Effects of Antiparasitic Treatment for Argulosis on Innate Immune System of a Cyprinid Fish (Fathead Minnow; *Pimephales promelas*, Rafinesque 1820)

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Dedicated to my parents,

in gratitude for the immense support

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Abbreviations

Ca: Calcium

cDNA: complementary DNA

DNA: Deoxyribonucleic Acid

Mg: Magnesium

HDFFDA: 2'7'-difluorodihydrofluorescein diacetate

MPO: myeloperoxidase

NE: neutrophil elastase

NET: Neutrophil Extracellular Trap

PCR: polymerase chain reaction

PMA: PhorbolMyristate Acetate

RNA: Ribonucleic Acid

ROS: reactive oxygen species

Spp.: species

TMB: Tetramethylbenzidine

TAE: TRIS-Acetat-EDTA-buffer

I. LITERATURE REVIEW

1. Introduction

Fish contributes notably to the protein supply of the growinghuman population as the global availability of terrestrial protein sources decline. The stagnating, at time even regressive, yield of fish from the sea and inland waters can't make up for the increasing protein demand.The production of fish and other aquatic organisms in aquaculture is therefore becoming more important and undoubtedly promising(Naylor et al., 2000, Tidwell and Allan, 2001). Silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodonidella*) and common carp (*Cyprinuscarpio*) are the three most important freshwater fish species in the global aquaculture (FAO, 2012). The ability to utilize native starch for their nutrition is an advantage compared to other fish species like trout where food production is an additional cost and therefore to thrive on simple feeding speaks in their favor.

The fish farming of carp in Germany is undertaking efforts to keep up with the international market (Füllner, 2011, Mann, 1975). The production of carp remained constant in 2010 (Brämick, 2010) and since then has increased only slightly(Statistisches_Bundesamt, 2013, Statistisches_Bundesamt, 2012). The German aquaculture industry is currently facing several problems: Firstly, the sales number stagnate due to the availability of cheaper fish imports from foreign countries, andparallel to this are the increasing costs of feed, energy and labor(Füllner, 2011). Secondly, which mainly concerns the carp sales, carp hasbecome less popular with consumersin recent years. Thirdly, the animal welfare regulations concerning cormorants(*Phalacrocorax carbo)*, causehigh profit losses (Wedekind et al., 2012).Finally, current veterinary drug use regulations in Germany limit the range of medications approved

astreatment for multiple diseases in freshwater fish. On the basis of all these reasons, some farmers have already decided to give up their business and cease farming carp (comparative table in Appendix,Fülner 2011, Table 1).

The authorhopes to support the persistence of carpaquaculture ponds, since they contribute greatly to the cultural landscape and offer habitat for a variety of animals. Furthermore, it is important to help local production in order to reduce dependence on imports.This study attempts to initiate a discussion about antiparasitic veterinary drug approval regulations in the European Union that are needed for minor use in minor species such as cyprinid aquaculturewhich is not yet clearly defined in the laws (Wack, 2010).The stepsthat need to beundertaken totest new drugsinaquaculture, are numerous.At the beginning, basic information and research data on the subject is needed to allow advanced research.This process is followed by verification of the basic research done in the laboratory with safety and efficacy studies duringlive animal experiments, before one can make educated statements about the tested drug and follow up with clinical studies and drug marketing approval process, EU Directive 2009/53 (EU, 2009).

In approaching such a topic, it's important to find out which drugs are on the market. Therefore some research was conducted on salmon farming, as this industry is decades ahead of the carp farming industry in areas such as research and innovation, and has gained experience in antiparasitic treatment.The next step in the process is to conduct research to determine what medications could be used in German carp aquaculture.

The thesis aims are to test the possible side effect of different active compounds of drugs on the individual fish fitness to provide a basis for further testing. The hypothesis was: Antiparasitic drugs (ivermectin, doramectin, diflubenzuron) will not cause significant side effects in fish when applied in therapeutic doses.Therefore this study is investigating possible

side effects of antiparasitic drugs on the innate immune system and gene expression in a cyprinid fish laboratory animal model.

To achieve dissertation goals, a series of experiments were conducted on the cyprinid fishfathead minnow (*Pimephales promelas*, Rafinesque 1820), an animal frequently used as a model in toxicological studies and easily maintained in laboratories. This study focused on using*in vitro* experiments[1]in order to support animal welfare guidelines.The research approachused*ex vivo* and *in vitro* studies, therefore completely eliminating the application of treatments on live animals, and also minimizing live animalsuse as tissue sources for neutrophil function assays and gene expression analysis.

The outcome of this study onfathead minnowas model fish can be transposed to fish in general and thereforeallows conclusions to be made on which of the tested drugs will have the least negative effect on carp.The relevance of studies of one species to another species depends upon the percentage to which the hereditary physiologic processes are comparable (Styrt, 1989).Similarities of lymphocytes in morphology exist between teleost fish and even human counterparts. A phylogenetic relationship between vertebrate blood cells indicated(Weinreb, 1963), thereby supporting the discussion about extrapolation of results from model species.

The dissertation is prepared in classical format (monography) and has a total of 13 chapters. The first chapter will outlinethe importance of freshwater aquaculture and carp farming in Germany. In the second chapter therationale for theuse of a model species (fathead minnow) will be explained. The third chapter will provide a summary of the significance of the fish's innate immune system and describe how it is connected with the fish'sgeneral health and welfare, its ability to fightdiseases, and reproduce. The following chapter will focus on occurring parasites found in freshwater systems and review previous studiesconcerned with the use, efficacy and possible biological and ecotoxicological side effects of the chosen

[1] *In vitro* refers to the conduct of experiments on animals euthanized or on the use of organs from animals killed in advance, so that the animal isn't adding suffering orpain through this kind of experiment

antiparastic drugs. Finally, after describing extensively the hitherto existing knowledge, the conducted experiments will be discussed and explained, followed by a presentation of the results and critical discussion, conclusion and suggestions for further research.

2. Cyprinidaeaquaculture in Germany

The family cyprinidae is the largest freshwater fish group in the world, including over 200 genera and 2100 species.This chapter discusses the cyprinidaefarming in Germany with particular focus on common carp aquaculture, its future prospects and problems, and the importance of maintaining and continuing support for the carp farming industry.

2.1. Significance in Germany

The farming of carp[2] is the oldest practiced form of fresh water aquaculture in Central Europe(Thienemann, 1950). There are 1200 year old records of Karl the Great (742-814 AD) giving instructions on the carp ponds on his properties in Germany. Carp are efficient in their use of resources and therefore correspond to the principles of sustainability. Today, carp ponds are not only part of the cultural landscape;they fulfill a plurality of functions such as being a substitute biotope for many animals and plants, and as water reservoir and recreation landscape(Füllner, 2011).

In 2013, the 3.900 carp farmsin Germanproduced a total of 5700 tons of carp for human consumption. This correlates to an increase of 3,2 % in overall carp production as well as 8 %

[2]Whenever "carp" is mentioned, it is referred to the "common carp" *Cyprinus carpio*.

increase in farm numbers, with 14 new carp farms to previous year (Statistisches_Bundesamt, 2013). Over 90 % of farms in addition to numerous unrecorded small producers pursue carp farming as a sideline venture. The total production surface area of carp ponds in Germany is estimated to exceed 37,000 hectares and the main areas for carp fish farming are Aischgrund and Oberpfalz in Bavaria, as well as Lausitzin Saxony and Brandenburg(Europäisches_Parlament, 2014).

Table 1: Farms with production of aquaculture and quantity produced in 2013, table from the German Federal Office of Statistics(Statistisches_Bundesamt, 2013)

Characteristic	**Business**	**Volume produced**
	Number	**kg**
Production in Aquaculture total	6 119	25 516 987
Fish (total)	6 093	20 409 983
Common carp	**3 852**	**5 699 625**
Rainbow trout	2 598	8 333 793
Salmon trout	262	1 266 955
Char	283	1 529 421
Brook trout	469	700 685
European eel	17	706 683
African catfish	10	695 081
Shellfish	11	5 035 640

Caviar	35	57 884

2.2. Facilitiesforculturedspecies

Facilities for carp farming are designed as ponds whichserve specifically for the rearing of carp. On average, carp ponds 0.70 to 1.50 meters deep in order to mimic the highly productive littoral zone of a natural lake and contain no continuous water flow(Schäperclaus, 1967). The flat carp ponds warm up rapidly from solar radiation and can produce a natural food chain rich in proteinfor the fish. At the same time the produced oxygen by photosynthesis is beneficially as it is essential for the growth of the fish(Bohl, 1982).

The common carp (*Cyprinuscarpio*) was the first fish species to be domesticated and is to this day the most important species for fish farming due to its exceptional characteristics ideal for farming. Through targeted and selective breeding over centuries, today's carp differ greatly from the wild counterpart(Schäperclaus, 1967). When kept in optimum conditions they are fast growing fish, able to excel at high stocking rates exhibit an impressive reproduction rate.Management can influence growth rate, weight gain and survival rate mainly through feeding measurements and rearing facilities (Jha and Barat, 2005, Kucharczyk, Żarski et al., 2011). A single reproducing female carp ("Rogener") can produce more than 1 million offspring annually(Schäperclaus, 1967).

Carp can tolerate low water temperatures down to 0.5 ° C, which makes them a suitable fish for aqua farming from tropical to subtropical regions to temperate climates. In Germany, it is customary to slaughter fish after a period of three summers(Bohl, 1982). This way,subadultfish are marketed, while their growth has not been reduced by energy losses for

the investment of gonadsand production (Europäisches_Parlament, 2014). Particularly around Christmas time, carp is popular as a traditional food fish. However carp sales in total are declining and fluctuate strongly due to seasonal influences. Its marketing has also been aimed to anglers to increase carp numbers as stocking fish. Besides carp, other fish species are produced in polyculture ponds, such as tench, other cyprinids, pike, walleye and catfish(Füllner, 2011).

2.3. Methods of production

Carp farming is based on the optimal utilization of naturally growing food(García-Berthou, 2001). In addition, the yield per unit area can be increased through a supplementation of feed with mainly starchy, plant matter, such as grain, as mentioned in the dissertation by (Barthelmes, 1961). In the last 60 years,carp farming in Germany has progressed from a completely naturalsystem without human intervention to a fish farming system with production-oriented management (Mann, 1975). Carp ponds are often so called “sky ponds” as they are reliant on gathered rainwater or are fed by river that has been dammed.

In the water pond chains, the water can be held for several years and is used repeatedly. At times during the production months, the water level in ponds may have to be compensated for evaporation and possible seepage by feed water. A continuousflow is counter-productive for fish growth, since this reduces the heating of thewater body and thus hinders the emergence of natural food sources and nutrients are lost from the water. Additional management measures include: adaption of feeding in accordance with fish numbers, age and other species, regulating impurities with regular draining and complete fish stock removal and drying of the pond, and refilling and re-stocking of the fish population (Schäperclaus, 1967).

Carponlygrowatwater temperatures above13° C(Schäperclaus, 1967), therefore the lifetime of thefishis measuredinsummers in temperate regions. Pondrearingusually takes place in a three-summer-rhythm. Thecapture of the carp for theproductionpondsisduring autumn, and during spring for winteringponds. Throughstrategic management measures, such asregulardraining, stringing(impoundment), fertilizationorreed cutting, onecanaffect theamountand timing ofnatural food growth in the pondsand thereby optimizing theyield of fish per unit area. In terms of natural food, carp mainly feed on detritus, amphipods (*Echinogammarus sp.),* phantom midge larvae (*Chaoborusflavicans*), diatom mucilages, and plant debris (García-Berthou, 2001).

The carpis an omnivore, and unlike manyother fish, it isableto digestnative starch. This allows carp farmersto supplement with inexpensivefeed– if needed(Schäperclaus, 1967). In the firstyear of rearing,fullcompound feed isalso utilizedto minimize losses. Theuse of biocidesisneither possible nor necessarydue to thecomplex relationships and intricate food websincarp farming ponds. Theyield per unit areaincarp farming utilizing the natural occurring foodexclusivelyis estimated to range from 150kg to450 kgediblecarp/ha(large regional differences), and with additionalgrainfeedingfrom 1000kg to1300 kg/ha. Thestocking densities, thefeed conversion ratioandtheamount of energyremain well belowthose of otheraquaculturemethods(Europäisches_Parlament, 2014).

There are opportunities forincomeenhancement by introducing diversification. The monetaryyield per unit areaofcarp ponds can be increased by the simultaneousproductionof otherfish species(so-called: “Sidefish”)(Füllner, 2011). A very suitable species asside fishis the tench(*Tincatinca*), which hasa similarfood spectrumasthecarp, butalsoprefersmollusksandcounts as valuablefood fish. Alsoincarp polyculture, grass

carp(*Ctenopharyngodonidella*), silver carp(*Hypophthalmichthysmolitrix*)andbighead carp(*Hypophthalmichthysnobilis*) are suitable(Wedekind et al., 2011).

Thegrass carpis particularly significant, sincethis speciescan not onlybe marketedas afood fish, but also servesto regulate the growth of macrophytesin the pond. Suitablecarp pondsare also usedfor the production ofpike(*Esoxlucius*), pike-perch (*Sander lucioperca*) orEuropean catfish(*Silurusglanis*), which achievea good marketprice as food fish. Some promisingexperimentsalsosuggestthe production ofwhitefish(*Coregonusspp*) to be possibleinsuitableponds. Depending on wages, sturgeon(*Acipenserspp*), which producecaviar, areanother optionto increase thevalue of aformerlypurecarpfarms(Europäisches_Parlament, 2014).

3. Fathead minnow as chosen standard species for in vitro testing

The following chapter discusses the importance and significance of the fathead minnow use as a modelspecies in research on fish diseases and toxicology. The particular suitability of this species as a test fish to correlate findings in the physiology and pathology of fish in general and in various research questionsis examined. An explanation is given why issues can be examined on this species, which relate to basic research in various other species and that the results obtained can be transferredto other vertebrates under certain circumstances.

3.1. Fathead minnows as representatives of cyprinids

The Fathead minnow is a member of the large cyprinidae (Linnaeus, 1758) family(Woodling, 1985) and is a native species from North America. The fathead minnow lives in muddy pools of creeks, small rivers, lakes and ponds. The fathead minnow tolerates unsuitable conditions (e.g.: hot, low in oxygen, intermittent streaming) for most other fishes. The wild living minnows feed on debris and algae and theyspawn in still-water habitats in relatively flat waters (Luna, 2015).

3.2. The role of Fathead minnow as model organism in science

The freshwater fathead minnow, *Pimephales promelas* (Teleostei: Cyprinidae) was selected as the test fish, given its suitability as laboratory species as demonstrated over several decades(Lemke and Bowen, 1998, Miles-Richardson et al., 1999). The fathead minnow tolerates a wide range of water types, and has a relatively well-defined reproduction cycle(Ankley and Villeneuve, 2006). It is therefore relatively simple to grow successfully in laboratories throughout the world(Brauhn, 1975, Denny, 1987).

The Fathead minnow is one of the most abundant species in toxicological and immunological research(Beyers et al., 1999, Vittozzi and De Angelis, 1991). Due to the tolerance of the Fathead minnow towards handling and varying water quality, and the amount of existing knowledge relating to their biology, the fish has been used extensively since the 1950s to explore different aspects of ecotoxicological interests (Ankley and Villeneuve, 2006).In the

immunological research, for the species Fathead minnow, a wide range of assays was established (Palić et al., 2005a, Palić et al., 2005c).*P. promelas* was established as a model species to observe the reaction of the innate immune system of fish to different stressors (Palić et al., 2006a) as well as to observe immunmodulators(Palić et al., 2005a, Palić et al., 2006b). The species fathead minnow was used in a variety of toxicological studies (Jovanović et al., 2011, Whitley et al., 2012) and has due to all these studies a firm place in the aquatic toxicology research, especially in the nanotoxicological research it proved itself as responsible model species (Jovanović et al., 2011, Jovanović and Palić, 2012).*P. promelas* was also commonly used in endocrine disruption research (Harries et al., 2000, Miller et al., 2007, Orlando et al., 2004).

4. Immunology of fish

The following chapter addresses the immune system in fish and explains the importance of the innate immune system in fish. It illustrates the significance of the immune system being a meaningfulparameter to evaluate the general health of the fish as well as its ability to ward diseases and thrive. In addition, it discusses the necessity to investigate the possibly compromised immune system during the exposure of animals to certain substances e.g. parasitic drug treatment.

4.1. Innate and acquired immune system

The immune system is a defence weapon of the organism against a variety of possible intruders. The immune system of fish hasa non-specific and a specificcomponent (Ellis, 2001), which also referred to as the innate immunity and the adaptive immunity.The innate immune system consists ofof physical barriers, cellular and humoral components. Humoral parameters include growth inhibitors, different lytic enzymes and elements of the complement pathways, agglutinins and precipitins (opsonins, primarily lectins), natural antibodies, cytokines, chemokines and antibacterial peptides(Magnadóttir, 2006). Different external and internal factors can influence the activity of innate immune parameters.

The acquired immune system in fish has a specific and a non-specific component. The specific humoral components include specific antibodies. Cellular factors involved in the specific response are B-cells and putative T-cells. The non-specific effector cells are various leukocytes(Ellis, 2001). Especially areas of vaccine development, such asnew advances in the development of DNA-vaccines, precise knowledge and advanced research about the acquired immune response is essential(Buchmann et al., 2001b).

However, the focus of this study was on the innate immune system, since it is the one which provides the body with a fast acting defense mechanism. Collectively, compared to mammals, fish innate immunity appears highly evolved with potentially enhanced functionality, whereas fish adaptive immunity might be possibly less refined (Lieschke and Trede, 2009)and is known to be adversely affect by low water temperatures(Le Morvan et al., 1998, Rijkers et al., 1980).

Neutrophils are the primary phagocytic cells involved in host-parasite interaction. Therefore, the primary innate immunity defensive mechanism of vertebrates against a variety of pathogens such as bacteria, viruses, parasites and fungi includes adherence, phagocytosing,

killing and digestion them (Smith and Lumsden, 1983). The neutrophils use the NADPH oxidase in order to dispose pathogens by producing a charge across the plasma membrane and the membrane of the specific granules(Hampton et al., 1998). The thereby provoked compensating enzymes released into the vacuole from the granules produce a milieu that will kill and digest the trapped pathogens (Segal, 2005).

