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Assessment of zebrafish embryo toxicity of environmentally relevant antibiotics

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Abstract:

Antibiotics are essential drugs in modern medicine. After consumption antibiotics are excreted in unmetabolized form in the urine and reach our sewage treatment plants (STP). STP are not able to degrade all antibiotics leading to release of antibiotics into the aquatic environment. Aquatic animals are thus continuously exposed to antibiotics. This study involved assessment of the toxicity of eight antibiotics previously detected in the river Fyrisån, Uppsala, Sweden, in developing zebrafish (*Danio rerio*) embryos up to day six of age. The experiments included assessment of embryonal toxicity for the individual antibiotics as well as mixtures of all antibiotics. The mixtures were based on previously measured concentrations in river Fyrisån and tested in increasing concentrations up to 1000-times higher concentrations. In the toxicity assessment different lethal and sublethal endpoints were observed, such as early movements, heart rate, hatching time and length. These experiments were followed by behaviour study observing the swimming activity during alternating dark-light alternations. Lastly, a bioaccumulation study was performed on mixtures of antibiotics to determine if these antibiotics were bioaccumulative in zebrafish embryos. The results showed that these eight antibiotics, individually and as a mixture did not affect any of the endpoints. As for bioaccumulation, none of the eight antibiotics were bioaccumulating in zebrafish embryos. These antibiotics seem to be non-toxic during fish embryonal development. However, the results cannot determine the long-term effects of antibiotic exposure and thus further studies are needed to assess the potential toxicity of environmentally present antibiotics to fish.

Popular summary:

Antibiotics are used every day to treat infections in both humans and animals. Consumed antibiotics are in some degree released from the urine and can then enter our aquatic environments via sewage treatment plants. This can be a problem that leads to many consequences, such as development of antibiotic resistance in bacteria living in the aquatic environment. The presence of antibiotics in the environment are pressuring bacteria to acquire certain properties for them to be able to survive. Additionally, the presence of antibiotics in the aquatic environment can affect animals that are living in or feeding of these environments. Water living animals, such as fish, are continuously exposed to antibiotics released from our sewage treatment plants into the surrounding surface waters. In the present study we wanted to investigate the early life-stage toxicity of antibiotics on fish. Zebrafish embryos were used as a model to investigate the toxicity of eight different antibiotics, which were measured in Fyrisån, Uppsala, Sweden in a previous study. Also, we measured if antibiotics could accumulate in zebrafish embryos. Zebrafish embryos were exposed to eight antibiotics, both individually and in mixtures mimicking the concentrations measured in Fyrisån and up to 1000-times higher concentrations. The zebrafish embryos were exposed from fertilization up to six days of age. The endpoints included morphological, physiological, and behavioural effects. The results from the present study showed no toxicity on early development in zebrafish embryos, neither from the individual antibiotics, nor from the antibiotic mixtures. In a second experiment, the potential bioaccumulation of antibiotics after six days of exposure to the mixtures were measured. Here, both the water exposure solutions and exposed embryos were measured analytically to determine the antibiotic concentrations. The study showed that antibiotics are not bioaccumulating in the embryos. In conclusion, the results from the present study show that the investigated eight antibiotics, are not acute developmental toxic or bioaccumulative in zebrafish embryos, neither individually nor in environmentally relevant mixtures. However, wild aquatic organisms, such as fish, are continuously exposed to antibiotics and other anthropogenic chemical pollutants throughout their lives. Therefore, future studies should focus on long-term effects to reveal possible consequences later in life.

1. Introduction

1.1 General background

Antibiotics are one of the most essential groups of pharmaceuticals in modern medicine. They have been used as treatment for bacterial infections since the first antibiotic was discovered. It has also been used as prophylaxis before surgeries to reduce possible infection afterwards (1), as well as growth promotors when feeding animals in agriculture (2,3). The human consumption of antibiotics between the years 2000 and 2015 increased by 65% (4). However, the prescriptions of antibiotics in Sweden are decreasing. Between years 2017 and 2018 the prescription (38) in Sweden decreased by 4.2% (5) and during 2020 sales of antibiotics per 1000 residents were decreased by 17% compared to previous year (6). Antibiotics are recommended to be used in a responsible manner that minimizes the risk of resistance development in bacteria since antibiotic use and resistance are correlated (7). According to the organisation GoHealth$_{TM}$ Urgent care some common bacterial infections do not need to be treated with antibiotics. For instance, sinus infections, bronchitis, paediatric ear infection and sore throat (8) are not needed antibiotic treatment. Furthermore, no infection caused by viruses can be treated with antibiotics (9).

When antibiotics have been used, any leftovers are recommended to not be saved or used for later. Leftovers or usage of antibiotics that have not been consulted with a healthcare professional can cause resistance (10) and other health complications such as yeast infections and Clostridium difficile induced diarrhoea (9). A study by Grigoryan et. al (2007) showed that there is an association between leftover antibiotics in Europe and self-medication (11), which means that there are patients that save antibiotics for later use. The most common reason for self-medication in Europe were upper respiratory tract infections. Antibiotic treatment has been shown to influence the gut microbiota and that it takes different amount of time for different taxa of bacteria to recover in population (12,13). Therefore, it is important to only take antibiotics prescribed by a health care professional, since self-medication will influence the microbiota and might not be specific to the infection. As stated before, antibiotic treatment can trigger resistance in bacteria, and this can occur in the gut microbiota as well.

1.2 Antibiotic resistance and antibiotics in the environment

The use of antibiotics for treatment in humans and in agriculture are pressuring bacteria to acquire resistance genes for survival, thus giving bacteria the ability to survive treatment in pressured environments. Resistance to antibiotics is not something new, many resistant strains could be identified after a short period of time. An example is penicillin which was out for sale during 1941 and resistant bacteria were being identified already one year later (14). When antibiotics are consumed, rest products of the antibiotic treatment will be excreted in the urine, both in metabolized and unmetabolized forms. When antibiotics are consumed generally, around 30-90% are being excreted in the urine unmetabolized (15), which then ends up in our sewage water and the sewage treatment works. The concentrations of antibiotics become diluted when entering the sewage water, but the prevailing concentrations might still influence antibiotic resistance selection. This can occur since resistant bacteria can be selected at minimal selective concentration (MSC), which can be much lower than the minimal inhibitory concentration (MIC) (16). Therefore, there are risks for resistance selection in environments where the concentrations of antibiotics are equal or over MSC for a specific bacterium. It has not been proven that selection can occur in sewage water, but if the concentration is at or above MSC it can be of risk.

At the sewage treatment plant (STP), the sewage water goes through a treatment process to remove contaminants. Afterwards, it is released in the environment via STP outlet pipes. The concentration found in the environment differs depending on the country and where in the environment the screening takes place.

A data analysis of antibiotics in surface water was made by Sanseverino et. al (2018) where they summarized data from global studies of 43 different antibiotics. The antibiotics with the highest mean concentration were Azithromycin, Sulfamethoxazole and Sulfapyridine at around 1µg/L. These antibiotics were also the highest measured antibiotics, together with Ciprofloxacin, at concentrations slightly over 10µg/L (17). However, most of the antibiotics had a maximum concentration around 0.1µg/L and 1µg/L. Sanseverino et. al also did the same for wastewater treatment plants effluents around the world, where the highest measured mean concentrations were of Cephalexin and Ofloxacin with concentrations over 1µg/L and below 10µg/L. The highest concentration measured were of Ofloxacin, at around 10µg/L and Sulfamethoxazole and Ciprofloxacin, with concentrations just below 10µg/L.

1.3 Antibiotics groups and mechanism of action

1.3.1 Macrolides and Chloramphenicol

Macrolides are a group of antibiotics that can be effective against a wide range of bacteria and can be used as a substitute for people with an allergy to penicillin (18). This type of antibiotic is isolated from Streptomyces species and the overall structural size is quite big compared to other antibiotics (reviewed by Retsema and Fu 2001). Macrolides can have either 14, 15 or 16- membered macrolide rings (19) and are lipophilic molecules (20). The mechanism of macrolide antibiotics is that they binds to the 50S subunit of the ribosome to prevent protein elongation in a broad spectrum of bacteria (reviewed by Patel and Hashmi 2020) (21). Chloramphenicol inhibits the protein synthesis in a broad spectrum of bacteria when it binds to the 50S subunit of the ribosome and inhibits the peptidyl transferase (22,23).

1.3.2 Lincosamindes

Lincosamides are a group of antibiotics that have a structure built with amino acids and sugar groups attached to. This group also interacts with the 50S subunit of the ribosomes and interferes with protein production (24). Like macrolides, produced by Streptomyces species (25).

1.3.3 Sulfonamides

Sulfonamides are a group of antibiotics that have a similar structure to the enzyme para-aminobenzoic acid (PABA) and are involved in the folic acid metabolism. These molecules bind to another enzyme, Dihydropteroate synthase that catalyses the reaction from PABA to dihydrofolic acid and inhibits it. This group of antibiotics are bacteriostatic, which means that it inhibits the growth and division of bacteria instead of killing it. Sulfonamides will not interfere with mammalian cells since mammals utilize folate from their food. Therefore, cells are not in a need of PABA for endogenous production (26,27).

1.3.4 Folate antagonists
Trimethoprim is an example of a folate antagonist. It interferes with the dihydrofolate reductase and inhibits it, this enzyme is involved in the catalysation of dihydrofolic acid to tetrahydrofolic acid, thus, disrupting the folic acid metabolism in the bacteria. (26,28)

1.3.5 Nitroimidazole
Metronidazole is a type of nitroimidazole used to treat anaerobic infections like clostridium, prophylaxis before surgery, amoebiasis and giardiasis. The exact mechanism of Nitroimidazoles seem to be unknown but a review by Weir and Le (2020) are discussing that metronidazole will enter the cell and cause DNA stand damage and loss of helical structure which leads to inhibition of proteins in susceptible bacteria (29).

1.4 Effects on zebrafish
There are relatively few studies on toxicological effects of antibiotics on zebrafish embryos and larvae, but generally it seems like some antibiotics can affect embryo development. For instance, at concentrations of 20µg/L of tetracycline, it has been shown to affect the development and growth of zebrafish embryos and larvae, such as body length shortening, increased yolk sac and shrunken swim bladder (30). Sulphonamides, in this case Sulfamethoxazole, has been shown to increase the heartbeat and decrease the spontaneous swimming activity when larvae are exposed to concentration of 10µg/L (31). Sulfamethoxazole, enrofloxacin, cefotaxime and tetracycline at have all been reported to affect growth of embryos with shorter body length at concentrations of 100µg/L. A combined mixture with a total concentration of 100µg/L with all four antibiotics showed the same results (32).

1.5 Zebrafish as model organism
The zebrafish (*Danio rerio*) is a model organism widely used in science. Zebrafish are well explored and their whole genome has been sequenced. During a mating period, a female zebrafish can lay hundreds of eggs, which can be used for experiments. Zebrafish embryos are transparent, which makes it easy to follow their internal development of different organs for studies and other endpoints important for the study (33).

1.5.1 Zebrafish embryo development
The development time of zebrafish embryos increases if the temperature is high and decrease if the temperature is low. The below description of zebrafish embryo developmental timepoints by Kimmel et. al (1995) is at standard temperature of 28.5 °C (34).
The fertilized egg cell is considered a zygote when it is at the one-cell stage and will stay as a zygote until it starts to divide, this usually takes around 40 minutes to occur. Then the cell starts to divide and enters the two-, four-, eight-, 16-, 32-, and 64-cell stage with a duration of approximately 15-minute intervals between stages.

