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EFFECT OF ENDOCRINE DISRUPTING MIXTURES AND CHEMICALS ON THE OXYGEN CONSUMPTION RATE OF ZEBRAFISH LARVAE

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Degree project for Master of Science (120 hec) with a major in Ecotoxicology
ES2520, Examination Course in Environmental Science with emphasis on Ecotoxicology, 60 hec
Second cycle
Semester/year: Autumn/Spring 2021/2022
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Abstract

Chemicals affecting the endocrine system, so called endocrine disrupting chemicals (EDC's), have been steadily increasing in the environment over the last 40 years. EDCs are harmful chemicals since they can act through multiple endocrine pathways, contain a wide spectrum of health effects that can be very persistent and can cause effects already at a low doses. Furthermore, these chemicals enter the environment through several routes and are used in multiple common consumer products. Besides that, we are not exposed to one of them at a time but to various mixtures of EDC's simultaneously.

The Swedish Environmental Longitudinal Mother and child Asthma and allergy (SELMA) pregnancy cohort study identified a mixture of 20 EDC's and metabolites in blood and urine samples of women approximately 10 weeks pregnant. Using the ratios of concentration of the EDC's found in the women, mixtures of EDC's were designed. Among them is the growth mixture (mix G) that was associated with low birth weight. Mixture G contains chemicals such as phthalates, phenols and perfluorinated chemicals, which are known environmental contaminants.

Previous studies revealed that mix G can influence birth weight through dysregulating the expression of key genes involved in thyroid hormone (TH) signalling and thus by disrupting the thyroid axis. Environmental contaminants causing mutations or dysregulations in these genes can lead to IQ loss and modified brain structure in offspring, slow growth, insulin resistance and lipid accumulation. Previous studies have focused on the gene networks important for TH signalling and hence neurological effects. The presented study extends on that knowledge focusing on what the impact is of the EDC's in mix G on energy metabolism and how it is related to the low birth weight. This was achieved by studying the impact of mix G on metabolic rate, which is influenced by TH signalling. The standard metabolic rate can be measured as oxygen consumption in a resting state. Oxygen consumption is influenced by oxidative energy metabolism in the mitochondria, which TH can affect via nuclear pathways or non-nuclear pathways. Oxygen consumption was measured with a respirometry protocol optimized for zebrafish larvae. Zebrafish larvae are exposed to mix G and its individual chemicals (PFOA, PFOS, MEHP and Triclosan) for 48h after which their oxygen consumption was measured using a respirometry microplate system (Loligo® Systems). Even though the results do not show a significant difference with the control, the results suggest that mix G and its individual chemicals can affect the oxygen consumption rate. Pilot experiments were also performed on three week old zebrafish larvae, which show some interesting results.

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Popular science summary

In the pans that you cook with, the toys your kids/siblings may play with and the personal care products that you use, there are dangerous chemicals. These chemicals can disrupt your hormone system, and this system defines which gender you're assigned at birth but also determines the development of your organs, and your growth. The chemicals that can disrupt your hormone system (also called endocrine system) are called endocrine disrupting chemicals (EDC's). Over the past 40 years EDC's have been steadily increasing in the environment due to the production and use of these chemicals in everyday products like described above. The alarming concern about EDC's is that they do not act like any other chemical. EDC's can cause harmful effects at low doses already, unlike other chemicals that usually cause effects only at higher doses. Besides that, they can be very persistent, meaning they're hard to break down and can stay in the environment and your body for long time. In 2012, a Swedish study was performed with 2582 pregnant women, where they found about 20 different known EDC's in the blood and urine of these pregnant women. They designed three mixtures for three different research tracks; mix S for Sexual development, mix N for Neurodevelopment and mix G for Growth. In this thesis project the focus is on mix G, which is associated with low birth weight.

Experiments

Zebrafish larvae (or baby fish) are exposed to multiple concentrations of two versions of mix G; mix G0, mix G1, and 4 individual chemicals; PFOA, PFOS, MEHP and Triclosan. See the example of mix G1 in the figure 1, approximately 20 zebrafish larvae in each Erlenmeyer flask are exposed for 48 hours at 30°C.

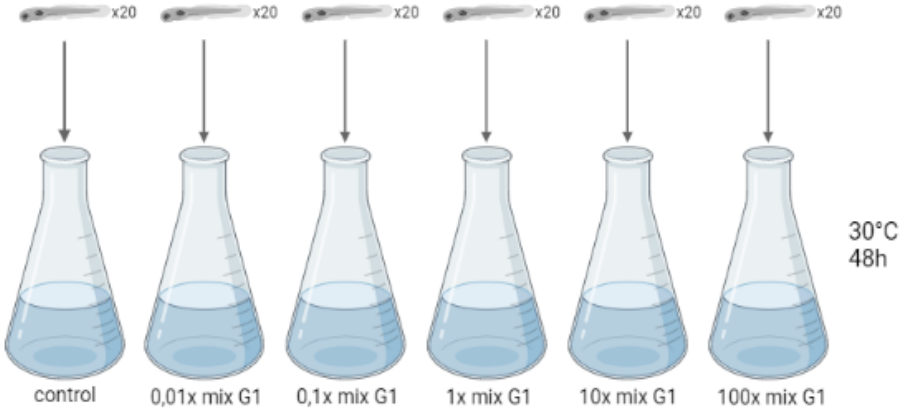

Figure 1: Exposure of 3 day old zebrafish to the different concentrations of mix G.

Figure 2: The respirometry microplate system from Loligo® systems.

After the exposure period, the zebrafish larvae are 5 days old and the oxygen consumption of each individual larvae is measured. This is done using a new 'respirometry' microplate system from Loligo® systems. One zebrafish larvae is put in one of the little pockets or wells with some water that can be seen in the second picture. When almost every well is filled, the plate is sealed with a plastic film and a block is placed on top so no oxygen can escape the well, the well becomes vacuum. There is an oxygen sensor spot in the well that measures the oxygen in the water and how fast it decreases, so how fast the zebrafish larvae consumes the oxygen.

Results

Oxygen consumption is measured because we think that mix G can interfere with the way our cells make energy. If this energy making is disrupted, the baby grows less and thus has a lower birth weight. Our cells need oxygen to make energy so we think we will see change in the oxygen consumption of the zebrafish larvae after exposure to the two versions of mix G. The results we found were that mix G1 increases the oxygen consumption rate of the zebrafish larvae but not significantly and mix G0 slightly decreases the oxygen consumption rate. The individual chemicals were tested to see if one or more of them would be the driver of the effect of one of the mixtures. PFOA and PFOS, both very persistent EDC's, would decrease the oxygen consumption rate of the zebrafish larvae slightly, like mix G0. They could thus be the chemicals that cause the effect of mix G. Triclosan showed a similar effect to mix G1, a slight increase in the oxygen consumption rate.

Triclosan can perhaps be the driver of the effect of mix G1, though more research with the mixtures and their chemicals is necessary to confirm this.

Conclusion

Mix G can affect the oxygen consumption rate of zebrafish larvae, but not significantly. The mechanism behind this needs to be researched more. If mix G is able to affect the energy system of the fetus, this can be dangerous for the development of the child. Low birth weight is also associated with diseases like diabetes and obesity later in life.

1. Introduction

1.1 Chemical mixtures

Chemical risk assessment and legislation is still largely focused on the management of single substances. Standardized procedures use single compounds and model species representing the most sensitive species creating individual thresholds (PEC/PNEC, ADI). These regulatory guidelines and values are not sufficiently protective and do not take into account the risk or effect of combined exposures to multiple substances. The risk & impact of mixtures exceeds the risk of each individual component. Several scientific committees from the EU have confirmed and performed research documenting interaction between individual chemicals resulting in higher or lower mixture toxicities also known as synergisms or antagonisms. They conclude that both, combinations of similarly acting substances and dissimilarly acting substances should be considered as possible concerns, "even if the exposure to all the substances involved is at a level below where no effects are predicted for the individual substances (i.e. below the individual Predicted No Effect Concentrations - PNECs)" – (European Commission, 2021). Furthermore, the risk assessment of mixtures can be performed for an extended time period to monitor the effects on certain biological groups for chronic toxicity data. This could reveal the potential recurring toxic pressures on certain biological groups and analyse for the drivers of the mixture effects. All in all, methods and approaches developed for assessing mixtures and their risks were more realistic, and for some endpoints also more sensitive, than single-substance risk assessments (Markert et al., 2020).

The European Commission has drafted up a report including the relevant legal requirements, guidance documents, methodologies and knowledge base as well as the remaining challenges for mixture risk assessment. The best approach for mixture risk evaluations is to integrate and compare (human) biomonitoring data and experimental models (European Commission, 2021). Furthermore, acute as well as chronic toxicity data must be used in mixture risk assessments. Nevertheless, there are still many uncertainties in mixture risk assessment due to data gaps of both exposure and ecotoxicological data. What needs to be considered for improving mixture risk assessments is that they can only be compared with other mixture risk assessments when consistent and comparable methods and designs are used. Moreover, high-quality ecotoxicological data should be accessible and regularly exchanged to improve future risk assessments (Markert et al., 2020).

1.2 Endocrine disrupting chemicals

Endocrine disrupting chemicals (EDCs) are chemicals that interfere with the endocrine system. According to the Swedish Environmental Longitudinal Mother and child Asthma and allergy (SELMA) pregnancy cohort study there are four main problems with EDCs and exposure to them. The first discloses how the adverse effects of EDCs can occur at a low dose range. This is because likewise, the endogenous endocrine system works with hormones at low concentrations (Bornehag et al., 2012). Additionally, most EDC's do not act like other 'traditional' toxicants that follow the typical dose-response curve of the higher the concentrations, the bigger the effect. Instead, EDC's have non-monotonic dose-response curves with different effects at high and lower concentrations and with more subtle side effects. Hence, effects may not be predictable or even detectable with standard established test methods on account of not being sensitive enough to detect also other negative side effects (Birgersson, 2022).

The second issue raised is the persistency of effects from EDC's. It could be that years after exposure to EDC's and the respective effects can still be detected. Especially when exposure already started in the early developmental stages (Birgersson, 2022). Hormone signalling is especially important during development in the prenatal and infancy period. Exposure to EDC's may be severely critical during that time in the development and cause long term effects (Bornehag et al., 2012).

A third issue is the amount of health effects found in different studies on EDC's. EDC's have been researched since the 1950's and a wide spectrum of health effects has been observed (Marlatt et al., 2022). EDC's can also work via multiple mechanisms, also described as key characteristics by La Merrill et al., (2020).
The fourth and last problem is the many diverse sources of EDC's in our life and how easily exposed we are to them. EDC's can enter and thus pollute the environment through multiple routes. Many commonly used consumer products consist of manmade endocrine disrupting chemicals that can leak into the surrounding environment during production and by use (and later the discarding) of these products (Bornehag et al., 2012). Exposure of humans and animals then happens through food and water intake, inhalation and direct (skin) contact. Additionally, EDC's have been increasingly identified in the placenta, breast milk, urine, blood and other human biological fluids. Unfortunately it is difficult to assess the full impact they can have on humans because some effects may develop latently and manifest at later ages (Braun, 2017).

1.3 The Selma Cohort, Mixture G and its individual chemicals

The SELMA cohort study researched the blood and urine of 2582 women in their 10th week of pregnancy. The blood and urine from their child were also collected as well as air and dust from the home environment during the pregnancy and infancy period. Their aim was to investigate "the importance of early life exposure to environmental toxicants with focus on EDCs during the pregnancy and infancy period and life style factors for the development of multiple chronic diseases/ disorders in offspring children" - Bornehag et al. (2012). They identified 20 endocrine disrupting chemicals and metabolites from which they made mixtures for four research tracks. One of the tracks focuses on alterations in expected growth, height and weight for which mixture G (mix G) was designed (Birgersson, 2022). Mix G is associated with low birth weight which is linked to a higher risk of metabolic diseases later in life (Liao et al., 2020). Mix G contains known environmental contaminants, they are the so called 'bad actors' associated to low birth weight which is why they are also tested as individual compounds to prove causality between the mixture and its effect. Additionally, they are tested separately to find a chemical group that could act as a driver of the effect, meaning the chemical component most associated to the effect or causing the biggest portion of the effect. Analysis of the drivers of toxicity may also be important for and guide future mitigation measures of the mixture components (Markert et al., 2020).
The first chemical group in mix G are per- and polyfluoroalkyl substances (PFAS). The two major components of this group investigated in this thesis are perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA). They are highly stable compounds that break down very slowly and thus over time can build up in humans, animals and the environment. They are man-made chemicals that have been in use since the 1940's for their stain-proof and grease-proof applications (U.S. EPA, 2022). PFAS have been found to have endocrine disrupting effects with thyroid disruptive properties and can severely affect the health of aquatic organisms (Birgersson, 2022). The main exposure routes to PFAS are by consumption of food and water but also by swallowing and inhaling contaminated air and dust. Infants are exposed more than adults due to exposure through placental transfer, breastfeeding, spending more time on the floor and thus closer to dust, putting things in their mouths, and children are able to drink and eat more compared to their body size/weight. PFOS and PFOA are measured as serum concentrations in the blood (Rappazzo et al., 2017).

The second chemical group in mix G are phthalates, specifically in this thesis the focus is on Mono-(2-Ethylhexyl) Phthalate (MEHP). MEHP is a metabolite from Di-2-ethylhexyl phthalate (DEHP), one of the most commonly used phthalates (Sant et al., 2021). Phthalates are used in plastic products for their ability to render plastic transparent, flexible and durable and they are used in a wide variety of industries. Phthalates are used as additives in most commercial products, this entails that they do

not form covalent bonds with the plastic and can thus more easily leach into the environment (Zhang et al., 2021). Exposure to DEHP is similar to PFAS chemicals like described above in the previous paragraph but excretion from the body is faster. After metabolism in the gastrointestinal tract to the more toxic metabolite MEHP, it is excreted from the body via urine withing 24 hours. However, repeated and long term exposures are the main causes of the persisting endocrine disrupting effects. MEHP is also able to pass the placenta and can be detected in the amniotic fluid, placental tissue and blood, and thus participate in the development and growth of the embryo (Qian et al., 2020).

The third and final individual chemical in mix G researched in this thesis is Triclosan. Triclosan is an antibacterial compound that disrupts bacterial lipid synthesis and cell membrane integrity (Braun, 2017). Triclosan was used in many personal care products and other consumer products like clothes and cleaning products. Exposure to Triclosan, like the other chemicals described above, happens mainly through oral routes, by consuming contaminated foods and beverages, but also via a dermal route, through direct absorption through the skin. Quite recently, triclosan has been banned from using it in commercial products in the European Union due to concerns about environmental impacts and its endocrine disrupting properties (Metcalfe et al., 2022). Like MEHP, Triclosan has a biological half-life of less than 24 hours and is primarily excreted via the urine, where it is also sampled and measured (Braun, 2017).

1.4 Thyroid hormone system

Where mix G and the individual chemicals described previously might start their effect is in the thyroid system. The following paragraphs will explain the thyroid hormone system and its function in energy metabolism and how that is related to low birth weight, oxygen consumption and respirometry research. Thyroid hormones (TH) have a plethora of effects. During developmental stages it's mainly important for growth and differentiation and later in life it regulates metabolic processes in almost every part of the body. There are two main thyroid hormones, T3 and T4. T4 is produced entirely by the thyroid gland and approximately 80% of T3 is generated (deiodination) from T4 in peripheral tissues by deiodinases (Cioffi et al., 2013). Excess production of TH, hyperthyroidism, causes a hypermetabolic state with one of the effects being weight loss. Conversely, reduced TH levels, hypothyroidism, respectively with a hypometabolic state has weight gain as one of its many metabolic effects (Iwen et al., 2013). Therefore, disruption of the thyroid hormone system is dangerous for energy metabolism and thus also alarming for birth weight and growth (the effect to which mix G is related).

There are two pathways through which TH influences energy metabolism: nuclear and non-nuclear. The nuclear pathway uses the thyroid hormone receptor (TR) in the nucleus of the cell. There are two genes that encode for several TR isoforms, though the two primary ones are TR alpha and TR beta (Cioffi et al., 2013). The two thyroid hormone receptors are modified posttranslationally by sumoylation, which is essential for TR crosstalk with other nuclear receptors (Weitzel & Alexander Iwen, 2011). TR are transcription factors that bind to specific DNA segments on TH target genes and in that way, TH and TR regulate gene transcription, including genes important in metabolic pathways. TR isoform expression and action is distinct per tissue, accordingly TH metabolism regulation is tissue specific (Cioffi et al., 2013). During developmental stages, TRα expression happens first, followed by expression and action of TR β. During development, there are specific windows of TR expression for specific tissues, for example sensory tissues like the ears and eyes expressing TR β during a specific time. In the liver and cardiac ventricles, TR β is predominantly expressed, whereas TRα is predominantly expressed in the brain and atria, and fat (adipose) tissue contains both TR isoforms (Mullur et al., 2014). Disruption of TR expression by EDC's can thus severely impact the development of these tissues.

Besides action via the nuclear pathway, TH can act via the non-nuclear pathway (outside the nucleus). This pathway has two main sites, the cytoplasm and the mitochondria. In the cytoplasm, this pathway does not use the TR but mainly uses signal-transducing pathways with protein kinases. It may also be called the nongenomic pathway and usually takes in a shorter amount of time. There are several receptors and proteins that cause a cascade of interactions, too much to specify in this thesis so for these pathways you are referred to these reviews; Cioffi et al. (2013), Weitzel & Alexander Iwen, (2011) and Mullur et al. (2014). The non-nuclear pathway in the mitochondria uses a combination of the genomic and non-genomic pathway. There are mitochondrial thyroid receptors in the inner mitochondrial membrane as well as TR that can bind to mitochondrial DNA affecting mitochondrial transcription and energy metabolism action (Cioffi et al., 2013).

1.5 Energy metabolism, ATP and oxygen consumption

Previous research from Birgersson (2022) and Mentor (2020) has found that mix G dysregulates the expression of key genes involved in thyroid hormone (TH) signalling and appetite/adipose tissue related signalling. Research by Mentor (2020) found that mix G affects the metabolic rate, however the responses differed per concentration and replicate experiments. This thesis is also based on the presumption that mix G affects the thyroid hormone system, which disrupts mitochondrial functions and pathways which thereby affects the standard metabolic rate and consequently body weight. TH is a key regulator of the standard metabolic rate (SMR). The standard metabolic rate is the minimal energy expenditure of cells, tissues and organs that is required to keep the organism alive. It doesn't include activity, digestion, growth or production of sexual products, only essential homeostatic activities. The SMR can be measured as oxygen consumption in a resting state. The key organelle to sustain the SMR is the mitochondria, it provides around 90% of the cellular energy supply mainly through ATP production (Chabot et al., 2016). The majority of ATP is produced by oxidative phosphorylation where molecules derived from carbohydrates, lipids and proteins are oxidized to activate the cofactors NADH and FADH2. For this process, oxygen is the mostly used oxidizing agent and the products are carbon dioxide (CO_2), water (H_2O) and heat (energy for the cofactors). These reducing cofactors donate electrons to a set of protein complexes in the inner mitochondrial membrane. These protein complexes, called the electron transport chain, then transfer protons from the mitochondrial matrix to the inner membrane space, creating a proton gradient. This proton gradient creates an electrochemical potential which is needed by the protein complex ATP synthase so that it can phosphorylate ADP to ATP (Salin et al., 2015). Compromission of this so called 'proton-motive force' can disrupt ATP synthesis, oxidation and thus oxygen consumption. The mitochondrial inner membrane and matrix contains thyroid receptors to which TH can bind and then increase the leak of protons and ions though the mitochondrial membrane. This can affect ATP production and consumption by other tissues, which may be witnessed in the oxygen consumption rate (Mullur et al., 2014). Though, oxygen consumption can also be affected independently from ATP production since it can also be used for other energy processes in cellular respiration and does not distinguish between them (Salin et al., 2015). The chemicals present in mix G and also individually tested (PFOA, PFOS, MEHP and Triclosan) in this thesis are known to disrupt mitochondrial function, oxidation and TH signalling (Braun, 2017), (Casals-Casas & Desvergne, 2011).

