Ecotoxicological characterization of the estrogenic mycotoxin zearalenone and environmental samples: Effects on zebrafish development and reproduction

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„Universitäten sind schöne Misthaufen, auf denen gelegentlich einmal eine edle Pflanze gedeiht.“

Albert Einstein (1879 - 1955)

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Summary

The aquatic environment is particularly susceptible to pollution, partly because there is considerable intentional release of chemicals into rivers, lakes and the sea (mainly through release of effluents), and partly because it receives a lot of accidental releases of chemicals (through spills, runoff, atmospheric deposition, etc.). Sewage treatment plants (STPs) release a complex mixture of natural and synthetic chemicals into the aquatic environment. It was estimated that 60'000 man-made chemicals are in routine use worldwide, most of them enter the aquatic environment and many of them via STPs.

Endocrine disruption occurs when specific chemicals interact with internal endocrine signalling pathways in organisms. In the aquatic environment, endocrine active substances are easily bioavailable to fish through aquatic respiration, osmoregulation, maternal transfer into eggs, dermal contact with contaminated sediments or ingestion of contaminated food. Endocrine disruption appears to be particularly widespread in freshwater fish throughout the world. Effects ranged from subtle changes in the physiology and sexual behaviour of fish to permanently altered sexual differentiation and growth, and reproductive impairment. Most attention has been directed towards identifying the main estrogenic chemicals, because many of the effects reported in wildlife appear to be a consequence of 'feminization' of males. Environmental estrogens can be anthropogenic, such as certain pharmaceuticals, pesticides and industrial chemicals or natural, such as phyto- and mycoestrogens.

Resorcyclic acid lactones (RALs) are the only known class of estrogenic mycotoxins and show estrogenic activity in the range of natural steroid estrogens. RALs occur as secondary metabolites of *Fusarium* spec. fungi growing on a variety of cereals. Zearalenone (ZON) belongs to the chemical group of RALs and is one of the worldwide most common mycotoxins. The occurrence of mycotoxins has been studied extensively in food, feed products, and domestic animals. Agricultural products around the world exhibited ZON contaminations of up to 69 % of the tested samples. However, only little is known about the ecotoxicological effects of ZON at concentrations found in the environment. Only a few short-term *in vivo* studies investigated ZON and its metabolites and observed effects on vitellogenin (VTG) induction, zona radiata proteins, the immune system, and growth of fish after exposure via injection or feeding.

In this research project, the ecotoxicological potential of ZON was evaluated. Effects on fish caused by an exposure to ZON were assessed using the zebrafish (*Danio rerio*) as a model organism. An embryo toxicity test was performed to investigate toxic effects of ZON on fish development. This study was complemented with a 30 day zebrafish early life-stage experiment to assess the influence of ZON on juvenile growth. In a second step the estrogenic potency of

ZON and its effects on adult fish were examined. A recombinant yeast estrogen screen (rYES) was used as an *in vitro* assay to determine effects on the activation of estrogen receptor-regulated genes. Zebrafish were exposed to ZON in a 42 day reproduction experiment enabling assessment of reproductive as well as physiological and morphological parameters. In a third step, the estrogenic potency of ZON was studied in a life-cycle experiment. This study was designed to investigate possible effects of continuous long-term exposure including a subsequent depuration period as well as possible transgenerational effects of F0 exposure on F1 generation. The offspring of the F0 generation that was exposed to ZON for 21 days was raised in a 140 day exposure experiment from embryo to adult. In a subsequent 42 day reproduction experiment, spawning groups of the F1 generation were either exposed to ZON (after growing up in clean water) or clean water (after growing up under ZON exposure) and effects on developmental, reproductive as well as physiological parameters were assessed.

Exposure to ZON had no effects on embryonic development up to 2000 ng/L, but a positive correlation between the body length and ZON concentration of up to 100 ng/L suggests a growth promoting effect for zebrafish exposed during 30 days. Although ZON revealed a moderate estrogenic potency *in vitro* (rYES), it exhibited a comparably strong effect on induction of VTG (1000 ng/L) and reproduction (100 ng/L) *in vivo* during 21 days exposure. It was further demonstrated that ZON influenced growth (1000 ng/L), sexual differentiation (320 ng/L), reproduction (1000 ng/L), and VTG levels (1000 ng/L) in zebrafish exposed from fertilization to day 140. Furthermore, ZON revealed a possible transgenerational effect on growth caused by short-term exposure of F0 and F1 generation each for 21 days to 1000 ng/L.

In the framework of this project, relevant data of the ecotoxicological relevance of ZON were collected. The estrogenic potential of ZON was confirmed *in vitro* and *in vivo* and detrimental effects on zebrafish reproduction were demonstrated. This indicates that ZON may have an effect on fish populations in the aquatic environment. Effects were observed at concentrations near the maximum measured values in surface waters (approximately double) and below the maximum values measured in effluents (approximately half). Even though to date there is not much data published presenting ZON concentrations in the aquatic environment and effects on different (native) fish species, this project demonstrated that ZON has the potential to pose a threat to fish in the aquatic environment.

In a second research project, the embryo toxicity and genotoxicity of biofilm and sediment from the catchment area of a STP was investigated. An embryo toxicity test with zebrafish and the comet assay (single cell gel electrophoresis) with primary cells isolated from the embryos were applied to investigate native biofilm for the first time. Biofilms were sampled from different

sections of the sewage system and sediments were taken from the rive Wyna directly at, upstream and downstream of the STP discharge Mittleres Wynental (Switzerland). The aim of this study was to verify the applicability of the aforementioned test system to investigate native biofilm and to evaluate the toxicity of the different sampling sites.

Clear differences in the embryo toxicity and genotoxicity of the biofilm from different sewage system sections as well as from the sediment sampling sites were found. Significant genotoxicity was determined in all biofilm and sediment samples. Temporal variability in toxicity were observed in some of the biofilm and sediment samples. Based on the results of this study it can be suggested that biofilm implemented in ecotoxicological bioassays such as the embryo toxicity test and comet assay with zebrafish can be a useful tool to assess (waste) water quality.

Chapter I

General Introduction

1 Ecotoxicological characterization of the estrogenic mycotoxin zearalenone

1.1 The phenomenon: Endocrine disruption

An endocrine disrupter has been defined as 'an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or subpopulations' (Vos et al. 2000). Endocrine disruption occurs when exogenous chemicals interact with internal endocrine signalling pathways in an organism (Cheek et al. 1998). Endocrine active substances (EASs) may affect the development or reproduction of organisms by interfering with normal synthesis, storage, release, transport, metabolism, binding, action or elimination of endogenous hormones (Kavlock & Ankley 1996).

In general, the reproductive physiology of vertebrates, both mammalian and non-mammalian, is similar, with the broad structure and function of the reproductive axis involving the hypothalamus, pituitary and gonads conserved. In all vertebrates, the release of a decapeptide gonadotrophin-releasing hormone (GnRH) from the hypothalamus stimulates the pituitary to secrete gonadotrophic hormones (GTH) that signal the gonads to synthesize steroid hormones. The basic biosynthetic pathways for steroid hormones and the active steroid hormones themselves are also well-conserved in both mammalian and non-mammalian vertebrates (Mills & Chichester 2005).

The aquatic environment is particularly susceptible to pollution, partly because there is considerable intentional release of chemicals into rivers, lakes and the sea (mainly through release of effluents from sewage treatment plants [STPs] and industries), and partly because it receives a lot of accidental releases of chemicals (through spills, runoff, atmospheric deposition, etc; Sumpter 2005). STPs (which often receive domestic, industrial and/or agricultural waste) release a complex mixture of natural and synthetic chemicals into the aquatic environment, following their partial or complete biodegradation during the treatment process. It is estimated that 60'000 man-made chemicals are in routine use worldwide and most of these enter the aquatic environment (Jobling & Tyler 2003).

Endocrine disruption appears to be particularly widespread in freshwater fish populations. There is little evidence, however, to suggest that fish are more susceptible to EASs relative to other

wildlife. There are many more similarities between the endocrine systems of fish and other higher vertebrates, with respect to the nature of the hormones, their receptors, and in the regulatory control of their endocrine system, suggesting that vertebrates are likely to be similarly sensitive to environmental EASs (Munkittrick et al. 1998). In the aquatic environment, EASs are easily bioavailable to fish through a variety of routes, including aquatic respiration, osmoregulation and maternal transfer of contaminants in lipid reserves of eggs (Van der Kraak et al. 2001). Dermal contact with contaminated sediments or ingestion of contaminated food (the major route of exposure to EASs in terrestrial animals) are additional exposure routes (Mills & Chichester 2005).

Endocrine disruption in wild freshwater fish populations has been reported in various parts of the world. Biological effects that have been attributed to the effects of endocrine disruptors include the inappropriate production of the blood protein vitellogenin (VTG; the female specific and estrogen-dependent egg yolk protein precursor) in male and juvenile fish, inhibited ovarian or testicular development, abnormal blood steroid concentrations, intersexuality and/or masculinization or feminization of the internal or external genitalia, impaired reproductive output, precocious male and/or female maturation, increased ovarian atresia, reduced spawning success, reduced hatching success and/or larval survival, altered growth and development and alterations in early development (Vos et al. 2000). Fish show high plasticity in phenotypic sexual differentiation and gonadogenesis can be a very complex and plastic process. Although sex determination is under genetic control, the final differentiation of the gonads in fish also depends on endocrine signals, i.e. estrogens and androgens (Arcand-Hoy & Benson 1998, Campbell & Hutchinson 1998, Devlin & Nagahama 2002). In most gonochoristic fish, the germ cells of the undifferentiated gonads are sexually bipotential (Kobayashi et al. 1991). During specific critical periods of development, changes in sex hormone levels can, therefore, affect the final sex independently of the genetic sex (Donaldson & Hunter 1982). Due to the lability of sex differentiation in fish, exposure to EASs during certain critical periods of development can lead to sex reversal (Andersen et al. 2003, Donaldson & Hunter 1982). These effects may arise due to disruption of a range of endocrine-mediated mechanisms (including receptor-mediated processes, and/or interference with steroid metabolism and/or excretion). Overall, current scientific evidence suggests that certain effects observed in freshwater fish can be attributed to cocktails of chemicals that mimic and/or disrupt hormone function/balance (Jobling & Tyler 2003).

Most attention has been directed to identify the main estrogenic chemicals, because many of the effects reported in wildlife appear to be a consequence of ‘feminization’ of males (Sumpter

2005). Environmental estrogens can be anthropogenic, such as certain pharmaceuticals, pesticides and industrial chemicals or natural, such as phyto- and mycoestrogens. The main causative chemicals found in the aquatic environment appear to be the natural steroid estrogens 17ß-estradiol (E2) and estrone (E1), and the synthetic steroid estrogen 17α-ethinylestradiol (EE2), all of which have been found in sewage effluent in low ng/L concentrations (Baronti et al. 2000, Desbrow et al. 1998, Snyder et al. 1999). The former two chemicals are natural hormones, and the last one is the active ingredient of the contraceptive pill. All are presumably excreted by people, and incompletely degraded in STPs, and hence are present in effluents (Munkittrick et al. 1998). Although these steroid estrogens are present at very low concentrations, such concentrations are biologically active (Gagnon et al. 1995, McMaster et al. 1991). It is likely that these estrogens act together, in an additive manner, to cause 'feminization' of fish (Van der Kraak et al. 1992). These estrogens bind to estrogen receptors (ERs) in exposed organisms with an affinity identical or similar to the endogenous estrogen hormone E2, and have the potential to exert effects at extremely low concentrations.

Man-made estrogen mimicking chemicals (xenoestrogens) are much weaker (less potent) estrogens, but sometimes they are present at much higher concentrations than the steroidal estrogens. In some specific locations, xenoestrogens appear to make a significant contribution to the overall estrogenic activity of effluents (Van der Kraak et al. 1998), and also appear to be possibly the major agent causing the adverse effects observed in fish living downstream of these effluents (Ankley et al. 1998). Biodegradation products of alkylpolyethoxylate detergents, such as nonylphenol and octylphenol, are also found in sewage effluent and wastewater from septic systems, but at high ng/L to low µg/L concentrations (Lye et al. 1999, Rudel et al. 1998). Some alkylphenols have been shown to bind to the estrogen receptor and stimulate a biological response similar to E2, although concentrations of at least 1000 fold more are required (Jobling & Sumpter 1993, Körner et al. 2000, White et al. 1994). Bisphenol A, which is used in epoxyresins, polycarbonate plastics and dental sealants (Sonnenschein & Soto 1998), exhibits estrogenic activity (Brotons et al. 1995, Metcalfe et al. 2001) and has been detected in sewage treatment effluent and septic system wastewater in low µg/L concentrations (Körner et al. 2000, Rudel et al. 1998). Some persistent environmental contaminants, such as polychlorinated biphenyls (PCBs) and pesticides (DDT and metabolites, chlordecone, methoxychlor) are also considered endocrine-disrupting, based largely on their ability to bind to estrogen receptors (Loomis & Thomas 1999, Nimrod & Benson 1997).

1.2 The mycotoxin zearalenone (ZON) and its environmental fate

Mycotoxins are naturally occurring secondary metabolites of fungi growing on a variety of cereals in the field or during storage. The resorcyclic acid lactones (RALs) are naturally occurring endocrine disrupting compounds and to date the only known class of estrogenic mycotoxins. RALs show estrogenic activity in the range of natural steroid estrogens. ZON belongs to the chemical group of RALs and is one of the worldwide most common mycotoxins (Fig. 1; Chelkowski 1998). The relative binding affinity (RBA) to the rainbow trout (rt) ER for ZON has a factor of 0.82 in relation to the natural steroid estrogen E2 (Matthews et al. 2000).

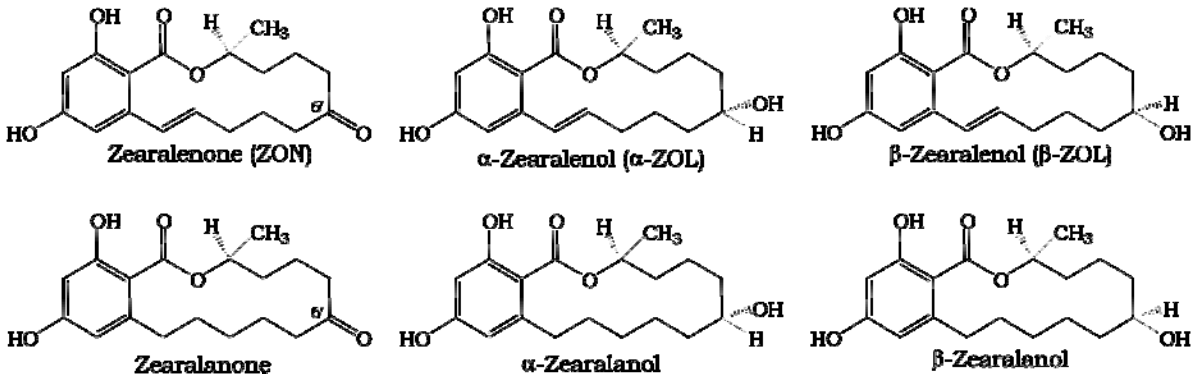

Fig. 1: Structural formulas of zearalenone and its metabolites

Several RALs occur as a result of *Fusarium* spec. infection of corn, wheat and other cereals. The most important mycoestrogen-producing fungus, *Fusarium graminearum*, is the world's major causal agent for red ear rot in corn or head scab in wheat, barley, rye, and oats. Airborne ascospores of *F. graminearum* infect the corn ear via silk channel and the other cereals while their heads are in flower. The fungus then appears as a pink to red mould on the kernels and, in severe infections, on other organs of wheat and corn such as the cob, husks and shank. The pathogen survives during the winter in plant residues such as wheat stubbles or corn stalks left on the field after harvest (Sutton 1982). The extent of *Fusarium* spec. infestation on crops and subsequent contamination with mycotoxins is dependent on factors such as crop rotation, soil cultivation, susceptibility of crop varieties and climatic conditions (Champeil et al. 2004). Climate change towards warmer and more moist conditions may provide even more fertile ground for fungi proliferation (Jenny et al. 2000).

The occurrence of mycotoxins has been studied extensively in food and feed products and domestic animals. Agricultural products around the world were contaminated by ZON up to

69 % of the tested samples with concentrations of up to 180 µg/kg (Rhyn & Zoller 2003). Due to its anabolic properties α-zearalanol (ZAL, Fig. 1), a ZON metabolite and the estrogenically most potent of the RALs, is licensed as growth promoter for cattle in USA and Canada (banned in EU) which increases the risk of exposure to RALs (Le Guevel & Pakdel 2001). It has been shown that RALs can cause severe reproductive and infertility problems in husbandry animals due to their high estrogenic potency (Gaumy et al. 2001, Kuiper-Goodman et al. 1987). In general the data point to a frequent and global occurrence of ZON in food and feed products.

ZON must be classified moderately water soluble (5.2 mg/L; Megharaj et al. 1997) and rather hydrophobic (K_{OW}: 3.7; Lemke et al. 1998). This indicates some environmental aqueous phase mobility and a certain potential for sorption and retention in soil systems (Koc: 3.9; Mortensen et al. 2006). Its stability during milling, food processing and heating (no significant loss in ZON during processing for 80 min at 100 °C) is considerable and its half-live in soil samples was reported to be 6.4 – 11.3 days (Mortensen et al. 2006, Ryu et al. 2003). Therefore it must be assumed that ZON is rather persistent in the environment. Data on biotic transformation of ZON indicated as main metabolic pathway the conversion to α- and β-zearalenol (ZOL; Fig. 1; El-Sharkawy & Abul-Hajj 1988, European Commission 2000, Kleinova et al. 2002, Mirocha et al. 1981). This is of relevance as the estrogenic activity of RALs decreases in the following order: α-ZOL (rtER-RBA: 2.67) > β-ZOL (rtER-RBA: 0.91) > ZON (Matthews et al. 2000).
On the basis of these physico-chemical properties, together with the findings from environmental studies, Bucheli et al. (2005) hypothesised that ZON and its metabolites are very likely emitted into the environment by several pathways: Toxins released from *Fusarium*-infested plants might contaminate the soil and infiltrate into groundwater, elute by surface runoff or subsurface drainage to surface waters or STPs, drift off on airborne fungi spores or soil particles; toxin residues in excrements of exposed livestock might enter agricultural soils and local waters via application of manure; food industry wastewater and human excretions might introduce toxin residues via sewage into surface waters.

Although the occurrence of ZON has been studied extensively in food and feed stuff, only little is known about its environmental distribution and impact (Rhyn and Zoller 2003, Pittet 1998). The occurrence of ZON was reported in Italian and Polish rivers in concentrations of 2 – 5 and 0.5 – 44 ng/L, respectively (Gromadzka et al. 2009, Lagana et al. 2004). It was further detected in US American (Kolpin et al. 2010) and Swiss rivers (Bucheli et al. 2008) below limits of quantitation. Up to 35 ng/L and 4 ng/g ZON was quantified in drainage water and soil, respectively, in a field study with wheat and maize (Hartmann et al. 2008a, 2008b). In Italian

waste water treatment plants and US American industrial wastewater effluents ZON concentrations of 1 – 10 and 95.5 – 220 ng/L, respectively, were measured (Lagana et al. 2001, 2004, Lundgren & Novak 2009). Given the comparably high ER-RBA, ZON and its metabolites might contribute to the overall estrogenic activity in the environment (Matthews et al. 2000).

Little is known about the ecotoxicological effects of ZON at levels found in surface waters and the consequence of exposure to aquatic organisms. Only a few short-term *in vivo* studies investigated ZON and its metabolites and observed effects on VTG induction, zona radiata proteins, immune system and growth in fish after exposure via injection or feeding (Arukwe et al. 1999, Celius et al. 2000, Keles et al. 2002).

1.3 The test organism zebrafish (*Danio rerio*)

The zebrafish belongs to the family of Cyprinidae, the most species-rich vertebrate family (Nelson 1994). The name *Danio* derives from the Bengaly name '*dhani*', meaning 'of the rice field'. Danios are included in the subfamily Rasborinae (Howes 1991).

The zebrafish is one of the most important vertebrate model organisms in genetics, developmental biology, neurophysiology and biomedicine (Amsterdam & Hopkins 2006, Grunwald & Eisen 2002, Rubinstein 2006, Vascotto et al. 1997). It has a number of attributes that make it particularly tractable to experimental manipulation. Females can spawn every 2 – 3 days up to several hundred eggs, generation time is short (3 – 4 months), eggs are optically transparent and development is rapid (precursors to all major organs developing within 36 h) (Kimmel et al. 1995). The sensitivity of zebrafish reproduction to exogenous estrogens and xenoestrogens appears to be comparable to that of other fish species (Kwak et al. 2001, Lange et al. 2001, Scholz & Gutzeit 2000, Yokota et al. 2000).

Danio rerio rarely exceeds 40 mm standard length. Its body shape is fusiform and laterally compressed, with a terminal oblique mouth directed upwards. The lower jaw protrudes further than the upper, and the eyes are central and not visible from above. The lateral line is incomplete extending to the pelvic fin base. It exhibits two pairs of barbels and five to seven dark blue longitudinal stripes extending from behind the operculum into the caudal fin (Barman 1991). The anal fin is similarly striped while the dorsal fin has dark blue upper edge, bordered with white. Males tend to have larger anal fins with more yellow colouration (Laale 1977, Schilling 2002). The sex of juveniles cannot be reliably distinguished without dissection. While gravid females tend to have more rounded body shape, the most reliable diagnostic feature is the presence of a genital papilla in front of the anal fin origin (Fig. 2; Laale 1977).

Fig. 2: *Danio rerio* male (left) and female (right)

The natural range of the zebrafish is centred around the Ganges an Brahmaputra river basins in north-eastern India, Bangladesh and Nepal. There is a wide range of temperatures within the natural range of zebrafish, from as low as 6 °C in winter to over 38 °C in summer. Zebrafish have typically described as inhabiting slow-moving or standing water bodies, shallow ponds, the edges of streams and ditches, particularly adjacent to rice fields (Jayaram 1999, Sterba 1962, Talwar & Jhingran 1991). However, they are also reported inhabiting the margins of rivers and streams with a low flow regime (Daniels 2002, Engeszer et al. 2007b, McClure et al. 2006). This association with rice cultivation may relate to the use of fertilisers that may promote the growth of zooplankton, a major component of the zebrafish diet (Spence et al. 2007). Behavioural observations of their vertical distribution indicate that they occupy the whole of the water column and occur as frequently in open water as amongst aquatic vegetation (Spence et al. 2006). Zebrafish are a shoaling species, a behaviour that appears to be innate; shoaling behaviour commences soon after hatching and fish reared in isolation quickly form shoals when placed together (Engeszer et al. 2007a).

The zebrafish is omnivorous, its natural diet consists primarily of zooplankton and insects, although phytoplankton, filamentous algae and vascular plant material, spores and invertebrate eggs, arachnids, detritus sand and mud have also been reported from gut content analysis (Dutta & Malhotra 1991, McClure et al. 2006, Spence et al. 2007). The composition of their diet indicates that zebrafish feed in the water columns, at the surface as well as from the substratum.

In the laboratory, domesticated zebrafish strains breed all year round whereas in nature spawning is more seasonal. However, larger females collected in January (outside the main spawning season) have been found to contain mature ova, indicating that reproduction may not be cued by season, but instead be dependent on food availability, which is likely to co-vary with season. Reproductive maturity appears to be related to size rather than age, wild and domestic zebrafish appear to reach reproductive maturity at similar sizes, despite having different growth rates (Spence et al. 2006).

Zebrafish show a distinct diurnal activity pattern, synchronised with the light/dark and feeding cycles. The first activity peak occurs immediately after illumination with two further peaks in the early afternoon and the last hour of light (Baganz et al. 2005, Plaut 2000). Spawning activity coincides with the first activity peak and usually commences within the first minute of exposure to light following darkness, continuing for about an hour (Darrow & Harris 2004). Zebrafish are group spawners and egg scatterers. The eggs are released directly over the substratum with no preparation of the substratum by either sex and there is no parental care. Females spawn onto a bare substratum, but when provided with an artificial spawning site, such as a plastic box filled with marbles or vegetation, will preferentially use it for oviposition (Spence & Smith 2005). Pairs of zebrafish left together continuously spawn at frequent but irregular intervals and a single female may produce clutches of several hundred eggs in a single spawning (Eaton & Farley 1974, Spence & Smith 2006).

Eggs are non-adhesive and demersal, with a diameter of approximately 0.7 mm. Egg survival is enhanced by incubation in a substratum that allows oxygenated water to circulate while protecting them from disturbance and cannibalism. Eggs become activated on contact with water and even in the absence of sperm, undergo a series of programmed developmental steps, but fail to develop beyond the first few cleavages (Fig. 3; Lee et al. 1999). Immediately after hatching the larvae attach to hard surface by means of small secretory cells in the epidermis of the head (Laale 1977). Attachment at progressively higher levels enables them to reach the surface to which the need to gain access in order to inflate their swim bladders (Goolish & Okutake 1999).

Zebrafish undergo a period a transitory hermaphroditism during juvenile development and thus are classified as undifferentiated gonochorists (Nakamura et al. 1998, Takahashi 1977). Approximately 10 days post hatch (dph) the differentiation of the gonads begins and all fish, irrespective of their definitive sex, develop ovaries. At approximately day 23 dph the ovaries of approximately half of the fish start to degenerate and proliferation of testes occurs. This process is completed at approximately 40 dph. In the remaining fish, the development and maturation of ovaries continues (Takahashi 1977, Uchida et al. 2002).

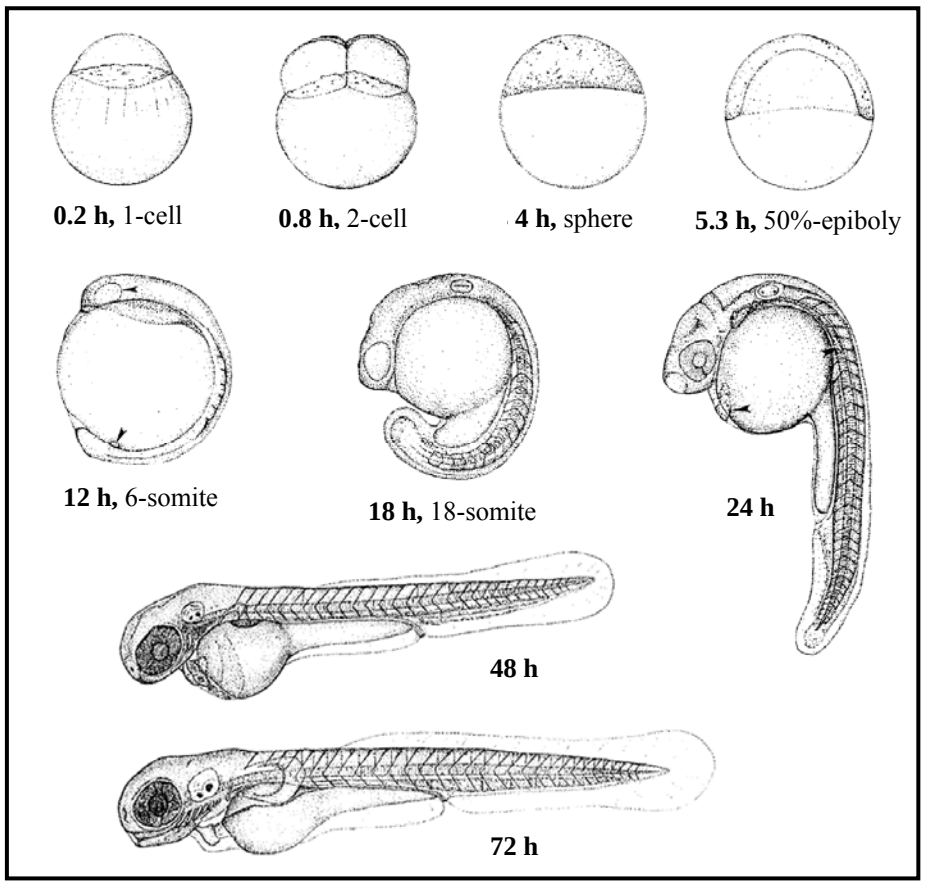

Fig. 3: Embryonic development of *Danio rerio* (modified according to Kimmel et al. 1995)

Zebrafish growth rate is most rapid during the first three month, after which it starts to decrease, approaching zero by about 18 months (Spence et al. 2007). Mean life span of domesticated zebrafish is 42 months, with the oldest known individual surviving for 66 months. However, instances of spinal curvature, a phenotype caused by muscle degeneration and commonly associated with senescence become apparent in domesticated and wild zebrafish after their second year in captivity (Gerhard et al. 2002, Kishi et al. 2003).

1.4 Early life-stage of fish – embryo toxicity assay

The developing fish is generally considered to be the most sensitive stage in the life-cycle of a fish. Many anthropogenic substances in the environment either exert a more toxic effect on embryos than on adult fish or affect only the sensitive early development (Lange et al. 1995, Nagel 2002, Nagel & Isberner 1998, Strmac et al. 2002, Von Westernhagen 1988). The early life-stage of fish provides an array of development parameters, which could serve as biomarkers of toxicant effects on ontogenesis and growth (Eaton & Farley 1974, Hutchinson et al. 1998, Luckenbach et al. 2001, Rosenthal & Alderdice 1976). The collected data could then be used for

estimating/extrapolating pollutant toxicity not only at individual but also at the level of fish populations (Ensenbach & Nagel 1997, Luckenbach et al. 2001, Triebskorn et al. 2000). The ability of growing embryos to discriminate between varying levels of pollution in aquatic systems has been well documented (Klumpp et al. 2002, Luckenbach et al. 2001, 2003).

Apart from mortality and malformation (obvious disadvantages for the fish and possibly for the population) there are additional endpoints in an embryo toxicity assay, such as hatching and heart rate, which can provide a further toxicological characterization of substances or an environmental sample. Hatching date is a sensitive endpoint, because environmental impacts and pollutants may influence the hatching by delaying or accelerating egg development (Dave & Xiu 1991). Hatching is a combined result of the activity of the hatching enzyme chorionase, increased perivitellin pressure, muscle contraction and active water uptake by the embryo (Denuce 1989). Pollutants may interfere directly with these processes and hatching failure may be due to various mechanisms that include the diminished activity of the embryo and abnormal distribution of the hatching enzyme chorionase (Rosenthal & Alderdice 1976) or the inability of the emerging larvae to break through the non-digestible outer part of the egg shell (Sinha & Kanamadi 2000). Furthermore, organisms exposed to pollutants assign a large part of their energy to metabolic pathways aimed at eliminating the pollutant (Wiegand et al. 2000), thus, less energy could be available for hatching (Osterauer & Kohler 2008). Heart rate determination is a simple reliable metric that has been successfully used to quantify the physiological and developmental stresses experienced by embryonic zebrafish as a result of exposure to a wide variety of waterborne chemicals and pollutants (Hallare et al. 2005, Hassoun et al. 2005). The heart rate continues to increase until heart valve maturation is complete at approximately fife days post fertilization. At this point the larval heart is in its adult conformation and its beat frequency begins to decrease gradually until about 60 days post fertilization (Barrionuevo & Burggren 1999).

1.5 Toxicological effects of the mycotoxin zearalenone on early development of zebrafish: A pilot study (Chapter II)

An embryo toxicity test with zebrafish was performed to investigate potential lethal and sublethal effects of ZON on early development and to assess its fish toxic potential in a waterborne exposure. Additionally, a 30 day zebrafish early life-stage experiment was conducted to study the influence of ZON on juvenile growth (Fig. 4).

