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Genotoxicity and effects on fertility, reproduction, development as well as histology in zebrafish (*Danio rerio)* in a multi-generation study –
is there a relationship?

Referat: Prof. Dr. Thomas Braunbeck

Korreferat: Prof. Dr. Henner Hollert

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Heppenheim, den 02.04.2013 ______________________________

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„In der lebendigen Natur geschieht nichts,

was nicht in der Verbindung mit dem Ganzen steht.“

Johann Wolfgang v. Goethe

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Summary

To date, it is unclear whether adverse effects by genotoxic anthropogenic pollutants in the aquatic environment are linked to the decline of fish populations observed in European and North American freshwaters. Therefore, there is a need for investigations into the relationship between genotoxic stress and detrimental effects on development and reproduction in fish. In order to contribute to this field, the present thesis investigates effects of the alkylating genotoxin methyl methanesulfonate (MMS) on genetic integrity, histological status and reproduction in zebrafish (*Danio rerio*). In addition, in their unexposed offspring (F1), larval development, histological status and reproduction as well as development in the F2 generation were examined at the population level in order to identify potential inheritable effects of genotoxicity.

First, methods for the use of primary gonad cells from zebrafish in the alkaline comet assay and histological sections of testis and ovary in the micronucleus test were developed. After *in vivo* exposure of adult zebrafish to MMS for up to two weeks, in the comet assay, concentrations-dependent genotoxic effects were detected in gonads, liver and gills. Likewise, the micronucleus frequency was elevated by MMS in all of these organs. Thus, the concentration range adequate for MMS exposure in the designated multi-generation experiment was identified. In the next step, zebrafish (F0) were exposed to MMS *in vivo* from fertilization until the age of one year. Mortality of F0 fish clearly depended on MMS concentrations. In exposed fish, times of first spawning were delayed and fertility was reduced. However, no unequivocal effects on growth were found. In F1 fish derived from MMS-exposed fish, teratogenic effects were increased, larval survival was reduced and sex ratio was shifted towards females. However, compared to the exposed F0 generation, fertility of the non-exposed F1 generation recovered. Development and survival rates recovered in the F2 generation. Significant genotoxic effects were found in the livers, gills and gonads of either sex of the F0 generation. Regarding histopathological aberrations and external lesions, mainly malformations of eyes, gills and liver and a number of neoplasia were observed in both the F0 and F1 generation. According to indirect measurement of MMS concentrations using ion chromatography, real concentrations in the replicate tanks were similar.

Taken together, chronic exposure of zebrafish to MMS led to DNA damage in somatic and generative tissues, induced the formation of a multitude of histopathological aberrations and affected survival, reproduction and development in exposed fish and their offspring. Regarding several endpoints, cessation of exposure allowed for recover over the generations. Combining these results with data of previous studies and transferring them to the environmental situation, there is considerable evidence that anthropogenic genotoxicants play a role in the decline of wild fish populations.

Zusammenfassung

Es ist bis heute ungeklärt, ob schädliche Effekte gentoxischer anthropogener Schadstoffe in der aquatischen Umwelt zu den Ursachen des Rückgangs der Fischpopulationen in europäischen und nordamerikanischen Süßgewässern zählen. Deshalb ist es angezeigt, den Zusammenhang zwischen gentoxischer Belastung und Beeinträchtigung von Entwicklung und Fortpflanzung bei Fischen weiter zu untersuchen. Um einen Beitrag zu diesem Forschungsfeld zu leisten, betrachtet die vorliegende Arbeit die Auswirkungen des alkylierenden Gentoxins Methylmethansulfonat (MMS) auf die genetische Integrität, das histologische Bild diverser Organe und die Fortpflanzung belasteter Zebrabärblinge (*Danio rerio*). Darüber hinaus wurden die Larvenentwicklung, das histologische Bild und die Fortpflanzung ihrer nicht belasteten Nachfolgegeneration (F1), sowie die Entwicklung der F2-Generation, auf Populationsebene untersucht, um potentielle erbliche Effekte der Gentoxizität auszumachen.
Zunächst wurden Methoden für die Verwendung primärer Gonadenzellen des Zebrabärblings im alkalischen Comet-Assay und histologischer Schnittpräparate von Hoden und Eierstöcken im Mikrokerntest entwickelt. Nach zweiwöchiger Belastung adulter Zebrabärblinge mit MMS *in vivo* wurden konzentrationsabhängige gentoxische Effekte im Comet-Assay mit Gonaden-, Leber- und Kiemenzellen gefunden. Somit wurde ein für den vorgesehenen Mehrgenerationen-Versuch geeigneter MMS- Konzentrationsbereich abgeschätzt. Anschließend wurde eine Generation von Zebrabärblingen (F0) *in vivo* von der Befruchtung bis zum Alter von einem Jahr belastet. Die Mortalität der F0-Fische hing deutlich von den MMS-Konzentrationen ab. Bei belasteten Fischen war das erste Laichen verzögert und die Fertilität reduziert. Es wurden jedoch keine eindeutigen Effekte auf das Wachstum gefunden. Bei von belasteten Fischen abstammenden F1-Fischen war die Häufigkeit teratogener Effekte erhöht, das Larvenüberleben reduziert und das Geschlechterverhältnis auf die Seite der Weibchen verschoben. Dennoch erholte sich die Fertilität der unbelasteten F1-Generation im Vergleich zur belasteten F0-Generation. Die Entwicklung und die Überlebensraten näherten sich in der F2-Generation den Kontrollen an. In Leber, Kiemen und Gonaden beider Geschlechter der F0-Generation traten signifikante gentoxische Effekte auf. Bezüglich histopathologischer Veränderungen und äußerlicher Läsionen wurden in der F0- und der F1-Generation v.a. Fehlbildungen der Augen, Kiemen und Leber sowie Neoplasien beobachtet. Die indirekte Messung der MMS-Konzentrationen im Aquarienwasser mittels Ionenchromatographie zeigte ähnliche Realkonzentrationen in den Belastungs-Replikaten.
Insgesamt führte die chronische Belastung von Zebrabärblingen mit MMS zu DNA-Schäden in somatischen und generativen Geweben, erzeugte eine Vielzahl histopathologischer Veränderungen und beeinträchtigte die Überlebensrate, Fortpflanzung und Entwicklung der belasteten Fische bzw. ihrer Nachkommen. Das Ende der Belastung ließ bei den nachfolgenden Generationen eine Erholung bezüglich mancher Endpunkte zu. Verbindet man diese Ergebnisse mit Daten vorhergehender Studien und überträgt sie auf die Umweltsituation, gibt es deutliche Anhaltspunkte, dass anthropogene Gentoxine eine Rolle beim Rückgang der Fischpopulationen spielen.

I

Introduction

I.1 Decline of fish populations

Industrialization brought economic prosperity, scientific progress, medical care, education, social framework and individual wealth to Europe, North America, Australia, Japan and an increasing number of newly industrializing countries. However, human health and wildlife are confronted to a multitude of threats, such as pollution of air (Brunekreef and Holgate 2002, Stern 1984) and water (Carpenter et al. 1998, Cooper 1993, Metcalfe 1989, Notovny and Olem 1994, van Dijk et al. 1994), eutrophication of water (Smith et al. 1999, van Dijk et al. 1994), soil acidification (Helyar and Porter 1989), forest dieback (Houston et al. 1992, Nihlgård 1985) and climate change (Forster et al. 2007). Regarding industrial pollution of forests, release of untreated wastewater and oxygen level in rivers, an amelioration of the situation can be observed over the last decades (de Witt et al. 1991, Fanta 1997, Hanley et al. 2006, Kiss 1985, van der Veen 1981). In addition to these issues, in the limnic environment of Europe and North America, a decline in fish populations has been observed over several decades to date (Burkhardt-Holm et al. 2002, Cook et al. 2003, de Lafontaine et al. 2002, Faller et al. 2003, Moyle and Williams 1990, Richter et al. 2003). It has been estimated that worldwide 20 % of the ca. 1.800 fish species are extinct or in serious decline (Moyle and Leidy 1992). The extinction of 4 % of the North American freshwater animals per decade resulted from a model calculation (Ricciardi and Rasmussen 1999). In the upper Danube River, fish populations, especially of chub (*Leuciscus cephalus*), barbel (*Barbus barbus*), pike (*Esox lucius*) and brown trout (*Salmo trutta fario*) were strongly reduced since the early 1990s despite stocking (Keiter et al. 2006, Wurm 2001). Similarly, the catch rates of brown trout (*Salmo trutta*) have been reduced to the half between 1987 and 2002 (Friedl 1999). A multitude of reasons has been suggested to play a role in this situation: reproductive failure, reduced recruitment of young stages, impaired health and fitness, e.g. caused by diseases such as proliferative kidney disease (PKD), reduced availability of food, increase of fine sediments, poor water and morphological quality and warming of rivers, changes of community composition, in parts due to appearance of exotic species, as well as anthropogenic chemical pollution with nutrients and synthetic compounds (Burkhardt-Holm et al. 2002, Fischnetz 2004, Richter et al. 2003). Wastewaters and surface run-off from industry, agriculture, traffic and private households, although widely treated in sewage treatment plants, constantly and intermittently contribute to the pollution of streams, lakes and groundwater. Especially industrial effluents from pulp and paper mills, steel foundries and organic chemical manufacturing facilities contain genotoxic substances (Claxton et al. 1998). In addition, persistent pollutants released to the water in the

past have been trapped in the sediments. Thus, a considerable load of heavy metals, polycyclic aromatic hydrocarbons (PAHs), halogenated organic compounds, pesticides, pharmaceuticals and nanomaterials is present in limnic environments (Bätscher et al. 1999, Harmon and Wiley 2010). There are endocrine disruptors such as organochlorine pesticides, polychlorinated biphenyls, dioxins, alkylphenolic chemicals and phthalates influencing sexual development and reproduction of aquatic organisms (Cooper and Kavlock 1997, Crisp et al. 1998, Tyler et al. 1998, Vandenberg et al. 2009). Another group of anthropogenic pollutants comprises genotoxic, mutagenic and carcinogenic compounds (Burkhardt-Holm et al. 2002, Fent 2003, Helma et al. 1994, Houk 1992, Marquardt 1994). In the present thesis, the focus is on the characteristics of genotoxic substances and the consequences to genotoxicants at the individual as well as at the population levels. In contrast to other endpoints such as endocrine disruption, genotoxicity is especially important for the investigation of the decline of fish populations because it may produce genetic damage that is inherited to future generations (Bickham et al. 2000, Depledge 1998). Thus, fish may be affected by genetic damage generations after the cessation of genotoxic exposure.

I.2 Genotoxic agents, mechanisms and repair

Genotoxicity is the interaction of DNA with chemical or physical agents such as alkylating agents, oxidative stress, metals, UV light and radiation (Fent 2003). Genotoxicity of metals such as Cd(II), Ni(II), Co(II), Pb(II) and As(III) is based on oxidative DNA damage and the impairment of DNA repair processes, leading to strand breaks and chromosomal aberrations (Hartwig 1995). Oxidative stress can give rise to oxidation of DNA bases as well as proteins and lipids, whereas the latter produces genotoxic compounds leading to increased micronucleus frequency, sister-chromatid exchange and chromosomal aberrations (Esterbauer 1993). Dependent on the wavelength, UV light produces either pyridine dimers leading to strand breaks and modification or deletion of bases or acts indirectly *via* production of radicals and reactive oxygen species (Griffiths et al. 1998). Low doses of gamma radiation cause strand breaks, oxidative base damage and chromosomal aberrations as well as decrease of expression of DNA repair genes (Sudprasert et al. 2006). Alkylating compounds bind covalently to nucleophilic centers in the DNA and other macromolecules (Friedberg et al. 1995). For example, this is true for nitrosourea and alkyl esters of methanesulfonic acid (Ayllon and Garcia-Vazquez 2001, Beckwith et al. 2000, Golding et al. 1997, Tates and den Engelse 1989, Wurdeman et al. 1989). Many organic compounds acquire genotoxic potential after bioactiva-

tion, e.g. by cytochrome P450 enzymes. This group includes polyaromatic hydrocarbons (PAHs), vinyl chloride and nitrosamines (Fent 2003, Mollerup et al. 2006, Phillipson and Ioannides 1984, Raucy et al. 1993). Genotoxicity affects multiple cellular levels. At the level of genes, in general, bases can be lost, added, linked or modified. In detail, effects on the genetic level are single or double strand breaks, apurinic and apyrimidinic (AP) sites, alkali-labile sites leading to strand breaks under alkaline conditions and crosslinks between both strands. In case of DNA adducts, electrophilic molecules covalently bind to the DNA. Finally, incomplete excision repair can lead to strand breaks (Tice et al. 2000). At the level of chromosomes, breaks, deletions, insertions, inversions, duplications and translocations can occur. In addition, at the level of genomes, chromosomes can get lost or be added. Moreover, defects in chromosomal distribution during mitosis or meiosis can arise. Generally, direct effects of genotoxicity are distinguished from indirect effects. In case of direct effects, genotoxic agents cause modifications of DNA integrity such as strand breaks or of base sequence. In contrast, indirect genotoxic effects refer to mechanisms at higher levels of cellular physiology such as reduction of DNA repair capacities (de Boeck et al. 1998).

There is evidence that genotoxic agents give rise to neoplasia, gene mutations leading to disease and teratogenicity as well as latent genetic damage that can be passed on over many generations and have adverse effects on their survival (Depledge 1996). Mutagenesis refers to the induction of mutations in the DNA and thus the alteration of the genetic code of an affected cell and frequently is closely related to genotoxicity. On the one hand, mutations are an elementary factor of evolution. On the other hand, genotoxic substances impose unpredictable risks on natural populations that are not comparable to the consequences of natural mutation rates.

In a wider perspective, tumorigenesis and carcinogenesis may arise from genotoxicity and mutagenesis. Approximately 90 % of the mutagens known are carcinogenic. Inversely, about 90 % of the carcinogenic compounds are also mutagenic (Fent 2003). As carcinogenesis is a multistage process than can develop over many years, the consequences of genotoxic exposure to organisms strongly depends on their life expectancy. Therefore, in contrast to long-lived organisms such as most vertebrates, short-lived individuals are more resistant to chronic exposure to low concentrations of genotoxins. However, also short-lived organisms can be affected by mutagenesis in germ cells, leading to genomic alterations that can be transferred to successive generations. Otherwise, populations with reproductive surplus are less threatened by extinction when somatic cells are more affected by genotoxicity than gametes (Fent 2003). As a consequence, various endpoints have come in the focus of genetic ecotoxicology:

gamete loss due to cell death, embryo mortality due to lethal mutations, abnormal development and teratogenesis, carcinogenesis as well as effects on genetic diversity as a consequence of heritable mutations. Relating these factors to reproductive success of natural biota is of crucial importance for the understanding of the fate of animal populations that are exposed to anthropogenic pollution (Anderson and Wild 1994). Since the ecological consequences of genotoxicity are possibly far-reaching, the investigation of genotoxic pollution of the aquatic environment is especially important in order to assess risks to wildlife (Chen and White 2004, Mitchelmore and Chipman 1998, Sun et al. 2004). Therefore, efforts need to be made to understand the relationship between genetic damage in aquatic species and population-relevant detrimental effects in the offspring (Attrill and Depledge 1997, Depledge 1996, White et al. 1999).

In fact, correlations between genotoxic effects and reproductive impairment have been shown in many groups of animals, i.e. polychaetes, nematodes, sea urchins, amphibians and fish (Anderson and Wild 1994). Mortality, malformation, and abnormal chromosome division of embryos of the Atlantic mackerel (*Scomber scombrus*) were associated with marine pollution (Longwell et al. 1992). Furthermore, in trout embryos and larval Pacific herring (*Clupea pallasi*), anaphase aberrations led to developmental aberrations (Hose 1998, Liguori and Landolt 1985). Exposure of Japanese medaka (*Oryzias latipes)* to physical and chemical genotoxicity caused dominant lethal mutations (Egami et al. 1983, Shimada and Egami 1984). Moreover, mortality and teratogenicity were increased in the offspring of mosquito fish (*Gambusia affinis*) that had been exposed to radionuclides (Theodorakis et al. 1997). In another study, multigeneration exposure of polychlorinated biphenyls (PCBs) and dioxins to mummichog *(Fundulus heteroclitus)* reduced population growth (Munns et al. 1997). It was also suggested that benzo[a]pyrene can reduce fertility and larval survival in fathead minnows (*Pimephales promelas*) two generations away from exposure (White et al. 1999). In addition, Diekmann and co-workers (2004a) predicted extinction of a zebrafish population after exposure to the genotoxicant 4-nitroquinoline oxide. Recently, in brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*), parental exposure to MMS was shown to cause morphological abnormalities in development of embryos and larvae (Devaux et al. 2011). Taken together, since reproduction is a population relevant process that can be affected by genotoxicity, genotoxicity assessment plays a major role in ecotoxicology. Moreover, with respect to the worldwide fish decline, genotoxicants might well be causative agents.

As a reaction to genotoxicity, there are various mechanisms of DNA repair in biota. DNA demethylation is a widespread mechanism of DNA repair (Gehring et al. 2009) that is espe-

cially required after exposure to alkylating agents. In general, four mechanisms for repair of DNA methylation are discussed in vertebrates: removal of methyl groups, removal and replacement of the affected base, conversion of the base into another base followed by removal and replacement, as well as excision of a group of bases around the affected base and replacement according to the opposite strand (Rai et al. 2008). In zebrafish (*Danio rerio*), it was shown that deaminase converts 5-methylcytosine to thymine and a specific thymine glycosylase repairs G:T mismatch. Hereby, demethylation is promoted by the non-enzymatic factor Gadd45 (Rai et al. 2008). DNA alkylation can also be repaired via enzymatic photoreactivation by DNA-photolyases, alkyltransferases and ligation of strand breaks (Friedberg et al. 1995). Moreover, DNA methylation is known to play a role in cancer development *via* inactivation of tumor suppressor genes (Esteller 2008). For example, tumor suppressor p53 is a transcription factor in cellular stress responses such as UV-B radiation (Sandrini et al. 2009). The factor regulates the expression of genes related to DNA repair including *XPC* and *DDB2* are also present in zebrafish hepatocytes (Ford 2005, Sandrini et al. 2009). For example, the genes *XPS* and *DDB2* are responsible for nucleotide excision repair using endonucleases. In addition, the genes *Apex1* and *Ogg1* are involved in the mechanism of base excision repair using DNA-glycosylases. An important mechanism of repair of double strand breaks is non-homologous end-joining (Ferguson et al. 2000) which is *inter alia* based on the gene *Ku80* in zebrafish (Sandrini et al. 2009). Furthermore, double strand breaks and inter-strand crosslinks are repaired by the mechanism of homologous recombination (Li and Heyer 2008) involving the genes *Nbs1* and *Rad51* in zebrafish (Sandrini et al. 2009). Defects in DNA repair mechanisms can lead to serious diseases and promote the proliferation of tumor cells (Leischner 2007).

I.3 Methyl methanesulfonate

The alkylating agent methyl methanesulfonate (MMS; Fig. I.1) was used as the test substance in the present thesis. MMS is an alkylmesilate, i.e. a methyl ester of methanesulfonic acid. As MMS is a direct-acting genotoxin independent of bioactivation, it has been widely used in the comet assay as a model genotoxin (e.g. Bony et al. 2010, Clements et al. 1997, Lee and Steinert 2003). Regarding its mode of action, MMS methylates adenine and guanine which leads to replication blockade, base mispairing and intramolecular DNA

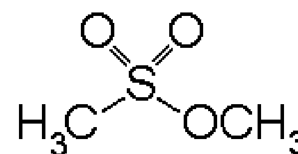


Fig. I.1: Chemical structure of methyl methanesulfonate (MMS).

crosslinking (Beranek 1990, Brookes and Lawley 1963). In detail, MMS almost exclusively alkylates the ring nitrogens of purine bases, i.e. N-7 of guanine (about 80 % of the total methylation damage), N-3, N-1 and N-7 of adenine (Beranek et al. 1980, Lee-Chen et al. 1993). Alkylation is counteracted via base excision repair and alkyltransferases (Lindahl and Wood 1999). From intermediates of base excision repair, single strand breaks can arise (Pascucci et al. 2005). Spontaneous hydrolyzation of alkylated bases as well as excision by glycosylases (Gates et al. 2004, Lindahl 1982, Paterson et al. 1984, Hanawalt and Sarasin 1986) lead to depurination and thus AP sites that are heat labile and can give rise to single strand breaks (Brookes and Lawley 1963, Lindahl and Anderson 1972). In this process, AP sites and adjoining nucleotides are excised by endo- and exonucleases and mended by polymerase-directed repair synthesis as well as ligase-mediated strand rejoining (Mirzayans et al. 1988). Alkylation of the opposite strand can produce double strand breaks (Rydberg 2000). However, heat-labile DNA methylated by MMS was shown to be converted into double strand breaks during sample preparation in the comet assay (Lundin et al. 2005). Thus, MMS does not produce double strand breaks *in vivo*. In addition, methylated bases block the replication fork and can impair replication (Lundin et al. 2005). Using EPI suite 4.0 (EPA's Office of Pollution Prevention Toxics and Syracuse Research Corporation, Environmental Protection agency, USA), a log k_{ow} of -0.66 was calculated. Thus, MMS is highly water soluble and adequate for genotoxic exposure to fish *in vivo*.

I.4 Genotoxicity testing

At present, a multitude of tests for genotoxicity testing is available. Toxicology is a discipline connecting medicine and chemistry and focuses on human health effects by industrial chemicals and pharmaceuticals. Therefore, methods for genotoxicity testing were initially developed for the investigation of human tissue and cells from animals serving as models for human toxicity, i.e. mainly rodents. Hereby, the following assays are commonly used: the comet assay (Anderson et al. 1998, Collins 2004, Oshida et al. 2008, Kumaravel et al. 2009, Singh et al. 1988, Speit et al. 2009), the micronucleus test (Fenech 2000, Tates et al. 1980, Tice et al. 2002), the bacterial Ames assay (Ames et al. 1975, Heil and Reifferscheid 1992, Maron and Ames 1983, Yamaguchi 1989) and the umu-assay with enzymatic supplementation imitating mammalian metabolism (S9; Hatanaka et al. 2001, Takemoto et al. 2002, Yamaguchi 1989), the UDS (unscheduled DNA synthesis) assay (Burlinson 1989, Hasheminejad and Caldwell 1994, Kennelly et al. 1994), the alkaline filter elution (Doerjer et al. 1988, Bradley and Kohn

1979, Kohn 1991), the alkaline unwinding assay (Daniel et al. 1985, Garberg et al. 1988), the dominant lethal test (Ehling et al. 1978, Epstein et al. 1972, Generoso et al. 1980, Generoso et al. 1986), the chromosome aberration test (Abbondandolo 1984, Hartmann et al. 2003, Matsuoka et al. 1979), the sister chromatid exchange assay (Roth et al. 1994, Wilcosky and Rynard 1990, Wolff 1977) as well as DNA fingerprinting assays able to detect DNA adducts such as randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP; Clerc et al. 1998, Vos et al. 1995). In contrast to medical toxicology, genetic ecotoxicology assesses the impact of anthropogenic influence on the natural environment. With respect to the fish decline, investigations have been focused on health effects of pollution of the aquatic ecosystem on fish. Many test methods have been adapted to the context of the natural aquatic environment and aquatic species: the comet assay (Bony et al. 2010, Diekmann et al. 2004b, Ferraro et al. 2004, Frenzilli et al. 2009, Keiter et al. 2006, Knopper and McNamee 2008, Kosmehl et al. 2004, Reifferscheid and Grummt 2000, Schnurstein and Braunbeck 2001, Suares Rocha et al. 2009), the micronucleus test (Diekmann et al. 2004b, Ferraro et al. 2004, Suares Rocha et al. 2009, Williams and Metcalfe 1992), Ames assay, umu assay and alkaline filter elution (Diekmann et al. 2004b, Dizer et al. 2002, Hamer et al. 2000, Reifferscheid and Grummt 2000), the alkaline unwinding assay (Shugart 1988), the dominant lethal test (Egami et al. 1983, Shimada and Egami 1984), the chromosome aberration test (Ferraro et al. 2004, Rishi and Grewal 1995) as well as the sister chromatid exchange assay (Alink et al. 1980, Kligerman 1979). RAPD and AFLP play a minor role in genetic ecotoxicology and are mainly used in fish for elucidation of phylogeny (Bardakci and Skibinsi 1994, Kocher et al. 1998, Sullivan et al. 2004).

As the comet assay and the micronucleus test are broadly used in medical toxicology as well as in genetic ecotoxicology and both assays are central to the present thesis, the backgrounds of these methods are regarded in detail in the following. The comet assay, also called single cell gel electrophoresis, was developed by Rydberg and Johanson (1978) and Östling and Johanson (1984). Singh et al. (1988) applied the method to mammalian cells. The most important advantage of the comet assay is its potential to detect DNA damage at the level of single cells from virtually every eukaryotic organism (Fairbairn et al. 1995). Dependent on the pH level before and during electrophoresis, there are two basic methods of the comet assay. In the alkaline version (pH > 13), single and double strand breaks, AP sites, alkali labile sites and crosslinks can be detected (McKelvey-Martin et al. 1993). Otherwise, the comet assay can be performed under neutral conditions. This is often applied to sperm because in the DNA structure of sperm manifold special proteins such as transition proteins make up a highly organized

and compact chromatin structure different from somatic cells (Laberge and Boissonneault 2005, Speit et al. 2009). This leads to a high background level of strand breaks under alkaline conditions, which is not the case under neutral conditions (Singh et al. 1989). Thus, it may seem likely that sperm cells have many alkali labile sites that turn into single strand breaks when the strands are separated under alkaline conditions. However, performing the comet assay under neutral conditions does not exclude single strand breaks from analysis. In contrast, the overall sensitivity of the assay is reduced (Speit et al. 2009). The comparison of studies using alkaline or neutral comet assay is further hampered by the use of different electrophoresis conditions. Therefore, as both methods are suggested to mainly measure single strand breaks, the alkaline as well as the neutral version of the comet assay can be applied to gametes (Speit et al. 2009). Bony et al. (2010) demonstrated that the alkaline comet assay is adequate for the detection of genotoxic effects in zebrafish spermatozoa. Hence, in the present thesis cells from liver, gills and gonads of both sexes of zebrafish were tested under alkaline conditions in the comet assay.

Other than the comet assay, the micronucleus test detects persistent genetic damage on the level of chromosomes in eukaryotic cells. Chromosomal mutations are of special importance with respect to carcinogenesis (Fenech 2000). On the one hand, chromosomal mutations can be a consequence of clastogenic effects, e.g. chromosomal fragmentation due to unrepaired double strand breaks. Misrepair of strand breaks can give rise to pathological chromosome rearrangements (Fenech 2000, Savage 1993). On the other hand, the spindle apparatus can be affected, leading to malsegregation of chromosomes during mitosis. In cells that replicated after such an event, acentric chromosomal fragments or lost chromosomes, termed micronuclei, can be located at a distance from the nucleus in metaphase (Fenech 2000). In the present thesis, micronuclei were observed in histological sections of liver, gills and gonads of both sexes of zebrafish.

I.5 Tumorigenesis and carcinogenesis

As mentioned above, tumorigenesis and carcinogenesis may result from genotoxicity as well as mutagenesis, and genetic damage such as mutations may be inherited. Therefore, the development of benign and malign neoplasia is investigated in the present thesis in the exposed fish and their offspring.

There are two groups of carcinogenic substances: genotoxins that directly react with DNA and compounds that have an epigenetic mode of action. The latter group comprises approximately

25 % of the known carcinogens (Fent 2003). Epigenetic carcinogens affect cell differentiation and mitosis. Some of these compounds act as hormones such as estradiol (Ahmad et al. 2000, Liehr 2000) or as tumor promoters such as polychlorinated biphenyls (PCBs; Laib et al. 1991, Uchiyama et al. 1974). In mammals, it has been shown that cancer is associated with diverse changes in epigenetics such as DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs, specifically microRNA expression (Sharma et al. 2010). Many substances require metabolization in order to become carcinogenic, e.g. aromatic amines, PAHs and nitrosamines (Laib et al. 1991, Leischner 2007, Lijinsky and Epstein 1970, Turesky et al. 1991).

Carcinogenesis is a process consisting of three steps (Fent 2003, Leischner 2007): First, the interaction of a carcinogenic with DNA is referred to as initiation. Unless genetic alteration is undone by DNA repair, this is an irreversible process. During initiation, mutations in protooncogenes occur and transform them into oncogenes. Protooncogenes regulate cell proliferation, motility and differentiation. As a consequence, a gain of function of the oncogene or its gene product can be observed (Leischner 2007). For example, mutations of genes of the ras family, which code for guanosine triphosphate (GTP) binding membrane proteins, which are important for cell proliferation, can cause uncontrolled cell division (Bailey et al. 1996, Bos et al. 1987). Mutations can cause defects of DNA repair mechanisms and thus genetic instability, leading to increasing heterogeneity of the tumor cell population. Activation of protooncogenes often involves amplification of gene loci, chromosomal translocations and point mutations.

In contrast to protooncogenes, mutations in tumor suppressor genes, which regulate cell growth, mostly cause a loss of function (Leischner 2007). For example, the gene p53 is responsible for the initiation of apoptosis in cells affected by heavy DNA damage. A reduced function of p53 enables these cells to divide and proliferate (Bhaskaran et al. 1999, Hollstein et al. 1991). In a second step, during promotion, a promoter such as epigenetic carcinogenis influences the initiated cell and allows for autonomous development and division (Fent 2003). Third, the affected cell transforms into a tumor cell. This step is called progression or realization. In general, a tumor or a neoplasia is an abnormal tissue formation that needs only initially to be influenced by a growth promoting factor (Leischner 2007). In these cells, control of growth, differentiation and apoptosis mechanisms can be affected. Benign tumors, characterized by slow growth in a delimited area, are distinguished from malign tumors that grow fast, develop multifaceted cell morphology, infiltrate other tissues and are prone to the formation of metastases. Malign tumors are also referred to as cancer. The presence of a tumor in an

organism can be ascertained using tumor markers such as oncofetal antigens, hormones, isoenzymes, organ specific proteins and intermediate filaments (Leischner 2007).

Dependent on their origin in a blastodermic layer, tumors are assigned to the epithelial (ectodermal and entodermal) or mesodermal group of neoplasia. Benign epithelial tumors derived from glandular epithelium and parenchyma are termed adenoma. Papilloma are benign epithelial tumors derived from squamous epithelium or urothelium. Approximately 90 % of all malign epithelium tumors are carcinoma. They emerge from squamous epithelium, glandular tissue in the case of adenocarcinoma or urothelium. Mesodermal tumors derive from musculature, connective tissue, vessel endothelia and blood cells. Examples for benign mesodermal tumors are fibroma, myoma, lipoma, chondroma and osteoma. Blastoma are benign mesodermal tumors derived from mesenchymal precursor cells. Malign mesodermal tumors are termed sarcoma. In addition to epithelial and mesodermal neoplasia, there are dysontogenetic tumors deriving from embryonic or fetal tissue. These comprise teratomes emerging from all three blastodermic layers, embryonic tumors such as hepatoblastoma that derive from precursor cells of the liver parenchyma, as well as hamartomes consisting of normally differentiated, but disordered cells (Leischner 2007). The process or carcinogenesis in somatic cells is illustrated in Figure I.2. Many different organisms are subject to the risk of carcinogenesis. Several studies have demonstrated carcinogenesis in a number of fish species (Bailey and Hendricks 1988, Bailey et al. 1996, Black and Baumann 1991, Bunton 1996, Cormier 1986, Cormier et al. 1989, Couch and Harshbarger 1985, Lee et al. 2000).

Some studies focused on carcinogenesis in zebrafish (Beckwith et al. 2000, Spitsbergen et al. 2000a, Spitsbergen et al. 2000b, Stern and Zon 2003). As zebrafish show tumor morphologies similar to mammals, the species is regarded as a model organism for vertebrate tumor biology (Amatruda et al. 2002, Feitsma and Cuppen 2008, Spitsbergen and Kent 2003, Tsai 1996). The zebrafish belongs to the Cyprinidae within the Cypriniformes and their natural habitat is the South East of Asia. In both sexes, sexual maturity is accomplished by 90 days of age (Driever et al. 1994, Heeb and Escher 2007). As they are tropical fish, spawning is possible over the whole year. Transparency of the chorion is advantageous for investigation of development. As the zebrafish is widely used in ecotoxicology, there are several test guidelines using it as test organism: OECD 203, 210, 215, 229, 230 and 234. Because of these beneficial properties, the zebrafish was selected as the test organism for the present thesis.

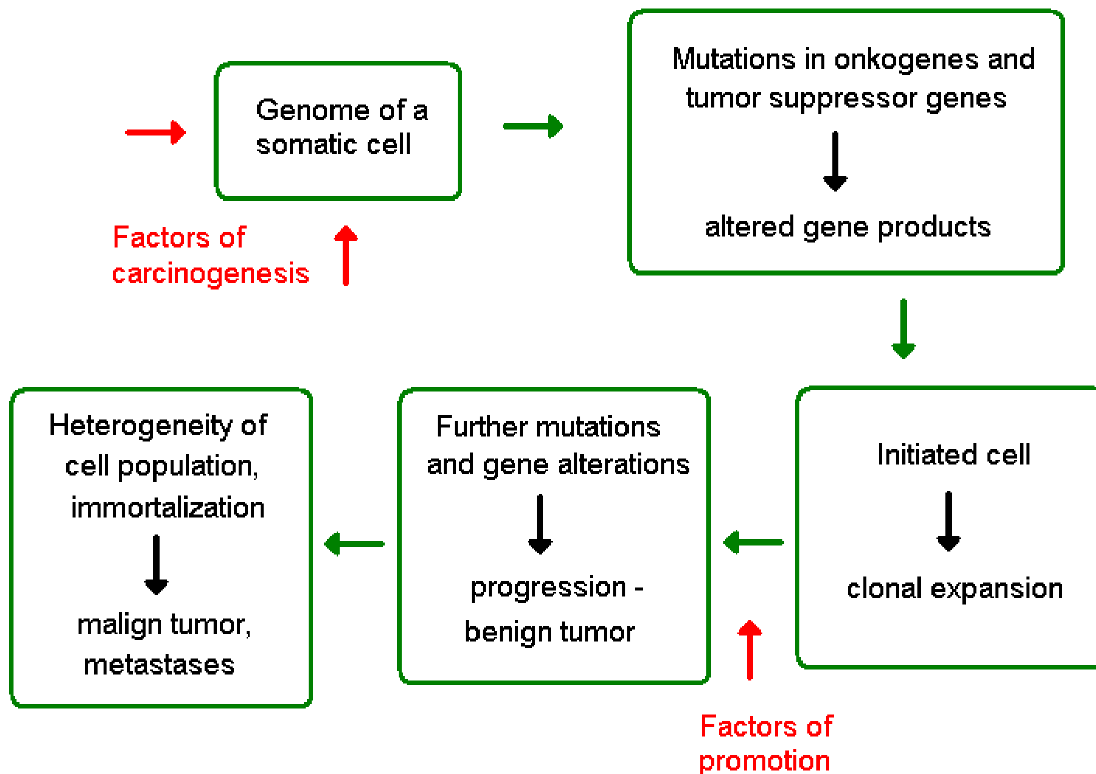


Fig. I.2: Steps in the process of carcinogenesis in somatic cells (based on Leischner 2007).

