Master's Thesis in Analytical Organic Chemistry

The Distribution and Elimination of Lufenuron in Atlantic Salmon (*Salmo salar*)

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June 2024

Acknowledgements

I am deeply indebted to my supervisors Rita Hannisdal and Aiofe Elizabeth Parsons at IMR for their guidance and support throughout the two years that I have worked on this thesis. I would also like to express my deepest appreciation to Associate Professor Monica Jordheim for her help with the writing of this thesis.

Many thanks to senior researcher Ole Samuelsen who generously provided his knowledge and expertise in the area. I would also like to give a big thanks to research fellow Kristine Ertnæs Brokke whose groundwork laid the foundation for this thesis.

Special thanks to the members of staff at IMR, especially senior engineers Annette Bjordal and Tore Tjensvoll for their assistance and help in the laboratory.

I would like to thank my family, especially my parents, for their endless support, motivation, and encouragement throughout the years of my studies. I would also like to thank my two dogs, Edmund and Stella, for their emotional support over the years.

Lastly, I'd like to dedicate this thesis to the loving memory of my late grandmother, Anna. Her unwavering support and encouragement have been a guiding light throughout my life. Though she is no longer with us, her presence continues to inspire me every day and I hope I have been able to make her proud of me so far.

Abstract

Salmon lice (*Lepeophtheirus salmonis*) are a growing problem in the Norwegian aquaculture industry with an estimated annual cost of sea lice management of 525 million USD in 2019 in Norway. Numerous lice treatments exist, yet chemical methods falter as lice increasingly resist today's commonly used pharmaceuticals. Therefore, it is of interest to investigate new potential pharmaceuticals that can be used as chemical treatments in Norway. One of these potential pharmaceuticals is lufenuron, a benzoylurea, that is already used as a salmon lice treatment in some countries, such as Chile. The purpose of this thesis was to determine the levels of lufenuron in different tissue types in Atlantic salmon (*Salmo salar*) over time and from this data determine several pharmacokinetic parameters of lufenuron in Atlantic salmon. Understanding lufenuron's pharmacokinetics is crucial, given its novelty as a salmon delousing agent and the current scarcity of comprehensive information. The concentration of lufenuron in the salmon tissue samples was determined using LC-MS/MS. The results showed that the concentration of lufenuron was highest in the skin and liver, while plasma had the lowest concentrations of lufenuron. The results also indicated that plasma is the main route of elimination, and the bile is the main excretory pathway for lufenuron in Atlantic salmon. In addition, the calculated half-lives of lufenuron in the different matrices suggested that lufenuron is very persistent in the tissues of Atlantic salmon compared to other veterinary drugs.

Abbreviations

AChE	Acetylcholinesterase
AUC	Area under the curve
EEA	European Economic Area
EFSA	European Food Safety Authority
EMA	Europe Medicines Agency
ESI	Electrospray ionisation
EU	European Union
HPLC	High-performance liquid chromatography
IMR	Institute of Marine Research
LOQ	Limit of quantification
LUF-d3	Lufenuron-d3
MRL	Maximum Residue Limit
MRM	Multiple Reaction Monitoring
MS	Mass spectrometer
nAChRs	Nicotinic acetylcholine receptors
NOMA	Norwegian Medical Products Agency
Q	Quadrupole
rpm	Revolutions per minute
SE	Standard error
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
γ-S-GTP	γ-S-Guanosine triphosphate

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1. Introduction

1.1. Salmon aquaculture in Norway

The salmon aquaculture in Norway has experienced significant growth since its beginnings in the 1970s.[1] Today, fish farming in Norway produces more than 1,5 million tonnes of fish per year, most of which is exported.[2] Salmon farming is one of the most important industries in rural Norway with an annual value of around 100 billion NOK in 2022.[3] It has grown from a niche market into a large-scale industry.[4]

The management of salmon aquaculture facilities are highly regulated by the Directorate of Fisheries and the Norwegian Food Safety Authority for environmental, fish health and food safety reasons. The major legislation that governs the salmon aquaculture is the Aquaculture Act, which has been modified over time with emerging issues. The Aquaculture Act contains a series of guidelines, regulations, management plannings and monitoring procedures. It also targets special issues and problems such as siting/licensing, waste management, escape prevention, fish health and the use of chemicals and drugs.[5]

As the industry has grown, concerns regarding the environmental impact of salmon production have also increased.[6] The environmental issue that has received the most attention is the maintenance of the wild salmon stock population in Norway, which spawns in the salmon streams rivers.[7] There are currently over 400 rivers with a local Atlantic salmon population in Norway, and this accounts for approximately 25% of the world's healthy salmon populations.[8] Therefore, the Norwegian authorities have taken a particular interest in protecting the wild Atlantic salmon populations and have identified the two most severe challenges for the preservation of the wild salmon populations as escapement from fish farms and high salmon lice densitites.[4]

1.2. Salmon lice

Salmon lice (*Lepeophtheirus salmonis*) are prevalent in the salmon aquaculture in Norway, and reducing the parasitic burden for wild salmon is one of the main concerns for the industry.[9] In 2019 it was estimated that the annual cost of sea lice management in Norway was 525 million USD.[10]

Salmon lice are ectoparasitic copepods that occurs naturally on wild salmons in the marine environment in the northern Atlantic Ocean. Their lifecycle has eight stages with each stage separated by a moult.[11] The moulting process is complex and a precisely coordinated event. The old cuticula is degraded by chitinases and carboxypeptidases during the pre-moult. These

enzymes are excreted by the epidermis and are found in the moulting fluid. Concurrently, a new and often larger cuticula is produced.[12] The lice reproduce sexually, and adult females can produce up to 11 pairs of egg-strings, where each egg-string have several hundreds of eggs.[13] The lice are planktonic during the first two naupliar stages and the following infectious copepod stage.[14] This means that they cannot swim against the current but are able to adjust their vertical depth. The next two stages are the chalimus stages where the lice are attached to the fish by a frontal filament. The final three stages are the two pre-adult stages and the subsequent adult stage. During these stages the lice can move around on the surface of the fish.[11] The lice feed on the mucous, skin and blood of their hosts and may cause lesions ranging from loss of scales to deep ulcerations.[15] Infected fish may be subjected to stress, osmoregulatory problems, anaemia, and secondary bacterial pathogens.[16, 17]

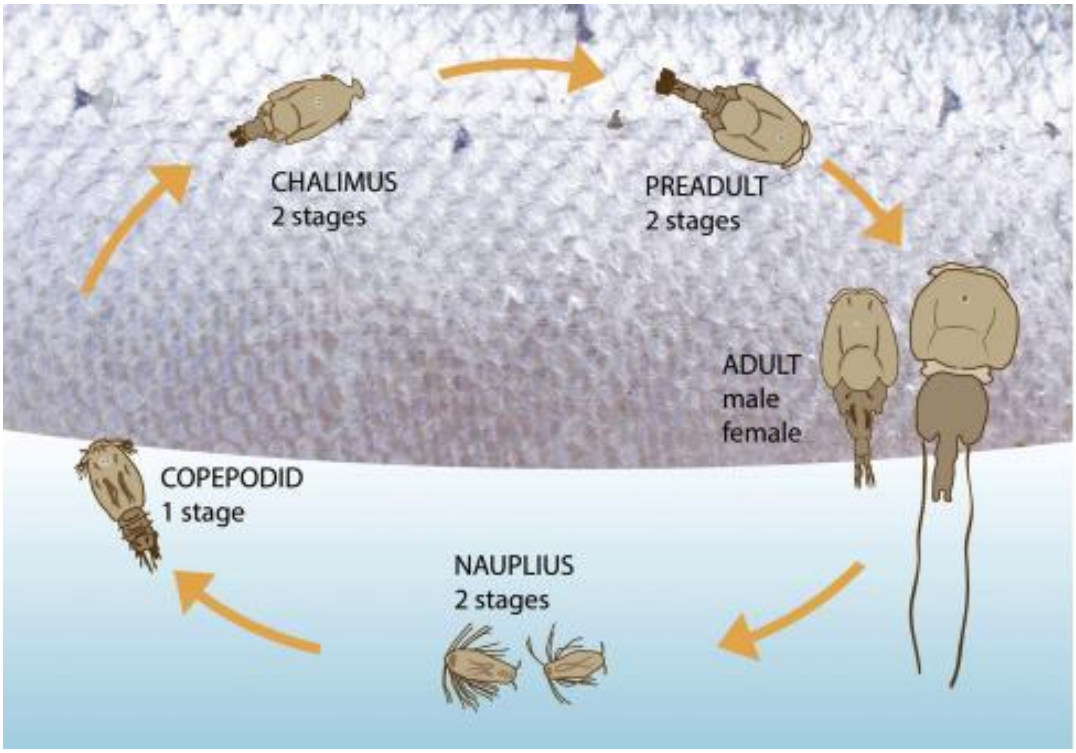


Figure 1. Life stages of salmon lice[18]

Under natural conditions salmon lice are not a major problem for wild salmon populations, but the high numbers of farmed salmon in the fjords year-round which acts as hosts for the parasite and the number of salmon lice has consequently increased.[19] This is mainly a problem for the wild smolts, young salmon, when they leave their rivers in the spring and migrate to the sea to grow.[20] The smolts must pass the fish farms on their way to the sea and these farms are high sea lice density areas.[21] It has been reported that if more than 10 salmon lice attach to the skin

of a smolt, they may die.[4] In the period 2010-2014 it was estimated that the annual loss of wild salmon due to salmon lice in Norwegian rivers was around 50,000 adult salmon. This is an annual loss of about 10% of wild salmon on a national level.[4]

A national programme for the control of sea lice was introduced in 1997 in Norway with the main purpose of the programme to keep the number of sea lice as low as possible in the spring.[22] A maximum of 0.2 mature female lice per fish on average are allowed in the wild smolt migration period and less than 0.5 lice per fish in the remaining season per regulations for fish farming in Norway. If this threshold is exceeded the farm is required to slaughter or treat their fish within two weeks.[4] The threshold is enforced by the Norwegian Food Safety Authority, who also requires the fish farmers to regularly count the salmon lice in their pens and report the highest mean count during a month. Farmers are required to report the means from samples of 10 fish from 50% of all active pens. In addition, all pens are to be counted for every two rounds of sampling to improve control.[4]

1.3. Treatment methods against salmon lice

Fish farms in Norway today uses a wide range of methods against salmon lice, such as cleaner fish and approved pharmaceuticals.[23] These treatment methods can constitute approximately 10% of the total production costs.[24] All pharmaceuticals that are used must be approved by NOMA following thorough testing. In addition, the use of these pharmaceuticals is strictly controlled to reduce the use of them as much as possible and to consider the potential environmental impact.[23,25] There is also a pollution issue to be considered when using chemical treatments against salmon lice. The chemical treatments used against salmon lice may have negative effects on shrimps and other crustaceans in the surrounding areas of the fish farms.[4,26] The salmon lice have over time developed resistance to the most used medicinal lice treatments.[27, 28] This has led to the increase in the use of biological methods such as cleaner fish to control salmon lice.[2,29]

In Norway, the most commonly utilised cleaner fish include the ballan wrasse (*Labrus bergylta*), goldsinny wrasse (*Ctenolabrus rupestris*), and corkwing wrasse (*Symphodus melops*).[13,30] Cleaner fish are seen as an environmentally friendly approach to delousing and are used as a parasitic control by many farming facilities.[31] There has been some concern raised about the poor welfare of the cleaner fish since they are generally used as a disposable tool in the production of salmon.[2] In addition, there are concerns that they could be a possible vector for transmitting disease.[24]

Freshwater treatment of salmon in well-boats is another biological method that has been utilised as an alternative to chemical bath treatments. However, there is a concern that the salmon lice may develop resistance towards freshwater. There are also mechanical and thermal delousing systems that has been recently developed, which can also be used as alternative to chemical treatments. They have been used in Norwegian salmon aquaculture since 2015.[32] However, these non-medicinal methods can have severe side-effects such as injuries and increased mortality compared to medicinal methods.[33] In addition, there are new management methods and technologies being developed such as closed sea-cages where the water is pumped in from the deep, plankton sheeting enclosing each cage to filter the surface water, tarpaulin wrapping around the upper part of each cage to direct the surface water around the cages, submerged lights and feeding to attract the salmons to deeper depths and submerged sea-cages that hold the salmon below the surface water.[24]

1.4. Chemical treatments used in Norwegian aquaculture

Chemical treatments are applied in two different ways: bath treatments and as additives in the feed.[11,34] Bath treatments involve the administration of drugs that are dissolved in water through complete immersion for a pre-determined time.[35] They are performed by either lining a sea-cage with tarpaulin and reducing the volume of water within the cage or by crowding and pumping the fish into a well-boat.[32,36] The advantage of bath treatments, especially when administered using well boats, is that all the lice is exposed to the same concentration of the drug. However, in open-pen treatments, the bath treatment dilutes immediately after being administered and can disperse and affect other organisms that are nearby. In addition, administration of the drug is labour intensive as the pens are treated one at a time and the drugs used are toxic so caution must be taken when administering the drugs.[37] In-feed treatments are administered by having the drug directly milled into the diet of the fish. The advantages of in-feed treatments are that it is less stressful to the fish and safer to administer. In addition, one can treat all pens at the same time.[38] However, since this treatment method is based on consumption of medicated feed it is possible that some fish are under-dosed due to sickness or hierarchies within the pen.[35] Another important aspect with in-feed treatments is that they must be able to be distributed to the mucus of the fish for a prolonged period to be effective against the lice.[39] Organophosphates, pyrethroids, hydrogen peroxide and imidacloprid have been used as bath treatments, while avermectins and benzoylureas are used as additives.[11,33] In 2023, the total consumption of anti-salmon lice agents in Norwegian aquaculture was 9,806 kg measured

as active ingredient. Table 1 shows the anti-salmon agents used in Norwegian aquaculture from 2014 to 2023 in kg measured active ingredient.[40]

Table 1. Agents used in Norwegian aquaculture against salmon lice from 2014-2023 in kg measured active ingredients.[40]

	2014	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**	**2022**	**2023**
Azamethiphos	4630	3904	1269	204	160	154	286	453	577	740
Cypermethrin	162	85	48	8	0	0	0	0	0	0
Deltamethrin	158	115	43	14	10	10	8	5	3	2
Diflubenzuron	5016	5896	4824	1803	622	1296	1000	240	519	571
Emamectin benzoate	172	259	232	128	87	114	117	101	79	74
Teflubenzuron	2674	2509	4209	293	144	183	1603	308	213	294
Imidacloprid	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3252	6564	6454
Hydrogen peroxide (100%) (tons)	31577	43246	26597	9277	6735	4523	5084	4060	3004	1571

1.4.1. Hydrogen peroxide

Hydrogen peroxide was introduced as a delousing agent in Norway in 1993 and was used until 1997. It was reintroduced in 2009 due to the development of resistance towards some of the other delousing chemicals such as organophosphates and pyrethroids in some Norwegian production zones. Unlike other delousing chemicals hydrogen peroxide dissociates into water and oxygen, and therefore does not bioaccumulate in the environment and hence is perceived to be environmentally friendly.[32,41] Hydrogen peroxide is a potent oxidising compound that disrupt membranes.[42] It is thought to cause mechanical paralysis in lice through the formation of gas bubbles inside the haemolymph, which causes the lice to detach from the salmon.[32] Its effect is rapid and most efficacious on pre-adult and adult salmon lice.[42] However, hydrogen peroxide is increasingly toxic to the salmon with treatment time, concentration, and temperature. It is administered either in-cage or by using a well-boat. The recommended dosage is 1.7 g L^{-1} for 20 minutes for temperatures below 8 °C and 1.3-1.5 g L^{-1} for temperatures between 8 and 13 °C. The use of hydrogen peroxide at temperatures above 13 °C is not allowed as the safety margins become too narrow.[32,43]

1.4.2. Organophosphates

Organophosphates were one of the first chemical treatments introduced as salmon delousing treatments since they are water soluble. They work by acting as an inhibitor of AChE, which causes overstimulation of the muscular and nervous systems and therefore leads to paralysis. Until 1995, more than 80% of all delousing treatments administered in Norway used organophosphates.[32] In 1974 metrifonate, also known as trichlorfon, was introduced in Norway as a treatment against salmon lice, however it is no longer in use. In 1986 dichlorvos was introduced.[22] Dichlorvos was the main chemical used in all countries with salmon farms until the early 1990s when widespread resistance to the chemical appeared.[44] Azamethiphos was introduced in 1994 and was 10 times more effective than dichlorvos, but also safer for mammals. The recommended dosage per treatment for azamethiphos is 0.1 mg L^{-1} for between 30 and 60 minutes. Azamethiphos was used in Norwegian salmon aquaculture from 1994 to 1999, and from 2008 onwards. It has a rapid effect that can be observed in a few hours, and it is effective in removing pre-adult and adult salmon lice. Its toxicity increases with temperature.[32]

1.4.3. Pyrethroids

Pyrethroids are synthetic analogues of natural pyrethrins and are extremely toxic to crustaceans, but also highly degradable and non-toxic to mammals.[32] Pyrethroids work by interfering with

nerve impulses by modulating the opening and closing of voltage-gated sodium channels in axons which leads to repetitive synaptic discharge, followed by paralysis and death. They are effective against all developmental stages of the salmon lice, but the full effect can only be determined after 1-2 weeks depending on the temperature.[42] The first available product was based on pyrethrum and contained a mixture of 6 compounds which was extracted from the plant *Chrysanthemum cinerariaefolium*. However, the proposed application of this treatment, a surface layer of a pyrethrum-oil mixture in the net-pen, was impracticable and it soon became replaced with synthetic compounds that could be administered in bath treatments.[22] In Norway, deltamethrin has been used for delousing on salmon farms since 1994 with a recommended dosage of 2 μg L^{-1} for 40 minutes.[32] Cypermethrin, another pyrethroid, was used from 1994 to 2017.[10]

1.4.4. Imidacloprid

Imidacloprid is a chloronicotinyl insecticide that was introduced as a pesticide in 1994. It functions as a competitive inhibitor at nAChRs of the nervous system of the insect, which results in the incapacitation of the normal nerve function.[45] It is effective on motile lice. Imidacloprid is the first chemical treatment with an active substance from a new chemical class that has been introduced to the Norwegian market in over twenty years. It has been used as a sea lice treatment on salmon farms in Norway since 2021.[33] The recommended dosage is 20 mg L^{-1} for 60 minutes. Imidacloprid must be used in a well-boat and the concentration in the outlet must be equal to or less than 0.3 μg L^{-1}.[46]

1.4.5. Avermectins

Avermectins are a group of compounds that were isolated from the soil microorganism *Streptomyces avermitilis*.[47] They modulate specific glutamate and gamma-aminobutyric acid-gated ion channels.[48] The influx of chloride ions results in hyperpolarisation which leads to disruption of nerve impulses, paralysis, and death. They are effective against all developmental stages, but the full effect of the treatment can only be determined after 2-3 weeks.[42] Emamectin benzoate is semisynthetic avermectin and can be used at low quantities within a treatment with a high efficacy.[47] It was introduced in 1999 as an oral medication with a recommended dosage of 0.05 mg kg^{-1} fish daily for 7 days.[22]