1.6 Respirometry in zebrafish larvae

This paragraph will explain why 'zebrafish larvae' is the model species used and why 'respirometry' is the method used in this thesis to accomplish the aims. The zebrafish has been increasingly used as a model species in many toxicological studies to assess the effect of xenobiotics on development and metabolic disruption. This due to their rapid external development and early-life transparency, and cost-effective breeding and husbandry. Their development is easily trackable and examinable in great

detail. Its genome has been completely sequenced and found 70% homology with the human nucleotide sequence and an impressive 84% of genes related to human disease share an orthologue in zebrafish (Vancamp et al., 2019). The metabolic system of zebrafish shares important similarities with that of mammals such as pathways involved in lipid metabolism (adipogenesis) and transport, appetite control by neuronal circuits and organs sensitive to insulin (Mentor, 2020). Furthermore, TH production and regulation has been conserved among many vertebrate species as well as the zebrafish. TH production is controlled by the hypothalamus-pituitary thyroid (HPT) axis, just like in all vertebrates. TH levels and TH circulation is also regulated by transmembrane TH regulators, like in other vertebrates, and deiodinase enzymes are also present in zebrafish to activate TH. Zebrafish also contain thyroid receptor genes and one encodes for TRα, which is highly homologous to the human TRα. As in most vertebrates during development, the expression of these deiodinases and TR varies over time and also per tissue. Additionally, in fish eggs the ratio of T4/T3 varies within the species, though generally there is more T4 than T3. Action of T3 occurs before maturation of the HPT axis and TH production by the larvae itself. It shows how important maternally derived THs are during the first stages of development. This is also true in humans. Furthermore, the gene repertoire in organs, tissues and cells induced by TH varies on the need at a given time point in development giving these tissues the ability to regulate TH concentrations autonomously in early development already. This also means that disruption of TH levels and timing also affects development of zebrafish larvae (Vancamp et al., 2019).

As explained in the previous paragraphs, energy metabolism can be altered by TH and this affects cellular respiration and thus the oxygen consumption rate. Mix G can affect key genes and mRNA expression related to TH. There are several studies that show that the EDC's in mix G can also individually affect gene (and mRNA) expression in thyroid hormone system. To find the physiological response belonging to this disruption, the oxygen consumption rate is measured. The oxygen consumption rate can be measured using respirometry. Respirometry is a simple method where the amount of oxygen consumed over a certain period of time is recorded. This is done in an airtight chamber where there is no oxygen in- and outflow for that certain period of time. Zebrafish larvae are exposed to multiple concentrations of Mix G and its individual chemicals and are then put in an airtight well with an oxygen sensor to measure the oxygen consumption rate. In this thesis I have tried to optimize and establish a respirometry protocol, and establish a physiological response to the effects of Mix G and its individual chemicals by measuring the oxygen consumption rate of exposed zebrafish larvae.

2. Aims

Assess embryo developmental metabolic effects of EDC mixtures derived from SELMA and their individual chemicals using respirometry.

Establish a respirometry protocol to measure the oxygen consumption rate in zebrafish larvae of 5 dpf and zebrafish larvae of three weeks old.

Asses the effects early exposure can have later in the development by performing respirometry measurements with the exposed zebrafish larvae that have grown three weeks old.

3. Methods

3.1 Chemicals

Mix G is associated with low birth weight (G standing for growth and metabolism) and it was composed of a mixture of 20 endocrine disrupting chemicals and metabolites. Mix G was designed based on geometric means of the measured and estimated urine serum concentrations in all SELMA mothers (Mentor, 2020). The concentrations of the mixtures are stated as factors of the geometrical mean of the exposure concentrations of the women (e.g. 0.1x mix G0). Thus the concentrations correspond with the exposure levels discovered in the SELMA mothers. The concentrations of the individual chemicals tested separately, correspond with their concentration in mix G1.
The two mixtures used in this project were built from mix G, named Mix G0 and Mix G1. Mix G0 is based on the 20 chemicals found in the SELMA mothers and mix G1 is based on 54 chemicals amongst which also the 20 chemicals from the SELMA mothers. The associated adverse health outcomes are the same for the two mixtures. Stock solutions and dilutions of the mixtures were stored at -18°C. Stock solutions and dilutions of the 4 separate chemicals used in the project, PFOA, PFOS, MEHP and Triclosan were stored at +4°C. Dilution series of the separate chemicals and mixes were made at room temperature. The 4 separate tested chemicals PFOA, PFOS, Triclosan and MEHP were picked based on previously performed research in *in vitro* systems as well as the quantity of previous (literature) research.

Table 1: The chemical components of mix G0 and mix G1 with their individual concentration in µmol/L.

Chemicals	**Mix G0 (µmol/L)**	**Mix G1 (µmol/L)**
MEP	0.23	29.7
MBP	0.20	26.4
MBzP	0.09	5.3
MEHP	0.12	19.0
MINP	0.18	
MINCH		0.5
Triclosan	0.03	0.3
2-OH-PH		1.3
DPP		0.5
3-PBA		0.1
PFOA	0.03	3.6
PFOS	0.09	9.7
PFHxS	0.03	3.0
HCB		0.1
p, p´- DDE		0.5

3.2 Fish husbandry and breeding

At the animal facility at Zoologen, University of Gothenburg, adult zebrafish (*Danio Rerio*) (wildtype, strain AB) were kept in 30L tanks with about 20 fish per tank in a temperature-controlled room (28-29°C) with photoperiods of 10 hours dark and 14 hours light. The Swedish Board of Agriculture approved animal use under the ethical permit 15986-2018.

Breeding tanks were prepared in late the afternoon with at least 2 clean plants per tank. The tanks were equipped with a false bottom through which the eggs could fall and be protected from the adults potentially eating them. One breeding tank is dropped in 1 big tank with the fish carefully, that the fish would not get hurt/stressed by the tank while dropping it in. This method was found to be less stressful for the fish compared to taking the fish out of the big tank and putting them in a smaller separate tank. The next morning the tanks are collected and care is taken that no fish would be taken out as well. The eggs were collected in the bottom of the tank and they were filtered from other dirt, rinsed with zebrafish embryo medium (ZEM) and transferred to petri dishes with ZEM. With a pipet, more dirt and bad (unhealthy + coagulated) eggs are taken out, then the petri dish is put in an incubator set at 30°C where the eggs develop. The next day more bad eggs are taken out with a pipet and a part of the water is replaced. The day after, 2 days post fertilisation (dpf), most larvae have hatched and they are put in a bigger tank or beaker with normal zebrafish water, but still in the incubator. If they would be kept and grown, from then on they would need to be fed everyday with rotifers. After two weeks they are fed with rotifers and artemia (brine shrimp).

3.3 Exposure

When diluting from the stock chemical or mixture, the first step was a 10x dilution in DMSO. Then further dilution of 10000x with 30 mL ZEM into an Erlenmeyer flask. The control is DMSO diluted 10000x with ZEM in an Erlenmeyer. The exposure concentrations are shown in table 2.
Larvae of 3 dpf are transferred to and exposed in Erlenmeyer flasks with about 20 larvae per Erlenmeyer flask. The Erlenmeyer flasks were put in the incubator with an aluminium foil lid for 48 hours. This method of exposure is adapted from previous research by Lina Birgersson (2022).
For the three week exposure, beakers were used. The larvae were exposed for 48h in the same way as described above. After those 48 hours, as much of the exposure solution as possible was removed and zebrafish water was added until approximately 350 ml. The aluminium foil lid was removed and the beakers were kept in the incubator for 3 weeks after exposure. The water was changed every day and at 23 dpf the respirometry experiment described below was performed.

Table 2: The different concentrations of the mixtures and chemicals used in the respirometry experiments and their respective concentrations as how they are portrayed throughout this thesis.

Chemical	Concentration (X SELMA)	Final Concentration (nM)
G1	0,01	1
	0,1	10
	1	100
	10	1000
	100	10.000
G0	0,01	1
	0,1	10
	1	100
	10	1000
	100	10.000
PFOA	0,01	0,0396
	0,1	0,396
	1	3,96
	10	39,6
	100	396
PFOS	0,01	0,096
	0,1	0,97
	1	9,7
	10	97
	100	970
Triclosan	0,01	0,003
	0,1	0,03
	1	0,3
	10	3
	100	30
MEHP	0,01	0,19
	0,1	1,9
	1	19
	10	190
	100	1900

3.4 Respirometry

To establish a respirometry protocol for this microplate system from Loligo® systems several different experiments were done. First the system calibration was tested. ZEM purged with air for at least 30 minutes is pipetted into the 80 µL wells. This is the 'air-equilibrated' water sample measurement. Afterwards, the oxygen-free water sample measurement takes place with ZEM purged with nitrogen gas for at least 30 mins. Calibration of the wells is a separate measurement per well per type of ZEM. Then egg oxygen consumption was measured with eggs of 1 day old (day of collection) and 2 days old (day after collection). The wells are first filled with 70 µL ZEM, then the egg is added, the wells are checked for air bubbles (which will be removed with a pipet) and the plate is sealed and the oxygen logging is started. Same was done with larvae of 3 dpf, 4 dpf and 5 dpf to look

at their oxygen consumption. Larvae were also exposed to different concentrations of the positive control cyanide to see how fast their oxygen consumption decreases. After this optimization, the real experiments started with the exposed larvae described below.

The Erlenmeyer flasks with the exposed larvae of 5 dpf are taken out of the incubator and allowed to cool down a little bit to the 28 °C in the room. First calibration of the system takes place with an air-equilibrated' water sample and an oxygen-free water sample as mentioned above. After the calibration, the wells are filled with 60 µL normal ZEM. The software automatically randomizes the wells and 'treatments', consequently the wells are filled with the larvae exposed to the respective concentrations. The transferring of the larvae goes together with some of the exposure solution, hence in the end the wells are completely filled until 80 µL. The wells are checked for air bubbles, which are removed with a pipet before the plate is sealed. The sensor spots are then normalized and the oxygen logging is started. The oxygen consumption is measured for ±30 minutes until the oxygen percentage in all the wells (except the blanks) has fallen below 70%. This is shown in the software by making the well (picture on the computer) red (see appendix for example). Respirometry experiments with the three week old larvae were performed with zebrafish water instead of ZEM and extra care was taken not to harm the larvae as we wanted to keep them alive for fixation afterwards. Fixation was performed by first anesthetizing the fish in MS-222 and subsequently fixating them in Davidson's fixative according to a protocol made by Lisa Baumann.

3.5 Data analysis

Raw data was collected in Excel and further analysis was done in R-studios. The replicates per concentration per experiment were all added up for the same chemical, so that every chemical and mixture had approximately 30-60 replicates per concentration. To assess the trustworthiness of the respirometry system, the data of the respirometry experiments was analysed in multiple ways. How the values of the wells differed from the other wells and the differences between plates (experiments) was looked at as well as how the standard deviations, errors and p-values changed when removing outliers.

The data was tested for normality by use of the Shapiro-Wilk test and a Q-Q plot and for homogeneity of variance by use of Levene's test. Then the data was tested for significance by a one-way ANOVA and if homogeneity of variance was violated (according to Levene's test) a Welch's one-way ANOVA was performed. Subsequently, a Tukey multiple comparison was done and differences between means were deemed significant at $P < 0.05$. The post-hoc test for the Welch's one-way ANOVA is a pairwise t-test without presumption of equal variances. If the normality assumption was violated (the Shapiro-Wilk test is significant), a Kruskal-Wallis test was performed and a pairwise Wilcox test was used as a post-hoc test. The Kruskal-Wallis test is a non-parametric alternative to an one-way ANOVA, which means that it doesn't assume that the data comes from a particular distribution. The test looks at medians rather than means.

4. Results

4.1 Protocol optimization

The air-equilibrated water sample showed oxygen at a mean starting concentration of: 8,241 mg/L. The oxygen-free water sample showed no oxygen present in the sample when added to the wells. This shows that this method of adding/removing oxygen works. The oxygen-time graphs are presented in the appendix section 1.1.

Eggs of 1dpf (which is the day of collection) do not show any oxygen consumption over a period of 45 minutes, which can be seen in figure… in the appendix section 1.1. Eggs of 2dpf do show oxygen consumption over a period of 30 minutes, which can be seen in figure .. in the appendix section 1.1. At 3dpf the eggs have hatched and the larvae also show oxygen consumption over a period of 30 minutes, which can be seen in figure.. in the appendix section 1.1. Also larvae of 4dpf and 5dpf show oxygen consumption over a period of 30 minutes. The means of the oxygen consumption rate (MO2) are shown in the table below.

Table 3: Mean oxygen consumption rate of eggs and larvae of different ages.

Eggs/larvae (dpf)	Mean oxygen consumption rate (pmol/min)	N
1	0	19
2	144,14	12
3	228,12	14
4	286,48	12
5	680,92	34

In the oxygen-time graphs in the appendix, the blanks are all shown in the colour black. The blanks are wells without organisms to make sure there is not background oxygen consumption by maybe bacteria in the system. In all the data the blanks show a fairly straight line, which confirms that there was no background oxygen consumption during the experiments. The oxygen consumption of the organisms also starts around the same level as the blanks to make sure that there is an equal amount of oxygen at the start of the experiment for each organism.

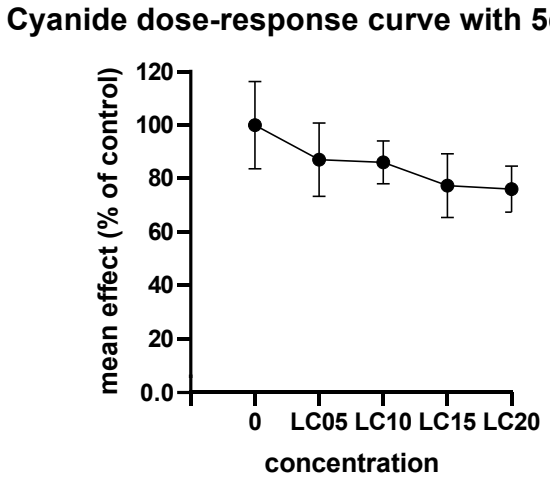

Figure 1: Positive control Cyanide respirometry experiment on 5dpf zebrafish larvae over approx. 45 minutes. The dots represent the mean fraction of the control (%) and the error bars represent the standard error of the mean.

The concentrations of cyanide are derived from the Lethal Concentration (LC) of cyanide that is expected to kill 50% of the tested organisms = LC50. The dots in the graph (figure 3) represent the mean effect and the effect is calculated per replicate by dividing its oxygen consumption rate by the

mean oxygen consumption rate of the control, as explained in the methods. The bars represent the standard error of the mean (S.E.M.) which is calculated from the effect data. There is no significance in the data set but we can assume homogeneity of variance in the data set and a normal distribution since Levene's test and the Shapiro-wilk test, respectively, are not significant. The summary of the data analysis is in the appendix section 1.1.

4.2 Mix G0 and G1, and individual chemicals experiments

4.2.1 Mix G0

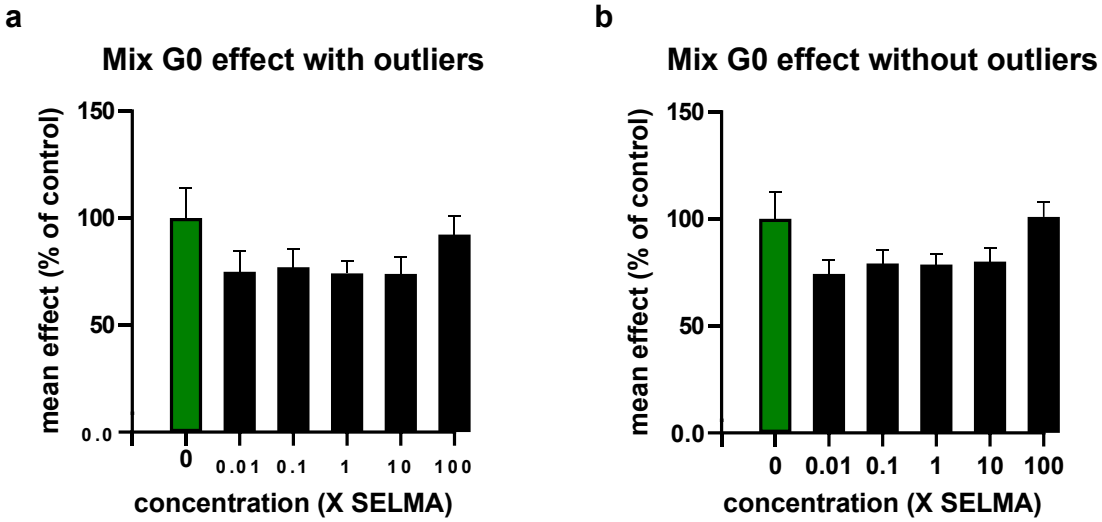

Figure 2: The mean effect of mix G0 in different concentration on respirometry of 5dpf zebrafish larvae after exposure of 48h is shown mean fraction of the control (%), results with vs. without outliers. The error bars represent the standard error of the mean (S.E.M.).

As can be seen in figure 4, the trend of the effect of mix G0 is a U-shaped dose response graph, which it stays even when removing outliers (bar graph b). Removing the outliers seems to make the means of the latter concentrations slightly higher; the means of the concentrations from 0,1X to 100X become slightly higher. A table with the means, STDEV, standard errors and medians is present in the appendix section 1.2 (for every mix and chemical).

Table 4: G0 effect data analysis summary.

Tests	mix G0+ p-values	mix G0- p-values
ANOVA	0,225	0,0586
Levene's test	0,005752*	0,01561*
Shapiro-Wilk test	< 2,2E-16*	1,065E-09*
Welch's ANOVA	0,3566	0,05806
Kruskal-Wallis test	0,4357	0,2248

Significance is portrayed by the star in the figures as well as the tables. As can be seen in table 4 and figure 2, the one-way ANOVA of mix G effect data does not show any significance. It only shows significance for Levene's test and the Shapiro-Wilk test, which is why Kruskal-Wallis test was performed. The p-value of the other one-way ANOVAs is almost significant, this is because the difference between the control and 0.01x concentration for the mix G0 effect without outliers is almost significant. However, because the data is not normal or homogeneous, only the Kruskal-Wallis

test counts which is not close to significance. When comparing the p-values of two data sets, the second data set without the outliers looks to be slightly improved since the p-values that you want to go down (ANOVA's + Kruskal-Wallis) decrease and the p-values that you'd want to go up (Levene's and Shapiro-Wilks) also increase.