4.2. Similarity in neutrophil function between species

The innate immune system is phylogenetically very old.Many mechanisms are shared with all animals (Lieschke and Trede, 2009); some mechanisms are also shared with plants (Hippeli et al., 1999). Comparative ultrastructural and histological studies on the lymphoid tissues of lower vertebrates[3] have allowed to obtain a picture of the emergence and evolutionary organization of vertebrate lymphoid organs(Zapata and Amemiya, 2000). The review of (Zapata et al., 1995)established phylogenetic correlations between lymphoid organs in the most primitive vertebrates and in mammals.

Due to those correlations inphylogeny[4] of the development of the innate immune system, one can assume that the innate immune system behaves similarilyin different species. So it is reasonable to state that it is therefore likely that findings on a fathead minnow could be extrapolated to similar effects in other cyprinid species, including carp.

[3] The use of the term “lower vertebrates” indicates representatives of taxa that diverged before the emergence of endothermic (warm blooded) vertebrates.

[4]Theevolutionarydevelopmentandhistory of a species or trait of a species or of a highertaxonomicgrouping of organisms.

5. Parasitoses

The following chapter discusses the various parasites that are found in freshwater aquaculture. In particular, it specifically deals with the crustacean parasites[5]. It discusses their biology, their negative effect on the host, and possible measures to combat them.

5.1. Ecology and Parasitism

Parasitic diseases in European aquaculture continue to pose economic and ecological threats to farmed and wild fish populations. Parasitic infestation caused costs to global salmonid industry of approximately US$ 480 million per year (Shinn et al., 2015).In the early stages of infection, an increase in the frequency of fish jumping and decreased appetite of the fish have been observed. The primary infection with ectoparasites can open the door to secondary or super- infections caused by bacterial and viral pathogens (Tully and Nolan, 2002). As the infection progresses, fish may exhibit shoaling behavior. When the infection advances, there may be large-scale mortalities. The review of Timi et al. (2015) showed that marine parasites play many roles in fisheries and aquaculture and present problems to be solved(Timi and MacKenzie, 2015).

Specifically, the infection with ectoparasites as *A. foliaceus* in carp can increase production losses in affected carp farms(Sahoo et al., 2013).Since carp develop specific immunity against some parasites during the course of their life, diseases caused by parasitesare

[5]Crustaceans form a very large group of arthropods, usually treated as a subphylum, which includes such familiar animals as crabs, lobsters, crayfish, shrimp, krillbut also many parasitic living animals MARTIN, M. F. 37. On the Morphology and Classification of Argulus (Crustacea). Proceedings of the Zoological Society of London, 1932. Wiley Online Library, 771-806, WALKER, P. D., FLIK, G. & BONGA, S. W. 2004. The biology of parasites from the genus Argulus and a review of the interactions with its host. *Host-parasite interactions,* 55**,** 107-129.

often typical juvenile diseases. So usually carp less than one year old show clinical signs of illness, older animals, however, are often symptom-free(Buchmann et al., 2001a).

In addition to influencing various organ systems, e.g. in the form of nutrient removal, mechanical displacement, or elimination of toxic metabolic products, a parasite can change the behavior of a host decisively and thus steer its own development in certain ways(Zander, 2013).For aquaculture, these are important findings because close proximity of fish and relatively higher densities of animals in stagnant water ecosystems such as carp ponds, provide favorable conditions for the development and transmission of parasites. For instance, stocking is made up of only a few different species of fish, comparable to monoculture rearing, which is favorable for the spread of diseases. On the other hand, a relatively high stocking rate increases the relative growth of the fish. These conditions lead to an optimal transfer of parasites from fish to fish and enhance the outbreak of epidemics (Zander, 2013). This clearly shows the importance of understanding the different communities below and above water to develop more therapeutic and prophylactic strategies to obtaining a healthy food fish production.

5.2. Prevalence of *Argulus foliaceus* in Germany

The parasitological investigation of (Kappe, 2004) showed the presence of *A. foliaceus* in carp fisheries in Saxony, Germany in spring as well as autumn. The actual loss incurring due to this disease has gone mostly unnoticed, especially because little data has been collected. Many freshwater carp fisheries suffer infection problems, however, no attempt has been made to date to quantify or establish the exact nature of the perceived problem in Germany. In India, this loss was estimated to be US$ 615 per ha/year (Sahoo et al., 2013). Comparably the losses due to sea lice infestation in salmon aqua farming, which is a similar problem, is known

to be very high (Costello, 2009) and a study in the UK of trout farming showed as well the relationship between infestation with ectoparasites and losses in production numbers (Taylor et al., 2006). Parasite infestations leads to a range of problems for the infested fish and affects the immune system, e.g. alters the macrophage function (Lewis, 2013).

5.3. Survey of the most prevalent ectoparasites and their treatment methods

Thecommercialcarpproductionworldwide is subject tomajor financiallossesdue to variousdiseases. According to a studyby Hossain et al., 2013, theaverageeconomiclosseswere causedbythe various types ofdiseases caused by various pathogens such asbacteria, fungi, protozoa, monogenea, crustacea; and alsomalnutrition, lack of oxygen,andpoor watervaluesataround 34 percent(Hossain et al., 2013). The most commonly encountered ectoparasitoses are Skin worms (Gyrodaktylus spp.), Gill flukes (Dactylogyrus spp.), Fish leeches (Pisciculageometra) and Crustaceans.

Thelosses caused byinfestationwithCrustacea aloneare estimated to beabout one third of total losses. It should be noted that this figure is from a studyconducted in India,whereclimaticeventsaresignificantlydifferent to thoseinCentral Europe. Nevertheless,one can saythat these figuresgivea good overview ofthe importance of parasitoses in aquaculture. For Germany,comparablestudieshave not yet beenconducted, themost recent reportby the FederalStatistical Officeis concernedexclusivelywiththeamount of fishproduced, ignoringthe losses(Statistisches_Bundesamt, 2012), onlythe dissertation of (Kappe, 2004) speaks aboutthe

occurrence of a variety of parasites in spring as well as autumn in carp ponds in Saxonia, Germany, which shows the definite necessity for future and further research.

There are many biological ways to reduce the infestation of *Argulus spp.* as well as prevention procedures. Gault et al. (2002) suggests that control may be possible by providing artificial substrates on which the parasite can lay its eggs and which could be removed afterwards(Gault et al., 2002). Draining and drying the fish ponds followed by a treatment of the empty ponds with quicklime respectively slaked lime is also effective.An initial step in the development of practical and effective management strategies is the identification of risk factors, as was done in the study of (Taylor et al., 2006) in trout fisheries. They categorized the following factors as risky and recommended to minimize them in order to minimize the parasitic burden: the existence of an algal bloom, time taken to remove and replace entire stock of trout [fast (<10.4 weeks)/ slow (>10.4 weeks)] and whether the lake water level dropped by over 30 cm in the summer. Although these risk factors are associated with the outcome, namely presence of problems with*Argulus spp*, they do not necessarily have any direct causal effect.

Here is a list of other methods and means with instructions for use and legal grounds for a ban in Germany in farmed fish. It is important to distinguish whether the substance is to be used for disinfection or as a therapeutic agent. The following list was taken fromFeneis (2008)(Feneis, 2008):

Quicklime (calcium oxide) is legally permitted for use because the substance has both an E-number (E 529) as well as a standard authorization.

Chloramine-T is not listed in the Annexes of EU Regulation 2377/97 and therefore may not be used.

Formalin (formaldehyde) for fish: listed in 2377/97, has only a standard authorization, no reference and is therefore, strictly speaking, not permitted. Indications especially in Costia (salmonids) - as a 35-40% solution, as a bath treatment: 250 ppm (at high pH) or 170 ppm (at low pH) for 30-60 minutes, as a lasting immersion: 20 ppm for 12 hours.

Potassium permanganate is a powerful oxidizing agent, is not listed in 2377/90. There is no legal application with potassium permanganate in Germany.

Saline (sodium chloride) in fish: indication in ectoparasites (protozoa), *Chilodonella spp.*, *Trichodina spp.*, *Ichthyobodonecatrix* and gill amoebas. 1-2.5% strength depending on the species and age: 10-25 g NaCl / l water, 30-60 minutes (especially for trout fry during first few feedings thereafter in new, clean pond or basin).

Caustic soda (sodium hydroxide)is not listed by name in an annex to Regulation 2377/90 (EEC). The use of sodium in food-producing animals is allowed, since it is a substance with an E-number (E 524). The effect is mainly reached through the elevated pH.

Peracetic acid: The active ingredient is in the Annex II of Regulation 2377/90 (EEC) and is therefore legal for all food-producing species e.g. *Upersan® dip* (Kesla AG) (veterinary medicinal product, therefore the first choice in the treatment), *Wofasteril®* E 400 (Kesla AG) (admission because approved in the human area), Perotan, not permitted for the treatment of fish. Example for the treatment of fish: Against ectoparasites,particularyskin flukes, 2, 5 ml / m^3

every 8 hours (spray on the water surface) for 6 days. Be sure to observe the safety instructions! Very dangerous!

Virkon-Aquatic (potassium monopersulfate): Disinfectant with excellent cleaning effect. It is highly effective virucidal, fungicidal and bactericidal substance. Active ingredient: potassium monopersulfate 497 mg / g. Contains Potassium persulfate but potassium monopersulfate is not listed in Annexes I-IV of 2377/90 and therefore may not be used for the treatment of fish. There is currently no available drugin Germany with potassium monopersulfate as the active ingredient.

Hydrogen peroxide is listed in Appendix II in 2377 and therefore allowed, but no veterinary drug is currently available in Germany, in which hydrogen peroxide is included as an active ingredient.

Sodium, e.g.Oxiper is not listed in Annexes I-IV of 2377/90 and therefore may not be used for the treatment of fish.

Potassium permanganate: highly effective against juvenile and adult parasites of the genus *Argulus coregoni*. It leads to death in 100 percent of cases at the dosage of 0.01 g per liter of water, according to (Hakalahti-Siren et al., 2008). Concentrations of 0.001 and 0.0001 g per liter had no apparent effect. The tested developmental stages of *A. coregoni* were two different juvenile stages and adult parasites.

5.4. Biology and pathogenesis of*Argulus spp*.

Argulus spp.(derived from Latin: hundred eyed watchman) are branchiuran crustaceans with a direct life cycle. They spend most of their life time as ectoparasites on fish(Taylor et al.,

2006). Carp lice are among the most common parasitic crustacean found in fish farming and require treatment since they affect both the economy of the fish farm and the health of the fish(Langford et al., 2014). Carp lice treatments are required in aquaculture because slow moving or stagnant water finfish, such as carp, but also stream fish such as trout, when raised in ponds, are typically vulnerable to ectoparasitic infections, particularly in fish farming where water quality can be poor, stress factors are typically high, and space is often confined.

The genus *Argulus*, the carp louse, includes approximately 50 species and it belongs to the sub-tribe of the crustaceans. It has a strong dorsoventral flattened body consisting of two sections, a cephalothorax and a pleon with a size of 3-15 mm. The carp louse attaches itself to its host with two suction discs and the clamp-like limbs. With the help of aproboscis between the mandibles, thehosts' skin is punctured. This probiscis is surrounded by venom glands and a pipe, which is used for sucking up blood and body fluids(Pasternak et al., 2000, Schlüter, 1979, Tam, 2008, Walker et al., 2004).

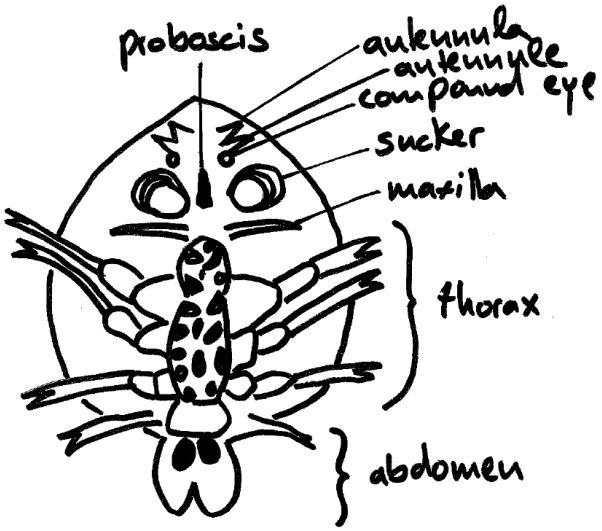
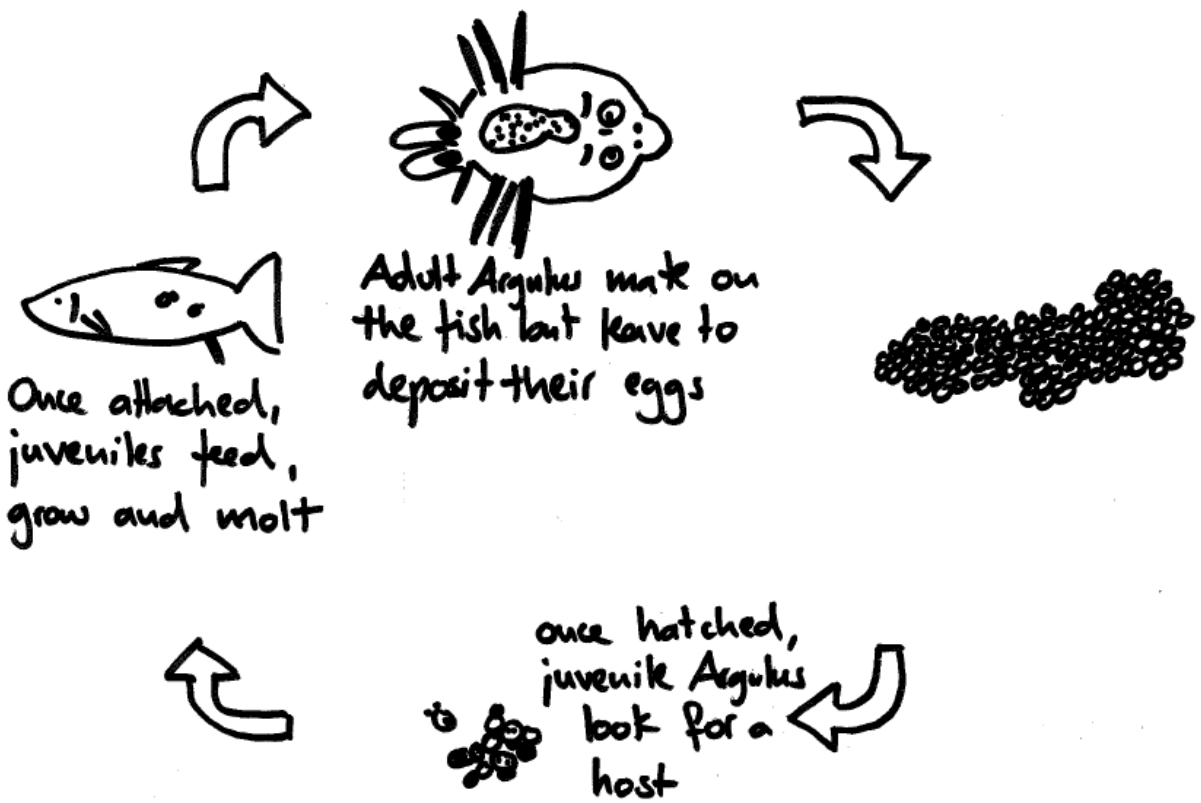


Figure 1: Schematic representation of Argulus foliaceus (ventral view), after a schematic Figure drawing of Noaman et al. (2010) (Noaman et al., 2010)

Due to their ontogenesis and thereby connected plurality of moltings, the parasite needs to feed on blood after every molting. Without a blood meal, *Argulus foliaceus* doesn't survive longer than 15 days.For reproduction, the carp louse usuallyleaves the host and reproduction takes place in the open water(Avenant-Oldewage and Swanepoel, 1993). Eggs are laid after leaving the fish and are attached to plants or stones. The eggs develop over a nauplius and six copepod stages and then after sevenmoultingsthe parasite reaches adulthood (Harrison et al., 2006). In some cases several hundred*A. coregoni*eggs have been found on the same stone for in fish ponds (Mikheev et



representation of life cycle of *Argulus foliaceus*

Male argulidae spend much less time on the host as females(Schlüter, 1979). In recent studies examining the host behavior, lice exhibit a three times higher activity during dark period compared to light. This is in contrast to the higher activity of the host animals during light, as the hosts of the fish lice rest at night, which makes them easy targets for blood collection(Mikheev et al., 2001).In addition to the direct damage by sucking blood, compounds present in parasite salivacan cause a reduction in lymphocyte counts and a general loss of vitality. Furthermore, since the carp louse's limbs constantly moveduring feeding, significant rubbingcan occur, which mechanically irritates the skin of the fish and could increase the production of mucus. In the skin areas damaged by parasites, secondary fungal infections may occur. The degree of damage is influenced strongly by the size of the fish and the degree of infestation, and high infestation levels (>50 lice per fish) were observed to cause direct mortalities(Weismann et al., 2004).

Argulus spp is a non-host-specific freshwater inhabitant and is located typically at the base of the fin, the mouth and the gill cover(Mikheev et al., 2001, Schlüter, 1979). Fish lice are macroscopically visible. Severe infestation leads to increased incidences oflesion of the integument, serious secondary infection with bacteria and fungi and to high mortality. They are also known for the spreading of the spring viremia of carp virus, which is a notifiable disease in Germany as it is considered highly lethal(Cusack and Cone, 1986, Walker et al., 2004).

The parasites feed on hemorrhagic liquids. Infected fish show lethargy, anorexia, aimless swimming and changes in the school size(Walker et al., 2004). At the cellular level, elevated monocyte and granulocytelevels can be found, indicating an ongoing immune response. Nevertheless, the immune response is less than what would be expected, which suggests that the parasite produces immunosuppressive secretions (Moller, 2012). The poikilothermic parasites are strongly dependent on the temperature of the water (Koyun, 2011). At an optimum of 28 ° C they reproduce three times faster than at 16 ° C (Schlüter, 1979). Treatment

against *Argulus spp.* infections therefore must involve a holistic treatment with various points of attack:

1. The capture of eggs laid by suspending colorful boards that are placedat different depths in the water, which the females will lay their eggs on it. The boards are then collected and changed regularly to remove the laid eggs from the system (Gault et al., 2002, Harrison et al., 2006).
2. Draining the ponds and treating the empty basins with disinfectants to kill all the remaining parasites as the persistence of only a handful of gravid females will set the reproductive cycle in motion again (Hakalahti-Siren et al., 2008, Mikheev et al., 2001, Pasternak et al., 2000)
3. As a last resort the use of chemical treatments to combat an existing parasite infection in the population is an option. For this purpose there are a variety of possible agents available. Firstly, agents that will be discussed later are an option, andsecondly, organochlorine and organophosphate pesticides have proved to be most effective against the parasite (Triclorphon).Pyrethroide compounds are also very effective and even more toxic to aquatic invertebrates (Avenant-Oldewage and Swanepoel, 1993). But due to their high environmental toxicity, they are unsuitable for the use in fish ponds.

