Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Ellen Karine Refne Mathisen

The effect of glucosinolates in the salmon louse (Lepeophtheirus salmonis) larvae and on their gene expression

Master's thesis in Biology
Supervisor: Atle M. Bones
Co-supervisor: Prashanna Guragain
June 2022

NTNU
Kunnskap for en bedre verden

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Acknowledgement

The work presented in this thesis was primarily conducted with the Cell, Molecular Biology and Genomics group (CMBG) and Taskforce Salmon Louse (TFSL) at the Norwegian University of Science and Technology (NTNU), Department of Biology (IBI). The handling of experiments with live salmon lice was conducted at NTNU Sea Lab, and the molecular work and analysis were performed at the CMBG labs.

I want to thank my supervisor Atle M. Bones, for the opportunity to work on this project. I would also like to thank my co-supervisor, Prashanna Guragain. A special thank you to Anna S. Båtnes, Stine Østerhus, Cecilie Miljeteig and the rest of the Taskforce Salmon Louse group for assistance and help.
Finally, I would like to thank all my friends and family for all the support and encouragement. I would also like to thank my fellow master's students for all the great tea breaks, the necessary ones, and the unnecessary ones; it was a blast!

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June 2022

Trondheim, Norway

Abstract

The salmon louse (*Lepeophtheirus salmonis*) is an ectoparasite that infects salmonid species like Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta*), arctic char (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*). The infection of *L. salmonis* causes substantial economic losses in the salmon fish farming industry and affects the health and welfare of farmed and wild salmonids. Biopesticides are compounds used to manage agricultural pests through specific biological effects, and the use of biopesticides could prove promising in the aquaculture industry. Glucosinolates are a large group of secondary plant metabolites. They are primarily found in the family Brassicaceae and are part of the plant's defence system against herbivores. Using glucosinolates as biopesticides could be a part of the solution to conquering the salmon lice problem.

In this project, the glucosinolate sinigrin and its derivative AITC were studied for their effects on salmon louse larvae and the expression of selected genes. The expression of genes related to immune responses, cell cycle, negative control of cellular proliferation, DNA replication and cellular division, and detoxification in *L. salmonis* copepodites were explored. The study showed that the number of active lice decreased with higher concentrations of sinigrin and with longer time periods. EtOH and AITC completely immobilised the lice. Genetic analysis showed that only three genes were significantly up-regulated, one due to sinigrin and two due to AITC. Exposed to AITC, the copepodites showed an up-regulation of the genes *CYP2j3* and *TNFaf-4*. Exposed to sinigrin, the copepodites showed an up-regulation of the gene *GST-mu3*. *CYP2j3* is a member of the cytochrome P450 family, which participates in metabolising endogenous and exogenous compounds. *TNFaf-4* modulates various responses, including inflammatory and immune-regulatory responses, antiviral responses, cell proliferation and growth inhibition. This study did not conclude whether sinigrin and/or AITC affect *L. salmonis* copepodites. Therefore, more research is needed to explore the possibilities of using glucosinolates as biopesticides against salmon lice.

Sammendrag

Lakselusen (*Lepeophtheirus salmonis*) er en ektoparasitt som infiserer ulike laksearter som laks (*Salmo salar*), sjøørret (*Salmo trutta*), røye (*Salvelinus alpinus*) og regnbueørret (*Oncorhynchus mykiss*). Lakselus utgjør en økonomisk trussel mot oppdrettsnæringen og lakslusinfeksjoner har en negativ påvirkning på både oppdretts- og villaksens helse og velferd. Biopestisider har en naturlig biologisk opprinnelse og kan brukes som et hjelpemiddel for å regulere skadedyr og sykdom i landbruket. Biopestisider kan muligens også være nyttige i havbruksnæringen. Glukosinolater er en gruppe sekundære plantemetabolitter. De finnes hovedsakelig i familien Brassicaceae og fungerer som en del av plantens forsvar. Å benytte seg av glukosinolater som et biopestisid er derfor en mulighet, og kan kanskje være en del av løsningen på lakselusproblemet.

I dette forsøket ble glukosinolatet sinigrin og derivatet AITC brukt for å undersøke effekten de kan ha på lakseluslarver og uttrykket av et utvalg gener. Gener relatert til immunresponser, cellesyklusen, negativ regulering av celleproliferasjon, DNA-replikasjon og celledeling, og detoksifisering ble undersøkt. Studien viste at antallet aktive lus avtar med høyere konsentrasjoner av sinigrin og lenger eksponering. EtOH og AITC immobiliserte alle lusene i prøven. Genetiske analyser viste at tre gener var signifikant opp-regulert. Kopepoditter eksponert for AITC hadde en opp-regulering av genene *CYP2j3* og *TNFaf-4*, mens hos kopepoder eksponert for sinigrin var genet *GST-mu3* opp-regulert. *CYP2j3* er et gen i cytokrom P450 familien. Gen i denne familien bidrar til metaboliseringen av endogene og eksogene forbindelser. *TNFaf-4* modulerer en rekke responser som inflammatoriske og immun-regulerende responser, antiviral responser, celleproliferering og vekstinhibisjon. Dette studiet konkluderer ikke om sinigrin og/eller AITC påvirker *L. salmonis* kopepoditter, og mer forskning må til for å kunne utforske mulighetene ved bruken av glukosinolater som biopestisider.

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Abbreviations

AC	Allyl cyanide
ADT3	ADP/ATP carrier protein
AITC	Allyl-isothiocyanate
Ala	Alanine
ANOVA	Analysis of variance
ATC	Allyl thiocyanate
cDNA	Complementary deoxyribonucleic acid
CETP	1-cyano-2,3-epithiopropane
CYP	Cytochrome P450
CYP18a1	Cytochrome P450 18a1
CYP2j3	Cytochrome P450 2j3
DAIA-2	Death-associated inhibitor of apoptosis 2
DCP	Dedicator of cytokinesis protein 7
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
eEF1α	Translation elongation factor 1α
EtOH	Ethanol
ITC	Isothiocyanate
gDNA	Genomic DNA
GST	Glutathione-S-transferase
GST-mu3	Glutathione S-transferase Mu3
GLs	Glucosinolates
HCl	Hydrochloric acid
Ile	Isoleucine
Leu	Leucine
Met	Methionine
PCR	Polymerase chain reaction
Phe	Phenylalanine
PRC	Protein regulator of cytokinesis 1
qPCR	Quantitative polymerase chain reaction

RNA	Ribonucleic acid
RNA-Seq	RNA-sequencing
SCS	Suppressor of cytokine signalling 4
TNFaf-4	TNF receptor-associated factor 4
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
W-o-L	Window-of-Linearity

1 Introduction

The salmon louse (*Lepeophtheirus salmonis*) presents a major problem to the aquaculture industry. The parasite infests farmed and wild salmonid fishes, causing damage to the fish and the salmon farming industry (Humble et al., 2019). The salmon louse is primarily kept in check using a combination of medical and non-medical approaches. The use of biopesticides could be a promising solution in biological control against salmon lice (Jodaa Holm et al., 2016).

Glucosinolates are specialised metabolites produced by plants in the Brassicaceae order, which protect the plants against herbivores (Bischoff, 2016; Blažević et al., 2020; Sønderby, Geu-Flores, & Halkier, 2010). Sinigrin is a glucosinolate and is hydrolysed by myrosinase to form allyl isothiocyanate (AITC) (Mazumder, Dwivedi, & du Plessis, 2016; Wittstock, Kurzbach, Herfurth, & Stauber, 2016). AITC is a compound active in the plant's defence, able to modulate an array of pathways. In humans, these pathways include the inhibition of CYP enzymes, induction of phase-II-enzymes, modulation of cell cycle regulators, induction of apoptosis and inhibition of metastasis (Hara, Yatsuzuka, Tabata, & Kuboi, 2010; Kumar et al., 2015). It may also function as a herbicide in high concentrations, thus presenting an alternative to current weed control treatments.

This thesis was conducted as an attempt to contribute to the counteraction to the harmful salmon-arthropod interactions currently found in salmon farming and as a step towards a solution to the salmon lice problem. Copepodites of *L. salmonis* were therefore exposed to sinigrin and AITC to investigate the effects these compounds may have on the larvae and their gene expression.

2 Background

2.1 *Lepeophtheirus salmonis*

2.1.1 Background and life cycle

The salmon louse (*Lepeophtheirus salmonis*) is a copepodite species belonging to the Caligidae family (Krøyer, 1837). In the Northern hemisphere, it is a marine salmonid ectoparasite that infests both wild and farmed salmonids. Salmon lice is a parasite specialised to Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta*), arctic char (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) (Bjørn, Finstad, & Kristoffersen, 2001; Dawson, Pike, Houlihan, & McVicar, 1997; O'Donohoe, Kane, Jackson, & McDermott, 2016; Wootten, Smith, & Needham, 1982). Since these species are economically and ecologically important, the salmon louse presents a severe aquaculture sector problem (Liu & Bjelland, 2014).

There are eight developmental stages in the life cycle of *L. salmonis* (Fig. 1). There are three planktonic stages and five parasitic stages (Fig. 1) (Hamre et al., 2013). Moulting separates each stage, which is as follows: nauplius 1 and 2, copepodite, chalimus 1 and 2, preadult 1 and 2 and sexually mature adult stage. The life cycle is divided into a free-living stage and an attachment stage. The two nauplii stages belong to the former, while the infective copepodite stage, chalimus, preadult and adult stages belong to the latter (Hamre et al., 2013).

The parasitic stages of the salmon lice feed on skin, mucus, and blood, often leading to eroding of the skin barrier of the fish (Frost & Nilsen, 2003; Mustafa et al., 2000). High infestation results in chronic stress, osmotic imbalance and damage to the host, further leading to mortality by secondary microbial infections (Liu & Bjelland, 2014).

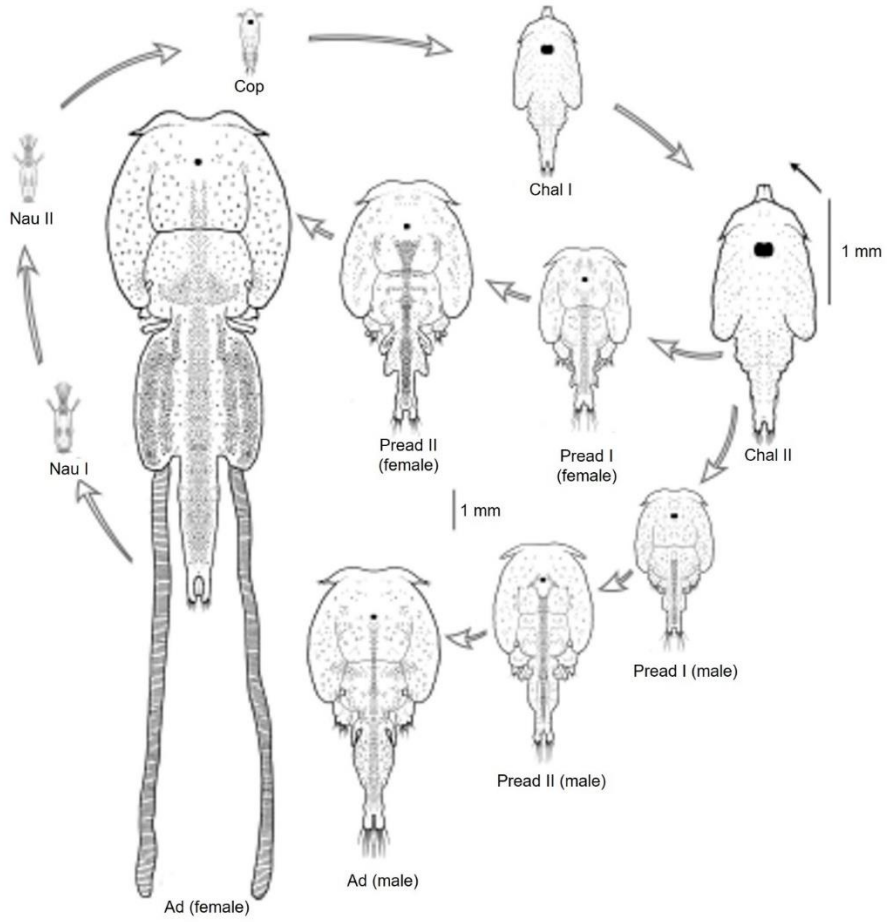

Figure 1: The salmon louse life cycle

The salmon louse *Lepeophtheirus salmonis* has a life cycle consisting of eight developmental stages. The two naupliar stages precede the infective copepodite stage, followed by two chalimus stages and two preadult stages until the salmon louse reaches the sexually mature adult stage. Female adults produce eggs organised in strings connected to their abdomen, which the nauplii larvae hatch from. The nauplii stages are the free-living stages of the life cycle. For the remainder of the life cycle, the salmon louse lives in an attachment stage. Adapted from UiB Open Research Data (Sea Lice Research, 2020).

2.1.2 Consequences in salmonid farming

Fish farming is the world's fastest-growing food-producing industry (FAO, 2021). Over the last 70 years, it has evolved, and salmon farming represents now 90% of the fish farming market (Luthman, Jonell, & Troell, 2019). The production value of Atlantic salmon and other salmonids summed up to 13.1 billion euros in 2018 (FAO, 2020; Guragain et al., 2021). However, the industrialisation of fish farming has created substantial environmental issues. Among them is the ongoing issue of sea lice proliferation, which harms farmed salmonids and has ramifications for the wild salmon and trout populations (Kristoffersen et al., 2018; Svåsand et al., 2017).

Sea lice are marine arthropods, and arthropods are the largest invertebrate phylum accounting for over 80% of all animal species on earth (Ghafor, 2020; Zhang, 2013). Arthropods cause losses in both agriculture and aquaculture, but the former has received more attention (Guragain et al., 2021). Currently, pest control in fish farming is a major challenge. The financial losses caused by pests, especially salmon lice, are substantial. Financial losses due to the lice infestation are estimated to be 9% of overall production revenue (Abolofia, Asche, & Wilen, 2017; Guragain et al., 2021). In 2020, the first-hand value of salmon in Norway was estimated to be around 64 billion NOK (Fiskeridirektoratet, 2021). The cost of combating the salmon lice is estimated to be approximately 5 billion NOK (Iversen et al., 2019). Salmon lice levels in Norway are regulated by law using a traffic light system (Guttormsen, 2015). This is done to protect wild salmon and secure animal welfare, but at the same time, it increases the intensity of treatment and handling, driving up costs and causing fish welfare issues (Guragain et al., 2021). Medical and non-medical treatments, cleaner fish, net cleaning, stress, increased mortality, reduced weight gain, fish handling costs, and higher feed consumption ratios all contribute to an increase in production costs caused by salmon lice (Iversen et al., 2019; Iversen, Hermansen, Nystøyl, Marthinussen, & Garshol, 2018).