4.2.2 Mix G1

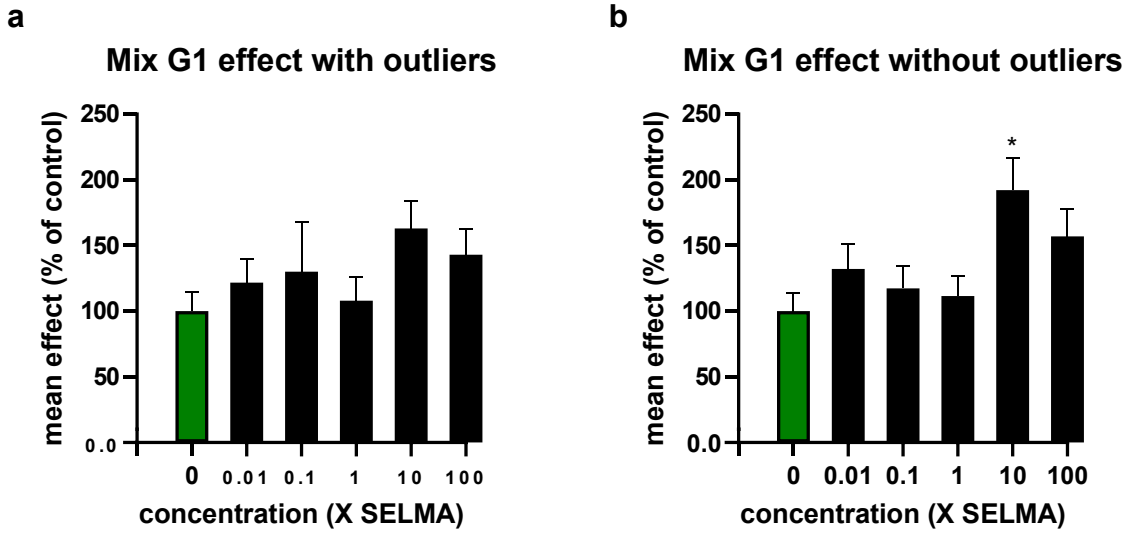

Figure 3: The mean effect of mix G1 in different concentrations on respirometry of 5dpf zebrafish larvae after exposure of 48h is shown as mean fraction of the control (%), results with vs. without outliers. The error bars represent the standard error of the mean (S.E.M.).

The general trend of effect that mix G1 has, which can be seen in figure 5, is that it can slightly increase the oxygen consumption rate of zebrafish larvae of 5dpf. Additionally, in both bar graphs the effect goes down with the 100X SELMA concentration. It could be interesting to do more research around those end concentrations seeing as there might be the most effect, 1X to 100X maybe even 1000X.

Table 5: G1 effect data analysis summary.

Tests	**mix G1+ p-values**	**mix G1- p-values**
ANOVA	0,335	0,00515*
Levene's test	0,6083	0,008115*
Shapiro-Wilk test	< 2,2E-16*	1,373E-08*
Welch's ANOVA	0,1911	0,023*
Kruskal-Wallis test	0,08421	0,03067*
Significant conc.		10X - 0

As can be seen in figure 5 and table 5, the difference between the control and the 10X concentration is significant. According to the first ANOVA with assumption of normality and equality of variance the difference between 10X and 1X was also significant, however this ANOVA cannot be used as Levene's test and the Shapiro-wilk test were also significant. According to Welch's ANOVA and the Kruskal-Wallis test and the pairwise tests after, only the 10X – control difference is significant.
When comparing the p-values of two data sets, the p-values of the ANOVAs and the Kruskal-Wallis tests are smaller in the second data set without the outliers. The Shapiro-Wilk test p-value increases which could mean that the distribution of the data becomes more normal, which is an improvement.

However, Levene's test becomes significant and thus the variance across the concentrations is statistically significantly different. This was not the case in the data set of mix G1 with the outliers which is a drawback for the data set without outliers.

4.2.3 PFOA

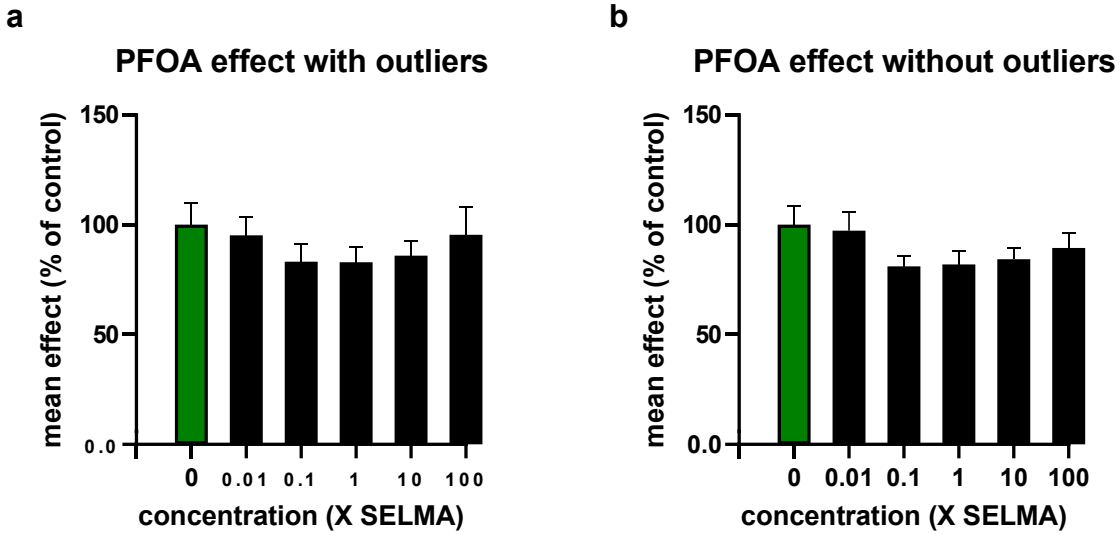

Figure 4: The mean effect of PFOA in different concentrations on respirometry of 5dpf zebrafish larvae after exposure of 48h is shown as mean fraction of the control (%), results with vs. without outliers. The error bars represent the standard error of the mean (S.E.M.).

The general trend that can be seen in figure 6 is that PFOA may very slightly decrease the oxygen consumption rate. In both bar graphs at the end, the effect of the 100X concentration is slightly higher compared to the other concentrations. Unfortunately there is no significance in the data and again the data set violates the homogeneity of variance test and the normality test, as can be seen in table 6.

Table 6: PFOA effect data analysis summary.

Tests	**PFOA+ p-values**	**PFOA- p-values**
ANOVA	0,646	0,263
Levene's test	0,1434	5,76E-04*
Shapiro-Wilk test	6,07E-15*	2,68E-04*
Welch's ANOVA	0,6303	0,3093
Kruskal-Wallis test	0,7125	0,5585

When comparing the p-values of the two different data sets in table 6, the same can be said for PFOA as was said for mix G1. The Shapiro-Wilk test increases for the data set of PFOA without outliers, which is an improvement, but Levene's test becomes significant. The variances across the concentrations are significantly different which is a drawback of the data set without outliers.

4.2.4 PFOS

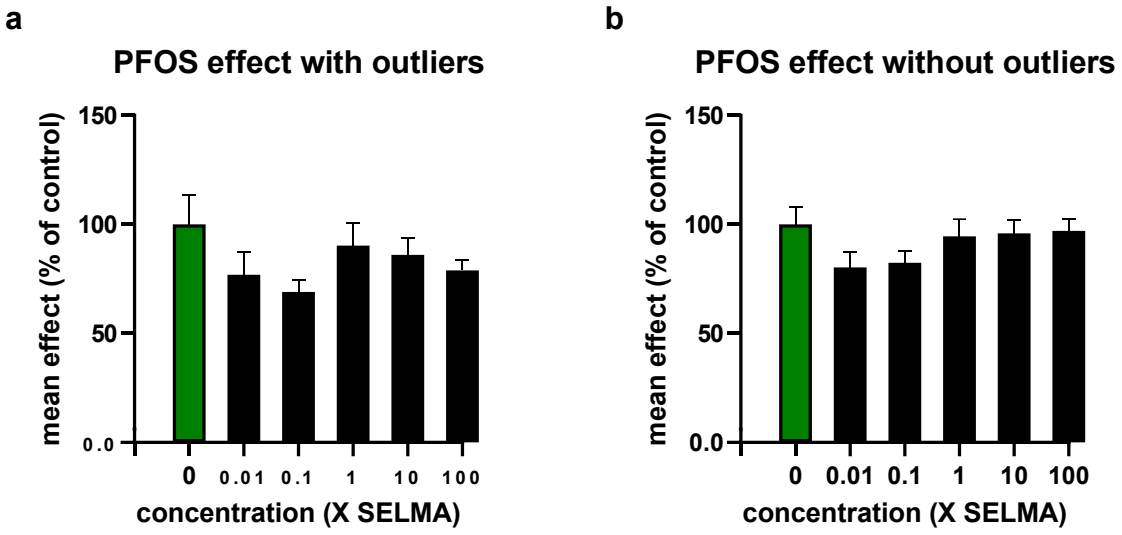

Figure 5: The mean effect of PFOS in different concentrations on respirometry of 5dpf zebrafish larvae after exposure of 48h is shown as mean fraction of the control (%), results with vs. without outliers. The error bars represent the standard error of the mean (S.E.M.).

In bar graph a in figure 7, the PFOS effect with outliers shows that it could possibly decrease the oxygen consumption rate of zebrafish larvae. However, when looking at bar graph b in figure 7 with the PFOS effect without the outliers this effect is not really there anymore. Though it may look as there is more effect in the first graph of PFOS with the outliers, there is no statistically significant difference between any concentrations as can be seen in table 7.

Table 7: PFOS effect data analysis summary.

Tests	**PFOS+ p-values**	**PFOS- p-values**
ANOVA	0,275	0,252
Levene's test	0,1091	0,1143
Shapiro-Wilk test	< 2,2E-16*	9,67E-09*
Kruskal-Wallis test	0,2051	0,1169

As can be seen in table 7, the data sets do have homogeneity of variance as Levene's test is not significant. This is an improvement of the PFOS data sets compared to the data sets of the other chemicals and mixtures. However the data is not normally distributed since the Shapiro-Wilk test is significant which means that the Kruskal-Wallis test needs to be used. The distribution of the data does become more normal with the second data set of PFOS without outliers since the p-value has increased. When comparing the other p-values between the two data sets, there is not a huge difference between them; Levene's test is increased slightly and the Kruskal-Wallis test is decreased slightly for the PFOS data set without outliers which an improvement.

4.2.5 MEHP

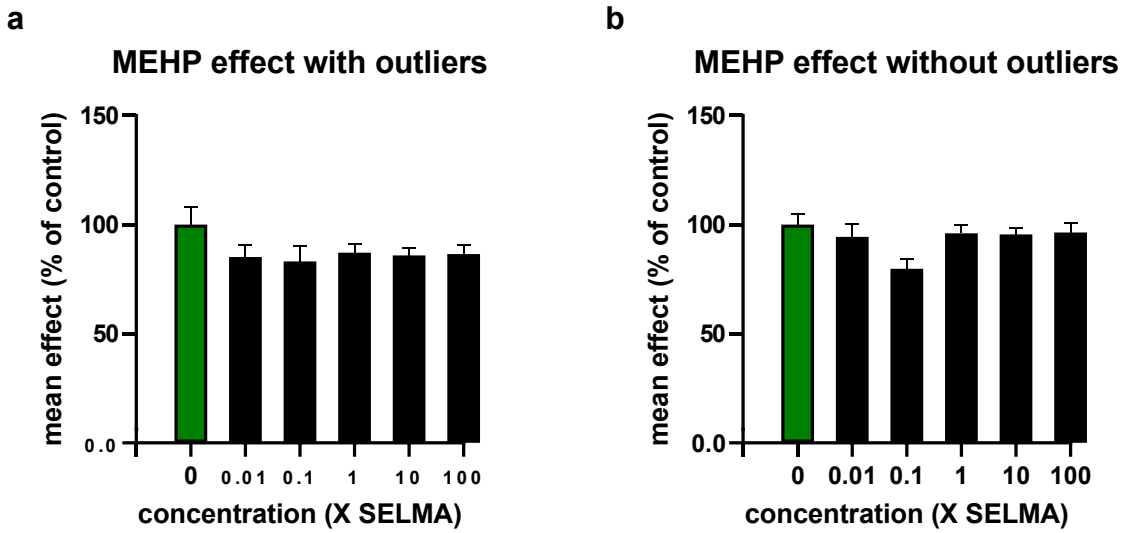

Figure 6: The mean effect of MEHP in different concentrations on respirometry of 5dpf zebrafish larvae after exposure of 48h is shown as mean fraction of the control (%), results with vs. without outliers. The error bars represent the standard error of the mean (S.E.M.).

The trend that can be seen in figure 8 is that MEHP may cause a very slight decrease in the oxygen consumption rate of zebrafish larvae of 5dpf. This trend is straightened out from concentration 1X to 100X in bar graph b for MEHP without outliers, coming closer to the control effect again.

Table 8: MEHP effect data analysis summary.

Tests	MEHP+ p-values	MEHP- p-values
ANOVA	0,329	0,0447*
Levene's test	0,06832	0,01413*
Shapiro-Wilk test	< 2,2E-16*	2,95E-04*
Welch's ANOVA	0,7072	0,03052*
Kruskal-Wallis test	0,4353	0,1034

As can be seen in table 8, the difference in effect for concentration 0,1X and the control of MEHP was significant in the two ANOVAs. However since the Shapiro-Wilk test is also significant, the Kruskal-Wallis test has to be used which does not show any significance for the data. The Shapiro-Wilk test p-value does increase when removing the outliers, which is an improvement of the data set. However, Levene's test decreases, meaning the variances are not homogeneous which is a drawback of the second MEHP data set without outliers.

4.2.6 Triclosan

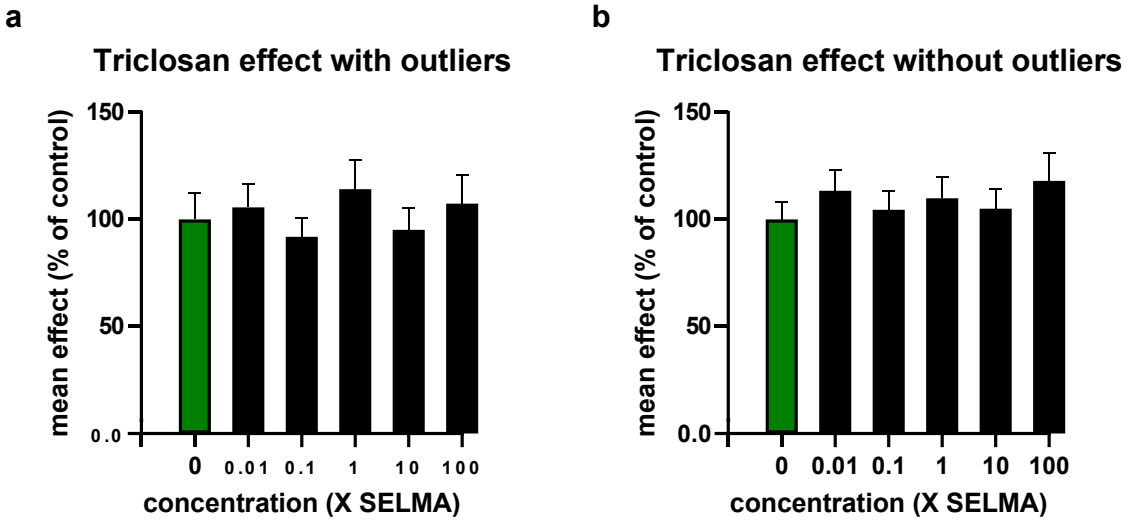

Figure 7: The mean effect of Triclosan in different concentrations on respirometry of 5dpf zebrafish larvae after exposure of 48h is shown as mean fraction of the control (%), results with vs. without outliers. The error bars represent the standard error of the mean (S.E.M.).

As can be seen in figure 9 bar graph a with the outliers is that the effects of the different concentrations of Triclosan are fluctuating around the control effect (100%), a trend cannot really be found. Whereas in bar graph b in figure 9, we can see an evident trend of slight increase in effect as the concentration also increases. Though the middle concentrations are still fluctuating, they are above the 100% control bar.

Table 9: Triclosan effect data analysis summary.

Tests	**Tri+ p-values**	**Tri- p-values**
ANOVA	0,79	0,816
Levene's test	0,5158	0,4146
Shapiro-Wilk test	< 2,2E-16*	1,36E-12*
Kruskal-Wallis test	0,9535	0,9405

As can be seen in table 9, the ANOVA and the Kruskal-Wallis test are not significant. Nor is Levene's test, which means that the variances are homogeneous. The Shapiro-Wilk test is significant, so the data is not normally distributed, but the p-value becomes higher when removing the outliers (Tri- p-values) which is an improvement of the Triclosan data set without outliers.

