universität wien

# DIPLOMARBEIT

Titel der Diplomarbeit

## DIFFUSION OF TOXINS INTO MEDAKA EMBRYOS

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)



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# 1 INTRODUCTION

## 1.1 MEDAKA FISH - *ORYZIAS LATIPES*

### 1.1.1 General features of medaka fish

Medaka fish (Oryzias latipes) is a small, egg laying teleost, which can be found in fresh and brackish water, as for example in paddy fields, which it inhabits mainly in Japan (Naruse et al., 2004). Its eggs are known to be resistant to a wide range of variations in temperature and salinity (Lynn Lamoreux et al., 2005). A photoperiod of 13 hours ensures an ovulation 1 hour before light appearance every morning. The embryonic development, from fertilization up to hatching, is completed within 10 days, assuming a temperature of 26 ℃ (Iwamatsu, 2004). With focus on certain distinct features and progresses during development, Iwamatsu subdivided the embryonic development of medaka fish into 39 stages. Medaka is especially suited for observation of the fish embryogenesis, because its eggs stay transparent during the whole process.

### 1.1.2 The chorion

In contrast to zebrafish, during its embryonic development the medaka fish is well protected inside a tough chorion (Furutani-Seiki and Wittbrodt, 2004). The glycoproteins, ZI-1,2 (74-76-kD) and ZI-3 (49-kD), of which the inner layer of the oocyte chorion mainly consists, become crosslinked after fertilization (Iuchi, 1995). Prior to this chorion hardening process, the ZI-1,2 is cleaved into proteins of 58-62-kD, as described by Iuchi and colleagues.

Furutani-Seiki and Wittbrodt described that the chorion can be a problem for the efficiency of several applied methods for manipulation, like for example bead implantations, but the dechorionization with hatching enzyme after epiboly, after which the embryo shows still a normal development, ensures the efficiency of the method (Furutani-Seiki and Wittbrodt, 2004).

### 1.1.3 The role of medaka fish as model system for vertebral development

Medaka fish is a favorable model system for studies of developmental and evolutionary processes on molecular basis. Because of the high amount of mutant stocks available, the possibility to determine a lot of gene functions is provided. Therefore, medaka genomics is important to find novel genes and to understand pathways in vertebrate organisms. Medaka has a lot of advantages in common with zebrafish for the use as laboratory model, but advantages of its own, too. As for example, there are different fertile, highly polymorphic inbred strains available, which provide the possibility of mutagenesis screening and genetic mapping (Naruse et al., 2004).

However, there are also differences between the two model organisms. During their evolution, the medaka and zebrafish genome was widely duplicated. Therefore, several mutations of the same loci seem to have different or unique effects, which provide the possibility to decipher their species-specific patterns. Medaka is the only fish so far, in which only one single genetic locus, namely DMY, determines the sex. Therefore it's the only fish organism in which the sex determination is the same as in mammals. So the molecular basic of sex determination can be researched by using this model system. The medaka fish genome is fully sequenced (800-Mb) and genetic and physical maps are available (Roest Crollius and Weissenbach, 2005).

## 1.2 PUROMYCIN

### 1.2.1 Puromycin - a selection marker *in vitro*

The antibiotic puromycin is widely used in cell culture. Because of its translation-inhibiting effect (Azzam and Algranati, 1973), it is used as a selection marker for transfection (Iwaki and Castellino, 2008) and for various cell lines of certain animals, like mouse and rabbit (Croons et al., 2008). It is also used for inhibition of several puromycin-sensitive aminopeptidases to determine their functions (Karsten et al., 2006). Alternatively to its common use *in vitro*, no application *in vivo* is known.

### 1.2.2 Puromycin - a selection marker *in vivo*?

The antibiotic puromycin as a selection marker *in vivo* would bring a lot of advantages. An example would be the selection of transgenic fishes in mutagenesis screening. Constructed plasmids containing the modified gene of interest and the puromycin resistance gene would be injected into the early medaka fish embryos (one- or two cell-stage) by DNA- microinjection. In most cases, transient expression would occur. But in few cases, a random insertion into the medaka genome would happen, and therefore the embryo would gain the puromycin resistant gene *pac* permanently. The selection of these transgenic embryos would then occur by addition of a certain concentration of puromycin into the embryo rearing medium (ERM), where the embryos would be put after the DNA-microinjection treatment. This certain concentration of puromycin would then cause the death of all non-transgenic embryos. For the establishment of stable transgenic lines, this process would be repeated with embryos of each next F-generation.

The goal of this diploma thesis was to find the right conditions for an establishment of the antibiotic puromycin as selection marker for transgenic medaka fish embryos *in vivo*.

# 2 MATERIALS AND METHODS

## 2.1 IN VIVO TECHNIQUES

### 2.1.1 Medaka fish embryos – treatment with sodium azide

Preparation of the fertilized medaka fish eggs

Medaka fish eggs were collected after mating and transferred into a Petri dish containing 1xERM. To avoid a temperature shock, 1xERM at room temperature was used instead of pre–cooled.

To separate the eggs, they were rolled gently on a Whatman paper. Remaining traces of attachment filaments was removed manually using tweezers and a dissecting microscope. Fertilized and living embryos were separated into groups of about 20 and each of these groups was transferred together with the 1xERM into a well of 6–well plates, using plastic Pasteur pipettes. The amount of groups per 6–well plate accords with the planned experimental approach. The 6–well plates were put into the incubator at 27 ℃ until the medaka fish embryos reached the aimed developmental stage.

Preparation of the sodium azide concentrations

A stock of sodium azide with a concentration of 20 g/L in dd$H_2O$ was prepared. Out of this stock, the sodium azide concentrations needed by the different experimental approaches, were prepared by dilution into dd$H_2O$ to a final volume of 4 ml into 15 ml tubes. Each tube was vortexed for several seconds to guarantee a homogenous solution. The different sodium azide concentrations were prepared shortly before use and put at room temperature.

Sodium azide treatment and incubation time

The 6–well plate containing the groups of about 20 wild type embryos was taken out of the incubator. The 1xERM was sucked off by a water jet vacuum pump, and was replaced by the prepared concentration of sodium azide with a volume of 4 ml per well. Depending on the experimental approach, one or several wells were incubated with the same or different concentration of sodium azide, and the incubation time differed between exactly one or two hours. During this time, the 6–well plate was put on the rocker at a gentle pace.

After the incubation time, the solution was sucked off by a water jet vacuum pump and was replaced by 1xERM. After repeating this process three times as washing steps, the embryos were transferred into new wells containing 1xERM with a plastic Pasteur pipette.

Recording the death rate values

Concerning their death rate, the medaka fish embryos were monitored immediately after the washing steps under a dissecting microscope. The dead embryos were noted and removed. The survived ones were put back in the incubator at 27 ℃. Each following 24 hours, up unt il 196 hours, the monitoring was repeated and the 1xERM was replaced with a fresh one. Each time, the dead embryos were noted and removed.

The death rate hardly changed after the first 48 hours. Because of this observation, the values taken for the figures were death rate values of 48 hours or later.

### 2.1.2 Dechorionization – treatment with hatching enzyme

Plate preparation

The wells of a 6-well plate were covered with 1,5 % agarose, dissolved in embryo rearing medium, 1xERM. After a short cooling time, the screwcaps of a 1,5 ml test tubes were put in the middle of each well, upside down. The wells were filled with 1,5 % agarose/1xERM media until the the screwcaps were almost covered. After cooling, the screwcaps were removed and the small well inside the hardened agarose filled with 1xERM until needed.

Dechorionization

After the 1xERM was removed completely out of the small wells, and the embryos which were to be dechorionized were put into the wells. It is important that the embryos won't lie on top of each other. Aliquots of 100 µl hatching enzyme solution were transferred into the small wells, in which the embryos now were fully covered. For protection against dehydration, a small piece of filter paper was soaked with water and was laid next to each small well. The 6-well plate was closed with its lid and was put into the incubator at 27 ℃. The embryos were firstly observed after 30 minutes and then each following 15 minutes, for the appearance of small craters in the chorion. When the hard layer of the chorion was dissolved completely, the reactions were stopped by addition of 1xERM to the small wells.

The soft inner layer of the chorion was now carefully removed with 2 sharp tweezers. Because of the observation that the dechorionized embryos must not get in contact with air or hard plastic, the transfer was done by using plastic Pasteur pipettes.

### 2.1.3 Microinjection in medaka fish

Preparation of the Petri dish

1,5 % agarose was dissolved in 1xYamamoto's by using the microwave oven. The heated medium was filled into a medium-sized Petri dish and covered the dish for a few millimetres. A mold with 6 furrows was added immediately to the liquid media to create furrows when cooled down. After the plate has cooled down and the mold has been removed carefully, the plate was filled with 1xYamamoto's and stored at 4 ℃ until injection.

Preparation of injection needles

Capillaries of 1 mm (Clark Electromedical Instr., GC100-10 borosilicate glass capillaries, standard wall without filament, 1.0 mm O.D. x 0.58 mm I.D) were pulled on a Sutter needle puller. Following settings were used: pressure 800, heat 560, pull 50, vel 80, time 200.

Preparation of the injection solution and injection

Medaka eggs were transferred to the pre-cooled 4 ℃ prepared Petri dish immediately after collection and separation, to arrest their development. The construct of interest was mixed with 1xYamamoto's and transferred to the glass capillary needle. An estimated volume of less than 10 % of the cytoplasm volume was injected through the chorion into the embryos of the one- or two-cell stage. For the right orientation of the embryos and to open the capillary needle, tweezers were used. The pressure settings for the Eppendorf microinjector were about 80-100 hPa holding pressure and 300-400 hPa injection preassure, depending on the actual diameter of the needle opening.
After injection, the Petri dish was filled with 1xERM instead of 1xYamamoto's and put into the incubator at 27 ℃.

### 2.1.4 Collection of medaka fish eggs

Collection of the eggs

First, a male was transferred to each fish tank which contained usually 3 females. The room was left for exactly 30 minutes. After this time, the embryos were collected from the belly of the females into a Petri dish containing pre-cooled 1x ERM or 1x ERM at room temperature, depending on which experiment was done.

Separation of the eggs

The eggs were separated by rolling them gently on a Whatman paper. The remaining attachment-filaments were removed with tweezers. The eggs were transferred back into the Petri dish and stored in the incubator at 27 ℃ until needed.

### 2.1.5 Fish strain and breeding conditions

The medaka strain Cab was used in all experiments. As constant conditions, a light period of 14 hours and a darkness period of 10 hours were maintained at a temperature of 28 ℃.

## 2.2 SOLUTIONS

### 2.2.1 1xERM

(Autoclave; room temperature)

1xERM; 1L:

NaCl 17 mM

KCl 0,4 mM

$CaCl_2$ x $H_2O$ 1,0 mM

$MgSO_4$ x $7H_2O$ 0,65 mM

### 2.2.2 10xYamamoto's

(No sterilization; room temperature)

10xYamamoto; 1L

NaCl 1,28 M

KCl 27 mM

$CaCl_2$ 14 mM

$NaHCO_3$ 2,4 mM

adjust to pH 7,3

### 2.2.3 Puromycin

(PAA; P11-019)

Formula: $C_{22}H_{29}N_7O_5$ x 2HCl

Molecular Weight: 544,2 g/mol

### 2.2.4 Sodium Azide

(Carl Roth; K305.1)

Formula: $NaN_3$

Molecular Weight: 65,01 g/mol

### 2.2.4 Triton X-100

(Carl Roth; 3051.2)

Formula: $C_{33}H_{60}O_{10}$

Molecular Weight: 624 g/mol

# 3 RESULTS

## 3.1 PUROMYCIN

### 3.1.1 Puromycin survival experiments with wild type medaka fish embryos

The first step to establish a method for puromycin as selective marker for medaka fish would be to find the minimal concentration of puromycin, which would cause a 100 % death rate in embryos which do not express the resistance gene. In my first experiment, I varied the concentration of puromycin in the fish growth media, the embryo rearing medium (ERM) from 10 µg/ml to 100 µg/ml. For comparison, the concentration used in cell culture experiments ranges from 1-10 µg/ml.