In summary, one can say that *Argulus* infections are rarely a problem in natural fish populations, but mainly a concern to fish farmers.

6. Antiparasitic drugs

The following chapter offers an overview of the existing anti-parasitic drugs in aquatic animals, specifically finfish. It presents previous research and gives suggestion, on which of the existingsubstances are best suited and efficient, as active compounds in effectively and gently treating Argulosis infection.

6.1. Current treatment situation worldwide

An international inventory of all chemotherapeutic agents used in salmon farming has been carried out by Roth(Roth, 2000). It shows that in 2000, eleven active ingredients which could be divided into five types of pesticides are used against the salmon louse in commercial salmon farms. There are two organophosphates: dichlorvos and azamethiphos; three pyrethrine: pyrethrum, cypermethrin and deltamethrin; an oxidizing agent: oxygen peroxide; three avermectins: ivermectin, emamectin benzoate and doramectin; and two urea derivatives (benzoylphenylureas): teflubenzuron and diflubenzuron. According to this study, there is a high variability on the amount used and available of each active substance in different countries. Each country where salmon is cultivated regulates the use of these pesticides. A veterinary prescription or reclassification is usually necessary to apply it. As a regular registration is very costly for manufacturers, there are very few drugs on the market and allowed in general. To avoid resistance of lice to the used pesticides, there are different maximum numbers of treatments with antiparasitics during a growth cycle in the various countries. For example, in Canada, up to three consecutive treatments are permitted whereas in Chile, up to eightare allowed (Burridge et al., 2010).

According to Burridge et al. (2010), the use of azamethiphos and teflubenzuron has been discontinued, as some parasites have developed resistances to azametiphos and teflubenzuron and is therefore no longer produced as sea louse parasiticide (Burridge et al., 2010). The antiparasitics against louses lack specificity, which is why they can also harm non-target organisms. The data on environmental impact and potentially toxic effects are often confidential and not available to the public. In the European Union there are standards, which state the maximum values of all active substances that may be detected in food-producing animals (Maximum Residue Level, MRL). Due to different regulations regarding the disclosure of the use of medicines in the various countries, it is very difficult to make general statements about the amount and type of global use of drugs in aquaculture. Especially the governments of Chile and some provinces of Canada publicize any of their data(Burridge et al., 2010).

The legal situation in Germany and Austria allows just very limited use of veterinary drugs in aquaculture. The project group of Weisman et al. (2004) studied the optimization of the existing treatment methods against ectoparasites in fish for controlling *Argulus foliaceus*-infestations in carp and trout(Weismann et al., 2004). They used the chemicals formalin (in a concentration of 300 mg/l ppm) and sodium chloride (20 g/l), peracetic acid (Perotan®, 40 mg/l), chlorinated lime (20/30/40 mg/l), chloramine T (40 mg/l) , hydrogen peroxide (40 mg/l) and Aquahumin (300 mg/l) and incubated therein from fish isolated Argulus foliaceus. Three hour incubation in 20 g/l sodium chloride and a four-hour incubation in 40 mg/l bleaching powder killed all parasites. After three hours of incubation at 40 mg/lChoraminT the survival rate of the parasites was reduced by about 50%. Formalin (20% stock solution) and Aquahumin® in concentrations <300 mg/l and hydrogen peroxide in concentrations Perotan® and <40 mg/l had no effect, since the same number of parasites survived as in the control.

The tests carried out in in-vivo experiments were disinfectant baths against *A. foliaceus* infestations in rainbow trout and carp. Lime chloride in concentrations of 30 and 40 mg/l for rainbow trout was already at a contact time of 1 h lethal. Three hour baths in 20 g/l sodium

chloride were endured by the rainbow trout significantly better, since the loss rate was only about 10%. Using this method, all the parasites have been killed.

Also free floating fish lice in the aquarium were no longer observed after sodium chloride bath. Carp on the other hand could not tolerate sodium chloride baths. Sodium chloride was for 8 ± 3 cm big carp after 1 h lethal, for 17 ± 4 cm big carp after 2 - 3 h. Four-hour baths in 40 mg/l bleaching powder resulted in carp to a failure rate of survival of approximately 30%. This bath method achieved that the parasites could be killed completely. Upon completion of bleaching powder bath, the carp showed significant behavioral changes, such as decreased reactions and disturbed swimming behavior. This normalized in most fish within 3 to 5 hours. However, within 3 days of the end of the bath further losses occurred. With a short time bath of 2 minutes in 75 g/l sodium chloride, *A. foliaceus* could be completely removed from both the carp and the rainbow trout. Through the saline solution, the fish lice fell from the fish skin and remained on the floor of the basin are, but they were not dead. When a sodium chloride concentration was just 50 g/l, single fish lice remained on the fish.

The comparative study by Weisman et al. (2004) showedthat *A. foliaceus* is very resistant to available chemicals that are approved for use in food fish production. Of the chemicals tested only chlorinated lime and sodium chloride were effective, both only with a relatively long exposure time and high concentrations, and treatment was bathing at the tolerance limit of rainbow trout and carp. Bleaching powder baths were lethal and thus unsuitable in the effective concentrations for the rainbow trout. Also in carp, the four-hour baths in 40 mg/l bleaching powder resulted in losses and significant behavioral changes in fish. Sodium chloride baths in the effective concentration of 20 g/l, and the effective exposure time of 3 hours were relatively well tolerated by the rainbow trout, since the losses were small. This method thus provides the most reliable option for combating *A. foliaceus* in rainbow trout aquaculture. In contrast, sodium chloride baths for carp were not suitable because the carp

responded to sodium chloride much more sensitive than rainbow trout. As with the methods described the tolerance limit of the fish has been reached.

So if taking this information into account, depending on their physiological state, the weakness of the carp by the infestation and the infestation degree, the losses due to mortality can be even higher. Furthermore it must be considered that these baths must be repeated in order to kill the hatching larvae and juvenile stages, which can result in additional losses of fish. Therefore, these methods are only partially suitable for controlling *A. foliaceus*infection in carp ponds. The investigations also showed that short-term exposure in sodium chloride (75 g/l, 2 min) removes the parasites from 100% of the fish. However, the released fish lice are only weakened, but remain viable after the short-term baths,therefore, this method is only suitable to remove the parasites from the fish, but not to remove them from the water system.

In the paragraph above it was explained, which substances are permitted to use on food delivering fish. It was clearly shown that the permitted treatments are not sufficient to successfully combat parasitic infestations. Therefore, alternatives have been sought.

The drugs presented below were chosen because others, e.g. Organophosphates are known for their toxicity for environment and vertebrates (Chowdhury et al., 2006). Previous studies showed that the active ingredients in ivermectin(Davies and Rodger, 2000, Hemaprasanth et al., 2012), doramectin(Hemaprasanth et al., 2012, Hemaprasanth et al., 2008), emamectinbenzoat(Braun et al., 2008) and Diflubenzuron(Bouboulis et al., 2004, Erdal et al., 1997) have the highest potential to be efficient while being biocompatible with the fish. In order to minimize stress for the fish, the tests in this study were only performed *in vitro* and to abstain from *in vivo* animal experiments.

Due to the better practice and reduced risk ofstress for the animals, the focus was on agents that could be administered via dietary treatment instead of treatments that involved handling procedure e.g. injection or bath treatment which are known to enhance the capability

of leucocytes (Saeij et al., 2003). The aim is to develop a recommendation on which active ingredient would be best suited as a treatment against *Argulus foliaceus* infestation in *Cyprinuscarpio*. It must beconsidered that food treated with medication may encounter less acceptances among some fish when offered with untreated food. Therefore attempts have been made to produce particularly tasty medicated feeds (Wedekind et al., 2011).

6.2. Ivermectin

Ivermectin is a drug of the group of avermectins and is one of the macrocyclic lactones. The structural formula is shown in Fig. 3. Avermectins are neurotoxic and have been used successfully in the treatment of a number of parasitic diseases of terrestrial mammals(Gokbulut et al., 2005, Pérez et al., 2002, Toutain et al., 1997) as well as in many human diseases (Omura, 2008) such as the river blindness.It acts on glutamate-gated chloride channels, which are common in nematodes, insects and ticks, thereby paralyzing pharyngeal and somatic muscles(Arena et al., 1992). Due to the low solubility of the drug, ivermectin is used primarily as an oral therapeutic agent.

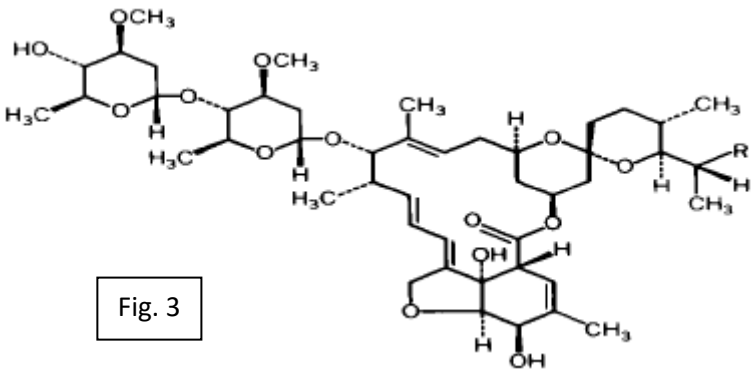


Fig. 3

Studies on toxicity and pathological effects of orally and intraperitoneally administered ivermectin on sea bass (*Dicentrarchuslabrax)*showed no toxicity while administered via the feed at 0.5 and 0.7 mg/kg. However, lower doses administered via oral intubation and via

injection have caused toxic effects on the treated animals. More toxicity was observed in 11 °C cold water than in 20 °C cold water (Athanassopoulou et al., 2002).

Ivermectin is poorly absorbed in the fish organism and is excreted at a high percentage by the faeces. The highest concentration was found in fatty organs such as the liver. Ivermectin remains in the tissue of the treated fish for a certain time and is then excreted mainly in unchanged form(Davies and Rodger, 2000).Ivermectin can therefore enter the water environment from the excretion of the fish and leftover treated feed. It has a strong affinity for lipids and to soil sediments, as well as other organic material. Risk analyses have shown that ivermectin accumulates in the sediment and that this is the reason why the resident micro-organisms are exposed to a greater risk than the organisms of the pelagic (distant shore waters, deep sea) environment.

Ivermectin has been found in some individual tests to be toxic to microorganisms in the soil, but there is so far no evidence that the treatment of fish with the agent in field trials would affect multiple species of seabed inhabiting populations.Ivermectin's toxicity levels are not considered dangerous and can be used without damaging the mesocosm(Sanderson et al., 2007) .

The compound ivermectin is predominantly used in the form of in-feed formulations on salmonids against copepods in the family Caligidae: *Lepeophtheirus salmonis*, *Caliguselongatus*and *C. rogercresseyi*. Ivermectin is well-documented as very effective on all developmental stages of the parasites. The duration of effect can be up to 10 weeks but the safety margin for ivermectin is narrow. The increasing number of resistant parasites strains is worrying for the salmon aquaculture industry(E Horsberg, 2012).

6.3. Doramectin

Doramectin, 25-cyclohexyl-5-O-dimethyl-25-de (1-methylpropyl) avermectin A1a, belongs to the group of the avermectins (Fig. 4). It is the fermentation product of a soil-dwelling Streptomyces avermitilis strain(Goudie et al., 1993). Like all avermectins, it is chemically seen a macrocyclic lactone. Its structure differs from ivermectin by an additional nonpolar cyclohexyl group at position 25 and a double bond between C 22 and C 23(clinipharm.ch, 2015). The lipophilic cyclohexyl group gives a longer tissue half-life compared to ivermectinand thus longer duration of effectiveness (Pérez et al., 2002, Toutain et al., 1997). It is extensivelyused in a variety of species as an antiparasitic treatment (Escudero et al., 1999, Gokbulut et al., 2005, Logan et al., 1996, Pérez et al., 2002, Toutain et al., 1997).

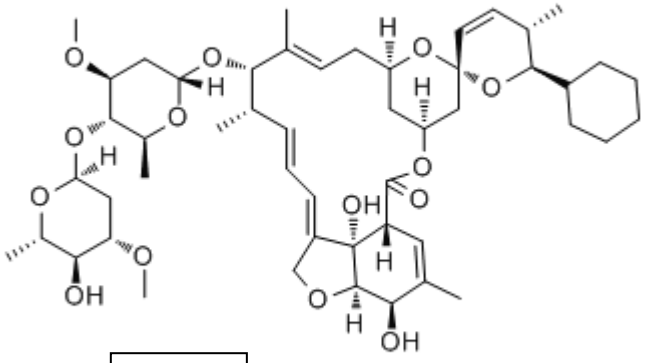


Fig. 4

It was chosen to be one of the tested drugs in this study because of it proven efficacy in Indian major carp against lice (Hemaprasanth et al., 2012, Hemaprasanth et al., 2008). Doramectin exhibited as well in toxicity tests just a very low toxicity toward *D. magna*, which is five orders of magnitude values lower than the toxic reference compound $K_2Cr_2O_7$ (Kolodziejska et al., 2013).

6.4. Diflubenzuron

Diflubenzuron is a chitin synthesis inhibitor, its structural formula shown in Fig. 5. At the moment it is used as an antiparasitic drug in salmon farms(Erdal et al., 1997). Little is known about the pharmacokinetics in other fish species. (Erdal, 2012) conducted a pharmacokinetic study in Atlantic cod (population living in the Baltic Sea). In this study, the conclusion was made that the middle fabric level of PCA (p-chloroaniline), which has an active ingredient that is an important metabolite in Atlantic cod called diflubenzuron, in fillet, skin and liver is 36.1 and 106 ng / g, which is surprisingly low compared to the levels in Atlantic salmon, where it was measured at 2240 ng / g. It showed to be efficient in eradicating lice (isopod, Ceratothoaoestroideson) from Sea bass in the form of in-feed-treatment (medicated pellets of diflubenzuron PC90 at a dosage of 3 mg/kg body weight per day for 14 days) (Bouboulis et al., 2004).

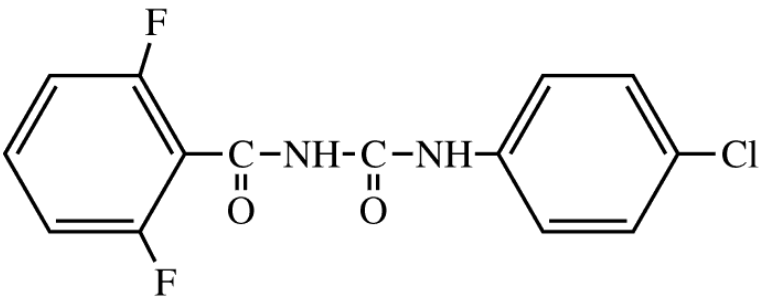


Fig. 5

6.5. Emamectin Benzoate

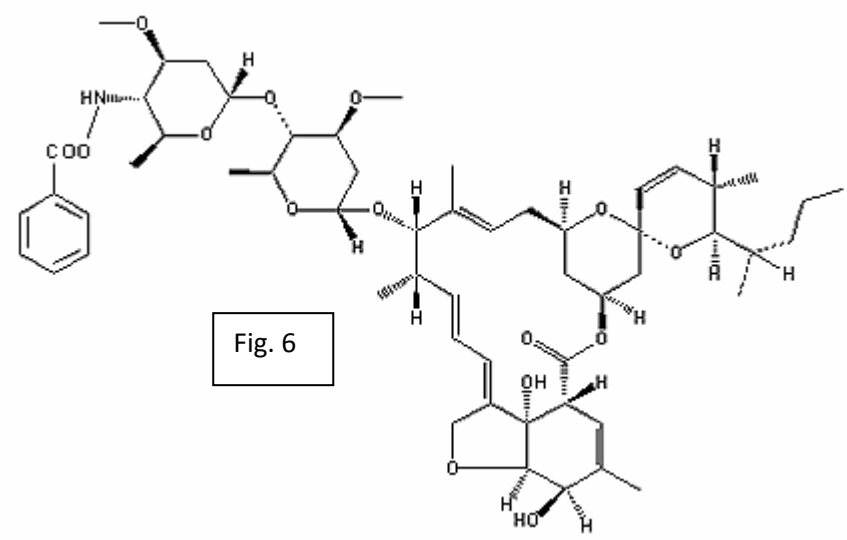


Fig. 6

Emamectin benzoate (Fig. 6) belongs to the group of avermectins, which have a wide range of use in treating livestock for parasites (Stone et al., 2000). The mechanism

of action is based on specific binding to glutamate-activated chloride channels and a resulting increase in membrane permeability to chloride ions, which produces an interruption of physiological.

Emamectin benzoate is administered through the diet. It is detectable in the fish up to 70(Sevatdal et al., 2005)to 90 (Kim-Kang et al., 2004)days after treatment. The toxicity to fish is low. Braun et al. (2008) described in a studyon *Cyprinuscarpio* that dietary treatment with emamectin benzoate (Slice®) was tolerated well in all tested concentration levels and the fish displayed no abnormities (Braun et al., 2008).

The drug is not known to accumulate in the sediment (Telfer et al., 2006) or to have any negative effects on the surrounding environment of salmon cages. Studies have also demonstrated that no interactions are to be expected with planktonic copepods (Willis and Ling, 2003), since the reached concentration is too low to have an toxic effect.

Emamectin benzoate was the treatment of choice in salmon farming against sea lice infection for many years. Unfortunately, in recent years, usage patterns of sea lice treatments have changed as resistance by sea lice to traditional treatments has increased(Igboeli, 2013). E.g. in Norway, the use of emamectin benzoate has therefore been reduced from 81 kg annual average use per fish farm in 2008 to 36 kg in 2012 (Macken et al., 2015). Emamectin benzoate is known by the commercial name of Slice® (Fa. Schering-Plough Animal Health, USA) approved in Norway and Scotland to combat sea lice infestation of Atlantic salmon. That active ingredient proved to be efficient in carp lice eradicating in *Cyprinuscarpio*(Braun et al., 2008), in goldfish and koi carp with infestations of *Argulus spp*(Hanson et al., 2011) as well as in the control of *Argulus coregoni* (Crustacea: Branchiura) in rainbow trout *Oncorhynchus mykiss* (Hakalahti et al., 2004).

