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Fulvic acid as water additive in aquaculture – A new way to deliver an immunostimulant to juvenile fish and fish larvae

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von
Diplom Biologe Thora Lieke

Präsidentin der Humboldt-Universität zu Berlin

Prof. Dr.-Ing. Dr. Sabine Kunst

Dekan der Lebenswissenschaftlichen Fakultät
der Humboldt-Universität zu Berlin

Prof. Dr. Dr. Christian Ulrichs

Gutachter/innen

1. Prof. Werner Kloas
2. Prof. Thomas Braunbeck
3. Prof. Helmut Segner

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Content

Summary

Aquaculture has become imperative to cover the dietary protein demands, and more than 53 % of the total fish production is already provided by aquaculture (FAO 2020a). However, inevitable handling, capture, transport, and confinement impose stress on the fish, which subsequently reduces the growth performance and the immune response, resulting in yield losses and leaving fish vulnerable to infections. Many therapeutics historically used to treat fish diseases have been banned inside the EU because of environmental and human health concerns which raised a therapy crisis (EG1272 2008, Andersen *et al.* 2009). The following search for alternative environmentally-friendly approaches has led to a plethora of studies on feed additives, including herbal extracts, vitamins, as well as pre- and probiotics (as reviewed by Lieke *et al.* (2019)). One such class of additives that has been studied are humic substances (HS). HS arise from the degradation of biomolecules and are divided based on their solubility into humus (insoluble), humic acids (insoluble at acid pH), and fulvic acids (soluble at any pH) (Stevenson 1982). They have recently gained attention as feed additives in terrestrial and aquatic animals. Increased vitality traits, such as growth and immunity, and reduced diseases were reported in poultry, rats, and mice (Yasar *et al.* 2002, Islam *et al.* 2005, Vetvicka *et al.* 2010). Supplementing fish feed with HS increased the growth performance and the immune response, and decreased the development of skin lesions and severity of infections (Nakagawa *et al.* 2009, Gao *et al.* 2017, Yamin *et al.* 2017c). However, there is another possible application route for fish than the feed: the waterbody. Supplementing the water with HS reflects the exposure occurring in nature, as up to 95 % of the dissolved organic matter in aquatic ecosystems are HS (Thurman 1985, Haitzer *et al.* 1998, Steinberg 2003b). Another benefit of this route of exposure is that pre-feeding larvae, which show exceptional high mortality (Sifa and Mathias 1987, Vadstein *et al.* 2013), can be conditioned as well.

Due to different raw materials and degradation pathways, HS are a chemically very heterogenic class of substances (Steelink and Tollin 1962, Meinelt *et al.* 2007, Stern *et al.* 2018, Savy *et al.* 2020). As a consequence, in addition to the beneficial effects, hazardous effects such as damage by oxidative stress (Chen *et al.* 2002a, Hseu *et al.* 2008, Saebelfeld *et al.* 2017) and inflammation-related diseases (Chen *et al.* 2002a, Hseu *et al.* 2008, Jiang *et al.* 2021) were reported. To allow a comparison between different humic substances and to evaluate their beneficial potential for use in aquaculture, determination of the chemical properties and referring the biological effects to these properties is therefore crucial. The fulvic acid (FA) chosen in the present study is characterized by a molecular mass of 800 g/mol, and a high aromatic, especially phenolic content (30 % aromatic carbon with 26 % phenolic groups) as revealed by ^{13}C-NMR spectroscopy. The FA has a high electron donor capacity (EDC), which reflects its ability to act as an antioxidant and protect from oxidative damage (Katalinic *et al.* 2006, Aeschbacher *et al.* 2012). At the same time, the FA has a high amount of persistent free radicals which have the potential to induce oxidative stress themselves (Dellinger *et al.* 2007, Lieke *et al.* 2018).

The present study aimed to determine if the waterbody is a way to apply FA to fish in aquaculture and if it can be used to improve the growth, development, and energy conversion of juvenile fish and larvae. Furthermore, if the FA exerts oxidative stress itself, and how FA affects the innate immune response and the response to inflammation. The last aim was to analyze if pre-exposure of juvenile fish to FA can help to reduce the effects of handling stress.

Zebrafish larvae (*Danio rerio*) were exposed to FA concentrations from 1 mg C/L to 500 mg C/L for up to 144 h and the effects on hatching, mortality, blood circulation, formation of hematomas, and edemas were recorded. Furthermore, the concentration of reactive oxygen species (ROS) and effects on the transcription of genes involved in growth (Gh, Igf-1, He-1α), innate immunity (Lyz, Mpx), and oxidative stress response (Keap1, Nrf2, Cat, Gpx, Sod-1, Sod-2) were analyzed by qRT-PCR after 96 h of exposure. Lastly, juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed for 4 weeks to two non-harmful concentrations (5 mg C/L and 50 mg C/L), and the effects on growth, innate immune response, oxidative stress protection, and effects of handling-stress were determined.

Exposing zebrafish larvae to FA concentrations between 20 mg C/L and 200 mg C/L significantly accelerated the hatching of exposed larvae. While around 40 % of the control larvae hatched after 72 h, the number increased to over 62 % in these exposure groups. Higher (≥ 300 mg C/L) and lower (< 20 mg C/L) concentration did not affect the hatching time. The overall hatching success was > 80 % after 96 h and did not differ between the groups. Genes involved in hatching (He-1α) and growth (Gh) showed increased transcription after exposure to 5 mg C/L and 50 mg C/L for 96 h. The transcription of Igf-1 was not affected by these concentrations. Exposing juvenile rainbow trout to the same concentrations (5 mg C/L and 50 mg C/L) of FA resulted in increased growth performance (weight and length gain). Devlin et al. (1995) showed, that the transcription of Gh in fish embryos affects the rate or efficiency (or both) of which stored yolk energy is converted into body mass. An improved conversion could result in increased growth and development of the larvae, resulting in the observed accelerated hatching. This is supported by a decrease of 20 % in the feed conversion ratio (increased efficiency) in rainbow trout exposed to 50 mg C/L. Our results indicate, that FA exposure promotes development and growth by activating genes involved in the growth hormone/insulin-like growth factors (GH/IGFs) axis (De Azevedo Figueiredo *et al.* 2007, Dang *et al.* 2018).

Starting after 72 h of exposure, concentrations of ≥ 400 mg C/L and after 96 h of exposure, concentrations ≥ 300 mg C/L had detrimental effects on the larvae (increased formation of edema and hematomas, impaired blood circulation, and mortality). Furthermore, the concentration of ROS inside the larvae after 96 h of exposure to 300 mg C/L and 500 mg C/L was significantly increased. 500 mg C/L also increased the transcription of genes involved in the detection and protection of oxidative stress (Sod-1, Cat, Keap1, Nrf2). Interestingly, exposure to 50 mg C/L increased the transcription of Sod-2 and Nrf2, indicating oxidative stress at this concentration as well. However, no detrimental effects nor an increased concentration of ROS was determined at this concentration. Similar results were found in the gills of rainbow trout, where the ROS concentration was decreased and the total oxygen scavenging

capacity (TOSC) was increased after FA exposure. Additionally, we showed that pre-exposure of larvae to 5 mg C/L and 50 mg C/L diminished a copper-induced inflammation, while high concentrations (300 mg C/L and 500 mg C/L) even enhanced the inflammatory response. Because of the high EDC, the FA acts itself as an antioxidant, and implementation of the FA into the mucus or epidermis of the gills could account for the increased TOSC and ROS protection. This could explain the anti-inflammatory properties observed at 5 mg C/L. However, the transcriptional results indicate, that FA also exerts mild oxidative stress at 50 mg C/L, probably due to the free radical content. According to the allostasis concept, mild oxidative stress activates defensive mechanisms (Sterling 1988, Minois 2000, Sterling 2012), which could help to protect the larvae from copper-induced inflammation. At high concentrations, the oxidative stress and the allostatic load to cope with it becomes too high, resulting in the observed detrimental effects.

Exposing zebrafish larvae to 5 mg C/L and 50 mg C/L increased the transcription of Lyz and Mpx. Lysozyme (Lyz) catalyzes the cleavage of the peptidoglycan layer in bacterial cell walls, while the myeloperoxidase (Mpx) is involved in the respiratory burst. Both are important parts of innate immunity. We did not find an increased respiratory burst activity after induction by phorbol 12-myristate 13-acetate (PMA) in larvae exposed to FA, however, Lyz and Mpx both mark neutrophils, and the increased transcription might hint towards an increased number of neutrophils. Lysozyme activity was also increased in the gills of rainbow trout after exposure to 50 mg C/L indicating a better protection from bacteria. Furthermore, we found increased respiratory burst activity and phagocytosis (rate and index) in head kidney leucocytes after exposure to 5 mg C/L and 50 mg C/L. Nevertheless, the leucocytes where still inducible to produce more ROS upon PMA exposure, showing that the FA activates the cells but does not deplete their capacity to produce ROS. Taken together, our results show, that exposure to FA stimulates the innate immune response of larvae and fish, which could help to decrease infections.

The results clearly demonstrated that bath application is an easy route to apply immunostimulants to fish. Especially for the pre-feeding larvae stage, this is a promising way of application, as feed additives can’t be used at this developmental stage. The FA used in these studies improved growth, antioxidative protection, and stimulated the innate immune response of larvae and juvenile fish when applied at low to medium concentrations. It helped to diminished inflammation comparable to diclofenac and ibuprofen. However, at high concentrations, the detrimental effects outweigh the protective once and the FA becomes hazardous and is not recommended for the use in fish.

Zusammenfassung

Aquakultur ist essentiell geworden, um den Bedarf an tierischem Protein zu decken. Mehr als 53 % des heute konsumierten Fisches wird bereits in Aquakultur produziert (FAO 2020a). Gleichzeitig stellen das unvermeidbare Handling, die Haltung selber, das Einfangen und der Transport große Stressoren für die Tiere dar. Diese verringern das Wachstum und reprimieren das Immunsystem, was zu Ertragsausfällen führt und die Tiere anfällig für Infektionen macht. Viele Therapeutika, die traditionell genutzt wurden, um Fischkrankheiten zu behandeln, wurden innerhalb der EU auf Grund von Bedenken hinsichtlich der Auswirkungen auf die Umwelt und die menschliche Gesundheit verboten, was zu einem Therapienotstand geführt hat (EG1272 2008, Andersen *et al.* 2009). Als Konsequenz wurde vermehrt nach umweltfreundlichen Alternativen geforscht und eine Vielzahl an Studien zum Einsatz von Futterzusatzmitteln wie beispielsweise Pflanzenextrakten, Vitaminen und Pre- bzw. Probitotika veröffentlicht (zusammengefasst in Lieke *et al.* (2019)). Eine der untersuchten Substanzgruppen sind Huminstoffe (HS). HS entstehen durch Degradation von Biomolekülen und werden nach ihrer Wasserlöslichkeit bei unterschiedlichen pH-Werten eingeteilt in Humus (unlöslich), Huminsäuren (unlöslich bei azidem pH) und Fulvosäuren (löslich bei allen pH-Werten) (Stevenson 1982). HS haben kürzlich das Interesse als Futterzusatzmittel für verschiedene terrestrische und aquatische Organismen geweckt. In Geflügel, Ratten und Mäusen verbesserte die Zugabe von HS die Vitalität, das Wachstum und verschiedene Immunparameter und verringerte das Auftreten von Krankheiten (Yasar *et al.* 2002, Islam *et al.* 2005, Vetvicka *et al.* 2010). Der Zusatz zum Fischfutter verbesserte ebenfalls das Wachstum, stimulierte die Immunantwort und verringerte die Schwere von Infektionen (Nakagawa *et al.* 2009, Gao *et al.* 2017, Yamin *et al.* 2017c). In Fischen gibt es zusätzlich zum Futter eine weitere mögliche Applikationsroute: das Wasser. Bis zu 95 % der gelösten organischen Materie in aquatischen Ökosystemen sind HS, so dass diese Art der Applikation die natürlich vorkommende Exposition reflektiert (Thurman 1985, Haitzer *et al.* 1998, Steinberg 2003b). Ein weiterer Vorteil liegt darin, dass die Larven, welche noch kein externes Futter aufnehmen und eine besonders hohe Sterblichkeit aufweisen (Sifa and Mathias 1987, Vadstein *et al.* 2013), mit dieser Applikationsmethode ebenfalls behandelt werden können.

Aufgrund des unterschiedlichen Ausgangsmaterials und verschiedener Degradationswege sind HS eine sehr heterogene Substanzklasse (Steelink and Tollin 1962, Meinelt *et al.* 2007, Stern *et al.* 2018, Savy *et al.* 2020). Neben den nützlichen Effekten wird daher auch immer wieder von schädlichen Effekten wie oxidativem Stress und Entzündungskrankheiten berichtet (Chen *et al.* 2002a, Hseu *et al.* 2008, Saebelfeld *et al.* 2017, Jiang *et al.* 2021). Daher ist es essentiell, die chemischen Eigenschaften zu bestimmen und diese auf die biologischen Effekte zu beziehen, um einen Vergleich zwischen verschiedenen HS zu ermöglichen und ihr Potenzial für die Aquakultur abschätzen zu können. Die

Fulvosäure (FA), welche in der vorliegenden Arbeit verwendet wurde, besitzt eine Molekularmasse von 800 g/mol und zeichnet sich durch einen hohen Anteil an aromatischen, insbesondere phenolischen Verbindungen aus (30 % aromatischer Kohlenstoff mit 26 % phenolischen Gruppen), wie die ^{13}C-NMR-Spektroskopie gezeigt hat. Weiterhin besitzt die FA eine hohe Elektronen-Donor-Kapazität (EDC), was ihre Fähigkeit widerspiegelt als Antioxidans zu wirken und somit vor oxidativen Schäden zu schützen (Katalinic *et al.* 2006, Aeschbacher *et al.* 2012). Gleichzeitig verfügt die FA aber über einen hohen Anteil an stabilen freien Radikalen, welche das Potential besitzen selber oxidativen Stress zu induzieren (Dellinger *et al.* 2007, Lieke *et al.* 2018).

Ziel der vorliegenden Arbeit war es zu bestimmen, ob der Wasserkörper ein möglicher Applikationsweg ist, um Fische in der Aquakultur mit FA zu exponieren und ob die FA genutzt werden kann, um Wachstum, Entwicklung und Energieumsetzung von Jungfischen und Fischlarven zu verbessern. Weiterhin wurde determiniert, ob die FA selber oxidativen Stress ausübt oder ob sie davor schützt, wie sie die angeborene Immunantwort beeinflusst sowie welche Effekte sie auf eine Entzündungsreaktion hat. Das letzte Ziel war es zu bestimmen, ob sich durch die Haltung in FA die Stressreaktion auf das Handling verringern lässt.

Larven vom Zebrabärbling (*Danio rerio*) wurden Konzentrationen von 1 mg C/L bis 500 mg C/L für bis zu 144 h gegenüber der FA exponiert und die Effekte auf Schlupf, Mortalität, Blutkreislauf, Hämatom- und Ödemenbildung untersucht. Weiterhin wurde die Konzentration an reaktiven Sauerstoffspezies (ROS) in den Larven gemessen sowie die Transkription von Genen, die am Wachstum (Gh, Igf-1, He-1α), der angeborenen Immunabwehr (Lyz, Mpx) und der Antwort auf oxidativen Stress (Keap1, Nrf2, Cat, Gpx, Sod-1, Sod-2) beteiligt sind, mittels qRT-PCR nach 96 h Exposition untersucht. Zuletzt wurden junge Regenbogenforellen (*Oncorhynchus mykiss*) für 4 Wochen in zwei der zuvor als förderlich determinierten Konzentrationen (5 mg C/L und 50 mg C/L) gehalten und die Effekte auf Wachstum, angeborene Immunantwort, Schutz vor oxidativem Stress und die Auswirkungen von Handling-Stress untersucht.

Zebrabärblingslarven, welche mit Konzentration zwischen 20 mg C/L und 200 mg C/L FA behandelt wurden, schlüpften signifikant schneller. Während nach 72 h rund 40 % der Kontrolltiere geschlüpft waren, stieg die Schlupfrate in den Expositionsgruppen auf über 62 % an. Höhere (≥ 300 mg C/L) und niedrigere (< 5 mg C/L) Konzentrationen beeinflussten den Schlupfzeitpunkt nicht. Gleiches gilt für die Gesamt-Schlupfrate, welche nach 96 h in allen Gruppen bei > 80 % lag. Die Exposition mit 5 mg C/L und 50 mg C/L der FA steigerte zudem die Expression von Genen, die an Wachstum (Gh) und Schlupf (He-1α) beteiligt sind, während die Expression von Igf-1 nicht beeinflusst war. In jungen Regenbogenforellen war nach Exposition mit den gleichen Konzentrationen (5 mg C/L und 50 mg C/L) gesteigertes Wachstum, sowohl in Gewicht als auch in Länge, zu beobachten. Devlin *et al.* (1995)

zeigten, dass die Transkription von Gh in Fischembryonen die Rate bzw. Effizienz (oder beides), mit der die im Eigelb gespeicherte Energie in Körpermasse umgewandelt wird, beeinflusst. Eine verbesserte Umwandlung könnte in gesteigertem Wachstum und Entwicklung der Larven resultieren, was dann zum beobachteten beschleunigten Schlupf führt. Eine Verbesserung der Quote mit der das Futter in Wachstum umgewandelt wurde (FCR) wurde ebenfalls in Regenbogenforellen beobachtet, wobei 50 mg C/L FA die Effizienz um ca. 20 % steigerte. Die Ergebnisse zeigen, dass die FA das Wachstum und die Entwicklung der Fische steigert und dass dies durch die Aktivierung von Genen der Wachstumshormon/Insulinähnliche-Wachstumsfaktor (GH/IGF)-Achse geschieht.

Konzentrationen ≥ 400 mg C/L hatten nach 72 h Exposition und Konzentrationen ≥ 300 mg C/L nach 96 h schädliche Effekte auf die Larven (Bildung von Ödemen und Hämatomen, Einschränkungen des Blutkreislauflaufes und Mortalität). Zudem konnte in den Larven eine erhöhte Konzentration an ROS nach 96 h Exposition mit 300 mg C/L und 500 mg C/L nachgewiesen werden. Weiterhin aktivierte 500 mg C/L die Transkription von Genen, die an der Detektion und Abwehr von oxidativem Stress beteiligt sind (Sod-1, Cat, Keap1, Nrf2). Interessanterweise führte die Exposition mit 50 mg C/L ebenfalls zu einer erhöhten Transkription von Sod-2 und Nrf2, was auf das Vorhandensein von oxidativem Stress bei dieser Konzentration hinweist. Bei dieser Konzentration wurden jedoch weder erhöhte ROS-Konzentrationen in den Larven, noch irgendwelche schädlichen Effekte beobachtet. Ähnliche Ergebnisse wurden in den Kiemen der Regenbogenforellen gefunden, wo die ROS-Konzentration verringert und die Kapazität Sauerstoffspezies zu binden (TOSC) erhöht war. Die Exposition mit 5 mg C/L und 50 mg C/L der FA konnte zudem eine durch Kupfer induzierte Entzündungsreaktion verhindern, wohingegen hohe Konzentrationen (300 mg C/L und 500 mg C/L) die Entzündungsreaktion weiter steigerten. Durch die hohe EDC kann die FA selber als Antioxidans wirken und eine Aufnahme der FA in den Schleim oder die Kiemenepithelien könnte für den gesteigerte Schutz vor ROS bei 5 mg C/L verantwortlich sein. Die Transkriptionsergebnisse weisen jedoch darauf hin, dass FA bei 50 mg C/L einen leichten oxidativen Stress ausübt, der vermutlich auf die stabilen freien Radikale der FA zurückzuführen ist. Nach dem Allostasis-Konzept (Sterling 1988, Minois 2000, Sterling 2012) aktiviert milder Stress die Schutzmechanismen. Diese Aktivierung könnte helfen, die Larven vor der kupferinduzierten Entzündung zu schützen. Bei hohen Konzentrationen der FA wird der oxidative Stress und die allostatische Last jedoch zu hoch, was in den beobachteten schädlichen Effekten resultiert.

Die Exposition der Zebrabärblingslarven mit 5 mg C/L und 50 mg C/L steigerte weiterhin die Transkription von Lyz und Mpx. Lysozym (Lyz) katalysiert die Spaltung der Peptidoglykanschicht in bakteriellen Zellwänden, während die Myeloperoxidase (Mpx) am respiratorischen Burst beteiligt ist. Beides sind wichtige Bestandteile der angeborenen Immunabwehr. In Fischlarven konnte keine

Veränderung der respiratorischen Burst-Aktivität durch FA Exposition nach Induktion mit Phorbol-12-myristate 13-acetat (PMA) festgestellt werden. Jedoch sind Lyz und Mpx beides Marker für Neutrophile und eine erhöhte Transkription könnte auch auf eine erhöhte Anzahl an Neutrophilen zurückzuführen sein. Im Gegensatz dazu zeigten die Leukozyten der Kopfniere von exponierten Fischen sowohl eine gesteigerte respiratorische Burst-Aktivität als auch Phagozytoseaktivität (Rate und Index). Die respiratorische Burst-Aktivität konnte durch Zugabe von PMA noch weiter gesteigert werden, was zeigt, dass die FA die Zellen zwar aktiviert, aber nicht erschöpft. Weiterhin war die Lysozymaktivität in den Kiemen der Regenbogenforellen nach Exposition mit 50 mg C/L signifikant gesteigert. Zusammengefasst zeigen die Ergebnisse, dass die Exposition mit FA die angeborene Immunantwort von Larven und Fischen stimuliert, was bei der Abwehr von Infektionen helfen könnte.

Die Ergebnisse der Arbeit beweisen, dass Badapplikation ein einfacher Weg ist um Immunstimulanzien bei Fischen einzusetzen. Insbesondere für die empfindlichen Larvenstadien, welche nicht mit Futterzusätzen behandelt werden können, ist dies eine vielversprechende Behandlungsmöglichkeit. Die in der vorliegenden Arbeit verwendete FA steigerte Wachstum, antioxidative Schutzmechanismen und stimulierte verschiedene Parameter der angeborenen Immunantwort in Larven und Jungfischen, wenn sie in geringen bis mittleren Konzentrationen eingesetzt wird. Zudem vermindert sie Entzündungen auf vergleichbare Art wie Diclofenac und Ibuprofen. In hohen Konzentrationen überwiegen jedoch die schädlichen Effekte und die FA wird gefährlich. Der Einsatz hoher Konzentrationen ist daher für Fische nicht empfehlenswert.

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1 General introduction

1.1 Importance of aquaculture and current challenges

In 2018 over 178 Mio t fish per year were produced worldwide and the percentage of overfished fish stocks has increased from 10 % in 1974 to over 34 % in 2017 (Figure 1-1) with extremes of over 60 % at an unsustainable level in the Mediterranean and the Black Sea (FAO 2020a). To cover this increasing demand for fish meat, over 53 % of the fish for human consumption is already produced in aquaculture (OECD/FAO 2020), with more than 62 % being freshwater species. But aquaculture also plays an important role in restocking and conserving overfished stocks and endangered wild fish populations (Braithwaite and Salvanes 2010, Cámara-Ruiz *et al.* 2019).

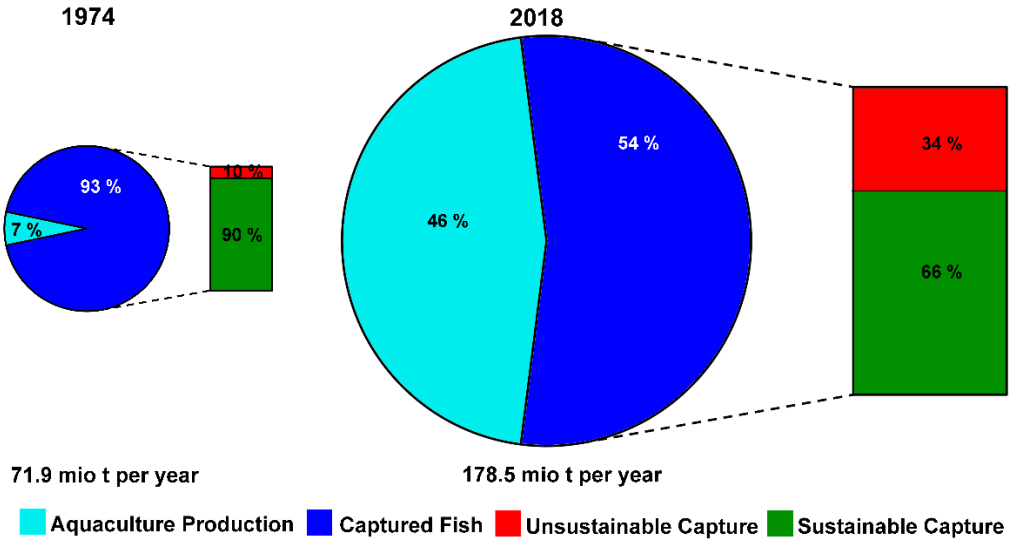

Figure 1-1: Total fish produced by capture and aquaculture in 1974 and 2018 and the changes in the sustainability of fish stocks. Based on data from the Food and Agriculture Organization of the United Nations (FAO 2016, FAO 2020a).

In aquaculture, as in any intensive animal rearing, the appearance of stressors is inevitable (Lockridge 1981). In sustainable aquaculture facilities, environmental factors such as water quality, temperature, and oxygen concentration are tried to be kept at optimal levels to ensure animal welfare and ideal growth performance. However, capture, handling, confinement, and transport are inevitable processes that stress the fish and often lead to wounding. High stocking densities, which are often required to decrease dominance hierarchies, can add to the stress and favor the spreading of pathogens (North *et al.* 2006, Rana *et al.* 2009). Simultaneous and ongoing occurrences of different stressors can depress the immune system and make fish more susceptible to diseases (Subasinghe *et al.* 2001, Pulkkinen *et al.* 2010). Apart from the high demand, aquaculture has to deal with prejudices because of excessive use of antibiotics and contaminations with hazardous compounds, especially in fish from countries with less stringent regulations or monitoring than the EU (Pham *et al.* 2015, Liu *et al.* 2017). Consumer standards for food quality, freshness, and safety for human health, but also the awareness to minimize negative impacts on the environment has increased over the last decades (Jung

et al. 2001, Romero *et al.* 2012, Lundborg and Tamhankar 2017). Classical therapeutics contain metals (mostly copper), triphenylmethane dyes, acriflavine, or formalin and are labeled as toxic and environmentally harmful by EU regulation (EG) 1272/2008. Many of them have been banned to use in fish intended for human consumption within the EU, and some are banned in the US and Canada as well (Culp and Beland 1996, Srivastava *et al.* 2004, Andersen *et al.* 2009, Lieke *et al.* 2019). The downsides of many 'traditional' chemical therapeutics as well as the lack of substances allowed in aquaculture have led to research focusing on alternative therapeutics. A prophylactic stimulation of the immune system and stress resistance by immunomodulatory substances can make fish less vulnerable to infections by activating the natural defenses.

1.1.1 Humic substances as immunostimulants

Humic substances (HS) are the major components of natural organic matter and arise from the physical, chemical, and microbial degradation of biomolecules. In aquatic ecosystems, HS account for up to 95 % of the dissolved organic matter (Stevenson 1982, Thurman 1985, Haitzer *et al.* 1998, Steinberg 2003b). Natural concentrations range normally between 0 and 50 mg C/L but can exceed 250 mg C/L in tropical water bodies (Suhett *et al.* 2013b). Due to the diversity of the organic raw material and the degradation pathways, HS have very heterogenic structures and different building blocks. They differ not only in the total amount of organic carbon (TOC) but in the fraction that can pass a 0.45 µm membrane filter (dissolved organic carbon, DOC). Based on their solubility in water HS are divided into humins (insoluble at any pH), humic acids (insoluble at acidic pH), and fulvic acids (soluble at all pH conditions) (Pettit 2004). Figure 1-2 shows the structure of a fulvic acid (FA).

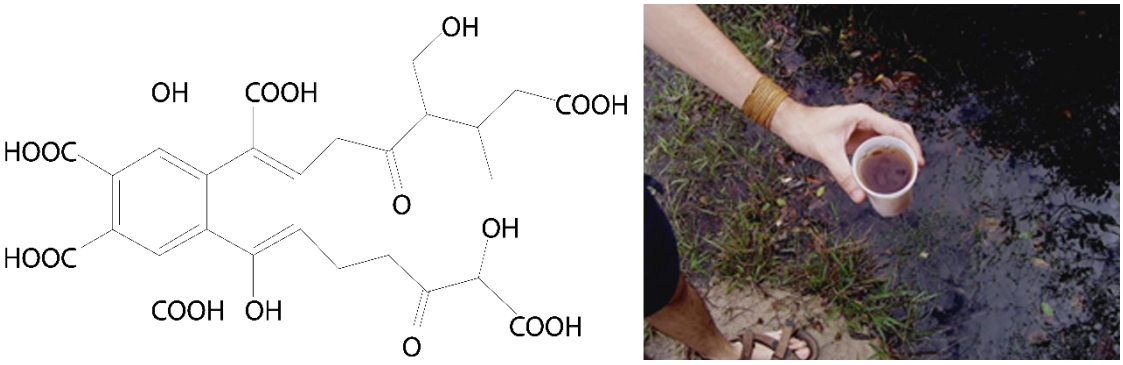

Figure 1-2: Schematic structure of a fulvic acid. Redrawn after ©ChemicalBook; Humic substance rich water in the Rio Grande do Norte, Brazil ©Christian E. W. Steinberg

The composition of the building blocks, especially the proportion of aliphatic and aromatic carbon groups determines the chemical properties, such as the electron acceptor and donor capacity, the total oxyradical scavenging capacity (TOSC), and the presence of persistent free radicals (Scott *et al.* 1998, Chen *et al.* 2002b, Lieke *et al.* 2018). Due to this structural diversity, reports on the biological effects are often contradictory (Meinelt *et al.* 2007) and cannot be compared easily when structural analyses are not provided. Anti-inflammatory effects such as decreases in edema and rheumatoid arthritis were

found in different *in vitro* and *in vivo* studies (van Rensburg 2015, Szot *et al.* 2019, Rusliandi *et al.* 2020). On the other hand, genotoxicity and oxidative DNA damage were reported after exposing human lymphocytes to HS, and HS exposure has been associated with Blackfoot and Kashin-Beck disease (Chen *et al.* 2002a, Hseu *et al.* 2008, Jiang *et al.* 2021).

