were reared as described in 2.4.2.

The F1-generation was terminated in June 2007, i. e. most of the individuals were killed and samples were taken for histology and microarrays. The fish that were used in the tests of "Batch fecundity" and "Proportion of spawning females", and that were used to establish the F2-generation, were kept under exposure and fed to satiation. The males and females were kept separately.

2.1.2. The F2-generation

The combined effects of toxicant exposure and density stress on individual-level endpoints were tested in a 3 x 2 experimental design, where individuals in two exposure groups, termed "Control" and "Mjøsa", were reared at three levels of population density (Figure 2), each with 5 replicates. In the following, the different density – exposure combinations will be referred to as "treatments".

Recommended rearing density in zebrafish laboratories is 9 individuals per litre. As the fish were to be kept in 6 litre tanks in the juvenile and adult stages, it was decided that the medium density should correspond to standard rearing density, and that low and high density should be

half and twice this density, respectively. Thus, in the low-density tanks there were 25 individuals, 50 in the medium-density tanks and 100 in the high-density tanks.

Food was intended to be the limiting resource in this experiment, but rearing density can also affect individuals in other ways such as through social stress (Smith et al. 1978). In this experiment, density stress is taken to be the combined effect of these.

The treatment groups were established by breeding seven pairs from each of the Control-Control and Mjøsa-Mjøsa exposure groups in the F1-generation (see Figure 2) according to the breeding procedure described in section 2.4.1. The previous experiments had shown that the mortality rate in the larval stage could be somewhat unpredictable. Moreover, as the larval feed was difficult to distribute in a controlled manner, we decided that it was not feasible to test the effect of population density in the larval stage. To make sure that we obtained a sufficient number of individuals required to test the effect of density in the juvenile and adult stage, the experiment was initiated with a large surplus number of eggs. The eggs from each of the exposure groups were pooled and 160 eggs were transferred to each of 10 parallel containers. These parallel containers were maintained for four weeks. The larvae were taken care of as described under 2.4.2.

On 26 days post fertilization (dpf), the juveniles within each exposure group were randomly assigned to 3x5 tanks – three density levels with 5 replicates in each. During the experiment the fish in the low density-tanks were fed to satiation (traces of feed left after five minutes) four times per day. An amount of food equivalent to satiation feeding of 25 fish was given to the tanks with 50 and 100 individuals. The amount of food corresponding to satiation-feeding of 25 fish was calibrated weekly. Feed was distributed in a way that gave all the individuals in a tank equal access to the food. For a detailed description of the feeding routine, see Appendix 2.

2.2. The model organism

Zebrafish *Danio rerio* (Hamilton) is a small cyprinid, found in shallow water bodies such as shallow pools, floodplains and edges of slow-flowing, turbid rivers. The natural range of the zebrafish is around the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh and Nepal (Engeszer et al. 2007; Spence et al. 2008).

Over the past 20-30 years, the zebrafish has gained enormous popularity as a model organism in genetics, biomedicine and developmental research. It is increasingly used in toxicogenomic

studies (P. Aleström, pers. comm.), and it was thus well-suited as a model organism in the ZEBPOP-project. However, it remains underexploited as a model species in ecology (Engeszer et al. 2007), and relatively few studies have investigated what factors affect life-history traits such as growth, age and size at maturity and spawning activity, in the wild or in the laboratory (but see e.g. (Bang et al. 2004; Darrow & Harris 2004; Engeszer et al. 2007; Gerlach & Lysiak 2006; Spence et al. 2007; Spence et al. 2008).

The zebrafish is in the r-strategist end in the K- to r-continuum. It is iteroparous, can produce a large number of offspring, and has no parental care. In textbooks on zebrafish laboratory husbandry, the zebrafish is usually described as a hardy fish that is easily bred all year round and has a short generation span and high fecundity (see e. g. (Westerfield 2000)).

Wild type zebrafish previously bought in a pet store gave rise to the first experimental generation.

2.3. Laboratory conditions

The fish were kept in transparent plastic tanks in closed recirculation systems (Figure 3) (Marine Biotech. Inc., Massachusetts, USA.). The systems were equipped with particle filters, a charcoal filter and suspended biofilters. In addition the water was irradiated by a UV-lamp. The water temperature in the systems was set to 28 °C. The pressure in the systems was set to ca. 25 litres per minute, equivalent to 6 water circulations pr. hour. Every day 10 % of the system water was replaced with new conditioned water.

Figure 3: Recirculation system with fish tanks in the Aleström zebrafish laboratory.

Conditioned water was produced by running filtered (5 micron) tap water through a reverse osmosis machine, and adding 150 mg/l synthetic sea salt (Instant Ocean, Marineland, USA), 75 mg/l sodium bicarbonate (Sigma, Schnelldorf, Germany) and 25 mg/l calcium sulphate (Sigma, Schnelldorf, Germany). The acidity was adjusted with 1 M HCl to pH 7.5, and conductivity was maintained at ~500 µS/cm. NO_2 was kept below 0.1 ppm, NO_3 below 50 mg/L, NH_3/NH_4^+ below 0.025 ppm. General hardness and carbonate hardness were kept at levels around 4 and 5 °dH, respectively. Tests of water quality were run weekly.
The temperature in the fish room was ~26 °C, the humidity was kept between 60 and 70 %. A 13.5:10.5 light:dark cycle was maintained using an automatic timer.

2.4. Experimental procedures

2.4.1. The breeding procedure

Spawning in domesticated zebrafish is influenced by photoperiod, starting within the first minute of exposure to light in the morning, and continuing for about an hour (Spence et al. 2008). In the afternoon on the day before spawning, the fish were placed in breeding tanks with conditioned water. A barrier was separating the males and the female during the night, and removed when the lights were turned on in the morning. The fish were then left undisturbed for half an hour. Subsequently, the fish were transferred back to the rearing tanks, and the eggs were collected in a sieve.

Breeding tanks consisted of an inner and an outer tank. The bottom of the inner tank was a lattice that allowed the eggs to slip through to the bottom of the outer tank, where they were safe from being eaten by their parents. Marbles were placed in the tank to give the female a visual cue that the site was suited for oviposition. As zebrafish females can be choosy with respect to mates, two males were placed in each tank along with one female, thus increasing the probability that the female would release her eggs.

2.4.2. Raising larvae

Initially, the larvae were kept in ¾ L transparent plastic tanks (11x5.5x11 cm) filled with water from the circulation systems ("old aquarium water"), water level ~5 cm. They were transferred to 2 L (25x6x17 cm) and 6 L tanks (26x19x16 cm) when they had reached a certain size. From hatching until 14 dpf, the larvae were too fragile to be kept in tanks that were connected to the circulation systems, and from 14 dpf until 26 dpf the water flow through the tanks was kept at the minimum level. During this period, feces, excess food and dead larvae were removed every day using pipettes, and ~50% of the water was replaced daily with water from the circulation systems. From 27 dpf, the juveniles were kept in the circulation systems with normal water flow, as described under section 2.3.

2.4.3. Toxicant treatment of larva feed (powder)

Two batches of SDS100 fodder were prepared; Mjøsa and Control. To ensure that the powder feed was initially free of toxicants, it was analyzed and it was found to contain less than 1ng/g wet weight of all components. The fat content of the feed was 10%. The toxicant mixture was added abundantly to obtain the same concentration as in the burbot liver oil. The Control feed was only treated with the solvent, cyclohexane (CHX). Plentiful amounts of the CHX and mixtures were added to the batches of baby powder to ensure that everything was soaked.

Subsequently, the solvent was evaporated under a gentle stream of N_2 until the feed was completely dried.

2.4.4. Toxicant treatment of adult feed (artemia)

The adult food, artemia or brine shrimp (*Artemia salina*) is a small marine crustacean. The artemia nauplii (larvae) are widely used as fish food in aquariums due to their high content of protein, lipids and unsaturated fatty acids. The brine shrimp are bought as metabolically inactive cysts that hatch when soaked in salt water for approximately 24 hours. The toxicant mixtures were added to the salt water solutions in which the artemia was prepared, to be absorbed by the fatty tissue of artemia nauplii.

2.4.5. Feeding regime and exposure

The fish were exposed through the feed. This is a realistic way of exposure to persistent organic pollutants, as POPs are typically lipid soluble. Zebrafish larvae begin feeding on 6 dpf. From 6 dpf - 13 dpf they were fed SDS100 powder feed (Special Diets Services, Scanbur AS, Nittedal, Norway) four times per day (9 am, 11 am, 1 pm and 3 pm). From 14 dpf - 21 dpf they were fed powder feed twice daily and artemia twice daily, and during the third week (19-25 dpf) SDS100 was given once per day and brine shrimp three times per day. Brine shrimp was given four times per day from 26 dpf and onwards. For a detailed description of the preparation of the artemia and the feeding procedure, see Appendix 2.

2.5. Recording endpoints

2.5.1. Survival

Mortality was monitored until the experiment was terminated by inspecting the tanks for dead individuals every day and recording the numbers.

2.5.2. Size

On 248 dpf, the fish were killed, measured (total length) and weighed.

2.5.3. Onset of maturity

To obtain an estimate of the onset of maturity in the different exposure groups, possible spawning activity in the replicates was monitored from when the fish had started to assume a more adult shape. Two to three times per week, small, open plastic boxes (12x8.5x3.5 cm) filled with marbles were placed in the fish tanks, left overnight and checked for eggs the following day.

2.5.4. Proportion of breeding females

Breeding trials were run with individuals from the Control-Control and Mjøsa-Mjøsa exposure groups in the F1-generation during four weeks in the fall of 2007 and six weeks in the spring of 2008. During these periods, seven breeding pairs (fall 2007) or 14 breeding pairs (spring 2008) were bred twice a week according to the breeding procedure described in 2.4.1. Between the breeding trials, the fish that were tested were kept in separate "male" and "female" tanks, 10-15 individuals pr. 2L tank, and fed to satiation with exposed or control feed.