I.6 Histology of fish gonads

In gametes, DNA repair capacity has been shown to be limited dependent on cell stage (Anderson and Wild 1994, Hales et al. 2005). During spermatogenesis, enzymes required for DNA repair present in cytoplasm are released from the cells (Aitken and de Iuliis 2007, Vogel and Natarajan 1995). Thus, late spermatids and spermatozoa are deficient in DNA repair (Devaux et al. 2011). In contrast, oocytes are capable of DNA repair (Ashwood-Smith and Edwards 1996, Dahms and Lee 2010). In addition to exposure during gametogenesis, gametes are easily exposed to genotoxic agents in the water. Both aspects are relevant for fish, since the majority of aquatic animals release their gametes directly into the water (Jha 2008).

For the investigation of multi-generational effects of genotoxicity, gonads are of special importance as a link between the generations. Herein, genetic damage can be assessed that is passed on to the next generation.

Gonads consist of generative as well as somatic cells. Generative cells are gametes and their precursor stages. Somatic cells in the gonads support metabolism, activities and development of the generative cells.

In teleost ovaries, during ovulation eggs are released into a lumen, the ovocoele. This ovary type is termed cystovarian (Hoar 1969). Ovarian follicles consist of an oocyte surrounded by

the chorion and somatic follicle cells. The latter form an inner layer of granulosa cells including the micropylar cell and an outer layer of theca cells, including capillaries and fibroblasts. A basement membrane lies in between both layers. Through pore canals penetrating the chorion, oocytes and granulose cells are in contact (Takashima and Hibiya 1995). During development of teleost ovaries, oocytes undergo a series of growth phases. First, in the chromatin-nucleolus phase, oogonia undergo leptotene, zygotene and pachytene of the first meiotic division. Oogonia are the smallest oocytic cells and are located within the ovarian germinal epithelium covering the female gonad (Johnson et al. 2009). Second, in the perinucleolar phase, diplotene is arrested and multiple nucleoli migrate to the nuclear periphery for the production of ribosomal RNA. Third, in the cortical alveoli phase, vesicles and oil droplets appear. In zebrafish, this stage begins concurrently with the vitellogenic phase (Takashima and Hibiya 1995, Yamamoto 1977). During the latter, hepatic vitellogenin is taken up and yolk is accumulated in the cytoplasm of the primary oocytes, leading to growth. Finally, in the maturation phase, the first meiotic division is completed. The second meiotic division is arrested in the metaphase. The secondary oocytes are released from the follicles during ovulation. After spawning and fertilization, the second meiotic division continues (Takashima and Hibiya 1995). A histological overview of a section of a female zebrafish gonad is given in Figures I.3 and I.4.

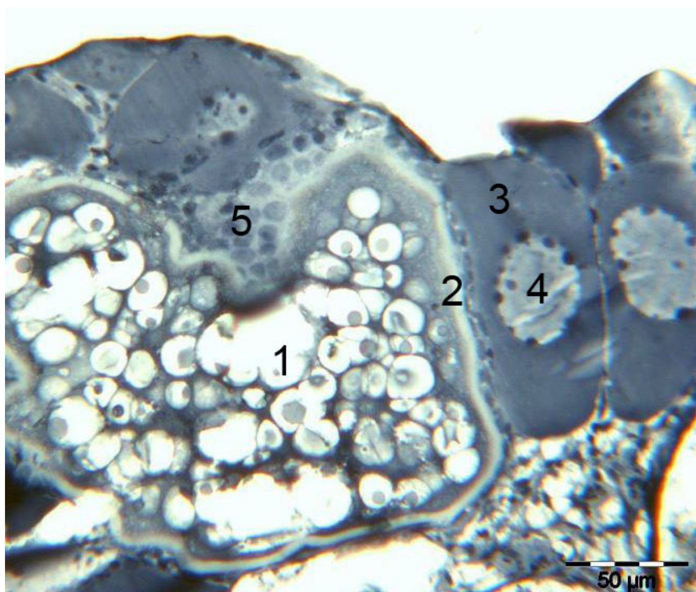


Fig. I.3: Histological section of female zebrafish gonads: secondary oocyte with lipid and oil droplets (1), chorion of a secondary oocyte (2), primary, perinucleolar oocyte (3) with germinal vesicle (= nucleus; 4) with small, dark nucleoli in verge and tissue consistent of somatic theca and granulosa cells as well as oogonia (5). Embedded in Spurr, stained with Weigert's hematoxylin. Scale bar: 50 µm.

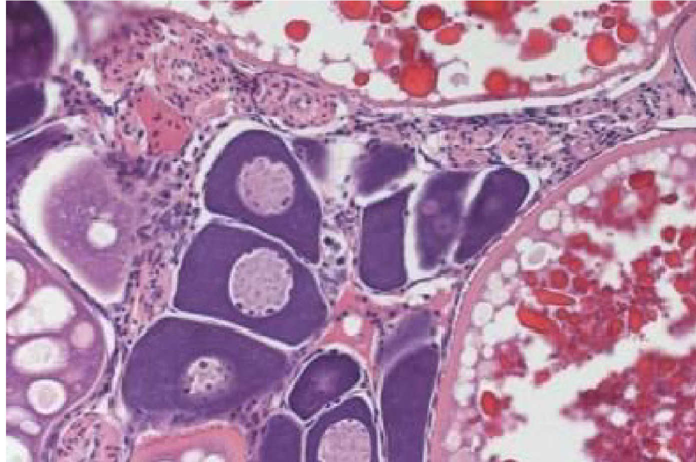


Fig. I.4: Histological section of female zebrafish gonads: secondary oocytes on the top and on the right as well as primary, perinucleolar oocytes in the center surrounded by tissue consistent of somatic theca and granulosa cells as well as oogonia. Embedded in paraffin, stained with hematoxylin and eosin. Original magnification: 20 x. Picture taken from Johnson et al. (2009).

According to Callard (1991), the teleost testis is lobular, i.e. it forms an open-ended compartment. Lobules are surrounded by a basement membrane and a layer of myoid cells. Generative cells are located inside spherical units, so called spermatocysts, formed by somatic Sertoli cells. Sertoli cells are few in number and usually adjacent to lobular septa (Johnson et al. 2009). Their functions are phagocytosis of degenerating germ cells and formation of the blood-testis barrier (Takashima and Hibiya 1995). Somatic Leydig cells are also present in low numbers. They can be found in the interlobular interstitium and have steroidogenic function (Billard 1986, Johnson et al. 2009, Takashima and Hibiya 1995). Development of teleost testis comprises meiosis, spermiogenesis and spermiation. Spermatogonia undergo complete meiosis without interruption and become primary spermatocytes. By the second meiotic division, they become secondary spermatocytes. In the next step, the cells divide into haploid spermatids. During spermiogenesis, spermatids evolve into spermatozoa featuring head, midpiece and flagellum. Finally, spermatozoa are released from the spermatocyst during spermiation (Takashima and Hibiya 1995). A histological overview of a section of a male zebrafish gonad is given in Figures I.5 and I.6.

Zebrafish undergo juvenile hermaphroditism, i.e. up to the age of 15 to 20 days after hatching, both male and female zebrafish exhibit ovaries with developing oocytes (Maack and Segner 2003, Takahashi 1977). However, during development of male zebrafish larvae, oocytes degenerate and spermatogonia are activated, which leads to the histological appearance of intersex gonads. Usually, transformation into testes is completed by 40 days after hatching (Takahashi 1977).

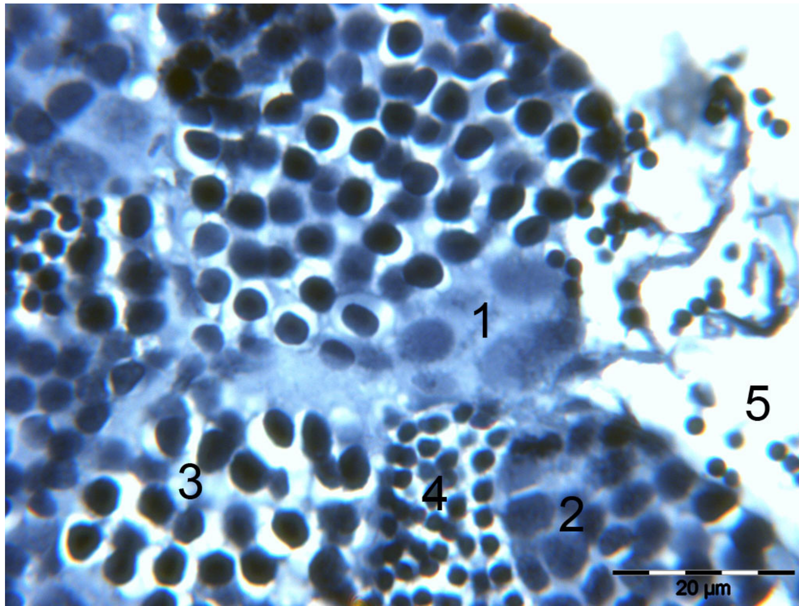


Fig. I.5: Histological section of male zebrafish gonads: spermatogonia (1), primary spermatocytes (2), secondary spermatocytes (3), spermatids (4) in separate spermatocysts and spermatozoa (5) outside of the tissue. Embedded in Spurr, stained with Weigert's hematoxylin. Scale bar: 20 µm.

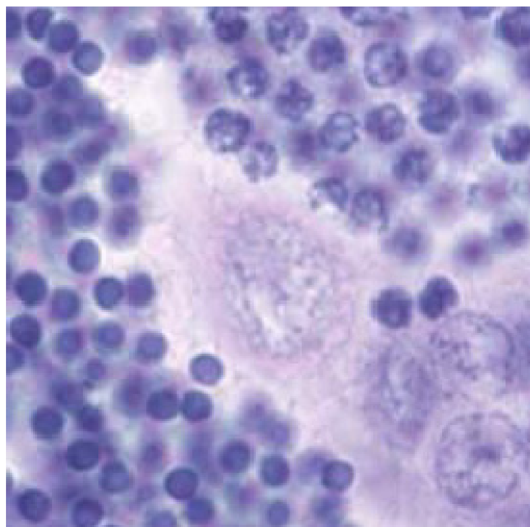


Fig. I.6: Histological section of male zebrafish gonads: spermatogonia in the center and in the lower right corner, primary spermatocytes in upper right corner and secondary spermatocytes on the left side. Embedded in paraffin, stained with hematoxylin and eosin. Picture taken from Johnson et al. (2009).

I.7 Current regulatory efforts towards restriction of aquatic pollution

Since anthropogenic pollution of aquatic ecosystems has been identified as the source of poor water status affecting wildlife as well as human health, political measures have been taken to protect aquatic ecosystems from excessive further pollution. In the European Union (EU), national water policies are harmonized with respect to a sustainable water use and good water quality. The Water Framework Directive (EU 2000) defines criteria for good biological, hydromorphological, physical-chemical quality and stipulates that member states restrict the influent of pollutants into water bodies. However, in the EU, there is lack of data on the risks for human health and ecotoxicological potential of thousands of chemicals that are industrially produced or imported and widely used (Rovida et al. 2011). Therefore, a European Com-

munity regulation directing Regulation, Evaluation, Authorization and Restriction of Chemicals (REACH; EC 1907/2006) was adopted by the European Commission and came into force on 1 June 2007. For substances that were available before 1981 applies the following: Until 30 November 2010, testing of chemicals of more than 1000 t/a had to be accomplished. Until the same deadline, substances that are highly carcinogenic, mutagenic or toxic to reproduction (CMR) had to be tested if their tonnage exceeded 1 t/a as well as substances toxic to the environment that exceeded 100 t/a. For chemical tonnages above 100 and 1 t/a, 30 May 2013 and 30 May 2018 have been fixed as deadlines for registration. For chemicals introduced into the market after 1981 applies that they have to be tested as soon as possible (Rovida et al. 2011). In general, producers are in charge for the conduction of the tests. The test battery includes testing of carcinogenicity in rodents, bioaccumulation in fish as well as acute and long-term toxicity to fish (ECHA 2011).

Compared to the EU, regulation of chemicals is less restrictive in the United States of America. However, the U.S. Environmental Protection Agency (EPA) developed essential principles for a reform of the American legislation of chemicals management. Collaborating with the Congress, the public, environmental community and the chemical industry, it is the aim of the Agency to reauthorize the Toxic Substances Control Act (EPA 2012).

I.8 Previous studies into the relationship between genotoxicity, reproductive toxicity and carcinogenesis

To date, there are several studies dealing with the relationship between genotoxicity and effects on fish populations. In an early approach, zebrafish embryos were exposed to benzo[a]pyrene (BaP; Diekmann et al. 1999). However, no effect of reproduction could be found at the population level because high fertility of males compensated potential effects.

In another study, zebrafish were investigated for genotoxic and reproductive effects after exposure to 4-nitroquinoline oxide (4-NQO) in a full life-cycle test over two generations (Diekmann et al. 2004a, b). Hereby, activation of DNA repair and elevated rates of strand breaks and micronuclei were observed. At the population level, a reduction of fertility of females was found. Based on a model calculation, this would have led to extinction of the population. Hence, a correlation between genotoxicity and reduction of reproduction in zebrafish could be established. However, more evidence was deemed necessary to clarify if there is a causal relationship. It has to be considered that mutations and apoptosis of gametes as well as metabolic alterations in organs involved in reproduction could be a reason for reduction of reproductive

capacities. Thus, also synthesis of vitellogenin or choriogenin could be affected (Diekmann et al. 2004b). In addition, compensation of reproductive effects by an inappropriate sex ratio in the spawning groups should be avoided (Diekmann et al. 2004a). This aspect is accommodated in the present thesis by composing spawning groups half-in-half of male and female fish.

In another study, effects of exposure of mummichog (*Fundulus heteroclitus*) to polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins (PCDDs) at the population level were predicted in a model calculation (Munns et al. 1997). Hereby, linear relationships between increasing exposure concentrations and decreasing population growth were shown. In one part of the study, dioxins were administrated to the fish *via* food. As a consequence, a reduction of survival and fertility of adults was observed. In the other part, fish were exposed to PCBs in natural habitats which led to a significant reduction in larval survival. In contrast to their parents, these larvae were not exposed themselves (Munns et al. 1997).

White et al. (1999) exposed fathead minnow (*Pimephales promelas*) to BaP in a life-cycle experiment and raised the offspring without exposure. Thus, effects in the offspring could be due to inherited gene mutations. In fact, concentration-dependent effects on egg number, hatching frequency and larval survival were found. However, fish were mated with their siblings. Hence, effects of BaP were probably masked by inbreeding leading to detrimental genetic homogeneity. As a consequence, a causal relationship between genotoxicity and population relevant effects could not be verified clearly. In the present thesis, this aspect is also addressed by mating the generation descendent from exposed fish irrespectively of their individual parents. Furthermore, new aspects such as the focus on genotoxic effects on the gonads after chronic exposure play a central role herein.

Regarding methodological aspects, the most important previously published literature are the protocol for isolation of primary cells from liver and gills of zebrafish by Schnurstein and Braunbeck (2001) as well as the comet assay with zebrafish sperm by Bony et al. (2010).

I.9 Design of the present thesis

The objective of the present study is to make a contribution to the clarification of the following questions:

1. How is anthropogenic pollution of the aquatic environment involved into the decline of fish populations?

2. Is there evidence for a causal relationship between genotoxic, reproductive and histopathological effects on parental fish on the one hand and survival, reproduction and histological appearance of the offspring?
3. Are these effects reversible in the next generation?

To this end, an approach to the wildlife situation including a multi-generational experiment was performed:

- On a laboratory scale, a generation of zebrafish (F0) was exposed to the model alkylating MMS in a flow-through aquaria system. Real concentrations of MMS were analyzed indirectly using high performance liquid chromatography (HPLC).
- In F0 fish, genotoxic effects of MMS were investigated in liver, gills and gonads using the comet assay for acute DNA damage and the micronucleus test for chromosomal fragmentation. Being a link between generations, special emphasis was put on genotoxic effects in the gonads.
- In the next step, parameters of reproduction such as egg number and fertilization frequency as well as larval survival and development were observed in the F1 generation. The F1 generation was raised without exposure in order to allow recovery of potential inherited DNA damage.
- Fish from the F0 and F1 generation were investigated for histopathological aberrations, such as formation of neoplasia, due to MMS exposure and inherited mutations.
- In the F2 generation, parameters of reproduction as well as larval survival and development were investigated. F2 larvae were not exposed to MMS either.
- Prior to the multi-generational study, a protocol for the comet assay with primary cells from zebrafish gonads was developed based on existing protocols for primary somatic cells from zebrafish. Analogously, a protocol for preparation and analysis of histological sections from the zebrafish gonads was developed. In addition, appropriate test concentrations for chronic exposure of the fish to MMS were derived from pre-tests. In these experiments, zebrafish were exposed under semistatic conditions to MMS for two weeks. Comet assay and micronucleus test were also performed using these fish.

II

Assessment of genotoxicity in gonads, liver and gills of zebrafish *(Danio rerio)* by use of the comet assay and micronucleus test after *in vivo* exposure to methyl methanesulfonate

II.1 Abstract

For the assessment of ecological risks of genotoxins in the aquatic environment, understanding of the relationship between genetic damage found in experimental species and detrimental effects at the population level is indispensable. Although the detection of genetic damage in gonads and germ cells of fish is essential for risk assessment, only few such studies have been conducted. Therefore, adult zebrafish *(Danio rerio)* were exposed *in vivo* to 2 - 16 mg/L methyl-methanesulfonate (MMS) for up to two weeks. Using the alkaline comet assay, genotoxic effects were investigated in primary cells derived from gonads and, for reference, in liver and gills. In addition, frequencies of micronuclei were examined in histological sections of the same organs. In the comet assay, concentrations-dependent genotoxic effects caused by MMS were detected for all organs tested, with liver and gills being more sensitive to genotoxicity than the gonads. Likewise, the micronucleus frequency was elevated by MMS in all of these organs. However, micronucleus formation was reduced at the highest test concentration, probably due to inhibition of DNA replication by MMS or the onset of acute toxicity. In conclusion, the alkaline comet assay and the micronucleus test proved appropriate for the detection of genotoxicity in primary male and female gonad cells and histological sections of the gonads from zebrafish.

II.2 Introduction

Over several decades, a decline in fish populations in European and Northern American freshwater ecosystems has been observed (Burkhardt-Holm et al. 2002, Cook et al. 2003, de Lafontaine et al. 2002, Faller et al. 2003, Keiter et al. 2006). In the Swiss midlands, e.g., between 1987 and 2002, the catch rates of brown trout and grayling was reduced by 50 % (Friedl 1999), and severe impairment of brown trout health has been identified (Burkhardt-Holm et al. 2002). Poor water quality, the presence of endocrine disruptors, diseases such as PKD (Proliferative Kidney Disease), morphological quality and warming of rivers as well as lack of food caused by changed community composition have been considered as reasons for the fish decline. Apart from these factors, general anthropogenic chemical pollution has been suggested to play a major role (Burkhardt-Holm et al. 2002, Fischnetz 2004). Streams are constantly contaminated by sewage treatment plants, rainfall and surface run-off mainly from agriculture. Thus, heavy metals, polycyclic aromatic hydrocarbons (PAHs), pesticides and persistent halogenated organic pollutants are released into freshwater ecosystems (Bätscher et

al. 1999). Many of these compounds are endocrine disruptors, others have genotoxic, mutagenic and carcinogenic potentials (Burkhardt-Holm et al. 2002, Fent 2003, Helma et al. 1994, Marquardt 1994). In aquatic systems, genotoxicity plays a major role because of its far-reaching ecological consequences (Chen and White 2004, Mitchelmore and Chipman 1998, Sun et al. 2004). Especially for the assessment of ecological risks of genotoxins in the aquatic environment, it is crucial to understand the relationship between genetic damage in endangered species and population-relevant detrimental effects in the offspring (Depledge 1996, White et al. 1999). It has been suggested that environmental pollution by genotoxins might affect the reproductive capacity of numerous species and that even a low level of pollution might lead to a destabilization of the ecosystem (Würgler and Kramers 1992). In addition to somatic mutations, heritable mutations and non-genetic modes of toxicity have been identified as the link between chemical contamination and population reduction (Bickham et al. 2000). In many groups of animals, a correlation has been shown between genotoxic effects and reproductive impairment (Anderson and Wild 1994, Devaux et al. 2011, Diekmann et al. 2004a, b, Lacaze et al. 2011, Lewis and Galloway 2009). In this context, gamete loss by cell death, embryo mortality, congenital mutations and changing genetic diversity have been observed. Especially gametes are prone to genotoxicity because of their low DNA repair capacity (Anderson and Wild 1994). This is of special importance for species releasing their gametes directly into the water, which holds true for the majority of aquatic animals (Jha 2008).

Since the genetic integrity of the gonads is essential for reproductive success, it is necessary to be able to detect DNA alterations such as fragmentation and chromosomal aberrations not only in somatic cells, but also in generative tissues of fish exposed to genotoxins. In human and mammalian toxicology, the comet assay or single cell gel electrophoresis has repeatedly been used with germ cells (Brendler-Schwab et al. 2005, Chatterjee et al. 2000, Cordelli et al. 2003, Speit et al. 2009). In contrast, in aquatic ecotoxicology, the comet assay has almost exclusively been applied to cells from various somatic tissues (Della Torre et al. 2010, Guilherme et al. 2012, Jha 2008, Santos and Martinez 2012). Only very few studies used sperm from wild species (Cabrita et al. 2005, Gwo et al. 2003). However, both of these studies were focused on aquaculture and do not have any relevance for ecotoxicology. Another genotoxicity assay, the micronucleus test, has not been adapted to the analysis of germ or gonad cells at all. In field fish, the micronucleus test has predominantly been performed using erythrocytes (Al-Sabti and Metcalfe 1995, Böttcher et al. 2010, Bony et al. 2010, Suares Rocha et al. 2009).

With reference to the specific shortcomings of the comet assay, Jha (2008) emphasizes the importance of its application to gametes of aquatic species. In addition to recent investigations on genotoxicity in fish sperm (Bony et al. 2010, Devaux et al. 2011), the adaption of the comet assay and micronucleus test to gonadal cells of both sexes as described in the present study is a step in this direction. Thus, the present study was designed to demonstrate that genotoxicity can be detected in primary cells and histological sections of testis and ovaries of zebrafish using the comet assay and the micronucleus test. In addition to gonads, liver and gills of exposed fish were investigated as a reference from somatic tissues. Hepatocytes are of special interest because of their central role in the metabolism of xenobiotics, and gill cells are of particular concern, because these organs are directly exposed to the environment. For primary cells from these organs, a protocol for genotoxicity assessment is already available (Braunbeck and Storch 1992, Schnurstein and Braunbeck 2001). Data on genotoxic effects in these cells should then serve as a basis to rule out systemic toxic effects and to reveal whether effects in gonad cells are really caused by genotoxicity.

As test substance, methyl methanesulfonate (MMS) was selected. This compound is known for its genotoxic potential (Bony et al. 2010, Clements et al. 1997, Lacaze et al. 2010) and has repeatedly served as a model substance for alkylating effects by environmental and occupational pollutants (Soloman and Faustman 1987).

II.3 Materials and methods

II.3.1 Chemicals

Medium M 199 (Hanks modification: 20 mM HEPES), Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS), penicillin-streptomycin solution (stabilized, penicillin: 5000 units/ml, streptomycin: 5 mg/ml), trypsin (trypsin-EDTA solution: 0.5 g/L porcine trypsin, 0.2 g/L EDTA x 4 Na), Triton X-100, dimethylsulfoxide (DMSO), ethidium bromide, fluorescein diacetate, methyl methanesulfonate (99 %) and all other chemicals of analytical grade were supplied by Sigma-Aldrich (Deisenhofen, Germany). Normal (NMA) and low-melting-point agarose (LMA) were purchased from Biozym (Oldendorf, Germany).

II.3.2 Fish embryo pre-testing

In order to estimate an upper limit for exposure of adult zebrafish (*Danio rerio*; West Aquarium wild type strain) to MMS for two weeks, zebrafish embryos were exposed to MMS first. In doing so, mortality of adult fish should be avoided in the subsequent experiment.
In the prolonged acute fish embryo test based on Nagel (2002), zebrafish embryos were incubated from four or eight cell stage in artificial water containing 1.9 to 50 mg/L MMS over 144 h. For the controls and each test concentration, 20 embryos were kept in a 24-well plate (TTP, Renner, Darmstadt, Germany). In plates with test concentrations of MMS, 4 additional wells were used for internal negative controls. For preparation of artificial water, 20 ml of calciumchloride-2-hydrate (20 mM), magnesium-7-hydrate (5 mM), sodium hydrogen carbonate (7.5 mM) and potassium chloride (0.37 mM) each were added to 1.92 L aqua bidest. Before use, artificial water was aerated, pH adjusted to 7.6 – 7.9 and kept at 27 °C. During the test, plates were incubated at the same temperature. As positive control, 3.7 mg/L 3,4-dichloranilin was used. Every 24 h, the embryos were examined for sublethal and lethal effects. As sublethal effects were regarded: yolk sack edema, pericardial edema, deformation of somites, lack of blood circulation, malformation of tail and delay of development. The following effects were considered lethal: tail not detached, lack of somites, lack of heartbeat and coagulation.
Based on DIN 38415-6 (2001), a test was considered to be valid if 90 % of the embryos in the negative control did not show any effects. In the positive control, the rate of affected embryos in the positive control should range between 20 % and 80 %. In the following, results are only given if these requirements were fulfilled.

II.3.3 Adult fish test conditions

Sexually mature zebrafish were raised in the fish facilities of the Aquatic Ecology and Toxicology Group Heidelberg. For each exposure group and the control, two adult groups of three male and three female fish were kept in 4L aquaria supplied with well-aerated tap water under semistatic conditions with a complete water renewal every 48 h (O_2 saturation > 92 %, hardness: 231 - 285 mg/L $CaCO_3$, pH: 7.9 - 8.4, conductivity: 577 - 679 µS/cm, ammonium ≤ 0.5 mg/L, nitrate ≤ 5 mg/L, nitrite ≤ 0.05 mg/L) at a constant temperature of 25 ± 1°C and a 12 h light / 12 h dark photoperiod. Temperature, pH, conductivity and O_2 saturation were checked daily (Multi 350i, WTW, Weilheim, Germany); hardness, ammonium, nitrate and nitrite were

measured weekly (AquaMerck test kits, Merck, Darmstadt, Germany). Fish were fed *ad libitum* with freshly bred *Artemia* nauplii (Great Salt Lake Artemia Cysts, Sanders Brine Shrimp Company, Morgan, Utah, USA) twice daily and commercial fish food (Tetramin fish food for ornamental fish, Tetra, Melle, Germany) once daily. Fish were exposed to 0, 2, 4, 8 and 16 mg/L MMS for two weeks. Pre-tests using the same methods as in the present study showed that lower concentrations failed to produce clear effects.
In other studies, zebrafish were exposed to MMS *in vivo* under similar conditions. However, water was renewed daily (Bony et al. 2010, Deventer 1996). Bony et al. (2010) argue that a daily water renewal can reasonably guarantee the quality of exposure. Analytical quantification of hydrolysis rates of MMS using ion chromatography suggest that MMS has a half-life of 40 h at 25 °C in the aquarium water used (Faßbender et al., unpublished results). Therefore, it can be assumed that water renewal every 48 h leads to a regular peak exposure. Since under field conditions toxicant concentrations are subject to fluctuations, the semistatic MMS exposure scenarios used in the present study mimics regular pulse exposure of alkylating agents in the aquatic environment.

II.3.4 Preparation of primary cell cultures from liver, gills and gonads

After two weeks of exposure, cells from liver, gills and gonads were isolated from three male and three female fish per exposure group and the control. Because of the onset of mortality at 16 mg/L, fish exposed to the highest test concentration were already sacrificed after 11 days.
The organs from three animals of each sex were pooled. In some cases, deviating numbers of fish were pooled due to the difficulty to clearly identify the sex of all fish prior to exposure and because of mortality. For cell isolation, a modified perfusion technique based on the protocols for the preparation of primary hepatocytes and gill cells in the comet assay by Braunbeck and Storch (1992) as well as Schnurstein and Braunbeck (2001) was applied: After anesthesia in a saturated solution of benzocaine (ethyl-*p*-aminobenzoate), the body cavity was opened ventrally, and blood was removed by perfusion with PBS using a peristaltic pump (MS-4 Reglo 8-100, Ismatec, Zürich-Glattbrugg, Switzerland) and pointed glass capillaries (o.d. 0.2 mm; i.d. 0.08 - 0.1 mm). The equipment for dissection is depicted in Figure II.1.
Liver, gills and gonads were dissected and rinsed in Ca^{2+}- and Mg^{2+}-free PBS. Liver and gills were incubated for 15 min in 0.05 % trypsin in PBS without $Ca^{2+}$and Mg^{2+}, but supplemented with 0.02 % EDTA. In contrast, gonads were incubated without enzymes in Ca^{2+} and Mg^{2+}-free PBS supplemented with 0.02 % EDTA. Using a glass rod, organs were passed through a

250 µm nylon gauze (Clear Edge, Geldern, Germany) into a stopping solution of PBS with 10 % fetal calf serum. All organs except for the female gonads were additionally passed through a 71 µm nylon gauze. The second filtration step was omitted for female gonads in order to not destroy larger oocytes. After filtration, liver and gonad cells were centrifuged for 10 min at 100 g and 20 °C. Gill cells were centrifuged at 200 g. The pellets were then resuspended in 200 µl erythrocyte lysis buffer (Betti et al. 1993). After 5 min of incubation, lysis was stopped with 800 µl M199 medium supplemented with 350 mg/L $NaHCO_3$, 10 % fetal calf serum and 10 ml/L penicillin-streptomycin solution. Cells were again centrifuged to remove cell debris and resuspended in M199 medium supplemented with 350 mg/L $NaHCO_3$, 10 % fetal calf serum and 10 ml/L penicillin-streptomycin solution at a density of 10^5 cells/ml.

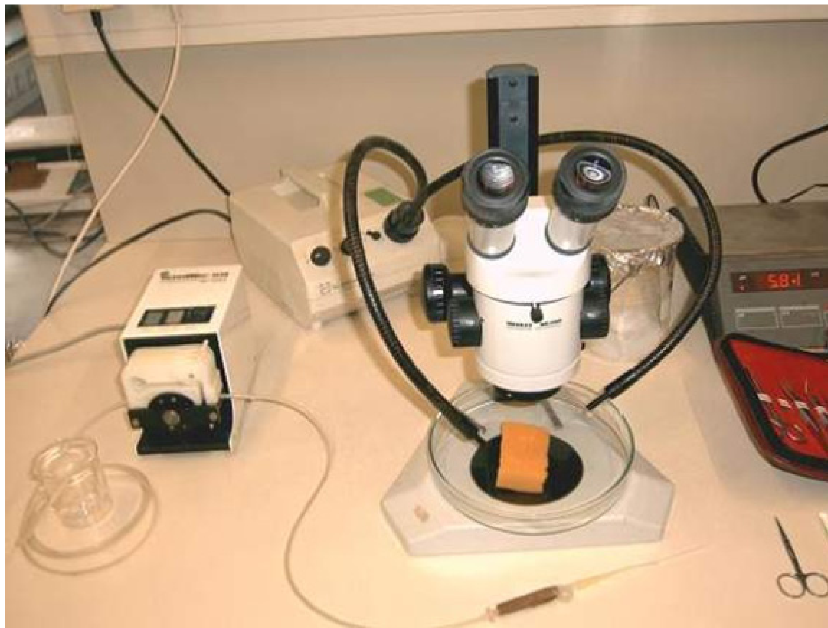


Fig. II.1: Equipment for zebrafish dissection and organ removal: binocular microscope equipped with illumination and foamed plastic for fish fixation with needles as well as pump and glass capillary for perfusion.

II.3.5 Viability testing

For the measurement of the percentage of living cells, the fluorescein diacetate (FDA) assay was performed according to Strauss (1991) in the adaption of Schnurstein and Braunbeck (2001). For the staining solution, 40 µl of an ethidium bromide solution (200 µg/ml in aqua bidest) and 6 µl of an FDA solution (5 mg/ml in acetone, stored at 4 °C) were added to 960 µl unsterile PBS. In the next step, 30 µl staining solution were given to 30 µl cell suspension, incubated for 30 s, spread on a glass slide and covered with a cover slip. Using a fluorescence microscope (Aristoplan, Leitz, Wetzlar, Germany) with 340-fold magnification at a wave-length of 435 nm, a total of 500 cells were examined in two replicates for each cell prepara-tion. As FDA is dissociated by cytosolic esterases into fluorescein and acetate, vital cells were

stained green under fluorescence. Dead cells appeared red due to ethidium bromide intercalating in the nuclear DNA. A comet assay was performed only if cell viability exceeded 90 % relative to the controls.

II.3.6 Comet assay

The comet assay was performed under alkaline conditions according to Singh et al. (1988) with modifications detailed by Schnurstein and Braunbeck (2001). Lysis conditions followed the protocol by Kosmehl et al. (2004). Fully frosted glass slides were degreased in 99 % ethanol. 1 % and 0.5 % NMA were dissolved at 300°C in PBS and kept liquid at 150 °C. The slides were covered with 1 % NMA, which was scraped off after hardening. The slides were dried for 5 min at 37 °C in order to enhance the adhesion of the following layers. 0.5 % NMA was dispersed on the slides before they were hardened for 3 min on ice and dried for 10 min at 37 °C. For embedding of the cells, 0.7 % LMA was dissolved at 300°C in PBS and kept liquid at 37 °C. 90 µl of cell suspension were added to 90 µl LMA. In the next step, 90 µl of the mixture were spread on the prepared slides, solidified on ice for 3 min and incubated at 37 °C for 5 min. In the next step, 60 µl of a 0.7 % LMA solution were added as a covering layer, solidified and dried as described before. For lysis, the slides were incubated for 2 h in lysis solution. Hereby, cell membranes were disrupted and proteins extracted under alkaline conditions. Autolysis and UV damage were minimized by keeping the slides in darkness at 4 °C. The following steps were carried out under red light. After lysis, the slides were incubated for 20 min in ice-cold electrophoresis buffer, so that DNA could be unwound and single strands were separated. Electrophoresis was carried out for 20 min at 25 V and a maximum of 310 mA (Power Supply Model 200/2.0, Bio-Rad, Richmond, USA) in the same buffer. Afterwards, the slides were neutralized in tris buffer for 2 min to restore the helical structure of the the DNA (McKelvey-Martin et al. 1993). The slides could be stored in the dark for up to 2 weeks at 4°C. For staining, 60 µl of an ethidium bromide solution (20 µg/ml in aqua bidest.) were spread on each slide. For each cell suspension prepared from the organs of three fish, 100 cells on each of two slides were evaluated at 400 x magnification for DNA migration by using the comet image analysis software Komet 5.5 (Kinetic Imaging Ltd., Liverpool, UK). For statistical analysis with SigmaPlot 11.0 (Systat, Erkrath, Germany), the relative percentage of DNA in the tail was measured. Data were not normally distributed, and statistical differences were analyzed using ANOVA-on-ranks (Kruskal-Wallis) in combination with the *post-hoc*

test according to Dunn in order to identify statistical power of difference. Tab. II.1 shows the solutions used in the comet assay.

Tab. II.1: Solutions used in the comet assay.