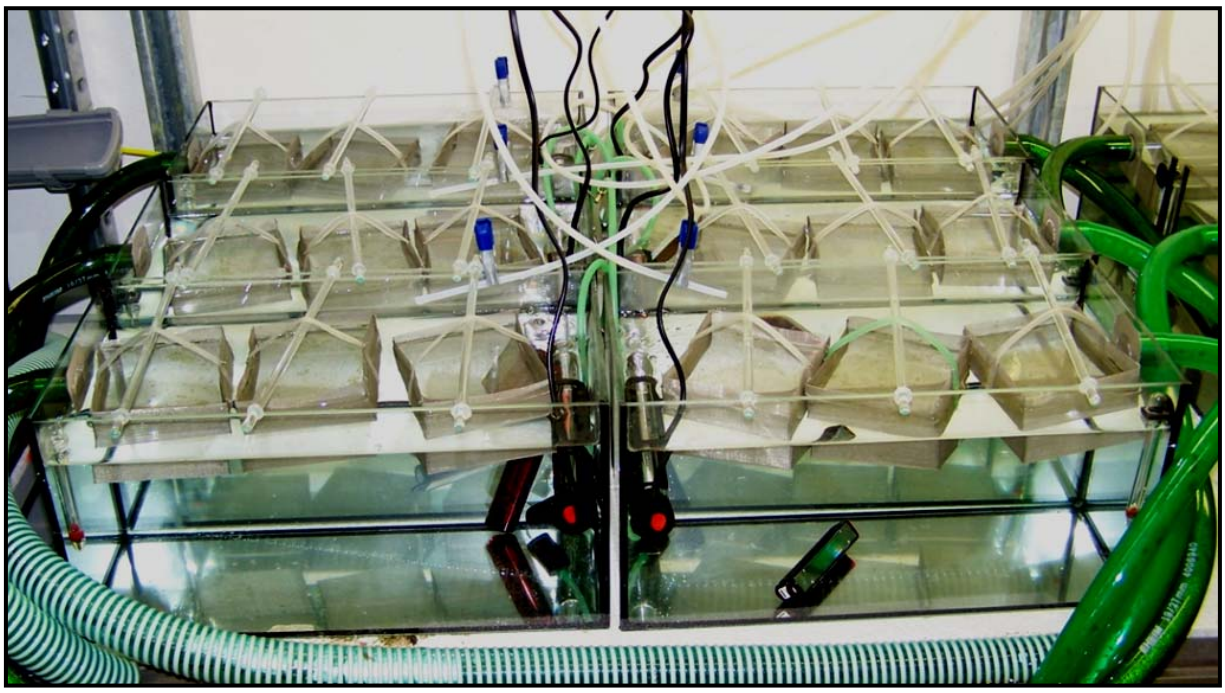

Fig. 4: Exposure system for early life-stage experiment.

1.6 Short-term exposure to the environmentally relevant estrogenic mycotoxin zearalenone impairs reproduction in fish (Chapter III)

Several studies in zebrafish have shown that estrogens such as the natural E2 or the synthetic EE2 induce the yolk precursor protein VTG, affect gonad development, induce atresia of oocytes and inhibition of spermatogenesis, and have an impact on egg production and viability, fertilization success, sexual differentiation and sex ratios (Andersen et al. 2003, Brion et al. 2004, Hill & Janz 2003, Maack & Segner 2004, Nash et al. 2004, Örn et al. 2003, Schäfers et al. 2007, Segner et al. 2003, Van den Belt et al. 2003b, Van der Ven et al. 2007).

The estrogenic potency of ZON and its effects on fish were investigated in a waterborne exposure. A 42 day reproduction experiment was conducted exposing spawning groups of adult zebrafish to ZON via water. Reproduction (spawning frequency, fecundity, fertility and hatch) as well as physiological (VTG induction) and morphological (histomorphometry of gonads) parameters were assessed to evaluate effects of endocrine disruption (Fig. 5). A recombinant yeast estrogen screen (rYES) expressing the human ERα was used as an *in vitro* assay with the endpoint 'activation of ER-regulated genes' to assess the relative estrogenic activity of ZON compared to E2.

Fig. 5: Exposure system for reproduction experiment.

1.7 Life-cycle exposure to the estrogenic mycotoxin zearalenone affects zebrafish (*Danio rerio*) development and reproduction (Chapter IV)

To fully assess the potential of EASs to disrupt reproduction and cause transgenerational effects, multigenerational full life-cycle exposure are needed that consider all relevant life stages and developmental paramteters. A few studies involving EE2 covered the full life-cycle of fish, and have tested the impact on reproductive parameters and observed decreased number of eggs and fertilization rate (Fenske et al. 2005, Larsen et al. 2008, Metcalfe et al. 2001, Nash et al. 2004, Schäfers et al. 2007, Segner et al. 2003, Van den Belt et al. 2003b).

The estrogenic effects of ZON on zebrafish were investigated in a life-cycle exposure. The study was designed to investigate possible effects of continuous long-term exposure including a subsequent depuration period as well as possible transgenerational effects of F0 exposure on F1 generation. For this experiment F1 fish of a parental generation were used, which was exposed to ZON for 21 days (Chapter III). With these fish, a 140 day exposure experiment from embryo to

adult was conducted. In a subsequent 42 day reproduction experiment spawning groups of the adult fish were either exposed to ZON (after growing up in clean water) or clean water (after growing up under ZON exposure). To evaluate effects of endocrine disruption, developmental (mortality, growth sex ratio and gonad histomorphometry), reproduction (spawning frequency, fecundity, fertility and hatch) as well as physiological (VTG induction) parameters were assessed (Fig. 6).

Fig. 6: Exposure system for life-cycle experiment.

2 Ecotoxicological characterization of environmental samples

2.1 Pollution of surface waters

In general, STPs are considered to be a major point source of anthropogenic contamination of river systems (Ternes 2007). The composition of STP effluents can be a complex mixture of more than 100'000 different chemicals which can exhibit detrimental effects on fish, resulting in impaired health status, increased mortality or decreased reproductive success in fish populations (Clements 2000, Kime 1999, Sumpter 1997).

Many anthropogenic chemicals in the water phase adsorb to suspended particles and are integrated into the sediment. Therefore, sediments are a sink but also a potential source for persistent toxic substances (Ahlf 1995, Burton 1991, Hollert et al. 2003, Schwarzenbach et al. 2006). A much higher amount of contaminants are stored in the sediments of many rivers than are introduced annually from external sources (Baker 1991, Power & Chapman 1992).

Sediment-bound contaminants can affect organisms that live over it directly or indirectly via the food web, and can also be remobilized during flood events (Mac et al., 1984; Power and Chapman, 1992; Hollert et al., 2000). In sediments which are contaminated by STP effluents and industrial waste, genotoxic substances are frequently detected (Aouadene et al. 2008, Claxton et al. 1998, Klee et al. 2004, Kosmehl et al. 2004). These genotoxic substances can affect the reproductive success of organisms: DNA damage leads to dieback of gametes, developmental disorders, embryo mortality, and hereditary mutations, and can directly influence the population structure and size (Anderson & Wild 1994). Furthermore, it was reported that increased DNA damage, induced by genotoxic substances, can affect gene flow in populations and hence reduce genetic diversity (Theodorakis et al. 2000, 2001).

Not only sediment but also biofilm is particularly interesting as indicator for environmental pollution, as it interacts with toxic substances and integrates over longer periods. Biofilm is a complex biocoenosis of microorganisms (bacteria, fungi, algae) on a solid surface at the boundary layer to a liquid phase (Sabater et al., 2007). Apart from natural boundary layers such as soil, sediments, plants, animals and mucosa, it can be found on technical installations such as canalizations. In biofilms, the cells of the microorganisms are embedded in a secreted mucilage matrix of extracellular, polymer substances (EPS). A multitude of organic and inorganic compounds are integrated and particular substances are embedded in the EPS (Geesey et al. 1994). Transport and exchange processes that take place between the EPS and the ambient medium determine which substances are integrated in the matrix and which are not (Geesey et al. 1994, Stanimirova et al. 2008).

2.2 Embryotoxic and genotoxic potential of sewage system biofilm and river sediment in the catchment area of a sewage treatment plant in Switzerland (Chapter VI)

The direct exposure of a test organism to an environmental sample closely resembles natural conditions and can therefore be of high ecological relevance. In several studies, zebrafish eggs have been exposed to native sediment to assess the bioavailable toxic potential of samples (Ensenbach, 1998; Hallare et al., 2005; Hollert et al., 2003). In contrast to sediment, the toxic potential of biofilm has to date not been investigated in bioassays. In the comet assay (a commonly used technique to detect DNA strand breaks in cell lines and primary cells) the bioavailable genotoxic potential of sediment can be assessed with primary cells from zebrafish embryos previously directly exposed to the sediment samples (Kosmehl et al. 2006, 2008).

Both an embryo toxicity test with zebrafish and the comet assay with primary cells isolated from the embryos were applied in combination to assess the toxicity of native sediments from a river directly at, upstream and downstream of a STP discharge in Switzerland (Fig. 7). For the first time, this test system was used to investigate native biofilms from different sections of the connected sewage system. The aim of the study was to evaluate the applicability of the test system to investigate biofilms and to evaluate the embryotoxic and genotoxic potential of the samples in a ranking. This ranking was used (1) to compare the hazard potentials of the different sampling sites, (2) localize possible toxic discharges into the sewage system, and (3) to investigate temporal variability from samplings in autumn and spring.

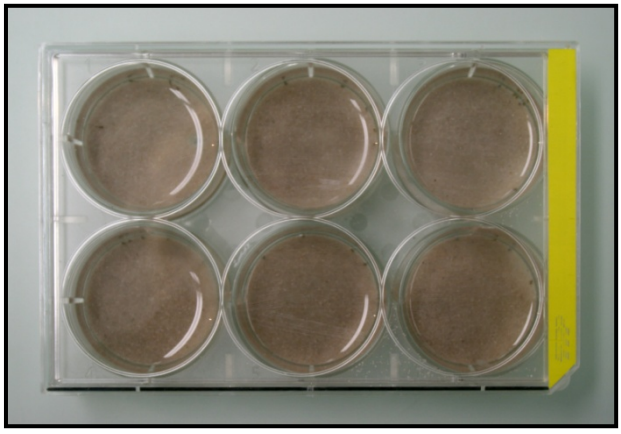

Fig. 7: Exposure in 6-well plates

Chapter II

Toxicological effects of the mycotoxin zearalenone on early development of zebrafish: A pilot study

Abstract

Zearalenone (ZON) is one of the worldwide most common mycotoxins and exhibits estrogenic activity in the range of natural steroid estrogens. The occurrence of ZON has been reported in soil, drainage water, wastewater effluents and rivers, but its ecotoxicological effects on fish have hardly been investigated.

In the present study, effects on zebrafish development and growth were investigated in a 96 h embryo toxicity test as well as in a 30 day early life-stage experiment. No effects on mortality, hatch rate, heart rate, and growth were observed after exposure from fertilization until hatch up to 2000 ng/L ZON. A positive correlation between body length and ZON concentration (25 – 100 ng/L) was detected after exposure from fertilization until 30 days. This might indicate a growth promoting effect of the mycotoxin.

The measured values in surface waters are below the LOEC (lowest observed effect concentration) we determined to cause effects on fish early life-stage. Thus, it is rather unlikely that ZON influence early development of fish directly, but it might contribute to an overall estrogenic activity in the environment and under certain circumstances could affect fish in their natural habitat.

1 Introduction

Zearalenone (ZON) is one of the worldwide most common mycotoxins and belongs to the chemical group of the resorcyclic acid lactones (RALs; Chelkowski 1998). RALs exhibit estrogenic activity in the range of natural steroid estrogens (Le Guevel & Pakdel 2001, Matthews et al. 2000). Several RALs occur as a result of *Fusarium* spec. (primarily *F. graminearum*) infection of corn, wheat and other cereals. Agricultural products around the world were contaminated by ZON up to 69 % of the tested samples with concentrations of up to 180 µg/kg (Rhyn & Zoller 2003). Climate change towards warmer and more moist conditions may provide even more fertile ground for fungi proliferation and lead to an increase of mycotoxin contamination (Jenny et al. 2000). Due to its anabolic properties, α-zearalanol, a ZON metabolite, is licensed as growth promoter for cattle in USA and Canada (banned in EU) which increases the risk of environmental exposure to RALs (Le Guevel & Pakdel 2001).

ZON must be classified moderately water soluble (5.2 mg/L; Megharaj et al. 1997) and rather hydrophobic (K_{OW}: 3.7; Lemke et al. 1998). This indicates some environmental phase mobility and a certain potential for sorption and retention in soil systems (K_{OC}: 3.9; Mortensen et al. 2006). Its stability during milling, food processing and heating (no significant loss in ZON during processing for 80 min at 100 °C) is considerable and its half-live in soil samples was reported to be 6.4 – 11.3 days (Mortensen et al. 2006, Ryu et al. 2003). Therefore it must be assumed that ZON is rather persistent in the environment. Data on biotic transformation of ZON indicates the conversion to α- and β-zearalenol (ZOL) as main metabolic pathway (El-Sharkawy & Abul-Hajj 1988, European Commission 2000, Kleinova et al. 2002, Mirocha et al. 1981). This is of relevance as the estrogenic activity of RALs decreases in the following order: α-ZOL > ZON > β-ZOL (Coldham et al. 1997, Le Guevel & Pakdel 2001).

Although the occurrence of ZON has been studied extensively in food and feed stuff, only little is known about its environmental distribution and impact (Pittet 1998, Rhyn & Zoller 2003). The occurrence of ZON was reported in Italian and Polish rivers in concentrations of 2 – 5 and 0.5 – 44 ng/L, respectively (Gromadzka et al. 2009, Lagana et al. 2004). Up to 35 ng/L and 4 ng/g ZON was quantified in drainage water and soil, respectively, in a field study with wheat and maize (Hartmann et al. 2008a, 2008b). In Italian waste water treatment plants and US American industrial wastewater effluents, ZON concentrations of 1 – 10 and 95.5 – 220 ng/L, respectively, were measured (Lagana et al. 2001, 2004, Lundgren & Novak 2009).

Little is known about the ecotoxicological impact of ZON as an environmental estrogen at levels found in surface waters and the consequence of exposure to aquatic organisms. Only few short-term *in vivo* studies investigated ZON and its metabolites and observed effects on vitellogenin

induction, zona radiata proteins, immune system and growth in fish after exposure via injection or food (Arukwe et al. 1999, Celius et al. 2000, Keles et al. 2002).

The zebrafish was selected as a model since it is easy to keep and readily reproducing in the laboratory, has a rapid development and short generation time, and is widely recommended to be used in many standard ecotoxicological tests in OECD guidelines. Several studies in zebrafish (*Danio rerio*) have shown that estrogens such as the natural 17β-estradiol (E2) or the synthetic pharmaceutical 17α-ethinylestradiol (EE2) have an impact on egg viability and production, fertilization success, sexual differentiation and sex ratios (Andersen et al. 2003, Brion et al. 2004, Hill & Janz 2003, Maack & Segner 2004, Nash et al. 2004, Örn et al. 2003, Schäfers et al. 2007, Segner et al. 2003, Van den Belt et al. 2001, 2003b, Van der Ven et al. 2007).

The early life-stage is generally considered to be the most sensitive period in the life cycle of a fish (Hutchinson et al. 1998, Lange et al. 1995, Luckenbach et al. 2001, Nagel 2002). In early life-stage tests, toxicant effects on ontogenesis and growth can be examined through diverse endpoints and extrapolated to assess effects at population levels (Ensenbach & Nagel 1997, Luckenbach et al. 2001, Triebskorn et al. 2000). In the present study, an embryo toxicity test with zebrafish was performed to investigate lethal and sublethal effects of ZON and to assess its fish toxic potential in a waterborne exposure. Additionally, a 30 day zebrafish early life-stage experiment was conducted to assess the influence of ZON on juvenile growth.

2 Materials and Methods

2.1 Experimental animals

All procedures concerning experimental animals were performed in compliance with the Swiss protection of animals act. The zebrafish used for egg production originated from our fish breeding stocks, held at the University of Basel. They were kept in aerated 60 L glass tanks supplied with a constant flow of 5 L/h conditioned water (1 : 1 mix of charcoal filtered tap water and reverse osmosis water) at a temperature of 26 ± 1 °C, pH of 8.0 ± 0.2 and an artificial light : dark photoperiod of 16 : 8 h. The fish were fed twice per day once with dry pellet (SilverCup, H. U. Hofmann AG, Switzerland) and once with frozen brine shrimps (*Artemia salina*, 3F Frozen Fish Food BV, Netherlands).

2.2 Egg production

Approximately 2 h before the dark period, spawning groups of two female and four male zebrafish were transferred into 12 L spawning tanks. All tanks were aerated and tempered to 26 ± 1 °C using submerged electrical heaters and a light : dark photoperiod of 16 : 8 h was maintained. The spawning tanks were equipped with a stainless steel mesh (mesh size: 2.5 mm) to prevent fish from eating their eggs. Artificial weed (Clear water coarse filtering wool, Dennerle GmbH, Germany) was attached to the centre of the steel mesh to provide a spawning stimulation. Oxygen was always above 7.4 mg/L (Oxi 315i, WTW, Germany), pH at 7.8 – 8.2 (pH 315i, WTW, Germany), and conductivity at 240 – 260 µS/cm (Cond 315i, WTW, Germany). On the following morning, 0.5 – 1 h after beginning of the light period, all fish were transferred back to the stock tanks and the eggs were collected into petri dishes for microscopical analysis.

2.3 Test chemical

A stock solution of 2 mg/L ZON (purity: 99 %, Sigma-Aldrich GmbH, Switzerland) was prepared in purified water (Elgastat, Option 4 water purifier, Elga Ltd, England) and stored in a glass bottle at 4 °C in the dark. This stock solution was diluted to prepare the test concentrations (15.6 – 2000 ng/L) for the embryo toxicity test and to prepare the supply solution (25 µg/L) for use in the exposure system of the early life-stage experiment.

2.4 Embryo toxicity test with zebrafish

The embryo toxicity test was conducted with 3 – 8 independent experiments (n = 3 – 8) according to the adapted test protocol of Schulte & Nagel (1994) and the German DIN 38415 – T6. ZON was applied at 8 – 9 concentrations, each with 20 – 24 wells of a 24-well plate (Becton Dickinson Labware, USA) and 2 mL dilution per well.

To prepare the test dilutions, the ZON stock solution was diluted with artificial water (ISO 7346/3, stock solutions of 58.8 mg/L $CaCl_2 \times 2\ H_2O$, 24.6 mg/L $MgSO_4 \times 7\ H_2O$, 12.6 mg/L $NaHCO_3$ and 5.5 mg/L KCl, diluted 1:5 with purified water), aerated to 100 % oxygen saturation. For the control 20 – 24 wells were filled with the same amount of artificial water.

Within two hours post fertilization (hpf) one fertilized and normally developing fish egg was transferred into each well using a plastic pipette. The 24-well plates were closed with lids and incubated in a warming cupboard at 26 ± 1 °C and saturated humidity to avoid evaporation from the wells.

Table 1: Toxicological endpoints for the evaluation of embryo toxicity and mortality (hpf: hours post fertilization).

Toxicological endpoint	24 hpf	48 hpf	72 hpf	96 hpf
Coagulation	●	●	●	●
Epiboly stage	●	●	●	●
No somites	●	●	●	●
Undetached tail	●	●	●	●
No heartbeat	—	●	●	●
No circulation	—	○	○	○
No pigmentation	—	○	○	○
No movement	○	○	○	○
Development retardation	○	○	○	○
Edema	○	○	○	○
Malformation	○	○	○	○
Hatch	—	—	○	○

● = lethal criterion used to determine mortality rate
○ = documented but not evaluated as lethal criterion
— = not documented

The eggs were exposed to the ZON concentrations during the period of 2 – 96 hpf and inspected at 24, 48, 72 and 96 hpf for lethal and sub lethal effects using an inverse microscope (Olympus CKX41, Olympus, Germany) at 40x and 100x magnification. The toxicological endpoints evaluated in the test are given in Table 1. For assessing the heart rate, the number of heartbeats

was counted for a period of 15 s and beats per minute were calculated. Digital photomicrographs were taken (Altra 20, Soft imaging system GmbH, Germany) at 40x and 100x magnification from hatched zebrafish larvae at 96 hpf and body length was measured using Adobe Photoshop CS3 Extended version 10.0.1 (Adobe Systems Inc., USA).

2.5 Early life-stage experiment: Exposure system

The zebrafish (egg to juvenile) were exposed in a flow-through system in 12 L glass tanks with three replicate tanks for each exposure group and the water control. All tanks were aerated and tempered to 26 ± 1 °C using submerged electrical heaters, and a light : dark photoperiod of 16 : 8 h was maintained during the duration of the experiment. Flow indicators (Sho-Rate 1355, Brooks Instrument, Netherlands) were used to regulate the flow of 2 L/h conditioned water into glass mixing chambers, equipped with magnetic stirrers. The ZON supply solution (25 µg/L) was dosed into the three mixing chambers of the exposed groups by means of a computer controlled dispenser (Microlab 541C Dispenser, Hamilton, Switzerland) at a speed of 2, 4 and 8 mL/h resulting in nominal exposure concentrations of 25, 50 and 100 ng/L, respectively. Each tank was supplied from the mixing chambers through Teflon tubing (Carl Roth GmbH + Co. KG, Germany) using modular valve positioner (MVP, Hamilton, Switzerland) with a flow rate of 1 L/h.

Dissolved O_2, pH, conductivity and temperature was monitored every fourth day. O_2 was always above 7.5 mg/L, pH at 7.9 – 8.3, conductivity at 210 – 220 µS/cm and temperature at 25 – 27 °C.

2.6 Early life-stage experiment: Experimental design

Group A was introduced at day 0 and group B at day 10 into the exposure system. For group A 115 and for group B 90 eggs per replicate were transferred within 4 hpf into mesh boxes. The mesh boxes (100 × 100 × 50 mm), consisting of stainless steel mesh (mesh size: 0.355 mm), were suspended in the 12 L exposure tanks. These fish were raised under the described exposure conditions for 30 and 20 days, respectively. From hatch till 8 days post hatch (dph), the larvae were fed dry powder (Sera Micron, Sera, Germany) and from 8 to 30 dph dry flakes (TetraMin Baby, Tetra GmpH, Germany) and freshly hatched *Artemia salina* nauplia twice a day.

On day 30 of the early life-stage experiment all juvenile zebrafish (group A and B) were euthanized in 150 mg/L MS222 buffered with 300 mg/L $NaCO_3$ to maintain a neutral pH. The total length of juvenile fish was measured to the nearest mm.

2.7 Chemical analysis

To analyse the ZON concentrations in the stock solution and in the exposure aquaria, 100 mL water samples were collected before the start of the experiments and on day 0, 15 and 30 (all replicate tanks) of the early life-stage experiment, respectively, into solvent cleaned glass bottles and sent within 24 h to the research institute for chemical analysis (Agroscope Reckenholz-Tänikon Research Station ART, Switzerland). An isotope labelled internal standard (D_6-ZON) was added to the water samples prior to solid-phase extraction (SPE; SupelcleanTM Envi-18 cartridges, 6 mL, 500 mg, Supelco, USA) and analysis by LC-MS/MS (negative electrospray ionization; Hartmann et al. 2007).

2.8 Statistical analyses

To investigate effects of the ZON exposure, data of exposed groups were compared to control group using SigmaPlot version 9.01 and SigmaStat version 3.1 (Systat Software Inc., USA). Data of mortality at 96 hpf, heart rate at 48 hpf, hatch rate at 72 hpf and body length (embryo toxicity test and early life-stage experiment) met the assumptions of normality and equality of variance. Hence, these data were analysed with a one-way analysis of variance (ANOVA) followed by Dunnett's test. Data of heart rate at 72 hpf was $\log_{10}$ transformed prior to analysis to meet the assumptions of normality and equality of variance. Performing standard transformation of mortality at 48 hpf and hatch rate at 96 hpf data could not provide for the assumption of normality and equal variance, and therefore were analyzed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's method for multiple comparisons. Analysis of correlation between body length and nominal ZON concentration was conducted using Pearson Product Moment Correlation.

3 Results

3.1 Aqueous ZON concentration

To verify the test concentrations, ZON was quantified in the stock solution and in the exposure tanks during the early life-stage experiment. ZON concentration in the stock solution for the embryo toxicity test was 1.70 ng/L which corresponds to 85 % of the nominal value. Mean ZON concentrations in the early life-stage experiment ranged between 70 and 84 % of the nominal value and were constant during the entire experiment. In the control group, no ZON could be determined (< 1 ng/L; Table 2).

Table 2: Measured concentrations of zearalenone (ZON) for exposure groups and controls (mean ± SEM, n = 3).

Nominal	**Mean measured concentrations [ng/L]**		
ZON	**day 0**	**day 15**	**day 30**
Control	BD[a]	BD[a]	BD[a]
25 ng/L	20.2 ± 0.10	18.1 ± 0.35	18.2 ± 0.27
50 ng/L	42.2 ± 0.30	38.6 ± 0.20	39.7 ± 0.33
100 ng/L	71.4 ± 0.95	70.2 ± 1.26	74.9 ± 1.76

[a] BD = below detection limit (< 1 ng/L)

3.2 Embryotoxicological effects of ZON

Zebrafish embryos developed normally in the control groups of all experiments, defined by the criteria of Kimmel et al. (1995). Developmental abnormalities were always observed in ≤ 1 fish (≤ 5 %) per concentration. The observed effects in the exposed groups showed no concentration response relation.

At 24 hpf only few developmental abnormalities such as malformations and edemas were observed in some exposure groups with a maximum of 1 – 2 fish (≤ 5 – 10 %) per concentration. An undetached tail was observed in one embryo, as indication of development retardation. At 48 hpf the effects no heartbeat and no circulation were observed in a few embryos but not more than 1 – 2 fish (≤ 5 – 10 %) per concentration. Also edemas and malformations appeared occasionally with a maximum of 1 – 2 fish (≤ 5 – 10 %) per concentration. At 72 hpf effects such as no heartbeat, no circulation, edemas and malformation appeared in some experiments in some concentrations with a maximum of 1 – 3 fish (≤ 5 – 15 %) per concentration. At 96 hpf the effects and their frequency were similar to those at 72 hpf.

3.3 Embryo toxicity: Mortality, hatch rate, heart rate, body length

The mortality in the control group showed means of 4.5 and 4.4 % at 48 and 96 hpf, respectively. Similarly the mortality in all ZON exposed groups revealed means of 1.6 – 5.2 and 1.3 – 6.7 % at 48 and 96 hpf, respectively, with no significant differences between groups (Fig. 1 A, B).

The hatch rate in the control group displayed means of 56.5 and 93.1 % at 72 and 96 hpf, respectively. Similarly the hatch rate in all ZON exposed groups showed means of 49.0 – 62.6 and 93.8 – 98.8 % at 72 and 96 hpf, respectively, with no significant differences between groups (Fig. 1 C, D).

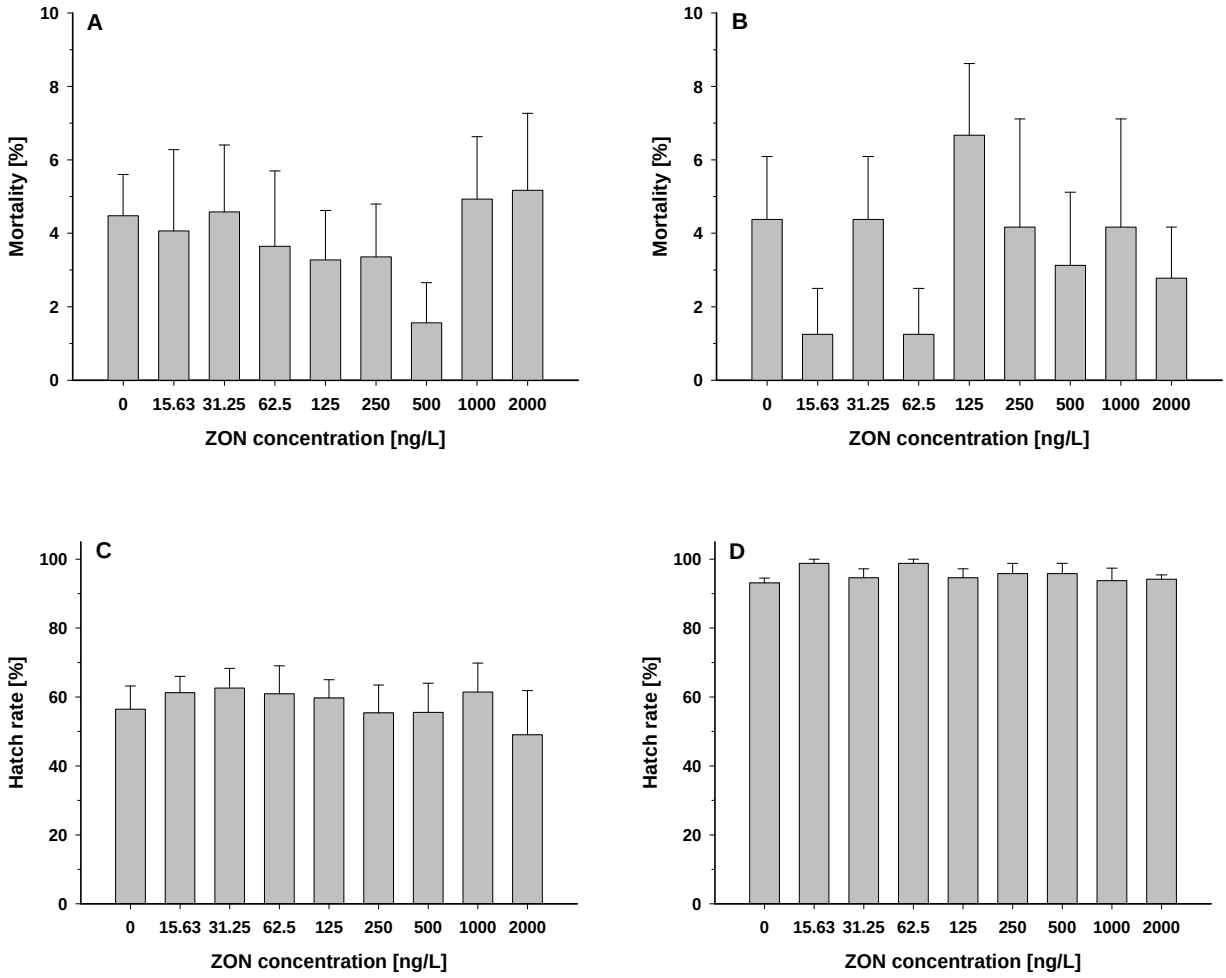

Fig. 1: Embryo toxicity: Mean ± SEM for mortality at (A) 48 hours post fertilization (hpf; n = 6 – 8) and (B) 96 hpf (n = 3 – 4), and for hatch rate at (C) 72 hpf (n = 6 – 8) and (D) 96 hpf (n = 3 – 4).

The heart rate in the control group revealed means of 140.1 and 151.4 beats/min at 48 and 96 hpf, respectively. Similarly the heart rate in all ZON exposed groups displayed means of 130.3 – 135.1 and 140.1 – 145.5 beats/min at 48 and 96 hpf, respectively, with no significant differences between groups (Fig. 2 A, B). Body length at 96 hpf showed a mean of 3.8 mm for

the control group and was almost identical in the ZON exposed fish (mean of 3.8 mm) without significant differences between groups (Fig. 2 C).