Non-medical and medical treatments have been used to control and manage the salmon lice problem. The most common delousing method is medical treatments due to its consistency and efficiency (Aaen, Helgesen, Bakke, Kaur, & Horsberg, 2015). The expense of medical treatments, on the other hand, is relatively significant, projected to be approximately 1-2 billion NOK in 2014 (Iversen et al., 2018). The widespread use of chemical agents in medicinal

therapies has also led to a parasitic shift toward drug resistance (Aaen et al., 2015). A strong enough dosage must be delivered to eradicate the parasites effectively, but this must be balanced against the host's capacity to withstand the chemical treatment. A decrease in dosage may limit the impact on the parasites, resulting in resistance selection (Kunz & Kemp, 1994). In arthropods, various genetic resistance mechanisms have been documented (Brattsten, Holyoke, Leeper, & Raffa, 1986; Aaen et al., 2015). These include point mutation in a target gene, upregulation of detoxification metabolism, efflux pumps in the intestines of parasites, changes in the thickness of the cuticle, and other mechanisms to reduce chemical penetration (Brattsten et al., 1986; Aaen et al., 2015). Resistance to many delousing agents and unsuccessful treatments have been reported (Fjørtoft et al., 2020; Grøntvedt et al., 2015; Aaen et al., 2015). There are also risks of chemical agents used against salmon lice affecting non-target species, such as lobster (Olsvik, Samuelsen, Agnalt, & Lunestad, 2015), shrimp, other crustaceans and bivalves (Guragain et al., 2021). Many non-target species have previously been negatively affected by chemotherapeutic drugs at lower doses than those employed in salmon lice treatments (Guragain et al., 2021; Macken, Lillicrap, & Langford, 2015; Urbina, Cumillaf, Paschke, & Gebauer, 2019).

2.2 Glucosinolates

2.2.1 Biosynthesis

Glucosinolates (GLs) are a large group of plant secondary metabolites (Blažević et al., 2020; Zinoviadou & Galanakis, 2017). They are primarily found in the Brassicaceae family (Bischoff, 2016; Halkier & Gershenzon, 2006; Sønderby et al., 2010). Glucosinolates are characterised by their β-D-glucopyranose residue and the presence of a sulphate group and a glucose molecule linked by a thioglucoside bond within one molecule (Bischoff, 2016; Blažević et al., 2020; Wittstock et al., 2016). Furthermore, GLs derive from amino acids. They can be classified according to their amino acid precursor: aliphatic glucosinolates (Ala, Leu, Ile, Val and Met), benzenic glucosinolates (Phe and Tyr) and indolic glucosinolates (Trp) (Sønderby et al., 2010).

The biosynthesis of the glucosinolates occurs through three distinct stages:

i. Chain elongation of the selected precursor amino acid
ii. Formation of the core glucosinolate structure

iii. Secondary modifications of the amino acid side chain

Only methionine (Met) and phenylalanine (Phe) undergo chain elongation (Sønderby et al., 2010). Met is deaminated, forming 2-oxo acid. The 2-oxo acid forms a substituted 2-alkylmalate derivate by condensing with acetyl-CoA. The 2-alkylmalate derivative is isomerised to a 3-alkylmalate derivative, which undergoes oxidative decarboxylation, producing a modified 2-oxo acid (homoketo acid). This contains one additional methylene group than the starting compound (Fig. 2). Following this, the compound could be transaminated into a homoamino acid to proceed to stage ii or continue several rounds of chain elongation (up to nine cycles have been observed in plants) (Halkier & Gershenzon, 2006).

The amino acids, or chain-elongated homoamino acids, are further metabolised by cytochromes P450 from the CYP79 family during core structure formation (ii). This results in aldoximes. CYP83 proteins further convert the aldoximes into intermediates. Because of their volatility, the intermediates remain unidentified. Glutathione S-transferase then conjugates the intermediates with glutathione (GTH). The conjugate is broken down into thiohydroximates. S-glucosyltransferases convert thiohydroximates to desulfo-glucosinolates. Sulfotransferase then transforms the desulfo-glucosinolate molecule to the core glucosinolate structure (Fig. 2) (Blažević et al., 2020; Halkier & Gershenzon, 2006).

Secondary amino acid side chain changes may further modify the core glucosinolate structure (iii). Secondary modifications of aliphatic glucosinolates include oxygenations, hydroxylations, alkenylations and benzoylations. On the other hand, indolic glucosinolates may go through hydroxylations and methoxylations. Secondary changes to the amino acid side chains result in glucosinolate structural variations (Blažević et al., 2020; Halkier & Gershenzon, 2006).

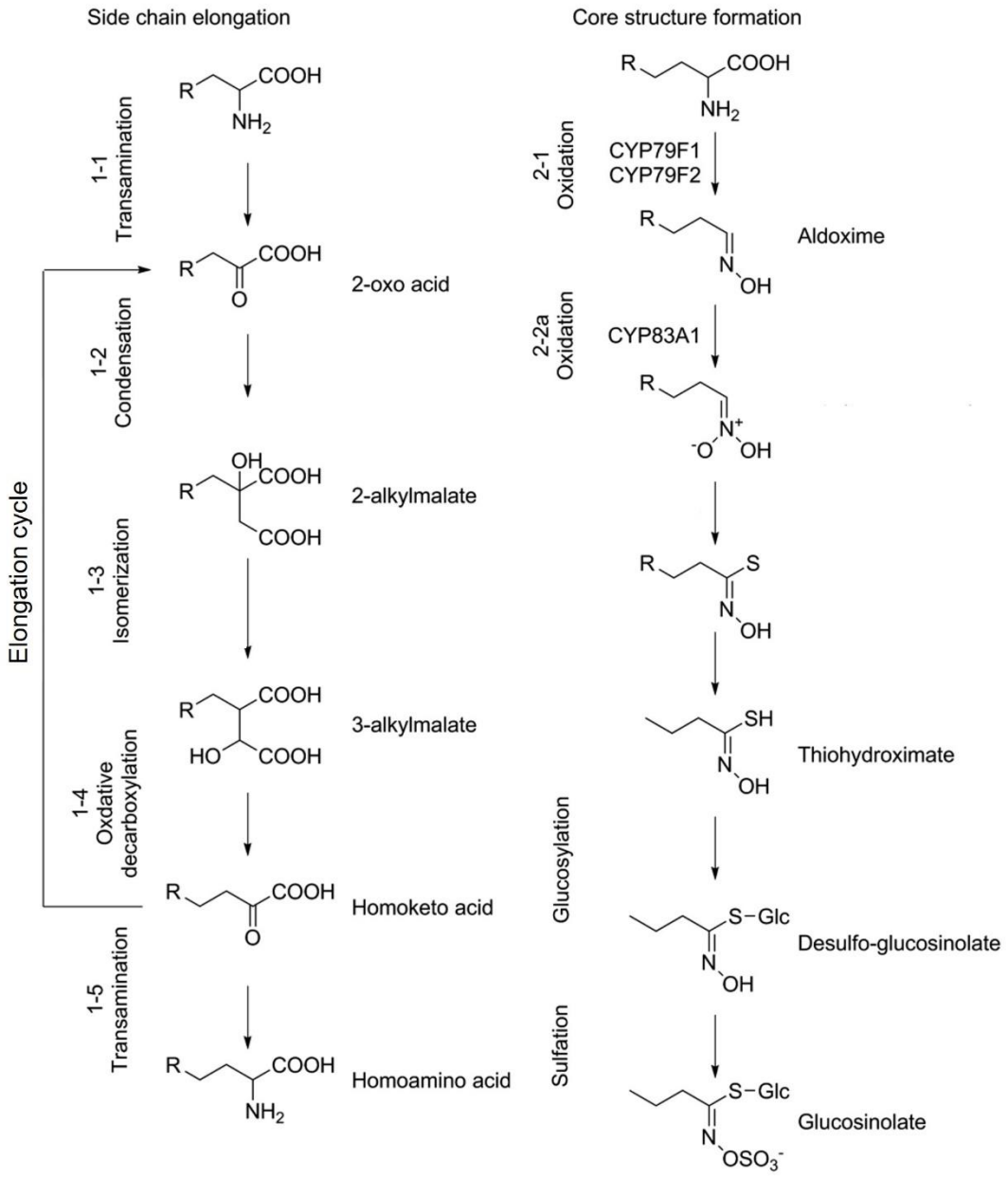

Figure 2: The biosynthesis pathway of aliphatic glucosinolates

The conversion of amino acids into the core structure of a glucosinolate goes through several stages. The side-chain elongation has five stages: 1-1: Transamination of amino acid to 2-oxo acid. 1-2: Condensation of 2-oxo acid to alkylmalate. 1-3: Isomerisation of 2-alkylmalate to 3-alkylmalate. 1-4: Oxidative decarboxylation of 3-alkylmalate to a homoketo acid. 1-5: Transamination of homoketo acid to homoamino acid. Following the side-chain elongation, the glucosinolate's core structure is formed by the oxidation of homoamino acid by the CYP-family proteins into aldoxime. Aldoxime is further oxidated into an unidentified intermediate, leading to the formation of thiohydroximate. Glucosylation of thiohydroximate forms desulfo-glucosinolate, which is further sulphated into the core structure of glucosinolates. Other enzymes than CYPs involved in the process and side-chain modification of glucosinolates are not shown. Adapted from Kakizaki & Ishida (2017).

2.2.2 Breakdown

Glucosinolates, together with the enzyme myrosinase (β -thioglucosidase glucohydrolase), can form a "chemical bomb" (Wittstock et al., 2016). Myrosinase is a thioglucosidase that catalyses the first step of glucosinolate bioactivation. This first step produces an unstable aglycone. The produced aglycone spontaneously rearranges, creating a poisonous isothiocyanate (ITC) (Parchem, Piekarska, & Bartoszek, 2020; Wittstock et al., 2016). Instead of ITCs, alternative products are produced in the presence of specifier proteins (Fig. 2.). These include ephionitriles, thiocyanates, and nitriles (Parchem et al., 2020; Wittstock et al., 2016). The products liberated after GL degradation due to myrosinase activity, especially ITCs, show a broad biocidal activity, including insecticidal, nematicidal and fungicidal effects (Parchem et al., 2020).

As previously stated, the combination of glucosinolates and myrosinases results in a "chemical bomb". Plants use this as a defence system against various herbivores, including insects and aquatic herbivores (Halkier & Gershenzon, 2006; Newman, Kerfoot, & Hanscom, 1996). The components are preserved in separate cells to avoid the "bomb's" premature detonation, potentially harming intact and undamaged tissue. When these cells are disrupted, either mechanically or due to herbivore assault, the myrosinase protein complex is released, making it accessible to GLs. This causes the GL hydrolysis intermediates to be liberated, which then subsequently are transformed into the end products (Fig. 3) (Wittstock et al., 2016).
The toxicity of the released ITCs is based on two main mechanisms:

(I) The inhibition of ATP synthesis

(II) The inactivation of intracellular enzymes.

Both mechanisms are caused by ITCs' ability to break down disulphide bonds and react with the amine groups in the protein structure (Parchem et al., 2020; Vig, Rampal, Thind, & Arora, 2009; Wittstock et al., 2016).

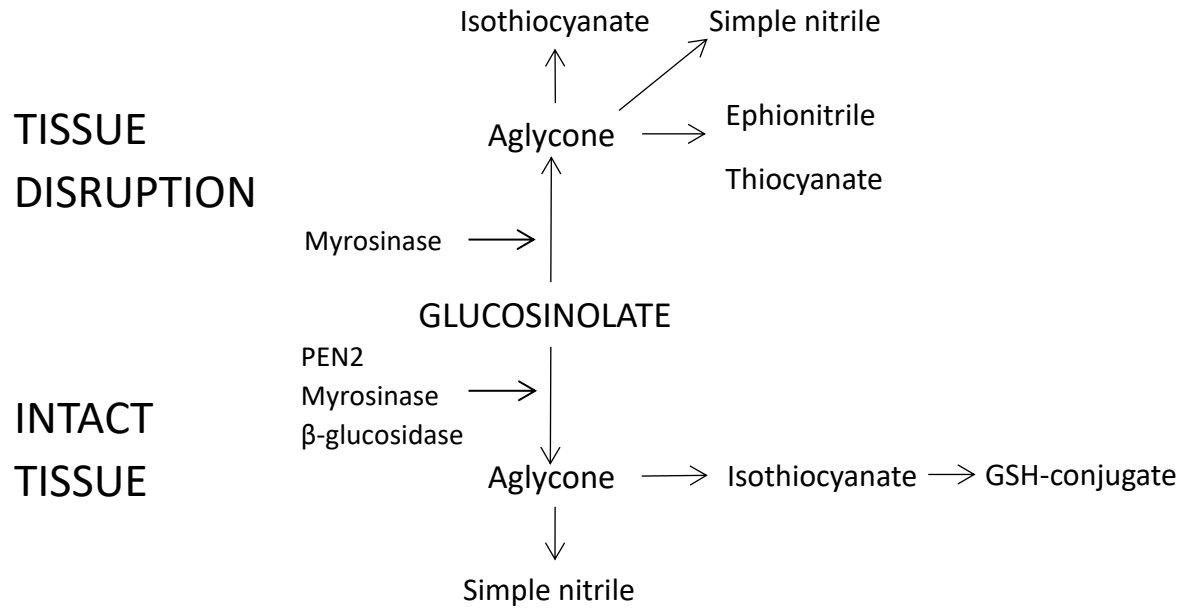

Figure 3: Different pathways of glucosinolate breakdown

The breakdown of glucosinolates can be triggered by tissue disruption (top) or chemical presence/pathogen attack (bottom). The aglycones formed are unstable and can spontaneously rearrange to isothiocyanate or simple nitriles with the help of specifier proteins. PEN2=penetration 2, GSH: glutathione. Adapted from Wittstock et al. (2016).

2.2.3 Sinigrin and allyl isothiocyanate

Sinigrin (allyl-glucosinolate or 2-propenyl-glucosinolate) is a natural aliphatic glucosinolate, and a major glucosinolate (Mazumder et al., 2016). Sinigrin may be hydrolysed to produce four aglycones: allyl isothiocyanate (AITC), allyl cyanide (AC), 1-cyano-2,3-epithiopropane (CETP) or allyl thiocyanate (ATC) (**Fig. 4**) (Shofran, Purrington, Breidt, & Fleming, 1998). The product hydrolysed is determined by the processing parameters. For instance, AITC is usually produced at a neutral pH, while AC production occurs at pH 4 (Bones & Rossiter, 1996; Shofran et al., 1998).

AITC is the source of the pungent flavour ingredient naturally found in the Brassicaceae family plants, such as mustard, wasabi, and horseradish. AITC is an unstable compound shown to gradually decompose in water at 37°C and at room temperature (Kawakishi & Namiki, 1969). Furthermore, AITC may undergo various chemical reactions such as hydrolysis, oxidation, thermal degradation, and protein reactions. The carbon atom found in the isothiocyanate

part of AITC cleaves the disulfide bond in cysteine when AITC reacts with proteins. This is followed by a polymer formation. (Mercer & Rodriguez-Amaya, 2021; Weerawatanakorn, Wu, Pan, & Ho, 2015).

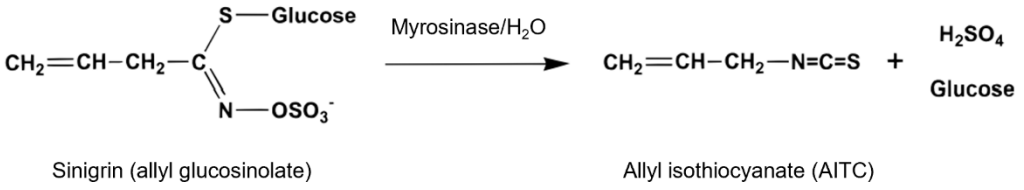

Figure 4: Enzymatic conversion of sinigrin to AITC

Sinigrin (allyl-glucosinolate) hydrolysed to the aglycone allyl isothiocyanate (AITC). This happens at a neutral pH. AITC is the source of the pungent flavour ingredient in plants found in the family Brassicaceae and is a part of the plant's defence system. Adapted from Tsao, Yu, Friesen, Potter, & Chiba (2000).