1.1.2 Application methods

Immunostimulants are traditionally applied as feed additives and include vitamins, pre-, pro-, and parabiotics, and secondary metabolites from plants (detailed information can be found in our review Lieke *et al.* (2019)). The use of HS in terrestrial animals as feed additives or in drinking water increased growth and immunity and reduced the appearance and severity of diseases (Yasar *et al.* 2002, TeraVita 2004, Islam *et al.* 2005). Supplementing the feed of aquatic species with HS is a relatively new practice. White shrimp (*Litopenaeus vannamei*) that were challenged with *Vibrio parahaemolyticus* had significantly increased survival rates when fed 2 g/kg of FA (Fierro-Coronado *et al.* 2018). Adding 1 % of humus extract to the feed of ayu fish (*Plecoglossus altivelis*) decreased the development of skin lesions during infection with *F. psychrophilum* (Nakagawa *et al.* 2009). Supplementing feed of juvenile loach (*Paramisgurnus dabryanus*) with 1.5 % FA for 60 days resulted not only in elevated growth performance but in an increase of intestinal protease activity, antioxidant activity, lysozyme activity, complement 3 content, immune globulin M content, acid phosphatase activity, and alkaline phosphatase activity (Gao *et al.* 2017). Infections with *Aeromonas salmonicida* and *Gyrodactylus turnbulli* were significantly reduced in common carp (*Cyprinus carpio*) and guppy (*Poecilia reticulata*) after oral application of different HS-rich substances (Kodama *et al.* 2007, Yamin *et al.* 2017b, Yamin *et al.* 2017c).

The commodity agricultural raw materials price index has increased by over 70 % within the last 20 years (Data from Barrientos and Soria (2021)) and the aquafeed production is subject to these common global market variations with particularly susceptibility of smallholders or rural farmers (Rana *et al.* 2009). Supplementation of feed with stimulants often requires additional processing which increases the feed production costs even further. Cost-effective supplements and application methods, which require no additional processing could help to increase both, animal welfare and economic efficiency.

Under natural conditions, freshwater fish are exposed to HS over the water body rather than by direct ingestion. HS would therefore interact directly with skin mucus and gill surface, which are both main entrance portals for pathogens. Yamin *et al.* (2017a) showed that one HS-like component present in the water was also detectable in the blood of tilapia raised in the system, showing that some HS are taken up via the epithelia. This enables a new method of application for fish, where the designated HS is added directly to the water avoiding increased production costs and quality losses due to instability. In contrast to the feed application method, where the doses vary between fish in each tank due to

feed competition, bath application targets all fish in the same way. It furthermore would allow the use of an immunostimulant on pre-feeding yolk-sac larvae, which are exceptionally susceptible and have high mortality rates (Sifa and Mathias 1987, Vadstein *et al.* 2013). Nevertheless, studies on bath application with HS are scarce. Treating goldfish (*Carassius auratus auratus*) which were pre-damaged (skin lesions, necrosis on the fins) by handling and infected with different parasites, for 1 h daily over 4 days and for 1.5 h to for 12 days with 10 mg/L of a sodium-humate, Heidrich (2005) found significantly decreased mortality and increased wound healing. However, no long-term application was studied. Exposing young swordtail (*Xiphophorus helleri*) for a total of 21 weeks to 5 mg /L of synthetic HS did not affect the initial growth rate, but decreased growth impairment following 2 weeks of daily netting stress (Meinelt *et al.* 2004). There was no difference between the lowest (5 mg/L) and highest (180 mg/L) concentrations applied. Clearly, more research is needed to evaluate if bath application is a suitable way to apply HS and if they have beneficial effects on growth, stress perception, and immune response of fish and fish larvae.

1.2 Physiological Processes

1.2.1 Physiological responses to stress

Stress is associated with a disturbance of the *status quo* by intrinsic or extrinsic stimuli (stressor). This can be sudden or extreme changes in the physical environment, animal interactions, and human interference (Wendelaar Bonga 1997). With this definition, stress is labeled as negative events only. However, the *status quo* or "homeostasis" is a static concept and in real life, organisms are constantly exposed to changing conditions and have to deal with and learn from them to cope with recurring situations (Wendelaar Bonga 1997, McEwen 2017). The concept of allostasis includes the reaction to stressors and the mechanisms that are activated to cope with a stressor ("achieving stability through change") (Sterling 1988, Sterling 2012, McEwen 2017). Exposure to mild stress (eustress) is even more beneficial to organisms than just maintaining homeostasis as it prepares the system to deal with stressors before they even arise (Minois 2000, Sterling 2012). Only if the costs (allostatic load) to deal with a stressor become too high, stress becomes detrimental (distress) (McEwen and Stellar 1993).

The stress response can be divided into three phases. The perception of a stressor activates the hypothalamus-sympathetic system-chromaffin cell (HSC) axis resulting in a release of catecholamines (such as adrenalin and noradrenalin) within seconds from the chromaffin cells. At the same time, the hypothalamus-pituitary-interrenal (HPI) axis is activated starting a hormonal cascade via corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) which results in the release of corticosteroids (in fish mainly cortisol) within few minutes from the interrenal cells (Wendelaar Bonga 1997, Mommsen *et al.* 1999, Pankhurst 2011). The rise of the cortisol concentration in the blood is a well-accepted parameter to determine the stress level of an organism (Barton *et al.* 1980, Pottinger

2008). The activation of these two axes is referred to as the primary stress response. In the secondary stress response, the catecholamines regulate the rapid mobilization of energy sources such as glucose and lactose to react to the immediate stressor, while cortisol adjusts the slower energy allocation to the new demands to restores the pre-stress conditions (e.g., gluconeogenesis) (Sadoul and Geffroy 2019). The interrenal steroidogenic cells, which release cortisol are located in the head kidney, which is also a secondary lymphoid organ of the immune system (Weyts *et al.* 1999, Gorissen and Flik 2016). It is therefore unsurprisingly that the stress response also affects the immune response. Acute and mild stress was shown to increase the number of leucocytes and the phagocytic activity (Maule and Schreck 1990, Dhabhar and McEwen 1997, Ruis and Bayne 1997). This activation allows the fast response to wounding which might occur due to the stressor (e.g., encountering a predator) or subsequent pathogen invasion.

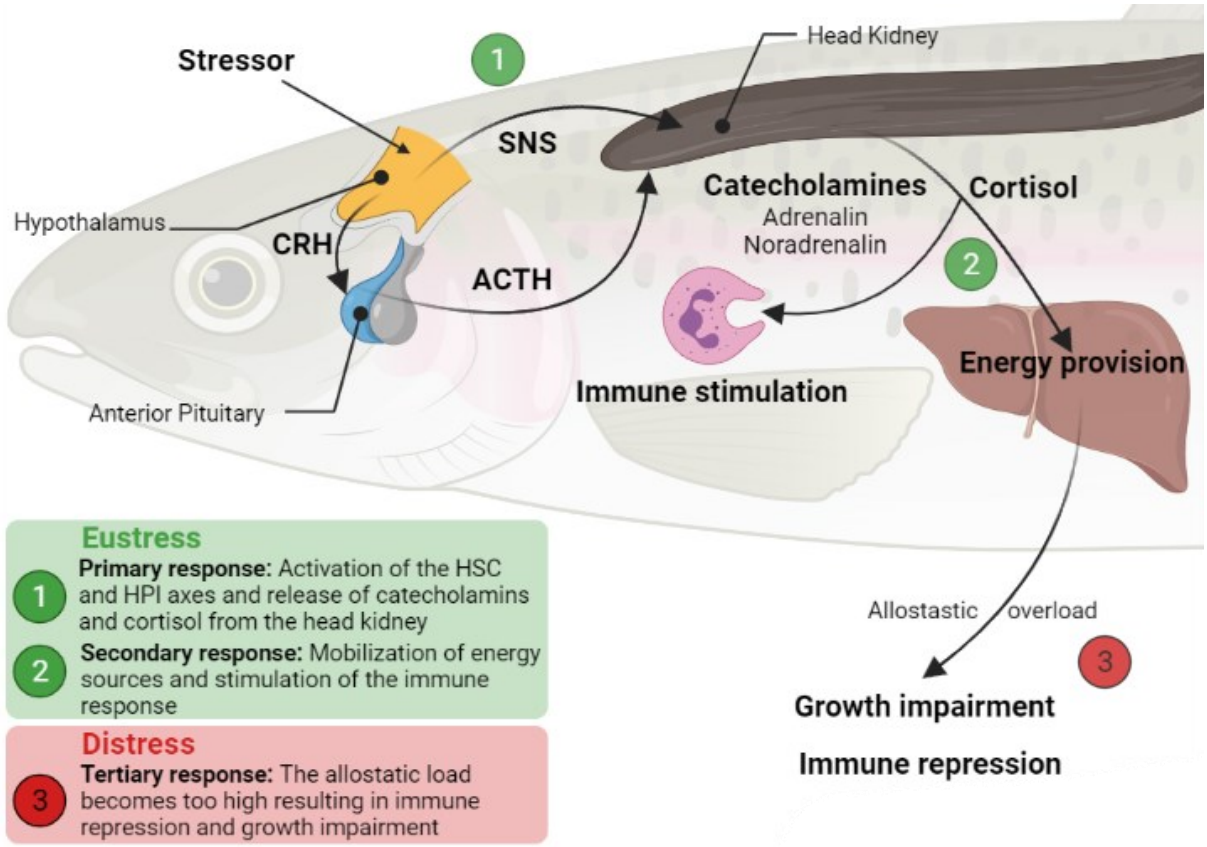

Figure 1-3: Stress response in fish. ACTH: adrenocorticotropic hormone, CHR: corticotropin-releasing hormone, HPI: hypothalamus-pituitary-interrenal, HSC: hypothalamus-sympathetic system-chromaffin cell, SNS: sympathetic nervous system. Created with BioRender.com

Long-term consequences of repeated strong or prolonged stress result in the tertiary phase and are associated with the whole-animal performance (Barton 2002). The energy that is normally used for growth and reproduction is allocated to processes needed to deal with the stressor leading to decreased growth performance and delayed development compared to non-stress conditions (Barton *et al.* 1987, Pickering 1990, Pickering 1993). Furthermore, the immune system is suppressed including

reduced numbers of lymphocytes and decreased phagocytic activity (Maier *et al.* 1994, Schreck and Tort 2016) as the importance to cope with the immediate stressors is stronger than preparing for a potential wounding or infection. This tertiary response is especially crucial for aquaculture as it not only decreases the development and yield performance but leaves the animals susceptible to diseases. Decreasing stressful conditions and increasing the stress resistance of animals are therefore desirable goals to ensure animal welfare.

1.2.2 Reactive oxygen species, oxidative stress, and defense mechanisms

Reactive oxygen species (ROS) are small oxygen-containing molecules with high reactivity generated by partial reduction of molecular oxygen. ROS play an important part in the innate immune response, act as signaling molecules, and can be involved in signal transduction pathways and the activation and deactivation of enzymes (Holmström and Finkel 2014, Franchina *et al.* 2018). Low levels of oxidative stress, termed oxidative eustress, are therefore essential, and exposure to mild stress is beneficial as it activates the cellular defense mechanisms (Minois 2000, Niture and Jaiswal 2010, Sies *et al.* 2017). The protective concentration, however, might be only slightly lower than detrimental concentrations. Overproduction or excessive external ROS damage macromolecules such as nucleic acids and proteins, leading to cellular toxicity, immunogenicity, inflammation, and gene mutations (Scott Obach and Kalgutkar 2010). Controlling the internal ROS concentration is therefore essential and cells have several mechanisms to scavenge ROS. The Kelch-like ECH-associated protein 1 (Keap1) and the nuclear factor erythroid 2–related factor 2 (Nrf2) are the central regulators that control the ROS concentration (Figure 1-4 A). Under normal conditions, Nrf2 builds a heterodimer with Keap1 leading to degradation of the heterodimer via ubiquitination. Upon oxidative and electrophilic stress, cysteine residues in Keap1 are arylated or oxidated leading to conformation changes and detachment from Nrf2 (Zhang and Hannink 2003, He and Ma 2009). This allows the translocation of Nrf2 into the nucleus where it activates the transcription of xenobiotic response elements (XRE) and antioxidant or electrophile response elements (ARE or ERE). ARE and ERE coordinate the induction of more than 200 genes involved in the detoxification of xenobiotics and protection against oxidative stress (Niture and Jaiswal 2010). Among those defensive enzymes are catalase (Cat), superoxide dismutases (Sod-1 and Sod-2), and glutathione peroxidase (Gpx) (Figure 1-4 B). The Sods catalyze the conversion of the superoxide anion ($O_2 \cdot^-$) to hydrogen peroxide (H_2O_2), which is then converted to oxygen and water by Cat or to water by Gpx (using glutathione as a substrate).

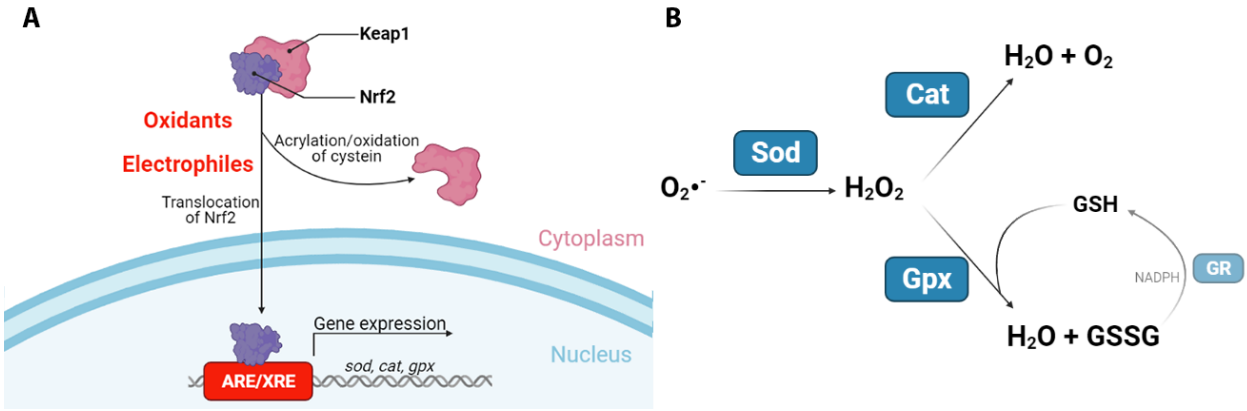

Figure 1-4 A: Nrf2-Keap1 pathway to detect oxidative and electrophilic stress. Arylation or oxidation of cysteine residues in Keap1 results in destabilization of the heterodimer and translocation of Nrf2 into the nucleus. Here Nrf2 activates antioxidative response elements (ARE) or xenobiotic response elements (XRE), and thereby the gene expression of defensive mechanisms, such as superoxide dismutase (Sod), catalase (Cat), and glutathione peroxides (Gpx). B: Enzymatic inactivation of reactive oxygen species by superoxide dismutase (Sod), catalase (Cat), and glutathione peroxidase (Gpx). Glutathione (GSH) is regenerated by glutathione-reductase (GR). Created with BioRender.com

In addition to the enzymatic scavengers, non-enzymatic antioxidants are involved in the protection against oxidative stress. These are for example glutathione, vitamin E, A, and C, uric acid, flavonoids, and polyphenols (Katiyar and Elmets 2001, Du *et al.* 2009, Gülçin 2010). Together with the enzymes, they determine the antioxidative or total oxyradical scavenging capacity (TOSC). As xenobiotics, HS could add to the oxidative stress imposed on cells (Matsuo *et al.* 2006b, Saebelfeld *et al.* 2017). On the other hand, the TOSC of HS could add to the protection of exposed cells making them less susceptible to oxidative damage. The effect (protective or detrimental) is defined by the chemical properties and is probably different for different HS. However, it is also possible that the effects shift due to different concentrations of the same HS.

1.2.3 Inflammation and the innate immune response

Due to the close contact and exchange with their environment, fish are exposed to a wide range of pathogenic and non-pathogenic microorganisms. For defense, they rely heavily on their innate immune system.

The innate immune system is the first line of defense against infections and is involved in wound-healing. In addition to the physical barriers like skin and mucus, which protect directly against pathogens, it is divided into the humoral and cellular components (Magnadóttir 2006). The humoral components include the complement system, cytokines, and lytic enzymes such as lysozyme. Lysozyme is found in the plasma, lymphoid tissue, gills, skin, and mucus of fish. It cleaves the β-1,4 linkages in the bacterial cell wall, provoking lysis (Grinde *et al.* 1988, Grinde 1989). Lysozymes of fish have been reported to kill both, $Gram^+$ and $Gram^-$ bacteria, although permeabilization of the outer membrane of $Gram^-$ bacteria requires additional components of the innate immune system such as lactoferrin and

cathelicidins (Yousif *et al.* 1994, Callewaert and Michiels 2010). Upregulated translation of lysozyme genes was found after pathogen contact or stimulation with LPS showing the importance of lysozyme in antibacterial defense (Hikima *et al.* 2003, Paulsen *et al.* 2003, Caipang *et al.* 2008).

Key cells of the innate immune system are the phagocytic cells, mainly neutrophils, and macrophages, which patrol randomly around the body areas (Figure 1-5). Phagocytes possess a variety of different pattern recognition receptors (PRRs), allowing them to detect different molecular patterns. It can be distinguished between self-associated molecular patterns (SAMPs), damage or danger-associated molecular patterns (DAMPS or alarmins), and pathogen or microbe-associated molecular patterns (PAMPs or MAMPs) (Krysko *et al.* 2011, Tang *et al.* 2012). PAMPs include a broad spectrum of microbial and non-self molecules such as cell-wall components, glycan-based polymers, and peptides, while DAMPs include endogenous molecules released from stressed or dying cells such as heat-shock proteins (HSPs), uric acid, and cytosolic proteins (Rubartelli and Lotze 2007, Schaefer 2014). Detecting a PAMP or DAMP, neutrophils are activated, and cells engulf pathogens and apoptotic cells and destroy them by the production of ROS inside the phagolysosome (respiratory burst) (Gordon 2016). Furthermore, they release ROS and proinflammatory cytokines as external signal molecules which recruit more immune cells to migrate into the affected area (Kany *et al.* 2019).

If the immune system is working correctly, an anti-inflammatory phase is initiated after clearance of the threat to prevent chronic inflammation and further tissue damage. This is mediated by specialized lipids, proteins, and arachidonate-derived eicosanoids (Serhan and Savill 2005, Norling and Serhan 2010). Anti-inflammatory cytokines inhibit further neutrophil recruitment and can polarize macrophages from pro-inflammatory (classically activated) M1 to anti-inflammatory (alternatively activated) M2 macrophages (Opal and DePalo 2000, Murray and Wynn 2011, Bohlson *et al.* 2014, Schauer *et al.* 2014). These produce anti-inflammatory cytokines, such as Il-10 and Tgf-β, and help to resolve the inflammation repair the damaged tissue (Yao *et al.* 2019).

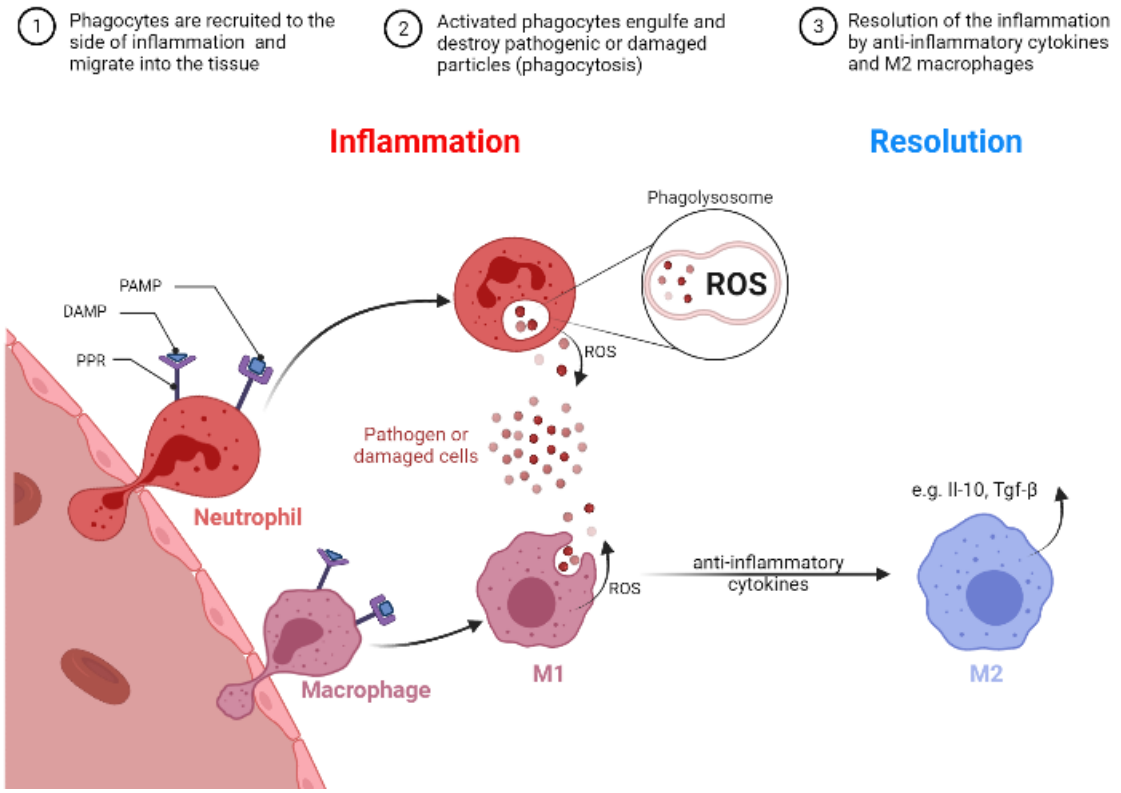

Figure 1-5: Inflammation and resolution after wounding or infection. PPR: pattern recognition receptors on phagocytes detect pathogen and damage-associated molecular patterns (PAMP and DAMP). Activated neutrophils and classically activated macrophages (M1) remove pathogenic and damaged cells. Resolution is initiated by anti-inflammatory cytokines which also polarize M1 to M2 macrophages. Created with BioRender.com

As mentioned above, pro- and anti-inflammatory properties have been reported for HS (Yang *et al.* 2002, van Rensburg 2015). However, the mode of action is still unclear. To ensure a safe application, the determination of the inflammatory properties is crucial.

1.3 Study organisms

1.3.1 Zebrafish (*Danio rerio*)

The zebrafish (*Danio rerio*) is a cyprinid native to South Asia and was first described in 1822. Because of the easy and relatively cheap maintenance and the high number of offspring the zebrafish has become a popular model organism in the last decades. The generation time is approximately 3 months, and the female produces 200-300 eggs per spawn. The genome is fully sequenced (Howe *et al.* 2013), and several mutants and transgenic fish lines have been established. Experiments with pre-feeding larvae (depending on the culture conditions around 144 hpf, hours post fertilization) do not require animal experiment permission inside the EU (European Union Directive 2010/63/EU). Together with the optical transparency of zebrafish larvae (Figure 1-6), allowing *in vivo* monitoring and the extremely fast developing rate, this enables fast screening. While the innate immune responses develop within 1 day after fertilization, the adaptive immune system is functionally matured after 4-6 weeks (Novoa and Figueras 2012). This allows studying the innate immune response independently from the adaptive

response. For the toxicity tests (OEDC 236) and range finding, as well as to study the effects of HS on the oxidative and immune response, the zebrafish was therefore chosen as a model organism.

Figure 1-6: Zebrafish (*Danio rerio*) 72 hours post-fertilization (hpf) and 96 hpf.

1.3.2 Rainbow trout (*Oncorhynchus mykiss*)

The rainbow trout (*Oncorhynchus mykiss*) is a salmonid and is native to the Pacific drainages of North America. It has been introduced to waters worldwide since 1874 for angling and aquaculture. Spawning and growth normally occur at temperatures between 9 °C and 14 °C. However, different domesticated strains have been selected for higher temperature stress resistance, such as the breeding line BORN, which is often used in aquaculture in Germany and which was used in our study. In 2018 3.6 Mio t of salmon, trout, and smolt were produced in aquaculture, of which 24 % were trout (FAO 2020b). Salmon and trout account for about 18 % of the total value of internationally traded fish products and with a price of 6,415 USD/t, they are even more expensive than sturgeon and paddlefish raised in aquaculture (5,626 USD/t) (FAO 2020a). In 2005, trout farming in Germany reached 24,000 t per year worth 113 Mio €, making it the most important branch of aquaculture production (FAO 2021). The high economic and nutritional value, as well as the fast growth, make rainbow trout an attractive fish for aquaculture. However, cultured trout is susceptible to stress and many disease-causing bacteria, parasites, fungi, and viruses (Noble and Summerfelt 1996). In aquaculture, up to 50 % of production loss is caused by diseases and with around 10 % it is the main single cause of financial losses (Meyer 1991, van West 2006, Assefa and Abunna 2018). Therefore, rainbow trout were chosen to perform the experiments on juvenile fish.

2 Aims of the study

HS are used successfully as feed additives in agriculture because of their beneficial effects on growth and disease prevention over the last decades. In the last years, they have gained interest in aquaculture, due to the increasing demand for safe and sustainable food production. Nevertheless, testing the natural way of application, over the water body, has been neglected. Furthermore, contradictory reports about the inflammatory effects of HS are reported, which can be attributed to the heterogeneity of this substance group.

In pre-experiments (unpublished) we tested different HS for their basic physio-chemical properties and their potential to be used in aquaculture systems and found fulvic acid (FA) extracted from groundwater in the Netherlands to best meet the requirements (e.g., full solubility, no precipitation, no influence on pH and oxygen levels). This FA was then used in all subsequent experiments. The chemical characterization of the FA was published in the article here referred to as the 5th chapter. For easier understanding of all chapters, excerpts of the results are listed in Table 2-1.

Table 2-1: Excerpts of the chemical characterization of the fulvic acid. Details are given in Lieke *et al.* (2021b) (Chapter 5).

Carbon	96.7 g/L
Oxygen	130.5 g/L
Nitrogen	1.6 g/L
Hydrogen	12.2 g/L
Sulphur	2.0 g/L
Aromatic content	29.2 %
Phenolic content	7.6 %
Electron donor capacity	380 µmol/g
Electron acceptor capacity	270 µmol/g
Electron paramagnetic resonance (measurement of persistent free radicals)	$4.6*10^6$ /g carbon

The study aimed to evaluate if bath application is a way to apply immunostimulants to larvae and juvenile fish. Because of the transparency and short developmental time, zebrafish larvae were used to determine FA concentrations that are safe for the application following the OECD236 guidelines and which impacts can be determined on the larval stage. Rainbow trout were chosen as an important aquaculture fish with high economic value and sensitivity to stress and diseases to determine the effects of FA exposure on juvenile fish.

The following questions were addressed in this thesis:

1. **Can FA be used as a bath stimulant to increase the growth and energy conversion of fish?**
2. **Does FA exert oxidative stress and does this change in a dose-dependent manner?**
3. **What are the inflammatory properties of FA?**
4. **Does FA exposure reduce the handling-induced stress effects?**

According to the present knowledge we expect beneficial effects on growth, increased resistance against handling stress, and stimulation of the innate immunity. Based on the chemical characterization, we furthermore expect the FA to have anti-oxidative and anti-inflammatory properties due to the high content of phenolic structures with a potential to become detrimental at high concentrations due to the PFR content. The gained results should help to establish an easy and

inexpensive application method for the application of immunostimulants in aquaculture. It should increase both, animal health and yield while allowing an application to the sensitive pre-feeding larvae.

2.1 Chapters

The thesis is divided into three chapters which are published or submitted articles in “Scientific Reports”, “Science of the Total Environment”, and “Chemosphere”. The articles were reformatted and pages, figures, and tables renumbered.

Chapter 3: Lieke, T., C. E. W. Steinberg, S. Bittmann, S. Behrends, S. H. Hoseinifar, T. Meinelt, K. Knopf and W. Kloas (2021). "Fulvic acid accelerates hatching and stimulates antioxidative protection and the innate immune response in zebrafish larvae." https://doi.org/10.1016/j.scitotenv.2021.148780.
The effects of FA exposure on zebrafish larvae were evaluated using the Fish Embryo Acute Toxicity Test (OECD 236) and larvae exposed to low to medium concentrations of FA had accelerated hatching, while detrimental effects were observed at high concentrations. The concentration of ROS in the larvae was significantly increased after exposure to high concentrations, while the inducibility of the leukocytes to perform a respiratory burst was not affected by any concentration. qRT-PCR analysis showed that expression of Gh, He-1α, and Igf-1 was increased by low and medium concentrations, accelerating the hatching. The increased transcription of Lyz and Mpx hints towards the activation of the innate immune system. Medium and high concentrations furthermore increased the transcription of antioxidative enzymes (Cat, Sod-1, Sod-2, Gpx, Nrf2, Keap1).

Chapter 4: Lieke, T., C. E. W. Steinberg, T. Meinelt, K. Knopf and W. Kloas (submitted). "Modification of the chemically induced inflammation assay reveals the Janus face of a phenol rich fulvic acid."
We adapted the chemically induced inflammation assay by (d'Alençon *et al.* 2010) to the use of wild-type zebrafish and used this modified assay to determine the inflammatory effects of fulvic acid. At low to medium concentrations, the FA is anti-inflammatory, similarly reducing the copper-induced inflammation as diclofenac. However, at high concentrations, the FA becomes pro-inflammatory and amplifies the inflammation reaction.

Chapter 5: Lieke, T., C. E. W. Steinberg, B. Pan, I. V. Perminova, T. Meinelt, K. Knopf and W. Kloas (2021). "Phenol-rich fulvic acid as a water additive enhances growth, reduces stress, and stimulates the immune system of fish in aquaculture." Scientific Reports **11**(1).https://doi.org/10.1038/s41598-020-80449-0.
Exposing juvenile rainbow trout to FA for 4 weeks, improved growth parameters (weight, length, feed conversion). Furthermore, treated fish had lower cortisol levels after being exposed to handling stress, increased phagocytic activity of leukocytes in the head kidney, and a higher oxygen scavenging capacity and lysozyme activity in the gills.