The cell then enters the blastula period which lasts around three hours where the egg cell continues to divide. Thereafter the developed blastula will enter the gastrula period where gastrulation will occur (34). Gastrulation is the development of a one-dimensional cell layer into a three-dimensional structure built of multiple cell layers (35). When this period is finished, the embryo is about 10 hours old, and will then enter the segmentation period. The embryo

starts to form and stays in this stage until it reaches 24 hours post fertilization (hpf). At this stage, the embryo starts having early movements. From 24hpf and 48hpf the embryo will enter the pharyngula period, during which the pigmentation of the embryo happens. From around 48hpf to 72hpf the embryo will enter the hatching period where the embryo will leave its egg and become a larva. Lastly, the larvae enters the early larval period 3 days post fertilization (dpf). During this period the swim bladder will inflate and the larvae starts to increase its swimming activity (34).

1.6 Aim of this study

The aim of the present study was to assess the embryo toxicity of eight different antibiotics. The selected antibiotics were based on previous measured antibiotics in river Fyrisån, Uppsala, Sweden. The toxicity was measured both for individual antibiotics and for environmental mixtures. Chemical markers were read for verification of actual exposure concentrations and measurement of bioaccumulation and bioconcentration factors were calculated for the mixtures to determine the level of bioaccumulation in embryos.

2. Materials and methods

2.1 Experimental antibiotics

Eight different antibiotics were assessed for zebrafish embryo toxicity in the present study. The antibiotics were selected based on a previous study of analytical measurements in surface water from river Fyrisån, Uppsala, Sweden, during a 3-year period from 2017 to 2019 (Örn et. al 2019) (36).

Table 1. Modified table from Örn et. al. 2019 (36) showing the concentrations of eight antibiotics measured at three different time points, 10m downstream of the Uppsala STP outlet.

Antibiotics	10m from STP 2017 (ng/L)	10m from STP 2018 (ng/L)	10m from STP 2019 (ng/L)
Chloramphenicol	<LOQ	1.3*	<LOQ
Trimethoprim	13	57*	9.2
Sulfamethoxazole	36*	<LOQ	20
Erythromycin	13	18*	0.72
Roxithromycin	0.8	4.9*	0.11
Clarithromycin	12	398*	3.8
Clindamycin	47	390*	8.4
Metronidazole	4.6	36	64*

** Selected environmental antibiotic concentration for toxicity assessments in the present study.*

2.1.1 Antibiotic stocks

The antibiotic stocks were prepared using commercial antibiotic salt which was dissolved in 99% methanol.

Table 2. Chemical information about each individual antibiotic and the final stock concentrations, prepared stock solutions and tested concentrations.

Antibiotic	CAS number	Supplier	Stock concentration mg/L	Tested concentration µg/L	Antibiotic group
Chloramphenicol	56-75-7	Sigma-Aldrich	1346	135	Chloramphenicol
Trimethoprim	738-70-5	Sigma Aldrich Fluka	1133	113	Folate antagonist
Sulfamethoxazole	723-46-6	Sigma-Aldrich	1816	182	Sulfonamide
Erythromycin	114-07-8	Sigma-Aldrich	1725	173	Macrolide
Roxithromycin	80214-83-1	Sigma-Aldrich	1085	109	Macrolide
Clarithromycin	81103-11-9	Sigma-Aldrich	1520	152	Macrolide
Clindamycin	21462-39-5	Sigma-Aldrich	1346	135	Lincosamide
Metronidazole	443-48-1	Sigma-Aldrich	1530	153	Nitroimidazole

2.2 Egg collection and zebrafish care

2.2.1 Adult care

The adult zebrafish were kept in 54-L tanks containing 26°C carbon filtered tap water at the Fish lab at the department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. The fish were fed 1-2 times daily with commercial flakes (SERA Vipan) and were held at a 12:12 h light to dark regime.

2.2.2 Egg collection

The day before egg collection four 10-L aquariums were filled with carbon filtered tap water (26°C) up to ¼ of the aquarium. In each aquarium, a stainless-steel mesh cage was placed containing silk ribbons and glass marbles to help stimulate spawning of the zebrafish. In each cage 8-12 adults were placed overnight. The next morning at onset of light the cages with the fish were moved to new 10-L aquariums allowing collection of newly laid and cleaner eggs. After around 60 minutes of spawning, the newly laid eggs were collected and examined using a stereo microscope (LEICA EZ4D). The examination was made to select healthy and fertilized eggs. Fertilization was considered successful in eggs starting their cell division, which was why eggs in the 4-36 cell stage were selected. The selected eggs were then placed in Petri dishes containing the different antibiotic exposure solutions to enable immediate exposure. The

exposed eggs were then transferred individually into transparent 96-well rounded microtiter plates along with 250µL of the exposure solution (composed of either individual or mixture antibiotic solution in carbon filtered tap water at 26°C).

2.3 Fish embryo toxicity test (FET)

The toxicity testing in the present study was based on the standardized OECD method (38), Fish Embryo Toxicity test (FET) with modifications based on Carlsson et. al (2013) as well as behaviour and length measurement (38).

2.3.1 Individual antibiotics

Individual antibiotics were exposed to embryos (n=22 for each treatment group except Chloramphenicol, Trimethoprim and Sulfamethoxazole with n=20) in concentrations between 100-200µg/L (Table 2) together with control containing 1:10000 dilution of methanol. Eggs exposed to antibiotics were placed in two 96-well plates and endpoints were then observed from 24hpf to 144hpf (Table 3).

2.3.2 Mixture antibiotics

Four mixtures were prepared, one containing environmental concentrations (Table 1) and the rest were increased by 10-folds up to 1000-times higher the environmental concentration. A control was prepared containing 1:10000 dilution of methanol. Embryos (n=24 for the control and n=18 for the different mixtures) were placed on a 96-well plate and endpoints were then observed from 24hpf to 144hpf (Table 3).

2.3.3 Positive control

The positive controls exposed to embryos were PCB-126 (20µg/L), Tramadol (10µg/L) and Albendazole (50µg/L). Two different controls were used for the testing, one containing 1:10000 dilution of methanol since PCB-126 and Albendazole were dissolved in methanol and diluted 1:100000. Tramadol, as well as control 2, was dissolved in carbon filtered tap water. Embryos (n=12 for each control group and n=24 for PCB-126, Tramadol and Albendazole) were placed on a 96-well plate and endpoints were then observed from 24hpf to 144hpf (Table 3).

2.3.4 Plate design

For the layout of the plate, each treatment group were placed diagonally on the 96-well plate to achieve a randomized block design for evenly distributed edge interference. Parafilm© was added on top of the plate to prevent evaporation of the medium. The plate was then incubated in 26 ± 0.5°C.

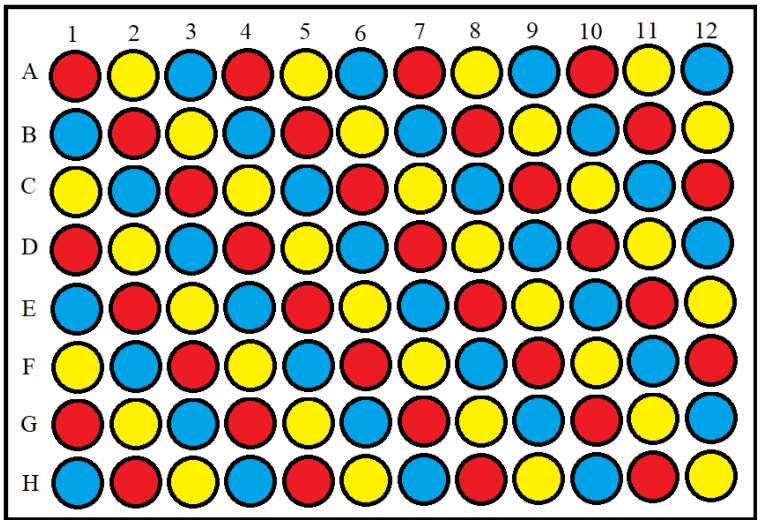

Figure 1. **Diagonal plate design.** An example on how the 96-well plate design looks like, with each colour representing different treatment groups.

2.3.5 Lethal and sublethal Endpoints

At three timepoints during the development, 24, 48 and 144hpf, sublethal and lethal endpoints were examined in the zebrafish embryos (Table 3) using stereo microscope (LEICA EZ4D), as well as a reversed microscope (Olympus CKX41) to measure the heart rate. A time laps camera (Canon Eos 500D) taking one photo each hour from 48 to 144hpf was used to determine the hatching time of each individual embryo. Movements were counted for 30 seconds and multiplied by two in order to get movements per minute. For heart rate, 30 beats were counted with a timer on, then calculated to get heartbeats per minute.

Table 3. Measured endpoints in the FET test at different hours post fertilization (hpf). Modified table from Carlsson et. al (2013) (38). “x” determines at which timepoint each endpoint was observed at.

Endpoints	Description	hpf		
Lethal categorical		24	48	144
Coagulation	Dead embryo, dissolved structures	x	x	x
Lack of heartbeat	No heartbeat is detectable		x	x
Sublethal categorical				
Tail deformation	Atypical shape; shorter and/or curved	x	x	x
Eye deformation	Atypical shape and/or size, no existing lens	x	x	x
Head deformation	Atypical shape and/or size	x	x	x
Reduced pigmentation	Visible reduction of pigment in eyes and/or body		x	
Oedema	Oedema is present		x	x
Lack of circulation	No existing circulation in tail artery		x	
Side-laying	Embryo lies on the side, not able to change position			x
Unhatched	Embryo is still in the egg			x
Sublethal continuous				
Movements	Number of movements for 60 seconds	x		
Heart rate	Number of heartbeats for 60 seconds		x	
Behaviour	Swimming distance at dark-light alternating exposure			x
Length	Measuring the length of each embryo			x
Hatching time	One photo taken every hour to determine the hatching time		48-	144

The behaviour analysis was done at 6 days post fertilization (dpf), monitoring each individual embryo using ViewPoint Zebrabox®, version 3.2, to record the swimming activity. The protocol included a 10-minute acclimatization phase followed by two alternating five-minute dark and five-minute light cycles, giving a total runtime of 30 minutes (including acclimatization phase). All data collected during this period were recorded in 10-second intervals, including the swimming distance. After the behaviour measurements, the embryos were each given 5µL Ethyl 3-aminobenzoate methanesulfonate (MS-222), 30g/L, as anaesthesia to take pictures and measure length. The lengths of the embryos were measured at 6dpf using ImageJ version 1.53g, which converts number of pixels between two areas to the chosen scale, in this case millimeters. The length was measured from tip of mouth to end of dorsal fin of the embryo (Figure 2).

Figure 2. Measurement of zebrafish embryo.
Showing how embryos were measured.

2.4 Bioaccumulation

For measurements of antibiotic bioaccumulation, a modified version of Vogs et. al (2019) protocol was used (39).