4.2.7 Total comparison

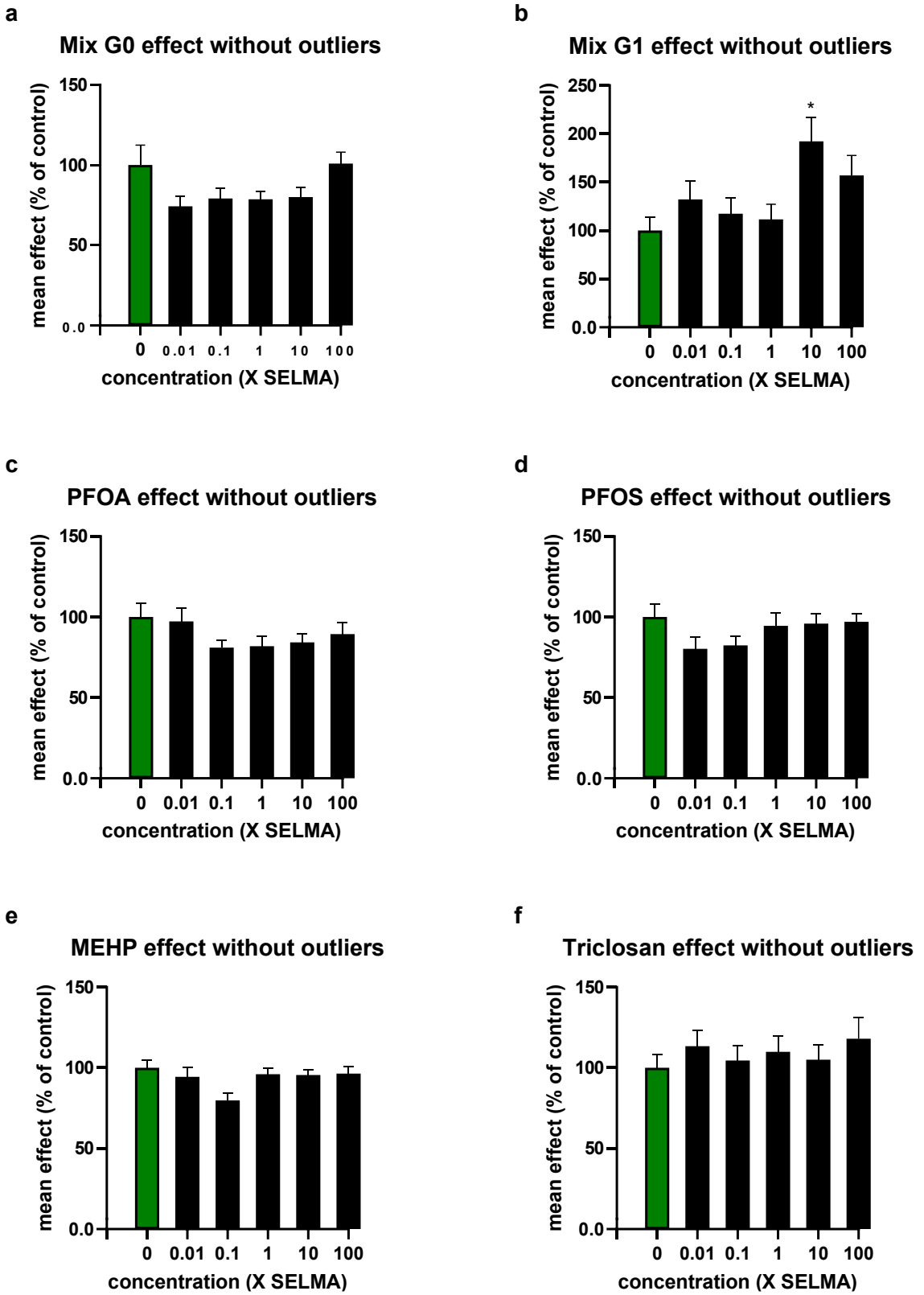

Figure 10: Comparison of the dose-response curves of the mixtures and chemicals without outliers

Figure 10 is a comparison of all the mixtures (mix G0 and G1) with the individual chemicals. It shows how mix G0 and PFOA have a similar shape for their dose-response graph, a U shape. The beginning of the curves of PFOS and mix G0 are also very similar, though in the end of the PFOS curve the effect

flattens out at control level (around 100%). MEHP also has a very slight U-shape in the beginning of the curve, however, like PFOS the end flattens out. The beginning of the curves of mix G1 and Triclosan are similar; an increase of effect at 0,01X, but a decrease again at 0,1X.
Here we only focus on the curves without the outliers as the difference with the curves with the outlier is not significant and they are quite similar.

4.3 Respirometry mixture experiments with larvae of three weeks

The data from these experiments is considered as a pilot study seeing as it is not complete and also involved some protocol optimization to consider when the larvae do not fit into the wells of the microplate anymore.

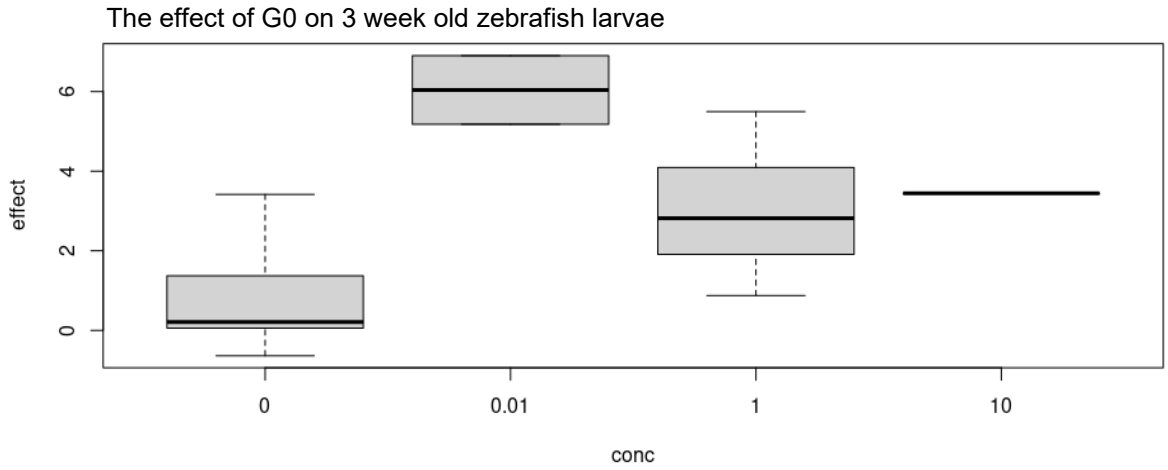

Figure 81: Box-Whiskers plot showing the effect of mix G0 on zebrafish larvae of 3 weeks old.
The thick black horizontal line in the middle is the median, the vertilcal lines show the distribution of the data and the bars represent the standard deviation.

Due to a very high mortality rate of the larvae during those three weeks but also during the respirometry experiment, replicates and concentrations are missing in the data sets. There is no 0,1X concentration, nor an 100X concentration for mix G0. As can be seen in figure 11, mix G0 only has 2 replicates for the 0,01X concentration and only 1 data point for the 10X concentration. The control has 19 replicates and the 1X concentration has 6 replicates, those amounts of replicates are better for a trustworthy data analysis.

Table 10: Summary of the data analysis of the effect of mix G0 on 3 week old zebrafish larvae.

Test	p-value	Significant concentrations	Tukey's p-value
ANOVA	5,35E-06*	0,01X – 0	<0,001
Levene's test	0,4698	1X – 0	0,0017
Shapiro-Wilk test	0,0554	0,01X – 1X	0,0212

As can be seen in table 10, the difference between the concentrations tested of mix G0 are significant. However, the little amount of replicates for the 0,01X concentration makes that significance less trustworthy and would need to be researched more.

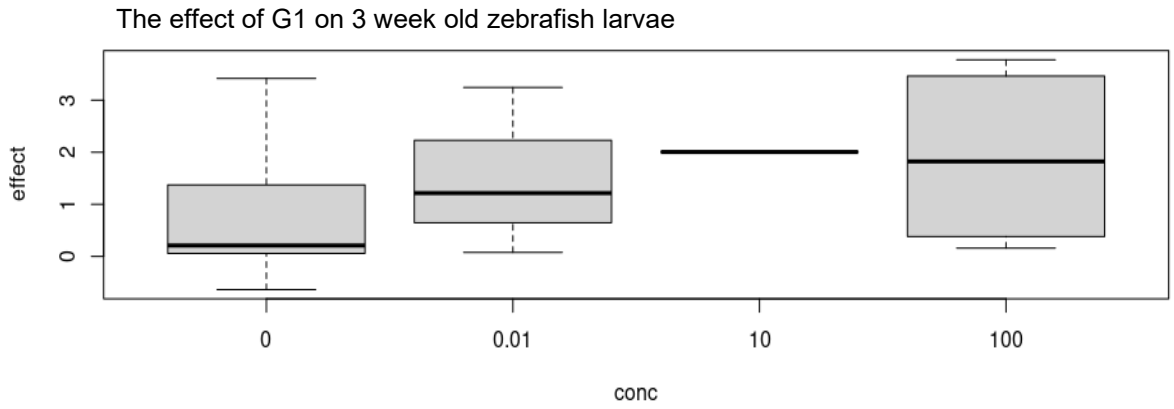

Figure 92: Box-Whiskers plot showing the effect of mix G1 on zebrafish larvae of 3 weeks old.
The thick black horizontal line in the middle is the median, the vertilcal lines show the distribution of the data and the bars represent the standard deviation.

As can be seen in figure 12, the two middle concentrations of mix G1 are missing, 0,1X and 1X, which makes this curve slightly misleading. Now it looks like there is a trend of an increase of effect with an increase in concentration, however the middle part of this trend is unknown.
The control has again 19 replicates, the 100X concentration has 6 replicates and the 0,01X concentration has 3 replicates. The 10X concentration unfortunately only has one data point, as seen in figure 12. The data is not significant, there is no significant different between concentrations, but the data is normally distributed and the variances are homogeneous, this is visible in section 1.3 in the appendix.

5. Discussion

This project aimed to assess the effects of environmentally relevant mixtures of endocrine disrupting chemicals, and 4 of their individual chemicals on the oxygen consumption rate of zebrafish larvae. A microplate respirometry system from Loligo® systems was used and its protocol optimized to measure the oxygen consumption of the individual zebrafish larvae in real time.

5.1 The mixtures and chemicals effects on the oxygen consumption rate of zebrafish larvae of 5dpf

Many studies just use the data without outliers but this thesis also shows the effect removing the outliers has on the data. As can be seen in the tables in the results section, the statistics usually become 'better' when the outliers are removed but we have to keep in mind that these are live animals and this could be their real oxygen consumption rate. This is why both graphs are shown however the data sets and statistics need to be more/better analyzed in order to conclude anything about reliability, unfortunately this was not managed in time. In the following discussion, the results without outliers will be discussed since the assumption is that the outliers are purely mechanistic. For example because the live organisms are transferred manually with a pipet from a safe environment in a group to alone in a small well.

Mix G1 shows a general trend of an increased effect with the concentrations when comparing to the control effect (figure 5). In previous master thesis research by F. Bodin, a significant increase in ATP concentration with increasing concentrations of mix G1 was found in mitochondrial isolates from rainbow trout livers. The increase in oxygen consumption rate could be the whole-body effect of the found increase in ATP concentration. When ATP production is increased, the mitochondria needs more oxygen to oxidize more substrates so that the given amount of ATP produced can be sustained (Roussel & Voituron, 2020). This may result in increased oxygen consumption rates for the whole organism. Increased ATP production and oxygen consumption may also induce the increase of reactive oxygen species (ROS). The electrons (that were donated by the cofactors NADH and FADH2) passing through the electron transport chain are at last accepted by oxygen, creating ROS. ROS in a small amount, are a sign of a well-functioning mitochondria, however in high concentrations they can cause severe damage because they are highly reactive molecules (Roussel & Voituron, 2020). Unpublished results by L. Birgersson (2020) found a significant decrease in membrane integrity of zebrafish liver cells with increasing concentrations of mix G1. This may be an effect of the increase in ROS. We can also see a decrease in the oxygen consumption rate at the 100X concentration of mix G1 compared to the 10X concentration. Though the effect is still higher compared to the control effect (figure 5), it may be the start of a reaction on too much ROS. However this would need to be researched more for example by use of a glutathione essay.
Furthermore, Mix G1 significantly altered mRNA expression of thyroid-related genes at the 100X concentration, and significantly decreased locomotion after one month of exposure compared to the control (Birgersson, 2022). Therefore, it may be interesting to perform more research with the higher concentrations, from 1X or 10X onward to perhaps 1000X.

Mix G0 has a non-monotonic, U-shaped dose-response curve (figure 4). Mix G0 also had a significant effect at lower concentrations (0,01X, 0,1X and 1X) on mRNA expression of thyroid related genes and significantly increased ATP levels at the 0,1X concentration (Birgersson, 2022). Seeing as the decrease in oxygen consumption also already starts at the 0,01X concentrations, it may be interesting to perform more research on the lower concentrations of Mix G0. Mix G1 has the opposite effect of mix G0 (fig. 10); mix G1 causes an increase in oxygen consumption rate whereas the oxygen consumption rates caused by different concentrations of mix G0 is generally decreased. Mix G0 is based on the

first round of chemical analysis of 20 different compounds found in the pregnant women and mix G1 is based on a second round of chemical analysis of 54 different compounds (Birgersson, 2022). Mix G1 is thus more complex than mix G0, which could explain why the effects are significantly different. Interestingly, mix G1 contains more chemicals known to interfere with the thyroid system (2-OH-PH, HCB and p, p'-DDE). As described in the introduction, thyroid hormone T3 can bind directly to TR in the mitochondria and interfere with membrane permeability and the electron transport chain. Mix G1 might contain chemical analogues to T3, that are not present in mix G0, which may be why the effect of mix G1 is more pronounced (and significant) compared to the control than the effect of mix G0. Thus, more of the chemicals in mix G1 need to be researched in order to determine what the drivers of its effect are.

PFOA shows a general trend of a decreased effect compared to the control in a minor U-shape (figure 6). PFOS showed with lower concentrations a decreased effect, but with the higher concentrations this effect was levelled back to the control level (figure 7). PFOA and PFOS can act as mitochondrial uncouplers, increasing the permeability of the mitochondrial membrane and disturbing the proton gradient and thereby the electrochemical potential. The decrease of this potential is known to cause an increase in oxygen consumption as the mitochondria membrane becomes more permeable for protons and perhaps even starts swelling (Shabalina et al., 2016). Shabalina et al. (2016) even found that PFOS induced a much greater decrease in membrane potential than PFOA and an increasing rate of oxygen consumption. Though at higher concentrations the effect became inhibitory and further depolarization causes a decrease in oxygen consumption (Shabalina et al., 2016).
This increase in oxygen consumption is however not seen in the current study for PFOA (fig. 6) and PFOS (fig. 7). This could be due to the fact that in most literature oxygen consumption of the mitochondrial isolate or cells are studied, not whole-body oxygen consumption which is measured in our system. Mitochondrial function, efficiency and in turn respiration can differ significantly among tissues in the same individual (Salin et al., 2015). Besides that, the type of exposure, exposure time and uptake is also completely different from whole-organism exposure. This makes it difficult to fully connect the whole-body physiological response to the cellular response found in previous studies.

MEHP shows an overall very minor decrease in the oxygen consumption rate, with a dip for the 0,1X concentration (figure 8). Traore et al. (2021) found that MEHP can decrease the ability of the mitochondria to consume dissolved oxygen. MEHP has also been found to have similar effects to PFOA and PFOS, like the collapse of the mitochondrial membrane potential, mitochondrial swelling, and ROS production (Park et al., 2020). Moreover, MEHP can interfere with the expression of genes involved in oxidative metabolism (Chen et al., 2020). Unpublished results from L. Birgersson (2020) found a significant decrease in cell viability and membrane integrity which corresponds with the literature described above. It has been hypothesized that MEHP may have an effect on one of the proteins or components in the electron transport chain which could diminish the mitochondria's ability to consume dissolved oxygen (Traore et al., 2021). This may be why there is a minor decrease in the oxygen consumption rate found, however this would need to be researched more.
Though MEHP has the highest concentration in the mixtures compared to the other chemicals, its effect is the least pronounced. Perhaps since MEHP is already a metabolized product but this does not match with previous studies finding significant effects after MEHP exposure.

Triclosan, unlike the other individual compounds, shows a general trend of an increased effect with the concentrations when comparing to the control effect (figure 9). However, like the other individual compounds, Triclosan has also been found to impair mitochondrial membrane integrity, and decrease the mitochondrial membrane potential (Du et al., 2021). Du et al. (2021) suggested that energy metabolism shifted form oxidative phosphorylation to glycolysis, the anaerobic version which

is much less efficient. The presence of oxygen prevents this shift which may be why a minor increase is found in the oxygen consumption rate after exposure to Triclosan.
Triclosan and mix G1 are the only substance and mixture suggesting an increase in effect compared to the control (figure 10). Furthermore, seeing as the beginning of their curves looks very similar (until the 0,1X concentration), there is a possibility that Triclosan is one of the drivers of effect in mix G1. Triclosan also had a higher concentration in mix G1 compared to mix G0.

For mix G0, the other individual compounds share more similarities like a decrease in general effect compared to the control (PFOA, PFOS, MEHP). As mentioned in the results, PFOS and PFOA show a similar curve to mix G0, the U-shape (figure 10). The 0,01X concentration of mix G0 and PFOS shows a similar drop in oxygen consumption rate compared to the control. The same accounts for the minor increase again at the 0,1X concentration. Therefore it may be possible that PFOS (and PFOA) are the main drivers of toxicity in mix G0. PFOA and PFOA also have a slightly higher concentration in mix G0 than Triclosan. Mix G0 was also found to have greater effect on mRNA expression of thyroid-related genes at lower concentrations by (Birgersson, 2022), which also corresponds to mix G0 (and PFOS) showing the effect at the lower concentrations and eventually forming a U-shape.
The U-shape curve of most of the chemicals and mix G shows that the effect of the higher concentrations levels back to the control effect. Perhaps tolerance mechanisms are activated but there is no proper explanation for this yet and shows the strange, non-monotonic responses that endocrine disrupting chemicals and mixtures can cause.

If oxygen consumption is disrupted during developmental stages this can negatively affect the growth of the organism. Diminished intrauterine growth and low birth weight are the very parameters with which mix G is associated and they are related to a higher risk for metabolic diseases later in life (Birgersson, 2022). Oxidative stress, the imbalance between free radicals (e.g. ROS) and antioxidants in cells and tissues, can cause two major physiological responses during prenatal development. First the growth of the fetus is slowed down and this causes metabolic adaptations. Substrate/nutrient availability becomes prioritized for the essential organs such as the heart and brain. This compromises the growth of the skeletal muscle and the liver, but these two tissues are responsible for 40-50% of the total foetal oxygen consumption and thus the two most important metabolic organs in the fetus (Pendleton et al., 2021). Neonates with low birth weight are usually born with lower muscle and liver mass and within the first weeks after delivery they increase their body mass excessively, also known as catch-up growth. However, muscle mass is usually not adequately increased, which lead to a modified metabolic phenotype with increased adiposity causing metabolic strain and increased risk of metabolic and hepatic diseases later in life (Pendleton et al., 2021). Thus, disrupted oxygen consumption may cause and be caused by oxidative stress which can lead to distorted development.

5.2 Mixture effects on three week old zebrafish larvae

The effects on three week old zebrafish larvae are considered as pilot data. This because it was still part of the protocol optimization and the amount of concentrations are not complete. The original idea was to perform respirometry experiments 30 days post exposure, however these larvae did not fit in the microplate respirometry system from Loligo®. Larvae of 21 days old did fit in wells, though at this age there may still be a couple that grew more and thus do not fit. Therefore, my recommendation of the maximum age of zebrafish larvae that fit in the wells is actually 19 days. During those 3 weeks, a lot of the larvae died which is one of the reasons why the data is incomplete. Unfortunately the high mortality rate cannot be directly accredited to exposure of the mixtures since this was not properly tested. Besides, there could be multiple reasons why the larvae did not survive, like low water quality, low food quality etc. Yet, the mortality in the control was not as high as the

mortality in the beakers with the exposed larvae. This is also seen in the amount of replicates the control has, 19 being much more than any of the mixture concentrations.
Nevertheless, respirometry experiments were performed on the larvae of 3 weeks that survived and fit into the wells and some interesting results came forward. Mix G1 shows a trend of a slight increase of effect, though nothing significant unlike its results on 5dpf zebrafish. The effect of mix G0 showed a significant increase compared to the control. This is the opposite of what was found in 5 dpf zebrafish larvae (decreased non-significant effect) and shows that chronic effects of the mixtures would be interesting follow-up research.

5.3 Protocol optimization

The results show why we chose to perform respirometry experiments on larvae of 5 dpf. Though, respirometry experiments on eggs is possible in eggs of 2dpf, it was chosen to not do this as the yolk sack of the embryo can contain the chemicals and prevent them from affecting the embryo. The embryo consumes its yolk for growth and differentiation, however this does not guarantee 100% exposure and it also means that exposure happens very slowly and it gradually but slowly increases (Halbach et al., 2020). Consequently, it is unknown when exposure starts, how much the embryo is exposed when or when it is fully exposed. Moreover, we also wanted to stay with the exposure protocol created by Lina Birgersson to be able to reference their data here as well.

