

Exposure of whitefish (*Coregonus lavaretus*) eggs to native or chemically spiked sediments from Lake Thun does not lead to abnormal gonad development

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ABSTRACT

A high number of whitefish, *Coregonus lavaretus*, from Lake Thun, Switzerland, display gonad malformations. We tested the hypothesis that exposure to sediment-borne contaminants during the embryonic life results in the development of malformed gonads later in ontogeny. The investigated contaminants were 2,4,6-trinitrotoluene (TNT), which may leak from residues in the lake sediments as consequence of former ammunition dumping into Lake Thun, as well as sulfonated naphthalene formaldehyde condensates (SNFC), which are introduced into the lake from wastewater disposals of a nearby tunnel construction site. Experimentally, whitefish eggs were exposed during 52 days from fertilization until hatching to a) an artificial sediment (control), b) an artificial sediment spiked with TNT (0.5mg·kg⁻¹ dry weight), c) SNFC compounds dissolved in water (3μg·l⁻¹ of each naphthalene-1-sulphonate,

naphthalene-2-sulphonate, naphthalene-1,5-disulphonate, naphthalene-2,7-disulphonate), and d) sediment from Lake Thun sampled in an ammunition dumping area. To mimic *in situ* exposure of the eggs to the sediment-water-interface under laboratory conditions, we developed a novel contact incubation technique. After hatching, fish were reared in tap water for three years until they reached sexual maturity, and were then examined for the presence of gonad malformations. No malformations were observed in the control, in the TNT and SNFC treatment groups. In fish incubated during the embryonic stage on Lake Thun sediment, 2 out of 117 adult males (1.7%) displayed malformed gonads, which is significantly lower than levels of gonad malformations in wild whitefish from Lake Thun (on average 29% in males, 12% in females). The results from our experiment provide no evidence that sediment contamination with TNT or SNFC compounds is a causative factor for the induction of gonad malformations in Lake Thun whitefish.

INTRODUCTION

Lake Thun is a prealpine, oligotrophic lake of 47.7km² with a volume of 6.4km³, a hydraulic residence time of 684 days, and a mean and maximal depth of 136m and 217m, respectively. The catchment area is 2451km² with a mean altitude of 1748 metres above sea level (a.s.l.) and a maximum level of 4272 metres a.s.l., respectively.

Whitefish (*Coregonus lavaretus*) is the main fish species of Lake Thun. This species is exploited by commercial fishermen, with an annual catch between 20 to 50 tons. Four coregonid morphs given the local names 'Albock', 'Balchen', 'Brienznig' and 'Kropfer' are living sympatrically in Lake Thun (Douglas et al. 1999; Fatio 1890; Steinmann 1950; Wagler 1937). While Balchen and Kropfer are rare, Albock and Brienznig are abundant and amount for most of the yield

of the commercial fishermen. With respect to spawning, the morphs are distinguishable on the basis of their spatial and temporal preferences (Kirchhofer and Tschumi 1986; Ruffli 1978; Steinmann 1950). Brienznig and Kropfer start to spawn in August and prefer spawning sites at 40-200m depth. The spawning season of Albock and Balchen begins in December. Balchen prefer littoral shallow waters for spawning whereas Albock spawn on steep slopes reaching down to 30m depth.

In the year 2000, commercial fishermen observed a large number of coregonid fish from Lake Thun displaying morphologically altered gonads. Bernet et al. (2004) described in detail the morphological deviations which included:

- (1) adhesion or fusion of the gonads, or parts thereof, to the peritoneal wall and the musculature,
- (2) compartmentation of the gonads into several separated lobes, connected with each other by the sperm duct (males)

- and the ovarian envelope (females), a condition which is also termed "segmented gonads" (Hecker et al. 2006),
- (3) atrophy or aplasia, when one or both gonad strands are undeveloped (atrophy) or totally absent (aplasia),
 - (4) simultaneous hermaphroditism and intersex condition, where testicular and ovarian tissue occur in the same individual,
 - (5) asymmetry of the left and right gonad strands in respect to their volume, whereby only differences >50% between left and right were counted, and
 - (6) constriction of the gonads when they are segmented into several lobes, but – in contrast to compartmentations - testicular (in males) or ovarian tissue (in females) is still present in the intersection parts and the lobes are not locally separated from each other.

Macroscopic and histological illustrations of these morphological gonad alterations are published by Bernet et al. (2004, 2008).

Epidemiological investigations on the presence of gonad malformations in whitefish from 3 Bernese lakes in the years 2004-2005 indicated that from the aforementioned malformation types only fusions to the lateral musculature, intersex gonads, compartmentations (or segmentations) of the gonad strands, and atrophy/aplasia of gonad strands showed an enhanced frequency in Lake Thun's whitefish compared to gonad development in whitefish from the other 2 lakes. Constrictions and asymmetries, however, were found in comparable frequencies in coregonids from all three lakes (Bittner et al. 2009). Based on this, Bittner et al. (2009) suggest that only fusions, compartmentations, intersex gonads and atrophy/aplasia represent malformations *sensu stricto*, while the other morphological alterations, constrictions and asymmetries, represent baseline variations of normal gonad morphology. Based on this definition, whitefish from Lake Thun show significantly higher prevalences of gonad malformations than whitefish from the two other lakes with the following frequencies: 29.1% (6%-67%; 16 samplings with a total (N) of 903 males) of coregonid males and 11.8% (0%-43%; N=574) females from Lake Thun showed malformed gonads, compared to 7% (0%-18%; 9 samplings; N=430) in males and 0.7% (0%-2%; N=574) in females from Lake Brienz and 4.5% (0%-57%; 8 samplings; N=356) in males and 3.2% (0%-55%; N=184) in females from Lake Biel (Bittner et al. 2009).