However,the production of medicated feed by the veterinarian is banned through the 11 AMG novella in 2001, therefore Germany has banned the use ofSlice®. This is why the effect of

Slice® on the immune system of fish was not investigated in this study, although it may have proven to be a good choice as a possible treatment.

7. State of science

The current state of research relates mainly to the scientific investigations concerning many methods of controlling parasites in salmon aquaculture. In salmon farming in America and in Europe, sea lice (*Lepeophtheirus spp, Caligusspp*) are the causative agent of the greatest economic damage. According to an extrapolation of Costello in 2009, the infestation with *Lepeophtheirusspp*and*Caligusspp*caused worldwide costs of approximately € 305 million(Costello, 2009). For economic reasons, and due to the substantial damage by these parasites in the affected host animals, there is much literature on the salmon louse available.

Some studies deal with the problem of sea lice infestation in salmon and its possible therapy. For pharmacotherapy, there are studies of emamectin benzoate (Jones et al., 2013), (Kim-Kang et al., 2004) and teflubenzuron(Ritchie et al., 2002), also concerning their bioavailability(Kim-Kang et al., 2004), the development of resistance (Igboeli, 2013) and their evaluation for other fish species (Olsvik et al., 2013). The louse infestation with the resulting lesions of the skin is a stress for the animals concerned and increases the susceptibility to secondary infections such as bacteria or fungi. The mortality of affected animals was significantly increased (Bandilla et al., 2006).

A study by Bouboulis et al. (2004) shows that in European sea bass (*Dicentrarchuslabrax*), which were experimentally infected with the isopods *Ceratothoaoestroides* (parasitically living in fish) and administered diflubenzuron as pellet feed treatment with 3 mg / kg body weight for 14 days, were free of parasites after this period(Bouboulis et al., 2004). Pre-adult and adult

parasites were killed. Within 15 days, no adverse effects of the drug and no reinfection was observed.Another study shows that one can use successfully therapeutics from salmon for trout who live in fresh water (Hakalahti et al., 2004). This finding further suggests that it is basically a correct assumption to use drugs from salmon living in saltwater, even for the freshwater cultured fish.

With regard to the use of various active ingredients for carp there are few studies, for example by Braun et al. (2008) who had already tested two drugs to carp, which are known todayto be effective and well tolerated(Braun et al., 2008). However, this work was incomplete because the efficacy study was conducted only at 20.6 ° C and tolerability study only 14.9 ° C. In fish as poikilothermic, so called cold-blooded animals, the ambient temperature is the decisive factor for the metabolism. So it is likely that at higher temperatures, as might be expected in the German summer in the earthen ponds of carp hosts, toxic effects of the drugs which are compatible with around 20 ° C , may occur quite possible at for example 30 °C,suggesting a considerable need for research of side effects on different water temperatures.

II. MATERIALS AND METHODS

1. *In vitro*experiments: testing of antiparasitic drugs effect on neutrophil function

1.1. Preparation and description of the function assays

Preliminary testing of antiparasitic drugs effect on fathead minnow neutrophil oxidative burst activity, neutrophil degranulation and NET release.

1.2. Animal care

Adult minnows were held at the ChairforFish Diseases and Fisheries Biology at the Faculty of Veterinary Medicine, Ludwig-Maximilians University, Munich in Bavaria, Germany. The average weight of fish was approximately 4.5 g. The fish were housed in a recirculating water system of 300 liters capacity with a filtration system. As food we used dried flake food,mixed in a ratio of 2:3 from Tropical® Breeder Mix and D- Vital Plus from Tropical Heimtierbedarf GmbH Germany. Fish were fed twice a day. Animals were cared for in accordance with German guidelines for laboratory animal care (§ 11 of the Animal Welfare).

1.3. Extraction of neutrophils

Fathead minnows were euthanized with an overdose of MS-222 (Pharmaq, U.K.). Kidneys from eight fish were extracted from the fish and pooled immediately in a sample. By mashing the organs with a tissue grinder, standard neutrophil suspensions were prepared as described previously (Palić et al., 2005b). After washing step with Hank's balanced salt solution (HBSS) (Thermo fisher), a purification step was used by pipetting cell suspension on top of lymphoprep solution at density 1.077 +/- 0.001 g/ml and osmolality of 290 +/- 15 mOsm (Fresenius Kabi Norge AS for Axis-Shield PoC AS, Oslo, Norway) in order to form a differential density gradient. Collected neutrophils were then washed for a second time. Cellswere counted with Coulter cell counter (Beckman Coulter, Z2, Coulter Particle Count and Size Analyzer)and adjusted in all the samples to 2 x 10^7 cell mL^{-1}. Then, neutrophil assayswere carried out.

1.4. Neutrophil function assays

In this study, three neutrophil function assayswere undertaken to assess the impact of some active ingredients of antiparasitic drugs (ivermectin, doramectin, diflubenzuron) on the innate immune response of fathead minnows (*Pimephales promelas*, (Rafinesque, 1836)). Neutrophil function is used as a valuable parameter to evaluate the health status of individuals and populations (Smith and Lumsden, 1983). The neutrophil function is measured on the basis of the degranulation of primary granules (MPO assay), the oxidative burst activity (HDFFDA assay) and the release of neutrophil extracellular traps (NETs).

For the preliminary test of antiparasitic drugs effect on fathead minnow neutrophil function, four independent experiments were conducted with 4 samples (N=4, each sample comprised of pooled neutrophils from 8 fish).

Three antiparasitic drugs were tested:

Ivermectin (308ng/ml desired plasma concentration, with 1 µl Ivomec®, Merial, Injektionslösung zur subkutanen Injektion in 1 ml HBSS with Calcium and Magnesium)
Doramectin (125ng/ml desired plasma concentration, with 1 µl Dectomax®, Zoetis injectable solution 1% in 1 ml HBSS with Calcium and Magnesium)
Diflubenzuron(200 ng/ml desired plasma concentration, with 1 µl Aradol Plus 250®, JBL solution for pouring into water in 100 µl HBSS with Calcium and Magnesium)
Those solutions were prepared by vortexing drug with HBSS.

All assays were also tested without cells to exclude spectrophotometric interference of the compounds and chemicals themselves. None of the tested chemicals interfered with photometric readings.

1.4.1. Neutrophil oxidative burst assay

Polymorphonuclear neutrophils generate reactive oxygen species (ROS) during phagocytosis of pathogens such as bacteria, fungi or toxic substances. This process is referred to as 'oxidative burst' or 'respiratory burst' and plays an important role in host defense. The ROS production helps to degrade internalized pathogens (Chen and Junger 2012). One of the ROS, hydrogen peroxide, is used by the degranulation of primary granules (s. above) to build

hypochlorous acid and chloride anion. These close interactions between the chemical process and products show the complexity in the neutrophil host defense.

The process of respiratory burst is the first defense mechanism of neutrophils when killing pathogens. By inducing a charge change across the membrane which is compensated by pumping electrons in the inside, these electrons react with oxygen and create together ROS (reactive oxygen species). ROS stimulates the release of digesting enzymes(Segal, 2005). The oxidative burst induces as well a selective release of granular proteins into the cytoplasm through an unknown mechanism to be the first part of a chain of action, activating a line of other mechanisms (Papayannopoulos et al., 2010).

HDFFDA: quantitative assay to monitor the oxidative burst (H_2O_2-production). HDFFDA diffuses during incubation into cells, where it oxidates; and the oxidative product is measured by spectrofluorometer.

Neutrophil oxidative burst activity was tested with a spectrofluorometry assay based on HDFFDA (2'7'-difluorodihydrofluorescein diacetate) on fathead minnow granulocytes from kidney to determine the amount of extracellular reactive oxidative products. Cells were exposed to standard stimulant (PhorbolMyristate Acetate: PMA). This assay is based on the principle that HDFFDA diffuses into the cell during incubation time and oxidates there with H_2O_2 and other electron-bearing radicals such as superoxides to form afluorescent product which can be quantified with the spectrofluorometer.

Plate Set-up was as below, all wells were filled in duplicates and the numbers given in µl, cells 1- 4 are the standard cell suspensions, N=4:

	PMA	HBSS	Iveremectin	Doramectin	Diflubenzurone
1	25 HBSS +50 PMA + 25 cells	75 HBSS + cells 25	3 Working solution (w.s.) + 72 HBSS + cells 25	1 working solution + 74 HBSS + cells 25	1,2 working solution + 73,8 HBSS + cells 25
2	25 HBSS +50	75 HBSS	3 w.s. + 72 HBSS	1 w.s. + 74 HBSS	1,2 w.s. + 73,8

	PMA + cells 25	+ cells 25	+ cells 25	+ cells 25	HBSS + cells 25
3	25 HBSS +50 PMA + cells 25	75 HBSS + cells 25	3 w.s. + 72 HBSS + cells 25	1 w.s. + 74 HBSS + cells 25	1,2 w.s.+ 73,8 HBSS + cells 25
4	25 HBSS +50 PMA + cells 25	75 HBSS + cells 25	3 w.s. + 72 HBSS + cells 25	1 w.s. + 74 HBSS + cells 25	1,2 w.s. + 73,8 HBSS + cells 25

After the cells, 50 µl of HDFFDA working solution (37,5 µl stock in 2465,5 µl of HBSS with Ca, Mg) was added in each well.

After incubation for 60 minutes in the dark, the plate was read in spectrofluorometer (Spectra Max M5, Molecular Devices). The index of stimulation of oxidative burst was calculated based on the following formula:

Average Test-Substance / average HBSS.

1.4.2. Degranulation of neutrophil primary granules

The degranulation of primary granules is based on the release of myeloperoxidase (MPO) stored in azurophilic granules of neutrophilic granulocytes. MPO is the most abundant proinflammatory lysosomal protein and it plays an important role in the antimicrobial actions that occur after the stimulation of neutrophils. After the stimulation of neutrophils, various produced reactive oxygen species such as superoxide (O_2^-) can interact with available hydrogen ions in a reaction catalyzed with superoxide dismutase to produce hydrogen peroxide. Continued with myeloperoxidase (MPO) mediated halogenation, this reaction creates hypochlorus acid, which is toxic for pathogens.

MPO is an electron-dense product in the azurophile/primary granules, which degranulates into the phagocytic vacuole and releases the MPO. Released MPO catalyzes the

reaction of reduction between hydrogen peroxide (H_2O_2) and chloride ion (Cl^-) to produce hypochlorous acid ($HOCl^-$). Hypochlorous acid is very reactive to surrounding tissues and cells, and is used by the neutrophil to kill bacteria and other pathogens in the phagolysosome or externally(Segal, 2005).

Degranulation (exocytosis) of fathead minnow neutrophil primary granules was tested with a colorimetric assay of release of myeloperoxidase based on reduction of Tetramethylbenzidine (TMB) in presence of hydrogen peroxide. The assay was modified by using ready-made liquid 3,3′,5,5′-Tetramethylbenzidine (TMB) working solution (2.5 mM in H2O2, Sigma-Aldrich, Germany) instead of powdered TMB. Fathead minnow granulocytes were collected from kidney to determine the relative degranulation rate of myeloperoxidase in this assay. Cells were exposed to standard stimulant (Calcium Ionophore, A23187, CaI, 5 µg mL^{-1}, Fisher Bioreagents, Switzerland) and the test substances.In this assay, it is the TMB that is developing the color. TMB can act as hydrogen donor for the reduction of hydrogen peroxide to water by myeloperoxidase. The resulting diamine causes the solution to take on a blue color. The reaction can be halted by addition of sulfuric acid which turns the reduced TMB solution yellow.

Plate set-up was as below, all wells were filled in duplicates and the numbers given in µl, cells 1- 4 are the standard cell suspensions, N=4:

	Calcium ionophore (CaI)	HBSS	CTAB[6]	Ivermectin	Doramectin	Diflubenzuron
1	50 CaI + 75 HBSS + 25 cells	125 HBSS + 25 cells	125 CTAB + 25 cells	4,6 Ivermectin-dilution + 120,4 HBSS + 25 cells	2 dilution + 123 HBSS + 25 cells	1,8 dilution + 123,2 HBSS + 25 cells
2	50 CaI + 75 HBSS + 25 cells	125 HBSS + 25 cells	125 CTAB + 25 cells	4,6 Ivermectin-dilution + 120,4 HBSS + 25 cells	2 dilution + 123 HBSS + 25 cells	1,8 dilution + 123,2 HBSS + 25 cells

[6]CTAB: 0, 02 %, Cetrimonium bromide, a detergent to lyse cell membranes and therefore release all myeloperoxidase in supernatant to be used as a positive control (100% of enzyme).

3	50 CaI + 75 HBSS + 25 cells	125 HBSS + 25 cells	125 CTAB + 25 cells	4,6 Ivermectin-dilution + 120,4 HBSS + 25 cells	2 dilution + 123 HBSS + 25 cells	1,8 dilution + 123,2 HBSS + 25 cells
4	50 CaI + 75 HBSS + 25 cells	125 HBSS + 25 cells	125 CTAB + 25 cells	4,6 Ivermectin-dilution + 120,4 HBSS + 25 cells	2 dilution + 123 HBSS + 25 cells	1,8 dilution + 123,2 HBSS + 25 cells

The plate was shaken for 5 seconds and after that incubated for 20 minutes at room temperature. Then, 100 µl of TMB (Liquid Substrate System peroxidase substrate, Sigma Aldrich)was added and exactly 4 minutes later 50 µl of sulfuric acid solution to stop the colorimetric reaction. Next, plate was centrifuged with 600 G for 2 minutes. After centrifugation, 200 µl of supernatant was transferred into a new plate and put in the spectrophotometer for reading.

The percentage of degranulation was calculated based on the following formula:

((average of test substance – average of HBSS value) / (average of CTAB value – average of HBSSvalue))*100

1.4.3. Neutrophil extracellular traps release assay

The neutrophil extracellular traps (NETs) release assay is based on the ability of neutrophils to release decondensed chromatin from their nucleus after activation of NETosis pathway through reactive oxygen species. These chromatin threads form extracellularstrucures that resemble nets to trap and kill pathogens and the process has been named ‘neutrophil extracellular traps’ formation (NETs) (Papayannopoulos et al. 2010). NETs contain

antimicrobial factors including histones, neutrophil elastase, cathepsin G and MPO (Brinkmann et al., 2004).

The final state of Neutrophil defense mechanism against invading pathogens is the newly discovered NET (Neutrophil Extracellular Trap) release. The NET mechanism is the last defense a cell can accomplish. It ejects its DNA intertwined with histones and granule proteins to tackle the pathogens. Fish, as well as other vertebrates, do have this defense mechanism in common (Palić et al., 2007). The formation of NETs is activated by neutrophil elastase (NE) which is produced inazurophilic granules and transferred to the nucleus, where it partially degrades some histones, bringing forward chromatin decondensation and becoming with MPO independent from that enzymatic activity(Papayannopoulos et al., 2010).

Neutrophil extracellular trap (NET) release was measured from fathead minnow kidneys neutrophils upon stimulation with phorbolmyristate acetate (PMA). The NET assay was performed by inocculating 10 µL of standard neutrophil cell suspension into 96 well plates and stimulated with 90 µL of the three different active ingredients in HBSS+. For the positive control 10 µL of standard cell suspension was seeded into 96 well plates prefilled with 40 µL HBSS+ and 50 µL of the standard stimulant, (PMA - positive control) of 1 µg mL-1. For the negative control 10 µL of standard cell suspension were seeded into 96 well plates preloaded with 90 µL HBSS+.

Thereafter, NETs were digested with Micrococcal Nuclease (MNase) and NETs release quantified using picogreen DNA quantification assay.

Plate Set-up was as below, all wells were filled in triplicates and the numbers given in µl, cells 1- 4 are the standard cell suspensions, N=4:

	PMS	HBSS	Ivermectin	Doramectin	Diflubenzuron
1	50 PMA + 40 HBSS + 10 cells	90 HBSS + 10 cells	3 dilution + 87 HBSS + 10 cells	1 dilution + 89 HBSS + 10 cells	1,2 dilution + 88,8 HBSS + 10 cells
2	50 PMA + 40	90 HBSS	3 dilution + 87	1 dilution + 89	1,2 dilution + 88,8

	HBSS + 10 cells	+ 10 cells	HBSS + 10 cells	HBSS + 10 cells	HBSS + 10 cells
3	50 PMA + 40 HBSS + 10 cells	90 HBSS + 10 cells	3 dilution + 87 HBSS + 10 cells	1 dilution + 89 HBSS + 10 cells	1,2 dilution + 88,8 HBSS + 10 cells
4	50 PMA + 40 HBSS + 10 cells	90 HBSS + 10 cells	3 dilution + 87 HBSS + 10 cells	1 dilution + 89 HBSS + 10 cells	1,2 dilution + 88,8 HBSS + 10 cells

Next, plate was shaken for 10 seconds and then incubated for 2 hours. After incubation time, 50 µl MNase was added to each well, incubated for 20 minutes at 37°C and was inactivated when 100 µl of EDTA (5 mMol) was added. Finally, plate was centrifuged at 4 °C, 400 G for 5 minutes.

Thereafter, 50 µl supernatant, 50 µl buffer and 50 µl picogreen(Quant-iTTM, Thermo Fisher Scientific, US) solution was pipetted in a black plate and incubated for 10 minutes. Piccogreen dyes dsDNA fluorescently.

Plates for all assays were read in a plate reader (Spectra Max M5, Molecular Devices, SOFTmax Pro 6.2.2.).

Amount of NETs released from neutrophils was calculated on the formula below:

(Average of the triplets from substance 1) / (average of the triplets from HBSS).

1.5. Gradient study

The conducted preliminary testing showed that the active ingredient diflubenzuron has the biggest impact on the oxidative burst activity, degranulation of primary granules, and NET production (compare results 1.4.).Thus, a gradient study was performed to confirm the findings in the single studies as well as to examine whether the increase of the stimulation with gradually increased concentrations of the tested compound diflubenzuron would trigger an

increases reaction of the innate immune system.Therefore, the same assays (HDFFDA-assay, MPO-assay and NET-assay) as described earlier were carried out testing four different concentration of diflubenzuron.

Diflubenzuron (200 ng/ml desired plasma concentration, Aradol Plus 250®, JBL solution for pouring into water) in HBSS with Calcium and Magnesium) in four different concentrations: 2 ng/ml, 20 ng/ml, 200 ng/ml, 2000 ng/ml.Those solutions were prepared by vortexing drug with HBSS.