2.5.5. Batch fecundity

To test whether there were differences in batch fecundity between the females from the Control-Control and Mjøsa-Mjøsa exposure groups in the F1-generation, we bred 14 pairs from each of the exposure groups and counted the number of eggs laid by each female. To control for the size of the female, we transferred each of the females to a small plastic tank filled with water to a level of ~ 2 cm, placed the tank on a grid and photographed it from a fixed height. The photos were imported into the imaging software ImageJ (Rasband 1997), and the standard length of the females was measured by drawing a segmented line along the spine from the snout to the posterior end of the last vertebra.

2.6. Statistical analyses

All statistical analyses were conducted in R version 2.6.0. (R Development Core Team (2007)). The replicate tanks were taken to be the experimental units.

2.6.1. Survival

The survival curves of the different exposure groups were estimated by the Kaplan-Meier method see (Bølviken & Skovlund 1994). The Cox proportional hazard model was used to test and quantify the differences in mortality between the exposure groups see (Bølviken & Skovlund 1994). The cluster term in the coxph-function was used to identify replicates.

2.6.2. Size

The length and body mass data were tested for normality using the Shapiro-Wilks test, and the data were found to be normally distributed. Plotting of residuals from the model vs. fitted values revealed that the body mass data were heteroscedastic, and ln-transformation was performed to mend this.

The mean was calculated for each of the five replicates within a treatment, and confidence intervals for a treatment was estimated based on these values. The mean values of the replicates were not as close to a normal distribution as the data on the individuals. However, parametric tests were still used to test for differences among the groups, as nonparametric tests are more conservative (i. e. give higher p-values). This was justified by the fact that the original data were normally distributed. Analysis of variance was used to test for differences among the treatment groups and check for interactions between the explanatory variables. Linear regression was used to analyse the effect of exposure and population density on length and weight. Density was log-transformed to make the three levels (25-50-100) increase linearly.

2.6.3. Onset of maturity

Whether there were differences in time to sexual maturity between the treatments was analysed with the logrank test (Bølviken & Skovlund 1994).

2.6.4. Proportion of breeding females

The same individuals were tested repeatedly in the different breeding trials, and the observations are thus not independent. Neither could the females be identified individually, since they were kept in a common tank between the breeding trials. It was therefore not appropriate to statistically test whether the proportions of females in each exposure group that spawned over the course of the breeding trials were significantly different from each other.

2.6.5. Batch fecundity

Batch fecundity was analysed by a log-linear model where ln(number of eggs) was modeled as a function of exposure groups and female length.

3. Results

3.1. Survival

The critical period for juvenile survival, i. e. the period where mortality was highest throughout the experiment, was between 6-20 days post fertilization (Figure 4). In the period 0-8 dpf, the Mjøsa-exposed juveniles had significantly lower survival probability than Control (Table 2). When the period 0-28 dpf was analysed as a whole, the survival probability of Mjøsa juveniles was no longer significantly different from Controls (Table 2). After 28 dpf the mortality in either of the exposure groups was negligible (i. e. 2-3 deaths).

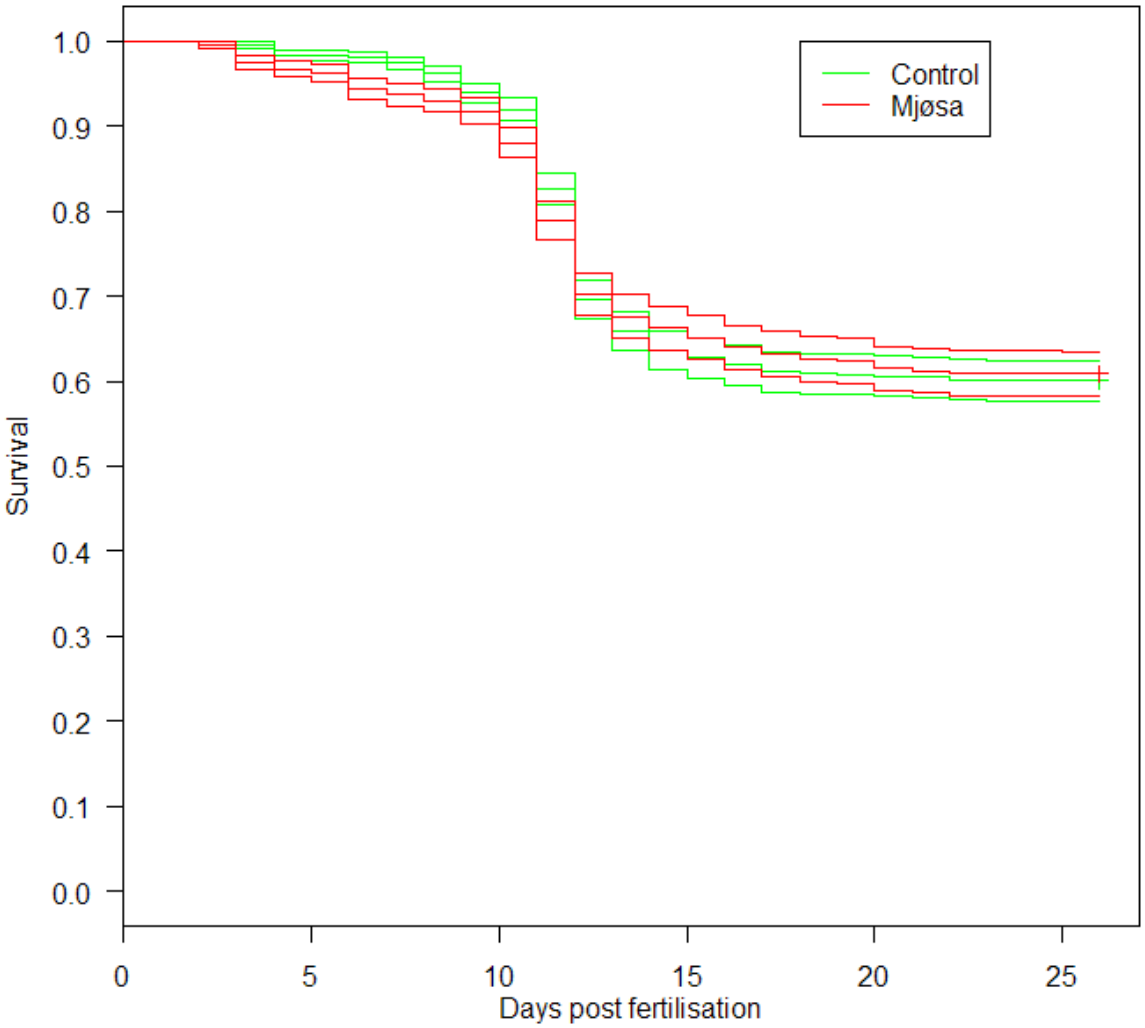

Figure 4: Kaplan-Meier plot showing survival of juvenile zebrafish; control fish and POPs-exposed fish. Mjøsa: mixture of POPs obtained from burbot from Lake Mjøsa. Control: n = 1600, Mjøsa: n = 1300.

Table 2: Survival of exposed and unexposed zebrafish analyzed by Cox' proportional hazard regression. Mjøsa = mixture of POPs obtained from burbot from lake Mjøsa. The parameter exp(β) is the hazard ratio: exp(β) > 1 means that the parameter has a negative effect on survival, 0 < exp(β) < 1 means that the parameter has a positive effect on survival. For instance, the hazard ratio of Mjøsa/ Control the first 8 days is 1.34 – this means that exposed fish have 1.34 higher risk of dying at any time step during the first 8 days than control fish.

	exp(β)	95 % C. I.	z	p-value
R^2 = 0.019				
time < 8 dpf				
Mjøsa	1.34	(0.949, 1.90)	1.66	**0.096**
R^2 = 0.0				
time < 28 dpf				
Mjøsa	0.997	(0.801, 1.24)	-0.28	0.98

3.2. Growth

For females, there was a negative effect of population density on the body mass ($F_{1,26}$ = 674, p < 0.0001) (Figure 5). There was no significant effect of exposure ($F_{1,26}$ = 0.08, p = 0.78). The length of females was negatively affected by population density on ($F_{1,26}$= 839, p < 0.0001). There was a negative effect of exposure ($F_{1,26}$=6.8, p =0.015). There was no interaction between exposure and population density ($F_{1,26}$ = 0.003, p = 0.95). (See Table 3 for regression coefficients).

For males, there was a negative effect of population density on body mass of males ($F_{1,26}$ = 640, p < 0.0001) (Figure 5). There was no significant effect of exposure ($F_{1,26}$ = 2.2, p = 0.15). There was a marginally significant difference in body mass between Mjøsa and Control in the medium density group –Mjøsa had lower body mass than Control (t = 2.3, CI_{diff} = (-0.002, 0.13), p=0.055). Length of males was negatively affected by population density ($F_{1,26}$ = 442, p < 0.0001). There was no significant effect of exposure ($F_{1,26}$ = 0.3, p = 0.6). (See Table 4 for regression coefficients.)