1.4.6. Benzoylureas

Benzoylureas inhibit chitin synthesis and therefore their effect is restricted to the moulting stages of the lice and will have no effect on adult lice.[11,49,50] There are also some subtle effects on reproduction and egg hatch. They are based on the chemical formula $C_{14}H_{12}N_2O_2$. The key

enzyme for chitin synthesis is chitin synthase and this is the enzyme that is inhibited by the benzoylureas.[51]

Diflubenzuron was the first of the benzoylureas to be discovered and was first introduced as a pesticide in 1975 and around 20 years later it was approved as a sea lice treatment. It is administered as a medicated feed with a standard dosage of 3 mg kg^{-1} fish daily for 14 days. Teflubenzuron was introduced in 1986 as a crop pesticide and in 1996 it was introduced as a sea lice treatment in Norway.[52] The recommended dosage for teflubenzuron for Atlantic salmon is 10 mg kg^{-1} fish daily for 7 days.[53, 54] In 2001 the use of teflubenzuron and diflubenzuron was stopped due to environmental concerns. They were however both reintroduced in 2009 after the chemical treatments that replaced them had become less effective.[55]

As mentioned earlier, there is a disadvantage with using benzoylureas as they are not environmentally friendly and can harm other chitin-synthesising organisms that live close to the fish farms.[56] Benzoylureas can enter the aquatic environment either through uneaten fish food pellets or as a digestive byproduct from the fish and end up in sediments at the seabed floor.[57] Benzoylureas are generally very poorly absorbed in the intestine of the fish.[58] In addition, the degradation rates of benzoylureas are slow. Benzoylureas have been found at the bottom and close to the fish farms for a long time after treatment.[57]

1.4.6.1. Chitin

Chitin is a structural polysaccharide of β-(1,4)-linked *N*-acetylglucosamine residues synthesised from the disaccharide trehalose.[12,59] It is found in the cuticle of insects and in the exoskeletons of crustaceans. It is also found in protozoa, fungi, algae and in nematodes. Chitin is not present in vertebrates and higher plants making its biosynthetic pathway an attractive target for the action of insect-specific insecticides.[60,61] Chitin exists as two different crystalline forms, α and β-chitin. There is also a third form, γ-chitin, that is a combination of the α and β forms. The α form is the most common form and is the form that is present in insect cuticles and exoskeletons of crustaceans. They are arranged in anti-parallel fashion and are very stable.[51]

Chitin synthesis is a complex process that take place in the polarised epidermal cells where trehalose enter through the basal lamina from the haemolymph and the polymerisation occurs at the apical plasma membrane. There are eight enzymatic steps that are needed to convert trehalose into chitin. It first starts with the hydrolysis of trehalose into two units of D-glucose. The glucose then enters glycolysis to produce fructose-6-phosphate. This is then diverted to the hexosamine pathway to produce, via four enzymatic steps, UDP-GlcNAc. This is then

polymerised into chitin. The key enzyme in this process is chitin synthase which is responsible for the polymerisation of UDP-GlcNAc into chitin.[51, 62]

1.5. Lufenuron

Lufenuron is a benzoylurea that was discovered in 1980 and introduced as a crop pesticide and flea control for dogs around 1990.[57, 63] Its chemical structure is shown in figure 2. Lufenuron was previously approved for restricted indoor use and in outdoor bait stations as an insecticide in the EU in 2010. However, the approval as an insecticide expired in 2019 and has not been renewed.[64] As a veterinary drug, a MRL of 1350 ng g^{-1} for fish was established in the EU.[65, 66] Medicated feeds with lufenuron have been applied against salmon lice in some countries outside the EU.[33] For example, it has been used as a sea lice treatment for farmed salmonids in Chile since 2016.[52,67]

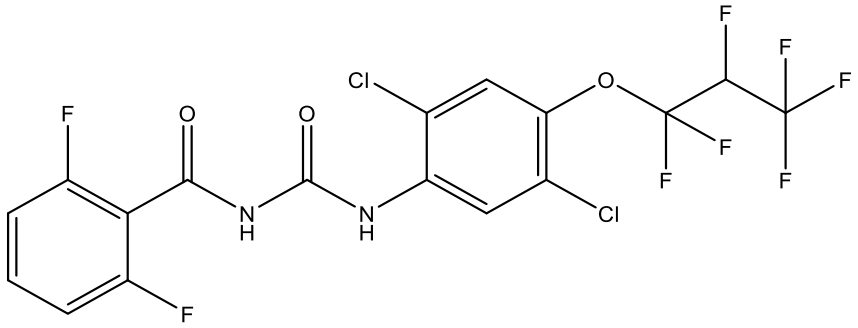


Figure 2. Chemical structure of lufenuron

The mechanism of action of lufenuron is not precisely known.[68,69] It is thought that the drug does not directly inhibit chitin synthesis, but that it inhibits the γ-S-GTP stimulated uptake of Ca^{2+} ions by chitin microfibre-containing excretory vesicles. This disrupts the vesicle fusion with the outer cell membrane and cuticle formation in arthropods. As with other benzoylureas, lufenuron is the most effective at the moulting stage. [70] An advantage of lufenuron over the other benzoylureas is that it is administered in the hatchery, which limits the release of this in-feed chemical into the environment.[71]

1.6. Food safety

The Norwegian Food Safety Authority and the Institute of Marine Research, IMR, monitors the content of undesirable substances in salmon.[23] EU legislation requires that all food producing animals should be monitored for certain substances and residues.[72] As Norway is a member of the European Economic Area, EEA, they are subject to EU regulation with regards to the use

of pharmaceuticals in animals. The European Medicines Agency, EMA, is the agency responsible for the evaluation of the pharmaceuticals and they make recommendations to the European Commission based on this data. The European Commission then determines the Maximum Residues Limits, MRL, values of the different pharmaceuticals based on these recommendations. The withdrawal period, i.e. the minimum period between the last dose is given and the slaughter of the animals, is determined by Norwegian Medical Products Agency, NOMA. The withdrawal period is established to ensure that drug residues and possible metabolites are in low enough levels so that the food source is safe enough to be consumed. [73]

1.7. Pharmacokinetics

Pharmacokinetics is the study of how xenobiotics move within the body after their administration. There are four fundamental processes that influences the *in vivo* pharmacokinetics of a compound. These are absorption, distribution, metabolism, and excretion, also known as ADME.[74] Absorption of a compound may occur via various routes in fish such as the skin, gut, and gills. After absorption, the compound will distribute into the interstitial and intracellular fluids to different extents. The distribution is largely dependent on the relative affinity of the compound for the blood and different body tissues.[75] The major pathway for elimination of most drugs is metabolism and elimination of the parent drug. Metabolism occurs via enzymatic processes that transforms lipophilic compounds into more hydrophilic metabolites to facilitate their excretion into bile or urine.[74] The majority of fish species excrete nitrogenous waste as ammonia via the gills and skin, unlike mammals who either store or converts it into urea and ureic acid. The gills, skin, kidneys, and liver (via the bile) all seem to be involved in the excretion of pharmaceutical drugs in fish. The relative contribution of each route is most likely dependent on both the fish species and factors relating to the specific drug.[75] One pharmacokinetic parameter that is useful to characterise the *in vivo* disposition of a compound is half-life. Half-life is the measure of time it takes for a compound to decrease to half of its initial concentration in the fluid or tissue in which it is measured. Another important pharmacokinetic parameter is the area under the curve, also known as AUC. AUC is the total body exposure of the compound which is determined by the area under the concentration versus time curve.[74]

1.8. Research aim

As lufenuron is a new treatment option for salmon delousing there is limited knowledge about its pharmacokinetics in salmon. The aim of this research was to better understand the distribution and elimination of lufenuron in Atlantic salmon by quantifying the levels of

lufenuron in liver, bile, skin, plasma and muscle of salmon that have been fed lufenuron-medicated feed following a standard medication. This valuable data will provide important information about the pharmacokinetics of lufenuron in Atlantic salmon and ultimately food safety.

2. Materials and methods

2.1. Lufenuron exposure and sampling

The tissue samples that were analysed in the present work originated from a previous laboratory study performed at the IMR in which juvenile Atlantic salmon with a mean weight of 40 ± 7 g were fed lufenuron medicated fish food pellets at a dose of 5 mg kg^{-1} fish biomass daily for 7 days. The aim of this experiment was to determine the concentration and the elimination time of lufenuron in different salmon organs and tissues after a treatment regime that reflected a standard treatment regime. In brief this experiment involved 400 individual salmon divided into four 1.5 m diameter tanks that contained 1000 L of water at a mean temperature of 9 °C. The medicated feed was made by preparing a premix of 4.5 g of maizena (Unilever Norge AS) and 0.5 g of lufenuron (Sigma Aldrich). The premix was surface coated on a batch of 500 g of 3 mm pellets (Skretting AS) using a few drops of cod liver oil (Møllers; Orkla Health). The feed for the control group was produced in the same way using a premix without lufenuron. The salmon was humanly killed at each sampling point using Finquel as an anaesthetic and then killed with a stroke to the head.[76] The total mass of each fish was determined by weighing the fish on a scale after being euthanised.

The experiment ran from 15 July 2019 to 5 March 2021 and their tissues were subsequently sampled at various time points over a 17-month period following the 1-week lufenuron treatment. The lufenuron was given in the freshwater phase before the salmon was transferred to seawater. Sampling of the fish occurred on day 0, 2, 4, 6, 7, 8, 10, 14, 22, 38, 62, 146, 255, 380 and 510 after administration of the first dose. For the current masters project liver, skin, bile, and plasma sampled at days 4, 7, 38, 62, 146 and 255 after the start of the medication were chosen to be analysed. In addition, previously analysed data for the tissue liver, skin and muscle were also included. For liver, the days 8, 14, 380 and 510 were previously analysed and for the skin the days 22, 38, 62 and 146 were previously analysed. For the muscle, all days except day 0 were previously analysed.

2.2. Chemicals and stock solutions

The chemicals used were acetonitrile, acetone, and tetrahydrofuran of HPLC grade. Lufenuron of analytical quality (CAS nr. 103055-07-8) and stable isotope lufenuron, lufenuron-d3, (CAS nr. unlabelled 103055-07-8) were used. Lufenuron-d3 were used for the internal standard. Milli-Q water was also used. The stock solution for the standard curve was made by solving 10.00 ± 0.04 mg lufenuron with tetrahydrofuran in a 10 mL volumetric flask. For the internal standard, 10.00 ± 0.004 mg lufenuron -d3 was solved with tetrahydrofuran in a 10 mL volumetric flask to make the stock solution.

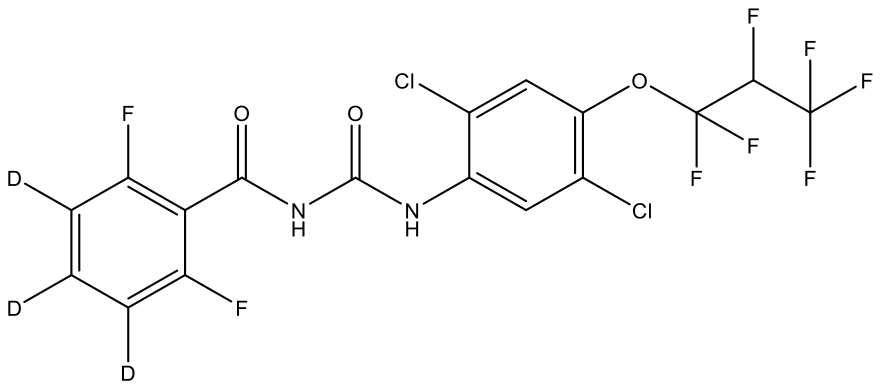


Figure 3. Structure of lufenuron-d3.

2.3. Preparation for chemical analysis

The level/concentration of lufenuron in the tissue samples were quantified using an existing method at IMR using liquid chromatography-tandem mass spectrometry (LC-MS/MS (QQQ)).

The samples were homogenised before analysis and each sample was weighed to between 0.1-0.5 g depending on how much material was available of each sample. Homogenised blank salmon muscle tissue was used for the blank, the standard curve and the control samples. An internal control material was used for the controls. The working solution for the standard curve and control samples was made by first pipetting 50 µL from the 1.0 mg mL^{-1} lufenuron stock solution into a 5 mL volumetric flask and adding acetonitrile/water (1:1). Of this solution, 1 mL of this solution was then transferred to a 10 mL volumetric flask and acetonitrile/water (1:1) was added to the flask. The internal standard work solution was made by pipetting 50 µL from the 1.0 mg mL^{-1} lufenuron d-3 stock solution into a 5 mL volumetric flask and adding acetonitrile/water (1:1). 500 µL from this solution was pipetted into a 10 mL volumetric flask and acetonitrile/water (1:1) was added. The samples were spiked according to tables 1-3 in the appendix depending on the matrix being analysed.

After spiking, 5 mL of acetone was added to each sample and the samples were then shaken for 10 minutes at 2500 rpm. After this the samples were placed in an ultrasonic bath for 15 minutes, and then centrifuged for 3 minutes at 4000 rpm. The supernatant was transferred to 10 mL tubes and dried at 40°C using nitrogen gas. 250 µL of acetonitrile/water (75:25) was added to each sample and the samples were then filtered through a 0.45 µm syringe filter to a 2.0 mL HPLC vial with an insert. The samples were all either analysed the same day as the preparation or the next day.

2.4. LC-MS/MS (QQQ)

The LC-MS/MS works by pumping the sample through the column containing the stationary phase by the mobile phase at high pressure. The chemical interactions between the molecules of the sample, the stationary phase and the mobile phase allows for different retention times which gives separation of the different molecules in the sample.[77] The mobile phases used were acetonitrile, solution A, and purified water, solution B, following the timetable in table 2 with a flow of 0.4 ml min^{-1}.

Table 2. Gradient and mobile phases for analysis

Time (min)	Solution A (%)	Solution B (%)
0	20	80
0.2	20	80
3	98	2
5	98	2
5.1	20	80
7	20	80

After being separated by the liquid chromatograph the eluent is directed to the mass spectrometer for detection. The eluent from chromatographic column contains the separated target analyte, the elution solvent, and other dissolved components. The target analyte must be ionised efficiently to be detected by the tandem MS analyser. For this method ESI is the technique used to produce the ions. It is a technique that creates ion species in solvent and converts them into ion species into the gas phase by passing the eluent through a capillary that is maintained at high voltages. The high voltage disperses the eluent stream, forming a charged droplet that undergoes further desolvation and emits a mist of highly charged spray. The

application of a stream of gas and temperature, typically 250-350 ℃, is used to assist in the desolvation and nebulisation of the spray that is formed. For ESI soft ionisation is performed. Soft ionisation refers to the formation of ions without breaking any chemical bonds. This means all covalent interactions are kept intact during the ionisation process. The ESI can be operated in either positive or negative ionisation mode depending on whether the target analyte is prone to accept a proton or lose a proton, respectively.[78] ESI was operated in negative ionisation mode for the method used in this thesis.

The charged particles then migrate under high pressure through a series of mass analysers by applying magnetic fields.[77] MS/MS employs two stages of mass analysis to selectively examine the fragmentation of ions in a mixture. One to preselect an ion and the other to analyse the induced fragments. A QQQ instrument consists of three quadrupoles arranged in a series. Each quadrupole consists of four circular rods placed in parallel to which an oscillating electric field is applied. Q1 and Q3 are responsible for filtering sample ions according to their *m/z* ratio, where *m* is the mass of the ion and *z* is the charge, based on the stability of their paths in the electric field. Q2 serves as a nonlinear collision cell and once they reach Q2 the ions are accelerated by the electric field and collide with a neutral gas to produce small fragments. QQQ have four different acquisition modes: product ion scan, precursor scan, neutral loss scan and MRM.[79] The analyte for this thesis was detected using MRM mode. MRM consists of selecting a fragmentation reaction. Q1 filters a specific precursor ion of interest and therefore ions generated in the ion source that have a different *m/z* ratio cannot pass Q1. Q2 is optimised to produce a characteristic product ion by collision of the precursor ion with a neutral collision gas. The generated product ions are then transferred into Q3 where only specific *m/z* is allowed to pass. All the other product ions are filtered out of Q3.[79] The mass to charge ratio (*m/z*) is 509/488.9 for the quantifier transition and 509/325.9 for the qualifier transition for lufenuron and 512/325.9 for the quantifier transition for LUF-d3, the internal standard.

The concentration of lufenuron was quantified using LC-MS/MS 6410 and the data was treated using the Masshunter software. The standard curve used to quantify the concentration of lufenuron in the samples are shown in figure 4.

The column used was SB C18, 2.1x50 mm, 1.8 µm, where the stationary phase is a nonpolar hydrocarbon. The injection volume was 2 µl and the column temperature was set to room temperature. The parameter settings for the mass spectrometer were gas temperature: 300 ℃, gas flow: 11l min^{-1}, capillary voltage: 4000 V and charging voltage: 500 V. The quantifier transition is used to quantify the analyte, while the ratio between the quantifier and qualifier

transition is used to identify the analyte. The LOQ for this method is set to 1.0 ng g^{-1} and the linearity is 1.0-15000 ng g^{-1} with a relative standard deviation of less than 20%.

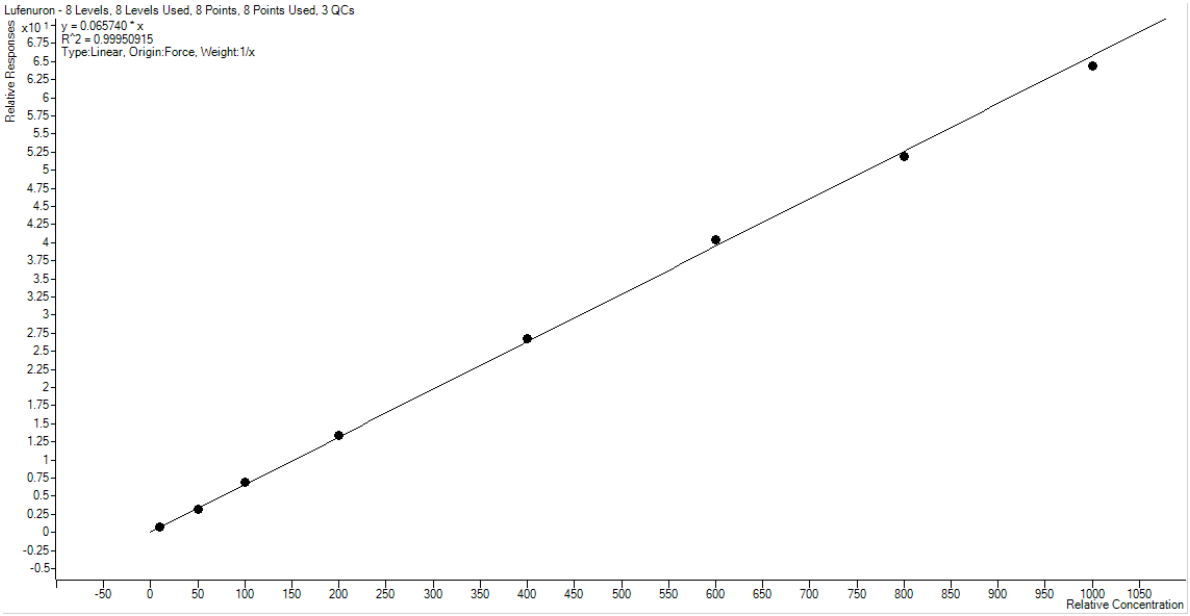


Figure 4. Standard curve used to quantify lufenuron concentrations in tissue samples.