The phenomenon of increased prevalences of gonad malformations in coregonids from Lake Thun raised the question about the causes. One etiological factor that might be involved in the induction of the gonad malformations are chemical contaminants. This is of particular relevance as Lake Thun serves as reservoir of drinking water for nearly half a million people. One possible source of chemical contamination of Lake Thun are explosives from ammunition deposits in the lake sediments. From the 1920s until the 1960s, the Swiss Army and a nearby ammunition manufactory dumped approximately

4,600 tons of ammunition into the lake. One of the main ammunition constituents 2,4,6 trinitrotoluene (TNT) and its major transformation products (aminodinitrotoluenes (2- or 4-ADNT), diaminonitrotoluenes (2,4- or 2,6-DANT), trinitrobenzene (TNB)) can have toxic effects on a variety of aquatic indicator species (see review by Talmage et al. 1999). Of particular concern is that TNT as well as some other explosive constituents were reported to have weak endocrine activities and thus may have the potential to interfere with the gonad development in fish (European Commission 2000).

A second potential source of chemical contamination of Lake Thun is wastewater from the tunnel works in the nearby Lötschberg mountain range. The mining construction work, executed from 1994 to 2006, led to the release of wastewater into the river Kander, which is the main tributary to Lake Thun. Chemicals applied for the tunnel works include a wide range of substances such as explosives (mainly polyisobutenes), concrete plasticisers (mainly sulphonated naphthalene formaldehyde condensates) and flocking agents for the wastewater treatment, mainly acryl amides (Cantonal Office for Water and Soil Protection, unpublished). Although the wastewater was treated before release into the river Kander, and although it was diluted during the 25km passage from the effluent downstream to Lake Thun, environmental fate models indicate that substances used in high tonnages could have reached effective concentrations in the lake (Brem and Gälli 2005). One group of chemicals used in high quantities (6,000 tons) for the tunnel construction were sulfonated naphthalene formaldehyde condensates (SNFC). A worst case scenario model predicted effective concentrations of SNFC compounds in the river Kander of $26.2\mu\text{g}\cdot\text{l}^{-1}$, and of $3\mu\text{g}\cdot\text{l}^{-1}$ in Lake Thun.

The sediments of Lake Thun can be a relevant source of chemical exposure of whitefish, as the sediments contain the dumped ammunition, and as they act as a sink for lipophilic chemicals. The life stage of whitefish that is in direct contact with sediments is the embryonated egg stage, a particularly vulnerable life stage. While spawning and fertilization of whitefish takes place in the littoral and pelagic zones, the fertilized eggs sink down to the sediments. Embryonic development on the sediments can last for about 3 months. After hatch, the larvae leave the sediment zone and swim up to the water surface.

In the present study, we tested the hypothesis whether exposure of whitefish eggs to Lake Thun-relevant contaminants during the embryonic development leads to altered morphological gonad development later in their life. To simulate sediment exposure, we developed a methodology to incubate whitefish eggs in contact with sediments under laboratory conditions. We tested three exposure conditions, i.e. (i) native sediments collected in Lake Thun, (ii) artificial sediments spiked with TNT (as a representative substance for the ammunition dump) and (iii) SNFC compounds dissolved in water (as a representative substance for the tunnel works). The control group was exposed to an artificial sediment.

MATERIAL AND METHODS

Exposure system

Eggs from whitefish are usually incubated in Zug jars. Zug jars are bottle shaped glass bodies without lid and bottom, standing upside-down. Water is constantly flowing through in bottom-up direction. The whitefish eggs placed in the Zug jars are gently moved by the water flow.

The Zug jar incubation method was unsuitable for our aim of sediment exposure of whitefish eggs. This was due to two reasons. First, the Zug jar installation does not allow to establish a sediment-egg interaction. To simulate the situation at the bottom of the Lake, the fertilised whitefish eggs have to be in direct contact with sediments, and this contact must be maintained over the entire embryonic development until hatch. Second, the strong water exchange as used with the flow-through Zug jar incubation technique would dilute the contaminant concentrations in the sediment of our exposure system over time.

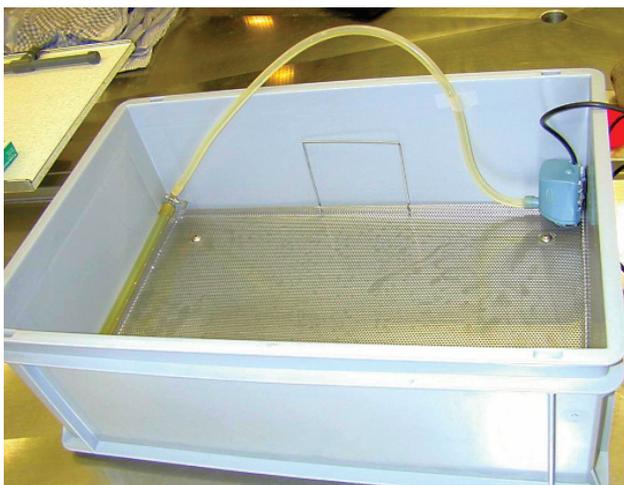


Figure 1. Plastic box for egg-sediment incubation, with water pump and perforated chrome-plated steel sheet tray. The whitefish eggs were seeded onto the tray.