As can be seen in figure 3, cyanide shows a decrease in oxygen consumption rate with higher concentrations of cyanide. This is expected from the positive control and shows that the respirometry system works correctly. It also shows that the magnitude of the oxygen consumption rate does not change very much per concentration. This is also confirmed by the one-way ANOVA, it is not significant. This means that the graph just shows a trend, which may also explain why the following results with the mixtures and chemicals do not show much significance but more small trends.

The mixtures and chemicals tested earlier (mix G1, G0, PFOA) have approx. 30-45 replicates and the other three chemicals (PFOS, MEHP and Triclosan) have approx. 45-60 replicates. Tables with the amount of replicates are shown in the appendix. This is because the protocol got more familiar and normalization was done before every experiment (from the last PFOA experiments onwards). Therefore, less wells got strange values (e.g. outliers in the minus or extremely high) and/or failed, hence the number of replicates is higher.
The results of the tested mixtures and chemicals did not show any significance, expect for the 10X G1 concentration without outliers. A study by Parker and Perry (2021) also did not find significant differences in their data when using 4dpf larval zebrafish in the Loligo® microplate respirometry system. They, however, did find significant difference between treatments with zebrafish larvae of 7dpf (Parker & Perry, 2021). A study by (Wang et al., 2021) has also measured the oxygen consumption rate as picomole per minute (pmol/min) in 4 dpf zebrafish larvae and had found oxygen consumption rates in a similar magnitude as our results. This confirms that our measurements are also in the correct magnitude.

Many papers using the Loligo® Microplate respirometry system have put the microplate in a flow-through water bath and on a shaker. This to ensure temperature control and constant movement in the wells to prevent settling of the organisms and water. As our experiments were done in a temperature controlled room, the temperature was already controlled so the water bath is perhaps not necessary. However for future use of this system, to put it on a shaker may be an improvement of the protocol. Besides that, the respirometry measurements also have to be done in the dark as the oxygen sensors are sensitive to light. It is possible that the larvae are less active in the wells because

of the dark and that the results are therefore not significantly different from the control. Light vs. dark respirometry experiments would need to be done in order to clarify this speculation.

In the Loligo® Microplate respirometry system, technically, oxygen uptake is measured. Usually it is assumed that uptake is the same as consumption, however this is not necessarily the case. Oxygen uptake is the removing of oxygen from the water by the gills, whereas oxygen consumption is the utilization of the oxygen by other tissues or organs. There is a lag between oxygen uptake at the gills and its utilization (or consumption) (Chabot et al., 2016). Oxygen consumption depends on a couple of factors like temperature, exercise, food, metabolism but also hormones. This is also why the variation in the data can be so high and perhaps why the data is not normally distributed. Moreover, as mentioned earlier to relate changes in oxygen consumption/uptake back to energy metabolism and specific cellular processes is very difficult and these reviews specify these challenges; Chabot et al. (2016), Nelson (2016), Lai et al. (2008).

5.4 Further research

Further research should aim to relate the whole-body effects more directly to specific cellular processes and disruption of the thyroid hormone system. This can be done e.g. by various fluorescence assays to visualize thyroid hormone receptors binding/activation. ROS measurements can be done by a glutathione assay and the mitochondrial membrane potential can be detected with fluorescence in order to investigate the effect of the different concentrations on the mitochondria. In order to make stronger conclusions about the oxygen uptake, heartrate and blood pressure could also be measured as well as perhaps the waste of the fishes.
The non-monotonic (U-shaped) effect of the mixtures and chemicals should also be researched more, why the oxygen consumption rate returns to the control level with the higher concentrations.
Finally, chronic effects of the mixtures should also be a focus in future research. The three week pilot data shows some interesting results and it would be good if this is continued with some more reliable/structured experiments looking at multiple factors at the same time like heart rate, mortality and deformities as well as performing histology and PCR afterwards.

6. Conclusion

In conclusion, this project has shown that endocrine disrupting chemicals and mixtures display trends of effects on whole-body oxygen consumption of 5dpf zebrafish larvae. The exact cellular and hormonal effect behind this, however, remains to be established. Nevertheless, these results aid in the visualization of the complete picture of the physiological response of the SELMA mixtures, which is important for mixture risk assessment.

7. Acknowledgements

First of all I would like to give a big thank you to my supervisor Joachim Sturve and all the other people from the fishtox group who I shared many fun fika's with and could always go to with my questions.
I want to thank Francesca especially for all the help with the zebrafish, you're so supportive and extremely hard-working which is really inspiring and I wish you all the best!

I would also like to thank all the amazing people and the master students at Zoologen who are a great company during lunch and especially at afterwork beers.

Last but not least, I want to thank my family and friends and especially my boyfriend Alex who have been supportive during this time had to listen to me after a long day in the lab talking about my difficulties even though you did not understand them sometimes.

Thank you!

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9. Appendix

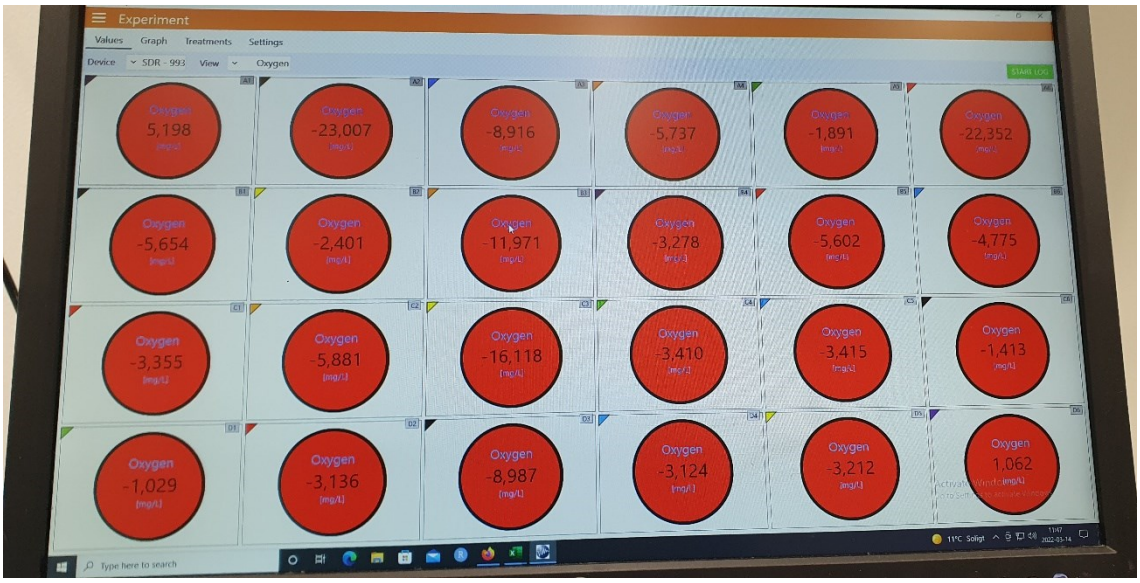

Figure 10: Software showing the oxygen in the wells has fallen below 70% by making the 'well' on the computer red.

Section: 1.1 protocol optimization

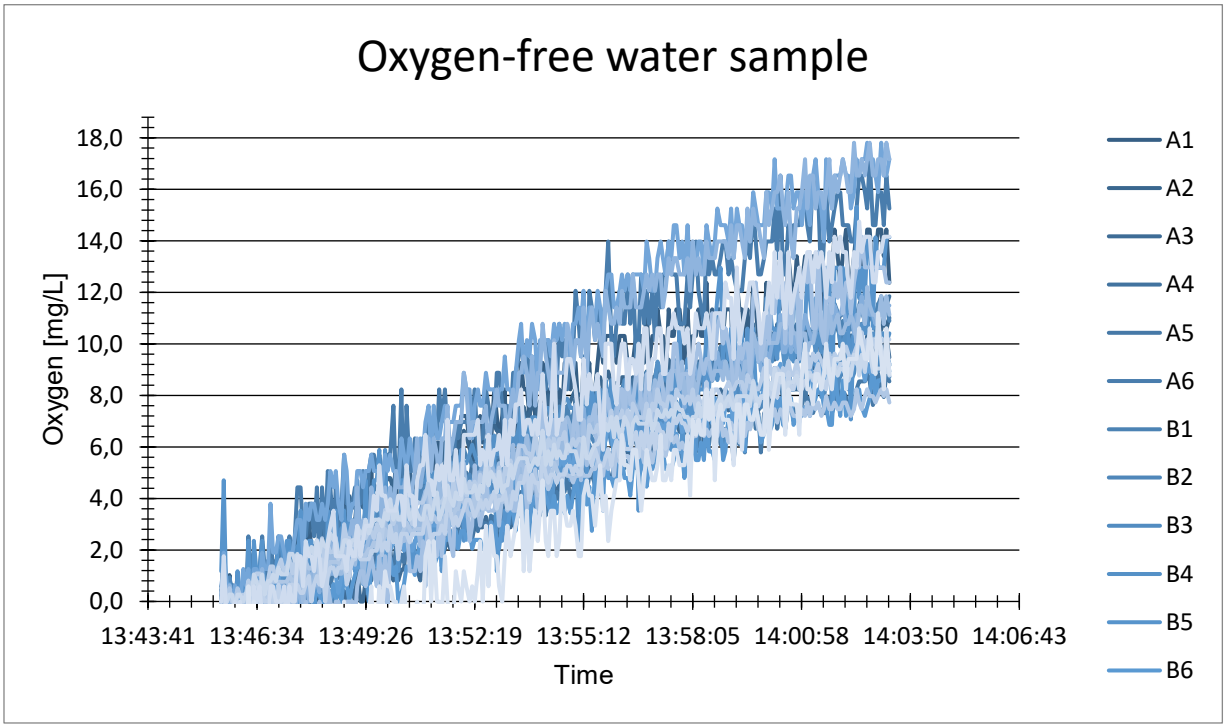

Figure 2: Oxygen-free water sample measurement over approx. 15 minutes. Measured without sealing the plate, therefore there is an increase in oxygen. But the focus of this figure is that the oxygen-free water sample is really oxygen free. Different colours to show the different lines, but all the wells were empty.

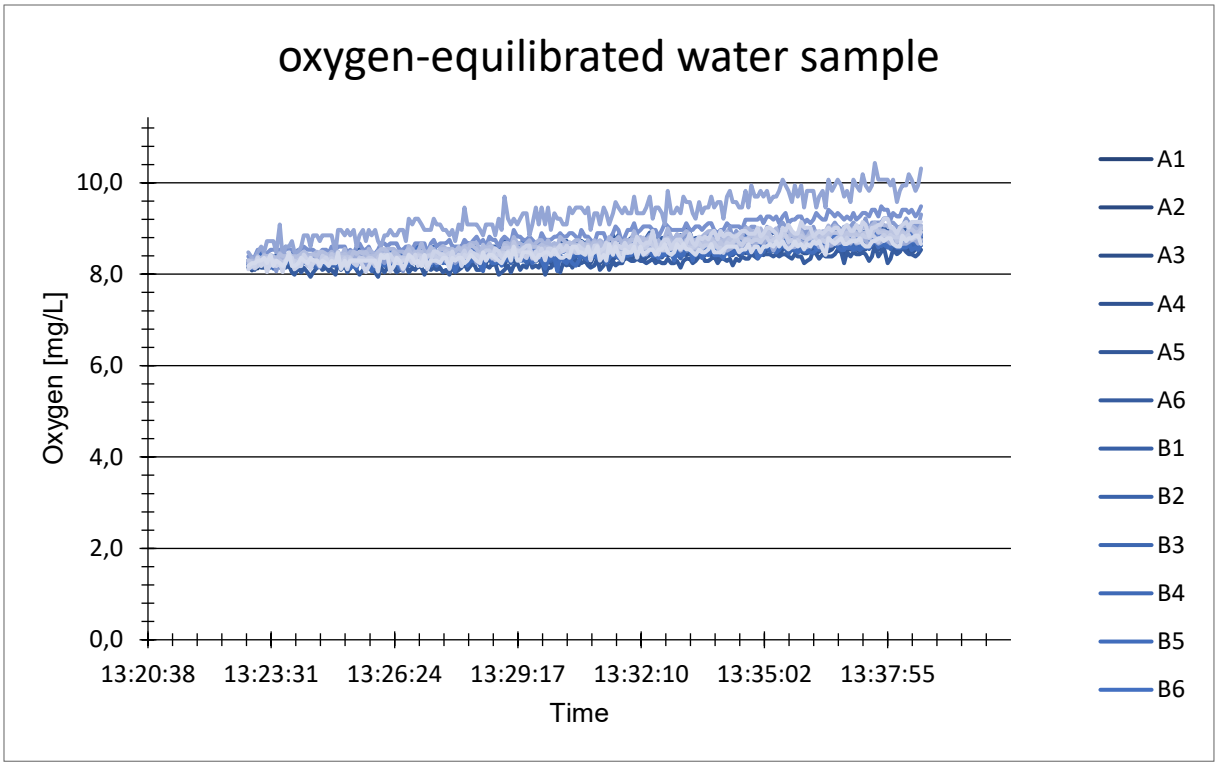

Figure 3: Oxygen-equilibrated water sample measurement over approx. 15 minutes. Measurement was again done without sealing the plate. Focus of the figure is that this water sample has a high oxygen concentration. Different colours to show the different lines, but all the wells were empty.

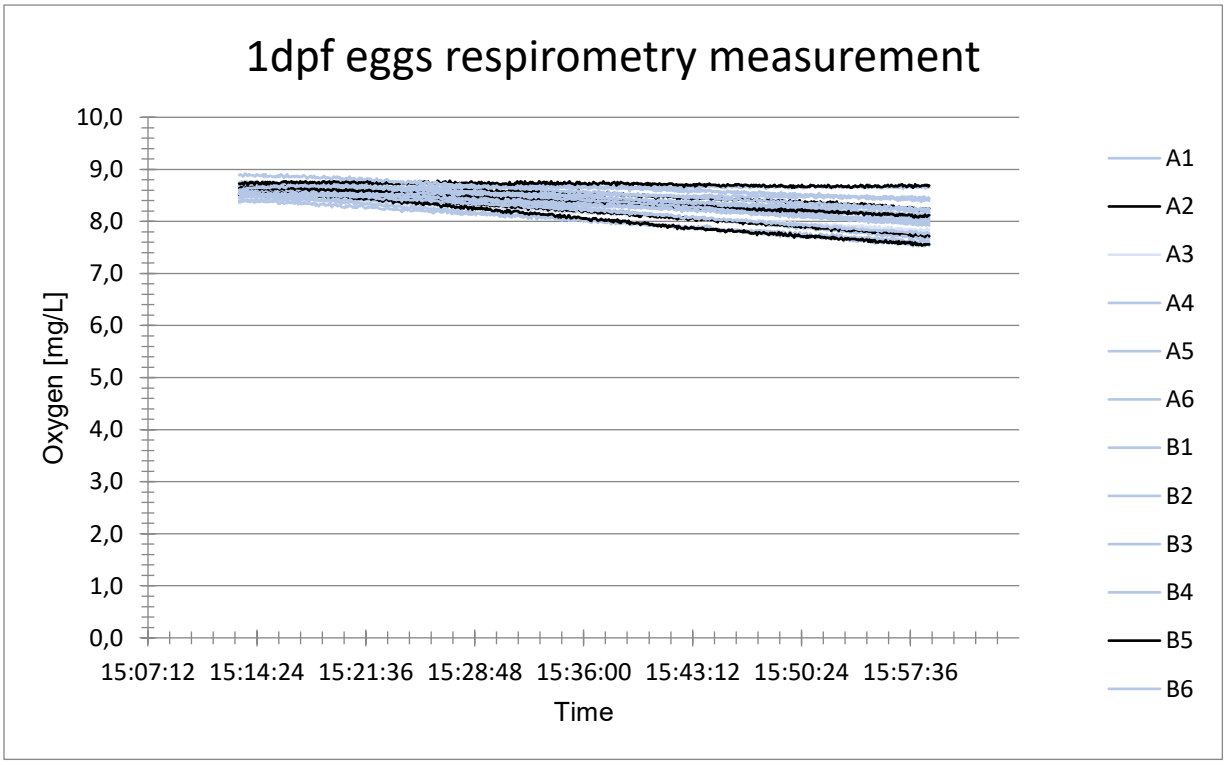

Figure 4: Respirometry experiment with 1dpf eggs over approx. 30 minutes. Blanks are shown as the black lines and the eggs are shown as the light blue lines.

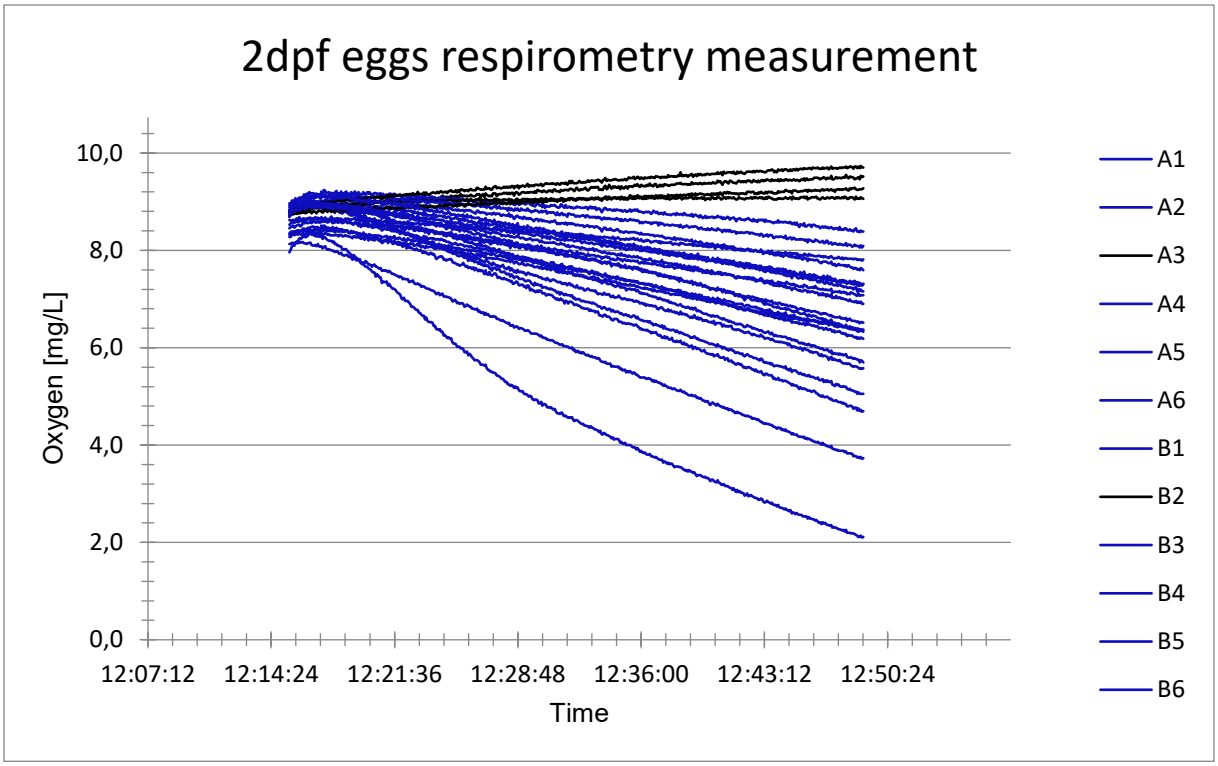

Figure 5: Respirometry experiment with 2dpf eggs over approx. 30 minutes. Blanks are shown as the black lines and the eggs are shown as the blue lines.