3 Fulvic acid accelerates hatching and stimulates antioxidative protection and the innate immune response in zebrafish larvae

Thora Lieke [a,b,*], Christian E.W. Steinberg [b,c], Sandra Bittmann [a], Sascha Behrens [a], Seyed H. Hoseinifar [d], Thomas Meinelt [a], Klaus Knopf [a,b], Werner Kloas [a,b]

[a] Department of Ecophysiology and Aquaculture, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, 12587 Berlin, Germany

[b] Humboldt University of Berlin, Faculty of Life Sciences, 10115 Berlin, Germany

[c] Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, 650500 Kunming, China

[d] Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, 49138-115739 Gorgan, Iran

* Corresponding author (lieke@igb-berlin.de), Phone: +49 (0)30 64181 641, ORCID ID: 0000-0002-4345-171

Keywords: Humic substance, Zebrafish (*Danio rerio*), Embryonic development, Reactive oxygen species (ROS), Oxidative stress

3.1 Highlights

- Fulvic acid between 20 and 200 mg C/L accelerates hatching of zebrafish larvae
- At ≥ 300 mg C/L oxidative stress causes tissue damage and mortality
- Hatching is mediated by induced expression of *gh, igf-1, and he1-α*
- 5 mg C/L and 50 mg C/L of the fulvic acid induced expression of *lyz* and *mpx*
- The antioxidative protection is activated via the *keap1-nrf2* pathway

3.2 Abstract

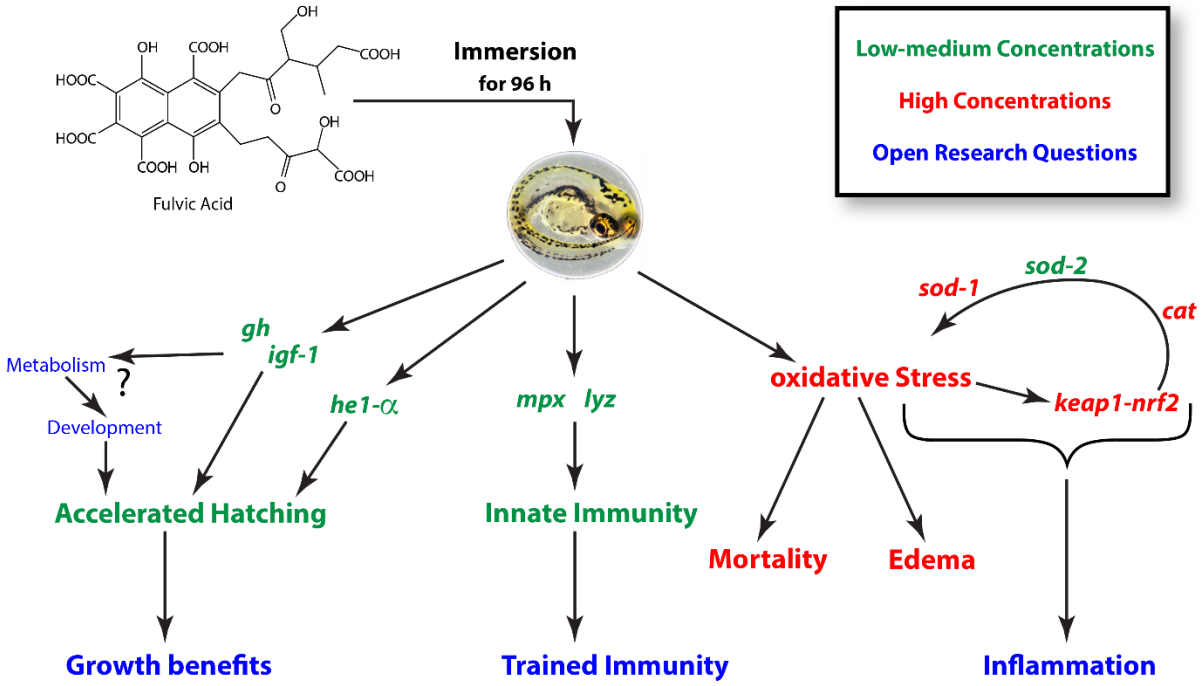

Figure 3-1: Graphical Abstract

Aquaculture plays a pivotal role in covering dietary animal protein demands and restocking endangered fish populations. However, high mortality takes place at the earliest life stages: prior and immediately after hatching. Improving growth and health parameters by immunostimulants is widely used in older fish, but rarely studied in larvae. Fulvic acids (FAs) are natural substances found in soil and water. Using zebrafish as a model organism, we evaluated the effects of exposure to a FA at concentrations ranging from 1 to 500 mg C/L (mg dissolved organic carbon per liter) on embryonic development. Furthermore, the concentration of reactive oxygen species (ROS) inside the larvae as well as the molecular mechanisms involved in growth, immune response, and antioxidative protection were determined at 5, 50, and 500 mg C/L. 20 to 200 mg C/L accelerated the hatching, which was mediated by increased expression of *ifg-1*, *gh*, and *he1-α*. Furthermore, *lyz* and *mpx* were significantly increased at 5 and 50 mg C/L. A concentration of 500 mg C/L induced genes involved in the protection against ROS (*nrf-2, keap-1, cat, sod-1*), increased the concentration of ROS inside the larvae and caused tissue damage and mortality. Interestingly, 50 mg C/L activated ROS protection as well (*nrf-2, sod-2*),

while no increase of ROS was found in the larvae. Our results show, that FA at low to medium concentrations can increase the health of larvae, but becomes detrimental at higher concentrations.

3.3 Introduction

Overfishing, climate changes, and water pollution are threatening the world's fish stocks. According to the FAO (2020b)) on average over 30% of the marine fish stocks are overfished and over 50% are fished at their maximal capacity. In European waters, overfishing is even higher with more than 50% of stocks being outside of safe biological limits (Froese *et al.* 2018). Aquaculture has become essential not only to cover the demand for dietary animal proteins but also for re-stocking and conserving overfished stocks and endangered fish populations (Braithwaite and Salvanes 2010, Cámara-Ruiz *et al.* 2019). Due to environmental and health concerns on the use of therapeutics, research on the use of natural immunostimulants as feed additives has increased. The benefits of the application include faster growth, improved feed conversion, and stimulation of innate and adaptive immune responses (Wang *et al.* 2017, Lieke *et al.* 2019, Hoseinifar *et al.* 2020). The natural survival rate for fish until adulthood is often lower than 1% with exceptionally high mortality in the early stages (Sifa and Mathias 1987, Vadstein *et al.* 2013). This critical period includes the embryo stage, the newly hatched pre-feeding stage (yolk-feeding larvae), and the juvenile metamorphosis (Hjort 1914, McCasker *et al.* 2014). Besides environmental factors such as temperature, predators, and food availability, pathogens are the main cause of mortality (Sifa and Mathias 1987, Rojo-Cebreros *et al.* 2018). The surface of the fish eggs is colonized by bacteria and fungi, and the freshly hatched larvae are exposed to this biofilm during a time of shifting and developing functions and organs (Hansen and Olafsen 1999, Bergh 2000). At the same time, the immune system is not yet fully developed, leaving the larvae vulnerable.

Supplementing the feed for fry to boost their growth and the immune response is already practiced (Bricknell and Dalmo 2005, Davis 2015, Zhang *et al.* 2019). However, this approach is unsuitable for yolk-feeding larvae. At this early life stage, the use of antibiotics is the remedy of choice in case of bacterial problems. On the other hand, the drawbacks of antibiotic treatments are well known (Romero *et al.* 2012, Santos and Ramos 2018). Due to resistance development, antibiotics should be used as little as possible. In addition, the development of the intestinal flora of the fish larvae, which is strongly influenced by the bacteria from the environment, is negatively affected by antibiotics (Hansen and Olafsen 1999, Olafsen 2001). Few studies have therefore tried to use water-soluble stimulants to affect the immune response of larvae. Exposing Atlantic halibut (*Hippoglossus hippoglossus*) yolk-feeding larvae to lipopolysaccharides (LPS) significantly increased the survival after a second exposure to high LPS concentrations (Dalmo *et al.* 2000). Zebrafish (*Danio rerio*) larvae raised from eggs exposed from 4 hpf (hours post fertilization) in β-glucan immersion showed significantly

improved innate immune levels and delayed mortality after infection with *Vibrio anguillarum* compared to non-exposed larvae or those exposed after hatching (72 hpf) (Oyarbide *et al.* 2012).

Humic substances (HSs) are complex compounds occurring from degrading organic material. They represent up to 95% of the dissolved organic matter (DOC) in freshwater aquatic ecosystems (Thurman 1985, Haitzer *et al.* 1998) and have been used as feed additives in fish (Abdel-Wahab *et al.* 2012, Gao *et al.* 2017). Concentrations normally range between 0.5 and 50 mg C/L but can exceed even 250 mg C/L in tropical lagoons (Steinberg 2003b, Suhett *et al.* 2013a). Although the natural exposure of HSs and especially fulvic acids (FAs) to aquatic organisms happens over the water body, to our knowledge, the only research that has been conducted on the long-term effects of HSs bath exposure to fish comes from our group. We recently showed that the phenol-rich fulvic acid (FA) used in this study can be used as a bath treatment to improve growth performance, stress resistance, and the immune response in rainbow trout (*Oncorhynchus mykiss*) fingerlings (Lieke *et al.* 2021b). The present study is the first attempt to use FA as an immunostimulant for the pre-feeding larvae stages. Our hypothesis is that the same FA can be used to improve the condition of larvae before the external feeding stage and that the effects can be traced back to the biomolecular level. Because of the rapid development, the transparency of the larvae, allowing monitoring of internal development, and the fully sequenced genome, we decided to use the zebrafish as a model organism.

3.4 Material and Methods

3.4.1 Fulvic acid

To ensure reproducibility and to exclude effects by contaminations, which may occur when extracting HS from environmental isolates, we used a commercially available FA (FulvoFeed, HuminTech). It contains 96.7 g C/L, is completely water-soluble, and is indicative of a high content of persistent organic radicals and phenolic compounds. More details of the chemical characterization can be found in Lieke *et al.* (2021b)

3.4.2 Husbandry and general experimental conditions

Parental zebrafish were raised and maintained in the facility of Leibniz-Institute of Freshwater Ecology and Inland Fisheries under recommended conditions (26 °C, 16 h/8 h light/dark cycle, lights on at 6:00 am, 7.5 ± 0.5 mg /L oxygen, pH 7.0 ± 0.3, 120 ± 20 µS/cm, 20.63 mg/L $NaHCO_3$ (S3 Chemicals, Germany), 18.75 mg/L sea salt (Tropic Marin, Switzerland), 26.81 mg/L $CaCl_2*2H_2O$ (Aquafair, Germany)). Zebrafish were kept in 60 L transparent tanks at group sizes of 30 (1:2 female: male ratio) and fed 3 times daily with commercial flake food (TetraMin Flakes, Tetra, Germany) and 2 times with freshly hatched *Artemia salina* nauplii.

The exposure in all experiments followed the guideline of the Fish Embryo Acute Toxicity Test OECD 236 (2013). Eggs were collected directly after fertilization and cleaned with dilution water (deionized water with 294.0 mg/L $CaCl_2*2H_2O$, 123.3 mg/L $MgSO_4*7H_2O$ (Carl Roth, Germany), 63.0 mg/L $NaHCO_3$, 5.5 mg/L KCl (Merck Germany)). Using a 1 mL pipette healthy eggs were randomly distributed within 60 min after fertilization into crystallization dishes containing either dilution water (control) or FA (details on the used concentration and exposure period are given in the following method paragraph). Afterward, larvae were individually transferred into separated wells of 24-well plates containing 2 mL of solution (control or FA). Due to the color of FA, the experimenter could not be blinded to the treatment group. The plates were sealed with SealPlate sealing film (Carl Roth) and eggs were incubated at 26 ± 0.5 °C (16 h/8 h light/dark cycle; IPP 110plus (Memmert, Germany)). The condition of larvae was checked with an IMT-2 inverse Microscope (Olympus, Japan) every 24 h. Basic requirements for inclusion of larvae in all experiments were: overall survival in the control ≥ 90%, hatching rate in the control after 96 h ≥ 80%, and mortality in the positive control (4 mg/L 3,4-dichloro-aniline) ≥ 30%. The pH and oxygen content in all test solutions were pH 7.0 ± 0.2 and 7.5 ± 0.5 mg/L oxygen, respectively at the start and end of the exposure.

3.4.3 Toxicity test

The toxicity test was performed following the Fish Embryo Acute Toxicity Test OECD 236 (2013). Effects were monitored in two independent experiments. Firstly, exposing larvae to low and medium concentrations (0, 1, 2, 5, 10, 20, and 50 mg C/L) and secondly, to medium to high concentrations (0, 50, 100, 200, 300, 400, and 500 mg C/L). According to the OECD 236 guideline, mortality (lack of heartbeat or coagulation), somite formation, detachment of tail-bud from the yolk sac were recorded every 24 h. Additionally, we monitored blood circulation, the appearance of edema or hematomas, and hatching. As there were no negative effects observed for exposure to 0-50 mg C/L of FA until 144 hpf in pre-tests, data was only recorded until 96 hpf for these groups but for 144 h for 50-500 mg C/L and the control. Per group, 30 larvae were exposed and the percentage frequency of the endpoints described above was calculated for each replicate (n=5 per group, percentage frequency was used as an experimental unit).

3.4.4 Concentration of reactive oxygen species and inducibility of respiratory burst

To determine the oxidative stress exerted by FA, reactive oxygen species (ROS) concentration was measured after 96 h exposure to FA. Based on the results of the toxicity tests, the concentration of ROS in the larvae was determined after exposure to FA at 0 mg C/L (Control), 5 mg C/L (low), 50 mg C/L (medium), 300 mg C/L (start of adverse effects), and 500 mg C/L (high concentration). Larvae were washed in dilution water to remove FA residues and transferred with 100 µL dilution water into 96-well fluorescence plates (ELISA plate black med.bind. F, Sarstedt, Nürnberg, Germany). Dead and

retarded larvae were excluded from the test. The ROS concentration was measured by the development of fluorescence after oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Sigma-Aldrich, USA) to dichlorodihydrofluorescein (DCF). According to Goody *et al.* (2013) H2DCF-DA was dissolved in DMSO with a final concentration of 1 mg/mL and stored at -20 °C (for light protection, aliquots were wrapped in aluminum foil). Detection solution was prepared on the day of the experiment by diluting the fluorescence dye with dilution water to a final concentration of 1 µg/mL H2DCF-DA and stored in the dark on ice. 100 µL of the detection solution was added to each well; the plate was immediately covered with aluminum foil and shaken at 200 rpm for 15 sec on a horizontal shaker (MTS 2/4 digital micro-shaker, IKA, Germany) to homogenize the solution. The concentration of the forming DCF is directly proportional to the amount of produced ROS and was detected using a plate reader (Infinite M200, Tecan, Switzerland). Using Magellan 7.2 Software, the following settings were programmed: temperature 26 ± 1 °C, kinetic cycle: duration 4 h, interval: 15 min; shaking: 5 sec, orbital, amplitude 1mm, frequency: 432 rpm; wait: 20 sec; reading mode: top, number of reads: 5, gain value: 100, multiple reads per well: 4x4, circle, border 250 µm; excitation wavelength: 485 nm, excitation bandwidth: 9 nm; emission wavelength: 528 nm, emission bandwidth: 20 nm. Respiratory burst activity was measured as described above after induction with 200 ng/mL PMA (phorbol 12-myristate 13-acetate, Sigma). Following the recommendation given by Goody *et al.* (2013)), each FA treatment was tested using 24 individual larvae as an experimental unit (uninduced and PMA-induced). Only larvae showing no detrimental effects (toxicity test) after the exposure to FA were used for the measurement of ROS. To account for this, a total of 30 larvae per group were exposed to FA as described above and 24 healthy larvae from each group were randomly selected for the subsequent measurement.

3.4.5 Gene expression analysis

40 larvae per group were exposed until 96 hpf to 0, 5, 50, and 500 mg C/L of FA and washed in DIN water to remove any FA residues from the surface. Per group, 30 healthy larvae were randomly selected (Dorts *et al.* 2016), pooled, snap-frozen in liquid nitrogen, and stored until analysis at -80 °C. Five independent repeats (experimental unit) per treatment were conducted.

Larvae were homogenized with a tissue lyser (Qiagen, Germany) using one stainless steel bead per sample (5 mm bead, Qiagen) at a frequency of 18 s^{-1} for 2 x 1.5 min. RNA was extracted using the ReliaPrep™ RNA Miniprep System (Tissue, Promega, USA) in an elution volume of 20 µL per sample. Quality and quantity were determined by gel electrophoresis and by a NanoDrop1000 spectrophotometer (Thermo Scientific, USA). RNA was immediately diluted to 125 ng/µL in a volume of 10 µL RNAase free water and reversely transcribed into cDNA using AffinityScript-RT (Agilent Technologies, USA). Reactions were performed as specified by the manufacturer using the

Mastercycler® nexus (Eppendorf, Germany). In a second step, the expression of 12 different genes (Table 3-1) was analyzed by real-time quantitative PCR (qRT-PCR) (Stratagene Mx3005P, Agilent Technologies). To account for differences between biological samples, primer efficiencies were determined using a pooled cDNA template composed from all samples (3 µL/sample). Samples were analyzed in duplicates and elongation factor 1- alpha (*ef1α*) was used as reference gene. Gene expression was calculated following Pfaffl (2001, 2006). Primers were designed using Clone Manager 7 or the sequence taken from the listed source in Table 3-1; all primers were synthesized by TIB MOLBIOL Syntheselabor GmbH (Germany). Genes are written in lower italic letter, the protein with first letter uppercase and non-italic following the ZFIN Zebrafish Nomenclature Conventions.

3.4.6 Statistical analysis

The data-sets were analyzed using RStudio 1.1.453 software (https://rstudio.com/products/rstudio/download/Libraries: PMCMRplus; dplyr; ggplot2; tidyr; car; openxlsx; tidyverse; gridExtra; grid). Normal distribution and homogeneity of variance were analyzed using Shapiro-Wilk test and Levene's Test. Differences between means of treatment groups were analyzed by one-way ANOVA with Tukey-HSD post-hoc (all pairs) or Dunnett post-hoc (against the corresponding control) when assumptions for parametric testing were met. In the case of non-parametric testing, the Kruskal-Wallis test was used followed by Dunn post-hoc. Statistical analysis of gene expression was performed on log2 values.

The supplement summarizes details of sample sizes, degrees of freedom, and p-values for the gene expression. Differences were considered statistically significant at $p < 0.05$. Graphs are Tukey boxplots, with outliers shown as dots. Data were collected when inclusion criteria were met (see above). No data were excluded from the statistical analysis.

Table 3-1: Primer for RT-qPCR

Gene	F-Sequence	R-Sequence	Description	Reference
cat	AGTGCTCCTGACGTCCAGCCA	TGAAGAACGTGCGCACCTGGG	Catalase	2
ef1- α	TGACTGTGCTGTGCTGATTG	TTTCACTCCCAGGGTGAAAG	Reference Gene	1
gh	CTAGAGCATTGGTGCTGTTG	TCCTCAGGCATAAGACCTTC	Growth hormone	1
gpx	AGATGTCATTCCTGCACACG	AAGGAGAAGCTTCCTCAGCC	Glutathione peroxidase	1
he1-α	AACTGGTGGCAAACAGGTGGTCTC	TCGCTCCTGGATTGCTCATGGTAGA	Hatching enzyme 1 α	6
igf-1	GGCAAATCTCCACGATCTCTAC	CGGTTTCTCTTGTCTCTCTCAG	Insulin-like growth factor 1	6
keap1	TGTAAATGCGAGCGGAGGG	GGATGTGCTCTTTTCACCCTTAT	Kelch-like ECH associating protein 1	4
lyz	TTGTGTCTGGCGTGGATGTC	GCACTCGGTGGGTCTTAAAC	Lysozyme	1
mpx	GCTGCTGTTGTGCTCTTTCA	TTGAGTGAGCAGGTTTGTGG	Myeloperoxidase	5
nrf2	CTCCCAGAGTTGCAGCAGT	CACTTCTGTTTGAGCCGAGC	Nuclear factor erythroid 2-related factor 2	4
sod-1	CCGTCTATTTCAATCAAGAGGGTG	CATGAGGGTTGAAGTGCGGA	Superoxide dismutase 1	4
sod-2	CGCATGTTCCCAGACATCTA	GAGCGGAAGATTGAGGATTG	Superoxide dismutase 2	2

1: this study; 2 Brammell and Wigginton (2010)), 3: Yousefi *et al.* (2018)), 4: Liu *et al.* (2015)), 5: Hsu *et al.* (2004)), 6: Mu *et al.* (2016))

3.5 Results

3.5.1 Toxicity tests

Freshly fertilized eggs were exposed to FA continuously for 96 h and 144 h, respectively. The development of the larvae was monitored every 24 h. No significant effects were determined after 24 h and 48 h of exposure. Exposure to FA resulted in a biphasic hatching response at 72 h (Table 3-2). Concentrations between 20 mg C/L and 200 mg C/L (20 and 50 mg C/L when testing 1 to 50 mg C/Land 50 to 200 mg C/L when testing 50 to 500 gm C/L) significantly increased the percentage of hatched larvae compared to the control. While around 38% of non-exposed larvae hatched after 72 h, the percentage significantly increased above 63% in larvae exposed to concentrations from 20 mg C/L to 200 mg C/L. Concentrations higher than 200 mg C/L and lower than 20 mg C/L did not alter the hatching rate significantly compared to the control group. Overall hatching success of all groups after 96 h was at least 85% without significant differences between exposure groups and controls. The formation of edema started to increase significantly after 96 h in larvae exposed to 300 to 500 mg C/L. After 120 h, the edema formation in the 300 mg C/L larvae was not significant anymore. The decreased percentages of edema formation can be explained by resolved edema or the death of the larvae. The same was observed in the 400 mg C/L where the percentage of edema dropped from 15.8% after 120 h ($p<0.05$) to 15.0% ($p>0.05$). Impairment of the blood circulation started in the 400 mg C/L and 500 mg C/L larvae after 72 h of exposure but was not significant in the 400 mg C/L larvae after 96 h ($p=0.08$). Starting after 72 h of exposure to 500 mg C/L caused the formation of hematomas; furthermore, the mortality significantly increased to 16% (120 hpf) and 30% (144 hpf).

Table 3-2: Results of toxicity tests. Data are expressed as the arithmetic mean ± standard deviation (SD). p<0.05 (*), p<0.01 (**), and p<0.001 (***); significances were analyzed against the control using Kruskal-Wallis-Test and Dunn post-hoc; n=5 (30 larvae per repeat)

72 h exposure					
Fulvic acid concentration in mg C/L	**Hatched %**	**Edema %**	**Impaired Blood circulation %**	**Hematomas %**	**Mortality %**
0	42.8 ± 11.8	0.8 ± 1.9	0.8 ± 1.1	0.4 ± 0.9	0.0 ± 0.0
1	54.7 ± 16.8	1.3 ± 1.8	0.7 ± 1.5	0.7 ± 1.5	0.7 ± 1.5
2	54.8 ±21.4	4.0 ± 4.3	2.0 ± 1.8	1.4 ± 1.9	0.7 ± 1.5
5	63.8 ± 22.8	2.7 ± 4.3	0.7 ± 1.5	0.0 ± 0.0	2.0 ± 4.5
10	62.7 ± 16.7	2.7 ± 2.8	0.7 ± 1.5	0.7 ± 1.5	0.0 ± 0.0
20	74.7 ± 11.0*	1.3 ± 3.0	0.7 ± 1.5	0.0 ± 0.0	2.0 ± 3.0
50	74.0 ±12.1*	1.3 ± 1.8	0.7 ± 1.5	0.0 ± 0.0	0.7 ± 1.5
96 h exposure					

Fulvic acid concentration in mg C/L	Hatched %	Edema %	Impaired Blood circulation %	Hematomas %	Mortality %
0	86.1 ± 22.9	2.1 ± 3.2	1.7 ± 1.6	0.3 ± 0.9	0.7 ± 1.1
1	96.1 ± 3.3	2.8 ± 2.5	2.2 ± 2.7	0.6 ± 1.4	1.1 ± 1.7
2	98.3 ± 1.8	2.8 ± 3.3	2.8 ± 2.5	0.0 ± 0.0	0.6 ± 1.4
5	97.2 ± 6.8	3.3 ± 4.2	2.2 ± 4.0	0.0 ± 0.0	1.1 ± 2.7
10	99.4 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20	97.8 ± 2.7	1.1 ± 1.7	1.1 ± 1.7	0.0 ± 0.0	1.7 ± 2.8
50	98.3 ± 1.8	1.7 ± 1.8	3.3 ± 3.0	0.0 ± 0.0	0.6 ± 1.4
72 h exposure					
Fulvic acid concentration in mg C/L	**Hatched %**	**Edema %**	**Impaired Blood circulation %**	**Hematomas %**	**Mortality %**
0	36.7 ± 11.3	0.8 ± 1.1	0.4 ± 0.9	0.4 ± 0.9	1.3 ± 1.1
50	62.7 ± 14.4*	0.7 ± 1.5	0.7 ± 1.5	0.0 ± 0.0	0.7 ± 1.5
100	72.0 ± 12.4**	2.0 ± 1.8	0.7 ± 1.5	0.0 ± 0.0	1.3 ± 1.8
200	70.7 ± 10.4**	2.0 ± 3.0	2.7 ± 2.8	0.0 ± 0.0	0.7 ± 1.5
300	55.3 ± 12.8	4.0 ± 5.5	2.7 ± 6.0	0.7 ± 1.5	1.3 ± 3.0
400	39.9 ± 17.9	9.7 ± 11.0	8.1 ± 5.4*	4.7 ± 6.9	2.0 ± 3.0
500	50.8 ± 16.0	11.3 ± 9.6	18.0 ± 10.7**	12.5 ± 9.1**	6.0 ± 8.3
96 h exposure					
Fulvic acid concentration in mg C/L	**Hatched %**	**Edema %**	**Impaired Blood circulation %**	**Hematomas %**	**Mortality %**
0	85.6 ± 13.8	0.7 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 0.8
50	97.2 ± 2.5	0.6 ± 1.4	0.6 ± 1.4	0.6 ± 1.4	0.6 ± 1.4
100	97.8 ± 1.7	2.2 ± 1.7	0.6 ± 1.4	0.0 ± 0.0	1.1 ± 1.7
200	94.9 ± 9.4	2.2 ± 2.7	1.1 ± 1.7	0.0 ± 0.0	1.1 ± 1.7
300	95.2 ± 4.5	6.7 ± 3.0*	3.3 ± 5.2	0.0 ± 0.0	2.1 ± 4.0
400	93.0 ± 8.2	7.9 ± 5.3*	6.8 ± 6.6	2.2 ± 4.0	2.8 ± 4.4

500	93.8 ± 7.1	11.8 ± 7.5**	22.1 ± 6.6***	8.6 ± 8.1**	8.9 ± 9.3
120 h exposure					
Fulvic acid concentration in mg C/L	**Hatched %**	**Edema %**	**Impaired Blood circulation %**	**Hematomas %**	**Mortality %**
0	94.8 ± 6.3	1.1 ± 1.1	0.5 ± 1.0	0.0 ± 0.0	1.0 ± 1.2
50	97.5 ± 3.2	0.8 ± 1.7	1.7 ± 1.9	0.8 ± 1.7	1.7 ± 1.9
100	96.7 ± 2.7	2.5 ± 1.7	1.7 ± 1.9	0.8 ± 1.7	1.7 ± 1.9
200	97.5 ± 3.2	2.5 ± 3.2	0.8 ± 1.7	0.0 ± 0.0	0.8 ± 1.7
300	95.0 ± 5.8	4.2 ± 4.2	1.7 ± 3.3	0.0 ± 0.0	2.5 ± 5.0
400	90.8 ± 5.7	15.8 ± 3.2*	15.0 ± 11.1*	0.8 ± 1.7	8.3 ± 6.4
500	85.8 ± 13.4	27.5 ± 6.9**	30.0 ± 4.7*	5.8 ± 5.0**	22.5 ± 8.3*
144 h exposure					
Fulvic acid concentration in mg C/L	**Hatched %**	**Edema %**	**Impaired Blood circulation %**	**Hematomas %**	**Mortality %**
0	94.8 ± 6.3	0.6 ±1.0	0.5 ± 0.1	0.0 ± 0.0	0.5 ± 1.0
50	97.5 ± 3.2	0.0 ± 0.0	0.8 ± 1.7	0.8 ± 1.7	1.7 ± 1.9
100	96.7 ± 2.7	1.7 ± 1.9	1.7 ± 1.9	0.0 ± 0.0	1.7 ± 1.91
200	97.5 ± 3.2	2.5 ± 3.2	0.8 ± 1.7	0.0 ± 0.0	0.8 ± 1.7
300	95.0 ± 5.8	5.8 ± 5.0	4.2 ± 6.3	0.0 ± 0.0	2.5 ± 5.0
400	90.8 ± 5.7	15.0 ± 9.6	23.3 ± 11.5*	0.0 ± 0.0	8.3 ± 6.4
500	85.8 ± 13.4	25.8 ± 5.7*	28.3 ± 6.4*	0.8 ±1.7	32.5 ± 4.2**

3.5.2 Concentration of ROS and inducibility of respiratory burst

ROS concentration in larvae exposed for 96 h to 5 and 50 mg C/L FA was unaffected but increased by 2 and 2.6 folds in the 300 and 500 mg C/L groups (Figure 3-2). The relative fluorescence values after PMA induction increased by 2060 RFU in all groups without differences between the treatments. However, due to higher base values in uninduced larvae, the 300 and 500 mg C/L groups showed significantly higher RFUs compared to the control.