2.4.1 Preparation of embryo- and water samples

Newly fertilized zebrafish eggs (n=55) were collected and placed in Petri dishes. The eggs were exposed to two different concentrations of the antibiotic mixture described above in a total volume of 50 mL, i.e., the environmental relevant mixture concentration and the 1000x environmental concentration. Three replicates were used for each treatment group, including a control treatment only containing carbon filtered tap water at 26°C and 1:10000 dilution of methanol. Water samples (1 mL) from each of the replicate Petri dishes were collected at day 0, 2, 4 and 6. Sampling of the embryos occurred on day 6. The embryos were sampled in Eppendorf tubes and as much water as possible was removed. Samples were then stored at -22°C until chemical analyses.

2.4.2 Embryo samples

The samples were thawed in room temperature. Embryo samples were then washed two times with 500µL of double distilled water (MilliQ) together with slow and gentle shaking of the tube. Afterwards, all liquids were removed before adding 500 µL acetonitrile (ACN) + 0.1% formic acid (FA) and 10µL of internal standard. Embryo samples were then homogenized with a handheld electric pellet pestle and incubated for an hour in room temperature. Afterwards, samples were centrifuged (Eppendorf centrifuge 5424R) for 10 minutes in 21°C at 14.000 rpm and the supernatant was collected afterwards. 200µL of the supernatant were added in glass vails (300µL fixed insert vials) and stored in fridge overnight. Three blank samples were prepared the same way as the embryo samples.

2.4.3 Water samples

Water samples were taken out from the freezer to thaw in room temperature, then 500µL were transferred to glass vials (Agilent Technologies, Vial, 2mL screw) and 10µL of internal standard was added to the sample.

2.4.4 Chemical analysis

The embryo and water samples were analysed using liquid chromatography tandem-mass spectrometry (LC-MS/MS) with an LC system from Thermo Fisher Scientific, San Jose, CA, USA, and a triple-stage quadrupole MS/MS TSQ Quantiva (Thermo Fisher Scientific). An Acquity UPLC BEH-C18 column (Waters, 100mm x 2.1 i.d., 1.7 µm particle size from Waters Corporation, Manchester, UK) was used as an analytical column. Injection volume was 10 µL for all samples. A heated electrospray ionization (HESI) was used to ionize the target compounds. The spray voltage was set to static: positive ion (V) 3500.00. Nitrogen (purity >99.999%) was used as a sheath gas (50 arbitrary units), auxiliary gas (15 arbitrary units) and sweep gas (2 arbitrary units). The vaporizer was heated to 400°C and the capillary to 325°C. Data were evaluated using TraceFinder™ 3.3 software (Thermo Fisher).

2.5 Statistical analysis

All collected data were analyzed using Minitab® 19 to determine significant differences between treatments. Continuous data, such as movements and heartbeats, were tested for normality distribution to determine what test should be used for analyzing. If the data were normally distributed, a one-way ANOVA were followed by a Dunnett's post-hoc tests to determine significance. For sublethal and lethal categorical data (affected/not affected) the Chi^2 test or Fisher's exact test was used to determine differences. Differences were considered significant at $p<0.05$.

2.5.1 Bioconcentration factor calculation (40)*:*

The weight of one individual larvae was assumed at 0.35mg for the calculation (40).

$$BCF = \frac{C_{Larva}}{C_{Water}}$$

C_{Larva} = Measured concentration of antibiotic in embryos (in mg per kg larvae).
C_{Water} = Measured concentration of antibiotics in water (day 6) (in mg per kg water).

3. Results:

The toxicity testing in the present study was based on the standardized OECD method (37), Fish Embryo Toxicity test (FET). However, the Original FET-test only covers lethal endpoints up to 96 hours post fertilization (hpf). In the present study the toxicity test was extended to 144hpf and included additional sublethal endpoints based on the experimental design by Carlsson et. al (2013) as well as swimming activity behaviour and length measurements (38).

3.1 FET-test of individual antibiotics

First experiment was conducted for the purpose to assess the toxicity of the antibiotics individually and determine if they have any toxic effects on zebrafish embryos. This is also done to identify possible drivers of toxicity. Each antibiotic was tested individually at the highest concentration possible depending on the stock concentration (Table 2). These concentrations were used for fish embryo toxicity test (FET). The assessment of zebrafish toxicity started with exposing embryos to the individual antibiotics at the highest concentration possible from the antibiotic stocks (Table 2). Each stock was diluted 1:10000 in carbon filtered tap water at 26°C and eggs were placed in the prepared solution. A control was made containing same concentration of methanol as the other samples (1:10000 dilution). Eggs were moved to a 96-well plate in a diagonal placement (Figure 1) and incubated at 26°C during the whole experiment (144h). Different endpoints were observed at different timepoints (Table 3) to determine any effects by individual antibiotic exposure.

3.1.1 Lethal categorical and sublethal categorical endpoints

Lethal categorical and sublethal categorical endpoints were observed at all timepoints (24-, 48 and 144hp (Table 3) in a stereomicroscope. All the different lethal categorical and sublethal categorical endpoints were summed as seen in Figure 3. For lethal categorical and sublethal categorical endpoints, no effects were recorded at timepoint 24- and 48hpf. At 144hpf, two embryos were coagulated, one in the Clarithromycin treated group and one in the Trimethoprim treated group with no significance. At the same timepoint, several embryos were recorded as side-laying in each treatment group, however not significantly different to the control group. All embryos categorized as lethally or sublethally affected were excluded in the behavioural study.

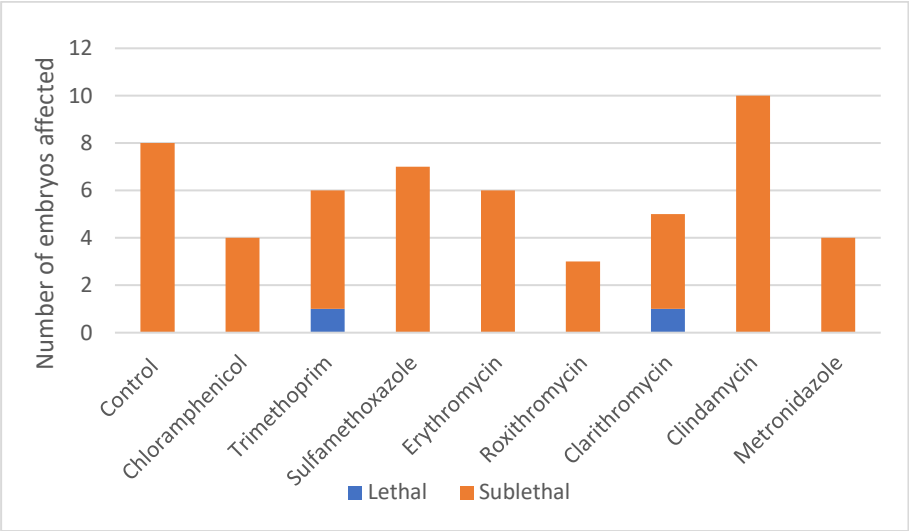

Figure 3. Lethal and sublethal categorical endpoints after individual antibiotic exposure. Graph showing number of affected embryos in each treatment group of individual antibiotics. Orange represents sublethal categorical and blue represents lethal categorical endpoints.

3.1.2 Early movements

At 24hpf the early movements were observed using a stereomicroscope. The movements were counted for 30 seconds, and the value were multiplied by two to get movements per minute. The mean early movements in embryos in all treatment groups varied between 5 and 7 movements per minute. The highest average number of movements was recorded for the Erythromycin treated group, whereas the lowest were the control and Metronidazole groups (Figure 4A). None of the results were significant compared to the control.

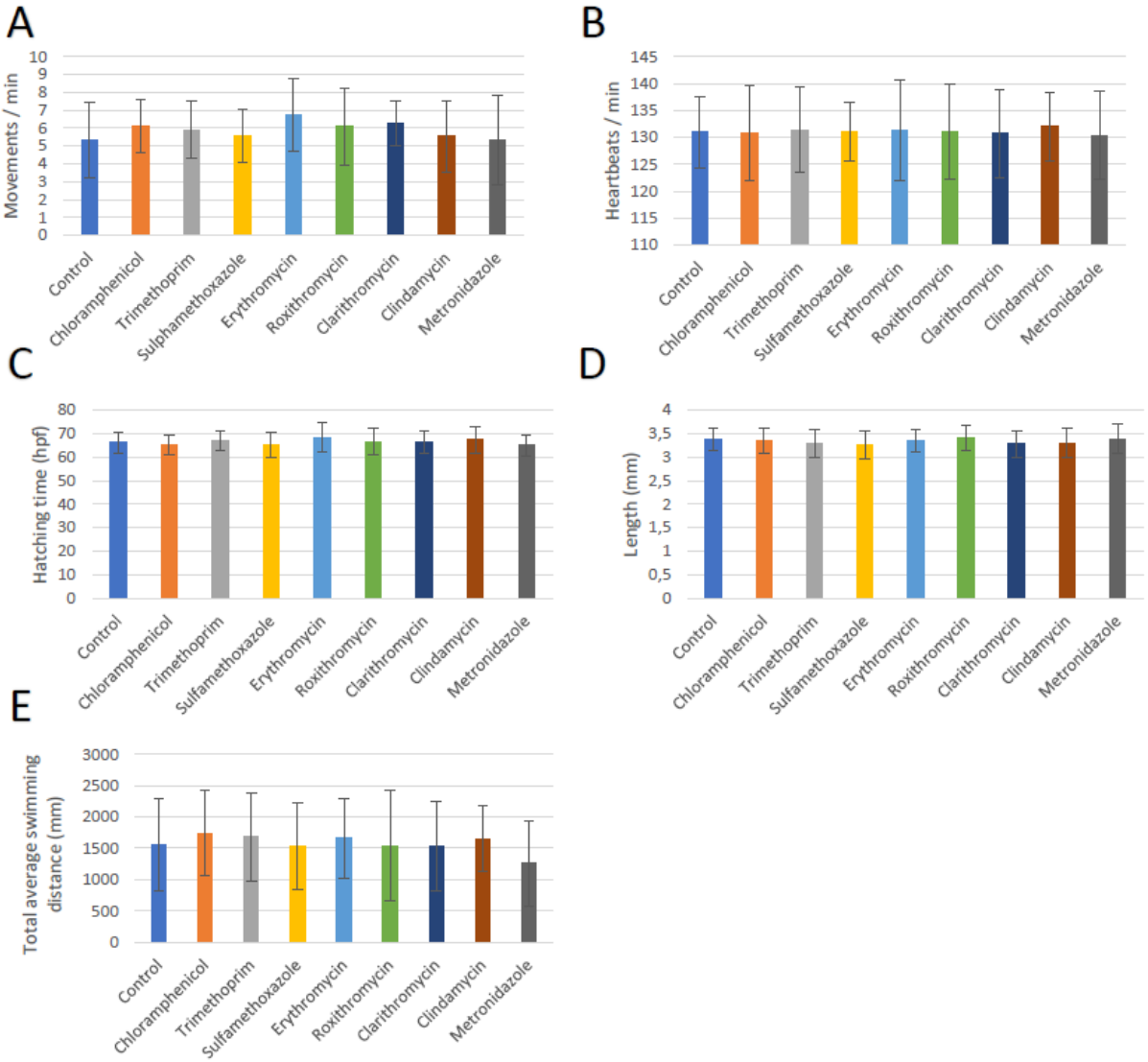

Figure 4. **Sublethal continuous endpoints observed in zebrafish embryos exposed to individual antibiotics.** A) Graph showing number of movements per minute for each antibiotic treatment group. B) Graph showing measured heartbeats per minute for each antibiotic treatment group. C) Graph showing the hatching time for each antibiotic treatment group. (hpf=hours post-fertilization). D) Graph showing mean length in embryos for each antibiotic treatment group. E) Graph showing the total average swimming distance in each treatment group of individual antibiotics.