After collecting, 3 groups of 12 embryos each (blastodisc stage) were selected under a dissecting microscope and transferred into 3 wells of a 6-well plate, containing ERM. The plate was put in the incubator at 27 ℃. 5 hours post-fertilization (between early and late morula stage), two different concentrations of puromycin (10 µg/ml or 100 µg/ml), were added to the ERM of 2 wells. The proper concentration of puromycin for each experiment was diluted from a stock solution of 100 mg/ml. In the third well, an untreated wild type control group was incubated. Each well had an overall volume of 4 ml. After a short time on the rocker at room temperature, to ensure a homogeneous distribution, the plate was put back into the incubator at 27 ℃. The embry os stayed in the solution for 48 hours. They were monitored every 24 hours, at which time the medium was renewed and dead embryos were removed to ensure they didn’t distort the results. The death rate values after 48 hours are shown in Figure 1.

| Puromycin [µg/ml] | Number of Embryos | Dead Embryos after 48 h | Death Rate (%) |
|---|---|---|---|
| 0 | 12 | 5 | 42 |
| 10 | 12 | 4 | 33 |
| 100 | 12 | 12 | 100 |

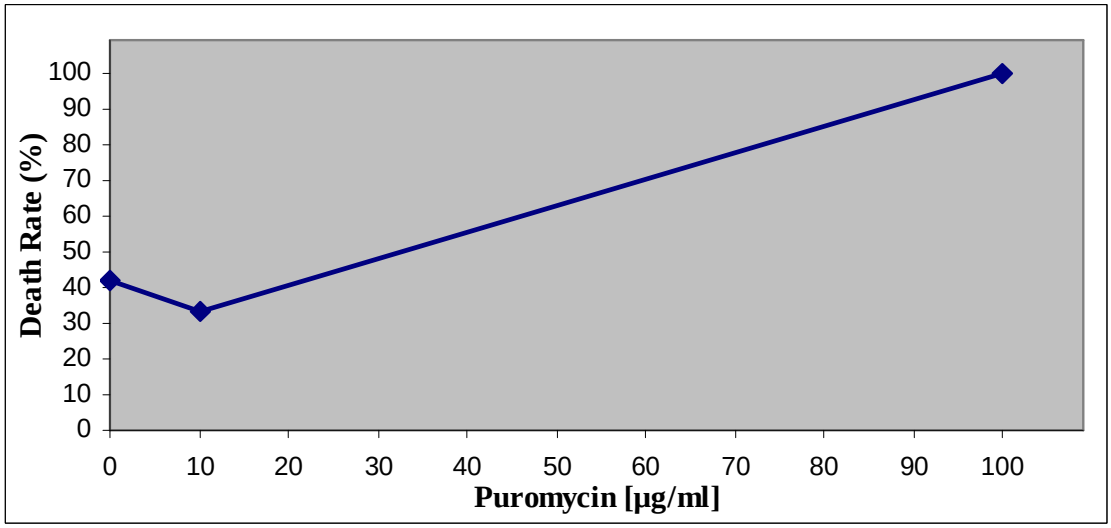



FIG. 1. **Changes in the death rate of wild type medaka fish embryos, after 48 hours of treatment with different concentrations of puromycin.** Embryos 5 hours post-fertilization were incubated with the indicated concentration of puromycin. Dead embryos were counted after 48 hours and the death rate determined. Detailed results of the experiment are shown in the table; a graphic presentation of the data is shown below.

The death rates for puromycin treated embryos were 30 % and 100 % for 10 and 100 µg/ml, respectively. The concentrations of puromycin used in this experiment were 10 to 100 fold higher than normally used in cell culture to kill wild type mammalian cells. However, the wild type control group of embryos showed an abnormal high death rate, which challenged the results of the experiment. In the following experiments, a death rate of 100 % at a concentration of 100 µg/ml could not be reproduced, not even at higher puromycin concentrations. Therefore, the applied concentration of puromycin most probably did not suffice to kill the embryos.

The envelope of the embryo, the chorion, could play an important role in diffusion of puromycin because of its main role of protecting the developing embryo as a mechanically barrier. Iuchi and colleagues showed that within 6 hours at room temperature, the elasticity and therefore the chorion hardening, correlated to the egg toughness reached its maximum value (Iuchi, 1995). The uptake of small molecules by the medaka fish embryo could be limited by the

chorion. There could be differences in the diffusion rates through the chorion, which could cause the differences in death rates observed in the puromycin experiments. The treatment of medaka embryos at a very early age, before the chorion hardening reached its maximum 6 hours after fertilization, could be of interest. Compared to the treatment of older embryos, it could enable a better diffusion of puromycin, and therefore a higher death rate. In the next experiments, the embryos were therefore treated with puromycin at different times shortly after fertilization (Fig. 2). The treatment of medaka fish embryos at 40 minutes to 2 hours post-fertilization should cover time points before the chorion would finish hardening.

The general experimental approach was similar to the first experiment, but this time the concentration of puromycin ranged from 80 µg/ml to 320 µg/m, which is 80 to 320 times higher than the concentration normally used in cell culture. More embryos were required, because of the fact, that medaka fish embryos at 4 different ages were compared at 3 different concentrations of puromycin, including the wild type control group without puromycin. Small groups of embryos, from 12 to 20, were put into wells of 6-well plates after collecting, containing ERM. They were put into the incubator at 27 ℃ until they reached their right age. Then the media were replaced by the different mixes of puromycin and ERM, with an overall volume of 4 ml per well. After a short time on the rocker, to ensure a homogenous distribution, the embryos were put back into the incubator. Again, the death rate values noted after 48 hours of treatment were taken (Fig. 2). To be able to treat 40 minutes old embryos with puromycin, it was necessary to prepare the solution during the mating and put them into it immediately after collection and rolling. Dead and unfertilized embryos were then removed afterwards under the dissecting microscope. This caused a variation in the number of embryos used for the experiment.

| Age of Embryos before treatment | Puromycin [µg/ml] | Number of Embryos | Dead Embryos after 48 h | Death Rate (%) |
|---|---|---|---|---|
| 40 minutes post - fertilization | 0 | 20 | 0 | 0 |
| | 80 | 14 | 11 | 79 |
| | 320 | 12 | 9 | 75 |
| 60 minutes post - fertilization | 0 | 20 | 0 | 0 |
| | 80 | 20 | 15 | 75 |
| | 320 | 20 | 10 | 50 |
| 90 minutes post - fertilization | 0 | 20 | 4 | 20 |
| | 80 | 20 | 6 | 30 |
| | 320 | 20 | 10 | 50 |
| 120 minutes post -fertilization | 0 | 20 | 0 | 0 |
| | 80 | 20 | 4 | 20 |
| | 320 | 20 | 15 | 75 |

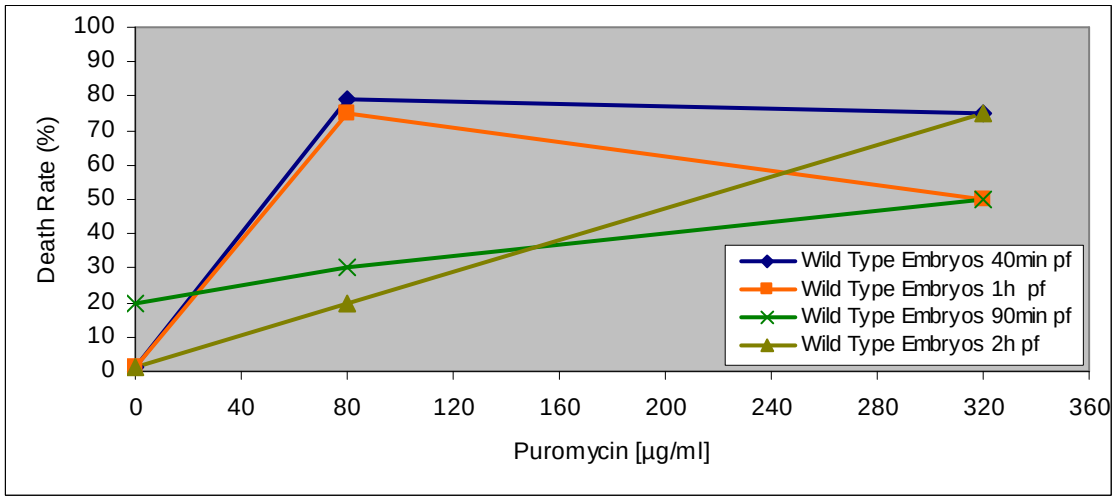



FIG. 2. **Comparison of changes in the death rates values of wild type medaka fish embryos, after 48 hours of treatment with different concentrations of puromycin.** Embryos 40, 60, 90 and 120 minutes post-fertilization were incubated with the indicated concentrations of puromycin. The death rate values were observed every 24 hours, the solutions renewed and dead medaka fish embryos removed. Dead embryos were counted after 48 hours and the death rate determined. Detailed results of the experiments are shown in the table.

This experiment showed no evidence that the death rate of medaka fish embryos after treatment with puromycin is higher at earlier developmental stages. Even though the comparison of the death rate values at 80 µg/ml puromycin would indicate such a trend, the values at the higher concentration of 320 µg/ml were similar. The results showed variations and no death rate of 100 %, even by using higher puromycin concentrations than in the first experiment (Fig. 1). Therefore, different stages of chorion hardening showed little effect on the death rate.

If this might be because the puromycin concentrations are still to low or puromycin hasn't a strong toxic effect on medaka fish cells, or since the chorion or other barriers and protection mechanisms still prevent diffusion, is unclear.

### 3.1.2 Puromycin survival experiments with medaka fish embryos after DNA-microinjection.

The treatment of medaka fish embryos with puromycin, after DNA microinjection of the DNA construct containing the puromycin resistance gene *pac*, would be another important test to establish a method for puromycin as selective marker. The DNA microinjection of the resistance gene should make these transgenic embryos viable in the media, which would contain a certain concentration of puromycin. In contrast, embryos without DNA microinjection should be killed.

The puromycin survival experiments so far did not result in a sufficient uptake of puromycin to kill the embryos. However, the penetration of the chorion by the glass capillary needle during DNA-microinjection might be able to improve the puromycin uptake. This mechanical disruption of the chorion could cause a higher death rate, because of the possibly altered diffusion rate of puromycin through the created hole into the embryo, or through the additional stress caused by the injection. For the next experiments, a DNA expression plasmid for gfp (pSAgfp) was injected into embryos at the one-cell-stage at a concentration of 80 ng. With this construct, it was possible to screen the embryos under the UV-microscope after DNA-microinjection and gfp-positive embryos were selected for further treatment with puromycin. The results of their death rate values after 48 hours of puromycin treatment were compared to that of embryos treated with puromycin at the same age without DNA-microinjection.