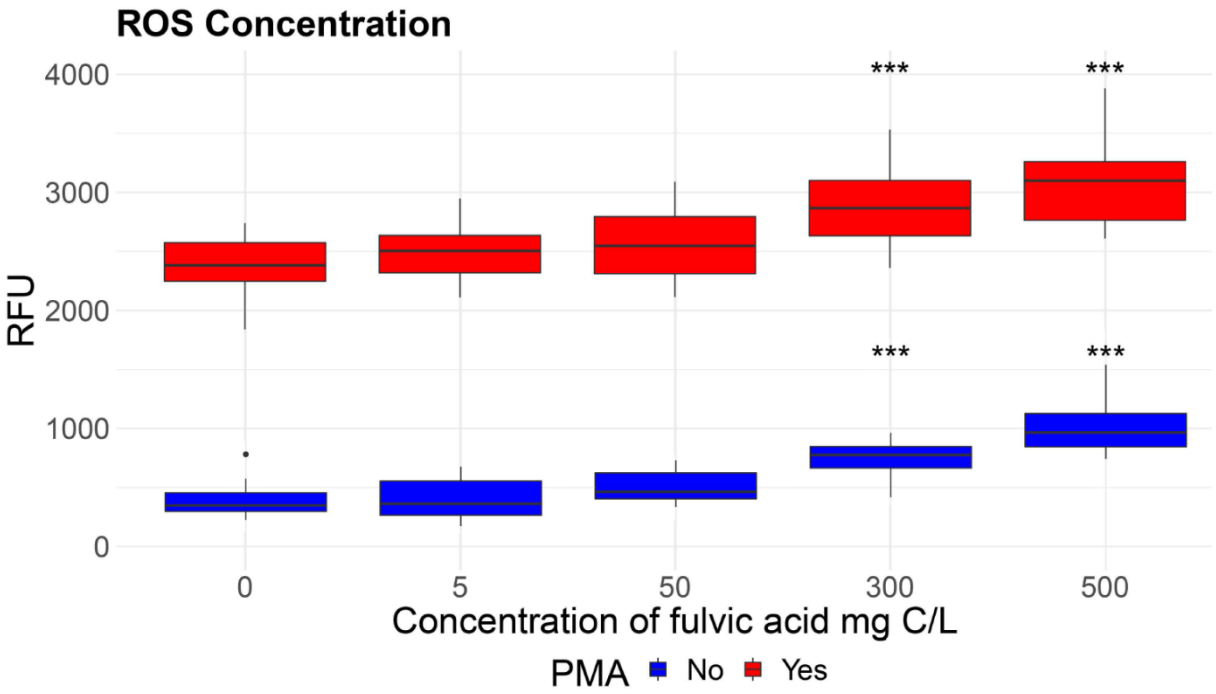

Figure 3-2: Concentration of reactive oxygen species (ROS) in larvae exposed for 96 h to fulvic acid before (No) and after (Yes) respiratory burst induction by phorbol 12-myristate 13-acetate (PMA). RFU-relative fluorescence unit; * - p<0.001 (Kruskal-Wallis-Test), n=24.**

3.5.3 Gene expression

Exposure to low (5 mg C/L) and medium (50 mg C/L) concentrations of FA increased the expression of the growth hormone *gh* by 1.3 and 2.4 folds (Figure 3-3). At the same time, the hatching enzyme *he1-α* increased to 1.5 and 2.1 folds. In larvae exposed to 500 mg C/L *gh* decreased to 0.8 folds (80% of the control expression), while *he1-α* decreased to 0.4 folds (40% of the control expression). *Igf-1* significantly decreased to 0.5-fold in this group but was unaffected in larvae exposed to the low and medium concentrations. Exposure to 5 mg C/L and 50 mg C/L FA significantly increased the expression of *lyz* by 1.3- and 1.6-fold (Figure 3-4). *mpx* followed the same trend and increased by 1.5- and 2.0-fold. In larvae exposed to 500 mg C/L, both genes were not significantly affected, however, mpx showed a trend towards an inhibited gene expression. Exposure to 500 mg C/L increased the expression of *keap1* by 1.6-fold and *nrf2* by 1.9-fold (Figure 3-5). An increase of *nrf2* by 1.6-fold was also observed in larvae exposed to 50 mg C/L, while both genes were not significantly affected after exposure to 5 mg C/L. In the high concentration group, the antioxidative enzymes cat and sod-1 were significantly increased (2.1 and 1.7-fold). However, gpx, which catalyzes the formation of hydrogen peroxide to water was not affected. Interestingly, exposure to 50 mg C/L resulted in an increase of *sod-2* by 1.5-fold, while it was unaffected in all other exposure groups.

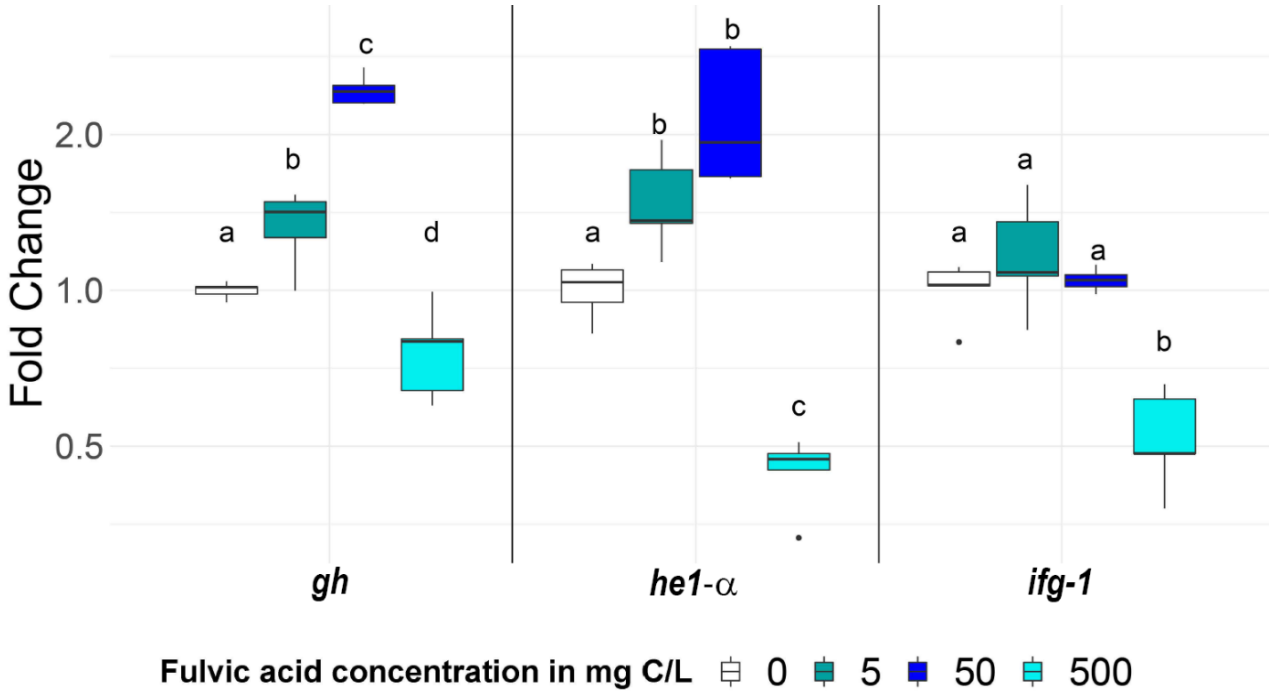

Figure 3-3: Gene expression of growth-related genes in larvae exposed for 96 h to fulvic acid; gh: growth hormone, he1-α: hatching enzyme 1, igf-1: insulin-like growth factor 1, different letters within one gene represent significant differences between the exposure groups; ANOVA-Tukey-HSD, n=5 (30 larvae pooled per repeat).

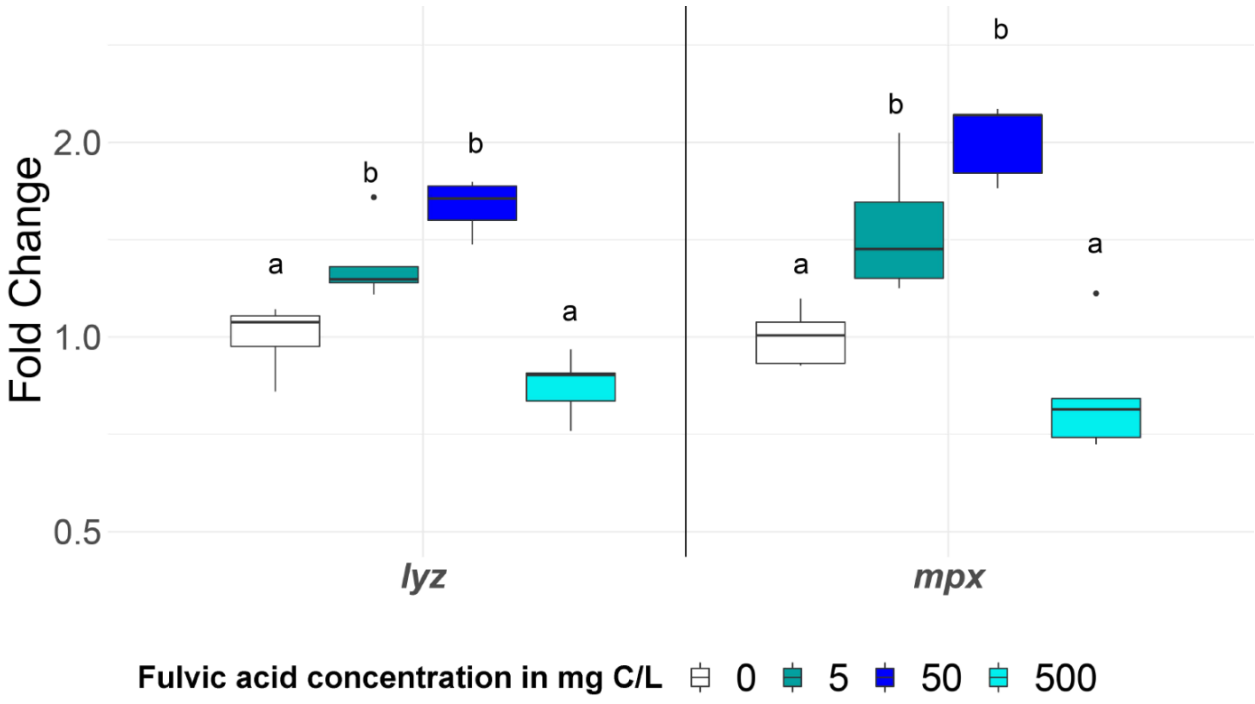

Figure 3-4: Gene expression of immune related genes in larvae exposed for 96 h to fulvic acid; lyz: lysozyme, mpx: myeloperoxidase, different letters within one gene represent significant differences between the exposure groups; ANOVA-Tukey-HSD, n=5 (30 larvae pooled per repeat).

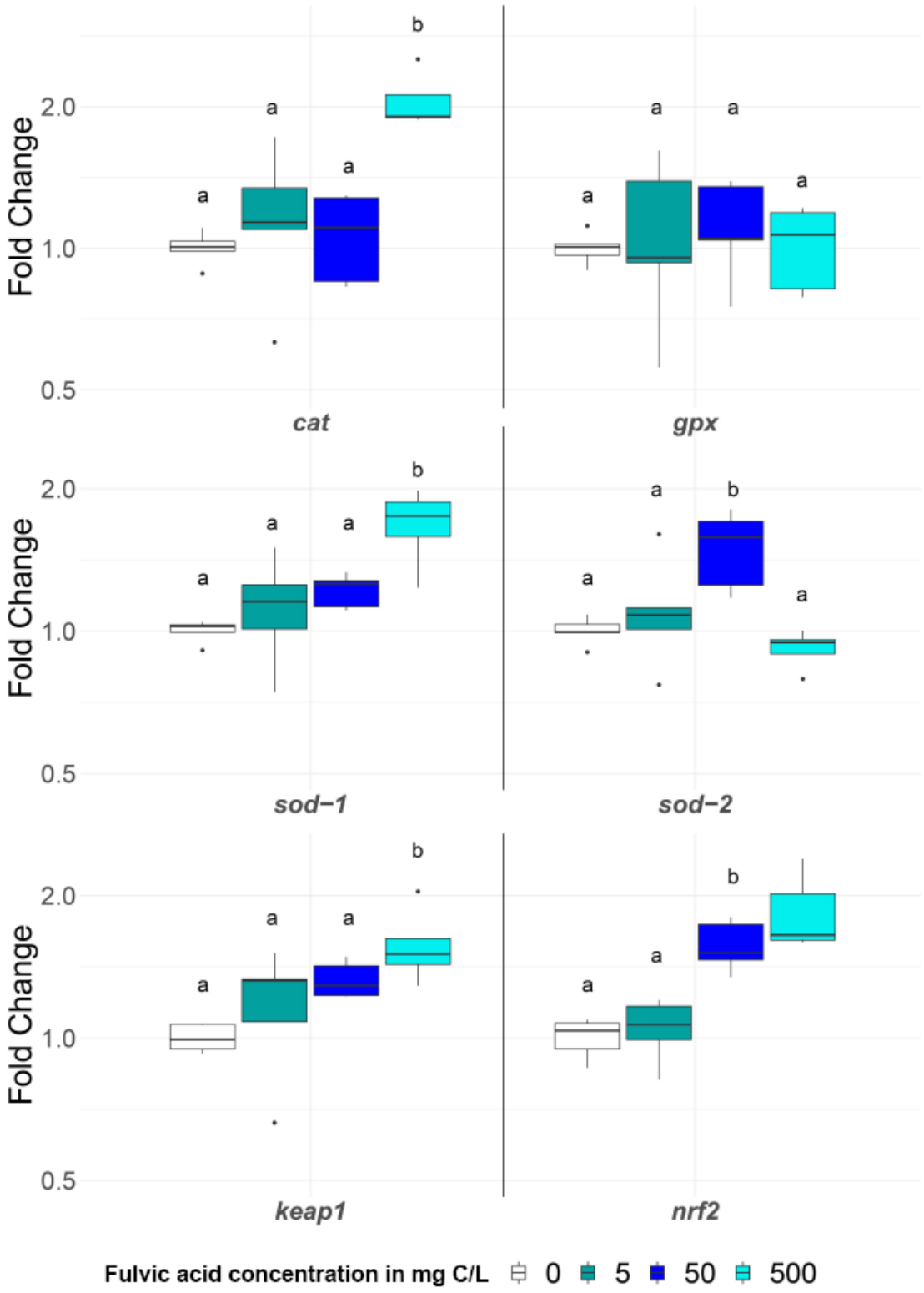

Figure 3-5: Gene expression of antioxidant related genes in larvae exposed for 96 h to fulvic acid; cat: catalase, gpx: gluthation peroxidase, sod-1 and 2: superoxide dismutase 1 and 2, keap1: kelch-like ECH associating protein 1, nrf2: nuclear factor erythroid 2-related factor 2, different letters within one gene represent significant differences between the exposure groups; ANOVA-Tukey-HSD, n=5 (30 larvae pooled per repeat).

3.6 Discussion

3.6.1 Growth and hatching

In larvae exposed to FA concentrations between 20 and 200 mg C/L hatching was accelerated. Similar results were reported after exposure to the immunostimulant pectin isolated from *Arthrospira* (*Spirulina*) *maxima* (Edirisinghe *et al.* 2019). However, stress by chemicals and low oxygen concentration can also lead to accelerated hatching (Cloud 1981, Latham and Just 1989, Schreck *et al.* 2001), enabling the larvae to escape from unfavorable conditions. Oxygen concentration in all test solutions was 7.5 mg/L; external low oxygen can therefore be excluded as a reason for the accelerated hatching. The ROS concentration measured inside the larvae after exposure to 5 and 50 mg C/L was not elevated. Oxidative stress is therefore also unlikely to cause accelerated hatching.

In both exposure groups, 5 and 50 mg C/L, *gh,* and *he1-α* expression were significantly induced, while *igf-1* remained unaffected. Growth in vertebrates is a complex interaction of molecular and biochemical factors. Igf-1 and Gh are two pivotal protein members of the growth hormone/insulin-like growth factors (GH/IGFs) axis that plays an important role in the regulation of fish growth (De Azevedo Figueiredo *et al.* 2007, Dang *et al.* 2018). Gh is produced in the pituitary and triggers the release of Igf-1 from the liver. Exogenous Gh has been used to increase the growth performance in many species, with importance for aquaculture. The growth performances of *gh*-transgenic lines of Coho salmon (*Oncorhynchus kisutch*) and Arctic charr (*Salvelinus alpinus*) were on average 11 and 14 times higher than that of non-transgenic fish (Devlin *et al.* 1994, Pitkänen *et al.* 1999). Administrations by injection or implants have shown, that Gh affects not only the growth directly, but also indirectly. Fish with increased levels of Gh showed elevated appetite and improved feed conversion efficiency as demonstrated by Hallerman *et al.* (2007)). Devlin *et al.* (2001)) showed, that the genetic background strongly determines how strong the fish response to Gh injection. While the specific growth rate increased only by 9.5% (from 2.09 to 2.29) in domesticated rainbow trout, that of wild trout increased by 166% (from 0.68 to 1.81). Especially less domesticated fish species or undomesticated wild populations would therefore benefit from an early treatment with FA.

The hatching enzyme He is a zinc metalloprotease related to the astacin enzyme family (Sano *et al.* 2008). He-2 is expressed in a very low concentration and is nonfunctional for hatching, while He-1 catalyzes the cleavage of the chorion, initiating hatching. Expression of He-1 is furthermore involved in embryo development and decreased *he-1α* gene expression correlates with delayed embryonal development and hatching (Mu *et al.* 2016, Castro *et al.* 2021). Exposure to 500 mg C/L decreased the expression of *gh*, *igf-1,* and *he-1α*, however, we did not find a delay in hatching. However, we did find an increased translation of *he1-α* in the 5 mg C/L and 50 mg C/L larvae, together with accelerated hatching in the 20 mg C/L to 200 mg C/L groups. Hence, it can be assumed that an increased expression

of *he1-α* is associated with accelerated hatching as observed in the present study. Moreover, overexpression of *gh* in fish embryos affected the rate or the efficiency (or both) of which stored yolk energy is converted into body mass (Devlin *et al.* 1995). It can be assumed, that exposure to 5 and 50 mg C/L FA increases the conversion of the yolk into body mass as well, resulting in improved metabolism and growth of the larvae. The faster development and the increased expression of *he-1α* would then induce the earlier hatching. However, if FA indeed increases the growth of larvae will require further experiments, focusing on the biomass, the body length, and the yolk size during exposure. The same applies to verifying if the accelerated hatching and increased metabolisms act as a jump-start for the larvae and result in better growth performance in older fish.

3.6.2 Immune response

The *lyz* gene codes for lysozyme, an enzyme catalyzing the cleavage of β 1-4 linked N-acetylmuramic acid and N-acetyl-D-glucosamine. The *mpx* gene encodes a myeloperoxidase, involved in the respiratory burst. Both are important parts of innate immunity and mark neutrophils. 5 mg C/L and 50 mg C/L of FA induced the expression of *lyz* and *mpx*. Increased activity of lysozyme and myeloperoxidase were reported as a result of immune stimulation via parabiotic feed additives (as reviewed by Choudhury and Kamilya (2018))). Anti-bacterial activities, including a significant increase in *lyz* expression, were also reported after exposing zebrafish larvae to a phenolic-rich extract from *Clerodendrum cyrtophyllum* (Nguyen *et al.* 2020). Furthermore, stimulation with LPS resulted in increased expression of *lyz* and *mpx* (Oyarbide *et al.* 2012). This aligns with our previous findings in juvenile rainbow trout where FA significantly increased the lysozyme activity in the gills and the phagocytic activity of head kidney leucocytes (Lieke *et al.* 2021b). Interestingly, the respiratory burst activity of leucocytes in the zebrafish larvae was not affected by any exposure concentration. Larvae exposed to 5 and 50 mg C/L FA seem to have a higher number of leucocytes, as indicated by increased expression of the marker *mpx*, however, these leucocytes are apparently not activated. To verify these findings, further studies, evaluating the cell composition and the number of cells by flow cytometry are needed. It would be especially interesting to determine the effects on the different phagocytic cell populations, such as the macrophages and neutrophils, as these are apparently not activated to perform a respiratory burst, but might increase in number or phagocytic activity. An increased number of leucocytes together with increased lysozyme activity could enable the larvae to defend themselves better against infections and diseases.

The immune and neuroendocrine systems are highly interdependent. While stress hormones, such as cortisol and catecholamines inhibit several parameters of the immune response, Gh has been shown to enhance different immune functions (Harris and Bird 2000). Gh shares a similar structure with important immune-related proteins such as the interferons, the interleukins IL-2, IL-4, and IL-5, and

the colony-stimulating factors CSF-1, CSF-2, and CSF-3 (Sprang and Fernando Bazan 1993). Plasma lysozyme activity and the production of superoxide anions in peripheral blood leukocytes of rainbow trout were stimulated after intra-peritoneal Gh administration (Yada *et al.* 2001, Yada 2007). Furthermore, increased phagocytosis, superoxide anion production, natural killer cell activity, and antibody production were reported (Sakai *et al.* 1996, Yada *et al.* 1999, Harris and Bird 2000). The increase of *lyz* and *mpx* expression in larvae exposed to 5 and 50 mg C/L could therefore be caused by the stimulated *gh* expression.

Mihai *et al.* (2011)) introduced the concept of "trained immunity". Components of the innate immune system are activated by a primary infection, resulting in a heightened response to a secondary infection (by the same microorganism or by a different one). Early-life challenges can have important impacts on the responses later in life. Zebrafish that have been infected with *Aeromonas salmonicida achromogenes* at an early age had higher expression levels of immune-related genes including *lyz* and *mpx* before a second infection (Cornet *et al.* 2020). They also responded with a fast and strong stimulation of *mxp* after the second infection. It can be hypothesized that induction of the immune system of early life stages as observed by FA exposure could therefore help to train the immune response and might help to prevent or reduce infections of older fish. Further investigations are needed to verify this hypothesis and to give a deeper insight into the mechanisms.

3.6.3 Oxidative stress response

The variety of reactive functional groups, hydrophilic and hydrophobic sides, and free radical moieties, enable the HSs to interact and react with a wide range of compounds and biological structures. Based on the redox-active functional moieties in their structure, they can be natural reductants or oxidants (Aeschbacher *et al.* 2012, Lv *et al.* 2018). While phenolic moieties add to the electron-donor capacity (EDC), quinone moieties add to the electron-accepting capacity (EAC). However, this can be shifted by chemical or microbial reduction pretreatment (Peretyazhko and Sposito 2006) or by temperature (Tan *et al.* 2017).

This chemical diversity of HSs explains the many contradictory results published over years. Oxidative stress can cause tissue damage, inflammation, and apoptosis when the defensive mechanisms are depleted (Day 1991, Cheng *et al.* 1999, Glover and Wood 2005). In the present study, FA at concentrations from 300 mg C/L upwards had severe negative effects on the development of the larvae. An impairment of the blood circulation cuts off the distal areas from a sufficient supply of oxygen and nutrients causing retardation and mortality. The formations of hematoma and edema furthermore indicate strong oxidative stress. Indeed, at high concentrations, the ROS concentration in the larvae was significantly increased. This could be caused by FA directly, as it contains a relatively high amount of persistent free radicals, which have been shown to cause oxidative stress and damage

at high concentrations (Balakrishna *et al.* 2009, Lieke *et al.* 2018). A high electron acceptor capacity (EAC), as mediated by high amounts of quinones, can also cause oxidative stress, however, in our FA, the EAC is much lower than the EDC. At lower concentrations, the phenol-rich structures protect from oxidative stress, a finding in good agreement with our previous study in rainbow trout in which no oxidative stress occurred in the gills (Lieke *et al.* 2021b).

Exposure of organisms to xenobiotics causes oxidative and electrophilic stress (Niture and Jaiswal 2010). If not coped with, damage to macromolecules such as nucleic acids and proteins can lead to cellular toxicity, immunogenicity, inflammation, and gene mutations (Scott Obach and Kalgutkar 2010). As electrophiles and ROS are being generated by metabolisms and organisms are constantly exposed, the cells have mechanisms to cope with stress. One of the key transcription factors is Nrf2. Under normal conditions, Nrf2 builds a heterodimer with Keap1 leading to degradation of the heterodimer via ubiquitination (Suzuki and Yamamoto 2015). Arylation or oxidation of cysteine residues in Keap1 upon cellular stress led to dissociation of the heterodimer.

Exposure to 500 mg C/L significantly increased the expression of keap1 and nrf2 by 1.6-fold and 1.9-fold, respectively. An increased ratio of Nrf2 over Keap1 decreases the formation of the heterodimer, allowing translocation of Nrf2 into the nucleus. As a transcription factor, Nrf2 is here involved in the activation of a cascade of xenobiotic response elements (XREs) and antioxidant or electrophile response elements (AREs or EREs). AREs and EREs coordinate the induction of more than 200 genes involved in the detoxification of xenobiotics and protection against oxidative stress (Niture and Jaiswal 2010). Among those defensive enzymes are catalase (Cat), superoxide dismutases (Sod-1 and Sod-2), and glutathione peroxidase (Gpx). cat and sod-1 were significantly increased in larvae exposed to 500 mg C/L (2.1 and 1.7-fold). This confirms the findings of increased ROS concentration inside the larvae exposed to high FA concentrations. The overserved damages, such as tissue damage and edema formation can therefore be attributed to oxidative stress. At this concentration, the protective effect of the quinones can no longer cope with electrophilicity and oxidative stress caused by the high amounts of PFRs. The expression of Gpx, which catalyzes the formation of hydrogen peroxide to water was not affected. However, for this reaction, glutathione (GSH) is needed, which is a strong antioxidant itself and might react directly. Furthermore, GSH is required as a substrate in phases 1 and 2 of the xenobiotic metabolism pathway and the regeneration via the glutathione reductase requires energy in form of NADPH. As dealing with a strong stressor and upregulation of several defensive genes is highly energy consuming, the regeneration of GSH might not occur, explaining why *gpx* was not affected in larvae exposed to 500 mg C/L.

Interestingly, exposure to 50 mg C/L increased the expression of *nrf2* as well, accompanied by an increase in *sod-2*, while the other genes involved in detection and protection from oxidative stress

were not significantly affected. Apparently, FA exerted oxidative stress at this concentration as well, but to a lower extent and without having negative physiological effects. Low concentrations of ROS exert mild oxidative stress, which induces the activation of defense mechanisms leading to the protection of cells (Minois 2000, Niture and Jaiswal 2010). The protective concentration, however, might be only slightly lower than detrimental concentrations. Activation of Nrf2 inhibits the transcription of several pro-inflammatory cytokines, such as IL-6 and Tnf-α, and promotes the transcription of anti-inflammatory cytokines, such as IL-10 (Ahmed *et al.* 2017, Helou *et al.* 2019). Together with the activation of Sod-2, this could reduce inflammations in larvae exposed to low or medium concentrations of FA. Indeed, in vitro suppression of the release of IL-6 and Tnf-α has been reported after exposure to HSs (Szot *et al.* 2019, Rusliandi *et al.* 2020). However, more studies are needed to verify if breeding fish larvae in FA can help to prevent inflammations in vivo.

3.7 Conclusion

The application of immunostimulants via feed has become a widely used practice to improve the condition of fish in aquaculture and reduce the use of therapeutics. However, this approach is not applicable for the vulnerable pre-feeding life stages. At this early stage protection against diseases depend on the innate immune response of the larvae. Raising larvae in 5mg C/L and 50 mg C/L of FA stimulated the expression of genes involved in the innate immune defense, which can result in strengthened protection against diseases. This early stimulation of the innate immune system might even improve the protection of older fish. However, a challenge-experiment using a virulent fish pathogen would be needed to verify if the increased gene expression translates into improved protection against diseases. Additionally, the accelerated hatching enables the larvae to switch faster to exogenous feeding, allowing the use of feed additives to further boost overall health. Exposure to concentrations ≥ 300 mg C/L of FA resulted in oxidative stress, causing tissue damage and mortality. Interestingly, at medium range concentrations (50 mg C/L), the oxidative defense mechanisms were activated as well without any obvious downsides. This might be a hint towards an anti-inflammatory effect of the FA but will require future studies. Implementing FA into the general maintenance of fish hatchery is a natural way to increase overall health and can help to increase survival at this critical life stage.

3.8 Compliances with Ethical Standards

Experiments were performed following the European Directive 2010/63/EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes and the German AnimalWelfare Act. Experiments with fish larvae until the start of exogenous feeding do not require special permission by the animal experimental ethics committee of the Berlin State Office for Health and Social Affairs (LaGeSo).

3.9 Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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3.11 Authors Contribution

All authors contributed to the conceptualization of the paper. Physiological experiments were performed by S. Be. and T. L. Molecular analysis was performed by S. Bi. and T. L. and supervised by S. H. H. Sample analysis, data analysis, writing of the original draft, graph creation, and editing were performed by T.L. Funding was acquired by C. E. W. S., T. M., and T. L. The study was supervised by K. K. and W. K. All authors contributed to reviewing the manuscript draft.

3.12 Competing Interests

The authors declare that there are no conflicts of interest. The founding sponsor had no role in the design, content, or decision to publish the paper. Mention of trade names or commercial products in this article is solely for providing specific information and does not imply recommendation or endorsement by the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Humboldt University, or the Federal Ministry for Economic Affairs and Energy.