2.2.4 The effects of sinigrin and AITC

ITCs can modulate an array of pathways such as inhibition of CYP enzymes, induction of phase II enzymes, the modulation of cell cycle regulators, the induction of apoptosis and the inhibition of metastasis (Kumar et al., 2015). In Atlantic salmon (*Salmo salar*), the ingestion of GLs has been shown to lead to an upregulation of immune genes, particularly those involved in innate antiviral responses (Jodaa Holm et al., 2016). GLs have also been demonstrated to influence genes involved in the cell cycle, negative control of cellular proliferation, DNA replication and cellular division, as well as the activation of detoxifying genes in Atlantic salmon (Skugor et al., 2016).

Furthermore, glutathione-S-transferases (GSTs) and other general phase I and phase II detoxification enzymes have been proposed as detoxification systems in insects (Schramm, Vassão, Reichelt, Gershenzon, & Wittstock, 2012). GSTs are present in nearly all living organisms, including insects and marine organisms (Brattsten, 1992; Roncalli, Cieslak, Passamaneck, Christie, & Lenz, 2015). GSTs are typically small proteins (200-250 amino acids) that get activated in response to oxidative stress or exposure to various toxins (Roncalli et al., 2015). Hasegawa et al. (2010) observed that AITC induced GST expression in *C. elegans* (Hasegawa, Miwa, Tsutsumiuchi, & Miwa, 2010). Additionally, Roncalli et al. (2015) discovered

that GSTs were involved in detoxification in the crustacean *Calanus finmarchicus* (Roncalli et al., 2015).

Members of the cytochrome P450 gene family may also be involved in metabolising harmful substances. CYPs constitute a large gene family present in prokaryotes and eukaryotes. Metazoan CYPs are membrane-bound that catalyse a wide variety of processes that metabolise both endogenous and exogenous substances. They also play a role in metabolic detoxification by contributing to the organism's biochemical defence against xenobiotics (Humble et al., 2019).

2.3 Project aims

The aims were to study the effects of the glucosinolate sinigrin and its derivative allyl isothiocyanate (AITC) on the salmon lice *L. salmonis* and their effect on the expression of a set of selected genes.

Research questions related to the project were:

A. Does sinigrin affect the gene expression of genes related to immune responses, cell cycle, negative control of cellular proliferation, DNA replication and cellular division, and detoxification in copepodites of *L. salmonis*?
B. Does AITC affect the gene expression of genes related to immune responses, cell cycle, negative control of cellular proliferation, DNA replication and cellular division, and detoxification in copepodites of *L. salmonis*?

3 Materials and methods

3.1 Salmon lice material

In the copepodite stage, three biological replicates consisting of 50 lice were collected for treatment in each concentration. The salmon lice were collected in 1.5 mL microcentrifuge tubes with lids. The lice were kept in approximately 250 µL of seawater before the correct concentration of chemicals was added to each sample. Then, the sample was topped up with seawater until it reached a total volume of 500 µL. The lice were then maintained in the microcentrifuge tubes for their designated time (1, 6 or 24 hours). The liquid was removed, and the samples were flash-frozen in liquid nitrogen. The samples were stored at -80°C.

3.1.1 Salmon lice maintenance

All experiments and fish handling were conducted according to Norwegian animal research and welfare regulations.

Atlantic salmon (*Salmo salar*) (approximately 200 g) were kept as host fish in 400 L tanks with a filtered seawater flow (250-450 L/h). The tanks were kept in a climate-controlled room with a stable temperature of 10°C. Temperature, salinity, water oxygen levels, parasite pressure, developmental progression and general fish health and welfare was monitored daily. The laboratory strain of the salmon louse, LsGulen (Hamre, Glover, & Nilsen, 2009), was kept at NTNU SeaLab on the Atlantic salmon hosts. The strain was maintained by picking salmon louse egg strings from anaesthetised fish. The anaesthetised fish were either returned to the tanks or euthanised, depending on their general health, time as host and size. The picked egg strands were placed in tubes in a salmon louse hatchery with one single strand in each tube (Fig. 5). The strings were monitored daily. When the louse reached the copepodite stage, the host salmon was reinfested. This was done by turning off the seawater flow to the fish tanks and reducing the water level to one-third of the original level. Then the copepodites were spread evenly in the tank. After 30 minutes, the seawater flow was put back to normal.

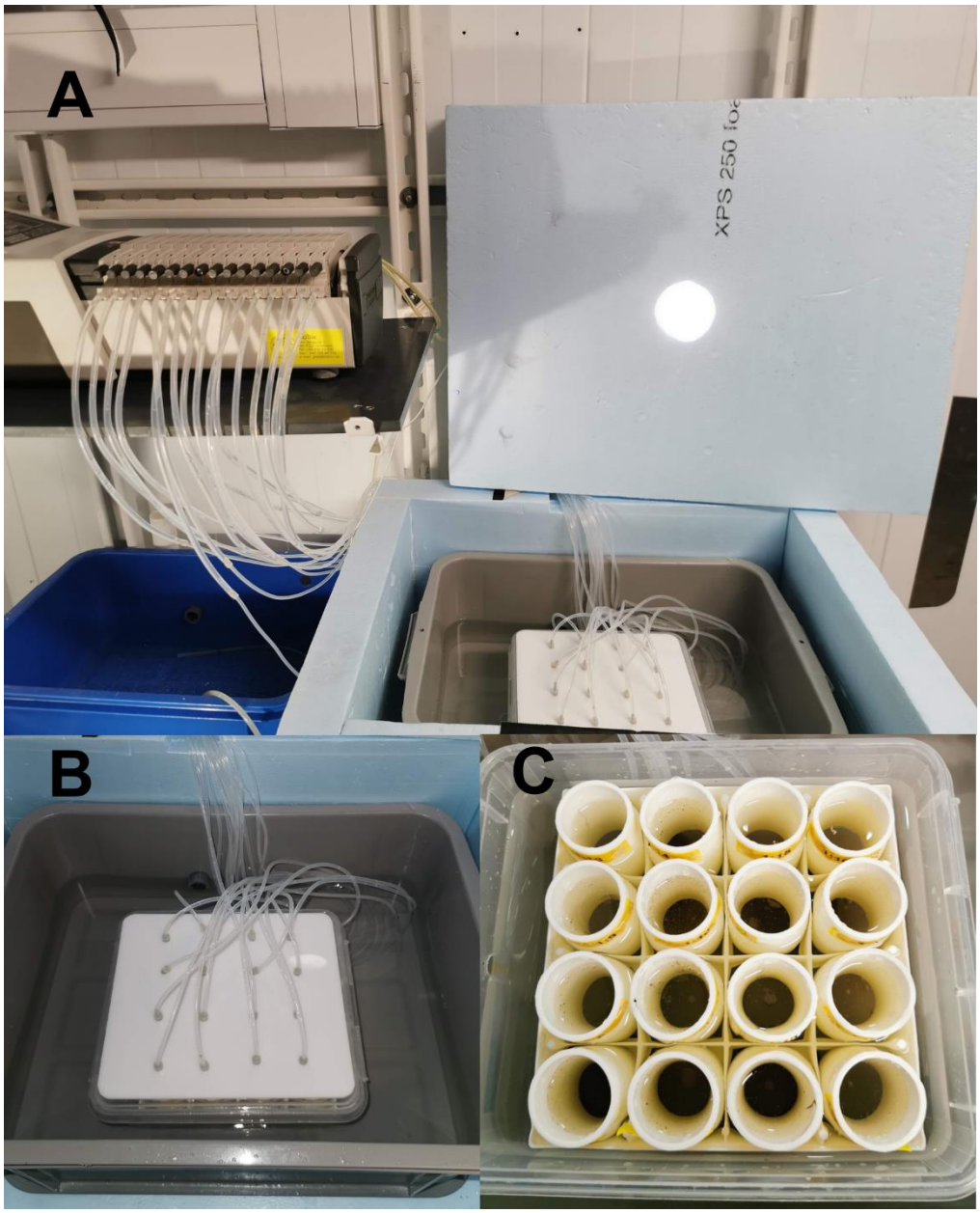

Figure 5: Salmon louse hatchery at NTNU SeaLab

A: A salmon louse hatchery made of a Styrofoam box with an inside light through the lid, a plastic tray with tubes and a peristaltic pump.

B: Through the inlets in the plastic box lid, the peristaltic pump pumps a flow of 3 mL per minute of filtered seawater (10 °C) into each of the tubes underneath. The filtered seawater (10 °C) flows through the tray from a tube connected to a pump and is drained through an outlet ~10 cm up on the back wall (not shown).

C: A rack supporting 16 PVC flow-through tubes (10 cm long) was placed inside the plastic box. The tubes were sealed at the bottom with 60 µm mesh. Pipette tips were placed through the lid to direct the seawater flow into each tube. A 2 cm space between the tube bottom and the box was left underneath the rack, allowing flow-through water to exit the tubes and the box. Excess seawater was drained through an outlet halfway up on the box's wall (not shown).

3.2 Exposure of *L. salmonis* to sinigrin and allyl isothiocyanate

Egg strings from the LsGulen strain were picked and incubated as described in chapter 3.1.1. The hatching was observed daily, and the nauplii were kept in the hatchery for 4-5 days until they were transformed into copepodites. There were three biological replicates for each concentration of treatments and time point in a single experiment. The time intervals were one hour, six hours and twenty-four hours. Each replicate was made up of 50 copepodites. The salmon lice were soaked in either sinigrin ($C_{10}H_{16}KNO_9S_2xH_2O$, CAS 3952-98-5) or allyl isothiocyanate (AITC, CH_2CHCH_2NCS, CAS 57-06-7). In addition, there were controls with the solvents DMSO and EtOH and a wild-type control in seawater.

The sinigrin was solved in 1% DMSO, and the solubility of sinigrin in DMSO is 72 µg/µL. AITC was solved in 90% EtOH, and the solubility of AITC in EtOH is 253.25 µg/µL. The concentrations of sinigrin in this experiment were 5 µg/mL, 7.5 µg/mL and 10 µg/mL, and the concentrations of AITC were 1 µg/mL, 2.5 µg/mL, and 5 µg/mL Only the highest concentrations of EtOH and DMSO were controlled for.

The soaking was conducted in a 0.5 mL microcentrifuge tube with lids containing a total volume of 500 µL.

3.3 Quantification of active and immobilised lice

3.3.1 Observation of active lice

Lice from one tube per concentration and time point were transferred from the microcentrifuge tubes to a petri dish. The activity was measured by observing the number of active lice in the sample. Activity in this experiment was counted as a movement in the form of swimming. The lice were observed for 5 minutes, and lice that did not swim were categorised as immobilised.

3.3.2 Neutral red

A stock solution of neutral red staining (powder) was made by dissolving 0.05 mg in 1 mL of distilled water. 45 µL of the neutral red staining was added to samples of 45 *L. salmonis* copepodites in 500 µL seawater. Copepodites were killed for control using a heat block at 60°C. A control with no dye was also performed. The lice were incubated in the dye for 20 minutes in a cold-water bath in a Styrofoam box. Three replicates were killed by flash-freezing them in liquid nitrogen. The rest of the lice were killed using a heat block at 60°C. The dead

lice were transferred onto a 60 µm mesh and rinsed with filtered seawater to wash away any excess dye. The flash-frozen copepodites were thawed before the rinsing. All the samples were transferred onto filter paper in a petri dish. The flash-frozen samples were thawed before being transferred to the filter paper. One flash-frozen sample was exposed to 1 M HCl to see if it affected the colour development. The stained samples were analysed using the microscope ZEISS AxioZoom v1.6.

3.4 Gene expression analysis

3.4.1 RNA isolation and cDNA synthesis

Total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The samples were transferred directly from -80°C into TissueLyser II (Qiagen) adapter set. One 5 mm steel bead (Qiagen) was added to each sample tube. The adapter set and the metal beads were cooled to -80°C before use. The tissue was homogenised in a TissueLyser II (25.0 Hz for 2.5 minutes). Quickly following the homogenisation, 600 µL Buffer RLT Plus lysis buffer with 2-mercaptoethanol was added to each sample. The samples were lysed again in the TissueLyser II before centrifuging at maximum speed for 3 minutes. Genomic DNA (gDNA) elimination and RNA washing were performed according to the RNeasy mini kit manufacturer's instructions. The RNA was eluted twice, using the primary eluate for the second elution. This was done to maximise the yield. The RNA concentration, and the eluates purity, were determined using NanoDropTM One/One© Microvolume spectrophotometer (Thermo Fisher Scientific).

cDNA synthesis was performed using QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Samples were diluted five times by adding RNase-free water. The cDNA was stored at -20°C.

3.4.2 RT-qPCR

The quantitative real-time polymerase chain reaction (qPCR) analysis was performed using the LightCycler 480 SYBR Green 1 Master Kit (Roche Life Science, 04 887 352 001) following the manufacturer's instructions.

15 µL of master mix consisting of RNase-free water, forward and reverse primers, and LightCycler 480 Probes Master were added to 5 µL of diluted cDNA (Appendix, Table A.2). *ADT3* (Eichner, Øvergård, Nilsen, & Dalvin, 2015) and *eEF1α* were used as reference genes. Primer sequences for the reference genes *ADT3* and *eEF1α* and the genes of interest *DAIA-2*, *SCS*, *GST-mu3*, *CYP2j3*, *CYP18a1*, *PRC*, *TNFaf-4* and *DCP* are given in Table 1. Each 96-well plate included at least one reference gene and two no-template controls (NTC) per primer pair. The plates were run on a LightCycler96 (Roche Life Science).

3.5 Statistical analysis

LinRegPCR v.2015.4 (Academic Medical Centre, Amsterdam, the Netherlands) was used to determine each primer pair's PCR efficiencies and Cq-values. Fluorescence intensity was grouped according to the primer before the software estimated the baseline fluorescence, further used for the baseline correction. The exponential increase in fluorescence cycles was determined by identifying a Window-of-Linearity (W-o-L) in the log-transformed fluorescence curves. Linear regression was used to calculate the PCR efficiency from the W-o-L for each primer pair.

The resulting data from LinRegPCR was exported to qBase+ v3.3 (Biogazelle, Zwijnaarde, Belgium). In qBase+, the input cDNA in the PCR reaction was normalised according to the reference genes. The relative gene expression was calculated following the procedure explained by Hellemans et al. (2007) (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007). The average PCR efficiency for each amplicon group, the Cq values and sample names were input for the calculation. The samples from the same concentrations and timepoints were paired. A one-way analysis of variance (one-way ANOVA) with a significance level of 0.05 was performed. The Tukey-Kramer posthoc analysis was used to compare all the possible pairings of the different genes. Following this, qBase+ produced a table of pairwise comparisons of all the possible combinations of relative gene expressions scaled against the expression in the seawater control.

4 Results

4.1 Salmon louse activity

4.1.1 Number of active lice

The number of active lice was observed for each concentration at each time point. In the seawater control, 40 lice were active after 1 hour, 35 lice were active after 6 hours, and 30 lice were active after 24 hours (**Fig. 6**). The DMSO control showed a similar trend, with 35 active lice after 1h and 30 active lice after 6h and 24h (**Fig. 7**). No active lice were detected in EtOH control at any time point (**Fig. 6**).