Solution	Composition
Lysis stock solution	146.5 g NaCl (99.5 %, p.A., Grüssing, Filsum, Germany), 37.2 g EDTA (disodium salt dehydrate, p.A., AppliChem, 8 g NaOH (p.A. Merck), 1.2 g tris (Carl Roth, Karlsruhe, Germany), in 750 ml aqua bidest; adjust pH with 1 M HCl to 10; fill up to 890 ml with aqua bidest.
Lysis solution	178 ml lysis stock solution, 20 ml DMSO (99 %, Grüssing), 2 ml Triton X-100 (Sigma-Aldrich)
Electrophoresis buffer	24 g NaOH (p.A., Merck), 0.74 g EDTA (disodium salt dihydrate, p.A., AppliChem) in 2 L aqua bidest.
Tris buffer	48.5 g tris (Carl Roth) in 1 L aqua bidest., adjust pH with 25 % HCl to 7.5

II.3.7 Micronucleus test on histological sections

For the preparation of histological sections for the micronucleus test, three male and three female fish from each exposure group and the control were anaesthetized as described above. After perfusion with fixative (1.5 % glutardialdehyde, 1.5 % paraformaldehyde, 2.5 % poly-vinylpyrrolidon (PVP) in 0.1 M phosphate buffer), liver, gills and gonads were dissected and further incubated in 2.5 % glutardialdehyde, 4 % PVP, 0.05 $CaCl_2$ in 0.1 M cacodylate buffer for 1 h at 4 °C. All organs were washed twice in 0.1 M cacodylate buffer for 10 min, dehydrated in an ascending ethanol series and incubated in ascending concentrations of Spurr's medium in ethanol. Spurr´s resin was prepared according to Spurr (1969). As a modification according to Ellis (2006), the toxic vinylcyclohexene dioxide was replaced by 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate (ERL-4221D; Serva, Heidelberg, Germany). Until polymerization, the preparations were incubated at 70 °C. Semithin sections of 1 µm thickness were cut using glass knifes on a Reichert OM-U 2 ultramicrotome (Vienna, Austria; Fig. II.2). Spurr was removed from the slides overnight by incubation in a NaOH-ethanol solution (1 g in 200 ml) at 4 °C, followed by a descending ethanol series and distilled

water. In order to specifically visualize euchromatin, the slides were stained with Weigert's iron hematoxylin according to Weigert (1904). The slides were then dehydrated as described above and coverslipped with DePeX (Serva). In order to determine micronucleus frequencies, micronuclei were counted in 2000 cells on each slide using an Aristoplan light microscope with 1,120-fold magnification (Leitz). Chromatin fragments were considered as micronuclei, if they had a diameter of not more than 30 % of that of a nucleus of the same cell type and were lying close to the nucleus and in the same focus plane. For normally distributed data, statistical differences in micronucleus frequency between groups were analyzed by One-Way-ANOVA in combination with the *post-hoc* test according to Holm-Sidak in order to identify statistical power of difference.

Fig. II.2: Ultramicrotome used for preparation of semithin sections of liver, gill and gonad samples embedded in Spurr's resin.

II.4 Results

II.4.1 Fish embryo pre-testing

From 16.4 mg/L MMS, a clear concentration-response relationship was found after 24 to 144 hpf (Fig. II.3). Concentrations lethal for half of the embryos (LC_{50}) were determined between 24 mg/L after 144 hpf and 41 mg/L after 24 hpf. Concentrations causing sublethal or lethal effects in half of the embryos (EC_{50}) were lower than the latter and determined between 16 mg/L after 144 hpf and 37 mg/L after 24 hpf. LC and EC values are given in Table II.2. Exemplarily, zebrafish embryos showing effects due to MMS exposure are shown in Figure II.4.

Based on these results, adult zebrafish were subsequently exposed to up to 16 mg/L MMS for two weeks.

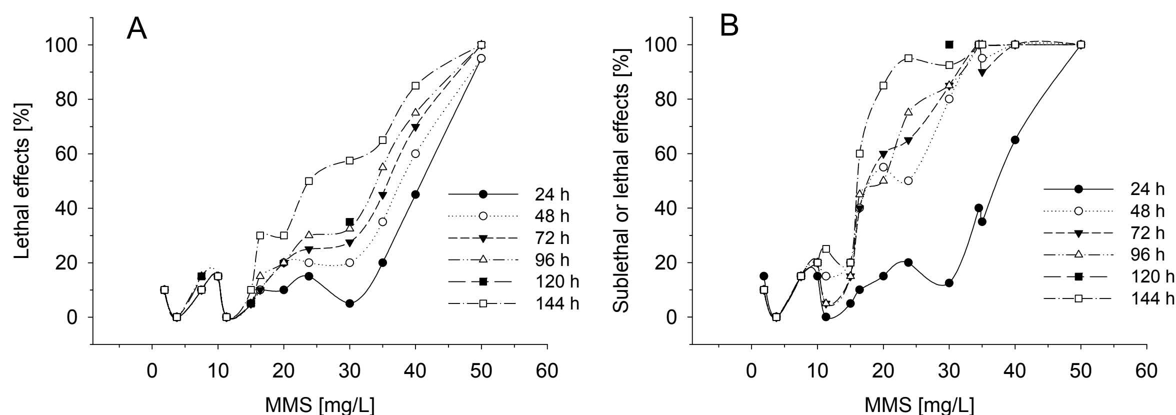


Fig. II.3: Lethal effects (A) and sublethal as well as lethal effects combined (B) on zebrafish embryos after exposure to MMS up to 144 hpf. Values of three tests using different concentrations are shown.

Tab. II.2: Concentrations lethal for half of the embryos (LC_{50}) and concentrations causing sublethal or lethal effects in half of the embryos (EC_{50}) after exposure to MMS over 144 hpf (hours post fertilization). At 120 hpf, not enough data were collected to calculate LC and EC values.

hpf	LC_{50} [mg/L]	EC_{50} [mg/L]
24	41	37
48	38	18
72	36	17
96	34	20
144	24	16

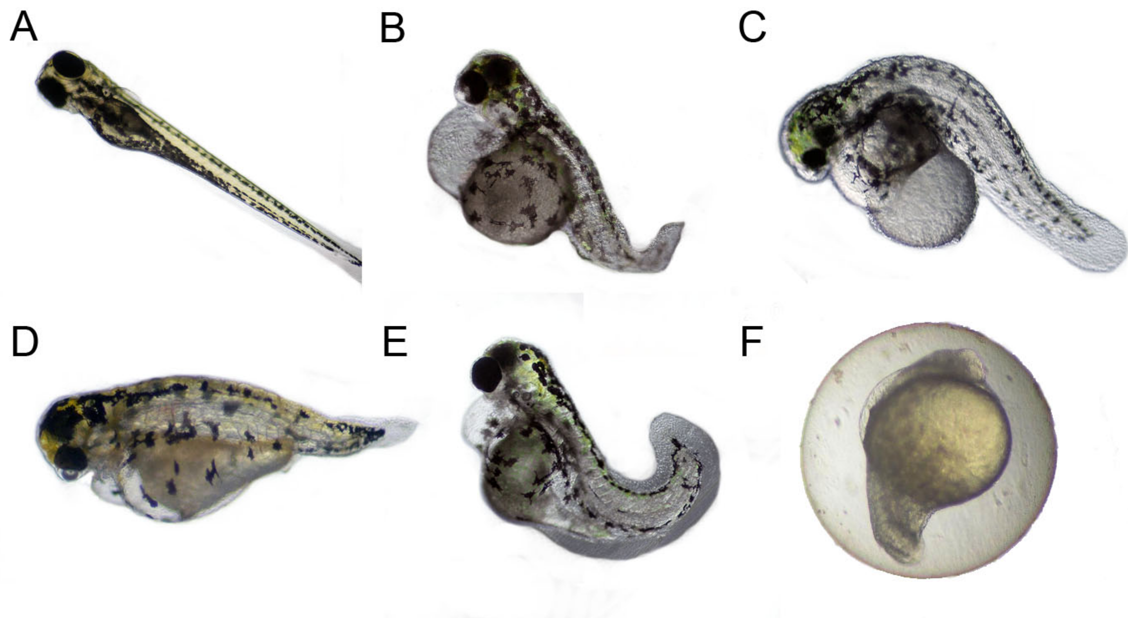


Fig. II.4: Zebrafish embryos after exposure to MMS. A: Untreated control at 96 hpf. B: Embryo exposed to 16.4 mg/L at 96 hpf showing pericardial edema and tail malformation. C: Embryo exposed to 23.8 mg/L at 96 hpf featuring pericardial and yolk sack edemata and tail malformation. D: Embryo exposed to 30 mg/L at 144 hpf showing effects similar to C and eye malformation. E: Embryo exposed to 40 mg/L at 144 hpf featuring effects similar to C. Additionally, in fish B – E reduction of heartbeat and reduction or lack of blood circulation were observed (all effects sublethal). F: Embryo exposed to 50 mg/L at 24 hpf with yolk sack edema and tail not detached (lethal effect).

II.4.2 Cell viability and fish survival

For all test concentrations, cell viability of primary cultures exceeded 90 % relative to the control. Hence, genotoxicity testing was performed in liver, gills and gonads at a concentration range from 2 to 8 mg/L. Appearance of primary cells in the FDA assay is shown in Fig. II.5. Since exposure to the highest test concentration of 16 mg/L unintentionally led to mortalities, genotoxicity was already analyzed after 11 days, and all individuals were used for the FDA and comet assays.

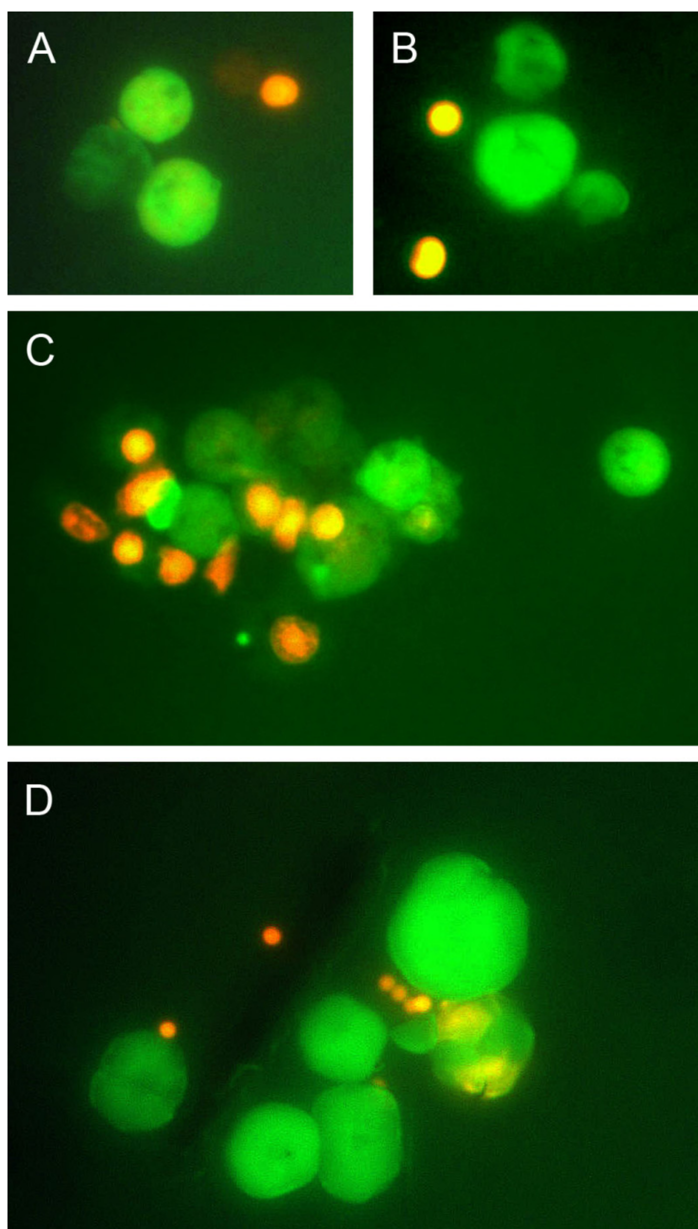


Fig. II.5: Control primary cells from male liver (A), female gills (B), female gonads (C) and male gonads (D) of zebrafish in the FDA assay (magnification 630 x). Living cells are stained green with fluorescein and the nuclei of dead cells are stained red with ethidiumbromide.

II.4.3 Comet assay

Over all tissues analyzed, the proportion of DNA in the tail of control cells ranged between 9 and 14 %. The appearance of control nucleoids is shown in Fig. II.6. For all organs studied, effect levels did not show any sex specificity. Effects in gills and gonad cells were generally less pronounced than in liver cells.

For all MMS concentrations tested, primary cells from liver and gills of both sexes of zebrafish showed a clear-cut dose-dependent increase in the percentage of DNA in the tail. In contrast, 2 mg/L MMS caused no significant effect in gonad cells of either sex. However, exposure to ≥ 4 mg/L MMS led to a dose-dependent increase in the percentage of DNA in the tail in gonad cells (Fig. II.7). The strongest effects were found in male and female liver cells after exposure to 16 mg/L MMS with a maximum of 62 and 68 % DNA in the tail, respectively. However, it should be born in mind that at 16 mg/L MMS fish were close to mortality.

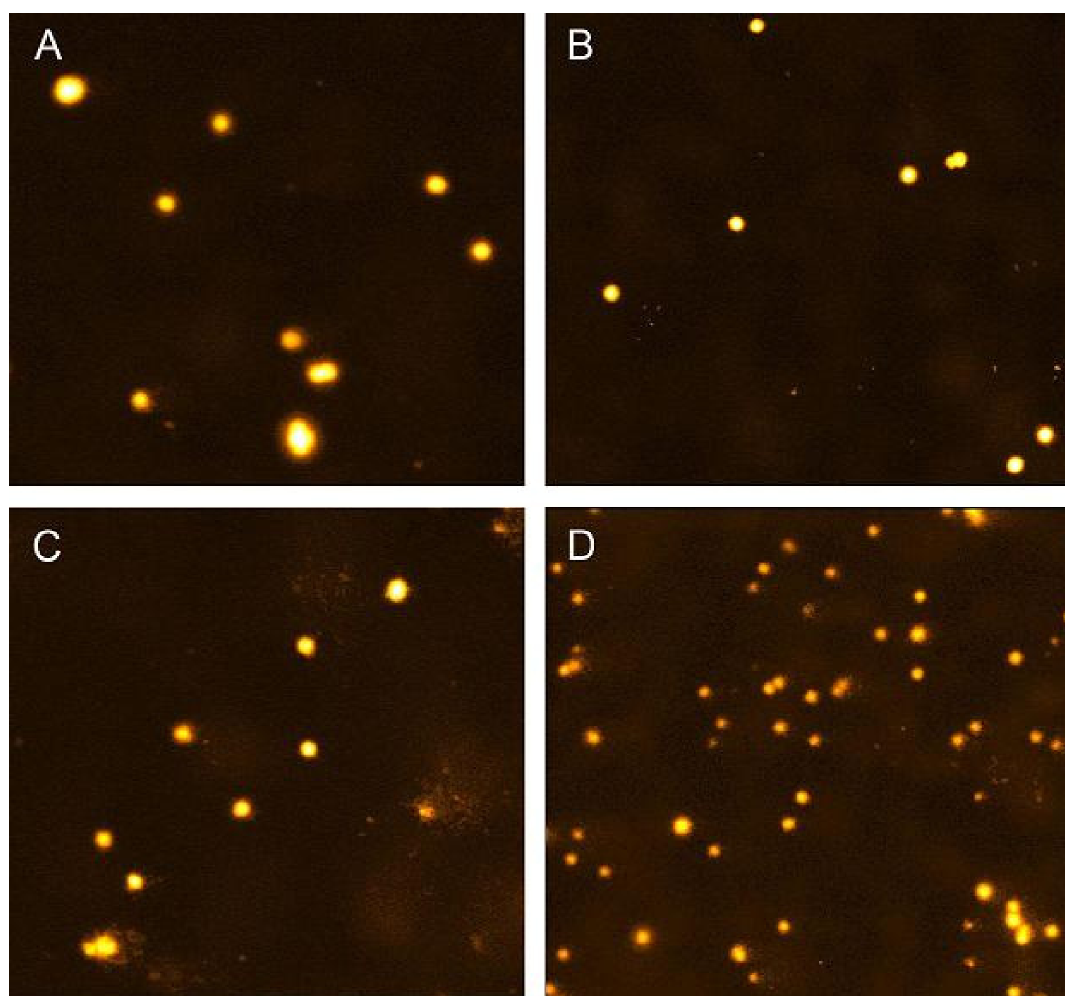


Fig. II.6: Nucleoids of control primary cells from male liver (A), female gills (B), female gonads (C) and male gonads (D) of zebrafish in the FDA assay (magnification 800 x).

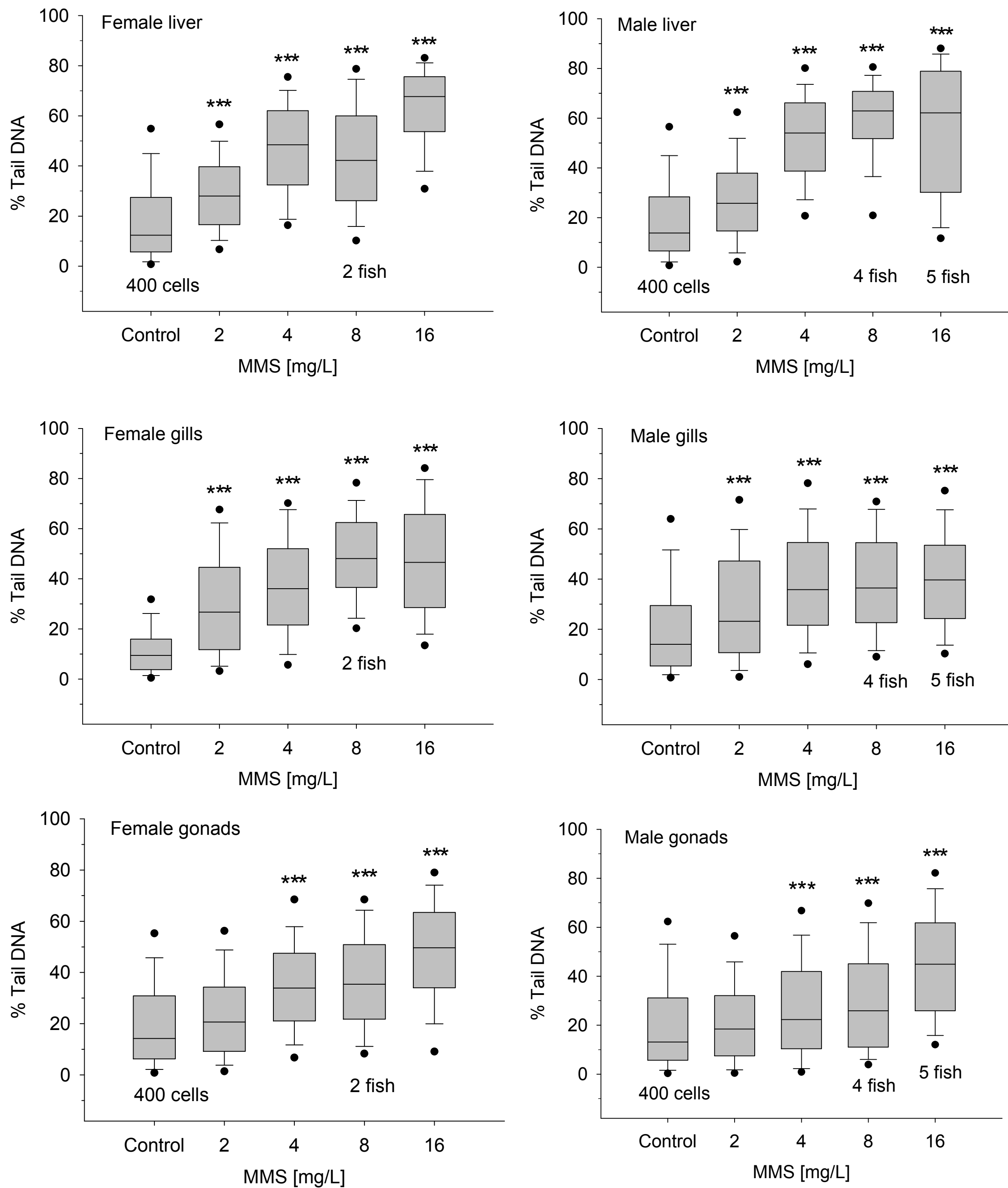


Fig. II.7: Percentage of tail DNA in the comet assay in primary cells from liver, gills and gonads of male and female zebrafish after two weeks of exposure to 2 - 8 mg/L methyl methanesulfonate (MMS) *in vivo*. In the case of 16 mg/L, fish were exposed for only 11 days. In each concentration and the controls, organs of 3 fish were pooled. For each box, data from two replicate slides with 100 nuclei each are unified. Data are given as means within box plots displaying the following percentiles: 25 and 75 (shaded box), 10 and 90 (whiskers) as well as 5 and 95 (points). *** $p < 0.001$ for significant differences from controls (ANOVA-on-ranks followed by *post-hoc* test according to Dunn).

II.4.4 Micronucleus test

In zebrafish hepatocytes, a significant increase in micronucleus frequency was found at 2 and 4 mg/L MMS irrespective of sex (Fig. II.8). At 8 mg/L, however, the elevation was not significant. MMS caused significant effects in male and female gill and gonad cells from2 and 4 mg/L, respectively. Whereas micronucleus frequency in gill cells increased in a concentration-dependent manner, liver and gonad cells showed a clear maximum at 4 mg/L followed by lower micronucleus frequencies at 8 mg/L. In Figures II.9 and II.10, the appearance of stained histological sections of zebrafish liver, gills and gonads is shown.

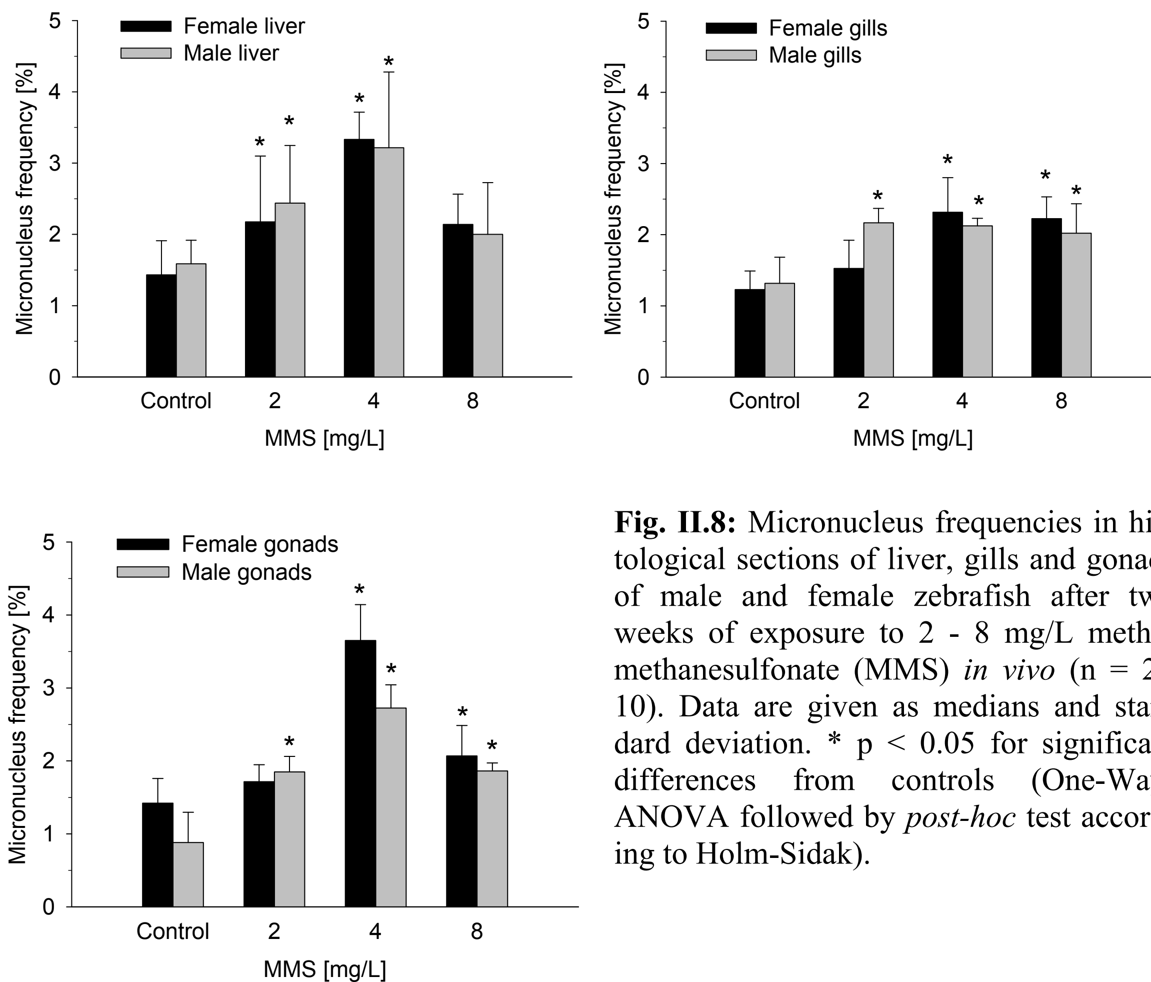


Fig. II.8: Micronucleus frequencies in histological sections of liver, gills and gonads of male and female zebrafish after two weeks of exposure to 2 - 8 mg/L methyl methanesulfonate (MMS) *in vivo* (n = 2 - 10). Data are given as medians and standard deviation. * $p < 0.05$ for significant differences from controls (One-Way-ANOVA followed by *post-hoc* test according to Holm-Sidak).

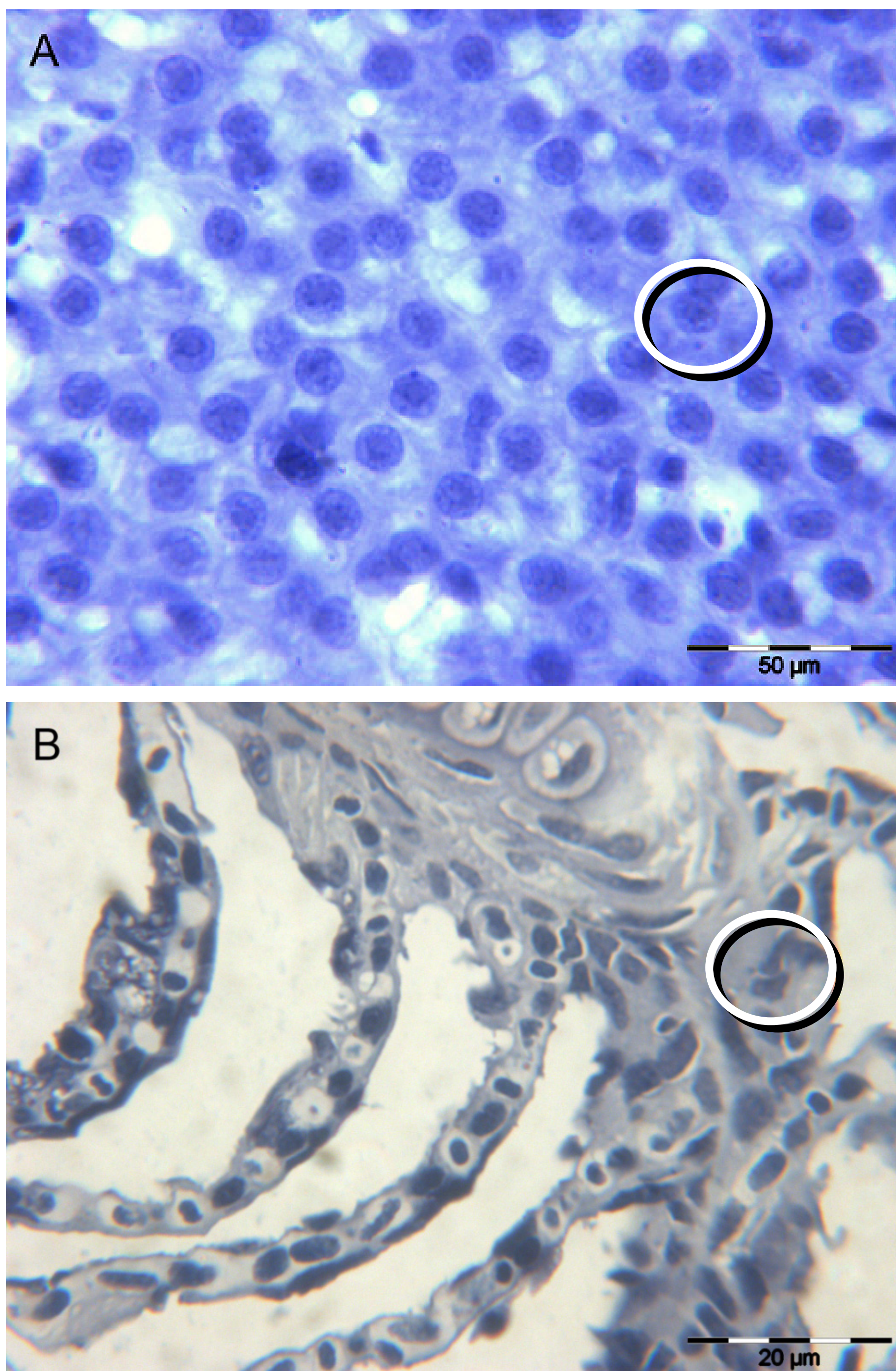
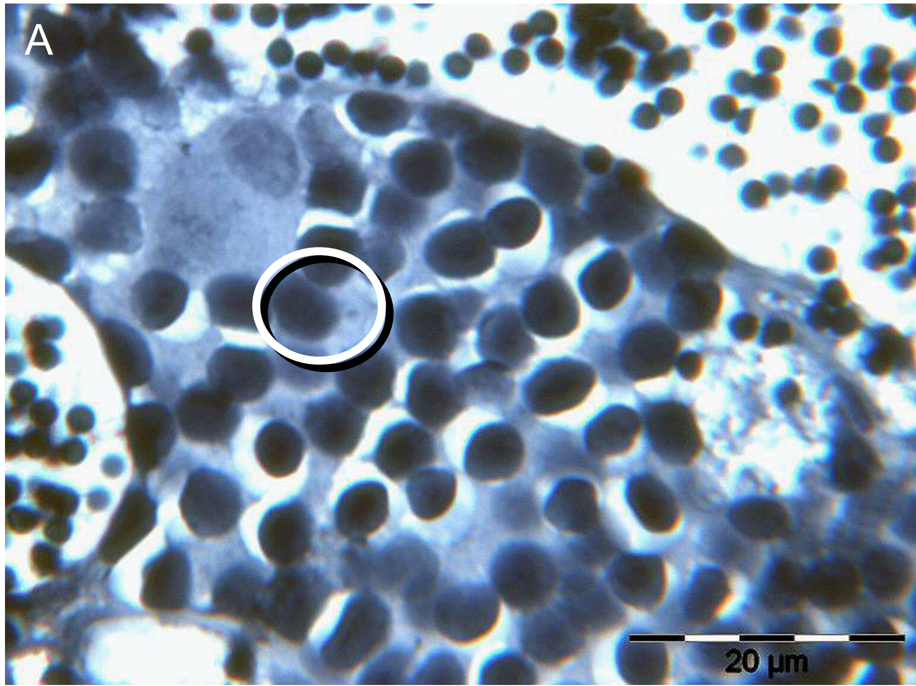


Fig. II.9: Histological sections of female liver (A) and male gills (B) from control zebrafish stained with Weigert's hematoxylin. In a hepatocyte and an interlamellar cell micronuclei are visible.



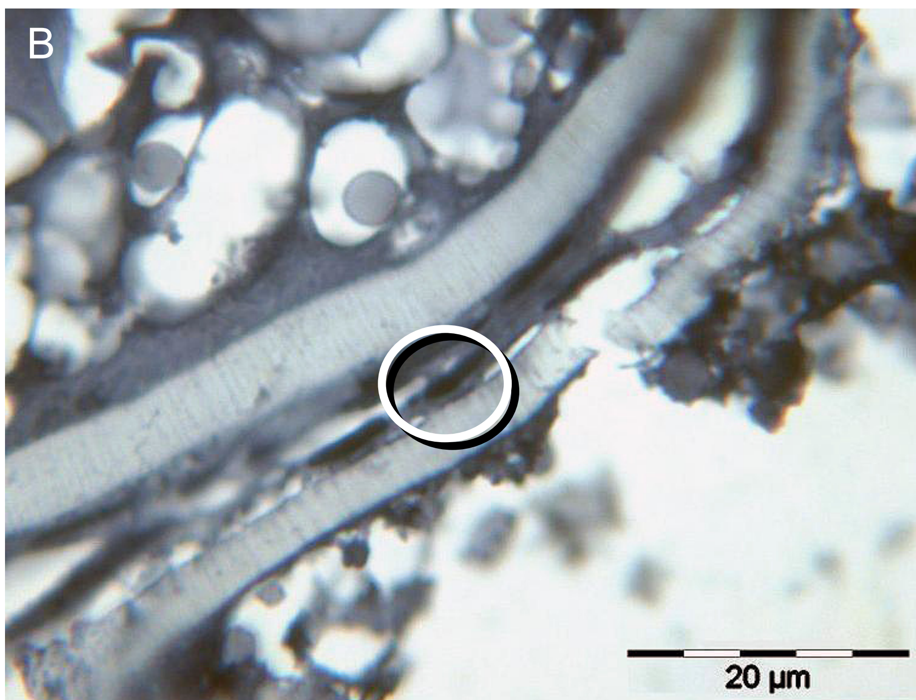


Fig.II.10: Histological sections of gonads from male (A) and female (B) zebrafish from controls stained with Weigert's hematoxylin. In spermatogonia and follicle cells micronuclei are visible.

II.5 Discussion

Since gonadal cells, especially gametes, link generations, investigations into genotoxic effects in these cells are of major interest for the understanding of potential reasons for the reduction of wild fish populations. The model genotoxicant MMS is regarded as representative for a number of alkylating anthropogenic compounds in the aquatic environment. Alkylating agents range among the most potent and abundant DNA-damaging agents in the aquatic environment: known examples are allylic reagents (Kuehl et al. 1994) and mucohalic acids (Gómez-Bombarelli et al. 2011a). Mucochloric acid, e.g., is a chlorination product of tap water (Gómez-Bombarelli et al. 2011b). Thus, knowledge on genotoxicity of MMS to fish at a laboratory scale can be useful to assess genotoxic hazards by environmental alkylating agents to aquatic animals.

The concentration range used for exposure of adult zebrafish in the present study was based on the results of the embryo toxicity test. As anticipated, clear effects were found in the comet assay and micronucleus test in adult fish. However, mortality at the highest concentration required the use of all survived fish in the comet assay at 16 mg/L. Thus, no fish could be used for the micronucleus test at that concentration. If a larger distance had been kept between the concentration range of the fish embryo toxicity test and exposure of adult fish, mortality could have been avoided. Despite this, the concentration range used was appropriate for the purpose of the current study, i.e. the investigation of genotoxicity in primary gonad cells.

Only gametes are able to transfer genotoxic effects to the next generation. However, in the present study, primary cells and histological sections from the gonads were analyzed without discriminating between gametes or their precursor stages and somatic cells such as Sertoli, interstitial and Leydig cells as well as granulosa and theca cells. Since genotoxicity in somatic gonad cells can lead to malfunction of these cells, tumor formation (Takashima and Hibiya 1995) and cell death, it cannot be excluded that genotoxic effects on somatic cells specifically in the gonads can have a negative impact on generative cells.