2.5. Statistics

All the statistical analyses were carried out in RStudio 4.2.1.[80] The residue levels of lufenuron measured in each tissue are presented as scatterplots using the R package *ggplot2*.[81] The graphs for elimination time and growth versus elimination were plotted using Excel. A Generalised Linear Model (GLM) was also performed on the data in RStudio using the glm function to investigate if there was any significant difference between the different tissue types and to pinpoint any days post administration that may be significant. The concentration of lufenuron in the tissue was used as the response variable, while the variables tissue type and days post administration were used as the explanatory variables. The model includes tissue type and day post administration as the main effects and a two-way interaction between them. The concentration data was normalised by converting to the natural log. Following model simplification and to compare the slopes in the interaction between tissue and days post administration, pairwise comparisons were performed using the estimated marginal means of linear trends function (emtrends) within the emmeans R package.[82] The half-life was calculated in using the R package *PKNCA*.[83] The AUC was calculated using the trapezoidal rule.

3. Results

Chromatographs showing the measured lufenuron, the internal standard, the qualifier transition and the ratio between the quantifier and qualifier are shown for a point on the standard curve (figure 5) and for a sample with a low measured lufenuron concentration (figure 6).

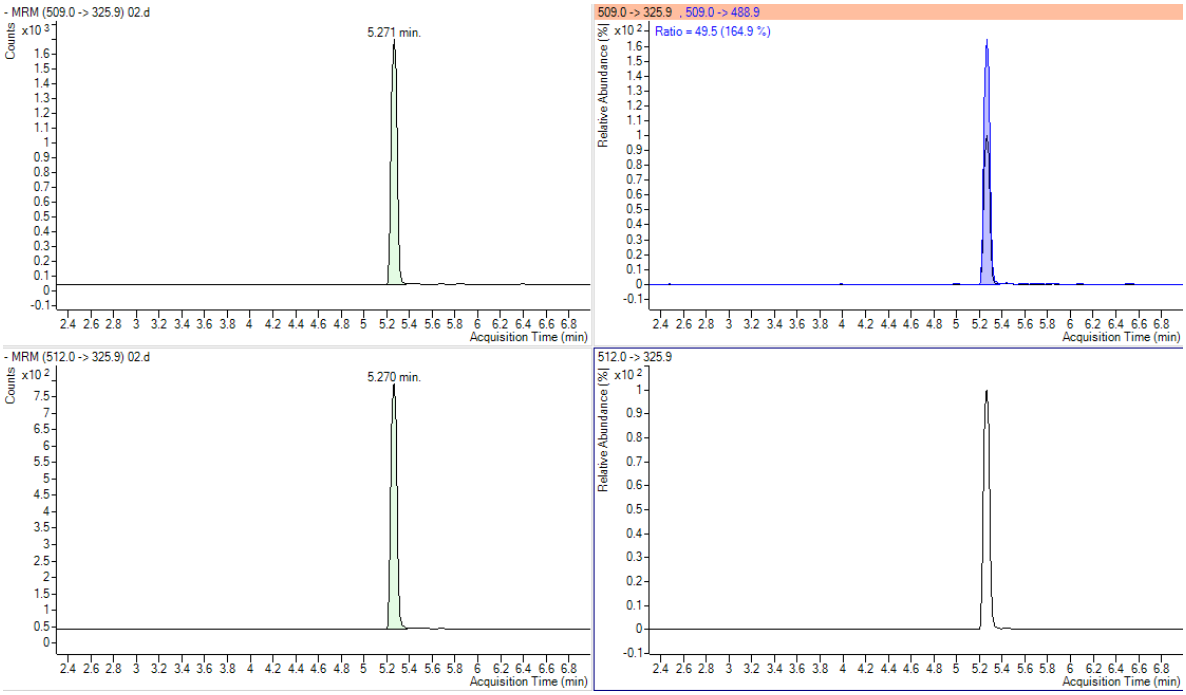


Figure 5. Chromatographs for a point on the standard curve. On the left the chromatographs for lufenuron (top) and LUF-d3 (bottom), while on the right the ratio between the quantifier and qualifier transition (top) and the qualifier transition (bottom).

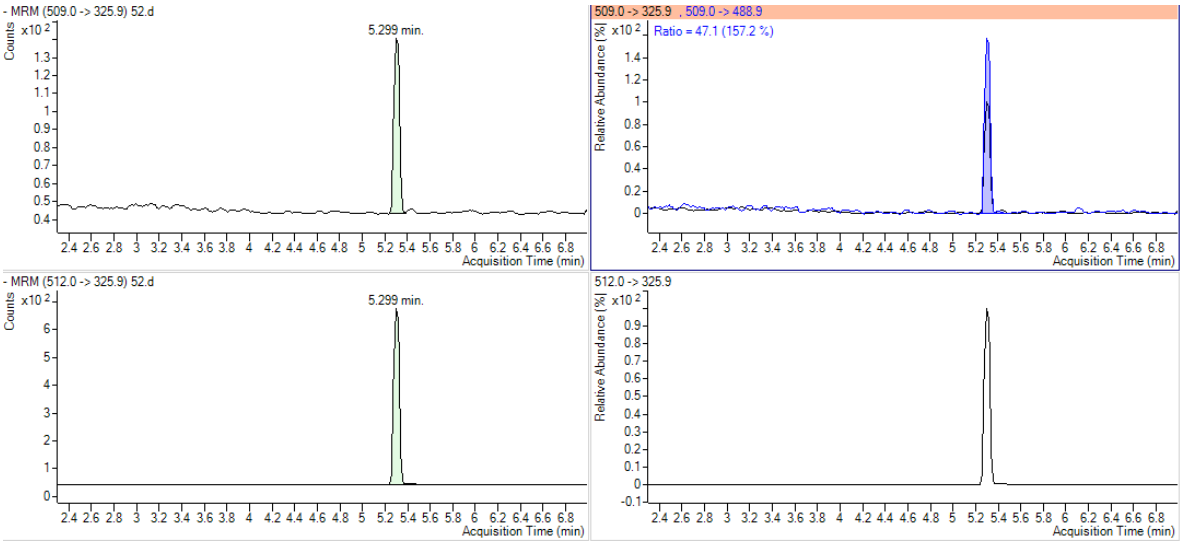


Figure 6. Chromatographs for a sample with a low measured lufenuron concentration (Bile sample on day 255). On the left the chromatographs for lufenuron (top) and LUF-d3 (bottom), while on the right the ratio between the quantifier and qualifier transition (top) and the qualifier transition (bottom).

A GLM model was performed on the obtained data which showed that a two-way interaction between tissue type and days post-administration had a significant effect on the measured concentration of lufenuron (GLM, Adjusted R^2 = 0.92, $F_{9, 359}$ = 471, p-value ≤ 0.001). Specifically, the measured concentration of lufenuron decreased with increasing number of days post-administration, though the rate in which this decrease occurred was dependent on the tissue type (Figure 7). Lufenuron levels decreased significantly faster in plasma than in skin, liver and muscle tissues ($p<0.001$), but not bile ($p>0.05$). Similarly, the lufenuron levels decreased significantly faster in bile than in skin, liver and muscle tissues ($p<0.01$), but not plasma ($p>0.05$). There was no significant difference in the rate of in lufenuron decreased between skin, muscle and liver ($p>0.05$).

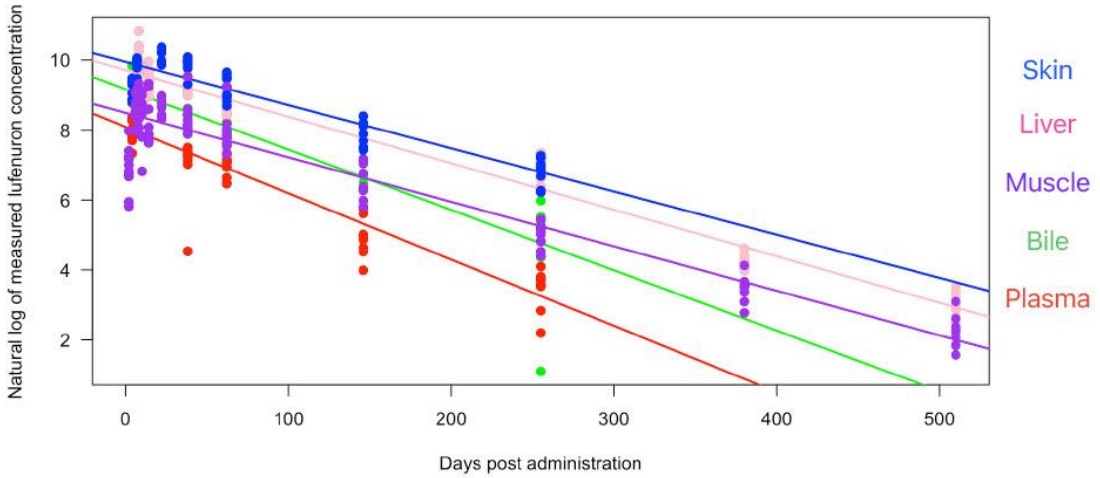


Figure 7. Visualisation of the GLM model with the natural log of the measured lufenuron concentration versus days post administration. The muscle samples are the purple dots, liver is pink, plasma is red, skin is blue, and bile is green. The lines are the regression lines.

3.1. Liver samples

The concentration of lufenuron in the analysed liver samples are shown in table 3. The means and standard deviations are shown for each day. The highest mean concentration was 29152 ± 10343 ng g^{-1} and occurred on day 8. In addition, the highest overall concentration of lufenuron in the liver also occurred on day 8 with 50632 ng g^{-1}.

Table 3. Measured concentration of lufenuron (ng g^{-1}) in salmon liver tissue sampled at various time points post lufenuron administration.

	Days post-lufenuron adminstration								
Sample ID	**Day 4**	**Day 7**	**Day 8**	**Day 14**	**Day 38**	**Day 62**	**Day 255**	**Day 380**	**Day 510**
1	6467	12529	28452	13407	13402	4905	1235	36	20
2	11959	11209	32591	12573	10482	6533	1546	81	27
3	9733	10963	50632	16006	9359	7477	750	73	24
4	11838	15346	32940	12955	8008	9373	1148	53	18
5	6834	10987	16371	20929	12483	7082	937	80	17
6	9047	17626	32822	8992	11294	6471	1086	31	14
7	10948	14804	14960	14308	9525	5481	1391	70	34
8	6685	10750	20380	16467	21180	5869	841	56	29
9	11295	12375	33566	7666	9346	6431	593	56	27
10	N/A	11319	28801	12031	8701	4546	809	101	12
Mean	9423	12791	29152	13533	11342	6417	1034	64	22
Standard deviation	2271	2348	10343	3780	3816	1386	301	22	7
Relative standard deviation (%)	24	18	36	28	34	22	29	34	32

Figure 8 shows the levels of lufenuron in salmon liver tissue measured over time in both the initial analysis (day 8, 14, 380 and 510) and the current analysis (day 4, 7, 38, 62 and 255) and demonstrated that the concentration of lufenuron in liver peaked at day 8.

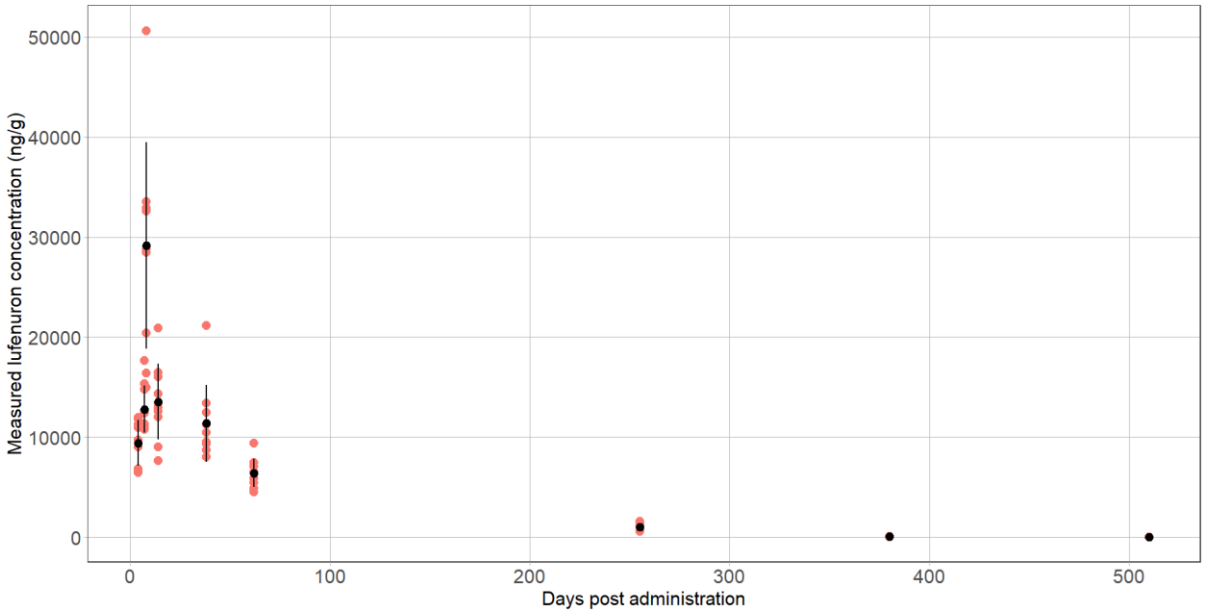


Figure 8. Plot showing the concentration (ng g^{-1}) of lufenuron measured in salmon liver at multiple time points post lufenuron administration. The black dots show the mean measured concentration per time point and the black lines shows the standard deviation. Each red dots represents a single sample.

3.2. Bile samples

The concentration of lufenuron in the analysed bile samples is shown in table 4. The highest mean concentration of lufenuron measured bile was 12935 ± 3725 ng g^{-1} and, as with the liver samples, this was collected at day 7. The highest overall concentration of lufenuron measured in bile was 18822 ng g^{-1} and this sample was collected on day 4.

Table 4. Measured concentration of lufenuron (ng g^{-1}) in salmon bile sampled at various time points post lufenuron administration.

	Days post-lufenuron administration					
Sample ID	**Day 4**	**Day 7**	**Day 38**	**Day 62**	**Day 146**	**Day 255**
1	6910	10232	2791	2701	657	250
2	2362	18326	5141	3740	804	165
3	18822	10682	5520	N/A	N/A	78
4	10796	12500	3158	N/A	N/A	3
5	N/A	N/A	N/A	N/A	N/A	228
6	N/A	N/A	N/A	N/A	N/A	221
7	N/A	N/A	N/A	N/A	N/A	395
8	N/A	N/A	N/A	N/A	N/A	162
9	N/A	N/A	N/A	N/A	N/A	165
Mean	9723	12935	4153	3221	731	185
Standard deviation	6977	3725	1377	735	104	110
Relative Standard Deviation (%)	72	29	33	23	14	60

Figure 9 shows the levels of lufenuron in salmon bile measured over time and demonstrated that the concentration of lufenuron in bile peaked at day 7. In addition, the calculated relative standard deviation shows that there is a large spread in the data for some of the days.

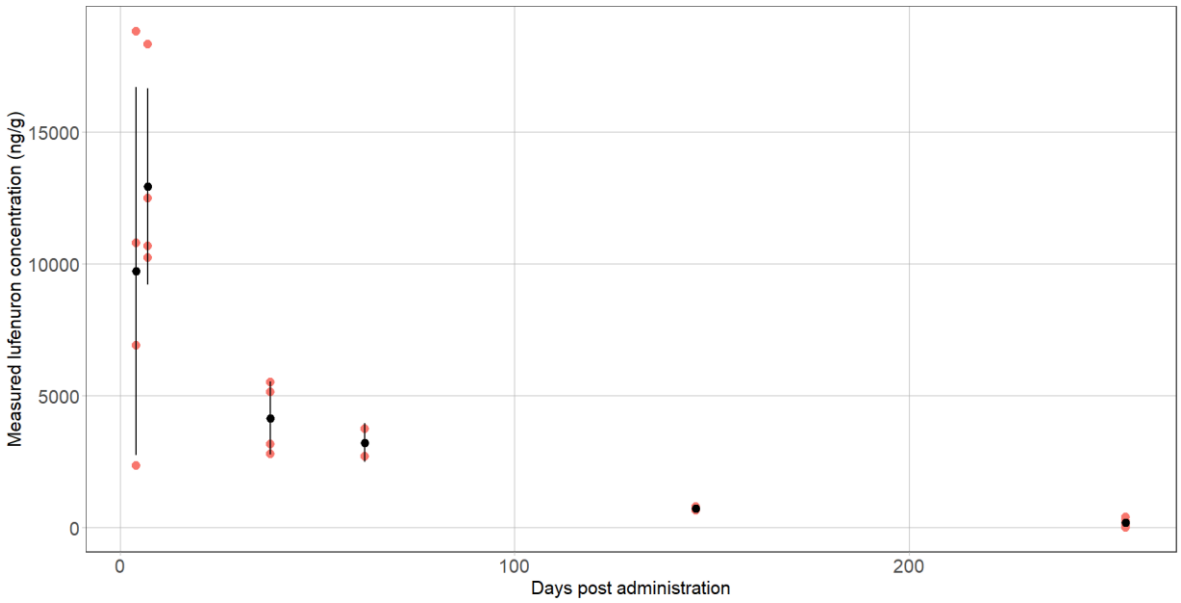


Figure 9. Plot showing the concentration (ng g^{-1}) of lufenuron measured in salmon bile at multiple time points post lufenuron administration. The black dots show the mean measured concentration per time point and the black lines shows the standard deviation. Each red dots represents a single sample.

3.3. Skin samples

The concentration of lufenuron in the analysed skin samples is shown in table 5. The highest mean concentration of lufenuron measured in skin was 25271 ± 4518 ng g^{-1} and occurred at day 22. In addition, the highest overall concentration of lufenuron measured in skin was also found on day 22 with 32284 ng g^{-1}. The mean concentration of lufenuron measured in skin were below the MRL value at day 255 with 1045 ± 348 ng g^{-1}. However, three of the samples had levels above the MRL.

Table 5. Measured concentration of lufenuron (ng g^{-1}) in salmon skin tissue sampled at various time points post lufenuron administration.

	Days post-lufenuron administration						
Sample ID	**Day 4**	**Day 7**	**Day 22**	**Day 38**	**Day 62**	**Day 146**	**Day 255**
1	6543	22067	26704	11031	6816	2191	1439
2	7030	17780	20968	22412	12987	1822	504
3	8568	23338	19108	21375	5946	3329	940
4	12687	20872	27017	21048	13222	4458	530
5	13137	19198	32284	20725	14335	1689	798
6	11572	18859	27774	18432	15581	1831	1368
7	7076	17655	29843	21152	8010	2668	1368
8	11008	12995	19964	20136	8163	4431	1344
9	N/A	N/A	27392	17480	10225	3676	1029
10	N/A	N/A	21653	23982	7637	1307	1126
Mean	9703	19096	25271	19777	10292	2740	1045
Standard deviation	2705	3186	4518	3579	3463	1163	348
Relative Standard Deviation (%)	28	17	18	18	34	42	33

Figure 10 shows the levels of lufenuron in salmon skin tissue measured over time in both the initial analysis (day 22, 38, 62 and 146) and the current analysis (day 4, 7 and 255) and demonstrated that the concentration of lufenuron in skin peaked at day 22.

