Master's Thesis

Does dehydroabietic acid (DHAA) at environmentally relevant concentrations cause developmental defects in brown trout larvae (*Salmo trutta lacustris*)?

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Könönen Iina: Aiheuttaako dehydroabietiinihappo (DHAA) ympäristön kannalta relevanteissa pitoisuuksissa kehityshäiriöitä järvitaimenen poikasissa (*Salmo trutta lacustris*)?

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Hakusanat: aikaisen kehitysvaiheen toksisuus, aryylihiilivetyreseptori, hartsihappo, sellutehtaan jätevedet, vesistöt

Hartsihapot ovat puun luonnollisia uuteaineita, joita päätyy vesistöihin sellu- ja paperitehtaiden jätevesien mukana. Niiden on osoitettu olevan kaloille ja muille vesieliöille haitallisia yhdisteitä. Etenkin aikaiset kalanpoikasten kehitysvaiheet ovat herkkiä vierasaineille, sillä altistuminen voi aiheuttaa kehityshäiriöitä. Dehydroabietiinihappo (DHAA) on yksi sellutehtaiden jätevesien yleisimmistä ja seuratuimmista hartsihapoista. Tämän tutkimuksen tarkoituksena oli tutkia ympäristön kannalta relevanttien DHAA-pitoisuuksien vaikutuksia järvitaimenen (*Salmo trutta lacustris)* vastakuoriutuneiden poikasten kehitykselle. Laboratoriossa suoritettiin 13 päivän DHAA-altistus, jonka aikana seurattiin poikasten kuolleisuutta. Altistuksen jälkeen poikasista todettiin ruskuaispussitaudin oireita, kuten ruskuaispussin ja sydänpussin edeemia. Myös poikasten kasvua ja energian kulutusta seurattiin mittaamalla poikasten pituus ja tasomainen ruskuaispussin pinta-ala altistuksen jälkeen. DHAA on polyaromaattisten hiilivetyjen esiaste, joten sen aryylihiilivetyreseptorin (AhR) aktivaatiota tutkittiin *cyp1a*-geenin ekspression avulla yhden päivän DHAA-akuuttialtistuksen jälkeen. Tutkimuksessa positiivisena kontrollina käytetty reteeni aiheutti poikasissa suurimmat vaikutukset. Epämuodostumia havaittiin kaikissa tutkituissa DHAA-pitoisuuksissa. Suuren toistokäsittelyjen välisen vaihtelun vuoksi selkeitä DHAA-annosriippuvaisia johtopäätöksiä ei voitu tehdä poikasten pituuden ja ruskuaispussin pinta-alan osalta, kun saatuja tuloksia verrattiin nollakontrolliin. Mikään käsittelyryhmä ei aiheuttanut tilastollisesti merkitsevää AhR-aktivaatiota, joten epämuodostuvat syntyvät luultavasti jollain muulla mekanismilla. Tulokset kuitenkin antavat syyn uskoa, että DHAA voisi aiheuttaa kehityshäiriöitä järvitaimenen poikasissa. Sellutehtaiden jatkuvat päästöt vesistöihin ja kalanpoikasten krooninen altistuminen jätevesien komponenteille voi siten aiheuttaa kehityshäiriöitä kalanpoikasille sellu- ja paperitehtaiden alapuolisissa vesistöissä. Lisää tutkimusta tarvitaan, jotta mekanismi mahdollisten epämuodostumien syntyyn selviäisi.

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Könönen Iina: Does dehydroabietic acid (DHAA) at environmentally relevant concentrations cause developmental defects in brown trout larvae (*Salmo trutta lacustris*)?

Master's thesis: 41 p., 7 appendices (7 p.)

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Resin acids are natural wood extractives released into aquatic environments along with pulp and paper mill waste water effluents. Resin acids have previously been shown to cause adverse effects on aquatic organisms, such as fish. Especially the early-life stages of fish are vulnerable, since the exposure may lead to developmental defects. One of the most abundant and monitored resin acid in pulp mill effluents is dehydroabietic acid (DHAA). The aim of this study was to find out the effects of DHAA to newly hatched brown trout larvae (*Salmo trutta lacustris*) at environmentally relevant concentrations. Measured endpoints following the 13-day sub-chronic DHAA laboratory exposure were the symptoms of blue sac disease: mortality and deformities, e.g., yolk sac edemas and pericardial edemas. Also, the body length and planar yolk sac area of the larvae were measured. As DHAA is a precursor for polycyclic aromatic hydrocarbons, the aryl hydrocarbon receptor (AhR) activation was measured as *cyp1a* gene expression after 1-day acute DHAA exposure. Following the laboratory exposures, the positive control treatment, retene, caused most of the observed effects of measured endpoints. Yolk sac and pericardial edemas were detected even in the lowest DHAA concentrations. Due to large variations between replicate treatments, no clear DHAA dose dependent trend was found in body length or planar yolk sac area of the larvae compared to negative solvent control (DMSO) treatment. None of the treatments caused statistically significant AhR activation. However, the results of this study showed the potential of DHAA to cause adverse effects on developing *S. trutta* larvae. The continuous resin acid load in aquatic environments and chronic exposure of DHAA may harm the normal development of fish larvae downstream from pulp and paper mills. Further research on the mechanisms leading up to developmental defects and deformities is needed.

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ABBREVIATIONS

AhR aryl hydrocarbon receptor

BSD blue sac disease

CYP1A a protein from cytochrome P450 family 1, subfamily A

DMSO dimethyl sulfoxide

DHAA dehydroabietic acid

EROD 7-ethoxyresorufin deethylase

PAH polyaromatic hydrocarbon

RIN RNA integrity number

(RT-)qPCR real-time quantitative polymerase chain reaction

SPE solid phase extraction

1 INTRODUCTION

In the field of pulp and paper industry, processes and wastewater treatment methods have improved over past decades, but still the concentrations of xenobiotic compounds released into aquatic environments may reach harmful levels for aquatic organisms, e.g., for fish, mussels, and *Daphnia magna*, causing acute and sub-lethal effects (Sandbacka *et al.* 2000, Gravato *et al.* 2005, Orrego *et al.* 2010). Especially the early-life stages of organisms are vulnerable to xenobiotics, and exposure may lead to delayed development and developmental defects (McKim 1977).

Resin acids are an example of wood extractives that are discharged in aquatic environments along with pulp and paper mill effluents (Ali and Sreekrishnan 2001). One of the most abundant and monitored resin acid in pulp mill effluents is dehydroabietic acid (DHAA) (Leppänen and Oikari 1999a). DHAA is converted into tricyclic polyaromatic hydrocarbon (PAH) compound, retene, via microbial metabolic processes in aquatic sediments (Ramdahl 1983, Leppänen and Oikari 1999b). Treated pulp and paper mill effluents commonly contain 40-2500 µg/l resin acids and receiving downstream surface waters 4-14 µg/l (Gravato and Santos 2002).

Dioxin-like compounds are planar chlorinated aromatic compounds, that can bind to aryl hydrocarbon receptor (AhR) and activate genes that encode several biotransformation enzymes, such as cytochrome P450 family member *cyp1a* gene (Billiard *et al.* 1999, Teraoka *et al.* 2003, Bauder *et al.* 2005). In phase I metabolism, biotransformation monooxygenation enzymes, that *cyp1a* gene encodes, increase the water-solubility of parent compound and make it easier to excrete out of the organism (Schlenk *et al.* 2008). Prolonged activation of AhR, and the induction of biotransformation oxygenation enzymes can lead to developmental defects and deformities, such as arrested growth, fin rot, yolk sac edemas, and hemorrhages,

which are symptoms of blue sac disease (BSD) in fish larvae (Billiard *et al.* 1999). DHAA may cause dioxin-like effects in fish larvae as it has structural similarities with PAH compounds that have previously found to induce *cyp1a* gene and cause developmental defects in fish (Billiard *et al.* 1999, Hodson *et al.* 2007).

Some studies have shown that DHAA can activate AhR in fish (Pacheco and Santos 1997, Gravato and Santos 2002). Pacheco and Santos (1997) have found in their study that DHAA activated AhR in glass eel (*Anguilla anguilla*) after a 3-day exposure at concentration of 90 µg/l. A study of Gravato and Santos (2001) showed that following 2-hour exposure to DHAA at concentration of 7.5 µg/l, DHAA caused an increase in liver 7-ethoxyresorufin deethylase (EROD) activity and liver P450 content in sea bass (*Dicentrarchus labrax*). Oikari *et al.* (1983) reported that the DHAA concentration of 20 µg/l is the minimum effect concentration for rainbow trout (*Salmo gairdneri*) following 30-day exposure to DHAA, causing other sub-lethal physiological effects, such as increased relative weight of spleen, and liver and gill lactate dehydrogenase (LDH) shifts to adult fish. Based on previous study results, it can be hypothesized that DHAA may have an influence on the normal fish larvae development at environmentally relevant concentrations (Billiard *et al.* 1999, Gravato and Santos 2002, Pandelides *et al.* 2014).

The aim of this master's thesis was to assess the effects of DHAA to developing brown trout (*Salmo trutta lacustris*) larvae at concentration range that is reported to be environmentally relevant for resin acids in aquatic environments (Gravato and Santos 2002). Till date, very few developmental toxicity studies following DHAA exposure have been published, and the few available focuses mainly on juvenile fish (Oikari *et al.* 1983, Pacheco and Santos 1997, Gravato and Santos 2002), while only a few studies have been performed on fish larvae and no tests with *Salmo trutta* spp. The aim of this master's thesis was to generate toxicity data in order to fill this knowledge gap, and for environmental risk assessors to use this data to improve their assessments.

The study was partially based on OECD guidelines for early-life stage (ELS) toxicity test on fish development (OECD 210/2013). A 13-day sub-chronic laboratory exposure of pure DHAA using newly hatched brown trout (*S. trutta*) larvae was performed at the University of Jyväskylä, from the end of April till early May, 2017. The study was a part of a survey for the ecological risk assessment process being carried out in Äänekoski, central Finland, where a new bioproduct extraction plant is currently being constructed.

The research questions of this study were; Does DHAA have an effect on the development of *S. trutta* larvae measured as BSD symptoms? And, does DHAA exposure at environmentally relevant concentrations cause AhR activation? Measured endpoints following the 13-day DHAA exposure were the symptoms of BSD: mortality and deformities; pericardial edemas, yolk-sac edemas and hemorrhages. The body length and planar yolk sac area of the larvae were also measured. In addition, the activation of AhR was measured using real time quantitative polymerase chain reaction (RT-qPCR) technique to quantify the expression of the *cyp1a* gene following 1-day acute exposure to DHAA. The hypotheses of this study were that the higher DHAA concentrations would lead to increased mortality and deformities of the *S. trutta* larvae. Based on few previous studies, it could be expected that the activation of AhR and increased *cyp1a* expression could be followed by the DHAA exposure.

2 BACKGROUNDS

Pulp and paper mill industry is one of the most water consuming and waste water producing practices in the industrial sector (Kreetachat *et al.* 2007). More than 250 different chemical compounds have been identified in pulp mill effluents, some of which are natural extractives of wood and others are synthetic by-products (Ali and Sreekrishnan 2001, Mark Hewitt *et al.* 2006). In industrial pulping processes, resin acids are extracted from the lignin fibres and discharged with the effluents of pulp and paper mills into aquatic environments (Ali and Sreekrishnan 2001). Even after a proper waste water treatment of the effluents, resin acids accumulate in the aquatic environments in harmful concentrations (Kostamo and Kukkonen 2003).

2.1 Pulp and paper mill industry and its effluents

Pulp and paper industry utilizes several kinds of techniques and raw materials in various processes needed to produce the desired final product (Ali and Sreekrishnan 2001). Thus, the waste waters from pulp and paper mills are a complex mixture of many different chemicals and wood resins that are discharged through different production processes (Ali and Sreekrishnan 2001). However, there are five common steps in wood refining processes: debarking, pulping, bleaching, washing and the final treatment of the end product (Ali and Sreekrishnan 2001). Pulp mills usually consume coniferous, soft wooden trees, like pines (*Pinus* spp.) and spruces (*Picea* spp.) as a raw material for production of sulphate pulp (Holmbom and Ekman 1978).

The waste water effluents from pulp and paper mills commonly consist of suspended solids, dissolved organic colloids, lignin compounds, bleaching chemicals and dissolved inorganic compounds (Ali and Sreekrishnan 2001). In addition to chemical compounds, the thermal load of the treated effluents and condensation waters can be harmful to the receiving aquatic environments (Ali and Sreekrishnan 2001). Nowadays, in western industrial countries, persistent

halogenated compounds, such as chlorophenols, dioxins and furans, no longer pose an environmental problem, since the pulp bleaching techniques have been changed to elemental chlorine free or total chlorine free, that are more environmentally friendly (Ali and Sreekrishnan 2001, Orrego *et al.* 2010). More of a concern in modern pulp and paper industry are wood extractives, such as resin acids and wood sterols that can have many adverse effects on aquatic organisms (Peng and Roberts 2000, Kostamo and Kukkonen 2003). In addition to monitoring, e.g., the biological oxygen demand (BOD) and chemical oxygen demand (COD) of waste waters, the toxicity of effluents to aquatic organisms is also an important parameter to measure in order to identify the environmental impacts of pulp mill discharges (Peng and Roberts 2000).