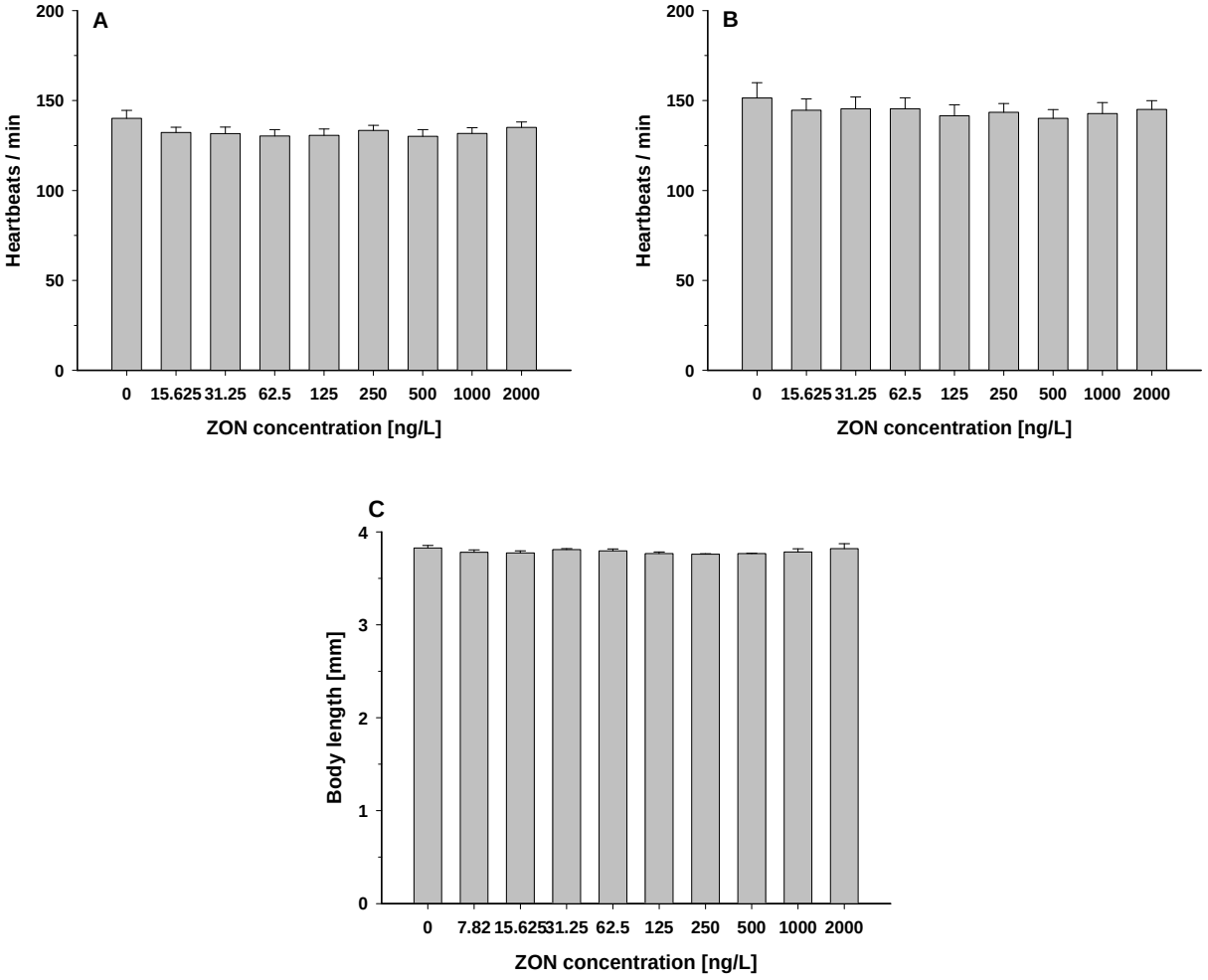

Fig. 2: Embryo toxicity: Mean ± SEM for heart rate at (A) 48 hours post fertilization (hpf; n = 7 – 8) and (B) 72 hpf (n = 7 – 8), and for body length at (C) 96 hpf (n = 3).

3.4 Early life-stage experiment

After exposure for 20 days up to 100 ng/L ZON, body length of zebrafish showed means of 5.3 – 5.4 mm and were similar to control group (5.3 mm) without significant difference between groups (Fig. 3 A). After exposure for 30 days, body length revealed means of 6.1, 6.5 and 6.7 mm at 25, 50 and 100 ng/L ZON, respectively, and 6.0 mm in the control group without significant differences between groups (Fig. 3 B). However, analysis of correlation revealed a positive correlation between ZON concentration and body length ($r = 0.695$, $p = 0.0121$).

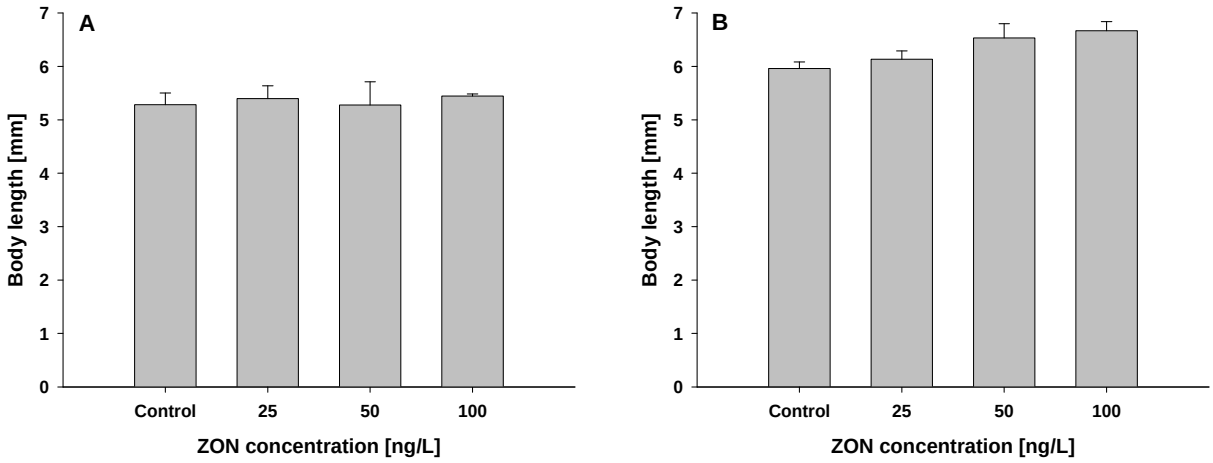

Fig. 3: Early life-stage experiment: Mean ± SEM for body length at (A) 20 days post fertilization (dpf) and (B) 30 dpf (n = 3).

4 Discussion

4.1 Embryo toxicity: Evaluation of effects

Only few deviations from normal development were observed in the various ZON concentrations without showing a relation between concentration and response. No indication of retarded development was observed at 24 hpf, except for one single embryo with an undetached tail. In the exposure period from 48 to 96 hpf, the effect no heartbeat appeared only occasionally. Evaluating these findings together with the results for mortality (as effects such as undetached tail and no heartbeat were determined as mortal) and hatch rate which showed no differences to the control, we suggest that ZON does not affect development up to a concentration of 2000 ng/L.

Developmental abnormalities such as edemas and malformations appeared in very few embryos in various ZON concentrations without significant differences to control. These deviations from normal development are probably due to inter-individual variation and therefore indicating no teratogenic potential for ZON up to 2000 ng/L.

Since developmental events in zebrafish were not arrested and embryos developed synchroniously in all concentrations, we can deduce that ZON exert no apparent threat to embryonic fish development until hatch up to a concentration of 2000 ng/L.

4.2 Mortality, hatch rate, heart rate and body length

The mortality in the control groups at 48 hpf was in all experiments below 10 %. Therefore the requirement of DIN 38415 – T6 for a valid test was met and the effects can be attributed to the ZON exposure.

Mean cumulative mortality at the end of the embryo exposure in control and exposure groups was similar with no significant differences between groups. Consequently there was no evidence that exposure for 96 h, from fertilization to hatch, up to a concentration of 2000 ng/L ZON affected survival of zebrafish. This is in accordance with data from toxicological studies demonstrating that apart from estrogenic effects ZON exhibits a low acute toxicity in many animal species (Kuiper-Goodman et al. 1987).

Similarly hatch rate at 72 and 96 hpf was for all exposure groups in the same range as for the control group and no significant difference between groups were found. Hatching date is a sensitive endpoint of the fish egg assay, because environmental impacts and pollutants may influence the hatching date by delaying of accelerating egg development or they may interfere directly with these processes, e.g. through the inhibition of the chorionase (Dave & Xiu 1991).

Furthermore, organisms exposed to pollutants assign a large part of their energy to metabolic pathways aimed at eliminating the pollutant, thus, less energy could be available for hatching (Wiegand et al. 2000). As we could not observe early or late hatch caused by the exposure to ZON there was no indication of influence on development time.

The heart rate in all exposure groups was slightly lower than in the control group but without significant differences among groups. Heart rate determination is a reliable metric that has been successfully used to quantify the physiological and developmental stresses experienced by embryonic zebrafish as a result of exposure to waterborne chemicals and pollutants (Craig et al. 2006, Hallare et al. 2005, Hassoun et al. 2005). From our results we conclude that exposure to ZON up to 2000 ng/L did not induce significant stress to the developing fish.

In addition, body length of the hatched larvae at 96 hpf was almost identical in the exposure and control groups. Consequently there was no evidence that exposure from fertilization to 96 hpf up to a concentration of 2000 ng/L ZON affected growth of zebrafish.

4.3 Early life-stage experiment

After exposure from fertilization until 30 dpf all exposure groups showed a trend for increased body length compared to control but without significant differences between groups. This was not the case at 20 dpf.

Several authors reported a significant increase in length and weight of zebrafish after exposure from 0 to 240 and 24 to 64 dpf to 0.5 – 2 ng/L and 2 – 10 ng/L EE2, respectively, as well as a concentration dependent increase of length and weight after exposure from 0 to 42 dpf and F0 generation to 27 – 272 ng/L E2 (Örn et al. 2003, Soares et al. 2009, Van der Ven et al. 2007). In contrast no effect on length and weight was found in zebrafish exposed from 0 to 75 and 0 to 270 dpf to 3 – 5 ng/L EE2 as well as 5 ng/L E2, respectively (Fenske et al. 2005, Nash et al. 2004). Other studies observed a significantly reduced length and weight of zebrafish after exposure from 0 to 60 – 90 dpf to 1.7 – 25 ng/L EE2 (Hill & Janz 2003, Schäfers et al. 2007, Van den Belt et al. 2003b). In summary, the cited studies show very diverse effects of the steroid estrogens E2 and EE2 on zebrafish. The observed estrogenic effects ranged from promoting growth to having no effect, or to inhibiting growth. Taken together these results show no pattern or tendency, such as concentration or duration dependence of positive or negative effect on growth of zebrafish, or give evidence under which conditions estrogens influence growth or not. However the observed positive influence on growth of zebrafish exposed to ZON for 30 days corresponds to the growth-promoting (anabolic) potential of E2 in early life of fish (Bell 2004, Mandiki et al. 2005).

4.4 Environmental relevance

ZON was reported to occur in surface waters and effluents in concentrations of 0.5 – 44 ng/L and 1 – 220 ng/L, respectively (Gromadzka et al. 2009, Lagana et al. 2001, 2004, Lundgren & Novak 2009). The maximum observed environmental concentrations are 9 – 45 times lower than the highest applied concentration which revealed no effect in the present embryo toxicity studies (2000 ng/L). The maximum observed surface water concentration is 44 % of the highest concentration applied in the early life-stage experiment, which showed no significant effect compared to control but a positive correlation between ZON concentration (25, 50 and 100 ng/L) and body length.

Assessing the environmental relevance of these results, the risk for early development of fish to be affected by ZON in the natural environment appears to be rather low. However, the actual exposure in a given catchment depends on many factors, such as *Fusarium* infection rates, meteorology and hydrodynamics (Bucheli et al. 2008, Hartmann et al. 2008b). Therefore, taking into consideration the highest detected concentrations, a possibly higher species sensitivity, regional and temporal limited higher concentrations (caused by effluent discharge in combination with low flow conditions, or runoff from *Fusarium* infected fields), and mixture effects in combination with other estrogen active substances, ZON might contribute to the overall estrogenic exposure in the environment.

5 Conclusions

No effects on embryonic development of zebrafish caused by exposure up to 2000 ng/L ZON from fertilization until hatch was observed. However, a positive correlation between the body length and ZON concentration for zebrafish exposed from fertilization until 30 days up to 100 ng/L suggests a growth promoting effect of ZON.

There is not much data available for ZON concentrations in the environment and the measured values in surface waters are below the LOEC (lowest observed effect concentration) we determined in this study to cause effects on fish early life-stage. Thus, it is rather unlikely that ZON impair early development of fish directly. However, ZON might contribute to an overall estrogenic contamination in the environment and under certain circumstances (higher species sensitivity, spatially and temporally limited events and combination with other estrogen active substances) there might be a certain risk for fish to be affected by ZON in their natural environment.

Further studies are needed to investigate the potential consequences for fish caused by exposure to ZON for longer periods. Furthermore it is of great importance to investigate effects on different life-stages as well as estrogen related and population relevant endpoints (e. g. reproduction).

Acknowledgements

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Chapter III

Short-term exposure to the environmentally relevant estrogenic mycotoxin zearalenone impairs reproduction in fish

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Abstract

Zearalenone (ZON) is one of the worldwide most common mycotoxins and exhibits estrogenic activity in the range of natural steroid estrogens such as 17β-estradiol (E2). The occurrence of ZON has been reported in drainage water, soil, wastewater effluents and rivers, but its ecotoxicological effects on fish have hardly been investigated.

In this study, the estrogenic potency of the ZON was compared to E2 in a recombinant yeast estrogen screen (rYES) and the effects of waterborne ZON exposure on reproduction, physiology and morphology of zebrafish (*Danio rerio*) were investigated in a 42 day reproduction experiment. E2 as well as ZON evoked a sigmoid concentration response curve in the rYES with a mean EC_{50} of 2 µg/L and 500 µg/L, respectively, resulting in an E2 : ZON EC_{50} ratio of 1 : 250. Exposure to ZON for 21 days reduced relative spawning frequency at 1000 and 3200 ng/L to 38.9 and 37.6 %, respectively, and relative fecundity at 100, 320, 1000 and 3200 ng/L to 74.2, 41.7, 43.8 and 16.7 %, respectively, in relation to the 21 day pre exposure period. A 4.4 and 8.1 fold induction of plasma vitellogenin (VTG) was observed in male zebrafish at 1000 and 3200 ng/L ZON, respectively. Exposure to ZON did not affect fertility, hatch, embryo survival and gonad morphology of zebrafish.

The results of this study demonstrate that although ZON possesses a moderate estrogenic potency *in vitro*, it exhibits a comparably strong effect on induction of VTG and reproduction *in vivo*. This indicates that ZON might contribute to the overall estrogenic activity in the environment and could therefore pose a risk for wild fish in their natural habitat.

1 Introduction

Zearalenone (ZON) belongs to the chemical group of the resorcyclic acid lactones (RALs) and is one of the worldwide most common mycotoxins (Chelkowski 1998). RALs show estrogenic activity in the range of natural steroid estrogens and the relative binding affinity (RBA) to the estrogen receptor (ER) for ZON has a factor of 0.82 in relation to the natural steroid estrogen 17β-estradiol (E2; Matthews et al. 2000).

Several RALs can co-occur as a result of *Fusarium* spec. (primarily *F. graminearum*) infection of corn, wheat and other cereals. The extent of *Fusarium* spec. infestation on crops and subsequent contamination with mycotoxins is dependent on factors as crop rotation, soil cultivation, susceptibility of crop varieties and climatic conditions (Champeil et al. 2004). Climate change towards warmer and more moist conditions may provide even more fertile ground for fungi proliferation (Jenny et al. 2000). The occurrence of mycotoxins has been studied extensively in food and feed products and domestic animals. Agricultural products around the world show contamination rates for ZON up to 69 % of the tested samples with concentrations of up to 180 µg/kg (Rhyn & Zoller 2003). Due to its anabolic properties α-zearalanol (ZAL), a ZON metabolite, is licensed as growth promoter for cattle in USA and Canada (banned in EU since 1985) which increases the risk of exposure to RALs (Le Guevel & Pakdel 2001).

ZON must be classified moderately water soluble (5.2 mg/L; Megharaj et al. 1997) and rather hydrophobic (K_{OW}: 3.7; Lemke et al. 1998). This indicates some environmental phase mobility and a certain potential for sorption and retention in soil systems. Its stability during milling, food processing, heating, etc. is considerable and it must be assumed that ZON is rather persistent in the environment (Ryu et al. 2003). Data on biotic transformation of ZON indicates as main metabolic pathway the conversion to α- and β-zearalenol (ZOL; El-Sharkawy & Abul-Hajj 1988, European Commission 2000, Kleinova et al. 2002, Mirocha et al. 1981). This is of relevance as the estrogenic activity of RALs decreases in the following order: α-ZOL > ZON > β-ZOL (Coldham et al. 1997, Le Guevel & Pakdel 2001).

The occurrence of ZON was reported in rivers in Italy, Poland and USA in concentrations of 2 – 5, 0.5 – 43, and up to 8 ng/L, respectively (Gromadzka et al. 2009, Kolpin et al. 2010, Lagana et al. 2004). Hartmann et al. (2008a, 2008b) demonstrated in a field study with wheat and maize the occurrence of ZON in drainage water and soil up to 35 ng/L and 4 ng/g, respectively. Concentrations of 1 – 10 and 95.5 – 220 ng/L ZON were measured in Italian wastewater treatment plants and US American industrial wastewater effluents, respectively (Lagana et al.

2001, 2004, Lundgren & Novak 2009). Given the comparably high ER-RBA, ZON and its metabolites might contribute to the overall estrogenic activity in the environment.

Little is known about the ecotoxicological impact of ZON as an environmental estrogen at levels found in surface waters and the consequence of exposure to aquatic organisms. Endocrine active substances (EASs) exert their effects by mimicking or antagonizing endogenous hormones, influencing the natural hormone synthesis, metabolism or elimination, and therefore have the potential to interfere with reproduction and development (Jobling & Tyler 2003b, Sumpter 2005). Primary concerns are on substances with estrogenic activity which can be anthropogenic, such as certain pharmaceuticals, pesticides and industrial chemicals, natural, such as phyto- and mycoestrogens or both, such as steroid estrogens. The few *in vivo* studies that investigated ZON and its metabolites revealed effects on vitellogenin (VTG) induction and zona radiata proteins, immune system and growth in fish (Arukwe et al. 1999, Celius et al. 2000, Keles et al. 2002). Exposure of fish to estrogens result in induction of the yolk precursor protein VTG in male and juvenile fish, making it a suitable specific biomarker for detecting estrogenicity (Sumpter & Jobling 1995).

Several studies in zebrafish (*Danio rerio*) have shown that estrogens such as the natural E2 or the synthetic pharmaceutical 17α-ethinylestradiol (EE2) affect gonad development and have an impact on egg viability and production, fertilization success, sexual differentiation and sex ratios (Andersen et al. 2003, Brion et al. 2004, Hill & Janz 2003, Maack & Segner 2004, Nash et al. 2004, Örn et al. 2003, Schäfers et al. 2007, Segner et al. 2003, Van den Belt et al. 2001, 2003b, Van der Ven et al. 2007). Investigations that focus on a relationship between exposure conditions, biomarker changes and reproductive effects can provide information to evaluate the environmental relevance of estrogens (Arcand-Hoy & Benson 1998, Miller et al. 2007, Thorpe et al. 2007).

The aim of this study was to investigate the estrogenic potency of ZON and its effects on fish in a waterborne exposure. A recombinant yeast estrogen screen (rYES) was used as an *in vitro* assay with the endpoint 'activation of ER-regulated genes' to assess the relative estrogenic activity of ZON compared to E2. For the *in vivo* experiment we used the zebrafish, a widely used species in many standard ecotoxicological tests in OECD guidelines, as a model. We conducted a 42 day reproduction experiment exposing spawning groups of adult zebrafish to ZON via water. Reproduction (spawning frequency, fecundity, fertility and hatch) as well as physiological (VTG induction) and morphological (histomorphometry of gonad) parameters were assessed to evaluate effects of endocrine disruption.

2 Materials and Methods

2.1 Experimental animals

All procedures concerning experimental animals were performed in compliance with the Swiss protection of animals act. The zebrafish used in this study were approximately 10 months old at the start of the experiment and originated from our fish breeding stocks, held at the University of Basel. Prior to the onset of the experiment the fish were kept in aerated 60 L glass tanks supplied with a constant flow of 5 L/h conditioned water (1 : 1 mix of charcoal filtered tap water and reverse osmosis water) at a temperature of 27 ± 1 °C, pH of 8.1 ± 0.2 and an artificial light : dark photoperiod of 16 : 8 h. The animals were fed twice daily once with dry pellet (SilverCup, H. U. Hofmann AG, Switzerland) and once with frozen brine shrimps (*Artemia salina*, 3F Frozen Fish Food BV, Netherlands).

2.2 Test chemical

A stock solution of 0.5 mg/mL zearalenone (ZON, purity: 99 %, Sigma-Aldrich GmbH, Switzerland) was prepared in acetone (purity: 99.9 %, Carl Roth GmbH + Co. KG, Germany) and stored in a glass bottle at 4 °C in the dark. This stock was used to prepare a solvent free supply solution every 3 days for use in the exposure system. The supply solution was prepared by coating a 2 L glass bottle with 4 mL of stock solution and removing the solvent under a gentle stream of N_2. The bottle was then filled up to 2 L with conditioned water to give a concentration of 1 mg/L ZON and stirred over-night with a magnetic stirrer prior to introduction to the exposure system.

2.3 Exposure system

The zebrafish were exposed in a flow-through system in 12 L glass tanks with four replicate tanks for each ZON concentration and the water control. All tanks were aerated and tempered to 26 ± 1 °C using submerged electrical heaters and a light : dark photoperiod of 16 : 8 h was maintained during the duration of the experiment. Flow indicators (Sho-Rate 1355, Brooks Instrument, Netherlands) were used to regulate the flow of 6 L/h conditioned water into glass mixing chambers, equipped with magnetic stirrers. The ZON supply solution was dosed into the four mixing chambers of the exposed groups by means of a computer controlled dispenser (Microlab 500 series Dispenser, Hamilton, Switzerland) at a speed of 600, 1920, 6000 and 19200 µL/h giving nominal exposure concentrations of 100, 320, 1000 and 3200 ng/L. Each

exposure tank was supplied from the mixing chambers through Teflon tubing (Carl Roth GmbH + Co. KG, Germany) using peristaltic pumps (Ismatec IPN 24, Ismatec, Switzerland) with a flow rate of 1 L/h and allocated to the different concentrations and negative control, respectively, using a random generator (www.randomizer.org).

Every tank was equipped with a spawning tray consisting of a stainless steel tray (265 × 185 × 30 mm) covered with a stainless steel mesh (mesh size: 2.5 mm). To minimize the handling stress for the fish two stainless steel cable ties were attached to the trays for manipulation from outside the water. Artificial weed (Clear water coarse Filtering wool, Dennerle GmbH, Germany) was attached to the centre of the steel mesh and surrounded by a few stained glass stones to provide an appropriate spawning stimulation.

Dissolved O_2, pH, conductivity and temperature was monitored every fourth day. O_2 was always above 7.4 mg/L, pH at 7.8 – 8.2, conductivity at 240 – 260 µS/cm and temperature at 25 – 27 °C.

2.4 Experimental design

Each exposure tank (four replicates per ZON concentration and control) was stocked with a spawning group consisting of two female and four male zebrafish. After an acclimatization period of one week the experiment started with a pre exposure period of 21 days to establish the baseline reproduction for each group, followed by a 21 day exposure period. During the whole time the animals were fed dry pellets once and 2 mL of freshly hatched *Artemia salina* nauplia twice per day.

Every morning the spawning trays were removed from the exposure tanks, to check for eggs and replaced with clean spawning trays. The fish eggs were transferred to petri dishes for microscopical analysis, to determine fertilization success. From each clutch a maximum of 50 fertilized eggs were randomly selected and transferred to an egg cup consisting of a glass tubing (80 × 60 mm) closed with a stainless steel mesh (mesh size: 0.355 mm) at the bottom side, and suspended in 21 L glass tanks filled with conditioned water at a temperature of 25 – 27 °C. Five days post fertilization the eggs were analyzed for vitality and hatch.

2.5 Estrogenic activity

A rYES expressing the human estrogen receptor alpha (hERα; Routledge & Sumpter 1996) was used to assess the relative estrogenic potency of ZON in comparison to E2 (purity: 98 %, Sigma-Aldrich GmbH, Switzerland) and to determine estrogenic activity in each aquarium in relation to an internal ZON standard.

The E2 and ZON standards were prepared in ethanol (purity: 99.8 %, Carl Roth GmbH + Co. KG, Germany) in a concentration of 54.5 µg/L and 20 mg/L, respectively. To assess the concentration-response curve and the relative estrogenic potency of ZON, 12 independent experiments were performed.

Samples of 1 L aquarium water were collected on day 4, 7, 21 (one replicate per concentration and control) and 12 (all replicates) of the exposure period into solvent cleaned glass bottles, spiked with 5 mL methanol (Sigma-Aldrich GmbH, Switzerland) and extracted onto pre-conditioned Sep-Pak Classic C18 cartridges (Waters corp., USA). The cartridges were air dried and stored until further processing at -40 °C. After defrosting and air drying, the cartridges were eluted into solvent cleaned amber glass tubes using 5 mL methanol. The methanol extracts were dried under a N_2 stream and redissolved in 1 mL ethanol (Sigma-Aldrich GmbH, Switzerland). E2 and ZON standard were tested in a serial dilution of 12 steps and the ethanol extracts of the water samples in a serial dilution of 5 steps, each in duplicate. The rYES was conducted following the methods described by Routledge & Sumpter (1996).

2.6 Chemical analysis

To measure ZON concentrations in the exposure tanks, 100 mL water samples were collected on day 4, 21 (one tank per concentration and control) and 12 (all replicates) of the exposure period into solvent cleaned glass bottles and sent within 24 h to the chemical laboratory (Agroscope Reckenholz-Tänikon Research Station ART, Switzerland). A HPLC-negative electrospray ionization (-ESI)-MS/MS analytical method with a preparatory solid-phase extraction was used to analyze the water samples following the methods described by Hartmann et al. (2007).

2.7 Fish sampling and analysing

On day 42 of the experiment all adult zebrafish were euthanized in 150 mg/L MS222 buffered with 300 mg/L $NaCO_3$ to maintain a neutral pH. The fish were wet weighed to the nearest 0.01 g and total length determined to the nearest mm. A 5 – 10 µL blood sample was taken by cardiac puncture using a heparinised (1000 Units heparin/mL, Heparin ammonium salt, Sigma-Aldrich GmbH, Switzerland) insulin syringe (Becton Dickinson & Company, USA), transferred in an Eppendorf reaction tube and centrifuged for 5 min. at 4 °C and 9300 g. The 3 – 5 µL plasma per sample was transferred to a new reaction tube and stored at -80 °C in the dark for later analysis of VTG concentration using a commercially available zebrafish ELISA kit (Prod. No. V01008402, Biosense Laboratories, Norway). The abdominal cavity was opened with dissecting

scissors and the whole fish fixed for 24 h in Bouin's fixative (Sigma-Aldrich GmbH, Switzerland).

For histological examination the head and tail of the fixed fish were removed and the trunk cut into four slices (~ 3 mm thickness) using a microtome blade (SEC 35, Microm International GmbH, Germany). Subsequently the samples were dehydrated in an ascending ethanol series, incubated in Roti Histol (Carl Roth GmbH + Co. KG, Germany) and paraffinized (Roti-Plast, Carl Roth GmbH + Co. KG, Germany). All four samples per fish were embedded in one standard embedding cassette. This enabled simultaneous whole body transverse sectioning of different areas. Transverse histological sections (9 µm thickness) were stained with standard hematoxylin and eosin (H&E, Carl Roth GmbH + Co. KG, Germany).

Digital photomicrographs were taken (Nikon ACT-1 version 2.63, Nikon Corporation, Japan) from male and female gonads and analyzed using Adobe Photoshop CS3 Extended version 10.0.1 (Adobe Systems Inc., USA). For the male gonads four photos, two per testis, of a routine section were taken at 400x magnification. The development stage of the sperm cells was classified in three major classes according to Dietrich & Krieger (2009), namely spermatogonia, spermatocytes and spermatides. The photos were analyzed by measuring the surface area of the three different classes and calculating the relative proportion of each class. For the female both gonads on a routine section were photographed completely at 40x magnification. The developmental stage of the oocytes was classified into three major classes according to Dietrich & Krieger (2009), namely perinucleolar, cortical alveolar and vitellogenic oocytes. The photos were analyzed by counting the cells and determining the relative ratio of each class.

2.8 Statistics

To investigate effects of the ZON exposure, data of exposed groups were compared to control groups using SigmaPlot version 9.01 and SigmaStat version 3.1 (Systat Software Inc., USA). For data of reproductive output the ratio of the 21 day exposure period to the 21 day pre-exposure period was calculated resulting in relative spawning frequency, fecundity, clutch size, hatch rate and embryo survival. Data of reproductive output ratio, body weight, length and histomorphometry met the assumptions of normality and equality of variance permitting to use one-way analysis of variance (ANOVA) followed by a Dunnett test. Data of fertilization rate and plasma VTG concentrations were $\log_{10}$ transformed prior to analysis to meet the assumptions of normality and equality of variance. Analysis of correlation between relative fecundity and $\log_{10}$ transformed VTG concentration was conducted using Pearson Product Moment Correlation.

3 Results

3.1 Estrogenic activity of ZON and aqueous concentration

To determine the relative estrogenic potency we compared ZON to E2 using a rYES. E2 as well as ZON evoked a sigmoid concentration response curve with a mean EC_{50} of 2 µg/L and 500 µg/L, respectively, resulting in an E2 : ZON EC_{50} ratio of 1 : 250 (Fig. 1). Both curves ran almost parallel and provoked comparable maximal receptor activation.

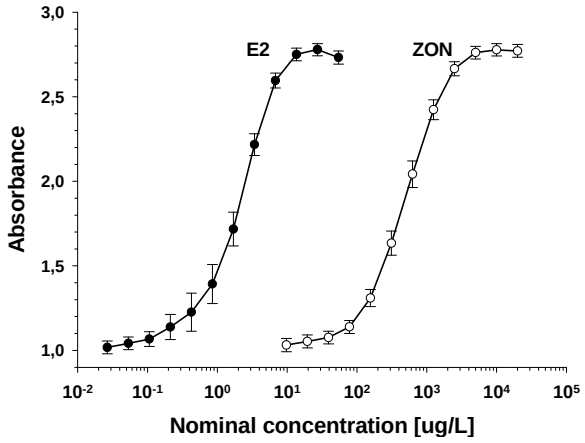


Fig. 1: Concentration response curve for 17β-estradiol (E2) and Zearalenone (ZON) in the recombinant yeast estrogen screen (mean ± SEM, n = 12).