3.1.3 Heartbeats

At 48hpf heart rate was measured using a reversed microscope. First, 30 heartbeats were counted with a timer, and then the heartbeat per minute was calculated using the time it took for 30 beats to beat. The mean number of heart beats in all different antibiotic treatment groups were around 130. The highest mean heart rate measured was for the Clindamycin treated group whereas the lowest measured was for Metronidazole (Figure 4B). The heart rates from the different antibiotic groups were not significantly different from the control.

3.1.4 Hatching times

A camera was set up and a time-lapse was started, after measuring the heart rate, at 48hpf until 144hpf. The mean hatching times varied between 60 and 70 hpf in all individual antibiotic

treatment groups. The highest hatching time recorded was in the Erythromycin treated group, and the lowest was recorded for the Metronidazole treated group (Figure 4C). There was no significance between any groups compared to the control.

3.1.5 Behaviour

Behaviour was measured at 144hpf and exposed embryos to alternating dark-light periods, each for five minutes. Total runtime was 30 minutes including a 10-minute acclimatization phase. The values were shown in 10s intervals for the 5min periods, thus the distance is in mm/10s. During the darkness periods, the embryos were much more active and swam longer than during the light period where the embryos were less active. Embryos from all individual antibiotic treated groups were swimming around 20mm/10s at each darkness period in average (Figure 5). The highest mean measured activity was in the Clindamycin treated group during the first darkness period (mean 23mm/10s), whereas the group with least activity in darkness was the metronidazole treated group (mean 16mm/10s). During the light periods, the swimming activity were around 5mm/10s for all treatment groups. No significant differences were recorded for swimming activity.

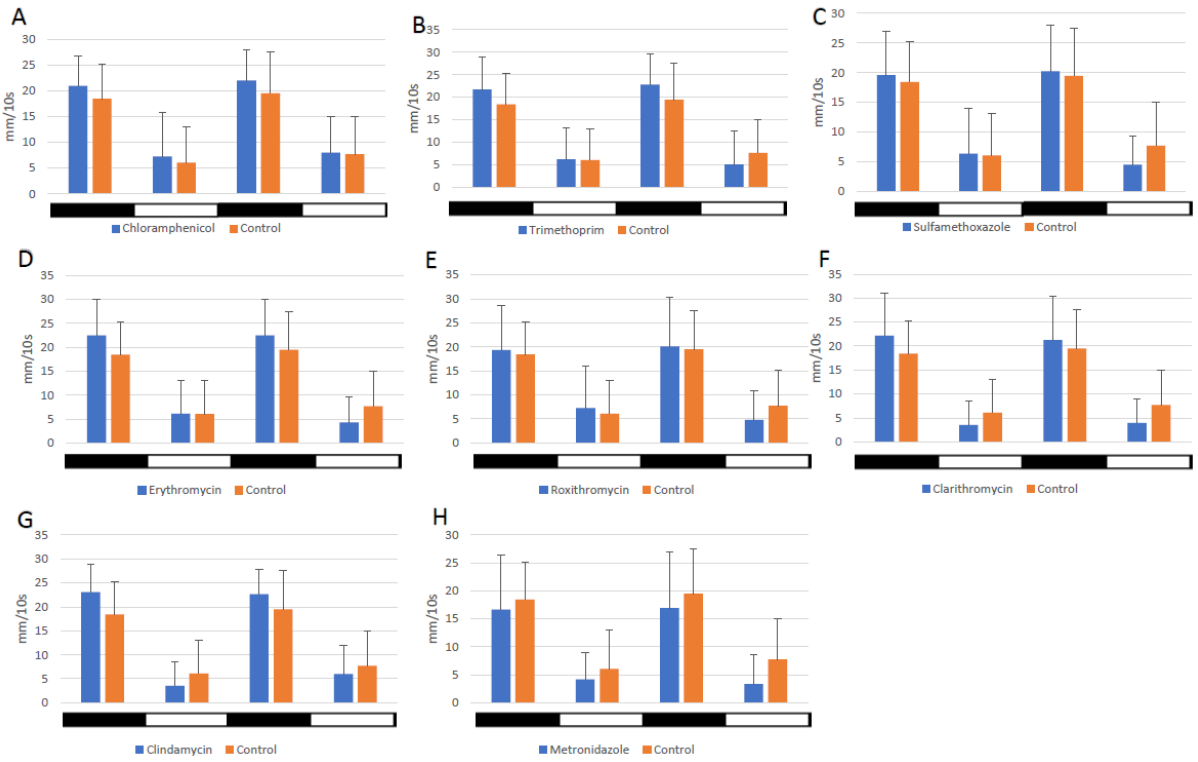

Figure 5. Behaviour study of embryos exposed to individual antibiotics. Graphs showing swimming distances during dark and light alternating period for individual antibiotic treatment groups compared to the control. The black and white bar below the graph represent dark and light phases respectively for the swimming distances. The orange bars represent the control whereas the blue bars represent the individual antibiotics. The different figure letters represent: A) Chloramphenicol, B) Trimethoprim, C) Sulfamethoxazole, D) Erythromycin, E) Roxithromycin, F) Clarithromycin, G) Clindamycin and H) Metronidazole.

The total swimming distance was calculated from the 20 minutes of dark-light alternating periods to determine if the total distance was affected. The highest average total swimming distance was observed in the Chloramphenicol treated group whereas the lowest was observed

in the Metronidazole treated group (Figure 4E). There was no significant difference between the groups compared to the control.

3.1.6 Lengths

Lastly, the embryos were anaesthetised using MS-222. Photos of embryos were taken after behaviour study at 144hpf using a microscope and then measured in the program ImageJ. The lengths of the embryos in each antibiotic treatment group varied between 3 and 3.5 mm. The longest mean measured length was for the Roxithromycin treated group whereas the shortest mean length was measured for the Sulfamethoxazole treated group (Figure 4D). No significant differences between the treatment groups were measured.

3.2 FET-test of antibiotic mixtures

After the individual antibiotic exposure, mixture of all together in environmentally relevant concentrations were used to expose embryos. This was done in order to determine if the antibiotics together in a mixture cause an effect on zebrafish embryo development. In the previous study by Örn et. al (2019), they collected surface water at several sites downstream the Uppsala sewage treatment plant (STP) (36). The measured concentrations were the highest closest to the STP outlet (10m downstream the STP). In the present study, the highest measured surface water concentrations, measured at the 10m site during the 3-year period, were selected for each antibiotic (Table 1) and were used for toxicity assessments of mixtures. Calculations were made to determine the volume of each antibiotic stock solution that should be combined to achieve the desired concentrations in the final antibiotic mixture stock solution. The mixture was designed to replicate the environmental concentrations measured in river Fyrisån. The antibiotic mixture was tested at an environmentally relevant mixture concentration as well as in three increasingly 10-fold higher concentrations up to 1000-times higher.

Table 4. Information on tested concentrations for mixture antibiotics based on concentrations found in Fyrisån (Table 1).

Antibiotic	Mix Environment (ng/L)	Mix 10x Environment (ng/L)	Mix 100x Environment (ng/L)	Mix 1000x Environment (ng/L)
Chloramphenicol	1.3	13	130	1300
Trimethoprim	57	570	5700	57000
Sulfamethoxazole	36	360	3600	36000
Erythromycin	18	180	1800	18000
Roxithromycin	4.9	49	490	4900
Clarithromycin	398	3980	39800	398000
Clindamycin	390	3900	39000	390000
Metronidazole	64	640	6400	64000

Each mixture was diluted 1:10000 (except for the highest concentration) in carbon filtered tap water at 26°C and eggs were placed in the solution. A control was made containing same concentration of methanol as the other samples (1:10000 dilution). An extra control with dilution 1:1000 was not made for the highest concentration of mixture antibiotics (1000x mix), due to previous experience with this concentration of methanol in the lab showed no toxic

affect. It has also been studied before and 1:1000 dilutions have not been linked to any toxicity to fish and therefore we chose not to include a control for it (41,42). All treatment groups share one control group. Eggs were moved to a 96-well plate in a diagonal placement (Figure 1) and incubated at 26°C during the whole experiment (144h). Different endpoints were observed at different timepoints (Table 3) to determine any effects.

3.2.1 Lethal categorical and sublethal categorical endpoints

Lethal categorical and sublethal categorical endpoints were observed at all timepoints (24-, 48 and 144hp (Table 3) in a stereomicroscope. All the different lethal categorical and sublethal categorical endpoints were summed as seen in Figure 6. No effects on lethal categorical and sublethal categorical endpoints were recorded during timepoint 24- and 48 hpf. At timepoint 144 hpf two embryos from the Mix 100x group were side-laying, had heart oedema, tiny eyes, and yolk sac oedema. This was not significant compared to the control. These embryos were excluded from the behaviour study.

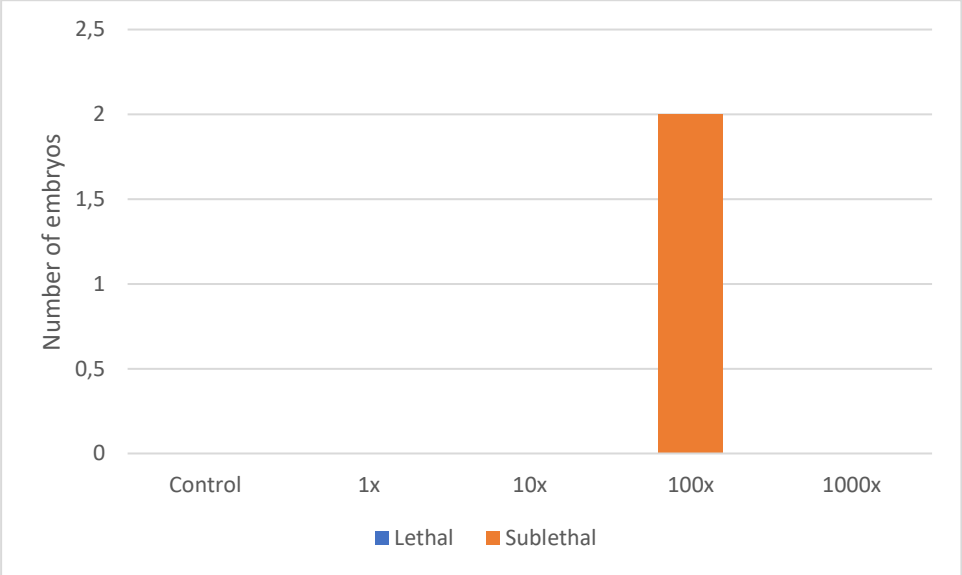

Figure 6. Lethal and sublethal categorical endpoints after antibiotic mixtures exposure. Number of affected embryos in the treatment groups. Orange represents sublethal categorical and blue represents lethal categorical endpoints.

3.2.2 Early movements

At 24hpf the early movements were observed using a stereomicroscope. The movements were counted for 30 seconds, and the value were multiplied by two to get movements per minute. The early movements in all mixture treatment groups varied between 3 to 5 movements per minute. The lowest number of early movements were measured in the environmental mix treatment group and the highest number of early movements was measured in the Mix 1000x treatment group (Figure 7A). No significant differences were observed for early movements in the mix treatment groups.