Two experiments with different concentrations of puromycin were done and compared (Fig. 3). In each experiment, a group of medaka embryos was treated with puromycin after DNA-microinjection, and compared with embryos, which were treated with the same concentrations of puromycin, but without injection. Each experiment had two control groups. These two groups weren't treated with puromycin, but in one group the construct was also injected. Following the DNA-microinjection protocol, the embryos were put in the incubator at 27 ℃ for 3 hours, to recover from the microinjection process. After removing dead ones

caused by the injection process, the embryos were screened for gfp-positive embryos under the UV-microscope. They were transferred in groups of 10 embryos each into 6-well plates containing ERM. Then the puromycin was added to the ERM of the plates. Different concentrations of puromycin, from 5 to 500 µg/ml, were used. After a short time on the rocker (at room temperature) to ensure a homogenous distribution, the plate was put back into the incubator at 27 ℃. Each well had an overall volume of 4 ml. The proper concentration of puromycin for each experiment was diluted from a stock solution of 100 mg/ml. After 24 hours, the embryos were monitored concerning their death rate and the solution was renewed. Dead embryos were removed to ensure they didn't distort the results. The values noted after 48 hours of incubation in the puromycin solution are shown (Fig. 3). Embryos at the same developmental stage were also treated with puromycin for comparison. To ensure reproducibility, they remained at 4 ℃ for the same time as the embryos used for DNA-microinjection, and were also put at 27 ℃ for 3 hours before applying the identical puromycin treatment.

a

| Age of Embryos before treatment | Puromycin [µg/ml] | Number of Embryos | Dead Embryos after 48 h | Death Rate (%) |
|---|---|---|---|---|
| 5 hours post-fertilization | 0 | 14 | 2 | 14 |
| | 5 | 14 | 2 | 14 |
| | 10 | 14 | 3 | 21 |
| | 20 | 14 | 1 | 7 |
| | 40 | 14 | 1 | 7 |
| | 80 | 14 | 3 | 21 |
| | 160 | 14 | 1 | 7 |
| 5 hours post-fertilization (+) DNA-microinjection | 0 | 10 | 6 | 60 |
| | 5 | 10 | 3 | 30 |
| | 10 | 10 | 1 | 10 |
| | 20 | 10 | 5 | 50 |
| | 40 | 10 | 3 | 30 |
| | 80 | 10 | 1 | 10 |
| | 160 | 10 | 5 | 50 |

b

| Age of Embryos before treatment | Puromycin [µg/ml] | Number of Embryos | Dead Embryos after 48 h | Death Rate (%) |
|---|---|---|---|---|
| 5 hours post-fertilization | 0 | 10 | 3 | 30 |
| | 80 | 10 | 3 | 30 |
| | 160 | 10 | 5 | 50 |
| | 320 | 10 | 1 | 10 |
| | 500 | 10 | 6 | 60 |
| 5 hours post-fertilization (+) DNA-microinjection | 0 | 10 | 5 | 50 |
| | 80 | 10 | 5 | 50 |
| | 160 | 10 | 7 | 70 |
| | 320 | 10 | 7 | 70 |
| | 500 | 10 | 8 | 80 |

c

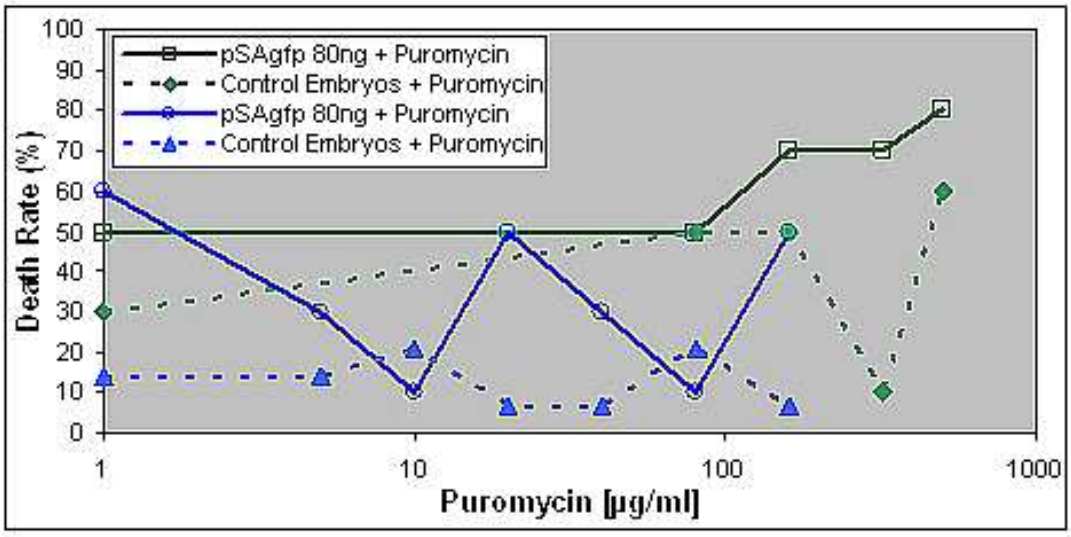



FIG. 3. **Changes in the death rate of medaka fish embryos treated with puromycin after DNA-microinjection.** Two experiments were merged together (c): In the first experiment (a), shown as blue graphs, the highest concentration of puromycin was 160 µg/ml and in the second experiment (b), shown as green graphs, 500 µg/ml. The interrupted lines show the group of medaka fish embryos without DNA microinjection before treated with puromycin at the same conditions.

Concerning their death rates, the results of both experiments showed high variations. The highest concentration of puromycin, 500 µg/ml, was 500 fold higher than that used in mammalian cell culture to kill wild type cells. No death rate value of 100 % could be reached. In both experiments, the medaka fish embryos treated with puromycin after DNA-microinjection reached not always a higher death rate than the embryos treated with puromycin without the previous application of DNA-microinjection. A high death rate of the microinjection control group in both experiments could be observed. This indicates an overall higher death rate of embryos caused by application of the DNA-microinjection method, even without puromycin. So far, it could not be shown if the stress due to the DNA-microinjection, or the penetration of the chorion during this process by the glass capillary needle, or experimental variations affect the death rates. Similar to the results in chapter 3.3.1, it could not be shown if there is a diffusion of puromycin into early medaka fish embryos, or if a certain concentration of puromycin could cause the strong effect on medaka embryo cells needed for a 100 % death rate. The experiments done in chapter 3.2.2 could not clearly show if the chorion has any influence concerning the diffusion rate of puromycin into the embryo. It is most likely the case, that the penetration of the chorion during the DNA-microinjection is repaired shortly afterwards. Further experimental approaches for the treatment of medaka fish embryos with puromycin are necessary to clarify these questions.

### 3.1.3 Injection of puromycin into early blastula stage medaka fish embryos.

The medaka fish embryos seem well protected against external influences, as showed by various toxicity-publications. One reason for not reaching a 100 % death rate, even at very high concentration of puromycin, up to 500 fold of cell culture concentrations, would be a reduced uptake into the medaka fish embryo. A solution to avoid the chorion as a possible barrier for the diffusion of puromycin would be the direct injection of different concentrations of puromycin into embryos at the early blastula stage, about 6 hours and 30 minutes post-fertilization. At this stage, it is possible to inject the puromycin-solution by using

the microinjection glass capillary needle, through the chorion and in between the cells of the blastoderm. For making the injection-solution visible, 0,1 % phenol-red was added. As a control group, wild type embryos at the same age without any treatment were observed.

Wild type medaka fish embryos were collected and put at 17 ℃ over night. They reached the early blastula stage (stage 10) in the next morning and were ready for injection of the different puromycin solutions. After injection, the embryos were observed under a dissecting microscope, and embryos which showed no homogenous distribution of the solution, indicated by the phenol-red, and embryos which had been damaged during the microinjection-process were removed. The embryos were put in the incubator at 27 ℃ and observed every 24 hours. The death rate values were noted, and the death rate values 48 hours after the injection were used for the evaluation (Fig. 4).

| Injection Solution | Puromycin [10 µg/ml] | Injection Solution | Puromycin [100 µg/ml] | Puromycin [900 µg/ml] |
|---|---|---|---|---|
| Puromycin [100 µg/ml] | 1 µl | Puromycin [1 mg/ml] | 1 µl | 9 µl |
| Phenol-Red 1% | 0,1 µl | Phenol-Red 1% | 0,1 µl | 0,1 µl |
| 10x Yamamoto's | 1 µl | 10x Yamamoto's | 1 µl | 1 µl |
| ddH2O | 7,9 µl | ddH2O | 7,9 µl | - |
| Total Volume | 10 µl | Total Volume | 10 µl | 10,1 µl |

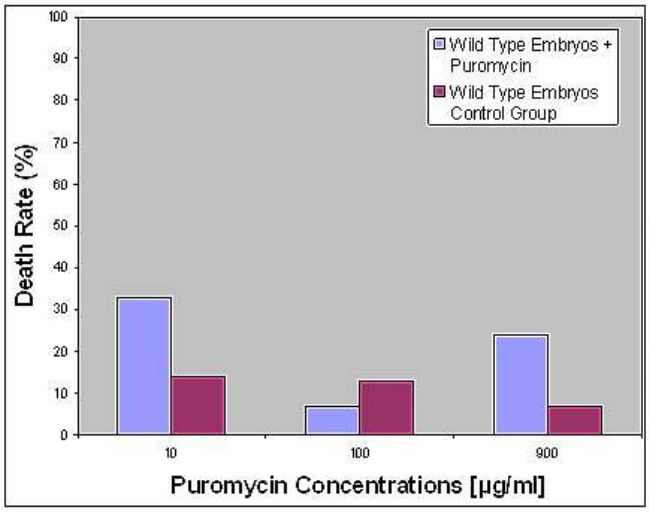
TABLE I. **Contents and volumes of the injection solutions used in the experiments.**

After injection, the puromycin concentrations have to be seen as relative concentrations, because of the fact that they would be diluted further due to the cytoplasm volume of the cell.

a

| Stage of Embryos before injection | Puromycin [µg/ml] injected | Number of Embryos | Dead Embryos after 48 h | Death Rate (%) |
|---|---|---|---|---|
| 10 - early blastula | 0 | 7 | 1 | 14 |
| | 10 | 12 | 4 | 33 |
| 10 - early blastula | 0 | 8 | 1 | 13 |
| | 100 | 15 | 1 | 7 |
| 10 - early blastula | 0 | 30 | 2 | 7 |
| | 900 | 21 | 5 | 24 |

b




c

FIG. 4. **Injection of puromycin into medaka fish embryos at early blastula stage.** Death rates of treated medaka fish embryos, indicated as the blue bars, are compared to their wild type control, indicated as the purple bars (b). Detailed results of the experiments are shown in the table (a). Visualization of the injection-solution through addition of 0,1 % phenol-red, showing a homogenous distribution (c).

The experiments showed very low death rates compared to the concentration used. Because of the fact, that the lowest concentration of puromycin injected showed the highest death rate, no toxic effect could be shown. The experiments showed no evidence that the chorion would be a limiting/inhibiting factor, which would prevent a sufficient diffusion rate and therefore a death rate of 100 % at high concentrations of puromycin. All the experiments done in chapters 3.1.1-3.1.3 showed high variations in their results, and it can be questioned if puromycin can really diffuse properly into the medaka fish embryo. The 100 % death rate was only reached once, in the first experiment, and could never be reproduced. However, if the puromycin itself has a strong toxic effect/impact on the early medaka fish embryo cells, could not be shown, too. Or the amount of concentration of puromycin used in the experiments was simply too low. In this case, the costs of further experiments would be too high, additionally to the limiting factor of solubility.

Further diffusion experiments with puromycin by additional protease K treatment, or dechorionization of the early medaka fish embryos by treatment with hatching enzyme, or treatment with trypan blue (data not shown), could not show any effect on the death rate, either. Puromycin does not seem to have the qualities needed for use as a selective marker for establishing transgenic lines, or the qualities needed for studying the characteristics of diffusion of toxic substances into the early medaka fish embryo.

## 3.2 ETHANOL

Based on the limitations of the puromycin experiments, the main question was if diffusion through the chorion and/or other membrane barriers into cells of the medaka fish embryo would be possible with other toxic substances. The characteristics of such a toxin should be availability, in terms of volume and concentration, and the price. Ethanol was chosen for the following experiments, to show effects on the medaka fish embryo after treatment. Ethanol is known for its toxic effects during development and has a variety of suggested mechanisms how it could damage the organism (Goodlett et al., 2005).