The waste water treatment methods have been evolved and the chemical load into aquatic environments have decreased (Pokhrel and Viraraghavan 2004). More closed water circulation and waste water treatment, including secondary biological treatment and tertiary treatment, are performed in order to decrease the amount of organic matter, nutrients (dissolved nitrogen and phosphorous species) and absorbable organic halogens (AOX) released into the aquatic environment along the other emissions (Ali and Sreekrishnan 2001, Kostamo and Kukkonen 2003). Activated sludge biological treatment of waste waters decreases the amount of organic matter and BOD by 85-95% and COD by 40-80%, whereas tertiary treatment reduces especially the brownish colour of pulp and paper mill effluents that basically originates from the lignin-degraded compounds and tannins (Kostamo and Kukkonen 2003, Kreetachat *et al.* 2007).

More than 90% of the resin acids are removed from the pulp and paper mill waste waters with biological activated sludge treatment, as Kostamo and Kukkonen (2003) have reported. Microbiological removal of resin acids is a possible option, since some mesophilic bacteria, such as *Pseudomonas* spp., *Escherichia coli* and *Flavobacterium* spp., are capable of degrading resin acids (Ali and Sreekrishnan 2001). In addition, thermophilic bacteria *Rubrivivax* spp. have been found to

degrade DHAA (Ali and Sreekrishnan 2001). Pulp and paper mill effluents usually have high temperatures and thus the usage of thermophilic bacteria, that require 30-60 ˚C temperature to thrive, could be a practical solution to further decrease resin acid emissions in pulp and paper mill effluents (Ali and Sreekrishnan 2001).

Modern bioproduct extraction plants are seeking to benefit the by-products and waste water components from pulp mill effluents, and turn waste into new products and biofuels (Lucia *et al.* 2007). For example, Äänekoski bioproduct extraction plant will produce a wide range of bioproducts, such as pine oil, turpentine, biocomposite materials, and biogas along with the sulphate pulp (Päätös 4/2015/1).

It has been shown with the toxicity tests of sediments contaminated by pulp mill effluents that the toxicity varies depending on whether the exposure have been performed in laboratory or in field (*in situ*) (Smolders *et al.* 2004, Vehniäinen *et al.* 2015). In the studies of Vehniäinen *et al.* (2015) and Costa *et al.* (2011), contaminated sediments has caused less toxic responses, measured as mortality and genotoxic responses in fish, in laboratory circumstances than when the test organisms had been exposed *in situ*. The whole toxicity of complex waste water effluents of pulp mills and contaminated sediments is not possible to reliable measure in laboratory, and therefore field exposures are needed to perform, in order to monitor the real effects of the effluents on aquatic organisms (Smolders *et al.* 2004).

2.2 Dehydroabietic acid and retene

DHAA (CAS-No. 1740-19-8) is a tricyclic resin acid found in coniferous tree resins (Cheremnykh *et al.* 2017) (Figure 1). The octanol-water partition coefficient (log K_{ow}) of DHAA is reported to be 1.74, so the compound is amphiphilic, having both hydrophobic and hydrophilic properties (Rissanen *et al.* 2003).

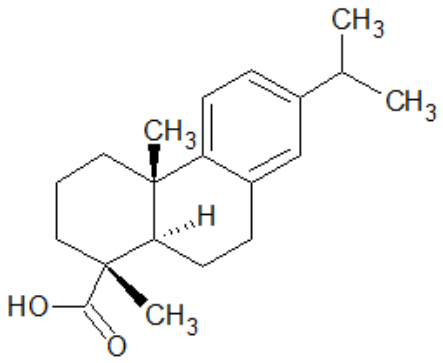

Figure 1. The structure of DHAA drawn with ACD/ChemSketch (Freeware version, Advanced Chemistry Development, Inc. ACD/Labs 12.01).

DHAA is a weak hydrophobic acid and the pH strongly affects the solubility and toxicity of DHAA (Ali and Sreekrishnan 2001). DHAA is the most water-soluble of the common resin acids (Peng and Roberts 2000). The solubility of DHAA to water is reported to be 5.11 mg/l measured with solid phase extraction method (Peng and Roberts 2000).

DHAA has an affinity to bind with particles in waste water effluents and accumulate in aquatic sediments (Ali and Sreekrishnan 2001, Kostamo and Kukkonen 2003, Meriläinen *et al.* 2006). Once bound and accumulated in sediments, DHAA can undergo desorption from the sediments via agitation caused by, e.g., erosion or dredging (Meriläinen *et al.* 2006). When DHAA is taken up by fish, it bioaccumulates in liver, blood, and bile, as the main elimination route of phase I metabolized DHAA is via bile (Pandelides *et al.* 2014).

Retene, i.e, 7-isopropyl-1-methylphenanthrene (CAS. No. 483-65-8), is a tricyclic alkyl substituted PAH compound (Figure 2). It is highly hydrophobic compound, having log K_{ow} of 6.4 (Hodson *et al.* 2007).

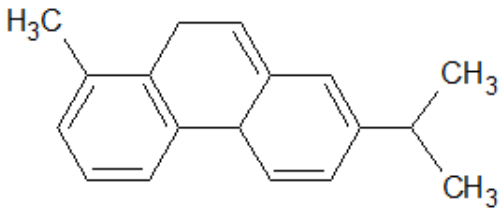

Figure 2. The structure of retene drawn with ACD/ChemSketch (Freeware version, Advanced Chemistry Development, Inc. ACD/Labs 12.01).

Retene is usually found in freshwater lake sediments in background concentration from 0.010 to 0.10 µg/g (dry weight), but it has been detected in sediments downstream from pulp and paper mills at concentrations as high as 3300 µg/g (dry weight) (Rämänen *et al.* 2010). As PAH compounds usually, retene is also formed from pyrolysis of organic material, e.g., from wood combustion (Ramdahl 1983). In anaerobic conditions of aquatic sediments, retene is naturally derived from DHAA by microbial metabolism reactions (Ramdahl 1983, Billiard *et al.* 1999, Hodson *et al.* 2007). Retene is considered as a labile PAH compound, since after taken up by organisms, it is rapidly metabolized by biotransformation reactions; hydroxylated to more water soluble benzylic alcohol by the catalysis of CYP1A enzyme, and excreted out of the organism (Billiard *et al.* 1999, Bauder *et al.* 2005).

2.3 Aryl hydrocarbon receptor and *cyp1a* gene

AhR is a transcription factor that regulates many biotransformation enzymes in phase I, II, and III biotransformation reactions (Goksøyr 1995, Doering *et al.* 2013). AhR activation occurs when an exogenous ligand binds with the transcription factor (Doering *et al.* 2013). That, in turn, leads to an induction of many downstream monooxygenation genes, such as *cyp1a* gene, and the translation of the respective

biotransformation enzymes (Bauder *et al.* 2005). Metabolic activation is a biological phenomenon that occurs when the biotransformation metabolites of the xenobiotic compound becomes more toxic than the parent compound during the phase I and II biotransformation reactions (Lech and Bend 1980).

Dioxins can cause toxicity in exposed organisms through prolonged induction of *cyp1a* gene, that encodes mixed function oxygenase (MFO) biotransformation enzymes (Billiard *et al.* 1999). Activation is prolonged because dioxins are persistent compounds having a strong binding affinity for the AhR and being a poor substrate for MFO metabolism (Billiard *et al.* 1999). The activation of AhR has been shown to cause adverse effects, such as hepatotoxicity, immune suppression, reproductive failure, carcinogenicity, and decreased growth on vertebrates (Doering *et al.* 2013). Other exogenous ligands, that have structural similarities with dioxins, such as planar substituted PAH compounds, may bind with the AhR and cause dioxin-like toxicity, as well (Bauder *et al.* 2005). Compounds that are more planar in their chemical structure have the greatest affinity to AhR (Doering *et al.* 2013). Planar 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has the strongest binding affinity to AhR (Teraoka *et al.* 2003).

AhR activation is commonly measured as gene expression of *cyp1a* gene by quantifying the amount of messenger RNA (mRNA) of *cyp1a* gene with real-time (RT-)qPCR technique (Lu *et al.* 2013). RT-qPCR technique requires the reverse transcription of the extracted RNA into complementary DNA (cDNA), the amplification of the cDNA using PCR and the real-time detection and quantification of the PCR-product (Nolan *et al.* 2006). The method is based on the fluorescent signal which is proportional to the amount of DNA produced in each PCR cycle (Nolan *et al.* 2006). Even though the AhR signalling pathway has been studied, e.g., in zebrafish (*Danio rerio*) and *Salmonidae* spp., the pathway is not fully understood (Lu *et al.* 2013).

2.3.1 Blue sac disease

Chronic exposure to dioxin-like compounds can cause BSD symptoms, such as decreased growth, yolk sac edemas, pericardial edemas, hemorrhages, and mortality in early-life stages of fish (Billiard *et al.* 1999). According to Billiard *et al.* (1999), the proposed mechanism for the symptoms of BSD is an oxidative stress mechanism caused by prolonged induction of CYP1A enzymes. Following continuous exposure to AhR agonist, the biotransformation reactions of exogenous AhR ligand can lead to an excess of reactive oxygen radicals and to breakdown of lipid membranes causing edemas and haemorrhaging (Billiard *et al.* 1999). Because the larval stage of fish has finite store of anti-oxidants, e.g., vitamin E in yolk sac, it is likely that fish larvae are especially vulnerable to oxidative stress and dioxin-like toxicity (Hodson *et al.* 2007).

If the monooxygenation gene activation, i.e., induction of *cyp1a* gene, is the link to dioxin-like toxicity and formation of the BSD symptoms, PAH compounds and PAH-like compounds, such as retene and DHAA respectively, might cause deformities in fish via AhR signalling pathway (Billiard *et al.* 1999). In phase I metabolism, PAH compounds are oxygenated and/or hydroxylated by monooxygenation reactions in order to increase the water solubility of the parent compound so that they become easier to excrete (Heintz *et al.* 2000). Since retene is rapidly metabolized and excreted, prolonged AhR associated gene activation would occur only if fish were chronically exposed to retene (Billiard *et al.* 1999). This could happen especially downstream from pulp and paper mill discharges, where retene is accumulated in the sediments (Meriläinen *et al.* 2006). The natural background concentrations of DHAA and retene or the acute concentrations released along with the pulp mill effluents are not high enough to cause acute toxicity on fish, but the continuously released and accumulated concentrations may cause adverse effects on fish (Kostamo and Kukkonen 2003).

2.3 Rainbow trout and brown trout

Rainbow trout (*O. mykiss*) and brown trout (*S. trutta*) are closely related salmonid species and they were selected for this study because of the relevance for boreal environments (FAO 2018). Both species have wide global ecological distribution and they are economically significant species (FAO 2018). *S. trutta* was chosen as the study species because it is naturally found in the Äänekoski area where the new bioproduct extraction plant is being constructed and the amount of pulp mill effluents released into the local watersheds is increasing (Päätös 4/2015/1).

3 MATERIALS AND METHODS

Before the initiation of the 13-day semi-static DHAA exposure of *S. trutta* larvae, a one-week semi-static pre-test was performed with *O. mykiss* larvae to find a suitable sub-lethal DHAA concentration range. The environmentally relevant concentrations chosen for this experiment were selected based on concentrations that have been reported to be sub-lethal (Oikari *et al.* 1983) and environmentally relevant (Gravato and Santos 2002), and also on the hypothesis that the early-life stages of animals might be more sensitive to xenobiotics than adults.

3.1 Pre-test

Twenty-four hours before the initiation of the pre-test, the exposure system was set up and the exposure bowls were pre-saturated with the appropriate DHAA concentrations. The system consisted of nine Pyrex glass bowls filled with one litre of lake water delivered from Lake Konnevesi, Central Finland (62°36'59.2"N 26°20'46.5"E) and DHAA (CAS. No. 1740-19-8, M = 300.444 g/mol, purity > 99%) with an aerating system pumping air under high pressure into each bowl via glass pipets attached to rubber tubing, thus maintaining constant oxygen saturation. The exposure concentrations were 1.6, 3.1, 6.25, 12.5, 25, 50, 100 and 200 µg/l of DHAA,

and dimethyl sulfoxide (DMSO) was used as negative control. No replicates were used in the pre-test. The exposure concentrations were made according to the Table 1. A 10 mg/ml DHAA stock solution had been made beforehand. A 1/16 stock solution was prepared by pipetting 10 µl of 10 mg/ml stock solution and 1500 µl of DMSO into a vial. The dissolution of solid DHAA and the 1/16 dilution were made into DMSO (CAS. No. 67-68-5, M = 78.138 g/mol, purity > 99.9%, Sigma Aldrich).