A combination of analytical measurement for ZON and *in vitro* analysis (rYES) for determination of estrogenic activity induced by ZON were used to quantify the content of mycotoxin in the exposure tanks. Analytically measured ZON mean concentrations ranged between 57.5 and 84.4 % of nominal values and were always lower than mean concentrations determined *in vitro*, expressed as estrogen equivalent (EEQ) for ZON, which ranged between 92.5 and 247.5 % of nominal values (Table 1). In the control group no ZON could be detected (LOQ = 1.5 – 2.4 ng/L) and estrogenic activity was below detection limit (LOQ ≈ 80 ng/L EEQ).

Table 1: Analytically measured concentrations of zearalenone (ZON) and *in vitro* (recombinant yeast estrogen screen, rYES) determined estrogen equivalent for ZON (EEQ) for exposure groups and control (mean ± SEM). Analytical chemistry: one replicate per group on day 4, 12, 21 (n = 3), all replicates on day 12 (n = 4); rYES: one replicate per group on day 4, 7, 12, 21 (n = 4), all replicates on day 12 (n = 4).

Nominal concentration	**Mean measured concentrations (ng/L)**			
ZON	**Analytical chemistry (ZON)**		**Recombinant yeast screen (EEQ)**	
	day 4, 12, 21	**day 12**	**day 4, 7, 12, 21**	**day 12**
Control	BD[a]	BD[a]	BD[a]	BD[a]
100 ng/L	73 ± 6	84 ± 2	248 ± 54	217 ± 55
320 ng/L	229 ± 9	233 ± 5	476 ± 75	656 ± 104
1000 ng/L	728 ± 89	575 ± 14	925 ± 66	1078 ± 76
3200 ng/L	2549 ± 407	2327 ± 66	3315 ± 317	4104 ± 165

[a] BD = below detection limit

3.2 Effects on mortality, weight, body length and gonad morphology

There was no evidence that exposure for 21 days to a concentration up to 3200 ng/L ZON affected survival, weight and body length of the male or female zebrafish. Similarly, we found no effect on gonad morphology (data not shown). The testes of all males in both control and ZON exposed fish were regular in structure and the tubules contained cysts showing a full spectrum of sperm cell differentiation stages with no significant differences between the groups in terms of the ratio of the various stages of development. None of the testes examined contained oocytes. Similarly, the ovaries of the control and ZON exposed fish showed a full range of the different oocyte developmental stages with no significant differences between the groups in terms of the proportions of oocytes at the various stages of development.

3.3 Effects on reproductive performance and VTG induction

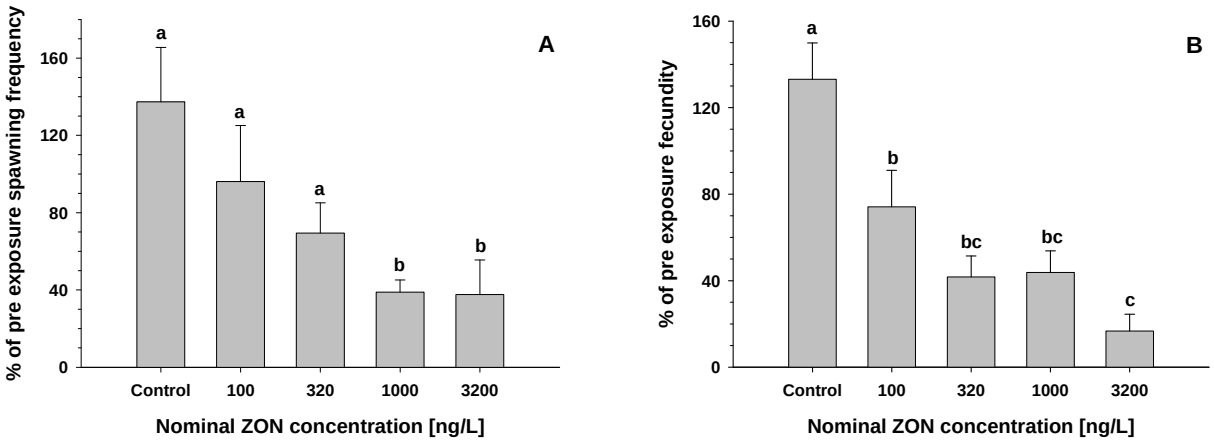
Details of the reproductive performance for each exposure group during pre-exposure and exposure period are presented in Table 2. Assessment of egg production demonstrated a concentration dependent decrease for relative spawning frequency as well as for relative fecundity during 21 days of exposure to ZON (Fig. 2 A, B). Mean relative spawning frequency during exposure period was 137.4 % for control group and significantly reduced at 1000 and 3200 ng/L ZON ($p < 0.05$) to 38.9 and 37.6 %, respectively, whereas the two lowest concentrations reached values of 96.1 and 69.4 %, respectively. Relative fecundity revealed a significant decrease in all breeding groups exposed to ZON ($p < 0.05$). Mean relative fecundity

for control was 133.1 % and the ZON exposed groups exhibited values of 74.2, 41.7, 43.8 and 16.7 % for 100, 300, 1000 and 3200 ng/L, respectively.

Data for relative clutch size displayed a slightly different pattern (Fig. 2 C). Whereas the two lowest and the highest ZON concentrations showed a negative concentration related response during exposure period, the value for 1000 ng/L reached with 109.5 % approximately the same value as control with 108.7 %. No effects on fertility, hatch and embryo survival after exposure to ZON up to a concentration of 3200 ng/L could be detected.

Table 2: Reproductive performance of zebrafish during 21 days pre-exposure and 21 days exposure to zearalenone (ZON) in the reproduction experiment (mean ± SEM; n = 4).

Exposure Group	**Reproductive performance**					
ZON	**Spawning frequency (spawnings per day)**		**Fecundity (eggs per day)**		**Clutch size (eggs per spawning)**	
	pre-exposure	**exposure**	**pre-exposure**	**exposure**	**pre-exposure**	**exposure**
Control	0.50 ± 0.10	0.63 ± 0.10	38.54 ± 9.7	54.89 ± 17.9	76.81 ± 11.9	81.21 ± 18.5
100 ng/L	0.57 ± 0.09	0.50 ± 0.09	69.37 ± 22.0	47.63 ± 13.8	131.11 ± 45.5	99.34 ± 30.5
320 ng/L	0.51 ± 0.05	0.37 ± 0.11	85.57 ± 21.6	37.06 ± 14.1	160.36 ± 30.1	92.09 ± 10.3
1000 ng/L	0.45 ± 0.03	0.18 ± 0.04	25.81 ± 5.2	9.93 ± 1.9	57.22 ± 12.0	56.93 ± 3.9
3200 ng/L	0.39 ± 0.04	0.17 ± 0.08	24.44 ± 6.8	4.65 ± 2.7	60.15 ± 14.8	18.18 ± 7.2



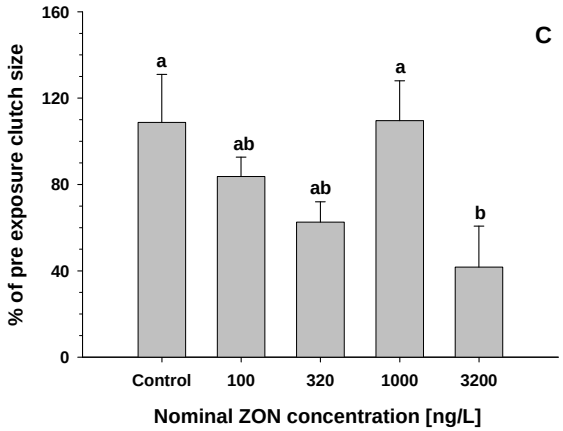


Fig. 2: Relative (A) spawning frequency, (B) fecundity and (C) clutch size for spawning groups of zebrafish (4 males, 2 females) exposed for 21 days to zearalenone (ZON) and water control in relation to pre-exposure period (mean ± SEM, n = 4). Significant differences between groups are denoted by different letters (a, b, c; $p < 0.05$).

After 21 days exposure, a concentration related increase in plasma VTG was observed in male zebrafish at ZON concentrations from 320 to 3200 ng/L (Fig. 3). There was a significant 4.4 and 8.1 fold induction of VTG at 1000 and 3200 ng/L ZON relative to control, respectively ($p < 0.05$). Analysis of correlation revealed a significant negative correlation between plasma VTG concentration and relative fecundity ($r = -0.558$, $p = 0.01$).

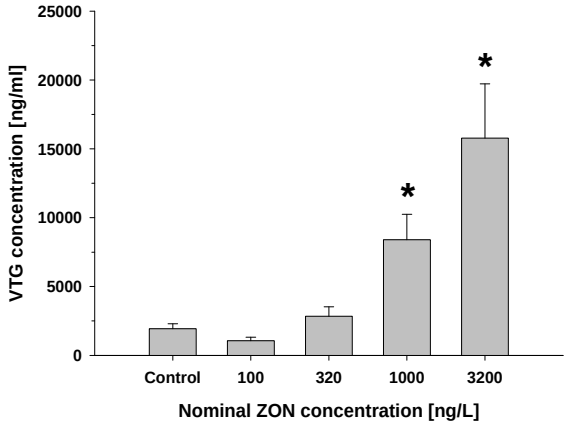


Fig. 3: Vitellogenin (VTG) concentration in plasma of male zebrafish exposed for 21 days to zearalenone (ZON) and water control (mean ± SEM, n = 4). Significant differences from control are denoted as * ($p < 0.05$).

4 Discussion

The estrogenic potency of ZON was compared to the natural steroid estrogen E2 *in vitro*, in a rYES, and its effects on morphology, reproduction and physiology of zebrafish were investigated *in vivo*, in a 42 day reproduction experiment. References on ZON and E2 provided in the following text are based on nominal concentrations.

4.1 Estrogenic activity of ZON

The estrogenic potency, as ability to bind to the ER, activate the receptor and express hormone controlled genes was determined in a rYES transfected with hER. ZON revealed a moderate estrogenic activity in the present study with an E2 : ZON EC_{50} ratio of 1 : 250. This is in accordance with Bovee et al. (2004) and Le Guevel & Pakdel (2001) who observed an E2 : ZON EC_{50} ratio of 1 : 205 – 391 in an rYES expressing the hERα and β. Le Guevel & Pakdel (2001) also applied a rYES expressing rainbow trout ER (rtER), resulting in a E2 : ZON EC_{50} ratio of 1 : 13.9.

Several studies reported E2 : ZON EC_{50} ratios for the binding affinity (BA) of E2 and ZON to human and fish ER (EC_{50} are calculated in g/L): 1 : 27.1 (hERα), 1: 14.6 (hERβ; Takemura et al. 2007) 1 : 129.9 (hER), 1 : 46.0 (rtER; Olsen et al. 2005, Tollefsen et al. 2002) and 1 : 350 (rtER; Arukwe et al. 1999). Ratios for hER : rtER EC_{50} are 1 : 1.3 (ZON), 1 : 3.7 (E2; Olsen et al. 2005) and 1 : 10 (E2; Le Drean et al. 1995). Data derived from rYES experiments imply that ZON in relation to E2 is approximately 14.8 – 28.1 fold more potent for rtER, a fish ER, than it is for hER, a mammal ER. Results from ER-BA studies, however, display a wider range for the estrogenic potency of ZON namely 14.6 – 129.9 and 46 – 350 times lower for hER and rtER compared to E2, respectively. The BA to rtER for E2 and ZON is 3.7 – 10 and 1.3 times lower than to hER, respectively, indicating only small difference in species sensitivity. Altogether the presented *in vitro* data suggest that relative estrogenic potency for ZON is 14.6 – 391 and 13.9 – 350 fold lower for hER and rtER, respectively, in relation to E2.

4.2 Aqueous ZON concentration

The quantified ZON concentrations for both methods analytical chemistry and rYES were stable in the exposure groups and undetectable in the control groups during the entire exposure period. The ZON concentrations determined in the rYES (92.5 – 247.5 % of nominal) were consistently higher than the analytically measured (71.5 – 84.4 % of nominal) values. An explanation for this

could be that in the rYES the overall estrogenicity is determined and, hence, other possible sources such as ZON metabolites could have contributed to a background estrogenic activity. Data on biotic transformation of ZON generally indicate as a main metabolization pathway the reduction of the 6′-keton to yield α- and β-ZOL. The estrogenic potency of α-ZOL in the rYES is approximately five fold higher than that of ZON (El-Sharkawy & Abul-Hajj 1988, Le Guevel & Pakdel 2001). Therefore, α-ZOL might have contributed to the high estrogenicity in the rYES.

4.3 Effects on mortality, weight, body length and gonad morphology

The absence of any mortality and the fact that fish were looking healthy and showed no signs of behavioural modification indicate that exposure to concentrations up to 3200 ng/L ZON up to 21 days does not have severe acute toxic effects on zebrafish. This is in accordance with data from toxicological studies demonstrating that apart from estrogenic effects ZON exhibits a low acute toxicity in many animal species (Kuiper-Goodman et al. 1987).

In this study we could not find an effect of exposure for 21 days to ZON up to a concentration of 3200 ng/L on gonad morphology. Brion et al. (2004) did not observe effects in adult female zebrafish in terms of the proportion of oocytes at the various stages of development and active spermatogenesis in adult male zebrafish after exposure for 21 days up to 100 ng/L E2. A factor of 32 calculated from these two maximal concentrations suggest that the results of Brion et al. (2004) are in accordance with our histology outcome considering a 13.9 – 350 weaker estrogenic potency for ZON in comparison to E2, resulting from *in vitro* data. At 2724 ng/L E2, a significant increase of spermatogonia surface area in testis and a lack of advanced maturation stages in ovary but no effect at 272.4 ng/L were stated in the study of Van der Ven et al. (2003). However, even based on the lowest estrogenic potency factor of 13.9 between ZON and E2 resulting from *in vitro* studies, a concentration of 2724 ng/L E2 corresponds to an approximately 11 fold higher ZON concentration than the maximum used in the present study.

4.4 Effects on reproductive performance

To assess potential effects of EASs on the population level their effects on the reproductive output needs to be understood. In control groups, an increased spawning frequency during the 21 day exposure period compared to pre-exposure period was observed, while in ZON exposed groups the spawning frequency decreased (significant at 1000 and 3200 ng/L). Relative fecundity revealed a significant decrease in all spawning groups exposed to ZON in comparison to control. With the exception of 1000 ng/L ZON, egg production was related to both, a

decreased spawning frequency and clutch size. Whether this impaired reproductive capacity was due to adverse effects on male and/or female reproductive function was not examined in the present study, since both sexes were exposed to the test chemical. As no differences in spermatocyte and oocyte development were histologically observable between the different treatments, one possible explanation for a reduced fecundity might be a disruption of mating behavior (Bjerselius et al. 2001, Gray et al. 1999).

Brion et al. (2004) demonstrated a decrease in relative fecundity during 21 days exposure of zebrafish to 25 and 100 ng/L E2 (following 21 days pre-exposure) to 80 and 75 %, respectively (n = 1). The authors reported that this effect was only related to a decreased spawning frequency. Van der Ven et al. (2007) observed a significant concentration dependent decrease of spawning frequency in zebrafish exposed to 27.2, 87.2 and 272.4 ng/L E2 for 21 days in comparison to control. However, this effect was counterbalanced by increased clutch size, so that fecundity during this period revealed no effect.

With a factor of 1 : 3.7 – 10 calculated from the lowest observed effect concentration (LOEC) of 1000 ng/L ZON in the present study relative to the maximal concentration of 100 (no significant effect) and 272.4 ng/L E2 reported by Brion et al. (2004) and Van der Ven et al. (2007), ZON revealed a comparably strong effect on spawning frequency. With a LOEC of 100 ng/L ZON resulting in a factor of 1 : 0.4 – 1, ZON demonstrated an even higher potency relative to E2 to affect fecundity.

Altogether the presented *in vivo* data suggest a factor of 1 : 0.4 – 10 for ZON relative to E2 to affect reproductive output. This is considerably lower than the estrogen potency factor of 1 : 13.9 – 350 for ZON relative to E2 calculated from *in vitro* data for rYES and ER-BA studies, indicating that ZON exhibits a comparatively strong effect on zebrafish reproduction. However, we could not detect any effect on fertility, hatch and embryo survival caused by exposure to concentrations up to 3200 ng/L ZON up to 21 days which is in accordance with Brion et al. (2004) and Van der Ven et al. (2007) who did not observe effects on fertilization rates and hatching success after exposure for 21 days up to 100 and 272.4 ng/L E2, respectively.

4.5 Effects on VTG induction

The observed concentration dependent increase of plasma VTG in male zebrafish after 21 days of exposure (significant 4.4 and 8.1 fold induction in 1000 and 3200 ng/L, respectively) clearly demonstrates the estrogenic potency of ZON to affect fish in waterborne exposure and the sensitivity of zebrafish to the mycotoxin, resulting in an abnormal VTG induction.

In the only other *in vivo* fish study with ZON, Arukwe et al. (1999) observed a elevation of plasma VTG in juvenile Atlantic salmon (*Salmo salar*) within seven days after a single intraperitoneal injection with 1 and 10 mg/kg ZON and a significant 32 fold induction in 10 mg/kg in relation to control. The authors compared this exposure to a single E2 injection of 5 mg/kg, resulting in a significant 87 fold induction of plasma VTG. A calculation based on the levels of VTG induction in this study (and on the assumption that 10 mg/kg E2 results in a 174 fold VTG induction) suggests that ZON is 5.4 fold less potent to induce VTG *in vivo* in relation to E2.

Olsen et al. (2005) and Tollefsen et al. (2003) found in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon primary hepatocytes an E2 : ZON EC_{50} ratio of 1 : 18.7 and 1 : 1438.9 for the potency to induce VTG, respectively. Based on this *in vitro* data a calculation suggests an 18.7 – 1438.9 fold lower potency for ZON to induce VTG in comparison to E2 which is 3.5 – 452.4 fold lower than the calculation based on the *in vivo* result from Arukwe et al. (1999). In contrast to the rather high difference in species sensitivity presented by these two *in vitro* studies, Van den Belt et al. (2003b) observed in adult male zebrafish and juvenile rainbow trout exposed for 21 days to 20 ng/L E2 a significant 130 and 30 fold, and to 100 ng/L E2 a 115 and 150 fold induction of plasma VTG, respectively, in relation to control, indicating only a minor difference in species sensitivity *in vivo*.

Assuming there is only a small difference in species sensitivity and taken a factor of 5.4, based on the study of Arukwe et al. (1999), our effect concentrations of 1000 and 3200 ng/L ZON correspond to 185 and 593 ng/L E2. This concentration is in the same order of magnitude as applied in the study of Seki et al. (2006) who demonstrated a significant VTG induction in blood of adult male zebrafish exposed for 21 days to 100 ng/L E2, as well as Brion et al. (2004) who reported a 4 and 2667 fold VTG induction, respectively, in whole body homogenate of adult male zebrafish in relation to control after 21 days exposure to 25 and 100 ng/L E2.

4.6 Evaluation of *in vitro* and *in vivo* results

A factor of 1 : 13.9 – 350 for the estrogenic potency of ZON relative to E2 calculated from *in vitro* data for rYES and ER-BA studies suggests an estrogenic potency that is one order of magnitude lower than the factor of 1 : 5.4 based on calculation from *in vivo* VTG induction. This high estrogenic potency *in vivo* corresponds to the comparatively strong effects on reproduction we observed for ZON in the present study.

In vitro results based on EC_{50} values of the rYES might substantially underestimate the estrogenic potency *in vivo*, especially for compounds where metabolisation or bioaccumulation

plays a crucial role during *in vivo* exposure. Van den Belt et al. (2004) found the relative estrogenic potency for EE2 *in vitro* (rYES) to be almost equipotent and *in vivo* (zebrafish VTG induction) to be about 30 times higher than E2. The authors suggested one explanation may be the lower susceptibility of EE2 for metabolization compared to endogenous steroids.

Hence, from the results of this study we suggest that ZON may not only be a potent active estrogen itself but that its rather strong estrogenicity *in vivo* is also a result of its metabolic products such as α-ZOL. This is in line with the observation of Kuiper-Goodman et al. (1987) who reported that the most sensitive species for reproduction alteration caused by ZON is pig and α-ZOL is the major metabolite in this species.

4.7 Environmental relevance

ZON was reported to occur in surface waters in concentrations of 0.5 – 43.7 ng/L (Gromadzka et al. 2009, Lagana et al. 2004). However, the actual exposure in a given catchment depends on many factors, such as *Fusarium* infection rates, meteorology and hydrodynamics (Bucheli et al. 2008, Hartmann et al. 2008b). This is about half the concentration we presented as LOEC for zebrafish reproduction in this study (reduced fecundity at 100 ng/L). Effluent concentrations of 1 – 220 ng/L ZON (Lagana et al. 2001, 2004, Lundgren & Novak 2009) are up to 2.2 times higher than determined LOECs for fecundity, spawning frequency (1000 ng/L) and VTG induction (1000 ng/L).

Evaluating the environmental relevance of this data taking into consideration the highest detected concentrations and possibly higher species sensitivity, long term/chronic exposure, a regional and temporal limited exposure to higher concentrations (caused by effluent discharge in combination with low flow conditions, or emission from Fusarium infected wheat fields) and mixture effects in combination with other EASs, ZON might pose a risk for fish in their natural environment.

5 Conclusions

The results of the present study confirm the estrogenic potential of ZON *in vitro* (rYES) and *in vivo* (zebrafish). We demonstrated that although ZON possesses a moderate estrogenic potency *in vitro*, it exhibits a comparably strong effect on induction of VTG and reproduction *in vivo* following waterborne short-term exposure.

Although there is not much data for ZON concentrations in the environment available and the measured values in surface waters are below the LOEC we determined to cause negative effects on fish, there might be a certain risk for wild fish to be harmed by ZON in their natural habitat. Further studies are needed to investigate the consequences of long term/chronic exposure (for entire life or even for several generations) to ZON as well as effects on different (more critical) life stages.

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Chapter IV

Life-cycle exposure to the estrogenic mycotoxin zearalenone affects zebrafish (*Danio rerio*) development and reproduction

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Abstract

Zearalenone (ZON) is one of the worldwide most common mycotoxins and exhibits estrogenic activity in the range of natural steroid estrogens. The occurrence of ZON has been reported in soil, drainage water, wastewater effluents and rivers, but its ecotoxicological effects on fish have hardly been investigated.

The consequences of continuous long-term ZON exposure, including a subsequent depuration period, as well as transgenerational effects of F0 short-term exposure on F1 generation were investigated. Effects on growth, reproduction activity, physiology and morphology of zebrafish (*Danio rerio*) were examined in a 182 day live-cycle experiment.

Live-long exposure to ZON for 140 days increased wet weight, body length and condition factor of female fish at 1000 ng/L and sex ratio was shifted towards female from 320 ng/L ZON. Only females at 1000 ng/L ZON revealed a 1.5 fold induction of plasma vitellogenin (VTG). Relative fecundity at 1000 ng/L recovered significantly during the depuration period. An increased condition factor in adult female F1 fish implies that exposure of F0 generation to 1000 ng/L ZON affect growth of F1 generation. A negative correlation between relative fecundity in the F1 generation (all groups exposed to 320 ng/L ZON) and the nominal ZON concentrations of the F0 exposure might indicate an influence of F0 exposure on reproductive performance of F1 generation. No exposure scenario affected fertility, hatch, embryo survival and gonad morphology of zebrafish.

Evaluating the environmental relevance of this data, the risk for fish to be harmed by exposure to ZON solely seems rather marginal but ZON might contribute to the overall estrogenicity in the environment.

1 Introduction

Endocrine active substances (EASs) exert their effects by mimicking or antagonizing endogenous hormones, influencing the natural hormone synthesis, metabolism or elimination, or modifying hormone receptor levels, and therefore have the potential to interfere with reproduction and development (Jobling and Tyler 2003, Sumpter 2005). Although sex determination in fish is under genetic control, the final differentiation of the gonads also depends on endocrine signals (Arcand-Hoy and Benson 1998, Campbell and Hutchinson 1998, Devlin and Nagahama 2002). Changes in sex hormone levels can affect the final sex independently of the genetic sex and exposure to EAS during development can lead to sex reversal (Andersen et al. 2003, Donaldson and Hunter 1982). Among the EASs, primary concerns are therefore on substances with estrogenic activity. These can be anthropogenic, such as certain pharmaceuticals, pesticides and industrial chemicals or natural, such as phyto- and mycoestrogens. Effects like inhibited testicular growth, testis abnormalities and intersex caused by estrogens have already been detected in wild fish populations (Milnes et al. 2006, Sumpter 2005).

Zearalenone (ZON) is one of the worldwide most common mycotoxins and belongs to the chemical group of the resorcyclic acid lactones (RALs; Chelkowski 1998). RALs show estrogenic activities that are in the range of natural steroid estrogens (Le Guevel and Pakdel 2001) and they occur as a result of *Fusarium* spec. (primarily *F. graminearum*) infection of corn, wheat and other cereals (Champeil et al. 2004). Agricultural products around the world show contamination rates for ZON of up to 69 % of the tested samples with concentrations of up to 180 µg/kg (Rhyn and Zoller 2003).

The occurrence of ZON was reported in Italian and Polish rivers in concentrations of 2 – 5 and 0.5 – 44 ng/L, respectively (Gromadzka et al. 2009, Lagana et al. 2004). It was further detected in US (Kolpin et al. 2010) and Swiss rivers (Bucheli et al. 2008) below limits of quantitation. Up to 35 ng/L and 4 ng/g ZON was quantified in drainage water and soil, respectively, in a field study with wheat and maize (Hartmann et al. 2008a, 2008b). In Italian waste water treatment plants and US American industrial wastewater effluents ZON concentrations of 1 – 10 and 95.5 – 220 ng/L, respectively, were measured (Lagana et al. 2001, 2004, Lundgren and Novak 2009).

Little is known about the ecotoxicological impact of ZON at levels found in the environment and the consequence of exposure to aquatic organisms. Only a few short-term *in vivo* studies investigated ZON and its metabolites and observed effects on vitellogenin (VTG) induction, zona radiata proteins, immune system and growth in fish after exposure via injection or food

(Arukwe et al. 1999, Celius et al. 2000, Keles et al. 2002). In a reproduction experiment, we documented recently a reduced fecundity in zebrafish exposed for 21 days to 100 ng/L and an increased plasma VTG concentration in males at 1000 ng/L ZON (Schwartz et al. 2010).

The zebrafish (*Danio rerio*) was selected in the present study as a model to assess estrogen-mediated responses to ZON in a waterborne exposure. This species is easy to keep and readily reproducing in the laboratory, has a rapid development and short generation time, and is widely used in many standard ecotoxicological tests in OECD guidelines. Several studies in zebrafish have shown that estrogens such as the natural 17β-estradiol (E2) or the synthetic 17α-ethinylestradiol (EE2) induce the yolk precursor protein VTG, affect gonad development, induce atresia of oocytes and inhibition of spermatogenesis, and have an impact on egg production and viability, fertilization success, sexual differentiation and sex ratios (Andersen et al. 2003, Brion et al. 2004, Hill and Janz 2003, Maack and Segner 2004, Nash et al. 2004, Örn et al. 2003, Schäfers et al. 2007, Segner et al. 2003, Van den Belt et al. 2003, Van der Ven et al. 2007). EE2 was selected as test chemical in many studies since it is a potent synthetic steroid estrogen, a common component of oral contraceptives and known to enter the aquatic environment via domestic wastewater (Metcalfe et al. 2001, Williams et al. 1999).

Reduced reproductive success may result from disruption of reproductive development; reduced female fecundity and male vitality, altered reproductive behaviour, and disruption of breeding dynamics (Balch et al. 2004). To fully assess the potential of EASs to disrupt reproduction and cause transgenerational effects, multigenerational life-cycle exposures are needed that include all relevant life stages and developmental parameters (Metcalfe et al. 2001, Van den Belt et al. 2003).

The aim of the present investigation was to study the estrogenic potency of ZON in respect to its effects on zebrafish in a life-cycle exposure. The study was designed to investigate possible effects of continuous long-term exposure including a subsequent depuration period as well as possible transgenerational effects of F0 exposure on F1 generation (Fig. 1). For this experiment F1 fish of a parental generation were used which was exposed to ZON for 21 days (Schwartz et al. 2010). With these fish we conducted a 140 day exposure experiment from embryo to adult. In a subsequent 42 day reproduction experiment spawning groups of the adult fish were either exposed to ZON (after growing up in clean water) or clean water (after growing up under ZON exposure). To evaluate effects of endocrine disruption, developmental (mortality, growth sex ratio and gonad histomorphometry), reproduction (spawning frequency, fecundity, fertility and hatch) as well as physiological (VTG induction) parameters were assessed. In the following we differentiate between the previously published ‘reproduction experiment F0’ (Schwartz et al. 2010) and the ‘juvenile exposure F1’ (raise of F1 generation under corresponding ZON exposure

for 140 days) and ‘reproduction experiment F1’ (reproduction experiment with F1 generation) presented here (Fig. 1).

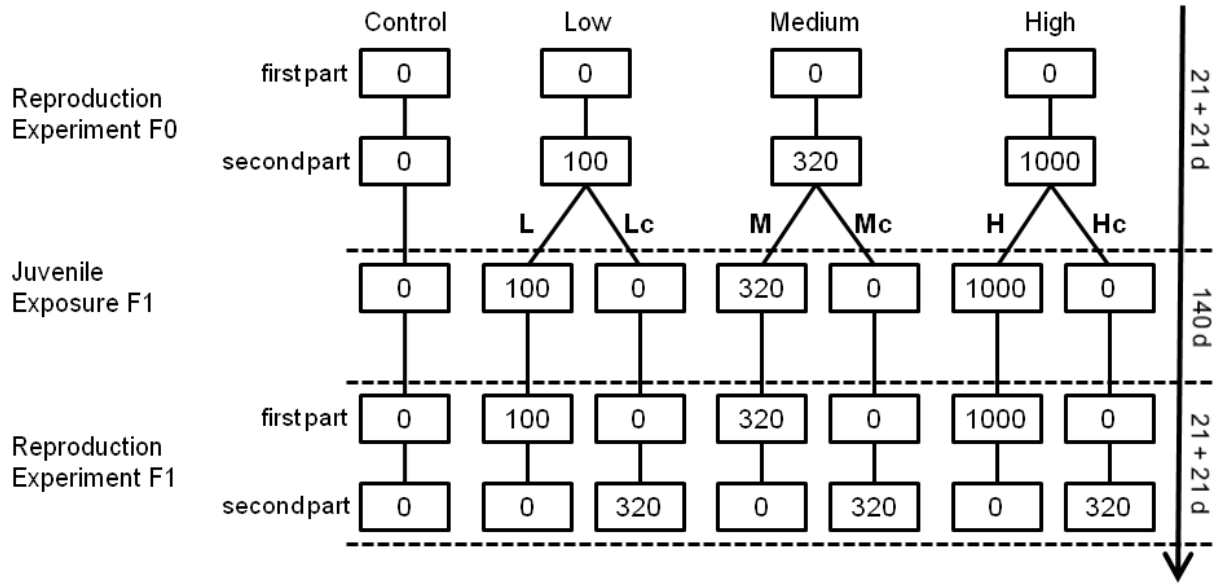

Fig. 1: Exposure scheme of the complete zearalenone (ZON) life-cycle study. Nominal concentrations in the respective phases of the experiments are stated in the boxes in ng/L ZON and exposure periods on the vertical arrow are indicated in days. Sampling points are illustrated as horizontal broken lines. Group L, M and H: life-long ZON exposure and subsequent depuration; group Lc, Mc and Hc: raised in clean water and short-term ZON exposure; Reproduction experiment F0: Schwartz et al. (2010).