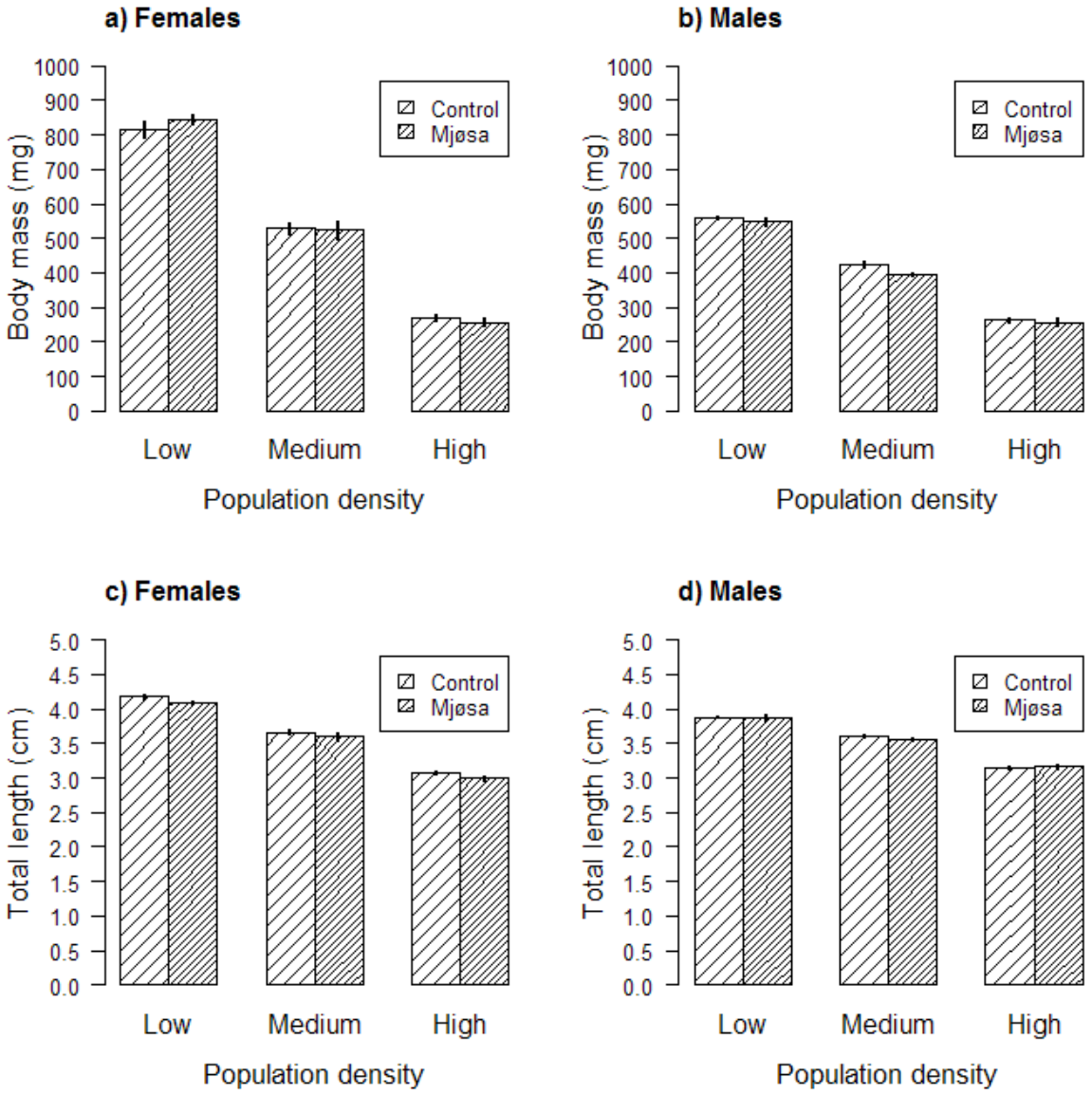

Figure 5. Body mass and length of zebrafish exposed to POPs from lake Mjøsa, reared at different densities. a) and c): females, b) and d): males. n = 75 in all combinations exposure x density x gender.

Table 3. Effect of population density and exposure on body mass and total length in female zebrafish exposed to POPs from Lake Mjøsa, analyzed by linear regression. p-values of significant explanatory variables are in bold type.

	β	S.E.	t	p-value
ln(Weight), females				
adj. R^2 = 0.96				
Exposure	-0.010	0.036	-0.279	0.782
ln(Density)	-0.830	0.032	-25.92	**<0.001**
Exposure:ln(Density)	-0.067	0.064	-1.048	0.304
Total length, females				
adj. R^2 = 0.97				
Exposure	-0.081	0.030	-2.65	**0.013**
ln(Density)	-0.792	0.039	-20.44	< **0.001**
Exposure:ln(Density)	-0.003	0.055	- 0.056	0.956

Table 4. Effect of population density and exposure on body mass and total length in male zebrafish exposed to POPs from Lake Mjøsa, analyzed by linear regression. p-values of significant explanatory variables are in bold type.

	β	S.E.	t	p-value
ln(Weight), males				
adj. R^2 = 0.96				
Exposure	-0.023	0.171	-0.168	0.868
ln(Density)	-0.546	0.031	-17.86	**<0.001**
Exposure:ln(Density)	-0.002	0.043	-0.046	0.964
Total length, males				
adj. R^2 = 0.94				
Exposure	-0.069	0.195	-0.352	0.727
ln(Density)	-0.525	0.035	-15.078	**<0.001**
Exposure:ln(Density)	0.014	0.049	0.277	0.784

The variation in growth in females was not affected by exposure or density ($p < 0.05$) (Figure 6).

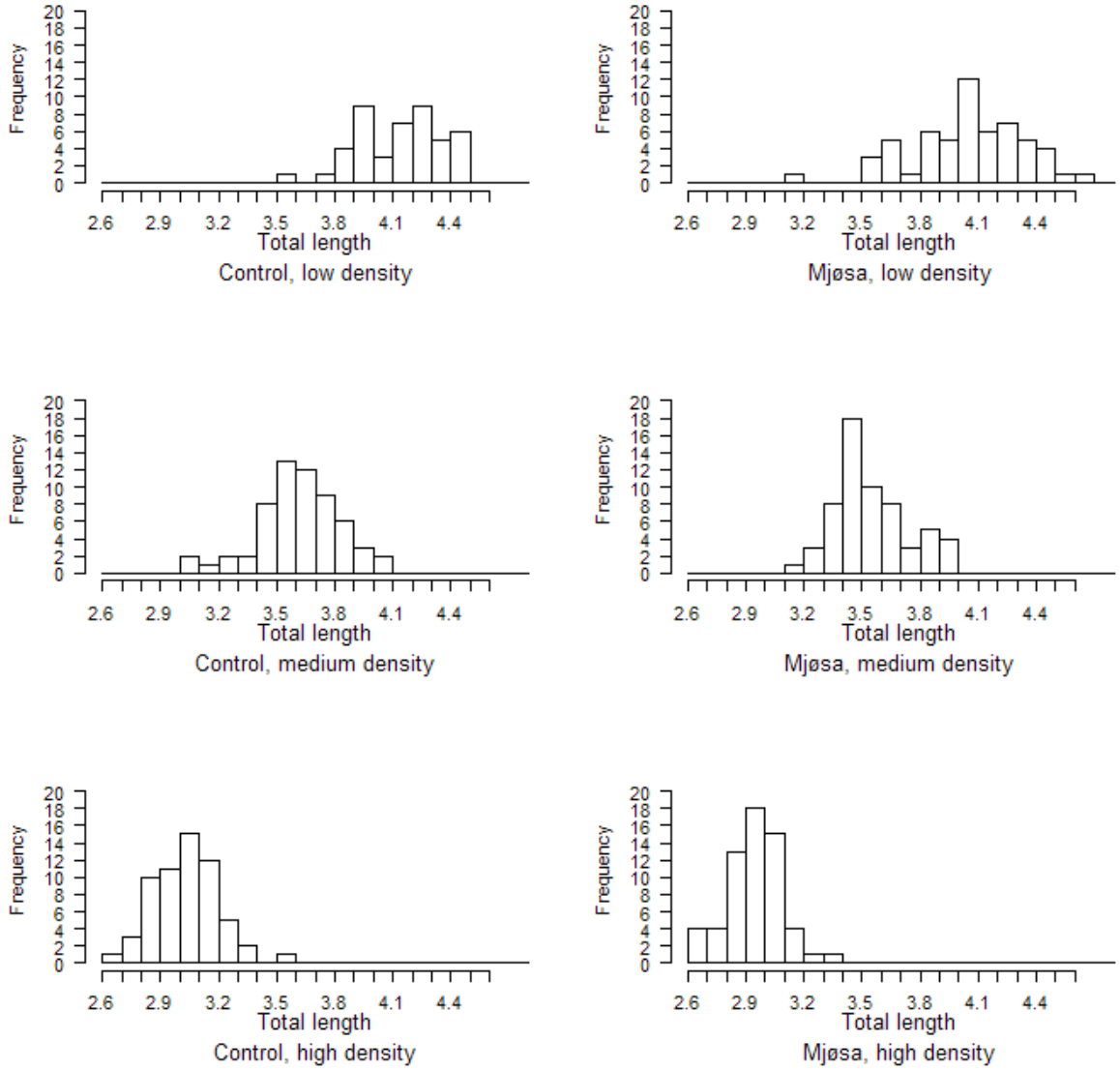

Figure 6: Length distributions in control and POPs exposed female zebrafish reared at different population densities.

3.3. Reproduction

3.3.1. Onset of maturity

In the low density group, Mjøsa fish had significantly later observed spawning than Control (Logrank test, $\chi^2_1 = 5.3$, $p = 0.021$). In the medium density group, there was no significant effect of exposure on time to first observed spawning ($\chi^2_1 = 0.2$, $p = 0.63$). Spawning was observed earlier in the low density tanks than in the medium density tanks (Figure 7). There was a positive effect of density on time to first observed spawning (where 'positive' means longer time), ($\chi^2_1 = 16.9$, $p < 0.001$). By the time the experiment was terminated, there was not observed any spawning in the high density tanks.

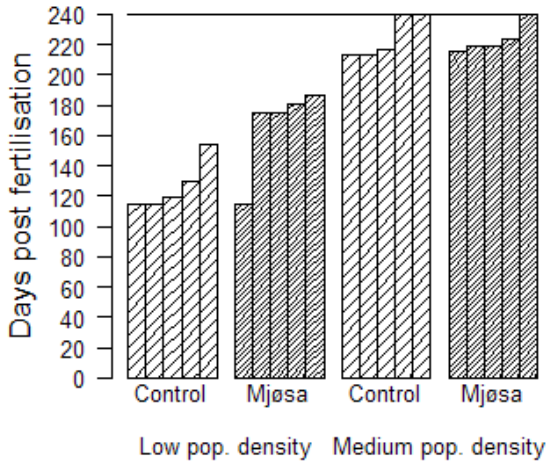

Figure 7. Time at first observed spawning in the replicates of zebrafish populations exposed to POPs. The experiment was terminated at 240 dpf, indicated by the horizontal line.