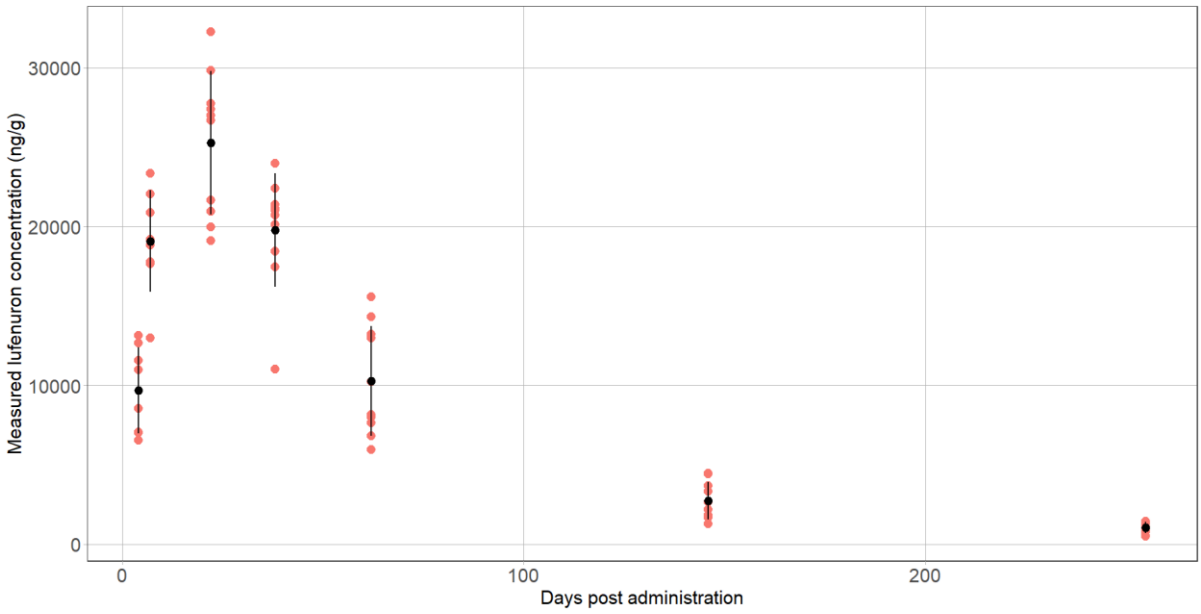


Figure 10. Plot showing the concentration (ng g^{-1}) of lufenuron measured in salmon skin at multiple time points post lufenuron administration. The black dots show the mean measured concentration per time point and the black lines shows the standard deviation. Each red dots represents a single sample.

3.4. Plasma samples

The concentration of lufenuron in the analysed plasma samples are shown in table 6. The highest mean concentration of lufenuron measured in plasma was 4633 ± 1031 ng g^{-1} and occurred at day 7. This day also had the highest overall concentration of lufenuron measured in plasma with 6335 ng g^{-1}.

Table 6. Measured concentration of lufenuron (ng g^{-1}) in salmon plasma sampled at various time points post lufenuron administration.

	Days post-lufenuron administration					
Sample ID	**Day 4**	**Day 7**	**Day 38**	**Day 62**	**Day 146**	**Day 255**
1	4315	3472	1405	1037	103	60
2	3914	5516	1810	1543	145	34
3	2747	3618	1453	1238	92	43
4	2383	6335	1260	2622	130	17
5	3942	4256	1714	1153	132	9
6	2208	4257	1738	1294	313	44
7	1532	5915	3429	640	274	36
8	2327	4371	1123	652	142	36
9	2421	3956	93	763	54	41
10	3109	N/A	1535	1203	151	45
Mean	2890	4633	1556	1215	154	37
Standard deviation	904	1031	822	576	80	14
Relative Standard Deviation (%)	31	22	52	47	52	38

Figure 11 shows the levels of lufenuron in salmon plasma measured over time and demonstrated that the concentration of lufenuron in skin peaked at day 7.

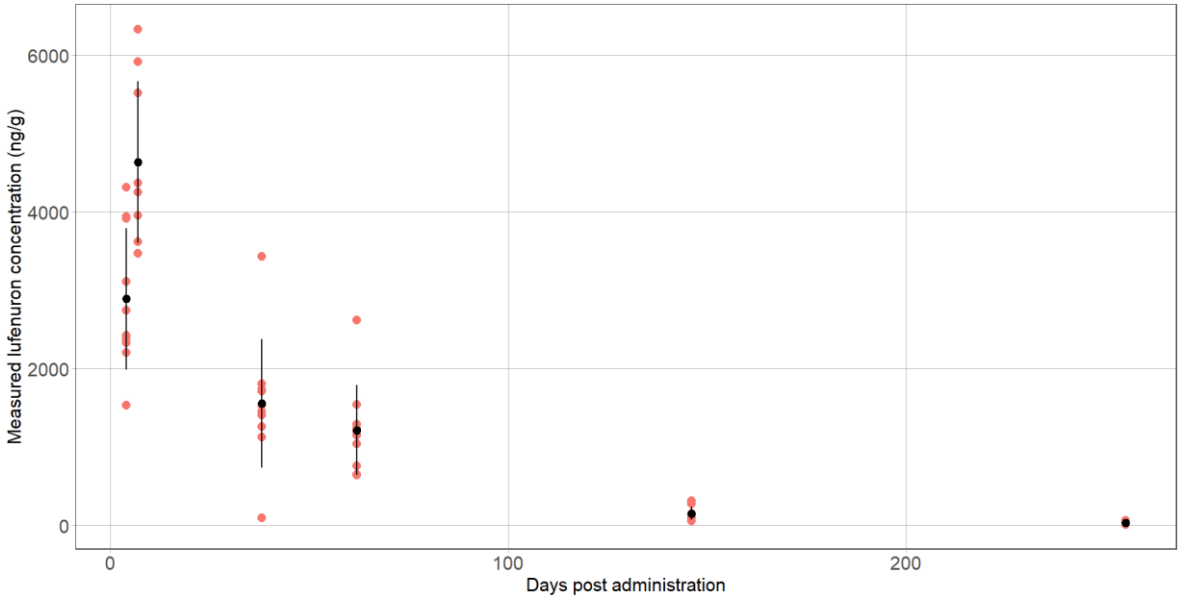


Figure 11. Plot showing the concentration (ng g^{-1}) of lufenuron measured in salmon plasma at multiple time points post lufenuron administration. The black dots show the mean measured concentration per time point and the black lines shows the standard deviation. Each red dots represents a single sample.

3.5. Muscle samples

The previously analysed data for the muscle for several days are shown in table 7. The highest mean concentration of lufenuron was found on day 7 with 7058 ± 2006 ng g^{-1}. The highest overall concentration of lufenuron was found on day 8 with 11339 ng g^{-1}. The mean concentration of lufenuron in the muscle were below the MRL value at day 146 with 747 ± 354 ng g^{-1}. Figure 12 shows the levels of lufenuron in salmon muscle tissue measured over time and demonstrated that the concentration of lufenuron in muscle peaked at day 7.

Table 7. Measured concentration of lufenuron (ng g^{-1}) in salmon muscle tissue sampled at various time points post lufenuron administration.

	Days post-lufenuron administration													
Sample ID	**Day 2**	**Day 4**	**Day 6**	**Day 7**	**Day 8**	**Day 10**	**Day 14**	**Day 22**	**Day 38**	**Day 62**	**Day 146**	**Day 255**	**Day 380**	**Day 510**
1	335	2267	6212	6256	11339	2438	2587	6661	3503	2340	398	176	29	10
2	2917	4516	4391	3964	8514	915	3240	4391	5425	2044	587	92	62	11
3	1309	1959	8576	9675	8782	5438	10332	4641	3398	1897	530	178	39	6
4	1099	2915	4478	7259	8730	4790	2208	4080	4649	10379	1223	233	35	5
5	383	2534	5017	4059	2776	6330	5434	6000	3972	2485	336	81	34	6
6	1644	4936	3008	8145	6851	5317	2113	7323	3146	3573	534	189	36	14
7	788	3686	8758	5811	3210	4739	2640	7635	4580	2889	572	123	35	22
8	913	3799	6487	8540	4120	9189	11347	5648	4725	1493	862	191	22	8
9	843	4602	4589	9270	8764	5639	2058	7953	13878	3043	1282	152	34	9
10	1402	1997	5929	7602	5134	8079	2203	6536	2656	2664	1146	124	16	9
Mean	1164	3321	5745	7058	6822	5287	4416	6087	4993	3281	747	154	34	10
Standard Deviation	745	1132	1847	2006	2869	2405	3537	1380	3235	2565	354	48	12	5
Relative Standard Deviation (%)	64	34	32	28	42	45	80	23	65	78	47	31	35	49

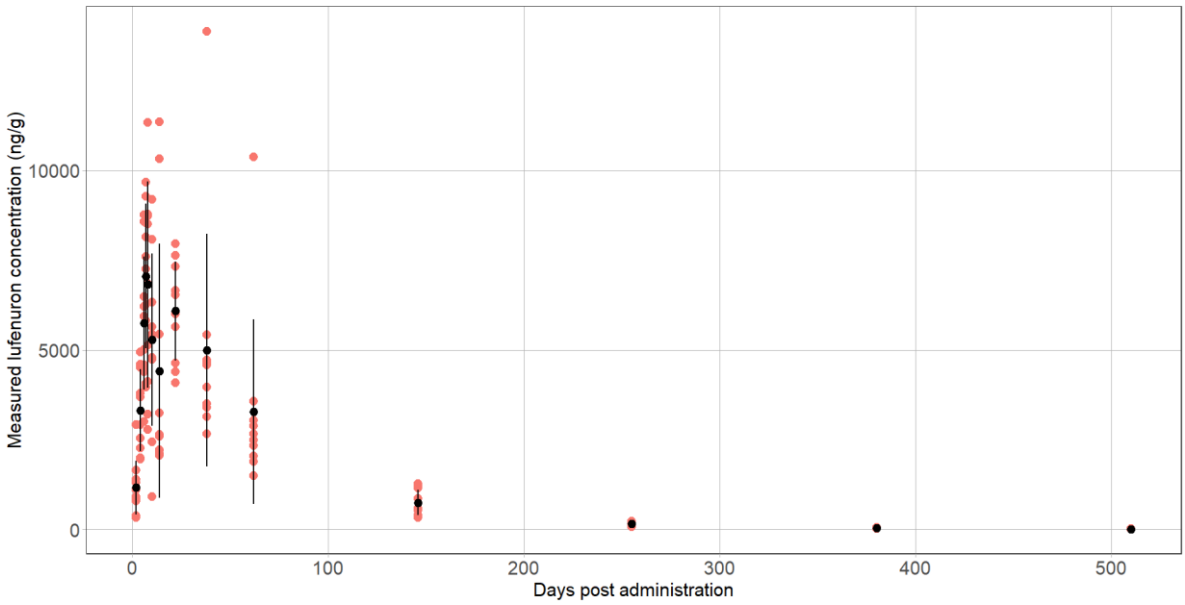


Figure 12. Plot showing the concentration (ng g^{-1}) of lufenuron measured in salmon muscle at multiple time points post lufenuron administration. The black dots show the mean measured concentration per time point and the black lines shows the standard deviation. Each red dots represents a single sample.

Table 8 shows the calculated concentration of lufenuron in 9/10-part muscle and 1/10-part skin, i.e. the ratio that the MRL value applies for, for some selected days. The data shows that the mean concentration of lufenuron were below the MRL value at day 146 with 946 ± 388 ng g^{-1}. However, two of the samples had levels that were above the MRL value.

Table 8. Calculated concentration of lufenuron in ng g^{-1} for a ratio of 9/10-part muscle and 1/10-part skin.

	Days post-lufenuron administration						
Sample ID	**Day 4**	**Day 7**	**Day 22**	**Day 38**	**Day 62**	**Day 146**	**Day 255**
1	2695	7837	8665	4256	2788	577	302
2	4767	5346	6049	7124	3138	711	133
3	2620	11041	6088	5196	2302	810	254
4	3892	8620	6374	6289	10663	1547	263
5	3594	5573	8628	5647	3670	471	153
6	5600	9216	9368	4675	4774	664	307
7	4025	6995	9856	6237	3401	782	248
8	4520	8986	7080	6266	2160	1219	306
9	N/A	N/A	9897	14238	3761	1521	240
10	N/A	N/A	8048	4789	3161	1162	224
Mean	3964	7952	8005	6472	3982	946	243
Standard Deviation	1014	1929	1517	2870	2465	388	60
Relative Standard Deviation (%)	26	24	19	44	62	41	25

3.6. Comparison between the percentage change in concentration

The different matrices can be compared by looking at the difference in the percentage change in the mean concentrations for two different days. The two sampling days that were chosen were day 7 and day 38. Day 7 is the last day in the feeding regime and day 38 is the first day after day 7 where there are results for all the matrices. The calculated results are shown below in table 9.

Table 9. Calculated percentage change between day 7 and day 38 for all matrices.

	Liver	**Bile**	**Skin**	**Plasma**	**Muscle**
Mean day 7 (ng g^{-1})	12791	12935	19096	4633	7058
Mean day 38 (ng g^{-1})	11342	4153	19777	1556	4993
Percentage change	-11	-68	4	-66	-29

All matrices had a negative percentage change except for skin which had a small positive change. Bile and plasma had the largest percentage changes overall with -68% and -66% respectively.

3.7. Elimination time

The half-life of lufenuron calculated for plasma, bile, liver, muscle and skin tissues was 39, 47, 52, 65 and 59 days, respectively (Figure 13a-e). The half-life in plasma is the biological half-life for lufenuron.

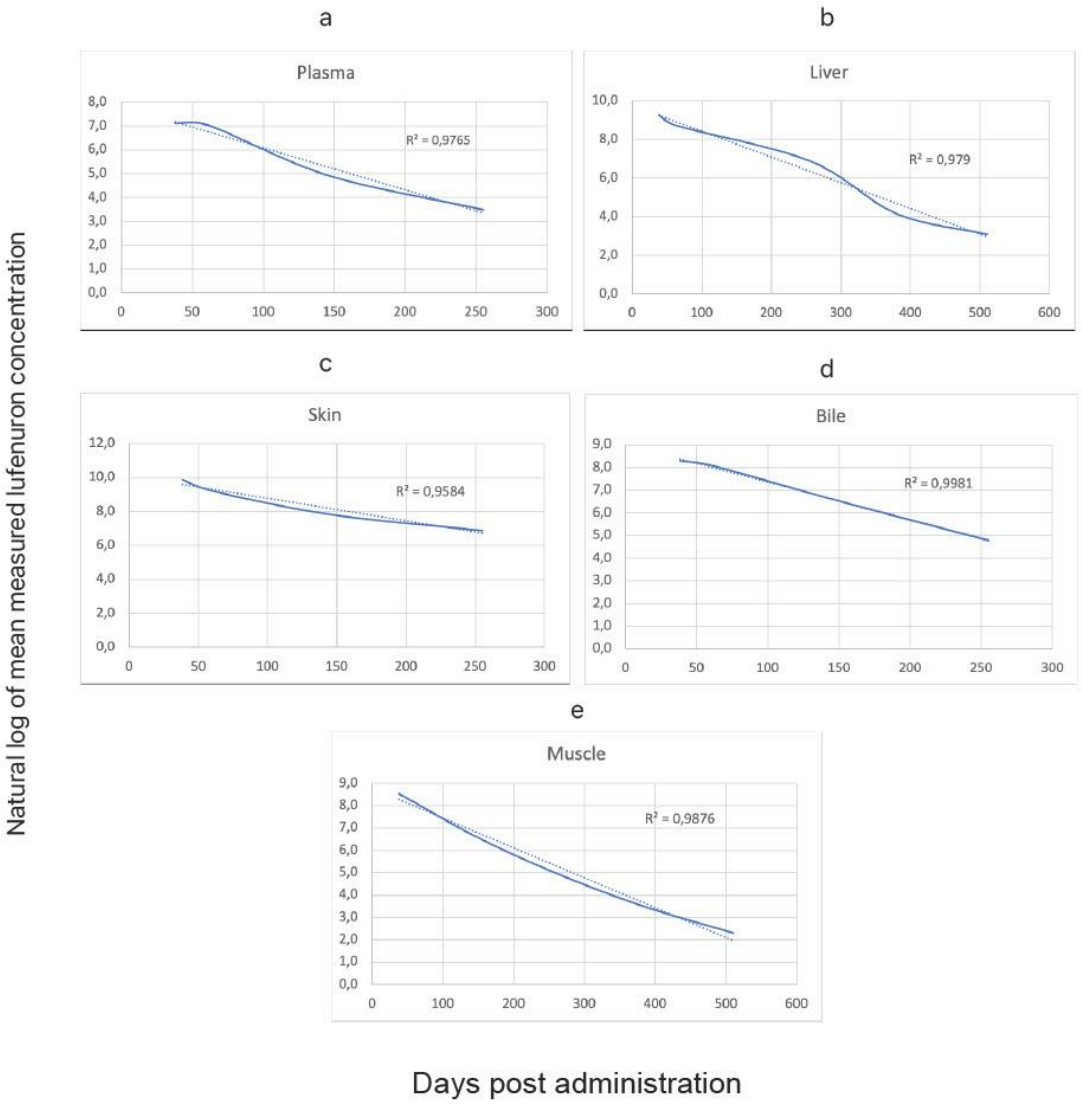


Figure 13. Elimination time of lufenuron in the different tissues with the x-axis as days post administration and the y-axis as the natural log of the mean measured concentration of lufenuron in the samples. Individual plots show elimination time in a) plasma, b) liver, c) skin, d) bile and e) muscle.

Table 10 shows the pharmacokinetic parameters AUC, t_{max} and C_{max} for all the matrices that were calculated along with the half-lives.

Table 10. Calculated pharmacokinetic parameters AUC, t_{max}, C_{max} and $t_{1/2}$.

	Plasma	**Liver**	**Skin**	**Bile**	**Muscle**
AUC (µg day g^{-1})	205	1487	1851	594	517
t_{max} (Day)	7	8	22	7	7
C_{max} (ng g^{-1})	4633	29152	25271	12395	7058
$t_{1/2}$ (Days)	39	52	59	47	65

3.8. Growth versus elimination

As the elimination time of lufenuron is rather long, it is of particular interest to investigate how much of the decrease in the concentration over time is due to the increase in mass. If no metabolism or elimination of lufenuron occurs the amount of lufenuron would stay the same over time but become more diluted as the fish grows over time. To calculate the concentration of lufenuron based on dilution due to growth the concentration at day 38 is multiplied by the mass at the same day. This gives the total amount of lufenuron present at day 38 and as mentioned before this amount should stay constant over time. To find the concentrations for the days after day 38 this amount is divided by the mass for these days. Table 11 shows the calculated concentrations for plasma. The data for the mass for all days are shown in table 12 with the mean mass as well.

Table 11. Calculation of the concentration of lufenuron based on dilution due to growth in plasma. The analysed values and mass are all mean values.