Table 1. Pre-test exposure concentrations and the pipetting volumes.

Stock solution (mg/ml)	Volume (µl) of stock solution per bowl (1 l)	Final concentration (µg/l) of DHAA per bowl (1 l)
10	20	200
	10	100
	5	50
	2.5	25
0.625 (1/16 of 10 mg/ml)	20	12.5
	10	6.25
	5	3.1
	2.5	1.6

On the first day of the experiment, 15 newly hatched *O. mykiss* larvae, supplied by Hanka-Taimen fish farm (Hankasalmi, Finland), were placed in every pre-saturated exposure bowl. The exposure was performed in a dedicated exposure room, the temperature set to 12 ˚C with a 16:8 light:dark photo periodicity. Exposure waters and chemicals were exchanged on a daily basis. The larvae were collected using a broad plastic pipet and placed in a 50 ml Falcon tube while the exposure water and chemicals were renewed.

The water quality parameters, i.e., the oxygen content, pH, and conductivity of the exposure water were monitored every other day from the lake water without chemicals. The temperature of exposure water was measured on a daily basis from the control bowls before the water exchanges.

3.2 Brown trout exposure

In the 13-day *S.trutta* experiment, five exposure concentrations of DHAA (1, 3.2, 10, 32 and 100 µg/l) as well as a DMSO control and positive control (retene, 100 µg/l) were used with three replicates (Table 2).

Table 2. The DHAA dilutions for 13-day exposure concentrations and the pipetting volumes into the exposure bowls.

DHAA stock (mg/ml)	DHAA stock + DMSO (µl) into vial	DHAA stock solution (mg/ml)	DHAA stock solution into 1 l (µl)	Final concentration in 1 l (µg/l)
10		10	10	100
10	320 + 680	3.2	10	32
10	100 + 900	1	10	10
1	100 + 900	0.32	10	3.2
3.2	100 + 900	0.10	10	1

Twelve *S. trutta* larvae were placed in every exposure bowl. The larvae were weighed so that there were approximately 750 mg of fish per 1 litre of water per day. The same water quality parameters were monitored and measured as in the pre-test.

Simultaneously with the 13-day *S. trutta* experiment, a 24-hour acute DHAA exposure was performed in order to investigate the *cyp1a* gene expression. The experimental setup was the same as in the 13-day sub-chronic DHAA exposure. The DHAA exposure concentrations were the same (1, 3.2, 10, 32, and 100 µg/l), and DMSO and retene controls were used. Three replicates were used from each treatment. Twelve *S. trutta* larvae were placed in every exposure bowl. The same water quality parameters were measured as in the pre-test and 13-day sub-chronic exposure.

3.2 Sampling

3.2.1 24-hour acute exposure

In the end of the acute 24-hour *S. trutta* exposure, one replicate group were sampled at time starting with the control group and proceeding from the lowest DHAA concentration to the strongest. Six larvae at a time were collected using a plastic pipet and transferred onto a net, gently dried with paper towel and then placed in a labelled RNase-free 1.5 ml Eppendorf tube and then quickly snap-frozen in liquid nitrogen. At the end of sampling, frozen samples were collected and randomly placed in storage boxes and stored at -80 ˚C.

3.2.2 13-day sub-chronic exposure

S. trutta larvae from the 13-day experiment were sedated in tricaine solution (MS-222, CAS. No. 886-86-2, 200 mg/l + $NaHCO_3$ 400 mg/l) for approximately 1.5 minutes. Every larva was photographed on a millimetre scale paper in groups of 3–7 larvae. The photographic files were transferred to a computer for later photo analysis. The photos of the larvae were analysed using ImageJ program (1.51j, National Institutes of Health, USA) to identify deformities and to measure the body length and planar yolk-sac area of the larvae.

3.2.3 Water samples

Water samples were taken twice during the 13-day experiment in order to quantify the real DHAA concentrations. Before the water exchange, half a litre of exposure water from each replicate bowl were combined into a 2.5 litre glass bottle. Also, one set of 'fresh' water samples were made straight into the glass bottles in order to determine the possible bioconcentration of chemicals into the larvae and adsorption to the walls of the exposure bowls. Samples were stored in a freezer at -20 ˚C.

For the analysis of retene concentration, 5 ml of exposure water from retene bowls were pipetted into 20 ml vials containing 5 ml of 99.4% ethanol. The retene samples were taken in a different manner because the measurement method of retene concentration was different to the one of DHAA. Retene sample vials were stored in refrigerator until the analysis.

3.3 RNA-extraction

The *cyp1a* gene expression was measured from three randomly selected, 24-hour exposed larvae from each DHAA, retene, and DMSO control bowl.

The RNA-extraction from frozen tissue was performed using RNA isolation TRI Reagent® (Molecular Research Center Inc., USA). Into each sample tube, 1 mL of TRI Reagent® was added, and the tissue was homogenized mechanically with a plastic homogenization pestle and stored on ice. The homogenized samples were transferred to new Eppendorf tubes and incubated at room temperature for 5 minutes. Phase separation was achieved by adding 200 µl of chloroform (CAS. No. 67-66-3, for analysis, M = 119.38 g/mol, EMSURE) to the homogenate and then shaken vigorously for 15 seconds by hand. The samples were incubated at room temperature for 3–10 minutes and then centrifuged (Hitachi Koki Co., Ltd, Himac CT 15 RE, VWR) with 12 000 x g for 15 minutes at 4 ˚C. The supernatant was pipetted into a new Eppendorf tube and 500 µl of isopropanol (CAS. No. 67-63-0, for analysis, M = 60.1 g/mol, EMSURE) was added to precipitate the RNA. The tubes were

vortexed gently and incubated at room temperature for 10 minutes, and centrifuged with 12 000 x g for 8 minutes at 4 ˚C. The supernatant was discarded and 1 ml of 75% ethanol was added to wash the RNA pellet. Tubes were vortexed and stored at -20 ˚C.

3.4 Dissolving the RNA, DNase-treatment and cDNA-synthesis

Extracted RNA was washed with 1 ml of 75% ethanol and the washing solution was removed by pipetting and completely evaporated for 30–60 minutes. When the precipitate started to turn transparent, the pellet was suspended in 100 µl of nuclease-free water (Thermo Scientific). The dissolved samples were placed on ice, heated at 60 ˚C in a heat block (Grant) for 3 minutes, and placed on ice.

The RNA purity, i.e., the absence of protein and DNA contamination, needed to be checked (Nolan *et al.* 2006). The RNA-concentration (ng/µl) and quality (A260:280 and A260:A230 ratios) were checked with NanoDrop Spectrophotometer ND100. Based on the concentration measurement, the samples were diluted with ratios 1+3 or 1+5 with nuclease-free water to obtain an RNA concentration within the range of 25–500 ng/µl, which is required concentration range for RNA integrity analysis. The diluted RNA samples were stored at -80 ˚C.

RNA integrity number (RIN) was confirmed (RIN between 7–10 being acceptable) using an Agilent 2100 Bioanalyser (Agilent Technologies, USA) following the standard protocol (Agilent Technologies© 2009, 2013) (Appendix 1).

The DNase treatment for the diluted RNA samples was performed using DNase Treatment Kit (Thermo Scientific). The reaction mixture for the removal of remaining genomic DNA was made according to the principles presented in Table 3. The total reaction mixture volume was set to 10 µl. The calculations for RNA and water pipetting volumes were calculated using equations (1) and (2).

Table 3. Pipetting volumes of the DNase treatment reaction mixture made into PCR-tubes for the removal of genomic DNA from the RNA samples.

Component	Volume (µl)	Final volume
Diluted RNA	Variable	1 µg
10x DNase I Reaction Buffer (with $MgCl_2$)	1	1x
DNase I enzyme	1	1 U
RNase free water	Variable	to 10 µl

$$V_{RNA} = \frac{1000}{c_{RNA}} \ , \tag{1}$$

where V_{RNA} is the volume of the diluted RNA sample and c_{RNA} is the measured concentration of the RNA.

$$\text{Example: } \frac{1000}{167.41 \text{ ng/µl}} = 5.97 \text{ µl}$$

$$V_{H_2O} = 8 \text{ µl} - V_{RNA} \text{ µl} \tag{2}$$

$$\text{Example: } 8 \text{ µl} - 5.97 \text{ µl} = 2.03 \text{ µl}$$

The tubes were spinned for 2 seconds and incubated in a PCR-machine (C1000 Thermal Cycler, Bio-Rad) for 30 minutes at 37 ˚C. After the incubation, 1 µl of 50 mM EDTA solution (DNase Treatment Kit, Thermo Scientific) was pipetted into the tubes. The mixture was heated at 65 ˚C for 10 minutes in order to end the DNase treatment reaction.

A supermix for cDNA-synthesis was made according to Table 4 using cDNA-synthesis Kit and protocol (Bio-Rad). The supermix was prepared into an Eppendorf tube so that there was at least one reaction volume excess for the spillage that pipetting small volumes usually causes.

Table 4. Preparing the supermix for cDNA-synthesis. Volumes are presented for one PCR reaction.

Component	Volume (µl)
5x iScript Reaction Mix	4
nuclease free H_2O	4
iScript Reverse Transcriptase enzyme	1

Following the DNase treatment, 9 µl of prepared supermix (Table 4) was pipetted into every PCR tube, the total reaction mixture volume being now 20 µl. The cDNA-synthesis was performed in PCR-machine using the protocol described in Table 5.

Table 5. The thermal cycling protocol for the cDNA-synthesis.

Phase	Temperature (˚C)	Time (min)
Priming	25	5
Reverse transcription	46	20
Reverse transcription inactivation	95	1

The cDNA samples were diluted with water with a ratio 1:10 for the qPCR reactions so that the final cDNA concentration was within a concentration range of 2.5–50 ng/µl. The samples were stored in a freezer at -20 ˚C.

3.5 qPCR

The qPCR measurements were performed using a fluorescent iQ™ SYBR® Green Supermix and protocol (Bio-Rad). Dry reverse and forward primers of eight genes: *cyp1a*, *ef1a*, *rl2*, *rl17*, *beta actin*, *gadph*, *ndufa* and *if5a1* (TAG Copenhagen A/S) were dissolved and diluted in sterile water to achieve a final concentration of 10 µM. A supermix was prepared for every gene into microcentrifuge tubes according to Table 6.

Table 6. Preparing the supermix for each gene. Volumes are presented for two qPCR reactions.

Component	Volume (µl)	Final concentration
iQ™ SYBR® Green Supermix	25	1x
Forward and reverse primers (10 µM)	1.5 each	300 nM each
sterile H_2O	12	-

Primer efficiencies were tested in a dilution series of a mixture consisting of four different samples (control, retene 100 µg/l, DHAA 3.2 µg/l and DHAA 100 µg/l) (Appendix 2). A sample stock solution was prepared by pipetting 30 µl of every sample in a microcentrifuge tube. The dilution series from the sample mixture was made with a 1:5 ratio in sterile water to generate the five concentrations. Five µl of mixture sample and 20 µl of prepared supermix (Table 6) were pipetted into the wells of a 96-well PCR-plate (Multiplate PCR plates, Low 96 well Clear, Bio-Rad) with two technical replicates for each gene, the final reaction volume being 25 µl. One negative control (No Template Control, NTC) of every gene included 5 µl of sterile water and 20 µl gene supermix. The plate was sealed with PCR sealer (PCR Sealers Microseal 'B' Film, Bio-Rad) and centrifuged for 7 seconds (Eppendorf Centrifuge 5430) before analyzing in a qPCR-machine (C1000 Touch Thermal Cycler, CFX96 Real-Time System, Bio-Rad). The qPCR protocol used for *cyp1a* gene amplification and quantification is presented in Table 7.

Table 7. The qPCR protocol for measurement of *cyp1a* gene expression.

	Temperature (˚C)	Time	
1. Denaturation	95	3 min	
2. Denaturation*	95	10 s	
3. Annealing*	58	10 s	
4. Extension*	72	30 s	plate read
5. Melting curve	65-95	5 s	plate read

*Amplification steps 2-4 repeated with 39 cycles.

The qPCR method requires reference genes to against which the level of *cyp1a* gene expression is determined (Kozera and Rapacz 2013). Reference genes commonly are housekeeping genes that have constant expression level in measured tissue and of which transcription should not be affected by experimental factors (Kozera and Rapacz 2013). Based on couple of qPCR run results, *ef1a* and *rl2* were selected as reference genes for *cyp1a* because the expression of those genes was the most consistent in every sample analyzed (Appendix 3).

All the cDNA samples were measured with the same protocol (Table 7) with the same reaction volume of 25 µl and three technical replicates of each sample. One randomly selected sample (DHAA 1 µg/l) was included on every plate in order to calibrate the data-analysis of the *cyp1a* gene activation. The program used for the gene expression data-analysis was Bio-Rad CFX Manager 3.1. An example of a *cyp1a* amplification curves is presented in Figure 3.