3.3.2. Batch fecundity

Batch fecundity (mean ± S. E.) for Control females was 434 ± 67, for Mjøsa females: 336 ±22.8 (Figure 8). When ln-transformed batch fecundity was modeled as a function of exposure and female size (standard length), size was a marginally significant explanatory variable ($t = 1.81$, $p = 0.08$) (Table 5). Effect of exposure was not significant.

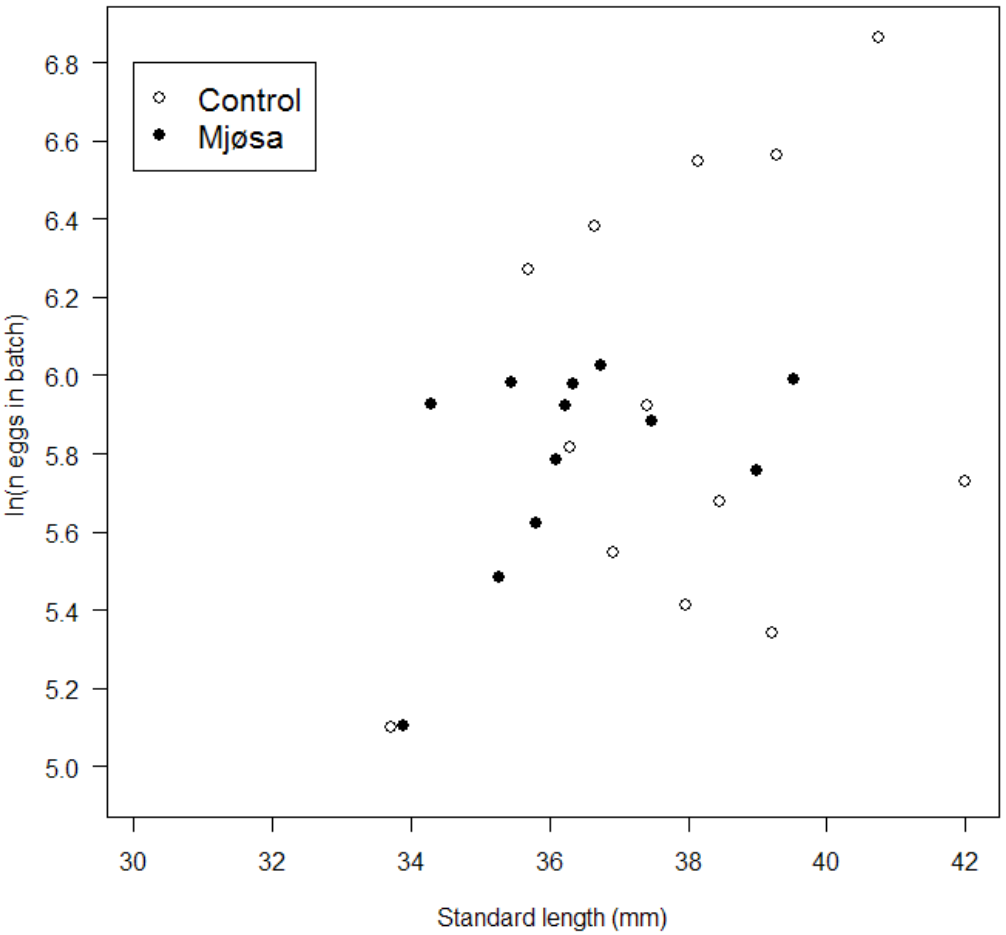

Figure 8. Relationship between standard length and ln-transformed batch size in control and POPs-exposed female zebrafish. Control: n = 14, Mjøsa: n = 12.

Table 5. Batch fecundity in POPs-exposed and control zebrafish analyzed by linear regression. p-values of significant explanatory variables are in bold type.

Parameter	β	S. E.	t	p-value
ln(n eggs), adj. R^2 = 0.16				
Standard length (mm)	0.08	0.044	1.810	**0.08**
Exposure	-0.024	0.179	-1.134	0.89

3.3.3. Proportion of spawning females

Exposure did not seem to affect the proportion of spawning females (Table 6). However, this was not tested formally (as explained in section 2.6.4).

Table 6. Proportion of control and POPs-exposed females spawning in repeated breeding trials. Trials run 21.08.2007-04.02.2008: n = 8, trials run 12.02.2008-04.03.2008: n = 14.

	Control	Mjøsa
21.08.2007	0.75	0.88
22.08.2007	0.75	0.25
18.09.2007	0.75	0.63
19.09.2007	0.38	0.5
25.09.2007	0.75	0.75
26.09.2007	0.25	0.38
09.10.2007	0.88	1
10.10.2007	0.25	0.25
21.01.2008	0.5	0.63
24.01.2008	0.5	0.38
28.01.2008	0.75	0.5
31.01.2008	0.5	0.5
04.02.2008	0.63	0.63
12.02.2008	0.64	0.5
19.02.2008	0.64	0.71
26.02.2008	0.71	0.79
02.03.2008	1	0.79
04.03.2008	0.36	0.43
Mean	0.61	0.58

4. Population model development

The purpose of constructing the model was to to integrate the individual-level effects of POPs measured in the laboratory to population-level effects.

4.1. Model structure

4.1.1. Population matrix

Age-structured models are commonly used in population modelling, i.e. the population is divided into age classes with age-specific demographic rates. In organisms with asymptotic growth, however, size can be a better predictor for individual survival probability and reproductive output than age (Caswell 2001). As will be elaborated on in section 6.1.2, in teleost fish, both fecundity and survival probability can be strongly correlated with size. It can thus be an advantage to use a size-structured model to simulate the population dynamics of fish. Size-structured matrix models for fish populations have been developed for instance for bluehead wrasse (*Thalassoma bifasciatum*) (Warner & Hughes 1988) and brook trout (*Salvelinus fontinalis*) (Marschall & Crowder 1996).

The population model was constructed as a size-structured projection matrix as the one in Table 7. Each row and column corresponds to a size class (to be defined below). The P_i denote the probability of surviving from one size class to the next within one time step, the G_i denote the probability of growing from one size class to the next within one time step, and the F_i denote fecundities (number of offspring per individual per time step). There are two versions of the model, one density-independent and one density-dependent. For each version there are two scenarios, termed Control and Mjøsa (exposure to POPs). The parameters P_1, the G_i and the F_i are all functions of both density and exposure, as will be explained in the following sections.

Table 7: Population model : size-structured projection matrix with survival probabilities (Pi), growth probabilities (Gi), and fecundities (Fi)

	J1	J2	J3	J4	A1	A2	A3	A4	A5
J1					F1	F2	F3	F4	F5
J2	P_1	$P_2*(1-G_2)$							
J3		P_2*G_2	$P_3*(1-G_3)$						
J4			P_3*G_3	$P_4*(1-G_4)$					
A1				P_4*G_4	$P_5*(1-G_5)$				
A2					P_5*G_5	$P_6*(1-G_6)$			
A3						P_6*G_6	$P_7*(1-G_7)$		
A4							P_7*G_7	$P_8*(1-G_8)$	
A5								P_8*G_8	P_9

4.1.2. The time step and the size classes

The time step in the model was set to 4 weeks. This time step was chosen because, in the laboratory, all the mortality occurred within 28 dpf, and it was convenient to describe the survival in this period with one parameter (P_1) for the entire period.

Growth trajectories for each of the fish were estimated using the von Bertalanffy growth function (Wootton 1998). Length at t days post fertilization was estimated as

$$L_t = L_\infty\left(1 - e^{\{-K(t-t_0)\}}\right)$$

where L_∞ is asymptotic length, K is growth rate and t_0 is the time at which the length of the fish is zero. According to Beverton and Holt, density-dependent growth mediated by competition for food is expected to reduce the asymptotic size (L_∞ or W_∞), but not to affect the growth rate K at which this size is approached (Lorenzen & Enberg 2002). Asymptotic length (L_∞) for the different experimental density groups was assumed to be the length of the longest fish in each density group. However, the growth rate K was estimated for each individual fish because it was thought likely that exposure could affect growth rate (6.1.2). The time step t_0 was taken to be 0 dpf. The K's were estimated by setting $t_0 = 0$, t = number of days before the time the experiment was terminated, L_t = the measured length of the fish at time t, L_∞ = the longest fish in each density group, and solving for K.

Size classes were based on the mean growth trajectory of the fish in the Control low-density group. The lower limits of the size classes were taken to be the estimated lengths of mean Control low density after 0, 4, 8, 12, … , 32 weeks (Figure 9). The time at first observed spawning in Control low density was approximately 16 weeks. This corresponds to an estimated size of 3.1 cm, which is within the limits of the fifth size class. For convenience, the fifth size interval was therefore taken to be the first adult size class. Thus, there were four juvenile size classes (J1 – J4) and five adult size classes (A1 – A5). The size limits are given in Table 8.

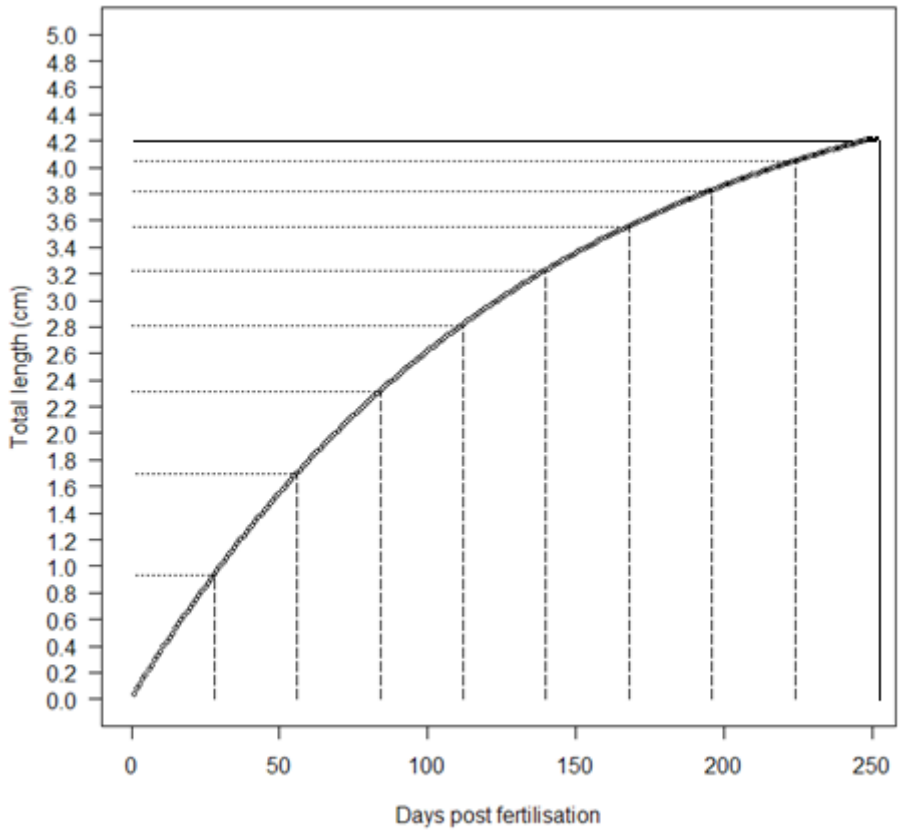

Figure 9: Size classes in the population model. The curve is the mean growth trajectory of fish from the experimental Control low density group. The vertical lines indicate 4-week time intervals. The horizontal lines indicate the corresponding size classes.