In contrast to organ-specific somatic cells, blood cells were excluded from analysis in the FDA and comet assays by perfusion with PBS and use of a cell-specific lysis buffer for erythrocytes. For the micronucleus assay with histological sections, this was done by perfusion of organs with fixative. Remaining erythrocytes could easily be identified in the sections and be excluded from analysis in the micronucleus assay. Thus, it could be made sure that the assessment of genotoxicity was restricted to organ-specific cells. In the micronucleus test, in

male gonads mainly spermatogonia and in female gonads mainly theca and granulosa cells were analyzed. The former is due to the restriction problem that in histological sections of testis tails of spermatozoa can easily be mistaken for micronuclei; therefore, analyses in this organ focused on other cell types. In female gonads, theca and granulosa cells are much more abundant than oocytes and their precursors. Thus, it would almost be impossible to reach sufficient statistical power, if only female gametes were analyzed. However, it was taken care to include as many gametes as possible into the investigation of micronucleus frequency.
To date, several studies have reported genotoxic effects in fish after *in vivo* exposure to MMS. For example, elevated rates of sister chromatid exchanges were documented in gill cells from the central mudminnow (*Umbra limi*; Kligerman 1979). In another study, zebrafish exposed to 1 µM (110 µg/L) MMS for up to three weeks showed significant effects in the comet assay with liver cells and spermatozoa (Bony et al. 2010). After two weeks of exposure, the percentage of DNA in the tail reached 16 and 26 % in spermatozoa and liver, respectively, and remained on this level for the next week. Compared to data for liver and male gonad cells in the present study, these values are quite low (cf. Fig. II.7). On the other hand, this confirms the results of the present study, because at much higher concentrations of 2 to 16 mg/L, stronger effects are plausible. The study of Bony et al. (2010) is an important precursor of the present study, as it shows effects in gonadal cells from zebrafish using the comet assay. Furthermore, results are corroborated by Devaux and co-workers (2011): Using the comet assay, significant DNA damage in spermatozoa from brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpines*) was found after exposure to MMS at the end of spermatogenesis. In another study, zebrafish were exposed to 0.8 - 80 µM (88 µg/L - 8.8 mg/L) MMS (Deventer 1996). At the highest concentration, the comet assay revealed a significant increase of tail length in liver and gill cells after 8 h of exposure. In both cell types, DNA damage increased up to 48 h of exposure, in liver cells more clearly than in gill cells. Exposure was stopped after 72 h, and subsequent 24 h of recovery in pure water led to a decrease of DNA damage. The highest concentration used by Deventer (1996) is only slightly higher than the second highest concentration used in the present study. On the other hand, a much longer exposure time of two weeks was applied without the possibility to recover from alkylating stress, apparently leading to an accumulation of DNA damage. Overall, comet assay results of the present study for liver and gill cells are in line with data available in the literature.
The micronucleus test has repeatedly been used for the assessment of the genotoxic potential of MMS or similar compounds in somatic cells: *In vivo* exposure of the African catfish (*Clarias lazera*) to ethyl methanesulfonate (EMS) for two weeks significantly increased the mi-

cronucleus frequency in erythrocytes (Odeigah and Osanyipeju 1995). Likewise, zebrafish erythrocytes exposed to 1 µM (110 µg/L) MMS for three weeks clearly showed induction of micronuclei (Bony et al., 2010). If compared to the comet assay, Deventer (1996) concluded a higher sensitivity for the micronucleus test in zebrafish. Apart from erythrocytes, only few studies have been carried out on the effect of genotoxic agents on micronucleus frequencies in fish gill and liver cells. In Nile tilapia (*Oreochromis niloticus*), *in vivo* exposure to a textile mill effluent for 9 days caused an increase of micronucleus frequency in gill cells to 12.83 ‰, compared to 1.93 ‰ in controls (Çavaş and Ergene-Gözükara 2003). In a similar experiment, Prussian carp (*Carassius gibelio*) exposed to $CdCl_2$ for 21 d showed maximum effects of 10 ‰ in gill cells, and micronucleus frequencies in liver cells of peppered cory (*Corydoras spaleatus*) reached 5 ‰ (Çavaş et al. 2005) indicating both species and organ specificity of genotoxic effects in primary cells from fish. This might also account for the fact that micronucleus frequencies in the present study with a maximum between 3 and 4 % are clearly higher than those reported in literature on other fish species.

The focus of previous micronucleus studies was on erythrocytes and other somatic tissues. In contrast, for no fish species studied so far, effects of exposure to genotoxicants on micronucleus frequency in gonad cells are available. Therefore, the present study is the first to describe a significant increase of micronucleus frequency in gonad cells. The induction of micronuclei in liver and gonad cells was concentration-dependent except for the highest concentration. This is probably due to the capacity of MMS to inhibit DNA replication. It is assumed that MMS leads to an arrest of replication forks (Lundin et al. 2005). Since cell division is required for the formation of micronuclei, a reduction in the number of dividing cells might constrain the formation of a great number of micronuclei. However, the onset of acute mortality at 16 mg/L MMS might also have been a factor reducing mitotic rates in exposed zebrafish.

Whereas exposure of somatic cells to genotoxicants may cause carcinogenesis, inheritable mutations and subsequent reproductive defects leading to impaired reproductive success and changes in population dynamics may arise from damage in germ cells (Belfiore and Anderson 2001, Bony et al. 2010). After intraperitoneal MMS injection, dominant lethal mutations have been documented in medaka (*Oryzias latipes*) spermatids. After mating of exposed males with unexposed females, both fertility and hatchability were reduced (Shimada and Egami 1984). Likewise, male Mozambique tilapia (*Tilapia mossambica*) exposed to MMS injection during spermatogenesis produced less offspring than controls, and up to 50 % of the embryos showed defects (Hemsworth and Wardhaugh 1978). Thus, there seems to be a correlation between

reduction in fertility and embryopathy due to dominant lethal mutations in male germ cells; MMS obviously affects the transition between generations and is, therefore, of special relevance in genotoxicity assessment in gonads.

In conclusion, the present study clearly documents that the alkaline comet assay as well as the micronucleus test are appropriate methods for the detection of genotoxicity in primary gonad cells and histological sections of the gonads, respectively. Further studies into the correlation between DNA damage and population-relevant parameters such as fertility of fish exposed to chronic genotoxicity as well as embryo toxicity and larval survival in subsequent generations are subject of the following chapters.

III

Developmental and reproductive effects as well as recovery in zebrafish after chronic exposure to an alkylating agent in a multi-generation study

In chapter I, it was demonstrated that genotoxic effects of MMS on primary cells from zebrafish gonads can be examined using the comet assay. In addition, it was shown that micronuclei can be detected in histological sections of the gonads. Based on this information, in chapter II, a multi-generation experiment using zebrafish is described. In order to simulate genotoxic exposure of wild fish in the freshwater environment, a generation of zebrafish (F0) was exposed to MMS. In these fish, survival, growth, sex ratio, fertility and reproduction were investigated. In the following generation (F1), which was not exposed to MMS, the same parameters as well as development were recorded. Moreover, in the F2 generation, survival and development were examined.

III.1 Abstract

There is still controversy whether adverse effects by genotoxic anthropogenic pollutants are linked to the decline of fish populations. Since anthropogenic chemical pollution is likely to be a key to the problem, investigations into the relationship between genotoxic stress and detrimental effects on development and reproduction in fish are required. For this end, zebrafish (F0 generation) were exposed *in vivo* to the alkylating model genotoxin methyl methanesulfonate (MMS) from fertilization to the age of one year in a continuous flow-through system. Survival of F0 fish was recorded, and F0 fish were mated over six months to check for reproductive capacities. In contrast to the F0 generation, F1 fish grew up without exposure in order to allow for regeneration. Embryonic and larval development of the F1 generation was recorded and F1 fish were mated. In both the F0 and F1 generation, sex ratios were determined, and weight and length were measured. In both the F1 and F2 generations, teratogenicity and survival were recorded. Mortality of F0 fish clearly depended on MMS concentrations. Times of first spawning in MMS-exposed F0 fish were delayed, and fertility of MMS-exposed F0 fish was reduced. No unequivocal effects on growth were found. In F1 fish derived from MMS-exposed fish, teratogenic effects were increased, larval survival was reduced and sex ratio was shifted towards females. However, fertility of the non-exposed F1 generation had recovered. Development and survival rates further recovered in the F2 generation. Anthropogenic genotoxicants may thus play a considerable role in the decline of wild fish populations.

III.2 Introduction

In European and Northern American freshwater ecosystems, a decline of fish populations has been observed over the last decades (Burkhardt-Holm et al. 2002, Fischnetz 2004, Friedl 1999). In addition to other factors, anthropogenic chemical pollution, especially by genotoxic, mutagenic and carcinogenic compounds, may have contributed considerably to this problem (Bätscher et al. 1999, Burkhardt-Holm et al. 2002, Fischnetz 2004, Helma et al. 1994). Thus, the investigation of genotoxic and mutagenic hazards in the field requires special regard (Chen and White 2004, Mitchelmore and Chipman 1998, Sun et al. 2004). In the assessment of the effects of genotoxic pollutants on aquatic wildlife animals, there has been a focus on the development of neoplasia, on gene mutations leading to teratogenicity and other diseases, as well as on latent genetic damage. As the latter is inheritable, it may affect survival at the individual and the population level (Bickham et al. 2000, Depledge 1996). Genotoxicity can be correlated with gamete loss, embryonic mortality and heritable mutations in fish (Anderson and Wild 1994).

The importance of reproductive impairment of fish due to aquatic pollution has been highlighted (Kime 1995). However, to date, only few studies available have addressed the relationship between genotoxicity and effects on fish populations. Theodorakis and co-workers (1997) found increased mortality and teratogenic effects in the offspring of mosquito fish *(Gambusia affinis)* after exposure to radionuclides *in situ*. In another study, the effects of multi-generation exposure of polychlorinated biphenyls (PCBs) and dioxines to mummichog *(Fundulus heteroclitus)* at the population level were modelled (Munns et al. 1997), and an inverse linear relationship between population growth rate and chemical concentrations was found. In one part of this study, adult fish were exposed to dioxins *via* food, leading to reduced survival of the adults and, hence, less offspring. In the same study, another fish population was exposed to PCBs *in situ* in a natural habitat, and a significant decrease in larval survival was observed. However, in contrast to their parents, these larvae had not been exposed to PCBs themselves. The authors concluded that maternal transmission of PCBs or their metabolites had occurred. However, a continuous multi-generation exposure only incidentally reflects the environmental situation, which is often characterized by fluctuating exposure due to discontinuous pollution, degradation and remobilization of pollutants from sediments. In a study by White and co-workers (1999), a generation of fathead minnow *(Pimephales promelas)* was exposed to benzo[a]pyrene (BaP), and the subsequent two generations were raised without exposure. Thus, effects in the offspring more than one generation away from the ex-

posed population could be shown to be due to genetic mutations and their inheritance. In fact, concentration-dependent adverse effects on the fertility of F1 fish and survival of F2 larvae were found. However, it cannot be excluded that BaP effects were obscured by inbreeding so that a causal relationship between genotoxicity and population relevant effects could not clearly be shown. Likewise, ambiguous results were provided by Diekmann and co-workers (1999) who tested the effects of BaP on zebrafish *(Danio rerio)* populations in a full life-cycle study. No population-relevant effect was found, because it was impossible to prepare high BaP concentrations, and potential effects were confounded by high spawning frequencies. In another life-cycle test, zebrafish exposed to 4-nitroquinoline oxide (NQO; Diekmann et al. 2004a) displayed a reduction of egg production that would have led to extinction of the fish at high NQO concentrations according to a mathematical simulation approach. In a parallel study on genotoxicity, an increase in DNA repair, DNA breaks and micronucleus frequency was evident in the same experiment (Diekmann et al. 2004b), thus providing evidence of a relationship between genotoxicity and impair of reproduction in zebrafish. However, it is still unclear to what extent genotoxic exposure may contribute to the decline of wild fish populations and if there is a causal relationship. Since in the study of Diekmann and co-workers (2004b) an inappropriate sex ratio of 4 females to 8 males (male surplus) in the mating groups might have concealed NQO effects on fertilization, the present study used an equalized sex ratio in the spawning groups.

Methyl methanesulfonate (MMS) has frequently been used as a model genotoxicant (Bony et al. 2010, Deventer 1996, Solomon and Faustman 1987). Although MMS itself is not relevant in the aquatic environment, it can be considered as a representative of several groups of environmental pollutants such as mucohalic acids (Gómez-Bombarelli et al. 2011a) and allylic reagents (Kuehl et al. 1994) due to its alkylating mode of action.

While the present chapter focuses on developmental and reproductive effects on zebrafish exposed to MMS, chapter IV correlates histopathological lesions to genotoxic lesions in liver, gills and gonads using the comet and the micronucleus assays.

III.3 Materials and methods

III.3.1 Chemicals

Methyl methanesulfonate (MMS, CAS Nr. 66-27-3, 99 %) was supplied by Sigma-Aldrich (Deisenhofen, Germany). It is well water soluble, up to a ratio of 1:5 at 25 °C (Budavari 1996). Although MMS tends to be hydrolyzed in water, MMS concentrations are stable, as long as the exposure medium is exchanged daily (Bony et al. 2010). Moreover, using ion chromatography, the half-life of MMS in water was determined to about 40 h at 25 °C (own data). Thus, regular water renewal every 48 h is expected to produce a regular peak exposure with limited degradation.

III.3.2 Test conditions

Water parameters were measured on a weekly basis by means of a Multi 350i electrode (WTW, Weilheim, Germany) and AquaMerck test kits (Merck, Darmstadt, Germany): temperature: 25±2 °C, pH: 7.0 - 8.3, O_2 saturation > 70 %, conductivity: 522 – 828 µS/cm, total hardness: 12 – 18 °dH, carbonate hardness: 6.5 – 9.5 °dH, ammonium ≤ 1.0 mg/L, nitrate ≤ 30 mg/L, nitrite ≤ 1.0 mg/L.

Zebrafish derived from West Aquarium wild type strain were bred in the laboratory. One to two hours after fertilization, 110 fertilized eggs (F0 generation) were exposed to 200 ml tap water containing 0, 2, 4 and 8 mg/L MMS in glass beakers at 26±1°C and a photoperiod of 12h:12h, which was maintained throughout the whole experiment. Daily, half of the exposure medium was renewed. Each treatment was run in two replicates. After 96 h, embryos were transferred to 10-L flow-through aquaria equipped with a triplicate water renewal. The water flow into each tank was regulated *via* rotameters (Rota Yokogawa, Wehr, Germany), whereas the flow of stock solutions (52, 104, 208 mg/L MMS) was adjusted by means of peristaltic pumps (Minipuls 3, Gilson, Wiesbaden, Germany). MMS stock solutions were renewed every 48 h. The flow through system is shown and depicted schematically in Fig. III.1.

From 6 to 11 days post fertilization (dpf), larvae were given liquid *Artemia* (Nobil Fluid, JBL, Neuhofen, Germany) 3 times a day. In addition, from 8 to 11 dpf, powder food (NovoTom, JBL) was given three times a day. From 12 to 16 dpf, larvae were fed liquid *Artemia* once and powder food three times a day. From 17 to 19 dpf, freshly bred *Artemia* nauplii (Great Salt

Lake Artemia Cysts, Sanders, Morgan, Utah, USA) were fed once, powder food three times and flakes (Tetramin, Tetra, Melle, Germany) two times a day. From 20 to 22 dpf, nauplii were fed twice in addition to powder food and flakes once a day. Finally, from the age of 23 dpf, larvae were fed nauplii two times and flakes once a day. The amount of food given was adjusted to the number of fish in the tanks.

Mortality was recorded daily. Because of high mortalities, the exposure to 8 mg/L MMS was terminated after 50 days. All fish considered moribund were sacrificed. Considering moribund fish as dead may lead to an overestimation of mortality; however, moribund fish are not likely to reproduce and can thus be regarded a loss to the population. Since one replicate of the 2 mg/L MMS treatment showed symptoms of fin rot at 169 dpf, all fish in this tank were sacrificed in a saturated solution of benzocaine (ethyl-*p*-aminobenzoate, Sigma-Aldrich).

Since all MMS-exposed spawning groups (cf. chpt. III.3.3) failed to spawn, and fish exposed to 4 mg/L MMS also showed mortalities, MMS concentrations were reduced from 2 and 4 to 0.2 and 0.4 mg/L, respectively, at 220 dpf. In the following text, exposure of fish to 2 and 4 mg/L from zygote and to 0.2 and 0.4 mg/L from 215 dpf is abbreviated as '2/0.2 mg/L' and '4/0.4 mg/L'. At 300 dpf, all remaining individuals exposed to 0.4 mg/L MMS were sacrificed because of mortality, macroscopic pathology and consistent lack of spawn. F0 control fish as well as fish exposed to 2/0.2 mg/L MMS were sacrificed at 370 dpf.

Since the reduction of exposure concentrations to 0.2 mg/L MMS allowed successful spawning, 120 F1 larvae could be transferred to 10-L aquaria after 96 h. In order to exclusively study the effects of potentially inherited DNA damage, F1 fish were not exposed to MMS. This scenario also allowed for studying potential recovery in the following F2 generation. F1 fish were counted daily and fed as described for the F0 generation. In order to reduce mortalities of young larvae in the overflow filters, water discharge was only started at 17 dpf, when triplicate renewal was initiated.

In the F1 generation, fish derived from control and 2/0.2 mg/L MMS-exposed fish were sacrificed at 230 dpf using benzocaine prior to determination of total length, weight and sex. Before weighing using accuracy scales (R 180 D-*D1, Sartorius, Göttingen, Germany), fish were wiped dry. Total length was recorded on millimeter paper.

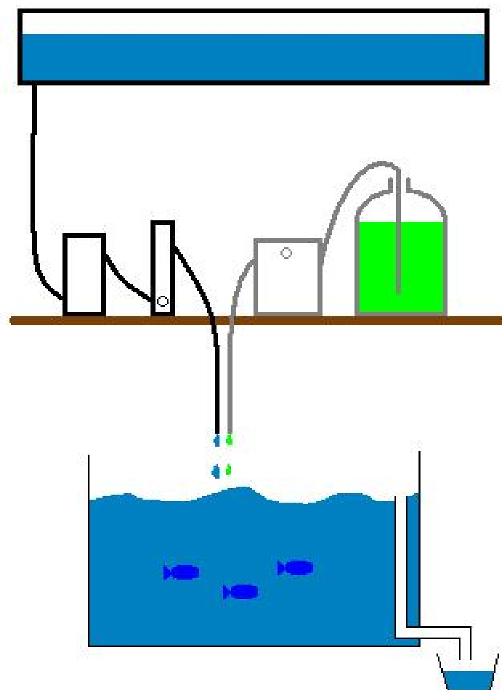


Fig. III.1: Flow through system comprehending tap water storage tanks, particle depositors and rotameters for water flow as well as MMS stock solutions (green) and pumps. In addition, tanks are aerated and heated. Cylinders are used for daily calibration of water and stock solution flow.

III.3.3 Reproduction and teratogenicity

For spawning, groups of 3 female and 3 male fish from a given tank were formed and transferred overnight to spawning aquaria filled with tap water at 26 °C and artificial spawning stimulant (Fig. III.2). Thus, first time spawning per exposure group was determined. One to two hours after spawning, eggs per spawning group were counted using a dissection microscope (Stemi 2000-C, Zeiss, Göttingen, Germany) or an Olympus CKX41 microscope (Olympus, Hamburg, Germany) and unfertilized eggs were discarded. In the next step, the fertilization rate was calculated. The development of the embryos and larvae was investigated for lethal and sublethal teratogenic effects 24, 48, 72 and 96 hours post fertilization (hpf). Based on Nagel (2002), the following effects were considered lethal: tail not detached, lack of somites, lack of heartbeat and coagulation. Sublethal effects recorded were: yolk sack edema, pericardial edema, deformation of somites, lack of blood circulation, deformation of tail and delay of development.

Spawning of the F0 generation was evaluated for a total of 26 experiments between days 154 and 341. When the F1 generation had reached an age of 3 months, F1 spawning was checked

for the first time analogously to the F0 generation. Overall, F1 spawning was analyzed in 16 experiments over a total of 142 days. First time spawning, egg number, fertility rate and embryonic development as well as teratogenicity were investigated in the F1 generation.

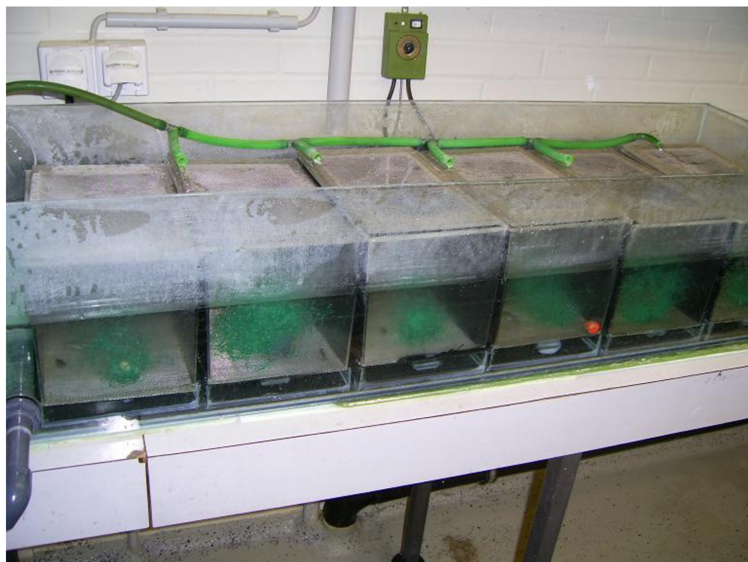


Fig. III.2: Spawning tanks with circulating water flow and artificial spawning stimulant. In order to prevent fish from feeding on their own spawn, eggs fall through a metal grid in the bottom of each container and are collected in a glass tray.

III.3.4 Statistics

Based on survival data in the F0 and F1 generation, Kaplan-Meier curves were generated according to Kaplan and Meier (1958) as well as Rich et al. (2010). Statistically significant differences between the groups were identified using log rank test and the post-hoc test according to Holm-Sidak. Kaplan-Meier curves were used in the present study because this tool allows for an exclusion of fish from statistical survival assessment that had obviously died because of reasons other than chemical exposure. In addition, Kaplan-Meier curves deal with the survival of fish at the endpoint of the experiment when the future point of death is unknown (Rich et al. 2012). The log rank test for independent data was used, since F0 fish were derived from diverse spawning groups consisting of several male and female fish. This was also true for F1 fish, because F0 spawning groups consisted of randomly assembled groups of three males and females.

Body weight data of each sex from each tank was compared using One-Way-ANOVA on ranks and the post-hoc test according to Dunn. Weight of both sexes in the F1 generation was compared using Mann-Whitney rank-sum rest. Length data were processed using the same statistical test methods. Prevalence of sexes within and between the F0 and F1 generation was compared using binomial test with approximation to normal distribution. Frequency of lethal as well as sublethal teratogenic effects was compared using Mann-Whitney rank-sum test.

Graphics were generated using SigmaPlot 11.0 (Systat, Erkrath, Germany) and Photoshop CS5 Extended 12.0 x32 (Adobe, San Jose, CA, USA).

III.4 Results

III.4.1 Mortality

Survival of the F0 fish in tanks 4 (2/0.2 mg/L) to 8 (8 mg/L) and the F1 fish descending from exposed parents was significantly reduced, if compared to controls (log-rank, Holm-Sidak, $p < 0.05$). Mortality in the F0 generation mainly occurred between the age of 96 hpf and 30 dpf, i.e. directly after transfer of the larvae to the 10-L aquaria (Fig. III.3 A). Already after 30 days of exposure, there was a clear concentration-dependent decrease of survival ranging from 50 % in the controls to 11 % in zebrafish exposed to 8 mg/L MMS. However, it should be noted that there was considerable variability between replicates; e.g. the two replicates of the controls showed significantly different mortality (log-rank test, Holm-Sidak test, $p < 0.05$). Later on, overall mortality was much lower in all tanks. However, all fish exposed to 8 mg/L MMS died until day 50, and 50 % of the fish in one tank with 4 mg/L MMS died until day 120. In one tank exposed to 2 mg/L MMS, there was a breakout of fin rot, and the exposure was terminated after 120 dpf.

In the F1 generation, until the age of two weeks post fertilization, mortality reached 28 % in the controls and 50 % in descendants from F0 fish exposed to 2/0.2 mg/L MMS in tank 3. For the rest of the experiment, mortality in F1 fish was below 31 % for controls and 53 % for fish derived from exposed parents (Fig. III.3 B). Altogether, mortality in the offspring of F0 fish exposed to 2/0.2 mg/L MMS was 20 % lower than in descendants from F0 controls. In addition, survival in tanks with fish descending from controls was significantly lower than in tanks with offspring of 2/0.2 mg/L MMS-exposed parents (log-rank test, Holm-Sidak test, $p < 0.05$).

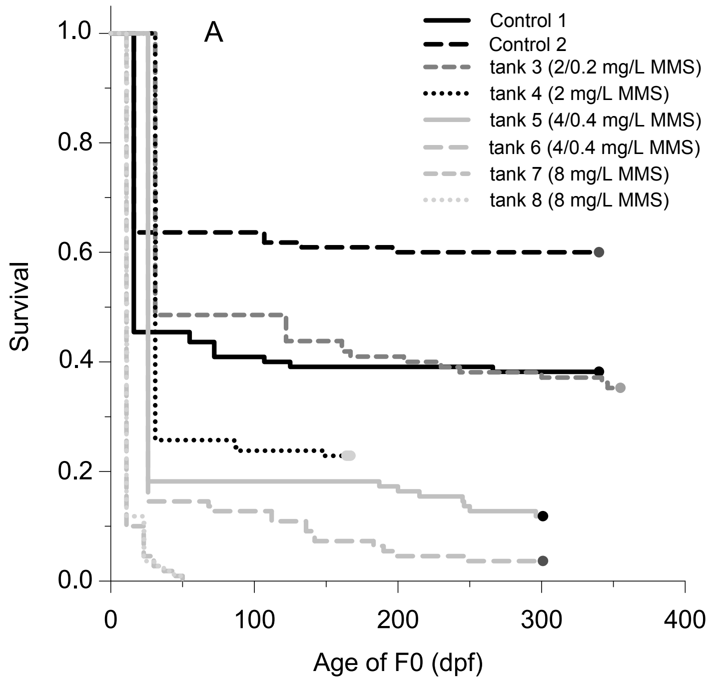
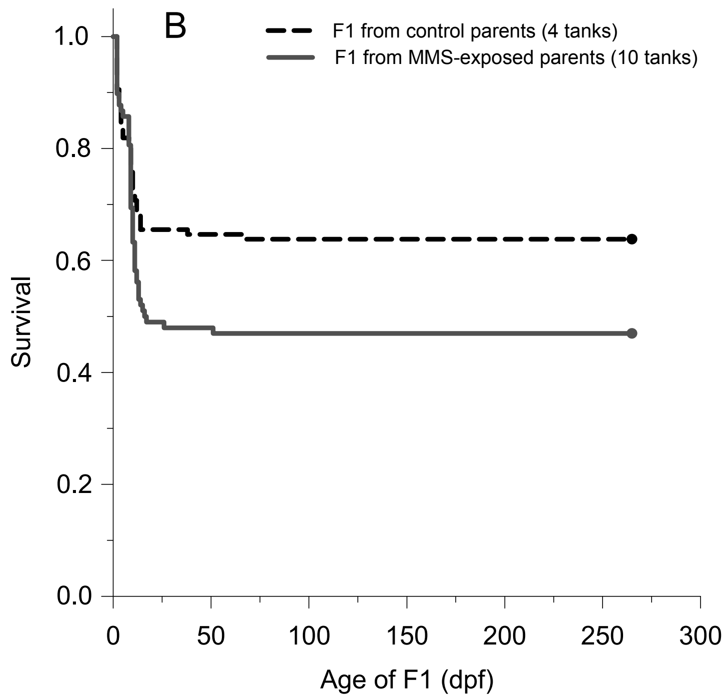


Fig. III.3: Kaplan-Meier survival curves for the F0 generation of zebrafish exposed to MMS (A) as well as the non-exposed offspring (F1; B). Due to disease and persisting mortality, fish in tanks 4 to 6 were sacrificed prematurely and censored from the curve at 169 dpf and 301 dpf, respectively. Survival data for the F1 generation are given as means from 4 control tanks and 10 tanks with offspring from fish exposed to 2/0.2 mg/L MMS (tank 3). For clarity, standard deviations are not shown (0.06 - 0.14 for controls and 0.14 – 0.29 for fish derived from exposed parents).



III.4.2 Growth

Body weight of either sex in the F0 generation did not show any significant difference between treatments (One-Way-ANOVA on Ranks, Dunn's test, $p < 0.01$; Fig. III.4 A). Weight of the F1 controls as well as the F1 fish with parental exposure was significantly reduced compared to the F0 controls (Mann-Whitney rank-sum test, $p < 0.001$). Weight of both sexes of zebrafish in the F1 generation descending from fish exposed to 2/0.2 mg/L MMS was increased compared to the F1 controls (Mann-Whitney rank-sum test, $p < 0.001$; Fig. III.4 B).
In the F0 generation, total length of fish exposed to 2/0.2 MMS was significantly reduced for females in one replicate tank (One-Way-ANOVA on ranks, Dunn's test, $p < 0.001$). Regarding the other groups, MMS exposure did not cause significant length reduction. Likewise, there were no significant differences between total length of the F1 controls and offspring of fish exposed to 2/0.2 mg/L MMS for both sexes (Mann-Whitney rank-sum test, $p < 0.05$).

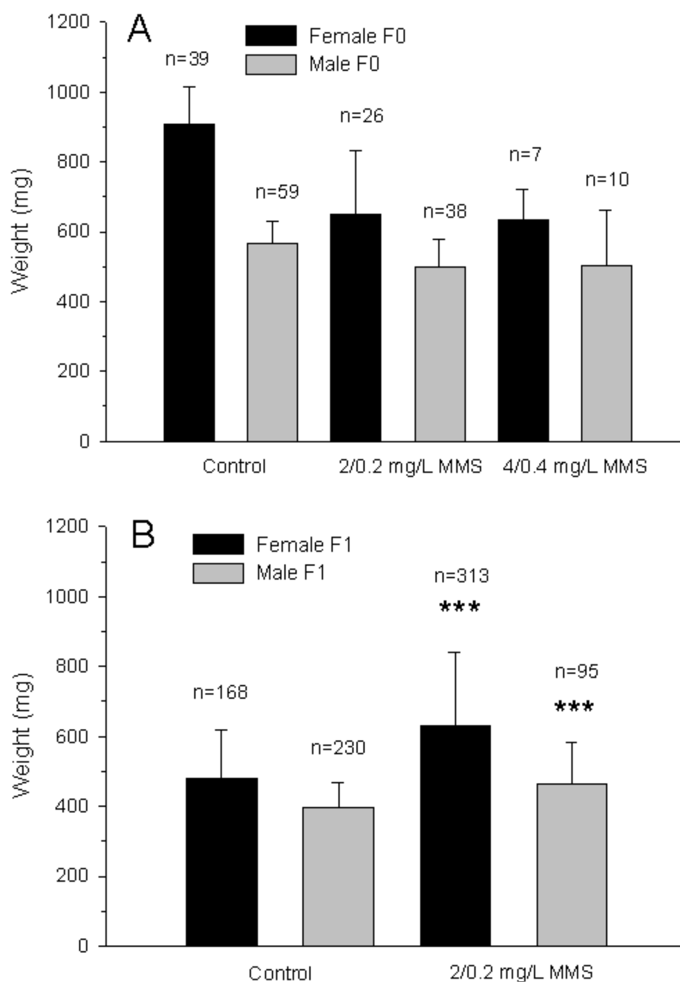


Fig. III.4: Average body weight of F0 zebrafish at 230 dpf combined from both replicate tanks of MMS treatments (2/0.2 and 4/0.4 mg/L) and control (A).
B: Average body weight of F1 fish descending from MMS exposed fish and controls at 230 dpf. Data are given as means ± SD and the number n of fish per group.
In the F0 generation, although statistically not significant (One-Way-ANOVA on ranks, Dunn's test, $p < 0.01$), there is a trend towards smaller body weight by MMS exposure.
In the F1 generation, weight of fish with parental exposure to 2/0.2 mg/L is increased compared to the controls (*** $p < 0.001$).

III.4.3 Fertility and fertilization

F0 controls spawned for the first time at 154 dpf (Fig. III.5 A). At that time, premature gonads were found in dead female MMS-exposed F0 fish. For the rest of the experiment, the F0 controls spawned in a regular and relatively homogenous pattern. Fertilization rates in F0 controls did not depend from time and almost exclusively ranged between 60 and 95 %. In F0 fish exposed to 2/0.2 mg/L MMS, the time point of the first spawning was delayed by 51 days relative to the controls. In addition, the number of eggs was clearly lower than in the controls. From 258 dpf, fish exposed to 2/0.2 mg/L MMS produced more eggs and spawned more frequently (Fig. III.5 B). However, egg production of exposed fish never reached control levels. Except for the first two sporadic spawning events of the F0 fish exposed to 2/0.2 mg/L MMS, between 47 and 100 % of their eggs were fertilized, i.e. fertilization rate was similar to F0 controls.

In the F1 controls, the first spawn took place at 116 dpf. Egg production in F1 controls was very similar to that of F0 controls. Fertilization rates were generally comparable to F0 controls. The offspring of fish exposed to 2/0.2 mg/L MMS spawned earlier than the F1 controls, i.e. at 92 dpf (Fig. III.5 C), however with a low fertilization rate of 12 %. Only at 104 dpf, fertilization rate in F1 fish derived from MMS-exposed fish reached 74 % and fluctuated between 20 and 80 % for the rest of the experiment. At 208 dpf, descendants from MMS-exposed fish produced even more eggs than corresponding controls (Fig. III.5 C).