2. Primary cell culture

Primary cell cultures are derived directly from the intact tissue or organ, usually by dispersion with proteolytic enzymes. Their major advantages are the degree to which differentiated characteristics are expressed and the fact that cells can be reorganized into a monolayer configuration, which provides easier access to the cells than in their complex cell structure as an organ. In addition, the isolation permits a precise control of the surrounding hormonal, substrate and physical conditions, which may alter cell and thus tissue function. So we can evaluate the effects of those exposures to the tissues (Hightower and Renfro, 1988).

At the present time, cultured fish cells are still extensively utilized for laboratory purposes and especially in diagnostic. Fish cell lines are also gaining importance in areas with impact beyond that of the diseases of fish, and are providing important contributions in studies relating to toxicology, carcinogenesis, genetic regulation and expression, like DNA replication and repair (Fryer and Lannan, 1994).

2.1. Kidney, Spleen and Liver sampling

Fathead minnows were euthanized with an overdose of MS-222 (Pharmaq, U.K.). Kidneys, spleen and liver from eight fish were extracted and made 16 samples of 8 fish.

2.2. Cell isolation

Cell dispersion was performed using a mechanical procedure modified from those previously developed for teleost fish(Wolf and Quimby, 1962). Standard quality control measurements were used as described (Lannan, 1994).

Fish were euthanized with an overdose of MS-222 as described previously. Fish were put on an disinfected plate and washed with HBSS with 100 U/ml Penicillin, 100 µg/ml streptomycin and 0,25 µg/mlAmphotericin B. Livers, kidneys and spleens were removed and washed with HBSS with Antibiotic and Antimycotic Solution, stabilized, Sigma-Aldrich, in same concentration as described above. Organs were minced with micropestles(Cao et al., 1996). Larger cell lumps were removed by pipetting through sieve (pore size 70 µm, Fisher Scientific). Cells were washed twice by centrifuging for 5 min at 50 G. Final pellet wasresuspended by manual pipetting (5-10 times) in 1 ml of HBSS and counted with Coulter cell counter (size of the counted cells between 4 and 16 µm). Afterwards dead cells were stained with Trypan Blue(Sigma Aldrich)counted with the hemocytometer (Flouriot et al., 1993) and percentage of viability of cells was calculated.

0,5 ml aliquots were pipetted in 12 well plates containing 3,5 ml of culture media at a density with proximately 1 x 10^6 viable cells per well (Kordes et al., 2002). As medium we used

L-15 (Leibowitz) from Sigma-Aldrich, supplemented with 10 % of fetal bovine serum (FBS) and with 100 U/ml Penicillin, 100 µg/ml streptomycin and 0, 25 µg/ml Amphotericin B.The resulting primary cell cultures were maintained for 48 h at 20 °C in the incubator. Initiation of attachment was checked by light shaking under light microscope and90 % of the culture media volume was changed daily.

2.3. Incubation with Diflubenzuron

The keynote of the establishing of the cell culture protocol was to document and examine the behavior of living cells in culture to exposure with the active component diflubenzuron. We decided to test for Diflubenzuron due to our findings in the neutrophil assays, as described above. We wanted to test if incubation with diflubenzuron in an expected plasma concentration of 200 ng/ml would interfere with the viability of the cells.

Concentration studies were done to see if the cells in culture would survive the single dose of our estimated plasma concentration, then, it was tested for the double, the fivefold and the tenfold estimated plasma concentration.

3. Gene expression of Fathead minnow cells exposed to active ingredient Diflubenzuron

3.1. Preparation and collection of cells

The liver and spleen-kidney cells from the primary culture were checked under the light microscope to confirmthat they were still vital. Then, they were harvested after incubation with diflubenzuron (double dose of the expected plasma concentration)by pipetting HBSS in the culture dish to loosen the attachment of the cells to the petri dish. The cells were pooled from two fish. Those cells were exposed to drug treatment independently, and were pooled after the treatment and as soon as the cells were harvested from the primary cell culture. In the case of each fish primary culture cells of the liver and kidney/spleen were divided in half, and only one half of the cells were treated with diflubenzuron while another served as control. This resulted in four experimental groups: liver treatment, liver control, kidney-spleen treatment and kidney-spleen control. Each experimental group had a total of six samples (n=6).

3.2. RNA extraction from cells obtained from cell culture

Each sample, consisting of the cells, pooled from two fish as previously described, was digested with 1 ml of TRI Reagent (SIGMA). To be sure that all cells were denaturized, pellet was additionally manually mortared. 200 µl of pure Chloroform was added in every one of the Eppendorf tubes, and then the tube was manually shaken for 30 seconds, followed by

incubation at room temperature for 15 minutes. After incubation, the tubes were centrifuged at 11.600 rpm at 4 °C for 15 minutes. Supernatant was collected and transferred to the new set of tubes. 500 µl of Isopropanol was added for RNA precipitation to the collected supernatant, vortexed and incubated for 10 minutes. Afterwards, supernatant was discarded and pellet wasresuspended in 1 ml of 75 % Ethanol. The mixture was spun at the same settings for 5 minutes. The supernatant was discarded. The pellet was soft spun, at 4 °C for 1 minute and with gentle pipetting, the last rest of Ethanol was removed. The pellet was air dried at room temperature for 10 minutes, then eluted in 20 µl of RNAse free water.

3.3. Quantification of RNA

Total RNA was quantified in spectrofluorometer (Spectra Max M5, Molecular Devices). The Micromax low volume plate protocol was followed for RNA quantification with a microplate (size 2 µl in 0,5 mm). In the results, the 260/280 and 230/260 ratio showed some protein contamination and some contamination with phenol or alcohol. Since phenol may inhibit the reaction in PCR, a cleanup step was performed, to get purer samples. All samples with a concentration of more than 100 ng / µl were used in the subsequent experiments.

3.4. Creation of cDNA from the purified RNA

To generate cDNA[7], 100 µl of primer solution (Universal Oligo primer dt15) were mixed with 900 µl of RNAse free water, solution was vortexed and a microplate for PCR was prepared. In that plate, samples and primers were pipetted and adjusted with water.Incubation at 70 °C (5 minutes) and 4 °C (5 minutes) followed. Mastermix, containing 5x reaction buffer (Promega), magnesium chloride ($MgCl_2$, 25 mM, Promega), dNTPs[8] (Promega), reverse transcriptase (Promega) and RNAse free water was added to get a final concentration of 3mM $MgCl_2$, 0.5 mM dNTPs, 25 ng/µl RNA.PCR was accomplished, using a cDNA program (5 min 25 °C, 1 hour 37 °C, 15 min 70 °C). That created double stranded DNA from the RNA. After PCR, samples were kept in the fridge.

3.5. Check for functional samples and primers

To check if the generated samples are functional, cDNA must be amplified to get a sufficient amount for electrophoresis.

The amplifying mastermix was prepared. It containedGreen Go Taq (Go Taq® q PCR Master Mix, 2x,Promega), a forward primer, a reverse primer and to replenish the volume RNAse free water to have 25 µl in total. Mastermix and sample were transferred to a microplate and put in the PCR machine to amplify. As primers elongation factor was used as that is expressed in every single cell and therefore an adequate control primer to see if the samples

[7]cDNA: complementary DNA (cDNA) is double-stranded DNA synthesized from RNA in a reaction catalysed by the enzyme reverse transcriptase

[8]dNTP: desoxynucleoside triphosphate is a molecule containing a nucleoside bound to three phosphates, a nucleotide

are functional (EF1 Alpha Original, reverse: 5'- CTC ATG TCA CGC ACA GCA AA -3', forward: 5'- AGC TTC TTG GCT CAG GTC AT -3') The samples were incubated first for 5 minutes at 70 °C and after that again for 5 minutes at 4 °C. Then the mastermix was added containing 5xBuffer, magnesium chloride, dNTP, reverse transcriptase and RNAse free water.

Gel for electrophoresis was prepared as described below:

Agarose and 1x TAE (TRIS-acetate-EDTA-buffer, 50 x solution, Fisher Scientific, Product of United States)were mixed and boiled in a microwave to obtain a homogenous mixture. Subsequently the mixture was allowed to cool down to 60 °C and ethidiumbromide solution (10 mg/ml in H_2O, Sigma) was added. Ethidiumbromid stains DNA by intercalating between the base pairs of DNA. Gel was poured in gel chamber between the electrophoresis aperture to harden. After hardening, all was doused and covered with 1x TAE. Per well in the gel, 12 µl of amplified sample was pipetted, as well as a marker. As marker, bench top 1 kb DNA ladder (Promega, USA) was used. Then the current of 100 V was applied. After 45 minutes of running, power was turned off and gel was removed, to read out theresultsunder UV-light (Bio Doc-H Imaging System, Benchtop 2UV, Transilluminator UVP, 302 nm) which in combination with the added marker makes the DNA visible. The reading showed that all tested samples are functional and can be used for further tests. The results are displayed in the results section.

The next step was to check if the primers are working. Therefore, same mastermix was prepared as described above, but each time with a different primer set. PCR was set at 58 °C cycle was performed.

Primers for the following genes were tested: CYP1A, catalse, glut.-s-transferase, glut. reductase and glut. synthase. Primers for genes of interest were obtained from Integrated DNA Technologies.Sequences are listed in table below.

Name	Forward primer	Reverse primer

Catalase	5'- TTA TCA GGG ATG CGC TTC TGT -3'	5'- TTC ACA TGA GTC TGC GGA TTT C -3'
Glutathione-S-transferase	5'- CCG GCA AGA GCT TCA CCA T -3'	5'- AGT GAA GTC GTG GGA AAT AGG C -3'
Glutathione reductase	5'-GTC CTA CTG TGC CGA TTG GT -3'	5'-GTC CAG GCG TTT ATG CAG TT-3'
Glutathione synthase	5'-CGG TCC AGA ACT ACC TGC TC -3'	5'-ACA CCA CGT CTT TGC CTT TC -3'

To check for the results, gel was prepared as described above and showed that all primers except primer for glut. reductase have single, clearly defined bands. Primer for glut.reductase showed faint double band, so different temperatures were tested until 56 °C gave the best result, so the primer for glut. reductase was run at 56 °C in further PCRs.

3.6. Gene expression analysisusing Q-PCR[9]

The cDNA was used for real-time PCR with primers designed for genes of interest (see table above). For all the real-time PCR reactions, master mixes were created, containing 12.5 µl of GoTaqqPCR Master Mix 2x containingCYBR Green® dye (Promega), 1 µl of 0.2 µM of forward and reverse gene specific primer, 2.5 µl of cDNA and 9 µl of nuclease free water.

For each gene of the ones in the table above, a qPCR was run with the test gene and the control gene. All samples were tested in duplicates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Kidney 1 treatment		Kidney 2 treatm.		kid. 3. treatm.		kid. 4. t.		k 5 t		k 6 t	
B	liver 1 treatment		l 2 t		l 3 t		l 4 t		l 5 t		l 6 t	
C	kidney 1 control		k 2 c		k 3 c		k 4 c		k 5 c		k 6 c	
D	liver 1 control		l 2 c		l 3 c		l 4 c		l 5 c		l 6 c	

[9]Q-PCR: quantitative real time PCR. A real-time polymerase chain reaction or quantitative polymerase chain reaction is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR), which is used to amplify and simultaneously detect or quantify a targeted DNA molecule.

The final cDNA concentration in the plate was 1 ng/µl. After set-up, plate was short spun and transferred to real-time PCR (Agilent Technologies StratageneMx 3000P). ROX was chosen as reference dye. The thermal profile set-up was set to 10 minutes at 95 °C, followed by 15 seconds at 95 °C, and annealing temperature according to pervious testing for 1 minute. A total of 45 cycles were run. The melting curve protocol contained 1 cycle at 95 °C for 1 minute, 55 °C for 30 seconds, and 95 °C for 30 s. The temperature was increased from 55 °C to 95 °C at a rate of 0.2 °C/s with fluorescence readings taken every 0.5 °C increase.Fluorescence readings were saved by the program at the end of each cycle. Normalized fluorescence data (Rn) from the Agilent Technologies StratageneMx 3000P machine were exported to the LinRegPCR software for analysis of quantitative real-time PCR data (version 2014.6). The software corrected the baseline of the data. Finally, relative gene expression for each gene of interest was calculated (relative mRNA concentration from the test gene divided through relative mRNA concentration). At the end, the results from the kidney and spleen samples as well as the liver samples treated with Diflubenzuron were compared with the negative control (non-treated) and described in charts as percentage of the control.

After the run, data was transferred to excel for analysis.

4. Statistics

Statistical testing for the neutrophil function assay data was done using Student's t-test in Excel for independent samples. Results with a *P*-value equal or below 0.05 were considered as statistically significant. For analyzing the gene expression results, the software LinRegPCR was used. For statistical comparison of those results, student t-test was used.

III. RESULTS

1. Neutrophil Function Assays

Neutrophil function was assessed with degranulation of primary granules, oxidative burst, and NETs release, after the exposure to ivermectin, doramectin and diflubenzuron *in vitro*.

1.1. Neutrophil Oxidative Burst Assay

Only the treatment with Diflubenzuron caused a significant increase of oxidative burst compared to non-stimulated controls in the *in vitro* test. The samples stimulated with doramectin and ivermectin didn't show an increase in the oxidative burst activity.

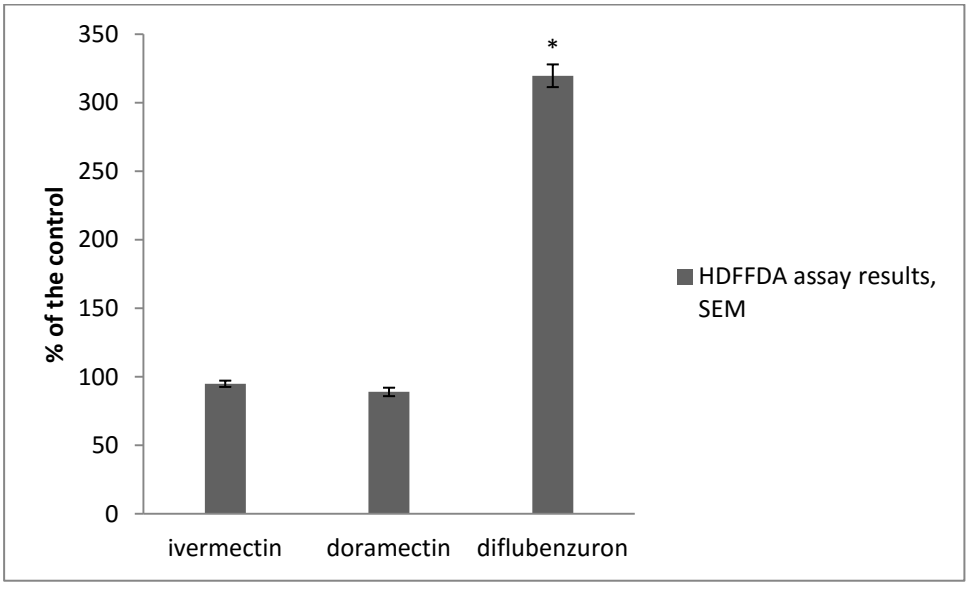


Figure 7: Respiratory burst of neutrophils exposed to ivermectin (concentration of 308 ng/ml), doramectin (concentration of 125 ng/ml), and diflubenzuron (concentration of 200 ng/ml)

1.2. Degranulation of Neutrophil Primary granules

The diagram below shows the averages of the values calculated with the different stimulants used:

None of the tested drugs increased the degranulation of primary granules significantly.

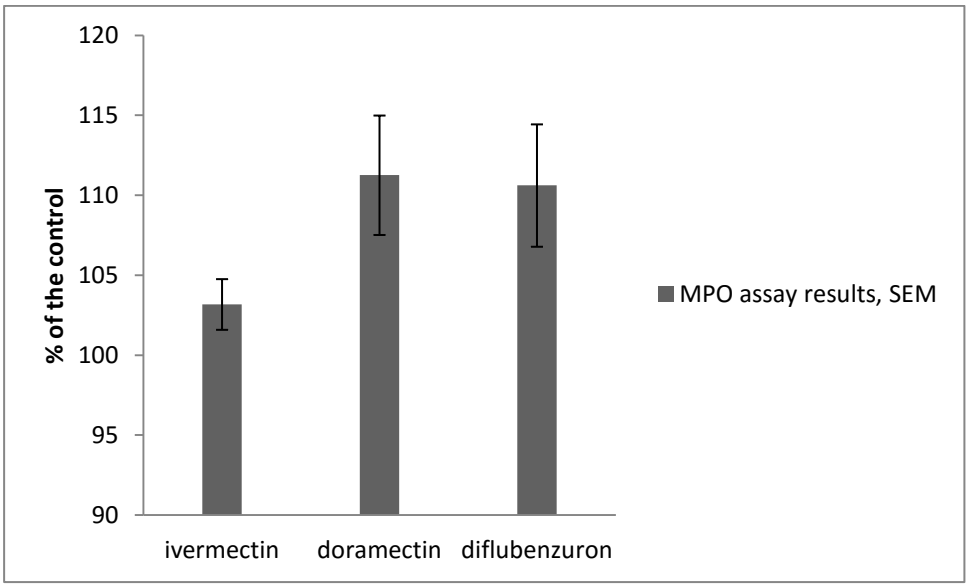


Figure 8: Percentage of degranulation of neutrophils exposed toivermectin (concentration of 308 ng/ml), doramectin (concentration of 125 ng/ml), and diflubenzuron (concentration of 200 ng/ml)

1.3. Neutrophil Extracellular Traps Release Assay

None of the tested drugs showed a significant increase of neutrophil extracellular trap release.