First it would be important, that the embryos would reach a death rate of 100 % after the treatment with high concentrations of ethanol. In these first experiments (data not shown), Medaka fish embryos between 45 minutes and 24 hours old were treated with different concentrations of ethanol, from 50 % to 1 %, as described in the text below. The embryos were incubated with ethanol only for 2 hours, before they were washed and put back into normal ERM. The first experiments showed that longer incubation times were too toxic. The concentration of 5 % ethanol showed high death rates at early developmental stages and none at later ones. This difference could be because the protection of the early embryo by the chorion is not fully established, or because the effect of toxic substances on earlier developmental stages is more crucial than on later ones. Therefore, the concentration of 5 % ethanol was chosen for further experiments (Fig. 5).

Medaka fish embryos were collected after the mating process and put into a Petri dish containing ERM at room temperature. After separation of fertilized eggs from the dead and unfertilized, small groups of embryos were transferred into 6-well plates also containing ERM, and were put into the incubator at 27 ℃. Exceptions were the experiments with medaka fish embryos at an age of 45 minutes post fertilization. The ethanol solution was added directly after transfer into the 6-well plate, without putting them into the incubator. When they reached their intended age, the different ethanol concentrations were added from a 100 % stock to the embryo rearing medium (ERM) of the well at room temperature to a total volume of 4 ml per well. For the incubation time, they remained on the rocker to ensure homogenous distribution. After the incubation, the death rate was observed under a dissecting microscope, the solution was replaced, the embryos washed at least 3 times with ERM, and then put into new Petri dishes at 27 ℃, containing again fresh ERM. After 24 hour s, dead embryos were noted and removed, and the solution renewed. The death rate values after 48 hours were used (Fig. 5).

| Age of Embryos before treatment | Incubation Time (h) | EtOH (%) | Number of Embryos | Dead Embryos after 48 h | Death Rate (%) |
|---|---|---|---|---|---|
| 45 minutes | 2 | 5 | 27 | 27 | 100 |
| 45 minutes | 2 | 5 | 21 | 21 | 100 |
| 1 hour | 2 | 5 | 13 | 13 | 100 |
| 1 hour | 2 | 5 | 15 | 15 | 100 |
| 2 hours | 2 | 5 | 25 | 25 | 100 |
| 3 hours 30 minutes | 2 | 5 | 22 | 11 | 50 |
| 4 hours 30 minutes | 2 | 5 | 16 | 11 | 69 |
| 24 hours | 2 | 5 | 16 | 1 | 6 |
| 24 hours | 2 | 5 | 15 | 0 | 0 |
| 24 hours | 2 | 5 | 35 | 0 | 0 |
| 24 hours | 2 | 5 | 35 | 0 | 0 |
| 24 hours | 2 | 5 | 15 | 1 | 7 |
| 24 hours | 2 | 5 | 15 | 0 | 0 |

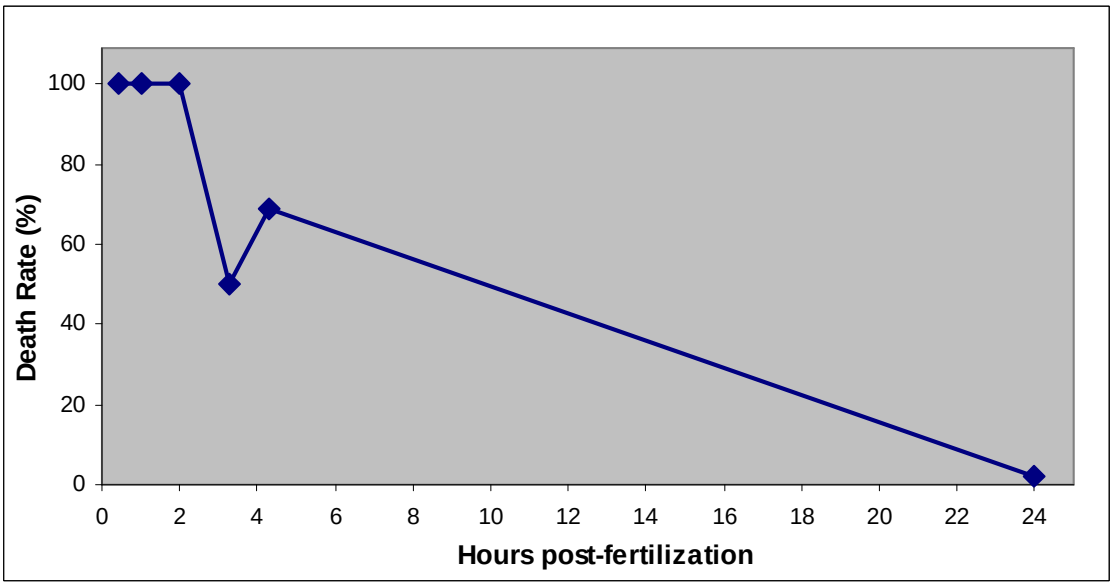



FIG. 5. **Death rates of wild type medaka fish embryos after treatment with 5 % ethanol** If the time point was measured at least two times, the average values were used for displaying the death rates in the figure. Detailed information is displayed in the table above.

The treatment of medaka fish embryos at early stages post-fertilization with an ethanol concentration of 5 % for 2 hours, showed a high death rate. Compared to that, the experiments with 24 hours old embryos, showed only an average death rate value of 2,2 %. The results indicate that the same concentration of ethanol differs strongly in its effects during embryonic development. The reason could be that in very early stages, the substances would be able to diffuse more easily into the embryo, because of the not yet finished development of the

chorion or other barriers. This could explain the variations of the death rate values when using 3 hours and 30 minutes or older embryos. At this time, the development of the chorion would be at an advanced state, whereas after 24 hours would be finished. To prove the role of the chorion as a protection barrier against the diffusion of toxic substances, experiments with embryos without the chorion should be done. Other diffusion barriers or changes in toxicity of ethanol during developmental stages could also cause the different death rates of the experiments.

The experiments with ethanol showed that the medaka fish embryo is suitable for experiments with toxic substances. In contrast to puromycin, a clear trend was observed. However, because of the fact that high concentrations of ethanol can denature proteins and thus alter the diffusion properties of the chorion, we searched for other toxic substances for the following experiments.

## 3.3 SODIUM AZIDE

To rule out the possibility that that the denaturing effect of high concentrations of ethanol could affect the experiments, another toxic substance was used in the following experiments. The goal of these experiments was to demonstrate differences in the death rate between early and late embryogenic stages similar to the experiments with ethanol. Sodium azide, $NaN_3$ was chosen for this purpose. This small molecule is highly soluble in water and toxic because of its cytochrome oxidase inhibiting effect by binding the heme cofactor irreversibly. Especially its effect on humans is well documented (Chang and Lamm, 2003).

### 3.3.1 Establishment of the conditions for experiments with $NaN_3$

The first experiments were made to define the concentration of sodium azide for the following experimental approaches. Concentrations from 1 to 100 mg/L of sodium azide, dissolved in $ddH_2O$, were used. Medaka fish embryos at different ages, from 45 minutes to 24 hours post-fertilization, were treated with these concentrations. The incubation time was two hours. The right concentration of $NaN_3$ was defined in terms of the effect which it should have on the different developmental stages of medaka fish embryos. It should cause a high death rate for embryos at early stages, and a significantly lower one at later stages.

The death rates 48 hours after the treatment with $NaN_3$ were used for the experiments. For explanation of the whole procedure, see Material and Methods.

| Age of Embryos (h) | $NaN_3$ [mg/L] | Number of Embryos | Incubation (h) | Death Rate (%) |
|---|---|---|---|---|
| 24 | 1 | 25 | 2 | 0 |
| 24 | 1 | 25 | 2 | 0 |
| 24 | 10 | 25 | 2 | 0 |
| 24 | 10 | 25 | 2 | 0 |
| 24 | 100 | 22 | 2 | 0 |
| 24 | 100 | 22 | 2 | 0 |
| 0,45 | 100 | 25 | 2 | 0 |
| 1 | 100 | 25 | 2 | 0 |
| 1,3 | 100 | 25 | 2 | 0 |

TABLE II. **Finding the right concentration of $NaN_3$ defined in terms of its effect on the medaka fish embryos.**
Embryos of different developmental ages were treated with different concentrations of $NaN_3$. The number of experiments done for each concentration differed.

All embryos survived using a concentration of sodium azide from 1 to 100 mg/L. Neither 45 minutes old medaka fish embryos nor 24 hours old were affected by the treatment. Therefore, the concentration of sodium azide was too low. The concentration used in cell culture experiments are for example 0.01 ng/ml to 10 mg/ml (Ishikawa et al., 2006). In the next experiments, the concentration of sodium azide was raised to 2000 mg/L of $NaN_3$, dissolved in $ddH_2O$. Different developmental stages of the medaka fish embryos, from 45 minutes to 48 hours post-fertilization, were treated with $NaN_3$ of 2000 mg/L. Two experimental

approaches were done, which differed in the incubation time of one or two hours, respectively. Again, the death rate values 48 hours after the incubation of the embryos were used for evaluation.

| Age of Embryos (h) | NaN3 [mg/L] | Number of Embryos | Incubation (h) | Death Rate (%) |
|---|---|---|---|---|
| 0,45 | 2000 | 17 | 2 | 100 |
| 1,45 | 2000 | 19 | 2 | 89 |
| 2 | 2000 | 22 | 2 | 86 |
| 2 | 2000 | 19 | 2 | 80 |
| 24 | 2000 | 38 | 2 | 13 |
| 24 | 2000 | 14 | 2 | 0 |
| 24 | 2000 | 18 | 2 | 0 |
| 24 | 2000 | 19 | 2 | 0 |
| 48 | 2000 | 27 | 2 | 4 |
| 2 | 2000 | 13 | 1 | 83 |
| 24 | 2000 | 14 | 1 | 0 |

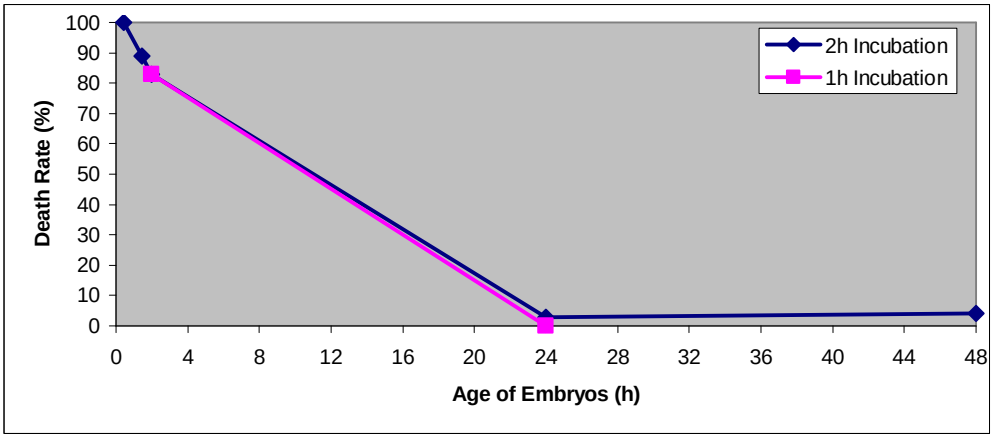



FIG. 6. **Finding the right concentration of sodium azide.**
Treatment of wild type medaka fish embryos of different developmental ages, treated with a concentration of 2000 mg/L sodium azide. Two different incubation times of $NaN_3$ were compared. Separate experiments were done at the same concentrations of $NaN_3$. The number of these experiments done differed between the developmental ages of the embryos. Detailed results of the experiments are shown in the table above.