4.1.3. Onset of maturity

To determine which size class that was the first adult (i. e. reproducing) size class in the different model versions, approximate size at maturity was estimated for each of the exposure x density treatments in which we had observed spawning in the experiment (Table 9). There had not been observed any spawning in the high-density group (100 individuals) by the time the experiment was terminated. It was assumed that size at maturity did not change further with increasing density.

The differences in size at maturity among the exposure and density groups were incorporated in the model as follows: In the experimental Mjøsa populations, size at maturity was approximately the same in low and medium density populations. Maturation in the model Mjøsa population was therefore simply delayed one size step compared to in Control low density. The Mjøsa matrix consisted of five juvenile size classes and four adult (reproducing) size classes, whereas the Control matrix consisted of four juvenile size classes and five adult size classes. . For the Control matrix, the difference in size at maturity that was observed

between the experimental low and medium density groups were incorporated in the model by making reproduction in the first adult size class conditional on total biomass. If the total biomass exceeded the total mass corresponding to 25 fish (low density) in the first adult size class, then there was no reproduction in the first adult size class, i. e. maturation was delayed one stage. For consistency, the fifth size class is termed A1 in both exposure groups.

Table 8: Limits of the size classes in a size-structured model of a zebrafish population,.

Size class	J1	J2	J3	J4	A1	A2	A3	A4	A5
Total length (cm)	0-1.0	1.0-1.8	1.8-2.4	2.4-2.9	2.9-3.3	3.3-3.6	3.6-3.9	3.9-4.1	4.1-4.2

Table 9: First reproducing size classes in the different exposure x density treatments, estimated from the experiment F2. (Note: There was not observed any spawning in the high (100) density group by the time the experiment was terminated.)

Exposure treatment	Control		Mjøsa	
Density treatment	Low (25)	Medium (50)	Low (25)	Medium (50)
Time to first observed spawning (days)	130	220	160	220
Estimated length at maturity (cm)	3.1	3.5	3.4	3.4

4.2. Model parameters

4.2.1. Survival probabilities (P_i)

The survival probabilities are based on the results obtained in the laboratory from the F1 generation. In this experiment, mortality occurred mainly in the larval stage, 0-28 dpf (J1). However, the survival probabilities in the subsequent stages were adjusted to reflect the fact that for individuals in most natural fish populations, survival probability increases with size.

As explained in 6.1, the results on larval survival in the F2 generation were not considered to be valid. Therefore, results from the F1 generation were used in the model. In the F1 generation, total survival in control larvae from control parents in the period 0-28 dpf was 0.57, whereas the corresponding figure for toxicant exposed larvae from exposed parents was 0.47 (Almås 2007). Density-dependent mortality is common in early life stages of fish, whereas growth and reproduction are the density-dependent traits more often observed later in life (Vandenbos et al. 2006). Thus, in the larval stage, survival (P_1) was assumed to be density-dependent, although the impact of population density on larval survival was not tested in the experiments.

$$P_1 = \frac{total\ larval\ survival\ in\ the\ given\ exposure\ group}{(1 + (a * total\ biomass\ at\ time\ step\ i)^b)}$$

The parameters a (strength of density dependence) and b (degree of density-dependent compensation) were set to 0.011 and 0.9, respectively.

In the subsequent juvenile and adult size classes, the survival probabilities were density-independent. The parameter values were chosen to reflect that typically in fish populations, individual survival probability increases with size. As we did not observe any difference in juvenile and adult survival between Control and Mjøsa in the laboratory, these probabilities were taken to be the same for the two groups. The survival probabilities are given in Table 10.

Table 10: Size-specific survival probabilities in the juvenile and adult size classes

Size class	J1	J2	J3	J4	A1	A2	A3	A4
Parameter	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9
Value	0.75	0.75	0.75	0.80	0.80	0.80	0.80	0.80

4.2.2. Growth probabilities *(G_i)*

The probabilities of growth from one size class to the next during one time step were modeled as functions of both density and exposure. Incorporation of density dependence based on the experiment in the laboratory into the growth probabilities was done by the following steps: For each fish in the experiment, the size at the beginning of each time interval was estimated from the growth trajectory. The estimated sizes of each of the fish at the beginning of each time interval were transformed into binary response variables based on whether they were larger than/the same as the lower size limit (= 1) for the interval in question or not (= 0). The probability of growing into size class *i* after (*i*-1)* 4 weeks was analyzed by logistic regression with the logit link function, which fits a linear combination of explanatory variables to a binary response variable (enter/not enter) by the maximum likelihood method (McCullagh and Nelder 1995). The response variable was whether the fish had entered the size class or not, and the explanatory variables were exposure and density. For the simulations, the parameter values estimated for each size interval were the values used in the growth probability functions.

The equation used for estimating G_i, the probability of entering size interval *i* after 4*(*i*-1) weeks, was:

$$G_i = \frac{e^{\beta_0+\beta_{i1}x_1+\beta_{i2}x_2}}{1+e^{\beta_0+\beta_{i1}x_1+\beta_{i2}x_2}}$$

where $x_1 = \begin{cases} 0 \text{ for control} \\ 1 \text{ for toxicant exposure} \end{cases}$, $x_2 = biomass$

The entries on the subdiagonal in Table 7, i.e. the probabilities of transition from one size class to the next during a time step, were calculated as a product of the respective survival probabilities and growth probabilities. The entries on the diagonal, i. e. the probabilities of staying within the current size class during a time step, were calculated as products of the respective survival probabilities and (1- the growth probability). It was assumed that an individual could not grow more than one size class during one time step.

4.2.3. Fecundities *(F_i)*

The contribution of eggs by the individuals in the model system was estimated using a variant of the Ricker stock–recruitment model. Zebrafish are known to eat their own eggs, and recruitment (here defined as number of eggs to enter the "larval pool", J1) is probably in part regulated through cannibalism. Although we were not able to observe the impact of egg cannibalism in the lab, this is still a reasonable adjustment in the model. The basic stock–recruitment model was $R = \alpha S e^{-\beta S}$, where *R* is recruitment, *S* is the parental stock, α describes maximum eggs per spawner (before density dependence), and β is the density-dependent parameter that determines the rate at which the stock–recruitment curve dampens as stock size increases (Richards et al. 2004).

Density-independent reproductive output (α_i) per individual in size class *i* during one time step was estimated based on the breeding trials in the laboratory:

$$\alpha_i = sex\ ratio * 4 * e^{(2.5+0.075*L_{Ti})}$$

where L_Ti is calculated as (upper limit of size class i - lower limit of size class i)/2. The factor $e^{(2.5+0.075*L_{Ti})}$ is the estimated batch fecundity of a female of length L_{Ti} (see Table 5). In the laboratory, zebrafish females are normally able to spawn approximately every 5 days (Westerfield 2000). For simplicity, in the model I assumed weekly spawning.

4.3. Model simulations

The initial abundance in the simulations were 100 individuals in the J1 stage (the larval stage), and 10 in all the other stages. The projection matrices for the density-independent versions of the model were constructed by setting all density dependence parameters equal to 0. This gave the matrices in Table 11 (control population) and Table 12 (exposed population). There was no stochasticity included in the simulations.

Table 11: Projection matrix for the Control population, density-independent model version

	J1	J2	J3	J4	A1	A2	A3	A4	A5
J1	0	0	0	0	336	421	489	569	613
J2	0.57	0.06	0	0	0	0	0	0	0
J3	0	0.69	0.06	0	0	0	0	0	0
J4	0	0	0.69	0.06	0	0	0	0	0
A1	0	0	0	0.69	0.06	0	0	0	0
A2	0	0	0	0	0.74	0.06	0	0	0
A3	0	0	0	0	0	0.74	0.06	0	0
A4	0	0	0	0	0	0	0.74	0.06	0
A5	0	0	0	0	0	0	0	0.74	0.80

Table 12: Projection matrix for the Mjøsa population, density-independent model version

	J1	J2	J3	J4	A1	A2	A3	A4	A5
J1	0	0	0	0	0	379	440	512	552
J2	0.47	0.10	0	0	0	0	0	0	0
J3	0	0.65	0.10	0	0	0	0	0	0
J4	0	0	0.65	0.10	0	0	0	0	0
A1	0	0	0	0.65	0.13	0	0	0	0
A2	0	0	0	0	0.67	0.13	0	0	0
A3	0	0	0	0	0	0.67	0.13	0	0
A4	0	0	0	0	0	0	0.67	0.13	0
A5	0	0	0	0	0	0	0	0.67	0.80

5. Results from population modelling

When density dependence was not taken into account, the number of individuals increased exponentially in both the Control and Mjøsa model populations. The population growth rates for the density-independent Control and Mjøsa populations, estimated by the dominant eigenvalue of the projection matrices (Table 11 and Table 12), are 2.6 and 2.0, respectively. In the density-dependent model version, the population abundance stabilised and gave zero growth rate. For this model version, the most relevant results are the stable size class distribution and the demographic rates resulting from the simulations of the Mjøsa and Control populations.