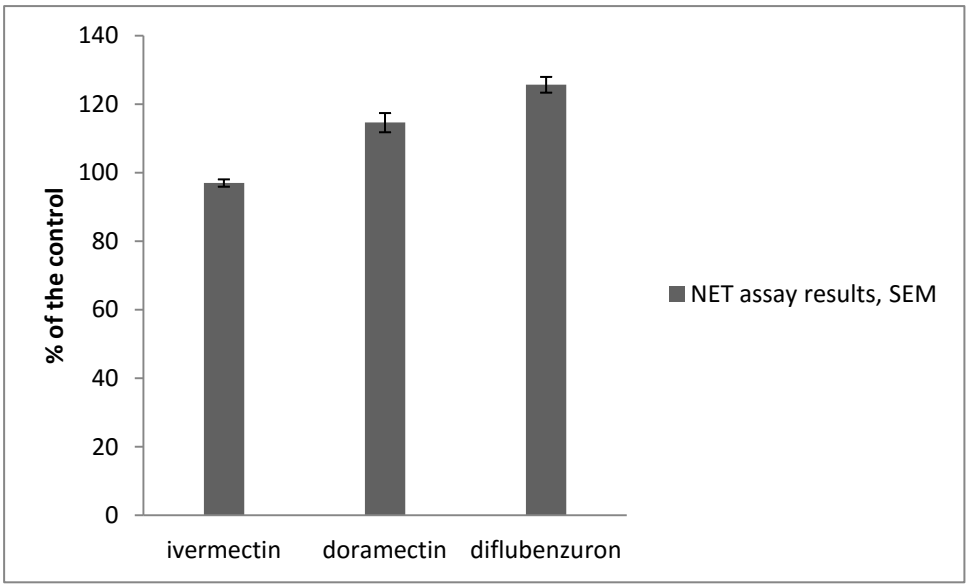


Figure 9: Percentage of neutrophil extracellular nets production of neutrophils exposed to ivermectin (concentration of 308 ng/ml), doramectin (concentration of 125 ng/ml), and diflubenzuron (concentration of 200 ng/ml)

1.4. Gradient Study

The gradient study was performed to confirm the findings in the single studies as well as to show the increase of the stimulation with gradually increased concentrations of the tested compound. The 200 ng/ml is the desired plasma concentration of diflubenzuron treatment, and the used concentrations are 100, 10 times lower, and also 10 times higher than this concentration (2, 20 ng/ml and 2 µg/ml, respectively).

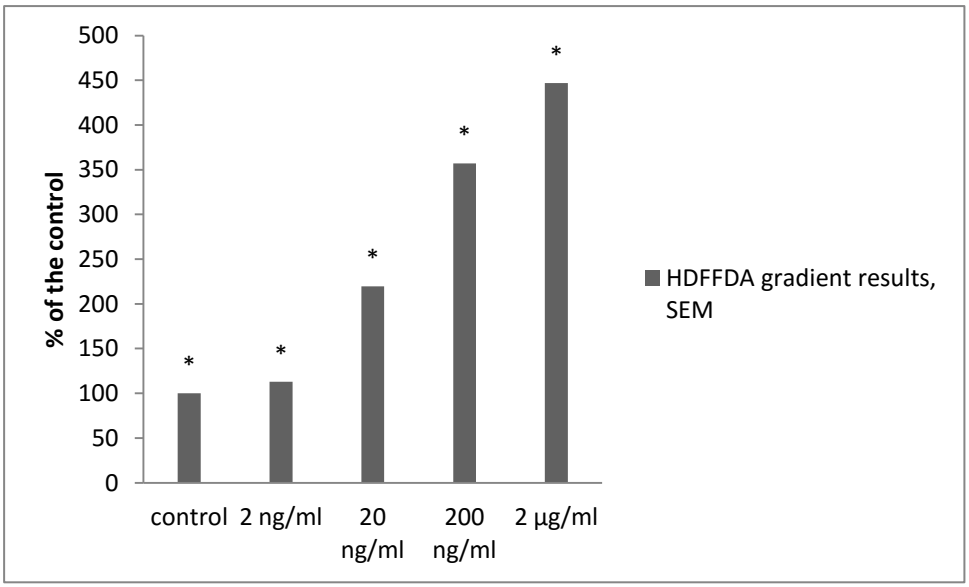


Fig. 10: Respiratory burst of neutrophils exposed to four different concentrations of diflubenzuron: 2 ng/ml, 20 ng/ml, 200 ng/ml, and 2 µg/ml

Respiratory burst activity shows statistical significance in all the tested concentrations

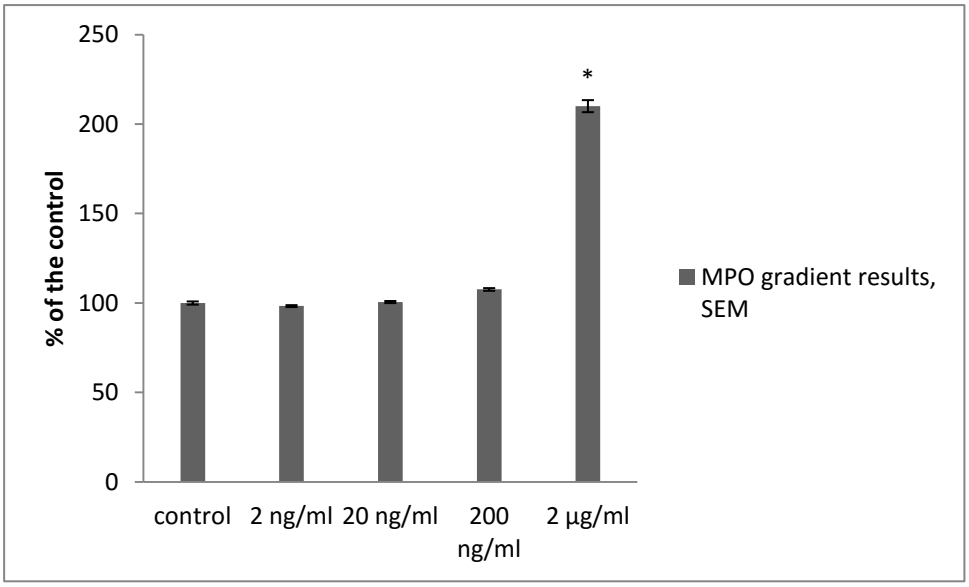


Fig. 11: Degranulation of primary granules of neutrophils exposed to four different concentrations of diflubenzuron: 2 ng/ml, 20 ng/ml, 200 ng/ml, and 2 µg/ml

The2 µg/ml concentrationshows statistical significant increase of degranulation.

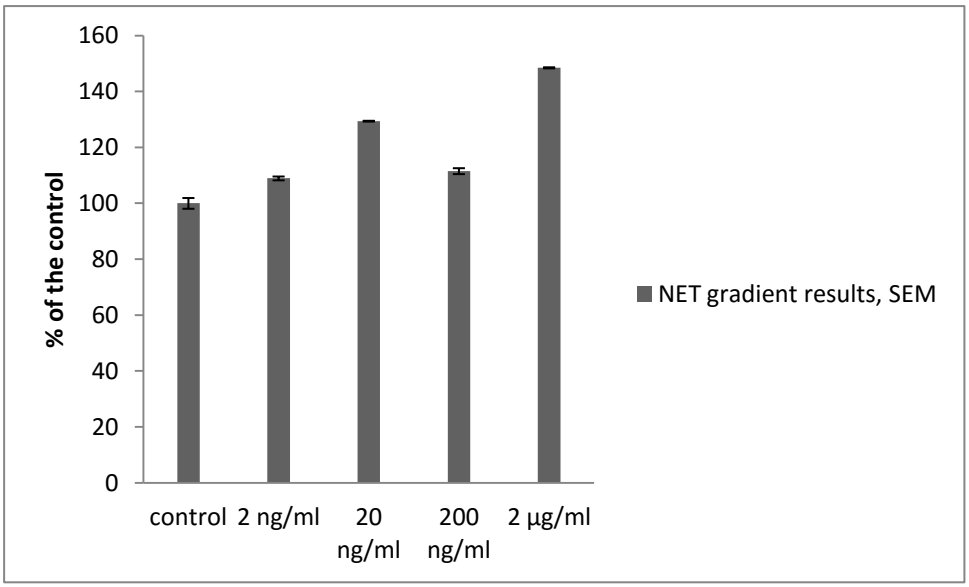


Fig. 12: Neutrophil extracellular traps of neutrophils exposed to four different concentrations of diflubenzuron: 2 ng/ml, 20 ng/ml, 200 ng/ml, and 2 µg/ml

The results do not show any statistical significance in the results of neutrophil extracellular trap release.

2. Results of cell culture

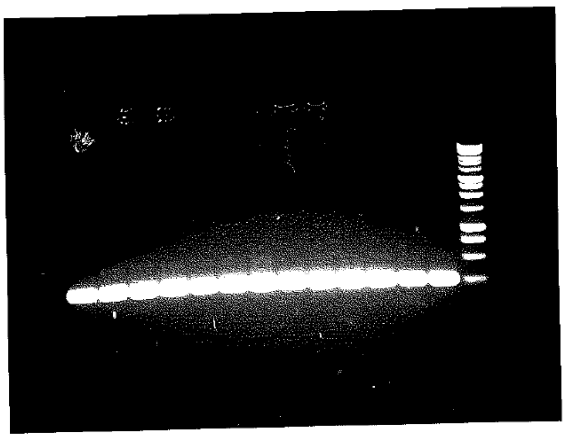
After the incubation of the cells with diflubenzuron in culture for12 hours, the cell layer in the petri dishes did not show any effect. Also, the cell treated with a single dose of diflubenzuron (200 ng/ml), after an incubation of 48 hours had similar surviving rates as the negative control group (between 10 and 40 percent of dead cells). The tenfold concentration caused cells mortality up to 70 percent. The fivefold concentration caused cells mortality of up to 50 %. The double concentration caused cells mortality of up to 20 %. Since the absolute cell number didn't allow such high losses in order to continue with the experiment, it was decided to incubate the cells in culture with the double dose of the expected plasma concentration, to

increase the possibility of detection of changes in gene expression and to follow the expected diflubenzuron metabolite pathway activation in the liver.

3. Results of gene expression analysis

3.1. Gel electrophoresis results

Annealing temperature of 58 °C was determined for all ofthe primers except for Gluta ıperature of 56 °C was determined.



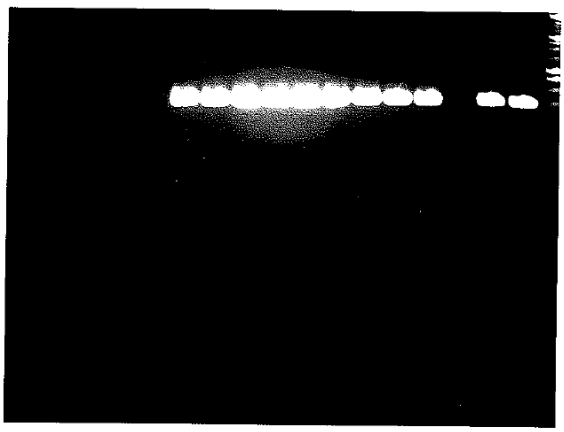
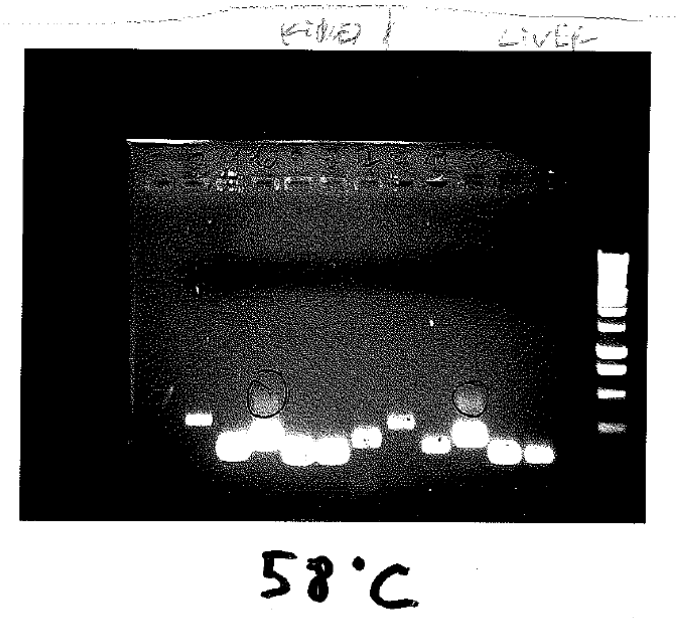
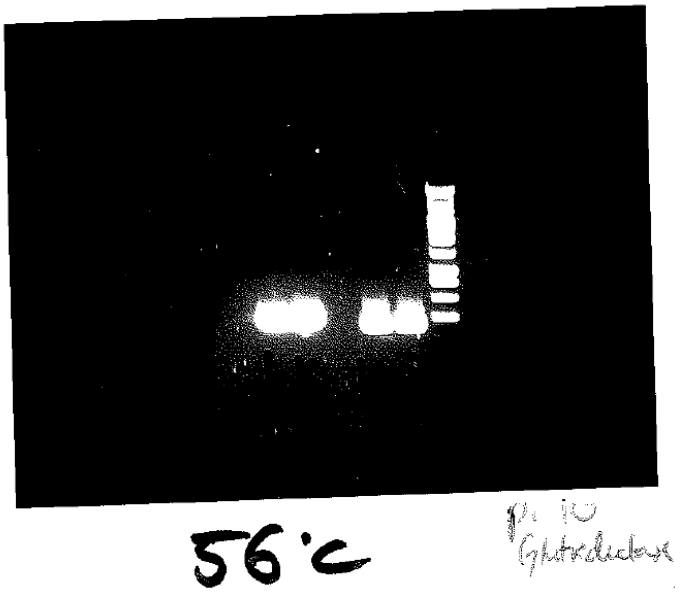


Figure 13: Test for functionality of samp ·iginal, reverse: 5'- CTC ATG TCA CGC ACA ;TC AT -3')

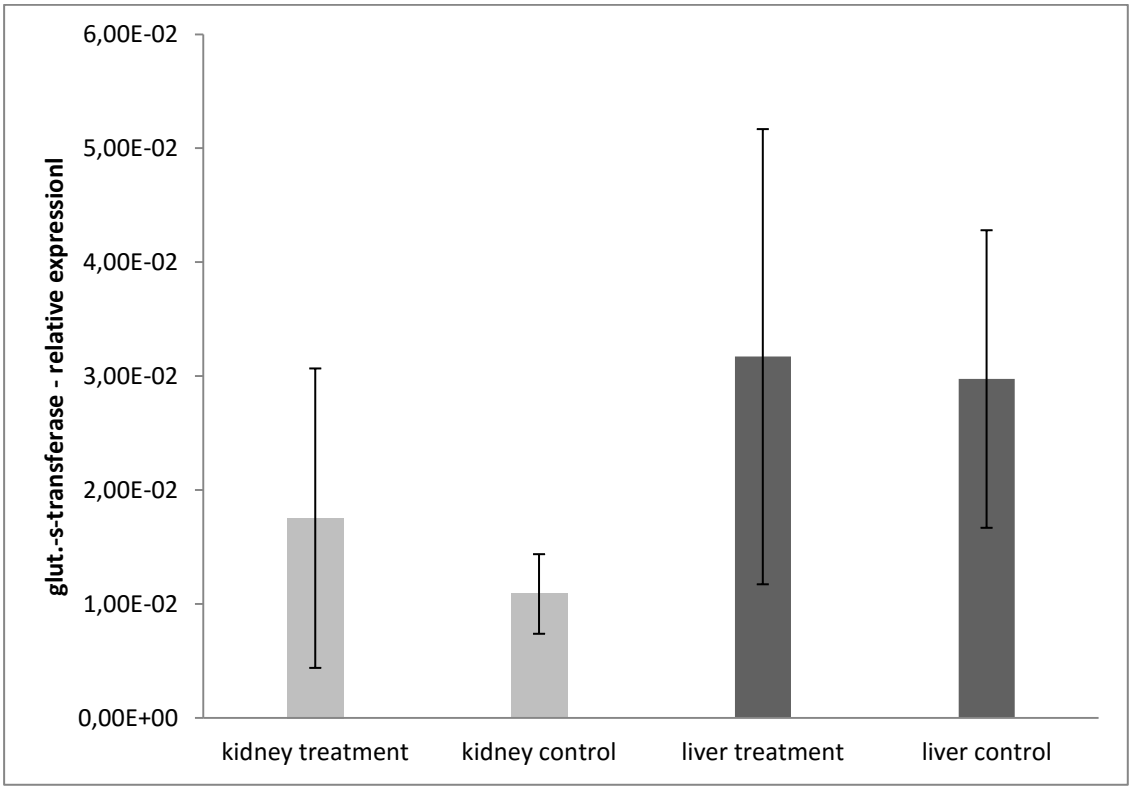
Figure 14: Test for functionality of primers. Primers for the following genes were tested: CYP1A, catalse, glut.-s-transferase, glut. reductase and glut. synthase. Primers for genes of interest were obtained from Integrated DNA Technologies. Sequences are listed in a table in chapter 3.5.





3.2. QPCR results

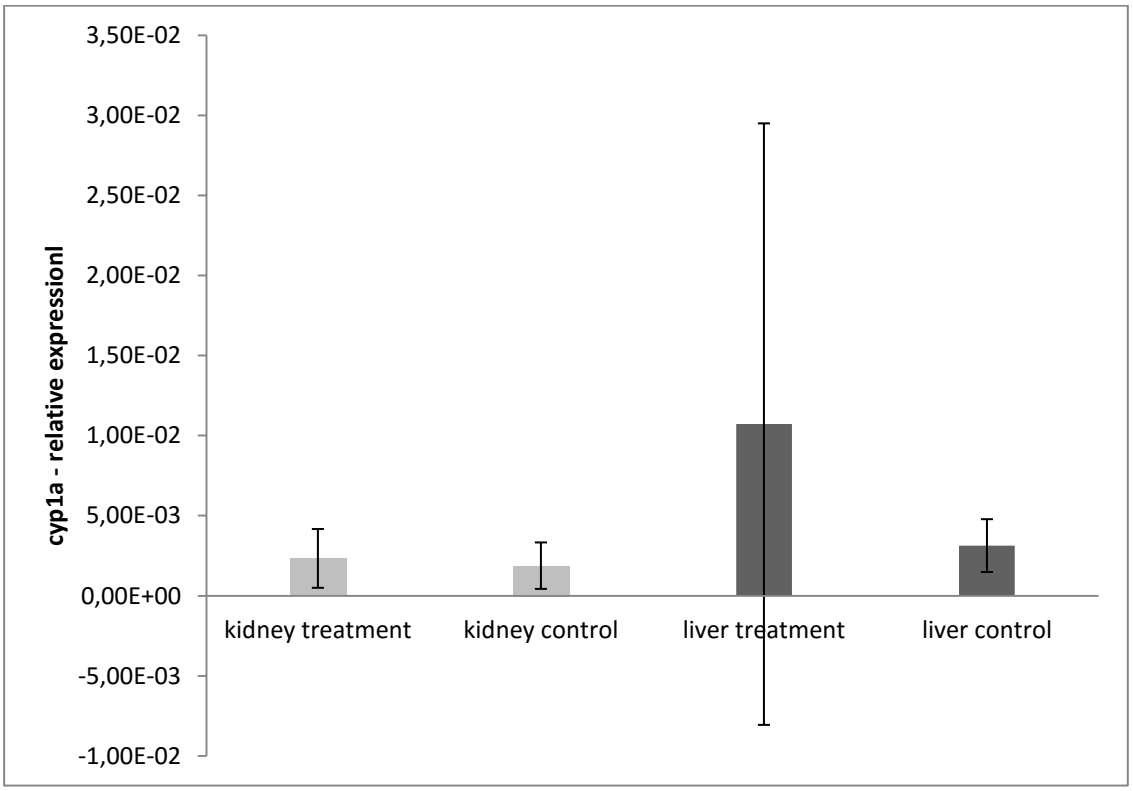
Gene expression analysis revealedthatafter 48 hours of fish cells exposure to 400 ng/ml of diflubenzuron, there was no significant difference in the expression of the genes for glutathione synthase, glutathione reductase, catalase, CYP A1 and glutathione-s-transferase, when the treatments were compared to the control.