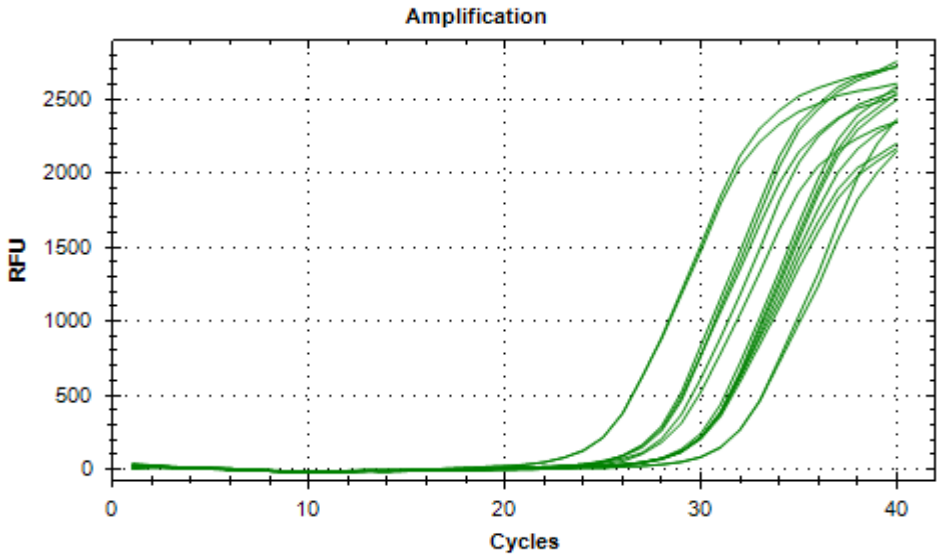

Figure 3. Amplification cycles for *cyp1a* gene in some cDNA samples of 1-day DHAA exposed larvae.

3.6 Water sample analyses

The water samples were extracted with solid phase extraction (SPE) method and the real DHAA concentration was analysed with gas chromatography combined with flame ionization detector (GC-FID). The sample volume used was 300 ml and the pH of the samples were adjusted to 4 with 1:9 acetic acid:water solution (anhydrous 100% acetic acid, CAS No. 64-19-7, EMSURE). The DHAA was bound into the membrane of Bond Elut C-18 LO sorbent, eluted with 6 ml of methanol (CAS No. 67-56-1, EMSURE), and collected into kimax tubes. Heneicosanoic acid (25 mg/50 ml) and betulin (25 mg/ml) standard solutions were added, 30 µl of each, into the kimax tubes. The methanol was completely evaporated at 55 ˚C in water bath with nitrogen flow. Onto dried samples, 200 µl of pyridine and 200 µl of silylation derivatization reagent BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) + 1% TMCS (trimethylchlorosilane) (CAS No. BSTFA 255561-30-2, CAS No. TMCS 75-77-4, Regis Technologies, Inc. USA) were added. The samples were incubated for an hour at 70 ˚C and transferred then into glass vials for the concentration measurement in GC-FID (6850 Series GC System, Agilent

Technologies). The injector temperature was 290 ˚C and detector temperature 300 ˚C. The starting oven temperature was 100 ˚C for 1.5 min and the temperature was raised 6 ˚C/min to 180 ˚C and after that 4 ˚C/min until the final temperature, 290 ˚C was reached. Holding time at 290 ˚C was 20 min. The total runtime was 62.33 min/sample. The column (Agilent HP-5 19091J-413E) dimensions were 30 m x 0.32 mm i.d. x 0.25 µm. The DHAA concentrations were calculated with equations (3) and (4).

$$c_{DHAA(vial)} = \frac{m_{std} * \text{peak area}_{DHAA}}{\text{peak area}_{std} * V_{sample}} \quad (3)$$

$$c_{DHAA(exp)} = \frac{c_{DHAA(vial)} * V_{sample}}{V_{SPE}}, \quad (4)$$

where $c_{DHAA(vial)}$ is DHAA concentration in vial (mg/l), m_{std} is the weight of standard (µg), peak area_{DHAA} is the integrated peak area of the DHAA peak in FID chromatogram, peak area_{std} is the integrated peak area of the standard compound peak, V_{sample} is the sample volume in vial (ml), $c_{DHAA(exp)}$ is the DHAA concentration in exposure bowl (g/l), and V_{SPE} is the sample volume extracted with SPE.

$$\text{Example: } c_{DHAA3.2(vial)} = \frac{15\ \mu g * 36.893}{588.199 * 0.4\ ml} = 0.376\ mg/l$$

$$c_{DHAA3.2(exp)} = \frac{0.376\ \frac{mg}{l} * 0.0004\ l}{302.49\ ml} \approx 0.5\ \mu g/l.$$

The DHAA peak from the FID chromatograms were identified with the measurement of gas chromatography combined with mass spectrometer (GC-MS) with one DHAA sample (Appendix 4).

Synchronous fluorescence spectroscopy was used to determine the retene concentrations from the positive control bowls. The technique is commonly used with determination of polynuclear aromatic hydrocarbons (Rubio *et al.* 1986). Retene samples were scanned using a spectrophotometer (LS55 Luminescence Spectrometer, Perkin Elmer) analysing wavelengths between 290–350 nm, the

Δλ being 50 nm (exitation slit 5 nm and emission slit 5 nm). Scanning speed was 150 nm. Retene peak appeared at 300 nm. Retene concentrations in samples and exposure bowls were calculated using an equation of obtained calibration curve (Figure 4) and equations (5) and (6).

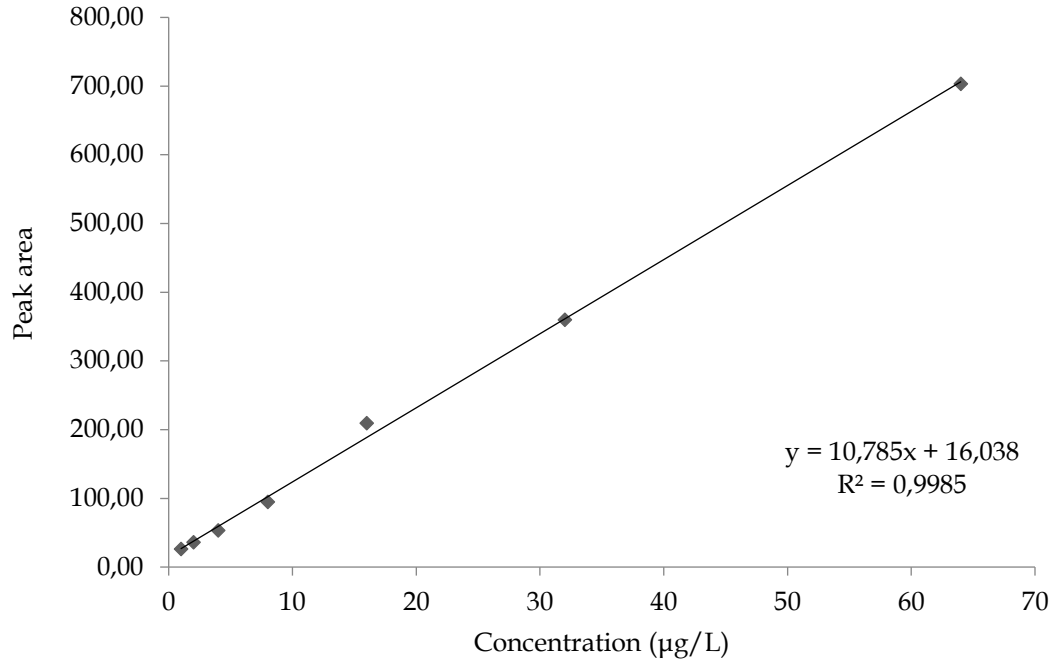

Figure 4. Retene standard curve obtained from synchronous fluorescence spectroscopy measurement of standard solutions.

$$c_{sample} = \frac{peak_{sum} - 16.038}{10.785} \quad (5)$$

$$c_{bowl} = c_{sample} * 2 \quad (6)$$

In equation (5), $peak_{sum}$ is the sum of the average peak values from three retene replicate samples from the wavelengths 290–315 nm. The average peak values from DMSO control are subtracted from the retene peak values.

Example: $\frac{266.95 - 16.038}{10.785} = 23.27$ µg/l

Example: 23.27 µg/l $* 2 = 46.53$ µg/l.

3.7 Statistics

IBM® SPSS® Statistics Version 24.0.0.1 (64 bit edition) was used to statistically analyse the obtained data. Firstly, the data of the body length and planar yolk sac area were checked if the three replicates of DHAA and control treatments could be combined as one treatment. The analyse was performed with Shapiro Wilk normality test and ANOVA (significance level $p < 0.05$). Combinations could be done if there were no statistically significant differences found between replicate bowls (1–3) with ANOVA. In most treatments, ANOVA did not pass ($p > 0.05$), and therefore the replicate data was not pooled in all cases. The combination was done, however, always when it was possible according to the ANOVA. To identify statistically significant differences in body length and planar yolk sac area between DHAA treatments and controls, a non-parametric Kruskal-Wallis test with Dunn's post-hoc pairwise comparisons using Bonferroni correction was performed.

Fisher's exact test was used to identify significant differences (significance $p < 0.05$) in mortality and deformity rates (%) comparing the DHAA treatments against the negative DMSO control.

Cyp1a gene expression data was Log10-transformed in order to get more normally distributed dataset. There was an outlier in retene treatment having a *cyp1a* expression of 6.9 whereas other expressions were between 0.6–1.7. The outlier was omitted from the analysis in order to make the standard deviation smaller. The statistic testing of the *cyp1a* expression was performed with Kruskal-Wallis test combined with Dunn's post-hoc pairwise comparisons using Bonferroni correction.

4 RESULTS

Most of the observed BSD symptoms and developmental defects were caused by the positive control, retene. DHAA caused deformities too, but the responses were, however, slighter than in the retene treatment. Retene was also the only treatment group that led to increased AhR activation after the 1-day acute exposure.

4.1 Mortality and deformities

In the pre-test, a slight increase (6.7%) in mortality was detected with *O. mykiss* in DHAA treatments 1.6, 25, 50 and 200 µg/l, and the larvae had deformities even in the lowest DHAA concentrations (Appendix 5 and 6). Increased, but non-significant mortality was found in *S. trutta* experiment at DHAA treatments 1, 10 and 100 µg/l when the mortality was compared to DMSO control (Figure 5). Retene treatment caused the highest mortality compared to control (Figure 5). The majority of the mortalities (71% of all of the deaths) were observed during the first week (days 1–7) of the exposure period, whereas in the last days (days 10–13) of the exposure period the mortality was lower (14% of all of the deaths) (Appendix 7).

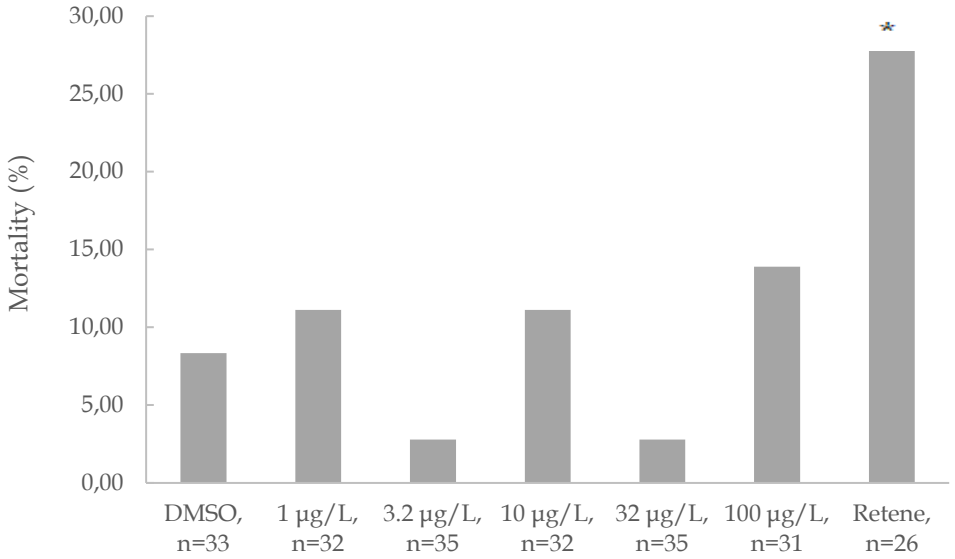

Figure 5. Mortality (%) of *S. trutta* larvae in 13-day DHAA and retene exposure. No significant percentile differences were found in mortality when DHAA exposed groups were compared to DMSO control. Mortality in retene group had a statistically significant ($p < 0.001$) difference with DMSO control (Fisher's exact test). Statistically significant difference from DMSO control is marked with *.