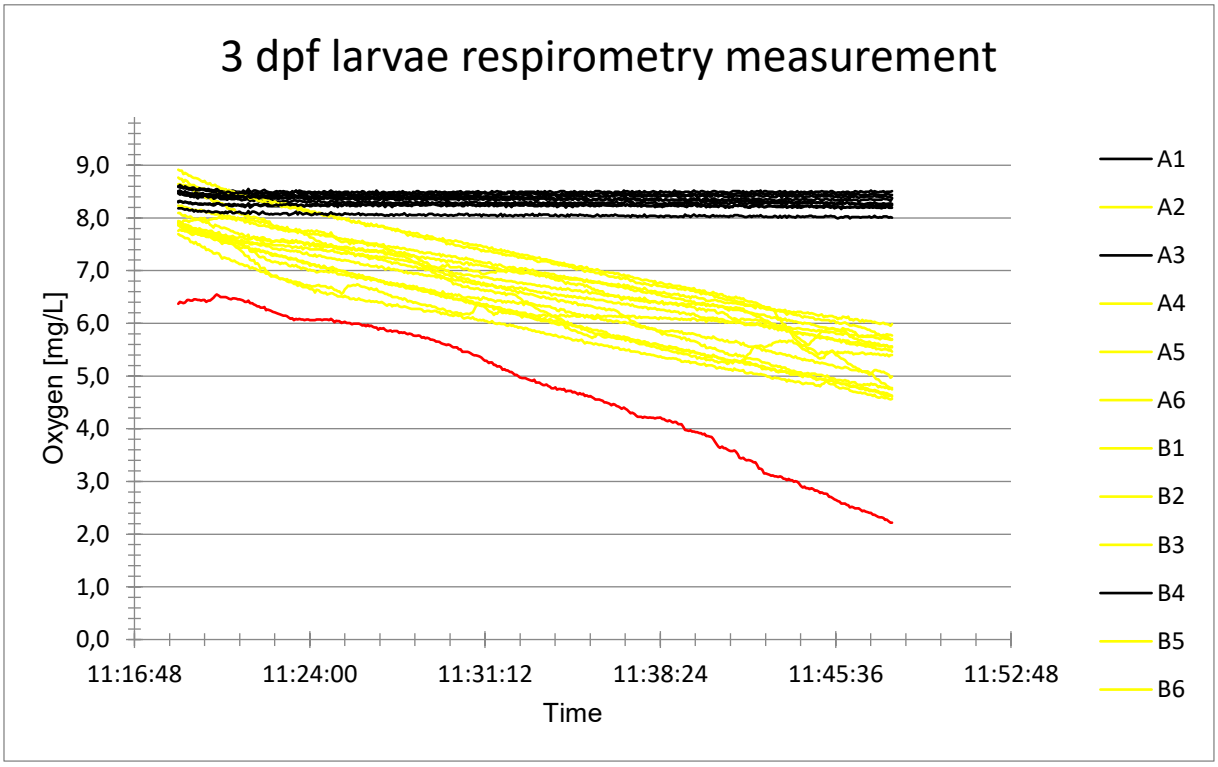

Figure 6: Respirometry experiment with 3dpf eggs over approx. 30 minutes. Blanks are shown as the black lines and the larvae are shown as the yellow lines. The red line shows a well that had two larvae instead of one.

Table 4: Summary of the cyanide data; concentration (LC), N, mean, standard deviation, median, standard error, and data analysis.

conc	count	mean	STDEV	median	st. error	Tests	p-value

0	9	1	0,489	0,98	0,163	ANOVA	0,62
LC05	6	0,871	0,335	0,774	0,137	Levene's	0,28
LC10	8	0,861	0,228	0,873	0,081	Shapiro-Wilk	0,57
LC15	7	0,774	0,315	0,893	0,119		
LC20	7	0,76	0,229	0,765	0,086		

Section 1.2 Respirometry experiments with 5dpf zebrafish larvae

1.2.1 Mix G0

Table 5: Summary of the effect data of mix G0 with outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	49	1	0,985	0,681	0,141
0,01	38	0,75	0,591	0,635	0,096
0,1	41	0,771	0,554	0,589	0,087
1	49	0,742	0,425	0,637	0,061
10	40	0,739	0,487	0,619	0,077
100	40	0,923	0,567	0,757	0,0897

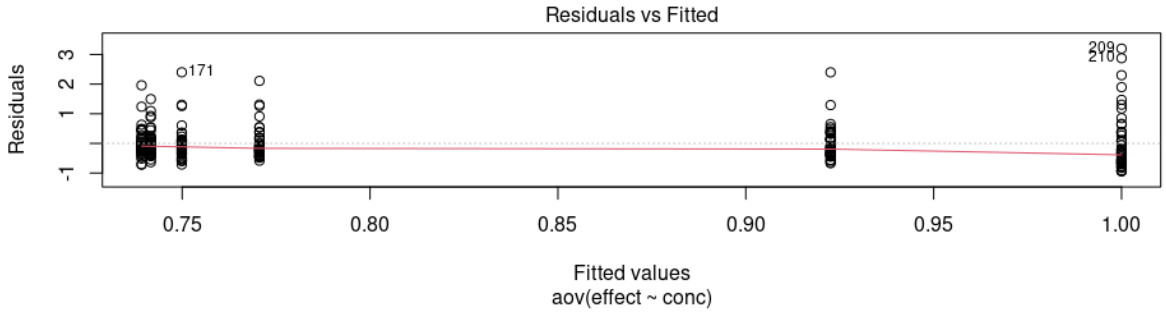

Figure 7: Residual plot of the effect data of mix G0 with outliers.

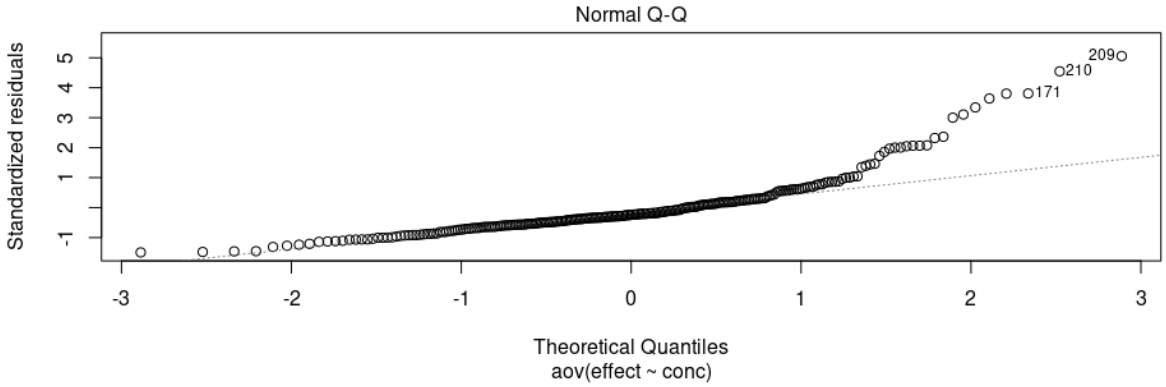

Figure 8: Q-Q plot of the effect data of mix G0 with outliers.

Table 6: Summary of the effect data of mix G0 without outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	46	1	0,842	0,763	0,124
0,01	35	0,743	0,375	0,764	0,063

0,1	38	0,791	0,4	0,704	0,065
1	45	0,787	0,329	0,714	0,049
10	38	0,801	0,383	0,75	0,062
100	38	1,01	0,439	0,907	0,071

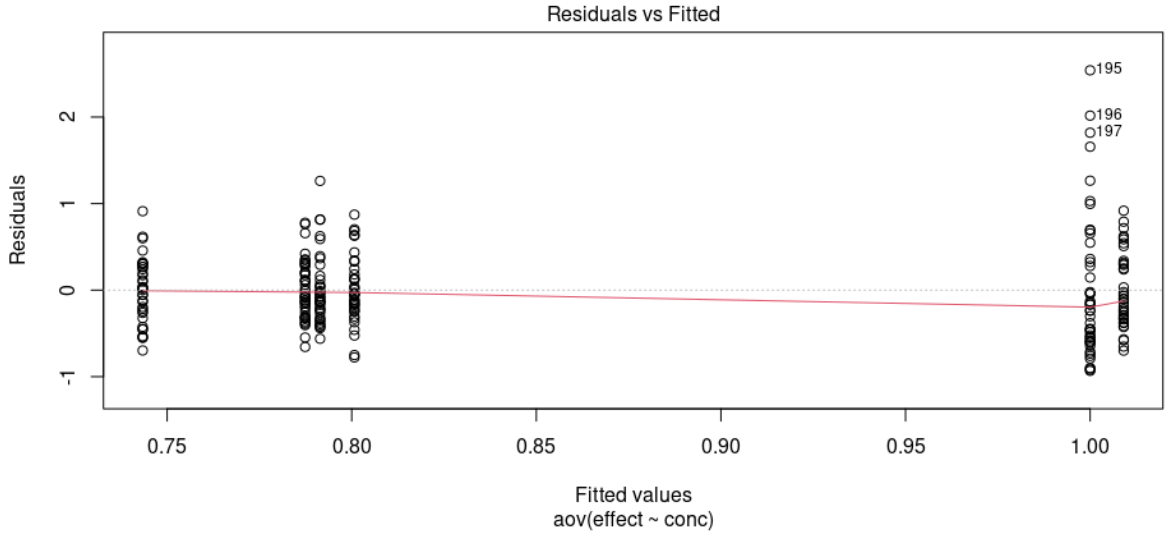

Figure 9: Residual plot of the effect data of mix G0 without outliers.

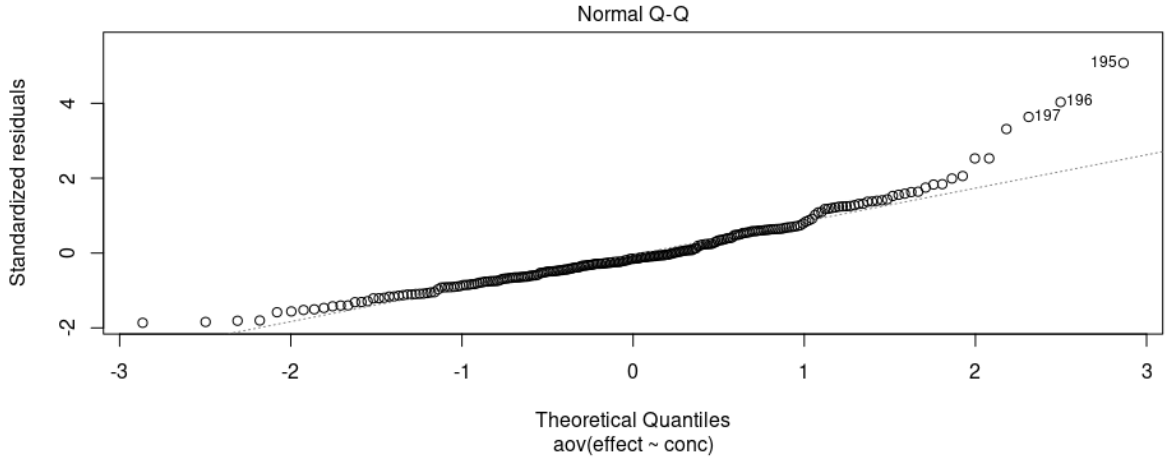

Figure 10: Q-Q plot of the effect data of mix G0 without outliers.

1.2.2 Mix G1

Table 7: Summary of the effect data of mix G1 with outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	34	1	0,872	0,722	0,1495
0,01	30	1,22	0,968	0,929	0,1768
0,1	30	1,3	2,08	0,664	0,3797
1	40	1,08	1,12	0,64	0,1766
10	34	1,63	1,22	1,24	0,2099
100	32	1,43	1,1	0,908	0,1946

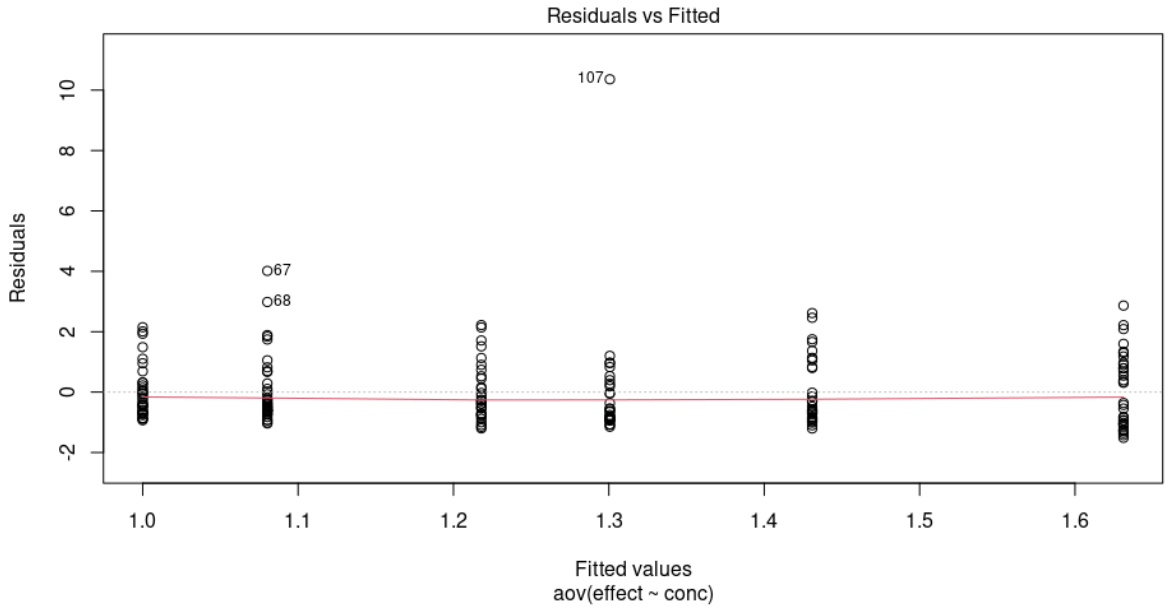

Figure 11: Residual plot of the effect data of mix G1 with outliers.

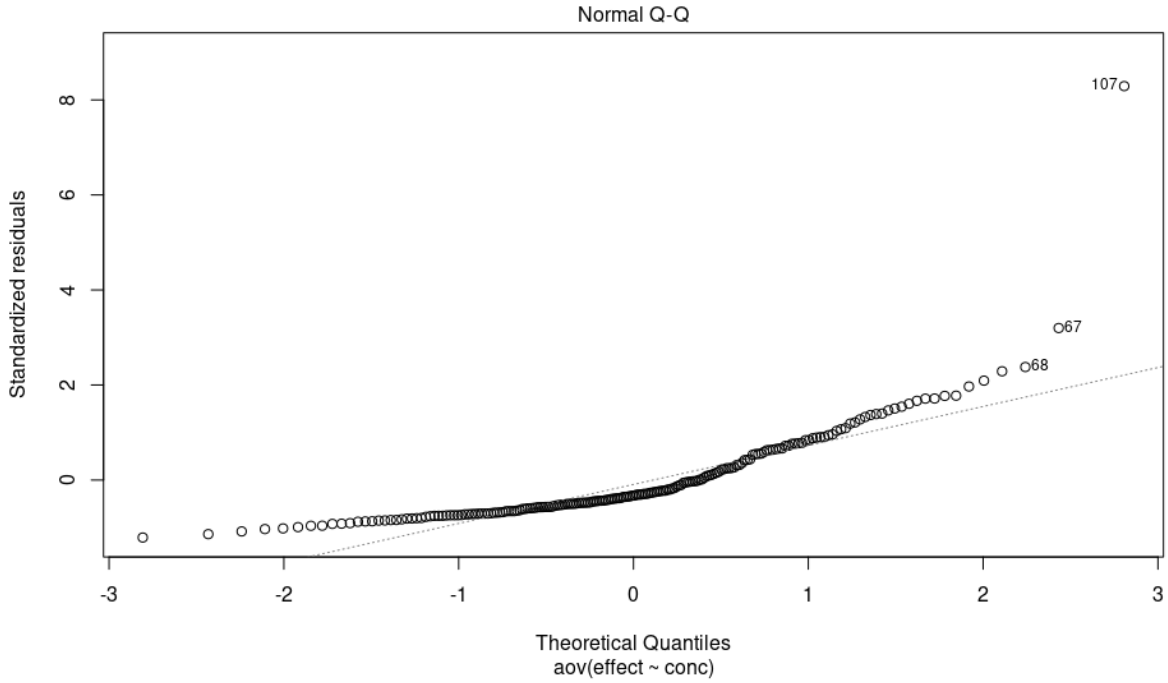

Figure 111: Q-Q plot of the effect data of mix G1 with outliers.

Table 8: Summary of the effect data of mix G1 without outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	31	1	0,771	0,764	0,138
0,01	28	1,32	0,988	1,09	0,187
0,1	29	1,17	0,894	0,81	0,166
1	38	1,12	0,967	0,748	0,157
10	33	1,92	1,41	1,5	0,245
100	30	1,57	1,13	1,06	0,206

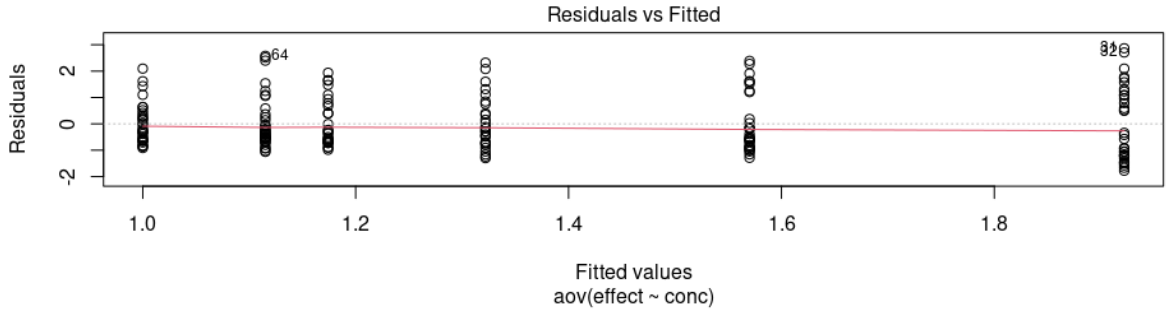

Figure 13: Residual plot of the effect data of mix G1 without outliers

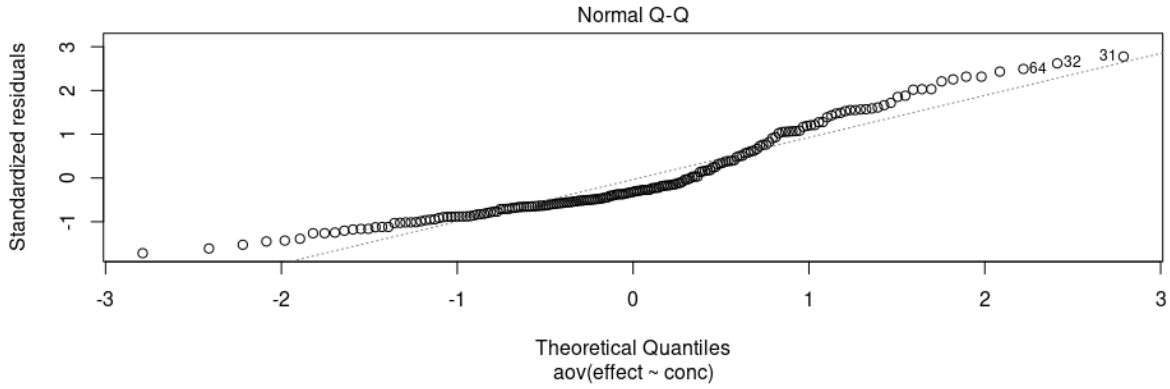

Figure 14: Q-Q plot of the effect data of mix G1 without outliers.