	% of control expression
kidney	161
liver	107

Fig. 15:Relative expression of the gene glut.-s-transferase in liver and kidney/spleen tissues after 48 hours exposure to 400 ng/ml of diflubenzuron, diflubenzuron treated tissues are compared with control tissues, SEM

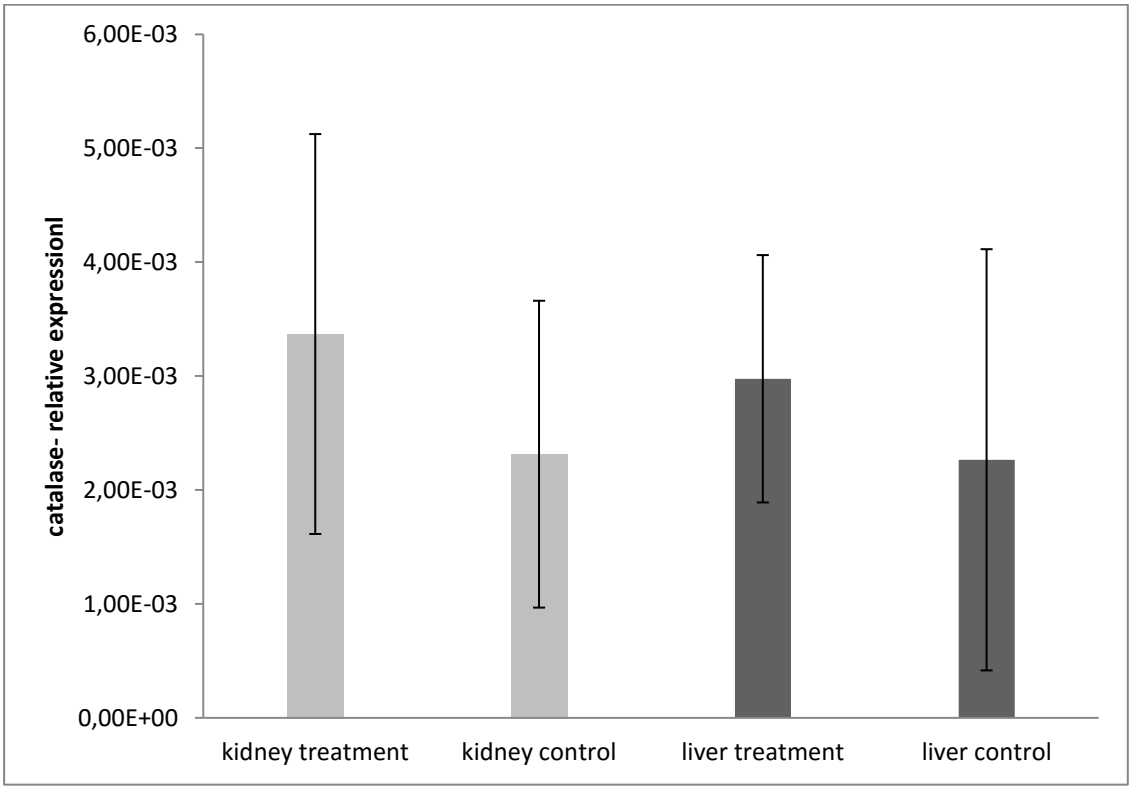
Figure 15shows the relative gene expression of Glutathione-S-transferase. Even though the expression in the kidney tissue of the treated cells is higher than in the non-treated control group, the results don't show statistical significance.



	% of control expression
kidney	124
liver	342

Fig. 16: Relative expression of the gene CYP A1 in liver and kidney/spleen tissues after 48 hours exposure to 400 ng/ml of diflubenzuron, diflubenzuron treated tissues are compared with control tissues, SEM

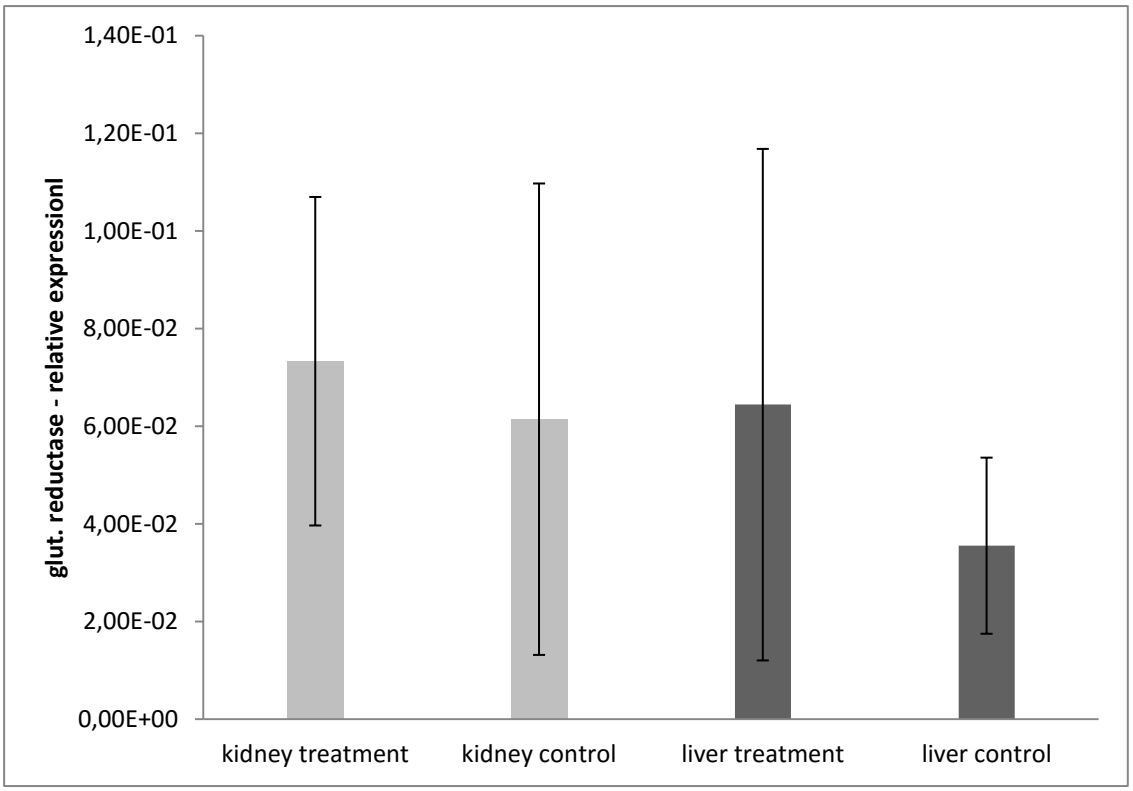
Graph 16shows the relative gene expression of CYP A1. Even though the expression of that gene in the kidney tissue of the treated cells is higher than in the non-treated control group, the results don't show statistical significance.



	% of control expression
kidney	146
liver	131

Fig. 17: Relative expression of the gene Catalase in liver and kidney/spleen tissues after 48 hours exposure to 400 ng/ml of diflubenzuron, diflubenzuron treated tissues are compared with control tissues, SEM

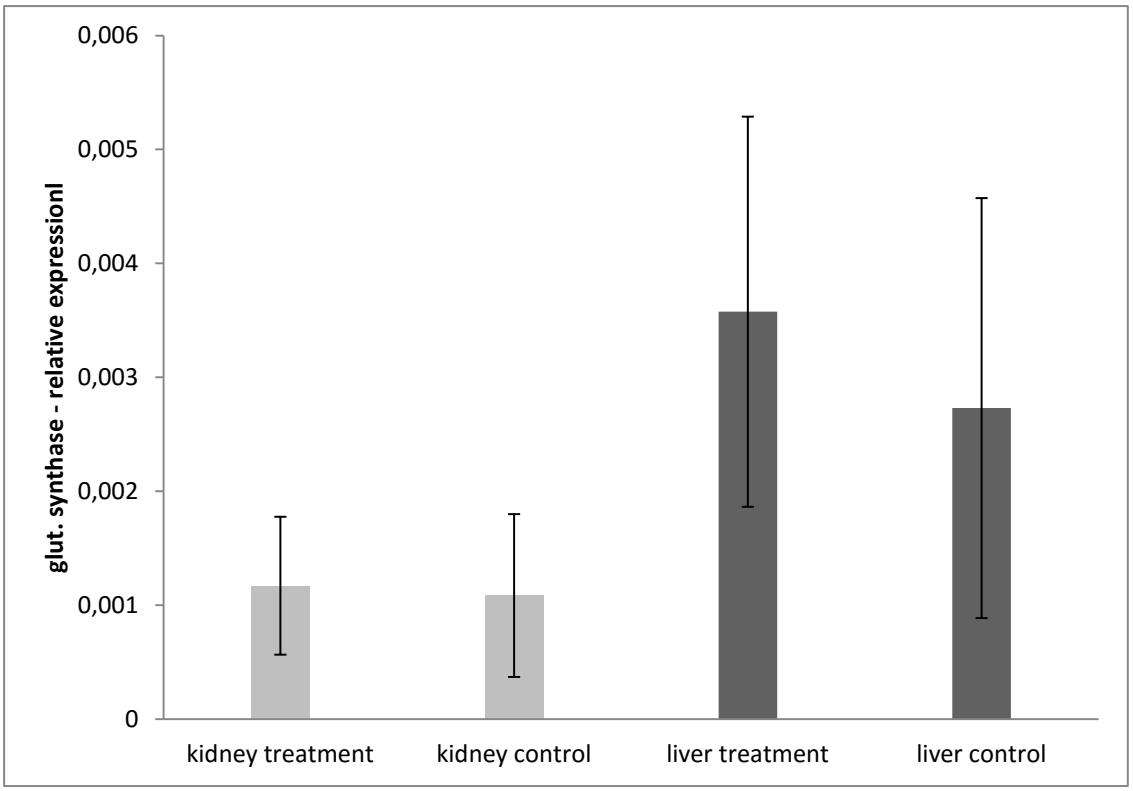
The results of the gene expression of the gene for catalase don't show statistical significance.



	% of control expression
kidney	119
liver	181

Fig. 18: Relative expression of the gene Glutathione reductase in liver and kidney/spleen tissues after 48 hours exposure to 400 ng/ml of diflubenzuron, diflubenzuron treated tissues are compared with control tissues, SEM

Even though the expression of that gene in the kidney tissue as well as liver tissue of the treated cells is higher than in the non-treated control group, the results don't show statistical significance. The results from the liver are nearly significant p-value = 0,065986.



	% of control expression
kidney	108
liver	131

Fig. 19: Relative expression of the gene glut.-synthase in liver and kidney/spleen tissues after 48 hours exposure to 400 ng/ml of diflubenzuron, diflubenzuron treated tissues are compared with control tissues, SEM

Figure 19shows the relative gene expression of Glutathione synthase. Even though the expression of that gene in the kidney tissue as well as liver tissue of the treated cells is higher than in the non-treated control group, the results don't show statistical significance.

IV. DISCUSSION

The results of the neutrophil assays have shown that the tested drugs ivermectin, doramectin and diflubenzuron stimulate an increase in neutrophil activity. This was investigated measuring the activity of oxidative burst, degranulation of primary granules and NET production. A concentration of 200 ng/ml of diflubenzuron significantly increased the fish neutrophil respiratory burst. This concentration is exactly the concentration that is expected to be found in the fish plasma when the fish is treated with a standard dose of in feed diflubenzuron. The accomplished results especially concerning the oxidative burst activity show statistical significance. The oxidative burstas well as the release of myeloperoxidase and the production of NETs showed an increase in neutrophil activity when challenged with diflubenzuron. Although the mechanisms of antiparasitic drug influence on neutrophils is not fully explained, the results indicates that the innate immune system is triggered and tries to protect the organism against the compound diflubenzuron. For the other two tested drugs, ivermectin and doramectin, no significant increase in activity could be measured. Several possible explanations may be responsible for these results. Those findings clearly show that ivermectin and doramectin don't provoke an increased activation of oxidative burst, degranulation of myeloperoxidase and production of NETs, when administered in a dose that leads to a plasma concentration which is 308 ng/ml for ivermectin and 125 ng/ml for doramectin.

On the one hand, itcan be seen as a proof that Ivermectin and Doramectin don'tinterfere with the organismal defenses. When the innate immune system doesn't classify a substance as harmful, it does not react with defensive measures(Segal, 2005).Hemaprasanth et al. (2008) observed in their study that doramectin controlled experimentally induced copepod (*Lerneacyprinacea*) infection in carpexcellent and without any noticeable adverse reactions or

toxicity to the fish host(Hemaprasanth et al., 2008).Athanassopoulou et al. (2002) studied the effect of in-feed treatment of ivermectin on sea bass (*Dicentrarchuslabrax*) and no signs of toxicity were observed (Athanassopoulou et al., 2002). Those findings support the first assumption, that doramectin and ivermectindo not provoke adverse effects in the fish host.

On the other hand, additional indicators of the immune function which have not been the focus of this study could be altered in their function and lead to different conclusions. Since ourexperiments have been the first to investigate the effect of those compounds on the innate immune system of fish, no comparative studies are available. Further study would include gradient studies with ivermectin as well as doramectin, to confirm the findings and to securethederivedinsights. It would be a possibility that higher concentrations would trigger a response in neutrophil function (oxidative burst, NETs release, degranulation of primary granules).

The results of the gradient study with the third tested compound diflubenzuron clearly indicate a positive correlation between the dose of the drug and the reaction of the immune system. It has been observed, that a higher dose of diflubenzuron leads to a higher neutrophil stress response. These findings indicate that diflubenzuron is less suitable for an antiparasitic treatment compared to the other tested compounds (e.g. ivermectin, doramectin). In other studies, diflubenzuron showed a good tolerance and no adverse effect on the fish were observed (Bouboulis et al., 2004). However, this study was accomplished using sea bass, so there might be species specific differences. The Bouboulis study didn't concentrate on the immune system, so such effects may have been overlooked.

Diflubenzuron was originally developed to replace the previous insecticides on phosphorester-, carbamate and hydrochlorofluorocarbon base through non-toxic and environmentally friendly compounds (Dieter, 1977). The data collected by this study suggest,however, that thedrughasan adverseeffectonthe treatedfish. Olsvik et al. (2013)

observed, when treating Atlantic cod with diflubenzuron, that there are small changes in gene transcription on the expression of genes involved in detoxification pathways. Five gene transcriptions (cyp3a, cpt1a, ptgs2, elovl5 and mapk1) responded significantly to diflubenzuron exposure in the dose-response study with increasing concentrations (3, 10 and 50 mg/kg fish). The Olsvik' data suggest that accumulated diflubenzuron at the levels studied would have no statistical relevant effect on wild Atlantic cod(Olsvik et al., 2013), since Olsvik et al. didn't discover distinct patterns in gene transcription in Atlantic cod liver in their time-series experiment (in-feed treatment at a rate of 3 mg/kg for 14 days). The observed statistical significant changes in gene expression are due to intravenous injection of an overdose of diflubenzuron. Thus, corresponding to the Olsvik study, the presented data of our study concerning the neutrophil activitysuggests effects on fish indeed, even when just observed when administered an overdose and lead to the conclusion, that diflubenzuron is not the most beneficial compound for treatment fish against parasites.

This study focused on three antiparasitics which were chosen for their estimated high efficacy while being assumed to have a low toxicity. Even though they pledged to be the most promising candidates for a successful antiparasitic treatment, other pharmaceuticals could have been valuable to be included in this study but had to be excluded due to limited study possibilities. Another limitationof this study is the initial assumption concerning the plasma concentration of ivermectin, doramectin and diflubenzuron in a cyprinid fish. It is difficult to establish a plasma concentration after drug administration which is due to the limited knowledge concerning those drugs and their pharmacokinetics in aquatic organisms would be only a guess, based on several publications, as explained in the paragraph below.

Little is known about the pharmacokinetics of ivermectin, doramectin and diflubenzuron in different fish species. This is the reason why a comparative study of the pharmacokinetics in different species was undertaken, to get indications, how those drugsrise in level and accumulate in the different tissues.Toutain et al. (1997) showed that the plasma concentration

of ivermectin and doramectin in cattle after subcutaneous injection with 200 µg/kg was 32 ng/ml (Toutain et al., 1997). Pérez et al. (2002) showed in a study about pharmacokinetics in horses that after oral administration of 0.2 mg/kg of ivermectin and doramectin, plasma concentration was up to 173 ng/ml (ivermectin) and 232 ng/ml (doramectin) (Pérez et al., 2002).Katharios et al. (2003) found that plasma concentration in sea bream was 308.4 ng/ml after injection with ivermectin of 100 µg/kg body weight (Katharios et al., 2002).No studies have to date been published on plasma concentration of doramectin in cyprinid fish.Concerning diflubenzuron, the assumption on the possible plasma level has been made due to the findings of Erdal(2012). He observeda concentration in filet and skin of 36.1 and 106 ng/g after an in feed treatment with diflubenzuron in a dose of 3 mg/kg once daily for 14 days(Erdal, 2012).

The results in the expression of the genes for glutathione reductase, glutathionesynthase, catalase, glutathione-s-transferase, catalase andCYP A1 after 48 hours exposure to diflubenzuron showed no significant difference in the expression compared to the control.