In order to simulate sediment exposure of whitefish eggs in the lake, we developed a new incubation system for the fertilised eggs based on a re-circulation technique for the water body. This system consisted of plastic boxes of 60·40·22cm in size containing a 4-5cm high layer of sediment (Figure 1). On top of the sediment, a chrome-plated steel sheet was placed. The steel sheet was perforated with holes of 2mm in diameter and had a pulled up rim of 1cm at every side. Fertilised whitefish eggs were seeded onto this steel sheet. The perforations of 2mm were slightly smaller than the size of a whitefish egg (2.2-2.5mm in diameter). Thus, the eggs settled in the holes of the perforated steel sheet (Figure 2). The advantages of using such a steel sheet are that (i) the eggs are in direct contact with the sediment, (ii) water exchange around

the eggs is possible in a way that anoxic conditions as they could occur in sediment incubations are avoided, and (iii) the handling of the eggs and/or the boxes during the incubation period is feasible, e.g. for treatments, cleaning and water exchanging measures. A small water pump with an adjustable water flow was set in every box. The water outflow of the pump was directed by a soft polyvinylchlorid tube of 1.5cm to a plastic pipe of 40cm length and 1.5cm in diameter, with multiple lined up perforations of 2mm in diameter. The plastic pipe was placed opposite the water pump. This generated a gentle water circulation all over the whole steel sheet area, and supplied the eggs with oxygenated water. Every box received trickle flow aeration by an air stone connected with an externally fixed air pump. We took care to ensure that water conditions in the experimental treatments were above anoxic levels. This simulated the oxygen conditions in Lake Thun, where on an average $8.1\text{mg}\cdot\text{l}^{-1}$ O_2 (68% oxygen saturation), min. $4.0\text{mg}\cdot\text{l}^{-1}$ O_2 (33.7%) and max. $10.9\text{mg}\cdot\text{l}^{-1}$ O_2 (91.1%) were measured over ground at depth of 210-213m over a 10-year monitoring period from 2000 to 2010 (unpublished data provided by the Bernese Cantonal Office for Water and Soil Protection).



Figure 2. Whitefish eggs in the eyed stage on the perforated chrome-plated steel sheet tray. The sediment layer is situated directly under the steel sheet. Thus the whitefish eggs are exposed in the sediment-water-interface layer.

The sizes of the boxes were suitable for experiments with 10-15kg sediment and 20-25l overlying water body, and to incubate up to 10,000 whitefish eggs.

Experimental setup

An experimental setup with four different treatment groups was designed:

- Control group: Eggs incubated on artificial sediment (for description see below). The aim was to test if or at what frequency whitefish develop malformed gonads independent of a contamination of the sediments. The treatment was run in duplicates.

- Lake Thun sediment group: Eggs were incubated on sediment sampled in Lake Thun at its deepest site. This is a site where ammunition has been dumped. The aim was to test if and at what frequency whitefish eggs exposed to this sediment develop malformed gonads in their adult life stages. The treatment was run in duplicates.
- Sediment spiked with 2,4,6 trinitrotoluene (TNT): Whitefish eggs were incubated on artificial sediment (see below) spiked with 0.5mg TNT·kg⁻¹ dry weight (wt). The aim was to test if and at what frequency whitefish eggs develop gonad malformations when exposed to TNT contaminated sediment during their egg stage. The treatment was run as a single group.
- Sulfonated naphthalene formaldehyde condensates (SNFC): Whitefish eggs were incubated in water spiked with different SNFC components. The experiment was run without sediment due to the fact that SNFC components show a prominent surfactant-like activity. The setup without a sediment matrix was chosen because we wanted to minimize the risk in a 3 year lasting experiment that the SNFC components adhere to an unpredictable extent to the sediment particles and thus would not have been available for the exposed whitefish eggs. The aim was to reveal if and at what level embryo exposure to the SNFC components has the potential to lead to gonad malformation in whitefish at the adult stage. The treatment was run as a single group.

Sediment preparation

Lake Thun sediment

On 5 December 2005, sediment from the top 50cm of Lake Thun at its deepest location (200-217 m; Coordinates SwissGrid: 623'500/169'500; WGS84: 1°21'49"/50°36'23") was sampled using a piston coring system described in detail by Kelts et al. (1986). The location chosen corresponds to one of the spots where ammunition was dumped. The overlying water was removed from the sediment fraction. The sediment was frozen at -20°C before further use. Prior to the experiment, the frozen lake sediment was thawed, and 6.5kg of wet sediment was filled in the box. The box was added with 15l tap water.

Artificial sediment

For the control groups and the treatment group spiked with TNT, we used an artificially composed sediment, prepared with the following components: 30% quartz sand (100-355µm), 20% quartz powder (Milisil W4), 34% dolomite powder, 0.5% lime powder (all components purchased from carlo Bernasconi AG, Bärschwil, Switzerland), 13.5% siliceous earth (Hänseler AG, Herisau, Switzerland), 2% alpha cellulose (Sigma-Aldrich GmbH, Schnelldorf, Germany). Each incubation box of the control group and the TNT spiked sediment group was filled with 8kg of this powder mixture. The different sediment fractions were homogeneously mixed and a continuous gentle water current was established during 48 hours. Thereafter the sediment was allowed to completely settle. Subsequently the

overlying water was removed. This resulted in sediment of 10.5kg wet weight.

The sediment was spiked with a nominal concentration of 0.5mg TNT·kg⁻¹ dry weight. To this end, a stock solution of 3mg 2,4,6 TNT per ml ethanol 100% was prepared. The TNT was kindly provided by armasuisse, Thun, Switzerland. 1.3ml of this stock solution were diluted with 8.7ml ethanol 100%. This solution was added to the wet artificial sediment and mechanically mixed. The spiked sediment was left undisturbed for 2h at room temperature to let evaporate the ethanol. On days 24, 34, and 43, five litres of water in the incubation box were renewed with tap water, and TNT concentration was refreshed with 0.7ml of the stock solution.

The box of the SNFC group was filled with 20l tap water. 120µg naphthalene-1-sulphonate (1-NS; purity: 50%), 85.7µg naphthalene-2-sulphonate (2-NS; purity: 70%), 70.6µg naphthalene-1,5-disulphonate (1,5-NdS; purity: 85%), and naphthalene-2,7-disulphonate (2,7NdS; purity: 100%) were mixed into the water. This amounted to nominal concentrations of 3µg·l⁻¹ for each SNFC component. All chemicals were commercially available from Sigma-Aldrich, Buchs, Switzerland, and ABRC GmbH, Karlsruhe, Germany. Five litres of the incubation water were renewed on days 24, 34, and 43 with tap water, and half of the dosage of the four SNFC chemicals was added to refresh the SNFC concentrations.