Following the 13-day DHAA exposure, *S. trutta* larvae were observed to have statistically significantly more yolk sac edemas in DHAA concentrations 10 and 100 µg/l than the untreated control larvae (Figure 6). However, observed yolk sac edemas in DHAA treated larvae were very small (Figure 7) and they were detected also in the lowest DHAA concentrations, and in 21% of the DMSO control larvae, as well (Figure 6).

Pericardial edemas were detected in all treatment groups, except in DMSO control and DHAA 32 µg/l (Figure 6). Retene treatment caused the highest occurrence of yolk sac edemas and pericardial edemas, and the larvae in the highest DHAA concentration (100 µg/l) had almost the same rate of edemas as the larvae in the retene treatment (Figure 6). Hemorrhages were not detected.

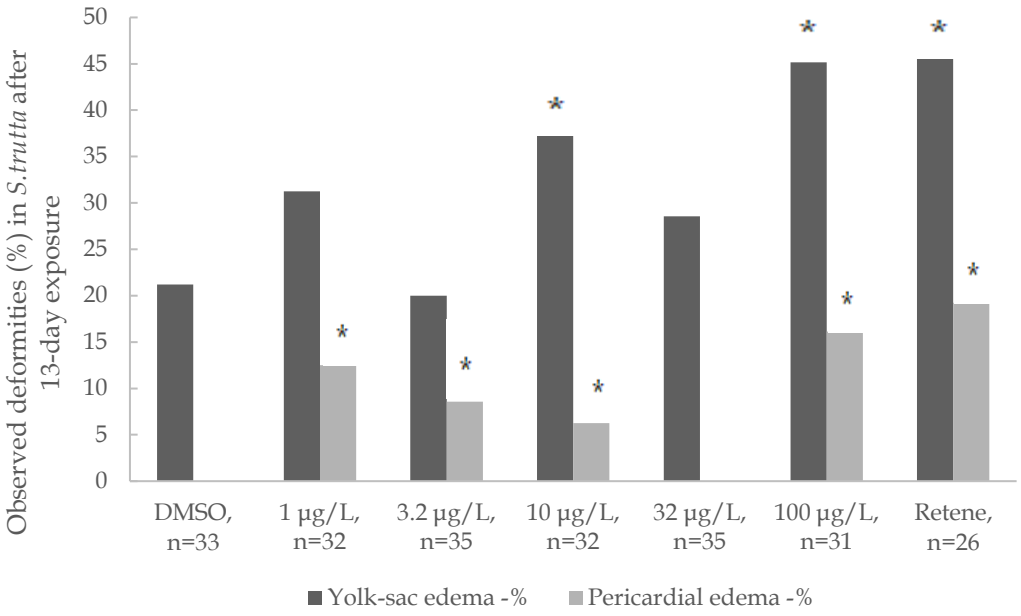

Figure 6. Observed occurrence of deformities (yolk sac edemas and pericardial edemas) in *S. trutta* larvae after the 13-day DHAA and retene exposure. Statistically significant percentile differences were found in increased yolk sac edema rates when DHAA and retene exposed groups were compared to DMSO control: DHAA 10 µg/l ($p = 0.013$), DHAA 100 µg/l ($p < 0.001$), and retene ($p < 0.001$) (Fisher's exact test). Statistically significant percentile differences were found in increased pericardial edema rates when DHAA and retene exposed groups were compared to control: DHAA 1 µg/l ($p < 0.001$), DHAA 3.2 µg/l ($p = 0.003$), DHAA 10 µg/l ($p = 0.029$), 100 µg/l ($p < 0.001$), and retene ($p < 0.001$) (Fisher's exact test). Statistically significant difference from control is marked with *.

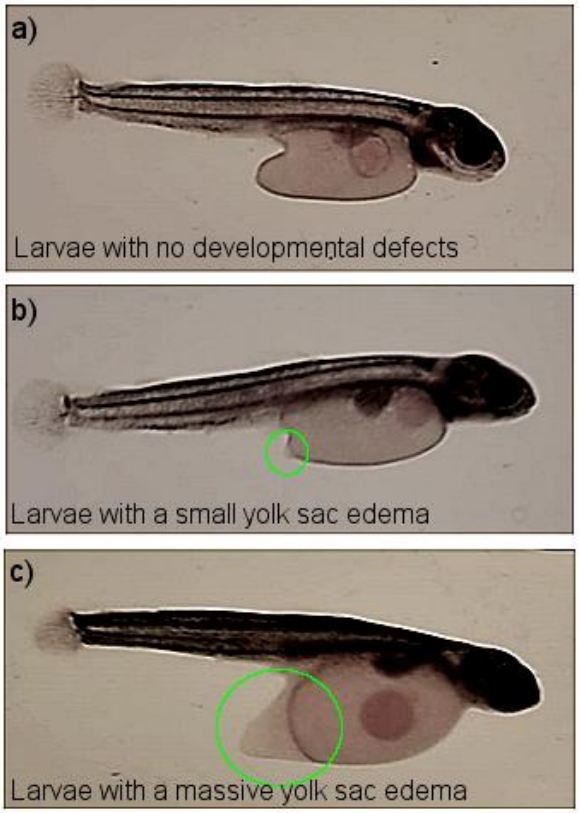

Figure 7. A *S. trutta* larva without deformities from DMSO control group (a), a larva with minor yolk sac edema from DHAA 100 µg/l treatment group (b), and a larva with larger yolk sac edema from retene treatment group (c). All photos are taken after 13-day exposure of DHAA and retene.

4.2 Body length and planar yolk sac area

The results from the analysis of body length are presented in several graphs, first by comparing the DHAA treatments to the negative DMSO control (Figures 8a–b) and then to positive retene control (Figures 9a–c), in order to make results easier to represent and interpret.

Statistically significant difference in body length was found in DHAA treatments 1, 10 and 32 µg/l (replicate bowls 1) compared to the DMSO control (replicate bowl 1), the DHAA treated larvae being shorter than control larvae (Figures 8a–b). However, other control replicate bowls (2–3) had statistically the same mean body length with all DHAA treatments and replicates (Figures 8a–b).

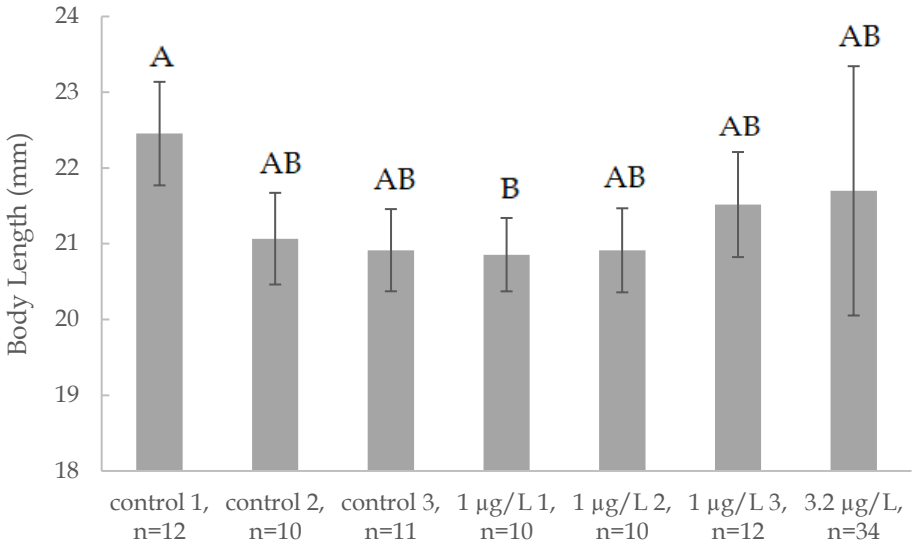

Figure 8a. Body length of the *S. trutta* larvae after 13-day exposure to DMSO control and DHAA concentrations 1 and 3.2 µg/l. Statistically significant differences (Kruskal-Wallis, test statistic = 81.823, df = 16, p < 0.000) were found between control (replicate bowl 1) and DHAA 1 µg/l (replicate bowl 1) (p = 0.020) (Dunn's post-hoc -test). The treatment groups at the same statistical level are marked with the same letter (A/B/AB).

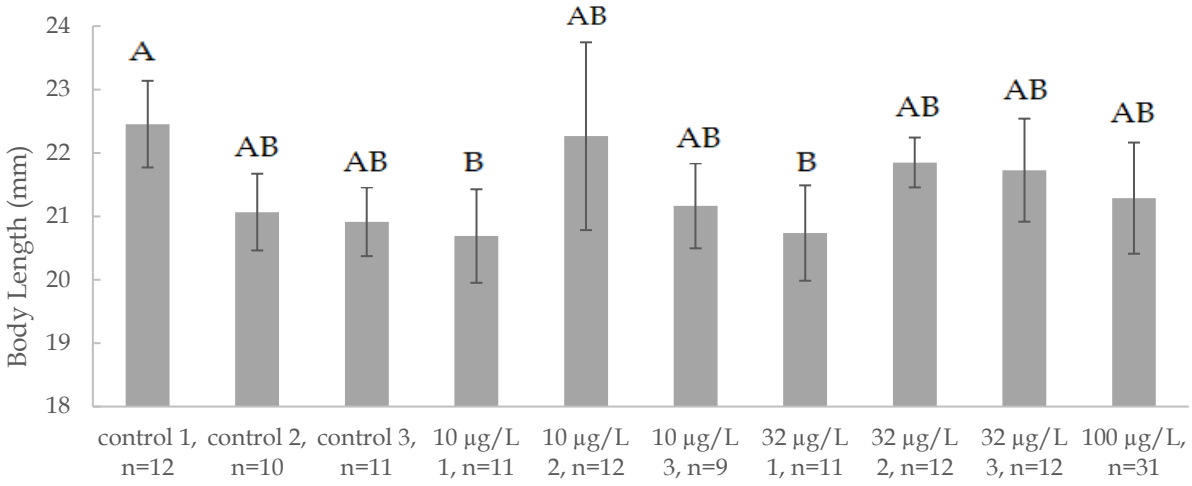

Figure 8b. Body length of the *S. trutta* larvae after 13-day exposure to DMSO control and DHAA concentrations 10, 32 and 100 µg/l. Statistically significant differences (Kruskal-Wallis, test statistic = 81.823, df = 16, p < 0.000) were found between treatments: control (replicate bowl 1) and DHAA 10 µg/l (replicate bowl 1) (p = 0.003), and control (replicate bowl 1) and DHAA 32 µg/l (replicate bowl 1) (p = 0.002) (Dunn's post-hoc -test). The treatment groups at the same statistical level are marked with the same letter (A/B/AB).

Retene treatment had a statistically significant effect on the body length of the larvae as the larvae in the retene treatment (replicate bowls 1–3) were shorter in body length when compared to the DMSO control or the DHAA treatment groups (Figures 9a–c).

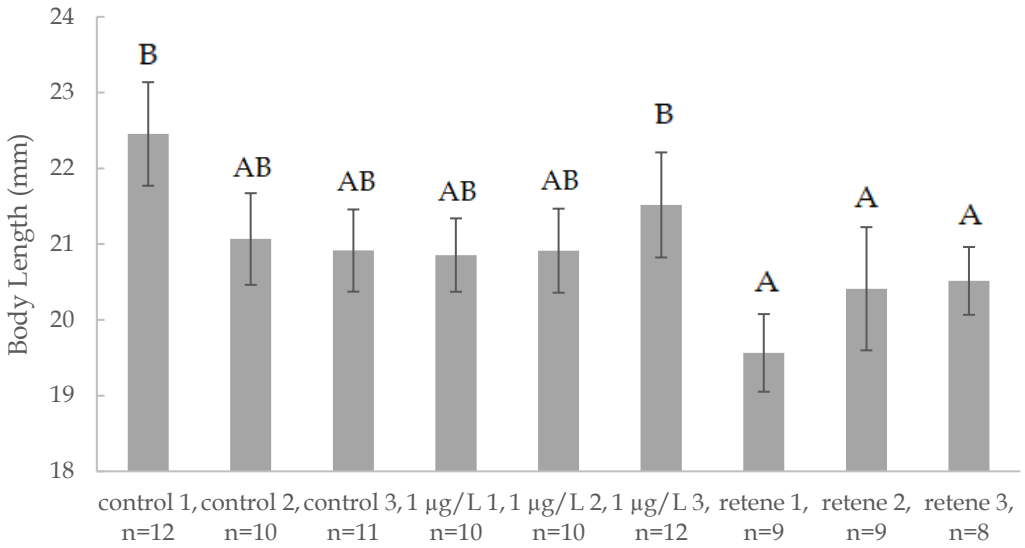

Figure 9a. Body length of the *S. trutta* larvae after 13-day exposure to retene, DMSO control and DHAA treatment 1 µg/l. Statistically significant differences (Kruskal-Wallis, test statistic = 81.823, df = 16, $p < 0.000$) were found between treatments: retene (replicate bowl 1) and control (replicate bowl 1) ($p < 0.000$), retene (replicate bowl 2) and control (replicate bowl 1) ($p = 0.001$), retene (replicate bowl 3) and control (replicate bowl 1) ($p = 0.001$), and retene (replicate bowl 1) and DHAA 1 µg/l (replicate bowl 3) ($p = 0.003$) (Dunn's post-hoc -test). The treatment groups at the same statistical level are marked with the same letter (A/B/AB).