At the 5 µg/mL concentration, lice exposed to sinigrin had 40 active lice at 1h, 30 lice were active at 6h, and 17 were active after 24h (**Fig. 7**). For higher concentrations, there was only one active louse after 1h in 7.5 µg/mL sinigrin (**Fig. 7**). There were no active lice at all the other time points and higher concentrations of sinigrin. In the AITC treatment, no active lice were observed (**Fig. 6**).

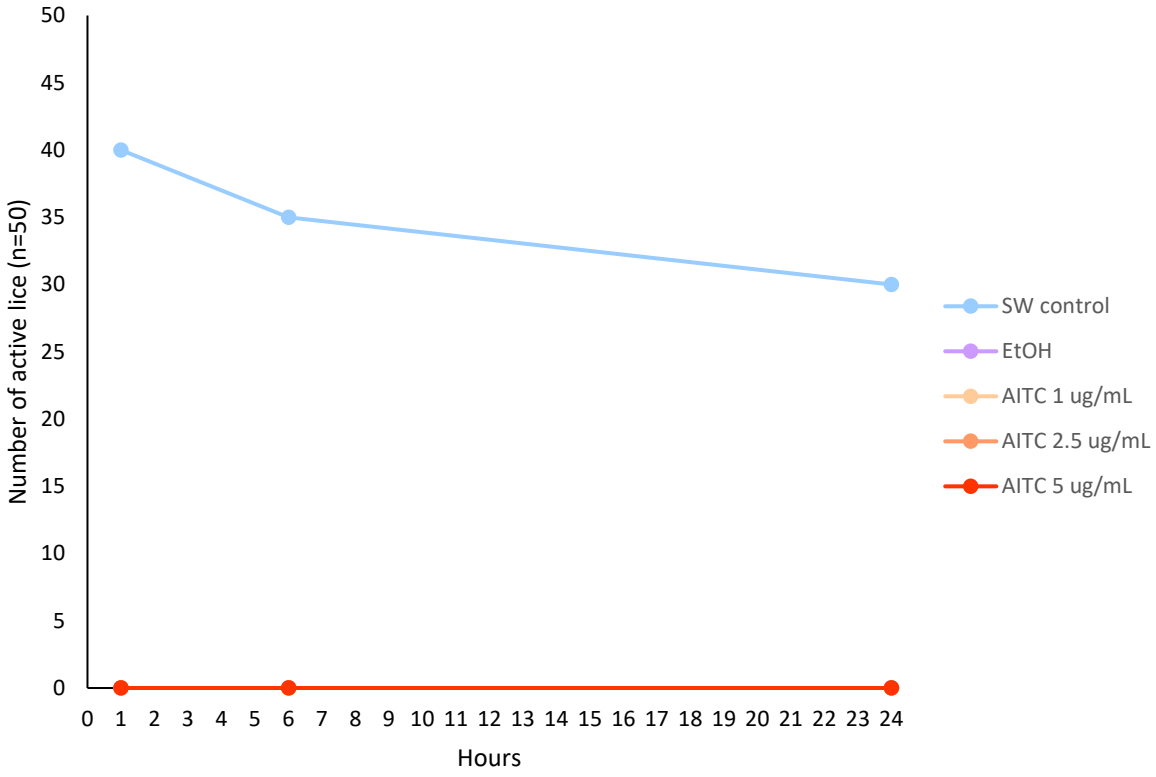

Figure 6: Number of active lice after exposure to AITC

The seawater control samples had 40 active lice after 1h, 35 were active after 6h, and 30 lice were active after 24h. No activity in the EtOH control or any of the AITC concentrations was observed.

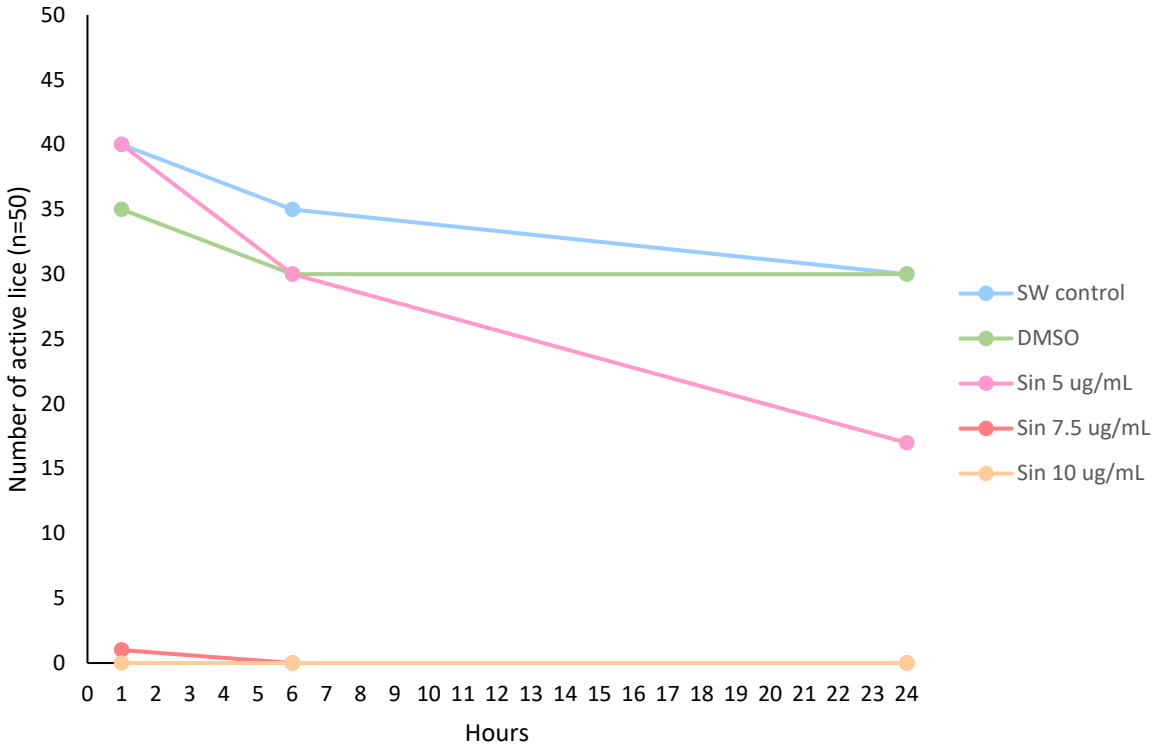

Figure 7: Number of active lice after exposure to sinigrin

In the seawater control, 40 lice were active after 1h, 35 were active after 6h, and 30 were active after 24h. The DMSO control had 35 active lice after 1h and 30 active lice after 6h and 24h. The sinigrin concentration of 5 µg/mL had 40 active lice at 1h, 30 lice were active at 6h, and 17 were active after 24h. In the 7.5 µg/mL concentration of sinigrin, there was 1 active louse after 1h and no active lice for the other time points. In the 10 µg/mL concentration, there were no active lice for any of the time points.

4.1.2 Neutral red

The colour of the neutral red treated copepodites were observed under a microscope (ZEISS AxioZoom v1.6). The control with the killed copepodites exposed to neutral red showed no colour (Fig. 8). Neither did the copepodites exposed to neutral red before death. The copepodites exposed to neutral red while alive and then flash-frozen and treated with HCl had a light pink colour. In the sample with both live and dead copepodites, the difference between the two was hard to distinguish (Fig. 8)

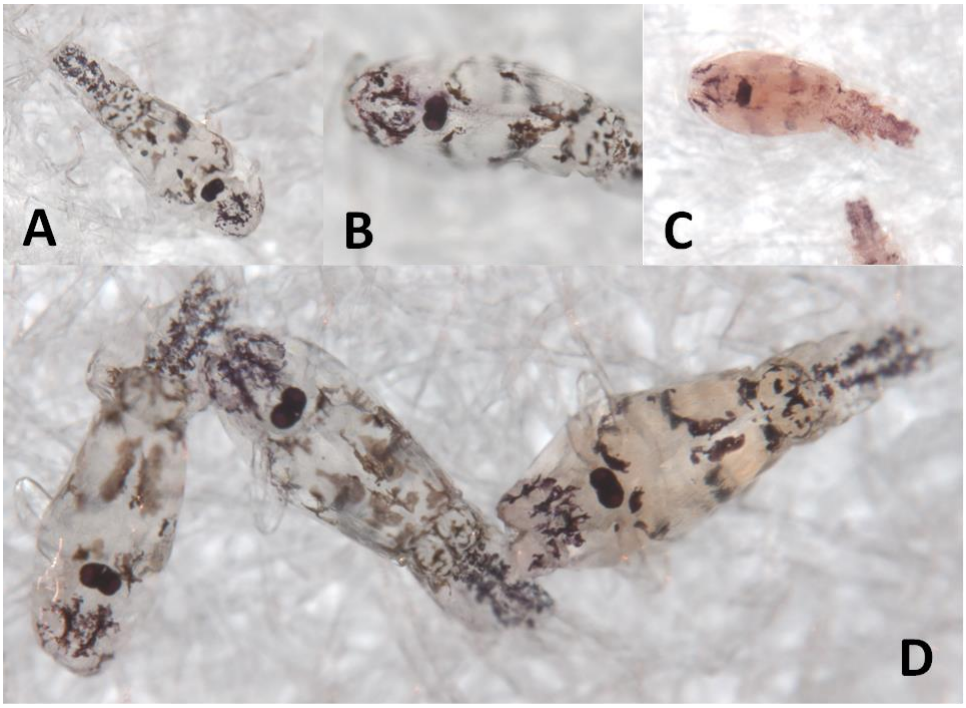

Figure 8: Staining of *L. salmonis* copepodites with neutral red

The appearance of neutral red treated *L. salmonis* copepodites under a microscope (ZEISS AxioZoom v1.6).

A: Killed copepodites exposed to neutral red after death

B: Copepodites exposed to neutral red before being killed with heat

C: Alive copepodites exposed to neutral red before flash-freezing and exposure to HCl

D: A combination of dead and live lice exposed to neutral red, killed with heat.

4.2 Gene expression analysis

The gene expression in the salmon lice was measured using qPCR. The genes of interest in the analysis were cytochrome P450 18a1 (*CYP18a1*), cytochrome P450 2j3 (*CYP2j3*), death-associated inhibitor of apoptosis 2 (*DAIA-2*), dedicator of cytokinesis protein 7 (*DCP*), glutathione s-transferase Mu3 (*GST-mu3*), protein regulator of cytokinesis 1 (*PRC*), suppressor of cytokine signalling 4 (*SCS*) and TNF receptor-associated factor 4 (*TNFaf-4*). Primer sequences can be seen in Table 1.

Table 1: qPCR primers. Primer sequences used in qPCR for amplification of each gene in the gene expression analysis

Gene	Direction	Sequence	Encoded protein
ADT3	Forward Reverse	CTGGAGAGGGAATTTGGCTAACGTG ACCCTGGACACCGTCAGACTTCACG	ADP/ATP carrier protein
CYP18a1	Forward Reverse	GGTAGGTCAACGCAAATGTC TTACGGTTACACCAGCAGTT	Cytochrome P450 18a1
CYP2j3	Forward Reverse	GACGCAAAAGTAGGGGACTA CCGGATTGAACTGATCTGGA	Cytochrome P450 2j3
DAIA-2	Forward Reverse	CTTCTCATGGAGCTGGAGTC CAGAACATTGCACCAGGAAC	Death-associated inhibitor of apoptosis 2
DCP	Forward Reverse	AGATCTGTCTCTCCATCACCT CCAACAAGGAAATGTTGTCGT	Dedicator of cytokinesis protein 7
eEF1α	Forward Reverse	GGTCGACAGACGTACTGGTAAATCC TGCGGCCTTGGTGGTGGTTC	Translation elongation factor 1α
GST-mu3	Forward Reverse	TTTCCGCAATGGCATAGTTC TTCTCCCGTAAACCAAGGAC	Glutathione S-transferase Mu3
PRC	Forward Reverse	GTTGAGACCGTTAGAGCAGT GCAAGTCACTTCAACCAAGT	Protein regulator of cytokinesis 1
SCS	Forward Reverse	GCCAGATCCGTGATATGTGA GCCACCTTGTGATTGTAGTG	Suppressor of cytokine signalling 4
TNFaf-4	Forward Reverse	TCCTCCAACTTCCTTCATCG GATTTGGACATGATACGGCG	TNF receptor-associated factor 4

The samples exposed to AITC showed a significant gene expression of the genes *CYP2j3* ($p<0.001$) and *TNFaf-4* ($p<0.001$) (Fig. 9). The expression of *CYP2j3* was shown to be highest in the 1 µg/mL concentration of AITC after 6h. While for *TNFaf-4*, the highest expression was found in the 1 µg/mL concentration of AITC after 24h. No expression was observed in the EtOH treated lice.

The samples exposed to sinigrin showed a significant gene expression of *GST-mu3* ($p=0.0061$) and *TNFaf-4* ($p=0.0107$) (Fig. 10). The gene expression of *GST-mu3* was highest in the seawater control after 1h, while the gene expression of *TNFaf-4* was highest in the seawater control after 6h. Gene expression was also observed in the 24h sinigrin 5 µg/mL concentration and the 1h and 6h seawater control. No expression was observed in any of the other concentrations or time points.

The analysis indicated no significant change in gene expression of *CYP18a1*, *DAIA-2*, *DCP*, *GST-mu3*, *PRC* or *SCS* in the AITC treated lice (Fig. 11). There was neither an indication of significant gene expression of the genes *CYP18a1*, *CYP2j3*, *DAIA-2*, *DCP*, *PRC* or *SCS* in the sinigrin treated lice (Fig. 12).

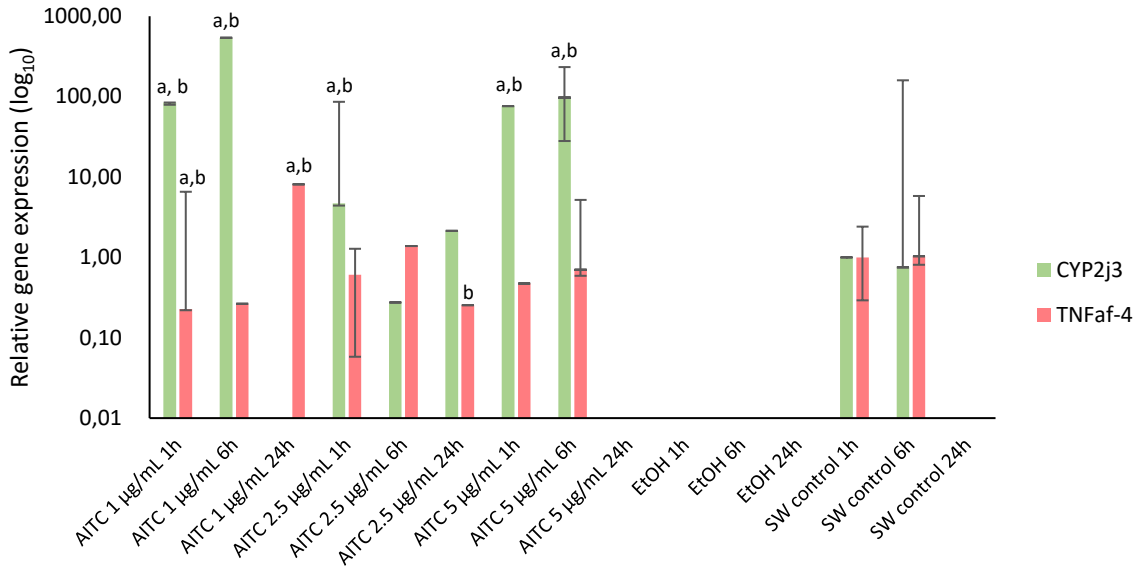

Figure 9: The relative gene expression in *L. salmonis* copepodites exposed to AITC

The relative gene expression levels of the genes *CYP2j3* and *TNFaf-4* ($p<0.001$) were found to be significantly up-regulated compared to the control treatments (seawater) and are denoted with different letters (a = significantly different from SW control 1h, b = significantly different from SW control 6h). The highest relative gene expression of *CYP2j3* was observed in 1 µg/mL concentration of AITC after 6h. The highest relative gene expression of *TNFaf-4* was observed in 1 µg/mL concentration of AITC after 24h. A one-way ANOVA with the Tukey-Kramer posthoc test compared the relative gene expression. Error bars show the standard error. n=150 for all treatments.