The overlying water in the incubation boxes of the TNT group was chemically analysed for TNT and its degradation product aminodinitrotoluene (ADNT) at days 24 and 31. Water of the SNFC spiked group was chemically analysed at termination of the sediment incubation at day 52.

Chemical water analyses

Water samples were analyzed for TNT and ADNT with high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (HPLC Agilent 1100 Series, Agilent Technologies, Palo Alto, CA; HTS PAL autosampler, CTC Analytics, Zwingen, Switzerland; API 4000 triple quadrupole mass spectrometer, Applied Biosystems, Foster City, CA). Direct coupling of an online solid-phase extraction (SPE) cartridge (two stacked cartridge pre-columns, 4·3mm, 5µm, same stationary phase as used for separation) to the LC-MS/MS was accomplished using a column switching technique.

Sample volumes of 1ml were injected into a loop. Samples were then transferred from the loop to the extraction cartridge using an auxiliary HPLC pump (Jasco PU980, Jasco®, Gross-Umstadt, Germany) and purified water as mobile phase at a flow rate of 1ml·min⁻¹ during 90s. After valve switching, the enriched compounds were eluted directly to the analytical column with the mobile phase used for analysis. The compounds were separated on a Synergi Fusion RP-80 (150·2 mm, 4µm, Phenomenex) HPLC column, fitted with a cartridge pre-column (4·2mm) filled with the same stationary phase. Elution was achieved using a mobile phase gradient starting from 100% water to 30% water and 70% methanol within 10min, followed by an isocratic hold of 5min.

The mass spectrometer was equipped with a turbo ion spray source and operated in negative mode (ion spray voltage, 4.5kV, 450°C) using multiple reaction monitoring (MRM) with the following ion transitions: TNT, m/z 226→196 with a collision energy of 18eV (and for confirmatory purposes, m/z 226→46, 45eV); ADNT, 196→119, 24eV (196→46, 41eV). Using these conditions, detection limits of 15 and 10ng·l⁻¹ were achieved for TNT and ADNT, respectively.

The analysis of 1-NS and 2-NS was based on sample enrichment and instrumental analysis by LC/MS (Varian 210 pumps/Varian 1200 MS). The volume of the aqueous sample was reduced from 20ml to approximately 0.5ml in a rotary evaporator. To remove precipitated minerals, the samples were filtrated over glass wool. The filters were washed (2ml) and the combined filtrate and wash solution were reduced to 0.55ml. LC was performed on a column from Macherery-Nagel (NUCLEOSIL 100-5 C18 Nautilus, 125mm·3mm i.D.; 5µm particle size) using a methanol/water (each containing 0.005% acetate buffer) flow gradient (0% MeOH/0.25ml·min⁻¹ at 0min; 0% MeOH/0.25ml·min⁻¹ at 10min; 35% MeOH/0.38ml·min⁻¹ at 12min; 60% MeOH/0.38ml·min⁻¹ at 25min; 60% MeOH/0.38ml·min⁻¹ at 27min; 0% MeOH/0.25ml·min⁻¹ at 29min; 0% MeOH/0.25ml·min⁻¹ at 45min). Mass spectrometry was performed by ionization in the negative electrospray mode (280°C drying gas, 6000V capillary voltage) and mass separation in the single ion mode (m/z 207, unit mass resolution). Quantification was done by external calibration. The limit of detection was 0.3µg·l⁻¹ for 1-NS and 2-NS (the limit of detection for 1,5-NdS and 2,7-NdS was much higher (250µg·l⁻¹) and prevented quantification of 1,5-NdS and 2,7-NdS in the exposure samples).

Fish stripping and egg fertilisation

Mature whitefish of the morph 'Albock' were caught in Lake Thun on mid-December 2005 on their spawning grounds by bottom gill nets. Fish were killed by a blow on their head. Eggs from females and milt from males were artificially stripped from every fish in separate Petri dishes. Donor fish were dissected and their gonads morphologically assessed. Only those eggs and semen in the Petri dishes that originated from donor fish without any of the Lake Thun typical gonad malformations (Bernet et al. 2004) were used for artificial fertilisation. Eggs from one female were fertilised with milt from one male. Fertilisation was induced by adding a few ml of lake water to a Petri dish. The Petri dishes were gently moved for few seconds. Few minutes later, the fertilised eggs were collected in a separate container, filled up with approximately 1l of lake water and left undisturbed for at least 30 minutes to allow for egg hardening. Subsequently, fertilised eggs were transferred temporarily for 2-4 days into a vertical-flow tray incubator, supplied with tap water of 8°C. During that time, any non-fertilized eggs were removed. For the subsequent experiment in the sediment incubator boxes, only viable eggs were used.

Egg incubation

After sediment preparation, the incubation boxes were placed into a dark climate chamber at 4°C. 9,000 fertilised whitefish eggs were transferred from the vertical-flow tray incubator into each incubator box. Temperature, dissolved oxygen, alkalinity, ammonia and nitrite concentration were monitored throughout the exposure period using commercially available kits (Aquamerck, Germany). The eggs were periodically treated against fungal infestations by transferring the steel sheets with the eggs into an anti-fungal treatment solution of Pyceze (Novartis, England; Dosage: 1ml·10l⁻¹). Dead eggs were removed every day or every other day.

Fish rearing

After 52 days or 340 day-degrees (sum of the daily mean water temperature), whitefish embryos started to hatch. Hatchlings were transferred initially to 150l flow-through aquarium tanks supplied with tap water, and later to 250l tanks. Hatchlings from the replicate incubation boxes were pooled. Fish were fed dry feed. Initially they were given Aglonorse Nr. 1 (0.6mm grain size) (EWOS AS, Bergen, Norway), and later Silvercup 501 (0.6mm) and 503 (1.2mm) (Hokovit, Bützberg, Switzerland). Water temperature throughout the year ranged between 7°C and 20°C. The rearing period lasted until fish were 1015-1028 days old and reached their first spawning season.