5.1. Population abundance – stable size distribution

Simulations run with the density-dependent models end up with stable abundances in all size classes in both the control and exposed populations (Figure 10). Abundances in the juvenile size classes are lower in the exposed population than in control, whereas there are little differences between the adult size classes in the exposed population.

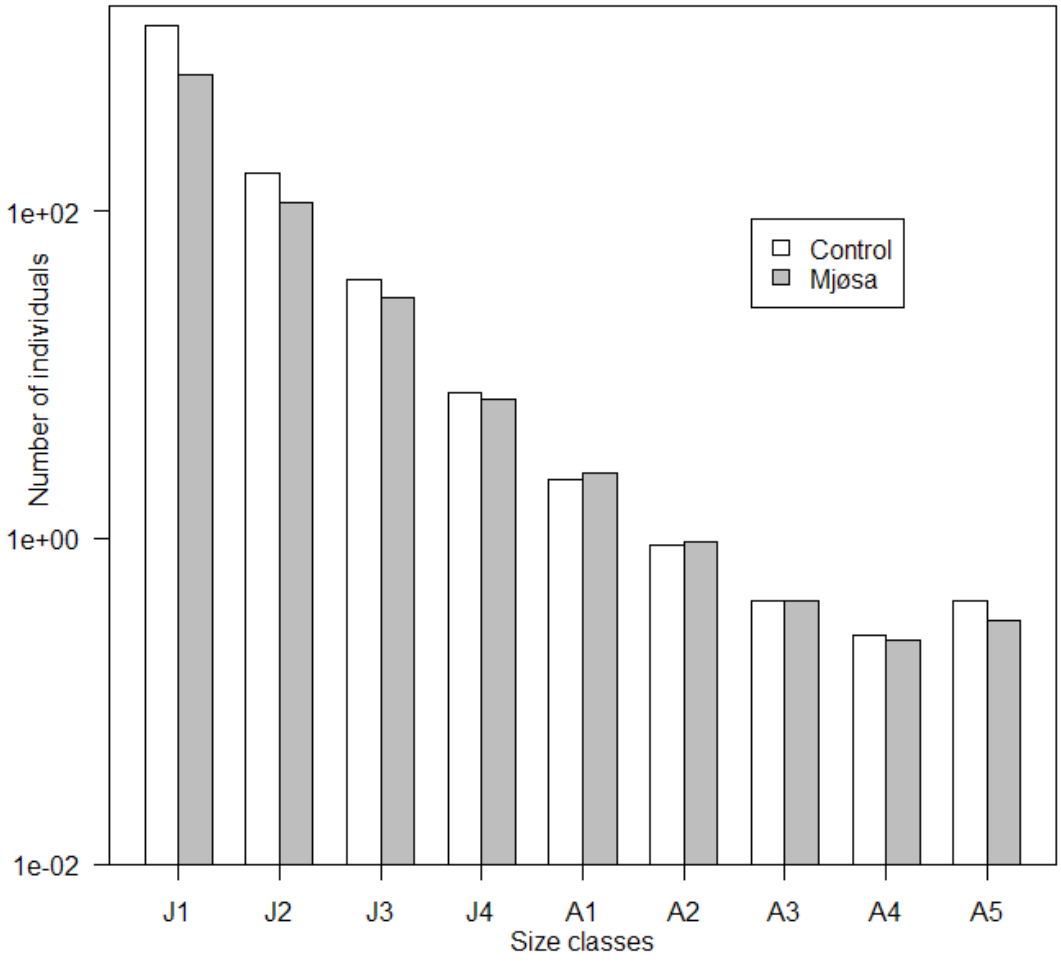

Figure 10: Stable abundances in the different size classes in simulated populations of control and toxicant exposed populations of zebrafish. Note log-scale on the y-axis.

5.2. Demographic rates calculated from the simulations

For the density-dependent model, the entries of the stable projection matrix (Table 13 and Table 14) are the long-term stable demographic rates as estimated from the simulations run with this model. These density-dependent demographic rates are based on simulations with a low-density population (25 individuals).

Table 13: Stable projection matrix for Control population with density-dependent growth based on maximum growth of 25 individuals

	J1	J2	J3	J4	A1	A2	A3	A4	A5
J1	0	0	0	0	262	328	381	442	477
J2	0.04	0.68	0	0	0	0	0	0	0
J3	0	0.07	0.68	0	0	0	0	0	0
J4	0	0	0.07	0.64	0	0	0	0	0
A1	0	0	0	0.11	0.63	0	0	0	0
A2	0	0	0	0	0.17	0.56	0	0	0
A3	0	0	0	0	0	0.24	0.48	0	0
A4	0	0	0	0	0	0	0.32	0.48	0
A5	0	0	0	0	0	0	0	0.32	0.80

Table 14: Stable projection matrix for the Mjøsa population with density-dependent growth based on maximum growth of 25 individuals

	J1	J2	J3	J4	A1	A2	A3	A4	A5
J1	0	0	0	0	0	309	359	417	449
J2	0.06	0.66	0	0	0	0	0	0	0
J3	0	0.09	0.66	0	0	0	0	0	0
J4	0	0	0.09	0.62	0	0	0	0	0
A1	0	0	0	0.13	0.65	0	0	0	0
A2	0	0	0	0	0.15	0.60	0	0	0
A3	0	0	0	0	0	0.20	0.54	0	0
A4	0	0	0	0	0	0	0.26	0.54	0
A5	0	0	0	0	0	0	0	0.26	0.80

5.2.1. Survival

Survival probabilities from J1 to J2 are highlighted in yellow. In the density-independent model, the Mjøsa population has lower survival probability from J1 to J2 than in control (cf. experimental results reported by Stavik (2007) and Almås (2007). In the density-dependent model, however, the survival probability from J1 to J2 in the exposed population is higher than in the control population. This means that there is overcompensating density dependence: toxicant-induced reduction in abundance/biomass is more than compensated by relief from density stress in the larval stage.

5.2.2. Growth

As is seen by comparing the subdiagonals in Table 13 and Table 14, juveniles in the exposed population have higher transition probabilities (i. e. grow faster) than juveniles in the control population. The opposite is the case for the adults, adults in the exposed population have lower transition probabilities than adults in the control population.

5.2.3. Fecundity

Fecundities are highlighted in green in Table 13 and Table 14. In the density-dependent model, as in the density-independent model, the fecundities are lower in the Mjøsa population than in the Control population. However, the reduction in fecundities in the Mjøsa population compared to Control is smaller in the density-dependent model than in the density-independent model. This means that there is some density-dependent compensation (relief from density stress) also in reproduction, although less than for larval survival.

6. Discussion

In the following, I will first discuss the results from the laboratory experiment. In Section 6.2, I will discuss the integration of these direct and indirect effects by the population model, and show how the use of a population model has given population-level results that could not have been deduced directly from the experimental results.

6.1. Individual-level effects (from experiments)

6.1.1. Survival

Survival in the period (0-8 dpf) was reduced in Mjøsa-larvae (i. e. larvae from Mjøsa-exposed parents). This is the period where the larvae have not yet started feeding, but depend on the resources provided by their mother in form of the yolk sac for growth and maintenance (Spence et al. 2008). It is known that both PBDEs (Lema et al. 2007), PCBs (Guiney et al. 1979) and trace metals (Bang et al. 2008) in the ovaries of fish are transferred from the female via the yolk to the offspring. Such maternal transfer of contaminants makes the early life stages susceptible to deleterious effects of toxic elements contained in the ovaries (Bang et al. 2008). The observation that larval survival is reduced by parental exposure suggests that parental exposure via the yolk sac is important to consider in life-time exposure studies. For many compounds, in particular POPs, which by definition can persist in the environment for a long time, the more realistic exposure regime is probably one where a population is exposed for several generations. For instance, although the use of DDTs was largely banned in Norway from 1970, and PCBs from 1980, these compounds were still found in considerable amounts in the burbot used in the present experiment.

Surprisingly, the total survival in the period 0-28 dpf was not affected by exposure. In both the previous experiments in the ZEBPOP project, exposure to the Mjøsa mixture was found to significantly reduce survival in this period (Almås 2007; Stavik 2007). The lack of an effect in the present experiment can be explained by the fact that because of a mistake made in the laboratory, there were fewer larvae in the Mjøsa containers than in the Control containers when the experiment was started (130 vs. 160). This was not discovered until later. Although dead larva and excess feed were removed, and 50 % of the water was changed every day, it is possible that the Mjøsa larvae had better conditions as they were fewer in each tank. It is thus possible that unintentionally, we tested the effect of density stress in the Control larvae. Since the rearing conditions were different for the two exposure groups, this result was not used in the population modeling.

We did not observe any effect of exposure on adult survival. As mentioned in the introduction, pollutants such as PCBs and PBDEs, which are two of the main types of compounds in terms of mass in the Mjøsa mixture, have low acute toxicity (Guiney et al. 1979; Petrovic et al. 2001). At the relatively low concentrations used in the present experiment, the observation that adult survival was not affected by exposure is reasonable.

There was no effect of density on adult survival for the range of densities tested in this experiment. Because of limitations in the laboratory, as described in section 2.1.2., we did not test effects of density on the larvae (age 0-28 dpf). In indeterminate growing organisms, early juvenile stages have been shown to have relatively low energy densities and high rates of energy store depletion, causing them to have the lowest capacity to survive food deprivation (Schultz & Conover 1997). It is thus possible that the observed negative effects of density on growth in older juveniles and adults also would have been manifested in the larval stage as lower survival, if they had been reared in different densities. Density-dependent survival is common in early life stages of fish, and thought to be important in population regulation (Shepherd & Cushing 1980; Vandenbos et al. 2006).

6.1.2. Growth

Exposure had a weak, but significant, negative effect on length in females. In males there was a tendency for exposed fish to have slightly lower body mass than controls, although these differences were not statistically significant.