2 Materials and Methods

2.1 Experimental animals

All procedures concerning experimental animals were performed in compliance with the Swiss protection of animals act. The zebrafish eggs used in this study derived from the reproduction experiment F0 which was conducted with adult zebrafish originating from our fish breeding stocks, held at the University of Basel (Schwartz et al. 2010).

2.2 Test chemical

A stock solution of 0.5 mg/mL zearalenone (ZON, purity: 99 %, Sigma-Aldrich GmbH, Switzerland) was prepared in acetone (purity: 99.9 %, Carl Roth GmbH + Co. KG, Germany) and stored in a glass bottle at 4 °C in the dark. This stock solution was used to prepare solvent free supply solutions every 4 days for use in the exposure system. The supply solutions were prepared by coating a 1 L glass bottle with 2 mL of stock solution and removing the solvent under a gentle stream of N_2. The bottle was then filled up to 1 L with conditioned water (1 : 1 mix of charcoal filtered tap water and reverse osmosis water) to give a concentration of 1 mg/L ZON and stirred over-night with a magnetic stirrer prior to introduction to the exposure system.

2.3 Juvenile exposure F1: Exposure system

The zebrafish (eggs to adults) were exposed in a flow-through system in 12 L glass tanks with four replicate tanks for each exposure group and the water control. All tanks were aerated and tempered to 26 ± 1 °C using submerged electrical heaters, and a light : dark photoperiod of 16 : 8 h was maintained during the duration of the experiment. Flow indicators (Sho-Rate 1355, Brooks Instrument, Netherlands) were used to regulate the flow of 6 L/h conditioned water into vitreous mixing chambers, equipped with magnetic stirrers. The ZON supply solution was dosed into the three mixing chambers of the exposed groups by means of a computer controlled dispenser (Microlab 541C Dispenser, Hamilton, Switzerland) at a rate of 600, 1920 and 6000 µL/h giving nominal exposure concentrations of 100, 320 and 1000 ng/L, respectively. Each tank was supplied from the mixing chambers through Teflon tubing (Carl Roth GmbH + Co. KG, Germany) using peristaltic pumps (Ismatec ISM 944D, Ismatec, Switzerland) with a flow rate of 1 L/h and allocated to the different concentrations and negative control, respectively, using a random generator (www.randomizer.org).

Dissolved O_2 (Oxi 315i, WTW, Germany), pH (pH 315i, WTW, Germany), conductivity (Cond 315i, WTW, Germany) and temperature was monitored once a week. O_2 was always above 7.0 mg/L, pH at 7.8 – 8.2, conductivity at 230 – 260 µS/cm and temperature at 25 – 27 °C.

2.4 Reproduction experiment F1: Exposure system

The adult zebrafish were exposed in a flow-through system in 12 L glass tanks with four replicate tanks for each exposure group and the water control. For group H only three replicates were used because in one replicate of the juvenile exposure F1 not enough male fish survived for assembling of a spawning group. All tanks were aerated and tempered to 26 ± 1 °C and a light : dark photoperiod of 16 : 8 h was maintained during the duration of the experiment. The exposure system for the first part of the reproduction experiment F1 had the same design as the system for the juvenile exposure F1 (see section 2.3). For the second part the ZON supply solution was dosed into the three mixing chambers of the exposed groups at a speed of 1920 µL/h giving just one exposure concentration of 320 ng/L.

Each tank was equipped with a spawning tray consisting of a stainless steel tray (265 × 185 × 30 mm) covered with a stainless steel mesh (mesh size: 2.5 mm). To minimize the handling stress for the fish, stainless steel cable ties were attached to the trays for manipulation from outside the water. Artificial weed (Clear water coarse Filtering wool, Dennerle GmbH, Germany) was attached to the centre of the steel mesh and surrounded by a few stained glass stones to provide an appropriate spawning stimulation.

Dissolved O_2, pH, conductivity and temperature was monitored every fourth day. O_2 was always above 7.2 mg/L, pH at 8.0 – 8.3, conductivity at 210 – 240 µS/cm and temperature at 25 – 27 °C.

2.5 Juvenile exposure F1: Experimental design

Between 80 and 440 eggs originating from one spawning event of each spawning group from the parental generation (F0) of the reproduction experiment F0 (Schwartz et al. 2010) were divided in halves (40 – 220 eggs) and transferred within four hours post fertilization (hpf) into egg cups. The egg cups, consisting of a glass tubing (80 × 60 mm) closed with a stainless steel mesh (mesh size: 0.355 mm) at the bottom side, were suspended in the 12 L exposure tanks. One half of each clutch was continuously exposed to the same ZON concentration as their parents (L = 100, M = 320, H = 1000 ng/L ZON) and the other half, as well as all eggs from control were reared in clean conditioned water (Lc, Mc, Hc, control). This F1 generation was raised under the described exposure conditions for 140 days until maturity (Fig. 1).

From hatch till 8 days post hatch (dph) the larvae were fed rotifers (from our internal culture) and from 8 to 10 dph rotifers and freshly hatched *Artemia salina* nauplia twice daily. From 11 dph till the end of the experiment they were fed freshly hatched *Artemia salina* nauplia twice and dry pellet (SilverCup, H. U. Hofmann AG, Switzerland) once per day.

2.6 Reproduction experiment F1: Experimental design

Each exposure tank was stocked with a spawning group consisting of two female and four male zebrafish from the juvenile exposure F1 experiment. After an acclimatization period of one week with the same exposure conditions as in the juvenile exposure, the first part of the reproduction experiment F1 started with unchanged exposure conditions for 21 days (control, Lc, Mc, Hc = 0 ng/L and L = 100 ng/L, M = 320 ng/L, H = 1000 ng/L ZON). For the second part, the following 21 day period, exposure conditions changed for Lc, Mc, Hc to 320 ng/L ZON (exposure period) and for L, M, H to 0 ng/L (depuration period; Fig. 1). During the whole experiment the animals were fed freshly hatched *Artemia salina* nauplia twice and dry pellet once per day.

Every morning the spawning trays were removed from the exposure tanks, to check for eggs, and replaced with clean spawning trays. The fish eggs were transferred to petri dishes for microscopical analysis, to determine fertilization success. From each clutch a maximum of 50 fertilized eggs were randomly selected, transferred into egg cups and suspended in 21 L glass tanks filled with clean conditioned water at a temperature of 25 – 27 °C. Five days post fertilization (dpf) the eggs were analyzed for vitality and hatch.

2.7 Estrogenic activity

A recombinant yeast estrogen screen (rYES) expressing the human estrogen receptor alpha (hERα; Routledge and Sumpter, 1996) was used to determine estrogenic activity in each aquarium in relation to a ZON standard. This ZON standard was prepared in ethanol (purity: 99.8 %, Carl Roth GmbH + Co. KG, Germany) in a concentration of 20 mg/L.

Samples of 1 L aquarium water were collected on day 10, 30, 50, 70, 90, 110 (one replicate per concentration and control) and 130 (all replicates) of the juvenile exposure F1 as well as on day 4, 7, 21, 25, 28, 42 (one replicate per concentration and control), 12 and 33 (all replicates) of the reproduction experiment F1 into solvent cleaned glass bottles, spiked with 5 mL methanol (purity: 99.9 %, Sigma-Aldrich GmbH, Switzerland) and extracted onto pre-conditioned (5 mL

methanol, 5 mL ultrapure water) Sep-Pak Classic C18 cartridges (Waters Corp., USA). The cartridges were air dried and stored until further processing at -40 °C.

After defrosting and air drying, the cartridges were eluted into solvent cleaned amber glass tubes using 5 mL methanol. The methanol extracts were dried under a gentle N_2 stream and redissolved in 1 mL ethanol (purity: 99.8 %, Sigma-Aldrich GmbH, Switzerland). The ZON standard was tested in a serial dilution of 12 steps and the ethanol extracts of the water samples in a serial dilution of 5 steps, each in duplicate. The rYES was conducted following the methods described by Routledge and Sumpter (1996).

2.8 Chemical analysis

To measure the ZON concentrations in the aquaria, 100 mL water samples were collected on day 10, 50, 90 and 130 (one tank per concentration) of the juvenile exposure F1 as well as on day 4, 12, 21, 25, 42 (one tank per concentration) and 33 (all tanks) of the reproduction experiment F1 into solvent cleaned glass bottles and sent within 24 h to the chemical laboratory (Agroscope Reckenholz-Tänikon Research Station ART, Switzerland). An isotope labelled internal standard (D_6-ZON) was added to the water samples prior to solid-phase extraction (SPE; SupelcleanTM Envi-18 cartridges, 6 mL, 500 mg, Supelco, USA) and analysis by LC-MS/MS (negative electrospray ionization; Hartmann et al. 2007).

2.9 Fish sampling and analysing

On day 140 of the juvenile exposure F1, all zebrafish were wet weighed to the nearest 0.01 g, total length was measured to the nearest mm and sex was determined based upon external secondary sex characteristics, such as coloration, size and marking of anal fin, body shape and visibility of an urogenital papilla. Mortality during the juvenile exposure F1 was calculated based on the number of eggs transferred in the tanks at the beginning of the experiment. Spawning groups were assembled and transferred to the subsequent reproduction experiment F1.

On day 42 of the reproduction experiment F1, all adult zebrafish were euthanized in 150 mg/L MS222 buffered with 300 mg/L $NaCO_3$ to maintain a neutral pH. The fish were wet weighed to the nearest 0.01 g and total length was measured to the nearest mm. A 5 – 10 µL blood sample was taken by cardiac puncture using a heparinised (1000 Units heparin/mL, Heparin ammonium salt, Sigma-Aldrich GmbH, Switzerland) insulin syringe (Becton Dickinson & Company, USA), transferred in an Eppendorf reaction tube and centrifuged for 5 min at 4 °C and 9300 g. The 3 – 5 µL plasma per sample was transferred to a new reaction tube and stored at -80 °C for later

analysis of VTG concentration using a commercially available zebrafish ELISA kit (Prod. No. V01008402, Biosense Laboratories, Norway). The abdominal cavity was opened with dissecting scissors and the whole fish was fixed for 24 h in Bouin's fixative (Sigma-Aldrich GmbH, Switzerland).

For histological examination the head and tail of the fixed fish were removed and the trunk cut into four slices (~ 3 mm thick) using a microtome blade (SEC 35, Microm International GmbH, Germany). Subsequently the samples were dehydrated in an ascending ethanol series, incubated in Roti Histol (Carl Roth GmbH + Co. KG, Germany) and paraffinized (Roti-Plast, Carl Roth GmbH + Co. KG, Germany). All four samples per fish were embedded in one standard embedding cassette. This enabled simultaneous whole body transverse sectioning of different areas. Transverse histological sections (9 µm thickness) were stained with standard hematoxylin and eosin (H&E; Carl Roth GmbH + Co. KG, Germany). Male and female gonads on the histological sections were verified for sex and analyzed following the methods described by Schwartz et al. (2010).

2.10 Statistical analyses

To investigate effects of the ZON exposure, data of exposed groups were compared to control group using SigmaPlot version 9.01 and SigmaStat version 3.1 (Systat Software Inc., USA). For the juvenile exposure F1 data of survival, male wet weight, body length, condition factor (weight × 100 / length^3) and sex ratio met the assumptions of normality and equality of variance. Hence, these data were analysed with a one-way analysis of variance (ANOVA) followed by Dunnett's test. Performing standard transformation of the female wet weight data could not provide for the assumption of normality and equal variance, and therefore were analyzed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's method for multiple comparisons.

For the reproduction experiment F1, the ratio of the second part (21 days exposure for group Lc, Mc and Hc and depuration for group L, M and H) to the first part (21 days pre-exposure for group Lc, Mc and Hc and exposure for group L, M and H) was calculated for data of reproductive output, resulting in relative spawning frequency, relative fecundity, relative clutch size, relative fertilization rate, relative embryo survival and relative hatch rate. The use of the relative reproductive output data is a possibility to deal with the considerable variation in the numbers of eggs spawned in a zebrafish population, which was already observed in other studies (e.g. Ensenbach & Nagel 1997, Nash et al. 2004). This method enables to obtain information about the change in these parameters caused by exposure to a substance and depuration

subsequently to exposure, respectively, without encountering the problem of high inter-individual variance.

Data of female wet weight, length, histomorphometry, fecundity, clutch size, embryo survival and plasma VTG concentration met the assumptions of normality and equality of variance permitting to use one-way ANOVA followed by Dunnett's test. Performing standard transformation of the male wet weight as well as relative spawning frequency, relative fertilization rate and relative hatch rate data could not provide for the assumption of normality and equal variance, and therefore were analyzed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's method for multiple comparisons.

Analysis of correlation between relative spawning frequency, relative fecundity, relative clutch size and nominal ZON concentration as well as between VTG concentration and relative fecundity was conducted using Pearson Product Moment Correlation.

3 Results and Discussion

3.1 Aqueous ZON concentration

A combination of analytical measurement and *in vitro* analysis (rYES) for determination of estrogenic activity induced by ZON were used to quantify the content of mycotoxin in the exposure tanks. During the juvenile exposure F1, analytically measured ZON mean concentrations ranged between 46 and 86 % of nominal values and were always lower than mean concentrations determined *in vitro*, expressed as estrogen equivalent for ZON (EEQ), which ranged between 91 and 206 % of nominal values. In the control and '0 ng/L' groups, no ZON could be detected (LOQ = 1 ng/L) and estrogenic activity was below determination limit (LOQ ≈ 80 ng/L EEQ; Table 1).

Similarly, during the reproduction experiment F1, analytically measured ZON mean concentrations ranged between 54 and 96 % of nominal values and were always lower than mean concentrations determined in the rYES, which ranged between 78 and 190 % of nominal values. In the control group, no ZON could be detected and estrogen activity was below determination limit (Table 1).

The quantified ZON concentrations for both methods analytical chemistry and rYES were stable during the entire juvenile exposure F1 and the reproduction experiment F1. An explanation for the consistently higher ZON concentrations determined *in vitro* compared to the analytically measured values could be that in the rYES the overall estrogenicity is determined and, hence, other possible sources such as primarily ZON metabolites could have contributed to a background estrogenic activity. Data on biotic transformation of ZON generally indicate the reduction of the 6′-keton to yield α- and β-ZOL as a main metabolization pathway (Galtier 1999). The estrogenic potency of α-ZOL in the rYES is approximately five fold higher than that of ZON (El-Sharkawy and Abul-Hajj 1988, Le Guevel and Pakdel 2001). Therefore, α-ZOL might have contributed to the high estrogenicity in the rYES.

In the following, references on ZON as well as literature data of E2 and EE2 provided in the text are based on nominal concentrations.

Table 1: Analytically measured zearalenone (ZON) concentrations and *in vitro* (recombinant yeast estrogen screen, rYES) determined estrogen equivalent (EEQ) for ZON (mean ± SEM). Analytical chemistry: one replicate per group on day 10, 50, 90, 130, on day 4, 12, 21 and on day 25, 33, 42; all replicates on day 33 (n = 4). rYES: one replicate per group on day 10, 30, 50, 70, 90, 110, 130, on day 4, 7, 12, 21 and on day 25, 28, 33, 42; all replicates on day 130, on day 12 and on day 33 (n = 4).

Nominal concentration[a]	Mean measured concentrations			
ZON	**Analytical chemistry (ng/L ZON)**		**Recombinant yeast screen (ng/L EEQ)**	
Juvenile exposure	10, 50, 90, 130	-	10, 30, 50, 70, 90, 110, 130	130
Control	BD[b]		BD[b]	BD[b]
Lc: 0 ng/L	BD[b]		BD[b]	BD[b]
Mc: 0 ng/L	BD[b]		BD[b]	BD[b]
Hc: 0 ng/L	BD[b]		BD[b]	BD[b]
L: 100 ng/L	46 ± 9		91 ± 28	206 ± 39
M: 320 ng/L	276 ± 38		368 ± 44	640 ± 53
H: 1000 ng/L	712 ± 55		933 ± 59	940 ± 35
Reproduction part 1	4, 12, 21	-	4, 7, 12, 21	12
Control	BD[b]		BD[b]	BD[b]
Lc: 0 ng/L	BD[b]		BD[b]	BD[b]
Mc: 0 ng/L	BD[b]		BD[b]	BD[b]
Hc: 0 ng/L	BD[b]		BD[b]	BD[b]
L: 100 ng/L	74 ± 2		190 ± 39	91 ± 7
M: 320 ng/L	281 ± 8		300 ± 56	360 ± 74
H: 1000 ng/L	959 ± 85		1030 ± 73	1187 ± 35
Reproduction part 2	25, 33, 42	33	25, 28, 33, 42	33
Control	BD[b]	BD[b]	BD[b]	BD[b]
Lc: 320 ng/L	174 ± 10	176 ± 3	313 ± 23	380 ± 32
Mc: 320 ng/L	189 ± 8	191 ± 11	309 ± 31	285 ± 26
Hc: 320 ng/L	176 ± 8	184 ± 11	251 ± 24	270 ± 23
L: 0 ng/L	BD[b]	BD[b]	BD[b]	BD[b]
M: 0 ng/L	BD[b]	BD[b]	BD[b]	BD[b]
H: 0 ng/L	BD[b]	BD[b]	BD[b]	BD[b]

[a] Group Lc, Mc and Hc: raised in clean water and short-term ZON exposure; group L, M and H: life-long ZON exposure and subsequent depuration.
[b] BD = below detection limit: Analytical chemistry < 1 ng/L, rYES < 80 ng/L

3.2 Juvenile exposure F1: Effects of ZON on mortality, weight and body length

Mean cumulative mortality at the end of the juvenile exposure F1 was 72 % for the control group, 62 – 75 % for Lc, Mc and Hc, and 69 – 80 % for L, M and H with no significant differences between groups (data not shown). Consequently there was no evidence that exposure for 140 days from fertilization to maturity up to a concentration of 1000 ng/L ZON affected survival of zebrafish.

Mortality occurred mainly during the first month of the juvenile exposure F1. The overall mortality was rather high compared to other studies that observed mean mortality rates from up to 47 % for control to 58 % for exposure groups, without significant differences, in zebrafish exposed from egg to adult up to 15.4 ng/L EE2 (Andersen et al. 2003, Fenske et al. 2005, Hill and Janz 2003, Nash et al. 2004). No effect on F1 mortality was observed after exposure of F0 and F1 generation up to 1.7 ng/L EE2 (Schäfers et al. 2007). Nonetheless our results for mortality indicate that long-term exposure up to 1000 ng/L ZON does not have acute toxic effects on zebrafish. This is in accordance with data from toxicological studies demonstrating that apart from estrogenic effects ZON exhibits a low acute toxicity in many animal species (Kuiper-Goodman et al. 1987).

Wet weight, body length and condition factor of the male fish did not show any effect caused by ZON exposure (data not shown). In female zebrafish the body length and the wet weight in group H (exposed to 1000 ng/L ZON) and similarly their condition factor, as well as in Hc (raised in clean water and F0 exposed to 1000 ng/L ZON) were increased compared to control (Fig. 2 A, B, C).

Several authors reported a significant increase in length and weight of male and/or female zebrafish after exposure from 0 to 240 and 24 to 64 dpf to 0.5 – 2 ng/L and 2 – 10 ng/L EE2, respectively, as well as a concentration dependent increase of length and weight after exposure from 0 to 42 dpf and F0 generation to 27 – 272 ng/L E2 (Örn et al. 2003, Soares et al. 2009, Van der Ven et al. 2007). In contrast, no effect on length and weight was found in zebrafish exposed from 0 to 75 and 0 to 270 dpf to 3 – 5 ng/L EE2 as well as 5 ng/L E2, respectively (Fenske et al. 2005, Nash et al. 2004). Other studies observed a significantly reduced length and weight of male and/or female zebrafish after exposure from 0 to 60 – 90 dpf to 1.7 – 25 ng/L EE2 (Hill and Janz 2003, Schäfers et al. 2007, Van den Belt et al. 2003). Two studies revealed an even divergent impact on zebrafish. Males exposed from 0 to 120 dpf to 0.05 ng/L EE2 showed a lower weight whereas at 5 ng/L EE2 a higher weight was found and exposure from 2 to 90 dph to 10 ng/L EE2 caused a lower weight and length but a higher condition factor compared to the control (Larsen et al. 2008, Xu et al. 2008).

In summary, the above cited studies show very diverse effects of E2 and EE2 on zebrafish. The observed estrogenic effects ranged from promoting growth to having no effect, or to inhibiting growth. Taken together these results show no pattern or tendency, such as concentration or duration dependence of positive or negative effect on growth of zebrafish, or give evidence under which conditions estrogens influence growth or not. However, the positive effect on growth of female zebrafish in group H parallels the growth-promoting (anabolic) potential of E2 in early life of fish (Bell 2004, Mandiki et al. 2005).

The consistently high and similar values for body length, wet weight and condition factor of female zebrafish in groups Hc and H in the present study, might imply that exposure of (only) F0 generation to a high ZON concentration affect growth in F1 more than continuous exposure of F0 and F1 generation to lower ZON concentrations as in the case of group L and M (Fig. 2 A, B, C). Therefore the results suggest a transgenerational effect of F0 exposure to ZON on F1 growth. Maternal transfer of EAS may occur in fish when pollutants are co-transported with VTG into the developing oocytes (Gray et al. 1999). However, we do not have data of ZON concentrations in the fish, and can therefore not test if in the present study such a mechanism has influenced growth in this extent over such a long period.

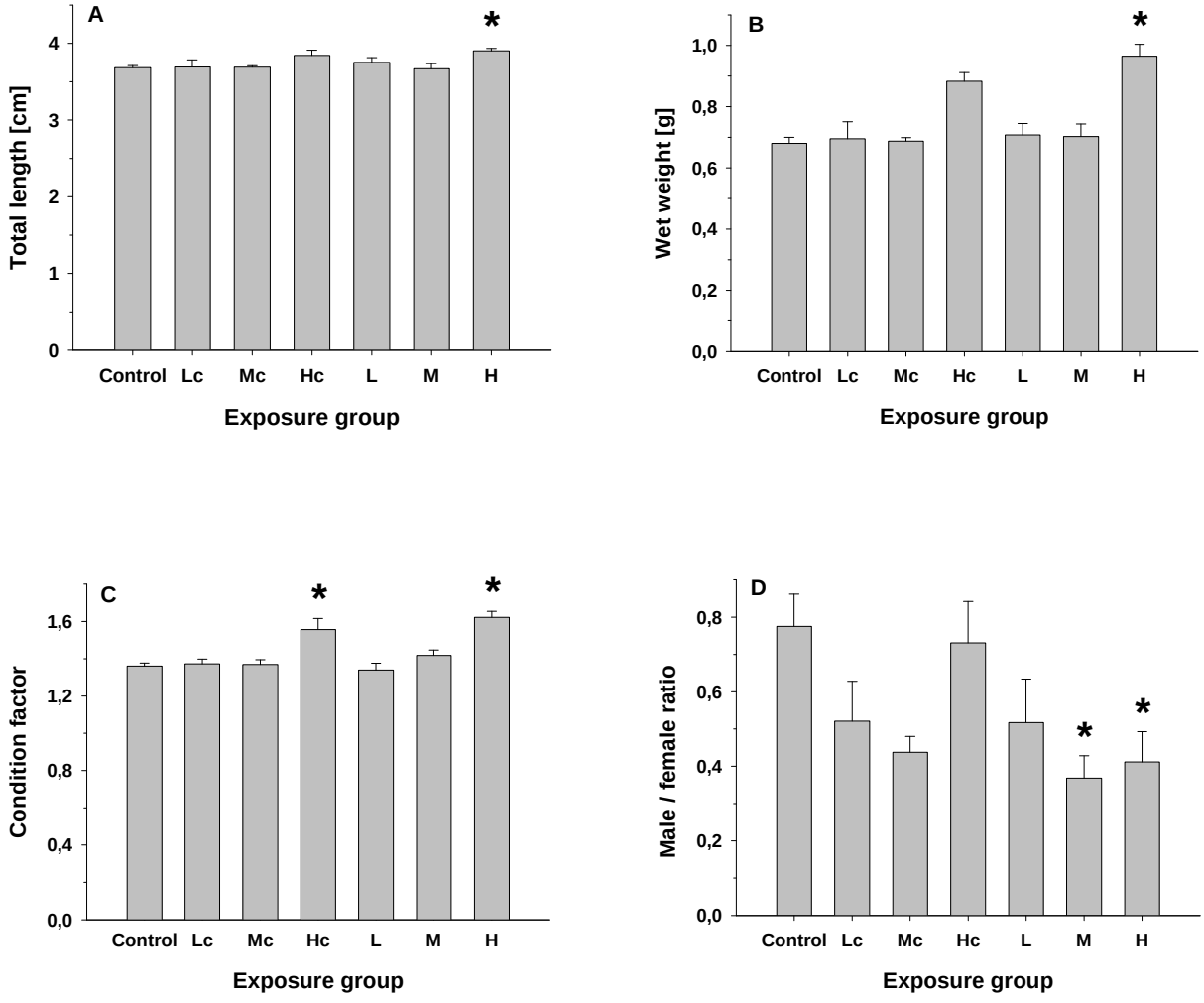

Fig. 2: (A) total length, (B) wet weight, (C) condition factor (weight × 100 / length^3) of females and (D) sex ratio of zebrafish raised in clean water (Lc, Mc, Hc) or exposed to (L) 100, (M) 320 and (H) 1000 ng/L zearalenone (ZON) for 140 days after exposure of parental generation, as well as water control (mean ± SEM, n = 4). Significant differences from control are denoted as * ($p < 0.05$).

3.3 Juvenile exposure F1: Effects of ZON on sex ratio

There was a higher proportion of female fish in all groups compared to control, which was significant in group M and H after 140 days exposure to 320 and 1000 ng/L ZON, respectively. The mean male/female ratio was the highest in the control (0.78) and reduced to 0.37 and 0.41 in group M and H, respectively (Fig. 2 D). The male/female ratios were clearly reduced in Lc, Mc and L (but not Hc) to 0.52, 0.44 and 0.52, respectively, although not significantly. This feminization effect caused by exposure to estrogens was reported by several authors who observed a shift of sex ratio towards females in zebrafish exposed from 0 to 60 – 120 dpf to 0.5 – 15.4 ng/L EE2 or from 0 to 21 dpf to 100 ng/L E2 (Andersen et al. 2003, Brion et al. 2004, Larsen et al. 2008). Other studies found, apart from an increased number of females and a decreased number of males, also an increased number of immature gonads in zebrafish exposed from 0 – 20 to 60 – 90 dpf to 0.1 – 25 ng/L EE2, indicating an arrested sexual differentiation (Hill and Janz 2003, Örn et al. 2003, Van den Belt et al. 2003, Xu et al. 2008). In the present study zebrafish were not investigated for gonadal conditions at the end of the juvenile exposure F1, because the fish were not sacrificed at this point. However, no negative gonadal effect was found later on (see below).

3.4 Reproduction experiment F1: Effects of ZON on mortality, weight, body length and gonad morphology

No mortality was recorded during the reproduction experiment F1 and hence exposure for (yet another) 21 days up to 1000 ng/L ZON did not affected survival of the adult zebrafish. Similarly there were no effects on body length and wet weight of the fish (data not shown). This corresponds with the reproduction experiment F0 where no mortality and no effects on growth could be detected after exposure up to 3200 ng/L ZON (Schwartz et al. 2010).

Also no effects on gonad morphology after exposure for 21 days up to 320 ng/L ZON (group Lc, Mc and Hc) could be observed, which is in line with the reproduction experiment F0 where no effects were observed up to 3200 ng/L ZON (Schwartz et al. 2010). As well no effects of exposure from 0 to 161 dpf up to a concentration of 1000 ng/L ZON and a subsequent 21 days depuration period (group L, M and H) on gonad morphology were observed (data not shown). The testes of all males in both control and ZON exposed fish were mature, regular in structure and the tubules contained cysts showing a full spectrum of sperm cell differentiation stages with no significant differences between the groups in terms of the ratio of the various stages of development. Similarly, the ovaries of the control and ZON exposed fish were mature and

showed a full range of the different oocyte developmental stages with no significant differences between the groups in terms of the proportions of oocytes at the various stages of development. Nash et al. (2004) and Xu et al. (2008) detected altered proportion of germ cell types in male zebrafish exposed from 0 to 75 and 6 to 90 dpf to 0.5 – 5 and 2 – 10 ng/L EE2 followed by a depuration period of 150 and 90 days, respectively. These authors furthermore found 43 and 75 – 80 % immature gonads after continuous exposure from 0 to 210 and 2 to 90 dpf to 5 and 2 – 10 ng/L EE2, respectively. In zebrafish exposed from 0 to 177 dpf to 10 ng/L EE2, solely gonads with mostly immature ovarian morphology were observed (Schäfers et al. 2007). However, the results of the present study indicate that even life-long exposure up to 1000 ng/L ZON (group L, M and H) does not arrest sexual differentiation of gonads as it was reported in the above cited studies with (partial) life-cycle exposure of zebrafish to estrogens.

3.5 Reproduction experiment F1: Depuration after life-long ZON exposure

Details of the reproductive performance for each exposure group during the first and the second part of the reproduction experiment F1 are presented in Table 2. Mean relative spawning frequency was 100 % for the control and increased to 200 % for group H. However, due to high variability between replicates the difference was not statistically significant. Group L and M showed values similar to the control (98, 116 %; Fig. 3 A). Analysis of correlation revealed a positive correlation between nominal ZON concentration and relative spawning frequency for L, M and H ($r = 0.878$, $p = 0.00002$). Relative fecundity exhibited values of 117, 128 and 233 % for group L, M and H, respectively, which was significantly increased for H compared to control (107 %; $p < 0.05$; Fig. 3 B). There was also a positive correlation between nominal ZON concentration and relative fecundity of these groups ($r = 0.843$, $p = 0.00008$). Data for relative clutch size was slightly but not significantly increased for group L, M and H to 119, 110 and 128 %, respectively, in comparison to control (106 %; Fig. 3 C).

Table 2: Details for reproductive performance of zebrafish during 21 days first part and 21 days second part of the zearalenone (ZON) reproduction experiment F1 (mean ± SEM; n = 4, for H: n = 3).

Exposure Group[a]	Reproductive performance					
	Spawning frequency (spawnings per day)		Fecundity (eggs per day)		Clutch size (eggs per spawning)	
	part 1	part 2	part 1	part 2	part 1	part 2
Control	0.92 ± 0.07	0.92 ± 0.07	169.6 ± 44.2	171.9 ± 33.9	181.1 ± 39.1	183.4 ± 27.0
Lc	0.82 ± 0.09	0.85 ± 0.09	175.5 ± 51.2	167.1 ± 55.8	206.5 ± 49.8	183.6 ± 46.9
Mc	0.93 ± 0.06	0.93 ± 0.03	261.3 ± 32.6	242.8 ± 34.6	278.4 ± 21.3	258.7 ± 29.2
Hc	0.71 ± 0.06	0.61 ± 0.06	105.4 ± 29.0	75.3 ± 33.3	160.4 ± 59.1	221.9 ± 52.9
L	0.82 ± 0.07	0.81 ± 0.08	171.4 ± 65.2	184.0 ± 53.2	198.7 ± 62.9	220.3 ± 53.4
M	0.79 ± 0.09	0.89 ± 0.06	188.0 ± 42.1	220.0 ± 32.8	230.6 ± 38.9	242.1 ± 24.6
H	0.35 ± 0.18	0.60 ± 0.27	31.3 ± 25.0	73.2 ± 60.0	60.0 ± 30.9	85.3 ± 53.6

[a] Group Lc, Mc and Hc: raised in clean water and short-term ZON exposure; group L, M and H: life-long ZON exposure and subsequent depuration.