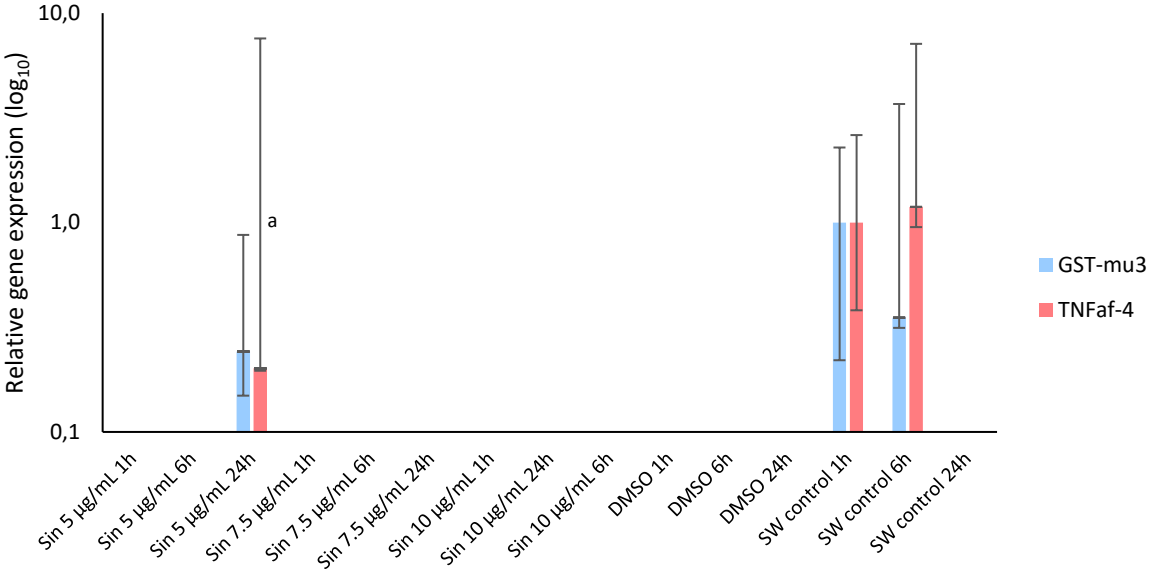

Figure 10: The relative gene expression in *L. salmonis* copepodites exposed to sinigrin

The relative gene expression levels of the genes *GST-mu3* ($p=0.0061$) and *TNFaf-4* ($p=0.0107$) were found to be significantly up-regulated compared to the control treatments (seawater) and are denoted with different letters (a = significantly different from SW control 1h. The highest relative gene expression of *GST-mu3* was observed in seawater control after 1h. The highest relative gene expression of *TNFaf-4* was observed in the seawater control after 6h. A one-way ANOVA with the Tukey-Kramer posthoc test compared the relative gene expression. Error bars show the standard error. n=150 for all treatments.

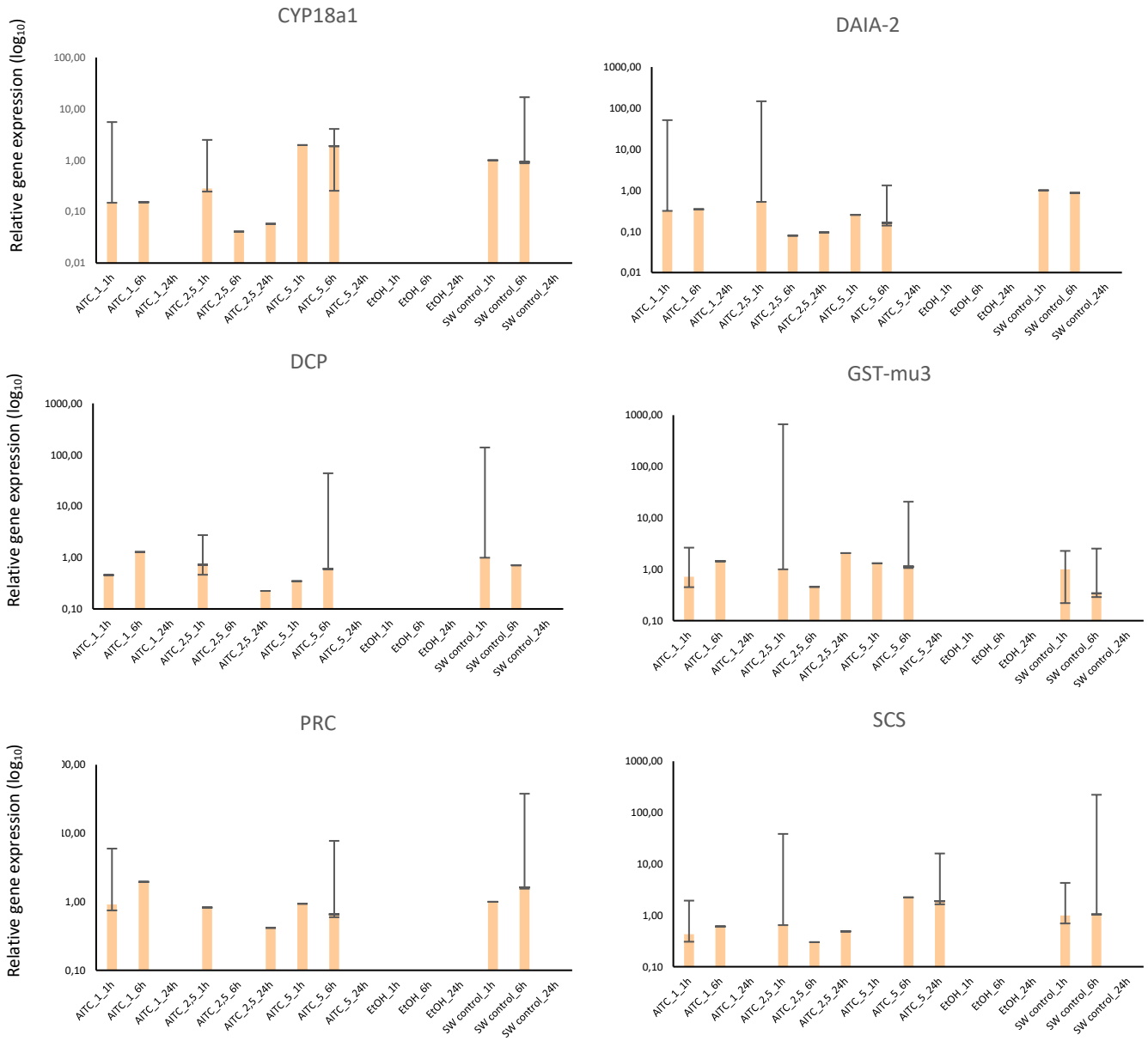

Figure 11: The relative gene expression in *L. salmonis* copepodites exposed to AITC

The relative gene expression levels of the genes *CYP18a1*, *DAIA-2*, *DCP*, *GST-mu3*, *PRC* and *SCS* ($p>0.05$) were found not to be significantly expressed compared to the control treatments (seawater). A one-way ANOVA with the Tukey-Kramer posthoc test compared the relative gene expression. Error bars show the standard error. n=150 for all treatments.

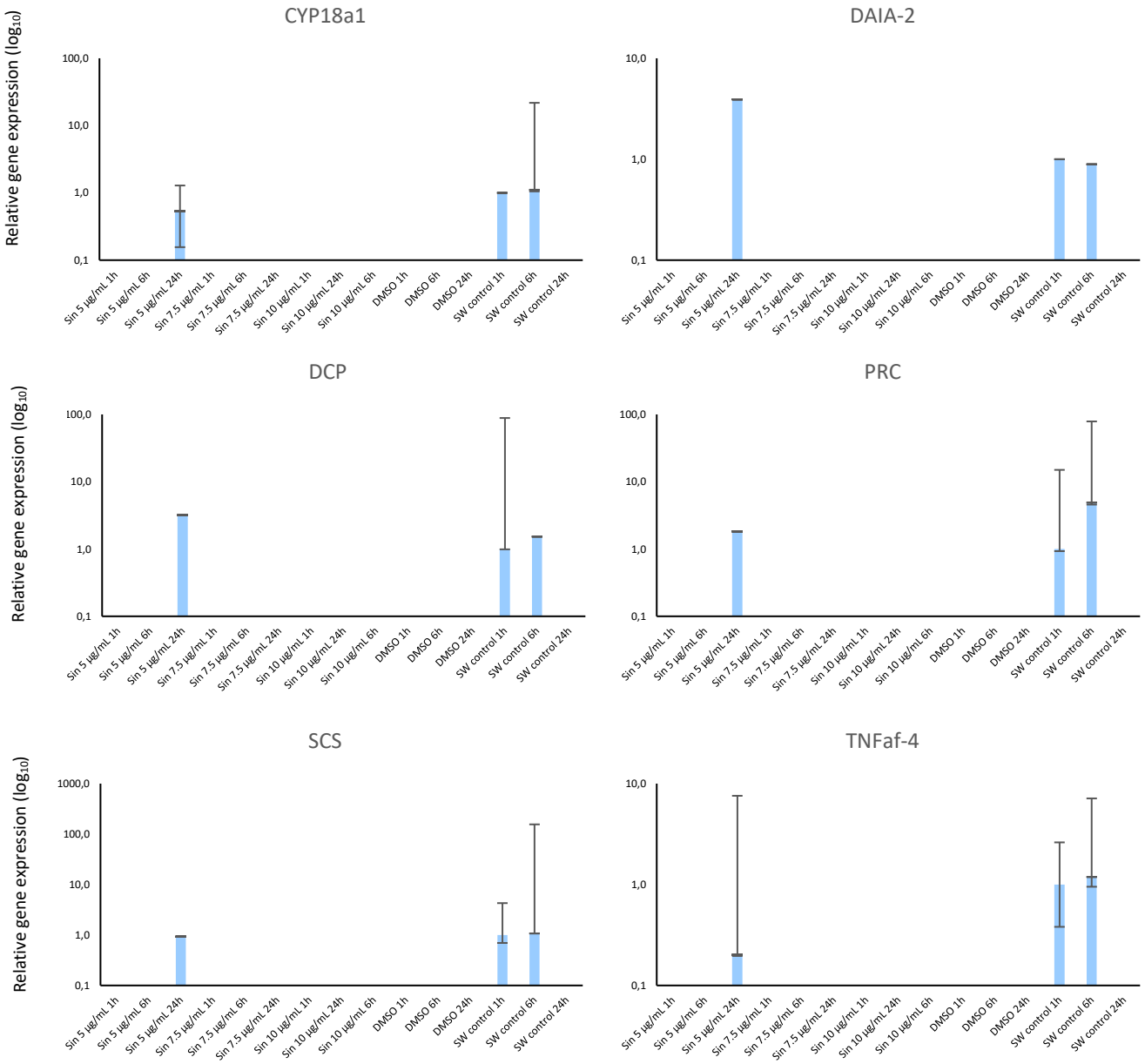

Figure 12: The relative gene expression in *L. salmonis* copepodites exposed to sinigrin

The relative gene expression levels of the genes *CYP18a1*, *DAIA-2*, *DCP*, *PRC*, *SCS* and *TNFaf-4* ($p>0.05$) were found not to be significantly expressed compared to the control treatments (seawater). A one-way ANOVA with the Tukey-Kramer posthoc test compared the relative gene expression. Error bars show the standard error. n=150 for all treatments.

5 Discussion

5.1 The number of active lice decreased with higher concentrations of sinigrin and longer time periods

During this study, the change in the activity of the lice was observed during the different time points (1h, 6h and 24h) after three different concentrations of AITC (1 µg/mL, 2.5 µg/mL, and 5 µg/mL) and sinigrin (5 µg/mL, 7.5 µg/mL, and 10 µg/mL). The results showed that the number of active lice decreased over time, hence increasing the number of immobilised lice. This is shown both in the control samples and in the exposed samples. For the seawater control, the number of active lice decreased from 40 after 1 hour to 35 after 6 hours and to 30 after 24 hours. In the DMSO control, there was also a decrease from 35 active lice after 1 hour to 30 active lice after 6 and 24 hours. This decline in active lice may be a result of the lice being kept in a closed microcentrifuge tube without a steady supply, or movement, of fresh seawater, resulting in a lack of enough dissolved oxygen.

Exposing lice to sinigrin also decreased the number of active lice. There was a higher number of lice active after 1h than at the later time points. The lowest concentration of sinigrin had the most active lice, with 40 active lice after 1h, 30 active lice after 6h and only 17 active lice after 24h. Here, the number of active lice follows the same trend as the control samples before a steep decline between 6h and 24h. This suggests that the concentration (which was the lowest in the study at 5 µg/mL) needs a longer time for the copepodites to accumulate a potentially harmful concentration. For the second concentration of the sinigrin (7.5 µg/mL), there was only 1 active louse after 1h and no active lice at the other time points. This suggests that the 7.5 µg/mL concentration inactivates the copepodites more than the lower concentration. This further supports the hypothesis that higher concentrations of sinigrin have more effect on the lice than the lower concentrations.

The other control treatment applied in this study was ethanol (EtOH). None of the lice exposed to this treatment showed any indication of being active at any of the time points. The same was observed for the lice samples exposed to AITC. AITC is not soluble in water, and for this experiment, the substance was dissolved in 90% EtOH. The total amount of EtOH in the different samples was 1.2% EtOH in the 5 µg/mL, 0.6% EtOH in the 2.5 µg/mL sample, and

0.24% in the 1 µg/mL sample. In the human cancer cell lines MDA-MB-231, MCF-7 and VNBRCA1, ethanol had a harmless effect on the cell lines in concentrations up to 2% (Nguyen, Nguyen, & Truong, 2020). Considering this, the concentrations exposed to the copepodites were lower, suggesting that the copepodites should not have been affected. In another human cell line, the HT22 cell line, however, it was observed that a concentration of 1% EtOH had a significant reduction in cell proliferation, thus suggesting an important degree of toxicity to the cells (Casañas-Sánchez, Pérez, Quinto-Alemany, & Díaz, 2016). This study indicates that the concentrations of ethanol in the samples could negatively affect the cell proliferation of the copepodites, possibly explaining the immobility. However, the effect of EtOH on arthropods may differ from the effect on different human cell lines.