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3.14 Supplement: Results of gene expression

ANOVA, n=5 with 30 larvae pooled per treatment, 4 treatments, df=3; followed by TukeyHSD-Test

3.14.1 cat

Comparison	**p-value**
0 mg C/L against 5 mg C/L	0.83757680
0 mg C/L against 50 mg C/L	0.98398232
0 mg C/L against 500 mg C/L	0.00067505
5 mg C/L against 50 mg C/L	0.96284764
5 mg C/L against 500 mg C/L	0.00361738
50 mg C/L against 500 mg C/L	0.00137410

3.14.2 gh

Comparison	**p-value**
0 mg C/L against 5 mg C/L	0.025544
0 mg C/L against 50 mg C/L	1.2170e-07

0 mg C/L against 500 mg C/L	0.024798
5 mg C/L against 50 mg C/L	1.7486e-05
5 mg C/L against 500 mg C/L	4.5307e-05
50 mg C/L against 500 mg C/L	2.3565e-09

3.14.3 gpx

Comparison	**p-value**
0 mg C/L against 5 mg C/L	0.99917
0 mg C/L against 50 mg C/L	0.95344
0 mg C/L against 500 mg C/L	1.00000
5 mg C/L against 50 mg C/L	0.97952
5 mg C/L against 500 mg C/L	0.99922
50 mg C/L against 500 mg C/L	0.95411

3.14.4 he1-α

Comparison	**p-value**
0 mg C/L against 5 mg C/L	0.04473872
0 mg C/L against 50 mg C/L	0.00015958
0 mg C/L against 500 mg C/L	8.2939e-05
5 mg C/L against 50 mg C/L	0.05322027
5 mg C/L against 500 mg C/L	6.3065e-07
50 mg C/L against 500 mg C/L	1.3930e-08

3.14.5 igf-1

Comparison	**p-value**
0 mg C/L against 5 mg C/L	0.57484084
0 mg C/L against 50 mg C/L	0.97763958
0 mg C/L against 500 mg C/L	0.00013665
5 mg C/L against 50 mg C/L	0.80382340
5 mg C/L against 500 mg C/L	1.2977e-05
50 mg C/L against 500 mg C/L	6.4815e-05

3.14.6 lyz

Comparison	p-value
0 mg C/L against 5 mg C/L	0.01349957
0 mg C/L against 50 mg C/L	5.4533e-05
0 mg C/L against 500 mg C/L	0.12496589
5 mg C/L against 50 mg C/L	0.05619939
5 mg C/L against 500 mg C/L	0.00012256
50 mg C/L against 500 mg C/L	1.0351e-06

3.14.7 mpx

Comparison	p-value
0 mg C/L against 5 mg C/L	0.0055179
0 mg C/L against 50 mg C/L	1.2017e-05
0 mg C/L against 500 mg C/L	0.0380453
5 mg C/L against 50 mg C/L	0.0191448
5 mg C/L against 500 mg C/L	3.1448e-05
50 mg C/L against 500 mg C/L	2.7400e-07

3.14.8 sod-1

Comparison	p-value
0 mg C/L against 5 mg C/L	0.7929849
0 mg C/L against 50 mg C/L	0.2896560
0 mg C/L against 500 mg C/L	0.0010456
5 mg C/L against 50 mg C/L	0.7938272
5 mg C/L against 500 mg C/L	0.0067850
50 mg C/L against 500 mg C/L	0.0429074

3.14.9 sod-2

Comparison	p-value
0 mg C/L against 5 mg C/L	0.870221
0 mg C/L against 50 mg C/L	0.010802
0 mg C/L against 500 mg C/L	0.854027
5 mg C/L against 50 mg C/L	0.049033
5 mg C/L against 500 mg C/L	0.427110
50 mg C/L against 500 mg C/L	0.002130

3.14.10 keap1

Comparison	p-value
0 mg C/L against 5 mg C/L	0.714868
0 mg C/L against 50 mg C/L	0.134921
0 mg C/L against 500 mg C/L	0.010513
5 mg C/L against 50 mg C/L	0.602984
5 mg C/L against 500 mg C/L	0.084508
50 mg C/L against 500 mg C/L	0.560029

3.14.11 nrf2

Comparison	p-value
0 mg C/L against 5 mg C/L	0.97115855
0 mg C/L against 50 mg C/L	0.00069757
0 mg C/L against 500 mg C/L	2.5523e-05
5 mg C/L against 50 mg C/L	0.00167294
5 mg C/L against 500 mg C/L	5.6116e-05
50 mg C/L against 500 mg C/L	0.32917335

4 Modification of the chemically induced inflammation assay reveals the Janus face of a phenol rich fulvic acid

Thora Lieke [a,b,*], Christian E.W. Steinberg [b,c], Thomas Meinelt [a], Klaus Knopf [a,b], Werner Kloas [a,b]

[a] Department of Ecophysiology and Aquaculture, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, 12587 Berlin, Germany

[b] Humboldt University of Berlin, Faculty of Life Sciences, 10115 Berlin, Germany

[c] Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, 650500 Kunming, China

* Corresponding author (lieke@igb-berlin.de), Phone: +49 (0)30 64181 641, ORCID ID: 0000-0002-4345-171

4.1 Highlights

- $CuSO_4$ induced inflammation activates leukocytes to produce reactive oxygen species
- The inflammation can be monitored using the fluorescence dye H2DCFDA

- Feasible alternative to using GFP-zebrafish strains to monitor *in vivo* inflammation
- Fulvic acid chances its inflammatory properties depending on the concentration

4.2 Abstract

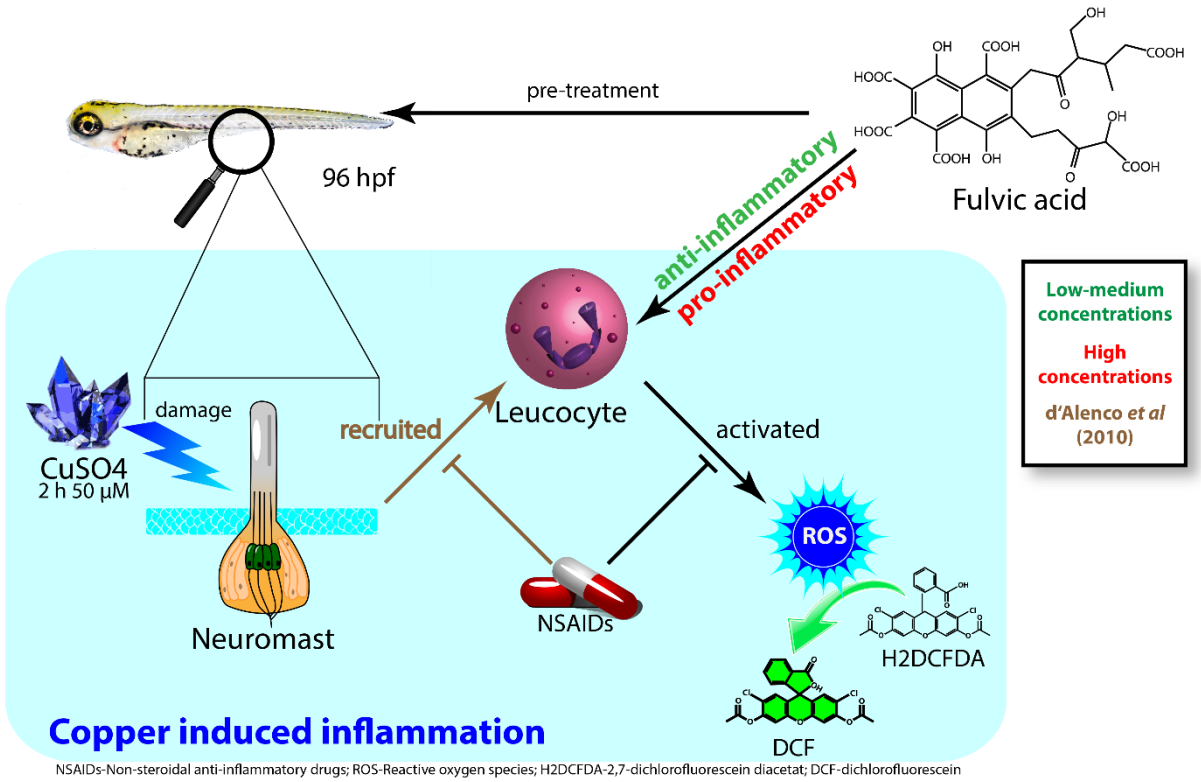

Figure 4-1: Graphical abstract

Inflammation is an essential process as a reaction towards infections or wounds. Exposure to hazardous environmental pollutants can lead to chronic inflammations, where the resolving phase is delayed or blocked. Very contradictory studies have been reported on the pro- and anti-inflammatory effects of humic substances (HSs) leading to significant disagreements between researchers. To a certain extent, this can be attributed to the chemical heterogeneity of this group of xenobiotics. Here we show for the first time that pro- and anti-inflammatory effects can occur by one HSs.

We adapted an assay that uses green fluorescence-labeled zebrafish larvae and $CuSO_4$ to indue an inflammation. In wild-type larvae, exposure to 50 µM $CuSO_4$ for 2h activated the leukocytes to produce reactive oxygen species, which can be monitored with a fluorescence dye (H2DCFDA). This allows not only the use of wild-type fish but also a temporal separation of copper exposure and inflammatory substance while retaining the high throughput. This modified assay was then used to evaluate the inflammatory properties of a fulvic acid (FA). The aromatic structure protects from inflammation at 5 and 50 mg C/L, while the persistent free radicals enhance the copper-induced inflammation at ≥ 300 mg C/L.

Keywords: Humic substance, Zebrafish (*Danio rerio*), Neuromasts, Copper sulfate, Anti-inflammatory, Respiratory burst, 2',7' –dichlorofluorescein diacetate (H2DCFDA)

4.3 Introduction

Inflammation is a normal reaction towards harmful stimuli and plays a critical role during infections or wounds. Inflammation follows a biphasic stage starting with the initiation phase, where cells are attracted towards the affected side and release pro-inflammatory signal molecules such as interleukin (IL)-1β, IL-8, TNF-α, and reactive oxygen species (ROS) (Mittal *et al.* 2014, Kany *et al.* 2019). These signal molecules recruit more immune cells towards the side of inflammation. In the resolving phase, anti-inflammatory molecules, e.g., IL-10, TGFβ (Opal and DePalo 2000, Cuneo and Autieri 2009) are released to end the inflammation. In chronic inflammation, the resolving phase is delayed or blocked. This can be caused by diseases, such as Alzheimer's disease or rheumatoid arthritis (Kuenzig *et al.* 2018, Newcombe *et al.* 2018), but also because of allergic reactions or exposure to hazardous environmental pollutants (Grunig *et al.* 2014, Prata *et al.* 2020).

Humic substances (HSs) are complex compounds mainly occurring from degrading organic material. They represent up to 95 % of the dissolved organic matter (DOC) in freshwater aquatic ecosystems (Thurman 1985, Haitzer *et al.* 1998, Steinberg 2003a). Concentrations range normally between 0 and 50 mg C/L but can exceed 250 mg C/L in tropical water bodies (Suhett *et al.* 2013b). Both, pro- and anti-inflammatory effects of HSs have been reported. HSs reduced the inflammatory effect of hyperglycemia on vascular endothelial cells, decreased edema, skin diseases, and rheumatoid arthritis (van Rensburg 2015, Szot *et al.* 2019, Rusliandi *et al.* 2020). On the other hand, the generation of excess ROS and oxidative damage has been reported as well, associated with the Blackfoot disease for example (Cheng *et al.* 1999, Lin and Yu 2011, Saebelfeld *et al.* 2017). HSs have very heterogenic structures depending on the organic raw material and the degradation pathways from which they are formed, which explain the contradicting reports on the inflammatory effects of HSs. We recently showed that fulvic acid (FA) exposure at concentrations ≥ 300 mg C/L significantly increased the ROS concentration in zebrafish larvae and caused tissue damage and mortality (Lieke *et al.* 2021a). However, at medium concentrations, the same FA induced the expression of anti-oxidative genes as well, without any detrimental effects. We hypothesize that the inflammatory properties do not only differ between different HSs but within one HS depending on the concentration.

In vivo studies on inflammation are difficult due to the opacity of the tissue. To bypass this problem, d'Alençon *et al.* (2010) developed an inflammation assay using the transgenic zebrafish strains lysC::DsRED2 and BACmpx::GFP. Copper is used to induce damage at the neuromasts along the lateral line and the migration of the fluorescent protein labeled myeloid leucocytes can be monitored in the living larvae. However, the use of genetically modified organisms (GMOs) requires special

precautionary measurements (e.g. disinfection procedures, waste inactivation, special training) to ensure environmental and human safety (Waigmann *et al.* 2012, Organisms 2013). Furthermore, in many countries working with GMOs is regulated and has to be approved by governmental agencies. The developed automated detection from d'Alençon *et al.* (2010) is furthermore unsuitable for HSs, as HSs chelate metals, which would prevent the inflammation induction by copper (McKnight *et al.* 1983, Benedetti *et al.* 1995). For their manual assay, each larva has to be fixated individually to record the fluorescence.

We hypothesize that the leucocytes are not only recruited by the copper-induced inflammation but are activated and react with the production of ROS. These ROS can be monitored using a fluorescence dye in normal wild-type zebrafish larvae. This modified assay can then be used to detect the inflammatory properties of the FA.

4.4 Material and Methods

4.4.1 Husbandry and general experimental conditions

Fertilized eggs were collected from parental zebrafish (*Danio rerio*) maintained at our facility under recommended conditions. DIN water (deionized water, 294.0 mg/L $CaCl_2*2H_2O$, 123.3 mg/L $MgSO_4*7H_2O$ (Roth, Germany), 63.0 mg/L $NaHCO_3$, 5.5 mg/L KCl (Merck Germany), pH 7.0 ± 0.2, DIN EN ISO 15088) was used as control. Exposure followed the guidelines of the Fish Embryo Acute Toxicity Test OECD 236 (2013). Healthy eggs were transferred individually into 24-well plates with 2 mL exposure solution (DIN water or FA) (sealed with SealPlate sealing film (Roth) and incubated for 96 h (26 ± 0.5 °C, 16 h/8 h light/dark cycle, IPP 110plus (Memmert, Germany)). Basic requirements that were met in all experiments were: overall survival ≥ 90 % (control), hatching rate after 96 h ≥ 80 % (control), and mortality ≥ 30 % in the positive control (4 mg/L 3,4-dichloro-aniline).

4.4.2 Detection of reactive oxygen species

The cell-permeant reagent 2',7' –dichlorofluorescein diacetate (H2DCFDA Sigma-Aldrich, USA) was used to detect the production of hydroxyl- and peroxyl radicals, and other ROS inside the larvae according to Goody *et al.* (2013) and Hermann *et al.* (2004). Briefly, larvae were individually transferred with 100 µL of DIN water into 96-well fluorescence plates (ELISA plate black med. bind. F, Sarstedt, Nürnberg, Germany). Plates were visually controlled using a stereomicroscope (Nikon SMZ 1270, Japan) with a ring light (Slim Ring-light Schott) attached to ensure proper plate assignment with exactly one larva in each well. 100 µL detection solution (1 µg/mL H2DCFDA in DIN water) was added to each well and the fluorescence was detected over a period of 4 h in a plate reader (Infinite M200, Tecan, Switzerland). The fluorescence is directly proportional to the amount of ROS.

4.4.3 Chemically induced inflammation assay

The chemically induced inflammation assay (ChIn) by d'Alençon *et al.* (2010) uses copper to induce an inflammatory reaction by damaging the neuromast cells at the lateral line and green fluorescence protein (GFP) labeled zebrafish strains to monitor infiltration of leukocytes. Our approach was to modify this assay to the use of wild-type zebrafish to monitor the anti-inflammatory properties of FA.

4.4.3.1 Copper concentration and time of exposure

To determine a proper working concentration, larvae raised in DIN water were first exposed to different concentrations (0, 10, 25, 50, and 100 µM) of copper ($CuSO_4$*5 H_2O, Sigma-Aldrich) for 2 h at 26.0 ± 0.5 °C. H2DCFDA was added and the emission of fluorescence was detected as described above for 4h and analyzed after 2, 3, and 4 h. In this first step, we determined the copper concentrations that activate the leucocytes to produce ROS as well as the period of incubation with the fluorescence dye. In the second step, larvae were exposed to 50 µM and 100 µM of copper for 1 and 2 h, respectively to determine the best copper exposition period. After copper exposure, larvae were washed by transferring them into a crystallizing dish containing at least 130 mL of DIN water before measuring the fluorescence. For each repeat and condition, 12 larvae were used and each test was repeated 3 times independently.

4.4.3.2 Verification of inflammation assay

To test if the assay described above can be used to determine the anti-inflammatory properties of chemicals, we used two agents, known for their anti-inflammatory properties. Diclofenac and ibuprofen are both non-steroidal antiphlogistics (NSAP) or non-steroidal anti-inflammatory drugs (NSAID), inhibiting the two isoenzymes of cyclooxygenase (COX-1 and COX-2). Both have been shown to behave against physical wounding in zebrafish as predicted (Mathias *et al.* 2007, Zhang *et al.* 2008, Loynes *et al.* 2010). Using 1.5 and 3 µM diclofenac, as well as 1 and 10 µM ibuprofen, d'Alençon *et al.* (2010), were able to significantly reduce the copper-induced infiltration of leucocytes in GFP zebrafish. To verify that copper stimulated the inflammatory ROS production rather than just causing oxidative stress (Husain and Mahmood 2019), we incubated 96 hpf larvae with diclofenac (1.5 and 3 µM) and ibuprofen (1 and 10 µM) for 1 h at 26 °C. Afterward, larvae were washed with DIN water and exposed to 50 µM copper (2 h, 26 °C). Production of ROS was detected as described above.

4.4.4 The inflammatory activity of fulvic acid

The inflammatory activity of FA was analyzed using the assay described above. We used a commercially available fulvic acid (FA, FulvoFeed®, HuminTech, Germany) because of its thorough characterization and to exclude effects by contaminations. It is fully water-soluble, with a carbon content of 96.7 g C/L, 88.2 % humic-like substances (94.4 % FA and 5.6 % humic acids), and 29.2 % aromatic carbon (of which 7.6 % are phenols) (Lieke *et al.* 2021b). Based on our previous study (Lieke *et al.* 2021a) larvae were

exposed for 96 h to 5, 50, 300, and 500 mg C/L of FA before copper exposure. To minimize chelation of copper with the FA, residues were removed from the larvae surface by transferring larvae into DIN water; subsequently, larvae were exposed to 50 µM copper for 2 h and the production of ROS was measured photometrically as described above. Larvae without FA and copper exposure were used as a negative control, 3 µM diclofenac was used as a positive control (larvae unexposed to FA, 1 h of incubation with diclofenac before copper exposure). A total of 24 larvae was used per group (4 independent repeats with 6 larvae).

4.4.5 Statistical analysis

The datasets were analyzed using RStudio 1.1.453 software (https://rstudio.com/products/rstudio/download/Libraries: PMCMRplus; dplyr; ggplot2; tidyr; car; openxlsx; tidyverse; gridExtra; grid; pwr; effectsize). Determination of the effect power and sample size are given in the supplement. Data were normalized to average fluorescence values of the control group (no exposure at all) to permit the calculation of fold induction. Differences between means were analyzed by one-way ANOVA with Tukey-HSD post-hoc after verifying that assumptions for parametric testing were met. Differences were considered statistically significant at $p < 0.05$. Graphs are Tukey boxplots.

4.5 Results and Discussion

4.5.1 Chemically induced inflammatory assay

To determine the anti-inflammatory property of the FA, we developed an assay based on the methods of ROS detection methods from Hermann *et al.* (2004) and Goody *et al.* (2013) and the ChIn from d'Alençon *et al.* (2010). Copper is used to induce an inflammatory reaction by damaging the neuromast cells at the lateral line and GFP labeled zebrafish strains to monitor infiltration of leukocytes. We showed that the recruited leucocytes are also activated by copper and produce ROS, which can be detected by the conversion of H2DCFDA to the fluorescence emitting DCF.

4.5.1.1 Copper concentration and time of exposure

After 2 h of incubation with H2DCFDA, none of the copper exposure groups showed any difference in the normalized relative fluorescence units (RFU) compared to the control (Figure 4-2). After 3 h incubation larvae exposed to both, 50 µM and 100 µM copper, showed a significantly increased signal (1.1-folds for 50 µM, $p=0.033$ and 1.1-folds for 100 µM, $p=0.002$). This increase became highly significant ($p<0.001$) after 4 h of incubation with the dye (1.2 for 50 µM and 1.3 for 100 µM). Neither 10 µM nor 25 µM copper exposure induced a significant increase in ROS production. During the experiments, none of the copper concentrations used resulted in larval mortality.

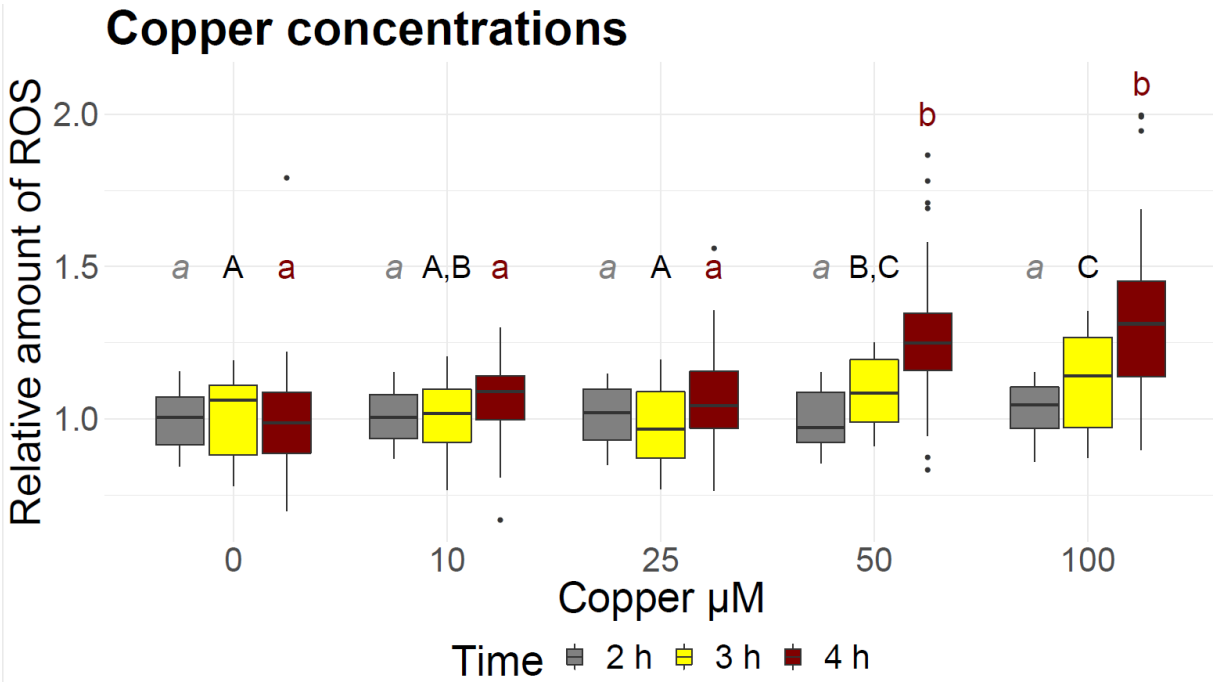

Figure 4-2: Relative amount of reactive oxygen species (ROS) in larvae exposed to different concentrations of copper. Fluorescence was measured after 2 h, 3 h, and 4 h of incubation with the fluorescence dye. Different letters of the same color represent significant differences within one time-point. ANOVA-HSD, n=36, df=4, p<0.05

Exposing larvae for 1 h to 100 µM Cu resulted in a significant increase in ROS production (1.6-fold), while 50 µM Cu did not induce any changes (Figure 4-3). After 2 h of incubation, both copper concentrations increased the ROS production significantly, similar to the experiments before. As 50 µM copper is probably less harmful to the larvae, we recommend using this concentration with 2 h of exposure rather than 100 µM. These results differ from those reported by d'Alençon *et al.* (2010), who found 40 min exposure with 10 µM $CuSO_4$ to induce the inflammation. Possible reasons are that the 56 hpf larvae used by d'Alençon *et al.* (2010) might be more sensitive to copper exposure than the 96 hpf larvae used in our experiment. Furthermore, copper toxicity decreases with increasing hardness and alkalinity, and the E3 water used by d'Alençon *et al.* (2010) differs from our DIN water in these conditions (E3: 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$, DIN water: 2 mM $CaCl_2$ and 0.5 mM $MgSO_4$). Lower concentrations of copper could also be sufficient to recruit the leucocytes to the side of inflammation, while activation to produce ROS might require higher concentrations. Finally, the detection of ROS by fluorescence dye might be less sensitive than the optical measurement of recruitment. Production of ROS might be induced by 10 or 25 µM copper, but with an intensity below the detection limit.

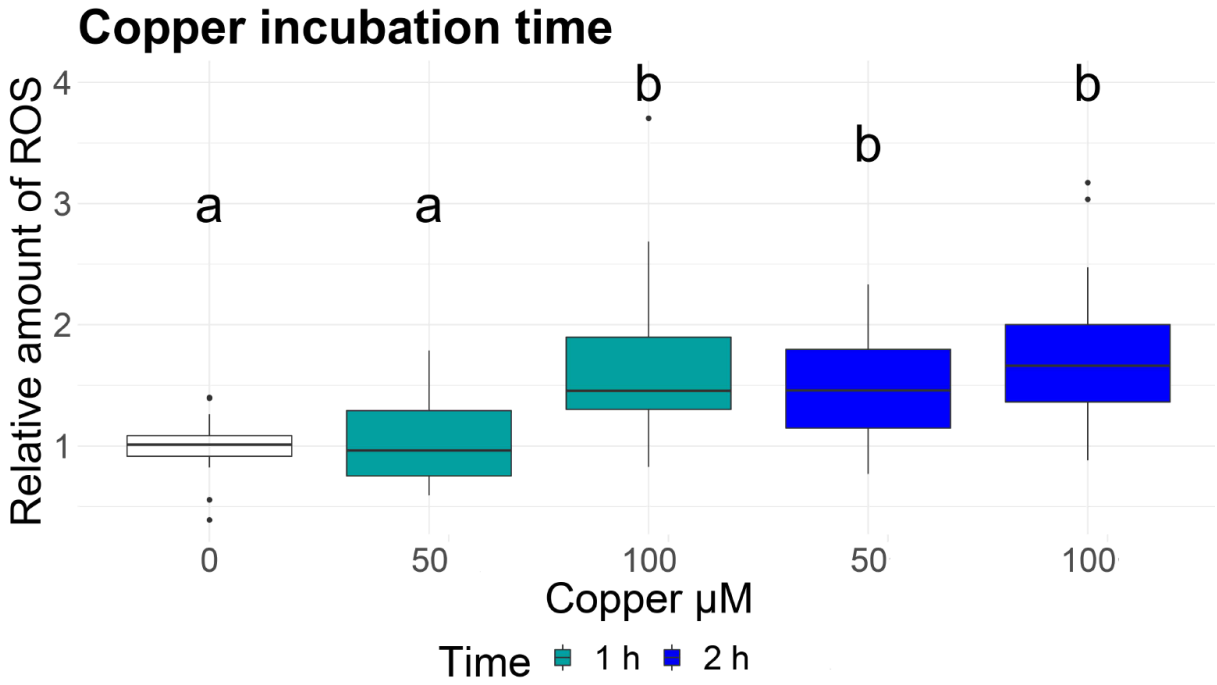

Figure 4-3: Relative amount of reactive oxygen species (ROS) in larvae exposed to different concentrations of copper for 1 h and 2 h. Fluorescence was measured after 4 h of incubation with the fluorescence dye. Different letters represent significant differences. ANOVA-HSD, n=36, df=4, p<0.05

4.5.1.2 Verification of the inflammation assay

Exposing larvae to 1.5 and 3 µM diclofenac, and 1 and 10 µM ibuprofen for 1 h before copper exposure, decreased the copper-mediated inflammation significantly ($p<0.01$, Figure 4-4). There was no significant difference to the unexposed control group.

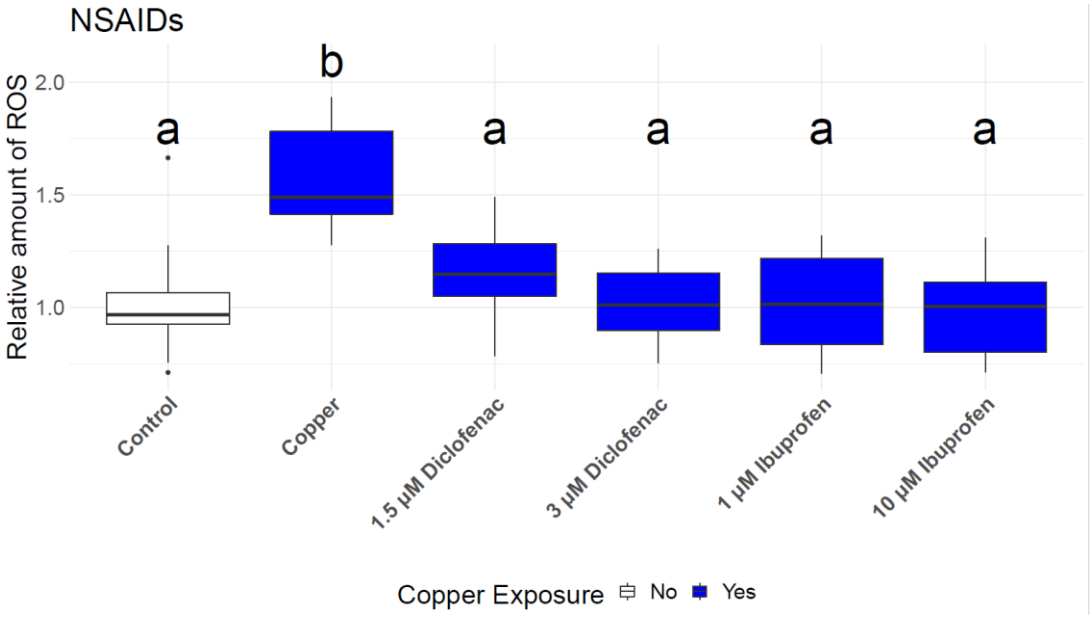

Figure 4-4: Relative amount of reactive oxygen species (ROS) in larvae treated with diclofenac or ibuprofen before exposed to copper. Fluorescence was measured after 4 h of incubation with the fluorescence dye. Different letters represent significant differences. ANOVA-HSD, n=24, df=5, p<0.05

This supports the findings from d'Alençon *et al.* (2010), where the same concentrations of diclofenac and ibuprofen suppressed the copper-induced migration towards the lateral line. Comparing transcriptional changes after ibuprofen and diclofenac exposure, Zhang *et al.* (2020) determined that ibuprofen led to higher dysregulation on hemodynamics than diclofenac. Larvae in our assay were exposed only for 1 h to the NSAIDs and 96 hpf are less susceptible than the 50 blastula-stage embryos (2-3 hpf) used to evaluate the effects of diclofenac and ibuprofen. Based on our observation ibuprofen and diclofenac can both be used as positive controls in the assay. Although no significant difference was observed between the two diclofenac concentrations, we decided to use 3 µM diclofenac as a positive control as the trend towards an increased reduction was visible.