Day	**Mass (g)**	**Lufenuron (analysed values ng g^{-1})**	**Amount lufenuron based on day 38 (ng)**	**Lufenuron (based on dilution due to growth) (ng g^{-1})**
38	63	1556	98028	1556
62	87	1215	98028	1127
146	164	154	98028	598
255	386	37	98028	254

Table 12. Mass of the whole sampled fish in g.[84]

	Days post-lufenuron administration													
Sample ID	**Day 2**	**Day 4**	**Day 6**	**Day 7**	**Day 8**	**Day 10**	**Day 14**	**Day 22**	**Day 38**	**Day 62**	**Day 146**	**Day 255**	**Day 380**	**Day 510**
1	33	35	31	42	39	58	40	47	73	112	144	179	1187	1614
2	34	37	42	36	35	24	39	39	58	86	147	398	746	1856
3	33	38	36	46	36	40	38	57	64	121	163	361	996	1963
4	34	35	46	38	36	38	43	40	80	78	146	259	1100	2992
5	33	37	40	39	35	41	35	42	55	76	145	571	1324	3296
6	36	39	63	39	37	41	57	40	52	75	176	459	1225	1418
7	33	38	41	43	34	45	37	40	65	92	150	372	1154	2272
8	37	38	38	40	44	38	39	63	60	84	218	460	1441	1962
9	44	37	38	39	37	40	66	50	56	73	143	195	815	1614
10	39	53	34	44	34	36	57	48	63	72	211	604	1196	1416
Mean	**36**	**39**	**41**	**41**	**37**	**40**	**45**	**47**	**63**	**87**	**164**	**386**	**1118**	**2040**

The analysed values and the values based on dilution due to growth were plotted against the days in a graph. Figure 14 shows the graph for the plasma.

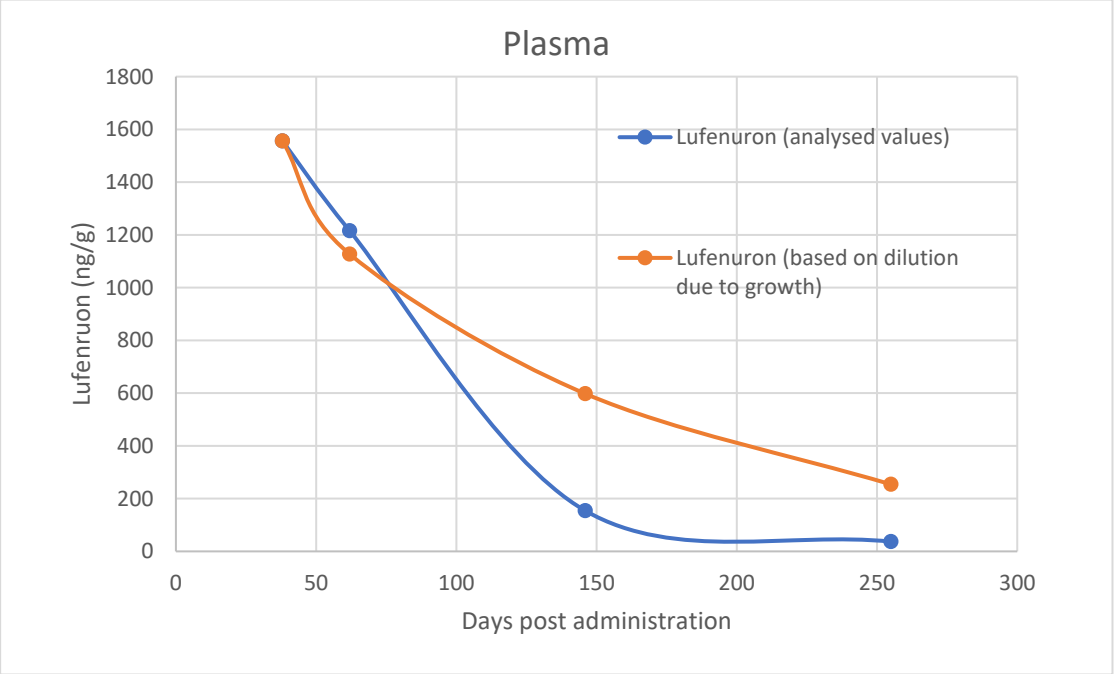


Figure 14. Concentration of analysed lufenuron values and lufenuron based on dilution due to growth in ng g^{-1} against days for the plasma.

The graph shows that for day 62 the analysed value is slightly higher than the predicted value, while for the days 146 and 255 the analysed values are much lower than the predicted values. The graph also shows that the curve for the analysed values is steeper than for the curve for the values based on dilution due to growth.

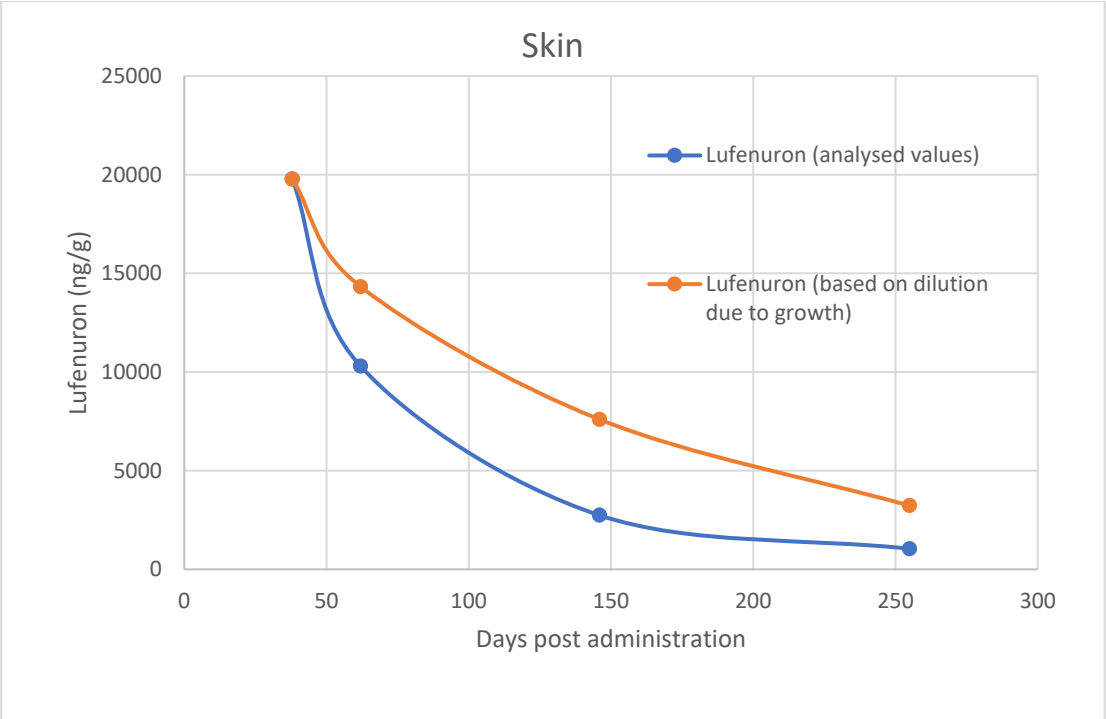


Figure 15. Concentration of analysed lufenuron values and lufenuron based on dilution due to growth in ng g^{-1} against days for the skin.

The graph for the skin is shown in figure 15. Unlike for the plasma all the analysed values are lower than the predicted values. In addition, the difference between the analysed value and predicted value at day 62 is much larger for the skin than for the plasma in terms of distance between the two points. On the other hand, for day 146 plasma have a much larger difference between the two points than the skin has.

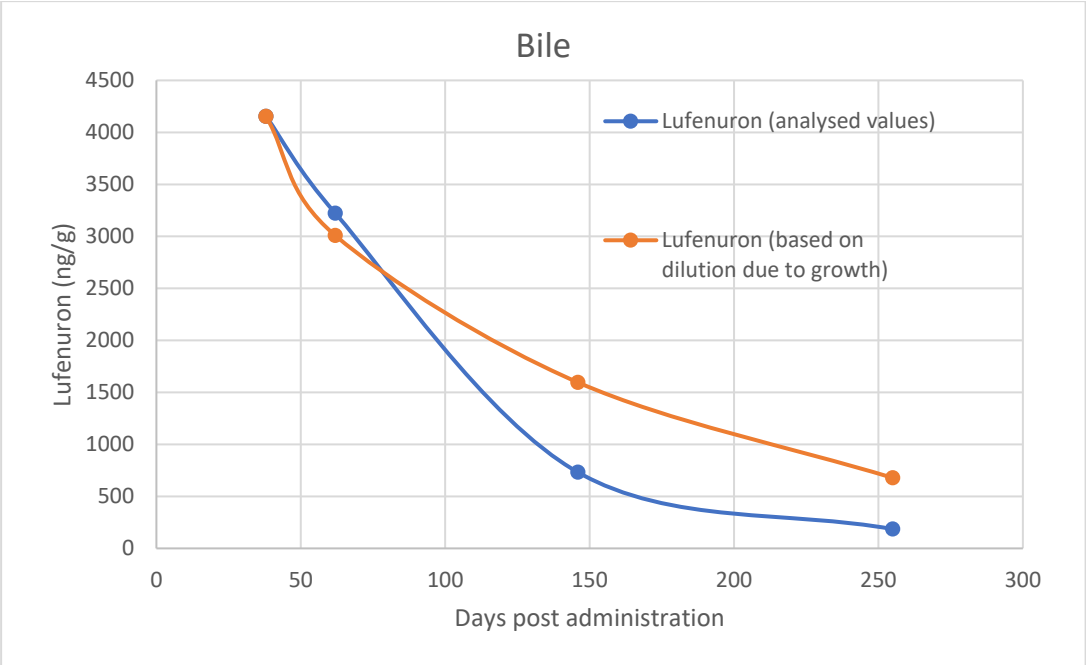


Figure 16. Concentration of analysed lufenuron values and lufenuron based on dilution due to growth in ng g^{-1} against days for the bile.

The graph for the bile is shown in figure 16. The graph is like that of the plasma graph with the analysed value for day 62 being slightly higher than the predicted value and the following two days, 146 and 255, being lower than the predicted values. The difference between the two points for day 146 is slightly smaller and the curve is also less steep than in the plasma graph.

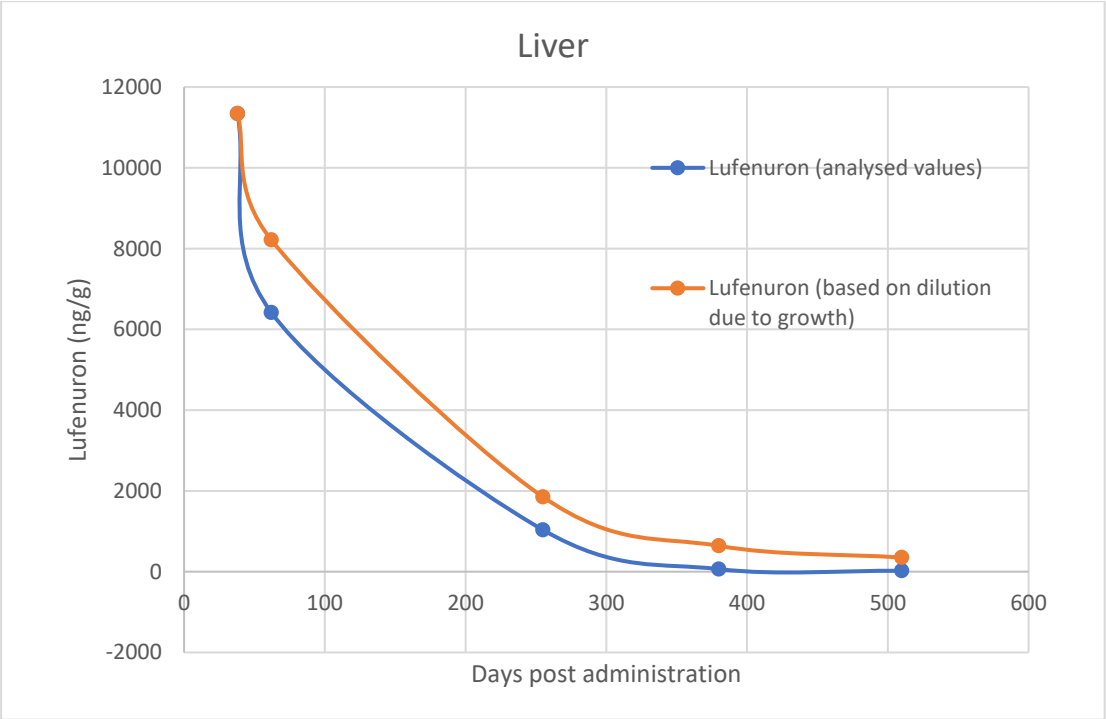


Figure 17. Concentration of analysed lufenuron values and lufenuron based on dilution due to growth in ng g^{-1} against days for the liver.

Figure 17 shows the graph for the liver. The graph shows that all the analysed values are lower than the predicted values, however the difference between the two points are somewhat small especially for days 255 and onwards. In addition, the steepness of the curve for the analysed values are also somewhat like that of the curve for the predicted values.

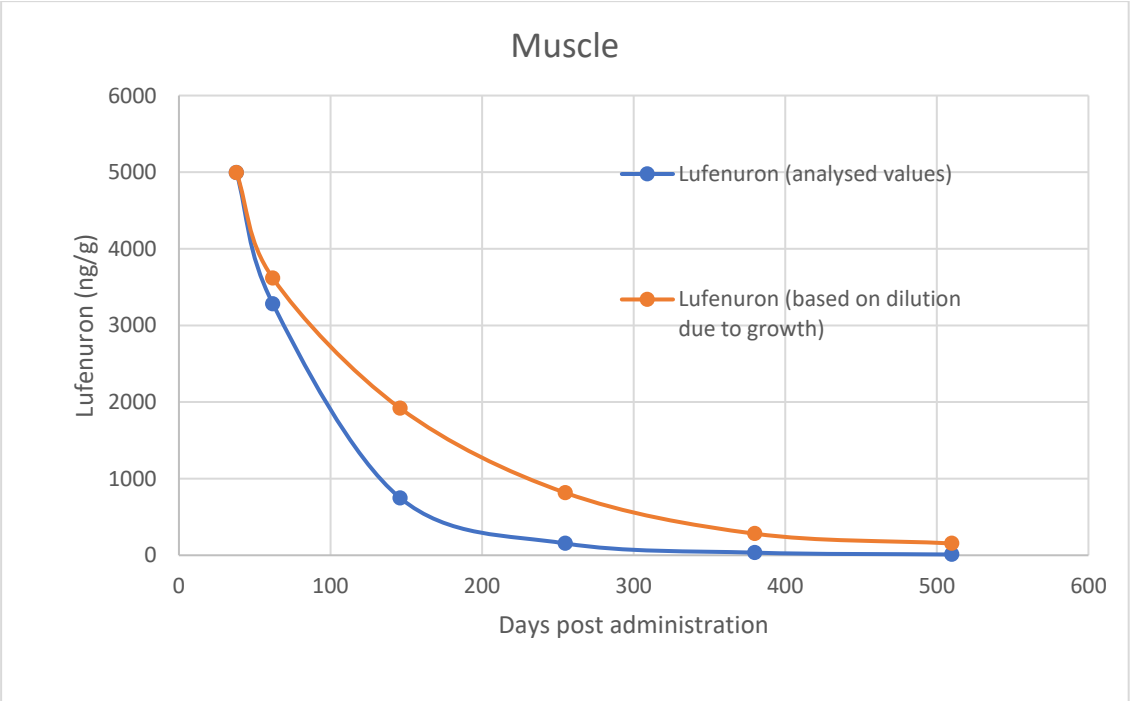


Figure 18. Concentration of analysed lufenuron values and lufenuron based on dilution due to growth in ng g^{-1} against days for the muscle.

The graph for the muscle is shown in figure 18. As with the liver, the analysed values are all lower than the predicted values for all days. However, the distance between the two points at day 62 is much smaller for the muscle than for the liver. On the other hand, the distance is larger at day 255 for the muscle than for the liver. In addition, the muscle graph has a point for day 146 which shows that there is a large difference between the analysed value and the predicted value at this day. The curve for the analysed values is also slightly steeper for the muscle than for the liver.

4. Discussion

The mean measured concentration of lufenuron in the liver reached a peak at day 8, which was the first day after the treatment had ended. The mean concentration peaking at day 8 is to be expected as the treatment period for the fish is 7 days and the concentration of the drug will build up over time in the different tissues of the fish. After day 8, the drug was slowly eliminated from the fish.

The results for the bile samples showed a similar pattern to that of the liver samples. The highest mean concentrations of lufenuron were measured in bile at day 7, however one can assume that if there were available samples from day 8 the peak mean concentration would have been on this day. The mean lufenuron concentrations measured in bile and liver tissues were similar at day 4 and day 7 which indicates that the drug was distributed similarly in the two matrices during the feeding regime. This is to be expected as the bile would be excreted from the liver and therefore if the concentration in the liver increases due to the fish consuming the drug, then the concentration in the bile must increase as well since the liver would be working on excreting the drug out of the fish. However, after the treatment has ended the concentration in bile appeared to decrease faster than in the liver. This could be because the lufenuron may not be quickly degraded in the liver and consequently persists in the liver at higher concentrations and for a longer time than in the bile.

The most variation in terms of the measured lufenuron concentrations was observed in the bile tissues (Figure 9). Some variation is expected in the measured concentrations at each sampling point as the fish are of different sizes and weights, and the amount of feed eaten by each fish is different, i.e. some fish might eat more or less than the other fish. This, however, mimics the environment in a real-world fish farm at an industrial scale. In addition, the bile samples were the matrix with the least number of samples per day with day 62 and day 146 only having two samples per day, which may have caused the wider spread in the data.

In an article Horsberg and Høy (1991) investigated the distribution of ^{14}C-Diflubenzuron in Atlantic salmon and observed high concentrations of diflubenzuron in the liver, kidney, brain, bile, fat, and cartilage after 12 hours after administration of the drug. They suggested that since the activity in the bile was high this indicated that the biliary route was the major excretion pathway of the drug.[58] It is reasonable to assume that the biliary route is also the major excretion pathway for lufenuron as well as both lufenuron and diflubenzuron are both benzoylureas with similar properties.

Lufenuron concentrations were the highest in the skin samples over time (figure 19) compared to all the other tissue matrices analysed here, with a peak concentration measured at day 22, two weeks after the final lufenuron dose. The mean concentration of lufenuron measured in the skin at day 4 (9703 ng g^{-1}) was like those measured in the bile and liver (9723 and 9423 ng g^{-1}, respectively). At day 7 and for all days after the treatment took place, the concentration of lufenuron measured in the skin was higher than in the other two tissues. When considering all matrices and all sampling days, the highest mean concentration of lufenuron was measured in liver at day 8. It is, however, reasonable to assume that if skin samples from day 8 were to be analysed, they will have a higher mean concentration than the liver samples as all subsequent days had a higher mean concentration than the liver samples.