At early stages, a high death rate of the medaka fish embryos was observed. At later stages, the treatment with sodium azide did not affect the survival. The difference in the duration of the incubation time did not seem to affect the death rate results, but closer observation of the embryos with a dissecting microscope showed fewer side effects like malformation and retardation for 1 hour treatment. Therefore, the treatment of the embryos with a concentration of 2000 mg/L of sodium azide, for an incubation time of 1 hour, was chosen for further experiments.

### 3.3.2 Sodium azide effects through embryonic development of medaka.

The experiments of the chapters 3.2 and 3.3.1 suggest clear differences in the diffusion rates during embryonic development. Therefore, experiments covering the whole embryonic development of medaka fish were initiated. These assays should show the differences between the early, middle and late developmental stages in terms of their death rate after treatment with the same concentration of sodium azide. Several experiments, covering all time points during the development of the embryo were needed. To strengthen the results, each experiment was repeated at least twice. A concentration of 2000 mg/L of sodium azide was used. Medaka fish embryos of different ages, from 45 minutes to 192 hours post-fertilization, were treated for an incubation time of one hour. In all experiments, the death rate values 48 hours after the incubation were used (Fig. 7). Statistical values are presented in an extra figure (Fig. 8).

Strongly deformed or retarded embryos were initially counted as viable, but then did not hatch. They did affect the death rate values for 48 hours, causing higher variations for each time point. Therefore, the death rate values of 5 days after the incubation with sodium azide were used for the experiments, too (Fig. 7). In later experiments, strongly deformed, but still living medaka fish embryos were counted as dead and removed because of the former observation, that they would not be viable.

| | | Death Rates for Experiments: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Age of Embryos (h)** | **Values taken** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **Mean Death Rate (%)** |
| 0,45 | 48 hours later | 69 | 100 | 69 | 100 | | | | | | | **85** |
| | 5 days later | 92 | 100 | 92 | 100 | | | | | | | **96** |
| 1 | 48 hours later | 79 | 83 | 48 | | | | | | | | **70** |
| | 5 days later | 79 | 83 | 70 | | | | | | | | **77** |
| 2 | 48 hours later | 83 | 96 | 63 | 75 | 50 | 67 | 86 | 80 | 77 | 89 | **77** |
| | 5 days later | 92 | 100 | 74 | 83 | 90 | 92 | 95 | 84 | 90 | 100 | **85** |
| 4 | 48 hours later | 88 | 70 | 75 | 86 | 60 | 80 | 60 | 60 | 50 | 60 | **70** |
| | 5 days later | 94 | 75 | 85 | 90 | 80 | 80 | 70 | 60 | 50 | 70 | **75** |
| 24 | 48 hours later | 0 | 0 | 4 | 4 | 13 | 0 | 0 | 0 | 0 | | **2** |
| | 5 days later | 0 | 0 | 4 | 4 | n.d. | n.d. | n.d. | n.d. | n.d. | | **2** |
| 48 | 48 hours later | 5 | 0 | 4 | | | | | | | | **3** |
| | 5 days later | 5 | 0 | n.d. | | | | | | | | **3** |
| 72 | 48 hours later | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | **0** |
| | 5 days later | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | **0** |
| 96 | 48 hours later | 0 | 0 | 0 | | | | | | | | **0** |
| | 5 days later | 0 | 0 | 0 | | | | | | | | **0** |
| 120 | 48 hours later | 0 | | | | | | | | | | **0** |
| | 5 days later | 0 | | | | | | | | | | **0** |
| 144 | 48 hours later | 96 | 75 | 100 | 0 | | | | | | | **68** |
| | 5 days later | 96 | 75 | 100 | 100 | | | | | | | **93** |
| 168 | 48 hours later | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | | | **100** |
| | 5 days later | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | | | **100** |
| 192 | 48 hours later | 100 | 100 | 100 | 100 | 100 | | | | | | **100** |
| | 5 days later | 100 | 100 | 100 | 100 | 100 | | | | | | **100** |

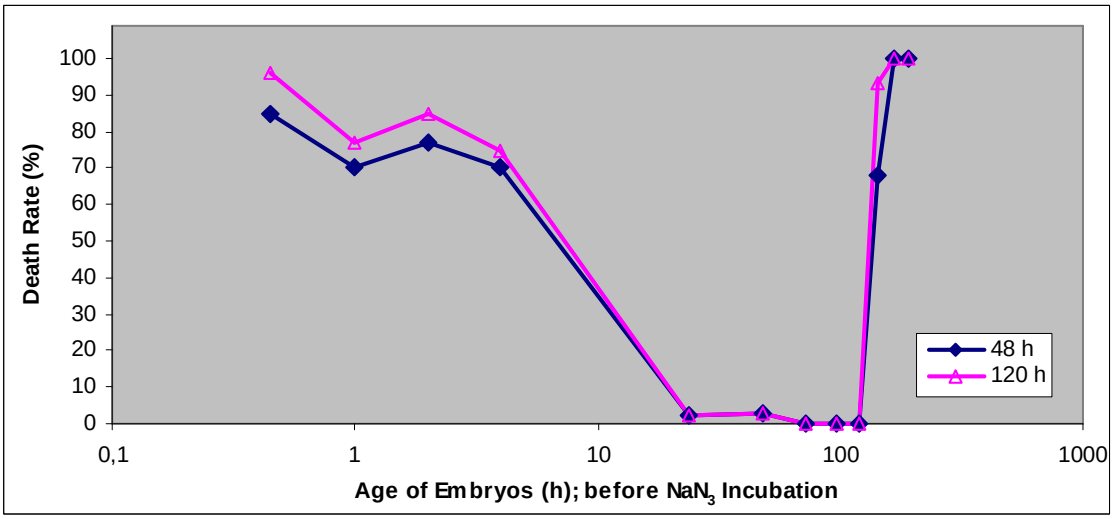



FIG. 7. **Sodium azide assay through the embryonic development of medaka fish.** The death rates of wild type medaka fish embryos after treatment with 2000 mg/L sodium azide for 1 hour are shown. Death rate values 48 (2 days) and 120 hours (5 days) after the treatment are compared. The number of separate experiments changed between the different developmental stages. The mean values of the results are shown in the figure. The average number of embryos per experiment is 20. Detailed information about the results of the experiments is shown in the table above.

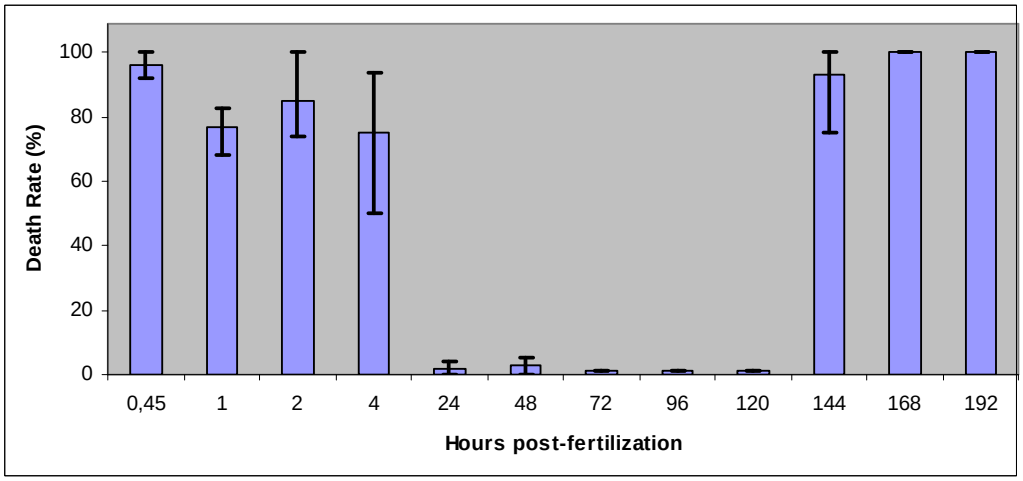



FIG. 8. **Statistics on the experiment shown in Figure 7.** The mean death rate values 120 hours after the incubation (1 hour) in sodium azide (2000 mg/L) are shown. The variations are shown as error bars. The number of experiments changed between the different time points through the embryonic development. Detailed information is shown in the table of Figure 7.

For the treatment of sodium azide (2000 mg/L), at very early (45 min - 4 h) and late (6-8 days) stages of the embryonic development, high death rates could be shown, whereas embryos between 24 hours and 5 days, mainly survived (Fig. 7). The variation of the results was quite low at most stages. The higher variations at the age of 4 hours and 6 days could indicate a period of transition, between strong and weak toxic effects of sodium azide on the medaka fish embryos (Fig. 8).

The sodium azide assay through embryonic development of medaka fish resulted in a strong toxic effect for sodium azide on early and late stages of embryonic development, whereas the middle stages were not affected. A explanation for this behaviour could be that the chorion limits the diffusion of sodium azide, after its hardening is finished, approximately 6 hours post-fertilization. Before this, the chorion would be permeable, resulting in a strong toxic effect. Due to the secretion of the hatching enzyme at late stages (7-10 days), the chorion would again become permeable for sodium azide. Alternatively, at later stages, the development of the respiratory system could increase the toxic effect of sodium azide because of its cytochrome oxidase-inhibiting characteristic. In order to show that the chorion is the reason for these differences, experiments are necessary in which the chorion is removed (see chapter 3.3.4).

### 3.3.3 Sodium azide assay - lower concentrations

A concentration of 2000 mg/L for sodium azide was used in chapter 3.2.2. Lower concentrations tested before (1-100 mg/L) showed no effect. Here also, concentrations in between these values were tested. Sodium azide concentration of 200 mg/L and 600 mg/L, dissolved in dd$H_2O$ were used for the experiments. Embryos of four different time points, 2 hours, 24 hours, 72 hours and 168 hours post-fertilization, were incubated with these concentrations. These four groups were chosen because of their strong differences in death rates. The incubation time was one hour. The death rate values for 5 days after the incubation with sodium azide are shown.

| Age of Embryos (h) | $NaN_3$ [mg/L] | Number of Embryos | Incubation (h) | Death Rate (%) |
|---|---|---|---|---|
| 2 | 200 | 24 | 1 | 50 |
| 24 | 200 | 14 | 1 | 0 |
| 72 | 200 | 20 | 1 | 0 |
| 168 | 200 | 20 | 1 | 15 |
| 2 | 600 | 24 | 1 | 75 |
| 24 | 600 | 14 | 1 | 0 |
| 72 | 600 | 20 | 1 | 0 |

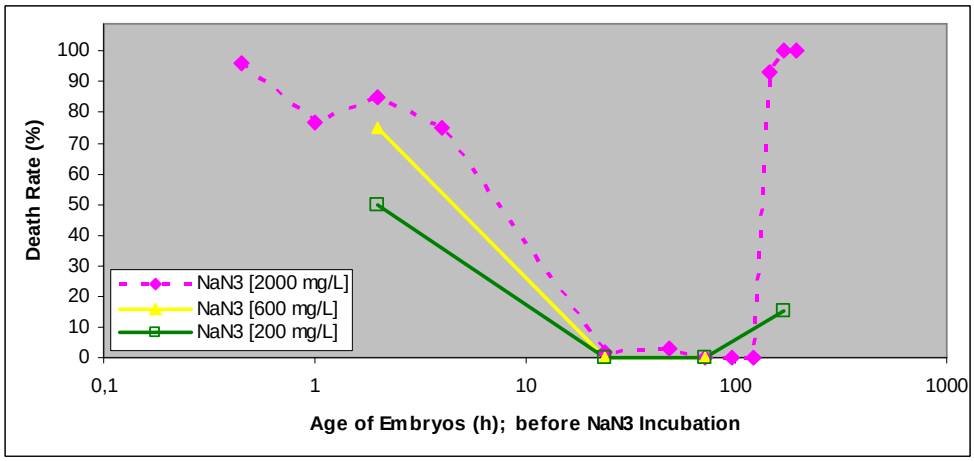



FIG. 9. **Death rates of medaka fish embryos after treatment with different concentrations of sodium azide.** For comparison, the death rate values through the embryonic development (see chapter 3.3.2) are shown as a dotted line. The two lower concentrations of sodium azide are 600 mg/L (yellow) and 200 mg/L (green).