1.2.3 PFOA

Table 9: Summary of the effect data of PFOA with outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	42	1	0,637	0,944	0,098
0,01	36	0,952	0,507	0,883	0,085
0,1	29	0,832	0,429	0,788	0,0796
1	45	0,83	0,457	0,733	0,068
10	35	0,859	0,386	0,795	0,065
100	37	0,955	0,783	0,848	0,013

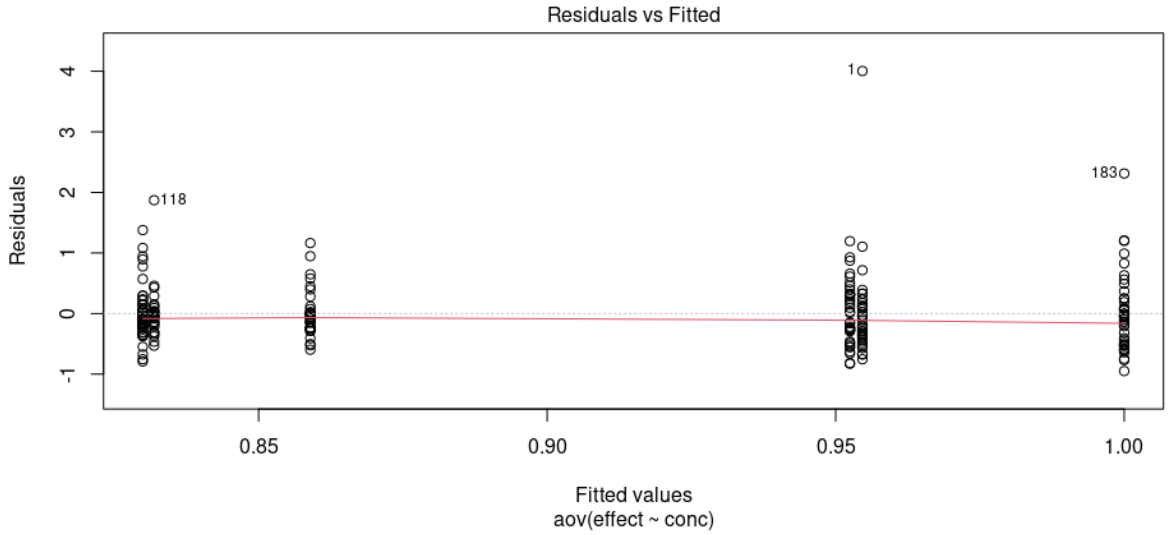

Figure 15: Residual plot of the effect data of PFOA with outliers.

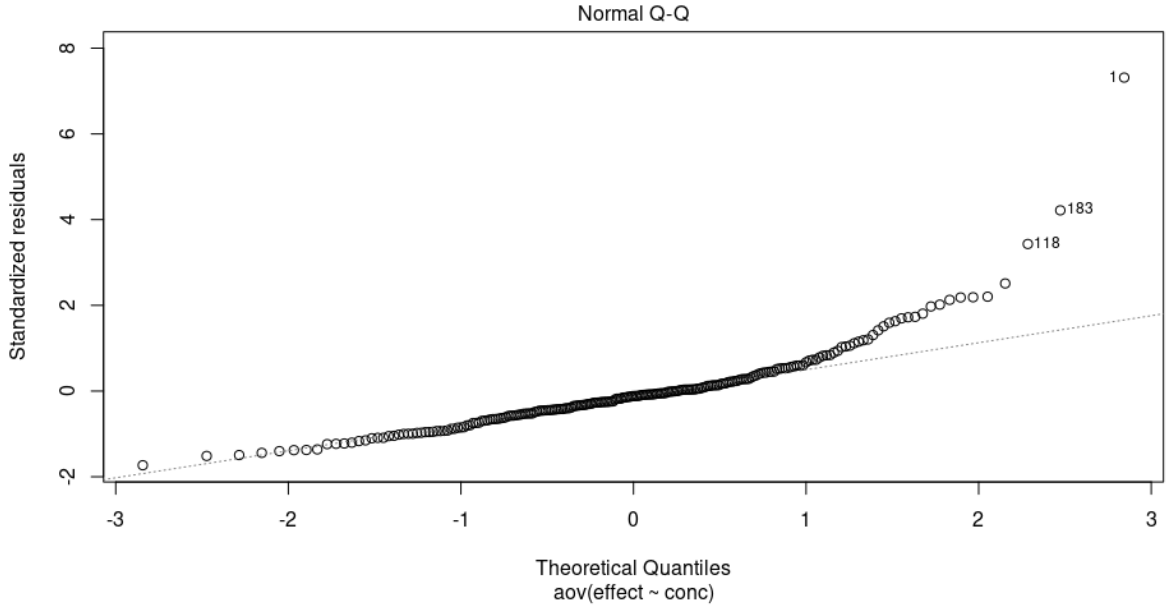

Figure 16: Q-Q plot of the effect data of PFOA with outliers.

Table 10: Summary of the effect data of PFOA without outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	41	1	0,559	0,994	0,087
0,01	35	0,973	0,499	0,853	0,084
0,1	28	0,811	0,252	0,825	0,048
1	43	0,819	0,4	0,776	0,061
10	33	0,843	0,307	0,828	0,053
100	36	0,894	0,425	0,855	0,071

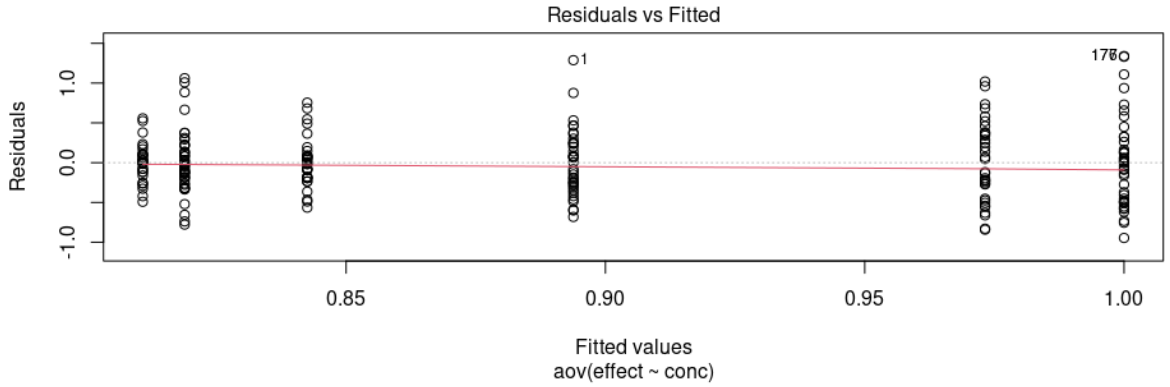

Figure 17: Residual plot of the effect data of PFOA without outliers.

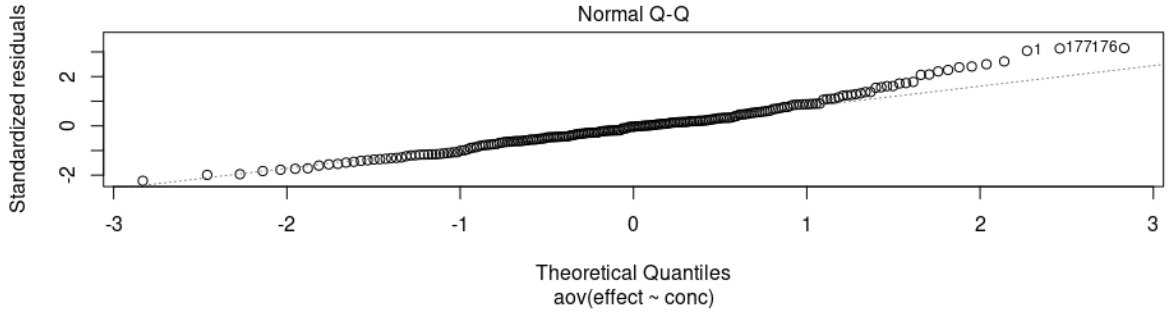

Figure 18: Q-Q plot of the effect data of PFOA without outliers.

1.2.4 PFOS

Table 11: Summary of the effect data of PFOS with outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	50	1	0,962	0,726	0,136
0,01	39	0,769	0,639	0,607	0,102
0,1	39	0,691	0,323	0,636	0,052
1	51	0,904	0,735	0,708	0,103
10	39	0,86	0,483	0,754	0,077
100	39	0,79	0,308	0,778	0,049

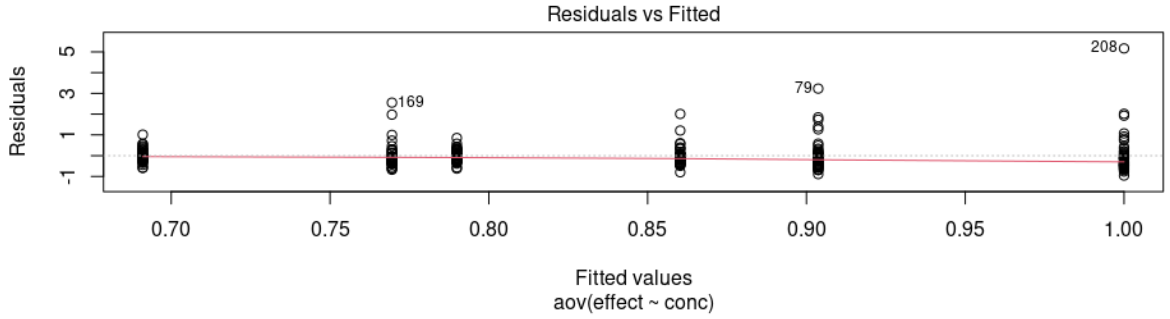

Figure 19: Residual plot of the effect data of PFOS with outliers.

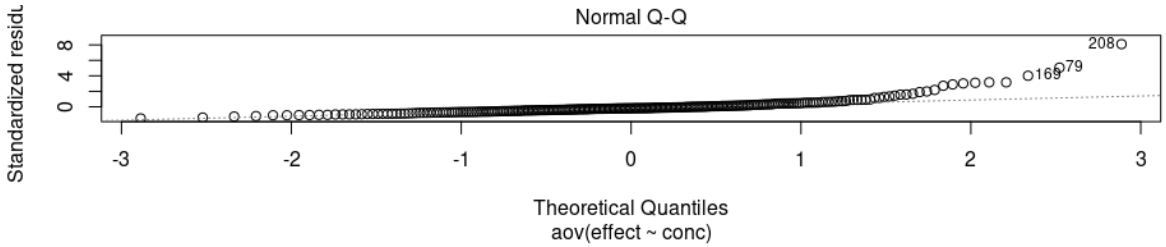

Figure 20: Q-Q plot of the effect data of PFOS with outliers.

Table 12: Summary of the effect data of PFOS without outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	47	1	0,552	0,831	0,081
0,01	37	0,803	0,44	0,751	0,072
0,1	38	0,824	0,348	0,782	0,057
1	48	0,945	0,548	0,869	0,079
10	37	0,959	0,363	0,927	0,0579
100	37	0,972	0,326	0,966	0,054

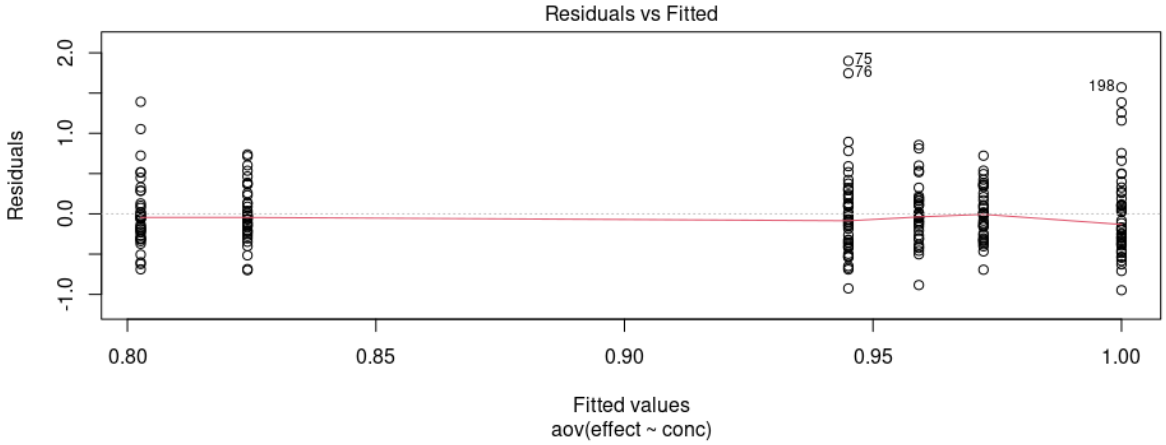

Figure 21: Residual plot of the effect data of PFOS without outliers.

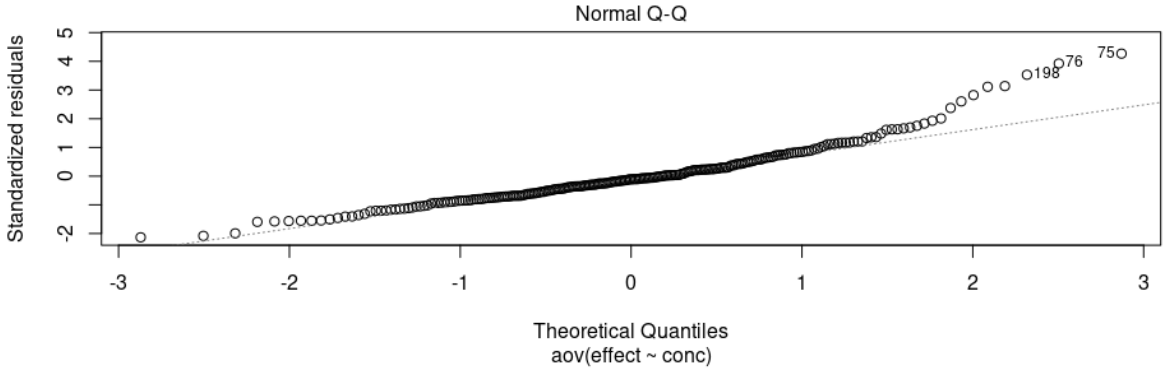

Figure 22: Q-Q plot of the effect data of PFOS without outliers.

1.2.5 MEHP

Table 10: Summary of the effect data of MEHP with outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	60	1	0,633	0,837	0,082
0,01	46	0,852	0,376	0,812	0,055
0,1	46	0,832	0,493	0,744	0,073
1	60	0,871	0,307	0,828	0,0397
10	46	0,859	0,23	0,815	0,034
100	42	0,866	0,283	0,839	0,044

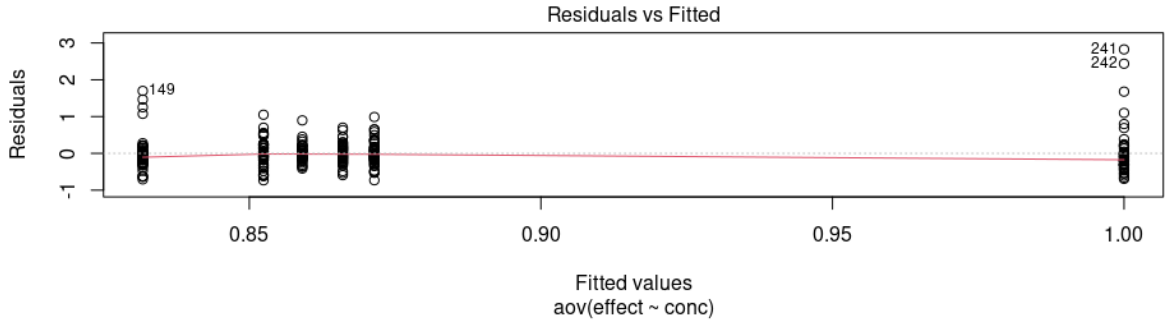

Figure 23: Residual plot of the effect data of MEHP with outliers.

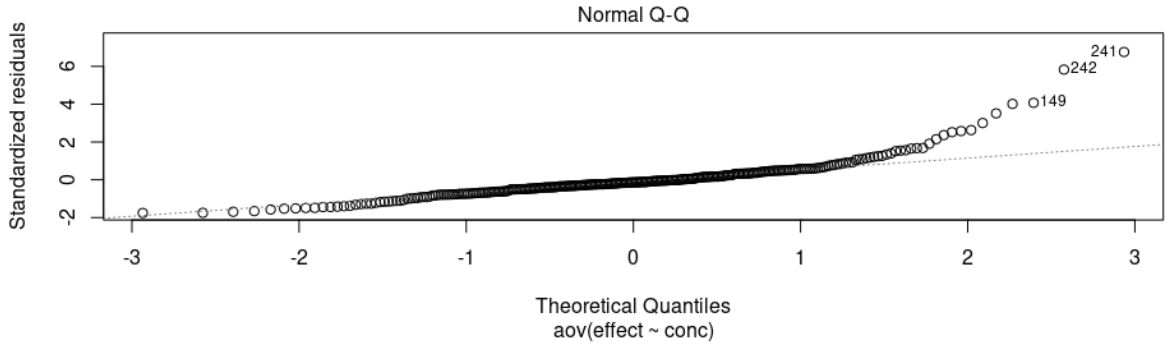

Figure 24: Q-Q plot of the effect data of MEHP with outliers.