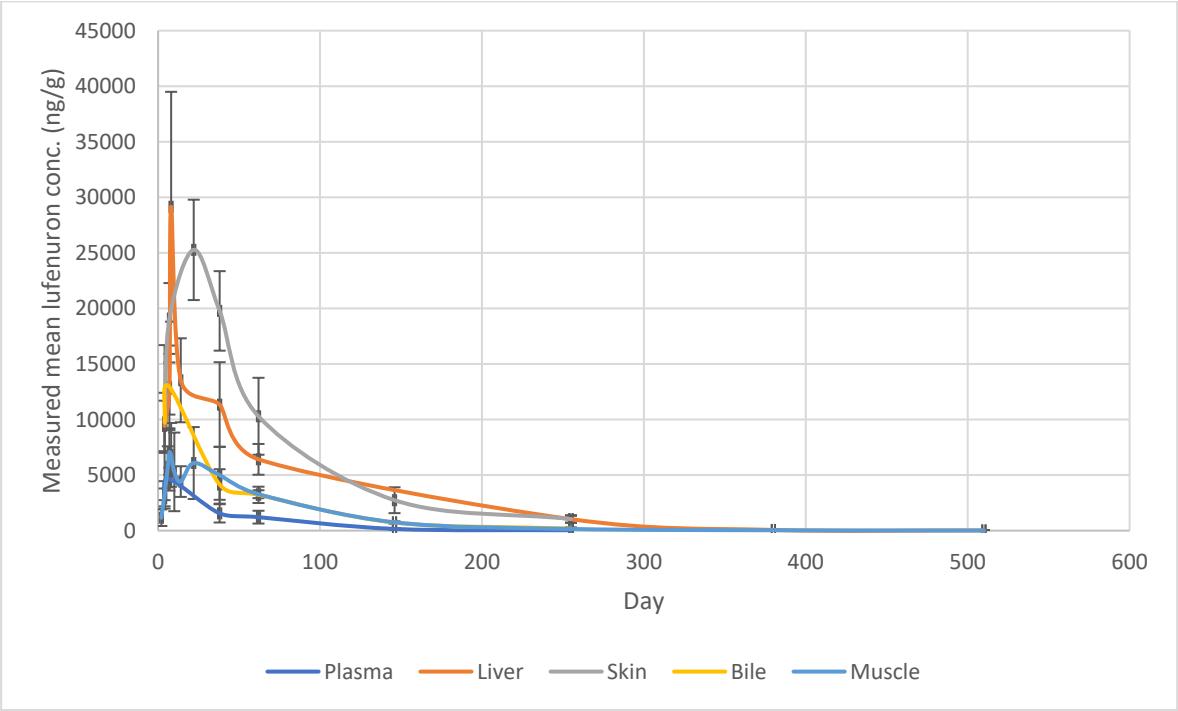


Figure 19. Graph showing the mean concentrations over time for all the matrices with error bars showing the standard deviation.

It is to be expected that the concentration of lufenuron were highest in the skin, as the skin is the point where the lice will attach to and infect the fish. Hence, the drug must be able to be distributed to the skin and persist there for a substantial amount of time to ensure that the salmon remains lice free. This explains the similar mean concentration of lufenuron observed in the skin, bile and liver at day 4 and the subsequent rapid increased concentration measured in the skin over the following days, as the drug is distributed to the site where it is needed. As mentioned earlier, the drug needs to be effective over a longer period to ensure that the salmon

remains lice free. Therefore, the concentration of lufenuron takes longer to decrease in the skin compared to the other matrices. In addition, while the mean concentration for day 255 for the skin was below the MRL as mentioned earlier, there were several samples where concentrations were above and on the border of the MRL. One could therefore suggest that the concentrations of lufenuron in the skin probably were likely lower than the MRL after day 255, in comparison with concentrations observed in the bile and liver which were below the MRL on day 255.

Despite the concentration data showing that the measured lufenuron concentration in the skin remained high over a long period of time, unpublished data from the same experiment showed that the lufenuron was effective against the salmon lice at day 146, but not at day 255.[84] This indicates that the duration of action for lufenuron is somewhere between 20.9 and 36.4 weeks, which is somewhat shorter than the duration of action reported by the drug manufacturer, Elanco, which is between 24 to 55 weeks.[67]

The concentration of lufenuron measured in plasma followed the same general trend as the other matrices with a peak concentration observed at day 7. As with skin and bile, it could be also argued that if samples from day 8 were to be analysed, a new peak concentration would be observed. In general, the concentrations of lufenuron in the plasma samples were the lowest at all sampling days compared to all the other matrices analysed. The minimal concentration suggests lufenuron's limited plasma distribution and swift elimination, as evidenced by the rapid decrease over time, indicating primary plasma-based excretion in fish.

The data for the muscle showed a similar trend to that of the rest of the matrices with a peak concentration of lufenuron measured at day 7. However, the concentration of lufenuron decreased from day 7 to day 14 and then increased again from 14 to day 22. From there on it followed the trend of the other matrices. In general, the concentration in the muscle was higher than that of plasma, but lower than bile, skin, and the liver.

Between day 7 and 38, bile and plasma had the largest percentage change in mean lufenuron concentrations. As mentioned before all the matrices except skin had a negative percentage change meaning that the concentration decreased. In addition, the skin had the smallest percentage change of all the matrices. There was a small increase in the concentrations of lufenuron in the skin from day 7 to day 38 which was probably due to the generally higher concentrations in the samples compared to the other matrices which took longer to be eliminated from the matrix. The calculated percentage changes support the previous discussion regarding the rapid decrease in lufenuron concentration observed in bile over time due to it being an

excretion pathway for the drug. In addition, there was also a rapid decrease in lufenuron concentrations measured in plasma. This makes sense as the plasma would be the main route of elimination for lufenuron in the fish.

The shortest half-life for lufenuron was calculated in plasma (39 days) indicating that lufenuron was easily distributed from the plasma to the other organ/matrices. To compare, a study carried out in Chile had determined the half-life of lufenuron in blood in salmon to be 54 days, slightly longer than our value.[66] One might expect the half-life to be the longest in the skin as high concentrations of lufenuron were observed in the skin over time compared to the other matrices and the skin is also the targeted area for the drug. Interestingly, however, we found that the half-life of lufenuron was the longest in the muscle (65 days), despite the significantly lower concentrations of lufenuron in muscle compared to skin and liver (figure 19). This indicates that the lufenuron persisted in the muscle for longer periods of time compared to the other matrices analysed here and this may be explained by the fact that lufenuron is very fat-soluble and therefore is retained in fat tissues.[66]

A challenge in determining how lufenuron was eliminated from the fish is the fact that lufenuron is more persistent than many other veterinary drugs which means that the growth of the fish would also influence the decrease in concentration over time.[66] In comparison with other veterinary drugs that are used on fish such as florfenicol, oxolinic acid and flumequine, lufenuron had a much longer half-life. The half-lives of these drugs in plasma were 30, 21 and 22 hours respectively, while the half-life of lufenuron calculated in the present study for plasma was 39 days.[29,30] In addition, the half-life of lufenuron calculated in the present study was also much longer than the half-lives for the two other benzoylureas that are also used as delousing agents, teflubenzuron and diflubenzuron. For diflubenzuron and teflubenzuron the half-lives in Atlantic salmon are 71.1 and 14.2 hours respectively.[54,52] However, the present work shows that the decrease in the concentration of lufenuron over time was not only due to the growth of the fish but also due the elimination of lufenuron over time.

The long half-lives of lufenuron in the tissues could possibly indicate that lufenuron might have a longer withdrawal period than other pharmaceuticals used as salmon delousing agents. However, even if the withdrawal period of lufenuron would be long it would still be shorter than the lifespan of the salmon in seawater. Previously calculated concentrations of lufenuron using the ratio used for the MRL values, i.e. 9/10-part muscle and 1/10-part skin, showed that the concentrations of lufenuron were below the MRL value at day 255. This means that a long withdrawal period would not be of much concern in terms of the production of Atlantic salmon

as food. However, if the salmon becomes diseased and must be slaughtered earlier than normal then the long withdrawal period would become a problem as there may be residues of the drug still in the fish. This could be a potential disadvantage of using lufenuron as a treatment option as one may have to wait longer to slaughter the salmon to ensure that there are no traces of the drug remaining above the MRL value in the fish. In addition, escaped salmons that have been treated with lufenuron could potentially be captured by someone unaware that the fish have undergone treatment, and this could lead to the person accidentally consuming too high levels of lufenuron.

As mentioned in the introduction the avermectin emamectin benzoate is also a drug used as an in-feed salmon lice treatment over 7 days, but it belongs to a different chemical group and has a different mode of action than lufenuron. In addition, it has a different recommended dosage than lufenuron. However, as the emamectin benzoate and benzoylureas, the chemical group which lufenuron belongs to, are the only in-feed treatments currently available for treatment it is worth comparing some of their properties. In addition, as lufenuron is not currently available as a treatment for salmon lice in Norway but emamectin benzoate is comparison between them could be of interest.

A study conducted by Sevatdal et al. (2005) investigated the distribution and elimination of emamectin benzoate in Atlantic salmon after they were fed emamectin-medicated feed at a dosage of 50 μg kg^{-1} daily for 7 days. They reported that the highest concentration was measured at day 7 in muscle and plasma, and this is in line with our findings where we also observed peak concentration of lufenuron at day 7 in muscle and plasma. Interestingly the previous study also showed that concentrations in plasma on day 7 were higher than the other analysed matrices. This is in stark contrast to the data in this thesis that showed that plasma had the lowest concentration of all the matrices. In addition, the calculated half-life of emamectin benzoate was shorter in the muscle compared to plasma (9.2 and 10 days, respectively).[39] This could suggest that lufenuron was more easily distributed into the muscle compared to emamectin benzoate as the mean concentration for lufenuron in muscle on day 7 was almost twice the mean concentration in the plasma. A one-dose distribution study was also conducted which showed that the emamectin benzoate reached the skin of the fish in high quantities compared to the other tissues. As mentioned earlier, the results showed that the skin samples had the highest concentrations overall which is comparable to the study.[39] The high concentrations in the skin for both emamectin benzoate and lufenuron is to be expected as mentioned before this is the area where the salmon lice would attach and infect the salmon. Furthermore, the study also

determined that the main excretory pathway for emamectin benzoate was through the bile, the same as for lufenuron.[39]

There were some methodological challenges that occurred during the work. The two main methodological challenges that occurred were the homogenisation of the skin samples and the amount of bile samples. The homogenisation of the skin samples proved to be a challenge as unlike the liver the skin samples were not easily broken down using a spatula and mixer. The samples for day 255 were homogenised using liquid nitrogen which made them sufficiently homogenised. However, as the samples for day 4 and 7 were small they were homogenised by breaking them down with a spatula while still frozen. Obviously, this made them less homogenised compared to the samples from day 255. If not homogenised enough the weighed samples would not be representative of the skin of the fish. The limited bile samples on certain days posed a methodological challenge leading to a broader data spread with fewer points, as previously noted.

5. Further work

For further work, it would be of interest to analyse the faeces samples that were also collected during the sampling period. The analysis of the faeces would help to determine the extent of the reabsorption of lufenuron in the intestine and could provide additional information on the excretory pathway of lufenuron. In addition, it would also be of interest to investigate whether any metabolites of lufenuron are detected in the tissues. The detection of the metabolites could help to gain knowledge about the metabolism of lufenuron in Atlantic salmon.

Another area that could be interesting to explore further is the environmental concerns regarding the use of lufenuron as a salmon delousing agent. As lufenuron belongs to the benzoylurea group, it would most likely be very persistent in the environment as with diflubenzuron and teflubenzuron. It would therefore be interesting to investigate the distribution and persistence of lufenuron in sediments around the salmon farm. It would also be of interest to investigate the effect of lufenuron on crustaceans as they would also be in proximity with the salmon farms.

6. Conclusion

The aim of this study was to better understand the distribution and elimination of lufenuron in Atlantic salmon by quantifying the levels of lufenuron in different tissues. The data showed that there was a difference in distribution between the tissues, with plasma having the lowest concentrations and the skin having the highest concentrations of lufenuron. The data indicated that lufenuron is mainly eliminated from the fish via the plasma and that the main excretory pathway for lufenuron is through the biliary route. In addition, the calculated half-lives showed that lufenuron is very persistent in Atlantic salmon, especially in the muscle, which could potentially be an issue with regards to food safety when it comes to disease or escaped farmed salmon that possibly contain levels of lufenuron that are higher than the MRL value. In conclusion, these findings have given a better understanding of the pharmacokinetics of lufenuron in Atlantic salmon.

7. References

1 B. Aarset and S.E. Jakobsen, *Mar. Policy*, 2009, **33**, 280–287.

2 Fish farming in Norway, https://dyrevern.no/dyrevern/fish-farming-in-norway/, (accessed August 2023).

3 Directorate of Fisheries, Akvakulturstatistikk: matfiskproduksjon av laks, regnbueørret og ørret, https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret/Matfiskproduksjon, (accessed May 2024).

4 J.O. Olaussen, *Mar. Policy*, 2018, **98**, 158–163.

5 Y. Liu, J.O. Olaussen and A. Skonhoft, *Mar. Policy*, 2011, **35**, 413–418.

6 H.B. Fjørtoft, F. Besnier, A. Stene, F. Nilsen, P.A. Bjørn, A.K. Tveten, B. Finstad, V. Aspehaug and K.A. Glover, *Sci. Rep.*, 2017, DOI:10.1038/s41598-017-14681-6.

7 J. Abolofia, J.E. Wilen and F. Asche, *Mar. Resor. Econ.*, 2017, **32**, 329–349.

8 T. Forseth, B.T. Barlaup, B. Finstad, P. Fiske, H. Gjøsæter, M. Falkegård, A. Hindar, T.A. Mo, A.H. Rikardsen, E.B. Thorstad, L.A. Vøllestad and V. Wennevik, *ICES J. Mar. Sci.*, 2017, **74**, 1496–1513.

9 Salmon lice, https://salmonfacts.com/salmon-and-environment/salmon-lice/, (accessed August 2023).

10 E.M. Jensen, T.E. Horsberg, S. Sevatdal and K.O. Helgesen, *PLoS One*, 2020, DOI:10.1371/journal.pone.0240894.

11 R. Hannisdal, O.J. Nøstbakken, H. Hove, L. Madsen, T.E. Horsberg and B.T. Lunestad, *Aquaculture*, 2020, DOI:10.1016/j.aquaculture.2020.735044.

12 C. Eichner, E. Harasimczuk, F. Nilsen, S. Grotmol and S. Dalvin, *Exp. Parasitol.*, 2015, **151–152**, 39–48.

13 B. Hjeltnes, G. Bornø. M.D. Jansen, A. Haukaas and C. Walde, *The Health Situation in Norwegian Aquaculture 2016*, Norwegian Veterinary Institute, 2017.

14 S. Dalvin, P. Frost, E. Biering, L.A. Hamre, C. Eichner, B. Krossøy and F. Nilsen, *Int. J. Parasitol.*, 2009, **39**, 1407–1415.

15 E.J. Branson, S.S. Rønsberg and G. Ritchie, *Aquac. Res.*, 2000, **31**, 861–867.

16 M. Guarracino, L. Qviller and A. Lillehaug, *Dis. Aquat. Organ.*, 2018, **130**, 1–9.

17 P.A. Olsvik, O.B. Samuelsen, A.L. Agnalt and B.T. Lunestad, *Aquat. Toxicol.*, 2015, **167**, 143–156.

18 E.B. Thorstad, C.D. Todd, I. Uglem, P.A. Bjørn, P.G. Gargan, K.W. Vollset, E. Halttunen, S. Kålås, M. Berg and B. Finstad, *Aquac. Environ. Interact.*, 2015, **7**, 91–113.

19 A.G. Murray, *Pest. Manag. Sci.*, 2016, **72**, 322–326.

20 O.B. Samuelsen, B.T. Lunestad, R. Hannisdal, R. Bannister, S. Olsen, T. Tjensvoll, E. Farestveit and A. Ervik, *Sci. Total Environ.*, 2015, **508**, 115–121.

21 S.M. Aaen, K.O. Helgesen, M.J. Bakke, K. Kaur and T.E. Horsberg, *Trends Parasitol.*, 2015, **31**, 72–81.

22 K. Grave, T.E. Horsberg, B.T. Lunestad and I. Litleskare, *Dis. Aquat. Org.*, 2004, **60**, 123-131.

23 *Aquaculture in Norway*, Norwegian Seafood Federation and Norwegian Seafood Council, 2011.

24 L.H. Stien, T. Dempster, S. Bui, A. Glaropoulos, J.E. Fosseidengen, D.W. Wright and F. Oppedal, *Aquaculture*, 2016, **458**, 29–37.

25 D.C. Love, S. Rodman, R.A. Neff and K.E. Nachman, *Environ. Sci. Technol.*, 2011, **45**, 7232–7240.

26 M.A. Tanani, K.S. Ghoneim and K.S. Hamadah, *The Florida Entomologist*, 2012, **95**, 928-935.

27 G. Carmona-Antoñanzas, S.N. Carmichael, J. Heumann, J.B. Taggart, K. Gharbi, J.E. Bron, M. Bekaert and A. Sturm, *PLoS One*, 2015, DOI:10.1371/journal.pone.0137394.

28 K.O. Helgesen, S. Bravo, S. Sevatdal, J. Mendoza and T.E. Horsberg, *J. Fish Dis.*, 2014, **37**, 877–890.

29 G.T. Haugland, K.O. Kverme, R. Hannisdal, M. Kallekleiv, D.J. Colquhoun, B.T. Lunestad, H.I. Wergeland and O.B. Samuelsen, *Front. Vet. Sci.*, 2019, DOI:10.3389/fvets.2019.00394.

30 K.O. Kverme, G.T. Haugland, R. Hannisdal, M. Kallekleiv, D.J. Colquhoun, B.T. Lunestad, H.I. Wergeland and O.B. Samuelsen, *Aquaculture*, 2019, DOI:10.1016/j.aquaculture.2019.734279.

31 A. Powell, J.W. Treasurer, C.L. Pooley, A.J. Keay, R. Lloyd, A.K. Imsland and C. Garcia de Leaniz, *Rev. Aquac.*, 2018, **10**, 683–702.

32 K. Overton, T. Dempster, F. Oppedal, T.S. Kristiansen, K. Gismervik and L.H. Stien, *Rev. Aquac.*, 2019, **11**, 1398–1417.

33 M. Aldrin, R.B. Huseby, L.C. Stige and K.O. Helgesen, *Aquaculture*, 2023, DOI:10.1016/j.aquaculture.2023.739749.

34 M.A. Urbina, J.P. Cumillaf, K. Paschke and P. Gebauer, *Sci. Total Environ.,* 2019, **649**, 1124–1136.

35 O.O. Igboeli, J.F. Burka and M.D. Fast, *Animal Frontiers*, 2014, **4**, 22–32.

36 M. Roth, R.H. Richards, D.P. Dobson and G.H. Rae, *Aquaculture,* 1996, **140**, 217-239.

37 J. Stone, I.H. Sutherland, C. Sommerville, R.H. Richards and K. J. Varma, *Aquaculture*, 2000, **186**, 205–219.

38 O.O. Igboeli, J.F. Burka and M.D. Fast, *Pest. Manag. Sci.*, 2014, **70**, 905–914.

39 S. Sevatdal, Å. Magnusson, K. Ingebrigtsen, R. Haldorsen and T.E. Horsberg, *J. Vet. Pharmacol. Ther.*, 2005, **28**, 101–107.