In general, an organism that is exposed to toxicants may be expected to have reduced growth because of the extra cost of metabolizing the toxicants (Bayne et al. 1979; Lawrence & Elliot 2003). Moreover, as growth in fishes is regulated by complex interactions of several hormones, including gonadal sex steroids (Lawrence et al. 2008), it is possible that pollutants with endocrine disrupting properties can affect growth directly, by interfering with normal endocrine function.

Another possible explanation for reduced growth is reduced appetite (Wootton, 1998). Feeding inhibition has been observed in juvenile rainbow trout (*Salmo gairdneri*) chronically exposed to TCDD (2,3,7,8-tetradibenzo-*p*-dioxin) (Mehrle et al. 1988). Certain PCB-congeners have properties similar to TCDD. However, judging from the amounts of feed that were left after the fish had finished feeding, there did not seem to be any difference in consumption between exposed and control fish, although this was not recorded systematically.

It is important to note that assumptions regarding size or length-weight relationships as indications of fish condition might not always hold in the case of exposure to contaminants

with endocrine disrupting properties, as abnormal growth and tumor formation are possible consequences of exposure to this type of chemicals (Lawrence et al. 2003).

Population density had a significant negative effect on growth. This was as expected, as teleost growth is known to be very flexible, and conspecific individuals of the same age may show big differences in size (Wootton 1998), depending on feeding history (Miglavs & Jobling 1989) and level of social interaction or stress (Smith et al. 1978; Vøllestad & Quinn 2003).

In the wild, as opposed to in confined laboratory 'habitats', individuals experiencing increased competition for resources may opt to disperse instead of competing for resources, or they may simply die from starvation (Einum et al. 2006). However, density-dependent growth has been reported from field studies of numerous fish species, for instance silver hake (*Merluccius bilinearis*) (Helser & Almeida 1997) , brown trout (*Salmo trutta*) (Jenkins et al. 1999; Lobón-Cerviá 2005) and salmon (*Salmo salar*) (Imre et al. 2005).

Even the individual-level response to toxicant stress may depend on population density (Arthur & Dixon 1994; Forbes et al. 2001). With respect to growth, we did not detect any statistically significant interaction between population density and toxicant exposure. It seems thus that over the range of population densities tested, growth become neither more nor less affected by toxicant exposure with increasing density.

Size in teleost fish is an important indicator of survival probability and fecundity (Wootton 1998). Survival probability is typically positively correlated with size, in particular in the larval and juvenile stages (Sogard 1997; Vandenbos et al. 2006; Wootton 1998). This size-dependent mortality is thought to operate through enhanced resistance to starvation, decreased vulnerability to predators, and better tolerance of environmental extremes (Sogard 1997). In temperate species such as brown trout (*Salmo trutta*) and Eurasian perch (*Perca fluviatilis*), overwinter survival probability in juveniles has been found to increase with size (body mass), as these species may lose mass during winter (Huss et al. 2008). In female fish, batch fecundity is usually strongly correlated with size (Wootton 1998).

Thus, an effect on growth, caused by any factor endogenous or exogenous to the population, may be translated into an effect on survival probability and/or fecundity. Through this relationship, density-dependent growth can be a potentially important regulatory mechanism in fish populations, and this is found to be the case for several species (Bardos et al. 2006; Lorenzen & Enberg 2002). If exposure reduces growth, this may in turn reduce density stress.

6.1.3. Reproduction

There are many ways in which toxicant exposure can affect fecundity in fish, for instance through the effect on growth, batch fecundity for a given size of the female, age/size at maturity trajectory and spawning frequency. In the present experiment, effects of exposure on fecundity were measured on three of these endpoints: age at sexual maturity, the relationship between female size and batch fecundity and the proportion of spawning females. Because breeding trials required a special set-up, it was not possible to obtain data on 'natural' spawning frequency. Due to termination of the experiment, the relationship between female size and batch fecundity, and the proportion of spawning females were not tested on fish from the F2-generation, and the results reported on these endpoints are from tests on females from the previous experiment (the F1-generation).

The observations of age at sexual maturity were made per tank, not per individual, and are therefore not as precise as desired. This must be taken into account when the validity of the results is assessed. Therefore, it was not possible to estimate any interaction between exposure and density with regards to onset of maturity.

In the low-density groups, spawning was observed later in the exposed fish than in the controls. In the medium population density groups there was no difference between the exposed and control fish in time to observed spawning. As mentioned under section 3.2, exposure reduced growth. It is possible that the delayed age at sexual maturity in the Mjøsa low-density group compared to the control is a consequence of the reduced growth in the exposed fish. However, as for growth, it is possible that the observed effect of exposure on age at sexual maturity is a direct consequence of endocrine disruption. Time to maturation may be very important for population growth rate or population abundance (Stearns 1993). An effect of exposure on time to maturation may thus have a large impact on the population.

Higher population density delayed the time to first observed spawning, i. e. spawning was observed later in the medium density group than in the low density group. By the time the experiment was terminated, there had still not been observed any spawning in the high density group.

Age and /or size at maturity in fish are generally not fixed, but may show plastic responses to environmental changes such as for instance population density (Wootton 1998). Zebrafish have been reported to show great variation in age at sexual maturity. Ten weeks has been

reported for a laboratory strain, whereas populations in the wild may take as much as ten months to reach sexual maturity (Spence et al. 2008). Thus, for zebrafish, size is probably a better indicator of degree of maturation than age. This has been reported for several other fish species (Wootton 1998). Density-dependent age at first maturity has been observed for instance in a Grand Banks population of American plaice (*Hippoglossoides platessoides*) females. An increase in growth rate and a decline in age at first maturity occurred when a period of heavy fishing reduced the abundance of the plaice. The length at maturity did not change significantly. The increased growth rate was probably a result of less competition for food/more food for the individuals that were left (Wootton 1998).

The proportion of females that laid eggs seemed not to be affected by exposure. However, as explained in 2.6.4, it was not feasible to statistically test whether the proportions of females in each exposure group that spawned over the course of the breeding trials were significantly different from each other.

In a study on fathead minnows (*Pimephales promelas*) (Muirhead et al. 2006), where adult fish were exposed to PBDE-47 over a 25-day period, it was found that exposure adversely affected spawning to the extent that the fish stopped reproducing after the first two weeks of exposure. PBDE-47 is one of the main compounds in terms of mass found in the Mjøsa mixture, however the amounts of PBDE-47 accumulated in the zebrafish tissue in the present study was considerably lower than the concentrations used in the study by Muirhead et al. (~6 µg/g lipid weight vs. ~50 µg/g whole-body tissue).

Batch fecundity is usually strongly correlated with length and weight of the female in teleosts (Wootton 1998). There are thus three ways in which exposure may affect batch fecundity in a female; by affecting the size-fecundity relationship, by affecting size only, or both. The relationship between female length and batch fecundity was not different between CC and MM treatments. As length was negatively affected by exposure, it is likely that exposed fish generally are less fecund because they are smaller. As there was considerable unexplained variation in the data, controlling for other factors such as female weight might have revealed effects of exposure on fecundity.

6.2. Population-level effects (from modelling)

The purpose of the modelling was to explore the effects of toxicant exposure on the population level by integrating the effects on the various demographic rates and incorporating density dependence, and to compare predictions from the density-dependent model with predictions from a corresponding density-independent model. The precise predictions of the models, e.g. in terms of abundance, are therefore not of interest in themselves.
The survival of the fish was affected by toxicant exposure in the larval stage, as reported by Almås (2007) and Stavik (2007). Furthermore, exposed fish grew more slowly and reached sexual maturity later than control fish. In addition, as reported by Almås (2007), the gender ratio was skewed towards males in the exposed populations. When density dependence is not taken into account, each of these factors will lead to a reduction in population growth rate in an exposed compared to an unexposed population. Such a reduction is seen in the simulations with the density-independent models.

The importance of density dependence over exogenous factors such as climate in governing the dynamics of a population has been much debated (Begon et al. 1990; Turchin 1999). Either way, a population model without density-dependent regulation will in the long run either increase exponentially or go extinct, unless the model parameters are fine-tuned to give zero growth rate. A density-dependent model is therefore more likely to represent a natural population. In the models presented here, density dependence was incorporated in both larval survival, juvenile and adult growth, and reproduction.

By relieving density stress, the negative effects of toxicant exposure observed in my experiments may result in an indirectly, positive effect on the population level.

In the simulations, when the populations had reached stability, the total reproduction (contribution to the larval pool) in the exposed population was lower than in the control population. This gave less competition among larvae in the simulated exposed population. Depending on the degree of compensatory mortality in the larval stage, less competition among larvae in an exposed population can result in higher larval survival compared to an unexposed population. The (density-independent) individual-level effect of exposure observed in the laboratory was a 17 % reduction of larval survival. With the chosen parametrisation of the density-dependent model, the resulting larval survival in the exposed population was actually 35 % higher than in the corresponding control population. This non-intuitive modelling result – indirectly positive effect of a toxicant on a demographic rate – is similar to

an actual observation in an experimental study of blowfly (*Lucilia sericata*) populations exposed to cadmium. In the blowfly study, where the larval stage was separated into three instars, survival of the third instar was higher in exposed populations (Moe et al. 2002). The reason was that reduced fecundity in the exposed adults and lower survival in the first-instar larvae gave less density stress in the third instar.

In the laboratory zebrafish populations there is also a possibility for a density-dependent compensation mechanism within the larval stage; higher initial mortality of toxicant-exposed larvae may result in reduced competition among toxicant-exposed larvae, and better survival of the older larvae. Such a mechanism, however, is not captured with the chosen time-step in the model.

As mentioned in section 6.1.2, the exposed fish in the present experiment had reduced size compared to controls. Individuals that are chronically exposed to toxicants may be expected to have reduced growth rate throughout their life due to the extra cost of metabolising the toxicant. However, when the transition probability from one size class to the next (i. e. somatic growth) is assumed to be density-dependent, it is seen in the model simulations that the exposed juveniles grow faster (have higher transition probabilities) than the unexposed juveniles. This means that the modelled density dependence resulted in an indirectly positive effect of the toxicant (overcompensation) for the juvenile growth rate.