The major drawback of the ChIn by d'Alençon *et al.* (2010) is the need for GFP zebrafish strains. With our modified assay, wild-type zebrafish larvae can be used to measure the inflammatory properties of chemicals. Exposing 96 hpf larvae to 50 µM copper for 2 h produced a significant increase in the ROS concentration, indicating an activation of the leucocytes. Treating larvae for 1 h with ibuprofen or diclofenac before copper exposure diminished the ROS production, showing that the copper induces inflammation and not only exerts oxidative stress. Overall, our assay can be used as an easy to handle alternative or useful addition to the ChIn assay and help to detect the effects of immunomodulatory compounds.

4.5.1.3 The inflammatory activity of fulvic acid

The modified ChIn assay was used to evaluate the inflammatory properties of a FA (Figure 4-5). In larvae exposed only to copper without FA conditioning, the ROS concentration increased 1.7 times, compared to the control (no FA, no copper exposure). In contrast, ROS concentrations in the larvae conditioned with 5 mg C/L and the 50 mg C/L FA differed neither significantly from the control without exposure to copper nor from the diclofenac group (1.3 and 1.1 times). However, larvae raised in 300 and 500 mg C/L showed significantly higher ROS concentrations than both control groups and the diclofenac group (2.8 and 3.2 times).

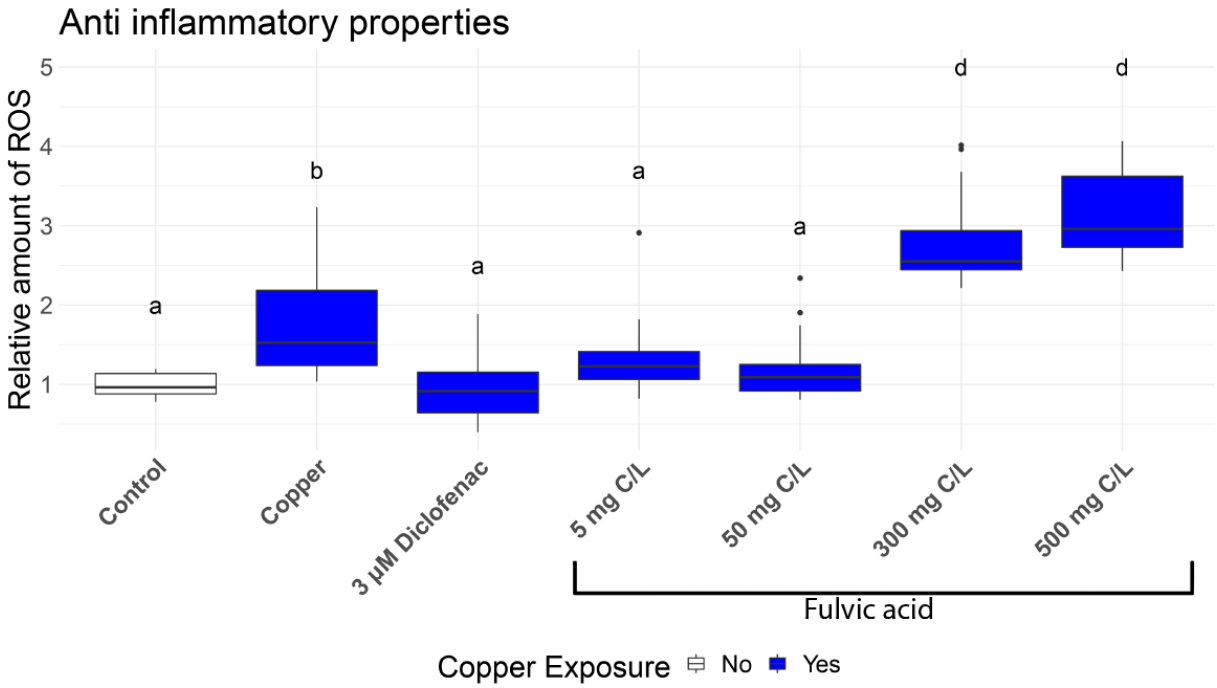

Figure 4-5: Relative amount of reactive oxygen species (ROS) in larvae exposed to different concentrations of fulvic acid for 96 h and copper for 2 h. Diclofenac larvae were exposed only to diclofenac for 1 h before being exposed to copper. Fluorescence was measured after 4 h of incubation with the fluorescence dye. Different letters represent significant differences. ANOVA-HSD, n=24, df=5, p<0.05

Anti-inflammatory effects of HS have previously been reported in terrestrial vertebrates. Topical application of oxifulvic acid reduced the cutaneous inflammation produced by dinitrofluorobenzene in mice in a comparable way as diclofenac sodium or betamethasone did (Van Rensburg *et al.* 2001). In lymphocytes, increased expression of the anti-inflammatory IL-2 was observed after exposure to 20 to 100 mg/L of a potassium humate (van Rensburg and Naude 2009). The classical and alternative complement pathway and pro-inflammatory cytokines TNF-α, IL-1ß, IL-6, and IL-10 were suppressed by 40 mg/L of the same substance. Contradicting these findings, pro-inflammatory effects and oxidative stress caused by HSs have been reported as well. Exposing juvenile pacu (*Colossoma macropomum*) to 20 to 80 mg C/L of a natural and a commercial HS for 10 days induced the concentration and activity of cytochrome P450 1A (CYP1A), which has been linked to oxidative damage and inflammation (Matsuo *et al.* 2006a, Tian *et al.* 2020). These results show that HSs, in general, can do both, exert oxidative stress, leading to tissue damage and inflammation, and protect against oxidative stress. However, as HSs differ structurally greatly from each other, biological results from different HSs cannot be compared without constraints, if structural characterizations are not provided (Meinelt *et al.* 2007).

Interestingly, we found in our previous studies that the same FA as used in the present study had beneficial effects, such as increased development of larvae and increased protection of the gills of juvenile fish when applied at low to medium concentrations, but exerted detrimental effects at high

concentrations (Lieke *et al.* 2021a, Lieke *et al.* 2021b). Similar results were found with vitamin E (α-tocopherol), which is normally labeled as anti-oxidant but possesses pro-oxidative properties at high concentrations (Pearson *et al.* 2006). d'Alençon *et al.* (2010) showed that pretreatment of larvae with diphenyleneiodonium (DPI), which inhibits the NADPH oxidase, reduces the number of recruited leucocytes as well, compared to larvae only exposed to copper, showing the involvement of ROS as signaling molecules in the ChIn. The FA has a high phenolic content and a high oxygen scavenging capacity (Lieke *et al.* 2021b), which might decrease the ROS level inside the larvae directly. However, the number of leucocytes recruited in pretreated DPI larvae in the d'Alençon *et al.* (2010) study was still ~4 times higher than in copper-untreated larvae, while FA pretreatment decreased the level of ROS to that of control larvae. This shows that although ROS scavenging of the FA might play a minor role, additional anti-inflammatory mechanisms are involved.

We found an increased expression of genes involved in anti-oxidative protection by medium concentrations of FA in our previous study and based on the current results, this could help in preventing the damage of the neuromast and subsequent inflammation by copper. Several studies have shown, that natural compounds, especially polyphenols mediate the transition from pro-inflammatory M1 macrophages to M2 macrophages, which are crucial for inflammation resolution (Aharoni *et al.* 2015, Dugo *et al.* 2017, Mendes *et al.* 2019). Although we did not find activation of the leucocytes in zebrafish larvae by FA exposure (Lieke *et al.* 2021a), we did find indications towards an increased number of neutrophils and thereby a shift in the composition of innate immune cells. As the used FA is well comparable to polyphenols (Lieke *et al.* 2021b), it would be an interesting future approach to investigate the overall cell composition, with special emphasis on the different macrophage sub-populations and shifts due to FA exposure.

At high concentrations (300 and 500 mg C/L) we previously found increased expression of anti-oxidative genes as well but combined with high ROS concentrations inside the larvae which caused tissue damage (Lieke *et al.* 2021a). The same concentrations significantly amplified the copper-induced inflammation in our modified assay. Jiang *et al.* (2021) found a strong correlation between the aromatic content and the associated free radicals of HSs and the inflammatory Kashin-Beck disease. Apparently, the phenolic moieties and the persistent free radicals in our FA become hazardous at high concentrations, causing pro-inflammatory effects. This shows that one HSs can exert both, pro- and anti-inflammatory effects and it is essential, to carefully evaluate the effects over a broad range of concentrations and to further compare the effects to the structure of the HS, before declaring any inflammatory properties.

Low-grade chronic inflammations are promoting numerous age-related diseases and can be caused by many factors including diet, disturbed sleep, physical inactivity, and exposure to pollution (Minihane

et al. 2015). Effective control of inflammation could help to resolve inflammations and protect individuals predisposed to such diseases. At the same time, it is essential to screen for pro- and anti-inflammatory properties of drugs, chemicals, and pollutants to ensure a proper risk assessment. The transparency and short life-cycle of zebrafish larvae allow easy monitoring of *in vivo* inflammation response., but also to study diseases associated with chronic inflammation. Our modification of the ChIn assay from d'Alençon *et al.* (2010) allows the use of wild-type zebrafish larvae instead of GMO-strains and monitors the activation of immune cells rather than their recruitment. It can be used as an alternative, when the use of GMOs is inconvenient, or as a complementary method to gain deeper insight into the mechanisms of inflammation. HSs can have very diverse chemical building blocks resulting in diverse biological effects between different HSs. Using the modified assay, we demonstrated that the anti-inflammatory effects of a phenol-rich FA turn pro-inflammatory due to the high content of free radicals at higher concentrations. The same chemical properties protecting against oxidative stress and inflammation at low to medium concentrations are responsible for the hazardous pro-inflammatory effects at higher concentrations.

4.6 Compliances with Ethical Standards

Experiments were performed following the European Directive 2010/63/EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes and the German AnimalWelfare Act. Experiments with fish larvae until the start of exogenous feeding do not require special permission by the animal experimental ethics committee of the Berlin State Office for Health and Social Affairs (LaGeSo).

4.7 Data availability

All data generated or analyzed during this study are included in this published article.

4.8 Acknowledgments

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4.9 Authors Contribution

All experiments, sample analysis, data analysis, writing of the original draft, graph creation, and editing were performed by T.L. Funding was acquired by C. E. W. S., T. M., and T. L. The study was supervised by K. K. and W. K. All authors contributed to reviewing the manuscript.

4.10 Competing Interests

The authors declare that there are no conflicts of interest. The founding sponsor had no role in the design, content, or decision to publish the paper. Mention of trade names or commercial products in this article is solely for providing specific information and does not imply recommendation or endorsement by the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Humboldt University, or the Federal Ministry for Economic Affairs and Energy.

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4.12 Supplements: Sample size determination based on the results of the pilot study

The first part of the modified chemically induced inflammation assay (Chapter 4.3.1 "Copper concentration and time of exposure" in the manuscript) was used as a pilot study to identify the sample size required for the assay.

To determine the effect of the copper exposure against the control, Cohens d (Cohen 1988) against the control group was calculated using the equation

$$d = \frac{mean_2 - mean_1}{SD_{pooled}} = \frac{mean_2 - mean_1}{\sqrt{\frac{sd_1^2 + sd_2^2}{2}}}$$

Table 4-1: Calculations of Cohen's coefficient against the control group at different concentrations and exposure periods of HD2DCFDA. Values are rounded to 2 digits

Copper exposure concentration	Mean	SD	Cohens d
2 h of incubation with the fluorescence dye			
0 µM	1.00	0.10	-
10 µM	1.01	0.08	0.11
25 µM	1.01	0.09	0.14
50 µM	1.00	0.09	0.00
100 µM	1.03	0.09	0.32
3 h of incubation with the fluorescence dye			
0 µM	1.00	0.14	-
10 µM	1.01	0.12	0.09
25 µM	0.98	0.13	0.17
50 µM	1.09	0.11	0.73
100 µM	1.12	0.17	0.77
4 h of incubation with the fluorescence dye			
0 µM	1.00	0.19	-
10 µM	1.06	0.13	0.40
25 µM	1.07	0.17	0.40
50 µM	1.28	0.23	1.35
100 µM	1.34	0.28	1.46

The theoretical number of larvae that has to be used to get significant results at different incubation periods with the fluorescence dye (2 h, 3 h, and 4 h) was calculated for larvae exposed to copper using R studio (t-test; Difference between two independent means (two groups)], α=0.05, β=0.2, and β=0.05). In the following simplified presentation, the low concentrations (10 µM and 25 µM copper) were excluded as they did not increase the relative fluorescence significantly until 4 h of exposure.

Table 4-2: Sample size required per group when using 2 groups for β =0.2 and β =0.05 after incubation with H2DCFDA.

β =0.2	**Theoretical minimal number of larvae per group when using 2 groups**		
Copper Concentration	**After 2h incubation**	**After 3h incubation**	**After 4h incubation**

50 µM	-	31	10
100 µM	155	28	9
β =0.05	**Theoretical minimal number of larvae per group when using 2 groups**		
Copper Concentration	**After 2h incubation**	**After 3h incubation**	**After 4h incubation**
50 µM	-	50	15
100 µM	255	45	13

As exposure to 50 µM and 100 µM copper gave similar results, we decided to use the lower concentration, as 100 µM might induce negative effects that are not evident at first. As a compromise between practical feasibility when using more groups and statistical certainty, we furthermore decided to use an incubation time with the dye of 4 h and a sample size of 24 per group for the anti-inflamatory tests (diclofenac and ibuprofen) and the FA.

5 Phenol-rich fulvic acid as a water additive enhances growth, reduces stress, and stimulates the immune system of fish in aquaculture

Thora Lieke [a, b,*], Christian E.W. Steinberg [b, c], Bo Pan [c], Irina V. Perminova [d], Thomas Meinelt [a], Klaus Knopf [a,b], Werner Kloas [a,b]

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[a] Department of Ecophysiology and Aquaculture, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany

[b] Humboldt University of Berlin, Faculty of Life Sciences, Berlin, Germany

[c] Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, 650500 Kunming, China

[d] Lomonosov Moscow State University, Leninskie Gory, 119991, Moscow, Russia

* Corresponding author (lieke@igb-berlin.de), Phone: +49 (0)30 64181 641, ORCID ID: 0000-0002-4345-171

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5.1 Abstract

Aquaculture has become imperative to cover the demands for dietary animal protein. Simultaneously, it has to overcome prejudices from excessive use of antibiotics and environmental impacts. Natural supplements are traditionally applied orally. In this study, we demonstrated another pathway: the gills. Humic substances are immunostimulants and a natural part of every aquatic ecosystem, making them

ideal to be used as bath stimulants. Five and 50 mg C/L of a fulvic acid-rich humic substance was added for 28 days to the water of juvenile rainbow trout (*Oncorhynchus mykiss*). This fulvic acid is characterized by a high content of phenolic moieties with persistent free radicals and a high electron exchange capacity. The high concentration of the fulvic acid significantly increased growth and reduced the food conversion ratio and the response to a handling-stressor. Phagocytosis and potential killing activity of head kidney leukocytes were increased, as well as the total oxyradical scavenging capacity (TOSC) and lysozyme activity in the gills. In conclusion, immunostimulation via gills is possible with our fulvic acid, and the high phenolic content improved overall health and stress resistance of fish.

5.2 Introduction

Fishes play an important role to cover the demands of animal protein, and FAO projected the aquaculture production to exceed capture for the first time in 2020 (FAO 2018). Apart from the increasing demand, aquaculture faces a major problem: tremendous prejudices of consumers because of the excessive use of antibiotics and other chemicals in the past and negative impacts on the environment. Stimulating the immune system with feed additives to help prevent diseases and the use of therapeutants is becoming a far-reaching practice in fish production (Reverter *et al.* 2014, Hoseinifar *et al.* 2018, Lieke *et al.* 2019, Zuo *et al.* 2019). There are several downsides to this application method: Firstly, complementing the feed requires additional processing, which increases the costs. Secondly, the concentration inside the fee is fixed and cannot be adjusted easily by the fish farmer depending on the current requirements. And thirdly, because of feed competition, not all fish inside the same batch ingest the same amount of additive, making the effects unpredictable (too low concentrations can have no effects, too high can have adverse effects). In fish, there is a second possible route of immunostimulant uptake: the gills. The large surface area of gills is not only necessary to guarantee sufficient oxygen uptake but is an entrance portal for microorganisms and xenobiotics (Haugarvoll *et al.* 2008, Noga 2011). Furthermore, the gill associated lymphoid tissue (Haugarvoll *et al.* 2008) plays an important role in the immune response and would be in direct contact with the bath stimulants. Nevertheless, research on stimulating the immune system via water treatment is still scarce (Jeney and Anderson 1993, Zhang *et al.* 2009). A long-term application, similar to the use of immunostimulants in feed, has not been studied yet. Possible reasons for those lacks are that bath stimulants have to cover several requests: complete water-solubility (excluding high molecular molecules such as ß-glucans from algae and fungi or chitin), non-irritant (excluding many herb extracts), and, due to the required large amounts, they have to be cheap to be economically realistic (excluding many vitamins).

Humic substances (HS) are part of natural organic matter and represent up to 95 % of dissolved organic matter (DOC) in aquatic ecosystems with concentrations normally ranging from 0.5 mg C/L to 50 mg C/L (Thurman 1985, Haitzer *et al.* 1998, Steinberg 2003b). They are “complex and

heterogeneous mixtures" (IHSS) and their structure can differ greatly depending on their origin: Comparing 20 HS Meinelt *et al.* (2007) found not only enormous differences between different lakes but also seasonal changes in the structure of HS within one lake. This chemical diversity is reflected by the heterogeneity of biological effects.

Beneficial effects of HS, when used as feed additives, include growth stimulation, reduced mortality, accelerated recovery after diseases, and stimulation of immune-related genes (Kodama *et al.* 2007, Gao *et al.* 2017, Yamin *et al.* 2017b, Fierro-Coronado *et al.* 2018). However, negative or contradictory effects are reported as well, including genotoxicity in blood lymphocytes, oxidative stress, and reduced offspring in *Daphnia magna* (Hseu *et al.* 2008, Steinberg *et al.* 2010, Saebelfeld *et al.* 2017). Only a few quantitative structure-activity relationships of humic substances have been conducted so far, but they showed that aromatic moieties and carboxyl/esterified functions have opposite effects in maize and *Saprolegnia parasitica* (Meinelt *et al.* 2007, Savy *et al.* 2020). Although not directly transferable to fish, these results showed that the structure of the HS plays a tremendous role in its biological effects. To compare the effects of different humic substances, the chemical characterization cannot be neglected.

Humic substances often occur as waste-products during drinking water production and because of their aquatic origin, they are ideal to be used as water additives. The present study aimed to close knowledge gaps on two main aspects: Firstly, can humic substances, applied as bath treatment, stimulate the immune system of fish, and secondly, which chemical structures are characteristic for the humic substance applied, being potentially responsible for the biological effects.

5.3 Material and Methods

5.3.1 Humic substance and chemical analysis

FF is a preparation of humic material that was obtained from HuminTech GmbH, Grevenbroich, Germany. It was extracted from groundwater in wetland/bog rich regions in northern parts of the Netherlands. Precipitation of FF was not observed in the concentrations used. Contents of carbon, nitrogen, hydrogen, and sulphur were determined by high-temperature combustion using a Vario MICRO cube (Elementar Analysensystem, Langenselbold, Germany). Oxygen content was calculated as the difference between the sum of elemental content (in mg/g) and total dry content (after freeze-drying the liquid FF). The quantitative solution-state ^{13}C NMR spectroscopy was performed using a Bruker DMS 400 NMR spectrometer operating at 100 MHz ^{13}C frequency, using 0.3 M NaOD/D_2O as a solvent. Spectral assignments were made according to Hertkorn *et al.* (2002). LC-OCD-OND was performed by DOC-Labor Dr. Huber, Karlsruhe, Germany. Mediated electrochemical reduction (MER) and oxidation (MEO) were performed at applied potentials of -0.70 V and +0.4 V (vs. Ag/AgCl reference electrode), respectively, to measure the EEC (including , EAC and EDC) (Zheng *et al.* 2019). EPR

spectrum was recorded on a Bruker X-band A300-6/1 EPR spectrometer (Bruker, Billerica, Massachusetts, USA) by loading liquid FF into a microcapillary (Weil and Bolton 2007, Liao *et al.* 2014, Lieke *et al.* 2018). Intensity and g-factor were calculated using the Bruker WinEPR Acquisition software 4.40 Rev.11.

5.3.2 Experimental setup

Eggs of rainbow trout (*Oncorhynchus mykiss*) were obtained from Uckermark-Fisch GmbH (Boitzenburg, Germany) and bread at Leibniz-Institute of Freshwater Ecology and Inland Fisheries until desired size. Fingerling rainbow trout (0+ years, 24.9 ± 2 g, 13.3 ± 0.4 cm) were randomly distributed into 9 tanks (40 L, n=30 per tank) of a flow-through system. After acclimatization, fish were exposed for 4 weeks to different concentrations (0, 5 (low), and 50 (high) mg C/L) of the FF, which were added constantly by a peristaltic pump. The throughput rate of water and FF were monitored twice per day to ensure constant exposure. Fish were fed 1.5 % of their weight daily (Aller Silver, Aller Aqua, Germany) and feeding was adjusted weekly to the calculated weight gain with an assumed feed conversion ratio of 1 (13.18 g/fish in total over 28 days). During the experiment, temperature (16.5 ± 0.2 °C), pH (8.5 ± 0.1), and dissolved oxygen (8.5 ± 0.5 mg/L) were monitored daily. Nitrite (0.3 ± 0.3 mg/L), nitrate (10.4 ± 2.0 mg/L) and ammonium (<0.02 mg/L) were measured three times a week. After taking base-line samples (t0;Figure 5-1) to determine the effects of the exposure, fish were stressed by netting and 30 s of air-exposure. Fifteen minutes after stress (t1), blood was sampled to determine the effects of exposure on the immediate stress response. These fish were removed from the experiment but not sacrificed. Two days after the baseline-sampling (t2), fish were sampled again to evaluate immune and stress response after handling.

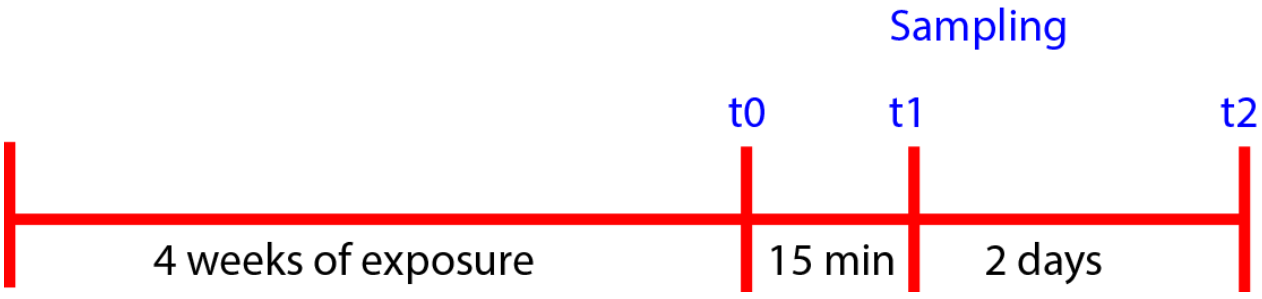

Figure 5-1: Timeline of experiment and samplings; t0: 28 days after start of experiment; t1: 15 min after the stressor (netting and 30-sec air-exposure); t2: two days after first sampling

5.3.3 Growth parameters

The weight and length of fish were determined at the beginning and the end of the experiment. FCR was calculated using equation 1; AGR was determined following equation 2 (Hopkins 1992); (n=3 tanks). Fulton's condition factor (K) was calculated for each individual using equation 3 following Fulton (1904) and Barnham and Baxter (2003) .

$$(1)\ FCR = \frac{feed\ given\ [g]}{average\ weigth\ gained\ [g]}$$

$$(2)\ AGR = \frac{average\ weigth_{end} - \text{average}\ weigth_{start}}{time}\ \left[\frac{g}{d}\right]$$

$$(3)\ K = 10^5 * \frac{weigth}{standard\_length^3}\ \left[\frac{g}{mm^3}\right]$$

5.3.4 Sampling of blood and tissues

Four fish from each tank were collected randomly at each time point and blood was taken within less than 5 min after catching from the caudal vein. Samples were centrifuged (2,000 x g, 5 min, 4 °C), frozen in liquid nitrogen, and stored at -80 °C. Fish were sacrificed immediately after blood sampling and liver, spleen, visceral fat, head kidney, and gills were dissected. Liver, spleen and visceral fat were weighted to calculate hepato-somatic-index (HSI), spleen-somatic-index (SSI, equation 4, n=36) and fat to body ratios (fat [g]/organs [g] and fat[mg]/total weight[g]); gills (n=12) were shock-frozen in liquid nitrogen and stored at -80 °C. Gill extracts were prepared by homogenization in tenfold volume (w/v) of ice-cold phosphate buffer (0.1 M, pH 7.2) and centrifuged at 10,000 x g for 20 min at 4 °C. The extract was used to determine immune and antioxidant parameters.

$$(4)\ HSI\ or\ SSI = \frac{weight(liver\ or\ spleen)\ [g]}{total\ weight\ [g]} * 1000$$

5.3.5 Cellular response of head kidney

Head kidneys from six randomly chosen fish per group (two per tank) were aseptically removed, passed through a 70 µM cell strainer, and placed in ice-cold washing medium (RPMI 1640, 25 mM HEPES buffer, 100 U/mL Penicillin-Streptomycin, 2 mM L-glutamine (all chemicals Biowest, France), 10 U/mL heparin (Carl Roth, Germany). Erythrocytes were removed from the cell suspension by density gradient centrifugation on Histopaque 1077 (Sigma-Aldrich, USA; 500 x g, 45 min, 4 °C). Leucocytes were washed twice in the washing medium and adjusted to 10^7 cells/mL in culture medium (washing medium without heparin). Cells were used to measure the respiratory burst activity by nitro blue tetrazolium (NBT) assay and to determine the phagocytic activity.

5.3.5.1 Nitro-blue tetrazolium (NBT) assay

The potential killing activity (reactive oxygen species (ROS) production) of head kidney cells was measured with NBT assay as described by Secombes (1990) and Chettri *et al.* (2010) with minor modifications. $1*10^6$ cells were added in 9 replicates to a 96 well plate (Nunclon delta, F, clear, ThermoFisher, USA). After incubation overnight at 17 °C, non-adherent cells were removed by washing twice with 100 µL culture medium. Subsequently, cells were incubated in culture medium with 1 mg/mL NBT for 90 min at 17 °C. PMA (1 µg/mL, Sigma Aldrich), and zymosan (3.2 mg/mL, Sigma Aldrich) were used in triplicates to induce the respiratory burst. Resulting formazan was dissolved in 100 µL 2 M KOH and 100 µL DMSO and optical density was measured at 620 nm.

5.3.5.2 Phagocytic activity

Phagocytic activity was measured as described by Crampe *et al.* (1998). 300 µL of the cell suspension were put on glass slides in duplicate. After 1 h incubation at 17 °C non-attached cells were rinsed with culture medium. Adherent cells were covered with 300 µL of heat-inactivated yeast cells (*Saccharomyces cerevisiae,* 10^8/mL in culture medium). After 1 h of incubation at 17 °C excess yeast cells were rinsed off and slides were fixed with 100 µL methanol. Slides were stained using the Pappenheim method (Giemsa stain solution, Merck, Germany; May-Grünwald-solution, Roth, Germany) (Begemann and Rastetter 1972)**.** For each slide, the number of phagocytized yeast cells was enumerated for 200 phagocytes and the rate of phagocytosis (R_P; equation 5) and phagocytic index (I_P; equation 6) were calculated.

$$(5)\ R_P = \frac{n_{ingesting}}{n_{total}}$$

$n_{ingesting}$= number of phagocytes ingesting any amount of yeast cells; n_{total}=total number of phagocytes

$$(6)\ I_P = \frac{\sum_{i=1}^{10} C(i)*i}{n_{ingested}}$$

i= number of yeast cells ingested; C(i) number of phagocytes ingesting i yeast cells; $n_{ingested}$=total number of yeast cells ingested

5.3.6 Lysozyme activity and protein content

Lysozyme activity of plasma and gill extracts was measured with the turbidimetric method (Siwicki 1993, Sitja-Bobadilla *et al.* 2008). Briefly, lyophilized *Micrococcus lysodeikticus* (Fluka, Sigma-Aldrich) (0.3 mg/ mL in 0.025 M phosphate-buffer pH 6.2) was used as substrate. Reduction in absorbance at 530 nm was measured from 0 to 7 min in 1 min intervals (Infinite 200, Tecan, Switzerland). One unit of lysozyme activity was defined as the amount of enzyme causing a decrease in absorbance of 0.001/min. Protein content was measured following Bradford (1976) and used as normalization to compare different sample types. Plasma albumin content was measured after precipitation of immunoglobulin with 12 % PEG (polyethylene glycol, Merck, (Siwicki 1993)). Immunoglobulin content was considered as the difference in total protein content and albumin content.

5.3.7 Reactive oxygen species (ROS) and total oxyradical scavenging capacity (TOSC)

Content of ROS and TOSC of gill extracts were measured following Amado *et al.* (2009). Briefly, the conversion of H2DCF-DA (2,7-dichlorodihydrofluorescein diacetate) to the fluorescent 2,7-dichlorofluorescein by ROS was quantified at 485 nm and 528 nm for excitation and emission, respectively, at 37 °C, for 45 min in 5 min-intervals using a microplate reader (Infinite 200, Tecan). Thermally activated 2,2-azobis dihydrochloride (ABAP, 4 mM) was used to generate peroxyl radicals to

measure TOSC. Using cubic spline interpolation, the area under each curve was integrated. ROS signal was normalized to protein content; TOSC was calculated following equation 7.

$$(7)\ TOSC = \frac{ROS\ Area_{background}}{ROS\ Area_{ABAP} - ROS\ Area_{background}}$$

5.3.8 Cortisol

Plasma cortisol levels were measured as duplicates using commercial ELISA kits (Cortisol Saliva ELISA, IBL) and following the manufacturer's instructions. The absorbance was measured at 450 nm using the microplate reader Infinite 200 and the analysis software SparkControl Magellan 2.2 (Tecan Group). Plasma was diluted 1:20 at t0 and t2 and 1:50 at t1 with buffer A from the kit.