Catalase plays an important role in the detoxification of oxygen-derived free radicals. Immune related increase of expression in catalase can be seen as an evidence of increased immune system activity, since the production of reactive oxygen species is one of the defenses of the immune system, as discussed above. Since those ROS do not only pose a threat to invading pathogens but to host tissues as well, the body produces antioxidant enzymes for its protection(Dorval and Hontela, 2003). The three most important antioxidant enzymes are the superoxide dismutase (SOD) that converts O_2 into H_2O_2which is detoxified into H_2O and O_2 by either catalase or peroxidases(Bachowski et al., 1998, Sun, 1990).In fish, a transition of antioxidant systems in liver by endosulfan, and the modulatory effect of preexposure to copper on the endosulfan-induced oxidative stress, have been reported by Pandey et al. (2001)(Pandey et al., 2001). The present results can be interpreted as indication that catalase production is not affected by treatment with diflubenzuron in a concentration up to 400 ng/ml.

The findings of this study didn't show a statistical significant increase in the gene expression of the gene CYP A1 (Cytochrome P 450) activity from the cells incubated with diflubenzuron compared to the control. Cytochrome P450s (CYP) include families from CYP1 to CYP 4, which are monoxygenases expressed at high levels in the liver and mainly involved in the oxidative metabolism of xenobiotics, including drugs, environmental pollutants, diet contaminants, fungal, and plant toxins. These enzymes play an important role in detoxification (Gonzalez, 1990).

Glutathione is ubiquitous in living cells. It has several important functions, including protection against oxidative stress. It is synthesized from its constituent amino acids by the consecutive actions of γ-glutamyl-cysteine synthetase and glutathionesynthetase(Anderson, 1998). The genes involved in the glutathione pathway (glutathione synthetase, glutathione-s-tranferase, glutathione reductase) didn't present statistical relevant up- or downregulation. Glutathione*S*-transferases (GSTs), include a family of eukaryotic and prokaryotic phase II metabolic isozymeswhich have the capability to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobioticsubstratesto effect detoxification (Hayes and Pulford, 1995).Glutathione reductase reduces oxidized glutathione (GSSG) to glutathione (GSH), a substrate for glutathione peroxidase. The consequences of oxidative stress are serious and, in many cases, are manifested by increased activity of enzymes involved in oxygen detoxification(Kimball et al., 1976).The interpretation of our collected results, which have been presented in the paragraph above, could be seen as indication that diflubenzuron does not trigger the tested genes to be upregulated or downregulated neither in the liver nor in kidney or spleen.However, the indicators of trends in increasing anti-oxidative metabolic pathways of liver enzymes suggest the possibility that potential higher doses could activate detoxifying mechanisms in significant amount.

The findings presented in this dissertation help to outline the effect of the tested compounds on the immune system, since there are no reports available on the effect of

ivermectin, doramectin and diflubenzuron on the immune system of cyprinid fish. According to the collected findings, it can be assumed thativermectin and doramectin will not negatively interfere with the ability of the immune system.

It has to be taken into account that the immune system of fish is adversely affected by low water temperatures (Le Morvan et al., 1998, Rijkers et al., 1980).In addition, a study on host immunity confirmed the immunosuppressive role of 11-ketotestosterone on fish immunity measured by complement activity (Rohlenová et al., 2011) which means that during the sexual activity of the fish, the immune system is less effective as normally. Therefore, it is especially important that drugs do not pose an additional stressor to theimmune system which has been shown by this study.

Conclusions and suggestions for future research

This thesis hopes to support the persistence of carp aquaculture ponds, since they contribute greatly to the cultural landscape and offer habitat for a variety of animals. Furthermore, it is important to help local production in order to reduce dependence on imports. Concerning this aim: What accomplished this study in terms of practicality?

For the practical application of the results observed it can be said, that it is relatively safe to use doramectin and ivermectin in fish as an antiparasitic treatment. These findings are in accordance with the results of Braun et al. (2008) who already tested drugs on carp, which are known to be frequently used in salmon aquaculture (emamectin benzoate and teflubenzuron), which were found to be effective and well tolerated (Braun et al., 2008).

The hypothesis was: Antiparasitic drugs (ivermectin, doramectin, diflubenzuron) will not cause significant side effect in fish when applied in therapeutic doses. Therefore this study

was investigating possible side effects of antiparasitic drugs on the innate immune system of cyprinid fish, followed by the *in vitro* study of cell culture of fathead minnow and the possible enhancement in gene expression through the administration of Diflubenzuron. Through the used methods and findings, this hypothesis could be proved true for ivermectin and doramectin, but false for diflubenzuron, since diflubenzuron showed significant increase in the oxidative burst activity. This hypothesis could be proven true as well for the gene expression studies, since no significant effect of diflubenzuron on the gene expression could be proven.

Concerning the problem of developing of resistances towards several active compounds, it can be seen as the ultimate problem. Even though through the findings gained in this study, it can be confirmed, that ivermectin and doramectin are from our point of view, a reasonable choice when treating parasites, one must be aware of the fact that every treatment can increase the possibility of generating resistant parasites.

Further research is also required on the parasite *Argulus spp.* to see if the dose is efficient in killing the parasite. However, the work previously done by Braun et al. (2008) was incomplete because the efficacy study was conducted only at 20.6 ° C and tolerability study only 14.9 ° C. In fish as poikilothermic, so cold-blooded animals, the ambient temperature is the decisive factor for the metabolism. So it is likely that at higher temperatures, as might be expected in the German summer in the earthen ponds of carp hosts, toxic effects of the drugs which are compatible with around 20 ° C, may occur quite possible at for example 30 °C.

The following conclusions can be drawn from the presented findings: *In vitro* studies on the innate immune system as the here presented neutrophil studies can give reliable first answers to questions concerning *in vivo* diseases and treatments. With studies on the gene expression, far-reaching and profound negative effects can be ruled out, when such investigations remain without significant results, as shown here.

V. SUMMARY

Keywords: Diflubenzuron, Ivermectin, Doramectin, Neutrophils, Immune Response, Fish

Parasitic diseases in European aquaculture continue to pose economic and ecological threats to farmed and wild fish populations. The primary infection with ectoparasites can open the door to secondary or super- infections caused by bacterial and viral pathogens. Specifically, the infection with *Argulus foliaceus* (fish louse) in freshwater cyprinid fish such as common carp (*Cyprinuscarpio*) can increase production losses in affected aquaculture operations, or stocked water bodies. However, current veterinary drug use regulations in Germany limit the range of medications approved as treatment against ectoparasites in freshwater fish. A comparable parasite *Lepeoptheirussalmonis* (salmon louse) treatment with Diflubenzuron, Ivermectin and Doramectin (pesticides), has been approved in Salmon aquaculture in various countries. In order to control parasite populations, fish farmers frequently reach for non-approved but available treatments without veterinary supervision, possibly leading to consequences such as environmental damage, emergence of drug resistance or unwanted side effects on cultured and wild fish. The focus of this study is to investigate possible side effects of Diflubenzuron, Ivermectin, and Doramectin treatments on the innate immune system of a cyprinide fish.

The overall research objective is to determine which antiparasitic drug and in what concentration and dose would present an effective treatment of the *A. foliaceus* without causing measurable side effects on fish neutrophil function. The first specific aim was to determine *in vitro* effects of commercial formulations of diflubenzuron, ivermectin and doramectin on fathead minnow (*Pimephales promelas*) neutrophil function. Treatment effects *in vitro* on oxidative burst, degranulation, and neutrophil extracellular trap (NETs) release

were studied. Application of ivermectin and doramectin (in estimated plasma concentrations of 308 and 125 ng/ml respectively) caused no significant stimulation of oxidative burst, degranulation of primary granules and NETs release. However, application of diflubenzuron (in estimated plasma concentrations of 200 ng/ml) caused a significant stimulation of oxidative burst. Diflubenzuron treated neutrophils showed up to three time's higher activity than the non-treated control. The second specific aim of the study was establishing a cell culture protocol of fathead minnow liver, spleen and kidney cells. In order to examine whether an incubation of the cells in culture of 48 hours with Diflubenzuron would alter the RNA expression in these organs, no significant change in expression of the tested genes (glutathione reductase, glutathione synthase, catalase, glutathione-s-transferase, catalase and CYP A1) compared to a control could be proven.

The observed effect indicates that the tested antiparasitic compounds have the potential to interfere with disease resistance in fish populations by modulating immune responses during treatment. Therefore, further study is required to find optimal therapeutic dose for effective and safe treatment against ectoparasites in cyprinide fish.

The following conclusions can be drawn from the presented findings: *In vitro* studies on the innate immune system as the here presented neutrophil studies can give reliable first answers to questions concerning *in vivo* diseases and treatments. With studies on the gene expression, far-reaching and profound negative effects can be ruled out, when such investigations remain without significant results, as shown here.

VI. ZUSAMMENFASSUNG

Stichwörter: Diflubenzuron, Ivermectin, Doramectin, Neutrophile, Immunantwort, Fisch

Parasitäre Krankheiten stellen in der europäischen Aquakultur weiterhin wirtschaftliche und ökologische Bedrohungen für Zucht- und Wildfischpopulationen dar. Eine primäre Infektion mit Ektoparasiten kann die Tür zu sekundären Infektionen oder Superinfektionen durch bakterielle und virale Erreger öffnen. Insbesondere kann die Infektion mit der Karpfenlaus (*Argulus foliaceus*) im Süßwasser bei Fischen wie den Karpfen (*Cyprinuscarpio*) zu erheblichen Produktionsverlusten in den betroffenen Aquakulturbetriebenführen.

Doch die aktuellenBestimmungen über die Zulassung von Arzneimitteln in Deutschland begrenzen die Auswahl an Medikamenten, die als Behandlung gegen Ektoparasiten bei lebensmittelliefernden Süßwasserfischen zugelassen sind. Ein vergleichbarer Parasit*Lepeoptheirussalmonis* (Lachslaus) wirdin der Lachszuchtindustrie in verschiedenen Ländern mit Diflubenzuron, Ivermectin und Doramectin behandelt. Um Parasitenpopulationen zu kontrollieren, greifen Fischzüchter häufig zu nicht zugelassenen, aber erhältlichen Arzneimitteln. DieseBehandlungen ohne tierärztliche Aufsichtkönnen unter Umständen zu Folgen wie Umweltschäden, Entstehung von Resistenzen oder sonstigeunerwünschte Nebenwirkungen bei kultivierten und wilden Fischen führen.

Der Schwerpunkt dieser Studie ist es, mögliche Nebenwirkungen von Diflubenzuron, Ivermectin und Doramectin Behandlungen auf das angeborene Immunsystem eines cypriniden Fischs zu untersuchen. DasForschungsziel ist es, festzustellen, welche antiparasitären Arzneimittel in welcher Dosis eine wirksame Behandlung von *Argulus foliaceus*gewährleisten,

ohne dass sich messbare Nebenwirkungen auf dieNeutrophilenfunktion derFische präsentieren.

Das erste spezifische Ziel war es, die *in vitro* Wirkung von kommerziellen Produkten mit den WirkstoffenDiflubenzuron, Ivermectinund Doramectin auf die Neutrophilenfunktion von Dickkopfelritzen (Pimephales promelas) bestimmen. Die Auswirkungen auf Indikatoren des angeborenen Immunsystems: oxidativen Burst, Degranulation und die Aktivierung der Neutrophilen Extrazellulären Traps (NETs) wurden untersucht.

Die Stimulation der neutrophilen Granulozyten mit Ivermectin und Doramectin (in Plasmakonzentrationen von 308 und 125 ng / ml) verursacht keine signifikante Stimulation des oxidativen Bursts, der Degranulation von primären Granula und NETs Ausstülpungen. Jedoch die Stimulation mit Diflubenzuron (in einer Plasmakonzentration von 200 ng / ml) führte zu einer signifikanten Stimulation des oxidativen Bursts. Mit Diflubenzuron behandelte neutrophile Granulozyten zeigten eine bis zu dreimal höhere Aktivität als die der nicht behandelten Kontrolle.

Das zweite spezifische Ziel der Studie war eine Etablierung eines Zellkulturprotokolls aus Leber, Milz und Nierenzellen der Dickkopfelritze. Diese wurden benötigt, um zu untersuchen, ob eine Inkubation der Zellen mit Diflubenzuronin der Kultur für 48 h die RNA-Expression in diesen Organen verändert. Es konnten keine signifikante Veränderung der Expression der untersuchten Gene nachgewiesen werden.

Die folgenden Schlussfolgerungen können aus den vorgestellten Ergebnissen gezogen werden: *In vitro*-Untersuchungen von Mechanismen des angeborenen Immunsystems, wie die hier vorgestellten Untersuchungen der Funktionsweise der neutrophilen Granulozyten geben zuverlässige erste Antworten auf Fragen bezüglich *in vivo* Erkrankungen und Behandlungen. Mit Studien zur Genexpression können weitreichende und tiefgreifende negative

Auswirkungen ausgeschlossen werden, sofern diese Untersuchungen ohne nennenswerte Ergebnisse bleiben, so wie hier gezeigt wurde.

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VIII. APPENDIX

1. References pictures of chemical structural formula

1. Fig. 3: http://medications.li/static/5470944a86b8e03c58f421b504482e07.gif
2. Fig. 4: http://www.medchemexpress.com/Doramectin.html
3. Fig.5: http://files.meistermedia.net/cpd/images/structures/largeview/diflubenzuron.gif
4. Fig 6: http://www.sinoharvest.com/pic/products/Emamectin-b1a.gif

2. Tables

2.1. Entwicklung der Kosten-Erlössituation in der sächsischen Karpfenteichwirtschaft im Zeitraum 1996 bis 2010 (aus FÜLLNER et al. 2011)

	ME	1996-1999	1999-2002	2007-2010
Abfischung	kg/ha	641	623	407
ErlöseausFischverkauf	€/ha	1459	1396	1327
SonstigeErlöse	€/ha	624	645	469
Direktkosten	€/ha	684	721	292
Summeallerkosten	€/ha	1952	2047	2186
Ergebnis ohne Lohn/Zins/Pacht	€/ha	505	4070	87
Betriebszweigergebnis	€/ha	131	-6	-390

3. Data releases

The data on the part of the neutrophil assays was presented in form of a talk at the DVG convention in Stralsund, 01.07.2015, and in form of a poster (attached below) at the convention Deutsche Fischereitage in Rostock, 25.08.20015 – 27.08.2015.

4. Poster

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Possible Side Effects of Antiparasitic Treatment of Argulosis in Common Carp (*Cyprinus carpio*) with Therapeutics Currently approved for Salmon Aquaculture

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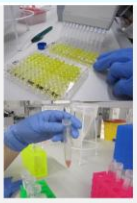
1. INTRODUCTION

Parasitic diseases in European aquaculture continue to be an economic and ecologic threat to farmed and wild fish populations.

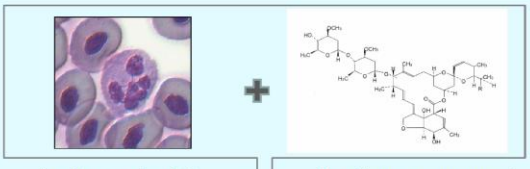
The primary infection with ectoparasites can open the door to secondary or super-infection with bacterial and viral pathogens. Specifically, the infection with Argulus foliaceus (fish louse) of common carp (Cyprinus carpio) can increase production losses in affected carp farms. However, current veterinary drug use regulations in Germany limit the range of medications approved as treatment against ectoparasites in freshwater fish. In order to prioritize some drugs compared to others, the focus of this study is to investigate possible side effects of Diflubenzuron, Ivermectin and Doramectin treatments on the innate immune system of a cyprinide fish. These results could serve as a base for recommendations to veterinarians as well as fish farmers. The choice of tested drugs was founded by the usage of treatments against a comparable parasite Lepeoptheirus salmonis (salmon louse) with Diflubenzuron, Ivermectin and Doramectin (pesticides) in various countries.

2. AIM OF THE STUDY

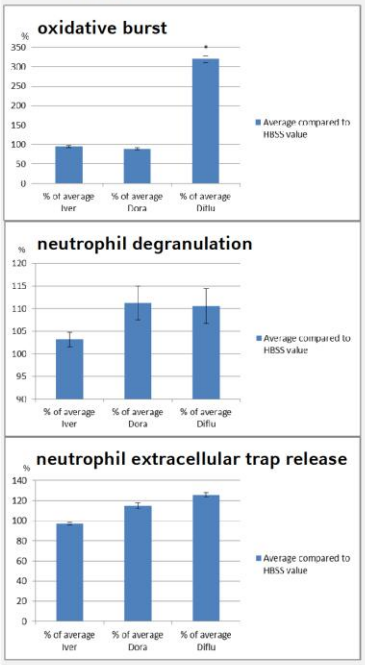
The overall research objective is to determine which antiparasitic drug and in what concentration/dose would present an effective treatment of the A. foliaceus without causing measurable side effects on fish neutrophil function. The first specific aim (presented here) is to determine in vitro effects of commercial formulations of Diflubenzuron (e.g. Aradol, JBL), Ivermectin and Doramectin on fathead minnow (Pimephales promelas) neutrophil function.



3. MATERIALS AND METHODS



4. RESULTS



Treatment effects in vitro on oxidative burst, degranulation, and neutrophil extracellular trap (NETs) release were studied.

Application of Diflubenzuron, Ivermectin and Doramectin (in an estimated plasma concentrations of 200, 308, and 125 ng mL-1, respectively) caused a significant stimulation of oxidative burst (marked in the graphs with *), and a noticeable stimulation of degranulation of primary granules and NETs release.

Diflubenzuron treated neutrophils showed up to three times higher activity then non-treated control. Observed effect indicates the potential of the above antiparasitic compounds to interfere with disease resistance in fish populations by modulating immune responses during treatment.

5. DISCUSSION

Other studies showed as well that the innate immune system is very fragile and reacts sensible to different impacts like different kinds of stress (handling, crowding, changement in pH or temperature) and toxic substances. So when actually treating fish, it is important to take into account that some active ingredients will stress the immune system more than others so one can chose more educated the more results we gain about those interactions. The study is limited to in-vitro findings, it would be interesting to confirm them in-vivo. Therefore, further studies are required to find optimal therapeutic doses for an effective and safe treatment against ectoparasites in cyprinide fishes.

6. CONCLUSION

Our major results show that the used active compounds do in fact influence the neutrophil behavior. Since the neutrophil granulocytes are a important part of the innate immune response, changes in their behavior allow us to draw the conclusion that this in-vitro findings will be reflected in an in-vivo model as well. So it is an acceptable statement to reason that the compounds Ivermectin, Doramectin and Diflubenzuron in particular manipulate the immune response of fish while being treated and so attenuate the ability of fish immune system to react appropriate when challenged with pathogens like bakteria, viruses and funghi.

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