Fish sampling

Fish were sampled for examination of their gonad morphology in December 2008 at an age of three years. After euthanization with an overdose of Fiquel (Argent Laboratories, Redmont, USA), fish were measured and weighed, dissected and macroscopically assessed for the presence of the gonad malformations: (i) fusions to the peritoneal wall, (ii) compartmentations, (iii) aplasia/atrophy and (iv) macroscopic intersex conditions (see Bernet et al. 2004). These morphological alterations are considered to represent malformations of gonad morphology in whitefish (Bittner et al. 2009).

RESULTS AND DISCUSSION

We applied a new technique for incubating coregonid eggs on horizontal trays with recirculation of the incubation water. The incubation system provided water parameters supporting normal development of the fish eggs: water temperature 5.2-5.6°C, dissolved oxygen 10.2-12.0mg·l⁻¹, oxygen saturation 95-101%, pH 7.3-7.7, nitrite (NO₂) 0.00-0.05mg·l⁻¹, ammonia (NH₃) 0.001-0.002mg·l⁻¹.

Compared to the conventional Zug jar incubation technique which usually results in more than 90% of the incubated eggs to hatch, the hatching success in the sediment incubation boxes was estimated to be about 50%. This reduced hatching success was to be expected,

because a re-circulation of the water without using UV-light or ozonising water treatments favours fungal infections of the eggs with *Saprolegnia* sp. Thus, it was essential to remove daily or at least every second day *Saprolegnia*-infected eggs. This makes the incubation technique time-and labour-intensive. Removal of infected eggs may be not sufficient to control fungal infection in the sediment incubation boxes, but additionally periodic antifungal baths as well as periodical water changes may be needed. In the current experiment, exchanging 25% of the water every 3-4 weeks controlled the incidence of fungal infection.

The advantage of the new incubation method is that it permits developmental exposure of whitefish eggs to the sediment-water interface. This methodology was a prerequisite for our study aim, i.e. to expose coregonid eggs to sediments, either control sediments or sediments spiked with chemicals, to evaluate possible effects on gonad development of whitefish.

Chemical analyses

According to chemical analyses, TNT concentrations present in the water body of the TNT treatment group were substantially lower than the nominal concentration used for spiking the sediment. Measured concentrations were $0.1\mu\text{g}\cdot\text{l}^{-1}$ for 2,4,6 TNT on day 24, and $0.15\mu\text{g}\cdot\text{l}^{-1}$ on day 31, in comparison to the nominal dosing concentration of $500\mu\text{g}\cdot\text{kg}^{-1}$ dry wt. The TNT transformation product ADNT was detected at concentrations of $3.6\mu\text{g}\cdot\text{l}^{-1}$ and $5.0\mu\text{g}\cdot\text{l}^{-1}$ at days 24 and 31, respectively. We assume that the bulk of the sediment-spiked TNT remained adsorbed to the sediment due to the strong interactions of TNT with organic matter and also with clay minerals (Brannon and Pennington 2002). Furthermore, TNT associated with soils and sediments undergoes transformation through reduction of the nitro groups on the ring, leading to the formation of aminodinitrotoluenes (2- or 4-ADNT), and subsequently diaminodinitrotoluenes (2,4- or 2,6-DANT) (Elovitz and Weber 1999). The adsorption and metabolic transformation are likely to be responsible for the low effective TNT concentrations in the water body of the incubation boxes, as detected analytically. Also in other sediment exposure studies with fish, rapid metabolic degradation of TNT, both outside and inside the fish, has been observed (Lotufo et al. 2010; Yoo et al. 2006). While uptake of TNT into the fish was rapid, bioconcentration remained low due to significant endogenous metabolism (Lotufo et al. 2010; Yoo et al. 2006). These observations are in agreement with the transcriptomics findings of Eisentraeger et al. (2007) who found that many genes involved in detoxification pathways were upregulated in TNT-treated HepG2 cells. The main target organs of TNT and metabolites in the fish appear to be liver and viscera (Lotufo 2011); with respect to fish gonads, no information is available.

In contrast to the findings with TNT, in the SNFC exposure experiment there was a good agreement between nominal and measured chemical concentrations. The levels of $3.2\mu\text{g}\cdot\text{l}^{-1}$ 1-NS and $3.7\mu\text{g}\cdot\text{l}^{-1}$ 2-NS in the water body corresponded well to the nominal spiking concentrations ($3\mu\text{g}\cdot\text{l}^{-1}$ each).

Gonad morphology

In the control group (eggs incubated on non-spiked artificial sediment), none of the fish that have been exposed to the sediment during the embryonic stage displayed Lake Thun-typical gonad malformations once they had developed to the adult stage. In the treatment group being exposed to sediment from Lake Thun during embryogenesis, 2 out of 200 adult fish displayed malformed gonads. These two fishes were males (2 out of 117 males), corresponding to 1.7 % malformed male fish (Figure 3). One of the two malformed males showed a normal left testicular strand, while the right one was missing (aplasia). The second male displayed compartmented testicular lobes. None of the females of Lake Thun sediment group (n=83) showed malformed ovaries. The frequency of gonad malformations as observed in the Lake Thun sediment exposure group is by far below the frequencies of malformed gonads in wild whitefish sampled on spawning sites in Lake Thun, which is on average 29.1% in males, and 11.8% in females (Bittner et al. 2009).

Whitefish exposed during embryogenesis to either TNT-spiked sediment or SNFC-dosed water showed no Lake Thun-typical gonad malformations at all when they had reached the mature stage. This was the case both for male and female whitefish (Figure 3). Also from the published literature, there is no clear indication that these chemicals would affect gonad phenotypic development. While to our knowledge, no information is available on toxic mechanisms or processes induced by SNFC, sparse information exists with respect to the processes potentially involved in TNT toxicity. In a study aiming to identify biomarkers of TNT exposure in rainbow trout, Ek et al. (2005) found that particularly enzymes and metabolites of the antioxidant defence system, such as glutathione reductase or the concentration of oxidised glutathione, were responsive. This is confirmed by the microarray study of Eisentraeger et al. (2007) on TNT-exposed HepG2 cells, where an upregulation of antioxidant genes were reported as well. Eisentraeger et al. (2007) had another interesting observation, that genes of extracellular matrix components were responsive to TNT exposure, because expression of extracellular matrix genes was also altered in Lake Thun whitefish displaying malformed gonads (Bittner et al. 2011).