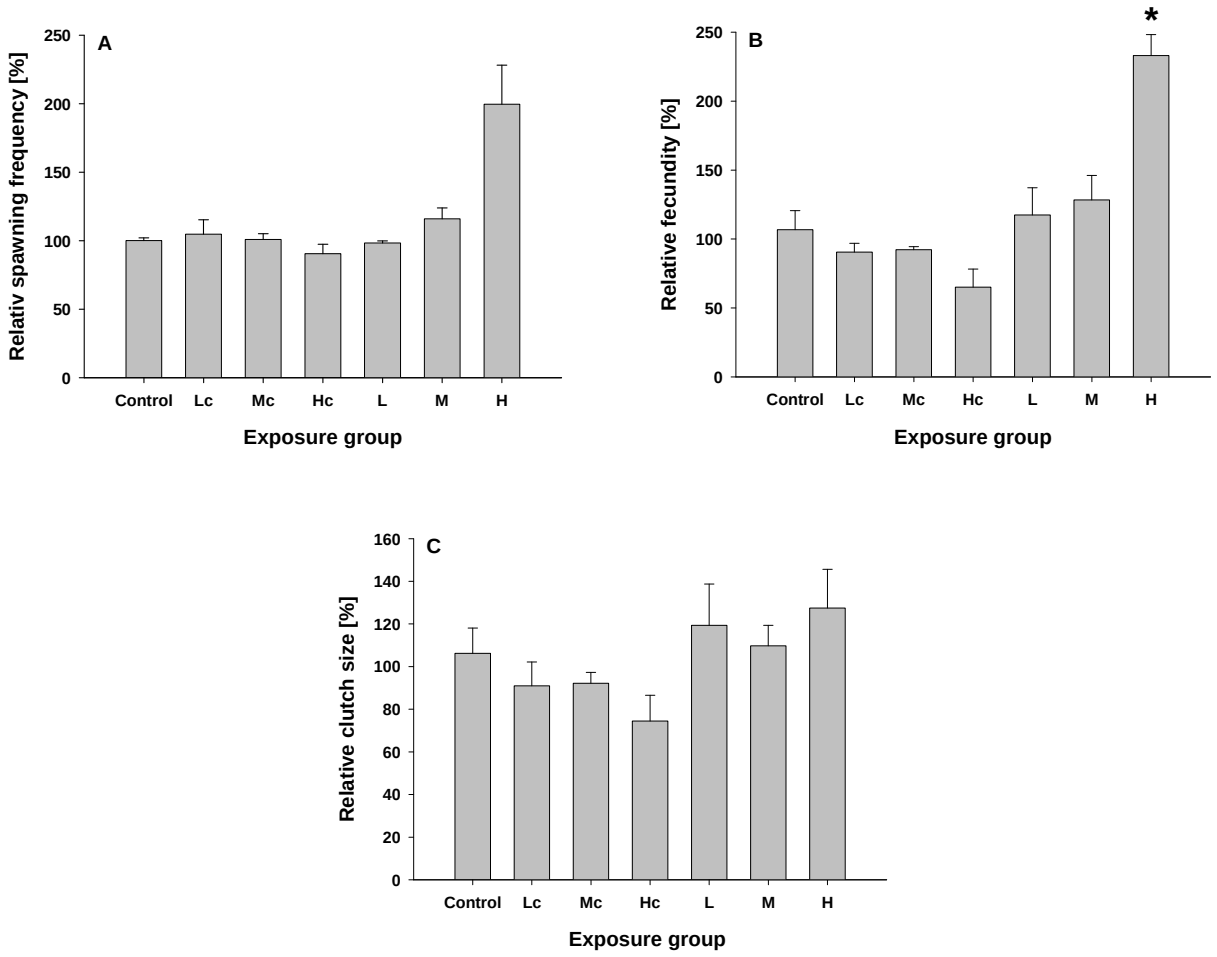

Fig. 3: Relative (A) spawning frequency, (B) fecundity and (C) clutch size (ratio of the second part to the first part of the reproduction experiment F1) for spawning groups of zebrafish (4 males, 2 females) exposed for 21 days to 320 ng/L zearalenone (ZON; Lc, Mc, Hc) or during a 21 days depuration after 161 days exposure to (L) 100, (M) 320 and (H) 1000 ng/L ZON, as well as water control (mean ± SEM, n = 4, for H: n = 3). Significant differences from control are denoted as * ($p < 0.05$).

Several studies observed a lower reproductive output after long-term exposure of zebrafish to estrogens. Nash et al. (2004) and Schäfers et al. (2007) found a reduced fecundity in fish exposed from 0 to 210 and 0 to 135 dpf to 5 and 1.7 ng/L EE2, respectively. Also a reduced fecundity was observed in a depuration period of 22 and 90 days after exposure from 0 to 118 and 6 to 90 dpf to 3 (fish never spawned while under exposure) and 0.4 – 10 ng/L EE2, respectively (Fenske et al. 2005, Xu et al. 2008). In the study of Nash et al. (2004) zebrafish exposed from 0 to 75 dpf up to 5 ng/L EE2 and 5 ng/L E2, with a subsequent depuration of 150 days showed no effect on fecundity, which was in contrast with the impact of continuous 0 to 210 dpf exposure to 5 ng/L EE2 causing a significant reduced fecundity. These data suggest a capacity for recovery of reproductive output during a depuration period after long-term exposure to estrogens. The observed increase of fecundity in group H indicates that some degree of recovery might occur during the 21 days depuration period after live long exposure to ZON. As the clutch size increased just slightly, the increase of fecundity was mainly related to an increase of spawning frequency. The correlation between nominal ZON concentration and relative fecundity of group L, M and H suggests that there might be also a slight recovery for L and M during the depuration period. As the ZON concentration was lower in these groups the change is obviously less prominent. Thus, the results suggest a potential for recovery of reproductive performance after live long exposure to ZON.

No effects on zebrafish fertility, hatch and embryo survival caused by exposure from 0 to 161 dpf up to 1000 ng/L ZON and subsequent 21 days of depuration (group L, M and H) could be detected (data not shown). Similarly no effects on fertilization and viability of offspring during exposure for 21 days up to 3200 ng/L ZON were found (Schwartz et al. 2010).

This is in contrast to several studies that observed effects on fertilization success and embryo viability in offspring after long-term exposure of zebrafish to estrogens. An increased embryo mortality at 12 and 80 hpf was observed after exposure from 2 to 90 and 0 to 240 dpf to 0.4 and 0.5 – 2 ng/L EE2, respectively, but no effects on fertilization (Soares et al. 2009, Xu et al. 2008). Nash et al. (2004) reported a reduced viability of eggs at 14 hpf in zebrafish exposed from 0 to 210 dpf to 0.5 – 5 ng/L EE2 and 5 ng/L E2. Even after 150 days depuration following exposure from 0 to 75 dpf to 0.5 and 5 ng/L EE2, the fertilization success was still reduced with an increased number of nonviable eggs. In the study of Schäfers et al. (2007), a reduced fertilization success after exposure from 0 to 135 dpf to 1.7 ng/L EE2 was observed. The data of the present study and the results of the reproduction experiment F0 (Schwartz et al. 2010) in relation to the results of the cited studies suggest that ZON is less endocrine disrupting and/or less toxic than the steroid estrogens E2 and EE2 with regard to fertility and embryo survival.

3.6 Effects of depuration on VTG induction after life-long ZON exposure

At the end of the reproduction experiment F1, only female zebrafish in group H revealed a significant 1.53 fold increase in plasma VTG concentration relative to control ($p < 0.05$; Fig. 4 B). There was also a correlation between female plasma VTG concentration and relative fecundity of L, M and H ($r = 0.721$, $p = 0.002$). Male fish did not show any significant differences for VTG concentration between the groups (Fig. 4 A).

These results, and the fact that in the reproduction experiment F0 (Schwartz et al. 2010) exposure for 21 days to 1000 ng/L ZON lead to a significant 4.4 fold VTG induction in male zebrafish, suggest that either a depuration period of 21 days is sufficient for recovery of normal VTG concentration or that life-long exposure leads to an adaption of metabolism and consequently to a normalization of VTG levels, or a combination of both.

The half-life of VTG concentrations in zebrafish was calculated to be 2.4 days after exposure from 0 to 25 dph to 15.4 ng/L EE2 (Andersen et al. 2003). Van den Belt et al. (2002) found in male zebrafish decreased plasma VTG level to 1/3 (still significantly elevated) in a 24 day depuration period after exposure for 24 days to 10 ng/L EE2. However, the VTG concentration in females decreased to 1/4 and was not significant anymore. These two studies indicate that a depuration period of 21 days may be long enough to recover VTG levels similar to control group after exposure of zebrafish to estrogens. In contrast to the short-term exposure of F0 zebrafish for 40 days (males: 0.5, 5 ng/L EE2, females: 5 ng/L EE2), Nash et al. (2004) observed no induction of plasma VTG in F1 males and females after exposure from 0 to 310 dpf up to 5 ng/L EE2 and suggested an acclimatisation and down regulation of vitellogenic response after long-term exposure to estrogens. As there is no data for VTG concentrations neither at the end of the juvenile exposure F1, nor at the end of the first part of the reproduction experiment F1, we cannot conclude here whether VTG levels in exposure group L, M and H were higher before the depuration period.

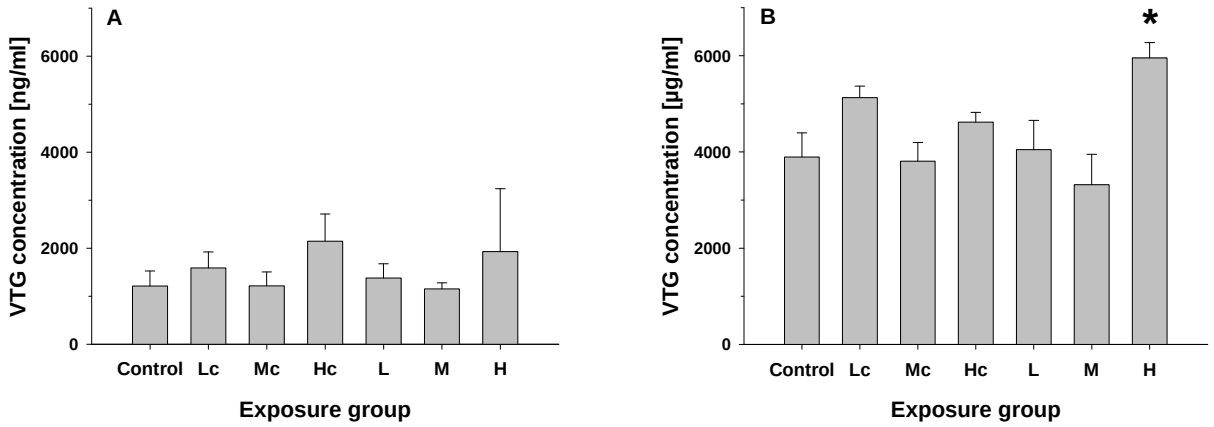

Fig. 4: Vitellogenin (VTG) concentration in the plasma of (A) male and (B) female zebrafish exposed for 21 days to 320 ng/L (Lc, Mc, Hc) or 161 days to (L) 100, (M) 320 and (H) 1000 ng/L zearalenone (ZON) with a subsequent 21 day depuration, as well as water control (mean ± SEM, n = 4, for H: n = 3). Significant differences from control are denoted as * ($p < 0.05$).

3.7 Reproduction experiment F1: Transgenerational effects of short-term ZON exposure

Details of the reproductive performance for each exposure group during the first and the second part of the reproduction experiment F1 are presented in Table 2. Mean relative spawning frequency was 105, 101 and 91 % for group Lc, Mc and Hc, respectively, and similar to control (100 %; Fig. 3 A). The mean relative fecundity for these groups exhibited values of 91, 92 and 65 %, respectively, and were considerably, but not significantly, lower than in the control group (107 %; Fig. 3 B). There was a negative correlation between the nominal ZON concentration of the F0 generation and relative fecundity of group Lc, Mc and Hc ($r = -0.618$, $p = 0.011$). Relative clutch size for these groups was slightly reduced to 91, 92 and 74 %, respectively, in comparison to control (106 %; Fig. 3 C).

In the study of Nash et al. (2004) fecundity of the F1 generation was not affected by F0 exposure for 40 days up to 5 ng/L EE2 and 5 ng/L E2. However, the data of the present study display the same tendency as in the reproduction experiment F0, although without being significant, where a significantly reduced relative fecundity was observed in adult zebrafish already from 100 ng/L ZON, but no effect on spawning frequency up to 320 ng/L and clutch size up to 1000 ng/L ZON (Schwartz et al. 2010). This result suggests that exposure of F0 to high ZON concentrations might affect the sensitivity of F1 generation, although the overall sensitivity on the level of reproductive performance decreased from F0 to F1.

No effects on zebrafish fertility, embryo survival and hatch caused by exposure to ZON for 21 days up to 320 ng/L were observed (data not shown), which corresponds with the reproduction experiment F0 where no effects could be detected after exposure up to 3200 ng/L

ZON (Schwartz et al. 2010). Similarly Brion et al. (2004) did not observe effects on fertilization and hatch in zebrafish at 85 dpf after exposure from 0 to 21, 21 to 42 dpf, as well as adults for 21 days up to 100 ng/L E2, and Van der Ven et al. (2007) found no effects on these parameters after exposure of adult fish for 21 days up to 272.4 ng/L E2.

3.8 Transgenerational effects of short-term ZON exposure on VTG induction

At the end of the reproduction experiment F1, females of group Lc and Hc displayed a slightly elevated VTG level (1.32 and 1.19 fold higher than control), although it was not significant (Fig. 4 B). Male fish did not show any significant differences for VTG concentration between the groups (Fig. 4 A).

These results are correspondent to the reproduction experiment F0 where no effect on male plasma VTG levels could be observed after 21 days exposure up to 320 ng/L ZON (Schwartz et al., 2010). But although there was no significant difference to control, mainly due to high variation, VTG concentration in males of group Hc was the highest among all groups (1.77 fold higher than control). This might suggest a possible transgenerational impact of F0 exposure to increase the sensitivity of F1 generation to ZON.

3.9 Environmental relevance

ZON was reported to occur in surface waters in concentrations of up to 44 ng/L (Gromadzka et al. 2009, Lagana et al. 2004), which is 14 % of the concentration calculated in the present study as LOEC (lowest observed effect concentration) for transgenerational effects on F1 after short-term exposure of F0 generation (condition factor at 320 ng/L, group Hc). It is as well 14 % of the concentration calculated as LOEC after short-term exposure of F0 and life-long exposure of F1 generation (male/female ratio at 320 ng/L, group M). The reported effluent concentrations of up to 220 ng/L (Lagana et al. 2001, 2004, Lundgren and Novak 2009) is still around 1.5 times lower than the LOECs of 320 ng/L for transgenerational and long-term effects. These LOECs of 320 ng/L are about three times higher than the LOEC of 100 ng/L for short-term exposure presented in the reproduction experiment F0 (Schwartz et al. 2010), suggesting a generally lower sensitivity of F1 generation to effects caused by ZON.

Evaluating the environmental relevance of this data, the risk for fish to be directly harmed by exposure to ZON in their natural environment seems rather marginal. However, the actual exposure in a given catchment depends on many factors, such as *Fusarium* infection rates, meteorology and hydrodynamics (Bucheli et al. 2008, Hartmann et al. 2008b). Hence, taking into

consideration the highest detected concentrations, a possibly higher species sensitivity, a regional and temporal limited exposure to higher concentrations (caused by effluent discharge in combination with low flow conditions, or emission from *Fusarium* infected wheat fields), and mixture effects in combination with other EASs, ZON might contribute to the overall estrogenic exposure in the environment.

4 Conclusions

The results of the present study confirm the estrogenic potential of ZON to influence sexual differentiation and reproduction in zebrafish caused by long-term exposure and a possible transgenerational effect on growth caused by short-term exposure of F0 and F1 generation.

Growth of fish was increased (1000 ng/L) after continuous F0 and F1 long-term exposure to ZON and sex ratio was shifted toward female (from 320 ng/L), demonstrating a feminizing effect. A positive correlation between the relative fecundity and the ZON concentration as well as an increased relative fecundity (1000 ng/L) indicates a recovery during the depuration.

The condition factor of female F1 fish with solely parental ZON exposure (1000 ng/L) was increased, suggesting that exposure of F0 influences growth in F1 generation. A negative correlation between the relative fecundity of F1 generation and the ZON concentration of the F0 exposure might imply that exposure of F0 to high ZON concentrations increases the sensitivity of F1 on the level of reproductive performance.

There is not much data available for ZON concentrations in the environment and the measured values in surface waters are below the LOECs we determined to cause negative effects on fish. Thus it is rather unlikely that ZON harm fish directly. However, ZON might contribute to an overall estrogenic contamination in the environment and under certain circumstances (higher species sensitivity, spatially and temporally limited events and combination with other EASs) there might be a certain risk for wild fish to be negatively affected by ZON in their natural habitat.

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Chapter V

Final Discussion

Ecotoxicological characterization of the estrogenic mycotoxin zearalenone

In this research project, the ecotoxicological potential of the estrogenic mycotoxin zearalenone (ZON) was characterized. Effects on fish caused by an exposure to ZON were assessed using the zebrafish as a model organism. Various experiments were conducted, investigating different exposure scenarios and life-stages of zebrafish as well as several endpoints. In the following the hazard (the potential to cause harm) of ZON is compared to common environmental estrogens and the risk (the likelihood of harm) for fish in the environment is estimated based on measured environmental concentration.

1 Effects of zearalenone on zebrafish – a summary

As a first pilot study (chapter II) an embryo toxicity test with zebrafish was performed to investigate toxic effects of ZON on fish development. This study was complemented with a 30 day zebrafish early life-stage experiment to assess the influence of ZON on juvenile growth.
In a second step (chapter III) the estrogenic potency of ZON and its effects on adult fish were investigated. A recombinant yeast estrogen screen (rYES) was used as an *in vitro* assay to determine effects on the 'activation of ER-regulated genes' representing the relative estrogenic activity of ZON in comparison to E2. During a 42 day reproduction experiment, spawning groups of zebrafish were exposed to ZON and reproduction (spawning frequency, fecundity, fertility and hatch) as well as physiological (VTG induction) and morphological (histomorphometry of gonad) parameters were assessed to evaluate effects of endocrine disruption.
In a third step (chapter IV) the estrogenic potency of ZON was studied in a zebrafish life-cycle experiment. This study was designed to investigate possible effects of continuous long-term exposure including a subsequent depuration period as well as possible transgenerational effects of F0 exposure on F1 generation. The offspring of the F0 generation which was exposed to ZON for 21 days was raised in a 140 day exposure experiment from embryo to adult. Spawning groups of this F1 generation were either exposed to ZON (after growing up in clean water) or clean water (after growing up under ZON exposure) in a subsequent 42 day reproduction experiment

and effects on developmental (mortality, growth, sex ratio and gonad histomorphometry), reproduction (spawning frequency, fecundity, fertility and hatch) as well as physiological (VTG induction) parameters were assessed.

ZON exposure revealed no effects on embryonic development of zebrafish up to a concentration of 2000 ng/L. However, a positive correlation between the body length and ZON concentration for zebrafish exposed from fertilization for 30 days up to 100 ng/L suggests a growth promoting effect during early development.

The estrogenic potential of ZON was confirmed *in vitro* (rYES) and *in vivo* (zebrafish). It was shown that although ZON possesses a moderate estrogenic potency *in vitro*, it exhibits a comparably strong effect on induction of VTG (1000 ng/L) and reproduction (100 ng/L) *in vivo* during exposure for 21 days.

It was further demonstrated that ZON influences growth (1000 ng/L), sexual differentiation (320 ng/L), reproduction (1000 ng/L) and VTG levels (1000 ng/L) in zebrafish during exposure from fertilization to day 140. Furthermore, ZON revealed a possible transgenerational effect on growth caused by short-term exposure of F0 and F1 generation each for 21 days to 1000 ng/L. A negative correlation between relative fecundity of F1 generation and the ZON concentrations of the F0 exposure might imply that short-term exposure of adult fish increases the sensitivity of the offspring on the level of reproductive performance.

2 Occurrence and potency of common environmental estrogens

To evaluate the relevance of ZON as environmental estrogen, its potential for estrogenic effects in fish was compared to other common and ecotoxicologically well described estrogens. The natural steroid estrogen 17β-estradiol (E2) was used as reference to compare the estrogenic potency of the environmental estrogens *in vivo* and *in vitro*.

2.1 Steroid estrogens

The synthetic steroid estrogen 17α-ethinylestradiol (EE2), a common component of oral contraceptives, and the natural steroid hormone E2 are mainly excreted by people and incompletely degraded in STPs (Guengerich 1990, Munkittrick et al. 1998, Tyler et al. 1998). Surface waters in Europe and USA showed concentrations of < 0.1 – 4.3 ng/L EE2 and 0.19 – 5.5 ng/L E2 (Baronti et al. 2000, Belfroid et al. 1999, Snyder et al. 1999). A survey of

139 streams in USA demonstrated that 5.7 % of them display more than 5 ng/L EE2 (Kolpin et al. 2002). In STP effluents in Europe and USA, concentrations of 0.2 – 15 ng/L EE2 and 0.48 – 48 ng/L E2 were measured (Baronti et al. 2000, Belfroid et al. 1999, Desbrow et al. 1998, Larsson et al. 1999, Snyder et al. 1999, Ternes et al. 1999).

Steroid estrogens have an estrogenic potency that is typically three orders of magnitude above that demonstrated by other environmental estrogens (Christiansen et al. 1998). The estrogenic potency in the rYES revealed an E2 : EE2 EC_{50} ratio of 1 : 1.13. Induction of VTG in rainbow trout primary hepatocytes resulted in an EC_{50} of 3.56 – 19.27 µg/L EE2 and 5.99 – 7.08 µg/L E2, yielding an E2 : EE2 EC_{50} ratio of 1 : 0.59 – 2.72 (Cosnefroy et al. 2009, Segner et al. 2003).

VTG induction was observed in zebrafish exposed to 0.5 – 3 ng/L EE2 (40 d) and 21.4 ng/L E2 (Nash et al. 2004, Rose et al. 2002, Segner et al. 2003). EC_{50} for VTG induction in female zebrafish was 6.22 ng/L EE2 and 174.9 ng/L E2 (21 d), resulting in an E2 : EE2 EC_{50} ratio of 1 : 0.04 (Van den Belt et al. 2004). Exposure of zebrafish to EE2 concentrations of 0.05 – 1.67 ng/L reduced fertilization rate (124 – 177 d); 0.5 – 2 ng/L resulted in a higher percentage of female (90 – 124 d); 1.1 – 5 ng/L inhibited or suppressed fecundity (177 – 210 d); and 3 ng/L induced changes in gonad histology (lifelong; Fenske et al. 2005, Larsen et al. 2008, Nash et al. 2004, Schäfers et al. 2007, Segner et al. 2003, Xu et al. 2008).

2.2 Bisphenol A (2,2-bis-(4-hydroxyphenyl)-propane

Bisphenol A (2,2-bis-(4-hydroxyphenyl)-propane [BPA]) is a widely used intermediate in the production of polycarbonate plastic and epoxy resins (Sonnenschein & Soto 1998). Concentrations of up to 21 µg/L BPA were reported in the aquatic environment (Crain et al. 2007), and Loos et al. (2010) found in water samples from the Danube River and its tributaries up to 68 ng/L BPA. River water analyses exhibited BPA concentrations of 3 – 55 ng/L in Belgium and Italy (Loos et al. 2007), 0.5 – 702 ng/L in Germany (Fromme et al. 2002, Kuch & Ballschmiter 2001), up to 12 µg/L in USA (Kolpin et al. 2002), and 0.058 – 19 µg/L in Japan (Ministry of Environment Japan 2004). In STP effluents, 0.26, 0.49 and 2.5 µg/L BPA were measured in Germany, Sweden and Austria, respectively (Fürhacker et al. 2000, Körner et al. 2000, Larsson et al. 1999). Industrial effluents in Canada revealed concentrations of 0.01 – 1.08 µg/L BPA (Lee & Peart 2000).

In vitro studies indicate BPA to be $1 – 3 \times 10^4$ times less potent than E2 (Körner et al. 2000, Metcalfe et al. 2001, Silva et al. 2002). The estrogenic potency in the rYES exhibited an E2 : BPA EC_{50} ratio of 1 : 10315. VTG induction in rainbow trout primary hepatocytes resulted in an

EC_{50} of 7082 ng/L E2 and 7419425 ng/L BPA, producing an E2 : BPA EC_{50} ratio of 1 : 1048 (Segner et al. 2003).

In vivo experiments with zebrafish demonstrated changes in gonad histology after exposure to 375 µg/L, and reduced fecundity and fertilization success after lifelong exposure to 1500 µg/L BPA (Segner et al. 2003). The EC_{50} for fertilization success after lifelong exposure of zebrafish was 1401.7 µg/L BPA (Segner et al. 2003). Exposing medaka to BPA resulted in induction of testis-ova at 10 µg/L (Metcalfe et al. 2001) and decreased fecundity and hatching success at 2282.9 µg/L (14 d; Shioda & Wakabayashi 2000). BPA revealed feminizing effects *in vivo* and induced VTG and/or zona radiate proteins in several fish species: carp, 100 µg/L (Mandich et al. 2007); fathead minnow, 160 µg/L (Brian et al. 2005, Sohoni et al. 2001); atlantic cod, 59 µg/L (Larsen et al. 2006); medaka, 1000 µg/L (Ishibashi et al. 2005); and rainbow trout, 500 µg/L (6 d; Lindholst et al. 2001).

2.3 Alkylphenol ethoxylates

Alkylphenol ethoxylates are widely used surfactants and antioxidants in the plastic industry (Soto et al. 1991). Nonylphenol (NP) and octylphenol (OP) are the major degradation products of alkylphenols in the aquatic environment (Ahel et al. 1994, Nimrod & Benson 1996, Rudel et al. 1998). In Belgian and Italian rivers concentrations of 0.32 – 2.5 µg/L NP and 0.02 – 0.11 µg/L OP were found and in the Danube river and its tributaries up to 0.24 µg/L NP (Loos et al. 2007, 2010). Up to 180 µg/L and 0.15 – 644 µg/L NP was measured in British and Spanish rivers, respectively (Blackburn & Waldock 1995, Sole et al. 2000). In STP effluent, concentrations of 0.1 – 3.7 µg/L NP were reported for UK, Germany, Switzerland and Italy (Ahel et al. 2000, Di Corcia et al. 1994, Körner et al. 2000) and up to 32 µg/L NP and 0.7 µg/L OP, and 330 µg/L NP were measured in USA and UK, respectively (Blackburn & Waldock 1995, Snyder et al. 1999).

4-tert-OP is the most potent of the alkylphenols followed by 4-tert-NP, being 1.5×10^3 and 1×10^4 times less potent than E2, respectively (Jobling et al. 1996, Routledge & Sumpter 1997). The estrogenic potency in the rYES resulted in an E2 : NP and E2 : 4-tert-OP EC_{50} ratio of 1 : 6745.8 and 1 : 1553.8, respectively (Segner et al. 2003, Van den Belt et al. 2004). VTG induction in rainbow trout primary hepatocytes revealed an EC_{50} of 5.99 – 7.08 µg/L E2 and 7483.43 – 8541.65 µg/L 4-tert-OP, resulting in an E2 : 4-tert-OP EC_{50} ratio of 1 : 1206.5 – 1249.3 (Cosnefroy et al. 2009, Segner et al. 2003).

Induction of VTG was observed in zebrafish males exposed to 30 – 100 µg/L NP (21 – 60 d; Hill & Janz 2003, Van den Belt et al. 2003b, Yang et al. 2006). EC_{50} for VTG induction in female

zebrafish were 174.9 ng/L E2 and 247012.4 ng/L NP (21 d), producing an E2 : NP EC_{50} ratio of 1 : 1412.3 (Van den Belt et al. 2004). Fertilization success in zebrafish showed an EC_{50} of 28.06 µg/L 4-tert-OP (lifelong; Segner et al. 2003) and exposure to 100 µg/L NP resulted in a shifted sex ratio in zebrafish and medaka (60 d; Hill & Janz 2003, Seki et al. 2003). VTG induction has been reported in several fish species: fathead minnow, 10 µg/L NP (Harries et al. 2000); medaka 11.6 µg/L NP and 11.4 µg/L OP (60 d; Seki et al. 2003); and rainbow trout, 20.3 µg/L NP (21 d; Jobling et al. 1996).

2.4 Phtalates

Phtalates are esters of phthalic acid and mainly used in the manufacture of plastics. The phthalates with the most evidence for estrogenic activity *in vitro* are butyl benzyl phthalate (BBP), dibutyl phthalate (DBP), and di-2-ethyl hexyl phthalate (DEHP; Murk et al. 2002). In a review of thousands of surface water measurements, most concentrations were below 1 µg/L for BBP and DBP (Staples et al. 2000), but surveys found DBP and DEHP concentrations of 0.12 – 8.8 µg/L and 0.33 – 97.8 µg/L in German, as well as 0.04 – 1.88 µg/L and 0.05 – 4.67 µg/L in Dutch surface waters, respectively (Fromme et al. 2002, Peijnenburg & Struijs 2006). In British and German sewage effluent < 1 – 2.8 µg/L BBP, < 1 – 14 µg/L DBP and < 2.4 – 182 µg/L DEHP were detected (Fatoki & Vernon 1990, Fromme et al. 2002).

BBP, DBP, and DEHP were *in vitro* 10^5 – 10^8 fold less potent than E2 (Murk et al. 2002). BBP was reported to induce VTG in fathead minnow and diethylphthalat (DEP) in carp exposed to 100 µg/L each (28 d; Barse et al. 2007, Harries et al. 2000).

2.5 Phytoestrogens

Isoflavones and coumestanes are the most prevalent groups of estrogenic compounds, present as phytoestrogens in high concentrations in legumes such as soy, clover, Lucerne beans and peas (Price & Fenwick 1985, Reinli & Block 1996). One of the major isoflavones is genistein, and coumestrol, the most common coumestan, is the phytoestrogen exhibiting the highest relative estrogenic potency (Bovee et al. 2004, Matthews et al. 2000). Up to 1 – 20 ng/L and 0.7 – 2 ng/L genistein and coumestrol were detected in Australian rivers (Kang & Price 2009), and concentrations of 3 – 5 ng/L and 24 µg/L genistein were found in an Italian and a Japanese rivers, respectively (Bacaloni et al. 2005, Kawanishi et al. 2004). Erbs et al. (2007) measured in drainage water of a test field containing red clover up to 14 ng/L genistein. Up to 0.1 –0.4 ng/L and 0.7 ng/L genistein and coumestrol were found in Australian STP effluents, respectively

(Kang & Price 2009). Pulp and paper mills exhibited high levels of isoflavones in their effluent with genistein concentrations up to 13.1 µg/L (Kiparissis et al. 2001). In plant-processing industries and STPs in USA, 0.158 – 151 µg/L genistein and 0.034 – 0.428 µg/L coumestrol were measured (Lundgren & Novak 2009).