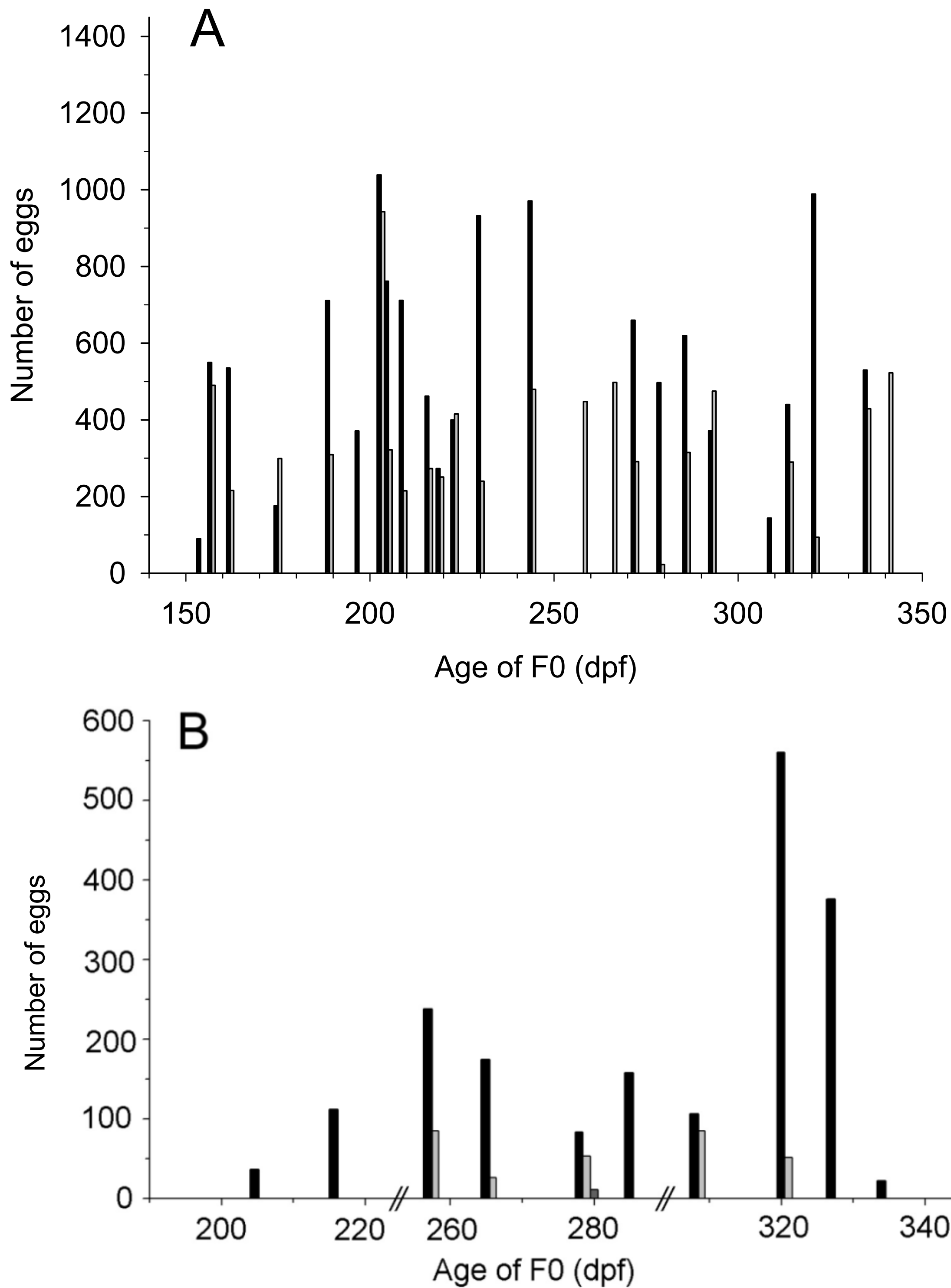
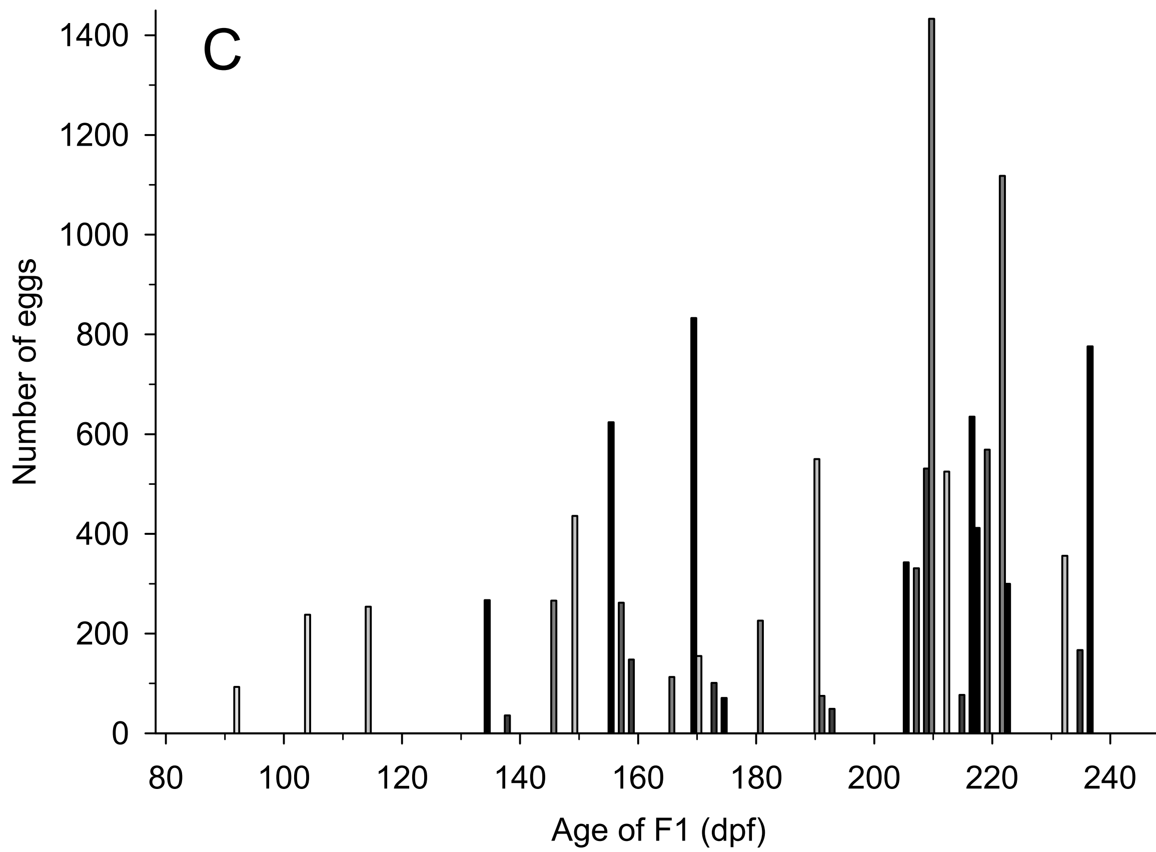


Fig. III.5 (this and the next page): Egg production of spawning groups from the F0 controls (A), the F0 fish exposed to MMS (B) as well as offspring of the latter (C). A: Egg production of the two replicate F0 controls (black and grey). B: Egg production of up to 3 spawning groups from one replicate tank with F0 zebrafish exposed to 2/0.2 mg/L MMS (up to 4 spawning groups at each spawning event; reduction of concentration at 216 dpf). C: Egg production of spawning groups from 7 replicate tanks with offspring from zebrafish exposed to 2/0.2 mg/L MMS (up to 4 spawning groups at each spawning event). Egg production refers to fertilized as well as non-fertilized eggs.



III.4.4 Sex distribution

In the F0 generation as well as in F1 controls, male fish were significantly more prevalent than females (Fig. III.6 A, B). In contrast, female fish were more prevalent in the groups descending from MMS exposed fish (binomial test with approximation to normal distribution, $p < 0.05$).

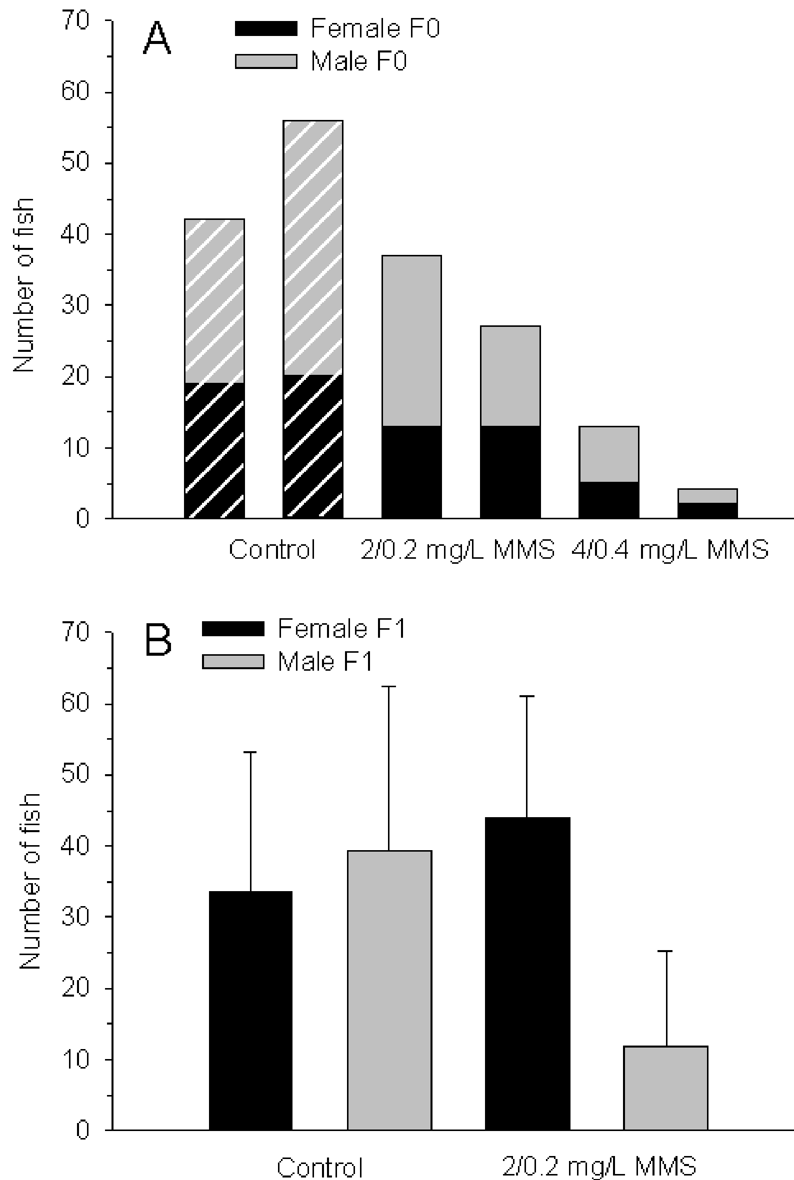


Fig. III.6: Sex distribution in the F0 generation of zebrafish exposed to 2/0.2 mg/L MMS at 360 dpf (A, two replicate tanks each, control bars are hatched) as well as their offspring after 265 dpf (B, whiskers indicate standard deviations for 5 control tanks and 8 tanks with offspring from exposed fish). For the total of all F0 groups and F1 controls, respectively, there is a significant prevalence of males over females (binomial test, $p < 0.05$). In contrast, F1 fish derived from MMS-exposed fish show a significant shift of the sex ration towards females (binomial test, $p < 0.05$).

III.4.5 Teratogenicity in embryos and early larval stages

Up to 96 hpf, there were significantly more lethal teratogenic effects in embryos and larvae descending from F0 fish exposed to 2/0.2 mg/L MMS than in the controls (Mann-Whitney rank-sum Test, $p < 0.05$). In the F0 controls, in 95 % of the spawn less than 10 % of the embryos showed lethal effects (Tab. III.1). In contrast, in 27 % of the spawn of exposed fish,

lethal effects were observed at frequencies higher than 10 %. Also the frequency of sublethal teratogenic effects was significantly elevated in exposed fish compared to the controls (Mann-Whitney rank-sum Test, $p < 0.001$). In no case, more than 6 % of the embryos in spawn from the F0 controls showed sublethal effects. In contrast, in 9 % of the cases, more than 10 % of the embryos two generations away from exposure were affected. Teratogenic effects in the F0 generation are exemplarily shown in Figure III.7.

In the F2 generation, there was no significant difference between the frequency of lethal teratogenic effects in the offspring of MMS-exposed fish and the controls (Mann-Whitney rank-sum Test, $p < 0.05$). In the controls as well as in offspring of exposed fish, in less than 10 % of the spawn more than 10 % of the embryos showed lethal effects up to 96 hpf (Tab. III.2). In contrast, there were significantly more sublethal effects in F2 embryos and larvae descending from fish that were exposed to MMS two generations before than in the F2 controls (Mann-Whitney rank-sum Test, $p < 0.05$). Identically to the generation before, in no case more than 6 % of embryos in spawn from the F1 controls showed sublethal effects. In contrast, in 6 % of cases, more than 10 % of the embryos were affected (Tab. III.2).

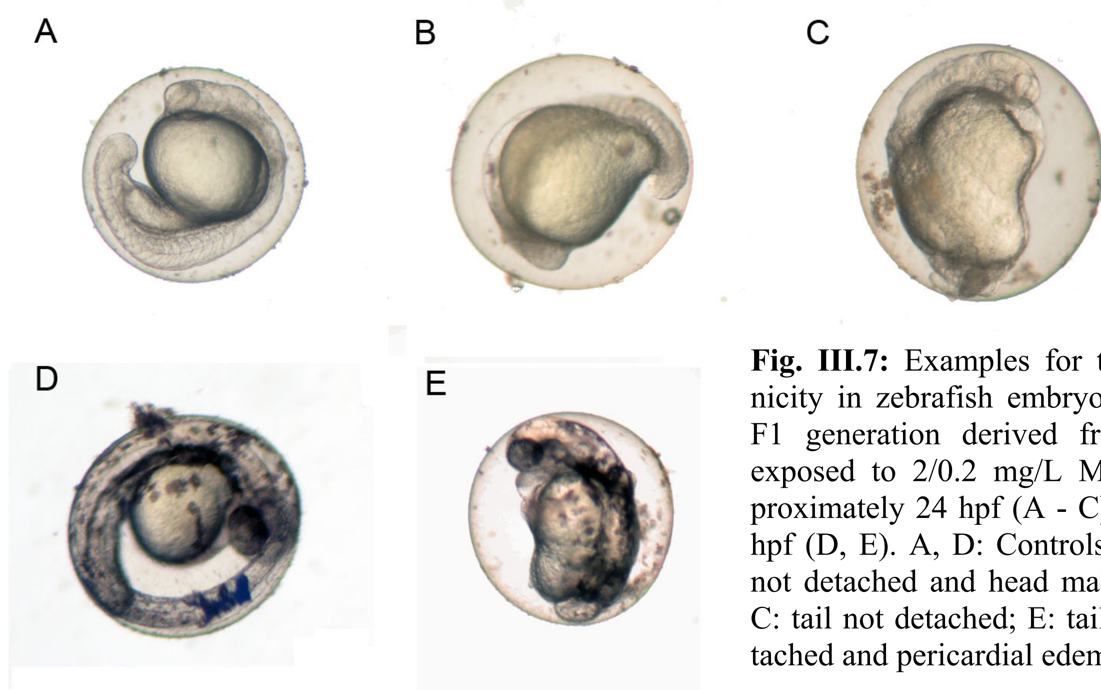


Fig. III.7: Examples for teratogenicity in zebrafish embryos of the F1 generation derived from fish exposed to 2/0.2 mg/L MMS approximately 24 hpf (A - C) and 48 hpf (D, E). A, D: Controls; B: tail not detached and head malformed; C: tail not detached; E: tail not detached and pericardial edema.

Tab. III.1: Frequencies of lethal and sublethal teratogenic effects in the spawn of the F0 generation in percent. Effects observed between 24 and 96 hpf are grouped; n.d.: normal development. Lethal effects are: tail not detached, lack of somites, lack of heartbeat and coagulation. Sublethal effects are: yolk sack edema, pericard edema, deformation of somites, lack of blood circulation, deformation of tail and delay of development.

	Spawn 1st control		Spawn 2nd control		Spawn 2/0.2 mg/L MMS	
dpf F0	lethal	sublethal	lethal	sublethal	lethal	sublethal
154	n.d.	n.d.	no spawn	no spawn	no spawn	no spawn
157	n.d.	n.d.	n.d.	n.d.	no spawn	no spawn
162	n.d.	n.d.	n.d.	n.d.	no spawn	no spawn
175	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
189	n.d.	n.d.	n.d.	n.d.	no spawn	no spawn
197	n.d.	n.d.	no spawn	no spawn	no spawn	no spawn
203	n.d.	n.d.	n.d.	n.d.	no spawn	no spawn
205	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
209	n.d.	n.d.	n.d.	n.d.	no spawn	no spawn
216	n.d.	n.d.	n.d.	n.d.	n.d.	1
219	n.d.	n.d.	n.d.	n.d.	no spawn	no spawn
223	n.d.	n.d.	n.d.	n.d.	no spawn	no spawn
230	n.d.	n.d.	n.d.	n.d.	no spawn	no spawn
244	n.d.	n.d.	n.d.	n.d.	no spawn	no spawn
258	no spawn	no spawn	1	<1	2	2
266	no spawn	no spawn	8	n.d.	5	1
272	1	<1	4	n.d.	no spawn	no spawn
279	12	n.d.	9	n.d.	23	n.d.
286	5	n.d.	8	n.d.	3	1
293	3	n.d.	n.d.	n.d.	no spawn	no spawn
309	n.d.	1	no spawn	no spawn	2	1
314	1	<1	<1	n.d.	no spawn	no spawn
321	9	6	3	1	5	1
328	no spawn	no spawn	no spawn	no spawn	11	<1
335	6	<1	2	<1	18	27
341	no spawn	no spawn	17	1	no spawn	no spawn

Tab. III.2 (this and the next page): Frequencies of lethal and sublethal teratogenic effects in the spawn of the F1 generation in percent. Spawn groups are taken from 4 control tanks and 7 tanks containing fish with parental exposure; tank numbers are given in the headings. Effects observed between 24 and 96 hpf are grouped; n.d.: normal development; bar: not tested. Lethal effects are: tail not detached, lack of somites, lack of heartbeat and coagulation. Sublethal effects are: yolk sack edema, pericard edema, deformation of somites, lack of blood circulation, deformation of tail and delay of development.

	Spawn control 4		Spawn control 8		Spawn control 9		Spawn control 13	
dpf F1	lethal	sublethal	lethal	sublethal	lethal	sublethal	lethal	sublethal
92	no spawn	no spawn	-	-	no spawn	no spawn	-	-
104	no spawn	no spawn	no spawn	no spawn	no spawn	no spawn	-	-
116	1	0	2	n.d.	1	<1	-	-
124	11	4	<1	<1	8	6	-	-
137	-	-	-	-	no spawn	no spawn	no spawn	no spawn
144	-	-	3	1	-	-	no spawn	no spawn
151	2	1	-	-	-	-	no spawn	no spawn
158	-	-	-	-	2	<1	no spawn	no spawn
164	-	-	1	2	-	-	7	3
172	-	-	-	-	-	-	no spawn	no spawn
179	9	1	-	-	<1	<1	no spawn	no spawn
192	-	-	1	n.d.	-	-	no spawn	no spawn
208	-	-	-	-	7	<1	2	1
214	5	0	-	-	-	-	no spawn	no spawn
220	-	-	2	1	-	-	n.d.	6
234	-	-	-	-	1	<1	no spawn	no spawn

dpf F1	Spawn 2/0.2 mg/L MMS 7 lethal	Spawn 2/0.2 mg/L MMS 7 sublethal	Spawn 2/0.2 mg/L MMS 10 lethal	Spawn 2/0.2 mg/L MMS 10 sublethal	Spawn 2/0.2 mg/L MMS 11 lethal	Spawn 2/0.2 mg/L MMS 11 sublethal	Spawn 2/0.2 mg/L MMS 12 lethal	Spawn 2/0.2 mg/L MMS 12 sublethal
92	-	-	no spawn	no spawn	no spawn	no spawn	5	n.d.
104	-	-	no spawn	no spawn	no spawn	no spawn	3	2
116	no spawn	no spawn	<1	2	no spawn	no spawn	-	-
124	no spawn	no spawn	no spawn	no spawn	no spawn	no spawn	-	-
137	0	n.d.	no spawn	no spawn	no spawn	no spawn	-	-
144	-	-	-	-	no spawn	no spawn	-	-
151	-	-	3	3	no spawn	no spawn	-	-
158	14	3	-	-	1	1	-	-
164	-	-	-	-	no spawn	no spawn	-	-
172	8	3	3	7	no spawn	no spawn	-	-
179	-	-	-	-	no spawn	no spawn	-	-
192	-	-	<1	1	n.d.	4	-	-
208	6	1	-	-	4	1	-	-
214	-	-	10	1	no spawn	no spawn	-	-
220	4	1	-	-	1	2	-	-
234	-	-	6	3	no spawn	no spawn	-	-

dpf F1	Spawn 2/0.2 mg/L MMS 14 lethal	Spawn 2/0.2 mg/L MMS 14 sublethal	Spawn 2/0.2 mg/L MMS 15 lethal	Spawn 2/0.2 mg/L MMS 15 sublethal	Spawn 2/0.2 mg/L MMS 16 lethal	Spawn 2/0.2 mg/L MMS 16 sublethal
92	-	-	-	-	-	-
104	-	-	-	-	-	-
116	-	-	-	-	-	-
124	-	-	-	-	-	-
137	n.d.	3	-	-	-	-
144	no spawn	no spawn	2	3	no spawn	no spawn
151	no spawn	no spawn	-	-	no spawn	no spawn
158	n.d.	9	-	-	no spawn	no spawn
164	no spawn	no spawn	2	2	no spawn	no spawn
172	8	13	-	-	1	3
179	-	-	15	n.d.	no spawn	no spawn
192	16	27	-	-	no spawn	no spawn
208	1	1	6	1	-	-
214	n.d.	3	-	-	<1	2
220	-	-	5	2	1	2
234	1	3	-	-	1	1

III.5 Discussion

MMS methylates DNA by nucleophilic substitution and leads to base mismatch and replication blockade (Beranek 1990, Lundin et al. 2005). Hydrolysis of methylated bases can produce AP-sites, which can give rise to single and double strand breaks (Lundin et al. 2005, Rydberg 2000). Chronic exposure using low concentrations may result in carcinogenesis, whereas acute exposure to high concentrations may lead to lethal mutations. In F0 zebrafish, MMS caused mortality in a concentration-dependent manner. In later stages of the experiment, however, in the exposure groups concentration-dependent mortality was further observed, whereas control fish numbers remained almost constant. Additional zebrafish embryo tests had shown that exposure to 16 mg/L MMS over 144 hpf did not induce any lethal effects (details not shown). Embryos of medaka *(Oryzias latipes)* were exposed to even higher concentrations (up to 82.6 mg/L MMS; Solomon and Faustman1987), and a concentration-dependent decrease of viability and development of malformations were found. At the concentrations used in the present study, however, teratogenic effects were not to be expected in the F0 generation; even more so, since in another study with adult zebrafish, 8 mg/L MMS did not cause any mortality after exposure for two weeks, whereas mortality was high at 16 mg/L (Faßbender and Braunbeck, under review). Thus, prolonged exposure to MMS starting immediately after fertilization apparently induces higher lethality than does acute exposure in later stages of embryonic development or adults. This might be caused by time-dependent accumulation of minor DNA damages during chronic exposure and further corroborates the conclusion that acute toxicity tests cannot provide an adequate substitute for chronic toxicity assessment (Nagel and Isberner 1998).

Since, however, there was also considerable lethality in F0 controls, mortality was not only due to MMS exposure. When zebrafish were exposed from fertilization to 4-nitroquinoline oxide (NQO) in a full life-cycle test, survival in the controls was higher than in the present study with at least 90 % after 42 days (Diekmann et al. 2004a). Only at the highest NQO concentration, survival was reduced to 12 %. Larvae were kept in 1-L vessels with 5-fold daily water renewal and were transferred to 5-L aquaria with double daily water renewal at 14 dpf. In the present study, mortality increased, when the larvae had been transferred to 10-L aquaria with triplicate daily water renewal after 96 hpf. Confrontation with continuous flow-through in combination with potential mechanical damage due to contact with the water outflow gauzes and difficulties in regular, uniform feeding might account for the increased mortality in the

experiment. Thus, quantification of the contribution of MMS to overall mortality is difficult. Therefore, larvae were not censored for the creation of Kaplan-Meier curves, in contrast to fish whose death was, e.g., due to fin rot. Nevertheless, MMS-induced mortality was clearly concentration-dependent in zebrafish. Since survival after exposure to 2/0.2 mg/L MMS was significantly different from controls in only one replicate, it remains unclear whether this concentration had an impact on survival or not. In contrast, higher concentrations of 4/0.4 and 8 mg/L MMS had clear detrimental effects on survival.
As a consequence of mortalities in F0 fish, water discharge in the F1 generation was only started at 17 dpf. Hence, larval mortality in F1 controls was considerably lower than in F0 controls. In the offspring of MMS-exposed F0 fish, larval survival was reduced by 20 % relative to the controls. This might be explained by the production of dominant lethal mutations in germ cells of F0 fish. In this case, individuals developing from these gametes still bore these mutations after homologous recombination. In addition, residual MMS concentrations might have been present in the yolk of F1 embryos. Increased mortality in the offspring of fish exposed to genotoxicity was found in mosquito fish exposed to radionuclides (Theodorakis et al. 1997), in fathead minnows exposed to BaP (White et al. 1999) and in zebrafish exposed to NQO (Diekmann et al. 2004a). Moreover, male *Tilapia mossambica* exposed to MMS during spermatogenesis (Hemsworth and Wardhaugh 1978) produced increased rates of lethal embryopathies. This is in line with the results of the present communication.

In body weight, no significant differences were found between F0 controls and fish exposed to MMS, although fish densities in the F0 tanks differed due to concentration-dependent mortality by MMS. Interestingly, body weight of F1 fish including the controls was lower than in F0 control fish. Potentially, this was due to higher fish densities in F1 tanks because of higher larval survival than in the F0 generation. In contrast, fish densities in F1 controls and F1 groups derived from exposed fish were similar. Thus, neither in the F0 nor in the F1 generation an effect of MMS on body weight was found. Regarding total length, a significant reduction in the F0 generation was only found in the females of one group exposed to 2/0.2 mg/L MMS that was affected by fin rot. Thus, it is likely that this effect is equivocal. In the literature, exposure of a generation of zebrafish to NQO led to length reduction at the highest test concentration whereat also high mortality was observed (Diekmann et al. 2004a). In fact, these fish did not reach sexual maturity, which resembles the situation of the fish exposed to 4/0.4 mg/L in the present study. In contrast, length and weight of the offspring of the zebrafish exposed to NQO did not differ significantly from the controls. In another study using brown trout, paternal exposure to MMS did not affect length and weight of the progeny at the

age of one year (Devaux et al. 2011). Hence, growth data in the present study is considered to be in line with literature data.

MMS exposure also caused sublethal stress leading to a complete failure of reproduction in fish exposed to 2 and 4/0.4 mg/L. The finding of fish that had died untimely during exposure and had premature gonads at the age of more than 3 months suggests that MMS may have caused a delay of sexual maturation. Usually, zebrafish are expected to reach maturity by 90 days (Heeb and Escher 2007). However, spawning was not checked before 154 dpf since earlier sex determination of MMS-exposed fish was not deemed safe. Control zebrafish spawned for the first time at 154 dpf; MMS-exposed zebrafish showed a delay of first spawning by 51 days. Spawning was obviously facilitated by the reduction of MMS concentrations at 215 dpf. Since only individual (moribund) fish could be examined for gonadal maturity, a spawning blockade of already mature fish might be an additional reason for spawning failure. Jobling et al. (2002) described a delay of spermatogenesis and high incidence of oocyte atresia in wild roach (*Rutilus rutilus*) from rivers polluted with treated sewage effluents in the context of endocrine disruption. The same conclusion has been drawn for delays in metamorphosis and sexual maturity of fish due to estrogenic pollutants in sewage treatment plant effluents (Keiter et al. 2006, van den Belt 2002, 2003). However, endocrine-disrupting effects by MMS have not been described so far. Therefore, the effects on sexual maturation in zebrafish in the present study are supposed to be a consequence of general chronic stress at repairing DNA and other macromolecules that were alkylated by MMS. It is also likely that general stress symptoms impaired the immune system of the fish so that a replicate exposed to 2 mg/L was affected by fin or tail rot. The outbreak of fin rot, which is caused by the myxobacterium *Cytophaga* spp., has been documented, e.g., in fish exposed to dioxins or retene (Billiard et al. 1999).

As a consequence of the reduction of MMS concentrations at day 215, zebrafish exposed to 2/0.2 mg/L eventually started reproduction in at least one replicate. Thus, the failure to spawn proved reversible, when the MMS-associated alkylating stress was reduced. However, reproduction in fish exposed to 2/0.2 mg/L MMS was not only delayed, but egg numbers as well as fertilization frequency were also reduced in comparison to the controls. Thus, although zebrafish is a pronounced r-strategist, there is a potential for extinction of populations with reduced fertility (Diekmann et al. 2004a, Schäfers and Nagel 1991). Over the subsequent months, egg production and fertilization frequency only gradually increased and reached levels comparable to controls at day 321. The fact that impairment of fertilization was only transient suggests

that female fish reached sexual maturity before males or overcame their spawning blockade earlier.
It has been documented in humans as well as in fish that significant DNA damage in sperm does not prevent from successful fertilization (Devaux et al. 2011, Donnelly et al. 2000). Thus, since in the current study both sexes were exposed to genotoxicity and fertilization frequency was reduced in exposed fish, the inability of oocytes to fertilization may also play a role in this context.

In the F1 generation, fish descending from MMS-exposed parents spawned 24 days earlier than F1 controls, even though the number of eggs and fertilization rate were low. Thus, in contrast to the parental generation there was no spawning delay in fish derived from MMS-exposed fish. Over the following weeks, the offspring of exposed F0 fish reached egg numbers and fertilization rates comparable to controls. Hence, as early as one generation after exposure to MMS, considerable regeneration of the reproductive parameters could be recorded. In the literature, the fertility of fathead minnows derived from parents exposed to BaP (White et al. 1999) or zebrafish descending from parents exposed to NQO (Diekmann et al. 2004a) was reduced. However, in these experiments, spawning was not delayed. Thus, both in the laboratory and in the environment, further studies are required to clarify if a delay of spawning can also be caused by other genotoxicants.

In the entire F0 generation and the F1 controls, the sex ratio was skewed towards males. In contrast, significantly more females were present in the offspring of MMS-exposed fish. Since the F1 animals derived from MMS-exposed fish were kept under identical conditions (temperature, nutrition, oxygen saturation and pH), external parameters can be excluded as confounding factors for sexual development (Baroiller et al. 2009). Although there are hints that outcrossing of zebrafish might provoke a high prevalence of female fish (Lawrence et al. 2008), such an effect can be ruled out, since F1 controls were bred under identical conditions and did not show a bias towards females. Feminization has frequently been reported as a consequence of exposure to steroid hormones, alkylphenols, bisphenol A, phthalates, pesticides and herbicides (Gross-Sorokin et al. 2006, Sonnenschein and Soto 1998, Vos et al. 2000), but such properties have never been assumed for MMS. Potentially, a sex-dependent increase of mortality in male embryos and larvae due to mutations or inbreeding effects may have played a role. Nonetheless, the feminizing effect observed in the F1 generation thus warrants further investigation.

In the offspring of MMS-exposed fish, frequencies of sublethal teratogenic effects until 96 hpf were increased. It was stated above that the onset of major mortality of F1 animals was later than 96 hpf and that the frequencies of lethal effects in F1 embryos were not elevated. These facts give rise to the suggestion that mainly lethal and not phenotypically visible DNA damage may have contributed to the elevated larval mortality after 96 hpf. Otherwise, more teratogenic effects would have been found up to 96 hpf. In the next generation, F2 larvae two generations away from exposure also showed higher rates of sublethal teratogenic effects than the respective controls, but no elevated frequency of lethal teratogenic effects, i.e. larval mortality. Parts of the mutations and other DNA damage formed in the gametes of F0 fish may have been passed on to the F2 generation *via* the F1 generation. Furthermore, higher fertility and fertilization frequencies were found in F1 fish than in the F0 generation. Overall, this indicates partial recovery of development and reproductive capacities between the F1 and the F2 generations due to repair of DNA damage and mutations as well as compensation by less affected F1 individuals bearing no or less serious DNA damage. In brown trout and Arctic charr, Devaux et al. (2011) observed a large array of morphological abnormalities in development of embryos and larvae after parental exposure to MMS. These effects comprehended jaw and spine deformation, yolk egg edemata and Siamese larvae. A positive correlation between the DNA damage level in sperm from parental fish and the incidence of skeletal abnormalities in the offspring led to the conclusion that DNA damage had been inherited. This confirms the teratogenic effects found in the F1 generation in the present study.

In chapter IV, it is shown that chronic exposure of zebrafish to MMS exerted genotoxicity on somatic and generative tissues and caused histopathological alterations. The present chapter demonstrated that fish were impaired in their survival, growth and reproductive capacities. Taken together, there is further evidence for a causal relationship between genotoxicity and impairment of fish populations. Regarding the next generation, due to the transfer of mutations and inherited DNA damage, the offspring was subject to elevated teratogenicity and mortality. Since the F1 generation was raised without exposure to MMS, their reproductive capacities recovered and development of the following generation was less impaired. On the one hand, these data confirm existing conclusions that genotoxic substances may well impair fish populations over several generations (Anderson and Wild 1994, Depledge 1996, Diekmann et al. 2004a, Munns et al. 1997, White et al. 1999). On the other hand, the results of the present study indicate the potential for recovery upon cessation of the contamination.

IV

Genotoxicity and histological aberrations in zebrafish after chronic exposure to an alkylating agent in a multi-generation study

In chapter II, effects of chronic exposure of a parental generation of zebrafish to MMS on survival, growth, sex ratio, fertility, reproduction and development in a multi-generation experiment were analyzed. Chapter III focuses on genotoxic effects in the exposed parental generation and histological aberrations as well as external lesions in the F0 and the F1 generation. Taken together, a multitude of ecotoxicological endpoints regarded in the present thesis serves as a model for health and reproductive status of wild freshwater fish exposed to genotoxic pollution.

IV.1 Abstract

There is a significant lack of understanding the mechanisms linking adverse effects of genotoxic anthropogenic pollutants to the decline of fish populations in aquatic ecosystems. Anthropogenic chemical pollution is probably a key aspect of the problem. For a better understanding, investigations into the relationship between genotoxicity, especially in the gonads, and histopathology are required in fish over several generations. For this end, zebrafish (F0 generation) were exposed *in vivo* to the alkylating model genotoxin methyl methanesulfonate (MMS) from fertilization to the age of one year in a continuous flow-through system. Primary cells from the F0 gonads, liver and gills were analyzed in the alkaline comet assay, and micronucleus frequency was measured in histological sections of the same organs. In the F0 and F1 generations, histopathological lesions and external aberrations including symptoms of neoplasia were recorded. Significant genotoxic effects were found in the livers, gills and gonads of either sex the F0 generation. As predominant histopathological features, malformations of eyes, gills and liver, especially spongiosis and steatosis hepatis, and a number of neoplasia were observed. External lesions were found in both the F0 and the F1 generation. Taken together, chronic exposure of zebrafish to MMS leads to DNA fragmentation and chromosomal damage in somatic and generative tissues and induces the formation of a multitude of histopathological aberrations, concertedly impairing health, survival and reproduction of exposed fish at the population level.

IV.2 Introduction

A considerable deterioration of fish health and a decline of fish populations have been observed in European and Northern American freshwater over the last decades (Burkhardt-Holm et al. 2002, Cook et al. 2003, de Lafontaine et al. 2002, Faller et al. 2003, Friedl 1999, Keiter et al. 2006), and anthropogenic pollution of the aquatic environment has been suggested to play a major role (Burkhardt-Holm et al. 2002, Fischnetz 2004). Numerous pollutants are known for their genotoxic, mutagenic and carcinogenic potentials (Black et al. 1992, Botalova and Schwarzbauer 2011, Gravato and Santos 2002, Helma et al. 1994). For example, Bony et al. (2010) found that vineyard pesticides (diuron and azoxystrobin) that are easily released to rivers caused genotoxic effects in zebrafish. This pollution is of special importance, since the ecological consequences of mutagenicity in the aquatic environment should not be underestimated (Chen and White 2004). Furthermore, *via* affection of the reproductive capacities of aquatic animals, multi-generational effects of pollution on the population level may be assumed (White et al. 1999, Würgler and Kramers 1992). Since such trans-generational processes may provide a link between pollution and population effects, the major focus of the present study has been put on genotoxicity in gonad cells and gametes. The latter may be particularly prone to genotoxicity because of their low DNA repair capacity and the fact that the gametes of aquatic organisms are usually released into the ambient water. Thus, they are directly exposed to pollutants (Anderson and Wild 1994, Jha 2008).

There are only few studies dealing with the effects of genotoxicity on fish populations. Detrimental effects of exposure to polychlorinated biphenyls (PCBs) and dioxins on reproduction and population growth of mummichog *(Fundulus heteroclitus)* were investigated by Munns et al. (1997). Likewise, effects on DNA damage and reproduction impairment in multiple generations of zebrafish *(Danio rerio)* and fathead minnow *(Pimephales promelas)* after exposure to 4-nitroquinoline oxide and benzo[a]pyrene (BaP) have been documented by Diekmann et al. (2004 a, b) and White et al. (1999), respectively. In addition, exposure of male brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpines*) to methyl methanesulfonate (MMS) has recently been shown to cause malformations during embryonic and larval development in their offspring (Devaux et al. 2011). Taken together, these studies provide evidence for the hypothesis that the development of fish populations is impaired by genotoxicants.