Another line of evidence that may point to an influence of TNT on gonad structure comes from reports on endocrine-disrupting activities of TNT. For instance, mRNA expression of steroidogenic regulatory protein (StAR), which is a key enzyme in the synthesis of (sex) steroids, was reduced in TNT-exposed American bullfrog (Paden et al. 2010). Also in eels (*Anguilla anguilla*), StAR expression was decreased under TNT exposure, and this was accompanied by TNT effects on P450 aromatase and the oestrogen receptors – thus pointing to a possible endocrine-disrupting activity of TNT (Della Torre et al. 2008). The fact, that chemically induced disruption of the endogenous hormone period during early stages of fish

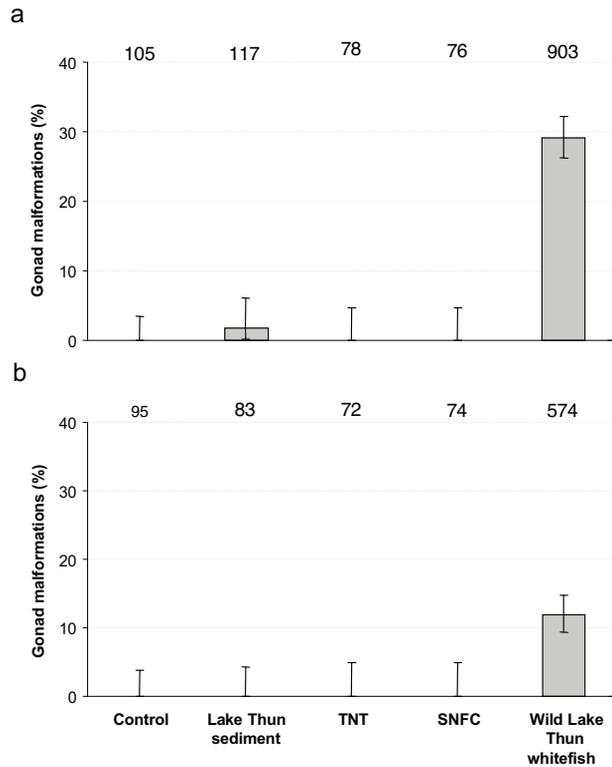


Figure 3. Frequencies of gonad malformations in males (a) and females (b) of the different treatment groups: Control = Eggs incubated on artificial sediment mixed with commercially available compounds; Lake Thun sediment = Eggs incubated on Lake Thun sediment sampled at a site, where ammunition has been dumped; TNT = Eggs incubated on artificial sediment spiked with 2,4,6 TNT at concentrations of $0.5 \text{ mg}\cdot\text{kg}^{-1}$ dry wt; SNFC = Eggs incubated in water in which $3 \mu\text{g}\cdot\text{l}^{-1}$ of each naphthalene-1-sulphonate, naphthalene-2-sulphonate, naphthalene-1,5-disulphonate and naphthalene-2,7-disulphonate have been mixed in. For comparison reasons, frequencies of wild whitefish from Lake Thun with malformed gonads are presented. These fish have been sampled during a spawning site monitoring during 2004 and 2005 (Bittner et al. 2009). Fish were classified as having malformed gonads if one of the following malformation types were present: Fusions to the peritoneal wall, compartmentations, aplasia/atrophy, hermaphroditism. No macroscopic intersex was observed. The error bars represent the 95% confidence intervals (CI95) of a binomial distribution of the frequencies.

development can have severe and lasting effects on their sexual development, is well established (Devlin and Nagahama 2002; Segner 2011). However, in order to affect gonad development of whitefish, it would be not sufficient that TNT disrupts the hormone system. Also the timing of exposure would be decisive. We know from research on salmonids that the timing of the critical period, when the developing gonad is sensitive to hormonal influences, can vary strongly even among closely related species. The results from our study suggest that, regardless if TNT had

an endocrine disrupting effect on the whitefish embryos, this did not lead to a lasting effect on sexual differentiation or gonad morphology.

Taken together, the results of this experiment provide no evidence that early life exposure to the tested chemicals or sediments lead to the formation of gonad malformations in adult whitefish. This finding is of relevance as it has been hypothesized that explosives from the ammunition dumping in Lake Thun, or SNFC compounds from the Loetschberg tunnel works could be responsible for the gonad malformations. However, we would like to emphasize that our experiments do not definitely exclude sediment contamination to be responsible for gonad malformations in Lake Thun whitefish. In our experiment, we tested only a few selected compounds while both the ammunition and the wastewater from the Loetschberg tunnel works contain a myriad of other chemicals. Also the possibility of chemical mixture effects and the effects of metabolites arising from the parent chemicals must not be overlooked. Similarly, although the Lake Thun sediment used in our experiment was sampled from an ammunition dumping area, this does not necessarily mean that this sample is representative for the sediment contamination by ammunition-derived chemicals.