Only the highest amount of EtOH (1.2%) was used in the control in this experiment. Therefore, the lower amounts of alcohol, and a lower total percentage of alcohol in the samples, were not explored. By not doing this, the question of the effect of EtOH in the lower percentages remains unexplored. This could have been controlled by using a dilution series. A dilution series would have allowed for closer monitoring of the copepodites' response to the ethanol, and the immobilisation could have been avoided or better explained. As it now stands, the effect observed in the samples exposed to the lower concentrations of AITC (and the lower total percentage of EtOH) cannot solely be attributed to the effects of AITC. Therefore, the level of activity and the effect of AITC on the copepodites remains inconclusive.

Furthermore, AITC could have been dissolved in another compound than EtOH to see if this would affect the lice differently. AITC is solvable in most organic solvents (O'Neil, 2006). Therefore, other solvents could also have been explored. AITC should, for instance, be solvable in vegetable oil. A dilution series should still have been performed to assert the effect of the vegetable oil on the copepodites. This would open the possibilities of finding a better solvent and exploring the different effects of AITC with a different solvent. As it now stands, the possibility of a synergistic or additive effect of AITC and EtOH on the copepodites cannot be ignored. Exploring these possible effects is important, as all possible interactions between AITC and the solvent need to be studied before AITC can even be suggested as a biopesticide for *L. salmonis*.

There was only one replicate for the quantification of the activity of the salmon lice. The use of one replicate was to limit the lice handling and reduce the amount of possible damage to the RNA. Consequently, the number of lice observed could be argued to not reflect the actual level of activity due to the low replicability. If the experiment were to be performed again, it would be recommended to have more replicates of the activity to better quantify the number of active lice. The copepodites could have been monitored differently to quantify the lice activity better. By putting them in a petri dish with a grid system and an overhead camera, the movement of the active lice could have been measured more in detail, making it easier to go back and check the activity. However, this approach would bring on additional analyses and data handling, and it still does not answer whether the lice are immobilised or dead.

5.2 AITC may lead to activation of detoxification metabolism in copepodites of *L. Salmonis*

Copepodites of the salmon louse *L. salmonis* were treated with three different concentrations of AITC (1 µg/mL, 2.5 µg/mL, and 5 µg/mL) for three different time points (1h, 6h and 24h). The gene expression of each concentration at each time point was measured using qPCR. The results showed that only two genes were differentially expressed in each treatment (normalised using the housekeeping genes *ADT3* and *eEF1α*). For the AITC treatment, the two genes that were significantly up-regulated were *CYP2j3* and *TNFaf-4*.

The *CYP2j3* gene is a member of the cytochrome P450 gene family. Members of this family participate in metabolising harmful substances, for instance, pesticides (Humble et al., 2019). Metazoan CYPs are membrane-bound and catalyse a wide range of reactions (Humble et al., 2019). These reactions metabolise endogenous and exogenous compounds, in this case, AITC. CYPs also contribute to the organism's defence against xenobiotics, involving them in metabolic detoxification (Humble et al., 2019). This suggests that the activation of *CYP2j3* in the copepodites may indicate that AITC is recognised as an exogenous molecule by the organism, hence triggering the detoxification process. This is further in line with the notion that the CYP superfamily includes genes classified as environmental response genes, as they code proteins involved in interactions external to the organism (Humble et al., 2019). Additionally, the other CYP gene expression in this study, *CYP18a1*, was also activated in some of the samples (Fig. 12). The results showed no significant gene expression (p=0.069), but this further suggests that some form of detoxification is activated in the copepodites.

The other gene found to be significantly up-regulated in the AITC treatment was the TNF receptor-associated factor 4 (*TNFaf-4*). This gene modulates various responses, including inflammatory and immune-regulatory responses, antiviral responses, cell proliferation and growth inhibition (Bradley & Pober, 2001). TNF pathways have been found to be activated in the skin of Atlantic salmon during infection with *L. salmonis* (Caballero-Solares et al., 2022; Tadiso et al., 2011). It has also been suggested that early and high activation of pro-inflammatory genes, such as genes in the TNF pathways, work as a mechanism of rapid louse-rejection (Fast, Johnson, & Jones, 2007; Robledo, Gutiérrez, Barría, Yáñez, & Houston, 2018). The activation of this gene in the copepodites suggests that some form of the immune system

is activated. This would further suggest that AITC could be seen as a threat to the organism's survival. This is not unexpected, as AITC is a result of the "chemical bomb" that forms when a glucosinolate and myrosinase react and has severe effects on insects and aquatic herbivores (Halkier & Gershenzon, 2006; Newman et al., 1996).

The results also show gene expression of *CYP2j3* and *TNFaf-4* in the seawater control samples. The relative gene expression is at a similar level between the two genes, but it is at a lower level for *CYP2j3* than in the AITC exposed samples. This may suggest that the AITC further increases the detoxification response but that the lice already experience some form of stress, even in the control samples. The expression of *TNFaf-4*, however, might be contributed to other effects. As mentioned earlier, the *TNFaf-4* gene modifies inflammatory and immune-regulatory responses, but it also plays a part in cell proliferation (Bradley & Pober, 2001). In this study, copepodites were used. This is still an early stage in the salmon lice life cycle (Hamre et al., 2013), and cell proliferation would therefore be expected as a part of the growth of the lice.

The up-regulation of *CYP2j3* in the control samples shows some stress present that may lead to the activation of the detoxification. However, the expression of *CYP2j3* is much higher in the AITC exposed samples than in the control samples. This indicates that the presence of AITC triggers the detoxification system more than what is present as a stressor in the controls. In contrast, the expression of *TNFaf-4* is primarily higher in the control samples, except for the AITC 24h sample. The reason for this could be related to the life cycle of the salmon louse, as discussed above.

A concern regarding the AITC exposed samples in this study revolved around the immobilisation observed when the copepodites were exposed to the EtOH control. The worry was that the immobilisation and gene expression observed in the AITC samples would be due to the EtOH, not the AITC. However, the control with the EtOH did not show any gene expression. It is, therefore, possible to argue that the response shown is due to the AITC and not the EtOH. Although this is observed, it still needs to be explored further before something conclusive can be said.

5.3 Challenges related to neutral red

In studies looking at marine zooplankton, the determination of dead animals can be challenging. Inspection of preserved animals can show some clear signs of death through visible signs of damage or decomposition, but this is a subjective and time-consuming method (Elliott & Tang, 2009). Additionally, this method does not easily distinguish between recently dead and live animals. Examination under the microscope can also be challenging because the immobilised organisms show little to no movement other than the occasional twitching of an antenna (Geest, Burridge, Fife, & Kidd, 2014). The challenges with determining the death of zooplanktons, including sea lice copepodites, also mean that it is hard to say for sure whether not the concentration applied kills the organisms.

A neutral red uptake assay can determine the number of viable cells in a culture and allows an in vitro quantification of xenobiotic induced cytotoxicity (Ates, Vanhaecke, Rogiers, & Rodrigues, 2017; Repetto, del Peso, & Zurita, 2008). The assay relies on living cells' ability to incorporate and bind the neutral red in their lysosomes (Ates et al., 2017; Repetto et al., 2008), thus showing an accumulation of red colour in the living cells and no colouration in the dead cells. The use of neutral red to stain copepodites was described by Dressel et al. in 1972 (Dressel, Heinle, & Grote, 1972), but there have been raised questions about the limitations of the method (Elliott & Tang, 2009). Neutral red staining has been applied to different zooplankton species such as *Acartia tonsa, Eurytemora affini* and *Acartia hudsonica* (Dressel et al., 1972; Elliott & Tang, 2009; Geest et al., 2014). The copepodites observed in the study performed by Elliott and Tang (2009) were coloured bright red in part or all of their tissues, while animals dead before staining appeared white, cloudy or light pink (Elliott & Tang, 2009). It has not yet been tested in *L. salmonis*.

In this study, none of the copepodites appeared bright red (Fig. 8). The live copepodites exposed to the neutral red dye appear no different than the copepodites killed before treatment with neutral red. The copepodites killed with liquid nitrogen and treated with HCl showed a pink hue in the entire animal, but it did not show the bright red colour described by Elliott and Tang (2009). The lack of difference in colourisation between the live and killed copepodites was also observed in the combined sample. The difference in staining between the two treatments was almost impossible to separate. Some individuals presented a light

pink colour in some parts of the body, but it was still not a strong enough foundation to conclude a difference between the two treatments. These results are in stark contrast to the results found by both Elliott and Tang (2009) and Geest et al. (2014). In the study by Geest et al. (2014), copepodites of *A. hudsonica* were exposed to deltamethrin, cypermethrin and hydrogen peroxide. They found that almost all organisms were stained with neutral red, including those immobilised at high test concentrations, which indicates that the organisms were still alive (Geest et al., 2014). These findings further highlight the importance of differentiating between immobilised and dead organisms.

Upon further inspection of the results and the methodology, it became clear that the compound used in the salmon lice experiment was a natural red dye, not a neutral red dye. The dye used was orcein, which is used to dye and demonstrate elastic fibres, hepatitis B surface antigen, copper-associated proteins, and sulphated mucins (Forrester, Dick, McMenamin, Roberts, & Pearlman, 2016; Henwood, 2003; Kanel & Korula, 2011). This dye is prepared by solving 0.1-1% orcein in 70% ethanol containing 1% HCl (Henwood, 2002, 2003). The use of orcein instead of a neutral red, like toluylene red, explains why the copepodites did not exhibit the same colourisation as the copepodites in the study by Elliott and Tang (2009) and Geest et al. (2014). The pink hue observed in the HCl treated lice can be explained by how the orcein stain is typically prepared in a solution with EtOH and HCl. The lack of colourisation overall can therefore solely be attributed to the use of the wrong materials. To acquire the correct dye and run new experiments testing the dye was deemed time-consuming. Consequently, the experiment of using neutral red was terminated, and the focus shifted to the quantification of active versus immobilised instead. Nevertheless, the use of neutral red as a way of determining live and dead copepodites in a solution holds promise and is something that should be explored further.

5.4 Gene expression of *GST-mu3* and *TNFaf-4* are highest in the seawater control of the sinigrin exposed samples

Samples exposed to sinigrin only had a significant up-regulation of the genes *GST-mu3* and *TNFaf-4*.The GST superfamily consists of enzymes involved in detoxification processes (Roncalli et al., 2015). However, the results show that the highest expression of *GST-mu3* can be found in the seawater control samples. These samples have not been exposed to anything other than filtered seawater and should therefore not have a detoxification process initiated. However, Roncalli et al. (2015) found a relatively high expression of GSTs in late copepodites and adult females in the crustacean *Calanus finmarchicus* (Roncalli et al., 2015). GSTs play a role during development, as shown in the insects *Mayetiola destructor*, *Lucilia cuprina* and *Agrilus planipennis* (Mittapalli, Neal, & Shukle, 2007; Pal, Sanil, & Clark, 2012; Qin et al., 2013; Rajarapu & Mittapalli, 2013). It is presumed that the peak in expression in these insects is a response to an increase in metabolic activity and apoptosis associated with the morphological changes that occur during the morphological rearrangements (Mittapalli et al., 2007; Pal et al., 2012; Rajarapu & Mittapalli, 2013). It could be possible that a similar process is happening in the copepodites of *L. salmonis*. This would explain why *GST-mu3* is expressed in the seawater control samples without toxicants. The expression of *TNFaf-4* in the control samples for sinigrin treatment can be contributed to the cell proliferation and not to the sinigrin.

On the other hand, if the expression of *GST-mu3* and *TNFaf-4* can be contributed to the morphological arrangements and cell proliferation in the copepodites, why are not the genes expressed in the other concentrations or at the other time points? If these were processes present at this developmental stage in the salmon lice, it would be expected that it was present regardless of treatment. The lack of gene expression of these genes at the other time points and treatments could suggest that they are not involved in cell proliferation or morphological rearrangements. This does not, however, explain why they are expressed. Therefore, it would suggest that the variations stem from elsewhere. One possible explanation for this could be variations in development between the copepodites. Even if the sea lice used in this study were all copepodites, they were not treated at the same time after moulting, meaning that some of the copepodites may be later in the developmental stages and some in the earlier stages. Therefore, the gene expression could vary whether the copepodite was closer to the nauplii stage or the chalimus stage.

There are also great differences between GLs and ITC regarding their specific biological effects (Zinoviadou & Galanakis, 2017). Sinigrin is a pure glucosinolate and a precursor to AITC (Mazumder et al., 2016). This means that it does not inhabit the same toxic or pathogenic effects as AITC. Considering this, it is unsurprising that the gene expression in the samples exposed to sinigrin is different. It is also not surprising that genes related to detoxification were not expressed. A study by Lozano-Baena et al. (2015) observed that the fly *Drosophila melanogaster* fed with Ethiopian mustard (*Brassica carinata*) containing sinigrin had no significant effect on *D. melanogaster* survival (Lozano-Baena et al., 2015). Furthermore, the study also showed that none of the concentrations they applied had any mutagenic or genotoxic effects on *D. melanogaster* (Lozano-Baena et al., 2015). This is in concordance with previous studies done with sinigrin, where sinigrin was found to be neither genotoxic nor cytotoxic in the *in vitro* hamster ovary cell line system (Musk, Smith, & Johnson, 1995). In contrast, toxicity tests performed on the nematode *Caenorhabditis elegans* showed that sinigrin was non-toxic up to the concentration of 80 g/L (Donkin, Eiteman, & Williams, 1995). Additionally, this study also showed that the addition of myrosinase increased the toxicity of sinigrin (LC_{50} = 0.5 g/L). The same study performed directly with AITC resulted in a lethal concentration of 0.04 g/L (Donkin et al., 1995). The difference in the effects of sinigrin and AITC is in concordance with the results found in the *L. salmonis* copepodites. The copepodites exposed to sinigrin had a different number of active lice and a different gene expression than those exposed to AITC. The lethality of either of the compounds was not determined in this study and is something that should be explored further.

5.5 The lack of gene expressions in many samples posed a challenge

The genes explored in this study span a variety of cellular processes. Many of the genes were not expressed, and only a very few were significantly differently expressed from the housekeeping genes (Fig. 9-12). The main challenge in this study was related to the Cq-values (Appendix, Table A.3). The Cq-values were in many of the samples very high. This could mean that the gene expression of the target genes was low, making it difficult to run an analysis. The low gene expression could mean that the stressors AITC and sinigrin did not affect the processes the genes are involved in. However, other factors could also affect the lack of gene expression. The difference in gene expression could stem from the distribution of cDNA in the samples being uneven, leading to lower Cq-values in the samples run with a large amount of cDNA and a higher Cq-value in the samples with lower content. Another consequence of the variations in Cq-values was the spread in the standard error. Many of the samples were excluded due to their high Cq-value. Therefore, there were little data left. By performing more experiments, this variation could have been counteracted. However, this study had limited lice resources due to the experiments being performed in a shared facility.