However, only very limited information is available on genotoxic effects on DNA integrity in reproductive tissues of fish. Genotoxic effects of vineyard pesticides and MMS were shown in fish spermatozoa using the comet assay (Bony et al. 2010, Devaux et al. 2011), and Faßbender

and Braunbeck (under review) demonstrated that genotoxicity can be assessed in primary cells from the gonads of both sexes of zebrafish after *in vivo* exposure to MMS. Based on the latter study, the purpose of the present study is to relate DNA fragmentation and chromosomal damage in somatic and generative cells in zebrafish after live-long genotoxic exposure to macro- and microscopically detectable changes.
Since genotoxicity, mutagenicity and carcinogenesis are closely interrelated, another aim of this study is the investigation of histopathological alterations, i.e. symptoms of neoplasia, (pre-)carcinogenesis and other structural aberrations in consequence of metabolic disturbances due to chronic genotoxic stress. In addition, especially with reference to DNA changes in reproductive organs, the consequences and inheritability of such structural aberrations to the next generation (even when bred without genotoxic exposure) is an important question.

Many environmental contaminants target the immune system, which results in an increasing number of infectious diseases and parasitoses (Brooks et al. 2012, Rice et al. 2001). Therefore, a difference has to be made between non-specific tumors related to infections, inflammatory processes, trauma and hyperplasia on the one hand and tumors that are directly due to neoplastic growth beyond the control of the host organism on the other hand (Mix 1986). Concerning the latter group of tumors, it is well known that environmental pollution can lead to genome mutations, which may cause neoplasia and carcinogenesis in fish (Mix 1986, Russell et al. 1957). E.g., buccal granulomatosis and external tumor-like disease were described in Elbe smelt (*Osmerus eperlanus)* and Northeast Atlantic fish, respectively (Möller 1988). Hepatic neoplasia and hepatocellular carcinoma in English sole *(Parophrys vetulus)* from Puget Sound could be related to high concentrations of PCBs and chlorinated butadienes (CBDs) (Malins et al. 1988). Likewise, unique degenerative conditions, storage disorders, foci of hepatocellular alteration (i.e. putative preneoplastic lesions), hepatocellular and biliary neoplasms as well as non-neoplastic proliferative conditions were found in the liver in English sole from Puget Sound (Myers et al. 1987). Hepatocellular carcinoma and fatty infiltration were described in Atlantic tomcod *(Microgadus tomcod)* from the Hudson and Pawcatuck Rivers (Cormier 1986, Cormier et al. 1989). Over the last years, different fish species, e.g. sheepshead minnow *(Cyprinodon variegatus)* and the limnic Japanese medaka (*Oryzias latipes*; Bunton et al. 1996), have been considered as model organisms for cancer research.

In addition, zebrafish has become a very successful model organism for the investigation of carcinogenesis in aquatic ecosystems (Amatruda et al. 2002, Feitsma and Cuppen 2008). Zebrafish develop almost every tumor type known from humans with remarkable morphologic

resemblance (Goessling et al. 2007, Liu and Leach 2011). A comparison between human and zebrafish genome sequences shows a conservation of tumor suppressors and oncogenes as well as cell-cycle genes (Amatruda et al. 2002). Thus, zebrafish developed papilloma and skin tumors after exposure to ethylnitrosourea, a direct-acting alkylating agent (Beckwith et al. 2000), and exposure of zebrafish to *N*-methyl-*N*'nitro-*N*-nitrosoguanidine led to both epitheli-al, e.g. hepatocellular adenoma or carcinoma in the liver, and mesenchymal neoplasia or tu-mors, e.g. osteoma or haemangioma in the gills (Spitsbergen et al. 2000a). Spitsbergen et al. (2000a, 2000b) demonstrated that zebrafish showed effects following exposure *via* different exposure routes including microinjection in embryos, dietary application or bathing. In sum, zebrafish has been documented to be a suitable model organism for carcinogenesis in ecotox-icological as well as medical research, which makes it easy to correlate neoplasia found in zebrafish to such known in humans.

In the current study, MMS was selected as the test substance because of its character as a model genotoxin (Bony et al. 2010, Deventer 1996, Solomon and Faustman 1987). Although MMS itself is not relevant in the aquatic environment, given its alkylating mode of action, it can be considered as a representative of several groups of environmental pollutants as muco-halic acids (Gómez-Bombarelli et al. 2011a) and allylic reagents (Kuehl et al. 1994).

IV.3 Materials and methods

IV.3.1 Test compound

Methyl methanesulfonate (MMS, CAS Nr. 66-27-3, 99 %) was supplied by Sigma-Aldrich (Deisenhofen, Germany). It is well water-soluble, up to a ratio of 1:5 at 25 °C (Budavari 1996). Despite a certain potential for hydrolysis, MMS concentrations may be deemed con-stant, provided that the exposure medium is exchanged once daily (Bony et al. 2010). In fact, in preliminary tests, the half-life of MMS in water (2, 4 and 8 mg/L) at 25 °C was determined by ion chromatography to be about 40 h (own data). *Via* constant flow-through, a total water renewal within 48 h was maintained, which was expected to produce a regular peak exposure with limited degradation.

IV.3.2 Test conditions

Water parameters were measured on a weekly basis by means of a Multi 350i electrode (WTW, Weilheim, Germany), and or AquaMerck test kits (Merck, Darmstadt, Germany): temperature: 25 ± 2 °C, pH: 7.0 - 8.3, O_2 saturation > 70 %, conductivity: 522 – 828 µS/cm, total hardness: 12 – 18 °dH, carbonate hardness: 6.5 – 9.5 °dH, ammonium ≤ 1.0 mg/L, nitrate ≤ 30 mg/L, nitrite ≤ 1.0 mg/L.

Zebrafish (*Danio rerio*) derived from the West Aquarium wild type strain were bred in the laboratory. One to two hours after fertilization, 110 fertilized eggs (F0 generation) were exposed to 200 ml tap water containing 0, 2, 4 and 8 mg/L MMS in glass beakers at 26 ± 1°C and a photoperiod of 12 h: 12 h, which was maintained throughout the whole experiment. Daily, 50 % of the exposure medium were renewed. Each treatment was run in two replicates. After 96 h, embryos were transferred to 10-L flow-through aquaria with a triplicate water renewal. The water flow into each tank was regulated *via* rotameters (Rota Yokogawa, Wehr, Germany), whereas the flow of stock solutions (52, 104, 208 mg/L MMS) was adjusted by means of peristaltic pumps (Minipuls 3, Gilson, Wiesbaden, Germany). MMS stock solutions were renewed every 48 h.

From 6 to 11 days post fertilization (dpf), larvae were given liquid *Artemia* (Nobil Fluid, JBL, Neuhofen, Germany) *ad libitum* 3 times a day. In addition, from 8 to 11 dpf, powder food (NovoTom, JBL) was given three times a day. From 12 to 16 dpf, larvae were fed liquid *Artemia* once and powder food three times a day. From 17 to 19 dpf, freshly bred *Artemia* nauplii (Great Salt Lake Artemia Cysts, Sanders, Morgan, Utah, USA) were fed once, powder food three times and flakes (Tetramin, Tetra, Melle, Germany) two times a day. From 20 to 22 dpf, nauplii were fed twice in addition to powder food and flakes once a day. Finally, from the age of 23 dpf, larvae were fed nauplii two times and flakes once a day.

Mortality was recorded daily. Because of high mortalities, the exposure to 8 mg/L MMS was discontinued after 50 days. All fish considered moribund were sacrificed. Considering moribund fish as dead may lead to some overestimation of mortality; however, moribund fish would definitely not be able to reproduce and can thus be regarded a loss to the population. Since one replicate of the 2 mg/L MMS treatment showed symptoms of fin rot at 169 dpf, all fish in this tank were sacrificed in a saturated solution of benzocaine (ethyl-*p*-aminobenzoate, Sigma-Aldrich).

Since all MMS-exposed spawning groups failed to spawn, and fish exposed to 4 mg/L MMS also showed mortalities, MMS concentrations were reduced from 2 and 4 to 0.2 and 0.4 mg/L,

respectively, at 220 dpf. At 300 dpf, all remaining individuals exposed to 4/0.4 mg/L MMS were sacrificed because of mortality, macroscopic pathology and consistent lack of spawn. F0 control fish as well as fish exposed to 2/0.2 mg/L MMS were sacrificed at 370 dpf.
Since the reduction of exposure concentrations to 0.2 mg/L MMS allowed successful spawning, 120 F1 larvae could be transferred to 10-L aquaria after 96 h. In order to exclusively study the effects of potentially inherited DNA damage, F1 fish were not exposed to MMS. This scenario also allowed studying potential recovery in the following F2 generation. F1 fish were counted daily and fed as described for the F0 generation. In order to reduce mortalities of young larvae in the overflow filters, water discharge was only started at 17 dpf, when triplicate renewal was initiated.

Chemical analysis of MMS concentrations in tank water was performed using ion chromatography. MMS is hydrolyzed in water to methanesulfonic acid (MSA). The latter can be detected *via* high performance liquid chromatography (HPLC), especially ion chromatography. Thus, information on MMS concentrations can be derived from MSA concentrations. Thereby, the negatively charged compound was transported by means of an eluent through a cationic column and was detected by its conductivity. An AS9-HC carbonate eluent anion-exchange column (4 x 250 mm; Dionex, Sunnyvale, CA, USA) with alkyl/alkanol quaternary ammonium ions was used in a DX-120 ion chromatograph (Dionex) equipped with a pump, a 50 µl sample loop and a self-regenerating suppressor (ASRS-ULTRA II, 4 mm, 25 mA, Dionex) for enhancement of analyte conductivity. Instruments were controlled with an EN308TC ethernet hub (Netgear, Santa Clara, CA, USA). In order to avoid particulate and chemical contamination of the analytical column, a guard column model AG9-HC (4 x 50 mm, Dionex) was connected upstream of the analytical column. Samples were conveyed to the injection valve using an autosampler AS 90 (PerkinElmer, Waltham, MA, USA). As eluent, an 11 mM Na_2CO_3 (99.5 %, Grüssing, Filsum, Germany) solution is pumped isocratically with a flow rate of 1 ml/min through the system. For detection, a DS4-1 conductivity cell (Dionex) was used at 35 °C, and data were analyzed with Chromeleon 6.8 (Dionex). In addition to the measurement of MSA in tank water, MSA standards of 5, 10 and 20 mM were recorded. Hereby, the areas under the conductivity peaks of MSA in the standards were assigned to the known concentrations. Based on these data, MSA concentrations in the samples from the tanks were calculated from the respective areas under the MSA peaks. Since one molecule of MMS is hydrolyzed to one molecule of MSA and the half-life of MMS in water is about 40 h at 25 °C (own data), MMS concentrations at the time point of sampling can be estimated after 3 to 4 days. Figure IV.1 shows the ion chromatography station used.

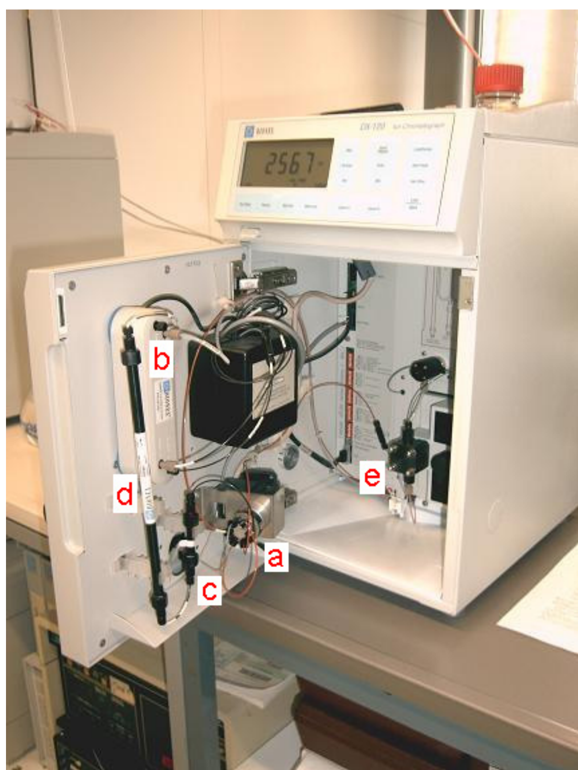


Fig. IV.1: Ion chromatography station comprehending automatic sampler, ion chromatograph and computer. The chromatograph consists of a pump, an injection valve with a sample loop (a), a self-regenerating suppressor (b), a guard column (c), an anion-exchange column (d) and a conductivity cell (e).

IV.3.3 Preparation of primary cell cultures from liver, gills and gonads

For analysis in the fluorescein diacetate (FDA) assay and comet assay in the F0 generation, 6 random fish of each sex were taken from both of the controls and from one replicate tank with 2/0.2 mg/L MMS at 357 dpf. For production of primary cell cultures from liver, gills and gonads, the respective organs from three animals were pooled according to the sex of the fish. For cell isolation, a modified perfusion technique based on the protocols for the use of primary hepatocytes and gill cells in the comet assay by Braunbeck and Storch (1992) as well as Schnurstein and Braunbeck (2001) was applied. In detail, the methodology of cell isolation and genotoxicity tests follows the protocol by Faßbender and Braunbeck (under review) for the analysis of primary cells from zebrafish gonads in the FDA and comet assay.

IV.3.4 Viability testing

For the measurement of the percentage of living cells, the fluorescein diacetate (FDA) assay was performed according to Strauss (1991) in the adaption of Schnurstein and Braunbeck (2001). For each cell preparation, a total of 500 cells was examined in two replicates. A comet assay was performed only, if cell viability exceeded 90 % relative to the controls.

IV.3.5 Comet assay

The comet assay was performed under alkaline conditions according to Singh et al. (1988) with modifications detailed by Schnurstein and Braunbeck (2001). Lysis conditions followed the protocol by Kosmehl et al. (2004). For each cell suspension prepared from the organs of three fish, 100 cells on each of two slides were evaluated for DNA migration by using the comet image analysis software Komet 5.5 (Kinetic Imaging Ltd., Liverpool, UK). For statistical analysis with SigmaPlot 11.0 (Systat, Erkrath, Germany), the relative percentage of DNA in the tail was measured. Data were not normally distributed, and statistical differences were analyzed using ANOVA-on-ranks (Kruskal-Wallis) in combination with the *post-hoc* test according to Dunn in order to identify statistical power of difference.

IV.3.6 Micronucleus test on histological sections

For the micronucleus test in histological sections in the F0 generation, 3 fish of each sex were taken from both of the F0 controls and from the remaining replicate tank with 2/0.2 mg/L MMS, as described for the sampling for the FDA and comet assays. The preparation of histological sections from liver, gills and gonads from zebrafish and their use in the micronucleus test was performed according to the protocol by Faßbender and Braunbeck (under review). In order to determine micronucleus frequencies, micronuclei were counted in 2000 cells on each slide using an Aristoplan light microscope with 1,120-fold magnification (Leitz, Wetzlar, Germany). Chromatin fragments were considered as micronuclei, if they had a diameter of not more than 30 % of that of a nucleus of the same cell type and were lying close to the nucleus and in the same focus plane. For normally distributed data, statistical differences in micronucleus frequency between groups were analyzed by One-Way ANOVA in combination with the *post-hoc* test according to Holm-Sidak in order to identify statistical power of difference.

IV.3.7 Histopathology

All F0 fish that had not been used for genotoxicity assessment were processed for histological analysis. In the F1 generation, fish derived from control and 2/0.2 mg/L MMS-exposed fish were sacrificed for histological analysis at 230 dpf. For this end, 10 fish per sex from each tank were used. If a lower number of fish of the respective sex were present, all available fish of this sex were processed. For conservation and decalcification, fish trunks were at least overnight incubated in Davidsons's fixative (Humason 1967). According to Humason (1967), 1 L of the fixative consisted of 220 ml formalin (37 %), 330 ml ethanol (96 %), 335 ml aqua bidest. and 115 ml glacial acetic acid. After conservation, the samples were dehydrated and infiltrated with paraffin (Paraplast Plus, Leica Biosystems, Nussloch, Germany) in an automatic Leica TP1020 tissue processor (Leica Biosystems) according to the following time schedule: 1 h in 80 % ethanol, 2 x 1 h in 90 % ethanol, 2 x 1 h in 96 % ethanol, and 2 x 1 h in 100 % isopropanol, 1 h, 4 h and again 12 h in 100 % xylene and finally 2 x 12 h in paraffin. In the next step, the samples were embedded in paraffin and cured using a Leica EG 1140 H paraffin dispenser (Leica Biosystems). For light microscopy, sagittal sections of 4 - 5 µm thickness with intervals of 40 µm were prepared with an HN 40 microtome (Reichert-Jung, Heidelberg, Germany). For stretching the sections, these were transferred to a water bath at 43 °C and subsequently to glass slides covered with glycerol albumen (Serva, Heidelberg Germany) for improved adhesion. After drying overnight at 38 °C, paraffin was removed by incubation in X-TRA Solv (Medite, Burgdorf, Germany) 3 x 10 min. Subsequently, a descending alcohol series with 2 x 5 min 100 % isopropanol and 3 min 96 %, 90 %, 80 % and 70 % ethanol each was applied. Slides were incubated for 1 min in aqua dest., stained with Mayer's acid hemalaun (Roth, Karlsruhe, Germany) for hematoxylin and erythrosine (HE) stain and blued under floating tap water for 10 min. According to Mulisch and Welsch (2010), the stain consisted of 1 g hemalaun, 0.2 g sodium iodate, 50 g potash alum, 50 g chloral hydrate and 1 g citric acid per litre aqua bidest. Stained slides were covered using X-TRA-Kitt (Medite). Aberrant morphological structures, e.g. preneoplastic lesions, were photographed with a Nikon DS Ri1 camera (Nikon, Düsseldorf, Germany) connected to a Nikon ECLIPSE 90i microscope using NIS Elements AR 4.00.05 64-bit software (Nikon). Alternatively, tissues were photographed with an Olympus Camedia C 5060 camera (Olympus, Hamburg, Germany) connected to an Olympus CKX41 inverted microscope using Analysis 5.0 software (Soft Imaging System, Münster, Germany). Pictures were processed using Adobe Photoshop CS5 (Adobe, Munich, Germany).

IV.4 Results

IV.4.1 Chemical analysis of MMS concentrations

Methanesulfonic acid (MSA) standards of 5 – 20 µM in fresh tap water were stable over 4 days (Fig. IV.2 A). In samples taken from aquaria, MSA concentrations decreased after sampling over a few days (Fig. IV.2 B). In replicate tanks, real concentrations were similar.

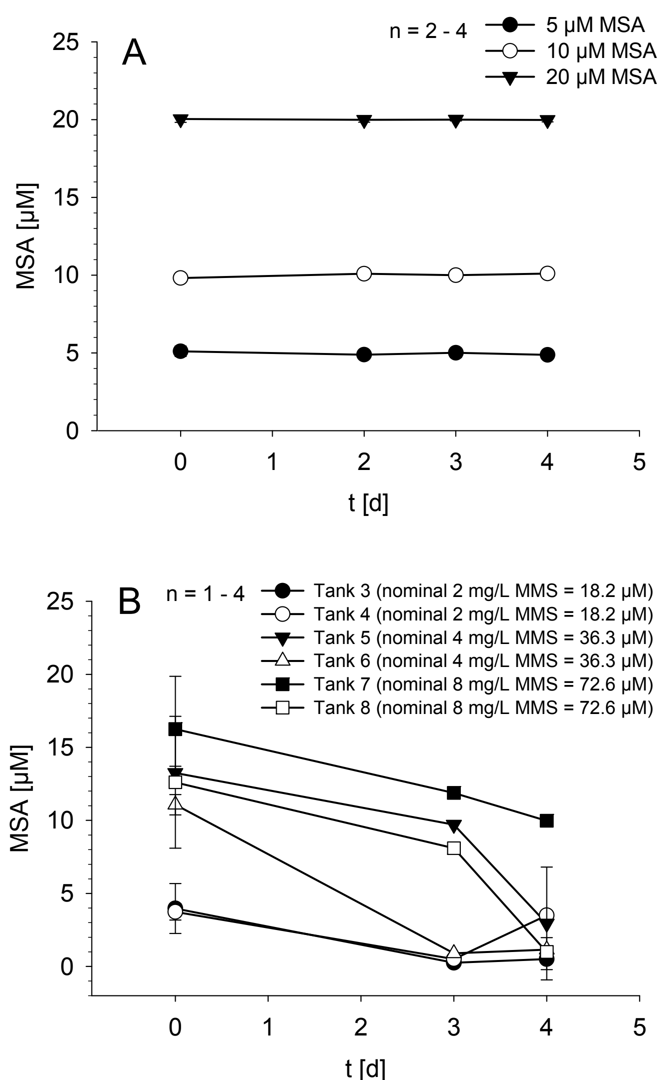


Fig. IV.2: Quantitative measurement of methanesulfonic acid standards (5, 10 and 20 µM MSA) in fresh tap water (A) using ion chromatography over 4 days and in tank water containing nominal MMS concentrations of 2, 4 and 8 mg/L (B) until day 4 after sampling. Data are given as means ± standard deviations.

IV.4.2 Genotoxicity

For all test concentrations, cell viability of primary cells from liver, gills and gonads from fish exposed to 2/0.2 mg/L MMS exceeded 90 % compared to the controls in the FDA assay. Hence, genotoxicity testing could be performed in all of these organs. In the comet assay, the percentage of DNA in the tail was significantly elevated in liver cells from both sexes as well

as female gills and male gonads (Fig. IV.3). In contrast, no significant effects were found in primary cells from male gills and female gonads. In histological sections from liver, gills and gonads of both sexes, micronucleus frequencies were significantly elevated compared to controls after exposure to 2/0.2 mg/L MMS (Fig. IV.4).

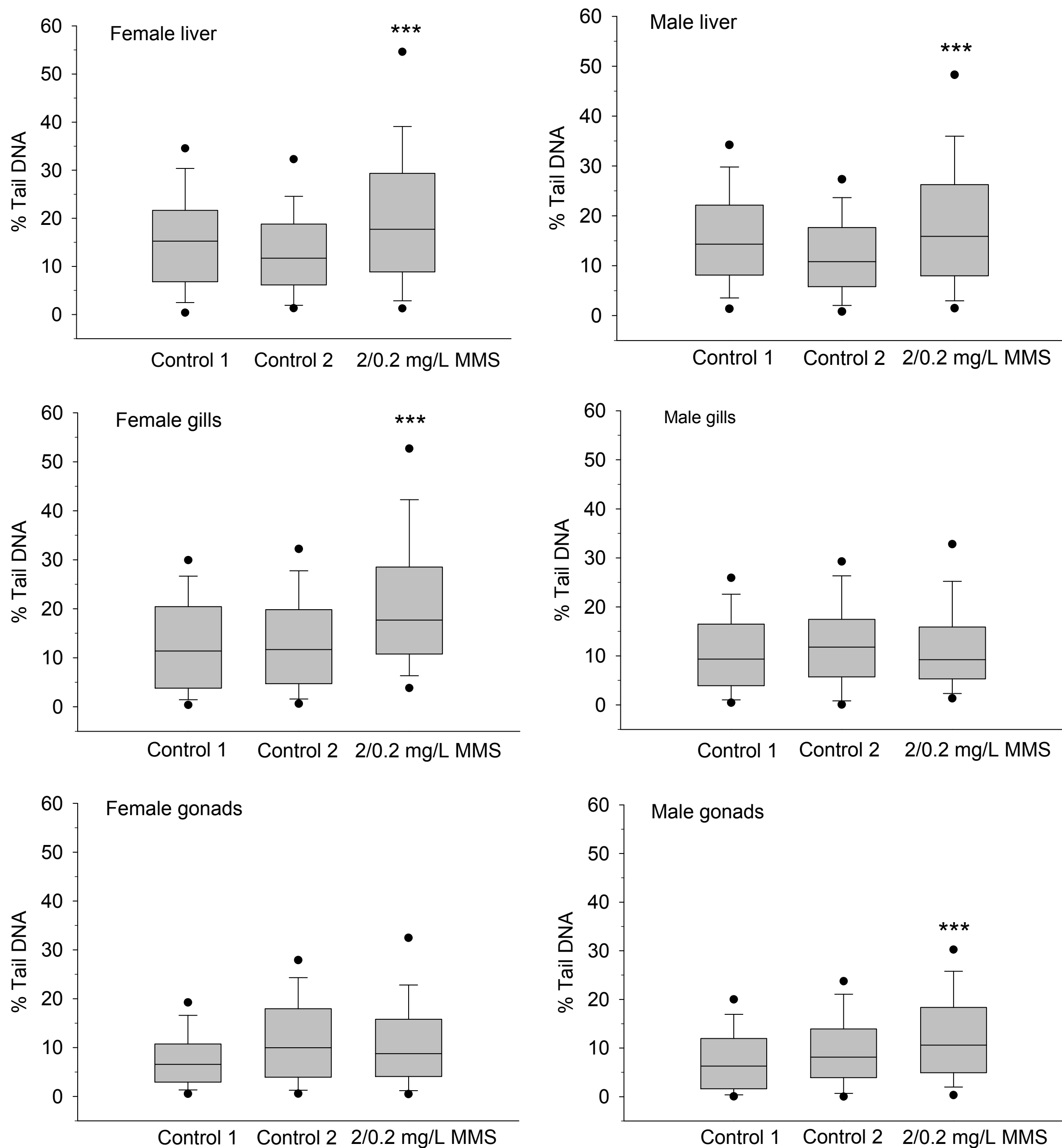


Fig. IV.3: Percentage of tail DNA in the comet assay in primary cells from liver, gills and gonads of male and female zebrafish after *in vivo* exposure to 2/0.2 mg/L MMS. For each concentration and the controls, organs of 3 fish were pooled. For the boxes, data from two replicate slides with 100 nucleoids each are pooled. Data are given as box plots, displaying the following percentiles: 25 and 75 (shaded box), 10 and 90 (whiskers) as well as 5 and 95 (points). Central solid lines represent medians. Three asterisks indicate significant differences from combined controls (Mann-Whitney rank-sum test, *** $p < 0.001$).

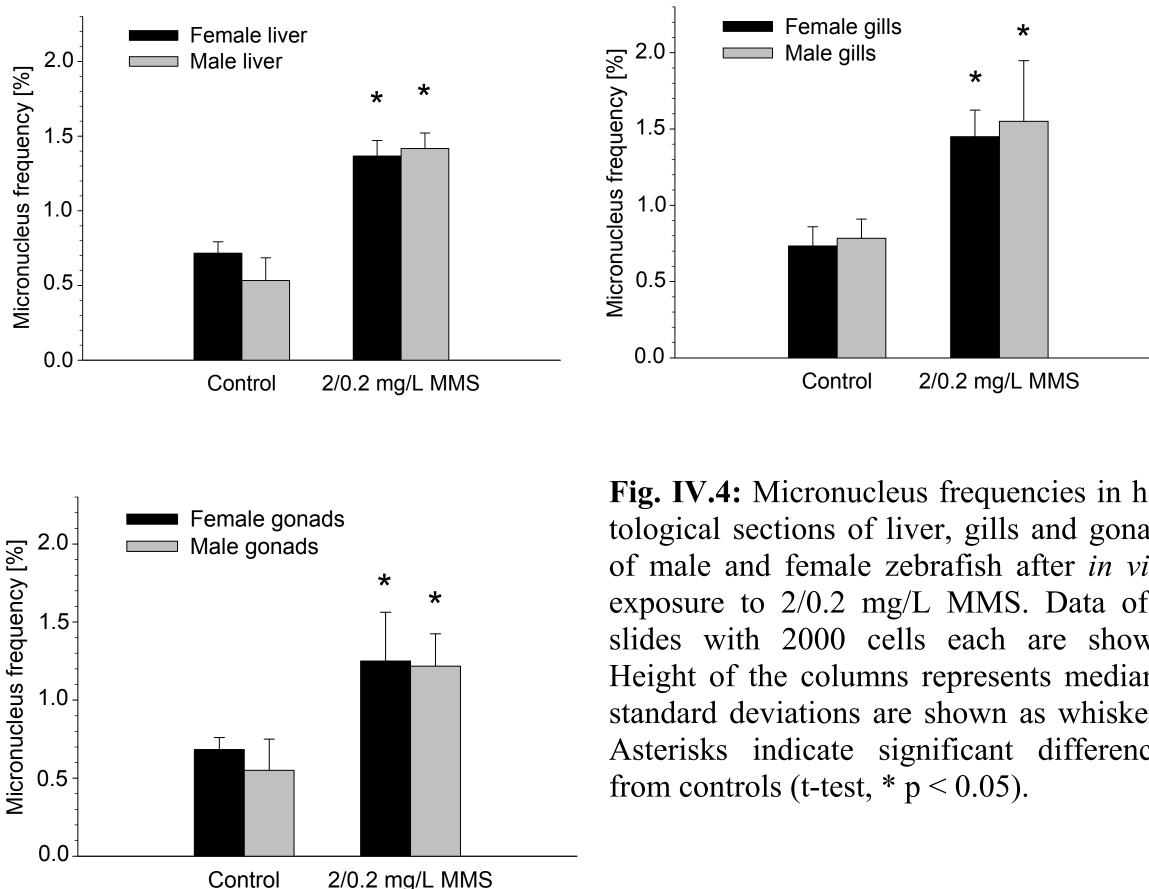


Fig. IV.4: Micronucleus frequencies in histological sections of liver, gills and gonads of male and female zebrafish after *in vivo* exposure to 2/0.2 mg/L MMS. Data of 3 slides with 2000 cells each are shown. Height of the columns represents medians; standard deviations are shown as whiskers. Asterisks indicate significant differences from controls (t-test, * $p < 0.05$).

IV.4.3 Histological aberrations in MMS-exposed fish and their offspring

IV.4.3.1 Overview of histological aberrations

In the F0 generation, the prevalence of histological lesions strongly increased with MMS concentrations (Fig. IV.5). However, the limited number of females available for histological analyses did not allow for a clear-cut decision with respect to relationships between aberrations and sex. Therefore, in the following, percentages of investigated fish are given irrespective of the sex.

In the F0 generation, the most prevalently affected organs were the eyes, followed by the gills and livers. Less frequently, aberrations were observed in the olfactory organs, kidneys, testes and ovaries.

The liver showed the widest range of histological aberrations of all organs. In the 2/0.2 mg/L exposure group, effects on liver histology were found in 8.0 % of the zebrafish. In the 4/0.4 mg/L exposure group, this was true for 29.4 % and for 40.7 % in the 2 mg/L exposure

group. Thus, there was a concentration-dependent increase of histological aberrations in the liver.

Presumably (pre-)neoplastic or (pre-)carcinogenic lesions were found in all groups of zebrafish exposed to MMS, with an increase in a concentration-dependent manner, whereas none were found in the controls. In the 2/0.2 mg/L exposure group, 4 % of the fish showed neoplastic structures in the liver. In 17.7 % of the fish exposed to 4/0.4 mg/L MMS, (pre-) neoplasia mainly in the liver (i.e. granuloma, spongiosis hepatis) were found. 22.2 % of the zebrafish constantly exposed to 2 mg/L exhibited tumors in the liver and musculature as well as neoplasia in liver and at the position usually taken by the swim bladder.
In the F1 generation, 44.4 % of the female and 11.1 % of the male examined zebrafish descending from parents exposed to 2/0.2 mg/L MMS showed histological alterations. The most affected organs in the F1 generation were ovaries, eyes and livers. Regarding (pre-)neoplastic lesions, eyes and livers, 6.5 % of the F1 fish examined were affected.

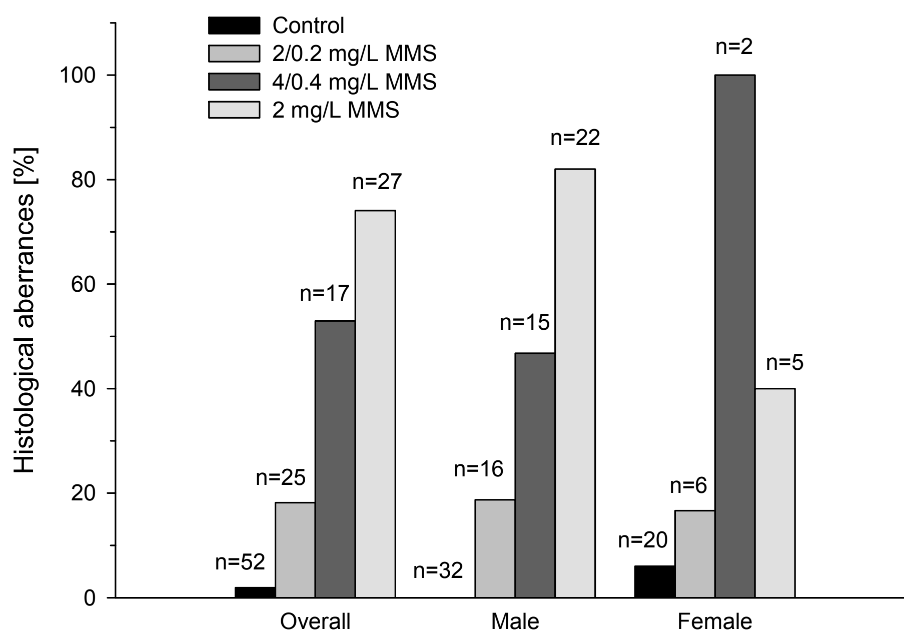


Fig. IV.5: Percentage of zebrafish of the F0 generation showing histological aberrations in controls and MMS-exposed groups after 355 dpf in the controls, 370 dpf in the group exposed to 2/0.2 mg/L, 301 dpf in the group exposed to 4/0.4 mg/L and 170 dpf in the group exposed to 2 mg/L.

IV.4.3.2 Eye lesions after exposure to MMS

Control fish were free of any eye lesions. In contrast, deformation of eyes was found in all exposure groups of the F0 generation in a concentration-dependent manner. Malformations of ocular bulbs and retinal layers were found in 5.9, 8 and 48.2 % of the individuals exposed to 2/0.2 mg/L, 4/0.4 mg/L, and 2 mg/L, respectively. According to the histology of retina cell layers described by Grehn (2008), the inner nuclear, inner plexiform and pigmented layers were malformed. In the F1 generation, 16.1 % of the examined fish with parental MMS exposure showed malformations of the eyes. Possible eye neoplasia were found in 3.2 % of the examined F1 individuals. Figures IV.6 and IV.7 exemplify malformations of ocular bulb and retinal layers in the F0 and F1 generation.