The two lower concentrations of sodium azide reached lower death rate values than the 2000 mg/L data at 2 and 168 hours post fertilization. At 24 and 48 hours post fertilization, they were equal. Interestingly, at early stages the medaka fish embryos seemed to be more vulnerable to low doses of sodium azide, whereas at 2000 mg/L the opposite was observed.

### 3.3.4 Sodium azide assay - addition of Triton X-100

Triton X-100 is a non-ionic detergent, which solubilizes membrane proteins and makes the cell membrane more permeable (Barbero et al., 1983). Therefore, it should allow a better diffusion of toxic substances into the cells of the developing medaka fish embryo.

In the first experiments the concentration of Triton X-100 for all following experimental approaches was determined (Fig. 10). It was tested at which maximum concentration Triton X-100 would not cause an increased death rate of the medaka fish embryos. This concentration should then improve the permeability through the chorion and other membranes of the medaka fish embryo, and thus affect the diffusion rate of sodium azide, resulting in a higher death rate of the embryos. Concentrations of 3 % to 0,003 % of Triton X-100 in the solution were tested. No sodium azide was added. The general experimental approach was similar to that of chapter 3.3.1-3.3.3 and is described in Material and Methods. The age of the medaka fish embryos before incubation with Triton X-100 was 24 hours post-fertilization and the incubation time was 1 hour. Again, the death rate 48 hours after the incubation of the embryos is shown (Fig. 10).

| Age of Embryos (h) | Triton X-100 (%) | Number of Embryos | Incubation (h) | Death Rate (%) |
|---|---|---|---|---|
| 24 | 3 | 14 | 1 | 100 |
| 24 | 1 | 12 | 1 | 100 |
| 24 | 1 | 24 | 1 | 100 |
| 24 | 0,5 | 24 | 1 | 100 |
| 24 | 0,3 | 13 | 1 | 100 |
| 24 | 0,25 | 24 | 1 | 100 |
| 24 | 0,1 | 24 | 1 | 100 |
| 24 | 0,1 | 13 | 1 | 100 |
| 24 | 0,06 | 13 | 1 | 100 |
| 24 | 0,03 | 13 | 1 | 0 |
| 24 | 0,03 | 22 | 1 | 77 |
| 24 | 0,01 | 22 | 1 | 0 |
| 24 | 0,003 | 22 | 1 | 0 |

TABLE II. **Detailed Information about the separate experiments with Triton X-100.**

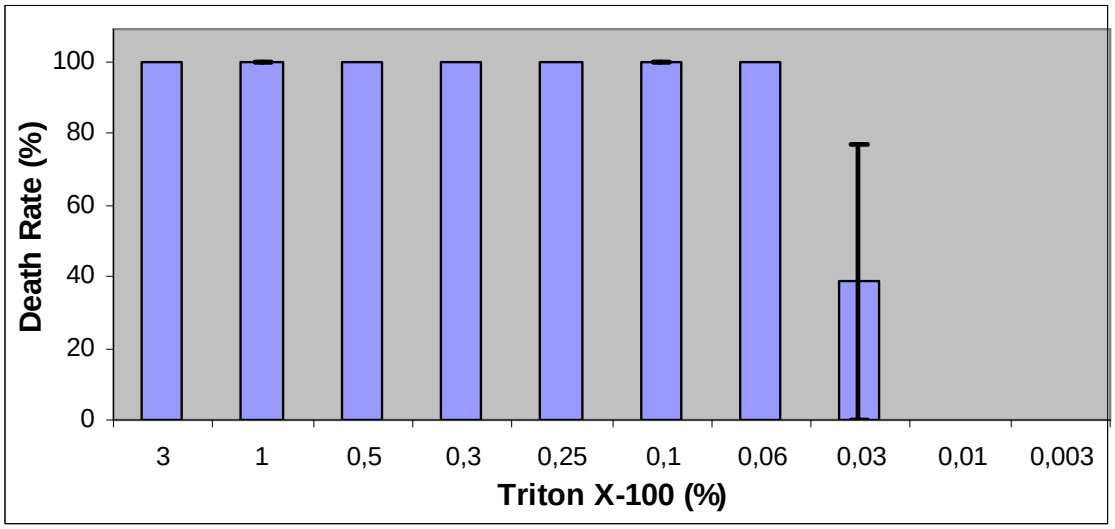



FIG. 10. **Effects of Triton X-100 on embryonic development.** Medaka fish embryos were incubated at several different concentrations of Triton X-100 dissolved in dd$H_20$ for 1 hour. Some experiments were done twice and the error bars show the variations. Detailed information of the separate experiments are shown in the table above (table II).

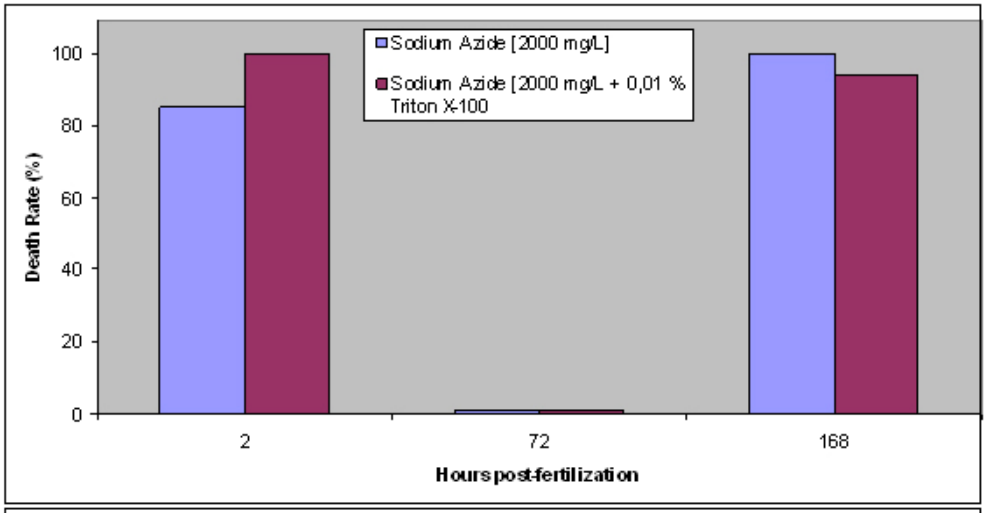
At concentrations from 3 % to 0,06 % of Triton X-100, the death rates often reached 100 % immediately after the incubation time. Treatment with 0,01 % Triton X-100 showed complete survival after the treatment, and was chosen for further experiments.

In these experiments, the embryos were treated with different concentrations of sodium azide in addition to 0,01 % Triton X-100. The death rate values were compared with that of embryos treated with the same azide concentrations, but without addition of Triton X-100, at chosen time points during embryonic development. The concentrations of sodium azide were 2000 mg/L, 200 mg/L and 60 mg/L. The incubation time was one hour. The three time points chosen were: 2 hours, 72 hours and 168 hours post-fertilization. The reason for choosing three groups was the differences in the death rates during the experiments of chapter 3.3.2. The results 48 hours after the incubation time are shown (Fig. 11).

a

| Age of Embryos (h) | Triton X-100 (%) | Number of Embryos | Incubation (h) | Death Rate (%) |
|---|---|---|---|---|
| 24 | 3 | 14 | 1 | 100 |
| 24 | 1 | 12 | 1 | 100 |
| 24 | 1 | 24 | 1 | 100 |
| 24 | 0,5 | 24 | 1 | 100 |
| 24 | 0,3 | 13 | 1 | 100 |
| 24 | 0,25 | 24 | 1 | 100 |
| 24 | 0,1 | 24 | 1 | 100 |
| 24 | 0,1 | 13 | 1 | 100 |
| 24 | 0,06 | 13 | 1 | 100 |
| 24 | 0,03 | 13 | 1 | 0 |
| 24 | 0,03 | 22 | 1 | 77 |
| 24 | 0,01 | 22 | 1 | 0 |
| 24 | 0,003 | 22 | 1 | 0 |

b




c

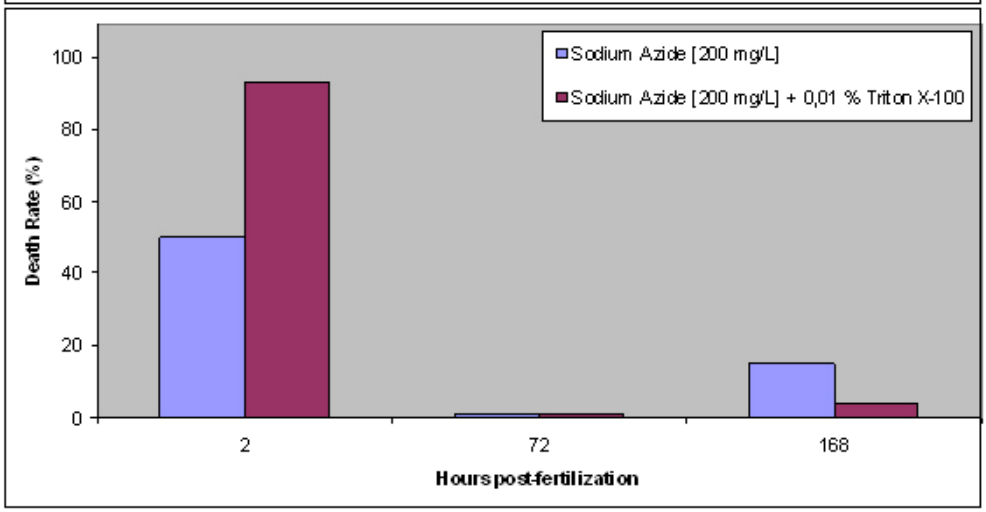



FIG. 11. **Comparison of death rates of medaka fish embryos after treatment with different concentrations of sodium azide +/- 0,01 % Triton X-100.** Death Rates of embryos after treatment with sodium azide concentrations of 2000 (b) or 200 mg/L (c) +/- addition of 0,01 % Triton X-100 were compared at 3 time points (2, 72 and 168 hours post-fertilization). The death rate values caused by sodium azide without addition of Triton X-100 are those of the experiments shown in Figure 7 and 9. Mean values are shown. Detailed information of the separate Triton-experiments done is shown in the table above (a).

The concentration of 2000 mg/L of sodium azide with 0,01 % Triton X-100, showed a slightly higher death rate at 2 hours and a slightly lower one at 168 hours post-fertilization. At a sodium concentration of 200 mg/L, the death rate at 2 hours post-fertilization was highly increased after addition of Triton, but again slightly lower at 168 hours pf. The death rate of 72 hours old medaka fish embryos remained the same. These results indicate that the concentrations of Triton X-100 used was able to produce a better uptake at very early stages, but was too low to show any effect at 72 or 168 hours post-fertilization. A reason for this could be the short incubation time of 1 hour in addition to the processing hardening of the chorion, which then provides a stronger barrier than at 2 hours post-fertilization.

### 3.3.5 Sodium azide assay - hatching enzyme treatment

The hatching enzyme of medaka fish embryos solubilises the chorion of the egg shortly before hatching. At this time, it is secreted from specific glands (Yamamoto, 1975). However, Ishida and colleagues observed, that the placement of medaka fish eggs into a hatching enzyme solution, independent of their developmental age, would dissolve the chorion (Ishida, 1944). Since that observation, the treatment of medaka fish eggs with a solution of hatching enzyme is a commonly used method for dechorionization of the embryos at various stages. The dechorionization of the medaka fish embryos, followed by treatment with sodium azide, should strongly influence the death rate. If the chorion is the main diffusion barrier of the embryo, the death rates of the different developmental stages should equal each other after removal of the chorion.