5.3.9 Statistical analysis

All data are expressed as the arithmetic mean ± standard deviation (SD). The data-sets were analyzed using RStudio 1.1.453 software (https://rstudio.com/products/rstudio/download/); significant differences between exposure and control were determined by Kruskal-Wallis Test (Hollander and Wolfe 20015) with a DunnTest (Many to one (Dunn 1964, Siegal and Castellan Jr. 1988) and BH (Benjamini and Hochberg 1995) adjustment; two-sided). Graphs are Tukey boxplots and levels of significance were expressed as p-values with $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

5.3.10 Compliances with Ethical Standards

Experiments were performed following the European Directive 2010/63/EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes and the German AnimalWelfare Act and were approved by the animal experimental ethics committee of Berlin State Office for Health and Social Affairs (LaGeSo, reference number G 0135/18).

5.4 Results

5.4.1 FulvoFeed (FF) is a small molecular fulvic acid with high phenolic moieties and EDC

The liquid FF has a dry content of 242.93 g/L and a carbon content of 97 g/L. As the analysis of elemental composition requires previous dry freezing, Table 5-1 shows directly measured results of dry content (mg/L) and calculated content of liquid FF(g/L).

Table 5-1: The elemental compositions of FF particles and solution.

	C	O	N	H	S
Dry FF (mg/g)	398.1	537.1	6.5	50.0	8.3
Liquid FF (g/L)	96.7	130.5	1.6	12.2	2.0

As natural products, humic substances are a mixture of different carbon-based structures. Liquid Chromatography – Organic Carbon Detection – Organic Nitrogen Detection (LC-OCD-OND) analysis showed, that the carbon-structures consists of 0.1 % bio-polymer, 4.2 % building blocks (low

molecular structural elements of HS), 7.6 % low molecular weight substances, and 88.2 % humic-like substances with an average molecular weight of 800 g/mol. Of these humics, 94.4 % are fulvic acids and 5.6 % are humic acids. As measured by ^{13}C-NMR spectroscopy, the amount of different structural groups was as follows: 29.2 % aromatic carbon (including 7.6 % phenolic carbon), 20.3 % carboxyl carbon, 5.4 % carbonyl carbon, 12.7 % carbohydrate carbon, and 28 % - non-substituted aliphatic carbon. Electron donor capacity (EDC) was 380 µmol/g and electron acceptor capacity (EAC) was 270 µmol/g. Electron paramagnetic resonance, as a measure of persistent free radicals, reveals a g-factor of 2.00453 and an intensity of $4.6*10^6$/g carbon.

5.4.2 Bath treatment increases growth and reduces feed conversion ratio (FCR)

Juvenile rainbow trout had a mean length of 13.3 ± 0.4 cm and a mean weight of 24.9 ± 2.0 g when starting the experiment. After 4 weeks, control fish had gained a length of 15.4 ± 0.4 cm (16.0 % increase) and a weight of 36.2 ± 3.1 g (45.0 % increase) (Figure 5-2 A and B). Exposure to FF led to increased growth: fish exposed to 5 mg C/L had a total length of 15.5 ± 0.52 cm (16.5 % increase) and a weight of 37.6 ± 3.65 g (48.8 % increase), while fish exposed to the high concentration (50 mg C/L) gained significantly more length and weight (18.2 % length and 56.4 % weight) resulting in a total length of 15.7 ± 0.6 cm and a weight of 38.2 ± 4.0 g. There was no mortality.

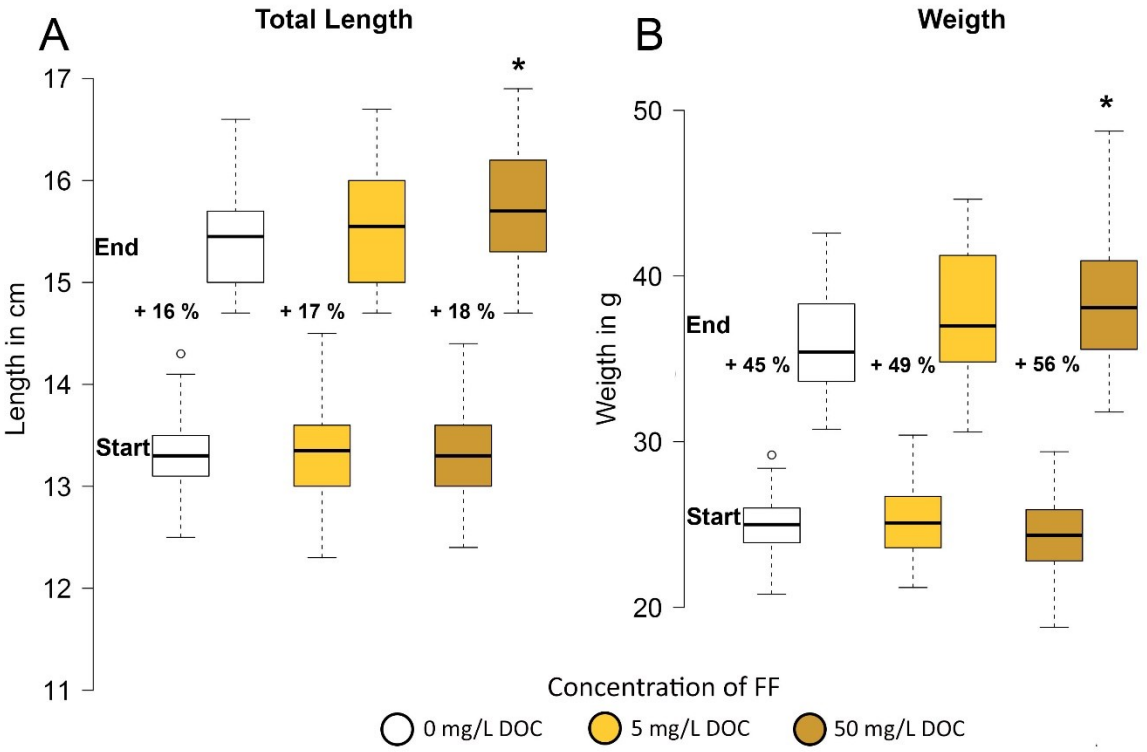

Figure 5-2: Mean total length (A) and mean weight (B) of juvenile rainbow trout at start and end (28 days) of exposure to FF. Percentages represent gains relative to the start value. *-p<0.05 against control.

At the same time, the mean absolute growth rate (AGR), was increased, from 0.401 ± 0.01 in control fish to 0.440 ± 0.05 in fish exposed to the low concentration of FF and significantly to 0.493 ± 0.05 ($p<0.05$) in fish exposed to the high concentration of FF. Furthermore, the mean feed conversion ratio (FCR) was reduced with a trend visible in the 5 mg C/L and a significant decrease ($p<0.05$) in the 50 mg C/L group (Control: 1.24 ± 0.03; 5 mg C/L: 1.13 ± 0.15, 50 mg C/L: 1.00 ± 0.10). There was neither a difference in the K factor (1.4 ± 0.1) nor the hepatosomatic index (HSI) (9.0 ± 1.9), the spleen somatic index (SSI) (0.9 ± 0.3) or the weight ratio of visceral fat to total weight (3.7 ± 2.0 mg/g) or visceral fat to organ weight (0.05 ± 0.02 mg/mg).

5.4.3 FulvoFeeds increases stress resistance

After 4 weeks of exposure, the plasma cortisol concentration was 8 ± 3 ng/mL showing no difference between the three groups. Fifteen minutes after netting and air-exposure, cortisol concentration in control fish increased approximately eleven times compared to the baseline value (85 ± 19 ng/mL), while that of fish exposed to 50 mg C/L FF increased only eight times (65 ± 13 ng/mL) (Figure 5-3). The trend to a lower cortisol concentration in response to stress was also detectable in fish exposed to 5 mg C/L of FF (76 ± 21 ng/mL). Plasma cortisol concentration in all fish at the 2nd-day post-stress was back to the baseline concentration without differences between the groups.

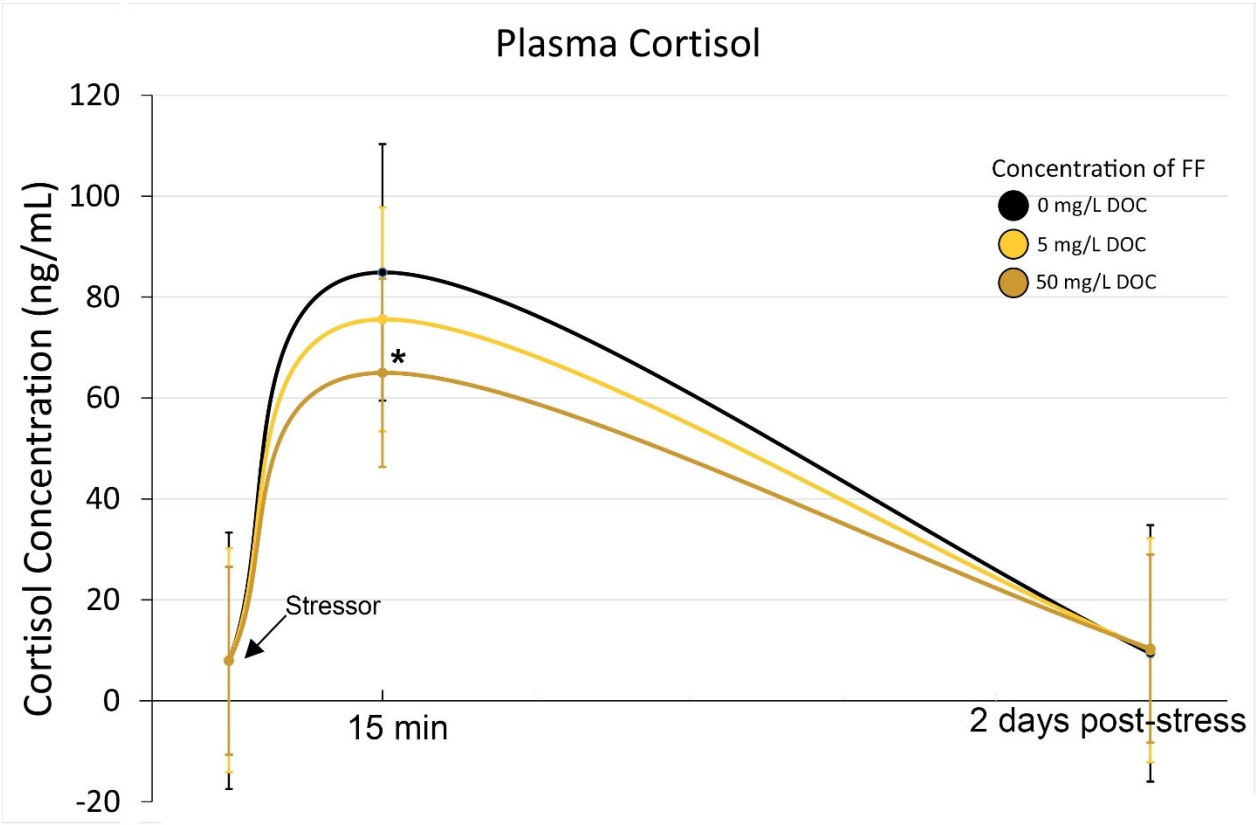

Figure 5-3: Plasma cortisol concentration after 28 days of exposure, 15 min post stressor (netting and 30 s air-exposure), and 2 days post-stress. N=12, mean ± SD; *-$p<0.05$ against control.

5.4.4 Exposure stimulates innate defense mechanisms in the head kidney but not in plasma

Phagocytes isolated from the head kidney of fish treated with both concentrations of FF had a significantly increased rate of phagocytosis and phagocytic index (Figure 5-4). While the cells from control fish had a rate of phagocytosis of 31.5 ± 13.3 % and a phagocytic index of 1.8 ± 0.2, cells from fish exposed to the low concentration had a rate of 57.6 ± 17.3 % and an index of 2.2. ± 0.3. In cells from fish exposed to the high concentration, the rate increased to 70.7 ± 9.9 % and the index to 2.3 ± 0.3. Two days after the stressor, the rate in control fish increased to 52.6 ± 10.5 % and the phagocytic index to 2.1 ± 0.2. Again, cells from FF exposed fish showed a significantly higher rate of phagocytosis (75.2 ± 7.4 % in the low exposure group and 80.5 ± 10.0 % in the high exposure group) and phagocytic index (2.3 ± 0.2 and 2.6 ± 0.1, respectively).

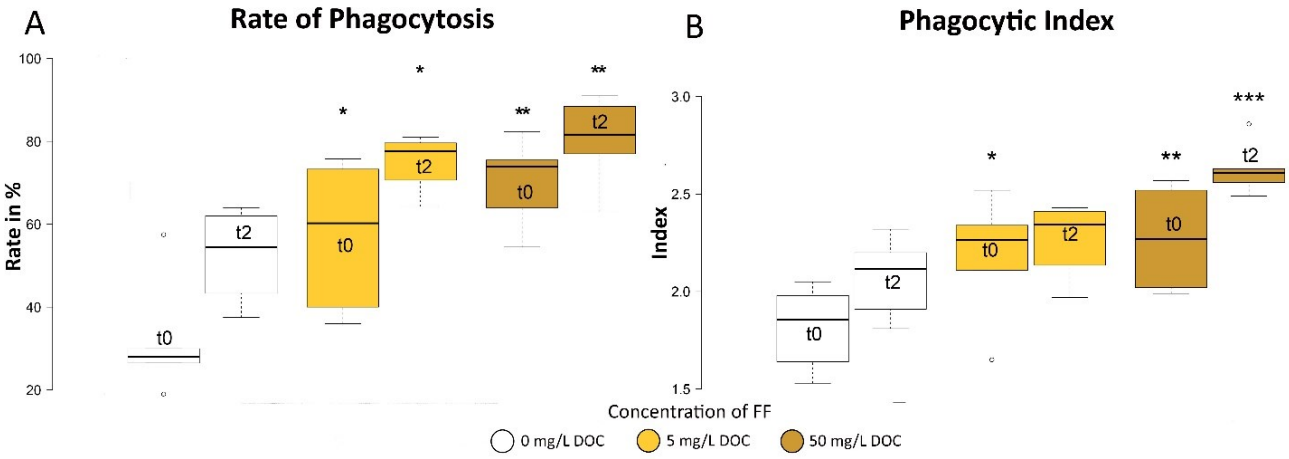

Figure 5-4: Rate of phagocytosis (A) and phagocytic index (B) of head kidney cells. t0: 28 days of exposure; t2: 2 days post stressor (t0). *-p<0.05; **-p<0.01; *-p<0.001. n=6.**

Before stressing, the head kidney cells from fish exposed to the high concentration of FF produced significantly higher amounts of reactive oxygen species (ROS) (OD (optical density) 620: 0.26 ± 0.05) compared to the cells from control fish (OD 620: 0.19 ± 0.06) (Figure 5-5). This trend was also visible in the cells from fish exposed to 5 mg C/L FF (OD620: 0.21 ± 0.07). After stimulation with phorbol 12-myristate 13-acetate (PMA), the ROS production in all groups increased by 68 ± 20 %, while zymosan increased the production by 145 ± 40 %. Again, the cells from the high FF concentration group showed significantly higher respiratory burst activity as compared to the control (OD 620: 0.42 ± 0.07 with PMA and OD 620: 0.58 ± 0.1 with zymosan). Cells from fish exposed to 5 mg C/L showed intermediate responses (OD 620: 0.35 ± 0.09 with PMA and OD 620: 0.47 ± 0.09 with zymosan). Two days post-stressor, there was no significant difference between the groups; neither before (OD 620: 0.14 ± 0.06) nor after induction of the respiratory burst (OD 620: 0.24 ± 0.10 with PMA, 0.40 ± 0.16 with zymosan).

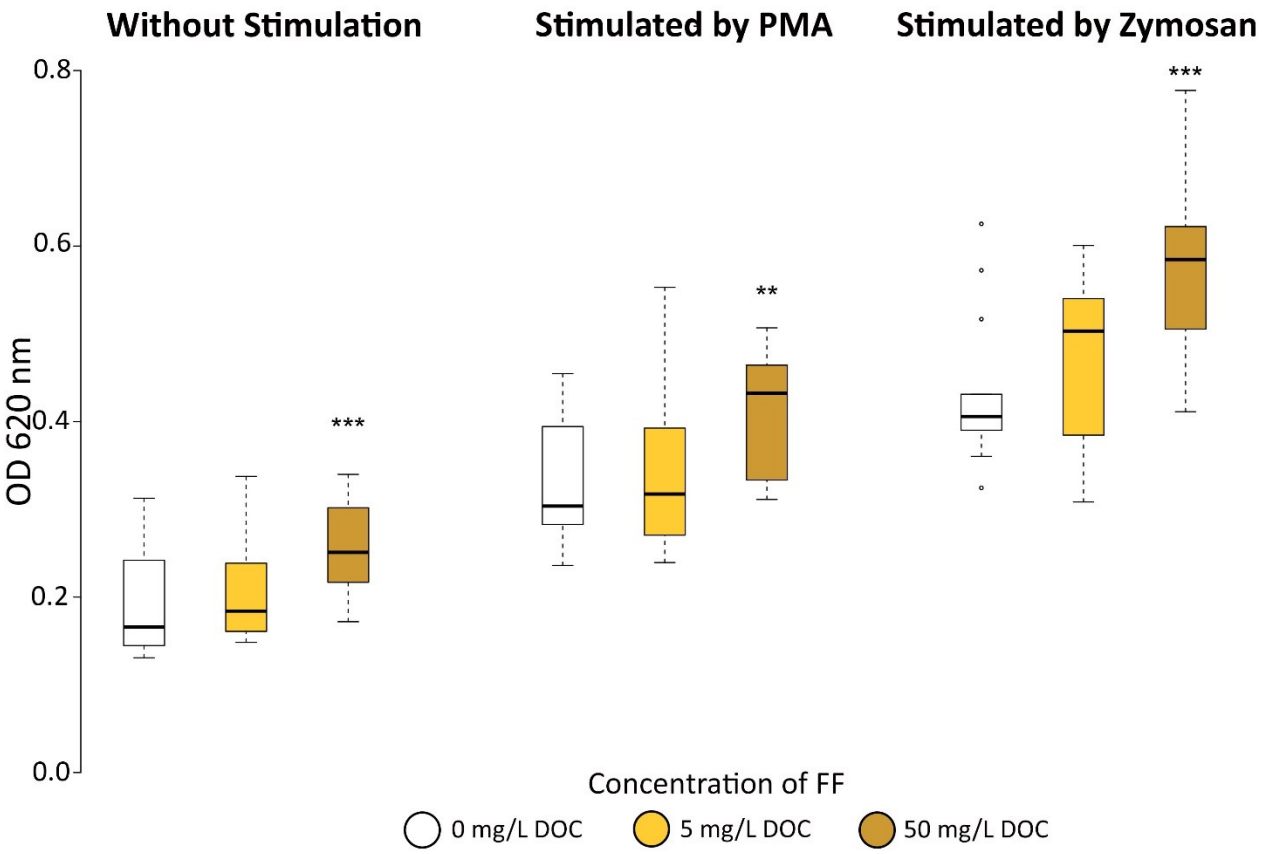

Figure 5-5: Production of reactive oxygen species of head kidney leucocytes measured by NBT (nitro blue tetrazolium) assay. (A) without additional stimulation; (B) using PMA (phorbol 12-myristate 13-acetate) as a stimulant; (C) using zymosan as a stimulant. Optical density (OD) was measured at 620 nm. **-p<0.01; *-p<0.001. n=6.**

The protein content of plasma in control fish was 22.3 ± 1.6 mg/mL and was not altered by exposure to FF. The same applies to the albumin: immunoglobulin ratio (1.9 ± 0.3) that was not affected by exposure. Furthermore, lysozyme activity of plasma was not changed by exposure (control: 44.0 ± 6.9 U/mg protein; 5 mg C/L: 42.1 ± 8.3; 50 mg C/L: 45.7 ± 7.0 U/mg protein).

5.4.5 Defense mechanisms of gills are stimulated by exposure to FF

The protein content of gill extracts (4.0 ± 0.3 mg/mL) was not altered by exposure to FF and used as normalization for lysozyme activity. The lysozyme activity of fish exposed to 50 mg C/L FF was significantly increased at the first sampling time point (Figure 5-6 A). The activity of control gills was 1.1 ± 0.2 U/mg protein, while fish from the high exposure group had an activity of 1.4 ± 0.2 U/mg protein. A trend towards an increased activity was detected in the low concentration group (1.3 ± 0.2). Two days after the stressor, the activity increased in all groups to an average of 1.4 ± 0.5 U/mg protein, without significant differences between the groups.

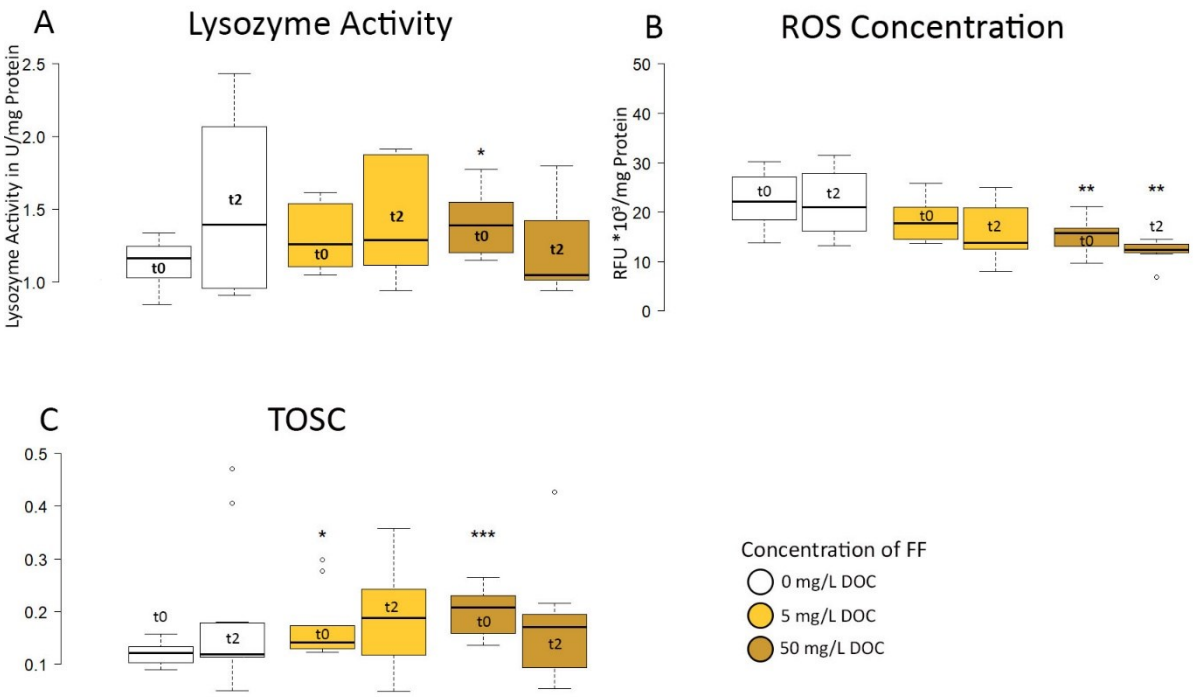

Figure 5-6: Defense mechanisms in gill supernatant. A: Lysozyme activity, B: Concentration of reactive oxygen species (ROS), C: Total oxyradical scavenging capacity (TOSC). t0-28 days of exposure; t2-2 days post-stressor (t0). *-p<0.05. **-p<0.01; *-p<0.001. n=12**

The concentration of ROS in gills decreased significantly in the fish exposed to high concentrations of FF as compared to the control group (Figure 5-6 B; Control: 22.4 ±5.6, 5 mg C/L: 18.4 ± 4.0, 50 mg C/L: 15.9 ± 3.4). This applies to both sampling time-points: after 28 days of exposure and 2 days after the first sampling (and the stressor). A trend towards a reduced ROS concentration was also visible in the group exposed to the low concentration of FF. At the same time, the total oxyradical scavenging capacity (TOSC) of fish after 28 days of exposure was significantly increased in both groups compared to the control (Figure 5-6 C, Control: 0.12 ± 0.02, 5 mg C/L: 0.17 ± 0.06, 50 mg C/L: 0.21 ± 0.04). Two days post stressor, there was no difference in the TOSC activity between the groups.

5.5 Discussion

5.5.1 Biological effects of humic substance have to be referred to chemical properties

FulvoFeed has an average molecular mass of 800 g/mol and contains primarily fulvic acids and a small amount of humic acids. Relatively small molecular masses (<3.5 kDa) can interact with plasma membranes, causing high biological reactivity (Muscolo *et al.* 2007, Pörs and Steinberg 2012a, Gilbert *et al.* 2018). This applies not only to plants but to aquatic animals as well. Fulvic acids are the low molecular weight fraction of humic substances and, therefore, their uptake over the gill epithelial surface or implementation into the mucus -or both- is highly possible.

The aromatic content of FF is around 30 %, which is in the higher range of aquatic fulvic acids. At the same time, its aromatic core is heavily substituted with phenolic groups which constitute 26% of the total aromatic carbon (Perdue (2009); Table 5-2 Supplement). The EPR spectrum indicates a high content of persistent free radicals (PFRs). This can be attributed to the high phenolic content and confirms the results from our NMR spectrum (Chen *et al.* 2002b). In previous studies, we showed that PFRs exert stimulation to several parameters including the growth of plants and neuro-behavior of *Caenorhabditis elegans* when applied at low concentrations and become toxic at higher concentrations (Liao *et al.* 2014, Lieke *et al.* 2018). Furthermore, FF has a high EDC (380 µmol/g), compared to DOC from compost (< 100 µmol/g) and a high EAC (270 µmol/g) compared to Suwannee River standard fulvic acid (<100 µmol). Both result in a high electron exchange capacity (EEC), reflecting its high ability to act as an electron shuttle (Scott *et al.* 1998, Yuan *et al.* 2012).

5.5.2 Increased resistance does not cause the growth stimulation

By adding FF for 4 weeks to the rearing water of juvenile rainbow trout, we were able to increase length and weight gain by 5 % and 10 %, respectively, and to significantly improve feed conversion by around 16 % in fish exposed to the high FF concentration. These results are comparable to those obtained in another study using immunostimulants as feed additives (Abdel-Tawwab *et al.* 2008). Against the background of the involvement of beneficial gut-bacteria in growth stimulation, it is intriguing that water supplementation with FF resulted in stimulated growth parameters as well, as there is little direct contact between the gut and the substance. As mentioned, uptake of FF and further distribution via blood is possible. The significant reduction of FCR indicates a better digestion efficiency and utilization of feed. Gao *et al.* (2017) reported increased intestinal digestive enzyme activity (protease, lipase, and amylase) on feed supplementation with fulvic acid, and chelation of mineral ions might promote the nutrient uptake and utilization of minerals in feed (Sanmanee and Areekijseree 2010). Eventually, resulting in an increased amount of energy gained from the feed, which can then be allocated for maintenance of homeostasis, growth, and defense mechanisms. An improvement of the FCR furthermore reduces the nutrient output (especially nitrogen and phosphorous) and the production of greenhouse gases, decreasing the impact on water bodies (if open systems are used) or wastewater production (in closed aquaculture recirculating systems) and the carbon footprint of aquaculture (Besson *et al.* 2016, Hasan and Soto 2017). Figure 5-7 shows a graphical summary of our results.

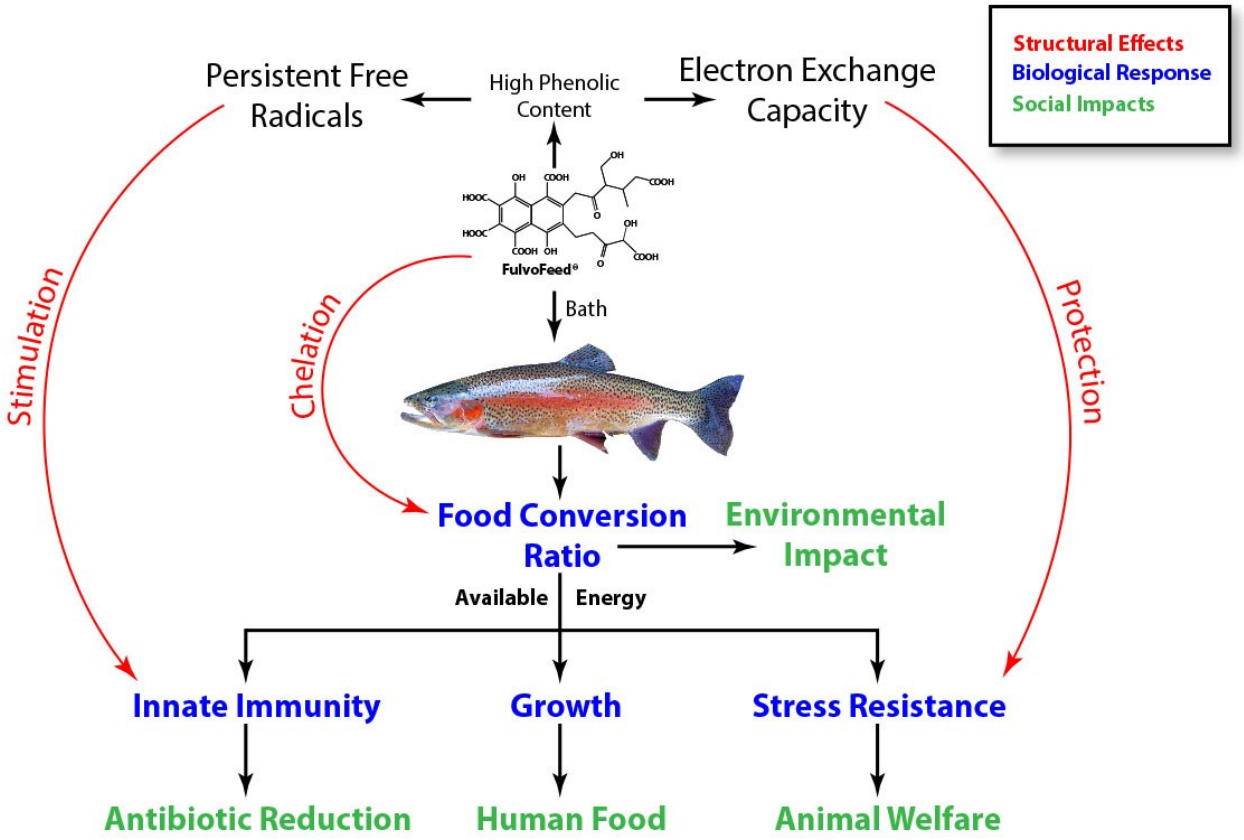

Figure 5-7 : Overview of results from this study

Stress, especially chronic one, and the allostatic load imposed to cope with it, significantly affects the energy metabolism and enhances the production of extra energy supply from body resources (Mommsen *et al.* 1999, Tort 2011). At the same time, stress leads to reduced or arrested feed intake and depressed immune system functions, impairing growth performance, and leaving the fish vulnerable to infections (Bly *et al.* 1997, Meinelt *et al.* 2004, Conde-Sieira *et al.* 2018). Cortisol is most widely used to define stress levels (Kalamarz-Kubiak 2018). Exposure to FF did not affect the cortisol concentration in blood, indicating, that it did not exert any stress on the fish. In the laboratory, fish were kept under ideal conditions and external stress was kept at a minimum; it is therefore unlikely, that a reduced stress during the exposure time accounts for the improvement detected in growth and FCR.