Cq-values might not be the optimal way of reporting the expression of the genes. Cq-values are the fractional numbers of cycles needed for the qPCR fluorescence to reach a quantification threshold (Ruiz-Villalba, Ruijter, & van den Hoff, 2021). Different circumstances like PCR efficiency, starting material, PCR artefacts, and pipetting errors sampling variations can influence these values (Ruiz-Villalba et al., 2021). A common rule of thumb for Cq-values is that with an input of 10 template copies in the reaction and a PCR efficiency between 1.8 and 2, a Cq-value of approximately 35 will be observed (Ruiz-Villalba et al., 2021). However, the difference between 1.8 and 1.9 in PCR efficiency can yield a very different number of cycles. So even though the Cq-value is related heavily to the PCR efficiency, the relation also heavily depends on the PCR efficiency, where amplification with a low efficiency takes more cycles to reach the quantification threshold (Ruijter et al., 2021). In this experiment, all genes had a PCR efficiency of over 1.8. However, most of the genes with a significantly different gene expression had a PCR efficiency of over 1.9 (the PCR efficiency for this experiment can be seen in Appendix, Table A.5). Therefore, excluding samples with a Cq-value over 35 might not have been the suitable threshold for this experiment.

The problems with the gene expression, or the lack thereof, could possibly be traced back to the RNA concentration in the samples after RNA isolation. However, this was controlled for by running the samples through a NanoDropTM One/One© Microvolume spectrophotometer. All the samples were run through NanoDrop before using them in the cDNA synthesis (Appendix, Table A.4). Although the RNA concentration was controlled for, it is still possible that RNA degradation could have affected the RNA concentration afterwards.

Another factor that could have contributed to the variation in RNA expression is the heterogeneity of the materials. The accuracy of the gene expression is dependent on the purity of the samples, which can be substantially impacted by the heterogeneity (Kukurba & Montgomery, 2015). By using samples consisting of multiple cell types, the heterogeneity can be considerable, thus impacting the measured gene expression. In the experiments performed in this study, the entire copepodite was used to extract the RNA. The gene expression could differ between the different cell types in the copepodites, affecting the final measured RNA concentration.

The *L. salmonis* transcriptome could have been explored through other techniques, like RNA-sequencing (RNA-Seq). In contrast to low-throughput methods such as qPCR, which are limited to measuring single gene transcripts, RNA-Seq provides a more detailed and quantitative view of the gene expression (Kukurba & Montgomery, 2015). However, an essential part of a successful RNA-Seq is high-quality RNA. Analysis with low-quality RNA can affect the sequencing results through uneven gene coverage and 3'-5' transcript bias, leading to incorrect biological conclusions (Kukurba & Montgomery, 2015). The employment of RNA-Seq as an analysis technique is often used to analyse differential gene expressions, allowing for the determination of the quantitative changes in expression between experimental groups (Stark, Grzelak, & Hadfield, 2019). Exploring RNA-Seq in addition to qPCR in this study could possibly have given a better explanation of how sinigrin and AITC affected the expression of the selected genes.

5.6 Challenges related to the use of biopesticides

In the management of sea lice, there seems to be a shift toward non-medical solutions in an effort to control the salmon lice infestation levels (Guragain et al., 2021; Overton et al., 2019). One of these methods is the use of biopesticides. Biopesticides are compounds used to manage agricultural pests through specific biological effects (Guragain et al., 2021; Sporleder & Lacey, 2013). The term refers to products that contain biocontrol agents such as genes or metabolites from animals, plants, bacteria, or certain minerals that control pests (Guragain et al., 2021; Sporleder & Lacey, 2013). The use of GLs as a management strategy would fall into this category, as AITC is a metabolite in the defence system of many plants. Biopesticides might possess several advantages compared to conventional pesticides, such as being less toxic to the environment and humans in addition to being biodegradable (Kaya & Vega, 2012; Sporleder & Lacey, 2013).

However, the risk of using biopesticides (as with any form of pesticide) is the possibility of the pest developing resistance against the compounds. Resistance to pesticides is defined as the development of an ability to tolerate doses of toxicants that would prove lethal to most individuals in a normal population of the same species (Aaen et al., 2015). This risk is also present in the use of AITC. The evolution of an organism's life history and virulence is a generalised adaption that the parasite employs to respond to management strategies. These strategies can impose specific selective pressures that can further drive the population in question towards resistance (Coates et al., 2021). This is well documented in terrestrial agriculture (Brattsten et al., 1986; Coates et al., 2021). Therefore, there are reasons to expect the same in marine agriculture (Guragain et al., 2021; Aaen et al., 2015). The fear and challenge related to resistance are that it can quickly render new methods obsolete, thus undermining the goal and efforts of sustainable pest control (Coates et al., 2021).

Another challenge that pest control is exposed to is the general emphasis in studies on lethality as an endpoint (Guedes, Rix, & Cutler, 2022). However, there has been a shift in focus, and now the importance of pesticide-induced hormesis has gained recognition (Guedes et al., 2022). Hormesis is a biphasic dose-response to an environmental agent, where the low dose has a beneficial or stimulatory effect while the high doses have a toxic effect (Hanniman & Sinal, 2005; Mattson, 2008). A hermetic response has been observed in many studies as a

response to insecticides (David Costantini, 2014; Guedes et al., 2022; Guedes, Walse, & Throne, 2017; Hanniman & Sinal, 2005). Sublethal exposures to insecticides may influence insecticide resistance (Guedes et al., 2017), further challenging the combat against pests. With the observation of hermetic responses to insecticides, it is reasonable to suggest that a hermetic response could be observed in *L. salmonis* as a response to pesticides. Hormetic responses can be seen as a molecular initiating event, where the initiation event is the exposure to the insecticide/pesticide. The outcome of this could, for instance, be an activation of the stress response pathways (Guedes et al., 2022). The activation of this pathway does not necessarily lead to an adverse outcome, but the outcome could include possible non-toxic and/or hormetic responses. Moreover, the extent of the stress responses in the organisms could impact the organism itself and its interactions (Guedes et al., 2022). This would be an interesting focus to apply to sea lice research.

It is not only the stress response pathway that may be activated when the organism is exposed to a pesticide. The induction of detoxification is also highly relevant and of interest regarding pesticides. The activation of detoxification systems is broadly recognised as an important pesticide resistance mechanism (Guedes et al., 2017). In these cases, the detoxification systems are often up-regulated and overexpressed. In terrestrial arthropods, insecticide resistance can be based on the enhanced expression of CYP genes (Humble et al., 2019). With these observations in terrestrial arthropods, it could be possible that an enhanced CYP gene expression could also lead to pesticide resistance in marine arthropods. However, the detoxification systems are often inducible, and this induction may still occur in resistant populations sublethally exposed to pesticides, further priming the organism against further exposure (Guedes et al., 2017). This priming may not only be reserved for the compound the organism was exposed to but may include similar compounds. This phenomenon is called hermetic priming or conditioning (David Costantini, 2014; D. Costantini, Monaghan, & Metcalfe, 2014; Guedes et al., 2017) and could possibly create considerable challenges for the salmon lice combatting.

The concept of hormesis regarding AITC as a pesticide is crucial. Even with the benefits of AITC as a biopesticide and the lower environmental and health risks associated with biopesticides (Kaya & Vega, 2012; Sporleder & Lacey, 2013), the risk of promoting resistance

is still present. This study shows that the induction of *CYP2j3* is highest in the lowest concentration and at the second time point (1 µg/mL, 6h). The gene expression increases from 1h to 6h, and there are no results available for the 24h time point. In the second concentration (2.5 µg/mL), the first time point (1h) was the highest. The following time point, 6h, has a lower expression before the expression increases again for the last time point (24h). However, the gene expression of *CYP2j3* in the 2.5 µg/mL concentration is lower for all the time points than in the 1 µg/mL concentration. The highest concentration (5 µg/mL) shows a similar trend to the lowest (1 µg/mL), with increasing gene expression over time. Still, there is the same issue as with the lowest concentration: data for the 24h time point is not available. The expression of *CYP2j3* in 5 µg/mL is lower than in the 1 µg/mL concentration. This illustrates a curve similar to the curve found in hormetic models, where the response to a chemical agent is biphasic, meaning that the response is higher in the individuals exposed to mild intensities but lower in the individuals exposed to higher intensities (David Costantini, 2014; Guedes et al., 2022; Hanniman & Sinal, 2005).

However, the hormetic trend observed in the results cannot be said to be complete without the presence of the later time points. The absence of results at the 24h time point in the 1 µg/mL and 5 µg/mL concentrations could be due to RNA degradation, either at the organism level before flash-freezing or later in the RNA-isolation process. Nevertheless, the risk of hormesis and resistance is still present, making it essential to take precautions when approaching studies exploring pesticides and biopesticides.

6 Conclusion

In this study, *L. salmonis* copepodites were exposed to various concentrations of the glucosinolate sinigrin and its derivative AITC for different time periods. The expression of genes related to immune responses, cell cycle, negative control of cellular proliferation, DNA replication and cellular division, and detoxification was explored through qPCR.

The number of active lice decreased over time in the seawater controls, and the sinigrin exposed samples. Some of the treatments did not show any activity at any time points. It was observed that the AITC treatment led to significant differences in gene expression of the genes *CYP2j3* and *TNFaf-4*. *CYP2j3* was shown to be up-regulated at several time points, suggesting an activation of the detoxification system in the copepodites. However, in this study, only two CYP genes were explored, and only one was shown to be significantly expressed. Therefore, it is necessary to perform more experiments to see if other genes in the CYP superfamily may be involved. Additionally, there is the question of the samples being activated by AITC or EtOH. This needs further exploration. The up-regulation of *TNFaf-4* suggests an activation of the immune system in the copepodites. Simultaneously, the gene was activated in the control samples, possibly suggesting involvement in cell proliferation. The copepodites are at an early stage in the salmon lice life cycle, thus possibly explaining the gene expression of *TNFaf-4* in the control samples, making it interesting to explore in further research. The sinigrin exposed samples showed an up-regulation of *GST-mu3* compared to the housekeeping genes. GSTs are involved in detoxification, but the expression was highest in the control samples where no stressor was present. However, GSTs are also a part of the developmental processes, which could explain the presence in the control samples.

This study aimed to see if sinigrin and/or AITC affect the gene expression of genes related to immune responses, cell cycle, negative control of cellular proliferation, DNA replication and cellular division, and detoxification in copepodites of *L. salmonis*. In conclusion, there seems to be an effect observed, but the uncertainty around the results is great, making it difficult to conclude if sinigrin and/or AITC affect the gene expression of a selection of genes. Therefore, the initial question is not answered, and more studies are needed before the effect can be fully understood.

6.1 Further research

The effects of biopesticides and their use in the aquaculture industry are interesting and could possibly aid in the salmon lice problem the industry is currently facing. This study explored the effect of the glucosinolates sinigrin and the precursor AITC, but the results were inconclusive and could benefit from further research. First and foremost, a proper protocol needs to be established so that the determination of death in the *L. salmonis* copepodites could be performed faster and more accurately. In this study, an attempt to use neutral red was explored, but natural red was used instead due to mistakes made in the laboratory. Therefore, more research on the use of neutral red in the determination of death in salmon lice copepodites would be interesting to explore.

The method used for quantifying the active lice in this study could also be explored further. By using cameras and taking videos, the movements could have been monitored more closely and over a longer time period. It would also be interesting to perform a recovery step with the lice that appeared immobilised. By placing the copepodites in fresh seawater and normal conditions after exposure to the stressors, one could observe whether the copepodites recovered or not, thus indicating immobility or death.

The results of this study did not give a conclusive answer to whether sinigrin and AITC affected the gene expression of the selected genes. There is an indication that genes related to detoxification might be activated, but further research is required. By focusing more on one group of genes and the processes they are involved in, a more detailed picture could be painted of the effect sinigrin, and AITC might have. By focusing on the detoxification system, for instance, the effect of glucosinolates as a biopesticide might be made more apparent.

A different approach to this study could also be explored. One other approach could be to use sealed 96-well (or another number of wells) plates on a shaker when exposing the copepodites to the stressors to simulate the movement of water. This approach would allow for more replicates with the same concentration and fewer copepodites in each well. It would also make it easier to observe the behaviour of the copepodites because of the smaller number in each well. This could prove beneficial as it makes it easier to compare the difference in movement and behaviour across concentrations directly.

Furthermore, the challenges related to using biopesticides are the possible development of resistance. Resistance to pesticides is well-documented in terrestrial agriculture and would therefore also be expected to be a part of the aquaculture industry. The fear of the development of resistance is the possibility of rendering new methods obsolete, undermining the goal of pesticides. It is important in further research to be observant of hermetic tendencies and sublethal exposure to avoid the development of resistance.

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Appendix – Supplementary tables

Table A.1: cDNA master mix. The volume of reagents used for cDNA synthesis with a total volume of 6 µL.

Reagent	Volume
Quantiscript RT	1 µL
RT buffer	4 µL
RT-primer mix	1 µL

Table A.2: qPCR Master mix. The volume of reagents used in the qPCR (Quantitative Real-time PCR) master mix with a total volume of 15 µL per reaction. 5 µL of cDNA was added to create a total volume of 20 µL. The primer mixes used are presented in Table A.3

Reagent	Volume
LightCycler® 480 Probes Master. 2X	10 µL
Forward/Reverse primer mix (5 µM each)	2 µL
Sterile MQ water	3 µL

Table A.3: Heat map showing the Cq-values of the different genes and treatments. The colours represent the number of cycles run before a real signal was detected from the samples. Over 35 cycles=red, between 30 and 35 cycles=yellow, under 30 cycles=green, and 0 cycles=blue.