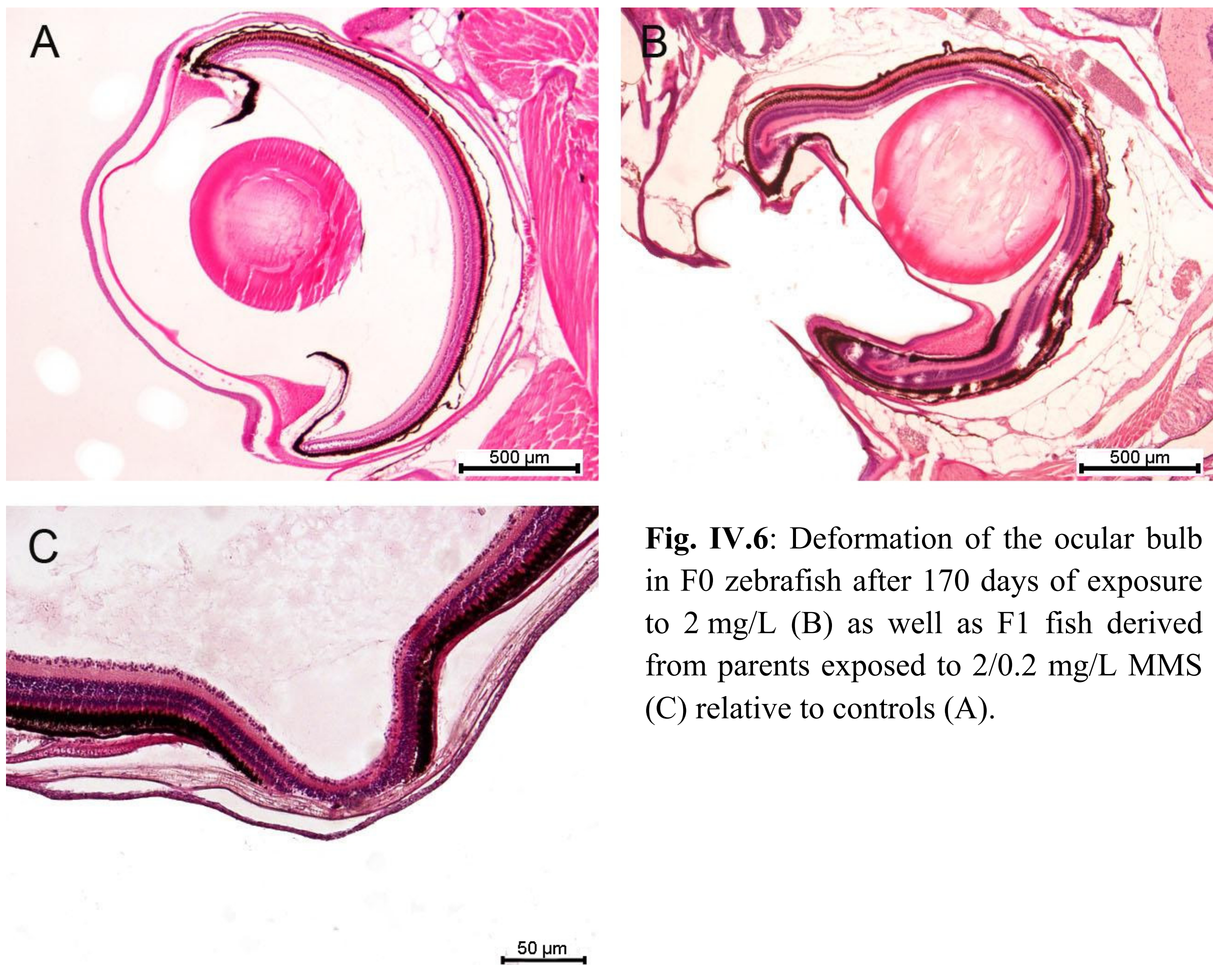


Fig. IV.6: Deformation of the ocular bulb in F0 zebrafish after 170 days of exposure to 2 mg/L (B) as well as F1 fish derived from parents exposed to 2/0.2 mg/L MMS (C) relative to controls (A).

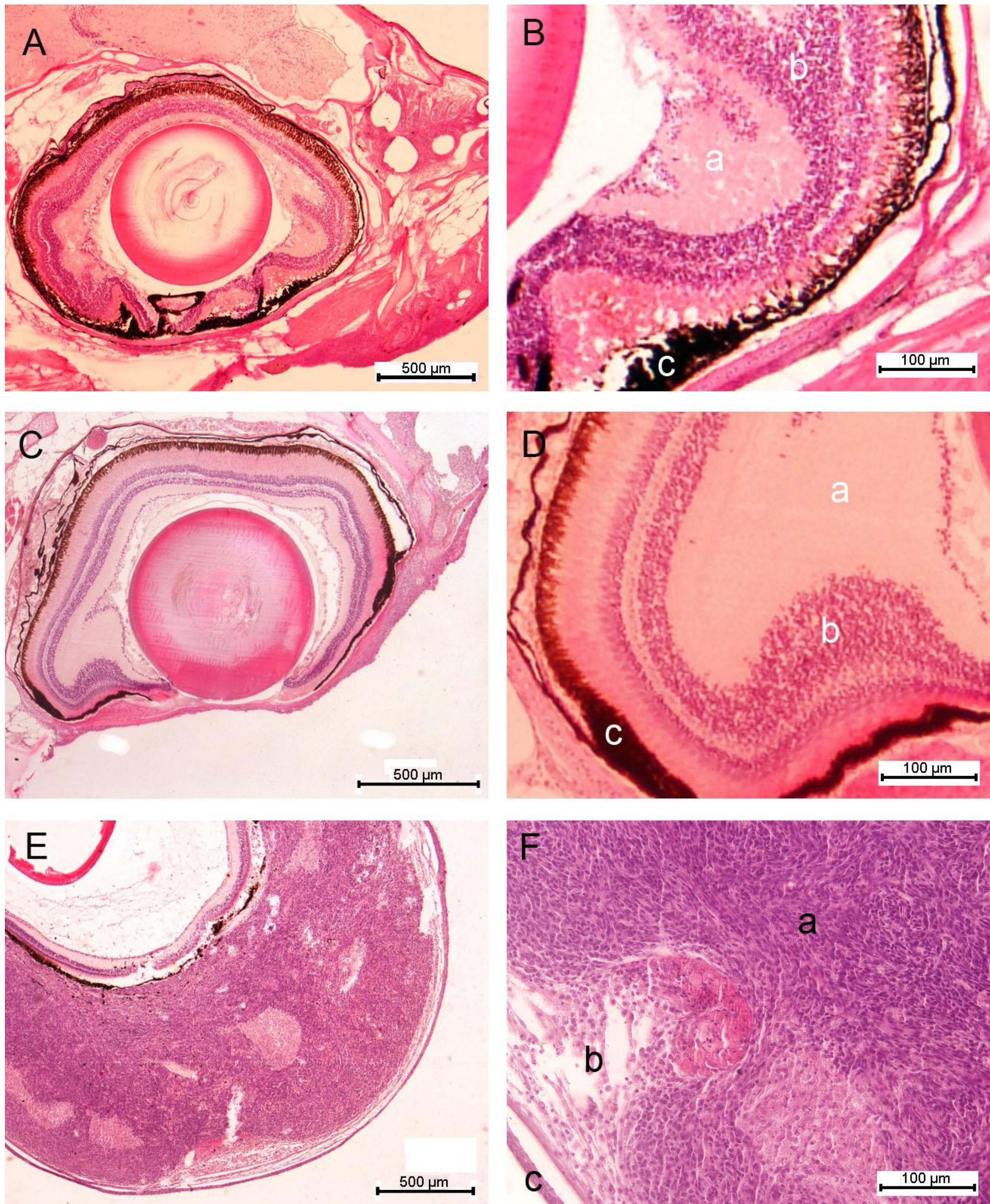


Fig. IV.7: Malformation of eyes in F0 zebrafish after 170 days of exposure to 2 mg/L (A – D) as well as F1 fish derived from parents exposed to 2/0.2 mg/L MMS (E, F). B, D (close-ups from A, C): proliferation of retinal layers: inner plexiform layer (a), inner nuclear layer (b) and pigmented layer (c). E, F: Extensive neoplasia of the sclera invading subcutaneous connective tissue (a) and the cutis (b); c: epidermis.

IV.4.3.3 Gill lesions after exposure to MMS

In the F0 generation, histological aberrations were exclusively found in the gills of 40.7 % of the zebrafish exposed to 2 mg/L MMS. Coalescence of adjacent secondary lamellae as well as secondary lamellae belonging to different primary lamellae and proliferation of gill epithelium were observed (Fig. IV.8). In the F1 generation, 6.4 % of the examined fish with parental exposure to 2/0.2 mg/L showed gill aberrations.

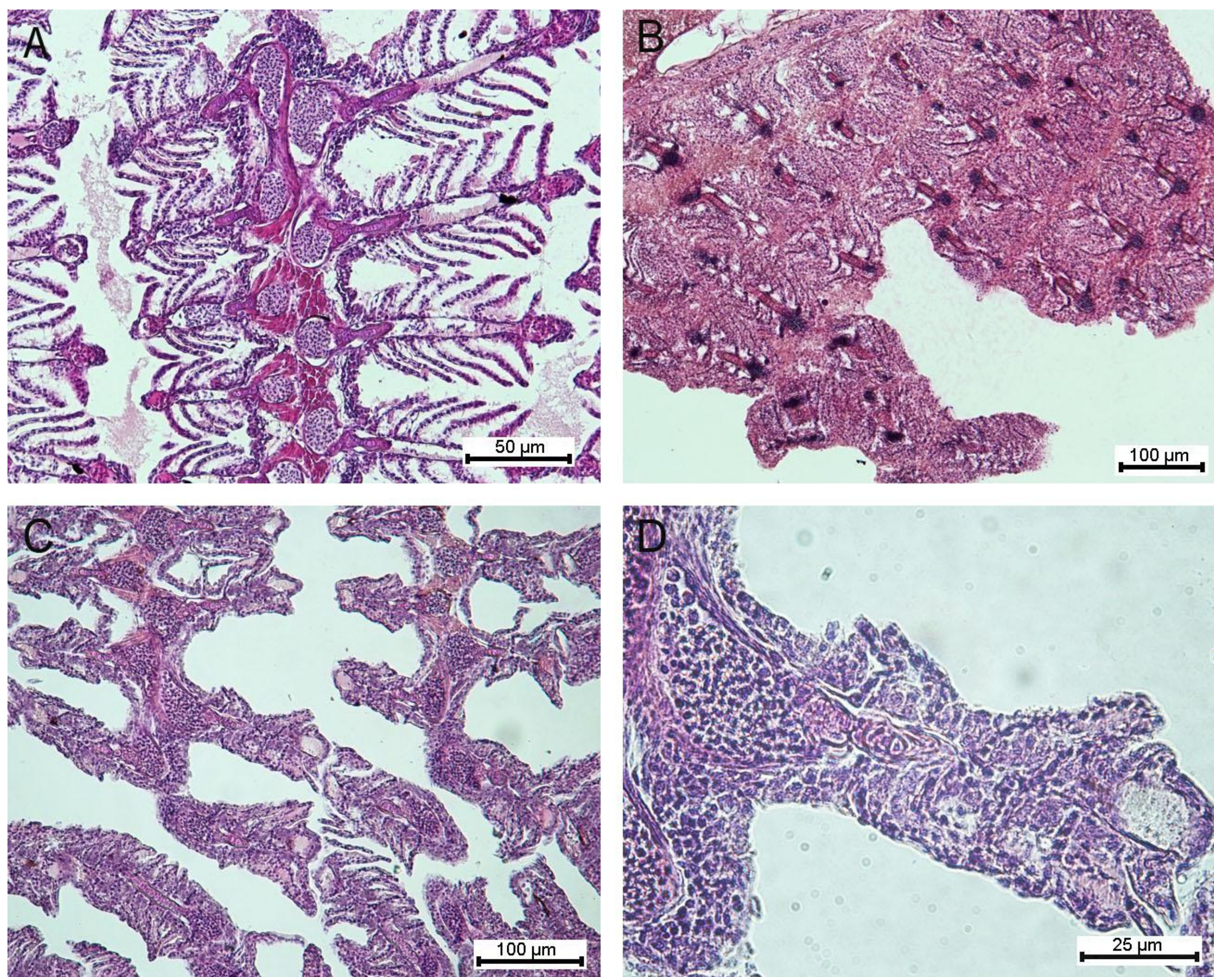


Fig. IV.8: Fusion of secondary gill lamellae in zebrafish after 170 days of exposure to 2 mg/L MMS (B) and proliferation of epithelium (C, D) in comparison to gills from controls (A).

IV.4.3.4 Liver alterations after exposure to MMS

Manifold histological aberrations were found in all exposure groups in the liver, whereas the control groups did not show any alterations. Fatty liver in hepatocytes (steatosis hepatis), was found in a total of 5.8 % of fish exposed to MMS (Fig. IV.9 B). Hereby, macro- and microvesicular steatosis hepatis co-occurred in the tissue, i.e. signet ring formed cells with displaced nuclei due to accumulation of triglycerides were found as well as fat accumulation without displacement of the nucleus. Cystic degeneration due to structural alterations in perisinusoidal liver cells (spongiosis hepatis) was found in 4.3 % (Fig. IV.9 C, D). Moreover, the following aberrations were found in individual cases: granuloma, i.e. inflammation related tubercular neoplasia, (Fig. IV.9 E) presumably tumorigenic tissue containing melano-macrophage centers (Fig. IV.9 F) and structural aberrations apparently associated with degradation of liver structure (Fig. IV.10 A). In case of the latter, an intensity gradient of staining of hepatocytes and their nuclei from the enteral surface of the liver to the inside was found. Additionally, in this case, a gradient of decreasing cohesion of the cells from the inside to the surface was observed. However, although a similar structure of liver tissue was not found in the controls, it can not be excluded that this is an artifact of incomplete fixation. Furthermore, abnormal staining of liver regions was observed (Fig. IV.10 B). In the liver of one fish exposed to 2 mg/L MMS, a possible neoplasia was found (Fig. IV.10 C). It presumably consisted of smooth muscular tissue encompassing a blood vessel, and might thus be considered as a possible leiomyoma.

In the F1 generation, histological aberrations in the liver similar to the effects in the F0 generation individuals were found at a frequency of 12.9 %. Spongiosis hepatis (Fig. IV.10 D) and different staining of liver regions were observed.

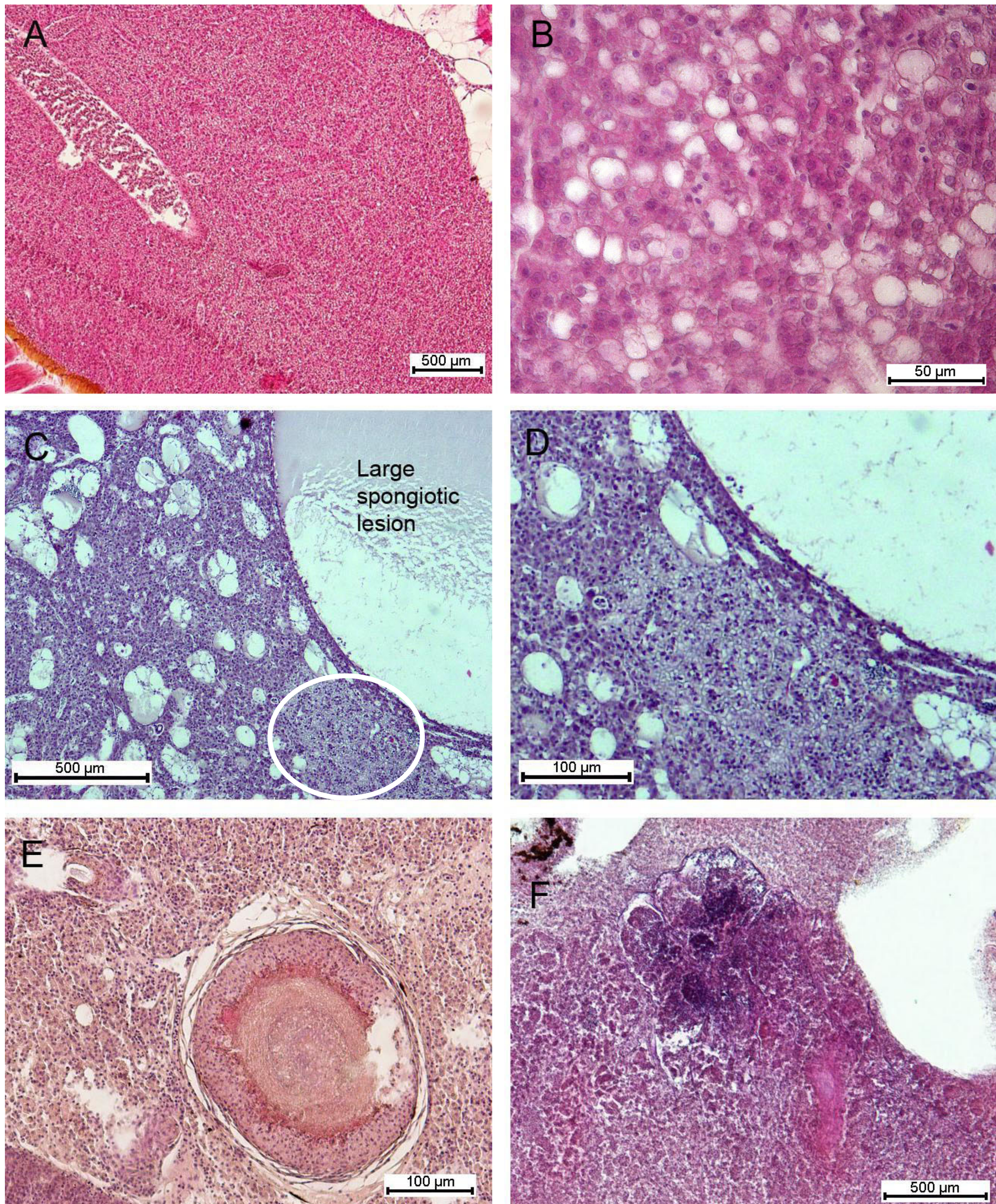


Fig. IV.9: Histological aberrations in the liver of zebrafish after exposure to 4/0.4 mg/L MMS at 301 dpf (C, E) and to 2 mg/L MMS at 170 dpf (B, D) in comparison to liver from controls (A). Fatty liver (steatosis hepatis; B), cystic degeneration (spongiosis hepatis) with a neoplastic nodule (marked with white ring in C and shown in detail in D), granuloma (E) and melano-macrophage center in a presumably benign tumor (E).

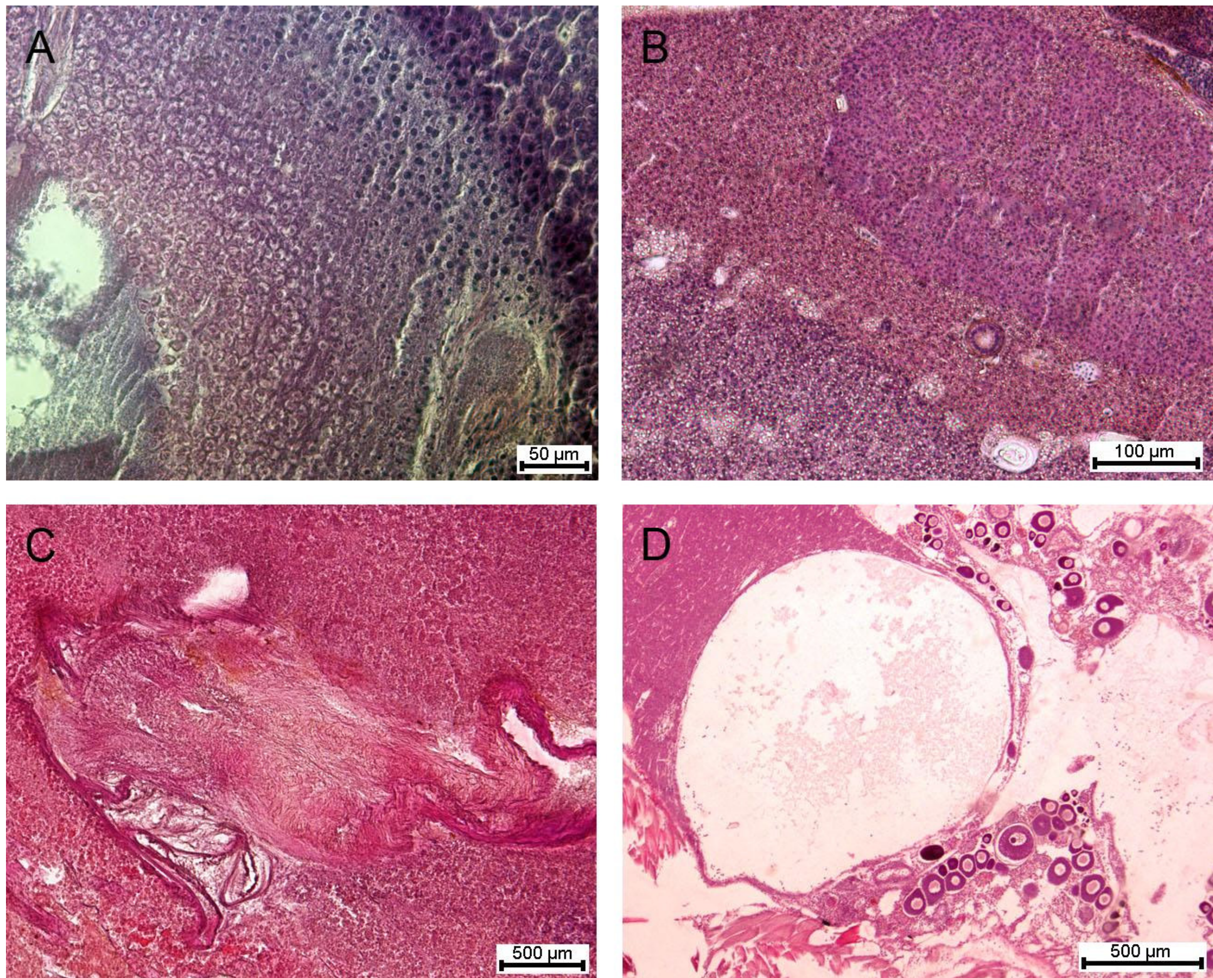


Fig. IV.10: Histological aberrations in the liver of zebrafish at 301 dpf after exposure to 4/0.4 mg/L MMS associated with degradation of liver structure (A) and structural alterations with different staining properties (B). C: Possible leiomyoma in the liver of a zebrafish after 170 days of exposure to 2 mg/L MMS. D: Liver cyst in a zebrafish of the F1 generation descending from MMS exposed parents.

IV.4.3.5 Histological aberrations in other organs after exposure to MMS

In all groups exposed to MMS, additional histological lesions were found in the kidney (8 % at 2/0.2 mg/L, 5.8 % at 4/0.4 mg/L, 7.4 % at 2 mg/L). Figure IV.11 B shows the middle kidney region of a zebrafish after exposure to 2 mg/L MMS. Compared to the control (Fig. IV.11 A), the interrenal parenchyma of the exposed fish was degenerated. In another fish, the volume of renal tracts was enlarged (Fig. IV.11 C).

Possible neoplasia in the olfactory organ were observed in 7.4 % of the fish exposed to 2 mg/L MMS (Fig. IV 12 B).

Effects on reproductive organs were only rarely found. In 3.7 % of the zebrafish exposed to 2 mg/L, underdeveloped testes were observed. At the same concentration, 11.1 % of the examined females showed ovarian underdevelopment.

Moreover, exposed individuals exhibited possible neoplasia consisting of not classifiable cell types in different body regions, e.g. at the position normally occupied by the swim bladder (Fig. IV.13 A) or dorsally to the gonads (Fig. IV.13 B). This was the case in 5.8 % of the examined fish at 4/0.4 mg/L and 7.4 % at 2 mg/L MMS.

In the F1 generation, histological aberrations in the ovaries were found at clearly higher frequencies than in the parental generation: 22.1 % of the examined female fish showed alterations in ovary structure. Underdeveloped ovaries lacking tertiary follicles (11.1 %; Fig. IV.13 C) and exhibiting an amorphous mass, which is suggested to be egg debris (11.1 %; Fig. IV.13 D), were found.

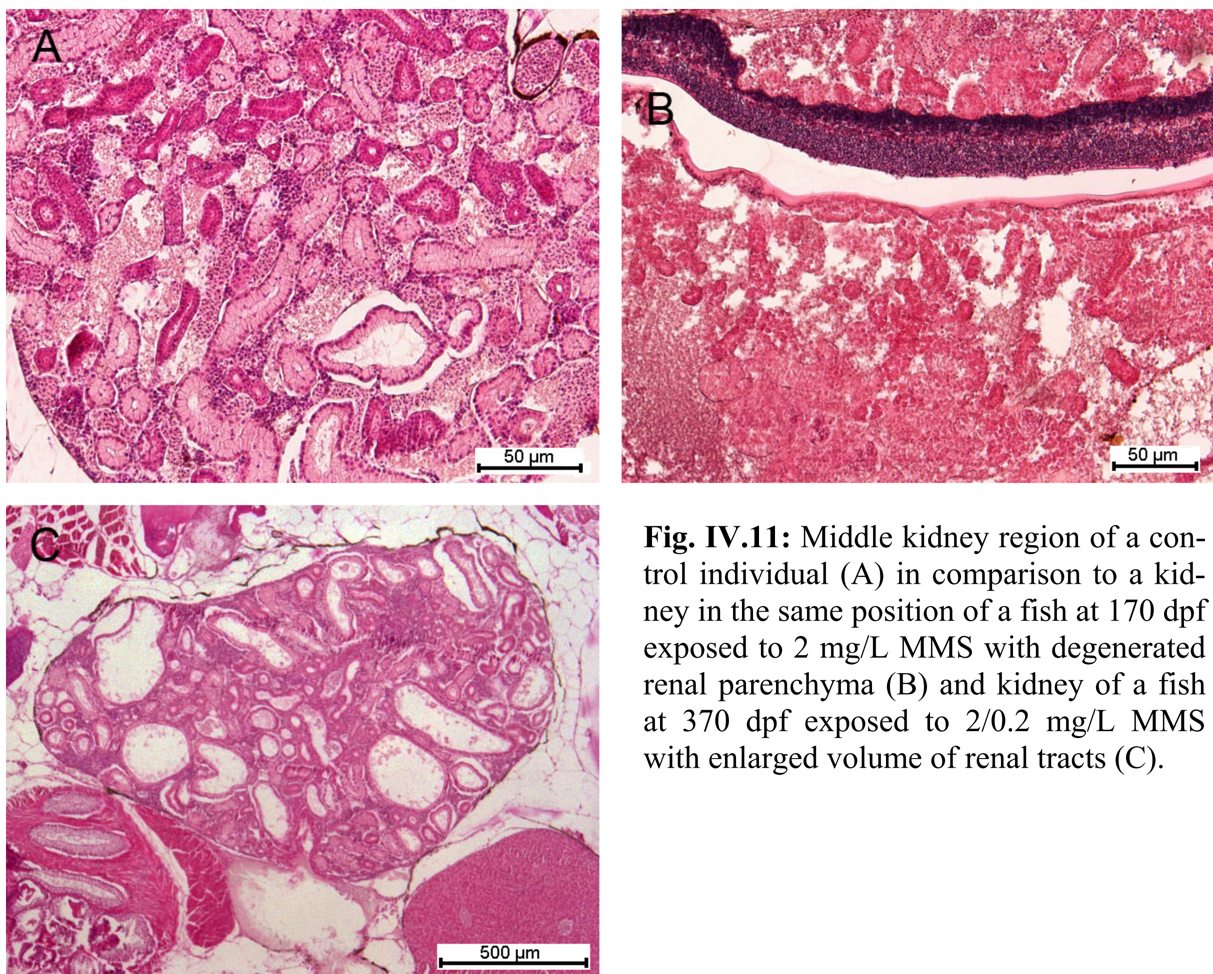


Fig. IV.11: Middle kidney region of a control individual (A) in comparison to a kidney in the same position of a fish at 170 dpf exposed to 2 mg/L MMS with degenerated renal parenchyma (B) and kidney of a fish at 370 dpf exposed to 2/0.2 mg/L MMS with enlarged volume of renal tracts (C).

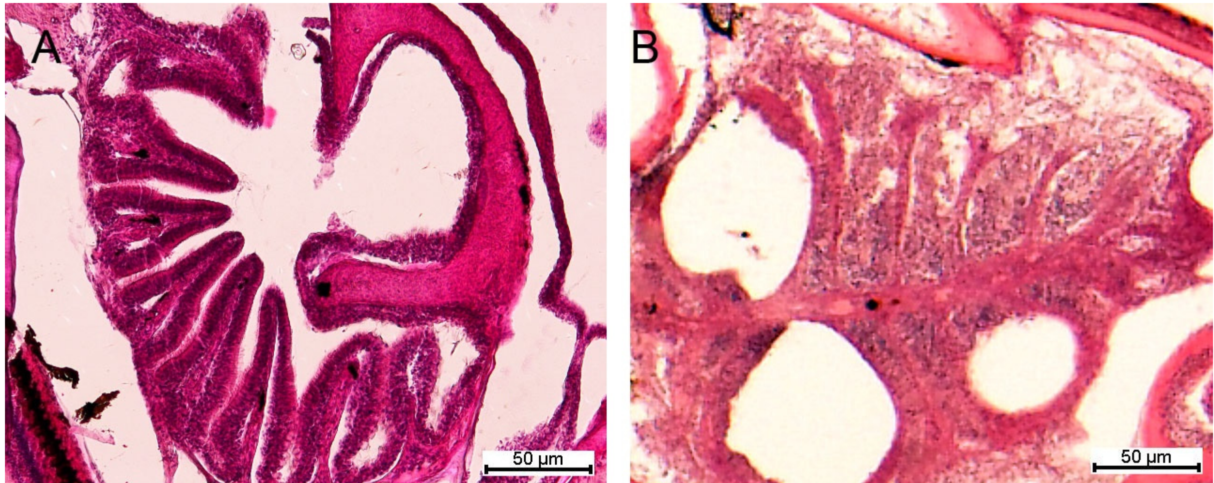


Fig. IV.12: Neoplasia of the olfactory epithelium in the olfactory organ of a zebrafish at 170 days of exposure to 2 mg/L MMS (B) in comparison to a control (A).

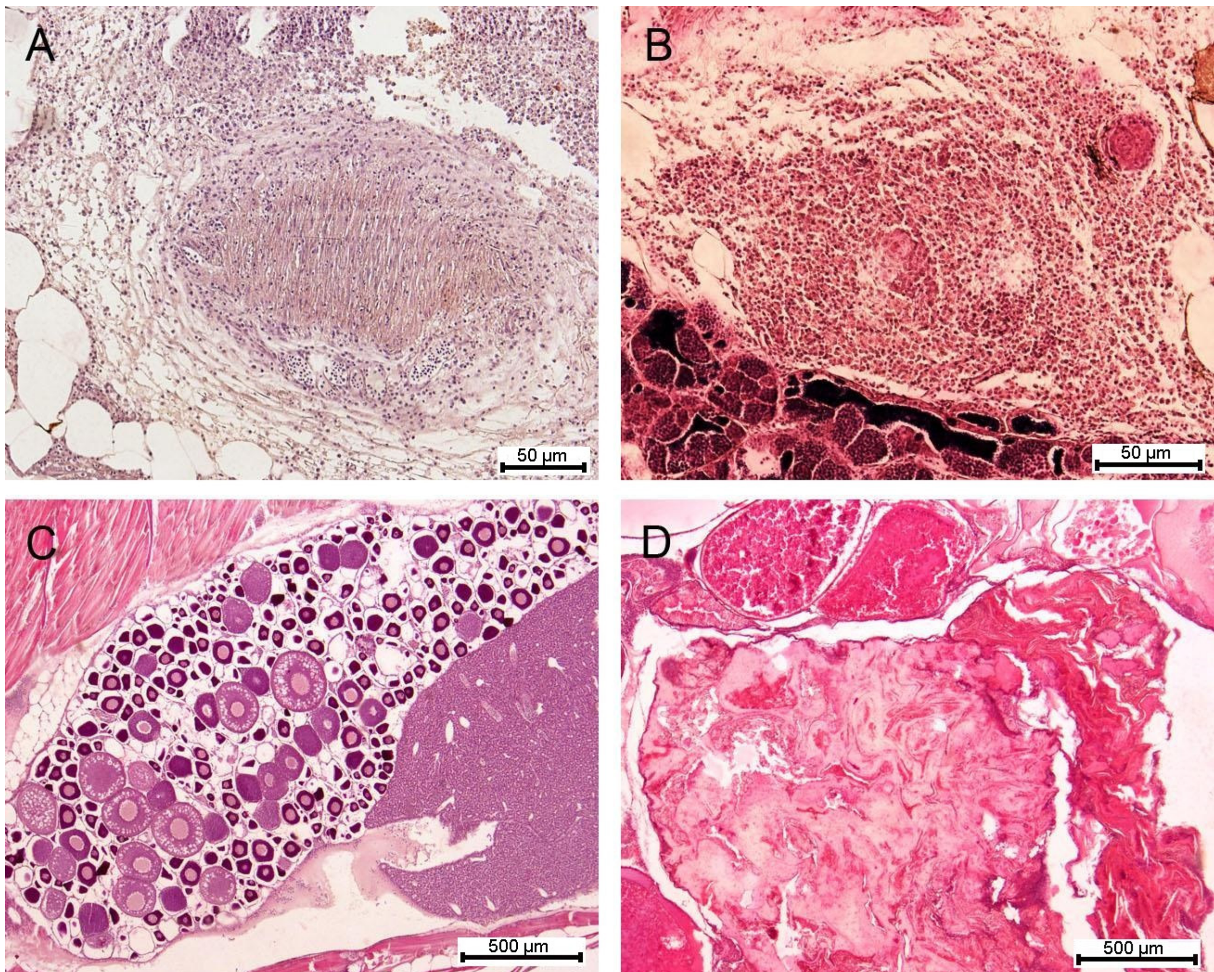


Fig. IV.13: Possible neoplasia originating from not assignable cell types in individual zebrafish after 203 days of exposure to 2 mg/L MMS in the position usually taken by the swim bladder (A) and after 301 days of exposure to 4/0.4 mg/L MMS dorsally to the testis (B). Effects on the ovary in the F1 generation: underdeveloped ovary (C) and egg debris (D).

IV.4.3.6 External lesions and coincidence of external and internal aberrations

In fish of the F0 (Tab. IV.1) and F1 generation (Tab. IV.2), the following external histopathological aberrations were found: skin neoplasia, redness (inflammation of the skin), swelling, scoliosis, exophthalmus and erection of scales. Figures IV.14 - 17 exemplarily show external aberrations in fish after exposure to 2/0.2, 4/0.4 and 4 mg/L MMS and the corresponding offspring.

Concurrent internal aberrations comprised spongiosis hepatis, neoplasia such as fibroma, granuloma and a further tumor with melano-macrophage centers, different staining of liver regions, liver cysts, enlarged renal collecting ducts and underdeveloped ovaries. Referred to the number of larvae at the beginning of exposure in the tanks, 5.5 % of the fish exposed to 2/0.2 mg/L MMS showed external aberrations. Moreover, 0.9 % of the fish showed external as well as internal aberrations. In one tank with 4/0.4 mg/L, 7.3 % of the fish featured external and 3.6 % internal aberrations. In the other replicate, 1.8 % of the fish showed external aberrations.

In the F1 generation, 3.1 % of fish taken from 3 tanks showed external aberrations. In one of these tanks, 2.6 % of the fish featured external as well as internal aberrations.