In our experiments, medaka fish embryos at a developmental age of 2 hours, 72 hours and 168 hours post-fertilization, were treated with a hatching enzyme solution. For detailed information, see Material and Methods. The chorion was dissolved and removed. The Petri dishes, in which the experiments were done, were agarose-coated in order to prevent contact with plastic, which would have damaged the dechorionized embryos. Except for that, the experiments were done like the others shown in the chapters of 3.3.

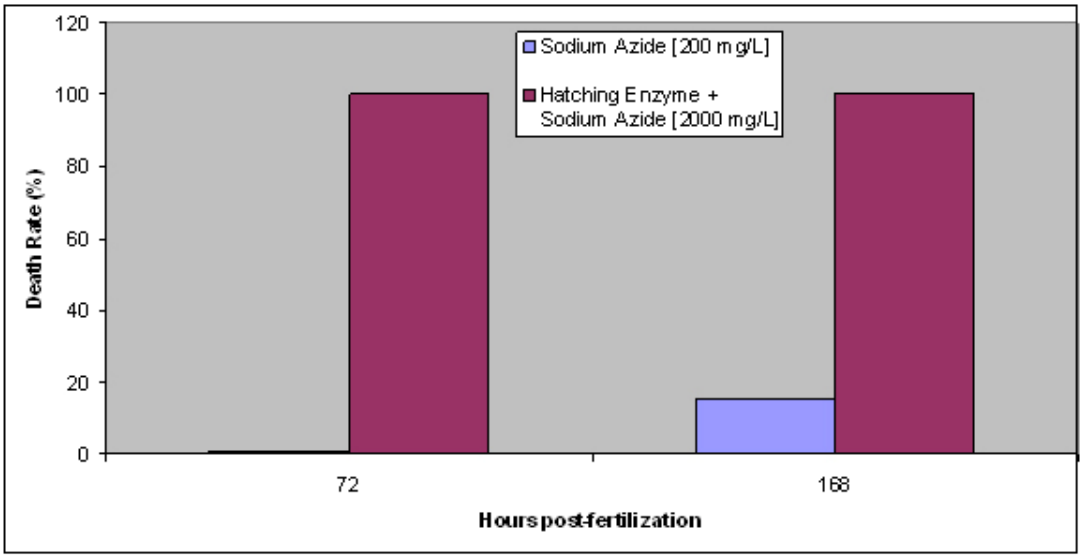
The concentrations of sodium azide used were 2000 mg/L, 200 mg/L or 60 mg/L. To ensure the dechorionized medaka fish embryos were viable, a wild type control group was also treated with the hatching enzyme solution, but no sodium azide was added. The incubation time was one hour (Fig. 12). However, no experiments were possible with 2 hours old embryos, because of the high death rates after hatching enzyme treatment and the high instability of the embryos at these stages when dechorionized. Even if some survived, no washing or medium replacement was possible after the sodium azide treatment, because of the damage it caused.

The dechorionization before treatment with sodium azide showed a strong effect on the death rates of the embryos. The results showed death rates of 100 % at 72 hours old embryos after dechorionization, whereas in all previous experiments at this stage low death rates were observed. Importantly, the control groups showed a completely normal development, even without the chorion (Fig. 12). These results finally indicated that the chorion played an important role in diffusion of toxic substances into the embryo. Its function as a diffusion barrier reduced especially the uptake of substances during the middle stages of the embryonic development.

a

| Age of Embryos (h) | $NaN_3$ [mg/L] | Number of Embryos | Incubation (h) | Death Rate (%) |
|---|---|---|---|---|
| 72 | 0 | 2 | 1 | 0 |
| | 2000 | 10 | | 100 |
| | 200 | 10 | | 100 |
| | 60 | 9 | | 100 |
| 168 | 0 | 2 | 1 | 0 |
| | 2000 | 11 | | 100 |
| | 200 | 10 | | 100 |
| | 60 | 11 | | 20 |

b




c

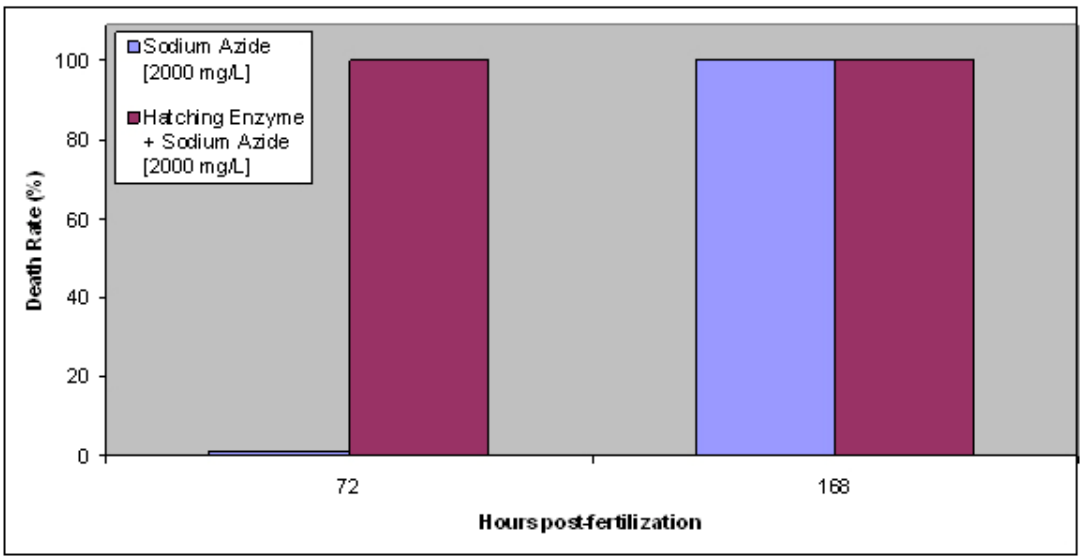



FIG. 12. **Death rates of dechorionized medaka fish embryos after treatment with sodium azide.** Embryos treated with hatching enzyme before incubation in different sodium azide solutions (b, c) were compared with results of former experiments (Fig. 7 and 9), which weren't treated with hatching enzyme before. Detailed results of the separate experiments are shown in the table above (a).

# 4 DISCUSSION

## 4.1 PUROMYCIN AS A SELECTIVE AGENT FOR MEDAKA FISH EMBRYOS *IN VIVO*

In cell culture experiments, puromycin is a common reagent used as selection antibiotic for transfection, and for selection of cell types harboring plasmids carrying puromycin resistance genes in gram positive bacteria and various animal and insect cells. However, our intention was to use puromycin for selection *in vivo*. For this purpose, it would be essential to determine a concentration of puromycin which reliably kills embryonic cells, whereas cells containing the puromycin resistance gene *pac* would survive.

Surprisingly, the embryos survived even at the highest concentration of puromycin that was used. Only in one experiment (see chapter 3.1.1) a death rate of 100 % was observed for 100 µg/ml of puromycin. However, this result could not be reproduced and in later experiments even at concentrations up to 500 µg/ml, the embryos partially survived. A possible explanation for these results could be that medaka cells are insensitive for puromycin. However since puromycin acts on a wide range of organisms covering bacteria up to mammals, this is unlikely. Another reason could be a diffusion barrier surrounding the embryo, which prevents sufficient uptake of the toxin. This diffusion barrier could be the chorion, because of its protective role during development (see chapter 4.2). Therefore, if a higher diffusion rate can be reached, it will result in higher death rates of medaka fish embryos. Penetration of the chorion by a glass capillary needle shows no effect on the toxicity of puromycin. Most likely, the small holes are repaired shortly after injection. A bypass of the chorion by injection of puromycin between the blastodermal cells of medaka fish embryos at early blastula stage (stage 10) shows no better uptake or effect. However, the reason for this might be more a concentration problem, because after the injection between the cells, the puromycin solution is strongly diluted.

Alternatively, the distribution of puromycin in the embryo could be biased by the yolk, which might be able to strongly enrich this toxin in the hydrophobic compartments of the embryos. Literature describes a fast hardening of the chorion (Iuchi, 1995); (Iwamatsu, 1995). In the short period until it is fully hardened, higher diffusion rates for toxins during the first hours of development would be suspected. This would result in higher death rates. When the chorion development is finished, the diffusion rate of toxins will be reduced, resulting in lower death rates. However, the experiments with puromycin did not show such a trend.

The results indicate that puromycin is not suitable for an *in vivo* selection of medaka fish embryos. The idea, that a certain concentration of puromycin will be able to enter the cells of the embryos by gradient diffusion could not be proven. One reason could be that puromycin can not enter the cells, even after bypassing the chorion, without additionally altering the cell membranes, to make them more permeable, like e.g. done in transfection *in vitro*. Nevertheless, further experiments with puromycin were not reasonable since even at the highest concentrations (limited by the solubility and the costs of puromycin) the embryos partially survived. The topic of my experiments therefore shifted to analyze the diffusion of small molecules into medaka fish embryos.

## 4.2 THE ROLE OF THE CHORION AS A DIFFUSION BARRIER

The chorion as semi-permeable membrane has a purpose as a physical and chemical defense mechanism against metals, pathogens, and xenobiotic chemicals (Finn, 2007). It is known for its protective role against an exposure to chemical substances (Braunbeck et al., 2005). After fertilization, the chorion undergoes structural changes and becomes more resistant to mechanical or chemical influence of the environment (Iuchi, 1995). According to Iuchi and colleagues, the elasticity of the medaka egg increases exponentially after fertilization. It reaches the maximum after about 6 hours post-fertilization (room

temperature). Chorion hardening is divided into two major parts: (a) reconstruction- and (b) insolubilization of the chorion proteins. The chorion gets fully insoluble within 120 minutes post-fertilization and an already 40-fold increased toughness after 60 minutes post-fertilization could be shown (Iwamatsu, 1995).

The experiments with puromycin left the question of how limited is the diffusion of toxic substances into the cells of the medaka fish embryo, especially in the early stages of embryonic development. During the process of chorion hardening, the embryo is more vulnerable, because during this short period of time the chorion can not fully maintain its protective function. This should result in a higher diffusion rate of toxic substances into the embryo, and therefore, dependent on its concentration, a higher death rate should occur. After the chorion is hardened, the diffusion into the embryo should be more limited, and therefore a reduction in the death rate, compared to the very early stages of embryonic development, should be observed. At late stages, shortly before hatching, the chorion weakens again and start to dissolve because of the secretion of hatching enzyme (Yamamoto, 1975). The chorion protects the fish embryos, but larvae or dechorionized embryos are more sensitive to exposure of toxic substances (Kashiwada et al., 2002); (Villalobos et al., 2000). Therefore, at the latest stages of embryonic development, the diffusion rate should increase again.

### 4.2.1 Ethanol as a model system for diffusion of small toxins into medaka fish during embryogenic development

A search for substances which would be able to prove the different diffusion rates of small toxic substances into the medaka fish embryo resulted in ethanol as applicable substance with the right properties. The role of ethanol as a developmental toxin and its effects are well described in the literature (Goodlett et al., 2005). The presented experiments with ethanol show the predicted differences in the diffusion rates of small toxic substances into medaka fish embryos. Embryos 0-4 hours post-fertilization, which are exposed for 2 hours to an ethanol concentration of 5 %, show a high death rate due to a high diffusion

rate. Embryos 24 hours post-fertilization show a reduced death rate at the same concentrations of ethanol, decreased to almost zero. Oxendine and colleagues performed similar experiments with medaka fish embryos (Oxendine et al., 2006). During their experiments with non-lethal concentrations of ethanol, they showed that ethanol uptake from the surrounding solution into the embryos is approximately the same at each stage of embryonic development. In their experiments, the earliest stage examined was 10, which is 6 hours post-fertilization. According to them, the concentration of ethanol in the embryo reaches 60-70 % of the concentration in the surrounding medium, when incubated for 24 hours. They showed that an exposure of the embryos to 2 % ethanol for 24 hours was lethal. In my experiments, I used a higher concentration of ethanol, but a shorter incubation time of 2 hours. However, I observed strong differences in the diffusion rates between the different stages of embryonic development. If the uptake of ethanol is the same for each developmental stage, the differences in the death rates would occur because of a stage-dependent sensibility of the medaka fish embryo. No comparable data concerning the first 6 hours of embryonic development are presented in the literature, and therefore the influence of chorion hardening on ethanol uptake, is unclear. Supposedly, because of the time the chorion needs to develop, there is a higher ethanol uptake during these first stages of embryonic development.