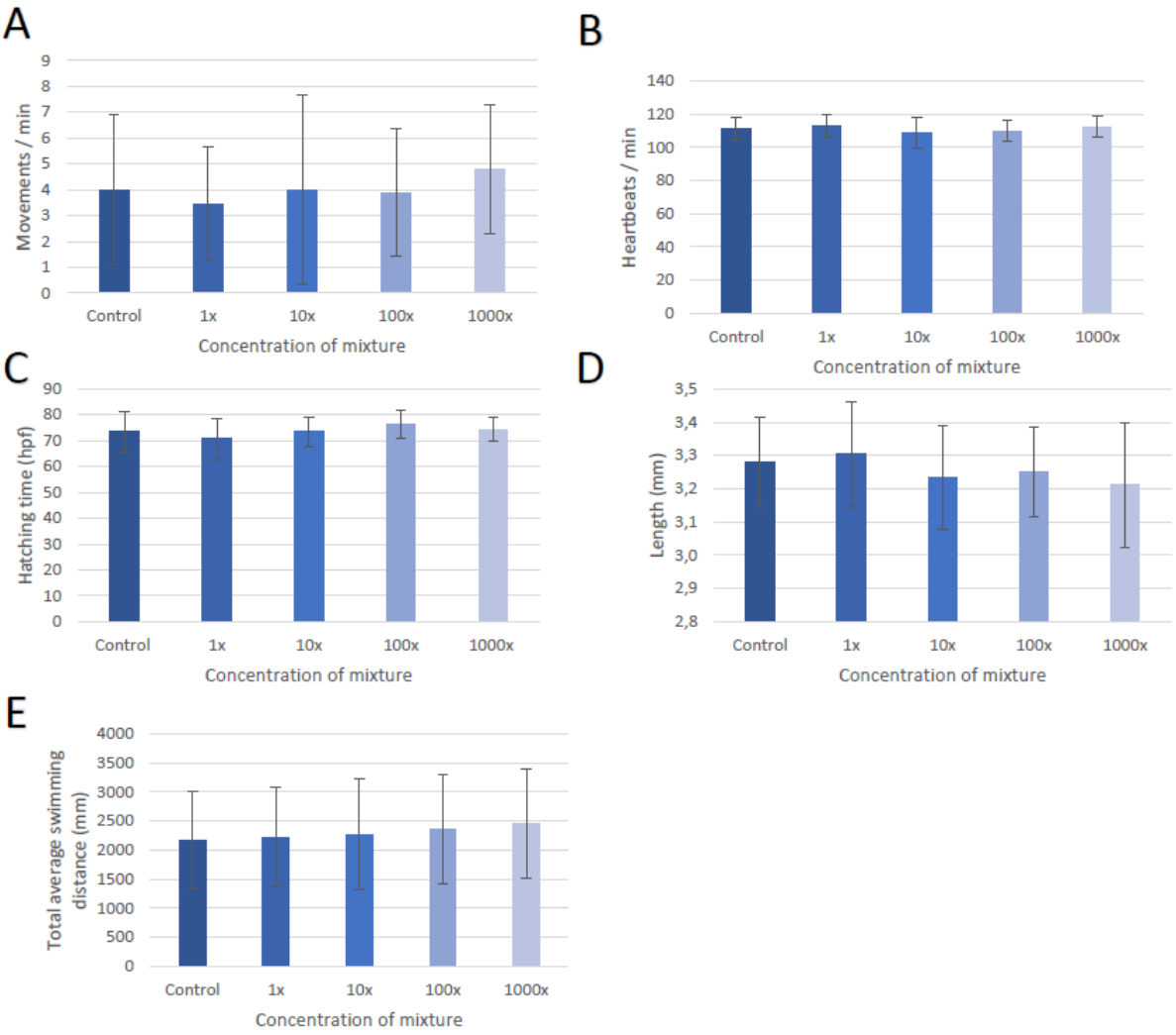

Figure 7. Sublethal continuous endpoints observed in zebrafish embryos exposed to antibiotic mixtures. A) Graph showing the number of early movements per minute in the control group and the different antibiotic mix treatment groups. B) Graph showing the heart rate (heartbeats per minute) for the control and the different antibiotic mixture groups. C) Graph showing the hatching time (hpf) for the control and the antibiotic mixture groups. D) Graph showing the length of embryos in the control group and the different antibiotic mixture groups. E) Graph showing total average swimming distance for each treatment group of mixture antibiotics.

3.2.3 Heartbeats

At 48hpf heart rate was measured with a reversed microscope. First, 30 heartbeats were counted with a timer, and then the heartbeat per minute was calculated using the time it took for 30 beats to beat. The mean number of heartbeats per mix treatment group varied between 108 and 113 beats per minute. The highest number was measured in the Mix 1x, whereas the lowest measured was in the Mix 10x group (Figure 7B). There were no significant differences in heartbeats between the groups and the control.

3.2.4 Hatching times

A camera was set up and a time-lapse was started, after measuring the heart rate, at 48hpf until 144hpf. The hatching time in all mix treatment groups varied between 70 and 80 hpf. The group with the highest average hatching time were the Mix 100x group whereas the lowest was measured in Mix 1x group (Figure 7C). No significant differences were recorded between the different mix treatment groups and controls.

3.2.5 Behaviour

Behaviour was measured at 144hpf and exposed embryos to alternating dark-light periods, each for five minutes. Total runtime was 30 minutes including a 10-minute acclimatization phase. The values were shown in 10s intervals for the 5min periods, thus the distance is in mm/10s. During the darkness periods, the embryos were much more active and swam longer than during the light period where the embryos were less active. The swimming activity for the different antibiotic mixture groups were approximately the same as for the control group, with mean 25mm/10s during the darkness periods and 10mm/10s during light periods. No significant differences in swimming activity were recorded between the different mix treatment groups and controls.

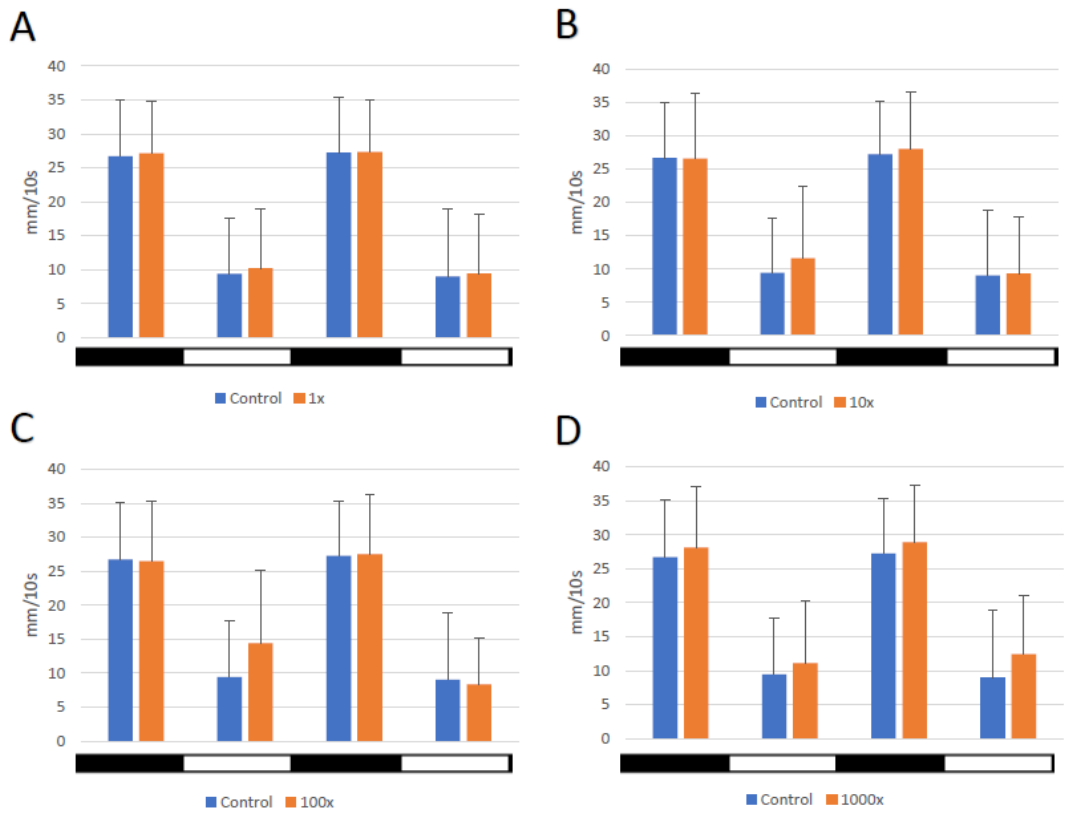

Figure 8. Behaviour study of embryos exposed to antibiotic mixture. Graphs showing swimming distances during dark and light alternating period for the different antibiotic mix treatment groups compared to the control. The black and white bar below the graph represent dark and light phases respectively for the swimming distances. The orange bars represent the control whereas the blue bars represent the antibiotic mixture group. The different figure letters represent: A) Mix 1x, B) Mix 10x, C) Mix 100x and D) Mix 1000x.

The total average swimming distance was calculated from the 20 minutes of dark-light alternating periods to determine if the total distance was affected. The control group had the lowest average distance, and the highest were 1000x mixture (Figure 7E). No significant difference was seen between these groups.

3.2.6 Lengths

Lastly, the embryos were anaesthetised using MS-222. Photos of embryos were taken after behaviour study at 144hpf using a microscope and then measured in the program ImageJ. The mean lengths of the embryos in all groups varied between 3.2 mm and 3.3 mm. The longest measured length was in the Mix 1x whereas the shortest length was measured in the Mix 1000x group (Figure 7D). No significant differences were recorded between the different mix treatment groups and controls.

3.3 FET-test of positive controls

Exposure to three different positive controls were used to validate the zebrafish embryo toxicity method. The compounds were PCB-126, Tramadol and Albendazole which has been shown in our lab to have effects on zebrafish embryos. The assessment of zebrafish toxicity started with exposing embryos to positive controls that has been shown in our lab to have effects on zebrafish embryos. PCB-126 at 20µg/L, Tramadol at 10µg/L and Albendazole at 50µg/L was used. Both Albendazole and PCB-126 were solved in methanol and diluted 1:10000 whereas Tramadol was solved in carbon filtered tap water. Control 1 contained 1:10000 dilution of methanol and Control 2 contained carbon filtered tap water. Eggs were moved to a 96-well plate in a diagonal placement (Figure 1) and incubated at 26°C for the whole experiment.

4.3.1 Lethal categorical and sublethal categorical endpoints

Lethal categorical and sublethal categorical endpoints were observed at all timepoints (24-, 48 and 144hp (Table 3) in a stereomicroscope. All the different lethal categorical and sublethal categorical endpoints were summed as seen in Figure 9. High mortality was recorded at 24hpf for the group exposed to Albendazole with 14 dead embryos. The remaining ten embryos suffered no mortality up to 144 hpf. No effects were recorded in any of the mixture treatment groups at 24 hpf or 48 hpf. At 144 hpf one embryo in the group exposed to Tramadol had yolk sac oedema, heart oedema and small eyes. In the group exposed to PCB-126 eleven embryos were affected by sublethal categorical and lethal categorical effects, where nine of the 11 were side-laying, nine had yolk sac oedema, eight had heart oedema, one had small eyes, two had malformed heads and one was coagulated. These observations with Albendazole and PCB-126 are significant compared to control (Figure 9).