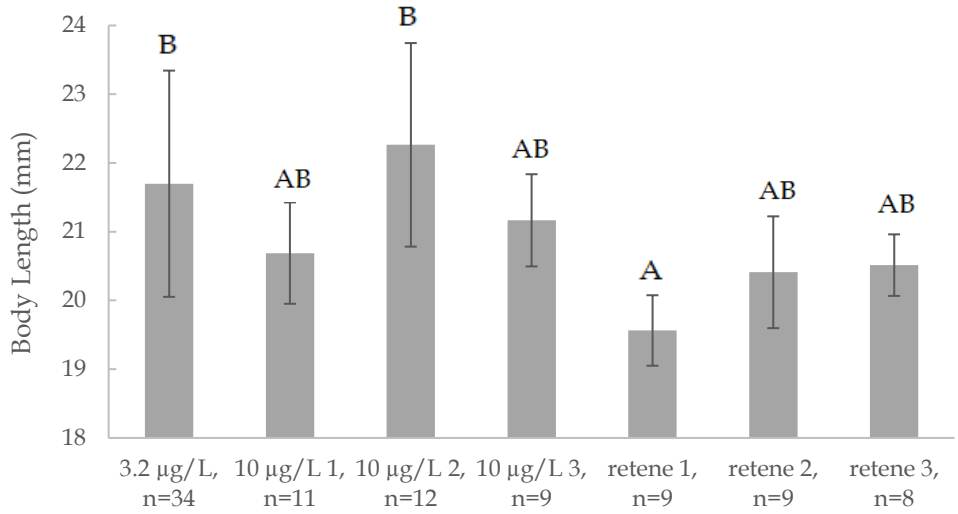

Figure 9b. Body length of the *S. trutta* larvae after 13-day exposure to retene and DHAA treatments 3.2 and 10 µg/l. Statistically significant differences (Kruskal-Wallis, test statistic = 81.823, df = 16, $p < 0.000$) were found between treatments: retene (replicate bowl 1) and DHAA 3.2 µg/l ($p = 0.001$), and retene (replicate bowl 1) and DHAA 10 µg/l (replicate bowl 2) ($p < 0.000$) (Dunn's post-hoc -test). The treatment groups at the same statistical level are marked with the same letter (A/B/AB).

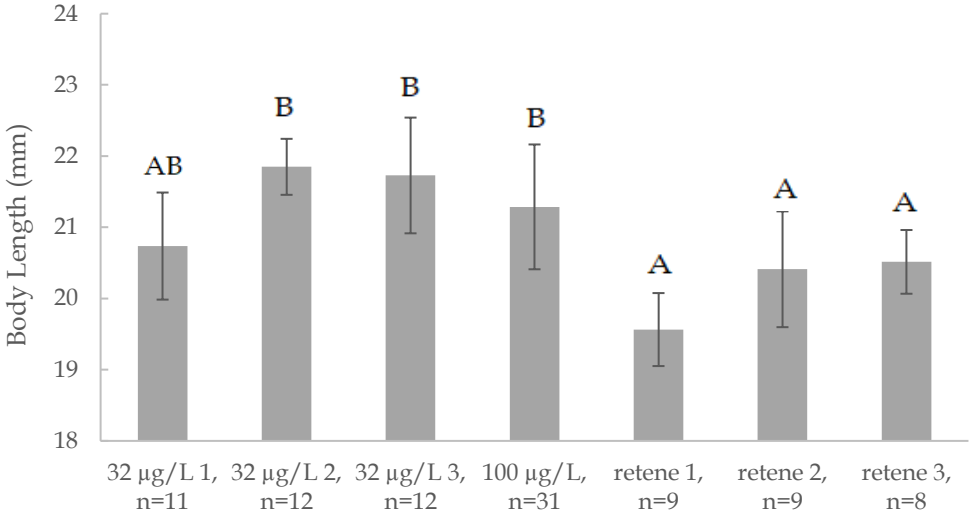

Figure 9c. Body length of the *S. trutta* larvae after 13-day exposure to retene and DHAA treatments 32 and 100 µg/l. Statistically significant differences (Kruskal-Wallis, test statistic = 81.823, df = 16, $p < 0.000$) were found between treatments: retene (replicate bowl 1) and DHAA 32 µg/l (replicate bowl 2) ($p < 0.000$), retene (replicate bowl 1) and DHAA 32 µg/l (replicate bowl 3) ($p < 0.000$), retene (replicate bowl 2) and DHAA 32 µg/l (replicate bowl 2) ($p = 0.024$), retene (replicate bowl 3) and DHAA 32 µg/l (replicate bowl 2) ($p = 0.032$), and retene (replicate bowl 1) and DHAA 100 µg/l ($p = 0.005$) (Dunn's post-hoc -test). The treatment groups at the same statistical level are marked with the same letter (A/B/AB).

As the Figure 10 shows, there was no clear DHAA dose dependent effect on planar yolk sac area when compared to DMSO control. However, DHAA treatment group 3.2 µg/l (replicate bowls 2–3) differed statistically significantly from DHAA treatments 1 and 100 µg/l, the larvae having larger yolk sacs in DHAA 3.2 µg/l treatment group (Figure 10).

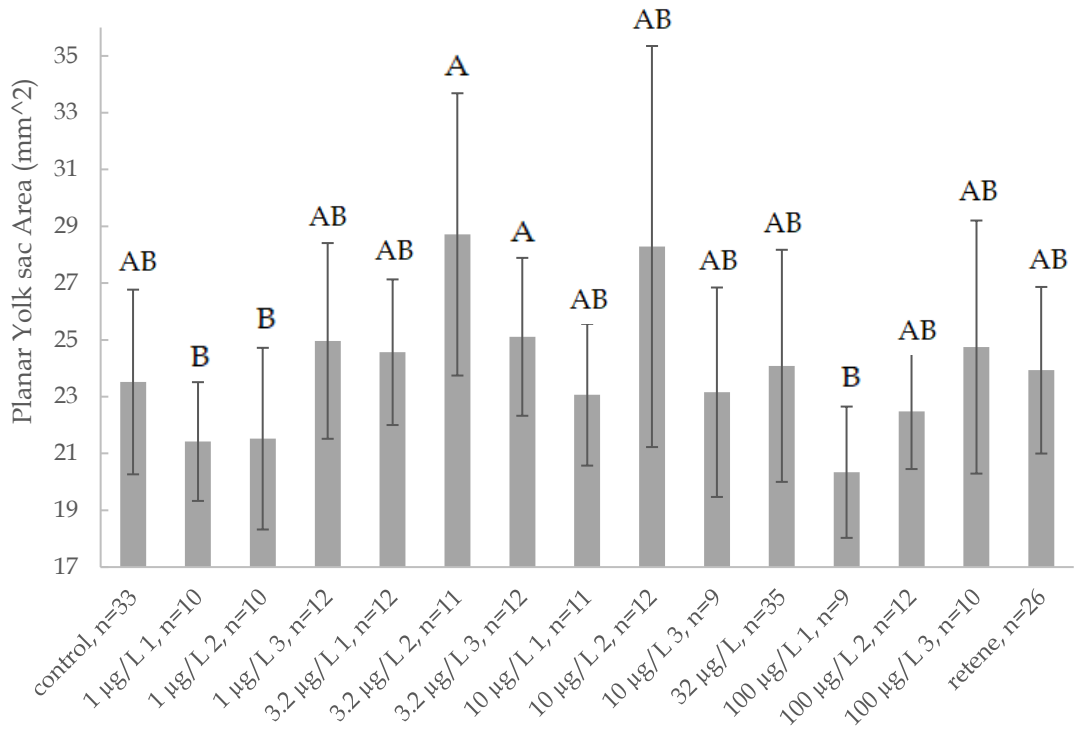

Figure 10. Planar yolk sac area of the *S. trutta* larvae after 13-day DHAA and retene exposure. Statistically significant differences (Kruskal-Wallis, test statistic = 48.257, df = 14, $p < 0.000$) were found between treatments: DHAA 1 µg/l (replicate bowl 1) and DHAA 3.2 µg/l (replicate bowl 2) ($p = 0.008$), DHAA 1 µg/l (replicate bowl 2) and DHAA 3.2 µg/l (replicate bowl 2) ($p = 0.006$), DHAA 3.2 µg/l (replicate bowl 3) and DHAA 100 µg/l (replicate bowl 1) ($p = 0.030$), and DHAA 3.2 µg/l (replicate bowl 2) and DHAA 100 µg/l (replicate bowl 1) ($p = 0.001$) (Dunn's post-hoc -test). The treatment groups at the same statistical level are marked with the same letter (A/B/AB).

4.3 Gene expression

The qPCR results did not reveal any AhR activation in DHAA treatment groups following the 1-day DHAA exposure (Figure 11). Only the DHAA treatment groups 32 and 100 µg/l had a slightly elevated, non-significant, mean *cyp1a* expression compared to DMSO control (Figure 11). Retene treatment had statistically significant difference in AhR activation with all the DHAA treatments but not, however, with the DMSO control (Figure 11).

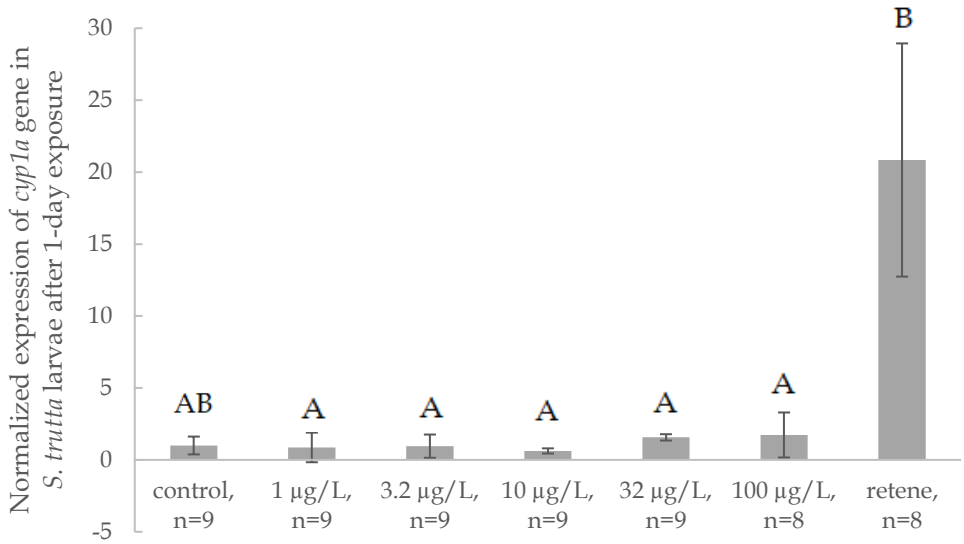

Figure 11. The expression of *cyp1a* gene in *S. trutta* larvae following 1-day acute exposure to DHAA and retene. The data is expressed as non-log transformed and normalized against control so that the control is set to 1. Statistically significant differences (Kruskal-Wallis, test statistic = 22.572, df = 6, p = 0.001) were found between treatments: retene and DHAA 1 µg/l (p = 0.001), retene and DHAA 3.2 µg/l (p = 0.007), retene and DHAA 10 µg/l (p = 0.004), retene and DHAA 32 µg/l (p = 0.003), and retene and DHAA 100 µg/l (p = 0.039) (Dunn's post-hoc test). The treatment groups at the same statistical level are marked with the same letter (A/B/AB).

4.4 Water analysis results

The measured concentrations of DHAA in the exposure bowls were considerably lower than the nominal DHAA treatment concentrations (Table 8). In the lower nominal DHAA concentrations (1–10 µg/l) the measured DHAA concentration was

relatively much closer to the nominal concentration than in the higher DHAA concentrations (32 and 100 µg/l) (Table 8). DHAA concentrations measured from the 'fresh' water samples without larvae were slightly higher than the water samples from bowls that had larvae in them (Table 8).

Table 8. DHAA concentrations in water samples analysed using gas chromatography combined with flame ionization detector (GC-FID).

Nominal DHAA concentration (µg/l)	DHAA concentration with fish (µg/l)	DHAA concentration without fish (µg/l)
1	0.0	0.2
3.2	0.5	2.9
10	1.1	5.6
32	3.7	8.7
100	13.8	14.9

Measured retene concentration of the sample without fish was very close to the nominal retene concentration, whereas the retene concentration of the exposure bowl with fish was about a half of the nominal concentration (Table 9).

Table 9. Retene concentrations in water samples with fish and without fish analysed with spectrophotometer.