40 Bruk av legemidler i fiskeoppdrett, 2001–2022, https://www.fhi.no/he/legemiddelbruk/fisk/bruk-av-legemidler-i-fiskeoppdrett/, (accessed December 2023).

41 J.F. Burka, K.L. Hammell, T.E. Horsberg, G.R. Johnson, D. J. Rainnie and D. J. Speare, *J Vet Pharmacol Ther*, 1997, **20**, 333–349.

42 O. Torrissen, S. Jones, F. Asche, A. Guttormsen, O.T. Skilbrei, F. Nilsen, T.E. Horsberg and D. Jackson, *J. Fish Dis.*, 2013, **36**, 171–194.

43 J.W. Treasurer and A. Grant, *Aquaculture*, 1997, **148**, 265-275.

44 P.G. Jones, K.L. Hammell, G. Gettinby and C.W. Revie, *J. Fish Dis.*, 2013, **36**, 209–220.

45 M.K. Rust, *Trends Parasitol.*, 2005, **21**, 232–236.

46 Vetrinærkatalogen, Ectosan Vet, https://www.felleskatalogen.no/medisin-vet/ectosan-vet-benchmark-animal-health-699283, (accessed April 2024).

47 L.A. Hamre, B.T. Lunestad, R. Hannisdal and O.B. Samuelsen, *J. Fish Dis.*, 2011, **34**, 453–457.

48 J.P. Arena, K.K. Liu, P.S. Paress, E.G. Frazier, D.F. Cully, H. Mrozik and J.M. Schaeffer, *J. Parasitol.*, 1995, **81**, 286-294.

49 V. Douris, D. Steinbach, R. Panteleri, I. Livadaras, J.A. Pickett, T. Van Leeuwen, R. Nauen and J. Vontas, *Proc. Natl. Acad. Sci. USA*, 2016, **113**, 14692–14697.

50 S.G. Salokhe, S.N. Mukherjee, S.G. Deshpande, V.P. Ghule and J.R. Mathad, *Curr. Sci. India*, 2010, **99**, 1256-1259.

51 D. Doucet and A. Retnakaran, in *Advances in Insect Physiology*, Academic Press Inc., 2012, vol. 43, pp. 437–511.

52 P. Junquera, B. Hosking, M. Gameiro and A. MacDonald, *Parasite*, 2019, **26**.

53 G. Ritchie, S.S. Rønsberg, K.A. Hoff and E.J. Branson, *Dis. Aquat. Org.*, 2002, **51**, 101-106.

54 S. Rath, L.G. Friedlander and R. Reuss, in *81st Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting,* Food and Agriculture Organization of the United Nations and World Health Organization, 2016, ch. 7.

55 O.B. Samuelsen, B.T. Lunestad, E. Farestveit, E.S. Grefsrud, R. Hannisdal, B. Holmelid, T. Tjensvoll and A.L. Agnalt, *Aquat. Toxicol.*, 2014, **149**, 8–15.

56 P.R.L. Soares, A.L. Corrêa de Andrade, T.P. Santos, S.C.B. Lucas da Silva, J. Freitas da Silva, A. Rodrigues dos Santos, E.H. Lima da Silva Souza, F. Magliano da Cunha, V.W. Teixeira, M.R.S. Cadena, F. Bezerra de Sá, L. Bezerra de Carvalho Júnior and P.G. Cadena, *Chemosphere*, 2016, **161**, 412–421.

57 H.M. Harðardóttir, PhD Thesis, University of Bergen, 2021.

58 T.E. Horsberg and T. Hoy, *Acta. Vet. Scand.*, 1991, **32**, 527–533.

59 U. Gangishetti, S. Breitenbach, M. Zander, S.K. Saheb, U. Müller, H. Schwarz and B. Moussian, *Eur. J. Cell Biol.*, 2009, **88**, 167–180.

60 R. Sun, C. Liu, H. Zhang and Q. Wang, *J. Agric. Food Chem.*, 2015, **63**, 6847–6865.

61 S.L. Lv, Z.Y. Xu, M.J. Li, A.L. Mbuji, M. Gu, L. Zhang and X.W. Gao, *Insects*, 2022, DOI:10.3390/insects13100963.

62 H. Merzendorfer, *Insect Sci.*, 2013, **20**, 121–138.

63 N. Ghazawy, *Journal of Orthoptera Research*, 2012, **21**, 141-148.

64 M. Anastassiadou, G. Bernasconi, A. Brancato, L. Carrasco Cabrera, L. Ferreira, L. Greco, S. Jarrah, A. Kazocina, R. Leuschner, J. O. Magrans, I. Miron, S. Nave, R. Pedersen, H. Reich, A. Rojas, A. Sacchi, M. Santos, A. Stanek, A. Theobald, B. Vagenende and A. Verani, *EFSA Journal*, 2020, DOI:10.2903/j.efsa.2020.6228.

65 Commission regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, 2023, *Offical Journal* L15, p. 1.

66 S. Rath, R. Reuss and H. Erdely, *Residue Monograph prepared by the meeting of the joint FAO/WHO Expert Committee on Food Additives (JECFA), 85th Meeting* 2017, Food and Agriculture Organization of the United Nations and World Health Organization, 2017.

67 Så lenge virker lufenuron, https://www.kyst.no/elanco-lufenuron-lusemiddel/sa-lenge-virker-lufenuron/629789, (accessed May 2024).

68 G.E. Abo-Elghar, P. Fujiyoshi and F. Matsumura, *Insect Biochem. Mol. Biol.*, 2004, **34**, 743–752.

69 S.R. Dean, R.W. Meola, S.M. Meola, H. Sittertz-Bhatkar and R. Schenker, *J. Med. Entomol.* 1998, **35**, 720-724.

70 *Small Animal Clinical Pharmacology*, ed. J.E. Maddison, S.W. Page and D.B. Church, Elsevier, 2008.

71 J.D. Poley, L.M. Braden, A.M. Messmer, O.O. Igboeli, S.K. Whyte, A. Macdonald, J. Rodriguez, M. Gameiro, L. Rufener, J. Bouvier, D.W. Wadowska, B.F. Koop, B.C. Hosking and M.D. Fast, *Int. J. Parasitol. Drugs Drug Resist.*, 2018, **8**, 174–188.

72 A. Bernhard, A.M. Azad and R. Hannisdal, *Monitoring program for pharmaceuticals, illegal substances and contaminants in farmed fish*, Institute of Marine Research, 2023.

73 Rester av legemidler i mat, https://www.fhi.no/kl/miljogifter/fremmedstoffer-i-mat/ulike-fremmedstoffer-i-mat/rester-av-legemidler-i-mat/?term=, (accessed May 2024).

74 J. Fan and I.A.M. De Lannoy, *Biochem. Pharmacol.*, 2014, **87**, 93–120.

75 C. Matthee, A.R. Brown, A. Lange and C.R. Tyler, *Environ. Sci. Technol.*, 2023, **57**, 8845–8862.

76 P.A. Olsvik, K.E. Brokke, O.B. Samuelsen and R. Hannisdal, *J. Fish Dis.*, 2024, DOI:10.1111/jfd.13880.

77 Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS), https://www.eag.com/app-note/liquid-chromatography-tandem-mass-spectrometry-lc-ms-ms/, (accessed April 2024).

78 A.G. Asimakopoulos, A. Bletsou, K. Kannan, N.S. Thomaidis, in *Mass Spectrometry for the Analysis of Pesticide Residues and Their Metabolites*, ed. D. Tsipi, H. Botitsi and A. Economou, John Wiley & Sons, Inc., 1st edn., 2015, ch. 5, pp. 113-130.

79 S. Lacorte, A. Agüera, M. Cortina-Puig, C. Gómez-Canela, in *Mass Spectrometry for the Analysis of Pesticide Residues and Their Metabolites*, ed. D. Tsipi, H. Botitsi and A. Economou, John Wiley & Sons, Inc., 1st edn., 2015, ch. 6, pp. 131-59.

80 RStudio (version 4.4.0.), Posit, 2024.

81 H. Wickham, ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag New York, 2016.

82 R.V. Lenth, emmeans: Estimated Marginal Means, aka Least-Squares Means, 2024.

83 W. Denney, S. Duvvuri and C. Buckeridge, Simple, Automatic Noncompartmental Analysis: The PKNCA R Package, B. Denney, 2015.

84 Institute of Marine Research, unpublished work.

8. Appendix

8.1. Tables

Table 1. The table shows the added amount of standard and internal standard (IS) to the samples. BUM= blank without matrix; BMM= blank with matrix; IKM= internal control material and N1-8 is the standard curve. The standard curve was used for liver and plasma samples.

	Added standard 10 µg mL^{-1} or 1 µg mL^{-1}* (µL)	Concentration in sample (ng g^{-1})	Added IS 500 ng mL^{-1} (µL)	Concentration of IS in sample (ng g^{-1})
BUM			100	250
BMM			100	250
IKM			100	250
K_{low}	20*	100	100	250
K_{medium}	80	4000	100	250
K_{high}	200	10000	100	250
N1	20*	100	100	250
N2	100*	500	100	250
N3	200*	1000	100	250
N4	40	2000	100	250
N5	80	4000	100	250
N6	120	6000	100	250
N7	160	8000	100	250
N8	200	10000	100	250
Unknown sample(s)			100	250

Table 2. The table shows the added amount of standard and internal standard (IS) to the samples. BUM= blank without matrix; BMM= blank with matrix; IKM= internal control material and N1-8 is the standard curve. The standard curve was used for the bile samples.

	Added standard 10 µg mL^{-1} or 1 µg mL^{-1}* (µL)	Concentration in sample (ng g^{-1})	Added IS 500 ng mL^{-1} (µL)	Concentration of IS in sample (ng g^{-1})
BUM			100	250
BMM			100	250
IKM			100	250
K_{low}	40*	200	100	250
K_{medium}	160	8000	100	250
K_{high}	400	20000	100	250
N1	40*	200	100	250
N2	200*	1000	100	250
N3	400*	2000	100	250
N4	80	4000	100	250
N5	160	8000	100	250
N6	240	12000	100	250
N7	320	16000	100	250
N8	400	20000	100	250
Unknown sample(s)			100	250

Table 3. The table shows the added amount of standard and internal standard (IS) to the samples. BUM= blank without matrix; BMM= blank with matrix; IKM= internal control material and N1-8 is the standard curve. The standard curve was used for the skin samples.

	Added standard 10 µg mL^{-1} or 1 µg mL^{-1}* (µL)	Concentration in sample (ng g^{-1})	Added IS 500 ng mL^{-1} (µL)	Concentration of IS in sample (ng g^{-1})
BUM			100	250
BMM			100	250
IKM			100	250
K_{low}	60*	300	100	250
K_{medium}	240	12000	100	250
K_{high}	600	30000	100	250
N1	60*	300	100	250
N2	300*	1500	100	250
N3	600*	3000	100	250
N4	120	6000	100	250
N5	240	12000	100	250
N6	360	18000	100	250
N7	480	24000	100	250
N8	600	30000	100	250
Unknown sample(s)			100	250

8.2. R code for GLM model

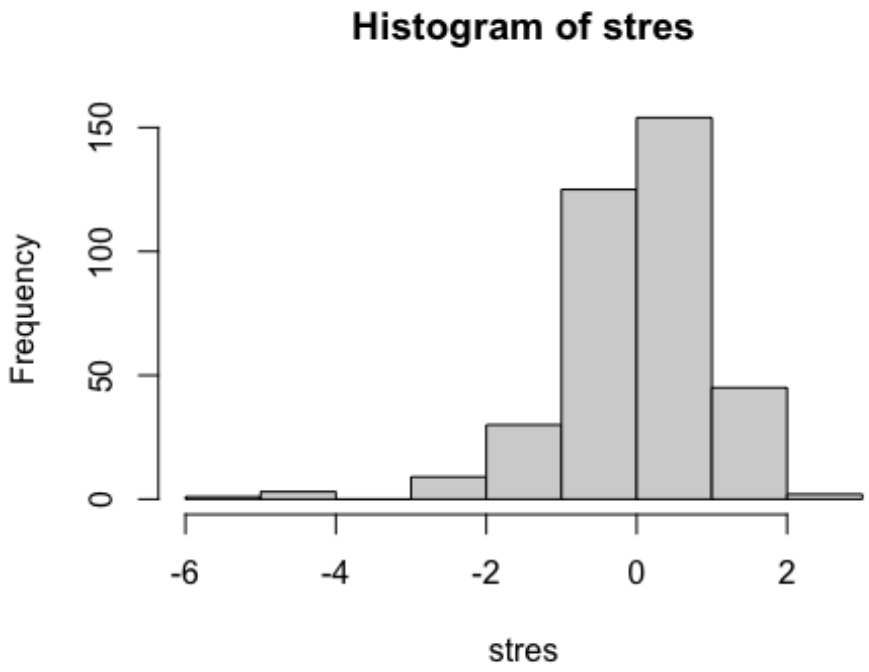
```
GLM_data<- readxl::read_excel("GLM data.xlsx")

#the "saturated" GLM: Starting model
model1 <- glm(log(GLM_data$Conc.) ~ Tissue + Day + Tissue:Day, family = ga
ussian (link=identity), na.action = na.exclude, data=GLM_data)
summary(model1)

##
## Call:
## glm(formula = log(GLM_data$Conc.) ~ Tissue + Day + Tissue:Day,
##     family = gaussian(link = identity), data = GLM_data, na.action = na
.exclude)
```

```
##
## Coefficients:
##                   Estimate Std. Error t value Pr(>|t|)
## (Intercept)       9.169351   0.180483  50.804  < 2e-16 ***
## TissueLiver       0.545233   0.199060   2.739 0.006469 **
## TissueMuscle     -0.673189   0.192346  -3.500 0.000524 ***
## TissuePlasma     -1.078842   0.212632  -5.074 6.27e-07 ***
## TissueSkin        0.780321   0.208530   3.742 0.000212 ***
## Day              -0.017267   0.001127 -15.326  < 2e-16 ***
## TissueLiver:Day   0.003956   0.001184   3.342 0.000920 ***
## TissueMuscle:Day  0.004532   0.001177   3.851 0.000139 ***
## TissuePlasma:Day -0.001697   0.001443  -1.176 0.240528
## TissueSkin:Day    0.004912   0.001432   3.430 0.000673 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.3853007)
##
##     Null deviance: 1772.13  on 368  degrees of freedom
## Residual deviance:  138.32  on 359  degrees of freedom
## AIC: 707.11
##
## Number of Fisher Scoring iterations: 2
```

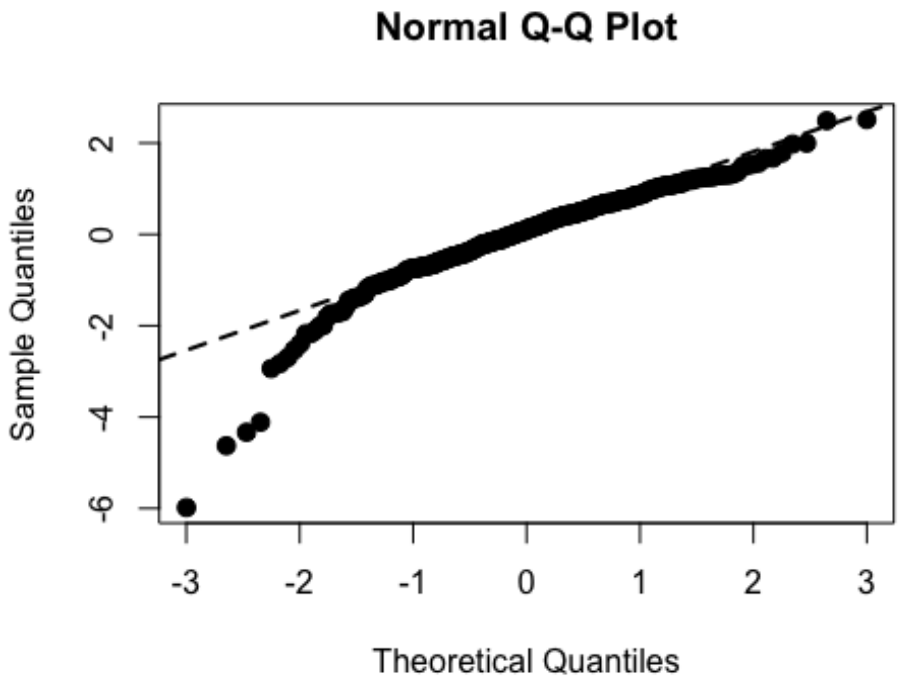
```
AIC(model1) #AIC=707
```

```
## [1] 707.1118
```

```
#Checking distribution of residuals:
stres<- (model1$residuals - mean(model1$residuals))/ sd(model1$residuals)
hist(stres)
```