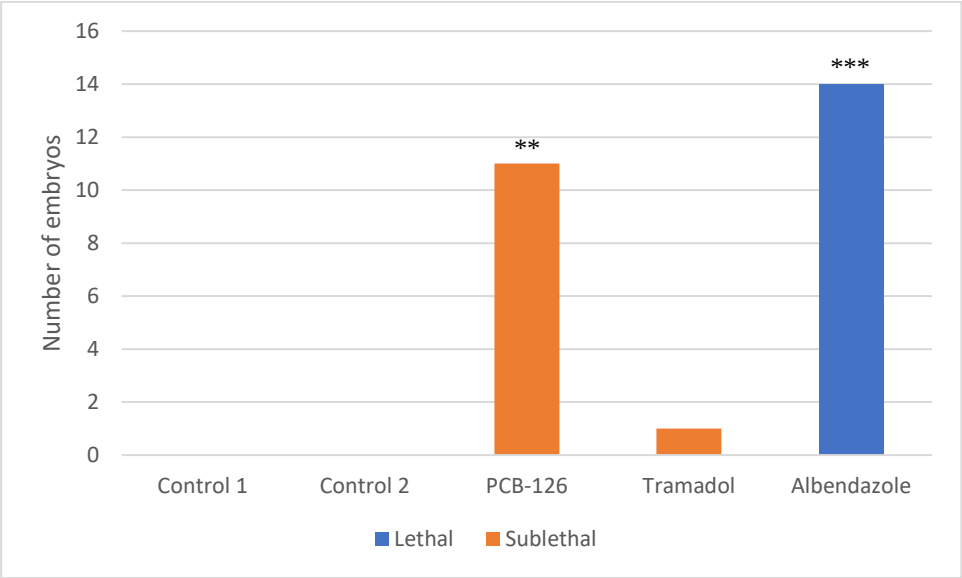

Figure 9. Lethal and sublethal categorical endpoints after antibiotic mixtures exposure. Number of embryos affected at 144 dpf after exposure to the positive control treatments PCB-126, Tramadol, and Albendazole. ** = $p<0.01$ and *** = $p<0.001$.

4.3.2 Early movements

At 24hpf the early movements were observed using a stereomicroscope. The movements were counted for 30 seconds, and the value were multiplied by two to get movements per minute. The mean measured early movements for the positive controls varied between two and four movements per minute. The highest mean number of movements were observed in the Tramadol treated group with approximately 4 movements/min whereas the lowest number of movements were observed in PCB-126 treated group having a mean of 2.6 movements/minute (Figure 10A). No significance was observed between treatment groups and controls.

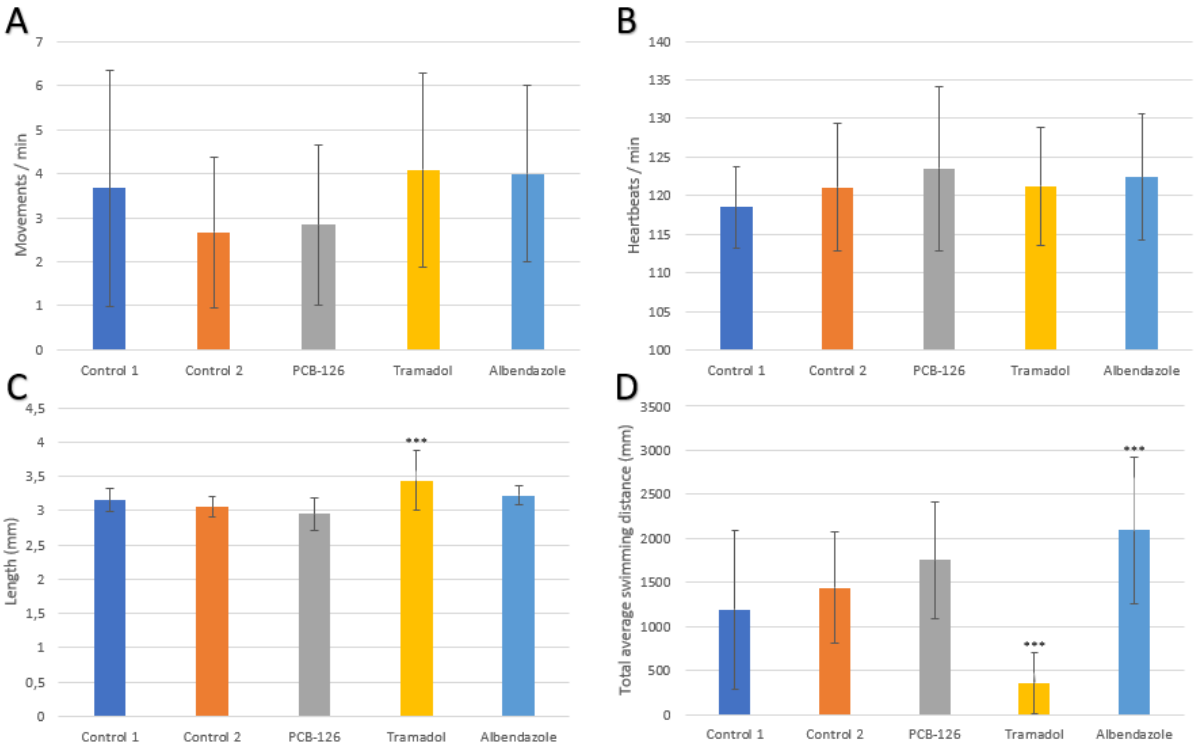

Figure 10 Sublethal continuous endpoints observed in zebrafish embryos exposed to positive controls. Control 1 is representative for PCB-126 and Albendazole, while control 2 is representative for Tramadol. A) Graph showing the number of early movements per minute in groups exposed to the positive controls PCB-126, Tramadol and Albendazole and two different controls. B) Graph showing the heart rate in groups exposed to the positive controls PCB-126, Tramadol and Albendazole and two controls. C) Graph showing lengths of embryos in groups exposed to the positive controls. D) Graph showing the total average swimming distance for each treatment group of positive controls. *** = P<0.001.

4.3.3 Heartbeats

At 48hpf heart rate was measured with a reversed microscope. First, 30 heartbeats were counted with a timer, and then the heartbeat per minute was calculated using the time it took for 30 beats to beat. The mean heart rates in the different positive control groups ranged between 115 and 125 heartbeats per minute. The highest mean heart rate was observed in the PCB-126 group with 123.5 heartbeats per minute, whereas the lowest mean heart rate was recorded in the Control 1 group with 118.5 heartbeats per minute (Figure 10B). No significant difference was observed.

4.3.4 Hatching

No hatching could be measured for the positive control due to technical failure with the camera.

4.3.5 Behaviour

Behaviour was measured at 144hpf and exposed embryos to alternating dark-light periods, each for five minutes. Total runtime was 30 minutes including a 10-minute acclimatization phase. The values were shown in 10s intervals for the 5min periods, thus the distance is in mm/10s. During the darkness periods, the embryos were much more active and swam longer than during

the light period where the embryos were less active. In the two control groups the mean swimming activity were similar, with approximately 15mm/10s during the darkness periods and 5mm/10s during the light periods. In the groups exposed to PCB-126 and Albendazole the swimming activity was higher than controls during both light and dark periods. In the group exposed to Tramadol the swimming activity was clearly affected, showing much less activity in both darkness and light compared with controls. Significant differences were observed for all treatment groups in different periods of the test (Figure 11).

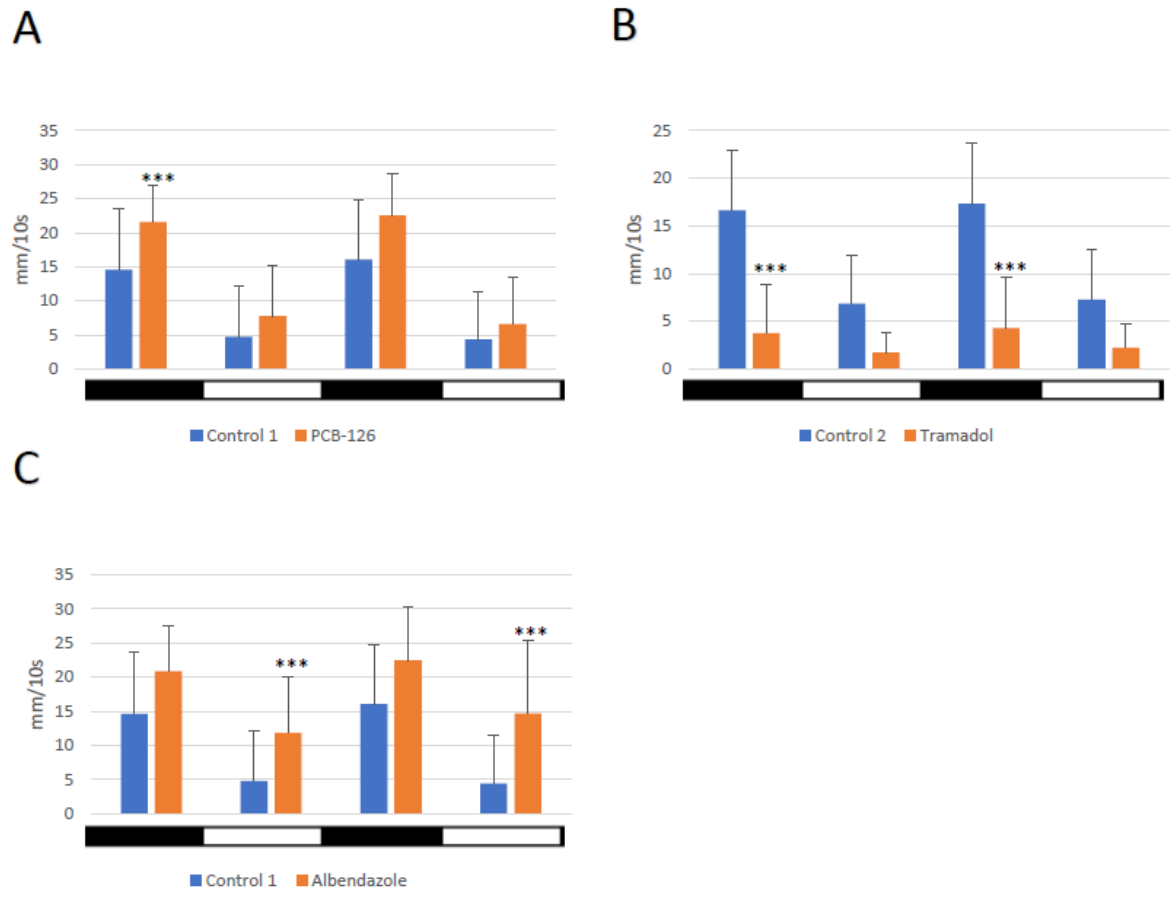

Figure 11. Behaviour study of embryos exposed to positive controls. Graphs showing swimming distances during dark and light alternating period in groups exposed to the different positive controls' PCB-126, Tramadol and Albendazole. The black and white bar below the graph represent dark and light phases respectively for the swimming distances. The blue bars represent the control whereas the orange bars represent the positive control group. The different figure letters represent: A) PCB-126, B) Tramadol, C) Albendazole. *** = P<0.001.