In the simulated populations, the reductions in size-specific fecundities for exposed vs. unexposed populations were smaller with the density-dependent model (Table 13 and Table 14) than with the density-independent model (Table 11 and Table 12). Thus, the model has simulated a density-dependent undercompensation of the toxicant-induced reduction in fecundities. In the model, the reduction in fecundities was caused by a skewed sex ratio, as reported from the F1 experiment (Almås 2007). Other authors have even reported of overcompensation in fecundity rates (Salice & Miller 2003). In their experiment the freshwater gastropod *Biomphalaria glabrata* was exposed to cadmium. At low levels of exposure, reproduction was higher in exposed individuals compared to controls. A possible explanation suggested by the authors is that mortality caused by exposure alleviated some stress by allowing the surviving snails greater access to resources.

6.3. Conclusions

In summary, the experimental results have shown that toxicant exposure may affect survival and fecundity indirectly through reduced growth. Such indirect, growth-mediated effects may not be detected by traditional toxicity tests, but may nevertheless have important consequences for the population-level responses to the toxicant. Moreover, toxicant-induced reduced growth at the individual level may also give relieve population density stress, which may in turn ameliorate the effects of exposure on the population level.

The modelling of toxicant-exposed and unexposed populations has demonstrated that population-level effects of exposure may differ considerably from the individual-level effects. Firstly, toxicant-induced reduction of demographic rates (larval survival, growth, and fecundity) may be ameliorated by relief from density stress. Secondly, the toxicant-induced reductions may in some cases (larval survival and growth) be more than compensated by density-dependent mechanisms. The effects of both toxicant and density on somatic growth rate were key elements in these mechanisms.

When results from toxicological experiments are used e.g. for ecological risk assessment, population modelling can be a very useful tool. One important reason is that it is possible to include density dependence, and thereby simulate indirect toxicant effects that would otherwise not have been possible to detect. Moreover, by using a size-structured population, it is possible to simulate the indirect effects of exposure on demographic rates via its effects on growth. It is also possible to model interactions between effects of toxicants, density stress and other external factors on growth. Size-selective mortality caused by population-extrinsic factors such as predation or climate often cannot be observed in an experiment, but it can be taken into account in a population model.

In this study, the contribution of each of the changes in vital rates to the estimated reduction in population growth rate is not known. Such contributions can be assessed by sensitivity analysis of the model. It is then possible to compare which toxicant impacts on demographic rates have the strongest impact on the population growth rate. This will be addressed in future work with the population models for toxicant-exposed zebrafish.

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Appendices

Appendix1: Preparation and assessment of toxicants

Livers from Burbots (*Lota lota*) weighing 1-2 kg, caught in Lake Mjøsa in August 2004, were transported on ice to the laboratory and stored at -20°C until processing at the Environmental Toxicology Laboratory at the Norwegian School of Veterinary Science. The laboratory is accredited for analysing the components reported here, according to the requirements of NS-EN ISO/IEC 17025:2000. Certified international reference materials (CRM 349 and 350, ICES cod liver oil and mackerel oil) are analysed regularly, with results within the given ranges. Spiked samples and the laboratory's own seal blubber reference sample are included in each series of analysis. POPs were extracted according to the method described by Brevik (1978), in addition to the use of ultraturrax® after adding the solvents for optimising the extraction. Liver tissues were homogenized before adding of solvents, ultrasound extraction and centrifugation. Aliquots of the samples were used for lipid determination and clean-up with sulphuric acid (SA), and extracts from the burbot livers were analyzed for POPs (Table 3). BDEs and HBCD were determined by GC-MS according to a method described by (Murvoll et al. 2005). PCBs, HCHs, HCB, chlordanes and DDTs were determined by GC-ECD. The detection limit for BDEs was 2 ng/ml, and for HBCD 3 ng/ml. For PCBs it was from 0,4 to 4 ng/ml and for HCHs, chlordanes HCB and DDTs it ranged between 0,5 and 3 ng/g. The same method was later on used to analyse the artemia and zebrafish after exposure.

Preparation of the Mjøsa mixture

To prepare the Mjøsa stock solution, livers were sliced with a scalpel and repeatedly frozen and thawed to room temperature, to separate the oil from the liver tissue, before centrifugation at 3 000 rpm for 10 min. 10 g of the clear oil was poured into a flask and diluted by adding 50 ml of cyclohexane (CHX) and 200 ml of concentrated sulphuric acid (SA) before shaking repeatedly together with freezing and thawing, separating the CHX from the acid and generating a organic phase containing the CHX and lipids. The acid phase was removed and the volume reduced under N_2, and new acid added. This was repeated until the volume was reduced to 1 ml. In each step the CHX/SA batch was given at least two days for the SA to separate from the organic phase. The CHX was replaced with acetone by adding 10 ml of acetone and reducing the volume under N_2 to 1 ml. This was repeated four times. The Mjøsa mixture was analyzed for POPs (table A1) with detection limits similar to the burbot analysis.

Measured toxins in burbot livers and the extracted mixture

The measured concentrations of POPs in burbot liver extracts from Lake Mjøsa (n = 20) and in the stock solution are given in Table A-15. In the Mjøsa mixture the dominating pollutants were BDEs and HBCD, but also considerable amounts of PCBs and DDTs were found in burbot liver and the resulting stock solution.

Table A-15: Contents of compounds in liver from Mjøsa burbot (Mjøsa Burbot) and in the Mjøsa mixture (Mjøsa stock). Unit: ng/g lipid weight.

	Mjøsa Burbot	Mjøsa Stock
Number (n)	20	
Lipid (% mean)	39.3-42.7	
Component		
HCB	41	43.8
HCHs		
α – HCH	5	3.4
β – HCH		12
γ – HCH	3.7	2.8
Chlordanes		
Oxy-chlordane	30	8.8
Cis-chlordane	28	23
Trans-nonachlor	91	75
DDTs		
pp-DDE	2207	1520
pp-DDD	161	113
pp-DDT	912	904
PCBs		
PCB-28	25	25
PCB-52	70	40
PCB-47	25	16
PCB-74	48	18
PCB-66	73	55
PCB-101	228	166
PCB-99	178	131
PCB-110	289	269
PCB-151	106	75
PCB-149	72	85

	Mjøsa Burbot	Mjøsa Stock
Number (n)	20	
Lipid (% mean)	39.3-42.7	
Component		
PCB-118	341	232
PCB-153	1270	918
PCB-105	164	85
PCB-141	78	52
PCB-137	39	27
PCB-138	932	608
PCB-187	90	58
PCB-183	131	83
PCB-128	129	144
PCB-156	95	84
PCB-180	562	334
PCB-170	198	120
PCB-189	20	13
PCB-194	60	30
PCB-206	17	19
PBDEs		
BDE-28	206	78
BDE-47	14917	15800
BDE-100	4572	4300
BDE-99	8890	8020
BDE-154	1479	1210
BDE-153	1351	1040
HBCD	3379	6060

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Appendix 2

Feeding protocol

9 am:

Preparation of artemia for feeding the next day:

- 2 1 L Erlenmeyer flasks were labeled "Mjøsa" and "Control" and each filled with 1 L conditioned system water
- 40 g Instant Ocean (Marineland, USA) and the amount of artemia cysts (Special Diets Services, Nittedal, Norway) required at the time were added to each flask
- 500 µl of either solvent (acetone) or toxicant solution were added to the corresponding flask using a automatic pipette (Eppendorf, Sydney, Australia)
- The flasks were placed in a plastic container filled with water to a level of approximately 10 cm, heated to 30 °C by a heating column
- Separate plastic tubes connected to air pumps (EHEIM, Deizisau, Germany) were placed in each of the flasks to provide proper aeration
- "Padding balls" (polyester) were put in the bottlenecks to keep the plastic tubes in place and prevent evaporation

Preparing the newly hatched nauplii for feeding

- the contents of the 1 L erlenmeyer flasks was poured through a sieve /sieved to remove the salt water
- The nauplii were transferred to labeled 250 ml erlenmeyer flasks that were filled completely with system water
- Empty shells/chorions float to the top and were removed (see figure 11.)
- Unhatched cysts collect at the bottom. To remove these, the hatched artemia were poured into the sieve, the erlenmeyer flask was rinsed, the hatched artemia was poured back into the flask, and system water was added to give a total volume of 150 ml
- The fish were fed using an automatic pipette to make sure that all the tanks received an equal volume of feed.
- While pipetting the feed, the Erlenmeyer flask was swirled in order to distribute the artemia evenly in the water.
- The feed was distributed in the fish tanks in a way that gave all the individuals in a tank equal access to the food.

- After feeding, ca. 7 g Instant Ocean was added to each 250 ml Erlenmeyer flask, system water was added to give a total volume of 200 ml and the plastic tubes providing air bubbles were put back in

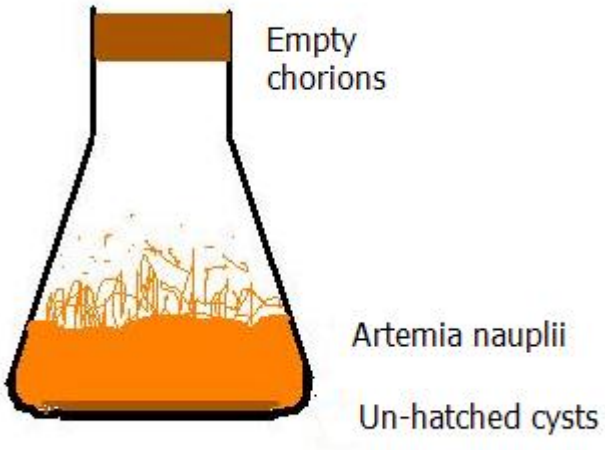

Figure 11: 250 ml erlenmeyer flask containing artemia nauplii

11 a. m., 1 p. m. and 3 p. m.:

- Before feeding, the artemia was sieved to remove the saltwater, and system water was added to give a total volume of 150 ml.
- After feeding at 11 a. m. and 1 p. m., ca. 7 g Instant Ocean was added to each 250 ml Erlenmeyer flask, system water was added to give a total volume of 200 ml and the plastic tubes providing air bubbles were put back in