The exposure period in the present experiment was restricted to the egg period. During this stage developing embryos from wild whitefish are in direct contact to sediments and sediment-borne contaminants. The underlying assumption is that the embryo exposure would result in a delayed effect on gonad morphology, i.e. that the embryo period is the sensitive phase during which environmental stressors can induce a persisting effect on fish development that becomes manifest only later in their ontogeny. In fact, the literature provides several examples of such delayed effects of early life exposure of fish. For instance, Papoulias et al. (2003) showed that treatment of Japanese medaka (*Oryzias latipes*) with the DDT metabolite o,p'-DDE during the egg stage had consequences for gonadosomatic index and gonad histology of adult, 107-day-old fish. Ottinger and Kaatari (2000) have shown that exposure of rainbow trout embryos to aflatoxin leads to immune dysfunction in later life stages. Similarly, Milston et al. (2003) observed that short-term early life exposure of Chinook salmon (*Oncorhynchus tshawytscha*) to o,p'-DDE caused humoral immunosuppression in the juvenile stage. Tiedeken and Ramsdell (2007) demonstrated that *in ovo* exposure of zebrafish (*Danio rerio*) to domoic acid had consequences on the toxicant susceptibility of the larval stage. Thus, it appears that early life exposure of fish indeed can result in late effects, although there exist no data whether this applies to whitefish and the stressors tested in the present experiment.

The experimental setup of this study must be seen as part of a series of experiments to elucidate the cause(s) of the malformed gonads in whitefish from Lake Thun. Whereas the present study tried to evaluate the role of chemical exposure during the embryonic stage, the other

studies set the focus to factors that are present during gonad development and differentiation in the period from hatching until sexual maturation. Bernet et al. (in preparation) reared hatchlings from different lakes (Lake Thun and Biel) over three years in different water sources (Lake Thun water, Lake Lucerne water and spring water as control) and fed them with zooplankton from Lake Thun or commercial dry feed. The study aimed at revealing the roles of the water, the food or the genetic constitution of the fish in the development of the gonad malformations. The results provide evidence that zooplankton from Lake Thun is the key factor in the development of gonad malformations. Malformed gonads were significantly more frequent in fish fed with zooplankton than in fish fed on dry food. Importantly, the frequency of gonad malformations in the zooplankton-fed fish was comparable to the frequency of gonad malformations in wild whitefish living in Lake Thun. The finding that the zooplankton from Lake Thun appears to contain a malformation-inducing factor corresponds with the finding from the present study which indicates that experimental treatments before the onset of feeding remain without effect on the development of gonad malformations.

CONCLUSION

We established a novel technology for the incubation of coregonid eggs, which enables to simulate under laboratory conditions the exposure of whitefish eggs to the sediment-water-interface, as it normally takes place in the lake. To this end, the whitefish eggs were placed on a steel sheet tray placed on top of the sediment. Dilution of spiked contaminants to the sediments was avoided by means of recirculation of the water body. This technique was applied to expose coregonid eggs to sediment from Lake Thun, to TNT-spiked sediment, and to SNFC-dosed water.

The exposure of whitefish eggs to sediment from Lake Thun, sampled at sites where ammunition was dumped, did not lead to gonad malformation in adult mature fish in the third year of life. Also exposure of whitefish eggs to TNT-spiked artificial sediment or to SNFC-containing water failed to induce the Lake Thun-typical gonad malformations in the whitefish. Thus, under the specific conditions of our experiment, we could not reveal an adverse effect of the embryo stage treatments on gonad morphology in adult fish.

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REFERENCES

- Bernet, D., T. Wahli, C. Kueng, H. Segner. 2004. Frequent and unexplained gonadal abnormalities in whitefish (central Alpine *Coregonus* sp.) from an Alpine oligotrophic lake in Switzerland. *Diseases of Aquatic Organisms* 61: 137-148.
- Bernet, D., A. Liedtke, D. Bittner, R.I.L. Eggen, S. Kipfer, C. Küng, C. Largiader, M. J.-F. Suter, T. Wahli, H. Segner. 2008. Gonadal malformations in whitefish from Lake Thun: defining the case and evaluating the role of EDCs. *Chimia* 62: 383-388.
- Bernet, D., T. Wahli, C. Küng, H.-R. Zieri, H. Segner. Zooplankton feeding induces gonad malformations in whitefish from Lake Thun, Switzerland. In preparation.
- Berset, J.D., U. Ochsenbein, M. Zeh. 2007. Are Lake Thun and Lake Brienz contaminated with explosive residues? *Chimia* 61: 532-532.
- Berset, J.D. 2007. Sprengstoffspuren in allen drei grossen Berner Seen. *GSA-Informationsbulletin*: 2-7.
- Bittner, D., D. Bernet, T. Wahli, H. Segner, C. Küng, C.R. Largiader. 2009. How normal is abnormal? - Discriminating between malformations and natural variation in gonad morphology of European whitefish *Coregonus lavaretus* (L.). *Journal of Fish Biology* 74: 1594-1614.
- Bittner, D., A.R. Cossins, H. Segner, L. Excoffier, C.R. Largiader. 2011. Identification of candidate genes and physiological pathways involved in gonad deformation of whitefish (*Coregonus lavaretus*) from Lake Thun, Switzerland. *International Journal of Environmental Research and Public Health*, submitted.
- Bogdal, C., M. Naef, P. Schmid, M. Kohler, M. Zennegg, D. Bernet, M. Scheringer, K. Hungerbühler. 2009. Unexplained gonad alterations in whitefish (*Coregonus* spp.) from Lake Thun, Switzerland: levels of persistent organic pollutants in different morphs. *Chemosphere* 74: 434-440.
- Brannon, J.M., J.C. Pennington. 2002. Environmental Fate and Transport Process Descriptors for Explosives, US Army Corps of Engineers Report ERDC/EL TR-02-01, Available at <http://el.erc.usace.army.mil/elpubs/pdf/trel02-10.pdf>
- Brem, D., R. Gälli. 2005. Beurteilung des Einsatzes von Bauchemikalien beim Bau der NEAT am Lötschberg – Stoffflussanalyse und grobe Abschätzung der ökologischen Risiken. Short report for the Amt für Gewässerschutz und Abfallwirtschaft des Kantons Bern, Abteilung Gewässer- & Bodenschuttlabor.
- Conder, J.M., T.W. La Point, J.A. Steevens, G.R. Lofuto. 2004. Recommendations for the assessment of TNT toxicity in sediment. *Environmental Toxicology and Chemistry* 23: 141-149.
- Douglas, M.R., P.C. Brunner, L. Bernatchez. 1999. Do assemblages of *Coregonus* (Teleostei: Salmoniformes) in the Central Alpine region of Europe represent species flocks? *Molecular Ecology* 8: 589-603.
- Della Torre, C., I. Corsi, S. Focardi, A. Arukwe. 2008. Effects of 2,4,6-trinitrotoluene (TNT) on neurosteroidogenesis in the European eel (*Anguilla anguilla*). *Chemistry and Ecology* 24: 1-7.
- Devlin R.H., Y. Nagahama. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 208: 191-364.