The total average swimming distance was calculated from the 20 minutes of dark-light alternating periods to determine if the total distance was affected. The lowest average swimming distance was the Tramadol treated group whereas the highest were the Albendazole treated group, both significant compared to their controls (Figure 10D).

4.3.6 Lengths

Lastly, the embryos were anaesthetised using MS-222. Photos of embryos were taken after behaviour study at 144hpf using a microscope and then measured in the program ImageJ. The mean lengths in the different positive control groups varied between approximately 3 to 3.5

mm. The longest mean length was measured in the Tramadol group (3.5mm) whereas the shortest mean length was measured for the PCB-126 group (3mm). One group were significantly different compared to its control was the Tramadol treated group, $p<0.001$ (Figure 10C).

3.4 Bioaccumulation

Last experiment was to determine if these antibiotics were bioaccumulating in zebrafish embryos as well as their concentration in the exposure medium (water samples) throughout the experiment. This was done in order to investigate the possibility of long-term effects antibiotics might have on zebrafish development. Bioaccumulation of some chemicals can become toxic or cause long-term effects (43,44). Measuring the concentration in the water samples this way gives an indication of stability and values needed to calculate the bioconcentration factor. Preparation of zebrafish embryos in petri dishes containing 1x mixture and 1000x mixture in 26°C carbon filtered tap water to analyse if bioaccumulation would occur. Water samples were taken at 0, 2, 4 and 6 days (stored in fridge after sampling) and embryo samples were taken at day 6 (stored in freezer after sampling). Samples of embryos were then thawed in room temperature until they were no longer frozen and then embryos were washed two times to remove any residues of antibiotics it might have had on their surface. All water was removed from the washed embryo samples and 500 µL ACN + 0.1% FA and 10µL of internal standard was added to the Eppendorf tube. The internal standard contains constant amount of compounds with similar structure as the ones analysed for and is used to compensate for any losses of chemical during sample preparation (45). Homogenization of the embryos were made with a handheld electric pellet pestle. Afterwards, the samples were incubated for an hour in room temperature (approximately 21°C) before centrifugation for 10 minutes in 21°C at 14.000 rpm. After the centrifugation, the supernatant was collected and 200µL of the supernatant were added in glass vails and stored in fridge overnight. Three blank samples were prepared the same way as embryos samples were to be able to determine if samples have been contaminated during process (no contamination was detected in this study). Water samples were also thawed in room temperature and 500µL of its content were transferred to glass vials and internal standard, 10µl, were also added to the samples. Samples were then analysed as seen in paragraph 2.4.4 using liquid chromatography tandem-mass spectrometry. Analyses of the exposure solutions for the 1x mixture showed that no levels of antibiotics could be detected over the limit of quantification (LOQ) except for Metronidazole and Clindamycin with an average concentration, for six days, of 140ng/L and 457ng/L respectively (supplementary data, Table 12). Similar as for the water sample, no antibiotics could be detected over the LOQ in embryos exposed to the antibiotic Mix 1x. Analyses of the exposure solutions for the Mix 1000x showed detectable antibiotics with values over LOQ (Table 5 and Figures 12). Similarly, embryos exposed to the Mix 1000x revealed detectable antibiotics (Table 5). The highest BCF value was 7.06 in the Clarithromycin treated group. For two treatment groups, Chloramphenicol and Roxithromycin, the BCF could not be calculated due to concentration in embryos in both groups were <LOQ, as well as the water sample for Roxithromycin was <LOQ.

Table 5. Concentrations (ng/L) of antibiotics measured in exposure solutions and embryos in the antibiotic Mix 1000x. The concentrations of antibiotics in the water exposure solutions were measured at start of exposure (Day 0) and then every second day (Days 2, 4, 6). Concentrations of antibiotics in embryos were measured on Day 6. The bioconcentrations factor (BCF) is the relative difference in concentrations of antibiotics in embryos compared with surrounding water exposure solution.

Antibiotics	Water samples (ng/L) Day 0	Water samples (ng/L) Day 2	Water samples (ng/L) Day 4	Water samples (ng/L) Day 6	Embryo samples (ng/embryo)	BCF average (Embryo/water)
Chloramphenicol	2200	2167	2333	2300	<LOQ	<LOQ
Trimethoprim	40667	40000	44667	44667	0.0442	2.83
Sulfamethoxazole	23000	22667	24667	24333	0.0038	0.45
Erythromycin	24000	11667	7100	7467	0.0047	1.80
Roxithromycin	2100	97	<LOQ	<LOQ	<LOQ	<LOQ
Clarithromycin	353333	156667	84667	84667	0.2091	7.06
Clindamycin	466667	456667	510000	503333	0.1879	1.07
Metronidazole	110000	106000	110000	110000	0.0069	0.18

Clindamycin, Chloramphenicol, Trimethoprim, Sulfamethoxazole and Metronidazole levels of antibiotics were more stable over time whereas Erythromycin, Roxithromycin and Clarithromycin decreased over time as seen in Figure 12A and 12B. Roxithromycin could not be detected at and after day 4 (Figure 12A).

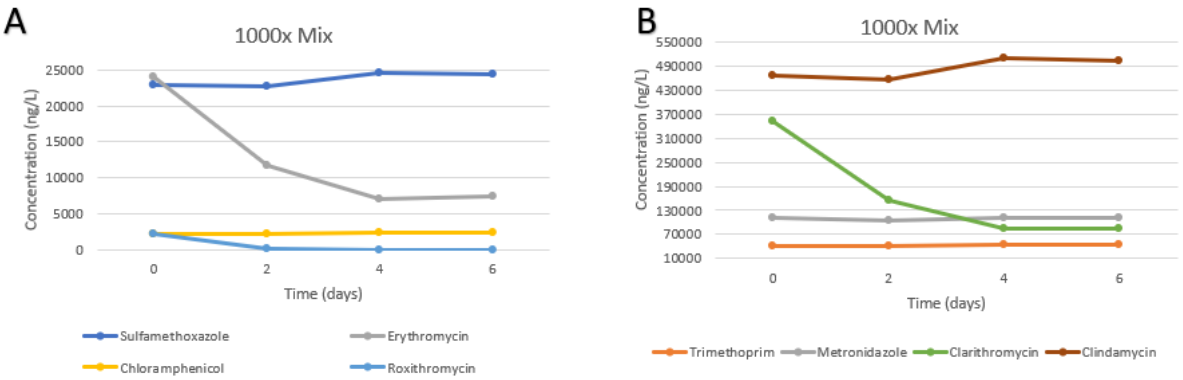

Figure 12. Concentration of antibiotics in the mixture over six days. A) Graph showing Sulfamethoxazole and Chloramphenicol which are stable overtime, and Erythromycin and Roxithromycin which decreases in concentration overtime. B) Showing Clindamycin, Metronidazole and Trimethoprim which are stable overtime, and Clarithromycin which is decreasing in concentration overtime.

4. Discussion:

This study was conducted to assess the toxicity of eight antibiotics at concentrations based on a previous study by Örn et. al 2019 on measurements of antibiotics in Fyrisån. in our study we exposed zebrafish (Danio rerio) embryos to these eight antibiotics individually to determine if they cause any morphological changes in embryos, affecting early movements, heartrate, hatching time, length, and their behaviour.

4.1 Individual antibiotics

In this study, we first assessed the toxicity of individual antibiotics. When we looked at lethal and sublethal endpoints, we did not observe any effects until 144hpf where we observed two coagulated embryos, both from different groups. There were many embryos that were side-laying in all groups including the control. However, no groups were statistically different compared to the control. As for sublethal continuous endpoints we did not observe any differences in the treatment groups. Also, in the behaviour study there were no significant differences. This made us assume that these eight antibiotics do not affect any of the measured endpoints individually up to 6dpf. In a previous study conducted by Lin et. al (2014), significant results on increased heartrate on embryos at the seventh day of exposure were reported (31), whereas in the present study, we only observed the heart rate at one timepoint; 48hpf which were not significant. A theory can be that the antibiotics are not penetrating the egg membrane (chorion) and can only affect the embryo when the embryos are hatched. Since Lin et. al (2014) has seen effects exposing Sulfamethoxazole at a later stage, that can be a reason for why we could not observe an effect on heart rate when exposing the embryos to Sulfamethoxazole. More studies must be done to determine if and which antibiotics penetrate the egg and if heart rate is affected after hatching. As for Clarithromycin, we could not observe any effect on embryos. A previous study conducted by Baumann et. al (2015), did not find a lethal effect on embryos when using concentration up to 2mg/L (46). For our study, the highest tested concentration for Clarithromycin was 152µg/L and we did not see any effects on other endpoints and no correlation with lethality. This can mean that Clarithromycin might not be toxic in very high concentrations. All concentrations we used for the eight antibiotics in our study were between 100-200µg/L (Table 2).

4.2 Mixture antibiotics

The other experiment we did was to make a mixture of the eight antibiotics at concentrations that previously had been measured in Fyrisån. We also increased the concentrations by 10-folds up to 1000-times higher concentration to assess the toxicity on embryos. For lethal categorical and sublethal categorical endpoints, two embryos in 100x concentration were side-laying, had heart oedema, yolk sac oedema and tiny eyes, which were not significant and could not be linked to the treatment. For sublethal continuous endpoints we observed no significant differences in movements, heart rate, hatching time, length, or in the behaviour study. Thus, the conclusion is that these eight antibiotics in a mixture up to 1000x concentrations found in environment are not toxic for the embryonal development or any other endpoints up till day six. If zebrafish is representable for wild fish species, we can expect similar lack of effects also in fish populations in Fyrisån. If the concentrations of these antibiotics increase in Fyrisån, it would probably not be acute toxic on fish embryo development based on the present zebrafish studies. However, other direct consequences and long-term effects from exposure of antibiotics might be possible, such as resistance development, affected gut microbiota and immune system. Mixtures and individual antibiotics have been reported to affect the embryonic development.

Qiu et. al (2020) observed that individual antibiotics and mixtures of antibiotics were affecting the length of embryos (32). They used different antibiotics compared to the antibiotics in the present study, which shows that other antibiotics might have negative effects on embryo development.

4.3 Positive control

We used positive control substances that we know affect the embryonal development in zebrafish embryos. The substances we chose were PCB-126, Tramadol and Albendazole. We observed that PCB-126 affected lethal and sublethal endpoints where in total 11 out of 24 embryos were affected. PCB-126 also affected the behaviour during the first darkness period with an increased activity compared to the control. As for Tramadol, we observed a significant effect on length where the embryos were much longer than the control, and for behaviour the embryos swimming activity were decreased significantly in both the darkness periods. The Albendazole significantly affected lethality where 14 out of 24 embryos were coagulated at 24hpf, as well as affected the behaviour where the embryos were significantly more active during both light periods. The use of the positive control substances thus shows that the methodology of the toxicity test on zebrafish embryos is accurate, and that the observed lack of toxicity of antibiotics is predicted properly.