The estrogenic potency of phytoestrogens is generally much weaker than that of steroidal estrogens but equal to other environmental estrogens such as BPA and NP (Erbs et al. 2007). VTG induction in rainbow trout primary hepatocytes revealed an EC_{50} of 5.99 µg/L E2 and 459.95 µg/L genistein, resulting in an E2 : genistein EC_{50} ratio of 1 : 76.69 (Cosnefroy et al. 2009).

A 215 and 620 fold induction of luciferase activity was detected after exposing a transgenic ERE-luciferase zebrafish line to 675.60 µg/L genistein and 272.39 µg/L E2, respectively (Sassi-Messai et al. 2009). Exposure to 1000 µg/L genistein caused gonadal intersex in male and atreitic oocytes in female medaka (Kiparissis et al. 2003).

3 Comparative hazard assessment of the estrogenic potential

To evaluate the estrogenic potency in fish, ZON was compared to the above described environmental estrogens. A ranking of the environmental estrogens according to their estrogenic potential *in vitro* and *in vivo* (if the corresponding data for the substance was available) is presented in the following.

E2 : EAS EC_{50} ratio for i*n vitro* estrogenicity (rYES):
E2 (1 : 1) > EE2 (1 : 1.13) > ZON > (1 : 13.9 – 350; chapter III) > OP (1 : 1553.8) > NP (1 : 6745.8) > BPA (1 : 10315)

E2 : EAS EC_{50} ratio for i*n vitro* VTG induction (fish cells):
EE2 (1 : 0.59 – 2.72) > E2 (1 : 1) > ZON (1 : 18.7 – 1438.9; chapter III) > genistein (1 : 76.69) > BPA (1 : 1047.7) > OP (1 : 1206.5 – 1249.3)

LOEC for *in vivo* VTG induction:
EE2 (0.5 – 3 ng/L) > E2 (21.4 ng/L) > ZON (1000 ng/L; chapter III) > NP (10 – 20.3 µg/L) > OP (11.4 µg/L) > BPA (100 µg/L) = BBP (100 µg/L) > genistein (675 µg/L, luciferase induction)

LOEC for *in vivo* effects on reproduction:
EE2 (0.05 – 5 ng/L) > E2 (27.2 ng/L) > ZON (100 ng/L; chapter III) > OP (28.06 µg/L) > genistein (1000 µg/L, intersex & atreitic oocytes) > BPA (1401 – 1500 µg/L)

According to these comparisons, ZON showed a weaker estrogen activity than the steroid estrogens EE2 and E2 but a stronger activity than OP, NP BPA, genistein and BBP. This result indicates a strong potential to act estrogenic in fish. From these data it can be concluded that ZON is of high relevance when environmental concerns on endocrine disrupters come into play.

4 Environmental risk assessment for ZON and common estrogens

To evaluate and compare the risk for fish in their natural environment, data of effects on fish were related to the concentrations found in the environment. The quantity of studies and sites for data of concentration in surface waters as well as bioaccumulation, biomagnification and metabolization mechanisms were not taken into consideration in this evaluation (sources for the data used in the following are already quoted in paragraph 2). For a basic risk characterization, each environmental estrogen was classified on the basis of environmental concentrations and LOECs from *in vivo* studies using three levels (Table 1).

Table 1: Classifications for the risk assessment

Risk characterization	Condition
Low	surface water and effluent concentrations < LOEC
Medium	only effluent concentrations ≥ LOEC
High	surface water concentrations ≥ LOEC

ZON exhibited surface waters concentrations of 0.5 – 44 ng/L which are about half the value presented as LOEC for zebrafish reproduction (100 ng/L, reduced fecundity) in this project. Measured effluents concentrations of 1 – 220 ng/L ZON are up to 2.2 times higher than LOECs for reproduction. Consequently, there is a medium risk for ZON to affect fish in the natural environment.
EE2 and E2 were present in surface waters at concentrations of 0.1 – 5 ng/L and 0.19 – 5.5 ng/L, respectively, which is for EE2 in the range of physiological responses in fish (0.5 – 3 ng/L, VTG induction) and effects on reproduction (0.05 – 5 ng/L), but below the LOEC for E2 (20 ng/L,

VTG induction; 27.2 ng/L, reproduction). The concentrations in effluents were 0.2 – 15 ng/L EE2 and 0.48 – 48 ng/L E2 which exceed also the LOEC for E2. Consequently, there is a high risk for EE2 and a medium risk for E2 to affect fish in the natural environment.

BPA was reported in surface waters at concentrations of 3 – 19000 ng/L and in effluents at 260 – 2500 ng/L, which in both cases is below the LOEC in fish (100 µg/L, VTG induction; 1401.7 – 1500 µg/L, reproduction). Therefore, it can be concluded that there is only a low risk for BPA to affect fish in the natural environment.

NP and OP were measured at concentrations of 0.15 – 644 µg/L and 0.016 – 0.111 µg/L in surface waters as well as 0.1 – 330 µg/L and up to 0.7 µg/L in effluents, respectively. This is in both cases above the LOEC for NP (10 – 20.3 µg/L, VTG induction; 100 µg/L, shifted sex ratio) but below the LOEC for OP (11.4 µg/L, VTG induction; 28.06 µg/L, reproduction) in fish. Thus, there is a high risk for NP and a low risk for OP to affect fish in the natural environment.

The limited data for phthalates demonstrated concentrations of 1 – 14 µg/L DBP, BBP and 0.05 – 97.8 µg/L DEPH in surface waters as well as 1 – 14 µg/L DBP, BBP and 2.4 – 182 µg/L DEHP in effluents, respectively, which is below the *in vivo* LOEC for BBP (100 µg/L, VTG induction). This implies only a low risk for BBP and, based on the assumption that the estrogenic potency is similar for DBP and DEHP, as well a low risk for DBP and a medium risk for DEHP to affect fish in the natural environment.

The phytoestrogens genistein and coumestrol were detected in concentrations of 1 – 24000 ng/L and 0.7 – 2 ng/L in surface waters as well as 0.1 – 151000 ng/L and 0.7 – 428 ng/L in effluents, respectively. Values in surface waters and in effluents are below the LOECs for genistein in fish (675.6 µg/L, luciferase induction; 1000 µg/L, intersex & atreitic oocytes). For coumestrol, effect data from fish are missing. These results suggest a low risk for genistein to affect fish in the natural environment.

Evaluating this data from a worst case point of view, that is taking into consideration the highest detected concentrations, there is a high risk for EE2 and NP to cause endocrine disruption in fish in their natural habitat. The risk for BPA, OP, BBP, DBP and genistein to affect fish is low and for coumestrol *in vivo* data is missing. According to the applied classification, there is a medium risk for ZON, E2 and DEPH (assuming similar potency for DEHP and DBB) to affect fish. However, taken into consideration a regional and temporal limited exposure to higher concentrations (above the LOEC) caused by effluent discharge in combination with low flow conditions (low dilution factor) or surface runoff from contaminated fields, it is conceivable that ZON, E2 and DEPH can affect fish in the natural environment.

For some of the discussed environmental estrogens, including ZON, data of environmental occurrence and concentrations is very limited. Therefore the significance of this risk assessment is also limited and has to be evaluated according to the described restrictions. Furthermore, a possible difference in species sensitivity has to be taken into consideration when extrapolating results from laboratory experiments with model organism to native wildlife. For example in a direct comparison of E2 induced VTG synthesis with zebrafish, fathead minnow and medaka, zebrafish was the least sensitive species (Seki et al. 2006) whereas no difference between zebrafish and rainbow trout was found (Van den Belt et al. 2003a).

5 Conclusions

In the present research project, the ecotoxicological significance of the estrogenic mycotoxin ZON was studied. The potential effects on fish caused by exposure to ZON were assessed using the zebrafish as a model organism. Different exposure scenarios and different life-stages of zebrafish were investigated in a series of experiments to investigate effects on various endpoints. The results of the experiments presented in this thesis provide crucial data for the ecotoxicological evaluation of ZON. The estrogenic potential of ZON was confirmed *in vitro* and *in vivo* and detrimental effects on zebrafish reproduction were demonstrated. The effects on zebrafish reproduction were observed at concentrations near the maximum measured values in surface waters (approximately double) and below the maximum values measured in effluents (approximately half). This might indicate a potential for ZON to affect fish populations in their natural environment. However, to date there is not much data published from ZON concentrations in the environment and effects on different (native) fish. Therefore, altogether we suggest a more detailed characterization of the ecotoxicological relevance of ZON in order to conduct a scientifically sound risk assessement.

Chapter VI

Embryotoxic and genotoxic potential of sewage system biofilm and river sediment in the catchment area of a sewage treatment plant in Switzerland

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Abstract

In the present study an embryo toxicity test with zebrafish and the comet assay with primary cells isolated from the embryos were combined to assess the toxicity of native biofilms from the sewage system of the sewage treatment plant (STP) Mittleres Wynental and native sediments from the river Wyna (Switzerland). The aim of the study was to evaluate the applicability of the test system to investigate biofilms and to rank the results of the biofilm and sediment sampling sites with regard to embryotoxic and genotoxic effects.
We demonstrated that the zebrafish embryo toxicity test in combination with the comet assay with embryo primary cells is a tool that can basically be applied for investigating the bioavailable toxic potential of native biofilms. By ranking the results for embryo toxicity and genotoxicity, we found clear differences in the toxic potential of the biofilm from different sewage system sections. Significant genotoxicity was determined in all biofilm samples and the sediments revealed a lower genotoxic potential upstream of the STP discharge compared to samples downstream and directly at the discharge. Temporal variability from samplings in autumn and spring were found for two of the five biofilms and for one of the three sediment samples.
Based on the results of our study we suggest that ecotoxicological bioassays (such as the embryo toxicity test and comet assay with zebrafish) using biofilm can be a useful tool to assess (waste) water quality.

1 Introduction

Fish decline in rivers is often discussed related to water pollution and degradation of aquatic habitats (Burkhardt-Holm et al. 2005, Keiter et al. 2006). In general, sewage treatment plants (STP) are considered to be one major point source of anthropogenic contamination of river systems (Ternes 2007). The composition of STP effluents can be a complex mixture of more than 100'000 different chemicals which can exhibit detrimental effects on fish, resulting in impaired health status, increased mortality or decreased reproductive success in fish populations (Sumpter 1997, Kime 1999, Clements 2000).

Many anthropogenic chemicals in the water phase adsorb to suspended particles and are integrated into the sediment. Therefore, sediments are a sink but also a potential source for persistent toxic substances (Burton 1991, Hollert et al. 2003, Schwarzenbach et al. 2006). Sediment-bound contaminants can affect organisms that live over it directly or indirectly via the food web, and can also be remobilized during flood events (Mac et al. 1984, Power and Chapman 1992, Hollert et al. 2000). In sediments which are contaminated by STP effluents and industrial waste, genotoxic substances are frequently detected (Klee et al. 2004, Kosmehl et al. 2004, Aouadene et al. 2008). These genotoxic substances can affect the reproductive success of organisms: DNA damage leads to dieback of gametes, developmental disorders, embryo mortality, and hereditary mutations, and can directly influence the population structure and size (Anderson and Wild 1994). Furthermore, it was reported that increased DNA damage, induced by genotoxic substances, can affect gene flow in populations and hence reduce genetic diversity (Theodorakis et al. 2000, 2001).

Not only sediment but also biofilm is particularly interesting as indicator for environmental pollution, as it interacts with toxic substances and integrate over longer periods. Biofilm is a complex biocoenosis of microorganisms (bacteria, fungi, algae) on a solid surface at the boundary layer to a liquid phase (Sabater et al. 2007). Apart from natural boundary layers such as soil, sediments, plants, animals and mucosa they can be found on technical installations such as canalizations. In biofilms, the cells of the microorganisms are embedded in a secreted mucilage matrix of extracellular, polymer substances (EPS). A multitude of organic and inorganic compounds are integrated and particular substances are embedded in the EPS (Geesey et al. 1994).

Several studies revealed that the sensitivity of fish embryos and larvae to some chemicals is greater than that of adults (Lange et al. 1995, Nagel and Isberner 1998, Nagel 2002, Strmac et al. 2002). In early life-stage tests, toxicant effects on ontogenesis and growth can be examined through many diverse endpoints (Ensenbach and Nagel 1997, Luckenbach et al. 2001). In

addition, the direct exposure of a test organism to an environmental sample closely resembles natural conditions and can therefore be of high ecological relevance. In several studies zebrafish (*Danio rerio*) eggs have been exposed to native sediment to assess the bioavailable toxic potential of samples (Ensenbach 1998, Hollert et al. 2003, Hallare et al. 2005, Colavecchia et al. 2006, Höss et al. 2010). In contrast to sediment, the toxic potential (toxicant load) of biofilm has to date not been investigated in bioassays.

The comet assay (single cell gel electrophoresis) is one of the most commonly used techniques to detect DNA strand breaks in cell lines and primary cells (Cotelle and Ferard 1999, Rojas et al. 1999, Kamer and Rinkevich 2002). By using primary cells from zebrafish embryos previously exposed directly to sediment samples, the bioavailable genotoxic potential of these samples can be assessed in the comet assay (Kosmehl et al. 2006, 2008).

In the present study, both an embryo toxicity test with zebrafish and the comet assay with primary cells isolated from the embryos were applied for the first time, to investigate native biofilms from different sections of a sewage system. Furthermore, this combination of test systems was used to assess the toxicity of native sediments from the river Wyna directly at, upstream and downstream of the STP discharge Mittleres Wynental (Switzerland). The STP was selected because previous studies demonstrated a negative effect of this STP on brown trout (*Salmo trutta fario*) embryo development (Escher 1999, Kobler 2004, Aqua-Sana 2006). The aim of the study was to verify the applicability of the test system to investigate biofilms and to evaluate the embryotoxic and genotoxic potential of the samples in a ranking. This ranking was used (1) to compare the hazard potentials of the different sampling sites, (2) localize possible toxic discharges into the sewage system, and (3) to investigate temporal variability from samplings in autumn and spring.

2 Materials and Methods

2.1 Sampling sites

The river Wyna has a total length of 32 kilometers and flows through the cantons Lucerne and Aargau, Switzerland, with an average flow rate in the downstream of about 1.76 m^3/s. The STP Mittleres Wynental is dimensioned for 8'500 inhabitant equivalents and located in an area of intensely used farmland and small industries at the lower reaches of the river Wyna (coordinates: N 47.334 E 8.112, WGS84). Three STPs upstream of the STP Mittleres Wynental are discharging wastewater into the river which can reach a proportion of up to 50 % of the river water (Kobler 2004).

Six biofilm samples (B1 – B6) were taken from the sewage pipelines of the STP Mittleres Wynental and three sediment samples from the river Wyna (S1 directly at the discharge of the STP, S2 100 m upstream and S3 200 m downstream, Fig. 1). The selection of the sampling location was done in cooperation with the cantonal authorities, based on their estimation in which sections problematic discharge could be expected.

2.2 Sampling and processing of biofilm and sediment

A first sampling of all sites, apart from B6, was conducted in October 2008 ('autumn samples') and a second sampling, including B6, in June 2009 ('spring samples').

At each sampling site, 0.2 – 0.3 L biofilm and 0.4 – 0.6 L sediment (0 – 5 cm depth) was taken from several points within an area of approximately 1 – 2 m^2 and 4 m^2, respectively, using a stainless steel spoon and a spattle. The biofilm and sediment samples were filled into solvent (ethanol and acetone) cleaned 0.5 and 1 L glass bottles, respectively, and closed with Teflon lids. The bottles were transported to the laboratory in a cool box equipped with cooling packs and stored at -40 °C for a maximum of two weeks until further processing. The frozen samples were then dried for one week in a freeze-dryer (Freezemobile 6, Virtis Inc. Gardier, USA), homogenized and stored at 4 °C in the dark.

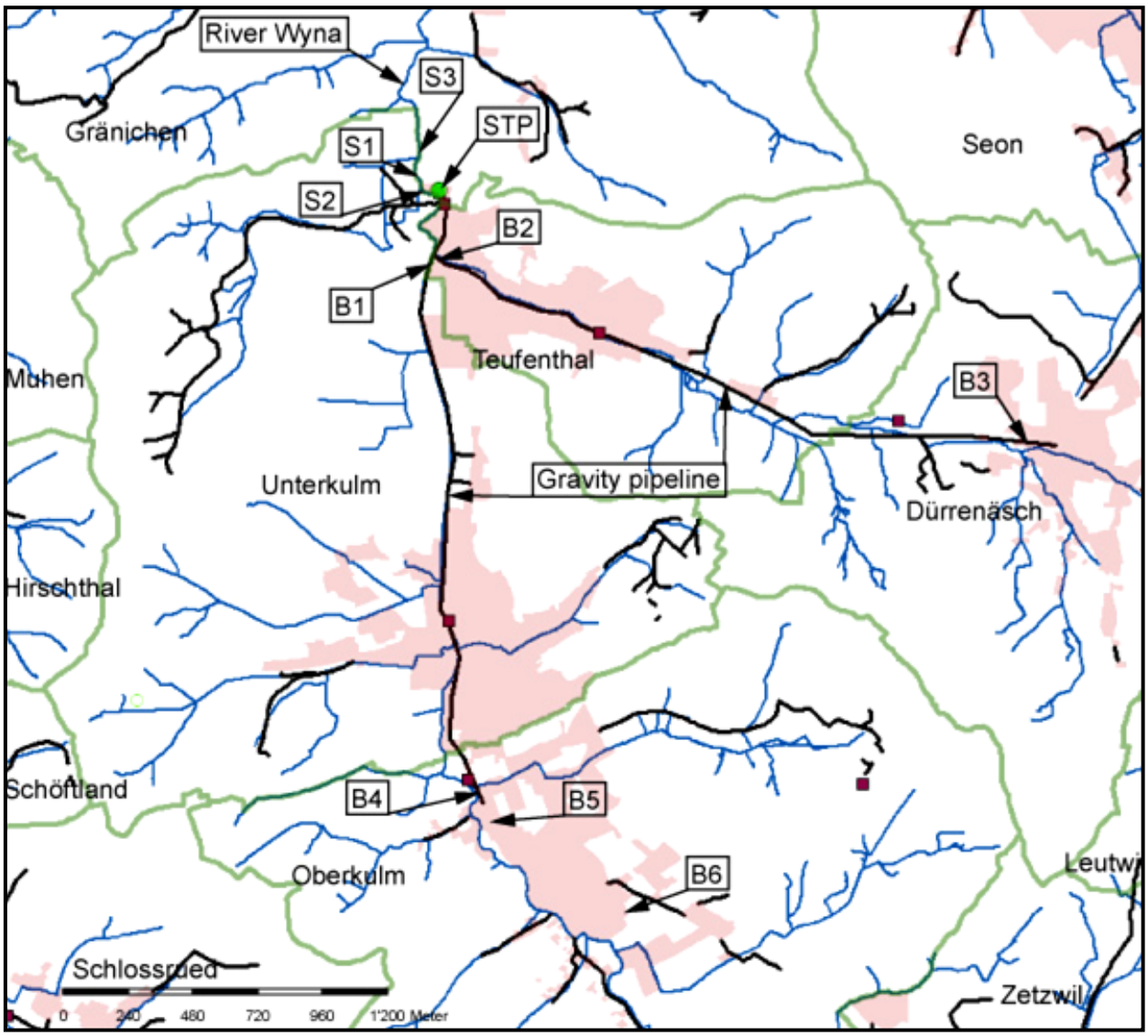


Fig. 1: Sampling sites for biofilm (B1 – B6) in the sewage pipelines and sediment (S1 – S3) in the river Wyna, in the catchment area of the sewage treatment plant (STP) Mittleres Wynental, canton Aargau, Switzerland.

2.3 Experimental animals

All procedures concerning experimental animals were performed in compliance with the Swiss protection of animals act. The zebrafish (*Danio rerio*) used for egg production originated from our fish breeding stocks, held at the University of Basel. They were kept in aerated 60 L glass tanks supplied with a constant flow of 5 L/h conditioned water (1 : 1 mix of charcoal filtered tap water and reverse osmosis water) at a temperature of 26 ± 1 °C, pH of 8.0 ± 0.2, water hardness of 142.4 mg/L $CaCO_3$ (8 °dH) and an artificial light : dark photoperiod of 16 : 8 h. The fish were fed twice a day once with dry pellet (SilverCup, H. U. Hofmann AG, Switzerland) and once with frozen brine shrimps (*Artemia salina*, 3F Frozen Fish Food BV, Netherlands).

2.4 Egg production

Approximately 2 h before the dark period, spawning groups of two female and four male zebrafish were transferred into 12 L spawning tanks. All tanks were aerated and tempered to 26 ± 1 °C using submerged electrical heaters and a light : dark photoperiod of 16 : 8 h was maintained. The spawning tanks were equipped with a stainless steel mesh (mesh size: 2.5 mm) to prevent fish from eating their eggs. Artificial weed (Clear water coarse filtering wool, Dennerle GmbH, Germany) was attached to the centre of the steel mesh to provide a spawning stimulation. Oxygen was always above 7.4 mg/L (Oxi 315i, WTW, Germany), pH at 7.8 – 8.2 (pH 315i, WTW, Germany), and conductivity at 240 – 260 µS/cm (Cond 315i, WTW, Germany). On the following morning, 0.5 – 1 h after the onset of the light period, all fish were transferred back to the stock tanks and the eggs were collected into petri dishes for microscopical analysis.

2.5 Embryotoxicity test

The embryo toxicity test was conducted with biofilm and sediment sampled in autumn and spring each with two independent experiments according to the adapted test protocol of Hollert et al. (2003) and the German DIN 38415-6. All samples were tested at 4 – 5 concentrations, each with 6 wells of a 6-well plate (Becton Dickinson Labware, USA) and 3 g sample dilution per well (Table 1). To prepare the dilutions, each sample of sediment and biofilm was mixed with silica sand (50 – 70 mesh particle size, Sigma-Aldrich, Switzerland) and homogenized with a mortar. The applied concentrations were determined in range finding experiments (data not shown). For the negative control, 6 wells were filled with 3 g pure silica sand. Approximately one hour before the exposure started, 7 mL artificial water (ISO 7346/3, stock solutions of 58.8 mg/L $CaCl_2 \times 2\ H_2O$, 24.6 mg/L $MgSO_4 \times 7\ H_2O$, 12.6 mg/L $NaHCO_3$ and 5.5 mg/L KCl, diluted 1:5 with purified water), aerated to 100 % oxygen saturation was added to each well.

Table 1: Sediment and biofilm dilutions and concentrations of the sample-silica sand mixture applied in the embryotoxicity test.

Sediment					
Dilution	1:2	1:4	1:8	1:16	1:32
Concentration [%]	50.0	25.0	12.5	6.3	3.1
Biofilm					
Dilution	1:32	1:64	1:128	1:256	1:512
Concentration [%]	3.1	1.6	0.8	0.4	0.2

Within two hours post fertilization (hpf), 5 fertilized and normally developing fish eggs were transferred into each well using a plastic pipette. The well plates were closed with lids and incubated in a heating cabinet at 26 ± 1 °C with saturated humidity to avoid evaporation from the wells. The eggs were exposed to the samples during the period of 2 – 96 hpf and inspected at 24, 48, 72 and 96 hpf for lethal and sub lethal deviation from normal development using an inverse microscope (Olympus CKX41, Olympus, Germany) at 40x and 100x magnification. For examination, the eggs were transferred into 24-well plates (Becton Dickinson Labware, USA) filled with artificial water. The toxicological endpoints evaluated in the test are given in Table 2 (Hollert et al. 2003). A line was extrapolated to the data using SigmaPlot version 9.01 (Systat Software Inc., USA), and the LC_{50} for mortality and EC_{50} for hatch rate (50 % effect concentration) were determined graphically from the linear section of the graph. The LC_{50} for mortality at 48 hpf and EC_{50} for hatch rate at 96 hpf (modification of DIN 38415-6) were used to determine embryo toxicity.

Table 2: Toxicological endpoints for the evaluation of embryotoxicity and mortality (hpf: hours post fertilization).

Toxicological endpoint	24 hpf	48 hpf	72 hpf	96 hpf
Coagulation	●	●	●	●
Epiboly stage	●	●	●	●
No somites	●	●	●	●
Undetached tail	●	●	●	●
No heartbeat	—	●	●	●
No circulation	—	○	○	○
No pigmentation	—	○	○	○
No movement	○	○	○	○
Development retardation	○	○	○	○
Edema	○	○	○	○
Malformation	○	○	○	○
Hatch	—	—	○	○

● = lethal criterion used to determine mortality rate
○ = documented but not evaluated as lethal criterion
— = not documented

2.5.1 Oxygen consumption of the samples

The oxygen concentration in the water can decrease during the experiment due to oxygen consumption caused by biological (degradation of organic material) and chemical reactions. Therefore, at the end of each experiment, the liquid of all wells from each sample concentration

was carefully pooled in a 25 mL glass beaker and O_2 concentration was measured (Oxi 315i, WTW, Germany).

2.6 Comet assay with primary cells

The comet assay was conducted with biofilm and sediment sampled in spring, according to the modified test protocol of Kosmehl et al. (2006). We conducted two independent experiments with two independent replicates (n = 2) each. Eight embryos were pooled for each replicate. An additional concentration of 0.2 % was used for all biofilm samples. At the end of the embryo toxicity exposure (96 hpf), the sample concentrations in which ≥ 80 % of the embryos survived were investigated in the comet assay. For the positive control, primary cells from embryos which developed on pure silica sand were exposed for 10 min to UV radiation in Eppendorf reaction tubes.

2.6.1 Cell isolation

The embryos were euthanized in 150 mg/L MS222 (Ethyl 3-aminobenzoat, methanesulfonic acid salt 98 %, Sigma-Aldrich GmbH, Switzerland) buffered with 300 mg/L $NaCO_3$ and washed in phosphate-buffered saline (PBS). MS222 does not cause primary DNA damage and is recommended for genotoxicity tests (Barreto et al. 2007). The eight embryos per replicate were transferred into a 5 mL glass tissue grinder (Potter-Elvehjem-type, Sartorius, Germany) with a defined pestle/wall distance of 50 to 70 µm in which 1 mL PBS-fetal bovine serum (Sigma-Aldrich GmbH, Switzerland) solution was added. The grinding step was conducted two times with careful pressure and a rotation of 90°. The resulting cell suspension was filtered through a 70 µm gauze (SEFAR, Switzerland) into an Eppendorf reaction tube and centrifuged for 10 min at 4 °C and 200 g.

2.6.2 Cell vitality test

A cell vitality test was conducted in preliminary experiments according to the modified test protocol by Borenfreund and Puerner (1984), to improve the cell isolation method and to avoid too much damage of the cells during the isolation process. Based upon the results of the experiments, two grinding steps with the homogenizer were selected as optimum. With the established method, the cell viability was consistently > 80 % (mean = 94 %, n = 8).

After cell isolation, the density was adjusted with PBS to 3×10^4 cells/mL. From this suspension, 200 µL was filled into each well of a 96-well plate and 50 µL of a 0.4 % toluylene red solution (2-Methyl-3-amino-7-dimethylaminophenazin, Carl Roth GmbH, Germany) in PBS was added. For the negative control, 8 wells were filled with 200 µL PBS and 50 µL toluylene red solution. After incubation for 10 h in the dark, the 96-well plate was centrifuged for 2 min at 200 g. The supernatant was discarded and the wells were again filled with 200 µL PBS and centrifuged for 2 min at 200 g. The supernatant was removed and the plate was dried for 15 min. Each well was filled with 50 µL lysis buffer (10 mL Ethanol, 200 µL acetic acid) and analysed in the plate reader (540 nm, Tecan Sunrise absorption-plate-reader, Tecan Group Ltd, Switzerland).

2.6.3 Electrophoresis

The slides (Superfrost Plus, Gerhard Menzel GmbH, Germany) were coated with 1.0 % (w/v) normal melting agarose (NMA; SeaKem LE Agarose, Biozym Scientific GmbH, Germany) in PBS. Following hardening on an ice cold metal plate for 3 min, the slides were dried for 24 h at room temperature.

After cell isolation, the density was adjusted with PBS to 3×10^4 cells/mL, gently mixed with 90 µL 0.7 % (w/v) low melting agarose (LMA; SeaPlaque GTG Agarose, Biozym Scientific GmbH, Germany) at 37 °C and spread onto a precoated slide. The slide was again put on the cold metal plate for 3 min and dried for 5 min at 37 °C. Finally, 100 µL 0.7 % (w/v) LMA was placed on top as a protective third layer, cooled for 3 min and dried for 5 min at 37 °C.

The slides were incubated in lysis solution containing 100 mM ethylene-diamine-tetraacetic acid (EDTA; purity: 99 %, Sigma-Aldrich GmbH, Switzerland), 2.5 M NaCl, 1 % (v/v) Triton X-100 (Sigma-Aldrich GmbH, Switzerland), and 10 % (v/v) dimethyl sulfoxide (pH 13.0; purity: 99.9 %, Sigma-Aldrich GmbH, Switzerland) for 3 h at 4 °C in the dark. For DNA unwinding, slides were immersed in a horizontal electrophoresis tank (Maxigel ECO 2, Apelex, France) containing ice cold alkaline buffer (12 g/L NaOH, 0.37 g/L EDTA) for 30 min in the dark. After electrophoresis for 25 min at 25 V and 0.3 A, the slides were neutralized with 400 mM Tris-HCl (purity: 99 %, Sigma-Aldrich GmbH, Switzerland) buffer at pH 7.4 for 2 min.

2.6.4 Image analysis and statistics

Immediately before scoring, the gels were stained with 75 µL of 20 µM ethidium bromide (purity: 95 %, Sigma-Aldrich, Switzerland) in purified water and embedded with a cover slip. All slides were examined at 340x magnification using a fluorescent microscope (Eclipse 400,

Nikon, Switzerland) equipped with an excitation filter for ethidium bromide (518 nm) and a camera (Nikon DXM-1200F, Nikon, Switzerland). For each concentration, 100 randomly selected nucleoids of two replicate slides (50 each) were photographed. Subsequently, the photos were analyzed with the software CometScore (TriTek, Sumerduck, USA) using the tail moment (product of the fluorescence intensity and the tail length) for statistical analysis.

Data of tail moments was analyzed by the Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn´s method for multiple comparisons (SigmaStat 3.1TM, Systat Software, Germany). In case of significant differences to the control ($p \leq 0.05$), the sample concentrations were scored as genotoxic. The lowest observed effect concentration (LOEC) was determined as the lowest test concentration, which caused statistically significant DNA damage. The induction factors (IFs) were calculated as quotient of the tail-moment means of all sample concentrations and the control.

For the genotoxicity ranking, the data was analyzed using the 3-step analysis (Seitz et al. 2008). This is a descriptive method that takes into account all information that can be obtained from the concentration-effect curve, allowing a comprehensive comparison of the genotoxicity of samples. According to this method, the evaluation of the samples was performed based on three steps: 1. LOECs (first the samples are ranked according to their LOECs); 2. Maximum IF (IFmax) in the concentrations significantly different from control (in case of identical rank in step 1, the samples are further ranked according to the IFmax in the concentrations with significant genotoxicity); 3. IFmax in the non significant concentrations (in case of identical rank in step 2, the samples are further ranked according to their IFmax in the concentrations without significant genotoxicity). However, only IFs can be compared which are based on identical concentrations.