### 4.2.2 Sodium azide as a model system for diffusion of small toxins into medaka fish during embryogenic development

For observation of the differences in the diffusion rates of small toxic substances into the medaka fish embryo during the embryonic development, one major disadvantage of ethanol is its protein denaturing feature when used at higher concentrations, which might affect the chorion. In order to confirm our results, we also performed diffusion experiments with sodium azide. Sodium azide ($NaN_3$) is a small colourless molecule and highly soluble in water. Its toxic effect is well described, especially in humans (Chang and Lamm, 2003). One of the different mechanisms, how it causes lethal toxicity, is the inhibition of the cytochrome oxidase by binding irreversible to the heme cofactor.

The sodium azide assay through the whole embryonic development of medaka fish indeed confirmed the results with ethanol. Embryos up to an age of 4 hours post-fertilization show a high death rate of 75 % or more when exposed for 2 hours to a sodium azide concentration of 2000 mg/L. In contrast, embryos of an age of 24 hours to 120 hours post-fertilization showed a low death rate. Embryos of an age of 144 hours post-fertilization up to the time they hatch showed a death rate of 100 %. Experiments done with lower concentrations of sodium azide of 200 mg/L still showed an increased death rate of 50 %, whereas in later stages, the death rate was reduced to a normal level.

To confirm our prediction that the diffusion rate of small toxic molecules into the medaka fish embryos depends on the different permeability of the chorion, medaka fish embryos were exposed to a hatching enzyme solution. Should the dechorionized embryos still show the same differences in their death rates, then it would be more likely, that the developmental stages differ in their sensibility to the toxins, and not because of the altered diffusion rates by the chorion. However, this time, the dechorionized medaka fish embryos 72 hours post-fertilization showed a death rate of 100 % at sodium azide concentrations of 2000 and 200 mg/L. Therefore, the diffusion rate of small toxic molecules into the medaka fish embryos seems to depend on the different permeability of the chorion.

## 4.3 ENHANCED DIFFUSION INTO MEDAKA FISH EMBRYOS

In general, embryos are to be expected to be more sensitive to the exposure and uptake of toxins, especially in early stages of development (Teraoka et al., 2003). Reliable and convenient methods for toxin and/or drug screening are of importance, to show their mechanisms, their dose-response relationships and in general, to determine their effect. Especially the fish model organism zebrafish (*Danio rerio*) has been used for a great variety of chemicals to evaluate their toxicity, because of their advantageous features compared to other vertebral

systems, like laboratory organ culture studies of rodents, for example. Zebrafish enables large scale screenings, and especially its size, husbandry and early morphology are of great advantage. It has a long history as a model organism and therefore provides a lot of morphological, biochemical and physiological information, additionally to a completely sequenced genome (Hill et al., 2005). However, zebrafish is not the only fish model used for embryo toxicity testing. Because of its short generation time up to three months, or its known temperature and salinity tolerances, the medaka fish (*Oryzias latipes*) also provides a good model. For example the stage dependent effects of carbaryl (Kashiwada et al., 2008), the distribution of nanoparticles (Kashiwada, 2006) or the toxicity of naphthoic acid isomers (Carney et al., 2008) are a few examples for the use of medaka in toxicity testing of several substances.

Our experiments would suggest, that during the toxicity testing of chemicals in medaka fish embryos, it should be noted that there seem to be differences during embryonic development, in terms of the toxin uptake into the embryo. This is caused by different diffusion rates through the chorion. However, there are several possibilities to enhance the diffusion and therefore to create an uptake of toxins regardless of the developmental stage of the embryos. One possibility is the treatment of medaka fish embryos with hatching enzyme, prior to the toxin exposure, like explained at the end of chapter 4.3.2. Except the treatment with hatching enzyme, the co- or pre-treatment of medaka fish embryos with other substances or under certain conditions, which will reduce the permeability of the chorion, can provide a better uptake of the toxin. Shibata and colleagues defined a single protein, called alveolin, which seems to plays the major role in chorion hardening *in vitro*, by hydrolyzing ZI-1,2 to 61-62-kDa intermediates (Shibata et al., 2000). They showed that the proteolytic activity of alveolin and supposedly therefore the whole hardening process can be influenced by variations in the pH value and the temperature. The change of one of these two factors for a short time during embryonic development could therefore provide a better permeability. The proteolytic activity of alveolin can even be strongly inhibited by chelating reagents such as EDTA, EGTA and *o*-phenanthroline, or its activity restored by addition of divalent metal ions, such as

$Co^{2+}$, $Mn^{2+}$ or $Zn^{2+}$. Therefore, by addition of chelating agents immediately after fertilization, a possible better uptake of the toxin of interest could be possible. Perhaps even the restoration of the proteolytic activity after the treatment with the toxin, by addition of the divalent metal ions could be done. However, since the protein alveolin is processed inside the chorion, the possible uptake of chelating agents and their effect has to be verified. Alternatively, a transgenic medaka fish line with an alveolin mutation could provide an exclusively strain for toxicity experiments, just like the use of specific morpholinos against the alveolin mRNA.

However, another possibility for enhanced diffusion into medaka fish embryos could provide their additional treatment with Triton X-100. Triton X-100 is a non-ionic detergent, which solubilizes membrane proteins and makes the cell membrane more permeable (Barbero et al., 1983). Therefore, the addition of low concentrations of Triton X-100 should make the chorion permeable without showing any negative effect on the embryonic development itself. After determination of a concentration, which causes no death rate by itself, the addition of Triton X-100 shows a certain effect in the early stages of development. A sodium azide concentration, at which embryos 2 hours post-fertilization normally showed a death rate of 50 %, causes a death rate of almost 100 %. Embryos 168 hours post-fertilization are not affected. At embryos 72 hours post-fertilization, the treatment with Triton X-100 shows no effect either, even at higher concentrations of sodium azide. However, the higher death rates at lower concentrations of sodium azide after the treatment with Triton X-100 indicate that his method provides a better diffusion rate into the embryos. As for embryos 72 hours post-fertilization, higher concentrations of Triton X-100 should be tested.

Summarizing, my experiments of the diffusion rates of small toxic molecules into the medaka fish during the different stages of embryonic development indicates strong toxic effects in the first two hours post-fertilization. During the middle and late stages, up to 144 hours post-fertilization, the toxicity related to the death rate of the embryos is strongly reduced. Shortly before hatching, an increase can be shown again. The reason for this might be that the chorion modulates the toxic effect by causing different diffusion rates during embryonic development. The dissolution of the chorion by dechorionization proves this theory, by showing equally high death rates of medaka fish embryos, which indicates equally high diffusion rates. The diffusion rates of small toxic substances into the embryo can be enhanced by additional treatment with Triton X-100.

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# ANHANG

## ZUSAMMENFASSUNG

Puromycin findet mehrere Anwendungsmöglichkeiten in den Methoden der heutigen Zellkultur. Aufgrund seiner Translations-inhibierenden Wirkung wird es vor allem als Selektionsmarker für Transfektion und ebenso zur Selektion von transgenen gram$^+$ Bakterien und verschiedenen transgenen Zelllinien von Tieren und Insekten eingesetzt. Neben diesen *in vitro* Methoden ist keine Benutzung von Puromycin *in vivo* bekannt. Im ersten Teil meiner Diplomarbeit wurde versucht, eine Methode zu entwickeln, welche Puromycin als Selektionsmarker für transgene Fische *in vivo* ermöglicht. Als Modellorganismus wurde Medaka (*Oryzias latipes*), ein kleiner Fisch welcher der Familie der Reisfische angehört, verwendet. Neben dem geläufigen Zebrafisch (*Danio rerio*), ist Medaka wohl der am häufigsten verwendete Fisch-Modellorganismus. Über DNA-Mikroinjektion würde neben dem DNA-Konstrukt, welches für das jeweilige Experiment gerade verwendet wird, auch das Puromycin-Resistenzgen *pac* in das Zytoplasma eines sich im Einzellstadiums befindlichen Medaka Embryos eingebracht werden. Die nachfolgende Selektion durch eine bestimmte Puromycinkonzentration im Medium würden nur transgene Embryonen, welche das Resistenzgen besitzen, überleben. Doch schon die Resultate der Experimente, welche die richtige Konzentration an Puromycin im Medium bestimmen sollten, zeigten dass Puromyzin als Selektionsmarker *in vivo* höchstwahrscheinlich nicht geeignet ist.

Die Resultate stellten uns vor die Frage nach dem generellen Prozess der passiven Diffusion von kleinen, toxischen Substanzen in die Zellen des Fischembryos. Zusätzlich wurde die Rolle des stark ausgebildeten Chorions von Medakas betreffend der Funktion während der Diffusion untersucht. Diffusionsexperimente mit Ethanol oder Natriumazid unter normalen

Bedingungen zeigten deutliche Unterschiede in den Diffusionsraten während der Embryonalentwicklung. Der Vergleich mit der Behandlung unter veränderten Bedingungen, wie durch die Dechorionisierung der Embryos mit Hatching Enzym oder die gleichzeitige Behandlung mit dem Detergent Triton X-100, konnten das Chorion als Ursache für die Modulierung der Diffusionsrate ausmachen. Das Chorion spielt also eine wichtige Rolle bei der Bestimmung der Toxizität von Chemikalien während der Embryonalentwicklung.

# ABSTRACT

The antibiotic puromycin has several common used applications in cell culture experiments. Because of its translation-inhibiting effect, it is used as a selection marker for transfection, for the selection of transgenic gram$^+$ bacteria and the selection of various transgenic cell lines in different animals and insects. Beside of its use in these applications *in vitro*, no known in use of puromycin *in vivo* is known.

In the first part of my diploma thesis I tried to find parameters for a method, which would allow puromycin as a selection marker *in vivo*, as selection marker for transgenic medaka fish embryos. Medaka (*Oryzias latipes*) is next to the known zebrafish (*Danio rerio*) the most used model organism in fish. Constructed plasmids, containing the gene of interest and the puromycin resistance gene *pac*, would be injected into the cytoplasm of a one- or two-cell-stage medaka fish embryo. The addition of a certain concentration of puromycin, which would have to be determined, into the embryo rearing medium (ERM) would only allow the transgenic embryos to survive. However, the experiments for the determination of the right concentration of puromycin in ERM already indicated, that puromycin seemed to be inappropriate as a selection marker *in vivo*.

The results of these first experiments with puromycin questioned the general process of passive diffusion of small toxic substances into the cells of medaka fish embryos. Especially the role of the strong medaka chorion in this process was of interest. Experiments with ethanol and sodium azide showed significant differences of diffusion, and therefore death rates during the embryonic development of medaka. The comparison with results after treatment with ethanol or sodium azide under modified conditions, like the dechorionization of embryos with hatching enzyme or additional treatment with the detergent Triton X-100, strengthen our observations that indeed the chorion modulates the diffusion rates into the embryos. Therefore, the chorion plays an important role in the determination of toxicity of chemicals during the embryonic development.

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