4.4 Bioaccumulation

When embryos were exposed to environmental concentration, no antibiotics could be detected. This is probably the case since the levels of antibiotics are very low in the water samples, and embryo concentrations are therefore below limit of quantification (LOQ). In the water samples, Metronidazole and Clindamycin, where the only ones in 1x concentration to have levels above LOQ. Clindamycin had high concentration levels at environmental concentration and were observed as stable in the experiment, thus, why it is detectable in the environmental concentration mixture. Metronidazole, however, are much higher than the concentration used, this could either be due to the stock concentration had more metronidazole or that the internal standard compound was not suitable for this antibiotic. Conclusion can be drawn that antibiotics in the levels found in environment are not enough to bioaccumulate in zebrafish embryos. In the experiment with antibiotic mix at 1000-times higher concentration than in the environment, we could determine that over the course of six days three of the eight antibiotics (Clarithromycin, Erythromycin and Roxithromycin) decreased in concentration. Roxithromycin could not be detected on the fourth day and sixth day, whereas Clarithromycin and Erythromycin decreased but were still detectable. The reason for the decreases of antibiotics can be degradation of the substances in the water. With these results we can now draw the assumption that embryos exposed with individual antibiotics and mixture antibiotics were not exposed to stable concentration over time for some of them for the antibiotics mentioned above. This is important to note since we do not know if these antibiotics can penetrate the egg. Therefore, when embryos are hatching the concentration of some antibiotics were much lower or not existing thus giving lower exposure for embryos. Measurements of antibiotics in the embryos showed that Clarithromycin accumulated most of the antibiotics while Roxithromycin and Chloramphenicol could not be measured since their values were below LOQ. Chloramphenicol and Roxithromycin would probably need to be higher in concentrations when exposed to embryos to be measured. Calculation of the BCF in embryos showed that the antibiotics are not bioaccumulating. Clarithromycin had the highest value of 7 as BCF and according to the European chemicals agency (47) BCF higher than 2000 are considered

bioaccumulative. Therefore, the results on BCF in the present study shows that the eight antibiotics tested in the Mix 1000x are not to be considered as bioaccumulative.

4.5 Ethics

Zebrafish embryos are not considered to be legislative research animals until they can eat by themselves according Swedish and EU research animal regulations. As proposed by European Centre for the Validation of Alternative Methods (ECVAM) (37) this occurs approximately on day 5 of age when using a recommended temperature for husbandry of zebrafish embryos at 28.5°C. Due to lower temperature (26°C) in our lab and after discussions with the Swedish Board of Agriculture the period until free-feeding of zebrafish larvae is considered six days, why the toxicity test used in the present study is considered as a non-animal test.

4.6 Limitations

Limitations with this study are that we tested the positive control by themselves and not together with our samples. If they had been made at the same time together with the others, the results would have been more accurate compared to what we have done since the environmental factors together with human error could be different from day-to-day. Another limitation is that the concentration of three antibiotics were not stable during the experiment which means the embryo, when hatched, was not exposed to the same concentration as at start of experiment. This is something to think about in future studies to change medium to have a stable exposure on embryos.

4.7 Conclusion

With the acquired results we can conclude that these eight antibiotics tested are probably not toxic to early life-stage developing embryos up to day six of age, as well as that these antibiotics are not able to bioaccumulate in embryos.

5. Acknowledgements

I would like to thank my supervisor Stefan Örn for letting me be in his lab and for the support and help he has shown throughout this project. Furthermore, I also would like to thank Oksana Golovko for letting me prepare my antibiotic stocks in her lab and perform the bioaccumulation experiment with her supervision and knowledge. Lastly, I would like to thank Kim Hanisch for helping me with the bioaccumulation experiments and answering all my questions about the chemical aspects of the experiment.

6. References

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7. Supplementary data

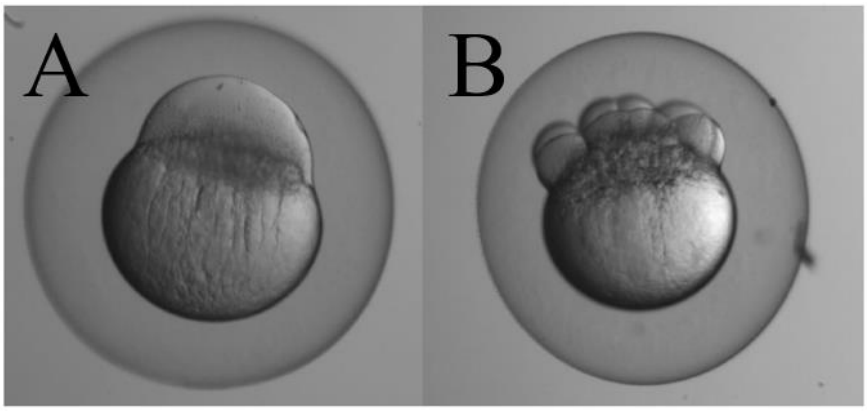

Figure 13. Embryo development. A) Egg cell in cell-stage 1 (zygote). B) Embryo in cell-stage eight.

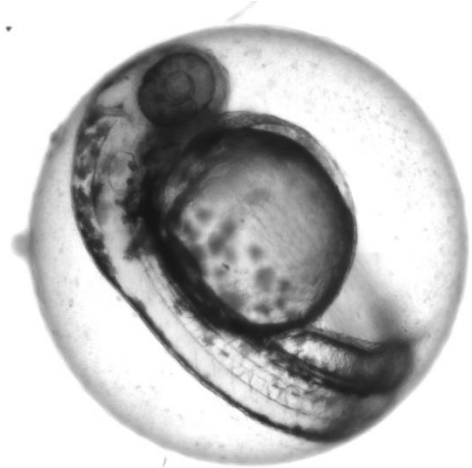

Figure 14. Embryo at 48hpf.

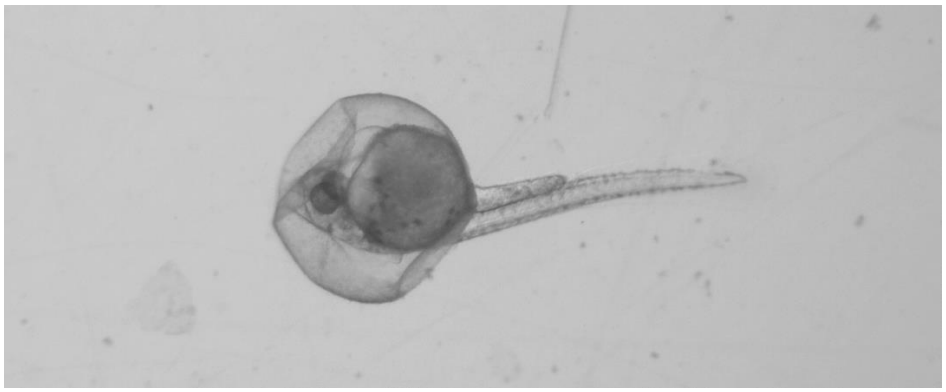

Figure 15. Hatching larva.

7.1 Individual antibiotics

Table 6. Values from the results in numbers from each sublethal continuous endpoints of the embryos exposed to individual antibiotics.

Antibiotics	Average movements/min	Average heartbeats/min	Average hatching time (hpf)	Average length (mm)	Average total swimming distance (mm)
Control	5.36	131	66.1	3.38	1550
Chloramphenicol	6.10	131	65.1	3.35	1750
Trimethoprim	5.90	131	67.2	3.28	1680
Sulfamethoxazole	5.60	131	65.2	3.26	1520
Erythromycin	6.73	131	68.5	3.35	1660
Roxithromycin	6.09	131	66.7	3.41	1540
Clarithromycin	6.27	131	66.3	3.29	1530
Clindamycin	5.55	132	67.4	3.30	1660
Metronidazole	5.36	130	65.0	3.39	1260

Table 7. Values from the results in numbers in the behaviour study from each dark-light alternating periods of the embryos exposed to individual antibiotics.

Antibiotics	Dark period 1 (mm/10s)	Light period 1 (mm/10s)	Dark period 2 (mm/10s)	Light period 2 (mm/10s)
Control	18.4	6.1	19.5	7.7
Chloramphenicol	21.0	7.3	22.0	8.0
Trimethoprim	21.7	6.3	22.8	5.1
Sulfamethoxazole	19.6	6.4	20.3	4.5
Erythromycin	22.5	6.2	22.5	4.4
Roxithromycin	19.3	7.2	20.1	4.8
Clarithromycin	22.2	3.6	21.3	4.0
Clindamycin	23.1	3.5	22.7	6.0
Metronidazole	16.7	4.2	16.9	3.4

7.2 Mixture antibiotics

Table 8. Values from the results in numbers from each sublethal continuous endpoints of the embryos exposed to antibiotic mixtures.

Antibiotics	Average movements/min	Average heartbeats/min	Average hatching time (hpf)	Average length (mm)	Average total swimming distance (mm)
Control	4.00	111	73.5	3.28	2170
1x Mix	3.44	113	70.6	3.30	2220
10x Mix	4.00	109	73.6	3.23	2270
100x Mix	3.89	110	76.4	3.25	2360
1000x Mix	4.78	113	74.3	3.21	2450

Table 9. Values from the results in numbers in the behaviour study from each dark-light alternating periods of the embryos exposed to antibiotic mixtures.

Antibiotics	Dark period 1 (mm/10s)	Light period 1 (mm/10s)	Dark period 2 (mm/10s)	Light period 2 (mm/10s)
Control	26.7	9.4	27.3	9.0
1x Mix	27.1	10.2	27.4	9.5
10x Mix	26.6	11.6	28.0	9.4
100x Mix	26.5	14.4	27.5	8.4
1000x Mix	28.1	11.1	28.9	12.4

7.3 Positive control

Table 10. Values from the results in numbers from each sublethal continuous endpoints of the embryos exposed to positive control treatments.

Antibiotics	Average movements/min	Average heartbeats/min	Average hatching time (hpf)	Average length (mm)	Average total swimming distance (mm)
Control 1	3.67	118	n/a	3.15	1190
Control 2	2.67	121	n/a	3.06	1440
PCB-126	2.83	124	n/a	2.95	1760
Tramadol	4.08	121	n/a	3.44	361
Albendazole	4.00	122	n/a	3.22	2090

Table 11. Values from the results in numbers in the behaviour study from each dark-light alternating periods of the embryos exposed to positive control treatments.

Antibiotics	Dark period 1 (mm/10s)	Light period 1 (mm/10s)	Dark period 2 (mm/10s)	Light period 2 (mm/10s)
Control 1	14.6	4.7	16.1	4.4
Control 2	16.6	6.8	17.3	7.3
PCB-126	21.6	7.8	22.5	6.6
Tramadol	3.8	1.8	4.3	2.2
Albendazole	20.9	11.8	22.5	14.7

7.4 Bioaccumulation

Table 12. Average LOQ values for each antibiotic and water sample concentration for 1x Mix.

Antibiotics	Average LOQ values (ng/L)	Water samples 1x Mix (ng/L) Day 0	Water samples 1x Mix (ng/L) Day 2	Water samples 1x Mix (ng/L) Day 4	Water samples 1x Mix (ng/L) Day 6
Chloramphenicol	0.1	<LOQ	<LOQ	<LOQ	<LOQ
Trimethoprim	0.4	<LOQ	<LOQ	<LOQ	<LOQ
Sulfamethoxazole	0.1	<LOQ	<LOQ	<LOQ	<LOQ
Erythromycin	0.31	<LOQ	<LOQ	<LOQ	<LOQ
Roxithromycin	0.24	<LOQ	<LOQ	<LOQ	<LOQ
Clarithromycin	0.25	<LOQ	<LOQ	<LOQ	<LOQ
Clindamycin	0.065	523	423	443	437
Metronidazole	0.066	143	127	140	150