3 Results

3.1 Embryotoxicity

Zebrafish embryos developed normally in the control groups of all experiments, defined by the criteria of Kimmel et al. (1995). In the following the results for mortality and hatch rate (3.1.1) as well as a qualitative description of the observed development abnormalities caused by exposure to the biofilm and sediment are given (3.1.2, 3.1.3, see also Table 2).

3.1.1 Mortality and hatch rate

The mortality in the control groups at 48 hpf was in all experiments below 10 %. Therefore the requirement of DIN 38415 – T6 for a valid test was always met and the observed effects can be attributed to the sediment and biofilm exposure.

In the biofilm samples, most eggs in most concentrations were already coagulated at 24 hpf and at 48 hpf many of the vital embryos had no heartbeat and were hence determined as mortal. In a few cases, some embryos which revealed a development retardation and were determined as mortal at 24 hpf could catch up the development retardation and therefore the mortality decreased until 48 hpf. The mean LC_{50} for mortality at 24 and 48 hpf and EC_{50} for hatch rate at 72 and 96 hpf for the two experiments with the samples from autumn and from spring are presented in Figures 2 and 3. The ranking based on the mean LC_{50} for mortality of all four experiments at 48 hpf is: B2 < B1 = B6 < B3 < B4 = B5, and for hatch rate at 96 hpf: B1 < B2 < B6 < B3 < B4 = B5 (Table 3).

In the sediment samples many embryos were not developed as far as in the control at 24 hpf and were therefore determined as mortal. However, they were able to catch up the development retardation until 48 hpf. Only in sample S1 was a high mortality determined at 48 hpf in one experiment. The mean LC_{50} for mortality at 24 and 48 hpf and EC_{50} for hatch rate at 72 and 96 hpf of the two experiments for the samples from autumn and from spring are presented in Figures 2 and 3. The ranking based on the mean LC_{50} for mortality of all four experiments at 48 hpf is: S3 < S2 < S1, and for hatch rate at 96 hpf: S3 < S2 < S1 (Table 3).

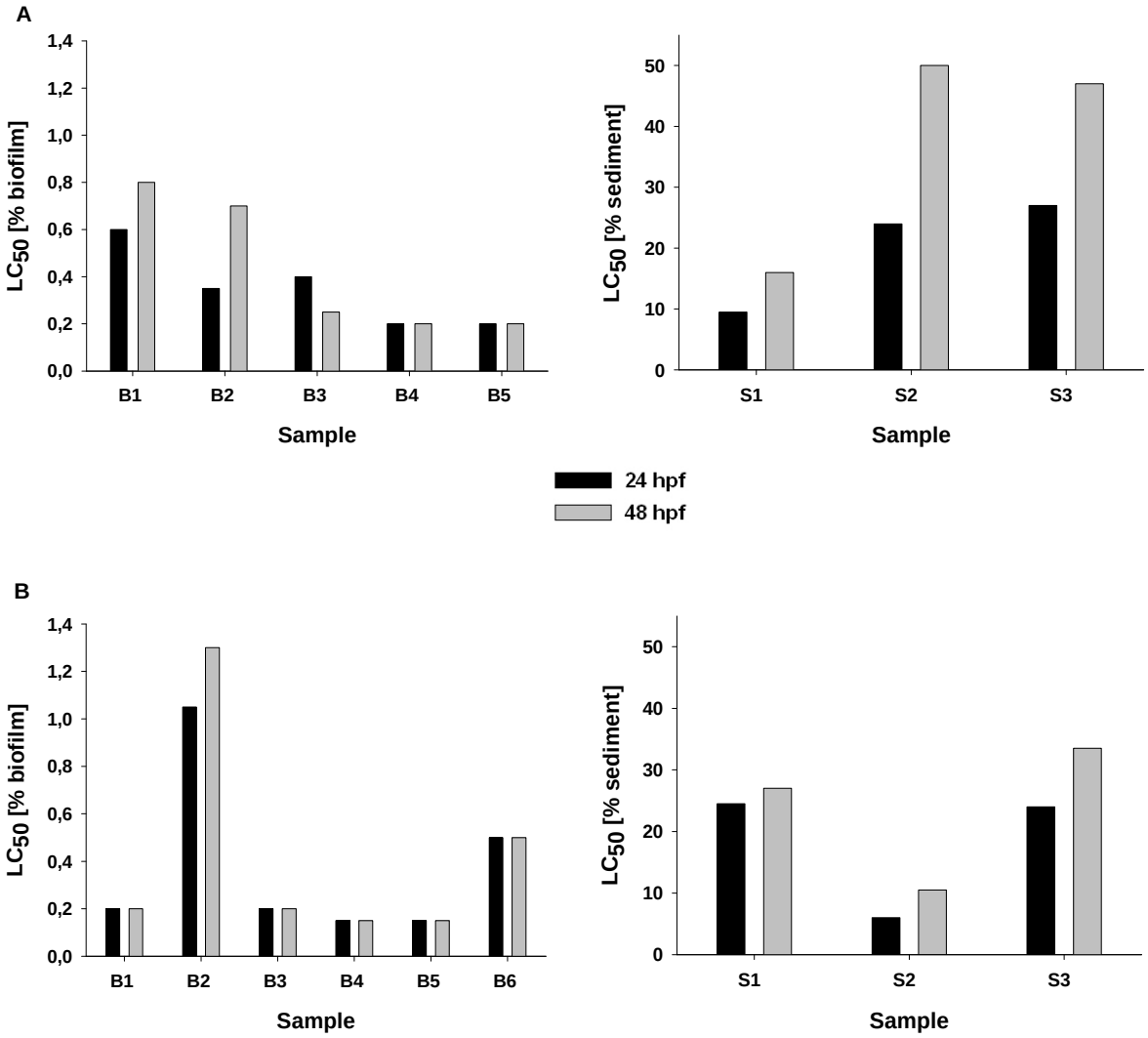
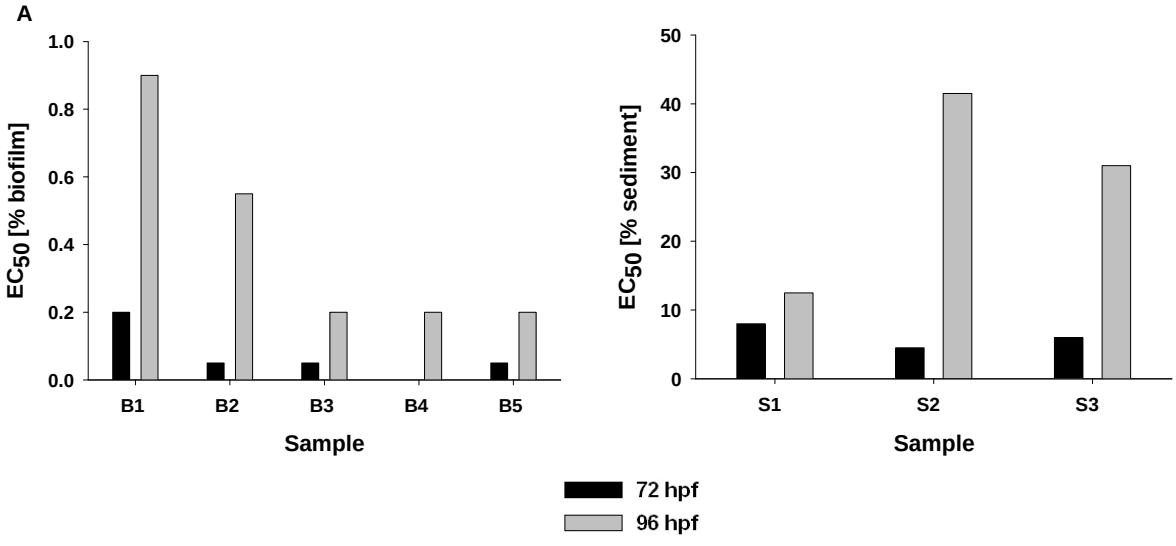


Fig. 2: Embryo toxicity of biofilm (B1 – B6) and sediment (S1 – S3): Lethal concentration of 50 % (LC_{50}) for mortality at 24 and 48 hours post fertilization (hpf). Mean of two independent experiments with samples from (A) autumn (October 2008) and (B) spring (June 2009).



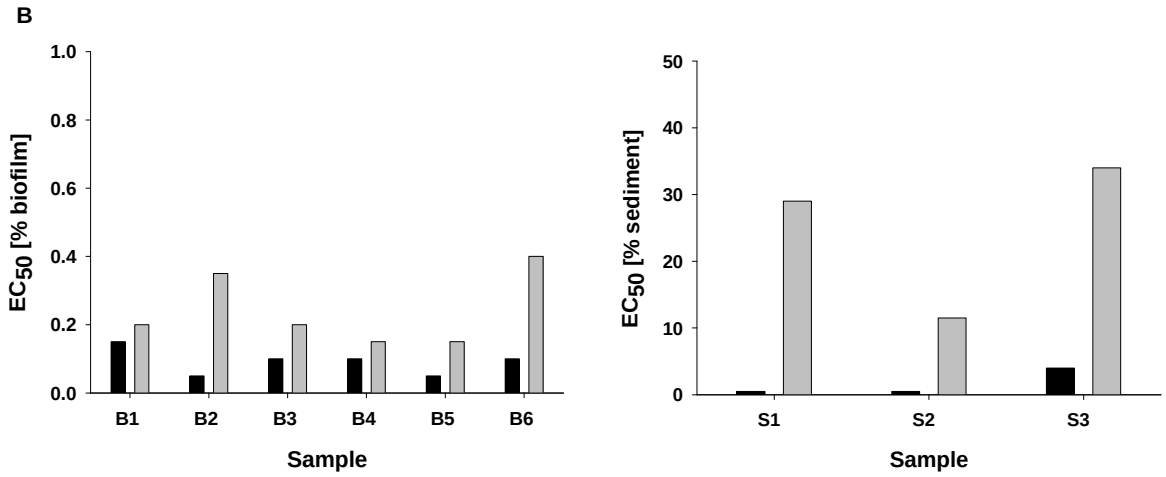


Fig. 3: Embryotoxicity of biofilm (B1 – B6) and sediment (S1 – S3): Effect concentration of 50 % (EC_{50}) for hatch rate at 72 and 96 hours post fertilization (hpf). Mean of two independent experiments with samples from (A) autumn (October 2008) and (B) spring (June 2009).

Table 3: Embryotoxicity of biofilm (B1 – B6) and sediment (S1 – S3): Effect concentration of 50 % (EC_{50}) for mortality at 48 hours post fertilization (hpf) and hatch rate at 96 hpf (sample concentration in %). Results from two experiments for samples from autumn (October 2008, 1 & 2) and spring (June 2009, 3 & 4).

EC_{50}	B1	B2	B3	B4	B5	B6	S1	S2	S3
Experiment	**Mortality 48 hpf**								
Autumn (1)	0.2	0.2	0.2	0.2	0.2	-	8	50	44
Autumn (2)	1.4	1.2	0.3	0.2	0.2	-	24	50	50
Spring (3)	0.2	1.3	0.2	0.2	0.1	0.5	37	5	37
Spring (4)	0.2	1.3	0.2	0.1	0.2	0.5	17	16	30
Mean	0.50	1.00	0.23	0.18	0.18	0.50	21.50	30.25	40.25
Experiment	**Hatch rate 96 hpf**								
Autumn (1)	1.0	0.2	0.2	0.2	0.2	-	7.0	33.0	12.0
Autumn (2)	0.8	0.9	0.2	0.2	0.2	-	18.0	50.0	50.0
Spring (3)	0.2	0.5	0.2	0.2	0.1	0.3	34.0	6.0	34.0
Spring (4)	0.2	0.2	0.2	0.1	0.2	0.5	24.0	17.0	34.0
Mean	0.55	0.45	0.20	0.18	0.18	0.40	20.75	26.50	32.50

3.1.2 Embryotoxicological effects of biofilm

At 24 hpf, the main observation in samples B1, B2 and B6 was an undetached tail up to 0.8 % biofilm. At 1.6 % and higher concentrations most embryos had no somites and were still in the epiboly stage or coagulated. In B2 from autumn, most embryos showed an undetached tail already at 0.8 % whereas in the spring sample this effect only appeared in higher concentrations.

In the samples B3, B4 and B5, most embryos had no somites and were in epiboly stage up to 0.8 %. At 1.6 % and higher only few egg survived.

At 48 hpf, in the B1 autumn sample effects such as no circulation and edemata were observed and many embryos had no heartbeat up to 0.8 %. In the spring sample, effects such as no somites and undetached tails appeared frequently up to 0.8 % and at 1.6 % and higher most eggs were coagulated. The embryos in B2 showed effects such as no heartbeat or no circulation up to 0.8 %. At 1.6 % and higher the embryos had no somites, were in epiboly stage or coagulated. In B3, B4 and B5 the embryos displayed undetached tails, no somites or were still in epiboly stage at 0.4 %. At higher concentrations all eggs were coagulated.

At 72 hpf, only few or no fish in all biofilm samples hatched. The embryos in the B1 autumn sample showed less development retardation up to 0.8 % than in the spring sample. At higher concentrations most eggs were coagulated in both samples. In B2 from autumn no lethal effects were observed up to 0.8 % and at higher concentrations most eggs already had coagulated earlier. In the spring sample effects such as no somites were observed at 3.1 % sporadically and at 0.4 – 0.8 % deformed tails appeared several times. Samples B3 and B4 showed effects such as no heartbeat at the lowest concentration whereas in higher concentrations all eggs already had coagulated earlier. In B3 from spring and B5 almost all eggs were coagulated.

At 96 hpf, effects such as undetached tails could be observed in all biofilm samples and no circulation was observed frequently in the vital embryos.

3.1.3 Embryotoxicological effects of sediment

At 24 hpf, most embryos exposed to S2 and S3 revealed development abnormalities such as undetached tails, absence of somites and epiboly stage at 12.5 % and higher. These effects appeared in S1 already at 6.3 %, and at 50 % many embryos coagulated. In S2 from spring undetached tails and epiboly stage appeared already at 6.3 %. Only very few edemata were observed.

At 48 hpf, S1 from autumn revealed effects such as no circulation, edemata and tail deformations already in concentrations of 3.1 %. In the S1 spring sample no circulation, edemata and deformed tails were observed frequently at 12.5 % and higher. From 25 % and higher, effects such as no heartbeat, undetached tail or coagulation appeared in the autumn and spring sample. In S2 and S3 from autumn, effects such as no heartbeat and no circulation appeared sporadically at 25 % and higher. In the spring sample abnormalities such as undetached tails and no circulation were already increased at 12.5 %.

At 72 hpf, all embryos in S1 were coagulated at 50 % and in lower concentrations effects such as no circulation, no heartbeat and edemata appeared frequently. The vital embryos in S2 from spring revealed development retardations in 12.5 % and higher. Most of the embryos in the sample S3 developed normally.

At 96 hpf, most embryos of the vital eggs in the samples S2 and S3 hatched. In the S1 autumn sample, the fish hatched up to 12.5 % whereas in the spring sample they hatched up to 25 %.

3.1.4 Oxygen consumption of samples

The oxygen concentration at the end of the experiment was generally higher in the sediment than in the biofilm samples and always above the limit of 2 mg/L, which was reported to have no negative effects on zebrafish development (Braunbeck et al. 2005). Only in the highest concentration (3.1 %) of the B1 spring sample and B3, B4 and B5 from spring and autumn, oxygen levels at 96 hpf were below 2 mg/L (data not shown).

3.2 Genotoxicity

For a conservative assessment, the ranking according to the 3-step analysis was based on the lower LOECs for genotoxicity of the two independent experiments. The mean IFmax of the significant genotoxic concentration for both experiments was calculated. In those cases where the concentrations in which the IFmax was measured were not identical for both experiments the lower concentration was used (Table 4; see appendix for the three-dimensional diagram).

The biofilm B1 only showed a significant genotoxicity at a concentration of 0.4 % in both experiments. In B2 and B6 significant genotoxicity in both experiments was detected at 0.4 % and higher and in one experiment at 0.2 %. B3, B4 and B5 revealed a significant genotoxicity in both experiments already at 0.2 %. The samples B2, B3, B4, B5 and B6 showed the same LOECs of 0.2 %. Accordingly, these samples were ranked based on their IFmax in the significant genotoxic concentrations. Only B3, B4 and B5 can be compared with each other, as they have the IFmax (13.95, 4.50 and 3.85) in the same concentration. Therefore B3 is more genotoxic than B4 and B4 is more genotoxic than B5. The ranking for the genotoxicity according to the 3-step analysis of the two experiments for the spring sample is: B1 < B2 = B6 < B5 < B4 < B3.

The sediment S1 revealed a significant genotoxicity in both experiments in all concentrations. In S2 and S3, significant genotoxicity was detected in both experiments at 12.5 % and higher. Sample S3 showed a significant genotoxicity in one experiment at 3.1 and 6.25 % and S2 at

6.25 %. S1 and S3 exhibited an identical LOEC but they could not be ranked based on the IFmax because they were different for the significant and non significant concentrations. The ranking for the genotoxicity according to the 3-step analysis of the two experiments for the spring sample is: S2 < S3 = S1.

Table 4: Genotoxicity of biofilm (B1 – B6) and sediment (S1 – S3): Lowest observed effect concentration (LOEC) in the comet assay for experiment 1 (LOEC 1) and experiment 2 (LOEC 2), mean maximum induction factor (IFmax) and sample concentration at which the IFmax was measured. The lower LOEC (bold) was used for the ranking.

	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**S1**	**S2**	**S3**
LOEC 1 [%]	**0.40**	0.40	**0.20**	**0.20**	**0.20**	**0.20**	**3.10**	12.50	12.50
LOEC 2 [%]	0.40	**0.20**	0.20	0.20	0.20	0.40	3.10	**6.25**	**3.10**
IFmax	7.35	8.00	13.95	4.50	3.85	21.70	7.30	7.65	14.65
Concentration [%]	0.40	0.80	0.20	0.20	0.20	0.40	12.50	12.50	25.00

4 Discussion

4.1 Applicability of the test system and evaluation of biofilm toxicity

For the first time, biofilm was investigated in an embryo toxicity test. The biofilms exhibited a 22 – 229 fold higher embryo toxicity (LC_{50}: 0.175 – 1 % at 48 hpf) than the river sediments. An obvious reason for this is that biofilm from a sewage system is exposed to the untreated wastewater and therefore it can be expected to exhibit a far higher toxic potential than river sediment which is exposed to treated and diluted wastewater. Another reason is the composition of biofilm. River sediments mainly consist of mineral and organic particles with toxic substances adsorbed, while biofilm is a biocoenosis of microorganisms embedded in an EPS matrix (Sabater et al. 2007). Hence, in the latter, organic and inorganic compounds are not only adsorbed to the surface but are also integrated in the EPS, and taken up, possibly accumulated and/or metabolized by the microorganisms (Geesey et al. 1994). Therefore, in biofilm the concentration and composition of substances is most likely different compared to river sediments and enables a higher toxic potential.

Although in the highest concentration of B1, B3, B4 and B5 the oxygen concentration at 96 hpf was below the limit which was reported to have no negative effects on zebrafish development (Braunbeck et al. 2005), the embryos were already coagulated at 48 hpf. It is rather unlikely that the O_2 concentration between 24 and 48 hpf was already below 2 mg/L. Furthermore, Hollert et al. (2003) found in preliminary experiments even 0.5 mg/L O_2 to be sufficient for normal development of zebrafish embryos. Therefore, we suggest that the toxicity of the samples caused the mortality rather than a low O_2 concentration.

Based on our results we suggest that the zebrafish embryo toxicity test is a tool that can basically be applied to investigate the toxic potential of native biofilm. However, direct comparisons with data from sediment investigations should be evaluated carefully, as the characteristics of the material are very different.

4.2 Evaluation of sediment toxicity

Sediment S2 and S3 showed a rather low toxic potential (LC_{50}: 30 – 40 % at 48 hpf), whereas S1 (LC_{50}: 22 % at 48 hpf), directly at the discharge of the STP, revealed a higher embryo toxicity. The embryo toxicity test was already applied in investigations of sediments from Southern German rivers. For the river Neckar and the Neckar catchment area an LC_{50} for mortality of ≥ 20 – 38 % and for a nature reserve connected to the Neckar values of 9 – 24 % sediment were reported. The rivers Danube and Rhine displayed an LC_{50} of ≥ 4 – 15 % sediment (overview:

Braunbeck et al. 2009). Hence, the embryo toxicity of the river Wyna sediments are in a similar range and rather low in comparison to Southern German river sediments.

4.3 Evaluation of the observed effects

Most of the deviations from normal development were general development retardations, distinguishable by epiboly stage, absence of somites, undetached tail, and no heartbeat or circulation. In all sediment and the biofilm samples B1 from autumn, B2 from spring and autumn and B6 from spring, most development retardations could be compensated until the end of the experiment. This observation was also reported from other studies with river sediments (Ensenbach 1998, Keiter et al. 2006). In the biofilms many embryos developed normally but did not hatch until 96 hpf. Furthermore, B1 from spring, B3, B4 and B5 from autumn and spring caused extreme development retardations in the vital embryos which could not be caught up. These immobile fish would be an easy prey for predators in the natural environment.

The embryos in the sediments revealed less pronounced development retardations than in the biofilms. As many embryos in S1 could not compensate the development retardation, it was evaluated as more toxic than S2 and S3 in which the LC_{50} for mortality and EC_{50} for hatch rate increased with exposure duration, suggesting mainly an effect on development time. In the lower sediment concentrations early hatch was frequently observed. As an explanation for an early hatch it was suggested that toxic substances in the water may cause an increased movement of the embryo and thereby penetrating the chorion earlier (Ensenbach 1998). Even though early hatched embryos do not show limitations of further development per se, the chorion protects the embryo from particles, mechanical influences and microorganisms (Stehr and Hawkes 1979; Schoots et al. 1982). In addition, the chorion is assumed to reduce the uptake of toxic substances (Van Leeuwen et al. 1985, Fent 1992, Braunbeck et al. 2005). Therefore, it is likely that an early hatched fish is exposed to environmental stressors too early, at a life-stage in which it is still very sensitive.

In this investigation, zebrafish embryos exposed to sediments frequently developed edemata. These findings concur with the results of other sediment studies (Strmac et al. 2002, Hollert et al. 2003). Smaller edemata often disappeared until a later point in time but larger persistent edemata are at least a handicap for the swimming ability of fish larvae. Malformations were only observed sporadically and thus it can be assumed that the sediment and biofilm samples had a minor teratogenic potential. From 72 hpf on, spine deformations appeared still rarely but more often. A strong spine curvature leads to an inability for fish to swim directed and therefore a survival under natural conditions would be impossible.

Although effects such as development retardation, late hatch, early hatch, edemata and malformation are no lethal criteria in the assessment of mortality they should be considered for the evaluation of the embryotoxic potential.

4.4 Temporal variability of toxicity

B3, B4 and B5 displayed similar embryo toxicity for the samplings in autumn and spring. However, data for spring revealed a considerably higher toxicity for B1 and a considerably lower toxicity for B2 compared to autumn. This result indicates a change in the toxic potential of the biofilms during a period of 8 months, possibly due to variation of the wastewater composition in the canalization or changed development parameters for biofilm such as temperature, pH, hydrodynamic conditions and colonizing microorganisms (Giaouris et al. 2005).

The sediment from site S2 from spring revealed a considerably higher toxicity compared to autumn. A possible explanation for this result is that the spring sample was very fine grained with a high proportion of organic matter whereas in autumn it was more sandy and gritty with a low proportion of organic matter. The finer the material, the greater is the surface at which substances can adsorb and therefore a higher toxic potential is likely. S1 was somewhat less and S3 somewhat more toxic in spring compared to autumn.

Although river sediments (Baker 1991, Power and Chapman 1992) and biofilms are long term sinks for many hazardous substances (Evans 2000), environmental and exposure conditions can vary over time and seasons. Therefore and from our results we conclude repeated samplings are necessary to obtain comprehensive evaluation results for environmental samples such as biofilms and sediments.

4.5 Localization of toxic discharges

Site B1 and B2 showed considerably lower embryo toxicity than B3, B4 and B5. As the former are near the STP, at the end of two canalization main branches and the latter are at the beginning of these branches, this result indicates a decrease of the toxic potential in the biofilm downstream along the pipelines. Possible explanations for this finding are (1) additional dischargers are located upstream and the toxic substances degrade in the pipelines, (2) less toxic wastewater is discharged along the pipeline and dilutes the more toxic wastewater coming from upstream, and/or (3) less toxic substances are integrated in the biofilm at site B1 and B2, since development parameters for biofilm and the substrate basis are different (Giaouris et al. 2005).

B4 and B5 were the most embryotoxic sites with an identical LC_{50} for mortality. The additional sampling site B6 upstream of B4 and B5 was included in the spring sampling to further localize potential polluters. B6 (LC_{50}: 0.5 % at 48 hpf) was less toxic than B1, B3, B4 and B5 (LC_{50}: 0.2, 0.2, 0.15 and 0.15% at 48 hpf) based on the spring samples. Therefore, data for B6 demonstrated that just a few hundred meters upstream of B4 and B5 considerably less toxic substances were present in the biofilm, although there are no industrial dischargers in between. Possible reasons for this result are that toxic material is integrated in the biofilm to a lesser extent before site B5 and B4, since development parameters for biofilm and the substrate basis are different (Giaouris et al. 2005) or a toxic discharge from connected private properties between the sampling sites occurs.

At the moment, cantonal authorities are evaluating the results of the present study. They are investigating the catchment areas of the different canalization sections and are trying to identify possible toxic dischargers into the sewage system.

4.6 Genotoxicity

As one mechanism leading to developmental disorders and embryo mortality, DNA-damage caused by genotoxic substances was investigated in the comet assay (Anderson and Wild 1994). A significant genotoxicity was observed in all samples and, apart from S2 and B1, in all tested concentrations. The tested samples were ranked according to the 3-step analysis. Five biofilm samples (B2, B3, B4, B5 and B6) exhibited identical LOECs and had to be ranked upon their IFmax. The IFmax of B2 and B6 were assessed at different concentrations and consequently they could not be ranked based on the IFmax. For the sediment samples S1 and S3 with identical LOECs a ranking based on the IFmax was not possible because the IFmax values were in different concentrations.

For application of the 3-step analysis, a ranking based on the IFmax requires identical concentrations for all samples. However, this is not always possible because of different embryo toxicity of samples. As an extension of the 3-step analysis we propose to rank the samples with the IFmax in different concentrations based on the concentration in which the IFmax was determined: A sample with the IFmax in a high concentration is less toxic than a sample with the IFmax in a lower concentration. Therefore, B6 (0.4 %) is evaluated to be more genotoxic than B2 (0.8 %) and similarly, S1 (12.5 %) is evaluated to be more genotoxic than S3 (25 %). In this way, a more detailed ranking for the genotoxic potential can be obtained and samples with an equal rank in the 3-step analyses before can be differentiated. With this extension the ranking for

genotoxicity is: B1 < B2 < B6 < B5 < B4 < B3 for the biofilm, and: S2 < S3 < S1 for the sediment.

4.7 Use of biofilm in ecotoxicological bioassays

Biofilms in waters interact with dissolved substances such as environmental chemicals and integrate the effects of environmental conditions over extended periods of time (Sabater et al. 2007). The ability of biofilms to act as interceptors of dissolved and particulate matter is based on their emerging physical and biological properties. The EPS offer potential binding sites for a variety of colloidal, organic, and inorganic species (Flemming 1995). By physical adsorption, biofilms remove substances from the water (Kaplan et al. 1987). Large molecules are trapped in the polysaccharide matrix and remain bound by weak physicochemical interactions (Flemming 1995).

In this study we demonstrated that biofilm can be implemented in ecotoxicological bioassays such as the embryo toxicity test and comet assay with zebrafish as a useful indicator for water quality. However, it has to be considered that biofilm not only accumulate but also metabolize substances and thereby alter their composition. Therefore, further studies are needed to elucidate these mechanisms and their significance related to the application in bioassays.

5. Conclusions

Native biofilm was for the first time investigated in an embryo toxicity test. We demonstrated that this test system is a tool that can basically be applied for investigating the toxic potential of biofilms. In combination with the comet assay using embryo primary cells, information about genotoxicity as one mode of action could be obtained.

Clear differences in the embryotoxic and genotoxic potential of the biofilm from different sewage system sections were found. Significant genotoxicity was determined in all biofilm samples and the sediments revealed a lower genotoxic potential upstream of the STP discharge compared to samples downstream and directly at the discharge. Temporal variability from samplings in autumn and spring were found in some of the biofilm and sediment samples.

Based on the results of our study we suggest that biofilm implemented in ecotoxicological bioassays such as the embryo toxicity test and comet assay with zebrafish can be a useful tool to assess (waste) water quality.

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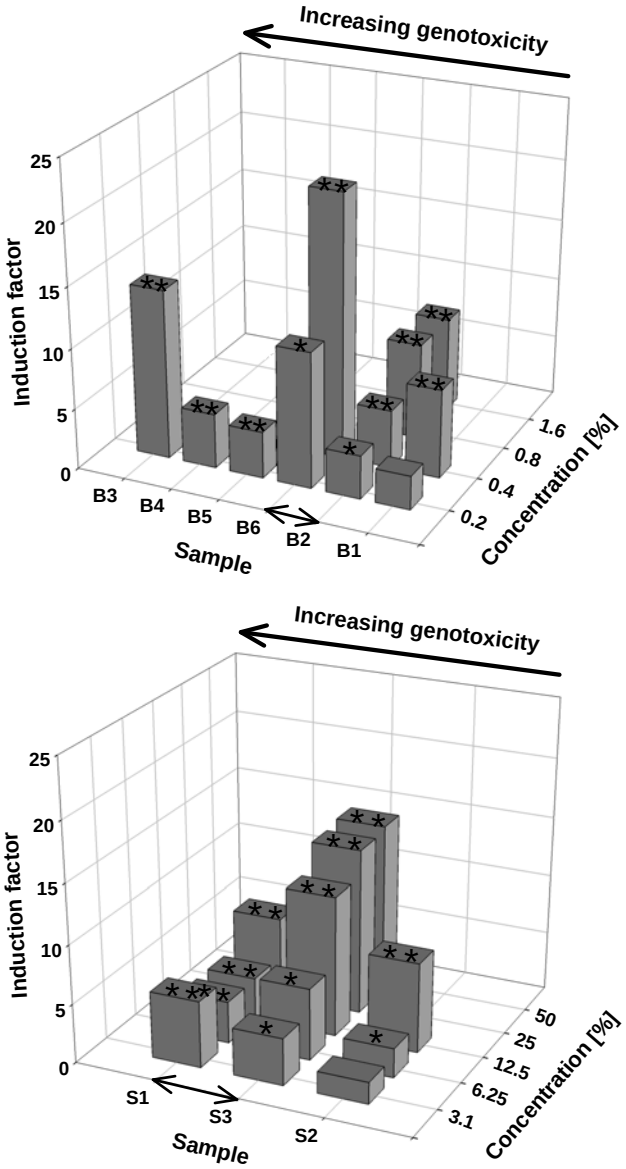
Appendix



Genotoxicity of biofilm (B1 – B6) and sediment (S1 – S3) in the comet assay with primary cells from zebrafish embryos according to the 3-step analysis. Mean of two independent experiments. Significant differences from control are denoted as * (one experiment) and ** (two experiments; $p < 0.05$). Samples with equal rank are indicated by two-headed arrows.

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