- Eisentraeger, A., G. Reifferscheid, F. Dardenne, R. Blust, A. Schofer. 2007. Hazard characterization and identification of a former ammunition site using microarrays, bioassays, and chemical analysis. *Environmental Toxicology & Chemistry* 26: 634-646.
- Ek, H., G. Dave, J. Sturve, B.C. Almroth, E. Stephensen, L. Förlin, G. Birgersson. 2005. Tentative biomarkers for 2,4,6-trinitrotoluene (TNT) in fish (*Oncorhynchus mykiss*). *Aquatic Toxicology* 72: 221-230.
- Elovitz, M.S., E.J. Weber. 1999. Sediment mediated reduction of 2,4,6-trinitrotoluene and fate of the resulting aromatic (poly)amines. *Environmental Science and Technology* 33: 2617-2625.
- Fatio, V. 1890. Faune des Vertébrées de la Suisse, Vol. V, 2e partie. Geneva: H. Georg, Libraire-éditeur.
- Kelts, K., U. Briegel, K. Ghilardi, K. Hsu. 1986. The limnogeology-ETH coring system. *Schweizerische Zeitschrift für Hydrologie* 48: 104-115.
- Kipfer, S., H. Segner, M. Wenger, T. Wahli, D. Bernet. 2009. Long term laboratory estrogen (E2) exposure of whitefish (central alpine *Coregonus lavaretus*) induces intersex but not the Lake Thun-typical gonad malformations. *Diseases of Aquatic Organisms* 84: 43-56.
- Kirchhofer, A., P. Tschumi. 1986. Age structure and growth of coregonid fish populations in Lake Thun. *Archives of Hydrobiology* 22: 303-318.
- Lotufo, G., W. Blackburn, S.J. Marlborough, J.W. Fleeger. 2010. Toxicity and bioaccumulation of TNT in marine fish in sediment exposures. *Ecotoxicology and Environmental Safety* 73: 1720-1727.
- Lotufo, G. 2011. Whole-body and body-part-specific bioconcentration of explosive compounds in sheephead minnows. *Ecotoxicology and Environmental Safety* 74: 301-306.
- Milston, R.H., M.S. Fitzpatrick, A.T. Vella, S. Clements, D. Gundersen, G. Feist, T.L. Crippen, J. Leong, C.B. Schreck. 2003. Short-term exposure of chinook salmon (*Oncorhynchus tshawytscha*) to o,p'-DDE or DMSO during early life history stages causes long-term humoral immunosuppression. *Environmental Health Perspectives* 111: 1601-1607.
- Ochsenbein, U., M. Zeh, J.D. Berset. 2008. Comparing solid phase extraction and direct injection for the analysis of ultra-trace levels of relevant explosives in lake water and tributaries using liquid chromatography-electrospray tandem mass spectrometry. *Chemosphere* 72: 974-980.
- Ottinger, C.A., S.L. Kaatari. 2000. Long-term immune dysfunction in rainbow trout (*Oncorhynchus mykiss*) exposed as embryos to aflatoxin B1. *Fish and Shellfish Immunology* 10: 101-106.
- Paden, N.E., J.A. Carr, R.J. Kendall, M. Wages, E.E. Smith. 2010. Expression of steroidogenic acute regulatory protein (StAR) in male American bullfrog (*Rana catesbeiana*) and preliminary evaluation of the response to TNT. *Chemosphere* 80: 41-45.
- Papoulias, D.M., S.A. Villalobos, J. Meadows, D.B. Noltie, J.P. Giesy, D.E. Tillitt. 2003. In ovo exposure to o,p'-DDE affects sexual development but not sexual differentiation in Japanese medaka (*Oryzias latipes*). *Environmental Health Perspectives* 111: 29-32.
- Rufli, H. 1978. Die heutigen sympatrischen Felchenpopulationen (*Coregonus* spp.) des Thuner- und des Bielersees und ihre Morphologie. *Schweizerische Zeitschrift für Hydrologie* 40: 7-31.
- Segner H. 2011. Reproductive and developmental toxicity in fishes. In: Gupta, R.C. (ed) *Reproductive and Developmental Toxicology*. Elsevier, Amsterdam, pp. 1145-1166.
- Steinmann, P. 1950. Monographie der schweizerischen Coregonen. *Schweizerische Zeitschrift für Hydrologie* 12: 340-491.
- Talmage, S.S., D.M. Opresko, C.J. Maxwell, C.J. Welsh, F.M. Cretella, P.H. Reno, F.B. Daniel. 1999. Nitroaromatic munition compounds: environmental effects and screening values. *Review of Environmental Contamination and Toxicology* 161: 1-156.
- Tiedeken, J.A., J.S. Ramsdell. 2007. Embryonic exposure to domoic acid increases the susceptibility of zebrafish larvae to the chemical convulsant pentylenetetrazole. *Environmental Health Perspectives* 115: 1547-1552.
- Wagler, E. 1937. Die Coregonen in den Seen des Voralpengebietes. IX. Die Systematik der Voralpen-Coregonen. *International Revue Hydrobiologie* 35: 345-446.
- Yoo, L.J., G.R. Lotufo, A.B. Gibson, J.A. Steevesn, J.G. Sims. 2006. Toxicity and bioaccumulation of 2,4,6-trinitrotoluene in fathead minnow (*Pimephales promelas*). *Environmental Toxicology & Chemistry* 25: 3252-3260.