Table 13: Summary of the effect data of MEHP without outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	57	1	0,376	0,947	0,0497
0,01	45	0,944	0,392	0,912	0,0585
0,1	42	0,798	0,28	0,813	0,0433
1	56	0,961	0,273	0,937	0,0365
10	45	0,955	0,214	0,922	0,0319
100	39	0,965	0,264	0,952	0,0422

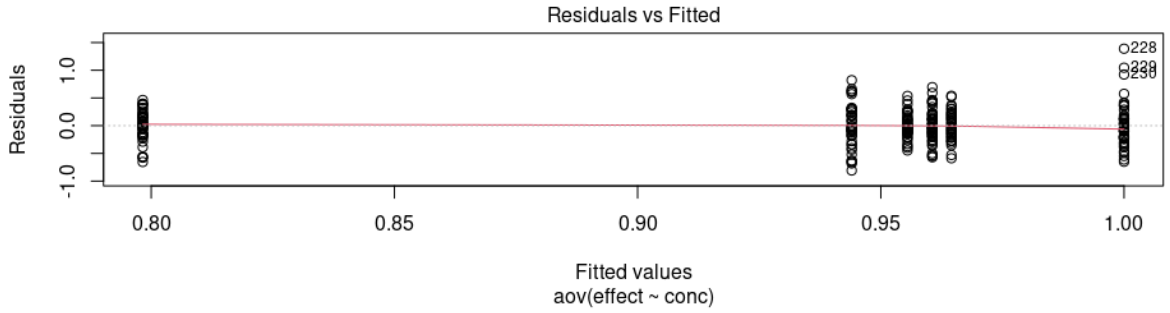

Figure 25: Residual plot of the effect data of MEHP without outliers.

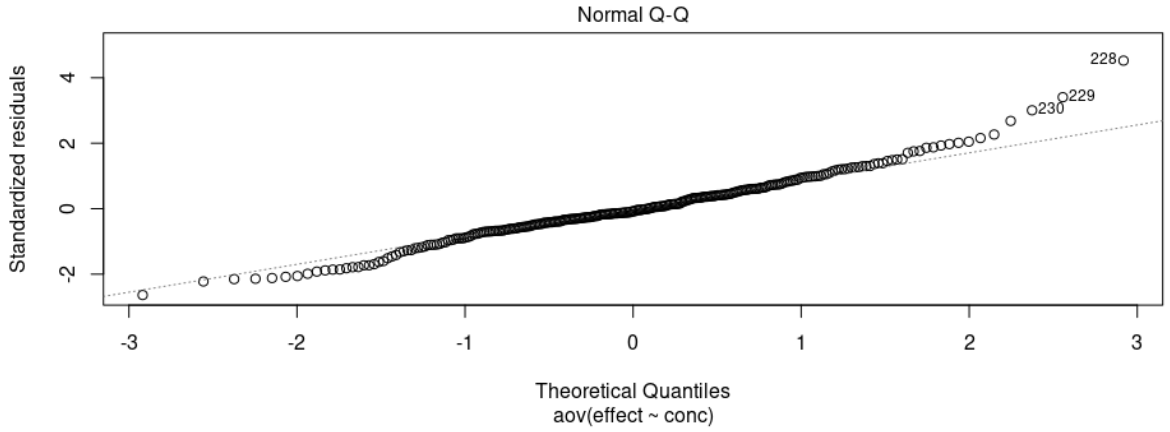

Figure 26: Q-Q plot of the effect data of MEHP without outliers.

1.2.6 Triclosan

Table 14: Summary of the effect data of Triclosan with outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	62	1	0,957	0,671	0,122
0,01	49	1,06	0,784	0,914	0,112
0,1	48	0,919	0,619	0,774	0,089
1	64	1,14	1,09	0,848	0,136
10	47	0,952	0,705	0,903	0,103
100	49	1,07	0,941	0,895	0,134

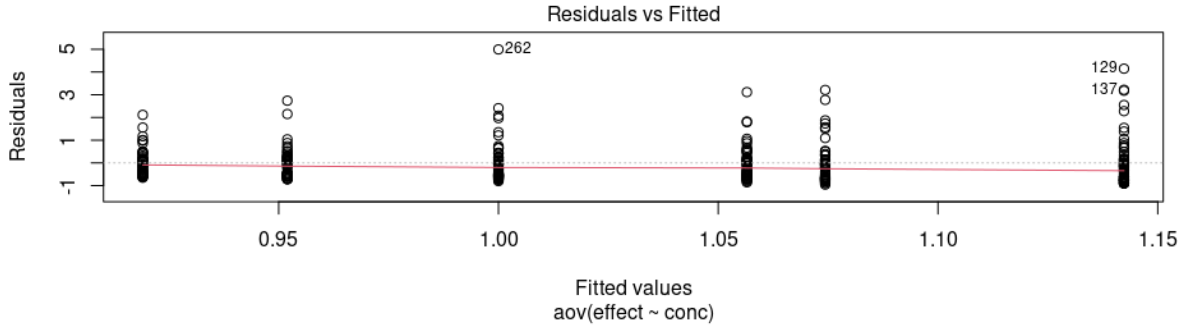

Figure 27: Residual plot of the effect data of Triclosan with outliers.

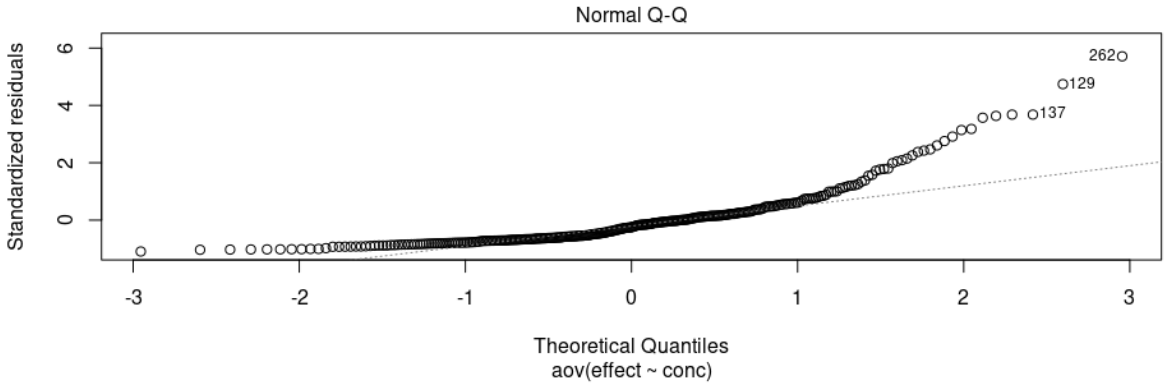

Figure 28: Q-Q plot of the effect data of Triclosan with outliers.

Table 15: Summary of the effect data of Triclosan without outliers; concentration (X SELMA), N, mean, standard deviation, median, standard error.

conc	count	mean	STDEV	median	st. error
0	58	1	0,633	0,72	0,083
0,01	46	1,13	0,65	0,964	0,096
0,1	46	1,05	0,611	0,914	0,09
1	59	1,1	0,75	0,994	0,098
10	45	1,05	0,6	1,07	0,089
100	47	1,18	0,895	1,05	0,131

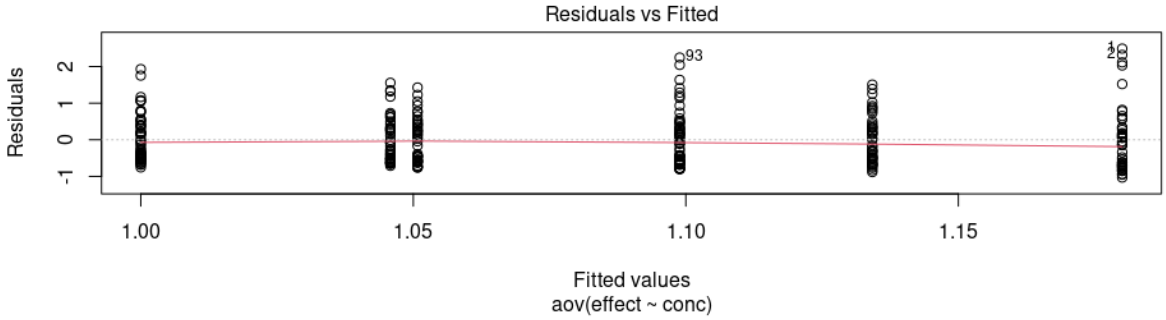

Figure 29: Residual plot of the effect data of Triclosan without outliers.

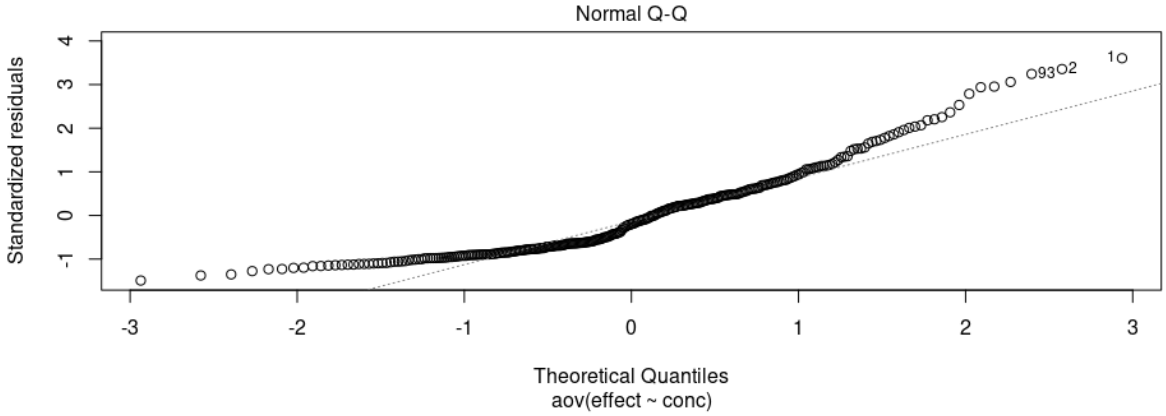

Figure 30: Q-Q plot of the effect data of Triclosan without outliers.

Section 1.3. Respirometry experiments with larvae of 3 weeks

Table 14: Summary of effect data of mix G0 on zebrafish larvae of 3 weeks.

conc	count	mean	STDEV	median	st. error
0	19	0,681347	1,043637	0,210309	0,239427
0,01	2	6,040226	1,218217	6,040226	0,86141
1	6	3,001401	1,640506	2,817612	0,669734
10	1	3,445079	-	3,445079	-

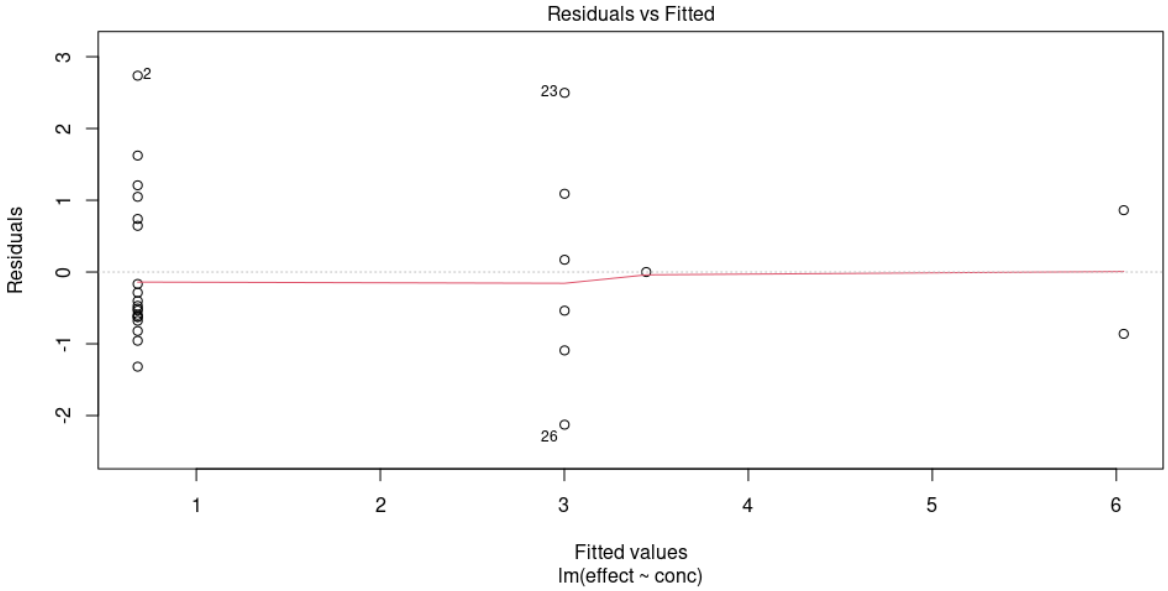

Figure 31: Residual plot of the effect data of mix G0 on zebrafish larvae of 3 weeks.

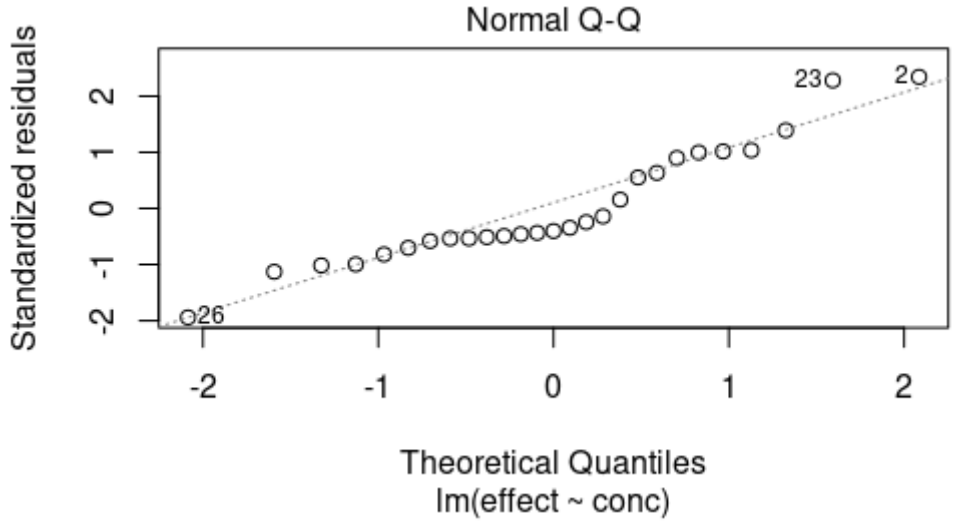

Figure 32: Q-Q plot of the effect data of mix G0 on zebrafish larvae of 3 weeks.

Table 15: Summary of effect data of mix G1 on zebrafish larvae of 3 weeks and data analysis.

conc	count	mean	STDEV	median	st. error	Test	p-value
0	19	0,681347	1,043637	0,210309	0,239427	ANOVA	0,162
0,01	3	1,512167	1,603808	1,215943	0,925959	Levene's	0,205
10	1	2,005643	-	2,005643	-	Shapiro-Wilk	0,067
100	6	1,903683	1,632021	1,82247	0,66627		

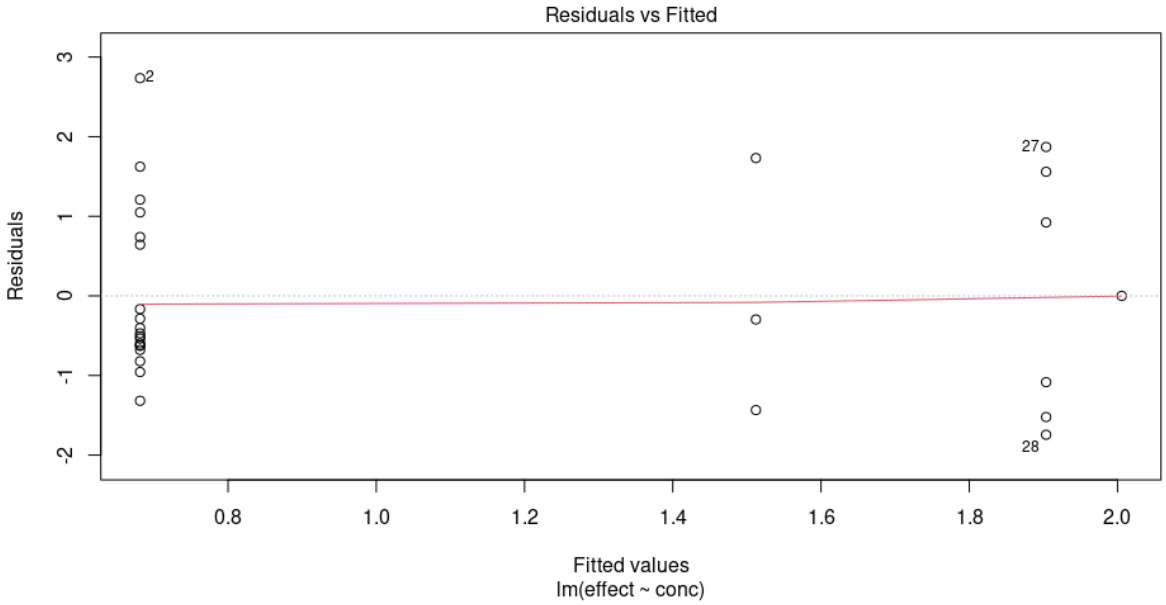

Figure 33: Residual plot of the effect data of mix G1 on zebrafish larvae of 3 weeks.

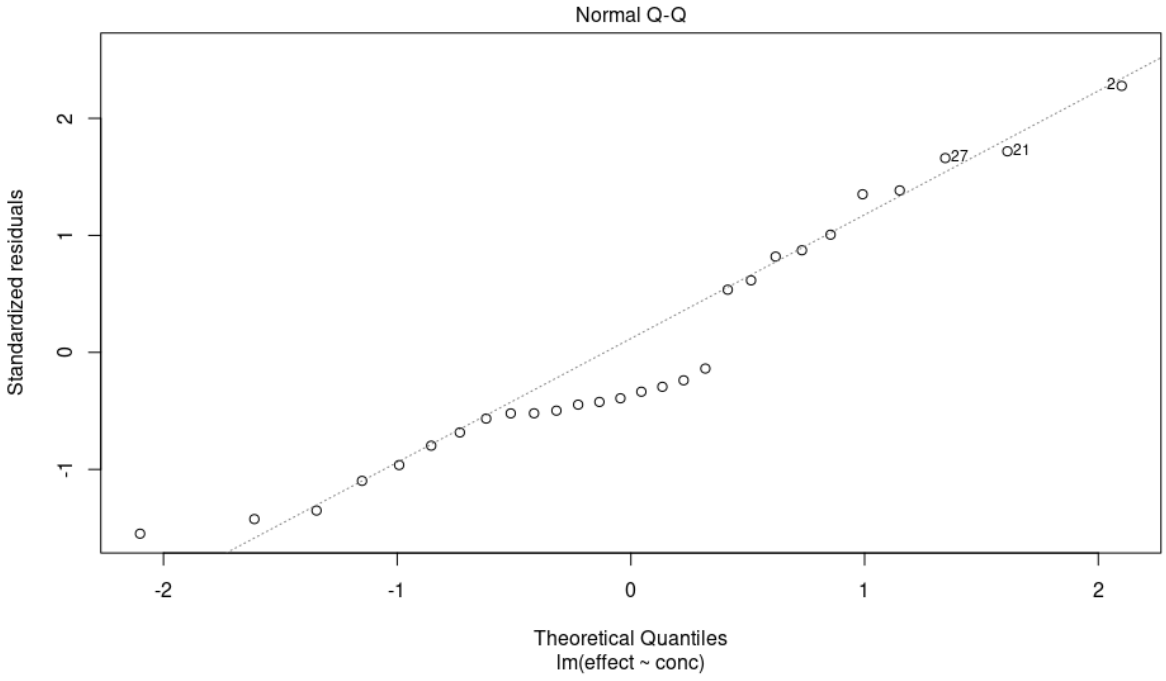

Figure 34: Q-Q plot of the effect data of mix G1 on zebrafish larvae of 3 weeks.