Nominal retene concentration (µg/l)	Retene concentration with fish (µg/l)	Retene concentration without fish (µg/l)
100	46.5	94.9

5 DISCUSSIONS

The experiment showed increased level of deformities (Figure 6) and statistically significant differences in body length (Figures 8a–b) of the DHAA exposed larvae when compared to control, following the sub-chronic DHAA exposure. These results show the potential of DHAA to cause developmental defects in *S. trutta* larvae at environmentally relevant and very low concentrations.

5.1 Mortality and deformities

As there was observed more mortality in the *S. trutta* experiment (Appendix 7) than in the *O. mykiss* pre-test (Appendix 5), it could be hypothesized that *S. trutta* is more sensitive species than *O. mykiss* for organic PAH-like chemicals and stressors. However, this may be due to the shorter exposure period in pre-test compared to 13-day *S. trutta* experiment. Laboratory tests are likely to induce the stress reactions in test animals and lead to increased mortality (Costa *et al.* 2011). Lower mortality in the last days of the *S. trutta* experiment (Appendix 7) may be due to the fish larvae being acclimated to the test conditions and the stress.

Significantly increased occurrence of BSD symptoms, such as yolk sac edemas and pericardial edemas, were found among both test species following the DHAA exposure, when compared to DMSO control treatment (Figure 6 and Appendix 6). As the observed edemas in DHAA treated larvae were very small (Figure 7), and the edemas were observed in DMSO larvae, too, prolonging the exposure period might reveal if the deformities are harmful for the development of fish larvae in long run. This could be tested in field, as downstream from pulp and paper mills the exposure to DHAA is continuous (Smolders *et al.* 2004). It has been found out with PAH compounds that smaller doses are enough to cause adverse effects when exposure time is extended (Heintz *et al.* 2000). There should not be any doubt that the same is true with DHAA, too.

Billiard *et al*. (1999) have exposed *O. mykiss* larvae to retene at concentration range of 32–320 µg/l for 42 days and found out that BSD symptoms in fish occurred at nominal retene concentration as low as 32 µg/l. The symptoms included increased *cyp1a* expression, yolk sac edemas, hemorrhaging and reduced growth (Billiard *et al.* 1999). These results (Billiard *et al.* 1999) are in accordance with this study where retene as a positive control caused BSD symptoms, decreased growth and increased (non-significant) AhR activation in a nominal concentration of 100 µg/l.

DHAA does not seem to cause AhR activation as there was no induction detected in *cyp1a* expression levels (Figure 11). The mechanism of the formation of yolk sac and pericardial edemas may be thus caused by some other mechanism than AhR associated toxicity. This could be due to, e.g., the effects on heart or kidneys as Incardona *et al*. (2004) have suggested in their study with early-life stages of *Danio rerio* exposed to different PAH compounds. Tricyclic PAH compounds have previously shown to cause cardiac dysfunction (Incardona *et al.* 2004), and as DHAA has tricyclic base structure (Figure 1), there might be heart-related mechanism behind the formation of edemas. The findings of Incardona *et al*. (2004) suggest that the effects of PAH compounds on kidney might lead to sub-lethal effects, e.g., deformities and decreased growth, and thus affect the survival of developing fish larvae (Incardona *et al.* 2004). Different PAH subclasses have found to act through distinct toxic mechanisms (Incardona *et al.* 2004), and as DHAA is a precursor of PAH compounds, it might act similarly as structurally related alkyl-substituted PAH compounds. However, as the results of this study revealed, the mechanism of DHAA and retene might not be the same.

5.2. Body length and planar yolk sac area

DHAA has been previously shown to affect the energy metabolism of fish and thus DHAA might interfere with the growth of fish larvae (Rissanen *et al.* 2003, Pandelides *et al.* 2014). DHAA treated larvae used approximately as much yolk energy to growth and energy metabolism as the untreated control larvae, as there

were no statistically significant differences in the planar yolk sac area when DHAA treatments were compared to DMSO control (Figure 10). Dioxin-like toxicity can result in larger yolk sacs due to decreased ability to absorb the yolk from yolk sac which, in turn, can lead to decreased growth and developmental delay (Vehniäinen *et al.* 2015). This kind of effect was not, however, detected in this study (Figure 10).

Retene treated larvae were statistically significantly shorter than the larvae in DHAA treatment groups or in the control treatment, but the consumption of yolk was at the same level with the DMSO control (Figures 9a–c and 10). This would indicate that the larvae in retene treatment did not use energy from the yolk sac to grow, but possibly to metabolize retene. However, the body length results are not completely comparable with yolk sac area because the replicate treatments were not pooled in the same way in the dataset of body length and yolk sac area, due to not normally distributed replicate treatments. The statistically significant differences in body length of the DHAA 1, 10 and 32 µg/l treated larvae compared to DMSO control (Figures 8a–b) indicate that DHAA could have an effect on the growth of *S. trutta* larvae even at very low concentrations.

5.3. Gene expression

The qPCR measurements did not reveal any significant *cyp1a* gene expression induction in DHAA treatments (Figure 11), which is in accordance with the study of Oikari and Lindström-Seppä (1990), where DHAA did not activate AhR in trout measured as EROD activity in liver. Billiard *et al.* (1999) have reported that following chronic 32-d exposure of juvenile trout to retene at concentration of 100 µg/l, retene caused continuous increase of *cyp1a* gene expression. In this present study, the exposure period of retene was only one day. Prolonged exposure time should have led to smaller standard deviation, and elevated the *cyp1a* expression of retene treatment above the significance level ($p < 0.005$). There could have been statistically significant difference in *cyp1a* expression levels between retene

treatment and DMSO control if parametric ANOVA test could have been able to use instead of non-parametric Kruskal-Wallis test.

Gravato and Santos (2002) showed in their study with sea bass (*D. labrax*), that high DHAA concentrations (from 270 µg/l up to 811 µg/l) did not activate AhR measured as liver EROD activity, whereas the lowest concentration (7.5 µg/l) with short acute 2-hour exposure time, caused a significant increase of AhR activation in *D. labrax*. They concluded that high DHAA concentrations might decrease the AhR activation and liver P450 content, and low concentrations might increase it (Gravato and Santos 2002). In this study, this kind of effect was not detected as the *cyp1a* expression was slightly, but non-significantly, elevated in the highest DHAA treatments (32 and 100 µg/l) compared to negative control, and not increased at all in the lowest DHAA concentrations (Figure 11).

In previous studies with retene, a BSD and AhR activation related correlation has been detected (Bauder *et al.* 2005). Even though DHAA did not activate AhR in this study, in long run and in field, DHAA will degrade into retene in aquatic sediments and may cause BSD symptoms through the AhR signalling pathway. Mixture effects are also possible, since there are usually several chemicals bioavailable at same time, some of which may act as inducers and others as inhibitors of the same signalling pathway (Scott and Hodson 2008).

5.4 DHAA concentrations

Low measured DHAA concentrations compared to nominal concentrations may be due to SPE sorbent becoming saturated with organic compounds during the water sample extractions. However, it was expected that the measured DHAA concentrations would be lower than the nominal concentrations, since as being an organic compound, DHAA tend to accumulate in organisms and adsorb to the humus particles in the exposure water, as well as, to the walls of exposure bowls (Jaffé 1991). In addition, the storage and freezing of the water samples for eight

months could have affected the DHAA concentration so that the results were not as reliable as they could have been if measured immediately after sampling. The water analysis results are not quite trustworthy as there were no comparable yield efficiency tests done with DHAA and the SPE method used.

It is necessary to measure the chemical concentrations of exposure water in order to know what are the real concentrations of chemical causing adverse effects in organisms (Van der Oost *et al.* 2003). In field, the amount of organic matter in water and sediment, and available binding sites in sediment particles have an influence on bioavailable, freely dissolved DHAA concentrations (Van der Oost *et al.* 2003).

It should be borne in mind, that DHAA is not the only toxicant in the pulp mill effluents or in the aquatic environments, and that the properties of DHAA may change during its way from the pulp mill into the aquatic environments. Before DHAA ends up in the environment along with the discharges, it mixes with other pulp mill waste water chemicals and waste water components, transforms in the microbiological processes in secondary treatment of waste water, binds with particles, dilutes when released into the watersheds, and slowly degrades in the anaerobic processes in the sediments (Mark Hewitt *et al.* 2006).

5.5 Critical overview of the experimental setup

The exposure conditions were held as constant as possible, and the stress the larvae experienced during the water changes and sampling was minimized. Some of the dead *S. trutta* larvae had ruptured yolk sacs, which could be caused by the pipetting the larvae during water exchanges (Appendix 7). The elevated mortality could have partially been due to that less stress tolerant larvae died during the pipetting transportation.

The experimental set up was randomized; the arrangement of exposure bowls circulated clockwise on the exposure room shelving in order to make lightning conditions and temperature variation equal for every replicate bowl. The results of

body length and planar yolk sac area were temperature adjusted in order to eliminate temperature as a confounding factor.

In this experiment, a whole larva was taken as a sample, and as Billiard *et al*. (1999) mentioned, the sample of whole larva may dilute the EROD-specific activity in liver by all other proteins than CYP1A. This could have been avoided by removing the yolk sac and the biasing yolk proteins (Billiard *et al.* 1999).

The standard deviation was large in every treatment group in the case of body length, planar yolk sac area, and *cyp1a* gene expression, as well (Figures 8–11). This was due to differences in individuals and the sensitivity to PAH-like xenobiotics. Differences led to outliers and not normally distributed dataset. Relatively small sample sizes and differences between individuals are considered as the main source of variation. All the samples were measured in the same manner and therefore the measurement and sample handling inaccuracies can be considered smaller, but still worth to mention, source of error.

The endpoints measured within the scope of this thesis, such as mortality and deformities, are not quite sensitive indicators of possible xenobiotic exposure as are the physiological sub-lethal effects, that, e.g., Oikari *et al*. (1983) studied in their study with *S. gairdneri,* which are considered as sensitive early signs of xenobiotic exposure (Van der Oost *et al.* 2003). The *cyp1a* expression, however, can be considered as a sensitive biomarker of PAH-like contamination (Goksøyr 1995).

6 CONCLUSIONS

In this study, according to the observed deformities, DHAA showed a potential to cause developmental defects in *S. trutta* larvae following the sub-chronic exposure to environmentally relevant DHAA concentrations. However, there was no observed DHAA dose dependent correlation with AhR activation and developmental defects, so the mechanism behind the formation of deformities is hypothesized to be distinct from the AhR mediated toxicity.

In aquatic environments single resin acids, such as DHAA, are a part of complicated mixture of pulp mill effluents and the real effects of them in aquatic environments is hard to extrapolate from single chemical toxicity tests performed in laboratory. In order not to underestimate the adverse effects that pulp and paper mill effluents might have on the fish larvae development, field exposures are needed to support the laboratory study results.

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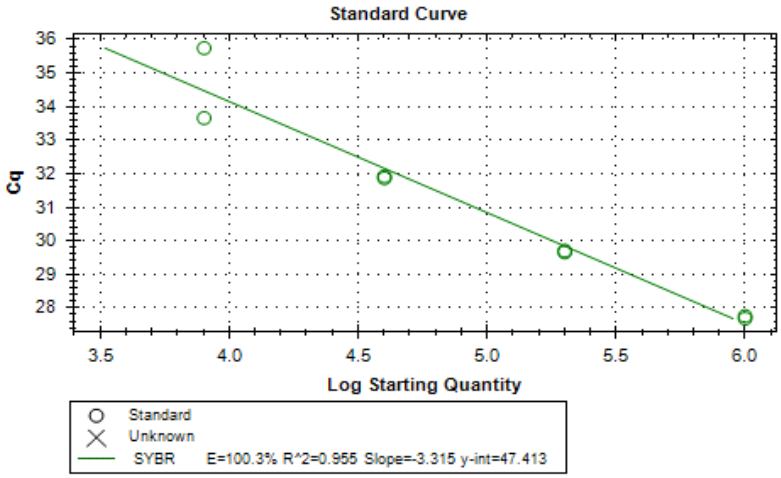
APPENDIX 1. RNA integrity numbers of the *S. trutta* RNA samples