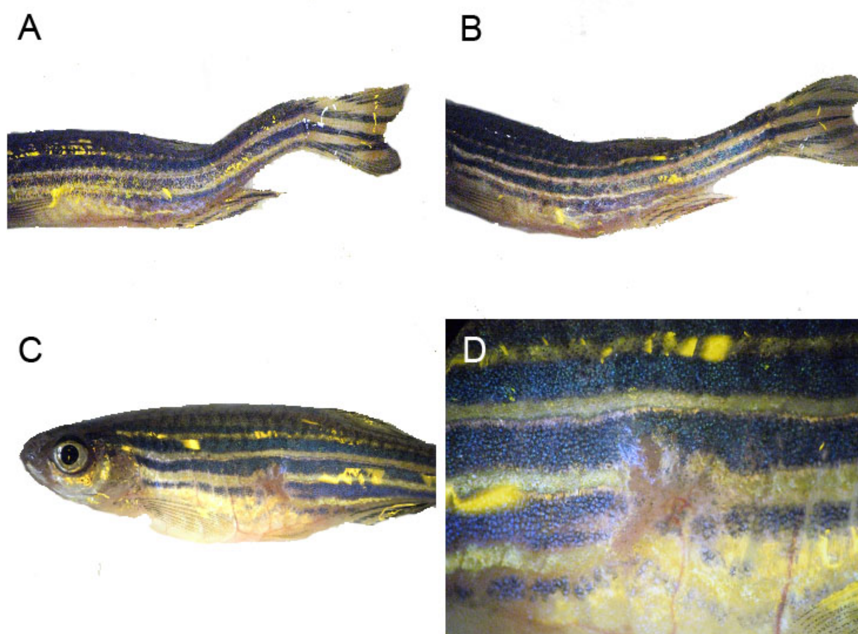


Fig. IV.14: F0 zebrafish showing external aberrations at 370 dpf after exposure to 2/0.2 mg/L MMS. A and B: Fish featuring scoliosis with tail bent up. C: Fish with skin neoplasia. D: Close-up view of the skin lesion in C.

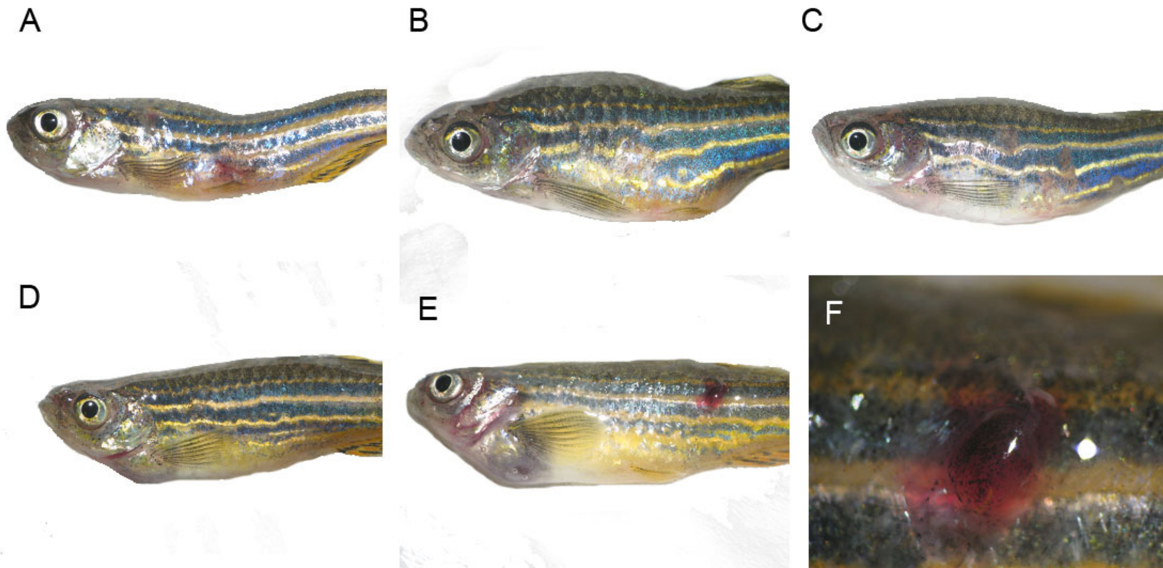


Fig. IV.15: F0 zebrafish showing external aberrations at 310 dpf after exposure to 4/0.4 mg/L MMS. A, B: Fish featuring scoliosis and skin neoplasia. C: Fish with skin neoplasia. D: Fish with swelling and redness of heart region. E: Fish featuring a skin neoplasia on the flank well supplied with blood, swollen heart region and redness. F: Close-up view of the neoplasia in E.

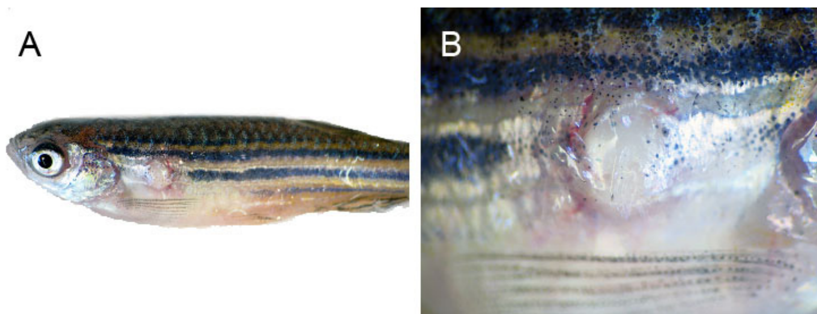


Fig. IV.16: F0 zebrafish showing external aberrations after exposure to 4 mg/L MMS at 187 dpf with round, elevated skin neoplasia.

Fig. IV.17: F1 zebrafish showing scale erection, redness and skin neoplasia on its belly.

Tab. IV.1: Externally visible histopathological lesions in the F0 generation of individual zebrafish after *in vivo* exposure to MMS. Internal histological aberrations in the same fish are also given.

Age (dpf)	MMS (mg/L)	Tank No.	External aberrations	Internal aberrations
67	4	6	Neoplasia on head	
112	4	6	Bilateral exophthalmus	
187	4	5	Round, elevated neoplasia on flank	Fibroma between liver and kidney
200	4	5	Skin neoplasia on both flanks	
201	2	3	Neoplasia on flank	
215	4	5	Thickened flank, tail bent, redness between pectoral fins	
240	2/0.2	3	Head bent up, redness between gills	
296	4/0.4	5	Redness on lateral trunk	
310	4/0.4	5	Neoplasia on flank well supplied with blood, swollen heart region, redness	
310	4/0.4	5	Scoliosis, skin neoplasia	Liver tumor with melano-macrophage centers, different staining of liver regions
310	4 /0.4	5	Scoliosis	Spongiosis hepatis, liver granuloma, neoplasia above ovary
310	4/0.4	5	Scoliosis, skin neoplasia	Spongiosis hepatis, liver cysts
310	4/0.4	5	Swollen heart region, redness	
370	2/0.2	3	Skin neoplasia	
370	2/0.2	3	Scoliosis, tail bent up	Renal collecting ducts enlarged
370	2/0.2	3	Scoliosis, tail bent up	
370	2/0.2	3	Scoliosis, tail bent up	

Tab. IV.2: Externally visible histopathological lesions in the F1 generation descending from zebrafish exposed to 2/0.2 mg/L MMS.

Age (dpf)	Tank No.	External aberrations	Internal aberrations
52	16	2 Fish with bright swellings on heads	
52	16	2 Fish with bloodshot heads	
105	15	Scales erected, redness, skin neoplasia on belly	
230	10	Scoliosis	
230	15	Skin neoplasia on flank	Spongiosis hepatis, underdeveloped ovary
230	15	Unilateral exophthalmus	Neoplasia in retina

IV.5 Discussion

IV.5.1 Analysis of MMS and its fate in the organism

MSA concentrations did not increase after sampling. In fact, an increase of MSA concentrations would have been expected based on the assumption of hydrolysis of MMS to MSA and the high stability of MSA shown in the standards, i.e. under sterile conditions. On the other hand, MSA has been reported to be readily biodegradable (Gernon et al. 1999) and the actual decrease of MSA concentrations in the tanks must be explained by microbial degradation of MSA in the tank water (Higgins et al. 1996, Higgins et al. 1997, Kelly and Murrell 1999). However, since MMS is partially hydrolyzed to MSA already in the tanks prior to sampling, the ratios between the different former MMS concentrations can be seen from MSA data at the time point of sampling (t = 0 d). Thus, since real concentrations in the replicate tanks with the same nominal concentration were similar, the flow-through system probably worked well and calibration was accurate (cf. Fig. IV.2). Apparently, concentration adjustment was less exact at the higher concentrations.

The major advantage of ion chromatography is its low cost. If the budget of a study allows for it, it should be tested if higher precision can be achieved using recently developed techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS, Xu et al. 2007). Altogether, ion chromatography provided useful results for a rough screening and comparison of exposure between tanks.

Regarding the fate of MMS in the organism, it can be assumed that it is rapidly distributed over the organs. Cumming and Walton (1970) showed that after intraperitoneal injection in male mice, in the liver MMS was found at high concentrations. The authors demonstrated that the genotoxin rapidly reached the testis at nearly the same dose as administered to the whole animal. A broad variety of enzymes is capable of metabolizing organic compounds and plays an important role in the detoxification of xenobiotics (Cormier et al. 1989). Regarding MMS, it is unknown if degradation is exclusively due to inorganic hydrolysis or if there is a process with enzymes being actively involved (Cumming and Walton 1970). By all means, the faster MMS is hydrolyzed under the specific conditions of the respective tissues, the more its potential to alkylate DNA is limited. The product methanesulfonic acid is rapidly excreted in the urine of rats without further metabolization (Trams et al. 1959). Since there are no data on metabolization and excretion of MSA in fish available in the literature, the compound is possibly excreted alike.

IV.5.2 Genotoxicity

There are only very few *in vivo* studies on genotoxicity in fish, which are comparable to the present approach. Bony et al. (2010) exposed zebrafish to 1 µM (110 µg/L) MMS for up to three weeks and analyzed genotoxicity in the comet assay. After two weeks, the percentage of tail DNA reached 26 % in liver cells and 16 % in spermatozoa. The MMS concentration studied by Bony et al. (2010) is approximately half the concentration of 0.2 mg/L applied in the later course of the present study. In fact, the effects observed by Bony et al. (2010) are similar to those in liver and male gonad cells described in the present study and, thus, serve as confirmation.

Medians of the percentage of tail DNA did not exceed 20 % after chronic exposure to 2/0.2 mg/L MMS in the present experiment (cf. Fig. IV.3). In a short-term test, Faßbender and Braunbeck (under review) found more than 20 % of DNA in the tail for liver, gills and gonads after two weeks of exposure of adult zebrafish to 2 mg/L MMS. DNA fragmentation in liver cells from female fish reached up to 68 % at 16 mg/L. Compared to the present study, after two weeks of exposure, values were higher for all organs examined. Hence, obviously, during chronic exposure to MMS in the current experiment including a reduction of test concentrations, DNA damage was widely repaired.

Bony and co-workers (2010) found a significant elevation of micronucleus frequencies in erythrocytes at 110 µg/L MMS, which is in line with the findings of the present study on mi-

cronucleus formation in liver, gill and gonad tissues. In another study, zebrafish were exposed to higher MMS concentrations than the fish examined for genotoxic effects in the present study: Deventer (1996) exposed zebrafish to 88 µg/L – 8.8 mg/L MMS for up to 72 h and found significant effects in the micronucleus test 6 – 12 days post exposure. Strong effects in the micronucleus test in the present study can be explained by the formation of micronuclei during the initial exposure to 2 mg/L. Although after the reduction to 0.2 mg/L few further micronuclei may have formed, the micronuclei already present have assumedly persisted until the end of the experiment.
In the present experiment as well as in a similar study, some effects appeared to be sex-specific. In the current study, no significant effects were found in the comet assay with male gill and female gonad cells. Faßbender and Braunbeck (under review) exposed zebrafish to 2 – 16 mg/L MMS for two weeks and found significant effects in the comet assay in liver, gills and gonads for both sexes. These effects were stronger than DNA fragmentation found in the comet assay after long-term exposure in the present experiment. Regarding the micronucleus test, micronucleus frequencies were clearly elevated in all organs from both sexes tested after chronic exposure to 2/0.2 mg/L MMS in the present experiment. In contrast, after two weeks of exposure to 2 mg/L MMS, Faßbender and Braunbeck (under review) found significant effects in the micronucleus test in liver of both sexes as well as male gills and gonads of zebrafish, but not in female gills and gonads. However, it is suggested that different results for the sexes are not due to a mechanism of sex dependence behind genotoxic effects in the present study but to the low level of damage near the detection limit. This is supported by the finding of significant effects in all organs tested from both sexes in the micronucleus test in the current experiment, bearing in mind that the micronucleus test is sensitive to chronic exposure to low-concentrated genotoxicants (Deventer 1996).

A comparison of the results of the present study and Faßbender and Braunbeck (under review) illustrates that, on the one hand, strong effects were caused by high concentrations during a shorter exposure of two weeks and on the other hand by low concentrations during chronic exposure. The comet assay is especially capable of measuring acute effects on DNA integrity at high concentrations, whereas the micronucleus test gives information on the long-term accumulation of chromosomal fragments lost during cell division. Probably, repair of DNA fragmentation in the nucleus is faster than decomposition of micronuclei in the cytoplasm. At least in humans, there is evidence that cytosolic nucleases can cause degradation of plasmid DNA (Lechardeur et al. 1999). Possibly, in addition to phagocytosis of micronucleated cells, micronuclei can also be degraded by enzymes in the length of time.

Little is known about transgenerational mutagenic effects of alkylating agents. Exposure of mice to MMS during post-meiotic spermatogenesis led to increased sterility in their offspring due to inherited translocations (Lang and Adler 1977). In a similar study, heritable translocations and dominant-lethal mutations were documented in mice after exposure to ethylene dioxide (Generoso et al. 1980). Paternal exposure of brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*) to MMS caused DNA damage in spermatozoa leading to morphological abnormalities during embryonic and larval development (Devaux et al. 2011). Concerning the increased mortality of zebrafish descending from the exposed generation in the present multigenerational experiment (chapter III, Faßbender and Braunbeck 2013), it can be assumed that heritable genetic damage played a considerable role in this case. In addition, gametes were exposed to MMS during their development inside the parental fish. Certainly, this also caused genetic damage in the F1 generation.

IV.5.3 Histopathology

Inherited mutations can result in a high risk of developing cancer, if they affect tumor suppressor genes, oncogenes, genes encoding proteins involved in DNA repair and cell cycle control as well as genes involved in stimulating the angiogenic pathway (Hodgson 2008). This is a possible link between genotoxic exposure of the parental generation on the one hand and malformations and carcinogenesis in the offspring on the other hand. In the current study, in addition to genotoxic effects, chronic exposure of zebrafish to MMS caused numerous histopathological aberrations in the F0 as well as the F1 generation. Neoplasia in different organs as well as other anomalies were found in all exposure groups investigated.

The occurrence of histological aberrations in eyes and gills of fish exposed to MMS can be explained by the fact that these organs were directly exposed to the surrounding medium. The most frequently observed effects in the F0 generation were structural alterations of the ocular bulb and malformation of the retina layers. Meira et al. (2009) observed photoreceptor apoptosis and severe retinal degradation in mice exposed to MMS. The authors state that alkylation-induced retinal degeneration is driven by the DNA repair protein alkyladenine DNA glycosylase (Aag). The enzyme removes alkylated bases, leading to apurinic and apyrimidinic (AP) sites, which are processed by base excision repair to strand breaks. In zebrafish, alkylated DNA bases are repaired by a deaminase, a glycosylase and the factor Gadd45 (Rai et al. 2008). Possibly, a mechanism involving a DNA glycosylase comparable to the case for mice is responsible for the histological aberrations in eyes of zebrafish observed in the present

study. Regarding effects in eyes of the F1 generation, in the literature, malformation of eye structures was observed by Rutledge et al. (1992) in mouse embryos after intraperitoneal injection of MMS and other alkylating agents into female mice directly after mating. The authors suggested that a nonconventional, epigenetic mechanism had linked mutagenic effects and malformations in the offspring. Similarly, the eye effects in the F1 generation found in the present study can in parts be explained by exposure of the gametes inside the parental fish. In addition, eye effects can be due to heredity transmission of genetic damage in gametes from MMS exposed fish to their offspring.

Regarding histological aberrations in the gills, fusion of secondary lamellae obviously was due to hyperplasia. However, it remains unclear if the division of epithelial, interlamellar, chloride or pillar cells was affected (Karlsson 1983). In another study, diverse forms of neoplasia and carcinogenic effects were found in a variety of organs of zebrafish 6 to 12 months after exposure of embryos for 1 h and fry for 24 h to the carcinogenic *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) *via* microinjection and bath exposure, respectively (Spitsbergen et al. 2000b). In the gills, branchioblastoma, chondroma, osteoma, osteochondroma and hemangiosarcoma were found. As a consequence of bath exposure to MNNG, 80 % of the fish showed neoplasia at the highest test concentration. In the present study, a maximum of 22.2 % of the fish exposed to 2 mg/L MMS exhibited (pre-)neoplastic lesions. In general, the abundance of (pre-)neoplastic lesions as well as histological lesions overall increased with MMS concentration, although the fish exposed to 2/0.2 mg/L were much longer in contact with MMS than the fish that were constantly exposed to 2 mg/L and sacrificed already at 169 dpf. This suggests that the frequency of histological aberrations may have decreased in the fish exposed to 0.2 mg/L after the reduction of MMS concentrations.

Concerning the gills, it should especially be taken into account that fusion of secondary lamellae reduces the surface of the respiratory epithelium, easily causing an oxygen deficit. It was shown that lack of oxygen can increase the ubisemiquinone free radical level in the catalytic mechanism of complex III in the respiratory chain (Fleury et al. 2002, Turrens et al. 1985). In water, these radicals lead to the formation of hydrogen peroxide, which can exert further genotoxicity. Thus, histological aberrations in the gills could have increased the genotoxic stress towards the fish.

In the F0 generation, next to eyes and gills, the liver was the most affected organ and showed diverse histological aberrations. The most frequent liver lesions were fatty infiltration (steatosis hepatis) and cystic degeneration (spongiosis hepatis).

Steatosis hepatis is a physiological response to many metabolic disorders and environmental contaminants and can be a pathological manifestation of detoxification (Cormier et al. 1989, Couch 1975, Longo et al. 1993). In general, a macrovesicular type of steatosis hepatis can be distinguished from a microvesicular type. In case of the first, a large triacylglycerol vacuole fills the cytoplasm and displaces the nucleus to the periphery, leading to the characteristic signet ring formation. This type is mostly due to abnormal nutrition (Reddy and Rao 2006). In contrast, microvesicular steatosis is characterized by numerous small lipid droplets in the cytoplasm (Reddy and Rao 2006) or in cisternae of the rough endoplasmic reticulum (Braunbeck et al. 1990) and is mainly caused by genetic or toxin-induced abnormalities in mitochondrial and peroxisomal β-oxidation of fatty acids (Reddy and Rao 2006). Braunbeck and coworkers (1990) described microvesicular steatosis after long-term exposure to lindane. At one test concentration, additionally, hepatic macrovesicular triglyceride droplets and glycogen depletion were observed. This resembles the situation in the present experiment, where macro- and microvesicular steatosis hepatis co-occurred in the same liver samples. Braunbeck et al. (1990) speculated that there is a functional connection between low glycogen contents and strong accumulation of microvesicular lipid. In order to investigate for glycogen depletion also in the present study, sections could have been stained using silver diamine (Singh 1964). Future research should focus on reasons for the co-occurrence of macro- and microvesicular steatosis.

It was suggested that fatty accumulation in the liver could be related to tumorigenesis in presence of a carcinogenic (Cormier et al. 1989). Massive accumulation of lipid was found in hepatocytes of Atlantic tomcod *(Microgadus tomcod)* from the Hudson and Pawcatuck Rivers, which were contaminated with chlorinated hydrocarbons such as DDT, Chlordane and PCBs (Cormier 1986, Cormier et al. 1989, Smith et al. 1979). Fatty accumulation makes available fatty acids and by-products of lipid synthesis. The role of unsaturated fatty acids in carcinogenesis could *inter alia* be due to their susceptibility to oxidation, which gives rise to epoxides and peroxides (Fang et al. 1996, Welsch 1987). In rats, there is evidence that unsaturated fatty acids promote carcinogenesis in the presence of carcinogenic compounds (Carroll and Khor 1971, Chan et al. 1983). Furthermore, it is known that carcinogenic alkylating chemotherapy agents can cause fatty liver in humans (King and Perry 2001, Zimmerman 1978). However, no correlation between a high dietary intake of polyunsaturated fats and an increased risk of breast, colon and prostate cancer has been found in long-time studies on humans (Holmes et al. 1999, Zock and Katan 1998). Altogether, it can be suspected that steatosis hepatis supported the tumorigenic effect of MMS in the present study.

Spongiosis hepatis is a structural alteration of the Ito or perisinusoidal liver cells (Bannasch et al. 1981, Couch 1991). These cells are located in the space of Disse between endothelial cells of the liver sinusoids and hepatocytes. Their main functions are lipid and vitamin A metabolism as well as collagen production and, thus, structural stabilization of the liver. In case of spongiosis hepatis, multilocular cyst-like complexes lacking an epithelial or endothelial lining are present. The cysts are variable in size and arise due to an overproduction or impaired degradation of acid mucopolysaccharides and extracellular proteins such as collagen (Bannasch et al. 1981). In addition, hepatocellular necrosis is suspected to enlarge the cysts (Couch 1991). Most likely, this histological aberration is caused by metabolic disturbances due to hepatotoxic and carcinogenic agents. It is unclear if spongiosis hepatis is directly linked to the occurrence of neoplasia (Bannasch and Zerban 1986, Couch 1991). Couch (1991) observed the transformation of chronic spongiotic lesions to less phenotypically consistent structures with the potential to become metastatic. In rat liver, spongiosis hepatis in association with hepatocellular carcinoma and neoplastic nodules was induced by different nitrosamines (Bannsch et al. 1981, Zerban and Bannasch 1983). Small lesions and cysts containing granular acidophilic material are frequently associated with neoplastic nodules in liver tissue (Bannasch et al. 1981). In fact, all spongiotic lesions in the present study were accompanied by granular material and neoplastic nodules as well (cf. Fig. IV.9 C). Hence, the hypothesis is corroborated that there is an association of spongiosis hepatis and the formation of neoplasia. In addition to rats, spongiosis hepatis has been induced in a number of fish species. For example, Couch (1991) exposed sheepshead minnows *(Cyprinodon variegatus)* for 6 weeks to *N*-nitrosodiethylamine, kept the fish further 140 weeks and finally observed the formation of spongiosis hepatis. In the present study, fish were kept for approximately one year after the onset of exposure. Obviously, this time frame was sufficient to cause the formation of spongiotic lesions in zebrafish. Braunbeck et al. (1992) exposed medaka *(Oryzias latipes)* for 48 h to diethylnitrosamine and observed cases of spongiosis hepatis at least 8 days later. Thus, spongiotic lesions can occur even earlier in fish. As the material inside the cysts was not investigated any further, future studies should make use of staining methods such as Alcian blue staining of mucopolysaccharides according to Gomori (1954), Masson-Goldner and the periodic acid-Schiff reaction.

In isolated cases, fish exposed to 4/0.4 mg/L exhibited liver granuloma in association with spongiosis hepatis and fibrosis (cf. Fig. IV.9 E). Granuloma are inflammatory responses with focal collections of macrophages, epithelioid cells and multinucleated giant cells and are often associated with necrosis (Adams 1976, Williams and Williams 1983). In the literature, granu-

loma formation in the liver has been documented in zebrafish after chronic exposure to perfluoroctane sulfonate, proposedly in consequence of a reduction of the immune response (Keiter et al. 2012) or after tuberculosis infection with *Mycobacterium marinum* (Swaim et al. 2006). Since the fish bearing granuloma were not affected by infectious diseases, granuloma probably represented an immune reaction to tissue alterations due to alkylating stress.

In one individual fish exposed to 2 mg/L MMS, a potential liver tumor in association with a melano-macrophage center was found (cf. Fig. IV.8 F). The altered tissue is clearly spatially differentiated from the adjacent liver tissue, which indicates that it could be a benign neoplasia. Melano-macrophage centers are normally found in hemopoietic tissues, i.e. spleen, kidney and liver. As part of the immune system in fish, occurrence of highly pigmented melano-macrophages is increased under stress conditions, in case of bacterial infections and in regions with chronic inflammatory lesions (Agius 1985, Agius and Roberts 2003, Roberts 1975, Vigliano et al. 2006).

In addition to several other functions, melano-macrophage centers play a role in antigen trapping and presentation to lymphocytes as well as detoxification and deposition of cell debris (Agius and Roberts 2003). It was shown that the centers are suitable as bioindicators for exposure of fish to hypoxia and chemical contamination (Fournie et al. 2001). As melano-macrophages are *inter alia* located in the liver of healthy fish, their presence in the regarded case may be independent from MMS exposure. In order to clarify if fish exhibiting possible (pre-)neoplastic or (pre-)carcinogenic lesions were in fact subject to inflammation and stimulation of their immune system, the concentration of the tumor necrosis factor should have been measured in cell suspensions (Goetz et al. 2004, Hogan and Vogel 2001). In the literature, hepatocellular carcinoma consisting of enlarged cells with low lipid contents and necrosis were found in Atlantic tomcods (*Microgadus tomcod*) from the Hudson River (Cormier et al. 1989). In liver of brown bullheads (*Ictarulus nebulosus*) from the Black River, Ohio, liver tumors were also observed (Fabacher and Baumann 1985). In other studies, zebrafish were exposed *in vivo* to the alkylating carcinogens *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and 7,12-diniethylbenz[a]anthracene (Spitsbergen et al. 2000a,b). Thereby, 9 % of the fish showed hepatocellular adenoma after microinjection, and benign as well as malign hepatocellular tumors were found in 7 % of the fish after bath treatment. Since in the present study only individual fish exhibited comparable alterations, it can again be discussed that a higher incidence would probably have been reached if the fish had been exposed to higher MMS concentrations for a shorter time.

In general, the hematoxylin and erythrosine (HE) stain does only allow for speculations about the cell type of tumor cells. Hence, for all potential tumors in the present study it remains unclear from which cell type they derived (e.g. cf. Figs. IV.9 F and 10 C). This could be clarified using antibodies specifically binding to intermediate filaments of the respective cell types (Miettinen et al. 1984).
In addition to the histological aberrations discussed above, liver regions sporadically showed different staining properties (cf. Fig. IV.10 B), which were in a single case associated with degradation of tissue structure after exposure to 2 mg/L MMS (cf. Fig. IV.10 A). Using the HE stain, the staining color depends on the charge of molecules and the pH value present in cytoplasm and nucleus (Mulisch and Welsch 2010). Therefore, HE-stained paraffin wax sections are adequate for rapid identification of basophilia (Cormier et al. 1989). Liver of female fish is usually stained darker than liver of males due to high content of basophilic endoplasmic reticulum (ER) for vitellogenin production (Islinger et al. 1999). However, as the differences in staining in the present study were found within the livers of individual fish, a shift in the ratio of ER and acidophilic lysosomes and autophagosomes due to cell death could be reflected in the staining of the tissue and thus show MMS-induced tissue degeneration. This suggestion is in line with the potential of alkylating agents to cause a regulated form of necrotic cell death (Zong et al. 2004). Furthermore, as the liver is the most important organ for storage of glycogen, staining with Best´s karmin could have elucidated if there were also glycogen gradients as a sign of metabolic disturbance in the liver regions (Mulisch and Welsch 2010). Moreover, the connective tissue-specific Masson-Goldner stain could have helped identifying present structures of the liver capsule.

In the kidney, degeneration of parenchyma was found, possibly associated with necrosis. This suggestion is supported by the finding that mouse embryo fibroblasts undergo necrosis as a self-determined process when they are exposed to alkylating stress (Zong et al. 2004). In addition, enlargement of renal tracts may result either from a possible tumor-induced atresia of the ureter or from mutations in osmoregulatory genes, e.g. ion and water transport proteins (Kalujnaia et al. 2007). The susceptibility of renal cells to genotoxicity caused by MMS was shown in mice (Oshida et al. 2008).

Underdeveloped testis and lack of mature spermatozoa is in line with the fact that alkylating agents injected in rats reduced fertility and finally led to sterility (Jackson et al. 1959). Furthermore, antifertility effects of MMS accumulating over time have been shown in rats. Hereby, it was stated that these effects were due to inhibition of stem-cell or spermatogonial de-

velopment as well as genetic damage in developing spermatids or spermatozoa (Jackson 1964). Most probably, this is also true for the affected fish in the present study. Amorphous yolk and egg debris found in ovaries of F1 fish resulted from irregular depletion of tertiary follicles. In another study, egg debris was documented in adult zebrafish that had previously been exposed to the endocrine disruptor 2,3,7,8-tetrachlordiebenzo-*p*-dioxin (TCDD) during early life (King Heiden et al. 2009). TCDD exposure also led to fewer vitellogenic follicles, increased atretic follicles and the occupation of 20 % of ovarian tissue by macrophages. In the present study, these effects were not found except for reduction of mature follicles mainly in the F1 generation. In contrast to TCDD, MMS is not known to be an endocrine disruptor. Instead, it can be assumed that MMS exposure has led to mutations during gametogenesis in the F0 generation. Affected genes responsible for gonadal development during early life or for oogenesis may thus have been transferred to the F1 generation, leading to visible histological effects as well as mortality.

In addition to internal histological aberrations, also external effects on tissue structure (cf. Tabs. IV.1 and IV.2) were found at relatively low frequencies when compared to the internal aberrations. External aberrations indicated inflammation or presence of tumors or skin disintegration. In the literature, exposure of zebrafish to ethylnitrosourea was shown to cause the formation of papilloma and skin tumors (Beckwith et al. 2000). In mice, it was demonstrated that MMS plays a considerable role in skin carcinogenesis (Fürstenberger et al. 1989, Haesen et al. 1993). Based on the results of the present study, it can be assumed that this is also true for zebrafish. In addition, scoliosis was found in a number of fish. Scoliosis is supposed to be caused by impairment of the central nervous system, neuromuscular junctions or ionic metabolism (Couch et al. 1977). In zebrafish, it was demonstrated that impairment of synthesis of fibrillar collagen causes scoliosis (Christiansen et al. 2009). It can be speculated that genes responsible for the physiologic function of these systems were affected by MMS in the respective fish in the present experiment.

IV.5.4 Conclusions

The present study clearly documents that chronic exposure to MMS induces both transient and persistent DNA damage as well as a wide variety of histological aberrations including neoplasia in zebrafish. A transfer of DNA mutations to the offspring of exposed fish *via* the gametes is likely. Genotoxic exposure and the inheritability of histological aberrations with the potential to lead to carcinogenesis at the population level most probably have affected

health of the fish in the present study. This is of special concern as wild fish are exposed to genotoxic, mutagenic and carcinogenic pollutants in the aquatic environment. Bearing this in mind, especially eye and liver tumors affecting orientation, feeding and metabolism of fish have the potential to serve as biomarkers for ecological risks.

V

Final conclusions

Against the background of the decline of fish populations in European and North American freshwater, it was the objective of the present thesis to contribute answers to the questions

(1) if there is evidence for a causal relationship between genotoxicity and histopathology on the one hand and inheritability of detrimental effects on survival, reproduction and histology on the other hand,
(2) if there is evidence for reversibility of these effects in the offspring, and
(3) how anthropogenic pollutants in the aquatic environment are involved into the decline of fish populations.

In fish exposed to chronic alkylating stress, comet assay and micronucleus test revealed DNA damage in liver, gills and gonads. Moreover, times of first spawning were delayed and fertility was reduced. In the F1 generation, teratogenic effects were increased and larval survival was reduced. In both the F0 and the F1 generation, histopathological aberrations and external lesions were found as a consequence of direct and parental MMS exposure, respectively. It is evident that genetic damage was transferred to the offspring and caused mortality, developmental effects and histological aberrations. Thus, the present thesis documents that chronic alkylating stress can cause diverse detrimental effects on health and reproduction in zebrafish.
Previously, it was shown that continuous multi-generational genotoxic exposure of fish can cause a decrease and even extinction of populations (Diekmann et al. 2004a). In contrast, in the present study, exclusively the F0 generation was exposed to genotoxic stress, which allowed for recovery of fertility capacities in the F1 generation and larval survival and development in the F2 generation. Hence, the present study opens up the perspective of recovery for fish at least two generations downstream exposure. Transferring this perspective to the field, a reduction of anthropogenic pollution may give surviving fish populations the possibility to improve their health status and reproduction capacities.
The comparison of data from experiments using acute and chronic exposure reveals an important finding: Whereas test concentrations of 2 – 8 mg/L MMS did not cause mortality in the acute test scenario with adult fish exposed for two weeks (chpt. II), the same concentrations caused mortality and inability of fish to spawn using chronic exposure (chpt. III). This demonstrates the difficulty to extrapolate toxicological hazards of chronic xenobiotic exposure from acute toxicity data (Nagel and Isberner 1998) as well as to transfer information on susceptibility to toxicity of adult fish to larval and juvenile stages.

The finding of a shift of sex ratio towards females in the offspring of exposed fish gives rise to further questions. It is unclear how an alkylating agent can have this effect, which is know

for other groups of compounds (chpt. III, Gross-Sorokin et al. 2006, Sonnenschein and Soto 1998, Vos et al. 2000). Therefore, effects of parental alkylating or in general genotoxic exposure on sex ratio in the offspring deserve further investigation.
In the present thesis, spawning groups comprehended 3 fish per sex in order to avoid masking of effects on fertility due to imbalanced numbers of fish per sex in a spawning group. Thus, a definite genealogy indicating which individual fish descended from which individual is not provided, and some population relevant effects in the F1 and F2 generation may be confounded by inbreeding. In order to avoid any possible inbreeding, future research should aim at obtaining spawn from definite parents. In addition, White and co-workers (1999) recommended that F1 fish should be cross-bred within families as well as between families having the same exposure background. Future research should also aim at quantifying inbreeding effects.
The current thesis correlates a considerable number of endpoints, namely genotoxicity, especially in the gonads, growth, fertility, survival, development and histopathology. However, there are even more links between the generations that should be taken into account by future research elucidating the reasons for the decline of fish populations, e.g. sperm motility, which can be examined *via* computer-assisted sperm analysis (Kime et al. 2001, Wilson-Leedy and Ingermann 2007).

In general, it should be kept in mind that there is always a factor of uncertainty when data from laboratory experiments are transferred to the field situation. Thus, laboratory studies can only complement the investigation of health and reproductive potential of fish over the generations at known pollution hotspots or random sites in the aquatic environment.
There is a number of field studies on genotoxic pollution of aquatic ecosystems and affected health of fish exposed in their natural habitat (Bätscher et al. 1999, Burkhardt-Holm et al. 2002, Cormier 1986, Cormier et al.1989, Malins et al. 1988, Möller 1988, Munns et al. 1997, Myers et al. 1987). Taking into account the results of several laboratory studies (Anderson and Wild 1994, Diekmann et al. 2004a,b, Munns et al. 1997, White et al. 1999) including the present thesis and the field situation, weight-of-evidence suggests that a causal relationship between genotoxicity and inheritable impairment of fish populations is likely, which may well manifest in the current fish decline.
In the European Union, a reduction of chemical pollution can be anticipated as a consequence of the current EU regulation REACH as well as the Water Framework Directive (cf. cpt. I.7). These regulations will considerably extent our knowledge on the (eco-)toxicological potential of chemicals and restrict the production, use and release of chemicals dangerous to human

health and wildlife (ECHA 2011, Rovida et al. 2011). However, genetic diversity is reduced in the first place as past and ongoing genotoxic pollution has a selective effect and reduces the overall genetic diversity of fish populations by extinguishing phenotypes sensitive to genotoxic stress (Castaño et al. 2003).
Likewise, other biotic factors in aquatic ecosystems, e.g. food webs, require recovery. Abiotic factors need to be taken into account as well. Since many pollutants bind to sediments (Keiter et al. 2006) and have done so for many decades, a reduction of the discharge of pollutants into the aquatic environment will probably take time and will not immediately improve development and reproduction of wild fish populations.

Finally, the present thesis does not only increase our understanding of diverse detrimental effects of long-term genotoxic exposure to fish. It may also contribute to an increased awareness of more sustainable ways of stewardship of natural resources and wildlife.

VI

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