To mimic inevitable handling in aquaculture, we exposed fish to a strong acute stressor (netting and 30 s of air exposure). The cortisol response of fish, conditioned with 50 mg C/L FF was 24 % lower than that of unexposed fish. It can be reasonably assumed, that other stressors will be diminished as well. Further analyses, especially on molecular levels, are needed to work out the modes of action, but we were able to show that FF addition to the rearing water helps to increase the growth of juvenile rainbow trout and reduce cortisol response to an acute stressor.

5.5.3 No apparent oxidative stress was exerted to the gills

As ROSs are naturally formed by aerobic metabolism in organisms and are part of the potential killing activity of macrophages, cells have antioxidant defense mechanisms (measured as TOSC) to scavenge ROS and to maintain the cellular redox homeostasis. External oxidative stress can deplete this defense mechanism and evoke severe cellular damage. The electron paramagnetic resonance (EPR) spectroscopy showed a rather high concentration of unpaired spins in FF. However, the amount of ROS detected in the gill tissue after exposure to the high concentration of FF was significantly reduced, and the same trend was also visible in the low exposure group. Furthermore, exposure to both concentrations of FF significantly increased the TOSC of gill tissue. Together, this indicates that the applied FF did not exert an apparent oxidative stress on the gills; the overall effect is protective.

At first glance, this appears to contradict previous studies with freshwater amphipods *Gammarus sp.* and *D. magna* exposed to humic substances, where several endpoint markers of oxidative stress increased (Timofeyev *et al.* 2006, Saebelfeld *et al.* 2017). However, invertebrates are a lot more sensitive to xenobiotics than vertebrates (Xin *et al.* 2015) and the gills of fish are covered by mucus decreasing direct contact with FF. Especially in the described case with *D. magna*, the concentrations of humic substances used were similar to the highest exposure used in our study on fish but it might be too high for potential protective effects in those invertebrates.

Furthermore, Timofeyev *et al.* (2006) observed a 2-stage-response in gammarids with increased activity of antioxidative enzymes in the first stage, and cell damage, probably due to depletion of the TOSC, in the second phase. The increased TOSC after 28 days of exposure to the FF in our study indicates that depletion to cope with the overproduction of ROS generated by oxidative stress is highly unlikely. The high aromatic content and EEC might add to the TOSC by inactivating radicals. Similar effects were observed after exposing the stonewort *Chara hispida* to humic substances (Pörs and Steinberg 2012b).

Lastly, the effects found with one humic substance cannot necessarily be transferred to another humic substance. Both, the concentrations of FF and the exposure duration used in our study, did not evoke oxidative stress in rainbow trout as previously reported in invertebrates. However, whether or not the negative effects will appear at higher concentrations and/or longer exposure time has to be tested before any other long-term application can be recommended.

5.5.4 Fish are prepared to deal with pathogens

Phagocytes are an important part of the innate defense mechanisms and can reasonably be expected to increase the capacity to neutralize invasive microbes (Demers and Bayne 1997). Exposure to FF increased the number of active phagocytes in the head kidney by 83 and 124 %, respectively.

Furthermore, the number of particles digested by each phagocyte increased by 22 and 28 %, respectively, and the potential killing activity increased significantly. Altogether, this increases the microbiocidal capacity (Dupré-Crochet *et al.* 2013) allowing the fish to defend themselves against pathogens. Similar effects were determined when using an oral application of immunostimulants (Geng *et al.* 2011). As chronic stress has been found to decrease the efficiency of macrophages (phagocytosis rate and ROS production) (Fries and Tripp 1980, Narnaware *et al.* 1994, Sesti-Costa *et al.* 2010), the FF protective function is working in two ways simultaneously: by increasing stress resistance and by stimulating the phagocytic activity and the potential killing activity of leucocytes.

Lysozyme activity was significantly increased in the gills after exposure to the high concentration of FF compared to the control, indicating an improved protection against bacterial invasion. Two days post-stressing the lysozyme activity of all groups was increased due to acute stress-induced enhancement (Dhabhar and McEwen 1997, Tort 2011), however, without any difference between the groups. Unexpectedly, the plasma lysozyme activity was not affected by FF exposure. This contradicts findings from oral administration of immunostimulants including HS (Christybapita *et al.* 2007, Adel *et al.* 2015, Gao *et al.* 2017). However, none of these studies evaluated the effects on gill lysozyme activity.

In addition to these direct anti-bacterial properties (and thereby anti-inflammatory properties), lysozyme is involved in pro-inflammatory responses and in resolving inflammation. This is why the temporal and spatial balance of lysozyme activity is crucial after the invasion of pathogens as explained by Ragland and Criss (2017).

Although lysozyme activity was increased, we did not detect an increase in the ROS concentration in the gill tissue after exposure to the FF sample, which could indicate chemotaxis and similar activation of leucocytes as in the head kidneys. There are several possible modes of action to explain this: firstly, some humic substances have antibacterial properties, reducing the overall bacterial load exerted on the gills (Ansorg and Rochus 1978, Gao *et al.* 2017) reducing the need to activate the fish defenses. Secondly, increased lysozyme activity and degradation of water-borne bacteria might be “defense enough”, and, therefore, the recruitment of neutrophils and macrophages might not occur. Thirdly, the measurement of ROS concentration in the gill extract is only an indirect method to detect the potential killing activity of leucocytes. As TOSC was increased in gill tissues, ROS produced by leucocytes might have been scavenged. To determine, whether or not FF stimulates recruitment and activates gill leucocytes, further analyses including isolation of gill leucocytes and preparations of cell cultures are required. Determining transcriptional changes of pro-inflammatory signal molecules and amounts of leucocyte marker molecules can help to deepen the understanding of how FF modulates the lysozyme-mediated immune response, and whether or not pro-inflammatory effects are exerted.

Our study shows that defense mechanisms are activated by bath exposure to FF, which could help the fish to protect themselves against diseases. However, more research including challenge experiments is needed to evaluate if exposure to FF helps to reduce the susceptibility of fish against pathogens.

5.6 Conclusion

Consumers' interest in safe and sustainable food sources is rising. This concerns especially the use of antibiotics and harmful therapeutants, but the overall-impacts of agri- and aquaculture on resources and environment as well. Natural immunostimulants can help to increase growth and animal welfare while protecting from diseases by activating the host immune system. They are commonly applied orally; we showed that stimulation over the gills is another pathway for fish in aquaculture. Fulvic acids are part of aquatic ecosystems and bath application is a natural way to apply these immunostimulants. However, because of the heterogenic character of this substance group, chemical structures have to be determined before the biological application.

We demonstrated that the addition of FF to the water increased the growth of fish without adversely affecting the weight/ fat ratio and improved the FCR. Furthermore, the stress response was significantly lower in fish conditioned with FF after netting and air-exposure. Gills, which are the entrance portal of viral and bacterial pathogens, had significantly improved defenses in regards to lysozyme activity and protection against oxidative stress as compared to non-treated fish. The innate immunity was also significantly improved in terms of the increased activity and efficiency of leucocytes in the head kidney. The high aromatic contents of FF, especially the phenolic moieties, lead to a high EEC and protect against oxidative stress. At the same time, the PFRs in FF exert mild stress, which stimulates the immune system. Although the protection against a pathogen has to be determined yet, FF could help to reduce the use of chemical therapeutants and prevent fish from diseases. Overall, implementing FF to the water of fish is an easy and natural way to improve fish health and growth, and to decrease the impact of aquaculture on the environment.

5.7 Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

5.8 Acknowledgments

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the TOSC-assay and corresponding R programming and to Viola Schöning for her assistance with the cell culture assays.

5.9 Authors Contribution

All authors contributed to the conceptualization of the paper. Performance of the experiments, sample analysis, data analysis, writing of the original draft, graph creation, and editing were performed by T.L. 13 NMR spectroscopy analysis and interpretation were performed by I.P. Mediated electrochemical reduction and oxidation and electron paramagnetic resonance spectroscopy was performed by B. Pan. Funding was acquired by C.E.W.S., T.M., and T.L. The acquisition of the animal experiment permission and the study were supervised by K.K. and W.K. All authors contributed to reviewing of the manuscript draft.

5.10 Competing Interests

The authors declare that there are no conflicts of interest. The founding sponsor had no role in the design, content, or decision to publish the paper. Mention of trade names or commercial products in this article is solely for providing specific information and does not imply recommendation or endorsement by the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Humboldt University, or the Federal Ministry for Economic Affairs and Energy.

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5.12 Supplement

Table 5-2: Standard and reference fulvic acids from international humic substances society (IHSS) http://humic-substances.org/acidic-functional-groups-of-ihss-samples/; http://humic-substances.org/13c-nmr-estimates-of-carbon-distribution-in-ihss-samples/ and aquatic fulvic acids from rivers (Chin et al. 1994)

Fulvic Acid	Phenol content %	Aromatic content %	Reference
Suwannee River	2.91-2.84	24-22	IHSS
Elliot Soil I	nd	30	IHSS
Elliot Soil II	2.27	nd	IHSS
Pahokee Peat I	2.33	34	IHSS
Suwannee River	3.11	nd	IHSS
Pahokee Peat	1.78	nd	IHSS
Nordic Lake	3.18	31	IHSS
Missouri River FA		20.4	(Chin *et al.* 1994)
Yakima River FA		24.3	(Chin *et al.* 1994)
Ohio River FA		24.3	(Chin *et al.* 1994)
Minnesota groundwater FA		12.6	(Chin *et al.* 1994)

6 General discussion

Humic substances are ubiquitous in nature and comprise up to 95 % of the dissolved organic matter in aquatic ecosystems. They arise from chemical, physical, and microbiological degradation of organic material. Because of their beneficial effects on growth, disease resistance, and anti-inflammatory effects they are used in agriculture and balneotherapy (Van Rensburg *et al.* 2001, Klöcking and Helbig 2005, Domínguez-Negrete *et al.* 2019). However, hazardous effects such as oxidative damage on macromolecules and diseases are associated with HSs as well (Yang *et al.* 2002, Steinberg *et al.* 2010, Saebelfeld *et al.* 2017). Due to the plethora of raw materials and formation pathways of HSs they can differ greatly in their structure and subsequently their biological functions (Chen *et al.* 2002b, Meinelt *et al.* 2007). To allow comparison between studies with different HSs, a general chemical characterization is essential.

6.1 Chemical characterization of the fulvic acid

The fulvic acid (FA) in this thesis is extracted from groundwater in the Netherlands. The carbon content (all carbon in the FA is dissolved) is 97 g/L. FA has an average molecular mass of 800 g/mol and aromatic content of around 30 %. Of this, 26 % are phenolic groups (7.9 % of total carbon), which is high for aquatic fulvic acids (Perdue 2009, Lieke *et al.* 2021b). This high aromatic content contributes to a high electron exchange capacity and determines the high amount of persistent free radicals (Scott *et al.* 1998, Yuan *et al.* 2012). Yamin *et al.* (2017a) showed, that some HSs are taken up into the blood of tilapia kept in HS-rich recirculating aquaculture systems (RAS). Furthermore, small molecules (< 3.5 kDa) can interact with plasma membranes and such interaction was shown for HSs (Muscolo *et al.* 2007, Steinberg *et al.* 2008). Uptake of the FA either by the gill surface or into the mucus is highly possible. Furthermore, HSs can interact with receptors, such as the aryl hydrocarbon receptor (AhR) and the resulting signal cascade controls complex transcriptional programs including those of the immune systems (Bittner *et al.* 2006, Rothhammer and Quintana 2019).

6.2 Fulvic acid accelerates hatching, stimulates growth, and improves the feed conversion ratio

Exposing zebrafish larvae for 96 h to FA concentrations between 20 mg C/L and 200 mg C/L significantly accelerated the hatching, but did not affect the overall hatching rate (Lieke *et al.* 2021a). Lower and higher concentrations had no significant effect. Stress by low external oxygen concentrations was excluded as a reason as the oxygen saturation was > 90 % at the end of the experiment (Cloud, 1981; Latham and Just, 1989; Schreck et al., 2001). Furthermore, the concentration of ROS inside the larvae was not elevated. However, the gene expression of the growth hormone (Gh)

and the hatching enzyme (He-1α) was significantly increased. Expression of the insulin-lieke growth factor 1 (Igf-1) was not affected by these concentrations.

Gh is produced in the pituitary and triggers the release of Igf-1 from the liver. Both play an important role in the regulation of fish growth (De Azevedo Figueiredo *et al.* 2007, Dang *et al.* 2018). Transgenic fish lines with higher Gh levels had significantly increased growth performance compared to non-transgenic fish lines. This aligns with our results after exposing juvenile rainbow trout for 4 weeks to the FA. We found significantly increased length and weight in trout exposed to 50 mg C/L and a small increase in those exposed to 5 mg C/L (Lieke *et al.* 2021b). Based on the gene expression results, it can reasonably be assumed, that this is caused by increased Gh transcription. Elevated levels of Gh have been associated with improved feed conversion ratio (FCR) and yolk energy conversion efficiency (Devlin *et al.* 1995, Hallerman *et al.* 2007). Both align with the accelerated development we observed in fish larvae and the improved growth performance in juvenile fish. An increased yolk energy conversion efficiency could result in faster development of the larvae and subsequently in accelerated hatching. At protected artificial living conditions such as aquaculture hatcheries, an accelerated hatching and faster development could lead to earlier onset of external feeding resulting in faster growth. Future studies should focus on behavior monitoring of larvae (e.g., body size, predatory movements) and whether the growth performance of juvenile/adult fish is indeed improved by the condition of larvae.

An increase in growth performance was also observed by Gao *et al.* (2017) after feeding juvenile loach (*Paramisgurnus dabryanus*) with dietary fulvic acid supplements. This was explained by the increased activity of digestive enzymes and changes in the intestinal microbiota. As mentioned, uptake of the FA over the gills might be possible and could affect the digestive enzymes or even the microbiota over the blood flow. Future studies are needed, to investigate if this mode of action also appears in the juvenile fish after bath exposure.

6.3 Oxidative stress and the protection from it

Stress is associated with a disturbance of the *status quo* by intrinsic or extrinsic stimuli (stressor). With this definition, stress is labeled as negative events only. However, the *status quo* or “homeostasis” is a static concept and in real life, organisms are constantly exposed to changing conditions and have to adapt to them (McEwen 2017). The concept of allostasis includes the reaction to stressors and the mechanisms that are activated to cope with a stressor (“achieving stability through change”) (Sterling 1988, Sterling 2012, McEwen 2017). Exposure to mild stress is even more beneficial to organisms than to just maintaining homeostasis as it prepares the system to deal with stressors before they even arise (Minois 2000, Sterling 2012). Only if the costs (allostatic load) to deal with a stressor become too high, stress becomes detrimental (McEwen and Stellar 1993).

This concept applies to oxidative and electrophilic stress (Minois 2000, Niture and Jaiswal 2010, Sies *et al.* 2017) and seem to apply to our FA as well. 50 mg C/L exerted mild oxidative stress as shown by increased transcription of Nrf2 and Sod-2. No indication of detrimental effects (neither damage of the larvae nor increased ROS concentration) was found in larvae after exposure for 96 h to 5 mg C/L and 50 mg C/L. Exposure to the same concentration increased the TOSC and decreased the ROS of the gills of juvenile rainbow trout. Polyphenols are strong anti-oxidants themselves and due to the high content in FA it could scavenge ROS directly when being implemented into the mucus or gills surface (Katiyar and Elmets 2001, Du *et al.* 2009). This would explain the increase in TOSC in the gills of rainbow trout after exposure to 5 mg C/L. However, the transcriptional results suggest, that at 50 mg C/L molecular pathways to protect against oxidative damage are activated as well. At low to medium concentration FA is protective.

Exposing zebrafish larvae to FA at concentrations of 300 mg C/L and 500 mg C/L significantly increased the ROS concentration inside the larvae (Lieke *et al.* 2021a). Oxidative stress can result in tissue damage, inflammation, and apoptosis (Day 1991, Cheng *et al.* 1999, Glover and Wood 2005). At these concentrations, detrimental effects, such as the formation of edema and hematomas and increased mortality were observed. At the same time, the transcription of Keap-1, Nrf2, Cat, and Sod-1 was increased. Although the defensive mechanisms were activated, the allostatic load was too high to cope with. At high concentrations (≥ 300 mg C/L) the hazardous effects of the FA outweigh the protective ones.

Under laboratory conditions, potential stressors are kept at a minimum, however, this is not always possible in hatcheries. Poor water quality, low oxygen, temperature changes, and high densities act as acute or chronic stressors (Piper 1982, Daskalova 2019). As FA exerts mild stress even at medium concentrations (50 mg C/L), it adds to the cumulative allostatic load imposed on the fish. Depending on the rearing conditions, hazardous effects might already appear at lower concentrations than in our studies. When applying the FA under practical conditions, the whole allostatic load has to be monitored to ensure safe application and beneficial effects.

6.4 Immune response and inflammatory properties

The innate immune system plays a pivotal role in protecting against infections and wounds. Apart from physiological barriers (epidermis, scales, mucus), fish have enzymatic and cellular mechanisms to protect themselves from pathogens or apoptotic and necrotic tissue (Erwig and Henson 2008).

The lysozyme activity in the gill of rainbow exposed for 4 weeks to 50 mg C/L FA was significantly increased. An increased gill lysozyme activity was also found shortly after infecting crayfish with the white spot syndrome virus (Wang *et al.* 2012). As the gills are in close contact with the environment

and entering-portal for many pathogens, the increased activity could help protect the fish against invasion. Transcription of *lyz* was also increased after exposing larvae to 5 mg C/L and 50 mg C/L of FA for 96 h. Increased mRNA levels of *lyz* were reported after intraperitoneal injection of LPS and heat-inactivated bacteria and are associated with increased antibacterial defenses (Jollès and Jollès 1984, Paulsen *et al.* 2003, Caipang *et al.* 2008). Interestingly, the lysozyme activity in the plasma of rainbow trout was not affected by any exposure. This contradicts reports from oral administration of phenol-rich immunostimulants or HS (Gao *et al.* 2017, Naiel *et al.* 2021), where the plasma lysozyme activity was increased. However, none of those studies determined the lysozyme activity in the gills.

Immune cells, mainly neutrophils patrol the body (Li and Ng 2012, Harvie and Huttenlocher 2015). Upon infection or wounding, the inflammatory response is activated by releasing cytokines that recruit more neutrophils and other innate immune cells. Pathogens and damaged cells are then destroyed by several mechanisms, including phagocytosis and the release of ROS (respiratory burst). The rate of phagocytosis of leukocytes isolated from the head kidney of rainbow trout was significantly increased after exposure to 5 mg C/L (by 83 %) and 50 mg C/L (by 124 %) of the FA. The same applies to the phagocytic index, which was increased by 22 % and 28 %, respectively. Increased phagocytosis is often reported after oral application of immunostimulants (Bridle *et al.* 2005, Anusha *et al.* 2014, Choudhury and Kamilya 2018). Increased phagocytosis was also reported after feeding rats with a humic acid (Habibian *et al.* 2010). However, the mode of action is still unclear. Phagocytes possess pattern recognition receptors (PRRs) that enable them to detect damage and pathogen-associated molecular patterns (DAMPs and PAMPs). One possible mode of activation might be that the mild stress exerted by the FA activates cells to produce DAMPs which then activate the phagocytes. Heat-shock proteins (HSPs) for example act as DAMPs by activating toll-like receptors (Asea *et al.* 2002, Seong and Matzinger 2004) and induced transcription of HSP 70 was reported after exposure to HSs (Timofeyev *et al.* 2004, Steinberg *et al.* 2006). Furthermore, as mentioned above, HSs activates the AhR, resulting in the release of HSP 90 (Heid *et al.* 2000). However, more research is needed to determine if this is indeed how FA activates the phagocytes. Exposure to 50 mg C/L of the FA furthermore increased the respiratory burst activity of both, unstimulated and stimulated head kidney leucocytes. This aligns with studies using human neutrophils, which showed increased respiratory burst activity after exposure to different HSs (Riede *et al.* 1991). As mentioned above, FA increased the TOSC and activated the transcription of anti-oxidative genes. As the phagocytes have to protect themselves from oxidative damage, these increased defense mechanisms might allow them to produce higher concentrations of ROS, while still keeping their redox balance. Regardless of the underlying mechanisms, the activation of the phagocytes increases the capacity of the fish to respond to an infection or wound (Demers and Bayne 1997, Dupré-Crochet *et al.* 2013). In zebrafish larvae, transcription of *mxp*, a myeloperoxidase, was significantly increased after exposure to 5 mg C/L and 50 mg C/L. Myeloperoxidase is involved in

the respiratory burst activity of neutrophils, and increased transcription could be an indicator, that the neutrophil activity is activated in larvae after exposure to FA similar as shown in head kidney cells. As *mpx* is also used as a marker gene for neutrophils (Meijer *et al.* 2008, Mathias *et al.* 2009, Buchan *et al.* 2019) the increased transcription could also be attributed to an increased number of neutrophils. Measuring the activity of the myeloperoxidase and analyzing the cell composition could help to shred light on the underlying mechanisms.

If the immune system is working correctly, the pro-inflammatory phase, where the cells are recruited to the affected tissue is followed by a resolving phase where anti-inflammatory molecules are released to end the inflammation (Opal and DePalo 2000, Cuneo and Autieri 2009). Exposing zebrafish larvae for 2 h to 50 µM $CuSO_4$ induces an inflammation which can be monitored by the ROS production of activated phagocytes. This assay was then used to analyze the inflammatory properties of substances. Exposing larvae for 96 h to 5 mg C/L and 50 mg C/L significantly decreased the copper-induced inflammation comparable to a 1 h treatment with 3 µM diclofenac. Based on the results of our previous studies, the anti-inflammatory effect could be caused by increased transcription of the anti-oxidative enzymes, which help to prevent the copper-induced damage. It is furthermore possible, that the increased activity and/or number of phagocytic cells help to resolve the inflammation faster than in unconditioned larvae. van Rensburg and Naude (2009) reported increased expression of anti-inflammatory and decreased expression of several pro-inflammatory cytokines after exposing lymphocytes to potassium humate. This could help to protect from the inflammation or help to resolve it. Anti-inflammatory cytokines, such as IL-4, IL-19, and TGF-β mediate the transition from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages and several polyphenol-rich natural compounds have been shown to promote this transition (Aharoni *et al.* 2015, Dugo *et al.* 2017, Grayfer *et al.* 2018, Mendes *et al.* 2019). Future studies are needed to determine which mode of action applies; however, our results clearly show, that FA bath application activates the innate immune response and protects from inflammation when applied at low to medium concentrations. At concentrations ≥ 300 mg C/L however, the FA becomes pro-inflammatory itself and amplifies the copper-induced inflammation. A possible explanation is, that the high content of persistent free radicals in the FA and the exerted oxidative stress at these concentrations outreach the protective mechanisms. Furthermore, at 500 mg C/L transcription of *mpx* tended to be decreased, indicating a lower activity or number of neutrophils.

6.5 Effects of FA condition on the stress response

The general response to a threatening situation is referred to as “stress” and activates the hypothalamus-pituitary-interrenal (HPI) axis, resulting in the release of corticosteroids (mainly cortisol) and catecholamines (such as adrenalin and noradrenalin) (Mommsen *et al.* 1999, Pankhurst 2011).

Elevated cortisol concentrations increase hepatic gluconeogenesis and alter the energy distribution (Mommsen *et al.* 1999, Schreck and Tort 2016). Acute or mild stress activates the defense mechanisms and sets the organisms to an "alert" state, where it is prepared to deal with the stress (Demers and Bayne 1997). Chronic stress and the allostatic load impair growth, the immune response, and reproduction (Barton *et al.* 1987, Schreck 2010). Exposing juvenile rainbow trout for 4 weeks to 5 mg C/L and 50 mg C/L did not affect the plasma cortisol concentration, showing that the exposure itself did not exert any strong stress. In rainbow trout, the blood cortisol concentration peaks 15 min after the initial acute stress (Barton *et al.* 1980). Stressing the rainbow trout by netting and 30 s of air exposure elevated the cortisol concentration after 15 min in control fish approximately eleven times compared to the baseline. Fish conditioned with 50 mg C/L had only eight times higher cortisol concentrations. The trend towards a lower response was also visible in fish conditioned with 5 mg C/L. Pre-exposure to FA reduced the impact of stress on fish. Similar results were reported by Meinelt *et al.* (2004) who raised swordtail fish (*Xiphophorus helleri*) to a synthetic HS and found lower impairment of growth after 2 weeks of daily netting.

Acute stress also activates the immune response (Tort 2011). Two days after the netting (t2), phagocytosis (rate and index) was elevated compared to the initial values (t0). Again, fish exposed to FA had higher rates and indices compared to unexposed fish. The respiratory burst activity showed no difference between the groups two days after the stressor and was comparable to the control baseline (0.19 OD at t0 and 0.14 at t2). Mounting the stress response is highly energy consuming (Schreck and Tort 2016) and apparently, the energy used to activate the leucocytes under non-stress conditions was allocated to other mechanisms upon netting stress. Handling and transport are inevitable stressors in aquaculture and the addition of FA before such procedures can help to reduce the stress of fish and subsequent impairments such as increased vulnerability to infections and impaired feed uptake.

6.6 Conclusion and outlook

Supplementing feed with immunostimulants is a widely used practice to improve growth and overall health in livestock. In fish, there is another possible route of stimulation: the water body. In contrast to feed, this allows easy application by the owner, reduces the change of molding of the feed and instability of the supplement, and guarantees that all fish in one batch receive the same dose. However, the immunostimulant has to fulfill certain criteria: it has to be completely soluble without precipitation to avoid changes in the concentration or clogging of pumps or pipes. This excludes high molecular molecules such as β-glucans or chitin, but also several humic substances, as chelation with carbonates can result in precipitation. Furthermore, the substance has to be non-irritant as the fish are constantly exposed. Finally, to be economically realistic, the substance has to be cheap, as larger amounts are needed for bath treatment than for feed supplementation.

We showed, that a phenol-rich FA extracted from groundwater can be used as a bath treatment to improve the condition of fish larvae and juvenile fish. The feed conversion efficiency was increased, allowing the fish to gain more energy by consuming the same amount of feed. This energy was then allocated to increase growth, and into different parameters of the innate defense systems comparably as was reported by immunostimulatory feed additives (Hoseinifar *et al.* 2015, Azimirad *et al.* 2016, Lieke *et al.* 2019). Gao *et al.* (2017) reported changed intestinal health in terms of digestive enzyme activity and altered microbiome after oral administration of a FA. It would be interesting to analyze if these mechanisms apply to our FA as well and if there are different effects when FA is applied as a bath or feed supplementation. It can be assumed, that the improved energy conversion applies to the yolk-sac larvae as well. If this resulted in increased metabolisms and also promotes their growth, is the content of future studies. Furthermore, if the FA treatment indeed gives larvae a "jump-start", it will be interesting to analyze if this growth improvement is lasting (and how long) or if it is lost after the end of the exposure.

Especially the sensitive gills had higher protection against invading organisms and oxidative stress, but activation of phagocytes was also found inside the body. If the FA conditioning can indeed protect against a virulent pathogen, would be an interesting approach for future studies. The same applies to the mucosal protection on the skin surface, as this contains several immune-related enzymes that provide the first line of defense against pathogens.

As the FA can become hazardous at high concentrations, analyzing long-term exposure is needed to ensure a safe application in aquaculture facilities. Especially, since the activation of antioxidative and immune parameters is cost-intensive, when not needed. However, as a short-term application, e.g., before handling or transport FA can help to reduce the adverse consequences of the stress impaired on the fish and the anti-inflammatory effects can help to deal with wounds that appear. Overall, we were able to show, that immunostimulation via bath is possible in fish and can be used in pre-feeding larvae and juvenile fish. It is therefore an alternative or addition to supplementing fish feed and can help to reduce the use of chemical therapeutics in aquaculture.

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11 Eidestattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Hilfen angefertigt habe. Beim Erstellen dieser Arbeit bestand keine Zusammenarbeit mit gewerblichen Promotionsberatern. Die dem angestrebten Verfahren zugrunde liegende Promotionsordnung habe ich zur Kenntnis genommen und die Grundsätze der Humboldt Universität zu Berlin zur Sicherung guter wissenschaftlicher Praxis wurden eingehalten. Die Dissertation oder Teile davon wurden nicht bereits bei einer anderen wissenschaftlichen Einrichtung eingereicht, angenommen oder abgelehnt. Weiterhin habe ich mich nicht anderwärts um einen Doktorgrad beworben bzw. besitze ich keinen entsprechenden Doktorgrad.

Berlin, August 2021

Thora Lieke