	CYP2j3		CYP18a1		DAIA-2		DCP		GST-mu3		PRC		SCS		TNFaf-4	
AITC_1_1h_1	20.242	26.102	28.083	28.480	27.496	27.153	31.412	29.942	24.234	0.000	30.801	30.892	25.983	26.198	30.662	30.356
AITC_1_1h_3	21.069	27.306	30.208	28.909	29.202	28.768	34.225	31.924	24.377	0.000	30.756	30.849	26.878	26.676	31.833	31.539
AITC_1_6h_2	36.046	0.000	0.000	0.000	42.184	42.890	40.894	0.000	0.000	0.000	39.359	0.000	0.000	0.000	0.000	42.275
AITC_1_6h_3	21.682	29.696	30.974	28.508	28.938	28.759	32.716	29.424	24.002	0.000	30.536	30.309	26.692	26.717	32.363	30.960
AITC_1_24h_2	36.104	0.000	37.517	37.300	44.835	43.968	46.350	44.160	0.000	0.000	38.063	0.000	0.000	37.212	38.379	29.979
AITC_25_1h_1	36.675	28.004	27.763	27.926	27.880	27.509	31.134	30.194	24.236	24.195	32.147	32.231	25.682	26.153	29.977	28.479
AITC_25_1h_2	38.593	27.979	27.365	28.670	27.028	26.916	31.782	31.415	23.247	23.231	28.883	30.624	0.000	25.598	29.918	41.315
AITC_25_1h_3	39.078	0.000	37.261	0.000	43.797	37.365	0.000	41.042	28.869	0.000	0.000	0.000	36.645	35.584	0.000	33.825
AITC_25_6h_3	37.329	33.763	31.468	33.742	31.747	32.243	38.896	38.151	26.782	26.675	35.238	0.000	28.017	29.131	36.466	29.880
AITC_25_24h_1	37.857	30.528	30.950	32.859	31.747	31.278	34.762	34.065	24.099	23.975	32.494	34.709	27.358	27.936	33.012	31.630
AITC_5_1h_1	21.209	30.028	26.269	27.227	30.339	30.505	34.352	34.022	25.228	25.323	32.696	32.793	25.756	25.689	32.639	30.983
AITC_5_1h_2	27.968	37.535	33.473	34.277	32.933	44.216	0.000	43.195	32.041	32.180	37.793	0.000	32.591	32.045	0.000	0.000
AITC_5_1h_3	35.439	38.562	37.663	37.166	40.502	36.965	0.000	0.000	0.000	0.000	0.000	37.865	0.000	0.000	40.748	46.176
AITC_5_6h_1	25.702	30.393	31.000	32.176	33.740	33.550	39.149	38.280	29.296	29.076	36.497	37.077	30.901	30.896	36.060	32.601
AITC_5_6h_2	21.311	28.984	25.946	27.335	30.972	31.467	33.827	33.544	25.684	25.778	32.487	34.451	25.528	25.627	33.197	29.358
AITC_5_6h_3	21.033	28.617	25.862	26.978	30.912	30.756	32.998	32.472	24.996	25.202	32.662	33.266	26.155	26.199	31.990	29.167
AITC_5_24h_1	25.669	30.069	33.047	33.481	33.516	33.758	36.193	35.776	29.063	29.321	35.792	37.389	31.370	31.293	35.379	32.731
DMSO_1h_1	23.987	31.408	28.757	31.225	30.866	26.365	35.706	32.410	28.242	25.158	32.177	29.654	27.763	21.996	32.626	32.101
DMSO_1h_2	29.838	35.184	33.917	35.291	37.406	31.482	35.714	46.034	35.963	28.142	34.106	33.516	34.543	27.201	0.000	37.115
DMSO_1h_3	22.452	31.826	26.915	28.589	28.435	26.314	32.951	30.731	26.003	19.903	30.255	29.963	26.668	23.865	29.804	28.142
DMSO_6h_1	30.510	31.104	27.104	29.253	28.947	28.625	38.411	34.514	34.369	32.828	27.021	29.104	29.928	29.451	31.025	31.728
DMSO_6h_2	34.586	0.000	33.107	34.756	34.363	33.913	41.090	0.000	41.703	39.877	33.512	35.163	36.014	33.441	43.795	35.758
DMSO_6h_3	35.463	0.000	32.035	32.643	32.711	32.438	43.266	0.000	40.692	34.274	31.428	33.113	34.713	36.143	44.619	33.422
DMSO_24h_1	35.455	37.532	33.881	34.596	34.996	36.455	0.000	43.070	42.245	40.518	35.027	35.514	0.000	36.292	35.913	35.401
DMSO_24h_2	36.419	37.864	35.651	37.851	37.556	36.533	35.638	47.585	42.373	0.000	37.679	0.000	37.509	59.443	0.000	35.151
DMSO_24h_3	36.962	0.000	36.611	37.748	38.867	40.881	0.000	0.000	0.000	45.341	37.531	37.844	36.440	0.000	0.000	36.162
EtOH_90_1h	36.107	37.725	0.000	0.000	42.641	35.794	37.493	49.297	41.631	42.010	29.495	0.000	0.000	0.000	36.606	47.766
EtOH_90_6h	36.170	0.000	0.000	0.000	41.785	41.894	44.364	0.000	42.955	0.000	35.819	0.000	0.000	35.795	37.606	33.291

EtOH_90_24h	35.558	36.650	34.899	35.181	33.530	33.454	44.754	35.455	40.547	34.361	32.464	36.561	34.109	31.773	33.303	31.612
sin_5_1h_1	36.327	34.692	35.593	34.248	33.364	33.848	39.867	38.419	39.852	42.789	35.782	36.649	33.602	33.881	0.000	33.488
sin_5_1h_2	0.000	0.000	35.914	34.733	32.871	35.361	36.383	44.766	0.000	0.000	0.000	0.000	34.694	36.332	0.000	35.636
sin_5_1h_3	0.000	0.000	38.265	0.000	42.797	44.070	0.000	41.721	0.000	0.000	0.000	0.000	0.000	0.000	0.000	43.336
sin_5_6h_1	38.244	35.523	34.818	35.720	34.206	34.940	37.735	36.728	39.617	42.312	43.971	36.469	36.361	34.876	42.493	35.092
sin_5_6h_2	0.000	0.000	0.000	40.153	40.536	40.744	39.598	40.300	0.000	0.000	0.000	0.000	37.114	0.000	0.000	37.473
sin_5_6h_3	0.000	0.000	37.277	36.164	34.110	34.661	36.252	40.099	0.000	0.000	36.249	0.000	0.000	35.605	37.182	44.626
sin_5_24h_1	31.679	30.368	28.171	28.958	23.992	25.926	0.000	32.905	27.475	27.952	33.176	36.312	25.961	26.012	37.764	33.073
sin_5_24h_2	0.000	0.000	39.209	40.079	42.283	41.743	0.000	43.474	0.000	0.000	42.885	0.000	0.000	0.000	0.000	0.000
sin_5_24h_3	33.999	33.202	27.495	28.537	25.322	26.788	27.224	32.246	26.942	27.660	36.537	35.520	27.085	27.239	32.215	31.142
sin_75_1h_1	0.000	36.306	0.000	46.167	37.503	43.196	0.000	45.093	0.000	38.277	35.228	36.908	35.208	37.180	0.000	0.000
sin_75_1h_2	0.000	0.000	38.470	38.311	36.322	42.761	0.000	39.725	0.000	41.415	36.173	36.228	37.067	34.436	0.000	44.899
sin_75_1h_3	0.000	0.000	37.316	0.000	37.060	34.952	0.000	46.435	41.261	0.000	36.764	0.000	35.983	34.961	0.000	0.000
sin_75_6h_1	37.413	0.000	37.529	35.863	33.126	33.338	0.000	38.302	0.000	41.803	35.478	0.000	33.536	33.122	37.520	0.000
sin_75_6h_2	0.000	0.000	0.000	0.000	39.431	40.497	0.000	45.029	0.000	0.000	40.475	0.000	0.000	0.000	37.599	0.000
sin_75_6h_3	33.980	0.000	0.000	43.868	41.533	43.378	0.000	43.045	0.000	0.000	40.176	0.000	0.000	0.000	0.000	0.000
sin_75_24h_1	0.000	35.604	36.251	35.184	33.826	36.302	0.000	41.206	36.919	41.011	35.783	0.000	33.812	35.027	0.000	42.181
sin_75_24h_2	31.195	0.000	0.000	0.000	41.276	44.617	41.585	0.000	0.000	0.000	0.000	45.655	0.000	0.000	0.000	37.345
sin_75_24h_3	0.000	0.000	0.000	0.000	38.983	45.297	0.000	37.153	0.000	42.088	40.310	0.000	0.000	0.000	0.000	0.000
sin_10_1h_1	37.799	45.336	39.692	0.000	36.501	37.688	0.000	41.925	39.057	0.000	36.173	0.000	36.272	0.000	0.000	0.000
sin_10_1h_2	45.399	36.601	37.260	36.774	34.754	35.655	40.495	43.875	39.715	42.220	36.847	37.626	34.238	35.422	36.974	0.000
sin_10_1h_3	29.456	31.385	32.902	32.394	29.529	29.788	37.042	38.894	24.634	26.550	32.646	36.892	25.376	25.856	34.099	0.000
sin_10_6h_1	37.755	0.000	34.763	35.715	36.435	35.022	44.753	42.445	0.000	0.000	46.620	36.216	37.211	35.826	34.325	35.938
sin_10_6h_2	37.423	0.000	36.629	44.003	36.291	35.748	0.000	0.000	0.000	0.000	42.255	38.031	37.529	36.536	35.109	0.000
sin_10_6h_3	0.000	0.000	34.327	37.140	43.084	34.690	0.000	0.000	0.000	40.616	39.267	35.302	36.998	36.890	32.718	0.000
sin_10_24h_1	0.000	0.000	36.030	38.189	44.134	37.132	0.000	45.193	42.178	40.362	37.516	37.032	36.883	37.156	33.304	0.000
sin_10_24h_2	37.852	0.000	37.355	36.581	42.273	35.702	39.984	46.129	0.000	41.147	38.414	37.187	36.920	0.000	36.642	0.000
sin_10_24h_3	0.000	0.000	0.000	0.000	42.115	36.133	41.559	0.000	0.000	0.000	42.666	0.000	0.000	36.477	36.950	41.653
wc_1h_1	29.067	29.226	26.626	21.041	27.757	26.586	29.912	34.570	23.062	24.119	33.594	31.714	25.326	24.971	28.209	29.125
wc_1h_2	35.643	36.356	31.928	31.587	35.720	34.014	35.528	40.599	31.075	31.431	0.000	43.529	31.572	31.656	35.508	35.419
wc_1h_3	31.437	31.894	28.829	27.625	28.215	23.125	31.555	37.352	23.742	24.459	35.000	29.825	25.595	24.956	26.588	31.739

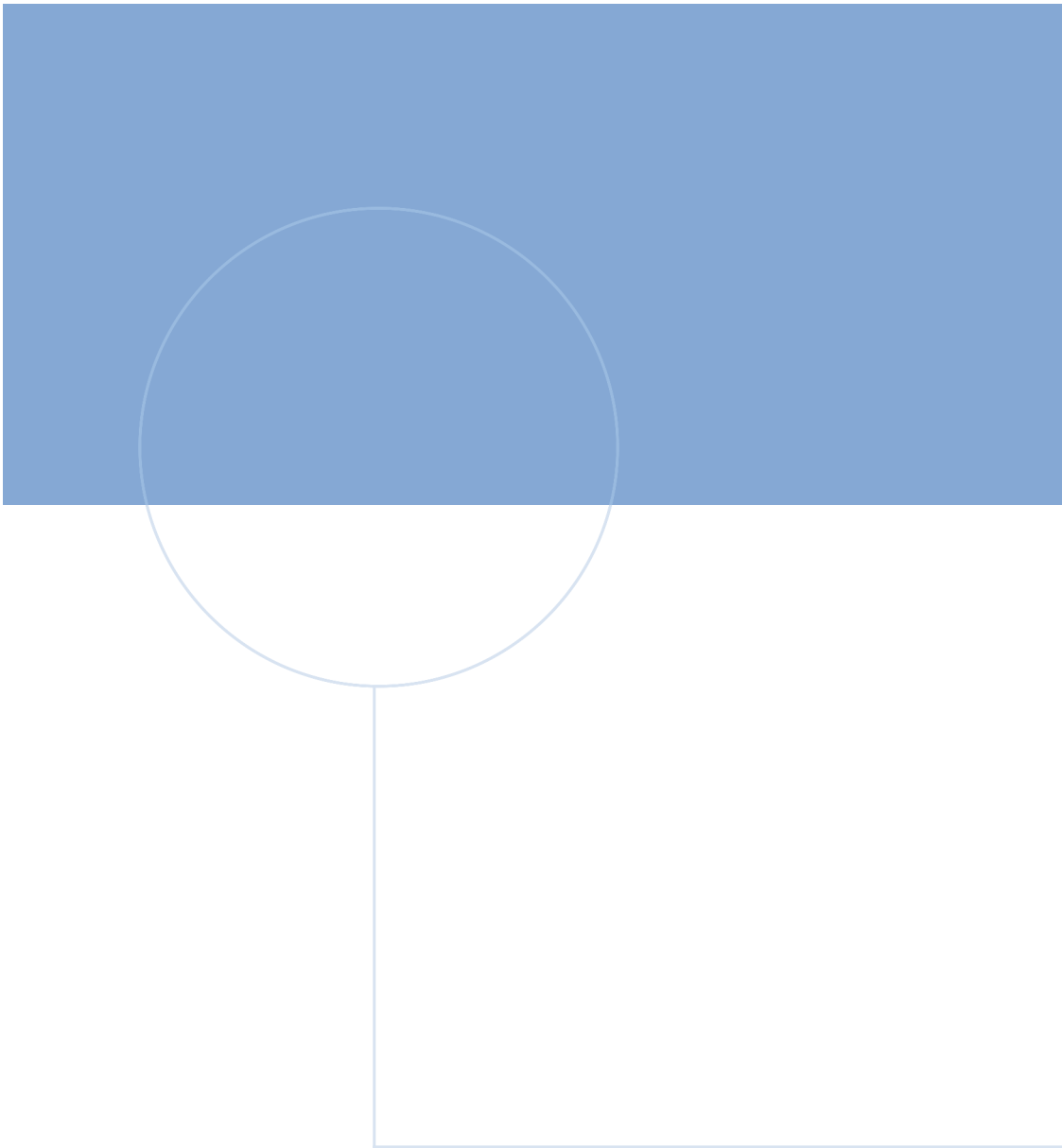
wc_6h_1	0.000	0.000	37.545	32.758	42.354	35.147	0.000	45.309	0.000	0.000	40.598	0.000	0.000	36.145	0.000	0.000
wc_6h_2	31.172	30.770	27.118	27.523	30.622	27.327	33.711	37.241	25.796	27.053	43.316	31.401	27.248	26.314	29.841	36.295
wc_6h_3	33.183	31.996	26.730	27.248	29.829	23.840	31.312	37.979	26.980	27.876	41.935	31.113	26.152	25.754	29.839	35.649
wc_24h_1	36.045	36.632	32.126	27.982	35.781	32.571	35.527	44.524	31.393	32.915	0.000	37.586	32.192	30.779	34.530	0.000
wc_24h_2	37.346	37.360	36.118	35.115	37.265	36.461	37.418	0.000	34.234	33.675	0.000	0.000	34.266	34.144	36.609	36.142
wc_24h_3	35.276	34.180	33.035	33.938	35.779	29.751	35.650	39.892	29.803	30.477	41.064	37.157	30.015	29.419	35.366	0.000

Table A.4: NanoDrop values. The RNA concentration, and the eluates purity, were determined using NanoDropTM One/One© Microvolume spectrophotometer (Thermo Fisher Scientific)

Sin 5 µg/mL	ng/µl	AITC 1 µg/mL	ng/µl	SW	ng/µl
1h	55,6	1h	21,1	1h	30,8
	64,4		19,0		74,8
	80,85		35,2		37,2
6h	47,1	6h	9,7	6h	86,5
	82,55		41,4		86,4
	60,55		48,2		99,3
24h	62,8	24h	14,8	24h	88,5
	107,3		24,8		59,8
	72,4		14,1		59,9
Sin 7,5 µg/mL		AITC 2,5 µg/mL		DMSO	
1h	72,4	1h	26,6	1h	49,9
	47,2		54,7		47,8
	43,5		112,9		85,5
6h	25,9	6h	20,6	6h	29,2
	47,4		19,2		27,2
	91,8		22,8		37,5
24h	98,8	24h	9,0	24h	32,9
	80,4		16,4		54,5
	58,0		34,6		90,7
Sin 10 µg/mL		AITC 5 µg/mL		EtOH	
1h	62,9	1h	96,3	1h	48,1
	77,9		90,0	6h	49,2
	107,0		102,0	24h	35,0
6h	47,0	6h	62,2		
	34,9		109,4		
	24,3		90,8		
24	61,3	24h	58,9		
	66,0		16,2		
	77,6		7,7		

Table A.5: PCR efficiency. The measured PCR efficiency of each of the explored genes.

Gene	PCR efficiency
ADT3	1,912
CYP18a1	1,876
CYP2j3	1,951
DAIA-2	1,872
DCP	1,887
eEF1α	1,869
GST-mu3	1,829
PRC	1,836
SCS	1,897
TNFaf-4	1,916



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