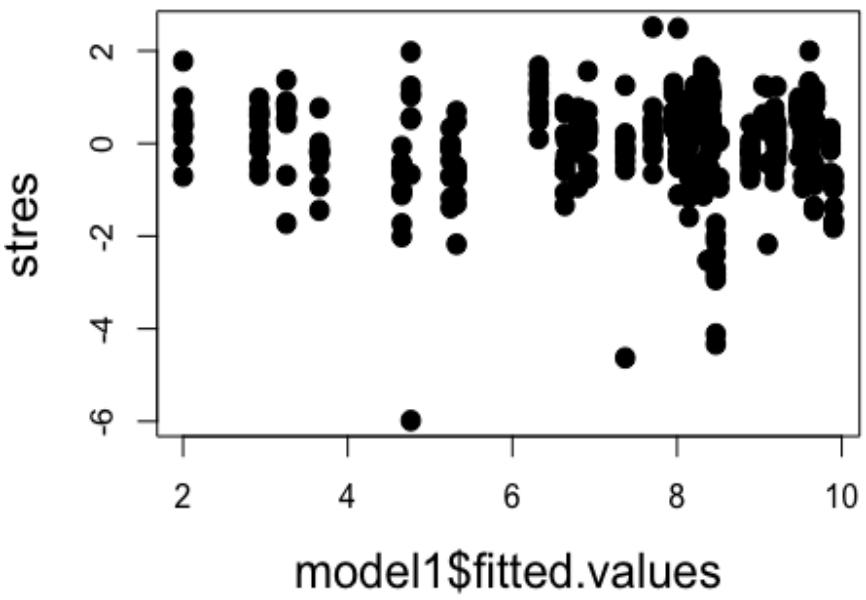
```r
qqnorm(stres, cex = 1.8, pch = 20)
qqline(stres, lty=2, lwd = 2)
```




```r
shapiro.test (stres)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  stres
## W = 0.91685, p-value = 2.074e-13
```

```r
plot(stres ~ model1$fitted.values, pch = 20, cex = 2, cex.lab = 1.5)
```




```
#Investigating the model
model1

##
## Call:  glm(formula = log(GLM_data$Conc.) ~ Tissue + Day + Tissue:Day,
##     family = gaussian(link = identity), data = GLM_data, na.action = na
.exclude)
##
## Coefficients:
##      (Intercept)       TissueLiver      TissueMuscle      TissuePlasma

##         9.169351          0.545233         -0.673189         -1.078842

##       TissueSkin               Day   TissueLiver:Day  TissueMuscle:Day

##         0.780321         -0.017267          0.003956          0.004532

## TissuePlasma:Day    TissueSkin:Day
##        -0.001697          0.004912
##
## Degrees of Freedom: 368 Total (i.e. Null);  359 Residual
## Null Deviance:       1772
## Residual Deviance: 138.3     AIC: 707.1

drop1(model1, test = "F")

## Single term deletions
##
## Model:
## log(GLM_data$Conc.) ~ Tissue + Day + Tissue:Day
##            Df Deviance    AIC F value    Pr(>F)
```

```
## <none>          138.32 707.11
## Tissue:Day  4   159.47 751.62  13.723 2.007e-10 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
summary(model1)
```

```
##
## Call:
## glm(formula = log(GLM_data$Conc.) ~ Tissue + Day + Tissue:Day,
##     family = gaussian(link = identity), data = GLM_data, na.action = na
.exclude)
##
## Coefficients:
##                    Estimate Std. Error t value Pr(>|t|)
## (Intercept)       9.169351   0.180483  50.804  < 2e-16 ***
## TissueLiver       0.545233   0.199060   2.739 0.006469 **
## TissueMuscle     -0.673189   0.192346  -3.500 0.000524 ***
## TissuePlasma     -1.078842   0.212632  -5.074 6.27e-07 ***
## TissueSkin        0.780321   0.208530   3.742 0.000212 ***
## Day              -0.017267   0.001127 -15.326  < 2e-16 ***
## TissueLiver:Day   0.003956   0.001184   3.342 0.000920 ***
## TissueMuscle:Day  0.004532   0.001177   3.851 0.000139 ***
## TissuePlasma:Day -0.001697   0.001443  -1.176 0.240528
## TissueSkin:Day    0.004912   0.001432   3.430 0.000673 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.3853007)
##
##     Null deviance: 1772.13  on 368  degrees of freedom
## Residual deviance:  138.32  on 359  degrees of freedom
## AIC: 707.11
##
## Number of Fisher Scoring iterations: 2
```

```
summary.lm(model1)
```

```
##
## Call:
## glm(formula = log(GLM_data$Conc.) ~ Tissue + Day + Tissue:Day,
##     family = gaussian(link = identity), data = GLM_data, na.action = na
.exclude)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -3.6676 -0.3148  0.0637  0.4043  1.5409
##
## Coefficients:
##                    Estimate Std. Error t value Pr(>|t|)
## (Intercept)       9.169351   0.180483  50.804  < 2e-16 ***
## TissueLiver       0.545233   0.199060   2.739 0.006469 **
## TissueMuscle     -0.673189   0.192346  -3.500 0.000524 ***
## TissuePlasma     -1.078842   0.212632  -5.074 6.27e-07 ***
## TissueSkin        0.780321   0.208530   3.742 0.000212 ***
```

```
## Day              -0.017267   0.001127 -15.326  < 2e-16 ***
## TissueLiver:Day   0.003956   0.001184   3.342 0.000920 ***
## TissueMuscle:Day  0.004532   0.001177   3.851 0.000139 ***
## TissuePlasma:Day -0.001697   0.001443  -1.176 0.240528
## TissueSkin:Day    0.004912   0.001432   3.430 0.000673 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.6207 on 359 degrees of freedom
## Multiple R-squared:  0.9219, Adjusted R-squared:   0.92
## F-statistic: 471.1 on 9 and 359 DF,  p-value: < 2.2e-16
```

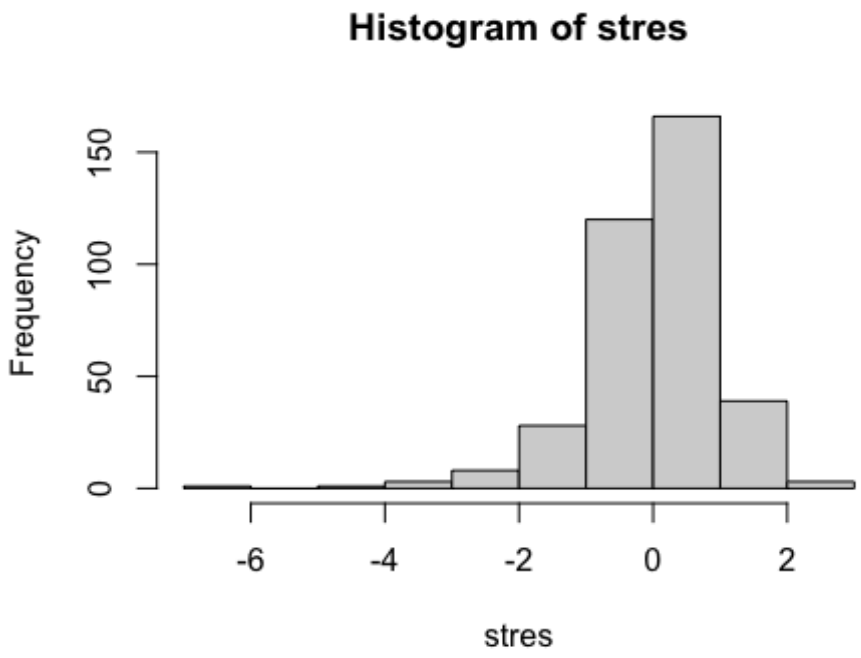
```
anova(model1, test="F")
```

```
## Analysis of Deviance Table
##
## Model: gaussian, link: identity
##
## Response: log(GLM_data$Conc.)
##
## Terms added sequentially (first to last)
##
##
##            Df Deviance Resid. Df Resid. Dev        F    Pr(>F)
## NULL                         368    1772.13
## Tissue      4   240.49       364    1531.64  156.043 < 2.2e-16 ***
## Day         1  1372.16       363     159.47 3561.282 < 2.2e-16 ***
## Tissue:Day  4    21.15       359     138.32   13.723 2.007e-10 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

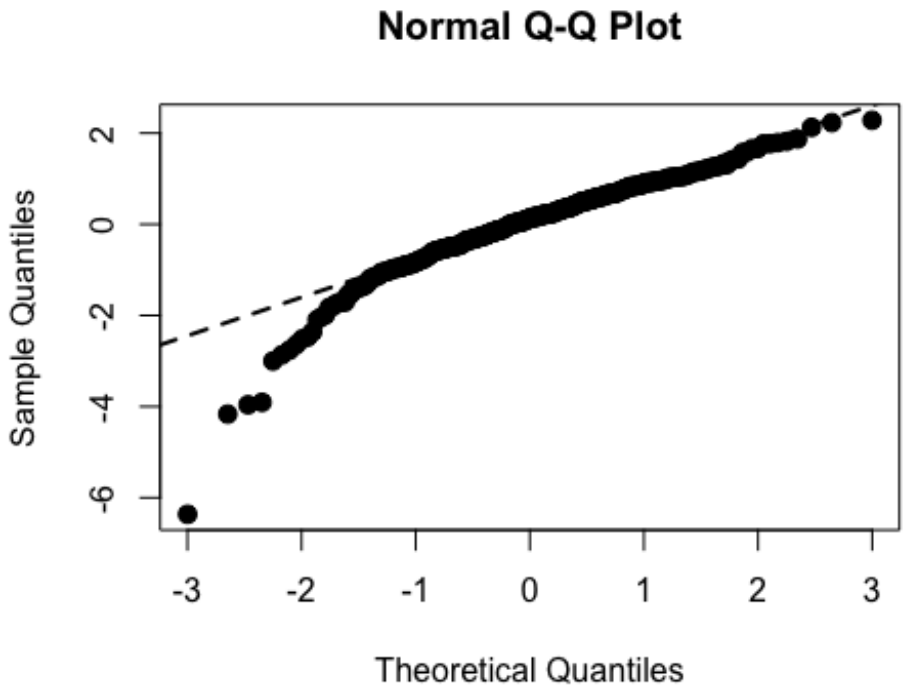
```
#Model refinement by stepwise deletions
model1 <- glm(log(GLM_data$Conc.) ~ Tissue + Day, family = gaussian (link=
identity), na.action = na.exclude, data=GLM_data)
AIC(model1) #AIC=752
```

```
## [1] 751.6151
```

```
#Checking distribution of residuals:
stres<- (model1$residuals - mean(model1$residuals))/ sd(model1$residuals)
hist(stres)
```



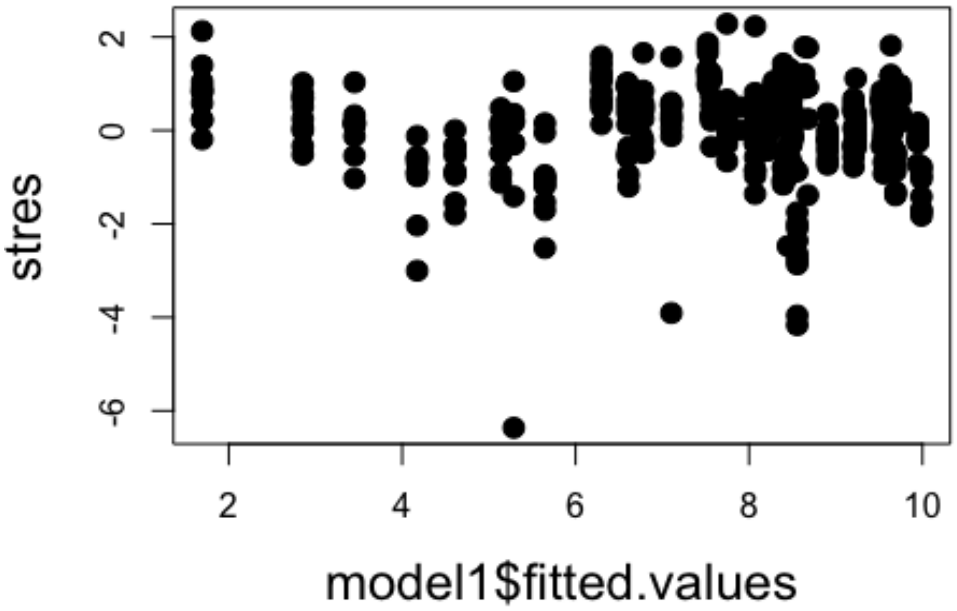

```
qqnorm(stres, cex = 1.8, pch = 20)
qqline(stres, lty=2, lwd = 2)
```




```
shapiro.test (stres)

##
##  Shapiro-Wilk normality test
##
```

```
## data:  stres
## W = 0.91746, p-value = 2.362e-13
```

```
plot(stres ~ model1$fitted.values, pch = 20, cex = 2, cex.lab = 1.5)
```




```
#Investigating the model
model1
```

```
##
## Call:  glm(formula = log(GLM_data$Conc.) ~ Tissue + Day, family = gauss
ian(link = identity),
##     data = GLM_data, na.action = na.exclude)
##
## Coefficients:
##  (Intercept)   TissueLiver  TissueMuscle  TissuePlasma    TissueSkin
##      8.73237       1.01063      -0.14927      -1.11456       1.31028
##          Day
##     -0.01351
##
## Degrees of Freedom: 368 Total (i.e. Null);  363 Residual
## Null Deviance:       1772
## Residual Deviance: 159.5     AIC: 751.6
```

```
drop1(model1, test = "F")
```

```
## Single term deletions
##
## Model:
## log(GLM_data$Conc.) ~ Tissue + Day
##        Df Deviance     AIC F value    Pr(>F)
## <none>      159.47  751.62
```

```
## Tissue  4   416.82 1098.14  146.45 < 2.2e-16 ***
## Day     1  1531.64 1584.37 3123.38 < 2.2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
summary(model1)
```

```
##
## Call:
## glm(formula = log(GLM_data$Conc.) ~ Tissue + Day, family = gaussian(lin
k = identity),
##     data = GLM_data, na.action = na.exclude)
##
## Coefficients:
##                Estimate Std. Error t value Pr(>|t|)
## (Intercept)   8.7323683  0.1355096  64.441  < 2e-16 ***
## TissueLiver   1.0106346  0.1501747   6.730 6.64e-11 ***
## TissueMuscle -0.1492670  0.1447519  -1.031    0.303
## TissuePlasma -1.1145560  0.1583357  -7.039 9.72e-12 ***
## TissueSkin    1.3102800  0.1558963   8.405 9.86e-16 ***
## Day          -0.0135092  0.0002417 -55.887  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.4393204)
##
##     Null deviance: 1772.13  on 368  degrees of freedom
## Residual deviance:  159.47  on 363  degrees of freedom
## AIC: 751.62
##
## Number of Fisher Scoring iterations: 2
```

```
summary.lm(model1)
```

```
##
## Call:
## glm(formula = log(GLM_data$Conc.) ~ Tissue + Day, family = gaussian(lin
k = identity),
##     data = GLM_data, na.action = na.exclude)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -4.1889 -0.3196  0.0843  0.4286  1.5020
##
## Coefficients:
##                Estimate Std. Error t value Pr(>|t|)
## (Intercept)   8.7323683  0.1355096  64.441  < 2e-16 ***
## TissueLiver   1.0106346  0.1501747   6.730 6.64e-11 ***
## TissueMuscle -0.1492670  0.1447519  -1.031    0.303
## TissuePlasma -1.1145560  0.1583357  -7.039 9.72e-12 ***
## TissueSkin    1.3102800  0.1558963   8.405 9.86e-16 ***
## Day          -0.0135092  0.0002417 -55.887  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
```

```
## Residual standard error: 0.6628 on 363 degrees of freedom
## Multiple R-squared:   0.91,  Adjusted R-squared:  0.9088
## F-statistic: 734.2 on 5 and 363 DF,  p-value: < 2.2e-16

anova(model1, test="F")

## Analysis of Deviance Table
##
## Model: gaussian, link: identity
##
## Response: log(GLM_data$Conc.)
##
## Terms added sequentially (first to last)
##
##
##        Df Deviance Resid. Df Resid. Dev       F    Pr(>F)
## NULL                     368    1772.13
## Tissue  4   240.49       364    1531.64  136.86 < 2.2e-16 ***
## Day     1  1372.16       363     159.47 3123.38 < 2.2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

# The final model includes a two-way interaction
#The final model
model1 <- glm(log(GLM_data$Conc.)~ Tissue + Day + Tissue:Day, family = gau
ssian (link=identity), na.action = na.exclude, data=GLM_data)
summary (model1)

##
## Call:
## glm(formula = log(GLM_data$Conc.) ~ Tissue + Day + Tissue:Day,
##     family = gaussian(link = identity), data = GLM_data, na.action = na
.exclude)
##
## Coefficients:
##                   Estimate Std. Error t value Pr(>|t|)
## (Intercept)       9.169351   0.180483  50.804  < 2e-16 ***
## TissueLiver       0.545233   0.199060   2.739 0.006469 **
## TissueMuscle     -0.673189   0.192346  -3.500 0.000524 ***
## TissuePlasma     -1.078842   0.212632  -5.074 6.27e-07 ***
## TissueSkin        0.780321   0.208530   3.742 0.000212 ***
## Day              -0.017267   0.001127 -15.326  < 2e-16 ***
## TissueLiver:Day   0.003956   0.001184   3.342 0.000920 ***
## TissueMuscle:Day  0.004532   0.001177   3.851 0.000139 ***
## TissuePlasma:Day -0.001697   0.001443  -1.176 0.240528
## TissueSkin:Day    0.004912   0.001432   3.430 0.000673 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.3853007)
##
##     Null deviance: 1772.13  on 368  degrees of freedom
## Residual deviance:  138.32  on 359  degrees of freedom
## AIC: 707.11
```

```
##
## Number of Fisher Scoring iterations: 2
```

```
summary.lm (model1)
```

```
##
## Call:
## glm(formula = log(GLM_data$Conc.) ~ Tissue + Day + Tissue:Day,
##     family = gaussian(link = identity), data = GLM_data, na.action = na
.exclude)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -3.6676 -0.3148  0.0637  0.4043  1.5409
##
## Coefficients:
##                    Estimate Std. Error t value Pr(>|t|)
## (Intercept)        9.169351   0.180483  50.804  < 2e-16 ***
## TissueLiver        0.545233   0.199060   2.739 0.006469 **
## TissueMuscle      -0.673189   0.192346  -3.500 0.000524 ***
## TissuePlasma      -1.078842   0.212632  -5.074 6.27e-07 ***
## TissueSkin         0.780321   0.208530   3.742 0.000212 ***
## Day               -0.017267   0.001127 -15.326  < 2e-16 ***
## TissueLiver:Day    0.003956   0.001184   3.342 0.000920 ***
## TissueMuscle:Day   0.004532   0.001177   3.851 0.000139 ***
## TissuePlasma:Day  -0.001697   0.001443  -1.176 0.240528
## TissueSkin:Day     0.004912   0.001432   3.430 0.000673 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.6207 on 359 degrees of freedom
## Multiple R-squared:  0.9219, Adjusted R-squared:   0.92
## F-statistic: 471.1 on 9 and 359 DF,  p-value: < 2.2e-16
```

```
model1
```

```
##
## Call:  glm(formula = log(GLM_data$Conc.) ~ Tissue + Day + Tissue:Day,
##     family = gaussian(link = identity), data = GLM_data, na.action = na
.exclude)
##
## Coefficients:
##      (Intercept)       TissueLiver      TissueMuscle      TissuePlasma

##         9.169351          0.545233         -0.673189         -1.078842

##       TissueSkin               Day   TissueLiver:Day  TissueMuscle:Day

##         0.780321         -0.017267          0.003956          0.004532

## TissuePlasma:Day    TissueSkin:Day
##        -0.001697          0.004912
##
## Degrees of Freedom: 368 Total (i.e. Null);  359 Residual
```

```
## Null Deviance:       1772
## Residual Deviance: 138.3     AIC: 707.1

#Pairwise comparisons
library(emmeans)

## Welcome to emmeans.
## Caution: You lose important information if you filter this package's re
sults.
## See '? untidy'

emtrends(model1, pairwise ~ Tissue, var="Day", type="response")

## $emtrends
##  Tissue Day.trend       SE  df lower.CL upper.CL
##  Bile     -0.0173 0.001127 359  -0.0195  -0.0151
##  Liver    -0.0133 0.000363 359  -0.0140  -0.0126
##  Muscle   -0.0127 0.000340 359  -0.0134  -0.0121
##  Plasma   -0.0190 0.000902 359  -0.0207  -0.0172
##  Skin     -0.0124 0.000884 359  -0.0141  -0.0106
##
## Confidence level used: 0.95
##
## $contrasts
##  contrast         estimate       SE  df t.ratio p.value
##  Bile - Liver    -0.003956 0.001184 359  -3.342  0.0081
##  Bile - Muscle   -0.004532 0.001177 359  -3.851  0.0013
##  Bile - Plasma    0.001697 0.001443 359   1.176  0.7654
##  Bile - Skin     -0.004912 0.001432 359  -3.430  0.0060
##  Liver - Muscle  -0.000576 0.000498 359  -1.158  0.7755
##  Liver - Plasma   0.005653 0.000972 359   5.814  <.0001
##  Liver - Skin    -0.000956 0.000956 359  -1.000  0.8552
##  Muscle - Plasma  0.006229 0.000964 359   6.463  <.0001
##  Muscle - Skin   -0.000380 0.000947 359  -0.401  0.9945
##  Plasma - Skin   -0.006608 0.001263 359  -5.233  <.0001
##
## P value adjustment: tukey method for comparing a family of 5 estimates
```