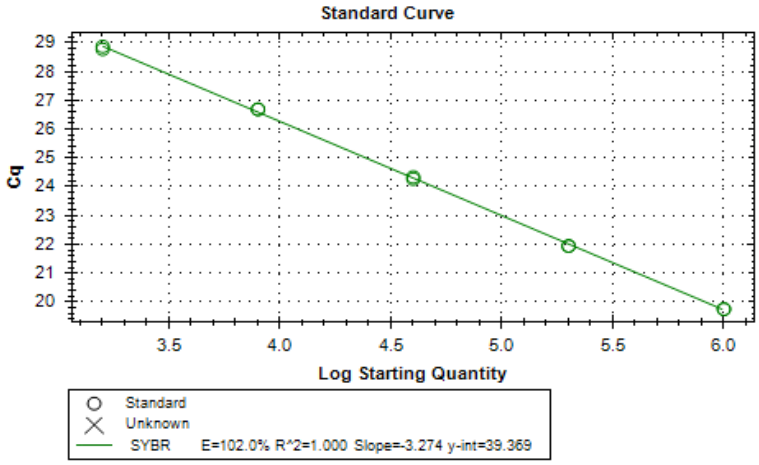
Replicate	Treatment	RIN	Replicate	Treatment	RIN	Replicate	Treatment	RIN
1	DMSO	9.8	2	DMSO	9.8	3	DMSO	8.7
1	DMSO	9.9	2	DMSO	9.6	3	DMSO	9.8
1	DMSO	9.3	2	DMSO	7.9	3	DMSO	9.8
1	1 µg/l	10	2	1 µg/l	10	3	1 µg/l	9.8
1	1 µg/l	10	2	1 µg/l	NA	3	1 µg/l	10
1	1 µg/l	8.6	2	1 µg/l	NA	3	1 µg/l	8.6
1	3.2 µg/l	8.1	2	3.2 µg/l	9.5	3	3.2 µg/l	9.8
1	3.2 µg/l	10	2	3.2 µg/l	10	3	3.2 µg/l	9.6
1	3.2 µg/l	10	2	3.2 µg/l	NA	3	3.2 µg/l	9.7
1	10 µg/l	9.7	2	10 µg/l	9.5	3	10 µg/l	9.9
1	10 µg/l	10	2	10 µg/l	NA	3	10 µg/l	10
1	10 µg/l	10	2	10 µg/l	NA	3	10 µg/l	10
1	32 µg/l	10	2	32 µg/l	7.5	3	32 µg/l	7.9
1	32 µg/l	9.8	2	32 µg/l	NA	3	32 µg/l	8.3
1	32 µg/l	8.2	2	32 µg/l	NA	3	32 µg/l	9.9
1	100 µg/l	9.8	2	100 µg/l	NA	3	100 µg/l	7.4
1	100 µg/l	10	2	100 µg/l	9.7	3	100 µg/l	10
1	100 µg/l	9.1	2	100 µg/l	NA	3	100 µg/l	9.6
1	Retene	9.7	2	Retene	9.9	3	Retene	10
1	Retene	9.9	2	Retene	9.7	3	Retene	10
1	Retene	9.1	2	Retene	9.4	3	Retene	9.8

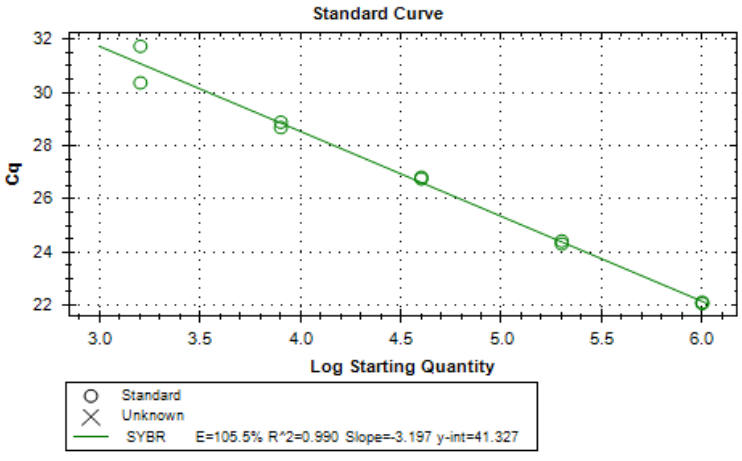
APPENDIX 2. Primers used in the qPCR-measurement

Primer	Sequence	T_M (°C)	Primer pair efficiency (%)	PCR-product length (bp)
cyp1a forward	5′- CAG TTC GCC AGG CTC TTA TCA AGC-3′	60.8		
cyp1a reverse	5′-GCC AAG CTC TTG CCG TCG TTG AT-3′	58.8	100.3	94
rl2 forward	5′-TTG AGA CAT GCA TGG CCA CAG T-3′	54.8		
rl2 reverse	5′-CGG TTC CTG CCA GCT TTA CCA AT-3′	57.1	102.0	80
ef1a forward	5′-CTT TGT GCC CAT CTC TGG TT-3′	51.8		
ef1a reverse	5′-TTA CGT TCG ACC TTC CAT CC-3′	51.8	104.5	90

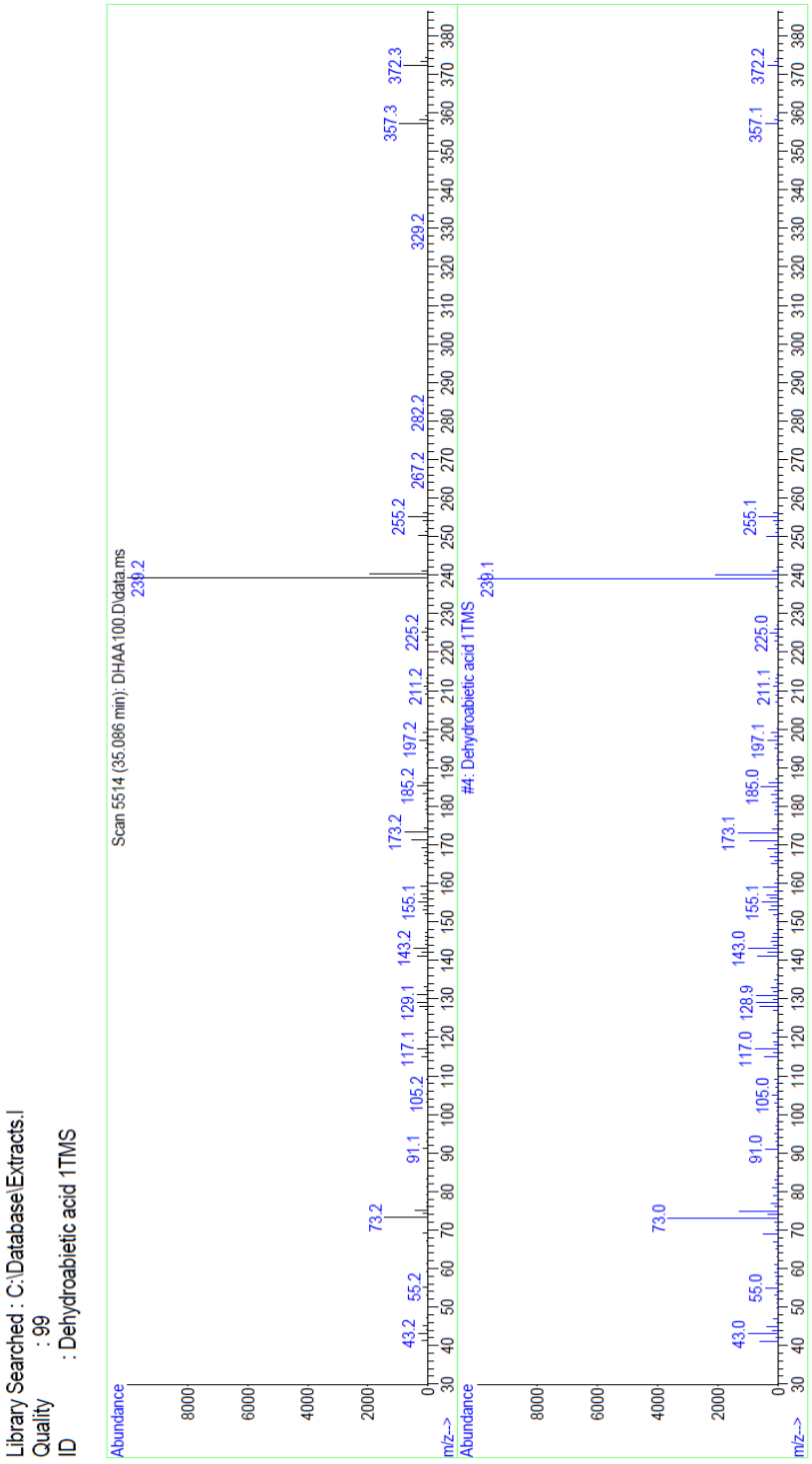
APPENDIX 3. Standard curves of the *cyp1a* gene and reference genes







APPENDIX 4. Identification of DHAA peak from mass chromatogram

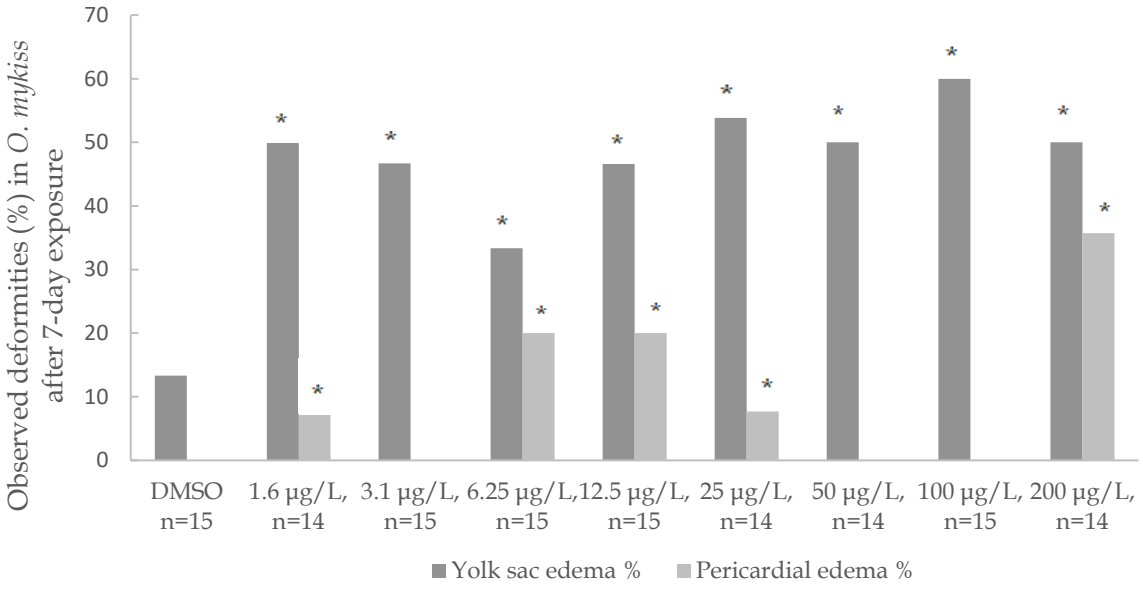


APPENDIX 5. Mortality table of the pre-test with *O. mykiss*

Treatment	Date	Mortality (%)	Yolk sac edema	Pericardial edema
1.6 µg/l	9.4.	6.7	+	-
25 µg/l	7.4.	6.7	+	-
50 µg/l	10.4.	6.7	-	+
200 µg/l	11.4.	6.7	+	-

The sign '+' indicates the occurrence of edema and '–' the absence of edema.

APPENDIX 6. Observed deformities in *O. mykiss* in the pre-test



Occurrence of deformities in *O. mykiss* larvae following 7-day DHAA exposure. Statistically significant differences (%) were found between control and all DHAA treatments ($p < 0.001$) when yolk sac edema rates were compared (Fisher's exact test). Statistically significant differences (%) were found between control and DHAA treatments 1.6 µg/l ($p = 0.014$), 6.25 µg/l ($p < 0.001$), 12.5 µg/l ($p < 0.001$), 25 µg/l ($p = 0.014$), and 200 µg/l ($p < 0.001$) when pericardial edema rates were compared (Fisher's exact test). Statistically significant difference from control is marked with *.

APPENDIX 7. Mortality table of the 13-day experiment with *S. trutta*

Replicate	Treatment	Date	Rupted yolk sac	Yolk sac edema	Pericardial edema
1	control	24.4.	-	-	-
1	control	2.5.	-	-	-
1	1 µg/l	23.4.	-	-	-
1	1 µg/l	25.4.	-	+	-
1	10 µg/l	27.4.	-	+	-
1	32 µg/l	24.4.	-	-	-
1	100 µg/l	21.4.	-	-	-
1	100 µg/l	28.4.	-	+	-
1	100 µg/l	30.4.	-	+	-
1	retene	22.4.	+	-	-
1	retene	24.4.	-	+	-
1	retene	28.4.	+	-	-
2	1 µg/l	22.4.	-	-	-
2	1 µg/l	22.4.	-	-	-
2	3.2 µg/l	23.4.	+	-	-
2	retene	23.4.	-	+	-
2	retene	26.4.	-	+	-
2	retene	28.4.	+	-	-
3	control	28.4.	-	-	-
3	10 µg/l	26.4.	-	+	+
3	10 µg/l	27.4.	+	-	-
3	10 µg/l	29.4.	-	+	-
3	100 µg/l	23.4.	-	+	-
3	100 µg/l	24.4.	-	+	+
3	retene	26.4.	-	+	-
3	retene	26.4.	+	-	-
3	retene	27.4.	+	-	-
3	retene	1.5.	-	+	+

The sign '+' indicates the occurrence of edema and '–' the absence of edema.